EXPERT OPINION

Hsp60 chaperonopathies and chaperonotherapy: targets and agents

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Introduction: Hsp60 (Cpn60) assembles into a tetradecamer that interacts with the co-chaperonin Hsp10 (Cpn10) to assist client polypeptides to fold, but it also has other roles, including participation in pathogenic mechanisms.

Area covered: Hsp60 chaperonopathies are pathological conditions, inherited or acquired, in which the chaperone plays a determinant etiologic-pathogenic role. These diseases justify selection of Hsp60 as a target for developing agents that interfere with its pathogenic effects. We provide information on how to proceed.

Expert opinion: The information available encourages the development of ways to improve Hsp60 activity (positive chaperonotherapy) when deficient or to block it (negative chaperonotherapy) when pathogenic. Many questions are still unanswered and obstacles are obvious. More information is needed to establish when and why autologous Hsp60 becomes a pathogenic autoantigen, or induces cytokine formation and inflammation, or favors carcinogenesis. Clarification of these points will take considerable time. However, analysis of the Hsp60 molecule and a search for active compounds aimed at structural sites that will affect its functioning should continue without interruption. No doubt that some of these compounds will offer therapeutic hopes and will also be instrumental for dissecting structure–function relationships at the biochemical and biological (using animal models and cultured cells) levels.

Keywords: autoimmunity, cancer, carboranylphenoxyacetanilide, chaperonopathies, chaperonotherapy, chemical compounds, Cpn60, electrophilic compounds, epolactaene, functional domain, GroEL, Hsp60, inflammation, mizoribine, structural domain

Expert Opin. Ther. Targets [Early Online]

1. The chaperonopathies and their treatment

Chaperonopathies are diseases in which molecular chaperones play an etiologic–pathogenic role [1]. The pathogenic chaperone may be normal (or apparently normal, considering our current means of molecular analysis) or structurally altered (mutation or aberrant post-translation modification) and belong to any of the known Hsp-chaperones groups, **Table 1**. The structural alteration of a pathological chaperone may affect any of its functional domains or modules, **Figure 1**. The impact on function will be strongest on, or will exclusively affect, the particular function of the module in which the structural alteration is located.

From the clinicopathological standpoint, quantitative chaperonopathies are characterized by an increase or decrease of the pathological chaperone in the affected cell and tissue [2]. If the increased or decreased chaperone is the etiologic–pathogenic factor, the disease is a primary chaperonopathy. However, in various pathological conditions, one or more chaperones are increased or decreased, but they are not

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Article highlights.

- **Chaperonopathies.** Chaperonopathies are diseases recently organized in a coherent nosological group in which the determinant etiologic–pathogenic factor is a molecular chaperone. In some chaperonopathies, factors other than the pathogenic chaperone may also be involved. The pathogenic chaperone may appear normal at the molecular level (at least considering today's methods of detection of protein abnormalities) or be abnormal. In the latter case, the pathogenic chaperone has structural alterations due to mutation or aberrant-post-translational modification.
- **Types of chaperonopathies.** Chaperonopathies can be classified into genetic (hereditary) and acquired or, considering mechanism and levels of concentration and activity, into by defect, excess or mistake.
- **Hsp60 chaperonopathies.** A chaperone that can cause disease is Hsp60. In humans, its canonical residence is the mitochondrion, but it can also reside and work outside this organelle, in the cytosol, cell membranes, extracellular space, biological fluids (plasma, cerebrospinal fluid) and secretions (saliva, urine). The canonical function of Hsp60 in the human mitochondrion (also named Cpn60) consists of assisting newly synthesized polypeptides in folding correctly. However, Hsp60 plays many other roles beyond the mitochondria that are unrelated to protein folding.
- **Hsp60 functional oligomers and interaction with Hsp10.** To exercise its typical chaperoning role, Hsp60 assembles into an oligomer with a well-defined quaternary structure which, in turn, interacts with an oligomer of the co-chaperonin Hsp10 (Cpn10). The whole process depends on specific sites on the Hsp60 and Hsp10 molecules, most of which have been mapped, and requires ATP hydrolysis.
- **Hsp60 functional structural modules.** The Hsp60 molecule is composed of various functional modules with distinct roles. If any of these modules is altered by a mutation, post-translational modification, or by the binding of a specific chemical compound, its functions may be seriously disrupted. This might lead to failure of the entire molecule with ensuing disease.
- **Genetic Hsp60 chaperonopathies.** Hereditary spastic paraplegia SPG13, and MitCHAP-60 disease are genetic Hsp60 chaperonopathies by defect, in principle amenable to chaperonotherapy by gene or protein replacement.
- **Acquired Hsp60 chaperonopathies.** Examples of Hsp60 acquired chaperonopathies include diverse conditions characterized by chronic inflammation and autoimmunity, and various types of cancer. In these pathologies, Hsp60 plays a pathogenic role as autoantigen, or as inducer of inflammatory cytokines, or as facilitator of cancer development and growth.
- **Hsp60 as therapeutic target.** As seen earlier, there are many Hsp60 chaperonopathies and candidates to be classified as such, pending more studies. Because of the variety of disorders in which Hsp60 is likely to play a determinant role, and because of its wide range of roles and distribution throughout the organism, the development of therapeutic means to either replace or improve the activity of a defective Hsp60 (positive chaperonotherapy) or to eliminate or block it when pathogenic (negative chaperonotherapy) are amply justified. However, because Hsp60 is present in all cells and cell compartments, it will be of the essence to develop very specific compounds and delivery systems targeting only the pathogenic molecule in the abnormal cell, for instance tumor cell.
- **From the present to the future.** This article provides information and suggestions on how and why to proceed with the analysis of human Hsp60 in its various forms and locations to understand its molecular features, normal and abnormal, and the mechanisms by which it causes pathology, and to verify its real value as biomarker of disease. These investigations should provide, for instance, information on how to monitor and control (block or eliminate) this chaperonin when it becomes a pathogenic factor, or to replace it or boost its activity when defective.

This box summarizes key points contained in the article.

etiologic–pathogenic factors; these are secondary chaperonopathies, in which the quantitative alterations of the chaperones are the consequence and not the cause of the disease. In secondary chaperonopathies, the levels of the chaperones affected may serve as indicators (biomarkers) of disease status and response to treatment.

The molecular chaperones and their co-chaperones and cofactors of any given organism constitute the chaperoning system [2]. The components of the chaperoning system form functional teams (chaperoning machines) and networks, involved not only in protein homeostasis (the canonical function of chaperones) but also in various other cellular processes that are not related to protein quality control. Therefore, failure of a single chaperone type may have far-ranging consequences, affecting diverse physiological mechanisms in various tissues, since components of the chaperoning system are present throughout the organism, inside and outside cells, and in circulation.

Chaperonopathies have been classified considering their biologic and molecular mechanisms into by defect, by excess or by mistake, **Table 2** [3]. This classification is useful to establish a diagnosis with physiopathological implications and, thus, aids in decisions on treatment. Further, the classification is instrumental to conduct a differential diagnosis exercise that will lead to an accurate identification of syndrome and disease, which is key to proper patient management.

Another way of classifying chaperonopathies that will also help the physician and, therefore, the patient, is based on whether they are inherited (genetic) or acquired. **Table 3** displays information about the human Hsp60 gene and protein and gives examples of its genetic chaperonopathies [2]. While **Figure 2** outlines the molecular anatomy of Hsp60 (see further), **Figure 3** shows the location of the two amino acids in Hsp60 whose mutation cause disease as described in **Table 3**. The mutations V98I, causing spastic paraplegia SPG13, and D29G, causing MitCHAP-60 disease, were

Table 1. Subpopulations of Hsp-chaperones classified according to apparent molecular weight.

MW (kDa) range	Classical family	Examples of other Hsp-chaperones implicated in the causation of chaperonopathies
200 or higher	None	Sacsin
100 – 199	Hsp100-110	
81 – 99	Hsp90	Paraplegin [SPG7]
65 – 80	Hsp70/DnaK	Spastin [SPG4]; LARP7
55 – 64	Hsp60 (chaperonins Groups I and II, e.g., Cpn60 and CCT, respectively)	Myocilin; protein disulphide-isomerases or PDIs
35 – 54	Hsp40/DnaJ	AIP; AIPL1; torsin A; clusterin; DNAJC19 (TIM14)
34 or less	sHsp (crystallins)	Hsp10 (Cpn10); Alpha hemoglobin-stabilizing protein or AHSP; cyclophilin type peptidyl-prolyl *cis-trans* isomerases or PPIs; alpha-synuclein; HSPB11

Adapted from [2,105].

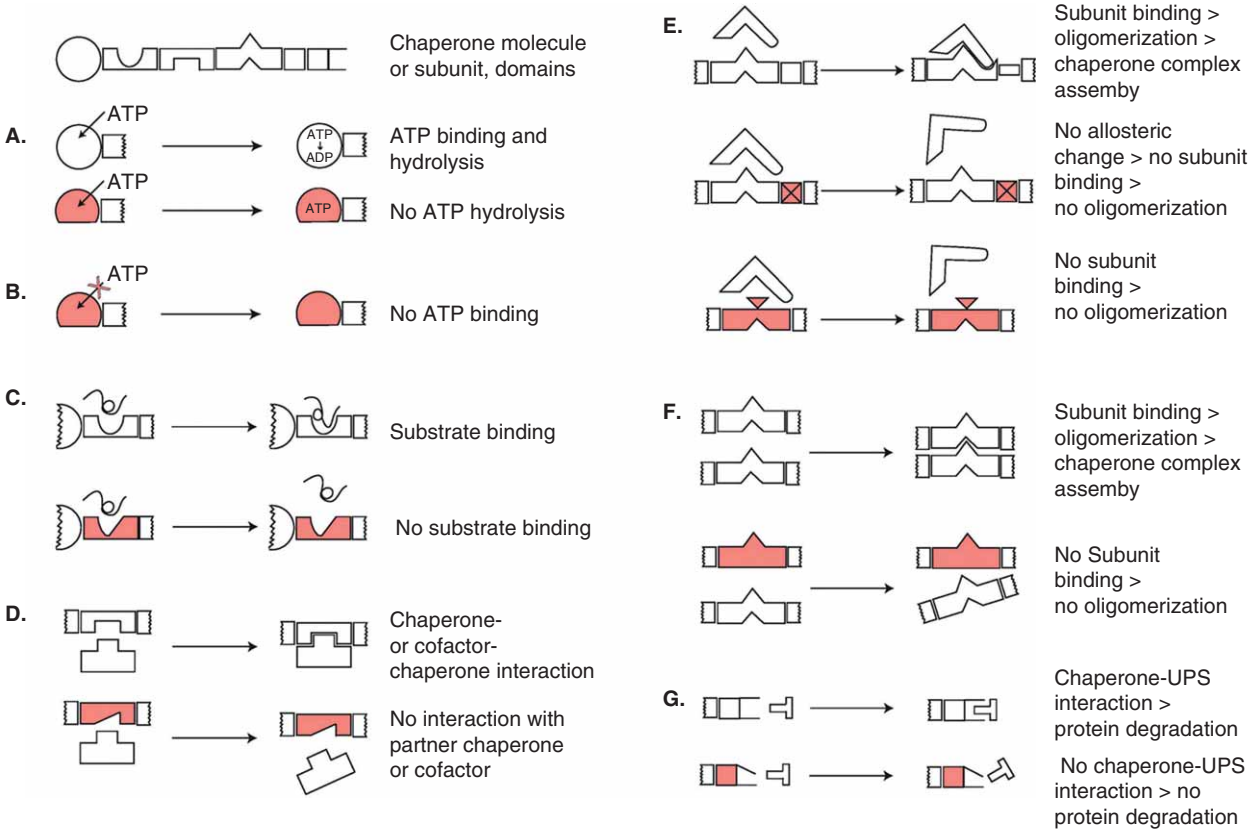

Figure 1. Schematic representation of the structural–functional domains or modules of a typical molecular chaperone. The key, starting with the circle, from left to right in the top-left scheme is ATP-binding-ATPase, substrate binding, chaperone or cofactor–chaperone interaction (needed for the assembly of the chaperoning networks, that is, interaction with other chaperones and chaperoning teams), oligomerization (needed for the formation of oligomers, that is, the chaperoning complex or team such as the homoheptamer formed by Hsp60 or the hetero-octamer characteristic of CCT); hinge (needed for allowing the allosteric changes accompanying all functions of the chaperone molecule – several of these domains are usually present); ubiquitin–proteasome interaction (needed for interaction with the ubiquitin–proteasome system for protein degradation). Filled forms represent structurally altered domains due to mutation or post-translational modification, or to the binding of a chemical compound. Note: Not all chaperones have all these functional modules, and they are not necessarily distributed along the molecule as shown in this schematic drawing.

UPS: Ubiquitin-proteasome system.

Adapted from [2].

Table 2. Classification of chaperonopathies according to pathogenic mechanism.

Chaperonopathies by	Mechanism, features
Excess	Quantitative, e.g., due to gene dysregulation and overexpression Qualitative, e.g., gain of function
Defect	Quantitative, e.g., gene downregulation Qualitative, e.g., due to structural defect genetic or acquired
Mistake	Normal chaperones can contribute to disease, e.g., some tumors that need chaperones to grow; autoimmune conditions in which a chaperone is the autoantigen; and possibly also prion diseases in which chaperones may be required for propagation.

Adapted from [2,3].

Table 3. Human Hsp60 gene and protein, and mutations.

Gene	Name	Cytogenetic location	Structure	Protein
HSPD1 HGNC:5261	Hsp60; HSP60; HSPD1; heat-shock 60-Kd protein 1; chaperonin, 60-Kd; CPN60; Cpn60; GroEL, *E. coli*, homolog of	*2q33.1*	12 exons (first non-coding) Variants: 2	UniProtKB/Swiss-Prot P10809 573 aa Isoforms: 2
Mutation	**Disease (mutation phenotype) name (and synonyms)**	**Phenotype MIM number**	**Gene locus MIM number**	
Val98Ile (V98I) Gln461Glu (Q461E)	Spastic Paraplegia 13, autosomal dominant; SPG13	605280	118190	
Asp29Gly (D29G)	Leukodystrophy, hypomyelinating, 4; HLD4; mitochondrial HSP60 chaperonopathy; MitCHAP-60 disease	612233	118190	

Adapted from [2,106,107], and the following Databases: Hugo Gene Nomenclature Committee (HGNC; http://www.genenames.org/); Online Mendelian Inheritance in Man (OMIN; http://omim.org/); UniProt (http://www.uniprot.org/); National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/guide/); and PubMed-NCBI (http://www.ncbi.nlm.nih.gov/pubmed).

described several years ago [4-6]. D29 is at a position located in the equatorial domain interface and does not seem to be involved directly in the interactions between Hsp60 monomers. However, aspartic acid (D) is medium sized, acidic, acyclic, negative and polar, whereas glycine (G), which replaces it in the mutant, is acyclic, aliphatic, hydrophobic, small sized and neutral. It is expected that these differences between the wild type and the mutant residues will affect charge distribution and equatorial domain interaction, resulting in impairment of the Hsp60 functions. Valine (V) is acyclic, aliphatic, hydrophobic, medium sized and neutral, whereas isoleucine (I), which replaces it in the mutant, is acyclic, aliphatic, hydrophobic, large and neutral. The 98th position is critical because it is very close to the end of the sequence 85-AGDGTTTATVL-95 implicated in ATP and Mg^{+} binding [7]. It is, therefore, probable that isoleucine instead of valine at that position will have a strong impact (e.g., steric hindrance due to its large size) on nucleotide and ion binding, which will inactivate the chaperonin. See also **Figure 1A and B**.

Acquired chaperonopathies affecting Hsp60 are discussed later. Chaperonotherapy includes any therapeutic means targeted to a chaperone with the purpose of correcting a chaperonopathy [8]. When the chaperone is deficient, the aim is to replace it or to boost its functionality, and we can refer to this therapeutic modality as positive chaperonotherapy. If, on the contrary, the chaperone favors disease rather than protection of the cell and the organism, the therapeutic strategy aims at eliminating or blocking the pathological chaperone. This latter therapeutic modality may be referred to as negative chaperonotherapy. These basic premises are important in practice because they offer the clinicians and pathologists a stand point that provides a good perspective of the field and its subfields and, thus, enables them to envisage what is best to control the disease at hand and treat the patient more effectively. Further, these basic ideas allow a better organization of data than would be possible without a scaffold on to which new information can be added and, consequently, these ideas will show possible routes for investigation and for developing novel treatments. The concept that a chaperone may be a determinant etiologic–pathogenic factor in some diseases should open a new window to look at patients and discover pathogenic mechanisms until now either misinterpreted or simply ignored. No doubt that medical and pharmacological research will benefit from this novel outlook. In this article, we present a brief account on Hsp60 chaperonopathies and on the possible ways that might be followed for developing

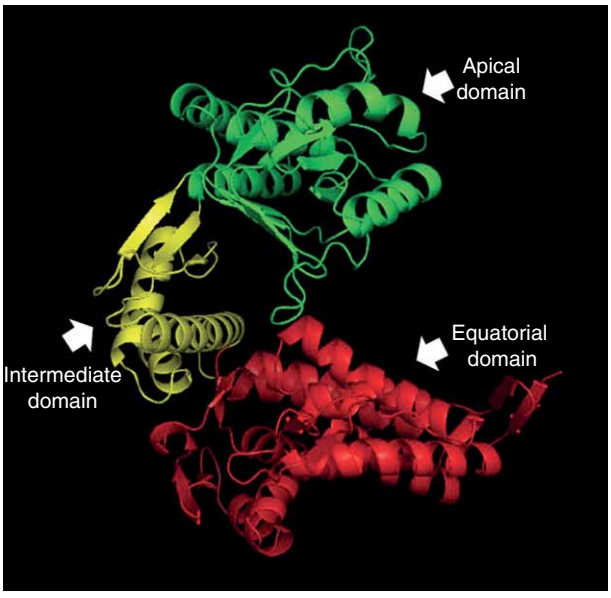

Figure 2. The anatomy of the human Hsp60: structural domains. The cartoon represents a three-dimensional model of the human Hsp60 monomer showing its three structural domains: apical in green, intermediate in yellow and equatorial in red. These structural domains are not the same as the functional domains or modules schematized in **Figure 1** but contain them. The material and methods for constructing the images in this **Figure 2** and subsequent **Figures 3,4,5,6,7,** were as follows: The amino-acid sequence of the human Hsp60 was retrieved from the PubMed website (http://www.ncbi.nlm.nih.gov/genbank/), using the accession number NM_002156. The three-dimensional model of the Hsp60 monomer was build using a fully automated protein structure homology-modeling server named SWISS-MODEL (http://swissmodel.expasy.org/) [203-205] accessible via the ExPASy web server (http://www.expasy.org/). The three-dimensional model of the tetradecameric GroEL alone and of the Gro-EL/GroES complex (**Figures 8,9,10,11**) were obtained from http://www.rcsb.org/pdb/home/home.do with PDB codes 4AAR and 1PQC, respectively [206,207]. All models were visualized and modified by PyMol (http://www.pymol.org).

treatments targeting the chaperonin. For instance, the diseases referred to in **Table 3** are caused by severe impairment of Hsp60 functions and should be considered candidates for positive chaperonotherapy, using the normal Hsp60 gene or protein to replace the defective molecule in the patients.

2. The concept of chaperonopathy by mistake: the case of Hsp60

Chaperonopathies by mistake occur when a normal (or apparently normal since some changes, for instance some post-translational modifications, may be difficult to detect with technology available today) chaperone functions to favor a pathogenic mechanism leading to a disease. Examples are some forms of autoimmune and chronic inflammatory diseases as well as some forms of cancer. In the following paragraphs, we report information concerning diseases in which Hsp60 act as a) autoantigen, participating in the mechanism of autoimmunity; b) chaperokine, inducing cytokine production and leading to chronic inflammation; and c) factor promoting cancer initiation and/or growth, and/or local spread, and/or distant spread (metastasis), and/or resistance to treatment, or any other procancer mechanism [2].

The participation of Hsp60 in many of these diseases has been inferred from observations of very close correlations between abnormalities in the chaperonin and disease onset, progression, response to treatment and patient status with time and between the histological location of the chaperonin with levels of inflammatory cells and levels of cytokines in the pathological tissue. The chaperonin abnormalities are not only quantitative, that is, increase or decrease, but also in distribution, for example, the chaperonin may appear or increase above normal levels in places other than the canonical location inside the mitochondria, such as the cytosol, vesicles, the plasma membrane and blood; or it may appear at detectable levels in cells in which it is normally undetectable. All these observations strongly implicate Hsp60 in pathogenesis; but in most instances, a direct demonstration of cause effect is still incomplete, and research must continue to elucidate this critical issue. Nevertheless, it is safe to say that the future is promising, as indicated by a series of reports, some of which have been highlighted in the Bibliography.

3. Hsp60 and autoimmune diseases

Both, microbial and human Hsp60 have been proposed to have a role in the pathogenesis of autoimmune diseases. The mechanism by which Hsp60 may act as autoantigen is based on molecular mimicry due to the high sequence similarity between human and foreign Hsp60 from bacteria and parasites that colonize humans, which leads to anti-Hsp60 antibody cross-reactivity [2] For example, mycobacterial Hsp65 (mHsp65), the active factor in complete Freund's adjuvant used to induce autoimmune disease in experimental animals, stimulates innate immunity via TLR pathways [9]. Another example pertains to the Hsp60 from *Chlamydia trachomatis* serovar D, which was compared with the human counterpart [10]. The amino-acid sequences were aligned and a high percentage of identity was found in 17 regions that 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 [11]. **Table 4** shows a list of diseases in which molecular mimicry between microbial and human Hsp60 may be involved in generation of autoimmunity.

The high structural similarity between Hsp60 and other human proteins (e.g., myelin-associated protein, glutamic

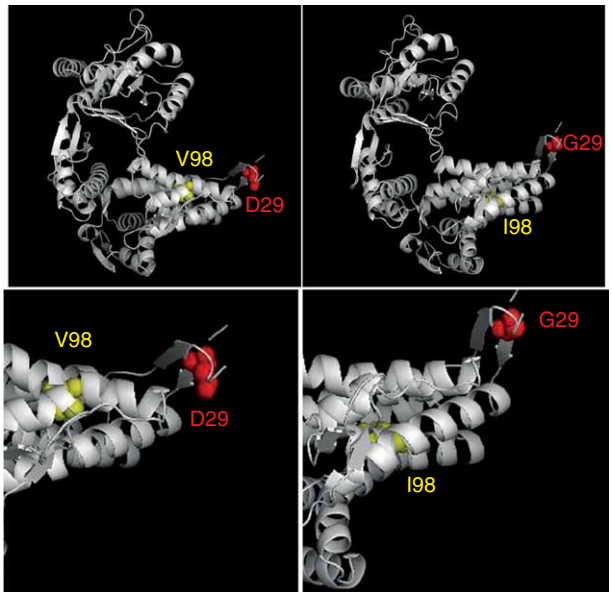

Figure 3. Mutations that cripple Hsp60 and cause disease in humans. The top panels represent the whole Hsp60 molecule, whereas the bottom panels show enlarged the portion of the molecule in which the mutations occur. Left panels: wild type Hsp60, with the amino acids V98 (yellow) and D29 (red) highlighted. Right panels: the mutants V98I, causing spastic paraplegia SPG13 (yellow), and D29G, causing MitCHAP-60 disease (red), are highlighted. These mutations appear to alter the molecular anatomy of Hsp60 to the point of making it functionally defective (see text, **Table 3**, and **Figure 1A** and **B**).

acid decarboxylase and acetylcholine receptor) suggested that Hsp60, when released into the circulation, may serve as autoantigen for generating an autoimmune response [12]. In turn, it may trigger autoimmune pathological conditions, too, like multiple sclerosis, type 1 diabetes, atherosclerosis and *Myasthenia gravis*. **Tables 5** and **6** contain lists of diseases in which cross-reactivity between human Hsp60 and other human molecules may be involved in generating autoimmunity.

4. Hsp60 and chronic inflammatory diseases

Hsp60 has been shown to activate inflammatory cells that are induced to produce cytokines and other mediators of inflammation. Hence, Hsp60 is able to function as "chaperokine" [13]". For example, it can activate human monocyte/macrophage cells by binding to CD14 with subsequent activation of p38MAPK, in turn stimulating these cells to synthesize proinflammatory cytokines (such as TNFa, IL-12 and IL-15) and nitric oxide [14,15]. Hsp60 can bind to TLR2 and TLR4 expressed on T cells and, thereby, contribute to either induction or termination of immune responses by controlling the activities of innate and adaptive immune cells [16]. Moreover, Hsp60 can bind to the neutrophils' plasma membrane, enhancing production of oxidants and release of proteases [17].

Expression of Hsp60 can be triggered by various stressors typical of chronic inflammatory diseases, as shown for example for atherosclerosis [18]. Importantly, the expression of Hsp60 in stressed vascular endothelial cells is always accompanied by the simultaneous expression of adhesion molecules. This situation then allows for attachment of Hsp60-reactive T-cells to target endothelial cells.

We found elevated levels of Hsp60 in the macrophages of the lamina propria in the large-bowel mucosa of patients with ulcerative colitis (UC) [19]. The number of Hsp60-positive macrophages diminished after therapy in parallel with a reduction of mucosal inflammation and with amelioration of the patients' symptoms, thus providing evidence that Hsp60 is associated with inflammation in UC. Moreover, we found Hsp60 in the neutrophils of the lamina propria in the airways mucosa of patients with chronic obstructive pulmonary disease (COPD) [20]. We also found a correlation between the number of Hsp60-positive cells and the number of neutrophils in patients with COPD, suggesting that this chaperonin plays a role in controlling neutrophil functions and, in turn, bronchial inflammation. **Table 6** shows a list of chronic inflammatory conditions in which Hsp60 has been implicated as a stimulator of inflammation. Some of the pathologies listed in **Table 6** are autoimmune conditions. This is due to the fact that autoimmune and inflammatory chronic diseases can overlap.

5. Hsp60 and cancer

Hsp60 levels change during the carcinogenetic steps in various organs, such as oral cavity [21], uterine cervix [22], large bowel [23], prostate [24] and the bronchial tree [25,26]. This implies that this chaperonin is involved, directly or indirectly, in carcinogenesis of these organs. **Table 7** lists examples of tumors in which Hsp60 levels have been found changed, in comparison with the normal tissue counterparts. Also, cellular distribution of Hsp60 changes during carcinogenesis: the chaperonin accumulates in extramitochondrial sites, such as cytosol, plasma membrane and secretory vesicles [27,28]. Data from various laboratories indicate that extramitochondrial Hsp60 may have some direct role in tumor transformation of normal tissues as well as in tumor progression. For instance, (i) cytosolic Hsp60 is involved in caspase-3 cleavage and apoptosis activation in non-tumor [29,30] and in tumor [31,32] cells; and (ii) cytosolic Hsp60 is secreted by tumor cells by both, the classic (Golgi's) and alternative (exosomal) pathways [28]. It remains to be established how the Hsp60 molecule reaches the cytosol, plasma membrane and secretory vesicles. Questions still unanswered are the following: Does Hsp60 migrate out of the mitochondria into the cytosol, or does it remain in the cytosol after being synthesized, or both?

Table 4. Examples of diseases in which molecular mimicry between microbial and human Hsp60 may be involved in generation of autoimmunity.

Organ/system	Disease	Pathogen	Ref.
Central nervous system	Multiple sclerosis	Unknown	[108]
Heart	Ischemic disease	*Chlamydia pneumoniae*	[109]
Lung	Asthma	*Chlamydia pneumoniae*	[110]
Muscle (neuromuscular junction)	Myasthenia gravis	*Chlamydia trachomatis*	[111,112]
Vessels	Atherosclerosis	*Escherichia coli* and *Chlamydia pneumoniae*	[113]
		Helicobacter pylori	[114,115]
		Porphyromonas gingivalis	[116]

Adapted from [2].

Table 5. Examples of diseases in which cross-reactivity between human Hsp60 and other human molecules may be involved in generating autoimmunity.

Organ/system	Disease	Human molecule with high similarity to Hsp60	Ref.
Adrenal gland	Addison's disease	17 alpha Hydroxylase	[117]
		21 Hydroxylase	[118]
Central nervous system	Multiple sclerosis	Myelin-associated protein	[119]
		Neurofilament triplet M protein	[120]
Cerebellum	Paraneoplastic cerebellar degeneration	Cerebellar degeneration associated protein	[121]
Heart	Coxsackie myocarditis	Cardiac myosin heavy chain	[122]
Kidney	Basement membrane disease	Laminin beta 2 chain	[123]
	Glomerulonephritis	Myeloperoxidase	[124]
Liver	Chronic active hepatitis	Cytochrome P450	[125]
	Primary biliary cirrhosis	Pyruvate dehydrogenase	[126]
		NADH dehydroreductase	[127]
		Dihydrolipoamide dehydrogenase	[128]
Lung and kidney	Goodpasture's syndrome	Laminin beta 2 chain	[123]
Neuromuscular junction	Myasthenia gravis	Acetylcholine receptor	[129]
Pancreas	Diabetes insulin dependent	Glutamic acid decarboxylase	[130]
Skin	Pemphigoid	Bullous pemphigoid antigen	[131]
	Scleroderma	KU autoimmune antigen	[132]
Synovial joints	Rheumatoid arthritis	Cytokeratin	[118,133]
Thyroid	Hashimoto's thyroiditis	Thyroglobulin	[134]
Various Systems	Systemic lupus erythematosus	DNA-binding protein	[135]
		Hsp90	[136]

Adapted from [2].

The quantity of Hsp60 contained in tumor-derived exosomes depends on the action of some stressors [33,34], and the Hsp60-carrying exosomes appear to be involved in modulating antitumor immune response [33].

Hsp60 has been found to accumulate in lymph node-invading tumor cells as well as in neoangiogenic islets in primary tumors [35], both findings suggesting involvement of Hsp60 in lymph node and distant organ metastasis.

Azacytidine, a chemotherapeutic agent, has been found to induce an increase of Hsp60 levels in tumor cells, and this increase has been related to an increased tumor resistance to therapy [36].

These data and many others from diverse laboratories let us to postulate that some forms of tumor may now be considered chaperonopathies by mistake (or by collaborationism, since the chaperone collaborates with the tumor, i.e., the "enemy"). In these cases, Hsp60 participates actively in tumor formation and progression by protecting tumor cells from external environment stressors and by favoring their proliferation. As a consequence, the inhibition of the protumoral Hsp60 effects (negative chaperonotherapy) may be a new therapeutic approach for treatment of some tumors.

6. Hsp60 can interact directly with molecules in various cell compartments

Hsp60 is classically described as a mitochondrial protein localized to the mitochondrial matrix, constitutively expressed

Table 6. Examples of chronic inflammatory conditions in which Hsp60 has been implicated as a stimulator of inflammation.

Organ	Disease	Ref.
Heart	Heart failure; Coronary vascular disease	[137-140]
Kidney	Glomerulonephritis	[141]
Large bowel	Inflammatory bowel diseases	[19,142,143]
Lung	Chonic obstructive pulmonary disease	[20]
Oral cavity	Periodontitis	[144,145]
Pancreas	Type 1 diabetes	[146-151]
Skin	Scleroderma, pemphigoid; Psoriasis; Dermatomyositis	[152,153]
Synovial joints	Rheumatoid arthritis; Juvenile idiopathic arthritis	[154-157]
Vessels	Vasculitis; Atherosclerosis	[158-167]

Adapted from [2].

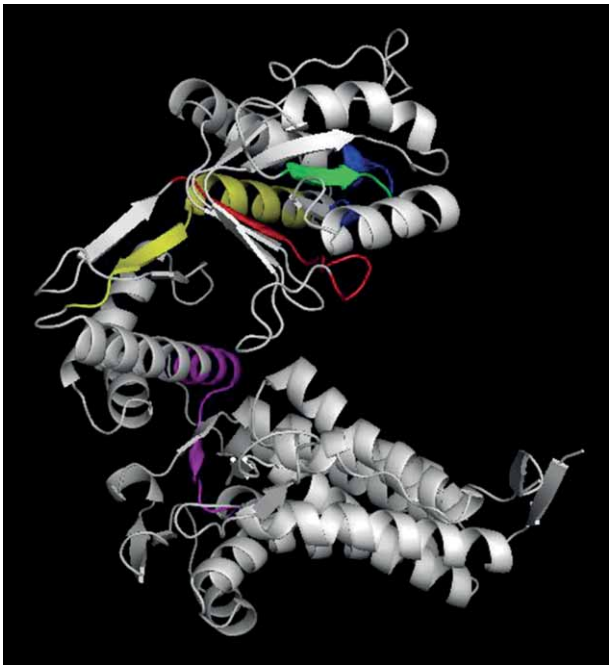

Figure 4. Highly conserved sequence segments in human Hsp60 that have very similar counterparts in many other Hsp60 and GroEL molecules from a variety of eukaryotic and bacterial species. Shown are five highly conserved sequence segments: 191 – 203 in red, 246 – 253 in green, 275 – 286 in blue, 363 – 382 in yellow, and 402 – 416 in magenta. These molecular segments are potentially immunogenic and antigenic epitopes and, because of their similarity in many species, could be cross-reactive sites for anti-Hsp60 antibodies and drugs.

under normal conditions, and induced by heat shock, mitochondrial damage, mtDNA depletion and other kinds of stressors [37]. However, a number of studies demonstrated that Hsp60 can also localize to extramitochondrial sites such as the cytosol and the plasma membrane, and circulate in the peripheral blood [38,39].

Although the complete list of client proteins for mitochondrial Hsp60 is not fully known, several studies demonstrated that Hsp60 can interact with other proteins also in the extramitochondrial environment. **Table 8** shows a list of proteins involved in several cellular pathways with which Hsp60 is able to interact directly.

In mitochondria, Hsp60 works as a folding machine, interacting with ATP, Hsp10 and mtHsp70 [40,41]. Aberrant folding, for example, misfolding, is involved in a number of severe conditions such as Parkinson's and Alzheimer's diseases, familiar amyloidotic polyneuropathy and bovine spongiform encephalopathy, as well as its human counterpart, Jakob–Creutzfeldt Disease [42,43]. In addition, atypical mitochondrial diseases with multisystem failure have been linked to a deficiency in Hsp60 [44].

The substrates of the Hsp60/Hsp10 system are poorly defined, but the combination of deficiency of mitochondrial enzymes and proteins that are highly dependent on this chaperoning system for folding is the likely trigger for many diseases [44,45]. For instance, Hsp60 is important for the correct folding and assembling of dihydrofolate reductase [46] and carbonicanhydrase II (HCAII) [47]. HCAII is the key enzyme for acid–base balance, respiration, carbon dioxide and ion transport, and also bone resorption, ureagenesis, gluconeogenesis, lipogenesis and body fluid generation in various tissues [48]. Hsp60 specifically associates with mitochondrial ATP synthase, the enzyme necessary to produce and hydrolyze ATP [49]. It was demonstrated that this interaction occurs also on the endothelial cell (EC) surface and, as a consequence from the therapeutic point of view, it is important to protect ATP synthase from degradation on EC membranes. Cell surface ATP synthase acts to bind several ligands and to control EC proliferation and differentiation. Its alteration causes damages to EC homeostasis and plays a role in vasculitis pathogenesis [49]. Hsp60 interacts also with many factors involved in apoptotic pathways and cell cycle regulation [37]. The actions of Hsp60 could lead to the modification of those proteins, and in this manner it could have a role in many diseases, including carcinogenesis.

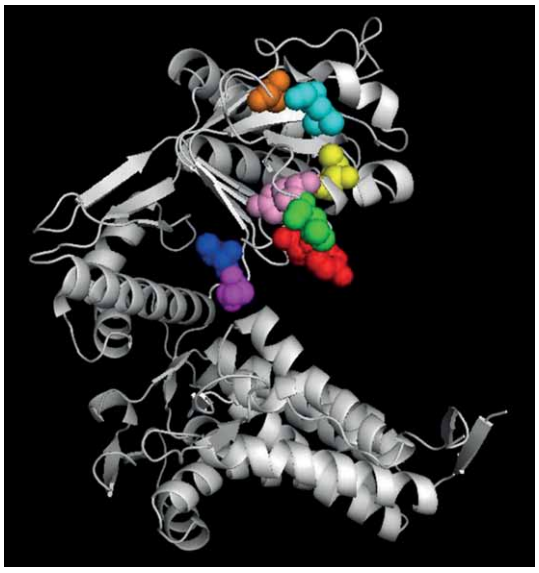

Figure 5. Amino acids involved in substrate binding in Hsp60. Shown are hydrophobic/aromatic positions possibly involved in substrate binding: Y199 red, Y203 blue, F204 magenta, L234 cyan, L237 orange-brown, L259 yellow, V263 pink and V264 green. Substrate (client polypeptide in need of assistance for folding) binding is a key step in the chaperoning process by Hsp60 and any alteration (or blocking) of the structures involved is likely to impair protein folding.

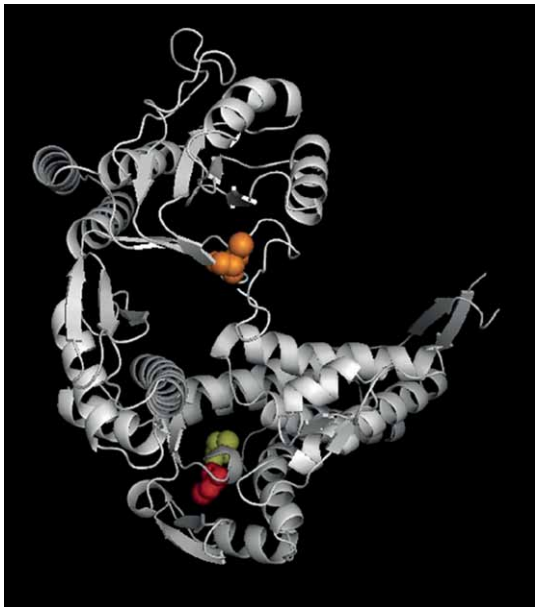

Figure 6. Cysteine residues are present in human Hsp60 but not in GroEL. The residues C237 (orange-brown), C442 (red), and C447 (lemon-green) are shown. These residues have no counterpart in GroEL and constitute convenient nucleophilic binding sites for electrophilic drugs (see Section Hsp60 inhibitors: new drugs for old diseases).

Hsp60 seems to contribute to tumor cell survival, but this is controversial. For example, it is known that Hsp60 interacts with procaspase-3 both in mitochondria and cytosol [29-32]. In diverse cancer cells, it was demonstrated that Hsp60 in combination with Hsp10 accelerates maturation of caspase-3 with concomitant mitochondrial activation of caspase-3 and release of cytochrome c and Hsp60, participating in caspase-3 activation by acting as a chaperone to promote maintenance of procaspase-3 in a protease-sensitive state [29,30].

By contrast, other studies showed that Hsp60 may have an antiapoptotic function through the binding of Hsp60 with caspase-3 without activation of the latter [31,32]. In addition, in cardiac myocytes, Hsp60 has antiapoptotic roles by forming a macromolecular complex with Bax and Bak and, thereby, blocking their ability to induce apoptosis [50].

Since Hsp60 is abundantly expressed in many human tumors [51], Hsp60 may be considered a cancer-specific gene product, a biomarker, involved in protection from apoptosis of transformed cells. Hsp60 binds survivin, stabilizing its levels, and curbing p53 function [52]. Elimination of Hsp60 results in loss of the mitochondrial pool of survivin, p53 increase and activation of p53-dependent apoptosis in tumor cells [52].

In addition, Hsp60 has a role in metastasization [53]. It was demonstrated in a patient with metastatic head and neck squamous cell carcinoma that the role of Hsp60 in metastasization occurs through activation of β-catenin, which has also been implicated in promoting metastasis in various other types of tumors [54]. Co-expression patterns of Hsp60 and β-catenin in this model support a mechanism of β-catenin activation by Hsp60 overexpression [53]. Hsp60 can also be found on the surface of normal and tumor cells, and it appears to be involved in the immune system activation and tumorigenesis [38]. Surface Hsp60 has been found associated with α3β1-integrin, a protein involved in the adhesion of metastatic breast cancer cells [55]. Several aspects of tumor progression and development, including proliferation, modulation of differentiation, invasion of surrounding tissues and metastasis have been shown to be dependent on α3β1-integrin activity. Induction of α3β1-integrin activity results in enhanced motility and adhesion of breast cancer cells. In addition, α3β1 integrin stimulates endothelial cell proliferation and angiogenesis. Therefore, therapeutic means that selectively inhibit α3β1-integrin activation or ligand binding, by targeting integrin or its associated Hsp60, may inhibit tumor progression by disrupting the function of α3β1-integrin in tumor cells and tumor vasculature [55].

The surface-exposed Hsp60 is also an agonist of the microglial TREM 2 receptor. TREM 2 is a receptor expressed by osteoclasts, and by myeloid and microglial cells, it has a protective role in bones and brain. Mutation of TREM 2 determines genetic disorders affecting bones and brain. Hsp60 present onto the surface of astrocytes and neuroblastoma cells interacts with TREM2 in pathological conditions, activating phagocytosis by microglial cells, while in normal

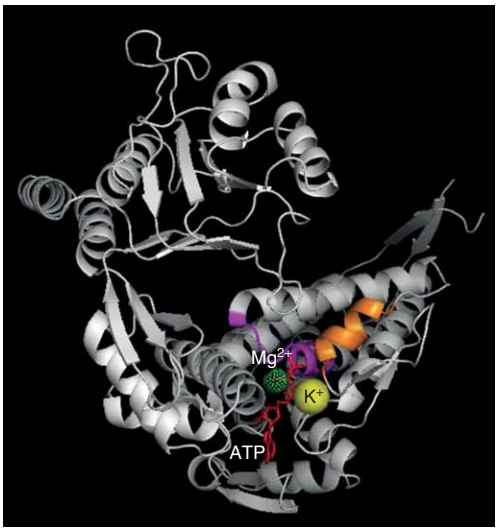

Figure 7. ATP and Mg^{+}/K^{+} binding sites on Hsp60. Shown are two highly conserved sequences: 52-DGVTVAKEI-60 (orange-brown), and 85-AGDGTTTATVL-95 (magenta) corresponding, to the binding sites for Mg^{+}/K^{+} (green and yellow spheres, respectively) and ATP/ADP (red).

conditions the TREM 2/Hsp60 complex has a role in coordinating the functions of the various brain cell types [56].

Hsp60 also occurs in the extracellular space and in circulation exported outside the cells via microvesicles such as exosomes [28]. It is assumed that circulating Hsps can have immunostimulating as well as immunosuppressing effects, depending on the circumstances in which the Hsps interact with other cells. Extracellular Hsp60 can interact with a variety of receptors present on the plasma cell surface, such as CD14, CD40, CD91 and TLRs [57,58]. Moreover, it was demonstrated that Hsp60 is an inductor of inflammatory adipocyte activity. Hsp60 binds an adipocyte receptor and, thereby, Hsp60 influences the proinflammatory capacity of adipocytes, thus contributing to obesity-associated inflammatory disease leading to diabetes [59,60].

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) has previously been identified as a novel receptor for Hsp60, and we hypothesize that LOX-1 through binding to extracellular Hsp60 promotes microglia-mediated neuroinflammation. LOX-1 is essential in microglia for promoting an inflammatory response in the presence of soluble neuronal-injury signals such as extracellular Hsp60, thereby linking neuroinflammation and neurotoxicity [61].

In conclusion, knowledge about these interactions is essential to developing new treatments for Hsp60 chaperonopathies. The Hsp60 interactions in many cases can promote disease. In this case, treatment ought to consider inhibition blocking of the Hsp60 interactions (negative chaperonotherapy). In the other cases, when the interaction of Hsp60 with another molecule is antidisease, treatment ought to stimulate, namely facilitate that interaction (positive chaperonotherapy).

The list of molecules that interact directly with Hsp60 (**Table 8**) is very important because it indicates possible targets for therapeutics focusing on Hsp60, either to block the interaction or to favor it.

7. Hsp60 structural features key for function and potential targets for therapeutics

In this section, we provide images, cartoons, of the Hsp60 molecule that represent approximations of what the real molecule might look like. We emphasize features of the Hsp60 molecule that can help to a) understand structure–function relationships, including the assembling of a functional chaperoning machine; b) infer the consequences that amino acid changes (mutation or post-translational modification) might have on these structure–function relationships; c) visualize possible focal targets for the binding of chemical compounds with predictable high probability of profound functional effects – this would be useful in therapeutics; d) likewise, identify sites on to which one could ligate reactive compounds that can help in following Hsp60 as a biomarker – this would be useful in diagnosis and disease monitoring as well as in research; and e) understand why anti-Hsp60 antibodies from any given organism can cross-react with the Hsp60 from many others.

We hope that these simple images will help nonstructural biologists, physicians and pathologists, in figuring out what portions of the molecule, that is, one or a few amino acids, build sensitive spots amenable to binding by antibodies and/or by specifically designed chemicals that will produce a desired effect, such as inhibition of a given function in a pathogenically active Hsp60, or the functional boosting of a defective chaperonin. For a more accurate description of the chaperonin GroEL and the ATP-driven mechanism of protein folding (see below), the reader may consult [62-64].

Hsp60 (also called Cpn60) and its co-chaperone Hsp10 (Cpn10) represent the protein folding apparatus of mitochondria, and the process in which they participate has been studied using the bacterial homologues GroEL and GroES, respectively [65]. The original experiments performed on *Escherichia coli* led to the characterization of GroEL and GroES and, by extension, facilitated the understanding of the structure and function of the eukaryotic homologs Hsp60 (Cpn60) and Hsp10 (Cpn10) [66]. Human Hsp60 in the mitochondria, similarly to bacterial GroEL, forms a ring-shaped homo-oligomer of seven subunits, and two rings associate to form a tetradecamer shaped like a barrel with a central cavity, which can accommodate polypeptide substrates of up to 50 kDa or so. The preferred substrates of Hsp60/Hsp10 complex are unfolded proteins, whose folding is catalyzed in an ATP-dependent manner. Shortly after GroEL binds one ATP molecule, the client polypeptide to be folded interacts with the inner side of the central cavity by means of hydrophobic residues and ATP hydrolysis induces conformational changes

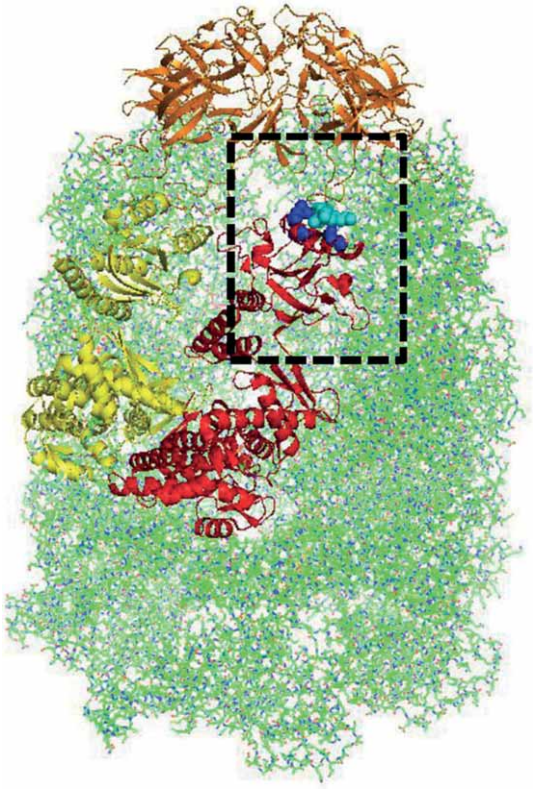

Figure 8. Amino acids involved in the interaction between GroEL and its co-chaperone GroES. The cartoon represents a three-dimensional model of the GroEL/GroES complex from *Escherichia coli* showing two monomers (yellow on the left, and red on the right) of the top ring of GroEL, and GroES (orange-brown) on the top. Framed and highlighted are residues that are thought to be crucial for the interaction between GroEL and GroES as follows: the hydrophobic conserved residues L234 and L237, and N265 on the surface of GroEL are shown in blue, while the hydrophobic residues I25, I26, L27, and A31 on the surface of GroES are shown in cyan.

(from *cis* to *trans* conformation). After these changes, GroES and GroEL separate and the folded protein is released. GroES, and presumably also Hsp10 in the mitochondria, act as a cap covering the central cavity of the tetradecamer, opening and closing the structure coordinating the behavior of the Hsp60 monomers and regulating ATP hydrolysis [65,67,68].

In **Figure 2**, it is shown that each Hsp60 monomer is constituted of three structural domains named apical, intermediate and equatorial; it is important to realize that these domains are distinct from the functional domains or modules schematized in **Figure 1**, but they contain them.

On the basis of data from the GroEL crystal structure and from the alignment of many diverse Hsp60 sequences from different species, including humans, various authors, including us, have identified highly conserved sequence segments and residues, some of which are shown in **Figure 4**. The apical domain includes two conserved sequences: 246-PLLII-AED-253 (green), which contains five aliphatic residues, and 275-AVKAPGFGDRRK-286 (blue) that is enriched in charged residues. The conserved sequence 191-EGMQFDR-GYISPY-203 (red) traverses the transition region from the intermediate to the apical domain and includes several aromatic residues for substrate binding sites. Another two conserved segments are 363-EKLQERLAKLAGGVAVIKVG-382 (yellow), which connects the intermediate with the apical domain; and 402-ATRAAVEEGIVPGGG-416 (magenta), which connects the intermediate with the equatorial domain. The charged residues at positions 275 – 286 (blue) and 363 – 382 (yellow) are exposed to the central cavity in the *cis* conformation while the glycine triplet at positions 402 – 416 (magenta) binds ATP/ADP [69,70]. These similarities among Hsp60 molecules from a variety of species are at the basis of the immunological cross-reactions observed in many diseases in which Hsp60 plays a role (see Section 3: Hsp60 and autoimmune diseases).

Hydrophobic/aromatic residues that contribute to substrate binding are also highly conserved and are located in the apical domain. These positions are: Y199, Y203, F204, L234, L237, L259, V263 and V264 (**Figure 5**) [71,72]. Since substrate binding is in fact a *sine qua non* condition for folding, any alteration (mutation, post-translational modification, modification or blocking with chemical compounds) of the Hsp60 residues involved in this process may result in a serious functional defect (see **Figure 1C**), leading to protein misfolding and aggregation. Defects in substrate binding as well as any of the other Hsp60 defects outlined in **Figure 1** may lead to disease (e.g., protein misfolding conditions). On the contrary, the substrate-binding structures or any of the other critical functional modules in Hsp60 offer an opportunity for therapeutics if the chaperonin is actively involved in pathogenesis. In the latter case, inhibition-blocking of Hsp60 (negative chaperonotherapy) may be a way to stop or delay disease progression (see, for example, Section 8: Hsp60 inhibitors: new drugs for old diseases).

The human Hsp60, but not GroEL, has cysteine residues (Cys237, Cys442 and Cys447), shown in **Figure 6**, that are of interest for developing Hsp60-binding compounds (see discussion later).

The equatorial domain contains the residues at positions 52 – 60 and 85 – 95 implicated in the binding of ATP/ADP and Mg^{2+}/K^{+} ions (**Figure 7**) [7,72]. These sites are essential for the functioning of Hsp60. Therefore, any alteration of these sites (mutation [see, e.g., **Figure 3**], post-translational modification) or blocking them with a chemical compound will most likely inactivate Hsp60. See also **Figure 1A and B**.

The apical domain contains the contact positions for GroES binding represented by the hydrophobic residues L234 and L237, and by N265 (**Figure 8**, blue). These residues contact GroES at the conserved hydrophobic residues I25, I26, L27 and A31 (cyan). The interaction between GroEL and GroES is necessary for the typical chaperoning process

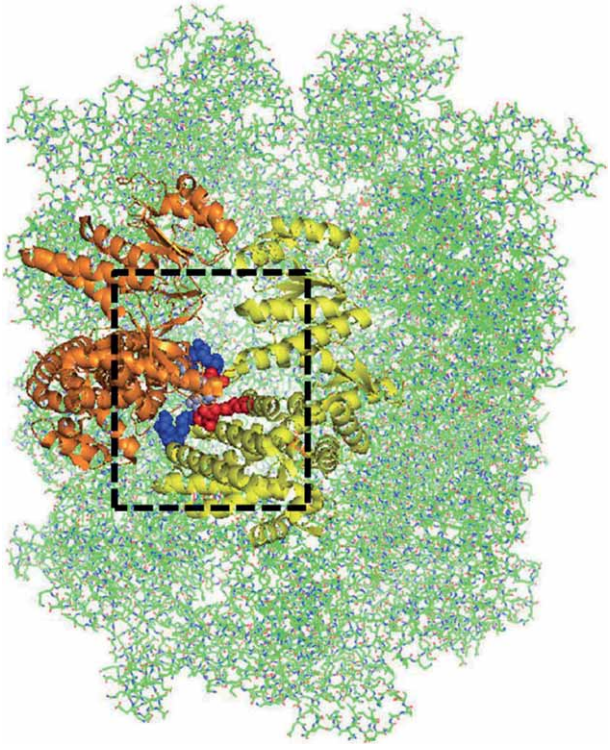

Figure 9. Intermonomer–intraring interaction in GroEL. The cartoon represents a three-dimensional model of GroEL from *E. coli*, showing only two monomers in one ring for the sake of clarity, in the GroEL tetradecamer, one in yellow (monomer to the right) and one in orange-brown (monomer to the left). The contact residues (framed) are at the equatorial domain interface between the two monomers. Hydrophobic residues I6, L73, L513, T517and V521 (all in red) interact with hydrophobic residues V39, L40, I49 and I60 (all in light violet) on the opposite surface. The charged residues K4-E518 and E61-R36 (all in blue) also participate in the intermonomer–intraring interactions.

during polypeptide folding and depends on the correct match of amino acids in the interphase between the two molecules. It follows that disruption of this interaction by modifying (e.g., mutation) or blocking (e.g., by using a chemical compound) one or more of these essential residues will most likely cripple Hsp60, impairing its chaperoning ability. See also **Figure 1D**.

Contacts between equatorial domains of contiguous monomers are unchanged both in *cis* and in *trans* conformations because the major rearrangements in the structure involve mostly the apical and the intermediate domains. The interaction is established by the conserved hydrophobic residues I6, L73, L513, T517 and V521, which interact with the conserved hydrophobic residues V39, L40, I49 and I60 on the opposite surface (**Figure 9**). The interaction is completed by opposite charge interactions between K4-E518 and E61-R36. Tight assembly of rings is necessary for the correct formation and functioning of the GroEL tetradecamer. Consequently, disruption of intermonomer intraring interaction (by alterations or blocking of the residues involved) will most probably result in a functional defective Hsp60. See also **Figure 1F**, which pertains to the formation of homo-oligomers such as those of Hsp60.

Intermonomer contacts of the apical domain and intermediate domains are essential for allosteric switch between *cis* (open cavity to receive the substrate molecule) and *trans* conformations. In the *cis* conformation Y203, V263 and V264 interact from one face with D304, the unaligned position 305, and G306 (**Figure 10**). In the *trans* conformation, only two contacts at the apical domain interface are highly conserved. These contacts involve E257 on one surface and R268 and G269 on the opposite surface. The intermediate domain interacts with the contiguous apical domain in the *trans* conformation and with the contiguous equatorial domain in the *cis* conformation. Conserved residues at positions 181 – 183 of contiguous intermediate domains are in contact only in *trans,* while within the peptide 383 – 389 the same residues contact different regions of the neighboring monomer in the *cis* and in the *trans* conformation (**Figure 10**, black) [7]. As mentioned earlier (**Figure 9**), tight assembly of rings is necessary for the correct formation and functioning of the GroEL tetradecamer. Consequently, disruption of intermonomer–intraring interaction (by alterations or blocking of the residues involved) will most probably result in a functional defective Hsp60. See also **Figure 1F**, which pertains to the formation of homo-oligomers such as those of Hsp60.

Interactions between rings occur via conserved hydrophobic residue V464, moderately conserved charged residues K105, E461 and E467, and the small residues A108, A109 and S463 (**Figure 11**). The opposite charged residues are E434 and D345. These residues contribute to the salt-bridge K105-E434 and to allosteric switch [7,73,74]. As correct intraring interaction and allosteric changes are essential for building a fully functional Hsp60 chaperoning machine, so is the interaction between the two rings of the tetradecamer. Several residues are crucial for the correct assembling of the two-ringed machine and, therefore, alterations of these residues (mutation, post-translational modification, modification or blocking with chemical compounds) can result in a failure of tetradecamer formation. See also **Figure 1E**, pertaining to allosteric changes, and **Figure 1F**, pertaining to the formation of homo-oligomers such as those of Hsp60.

8. Hsp60 inhibitors: new drugs for old diseases

In order to develop inhibitors of human Hsp60, it is crucial to focus on structural differences between the widely studied prokaryotic Hsp60 (GroEL) and its corresponding eukaryotic Hsp60 (Cpn) [75,76]. For example, in contrast to GroEL the eukaryotic Hsp60 possesses three cysteine residues (Cys237, Cys442 and Cys447; **Figure 6**), which represent ideal nucleophilic binding sites for electrophilic drugs.

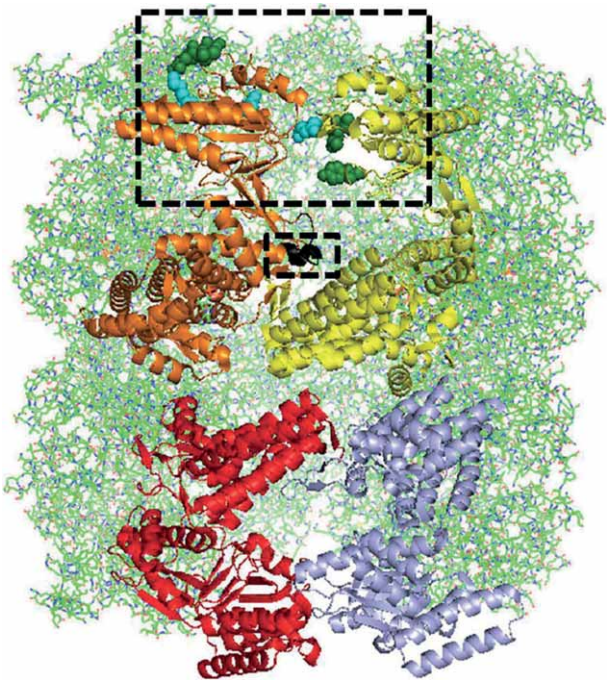

Figure 10. Intraring interactions in the GroEL tetradecamer. The cartoon represents a three-dimensional model of GroEL from *E. coli*, showing only four monomers for the sake of clarity, of the two rings (orange-brown and yellow, top ring; and red and light violet, bottom ring), in the tetradecamer. Large frame: intermonomer–intraring contacts of Y203, V263 and V264 in the apical domain (forest-green color on orange-brown monomer) interact with D304, the unaligned position 305, and with G306 (forest-green color on yellow monomer). E257 (cyan on yellow monomer) on one surface interacts with R268 and G269 (cyan on orange-brown monomer) on the opposite surface. Small frame: conserved residues at positions 181 – 183 and 383 – 389 of the contiguous intermediate domains are shown in black.

Only two strategies have been applied up to now to develop Hsp60 inhibitors. The first one aims at inhibiting ATP binding and hydrolysis, thus, affecting those ATP-dependent conformational changes crucial for the protein folding function [77-79] (see **Figure 1A and B**, and **Figure 7**). The second strategy aims at targeting the Hsp60s cysteine residues (see **Figure 6**) either as oxidizable sites [80] or for covalent binding of the desired compound [81-83].

Ideally, when testing a novel compound targeting Hsp60, its binding capability and docking site should be assessed together with its efficacy in inhibiting ATP binding and hydrolysis, and protein folding activity. Unfortunately, these issues are rarely addressed comprehensively [79,81], thus past studies leave several unanswered questions concerning currently available Hsp60 inhibitors. This lack of experimental information can be partially remedied by resorting to biomolecular computational studies which, for instance, have been conducted *in silico* to model the ATP-binding pocket of Hsp60 in humans, *E. coli* and *Brugia malayi* [84].

Several compounds, including 1,25-dihydroxyvitamin d3 [85], bifenthrin [86], bortezomib [87], metformin [88] and morphine [89] have been correlated with an overexpression of Hsp60, as well as of other heat-shock proteins, generating in some cases an antitumor effect. Treatment with the drug bortezomib led to upregulation of the cell-surface expression of Hsp60 thus triggering an immune response resulting in phagocytosis of the tumor cell by dendritic cells [87]. Popular drugs, such as mefipristone (RU486), have been correlated with diminished levels of expression of the bacterial gene for Hsp60, leading to the beneficial inhibition of *Chlamydophila pneumoniae* infections [90]. Similarly, circulating levels of Hsp60 were reduced in HIV patients after receiving combination antiretroviral therapies (cART) [91]. Besides these correlations of a given compound with the expressed levels of Hsp60 without demonstrating a direct interaction, only a few studies have been devoted to chaperonin-targeting drugs.

Among ATPase activity targeting compounds, mizoribine – an imidazole-based immunosuppressant (**Figure 12**), can form a complex with Hsp60 and, thus, affect the protein-folding activity of the chaperonin [77,78]. A recent study showed that mizoribine slowed down the folding cycle by affecting the ATP hydrolysis cycle. Additionally, inhibition of the dissociation of the co-chaperonin Hsp10 from the Hsp60/Hsp10 complex was suggested to have a key role in the mizoribine activity. Interestingly, the inhibitory functions were found different when studying Hsp60/Hsp10 or GroEL/GroES systems, with the latter being not significantly affected by mizoribine [79].

After the discovery of the interaction of mizoribine with Hsp60, another heterocyclic compound, EC3016 (**Figure 12**), was reported to block ATP binding and hydrolysis thus affecting the protein-folding function of Hsp60 [78]. However, no updates about the potential pharmacological use of this pyrazolo-pirimidine derivative have appeared since the first report.

In addition to the above ATPase activity-targeting drugs, other compounds (**Figure 13**) have been reported to directly interact with cysteine residues of Hsp60 (**Figure 6**). For instance, avrainvillamide and its dimer, whose inhibiting activity of Hsp60 functions have not been yet demonstrated, can alkylate the Hsp60 cysteine residues through the electrophilic 3-alkylidene-3H-indole 1-oxide in moiety [83]. Epolactaene, which covalently binds to Cys442, was discovered to inhibit the chaperoning activity of human Hsp60 [82]. While the epoxide moiety is the most likely binding site to covalently trap the thiolic ends of cysteine residues, an analysis of structure–activity relationship on epolactaene derivatives showed that both the cyclic amide (lactam) and the αβ-unsaturated ketone are crucial moieties for inhibiting the chaperone activity [81]. Recently, also the tertiary butyl ester epolactaene derivative, ETB, was shown to target mitochondrial transcription in fission yeast [92].

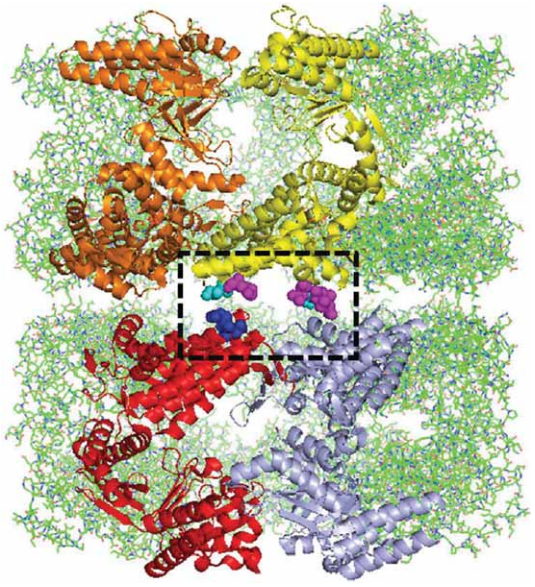

Figure 11. Interring interactions in the GroEL tetradecamer. For the sake of clarity, only four monomers are shown of the *E. coli* GroEL two rings (orange-brown and yellow, top ring; and red and light violet, bottom ring). Frame: the interactions between rings involve a) V464, and the charged residues K105, E461 and E467 (all in magenta on yellow monomer); and b) the small residues A108, A109, and S463 (cyan on yellow monomer). On the red monomer are shown E434 and D345 (both blue), which contribute to the salt-bridge K105-E434 and to allosteric switch.

Besides acting as nucleophilic counterpart for the electrophilic epolactaene or its related derivative ETB, cysteine residues of Hsp60 have been targeted also for sulfation by suvanine, a sesquiterpene natural product of marine origin, which was screened for interaction with Hsp60 through chemical proteomics [93]. Additionally, given the typical thiol/disulfide redox reaction of its cysteine residues, Hsp60 was found to interact also with gossypol, a polyphenolic drug that induces apoptosis through oxidative stress [80].

Also other electrophilic compounds have been proven to target Hsp60, although not in a specific or protein-selective fashion. For instance, proteomic analysis revealed that 4-hydroxynonenal (HNE; **Figure 14**), an electrophilic α,β-unsaturated aldehyde, targets Hsp60, together with several other proteins involved in stress signaling, in a dose-dependent increase in labeled proteins with increased sequence coverage at higher concentrations [94]. Also in this case, the binding site was not elucidated, although, by considering the electrophilic nature of the aldehyde, involvement of Hsp60 cysteine residues cannot be excluded.

Recently, carboranylphenoxyacetanilide, a hypoxia-inducible factor 1 alpha (HIF-1α) inhibitor, was proven to bind Hsp60 as a primary target, although the exact binding site was not identified [95]. Interestingly, a derivative of carboranylphenoxyacetanilide (R^1 = CH_2CH_3, R^2 = $B(OH)_2$, R^3 = OH; **Figure 15**) showed a chaperone inhibition activity two times higher than that of ETB [96,97].

As a perspective, besides targeting the chaperonin's ATP-binding site or cysteine residues, other regions of Hsp60 can be surveyed to develop novel inhibitors. For instance, by taking advantage of recent knowledge gained about the mechanism of refolding denaturated proteins [41], one can envisage to target the site of interaction between the mithocondrial chaperonin and its co-chaperonin (see **Figure 8**). Alternatively, compounds can be developed to target the ability of Hsp60 to form complexes with proteins involved in the apoptotic cascade (see **Table 8**). For example, metal complexes of pyridyl-substituted 1,2,4-oxadiazole [98,99], a five-membered heterocycle often present in organic materials [100,101] and biologically active compounds [102-104] have recently shown their potential antitumor activity against human hepatoblastoma HepG2 and colorectal carcinoma HT29 cells, [98], and their effect on Hsp60 and Hsp60/pC3 complex formation is currently under investigation by our group.

9. Conclusions

Hsp60 plays diverse roles in various cellular and extracellular locations; some are physiological, normal roles but others are not. In these latter instances, Hsp60 is an etiologic–pathogenic factor and should be eliminated or blocked. The various roles of Hsp60 depend on intrinsic functions of the molecule, which in turn depend on the integrity of specific structures within the entire molecule. In addition, some Hsp60 functions are linked to the correct assembly of multimolecular machines, including Hsp60 monomers and also other molecules. Thus, disruption or blocking of certain spots in the monomer will have an impact on its functions, including oligomerization and interaction with other molecules (networking), and thus interfere with one or more of the roles played by the chaperonin inside and outside the cell. This dependency of intrinsic and overall functions on the integrity of specific structural sites opens the road to developing compounds that will bind those sites and, thereby, block Hsp60 when it is a pathogenic factor. These compounds may have therapeutic potential if devoid of unwanted side effects and if amenable to be delivered into sick cells with an Hsp60 chaperonopathy.

10. Expert opinion

Hsp60 is a type I chaperonin present in virtually all species and cells across the phylogenetic Domains Bacteria and Eukarya, but it occurs only in a small minority of the members of the third Domain, Archaea. In humans, the canonical residence is the mitochondrion, but it can also reside and work outside this organelle, in the cytosol, cell membranes, extracellular space, biological fluids (plasma, cerebrospinal fluid) and secretions (saliva, urine). The canonical function of Hsp60 in the human mitochondrion (also named Cpn60) is assisting newly

Table 7. Examples of human tumors in which human Hsp60 levels have been found changed in relation to disease status (i.e., tumor grading or staging).

Tissue/organ	Tumors	Ref.
Adrenal gland	Adrenal Cushing's tumor	[168]
Bladder	Transitional cell carcinoma; carcinosarcoma	[169-171]
Bone marrow, blood, lymph nodes	Acute myeloid leukemia; Hodgkin's lymphoma	[155,172-174]
Brain	Astroglyoma; glioblastoma; meningioma	[175-179]
Breast	Breast ductal carcinoma	[180,181]
Esophagus	Oesophageal carcinoma	[182,183]
Large bowel	Adenocarcinoma	[23,169,184-187]
Liver	Hepatocellular carcinoma	[188]
Lung	Bronchial carcinoma	[25,26,189,190]
Ovary	Serous ovarian cancer	[191]
Prostate	Prostate carcinoma	[24,192-194]
Stomach	Gastric maltoma	[195]
Uterus	Exocervical carcinoma	[22,196]

Adapted from [2].

Table 8. Molecules found to interact directly with Hsp60.

Hsp60 localization	Molecule interacting with Hsp60	Ref.
Mitochondria	Aldehyde dehydrogenase 2	[37]
	ATP	[40]
	ATP synthase	[197]
	Dihydrofolatereductase	[37,46]
	Hsp10	[37,41]
	Human Carbonicanhydrase II	[37,198,47]
	mtHsp70	[37]
	Pro-caspase3	[28-31,37]
	Survivin	[52]
Cytosol	Bax/Bak	[50]
	Beta-catenin	[53]
	p53	[52]
	Pro-caspase3	[28-31,37]
Plasma membrane	Adipocyte receptors	[59,60]
	Alpha-3-Beta-1 integrin	[55]
	ATP synthase	[197]
	CD14	[57,58]
	CD40	[57,58]
	CD91	[57,199]
	Endothelial ATP synthase	[197]
	Microglial lectin-like oxidized low-density lipoprotein receptor-1	[61]
	Microglial TREM 2 receptor	[56]
	Toll-Like Receptors	[57,58,200-202]

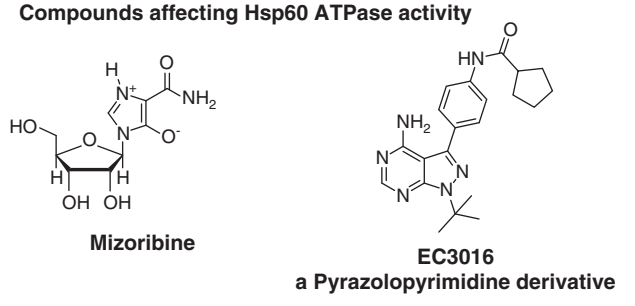

Figure 12. Proven Hsp60 inhibitors affecting ATP binding and hydrolysis. See also **Figure 1.A and B**.

synthesized polypeptides in folding correctly. For this chaperoning function, Hsp60 assembles into an oligomer with a well-defined quaternary structure which, in turn, interacts with an oligomer of the co-chaperonin Hsp10 (Cpn10). The whole process requires ATP hydrolysis. While this is the classical view of Hsp60 and its properties and function, now we know that it plays other roles unrelated to protein folding in a variety of locations beyond mitochondria. We also know that Hsp60 is implicated in the pathogenesis of diverse pathological conditions. As such, the chaperonin has become the subject of studies aiming at determining its precise role as etiologic-pathogenic factor and also aiming at inventing new diagnostic and therapeutic ways centered on it.

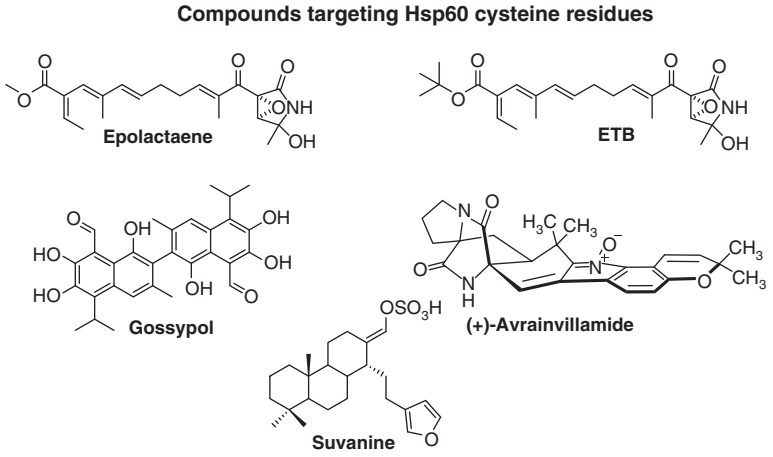

Figure 13. Compounds interacting with Hsp60 cysteine residues through covalent binding (epolactaene, the tertiary butyl ester epolactaene derivative ETB, and avrainvillamide), redox processes (gossypol) or sulfation (suvanine). See also **Figure 6**.

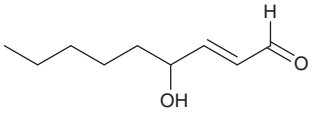

Figure 14. HNE (4-hydroxynonenal), an electrophilic compound targeting proteins involved in stress signaling.

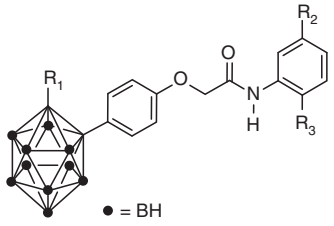

Figure 15. General structure of carboranylphenoxyacetanilide derivatives. The most active Hsp60 inhibitor found had this structure: $R^1 = CH_2CH_3$, $R^2 = B(OH)_2$, $R^3 = OH$.

In short, Hsp60 is an appropriate, convenient and obligatory target for therapeutics. First, it has a variety of functions in diverse locations inside and outside the cells and, therefore its malfunction can result in pathologic conditions affecting almost any tissue or organ. Second, Hsp60 intrinsic functions (e.g., formation of oligomeric rings and double-rings, ATP binding and hydrolysis, substrate recognition, allosterism, interaction with other chaperones forming functional networks) depend on the integrity of defined sites in its molecule, many of which have been mapped. Third, it has been implicated in a variety of disorders in which it most likely plays a primary pathogenic role thus justifying efforts to develop anti-Hsp60 blocking agents. Last, there is already a considerable amount of information on almost all aspects of its structure and function, and on its pathogenicity, which encourages and facilitates research on compounds that bind Hsp60 at predefined sites with predictable impact on the molecule and its functions.

On the contrary, it is also true that there are still many aspects of the Hsp60 molecule and its interactors that are incompletely understood. These obscure patches in the available knowledge are difficult to negotiate when planning experiments and strategies to develop pertinent drugs. In other words, while the information available is encouraging and justifies efforts to study Hsp60 and to develop ways to control its activity, for example, to promote it (positive chaperonotherapy) when deficient or to block it (negative chaperonotherapy) when pathogenic, many questions are still unanswered and obstacles are obvious. For instance, more information is needed to establish a) when (e.g., at what age) and why autologous Hsp60 becomes a pathogenic autoantigen and participates in autoimmune pathology; b) when (e.g., at what age or stage of disease development) and why Hsp60 induces cytokine formation leading to chronic persistent inflammation; and c), when (e.g., at what tumor stage) and why it turns in favor of a tumor rather than protecting the host (i.e., protect the patient's cells and tissues from tumorigenesis). Clarification of these points will take intensive research over considerable periods of time. However, specific analysis of the Hsp60 molecular features and a search for active compounds aimed at defined structural sites that might affect its functioning should continue without interruption. No doubt that some of these compounds will offer therapeutic hopes. Further, it is likely that the compounds will be

useful tools for dissecting structure–function relationships at the biochemical level, and also at the biological level, using model systems *in vivo* (e.g., mouse) and *in vitro* (e.g., human cell primary cultures, and cell lines). We can, then, say that although more research is needed to elucidate basic aspects of Hsp60 structure, function and biology, and to develop delivery systems for active compounds that will target only the pathological molecule in the sick cell, what is already available allows us to predict that significant developments will occur in the near future. These advances pertain to the use of Hsp60 as a) biomarker for diagnosis and assessing prognosis, and monitoring disease status and response to treatment; and b) target for developing new drugs that will modulate or block its activity.

Declaration of interest

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