Heat Shock Proteins 9
Series Editors: Alexzander A. A. Asea · Stuart K. Calderwood

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Heat Shock Protein-Based Therapies

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Volume 9

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Heat Shock Proteins: key mediators of Health and Disease. Heat shock proteins (HSP) are essential molecules conserved through cellular evolution required for cells to survive the stresses encountered in the environment and in the tissues of the developing and aging organism. These proteins play the essential roles in stress of preventing the initiation of programmed cell death and repairing damage to the proteome permitting resumption of normal metabolism. Loss of the HSP is lethal either in the short-term in cases of acute stress or in the long-term when exposure to stress is chronic. Cells appear to walk a fine line in terms of HSP expression. If expression falls below a certain level, cells become sensitive to oxidative damage that influences aging and protein aggregation disease. If HSP levels rise above the normal range, inflammatory and oncogenic changes occur. It is becoming clear that HSP are emerging as remarkably versatile mediators of health and disease. The aim of this series of volumes is to examine how HSP regulation and expression become altered in pathological states and how this may be remedied by pharmacological and other interventions.

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Preface

The book *Heat Shock Protein-Based Therapies* provides the most up-to-date review on new and exciting therapies that utilize mechanisms based on the heat shock response and targeting various stress proteins as a promising therapeutic strategy for a wide variety of human disorders and diseases including cancer, neurodegenerative disorders (Alzheimer's disease, multiple sclerosis), and infectious diseases (HIV, periodontal disease).

This book is divided into therapies based on various heat shock proteins (HSP). Part I begins with therapies based on mechanisms dependent on HSP27 and covers plant-based therapies and their use for cancer. Part II deals with how HSP60 is targeted on Alzheimer's disease and periodontal disease. Part III covers novel ways of targeting HSP70 in therapies including infectious diseases, antiviral drug therapy, cancer, neurodegenerative disorders, and nanotechnology. Part IV comprehensively covers HSP90-based therapies in cancer, neurodegenerative disorders, and drug development.

Heat Shock Protein-Based Therapies is written by leaders in the field of heat shock protein research in clinical research, basic research, translational research, and pharmaceuticals. The contributed chapters review present cutting-edge research activities and importantly project the field into the future. The chapters systematically and in a stepwise fashion take the reader through the fascinating sequence of events by which mechanisms dependent on heat shock proteins are targeted and provide answers as to the biological significance of HSP to human health and disease.

This book is a must-read for undergraduate, graduate, postgraduates, and experts in the field of neuroscience, medicine, oncology, immunology, dentistry, microbiology and infectious diseases, autoimmunity, pharmacology, pathology, phytomedicine, drug development, biotechnology, and pharmaceutical industry.

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Part I
Heat Shock Protein HSP27-Based Therapies

Chapter 1
HSP27 as a Therapeutic Target of Novel Inhibitors and Dietary Phytochemicals in Cancer

Elena Aréchaga-Ocampo and César López-Camarillo

Abstract Heat shock proteins (HSP) are a family of evolutionary conserved proteins induced by cellular stressors. HSP are essential players in the development and progression of cancer inducing resistance to conventional treatment in a wide range of human tumors. Overexpression of HSP27, a member of HSP family, is associated with apoptosis inhibition, enhanced migration and metastasis and several clinical features of cancer including drug resistance promotion. At present, HSP27 could be a promising strategy to enhance sensitivity of tumors to cancer treatment. A plethora of novel compounds present in the diet, including flavonoids, can efficiently inhibit the growth of tumor cells by acting as natural "chemopreventers". Many works reported the efficient targeting of HSP27 by using small inhibitors and dietary natural compounds in order to enhance the effectiveness of cancer therapies. In this chapter, we reviewed the current status of treatments based in dietary components targeting HSP27 as a novel strategy to circumvent chemotherapy cytotoxicity in cancer patients. Moreover we addressed the current status of HSP27 overexpression in many types of human cancers and highlighted the prominent role of targeting HSP27 as a novel therapeutic strategy in cancer.

Keywords HSP27 • Cancer • Dietary phytochemicals • Therapeutic target • Chemotherapy

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Abbreviations

5-FU	5-fluorouracil
AA	Anaplastic astrocytoma
AR	Androgen receptor
Asp17	Asparagine 17
CRPC	Castration-refractory prostate cancer
CSCs	Cancer stem cells
DRSP	Drug-resistant sphere
ESI MS/MS	Electrospray ionization/multi-stage mass spectrometry
GBM	Glioblastoma multiforme
HCC	Hepatocellular carcinoma
HER2	Human epidermal receptor 2
HSF1	Heat shock factor protein 1
HSP27	Heat shock protein 27
NSCLC	Non-small cell lung cancer
OSCC	Oral squamous cell carcinoma
Phe55	Phenylalanine 55
p-HSP27	Phosphorylated HSP27
siRNA	Small interfering RNA
TDP	1,3,5-trihydroxy-13,13-dimethyl-2H-pyran [7,6-b] xanthone
TRAIL	Tumor necrosis factor-alpha-related apoptosis-inducing ligand

1.1 Introduction

Heat shock proteins (HSP) are a family of evolutionary conserved proteins induced by cellular stressors. They were serendipity discovered five decades ago in the common fruit fly Drosophila melanogaster [1]. Ferruccio Ritossa published the first observation that cells could mount a specific transcriptional activity when exposed to elevated temperatures that was dubbed as the heat shock response. This discovery led to the identification of the so-called HSP, which impact many areas of current biology and medicine, as well as to the understanding of its involvement in human diseases [2]. Differential expression of individual HSP occurs in a broad range of neoplastic processes. In cancer, the HSP facilitate rapid cell division, metastasis, and the evasion of apoptosis induced by cytotoxic anti-neoplastic drugs [3]. Therefore, HSP are essential players in the development and progression of cancer and they are prime therapeutic targets. In particular, many tumors including breast, prostate, ovarian and renal cancer as well as osteosarcoma and leukemias have been shown to unusually express high levels of HSP27 [4]. Activation of HSF1 during cell stress induces the up-regulation of HSP27 in many cancer types [5] and is associated with drug resistance promotion, apoptosis inhibition, enhanced migration and metastasis, more aggressive tumors behavior, and poor patient outcomes [6, 7]. In this chapter,

we reviewed the current status of HSP27 overexpression in many types of human cancers and highlighted the prominent role of small inhibitors and dietary natural compounds targeting HSP27 as a novel therapeutic strategy in cancer.

1.2 Chemopreventive Dietary Phytochemicals in Cancer

Chemotherapy constitutes the standard regimen used for treatment of many types of human cancers. However, non-desirable side effects in normal cells related to these cytotoxic treatments limits its rationale use. Therefore, novel natural compounds and complementary medications including herbal molecules have become more frequently used for the prevention and treatment of cancer [8]. A plethora of novel compounds present in the diet, including flavonoids, can efficiently inhibit the growth of tumor cells by acting as natural "chemopreventers". The potent antioxidant activity of these compounds has recently received a great interest, because oxidative stress participates in the initiation and progression of cancer. Phytochemicals are capable of preventing oxidative damage to DNA, thus a wide use of natural food-derived molecules is receiving greater attention as potential anti-carcinogens. Epidemiological evidences from observational and prospective studies, mainly in Asian and European populations indicate that dietary compounds may substantially alter the natural history of carcinogenesis. A large body of epidemiologic data supports the fact that diet and nutrition play a key role in carcinogenesis. In fact, an inverse correlation between a high consumption of fruits and vegetables and the incidence of some cancers has been widely documented in diverse studies [9]. In this section, we reviewed the current status of treatments based in dietary components targeting HSP27 as a novel strategy to circumvent chemotherapy cytotoxicity in cancer patients.

1.2.1 HSP27 Targeting by Resveratrol Sensitize Breast Cancer Cells to Doxorubicin Therapy

Breast cancer represents the neoplasia with the highest incidence and mortality that affects women worldwide [10]. Breast carcinomas represent a heterogeneous group of tumors that are diverse in behavior, outcome, and response to therapy. Despite advances in screening, diagnosis, and therapies, the percentage of patients with complete pathological response to standard chemotherapy-based treatments is very low. The use of chemopreventive natural phytochemicals represents a promising strategy in the search for novel therapeutic agents in breast cancer. Resveratrol (3,4′,5-trans-trihydroxystilbilene) is a dietary polyphenol found in fruits, vegetables and medicinal plants that exhibits chemopreventive and anti-tumor effects in a wide spectrum of human cancers. Using a proteomic approach based on two-dimensional

electrophoresis and ESIMS/MS tandem mass spectrometry, Diaz Chavez et al. reported that resveratrol (250 μM) treatment for 48 h significantly modulated the expression of 16 proteins in MCF-7 breast cancer cells (fold change >1.5; p value <0.05). Particularly, resveratrol treatment at 100, 200, 250 μM resulted in an efficient down-regulation of HSP27 protein [11]. It has been well established that HSP27 is frequently overexpressed in human cancer cells resulting in apoptosis inhibition and resistance to anti-neoplastic therapy [12]. Interestingly, resveratrol was able to induce apoptosis in MCF-7 cells. Cell death was associated with a significant increase in mitochondrial permeability transition, cytochrome c release in cytoplasm, and caspases-3 and -9 independent cell death. These data suggested that resveratrol might exert its effects in cell death and chemotherapy resistance by targeting HSP27. The potential chemosensitizing effect of resveratrol was demonstrated by treating MCF-7 cells with increasing concentrations of the polyphenol in combination with doxorubicin therapy. Combined therapy caused a potent effect in MCF-7 cells viability. Moreover, targeted abrogation of HSP27 expression using specific short-harping RNAs exerted a synergistic effect in cytotoxicity caused by doxorubicin. These data indicate that resveratrol inhibits HSP27 expression resulting in a cooperative effect on doxorubicin-induced cell death. The potential modulation of HSP27 using natural alternative agents, as resveratrol, may be an effective adjuvant in breast cancer therapy.

1.2.2 Ginko biloba *EGb761 Inhibits Migration by Targeting HSP27 in Non-small Cell Lung Cancer*

Lung cancer remains as one of the most aggressive cancer types with nearly 1.6 million new cases worldwide each year [13]. Non-small cell lung cancer (NSCLC) is the most common subtype of lung cancer, comprising three major histological subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Overall 5-year survival rates for lung cancer are consistently low (7.5–16 %). Approximately 40 % of patients with advanced unresectable disease at the time of diagnosis have a poor prognosis. The treatment choice for unresectable stage III NSCLC is platinum-based chemotherapy regimen and thoracic radiation. Considering that the limited therapeutic options for NSCLC, novel therapeutic targets and drugs are urgently needed. The extracts from the leaves of *Ginkgo biloba* have been widely used for centuries due to their antioxidant properties. A standard *G. biloba* extract, EGb761 (commercial name), contains 22–27 % flavonoids and 5–7 % terpenoids that constitute the most active molecules. At cellular level EGb761 may scavenge free radicals and neutralize ferry ion-induced peroxidants. In addition, EGb761 displays beneficial effects by blocking exacerbated cell proliferation, angiogenesis, and apoptosis in cancer cells [14, 15]. Importantly, phase II combined treatment involving 5-fluorouracil (5-FU) and EGb761 has been tested in patients with pancreatic or colorectal cancer, and has shown promising results [16, 17]. Recently,

Tsai et al., analyzed the cellular effects of EGb761 in NSCLC cell lines, and the HSP27 expression in patients with NSCLC in relationship with clinical outcomes. In NSCLC tumors HSP27 is overexpressed in comparison to normal lung tissue and is a poor prognostic indicator of NSCLC. Kaplan–Meier survival curve showed that NSCLC patients with low HSP27 expression had significantly better survival time than those with a high expression. EGb761 did not have cytotoxic neither apoptotic effects in A549/H441 cell lines. Interestingly, EGb761 inhibited HSP27 expression and migration of NSCLC cell lines. Moreover, HSP27 abrogation by using specific HSP27-siRNA significantly inhibited the migratory ability of A549/H441 cell lines. Mechanistic studies showed that EGb761 treatment activated the AKT and p38 pathways but not affected the PI3K, ERK, and JNK pathways [18]. These data showed that EGb761 could decrease the migration of A549/H441 by inhibiting HSP27 expression most likely through AKT and p38 MAPK pathways activation.

1.2.3 Targeting of HSP27 by Quercetin in Oral Cancer Cells

Oral squamous cell carcinoma (OSCC) is one of the most common and lethal head and neck malignancies worldwide [13, 19]. Treatment failure in oral squamous cell carcinoma (OSCC) leading to local recurrence(s) and metastases is mainly due to drug resistance. Cancer stem cells (CSCs) are thought be responsible for the development of drug resistance. However, the correlations between CSCs, drug resistance, and new strategy against drug resistance in OSCC remain elusive. Quercetin is a phytochemical found in apples, onions, teas, red wines, and many other foods. It can inhibit the growth and proliferation of cancer cells. Quercetin cancer-preventive effects have been attributed to various mechanisms, including the induction of cell-cycle arrest and/or apoptosis, as well as its antioxidant functions [20]. Using a drug-resistant sphere (DRSP) model of SCC25 oral cancer cells, Chen et al. [21] showed that quercetin suppresses drug-resistant spheres via the p38 MAPK-HSP27 apoptotic pathway in oral cancer cells. The molecular characterization of DRSPs revealed the upregulation of the drug-resistance-related genes ABCG2 and MDR-1 and of CSC-representative markers, suggesting that DRSPs have greater resistance to cisplatin and stronger CSC properties. Moreover, overexpression of phosphorylated (p-HSP27) via the activation of p38 MAPK signaling was found in DRSPs. Remarkably, targeting of HSP27 decreased cisplatin resistance and drug-induced apoptosis in DRSPs. Furthermore, quercetin treatment suppressed p-HSP27 expression resulting in apoptosis activation. Importantly, inhibition of tumor growth and attenuation of cisplatin resistance by quercetin was demonstrated in vivo using a xerograph model. These data showed that the p38 MAPK–HSP27 axis plays an important role in CSCs-mediated drug resistance in OSCC and that a combination of quercetin and cisplatin regimen can reduce tumor growth and decrease drug resistance in OSCC.

1.2.4 *Targeting of HSP27 Induces Apoptosis Upon Temozolomide and Quercetin Treatments in Glioma Cells*

Gliomas are the most common and devastating brain tumors. The most malignant group is represented by glioblastoma multiforme (GBM, WHO grade IV) and anaplastic astrocytoma (AA, WHO grade III) [22]. Prognosis for patients is poor even with aggressive treatment including surgical resection, chemotherapy, and radiation. Unfortunately, the average patient survival remains less than 2 years. Temozolomide is an alkylating drug used for treatment of grade IV astrocytoma, relapsed grade III anaplastic astrocytoma and melanoma [23]. In an interesting study, Jakubowicz-Gil et al. showed that silencing of HSP27 or HSP72 expression using specific siRNAs in GBM T98G and anaplastic astrocytoma MOGGCCM cells, increases their sensitivity to apoptosis induction upon temozolomide and/or quercetin treatment. After subsequent quercetin and/or temozolomide treatment, the level of HSP within the cells remained unchanged, indicating that the effect in cell death was specific for HSP27 and HSP72 downregulation. Notably, no autophagy neither necrosis was detected upon drugs treatments in T98G and MOGGCCM HSP27 or HSP72-deficient cells. Cell death was correlated with a decreased mitochondrial membrane potential, release of cytochrome c in the cytoplasm, and activation of caspase 3 and caspase 9 indicative of internal pathway involvement [24]. These findings indicate that molecular chaperones HSP27 and HSP72 are responsible, at least in part, for glioma cells resistance to programmed cell death. Thus, targeting of HSP using temozolomide and quercetin seems to be a potent and promising combination that might be useful in glioma therapy.

1.2.5 *Targeting of HSP27 by Xanthones from* **Garcinia oblongifoliain** *in Hepatocellular Carcinoma*

Hepatocellular carcinoma (HCC) is one of the most aggressive malignancies and leading cause of cancer-related death worldwide which accounts for approximately 500,000 deaths annually [25]. HCC is characterized by poor prognosis and recurrence after liver resection. With advances in the knowledge of molecular mechanisms of carcinogenesis, a lot of new diagnostic and therapeutic molecular targets may provide novel therapies in HCC [26]. Some of these novel therapeutic modalities are represented by dietary phytochemicals. In an recent study, Fu et al., showed that 1,3,5-trihydroxy-13,13-dimethyl-2H-pyran [7,6-b] xanthone (TDP), isolated from the traditional Chinese medicinal herb, *Garcinia oblongifolia*, effectively inhibited cell growth and induced the caspase-dependent mitochondrial apoptosis in HCC. Using a proteomic approach based on two-dimensional gel electrophoresis and mass spectrometry identified the molecular targets of TDP in HCC cells. Particularly, HSP27 protein was one of the most significantly

down-regulated proteins by TDP treatment. Functional studies showed that HSP27 mediated cell death induced by TDP in vitro. Furthermore, a nude mice model also demonstrated the suppressive effect of TDP on HCC [27]. These data suggests that TDP induces apoptosis by repressing HSP27 expression, which was associated with the caspase-dependent pathway. Therefore, TDP may be a potential novel anti-cancer drug, especially to cancers with an aberrant high expression of HSP27. In other related study, it was demonstrated that TDP effectively stimulated HSP27 to form aggregates ex vitro, leading to suppression of its chaperone activity [28]. Mechanistic investigations shown that complexes were degraded by the ubiquitin-proteasome pathway. Moreover, TDP directly interacted with Asp17 and Phe55 in chain C of HSP27. In conclusion, this report indicates that HSP27 is a direct target of TDP in its anti-cancer activity, which provides strong support for a clinical application.

1.3 HSP27 as a Therapeutic Target in Human Cancers

HSP have been found overexpressed in a wide range of human tumors inducing resistance to conventional treatment; therefore they represent hopeful therapeutic targets in cancer [29, 30]. Many studies showed that high expression of HSP27 in cancer has been associated with metastasis [31–33] and has been described as differential prognostic marker [34–36]. HSP27 studies in animal models shown increased tumourigenic potential of rodent cells transplanted in syngeneic hosts [33, 37, 38]. Tumorigenic potential of HSP27 is explained at least in part, through their cytoprotective activity. In addition, the overexpression of HSP27 seems to be correlated with the induction of chemo- and radio-resistance in cancer cells. Studies in vivo e in vitro in cancer models shown that HSP27 is a potential gene target because its inhibition enhances the effectiveness of anti-cancer chemotherapies [39]. In this section, we reviewed the studies using inhibitors of HSP27 as a strategy to improve the chemotherapy or antibodies based treatments in many types of cancers

1.3.1 HSP27 Targeting in Breast Cancer

Clinical-pathological studies in breast cancer reveals that overexpressed HSP27 correlates with small tumor size, low proliferation index and short survival for patients node-negative [40]. In vivo analysis shown that HSP27 overexpression increases the metastatic potential of human breast cancer cells inoculated into athymic nude mice [41]. Moreover, in breast tumors the expression of estrogen receptors is correlated with up-regulated HSP27 expression, now it know that the HSP27 gene contains an imperfect estrogen-responsive element and can be induced by estrogen treatment in breast cancer cells [42]. Despite the chemo-protective effects of HSP, studies attempting to correlate HSP27 protein level in

breast cancers after therapy with tumor progression and clinical outcome have not been entirely clear. HSP27 overexpression confers resistance to doxorubicin and paclitaxel through inhibits apoptosis in human breast cancer cells [43, 44]. However, increased levels of HSP27 expression were not significantly associated with response to tamoxifen, time to treatment failure, or survival in an estrogen receptor-positive breast cancer population [45]. Studies in vitro demonstrated that downregulation of HSP27 in breast cancer cells expressing the human epidermal receptor 2 (HER2) resulted in increased responsiveness to Herceptine (trastuzumab) therapy, a monoclonal antibody specific to HER2, widely used against HER2-overexpressing metastatic breast cancers. Kang, et al. showed that HSP27 could enhance HER2 protein stability, which results in low susceptibility to treatment with Herceptine [46]. Moreover, the authors demonstrated by co-immunoprecipitation analysis that HSP27 can bind to HER2, which could be a mechanisms for obstructed the downregulation of HER2 by trastuzumab.

1.3.2 HSP27 Inhibition in Prostate Cancer

In patients with prostate cancer the overexpression of HSP27 is associated with poor clinical outcome following hormonal therapy [47]. Some studies showed that expression of HSP27 is upregulated by hormonal ablation and chemotherapy and is associated with castration-refractory prostate cancer (CRPC). Rocchi et al. showed that HSP27 expression was low or absent in prostate tumor tissues from patients untreated, but starts increasing 4 weeks after androgen ablation, to become uniformly highly expressed in CRPC [48]. As HER2 in breast cancer, in prostate tumors HSP27 regulate the expression of androgen receptor (AR) [49]. Therefore HSP27 is an independent predictor of clinical outcome because a low expression of HSP27 is associated with a delay in prostate tumor progression. Experimentally, HSP27 antisense oligonucleotides can enhance apoptosis and delay tumor progression in prostate cancer. Many studies shown that the use of HSP27 antisense oligonucleotides is an effective strategy for successful conventional therapy in some cancers including prostate, bladder and pancreas [50].

1.3.3 HSP27 in Leukemia and Lymphomas

HSP27 protein expression was evaluated in 98 adult patients with newly diagnosed acute myeloma leukemia for to identify prognostic factors alternative or additional to drug-resistance and apoptosis proteins. The expression of HSP27 was analyzed by immunocytochemistry and western blot and the results showed that it was expressed in a median of 15 % of total patients. In the multivariate analysis, HSP27 correlated with poor-risk cytogenetic [51]. Expression of HSP was associated with major adverse prognostic factors in acute myeloid leukemia. In pediatric patients,

HSP27 is highly expressed in bone marrow mononuclear cells of newly diagnosed acute myeloid leukemia-M4/M5 subtypes and leukemia cell lines. Knockdown of HSP27 expression allow apoptosis induced by anthracycline and cytarabine-based induction chemotherapy regimen. Downregulation of HSP27 increased the chemosensitivity of leukemia cells and the cytotoxicity anticancer drug-induced [52]. HSP27 confers resistance to bortezomib, a proteasome inhibitor that is currently used in clinical for the treatment of lymphoma cells. Blocking HSP27 using an antisense strategy enhanced the cells sensitivity allow apoptosis induced by bortezomib [53]. The cytoprotective effect from HSP27 in cancer has been studied in lymphomas and multiple myelomas. HSP27 is overexpressed in dexamethasone resistant cell lines. Specific down-regulation of HSP27 demonstrated that it could sensitize dexamethasone-resistant multiple myeloma cell lines and primary patient cells to treatment with this drug. Downregulation of HSP27 by siRNA restored the sensitivity to dexamethasone treatment by enhancing apoptotic response by triggering the release of mitochondrial protein Smac, followed by activation of caspase-9 and caspase-3 [54]. Therefore HSP27 may play a role in the inhibition to apoptotic pathways activation in order to decrease the cytotoxic action of chemotherapy drugs.

1.3.4 HSP27 Targeting in Lung Cancer Cells

Tumor necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL) has recently emerged as a cancer therapeutic agent however most tumor cells, including A549, are resistant to TRAIL treatment. Zhuang et al. investigate the effect of HSP27 abrogation using specific siRNA on drug sensitization of A549 cells to TRAIL treatment. Results showed that combination of HSP27 siRNA with TRAIL induced caspases activation and apoptosis in TRAIL resistant A549 cells. Combined treatment with HSP27 siRNA and TRAIL also increased JNK and p53 expression and activity [55]. Collectively, these findings indicate that targeting of HSP27 can sensitized the cells to TRAIL-induced apoptosis.

1.3.5 Targeting HSP27 in Cervical Cancer

A novel mechanism underlying the TRAIL sensitizing activity of the small molecule LY303511, an inactive analog of the phosphoinositide 3-kinase inhibitor LY294002, was reported in HeLa cells that are refractory to TRAIL-induced apoptosis. On the basis of the fact that LY303511 was derived from LY294002, itself derived from quercetin, and earlier findings indicating that quercetin and LY294002 affected HSP27 expression, authors investigated whether LY303511 sensitized cancer cells to TRAIL via a conserved inhibitory effect on HSP27. Data showed that upon treatment with LY303511, HSP27 was progressively sequestered in the nucleus,

thus reducing its protective effect in the cytosol during apoptosis. Remarkably, LY303511-induced nuclear translocation of HSP27 was linked to its sustained phosphorylation via activation of p38 kinase and MAPKAP kinase 2 and the inhibition of PP2A. Furthermore, genetic manipulation of HSP27 expression affected the TRAIL sensitizing activity of LY303511, which further corroborated the HSP27 targeting activity of LY303511. These data suggest a novel mechanism of small molecule sensitization to TRAIL through targeting of HSP27 functions, which could have therapeutic implications for overcoming chemotherapy resistancein HeLa tumor cells [56].

1.4 Conclusion

HSP27 expression is enhanced in many tumor cells, and it is involved in tumor progression and the development of treatment resistance in various tumors. Therefore, it is tempting to conclude that using conventional therapy for cancer treatment together with an inhibitor of HSP27 would have additive or synergistic anti-cancer effects. The phytochemicals derived from dietary compound represents potent novel inhibitors of the expression or activation of HSP27, which have prominent roles by affecting the hallmarks of cancer (Fig. 1.1). Moreover, phytochemical intervention

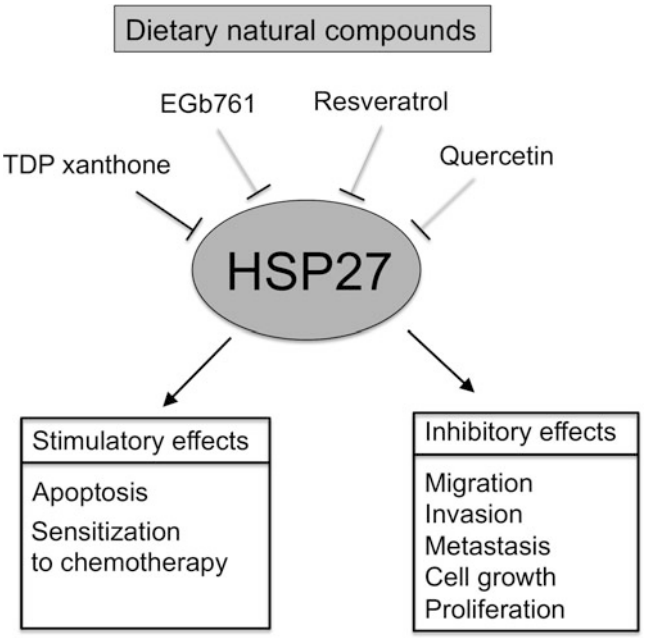

Fig. 1.1 Dietary compounds targeting HSP27 expression. HSP27 inhibition could induce stimulatory and inhibitory effects on the hallmarks of cancer

in combination with conventional chemotherapy induced strong drug sensitivity, mainly allowing apoptosis, at less in part, through to HSP27 inhibition. Therefore phytochemicals in combination with standard chemotherapy could represent a novel strategy for cancer treatment in tumors with overexpression of HSP27 chaperone.

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Chapter 2
Heat Shock Protein 27 (HSP27, HSPB1) Is Up-Regulated by Targeted Agents and Confers Resistance to Both Targeted Drugs and Chemotherapeutics

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Abstract The Heat Shock Protein of 27 kDa (HSP27) is a molecular chaperone with anti-apoptotic properties and a role in cytoskeleton stability. Not surprisingly, HSP27 is often increased in cancers and associated with poor patients' survival and resistance to conventional chemotherapy. Conversely, the role of HSP27 in response to therapies targeted towards oncogenic kinases was poorly characterized. In this chapter we review the findings on the role of HSP27 in resistance to both chemotherapeutics and targeted drugs and in the pro-metastatic phenotype, focusing on cancers where the tyrosine kinase receptor encoded by the MET oncogene is

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activated, namely, gastric and ovarian cancers. The inhibition of the MET receptor kinase in the MET addicted gastric cancer cells triggered HSP27 increase in a MEK/ERK dependent manner and in turn limited the effectiveness of inhibitors both in vitro and in vivo. Furthermore, in ovarian cancer cells HSP27 is required for metastasis upon MET activation in vivo and modulates the sensitivity to the first line chemotherapeutics Cisplatin and Paclitaxel. Altogether, these findings suggested that HSP27 induction by targeted agents might impact the success of targeted and conventional therapies and that it might be a suitable therapeutic target in combination treatments.

Keywords HSP27 • Oncogene addiction • Ovarian cancer • Gastric cancer • Targeted therapies • Chemotherapeutics

Abbreviations

CDDP	Cisplatin
HGF/SF	Hepatocyte growth factor/scatter factor
HSP27	Heat shock protein of 27 kDa
PTX	Paclitaxel
RTK	Receptor tyrosine kinase
sHSP	Small heat shock protein

2.1 Introduction

Heat shock proteins (HSPs), because of their multifaceted properties have crucial roles in tumor onset and progression. Therefore it is not surprising that they are often over-expressed in cancer cells, which can sometimes also become dependent for their transformation and survival on one or more HSP or on HSF1, the master transcription factor that regulates HSP expression ("non-oncogene addiction") [1].

The small heat shock protein of 27 kDa (HSP27) belongs to the family of the small HSPs and is markedly increased in many cancers, namely carcinomas of the breast [2], ovary [3], prostate [4] and others [5]. Its expression has been often associated to the malignant properties of these cells, e.g. increased tumorigenicity, metastatic behavior, apoptosis inhibition and chemotherapy resistance [6].

HSP27 is able to interfere with the cellular apoptotic machinery at multiple levels: HSP27 can prevent the assembly of the apoptosome by binding and consequently sequestering cytosolic cytochrome c once it is released from mitochondria upon stress; it can impair the cleavage, i.e. activation, of the procaspase-3, most likely through its ability to prevent caspase-9 from gaining access to the residues whose cleavage is necessary for procaspase-3 activation [7]. Furthermore, during stress, HSP27 protects the activation of Akt most likely via a PI3K-dependent

mechanism [8, 9]. Besides its role in stressed cells, HSP27 is also a regulator of cytoskeletal structural stability [10, 11] and thus might help promoting cell motility and invasiveness and thereby cancer metastasis.

Moreover, HSP27 over-expression is a well-known mechanism of cancer resistance to chemotherapeutics [6]. Conversely, the role of HSP27 in response to targeted-therapies was poorly characterized. As new drugs for cancer therapy are continuously emerging based on molecular targeting and are frequently associated with chemotherapeutics in combination treatments, it is important to characterize HSP27 role in the response of cancer cells to targeted drugs.

2.2 HSP27 Is Upregulated by Inhibitors of Oncogenic Tyrosine Kinases (TKs) and Confers Resistance to TK Targeted Therapies

The oncogenic tyrosine kinases (TKs) are among the best candidates for the development of targeted drugs, as they are either mutated or amplified or aberrantly expressed in a variety of cancers and they can sustain cancer onset and progression. Importantly, in some tumors, cancer cells become addicted to a particular driver TK oncogene, whose activity is required for cell survival and proliferation ("oncogene addiction") [12]. Among the TKs identified as suitable targets for therapy, the MET oncogene encoded tyrosine kinase receptor for the Hepatocyte Growth Factor/Scatter Factor (HGF/SF) is aberrantly expressed in cancer and promotes cell proliferation, dissociation, motility, invasion of the surrounding matrix and protection from apoptosis [13].

In a number of gastric carcinoma cells, such as GTL-16 cells, the gene encoding MET is amplified and thus the receptor is overexpressed and constitutively active. As a result, these cells are addicted to MET activity [14]. It has been shown that the GTL-16 cells become quiescent following treatment with MET inhibitors, such as the highly selective JNJ-38877605 (Fig. 2.1a), as a consequence of MET inactivation [14, 15]. However, the cells were not committed to death upon MET inhibition (Fig. 2.1a) and they started re-growing following drug washout.

Importantly, the treatment of these and other MET addicted cells with MET small molecule inhibitors resulted in the induction of HSP27 expression [15 and Fig. 2.1b]. Blunting of HSP27 increase with RNA interference turned the anti-proliferative effect of MET inhibition towards cell death in vitro and in vivo, as shown by the reduced percentage of alive cells, and by the induction of PARP cleavage following drug treatment [15 and Fig. 2.1 c, d]. This implies that the combination of HSP27 silencing with MET inhibitors could control more steadily the growth of MET addicted cells.

The study of the downstream signals affected by MET blockade [15] allowed the dissection of the molecular pathway leading to HSP27 induction upon MET inhibition. Both AKT and ERK-dependent signaling pathways were dampened by

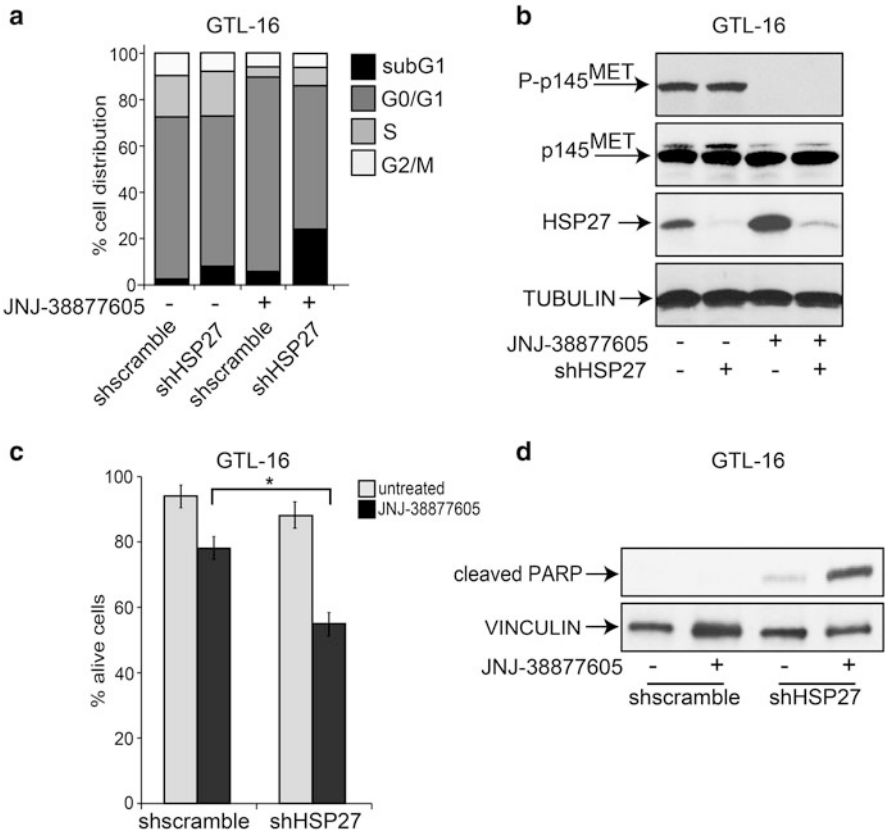

Fig. 2.1 HSP27 is up-regulated by inhibition of the MET tyrosine kinase and protects cancer cells from apoptosis. (**a**) Cell cycle analysis of the MET addicted GTL-16 cells transduced to express either control (shCTRL) or HSP27 specific (shHSP27) short hairpin RNAs and, when indicated, treated with the MET TK inhibitor JNJ-38877605 (0.1 μM) for 24 h; percentages of cells in each phase of the cell cycle are shown and demonstrate that while the MET inhibitor blocks cells in the G1 phase, HSP27 suppression increases the percentage of apoptotic cells accumulated in subG1; (**b**) Western blot analysis showing that MET inhibition in the same cells induces increased HSP27 expression, that is suppressed by the HSP27 specific shRNA; (**c**) percentage of alive (DAPI and AnnexinV double negative) GTL16 cells expressing either the control or the HSP27 specific shRNA as above, after cell treatment with the MET inhibitor; these data and the increased of PARP cleavage (**d**) confirm that the combination of HSP27 suppression and MET inhibition is necessary to drive the death of MET addicted cells

MET inhibition in MET addicted cells. Intriguingly, the inhibition of MEK/ERK axis was sufficient to recapitulate the HSP27 increase triggered by MET inhibitors, whereas the inhibition of AKT pathway did not affect HSP27 levels per se. In agreement, the ectopic expression of a constitutively active Ras (K-Ras G12V) in the MET-addicted cells rescued the inactivation of the MEK/ERK pathway due to MET inhibition, and consequently impaired the HSP27 increase. These data suggested

that HSP27 induction following inhibition of the MEK/ERK pathway might be more widespread than expected and thus might interfere with different RTK targeted therapies. To test this hypothesis, the EGFR-addicted cell lines A431 and SW48 were studied. EGFR inhibition blunted ERK phosphorylation and consequently resulted in the increase of HSP27 expression. More importantly, HSP27 silencing sensitized both the A431 and SW48 cells to EGFR inhibition. Altogether these data showed that inhibition of MET and EGFR in the susceptible cell lines results in the increased expression of HSP27 that in turn might reduce the efficacy of the TK-targeted therapies.

2.3 HSP27 Is Required for Invasion and Metastasis Triggered by the MET Oncogene Encoded TK Receptor

The knowledge that HSP27 might promote cell motility and invasiveness by regulating cytoskeletal stability, as mentioned above, together with the frequent report of increased expression of HSP27 in human cancers at advanced stage, suggested that HSP27 might play a role in the MET driven invasion and metastasis of cancer cells. The MET receptor, indeed, upon binding to its ligand HGF, activates downstream signaling leading to cancer cell invasion and metastasis [16].

The contribution of HSP27 to the MET triggered invasive phenotype was studied in ovarian cancer cells where HGF induced the phosphorylation of HSP27 by activating the p38MAPK. HSP27 was necessary for the remodeling of actin filaments induced by HGF and motility in vitro depended on the p38MAPK-MK2 axis. In vivo, HSP27 silencing impaired the ability of the highly metastatic, HGF-secreting ovarian cancer cells to give rise to spontaneous metastases (Fig. 2.2). This was due to defective motility across the vessel wall and reduced growth. Indeed, HSP27 silencing impaired the ability of circulating ovarian cancer cells to home to the lungs and to form experimental haematogenous metastases and the capability of cancer cells to grow as subcutaneous xenografts [17]. Altogether, these data showed that HSP27 is required for the pro-invasive and pro-metastatic activity of HGF and suggest that HSP27 might be not only a marker of progression of ovarian cancer, but also a suitable target for therapy.

2.4 HSP27 Interferes with the Effectiveness of Chemotherapeutics

As mentioned above, it was known that HSP27 over-expression might cause resistance to chemotherapeutics. The role of HSP27 in the response to platinum compounds and taxanes was investigated in ovarian cancer where these drugs represent the first-line treatment. The SK-OV-3 and TOV-21-G ovarian cancer cell

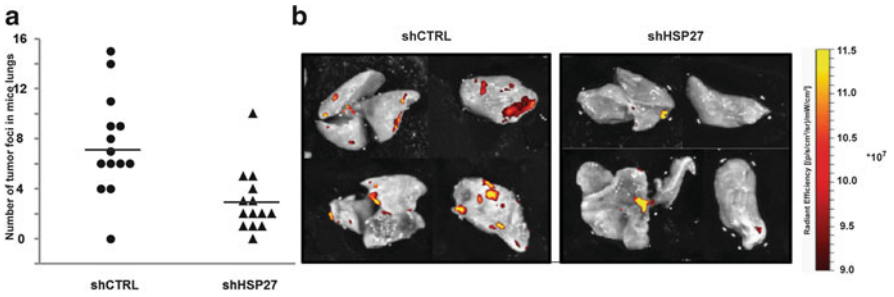

Fig. 2.2 HSP27 silencing impairs the ability of HGF-secreting ovarian cancer cells to give rise to spontaneous metastases. SK-OV-3 cells were engineered to secrete HGF and to express either control (shCTRL) or HSP27 specific (shHSP27) short hairpin RNAs driven by lentiviral vectors carrying also the GFP transgene. Engineered cells were injected subcutaneously in immune-compromised mice and gave rise to lung spontaneous metastases. (**a**) Quantification of lung metastatic foci, acquired by IVIS Lumina II imaging system. The average of metastatic colonies in mice lungs treated with cells silenced for HSP27 were 2.9 ± 0.7 (s.e.m.), while the average of metastatic colonies in control mice lungs were 7.5 ± 1.0. The two populations were significant different by Student's *t*-test $p < 0.05$; (**b**) Representative ex vivo fluorescent images of the lungs (left and right lungs are separated) of mice with metastatic foci. Fluorescent intensity is expressed as radiance efficiency (p/s/cm^2/sr/mW/cm^2)

lines were studied, as they are representative models of serous and clear cell ovarian carcinomas, respectively. Both cisplatin (CDDP) and Paclitaxel (PTX) exert their cytotoxic effect on dividing cells, causing DNA damage detected during DNA replication and preventing the formation of normal mitotic spindle, respectively. Indeed, cell proliferation rate might affect the responsiveness of cancer cells to these drugs. Notably, HSP27 knockdown did not affect the in vitro growth of either SK-OV-3 or TOV-21-G cell lines.

The loss of HSP27 sensitized the SK-OV-3 ovarian cancer cells to PTX triggered apoptosis in vitro (Fig. 2.3a). In vivo, it was shown that HSP27 suppression resulted in the sensitization of xenografts to low doses of PTX [17]. This was expected also because HSP27 protects microtubules from bundling caused by the drug [17].

Furthermore, HSP27 knockdown sensitized TOV-21G ovarian cancer cells to CDDP induced apoptosis (Fig. 2.3b). Interestingly, the phosphorylation levels of AKT were reduced following CDDP exposure only in HSP27 depleted cells, suggesting a role for this small heat shock protein in protecting ovarian cancer cells from death through the stabilization of AKT activity (Fig. 2.3c). Accordingly, a PI3K/AKT pathway inhibitor alone was able to sensitize ovarian cancer cells to CDDP-induced apoptosis (Fig. 2.3d). As HSP27 is over-expressed in ovarian cancer cells and the PI3K/AKT pathway is frequently correlated with chemotherapeutics resistance, these findings highlighted the possibility to target HSP27 to circumvent chemotherapeutics resistance in ovarian cancer.

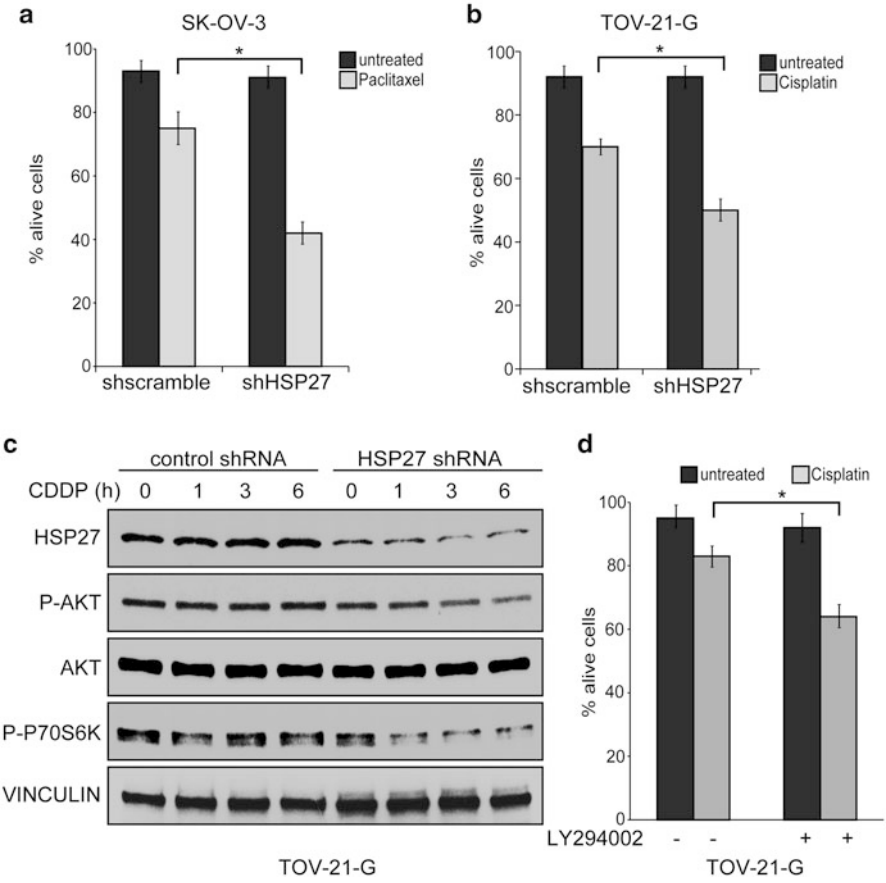

Fig. 2.3 Sensitization of ovarian cancer cells to chemotherapeutics by HSP27 suppression. (**a**) Percentage of alive (DAPI and AnnexinV double negative) SK-OV-3 cells expressing either the control or the HSP27 specific shRNA as in the Legend to Fig. 2.1, after cell treatment with the chemotherapeutic Paclitaxel; (**b**) percentage of alive (DAPI negative and AnnexinV) TOV-21-G cells expressing either the control or the HSP27 specific shRNA as above, after cell treatment with the chemotherapeutic cisplatin; (**c**) Western blot analysis of the phosphorylation of AKT and of its substrate p70S6 kinase in TOV-21-G cells upon treatment with cisplatin (CDDP) of control or HSP27 silenced cells; (**d**) percentage of alive (DAPI negative and AnnexinV) TOV-21-G treated with the PI3K inhibitor, that inhibits the PI3K-AKT pathway, upon treatment with cisplatin

2.5 Conclusions and Perspectives

The finding of HSP27 increased expression in cells treated with targeted agents and the increased efficacy of these agents after HSP27 silencing indicated that this increase might limit the effectiveness of targeted therapies. As targeted agents and conventional chemotherapeutics are often simultaneously administered in

combination therapies, altogether the findings summarized above suggest that targeting HSP27 could be a therapeutic strategy as an adjunct to either targeted therapies or conventional chemotherapies or both. Antisense [18, 19] and peptide aptamer [20] strategies have shown that targeting HSP27 increases cancer cell death in vitro and in vivo in preclinical models. More importantly, an antisense drug inhibiting HSP27 is available for human therapy and is currently tested in Phase II clinical trials (http://clinicaltrials.gov/ct2/results?term=OGX427&Search=Search). Therefore HSP27 knockdown in combination with other agents can be envisaged as a viable and effective therapeutic approach for clinical applications.

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Chapter 3
Heat Shock Proteins and Cancer: Plant Based Therapy

Evren Önay-Uçar

Abstract Cancer is one of the major causes of mortality in the world. Each year approximately 13 million people suffer from cancer disease, and approximately 60 % of them die because of cancer. Besides most of the patients response harmful side effects of chemo- and radiotherapies. Therefore the establishment of new therapeutic strategies for the treatment of cancers will be required. A number of studies have shown that some HSP are induced in specific tumor cells. For example, increased levels of HSP105, HSP90, HSP70, HSP60, HSP27 have been detected in colon cancer, lung cancer, hepatocellular carcinoma, colorectal cancer, and gliomas, respectively. Elevated HSP levels in tumor cells are suggested to be responsible for increased chemotherapy resistance and poor prognosis. Suppression of HSP expressions in cancer cells is a new strategy for the treatment. It is well known that some plant extracts and their flavonoids significantly decrease HSP expression, and induce apoptosis of cancer cells. In addition, using of the HSP inhibitors in association with classical chemotherapy increases the sensitivity of cancer cells to the cytotoxic drugs. Therefore, some plants and their biologically active natural compounds have been investigated for their possible contribution to cancer therapy. The current chapter reviews the role of HSP in different cancer types and suppressing HSP with some natural products.

Keywords Cancer • Plant • Heat shock proteins

Abbreviations

17-AAG	Tanespmycin
17-DMAG	Retaspmycin
CDDP	Cisplatin
CRC	Colorectal carcinoma

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Dox	Doxorubicin
EGCG	Epigallocatechin-3-gallate
ERK	Extracellular signal-regulated kinase
GA	Geldanamycin
Grp	Glucose-regulated protein
HBV-related HCC	Hepatit B virus-related hepatocellular carcinoma
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein
JNK/SAPK	Jun-amino-terminal kinase/stress-activated protein kinase
PA	Peptide aptamer
PDTC	Pyrrolidine dithiocarbamate
PEITC	Phenethyl isothiocyanate
PES	2-phenylethynesulfonamide
Phen	1,10-phenanthroline
PTMs	Posttranslational modifications
RCC	Renal cell carcinoma
RP101	Brivudine
siRNA	Small interfering RNA
TF	Theaflavins
TR	Thearubigins
ZER	Zerumbone

3.1 Introduction

One of the major causes of mortality in the world is cancer. According to known statistical and epidemiological data, in 2008 approximately 7.6 million people have died from cancer in all over the world and it is estimated that by 2030 there will be 22 million new cancer cases every year (http://www.cancerresearchuk.org/cancer-info/cancerstats/world/; [1]). Although the most common cancer treatments such as surgery, radiotherapy, and chemotherapy are used for cancer therapy, these treatments are not enough to cure all cancer types. When it is considered that the cancer cases will increase in the future, the establishment of new therapeutic strategies for the management of the cancers will be essential.

Heat shock proteins (HSP, also called stress proteins) are one of the largest components of the cytoplasmic network. The evolutionary conserved HSP are classified into different families by their molecular weight: HSP100, HSP90, HSP70, HSP60, HSP40, and small HSP [2, 3]. These proteins are responsible for maintaining protein homeostasis in the cell. They generate dynamic complexes by forming non-covalent bonds with each other, other proteins and all of the cytoplasmic network components, and play an important role in preventing damage occurring in various proteins [4]. Heat shock proteins can be overexpressed in all organisms to protect themselves from environmental stresses such as heat, oxidative stress, and ischemia. They mediate the refolding or degradation of stress-damaged

proteins, thus protect the cells from potential deleterious effects and promote the cell recovery [5]. Besides, HSP have a lot of functions in the cell, they play some roles in cell division, apoptosis inhibition, and metastasis of cancer cells [6, 7]. They also play the regulatory roles in cell viability and death. Some stress-induced HSP, especially HSP27 and HSP70, protect the cells against apoptosis and necrosis [8, 9].

HSP also play important roles in the development of various diseases, especially in cancer and neurodegenerative diseases. A lot of studies indicated that the expressions of some HSP are elevated in many cancer types. Therefore some researchers have reported that these proteins are used as a biomarker in some cancer types [10–15]. It is revealed that HSP105, HSP90, HSP70, HSP60 and HSP27 are increased in colon cancer, lung cancer, hepatocellular carcinoma, colorectal carcinoma, and gastric cancer, respectively [11–13, 16, 17]. It is also known that the elevated HSP in tumor cells are suggested to be responsible for increased therapy resistance and poor prognosis [16, 18, 19]. A lot of studies have indicated association between HSP and chemotherapeutic drug resistance in cancer cells [10, 20–24].

As a result, a new strategy for the cancer treatment has been put forward to reduce the HSP expressions, and to decrease the resistance of chemotherapy and radiotherapy in cancer cells. Many studies have been performed to prove the accuracy of this new strategy and "a new HSP target". Recent studies showed that HSP inhibition by using antisense oligonucleotides or inhibitors has revealed successful results in clinical trials related to cancer treatment [25–30]. Besides, several plant extracts and some natural compounds have been used to suppress HSP expression in cancer cells for a long time [31–37]. In this chapter, HSP suppression using plant extracts and natural compounds will be discussed in detailed.

3.2 The Role of HSP in Cancer

Recent studies indicated that different HSP have been altered in different cancer types. The studies related to association between overexpressed HSP and cancer behaviour are still going on. The list of elevated HSP levels in different cancer types is shown in Table 3.1. Kai and his coworkers showed that HSP105 was increased in colorectal cancer and pancreatic adenocarcinoma patients [11]. Similar study on pancreatic adenocarcinoma patients revealed that HSP105 was remarkably increased in carcinogenic tissue versus normal tissue [38]. In a clinical study of prostate cancer patients, it has been found that HSP70 expression is increased in cancer patients in comparison to normal individuals [47]. HSP27, which is found abundantly in human serum, is suggested as a potential diagnostic marker in breast cancer [14]. Overexpressed HSP27 has been found in metastatic hepatocarcinoma tissues when compared to non-metastatic tissue [66]. Similar results were also obtained in gastric cancer [57]. HSP27 was found to be upregulated in colorectal carcinoma (CRC) versus normal cells [54]. A number of reports have been revealed that HSP27 has been upregulated in primary nervous system tumors, human astrocytoma, glioma and brain tumour [60, 62–64].

Table 3.1 Elevated heat shock proteins (HSP) in cancer

HSP	Cancer type	Findings	References
HSP105	Colorectal cancer and other several cancer types	HSP105 is overexpressed	Kai et al. [11]
	Pancreatic ductal and colon adenocarcinoma	HSP105 is overexpressed	Nakatsura et al. [38]
HSP90	Bladder carcinoma	HSP27, HSP60, HSP70 and HSP90 are overexpressed	Ischia and So [15]
	Breast cancer	HSP90 is overexpressed	Yano et al. [39], Pick et al. [40]
	Hepatocellular carcinoma	HSP27, HSP70, HSP90, Grp78, and Grp94 are increased in HBV-related HCC	Lim et al. [41]
	Leukemia	Upregulated HSP90 reflected aggressiveness of the cancer	Zackova et al. [42]
	Lung cancer	HSP90 is overexpressed	Didelot et al. [16]
HSP70	Breast cancer	HSP70 is upregulated	Ciocca et al. [43]
	Cervical squamous cell carcinoma	HSP70 can be use as diagnostic marker	Garg et al. [44]
	Hepatocellular carcinoma	Increased HSP70 is sensitive marker in early HCC	Chuma et al. [45], Takashima et al. [17]
	Intrahepatic cholangiocarcinoma	HSP70 is overexpressed	Lagana et al. [46]
	Prostate cancer	HSP70 is overexpressed	Abe et al. [47]
HSP60	Colorectal cancer	HSP60 is overexpressed	Cappello et al. [12]
	Lung cancer	HSP60 is overexpressed	Rubporn et al. [48]
	Prostate cancer	HSP60 and HSP10 are overexpressed	Cornford et al. [49], Cappello et al. [50]
HSP27	Breast cancer	HSP27 is upregulated	Conroy et al. [51], So et al. [14], Zhu et al. [52]
	Colorectal cancer	HSP27 is overexpressed	Yu et al. [53], Pei et al. [54]
	Endometrial cancer	HSP27 is upregulated	Geisler et al. [55]
	Gastric cancer	HSP27 is overexpressed	Kapranos et al. [56], Ryu et al. [13], Chen et al. [57]

	Gliomas and other brain tumours	HSP27 is overexpressed	Hitotsumatsu et al. [58], Hermisson et al. [59], Zhang et al. [60], Graner and Bigner [61], Cao et al. [62, 63], Liang et al. [64]
	Head and neck squamous cell carcinoma	HSP27 is overexpressed in metastatic lymph nodes	Zhu et al. [52]
	Hepatocellular carcinoma	HSP27, HSP70 and Grp78 are increased	Luk et al. [65], Song et al. [66], Chen et al. [67]
	Intrahepatic cholangiocarcinoma	HSP27 is related to aggressive tumor behaviour	Romani et al. [68]
	Acute myeloid and lymphoblastic leukemia	HSP27 is overexpressed	Yang et al. [69]
	Ovarian cancer	HSP27 level is increased	Arts et al. [70], Ciocca and Calderwood [23]
	Prostate cancer	HSP27 is overexpressed	Cornford et al. [49], Garrido et al. [71], Zhang et al. [72], Parcellier et al. [73], So et al. [14], Andrieu et al. [24])
	Renal cell carcinoma	HSP27 expression is significantly increased	Erkizan et al. [74]

Several studies in different tumour models exhibited that there is an association between HSP expression and multidrug resistance [75] and increased tumorogenesis-related apoptosis [76]. Studies have revealed that the upregulated HSP cause to increased resistant against anticancer drugs, such as Cisplatin (CDDP), Doxorubicin (Dox), Vincristine, Paclitaxel etc. in cancer cells [10, 77–82]. Besides, some studies have demonstrated that overexpression of HSP eliminates the lethal effect of gamma radiation in cancer cells [21, 82, 83].

Especially, HSP27 and HSP70 are found abundantly in malignant cancer cells, and they cause chemotherapy resistance [84]. In human ovarian tumor cell line, it has been shown that HSP27 and HSP70 are linked with the resistance to Cisplatin (CDDP). CDDP is a compound containing platinum and a potential anti-cancer agent. It has been widely used in the treatment of several malignant tumors such as testicular, head and neck, esophageal, lung, ovaries, and bladder cancers etc. The long-lasting CDDP treatment is limited, because of the risk of developing the resistant cells. Then these resistant cells become malignant. According to the western blot analysis increased expression levels of HSP27 and HSP70 are critical for the resistance mechanism of CDDP [22]. Upregulated HSP70 and HSP90 are also enhanced drug sensitivity in ovarian cancer [85].

In a study with 300 breast cancer patients, an association between tumour aggressiveness and HSP27 localization has been found [86]. The increased levels of HSP27 and HSP70 in breast cancer indicate that the cancer cells' resistance against to chemotherapy especially to Dox and apoptosis is increased [10, 23, 26, 78, 87, 88]. A recent report has indicated a relationship between HSP27 and Herceptin sensitivity in breast cancer cells. Overexpressed HSP27 has reduced Herceptin susceptibility in these cells [81]. Upregulated HSP70 is also essential for survival of tumorogenic breast cancer cells, and the decreasing of HSP70 activates tumor-specific cell death program (apoptosis) [20]. Especially, overexpressed HSP70 has been revealed to connect with weak prognosis and treatment resistance of breast cancer, servical cancer and hepatocellular carcinoma cells [6, 17, 45]. It has been shown that the overexpression of this protein in breast cancer is an indicator of failed treatment [43].

Additionally, it is known that HSP27 and HSP72 expressions are upregulated in prostate cancer [49, 89]. Recent immunohistochemical studies have shown that there is a correlation between HSP27 expression and prostate cancer aggressiveness, progression, and the development of the phenotype that does not respond to the hormone therapy [14]. HSP27 expression is induced to respond to the hormone or chemotherapy, thus it suppresses therapy with induced-apoptosis [79, 90]. The overexpressions of HSP27 and HSP70 in human prostate cancer have been shown to provide resistance to apoptosis and chemotherapy [24, 91]. Garrido and Parcellier have also found that increased HSP27 levels protect prostate cancer cells by increasing tumor proliferation and decreasing apoptosis, thereby facilitating tumor progression [71, 73]. Similar results were obtained for HSP72 in prostate cancer. Prolonged downregulation of HSP72 in PC-3 cells enhanced the sensitivity of cells to radiotherapy, and chemotherapy agents such as CDDP, vinblastin and taxol [89].

HSP27 has also been determined in several brain tumors, and there is a correlation between its expression and the degree of tumour malignancy [58, 92]. In neuronal cells in vivo overexpression of HSP27 exhibits neuroprotective properties by HSP27-mediated inhibition of apoptosis [93]. Induced HSP27 is also important for ranking histologically, which is related with weak prognosis in hepatocellular carcinoma [6]. The overexpressed HSP27 inhibits etoposide-induced apoptosis in human leukemic cells [94].

Besides, some studies have proved that overexpressed HSP70 is prior condition for the survival of various cancer types and suppressed HSP70 in tumour cells has been caused to cell death [20]. It is known that some inducible HSP (especially HSP27 and HSP70) protect the cells against apoptosis [84] and necrosis [19, 79, 95–99]. Recent studies have indicated that these proteins inhibit apoptosis via preventing caspases in different stages [100, 101]. The antiapoptotic effects of HSP27 and HSP70 have clarified by associating cytochrome C release from mitochondria, formation of apoptosome, and caspase [76, 101–103].

Considering all these studies, decreasing the HSP level in cancer cells would be beneficial for the treatment of cancers and the development of new therapeutic approaches targeted to HSP.

3.3 Why Are HSP Induced?

There are a variety of physiological, pathological and environmental factors such as growth factors, cell differentiation, tissue development, viral, bacterial, parasitic infections, ischemia, heat shock, heavy metals, ethanol, antibiotics etc. that induce HSP expression in the cells [104].

The transcription of *hsp* genes is provided through the interaction of heat shock transcription factor (HSF) and heat shock element (HSE). It has been determined four different HSFs up to now: HSF1, HSF2, HSF3 and HSF4 [3]. All HSFs are induced during development and adaptation of the cells, but only HSF1 regulates the HSP synthesis. HSF1 is activated when the cell exposed to stress, and regulates the expression of *hsp* genes [3, 103, 105].

The mechanism of *hsp* gene activation is still remaining not fully understood, but it is well known that the HSE and HSF1 carry impontant role in this mechanism. Although under normal circumstances HSFs are found in the cytoplasm as inactive monomers, which are linked to HSP70 and HSP90, they can only bind to DNA under stress conditions [106]. Under stress conditions HSF is subjected to a number of posttranslational modifications (PTMs), and converted to three phosphated form (homotrimer), and then transferred from cytoplasma to nucleus for binding to the DNA. After binding of HSF to promoter regions of *hsp* genes called as HSE, the gene transcription is get started in nucleus [103, 107, 108].

Several factors can lead to HSF activation. The most important clue for HSF activation is connection balance between the HSP molecules (like HSP70) and HSF

and stress caused- unfolded proteins. Any increase in the unfolded proteins entity, this balance changes the direction of the unfolded protein-HSP balance, so the HSF monomers are released from this complex [3].

HSF1 is a constitutively expressed protein, which is located in the cell nucleus and cytosol. Its molecular weight is 75 kDa, and which is found in a complex of approximately 200 kDa as inactive monomers [109]. Both DNA-binding and transcription activities of HSF1 monomers are suppressed with negative control, these monomers bind normally to HSP70 and HSP90 in the cell [105]. In physiological stress conditions such as high temperature and ischemia, HSF1 is separated from HSP via activation of kinases. This HSF1 monomers are hyperphosphorilated by ERK1, JNK/SAPK and p38 protein kinase [110] and they form a homotrimer approximately 700 kDa [105]. This active trimer is transferred from cytosol to the nucleus and binds to *hsp* genes [104]. The HSF1-DNA binding is closely associated with HSE, which is found in upstream promoter region of *hsp* genes. When the homotrimer is phosphorilated again by kinases, the *hsp* gene transcription is triggered in the nucleus. Following the transcription, mRNA of HSP is moved to cytosol and the synthesis is completed there. At the end of these events, HSF1 is retransferred to the cytosol, and created a complex with newly synthesised HSP for interrupting of HSP synthesis (Fig. 3.1).

In disease conditions, it is thought that the induction of HSF1 reduces protein damages by increasing *hsp* gene expression. Cell culture studies have revealed that the treatments of hypoxia, ethanol and sodium arsenite increase HSF1-DNA binding

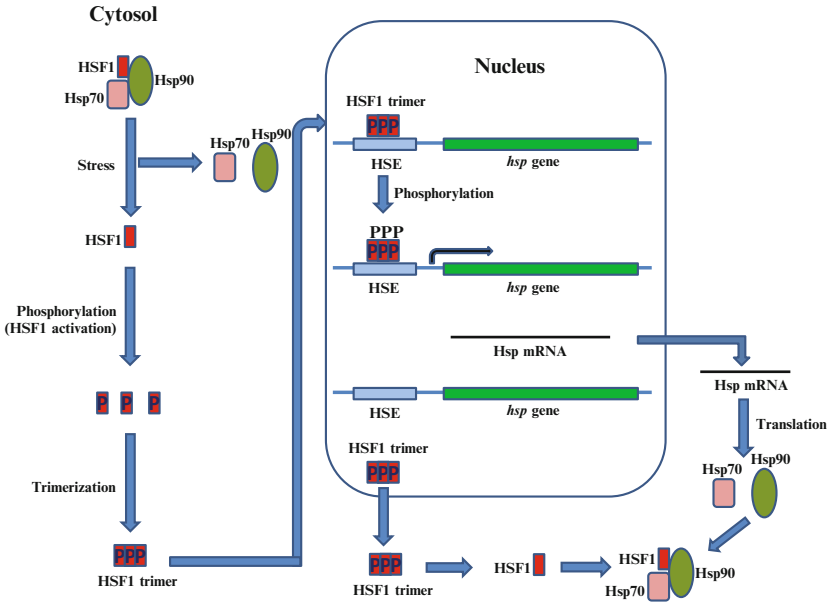

Fig. 3.1 The regulation mechanism of stress-induced *hsp* gene expression

and HSP70 level within the cell. High temperature weakens the association between HSF1 and HSP90, since the stress caused-denaturated proteins compete with HSFs to bind HSP90 [111]. The increasing of inducible HSP in cell suppresses the HSF1 activation via a regulatory mechanism [112].

The studies showed that overexpressed *hsp27* gene provides temporary resistance against lethal heat shock and increases the stability of actin filaments in the cell [113–115]. HSP27 also immobilizes the mitochondria [9]. Overexpressed HSP70 in pulmonary endothelial cells induced endotoxins and besides reduced apoptosis [116]. This suppression is obtained with interrupting procaspase-3 transformation to active caspase-3 [117]. Proapoptotic signals, such as Fas, decrease the HSF1-DNA connection in heat shock conditions, because HSF1 is not hyperphosphorylated [3]. It has been shown that HSP inhibit apoptosis via prohibiting to the different phases caspases [9, 100]. HSP27 plays a role in cellular redox state [96] and in preventing cellular damage as a result of declining of ATP [95]. The antiapoptotic effects of HSP27 and HSP70 are associated with the release of cytochrome C from mitochondria, caspase activation, and the formation of apoptosome [100, 101].

3.4 HSP Inhibition and Clinical Trials

Nowadays, many researchers focus on HSP inhibition as one of the important pharmacological approaches in cancer therapy. For that purpose, different methods such as antisense oligonucleotide, some natural agents, siRNA (small interfering RNA) applications etc. are used to suppress HSP in the cancer therapy [30]. Basically, there are three different strategies for suppressing of HSP: (1) Direct inhibitors, (2) Peptide aptamers binding to HSP, (3) Antisense oligonucleotides [30].

Because of the relationship between upregulated HSP and the treatment effectiveness, the HSP suppression is among the strategic targets in cancer treatment. The HSP inhibitors, which have been proposed for the cancer treatments, have been used either alone or with chemotherapic agents. According to the US National Istitutes of Health, today in all over the world, 168 studies are carried out with the title of “HSP”, and 75 clinical trials of them are related with “HSP inhibitors” for the treatment of different cancer types (http://clinicaltrials.gov/). These active clinical trials are generally related with HSP90 suppression. The importance of HSP90 is associated with the number of successful studies [7, 25, 26, 29, 118].

It is well known that HSP90 is responsible for maintaining the correct folding and stability of over 100 client proteins in cancer survival [119]. The inhibition of HSP90 causes to cell death (apoptosis). The natural products geldanamycin (GA), and semi-synthetic derivatives tanespimycin (17-AAG) and retaspimycin (17-DMAG) are known as HSP90 inhibitors and they evaluated alone or in combination with other drugs for the treatment of breast cancer in Phase 1 and Phase 2 clinical trials [25, 26, 29]. The other natural products such as radicicol

analogues, cyclopropara dicicol and radicicol oximes are also used in preclinical trials to inhibit HSP90 [118, 119]. A recent study showed that the use of VER-155008 and 17-DMAG inhibitors to suppress HSP70 and HSP90 significantly increased antiproliferative and proapoptotic effects in acute myeloid leukemia [120].

The previously mentioned different strategies are also used to suppress the HSP27 expression in several clinical studies. These experiments revealed that quercetin and brivudine (also called RP101) directly inhibited HSP27 expression in several cancer cells [27, 79], the use of antisense oligonucleotides reduced HSP27 expression in cancer cells [121]. Besides, when peptide aptamers PA11 and PA50 are used for inhibiting of HSP27, chemo/radio-therapy efficacy is increased in HeLa cells [28].

Antisense oligonucleotides induce apoptosis of tumor cells by suppressing HSP [34]. When HSP27 expression is reduced about 40 % in HeLa cells by using antisense technology, these cells become more sensitive to apoptotic inducers [122]. Similarly, HSP27 supression decreases the potential of creating tumor from the prostate cancer cells, and increases the sensitivity of cells to anticancer drugs such as paclitaxel [79]. In a pancreatic cancer study, upregulated HSP27 caused to resistance of gemcitabine. When HSP27 expression was suppressed with siRNA, the cells became more sensitive to gemcitabine [123].

Recently, the silencing of HSP27 and HSP90 are one of the new targets to sensitize prostate cancer cells to chemotherapy and radiotherapy [118, 124]. Some experiments indicated that suppressed HSP70 by using quercetin, antisense oligonucleotide or siRNA increased apoptosis in prostate cancer cells [14, 34]. HSP27 antisense oligodeoxynucleotides and siRNA that target the human translation initiation site were reported to potentely inhibit HSP27 expression in human prostate PC-3 cells with increased caspase-3 cleavage, apoptosis and 87 % suppression of cell growth [79, 125]. Besides, targeting HSP27 by the second-generation antisense oligodeoxynucleotides (OGX-427) inhibited HSP27 expression and enhanced drug sensitivity in several xenograft models [79, 126].

Several studies emphasized that some synthetic antioxidants reduced increasing of HSP expression in the cells. Gorman and his colleagues have shown that certain antioxidant compounds (such as pyrrolidine dithiocarbamate (PDTC) and 1,10- phenanthroline (Phen)) prevented *hsp* gene induction and inhibited HSP27 and HSP70 in HL-60 cell line. The combination of antioxidant treatment led to cell death, which were exposed to heat stress [31]. There are also some evidences that several chemicals, such as benzylidene lactam, triptolide, emunine etc., inhibit *hsp* gene expressions by interacting with HSFs [108]. It is known that 2-phenylethynesulfonamide (PES, pifitrin-μ) is a specific inhibitor of stress-inducible HSP70, the usage of PES induces cell death in primary effusion lymphoma [127].

3.5 Plant-Based HSP Inhibitions

Nowadays in addition to synthetic drugs, a wide range of natural products are used in the treatment of cancer as supportive (supplemental) products. The use of medicinal plants in the treatment of cancer has a long history. They have been used since ancient times for the treatment of several diseases. Currently, more than 60 % of the anticancer agents is obtained from natural sources, such as plants, water organisms and microorganisms [128]. According to the clinical trials web page, at least 106 studies related to plant-based cancer therapy are being conducted (http://clinicaltrials.gov/).

In the literature, a lot of studies have been found related to HSP inhibitions by using plant extracts or natural products in different cancer cells (see Table 3.2). For example, Morino and his coworkers have pointed out that some flavonoids reduced the expressions of HSP27, HSP40, HSP60 and HSP70 in different tumor cell lines [144]. Similarly, in 2002, Rusak and his coworkers have revealed that quercetin, kaempferol, taxifolin, and isorhamnetin flavonoids are significantly decreased HSP27 and HSP70 gene expressions in heat-stressed leukemia cells [132]. Proteomic-based results indicated that resveratrol caused suppression of HSP27 and thus induced apoptosis in breast cancer (MCF-7) cells. Besides, inhibition of HSP27 expression by specific siRNA transfection also enhanced the chemotherapeutic effects of Dox in this cell [37].

It is well known that several natural compounds inhibit HSP expression in cancer cells. Boesenbergin A is a natural compound isolated from *Boesenbergia rotunda* and has apoptotic effect on the cancer cells. Boesenbergin A treatment is caused significantly suppressing of HSP70 in human T4-lymphoblastoid cells [36]. Phenethyl isothiocyanate (PEITC), a natural compound found in some plants, significantly reduced HSP27, HSP70, HSP90 and HSF-1 expressions in MCF-7 and MDA-MB-231 breast cancer cell lines [134]. Triptolide from *Triptergium wilfordii* suppressed HSP70 expression via inhibiting heat shock response in HeLa cells [141]. Zerumbone (ZER), an antioxidant isolated from *Zingiber zerumbet Smith* inhibited HSP27 expression in lung adenocarcinoma cells and also increased radiosensitization of this cell [143]. The plant polyphenols also show similar effects on cancer cells. For example, epigallocatechin-3-gallate (EGCG), one of the major polyphenols in green tea, specifically suppressed the expressions of HSP90 and HSP70 in MCF-7 human breast cancer cells by inhibiting the promoter activity of HSP90 [130]. EGCG has also been shown to induce apoptosis in human urinary bladder carcinoma cell line (TSGH-8301) by suppressing of HSP27 [131]. In an other study high-dose green tea polyphenols caused to downregulation of HSP27 and HSP90 mRNAs in mouse kidney and liver [145]. Black tea polyphenols, theaflavins (TF) and thearubigins (TR), downregulated the HSP90 expression and induced apoptosis in human leukemic U937 and K562 cells [140]. Lycopene and its derivative apo-14′-lycopenoic acid inhibited HSP70 and HSP90 expression in acute monocytic leukemia cells [133]. Deguelin isolated from *Mundulea sericea* induced

Table 3.2 Downregulated HSP by use of natural compound/plant extract in cancer

Plant extract/natural compound	Cancer cell/type	Findings	References
Boesenbergin A	T4 Lymphoblastoid cells	HSP70 ↓	Ng et al. [36]
Cimicifuga foetida extract	Breast cancer cells (MCF-7 cell line)	HSP27 ↓	Soler et al. [129]
Deguelin	Head and neck squamous cell carcinoma	HSP90 ↓, apoptosis ↑, autophagy ↑	Yang et al. [69]
EGCG	Breast cancer cells (MCF-7 cell line)	HSP70 ↓, HSP90 ↓	Tran et al. [130]
	Urinary bladder carcinoma	HSP27↓, apoptosis ↑	Chen et al. [131]
Kaempferol	Leukemia cell line (HL-60)	HSP27 ↓, HSP70↓	Rusak et al. [132]
Lycopene	Acute monocytic leukemia cell	HSP70 ↓, HSP90 ↓	Catalano et al. [133]
PEITC	Breast cancer cell	HSP27↓, HSP70 ↓, HSP90 ↓, HSF-1 ↓	Sarkars et al. [134]
Quercetin	Breast cancer	Dox efficacy↑	Staedler et al. [135]
	HeLa cell line	HSP27 ↓, HSP70↓, CDDP-induced apoptosis ↑	Jakubowicz-Gil et al. [136, 137]
	Leukemia cell line (HL-60)	HSP27 ↓, HSP70↓	Rusak et al. [132]
	Lung cancer cell line (A549)	HSP27↓, CDDP and gemcitabine efficacy ↑	
	Neuroblastoma and Ewing's sarcoma	HSP27↓, Dox efficacy ↑	Zanini et al. [138]
	Prostate cancer	HSP70↓	Kagaya et al. [32], Asea et al. [33], Jones et al. [34]
Resveratrol	Breast cancer cells (MCF-7 cell line)	HSP27↓, apoptosis ↑, Dox efficacy↑	Diaz-Chavez et al. [37]
Taxifolin, isorhamnetin	Leukemia cell line (HL-60)	HSP70↓	Rusak et al. [132]
Taxol	Ovarian and uterine cancer cells	HSP27↓, etoposide, colcemid and vincristine efficacy↑	Tanaka et al. [139]
Theaflavin and thearubigin	Leukemia cell lines (U937 and K562)	HSP90 ↓, apoptosis ↑	Halder et al. [140]
Triptolide	HeLa cell line	HSP70↓	Westerheide et al. [141]
Viscum album extract	Glioma cell line	HSP27 ↓, 14-3-3 β ↓, ζ ↓, γ ↓, apoptosis ↑	Önay-Uçar et al. [35]
Withaferin A	Pancreatic cancer	HSP90 ↓	Yu et al. [142]
Zerumbone	Lung adenocarcinoma cells	HSP27↓, radiosensitization ↑	Choi et al. [143]

↑: upregulation, ↓: downregulation

apoptosis and autophagy in head and neck squamous cell carcinoma ans is proposed as a potential HSP90 inhibitor [69]. Taxol has been suggested to overcome drug resistance to etoposide, colcemid and vincristine in ovarian and uterine cancer cells in vitro by inhibiting HSP27 expression [139]. These examples can be multiplied.

There are strong evidences related to suppressed HSP expression by using some plant extracts [35, 129]. Various *Viscum album* (mistletoe) extracts are widely used as complemantary cancer therapies in Europe [146, 147]. We checked antioxidant activity of the methanolic extract of *Viscum album* [148]. Our further studies revealed that *Viscum album* methanolic extract decreased the expression level of HSP27 and some 14-3-3 isoforms in glioma cells, pretreated with the extract before heat shock, and increased apoptosis via caspase-3 activation [35]. 14-3-3 proteins are considered as HSP, because the expression of some isoforms are induce via a process mediated by heat shock transcription factor [149]. In another study, it was reported that *Cimicifuga foetida* extract reduced HSP27 expression in MCF-7 cells [129].

There are a lot of studies explained that how quercetin affects HSP induction in the cells. Plant-derived flavonoid quercetin is an antioxidant molecule and regarded as an HSP inhibitor [150]. It suppressed heat shock induced-HSP70 expression in prostate cancer cells [32–34]. Quercetin also repressed heat shock induced-HSP27 and HSP70 expressions in HeLa cells [136]. This flavonoid reduced *hsp* gene expression at trancription level via preventing between HSF and HSE linkage [32, 33, 136, 151, 152] and inhibited heat shock response by preventing the formation of HSF trimers [153]. Quercetin acts on early steps of HSP synthesis, by blocking the additional modifications necessary for activation of HSFs, like posttranslational phosphorylation or by causing conformational changes of the factor, and by inhibiting its interaction with other DNA-binding proteins in the promoter region [136, 154]. Quercetin reduced the intracellular HSF1 level, especially constitutive phosphorylated forms [153], and thus connection to DNA [155, 156]. Quercetin inhibits not only HSF1 activation, but also many protein kinase activities [108].

Antioxidant compounds, such as quercetin and other bioflavonoids are useful for not only establishing positive and negative regulatory mechanisms for HSP expression but also for the clinical improvement of hyperthermic therapy of tumors [152]. In addition, many studies have demonstrated that some flavonoids exhibited a synergistic antitumour effect with chemotherapeutics [137, 157]. Quercetin sensitises HeLa cells to cisplatin and increases the level of apotosis. The significant decrease in HSP27 and HSP72 expression after the treatment correlates with the highest sensitivity of HeLa cells to cisplatin-induced apoptosis [137]. Additionally, it is well known that while the quercetin enhanced Dox efficacy in highly invasive breast cancer, it helped to reduce the cytotoxic side effects of Dox in non-tumoral cells [135]. The heat shock-induced stress proteins increased Dox resistance, but quercetin treatment caused a decrease in HSP expression and as a result the cells become more sensitive to drug in neuroblastoma and Ewing's sarcoma cells [138]. Besides, the quercetin caused to the suppression of HSP27 in lung cancer cells (A549). Using it combined with CDDP or gemcitabine, leaded to reduction of

the survival rate of lung cancer stem cells [158]. These findings indicate that other natural antioxidant compounds may also have potential for suppressing HSP expression.

3.6 Conclusion

Today, the researchers working on cancer therapy focused on HSP suppression, as it is well know that the HSP levels are elevated in many cancer types. Overexpressed HSP causes inhibition of programmed cell death, and increases resistance to the chemotherapeutic drugs [16, 19, 52]. Therefore, the inhibition of HSP has become an interesting strategy in cancer therapy. A lot of studies have also emphasized that HSP inhibition is gaining importance in cancer treatment [15, 25, 28, 82, 118, 119, 159]. Although some HSP inhibitors are used in several clinical trials, new agents that target HSP inhibition should be investigated for the treatment of cancer.

As described in this chapter, the suppressive effect of some plant extract or natural products on HSP expression may provide the development of new approach in cancer therapy. Especially downregulation of HSP can enhance the impact of chemotherapy or may reduce the side effects of applied drugs through medicating with low doses of chemotherapy agents to the patients. Considering all these studies, it is understood that the rate of success in the cancer treatment may have been boosted via new drug development, which has targetted to inhibition of HSP expression. Thus, the cancer cells may have been sensitized against the chemotherapeutic or radiotherapeutic agents. In summary, all data indicate that suppressing HSP by natural products may be a promissing wat to enhance apoptosis, and improve treatment efficacy, alongside with minimizing of toxic side effects in the cells. Future studies targeting these proteins for development of chemosensitizers may help to achieve more effective cancer treatment methods in combinational therapy.

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Part II
Heat Shock Protein HSP60-Based Therapies

Chapter 4
Chaperonotherapy for Alzheimer's Disease: Focusing on HSP60

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Abstract This review will analyze growing evidence suggesting a convergence between two major areas of research: Alzheimer's disease (AD) and chaperonopathies. While AD is a widely recognized medical, public health, and social problem, the chaperonopathies have not yet been acknowledged as a related burden

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of similar magnitude. However, recent evidence collectively indicates that such possibility exists in that AD, or at least some forms of it, may indeed be a chaperonopathy. The importance of considering this possibility cannot be overemphasized since it provides a novel point of view to examine AD and potentially suggests new therapeutic avenues. In this review, we focus on the mitochondrial chaperone HSP60 and discuss some of its biological, molecular, and pathological facets as they pertain to AD. We further illustrate how HSP60 may be an etiologic-pathogenic factor in AD and, as such, it could become a novel, effective therapeutic target. This possibility is discussed both in the light of negative chaperonotherapy, namely the development of means to inhibit HSP60 in the event its excessive activity is a disease-promoting event in AD, as well as positive chaperonotherapy, that is boosting its activity if, on the other hand, it is demonstrated that HSP60 insufficiency is a key feature of AD with such pathological consequences as causing mitochondrial dysfunction.

Keywords Chaperoning system • Chaperonopathies • Negative chaperonotherapy • Positive chaperonotherapy • Alzheimer's disease • Amyloid precursor protein (APP) • Amyloid peptide Aβ • Plaques • Protein Tau • Intracellular tangles • HSP60 • Chaperonin • HSPD1 • HSP60 inhibitors • Methylene blue • Mizoribine • Pyrazolopyrimidine EC3016 • Avrainvillamide • Epolactaene • Carboranylphenoxyacetanilide

Abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
AD	Alzheimer disease
ALCAR	Acetyl-L-carnitine
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Aβ	Amyloid-beta peptide

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DLS	Dynamic light scattering
ETB	Epolactaene tertiary butyl ester
HNE	4-hydroxynonenal
HSP	Heat shock proteins
LOX-1	Lectin-like oxidized low-density lipoprotein receptor-1
MAP	Mitogen-activated protein
MB	Methylene blue
MHC	Major histocompatibility complex
NEF	Nucleotide exchange factor
SALS	Multi angle laser scattering
SANS	Small angle neutron scattering
SAXS	Small angle X-ray scattering
SLS	Static light scattering
TGF-β1	Transforming growth factor beta 1

4.1 Introduction

The concept of the "chaperoning system" is relatively new and describes a system that includes all molecular chaperones, co-chaperones and co-factors of an organism [1]. The science that studies this system may be called chaperonology, which also includes the study of diseases caused by chaperoning system malfunction [2]. In broader terms, the chaperoning system is a physiological set of molecules necessary to maintaining protein homeostasis by governing proper protein folding and sub-cellular trafficking in a highly regulated fashion so that its dysfunction unequivocally results in pathological conditions [3].

Chaperones are proteins that have been highly conserved throughout evolution. They constitutively assist nascent proteins to fold correctly and to refold if partially denatured by aberrant conditions such as cellular stress. They also drive proper protein translocation across cell organelles and, if a protein is irreversibly damaged, enhance its degradation [4]. Investigations in the past recent years have shown that molecular chaperones have also extra-chaperoning roles, such as participation in immune system regulation [5, 6], apoptosis [7], and carcinogenesis [8].

Many heat shock proteins (HSP) function as molecular chaperones as they play crucial roles in the biosynthesis, folding/unfolding, transport and assembly of other proteins [9]. HSP were first discovered as a group of molecules whose expression is induced by heat shock [10, 11]. HSP are evolutionarily conserved and can be found in every organism and cell type ([12, 13]; and see later). Despite their original name, a wealth of evidence has demonstrates that HSP can be also induced by a variety of different stressors, such as hypoxia, ischemia, heavy metals, ethanol and infections as well as several diseases [4, 13]. The term HSP, which is commonly given to molecular chaperones, is indeed inadequate because not all chaperone genes are heat inducible. Moreover, a number of human genes that are not members of the chaperone system are induced by heat. For example, Alpha Hemoglobin Stabilizing

Protein is a chaperone whose substrate (client) is alpha hemoglobin but it is not encoded by a heat shock gene. On the other hand, HSP32, which is the inducible isoform of heme oxygenase 1, is highly expressed in tissues responsible for heme metabolism, and is an anti-oxidative defense mechanism under stress conditions [14] but a chaperoning function for this canonic heat shock protein has not been demonstrated. Nevertheless, the terms HSP and chaperone are still commonly used as synonyms in official protein databases as well as scientific literature.

Molecular chaperones have been traditionally classified on the basis of their various characteristics, including molecular weight, as superheavy (e.g., sacsin); heavy (e.g., HSP110), HSP90, HSP70, HSP40, and the small HSP (sHSP) [13]. A new terminology has recently been proposed based largely on the more consistent nomenclature assigned by the HUGO Gene Nomenclature Committee and used in the National Center of Biotechnology Information Entrez Gene database for the heat shock genes [15]. However this terminology has not been generally adopted in the scientific literature, yet.

According to the origin and mobility, a molecular chaperone can be further defined as: (i) *Autochthonous* if it resides and functions in the cell in which it was originated; (ii) *Imported* if its place of residence is not the same as that of its origin; (iii) *Sessile* if it is anchored to another structure, i.e. cell membrane; (iv) *Mobile* if it travels in body fluids or in the intercellular environment [13]. In addition, chaperones are classified according to their capacity to form functional complexes with other molecules. Accordingly, chaperones can be classified as: (i) *Single* when a molecular chaperone performs its role alone, and (ii) *Social* when it forms an association, a "*Chaperoning Team*" with other chaperones and co-chaperones and/or or co-factors. In a *Chaperoning Team* the molecules interact with each other. For example, HSP60 interacts with its co-chaperone HSP10 to form a chaperoning team in mitochondria, and HSP70 interacts with the co-chaperone HSP40 and also with the co-factor Nucleotide Exchange Factor (NEF) to form a cytosolic chaperoning machinery [16]. In addition, a *Chaperoning Team* can be a member of a *Chaperoning Network* involving more than one team and, sometimes, also other molecules. Moreover, molecular chaperones can associate with other molecules that are not chaperones to form complexes with functions unrelated to the canonical role in the control of protein homeostasis. An example of such complexes is that formed between HSP60 with caspase 3 during apoptosis [17, 18].

The chaperoning system is a major component of the anti-stress mechanisms in human cells. As such, the chaperoning system has a crucial role in cell homeostasis and its malfunctions have been demonstrated as etiological factors in several human disorders, which are thus collectively known as chaperonopathies [13]. The study of the chaperoning system in normal and abnormal conditions is therefore necessary to devise novel treatments for the chaperonopathies, as we will discuss in the following sections.

4.2 Chaperonopathies and Chaperonotherapy

There has been so much research on molecular chaperones [10] since their discovery that it would seem justified that all these studies and the information they provided have been unified within a subfield of biomedicine named chaperonology [2]. This unification should bring various advantages in many fronts from learning and teaching to diagnosis and treatment and, last but not least, funding [19]. Within chaperonology, various subdivisions are emerging, such as chaperonomics, chaperonopathies, and chaperonotherapy.

As said in the previous section, at the beginning of their history, chaperones were seen as cytoprotective, part of a defense mechanism against the deleterious effects on cells of stressors, e.g., heat-shock, chemical injury, sudden pH or osmolarity changes, inflammation, ischemia-hypoxia, etc., in Bacteria, Archaea, and Eukaryotes [20–24]. However, in the course of time, it was observed that various pathological conditions seemed associated with some kind of failure or malfunction of one or more chaperones [4, 25]. These conditions were recently grouped as a coherent nosological category under the name of chaperonopathies [13].

Since it was realized that chaperones may be at the center of mechanisms that cause cell damage, tissue abnormalities, and disease, it was also realized that therapeutic means ought to use chaperones either as remedies or as targets for treatment agents [2], and we, in our research, have focused on the chaperonin HSP60 (also termed Cpn60 or, accordingly to the new terminology, HSPD1) [26–28]. This therapeutic approach, involving chaperones as central players or targets, i.e., chaperonotherapy, includes two modalities. When the chaperone is a primary etiologic-pathogenic factor and its activity must be blocked or at least partially inhibited, we may speak of negative chaperonotherapy. On the contrary, when the chaperonopathy is caused by a defective chaperone due, for example, to a function-crippling mutation, therapy ought to aim at replacing the defective molecule or, at least to boost its activity; this would represent a form of positive chaperonotherapy.

In the following sections of this chapter we focus on HSP60 chaperonopathies in which HSP60 is an etiologic-pathogenic factor, and HSP60 chaperonotherapy, all within the context of Alzheimer's disease (AD).

4.3 Alzheimer's Disease and Molecular Chaperones: Biophysical Remarks

AD is a chronic and progressive condition, which affects about 5 % of the population over age 65, and represents the most common cause of dementia in the elderly population. AD affects nearly seven million people in Europe and, if effective therapeutic strategies are not found, the number of people suffering from this devastating disease can be expected to double every 20 years [29].

From a molecular point of view, AD is characterized by the accumulation of a 40-42-amino acids peptide, the amyloid-beta peptide (Aβ), in insoluble cerebral plaques in the form of amyloid fibrils. These consist of 2–6 unbranched protofilaments, each about 2–5 nm in diameter, characterized by a cross-β spine, with β-strands perpendicular to the fibril axis and β-sheets along the length of the fibril [30]. The amyloid peptide Aβ results from a specific proteolytic pathway of a large transmembrane glycoprotein, the amyloid precursor protein (APP) [31]. According to literature, the amyloid aggregation process follows typical nucleation-polymerization kinetics, characterized in each phase by structural intermediates with specific dimensions, morphologies, and cytotoxic activity [32, 33]. The peptide assembly in several metastable non-fibrillar forms, known as prefibrillar forms, always precedes mature fibril formation. Substantial evidence suggests that small prefibrillar oligomers, which form at the beginning of the aggregation process, are the crucial species in the onset of the disease and in neuronal cell degeneration [34]. The oligomers, about 10 nm in size, can interact with cell membranes, impairing their structural organization, destroying their selective ion permeability, and leading to metabolic alterations (oxidative stress, Ca^{2+} homeostasis) that may eventually culminate in neuronal cell death. Analysis of the binding of various amyloid oligomers with the amyloid oligomer-specific polyclonal antibody A11 revealed that, regardless of the primary protein structure, the amyloid oligomer represents a generic conformation, and suggested that toxic β-aggregation processes possess a common mechanism of toxicity in which the role of plasma membranes is critical [35]. On the one hand, the cytotoxic effect results from Aβ/plasma membranes direct interaction, leading to calcium homeostasis variations and reactive oxygen species production due to oxidation of the membrane itself [36]. Studies with the transgenic mouse model Tg2676 of AD have shown that lipid rafts constitute a site where Aβ oligomers can accumulate and cause toxicity [37]. This hypothesis validates the role assumed by an intraneuronal Aβ assembly in amyloid toxicity and pathogenesis in AD, together with the widely established toxicity of the “extracellular” insoluble Aβ aggregates.

Several studies using Aβ1-40 and Aβ1-42 specific antibodies demonstrated that accumulation of Aβ inside neurons could originate from intracellular cleavage of APP and from Aβ internalization from the extracellular milieu [38]. Some mechanisms of Aβ endocytosis involved receptors that bind apolipoprotein E (apoE) and belong to the Low-Density Lipoprotein Receptor family, primary carriers of cholesterol in the brain [38]. Allelic variation in the apoE gene is the major risk factor for sporadic AD and recent studies showed a strong link between cholesterol homeostasis, apoE and Aβ intracellular degradation [39, 40]. Moreover, cholesterol has a direct role in AD by inducing changes in the structure and fluidity of the phospholipid bilayer and by modulating the incorporation and pore formation of Aβ into cell membranes [40].

The second neuropathological hallmark of AD is the accumulation in neurofibrillary tangles of the microtubule-binding protein Tau. Tau proteins normally stabilize microtubules, and it is suggested that they are hyperphosphorylated in pathogenic

conditions and released from microtubules in paired helical filaments, which are found at autopsy in AD brains [41].

Therapeutic strategies in amyloid diseases include at least four broad approaches: (i) blocking the production of the amyloidogenic peptide or protein; (ii) blocking its "misfolding" or transformation from a nonpathogenic monomer to toxic oligomers and fibrils; (iii) blocking the toxic effects of amyloid; and (iv) modulating an auxiliary cellular pathway in a manner that would affect beneficially one or more of the foregoing approaches [42]. Chaperones are the class of molecules that exercise their function at all the above-mentioned levels; hence their potential role in the therapeutic approach of AD can be proposed. As mentioned earlier, molecular chaperones play essential roles in many cellular processes, including protein folding, targeting, transport, and protein degradation and disaggregation of toxic aggregates by clearance mechanisms ([41]; and see earlier). This explains why molecular chaperones are essential in the cellular defenses against protein aggregation caused by misfolding both at intra- and extra-cellular levels and are potentially powerful suppressors of neurodegeneration. Moreover, chaperones regulate protein functions in order to protect against oxidative stress due to the toxicity of amyloid aggregates and their expression is highly increased under conditions of an amyloid challenge [41].

The HSP with chaperone activity belong to various groups, including HSP60, HSP70, HSP90, HSP100, and the sHSP. Based on their mechanism of action, three functional subclasses can be distinguished: (i) *Folding* chaperones (e.g., DnaK and GroEL in prokaryotes; and HSP60, HSP70, and the HSPB group of HSP including HSP27 and HSPB1 in eukaryotes) induce refolding/unfolding of their substrates, with conformational changes depending on adenosine triphosphate (ATP)-binding and hydrolysis; (ii) *Holding* chaperones (e.g., HSP33, and HSP31) that bind partially folded proteins and present them to the subsequent *Folding* chaperones action; and (iii) *Disaggregating* chaperones (e.g., ClpB in prokaryotes and HSP104 in eukaryotes) are able to solubilize proteins that have pathologically formed aggregates [43].

Recent in vitro studies have attributed to small HSP, chaperones and other stress-related proteins, and small molecules with a chaperone-like activity an important role in AD, although their specific mechanisms of action in AD is yet to be clarified [4, 12, 44, 45].

In the brains of patients affected by AD, an increased level in the expression of HSP70 has been reported [46], suggesting a potential role of HSP70 in pathogenesis. The increase of HSP70 levels could be related to an increase in the expression of the transforming growth factor beta 1 (TGF-β1), an enzyme that is considered responsible for the degradation of Aβ [47]. Accordingly, a "sink hypothesis" has been proposed that posits that cellular toxicity in AD may develop because chaperones and other proteins are being sequestered on the amyloid fibrils and thus re-directed away from their normal cellular tasks [48].

Another HSP, HSP104, seems to strongly inhibit Aβ1-42 amyloidogenesis, raising hopes for developing ways to use HSP104 as a therapeutic agent [49].

Despite all the above evidence, the influence of chaperones on the amyloid aggregation process and the specific role of all the components involved in the AD pathogenic pathway, remain unclear and need to be further investigated. Moreover, additional progress in the understanding of the role of intraneuronal Aβ and dysfunction of intra- and extra-cellular membranes will require the biophysical characterization of the direct interactions involving chaperones, Aβ peptide and cell membranes.

In this respect, the application of biophysical methods is advancing our knowledge on the molecular mechanisms of chaperone action and interaction with other molecules involved in AD. Therefore, a biophysical approach to these aspects of AD represents a fundamental step toward the understanding of the molecular basis of a future chaperonotherapy for this disorder. Since chaperones and Aβ peptide can directly interact in the extracellular space, thus influencing the Aβ amyloid aggregation process, it is tempting to speculate that, in response to the generation of intracellular Aβ oligomers, a small fraction of cytosolic chaperones could be targeted to lipid rafts and become associated with Aβ oligomers before their ultimate secretion into the extracellular space [50]. In addition, in vitro studies have shown that HSP70 can be released by glia and thus enhance neuronal stress tolerance [51]. Surprisingly, the anti-oligomer antibody A11 reacted with several purified HSP, including HSP70 and HSP90 [51], thus supporting the hypothesis of a direct chaperone-peptide Aβ interaction, which is of particular importance in the case of intracellular proteins accumulation (Parkinson's disease, spinocerebral ataxia) and toxicity caused by intraneuronal Aβ. The in vitro inhibition of protein aggregation by chaperones may be regulated by mechanisms of protein refolding or holding, to be discriminated by means of specific chemical-physical experiments of fibrillogenesis kinetics as a function of amyloid protein/chaperone relative concentration [52]. In the *holding* model, chaperones bind the misfolded Aβ monomer without releasing it or without causing structural variation. The anti-aggregation effect would in this instance arise from the Aβ peptide reduced concentration that results in a lower amount of Aβ amyloid aggregate (Fig. 4.1a). In the *refolding* model, the chaperone action consists in inducing the Aβ peptide conversion into an altered monomeric form, less competent than the misfolded one to undergo an on-pathway amyloid route. The altered monomer is then released, allowing the subsequent re-use of the chaperone (Fig. 4.1b).

The crosstalk between molecular chaperones and the ubiquitin–proteasome system has been considered important in the Tau deposition mechanism [41]. Immunofluorescence studies in cultured cells showed interactions between Tau proteins and HSP70 and HSP90. These chaperones were claimed to prevent Tau aggregation by maintaining it in a soluble and functional conformation [53]. However, according to other reports, HSP70 proteins were considered to cause both acceleration and slowing of Tau degradation. A recent study shed further light on this seemingly paradoxical mechanism by demonstrating that homologous variants in the HSP70 family can have opposing effects on Tau clearance kinetics [54]. Specifically, nuclear magnetic resonance spectroscopy demonstrated that HSP72 had greater affinity for Tau in comparison with heat shock cognate 70 (HSC70).

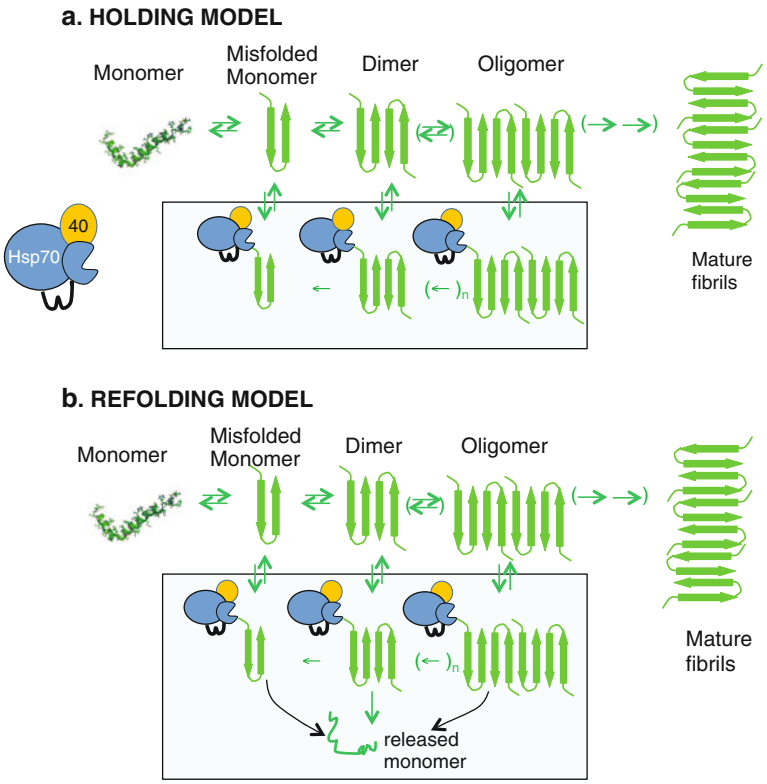


Fig. 4.1 Model for chaperone differential action on Aβ-peptide aggregation pathway. (**a**) Holding mechanism: the misfolded peptide remains on the chaperone surface upon binding, thus reducing the concentration of proteins competent for on-pathway aggregation. (**b**) Refolding mechanism: a conformational change in Aβ peptide occurs, which is thus released, allowing the reuse of the chaperone (Source Adapted from Evans et al. [52])

However, HSC70 is more abundant than HSP72 in brain tissues. Hence, because HSC70 is the predominant variant of HSP70 in the brain, a slower Tau clearance is more likely to occur. HSP72 was not induced in the AD brain, suggesting a mechanism for age-associated onset of the disease.

The results described above underline the importance of a biophysical and molecular approach to study in vitro the interplay between biomolecules at a simplified level, because the interaction mechanisms of chaperones and proteins related to AD could be far more complex in vivo, rendering mechanistic studies difficult to accomplish. First, the use of biophysical tools (like Small Angle X-ray Scattering, SAXS; and Multi Angle Laser Scattering, SALS) can address some unanswered questions concerning stability, oligomeric structure and folding activity of chaperones in solution, under conditions resembling those in vivo [55]. Many chaperones assemble in multimeric species that represent the functional

form of the protein (see earlier chaperoning teams, networks, and complexes). Emblematic in this respect is the molecular chaperone HSP60. HSP60 forms a ring-shaped heptameric quaternary structure, two of which associate to form a barrel-shaped tetradecamer, which is the functional macromolecular chaperoning complex. However, studies in vitro have shown that, differently from all other chaperonin homologs that exist only as tetradecamers composed of two 7-membered rings, the mammalian mitochondrial HSP60 can also occur as a single ring, which under specific conditions dissociated into monomers [56]. What about the potential interaction of HSP60 with Aβ peptides involved in AD? Which oligomeric species could be effective in influencing the Aβ fibrillogenesis pathway and which role could thereby HSP60 play? The answers to these questions will certainly help further studies that may shed light about the functions of HSP60, its role in AD pathogenesis, and its use as target for novel therapies.

Collectively, the study of chaperones/Aβ direct interactions and the validation of possible defense pathways based on clearance effects are the scaffold for a potential therapy aimed at using chaperones in order to remove oligomeric species, considered as responsible for amyloid toxicity.

In order to test chaperones with potential therapeutic activity, the chemical-physical characterization of such molecules/protein interactions is critically important. This is necessary so as to identify chaperones that can be considered good candidates for treatment and prevention of AD. As a secondary objective, the implementation of multiple forms of biophysical, cellular, and in vivo assays, that are essential to fully correlate interactions of a compound with its target, should be also pursued, in the presence or absence of membrane systems that, as previously mentioned, play a critical role in amyloid toxicity and neurodegeneration. Static and Dynamic Light Scattering (SLS, DLS) measurements are suitable techniques used to monitor protein aggregation processes in the presence of chaperones, characterizing the hydrodynamic radius as well as the radius of gyration, the aggregation number, and shape properties of the aggregates. These biophysical tools have been successfully used to characterize the effect of proteins with a disordered structure and chaperone-like activity like caseins on the fibrillogenesis of Aβ1–40 peptide [57]. Biophysical techniques involving polarized light such as circular dichroism in FAR and NEAR UV have been very useful to investigate variations in secondary and tertiary structures induced by the HSP20, HSP27, and αB-crystallin chaperones on Aβ peptide, both in monomeric and in monomer/oligomer equilibrium forms as well as during fibrillogenesis [58]. Electron microscopy and fluorescence spectroscopy with specific dyes as thioflavin T have been very effective in revealing that HSP70 and HSP90 inhibit early stages of Aβ1-42 aggregation in vitro [52]. Furthermore, in solution SAXS/SANS can provide detailed information concerning the key players of Aβ aggregation process, as already evidenced using recombinant Aβ proteins [59], and evaluate the effects thereupon of the presence of anti-aggregants or chaperones. Biophysical characterizations have to be taken into account when working to develop an anti-AD HSP-based chaperonotherapy. In the following paragraphs we propose a model for examining a putative anti-AD chaperonotherapy based on HSP60 as molecular target.

4.4 HSP60: Biomedical and Molecular Aspects

As mentioned earlier, HSP60 is classically described as a mitochondrial protein, constitutively expressed under normal conditions and induced by various types of stressors as heat shock, oxidative stress, and DNA damage [16]. Inside mitochondria, HSP60 acts as a folding machine, together with HSP10, for the correct folding of several mitochondrial proteins [60, 61]. HSP60 and HSP10 are often referred to as "chaperonins," being HSP60 the chaperonin and HSP10 its co-chaperonin. A series of studies, published over the past several years, have demonstrated new sub-cellular localizations and functions for HSP60, describing it as a ubiquitous molecule with multiple roles in health and disease [62, 63].

HSP60 has both pro-survival and pro-death functions depending on tissue, cell type, and apoptosis inducers [18, 64]. The cytosolic HSP60 is mainly anti-apoptotic, as it binds to pro-apoptotic Bax in rat cardiac myocytes during hypoxia, preventing its translocation to the outer mitochondrial membrane and the triggering of the apoptotic cascade [64, 65]. Cytosolic accumulation of HSP60 is a common phenomenon during apoptosis induction and it may occur either with or without mitochondrial release [18]. It has been demonstrated that HSP60 may associate with Bak or Bcl-XL in normal heart tissues, inhibiting their apoptotic potentials and consequent cytochrome c release [66]. Additional evidence supporting a pro-survival role of HSP60 is its up-regulation in several cancers, including prostate, colorectal, and cervical cancers and osteosarcoma [8]. HSP60 may participate actively in tumor progression as suggested by its accumulation in the cytosol and plasma membrane of cancerous cells, reaching the extracellular space via secretory vesicles [67, 68] that in turn can induce anti-tumor immune responses [69]. Also, HSP60 has a role in metastatic transformation through activation of β-catenin [70]. Surface HSP60 has been found associated with α3β1-integrin, a protein involved in the adhesion of metastatic cancer cells [62, 71]. Acute ablation of HSP60 by small interfering RNA (siRNA) in tumor cell lines was associated with increased stabilization of p53 and increased expression of pro-apoptotic Bax [72]. In addition, hyperacetylation of HSP60 in osteosarcoma 143B cells seems to be associated with the anticancer activity of geldanamycin [73]. This evidence supports the idea that HSP60 can be used as target for antitumor therapy and promises the prospect for novel drugs design [55].

On the other hand, there exists published evidence supporting a death-promoting function for HSP60. Mitochondrial HSP60 binds to pro-caspase 3 in Jurkat and Hela cells, accelerating the maturation of pro-caspase 3 by upstream activator caspases during apoptosis [74, 75]. In agreement with these observations, positive HSP60 expression in esophageal squamous cell carcinoma correlates with good prognosis for the patients [76]. Collectively this evidence suggests that there is a delicate equilibrium regulating the pro- and anti-apoptotic functions of HSP60 in cells and tissues that depends on mechanisms yet to be fully understood.

HSP60 has been also involved in mechanism of cell aging [77]. Senescent cells in culture become flat and enlarged and can be maintained in a viable state for

long periods, but cannot be induced to divide by normal mitogenic stimuli [78]. Senescence-induced resistance to apoptosis leads to an increase in the number of senescent cells inside tissues, with consequences on the feebleness of tissue integrity and on neoplastic transformation, likely promoting the development of late-life cancers [79]. During replicative senescence of normal human skin fibroblasts, the levels of HSP60 increase and form a complex with a MAP kinase, named MOK, involved in signal transduction to the nucleus [80, 81]. Furthermore, a correlation between increased levels of HSP60 and senescence of skin fibroblasts was shown to involve interaction between HSP60 and mtHSP70 [82].

HSP60 may be involved in a number of autoimmune processes on the account that it may act as autoantigen because of the high sequence similarity (molecular mimicry) between human and foreign HSP60 from bacteria and parasites that colonize humans, which leads to anti-HSP60 antibody cross-reactivity [13]. For example, in our laboratory HSP60 from *Chlamydia trachomatis* serovar D was compared with human HSP60 and a high percentage of identity was found in 17 regions [83]. These regions could be presented to T cells by the MHC class I molecules, triggering an autoimmune response. This led us to postulate a crucial role of molecular mimicry between Chlamydial and human HSP60 in the pathogenesis of some autoimmune diseases [63]. HSP60 has high structural similarity also with other human proteins such as myelin-associated protein, glutamic acid decarboxylase, and acetylcholine receptor, further suggesting that circulating HSP60 may serve as autoantigen for the generation of an autoimmune response [84]. Consequently, HSP60 may trigger autoimmune pathological conditions, including some affecting the nervous system such as multiple sclerosis and myasthenia gravis [13, 85].

There is also evidence for the ability of HSP60 to activate inflammatory cells that are induced to produce cytokines and other inflammatory mediators. Accordingly, HSP60 has been proposed to function as a “chaperokine” [86]. Extracellular HSP60 can interact with a variety of receptors present on the cellular plasma-membrane surface, such as CD14, CD40, CD91 and TLRs [87, 88]. In addition, it has been demonstrated that HSP60 is an inducer of inflammatory adipocyte activity. HSP60 influences the pro-inflammatory capacity of adipocytes binding an adipocyte receptor, contributing to obesity-associated inflammatory disease leading to diabetes [89]. HSP60 has been also involved in the pathogenesis of chronic inflammatory diseases such as Crohn’s disease [90], ulcerative colitis [90, 91], chronic obstructive pulmonary disease [92], and atherosclerosis [93].

In brain, HSP60 is endogenously expressed in astrocytes, neurons, microglia, oligodendrocytes, and ependymal cells [94]. This distribution suggests an active participation of this chaperonin in many functions of the brain, both in normal

and pathological conditions. HSP60 exposed onto the surface of astrocytes and neuroblastoma cells interacts with TREM2, a receptor that, if mutated, is responsible for genetic disorders affecting bones and brain [95]. Extracellular HSP60, through binding to lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), may promote microglia-mediated neuroinflammation and a prolonged delay in the induction of HSP60 in brain injured regions [96, 98]. For example, expression of HSP60 has been found increased in the brainstem after subarachnoidal hemorrhage, forebrain or focal cerebral ischemia, and neonatal hypoxia-ischemia [98]. Neural expression of HSP60 increases over the course of development, a trend consistent with the changes of mitochondrial content in the brain [94].

Given that HSP60 plays a critical role in assisting the correct folding of other mitochondrial proteins and enzymes, a deficiency in its function or expression, together with increased vulnerability to oxidative stress, may lead to severe conditions caused by protein misfolding and aggregation [97]. Thus, HSP60 deficiency might be a common cause of mitochondrial dysfunction, which is a significant observation since AD has been classically described as a disorder aggravated by oxidative stress and/or mitochondrial defect characterized by protein conformation abnormalities [98, 99]. Expression of HSP60 is significantly decreased in the parietal cortex of AD subjects and in the cerebella of a rat model of AD, suggesting a defect in the protective role of this chaperonin in the AD brain [99, 100].

In support of the neuroprotective effects of HSP60, it has been demonstrated that in a human neuroblastoma cell line, induced expression of HSP60 prevented intracellular β-amyloid-induced inhibition of complex IV and consequently reduced apoptosis [45]. Aβ25–35 induced oxidation of HSP60 in fibroblasts derived from AD patients [101], and HSP60 was significantly oxidized by Aβ1–42 leading to a loss of function of HSP60, causing an increase in protein misfolding and aggregation [102].

HSP60 levels were found elevated in lymphocytes from AD patients when compared to controls [103]. An amyloid beta-HSP60 peptide-conjugate vaccine led to the induction of anti-Aβ-specific antibodies, associated with a significant reduction of cerebral amyloid burden and of the accompanying inflammatory response in the brain of a mouse model of AD [104]. On the contrary, other authors have attributed a deleterious effect to the elevated expression of HSP60 in AD. For example, it was shown that, in vitro, HSP60 mediates the translocation of APP to the mitochondria leading to dysfunction of this organelle [105].

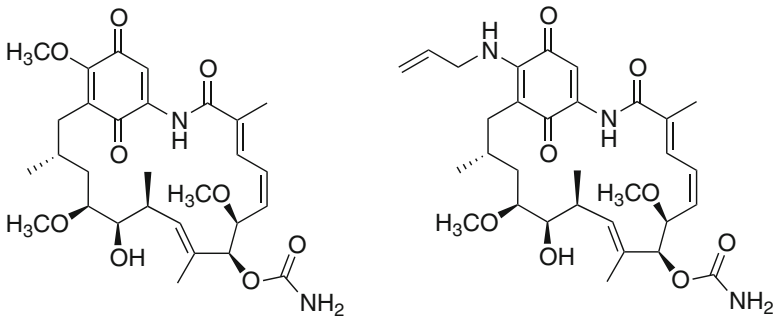
In summary, compelling evidence strongly indicates that HSP60 can be a likely candidate as target for either positive or negative chaperonopathy of AD. However, it has to be borne in mind that this chaperonin is a multifaceted protein involved in numerous physiological functions and pathological mechanisms, all of which may limit the therapeutic use of its targeted inhibition. In the last part of this chapter, we discuss the potential use of HSP60 inhibitors in AD therapy.

4.5 HSP60 Inhibitors and Their Potential Applications in Alzheimer's Disease Chaperonotherapy

The search for small molecules capable of reducing the toxic effects of Aβ and/or Tau protein aggregation have followed a variety of approaches and lead to the identification of many prospective drugs, whose description is beyond the scope of this chapter (the interested reader can consult [106–109]).

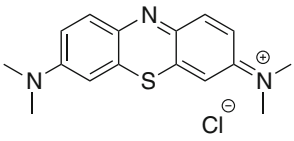
Recently, the link between HSP and AD has been established, with particular emphasis on the effect of HSP70 and HSP90 (see for example: [47, 52, 54, 110–115]). The demonstrated involvement of HSP in the progression of AD prompted a switch in the focus of much HSP-related research from the development of anti-tumoral compounds toward the treatment of neurodegenerative diseases [116, 117]. Consequently, a number of HSP-interacting compounds have been proposed as prospective drugs for the management of neurodegenerative diseases. These include geldanamycin (1) (Fig. 4.2, left), a Streptomyces-derived HSP90 inhibitor with the ability of clearing Tau aggregation [118], and 17-Allylamino-17-demethoxygeldanamycin (17-AAG) (2) (Fig. 4.2, right), a novel HSP90 inhibitor that showed less toxicity than the parent compound in a mouse model of spinal and bulbar muscular atrophy [119].

A high-throughput screening assay revealed that methylene blue (MB) (3) (Fig. 4.3) acts as inhibitor of HSP70 ATPase activity. MB (3) reduced Tau levels, both in cells and brain tissue [120]. In this case, targeting an HSP activity resulted in a very effective strategy and, consequently, MB has passed phase II clinical trials and has been considered for phase III trials for the treatment of AD patients [121]. Celastrol (4) (Fig. 4.4), a HSP90-inhibitor [122], was effective in inducing a set of neuroprotective HSP in cultures derived from cerebral cortices, including HSP70, HSP27 and HSP32, suggesting its potential usefulness to treat AD [123]. Despite the failure of previous clinical trials, also acetyl-L-carnitine (ALCAR) (5)



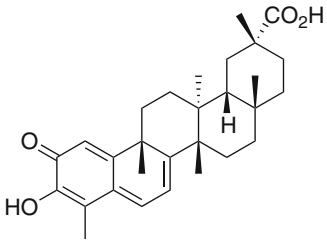
***geldanamycin* (1)** **17-AAG (2)**

Fig. 4.2 Streptomyces-derived HSP90 inhibitor geldanamycin (1) and its less toxic derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) (2)



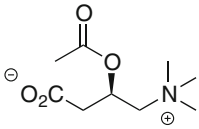
***MB* (3)**

Fig. 4.3 Structure of methylene blue (MB) (3), inhibitor of ATPase activity for HSP70. MB reduces Tau levels in cells and brain tissue



***celastrol* (4)**

Fig. 4.4 HSP90 inhibitor celastrol (4), a pentacyclic triterpenoid isolated from the root extracts of *Celastrus regelii,* effective for inducing HSP70, HSP27 and HSP32



***ALCAR* (5)**

Fig. 4.5 Structure of acetyl-L-carnitine (ALCAR) (5), which exerts protective effects against Aβ peptide by up-regulating GSH and HSP

(Fig. 4.5) was found to exert protective effects against Aβ1-42 toxicity and oxidative stress by up-regulating the levels of GSH and HSP. This evidence renewed the potential for ALCAR (5) in the management of Aβ1-42-induced oxidative stress and neurotoxicity [124].

Despite the compelling evidence supporting the involvement of HSP60 in AD progression [105], none of its known inhibitors or regulators has been tested to determine its effects as potential therapeutic agents in AD. On the other hand, the HSP60's role in tumor-cell lifecycle has been widely assessed and its anti-apoptotic role unraveled [27, 55, 125]. These studies pointed out the possibility of targeting HSP60 as a therapeutic anticancer approach. For example, the exposure of tumor cells to some recently characterized copper complexes showed antitumor activity that was correlated to decreased levels of HSP60 [126]. Despite various studies pointing toward targeting HSP60 as a promising therapeutic strategy, only a few compounds have been characterized in some detail as HSP60 inhibitors. However, for most of this inhibitors their mechanism of action remains unclear.

In the development of new HSP60 inhibitors, as well as in the study of the mechanism of action of known inhibitors, it is crucial to pay attention to the structural differences between the eukaryotic HSP60, and the more widely studied prokaryotic homolog GroEL. For example, only the human HSP60 possesses three cysteine residues (Cys237, Cys442 and Cys447), which represent ideal sites for drug interaction due to their nucleophilicity and tendency to be oxidized [127].

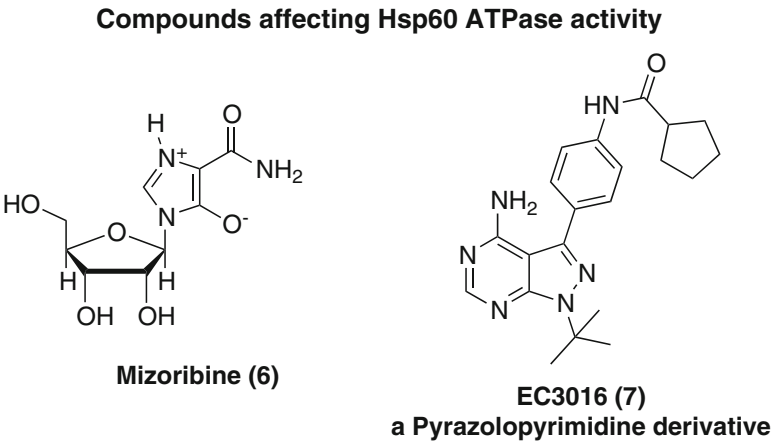


Fig. 4.6 Compounds affecting ATP-related activity of HSP60

As of today, only two strategies have been followed for the design of new HSP60 inhibitors [127]. One strategy aims at targeting HSP60 cysteine residues either as oxidizable sites [128] or for covalent binding, presumably through interaction with an electrophilic compound [129–131]. The other approach targets ATP binding and hydrolysis sites-functions, thus affecting those ATP-dependent conformational changes of HSP60 which are crucial for the protein folding function [132–134].

Among compounds targeting the ATPase activity of HSP60, mizoribine (6) – which is an imidazole-based immunosuppressant (Fig. 4.6, left) – can form a complex with HSP60 thus affecting its protein-folding activity [132, 133]. A recent study further showed that mizoribine (6) slowed down the folding cycle by affecting ATP hydrolysis. Additionally, mizoribine (6) activity was related to the inhibition of the dissociation of the co-chaperonin HSP10 from the HSP60/HSP10 complex. In the case of mizoribine (6), there was a significant difference in the activities observed with prokaryotic and eukaryotic HSP60 in that GroEL/GroES systems were not significantly affected by mizoribine (6) [134].

Another azaaromatic heterocyclic compound, pyrazolopyrimidine EC3016 (7) (Fig. 4.6, right), was reported to block ATP binding and hydrolysis thus affecting the protein-folding function of HSP60 [133]. Despite its activity, no update on the use of EC3016 (7) has appeared since the first report of its HSP60 inhibitory function.

Other compounds have been reported to interact with cysteine residues of HSP60 (Fig. 4.7) [127]. For instance, avrainvillamide (8), can alkylate HSP60's cysteine residues through the electrophilic 3-alkylidene-3H-indole 1-oxide moiety [131]; however, its activity in inhibiting HSP60 functions has not been demonstrated yet.

HSP60-interacting molecules were also found among natural compounds, such as epolactaene (9), which covalently binds to the Cys442 residue and thus inhibits the chaperoning activity of human HSP60 [130]. Recently, other derivatives of epolactaene (9), such as the epolactaene tertiary butyl ester (ETB) (10), were shown to target mitochondrial transcription [135].

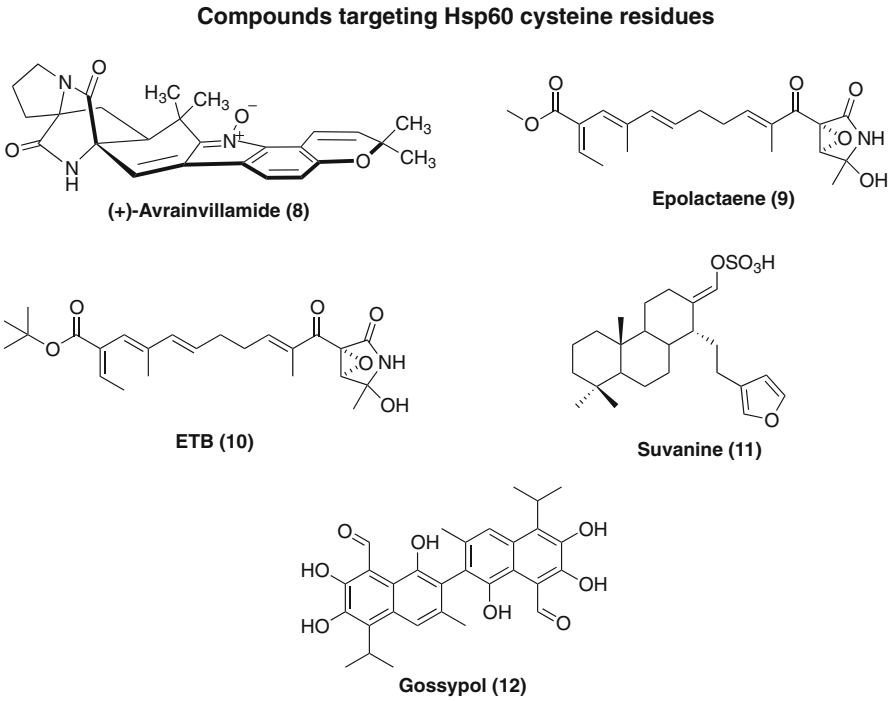


Fig. 4.7 Compounds interacting with cysteine residues of HSP60

A structure-activity relationship analysis performed on epolactaene derivatives demonstrated that both the cyclic amide (lactam) and the α-β unsaturated ketone are critical moieties for inhibiting the chaperone activity of HSP60 [129]. However, considering the entire molecular structure, one can predict more than one electrophilic site able to covalently bind the nucleophilic thiolic ends of cysteine residues. In particular, the tetrasubstituted carbon of the epolactaene epoxide moiety should be the most likely binding site for the nucleophilic cysteine residues due to the electron-withdrawing effect of the two carbonyl groups. Indeed, a recent study on the reaction mechanism of epolactaene with a series of thiols (R'SH), including free (non proteinic) cysteine, evidenced the active site of epolactaene and pointed out its ability to promote disulfide formation (Fig. 4.8) [136]. Current studies from our group are examining the mechanism of action of epolactaene (9) in the HSP60 assembly using both computational and experimental approaches. On the basis of the epolactaene (9) chemical behavior, and the requirement of Cys442 for epolactaene to exert ts inhibitory activity, one could suggest that once within the HSP60 structure, epolactaene (9) can bind either one of the two Cys442 and Cys447 residues, and subsequently involve the other in the formation of a Cys442-S-S-Cys447 disulfide bridge.

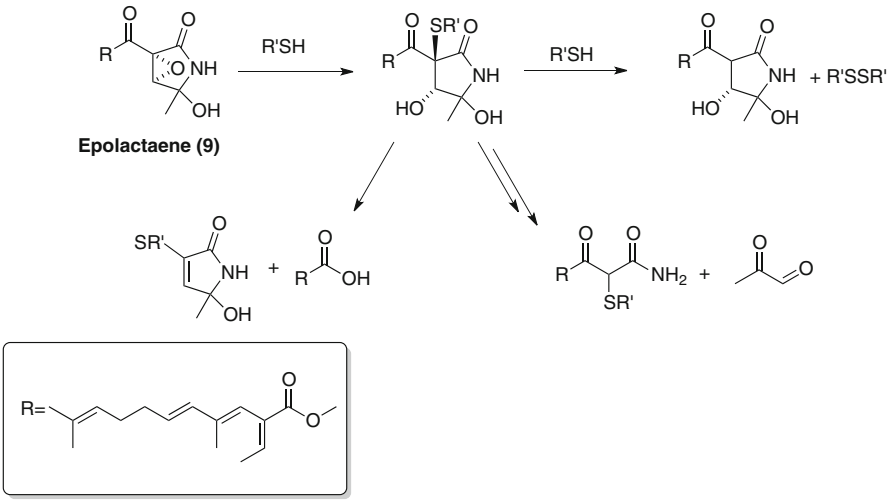
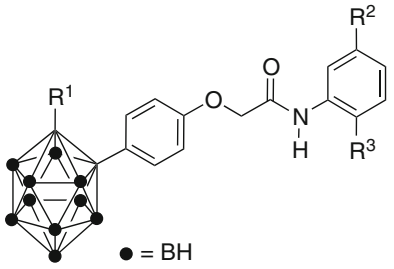


Fig. 4.8 Proposed mechanism for the reaction of epolactaene derivatives with thiols. The epoxide moiety of epolactaene (9) is proposed as binding site for cysteine, due to the electron-withdrawing effect of the two carbonyl groups. The covalent adduct decomposes producing disulfide (R'SSR') formation

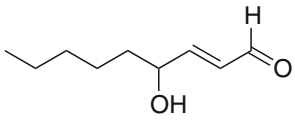
Fig. 4.9 General structure of carboranylphenoxyacetanilide (13). The most active HSP60 inhibitor had R1 = CH_2CH_3, R2 = $B(OH)_2$, R3 = OH



Carboranylphenoxyacetanilide derivatives (13)

Recently, a chemical proteomics screening performed on several natural compounds interacting with HSP60, showed that cysteine residues on HSP60 were targeted for sulfation processes by suvanine (11), a sesquiterpene natural product of marine origin [137]. Additionally, the typical thiol/disulfide redox reaction of its cysteine residues was supposedly responsible for HSP60 interaction with gossypol (12), a polyphenolic drug which induces apoptosis through oxidative stress [128].

Interestingly, a hypoxic-inducible factor 1 alpha inhibitor (13) containing a pharmacophorically unusual carboranyl moiety was found to primarily target HSP60, although the actual binding site is still undefined [138]. Recently, one of carboranylphenoxyacetanilide derivatives (13) (Fig. 4.9; R1 = CH_2CH_3, R2 = $B(OH)_2$, R3 = OH) showed a chaperone inhibition activity that was two times higher than that of ETB (10) [139, 140].



HNE (14)

Fig. 4.10 Structure of 4-hydroxynonenal (HNE) (14) targets HSP60, as well as other proteins involved in stress signaling, in a dose-dependent manner

Another proteomic analysis performed on compounds targeting proteins involved in the stress response (HSP60, HSP70, HSP90, and 78-kDa glucose regulated protein) revealed that 4-hydroxynonenal (HNE) (14) (Fig. 4.10) targets HSP60, with a dose-dependent increase in labeled proteins with increased sequence coverage at higher concentrations [141]. Also in this case, the binding site was not discovered or inferred; however, by considering the presence of an electrophilic α-β-unsaturated aldehyde moiety, one can hypothesize that the nucleophilic cysteine residues are the most likely binding sites.

In general, even if current studies are focused on targeting the chaperonin's ATP binding site or cysteine residues, other regions of HSP60 can be surveyed to develop novel inhibitors [127]. For instance, one can envision the targeting of the site of interaction between the mitochondrial HSP60 and its co-chaperonin (HSP10), which is crucial for the refolding of denatured client proteins [61]. Alternatively, compounds can be developed to target the ability of HSP60 to form a complex with APP, thus avoiding its translocation.

Ideally, besides testing the efficacy in inhibiting ATP binding and hydrolysis and the chaperonin's protein folding activity, a thorough study on the development of new HSP60 inhibitors should also address the binding capability and define the docking site [127]. Unfortunately, these issues are rarely addressed in comprehensive studies [129, 134], thus leaving several unanswered questions concerning current HSP60 inhibitors. As a consequence, the lack of information on the mechanism of action of several promising HSP60-targeting drugs promotes the supporting role of biomolecular computational studies. A valid and recent example of this approach is the *in silico* study performed to model the ATP-binding pocket of HSP60 in humans, *Escherichia coli* and *Brugia malayi* [142].

4.6 Conclusion

The evidence of the involvement of HSP60 in the pathogenesis of AD is relatively new and further investigations are ongoing to better clarify the molecular mechanism(s) by which HSP60 contributes to the onset and/or the progression of this disorder. A better knowledge of the role of HSP60, as well as of other HSP/chaperones, in AD pathogenesis will further promote these molecules as candidates for the future development of a novel, effective chaperonopathy in AD patients. For example, recent data encourage the hypothesis that a negative

chaperonotherapy could be a way to reduce β-amyloid protein accumulation [105]. The road thus appears to be paved for new therapeutic solutions for AD centered on the novel concept of chaperonotherapy.

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Chapter 5
Secreted and Circulating Cell Stress Proteins in the Periodontal Diseases

Brian Henderson, Nikos Donos, Luigi Nibali, and Frank Kaiser

Abstract The periodontal disease are the major source of human inflammatory pathology and so understanding the molecular and cellular pathology of periodontitis, and its response to treatment, is vital and secreted biomarkers may be useful. One key group of biomarkers are secreted cell stress proteins – now recognised to be potent intercellular signalling molecules. Periodontitis is not an infectious disease, but is driven by oral bacteria and so both bacterial and host cell stress proteins could be potential disease biomarkers. Both bacterial and human cell stress proteins have signalling actions on human leukocytes which could promote and/or inhibit periodontitis. Bacterial cell stress proteins have not been looked for in the circulation but there are antibodies to HSP60 and HSP90 in the blood of periodontal patients. So far little is known about circulating host cell stress proteins, but one treatment study reveals that patients have lower circulating levels of HSP10 and BiP, both anti-inflammatory molecules, than healthy controls, with successful treatment resulting in both proteins reaching normal levels, suggesting these proteins are natural anti-inflammatory signals in periodontitis. Another anti-inflammatory protein, HSP27, has, in unpublished studies, been shown to have diagnostic potential. These findings argue for much more detailed analysis of circulating cell stress proteins in the periodontal diseases.

Keywords Periodontitis • Gingivitis • Inflammation • Biomarker • Cell stress proteins

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Abbreviations

BiP	Immunoglobulin-binding protein
DAMPS	Danger- or damage-associated molecular patterns
ER	Endoplasmic reticulum
HSP	Heat shock protein
IL	Interleukin
LPS	Lipopolysaccharide
NHAMES	National Health and Nutrition Examination Survey
PD	Periodontal disease
PFC	Protein folding catalysts
TNF	Tumour necrosis factor

5.1 Introduction

Proteostasis is now recognised as a key cellular system, with emergent properties, responsible for cellular and tissue homeostasis. Alterations in proteostatic networks can lead to cell and tissue pathology and so, therefore, proteostasis, and its key components, molecular chaperones and protein-folding catalysts (PFCs), are now recognised as pathological elements and therapeutic targets in human disease [1, 2]. A possible emergent property of the intracellular proteostatic network is the secretion of molecular chaperones and PFCs by cells and the finding that these secreted proteins have potent intercellular signalling actions [3, 4], particularly influencing the activity of the innate and adaptive immune systems [5]. Secreted molecular chaperones and PFCs (collectively, but sometimes incorrectly termed in this chapter – cell stress proteins) have been found in the circulation in a range of diseases and are increasingly being used as biomarkers in human disease states. Currently we have most information about the role of circulating cell stress proteins in the cardiovascular diseases [6]. This chapter will concentrate on the most common acute and chronic inflammatory conditions of humanity, which afflict nearly everyone at one or other times in their lives. These are the periodontal diseases (PDs) which afflict the gingivae (gums) and the tissues supporting the teeth – the periodontal ligament and alveolar bone. In addition to being the most common of mankind's chronic diseases the PDs, because of their prevalence, and because of the ease of access of the diseased tissues, can function as an easily-studied natural model of inflammation in the human. Before describing these conditions and the evidence for a role for secreted molecular chaperones, a brief description of the biological roles of secreted cell stress proteins will be provided.

5.2 Secretion of Cell Stress Proteins

The major discoveries of the protein-folding mechanisms of cell stress proteins, such a HSP60, HSP70 and HSP90 in the 1980s and 1990s, overshadowed the findings, beginning in the mid-1980s [7], that cell stress proteins were secreted from cells and that these secreted proteins had cell signalling functions. This was first shown for the redox protein, thioredoxin [8], which was found to be a secreted T cell-stimulating cytokine-like signal. A few years later, the peptidylprolyl isomerase, cyclophilin A, was reported to be a secreted product of LPS-activated human monocytes with potent pro-inflammatory activities [9]. This was the start of the discovery of the cell signalling actions of secreted cell stress proteins. Currently, around 20 cell stress proteins have been shown to have intercellular cell signalling activity with a growing variety of cells (Table 5.1). This field of study is still in its infancy, with most studies focusing on the influence of cell stress proteins on leukocyte populations. At the time of writing, a story seems to be emerging with cell stress proteins having either pro-inflammatory or anti-inflammatory activities [5] (Table 5.1). The finding that cell stress proteins are secreted has been heavily criticised over the years by individuals who are unaware of the myriad of secretion mechanisms open to the eukaryotic and prokaryotic cell [10]. However, it is quite clear that modifiable secretion of cell stress proteins occurs, as exemplified by the differences in levels of some of these proteins in humans suffering either acute or chronic disease states [6].

Table 5.1 Identity of secreted/circulating cell stress proteins in humans

Cell stress proteins	Pro-inflammatory activity	Anti-inflammatory activity
Ubiquitin	No	Yes
ISGN15	Yes	No
HSP10	No	Yes
Trx80	Yes	No
Thioredoxin (Trx)	No	Yes
Glutaredoxin	?	?
Cyclophilin A	Yes	No
Cyclophilin B	No	Yes (?)
HSP27	No	Yes
Peroxiredoxins	Yes	No
HSP40	No	Yes
Protein disulphide isomerase (PDI)	?	?
HSP60	Yes	No
HSP70 (HSPA1A)	Yes	No
BiP (HSP5)	No	Yes
HSP90	?	?
Gp96	Yes	No
Clusterin	Yes	No

See Henderson and Pockley [6] for references

5.3 Cell Signalling Actions of Extracellular Cell Stress Proteins

From the original discovery of the cell stress response in 1962, up until the beginnings of our understanding of the protein-folding and intracellular-mechanisms of cell stress proteins in the late 1980s/early 1990s, the prevalent paradigm was that cell stress proteins were intracellular proteins involved only in controlling the folding of intracellular proteins. In spite of a lack of interest by the pioneers of this field, it has become established that secreted molecular chaperones function as intercellular signalling molecules with a bewildering range of functions. Most attention on the signalling actions of cell stress proteins has been on eukaryotic proteins, but there is a growing literature on secreted bacterial cell stress proteins which reveals that these molecules have a complex range of activities relevant to bacteria-host interactions and to bacterial virulence, with many of these functions being unrelated to the control of cytokine synthesis [11].

In the eukaryotic field of cell stress proteins, there has been criticism of the findings that these proteins are able to activate leukocytes and induce cytokine synthesis [12]. This has largely centred around the problem, faced by all workers generating recombinant proteins in *E. coli*, that phlogistic bacterial components, such as lipopolysaccharide (LPS), may contaminate the recombinant protein under study. Curiously, this is one area that individuals working on the cell signalling actions of cell stress proteins have successfully addressed [13]. The counterargument to this charge is provided by studies showing cell signalling activity of cell stress proteins expressed in eukaryotic cells [14], and by the finding that a number of cell stress proteins such as HSP10, HSP27 and BiP have anti-inflammatory actions and, indeed both HSP10 [15] and BiP [16] are in clinical trial as anti-inflammatory agents.

There is now convincing evidence for the role of secreted cell stress proteins as secreted signals involved in the interactions between immune cells and cells of the vasculature [3–5]. Many of the studies of the cell signalling actions of cell stress proteins have focused on the ability of these proteins to induce the synthesis of either, or both, pro- and anti-inflammatory cytokines. This raises an obvious question. Cell stress proteins, like cytokines, can induce target cell populations to synthesise cytokines. As cell stress proteins are a group of secreted signals, can they form networks similar to the networks that cytokines generate? A more fundamental question is whether cell stress proteins and cytokines form interacting networks. Answers to these questions are likely to alter our perception of the homeostatic and pathogenic roles of secreted cell stress proteins. The possibility that cell stress protein/cytokine reciprocal networks play roles in the pathology of inflammatory diseases, such as the PDs, will be considered.

5.4 The Periodontal Diseases (PDs)

An emerging paradigm which, like dark matter and dark energy in cosmology, will alter our perception of human health and disease is the fact that vertebrates are supra-organisms containing huge numbers of resident microorganisms. It is estimated that for every cell in the human body there are 10 bacteria [17]. These bacteria inhabit the skin and the exterior mucosal surfaces of the oral cavity, airways, gut and genitourinary system. In addition to their enormous numbers, these bacteria, which are collectively known as the microbiota, are extremely heterogeneous, with each individual human being colonised by several hundred bacterial phylotypes [17]. Evidence is only now emerging for the possibility that the microbiota may have some impact on the health of the skin or gut, although it is well-established that the vagina is subject to the microbiota-associated disease – bacterial vaginosis [18]. However, the oral microbiota, and specifically those bacteria associated with the gingivae, are linked with an enormous prevalence of oral inflammatory disease in terms of the PDs. The PDs represent a spectrum of conditions ranging from the early signs of disease, termed gingivitis [19], which is the mildest form of PD, and one not associated with tissue damage, to chronic periodontitis [20], aggressive periodontitis [21], periodontitis as a manifestation of systemic diseases [22] and necrotising periodontal diseases [23]. The two major PDs are chronic periodontitis and, the less common, aggressive periodontitis. Currently, it is difficult to objectively discriminate these two forms of disease other than through the application of defined diagnostic criteria. These conditions are serious causes of human morbidity, but not life-threatening. However, a related necrotizing stomatitis, known as NOMA or *cancrum oris,* which is associated with malnutrition, has, if untreated, a high mortality rate and can cause severe facial disfigurement [24].

It has been known for years that the PDs are extremely common. However, a recent analysis of the National Health and Nutrition Examination Survey (NHANES) in the USA, has shown just how common they are, with the estimate that periodontitis (mainly chronic and, the less common, aggressive periodontitis) affects over 47 % of adult Americans [25]. This huge prevalence of periodontitis represents an enormous burden on national health systems. For example, the National Health Service in England spends £3.3 billion on dental treatment, a good proportion of this on the PDs [26]. Such cost is due to the fact that current treatment for periodontitis is labour-intensive, requiring a combination of dental auxiliary team and specialist clinicians to improve oral hygiene and provide different forms of non-surgical and surgical treatments to the diseased gingivae. The cost of the PDs worldwide must be in the order of 10–100 billions of dollars – a figure that will clearly increase dramatically with a growing global middle class who want to retain their teeth [27].

The enormous prevalence of the PDs, and the human suffering they produce, makes it an urgent matter to understand the causation of these conditions. Clearly, disease is linked to the bacteria populating the gingiva and, in particular, populating

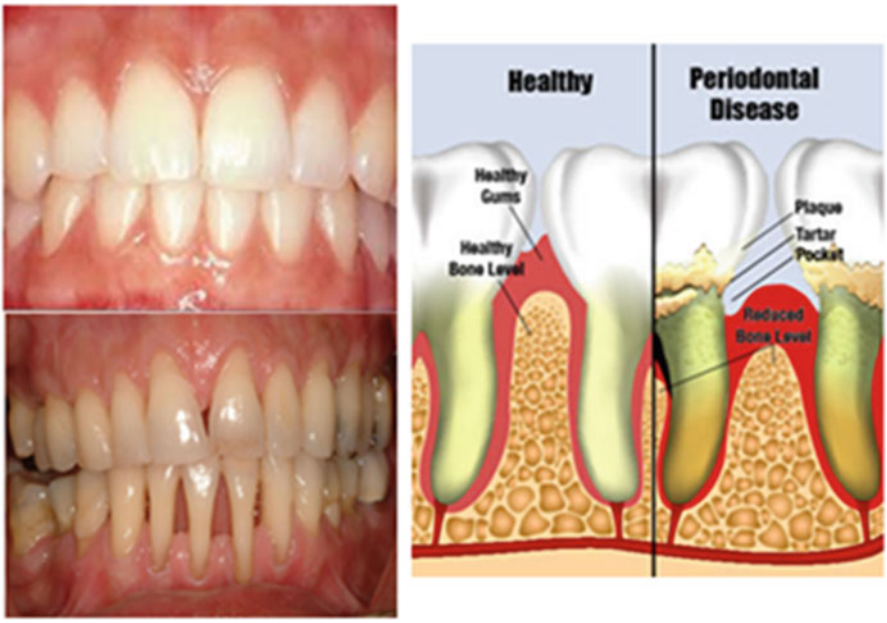

Fig. 5.1 The pathology of periodontitis. The photograph on *upper left* shows healthy gums and their relationship to the teeth. At the margin between tooth and gum there is a raised sulcus which attaches the teeth firmly to the gums preventing bacterial ingress. In periodontitis, this natural connection is lost and pockets begin to form between the teeth and the gums in which mixed bacterial biofilms accumulate resulting in damage to the periodontal ligament and alveolar bone. In the *lower* photograph the consequences of this damage to the alveolar bone is evident. The diagram on the *right* shows this process of gingival and bone pathology in a diagrammatic fashion

the gingival pockets that are a key sign of this disease (Fig. 5.1). However, periodontitis is not an infectious disease in the sense of being able to be transmitted. Rather, it is due to some alteration in the homeostatic and dynamic networks that exist between the gingivae and its associated tissues and the bacteria colonising the gums [28]. Having said this, there are some oral organisms that appear to aid in the genesis of periodontitis such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* [29]. In addition to the inflammation in the gingivae and the loss of attachment of the gingivae to the tooth, resulting in the production of pockets in which bacteria can grow, the disease, in this case, specifically periodontitis, also causes destruction of the alveolar bone and periodontal ligament that attach the teeth to the jaw bones. The long-term result of periodontitis is tooth mobility and eventually the loss of teeth. Now this is not a lethal effect in the human, but it is likely to be lethal in any other toothed vertebrate. An unexplored potential of the PDs is that it is possible both to experimentally generate the mildest form of the PDs, gingivitis, and to 'cure' the chronic forms of periodontitis. Thus if volunteers cease dental cleansing in one half of the mouth, this sector develops gingivitis within a few days and this inflammation can be maintained for several weeks [30]. Changes in

the tissues of the non-cleansed gingiva can then be compared to the dentally healthy gingiva. Reintroduction of whole-mouth dental cleansing rapidly results in cessation of the gingivitis and thus allows study of both the induction and the resolution of acute inflammation in the human. Likewise, treatment of the chronic condition, periodontitis, 'cures' the disease – at least for a time – due to the removal of the bacteria that are having a part to play in the gingival pathology. This allows study of the least understood phase of inflammation – resolution. Artificially inducing resolution of chronic inflammation is the Holy Grail of inflammation research [31]. However, subtle, but crucial, differences between animals and humans make it unclear if resolution mechanisms in rodents are informative of human inflammation. Properly constituted trials of patients undergoing treatment of periodontitis could produce an excellent human model for the resolution of inflammation.

5.5 Cell Stress Proteins in the Periodontal Diseases

The periodontal diseases represent an unusual spectrum of diseases and one that may be a paradigm for the idiopathic diseases of humanity. To preface this argument it is important to realise that inflammation is an integral part of the pathology of all idiopathic diseases including cancer [32]. A key question is the nature of the signals inducing inflammation in idiopathic disease. These idiopathic diseases do not have the clear-cut pro-inflammatory signals emanating from infectious agents, such as the pathogen-associated molecular patterns (PAMPs), generated by bacteria [33], to account for inflammation. A key consideration in human disease states in which cytokines are key effectors of pathology – and this describes most, if not all diseases – is the nature of the cytokine-stimulating activity or activities. A new paradigm, based on Polly Matzinger's danger signals [34], is that cell and matrix components released as a result of tissue damage can act as endogenous cytokine inducers, thus driving inflammation and tissue pathology. These endogenous signals have been christened Danger- or Damage-Associated Molecular Patterns (DAMPs) after the bacterial PAMPs [35]. Secreted cell stress proteins were originally classed by Matzinger as DAMPs [34]). However, this has been challenged [6, 36] on the basis that cell stress proteins are found in the circulation of the healthy individual and, as such, are not damage-associated. Thus it is postulated that the interrelationships between cell stress proteins and tissue pathology is much more complex than the simple release of materials from damaged cells.

For the past century a search has been made for infectious agents as causative factors in idiopathic disease (e.g. [37]). One of the most active diseases in this respect has been rheumatoid arthritis [38] which, pathologically, closely resembles periodontitis. In recent years, one of the bacteria believed to be involved in the pathology of periodontitis, *Porphyromonas gingivalis*, has also been shown to be a likely causative agent of the autoantibodies that drive rheumatoid arthritis [39]. This is the strongest evidence for the hypothesis that the periodontal diseases

are contributory factors to multiple systemic diseases [40]. Further, this putative relationship between periodontitis and other systemic diseases is one brick in a new hypothesis that suggests that bacterial dysbiosis is an important causative factor in idiopathic disease [41]. Thus with regard to the role of secreted cell stress proteins in periodontitis we need to consider that both bacteria and host proteins are contributing to pathology.

5.6 Secreted Bacterial and Host Cell Stress Proteins in Periodontitis

5.6.1 Bacterial Cell Stress Proteins

Interest in bacteria cell stress proteins in periodontitis goes back to the early 1990s and focuses on the incredible immunogenicity of these proteins [42] and the role that this could play in the chronic inflammation of this disease. Indeed, there is a long history of immunogenicity to bacterial cell stress proteins such as HSP60, HSP70 and HSP90 and the induction of cross-reactivity to human homologues being a mechanism driving T cell responses in diseases such as rheumatoid arthritis, diabetes and atherosclerosis [43]. Surprisingly, it is proposed that this immunogenic response to specific cell stress proteins may be a therapeutic target in these diseases [44]. An obvious question is why the immune system responds to highly conserved proteins such as HSP60. An answer which has come, in part, from the bacteria involved in periodontitis, is that cell stress proteins, as explained, can be very potent signals for immune cells. In the periodontal context this was first shown by the lead author, who found that the HSP60 protein of *A. actinomycetemcomitans* was secreted by this bacterium and functioned as a very active signal inducing the formation of the bone-resorbing myeloid cell population known as osteoclasts. Furthermore this protein worked on bone from TLR4-negative mice, suggesting a novel mechanism of action of this cell stress protein [45, 46]. Curiously, the homologous proteins from the major pathogen, *Mycobacterium tuberculosis*, which has two paralogous proteins, either have no effect on bone (HSP60.2) or actually inhibit the formation of osteoclasts (HSP60.1) [47]. It is possible that similar differences may be seen with the cell stress proteins of the phylogenetically-distinct bacteria found in the periodontal pocket. Other workers have found that the HSP60 protein of *A. actinomycetemcomitans* can promote vascular endothelial cell proliferation [48]. Another group has reported that this same protein has a range of effects on epithelial cells [49–51]. In contrast to the *A. actinomycetemcomitans* protein, the *P. gingivalis* HSP60 protein stimulates NF-kB in human monocytes via interaction with TLR2 and TLR4 [52] and this protein also stimulates IL-1 expression by an oral keratinocyte cell line [53]. The only other HSP60 protein to be examined is that from the key oral bacterium, *Fusobacterium nucleatum*, which has pro-atherogenic affects [54]. Finally, the HSP90 protein of *P. gingivalis*

has been reported to induce CXCL8 synthesis by cultured monocytes and vascular endothelial cells [55]. Of interest, in this respect, is the report that leukocytes from patients with periodontitis were less responsive to bacterial cell stress proteins than those from healthy individuals [56].

Given that the periodontal pockets in patients with periodontitis may contain hundreds of different bacterial phylotypes, all of which may be secreting cell stress proteins, with each protein having a different spectrum of biological activity, the role of bacterial cell stress proteins in periodontitis is likely to contribute to the pathogenesis of disease. So far, no one has reported circulating levels of these bacterial proteins, and it is not clear if these proteins could enter into the bloodstream. However, it is established that periodontal patients have both B and T cell responses to these bacterial cell stress proteins, which may play some role in tissue pathology. Immune reactivity to bacterial HSP60 proteins could be important in the pathology of periodontitis and diseases related to periodontitis. Indeed one of the earliest studies suggested salivary antibodies to mycobacterial HSP60 could diagnose gingivitis and even suggested a link with atherosclerosis [57]. In a small treatment study, levels of antibodies to *P. gingivalis* HSP60 declined following therapy [58]. The natural assumption is that immunity to cell stress proteins is pathogenic. However, circulating levels of IgG antibodies to *P. gingivalis* HSP90 are inversely correlated with disease status – in other words these antibodies appear to be protective, suggesting some pathogenic role for the HSP90 protein [59], such as its ability to induce CXCL8 synthesis [55].

There is reasonable evidence for the hypothesis, propounded by Georg Wick in the 1990s, that cross-reactive immunity to host HSP60, due to immune responses to bacterial HSP60, is a key factor in atherogenesis [60]. This is of interest in the context of the past 20 years of research in which it has been proposed that periodontitis is a risk factor in atherosclerosis [61]. There is evidence that immunogenicity to *P. gingivalis* HSP60 [62], or to a specific HSP60 epitope [63], is associated with autoimmune and cardiovascular systemic disease states. Indeed, there is evidence that bacterial HSP60 proteins can directly induce atherogenic changes in vascular endothelial cells [64, 65]. In animals, induction of oral tolerance to self HSP60 or HSP60 epitopes can reduce atherogenesis [66, 67]. Thus it is interesting that a recent study has reported that sublingual vaccination of apolipoprotein E-deficient mice with the HSP60 protein from *P. gingivalis* significantly reduced the formation of atherosclerosis [68].

5.6.2 Self Cell Stress Proteins

Do self cell stress proteins play any role in periodontitis? Given the chronic nature of periodontitis and the added stimuli of bacterial factors, it would be assumed that the major stress responses in the various cellular compartments (endoplasmic reticulum (ER), cytosol, mitochondria, etc.) of gingival cells would be upregulated leading

to increased secretion of these proteins. The literature on this subject is sparse but it is reported that levels of ER stress proteins are elevated in gingival biopsies for periodontitis compared to gingivitis patients [69].

It appears that to date, only a few studies have focused on the circulating levels of cell stress proteins in periodontitis [70–72]. The report by Shamaei-Tousi and colleagues is a study of patients with periodontitis versus periodontally-healthy controls and also includes a treatment group to see what effect this has on circulating analytes. The circulating cell stress proteins measured were HSP10 and BiP which, as stated, are both in clinical trial as anti-chronic inflammatory agents, as well as HSP60, which is normally regarded as a pro-inflammatory signal when secreted. Now, the transcription of HSP10 and HSP60 are controlled by a single bidirectional promoter which generates twice as much HSP60 as HSP10 [73]. This reflects the fact that the HSP60 oligomer is a tetradecamer whereas HSP10 is a heptamer.

Circulating levels of HSP10 and of BiP were significantly higher in dentally-healthy controls than in patients with periodontitis (Fig. 5.2) suggesting that these two cell stress proteins were either not being secreted or were being removed from the circulation in patients with periodontitis [71]. Indeed, there was an

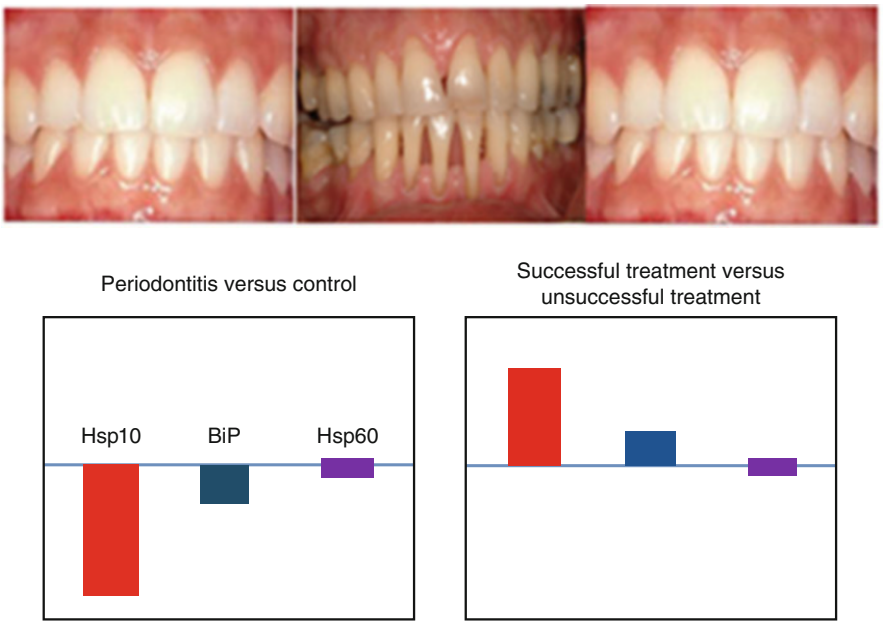

Fig. 5.2 Circulating levels of cell stress proteins in periodontal patients. This diagram attempts to encapsulate the data from a study of circulating levels of HSP10, HSP60 and BiP in controls and periodontal patients and in periodontal patients following successful treatment. Levels of HSP10 and BiP in the circulation of periodontal patients were lower than found in healthy controls. In contrast, HSP60 levels were not really any different in the two groups. Successful treatment of periodontitis was associated with significant increases in levels of HSP10 and in a less significant increase in BiP. There was no change in HSP60

inverse relation between circulating levels of HSP10 and the clinical periodontal parameters being measured. While most individuals showed the presence of HSP10 and BiP in their circulation only around half exhibited circulating HSP60. There was no difference in the proportion of controls or patients with zero or levels of HSP60 > 1 μg/ml. Inexplicably, significantly more periodontal patients than controls had levels between 1 ng and 1 μg. It is not clear what this means. What was more surprising was that there was no correlation between circulating HSP10 and HSP60 levels in either controls or patients. As HSP10 production is half that of HSP60, but circulating levels of these two proteins do not correlate, this suggests that the control over circulating levels of these two proteins is much more complex than was envisaged envisage. It must be controlled by levels of removal of the protein rather than by the degree of production. Such removal could occur by various mechanisms, including by binding to target cells and uptake of the protein by receptor-ligand uptake mechanisms. There may also be selective binding proteins which could prevent antibody binding.

Were the lower levels of HSP10 and BiP a response to the inflammation? One hypothesis is that these proteins are interacting with pro-inflammatory target cells, in an attempt to dampen their activity, and control inflammation, and, in essence, are being used up by the disease process. Support for this hypothesis would occur if treatment of the periodontitis resulted in a renormalisation of the circulating levels of these cell stress proteins. Of the 80 periodontal patients half were given an extremely detailed treatment regime which was designed to clear up all signs of disease for a prolonged period. The other patients were provided with a routine treatment schedule which did not produce such clear-cut changes in local pathology. Patients showing clear-cut signs of improvement also had elevated levels of HSP10 and, to a lesser extent, BiP. There was no change in HSP60 levels in blood. This is shown diagrammatically in (Fig. 5.2). In contrast, those patients not showing clinical improvement did not exhibit changes in HSP10 or BiP levels. As explained, human HSP10 has anti-inflammatory properties in mice [74] and men [15]. The renormalisation of the levels of this protein in the human circulation after successful periodontal therapy strongly suggests that this protein is being utilised as a natural anti-inflammatory. Indeed, this protein was initially identified in the 1970s as a circulating immunosuppressive factor generated in women in the first trimester of pregnancy. It is postulated that its main function is to protect the implanted ovum from immune attack (reviewed by [75]). So it is possible that periodontitis could be even more serious a disease without the intervention of circulating HSP10 and BiP.

Another case-control study found increased HSP60 levels in the circulation of patients with mild periodontitis compared with healthy individuals with an apparent gradient increase by disease severity [72]. This finding did not match the similar immunohistochemical HSP60 expression in gingival tissues between the same two groups and was also in contrast with previous studies [70], possibly due to differences in case selection and laboratory assays used and leaves a question mark on the meaning of HSP60 levels in periodontitis.

In unpublished studies of circulating levels of another potential anti-inflammatory cell stress protein, HSP27, we have found that this protein in the

circulation is not simply a biomarker but is also a diagnostic marker allowing discrimination between the two major forms of this disease – chronic and aggressive periodontitis. This is one of the first reports of a cell stress proteins acting as a diagnostic marker and suggests that HSP27 is playing a discriminatory function in these two forms of periodontitis.

5.7 Conclusion

Studies of the role of secreted and circulating cell stress proteins in tissue pathology and as markers of human pathology and still in their infancy and it is surprising, given our lack of real knowledge of these proteins, that it has already been established that some of these proteins (e.g. HSP10 and BiP) have therapeutic potential and that others, for example, cyclophilin A are therapeutic targets in inflammatory disease [76]. To fully appreciate the role of circulating cell stress proteins in human disease, and in particular, human inflammatory disease, requires human pathologies that can be successfully treated, at least in the short time. Periodontitis is one such condition. Given its prevalence and relative ease of treatment this disease makes an ideal model for human inflammatory disease studies. It is hoped that more groups will see the potential of this disease and use it to elucidate the role of secreted cell stress proteins in human pathology.

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Part III
Heat Shock Protein HSP70-Based Therapies

Chapter 6
The Role of Heat Shock Protein 70 in Infection and Immunity

Jose Rey-Ladino, Abiola Senok, Abdullah Sarkar, and Ahlam Al Shedoukhy

Abstract Heat shock protein 70 (HSP70) has been the subject of intense research concerned with infectious diseases and the immune response. HSP70 is found to be associated with both host and microbial cell surface membranes where it appears to assist in the attachment and colonization of host cells by pathogens. Following infection, HSP70 readily promotes microbial survival, although in certain circumstances such as during some viral infections, it inhibits microbial growth. Regarding immunity, HSP70 induces the activation of both innate and acquired immune responses. These unique immune capabilities of HSP70 are broadly employed for the design of novel vaccines against a variety of infectious diseases.

Keywords Infectious diseases • Parasites • Infection and immunity • Vaccines • Heat shock proteins and infection • HSP70 • Adjuvants

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Abbreviations

APCs	Antigen presenting cells
BCG	Bacillus Calmette–Guérin
CFA	Complete Freund's adjuvant
CMV	Cytomegalovirus
DnaK	Bacterial HSP70
DTH	Delayed type hypersensitivity
GroEL	Bacterial HSP60
H2O2	Hydrogen peroxide
HIV-1 and HIV-2	Human immunodeficiency virus 1 and 2
HIV-p24	Human immunodeficiency virus protein p24
HSP	Heat shock protein
HSV1	Herpes simplex 1
HTNV	Hantaan virus
KMP11	Kinetoplasmid membrane protein 11
LCMV	Choriomeningitis virus
LPS	Lipopolysaccharide
NO	Nitric oxide
NP	Nucleocapsid protein
Pf72	*Plamodium falciparum* HSP72
pfHop	*P. falciparum* organizing protein complex
PfHSP 70-1 and PfHSP 70-2	*P. falciparum* HSP70-1 and 2
PPD	Purified protein derivative
ROI	Reactive oxygen intermediates
RSV	Respiratory syncytial virus
SV40	Simian virus 40
TAP	Transporter associated with antigen processing
TC1	*T. cruzi* antigen 1
TLR	Toll-like receptor
VSV	Vesicular stomatitis virus

6.1 Introduction

Heat shock protein 70 (HSP70) is a family of ubiquitous molecules expressed by most organisms from microbes to mammals [1]. HSP70 is one of the most conserved proteins known. Analysis of the amino acid sequence demonstrates that human HSP70 is 72 % identical to *Leishmania amazonensis*, 73 % identical with *Drosophila* HSP70 and 47 % identical to *E. coli* dnaK (bacterial HSP70). Furthermore, *L. amazonensis* HSP70 is 95 % identical to *L. donovani* or *L. major* HSP70, and it is 85 % identical to the more distant HSP70 from *Trypanosoma cruzi*

[2, 3]. HSP70 proteins are expressed constitutively and further induced in response to a variety of stress conditions, including heat shock, oxidative stress, ischemia-reperfusion injury, radiation, chemicals,: nutrient deprivation and infections. The main function of HSP70, as well as other heat shock proteins (HSP), is to protect cells from injury by promoting the refolding of denatured proteins [4].

Studies of host pathogen interaction and expression of HSP at infection have led to hypothesize that HSP are important for the survival of intracellular pathogens such as *Plamodium*, *Leishmania*, *Mycobacteria*, and *Toxoplasma*. From the microbial viewpoint, host cells such as phagocytes represent a hostile environment due to the presence of toxic molecules including low pH, nutrient deprivation, proteases, nitric oxide (NO), reactive oxygen intermediates (ROI) and high temperatures. Experimental evidence indicates that HSP are important for microbes to survive within these toxic environments of host cells. With regard to HSP70, it promotes thermotolerance, a condition that allows microbes to withstand a secondary, more severe, heat shock treatment [5]. Indeed, *E. coli* strains lacking HSP70 are highly sensitive to heat shock, but become resistant to heat following transfection with HSP70 from *Plamodium falciparum* [6]. In the context of infection, experimental evidence also demonstrates that HSP70 contributes to intracellular survival of pathogens. Disruption of *P. falciparum* HSP70, as well as inhibition of the ATPase activity of HSP70, severely affects development of malarial parasites within erythrocytes [7, 8]. Of note, optimal function of HSP70 requires the presence of HSP70 partners including HSP40, HSP60 as well as HSP90 [9].

HSP in general are among the most immunogenic antigens found in nature, stimulating both innate and antigen-specific immunity. With respect to innate immunity, HSP70 is secreted from host cells into the extracellular milieu. Extracellular HSP70 triggers innate immunity via activation of antigen presenting cells (APCs) [10–12]. Regarding adaptive immunity, HSP70 is an abundant antigen of both B and T cells. *P. falciparum* HSP70 is expressed by most parasites within their life cycle and it is recognized by sera from malaria patients [13]. Sera from *S. mansoni*-infected individuals contain antibodies recognizing *S. mansoni* or *S. japonicum* HSP70 [14, 15]. Both antibodies and T cells recognize *M. tuberculosis* HSP70 as determined in patients with tuberculosis [16]. Interestingly, antibodies and T cells that recognize HSP70 have been identified also in apparently healthy individuals, suggesting that HSP70 may provoke autoimmunity due to molecular mimicry.

The immunogenicity of HSP70 and its capacity to activate antigen-specific immunity have made this protein an ideal candidate for vaccine development. Vaccines employing HSP70 have been tested against various infectious conditions. Protective immunity and resistance to infection have been observed to develop in *Salmonella* [17] and cytomegalovirus [18]. However, no protection has been experienced with vaccines against various fungal infections [19]. This review will examine current issues on the role that HSP70 plays in the infection process and its importance in immunity against microbial infection. It will also examine existing evidence suggesting that HSP70 can be a potential vaccine candidate adjuvant.

6.2 The Role of HSP70 in Infection

6.2.1 HSP70 and Bacterial Infections

Salmonella typhimurium is a common bacterium causing diarrhea in humans in many countries around the world. Evidence has shown that colonization of cells by bacteria requires the assistance of stress proteins. Treatment of *S. typhimurium* with the toxic H_2O_2 results in the induction of at least 30 proteins [20]. HSP70 (DnaK) is one of the 30 HSP proteins induced by H_2O_2, and is also induced by heat shock [21]. A study shows that S. *typhimurium* overexpresses both HSP60 (GroEL) and the HSP70 (DnaK) during infection of macrophages and the presence of these HSP is essential for survival of the pathogen within the infected cell [22, 23]. Furthermore, DnaK/DnaJ mutants of S. *typhimurium* could not survive or proliferate within macrophages, and the bacteria were unable to invade epithelial cells in vitro and could not secrete any of the invading proteins encoded within a Salmonella pathogenicity island 1 [24]. Interestingly, Monocytic cell line J774A.1 infected with virulent *S. choleraesuis* died spontaneously due to TNF-α production by the infected cell. Induction of HSP70, however, resulted in significant survival of the infected monocytes [25]. Thus S. *typhimurium* HSP70 (DnaK) and its co-chaperone DnaJ play a significant role in *Salmonella* infection [24].

Mycobacterium tuberculosis infection of human THP-1 cells induced expression of at least 16 proteins. Some of these proteins are also expressed by the bacteria extracellularly in culture medium in the presence of heat shock, H_2O_2 or low pH [26]. Furthermore, *M. tuberculosis* overexpressing HSP70 was shown to express full virulence at the initial stage of infection. The bacterium, however, was significantly impaired in its ability to persist during subsequent chronic phase of infection [27].

Helicobacter pylori is a gram-negative bacterium causing gastric ulcers. The microbial HSP70 as well as HSP60 is shown to associate with the microbial cell membrane and their presence mediates attachment of the bacteria to gastric epithelial cells. Interestingly, though, both HSP70 and HSP60 are increased following acid shock of cells in vitro and their expression correlates with increased inflammation of the gastric mucosa [28].

Chlamydia trachomatis is known for leading the burst of sexually transmitted infections globally causing pelvic inflammatory disease and infertility [29]. Chlamydial HSP70 has been found expressed on the cell surface of elementary bodies – the infectious forms of *C. trachomatis*. Expression of HSP70 on the cell membrane of bacteria, however, does not seem to help attachment of bacteria to host cells and it may be involved in host immune recognition [30, 31].

Yersinia enterocolitica is a facultative intracellular pathogen that invades epithelial cells of the intestine causing acute diarrhea in humans. At least 16 proteins, including *Y. enterocolitica* HSP70 (DnaK) were selectively induced in the macrophage-like J774-1 cells infected with *Y. enterocolitica*. This HSP70 (DnaK) was invariably induced by the bacteria in vitro in response to heat shock (HS) at

42 °C or following oxidative stresses. Thus, *Y. enterocolitica* HSP70 is expressed as a global stress response of the bacteria to the hostile environment of the macrophage [32].

Bordetella pertussis produces two toxins including adenylate cyclase-hemolysin and pertussis toxin. Expression of either adenylate cyclase-hemolysin or purified bacterial toxins reduces the expression of HSP70 in *B. pertussis* infected macrophages, suggesting that HSP70 may be involved in host protection against *B. pertussis* [33].

Staphylococcus aureus HSP70 plays a dual role in infection of host cells. From one side HSP70 is a receptor for *S. aureus* attachment and internalization by host monocytes [34]. From other side, HSP70 inhibits apoptosis of host monocytes induced by *S. aureus*. Indeed, human peripheral blood monocytes die by apoptosis following phagocytosis of *S. aureus*. However, induction of HSP70 expression renders monocytes resistant to *S. aureus*-induced apoptosis [35].

6.2.2 HSP70 and Parasitic Infections

HSP appear to play a major role in the survival of parasites following invasion of mammalian cells at 37 °C. In effect, parasites must adapt to the high temperatures of host cells and to the presence of stressing conditions of oxygen radicals, nitric oxide, lack of nutrients, toxic compounds, etc. In this regard, *Trypanosoma*, *Leishmania*, *Plasmodium* and *Schistosoma* have all been found to express constitutive or induced forms of HSP at high concentrations [36].

Trypanosoma is a unicellular parasite transmitted by the bite of an insect vector causing Chagas disease in Central and South America. *Trypanosoma* Also causes sleeping sickness in Africa. The epimastigote developmental stage of *Trypanosoma* is transmitted by the insect vector upon feeding on host blood. Within the mammalian host, epimastigotes differentiate into flagellated trypomastigotes. A stress response may play a role during parasite transition from insect vector to mammalian host and may trigger expression parasite HSP. Indeed, mRNAs of *T. brucei* HSP70 and HSP83 are augmented 100-fold in trypomastigotes exposed at 37 °C as compared with forms found in the insect vector at approximately 24 °C [37]. At least six *hsp70* genes, which are transcribed as long polycistronic molecules, have been described in *T. brucei* [36]. Furthermore, epimastigotes of *T cruzi* express 10 major proteins ranging from 60 to 83 kDa upon heat shock at 41 °C [38]. Approximately 11 genes encoding HSP70 proteins have been identified in the *T. cruzi* proteome. Some of these are highly expressed in epimastigotes whereas some others are expressed in trypomastigotes [39].

Leishamnia is an intracellular protozoan parasite transmitted by the bite of sandflies. *Leishmania* causes a wide spectrum of diseases including cutaneous, mucocutaneous and visceral leishmaniasis. The parasite life cycle includes two forms: a flagellated promastigote surviving within the alimentary tract of the insect vector and the amastigote (without flagellum) living within the parasitophorous

vacuole of infected macrophages. *Leishmania* contains multiple copies of *hsp70* genes, with absolute copy number varying among strains with *L. major* containing at least 14-associated *hsp70* genes [40].

Leishmania has to survive within host macrophages and requires adaptation to this new environment following infection. Both *L. donovani* HSP70 and HSP60 were found expressed in murine macrophages following infection [41]. Heat shock (HS) treatment of *L. chagasi* promastigotes makes leismanial parasites resistant to macrophage-induced oxidative stress [42]. Similarly, HS treatment of *L. tarentolae* promastigotes causes the parasite to develop resistance to the leishmanicidal effects of pentavalent antimonials [43]. Interestingly, Balb/c mice inoculated with *Leishmania infantum* lacking *hsp70* genes did not develop *Leishmania*-associated pathology or disease. Instead, these mice develop a Th1 immunity and resistance to *L. infantum* infection suggesting that *L. infantum* HSP70 may be associated with leishmanial pathogenesis [44, 45].

Malarial disease is caused by intracellular parasites of the genus *Plasmodium*. Within this genus, *Plamodium falciparum* is the most common species identified, causing nearly 75 % of all malaria cases. *P. falciparum* is transmitted by a female mosquito of the genus *Anopheles*. Most HSP70 studied in malaria are referred to the intracellular erythrocytic stage of the parasite. At least six HSP70 (pfHSP70) isoforms have been described in *P. falciparum* [46, 47]. The proteins encoded by these genes are constitutively expressed at all blood stages of *P. falciparum*. A member of the HSP70 family of 75 kDa is expressed on the surface of merozoites, and it is recognized by the immune response [48]. Another *P. falciparum* HSP70-1 (Pf72/HSP70-1) is a major immunogen expressed in infected erythrocytes and found experimentally to protect Saimiri monkeys against malarial infection [49].

P. falciparum HSP70 functions to promote parasite survival within host cells. Disruption of *P. falciparum* HSP70 and HSP90 complexes and inhibition of the ATPase activity of HSP70 inhibits development of parasites in infected erythrocytes. Exposure of parasites at 41 °C, the equivalent to malaria-induced febrile disease in the host, promotes parasite development in human erythrocytes [7, 8]. Furthermore, transfection of *E coli* lacking HSP70 (DnaK) with *P. falciparum* HSP70 causes thermosensitive *E coli* to become thermoresistant [6]. Together these observations suggest that HSP70 promote parasite survival possibly by inducing thermotolerance.

It is becoming clear that, *P. falciparum* HSP70 works together with other molecular chaperones during malarial infection. Within erythrocytes *P. falciparum* HSP70 associates with HSP90 and form the HSP70-HSP90 organizing protein (pfHop) complex. This protein conglomerate promotes parasite survival via chaperoning signal transduction pathways [50]. Furthermore, gene analysis demonstrated that within erythrocytes, HSP70 and HSP90 associate with co-chaperone HSP40 [51]. In addition, HSP70 was found exported from the parasite to the erythrocyte cytosol where it associates with HSP40 [52], and both HSP70 and HSP40 form the *P. falciparum* virulence factor (pfEMP1) complex. The pfEMP1 complex provides adherence to the infected erythrocyte [52, 53]. Thus, *P. falciparum* HSP70 and its associated molecular chaperones work in concert to promote parasite survival and transmission.

Toxoplasmosis gondii is a protozoan parasitic that invades mammalian cells causing neurologic diseases. It can cross the placenta and infect the fetus, causing abortion [54]. At least five HSP70 isoforms have been identified in *T. gondii* [40]. The role played by *T. gondii* HSP70 in parasite survival has not been completely defined. One study shows that *T. gondii* HSP70 assists the conversion of parasite from the bradyzoite to the tachyzoite stage, and that this effect occurs primarily during reactivation of chronic toxoplasmosis [55]. Another study shows that *T. gondii* HSP70 induce maturation of dendritic cells, and that maturation involves Toll-like receptor 4 (TLR4)-mediated signalling pathway. These mature DCs are able to prime Th1 T cell responses and promote resistance against *T. gondii* infection [56]. Interestingly, *T gondii*-infected mice develop antibodies against *T. gondii* HSP70, which cross-react with human HSP70 suggesting autoimmune recognition [57].

Schistosomiasis is a human disease caused by helminths of the genus Platyhelminths and highly prevalent in Africa, the Middle East and Asia. Children with schistosomiasis develop anemia, malnutrition and learning difficulties [58]. Four genes corresponding to HSP70 have been cloned from schistosomes. The HSP70 proteins are expressed in larvae and in adult organisms. At least one of these *hsp70* genes is inducible in *S. mansoni* [59]. Interestingly, fully-differentiated schistosomula – the stage found in humans – can induce expression of the *hsp70* gene. Furthermore, the *S. mansoni hsp70* gene is expressed constitutively in miracidia, the parasite stage in snails but not in cercaria (the developmental form that infects humans). In cercaria, however, the gene is induced at 42 °C [36, 59]. These observations suggest that the expression of *Schistosoma* HSP70 may help parasite transmission from low temperatures to high host temperature (37 °C).

6.2.3 HSP70 and Fungal Infections

Histoplasma capsulatum is a dimorphic fungus that survives as a multicellular filamentous stage (mycelia) at temperatures close to 25 °C, and as unicellular (yeast) at 37 °C. The transition between mycelia and yeast can be reversibly induced in the laboratory by shifting these temperatures. Interestingly, mRNA studies have shown that *H. capsulatum* maintains mRNA processing at high temperatures when phase transition from mycelia to yeast is induced in a culture at 42 °C. Since HSP are abundant in mycelial cells at normal temperatures and during phase transition [36], it is suggested that HSP, particularly HSP70, may promote normal mRNA processing and normal cell function at 37 °C by protecting the splicesome [60, 61]. In the yeast *Candida albicans* optimal expression of HSP appears to vary between strains with different degree of virulence. Non-virulent strains express maximum transmission of *hsp70* and *hsp82* genes at 34 °C. On the contrary, virulent organisms expressed maximal transcription of *hsp* genes at 37 °C [62]. Of note, in *Cryptococcus neoformans* HSP70 is associated with the fungal cell surface, where it may have a role in interaction of the yeast and host cells [63].

6.2.4 HSP70 and Viral Infections

HSP70 is one of the most studied HSP with respect to the role of chaperones in the biology of viruses. HSP70 induces replication of various DNA viruses including herpes virus (HSV1) [64, 65], vaccinia virus [66], adenovirus [67, 68], simian virus 40 (SV40) and others. HSP70 also assist positive- and negative-stranded RNA viruses in infection of host cells. In measles (negative-strand RNA virus), HSP70 interacts with virus nucleocapsid N protein and assists viral capsid formation and optimal viral replication [69]. Furthermore, transgenic mice overexpressing *hsp72* gene in neurons showed augmented measles viral burden in the brain of mice following viral infection [70].

HSP70 assists virus replication at various levels. In human cytomegalovirus (CMV)-infected cells, HSP70 localizes to the nucleus early in infection and then translocate to the cytoplasm late after infection [71]. In the Hantaan virus (HTNV) infection of Vero E6 cells, HSP70 is also shuttled to the cell nucleus and then to the cytoplasm. Within the cytoplasm HSP70 associates with HTNV nucleocapsid protein (NP) resulting in control of expression levels of viral structural proteins and virus assembly [72].

Virus infection, replication and assembly may require the assistance of various molecular chaperones. In polyomavirus, HSP70 interacts with capsid proteins VP1, VP2 and VP3 in an ATP-sensitive manner within the cytoplasm of various host cells. When bound to VP1, HSP70 inhibits the assembly of viral capsids. However, in the presence of ATP and DnaJ and GrpE chaperones, VP1 assembles into complete uniform capsids [73].

In simian virus 40 (SV40), infection of mouse cells by SV40 results in the induced expression of HSP70 and HSP90 [74]. In mouse keratinocytes, HSP70, HSP60, as well as HSP90 were found induced following SV40 infection. However, induction of these chaperones was accompanied by down-regulation of small HSP27 [9]. Thus, diverse chaperone families take part in virus replication but their activation and/or inhibitory activity on viruses may depend on the infected host cell and the conditions of infection.

Remarkably, HSP70 has been identified as a virion component in various RNA viruses, including influenza A virus, vesicular stomatitis virus, rabies virions, and HIV-1 particles [9]. RNA viruses have developed a unique adaptation pathway for multiplication, and HSP70 may be directly involved in this replication pathway.

Rotavirus causes gastroenteritis and watery diarrhea and children around the world are most susceptible. Rotavirus infection of epithelial cells lining the gastrointestinal tract is assisted by host HSP70. HSP70 is part of a receptor complex that binds rotaviruses [75]. Attachment of viral particles to HSP70 occurs via rotaviral structural protein VP5 [9]. Following rotavirus infection, levels of intracellular HSP70 are subsequently induced, resulting in augmented production of rotavirus structural proteins VP2, VP4, and VP6. Interestingly, inhibition of intracellular HSP70 in rotavirus-infected cells results in significant reduction of viral particles produced [76]. Thus, HSP70 has a double role in rotavirus infection, a viral receptor component, and as a promoter of viral replication.

Human immunodeficiency virus 1 and 2 (HIV-1 and HIV-2) target and kill $CD4^+$ T helper cells. Evidence shows that HSP70 plays a role in HIV infection and replication. Increased levels of HSP70 are observed in human lymphoma cells chronically infected with HIV-1 as well as in lymphocytes from HIV-1 infected patients [77, 78]. Within infected cells, HSP70 facilitates the import of HIV-1 pre-integration complexes into the cell nucleus, leading to virus integration in host chromosomes [79]. Interestingly enough, in macrophages, the presence of recombinant HSP70 significantly diminished replication of HIV-1 [80]. However, the presence of co-chaperone HSP40 induces viral gene replication, suggesting that coordination between HSP70 and its co-chaperone HSP40 decides either inhibition or activation of HIV-1 replication [81].

Regarding replication inhibition, various studies showed that in some circumstances HSP70 is involved reduction of viral infections. For example, increased expression of HSP70 by heat treatment significantly reduced virus replication in neurons infected with vesicular stomatitis virus (VSV) [82]. Furthermore, constitutive expression of *hsp70* genes in neurons led to the clearance of VSV particles from mice brain, resulting in reduced mice mortality. Interestingly, this effect correlated with the secretion of HSP70 by VSV-infected neurons and with the enhanced expression of type I interferons [82]. Another study showed that expression of HSP70 correlated with protection against influenza virus. This mechanism of protection involves the polymerase activity, which negatively regulates viral transcription [83, 84]. Negative effects of HSP70 in viral replication have also been reported in rotaviruses [76] as well as in respiratory syncytial viruses [85]. The mechanisms associated with HSP70 down-regulation of viral replication are not well understood. They may be explained, in part, in the context of a global heat shock response. Heat treatment of cells down-regulates the NF-kB signalling pathway, leading to replication inhibition of some viruses such as HIV-1 [9, 65].

6.3 HSP70 and the Host Immune Response to Infection

6.3.1 HSP70 as Antigen

HSP in general are among the most immunogenic antigens found. It is suggested that the immunogenicity of these proteins is a direct consequence of their abundance, which by virtue of mass action leads to the processing and presentation by antigen-presenting cells [13]. It is also suggested that the immunogenicity of at least some HSP may be related to functional association with the MHC-processing machinery [86]. Furthermore, invading microbes undergo stress due to primary host defence mechanisms, which causes up-regulation of microbial HSP, making them targets of immunity [87].

In any case, HSP appear to be highly immunogenic in their own right. Antibodies and T cells that recognize HSP have been identified in a variety of infections, and also in apparently healthy individuals. The later findings have led to the suggestion

that HSP may play an important role in immune surveillance. Thus, anti-HSP immune responses appear to be regularly induced as a result of frequent contact with low virulence organisms. Repeated contact with low virulence pathogens impels the immune system to focus on regions of HSP conserved in the microbial world. This may provide a mechanism for rapid and specific responses to eventual encounters with more highly virulent microbes [86].

With respect to HSP as immunogens of infection, evidence indicates that HSP70 and in some cases HSP90 are major targets of the immune response in parasitic infections. For example, *P. falciparum* HSP70-1 and 2 (PfHSP 70-1 and PfHSP 70-2) are two abundant antigens of *P. falciparum* HSP70 that are expressed at all stages of the parasite life cycle. Although they share 64 % of amino acid identity, antibodies raised against either of them do not show cross-reactivity indicating that the common sequences are non-immunogenic [13]. The PfHSP 70-1 antigen is expressed on the surface of infected hepatocytes, where it is the target of antibody-dependent cell-mediated cytotoxicity.

As mentioned, a major HSP70-related immunogen Pf72/HSP70-1, which is present in blood stages of *P. falciparum*, has been found to protect *Saimiri* monkeys against infection. Fifty-two percent of individuals living in an endemic zone in West Africa have antibodies to this antigen. Furthermore, T cells specific for epitopes within the C-terminus of this protein are found in individuals continuously exposed to the parasite. The same epitopes are not recognized by T cells of non-exposed Europeans. However, since some of these T cell epitopes are also present in the homologous human HSP70, the use of this antigen in vaccine development against malaria remains controversial [49]. An antigen of *P. falciparum* which shares 55 % of amino acid identity with PfHSP 70–1 and 72 % identity with grp78 is also recognized by sera from infected patients [88].

In *T. cruzi*, the antigen TC1 that belongs to the HSP70 family has been cloned from the λ gt11 expression library. Antibodies against this antigen do not cross react with human HSP70, despite 73 % of amino acid homology between the proteins [89]

A HSP70 molecule from *S. mansoni* is recognized by sera from *S. manosni*-infected individuals. The same molecule is not recognized by sera from patients infected with *S. japonicum*, indicating that the two groups of sera recognize different epitopes within the two HSP70s [14, 15]. HSP70 is also a major B cell antigen in patients infected with either *Brugia mamayi* or *Onchocerca volvulus*. The immunogenic domain in both these cases has been localized to the HSP70 carboxy-terminus [90].

HSP have been also been found to be major targets of the immune response in bacterial infections. Mice immunized with either *M. tuberculosis* or *M. leprae* produce antibody responses to a limited set of proteins. Amongst these are represented at least four stress protein groups: HSP70, HSP60, HSP18 and HSP12 [13]. HSP70 was initially identified by a mAb raised against an extract of *M. leprae*. HSP70 was then found to be recognized by both T cells and antibodies in patients with leprosy [91]. Similarly, HSP70 from *M. tuberculosis* was also identified with a mAb raised against *M. tuberculosis*. This HSP70 was recognized by antibodies and T cells from patients with tuberculosis [16]. Furthermore, $CD8^+$ T-cell clones

isolated from patients with tuberculosis proliferate in response to HSP70 from mycobacteria, *E. coli*, and human. Interestingly, mice infected with *M. tuberculosis* develop a strong antibody response to mycobacterial HSP70 and little or no response to murine HSP70. However, immunization of mice with the mycobacterial HSP70 induces antibodies that cross-react with self HSP70 [92].

6.3.2 The HSP70 as Chaperone of Antigenic Peptides and Proteins

A method for immunization against cancer exploiting the peptide-binding capabilities of HSP was explored in the 1990s. Mice immunized with HSP70 purified from Balb/c Meth A sarcoma cells were found to be protected against an otherwise lethal challenge with tumor cells. Protection was specific since it was not protective when mice were immunized with either (i) HSP70 from normal tissue, or (ii) HSP70 treated with ATP which removed HSP70-bound immunogenic peptides. Thus, protection appeared to require a combination of HSP70 and co-purifying bound peptides. Further analysis indicated that HSP70 bound peptides were in the range of 1,000–5,000 Da [93]. It was suggested that HSP-chaperoned peptides were efficiently processed endogenously by antigen-presenting cells (APCs) and presented in the context of MHC class I molecules.

Further evidence indicated that the chaperoning capacity of HSP is not limited to immunopeptides, but also to entire immunogenic proteins attached to HSP. HSP70 from *M. tuberculosis* fused to the human immunodeficiency virus protein p24 (HIV p24) elicited both humoral and cellular immune responses against p24 following immunization of mice with the HSP70-p24 complex in the absence of adjuvant [94]. Another experiment showed that mice immunized with HSP70 fused to the Hantaan virus nucleocapsid protein (NP) elicited significantly higher levels of NP-specific antibodies, IFN-gamma-producing cells and cytotoxic T lymphocytes than mice immunized with NP protein alone [95]. In a series of experiments with various chaperones involved in chaperoning antigens including gp96, HSP90, and HSP70, it was proposed that the complex of chaperone-peptides are internalized via the CD91 receptor into endosomal compartments, where they are targeted for presentation. Furthermore, some peptides HSP-peptide complexes were found to enter an acidic compartment and loaded onto MHC class II where peptides are presented to $CD4^+$ T cells [96].

6.3.3 HSP70 and Cross-Presentation of Antigens

More recent evidence demonstrated that HSP preferentially enter the MHC class I processing pathway via cross-presentation [97–99]. In this pathway, antigens are taken up by dendritic cells (DCs), and following internalization, they are processed and loaded onto MHC class I molecules and presented to $CD8^+$ T cells, which

destroyed pathogen-infected cells [100]. The mechanisms of cross-presentation by HSP70 or by HSP in general have not been completely defined. Interaction of the HSP-peptide complex with CD91 results in the internalization of the complex into a non-acidic compartment. Transfer of the complex to the cytosol allows peptides to be processed by the proteosome and transported into the ER by the transporter associated with antigen processing (TAP), which assist peptide loading onto MHC I molecules [101, 102]. This cross-presentation model has been tested in *M. tuberculosis* HSP70 and OVA peptide (OVA257-264). Cross-presentation of OVA peptide occurred via MHC-I in B cells. Processing was dependent on linkage of OVA peptide to HSP70 and was a CD91-dependent process [98].

6.3.4 HSP70 and Activation of Innate Immunity

In addition to chaperoning peptides and proteins for antigen presentation, HSP in general and HSP70 in particular possess intrinsic mechanisms that trigger innate immunity. When conjugated to poorly immunogenic peptides or oligosaccharides, HSP enhance the immune response to these relatively weak antigens. For example, immunization of mice with the polypeptide (NANP 40) [*P. falciparum* circumsporozoite protein] conjugated to the mycobacterial HSP70 or HSP60, resulted in a strong antipeptide IgG antibody response. This response was similar to the response observed when a purified protein derivative (PPD) is used as a carrier, in spite of the fact that no conventional adjuvant is used in the case of the HSP70-conjugated peptides [103]. Furthermore, priming with Bacillus Calmette–Guérin (BCG) prior to immunization was required in cases when HSP60 was used as carrier. However, priming with BCG was not required when HSP70 was employed [103].

The mechanism by which HSP70 provides adjuvant effects has been the focus of intense research. Recent studies showed that HSP70 possesses intrinsic adjuvant capabilities and that this protein can trigger activation of innate immunity. The following findings support the idea that HSP70 activates innate immunity: (i) HSP70 has been found localized on the cell surface membrane; (ii) HSP70 is secreted from the cell into the surrounding environment of cells [104–107]; (iii) secretion of HSP70 occurs in response to cytokines IFN-γ and IL-10 treatment [108]; (iv) HSP70 activates APCs following TLR4 and TLR2 engagement. Activation of APCs by HSP70 is NF-kB-dependent leading to proinflammatory cytokine production [12]; (v) extracellular HSP70 is internalized by APCs via cell surface receptors including CD40, CD91, LOX-1 and CD94 [109]. Together, these and previous observations demonstrate that HSP70 is not only a chaperone but also an inducer of cytokine production by APCs. HSP70 is a chaperokine [10, 11].

The induction of innate immunity by HSP70 has been observed in various models of host microbial interaction. For example, purified HSP70 from *T. gondii* or *T. cruzi* induced maturation of DCs. These DCs increased the expression of costimulatory molecules CD40, CD80, CD86, and activated DCs produced increased amounts of proinflammatory cytokines IL-12 and TNF-α two key cytokines involved in Th1 priming [56, 110].

Of note, it has been argued that the inflammatory properties of HSP70 are not due to HSP70 itself, but to lipopolysaccharide (LPS) contamination, which remains bound to the protein following purification from *E. coli*. In fact, experiments have shown that LPS-free HSP70 is immunosuppressive and that rather than inducing stimulation, HSP70 inhibits T cells and reduces the capacity of DCs to produce inflammatory cytokines [111]. Furthermore, a recent observation shows that *Francisella tularensis* HSP70 inhibits alkaline phosphatase in mice lungs infected with *Francisella*, suggesting that *Francisella* HSP70 could down-regulate host immunity by interfering with host cell signalling pathways [112].

Challenging the argument of inhibitory effect of HSP70, a recent observation shows that LPS-free HSP70, expressed in baculovirus expression vector system containing no LPS, invariably induced activation of mouse splenocytes and enhanced production of proinflammatory cytokines [113]. The apparent contradiction of HSP70 as an immunostimulatory or as an immunosuppressive molecule is still a matter of controversy. However, it is clear that LPS-contamination alone does not explain the multiple observations associated with the activation of the immune response by HSP70.

In addition to activating APCs, HSP70 also activates natural killer cells (NK cells) as shown in various cancer models. Little is known, however, on the role of HSP70-mediated activation of NK cells during infection. In malaria, HSP70 is recruited to the surface of *P. falciparum*-infected erythrocytes [114]. The infected erythrocytes become targets of NK cell-mediated cytotoxicity via granzyme B [115]. It should be noted that NK cells, which produce IFN-γ are important in the control of intracellular infections. IFN-γ is a primary cytokines involved in development of Th1 type immunity.

6.4 The Potential of HSP70 in Vaccine Development

The ability of HSP70 to chaperone antigenic peptides and proteins as well as its unique adjuvant capabilities are attractive features for vaccine development. In addition, HSP70 non-conserved amino acid sequences are potential vaccine candidate antigens. In this context, both host and pathogen HSP70 has been tested in various vaccine infection models.

6.4.1 HSP70 as Antigen

Bacterial HSP70 as a Vaccine Antigen HSP70 has been employed in vaccine preparations tested in vaccines against various bacterial infections. Mice immunized with *Salmonella typhi* HSP70 and complete Freund's adjuvant (CFA) developed significant increased levels of antibodies and a mixed Th1/Th2 response against *S. typhi*. Immunized mice displayed 70–90 % protection against *S. typhi* [17]. In leprosy, immunization with the C-terminal fragment of *M. leprae* HSP70 resulted

in increased delayed type hypersensitivity (DTH) against both C-terminus and the whole HSP70 molecule. In vitro, lymph node cells from the immunized mice recognized and proliferated in response to both C-terminus and whole the HSP70, suggesting protective immunity [116]. In *Helicobacter pylori*, immunization with DNA from *H. pylori* HSP70 triggered Th1 immunity against *H. pylori*. Immunized mice showed less microbial load and less gastric mucosal inflammation than non-immunized control mice [117].

Protozoal HSP70 as a Vaccine Antigen Antigenic capabilities of HSP70 has also been tested in vaccines against various protozoal infections. In toxoplasmosis, mice immunized with DNA containing *T. gondii hsp70* gene developed Th1 type immunity as determined by cytokine responses in vitro. As compared to controls, immunized mice showed a significant reduction of parasite loads in the brain following infection challenge with *T gondii* infecting doses [118].

In the *L. donovani* infection model, Balb/c mice immunized with *L. donovani* HSP70 and HSP83 proteins in the presence of adjuvant monophosphoryl lipid A (MPLA) developed significant levels of Th1 type immunity. Vaccinated mice were resistant to *L. donovani* infection [119]. Similar Th1 protective results were observed in mice immunized with *L. donovani* HSP70 and the major leishmanial surface glycoprotein gp63 as antigen [120]. However, immunization of BALB/c or C57BL/6 mice with *L. major* HSP70 resulted in a mixed Th1/Th2 development and no protection against *L. major* infection was observed [121]. In Chagas disease, however, mice immunized with *T cruzi* HSP70 developed HSP70-specific $CD4^+$ T cells producing IFN-γ, IL-2 and TNF-α suggesting Th1 type protective immunity [122]. Interestingly, $CD8^+$ cytotoxic T cells recognizing T cruzi HSP70 epitopes were identified in Chagas disease patients indicating presence of protective immunity [123].

Fungi HSP70 as a Vaccine Antigen In fungi models of infection, immunization with fungal HSP70 was not found to induce resistance against these infection types. Mice immunized with *C. albicans* HSP70 showed high levels of IgG antibodies and cell-mediated immune responses against HSP70. However, no protection against *C. albicans* infection was detected [19]. Similar results were observed following immunizations with *Histoplasma capsulatum* HSP70 [124]. Interestingly, immunization of mice with *H. capsulatum* HSP60 did confer significant immune protection and resistance to infection against *H. capsulatum* [125]. These results demonstrate that different HSP may be recognized differently by the immune response and this results in particular immune protection capabilities to fungal infections.

6.4.2 HSP70 as Adjuvant

HSP70 as Adjuvant in Vaccines Against Bacteria The ability of HSP70 to chaperone antigenic molecules and its capacity to trigger activation of the immune

response has been employed in various experimental vaccines. Mice immunized with *L. monocytogenes* HSP70 loaded with *Listeria* antigenic peptides were found to develop $CD8^+$ T cells producing IFN-γ requiring the help of $CD4^+$ T cells for expansion [126]. In tuberculosis HSP70 as well as HSP60, in combination with Bacillus Calmette–Guérin (BCG) as an antigen, induced more robust immunity and conferred greater protection to immunized mice than BCG alone [127]. Furthermore, *M. tuberculosis* HSP70 specifically stimulates antigen-primed cells to produce proinflammatory cytokines in vitro [128]. However, this effect was not observed with human HSP70, suggesting that adjuvanticity of HSP70 may be selective to some, but not to all HSP70s [129]. An expression system based on HSP70 fused to diverse antigen-encoding sequences, were developed recently. Mice immunized with the HSP70/antigen complexes efficiently elicited antigen-specific $CD8^+$ T cell responses without the need of adjuvant [130].

HSP70 as Adjuvant in Vaccines Against Protozoal Parasites A parasitic DNA vaccine containing *L. amazonensis* HSP70 and *L. amazonensis* gene encoding P4 nuclease were tested in a vaccine in BALB/c mice. Mice immunized with P4 and HSP70 vaccine developed modest protective immunity and little resistance to infection against *L. amazonensis* [131]. In Chagas disease, mice immunized with *T. cruzi* HSP70 fused to the kinetoplastid membrane protein 11 (KMP11) antigen from *T cruzi* developed $CD8^+$ T cell cytotoxic responses against cells expressing KMP11 antigen [132]. Another experiment showed that C57BL/6 mice immunized with *P. falciparum* EB200 antigen and both Cholera toxin (CT) and *T. cruzi* HSP70 as adjuvants develop high antibody levels against EB200 and enhanced secretion of IFN-γ by splenocytes in vitro, suggesting that CT and HSP70 can work synergistically to improve immunogenicity [133, 134].

HSP70 as an Adjuvant in Vaccines Against Viruses *M. tuberculosis* HSP70 and the HIV-1 p24 protein antigen were tested in a vaccine against HIV-1. Immunized mice develop antibodies against P24 protein and their immune cells responded to P24 antigen in vitro [94]. Another study showed that mice immunized with *M. tuberculosis* HSP70 non-covalently bound to MHC class II influenza A peptide responded by increasing T cell responses against influenza A peptide [135]. In choriomeningitis virus (LCMV), mice were immunized with an epitope from LCMV and recombinant HSP70 as adjuvant. Immunized mice developed high levels of memory $CD8^+$ T cells. Infection challenge with LCMV resulted in Virus titres reduced by 10–100 fold as compared to control non-immunized mice groups [18]. Regarding respiratory syncytial virus (RSV), RSV antigen G1F/M2 was chemically linked to HSP70. Mice immunized with G1F/M2-HSP70 conjugate developed significantly higher levels of antibodies against G1F/M2 and $CD8^+$ cytotoxic T cells than mice immunized without HSP70 [85]. Together, these results demonstrate that HSP70 is an adjuvant that significantly enhances both humoral and $CD8^+$ T cells against chaperoned peptides derived from virus.

6.5 Conclusion

Studies concerned with the role of HSP70 in microbial infections revised here demonstrate that: (i) microbial HSP70 can associate with microbial cell surface where it may assist pathogen invasion of host cells; (ii) HSP70 can play dual roles, as a host receptor of microbes and as a chaperone for microbial survival within host cells; (iii) HSP70 promotes microbial survival by helping microbes cope with the toxic environment of host cells; (iv) HSP70 assists in microbial invasion and survival, and HSP70 works in concert with other associated molecular chaperones, including HSP90 and HSP40; (v) HSP70 is immunogenic, and is recognized by both antibodies and T cells in infected as well as in apparently healthy individuals; (vi) HSP70 induces the activation of acquired immunity and (vii) HSP70 is an efficient adjuvant that enhances both humoral and cell-mediated responses against various intracellular infections. Nevertheless, in certain conditions HSP70 cannot trigger the immune response, but on the contrary, down regulates immunity. Furthermore, HSP70 has the potential to provoke autoimmune reactions due to molecular mimicry between host and microbial HSP70s. Future research will be required to unquestionably clarify the potential of HSP70 as a vaccine adjuvant and as an antigen.

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Chapter 7
Potential Cytoprotective Effects of Heat Shock Proteins to Skeletal Muscle

John P. Vardiman, Philip M. Gallagher, and Jacob A. Siedlik

Abstract Heat shock proteins (HSP) are chaperone molecules that are known to facilitate protein synthesis, protein assembly, provide cellular protection and regulate intracellular signaling. These cytoprotective effects have been linked to increases in HSP70 and HSP27p concentrations but there has been little progress in determining the specific role of HSP in human skeletal muscle adaptations. Short wave diathermy (SWD) and ultrasound are treatments commonly used to stimulate deep heat increases in skeletal muscle with limited research examining the effects of increased muscle temperature on muscle damage induced injury severity. Current research cannot definitively identify the mechanistic roles of HSP in mitigation of muscle damage even though they are commonly cited as mechanism of action for prevention of damage in heat-treated muscle. This article will examine the role of HSP induction in skeletal muscle as a therapeutic countermeasure for reduction of muscle atrophy during prolonged periods of immobilization as well as mechanisms for accelerated repair of injured muscle fibers through increased total protein concentrations.

Keywords Cytoprotection • Heat shock protein • Skeletal muscle • Cytokines • Signaling pathway • Therapeutic modalities • Heating

Abbreviations

HSF-1	Heat shock transcription factor-1
HSFs	Heat shock factors
HSP	Heat shock proteins
HSP70	70-kDa HSP
HSP72	70-kDa HSP
IL	Interleukin
SOL	Soleus

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7.1 Introduction

Early studies on muscle damage, and exercise-induced muscle damage in particular, suggested that the delayed onset of muscle soreness was due to micro tears in the muscle [1]. Though the hypothesis was later proven to be true [2], it does not fully explain the mechanisms of muscle damage. Proske et al. [3], showed that eccentric contractions and unaccustomed loading of skeletal muscle leads to severe disruptions of the sarcomeres, the sarcoplasmic reticulum, transverse tubules, and individual myofibrils, triggering an immediate, inflammatory immune response. Incurred damage to the sarcoplamic reticulum leads to an increased intracellular calcium concentration [4] that has been shown to activate calpains and further the degradation of cytoskeletal proteins [5]. Other consequences of muscle damage include swelling [6], disruption of contractile proteins [7–9], and extracellular matrix damage [10] with eventual apoptosis and cell death [3].

Many studies have focused on apoptosis activation by muscle damage and the role that apoptosis has in exacerbating muscle injury [11–13]. Following muscle damage there is an increase in local blood flow, plasma CK-8, α-actin [14], and HSP [15] along with an influx of neutrophils to the damaged area. Neutrophil invasion causes secondary injury through the release of free radicals and proteases, leading to pro-apoptotic signaling by way of increasing JNK [11, 13, 16], p53, [17] and caspase [18] activity. The levels of heat shock protein (HSP) expression appear to be positively related to the magnitude of damage to skeletal muscle [13, 19, 20]; however, there have been mixed results in experimental designs with some studies showing modest changes in HSP72 expression in the soleus (SOL) muscle [21, 22] and others unable to replicate these increases following a bout of downhill running [23]. The purpose of this chapter is to briefly discuss the cytoprotective effects of heat shock proteins and how they can be manipulated as part of therapeutic modalities with a primary focus on the role of HSP70 in skeletal muscle.

7.2 Induction of Heat Shock Proteins Following Skeletal Muscle Damage

The production of HSP in skeletal muscle is primarily stimulated by heat stress, oxidation and a high level of muscle contraction [24, 25]; however, the role these proteins play in attenuating damage to skeletal muscle is poorly understood. HSP70 is a family of stress proteins that are the most highly conserved and temperature sensitive of all the HSP [26]. It has been shown that the expression of HSP increases in response to muscle damage [19, 27–30]. Investigations involving humans have shown that single bouts of eccentric contractions can initiate a similar response in intramuscular (biceps brachi and vastus lateralis) HSP70 concentrations [19, 31]. Furthermore, eccentric exercise prior to unloading has been shown to attenuate muscle damage during subsequent reloading of skeletal muscle. HSP70 appears to

play a role in the repeated bout effect that is seen with adaptation to lengthening contractions. Thompson et al. [19] investigated the HSP response to a repeated eccentric stimulus. Upon reloading there were substantial decreases in the HSP70 response to the second bout of eccentric induced muscle damage. These authors also found changes in HSP70 concentrations were accompanied by a significant reduction in serum creatine kinase levels [19]. These data suggest that HSP70 not only mediates adaptation to exercise, but plays a role in preventing acute and chronic injury to myofilaments during bouts of unaccustomed loading.

The increase in HSP synthesis following muscle damage is thought to be triggered by two factors: (1) the proteolysis that occurs following eccentric contractions [19, 31] and (2) elevations in plasma IL-6 [32]. Ingalls et al. [33], reported that exercise induced muscle damage stimulated an increase in HSP70 expression in mice, which was thought to be related to a decline in actin and myosin heavy chain proteins as a result of muscle injury. These data support a role for HSP in the degradation process of damaged myofibrillar proteins but further research in the area is required to identify the particular mechanistic relationship of this process.

A recent study by Welc et al. [34] found that both heat shock factor-1 (HSF-1) and AP-1 play major roles in hyperthermic induction of IL-6. It is possible that HSF-1 may also be involved in the induction of IL-6 under other stress conditions. The data in this study suggests that HSF-1 regulates IL-6 activity even under physiologic conditions where HSF-1 is thought to be inactive. The regulatory link between the IL-6 and HSF-1 indicates that there may be a role for heat shock factors as mediators of the inflammatory response in skeletal muscle absent heat stress.

7.3 Attenuation of Skeletal Muscle Damage by Heat Shock Proteins

While a plethora of data is available exhorting the cytoprotective role HSP70 plays in a variety of other cell types [26], limited data exists in skeletal muscle. The available data does, however, suggest that HSP70 induction can attenuate the severity of muscle damage [12, 35, 36]. Most research has focused on the chaperoning functions of HSP70 and its ability to regulate protein folding and, subsequently, cellular repair processes in response to stress [37]. HSP70 has been implicated in protecting skeletal muscle from ischemia-reperfusion injury [38], and lengthening contractions [39]. Further, in a model using C2C12 skeletal muscle cells, brief exposure to heat shock treatment resulted in a significant increase in HSP expression and subsequent protection against exposure to the calcium ionophore, A23187 and the mitochondrial uncoupler, 2,4-dinitrophenol [12]. Similar results were seen in heat stressed rat skeletal muscle in the presence of a cardiotoxin. Induction of HSP70 stimulated not only satellite cell proliferation, but also protein synthesis during the regeneration of injured skeletal muscle [40]. HSP70 overexpression has also been shown to reduce histological evidence of muscle damage. A recent

investigation, cryolesioned the soleus and tibialis anterior muscles to induce injury, and analyzed these muscles up to 3 weeks following the bout of muscle damage [35]. Histological analysis showed that muscles from HSP70 expressing mice had reduced necrosis and preserved cross-sectional area, as compared to non-treated controls [35]. Collectively, these data imply that HSP70 is associated with reduced muscle damage that may be attributed to an increase in skeletal muscle proliferation.

Limited data is available concerning the effect of heat shock during unaccustomed loading. Interestingly, following 28 days of unloading, 7 days of reloading did not result in recovery of HSP70 protein levels and this continued impairment upon reloading is directly related to the continued suppression of HSF-1 [41]. It appears that longer periods of reloading (14–28 days) may be needed following unloading, to stimulate upregulation of HSP70 and recovery of muscle mass [42]. Exercise may accelerate this recovery. It was recently shown that intensive treadmill running significantly upregulated HSP expression in as little as 6 days following 4 weeks of unloading [15]. This rapid increase in HSP content stimulated by exercise during reloading may contribute to accelerated recovery from atrophy. Selsby et al. [43] has recently reported that heat shock used during reloading attenuated oxidative stress, and improved the rate of skeletal muscle re-growth. Significant muscle remodeling occurs during reloading, leading to muscle hypertrophy and the restoration of muscle function. Previous investigations have shown that a single bout of hyperthermia is capable of inducing increases in muscle hypertrophy and protein synthesis [44, 45]. Furthermore, Goto et al. [46] have shown an increase in muscle-to-body weight ratio following single bouts of heat stress.

The role of JNK appears to be pivotal [47] for the intrinsic pathway of apoptosis. We [23], and others [13] have shown increased JNK expression following muscle damage. JNK is a known regulator of Caspase-3 [48], which is significantly upregulated following muscle damage [49]. It is has been shown that this change in Caspase-3 activity is directly linked to the loss of actin filaments from the sarcolemma [50] and serves as an upstream regulatory factor for accelerating muscle proteolysis [50, 51].

HSP70 inhibits JNK and as a result reduces downstream signaling of apoptosis [47]. In support of this, HSP70 has been associated with inhibiting Caspase-3 activation and preventing the formation of the apoptosome [52]. However, it is not known if HSP70 mediates this process following muscle damage.

7.4 Clinical Modalities to Induce Heat Shock Proteins in Skeletal Muscle

Ultrasound and short wave diathermy (SWD) are common modalities for deep heating of skeletal muscle tissue. These modalities are most commonly used to treat large muscle areas and to target tissues from 2 to 5 cm. In rats, heat treatment (HT) has been shown to preserve muscle size during unloading experiments [53] and

improve the recovery of atrophied muscle [46]. We [23, 54], and others [55], have shown that various heat treatment modalities activate heat shock proteins in skeletal muscle.

Consistent with the current literature, our data suggests increases in HSP70 concentrations are associated with a need to maintain homeostasis and prevent future/further damage to the cell. For example, we saw that increasing HSP expression prior to muscle damage appears to protect skeletal muscle from injury. In addition, we suggest that heat shock prior to damaging exercise may facilitate recovery from exercise by increasing the total protein concentration and the expression of MHCneo in vivo. Heat treatment 48-h prior to damaging exercise enhanced muscle adaptation by increasing total protein content and MHCneo expression independent of Akt, p70s6k, and JNK signaling [23]. These findings are supportive of the majority of studies showing elevated HSP can have a positive effect on skeletal muscle and advance the idea that induced over expression of HSP prior to muscle damage may mitigate muscle fiber injury. That said, further research is required to identify the precise mechanism(s) by which HSP influence skeletal muscle regrowth and regeneration.

7.5 Pharmaceutical Induction of Heat Shock Proteins

The potential cytoprotective effects of the heat shock response are an attractive target for pharmacological therapies. This is particularly relevant for a number of neurodegenerative diseases associated with protein misfolding and subsequent aggregation [56] such as Alzheimer's disease, Amyotrophic Lateral Sclerosis (ALS), and Parkinson's disease. Hydroxylamine derivatives like bimoclomol and arimoclomol are co-inducers of the heat shock response by way of prolonging the activation of heat shock factor-1 (HSF1) [56–58]. Bimoclomol has been particularly effective in treatment of diabetes mellitus and cardiovascular diseases [59], but has shown few cytoprotective effects within skeletal muscle.

Arimoclomol has been shown to be effective in mouse models of motor neuron degeneration [60, 61] and, moreover, found to be well tolerated and safe in Phase II clinical trials of ALS patients [62]. Of particular interest for skeletal muscle applications, Kalmar et al. [60] found arimoclomol treatment improved muscle innervation in the periphery of $SOD1^{G93A}$ mice prior to central effects within the spinal cord. While this is of specific importance to treatment of ALS due to the different stages of disease progression, it also illuminates the ability of drug therapy to co-induce HSP expression within the skeletal musculature. An important note regarding the mechanism of the HSP co-induction through arimoclomol is the fact that the prolonged activation of HSF-1 only occurs in cells where HSF-1 is already activated [61] (i.e. only cells that are already stressed), providing for a very targeted response. Arimoclomol has exciting possibilities as a drug therapy targeting skeletal muscle but more research will be required to understand the positive and negative consequences of drug administration.

7.6 Conclusion

This chapter has covered the most relevant cytoprotective features of HSP, particularly HSP70, as it relates to human skeletal muscle. While the cytoprotective effects are observed in response to therapeutic modalities such as SWD and Microwave Diathermy, the specific mechanism underlying this phenomenon is unclear. This should not deter clinicians or other relevant practitioners from utilizing these modalities, but does identify the need to elucidate the exact role elevated HSP in human skeletal muscle in various conditions including muscle damage and exercise. Successful studies examining the mechanistic properties of HSP in skeletal muscle will further our current understanding of the role heat shock proteins play as chaperones and help identify other clinically relevant applications for use of heat therapies.

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Chapter 8
Heat Shock Proteins in Triple-Negative Breast Cancer (TNBC) Treatment

Punit Kaur, Tarundeep Singh, Moses Galukande, Sunil Krishnan, and Alexzander A.A. Asea

Abstract Triple-negative breast cancer (TNBC) accounts for approximately 15 % of breast cancer diagnoses, and exhibits substantial overlap with basal-type and BRCA1-positive breast cancer. Large population based studies have identified a higher proportion of triple-negative breast tumors among premenopausal African American women, and a suggestion that increased parity, younger age at first term pregnancy, shorter duration of breast feeding, and elevated hip-to-waist ratio might be particular risk factors. In recent years, a greater understanding of the biology of this disease has led to the development of numerous and varied therapeutic approaches. Neoadjuvant trials using conventional cytotoxic agents such as cisplatin have demonstrated TNBC to be a relatively chemosensitive disease. Drugs developed to inhibit the angiogenic process may be particularly effective in TNBC. The molecular chaperone heat shock protein (HSP90) is required for the conformational maturation and stability of a variety of proteins in multiple pathways, such as epidermal growth factor receptor (EGFR), AKT, Raf, cdk4, etc. Therefore, an HSP90 inhibitor may show therapeutic benefit in TNBC by targeting multiple pathways. In terms of therapy, an increasingly rational drug development effort has resulted in agents against new molecular targets that are active against

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only those tumors with the targeted molecular alteration or phenotype including receptor and non-receptor tyrosine kinase inhibitors, human epidermal growth factor receptor (HER1, HER2, HER3, insulin like growth factor receptor, c-met, fibroblast growth factor receptor and HSP90 inhibitors), intracellular signaling pathways (PI3K, AKT, mTOR), angiogenesis inhibitors and agents that interfere with DNA repair poly (adenosine diphosphate ribose) polymerase 1 (PARP) inhibitors. The overall management of breast cancer will increasingly require an understanding of breast cancer heterogeneity, the biological nature of any given tumor as well the existence of increased personalized treatment options. In the current review, focus is directed towards novel targeted strategies for TNBC.

Keywords Androgen receptor • Angiogenesis • *BRCA1* • Cancer stem cells • Chemotherapy • Clinical trials • HSP90 • mTOR inhibitors • Targeted therapy • Triple-negative breast cancer • Tyrosine kinase inhibitors • VEGF

Abbreviations

AR	Androgen receptor
CKs	Cytokeratins
CM	Cyclophosphamide and methotrexate
CSC	Cancer stem cells
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HR	Hormone receptor
HSP	Heat shock protein
IHC	Immunohistochemistry
mTOR	Mammalian targets of rapamycin
ORR	Overall response rate
OS	Overall survival
PARP	Poly (adenosine diphosphate-ribose) polymerase 1
pCR	Pathologic complete response
PFS	Progression free survival
PgR	Progesterone receptor
SES	Socioeconomic status
TN	Triple-negative
TNBC	Triple-negative breast cancer
TPBC	Triple-positive breast cancer
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

8.1 Introduction

Triple-negative breast cancer (TNBC) is characterized by a lack of estrogen receptor (ER) and progesterone receptor (PgR) expression and lack of gene expression for human epidermal growth factor receptor 2 (HER2) [1–3]. The aggressiveness of TNBC is further indicated by the fact that: (i) the peak risk of recurrence occurs within the first 3 years after initial treatment of the disease with the majority of deaths occurring in the first 5 years [4] and (ii) after diagnosis of metastatic disease a significantly shorter survival is observed in TNBC [4–9]. Conversely, the risk for late recurrences (i.e. beyond 5 years of diagnosis) is decreased by 50 % compared with $HER2^+$ disease [10]. TNBC is typically observed in young African-American women and Caucasian women who carry a mutation in the BRCA1 gene [11]. While some TNBC respond to chemotherapy, subsets of TNBC are chemotherapy resistant and highly metastatic carrying with it an extremely poor prognosis [11]. The majority of carcinoma cells express a $CD44^{low}/CD24^{high}$ phenotype; however, a small subpopulation of carcinoma cells present within human breast cancers that exhibit a $CD44^{high}/CD24^{low}$ antigenic phenotype that are highly enriched for cancer stem cells (CSC), also termed as tumor-initiating cells [12].

Immunohistochemistry (IHC) staining of cytokeratins (CKs) further subclassifies tumors into clinically meaningful pathological subtypes [13]. "Basal like" refers to the 8–20 % of breast tumors that stain positively for high molecular weight CKs (CK5/6, CK14, and CK17) since these CKs are expressed in the basal/myoepithelial cells of normal breast ducts [14]. Sporadic basal like tumors share key features with BRCA1 mutation-associated hereditary breast cancers, including morphology, a frequent TN status, and expression of basal CKs, p53, P-cadherin, and HER1 and epidermal growth factor receptor (EGFR). Conversely, breast cancers in women carrying BRCA1 mutations are frequently basal like as well as TN [15]. Key IHC based biomarkers, including ER-α and HER2, were represented on the mRNA array. Several investigators have sought to identify clinically useful markers characteristic of the basal like breast cancer subtype. Nielsen et al. indicated that the majority of basal like breast tumors illustrated low-to-absent expression of estrogen receptor (ER) and HER2, with high expression of HER1, basal CKs 5/6, and c-Kit. Interestingly, a survival analysis of over 900 cases illustrated a shorter disease specific survival among cases that expressed the basal CKs 5/6 and 17 [16]. All basal like tumors tested were ER^- and $HER2^-$ and illustrated immunoreactivity for vimentin (17/18), luminal CKs 8/18 (15/18), EGFR (13/18), and CKs 5/6 (11/18). Compared with the endocrine sensitive, luminal A (ER^+ and/or $PgR^{+/}HER2^-$) breast tumors, basal like ($ER/PgR/HER2^-$) breast tumors harbored a higher number of *TP53* mutations (44 % vs. 15 %; $P < .001$), higher mitotic index (odds ratio [OR], 11.0; 95 % CI, 5.6–21.7), more marked nuclear pleomorphism (OR, 9.7; 95 % CI, 5.3–18.0), and higher combined grade (OR, 8.3; 95 % CI, 4.4–15.6) [17]. An association between the TN phenotype and breast cancers harboring germline mutations in the *BRCA1* gene has been well described and confers an approximately 80 % lifetime risk of breast

cancer among carriers [18, 19]. The large majority of BRCA1-associated breast cancers express the TN phenotype in addition to "basal like" CKs 5, 14, 17 and HER1/EGFR [20–23]. Immunohistochemical staining was used to classify specific subtypes in approximately 500 tumors, and "basal like" tumors were defined as TN (ER/PgR/HER2$^-$) and CKs 5/6 positive and/or HER1$^+$. Results indicated that those with basal like tumors were more likely to be African American compared with non–African American (26 % vs. 16 %) and premenopausal compared with postmenopausal (24 % vs. 15 %). There was a particularly high prevalence of basal like tumors among premenopausal African American women compared with postmenopausal African American women and non–African American women of any age (39 % vs. 14 % and 16 %; $P < .001$). Preclinical studies have illustrated that the small heat shock protein, αB-crystallin, is commonly expressed (45 %) among basal like breast tumors as determined by microarray analysis and independently predicts shorter survival [24]. Interestingly, overexpression of αB-crystallin induces neoplastic changes in mammary acini (single layers of polarized, growth arrested mammary epithelial cells surrounded by a hollow lumen), transforms immortalized human mammary epithelial cells, and increases cell migration and invasion in vitro.

8.2 Biology of ER$^-$ Subtypes of Breast Cancer

African-American women are more likely to present at a younger age as 33 % of African-Americans were diagnosed at age <50 years compared with 21.9 % of white women [25]. After the Women's Health Initiative reported the link between breast cancer and hormone replacement therapy in a randomized control trial, the subsequent mass cessation of hormone replacement therapy in 2002 is felt to have contributed to a decrease in breast cancer incidence over the next 2 years [26, 27]. For ER$^-$ tumors, incidence rates decreased 4.7 % for whites but increased 4 % for African-Americans [28], which includes the higher rate of estrogen only therapy among African-Americans and the higher rates of obesity among African-American women. African-American women diagnosed with breast cancer are more likely to die than whites [25].

Socioeconomic status (SES) contributes to breast cancer disparities, seen in lower levels of mammographic screening together with higher overall mortality and 5-year case specific probability of death among African-American compared to white women [29]. In young African-American women, ER/PR$^-$ tumors showed a significantly higher frequency of hypermethylation in four of five designated genes; cyclin D2 methylation was significantly associated with shorter survival [30]. In African-American women, reproductive factors, including younger age at menarche and early age at first pregnancy have been shown to protect against ER/PR$^+$ breast cancer but may be positively associated with ER/PR$^-$ tumors [31]. Breastfeeding for 6 months versus never breastfeeding were also protective for TN disease [32]. TN breast cancer was associated not only with race/ethnicity of African descent but also with having a lower SES [33]. SES may actually operate through lifestyle risk

factors in influencing ER^- breast cancer. Healthy diets [34], high phytoestrogen intake [35], high folate intake [36], fiber intake [37], and possible calcium intake [38] may protect against ER^- tumors. Also, postmenopausal women consuming dietary folate equivalents of total folate over 10 years appear to derive a greater benefit for ER^- than ER^+ breast cancers [36]. However, a small case study of 91 women with breast cancer found that low serum vitamin D level and vitamin D deficiency were associated with increased risk for TN tumors [39]. Strenuous to moderate levels of physical activity may lower ER^- breast cancer risk [40], with exercising during adolescence and within the last 10 years being associated with decreased risk of ER/PR^- breast cancer in both premenopausal and postmenopausal women [41].

8.3 Current Therapeutic Options

8.3.1 Chemotherapy

Of those African-American women who do receive adjuvant therapy, a higher percentage tends to experience premature termination of chemotherapy than their white counterparts, leading to suboptimal treatment in a curative setting [42]. Neoadjuvant chemotherapy has consistently demonstrated higher overall response rates (ORR) for TNBC than non-TNBC [7, 43, 44]. Two of the most important ongoing randomized trials are CALGB40603, evaluating the benefit of carboplatin added to paclitaxel and anthracyclines, and the TN trial evaluating carboplatin against docetaxel and importantly will provide rich resources to identify potential tissue biomarkers to help define subgroups of patients with TNBC most likely to benefit from platinum agents [45, 46]. Limited data suggests BRCA1 mutation-associated TNBC may have particular sensitivity to platinum agents and relatively less sensitivity to taxanes [21, 23, 47]. Among 55 TNBC patients who received six cycles of FEC100 (fluorouracil/epirubicin 100 mg/m^2/cyclophosphamide), 12 BRCA1 carriers were identified. The pathologic complete response (pCR) rate for the 12 TN BRCA1 carriers was 17 % compared with 42 % in the 55 sporadic TN non-carriers. Although most studies support a benefit for anthracycline based regimens, an analysis from the MA5 study comparing adjuvant cyclcophosphamide, epirubicin, fluoruracil (CEF) to CMF showed an improvement in 5-year overall survival (OS) in the CMF arm for TNBC (71 % vs. 51 %), whereas the CEF arm was superior in all other subgroups. In a retrospective study of 151 patients receiving neoadjuvant anthracycline and taxane based therapy, those patients with TNBC (14 %) had significantly higher pCR rates compared to non-TNBC (38 % vs. 12 %) [43, 44, 48, 49]. In the 129 hormone receptor (HR) negative patients, the pCR rate was 33.3 % compared to 7.6 % in the HR positive group. It was reported that patients who achieved a pCR had a slightly worse prognosis than those who did not achieve pCR. One of the characteristics of TN tumors is their high chemosensitivity,

but with a short time to progression and survival. Preclinical data demonstrate that intact BRCA1 function contributes to anti-microtubule agent sensitivity [50]. The use of nab-paclitaxel may warrant further testing for TNBC with high Caveolin-1 expression [51].

A large trial of 4,950 patients randomized to receive adjuvant doxorubicin and cyclophosphamide followed by docetaxel or paclitaxel given weekly or once every 3 weeks demonstrated an overall improvement in 5-year DFS and OS of 27 % and 32 % [52]. A subset analysis of the BCIRG001 trial evaluated the benefit of docetaxel versus fluorouracil when added to doxorubicin and cyclophosphamide (TAC vs. FAC) in molecular subgroups [53–55]. The GEPARDUO trial also evaluated the pCR rate in 913 women randomized to receive preoperative doxorubicin and docetaxel for four cycles or doxorubicin and cyclophosphamide for four cycles followed by docetaxel for four cycles [56]. The pCR rate was only 10.6 % in all patients combined, with an improvement from 7.0 % to 14.3 % with the three drug regimen. However, the ER− subgroup was three times more likely to achieve a pCR compared with the ER+ subgroup (22.8 % vs. 6.2 %). In addition, the NSABP B28 trial compared doxorubicin and cyclophosphamide with or without four cycles of paclitaxel in 3,060 patients and found no statistically significant difference in the relative risk of recurrence and OS based on HR status [57]. Among 44 BRCA1 carriers identified in a registry of 3,479 patients, only 6 of 15 who received docetaxel and doxorubicin had a complete or partial response, compared to 29 of 29 who received non-taxane, DNA damaging regimens [58]. Mutations in BRCA1 were found in 5 of the 19 (26 %), and all 5 were in the TNBC subgroup, suggesting that BRCA1 mutation might confer decreased response to docetaxel. In the CALGB9342 trial, which evaluated three different doses of paclitaxel for metastatic breast cancer, there was no statistically significant difference in ORR or time to treatment failure between TNBC and HR positive tumors [46]. However, the OS was significantly worse for the TNBC compared to HR positive [59]. Over 90 % of the patients were $HER2^-$, and more than a third were ER^- and PR^-, suggesting a majority of the HR negative patients were likely TN.

In CALGB49907, standard adjuvant chemotherapy (either CMF or AC) was compared to capecitabine in women over age 65 to determine non-inferiority [60]. After 600 patients were enrolled, the trial found capecitabine was inferior to standard chemotherapy with a hazard ratio (HR) of 2.09. Importantly, a planned subgroup analysis revealed that the benefit of standard chemotherapy was most pronounced in HR negative patients compared to HR positive 3.04 for relapse-free survival, 2.62 for OS. In a combined subgroup analysis, 857 total patients received capecitabine alone, of which 208 patients had TNBC. The ORR and PFS in the capecitabine monotherapy arm were 25 % and 4.2 months in the overall population, but only 15 % and 1.7 months in the TNBC subgroup. Chemotherapy included single agent taxanes, anthracycline-based regimen, and capecitabine in RIBBON I and taxanes, capecitabine, gemcitabine, and vinorelbine in RIBBON II with improved progression free survival (PFS) and ORR. The phase II trial of sunitinib, given in a dose of 50 mg a day for 4 weeks followed by a 2-week break,

enrolled patients with chemotherapy pre-treated metastatic disease [61]. Twenty patients (31 %) had TNBC: in this subset the ORR was 15 %.

The benefit of polychemotherapy in ER-poor breast cancer was evident in the 2005 overview meta-analysis by the Early Breast Cancer Trialist's Collaborative Group (EBCTCG). In over 6,000 women with ER-poor disease enrolled in 46 polychemotherapy trials that began prior to 2000 (but did not include taxanes), a substantial reduction in risk of recurrence and death from breast cancer was seen in younger (10-year hazard ratio 0.73 and 0.73, respectively) and older patients (10-year hazard ratio 0.82 and 0.86, respectively). In comparing the low dose cyclophosphamide, doxorubicin, fluorouracil (CAF) regimen from CALGB8541 to the dose dense regimen of doxorubicin, cyclophosphamide followed by paclitaxel (AC-T) in CALGB9741, the relative reduction in risk of recurrence was 55 % for ER^- tumors, and 26 % in ER^+ tumors [46]). The absolute improvement in risk of recurrence at 5 years was 22.8 % for ER^- tumors and only 7 % for ER^+ patients treated with tamoxifen.

A study evaluating preoperative chemotherapy in basal like breast cancer treated with 12 weeks of weekly paclitaxel followed by four cycles of fluorouracil, doxorubicin, cyclophosphamide (FAC) revealed a pCR rate of 45 % [62]. The pCR rates of the Luminal A/B ($n = 30$), normal breast like ($n = 10$), and HER2+ ($n = 20$) molecular subtypes were 7 %, 0 % and 45 %, respectively [62]. This supports the conclusion that basal like breast cancer is more highly sensitive to paclitaxel and doxorubicin chemotherapy, and correlates with similar results seen with histological markers defining TNBC. Among the TN tumors that were classified as basal like, the clinical ORR was 85 % and the pCR rate was 27 %.

8.3.2 Histone Deacetylase (HDAC) Inhibitors

Preclinical studies have shown that pharmacologic inhibition of these mechanisms i.e., DNA methyltransferase and histone deacetylase (HDAC) inhibitors result in re-expression of functional ER mRNA and protein. Specifically, treatment of three ER^- breast cancer cell lines (MDA-MB-231, MDA-MB-435, and Hs578t) and a xenograft model with an HDAC inhibitor (Scriptaid) resulted in both significant growth inhibition and re-expression of estrogen responsive genes, namely PgR. It renders cells sensitive to hormonal manipulation with an aromatase inhibitor [63]. A clinical study evaluating tamoxifen plus the HDAC inhibitor vorinostat (suberoylanilide hydroxamic acid; SAHA) in patients with heavily pretreated, endocrine-resistant ER^+ breast cancer illustrated four major responses and five patients with standard deviation for > 12 months. Preclinical models have illustrated re-expression of ER-α in ER^- breast cancer cell lines following treatment with trichostatin A and Scriptaid, thereby potentially opening up an avenue for therapy with endocrine agents [63].

8.3.3 EGFR Inhibitors

There are many other drugs in development and in early stage trials, which interfere with signaling events downstream of vascular endothelial growth factor (VEGF) through the use of tyrosine kinase (TK) inhibitors and other small molecules. Sunitinib is an oral tyrosine kinase inhibitor that inhibits vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), stem cell factor receptor (c-kit), and colony stimulating factor-1 receptor. In a phase II study, Burstein et al. examined the effect of sunitinib in 64 patients previously treated with an anthracycline and a taxane, 20 of them being TNBC [61]. In the metastastic setting, metronomic dosing of cyclophosphamide and methotrexate (CM) has shown reduction in serum levels of VEGF, with ORR ranging from 12 % to 21 % [48, 64]. Torrisi et al. reported the results of 30 patients with locally advanced TNBC who were treated with four cycles of neoadjuvant epirubicin, cisplatin, and fluorouracil, followed by 4–6 months of adjuvant cyclophosphamide and methotrexate (CM) and at 2 years of median follow up, 87.5 % of patients were disease free [65]. The Eastern European Cooperative Oncology Group (ECOG) had 2100 randomized patients with chemotherapy naive, predominantly $HER2^{-}$ metastatic breast cancer, to receive weekly paclitaxel (3 of every 4 weeks), with or without bevacizumab. Miller et al. evaluated 722 metastatic breast cancer patients (90 % $HER2/neu^{-}$) to receive paclitaxel either alone or in combination with bevacizumab [66]. Although OS was similar in both groups, the paclitaxel plus bevacizumab group experienced a significant higher PFS compared with the paclitaxel alone group (11.8 vs. 5.9 months, $p < .001$).

Dysfunction or lack of protein phosphatase and tensin (PTEN) has been linked to activation of mammalian targets of rapamycin (mTOR): proteins involved in the regulation of many cell functions including endothelial proliferation and angiogenesis [67]. A phase II randomized study evaluated two everolimus oral mTOR inhibitor regimen for first line or second line treatment in 59 metastatic breast cancer patients, of which 20 patients were $HER2^{-}$ [68]. The inhibitor PU-H71 demonstrated impressive response in TN breast cancer disease in preclinical studies [69]. Activation of the PI3K pathway either by frequent PTEN alteration, elevated PIK3CA expression or oncogenic mutation has been identified in TNBCs and is also associated with a poor prognosis [70].

A phase II study by the Translational Breast Cancer Research Consortium evaluated 102 TNBC patients, randomized to receive cetuximab alone, with addition of carboplatin upon progression, or cetuximab plus carboplatin throughout. Thirty-one patients received single agent cetuximab, with two partial responses; 71 received cetuximab plus carboplatin, with 13 patients showing response to therapy. Small molecule tyrosine kinase inhibitors directed at EGFR, such as erlotinib, have also been evaluated. Dickler et al. examined the effect of single agent erlotinib in 69 patients with advanced breast cancer with progression after conventional chemotherapy with 15 TNBC patients [71].

8.3.4 PARP Inhibitors

Poly (adenosine diphosphate ribose) polymerase 1 (PARP) inhibitors selectively target cancer cells that are deficient in *BRCA-1* and/or *BRCA-2*. A phase II study combining gemcitabine and carboplatin (GC) with or without BSI-201, an intravenous PARP1 inhibitor, was reported. The basis for using GC comes from a phase II study that showed RR ranging from 27 % to 32 % in HER2/neu non-overexpressing patients with metastatic breast cancer [72]. Fong et al. reported results of a phase I trial with the use of olaparib (AZD2281), an oral inhibitor of PARP [73, 74], which included patients with refractory breast cancer and with *BRCA-1* or *BRCA-2* mutations. Of the three breast cancer patients with *BRCA-2* mutations, one had complete remission and another had stable disease for 7 months. Carboplatin was administered in patients with brain metastases (comprising up to 46 % of all patients with TNBC) at an AUC of 2 on days 1 and 8 of a 21-day cycle, and gemcitabine was administered at 1,000 mg/m^2 on days 1 and 8. On the experimental arm, BSI-201 was dosed at 5.6 mg/kg on days 1, 4, 8 and 11. In a paired correlative analysis, PARP1 gene expression profiling has been performed in 50 patients included in the study to date and compared to non-TNBC controls; these analyses (though preliminary) overwhelming suggest that the moiety is upregulated in TNBC.

8.3.5 Src Inhibitors

Preclinical data indicate that BlBCs are particularly sensitive to inhibition of scr kinase however, the antitumor activity of the dual abl/src kinase inhibitor dasatinib, was modest (<5 % responses) when given as monotherapy to heavily pretreated patients with TNBC. In a phase II study, Finn et al. examined the effect of single agent dasatinib in 44 patients with advanced TNBC. Even though dasatinib showed a modest clinical benefit (9.3 %), these results are encouraging because it has shown to be active in TNBC patients.

8.3.6 Antiandrogens

Preclinical in vitro studies have demonstrated that high concentrations of androgen stimulate the proliferation of estrogen responsive human breast cancer cell lines MCF-7 and EFM-19 with measurable effects observed at concentrations of 10^{-9} with the highest effects seen at concentrations of 10^{-6} [75]. Androgen-induced proliferation was also demonstrated in the AR^+, ER^-/PR^- breast cancer cell line, MDA-MB-453 when incubated with the synthetic, nonmetabolized androgen, mibolerone [76]. Investigators demonstrated that higher androgen receptor (AR)

levels were associated with older age at diagnosis, expression of ER or PR, and lower nuclear grades. ER^- and/or PgR^+ cancers had the highest AR expression; TNBC had the lowest AR levels.

Bicalutamide (Casodex, AstraZeneca) is a widely available, orally active, nonsteroidal antiandrogen that competitively inhibits the action of androgens by binding to cytosol ARs in target tissues. The ongoing phase II trial for bicalutamide given in patients with advanced breast cancer that is AR(+), ER(−)/PR(−). The subset of ER/PR(−) breast tumors which express AR may benefit from hormone manipulation which targets androgen rather than estrogen. Targeted endocrine therapy is a mainstay of breast cancer treatment in patients with ER/PR(+) tumors. The development of effective AR targeted therapy for patients with AR(+), ER/PR(−) tumors may offer a well-tolerated alternative for these patients. Additional study into the role of the AR is warranted for both ER positive and negative disease because this commonly expressed steroid HR may serve as a useful therapeutic target across more subtypes of breast cancer. The identification of a novel target such as the AR and the development of a specific, minimally toxic therapy for patients who express this target epitomizes the dawning era of personalized medicine.

8.3.7 Antiangiogenic Agents

Chemotherapeutic agents such as paclitaxel have been shown to have antiangiogenic activity, which is enhanced by altering the schedule of administration or giving the chemotherapy in combination with antivascular agents. Data demonstrating an antiangiogenic effect exist for many drugs including taxanes (89), anthracyclines (90), and cyclophosphamide (91). Patients with TNBC had significantly higher VEGF levels ($P < 0.001$) and significantly shorter recurrence free and OS with a shorter time from diagnosis to relapse and from relapse to death compared with non-TNBC. Wild type p53 inhibits angiogenesis by regulating a natural inhibitor, thrombospondin-1, and by downregulating the promoter of VEGF. Mutated p53 is associated with higher plasma and intratumor VEGF levels, and the combination of both mutated p53 and high VEGF levels has been associated with poor outcome. Many oral, multitargeted, TKIs antagonize tumor angiogenesis by their action on the TK associated with the intracellular domain of the VEGFR. A phase III study testing docetaxel with or without IMC-1121 in patients with metastatic breast cancer is ongoing. VEGF-Trap (aflibercept) is a decoy receptor for VEGF, designed to prevent the interaction between VEGF and its receptor [77]. While bevacizumab binds soluble VEGF in the extracellular compartment, various small molecule tyrosine kinase inhibitors (TKIs) have been designed to antagonize tumor angiogenesis through binding the intracellular domain of cell surface receptors. Sunitinib represents one such agent, binding multiple moieties including VEGFR1, VEGFR2, PDGFR and KIT [78].

The 2-methoxyestradiol is a physiological inhibitor of angiogenesis, which suppresses cytokine induced angiogenesis and human breast cancer growth by

up to 60 % in preclinical studies [79]. Preclinical data suggest that medroxyprogesterone acetate (MPA) binds to the glucocorticoid receptor, increasing Nm23, thrombospondin-1, and plasminogen activator inhibitor-1 [80]. An ongoing trial is evaluating high dose MPA alone and in combination with CM in patients with metastatic TNBC.

Sorafenib is a potent multikinase inhibitor with antiangiogenic and antiproliferation activity has shown modest activity in patients with advanced breast cancer. The SOLTI-0701 trial evaluated the combination of sorafenib (400 mg twice daily) with capecitabine or placebo in patients with metastatic breast carcinoma. Thirty percent of patients had TN disease. Median PFS was extended in patients treated with the combination of sorafenib–capecitabine in comparison with the combination sorafenib–placebo. The second trial evaluated sorafenib in combination with paclitaxel or placebo, as first line therapy in patients with locally recurrent or metastatic breast cancer. Forty percent of patients had TN disease.

8.3.8 Anti-tubulin Agents

A new agent that has been added to the armamentarium of drugs available for the treatment of breast cancer is ixabepilone. A subset analysis of women with TNBC identified an improved ORR for this combination of 31 % versus 15 % and PFS of 4.2 months versus 1.7 months. In the neoadjuvant setting, treatment with ixabepilone led to a pCR in 26 % of the 42 women with TNBC [81]. A retrospective analysis of this study analyzed the expression of bIIItubulin, a b-tubulin, whose expression is correlated with resistance to taxanes. Another novel mitotic inhibitor currently being studied for the treatment of breast cancer is eribulin in a phase III trial for refractory metastatic breast cancer [82].

8.3.9 Heat Shock Protein Inhibitors

Heat shock protein 90 (HSP90) is a molecular chaperone that regulates multiple signal transduction pathways by maintaining stability and activity of client proteins such as EGFR, AKT, Raf, and Cdk4; therefore, targeting HSP90 function may provide an opportunity to inhibit tumor progression of TNBCs through EGFR, PI3K, and other proteins [69, 83]. Elevated HSP90 expression seems to be a trait of breast cancer and may be an integral part of the coping mechanisms that cancer cells exhibit *vis-à-vis* stress and have shown positive association between HSP90 expression and grade, nodal positivity, tumour size, ER, c-erbB-2 and decreased survival [84]. A series of 2-amino-5,7-dihydro-pyrrolo[3,4-d]pyrimidine-6-carboxylic acid amide compounds were previously described as potent and specific HSP90 inhibitors; the detailed structure activity relationship (SAR) has also been reported [85].

Inhibitors of the HSP90 are required to maintain HER2 integrity and function and have is active against HER2 breast cancer due to enhanced receptor degradation. Geldanamycin and radicicol, bind to HSP90 at the NH2 terminal ATP pocket and inhibit the ATPase activity of HSP90 subsequently leading to client protein degradation through ubiquitin ligase machinery [86]. Geldanamycin based HSP90 inhibitors, 17-AAG (also called Tanespimycin, KOS-953, and IPI-504) and 17-DMAG (alvespimycin, KOS-1022) were clinically developed for this subset and demonstrated responses even (and in particular) in patients with progressive disease after trastuzumab therapy [87]. Several small molecular weight HSP90 inhibitors, including SNX-5422, CNF2024, STA 9090, and AUY 922, are currently in clinical trials in various tumor types [88]. Clinical data with the HSP90 inhibitor tanespimycin has demonstrated antitumor activity and tolerability in combination with trastuzumab in patients with trastuzumab refractory breast cancer [87]. HSP90 may not be as crucial for maintaining the malignant phenotype in TNBC, or alternatively, HSP90 oncoproteins essential in TNBC may not be efficiently downregulated by doses of HSP90 inhibitors that can be safely administered in vivo.

HSP90 regulates multiple signal transduction pathways by maintaining stability and activity of client proteins such as EGFR, AKT, Raf, and Cdk4; therefore, targeting HSP90 function may provide an opportunity to inhibit tumor progression of TNBCs through EGFR, PI3K, and other proteins [69, 83]. The correlation of AKT degradation and HSP70 induction between peripheral blood lymphocytes (PBL) and xenograft tumor was also determined and reveals a differential modulation of HSP90 activity between host and tumor tissues in response to drug treatment suggesting that AKT degradation in PBLs can potentially be used to guide clinical studies for TNBC.

HSP70 overexpression was found to be associated with drug resistance in both HER2+ and TNBC tumors, which is implicated in tumor cell proliferation, differentiation, death, invasion, metastasis, and immune recognition [89]. In TNBC tumors, a list of different proteins was found to be overexpressed in tumors resistant to neoadjuvant chemotherapy. Periostin precursor (OSF-2), RhoA, actinin α4, cathepsin D preproprotein, and annexin 1 predicted a poor response of TNBC to treatment. Periostin interacts with multiple cell-surface receptors (most notable integrins) and signals via the PI3-K/Akt and other pathways to promote cancer cell survival, epithelial-mesenchymal transition, invasion, and metastasis, which may contribute to the periostin-mediated drug resistance in TNBC [81]. In the TN group, tumors developed metastases expressed more 14-3-3, which is highly conserved protein family takes part in many signal transduction pathways regulating cell proliferation, apoptosis, and differentiation and HSP70 than those without metastasis.

GRB7, a calmodulin-binding protein that binds phosphorylated tyrosine residues (e.g., EGFR, HER2), and the small heat shock protein α-basic-crystallin (αB-crystallin) have been associated with inferior outcome among patients diagnosed with TN and basal like breast tumors [24].

8.4 Therapeutic Interventions

Animal models that mimic attributes of ER^- breast cancer have been used to identify agents or regimens to treat and prevent these cancers. Human basal like/TN signatures are approximated in tumors in (1) SV40-T-antigen expressing and (2) BRCA1 knockout mouse models [90]. The latter association is expected, given the prevalence of basal like/TN features among human BRCA1 mutation-associated breast cancers [15], although only 40 % of mouse BRCA1-knockout tumors resemble human TN tumors. Basal like/TN breast cancers lack established targets like ER or HER2 that can serve as a focus for treatment; therefore, the main goal in developing treatment is to move away from empirical chemotherapy toward drugs with highly specific activity against these aggressive ER^- subtypes.

The genetically engineered mouse (GEM) models are of importance for studying the in vivo functions of BRCA1, its role in tumorigenesis, its genetic interactions, and for testing potential therapies [91]. However, most homozygous mouse mutants turned out to be embryonic lethal and heterozygous mutants were not tumor prone. To overcome these limitations, conditional mutagenesis of Brca1 in mammary gland epithelium has been used to model hereditary breast cancer. Tissue specific Cre recombinase expression, driven by the mouse mammary tumor virus long terminal repeat (MMTV-LTR), whey acidic protein (WAP), CKs 14 or β-lactoglobulin (BLG) promoter, has been used to delete Brca1 in mammary epithelial cells [92]. Mammary tumors arising in conditional BRCA1-deficient mouse models have been characterized for several properties, including histopathology, p53 mutation status, ER expression, HER2 expression, genomic instability and tumor incidence [91].

The aggressiveness of TNBC was studied by producing a stable cell line by transfecting 4 T1 cells with empty vector control and sorted for cells that do not express HER2, ER or PgR and triple-positive breast cancer (TPBC) by transfecting 4 T1 cells with rat HER2, ER and PgR genes and sorted for cells with high expression of ER and PgR as a control by flow cytometry [93]. We isolated CSC by sorting for CD24+/ALDH-1+/CD44 high-expressing cells from TNBC (TNBC-CSC) and TPBC (TPBC-CSC) stable cell lines and were implanted into naive female BALB/c mice, which metastasized to the lungs to a significantly greater extent than TNBC, TPBC-CSC, TPBC or parental 4 T1 cells [93]. To negate the possibility that H&E staining of lung tissues might inadvertently miss micro-metastasis, we used the clonogenicity assay and revealed that TNBC-CSC and TNBC developed approximately fivefold and twofold more metastatic foci than the parental 4 T1 cells, respectively [93].

8.5 Conclusion

TNBC is a composite of a heterogeneous group of multiple molecular subtypes of breast cancer. TN breast cancer represents a unique subgroup, with a specific molecular profile, an aggressive behavior pattern, a relative lack of effective

therapies and a poor prognosis. A large number of therapies have been developed to date for specific molecular targets used as monotherapy or combined with traditional chemotherapy. Currently, there is no standard of care for its management. Most of these tumors are sensitive to systemic therapy, although their prognoses are still poor. Neoadjuvant chemotherapy has consistently demonstrated higher ORR for TNBC than non-TNBC, and pCR predicts improved long term outcomes for TNBC. Cytotoxic chemotherapy remains the backbone of current treatment strategies for TNBC because of a lack of known specific therapeutic targets. The benefits of chemotherapy for TNBC have now been clearly demonstrated in multiple studies in the early and advanced stages. Taxanes and anthracyclines are active in TNBC and remain important agents, but have not shown specific benefit over other subgroups. Capecitabine has limited reported data as monotherapy in TNBC, but some reports raise concerns that it may be less active in TNBC compared to HR positive breast cancer. For example, limited data suggests BRCA1 mutation-associated TNBC may have particular sensitivity to platinum agents and relatively less sensitivity to taxanes. Improved knowledge of the role of BRCA1 and the discovery of metabolic pathways has led to the development of other therapeutic strategies.

Angiogenesis inhibitors target a critical pathway in tumor development and may play an important role in rapidly proliferating tumors such as TNBC. A number of trials are ongoing, and in particular, the trials with bevacizumab should provide more definitive data in the near future. Additional study into the role of the AR is warranted for both ER positive and negative disease because this commonly expressed steroid HR may serve as a useful therapeutic target across more subtypes of breast cancer. As we gain a deeper understanding of the biologic processes driving TNBC, the arena of targeted therapeutic agents will continue to evolve, including strategies targeting the DNA repair enzyme PARP1 as well as EGFR, HDAC, angiogenesis, Src, and beyond.

The discovery of the CD44/CD24 phenotype and its relation with unfavorable prognosis in TN breast cancer disease also makes CD44 targeting an attractive therapeutic alternative [94]. The malleability of epigenetic changes as they interact with factors such as diet [95] and physical activity might explain differential cancer presentations and outcomes in African-American racial group. This line of research will enable promotion of the use of specific targeted therapies and will allow progress in the development of an early treatment that may change the aggressive course of the disease.

Most of these agents are still under further clinical validation, either alone or in combination with conventional systemic therapy. Continued research aimed at more fully characterizing the molecular and epidemiologic factors, as well as patterns of metastases observed among TNBC will advance the development of prevention and treatment strategies aimed at improving outcomes for patients diagnosed with this aggressive disease. The use of newer molecular techniques have and will continue to be very valuable in identifying potential new molecules important for survival of neoplastic cells and that could potentially be targeted in the treatment of women with TNBC.

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Chapter 9
Heat Shock Proteins in Multiple Sclerosis Pathogenesis: Friend or Foe?

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Abstract Multiple Sclerosis is a complex chronic inflammatory, neurodegenerative disease conditioned by genetic, epigenetic and environmental factors. Main pathological features of MS include areas of focal demyelination of white matter characterized by gliosis, neuron and oligodendrocyte loss. Neurodegenerative as well as immune-mediated processes play a role in the pathogenesis of this disease. One of these immunogenic factors could be represented by the heat shock proteins. HSP exhibit cytoprotective and cytostimulatory effects due to their molecular chaperones role, in many brain model misfolding diseases such as Alzheimer's and Parkinson's diseases, whereas still no unambiguous results have been reported for autoimmune disorders of the central nervous system such as Multiple Sclerosis. Actually, either positive or negative HSP role seems to depend on HSP family and on their intracellular or extracellular localization. It will be interesting to study drug treatment which overexpress or inhibit HSP production in order to gain much more information about the role of the HSP in this disease.

Keywords Autoimmunity • Central nervous system • Chaperone activity • Demyelination • Heat shock proteins • Multiple sclerosis

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Abbreviations

17AAG	17-(Allylamino)-17-demethoxygeldanamycin
APCs	Antigen-presenting cells
CNS	Central nervous system
CSF	Cerebrospinal fluid
EAE	Experimental autoimmune encephalomyelitis
GA	Geldanamycin
HSE	Heat shock elements
HSF	Heat shock factor
HSP	Heat shock proteins
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NAWM	Normal appearing white matter
NEF	Nucleotide exchange factors
OPCs	Oligodendrodyte precursor cells
PBMCs	Peripheral blood mononuclear cells
pc	Peptide complexes
PLP	Proteolipid protein
PP	Primary progressive
RR	Relapsing-remitting
SP	Secondary progressive
SRs	Scavenger receptors
TLRs	Toll-like receptors

9.1 Introduction

In the last years many studies have focused their attention on the neuroprotective role of the Heat Shock Proteins (HSP). In particular, it has been demonstrated that they can be neuroprotective in the so called conformational misfolding diseases. Their protective effect is believed to be mainly related to their chaperone role. No clear results were instead observed for immune disorders of the central nervous system (CNS) such as multiple sclerosis (MS), which is a chronic inflammatory disease of the central nervous system. The main feature of this disease is the demyelination of the white matter, but other morphological manifestations are: inflammation, axonal loss, and gliosis. MS is a complex pathology conditioned by both genetic, epigenetic and environmental factors such as gender, sexual hormones, ethnic origin, geographical latitude of residence, smoking, pathogen exposure and vitamin D levels [1–5].

Demyelinating lesions are predominantly located in the white matter even if they have been also found in the gray matter [6] and contain clonally expanded $CD8^+/CD4^+$ T cells [7–10], αβ-T cells [11], and monocytes [12, 13]. The disease is characterized by disseminated areas of focal destruction of myelin and axonal loss [14]. The progression of these lesions is one of the most typical MS features. The lesions are characterized by infiltration of cells and soluble factors of the immune system (e.g. T cells, B cells, macrophages, microglia, cytokines, chemokines) [15–17].

The majority of patients (nearly 85–90 %) experience a sudden onset of symptoms with subsequent several episodes of acute attacks followed by partial or complete recover and variable periods of remission. In the remaining 10–15 % of patients the MS course is progressive since the onset (PP). Most of patients with a relapsing-remitting disease course (RR) at onset will experience during the years a change in the disease course that will become progressive. This second phase of the disease is called secondary progressive (SP) [18]. Pathogenic studies have clearly indicated that axonal injury is a key feature of MS and the extent of axonal damage is also correlated to the degree of inflammation in the relapsing phases of the disease. A close relationship between inflammation and degeneration has also been described for all the disease stages of MS. Nevertheless, the interdependence between focal inflammation, diffuse inflammation and neurodegeneration still remains unclear.

In MS, myelin antigens such as myelin basic protein (MBP), one of the most immunogenic proteins of the CNS, synthesized in the CNS only by oligodendrocytes, proteolipid protein (PLP), the most abundant component of CNS myelin and one of the major targets of the autoimmune response [19], myelin oligodendrocyte glycoprotein (MOG), myelin-associated glycoprotein (MAG), and non-myelin antigens such as αB-crystallin (HSPB5), transaldolase, and CNPase are believed to be targets of pathogenic T cells [20–27]. T-cell activation induces inflammatory cytokines secretion (e.g. IFN-γ, TNF-α, IL-1β and IL-6) [28]. Cumulative data indicate that once damage to the CNS has occurred, sensitization to other antigens may also arise, contributing to the development of chronic disease.

Currently, it is not possible to prove without any doubt that T cells for myelin antigens are really pathogenic in patients with MS, or whether they are produced secondary to the release of myelin as a consequence of the demyelinating process. In order to prove the disease-relevance T cell responses directed against the above described antigens it would be useful to develop an adequate animal model. To date many results have been obtained by using as animal experimental model autoimmune encephalomyelitis (EAE), a T-cell mediated autoimmune disease of the CNS [29–34], which can be induced in rodents and primates by myelin antigen administration (e.g. MOG, PLP, MBP) [27, 35, 36]. However, this model is not completely equivalent to MS and does not prove that autoimmune reactivity against myelin antigens causes MS.

Non-CNS-specific antigens expressed in response to, or as a consequence of the inflammatory insult to the CNS could also be involved in MS progression. One of these immunogenic factors could be represented by the heat shock proteins.

Table 9.1 HSP family nomenclature

Old names	Molecular mass (kDa)	New names
Small HSP	34 or lower	HSPB
HSP27		HSPB1
αA-crystallin		HSPB4
αB-crystallin		HSPB5
HSP40	35–54	DNAJ
HSP40		DNAJB1
HSP60	55–64	HSPD1
HSP70	65–80	HSPA
HSP72		HSPA1A
HSC70		HSPA8
Grp75		HSPA9
Grp78		HSPA5
HSP90	81–99	HSPC
HSP90		HSPC1
HSP90β		HSPC3
HSP100	100 or higher	HSPH

9.2 Heat Shock Proteins (HSP)

HSP are classified into different families on the basis of molecular mass: HSP110, HSP90, HSP70, HSP60, HSP40, and the small HSP families. In 2009, Kampinga and coworkers proposed new guidelines for the nomenclature of human HSP families as well as for the human chaperonin families [37] (Table 9.1).

9.3 Heat Shock Proteins in the Central Nervous System

HSP expression in CNS has been detected in multiple cell types, including neurons, glia and endothelial cells [38]. Their role in the CNS is difficult to define due to the large number of cell types, which constitutes this organ. Literature data reported that the expression of HSP is induced in many pathological conditions, such as cerebral ischemia, neurodegenerative disease, epilepsy and trauma [39]. Hence, HSP can exert two neuroprotective roles: on one hand, they prevent protein aggregation and misfolding via their chaperone activity; on the other hand they can inhibit apoptosis in both intrinsic and extrinsic pathway [40–43].

9.4 Role of Extracellular

Noteworthy HSP exist not only as intracellular proteins, but almost all the HSP are released in the extracellular environment [44, 45] and they can enhance resistance to stress as a consequence of binding to stress-sensitive recipient cells such as neurons

Table 9.2 Intracellular and extracellular HSP70 functions

Location	Functions
Intracellular	Cytoprotection
	Chaperone function
	Apoptosis inhibition
Extracellular	Immune response mediator
	Antigene adjuvant
	APC maturation and innate immune response induction

[46]. For example, glial cells produce and release HSP, including the HSPA8 and the HSPA1A [47], which are rapidly taken up by neurons. Actually, motoneurons express high HSPA8 level, whereas they are not able to induce HSPA1A under stress conditions [48]. For this reason, the supply of exogenous HSP into the CNS [49], or its pharmacological induction, can reduce neuronal death in several neurodegenerative diseases [50]. As they lack any exocytosis signal to explain their presence in the extracellular milieu, at the beginning it was believed that their release was due to cellular necrosis, but it is now well known that they are released through exosomes [51, 52], extracellular vesicles, which originates from the fusion of multivesicular bodies with the plasma membrane. Recently, for HSPA1A, HSPB and HSPD1 it was also demonstrated that they can be released through membrane vesicles, lipid particles that originate directly from the plasma membrane (Tinnirello et al. unpublished results).

Otherwise to the intracellular counterpart, the extracellular HSP are involved in enhancing the adaptive immunity and in the induction of the innate immune response through the interaction with macrophages or dendritic cells [45]. Extracellular HSP are also able to signal danger to inflammatory cells and aid in immunosurveillance by transporting intracellular peptides to immune cells [45]. Two are the main characteristics which make HSP able to initiate or perpetuate autoimmune diseases: their phylogenetic conservation, (e.g., immune responses to bacterial HSP cross-react with mammalian HSP [53]); and their ability to evoke strong immune responses. In both cases HSP interaction with the target cells is mediated through membrane receptors, such as, toll-like receptors (TLRs) and scavenger receptors (SRs) [54].

The TLR family consist of extracellular and intracellular members, and two of them, TLR2 and TLR4, have been demonstrated to be involved in neurodegeneration [55–60], and might be also involved in MS pathology [61–65]. Their possible role in MS was demonstrated in EAE model. In induced mice, TLR4 knockout increased the severity of the disease symptoms due to a Th17 increased activity [66] (for reviews on TLR role in MS see [63, 64]).

Table 9.2 summarizes currently known functions of HSP in the nervous system.

9.5 HSPA1A and Multiple Sclerosis: A Negative Role?

Immune activation was already observed within the CNS in many pathological conditions such as ischemia, neurodegenerative diseases, immune-mediated disorders, infections and trauma, and it often contributes to neuronal damage. HSP, due to their evolutionary conservation and their high immunogenicity, can act as potential autoantigen, amplify and/or modify an autoimmune response. In particular, it has been demonstrated that the extracellularly released HSP are able to induce the innate immune system through their interactions with cell surface receptors, including Toll-like receptor, leading to the expression of proinflammatory cytokines [67, 68], chemokines [69, 70], and to the activation of dendritic cells [71, 72]. In the same way, the extracellular HSP enhance antigen presentation of bound polypeptides, activating also the acquired immune system. Increased expression of HSP has been observed in autoimmune forms of arthritis and diabetes, confirming their immunogenic role, and indeed HSP-reactive T cell lines have been found in these diseases.

One of the most conserved HSP is the HSPA family [73]. Among the principal protein involved in the formation of the immunogenic complex is HSPA1A [74–79]. Both extracellular and intracellular HSP play a neuroprotective role in many neurodegenerative diseases (e.g. Parkinson, Alzheimer and polyQ diseases) as they reduce the misfolded proteins. Even so a comparable protective role does not concern MS, where the extracellular HSPA1A intensifies the immune response by acting both as an adjuvant for myelin peptides presentation and as a proinflammatory cytokine [80].

As previously described, MS is a multifactorial disease, also characterized by an inappropriate immune T cell-mediated response to CNS myelin antigens. Accessory molecules represented by either class I or class II components of the major histocompatibility complex (MHC) are needed to activate T cells. Numerous studies reported that HSPA1A enhances antigen presentation through the MHCI antigen presentation pathway. Furthermore, Mycko and coworkers [81] demonstrated that HSPA1A is also able to promote antigen presentation, via the MHC class II-dependent pathway.

Several studies have been carried out on different HSP belonging to the family of HSPA both in vivo, in patients affected by MS, and in animal model miming the disease. Under normal condition, an HSPA8 chaperone role was observed for MBP, one of the two major myelin proteins of the myelin sheath [82]. In contrast, PLP, the other main component of the myelin sheath, is not likely to require chaperoning by HSPA8. It is plausible that HSPA8 should be similarly required for remyelination during the process of lesion repair in the remitting phase of RR MS. During this phase the association of the chaperone with myelin proteins on cell membrane could function as an additional target of the immune response blocking remyelination. This process could also be diminished by a reduction in intracellular HSPA8 content. Indeed, the HSPA8 content in normal brain tissue has been found to be 30–50 % higher than that in autopsy tissue from MS lesions, with chronic lesions showing the highest gap in expression [29, 30]. This reduction could be responsible for permanent loss of myelin from the lesions [82].

In MS, the immune response which occurs in the CNS leads to inflammatory and oxidative condition responsible for the overexpression of most of the inducible HSP, including HSPA1A, both within the lesion and its edge. Immunoreactivity for HSPA1A was strongly positive on reactive astrocytes and some macrophages at the leading adge of both early and chronic active lesions [82]. This overexpression was also observed in EAE animal model [29–34], and it could be interpreted as the activation of an endogenous neuroprotective mechanism [30, 33, 83].

In contrast, Cwiklinska and co-workers detected complexes of HSPA1A with both MBP and PLP in human MS lesions. Both complexes resulted highly immunogenic and in EAE model they are able to induce a specific T cell response [83–85]. Conversely, no coimmunoprecipitation was observed in control brain tissues, supporting the specificity of the complexes in MS. Completely different results were obtained by Lund et al. [85] who demonstrated that HSPA1A was associated with MBP in normal appearing white matter (NAWM) of both MS and normal human brain. These authors demonstrated that HSPA1A addition to MBP enhanced its uptake by antigen-presenting cells (APC) and its presentation via MHC II. Moreover, and in an adjuvant-like mechanism enhances an immune response to myelin antigen [81, 83].

Chiba and colleagues [86] examined antibody titers against various types of HSP in cerebrospinal fluid (CSF) of patient with either MS or other motor neuron diseases. These authors found an increased amount of IgG antibodies against both HSPA8 and HSPA1A, but no autoantibodies against other HSP, including HSPB1, HSPD1 or HSPC1 [86, 87]. In addition, remarkable higher anti-HSPA1A levels were found in patients with progressive MS compared to patients in a stable state of the disease.

Yokota et al. [88] pointed out that CSF obtained from patients with high anti-HSPA1A titers have a higher HSPA1A-induced IL-8 production in monocytic THP-1 cells, and as a consequence an increased extracellular HSPA1A level stimulated an inflammatory responses. In contrast, according to other studies no differences were observed in serum of MS patients when compared to healthy donors (HD) [84, 85, 89]. In agreement with these data, Cwiklinska et al. demonstrated that HSPA1A was not overexpressed in ex vivo PBMCs, obtained from MS patients, whereas upon cell stress HSPA1A was found to be significantly overexpressed as compared with healthy controls, and this overexpression was due to an increase in HSF1 nuclear translocation dependent on the A group of PKC isoenzymes [90]. However, this result was not unambiguous.

Recently, Mansilla and co-workers, contrary to previous studies, found an up-regulated level of HSPA1A in peripheral blood samples of MS patients compared to healthy donors. They also demonstrated that HSPA1A level increased just slightly following thermal stress in MS $CD4^+$ T lymphocytes compared to HD. An analogous result was obtained for $CD8^+$ lymphocytes and macrophages of MS patients [91]. γδ T cells are another type of T cells founded within demyelinated brain lesions of RRMS patients which are able to respond to HSPA1A. These cells produce a large amount of IL-17 [92], a potent proinflammatory cytokine involved in MS pathogenesis, and in other autoimmune diseases [93, 94]. Lund

and co-workers found an adjuvant-like effect of HSPA1A associated MBP-derived peptides. Based on these results, the authors hypothesized that a small dose of HSPA1A-MBP peptide secreted by stressed oligodendrocytes stimulated an in vivo adaptive immune response specific for the associated autoantigen. According to these data it is possible to hypothesize that a deregulated HSPA1A expression could be involved in the pathogenesis of MS due to a contribution to the chronic inflammation of the environment, and/or to the facilitation of myelin autoantigen presentation. In addition, in vivo experiments in animal model showed that HSPA1A is involved in EAE resistance as demonstrated by hsp70.1 knock-out in mice immunized with $MOG_{35–55}$ peptide [79]. The results obtained by Mycko and coworkers using hsp70.1$^{-/-}$mice demonstrated that HSPA1A is essential for the induction of the autoimmune response to the peptide $MOG_{35–55}$ [79].

In conclusion, these data demonstrate that HSPA1A is intracellularly overexpressed in the CNS of MS patients and this overexpression could have a neuroprotective function in neurons and oligodendrocytes in an inflammatory environment. Notwithstanding, intracellular HSPA1A is released into the extracellular milieu where it is accountable for the induction or the worsening of an immunological response depending on its cytokine-like property as well as its myelin-peptide adjuvant capacity.

A different result was obtained by Galazka et al. using an animal model [95]. In fact, they demonstrated that mice immunization with an HSPA1A fraction associated with peptide complexes (pc) isolated from animals with EAE, reduced the subsequent induction of EAE. On the contrary, there was no induction of EAE using HSPA1A-pc isolated from HD. These various results suggest substantial differences in the peptide that binds HSPA1A in normal versus pathological CNS. On the other hand, in EAE, pharmacological induction of HSPA1A (e.g. with geldamycin) suppresses the glial inflammatory response and ameliorate the pathology [96]. Among other drugs that can suppress EAE by means of induction HSPA1A there are triptolide [97], and its less toxic derivative (5R)-5-hydroxytriptolide [98] and celastrol.

In some cell types HSPA1A is responsible for NF-κB inhibition, a transcription factor involved in the proinflammatory response [97–99]. Indeed, NF-κB is responsible for the transcription of various cytokines relevant to MS pathogenesis, and an increased activity of this factor was observed in microglia and in invading macrophages of MS patient active lesions [100]. Taking together all these results, it is still unclear whether HSPA1A plays a protective or injurious role in MS.

9.6 The HSPC Family

Like other chaperones, HSPC1 exhibits potent protective capability such as prevention of non-specific aggregation of non-native proteins [101]. The proteins of this family seems to play an important role in the etiology of some autoimmune diseases such as rheumatoid arthritis [102], systemic lupus erythematosus [103] or type I diabetes [104].

9.7 HSPC and Neurodegenerative Diseases: Focus on Multiple Sclerosis

In several neurodegenerative disorders associated with protein aggregation, comprising Alzheimer disease and Parkinson's disease, HSPC1, conversely to HSPA1A retains the functional stability of aberrant neuronal proteins, and thus supports the accumulation of toxic aggregates [105, 106]. Oligodendrocyte precursor cells (OPCs), which retain the characteristics of multipotential CNS stem cells [107], have been found in the adult human CNS [108–110], and are normally involved in remyelination [109]. However, in MS, this process fails suggesting that OPCs are for some reason ineffective. Indeed, repair of demyelinated plaques is possible only during the initial phases of the disease. However, when MS becomes chronic this capacity is lost and there is no more remyelination in the CNS [111]. Cid et al. [112, 113] identified antibodies in the CSF patients that recognize antigens on OPCs in culture conditions. These authors demonstrated that the antibodies recognize the β isoform of HSPC (HSPC3), a protein which is expressed or overexpressed specifically on the OPC surface [114], particularly in MS patients who are in remission [113]. These antibodies did not recognize cytosolic HSPC3 and were not found in control subjects or patients with other types of inflammatory diseases [112, 114]. The authors further proved that the recognition between the antibody found in the CSF and the HSPC3 on the OPCs is responsible for complement fixation, which is responsible for complement-mediated OPC death [113]. Taken together, this is a possible explanation for OPC death and explains their remarkable decrease during the disease [112, 114].

The involvement of also α isoform of the HSPC family (HSPC1) in MS is demonstrated by several data. In fact, HSPC1 inhibition by geldanamycin (GA) treatment blocked the release of cytokines from activated monocytic cells [115–117]. Furthermore, Murphy et al. observed that GA reduced astrocyte nitric oxide synthase 2 expression and activity, and also reduced both incidence and severity of EAE [118]. However, the therapeutic potential of GA is limited by its toxicity [119].

Dello Russo and co-workers [96] preformed in vitro experiments with the less toxic GA derivative 17-(Allylamino)-17-demethoxygeldanamycin (17AAG), and their results confirmed that there was a reduction in astrocyte inflammatory responses and a lower inhibitory effect on microglia activation. On the other hand, in vivo experiments on EAE mice with a new formulation of 17-AAG (named EC72), showed a decrease in the incidence of pathology when the drug was administered before the appearance of clinical symptoms. EC72 also induced a clinical recovery in mice that were already ill. Instead, it was not observed a significant reduction in T cell activation. Similar results were obtained in vitro with 17-AAG when added to T-cell during re-stimulation with MOG, whereas no reduction in IFN-γ production was observed. On the contrary, the treatment exhibited an inhibitory effect on IL-2 production. All these findings suggested a selective effect on T-cell

derived cytokines [96]. Taken together, these results suggest that HSPC1 inhibition could reduce or delay the clinical development of demyelinating disease.

9.8 HSPD1 and the Immune System

Various kinds of cell types can release HSPD1, as demonstrated for other HSP, in the extracellular environment, under normal physiological conditions. This chaperonin, both foreign and self, represents an antigen for B and T cells [120], indeed in a number of different autoimmune and inflammatory diseases, such as type I diabetes [121, 122], rheumatoid arthritis [123, 124], and MS [21, 125, 126] were found autoantibodies to self-HSPD1. However, it has been shown that HSPD1 can regulate immune responses in animal models for MS [32, 127]. In particular, the chaperonin may exert inflammatory activity through a signal via monocytes, B cells and effector T cells, whereas anti-inflammatory properties depend on B cells, Tregs and anti-ergotypic T cells [120].

9.9 HSPD1 in Multiple Sclerosis and in EAE

As described above EAE represents the best studied animal model of MS. Depending on the species used and the age at time of sensitization, EAE may shows up as an acute episode or may develop into a more chronic syndrome with period of exacerbation and remission. Gao et al. tested the hypothesis that inflammation in the CNS is associated with an altered expression of HSP, which may be target for the development of chronic disease [32]. In animals with acute EAE in the white mater it was observed an increased immunoreactivity for HSPD1, especially in the cytoplasm of infiltrating macrophages, as opposed to normal mice, which showed HSPD1 immunoreactivity exclusively within mitochondria [32]. On the other hand, in the chronic phase of EAE the HSPD1 immunoreactivity was localized in both astrocytes and oligodendrocytes, whereas a small increase in its level was observed in the spinal cord [32].

In early MS lesions it has been observed some remyelinization of axons due to oligodendrocyte proliferation at the border of demyelinating plaque [128, 129]. Nevertheless, this process is incomplete and, with the progression of the disease, oligodendrocytes are depleted and consequently remyelinization is impaired. On the other hand, studies on MS patients have demonstrated HSPD1 reactivity in immature oligodendrocytes, whereas no staining was present in interfascicular oligodendrocytes or in other cell types of MS tissues [21, 125].

In vitro experiments confirmed that in the astrocytes predominate HSPA family members [130] but it is not present HSPD1, which is localized in oligodendrocytes [125, 131, 132]. These reactive cells are located at the margins of chronic lesions in

areas of demyelination containing TCR γδ lymphocytes [21, 133, 134], which are present also in the CSF of MS patients [135]. Since γδ T cells are present in brains of MS patients and in other neurological disease brain and CSF [135, 136] their presence per se is not disease specific. However, the simultaneous presence of both T-cells and HSPD1 is specific for MS plaques. The expression of this chaperonin and the entrance of TCR γδ cells in this area of MS lesion might explain the selective depletion of oligodendrocytes through TCR γδ cell activation by HSPD1.

9.10 HSPB in the Nervous System

Almost all the small HSP (HSPB) are constitutively expressed at low levels in the brain [137]. Cellular stress induces the expression of HSPB1 and HSPB5. Although HSPB role is not well known, it has been demonstrated that HSPB1 and HSPB5 have been involved in several neurological disorders. Many researchers observed that HSPB are present in the extracellular environment, indeed they activate immune cells during inflammation (i.e. macrophage or macrophage-like cells) [138, 139]. The immunogenic role is executed by the extracellular HSPB through specific receptors, such as TLRs or other SRs [45, 54]. Extracellular HSPB release is frequently mediated by exosomes [140, 141], also in pathological conditions (e.g. Alzheimer disease) [142, 143].

9.11 HSPB in Autoimmune Diseases: Multiple Sclerosis

Several studies suggested that both HSPB5 and HSPB1 were present in demyelinating plaques of MS brain [144]. Only few data exist on the involvement of HSPB1 on MS. In particular, Ce et al. evaluated HSPB1 blood levels during both relapse and remission phases in acute MS patients. The authors observed an outstanding increase of HSBP1 during MS relapse phase. In contrast, serum HSPB1 levels were slightly increased during the remission phase [145]. These observations suggested that the overexpression of this protein during MS might have a protective role by inhibiting misfolding of proteins and aggregation of toxic substances. However, the authors concluded that their data were not sufficient to explain the exact role of HSPB1 elevation during MS.

More data exist on HSPB5 involvement in MS pathogenesis. van Noort et al. pinpointed this molecule as the most immunodominant myelin T cell antigen in MS [146]. Indeed, HSPB5 is the major target of $CD4^+$ T-cell immunity, particularly when it accumulates to relatively high levels [26, 147, 148]. Their hypothesis was based on the reactivity of PBMCs from both MS patients and HD to proliferate in response to myelin fraction containing HSPB5 obtained from MS brains. These findings suggested that HSPB5 might be an autoantigen in MS, and that immune cells attacked endogenous HSPB5 as part of the pathology of the

disease. This hypothesis was also supported by data that showed high levels of HSPB5 in astrocytes and oligodendrocytes localized in MS lesions [149, 150], and as demonstrated later this HSPB was the most abundant transcript in MS lesions [33]. HSPB5 level is also elevated in the blood of MS patients [151]. Recently, it was demonstrated that HSPB5 accumulates in the cytosol of CNS oligodendrocytes, but not in astrocytes or axons in what it is the so-called "preactive multiple sclerosis lesions" in normal appearing white matter [147].

These lesions are defined as clusters of activated microglia that appear also in the absence of any obviously blood-brain barrier impairment, leukocyte infiltration or demyelination [152–156]. In particular, HSPB5 was found at the interface between oligodendrocytes and microglia, as well as between the layers of the myelin sheath and axons, often in granule-like patterns of expression. In this way oligodendrocytes assist survival of other cell types by releasing HSPB5 via exosomes [141, 157]. Due to the observation that there is a migration of peripheral activated T cells into the CNS and also tissue specificity of the inflammatory process. The existence of preactive lesions was confirmed by several techniques of in vivo imaging [130, 158–160]. On the contrary, van Noort proposed that these observations could be also due to the interplay of two molecules, i.e. interferon-gamma (IFN-γ) and HSPB5 (160). IFN-γ promotes microglia and macrophages activation, enhancing tissue destruction. In addition, IFN-γ kills OPCs preventing the process of remyelination [161]. On the other hand HSPB5, as previously demonstrated by van Noort and colleagues, accumulates in olygodendrocytes and myelin in MS brain due to neurodegeneration processes [162]. According to this interplay model it is not required an abnormal immune system in MS patients as hypothesized from some researchers [161]. The large amount of HSPB5 in the CNS of MS patients begins to be presented by perivascular APCs. This event triggers a response by HSPB5-reactive memory T cells, which releases IFN-γ. In this way, IFN-γ modifies the originally protective effects of HSPB5 that becomes, through TLR2 signalling, pro-inflammatory.

In 2007, a study by Ousman et al. demonstrated that mice deficient in this HSPB suffered from more severe EAE than wild type mice, and that treatment with exogenous HSPB5 ameliorated the symptoms [163]. The increased severity of EAE was due to a much more inflammatory state of immune cells, to a higher level of immune cell infiltration into the brain and to a greater demyelination in mouse brain and spinal cord both in the acute and in the progressive phase of the disease. In this situation HSPB5 acts as a negative regulator of inflammation in EAE brain and as a potent modulator of glial apoptosis [164]. In fact, exogenous administration of HSPB5 in deficient mice decreased immune infiltration into the brain and shifted the phenotype of these immune cells to an anti-inflammatory state. The opposite results obtained between MS patients and EAE model confirm that there are relevant differences between the species and that EAE model is not completely equivalent to MS.

9.12 Different HSP Expression in MS Chronic Active vs Inactive Plaques and in Different Areas of the Active Lesion

The MS pathology is characterized by plaques and the most common is the chronic-active type, with lesion activity restricted to the lesion edge [14, 165]. In these lesions, the center lacks inflammatory activity and is composed of a demyelinated parenchyma, reactive astrocytes and glial scarring [14, 166]. Usually lesion activity is restricted to the marginal zone but may extend into adjacent NAWM.

Mycko and co-workers for the first time compared samples obtained from different MS lesions (i.e., chronic-active and chronic-inactive) and from regions with different activity (i.e., margin vs. center) together with adjacent white matter, and on those samples they carried out differential gene expression studies. As expected, the authors found significant differences between the marginal zone of active and silent lesions [84, 167] and also between lesion center and lesion margin, whereas lower differences were observed between center and margins of silent lesions.

Gene expression analysis displayed an upregulation of HSP in both margins and centre of chronic active lesions compared to NAWM. In particular, in the margin of chronic active plaques the HSPC1 is the most abundant HSP if compared with the centre region, and this could explain the heterogeneity of the pathological process in different regions of MS lesions. The finding of an upregulation of the HSF4 transcription factor, inside the margin and the center of chronic-active lesions compared to NAWM, suggests that it could be one of the principles responsible for HSP activation in active lesions [168]. In order to obtain a complete view of gene expression, Quintana and colleagues performed antigen microarray analysis to identify self-antigens in different clinical subtypes of MS. These studies demonstrated that unique autoantibodies HSP signature characterize RRMS, SPMS and PPMS [126]. Strikingly, antibody responses to HSP were decreased in both SPMS and PPMS, consistent with the less inflammatory nature of progressive MS.

9.13 Conclusion

In conclusion, we have analyzed the role of HSP in one autoimmune disease of the central nervous system, multiple sclerosis. Many experimental data have demonstrated that also non-CNS-specific antigens could be involved in MS progression and that HSP could be one of these immunogenic factors. In many pathologies characterized by CNS immune activation, such as in ischemia, neurodegenerative diseases, immune mediated disorders, infections, and trauma, extracellular HSP could be involved in this process as these proteins are able to activate both the innate and the adaptive immune responses. In addition, they may exert a dangerous role due to their phylogenetic conservation.

Extracellular HSP, through the interaction with specific cell surface receptors, are responsible for the expression of proinflammatory cytokines, chemokines and for the activation of dendritic cells, which are involved in antigen presentation to B and T lymphocytes. Among all the HSP the HSPA1A is the principal responsible for the immune response regulation. In fact, high levels of anti-HSPA1A autoantibodies were found in the CSF of MS patients, especially in those with progressive MS respect to patients with a stable state of the disease. Furthermore, in stable or progressive MS the antibodies anti-HSPA1A are always increased compared to healthy controls, and this increase corresponds to a higher production of IL-8 in THP-1 monocytes with consequent higher inflammatory levels. HSPA1A was found both in and around MS lesions and it could be involved in the induction or exacerbation of the immunological response due to its ability to act as a proinflammatory cytokine. There is also a physic contact between HSPA1A and BMP and PLP, as demonstrated by immunoprecipitation assays.

On the contrary, in EAE HSPA1A overexpression, pharmacologically induced has a protective role on the disease as it attenuates the inflammatory response, and also ameliorates the symptoms. A higher level of autoantibodies against HSPA8 was also observed.

A different role was observed for the intracellular HSPA proteins, which seem to have a neuroprotective function. In particular, it has been demonstrated that HSPA8 is a chaperone for MBP under physiological condition, and it seems to be involved in the remyelination during lesion repair in the remitting phase of MS. HSPA8 identified reduction in MS lesions could be responsible for permanent myelin loss in those areas. On the other hand, HSPA1A inhibits inflammatory response by blocking the function of NF-kB, one of the transcription factors responsible for the activation of cytokines relevant to MS pathogenesis.

Another HSP which has a positive effect on MS progression is HSPC3. Indeed, in the CSF have been found autoantibodies, which have the ability to recognize the protein, inducing OPC complement-mediated cell death. Also the chaperonin HSPD1 is responsible for oligodendrocytes depletion in MS patients.

On the contrary, controversial results were observed for HSPB. For example, HSPB5 is present at up to 20-fold higher levels in glial cells in MS-affected brain samples. What is interesting is the timing of its expression. Indeed, HSPB5 accumulates in oligodendrocytes not only at later stage of inflammation but also before any peripheral blood cells have entered the tissue, in the so called pre-active MS lesions. Therefore, initially HSPB5 induced innate immune response has a neuroprotective role, whereas its accumulation, caused by cell neurodegeneration, induces an adaptive immune response responsible for tissue damage.

In conclusion, HSP are definitely involved in MS with both positive and negative role, even if to date their exact role is still incompletely understood. It will be interesting to study drug treatment which overexpress or inhibit their production, although, as demonstrated, EAE not always corresponds to human behavior.

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Chapter 10
New Indications for HSP90 and HSP70 Inhibitors as Antiviral Drugs

Matthew K. Howe and Timothy A.J. Haystead

Abstract Viruses infect host cells and elicit a broad range of ailments. The viral genome is relatively small and therefore viruses are reliant on host factors throughout the viral lifecycle. The molecular chaperones, heat shock proteins 70 (HSP70) and 90 (HSP90), have been shown to be host factors that are utilized by a wide range of viruses, including HIV, influenza, polioviurs, and dengue virus for replication and propagation. There is an observed increase in HSP70 and HSP90 expression following viral infection. Additionally, HSP70 and HSP90 regulate anti-apoptotic pathways and assist in the proper folding of newly synthesized proteins during the viral lifecycle. The utilization of HSP70 and HSP90 in viral propagation is similar to the roles of these proteins in cancer progression. Small molecule inhibitors have been developed for both HSP70 and HSP90 as anticancer therapeutics, but there is recent evidence to suggest these inhibitors have indications as antiviral drugs. This chapter aims to highlight the roles of HSP70 and HSP90 in the lifecycle of numerous viruses. Furthermore, this will highlight the potential for already developed HSP70 and HSP90 inhibitors as antivirals and the development of further antiviral drugs targeting these proteins.

Keywords HSP70 • HSP90 • Virus • Antiviral target • Host factor • HSP70 inhibitor • HSP90 inhibitor

Abbreviations

17-AAG	17-*N*-allylamino-17-demethoxygeldanamycin
17-DMAG	1 7-Dimethylaminoethylamino-17-demethoxygeldanamycin
Aha1	Activator of heat shock 90 kDa protein ATPase homolog 1
Apaf-1	Apoptotic protease-activating factor-1
Azt	Azidothymidine
Bax	Bcl2-associated X protein

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Bcl-2	B-cell lymphoma-2
Cdc37	Cell division cycle 37
Cdk9	Cyclin-dependent kinase 9
CHIP	Carboxyl-terminus of HSP70 interacting protein
Cyp40	Cyclophilin 40
E1A	Early region 1A
FLIP(S)	FLICE inhibitory protein
GRP75//78/94	Glucose regulated protein 75/78/94
Hip	HSP70-interacting protein
Hop	HSP70-HSP90 organizing protein
JNK	c-Jun N-terminal kinase
pRB	Retinoblastoma protein
TPR	Tetratricopeptide repeat
VP1/2/3/4/7	Viral protein 1/2/3/4/7

10.1 Introduction

Heat shock protein 70 (HSP70) and 90 (HSP90) are protein chaperones that play essential roles in maintaining cellular homeostasis. HSP70 and HSP90 have many housekeeping functions, including the folding of nascent proteins, refolding of misfolded proteins, protein transport between cellular compartments, breakdown of unstable proteins, removal of protein complexes, and control of regulatory proteins [1, 2].

HSP70 is an evolutionary conserved protein that is found in species ranging from bacteria, plants, and humans [3]. There are several HSP70 family members, including the constitutively active and stress inducible isoforms. The inducible form of HSP70 and the constitutively active form of HSP70, heat shock cognate 70 (HSC70), are localized to the cytosol and nucleus, while the inducible form can also be found in lysosomes [1]. There are also HSP70 family members localized to the endoplasmic reticulum, GRP78, or to the mitochondria, Grp75 [1]. The stress inducible HSP70 (also called HSP70i, HSP72, HSP70-1, HSPA1A/HSPA1B) is present in low or undetectable levels in most unstressed normal cells and tissues; however, expression levels rapidly increase in response to stress conditions that can lead to changes in protein conformation and/or stability.

HSP90 is also an evolutionary conserved protein in bacteria and eukaryotes [4]. The HSP90 family members consist of cytosolic HSP90, mitochondrial TRAP1, endoplasmic reticulum GRP94, and chloroplast HSP90C [5]. Cytosolic HSP90 is an abundant protein, consisting of up to 1–2 % of cellular protein [5]. The cytosolic family member has two isoforms, HSP90α, the inducible isoform, and HSP90β, the constitutively active isoform [6]. HSP90 has a defined list of protein clients, which includes nuclear hormone receptors and many protein kinases [7, 8].

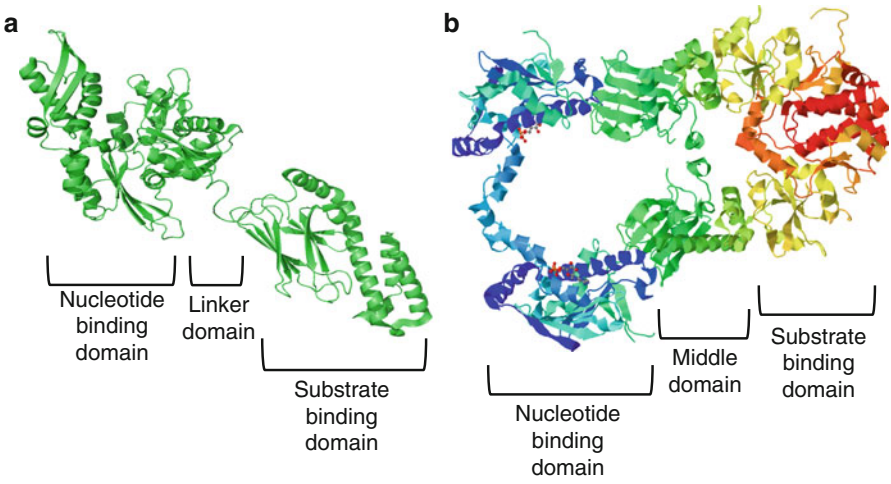


Fig. 10.1 Structure of full length *E. coli* HSP70 and HSP90. (**a**) Crystal structure of HSP70 in the ADP-bound conformation. The three functional domains of HSP70 are indicated with brackets. PDB: 2KHO, [9]. (**b**) Crystal structure of HSP90 dimer in the ADP-bound conformation. The three functional domains of HSP90 are indicated with *brackets* and ADP (represented as ball-and-stick model) in the nucleotide binding domain. PDB: 2IOP [10]

HSP70 and HSP90 are structurally distinct proteins (Fig. 10.1). Structurally HSP70 family members are similar; consisting of three functional domains, a nucleotide-binding domain (NBD) in the N-terminal region, a substrate-binding domain (SBD) in the C-terminal region, and a linker in the middle (Fig. 10.1a) [11]. The chaperone activity of HSP70 is a function of the C-terminus EEVD sequence in cooperation with other chaperones such as HSP40, HSP90, Hip, Hop, CHIP and Bag1 [11]. Much of HSP70 function and activity is regulated through interactions with co-chaperones. The J-domain co-chaperones, such as HSP40, bind to the NBD of HSP70 and assist in stimulating its ATPase activity [12]. The nucleotide exchange factor (NEF) co-chaperones, Bag-1, HSP110, and Hip, catalyze the release of ADP to complete the ATP cycle [12]. The TPR domain co-chaperone Hop, is involved in the formation of HSP70-HSP90 complexes and assists in substrate transfer [12]. CHIP, another TPR domain co-chaperone, also mediates HSP70-HSP90 binding but may also ubiquitinate some HSP70 substrate proteins through its ubiquitin ligase activity, which leads to protein degradation [13].

The HSP90 family members also contain a N-terminal NDB (Fig. 10.1b). The middle domain, between the N and C-terminal domains, is the site for protein client interactions and co-chaperone interactions [4]. While in the C-terminal domain, HSP90 dimerization occurs and co-chaperones such as Hop bind the EEVD motif [14]. HSP90 co-chaperones also assist in ATP hydrolysis as well as in client protein recognition and binding. The TPR domain proteins are the primary co-chaperones of HSP90 [15]. This group of co-chaperones includes HSP70, Hop, Cyp 40, and

FK506 binding protein [14]. The co-chaperone Aha1 binds the middle domain of HSP90 and increases its ATPase activity [16]. Cdc37 and p23 are co-chaperones that facilitate the interaction between HSP90 and client proteins [17, 18].

ATP hydrolysis is essential for both HSP70 and HSP90 function. ATP binding to HSP70 occurs through the β and γ phosphates being submerged in the NBD and stabilized through interactions with the NBD subdomains and K^+ and Mg^{2+} cations [19]. This is in contrast to HSP90, where the adenine portion of ATP is submerged in the ATP binding pocket and the α, β, and γ phosphates are in a solvent exposed conformation [19]. Upon ATP hydrolysis, both HSP70 and HSP90 undergo conformational changes that are regulated by nucleotide binding and the presence of substrate/client protein interactions [4, 20].

10.2 Targeting HSP70 and HSP90 in Cancer Therapy

Current research has shown that there is a higher level of HSP70 and HSP90 expression in malignant tumors compared to non-malignant tissue [14, 21]. HSP70 and HSP90 are up regulated in response to stress such as heat, hypoxia and nutrient deprivation, stressors that are present in the tumor microenvironment [14, 21]. Furthermore, in general there is a correlation in heat shock protein overexpression and resistance to cancer therapeutics, highlighting the role of these proteins in promoting cancer cell survival under conditions that are otherwise lethal [22]. The chaperone function of the heat shock proteins, assisting in the proper folding of proteins and the prevention of protein aggregates, is one method of providing cellular protection [22]. Both HSP70 and HSP90 regulate apoptotic pathways, both extrinsic and intrinsic apoptotic pathways [22, 23]. Extrinsically, HSP70 prevents apoptosis through inhibiting formation of the death-induced signaling complex (DISC), which consists of the death receptors, while HSP90 inhibits activation of the DISC through association with and transport of FLIP(S) to the DISC [23, 24]. Intrinsically, HSP70 and HSP90 prevent cytochrome c release from the mitochondria through preventing Bax translocation or through forming a complex with Bcl-2 [25, 26]. HSP70 also prevents apoptosis through JNK inhibition as well as through binding to Apaf-1, which blocks recruitment of procaspase-9 to the apotosome [27, 28]. Additionally, HSP90 also binds to Apaf-1, preventing apoptosis through caspase inhibition [29]. Furthermore, HSP70 and HSP90 both activate Akt, which regulates signaling pathways involved in cell survival [30]. This had led to the hypothesis that cancer cells are “dependent” on heat shock proteins for survival. Therefore much work has been done to develop inhibitors to target HSP70 and HSP90.

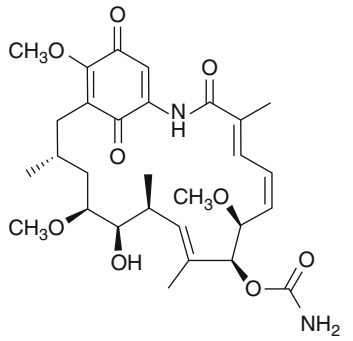
There has been a great deal of progress in targeting HSP90 for use in anti-cancer therapy. Currently there are 17 HSP90 inhibitors in clinical trials, from phase 1 to phase 3, for multiple types of cancer. The inhibitors are either derived from natural products such as 17-AAG and radicicol, or synthetic such as IPI-504, VER52296,

PU-H71, and SNX-5422 (Fig. 10.2) [14, 22, 31]. HSP90 inhibitors competitively bind the ATP binding site in the NBD, thereby preventing hydrolysis of ATP and HSP90 function in vivo [22].

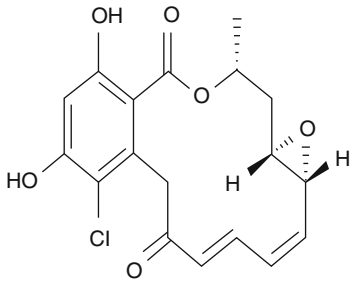
Several HSP70 inhibitors have been developed, with select compounds shown in Fig. 10.3. However, targeting HSP70 with small molecule inhibitors is faced with several challenges. First, there is close sequence identity between HSP70i and HSC70, and the conformational state of HSP70 may limit small molecule accessibility to the ATP binding site [32]. Furthermore, HSP70 has a high affinity for ADP, which makes it difficult to target the ATP binding site [19]. These complications have prevented current HSP70 inhibitors from discriminating between HSP70 family members and show little efficacy in vivo [19]. Therefore, several groups including our own have begun to develop small molecule inhibitors of HSP70 that target allosteric sites to regulate its function (Unpublished results and [33, 34]). Our group has identified a small molecule inhibitor that targets HSP70 at an allosteric site and is selective for the stress inducible HSP70 family member [35].

10.3 HSP70 and HSP90 in Viral Pathogenesis

Viruses are dependent on host factors for successful replication and propagation. Viruses utilize host factors such as protein chaperones because the genome of many viruses is limited to a few proteins; therefore HSP70 and HSP90 are utilized throughout the lifecycle of a variety of viruses' (Fig. 10.4). Upon infection of a host cell a virus rapidly induces the production and synthesis of numerous viral proteins in a small timeframe. At this step in the viral lifecycle, HSP70 and HSP90 are utilized to ensure proper folding and function of the newly synthesized viral proteins. This utilization of heat shock proteins is highlighted by viral capsid proteins being assembled into an intricate structure, which yields a potential for the formation of aggregates [36]. Proper folding and assembly of viral proteins illustrates the requirement for HSP70 and HSP90 in one step in the viral lifecycle. Furthermore, viruses need to rewire cellular signaling pathways to avoid host immune detection and to prevent apoptosis of a host cell to ensure production of mature virions. As described previously, protein chaperones are involved in various cellular pathways and have been shown to be an integral part of the viral life cycle. HSP70 and HSP90 are involved in the life cycle of many viruses including DNA viruses and positive and negative sense RNA viruses. The role of HSP70 and HSP90 in viral lifecycles is further demonstrated due to heat shock protein expression being induced following viral infection [37]. Table 10.1 highlights a subset of the work showing the role of HSP70 and HSP90 in the life cycle of numerous viruses. The role of HSP70 and HSP90 in a diverse set of viruses shows the potential for HSP70 and HSP90 as antiviral targets.



Geldanamycin



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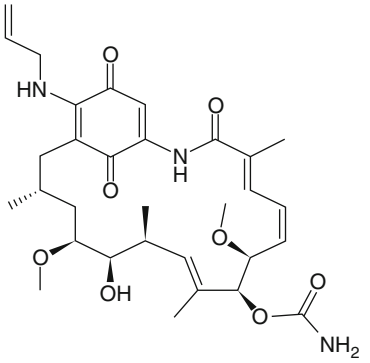
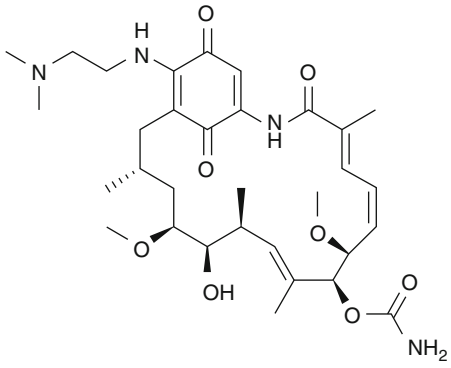


17 -AAG



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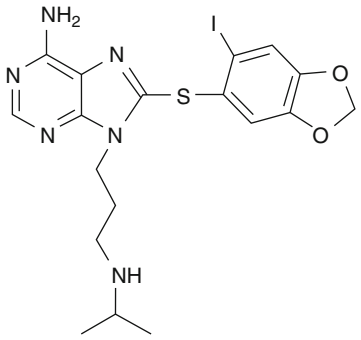
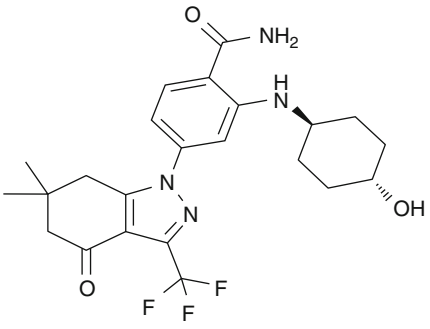
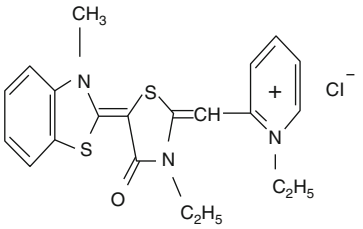


PU-H71

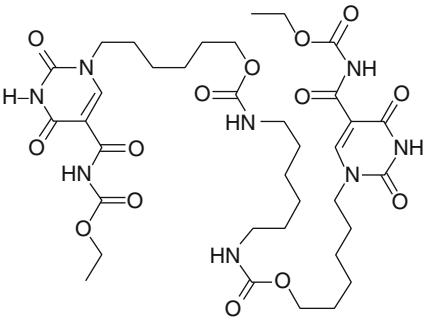


SNX-2112

Fig. 10.2 Chemical structure of select HSP90 inhibitors



MKT-077



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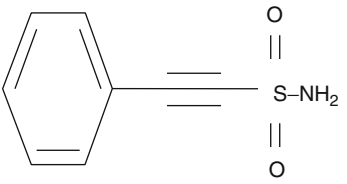
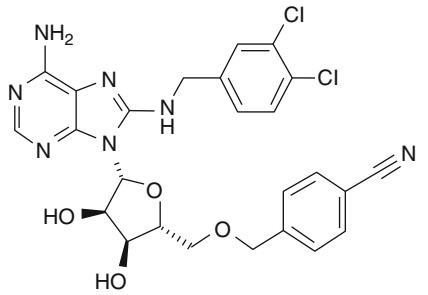
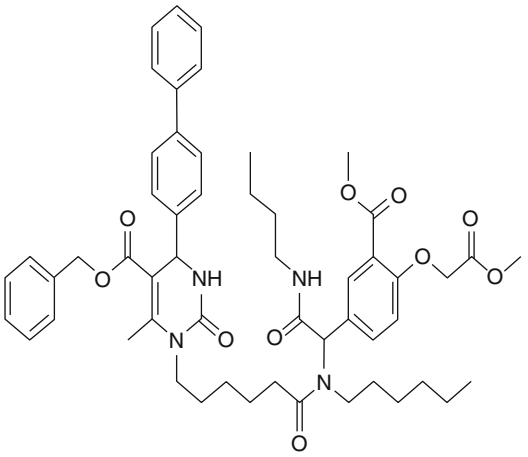


Pifithrin-μ



VER-155008



MAL3-101

Fig. 10.3 Chemical structure of select HSP70 inhibitors

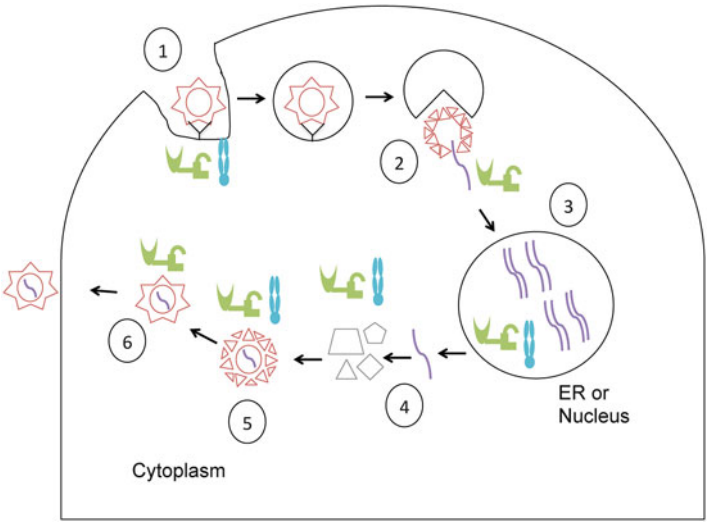


Fig. 10.4 HSP70 and HSP90 have roles in many aspects of viral lifecycles. (*1*) HSP70 and HSP90 are part of the receptor complex that mediates viral binding and internalization into host cells (ex: DENV and HIV). (*2*) After being internalized and released into the cytoplasm, HSP70 mediates disassembly of virions, which allows the viral genome to be released into the infected host cell (ex: HPV). (*3*) HSP70 and HSP90 co-localize with replication complexes of positive sense single stranded RNA viruses on the ER (ex: HCV and JEV). Additionally, HSP70 and HSP90 also mediate replication of DNA viruses in the nucleus through binding of the initiator protein to the origin of replication or proper localization of the viral polymerase, respectively (ex: VZV and HSV). (*4*) Following translation of viral proteins, HSP70 and HSP90 mediate proper folding and function of the newly synthesized viral proteins (ex: Vaccinia Virus). (*5*) Accurate assembly of the viral proteins and genome into virions is mediated by HSP70 and HSP90 (ex: Poliovirus). (*6*) HSP70 has been found to be a component of a mature virion, which is exported from the host cell (ex: Influenza A). Schematic of HSP70 represented in *green* and HSP90 dimer represented in *blue*. Presence of HSP70 and HSP90 schematic represents a role in that step of the viral lifecycle. Virus particle represented in *red*. Viral genome represented in *purple*. Viral proteins represented in *gray*

10.4 Indications for HSP90 and HSP70 Inhibitors as Antiviral Drugs

10.4.1 HSP90 as an Antiviral Target

Despitethenumber of HSP90 inhibitors that have been developed and the previous studies showing that HSP90 is involved in viral propagation, few small molecule HSP90 inhibitors have been tested for antiviral activity. One HSP90 inhibitor that has been shown to have antiviral activity is the natural product geldanamycin, which is a first generation HSP90 inhibitor. Given that there is an observed antiviral effect with geldanamycin, this shows the possibility for recently developed less toxic synthetic small molecule HSP90 inhibitors, such as PU-H71, SNX-5422,

Table 10.1 Summary of viruses that utilize HSP70 and HSP90

Virus family	Virus	HSP70 role	HSP90 role	Reference
Flaviviridae	DENV	Virus entryInduction following infection, suppression of interferon response	Virus entry	Reyes-Del Valle et al. [38], Padwad et al. [116]
	HCV	Folding of capsid and NS5A proteins	Maturation and preservation of NS3NS5A association and replication complex formation	Choukhi et al. [39], Ujino et al. [40], Okamoto et al. [41]
	JEV	Colocalizes with replication complex and with NS3 and NS5 proteins		Hui et al. [117]
	YFV	Induced late in infection		Lefeuvre et al. [42]
Hepadnaviridae	HBV	Proper folding and assembly of L and envelope proteins	Reverse transcriptase function	Cho et al. [43], Hu et al. [44]
Herpesviridae	HSV	Mediate binding of initiator protein with origin of replication	Proper folding and localization of HSV polymerase	Tanguy Le Gac and Boehmer [45], Burch and Weller [46]
	EBV	Virus dependent inductionInteraction with nuclear antigen-LP	Virus dependent inductionCell survival	Cheung and Dosch [47], Kitay and Rowe [48], Jeon et al. [49]
	VZV	Mediate replication through colocalization in replication complexes with viral DNA binding protein ORF29p	Mediate replication through colocalization in replication complexes with viral DNA binding protein ORF29p	Kyratsous and Silverstein [50]
Orthomyxoviridae	Influenza A	Component of virionLocalization of viral polymerase	Localization of polymerase subunits to nucleus	Sagara and Kawai [51], Manzoor et al. [52], Naito et al. [53]
Papillomavirus	HPV	Increased expression following infectionMediate genome replicationVirion assembly and disassembly		Liao et al. [54], Lin et al. [55], Chromy et al. [56]
Picornaviridae	Poliovirus	Forms complex with capsid P1 protein	Folding and maturation of capsid protein	Macejak and Sarnow [57], Geller et al. [58]

(continued)

Table 10.1 (continued)

Virus family	Virus	HSP70 role	HSP90 role	Reference
Polyomaviridae	SV40	Induction following infectionProper folding, localization, and assembly of capsid	Induction following infectionProper folding of large T antigen	Khandjian and Turler [59], Chromy et al. [56], Miyata and Yahara [60]
Poxviridae	Vaccinia virus	Induced following infectionFolding of viral proteins	Folding of core protein	Sedger et al. [61], Jindal and Young [62], Hung et al. [63]
Reoviridae	Reovirus	Folding of sigma1 viral protein	Folding of sigma1 viral protein	Leone et al. [64], Gilmore et al. [65]
	Rotavirus	Part of receptor complex mediating viral binding	Proper folding of nonstructural protein 3, leading to efficient viral replication	Dutta et al. [66], Guerrero et al. [67]
Retroviridae	HIV	Increased expression following infection. HIV transcription	HIV transcriptionReactivation from latency	Wainberg et al. [68], O'Keeffe et al. [69], Anderson et al. [70]
Rhabdoviridae	VSV	Component of virionFolding of viral G proteins	Stabilization of large subunit of VSV polymerase	Sagara and Kawai [51], de Silva et al. [71], Connor et al. [72]
	Measles virus	Replication, proper folding of N protein		Zhang et al. [73]
Togaviridae	CHIKV	Part of receptor complex mediating virus binding and entry	Interaction with viral proteins nsP3 and nsP4, mediating viral replication	Paingankar and Arankalle [74], Rathore et al. [75]

DENV dengue virus, *HCV* hepatitis C virus, *JCV* Japanese encephalitis virus, *YFV* yellow fever virus, *HBV* hepatitis B virus, *HSV* herpes simplex virus, *EBV* Epstein-Barr virus, *VZV* varicella zoster virus, *HPV* human papillomavirus, *SV40* simian virus, *HIV* human immunodeficiency virus, *VSV* vesicular stomatitis virus, *CHIKV* chikungunya virus

and ganetespib, to be used as antivirals (Fig. 10.2) [14, 22]. The current research using geldanamycin has further elucidated the role of HSP90 in viral pathogenesis, has shown that HSP90 is a *bona fide* anti-viral target, and indicates the potential for HSP90 inhibitors to be used as antiviral drugs. Geldanamycin has been used extensively as an anticancer agent and entered phase I clinical trials, which were subsequently suspended due to toxicity concerns [76]. Recent evidence highlighting a role for HSP90 in the lifecycle of many viruses has shown the potential for HSP90 as an antiviral target and therefore the use of HSP90 inhibitors as antiviral agents.

Several structural and nonstructural viral proteins from DNA and RNA viruses are dependent on HSP90 for proper folding, activity, and maturation, and are therefore classified as clients of HSP90. The HBV viral polymerase is a client of HSP90 and HSP90 activity is required for the polymerase function [44]. This is supported by treatment with geldanamycin, leading to an inhibition in reverse transcription of pregenomic RNA into DNA and incorporation of pregenomic RNA into nucleocapsids from decreased polymerase activity. Proper folding and localization of the rotavirus nonstructural protein 3 (NSP3) is dependent on HSP90 [66]. Treatment with 17-AAG or 17-DMAG, modified analogs of geldanamycin, results in reduced viral replication through loss of NSP3 nuclear localization [66]. The HCV viral proteins NS3 and NS5A are clients of HSP90 and require HSP90 for proper function [40, 41]. 17-AAG or geldanamycin treatment, leads to a decrease in HSV NS3 or NS5A protein levels, respectively, and subsequently a decrease in viral replication [40, 41]. HSP90 stabilizes the large subunit of the VSV polymerase (L protein), and treatment of infected cells with geldanamycin or radicicol leads to degradation of the L protein and a reduction in viral replication [72]. Another nonstructural protein that is an HSP90 client is the HSV polymerase, UL30, which is properly localized and stabilized by HSP90 activity [46]. HSP90 inhibition, with geldanamycin, leads to mislocalized and degraded UL30 and therefore reduced viral replication and titer [46]. The influenza A polymerase subunits PB1 and PB2 associate with and require HSP90 activity for proper function, which is supported by geldanamycin and 17-AAG treatment leading to degradation in PB1 and PB2 and therefore a reduction in viral titer [77].

Along with HSP90, HSP70 forms a complex that regulates the proper localization of the VZV DNA binding protein, ORF29p [50]. Disruption of this complex leads to improper localization and decreased VZV replication [50]. Another viral protein that is regulated by both HSP90 and HSP70 is the SV40 large T antigen (LT). HSP90 has been shown to interact with the SV40 LT, and treatment with geldanamycin leads to a reduction in protein levels of LT in COS7 cells [60].

The structural proteins of several viruses are regulated by HSP90 activity. In vaccinia virus, HSP90 associates with its client, viral core protein 4a, and also aids in the replication of the virus [63]. Geldanamycin treatment delays vaccinia virus replication and leads to a reduction in viral titer [63]. Another client that HSP90 properly folds, while in the immature conformation, is the reovirus protein sigma1. Geldanamycin treatment inhibits the formation of the mature sigma1 complex [65]. The viral capsid protein of the Picornaviridae family of viruses,

including poliovirus, rhinovirus, and coxsakievirus, is also a client of HSP90. The mature conformation of the capsid protein is formed following cleavage of a precursor protein, and interaction with HSP90 is required for successful cleavage of the precursor protein [58]. Furthermore, treatment with geldanamycin or 17-AAG inhibits poliovirus, rhinovirus, and coxsakievirus replication [58]. This occurs because the picornavirus capsid protein, P1, relies on HSP90 for proper folding [58].

HSP90 mediates HIV replication through formation of a complex, not through interaction with viral client proteins. HSP90 along with Cdc37 form a complex with Cdk9/cyclin T1, which allows for interaction of RNA polymerase II with HIV protein Tat, leading to viral transcription [69]. HSP90 inhibition with geldanamycin leads to disrupting the Cdk9/cyclin T1 complex and ultimately perturbing viral transcription [69]. As mentioned previously, HSP90 is involved in regulated intrinsic and extrinsic apoptotic pathways. Evasion of apoptosis through HSP90 is utilized by cancer cells that are faced with stresses such as hypoxia or anticancer treatment [22]. Following infection, viruses will utilize HSP90 to prevent apoptosis [78]. Furthermore, treatment with geldanamycin and its analogue 17-AAG have been shown to lead to cell death in NK/T-cell lymphoma cells infected with Epstein-Barr virus [49]. In human cytomegalovirus infected lung fibroblasts, geldanamycin treatment resulted in inhibited protein synthesis and viral titer, because HSP90 inhibition led to inactivation of the Akt signaling pathway, which is critical for human cytomegalovirus propagation [79].

Furthermore, our group has shown antiviral indications with the clinical candidate HSP90 inhibitor SNX-5422. Studies have shown that SNX-5422 potently inhibits HIV replication in CD4+ T cells, acting at several stages of the virus life cycle including viral trafficking, entry and integration (Unpublished Results). Additionally, a recent collaboration with the Vasudevan group (Duke-NUS) showed that SNX-5422 and a close structural analog (HS-10) potently inhibits CHIKV infection in *both* in vitro and in vivo models [75]. Novel interactions between CHIKV non-structural protein 3 or non-structural protein 4 with HSP90 were observed in pull down experiments, and silencing of HSP90 transcripts with siRNA were shown to disrupt CHIKV replication in cultured cells [75]. Finally, in a CHIKV mouse model, SNX-5422 dramatically reduced viral titers and reduced inflammation in severe infection as well as the associated musculopathy [75].

10.4.2 HSP70 as an Antiviral Target

While HSP90 inhibitors have been extensively developed and have already entered into numerous clinical trials, there are few HSP70 inhibitors that have been identified and developed [22]. HSP70 inhibitors that have previously been identified include MAL3-101, MKT-077, NSC 630668-R/1, Pifithrin-μ, and VER-155008 (Fig. 10.3) [80]. The binding site of these inhibitors on HSP70 varies, targeting the NBD or the SBD [32, 81, 82]. Additionally, many of these compounds do not discriminate between the HSP70 family members, specifically between inducible

HSP70 and the constitutively active HSC70. NSC 630668-R/1, is an ATPase activity inhibitor, but does not discriminate between HSP70 family members [82]. The binding site of NSC 630668-R/1 has not been confirmed, but it is hypothesized that the binding site is in the SBD of HSP70 [82]. MAL3-101 is a second-generation derivative of NSC 630668-R/1 and specifically inhibits J domain mediated HSP70 ATPase activity, suggesting a binding site that disrupts this interaction [83]. MKT-077 is a rhodacyanine dye that was identified to have anticancer activity, from a screen of compounds obtained from Fuji Photo Film Co., Ltd. [84]. MKT-077 binds the NBD of HSP70 and interacts with the mitochondrial HSP70 family member [85]. It was found that MKT-077 has selectivity for inhibiting proliferation in cancer cells and was tested in phase I clinical trials; however severe renal dysfunction was observed in patients, which halted additional trials [86, 87]. Pifithrin-μ was identified from a screen for compounds that inhibit p53-mediated apoptosis and induces cancer cell death [88]. While Pifithrin-μ is selective for inducible HSP70 through binding the SBD, it also has off target interactions with p53, which could be responsible for the observed anticancer activity [88]. VER-155008 displays anticancer activity and is the first HSP70 inhibitor that is an ATP mimetic [89, 90]. The compound binds the NBD of HSP70; however, it does not discriminate between HSP70 family members [89, 90].

As previously mentioned, our laboratory has also identified and is further developing a novel small molecule HSP70 inhibitor, called HS-72 that is an allosteric inhibitor selective for the inducible HSP70 family member [35]. Using HS-72 to inhibit HSP70, we have shown a dose dependent reduction in DENV infection, with no significant effect on cell viability (Unpublished results). Studies are ongoing to elucidate the mechanism of action that yields a reduction in DENV infection.

As previously discussed, HSP70 inhibitors have been developed for anticancer applications, while there has been little work done with the current inhibitors for use as antivirals. As Table 10.1 highlights, HSP70 has far reaching roles in viral pathogenesis, which indicates that HSP70 inhibitors would have potent antiviral activity. It has been observed in several viruses that there is an increase in HSP70 expression following infection. In Epstein-Barr virus, like HSP90, HSP70 mRNA is induced and subsequently HSP70 protein levels following infection [47]. HSP70 gene expression is specifically induced following adenovirus, HSV, and SV40 infection, while expression of other stress-associated and heat shock protein genes are not induced [91]. There is also an observed increase in HSP70 expression at the early stages of HIV infection, which leads to an increase in HSP70 present on the cell membrane [37]. The presence of HSP70 on the cell membrane may indicate a role in mediating binding and internalization of the virions. The initial increase in HSP70 expression is reduced during viral replication, but is increased once again at the late stages of the viral lifecycle that correspond with virion release [37]. Additionally, HSP70 is involved in folding, assembly, and localization of the HIV envelope protein and associates with the HIV p24 viral protein [92]. Furthermore, HSP70 is integrated into infectious HIV virions that are exported from cells, which correlates with increased HSP70 expression late in the viral lifecycle [37].

HSP70 mediates virus binding as part of the receptor complex for multiple viruses. As previously mentioned, HSP70 is found on the cell surface following HIV infection and possibly mediating viral binding and entry. HSC70 has been identified as a part of the receptor complex for rotavirus binding, and anti-HSC70 antibodies inhibit rotavirus infection [67]. The viral proteins VP4 and VP7, which are located on the surface of the virion, interact with HSC70 during viral binding [53]. The HSP70 family member, GRP78, has been identified as part of the receptor complex, along with integrin $\alpha_v\beta 3$, for coxsackievirus A9 and, GRP78 is essential both for coxsackievirus A9 binding and internalization into host cells [93]. Furthermore, both HSP70 and HSP90 have been shown to be part of the receptor complex that mediates dengue virus binding [38]. Antibodies against HSP70 or HSP90 inhibit dengue virus entry in cell lines and monocytes derived from human tissue samples [38].

In addition to mediating viral entry, HSP70 is involved in the viral life cycle in post-internalization steps. HSP70 associates with the viral capsid protein of adenovirus serotype 2 after the virus has been internalized into cells [94]. There is also a colocalization of HSP70 with adenovirus particles in the nucleus of infected cells [94]. This colocalization, between HSP70 and the hexon viral protein, regulates viral DNA import into the nucleus [95]. HSP70 is also involved in the replication steps of viral life cycles. Binding of the E1 helicase of human papillomavirus to the origin of replication is increased by interaction with HSP70 and is dependent on the ATPase activity of HSP70 [96]. HSP70 mediates replication of HSV-1 through association with the UL9 helicase protein. Association of UL9 with HSP70 increases affinity of UL9 binding to the HSV-1 origin of replication and facilitates viral replication [45]. In addition to aiding viral binding and replication, HSP70 also mediates viral protein folding. The HCV envelope proteins E1 and E2 assemble into a heterodimer, and aggregation can occur during this assembly [39]. GRP78 has been shown to interact with E1 and E2 to prevent the aggregation of these proteins [39]. Association with HSC70 mediates the proper folding of the polyomavirus capsid proteins, VP1, VP2, and VP3 [97]. This association occurs when HSC70 colocalizes with VP1, VP2, and VP3 in the nucleus following polyomavirus infection [97]. Nascent chains of the reovirus attachment protein, sigma1, associate with and are folded properly by HSP70 [64]. HSC70 as well as GRP78 associate with the large L envelope protein of HBV, which is a transmembrane protein, with cytosolic and ER domains [98]. The association with HSC70 and GRP78 maintains proper folding of the L protein as well as a transmembrane orientation [98].

Cellular transformation that occurs following viral infection is aided and regulated by HSP70 activity. This transformation involves the virus reactivating the cell cycle in quiescent cells. The involvement of HSP70 in this process has been shown in the SV40 virus [91]. The transforming activity of SV40 is through the viral large and small T antigen, TAg [91]. SV40 TAg contains a J domain, which allows for interaction with HSP70. During viral transformation and to initiate replication, SV40 TAg associates with pRB, p107, p103, and the E2F transcription factors in a manner dependent on interaction with HSP70 and HSP70 ATPase activity [91]. This initiates viral replication and reinitiates the cell cycle. The activity of the HPV E1A

viral protein is similar to that of SV40 TAg and E1A interacts directly with HSP70 during cellular transformation [91]. The role of HSP70 in cellular transformation during viral infection directly parallels its involvement in the transformation of cancer cells [99].

In addition to cellular transformation, HSP70 aids in the survival of cells following viral infection. As mentioned previously, HSP70 regulates extrinsic and intrinsic apoptotic pathways. The role of HSP70 aiding cell survival is also utilized by cancer cells faced with extracellular and intracellular stresses [22]. The induction of HSP70 that is observed upon infection with numerous viruses may have multiple functions, including serving as a mechanism to escape apoptosis. This is essential for viral propagation, and viruses are known to inhibit many apoptotic pathways, from inhibiting caspase activation, cytochrome c release, and activation of the death receptors [100]. HSP70 can prevent apoptosis in cancer cells through binding p53 [101]. The same interaction with p53 has been observed with SV40 TAg, a viral protein previously mentioned that interacts with HSP70 [100]. This interaction could provide viruses a HSP70 mediated mechanism for evading apoptosis following infection.

As mentioned previously, the SV40 TAg contains a J domain in its N-terminal domain, which allows for interactions with HSP70 [91]. Along with SV40 the poxvirus, Molluscum contagiosum virus consists of protein with a J domain [102]. It has been shown that TAg is involved throughout the viral lifecycle and mutations that disrupt the J domain interacting with HSP70 inhibit viral propagation [103]. This further highlights HSP70 being required by viruses for efficient replication and propagation, since these viral proteins contain J domains, which stimulate HSP70 ATPase activity.

Our studies, along with previously published results, highlight HSP70 and HSP90 as antiviral targets and shows the potential indications for therapeutic efficacy of HSP70 and HSP90 inhibitors as antiviral drugs.

10.5 Conclusion

Herein we have discussed several lines of evidence suggesting that HSP70 and HSP90 are viable antiviral targets for a wide variety of viral infections. In the case of HSP90, there is a two-decade clinical history with inhibitors targeting the protein for multiple indications in cancer [104]. Over 38 clinical trials for 17 HSP90 inhibitors have been conducted in the US alone, from phase 1 to phase 3, and many experts in the field expect FDA approval for one or more of the synthetic HSP90 inhibitors in the very near future. From our discussion therefore, it would seem that there might be much to gain by testing one or more HSP90 inhibitors for cancer for efficacy as antiviral drugs against viral infections such as HIV. Indeed, this may be low hanging fruit from a development perspective, since the human toxicities and pharmacological properties of most clinically relevant HSP90 inhibitors are very well understood. It is also likely that the therapeutic window to achieve antiviral

activity in vivo would be considerably lower than used to treat tumors. Typically, most cancer trials involve dosing patients are close to the maximum tolerated dose. One would imagine when used as an antiviral, an HSP90 inhibitor would be used at lower dose over an acute period to clear an infection. It is interesting to note that one of the first effective antivirals, AZT, for HIV came from a cancer program [105]. Additionally, in the case of AZT, dosing levels for antiviral activity were considerably lower than used to achieve efficacy for cancer [106].

A major advantage of targeting HSP70 and HSP90 as opposed to viral proteins is the issue of drug resistance. Although many successful antiviral drugs have been developed, they are all subject to development of drug resistance because of a high mutation rate that is seen in certain viruses, such as RNA viruses [107]. These mutations can lead to changes in viral proteins and render antiviral drugs ineffective [108]. To offset drug resistance, combination therapies have been developed in which two or more virally encoded proteins are targeted simultaneously. This has been very successful in managing long term HIV treatment although is not infallible, and even the most compliant of patients can develop resistance [109]. For these reasons, many groups have aimed to identify host factors that would be viable alternatives for antiviral therapy [110–112]. HSP70 and HSP90, acting in their cellular chaperone capacity as well as involvement in regulating a wide range of cellular pathways, may make them ideal host antiviral targets. The fact that neither protein would be under the direct genetic control of any invading virus renders them essentially immune from resistance arising through a single point mutation, as typically occurs with most antivirals targeting virally encoded proteins [113]. Indeed, there is some evidence that viruses may never develop resistance to HSP90 inhibitors. For example, poliovirus, rhinovirus, and coxsackie virus do not develop resistance to HSP90 inhibitors in vitro and poliovirus does not develop resistance in vivo [58]. Furthermore, there has been no reported resistance to HSP90 inhibitors that lack the chemical backbone that is found in 17-AAG, 17-DMAG, and IPI-504, when used as anti-cancer drugs [114]. Indeed, with respect to drug resistance, there are many parallels between cancer and viral infections where HSP90 is concerned, and this is also likely to also be true with HSP70. In cancer, cellular HSP90 and HSP70 are often exploited by oncogenes, which use its chaperone functions to stabilize the oncogenically expressed proteins [14, 22]. If one inhibits HSP90 or HSP70 function, the expressed oncogene becomes unstable and the cancer cells cease to grow [104, 115]. Drug pressure in this case is therefore not directed at the oncogene itself, but at the heat shock proteins. Similarly, in the case of HSP90 and HSP70 acting as antiviral targets, drug pressure would be outside the immediate genetic control of the virus. Under these circumstances, more than a single point mutation would therefore be required to promote resistance to an HSP90 or HSP70 inhibitor. Therefore, the host cell may have to involve a new drug transporter or adapt an existing metabolic enzyme to effect drug resistance when placed under selective pressure with an HSP90 or HSP70 inhibitor.

Our group is certainly not alone in thinking about HSP70 and HSP90 as antiviral targets. The development of HSP70 and HSP90 as antiviral targets and their inhibitors as antiviral drugs was the focus of a recent meeting at the NIH in late

2013 titled “Protein Homeostasis and Viral Infection: From Mechanisms to Therapeutics”. The meeting highlighted current work targeting both HSP70 and HSP90 in a variety of viruses, including HIV, poliovirus, DENV, and EBV. Much of the research thus far has been with geldanamycin and the second-generation derivatives, 17-AAG, and 17-DMAG. These compounds have been shown to elicit antiviral activity, which indicates that the potent and selective synthetic HSP90 inhibitors will also yield antiviral activity. As discussed, unlike their natural product counterparts, many synthetic HSP90 inhibitors have shown to be well tolerated in humans, with some close to FDA approval for certain cancers. HSP70 inhibitors have not been as extensively developed as HSP90 inhibitors, but current research showing the role of HSP70 in the life cycle of many viruses’ highlights the potential for theses inhibitors as well. Therefore, we predict that in the near future HSP90 inhibitors or HSP70 inhibitors could have immediate impact in the clinic as antiviral drug targets.

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Chapter 11
Potential Applications of Nanoparticles for Hyperthermia

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Abstract There is converging evidence that clinical mild temperature hyperthermia sensitizes tumors to conventional therapies as chemotherapy and radiation therapy. Coupled with an increasing understanding of the biological basis of this synergy there has been a parallel increase in the ability to achieve, maintain, measure and monitor temperature and its physiological and physical consequences. A new entrant in the arena of hyperthermia generation is nanotechnology which capitalizes on locally injected or systemically administered nanoparticles that home to tumors and are activated by extrinsic energy sources to generate heat. This chapter highlights the unique opportunities and challenges with implementing hyperthermia mediated by a variety of engineered nanoparticles for cancer therapy.

Keywords Nanoparticles • Hyperthermia • Radiation • Cancer • Immunity

Abbreviations

AMF	Alternating magnetic field
AuNRs	Gold nanorods
AuNSs	Gold-silica nanoshells
CTAB	Cetylmethylamunium bromide
EGFR	Epidermal growth factor receptor
EPR	Enhanced permeability and retention
HAuNS	Hollow gold nanoshells
MSH	Melanocyte-stimulating hormone

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MWCNTs	Multi walled carbon nanotubes
NIR	Near-infrared
PEG	Polyethylene glycol
SAR	Specific absorption rate
SPIONs	Superparamagnetic iron oxide nanoparticles
SWCNTs	Single walled carbon nanotubes

11.1 Introduction

The unifying theme across all chapters in this book is the concept that heat and other extrinsic sources of stress induce distinct responses in biological tissues that modulate their susceptibility to damage. Whereas understanding the mechanisms of these responses and exploiting them for therapeutic benefit is still an evolving science, there is an abundance of literature pointing to the clinical advantages of employing heat by itself or in combination with conventional cancer therapies. This dichotomy in the use of heat for therapeutic benefit derives from distinct biologic effects desired within tissues and stratifies thermal therapy into *thermoablation*, where the intent is to irreversibly coagulate all proteins and biomolecules in cells to destroy all tissues within an ablation zone, and *hyperthermia*, where the intent is to minimally raise the temperature of a region of the body to alter its physiology and biology and thereby prime it for the onslaught of another therapeutic modality, like chemotherapy or radiation therapy. We will focus on the latter in this chapter.

We define *hyperthermia* as temperatures that range between 41 °C and 45 °C, i.e., temperatures above fever range but below that considered to be ablative, temperatures that are not inherently lethal to cells and therefore are less prone to collateral damage if the envelope of thermal damage cannot be confined to the tumor alone. Among the first indications that temperature may have therapeutic value was the observation that erysipelas, a streptococcal skin infection associated with a febrile reaction, causes regression of tumors. Since there were no antibiotics to cure such potentially lethal infections in those days, deliberate inoculation of streptococci was not a viable therapeutic option but decades later, Dr. William Coley re-established the correlation between infection and cancer regression in sarcoma patients in 1891 [1] and extended his observations to the conscious injection of cocktails of attenuated bacterial cultures to induce a fever and prompt an anti-tumor response. Since then, there have been numerous studies that have documented that more safely administered hyperthermia has clinical utility in improving the response of tumors to conventional therapies [2–11].

The biological underpinnings of this phenomenon of synergy between ionizing radiation or chemotherapy and hyperthermia are described elegantly in other chapters of this book. A cursory glance at the underlying principles would suggest that there are physical, physiological, molecular, and immunological effects of mild elevations of temperature that, when combined with other anti-cancer therapies aid and abet those therapies. For instance, it is generally recognized that hyperthermia

causes a sustained improvement in blood flow preferentially within tumors (and not normal tissues) and this increased perfusion improves the delivery of chemotherapeutic agents to the poorly vascularized core of the tumor. Increased perfusion also implies more delivery of oxygen to the poorly perfused areas of the tumor where rapidly multiplying cancer cells have outgrown their blood supply – the resulting hypoxia, at its most elemental level, makes cells inherently resistant to radiation therapy which mediates many of its cytotoxic effects through activation of reaction oxygen species. This constellation of chaotic, underdeveloped, leaky blood vessels surrounding and penetrating a tumor also results in an acidotic and nutrient-deprived environment within the tumor that makes them more sensitive to heat-induced cellular damage [12]. This property of heat being more destructive to cells with a low pH, as with reducing hypoxia, permits synergism with radiation, which is less effective in cells in an acidotic milieu. Furthermore, physical pockets of higher temperature may cause some unintended coagulative necrosis and synergize with radiation- and chemotherapy-induced cell kill. Lastly, hyperthermia can also activate immunological responses to prime tumors for eradication by the host immune response via elaboration of self-antigens and cell surface display of these antigens (via the chaperone function of heat shock proteins described throughout this book) for recognition by antigen presenting cells that then activate tumor-specific effector T cell responses [13, 14]. A significant contributor to effectiveness of multiple courses of hyperthermia is thermotolerance, where the adaptive response of cells to heat involves cells surviving the onslaught of a given temperature applied for a given duration at specific intervals via activation of prosurvival responses including the heat shock response that blunt the effectiveness of a subsequent thermal insult. Subtle differences in normal tissue and tumor tissue thresholds for thermotolerance suggest that a more nuanced understanding of this parameter might permit not only preservation of healthy tissues during thermal therapy, but also achieve greater therapeutic efficacy. Collectively, an understanding of the tumor, stromal and normal tissue responses and adaptation to thermal stress should facilitate the rational combination of hyperthermia with chemotherapy and radiation therapy.

Indeed, that is what the preponderance of evidence in the literature would suggest. Rationally combined short durations of mild temperature hyperthermia in conjunction with optimally sequenced radiotherapy can substantially improve local control, overall survival and/or palliation of symptoms following treatment with radiation therapy without increasing toxicity [15–22]. Despite the scientific rationale and cumulative positive clinical experience with combination of hyperthermia and radiation therapy, hyperthermia is infrequently invoked as a complementary therapeutic modality in modern oncologic practice. We believe this is not due to the lack of effectiveness of the therapy but due to the difficulties in implementing hyperthermia in a controlled and specific manner. Understanding some of these challenges may provide some insight into ways to overcome these hurdles and pave the way for more widespread utilization and adoption of hyperthermia in clinical practice.

11.2 Conventional Hyperthermia Techniques

A variety of techniques have been employed to achieve hyperthermia in the clinic. They are distinguished either by their heat source, namely, laser light, radio frequency, high intensity focused ultrasound and microwaves, or by the extent of hyperthermia, namely, whole body, regional and local hyperthermia.

Whole body hyperthermia using hot water blankets and thermal chambers akin to tanning booths is employed primarily as an adjunct to chemotherapy for metastatic disease where focal therapy of each metastatic site is impractical [23]. Regional hyperthermia involves perfusing the peritoneal cavity with a warm solution of the chemotherapeutic agent for treatment of diffuse peritoneal carcinomatosis or extracorporeal circulation of a warm chemotherapeutic agent into a vascularly isolated limb with a sarcoma [24, 25]. Neither whole body hyperthermia nor regional hyperthermia is tumor-specific and therefore they both cause normal tissue side-effects that compromise some of their efficacy and feasibility/practicality. Local hyperthermia is tumor-focused and may be administered extrinsically, intratumorally or via a luminal structure within the tumor. Both luminal hyperthermia and interstitial/intratumoral hyperthermia tend to involve applicators or a grid of probes inserted into the body – they are therefore at least minimally invasive and hard to perform repeatedly. Heat sources can be activated by connecting them to extrinsic energy supply units or by remotely activating the implanted sources using an extrinsic transducer. The latter method would allow repetitive treatments after the initial minimally invasive implantation of the antennas that then emit heat and raise the temperature of the tumor from the inside outwards. Extrinsic heating can be achieved with electromagnetic radiation (microwave, laser and radiofrequency) [26] or high-intensity focused ultrasound [27]. These are minimally invasive or non-invasive techniques with extrinsic control of heating parameters. However, their tumor specificity is largely a direct consequence of physical collimation to focus energy on the tumor and there is often no sparing of or greater damage to normal tissue proximal to the tumor between the source and the tumor. In summary, contemporary methods of generating and maintaining tumor hyperthermia have unique advantages and disadvantages depending on the method employed but they all rely on physical discrimination between the tumor and adjacent normal tissue.

11.3 Nanoparticles for Hyperthermia

The search for an optimal method of hyperthermia that is accurate, precisely focused, minimally invasive, inherently capitalizes on the biology of the tumor rather than its physical confines, and with a uniform energy delivery pattern, has led to the investigation of nanoparticles as a way of delivering heat for hyperthermic treatment of tumors. The term ‘nanoparticle’ typically refers to a material with its longest dimension measuring less than 100 nm but the generally accepted

dimension in the scientific literature often sneaks up to about 300 nm. A common characteristic of these particles is that they have unique properties distinctly different from their bulk counterparts – for instance, at these sizes the surface area-to-volume ratio is considerably larger and that alone substantially increases the interaction cross section with biological tissues or with incident electromagnetic radiation. For the purposes of nanoparticle applications in hyperthermia, it is worth noting that these nanoscaled transducers can be engineered as specific absorbers of tuned electromagnetic radiation that is efficiently converted to heat, which in turn is coupled and transmitted to the tissues the nanoparticles reside within. The design of such nanoparticles can be controlled with unsurpassed precision, properties characterized with extreme accuracy and their interaction with biological systems can be modulated with physicochemical modifications of their surface. Furthermore, preferential accumulation of such nanoparticles within tumors allows maximum energy to be deposited in the tumor with minimal exposure of surrounding tissue [28].

The effectiveness of nanoparticle-mediated hyperthermia in delivering a specific amount of heat to a particular region depends on several factors. In general, these nanoparticles will require some degree of tumor specificity, excellent heating efficiency, favorable biocompatibility and toxicity profile, and preferably a simple method for imaging in vivo to facilitate image-guided therapy. Whereas different classes of nanoparticles have different advantages and disadvantages, some unique properties of all classes of nanoparticles that make them particularly attractive as a means to generate hyperthermia in tumors are outlined below.

First, nanoparticles are relatively easy to fabricate and such fabrication can be scaled up for clinical production without substantial capital investment. Metallic nanoparticles can be considered as devices rather than as drugs and this may hasten their transit through the regulatory process when compared to pharmaceutical agents. Second, nanoparticles can be administered to tumors via minimally invasive techniques. In general, these particles can be given intravenously, by inhalation or by topical application. In the case of intravenous administration, the main phenomenon responsible for enabling passive accumulation of nanoparticles in the tumor is the enhanced permeability and retention (EPR) effect wherein particles leak out of immature tumor neovasculature with large fenestrations (60–400 nm in size) and are inefficiently cleared by a rudimentary lymphatic drainage system. To fully capitalize on EPR-mediated passive accumulation, particles still need to evade the spleen and liver, where reticuloendothelial cells recognize these particles as foreign, engulf them and clear them from circulation. Ways to avoid sequestration in the liver and spleen include reducing the size of the particles to below 5.5 nm to divert particles from reticuloendothelial system clearance to renal clearance, maintaining a close-to-neutral charge on the surface of particles to minimize opsonization by the adsorption of plasma proteins (activating the complement cascade) and macrophage clearance, and decorating the surface of particles with substances that render them some stealth properties [29–32]. Commonly, the surface of nanoparticles is functionalized with a hydrophilic and biocompatible polymer which is protease-resistant, non-immunogenic and non-antigenic like polyethylene glycol (PEG) [33]. The resulting

increase in circulation time allows greater exposure of tumors to the nanoparticles and maximum probability of EPR-mediated passive accumulation. Third, nanoparticles can be decorated with targeting molecules (peptides, antibodies, etc.) homing to cancer-specific and cancer-associated antigens to achieve even greater tumor sensitivity [31]. The underlying premise is that these agents would facilitate diffusion through interstitial tissues into tumor parenchyma. In practice, however the greatest benefit with bioconjugation seems to be with cellular localization rather than dramatically enhanced delivery of nanoparticles preferentially to tumors in in vivo studies [34]. Once engineered for greatest potential for long circulation times and preferential tumor accumulation based on the principles noted here, the next step is to find the optimum method to convert the extrinsic energy source into heat focally within the tumor. For this purpose, several methods for delivering thermal therapy in an efficient and harmless manner are being tested, each one with advantages and also limitations.

Broadly speaking, there are two energy sources that can activate nanoparticles residing within tumors. Non-ionizing radiation in the form of a collimated laser beam focused on the tumor can activate particles with surface plasmon resonances tuned to the incident energy. This is discussed in greater detail in subsequent sections. The other method is to use alternating magnetic fields to activate ferromagnetic nanoparticles.

An attribute of nanoparticle-mediated hyperthermia that capitalizes on the metallic nature of systemically administered nanoparticles is the fact that they can be imaged non-invasively as well. This imaging can facilitate image-guided therapy where tissue distribution of particles is visualized, models are used for estimating the hyperthermia achievable within tumors, and possibly some adaptive treatment modifications are supported.

A final attribute of nanoparticle-mediated hyperthermia is the geography of heat distribution within tumors. In the case of most forms of hyperthermia where an extrinsic source is employed to generate heat with the tumor, there is some heterogeneity in the distribution of heat – cold spots are frequently noted along large vessel interfaces with the tumor because cooler blood (at body temperature) courses through these vessels and dissipates heat. This “heat-sink” effect results in under-dosing of heat along tumor vasculature. In contrast, however, heat generated from activated nanoparticles within tumors tends to have a different pattern. Since particles delivered intravenously have a decreasing gradient away from the vessel into the tumor parenchyma, they have hot spots along vasculature despite the dissipation of heat by blood coursing through. We think of this as “inside-out” hyperthermia originating from vasculature-tumor interfaces as opposed to classical “outside-in” hyperthermia originating from the outside. As noted in subsequent sections, this may provide some rationale for vascular disruption strategies using nanoparticles and/or cancer stem cell sensitization.

We will now highlight some of the types of nanoparticles typically used for hyperthermic treatment of cancers.

11.3.1 Gold Nanoparticles

The exposure of a metallic nanoparticle to light, at their resonance wavelength, can cause either light scattering or absorption. By specifically selecting the size, shape, and composition of gold nanoparticles, the proportion of light scattered relative to light absorbed can be optimized for the intended application [35]. This absorption cross section (σ_a) is the optical parameter that contributes directly to the photothermal heating efficiency, while the scattering cross section (σ_s) does not contribute to photothermal conversion. The absorption cross section is defined as the product of the geometric cross section area (σg) and the absorption efficiency (Qa) [29],

$$\sigma_a = \sigma_g Q_a,$$

which explains the heating efficiency's strong dependence on nanoparticle size. Thus, important features to consider in designing strategies to generate hyperthermia using nanoparticles are the wavelength of maximal absorption, the absorption cross-section, and the size of the particle [35].

Gold nanoparticles are currently being studied for use as imaging contrast agents, absorptive heating agents, and as dual imaging and therapeutic agents [35]. Specific geometries are outlined below.

11.3.2 Silica-Gold Nanoshells

Gold-silica nanoshells (AuNSs) are composed of an inner silica core and a thin outer layer of gold. This metal-dielectric structure results in a red shift of gold's characteristic plasmon absorption spectrum into the near-infrared (NIR) region of the electromagnetic spectrum – tuning this absorption to different incident light wavelengths is achieved by varying the size of the silica core and the thickness of the gold shell. Thus, the resonance of these particles can span from the visible to the NIR. NIR-activatable gold-silica nanoshells (AuNSs) are composed of an inner silica core, with 100 nm diameter approximately, and a thin outer layer of gold, ranging between 10 and 15 nm thickness. In the NIR region of the electromagnetic spectrum (700–850 nm), light penetrates the deepest in tissues due to lesser absorption by native molecules like melanin, water, oxy-hemoglobin, and deoxy-hemoglobin. Among plasmonic gold nanoparticles, silica-gold nanoshells have large absorption cross sections due to their spherical geometry, which can provide high photothermal conversion efficiencies. For instance, these nanoparticles can achieve up to 1 million times greater absorption cross section when compared to organic near Infrared dyes [36]. Collectively, AuNSs allow for tunable and highly efficient photothermal activation, can passively extravasate from the abnormal tumor vasculature (EPR effect) due to their nanoscale size, and are generally considered

relatively nontoxic and biocompatible. Even so, to further improve biocompatibility, the gold surface is easily modified by rigid and covalent attachment of PEG to its surface to suppress immunogenic responses and improve blood circulation time [29, 37].

In the initial demonstration of therapeutic efficacy in murine cancer models, tumor size and survival were monitored for 90 days following treatment with neither AuNSs nor laser illumination, treatment with AuNSs followed by illumination with NIR lasers, or sham irradiated after AuNS administration. Within 10 days of treatment, complete tumor resorption was observed in the combined treatment group and a majority of mice remained healthy and free of tumors after 90 days post treatment [37] whereas untreated and sham irradiated mice succumbed to their tumors within 3 weeks. Whereas this was photothermal ablation, a subsequent demonstration of hyperthermic radiosensitization used the same AuNS construct. Illumination of tumors laden with these AuNSs resulted in an increase in tumor perfusion (conceivably, increasing tumor oxygenation which results in greater tumor radiosensitivity) initially, a subsequent vascular disruption) due to perivascular sequestration of these relatively large 150 nm AuNSs that do not penetrate interstitium deeply) and a preferential sensitization of radioresistant cancer stem cells (presumably due to their relative abundance in perivascular niches) [33, 38–41]. Based on a favorable preclinical safety, tolerability and biocompatibility profile of AuNSs, these particles are being tested in clinical trials for head and neck cancer and prostate cancer using interstitial illumination with NIR lasers for thermoablation applications [33]. A similar approach, but without the need for interstitial applicators, is being undertaken with AuNSs for treatment of acne after topical application and laser illumination to ablate lesions that accumulate AuNSs. Similar applications for treatment of superficial tumors are also envisioned [31]. Unfortunately, given the limited penetration of NIR lasers into tissue, this approach is inadequate for non-invasive treatment of deep-seated tumors [31]. And due to the relatively large size (150 nm), these nanoparticles do not penetrate deep into tumor parenchyma but stay close to tumor vasculature.

11.3.3 Gold Nanorods (AuNRs)

Gold nanorods were developed during the same period as gold nanoshells, and are generally smaller than nanoshells (presumably allowing greater tumor penetration), cylindrical, and made of solid gold. A spherical solid gold nanostructure has a fixed optical absorption maximum in the 540 nm region and cannot be tuned to other wavelengths. However, shapes with some polarity generated by elongating them into rods with different aspect ratios (ratio of length of the rod to its diameter) are photothermally activatable. Owing to their distinctive rod shape, gold nanorods have two peaks of absorption, corresponding to their longitudinal and transverse resonances. The transverse resonance occurs at around 540 nm, while the longitudinal resonance can be tuned to specific wavelengths of light including

the NIR region by varying the aspect ratio [33, 35]. The typical manufacturing process for these rods is to include a surfactant in the reaction mixture to stretch them into rods without collapsing into spheres. The surfactant most commonly used for this purpose is CTAB (cetylmethylamunium bromide), which can pose some toxicity issues if present in large quantities in the systemically injected solution. Excessive removal of CTAB compromises the stability of the rods, so there is a fine balance that needs to be struck. Typically, removal of CTAB is done by replacing it with PEG and by centrifugation, filtration and possibly dialysis. In this case also, coating AuNRs with biocompatible PEG or a polysaccharide, like chitosan, can improve their circulation time [33]. Despite some potential issues with toxicity, AuNRs are more efficient than AuNSs in heat generation by NIR light stimulation and have a longer circulation half-life, which improve chances for tumor accumulation [33]. Investigating the photothermal heating efficiency of gold nanoparticles showed that AuNRs offer a superior absorption cross-section compared to AuNPs and AuNSs – heating per gram of gold is at least six times faster than nanoshells, when normalized for particle size differences [35]. With respect to cancer therapy applications, AuNRs have the advantage of being smaller (10 nm–50 nm) and having higher photothermal efficiency that has been used for ablation applications but could equally be used for hyperthermia applications.

11.3.4 Hollow Shells

Hollow gold nanoshells (HAuNS) consist of a hollow center with a thin outer gold shell. Their main advantage is that they can have the internal geometry adjusted to achieve better absorption across a broad size range. And from a cancer therapy standpoint, they can be made NIR plasmon resonant at sizes smaller than 100 nm [30]. These Au nanocages can be formed by synthesizing cobalt or silver nanoparticles with a shell of gold and then oxidizing the template material to end up with no solid material in the core, i.e., a hollow AuNS that is considerably smaller in size than a silica-gold AuNS. There are some safety concerns about residual Ag and Co that can remain inside the hollow Au nanostructures after the reaction, raising the potential for in vivo instability and cytotoxicity. Nevertheless, the smaller size of hollow gold nanoshells gives a delivery advantage for in-vivo photothermal ablation over larger gold nanoparticles [29, 30, 35]. There are studies reporting HAuNSs conjugated to antibodies against the epidermal growth factor receptor (EGFR), used to target and ablate EGFR-overexpressing cells, and HAuNSs conjugated to a melanocyte-stimulating hormone (MSH) analogs for in vivo ablation of xenografted subcutaneous murine melanoma tumors [35, 36]. As an added advantage, the hollow core of these nanoshells can be used as a delivery vehicle for drugs or enzymes [35, 42].

11.3.5 Matryoshka

Another method to create smaller gold nanoparticles that are photothermally activatable is to make multilayered nanoparticles consisting of a gold core, a thin silica epilayer, and finally a thin gold shell. The resulting Au/SiO2/Au particle has been called a nanomatryoshka, reminiscent of the Russian doll-within-a-doll. Relying on the strong coupling between the plasmons of the gold core and shell, known as plasmon hybridization, the plasmon resonance of the nanomatryoshka is tunable by controlling geometry and size – increasing the gold core size, keeping the intervening silica layer very thin, and growing thin gold shells results in shifting the plasmon resonance to longer wavelengths. The nanomatryoshkas have a low-energy plasmon subradiant mode at 783 nm and a high-energy superradiant plasmon mode at 560 nm [29]. More importantly, for clinical applicability, the size of an NIR-activatable nanomatryoshka is smaller than 100 nm, allowing it to have wider cancer therapeutic relevance than the larger AuNS which is unable to penetrate deep into tumors [29]. In early preclinical studies, when compared to standard gold nanoshells, these gold nanomatryoshkas exhibited improved photothermal therapy efficiency in orthotopic xenografts in nude mice (63 % vs. 39 %) and a greater uptake in tumors (nearly fivefold higher tumor accumulation when identical amounts of gold were injected intravenously) [29]. These results suggest that nanomatryoshkas have great promise for further development and clinical translation.

11.3.6 Magnetic Nanoparticles

As opposed to photothermal activation with the nanoparticles described above, ferromagnetic nanoparticles can be activated by an external alternating magnetic field (AMF). As with other nanoparticles, tumor-specific accumulation either passively or actively can permit AMF-mediated thermal activation for localized heating of the tumor while sparing surrounding healthy tissues. Interaction of AMFs with iron oxide nanoparticles and especially with Superparamagnetic Iron Oxide Nanoparticles (SPIONs) which possess a single magnetic domain (the sum of all the electron spins of all atoms in the nanoparticle aligned into a single giant magnetic moment) results in conversion of this energy to heat. Multiple theories explain the mechanism of conversion of AMF energy to heat. Néel relaxation causes energy to be released when the magnetic dipole of a particle flips between two stable orientations within a magnetic field that are separated by an energy barrier and Brownian relaxation invokes physical rotation of particles within an AMF field and collision with other particles. Heat can also be generated by frictional heating due to the physical rotation of an anisotropic magnetic particle and by hysteresis losses in a magnetic material. An AMF can also generate heat in non-magnetic material by dielectric losses in a low electrical conductivity material and eddy current losses

in a high electrical conductivity material – these forms of nanoparticle-independent heating can cause unwanted heat deposition in healthy tissues. These effects depend on particles size, shape and coercivity, and also on the applied AMF features, such as frequency and amplitude [43]. The magnetic field applied is typically within the range of 10 kHz–10 MHz, which can easily penetrate soft tissue and bones. The heat deposition depends on how effective the magnetic nanoparticle are at transforming the absorbed energy into heat, an attribute referred to as the Specific Absorption Rate (SAR) of the particle [44, 45]. SAR (in Watts per gram) is defined as:

$$\mathrm{SAR} = c\Delta T/\Delta t,$$

where c is the specific heat capacity, T is the temperature rise during the time interval (t) [43].

11.3.7 Iron Oxide

For the express purpose of producing localized hyperthermia in a tumor, the primary design specification of a nanoparticle is that of maximizing energy deposition to create direct cytotoxicity or prime tumors for sensitization to radiation therapy or chemotherapy. Unfortunately, the low SAR and/or the poor targeting of magnetic nanoparticles currently available for preclinical testing mandates extremely high concentrations of nanoparticles within the tumor with associated high concentrations in normal cells surrounding the tumor – this highlights the importance of finding methods to minimize normal tissue accumulation (and therefore, toxicity) and tumor-specific conjugation of particles for rapid and specific heating of tumor tissues [31, 43].

Magnetic hyperthermia is limited by the lack of a technology that effectively localizes and focuses heat to the tumor without heating surrounding healthy tissues. Also, a second limitation is the inability to accurately measure the heat deposited in the tumor relative to surrounding tissue. Another important parameter to take into consideration is the heat dose rate. The maximum temperature achievable occurs with the largest dose rate, requiring application of the highest field amplitude in the shortest time interval possible. This minimizes body temperature regulation and homeostasis back to baseline either by thermal washout (the heat sink effect) or by shivering and contraction of blood vessels close to the skin. The faster heat can be deposited locally within the tumor, the higher the probability of achieving a sustained rise in temperature before physiological responses dissipate the heat [45].

11.3.8 Doped Iron Oxide

To increase the efficiency of magnetic thermal induction, iron oxide nanoparticles can be doped with elements that can increase the SAR and enhance the local

delivery of heat [44]. Commonly, these are manganese, cobalt and zinc. By varying the dopant, the size of the nanoparticle/crystal, and the crystalline anisotropy, the SAR can be maximized. The magnetic thermal induction efficiency can be further enhanced by creating core-shell nanoparticles with a magnetically hard core encased within a magnetically soft shell and the exchange-coupling of these two materials tuned to maximize the specific loss power [46].

11.3.9 Carbon Nanotubes

Carbon nanotubes are long hollow structures with walls formed by sheets of single atoms of carbon arranged in a honeycomb-like manner. Single walled carbon nanotubes (SWCNTs) have a single roll of such sheets with diameters in the order of nanometers and lengths in the order of microns or larger. Multi walled carbon nanotubes (MWCNTs), on the other hand, have concentric tubes of SWCNT stacked within each other. They are both characterized by a broad electromagnetic absorption dynamic range that arises from their one-dimensional structure. This, in turn, results in a unique combination of electrical, thermal and spectroscopic properties that can be exploited for generation of hyperthermia. The potent thermal conductivity is determined by the arrangement of carbon atoms in the wall of the cylinder [47].

MWCNTs have superior thermal ablation properties relative to SWCNTs [28]. When administered intratumorally in mice, they serve as very efficient photothermal activators requiring merely short pulses of low laser power. MWCNT dose-dependent tumor regression and durable long term tumor regressions are achievable in this manner. Alternatively, these MWCNTs can be activated by tuned radiofrequency waves, potentially at the risk of some off-target toxicity [48]. Lastly, these nanostructures can be decorated with tumor-targeting peptides or antibodies, laden with superparamagnetic iron oxide or other such imaging vehicle, and modified to reduce their toxicity which is often a function of nanotube type, size, shape, and surface characteristics. In particular, when entrapped with iron for T2-weighted MR imaging, they can facilitate sequential MR imaging and hyperthermia without cross interference, since the iron content does not affect the heating properties and the hyperthermia does not affect the MR contrast. Therefore, it is possible to use these particles to perform local and specific hyperthermia guided by MR imaging [35, 44, 47, 49, 50].

11.4 Unique Opportunities

11.4.1 Tumor-Targeting

An exciting feature of accessing tumors with nanoparticle formulations is the possibility of enhancing tumor specificity above and beyond the EPR effect. Pref-

erential targeting via bioconjugation of nanoparticles with peptides and antibodies that recognize cell surface antigens on tumors allows greater accumulation within tumors and possibly lesser entrapment in the reticuloendothelial system. Identifying biomarkers expressed only on the surface of cancers and not normal cells is the key to allowing specific accumulation of nanoparticles selectively in cancers (including micrometastatic disease) and minimizing therapy-related toxicities. The challenge is that some biomarkers, like epithelial growth factor receptor (EGFR), are expressed not only in cancer cells but also in some normal cells, albeit at a higher concentration in cancer cells than in normal cells. This carries with it the possibility of potential toxicity from nanoparticle accumulation in nonmalignant sites. Another challenge with delivery of nanoparticles into tumors is the inability to penetrate deep within tumors due to the inhomogeneous and chaotic vasculature and blood flow of tumors. This also increases the interstitial pressure within tumors and forces out fluids from the tumor and prevents nanoparticle extravasation from the circulation into the tumor. Lastly, targeting tumors using cell surface receptor-targeted nanoparticles is constrained by the limited number of receptors to which the targeting particles may bind; an issue that compounds the problem of poor deep penetrability of nanoparticles [51, 52]. Not surprisingly, it is becoming more evident that nanoparticles decorated with targeting ligands only marginally improve the total nanoparticle accumulation in tumors when compared to untargeted nanoparticles although the intracellular and extracellular nanoparticle distributions can be significantly altered by targeting techniques [34]. A potential solution to this problem is to target biomarkers specific to tumor vasculature and circumvent the issue of high tumor interstitial pressure and poor deep penetration [52].

11.4.2 Image-Guided Therapy

A unique advantage of nanoparticles used for thermal therapy is their ability to also serve as imaging agents, either due to inherent properties of the therapeutic nanomaterial or via conjugation to, decoration with or encapsulation of imaging contrast agents. This combination of therapeutic and diagnostic capabilities within the same platform is termed theranostics. As noted previously iron oxide nanoparticles are often readily imaged by MRI [53], plasmon resonance of photothermally activatable nanoparticles permit imaging based on absorbance or scattering [54], gold particles can be visualized with x-ray fluorescence imaging based on characteristic x-ray emission [55], and gold nanoparticles can be visualized by photoacoustic imaging [56] or surface-enhanced Raman scattering imaging [57]. These visualization options for nanoparticles make them ideal for image-guided therapy applications and for dosimetric modeling of heat generated. Indeed, these same properties also allow nanoparticles to detect the present and extent of tumors often with greater accuracy and sensitivity than traditional contrast agents. In turn, the hope is that such imaging techniques will allow early detection of cancer in high-risk populations and guide therapeutic decisions in patients with more advanced stages of malignancy [15, 21, 22, 31].

11.4.3 Drug Delivery

The idea of using nanoparticles as efficient means of delivering drugs to disease sites has been around for a long time. In principle, the use of a nanoparticle to, at the same time, deliver an anticancer agent or a radiosensitizer to the tumor and generate hyperthermia can be a powerful combination. The restriction of access of the drug to the tumor would reduce toxicity to neighboring healthy cells. The versatility of nanoparticle structure and composition makes them amenable to delivery of drugs and oligonucleotides [31, 58, 59].

11.4.4 Enhancement of Traditional Therapies

A unique attribute of gold nanoparticles is the high atomic number (Z) of gold. High-Z materials are known to cause significant radiation dose enhancement via photoelectric interactions with ionizing radiation, which is directly proportional to Z^{3-4} [60]. Consequently, gold nanoparticles used for hyperthermia may also have applications as radiation dose enhancers, particularly if the amount of gold that accumulates in tumors is large and the location of such accumulated particles is close to tumor vasculature and/or internalized within cells where the secondary electron showers are more likely to cause oxidative stress and other events leading to cell death. This is probably more likely with gold nanorods than gold nanoshells given their smaller size and greater depth of penetration into tumor parenchyma.

11.4.5 Immune Modulation

Hyperthermia is a known immunomodulator that not only increases chemotaxis of T cells into the tumor microenvironment [61] but also mediates antigen presentation [62] for greater activation of effector T cells and more efficient elimination of cancers by the immune system. Other chapters in this book allude to the role of heat shock proteins in chaperoning tumor specific proteins from the interior of cells to the surface and also the extracellular space (as cytokines). It remains to be determined whether nanoparticle mediated hyperthermia has any distinct advantages over other forms of hyperthermia in terms of immune modulation given the nature of heat distribution within tumors heated with nanoparticles; i.e., does “inside-out” hyperthermia lead to greater perivascular thermal damage that enables more extravasation of effector T cells and does cellular internalization of nanoparticles create a greater stress that leads to more efficient display of autoantigens on tumor cell surface for recognition by T cells. A greater understanding of the immunomodulatory effects of nanoparticle-mediated hyperthermia may also permit its use as an adjunct to adoptive T cell transfer for more efficient delivery of cytotoxic T cells to primary and metastatic tumors.

11.4.6 Heat-Triggered Release of Nanoparticles

Among a variety of methods of delivering nanoparticles to tumors is the idea that smaller nanoparticles can be encapsulated in larger nanoparticles and deployed within the tumor microenvironment by an external trigger. In hyperthermia circles, this concept of triggered release has been most elegantly demonstrated with custom-designed liposomes that rupture at hyperthermia-range temperatures and release their payload of chemotherapeutic agents into the tumor. This thermosensitive liposome carrying doxorubicin has been tested in clinical trials (NCT00093444 and NCT00346229, www.clinicaltrials.gov) and is undergoing continued evaluation in the clinic. A similar strategy could be employed for delivery of nanoparticles to tumors where an external energy source heats the nanoparticle contained within a thermosensitive liposome and the heat generated ruptures the liposome and releases its contents (thermal therapy nanoparticle, imaging agent, chemotherapeutic agent, immunomodulatory agent and/or oligonucleotide) for efficient cell kill. The cytotoxic effects of such therapy would again be localized to the tumor and would, in principle, spare surrounding normal tissues.

11.5 Challenges

The preceding sections have documented the unique attributes of nanoparticles used for hyperthermia and highlighted the potential for distinct advantages when compared to other forms of hyperthermia. However, nanoparticle-mediated hyperthermia poses its own set of challenges for clinical translation.

First, scaling up production of nanoparticles from small batches used for preclinical studies to large batches needed for clinical trials is a challenge. Particles need to be precisely engineered and characterized for physical, chemical and biological properties. Synthesis, purification, characterization and analysis have to confirm batch-to-batch consistency.

Second, these nanoparticles need to be shown to be biocompatible and safe by themselves in animal models. Whereas the parent bulk metal may be considered safe for clinical use as in the case of gold, once it is formulated at a nanoscale the resulting unique properties can influence toxicity. Some features of biocompatibility testing are outlined below. It is customary to first establish sterility and lack of endotoxin contamination of the nanoparticle formulation before advancing to the clinic. Next, formulations should be stable in physiologic conditions in vivo without aggregation, dissociation of component layers such as targeting ligand, or non-specific dissolution of protective epilayers that shield more toxic internal components. Next, residual components from the fabrication steps should be accounted for. For instance, CTAB on the surface of gold nanorods should be minimized in the final formulation. Next, aspects of biological activity that are unrelated to the primary function of the nanoparticle may influence the biocompatibility and efficacy such

as complement activation and opsonization, sequestration in the liver and spleen, mutagenicity, hemolysis, allergic sensitization, and possible immune stimulation. The Nanotechnology Characterization Laboratory was specifically established at the NCI for assisting government, academia and industry with translating promising preclinical discoveries from the bench to the bedside by characterizing nanomaterials of interest. Their experiences, stemming from characterizing over 250 different nanomaterials from over 75 different investigators, with some common mistakes and oversights in nanomaterial formulation are outlined in an informative and eye-opening manuscript published recently [63]. On a similar note of caution, there is some concern that the elongated nature of carbon nanotubes that resemble asbestos fibers contributes to the formation of mesothelioma-like plaques when administered directly into the abdominal cavity [35, 64–67].

Third, a persisting challenge with adequate delivery of nanoparticles to the tumor via the systemic circulation is the issue of biodistribution. As already discussed here before, the accumulation within the tumor is attributed to the EPR effect wherein nanoparticles circulating in the blood stream gradually concentrate in tumors by extravasation from leaky tumor vasculature. However, they are also readily engulfed by the liver and spleen macrophages and efficiently cleared from circulation when their size exceeds about 5.5 nm [32]. Typically, less than 10 % of injected particles reach the tumor – this is a major limitation for hyperthermia applications if the efficiency of thermal induction is not large. For this reason, many magnetically activatable nanoparticles need to be directly injected into the tumor. The other possibility is that long nanotubes do not circulate long because of rapid clearance by the reticuloendothelial system, again necessitating direct injection over systemic administration. Lastly, the accumulation of nanoparticles in tumor cells is also dependent on surface charge and surface chemistry. Positively charged particles are more readily endocytosed than neutrally charged particles, while negatively charged nanoparticles spread throughout the tumor bulk more rapidly [68]. Even for validated and effective particles for photothermal therapy, there are in vivo studies showing that their size (around 130 nm) and shape significantly hamper their uptake in tumors, since the average rate for accumulation remains close to 5 % of total volume administered, while the concentration observed in the liver and spleen were 20 times higher than this [35]. These data suggest that optimizing biodistribution and finding better solutions for efficient delivery vectors for tumor uptake are critical components of clinical translation of nanoparticle-mediated hyperthermia from the bench to the bedside.

11.6 Conclusion

Hyperthermia has been recognized as a potent sensitizer of cancer to traditional therapies such as radiation therapy and chemotherapy for a long time. Nevertheless, enthusiasm for its widespread clinical use has been dampened by the challenges in administering, maintaining, monitoring, modeling and reporting hyperthermia.

The recognition that nanoparticles have unique attributes that can be exploited for clinical hyperthermia applications has fueled a growing interest in exploring potential clinically translatable options. The search for optimal particle structures, thermal activation strategies, image-guided interventions, theranostics, and multifunctional nanoparticles continues. This chapter provides an overview of the landscape of nanoparticle-mediated hyperthermia and outlines the promise, the successes, and the challenges of using nanoparticles for thermal therapy. A greater understanding of mechanisms of action and the operating parameters for the optimal integration of nanoparticle-mediated hyperthermia into clinical treatment paradigms will be critical as we advance lessons learned at the bench to practices adopted at the bedside. Nonetheless, recent advances in our understanding of fundamental properties of nanoparticles exploitable for hyperthermia foretell a bright future for the translation of nanoparticle-mediated hyperthermia to the clinic and the addition of this form of hyperthermia to the therapeutic armamentarium available for the fight against cancer.

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Part IV
Heat Shock Protein HSP90-Based Therapies

Chapter 12
Gene Therapy Against HSP90: Glucocorticoid Receptor-Assisted Cancer Treatment

Susanta Sekhar Adhikari*, Sujan Kumar Mondal*, and Rajkumar Banerjee

Abstract Heat shock protein 90 (HSP90) is an ideal target for cancer because of its involved participation in multiple cancer-implicated pathways that characterize various hallmarks of cancer. Many of the small molecules reported find limited human use because of non-specific collateral damage, and also because of stability & solubility issues. Here we intend to give a different strategy to eliminate HSP90 that too in cancer-specific manner. Towards this we show an interesting property of expressed glucocorticoid receptor (GR) in cancer cells. In these cells GR can be targeted selectively by a newly designed cationic lipid-based liposomal formulation (DX) for selective gene expression. We designed an artificial micro-RNA targeted to certain highly conserved domains in 3′-untranslated region of HSP90, and delivered it as plasmid with DX to different cells and tumor-bearing mouse-models. We observed targeted reduction of HSP90 expression with concomitant reduction of various pro-proliferative, HSP90 client proteins including various kinases and growth factor receptors. Interestingly, anti-apoptotic yet HSP90 client protein, p53 are up-regulated. Together it affected the significant reduction of tumor volume/mass. We hereby describe the development of first 'gene therapy' strategy to target HSP90 by manipulating cancer cell-associated GR, for effective tumor reduction with minimal collateral damage.

Keywords Glucocorticoid receptor • Gene delivery • Liposome • Cationic lipid • Dexamethasone • Artificial miRNA • Tumor

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Abbreviations

ADP	Adenosine diphosphate
Akt/PKB	Protein kinase B
AR	Androgen receptor
ATP	Adenosine triphosphate
Bcl2	B-cell lymphoma 2
Cdc37	Cell division cycle 37
Cdk	Cyclin-dependent kinase
Chk1	Checkpoint kinase 1
CMV	Cytomegalovirus
DAPK	Death associated protein kinase
Dex	Dexamethasone
DODEAC or DO	N,N-di-n-octadecyl-N,N-(2-hydroxyethyl) ammonium chloride
DX	Dexamethasone-associated cationic lipid-based liposomal formulation
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ErbB2	V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2
ERK1	Extracellular-signal-regulated kinase 1
FGF	Fibroblast growth factors
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPR30	G protein-coupled receptor 30
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive elements
GSK3β	Glycogen synthase kinase 3 beta
HDAC6	Histone deacetylase 6
Her2	Human epidermal growth factor receptor 2
HSBP1	Heat shock factor binding protein 1
HSE	Heat shock element
HSF	Heat shock factor
HSP90	Heat shock protein 90
hTERT	Human telomerase reverse transcriptase
IFNγ	Interferon gamma
IGF1	Insulin-like growth factor 1
JAK	Janus kinase
LBD	Ligand binding domain
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog
miRNA	Micro RNA
MRP1	Multidrug resistance protein 1
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NHR	Nuclear hormone receptor
PDK1	Pyruvate dehydrogenase kinase isoenzyme 1
P-gp	P-glycoprotein
Plk	Polo-like kinase
qRT-PCR	Quantitative real-time polymerase chain reaction
SAR	Structure activity relationship
SR	Sigma receptor
STAT	Signal transducer and activator of transcription
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
UTR	Untranslated region
VE-cadherin	Vascular endothelial cell-associated cadherin
VEGF	Vascular endothelial growth factor

12.1 Introduction

Heat shock proteins (HSP) are the 'Messiah' class of proteins chaperoning ill-folded protein factors. This hence necessitates ubiquitous expression of HSP in all cells. Under stressed conditions such as during acidosis, elevated temperature, hypoxia etc. cells express markedly enhanced HSP, especially HSP90 [1]. This 90 KD or so evolutionarily conserved chaperone protein has many (to date, clearly defined more than 300 or so) client proteins, which essentially needs chaperoning not only in the event of misfolding or mutation but also for their correct, regulated functioning. A comprehensive list of HSP90 client proteins and interactors (i.e., protein factors directly or indirectly interacting with HSP90) can be seen in a compilation [2]. Among these clients there are multiple protein factors including kinases that are directly involved in the maintenance and progression of cancer. Hence, HSP90 is essentially over-expressed to simultaneously chaperone multiple of these factors complicating the overall pathway-scenario in tumor microenvironment. This therefore critically confounds the management of cancer and its related therapy. Optimistically, in return cancer (or in broader sense tumor) shows overwhelming dependency on HSP90. This leads to what is called as 'HSP90 addiction'. As a result HSP90 inhibitors sensitizes cancer cells more than normal cells as HSP90 in cancer cells becomes more sensitive to these inhibitors [3]. Even though the delivery and execution of HSP90 inhibition should be clinical otherwise the attempt to eradicate cancer will be overshadowed by collateral, irreversible side effects. In overall, anticancer therapies aimed to target and inhibit HSP90 gain importance simply because they in turn are expected to target multiple factors implicated in cancer progression.

Among various client proteins of HSP90, glucocorticoid receptor (GR) holds very limited attention when 'cancer' is in perspective. Since, GR is essentially expressed in almost all cells it cannot be a viable target for cancer, at least theoretically. Mechanistically however, in the absence of hormonal or synthetic ligands in cytoplasm, HSP90 firmly holds GR while clutching its ligand-binding

domain (LBD) and strictly maintains the structural integrity of GR-LBD. HSP90 leaves GR only when the correctly fitting GR-ligand snuggles into the LBD, making GR nucleus-bound [4, 5] Ligand-bound GR as dimer 'transcriptionally regulate' the expression of various genes, including those of gluconeogenesis and these genes are regulated by a collection of glucocorticoid responsive elements (GRE) in genome [6, 7]. Gluconeogenesis is a cellular phenomenon predominantly happening in liver cell hepatocytes, which produce glucose from non-carbohydrate precursors and release it in blood stream for other organs' usage. If gluconeogenesis is the only important phenomenon that is associated with GR then one may question why GR is anyway ubiquitously expressed in all cells! The reason for this ubiquitous expression is not clear though. One of the GR synthetic ligands, dexamethasone (Dex) is a well-known anti-inflammatory agent having limited anticancer effects [8, 9]. Since, cancer is an inflammatory disease we believe any therapeutics intended to reverse inflammatory responses may be logically modified to generate novel anticancer therapeutics.

This chapter is intended to focus on how GR can be utilized for the purpose of selectively targeting cancer. We describe an anomalous behavior of cancer cell-expressed GR which is believed to be compounded by a surprisingly 'compromised' HSP90 in cancer cells [10]. The serendipitous discovery enabled us to design an anticancer liposomal delivery system, which could deliver and express in cancer cells a specially designed artificial micro RNA (miRNA) against HSP90 with high selectivity. The target selectivity was so pronounced that besides HSP90, multiple of its pro-proliferative client proteins were degraded rendering tumors to shrink significantly [11]. Although miles-to-go towards honing specificity in the modality but this study clearly documents the first gene therapy attempt against HSP90 and also attempts to shed some light on the role of GR and HSP90 in tumor microenvironment.

12.2 HSP90 in the Crossroad for Cancer Progression

It is now well documented that cancer cells need HSP90 in a 'big way' to maintain various illicit, unregulated pathways towards establishing various 'hallmarks' of cancer. Several of the crucial proteins involved in this juggernaut that propels the advancement of cancer are the active client proteins of HSP90. Most of these crucial client proteins acquire mutations, which could have been otherwise detrimental, are eventually not only tolerated but obtain 'gain of function' to drive oncogenesis. This happens only by the active support of HSP90 for which HSP90 needs to be over-expressed to buffer this calamitous situation so that the cancer maintains a pseudo-homeostasis in the body or specifically in the tumor-growing region. HSP90, which comprises less than 2 % of total cellular protein content in normal un-stressed conditions, expresses 3–4 % during stressed condition. Most of these excess HSP90s, now co-engage other co-chaperones, ATPase modulators and other protein accessories to form individual clusters, to interact with different client proteins in

what is called as chaperone cycle [12]. Subsequently, driven by crucially important multiple ATP hydrolyses in the N-terminal domain of HSP90, the regulation of client protein function and their 'turnover' occurs. Oncogenic mutations in client proteins such as various kinases lead to their higher conformational instability and hence require exaggerated chaperonic help from HSP90 to maintain say, increased kinase activity. This way inherent genetic variation/mutation can be buffered by increased level of HSP90 besides chaperoning usual client proteins [13, 14].

There are multiple instances in literature exhibiting that various protein factors involved in maintaining the hallmarks of cancer are actively chaperoned by HSP90 [This has been excellently reviewed recently by [15]]. Briefly, let's see how. Cancer cell proliferating kinases need HSP90 (and also sometimes HSP90's co-chaperone Cdc37) for their activation and stability. Any hostility against HSP90 such as through chemical inhibition renders these kinases unstable, forcibly degraded with suppression of activities. These kinases play important roles in cell cycle regulation and check points & cell division machinery. These are belonging to: (a) Cell Surface Receptor tyrosine kinases (ErbB2/Her2, insulin receptors and insulin-like growth factor I etc.) [16–18]; (b) Src Family tyrosine kinases (Src, Yes, Fes, Fps, Lck etc.) [19–21]; (c) Serine/Threonine kinases such as Raf Family protein kinases (Raf1, B-Raf) [22, 23]; (d) other Serine/Threonine kinases such MAPK-related protein kinases (MAK, MOK, MRK) [24]; (e) Cell cycle and division-related kinases such as Cyclin-dependent kinases (Cdk4 and Cdk6), other mitotic kinases (Plk, Aurora Kinase B, Chk-1) etc. [25–29].

Survival growth factors such as Insulin receptors, IGF-1 receptors, PDK1, Akt/PKB, mTOR etc. are all chaperoned by HSP90 [17, 18, 30–32]. One of the most important Factors involved for DNA damage response and cell apoptosis is p53. 50 % of cancer carries p53 mutation [33]. So, it is evident that 50 % of cancer still carries wild type p53. Then why is it not properly functioning to contain cancer growth? This is simply because wild type p53's activation is predominantly sequestered by many factors, many of them including mdm2 are critically chaperoned by HSP90. Evidences however show that cancer-causing mutated p53s interact with HSP90 persistently more than the corresponding wild types and exhibit higher binding affinities [34, 35]. Clearly, tumor-associated p53-mutants are HSP90 addicted and hence, down-regulation or inhibition of HSP90 will have more adverse effect on 'HSP90-addicted' subtypes and therefore on cancer cell survival. Other than p53, DAPK (death associated protein kinase) is another prominent HSP90 client, serine/threonine kinase protein, which mediates apoptosis in response to interferon-γ (IFN-γ) and hence acts as tumor-suppressor protein. Its expression is anyway lost in multiple tumors, however inhibition of HSP90 is expected to have deteriorating role on DAPK expression [36]. Expectedly, since DAPK expression contradicts the very survival of cancer cells, DAPK's expression unlike that of other pro-cancerous factor should not be HSP90-addicted. Hence, in cancer cells HSP90 inhibition-linked DAPK's degradation is not expected to out-weigh impact of the degradation of pro-cancerous factors.

Further, sex steroid receptors such as estrogen receptors (ER) and androgen receptor (AR) are chaperoned by HSP90 for its steroid hormone binding [5, 37].

This binding leads to subsequent transactivation and gene expression. In breast and prostate the activation of these respective receptors are highly implicated with cancer progression. The limitless replication potential in cancer primarily involves telomerase, a reverse transcriptase, which incessantly helps elongating and maintaining telomeric end of chromosomes in replicating cancer cells. The human catalytic subunit hTERT in telomerase complex is chaperoned by HSP90 and other co-chaperones [38]. HSP90-inhibition eventually leads to proteolysis of hTERT and retards the mechanism of telomeric DNA length maintenance [39, 40].

Towards strengthening the expansive potential of solid tumors angiogenesis plays a very critical role. Neovascularization and potent angiogenic ability of tumor cells determines the rate of tumor progression and metastasis. Vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF) bind to the respective tyrosine kinase receptors VEGFR1/2 and FGFR3/4 expressed on tumor-associated endothelial cells' surface and stimulate proliferation of endothelial cells and hence new blood vessels. These receptors are client proteins of HSP90 and their stability and functions are dependent on chaperone-function of HSP90 [41, 42]. Hence, HSP90's inhibition leads to reduction in levels of these receptors and thereby angiogenesis and tumor growth [43]. So, plainly speaking, cancer progression-associated HSP90 client proteins enact more crucial role in cancer than other HSP90 client proteins and hence targeted down-regulation or inhibition of HSP90 will significantly impact the overall growth of tumor.

So, aren't there any of the HSP90 client proteins, whose expression and functionality may be utilized for cancer elimination, yet its expression would not be greatly influenced by HSP90-inhibition? Theoretically if it is possible, then one can design novel anticancer therapeutics aimed to inhibit HSP90 targeted via that client protein. In this regard, let us look into one very interesting aspect of HSP90 chaperone property.

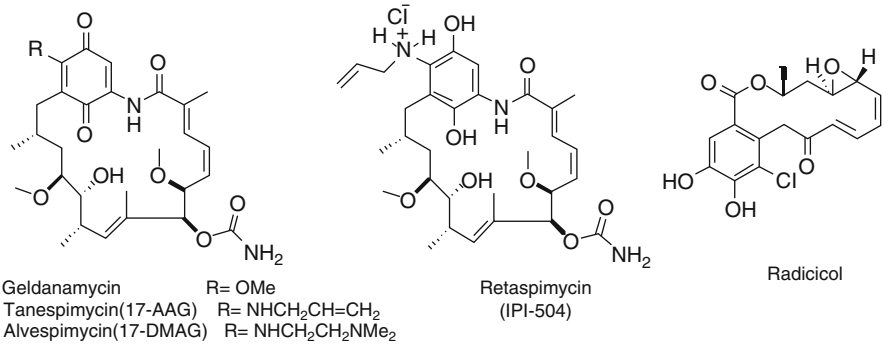
Glucocorticoid receptor (GR) is a client protein of HSP90. The structural integrity of GR-ligand binding domain (GR-LBD) is strictly monitored by HSP90, which allows a space of maximum two molecules of GR-ligand to sit on to LBD [4, 5, 44]. As the ligands bind to LBD, HSP90 release the GR. Ligand-bound GR then localizes in nucleus for further gene transactivation/repression. This interaction of HSP90 with client proteins is functionally related to ATPase cycle involved in the N-terminal domain of HSP90. Generally, binding and hydrolysis of ATP in N-terminal domain of HSP90 precede the release of chaperoned client proteins from it, however with a glaring exception in p53-HSP90 interaction, where ATP binding and not its hydrolysis is all that is needed to release p53 from HSP90 [45, 46]. Hence for GR, ligand binding and ATP hydrolysis in HSP90 seems to occur simultaneously. But most importantly, GR-LBD and HSP90 seemingly share a cellular symbiotic relation. How? It is shown that in one hand, release of GR-LBD by HSP90 involves ATP hydrolysis; on the other hand, GR-LBD stimulates ATPase activity (i.e., which leads to ATP hydrolysis in N-terminal domain) of HSP90 [47]. Conversely, one can hypothesize that the presence of cellular GR (or in other words, GR-LBD) stimulates the ATPase activity and hence influence prompt release of GR as well as other chaperoned client proteins. In cancer cells since chaperoning of HSP90 client

proteins that are implicated broadly with cancer dissemination is rampant, HSP90 needs more and more ATP binding and requires enhanced ATPase activity. When urgency exceeds supply, complete chaos occurs. Towards simultaneous chaperoning of multiple client proteins in cancer cells, HSP90 possibly needs more and more 'illicit' involvement of GR (or GR-LBD or other similar proteins whose identity or functioning are not clear) for strengthening and arming its ATPase activity. Naturally in this scenario, one can expect leniency in strictness about GR-LBD and HSP90 chaperone interaction. The essence of the present chapter is actually based on this hypothesis and an all-important observation related to this forbidden involvement of GR with HSP90, which allowed us to develop a novel anticancer therapeutics.

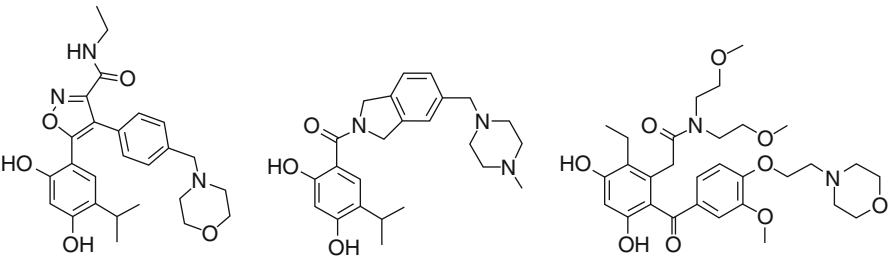
12.3 Chemist's Role in Harnessing Hsp90 in Cancer

There has been an upsurge of chemistry-related activities in developing small molecular weight HSP90 inhibitors. These inhibitors are primarily responsible for inhibiting the functional activity of the heat shock proteins. Among heat shock proteins the focus on HSP90 has increased due to its involvement in the several cellular phenomena and more importantly in disease progression. HSP90 keeps the death protein in an apoptosis resistant state by direct association. The wide range of functions of HSP90 results from its ability to chaperone several client proteins that exert a central pathogenic role in human diseases including cancer, neurodegenerative diseases and viral infection. Molecular chaperones are over expressed in wide varieties of cancer cells and in virally transformed cells and are responsible for controlling the activity of signalling proteins. Therefore inhibition of function of these chaperones is essential in controlling cancer cells. The availability of drugs that can specifically target HSP90 and inhibit its function thereby resulting in the depletion of client proteins, has made Hsp90 a novel and exciting target for cancer therapy. This section of the chapter briefly summarizes the current status of both first and second-generation HSP90 inhibitors based on their chemical classification and stage of clinical development.

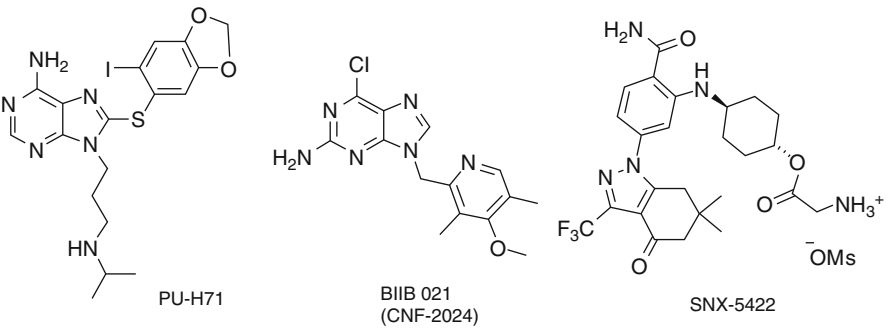
There are now 14 drug candidates that target HSP90 undergoing clinical trials in multiple indications as single agents or combination therapy. These compounds represent a diverse array of chemical matter stemming from natural product scaffolds to synthetic structure-based design. A close inspection of a diverse array of structures that advanced to clinical trials reflects that they can in general be classified according to their similarity to natural product geldanamycin (Scheme 12.I) and the unrelated natural product radicicol (Scheme 12.I) or to the resorcinol- (Scheme 12.II) and purine-scaffolds (Scheme 12.III). Only SNX-5422 falls outside any of these designations (Scheme 12.III). The ansamycins (geldanamycin and herbimycin) and the unrelated natural product radicicol were found to bind to the N-terminal pocket of HSP90 and inhibit its function. The discovery of HSP90 as the target of anticancer activity of geldanamycin has resulted in intense efforts from industry and academia to develop clinically viable small molecule HSP90 inhibitors [48].



Scheme 12.I Chemical structures of HSP90 inhibitor geldanamycin and its derivatives and natural product radicicol



Scheme 12.II Chemical structures of resorcinol-containing synthetic inhibitors



Scheme 12.III Chemical structures of purine and pyrazole-based synthetic inhibitors

12.4 Scheme I

Geldanamycin has interesting anti-tumor properties in vivo in animal models. However, issues like stability and hepatic toxicity impeded the translation of this compound in clinics. In an effort to improve tolerability and to address formulation issues, a number of semi-synthetic geldanamycin derivatives have been developed, including tanespimycin (17-AAG), alvespimycin (17-DMAG) and retaspimycin (IPI-504) as depicted in Scheme 12.I. Tanespimycin (17-allylamino-17-desmethoxygeldanamycin, 17-AAG), the first-in-class inhibitor of HSP90, derived from substitution of C-17 methoxy group of geldanamycin with an allylamino group to enter the clinic in 1999 is now in Phase II trials [49]. Unfortunately, 17-AAG is insoluble, difficult to formulate and lacks oral bioavailability. Alvespimycin (17-desmethoxy-17-N,N-dimethylaminoethylaminogeldanamycin, 17-DMAG), obtained from the substitution of the C-17 methoxy group of geldanamycin with N,N-dimethylaminoethylamine, was proved to be potent and to have improved water solubility. The presence of an ionisable amino group is responsible for its increased water solubility, better oral bioavailability and equal or greater anti-tumor activity compared to 17-AAG. This drug has entered Phase I clinical trials in various types of lukemia and solid tumors, and has demonstrated tolerable toxicity [50].

12.5 Scheme II

Retaspimycin (17-allylamino-17-desmethoxygeldanamycin hydroquinone hydrochloride, IPI-504), a 17-AAG analogue containing a reduced hydroquinone, prepared by reduction of 17-AAG with sodium dithionite followed by conversion to its hydrochloride salt, has improved water solubility properties thereby facilitating formulation for parental administration. However, hepatotoxicity problem has been observed as similar to 17-AAG and 17-DMAG. Geldanamycin-derived products are also being clinically tested in combination with other chemotherapeutic agents. Imatinib (STI-571, Gleevec) is an effective therapy for only chronic myeloid leukemia characterized by the expression of the oncoprotein, Bcr-Abl. Imatinib inhibits this active tyrosine kinase that renders the cancer cells resistant to apoptosis. Since Bcr-Abl is a Hsp90 client protein, a combination of 17-AAG with imatinib is being tested in Phase I clinical trials in Bcr-AbI positive leukemia with encouraging results [51]. Enhancement of 17-AAG activities has also been reported for proteasome inhibitors such as bortezomib [52]. This effect might result from the increase in protein misfolding induced by 17-AAG, together with the impaired clearance of proteins by the ubiquitin proteasome pathway [53].

A combination of 17-DMAG with arsenic trioxide has emerged as a promising therapeutic combination since they synergize to induce apoptosis and mitotic arrest in leukemic cells.

The natural product radicicol (Scheme 12.I), a macrocyclic antifungal antibiotic was found to bind to the N-terminal pocket of Hsp90 and inhibit its function. Radicicol displays potent *in vitro* activity but was largely inactive in tumor xenograft models probably because of its multiple electrophilic sites such as the strained epoxide and conjugated dienophiles that lead to metabolic deactivation in vivo [54]. However, this scaffold has led to rationally design resorcinol-containing synthetic inhibitors with improved solubility and in vivo potencies. The most advanced clinical compounds in this class are NVP-AUY922 (VER-52296), KW-2478, and AT13387 as well as STA-9090 (Scheme 12.II). STA-9090 is an unspecified novel resorcinol based triazole inhibitor advanced to multiple clinical trials in both advanced solid tumors and hematological malignancies.

12.6 Scheme III

A rational choice for HSP90 inhibitors targeting ATPase activity is purine-based compounds (Scheme 12.III). The first synthetic class of such scaffolds was the PU series, such as PU-H71 and PU-D28. These purine-based molecules were developed based on available X-ray crystal structures of HSP90 bound to ATP/ADP as well as to natural inhibitor geldanamycin and radicicol. These PU series molecules mimic the conformation of ADP in the pocket and have a higher affinity for HSP90 than ADP [55]. These molecules possess good solubility and permeability. CNF-2024 (BIIB021), a purine-scaffold induces Hodgkin's lymphoma cell death through inhibition of NF-kB signaling pathway [56] and is orally bioavailable. Interestingly, CNF-2024 was active in cancer models in which 17-AAG and other ansamycin derivatives were inefficient because of the expression by the tumor cells of multidrug resistant proteins (such as, P-gp, MRP-1) [57].

Analysis by computational chemistry and X-ray crystallography of selected ATP-binding proteins allowed the discovery of an orally bioavailable and effective pro-drug, 2-aminobenzamide derivative, SNX-5422, a potent Hsp90 inhibitor which is now in multiple Phase 1 clinical trials [58].

12.7 Synthetic Lipids, Liposomes and Cancer Targeting

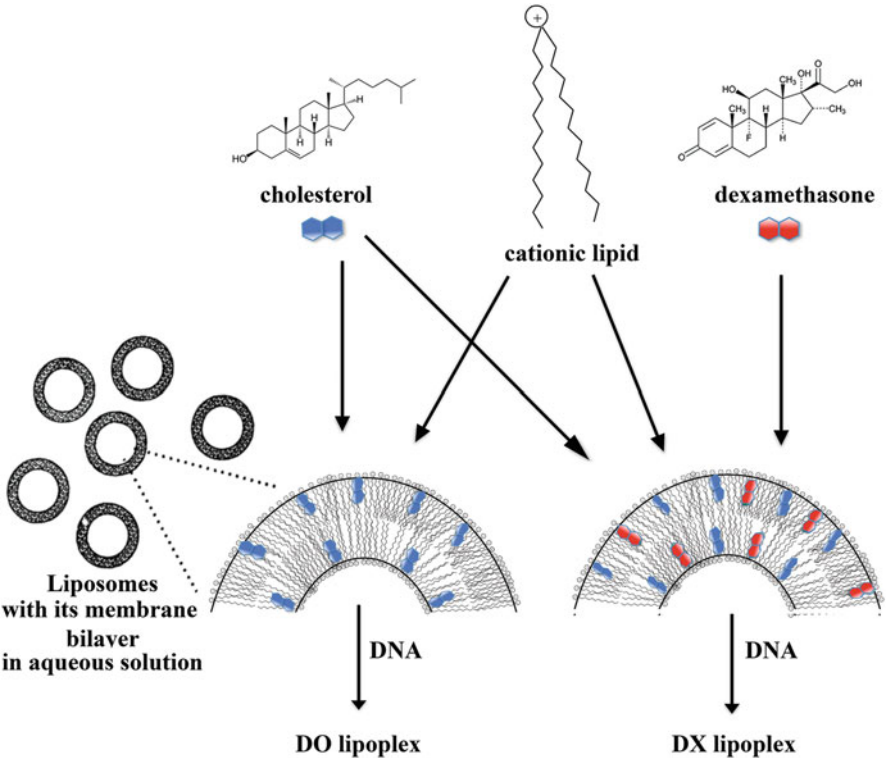
In this section, we would like to discuss in brief the status of the development of cationic lipids, their liposomal behavior and targeted gene delivery aspect. This is important to know to understand the basis of our present discussion, which relates to a new liposomal formulation-based anticancer therapeutics. We will also touch upon how select analogues of these cationic lipids if typically modified develop

into a novel class of selective anticancer molecules. Although under thorough investigation but we will discuss in brief that few of these molecules exhibit an interesting property of regulating HSP90 expression.

12.7.1 Synthetic Lipids and Liposomes

Any amphipathic molecule possessing typical hydrophilic/lipophilic balance (HLB) tends to make aggregates of different interesting characters in aqueous solutions. Typically, an aggregate forming molecule should contain at least a lipophilic moiety (such as a carbon chain length of varied size) and a hydrophilic functional 'head' group (such as an ionic or proton-carrying polar groups etc.). As an example, an ionic molecule conjugated with a single lipophilic carbon chain in aqueous solution can form aggregates called 'micelle' with a hydrophobic/lipophilic core. The same molecule aggregates to form 'reverse micelle' in organic solvent maintaining a hydrophilic pocket in the inside core. However, if instead of one, two lipophilic carbon chains are conjugated to ionic molecules with hydrophilic head group and suspended in aqueous solution, it forms bi-layered aggregates called 'liposomes'. Liposomes (or lipid based compartments) can be classically envisioned as stable lipophilic bubbles, which individually maintain an aqueous pocket [Please see Scheme 12.IV]. Because of this basic structural feature liposomes find many uses especially in pharmaceutical formulations, which can carry hydrophilic drugs in the aqueous pocket and lipophilic drugs in lipid bilayer. If the constituent lipid moiety of liposome carries a positive charge in the hydrophilic head-group region then the liposome 'cooperatively' assumes net positive surface charge. The lipid is called as a cationic lipid and therefore the liposome is called as cationic liposome. Hence, in addition to carrying drugs of different solubility properties, the cationic liposomal formulation bearing net surface positive charge can electrostatically complex with cargoes possessing net negative charges. DNA is a classic example that, because of carrying negatively charged phosphodiester bond among linking nucleotides, can easily complex with cationic liposomes [Scheme 12.IV]. This electrostatic complex is called lipid/DNA complex or 'lipoplex'.

Now, cellular surfaces are negatively charged because of negatively charged membrane-associated components such as proteoglycans, phosphonated fatty lipids, etc. Hence, for DNA the cellular entry is not easy. A lipoplex with net positive charge will have electrostatically more favorable disposition to enter cells, thereby allowing easy cellular entry of DNA cargo. Lipoplex upon treatment to cells are endocytosed and the lipid and the cargo are generally known to dissociate allowing DNA to enter nucleus for further gene expression. The expression of delivered gene following the whole sequential episodes of cellular entry, endocytosis, lipid-DNA dissociation and nuclear entry is collectively called as gene transfection. However, not all cationic lipids have the ability to induce efficient gene transfection. Huge body of evidences available in literature showcasing the structure activity relationship (SAR) study as to how varying functional groups in cationic lipids



Scheme 12.IV Schematic representation to show how targeted (DX, i.e., with dexamethasone) and non-targeted (DO, i.e., without dexamethasone) liposomes were formulated. Please note that liposomes acquire a bilayer-membrane in aqueous solution. A cross-section of this bilayer is depicted wherein different components such as cationic lipids and other steroidal co-lipids such as cholesterol and GR-ligand (here dexamethasone) are mutually and cooperatively associated to form different liposomes. Liposomes (with net positive charges) are then complexed with DNA (with negative charges) to form lipoplexes (i.e., lipid-DNA complexes)

affect lipids' gene transfection abilities. For details regarding liposome formation procedure, DNA complexation and delivery, pathways of gene transfection, details of SAR studies, please see representative reviews [59, 60].

Further to this, researchers have developed wide arrays of liposome-based drug/gene delivery reagents, which are target specific for many diseases including cancer [Please see representative, recent reviews such as, [61–63])]. In this context, we have also developed few cancer targeted, cationic lipid-based gene delivery systems. One of the delivery system was ornamented with a hanging ligand targeted to sigma receptor. This receptor is over-expressed on various cancer cell surfaces [64]. Towards targeting estrogen receptor (ER), the highly breast cancer-implicated receptor, we developed cationic lipid formulations with hanging estrogen ligand on liposome surface [65]. Estrogen receptor is a nuclear hormone receptor (NHR)

and is cytoplasmic (and also nuclear but not membrane-associated) in nature. But cells expressing ER also bear membrane-associated ER-like receptors (such as GPR30), which recognize ER-ligands and may also recognize ER-ligand-associated delivery systems. However, glucocorticoid receptor, another NHR, is predominantly cytoplasmic in nature in the absence of its ligand and its endogenous ligands such as hydrocortisone are believed to enter cells through passive diffusion. Moreover, GR is known to express in almost all cells irrespective of their origin and lineage and till now there is no evidence to suggest that there is any membrane-associated receptor that recognizes GR-ligand for cellular uptake. This hence makes GR a cellular target of subdued prominence for the fraternity that develops 'targeted delivery system', especially for cancer.

12.7.2 Cationic Lipid-Conjugated Small Molecule as Anticancer Entity

In this we will briefly discuss how liposome-forming cationic lipids (i.e., lipids of twin aliphatic carbon chain) of a range of specific length if conjugated to various pharmaceutically important molecules, makes a novel class of potent anticancer entities. Firstly, we conjugated a C-8 (i.e., eight aliphatic carbon chain) cationic lipid to 17β-estradiol (ES) at its 17-position using suitable spacer and formed a molecule called 'ESC8'. This molecule besides exhibiting potent killing of ER+ breast cancer cells also exhibited simultaneous autophagy and apoptosis through inhibition of mTOR kinase activity in triple negative breast cancer cells and in tumor xenograft [66]. This molecule shows potentiality to target and treat all stages of breast cancer. Additionally, C-10 analogue of ES (ESC10) showed potent anticancer activity against even ER+ melanoma and pancreatic cancer cells and tumor model [67]. Secondly, upon conjugating a C8-cationic lipid to haloperidol, an anti-psychotic drug and a potent sigma receptor ligand, we developed another anticancer agent (HPC8) that targeted sigma receptor over-expressing cancer cells of varied origin and elicit potent toxicity through Akt downregulation [68]. Thirdly, we once again showed that upon conjugating C8-cationic lipid with GR synthetic ligand, dexamethasone we could develop another anticancer agent (DX8) that acts through down-regulation of JAK/STAT3 pathway [69]. Others have also used the same concept to develop respective novel classes of anticancer agents [70, 71]. Incidentally, we found a rather interesting observation albeit preliminary, with cationic lipid analogues of dexamethasone upon treating to cancer cells. In cancer cells, we found that DX8 transactivates GR many folds more than even dexamethasone (Unpublished data). But more interestingly, DX8-treatment led to selective down-regulation of HSP90 expression in cancer cells, whereas naked dexamethasone visibly increased the expression (Fig. 12.1). This assumes more interest when it is revealed that this molecule synergizes anticancer effect of inhibitors of JAK/STAT3 pathway (unpublished data). Taken together the data indicate that expressed GR in cancer cells can possibly be targeted by modified GR-ligands to regulate HSP90.

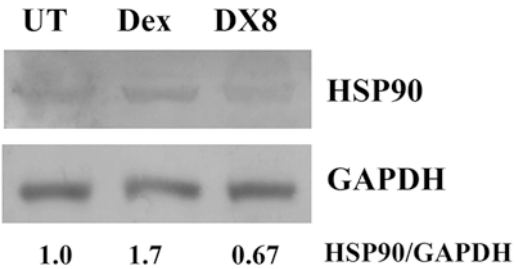


Fig. 12.1 Cationic lipid-conjugated Dex potentially regulates the expression of HSP90. B16F10 murine melanoma cells were first treated with free Dex or DX8 (this is a new derivative of Dex formed by conjugating a C8 chain length cationic lipid; for details of this structure please see Ref. [69]) or kept untreated (UT) for 24 h. Following this the cells were harvested and Western blot analysis was done on the respective cellular lysates. The level of expression of HSP90 was compared to that of GAPDH

This might lead to selective anticancer effect. Beyond this preliminary data we will now explain how a different modality of GR-targeting can elicit selective regulation of HSP90 in cancer cells.

12.8 Glucocorticoid Receptor (GR) as an Under-Utilized Cellular Target in Cancer

GR is a ubiquitously expressed nuclear hormone receptor residing in the cytoplasm as hormone-unbound state. It is expressed in most cells if not all, including cancer and non-cancer cells. Upon ligand (or glucocorticoid) binding GRα isoform translocates itself to nucleus for classical gene transactivation. The gene transactivation includes genes related to maintenance of glucose homeostasis and gluconeogenesis, anti-inflammation, immunosuppression etc. Among various species, the sequence homology of GR DNA binding domain (DBD) and its LBD is highly conserved. Obviously, among the same species sequence homology of GR-DBD and LBD do not vary among cells of normal and cancer lineage. As a result there is no evidence exhibiting differential affinity of GR-LBD to glucocorticoids in normal and cancer cells. Hence, in the context of selective targeting of cancer cells over normal cells, using GR as the cellular target and glucocorticoids as targeting ligand, one should become naturally skeptical as to how to obtain selectivity. Moreover, since GR is a cytoplasmic receptor and does not express on membrane surface it is rather difficult to design therapeutics, which could target any cell of interest expressing GR. There is also no universal evidence to believe that unlike other NHRs such as estrogen receptors (ER), or cell surface receptors such as sigma receptors (SR), GR is selectively over-expressed in cancer cells. For these legitimate reasons, among the fraternity who develops anticancer therapeutics, GR-targeting never received any attention.

12.9 GR-Mediated Gene Delivery: A Cancer Selective Phenomenon

We have shown previously that a surface-functionalized liposomal gene delivery system can target cancer cells by the virtue of over-expressing sigma receptors on cell membrane for selective gene expression [64]. We have also shown that using estrogen as ligand for a liposomal gene delivery system one can target ER-expressing breast cancer cells, although ER is a cytoplasmic receptor, for selective gene expression [65]. This happened because of targeting possibly via ER-like membrane receptors such as G-protein coupled receptor (GPR-30), which has high affinity for estrogens. Clearly, there existed helping factors, which helped selective uptake of these receptor-targeting liposomal gene delivery systems in cancer cells. No evidence is present in literature to believe that GR also has a GR-like membrane receptor, which will work in mutual harmony to pull in glucocorticoids or GR-ligands inside cells only to be attracted to LBD of GR residing in cytoplasm. To our knowledge, there is single evidence, wherein GR is over-expressed during phenotypic change from adenoma to carcinoma in adrenocortical tumor [72]. Moreover, there is an indication that among prostate cells all the cellular phenotypes including normal to aggressive cancer cells of different stages expresses GR. There is an exception to only one stage of prostate cancer (the cells of which is identified and preserved as LNCaP cells), where no GR expression is visible [9]. Barring these examples, it is unquestionably established that GR is ubiquitously expressed in most cells, generally with basal levels. Hence to begin with, there is no reason to believe that a GR-targeted delivery system could maintain cancer cell-selective delivery property because of GR's ubiquitous presence.

Initially, we intended to develop a general transfection agent, which could deliver genes in all cell types. Towards this, we hypothesized that GR could be used as the potential cellular target because of its ubiquitous presence. But we had no answer to the primary question as to how GR, which is not a cellular membrane but a cytoplasmic protein could be targeted? Do natural glucocorticoids enter cells only by passive diffusion or do they enter by attaching to some hypothetical GR-like membrane receptors, whose identity is yet to be known? We hence questioned that if this delivery agent is successfully developed as a GR-assisted, general transfection agent could this be used as a probe-formulation to discover those hypothetical GR-like membrane receptors (if any)?

Based on these 'intangible' notions we first developed a potential GR-targeted cationic lipid-based gene transfection formulation. Any cationic lipid-based gene transfection agent uses a cationic lipid and a co-lipid, such as cholesterol, DOPE etc. Cholesterol (Chol) lends stability to the liposomal formulation [73]. We have used Chol along with our previously developed cationic liposomal formulations for efficient gene transfection [74, 75]. We hypothesized that if we simply mix dexamethasone (Dex), a synthetic & steroidal GR-ligand with a closely similar structure as cholesterol, in the formulation we may achieve potent GR-targeting.

Because of structural similarity Dex may co-adjust with Chol in the lipid bilayer without compromising the stability and gene delivery ability of the formulation. Soon, we indeed found this to be true [Scheme 12.IV].

12.10 Scheme IV

The Dex containing formulation is also called as DX or 'targeted formulation'. We also made a 'non-targeted', Dex-less formulation called DO. Please see the representation of the formulations in Scheme 12.IV. The formulations bearing net positive charge are electrostatically complexed with negatively charged DNA and they are respectively named as 'DX' or 'DO' lipid-DNA complexes (or, lipoplexes). When DX lipoplex (i.e., which contains Dex) is treated to cancer and non-cancer cells we find the following interesting observations:

(a) DX transfects cancer cells much more efficiently than it does in non-cancer cells.
(b) DX-mediated transfection in cancer cells is significantly inhibited following GR antagonism and GR-siRNA-mediated GR down-regulation.
(c) No such inhibition of DX-mediated transfection is observed in GR-down-regulated/inhibited non-cancer cells.
(d) We observed simultaneous nuclear translocation of GR and DX in cancer cells, but it is not observed in non-cancer cells. No such effects were observed following DO-mediated transfection in either cancer or non-cancer cells (Fig. 12.2a).

Please see Mukherjee et al. [10] for the wholesome data as discussed above.

The observations as delineated above unraveled some invaluable points, which we believe is the mainstay of the current chapter. These observations may be interpreted as following:

(a) DX lipoplex could transfect cancer cells in a GR-selective manner.
(b) In cancer cells, DX lipoplex could recognize GR-LBD, as a result GR was activated and translocated to nucleus.
(c) In all probability, the GR-activation was performed by intact fraction of DX lipoplex, which continued to carry lipid and co-lipid components besides Dex.
(d) This means that GR-LBD in cancer cell-associated GR could not differentiate between 'Dex' molecule and DX lipoplex and allowed DX to bind to it. This led to nuclear localization of DX components that include cationic lipids.
(e) Non-cancer cells did not exhibit similar nuclear localization of DX. This indicates that GR-LBD in non-cancer cell-associated GR could not recognize DX.

To answer, why non-cancer cells' GR could not recognize DX, one can take in consideration the role of chaperone protein HSP90 in maintaining the structural and functional integrity of GR-LBD. In non-cancer cells the size of GR-LBD is strictly maintained thanks to HSP90's chaperone-guardianship. One should not

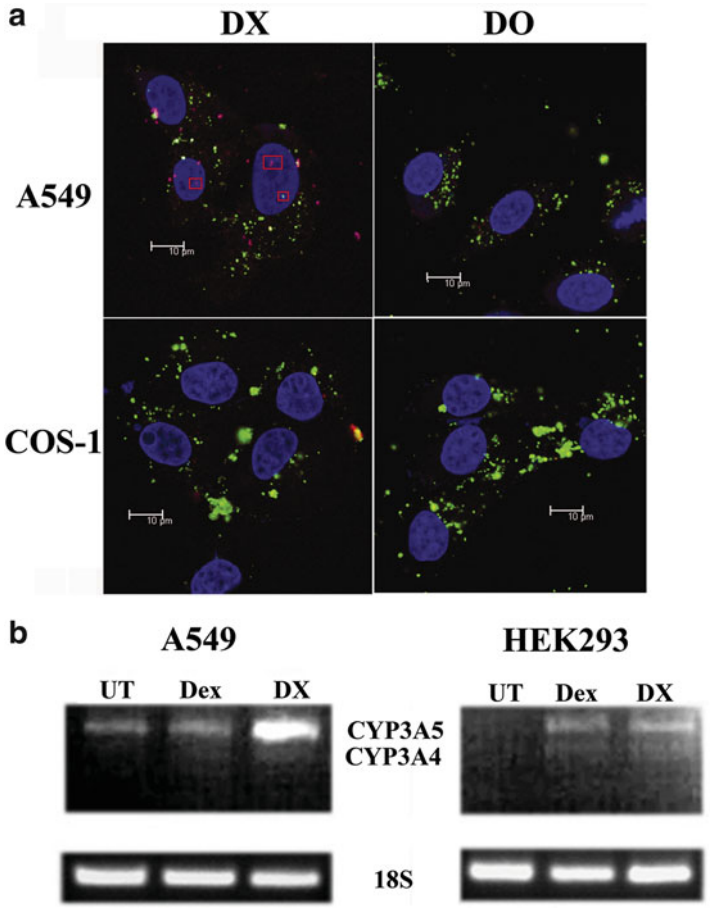


Fig. 12.2 DX-lipoplex mediated nuclear localization of its lipid component and regulation of GRE genes in cancer cells: A microscopic and reverse transcriptase (RT)-PCR studies. (**a**) A549 (*top row*, cancer cells) and COS-1 (*bottom row*, non-cancer cells) were treated with DX (*left column*) and DO (*right column*) lipoplex containing green-fluorescent lipid and red-fluorescent DNA. Cells were visualized by confocal scanning microscope to ascertain the presence of lipid and/or DNA inside the nucleus. Cells were also visualized following DAPI-based nuclear staining. *White* bar represents 10 μm. The *red* square depicts the localization of lipoplex inside the nucleus. These data shows that DX lipoplex could gain entry in the nucleus of only cancer cells. (**b**) Human cells, A549 (cancer) and HEK293 (non-cancer), were respectively treated with DX and Dexamethasone (Dex) or kept untreated (UT). The total RNAs of the cells were isolated and the m-RNA levels of a GRE-regulated gene, CYP3A5, and another gene, CYP3A4, were determined using RT-PCR technique. Dex has the ability to regulate the expression of only CYP3A5 isoform between these two, 3A5/3A4 isoforms. 18S RNA was used as internal loading control. The respective cDNAs obtained from RT-PCR were run in an agarose gel. These data shows that DX-lipoplex (carrying Dex) could induce cancer cell-specific promotion of many fold more CYP3A5 m-RNA expression than equivalent amount of free Dex (The figure panels were reproduced from [10] with permission from publisher)

forget that even a fraction of DX lipoplex contains multiple numbers of cationic lipids, cholesterol, Dex and DNA. The lipoplex fraction is at least few hundred folds, if not thousand folds bigger than the size of GR-LBD. Additionally, we observed that the DX lipoplex-mediated GR-responsive gene transcription in cancer cells was fivefold to eightfold more than what naked Dex could do (Fig. 12.2b). However, no significant difference in gene transcriptions was observed following DX or Dex-treatment in non-cancer cells.

After observing this DX-mediated, nuclear localization of lipid components followed by excessive GR-transactivation in cancer cells, one can postulate that essentially the chaperone activity of HSP90 in cancer cells is somewhat compromised and understandably, not diminished. Hence, hypothetically this compromised HSP90 allowed the audacious entry of intact DX lipoplex in cancer cells' GR-LBD. This led to unnaturally excessive GR transactivation in cancer cells [10]. To prove, if compromised activity of HSP90 in cancer cells is behind the selective nuclear localization of lipid components of DX lipoplex, we briefly treated non-cancer cells with HSP90 inhibitor geldanamycin and then treated the cells with DX. The lipid components could be then clearly located inside nucleus within few hours. Taken together, we logically hypothesized that since HSP90 in cancer cells cannot be in inhibited state (as it happens by geldanamycin treatment), its chaperone activity must be eternally compromised in cancer cells thereby allowing GR to recognize much larger sized DX lipoplex as a ligand.

Buoyed by the described cancer cell-selective gene transfection we further delivered anticancer p53 gene-coded plasmid to lung tumor subcutaneous model in mice to obtain selective tumor uptake of lipoplex and regression of tumor size [10]. Thus the Dex-associated cationic lipid formulation (DX) paved the development of a novel GR-targeted, cancer-selective, gene delivery agent.

12.11 Logic to Target Hsp90: A Cancer Selective Way

No doubt, the cancer cell-selective phenomenon as described above was primarily the handiwork of cellular HSP90 in cancer cells, wherein according to our hypothesis, its chaperone activity is suspected to be compromised. On the basis of this selective, suspected chaperone-activity of HSP90 in cancer cells, we logically questioned if HSP90 can be selectively targeted and say, degraded in cancer cells. Since, HSP90 as a chaperone is linked to many cancer implicated kinases downstream, its selective down-regulation should negatively impact the growth of cancer. If so, how is it possible to target and degrade HSP90 selectively in cancer cells?

As referred above, we have in hand a potent GR-targeted delivery system, which showed highly efficient cancer cell-selective gene transfection. We thought of designing a novel strategy by way of delivering a cargo aimed to adversely affect the expression or activity of HSP90. If HSP90's compromised chaperone-activity in cancer cells is true, then the same activity can be manipulated to affect HSP90's expression or activity. Towards this, one can target HSP90 by many ways

as delineated below. But caution should be maintained about the fact that the strategy should not jeopardize the GR-targeting ability of the liposome, as GR-targeting is the primary goal.

12.12 Targeting Hsp90: Possible Ways

12.12.1 Small Molecule

(a) **Hsp90 Inhibitor**
Among the small molecules, HSP90 inhibitors geldanamycin, radicicol and their analogues are well known candidates. The structure and their utility towards targeting HSP90 have already been discussed in an earlier section.

(b) **Trans-Repression of Heat Shock Element (HSE)**
This is a relatively new strategy, wherein molecules [or possibly co-repressor proteins (if any) may be delivered to cells of cancer lineage selectively to act as transcriptional repressor to repress the heat shock elements (HSE) belonging to promoter region of HSPs. The regulation of HSE impacts the turnover of HSP. HSE is hence a lucrative target for regulating the expression and effect of HSP90, especially for treating cancer.

Heat shock factors (HSF) are well known transcriptional regulators for HSEs for the production of HSP. Hence, inhibiting HSF or its activation is certainly a useful anticancer strategy. For the activation of HSF-1, the most important factor yet deciphered in HSE regulation, many other factors are involved. Many proximal negative regulators of HSF activation are well known. These include HDAC6, GSK3α, ERK-1, HSBP-1, etc. and HSP themselves [76–80]. As the list contains HSP, an indiscriminate up-regulation of negative regulators of HSF-1 may have contradicting effects on anticancer result. Moreover, long term and ill-targeted delivery of HSF-1 inhibitor is detrimental for health since HSF-1 protects against neurodegenerative disorders, ischemia etc. [81, 82]. Briefly, new strategies are developed which essentially use different molecular classes of HSF inhibitors that repress HSP activation. They include flavonoids (such as quercetin), quercetin-based prodrugs, benzylidene lactams, diterpene triexpoxide (triptolide), emetine derivatives etc. Some of their mechanisms of action are not clearly known, some act by inhibiting kinases that phosphorylates HSF-1, some act by interfering with transactivation of HSF-1 through acetylation, some sensitizes cancer cells following HSP inhibition, etc. [83–87]. However, for small molecule caution should be exercised for their non-specific delivery to non-cancer cells, including that in brain.

In relation to our newly developed liposomal formulation DX and the present strategy of targeting HSF-1, one thing should be noted. Non-cancer cells also take up GR-targeted formulation (DX) with equal efficiency compared to that exhibited by cancer cells. Moreover, in non-cancer cells the integrity of DX formulation is suspected to be ruptured as a result Dex is freed from lipoplex. This lipoplex-unbound Dex then behaves like free Dex and transactivates GR with same efficiency,

as a free Dex does in cytoplasm [10]. Hence, any small molecule cargo (such as, HSF-1 inhibitors) associated with this formulation would expectedly be freed in non-cancer cells and it might play havoc with the activity of HSP90 leading to collateral damage in normal tissues, unless these molecules are known to exhibit least cytotoxicity in normal cells.

As regard to regulation of HSF-1, GR plays a relatively lesser known, yet a vital role. A GR and HSF-1 crosstalk is discovered which reveals two important things, (a) with assistance from HSF-1, under stress GR's transactivation dramatically increases; (b) in presence of Dex, GR inhibits the cellular heat shock response by inhibiting HSF-1 binding to HSE [88–91]. Notably our previous observation showed that DX formulation has the ability to transcribe GRE-genes multiple folds higher than naked Dex treatment in cancer cells. Moreover, later in the chapter we will discuss that the level of HSP90 in cancer cells comes down upon DX treatment, indicating that DX might influence the expression of HSE-regulated genes (such as HSP90) besides GRE-regulated genes. Although subject to further proof, but going by the previous observation one can reason that with all probability DX will have similar, if not more, inhibitory effect than free Dex on HSF-1-binding to HSE in cancer cells. When DX will additionally carry other anti-HSP90 agent (such as an anti-HSP90 genetic cargo, as is described later) it should doubly impact the expression of HSP90 and an anticancer synergism may be expected.

12.12.2 SiRNA/ShRNA

siRNA or shRNA technology is used to target m-RNA of target protein. Hence, in order to target m-RNA of HSP90 one can design siRNA or shRNA complimenting and targeting m-RNA regions bearing domains of HSP90 preferably conserved among all the isoforms. SiRNAs are synthetic RNAs with perfect complimentary strands to target m-RNA. siRNA usually mediates transient degradation of m-RNA and sometimes causes degradation of off-target m-RNA bearing partial complementarity. This targeting and onset of degradation machinery to eradicate m-RNA occurs in cytoplasm. Hence, one needs an efficient cytoplasmic delivery system, for example cationic liposomes etc. However, cancer cell specific delivery has to be assured to eliminate any possibility of diminishing HSP90 in non-cancer cells. For this, delivery systems targeted to various receptors preferably over-expressed in cancer cells may be employed.

12.12.3 Targeting Cells via Over-Expressing Membrane Receptors

Many cancer cells overexpress receptors, but for efficient targeting and delivery of cargo, delivery systems are logically designed to target over-expressing membrane receptors. Folate receptor, sigma receptor, EGFR, VEGFR, integrin receptors etc.

are over-expressed on cell surfaces of cancer cells belonging to various phenotypes and on the surface of tumor-associated endothelial cells. So, toward delivering small molecules or protein factors selectively to cancer cells one can design delivery systems ornamented on their outer surface with protruding small molecule ligands or antibodies against these respective receptors. Liposomal delivery systems with small molecule ligands targeted to folate receptor, sigma receptor, integrin receptor etc. are well known or recently developed (some representative references including reviews are available, e.g., [64, 92–94]). Respective antibody or corresponding growth factor/protein-ligand linked liposomal delivery systems targeted to EGFR, VEGFR, transferrin receptor are also known (representative examples are [95–97]).

12.12.4 Targeting Cytoplasmic Receptors: A Logical Challenge

Delivery of cargoes exhibiting its cellular fate and action in cytoplasm logically does not need delivery systems targeted to intracellular, cytoplasmic receptors. However, if a delivery is, say, related to targeting estrogen receptors (ER), which is highly implicated in many of the breast cancer incidences, then the challenge assumes an interesting dimension. ER-ligands, natural or synthetic are known to receive help from membrane bound ER-like receptors such as GPR30 for their efficient uptake in breast cancer cells expressing ER. In that case the cellular delivery will be via GPR30, following which the delivery system in all probability is expected to bind to ER for further cytoplasmic activity. However, the integrity of the delivery system in cytoplasm may not remain uncompromised and the binding of delivery system to ER in intact form may not happen. Few years back we showed that estrogen ligand-associated cationic liposomal delivery system could exhibit ER-mediated gene transfection. As a result using anticancer gene as a cargo the delivery system could induce potent killing of ER-expressing breast cancer cells [65]. The killings in ER-negative breast cancer cells were minimal. Although the reason is not clear but these data indicate that somehow in cancer cells the ER-targeted lipoplex might remain intact for which lipoplex could bind to ER and the whole complex could have been ferried inside the nucleus for efficient reporter (or functional, exogenous) gene expression.

12.12.5 ShRNA/miRNA Cloned in Plasmid

For consistent release of silencing RNA in cytoplasm, the siRNA/ShRNA under the influence of an efficient promoter can be cloned in a plasmid. Cationic lipid-based delivery systems exhibiting efficient plasmid transfection can be conveniently used here to deliver and express exogenous gene constructs as RNA (in the form of shRNA or miRNA). This RNA will target the m-RNA of the target HSP90 protein. The delivery of plasmid will ensure sustained release of artificial RNA for a certain time, after which the efficiency vanishes. But the use of shRNA or

miRNA plasmid, producing this synthetic RNA albeit limited for a specified time, is advantageous in one way than using and delivering naked siRNA or shRNA. It will reduce the concept of frequent dosing. A set of siRNA/shRNA so delivered will target a set of m-RNA for the protein. But a plasmid can produce more copy numbers of the same RNA at a given time, repeatedly over a period, and will have an edge over regulating the protein expression turnover. Moreover, siRNA/shRNA for a therapeutic standpoint are needed in huge quantity (and hence are expensive). Additionally, it is a big concern to keep RNA in intact form from endogenous RNAase before its cellular delivery is completed. Hence, shRNA/miRNA cloned in a plasmid is advantageous in many ways. However, since plasmids are produced from bacterial culture it will contain bacterial CpG motifs, which are known to induce non-specific cytokine responses if dosed for a longer period. Moreover, one should positively get rid of possible bacterial toxin contaminants from plasmids before it is used for therapeutic purposes.

12.13 Our Approach

12.13.1 miRNA Plasmid: The Design Aspect

Micro-RNAs (or miRNA) are generally 19–24 nucleotide long regulatory RNA molecules. These are produced in cells of most of the eukaryotes from bigger long precursor RNA molecules using RNAseIII complex containing 'Drosha'. The resulting stem loop structure in cytoplasm undergoes 'Dicer'-mediated processing to generate double stranded dsRNA molecules. One of these strands incorporates with Argonaute-associated protein complex (miRNP) to finally interact with target m-RNA bearing partial complimentary sequence to miRNA. The complex then modulates the transcription/translation and hence regulates the corresponding protein expression from m-RNA [98–102]. In this chapter we will describe about artificial mi-RNAs (amiRNA) designed to express as m-RNA utilizing cellular machinery. It will then target m-RNA (with near complementarity) and affect the expression of target protein.

We selected a 21-nucleotide stretch RNA sequence in the 3′UTR region of HSP90 identical in both mouse and human. The idea is to use, in long run, the same construct in human after validating it in animal model. Moreover, the same construct can be possibly used in both allografts as well as human cancer cell-associated xenografts in animals. As depicted in Fig. 12.3a we designed the necessary amiRNA, which will bind mRNA belonging to the target region. Further, a pre-miRNA with flank and loop regions, originally derived from an effectively expressed and processed miRNA, let-7, was also designed (Fig. 12.3b). Finally, the amiRNA sequence was cloned under CMV promoter in pSilencer plasmid vector, which is widely used for miRNA over-expression. Experiments to validate the proper expression and function of amiRNA were performed. This included qRT-PCR of synthesized amiRNA following vector transfection in HEK293 cells as well

the luciferase-based cellular functional studies to see the effective down-regulation of HSP90 3′UTR-luciferase activity. This proved that the amiR-HSP90 plasmid is now ready to be used for further studies.

12.13.2 Complexation with Cationic Lipid

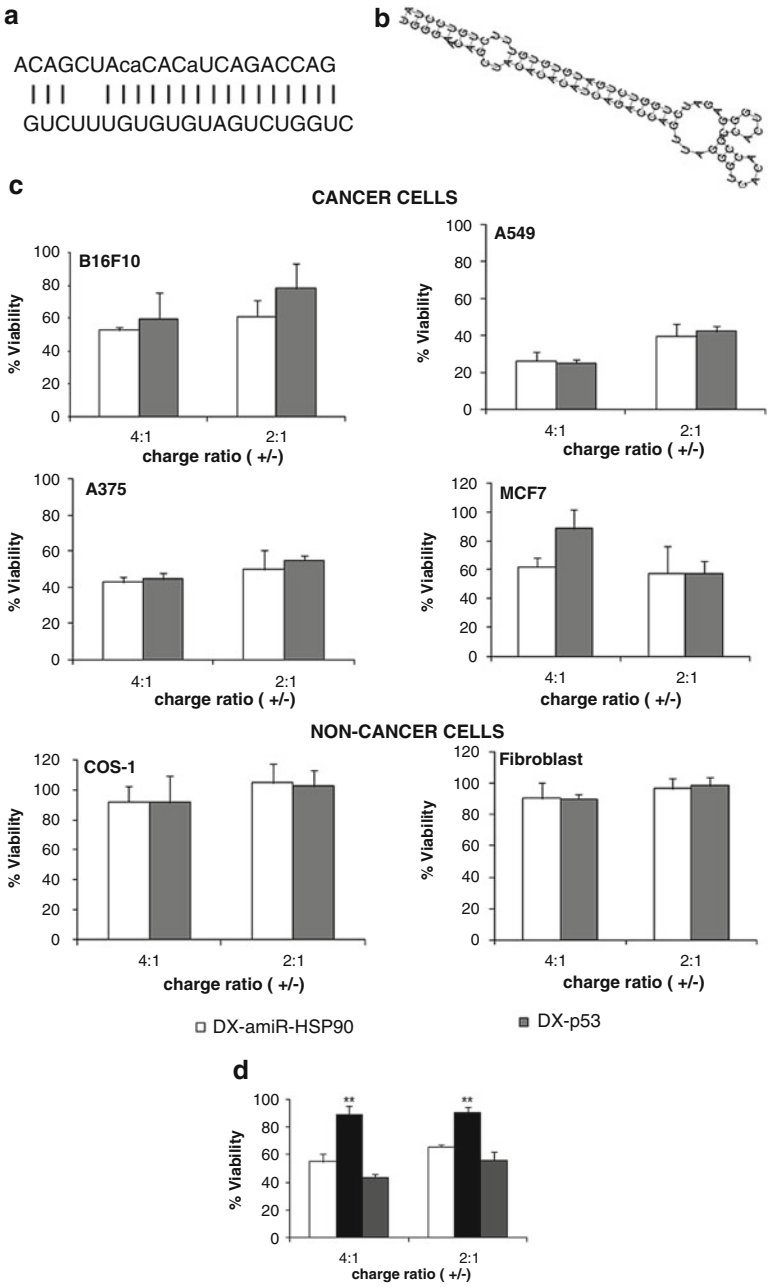
The cationic lipid formulation is the same GR-targeted formulation as we did for our previous work [10] and as described in earlier sections. This formulation DX, contains DODEAC (N,N-Di-n-octadecyl-N,N-dihydroxyethylammonium chloride), cholesterol and dexamethasone at a mole ratio of 1:1:0.75. DX being positively charged was electrostatically complexed with negatively charged amiR-HSP90 pDNA. This resulted in a serum stable, DNAase stable, lipid/DNA complex (or, lipoplex), which maintained a size in the range of 100–200 nm (Supplementary information in [11]). This empirically indicates that the lipoplex would keep DNA cargo intact while maintaining an ideal size for tumor targeting during blood circulation.

12.13.3 DX-amiRHSP90 Lipoplex Induces Selective Toxicity in Cancer Cells

DX-amiR-HSP90 lipoplex was then treated to various cancer and non-cancer cells. As a positive control we used DX-p53 lipoplex and as a negative control we used DX-β galactosidase (DX-β-gal) lipoplex and then we compared the toxic effects of each lipoplexes in various cancer and non-cancer cells. As representatively depicted in Fig. 12.3c, we found the following: (a) DX-amiR-HSP90 lipoplex and DX-p53 lipoplex had similar toxic effects in cancer cells and there were no toxic effects in non-cancer cells; (b) DX-β gal lipoplex had no toxicity in either cancer or non-cancer cells in the given condition (data not shown). The selective toxicity in cancer cells could be because of the selectively high DX-mediated gene transfection in cancer cells. There could be depletion (if any) of HSP90 levels or up-regulated expression of p53 in non-cancer cells, but these may have minimal adverse effect since these cells are perennially under highly regulated surveillance.

12.13.4 DX-amiR-HSP90-Induced Toxicity in Cancer Cells is GR-Mediated

For this to establish, we treated DX-amiR-HSP90 lipoplex to different sets of same cancer cell, first, wherein GR was down-regulated by pretreatment of GR-siRNA and the other where cancer cells were either pretreated with control, scrambled siRNA or kept untreated with any siRNA. The significant reduction in toxicity was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ACAGCUAcaCACaUCAGACCAG
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b
c
CANCER CELLS
B16F10
A549
A375
MCF7
NON-CANCER CELLS
COS-1
Fibroblast
% Viability
charge ratio (+/-)
4:1
2:1
DX-amiR-HSP90
DX-p53
d
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clearly observed in GR-depleted cancer cells (Fig. 12.3d). The reduction in toxicity was also observed in cancer cells when cells were pretreated with GR-antagonist RU486. No such observations were obtained in non-cancer cells. This clearly indicates that in cancer cells, expressed cytoplasmic GR protein is indeed an essential factor for processing DX-delivered amiR-HSP90 pDNA for their eventual killing.

12.13.5 Cancer Cell Selective Regulation of HSP90 Client Protein Kinases

HSP90 chaperones multiple kinases, which are highly implicated in cancer progression and sustenance. Down-regulation of HSP90 levels in cancer cells will adversely affect the functioning of each or many of these kinases since HSP90 is involved in chaperoning them simultaneously. A transient decrease in HSP90 will have more impact in cancer cells than in normal cells because of these over-working tendencies of HSP90 in cancer cells. So, let us see in cellular level what could happen to different kinase client proteins of HSP90 in cancer cells.

Upon DX-amiR-HSP90 lipoplex treatment there were clear down-regulation of HSP90 in both mRNA and protein levels in cancer cells (Fig. 12.4a). It is to be noted that first of all, it was not easy to show down-regulation of HSP90 proteins in cultured cancer cells. We could show the HSP90 down-regulation in freshly isolated tumor cells obtained from tumors created by those cultured cancer cells only. This indicates that tumor cells in tumor acquire certain properties, which make them more dependent on HSP90. If so, then subtle change in HSP90 level in tumor (or tumor-associated cancer cells) should have distinctly more effect than that expected in normal cultured cells. The data above shows that using DX-amiR-HSP90 lipoplex one can possibly try to dissect the role of HSP90 in tumor microenvironment.

However, the kinases such as AKT and MAPK of both freshly isolated tumor cells and cultured cancer cells showed signs of down-regulation with concomitant increase in cleaved caspase 3 level (Fig. 12.4b). This indicates that the anticancer effect was triggered by down-regulation of pro-proliferative, HSP90 client, kinase

Fig. 12.3 Design of the artificial micro-RNA (amiRNA) against *Hsp90* 3′UTR and viability studies using this amiRNA: (**a**) predicted binding pattern of the amiR to the *Hsp90* 30UTR, (**b**) predicted secondary structure of the pre-miRNA incorporating flanks and loop region of miR-let-7 and the amiRNA. This is then cloned in a plasmid (namely, amiR-HSP90) and then used to complex with DX liposome, (**c**) viability studies in cancer cells, B16F10, A549, A375 & MCF-7 and in non cancer cells, COS-1 & NIH3T3 fibroblast. Cells were continuously treated with DX-amiR-HSP90 (*white bar*) and DX-p53 (*gray bar*) lipoplexes for 96 h. Following this MTT assay was performed to assess the viability of respective cells. (**d**) Viability of A549 cells initially kept untreated or treated with respective siRNAs followed by 96 h treatment of DX-amiR-HSP90 lipoplex. *White*, *black*, and *gray* bars represent siRNA untreated, GR siRNA treated, and negative control siRNA treated cells, respectively (The figure panels were reproduced from [11] with permission from publisher)

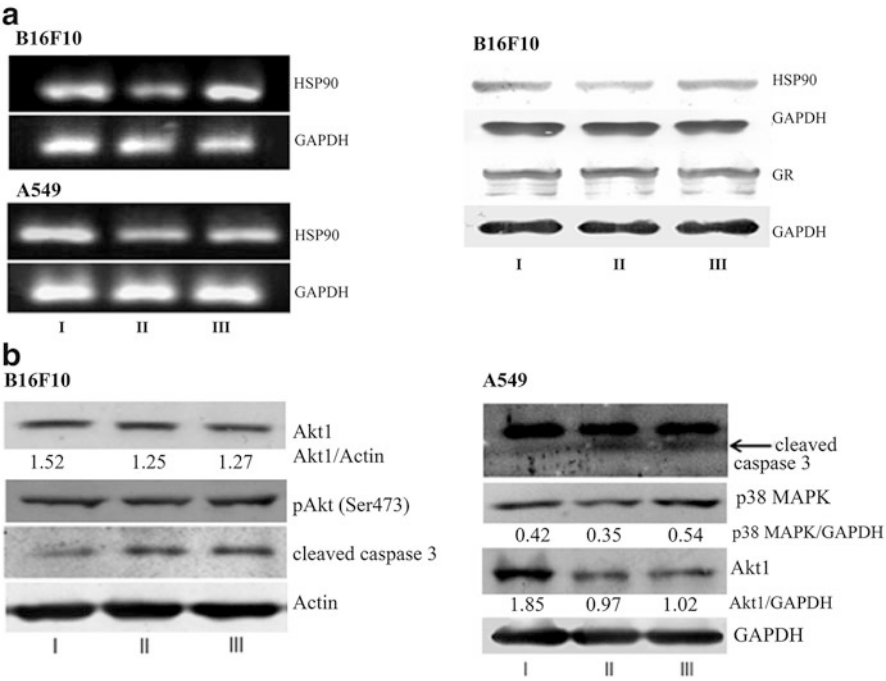

Fig. 12.4 Expression levels of HSP90, its client proteins and caspases in cancer cells: (**a**) Hsp90 m-RNA levels in both cancer cells, B16F10 and A549 (*left panels*) and Hsp90 and GR protein levels obtained after treatment of cultured tumor cells freshly isolated from the B16F10 tumor developed in mice (*right panel*). (**b**) In here, Western blot analysis of B16F10 and A549 cell lysates is exhibited. Cell lysates were obtained from cells either kept untreated (I), or continuously treated with DX-amiR-Hsp90 (II), and DX-βgal (III) for 36 h (m-RNA levels) or 48 h (protein levels). DX-βgal is a control lipoplex that carries a non-therapeutic control plasmid encoding $\tilde{\beta}$ galactosidase enzyme. This figure clearly shows that with down-regulation of m-RNA for HSP90 and levels for HSP90 proteins obtained from freshly isolated tumor cells, various HSP90 client proteins, especially kinases (Akt1, MAPK) are down-regulated. As a result, apoptosis is triggered in respective cancer cells as witnessed by the up-regulation of cleaved caspase-3 (Reproduced with publisher's permission from [11])

proteins leading to induction of intrinsic mode of apoptosis. In here, two additional things are to be noted: (a) Even if HSP90 down-regulation is unobservable (in cultured cancer cells), the down-regulations of kinase proteins were clearly observed; (b) DX-β-gal lipoplex also induced Akt down-regulation and formation of cleaved caspase-3, even though the anticancer readout was not as prominent as DX-amiR-HSP90 lipoplex treatment. The data so far could not directly explain these phenomena. However, as discussed earlier, Dex critically up-regulates GR-mediated inhibition of HSF-1-induced heat shock response and consequently down-regulates expression of HSPs [88]. We found that in cancer cells DX formulation transactivate GR many folds more than even Dex (Fig. 12.2b and ref. [10]). Hence, in view of the above observation regarding DX-β-gal lipoplex we hypothesize that since this

lipoplex consists of DX it could have induced Dex-alike, if not more, inhibitory effect on HSF-1 and hence on the expression HSP90 (even though it is not clearly observable), thereby resulting in down-regulation of HSP90 client protein Akt.

12.13.6 Effect of DX-Lipoplex In Vivo

After accomplishing DX-amiR-HSP90-mediated down-regulation of HSP90 and its client protein kinases in cancer cells we studied the effect of this lipoplex in tumor. Tumor is an extremely multifarious condition with multiple factors involved to maintain its complexities. Hence, treating tumor is not straightforward compared to killing cancer cells in culture. However, our understanding was that since HSP90 is involved in so many growth-regulating pathways of both tumor-associated epithelial and vascular endothelial cells through chaperoning several of its member proteins, tumor cells may be more dependent on HSP90, as many of these inter-related pathways are simultaneously switched on and may relentlessly cross-talk to make the environment more and more complex. As a result, even a subtle change in HSP90 levels may induce deep-running effects on tumor by affecting the growth of tumor-associated epithelial and vascular endothelial cells.

With this pre-notion we treated DX-amiR-HSP90 to two different models in mice bearing subcutaneous tumor of melanoma and lung cancer cells respectively. Clearly, as depicted in Fig. 12.5a, DX-amiR-HSP90 lipoplex had maximum effect in reducing the tumor growth. The control lipoplex (DX-βgal) also showed some tumor-growth inhibiting effect in both the models, indicating the possible GR-mediated heat shock response inhibiting effect of individual DX formulation entity.

The enormity of tumor size reduction can be seen by the representative images of tumor from individual treatment groups (Fig. 12.5b). The melanoma tumor was a very aggressive model wherein mice in untreated groups die within 30 days. We find that in this experiment even though untreated mice were dead by 25 days, the mice in other treated groups showed no signs of mortality for more than 45 days (data not shown).

Microscopic visualization of sections of tumors from DX-amiR-HSP90-treated mice revealed few interesting features (Fig. 12.5c). Tumor cells underwent apoptosis as revealed by TUNEL assay (green fluorescent areas). Additionally on marking tumor-associated vascular endothelial cells with VE-cadherin (red fluorescent areas) we found that these cells also underwent apoptosis (yellowish regions). To trace the reason for this effect we find that the tumor sections bearing vascular endothelial cells bore fewer vascular endothelial growth factor receptor-2 (VEGFR2) on its surface [11]. VEGFR2 is another client protein of HSP90. It is highly expressed in aggressive tumors, which exhibit features of immense angiogenesis. Evidently, if the HSP90 level was lowered it would eventually affect the level of VEGFR-2 and we think this is what we witnessed. With the lowering levels of VEGFR-2 on vascular cells there would be no uptake of vascular endothelial growth factors (VEGF). As a result the paracrine growth stimulation and vascular permeability in these cells

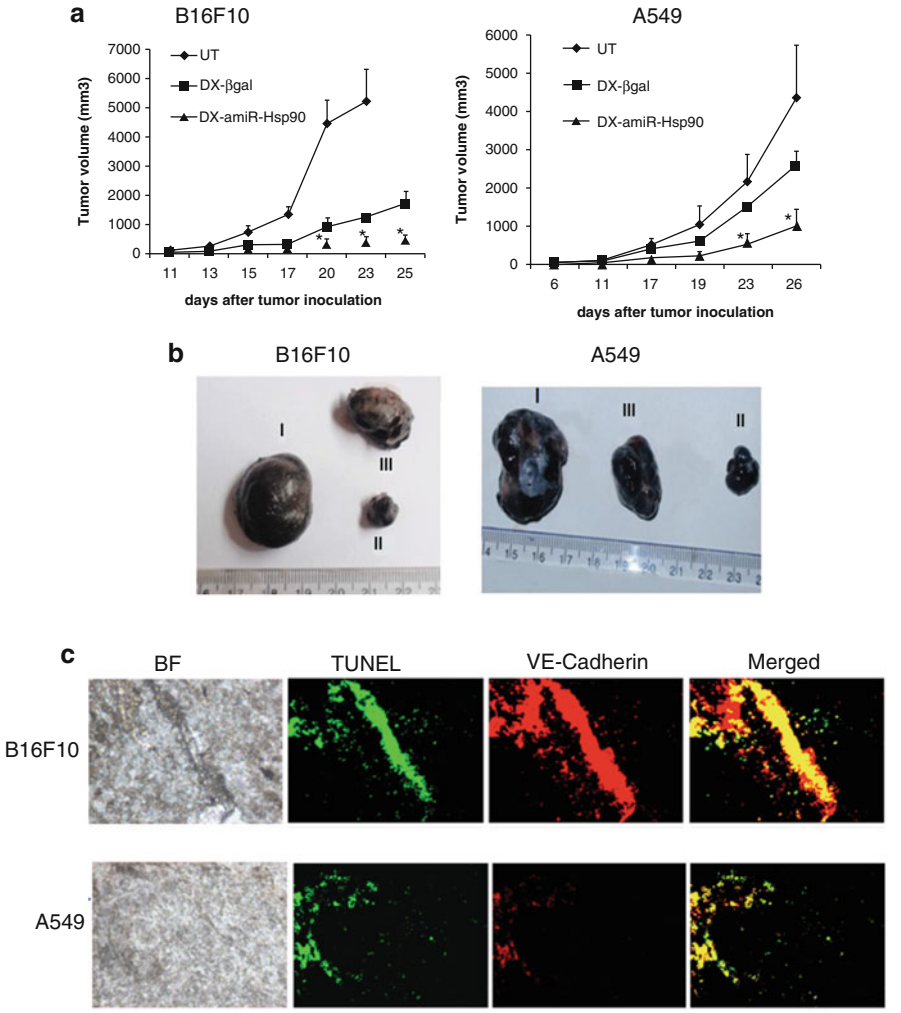

Fig. 12.5 Tumor growth curve, representative tumor sizes and microscopic images for signatures of apoptosis in sections of tumors: (**a**) Tumor growth curve after subcutaneous implantation of B16F10 (*left panel*) cells in C57BL6/J mice and A549 cells (*right panel*) in BALB/C athymic nude mice followed by intraperitoneal injection of 5 % glucose (UT, *black rhombus*), DX-βgal (*black square*), and DX-amiR-Hsp90 (*black triangle*) lipoplex at 6:1 cationic lipid/DNA charge ratio. Intraperitoneal injections started from day 8 for both the models. Seven injections were given with 2 days interval. Y-axis denotes the size of tumors as tumor volume in cubic mm, and X-axis denotes the number of days passed after tumor inoculation. The *asterisk* (*) denotes $p < 0.01$ while comparing with DX-βgal treatment. (**b**) This exhibits the image pictures of representative samples of B16F10 and A549 tumors excised after sacrificing the mice on day 25 and 26 respectively for models as described in section A. Herein, (I) denotes tumors from untreated, (II) denotes tumors from DX-amiR-Hsp90 lipoplex treated and (III) denotes tumors from DX-βgal lipoplex treated groups respectively. (**c**) Microscopic pictures of 10 mm tumor sections of DX-amiR-Hsp90 treated group from B16F10 and A549 tumor. First column from left shows the tissue architecture in bright field (BF), second column from left shows the apoptotic regions in TUNEL assay

would hinder, thereby reducing the diffusion of vital proteins (needed to support cell's migration) in the interstitial space within tumor. Tumor microenvironment supports the formation of micro-angiogenesis, which needs the help of this paracrine stimulation from vascular endothelial cells. Hence herein, reduction in VEGFR2 level sequestered overall angiogenesis, which led to eventual shrinkage of tumor.

Till now we saw downsizing of tumors in DX-amiR-HSP90 lipoplex treated tumor-bearing mice. But one may logically question if tumor inhibitory effect is really due to the real time expression of anti-miRNA against HSP90 in tumor or not. Clearly, there was no direct evidence to show that DX-lipoplex did have the ability to selectively express the genetic cargo in tumors, relieving other vital organs. For this, we chose to deliver a reporter plasmid using DX. The reporter plasmid contained gene clone of green fluorescent protein (GFP) under the influence of CMV promoter. It is expected that on a reasonable time scale upon gene delivery green fluorescent protein would functionally express in various organs. Therefore, we injected DX-GFP lipoplex in tumor bearing mice and after 24 h we collected the organs including tumors from sacrificed mice. Organs were sectioned and visualized under microscope. We observed practically no GFP expressions in any vital organs including lung, liver, kidney, spleen and heart. But, expression of GFP was abundant in tumors (Fig. 12.6). This proved that DX, which carries GR ligand Dex, has the ability to functionally express its genetic cargo in only tumors thereby nullifying any non-specific genetic expression and therefore any possibility of collateral damages in vital organs. This data can be related to potent expression of anti-miRNA against HSP90 in tumors if delivered by DX in vivo.

Next, we show that DX-amiR-HSP90 lipoplex delivery in vivo led to changes in protein levels of various pro-proliferative and apoptotic factors in tumor mass (Fig. 12.7). Down-regulation of HSP90 was logically evident. Possibly as a result, Protein kinase B or Akt was also down-regulated leading to down-regulation of its different phosphorylated forms. Expression of one of the vital HSP90 client proteins, Bcl-2 was inversely affected, as a result the cascade of events related to triggering of intrinsic pathway of cancer, such as elevation of cleaved form of caspase 9 and intact caspase 3 were observed. Cleaved caspase 3 was not evident though, but we observed another interesting feature. The expression of apoptosis-inducing p53 protein, a protein prone to undergo ubiquitination and degradation in tumor, was the highest in DX-amiR-HSP90 group.

p53 is a client protein of HSP90, so do Akt. Upon HSP90 down-regulation Akt was down-regulated but p53 was up-regulated. This contradiction although links to

Fig. 12.5 (continued) (*green fluorescent*), third column from left shows the regions of where endothelial cells are present and are visualized after staining with VE-cadherin antibody (*red fluorescent*), and extreme right column is merged pictures of second & third columns from left. All the images are taken at 10x objective magnification. This merged section shows that upon the treatment of DX-amiR-HSP90 signs of apoptosis could be detected clearly in tumor cells (*green areas*) as well as tumor-associated endothelial cells (*yellow areas*). The emergence of yellow area additionally indicates the anti-angiogenic role of DX-amiR-HSP90 lipoplex (Selective image and figure panels are reproduced from [11] with permission)

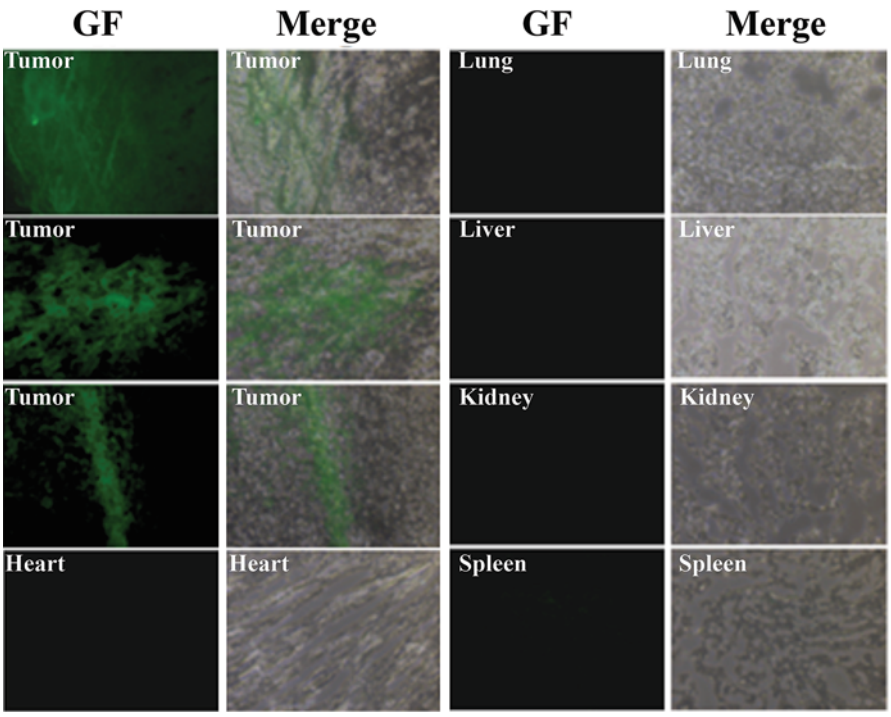

Fig. 12.6 Functional bio-distribution of DX-lipoplex in tumor-bearing mice: Sections (10 μ thickness) of tumors and different vital organs were obtained after sacrificing B16F10 tumor-bearing mice intraperitoneally injected with DX-associated green fluorescence protein (GFP)-plasmid. The sections from various organs and tumors of mice treated with lipoplex (DX-GFP) were mutually compared. The respective imaging of sections from individual organs and tumors from DX-GFP treated mice were obtained at same exposure time and magnification (10×). 'GF' represents green field and 'Merge' represents merging of respective sections visualized under bright field and under GF (Selective image panels were reproduced from [11] with permission). This data clearly shows that DX-lipoplex has a very selective ability to induce gene expression of its genetic cargo in tumor-associated cells. DX-lipoplex has apparently no capability to transfect normal cells of vital organs (Selective image panels are reproduced from [11] with permission)

eventual antitumor effect but could not be explained by the present data. Moreover, there are contradicting reports about the status of p53 in the tumor cells of interest here. B16F10, the melanoma cell, carries wild type p53 [103–106]. This p53 status is however also contradicted but it seems p53 maintains its function and retains its binding activity to the response elements in these melanoma cells [107]. Our data however indicates that p53 in this experimental set of B16F10 cells is possibly wild type, otherwise the up-regulation could have resulted in tumor aggression rather than tumor retardation. Others have also found that geldanamycin-induced inhibition of HSP90 leads to down-regulation of mutant p53 but up-regulation of wild type p53 [108, 109]. One can further speculate several other things: Firstly, Akt phosphorylates mdm2, which eventually ubiquitinates p53 for degradation.

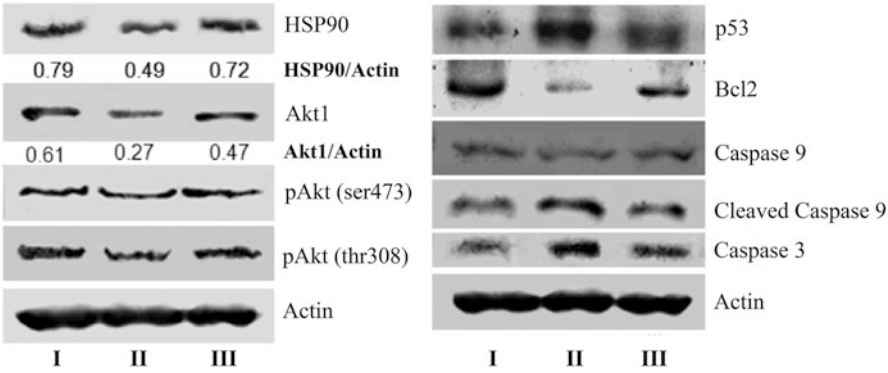

Fig. 12.7 Western blot analysis of tumor lystates: Differential expression of different regulators of apoptosis and proliferation in B16F10 tumor lysates from the tumors of untreated group (I), from groups treated with DX-amiR-HSP90 (II), and DX-βgal (III) respectively. As a whole, the figure clearly depicts that upon HSP90 down-regulation, levels of various regulators of apoptosis (Bcl-2, caspases) and that of protein kinase (Akt1) are changed. Pro-cancerous factors such as Bcl-2 and Akt-1 are down-regulated, at the same time signatures of apoptosis (i.e., the caspases) are up-regulated (Reproduced *in toto* from [11] with permission)

Here, as Akt was down-regulated, up-regulation of p53 was expected. It could be possible that up-regulation of p53 possibly out-weighed p53's down-regulation (if any) by HSP90 depletion. The second reason could be the following: There are two main isoforms of HSP90. HSP90α is a stress-induced one whereas HSP90β is constitutively expressed isoform and both their elimination is bad for any cell's survival. However, it is also known that ratio of α/β isoform changes predominantly in favor of α under stress condition, such as during malignant transformation [110]. The client proteins, such as survival signaling kinases (Akt), oncogenes (v-src, Bcr-Abl), whose up-regulated presence provides stability and perpetuity to this malignant transformation are mostly chaperoned by HSP90α. Interestingly, p53 is chaperoned by HSP90β [46]. Our construct was designed to target a common region of 3′UTR of mRNA of both α and β isoform. Although subject to realization but stoichiometric analysis should show larger proportions of α isoform than β isoform in the tumor. As a result, following amiR-HSP90 treatment, expectedly more of α isoform would be eliminated than β isoform and hence more of α-isoform-chaperoned client proteins would be down-regulated.

12.14 Any Other Possible Target for Gene Therapy and Other Approaches

In this subsection, we would like to emphasize some hypothetical strategies that may be adopted for targeting HSP90. The first one is, targeting the highly conserved middle domain of HSP90. It contains the catalytic Arg380 motif, which interacts

with the ATP γ̃phosphate trapped inside the closed, catalytically active N-terminal dimerized state. Hydrolysis of ATP is the key function for chaperone activity of Hsp90. Arg380 while residing in a highly conserved domain helps in this hydrolysis. The domain covering Arg380 is hence a lucrative region for gene therapy and can be targeted by suitably designed shRNA or miRNA.

Secondly, it is known that GR-LBD is chaperoned by HSP90. The release of this client protein (i.e., GR) from HSP90 requires ATP hydrolysis. In turn, the GR-LBD affects the nucleotide-binding affinity of HSP90. It increases affinity for ATP and decreases for ADP. Another client protein is p53. It remains in bound state with HSP90. Interestingly, p53-HSP90β dissociation requires binding of ATP but not its hydrolysis. Chaperoning of client proteins such as kinases requires ATP binding. So, GR could be a hypothetically potential target whose critical down-regulation can affect nucleotide binding to HSP90 and may have indirect effect on client protein kinases. However, as far as cancer treatment is concerned there are possible contradicting data against this theory of down-regulating GR. It is shown that Dex-treatment induces energetically unfavorable gluconeogenesis over glycolysis in cancer cells thereby rendering these cells drug sensitive [111]. This shows that presence of GR can be utilized for cancer treatment. Our recent discovery along this line is worth mentioning. We found that the formulation DX has the capability to induce many folds more GR-transactivation (which involves gluconeogenesis also) than even Dex (Fig. 12.2b). Hence, we hypothesize that DX (or any cationic lipid formulation with a GR ligand) will have more drug-sensitivity-inducing effect than Dex in cancer cells. This possibility is also worth testing.

Thirdly, our recent experience, as described above, allows us to recommend using this artificial miRNA strategy to specifically target conserved 3′UTR domains of HSP90α isoforms only. As discussed above, α-isoform is critically up-regulated in tumors and this isoform is critically involved in chaperoning various cancer implicated kinases, whereas the β-isoform is involved in chaperoning p53, the apoptosis-inducing gene. So, targeting α-isoform over β-isoform may have better implications toward treating cancer. However, it is also to be noted that, going by the HSP90 addiction theory, the dependence of mutated p-53 on β-isoform is more than its wild type form. Hence, depending on p53 status in cancer cells the strategy of utilizing certain design of miRNA may be conveniently adopted to obtain optimal anticancer outcome.

12.15 Conclusion

In this chapter, we described the development of a novel concoction of DX-amiR-HSP90 formulation that exhibited the first gene therapy against HSP90 in a cancer selective manner. The artificial miRNA against HSP90 directly inflicted down-regulation of HSP90 expression in only cancer cells. Notably, the strategy does not involve inhibition of HSP90 activity rather involved direct down-regulation of HSP90 protein expression. We know cancer cells are HSP90 ‘addicted’. As a result

of even slightest down-regulation of HSP90 expression, the pro-proliferative client proteins of HSP90 in cancer cells including various kinases showed down-regulated expression, which was possibly enough to reduce the tumor burden. This selective reduction of tumor burden was possible when we delivered the genetic cargo via GR. The use of GR or its antagonists for cancer treatment are limited. Our data suggests that GR can be selectively manipulated for cancer treatment. It also indicates that the GR-manipulation may have additional effect on HSP90 down-regulation possibly via inhibition of heat shock factors. Even GR-targeting molecules such as Dex if formulated with cationic lipid showed HSP90 down-regulatory effect, indicating that GR should come under the active scanner for targeting HSP90 for developing newer anticancer modalities.

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Chapter 13
Potential of HSP90 Inhibitors to Treat Neurofibromatosis-Related Tumors

Jeremie Vitte and Marco Giovannini

Abstract Neurofibromatosis type 1 and 2 (NF1 and NF2) are two distinct tumor predisposition syndromes in which affected patients develop benign or malignant tumors of the nervous system. NF1 is characterized by the development of neurofibromas, malignant peripheral nerve sheath tumors and gliomas resulting from mutations in the NF1 gene, encoding neurofibromin. NF2 patients primarily develop schwannomas, meningiomas and ependymomas resulting from mutations in the NF2 gene, encoding merlin. Current therapeutic options are limited to conservative monitoring, repeated surgical resection or radiotherapy. Unfortunately these options are not optimal for treating the recurring life-threatening tumors in patients affected by these genetic syndromes. Recent advances in the understanding of molecular pathways involved in the tumorigenesis of neurofibromatosis-related neoplasms and the development of robust mouse models has allowed for the identification of promising drug targets for chemotherapy, including HSP90. In an NF1 mouse model, apoptosis induction in cells with proteotoxic stress was achieved with a combination of mTOR and HSP90 inhibitors. In cell and mouse models of NF2, HSP90 inhibitors have been used to target the multiple signaling pathways activated. This chapter summarizes the rationale and recent developments for the use of HSP90 inhibitors as potential therapies for neurofibromatosis type 1 and 2 tumors.

Keywords Neurofibromatosis type 1 • Neurofibromin • Neurofibromatosis type 2 • Merlin • Schwannomin • HSP90 inhibitors

Abbreviations

EGF	Epidermal growth factor
ERM	Ezrin, radixin, moesin
FERM	Four-point-one, ezrin, radixin, moesin
GAP	GTPase-activating protein

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HSPs	Heat shock proteins
MPNSTs	Malignant peripheral nerve sheath tumors
mTOR	Mammalian target of rapamycin
NF1	Neurofibromatosis type 1
NF2	Neurofibromatosis type 2
ROS	Reactive oxygen species
UPR	Unfolded protein response
WHO	World health organization

13.1 Introduction

Heat Shock Proteins (HSPs) are a group of highly conserved chaperone proteins facilitating the proper folding of other proteins (client proteins) and the assembly of multiprotein complexes [1, 2]. In response to a variety of cellular stressors, HSP expression is increased – a key feature of the heat shock/stress response [3]. Cancer cells are challenged by multiple insults, including mutations and anti-cancer chemotherapy, which affect their metabolic and signaling pathways. Consequently, in an attempt to survive and adapt to stressful or potentially lethal conditions, cancer cells increase the expression of components of the chaperone machinery [4].

HSPs are categorized into four families depending on their molecular weight: HSP90, HSP70, HSP60 and small HSP (15–30 kDa) families. Among the HSPs, HSP90, HSP70 and HSP27 are the most common chaperones targeted by therapies to defeat proliferating cancer cells [5]. HSP90, one of the core components of the chaperone machinery, has been shown to maintain protein homeostasis and stabilize client proteins involved in apoptosis, angiogenesis, growth factor signaling and cell cycle control [4], all processes integral to cancer progression. With the elevated level of HSPs in cancer cells compared with neighboring cells and the heightened reliance on HSP activity due to the increased signaling burden, targeting HSPs may translate into selective targeting of cancer cells. Multiple compounds inhibiting the activity of HSP90 have been developed and are now in evaluation for anti-tumor activity in a variety of cancers [6]. Given that common signaling pathways and HSP client proteins are implicated in tumorigenesis for cancers and in neurofibromatosis, HSP90 inhibitors are also actively being studied as potential therapies for neurofibromatosis type 1 and 2 related tumors.

13.2 Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF1) is one of the most common inherited tumor predisposition syndromes, affecting 1 in 3,000 individuals worldwide [7]. The NF1 gene, located in chromosomal region 17q11.2, was identified in 1990 [8–10] and encodes neurofibromin, a GTPase activating protein (GAP) that stimulates the

conversion of Ras-GTP to Ras-GDP and thereby attenuates Ras signaling [11]. NF1 patients harbor constitutional heterozygous inactivating mutations at the *NF1* locus, reflecting either inherited or new germline mutations, the latter accounting for 30–50 % of cases [12]. Consistent with the classification of *NF1* as a tumor suppressor gene, somatic mutation of the remaining wild-type allele is presumed to initiate tumor development and can be detected in tumors [13, 14]. NF1 is characterized by a high phenotypic variability and age-dependent manifestations including nervous system neoplasms and extraneural symptoms. Affected children and adults have a predisposition to benign and malignant tumor formation; the hallmark lesion is the neurofibroma, a benign peripheral nerve sheath tumor. Histologically, the major cell type in neurofibromas is the Schwann cell, but they also contain fibroblasts, mast cells, axons and perineurial cells [7, 15]. Neurofibromas, classified as WHO grade I tumors, can develop as encapsulated dermal and subcutaneous masses or as plexiform neurofibromas deep within the body or near the nerve roots. Between 9 % and 13 % of NF1 patients develop malignant peripheral nerve sheath tumors (MPNSTs), which are highly aggressive, metastatic tumors with a poor prognosis and are classified as WHO grade II, III or IV [16]. The development of MPNSTs may involve additional cooperating genetic mutations implicating the p53, p16 and p27-Kip1 tumor suppressors [17–20]. NF1 patients also have an increased risk of developing other tumor types, including gliomas (mainly located within the optic nerve), myeloid leukemias, and pheochromocytomas. Other manifestations of the syndrome affect a variety of tissues such as skin (café-au-lait spots and axillary freckling), eye (Lisch nodules), bone (dysplasia of sphenoid bone or long bones, kyphoscoliosis), cardiovascular system (cerebral arteriopathy, pulmonary artery stenosis), and central nervous system (cognitive disabilities) [21]. Haploinsufficiency for the NF1 gene might underlie these non-tumor manifestations [22–24].

Neurofibromin, the NF1 protein, controls cell growth and proliferation by regulating activity of the proto-oncogene Ras. Null or inactivating mutations of neurofibromin lead to constitutive activation of Ras signaling [13, 14]. Consequently, high levels of activated RAF/MEK [25] and AKT/mammalian target of rapamycin (mTOR) are observed [26, 27] (Fig. 13.1a). In addition, neurofibromin also positively controls adenylyl cyclase activity, and intracellular cAMP levels [28, 29].

Despite the elucidation of a potential molecular pathogenic mechanism for the disease, the treatment approach for plexiform neurofibromas has been empiric, with surgery being the primary option. However, progressive lesions are still a major cause of morbidity. Even with the understanding of Ras protein regulation and downstream signaling pathways, there are no effective targeted therapies for Ras-driven cancer. The identification of aberrant molecular pathways in *NF1*-deficient tumors has led to preclinical testing and clinical trials [30] with agents targeting angiogenesis [31], mTOR [32, 33], or different tyrosine-kinases [34–37]. Thus far, no treatment has successfully tackled the formation or growth of NF1 tumors [38, 39].

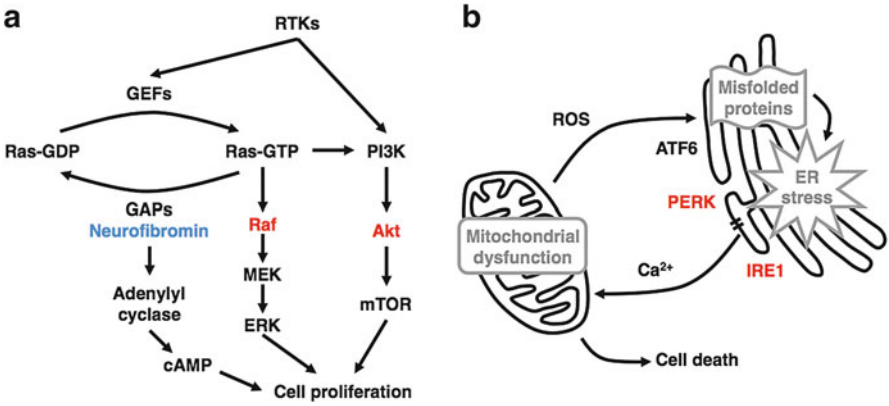

Fig. 13.1 (**a**) Neurofibromin functions as a negative regulator of Ras activity and positive regulator of Adenylyl cyclase. Inactivation of neurofibromin lead to increased activation of Ras resulting in high levels of Raf/MEK/ERK and Akt/mTOR signaling and cell proliferation. (**b**) HSP90 inhibition first induces ER (Endoplasmic Reticulum) stress by increasing misfolding of proteins and UPR (Unfolded Protein Response). Prolonged exposure to HSP90 inhibitor impairs the UPR by targeting two components: PERK and IRE1. ER stress starts a vicious cycle by triggering intraluminal calcium release, promoting mitochondrial membrane depolarization and ROS production. ROS then promotes protein misfolding and enhance ER stress. Thus, irresolvable ER stress and mitochondria dysfunction lead to apoptosis of the cells. Proteins in *red* are HSP90 client proteins

13.3 Neurofibromatosis Type 2

Neurofibromatosis type 2 (NF2) is a rare autosomal dominant genetic disorder affecting approximately 1 in 33,000 individuals worldwide and is characterized by the onset of a variety of neural tumors, with bilateral vestibular schwannoma as the most frequent manifestation [40]. The majority of NF2 patients present with hearing loss and deafness. NF2 patients also have high risk for developing multiple schwannomas in cranial, spinal, and peripheral nerves and meningiomas. The NF2 gene, located in chromosomal region 22q12.2, was identified in 1993 [41, 42]. Lack of expression of the functional *NF2* protein, merlin (or schwannomin) has been found in both sporadic and familial forms of vestibular schwannomas [41, 42]. Schwannomas, classified as WHO grade I tumors, are encapsulated tumors, comprised only of neoplastic Schwann cells [7, 15]. They develop in NF2 patients at earlier ages (third decade) than in sporadic vestibular schwannoma patients (sixth decade) [43, 44]. Meningiomas occur in half of all NF2 patients [45]; they occur earlier in life and are more aggressive than in patients where they arise sporadically [46, 47]. Although rare, NF2 patients also develop ependymomas with a higher incidence than the general population. Other manifestations of the disease include meningioangiomatosis, glial hamartia, peripheral neuropathy and posterior subcapsular cataracts [48]. Schwannomas in NF2 patients are benign, slow growing and do not progress to malignancy. However, depending on their location, they can

compress associated nerves and consequently trigger pain and nerve dysfunction. Meningiomas can cause severe symptoms due to compression of the brain or spinal cord (resulting in weakness or paralysis, migraines, impaired vision, speech or memory) and can progress to malignancy. The current standard of care for both tumor types is repeated surgical removal, which is accompanied with significant risk for neurological deficits as the tumors most frequently lie on nerves near the brain and spinal cord [49]. Other treatment options are conservative monitoring and radiation therapy, although malignant transformation of schwannomas and meningiomas due to radiation exposure is a significant concern [50–52]. Morbidity associated with NF2 is high due to the risk of recurrent tumors and the inoperable nature of a significant proportion of meningiomas. Therefore, development of effective chemotherapy would be most beneficial for overall NF2 tumor treatment [39].

Several *Nf2*-mutant mouse models have been generated to study disease pathogenesis, to delineate the molecular function of merlin, and to identify potential therapeutic targets and test candidate therapeutic strategies [53–56]. Consistent with the classification of *NF2* as a tumor suppressor gene, these models confirmed the requirement for *Nf2* loss in schwannoma and meningioma development and also revealed an unexpectedly broad role for merlin in development and tumorigenesis. Indeed, *NF2* mutations are also found in a variety of sporadic human cancers, including mesothelioma [57, 58] and renal adenocarcinoma [59, 60]. The *NF2* gene product, merlin, is a unique type of tumor suppressor, implicated in multiple signal transduction pathways, suggesting that a variety of signaling aberrations contribute to molecular pathogenesis in NF2. Merlin shares a highly conserved N-terminal FERM (Four-point-one, Ezrin, Radixin, Moesin) domain with the ERM (Ezrin, Radixin, Moesin) proteins which are thought to organize specific membrane domains by providing regulated linkage between membrane proteins and the actin cytoskeleton [61–63]. Merlin, like the ERM proteins, localizes to areas of cortical cytoskeleton remodeling, including cell-cell boundaries and membrane ruffles [64–66]. Merlin localization suggests that it functions in the transfer of extracellular signals to cytoplasmic kinases and proteins involved in cell proliferation [63, 67]. While the precise molecular mechanisms of merlin tumor suppression remain to be fully elucidated, loss of merlin appears to render the cell insensitive to inhibition of growth due to contact with other cells [65]. Merlin is also implicated in regulating the abundance and turnover of multiple cell surface receptors, such as those for Schwann cell mitogens and the EGF family of ligands [68–71]. A recent study suggested that merlin may play a role in suppressing tumorigenesis by translocating to the nucleus and inhibiting $CRL4^{DCAF1}$-dependent gene expression [72]. In addition, merlin may be involved in regulating the Hippo/Lats/YAP signaling pathway [73] although it remains unclear whether the mechanism of activation is $CRL4^{DCAF1}$-dependent or not [74]. Taken together, the multitude of proteins interacting with merlin underscores the activation of multiple signaling pathways, such as PI3K/Akt, Ras/Raf, Hippo/YAP and the cyclin-dependent kinases [67, 75] as found in NF2-related tumors.

Recently, advances in the understanding of schwannoma pathogenesis and the development of mouse models predictive of drug response in patients have

facilitated the emergence and validation of candidate NF2 drug targets [53, 76]. Consequently, targeted therapies are currently under evaluation for NF2 patients [30] based on the inhibition of VEGF [77] (*Clinical trial reference 2*), EGFR/ErbB2 [78] or mTOR (*Clinical trial reference 3, Clinical trial reference 4*). However, the involvement of multiple deregulated pathways remains a challenge for developing targeted therapeutics for NF2 tumors.

13.4 HSP90 Inhibition Induces ROS Formation Promoting Cell Death in Neurofibromatosis Type 1 and 2 Tumor Cells

A recent study used HSP90 inhibitors in a broader approach to target Ras-driven tumors. In an *Nf1/p53*-mutant MPNST mouse model, where both *Nf1* and *p53* genes are deleted on the same chromosome [79], the mTOR inhibitor rapamycin suppressed tumor growth by a cytostatic mechanism when used as a single agent [80]. Several agents that induce proteotoxic or ER (Endoplasmic Reticulum) stress, including the HSP90 inhibitor IPI-504, cooperated with rapamycin and promoted dramatic tumor regression [81]. Only the combination of the two drugs (rapamycin and an HSP90 inhibitor IPI-504 or AUY-922), both used at a sub-toxic dose, induced irresolvable ER stress, continuous autophagy, and progressive damage to the ER and mitochondria leading to massive cell death and tumor shrinkage. In *NF1*-deficient cells, the exposure to HSP90 inhibitors impaired the UPR (Unfolded Protein Response) by destabilizing two key stress-sensing components of the UPR: IRE1 and pPERK/PERK, as previously shown by Marcu et al. [82]. Thus, inhibition of HSP90 would induce apoptosis by two mechanisms: first by directly increasing the burden of misfolded proteins, and then by inactivating two of the three arms of the UPR [83] (Fig. 13.1b). ER stress is known to induce intraluminal calcium release, which triggers mitochondrial membrane depolarization and ROS production (Fig. 13.1b). In a vicious cycle, ROS then promotes protein misfolding and increased ER stress [84, 85]. Moreover, oxidative stress played a critical role in the response to the combination of rapamycin and IPI-504. Pre-treatment with the antioxidant vitamin C suppressed cell death and tumor regression that was induced by rapamycin and IPI-504 in the MPNST cellular and mouse models [81]. The sensitivity of MPNST cells to HSP90 inhibitors could also be attributed to the acquired dependency of *Nf1*-deficient cells to heat shock response proteins. Indeed, loss of *Nf1* activates HSF1 (heat shock factor 1), a master transcriptional regulator of the heat shock response that drives the expression of heat shock proteins, including HSP27, HSP70 and HSP90 [86]. Concomitant blockade of the PI3K/mTOR signaling axis may attenuate the HSF1-driven cellular heat shock response elicited by pharmacologic inhibition of HSP90 [87]. These findings provide a molecular framework for the investigation of novel combinatorial strategies designed to enhance the antitumor activity of targeted HSP90 inhibitors.

De Raedt et al. demonstrated the therapeutic potential of appropriate pharmaceutical agents that exhaust the cell-protective features of the ER stress response and trigger its pro-apoptotic component as it was previously suggested [88, 89]. The combination of mTOR and HSP90 inhibitors was the first to induce tumor regression in a MPNST mouse model and in Ras-driven NF1 tumor cells. Based on this encouraging data, a clinical trial was recently initiated to evaluate the potential benefit of ganetespib (HSP90 inhibitor) in combination with sirolimus (mTOR inhibitor) for patients with sporadic or NF1-associated MPNST (*Clinical trial reference 1*).

Interestingly, production of reactive oxygen species and subsequent induction of apoptosis was also demonstrated in the HEI193 human NF2 schwannoma cell line after in vitro treatment by curcumin, a molecule known to disrupt the chaperone functions of HSP90 [90–92]. However, the growth of resistant cells with increased expression of HSP70, a known mechanism to escape curcumin-induced apoptosis [93], was only moderately inhibited by a combination of curcumin and KNK473, an inhibitor of HSP synthesis [91]. As in NF1 models, new combinations of HSP90 and mTOR or other inhibitors could be more efficacious than single compounds in eliciting robust therapeutic effects

13.5 Use of HSP90 Inhibitors to Target Multiple Signaling Pathways Activated in Neurofibromatosis Type 2 Tumors

Many client proteins of HSP90 were found to be activated in human NF2-related tumors, including HER/ErbBs, Akt, Axl, Met, c-Raf, Cdk4 and PDGFR [94–97]. Other proteins activated in NF2 are regulated by HSP90 client proteins (e.g. YAP by Lats 1/2) [98, 99]. To simultaneously suppress these diverse activated proteins and signaling pathways, HSP90 represents an attractive therapeutic target, as its inhibition would induce proteasomal degradation of a multitude of client proteins (Fig. 13.2).

Proof of principle of this strategy was provided using preclinical cellular and mouse models of NF2 [76]. Using gene expression analysis, overexpression of HSP90 in schwannoma was confirmed, as previously shown for other tumor types [100], and thought to provide selectivity for targeting tumor cells over normal cells. NXD30001 is a novel small-molecule inhibitor (pochoxime A) developed on the radicicol scaffold that previously displayed a strong efficacy for HSP90 inhibition and specifically reduced proliferative activity in a number of tumor cell types [101, 102]. A separate study reported that the compound penetrated the blood–brain and blood–spinal cord barriers and accumulated in nervous tissues [103], a pre-requisite for treating some NF2-related tumors.

The inhibition of HSP90 demonstrated robust anti-proliferation activity at the nanomolar level in several *NF2*-deficient cell cultures (mouse and human primary and immortalized cells). HSP90 inhibitors also demonstrated a strong anti-tumor

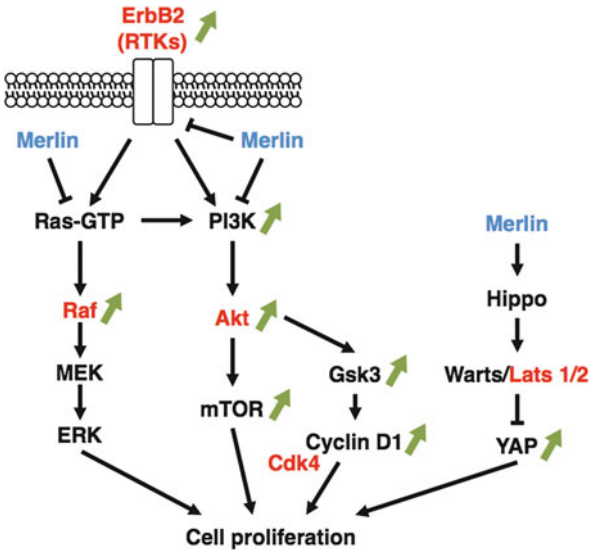

Fig. 13.2 Merlin negatively regulates transmembrane receptor signaling. Inactivation of merlin can affect a variety of signaling pathways such as Raf/MEK/ERK, Akt/mTOR and Hippo/YAP pathways. Therefore multiple proteins have been found upregulated in schwannomas, including ErbB2 and other Receptor Tyrosine Kinases (ErbBs, Axl), PI3K, Raf, Akt, mTOR, Gsk3, Cyclin D1 and YAP (*green arrows*). Several of these proteins are HSP90 client proteins (in *red*) or are regulated by HSP90 client proteins

activity in two NF2 mouse allograft models and in a genetically modified NF2 mouse schwannoma model (P0-Sch△(39–121)-27) [76]. The treatment of P0-Sch△(39–121)-27 mice with 17-DMAG showed a dose dependent reduction of Schwann cell tumor lesions. At the molecular level, NXD30001 induced the degradation of multiple HSP90 client proteins in *NF2*-deficient cells and suppressed their proliferation. Differential expression analysis showed that NXD30001 treatment induced modifications in the expression of genes implicated in cell proliferation, cell survival, vascularization and Schwann cell differentiation. Thus, transcriptional modulation of multiple biologic pathways may contribute to the efficacy of HSP90 inhibition against NF2-related tumorigenesis. Altogether, these data demonstrate that HSP90 inhibition induces significant anti-tumor activity against NF2-related tumor cells in vitro and in vivo, and represent a novel promising option for NF2 therapy.

13.6 Conclusion

Despite recent advances in the understanding of molecular pathology and in tools to evaluate potential drug targets, rational targeted therapeutic options for patients with neurofibromatosis type 1 and 2 are at an early stage of evaluation and none have yet proven clinically effective. Benign and aggressive tumors in NF1 and NF2 both represent challenges for the research and development of treatments. Additionally, patients with benign tumors or with multiple tumor occurrences require long-term treatments that can be challenging due to drug toxicity and adverse events.

HSP90 inhibitors offer a different therapeutic approach for these two genetic syndromes where the molecular pathogenetic mechanisms of tumor formation are not directly targetable by single drugs or rely on activation of multiple pathways. The ongoing development of a new generation of HSP90 inhibitors, with improved safety and efficacy, has prompted clinical trials for a variety of tumors. These compounds could be used alone or in combination with targeted therapies to block the capacity of tumors to adapt and to develop resistance to drugs [104] or to exploit vulnerabilities in already stressed cells, as in the case with the NF1 preclinical study above. To date, there are no approved HSP90 inhibitors, but this promising therapeutic strategy continues to generate interest among the research community [105, 106] and may provide much-needed options for neurofibromatosis patients.

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Chapter 14
Role of Heat Shock Protein 90 in the Cause of Various Diseases: A Potential Therapeutic Target

Subhankar Paul

Abstract Different classes of Heat shock proteins (HSP) play a diverse role in influencing proper assembly, folding, and translocation of cellular proteins. HSP90 is one such kind of molecular chaperone which has been implicated the formation of number of diseases like cancer and various kinds of neurodegenerations. The chaperone, HSP90 assists in folding, maturation and maintains the functional stability of many proteins that includes many oncoproteins like p53 as well as neuronal proteins like tau. It also regulates transcription factors including Heat shock factor-1 (HSF-1). In addition to its well characterized functions in malignancy, recent evidence from several laboratories suggests a role for HSP90 in maintaining the functional stability of neuronal proteins of aberrant capacity, whether mutated or over-activated, allowing and sustaining the accumulation of toxic aggregates. Preclinical studies have demonstrated that disruption of much client proteins chaperoned by HSP90 is a possible strategy to reduce tumorigenesis but could suppress many neurodegeneration both in vivo and in vitro. Thus, inhibition of HSP90 has been found to be a novel strategy to target such diseases and pave the novel way of battling with these lethal diseases.

Keywords Heat shock proteins • HSP90 • Cancer • Neurodegenerations • HSP90-inhibitors • Geldanamycin

Abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
17-DMAG	17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AR	Androgen receptor

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CTD	C-terminal domain
EGFR	Epidermal growth factor receptor
GA	Geldanamycin
HD	Huntington's disease
HIF	Hypoxia-inducible factor
HSF-1	Heat shock factor-1
HSP	Heat shock protein
HSR	Heat stress response
HTS	High throughput screening
NTD	N-terminal domain
PD	Parkinson's disease
polyQ	Polyglutamine diseases
SBMA	Spinal and bulbar muscular atrophy
SCA	Spinocerebeller ataxia
UPS	Ubiquitin–proteasome system

14.1 Introduction

Molecular chaperones, commonly known as heat shock proteins (HSP) serve as multifunctional molecules and their role includes assist in the folding of cellular proteins, intracellular disposition, and proteolytic turnover of many key regulators of cell growth, differentiation, and survival. HSP normally assist proteins in maintaining a conformation that allows appropriate folding, recognition, and modification by the ubiquitination systems or hydrolysis by the proteasome [1, 2]. HSP ensure that these proteins maintain their native conformations during stressed condition [2]. HSP comprise of several highly conserved families of related proteins. They prevent misfolding, aggregation of proteins and facilitate to achieving a correct conformation of a nonnative protein, often through an ATP-dependent manner. HSP typically recognize and bind to the exposed hydrophobic residues of non-native proteins through non-covalent interaction [3].

Mammalian HSP have been classified mainly in four families according to their molecular weight: HSP90, HSP70, HSP60 and small HSP (15–30 kDa) that include HSP27. HSP are expressed either constitutively or regulated inductively, and are present in different subcellular compartments. Large HSP (HSP60, HSP70, etc.) are ATP-dependent chaperones whereas small HSP (like HSP27) function in an ATP-independent fashion. Based on various research reports, it has been observed that HSP specifically recognize and bind to the exposed hydrophobic patches or residues of unfolded/partially folded (non-native) proteins, through hydrophobic interaction [1]. In addition, HSP are required for protein trafficking to target organelles and to facilitate the transfer of misfolded proteins to the proteasome for degradation [4]. HSP can be induced by various stresses such as heat shock, ischemia, hypoxia, heavy metals, and amino acid analogs [4]. Some HSP are expressed constitutively in unstressed cells [5].

Although various HSP have different mechanism of action, in the present chapter we will only focus on HSP90 and its role in various diseases and its use as therapeutic approach in such diseases. HSP90 has been reported in many studies as responsible agent for the cause of tumorigenesis and cancers [6]. Its role in cancer has received much attention in the last decade since HSP90 has been found to assist number of proteins (called 'client proteins') includes many oncogenic proteins in their folding, maturation, transportation, etc. In many cases such as breast cancer, HSP90 was found to be overexpressed along with its clients like p53.

Since number of neuronal proteins is also the clients of HSP90 (like 'tau' protein), it has also been implicated with various neurodegeneration like Alzheimer's disease [7], etc. In cells, the combined functions of molecular chaperones like HSP, the UPS (ubiquitin–proteasome system) and lysosome-mediated degradation pathway are normally sufficient to prevent the accumulation of misfolded proteins in cells. However, under certain pathological circumstances, the protein quality control machinery is overloaded and misfolded proteins can accumulate to a dangerous level. AD, PD, Prion disease and the polyglutamine (polyQ) disease are all characterized by the accumulation of this kind of proteins, mutations of which cause misfolding and subsequent aggregation leading to severe, inherited forms of diseases.

Inhibition of HSP90 has been reported to be the recommended strategy to find therapeutic avenue in these diseases and hence, several selective HSP90 inhibitors have been discovered and are currently undergoing clinical evaluation. Much of the recent progress in understanding the complex role of heat shock proteins in tumorigenesis has been made possible by the discovery of several natural product antitumor antibiotics like geldanamycin and their analogues that selectively inhibit the function of the chaperone HSP90. These agents have been used as probes to define the biological functions of HSP90 at the molecular level and to validate it as a novel target for anticancer drug action.

This chapter focuses on the role of HSP90 in the cause of these diseases like cancer and neurodegenerations. We also discuss here the potential use of HSP90 inhibitors in clinical areas as therapeutic agents.

14.2 Cellular Heat Shock Proteins 90 (HSP90)

HSP90 is a highly abundant and ubiquitous molecular chaperone (approximately 1–2 % of total protein present in the cytosol) which plays an essential role in many cellular processes including cell cycle control, cell survival, hormone and other signaling pathways [6]. HSP90 is an ATP-dependent molecular chaperone which is essential in eukaryotes. It is required for the activation and stabilization of a wide variety of client proteins and many of them are involved in important cellular pathway. In the last one decade, it has become a major therapeutic target for various diseases like cancers and in various neurodegenerative disorders. The structure of HSP90 consists of three domains i.e., the N-terminal domain, the middle-domain and the C terminal domain [8]. These domains are composed of nearly 732 amino acids [8]. HSP90 has two isomers HSP90α and

HSP90β, which are mainly present in the cytosol [9]. The N–terminal domain is an amino terminal domain and contains a fold known as bergerat fold, which possesses the ATP and drug-binding site [10]. The middle domain possesses a co-chaperone interacting motif, which provides binding sites for client proteins and co-chaperones. These binding sites play an important role in forming the active ATPase [8]. The C-terminal domain is the carboxy terminal domain, which possesses a dimerization motif. This is known as the second drug-binding site and the site where HSP90 also interacts with the other co-chaperones [8]. Dimerization of HSP90α monomer mediated through C-terminal, which is essential for its chaperoning function [11]. After dimerization, HSP90 binds to ATP in its open state facilitating the attachment of co-chaperones and client protein binding. The closed conformation is mediated by ATP hydrolysis [8] (Fig. 14.1).

14.3 Functions of HSP90 in Various Biological Processes

HSP90 is one of the most abundant cellular chaperone proteins comprising up to 1–2 % of total cellular protein and increases to 4–6 % of cellular proteins under stress. It increases by twofold to tenfold during environmental stress and this up-regulation could be explained as a part of the protective mechanisms that enhance cell survival [13]. HSP90 exerts essential housekeeping functions as a molecular chaperone, such as facilitating protein refolding, translocation of proteins between cellular compartments, suppression of protein aggregation, supporting functional maturation of signaling proteins and facilitating normal protein turnover [6]. It functions in a multi-component complex of chaperone proteins that may include p60/Hop, p50Cdc37, HSP40/HDJ2, p23, HSP70 and one of a variety immunophilins [14]. Unlike other chaperones, HSP90 distinguishes itself in that most of its known clients are protein kinases or transcription factors involved in signal transduction [14].

14.4 HSP90 Client Proteins and Diseases

More than ‘200’ client proteins covering almost all cellular processes have been identified so far (http://www.picard.ch/downloads/Hsp90interactors.pdf). Many of these client proteins are intimately associated in critical cellular functions that promote cell growth, proliferation and cell survival. A significant number of cancer associated proteins have been identified as HSP90 client proteins which includes apoptotic mediators (Bcl-2, Apaf-1), cell cycle regulatory proteins (CDK4, hTERT), tumor suppressors genes (p53), mediators of tissue invasion and metastatics (MMP2), transportation factors (HSF-1, HIF-1), signaling molecules (AKT, RAF-1), steroid hormones (androgen, progesterone, glucocorticoid receptors).

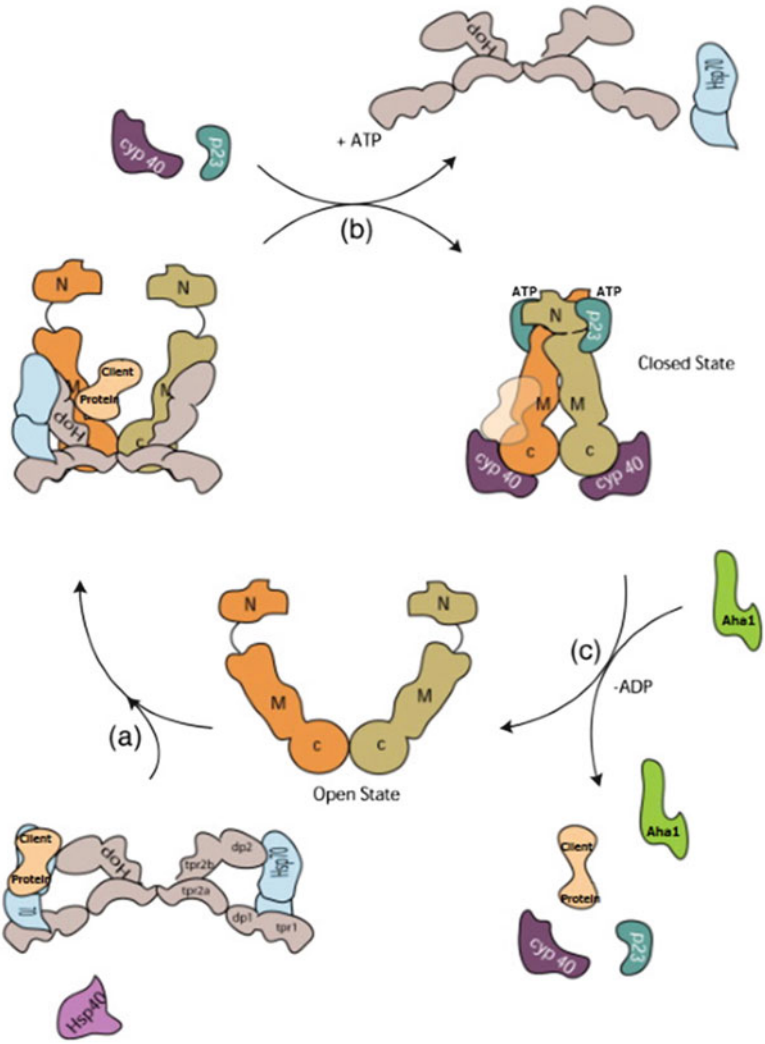

Fig. 14.1 Model of the conformational cycle of HSP90 (Adapted from [12])

Moreover, recent findings have revealed that HSP90 assist in the stability of many neuronal aberrant proteins, and thus it favors the process of accumulation of toxic protein aggregates [7, 15]. For example, in AD, in addition to β-amyloid aggregation, it has been found that there is an accumulation of abnormal species of hyperphosphorylated protein tau, which leads to the formation of toxic neurofibrillary tangles [7, 15]. This hyperphosphorylation is caused by abnormal kinases like CdK4, GSK-3β activities and the stability of function of them is maintained by HSP90 [16, 17]. Experiments suggest a role for HSP90 in maintaining the functional

stability of neuronal proteins, which are aberrant in nature, whether mutated or over-activated [18]. These phenomena results in the accumulation of toxic aggregates.

HSP90 functions by recruiting many co-chaperones viz, Cdc37, Hop, p23, HSP70 and HSP40 [19]. The complex formation is an energy requiring step and involves sequential ATPase cycles [20]. Client proteins of HSP90 are several kinases viz, AKT, B-Raf mutant, MET and CDK4; Transcriptional factors HIF-1A, ERA-receptors mutant p53th regulates cell proliferation and survival and chimeric fusion proteins [21].

14.5 HSP90 and Cancer Development

HSP90 is a molecular chaperone [22] that participates in the quality control of protein folding. More than 200 client proteins covering almost all cellular processes have been identified so far. It is involved in the maturation and stabilization of a wide range of oncogenic client proteins which are crucial for oncogenesis and malignant progression [23]. Indeed the cancer cells are particularly dependent on proper HSP90 function [24, 25]. Many of these client proteins such as p53, BCR-ABL, HER2, epidermal growth factor receptor (EGFR), CRAF, BRAF, AKT, MET, VEGFR, FLT3, androgen and estrogen receptors, hypoxia-inducible factor (HIF-1α), and telomerase are involved in critical cellular functions that promote cell growth, proliferation and cell survival. Many of these client proteins are mutated and/or overexpressed in cancers [26]. Moreover, the harsh environmental conditions found in tumors such as hypoxia, low pH, and bad nutritional status may tend to destabilize proteins, making them even more dependent on HSP90 activity [23, 27, 28].

It has also been observed that HSP90 is constitutively expressed at manifold levels in tumor cells compared to their normal counterparts [29, 30], suggesting that HSP90 is critically important for tumor cell growth and and/or survival and its inhibition would help to check the proliferation of cancer cells. Inhibition of HSP90 causes client protein degradation via the ubiquitin-proteasome pathway, and is a mechanism that might simultaneously down-regulate several redundant pathways crucial for cell viability and tumor development.

Therefore, in many cancers such as non-small cell lung cancer, oesophageal squamous cell carcinoma, pancreatic carcinoma and advanced malignant melanoma the over-expression of HSP90 has been observed [31–34]. In addition, studies showed that HSP90 stabilizes various key oncogenic proteins such as survivin, Akt, Erb-2 and HIF-1α in cancer cells [35–37].

Cancer cells are stressed cells and heavily depend on HSP90 chaperoning and thus show higher levels of expression of HSP90, therefore HSP90 has emerged as a target for cancer therapy [38]. Many drugs targeting ATP binding domain have been developed and are under clinical trials. Geldanamycin a prototype example of ansanamycins antibiotic showed exciting results disrupting multiple pathways but was toxic to normal cells and hence could not enter clinical trials [39].

Analogues of geldanamycin, 17-AAG and 17-DMAG were designed which was non toxic to human cells. HSP90 inhibitors found in literatures have mostly been discovered by structure based virtual screening, generating derivatives from already existing inhibitors or finding new scaffolds by HTS [40]. Recently Shepherdin, a novel anticancer agent was designed based on the interaction between HSP90 and surviving [41]. Survivin is a mitotic regulator and antiapoptotic protein involved in many pathways [42]. The structure of shepherdin was based on modelled interface between HSP90 and survivin.

14.6 HSP90 in Neurodegeneration

In addition to its well-characterized functions in malignancy, recent evidence from several laboratories suggests a role for HSP90 in maintaining the functional stability of neuronal proteins of aberrant capacity, whether mutated or over-activated, allowing and sustaining the accumulation of toxic aggregates. One such example is tau protein responsible in the cause of Alzheimer's disease [43]. In addition, HSP90 regulates the activity of the transcription factor heat shock factor-1 (HSF-1), the master regulator of the heat shock response, mechanism that cells use for protection when exposed to conditions of stress.

Recent evidences revealed a very crucial role for HSP90 in neurodegeneration. Many of the client proteins of HSP90 have been found the central cause of neurodegeneration. HSP90 maintains the functional stability of such neuronal proteins of aberrant capacity, thus, allowing and sustaining the accumulation of toxic aggregates [18, 44, 45]. Recent findings have also revealed that HSP90 assists in the stability of many neuronal aberrant proteins, and thus it favors the process of accumulation of toxic protein aggregates [18, 44, 45]. For example, in AD, in addition to β-amyloid aggregation, it has been found that there is an accumulation of abnormal species of hyperphosphorylated protein tau, which leads to the formation of toxic NFT [7, 17]. This hyperphosphorylation is caused by abnormal kinases like CdK4, GSK-3b activities, and the stability of function of them is maintained by HSP90 [17].

14.7 HSP90 Inhibitors Targeting Co-Chaperone/HSP90 Interactions

For the proper functioning of HSP90, a series of co-chaperones are required. Binding and leaving of the co-chaperones at various stages provide regulatory control to the chaperoning process of HSP90 [46]. Therefore, blocking the chaperone cycle at these stages by targeting different co-chaperone/HSP90 interactions is likely to achieve similar results with the direct inhibition of HSP90 [47]. Various co-chaperones targeted so far are cdc37/HSP90, HSP70/HSP90, HOP/HSP90 and Aha1/HSP90 [12, 48–50].

14.8 HSP90 Inhibitors as Cancer Chemotherapeutics

HSP90 plays an important role in the maintenance of multiple oncogenic pathways, and is essential for maintaining folding, stability, and conformation of several oncoproteins to help retain aberrant activity and sustain the malignant state [51]. Although directed toward a single molecular target, HSP90 inhibitors simultaneously block multiple signaling pathways implicated and are essential to maintain folding, stability and conformation of several oncoproteins to help retain aberrant activity and sustain the malignant state [52]. Because many of its clients include oncoproteins with important functions in the development and promotion of cancer, HSP90 is an important target in cancer therapy. Thus, HSP90 inhibition has been a promising strategy for targeting cancer therapy.

Small molecule HSP90, inhibitors bind to the ATP binding pocket and cause the catalytic cycle arrest of HSP90, in the ADP bound conformation, leading to the inactivation of chaperone activity, premature ubiquitination and proteasomal degradation of the client proteins [53]. This results in depletion of oncoproteins, cell cycle arrest and apoptosis [54]. Inhibition of HSP90 function has already been proved effective in killing cancer cells that developed resistance to kinase inhibitors [55]. One of the key inhibitors used for HSP90, inhibition is Geldanamycin (GA). Despite its potent anti-tumor effects, GA was never evaluated in clinical trials because of its poor "drug-like" properties including poor solubility, limited in vivo stability and significant hepatotoxicity in animals [56] and [57].

After the failure of GA in the clinical trials, research was more focused on its more water-soluble derivatives like 17-AAG. 17-AAG (17-N-Allylamino-17-demethoxygeldanamycin) has shown less toxicity but it shows less affinity for HSP90 than GA [58]. It is poorly water soluble which affects its formulation development during clinical trials [59].

Another analogue of GA is 17-DMAG (17-Dimethylaminoethylamino-17-demethoxygeldanamycin); this was considered to be very effective but like others, this drug could not be used for a prolonged period and it also showed limited metabolism which resulted in problems associated with drug clearance [43]. To resolve the problems that are associated with NTD (N-terminal domain) inhibitors, various CTD (C-terminal domain) inhibitors have been developed. Till date, a variety of drugs which have been identified as the HSP90 CTD-inhibitors such as Novobiocin, Clorobiocin, EGCG, Derrubone, etc. binds at the ATP binding site of HSP90, CTD (Fig. 14.2) (Shumaila and Paul 2014). Hence, in the present investigation, we attempted to find the residues involved in the ATP binding site in CTD and a potential inhibitor which can block this ATP binding site in the C-Terminal domain.

Many evidences suggested that HSP90 in tumor cells has greater affinity for HSP90-inhibitors like 17-AAG than that found in normal cells. This difference may result from the bulk of HSP90 being tied up in multiprotein complexes in tumor cells, whereas a substantial pool of free dimers with low ATPase activity and low drug affinity exists in normal cells [29, 30, 60].

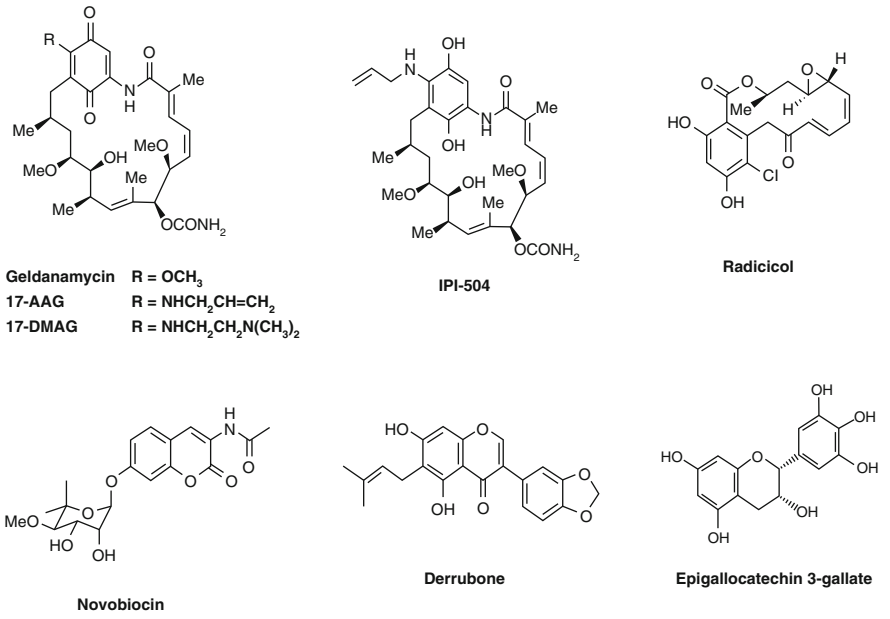

Fig. 14.2 Chemical structure of the naturally occurring HSP90 inhibitors (Adapted from [40])

Collectively, these findings implicate an important role for HSP90 in the development of Alzheimer's disease and other neurodegenerative diseases and suggest that HSP90-interfering drugs may represent a potential novel class of drugs to promote the survival of neurons. They also imply that, because of their specificity for high-affinity HSP90, small molecule HSP90 inhibitors may selectively target neurodegenerative disease processes without toxicity toward normal tissues.

14.9 HSP90-Inhibition: A Therapeutic Target in Neurodegeneration

It has been found that inhibition of HSP90 activates heat shock factor-1 (HSF-1), a transcriptional regulator of heat shock proteins, to induce the cellular expression of HSP70 and HSP40 and other molecular chaperonins, which in turn, trigger disaggregation of protein aggregates and protein degradation [61–63]. Moreover, direct inhibition of HSP90 function also facilitates mutant or aberrant protein degradation. These biological functions, therefore, propose HSP90 inhibition as dual therapeutic modality in neurodegenerative diseases. First, by suppressing aberrant neuronal activity, HSP90 inhibitors may ameliorate protein aggregation and its associated toxicity. Second, by activation of HSF-1 and the subsequent induction

Table 14.1 HSP90-inhibitors induce HSP70 expression and triggers neurodegenerative suppression [3]

HSP90-inhibitors	Disease
GA	AD, HD, PD
17-AAG	Spinal and bulbar muscular atrophy, PolyQ, SCA, HD
17-DMAG	HD, spinal and bulbar muscular atrophy
Celastrol	Polyglutamine disease, amyotrophic lateral sclerosis, AD
Herbimycins D−F	Proposed in neurodegeneration

of heat shock proteins, such as HSP70, HSP90 and thus the inhibitors may redirect neuronal aggregate formation, and protect against protein toxicity.

Moreover, there are various experimental findings which revealed that various HSP90-inhibitors when used in diseased cellular models the cellular expression level of HSP70 and HSP40 was increased by multi-folds. It was already observed that the overexpression of HSP70 and HSP40 suppressed various neurodegeneration conditions. Hence, inhibition of HSP90 using various inhibitors like Geldanamycin (GA), 17-allylamino-17-demethoxygeldanamycin (17-AAG), 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), radicicol, novobiocin, etc. (see the structures in Fig. 14.2) have been used to inhibit of protein aggregation progress. A list of the inhibitors name and working system of HSP90-inhibitors in various neurodegeneration was given in tabular form in Table 14.1. Moreover, it is worthy to mention that Kitson and his group recently reported the synthesis of 19 GA substitute HSP90 inhibitors which exhibited much less toxicity and when tested in human breast cancer and dopaminergic neural cells, they demonstrated them to act as potential therapeutic agent against both cancer and dopaminergic cells [64].

Protective effects of GA in a Drosophila model and in a mouse model of Parkinson's disease (PD) was observed [65, 66]. Few studies also proved that 17-AAG increased the degradation of androgen in SBMA mouse model and in a familial model of ALS [44, 67, 68]. 17-AAG treatment successfully suppressed neurodegeneration in a Drosophila model of SCA3 and Huntington's disease (HD), and it was found to be the most effective agent among the other HSF1-activating compounds in suppressing polyQ-related neurodegeneration in Drosophila model [69]. In the SBMA model system, 17-DMAG, and 17-AAG, preferentially caused HSP90 client protein degradation and upregulation of HSP70 and HSP40 [68]. In another study, 17-DMAG was found modulated the AR-HSP90 chaperone complex from a mature stabilizing form to a proteasome-targeting along with Hop [70]. Celastrol, a drug exhibited inhibition the interaction of HSP90 with its cochaperone cdc37 and also caused induction of HSP, moreover, in another study, it was shown that Celastrol could provide neuroprotection against polyglutamine toxicity, both in vivo and in vitro [71–73]. They were also found to reduce the β-amyloid amount in a transgenic mouse model of AD and HD [73, 74].

Sittler and colleagues reported for the first time that GA reduced the aggregation of mutant huntingtin protein by inducing molecular chaperones in cell culture [75]. The mechanism was that GA caused disruption of the complex between HSP90 and HSF1, the master stress-inducible regulator, which activates the heat stress response (HSR) in mammalian cells. Treatment with GA upregulated HSP70 and HSP40 expression and resulted in inhibition of huntingtin aggregation in a cell-culture model of HD and in a primary culture model of familial amyotrophic lateral sclerosis (ALS) [75, 76]. GA is also responsible for modulating α-synuclein pathology and its solubility and it was also found to decrease α-synuclein aggregation in neuroglioma-transfected cells and protects them against toxicity [77].

It was recently reported by Waza et al. that in SBMA model, one of the polyQ diseases, the administration of 17-AAG significantly ameliorated polyQ-mediated motor neuron degeneration by reducing the total amount of mutant androgen receptor (AR) [44]. 17-AAG accomplished the preferential reduction of mutant AR mainly by inhibiting HSP90 function. It was also proposed from experimental findings that 17-AAG induces the up-regulation of HSP70 and HSP40 in vivo and overexpression of them might support the degradation of mutant protein and thus suppress the disease severity.

14.10 Conclusion

Inhibition of HSP90 offers very selective binding, however, many facts yet to be explored regarding the molecular basis for their interactions. The precise knowledge is the key to understand how the client/HSP90 interaction is targeted for studying the structure and biochemistry of the molecular complexes.

A common cause of cancer pathogenesis and neurodegeneration was clearly observed through a common regulator HSP90 which was found deregulated from various experimental findings. A well established role of HSP90 thus has been observed in the cause and therapeutic target for the cancer and neurodegeneration using various HSP90 inhibitors like geldanamycin, its derivatives like 17-DMAG and others. Interestingly, the chaperoning function of HSP90 depends upon both cellular HSP70 and HSP40. Therefore, targeting HSP90 might be a therapeutic route for multiple diseases including neurodegeneration. Although few HSP90-inhibitors like geldanamycin, 17-AAG, 17-DMAG, Celastrol have been used so far in neurodegeneration, the actual number of existing HSP90-inhibitors is more. Therefore, other inhibitors also should be attempted. Moreover, the analogues of existing inhibitors and peptide-based novel inhibitors have also been proposed to be the HSP90-inhibitors [3]. It may be recommended to use such analogues in neurodegeneration conditions.

However, most of the details of the client/chaperone interactions are still unclear, and therefore, the strategy of targeting these associations is more challenging.

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Chapter 15
HSP90 Inhibitor-Based Strategies for Cancer Therapy: Advancing Toward Clinical Impact

David A. Proia and Richard C. Bates

Abstract The discovery of selective inhibitors of HSP90 two decades ago has enabled both a better understanding of the biology of HSP90 as well as its validation as a pharmacologic target for cancer. A number of HSP90 inhibitors have entered human clinical trials; to date, however, none have been approved for cancer therapy and thus the full potential of this class of agents remains to be realized. In this chapter we review the current status of HSP90 inhibitor development for cancer treatment, with particular emphasis on the second-generation, synthetic classes of compounds. In addition, we highlight various strategies currently being pursued that are designed to exploit specific cancer cell vulnerabilities and provide frameworks for optimization of HSP90 inhibitor-based strategies across a broad spectrum of cancer types.

Keywords Heat shock protein 90 • Cancer therapy • Small molecule inhibitors • Molecular chaperone

Abbreviations

5-FU	5-fluorouracil
17-AAG	17-allylamino-17-demethoxygeldanamycin
17-DMAG	17-dimethylaminoethylamino-17-demethoxygeldanamycin
ALK	Anaplastic lymphoma kinase
AR	Androgen receptor
BCR-ABL	Breakpoint cluster region- Abelson murine leukemia viral oncogene homolog
BRAF	v-Raf murine sarcoma viral oncogene homolog B
CRC	Colorectal cancer
EGFR	Epidermal growth factor receptor
EML4	Echinoderm microtubule-associated protein-like 4

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ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FLT3	Fms-related tyrosine kinase 3
GIST	Gastrointestinal stromal tumor
HER2	Human epidermal growth factor receptor 2
HIF-1α	Hypoxia-inducible factor 1 alpha
HSF1	Heat shock factor-1
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
IGF-1R	Insulin-like growth factor 1 receptor
JAK/STAT	Janus kinase/signal transducer and activator of transcription
KRAS	Kirsten rat sarcoma viral oncogene homolog
LV	Leucovorin
MAPK	Mitogen-activated protein kinase
MEK	Mitogen/extracellular signal-regulated kinase
mTOR	Mammalian target of rapamycin
NSCLC	Non-small cell lung cancer
ORR	Objective response rate
OS	Overall survival
PI3K/AKT	Phosphoinositide 3-kinase/AKT8 virus oncogene cellular homolog
PFS	Progression free survival
PR	Progesterone receptor
PSA	Prostate-specific antigen
TKI	Tyrosine kinase inhibitor
TNBC	Triple-negative breast cancer
UGT	UDP-glucuronosyltransferase

15.1 Introduction

By traditional measures, the molecular chaperone heat shock protein 90 (HSP90) represents an unlikely and enigmatic candidate for oncology drug development. Highly conserved and ubiquitously expressed, HSP90 plays an indispensable role in regulating the maturation and functional stability of a vast array of cellular substrates, known as ‘client’ proteins (refer to http://www.picard.ch/downloads for current database). The repertoire of HSP90 clients is diverse, and is particularly enriched for signal transducers such as kinases and transcription factors [1]. In this regard, HSP90 activity impacts a broad range of normal homeostatic and physiological processes, including cell growth and survival, immune modulation, and development. In addition, HSP90 is also an essential component of the cellular heat shock response and may be further transcriptionally induced as a consequence of proteotoxic stress [2]. Not surprisingly, deletion of HSP90 is embryonic lethal

and no mutations have been described nor are any polymorphisms known to exist that are suggestive of any association or causal relationship with cancer [3]. For these reasons, HSP90 was not originally considered an intuitively attractive target for the development of antineoplastic therapies.

In 1994, Whitesell and colleagues published a seminal study identifying the first small molecule inhibitors of HSP90 function [4]. This demonstration that natural product benzaquinone ansamycins (such as geldanamycin) were *bona fide* inhibitors of HSP90 with potent antitumor activity paved the way for a dramatic and exponential increase in our understanding of HSP90 biology and its relationship with malignancy over the past two decades. Most notably, it has emerged that the chaperoning functions of HSP90 can become subverted during tumorigenesis in order to facilitate malignant progression and maintain a transformed cellular phenotype [5]. Indeed, a large number of HSP90 client proteins have been implicated in the pathogenesis of human cancers, and are known to contribute to nearly every aspect of the oncogenic process including immortality, pro-survival/anti-apoptosis, metabolism, genomic instability and dissemination [6]. Often, these oncoproteins are expressed in labile states (e.g. mutant, translocated, or amplified) that are particularly reliant on the HSP90 machinery as a biochemical buffer for their stability and function [7]. Indeed, the buffering capacity of HSP90 against numerous environmental stresses, such as genotoxic, proteotoxic, and/or hypoxic insults, represents an important and broad-based mechanism by which tumor cells may co-opt HSP90 activity for selective advantage [8].

A number of additional considerations arose during the characterization and development of first-generation HSP90 inhibitors that helped establish the feasibility of targeting HSP90 as a viable therapeutic strategy for cancer. First, a unique characteristic of pharmacological HSP90 blockade is that inhibition of the chaperone leads to client protein degradation via the ubiquitin ligase-proteasome machinery. At the cellular level, this results in the simultaneous destabilization of literally hundreds of clients and client-driven signaling pathways. This stands in stark contrast to other molecularly targeted approaches, such as direct kinase inhibition, that selectively ablate only one (or a few) oncoproteins or signaling cascades. Moreover, the concomitant disruption of multiple signaling nodes provides a means to inhibit redundant pathways and feedback loops that can contribute to intrinsic and acquired drug resistance mechanisms [9–11]. Second, a common characteristic displayed by all HSP90 inhibitor compounds to date involves preferential and selective tumor retention characteristics [12]. While the underlying basis of this observation remain to be fully elucidated, HSP90 is frequently overexpressed in tumor tissues and a model has been proposed that the molecule exists in cancer cells as part of a highly active, multi-chaperone complex that exhibits greater affinity for targeted inhibitors than that observed in normal cells [13]. Recently, increased SUMOylation of HSP90, a consequence of cellular transformation, has been shown to sensitize both yeast and mammalian cells to HSP90 inhibitors [14] thus providing mechanism of tumor-selective HSP90 activation. Taken together,

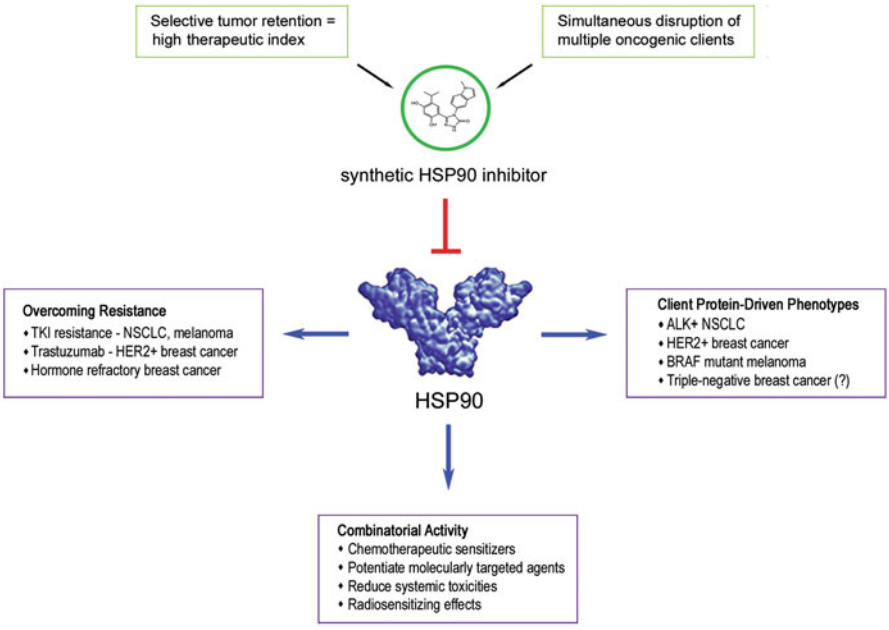

Fig. 15.1 Summary scheme showing exploitable therapeutic characteristics of next-generation HSP90 inhibitor compounds based on current outcomes from preclinical and clinical evaluations

the features of tumor selectivity and multimodal impacts on the malignant phenotype combine to provide an exploitable therapeutic index for this collection of compounds (Fig. 15.1).

Thus, pharmacologicalblockade of HSP90 represents an innovative and multifaceted approach for the development of novel antineoplastic agents and small molecule inhibitors of this molecular chaperone rank among the most actively pursued classes of agents in oncology. While evaluation of the prototypical inhibitor class of ansamycin-based compounds (including geldanamycin and its derivatives 17-AAG and 17-DMAG) delivered critical proof-of-concept evidence and validated HSP90 as a druggable target for cancer treatment [7], their clinical application was hampered, and ultimately halted, due to a number of pharmacological and safety limitations [15]. Since then, an increasing number of synthetic small molecule inhibitors of HSP90 have been developed based on a diverse variety of chemical scaffolds, including those presented in Fig. 15.2 [16, 17]. To a large extent these second-generation compounds have overcome many of the original limitations of, and show improved potency relative to, the ansamycin class of HSP90 inhibitors and a number have entered early stage clinical evaluation. The outcomes of these trials have so far resulted in the early termination of clinical development of some candidates based on the emergence of new, potentially confounding safety concerns.

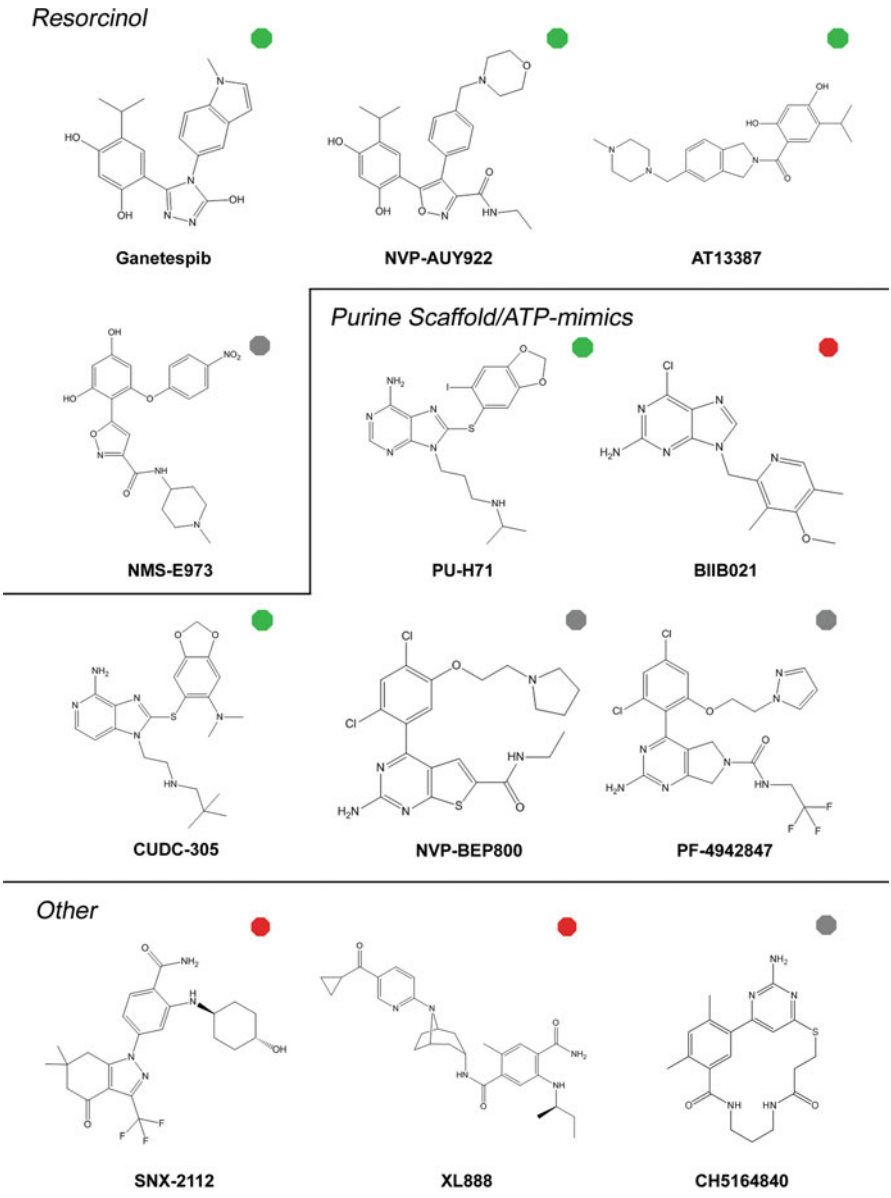

Fig. 15.2 Chemical structures of second-generation HSP90 inhibitor compounds. Key: *Green hexagons* currently under clinical evaluation in human solid tumors; *red hexagons* clinical development discontinued by sponsor; *gray hexagons* not yet entered clinical trials

For example, SNX-5422 (the prodrug of SNX-2112) has been discontinued based on reports of ocular toxicity and potential irreversible retinal damage [18], with treatment-related cardiac abnormalities also observed with this drug [19]. Others, including the potent resorcinol-based inhibitors NVP-AUY922 and ganetespib, have shown favorable tolerability in studies across a variety of human cancers [20, 21] and, for ganetespib in particular, encouraging signs of clinical activity [22]. At present, however, no HSP90 inhibitors have been approved for any human malignancy. This chapter will highlight recent developments in the therapeutic targeting of HSP90 in human solid tumors. Particular emphasis is placed on next-generation inhibitor compounds that are providing the framework for achieving the full translational potential of this promising group of anticancer agents. Targeting HSP90 is considered an equally promising avenue for intervention in hematological malignancies, including multiple myeloma (where buffering of proteotoxic stress is crucial for cell survival) and those cancers driven by sensitive HSP90 clients e.g., BCR–ABL in chronic myeloid leukemia, NPM–ALK in anaplastic large-cell lymphoma, FLT3 in acute myeloid leukemia, and ZAP70 in chronic lymphocytic leukemia [23].

15.1.1 Two Therapeutic Strategies: Client Protein-Driven Tumors Versus Combination Therapy

Many human cancers are known to be ‘oncogenically addicted’ to mutated, overexpressed, and/or chimeric kinases for growth and survival. Importantly, a number of these same oncogenic drivers are established HSP90 clients e.g., mutant EGFR (epidermal growth factor receptor) or ALK (anaplastic lymphoma kinase) translocations in non-small cell lung cancer (NSCLC); HER2 (human epidermal growth factor receptor 2) in breast cancer; mutated BRAF in melanoma; and mutant KIT in gastrointestinal stromal tumors (GIST). Accordingly, a reasonable approach for evaluating the potential of single-agent HSP90 inhibitor therapy involved the selection of tumor types expressing such modifications in order to capitalize on their client protein-driver dependence on HSP90. This premise has been supported by promising clinical activity observed in individuals with defined tumor phenotypes, most notably those exhibiting ALK-driven or HER2-amplified disease. Unexpectedly, similar translational benefit has not been achieved using HSP90 inhibitor monotherapy in other client-protein driven patient populations. This clinical experience has now led to the realization that the pleiotropic effects of HSP90 inhibitors may best be employed in the clinical setting as chemotherapeutic or molecularly-targeted agent sensitizers in order to provide superior antitumor efficacy, overcome resistance mechanisms, and reduce treatment-related toxicities, as summarized in Fig. 15.1 [5]. Here we discuss the potential utility and limitations of both of these strategies, with particular respect to individual cancer types and client protein sensitivity.

15.2 Non-small Cell Lung Cancer: Highlighting the Relationship Between EML4-ALK Client-Protein Dependence and Clinical Efficacy

NSCLC accounts for 85 % of all cases of lung cancer, which remains the leading cause of cancer-related deaths worldwide [24]. The high mortality is associated, in part, with the fact that a majority of patients present with advanced disease at the time of diagnosis with treatment options limited to systemic therapy. Platinum-based combination chemotherapy provides the foundation for current standard-of-care treatments in the clinical management of advanced NSCLC – a strategy that has largely reached a plateau of effectiveness in improving survival rates for lung cancer patients [25, 26]. Over recent years, an increased understanding of NSCLC biology has transformed lung cancer therapy from such broad-based cytotoxic use towards more tailored treatment approaches for certain patient sub-populations. A prime example involved the introduction of targeted inhibitors of the epidermal growth factor receptor (EGFR), erlotinib and gefitinib, which revolutionized and modified the principles of NSCLC treatment a decade ago [27]. The paradigm shift towards personalized therapies has provided meaningful improvements in overall survival and quality of life for lung cancer patients [28, 29].

NSCLC is characterized by a remarkable degree of genetic diversity and may be classified into distinct molecular subsets based on specific genomic alterations that drive tumorigenesis [29, 30]. This tumor type has long been considered a promising indication for the application of HSP90 inhibitors [6] since many of these oncogenic driver proteins are kinases that are established HSP90 clients, including EGFR, RAF, HER2, and, notably, the EML4-ALK fusion protein [31–36]. Indeed, EML4-ALK provides a compelling example of the relationship between client protein-driver dependence and clinical efficacy of targeted HSP90 inhibition. Approximately 3–7 % of NSCLC tumors are characterized by oncogenic gene rearrangements of ALK, most commonly with echinoderm microtubule-associated protein-like 4 (EML4), resulting in constitutively active kinases with transforming capacity [37, 38]. Crizotinib, a dual MET/ALK small molecule tyrosine kinase inhibitor (TKI), was the first ALK-targeted agent evaluated clinically and was granted accelerated approval in the United States for the treatment of patients with ALK-positive (ALK+) NSCLC [39, 40]. Crizotinib therapy improves overall patient survival compared with crizotinib-naïve controls [41], thereby providing clinical validation for targeting ALK in ‘oncogene-addicted’ lung tumors of this genotype. Since EML4-ALK is a highly sensitive client protein of HSP90 [31, 33], pharmacological blockade of the chaperone has been investigated as an alternative approach to direct kinase inhibition for therapeutic intervention in ALK-driven lung cancer. Confirming preclinical predictions, it was originally shown that ALK+ lung cancer patients could derive therapeutic benefit from targeted degradation of ALK via HSP90 blockade using the ansamycin compound IPI-504 [42]. This finding was supported by the results of a separate phase II study of ganetespib, wherein seven

of eight patients harboring EML4-ALK rearrangements had disease control lasting at least 16 weeks, with four of these individuals exhibiting objective responses to inhibitor monotherapy [22].

Recent experimental data with ganetespib suggest that HSP90 inhibition also offers a promising strategy for overcoming acquired TKI resistance in ALK+ lung tumors [43]. Despite its robust clinical success, durable responses to crizotinib therapy are hampered by the invariable development of acquired drug resistance, a common feature of many TKI drugs [44]. Relapses frequently occur due to a spectrum of newly acquired secondary mutations within the ALK kinase domain [45, 46]. To date, a variety of mutations at different amino acid sites have been reported in NSCLC patients who exhibited resistance to crizotinib, and a number of others that can mediate ALK TKI resistance have been identified through in vitro mutagenesis screens [39, 47, 48]. In preclinical models, ganetespib retained robust cytotoxic activity against crizotinib-resistant cell lines irrespective of the mutational site or specific amino acid substitution present [43]. Significantly, this finding was validated by clinically observed tumor responses in a relapsed NSCLC patient who had progressed after 1 year of crizotinib therapy. Despite the presence of a specific kinase domain mutation, a single cycle of ganetespib treatment resulted in a marked shrinkage of lung lesions, underscoring the therapeutic potential of the drug within this refractory population [43].

In addition, systemic resistance to ALK inhibitors may arise in the absence of secondary ALK kinase domain mutations [45]. Although the mechanism(s) remain to be fully elucidated, it has emerged that ligand-mediated activation of secondary and/or separate oncogenic signaling pathways, in particular EGFR and HER2 [49–51], is one process that can bypass the dependency of tumor cells on ALK signaling and contribute to a resistant phenotype. Strategies to counteract these types of acquired resistance in ALK-driven NSCLC have not yet been established and, within this context, the use of ALK-selective inhibitors with increased potency is unlikely to provide any clinical impact. Both EGFR and HER2 are established HSP90 clients and, as such, the broad spectrum of biological activity afforded by HSP90 inhibition represents a potential approach to counteract such compensatory mechanisms. In preclinical models, ganetespib exposure resulted in the simultaneous destabilization of EML4-ALK as well as EGFR in ALK+ NSCLC lines both in vitro and in vivo, with concomitant loss of multiple downstream effector signaling pathways [43]. The effects were distinct to those of crizotinib, with the multimodal activity of the HSP90 inhibitor sufficient to account for its superior relative potency and antitumor efficacy.

Interestingly, chromosomal rearrangements of two additional tyrosine kinases, ROS1 and RET, generate fusion proteins that are sensitive to HSP90 inhibition by ganetespib [43]. Similar to ALK, ROS1 has been reported to define a genomic subset of NSCLC with distinct clinical characteristics [52] and, while the incidence of ROS1 fusions is less than 2 %, preclinical and clinical data suggest that patients whose tumors harbor these rearrangements may benefit from crizotinib therapy [53, 54]. RET kinase fusions have also recently emerged as promising molecular targets in NSCLC [55], where they have been reported to segregate from genetic

modifications in EGFR, KRAS, HER2 and ALK [56]. As the clinical significance of these oncogenic drivers in NSCLC becomes realized, it is reasonable to suggest that HSP90 inhibition may warrant evaluation as a potential point of therapeutic intervention.

15.3 Non-small Cell Lung Cancer: Combining the Modalities of HSP90 and Tyrosine Kinase Inhibition to Improve EGFR therapy

ALK targeting is a prime example of genetics dictating treatment in lung cancer, but what about more frequent genetic alterations that may be targeted through HSP90 blockade? Mutations in EGFR, an established client protein of HSP90, define one of the most prevalent and actionable subgroups of NSCLC. It is estimated that 10–15 % of NSCLCs in Caucasians and up to 30 % in Asian populations harbor activating mutations in EGFR [57]. Aberrant activation of this receptor stimulates a variety of oncogenic signaling cascades, including the JAK/STAT, RAS/RAF/ERK, and PI3K/AKT pathways [58]. EGFR activity may also become dysregulated during tumorigenesis by other mechanisms, including gene amplification and/or receptor overexpression [59]. Importantly, EGFR is a validated therapeutic target in NSCLC, with three small molecule kinase inhibitors (erlotinib, gefitinib, and afatinib) currently approved for the treatment of advanced disease [60]. Each of these TKIs show preferential clinical efficacy in NSCLC patients with EGFR mutant-bearing tumors, although durable responses are rare due to the development of acquired resistance, which typically arises through the acquisition of a second site mutation (T790M) within EGFR [61, 62], or via activation of compensatory signaling pathways that bypass the receptor and restore downstream oncogenic signaling [63]. Mutant EGFR oncoproteins are reliant on the chaperone activity of HSP90 for their conformational stability and function [35, 64], and there are abundant experimental data showing sensitivity of EGFR-driven NSCLC to multiple second-generation HSP90 inhibitors in preclinical studies [65–70]. In addition, it has recently been demonstrated that the mature, wild-type receptor is also a *bona fide* HSP90 client in cancers that overexpress wild-type EGFR [71], suggesting potential utility for targeted HSP90 inhibition beyond the mutant receptor phenotype. Despite this, the clinical experience with selective HSP90 inhibitors has revealed only modest single-agent activity in molecularly defined subsets of both wild-type and mutant EGFR NSCLC patients [22, 42].

To date, efforts to combine EGFR TKIs with standard chemotherapies have not been associated with survival benefits in clinical trials [29]. Recently, attention has focused on combining the modalities of HSP90 blockade with selective EGFR tyrosine kinase inhibition for optimizing NSCLC tumor responses to EGFR TKIs. Ganetespib can potentiate the efficacy of both erlotinib and afatinib in preclinical models of NSCLC driven by activating EGFR mutations, with

combination treatment inducing tumor regressions and a capacity to overcome erlotinib resistance [72]. Similar combinatorial benefit has been reported when erlotinib is combined with SNX-2112 [73] or a new investigational HSP90 inhibitor compound CH5164840 [74]. Moreover, encouraging signs of clinical activity dosing NVP-AUY922 in combination with erlotinib in patients with acquired resistance to EGFR inhibitors have been observed in an ongoing phase II trial (NCT01259089). In the first stage of that study, 2 of 16 patients demonstrated a partial response; both of which had the T790M mutation.[1] Taken together, these data suggest that HSP90 inhibition alongside EGFR kinase blockade may represent a potential therapeutic pathway for improving patient outcomes and overcoming TKI resistance in EGFR-mutant NSCLC.

15.4 Non-small Cell Lung Cancer: Targeted HSP90 Inhibition Alongside Taxane Therapy Improves Patient Outcomes

For most individuals with advanced and unresectable NSCLC, treatment options are limited to platinum-based two-drug regimens consisting of either cisplatin or carboplatin in combination with an additional 'third-generation' cytotoxic agent (paclitaxel, docetaxel, gemcitabine, vinorelabine, or pemetrexed) [25]. One strategy that has been employed in order to try and improve objective response rates for these patients is through the use of molecularly targeted agents, such as bevacizumab and cetuximab, in combination with front-line chemotherapeutics [28, 76]. Benefit from such therapy combinations is typically observed within subsets of NSCLC patients and correlates with specific tumor histology and/or molecular phenotypes. It is now established that HSP90 blockade can potentiate the activity of a wide variety of chemotherapeutic drugs [77] and, given the pleotropic effects of HSP90 inhibition across a range of NSCLC phenotypes, these considerations provide a strong rationale for evaluating novel HSP90 inhibitors alongside traditional chemotherapy in this malignancy.

An encouraging validation of this is provided by ganetespib improving therapeutic outcomes alongside taxane therapy in NSCLC. Paclitaxel and docetaxel comprise the taxane family of antimitotic agents widely used in the treatment of multiple human cancers. While active in NSCLC therapy, their effectiveness is often hampered by a variety of significant and dose-limiting adverse side effects in clinical practice. Synergistic combinatorial benefit between HSP90 inhibitors and taxanes has previously been described in different cancer models [78–82] suggesting that their non-overlapping but complementary mechanisms of action are conserved across tumor types. Combinations of ganetespib with both taxanes

[1] Johnson et al. [75]

showed synergistic activity and enhanced antitumor efficacy in preclinical models of NSCLC [83]. The mechanistic benefit afforded by the addition of ganetespib to taxane regimens was found to be multifactorial and included loss of pro-survival signaling, direct impacts on the cell cycle machinery, and exacerbation of mitotic catastrophe [83, 84]. In early clinical trials, ganetespib was shown to be well tolerated as monotherapy [20, 22], lacking the severe liver toxicities characteristic of the ansamycin class of inhibitors and also adverse visual disturbances that have emerged as an important clinical concern for some newer HSP90 inhibitor drugs [85]. Together with demonstrated activity in oncogene-driven subsets of NSCLC patients, these findings prompted evaluation of the cytotoxic sensitizing property of ganetespib alongside docetaxel in refractory NSCLC in a recently completed phase II (NCT01348126; GALAXY-1) and ongoing phase III study (NCT01798485; GALAXY-2).

A number of considerations support the selection of docetaxel as the optimal front-line taxane candidate in these trials. Docetaxel is the only agent that is approved for both first- and second-line therapy in advanced NSCLC [26] and was also the first drug to establish superior efficacy and tolerability over other third-generation agents when used in combination with platinum compounds [25]. Meta-analyses of current treatment regimens have shown that docetaxel is associated with better disease control than paclitaxel combinations and tumor histology does not exert any influence over drug activity or efficacy, as opposed to other third-generation cytotoxics [25]. Significantly, the GALAXY-1 trial is the first randomized study to show improvement in efficacy through addition of a targeted HSP90 inhibitor to chemotherapy in cancer patients.[2] The strongest signals of efficacy were noted improvements in ORR, PFS, and OS observed in a large subset of adenocarcinoma patients with chemosensitive disease. Accordingly, it is this population of NSCLC patients that is presently being enrolled in the ongoing confirmatory GALAXY-2 study. Importantly, the combination of ganetespib with docetaxel was well tolerated and not associated with any excessive toxicity over that seen with docetaxel treatment alone. Moreover, an interesting finding to emerge was that the time to disease progression due to emergence of new metastatic lesions was prolonged in individuals undergoing combination treatment. While this observation warrants confirmation in additional trials, it strongly suggests that HSP90 inhibitor treatment exerted a significant biological impact on the metastatic dissemination and growth in these patients. This provides important validation of a large body of preclinical evidence to support an essential role for HSP90 in regulating multiple facets of the metastatic process [87–89].

[2]Ramalingham et al. [86].

15.5 Breast Cancer: Client Proteins Dictate Sensitivity to HSP90 Inhibitors

The cellular and molecular heterogeneity exhibited by tumors of the breast is such that breast cancer is no longer considered a single disease with variable morphology, but instead a collection of distinct neoplastic disorders each associated with their own pathological features and clinical outcomes [90]. Gene expression profiling has resulted in the classification of human breast cancer into at least five subtypes based on discrete molecular signatures [91–93]. These include normal breast-like, the hormone receptor-positive (estrogen and progesterone receptors; ER and PR) luminal A and luminal B subtypes, HER2-positive, and basal-like. Within this stratification, triple-negative breast cancers (TNBC) are an orphan grouping of tumors characterized by an absence of ER, PR and HER2 expression that primarily fall within the basal-like subtype, however the two definitions are not strictly synonymous [94, 95]. Given this degree of complexity, it is now apparent that pharmacologic targeting of a single pathway or individual component of an oncogenic signal cascade typically fails to translate to long-term efficacy, particularly for metastatic disease. A number of established HSP90 clients have been implicated in the pathogenesis of breast tumors, including the ER and PR steroid hormone receptors, EGFR and HER2 receptor tyrosine kinases, and intermediates of oncogenic signaling cascades (AKT and RAF1) [9]. Accordingly, there is considerable preclinical support for potential therapeutic use of HSP90 inhibitors in breast cancer [96–102].

Similar to ALK-driven lung tumors, HER2-positive breast cancer provides another impressive illustration of HSP90 inhibitor efficacy in client protein-driven disease. HER2 is a highly sensitive HSP90 client protein and overexpression of this receptor defines a clinically relevant subset of breast cancers exquisitely dependent on oncogenic HER2 signaling for growth and survival. In recent years the prognosis for HER2-positive patients has improved following the introduction of selective HER2-targeted agents (such as trastuzumab and lapatinib) as first-line treatments in this disease [103]. Trastuzumab in combination with chemotherapy is the current standard of care for metastatic HER2-positive breast cancer [103]; however the invariable development of resistance presents a significant and unresolved clinical problem. A variety of mechanisms have been proposed to account for the trastuzumab-resistant phenotype, including activation of compensatory growth factor signaling pathways, amplification of the PI3K/AKT cascade, and expression of truncated HER2 receptors that lack the antibody binding epitope [104]. By virtue of its multifaceted mode of action, HSP90 inhibition has been shown to overcome each of these various mechanisms in laboratory models of trastuzumab-resistant breast cancer [98, 101]. Interestingly, recent analyses have suggested a benefit for continued trastuzumab treatment even beyond progression [105] and other selective HER inhibitors such as lapatinib have shown efficacy in patients that have become refractory to trastuzumab therapy [104]. Thus, trastuzumab-resistant tumors appear to remain oncogenically reliant on HER2; raising the possibility that HSP90

inhibition might afford an effective therapeutic approach to abrogate acquired resistance to primary anti-HER2 treatment. In this regard, proof-of-concept was provided in a pivotal phase II trial that demonstrated meaningful clinical efficacy could be achieved by combining 17-AAG with trastuzumab in HER2-positive metastatic breast patients who had previously progressed on trastuzumab [106].

Despite the positive outcome of that study, the clinical development of 17-AAG has been discontinued. Thus a therapeutic opportunity clearly exists within this patient group for an effective and tolerable alternate HSP90 inhibitor. A phase I study evaluating BIIB021, either alone or in combination with trastuzumab (NCT00412412), showed only modest antitumor activity for this purine-based HSP90 inhibitor in a cohort of heavily pre-treated metastatic HER2+ patients [107]. Additional second-generation HSP90 compounds, including clinically advanced agents such as NVP-AUY922 and ganetespib that show superior potency and safety over 17-AAG, also exhibit strong preclinical activity in models of HER2+ breast cancer [99, 108]. NVP-AUY922 has undergone clinical evaluation as monotherapy for locally advanced and metastatic HER2+ breast cancer as part of a completed phase II study expansion arm (NCT00526045) and also as part of combination therapy alongside trastuzumab in trastuzumab-refractory advanced HER2+ breast patients (NCT01271920), essentially reflecting the successful 17-AAG trial. Ganetespib has shown preliminary signs of single-agent activity within the advanced HER2+ breast cancer population in a phase II window-of-opportunity trial (NCT01677455; ENCHANT). While no final data from any of these studies are yet available, the outcomes are likely to be instrumental in guiding the application of HSP90 inhibitor use for this malignancy.

Beyond the HER2+ phenotype, HSP90 blockade may also be considered a logical targeted approach for hormone-responsive, luminal-type breast tumors due to its well-defined role in the chaperoning of steroid receptors, including ER and PR [109]. For patients with estrogen-dependent disease, adjuvant endocrine therapy with ER antagonists such as tamoxifen and aromatase inhibitors are effective first-line treatments [110]. Despite the obvious benefits of these standard-of-care agents for the vast majority of individuals diagnosed with breast cancer, the development of acquired resistance is common, ultimately resulting in disease relapse and death. The mechanisms that underlie hormone refractory phenotypes remain incompletely defined although crosstalk between the ER and growth factor receptor pathways (involving HSP90 clients like EGFR, HER2 and IGF-1R) have been implicated in the resistance process [111]. Interestingly, tamoxifen resistance may arise even as the tumors themselves remain ER positive [112]. Within this context, early reports evaluating ansamycin-based HSP90 inhibitors revealed that chaperone inhibition could overcome endocrine therapy resistance in tamoxifen- and aromatase-resistant breast cancer cell lines [102, 113]. These findings provide a rational framework for investigating the clinical potential of this modality as an alternate, ligand-independent mechanism for sustained degradation of ER/PR in hormone receptor-positive breast tumors. In this regard, the results of a completed phase II trial of BIIB021 in combination with exemestane (NCT01004081) in patients whose hormone receptor-positive cancer had progressed on prior aromatase inhibitor therapy

are eagerly awaited. Further, a randomized phase II trial evaluating the addition of ganetespib to the approved ER antagonist fulvestrant in hormone receptor-positive metastatic breast cancer is currently recruiting participants (NCT01560416).

15.6 Breast Cancer: Will HSP90 Inhibition Be Effective in Triple-Negative Tumors?

In contrast to HER2 or hormone receptor-positive breast cancer, TNBC represents a heterogeneous collection of orphan status tumors that lack a defining molecular vulnerability to serve as a druggable target. TNBC shows a disproportionate mortality amongst breast cancer subtypes and patients with TNBC have a higher likelihood of visceral metastatic disease and early relapse [94, 95]. An absence of reliable predictive biomarkers, combined with the disappointing efficacy of conventional chemotherapy, highlights an urgent need for alternate treatment options for these patients. A variety of potential biological drivers have been incompletely validated in TNBC. Many of these are established client proteins of HSP90, including EGFR, KIT and IGF-1R as well as critical mediators of the RAS/RAF/ERK, PI3K/AKT and mTOR tumorigenic signaling pathways [94]. Indeed, and contrary to an earlier notion that TNBC tumors may not be sufficiently responsive to targeted HSP90 blockade, there is now accumulating preclinical evidence that supports therapeutic sensitivity of triple-negative cancer cell lines to HSP90 inhibition, shown using the second- generation compounds ganetespib and PU-H71, as well as a new investigational agent PF-4942847 [84, 97, 100].

A distinct clinicopathological feature of TNBC is the hematogeneous spread of metastases, showing preferential dissemination to the lungs and brain rather than to bone or soft tissues [94]. Ganetespib treatment negatively impacts orthotopic tumor growth, invasion and distal lung metastasis in experimental metastasis models of TNBC [84, 89]. At a molecular level this has been attributed to, at least in part, potent drug-induced reductions in the expression and activity of HIF-1α (hypoxia-inducible factor 1α) and concomitant target genes that are linked to TNBC progression [89]. Significantly, compelling anecdotal evidence of metastatic tumor responses in TNBC patients undergoing ganetespib therapy has also been obtained in the clinical setting [84]. As shown in the CT scans presented in Fig. 15.3a, four cycles of ganetespib monotherapy resulted in discernible shrinkage of large metastatic lung lesions in a heavily pre-treated TNBC patient as part of a phase I trial (NCT00688116). This cancer was highly aggressive – at the time of enrollment, the patients' disease had progressed with multiple bilateral pulmonary, liver and bone metastases following six prior, different chemotherapeutic regimens. An overall objective response was not confirmed as the patient was taken off study at the end of cycle 5 due to the detection of brain metastases. Figure 15.3b shows CT scans from another individual currently enrolled in the ongoing ENCHANT monotherapy trial in breast cancer (NCT01677455). In 2011 the patient received six cycles of

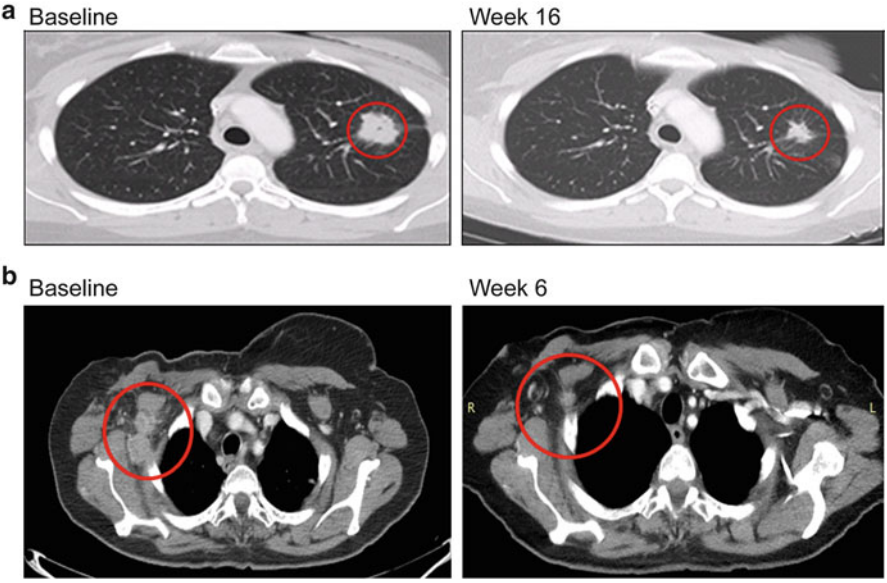

Fig. 15.3 Clinical activity of ganetespib in TNBC patients. (**a**) CT scans of a metastatic lung deposit taken prior to ganetespib treatment (*left panel*) and after 16 weeks on-therapy (*right panel*). *Circles* depict location and size of the tumor mass. (**b**) CT scans taken prior to ganetespib treatment and after 6 weeks on therapy. *Circles* depict the location and size of multiple axillary lymph node lesions (Images reproduced from [84])

adjuvant FEC chemotherapy (5-fluorouracil, epirubicin, and cyclophosphamide) but progressed with recurrent disease 2 years later, presenting with multiple axillary and supraclavical lymph node deposits and pulmonary metastases. Within 6 weeks of starting ganetespib treatment, the patient achieved a confirmed partial response, including the marked axillary lymph node tumor shrinkage shown in Fig. 15.3 and the patient remains on therapy at the present time. These robust clinical responses, observed in independent trials, suggest that the TNBC tumors were acutely reliant on the chaperoning function of HSP90, with one or more chaperone-dependent signaling pathways responsible for promoting metastatic growth and survival. Given the remarkable molecular heterogeneity of this collection of breast cancers, an ongoing clinical challenge remains to identify which specific client proteins may ultimately serve as predictive biomarkers for those individuals likely to respond to HSP90 inhibitor treatment.

Of note, basal-like breast (including TNBC) and ovarian cancers share striking similarities in terms of genomic and proteomic modifications [114]. The common findings of *MYC* amplification as well as *TP53*, *RB1* and *BRCA1* loss suggest that these represent may represent shared driving events for TNBC and ovarian carcinogenesis, and that common therapeutic approaches might be considered for both these diseases [114]. In addition, bioinformatic meta-analyses recently identified an HSP90-centric hub in ovarian cancer that was susceptible to inhibitor

treatment [115]. This finding was validated by the capacity of ganetespib to significantly reduce tumor growth and dissemination in xenograft models and spontaneous ovarian tumors in transgenic mice [115]. Of particular relevance, combining ganetespib with paclitaxel strongly augmented the antitumor efficacy of either agent alone – a result that supports a promising rationale for combination ganetespib and paclitaxel regimens presently being evaluated in two trials in recurrent and metastatic ovarian cancer (NCT01962948; NCT02012192). Further, combination ganetespib plus paclitaxel therapy is undergoing clinical assessment in women with locally advanced breast cancer as part of the ongoing I-SPY2 adaptive phase II study (NCT01042379).

15.7 Melanoma: Disrupting Oncogene Addiction and Overcoming Resistance

Cutaneous melanoma ranks among the most aggressive and treatment-resistant human cancers, and the worldwide incidence of this disease continues to increase [116]. Mutational activation of BRAF, resulting in dysregulation of the canonical MAPK (RAF/MEK/ERK) signaling cascade, is characteristic of over half of all malignant melanomas [117, 118]. This high frequency underscores a critical role for mutant BRAF activity in melanoma oncogenesis [119]. In this regard, mutated BRAF (most commonly $BRAF^{V600E}$) also provides an actionable target for molecular therapeutic approaches as evidenced by the recent approval of the first highly selective $BRAF^{V600E}$ inhibitor, vemurafenib, for patients with metastatic melanoma [120]. The conformational stability of mutant BRAF is reliant on the activity of HSP90 [121], the chaperoning function of which thus likely facilitates oncogene addiction in this malignancy. In support of this premise, a number of synthetic small molecule HSP90 inhibitors have shown robust activity in mutant BRAF-driven melanoma cell lines in vitro and in vivo, including NVP-AUY922, SNX-2112, and NVP-BEP800 [122–124]. Extending these observations, ganetespib exhibits superior potency and efficacy compared to vemurafenib in mutant BRAF–driven melanoma models, with drug exposure inducing the simultaneous destabilization of $BRAF^{V600E}$ as well as CRAF, AKT, and RAF/MEK/ERK signaling that is stimulated by mutant BRAF activation [125, 126]. These preclinical findings suggest that targeting the chaperone function of HSP90 represents a rational approach for intervention in mutant BRAF-driven melanoma. Initial clinical studies evaluating the first-generation compound 17-AAG in metastatic melanoma were disappointing, with no objective responses observed [127, 128]. A lack of durable target suppression and pharmacological limitations of the ansamycin inhibitor itself likely contributed to the absence of clinical activity in these trials; however this does not preclude the potential for newer agents with improved potency, durable activity, and favorable tolerability in this malignancy [129].

Indeed, emerging data strongly suggest that combining the modalities of HSP90 inhibition with either selective BRAF or MEK targeting warrants further investigation in melanoma displaying oncogenic addiction to mutated BRAF, particularly as a means to overcome mechanisms of resistance to targeted BRAF agents. Acquired resistance to vemurafenib represents a significant clinical obstacle to its long-term efficacy, and most patients relapse with drug-resistant disease within 6–8 months [130]. Unlike the case for EGFR or EML4-ALK described above, to date there is no evidence of secondary mutations in BRAF to account for a resistant phenotype. Instead, a variety of mechanisms have been identified that allow for either bypass or reactivation of MAPK signaling [119, 131, 132]. Despite the complexity of the signaling intermediates involved that lead to therapeutic escape from BRAF inhibitor treatment, all appear to converge on pathways that are sensitive to HSP90 inhibition. Both XL888, a tropane-derived small molecule HSP90 inhibitor whose clinical development has been halted by the sponsor [133], and ganetespib can overcome a diverse array of intrinsic and acquired vemurafenib resistance mechanisms in relevant melanoma models [125, 134]. NMS-E973, a new investigational inhibitor compound, has also shown activity in models of vemurafenib resistance, including inhibiting the growth of intracranially implanted melanoma xenografts in mice [135].

Given the exquisite dependence on MAPK signaling for both melanoma viability and drug resistance, pharmacological inhibition of MEK has also emerged as an important strategy for therapeutic intervention in mutant BRAF-driven melanoma [136]. While the role of MEK inhibitor monotherapy, given the advent of approved BRAF-targeted agents, remains to be determined [130] a number of combination trials investigating the dual blockade of mutant BRAF and MEK are currently underway, and early evidence suggests that this strategy may represent an effective approach to prevent or delay the onset of resistance due to MAPK reactivation [137, 138]. Within the resistance setting, we have shown that the combination of ganetespib with the allosteric MEK inhibitor TAK-733 provides superior cytotoxic activity and inhibition of MAPK reactivation compared to dual vemurafenib plus TAK733 treatment in melanoma cell lines. Moreover, these effects were recapitulated in vemurafenib-resistant tumors in vivo, where combination treatment induced significant tumor regressions [125]. Such data reinforce the potential utility of HSP90 inhibition as an alternative, and potentially complementary, strategy for treating tumors with acquired resistance to BRAF inhibitors.

15.8 Gastrointestinal Stromal Tumors and Prostate Cancer: All Clients Are Not Created Equal

ALK-rearranged NSCLC and HER2-positive breast cancer have provided the clearest demonstrations of clinical efficacy in HSP90 client oncoprotein-addicted cancers – however, not all tumor types that appear to meet the same criteria have

displayed similar sensitivity to this therapeutic approach. For example, GIST, the most common type of soft-tissue sarcoma, is a malignancy that may be considered particularly amenable to HSP90 inhibitor-directed therapy due to the high frequency (85 %) of driver KIT mutations [139, 140]. To date, however, the results of a phase I study evaluating IPI-504 in patients with metastatic and/or unresectable GIST and other soft-tissue sarcomas revealed that only 1 of 37 GIST patients exhibited a confirmed partial response; moreover genotyping from this individual failed to identify any KIT mutation present [141]. A subsequent placebo-controlled phase III trial in relapsed/refractory GIST (NCT00688766) was prematurely terminated due an excessive mortality rate in the IPI-504 arm, with treatment-related deaths resulting from liver failure, metabolic acidosis, renal failure, and cardiopulmonary arrest [142]. No results are yet available from an ongoing study of NVP-AUY922 monotherapy in this population (NCT01404650). While its clinical development has now been discontinued, BIIB021 underwent evaluation in a small cohort of refractory GIST patients [143] where metabolic partial responses were observed in 22 % of cases, although no RECIST-defined partial responses were achieved. This study raised the possibility that a lack of durable client protein suppression underscored such modest responses to HSP90 inhibitor monotherapy. Pharmacodynamic assessment showed that significant rebounds in FDG activity were seen in patients between doses, suggesting that although target inhibition was being achieved, it was not sustained. In agreement with this premise, prolonged inhibition of KIT or its downstream pathways has not been observed following ganetespib exposure in either preclinical GIST models or patient biopsies, and consequently only limited efficacy was obtained in a phase II trial evaluating weekly administration of ganetespib to GIST patients (NCT01039519).[3]

A number of considerations have also made prostate cancer an attractive disease indication for HSP90 targeting. Androgen ablation therapy is the foundation of current treatment for patients with locally advanced or metastatic disease and, importantly, it is now clear that advanced and recurrent tumors continue to rely on androgen receptor (AR) signaling, even in the castrate environment [145, 146]. The AR is an established HSP90 client, and the relationship between the chaperoning function of HSP90 with steroid receptor stability, conformation and modulation of ligand binding is well characterized [147]. Accordingly, a number of preclinical studies have provided experimental support for the potential utility of HSP90 inhibitors in prostate cancer [148–152]. Unfortunately, the clinical experience using such compounds in the single-agent setting has not met expectations, with minimal effects on PSA (prostate-specific antigen) levels or tumor burden being observed along with unacceptable toxicities [153, 154]. For these tumors, it appears that combining targeted HSP90 inhibitors alongside other AR antagonists or chemotherapeutics may represent a more feasible path to clinical efficacy. In addition, radiation therapy displays high control rates for low-risk, localized prostatic disease [155]

[3]Demetri et al. [144].

and there is emerging evidence to suggest that the radiosensitizing effects of HSP90 blockade may be exploitable as a valid adjunct to radiotherapy in this disease [156].

15.9 Colorectal Cancer: A Potential Chemosensitizing Role for HSP90 Inhibitors?

The clinical management of colorectal cancer (CRC) is largely dictated by the stage of the disease. Patients who present with early stage, localized tumors are amenable to curative resection surgery while adjuvant chemotherapy is indicated for patients with Stage III disease or higher, with the aim of improving survival and preventing recurrence [157]. Over the last decade, the addition of targeted biological agents (such as such as the anti-VEGF antibody bevacizumab and the EGFR antagonists cetuximab and panitumumab) to existing chemotherapeutic regimens has effectively doubled the survival outlook for patients with metastatic CRC [158, 159]. This type of approach clearly serves as an encouraging paradigm for providing continuing improvements for the treatment of advanced CRC.

Although there is a considerable literature showing robust activity of HSP90 inhibitors (both first- and second-generation) in preclinical models of CRC [160–163], meaningful responses to HSP90 inhibitor monotherapy within the clinical setting have proven far more modest. As a prime example, the most significant demonstration of efficacy as part of the initial phase I evaluation of ganetespib in solid malignancies involved a patient with metastatic CRC, who achieved a partial response while on-therapy [20]. In a subsequent phase II trial in heavily treated metastatic CRC patients (NCT01111838) no objective responses were observed, although 2/15 evaluable patients achieved durable stable disease. In general terms, this malignancy might not be expected to be highly responsive to targeted HSP90 therapeutic intervention since the proteins that show the highest frequency of alteration as part of colorectal tumorigenesis, APC and KRAS, are themselves not HSP90 clients. However, similar to what is observed following mutation of BRAF in melanoma described above, the canonical MAPK kinase cascade is the primary mitogenic pathway aberrantly stimulated by KRAS under pathological conditions [164] and this signaling axis is particularly sensitive to HSP90 modulation. In addition, mutations in BRAF have also been described in approximately 5–15 % of cases of CRC [165]. An important lesson gleaned from integrating molecularly targeted agents into existing therapies for CRC was that each of the biologics displayed minimal clinical activity as single agents and their full benefit was only realized when they were combined with standard treatment regimens [158]. It is reasonable to suggest then that this profile may be similar for any future application of selective HSP90 inhibitors in CRC.

For five decades, 5-Fluorouracil (5-FU) has played an indispensable role in CRC treatment, both in the curative and palliative settings and constitutes the backbone of the FOLFOX (5-FU/leucovorin (LV) plus oxaliplatin) and FOLFIRI

(5-FU/LV plus irinotecan) combination therapies that represent the standard first line cytotoxic regimens for metastatic CRC patients. Due to a short half-life and variations in bioavailability, 5-FU requires intravenous infusion – consequently the first oral prodrug formulation, capecitabine, has more recently received approval for adjuvant monotherapy use [166]. A synergistic interaction between 5-FU and NVP-AUY922 in vitro has been reported in bladder cancer cell lines; no correlative translation into in vivo efficacy was shown in that study [167]. In preclinical CRC models, ganetespib exerts robust antitumor and antiangiogenic activity, through the simultaneous destabilization of multiple key growth and survival pathways, perturbation of cell cycle regulation, and disruption of HIF-1α and STAT3 signaling [168, 169]. Perhaps most importantly, ganetespib co-treatment can significantly improve the efficacy of fluoropyrimidine therapy in CRC xenografts [168]. Using an inherently 5-FU-insensitive HCT116 xenograft model, capecitabine administered on a clinically relevant dosing schedule suppressed tumor growth by over half. Single-agent ganetespib treatment showed a similar, modest degree of tumor growth inhibition, comparable to what has previously been reported for 17-DMAG [163]. However, a combination regimen resulted in over 50 % tumor regression [168]. This represents the first demonstration of combinatorial benefit between a small molecule HSP90 inhibitor and fluoropyrimidine therapy in CRC-derived tumors. Moreover, these data provide a clear rationale for exploiting both the chemosensitizing activity as well as the capacity to overcome intrinsic fluoropyrimidine resistance that was conferred by the addition of a potent HSP90 inhibitor. In light of these considerations, a phase I trial evaluating ganetespib in combination with capecitabine and radiation in rectal cancer [NCT01554969] has been initiated.

15.10 Future Considerations

Since the initial clinical trials of the natural product-derived HSP90 inhibitors commenced only a decade ago, enormous progress has been made in the discovery and characterization of next-generation synthetic compounds with improved pharmaceutical and tolerability profiles. As outlined in this chapter, continued exploration of biologically informed drug combinations are likely to provide the most direct route to efficacious use of these agents for cancer treatment in the near term. Indeed, it is now possible to envision the eventual application of potent and safe HSP90 inhibitors as adjuncts to both chemotherapy and molecular-targeted strategies for a broad range of human malignancies. However a number of key considerations remain as obstacles on the path to widespread clinical use. Perhaps the most significant factor required for success involves the identification of specific predictive biomarkers within cancer types to stratify those patients most likely to receive benefit from therapeutic HSP90 blockade. In many ways, the pleiotropic effects of HSP90 inhibition represent a double-edged sword – such a wide and diverse range of clients represents an inherent therapeutic advantage, yet at the same time has emerged as a challenge for predicting individual response. Implicit

to this endeavor will be a more complete understanding of the relationships that exist between HSP90 and its oncoprotein clients and predicting what makes a client dependent on the chaperoning of HSP90. For example, it was recently discovered that 30 % of the various EML4-ALK gene rearrangement structures observed in NSCLC patients were insensitive to HSP90 blockade in preclinical studies [170], a finding with clear translational relevance to the application of HSP90 inhibitors in tumors driven by these client proteins. Further, the tumor retention characteristic of targeted HSP90 agents implies that predictive assays of drug sensitivity and pharmacodynamic profiling are likely to require assessment of client protein modulation within tumors themselves, since surrogate tissues are unlikely to reflect true effects of these drugs. Taking into consideration both the multimodal antitumor activity and rapid clearance of small molecule inhibitors from normal tissues and the blood compartment, traditional serum pharmacokinetics may offer only limited guidance for therapeutic dosing of these particular compounds [171]. A number of alternative and non-invasive methods are in development to circumvent this issue, including functional imaging using radiolabeled HSP90 inhibitors such as ^{124}I-PU-H71 [23].

In addition, tumor cells (even within the same cancer type) may display variable degrees of intrinsic 'resistance' to HSP90 inhibitory compounds. Pharmacological blockade of HSP90 elicits induction of the heat shock factor-1 (HSF1)-directed heat shock response [172], providing a compensatory mechanism which may mitigate sensitivity to targeted HSP90 inhibition. This cellular stress response is characterized by up-regulation of the inducible molecular chaperone heat shock protein 70 (HSP70). Indeed, elevated HSP70 expression is commonly used in the clinical setting as a pharmacodynamic biomarker for HSP90 blockade [173, 174]. However, in line with its cytoprotective cellular roles, HSP70 possesses strong antiapoptotic activity and experimental evidence has shown that silencing this protein dramatically increases tumor cell sensitivity to selective HSP90 inhibitors [175]. Similarly, knockdown of HSF1 increases tumor cell susceptibility to HSP90 inhibitor treatment [176]. Of interest, in addition to its master regulatory role in orchestrating the heat shock response, HSF1 also drives specific transcriptional programs that maintain the malignant phenotype [177, 178]. Hence, it has become evident that concomitant suppression of the heat shock response represents a potential method for optimizing the full therapeutic potential of HSP90 inhibitors [179].

In an earlier study, Zaarur and colleagues screened a chemical library, in part through evaluation of inducible HSP70 expression, and identified a group of structurally similar benzylisoquinoline alkaloid compounds that could inhibit the stress response and consequently sensitize tumor cells to the cytotoxic effects both HSP90 and proteasome inhibitors [180]. Using a similar experimental approach, we recently uncovered a clinically feasible strategy to overcome this limitation. By performing an immunoassay screen of over 300 late-stage or approved drugs, a number of relevant compounds were identified that effectively blocked HSP70 up-regulation in response to ganetespib treatment [181]. This proof-of-concept study found that targeted inhibitors of the PI3K/mTOR signaling axis could attenuate the

HSF1-driven cellular heat shock response at both the genomic and proteomic levels and, importantly, this finding was validated by the capacity of selective mTOR or dual PI3K/mTOR agents to potentiate the antitumor efficacy of ganetespib in multiple in vivo xenograft models [181]. Importantly, these observations provide a molecular framework for novel combinatorial strategies that add PI3K/mTOR inhibitors to exploratory HSP90 inhibitor-based treatment regimens. In this regard, a number of selective mTOR inhibitor drugs are already approved for a variety of solid malignancies and other investigational agents are undergoing late-stage clinical evaluation [182, 183]. The results of two ongoing human trials evaluating combinations of IPI-504 with everolimus in mutant KRAS-driven NSCLC and NVP-AUY922 with the novel PI3K inhibitor BYL719 in metastatic gastric cancer (NCT01427946 and NCT01613950, respectively) are therefore likely to be informative as to clinical applicability of this approach.

Finally, there is now unequivocal evidence to suggest that tumor-specific drug metabolism may also serve as a primary determinant of 'resistance' to particular chemical classes of HSP90 inhibitors. It has long been established that the efficacy of the benzoquinone ansamycin inhibitors is related to levels of NAD(P)H dehydrogenase quinone 1 (NQO1), an enzyme required to catalyze the reduction of these compounds to a more active state [184]. For the newer chemical classes of HSP90 inhibitors, however, the possible influence of metabolism underlying diminished cellular activity has thus far received less attention. Of note, two recent studies have uncovered differential sensitivities exhibited by tumor lines to the resorcinol-based inhibitors NVP-AUY922 and ganetespib [185, 186]. Demonstrated for both bladder and CRC-derived lines, cell fate following inhibitor exposure varied according to the chemical class of inhibitor used. While retaining full sensitivity to ansamycin inhibitors, a number of lines of each tumor type were found to be largely resistant to ganetespib treatment or exposure to NVP-AUY922. Both of these resorcinol-containing compounds are primarily metabolized by the UGT1A family of UDP-glucuronosyltransferase enzymes [122, 185], the primary catalysts of glucuronidation reactions in multiple human tissues [187]. Mechanistically, it was determined that rapid metabolism (via glucuronidation) of ganetespib within bladder cells expressing high basal levels of UGT1A enzyme expression was sufficient to account for the lack of HSP90 inhibitory activity [185]. Moreover, targeted knockdown of UGT1A in high-expressing bladder and colorectal lines could sensitize previously resistant cells to HSP90 blockade by ganetespib. This correlation between UGT1A expression and resorcinol-inhibitor resistance suggests that UGT1A detection in tumor biopsy specimens might ultimately allow the development of a specific biomarker with direct translational relevance for the clinical evaluation HSP90-based strategies for this chemical class of inhibitor. Equally, this consideration is likely to be important for the informed application of resorcinol HSP90 drugs for the treatment of neoplasms that arise from tissues known to express the greatest abundance and array of UGT enzymes, including the gastrointestinal (liver, stomach, small intestine, colon) and urinary (kidneys, bladder) tracts [188].

15.11 Conclusion

By exploiting unique characteristics of HSP90 biology and pharmacology, considerable progress has been made in the development of selective inhibitors of this chaperone for cancer therapy. Indeed, lessons gleaned from characterization of both first- and second-generation classes of targeted HSP90 compounds have now validated inhibitor-based intervention as a promising therapeutic strategy for a wide variety of human malignancies. The clinical experience has identified potential frameworks for realizing the translational potential of this group of anticancer agents, particularly as chemotherapeutic or molecularly targeted agent sensitizers with substantial capacity to promote superior antitumor efficacy, overcome resistance mechanisms, and reduce treatment-related toxicities. Small molecule modulators of HSP90 thus stand at a critical stage of development and, while a number of challenges remain to be overcome, this collection of antineoplastic agents appears poised to achieve their full therapeutic promise in the application of novel cancer management strategies.

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Chapter 16
Molecular Survival Strategies of Organisms: HSP and Small Molecules for Diagnostics and Drug Development

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Abstract Nature has developed evolutionary strategies to synthesize the vast number of what are called secondary metabolites or in short natural products. These include molecules that are able to inhibit heat shock proteins (HSP90). A prominent group of natural products are polyketides whose molecular cores are biosynthesized by the polyketide synthase (PKS) which is a sophisticated catalytic machinery which evolved through evolutionary processes. Manipulation of the PKS based on genetic engineering allows utilizing the biosynthetic machinery in combination with chemically synthesized unnatural building blocks and the initiation of what may be called a chemosynthetic evolution with the aim to obtain new derivatives based on the parental natural product. This review illuminates the progress in the development of such chemoenzymatic approaches exemplified for geldanamycin and how new analogues show promising and improved inhibitory properties towards HSP90 and hence cancer cell growth. Based on the understanding of the molecular interaction of new inhibitors with their target, we discuss methods to redesign target-oriented natural products as well as reasons why susceptibilities towards HSP differ.

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Moreover, the development of novel screening methods to identify new HSP90 inhibitors and the investigation of their physical properties e.g. by using AFM are presented. This report sheds light on different aspects of targeting HSP90 with geldanamycin as lead structure, a prerequisite to move on towards preclinical studies for *in vitro* or *in vivo* fighting of cancer cells.

Keywords Heat shock proteins • Atomic force microscopy • Mechanochemistry • N-acetyltransferase • Polyketide synthase • Protein microarray • Target-oriented

Abbreviations

17AAG	17- Demethoxy- 17- (2- propenylamino)-geldanamycin (tanespimycin)
ACP	Acyl carrier protein
AFM	Atomic force microscopy
AHBA	3-Amino-5-hydroxybenzoic acid
ALPHA	Amplified Luminescent Proximity Homogeneous Assay
AT	Acyl transferase
ATP	Adenosine triphosphate
CE	Capillary electrophoresis
Cy3	2-((1E,3E)-3-(1-(5-Carboxypentyl)-3,3-diMethylindolin-2-ylidene)prop-1-enyl)-1-ethyl-3,3-diMethyl-3H-indolium
DEBS	6-Deoxyerythronolide B synthase
EGCG	(−)-Epigallocatechin-3-gallate
FITC	Fluorescein isothiocyanate
Gda	Geldanamycin
HSC	Heat shock cognate
HSP	Heat shock protein
HtpG	High temperature protein G
IC50	Inhibitory concentration at 50 %
ITC	Isothermal titration calorimetry
KR	Ketoreductase, KS, ketosynthase
MST	Microscale thermophoresis
NAT	N-acetyltransferase
PDB	Protein data bank
PKS	Polyketide synthase
SEM	Scanning electron microscopy
SPR	Surface plasmon resonance
TIRF	Total internal resection fluorescence
TRAP	Tumor necrosis factor type 1 receptor-associated protein

16.1 Introduction

HSP90 is an essential component of the chaperone machinery enabling ATP-dependent post-translational protein folding [1]. This process is present in both lower and higher eukaryotes, as well as in bacteria [2]. Noxious environmental conditions, including heat, UV radiation, and oxygen deficiency, the presence of ethanol or heavy metals, but also cellular rearrangement during development elicit unfolded proteins associated with loss of function [3, 4]. To preserve resources, heat shock proteins restore the functionality of proteins as a rescue pathway of many damaged or unfolded client proteins without the need for *de novo* synthesis of the affected proteins [5]. This closely correlates to elevated intracellular HSP90 levels in cells and one can strongly assume that HSP90 serves as a cellular stress marker. Many diseases arising from folding defects are known including different forms of cancer, but also Alzheimer's and Parkinson's diseases, cystic fibrosis, retinitis pigmentosa or Huntington's chorea and other [6, 7].

This cellular key role of HSP90 was first observed in Drosophila [8, 9]. Later it was shown that heat shock proteins can be targeted by microbial secondary metabolites also known as HSP inhibitors. These may belong to a wider pattern of molecular concepts in the evolutionary battlefield. One strategy for the development of natural products is the biosynthesis via secondary pathways using a specific arsenal of enzymes. These enzymes are exposed to a high permutation rate so that a broad range of secondary metabolites with inhibitory properties can be synthesized. These metabolites exert a high selectivity for biological targets with the subsequent advantage to propagate the natural producer. Similar strategies are found for snake, snail and microbial toxins [10–12]. The group of conus snails produces complex venoms with 7–35 amino acids in length. Around 100 different venom peptides can be found in a single producer and remarkably each peptide can be modified post-translational to enhance diversity and hinder adaptation processes [13]. Due to the high selectivity of those toxins, the corresponding drug targets are of main interest for various therapies [14].

In 1970, it was reported that the soil dwelling actinomycetes *Streptomycetes hygroscopicus* produces the natural product geldanamycin (Gda), a benzoquinone ansamycin antibiotic, which was first described to have a high potency against mouse leukemia (L1210) and oral carcinoma (KB) cells (Fig. 16.1).

Later it was shown that HSP90, an intracellular protein with the molecular weight of 90 kDa, is the target of Gda, which competes with ATP for the N-terminal pocket of HSP90 and thereby inhibiting the ATPase activity of HSP90 at very low concentrations. As a result, the recycling of unfolded proteins, which occur due to elevated stress in cancer cells, is suppressed. In the past a "natural weapon" library has been assembled with Gda and related benzoquinone and ansamycin derivatives of fungal-derived HSP90 inhibitors (e.g. hypothemycin, radicicol, zearalenone, and structurally related monocillins and pochonins) being key members. They address HSP90 as a target and use HSP90 as an Achilles' heel. The toxicity of Gda elicited in target plant, bacteria or human cells does not exist in the Gda producer cells

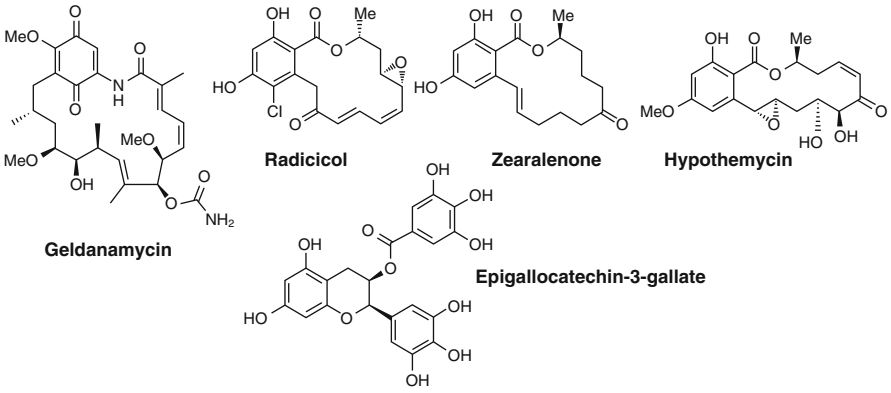

Fig. 16.1 Structures of important natural products that inhibit HSP90 and have potential in anticancer therapy

of *S. hygroscopicus*. This absence of susceptibility is due to certain amino acid exchanges in the N-terminal ATP binding domain of the bacterial HSP90 analogue HtpG. This resistivity of HtpG demonstrates that permutations of a few amino acids enable adaptation of heat shock proteins to HSP90 inhibitors. An interesting effect for plant metabolites on humans was reported for green tea, which produces (−)-epigallocatechin-3-gallate (EGCG) (Fig. 16.1). It targets HSP90 and shows cancer protective properties [15–17]. Traditionally, green tea was used a long time before this molecular knowledge was at hand. Primarily, this HSP90 inhibitor may be a result from the rhizosphere battlefield of the plant with organisms like insects, bacteria, fungi and others, but due to cultivation by men and consequently reduced toxicity this knowledge gains new significance for human health [18].

Many natural products including HSP90 inhibitors and others are biosynthesized by polyketide pathways that are found in bacteria, fungi and plants. Polyketide synthases (PKSs) are megaenzyme complexes that use acyl CoA building blocks as precursors to produce many clinically important drugs such as the tetracyclines and anthracyclines as well as erythromycin, rapamycin and lovastatin (Fig. 16.2). In bacteria three types of PKS are known, namely (a) the non-iterative type I PKS, (b) the iterative type II PKS, which are both multifunctional megaenzyme complexes, and (c) the acyl carrier protein-independent homodimeric type III PKS, also known as chalcone synthase-like PKS. Despite structural and mechanistic differences, all types of PKS biosynthesize polyketides by sequential decarboxylative condensations of the acyl CoA precursors. The ketoacyl synthase (KS) domain (for type I PKSs) or subunit (for type II and III PKSs) catalyzes the C–C bond-forming step [19].

Recently structural details of a polyketide synthase module were unraveled by cryo-EM and 3D reconstructions [20, 21] and earlier by Tang et al. [22] on the KS-AT didomain from the DEBS (6-Deoxyerythronolide B synthase) module. For this PKS type I from *Streptomyces venezuelae* it was demonstrated that the dimeric PKS

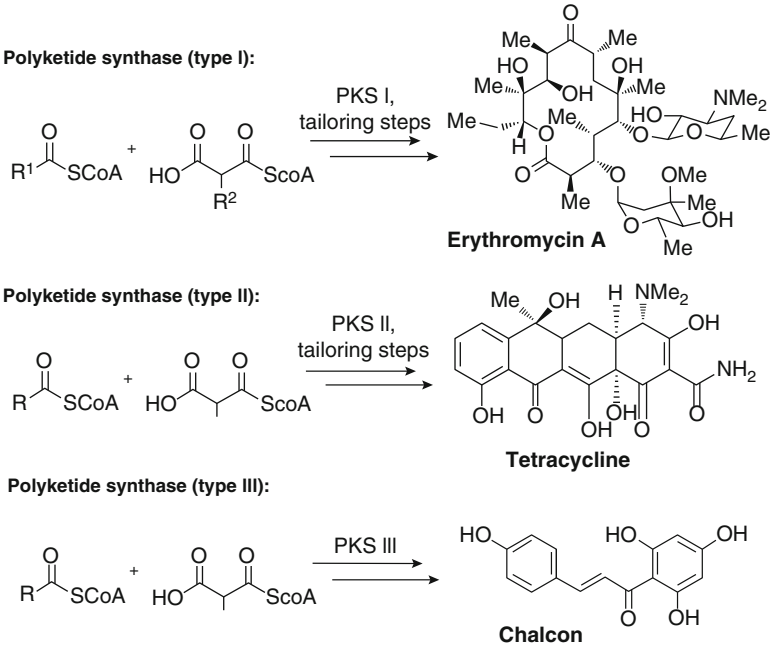

Fig. 16.2 Simplified overview on the three types of polyketide synthases and representative natural product examples

module with 328 kDa consists of a homodimeric ketosynthase (KS5) domain on the top which forms a single reaction chamber with acyltransferase (AT_5) and two reductases (KR_5) on each side and which is closed by a single acyl carrier protein (ACP) domain. This set-up enables successive two carbon extensions in a linear fashion and keto-group processing reactions on intermediates covalently tethered to carrier domains (Fig. 16.3).

One major goal over the last years was the identification of the corresponding genes of type I PKS, since understanding the structure and function of these extraordinary multienzyme complexes allows the manipulation and engineering of biosynthetic pathways with aim to generate novel natural product-based compound libraries. Standard recombinant DNA technology with genes that encode for PKS enzymes allowed to manipulate known PKS gene clusters either to produce the natural product in higher yields in the natural producer strain or heterologously expressed in host strains that otherwise do not produce polyketides. The manipulation of biosynthetic pathways, e.g. of PKS I, provides new synthetic opportunities. A combination of manipulated biosynthesis with chemical synthesis (see also Fig. 16.4) can provide new polyketide derivatives with improved or different biological properties that cannot be easily accessed either by chemo- (CHEM, Fig. 16.4) or biosynthetic (BIO, Fig. 16.4) approaches alone [23]. Today, it is possible to shape or predesign a binding profile of an ideal HSP inhibitor candidate using several tools

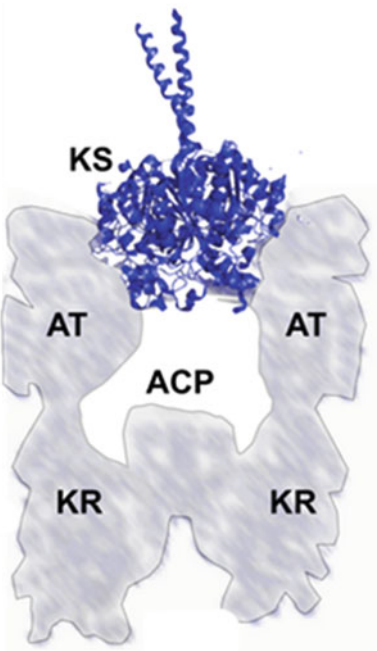

Fig. 16.3 Cartoon of the holo ACP model. The DEBS ACP structure is fitted into the pikromycin PKS module 5 from *Streptomyces venezuelae* according to the recently published structure by Dutta et al. [20] bearing the KS_5-AT_5-KR_5-ACP_5 domain architecture, with acyl carrier protein (ACP), acyltransferase (AT), ketosynthase (KS) and ketoreductase (KR) using pdb code 2HG4 for KS

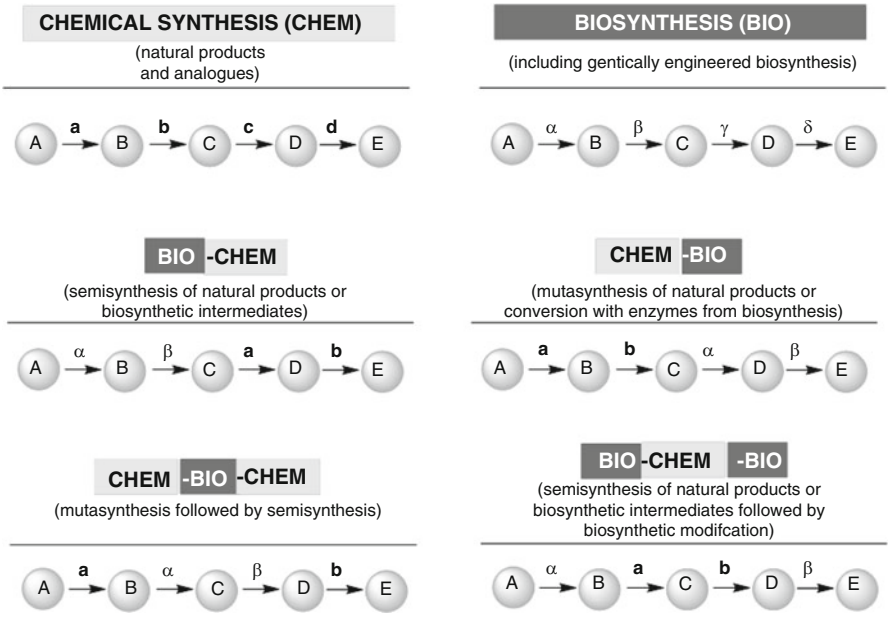

Fig. 16.4 Overview on chemo- and biosynthetic approaches towards natural products and derivatives (a–d = reagents or catalysts; α–δ = enzymes)

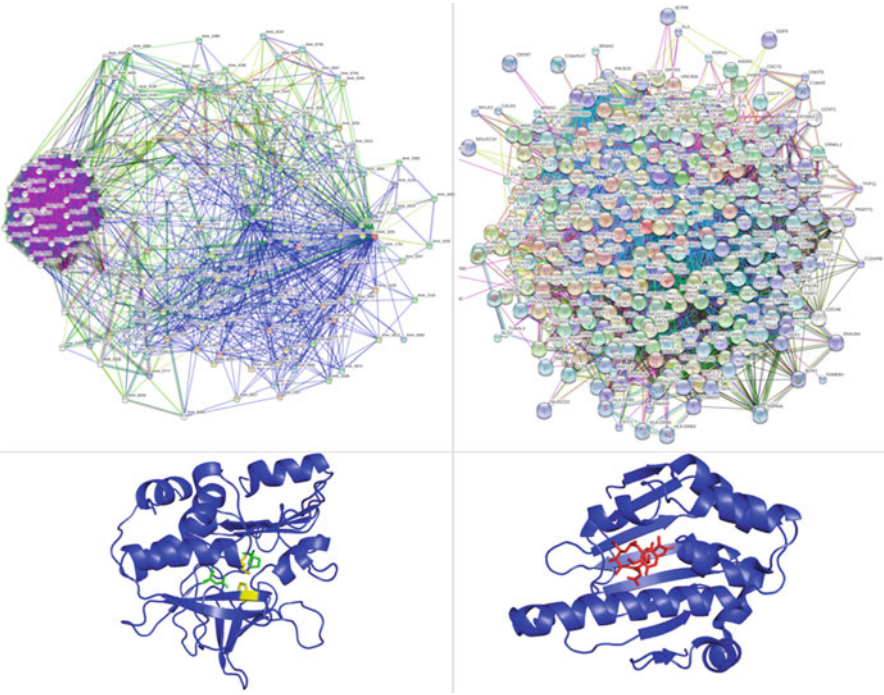

Fig. 16.5 Comparative interactome of two superfamily clusters of HSP inhibitor donor/acceptor systems. Lower panel (*left*): A key enzyme of the geldanamycin producer is the N-acetyltransferase from *S. hygroscopicus* and on the acceptor site (*right*) is the N-terminus of human HSP90 (pdb 3T0Z). The N-acetyltransferase enzyme was modeled by SWISS model using 64 homologues templates. Upper panel: The interactome of N-acetyltransferase; NAT (*left*) and hHSP90 (*right*) using String 9.1 software [25] with NAT (acc. AAO06919) and hHSP90 (acc. NP_001017963). Marbles or knots indicate interaction partners, and lines connections

including molecular modeling, isothermal titration calorimetry (ITC) for estimating the binding energies and atomic/single force molecule spectroscopy (AFM) [24].

Nature has established and evolutionary improved this complex machinery over a period of millions of years (Fig. 16.5). N-acetyltransferases are ubiquitous and are found in bacteria as well mammalian cells where they play different roles. In *S. hygroscopicus* N-acetyltransferase is one representative of a group of tailoring enzymes that finalize the biosynthesis of geldanamycin. There, it catalyzes the formation of the macrolactam ring. Similar transformations are found for secondary metabolites present in other bacteria or fungi while in mammalian cells N-acyltransferases have completely other functions and use completely other substrates. Therefore the interactome indicates that N-acyltransferases are linked to many other types of proteins due to homology or for direct interaction. Clearly, proteins are also under evolutionary pressure as is exemplified in the following for two very different proteins that are associated with geldanamycin: On one

hand the biological target HSP90 is a result of evolutionary optimization and so is the biosynthetic machinery such as the PKS type I as well as the key tailoring enzyme, the N-acyltransferase. HSPs are young on the evolutionary time scale but the molecular adaptations are not known yet. Although the enzymes are autonomous for each cell, molecular changes in both can have profound consequences for the survival of the producer/donor or acceptor site (HSP90, Fig. 16.5).

The evolutionary process required two important modifications in order to facilitate the production of Gda in *S. hygroscopicus*. First, the donator enzymes have to be permutated to enable the macrolactam ring formation and secondly the acceptors have to be modified to hinder their susceptibility to self produced toxic Gdas.

One may raise the question whether substantial synthetic modifications of a natural product lead structure (Fig. 16.6) can lead to an evolutionary process that is directed into a new direction. However it remains arguable if we will be able to generate better drugs by using the natural stamped way for anti HSP compounds. It becomes even more complicated since the recently drafted map of the human proteome identified a complex landscape of heat shock protein expression in different fetal and adult cells and tissues. For all contributing tissues and cells from fetal and adult it was shown that HSP90 is present but occurs in a low abundance together with other HSP types (Fig. 16.6), whereas in few tissues such as fetal ovary, liver, adult adrenal gland and placenta the expression rates exhibit highest level [26, 27]. This poses the question if HSP90 is the correct target for all cancer traits and which molecular fine-tuning is required to generate cancer specificity.

In this article we illuminate the HSP90 field on the basis of latest results and discuss the question what can be expected from HSP90 inhibitors. HSP90 is present in all cell types; this ubiquitary presence raises several issues concerning validation as biological target in cancer therapy. Will highly specific HSP90 inhibitors show strong side effects due to cell toxicity in almost all cell types and how could one develop strategies to increase cancer selectivity and reduce side effects?

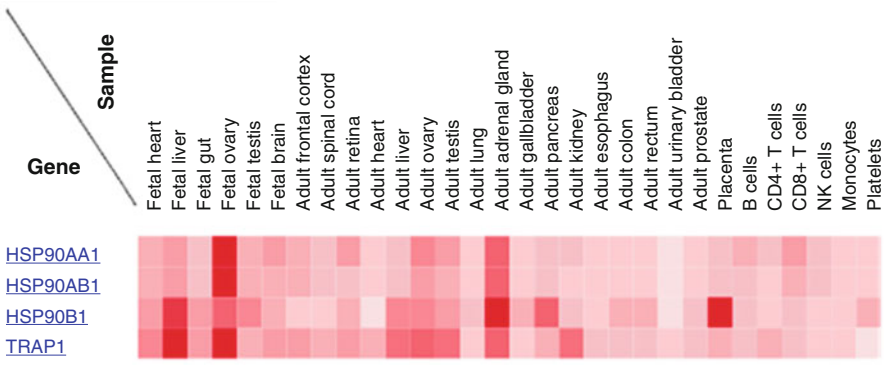

Fig. 16.6 Proteome heat map of HSP. In *red* high expression of HSP proteins is shown, whereas *lighter colors* indicate low abundance. Different intensities indicate different levels of protein titer (Courtesy provided by [26])

16.2 Natural Product Synthesis Is the Key Element for Future Drug Design

16.2.1 Principal Concepts of Chemo- and Biosynthetic Approaches and Their Combination

In many microorganisms, plants as well as animals, biosynthesis machineries such as polyketide synthases (see also Fig. 16.2) can be found that are responsible for the generation of complex natural products [28–31]. With the detailed genetic background now available the genetic manipulation of these biosynthesis machineries has become possible which also includes the production of those secondary metabolites shown in Fig. 16.1 that inhibit HSP90. Several strategies can be pursued to utilize these biosynthetic gene clusters for creating new derivatives of HSP90 inhibitors [32]. These include combinatorial biosynthesis (BIO, Fig. 16.4) and mutasynthesis (CHEM-BIO, Fig. 16.4), the former totally relying on genetic engineering while the latter combines the structural flexibility of chemical synthesis with the power of biosynthesis [33]. The new derivatives that result from these strategies can be further modified and hence even further diversified potential HSP90 inhibitors can be formed by finalizing the synthesis with semisynthetic steps (e.g. CHEM-BIO-CHEM, Fig. 16.4).

Chemical synthesis in combination with biosynthesis can be exploited to accelerate the "evolution" of small molecules as inhibitors of key proteins such as HSP90. Two strategies for creating new geldanamycin libraries may illustrate this, the first being one of the latest examples of semisynthetic derivatization (BIO-CHEM) of geldanamycin, while the second strategy is a mutasynthetic approach (CHEM-BIO).

The benzoquinone moiety has been made responsible for hepatocytotoxic side effects related to geldanamycin. It is argued that thiol nucleophiles react at the C19 position and this has been made responsible for undesired toxicity [34].

Therefore, Moody and coworkers developed a semisynthetic protocol that leads to blockage of this position in order to ameliorate toxicity (Fig. 16.7) [35]. Protein crystallography established that these derivatives bind to HSP90 with a *syn*-configured arylamide conformation. This conformation considerably differs from the preferred *anti* conformation found in geldanamycin and facilitates protein binding specifically to the N-terminal ATP site of HSP90. The new 19-substituted benzoquinone ansamycins showed distinct less toxicity to normal endothelial and epithelial cell systems (HUVEC and ARPE-19) than their parent quinones thus, potentially widening the therapeutic window of ansamycins.

An alternative synthetic strategy for "optimizing" geldanamycin relies on intervention into the biosynthetic machinery in *S. hygroscopicus*, the geldanamycin producer. The PKS starting building block of all ansamycin antibiotics including geldanamycin is 3-amino-5-hydroxybenzoic acid (AHBA) [36]. Having blocked the biosynthesis of AHBA by genetic engineering, a knockout mutant strain of *S. hygroscopicus* is available, which allows to feed derivatives of AHBA that are obtained by chemical synthesis. If these are fully processed by the geldanamycin

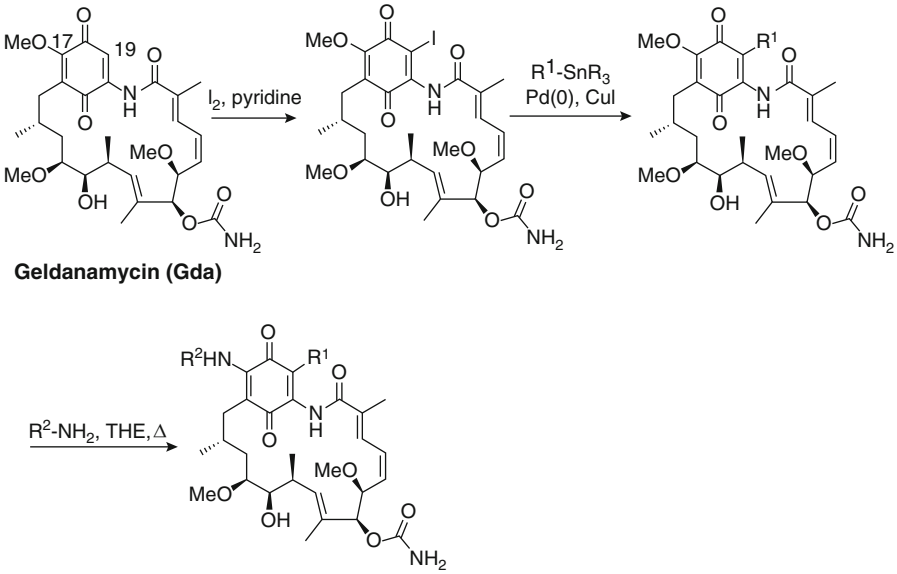

Fig. 16.7 Semisynthetic formation of new geldanamycin derivatives modified at C-17 and C-19 and their antiproliferative activities compared to geldanamycin [35]

biosynthesis machinery, via a strategy that is termed mutasynthesis [33] new geldanamycin derivatives became available. Figure 16.8 depicts four illustrative experiments and six resulting new geldanamycin derivatives which in two cases (one bromo- and fluorogeldanaymcin) show antiproliferative activity against a panel of cancer cell lines in a similar range to the natural product geldanamycin [37]. Remarkably, these derivatives do not contain a benzoquinone moiety but are highly potent [34]. Obviously, the quinone group or hydroquinone moiety is not essential for binding to the N-terminus of the ATP binding site of HSP90. Therefore, the mutasynthetic strategy has great potential for accessing compound libraries of natural products that are structurally very complex (Fig. 16.8).

Cancer cells become vulnerable through constant elevated HSP90 levels, which are induced through continuous stress. The reason for this phenomenon is that HSP90 is a multiplayer in the proteome network. The central physiological role of HSP90 and direct and indirect contact partners can be identified as part of a huge interactome network including up to 600 partners (Fig. 16.5) including proteins essential especially for the eukaryotic cellular function. HSP90 inhibitors abolish the continuation of this contact network with the known effect that susceptible cancer cells lose their viability. An inhibitor is expected to reach the intracellular target; thus the molecule must shuttle into the cell and remain there until it binds to the target as a donation for the acceptor HSP90. Mainly small molecules up to 1 kDa can overcome the membrane barrier by different pathways. In recent years several novel inhibitors were designed to target the ATP-binding site of HSP90 including

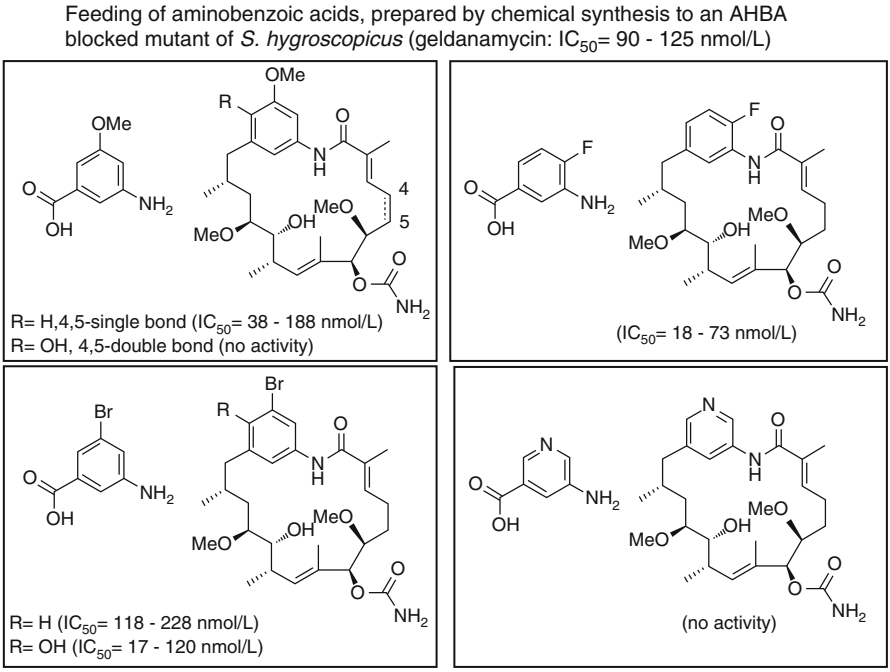

Fig. 16.8 Mutasynthetic formation of new geldanamycin derivatives and their antiproliferative activities towards different cancer cell lines compared to geldanamycin [37]

natural products or ATP analogues [32]. These have been obtained from predictions by molecular modeling and high throughput screenings (HTS) techniques, but due to the huge possible complexity of small molecules clear predictive *in silico* strategies are not available. In addition, some limitations of the natural products Gda exist which rigorously influence the binding affinity to HSP90 [32]. In some cases it is helpful to analyze the acceptor site here the ATP-binding site of HSP90.

16.2.2 Key Positions in the Structure of HSP90α

There are several key amino acids in HSP90α, which are known to be relevant for the binding of geldanamycin (Gda) and its derivatives such as 17-N-allylamino-17-demoethoxygeldanamycin or the free 17-amino derivative (17-AAG). According to Millson et al. [38] the amino acids listed below are important for the binding of ATP or Gda in the ATP-binding pocket of human HSP90α. These positions can either contribute to van der Waals' interactions or hydrogen bonding with the substrate/inhibitor or form hydrogen bonds with water. The numbering refers to residues present in human HSP90α [38].

- Van der Waals' interactions: N21, D54, A55, I96, M98, D102, N106, L107, V150
- Hydrogen bonds with the ligands: K58, D93, K112, F138
- Hydrogen bonds with water: G97, T184.

Remarkably, the bacterial chaperon HtpG found in *S. hygroscopicus* is not inhibited by Gda for which differences in positions 58, lysine to arginine (K58R) and 112, lysine to asparagine (K112N) can be made responsible. The human heat shock protein forms hydrogen bonds with Gda exactly via these positions, and because of this scenario the quinone ring of geldanamycin is stabilized and held in the binding pocket, as was demonstrated by Millson et al. [38] in co-crystallization studies of HSP90α and Gda. They also suspected that a change from valine to methionine at position 150 (V150M) inhibits binding of Gda. The most important contacts of Gda and the semisynthetic derivate 17AAG inside the ATP-binding pocket of human HSP90α are K58, D93, K112 and F138 [38, 39].

Although HSP show a high degree of similarity in the amino acid sequence, small deviations may results in larger effects with respect to inhibition, therapeutic access or resistance. Such small changes can be visualized by hydropathy plots whereby the amino acid sequence of the ATP/Gda -binding domains obtained from the first homologous 240 amino acids from human HSP90α, *Streptomyces hygroscopicus* HtpG and the human pathogen *Helicobacter pylori* HtpG are translated into the hydropathy index values and plotted as a function of the amino acid sequence according to Kyte and Doolittle [40] (Fig. 16.9). This N-terminal domain contributes to the binding of Gda or other small molecules to the ATP-binding pocket. The higher the score, the stronger is the hydrophobicity of the respective region. Hydropathy indices were calculated with a window size of nine in ExPASy ProtScale. The hydropathy plot of human HSP90α is shown in black, of HtpG from

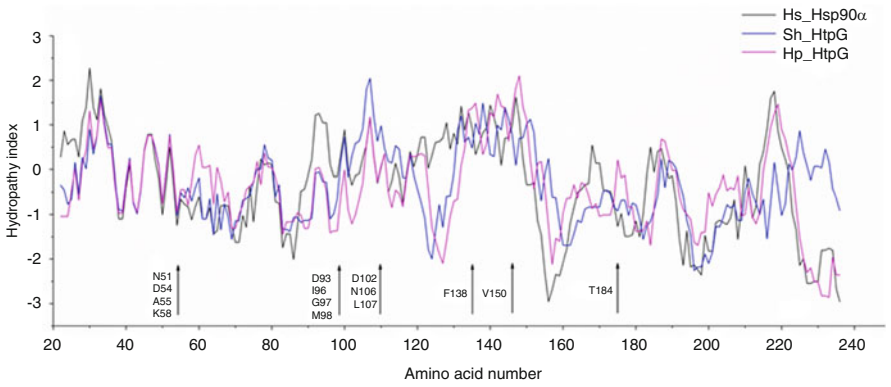

Fig. 16.9 Hydropathy index as function of the amino acid sequences according to Kyte and Doolittle [40]. For this hydropathy plot homologous areas of the N-terminal domain of the ATP-binding pocket were used (amino acid number 22–236). Hs_HSP90 (*black*): HSP90α of *Homo sapiens* (acc. NP_001017963), Sh_HtpG (*blue*): HtpG of *Streptomyces hygroscopicus* (AEM46060.1), Hp_HtpG (*magenta*): HtpG of *Helicobacter pylori* (NP_222917.1)

*Helicobacter pylo*ri in magenta and the HtpG from *S. hygroscopicus* is represented in blue (Fig. 16.9). *H. pylori* is made responsible for many stomach diseases.

In the region of amino acids 120–140 the hydrophobicity of HtpG from *H. pylori* is comparable to that of HtpG from *S. hygroscopicus*. In contrast, the hydrophobicity of the human HSP90α is larger. Since this region is deeply embedded in the N-terminal domain, it is likely that the differences of the hydrophobicity are affecting the binding of Gda. Furthermore, the hydrophobicity of HtpG from *S. hygroscopicus* and HSP90α from *H. sapiens* substantially differs in the area of amino acids 85 and 120 where several amino acids for binding of Gda are located. The graph of *H. pylori* runs balanced between the other two graphs and rather small differences in the hydropathy have to be noted. At the N-terminus between positions 210–240 the progression of the hydropathy indices differs between *H. sapiens* and *S. hygroscopicus* and *H. pylori*, respectively.

16.3 Protein Microarrays

Protein microarrays are miniaturized systems either with proteins used as capture probes or proteins used as targets which should be bound by specific capture probes [41]. They consist of a solid support, e.g. glass or synthetic material with a modified or coated surface. Different proteins such as antibodies, antigens, receptors and enzymes can be bound on several formats (modified glass surfaces, hydrogel, membranes, nanoplates, micro fluidic chips) using special printers (e.g. contactless GeSim Nano-Plotter^{TM}) or by *in-situ* synthesis. Capture probes will be arrayed in the form of microspots (<200 μm) in rows and columns. Every microspot contains only one kind of capture probes. These immobilized capture probes bind their specific target molecules out of a complex solution. The development of protein microarrays shares the principle of miniaturization, parallelization and automation with DNA microarrays. In principal, microarray technology enables both, the probing of the genome and the proteome. But arraying of proteins is more difficult than the arraying of DNA, because proteins have to maintain their correctly folded conformations. The fabrication of protein arrays is therefore particularly challenging and protein arrays lagged behind in development so far, because of the more complex coupling chemistry, the instability of the immobilized protein and the far weaker detection signals [42].

Protein microarray technology allows the analysis of protein function on a whole genome level [43, 44] and to screen large chemical libraries against biological targets fast and highly parallel. Genome-wide screens for protein function are of biological importance for many applications, e.g. analyzing protein expression profiles, monitoring protein-protein interactions, screening the substrates/inhibitors of protein kinases or chaperones and examining the protein targets of small molecules. Functional protein microarrays are fabricated by the immobilization of purified proteins. In contrast to analytical microarrays they have enormous potential in assaying for many biochemical activities (protein-protein, enzyme-

substrate, protein-lipid, protein-DNA interactions). Furthermore, they can be used to investigate drug-target interactions and to identify drugs. The goal of analyzing protein-protein interactions is to study biochemical activities of certain proteins of interest. For example protein microarrays hybridized with labeled inhibitors can be used to analyze ATP-binding proteins such as HSP90α or HtpG involved in many diseases such as cancer, Morbus Alzheimer or malaria. The analysis of such interactions can improve screening for active substrates in the pharmaceutical field. Functional protein microarrays are promising parallelized screening platforms in modern drug discovery processes, talented for analyzing huge numbers of drugs in a time, material and cost effective manner.

16.3.1 Identification of Novel Inhibitors Using Protein Microarrays

The demand to find effective and specific inhibitors for HSPs is constantly increasing. HSPs are involved in many diseases including cancer, Alzheimer's disease and malaria. In this context, huge libraries of potential HSP inhibitors are in development as outlined in Sect. 16.3 of this chapter. To allow the screening of this large amount of substances against different disease-related HSP, an easy, multiplexed, rapid/robust and efficient screening for novel inhibitors, which allows a high degree of parallelization, is needed.

The direct competitive protein microarray shown schematically in Fig. 16.10 conforms to these requirements. Different heat shock proteins can be spotted side by side on a nitrocellulose pad of the microarray with a low amount (300 pg protein/per spot). Also the ligands are needed in much lower quantity in comparison to conventional assays. The known inhibitor or putative candidate directly competes

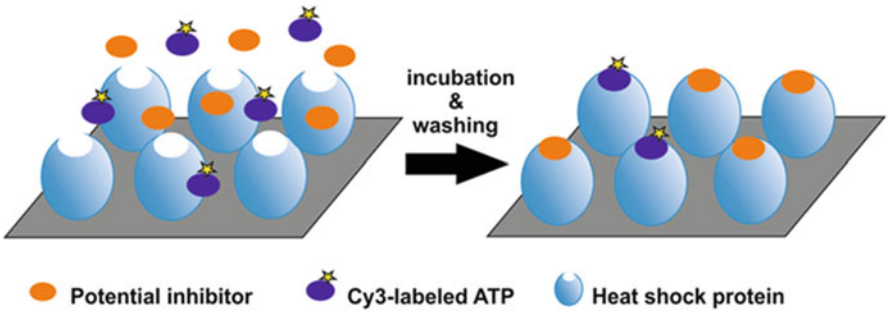

Fig. 16.10 Schematic representation of the direct competition assay using fluorescence-labeled ATP (Cy3-ATP) as reference. HSP are immobilized on the nitrocellulose membrane of the microarray. The proteins can either bind Cy3-ATP or a potential inhibitor with stronger affinity. After incubation and washing, fluorescence and the binding properties of the potential inhibitors are determined

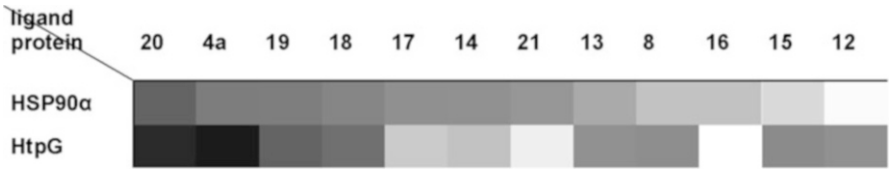

Fig. 16.11 Heat map of the calculated IC_{50} values of HSP90α from *H. sapiens* and of HtpG from *H. pylori* for several tested potential inhibitors (compare ligand numbers with (Schax et al. 2014b) screened in the direct competitive protein assay. Different shades from *white* over *gray* to *black* indicate the potential of the ligand as an inhibitor. *White* corresponds to strong inhibitory effects, whereas *black* indicates that ATP binds stronger to the ATP-binding pocket than the ligand

with the fluorescent-labeled ATP (Cy3-ATP) for the ATP-binding pocket of the HSP. After an incubation time and removal of the unbound compounds, the fluorescence intensities are measured. Depending on the inhibitory effects of the compounds, the fluorescence intensity varies.

With the help of this protein microarray, we could identify several novel inhibitors for human HSP90α and HtpG from *H. pylori*. The heat map (Fig. 16.11) represents some of the screened ligands, which have demonstrated to be effective. As we can see, the ligand 12 is, out of these compounds, the best inhibitor for human HSP90α. For HtpG from *H. pylori* number 16 shows the highest effects. Interestingly drug candidate 17-AAG, here ligand number 4a, does not inhibit the bacterial HtpG at all while a moderate inhibition of HSP90α was observed. As outlined in Sect. 16.3.2, HtpG differs from the human HSP90α only in a few key amino acids. Ligand 4a is a candidate showing specific selectivity, since it inhibits the human Hps90α but not the HtpG. The other non-benzoquinone geldanamycin derivatives and radicicol (8) showed strong inhibitory effects for both proteins with IC_{50} values in the low nanomolar range. These results demonstrate that the multiplexed HSP microarray represents a useful tool for the screening of small molecules binding selectively to different HSP (Schax et al. 2014a), e.g. the screening of compounds that are strong inhibitors of pathogen-related HSP and show no effect on human HSP is enabled. Thereby, site effects of potential drugs can be minimized.

16.3.2 In Vitro Screening Methods for HSP Inhibitors

Various *in vitro* methods have been developed to identify potential inhibitors for HSP90; (a) refolding assays and (b) binding assays including the novel microarray application shown in the section before (Franke et al. 2014, [32]). Folding assays address the function of HSP90 as a molecular chaperone and enable high-throughput screening approaches [45]. *In vitro* protein-targeted screening systems and detection methods include ATPase activity assay, proteolytic fingerprint assay combined with mass spectrometry, thermal shift assay, thermal scanning fluorescence meth-

ods, fragment screening using affinity capillary electrophoresis (CEfrag), surface plasmon resonance (SPR), thermophoresis, fluorescence polarisation technique, TIRF (total internal resection fluorescence), and aggregation assays ([23], Franke et al. 2014, [32]). Finally, Alpha (Amplified Luminescent Proximity Homogeneous Assay) Screen is a very sensitive non-radioactive homogeneous, but expensive assay technology that allows the screening of interaction on a high accuracy level using donor and acceptor beads, that is described for different applications [47]. Formats suitable for a colorimetric quantification are also frequently used; in the case of HSP90 this is achieved by detecting inorganic phosphate as a phosphomolybdate complex. Fluorescence polarization utilizes a geldanamycin derivative as HSP90 inhibitor that is labeled with fluorescein isothiocyanate (FITC). Microscale thermophoresis (MST) is a novel technique that allows the characterization of biomolecules in a label-free fashion. The method is able to detect changes in thermophoretic mobility induced by ligand binding. Several factors such as size, charge and solvation entropy influence the mobility of the biomacromolecule and it has been shown that this is a highly sensitive technique to distinguish between binding and non-binding. A special method to examine topography on single molecule level and to identify binding energies of small molecules is the atomic force microscopy as in described in detail in Sect.16.3.3.

16.3.3 Single Molecule Analysis, Mechanochemistry

Biomolecules such as proteins show relatively soft mechanical properties as well as very small dimensions (a few to tens of nanometers). Since biomolecules are very dynamic in their nature and may change shape and size during an investigation, *in situ* investigations for observing the characteristics of a molecule over time under physiological conditions are of great importance. While today the topography of proteins can be observed by means of different methods such as scanning electron microscopy (SEM), which require extensive sample preparation, AFM is so far the only method, being able to sense mechanical properties of biomolecules *in situ* [48].

The atomic force microscope (AFM), invented in 1986 by Binning and Quate possesses a versatile tool for investigating any biological as well as non-biological surfaces on a nanometric level [49]. To investigate surface topogryphies by means of AFM a mechanical sensor entitled as cantilever, which is a thin spring fabricated from silicon carrying a very sharp tip at its front, is brought into contact with a surface under defined and precisely controllable forces and scan velocities. As the tip is pressed onto the surface by a certain mechanical force (setpoint) and is moved above the surface the cantilever is deflected by the surface irregularities. The deflections of the cantilever from its resting position, detected using a laser beam and segmented photodiodes, are finally reconstructed in a three-dimensional (3D) image, giving the height profile of the sample surface. Here, we briefly communicate our latest results regarding force spectroscopic measurements as well as topographical investigations carried out on HSP90 [24]. As shown in Fig. 16.12a

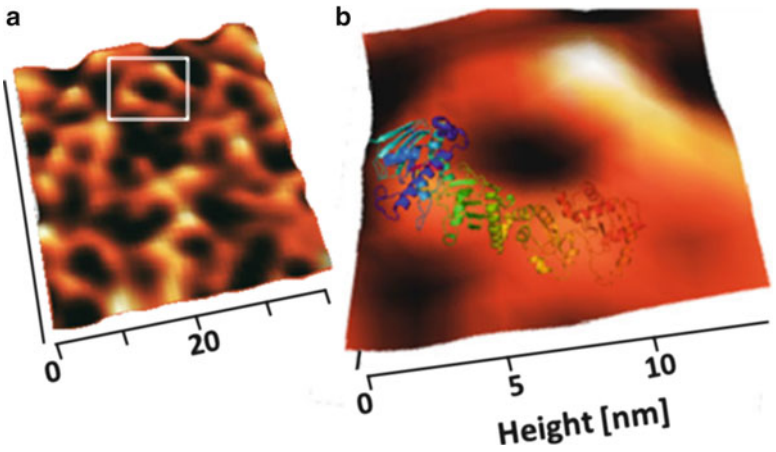

Fig. 16.12 AFM 3D high resolution image of HSP90 molecules topography. Illustration of HSP90 monolayer (**a**) showing morphological variations of the molecule. A single doughnut-like HSP90 with a length of 12, a width of 5–7 and a height of ca. 2 nm (**b**) One subunit of the HSP90 was superimposed by the HSP90 protomer obtained from pdb code: 2CG9, with N-terminal domain (*blue*), middle domain (*green*) and C-terminal domain (*orange*)

the topographical scans revealed a monolayer of single HSP90 molecules adsorbed onto mica surface. Doughnut-like as well as V-shaped structures were observed (Fig. 16.12a, b) and the variations in molecule shape were hypothesized to be based on different states of single HSP90 molecules. The alignment of the doughnut topography from HSP90 with the protomeric structure of HSP90 obtained from crystallography gives an excellent conformity (Fig. 16.12b).

Beside the imaging capabilities of the AFM, it can also be employed for detecting or/and applying mechanical forces at the tip-sample surface interface (force spectroscopy). For this, the sensitivity of the cantilever as well as its force constant need to be first measured for example using thermal noise method [50] to convert any cantilever deflection into interacting forces using the Hook's law. In force spectroscopy interacting forces between the tip and the sample surface can be measured at measurement points that are precisely defined by the investigator. At each point the tip is lowered onto sample surface and is pressed onto it until a predefined setpoint is reached. The sample surface (e.g. biomolecule) is allowed to react to the applied force and undergoes an indentation, while it also adheres to the sharp tip. Finally, the cantilever is moved away from the surface leading to an elongation of the biomolecule followed by unfolding of its domains before the tip and the sample are fully separated. The data is then collected in a so-called force-displacement (FD) curve, revealing the applied force on the y-axis as a function of the elongation, given on x-axis. Using the extension curve and by superimposing a Hertz-Model onto it and by knowing the tip dimension, the elasticity of the biomolecule can be measured quantitatively. The retraction curve though can be used to measure adhesion forces as well as to mechanically

characterize the unfolding events in the biomolecule. Then, the data is fitted with mathematical models such as the worm-like chain model [51] to characterize the contour length and persistence lengths of the biomolecule [48]. For example, using we were able to detect changes in HSP90 elasticity as well as size with respect to an inhibition by novel fluorogeldanamycin derivatives using this method in an unpublished work. Our results showed changes in both rigidity and volume of the HSP90 molecule in the inhibited state compared with the native state.

In conclusion, single molecule analysis by means of methods such as AFM-based single-molecule force spectroscopy provides information about interaction of small molecules with a known target and allows the determination of elastomeric behaviour of HSP or any other biomolecule and randomizing effects beside the binding site, it possesses a novel and strong tool to characterize inhibitors on single molecule level.

16.4 Perspectives

The requirement of suitable potent HSP90 inhibitor is not only a challenge concerning target orientation and cell viability, moreover the stability of the compound, its distribution and many other properties have to be considered.

With novel miniaturized assay systems like the protein microarray, but also other screening applications like luminescence-based refolding assays it is possible to test different target sites of the HSP90 molecule including the client binding site or the C-terminal site. Recently the HSP90 microarray application method was extended to test binding of putative denatured and non denatured clients of HSP90 on the microarray. Thus, this method could be used for screening of peptide libraries and/or search for novel inhibitors of the client binding site (Schax et al. 2014b). In combination with AFM single force spectroscopy, which is a highly miniaturized application we can analyze the mechanochemistry and the elasticity of the HSP90 molecules and the effect of inhibitors in detail so that binding energies and elasticity properties can be examined (Aliuos pers. com.).

The wide distribution of HSP90 and its important role in prokaryotic and eukaryotic cells and also in virus function is the reason that we can imply an HSP code with a selective susceptibility for different PKS products. Beside the anti cancer potential, these products have anti-fungal, anti-inflammatory and anti-viral activities [52], which requires the selection of the appropriate product line by target-oriented or site oriented screenings. As shown recently by Schax [53, 54], it is possible to find different inhibitor selectivity between human, pathogenic bacteria and plant. Furthermore, the HSP90 microarray technique can be used for diagnostic application; here cancer cell lines or tissues can be applied and fluorescently labeled ATP can be competed by novel synthesized Gda derivatives.

Opening the focus to several other HSP targets, targets like HSP70, the stress inducible HSC70 or mitochondrial HSP90 (TRAP, tumor necrosis factor type 1 receptor-associated protein) can also be analyzed on the protein microarray to select

compounds that are important for clinical diseases like cancer, cardiovascular, neurological, hepatic, and others, and can have a strong therapeutic potential [55–57].

16.5 Conclusion

Heat shock proteins (HSP) are molecular chaperones, which help the proteome to mature by folding of proteins to their native structures even under extreme environmental conditions. During evolution several natural product synthesizers used these traits to establish randomized survival strategies. This evokes highly toxic inhibitors or drugs lowering the foreign cellular HSP90 activities by bait and prey manner. Consequently these molecular pathways have been used to synthesize novel drug compounds with toxic effects for cancerous cells exhibiting increased HSP90 levels by mutasynthetic strategies. These compounds ideally lead to the death of the cancer cell or in special cases to the reduction of a pathogen by inhibition of HSP. Successful semisynthetic strategies have been elicited several novel geldanamycin derivatives with antiproliferative potential and provide a library with diversified macrocycles with novel exchanges and manipulations. Monitoring the activity of the synthesized library of macrocycles requires a robust and rapid test system. Miniaturization of assays via HSP microarrays was a key step forward to analyze complex libraries versus HSP proteome and enables to test the potential inhibitors simultaneously on bacterial as well as on human HSP. In addition, mechanochemistry using atomic force microscopy represents further miniaturizing on atomic level. This novel technique measures the effect of inhibitors on single HSP90 molecules and could be a novel technique to estimate binding energies obtained directly from pulling experiments. Heat-shock proteins have already been identified as target for different therapies; several presaged natural strategies can help for the synthesis of target-oriented straight-forward predesign of highly active compounds.

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Chapter 17
Targeting Heat Shock Proteins in Colorectal Cancer

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Abstract Colorectal cancer (CRC) causes over half a million deaths worldwide and has a particularly poor prognosis when diagnosed at an advanced stage. Heat shock proteins (HSP) have been found to be elevated in CRC patients and HSPB1, HSPA1A and HSPC1 has been shown to have some prognostic value. CRC, in common with all cancers, has important associated oncogene and tumor suppressor gene associations and we show how many of these interact directly with one or more of the HSP. We discuss the current chemotherapeutic options available to the clinician when presented with CRC and how these may be improved with a consideration of the role of HSP in the development of the tumor as well as the response to therapy. Direct manipulation of HSP has the potential to decrease the therapeutic dose of anti-tumor drugs and we propose novel strategies that have the potential to be adapted to the clinic.

Keywords HSP • Colorectal cancer • Chemotherapy • APC • KRAS • BRAF • p53

Abbreviations

AJCC	American Joint Committee on Cancer
APC	Antigen presenting cells
CRC	Colorectal cancer
CT	Computed tomography

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FAP	Familial adenomatous polyposis
HNPCC	Non-polyposis colorectal cancer
HSP	Heat Shock Proteins
TNM	Tumour node metastasis
UICC	Union for International Cancer Control

17.1 Introduction

17.1.1 Colorectal Cancer (CRC)

CRC affects about 1.2 million people worldwide with 60 % of the cases occurring in developed regions. About 600,000 deaths are attributed to CRC yearly, making it the fourth most common cancer death in the world [1]. In the United Kingdom, CRC is the fourth most common cancer with around 110 people diagnosed and 43 people dying from it every day [2]. Most cases of CRC are sporadic (80 %), while the remaining cases (20 %) include patients with a familial risk such as hereditary CRC. Risk factors for developing CRC are: increasing age, male sex, diabetes, ulcerative colitis or Crohn's disease and a first-degree relative with CRC. Environmental factors that play a role include high intake of red and processed meat, smoking, excessive alcohol intake, sedentary lifestyle and obesity [2].

CRC can be broadly divided into two groups based on the pre-dominant location of the cancer in the colon: proximal (right-sided) or distal (left-sided). Proximal CRC are typically less aggressive, more associated with microsatellite instability and have diploid DNA while the distal CRC behave more aggressively, commonly have chromosomal instability and aneuploid DNA [3]. Around 5–10 % of all CRC is inherited in an autosomal dominant manner [4]. Hereditary CRC have an earlier age of onset and behave differently from sporadic CRC with similar carcinogenesis pathway. Hereditary non-polyposis colorectal cancer (HNPCC) is the most common hereditary colon cancer with a high occurrence of concurrent extra-colonic cancers such as endometrium, ovary, stomach and small bowel. The second most common hereditary CRC is Familial Adenomatous Polyposis (FAP), which is inherited in an autosomal dominant pattern. FAP is characterised by multiple adenomas developing in the colon and rectum around pre-teen age and continuing to proliferate throughout adult life with an almost 100 % chance of malignant transformation.

17.1.2 Pathogenesis of Colorectal Cancer

The multistep model of adenoma-carcinoma sequence of colorectal carcinogenesis suggests that disease progression is driven by a step wise alteration of several proto-oncogenes and loss of function of tumour suppressor genes over many years [5]. However, as our understanding of carcinogenesis progresses, it has become clear

that this traditional model of CRC is over simplistic and is not true for all cases of CRC. It is now widely accepted that there are three distinct molecular carcinogenesis pathways leading to the development of CRC: (i) chromosomal instability; (ii) microsatellite instability; (iii) serrated or CpG island methylator phenotype (CIMP) pathway [6]:

(i) Chromosomal instability occurs when there is gross chromosomal aberration leading to aneuploidy. This is the most common pathway and the most common genetic alterations to be affected are adenomatous polyposis coli (APC), KRAS and p53 (details will be discussed in Sect. 17.2.3) [5];
(ii) A microsatellite is a repeated sequence of DNA that is 2–6 base pairs long and is unique to each individual. Microsatellite instability occurs when DNA mismatch repair system fails to correct errors during the DNA replication process resulting in frameshift mutations and base-pair substitutions [7, 8]. This is most commonly mediated through epigenetic gene silencing near the promoter of DNA mismatch repair genes, most commonly MLH1 and MSH2 [9];
(iii) The serrated pathway involves a widespread abnormal hypermethylation of CpG islands, now commonly termed (CIMP), near the promoters of many tumour suppressor genes [10]. Serrated CRC are strongly associated with BRAF mutations and are usually flat or sessile and hence harder to detect and remove completely [11].

These three different pathways are differentiated using their molecular profiles (Table 17.1) and each have a distinct clinical course, prognosis and management. Chromosomal instability and CIMP pathways are associated with both the sporadic and hereditary cancers whereas microsatellite instability is mostly related to hereditary CRC.

Although there are three distinct pathways that lead to CRC, it is of utmost importance to understand that it is a heterogeneous disease. In the landmark article by Wood et al. [12], the estimated number of mutations per tumour was between 49 and 111 [12], affecting around 20 signalling pathways [13]. Using comprehensive systematic analyses of genetic alterations, Wood et al. supports the view that

Table 17.1 Molecular classification of colorectal cancer

	CIN pathway	MSI pathway	CIMP pathway
CIN	+++	− − −	− − −
MSI	− − −	+++	+/−
CIMP status	− − −	− − −	+++
KRAS mutation	+++	+/−	− − −
BRAF mutation	− − −	− − −	+++
MLH1 status	Normal	Mutation	Methylated

Adapted from Noffsinger [11]
CIN chromosomal instability, *MSI* microsatelite instability
Key: +++ present, − − − absent, +/− may or may not be present

the development of CRC is a multistep process involving multiple genes, each conferring small advantages driving the progression of the disease [14], instead of the traditional step-wise progression of mutations as described by Fearon and Volgestein [5]. Driven by advances in bio-informatics and the human genome project, it is now possible to map out each individual tumour genome. Indeed, it is widely believed that every tumour is unique in its genomic make-up.

17.1.3 Clinical Presentation and Screening

Patients with CRC commonly present with a change in bowel habit, weight loss, anaemia, rectal bleeding, abdominal pain, bowel obstruction and abdominal or rectal mass. Clinical manifestations depend on the location of tumour for example anaemia is more common in right sided tumour, changes in bowel habit suggest a left sided tumour while rectal bleeding is often caused by recto-sigmoid disease [15]. CRC can spread via lymphatic, hematogenous or transperitoneal dissemination to other sites in the body. The most common metastasis sites are regional lymph nodes, liver, lungs and peritoneum. Many patients do not have any signs or symptoms until later stages of the disease with 20 % reported to have metastatic disease at time of diagnosis [16].

In response to the late presentation, screening tests for CRC have been implemented in several countries using either the faecal occult blood test, flexible sigmoidoscopy or colonoscopy [17]. In the United Kingdom, the National Health Service Bowel Cancer Screening Program invited asymptomatic 60–74 year olds to biennial screening using faecal occult blood testing and those with a positive result were followed-up with a colonoscopy. A systematic review conducted by Hewitson et al. [18] involving 320,000 participants in four randomised controlled trials showed that the faecal occult blood test reduced the mortality rate by 16 %. Since its inception in 2006, around 15,000 cancers were diagnosed with 35 % in stage I, showing that a screening program is able to detect CRC at a much earlier stage in an asymptomatic population [19].

17.1.4 Diagnosis and Investigations

It is recommended that patients presenting with symptoms suggestive of CRC and those who have a positive screening test should have endoscopic investigation. All polyps should be ideally removed and a biopsy should be taken from any suspicious lesions. Attempts should also be made to remove the lesion completely in a single segment with a clear margin. Diagnosis of CRC is made after pathological examination of the specimen. Once a diagnosis is made, further investigations are needed to determine the stage of the disease and suitable treatment. A baseline computed tomography (CT) scan is indicated for both colon and rectal cancer

Table 17.2 Definition of colorectal TNM by American Joint Committee on Cancer [23]

Primary tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through the muscularis propria into pericolic tissues
T4a	Tumour penetrates visceral peritoneum
T4b	Tumour directly invades or histologically adherent to other organs or structures
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1a	Metastasis in one regional lymph node
N1b	Metastasis in 2–3 regional lymph nodes
N1c	T1-2 lesions that lack regional lymph node metastasis but have tumour deposit(s)
N2a	Metastasis in 4–6 regional lymph nodes
N2b	Metastasis in >7 regional lymph nodes
Distant metastasis (M)	
M1a	Single metastatic site
M1b	Multiple metastatic sites

to monitor disease progression and to investigate any occurrence of synchronous metastatic disease [20, 21]. For rectal cancer, a pelvic magnetic resonance imaging (MRI) scan is recommended as additional pre-operative assessment due to increased sensitivity and specificity for evaluation of T stage and correlation with survival outcomes [22].

17.1.5 Staging, Biomarkers and Prognosis

CRC can be staged using the tumour, node, metastasis (TNM) system (Table 17.2) or the Dukes' staging system. Both systems describe how far the cancer has spread anatomically using the invasiveness of primary tumour, involvement of local lymph nodes and presence of metastatic disease as their parameters and are used interchangeably in clinical settings. The TNM system, maintained by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC), is the most widely used staging system worldwide due to its comprehensive definition and continuous expert review and is the adopted system in this article (Tables 17.2 and 17.3). The T and N stages are performed by pathologists whereas the M stage is from radiological imaging, most commonly a computed tomography (CT) scan. Staging of a cancer provides useful information regarding investigation, prognosis, treatment, survival rate and surveillance.

Table 17.3 TNM staging system with 5 year overall survival rate by American Joint Committee on Cancer [23]

Stage	TNM	5-year overall survival (%)
I	T1N0M0	74.1
	T2N0M0	
IIA	T3N0M0	52.4
IIB	T4aN0M0	
IIC	T4bN0M0	
IIIA	T1N1/1 cM0	50.0
	T2N1/1 cM0	
	T1N2aM0	
IIIB	T3N1M0	
	T4bN1M0	
	T1N2bM0	
	T2N2a-bM0	
	T3N2aM0	
IIIC	T4aN2aM0	
	T3N2bM0	
	T4aN2bM0	
	T4bN1M0	
	T4bN2M0	
IVA	Any T any N M1a	5.9
IVB	Any T any N M1b	

The TNM system may have limitations in predicting prognosis. Many studies have recognized other prognostic factors for CRC including perineural invasion [24], infiltrative growth patterns [25] and venous and lymphatic invasion [26]. Other prognostic markers such as KRAS mutation, BRAF mutation and mismatch repair deficiency [27] are also unaccounted for in the TNM staging system. Additionally, specific gene polymorphisms such as DPD/DYPD [28, 29], UGT1A1*28 [30] and also KRAS status [31] that can predict patients' response to chemotherapy drugs 5-FU, irinotecan and anti-EGFR therapy respectively, are not included in the TNM staging process. One of the reasons that these prognostic markers were not included in the AJCC Cancer Staging Manual (seventh edition) is due to the lack of large scale clinical validation and prospective randomised trials [27]. In the future, prognostic markers are expected to play a pivotal role in individualising management of CRC.

17.1.6 Treatment of Colorectal Cancer

Adjuvant treatment of CRC is stage dependent. Stage I is treated with surgery only and has a relative survival rate of >90 % [32]. Stage II is treated by surgery and/or chemotherapy if the tumour exhibits unfavourable histological features including grade 3 or 4 cancer, angiolymphatic invasion or a positive circumferential resection margin. Stage III and IV are treated with surgery and chemotherapy or

chemoradiotherapy. In addition, clinical trials involving new regimens to optimise the outcome of CRC are on-going. One such example is the FOxTROT trial (ISRCTN87163246). Currently a phase III clinical trial, FOxTROT aims to investigate (i) the feasibility, safety and efficacy of pre-operative chemotherapy; (ii) the benefit of combining panitumumab, a monoclonal antibody targeting the Epidermal Growth Factor Receptor (EGFR) with standard chemotherapy in patients with wild type (WT) KRAS tumours [33].

Treatments available for CRC are surgery, chemotherapy, radiotherapy (rectal cancer only) and palliative care. Surgery is the mainstay of therapy and is indicated for the removal of primary tumour, regional lymph nodes and resection of metastatic lesion/s or to relieve symptoms of bowel obstruction. Chemotherapy is used to prevent recurrence and treat micro-metastases that may evade detection or in palliative cases where surgery is not possible. The three main classes of chemotherapy are 5-fluorouracil (5-FU) (and its oral prodrug capecitabine), oxaliplatin and irinotecan. The mechanism of action of each chemotherapeutic agent will be discussed in Sect. 17.2.3. Combination therapy is used to treat CRC and the most common regimens are FOLFOX (5-FU and oxaliplatin), FOLFIRI (5-FU and irinotecan) and XELOX (capecitabine and oxaliplatin). Advanced CRC with liver metastasis is also treated with targeted therapy such as bevacizumab (anti-vascular endothelial growth factor (VEGF)) and cetuximab or panitumumab (anti-EGFR) [20, 21].

Neoadjuvant radiotherapy is used to treat advanced rectal cancer because of the high risk of local recurrence. This is due to the fact that the rectum is very closely related to the neighbouring pelvic structures without a layer of serosa acting as a physical barrier. Patients with local recurrence of rectal cancer suffer from deep pelvic pain, significant loss in quality of life and have a poorer prognosis with a 5 year survival rate of 9 % [34]. Chemoradiotherapy with 5-fluorouracil is the standard regimen although addition of oxaliplatin is also being used in a multicenter phase III clinical trial CAO/ARO/AIO-04 in Germany [35]. Patients with stage II and stage III rectal cancer are given combined chemoradiotherapy pre-operatively to downsize the tumour and to reduce the chances of local recurrence.

There has been a growing interest in the search for treatments that target multiple cell-signaling pathways simultaneously [36]. Resistance to single targeted therapy is common and can be immediate or delayed. The mechanisms of resistance are varied, including the stimulation of alternate survival pathways resulting in activation of downstream targets crucial for cancer progression or secondary mutation in drug receptor domains [37, 38]. Many signaling pathways (see Sect. 17.1.2) are simultaneously involved in the carcinogenesis of CRC, making it difficult for single targeted therapy to have meaningful clinical success. In a cancer as complex and as diverse as CRC, targeting a factor that is involved in numerous signaling and apoptotic pathways would seem to have greater potential for success.

17.2 Role of Heat Shock Proteins in Colorectal Cancer

17.2.1 Targeting Heat Shock Proteins in cancer

Heat Shock Proteins (HSP) have been suggested as diagnostic biomarkers, prognostic biomarkers and mediators of chemo- and radioresistance in cancer [39, 40]. HSP are unique targets in cancer due to their interaction with numerous client proteins, many of which play a key role in cancer [41]. Depending on their locations, HSP are involved in different processes of carcinogenesis and metastasis (Table 17.4). The most well studied HSP in cancer are HSPC1, HSPA1A and HSPB1.

Since HSP were identified as attractive therapeutic targets, numerous pharmacological compounds have been developed to be used in both the 'bench' and the 'bedside'. This article aims to highlight the methods that are presently used to target HSP in the clinical setting and their translational potential in the treatment of CRC.

There are three main methods of targeting HSP currently employed in clinical trials: (i) HSPC1 inhibitors; (ii) anti-sense oligonucleotides (ASO); (iii) HSP-based vaccination.

(i) HSPC1 is ATP dependent and seventeen inhibitors targeting its N-terminal ATP binding domain have been used in clinical trials for both solid and non-solid cancers with varying degrees of success [52]. In addition to the intrinsic importance of HSPC1 to tumour cells, targeting HSPC1 has an added attractiveness as HSPC1 inhibitors have selectivity for tumour cells and evidence suggests that inhibitors accumulate more readily in tumours compared with normal tissues due to a high affinity conformation of tumour HSPC1 [53, 54]. However, this tumour- selectivity has recently come under scrutiny [55].

(ii) ASO is a type of RNA based therapy that inhibits expression of a sequence specific gene by altering mRNA splicing, disrupting mRNA translation and stimulating mRNA degradation [56]. Currently HSPB1 is the only HSP- targeted via this method, and the HSPB1-specific ASO, OGX-427 (developed by Oncogenex technologes), is currently in clinical trials. Expression of HSPB1 is significantly higher in many cancers and has been suggested to play a role

Table 17.4 Functions of HSP based on their location in vivo

Location	Functions
Cytoplasm	Sustenance of mutant oncoproteins [42, 43]
	Stabilisation and re-folding of aggregated proteins [44]
	Anti-apoptotic functions [45]
Cell surface	Cancer cell invasion [46, 47]
	Tumour immunomodulation [48, 49]
Extracellular	Angiogenesis [50]
	Tumour immunomodulation [51]

in tumour invasiveness and metastasis, chemoresistance and radioresistance [40, 57]. Using gene-silencing, HSPB1 has also been implicated in mediating chemoresistance in pancreatic cancer [58] and melanoma cells [59]. OGX-427 has produced positive responses as a single agent in bladder cancer [60] and prostate cancer [61], in combination with chemotherapy in pancreatic cancer [62] and bladder cancer [63], and in combination with radiotherapy in head and neck cancer [64]. To date, OGX-427 has entered phase II trials for metastatic bladder cancer (Borealis-1™, Borealis-2™), superficial bladder cancer (OGX-427-BL01), advanced squamous, non-small cell lung cancer (Cedar™), advanced non-small cell lung cancer (Spruce™), pancreatic cancer (Rainier™) and advanced prostate cancer (Pacific™ & OGX-427-PR01) [65].

(iii) HSP-based vaccination takes advantage of the tumour immunomodulation ability of extracellular HSP which are involved in both innate and adaptive immunity by acting as both a chaperone and a cytokine. When released into the extracellular environment, the presence of HSP stimulates the production of cytokines and chemokines, mounting an innate immune response headed by natural killer (NK) cells [66–68]. In addition, the release of HSP-peptide complexes are internalised by antigen presenting cells (APC), including dendritic cells and macrophages [51], via the CD91 receptor [69] and re-presented by MHC I or MHC II molecules to stimulate an adaptive immune response mediated by CD4+ or CD8+ T-cells [70]. Unlike other vaccinations, HSP-based vaccination uses HSP-peptide complexes derived from the patient's own tumour antigens [71]. As the recognition that each cancer is unique grows, immune modulation via individualised vaccines will become more acceptable and welcomed. The side effect profile of HSP-based vaccines is encouraging and the most successful vaccination, Vitespen, an autologous gp-96-peptide complex, has shown success in phase III trials for renal cell carcinoma and melanoma [70].

In CRC, HSPC1, HSPA1A and HSPB1 expression have been correlated with increase chemo- and radio-resistance, invasiveness with consequently poorer prognosis [72–75]. However, evidence of HSP-based therapy in CRC is limited across all experimental models including cell lines, ex-vivo models and animal models. Published data focuses on HSPC1 inhibitors. There are currently only two clinical trials investigating HSPC1 inhibitors in metastatic CRC: a phase I study of NVP-AUY922 (Novartis Pharmaceuticals) in combination with cetuximab and a phase II study of STA9090 (Synta Pharmaceuticals Corp) [76]. Ex-vivo HSPA1A-activated autologous natural killer (NK) cells were used in a clinical phase I trial for colon and lung cancer (in Germany) which showed stable disease and a good safety profile [77]. In addition, vaccination with autologous tumour-derived gp96 (HSPC4) in 29 CRC patients following metastatic liver resection also demonstrated positive clinical results [78]. However to date, there are no follow up clinical trials for both studies. There have been no clinical trials examining HSPB1 inhibitors in CRC.

17.2.2 Expression of Heat Shock Proteins in Colorectal Cancer

Different families of HSP have been reported to be highly expressed in CRC cells and increase the risk of metastases, thus leading to a poorer prognosis [79, 80]. However, evidence of their expression in relation to disease grading or staging is controversial [73, 81, 82].

17.2.3 HSPA1A

There is an overexpression of intracellular HSPA1A in CRC cells when compared with normal cells [81]. Neutralisation of HSPA1A using AIF-Derived Decoy for HSP70 (ADD70) in animal models had antitumour effects [83]. A higher expression of HSPA1A in primary tumours was shown to be associated with lower partial response rate to chemotherapy in patients with unresectable liver metastasis [84]. Interestingly, a higher expression of HSPA1A was reported in CRC with microsatellite instability compared with microsatellite stable disease [85]. It was postulated that the immunomodulation property of HSPA1A resulted in enhanced antitumour immunity leading to a better prognosis in patients with tumour microsatellite instability. This view is controversial as a high level of extracellular soluble HSPA1A correlates to a poorer prognosis following an analysis of 142 patients' serum at 33 months follow up [80]. The prognostic value is improved when combined with acute phase proteins, C-reactive protein and C1 esterase inhibitor, [72] as well as when combined with extracellular soluble HSPA9. This is a mitochondrial HSPA family member, commonly known as mortalin or GRP75 [74]. Indeed, high serum level of HSPA1A is a consistent finding in other cancers including lung cancer [86] and prostate cancer [87]. It is worth noting that authors were unable to provide an explanation for the relationship between high soluble HSPA1A and poor survival in patients with CRC.

17.2.4 HSPB1

Similar to other HSP, HSPB1 is overexpressed in CRC cells compared with adjacent normal tissues [88, 89]. A higher level of HSPB1 is associated with a poor prognosis and its expression correlates to TNM staging [90]. In 175 primary CRC samples, the prognostic value of HSPB1 was further improved by combining with heterogeneous nuclear ribonucleoprotein K (hnRNP K) [89]. Tweedle et al. [75] further subcategorised the prognostic value of HSPB1 into rectal ($n = 205$) and colon ($n = 199$) cancer. The expression of HSPB1 has a strong association with poor cancer-specific survival in rectal cancer but not colon cancer and the level of expression was independent of pre-operative radiotherapy. Interestingly, this relationship was only observed in rectal cancer patients with a poor prognosis ($n = 205$) but not those with good prognosis ($n = 115$).

17.2.5 HSPC1

Expression of intracellular and extracellular HSPC1 is increased in CRC cells [73]. Chen et al. [79] investigated the role of HSPC1 in both intra- and extracellular environment and reported its function in cell migration via the chaperoning of IMH-2 epitope. Secreted HSPC1α enhances CRC cell migration and invasiveness via the NF-κB pathway affecting E-cadherin [91] and integrin αV [92]. These are surface membrane receptors that are important for cell-cell adhesion, resulting in interruption of both gap junctions and intercellular communication.

17.2.6 Other HSP

Expression of other HSP has also been studied in CRC. HSPD1, a mitochondria based HSP-, is reported to have diagnostic and prognostic significance as its expression correlates with tumour grading and regional lymph nodes metastasis [93]. Extracellular HSPD1 has also been identified as a serum marker for CRC using two-dimensional difference gel electrophoresis and Western blotting in 15 tumour samples. The utility of HSPD1 as a biomarker was further validated in cancer ($n = 112$) and normal ($n = 90$) groups and it was reported to be as effective as Carcinoembryonic antigen (CEA), although it appears to be more specific in late stage disease [94].

17.3 Interaction of Heat Shock Proteins with Common Aberrant Pathways in Colorectal Cancer

HSP are involved in stabilisation and regulation of several key proteins and pathways contributing to the tumourigenesis of CRC. Mutation in APC, KRAS and p53 are the most common genetic instabilities in CRC [5], This section describes the interactions between these key proteins and members of the HSP- family reported in tumourigenesis.

17.3.1 APC

Mutation in adenomatous polyposis coli (APC), a tumour suppressor gene, is responsible for the development of familial adenomatous polyposis (FAP) and is reported in 60–80 % of sporadic colorectal adenomas and carcinomas [95, 96]. APC is involved in many cellular processes including cell adhesion and migration, organization of actin and microtubule networks, spindle formation and chromosome segregation [97]. However, central to tumourigenesis caused by a mutation in the APC gene is the sustained stabilisation and activation of β-catenin, a proto-oncogene which is tightly regulated by the canonical Wnt/β-catenin signaling pathway [98].

APC, together with Axin, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3) form the 'destruction complex' which phosphorylates and ubiquitinates β-catenin for proteosomal degradation in the absence of Wnt signaling [99]. The destruction complex ensures continuous elimination of β-catenin, preventing its translocation to the nucleus and subsequent transcription of Wnt target genes. The evidence suggesting binding of HSP to β-catenin is few and controversial. More importantly, it appears that direct interaction between them is dependent on cell type and, to date, this relationship has not been studied in CRC.

In a study of breast cancer, β-catenin was immunoprecipitated from breast cancer biopsies and probed with HSP antibodies [100]. The association of HSPB1 and β-catenin was strong in the cytoplasm but not at the cell membrane. When murine mammary carcinoma cells were forced to express HSPB1 homolog (HSP25), β-catenin relocated from the cell membrane to the cytoplasm. At the cell membrane, β-catenin provides the link between cadherins to the actin cytoskeleton, hence modulating cell-cell adhesions [101, 102]. Silencing murine HSP25 resulted in a loss of migration ability in murine breast cancer cells [103]. It is hence possible to postulate that the association of increased HSP25 expression with increased invasiveness and poor prognosis is due to the loss of cell-cell adhesions as a consequence of β-catenin sequestration in the cytoplasm. Direct interaction between HSPD1 and β-catenin was also observed in squamous cell carcinoma [104]. HSPD1 was suggested as an alternative activator of the Wnt/β-catenin pathway in the absence of disruption of the 'destruction complex'. It was observed that overexpression of HSPD1 promotes metastasis through activating β-catenin and its down-stream effectors.

Although there is no evidence that HSPC1 binds directly to β-catenin, it interacts with several proteins in the Wnt/β-catenin pathway. HSPC1 was reported to stabilise GSK3 [105, 106], allowing phosphorylation of β-catenin for proteosomal degradation in breast cancer cell lines [107]. In contrast, Kurashina et al. [108] reported that inhibition of HSPC1 resulted in activation of GSK3 via deactivation of AKT, and subsequent down regulation of TVF7L2, a downstream effector of β-catenin. This data suggests that the role of HSPC1 in the Wnt/β-catenin is cell line dependent. In addition, among Wnt target genes in CRC, survivin and VEGF are also client proteins of HSPC1 (by cross referencing online databases of Wnt target genes [109] and HSPC1 interactors [110]). Survivin is highly expressed in CRC and has been reported to have prognostic value [111, 112]. The expression of survivin in CRC tissue has been showed to be dependent on the APC/β-catenin/TCF-4 signalling pathway [113].

17.3.2 KRAS

Mutation in Kristen-Ras (KRAS) is estimated to occur in around 35 % of CRC [114]. KRAS status is an important factor in determining tumour progression in CRC [115] and has been intensely investigated recently due to its relationship with resistance to anti-EGFR agents, cetuximab and panitumumab [31]. KRAS is

a proto-oncogene that is involved early in the signal transduction pathway starting from cell surface receptors to key cellular functions including cell proliferation, differentiation and death via the RAS-RAF-MEK-ERK-MAPK signal cascade [116, 117]. The most common KRAS mutation occurs in codons 12 and 13 where a mutant KRAS protein becomes insensitive to GTPase activating proteins (GAPs), hence remaining active in the GTP-bound state [118]. Many KRAS downstream proteins such as BRAF, SKT33 and Akt are dependent on HSPC1 for their functions [119–121]. In addition, expression of HSPB1 was found to be dependent on WT KRAS with activated PI3K/Akt pathway in CRC [122], further strengthening the relationship between KRAS status and HSP.

HSPC1 inhibitors have shown to preferably induce cell death in lung and CRC cell lines with KRAS mutation compared with those expressing WT KRAS [123, 124]. STK33, a serine/threonine kinase that suppresses mitochondrial apoptosis, was discovered to be essential in the survival of tumours driven by KRAS mutation but not those with WT KRAS across various cancer cell lines, including colon cancer [125] although this view is challenged by Babij et al. [126]. Nevertheless, by using a mass spectrometry-based approach, Azoitei et al. [119] discovered that the HSPC1/CDC37 complex binds to and stabilises STK33. This was further confirmed by co-immunoprecipitation. Interestingly, knock-down of HSPC1 down regulated SKT33 in both WT and mutant KRAS cell lines but increased apoptosis was only seen in those with mutant KRAS, lending evidence to the hypothesis that SKT33 does play a crucial role in survival for cancer with oncogenic KRAS addiction. This result was also replicated when HSPC1 inhibitors were used on primary colon cancer and metastatic colon cancer cells [119]. It is worth noting that inhibition of STK33 kinase activity does not affect survival in KRAS dependent tumours [126, 127]. Instead, it is the ubiquitination of STK33 and subsequent proteosomal degradation as a result of HSPC1 inhibition that is essential in inducing apoptosis.

17.3.3 BRAF

BRAF mutation can be found in approximately 10 % of CRC and its presence more than doubles the mortality rate [128]. Although KRAS testing is widely accepted as the standard practice in patients with metastatic CRC, KRAS mutation only accounts for 30–40 % of non-responders [27] and not all patients with WT KRAS respond to anti-EGFR treatment [129]. A retrospective study by di Nicolantonio et al. [130] showed that 14 % of patients with WT KRAS have a mutation in BRAF. In addition, none of the responders had a BRAF mutation and none of the patients with mutant BRAF responded to treatment. This highlights mutant BRAF as a potential mediator of resistance against cetuximab and panitumumab. HSPC1 inhibitors have been effective in down-regulating both mutant and WT BRAF [131, 132]. HSPC1 inhibitors are currently being tested in clinical trials for metastatic melanoma, a cancer with 48 % prevalence of mutant BRAF, displaying its potential in targeting cancers reliant on mutant BRAF for survival [133–135].

17.3.4 p53

The tumour suppressor protein p53 is dubbed the "guardian of the genome" due to its role in preventing cell growth and inducing apoptosis in the face of DNA damage [136, 137]. Under normal conditions, p53 is expressed in low level latent form that is unable to bind to the DNA. When activated, p53 functions as a transcription factor which prohibits cells with DNA damage from entering critical stages of DNA synthesis and mitosis, thus preventing the propagation of malignant transformation [138, 139]. Although mutation in p53 is the most frequent mutation in human cancer and has been reported in approximately 50 % of CRC, the term 'mutant p53' describes a heterogenous group of more than 1,500 different mutations (http://p53.curie.fr) which correspond with different levels of transactivation activity [140, 141]. This large number of documented mutations complicated its studies in clinical setting. Iacopetta et al. [142] analysed 2,867 CRC cases using the 'CRC-p53' International Collaborative Study database and categorized p53 into active (21–100 % activity) and inactive (0–20 % activity) groups. This study reported that 21 % of CRC expressed inactive p53. Interestingly, a significantly higher frequency of inactive p53 was observed in rectal cancers (32 %) compared with proximal colon cancers (22 %).

HSPA1A was the first HSP in the family to be identified as a chaperone for mutant p53 [143]. It was later observed that this interaction also includes HSPC1, co-chaperones cyclophilin 40 and p23 [144]. Dai et al. [145] demonstrated that HSF1 knock-down in mice carrying a germ line mutation of p53 remained cancer free, supporting the view that heat shock response is crucial to the tumourigenesis process mediated by p53. HSPA1A also stabilises mutant p53 by interfering with the Murine Double Minute Clone 2 (MDM2) but not the C-terminus of HSPA8-interacting (CHIP) degradation pathway [43]. Another member of the HSPA family, HSPA9 (mortalin) was also reported to participate in cytoplasmic sequestration of p53 in various cancers including CRC [146, 147], preventing the translocation of p53 to the nucleus for transcriptional activities.

Interaction between HSPC1 and p53 is well described [148, 149]. Mutant p53 was reported to be more reliant on HSPC1 than WT p53 for its stabilisation [150]. HSPC1 inhibits MDM2 mediated degradation of mutant p53 and HSPC1 inhibition using geldanamycin and 17-AAG were able to abrogate this effect, leading to mutant p53 degradation [42, 144, 151]. In contrast, Ayrault et al. [152] reported that with HSPC1 inhibitor, 17-DMAG induced apoptosis required intact p53 functions. Most of the studies on p53 status in relation to HSPC1 inhibitor sensitivity were done on p53 knock-down or single mutation cell lines and animal models. Therefore, while interpreting these results for use in clinical settings, it is important to note the diverse number of p53 mutations and its consequent variable level of transcriptional activity.

17.4 Role of Heat Shock Proteins in Overcoming Chemo/Radio-Resistance in Colorectal Cancer

HSP are associated with resistance to a variety of chemotherapeutic agents as well as conferring thermotolerance to cells subjected to hyperthermia. 5-FU, oxaliplatin and irinotecan remain the mainstay chemotherapeutic agents in the treatment of CRC. All three agents are genotoxic and are non-discriminatory between malignant and normal cells, rendering side effects of chemotherapy common and often unavoidable. When used in combination (FOLFOXIRI), the most optimistic overall response rate was 70 % in patients presenting with initially unresectable metastatic CRC, with 42 % achieving 5-year survival [153]. Although combination therapy offered a better clinical outcome, a significantly higher proportion of patients receiving the FOLFOXIRI regimen experienced grade 3 and 4 toxicity including myelosuppression and neurotoxicity [154]. Studies have been done using a combination of traditional chemotherapeutic agents and HSP inhibitors to target tumour cells and overcome chemoresistance. This should result in the same clinical efficacy using a lower dose of chemotherapy, hence reducing dose dependent side effects. In the following section, pre-clinical evidence of HSP interaction with 5-FU, oxaliplatin, and irinotecan in CRC cells will be extensively discussed, followed by role of HSP in anti-EGFR/VEGFR treatment and radiotherapy.

17.4.1 5-fluorouracil

Despite newer agents, 5-FU, remains the mainstay of treatment for CRC. 5-FU is an analogue of the RNA nucleotide uracil, with the hydrogen atom at C-5 position substituted by a fluoride atom. Once it enters the cell, 5-FU is converted into active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). 5-FU acts as an antimetabolite via two pathways: (i) disruption of DNA and RNA synthesis via incorporation of FUTP and FdUTP into RNA and DNA; and (ii) inhibition of thymidylate synthase, an enzyme that generates thymidylate, a nucleoside that is essential in DNA replication and repair [155]. The response rate for 5-FU is only 5–18 % when used as a single agent, which improves to 16–45 % when combined with leucovorin, a folinic acid agent [156].

One of the key determinants of response rate to 5-FU is the increased presence of thymidylate synthase in tumour cells [157, 158]. Level of thymidylate synthase increased when CRC cells were continuously exposed to 5-FU due to gene amplification [157], resulting in chemoresistance in patients who showed optimistic response initially. Thymidylate synthase was shown to interact with HSPC1 and HSPA1A [159]. When HSPC1 was inhibited by histone deacetylase inhibitor, thymidylate synthase increased binding with HSPA1A and was subsequently degraded via the proteosomal pathway [159]. The interaction of thymidylate

synthase with HSP (although only reported by one group) indicates that targeting HSP is a potential pathway to overcome 5-FU resistance.

By comparing the relative chemoresistance to 5-FU between two colorectal cell lines, HT29 and SNU-C4, and corresponding the level of increment of intracellular HSPA1A in response to 5-FU treatment, a higher HSPA1A content was associated with resistance to 5-FU [160]. However, our laboratory have discovered that following treatment by 5-FU, HT29 cells express an increase level of both HSPA1A and HSPB1 (unpublished data), most possibly mediated by hsf-1. We believe that either HSPA1A or HSPB1 could have mediated the resistant to 5-FU and further study to determine this is on-going.

HSPB1 was found to be overexpressed in 5-FU resistant cells in gastrointestinal cell lines using mRNA differential display analysis [161] and human CRC biopsies using tissue microarray [162]. Tsuruta et al. [163] investigated the level of HSPB1 in six different colorectal cell lines (LoVo, HCT15, WiDr, HCT116, HT-29 and SW480) and detected a correlation between HSPB1 and resistance to 5-FU. By down regulating HSPB1 using siRNA transfection technique in LoVo cell line, chemosensitivity to 5-FU was increased [163].

17.4.2 Oxaliplatin

Oxaliplatin is used with 5-FU in many chemotherapeutic regimens such as FOLFOX, FOLFIRI and XELOX. A 3rd generation platinum-based compound with a 1,2-diaminocyclohexane (DACH) carrier ligand, oxaliplatin forms DNA adducts which are recognised by mismatch repair complex, triggering apoptosis [164, 165].

The role of HSP in overcoming chemoresistance to oxaliplatin was investigated using HSPC1 inhibitors 17-AAG and 17-DMAG in-vitro [166, 167] and in-vivo [168]. Chemoresistance to oxaliplatin was suggested to be mediated by mutant p53 and addition of 17-AAG was able to overcome this resistance in three separate colorectal cell lines HCT116 (WT p53), HT29 and DLD1 (both mutant p53) [167]. However Moser et al. [168] made a similar observation with 17-DMAG though only in HCT116 but not in mutant p53 cell lines HT29 and SW620. This discrepancy between the two groups of cell lines result could be due to the use of a different HSPC1 inhibitor – the effective concentrations vary between the different inhibitors. The in vivo study using nude mice injected subcutaneously with SW620 cells showed that by combining 17-DMAG and oxaliplatin, growth of colorectal tumour, angiogenesis and hepatic metastasis were significantly reduced [168]. This goes to show the limitation of cell line studies in mimicking the complexity of the tumour milieu in vivo.

Down regulation of NF-κB has been suggested as the mechanism of action resulting in the positive effect as seen in the combination of 17-AAG and oxaliplatin [166]. HSPC1 is a known upstream regulator of NF-κB and HSPC1 interaction with IκB kinase (IKK) complexes has been reported in colonic epithelial cells [169]. The

relationship between the NF-κB pathway and HSPC1 has been reported in many cell types including lymphocytes, macrophages and cardiac cells [170–172].

17.4.3 Irinotecan

Irinotecan exerts its chemotherapeutic effects via inhibition of topoisomerase I, an enzyme that plays a key role in DNA replication. During DNA replication, the supercoiled DNA double helices relax to allow a replication fork to develop for new DNA to be synthesised. Topoisomerase I binds to one of the parent DNA molecules during this process to achieve a transient cleavage reaction, thus allowing the intact strand to pass through the nick and unwind without extreme torsional stress. Topoisomerase then re-ligates the broken strand and dissociates from the DNA molecule [173]. By inhibiting topoisomerase I, irinotecan suspends the re-ligation process, resulting in irreversible breakage of the double stranded DNA and cell cycle arrest which eventually leads to cell death [174]. In vivo, irinotecan must be hydrolysed or re-esterified to its active metabolite SN-38 to exhibit therapeutic effects. SN-38 is also available as a compound for laboratory use.

SN-38 induces the G2/M check point which under normal circumstances, delays mitosis to allow DNA repair to prevent transmission of damaged genetic material to daughter cells. By abrogating the G2/M checkpoint, the sensitivity of tumours with mutant p53 to topoisomerase I inhibitor was increased [175]. The G2/M checkpoint is regulated by several proteins including Chk1 and Wee1, both HSPC1 clients. By inhibiting Chk1 and Wee1 using 17-AAG, the cytotoxicity of SN-38 was improved, and the effect was more potent in cells with mutant p53 [226]. With this preclinical data, the same group conducted a phase I clinical trial using the combination of 17-AAG and irinotecan in 27 patients with refractory solid tumours, of which 6 were colorectal tumours [176]. Although there was no reported complete or partial response to the treatment, stable disease was observed. A higher number of patients with mutant p53 achieved stable disease compared with those with WT p53, which was consistent with the preclinical data. Furthermore, a decrease in phosphorylated Chk1, abrogation of G2/M checkpoint and increased apoptosis were also observed in tumour biopsies. There is no follow-on phase II study to date, possibly due to replacement of 17-AAG by newer HSPC1 inhibitors.

The role of other HSP in mediating chemoresistance in irinotecan has also been examined. Using two colorectal cell lines Colo320 (irinotecan resistant) and KM12C (irinotecan sensitive), Choi et al. [177] measured the levels of eight different HSP and identified HSPB1 as being significantly different between the two cell lines. Support for the role of HSPB1 was provided by specific knock-down of expression using ASO. Immunohistochemical staining of HSPB1 in CRC biopsies from 20 patients before the FOLXIRI regimen also correlated high expression of HSPB1 with poor clinical response although it is unclear how the authors attributed this to resistance against irinotecan and not 5-FU or both.

17.4.4 Cetuximab, Panitumumab and Bevacizumab

Anti-EGFR monoclonal antibodies – cetuximab (IgG1) and panitumumab (IgG2), bind to the extracellular domain of EGFR receptor, preventing its activation. When used as a monotherapy in metastatic CRC that did not response to other types of chemotherapy, cetuximab and panitumumab have approximately 10 % response rates. This was attributed to KRAS status [31]. There are no studies comparing the effect of cetuximab and panitumumab in combination with HSPC1 inhibitors in CRC, although a parallel study in breast and gastric cancer cell lines have shown greater antitumour effect occurs when combining transtuzumab (an anti-HER2 monoclonal antibody) and HSPC1 inhibitors compared with either agent alone [178]. In a phase II clinical trial, definite RECIST-defined responses in HER2 positive metastatic breast cancer was observed after addition of 17-AAG (Bristol-Myers Squibb) with conventional transtuzumab regimens [179]. More HSPC1 inhibitors are now used in clinical trials with transtuzumab in a similar group of patients [180], highlighting the promising future of combining anti-EGFR monoclonal antibodies and HSPC1 inhibitors. In addition, EGFR degradation after HSPC1 inhibitors has been observed in a mouse model which showed lower uptake of (64)Cu-DOTA-cetuximab following treatment [181].

EGFR (ErbB1) and HER2 (ErbB2) are both members of the ErbB receptor tyrosine kinase family and are known to form heterodimers for signal transduction [182]. HSPC1 is known to stabilise both HER2 [183] and EGFR [184, 185]. Although both HSPC1 and EGFR bind to HER2, the interaction between them is direct and independent of HER2 [184]. It is therefore reasonable to hypothesise that inhibiting HSPC1 will down regulate EGFR regardless of HER2 expression. This is relevant clinically, as the role of HER2 in CRC is an on-going debate and has yet to play a definite role in clinical decision [186].

Bevacizumab is a monoclonal antibody that neutralises circulating VEGF-A, preventing its binding to VEGFR-2 receptor on either endothelial or tumour cells [187]. Similarly, the combination of bevacizumab and HSP inhibitors has not been studied in CRC. However, the role of HSP in the regulation of angiogenesis is well established. In addition to stabilising VEGFR2 [188], HSP also regulate hypoxia induced factor (HIF)-1α, a proangiogenenic transcription factor [189, 190]. HSPC1 inhibitors have been reported to show antiangiogenic effects in CRC [191] and pancreatic cancer [192] whereas up-regulation of HSPC1 led to angiogenesis [193].

Based on these evidences, it is therefore reasonable to speculate that inhibition of HSPC1 in CRC will yield similar effects to anti-EGFR and anti-VEGF monoclonal antibodies. In addition, as stated above in Sect. 17.2.3, HSPC1 inhibitors are effective in diminishing the effect of mutant KRAS and BRAF. Thus HSPC1 inhibitors are an attractive alternative to either cetuximab or panitumumab as anti-EGFR agents.

17.4.5 Radiotherapy

High expression of HSP has long been known to result in thermotolerance in cells subjected to hyperthermia. The heat resistance phase of thermotolerance can be long lived and as a consequence, radiotherapy doses are scheduled to avoid repeated exposure during heat insensitive period [39]. Kampinga et al. [194] observed that HSPB1, HSPA1A and HSPC1 are all heat inducible and the level of expression correlates with the level of hyperthermia. In addition, enhanced rate of recovery following hyperthermia induced protein aggregation was observed in cell lines overexpressing HSPB1 [195], leading to the hypothesis that thermotolerance provided by HSP is due to their protein stabilisation and refolding ability. In support of the cytoprotection effect offered by HSPA1A, down regulation of OLA1 (Obg-like ATPase), a cytosolic ATPase that stabilises HSPA1A, also resulted in loss of thermoprotection in human cell lines [196].

HSPC1 inhibitors increase sensitivity of cells from glioblastoma, pancreatic cancer and prostate cancer cells to radiation therapy via client proteins degradation, G2/M accumulation, apoptosis and necrosis pathways [197, 198]. Inhibition of HSPC1 in conjunction with radiation therapy also correlates with decreased cell migration and tumour invasiveness in lung carcinoma and glioblastoma as a result of altered matrix-associated protein (FAK/p-FAK, Erk2, RhoA) expression and attenuated F-actin function [199]. Toxicities of combined HSPC1 inhibitors and radiation are cell type specific and the characteristics of cell type seem to determine the mode of cell death or senescence. The effect of combined treatment is also dependent on the drug-radiation sequence and length of drug exposure post-irradiation [200].

17.5 The Challenges of Targeting Heat Shock Proteins in Clinical Setting

There is considerable scientific evidence for targeting HSP to improve cancer treatment. However, only two clinical trials using HSPC1 inhibitors have shown meaningful clinical results [52]. Although generally devoid of side effects, the clinical success of HSP- based vaccination is also limited. Despite currently marketed only in Russia, two phase III randomised clinical trials testing Vitespen on metastatic melanoma [201] and renal cell carcinoma [202] have shown no differences in treated and untreated populations. Translating results from laboratory models to clinical settings have many setbacks as no functional model as yet can reproduce the complex tumourigenesis process in CRC [203]. Thankfully, after a decade of clinical trials, there are many lessons that should be learned and be adapted into the design of future trials to maximize the efficacy of HSP inhibitors. This section will examine clinical trial updates from HSPC1 inhibitors [reviews by Neckers and Workman [52] and Alarcon et al. [204] provide excellent updates] and

HSP- based vaccinations (review by Reitsma and Combest [205]). Phase II clinical trials using OGX-427 for bladder, prostate and pancreatic cancers are on-going and no publications are currently available.

Paradoxically, inhibiting HSPC1 can produce cytoprotective effects. Firstly, inhibiting HSPC1 is known to activate heat shock factor-1 (HSF1), the main transcription factor for HSP during the heat shock response [206]. Upon activation, HSF1 induces transcription of HSPA1A and HSPB1, both of which protect cancer cells and are associated with resistance. HSPC1 inhibitors targeting the C-terminal were reported to have no modulation effect on HSF1 [207] and recently there is increased interest in improving the effectiveness of these C-terminal inhibitors [208, 209]. Current research is examining inhibitors of HSF1, HSPA1A, HSPB1 and other co-chaperones such as p23, activator of HSPC1 ATPase-1 (Aha1) and Cell division cycle 37 homolog (Cdc37) to be used concurrently with HSPC1 inhibitors to improves its efficacy [52].

Secondly, inhibiting HSPC1 interferes with its immunomodulation function in both tumour cells and immune cells. Tumour cells present antigenic peptides via MHC I and MHC II molecules on the surface of cells where they may be recognised by CD8+ and CD4+ T-cells potentially leading to an anti-tumour immune response. This form of antigen presentation requires HSPC1 in the peptide 'loading' process. Inhibition of HSPC1 resulted in "empty" MHC molecules [210, 211]. In addition, HSPC1 inhibition also significantly depressed immature and mature dendritic cell functions including antigen uptake, processing and presentation to T-cells [212]. In contrast, Rao et al. [213] showed that HSPC1 inhibition improves T-cell recognition of tumour cells by down regulating Ephrin receptor A2 (EphA2). This is a receptor tyrosine kinase that is recognised as a self-protein which hinders anti-tumour immune response. It is highly probable that individual host immune systems will react differently to HSPC1 inhibition and the immune response will vary for different tumours. Host immune response to HSPC1 inhibition is difficult to assess in the laboratory environment as cell lines and xenograft models are unable to imitate the intricate human immune system. This could explain the disparity between positive pre-clinical findings and the equivocal clinical results.

Ciocca et al. [214] have shown encouraging results with reports of stable disease, partial response and disease free results in cancer patients ($n = 20$) using a vaccination prepared by combining hydroxyapatite (HA) and HSP. However, treatments involving HSP-based vaccination face barriers in production as well as patient factors. Extraction of autologous tumour peptide used in HSP-based vaccination can be technically difficult resulting in poor production of vaccines [201]. The success of vaccination based therapy is also dependent on the immune ability of individual patients which could be compromised due to factors such as prior surgery, chemotherapy or the disease itself. Another possible reason for limited success is the lack of an optimally defined dose in clinical trials [205]. Preclinical studies in mice have shown that doses higher or lower than the optimal dose have failed to immunise [215]. To date, there have been no recommendations for identifying susceptible patients who are more likely to respond to HSP-based vaccination. These are likely to improve with more experiences in on-going clinical trials.

17.6 Looking Ahead-Targeting HSP in Colorectal Cancer

There is a pressing need for new therapies to be discovered in the treatment of CRC, especially in unresectable metastatic disease which if left untreated, has a 5-year survival rate as low as 3.3 % [216]. Despite the challenges mentioned above, success in targeting HSP in CRC is achievable.

Targeting HSP is likely to be most effective in cancers where oncogenic drivers are sensitive HSP- client proteins. This aspect is most studied in HSPC1 inhibitors due to the large number of clinical trials. The hypothesis is supported by the relative success in targeting HER2 positive metastatic breast cancer [179] and non-small cell lung carcinoma with ALK rearrangement [217]. CRC that are driven by mutant KRAS [119] Sect. 17.2.3 and those caused by the CIN pathway (aneuploidy) [218] are suggested to be susceptible to HSPC1 inhibitors. Personalised medicine is already in practice in metastatic CRC to rule out patients with mutant KRAS who will not respond to anti-EGFR treatment [20, 21]. Other independent groups need to investigate the dependency of colorectal tumours with mutant KRAS on STK33 for survival as well as establish the sensitivity of STK33 as a client protein for HSPC1 [119]. If the results are reproducible, HSPC1 inhibitors will be prime candidates for clinical trials in this group of patients who will benefit from anti-EGFR treatment but do not respond to the current monoclonal antibody agents. Another prospective biomarker for CRC is HER2. The frequency of HER2 expression in CRC is controversial [186] and investigations regarding the clinical impact of membrane-bound and cytoplasmic HER2 need to be conducted before confirming validity as a biomarker. Due to its sensitivity to HSPC1 inhibition, $HER2^{+}$ CRC is likely to respond successfully.

Safe side effect profile and optimum delivery systems are common obstacles in the discovery and development of a new drug. Delivering chemotherapeutic agents accurately and precisely to the localised, affected areas can minimise systemic side effects. Numerous locoregional treatments are already available in clinical trials for unresectable liver metastases including drug-eluting DC Bead® with irinotecan (DEBIRI) [219, 220] and yttrium-90 microspheres delivering targeted radiation therapy [221, 222]. Both therapy delivery systems require transarterial embolization. This is a minimally invasive non-surgical procedure to deliver the drug-eluting beads or microspheres to the hepatic artery where they release chemo- or radiotherapy in a controlled manner. Superparamagnetic iron oxide nanoparticles (SPIONs) are biocompatible vehicles that are used for drug delivery in the management of cancer, enhancement of magnetic resonance imaging (MRI), magnetic hyperthermia and delivery of siRNA treatments [223, 224]. As well as using a magnetic field to maneuver SPIONs to target organs, tumour specific targeting ligands can be attached to the surface of SPIONs to ensure internalisation of chemotherapies only occur at tumour site. Recently, Bausero et al. [225] have successfully transfected HSPB1 siRNA into murine breast cancer model using SPIONs with positive results. Although still at early stage, the use of SPIONs as a delivery vehicle to target HSP specifically at colorectal tumour and metastasis is a very exciting concept specially

as it incorporates both chemo- and radio- therapies. Its ability to deliver siRNA also opens up new opportunities to target HSPA1A and HSF1.

17.7 Conclusion

HSP are attractive therapeutic targets due to their involvement in the propagation of tumourigenesis and cancer cell survival. The presence of readily available methods and agents such as HSPC1 inhibitors, HSPB1 antisense oligonucleotide and HSP-based vaccination that are already involved in clinical trials have enhanced its attractiveness and encourage interest in pursuing research to target HSP in CRC. Already there is a vast array of pre-clinical data describing (i) overexpression of HSP in relation to clinical prognosis; (ii) the role of HSP in sustaining frequently aberrant pathways in CRC; (iii) the role of HSP in mediating chemo- and radio-resistance and (iv) potentiation of current chemotherapeutic agents and radiotherapy in combination with HSP inhibitions In addition, although on a smaller scale compared to other cancers, clinical trials targeting HSP in CRC are on-going, which will provide new information and discovery in time to come.

Success will likely come from patient stratification using biomarkers to identify susceptible patients and when HSP- inhibitors are used in combination with other therapeutic agents that shows additive or synergistic effects. Newer drug delivery methods such as SPIONs and DC Bead® could be developed to deliver HSP targeting drugs to achieve loco-regional toxicity effects while limiting systemic side effects.

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