Abstract. Among the heat shock proteins (HSP), HSP27, HSP70 and HSP90 are the most studied stress-inducible HSPs, and are induced in response to a wide variety of physiological and environmental insults, thus allowing cells to survive to lethal conditions based on their powerful cytoprotective functions. Different functions of HSPs have been described to explain their cytoprotective functions, including their most basic role as molecular chaperones, that is to regulate protein folding, transport, translocation and assembly, especially helping in the refolding of misfolded proteins, as well as their anti-apoptotic properties. In cancer cells, the expression and/or activity of the three HSPs is abnormally high, and is associated with increased tumorigenicity, metastatic potential of cancer cells and resistance to chemotherapy. Associating with key apoptotic factors, they are powerful anti-apoptotic proteins, having the capacity to block the cell death process at different levels. Altogether, the properties suggest that HSP27, HSP70 and HSP90 are appropriate targets for modulating cell death pathways. In this review, we summarize the role of HSP90, HSP70 and HSP27 in apoptosis and the emerging strategies that have been developed for cancer therapy based on the inhibition of the three HSPs.

1. Introduction

Stress or heat shock proteins (HSPs) are a family of highly conserved proteins induced in response to a wide variety of physiological and environmental insults such as hypoxia, hyperoxia, exposure to UV light and chemicals, viral agents, surgical stress, nutritional deficiencies (e.g. glucose deprivation), emotional and mechanical stress, or other stresses, thus helping maintain cellular homeostasis under stress or allowing the cell to survive to lethal conditions (1-4).

Mammalian HSPs have been classified into six families according to their molecular size: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (15 to 30 kDa) including HSP27. Family members of HSPs are expressed either constitutively or regulated inductively, and are present in different subcellular compartments (5). High molecular weight HSPs are ATP-dependent chaperones, whereas small HSPs act in an ATP-independent fashion (5). As molecular chaperones, the function of HSPs is to regulate protein folding, transport, translocation and assembly, especially helping in the refolding of misfolded proteins or assisting in their elimination if they become irreversibly damaged after various stresses or environmental insults.

Cancer cells, with higher metabolic requirements and more abundant signal transduction pathways than normal cells, thereby have a higher need of chaperones than non-transformed cells to maintain cancer cells survival. In addition, by commanding over the folding and stabilization of relevant oncoproteins, HSPs stand at the crossroads of multiple important oncogenic pathways. Inhibition of HSPs hereby offers the unique advantage of depleting multiple oncoproteins while simultaneously attacking several pathways necessary for tumor progression (6). The most studied stress-inducible HSPs are HSP90, HSP70 and HSP27. Indeed, the expression and/or activity of the three HSPs is abnormally high in cancer cells and further increased after many different death stimuli (5,7). They are powerful anti-apoptotic proteins, associating with key apoptotic factors, and thereby blocking this cell death process at different levels (8). Preclinical trials have proved that overexpression of the HSPs increases tumor growth, metastatic potential, and resistance to chemotherapy in rodent models. The inhibition of HSP90, HSP70 and/or HSP27 is thus emerging as a novel strategy for cancer therapy. In this review, we will present our view on the role of HSP90, HSP70 and HSP27 in apoptosis (Fig. 1) and the emerging...
strategies that are being developed for cancer therapy in clinic based on the inhibition of these three HSPs.

2. Apoptosis

Mainly, two pathways of apoptosis can be distinguished, although crosstalk between the two signal transducing cascades is present: the intrinsic or mitochondrial pathway and the extrinsic or death receptors pathway. The two signal-transducing cascades meet at the point of caspase-3, an effector caspase that leads to the typical morphologic and biochemical changes of the apoptotic cell.

The intrinsic pathway involves intracellular stress signals that provoke the permeabilization of the outer mitochondrial membrane, resulting in the release of pro-apoptotic molecules normally confined to the intermembrane space. Outer mitochondrial membrane permeabilization leads to the release of caspase activators under control of the Bcl-2 (B-cell lymphocytic-leukaemia proto-oncogene) family of proteins. Bcl-2 proteins include anti-apoptotic members such as Bcl-2 and Bcl-xL, multi-domain pro-apoptotic members mainly Bax and Bak (9,10) and a series of BH3 domain-only pro-apoptotic proteins, such as Bid, that function upstream of Bax and Bak (11). One of the released mitochondrial molecules is cytochrome c, which interacts with cytosolic apoptosis protease-activating factor-1 (Apaf-1) and pro-caspase-9 to form the caspase-3 activation complex, apoptosome (12). Apoptosis inducing factor (AIF) and the DNase, endonuclease G (EndoG), are other mitochondria intermembrane proteins released upon an apoptotic stimulus. They translocate to the nucleus and trigger caspase-independent nuclear changes (13). Two additional mitochondrial proteins, Smac/Diablo and Htra2/Omi, activate apoptosis by neutralizing the inhibitory activity of the IAPs (inhibitory apoptotic proteins) that associate with and inhibit some of the activated caspases (14).

The extrinsic pathway is triggered through plasma membrane proteins of the tumor necrosis factor (TNF) receptor family known as death receptors, and leads to the direct activation of caspases, starting with the receptor-proximal caspase-8 or caspase-10 in the death-inducing signalling complex (DISC). Caspase-8 either directly activates the downstream cascade of caspases or cleaves Bid into an active truncated form named tBid that connects the extrinsic to the intrinsic apoptotic pathways through mitochondria permeabilization (15).

3. HSF1

The rapid induction of HSPs in response to multiple stress is collectively referred to as the heat shock response (HSR)(16). The HSR is mediated at the transcriptional level by heat shock transcription factors (HSFs), the upstream transcriptional regulators of HSPs (17). So far, the vertebrates HSFs that have been
identified include HSF1, 2, 3, 4 and HSFY, all of which exhibit a similar structure with a highly conserved amino-terminal helix-turn-helix DNA-binding domain and a carboxy-terminal transactivation domain (18-20). Different HSFs are differently regulated and have a different impact on transcriptional responses, which suggests their specialized functions in response to distinct stimulation (21,22). Among them, HSF1 is considered as the master transcription factor for the HSR (17,23). It not only regulates the expression of HSPs but also orchestrates the survival of cells in response to different forms of cellular stress (21,23). In physiological conditions HSF1 exists as an inactive monomer. HSP90 and HSP70 can bind to HSF1 in unstressed state to abrogate the transcription function of HSF1 and dissociate from it under the exterior cellular stress to activate HSF1 (24). Then the monomeric HSF1 trimerizes, phosphorylates and translocates to the nucleus. In the nucleus, HSF1 binds cis-acting DNA elements, termed heat shock elements (HSEs), which are present in heat shock genes, and activate transcription of the Hsp genes, e.g. HSP27, HSP70 and HSP90. HSF, heat shock factor; HSP, heat shock protein.

4. Targeting HSP27

**HSP27 structure.** HSP27 (HSPB1) belongs to a member of the small heat shock proteins (sHSP). The primary structure of HSP27 is highly homologous to other members of the sHSP family, containing the conserved α-crystallin domain and differing in the C- and N-terminal regions. HSP27 is expressed in all human tissues, including astrocytes and primary neuronal cells but mainly in skeletal, smooth and cardiac muscles (25) and shares with other members of the small HSP family the capacity to phosphorylated and oligomerize. Human Hsp27 can be phosphorylated on three serine residues 15, 78 and 82 and on threonine (Thr143) by a large number of kinases including MAPKAP kinases 2 and 3, p90Rsk, PKC, PKD and PKG (26). The phosphorylation is a reversible event that modulates the oligomerization of the protein: its dephosphorylation favors the formation of large oligomers (27,28). However, some studies have found that the ability to oligomerize is reduced in *in vitro* cell based assays by phosphorylation, and that *in vivo* oligomerization has been tied to cell-cell contact and is independent of phosphorylation.
Hsp27 can form oligomers of up to 1,000 kDa. This oligomerization is a highly dynamic process that seems to play a central role in regulating the chaperone activity of HSP27, the multimer being the binding competent state for affinity for client proteins (30). The dimer of HSP27 is the ‘building block’ for multimeric complexes. Particularly, phosphorylated and small oligomers of HSP27 are efficient in binding to F-actin and Daxx (31) and it is the phosphorylated form of HSP27 that protects from neurotoxicity (32).

The function of HSP27 in cancer. HSP27 has a strong protective effect on cells. High levels of HSP27 have been observed in many cancer types, and the tumorigenic potential of HSP27 has been observed in experimental models (33). Many clinical trials have also shown its association with promoting drug resistance, aggressive cancers, metastasis, and poor patient outcomes (34-37). The strong protective effect of HSP27 is mainly due to its vital function at apoptosis regulation. HSP27 is able to block apoptosis at different stages, because of its interaction with a number of partners implicated in the apoptotic pathways.

Numerous studies describe that HSP27 inactivates the caspase cascade through its binding with caspase-3 and cytochrome c released from mitochondria (38-40). Knockdown of HSP27 by small interfering RNAs displayed increased caspase-3 activation, thereby inducing more apoptosis (41). Other data also confirmed that HSP27 prevents apoptosis and induces resistance to chemotherapy through sequestration of cytochrome c when released from the mitochondria into the cytosol (38).

High intracellular levels of HSP27 can inhibit caspase activation by interfering upstream of the mitochondria (42). This effect seems to have connection with the ability of HSP27 to stabilize cytoskeletal elements including actin microfilaments, such as F-actin, to prevent the cytoskeletal disruption and Bid intracellular redistribution that precede cytochrome c release, which is also required for the activation of matrix metalloproteinase 2 (MMP2) (42). In myeloma cell lines, it has been reported that HSP27 activation blocks release of Smac (second mitochondrial-derived activator of caspase) from mitochondria (43). In stressed cells, HSP27 has also shown its importance in Akt activation, through binding the protein kinase Akt (7). In renal epithelial cells, HSP27 indirectly inactivates Bax and its translocation to mitochondria. This is due to an increase of PI3-kinase activity that activates Akt and promotes interaction between Akt and Bax (38, 44). The phosphorylated form of HSP27 directly interacts with death-domain-associated protein (Daxx), which connects Fas signaling to the protein kinase Ask1 that mediates caspase-independent cell death (7, 31).

Large oligomers of HSP27 have also been described to display anti-oxidant property, which is related to its ability to maintain glutathione in its reduced (non-oxidized) form to abolish the production of the potentially lethal burst of intracellular reactive oxygen species (ROS) that can occur (45, 46). These anti-oxidant properties of HSP27 particularly contribute to its cytoprotection in neuronal cells.

The function of HSP27 and the role that it plays in cancer were recently reviewed (38). These numerous reports account for the role of HSP27 in apoptotic cell death inhibition, which also emphasizes its properties in cancer therapy (44, 47).

The inhibition of HSP27 in cancer therapy. The strong cytoprotective function of HSP27, together with the fact that this protein is overexpressed in most cancers, makes this chaperone an attractive target in cancer therapy. Depletion of HSP27 in various animal models induces the regression of tumors (48). Though starting late, Hsp27 therapies have produced only few advances after tremendous efforts.

The antisense oligonucleotide OGX-427 is the only known specific inhibitor of HSP27 that can be safely administered in patients and is currently in phase II clinical trials (http://www.oncogenex.ca/). OGX-427 targets the human hsp27 translation initiation site (5’-GGGACGGCGCCGGCTGGTACTAC-3’) and prevents the translation of hsp27 mRNA, thereby decreasing the expression of the protein compared to untreated cells (49).

Less specific, the chemical molecule RP101 (also known as bromovinyldeoxyuridine, BVDU, brivudine) was reported to improve the efficacy of chemotherapy in pancreatic cancer through its interaction with HSP27 (50). RP101 is a nucleoside that binds via π-stacking with Phe29 and Phe33 of Hsp27 thereby inhibiting its function (50). Functioning as a chemosensitizing agent and preventing the development of resistance, RP101 recently completed a phase II clinical trial for the treatment of pancreatic cancer in combination with gemcitabine (Hidalgo M; http://clinicaltrials.gov/ct2/show/NCT00550004?term=NCT00550004&rank=1, 2011). However, overdosing caused an increase of the toxic side-effects of gemcitabine and thus the combination provided a 25% increase in survival only for patients that had a body surface area (BSA) ≥1.85 m² compared with gemcitabine combined with placebo (50). There were no side-effects caused by RP101, and more accurate dosing would likely improve the survival rates for all patients regardless of size (50). Development of second-generation candidates of RP101 are ongoing.

A strategy of peptide aptamers has also been used to target HSP27. Protein aptamers, small amino acid sequences, are designed to bind to a specific protein domain, thus inhibiting its function (51). Gibert et al have shown that peptide aptamers (PA11 and PA50) that specifically interact with HSP27 are able to disturb the dimerization and oligomerization of the chaperone, thereby acting as negative regulators of HSP27 anti-apoptotic and cytoprotective properties (52). PA11 prevents the HSP27 oligomerization, which leads to the inability of HSP27 to inhibit early stage protein aggregation and induces proteotoxic stress that ends in cell death. PA50, through a different mechanism, mainly inhibits HSP27 dimerization, disrupting the ability of HSP27 to participate in cell-signaling events thereby interfering with processes essential for cell survival. In xenograft models these peptide aptamers strongly reduced tumor cells growth (52). Similar to the small molecule inhibitors of HSP27, peptide aptamers are not effective on their own but are used to sensitize cancers to other therapies. The pre-clinical success of peptide aptamers suggests this avenue of cancer therapy has potential.

Different kind of inhibitors, which have been experimentally tested, like the flavonoid quercetin and the diterpene trioxipoxide, triptolide (24), act at the level of the HSF1 to block the transcription of heat shock proteins genes, thus inhibiting the heat shock response. However, such approaches are non-specific since through HSF1 inhibition, all the stress-inducible heat shock proteins can be blocked affecting important housekeeping functions in normal cells.
5. Targeting HSP70

**Human Hsp70: structure and general function.** HSP70 refers to a family of chaperone proteins that are 70 kDa. The HSP70 human genome superfamily consists of at least 13 members (53). There are four major proteins: constitutively expressed HSC70 (HSP73 or HSPA8), endoplasmic reticulum-localized GRP78/Bip, mitochondrial mHSP70 and stress-inducible HSP70 (HSP72 or HSPA1) (called here simply HSP70) (54).

HSC70 is ubiquitously expressed in practically all organs and tissues. Under normal conditions, it functions as ATP-dependent molecular chaperone that assists the folding of newly synthesized polypeptides, the assembly of multi-protein complexes and the transport of proteins across cellular membranes (55-57). Its levels are also increased under stress conditions showing the involvement in stress response (57). On the contrary, the expression of HSP70 is often not observed under non-stress conditions. Under stressful conditions, elevated HSP70 levels allow cells to cope with increased concentrations of unfolded or denatured proteins (58).

All of the proteins share homology and contain two distinct functional domains: a C-terminal peptide-binding domain (PBD) and the N-terminal ATPase domain (ABD), which were connected through a hydrophobic linker and both domains are important for substrate binding and stabilization. The PBD, which include a carbonyl-terminal chaperone EEVD motif, is responsible for substrate binding and refolding. The ABD, containing the ATPase pocket and binding J-domain-containing proteins, such as HSP40 that regulate the HSP70 ATPase activity, in turn, facilitates the release of the client protein after ATP hydrolysis. A conserved proline in the ATPase domain is essential to alternate HSP70 conformations in response to ATP binding and hydrolysis (59,60).

HSP70 chaperone activity is regulated by distinct co-chaperones, e.g. Hip, CHIP or Bag-1. These co-chaperones bind to HSP70 and modulate its chaperone function by increasing or decreasing HSP70 affinity for substrates through the stabilization of the ADP or ATP bound state of HSP70. They can be classified into three groups. i) The J-domain co-chaperones, like HSPA40, are a relatively large group that binds to the HSP70 ABD and stimulate the low ATPase activity of this chaperone (1). ii) The nucleotide exchange factor co-chaperones catalyze the release of ADP which is required for the completion of the HSP70 ATPase cycle. Members of this group are Bag-1, HSP110, or HSPBP1. iii) The TPR domain co-chaperones (Hop, CHIP) bind to the C-terminal EEVD motif presented in both HSP70 and HSP90. They are essential for combinational assembly of HSP70 and HSP90 complexes, required for the stabilization of HSP90 client proteins. CHIP, with ubiquitin ligase activity, has been implicated in the ubiquitination of at least some HSP client proteins (5,61).

The function of HSP70 in cancer. Similar to HSP27, HSP70 is also abundantly expressed in many tumor forms and is accompanied by increased cell proliferation, metastases and poor response to chemotherapy. Constitutively high expression of HSP70 enhances the ability of the cancer cells to survive to a range of lethal conditions. The cytotoxic effect of HSP70 down-modulation is particularly strong in transformed cells yet undetectable in normal, non-transformed cell lines or primary cells (62). This fact has been interpreted by assuming that tumor cells, as compared to their normal counterparts, exhibit a constitutively stressed phenotype with an enhanced dependency on the cytoprotective action of HSP70. HSP70 exerts the cytoprotective action probably through its ability to inhibit apoptosis. Gene ablation studies demonstrate that HSP70 plays an important role in apoptosis. Cells lacking hsp70.1 and hsp70.3, the two genes that code for inducible HSP70, are highly sensitive to apoptosis induced by a wide range of lethal stimuli (62). Ablation of the testis specific isoform of HSP70 (hsp70.2) results in germ cell apoptosis (63).

HSP70 can regulate apoptosis at the different levels from death receptors signaling to executors of cell death program affecting both upstream and downstream of the death-associated mitochondrial events.

At the level of death receptors, HSP70 can bind to the death receptors DR4 and DR5, thereby inhibiting the TNF-α-related apoptosis-inducing ligand (TRAIL)-induced assembly and activity of death inducing signaling complex (DISC) (64). HSP70 also appears to affect the Bid-dependent apoptotic pathway. HSP70 inhibits TNF-α-induced cell death and this protective effect is lost in Bid homozygous-deleted MEF cells. HSP70 can block the cleavage of Bid by activated caspase-8 (65).

At the premitochondrial level, HSP70 inhibits stress-activated kinases, such as apoptosis signal regulating kinase 1 (Ask1). In NIH3T3 cells, it was shown that downregulation of HSP70 facilitates H2O2-induced Ask1 activation and subsequent apoptosis (66). HSP70 also negatively interferes with MAPK family kinase activity, in particular, the p38 kinase and the c-Jun N-terminal kinase (JNK) (67). Studies have found that HSP70 inhibits the apoptosis induced by hyperosmolarity modulating JNK and ERK phosphorylation (68). HSP70 has been shown to contribute to stabilize the stress-activated kinases, such as non-phosphorylated protein kinase C (PKC) and Akt, by means of binding to the non-phosphorylated kinase via the kinase unphosphorylated carboxyl-terminus, priming the kinase for rephosphorylation and stabilizing the protein (69).

HSP70 has also been shown to affect some transcription factors involved in the expression of the Bcl-2 family. Bcl-2 family of proteins, playing a critical role in the regulation of apoptosis through controlling the release of caspase activators, are transcriptional targets of the tumor suppressor protein p53. The transcription of Bcl-2 is repressed by p53, whereas that of Bax is induced. HSP70 can form stable complexes with mutated p53, thus inducing apoptosis in response to DNA damage. HSP70 can also cover the nuclear localization sequence of p53, thereby preventing its nuclear import (70).

At the mitochondrial level, HSP70 blocks heat-induced apoptosis by binding to Bax to prevent its translocation to the mitochondria (71), thus preventing outer mitochondrial membrane permeabilization and inhibiting the release of mitochondrial apoptogenic molecules, such as cytochrome c and AIF (72). This HSP70 function relies on both its chaperone HSPA40, and its ATP hydrolytic domains.

At the post-mitochondrial level, downstream of the release of cytochrome c and upstream of the activation of caspase-3, HSP70 has been demonstrated to directly bind to Apaf-1 to prevent the recruitment of procaspase-9 to the apoptosome,
thus inhibiting apoptosis (73,74). This interaction depends on the ATPase domain of HSP70 (74).

HSP70 can prevent cell death under caspase inactivation. That is HSP70 can also prevent caspase-independent pathways (75,76). Indeed, HSP70 directly binds to AIF and inhibits AIF nuclear translocation, thereby inhibiting AIF-induced chromatin condensation (76-78). The ATPase function of HSP70 was described to be necessary for this interaction, which also depends on a region between amino acids 150 and 228 of AIF (76). HSP70 can also indirectly associate with EndoG to prevent DNA fragmentation through affecting AIF (79).

Moreover, HSP70 can also rescue cells from a later phase of apoptosis. During the final phases of apoptosis, the main characteristic is nuclear condensation and fragmentation, and the chromosomal DNA fragmentation is digested by the DNase CAD (caspase activated DNase) following activation by caspase-3. It has been reported that HSP70 has an important influence on the enzymatic activity and proper folding of CAD, which also depends on its cochaperones: HSP40 and the inhibitor of CAD(ICAD), suggesting that HSP70 plays a role in maintaining DNA integrity (80,81). Some studies also found that HSP70 could protect GATA-1, another final target of caspase-3, from caspase-3 cleavage (82). However, specific mechanism remains to be further studied.

HSP70 has been shown to promote cancer cell viability by safeguarding lysosomal integrity. In cysteine cathepsin-dependent death, HSP70 acts to inhibit lysosomal membrane permeabilization, thereby preventing the release of lysosomal constituents into the cytosol, which contains a group of proteases that are involved in apoptosis (83,84).

HSP70 is a crucial negative regulator of the mitochondrial pathway of apoptosis that can block cell death at several levels from death receptors signaling to executors of cell death program affecting both upstream and downstream of the death-associated mitochondrial events.

The inhibitors of HSP70

Despite the critical role of HSP70, as discussed above, in protein regulation and cancer progression, tremendous efforts have produced few advances in hsp70 inhibitors. Here, we explore HSP70 inhibitors though three basic categories: small molecule inhibitors, protein aptamers, and antibody treatments, also, the targets of drugs - targeting PBD, targeting ABD and targeting HSP70 co-chaperones are also discussed.

Small molecule inhibitors: a) Targeting the peptide binding domain (PBD). A small molecule inhibitor called 2-phenylethynesulfonamide (PES) or pifithrin-μ interacts with the C-terminal PBD of HSP70, disrupting the association between HSP70 and several of its cofactors such as HSP40 and client proteins, including pro-apoptotic proteins: APAF-1, p53 and others (85). This disruption leads to the aggregation of misfolded proteins, and the destabilization of lysosome membranes, thus inducing cell death (85). PES has been proven as a potent antitumor agent.

Neutralization of HSP70 functions could be achieved with peptides that mimic a domain of the AIF which is required for HSP70 binding. The AIF-derived peptides were designed carrying the AIF region from amino acid 150 to 228, which was previously defined as required for HSP70 binding in its PBD and lack AIF pro-apoptotic function (77). These peptides bind HSP70 and block its function (62,76). Experiments in vitro carried out on different cell lines, such as leukemia, colon and breast cancer lines, demonstrated that several of these peptides increase sensitivity to chemotherapy (62). Experiments in vivo: in syngeneic rat colon cancer and mouse melanoma models, demonstrated that AIF-derived decay for HSP70 (ADD70), an inhibitor of HSP70, reduced the tumor size and metastatic potential, and led to a complete and permanent cure after treatment with cisplatin (86).

b) Targeting the amino-terminal ATPase domain (ABD). ATP hydrolysis and ADP/ATP exchange play a central role in HSP70 chaperone activity. Therefore, disruption of HSP70-ATP interaction could lead to the inability of HSP70 to perform its functions.

15-Deoxyxypregualin (15-DSG), a natural immunosuppressive agent, disrupting HSP70-ATP interaction through binding to HSP70 and stimulating its ATPase activity, was the first compound described by Nadeau et al in 1994 (87). It binds ABD with its main structure, the dihydropyrimidine group. Screening for inhibitors of HSP70 ATPase activity and a subset of the National Cancer Institute drug collection brought about the identification of NSC630668, a dihydropyrimidine, which also effectively blocked protein translocation mediated by yeast HSC70 in vitro (88). Noteworthy is the second generation compound MAL3-101 and its subsequent modifications, which was described inhibiting HSP70 ATPase activity and blocking proliferation of SK-BR-3 cancer cells (89). Fortuitously, MAL2-11B, an intermediate in the synthesis of MAL3-101, was also shown to interfere with the activity of a viral J-domain of a chaperone-like protein, T-antigen, suggesting that it may be a new class of polyoma-virus inhibitors (90). However, the exact action mechanism of these molecules remains unclear.

VER-155008 is an adenosine-derived compound. It functions to inhibit the chaperone activity of HSP70 and other family members by binding the ATPase domain. Although further studies are necessary to determine its specificity and potency, some in vitro results are encouraging: inducing caspase-dependent apoptosis in BT474 breast cancer cells and non-caspase-dependent cell death in HCT116 colon cancer cells (91). This product has undergone pharmacokinetics studies in mice, but efficacy studies have yet to be reported.

Azure C, methylene blue and myricetin have been identified as inhibitors of HSP70 through a high-throughput screening for ATP Turner mediated by human HSP70, but their specificity for inducible HSP70 family has not yet been analyzed (92).

MKT-077, a cationic rhodacyanine dye analog, can also bind to the ABD of Hsp70 (93). Mechanistic studies indicate that MKT-077 localizes in the mitochondria where it inhibits the deleterious interaction of mitochondrial HSP70 with p53 by binding to the mt-HSP70 ABD (94). Studies on MKT-077 have generated significant excitement about this product and it has been explored on phase I clinical trial as an antitumor agent (95). However, MKT-077 was found to be nephrotoxic in solid tumor-bearing patients due to lack of binding specificity (95). Although the product does not specifically bind to HSP70 (i.e., it also binds to actin), such a drug-like compound deserves further investigation.
Apoptozole was discovered to induce apoptosis in the human embryonic carcinoma cell line while looking for small molecules that induced apoptosis in the imidazole compound library (96). It was shown to inhibit the ATPase activity of HSC70, but further information is required to define the precise molecular mode of action and the selectivity of this compound (97).

Sphingolipids, another group of HSP70 ABD binding agents, can bind and specifically inhibit HSP70 ATPase activity in vitro depending on the rate of ATP hydrolysis (98).

Protein aptamers. Peptide aptamers, targeting the ATP binding domain of HSP70 to attenuate the HSP70 function, were recently demonstrated as promising drugs in cancer therapy (51). The most potent aptamer, A17, binds to the ABD of HSP70 and disrupts the function of HSP70 in a biochemical assay in vitro (99). A17 increases the sensitivity to apoptosis induction by anticancer drugs (cisplatin and 5-fluorouracil) and, in vivo, has a strong antitumor effect (99).

Antibody treatments. The most promising strategy reported for developing HSP70 inhibitors utilizes the immune system, and it is the only HSP70-targeted therapy currently in clinical trials (clinicaltrials.gov). However, they are limited by the lack of tumor-specific markers (100). A recently developed monoclonal antibody, cmHsp70.1, successfully recognizes the extracellular motif, TKDNLLLGRFELSG (TKD) of membrane bound HSP70 (101). Furthermore, tumors express HSP70 in the membrane while normal (non-transformed) cells do not, thus making the TKD motif an excellent tumor-specific biomarker (101). CmHsp70.1 has successfully passed through a safety and efficacy phase I trial and it is currently in a phase II clinical trial for non-small cell lung cancer in combination with radio chemotherapy (102).

6. Targeting HSP90

HSP90 structure. HSP90, a highly abundant chaperone protein expressed by all eukaryotic cells, belongs to another important class of the HSP family (103). It is highly conserved throughout evolution and accounts for 1-2% of total cellular proteins, increasing upon induction from baseline levels to 4-6% (104). It is an ATP-dependent chaperone with various isoforms among which the most prominent members in humans are HSP90α (inducible form) and HSP90β (constitutive form) isoforms (now also called HSPC1 and HSPC3, respectively) which are encoded by separate, but highly conserved genes, and have different roles (105). The hsp90α was shown to be constitutively expressed at low level but strongly heat inducible. In contrast, the hsp90β gene (hsp90β1) is expressed constitutively at a much higher level and is only weakly inducible following a heat shock (105). HSP90 exists as a homodimer and contains three major regions (104,106): i) the amino (N)-terminal domain, with an adenosine triphosphate (ATP)-binding and hydrolyzing pocket, is responsible for the protein's ATPase activity, ii) the charged middle linker region involved in co-chaperones and client proteins recognition/binding, and iii) the carboxy (C)-terminal dimerization domain which directs HSP90 dimerization contains the tetratricopeptide repeat-binding (TRP) motif, EEVD. TPR-containing co-chaperones such as Hop (HSP organizing protein), bind to this motif regulating the ATPase function of HSP90 (17). ATP is required for HSP90 activity and it is possible to determine a potential conformational equilibrium of HSP90 (108,109). The available structural information for HSP90s shows that the C-terminal domains are involved in dimerization and that the dimer formation by these domains is independent of nucleotide binding or client proteins or co-chaperone interactions (108). On the other hand, the dimerization of the N-terminal domains is dependent on binding. The binding of ATP triggers the dimerization of the N-terminal domains and enables client protein binding/loading. HSP90-bound ATP is then hydrolyzed, and the energy released by ATP hydrolysis enables client protein folding (104). ATP hydrolysis results in a conformational changing from an elongated orientation in which the N-terminal domains dimerize to a wide, open V-shaped orientation releasing the client protein (108). There are a few contacts between the middle domains, a gap remains between the two middle domains, although each makes contact with the N-terminal domain of the other protomer upon dimerization of the N-termini (110).

The function of HSP90 in cancer. As a molecular chaperone, like HSP27 and HSP70, HSP90 helps nascent proteins adopt their biologically active conformations, correct the conformation of misfolded proteins, and helps incorrigibly misfolded proteins to be removed and degraded by the ubiquitin-proteasome system (104).

HSP90 functions as part of a multichaperone complex via association with a variety of co-chaperones and client proteins that rely on the complex for acquiring active conformation. It facilitates the maturation, stability, activity and intracellular sorting of more than 200 client proteins (104,111). These client proteins covering almost all cellular processes have been identified (for an updated list, see http://www.picard.ch/downloads/Hsp90facts.pdf). Many of these client proteins are involved in critical cellular functions that promote cell growth, proliferation and cell survival which are also important to maintain the cancer phenotype. HSP90 is overexpressed in cancer cells and several of its client proteins are signaling oncoproteins that represent nodal points in multiple oncogenic signaling pathways, including mutant cKIT, human epidermal growth factor receptor 2 (HER2/neu), mutant epidermal growth factor receptor (EGFR), the BCR-ABL fusion protein and BRAF (111-113). HSP90 client proteins are also involved in other hallmark processes of cancer, including induction of angiogenesis, mediation of apoptosis, and promotion of tissue invasion and metastasis (114). For example, HSP90 influences angiogenesis by chaperoning hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor receptor (VEGFR) in addition to governing nitric oxide synthase upregulation. HSP90 chaperones client proteins that are apoptotic mediators, including Bcl-2, Atpaf-1, the serine-threonine protein kinase AKT/PKB and surviving (114). Also, HSP90 may promote tissue invasion and metastasis through MMP-2 activation, digesting extracellular matrix proteins (114). Other client proteins of HSP90 that play a role in cell signaling processes include FAK (integrin pathway), IL6R (JAK/STAT3 pathway), IκB kinases (NFκB pathway), CDK 4,
6, 9, hTERT (cell cycle), p53 (tumor suppressor genes), and the steroid hormone receptors (estrogen receptor and androgen receptor) (115).

Because these oncogenic proteins substantially rely on the function of HSP90 for their maturation and/or stabilization, as well as regulation of their activated states (116), inhibition of HSP90 provides the unique advantage of causing depletion of multiple oncogenic client proteins, while simultaneously leading to blockade of many key cancer causing pathways, and hence leads to potent anticancer effect.

Over 20 co-chaperones regulate HSP90 activity mainly through the modulation between the interconversion of the ATP- and ADP-bound states. Some of these inhibit HSP90 ATPase activity, thus to be involved in client loading or the formation of mature HSP90 complexes, such as HSP70/HSP90 organizing protein (HOP), cell division cycle protein 37 (CDC37) and p23. Whereas, others enhance it, such as activator of HSP90 ATPase 1 (AHA1) and cyclophilin-40 (Cpr6 and Cpr7), hence leading to their use as activators of the HSP90 conformational cycle (104,117,118).

Lessons learned in oncology clinical trials and future directions for oncology drug development of HSP90 inhibitors. Many of HSP90 client proteins hold important functions in the development and promotion of cancer, as described above, thus, Hsp90 has a putative role in numerous cancers and deserves to be an attractive target for therapeutics. Targeting HSP90 as a therapeutic approach in treating cancer began with geldanamycin (GM), which exhibits antiproliferative activity by binding to the ATP-binding site of HSP90 and thereby preventing its function. However, GM has limited therapeutic potential owing to its hepatotoxicity. The discovery of GM sparked much interest in the inhibition of HSP90 as a strategy for the treatment of cancer, resulting in intense efforts from both industry and academic research institutes to develop clinically viable HSP90 inhibitors (119). Although the exact antitumor action of HSP90 inhibitors remains largely unknown, substantial number of molecules are currently in preclinical and clinical evaluations, and some have promising results. These inhibitors are summarized in Table I.

As illustrated above, the rationale for using HSP90 inhibitors in cancer therapy is well established. Pursuing after new easily administrable HSP90 inhibitors and their evaluation in clinical trials is a goal for many pharmaceutical companies. However, no HSP90 inhibitor has been FDA-approved to date. Lessons learned in oncology clinical trials give us strategies and future directions that may enhance therapeutic benefit and accelerate the drug approval process for safe and efficacious HSP90 inhibitors.

There have been hints that these inhibitors are minimally effective and with more side-effects as single agents against various cancer cell line, and that they may show tremendous promise when used as combination and dual treatment agents (120).

Inhibition of HSP90 activates the heat shock response, which compensatorily induces expression of several heat shock factors, including heat shock factor 1 (HSF1), members of the HSP70 family and HSP27, which are protective proteins that could counteract the pro-cell death effects of HSP90 inhibitors (121). Therefore, an interesting approach for combination studies in the future is to inhibit multiple heat shock proteins. Silencing HSF1, HSP70 and HSP27, has been shown to cause a marked increase in the sensitivity of cancer cells to HSP90 inhibition, and induction of apoptosis (112). However, it should be noted that the toxicity of the combination of HSP70 and HSP90 inhibitors is unclear, and remains to be understood.

Since targeted therapy was introduced into cancer treatment, it has brought hope, and it is increasingly believed that combination therapies targeting parallel signaling pathways that regulate iconic processes that are absolutely necessary for cancer cell survival and proliferation may provide better cancer therapy. As described above, many of HSP90 client proteins are involved in critical cellular functions, thus, the development of these HSP90 inhibitors may require close developing client protein inhibitors, e.g., RAF inhibitors (114).

To enhance the effectiveness of HSP90 inhibitors, combinatorial targeting of HSP90 cochaperones and/or of post-translational modifications that influence HSP90’s function is a potentially attractive approach (112). Then, a greater understanding of HSP90 cochaperones, and post-translational modifications of HSP90 is needed.

In addition, a better understanding of the HSP90 inhibitors is still the key. For HSP90 has various isoforms and each has different functions, specific inhibition for HSP90 inhibitors is expected. It has been suggested that HSP90α plays an essential and unique role in embryonic cell differentiation, and its inhibition blocks macrophagic differentiation in the already formed animal (122). Furthermore, only HSP90α was described to emerge in the extracellular (breast cancer and melanoma) and to play a role in tumor invasion and metastasis by promoting maturation of extracellular MMP-2 (123). Evidences from that the concentration of secreted MMP90α positively correlates with tumor malignancy in liver and breast tumor patients (124). Therefore, future drug discovery approaches should focus on looking for more specific inhibitors that only target the HSP90α isoform. Similarly, drugs that specifically disrupt the interaction of HSP90 with a given chaperone or client protein without affecting others could be interesting to avoid the side-effects associated to HSP90 inhibitors (5).

Clinical trial designs may ultimately be critical in determining if one HSP90 inhibitor has any clear clinical benefit to exploit. Several strategies can be applied to enhance the effectiveness of HSP90 inhibitors. Personalizing treatments is always one of the principles of clinical treatment. Personalizing treatments to match patients’ genetic profiles and targeting specific tumors/tumor types should be recognized as ways to increase the effectiveness of HSP90 inhibitors. As we enter the era of targeted therapy and personalised medicine, development of biomarkers to help to stratify patients, ascertain target inhibition, and monitor or predict response to HSP90 inhibitors is of vital importance if HSP90 inhibitors are to succeed. It is also possible that the therapeutic schedule of HSP90 inhibitors has not been optimized. Future effective target inhibition would benefit from a valuable method to optimise drug dosing and scheduling.
Table I. HSP inhibitors in clinical development as mono- or combination-therapy.

<table>
<thead>
<tr>
<th>Drug (HSP90 inhibitors)</th>
<th>Disease type</th>
<th>Stage of development</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Geldanamycin analogues</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tanespimycin (17-AAG)</td>
<td>Kidney tumors in Von Hippel-Lindau disease; relapsed or refractory anaplastic large cell lymphoma, mantle cell lymphoma, or Hodgkin's lymphoma</td>
<td>II</td>
</tr>
<tr>
<td>17-AAG</td>
<td></td>
<td>II</td>
</tr>
<tr>
<td>17-AAG + trastuzumab</td>
<td>Breast cancer</td>
<td>II</td>
</tr>
<tr>
<td>17-AAG + bortezomib</td>
<td>Multiple myeloma</td>
<td>II/III</td>
</tr>
<tr>
<td>17-AAG + gemcitabine</td>
<td>Recurrent advanced ovarian epithelial or peritoneal cavity cancer</td>
<td>II</td>
</tr>
<tr>
<td>17-AAG + bortezomib</td>
<td>Advanced solid tumors or lymphoma</td>
<td>I</td>
</tr>
<tr>
<td><strong>Alvespimycin (17-DMAG)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17-DMAG</td>
<td>Melanoma</td>
<td>I</td>
</tr>
<tr>
<td>17-DMAG</td>
<td>Prostate</td>
<td>I</td>
</tr>
<tr>
<td>17-DMAG + trastuzumab</td>
<td>Breast cancer</td>
<td>I</td>
</tr>
<tr>
<td>17-DMAG (KOS-1022) + trastuzumab</td>
<td>Ovarian</td>
<td>I</td>
</tr>
<tr>
<td><strong>Retaspimycin hydrochloride (IPI-504)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPI-504</td>
<td>Hormone-resistant prostate cancer</td>
<td>II</td>
</tr>
<tr>
<td>IPI-504</td>
<td>Relapsed and relapsed refractory multiple myeloma</td>
<td>I</td>
</tr>
<tr>
<td>IPI-504</td>
<td>Relapsed/refractory stage IIIb, or stage IV NSCLC</td>
<td>I/II</td>
</tr>
<tr>
<td>IPI-504 + docetaxol</td>
<td>Advanced solid tumors</td>
<td>I</td>
</tr>
<tr>
<td>IPI-504 + everolimus</td>
<td>KRAS mutant NSCLC</td>
<td>I/II</td>
</tr>
<tr>
<td>IPI-504 + trastuzumab</td>
<td>HER2(^*) breast cancer (study terminated)</td>
<td>II</td>
</tr>
<tr>
<td>IPI-493</td>
<td>Advanced malignancies; hematologic malignancies (study terminated)</td>
<td>I</td>
</tr>
<tr>
<td><strong>Resorcinol derivatives</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ganetespib (STA-9090)</td>
<td>Patients with unresectable stage III or stage IV melanoma who received prior tyrosine kinase inhibitor treatment (has 2 arms-mutant V600E BRAF arm and a wild-type BRAF arm); metastatic hormone-resistant prostate cancer previously treated with docetaxel-based chemotherapy; previously untreated metastatic HER2(^*) or triple negative breast cancer; stage IIIB/IV NSCLC; metastatic ocular melanoma; metastatic or unresectable GIST; refractory metastatic colorectal cancer</td>
<td>II</td>
</tr>
<tr>
<td>STA-9090</td>
<td>Advanced hepatocellular carcinoma; solid tumors, STA-9090 administered twice-weekly</td>
<td>I</td>
</tr>
<tr>
<td>STA-9090 as second- or third-line therapy</td>
<td>Metastatic pancreatic cancer</td>
<td>II</td>
</tr>
<tr>
<td>STA-9090 + docetaxel</td>
<td>Solid tumors</td>
<td>I</td>
</tr>
<tr>
<td>STA-9090 + dutasteride</td>
<td>Castration-resistant prostate cancer</td>
<td>II</td>
</tr>
<tr>
<td>STA-9090 + fulvestrant</td>
<td>HR(^*), metastatic breast cancer</td>
<td>II</td>
</tr>
<tr>
<td><strong>AUY922</strong></td>
<td>Advanced solid malignancies in older patients (≥75 years)</td>
<td>I</td>
</tr>
<tr>
<td>AUY922</td>
<td>Lymphoma; metastatic pancreatic cancer resistant to first line chemotherapy; NSCLC patients who received 2 previous lines of chemotherapy; refractory GIST</td>
<td>II</td>
</tr>
<tr>
<td>AUY922</td>
<td>HER2(^*) trastuzumab-resistant breast cancer [imaging component using 89Zr-trastuzumab positron emission tomography (PET) to study the effect of HSP90 inhibition on HER2 expression]</td>
<td>I/II</td>
</tr>
<tr>
<td>AUY922</td>
<td>ER(^*) hormone therapy refractory breast cancer (to study the effect of HSP90 inhibition by AUY922 on VEGF using 89Zr-bevacizumab PET)</td>
<td>I/II</td>
</tr>
</tbody>
</table>
7. Conclusions

Owing to the complicated pathogenesis, poor prognosis and resistance to treatments, cancer remains a notoriously unsolved medical issue and desperately requires efficacious drug candidates. By commanding over the folding and stabilization of relevant oncoproteins, HSPs are involved in vital mechanisms of cancerous cells, such as cell proliferation, differentiation, invasiveness, neoangiogenesis, metastasis and immune system recognition. Additionally, they have the added advantage of reducing the likelihood of the tumor acquiring resistance to any single therapeutic strategy. As a consequence, HSPs are emerging as interesting targets in cancer therapy, particularly HSP90, 70 and 27. Owing to the potent anti-apoptotic function of HSPs 27, 70 and 90 as well as their role in drug resistance, it is considered that their deletion may increase tumor cell susceptibility to apoptosis and fight against carcinogenesis or elicit drug sensitivity (4). This is one area, which although representing a challenging endeavor with potential risks, offers very promising alternatives for the treatment of cancer. Several HSP inhibitors such as 17-AAG, IPI-504 and BIIB021 are currently in clinical phase trials. However, specificity is still an important issue for all the tested HSP inhibitors. HSP90 inhibitors represented the best developed candidates to treat cancer. However, as we reviewed above, these inhibitors targeting HSP90 are minimally effective and with more side-effects as single agents. Consequently, developing drug candidate targeting multiple HSPs or the combination of different HSP inhibitors could be particularly appealing. In addition, combining HSP inhibitors with other validated drug candidates for target therapies might provide promising therapeutic benefits. Although many issues remain unresolved, scientists in the field still continue to strive toward a better understanding of the mechanisms of HSPs/HSR and other essential oncogenic pathways, hoping that this will eventually lead to successful drug candidates and significantly improve cancer clinical therapeutic index.

Acknowledgements

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