Heat Shock Proteins 9

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Heat Shock Protein-Based Therapies

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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

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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.

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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].

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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

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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].

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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).

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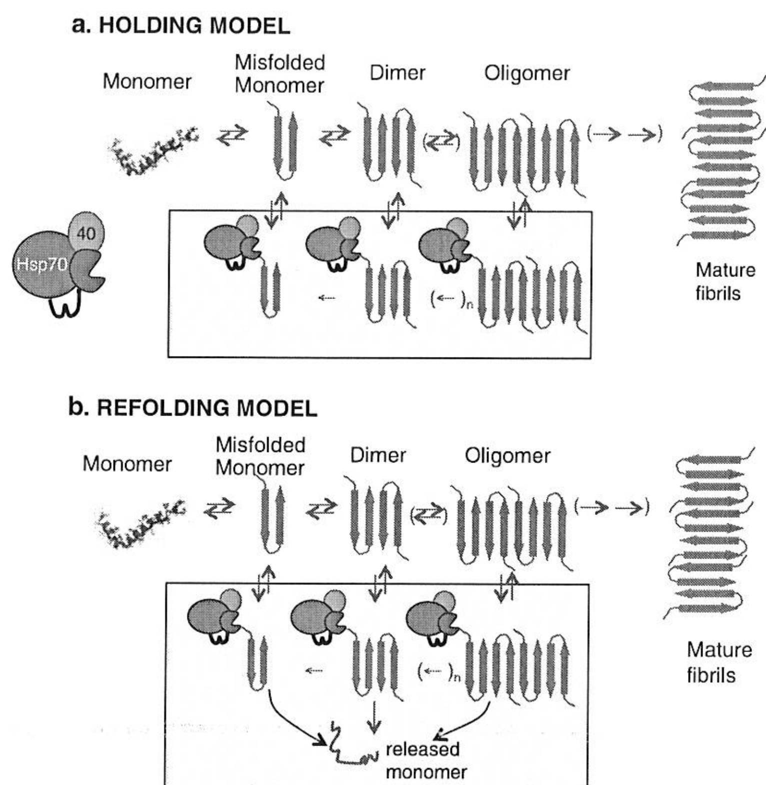

Fig. 4.1 Model for chaperone differential action on Aβ-peptide aggregation pathway. (**a**) Holding mechanism: the misfolded peptide remains on the chaperone surface upon binding, thus reducing the concentration of proteins competent for on-pathway aggregation. (**b**) Refolding mechanism: a conformational 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

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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.

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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

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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

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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.

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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)

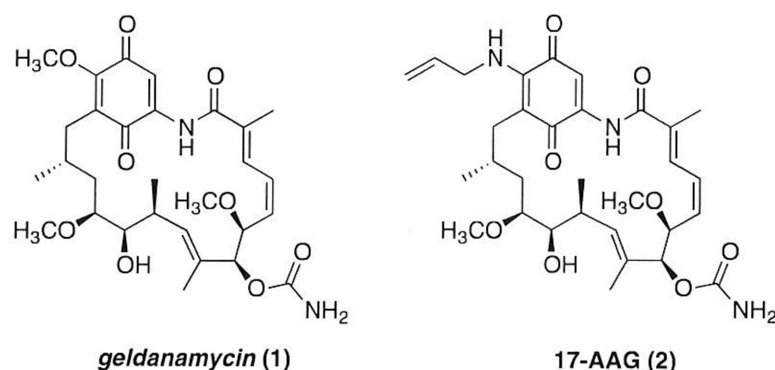

Fig. 4.2 Streptomyces-derived HSP90 inhibitor geldanamycin (1) and its less toxic derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) (2)

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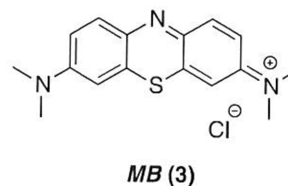

Fig. 4.3 Structure of methylene blue (MB) (3), inhibitor of ATPase activity for HSP70. MB reduces Tau levels in cells and brain tissue

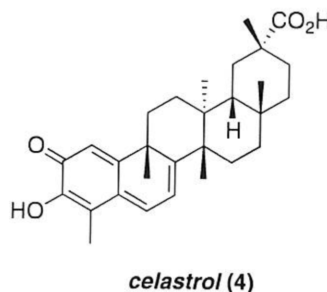

Fig. 4.4 HSP90 inhibitor celastrol (4), a pentacyclic triterpenoid isolated from the root extracts of *Celastrus regelii,* effective for inducing HSP70, HSP27 and HSP32

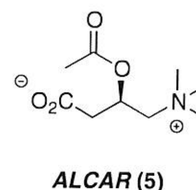

Fig. 4.5 Structure of acetyl-L-carnitine (ALCAR) (5), which exerts protective effects against Aβ peptide by up-regulating GSH and HSP

(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].

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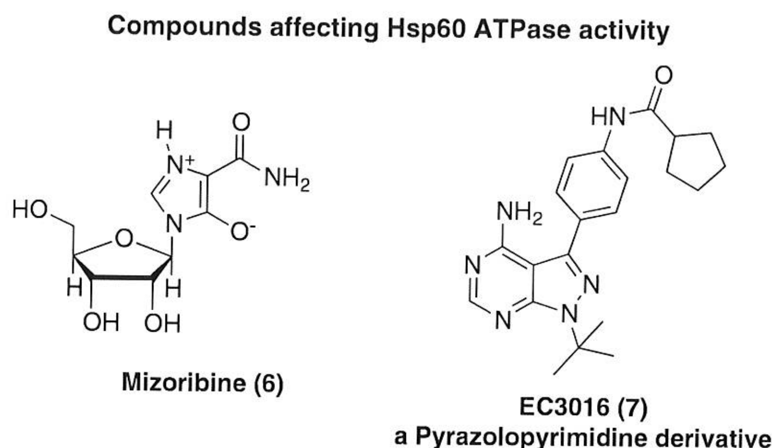

Fig. 4.6 Compounds affecting ATP-related activity of HSP60

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].

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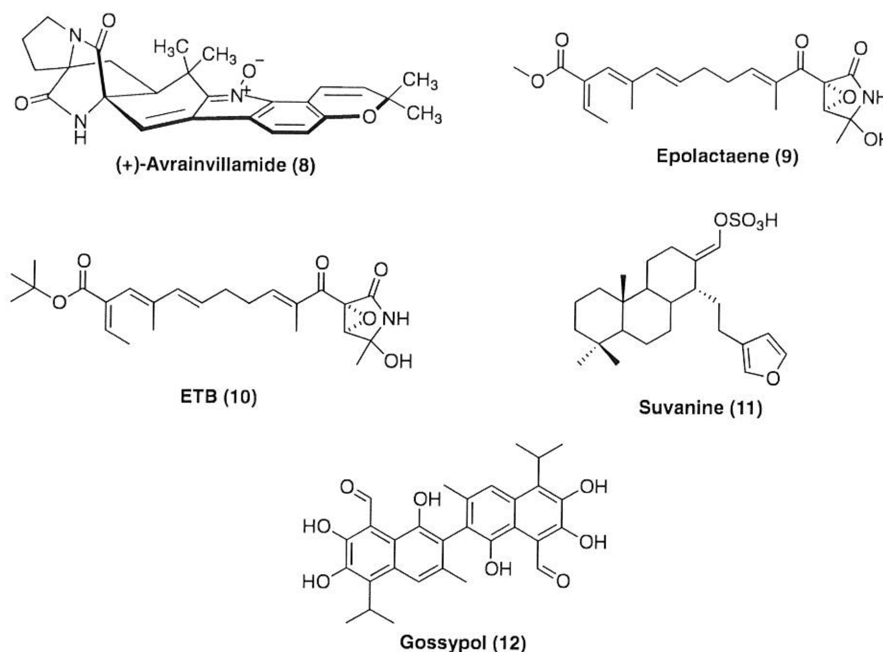

Fig. 4.7 Compounds interacting with cysteine residues of HSP60

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.

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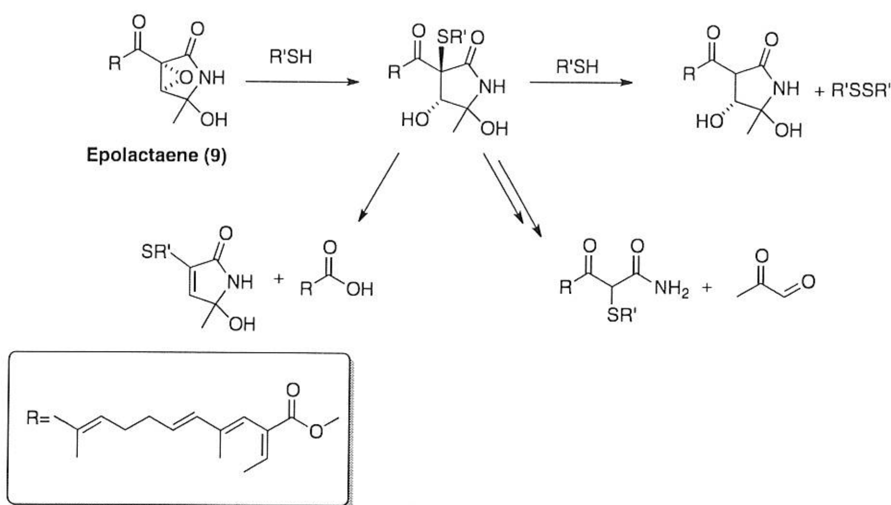

Fig. 4.8 Proposed mechanism for the reaction of epolactaene derivatives with thiols. The epoxide moiety of epolactaene (9) is proposed as binding site for cysteine, due to the electron-withdrawing effect of the two carbonyl groups. The covalent adduct decomposes producing disulfide (R'SSR') formation

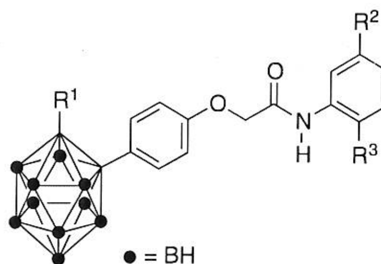

Fig. 4.9 General structure of carboranylphenoxyacetanilide (13). The most active HSP60 inhibitor had $R1 = CH_2CH_3$, $R2 = B(OH)_2$, $R3 = OH$

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].

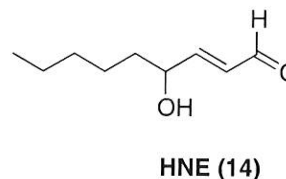

Fig. 4.10 Structure of 4-hydroxynonenal (HNE) (14) targets HSP60, as well as other proteins involved in stress signaling, in a dose-dependent manner

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

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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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