Molecular Chaperone Hsp90 as a Target for Oxidant-Based Anticancer Therapies

R. Beck1, N. Dejeans1, C. Glorieux1, R.C. Pedrosa2, D. Vásquez3, J.A. Valderrama3, P.B. Calderon1,4 and J. Verrax*,1

1Toxicology and Cancer Biology Research Group, Louvain Drug Research Institute, Université Catholique de Louvain, Belgium; 2Laboratorio de Bioquímica Experimental, Departamento de Bioquímica, Universidade Federal de Santa Catarina, Florianópolis, Brazil; 3Departamento Química Orgánica, Pontificia Universidad Católica de Chile, Santiago, Chile; 4Departamento de Ciencias Químicas y Farmacéuticas, Universidad Arturo Prat, Iquique, Chile

Abstract: Hsp90 is a molecular chaperone involved in the stabilization of many oncoproteins that are required for the acquisition and maintenance of the so-called six major hallmarks of cancer cells. Various strategies have, therefore, been developed to inhibit the chaperone activity of Hsp90 and induce cancer cell death through the destabilization of its client proteins. Among these strategies, we have shown that generation of oxidative stress leads to the cleavage and deactivation of Hsp90. Because cancer cells are often deficient in antioxidant enzymes and exhibit higher basal levels of reactive oxygen species (ROS) than their normal counterparts, inducing a selective oxidative stress may be a promising approach for cancer treatment. Thus, many redox-modulating agents have, therefore, been developed or are undergoing clinical trials and Hsp90 represents a new target for oxidative stress-generating agents. The purpose of this article is to review the current state of knowledge about Hsp90 and the use of oxidative stress-generating agents in cancer treatment. We will illustrate the review with some of our results concerning the effects of oxidative stress on Hsp90 using various oxidative stress-generating systems based on different quinones in combination with a well-known reducing agent (i.e., ascorbate). Our results show that oxidative stress provokes the cleavage of Hsp90 in CML cells, as well as the degradation of its client protein Bcr-Abl and the deactivation of its downstream signaling pathways, namely MAPK and STAT5. Overall, these results highlight the potential interest of using oxidative stress to target Hsp90.

Keywords: Hsp90, oxidative stress, Fenton reaction, adenosine, redox cycling, cancer therapy, protein cleavage.

I. HSP90: STRUCTURE AND FUNCTION

Generalities

Heat-shock proteins (HSPs) are a highly conserved family of molecular chaperones. The name “heat shock” was used because some members (e.g., Hsp70) can be induced by various stresses, such as temperature elevation, hypoxia or oxidative damage. The transcription of inducible HSPs is controlled by heat-shock factors (HSFs), mainly HSF1 [1]. The rapid induction of HSPs in response to stress is the result of a coordinated series of events including the heat shock response (HSR). Because HSP mRNAs lack introns, they are efficiently and rapidly transcribed without requirement of ATP-dependent post-translational modifications [2, 3]. Once present in the cell, induced HSPs provide protection against subsequent and potentially lethal stresses. Thus, HSPs regulate protein folding to ensure correct conformation and translocation, as well as to avoid the formation of toxic protein aggregates inside the cell [4]. It is noteworthy that HSPs also participate in the post-translational modification of signaling molecules, the assembly and disassembly of transcriptional complexes, and the modulation of immunogenic proteins [5].

HSPs are classified into families according to their molecular size, including Hsp100, Hsp90, Hsp70, Hsp60, and small HSPs (15-30 kDa). Although high molecular weight HSPs are ATP-dependent, small HSPs act in an ATP-independent manner [6]. HSPs act in concert with co-chaperones and other molecules to form large multiprotein complexes [4]. The Hsp90 family of proteins in mammalian cells consists of two different cytotoxic isoforms of Hsp90, Hsp90α (gene name: HSP90AA1) and Hsp90β (gene name: HSP90AB1), one endoplasmic reticulum paralogue, grp94 (gene name: HSP90B1), and one mitochondrial paralogue, grp78 (gene name: HSP90A1). Among chaperones, Hsp90 is unique because it is not needed for the biogenesis of most polypeptides [7]; instead most of its client proteins are conformationally labile signal transducers that have a crucial role in growth control, cell survival and developmental processes [8].

Hsp90 Structure and Function

Hsp90 is a constitutively expressed protein that represents 1-2% of the total protein content of the cell [9]. It is required for the stabilization and activation of numerous proteins referred to as “client proteins”. Client proteins need Hsp90 to exhibit a correct conformation or simply to prevent their degradation. If the activity of Hsp90 is impaired, for example, because of the presence of an inhibitor or lack of ATP, client proteins are deactivated or destabilized, depending on the nature of their dependence on Hsp90 [10]. Structurally, Hsp90 is a flexible dimer that mainly consists of alpha-alpha or beta-beta homodimers [11]. The monomers consist of three domains with distinct functions: An N-terminal nucleotide-binding domain, a middle client protein binding/ATP-hydrolysis regulating domain, and a C-terminal dimerization/nucleotide-binding domain (Fig. 1).

Dynamic Chaperone Cycle of Hsp90

The structure of the N-terminal nucleotide-binding domain of Hsp90 makes it a member of the GHKL (gyrase, Hsp90, histidine kinase, mutL) family of kinases/ATPases, together with other proteins, such as DNA topoisomerase II, mismatch repair enzymes, MutL, and histidine kinases [12]. The different members of this family share four highly conserved motifs that define an ATP-binding fold known as the Bergerat fold [13].

Upon ATP binding, Hsp90 undergoes a complex cycle of conformational changes that have often been compared to a “molecular clamp” [14]. Briefly, a short segment of the N-terminal domain changes its position and flaps over the binding pocket. This releases a short N-terminal segment, which, in a subsequent step, binds to the respective N-domain of the other subunit in the dimer, inducing a transient dimerization of both subunits. These rearrangements result in other conformational changes throughout the entire Hsp90 dimer, leading to a twisted and compacted conformation in which N- and M-domains associate. The association of N- and M-domains completes the active site of this “split ATPase” since a catalytic arginine residue (Arg-380 in yeast)
is thus correctly positioned for ATP hydrolysis to occur [15]. The ATPase activity of human Hsp90 is very slow: One molecule of ATP is hydrolyzed by the human protein every 11 minutes [16]. Interestingly, the affinity of the Hsp90 N-terminal nucleotide-binding domain for ADP is much stronger than for ATP: The Ks are 7.2 μM for ADP and 240 μM for ATP in human Hsp90 [17]. As a consequence, only a small modification of the ADP/ATP ratio is sufficient to affect Hsp90 function.

In addition to hydrolyzing ATP and undergoing conformational rearrangements, Hsp90 needs to associate with many co-chaperones to stabilize its client proteins. Indeed, in vivo, Hsp90 functions as the core component of a dynamic set of multiprotein complexes, involving a plethora of collaborating proteins or co-chaperones. In eukaryotic cells, more than 20 co-chaperones have been identified. Co-chaperones modulate Hsp90 function in several ways. They coordinate the interplay between Hsp70 and other chaperone systems (such as Hsp70); they stimulate or inhibit the ATPase activity of Hsp90; they recruit specific classes of clients to Hsp90; and, through their enzymatic activities, they contribute to various aspects of the chaperone cycle [18].

II. HSP90: THE CANCER CHAPERONE

Many tumors, solid [19-21] and hematological [22, 23], show increased levels of HSPs. In the particular case of Hsp90, the protein is expressed 2-10 fold more in cancer cells compared to their normal counterparts [24], making it an attractive target for the development of therapeutic inhibitors. By stabilizing and activating diverse client proteins involved in numerous signaling pathways, Hsp90 plays a pivotal role in the acquisition and maintenance of each of the six particular capabilities that characterize cancer cells, as described by Hanahan and Weinberg [25] (Fig. 2). For this reason, Hsp90 is often referred to as the “cancer chaperone” [26].

Hundreds of proteins have already been shown to interact with Hsp90 and the number of known client proteins of Hsp90 grows every day, already exceeding 200 (for review, see the frequently updated list on the website of Didier Picard’s laboratory http://www.picard.ch/downloads/Hsp90interactors.pdf). Many of them are kinases that are involved in proliferation and growth signaling. This is the case of HER2 (Human Epidermal Growth Factor 2, also known as Erbb2 or Neu), that was the first protein shown to be degraded following benzoquinone ansamycin treatment, although it was not then known that degradation occurred through Hsp90 inhibition [27]. HER2 is a member of the HER receptor family, which is situated in the plasma membrane and does not possess an external ligand-binding domain. HER2 participates, by homo- or heterodimerization, with other members of the HER family, in the activation of downstream signaling pathways, such as MAPKinase or PI3Kinase pathways. B-Raf is a typical example of proteins which depend more on Hsp90 when they harbor mutations. Wild-type unmutated B-Raf does not require Hsp90 for stability whereas activated mutated B-Raf does. Mutated B-Raf directly activates MEK or c-raf, activating the ERK MAPK pathway [28]. The selective degradation of mutated B-Raf by Hsp90 inhibitors illustrates the fact that mutated proteins are less stable than normal proteins and are thus more dependent on Hsp90. This also justifies the use of Hsp90 inhibitors as anticancer therapy because they will preferentially affect abnormal mutated proteins rather than normal ones.

Upon Hsp90 inhibition, by inhibitors or as the result of a decrease in ATP, Hsp90 client proteins are subsequently degraded by the proteasome [24]. Degradation of proteins by the proteasome is mainly mediated by the E3 ubiquitin ligase, CHIP (Carboxy-terminus of Hsc70 Interacting Protein) [29]. CHIP interacts with the C-terminal part of Hsp70 (and Hsp90) through its own N-terminal domain that contains a tetratricopeptide repeat (TPR) domain [30]. Ubiquitinylated proteins are then targeted and degraded by the proteasome.

III. HSP90 INHIBITORS

Hsp90 is a very promising and relevant target in cancer therapy because it is required for the stability and/or activity of many client proteins that are important for cancer cells. By inhibiting Hsp90, several pathways can therefore be targeted with a single inhibitor. This is of importance because a frequent cause of failure of targeted therapy is that the therapies inhibit only one signaling pathway, whereas several may be dysregulated at the same time in a cancer cell. In addition, oncoproteins are often expressed as mutant forms, which are more dependent on Hsp90 for stability than their normal counterparts. Cancer cells are also subjected to more stresses (for example due to acidosis, hypoxia and nutrient deprivation) than normal cells and are consequently more dependent on the activity of the Hsp90 chaperone machinery [31]. Finally, it has been suggested that Hsp90 inhibitors preferentially bind to Hsp90 in cancer cells rather than in normal cells, possibly because Hsp90 would be in an activated complex in cancer cells, having a greater affinity for nucleotides and inhibitors [32].

For these reasons, various Hsp90 inhibitors have been developed and several have entered clinical trials. First attempts with inhibitors focused mainly on compounds that bind to the N-terminal nucleotide binding pocket of Hsp90, such as ansamycins and radicicols. New approaches are now also being considered. These include inhibition of the C-terminal nucleotide-binding pocket of Hsp90 using derivatives of novobiocin [33]; inhibition of the interaction between Hsp90 and co-chaperones, such as Aha1.
[34] and p50cdc37 [35]; inhibition of the interaction of Hsp90 with client proteins [36]; Hsp90 inhibition by hyperacetylation through inhibition of HDACs [37]; and other mechanisms such as Hsp90 oxidation and cleavage by oxidative stress [38].

**N-Terminal Nucleotide-Binding Pocket Inhibitors**

Until now, all Hsp90 inhibitors that have been tested clinically rely on a common mechanism of action that involves competitive binding of a drug to the N-terminal nucleotide-binding site of Hsp90. Hepatotoxicity and the development of multidrug resistance via the expression of P-gp (P-glycoprotein) efflux pumps were the first issues encountered with the geldanamycin-derived inhibitors, 17-AAG (tanespimycin) and 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin, alvespimycin), which entered clinical trials [39]. Moreover, recent results from a clinical phase II study in patients with metastatic melanoma showed no effect of 17-AAG on tumor progression [40]. Another phase II study in patients with hormone-refractory metastatic prostate cancer showed no response to 17-AAG as a single agent [41]. In another phase II study that enrolled patients with papillary and clear cell renal cell carcinoma, no response to 17-AAG was observed either [42]. Because of the lack of effects of 17-AAG alone in phase II studies, recent studies have involved 17-AAG in combination with at least one or more chemotherapeutic agent. For instance, in patients with multiple myeloma and metastatic breast cancer, 17-AAG is now administered in combination with bortezomib (a proteasome inhibitor) or trastuzumab (an anti-HER2 monoclonal antibody). In this latter case, 17-AAG seems to be only active in HER2-positive breast cancers [43]. The development of 17-DMAG (alvespimycin) was discontinued because of an unfavorable toxicity profile [45]. Radicicol binds to the N-terminal nucleotide binding pocket of Hsp90 with very high affinity [44]. It has cellular effects similar to the ansamycins, including client protein degradation and subsequent cancer cell death, but it lacks hepatotoxicity. However, radicicol and its derivatives, such as KF29163, are not stable in the serum and consequently have no activity in vivo [45].

A potentially detrimental facet of N-terminal Hsp90 inhibition remains the induction of the HSR. All Hsp90 inhibitors that bind to the N-terminal ATP binding site induce the overexpression of HSPs, including Hsp27, Hsp40, Hsp70, and Hsp90. Because of the anti-apoptotic and pro-survival effects of HSPs, mainly Hsp70, their overexpression in cancer cells may alter the potential of N-terminal inhibitors as anti-cancer agents [46]. In conclusion, many Hsp90 N-terminal nucleotide-binding pocket modulators have been described and to date, 13 Hsp90 inhibitors are undergoing clinical evaluation in cancer patients, 10 of which have entered the arena in the past 3 years [47].

**C-Terminal Nucleotide-Binding Pocket Inhibitors**

C-terminal nucleotide-binding pocket has been studied far less than its N-terminal counterpart. The mechanism by which it is required for correct Hsp90 stabilization of client proteins is not clear. However, Marcu et al. reported that inhibition of this C-terminal ATP-binding domain with novobiocin and coumarin analogues results in the degradation of the Hsp90 client proteins through the ubiquitin-proteasome pathway [48]. Unfortunately, the concentrations required to achieve client protein degradation are relatively high (around 700 μM) compared with the use of N-terminal inhibitors. Thus, studies are now being conducted to discover new inhibitors of the C-terminal domain of Hsp90 with better affinities [33, 49].

**IV. OXIDATIVE STRESS AND DISRUPTION OF HSP90 CHAPERONE ACTIVITY**

**Oxidative Stress in Cancer Treatment**

Selectivity is a critical issue in cancer therapy. Knowledge of cancer cell features is, therefore, important to selectively target these cells. Among other particularities, most cancer cell types are deficient in antioxidant enzymes. This feature has been demonstrated in many laboratories using various models [50-53] and our team has obtained similar results by comparing normal murine hepatocytes to hepatoma cells and normal human leukocytes to chronic myelogenous leukemia (CML) cells [54, 55]. This antioxidant enzyme deficiency may represent an advantage for cancer cells, because a moderate increase in reactive oxygen species (ROS) can promote cell proliferation and differentiation [56]. Indeed, cancer cells have increased levels of ROS when compared to normal cells [57]. Deficiency in antioxidant enzymes...
that detoxify ROS is not the only reason to explain the higher levels of ROS. Cancer cells produce more ROS through several mechanisms: ROS generation by oncogenic stimulation (i.e., Bcr-abl), dysfunction of the mitochondrial respiratory chain leading to an increased leakage of electrons, increased metabolic activity [58], and loss of p53, which has been shown to upregulate several antioxidant genes [59]. Since cancer cells have higher levels of ROS and less antioxidant capacity, they are more vulnerable to ROS-generating agents. Targeting this biochemical feature of cancer cells with redox-modulating strategies is, therefore, a feasible therapeutic approach that may enable selectivity and overcome drug resistance. Thus, several agents that induce cancer cell death through redox mechanisms are currently undergoing preclinical and/or clinical evaluation.

Agents, such as arsenic trioxide (As$_2$O$_3$), which impair the function of the respiratory chain, are known to increase the production of superoxide [60]. This compound has been used as an anticancer drug for a long time in traditional Chinese medicine and has been shown to be effective in the treatment of newly diagnosed and relapsed acute promyelocytic leukemia. More interestingly, the anticancer action of As$_2$O$_3$ has been shown in many solid tumours. The exact mechanism of action of As$_2$O$_3$ is still incompletely understood and it may have several intracellular targets. In addition to impairment of the function of the respiratory chain, As$_2$O$_3$ also strongly inhibits the thioredoxin antioxidant system [61].

Some iron chelators have been described as exerting antitumor effects through redox mechanisms. For example, OCC-191 was shown to provoke inhibition of ribonucleotide reductase through localized generation of ROS and subsequent destruction of a tyrosine radical in the protein. Because this enzyme is important for the synthesis of deoxyribonucleotides from their ribonucleotide precursors in cancer cells, cancer cell treatment with OCC-191 leads to cell death [62, 63]. Adaphostin (NSC 680410) is another ROS-generating agent that induces cancer cell death [64]. It generates ROS by inhibiting complex III of the respiratory chain, leading to mitochondrial ROS generation [65]. In CML cells, adaphostin induces degradation of the BcR-Abl protein, and overcomes BcR-Abl-mutation-dependent resistance to imatinib [66]. In addition, adaphostin is effective against several types of cancer other than leukemia [67, 68].

Other compounds act by inducing glutathione (GSH) depletion, either by a direct reaction (isocyanates such as benzylisothiocyanate (BITC), phenylethylisothiocyanate (PEITC), sulforaphane, and aziridine derivatives (imexon) [69] or by inhibiting its synthesis (as with buthionine sulphoximine (BSO), an inhibitor of glutathione cysteine synthetase) [70]. Sulphasalazine, an inhibitor of the cystine/glutamate antiporter, may also cause GSH depletion by inhibiting the uptake of cysteine, the precursor of cysteine, which is a rate-limiting substrate for GSH synthesis [71]. Another thiol-based antioxidant is the thioredoxin system. Specific inhibitors of thioredoxin 1 and thioredoxin reductase 1 have recently been developed. For example PX-12 (1-methylpropyl 2-imidazolyl disulphide), a Trx-1 inhibitor, was shown to have potent antitumor activity in vivo [72].

**Oxidative Processes and Disruption of Hsp90 Signaling**

Interestingly, it can be observed that several Hsp90 inhibitors carry a quinone moiety and exhibit redox-active properties, suggesting a potential role for oxidative stress and Hsp90 inhibition. It has thus been reported that ansamycin antibiotics, such as geldanamycin, can produce superoxide anion, suggesting that, in addition to direct inhibition of Hsp90, the antioxidant effect of geldanamycin and its derivatives is also mediated through the production of ROS [73, 74]. For instance, Fukuyo et al. have reported that ROS production by 17-AAG and DMAP contributes to the antitumorigenic effect of these compounds by provoking the destabilization and the inactivation of mutant BRAF (V600E), thereby inhibiting the activation of mitogen-activated protein/extracellular signal-regulated kinase 1/2 (MEK1/2) [75]. However, the effects observed in this study seem independent of Hsp90 because Hsp90 inhibitors structurally unrelated to geldanamycin (such as radicicol and novobiocin), while inducing the degradation of Hsp90 client proteins, failed to induce BRAF(V600E) degradation.

The pro-oxidant properties of geldanamycin can also be explained by its electrophilic nature. Indeed, geldanamycin and its derivatives have been found to react nonenzymatically with glutathione (GSH) at physiological pH and concentrations, forming stable GSH adducts and depleting cellular GSH [76]. These results suggest that reactions with thiol groups of critical cellular proteins could be important for the mechanism of toxicity of these agents, but also that intracellular GSH levels could determine the sensitivity of cancer cells to this class of chemotherapeutic agents. In line with this suggestion, McCollum et al. have shown that up-regulation of heat shock protein 27 (Hsp27), in response to Hsp90 inhibition by 17-AAG, provokes an increased synthesis of GSH that induces a cellular resistance to 17-AAG [77]. Interestingly, it has also been shown that Hsp90 contains few very reactive cysteine residues and is therefore able to reduce some proteins such as cytochrome c, suggesting a role for Hsp90 in the maintenance of the redox status of the cytosol [78]. It is therefore tempting to speculate that the Hsp90 inhibitor geldanamycin or its derivatives, because of their electrophilic properties, could react with critical cysteine residues of Hsp90 and may thereby inhibit its ability to regulate cellular oxidative stress, resulting in cytotoxicity.

Taken together, these data illustrate the close relationships existing between Hsp90 regulation and cellular redox homeostasis. However, despite the existence of several publications on the topic, the precise role of oxidative stress, such as induced by geldanamycin and its derivatives, in the disruption of Hsp90 signaling complex still remains unclear [79]. In the next part of this review, a particular attention will therefore be given to the effects of an oxidative stress, generated by the use of combinations between quinone compounds and ascorbate, on Hsp90 integrity and function.

**Ascorbate/Menadione Redox Cycling and Oxidative Stress in Cancer Treatment**

a) **Generalities**

The combination of ascorbate and menadione (asc/men) is a ROS-generating system that induces the death of multiple cancer cell types. Ascorbate-driven menadione-redox cycling is initiated by electron transfer from ascorbate to quinone (Fig. 3). The rapid reoxidation of the semiquinone radical to its quinone form by molecular oxygen leads to the generation of ROS, such as superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$).

The first studies with asc/men were conducted in vivo, and showed that asc/men potentiated chemotherapeutic action in TLT (transplantable liver tumor) bearing mice [80]. The potentiation was not specific for particular classes of chemotherapeutic agents since all five classes tested (alkylating agents, antimetabolites, mitotic inhibitors, intercalating agents, and asparaginase) were potentiated by asc/men. Moreover, asc/men was also shown to potentiate radiotherapy [81]. In vitro, it was also demonstrated that addition of ascorbate and menadione was synergic and induced the death of cancer cells. The asc/men antitumor effects involved H$_2$O$_2$ production because the addition of catalase totally suppressed the toxicity of asc/men in cultured cancer cells [82]. From a mechanistic point of view, Verrax et al. have shown that asc/men-induced cell death was necrotic rather than apoptotic [83–85]. They also showed that cell death occurred by inhibition of glycolysis, provoking a marked depletion in intracellular ATP levels. Inhibition
of glycolysis is actually due to activation of the poly(ADPribose) polymerase (PARP) enzyme, which, following DNA damage, gets activated and depletes NAD+ levels [86]. Furthermore, Dejeans et al. showed that asc/men impaired calcium homeostasis and induced endoplasmic reticulum (ER) stress in cancer cells [87]. Recently, we also observed that the oxidative stress generated by asc/men combination causes the cleavage of Hsp90 [38], thereby representing a new molecular pathway by which this treatment can affect cancer cell viability.

Based on the encouraging preclinical data, the use of asc/men for the treatment of metastatic or locally advanced, inoperable transitional cell carcinoma of the urothelium (stage III and IV bladder cancer) has been granted by the FDA. Recently, an oral combination of ascorbate and menadione (Apatone©) was evaluated as an anti-cancer agent in a clinical study in prostate cancer patients who had failed standard therapy. Promising delay in biochemical progression of the disease was observed in Apatone-treated patients. Indeed, a significant increase in the PSA (Prostate Serum Antigen) doubling time was induced in patients by Apatone, suggesting that cellular antioxidant capacity is the main factor involved in the sensitivity of cells to the combination [91].

b)Asc/Men Effects on Hsp90

Given that Hsp90 requires ATP for its chaperoning activity [89], we first postulated that asc/men could inhibit the Hsp90 chaperone machinery because it inhibits glycolysis and depletes intracellular ATP levels [86]. In contrast to our hypothesis, however, inhibition of Hsp90 chaperone activity was likely not caused by ATP depletion but rather by the oxidative cleavage of the protein. Such a result is not a surprise because it is well known that ROS can oxidize proteins and provoke several types of damage, including cleavage of the polypeptide chain [90]. We, therefore, postulated that oxidative stress by asc/men, and especially H2O2, would induce Hsp90 cleavage. The molecular mechanism appears to involve the in situ formation of hydroxyl radicals due to a Fenton-type reaction (submitted). Indeed, the cleavage requires the presence of redox-active iron that is driven close to the protein by adenine nucleotides bound to the N-terminal nucleotide binding pocket of Hsp90. The in situ reaction between catalytically active iron and H2O2 leads to the formation of oxygen radicals (Fenton-type reaction) that form an Hsp90 protein radical, which, by rearrangement, causes rupture of the peptide backbone. As a consequence of this cleavage, Hsp90 activity is inhibited, provoking degradation of several client proteins [38, 55].

Interestingly, the cleavage seems rather specific for cancer cells. Indeed, by analyzing the effects of asc/men on the integrity of Hsp90 in a wide panel of cell lines, including both normal (HUVEC, Balb/c3T3, freshly prepared human leukocytes and freshly prepared murine hepatocytes) and transformed cells (TLT, Ishikawa, MCF-7, melanoma B16, FSAII and LLC), we observed that asc/men caused a cleavage of Hsp90 only in transformed cell lines, irrespectively of species (rodents or human) or tissue origin (breast, liver, skin, endometrial) [38]. This is in good agreement with our previous data showing that asc/men was cytotoxic against a human chronic myelogenous leukemia (CML) cell line, namely K562 cells, but was devoid of effect on human hematopoietic stem cells, their normal counterparts [84]. More recently, we obtained similar results by comparing the sensitivity of peripheral leukocytes derived from patients with CML and peripheral leukocytes derived from healthy donors, to the oxidative stress generated by asc/men. Again, we observed that asc/men was active against CML cells but was less cytotoxic in normal leukocytes [55]. This differential sensitivity may be explained by the decreased expression of the antioxidant enzyme catalase in CML cells compared to normal leukocytes. Such a difference in the activities of the three major antioxidant enzymes (i.e. superoxide dismutase, catalase and glutathione peroxidase) between normal and cancer cells had been previously observed between freshly isolated murine hepatocytes and murine hepatoma cells [54]. Furthermore, a correlation between the sensitivity of different cancer cell lines to asc/men and their levels of antioxidant enzymes had also been described by our group, suggesting that cellular antioxidant capacity is the main factor involved in the sensitivity of cells to the combination [91].

In CML cells, Hsp90 cleavage leads to the degradation of the oncoprotein Bcr-Abl and deactivation of several of its downstream pathways. Thus, Western-blot experiments revealed rapid dephosphorylation of c-Raf, ERK1/2 and Elk, three members of the MAPK family of proteins. In addition, we also observed dephosphorylation of STAT5 upon treatment of the cells with asc/men. Interestingly, Hsp90 cleavage did not induce Hsp70, an undesirable adverse effect that is observed with many classical Hsp90 inhibitors (Fig. 4).

Based on these data, we determined whether oxidative stress-induced Hsp90 cleavage could have implications in the field of cancer therapy. In CML, a common problem is the appearance of mutations within the Bcr-Abl protein that render the cells resistant

**Fig. (3).** The ascorbate-driven menadione redox cycling.
to classical inhibitors, such as imatinib [92]. Among the different mutations, T315I seems to be the worst because it renders the cells completely resistant to imatinib [93]. Our results showed that asc/men, by its oxidative stress and Hsp90 inhibition, overcame the resistance to imatinib of CML cells harboring Bcr-Abl mutations (either E255K or T315I). Indeed, asc/men induced degradation of Bcr-Abl rather than a classical pharmacological inhibition [38, 55]. Its activity is, therefore, not impaired by mutations within the kinase domain of the protein. All the mutated forms of Bcr-Abl were degraded to the same extent following exposure of cells to asc/men, suggesting that asc/men could be of interest in the treatment of resistant CML cells.

The effects of asc/men were also tested in a model of BaF3/Bcr-Abl-T315I cells growing as solid tumors. We observed that, compared to animals receiving i.p. saline, treatment of mice with asc/men (1 g/kg, 10 mg/kg, i.p.) significantly reduced the growth rate of tumors [55]. However, when we decided to test whether asc/men could reduce the proliferation of BaF3 cells expressing either wild-type (WT) or mutated Bcr-Abl in the blood of mice, we observed that asc/men treatment was not able to impair the proliferation of BaF3/Bcr-Abl cells, irrespective of the Bcr-Abl mutation. Since asc/men was administered in the same manner (i.p. injections) in these models, pharmacokinetic differences cannot explain this difference of effects and the most likely explanation is that red blood cells (RBC), which exhibit high catalase and glutathione peroxidase activities, can readily detoxify H$_2$O$_2$ and protect cells and tissues from the damage it induces [94]. A similar antioxidant protection was also observed by Chen et al. who reported that RBC completely protect lymphoma cells from H$_2$O$_2$ generated by pharmacologic concentrations of ascorbate [95]. Because Hsp90 cleavage could have broad implications, we decided to test whether other quinones such as dichlone, lawsone and anthraquinone could exert the same activity as menadione when administered in combination with ascorbate. It was observed (Fig. 5A) that only dichlone provoked Hsp90 cleavage when administered in combination with ascorbate, in a similar extent to that was observed with asc/men. The lack of effect of combinations of ascorbate/lawsone and ascorbate/anthraquinone on Hsp90 was correlated with the absence of cytotoxicity (Fig. 5B). No effect was observed when compounds were administered in the absence of ascorbate. The reason for which certain quinones react with ascorbate but not with others probably relies on the first half-redox potential of the quinone. Indeed, we have previously described that only quinones having a half-redox potential between -250 and +50mV are able to oxidize ascorbate, thus generating a redox cycling and the formation of ROS [84]. Thus, menadione and dichlone exhibit a first half-wave potential at -203 and -36mV, respectively, whereas lawsone has an half-wave potential at -
415mV and anthraquinone at -445mV [96]. This further confirms the critical role played by oxidative stress in the Hsp90 cleavage induced by asc/men.

Recently we have reported that the addition of ascorbate influences the cytotoxicity of the members of a series of 8-phenylaminopyrimido[4,5-c]isoquinolinequinones (Fig. 6) [97]. Within this series, a group of members unsubstituted at 6-position such as 5a-e showed a significant higher cytotoxic activity and induction of oxidative stress with ascorbate, as compared to their corresponding analogues 5d-f containing a methyl group at 6-position. The results were attributed to the difference between the redox capabilities of the corresponding quinone analogues, evaluated through their corresponding first half-wave potentials. Based on these precedents and assuming that the redox cycling process is related with Hsp90 cleavage, we examined the ability of quinones 5a-c and their analogues 5d-f to induce the cleavage of the Hsp90 protein, in the presence of ascorbate (Fig. 7). According to the biological evaluation, it was observed that the former compounds (5a-c) induced Hsp90 cleavage and the later (5d-f) were unable to provoke such effect on Hsp90. From Fig. (7) it can also be pointed out that an apoptotic inducer, namely sanguinarine, failed to induce Hsp90 cleavage, thereby confirming that the cleavage process is not the consequence of cell death. Therefore, we can conclude that an oxidative stress induced by a combination between ascorbate and the highly cytotoxic compounds 5a-c is involved in the cleavage of this oncologic target.

**V. CONCLUDING REMARKS**

Generation of oxidative stress is a promising experimental approach to induce cancer cell death. Among oxidative stress-generating systems, the combination of ascorbate and menadione (asc/men) induces cancer cell death through several mechanisms, including glycolysis inhibition, impairment of calcium homeostasis,
and Hsp90 cleavage. Hsp90 chaperone disruption leads to the degradation of several client proteins and the subsequent deactivation of their downstream signaling pathways. Hsp90 cleavage is also observed with quinones other than menadione, showing that the effect is not specific for one particular compound. These results could have broad implications because Hsp90 client proteins are mostly oncoproteins that are involved in the acquisition of several hallmarks of cancer. The combination of quinone compounds with ascorbate could, therefore, represents an innovative strategy for the treatment of cancer.

ACKNOWLEDGEMENTS

The authors thank Isabelle Blave and Veronique Allaeys for their excellent technical assistance. This work was supported by grants from the Belgian Fonds National de la Recherche Scientifique (3.4605.06), by the Fonds Speciaux de Recherche (FSR) Université Catholique de Louvain and by CONICYT-WBI (Belgium).

REFERENCES


Verrax, J.; De Gerlache, J.; Lans, M.; Roberfroid, M. Non-toxic potential


