Send Orders of Reprints at bspsaif@emirates.net.ae

2094 *Current Topics in Medicinal Chemistry,* 2012, *12,* 2094-2102

Biological Evaluation of 3-Acyl-2-Arylamino-1,4-Naphthoquinones as Inhibitors of Hsp90 Chaperoning Function

David Ríos[a,b], Julio Benites[a,b], Jaime A. Valderrama[b,c,*], Mirelle Farias[d], Rozangela C. Pedrosa[d], Julien Verrax[e] and Pedro Buc Calderon[a,b,e,*]

[a]*Facultad de Ciencias de la Salud, Universidad Arturo Prat, Iquique, Chile;* [b]*Instituto de EtnoFarmacología (IDE), Universidad Arturo Prat, Iquique, Chile;* [c]*Facultad de Química, Pontificia Universidad Católica de Chile, Santiago, Chile;* [d]*Laboratorio de Bioquimica Experimental, Departamento de Bioquimica, Universidade Federal de Santa Catarina, Florianopolis, Brasil;* [e]*Toxicology and Cancer Biology Research Group, Louvain Drug Research Institute (LDRI), Université Catholique de Louvain, Brussels, Belgium*

Abstract: Hsp90 is a chaperone that plays a key function in cancer cells by stabilizing proteins responsible of cell growth and survival. Disruption of the Hsp90 chaperone machinery leads to the proteasomal degradation of its client proteins. Hsp90 appears then as an attractive target for the development of new anticancer molecules. We have shown that ascorbate-driven menadione-redox cycling inhibits Hsp90 activity by provoking an N-terminal cleavage of the protein, inducing the degradation of several of its client proteins. Since the mechanism involves an oxidative stress, we explored the effect of a series of diverse donor-acceptor 3-acyl-2-phenylamino 1,4-naphthoquinones on Hsp90 integrity, in the presence of ascorbate. Results show that quinone-derivatives that bear two electroactive groups (namely quinone and nitro) exhibit the highest inhibitory activity (Hsp90 cleavage and cell death). The biological activity of the series mainly relies on their redox capacity and their lipophilicity, which both modulate the ability of these compounds to induce a cytotoxic effect in K562 cells. As observed with other redox cycling quinones, the protein cleavage is blocked in the presence of N-terminal Hsp90 inhibitors suggesting that the availability or occupancy of nucleotide binding site in the N-terminal pocket of Hsp90 plays a critical role. In addition the survival of cancer cells and their metabolic and redox homeostasis were strongly impaired by the presence of ascorbate. Since these effects were similar to that obtained by ascorbate/menadione and they were blocked by the antioxidant N-acetylcyteine (NAC), it appears that oxidative stress is a major component of this cytotoxicity.

Keywords: Cancer cell death, Hsp90, oxidative stress, protein cleavage, quinones, redox cycling.

INTRODUCTION

Hsp90 is a constitutively expressed protein that represents 1-2% of the total protein content of the cell [1]. It is required for the stabilization and activation of numerous proteins referred to as "client proteins". In cancer cells, Hsp90 is expressed 2-10 fold more as compared to their normal counterparts [2]. Thus, by stabilizing and activating diverse client proteins involved in numerous signaling pathways, Hsp90 plays a pivotal role in the acquisition and maintenance of some major capabilities that characterize cancer cells, as nicely described by Hanahan and Weinberg [3]. Upon Hsp90 inhibition its client proteins are subsequently degraded by the proteasome [2]. For this reason, Hsp90 is often referred to as the "cancer chaperone" [4], making it an attractive target for the development of therapeutic inhibitors. 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. Since cancer cells are addicted to oncoproteins, the inactivation of a single oncoprotein often leads to the stops of proliferation or death of the cancer cell. This is caused by the fact that cancer cells often inactivate several normal genes while oncogenes are overactivated and perform the functions of several inactivated genes at the same time [5]. In addition, oncoproteins are often expressed as mutant forms, which are more dependent on Hsp90 for their 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 [6]. 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 [7].

The understanding of the role of Hsp90 in promoting cellular malignant transformation is mostly derived from studies of Hsp90 inhibitors such as the natural product Geldanamycin [8,9] a benzoquinone ansamycin antibiotic, and the synthetic derivative 17-*N*-allylamino-17-demethoxy-geldanamycin: 17AAG [10-12] (Fig. 1). Geldanamycin binds

*Address correspondence to these author at the Vicuña Mackenna 4860, Casilla 306, Santiago, Chile; GTOX 7309, 73 avenue E. Mounier, 1200 Bruxelles, Belgium; Tel: +32-2-7647366; Fax:+32-2-7647359;
E-mails: pedro.buccalderon@uclouvain.be and jvalderr@uc.cl

1873-5294/12 $58.00+.00

to the ATP-binding pocket of Hsp90, specifically inhibiting ATPase activity and therefore it has been evaluated for its antiproliferative effects in oncogenesis *in vitro* and *in vivo* with promising application as a novel anti-cancer therapy [13,14]. In addition to the blockade of ATP binding, current Hsp90 inhibitors may also act by disrupting cochaperone/Hsp90 interactions, by antagonism of client/Hsp90 associations and by interfering with post-translational modifications of Hsp90 [15]. It should be underlined, however, that various side effects have slowed the clinical development of these molecules [11,16,17].

Recently we have reported that menadione redox cycling in the presence of ascorbate inhibited the Hsp90 chaperone machinery by the oxidative cleavage of the protein [18-21]. As a consequence of this cleavage, Hsp90 activity is inhibited, provoking the degradation of several client proteins which are critical for the survival of cancer cells [18-21]. The oxidative cleavage of a polypeptide chain is a damaging process initiated by oxygen radicals [22,23]. The mechanisms of peptide bond cleavage following α-carbon oxidation are summarized in (Fig. **2**). Briefly, the oxidative attack of the polypeptide backbone is initiated by the HO˙-dependent abstraction of the α-hydrogen atom from an amino acid residue to form a carbon-centered radical, which, in the presence of oxygen, is rapidly converted to an alkylperoxyl radical (ROO˙). Further reactions with $HO_2^{\cdot}$, the protonated form of superoxide anion ($O_2^{\cdot-}$), or transition metals such as ferrous iron (Fe^{2+}) leads to the formation of an alkoxyl radical (RO˙) which can undergo peptide bond cleavage by either the α-amidation or diamide pathways [22,23].

The aim of this work was therefore to study the effect of a series of diverse donor-acceptor 3-acyl-2-phenylamino-1,4-naphthoquinones (Fig. **3**) on Hsp90 integrity with a special focus on redox cycling and oxidative protein cleavage. The redox active 1,4-naphthoquinones were designed taking into account precedents on the ability of 2,3-substituted 1,4-naphthoquinones, in particular compound HTS1 (Fig. **3**), to inhibit Hsp90 [24] and on the possibility to modulate the redox capability of the pharmacophore to induce selective biological damage of K562 cancer cells, a stable human chronic myeloid leukemia (CML) cell line. To best characterize the effects of quinones, cell survival and metabolic (ATP content) and redox (GSH levels) homeostasis were also monitored in the absence and in the presence of ascorbate.

Chemistry

The preparation of the target compounds **3a-i** was initiated with the synthesis of 2-acyl-1,4-dihydroxynaphthalenes **2a-c** *via* solar photo-Friedel-Crafts acylation of 1,4-naphthoquinone **1** with aldehydes according to a recently reported procedure [25]. Compounds **2a-c** were then oxidized with silver (I) oxide in dichloromethane and the corresponding 2-acyl-1,4-naphthoquinones were *in situ* reacted with the selected phenylamines, in a one-pot procedure, to give the corresponding 3-acyl-2-phenylamino 1,4-naphthoquinones **3a-i** (Scheme 1). The structures of the new compounds were established on the basis of their IR, proton/carbon NMRs and high resolution mass spectra (HRMS).

Experimental

Chemical Synthesis

All reagents were commercially available reagent grade and were used without further purification. Melting points were determined on a Stuart Scientific SMP3 apparatus and are uncorrected. ^{1}H NMR spectra were recorded on Bruker AM-400 instruments in deuterochloroform ($CDCl_3$). ^{13}C NMR spectra were obtained in $CDCl_3$ at 100 MHz. 2D NMR techniques (COSY, HMBC) and DEPT were used for signal assignment. Chemical shifts are expressed in ppm downfield relative to tetramethylsilane (TMS, δ scale), and the coupling constants (*J*) are reported in Hertz. HRMS were obtained on a Thermo Finnigan spectrometer, model MAT 95XP. Silica gel Merck 60 (70-230 mesh) was used for preparative column chromatography and TLC aluminium foil $60F_{254}$ for analytical TLC. Anhydrous dichloromethane HPLC (99.8%) for electrochemical evaluations was obtained from Sigma-Aldrich. Acylhydroquinones were prepared by using a recently reported solar photoacylation procedure [25].

General Procedure for the preparation of 1,4-naphthoquinones 3a-i: A suspension of acylhydroquinone (1 mmol), Ag_2O (5 mmoles), anhydrous $MgSO_4$ (3 mmoles), the required amine (2 mmol) and dichloromethane (15 mL) was left with stirring at room temperature after completion of the reaction as indicated by TLC. The mixture was filtered and washed with CH_2Cl_2. Removal of the solvent under reduced pressure followed by column chromatography yielded pure quinone.

3-Acetyl-2-(phenylamino)-1,4-naphthoquinone (**3a**): Was obtained as a dark red solid (98% yield), mp 137-138 °C. IR (KBr, cm^{-1}) ν_{max} 3449 (O-H), 1683, 1645 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.69 (bs, 1H, NH), 8.10 (d, 1H, *J* = 7.4 Hz, 5- or H-8), 7.82 (d, 1H, *J* = 7.5 Hz, 8- or 5-H), 7.71 (t, 1H, *J* = 7.4 Hz, 6-H or 7-H), 7.56 (t, 1H, *J* = 7.4 Hz, 7- or 6-H), 7.31 (t, 2H, *J* = 7.3 Hz, phenyl), 7.23 (t, 1H, *J* = 7.1 Hz, phenyl), 7.04 (d, 2H, *J* = 7.5 Hz, phenyl), 2.63 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 202.9, 182.3, 181.7, 151.6, 139.4, 135.5, 133.6, 132.8, 131.4, 129.4 (2xC), 127.1, 126.8, 126.3, 124.7 (2xC), 112.3, 33.3. HRMS (M^+): m/z calcd for $C_{18}H_{13}NO_3$: 291.08954; found: 291.08909.

3-Acetyl-2-(4-methoxyphenylamino)-1,4-naphthoquinone (**3b**): Was obtained as a dark brown solid (58% yield), mp 139-140 °C. IR (KBr, cm^{-1}) ν_{max} 3448 (O-H), 1683, 1638 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.74 (bs, 1H, NH), 8.18 (d, 1H, *J* = 7.8 Hz, 5- or 8-H), 7.91 (d, 1H, *J* = 7.4 Hz, 8- or 5-H), 7.81 (t, 1H, *J* = 7.6 Hz, 6- or 7-H), 7.80 (t, 1H, *J* = 7.6 Hz, 7- or 6-H), 7.05 (d, 2H, *J* = 8.7 Hz, phenyl), 6.90 (d, 2H, *J* = 8.9 Hz, phenyl), 3.83 (s, 3H, OCH_3), 2.70 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 202.8, 182.4, 181.6, 158.6, 135.4, 133.7, 132.7, 132.0, 131.4, 126.8, 126.3, 125.9 (3xC), 114.6 (3xC), 55.6, 33.3. HRMS (M^+): m/z calcd for $C_{19}H_{15}NO_4$: 321.10011; found: 321.09956.

3-Acetyl-2-(4-nitrophenylamino)-1,4-naphthoquinone (**3c**): was obtained as a red solid (69% yield), mp 178-179 °C. IR (KBr, cm^{-1}) ν_{max} 3449 (O-H), 1677, 1644 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.83 (bs, 1H, NH), 8.18 (d, 2H, *J* = 8.8 Hz, phenyl), 8.12 (d, 1H, *J* = 7.8 Hz, 5- or 8-H), 7.85

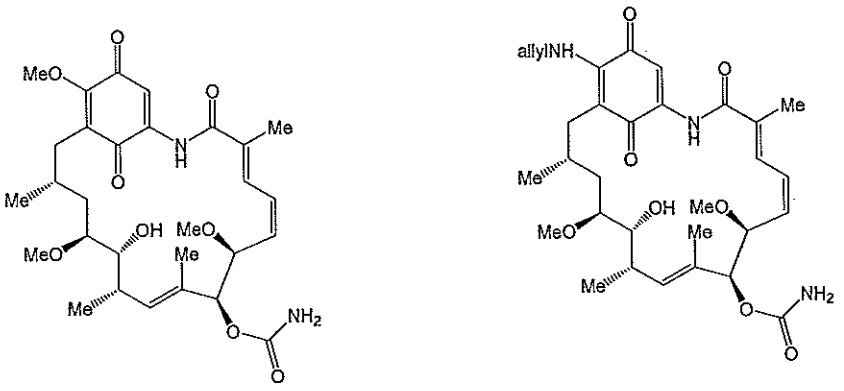


Fig. (1). Chemical structures of geldanamycin and its derivative 17-AAG.

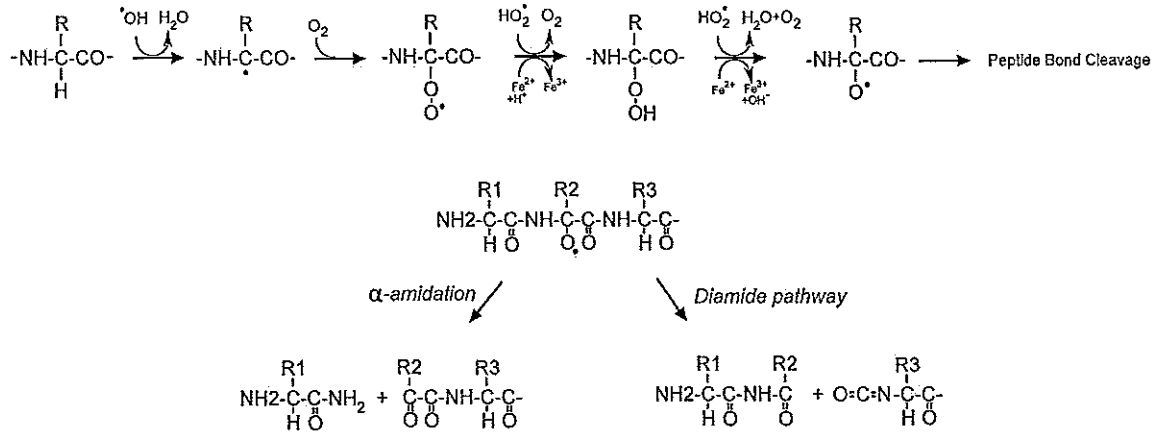


Fig. (2). Theoretical mechanisms of protein cleavage by an oxidative stress.

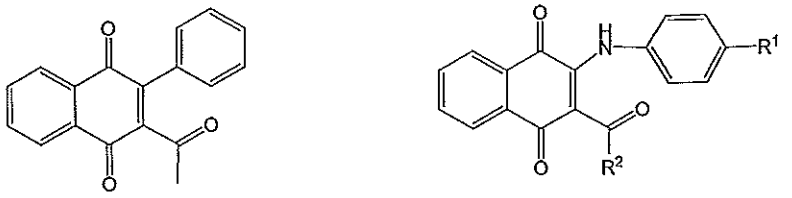


Fig. (3). Chemical structure of HTS1 and the designed substituted 1,4-naphthoquinones.

(d, 1H, J = 7.6 Hz, 8- or 5-H), 7.76 (t, 1H, J = 7.6 Hz, 6- or 7-H), 7.62 (t, 1H, J = 7.6 Hz, 7- or 6-H), 7.16 (d, 2H, J = 8.8 Hz, phenyl), 2.69 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 203.4, 181.8, 181.7, 150.8, 145.7, 135.8, 133.3, 133.1, 127.0, 126.4, 124.9 (3xC), 124.8 (3xC), 113.7, 33.4. HRMS (M^+): m/z calcd for $C_{18}H_{12}N_2O_5$: 336.07462; found: 336.07401.

3-(Furan-2-carbonyl)-2-(phenylamino)-1,4-naphthoquinone (**3d**): Was obtained as a red solid (80% yield), mp 223-224 °C. IR (KBr, cm^{-1}) ν_{max} 3449 (O-H), 1672, 1645 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (d, 1H, J = 7.7 Hz, 5- or 8-H), 8.07 (d, 1H, J = 7.7 Hz, 8- or 5-H), 7.79 (bs, 1H, NH), 7.74 (t, 1H, J = 7.5 Hz, 6-H or 7-H), 7.65 (t, 1H, J = 7.6 Hz, 7- or 6-H), 7.39 (bs, 1H, furyl), 7.04 (m, 3H, phenyl), 6.88 (m, 2H, phenyl), 6.79 (d, 1H, J = 3.5 Hz, furyl), 6.37 (m, 1H, furyl). ^{13}C NMR (100 MHz, $CDCl_3$): δ 182.4, 181.7, 181.2, 153.6, 146.0, 143.8, 137.1, 135.6, 133.1, 132.9, 130.0, 129.0 (2xC), 127.4, 126.8, 126.7, 125.6 (2xC), 118.0, 113.2, 112.7. HRMS (M^+): m/z calcd for $C_{21}H_{13}NO_4$: 343.08446; found: 343.08396.

3-(Furan-2-carbonyl)-2-(4-methoxyphenylamino)-1,4-naphthoquinone (**3e**): Was obtained as an orange solid (42% yield), mp 190-191 °C. ^{1}H NMR (400 MHz, $CDCl_3$) 8.16 (d, 1H, J = 7.7 Hz, 5- or 8-H), 8.13 (d, 1H, J = 7.6 Hz, 8- or 5-H), 7.80 (t, 1H, J = 7.6 Hz, 6- or 7-H), 7.71 (m, 2H, NH + 6- or 7-H), 7.45 (m, 1H, furyl), 6.87 (m, 3H, phenyl and furyl), 6.61 (d, 2H, J = 8.8 Hz, phenyl), 6.43 (m, 1H, furyl), 3.73 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 182.7, 182.0, 181.6, 159.0, 154.0, 146.2, 135.8, 133.0, 130.4, 127.8 (3xC), 127.0, 127.0 (2xC), 118.2, 114.4 (3xC), 112.9 (2xC), 55.8. HRMS (M^+): m/z calcd for $C_{22}H_{15}NO_5$: 373.09502; found: 373.09548.

3-(Furan-2-carbonyl)-2-(4-nitrophenylamino)-1,4-naphthoquinone (**3f**): Was obtained as an orange solid (50% yield), mp 247-248 °C. IR (KBr, cm^{-1}) ν_{max} 3448 (O-H), 1677, 1640 (C=O). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.74 (bs, 1H, NH), 8.10 (d, 1H, J = 7.5 Hz, 5-H or 8-H), 7.99 (d, 1H, J = 7.8 Hz, 8- or 5-H), 7.83 (d, 2H, J = 8.8 Hz, phenyl), 7.82 (m, 1H, 6- or 7-H), 7.77 (t, 1H, J = 7.4 Hz, 7- or 6-H), 7.68 (m, 1H, furyl), 7.09 (d, 2H, J = 8.8 Hz, phenyl), 7.06 (d, 1H, J = 3.5 Hz, furyl), 6.53 (dd, 1H, J = 3.6, furyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 181.3, 181.1, 180.0, 152.2, 146.9, 144.7, 143.6, 143.4, 134.7, 132.7, 131.9, 129.9, 125.9, 125.5, 123.8 (2xC), 123.3 (2xC), 119.0, 115.3, 112.4. HRMS (M^+): m/z calcd for $C_{21}H_{12}N_2O_6$: 388.06954; found: 388.04736.

3-(Thiophene-2-carbonyl)-2-(phenylamino)-1,4-naphthoquinone (**3g**): Was obtained as a red solid (49% yield), mp 210-211 °C. IR (KBr, cm^{-1}) ν_{max} 3483 (O-H), 1672, 1642 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.09 (d, 1H, J = 8.0 Hz, H-5 or H-8), 8.06 (d, 1H, J = 7.9 Hz, 8- or 5-H), 7.78 (bs, 1H, NH), 7.73 (t, 1H, J = 7.5 Hz, 6-H or 7-H), 7.64 (t, 1H, J = 7.5 Hz, 7- or 6-H), 7.46 (d, 1H, J = 4.4 Hz, thienyl), 7.34 (d, 1H, J = 3.1 Hz, thienyl), 7.00 (m, 3H, phenyl), 6.91 (t, 1H, J = 4.3 Hz, thienyl), 6.84 (d, 2H, J = 7.0 Hz, phenyl). ^{13}C NMR (100 MHz, $CDCl_3$): δ 185.3, 182.4, 181.7, 145.5, 143.4, 136.7, 135.6, 134.6, 133.6, 133.0, 132.9, 130.0, 128.9 (2xC), 127.7, 127.3, 126.9, 126.7, 126.2 (2xC), 113.9. HRMS (M^+): m/z calcd for $C_{21}H_{13}NO_3S$: 359.06161; found: 359.05876.

3-(Thiophene-2-carbonyl)-2- (4-methoxyphenylamino)-1,4-naphthoquinone (**3h**): Was obtained as an orange solid (40% yield) mp, 189-190 °C. IR (KBr, cm^{-1}) ν_{max} 3448 (O-H), 1678, 1650 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$) 8.09 (d, 1H, J = 7.7 Hz, 5-H or 8-H), 8.06 (d, 1H, J = 7.7 Hz, 8- or 5-H), 7.72 (t, 1H, J = 7.6 Hz, 6- or 7-H), 7.65 (bs, 1H, NH), 7.64 (m, 1H, 7- or 6-H), 7.47 (d, 1H, J = 4.9 Hz, thienyl), 7.33 (d, 1H, J = 3.8 Hz, thienyl), 6.91 (t, 1H, J = 4.8 Hz, thienyl), 6.75 (d, 2H, J = 8.7 Hz, phenyl), 6.49 (d, 2H, J = 8.9 Hz, phenyl), 3.66 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 185.4, 182.3, 181.5, 158.7, 145.5, 143.7, 135.4, 134.2, 133.5, 132.9, 132.6, 129.8, 128.9, 128.1 (2xC), 127.6, 126.6, 126.5, 113.9 (2xC), 113.5, 55.5. HRMS (M^+): m/z calcd for $C_{22}H_{15}NO_4S$: 389.07218; found: 389.06928.

3-(Thiophene-2-carbonyl)-2- (4-nitrophenylamino)-1,4-naphthoquinone (**3i**): Was obtained as a yellow solid (45% yield), mp 276-277 °C. IR (KBr, cm^{-1}) ν_{max} 3448 (O-H), 1678, 1647 (C=O). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.77 (bs, 1H, NH), 8.13 (d, 1H, J = 7.4 Hz, 5- or 8-H), 8.00 (d, 1H, J = 7.7 Hz, 8-H or 5-H), 7.93 (m, 2H, 6-H + 7-H), 7.87 (d, 2H, J = 9.0 Hz, phenyl), 7.86 (m, 1H, thienyl), 7.81 (d, 1H, J = 3.8 Hz, thienyl), 7.13 (m, 1H, thienyl), 7.12 (d, 2H, J = 9.0 Hz, phenyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 185.0, 181.6, 181.5, 145.4, 144.1, 144.0, 143.3, 136.2, 135.3, 134.9, 133.2, 132.2, 130.5, 128.3, 126.1, 125.6, 124.5 (2xC), 123.6 (2xC), 117.0. HRMS (M^+): m/z calcd for $C_{21}H_{12}N_2O_5S$: 404.04669; found: 404.04304.

Electrochemical Measurements

Cyclic voltammograms of compounds were obtained on a Bioanalytical Systems BAS CV-50W electrochemical analyzer. A small capacity measuring cell was equipped with a platinum disc as working electrode, an Ag/10 nM Ag (MeCN) reference electrode for non aqueous solvent, with a platinum wire auxiliary electrode, a mechanical mini-stirrer, and a capillary to supply an inert argon atmosphere. A 0.1 M solution of tetrabutylammonium tetrafluoroborate in dichloromethane was used as supporting electrolyte. The half-wave potential values, $E^I_{1/2}$ and $E^{II}_{1/2}$, evaluated from the voltammograms obtained at a sweep rate of 100 mVs-1, are summarized in (Table **1**).

Biological Assays

Cell Culture Conditions

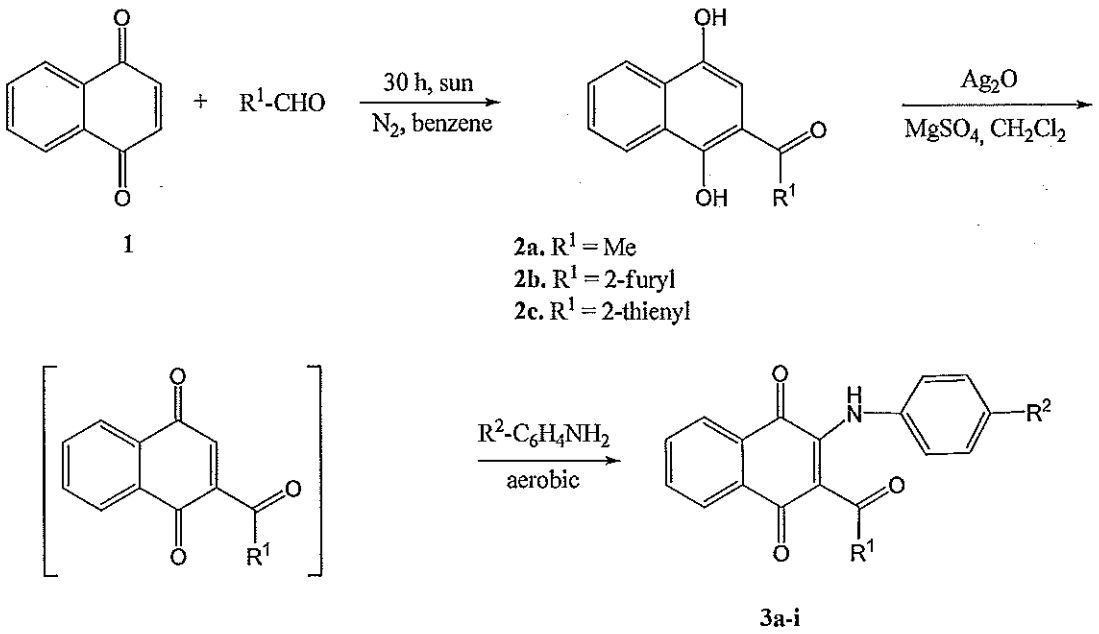
The chronic myeloid leukemia (CML) cell line K562 was a gift of Dr. F. Brasseur (Ludwig Institute for Cancer Research-LICR-Brussels) and maintained in RPMI medium supplemented with 10% fetal calf serum, streptomycin 100 mg/mL, penicillin 100 IU/mL, and gentamicin (50 mg/mL) at 37 °C in humidified 5% CO_2. Cells were incubated at 1 x 10^6 cells/mL and treated with ascorbate (2 mM) and quinones (10 μM), either separately or in association for the indicated length of time.

Cell Death Assessment

The effect of quinones alone or in the presence of ascorbate on the survival of human leukocytes was monitored using the erythrosine exclusion assay. Samples were withdrawn at defined times, centrifuged and suspended in medium. Cell viability was estimated by using the TC10 cell counter, following manufacturer's instructions. Briefly, an aliquot of cell suspension (10 μl) was added to 10 μl of 0.4 % trypan blue solution (from Bio-Rad) in a test tube and the mixture was mixed by gently pipetting up and down several times. Ten microliters were then transferred into the counting chamber and analyzed with the TC10 cell counter. Results are expressed as percentage of viable cells with regard to untreated cell cultures at each sampling time.

Western Blots Analysis

Appropriate protein amounts (40–50 μg) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to a nitrocellulose membrane. The membranes were blocked in blocking-buffer (5% non-fat dry milk, Tris 20 mmol/L, pH 7.6, NaCl 150 mmol/L, 0.01% Tween-20) for 1 h at room temperature, which was followed by incubation with primary antibody overnight at 4°C. The membranes were then washed and incubated for 60 min with a secondary antibody. Immunodetection was performed using the ECL detection kit (Amersham, UK) for HRP-coupled secondary antibodies. Mouse monoclonal primary antibodies against



N°	3a	3b	3c	3d	3e	3f	3g	3h	3i
R^1	Me	Me	Me	2-furyl	2-furyl	2-furyl	2-thienyl	2-thienyl	2-thienyl
R^2	H	OMe	NO_2	H	OMe	NO_2	H	OMe	NO_2

Scheme 1. Preparation of 3-acyl-2-phenylamino 1,4-naphthoquinones **3a-i**.

Hsp90α/β C-terminus were purchased from Santa Cruz Biotechnology (F-8) (Santa Cruz, CA), and mouse monoclonal primary antibody against β-actin (clone AC-15) was purchased from Abcam (Cambridge, UK). Mouse secondary antibodies were purchased from Dako (Glostrup, Denmark). Complete Mini protease inhibitor cocktail was purchased from Roche Applied System (Mannheim, Germany). β-actin served as a loading control.

Intracellular Levels of GSH and ATP

Intracellular GSH was estimated according to Griffith [26]. Briefly, treated cells were washed with cold PBS and immediately acidified with SSA (5%). The samples were submitted to two freeze-thaw cycles and centrifuged at 4°C (10.000 x g during 10 min). Ten micro liters of the supernatant were then placed in a reaction mixture containing 0.2 U/mL of GSR, 50 mg/mL of DTNB and 1 mM of EDTA at pH 7. The reaction was initiated by the addition of 50 mM NADPH and changes in absorbance were recorded at 412 nm. Results are expressed as nmol/mg protein. The protein content was calculated by using the method of Bradford.

ATP content was determined by using the Roche ATP Bioluminescence Assay Kit CLS II (Mannheim, Germany) according to the procedures described by the suppliers. The results are expressed as nmol/10^6 cells.

Statistical Analysis

Data were analyzed by using ANOVA followed by a post hoc Bonferroni test to determine the statistical significance among the different groups. The level of significance was set at $p < 0.05$ (*$p < 0.05$; **$p < 0.01$).

RESULTS AND DISCUSSION

We have recently shown that upon ascorbate/menadione (a 2,4-naphthoquinone derivative), K562 cells were harboring a cleavage of the Hsp90 chaperone protein [18-21]. Consequently, a loss of activation was observed in signaling proteins like STAT5, ERK, or RAF, that are located downstream from Bcr-Abl, a well-known Hsp90 client protein [19]. Interestingly, this cleavage seemed to be more specific for cancer cells than for normal cells [18]. This sensitivity may be explained by the fact that cancer cells are deficient in antioxidant enzymes as shown by the low levels of catalase in K562 cells as compared to leukocytes isolated from healthy donnors [21].

In agreement with our previous results, the (Fig. 4) shows that Hsp90 was cleaved in asc/men-treated cells, but other quinones (at 10 μM) did not affect the integrity of the Hsp90 protein in the absence of ascorbate. Only the compound **3f** was able to induce a slight protein cleavage. Nevertheless, when ascorbate was added to the incubation medium at the pharmacological doses of 2 mM [23], Hsp90 cleavage was clearly observed in several conditions. Indeed, in cells incubated in the presence of ascorbate and either compounds **3c**, **3f** and **3i**, a net cleavage was observed. A slight effect was observed for compounds **3d** and **3h**. The quinones **3a**, **3b**, **3e** and **3g** are practically devoid of such proteolytic activity. It

should be underlined that after 6h incubation, all the quinones, with the exception of compound **3f**, are not cytotoxic (Table **1**). The survival of K562 cells exposed to compound **3f** is 77% as compared to 100% of control untreated cells whereas cell survival in the presence of the other quinones was around 90%.

Interestingly, the first half-wave potentials ($E^{I}_{1/2}$) of compounds **3c**, **3f** and **3i**, namely -376.5, -349.8 and -375.7 mV respectively (Table **1**), are in a range that makes them redox-active molecules able to react with ascorbate to generate a redox cycling leading to the formation of ROS. In this context, only compound **3f** was able to exhibit a significant cytotoxicity to K562 cells when incubated for 6 hours only. Data in (Table **1**) show that the analogues **3c**, **3f** and **3i**, which all contain two electroactive groups, namely quinone and nitro, exhibit the more positive values of first half-wave potential ($E^{I}_{1/2}$) and so the highest oxidative capacity of the series. Indeed, the electroatractive effect of the 4-nitrophenylamino substituent bonded at 2-possition in compounds **3c**, **3f** and **3i**, appears to induce an increase of the electron acceptor capacity of the quinone nucleus compared to the other members of the series. This effect exhibited by the 4-nitrophenylamino group, probably facilitate the formation of the semiquinone radical specie that is involved in the redox cycling process that ultimately lead to the formation of ROS. It is noteworthy that although the analogues **3c**, **3f** and **3i** possess the same nitrogen group substituent of the naphthoquinone nucleus, compounds **3c** and **3f** having similar lipophilic properties (Log P = 2.14 and 2.68) exhibited greater inhibition activities than **3i** (Log P = 3.58). Therefore, it is reasonable to assume that the lower activity of compound **3i**, with respect to compounds **3c** and **3f**, is likely due to the higher lipophilicity of this compound. From these results it seems that the biological activity of the members of the screened series depends, in part, on their redox capacity and lipophilicity, which both modulate the ability to cause cytotoxicity.

According to previous reports [28-33], we founded that quinones endowed of redox properties are capable to generate a redox cycle driven by ascorbate. As a consequence, it results a cleavage of the chaperone Hsp90. Indeed, in K562 cells the association of ascorbate with a series of different quinones, including menadione, provoked the cleavage of Hsp90 [18,21]. The subsequent impairment of its chaperone function leads to the proteasomal degradation of several client proteins like Bcr-Abl, Akt, RIP,cRAF, Nemo (IKKγ) and hTERT [18,20,21]. Since the oncogenic protein Bcr-Abl, a constitutively active protein tyrosine kinase, is responsible for the transformation of normal cells to CML cells [34-39], we anticipate that the survival of K562 cells will be markedly affected by the cleavage of Hsp90 protein.

To best characterize the biological effects of this series of acyl-arylaminonaphthoquinones, we selected the most active member of the series, namely **3f**, and we compare its activity to menadione, a well-known 1,4-naphthoquinone derivative currently studied in our laboratory. A first attempt to characterize the cleavage of Hsp90 was the use of pharmacological inhibitors able to bind the C- and the N-termini nucleotide-binding pocket of the Hsp90 protein. The (Fig. **5**) showed unequivocally that Hsp90 was cleaved upon ascorbate/menadione and ascorbate/**3f** treatments. Interestingly, we observed in K562 cells that Hsp90 cleavage was inhibited by the occupancy of the N-terminal nucleotide binding pocket of Hsp90, as shown by geldanamycin, its 17-AAG derivative and the structurally unrelated radicicol inhibitor. Conversely, the cleavage remained unaffected by novobiocin, a pharmacological inhibitor that binds the C-terminal ATP-binding domain of Hsp90 [40]. This result confirms our hypothesis that the cleavage is taking place inside the nucleotide-binding pocket of the N-terminal domain of Hsp90.

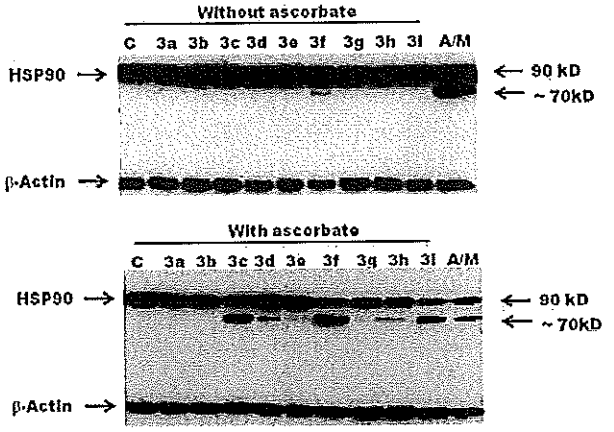


Fig. (4). Hsp90 cleavage by quinones K562 cells were incubated for 6 h in the presence of quinones at 10 μM without or with 2 mM ascorbate. Aliquots of cell suspension were taken and Western blots were performed as reported in the Biological section. The membranes were probed against mouse monoclonal antibody against Hsp90α/β C-terminus (F-8) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Typical results out of three separate experiments are represented. (C): control conditions; (A/M): ascorbate (2 mM)/menadione 10 μM).

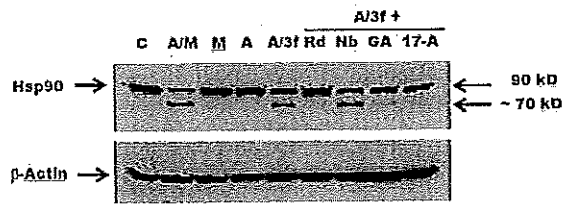


Fig. (5). Effects of Hsp90 inhibitors on protein cleavage K562 cells were incubated for 6 h at 37°C. Here are the experimental conditions: (C): control untreated cells; (A/M): 2 mM ascorbate and 10 μM menadione; (M): 10 μM menadione; (A/3f): 2 mM ascorbate and 10 μM quinone 3f; (A): 2 mM ascorbate. In lanes 6-9 cells were preincubated 1 h with different Hsp90 inhibitors and then exposed 6 h to A/3f. Hsp90 inhibitors include: 1 mM of novobiocin (Nb); 10 μM of geldanamycin (GA); 5 μM of 17-allylamino-17-demethoxygeldanamycin (17-A); and 10 μM of radicicol (Rd). Aliquots of cell suspension were taken and Western blots were performed as reported in the Biological section. The membranes were probed against mouse monoclonal antibody against Hsp90α/β C-terminus (F-8) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Menadione sodium bisulfite, sodium ascorbate, novobiocin and radicicol were purchased from Sigma (St Louis, MO, USA). Geldanamycin and 17-allylamino-17-demethoxygeldanamycin were purchased from Invivogen (San Diego, CA, USA). Typical results out of three separate experiments are represented.

Table 1. Cytotoxicity by Quinones and their Molecular Descriptors.

	K562 Cell Survival (%)#				Potential (mV)		LogP
	- Asc. 6h	- Asc. 24h	+ Asc. 6h	+ Asc. 24h	$E^{I}_{1/2}$	$E^{II}_{1/2}$	(calcd)
3a	90.6 ± 8	89.5 ± 3	91.5 ± 3	90.3 ± 3	-578.6	-1145.1	1.68
3b	88.3 ± 2	88.2 ± 6	91.0 ± 5	85.5 ± 4	-596.5	-1201	1.63
3c	88.0 ± 4	88.8 ± 3	87.5 ± 4	26.1 ± 1**	-376.5	-914.8	2.14
3d	89.0 ± 5	86.5 ± 2	90.5 ± 5	42.6 ± 4**	-578.3	n.d.	2.23
3e	86.0 ± 6	88.4 ± 3	93.0 ± 4	83.5 ± 2*	-609.2	-1185	2.18
3f	86.6 ± 6	40.3 ± 4**	76.5 ± 3**	18.0 ± 3**	-349.8	-902.5	2.68
3g	91.6 ± 2	89.5 ± 2	89.0 ± 1	87.0 ± 7	-552.0	n.d.	3.12
3h	85.6 ± 5	90.1 ± 3	88.5 ± 2	83.0 ± 2*	-600.8	-1106.6	3.07
3i	85.0 ± 5	91.0 ± 4	89.0 ± 3	33.0 ± 3**	-375.7	-875.2	3.58

(#) Cells were incubated for 6 and 24 h with 10 μM of quinones in the absence or in the presence of ascorbate (2 mM). At the indicated time, aliquots of cell suspension were taken and cell viability was determined by an automated cell counter, as described in the Methods Section. Treated cells were compared to control untreated cells taken as reference 100%. (*) = $p < 0.05$ as compared to control conditions. (**) = $p < 0.01$ as compared to control conditions. n.d. = not determined.

Table 2. Metabolic and Redox Homeostasis and Cancer Cell Survival Under Oxidative Stress.

	K562 Cell Survival	GSH	ATP
	(%)	(nmol/mg protein)	(nmol/10^6 cells)
.	92.6 ± 3	14.7 ± 0.9	30.4 ± 2.2
Ascorbate/Menadione	12.9 ± 2**	4.3 ± 0.7**	14.8 ± 1.2**
Ascorbate/Menadione + NAC	89.2 ± 2	13.4 ± 0.6	28.4 ± 1.2
Quinone 3f	40.8 ± 3**	11.4 ± 1.2*	25.5 ± 1.1*
Ascorbate/Quinone 3f	16.5 ± 2**	5.1 ± 0.8**	16.4 ± 1.5**
Ascorbate/Quinone 3f + NAC	91.6 ± 2	14.2 ± 1.1	31.1 ± 1.5

K562 cells were incubated for 6 h (ATP and GSH) and 24 h (cell survival) with 10 μM of quinones in the absence or in the presence of ascorbate (2 mM). When indicated, cells were preincubated 2 hours with 3mM of N-acetylcysteine (NAC). Afterwards, aliquots of cell suspension were taken and ATP and GSH were measured as described under Materials and Methods section. Cell survival was determined by an automated cell counter. Results are means values ± standard error of the mean (SEM) calculated from at least three separated experiments. (*) = $p < 0.05$ as compared to control conditions. (**) = $p < 0.01$ as compared to control conditions.

Since we have previously demonstrated that the cleavage of Hsp90 by ascorbate/menadione remained unaffected by various protease inhibitors [18], we explored non-enzymatic mechanisms of protein cleavage. As previously mentioned the oxidation of protein by ROS may lead to the cleavage of proteins [22,23]. Although no information about the occurrence of a non-enzymatic oxidative cleavage of proteins in living cells has been documented, we recently showed that the cleavage occurs by a localized generation of hydroxyl radical leading to peptide backbone cleavage [20]. The molecular mechanism appears to involve the *in situ* formation of hydroxyl radicals (HO˙), due to a Fenton-type reaction. Indeed, Hsp90 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 H_2O_2 leads to the formation of oxygen radicals (Fenton-type reaction) that oxidize Hsp90 to form a protein radical, which, by rearrangement, causes the rupture of the peptide backbone [20].

The toxicity profile of menadione is close and similar to **3f** when both quinones were associated to ascorbate. Indeed, (Table 2) shows a dramatic decrease in the survival of cancer cells and a strong impairment of their metabolic and redox homeostasis when K562 cells were exposed to ascorbate in the presence of either menadione (A/M) or **3f** (A/**3f**). The survival rates of K562 cells were decreased to about 15%, and the GSH levels, currently taken as a marker of redox status, were decreased by 71% (A/M) and 65% (A/**3f**). Moreover, the intracellular content of ATP, an index of cellular energy status, dropped by 52% (A/M) and by 47%

(A/3f). These markers were restored to normal values in the presence of the antioxidant molecule N-acetylcysteine (NAC). On this basis, we inferred that menadione and **3f** behave as similar oxidizing entities thus leading to the generation of a redox cycling process. In this context, the produced ROS would play a considerable role in the mechanism underlying the cytotoxicity by ascorbate/**3f** in K562 cells. Indeed, we observed a decrease in cell survival, a loss of redox and metabolic homeostasis and the cleavage of Hsp90. Since the protein cleavage is observed at doses of **3f** generally without a prominent toxicity, it may be assumed that Hsp90 cleavage is caused mainly by an *in situ* formation of ROS (like in the case of ascorbate/menadione) and not due to proteolysis preceding cell death.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

The authors express their gratitude to Isabelle Blave and Véronique Allaeys for their excellent technical support. Financial support from Belgian Fonds National de la Recherche Scientifique (FNRS), National Council for Scientific and Technological Development (CNPq) from Brazil and Fondo Nacional de Ciencia y Tecnología (FONDECYT Grant #1100376) from Chile.

REFERENCES

[1] Lai, B.T.; Chin, N.W.; Stanek, A.E.; Keh, W.; Lanks, K.W. Quantitation and intracellular localization of the 85K heat shock protein by using monoclonal and polyclonal antibodies. *Mol Cell Biol*, **1984**, *4*, 2802-2810.

[2] Neckers, L. Hsp90 inhibitors as novel cancer chemotherapeutic agents. *Trends Mol. Med.*, **2002**, *8*, S55-61.

[3] Hanahan, D.; Weinberg, R. The hallmarks of cancer. *Cell*, **2000**, *100*, 57-70.

[4] Neckers, L. Heat shock protein 90: the cancer chaperone. *J. Biosci.*, **2007**, *32*, 517-530.

[5] Weinstein, I.B.; Joe, A. Oncogene addiction. *Cancer Res.*, **2008**, *68*, 3077-3080.

[6] Whitesell, L.; Bagatell, R.; Falsey, R. The stress response: implications for the clinical development of Hsp90 inhibitors. *Curr. Cancer Drug Targets*, **2003**, *3*, 349-358.

[7] Kamal, A.; Thao, L.; Sensintaffar, J.; Zhang, L.; Boehm, M.F.; Fritz, L.C.; Burrows, F.J. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature*, **2003**, *425*, 407-410.

[8] DeBoer, C.; Meulman, P. A.; Wnuk, R. J.; Peterson,D. H. Geldanamycin, a new antibiotic. *J. Antibiotics*, **1970**, *23*, 442–447.

[9] Stebbins, C. E.; Russo, A. A.; Schneider, C.; Rosen, N.; Hartl, F. U.; Pavletich, N. P. Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell*, **1997**, *89*, 239–250.

[10] Sausville, E. A.; Tomaszewski, J. E.; Ivy, P. Clinical development of 17-allylamino, 17-demethoxygeldanamycin. *Current Cancer Drug Targets*, **2003**, *3*, 377–383.

[11] Heath, E. I.; Gaskins, M. A phase II trial of 17-allylamino-17-demethoxygeldanamycin in patients with hormone-refractory metastatic prostate cancer. *Clinical Prostate Cancer*, **2005**, *4*, 138–141.

[12] Ronnen E. A.; Kondagunta, G. V. A phase II trial of 17-(Allylamino)-17-demethoxygeldanamycin in patients with papillary and clear cell renal cell carcinoma. *Invest. New Drugs*, **2006**, *24*, 543–546.

[13] Banerji, U.; Judson, I.; Workman, P. The clinical applications of heat shock protein inhibitors in cancer-present and future. *Current Cancer Drug Targets*, **2003**, *3*, 385–390.

[14] Workman, P. Combinatorial attack on multistep oncogenesis by inhibiting the Hsp90 molecular chaperone. *Cancer Lett.*, **2004**, *206*, 149–157.

[15] Li, Y.; Zhang, T.; D. Sun. New developments in Hsp90 inhibitors as anti-cancer therapeutics: mechanisms, clinical perspective and more potential. *Drug Resist Updat.*, **2009**, *12*, 17–27.

[16] Breinig, M.; Caldas-Lopez, E.; Goeppert, B.; Malz, M.; Rieker, R.; Bergmann, F.; Schirmacher, P.; Mayer, M.; Chiosis, G.; Kern, M.A. Targeting heat shock protein 90 with non-quinone inhibitors: a novel chemotherapeutic approach in human hepatocellular carcinoma. Hepatology, **2009**, 50, 102-112.

[17] Taldone, T.; Gozman, A.; Maharaj, R.; Chiosis, G. Targeting Hsp90: small-molecule inhibitors and their clinical development. Curr. Opin. Pharmacol., **2008**, 8, 370-374.

[18] Beck, R.; Verrax, J.; Gonze, T.; Zappone, M.; Pedrosa, R.C.; Taper, H.; Feron, O.; Buc Calderon, P. Hsp90 cleavage by an oxidative stress leads to its client proteins degradation and cancer cell death. Biochem. Pharmacol., **2009**, 77, 375-383.

[19] Beck, R.; Dejeans, N.; Glorieux, C.; Curi Pedrosa R.; Vasquez, D.; Valderrama, J.A.; Buc Calderon, P.; Verrax, J. Molecular chaperone Hsp90 as a target for oxidant-based anticancer therapies. *Curr. Med. Chem.*, **2011**, *18*, 2816-2825.

[20] Beck, R.; Dejeans, N.; Glorieux, Ch.; Creton, M.; Delaive, E.; Dieu, M.; Raes, M.; Leveque, Ph.; Gallez, B.; Depuydt, M.; Collet, J.-F.; Buc Calderon, P.; Verrax, J. Hsp90 is cleaved by oxidative stress at a highly conserved N-terminal amino acid motif. *PlosOne* **2012**, *7*, e40795.

[21] Beck, R.; Pedrosa, R.C.; Dejeans, N.; Glorieux, C.; Leveque, P.; Gallez, B.; Taper, H.; Eeckhoudt, S.; Knoops, L.; Buc Calderon, P.; Verrax, J. Ascorbate/menadione-induced oxidative stress kills cancer cells that express normal or mutated forms of the oncogenic protein Bcr-Abl. An *in vitro* and *in vivo* mechanistic study. *Invest. New Drugs*, **2011**, *29*, 891-900.

[22] Stadtman, E. R.; Levine, R. L. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids*, **2003**, *25*, 207-218.

[23] Berlett, B. S.; Stadtman, E. R. Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.*, **1997**, *272*, 20313-20316.

[24] Kyle Hadden, M.; Hill, S. A.; Davenport, J.; Matts, R. L.; Blagg, B. S. J. Synthesis and evaluation of Hsp90 inhibitors that contain the 1,4-naphthoquinone scaffold. *Bioorgan. Med. Chem.*, **2009**, *17*, 634-640.

[25] Benites, J.; Ríos, D.; Díaz, P.; Valderrama, J. The solar-chemical-Friedel-Crafts heteroacylation of 1,4-quinones. *Tetrahedron Lett.*, **2011**, *52*, 609-611.

[26] Griffith, O.W. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.*, **1980**, *106*, 207-212.

[27] Verrax, J. and Buc Calderon, P. Pharmacologic concentrations of ascorbate are achieved by parenteral administration and exhibit antitumoral effects. *Free Rad. Biol. Med.*, **2009**, *47*, 32-40.

[28] Benites, J.; Rojo, L.; Valderrama, J.A.; Taper, H.; Buc Calderon, P. Part 1: anticancer activity of two euryfurylbenzoquinones on TLT, a murine hepatoma cell line. Role of vitamin C in the mechanism leading to cell death. *Eur. J. Med. Chem.*, **2008**, *43*, 1813-1817.

[29] Benites, J.; Valderrama, J.A.; Taper, H.; Buc Calderon, P. Part 2: Influence of 2-euryfuryl-1,4-naphthoquinone and its peri-hydroxy derivatives on both cell death and metabolism of TLT cells, a murine hepatoma cell line. Modulation of cytotoxicity by vitamin C. *Chem. Pharm. Bull.*, **2009**, *57*, 615-619.

[30] Vasquez, D.; Rodriguez, J.A.; Theoduloz, C.; Verrax, J.; Buc Calderon, P.; Valderrama, J.A. Synthesis and antitumor evaluation of 8-phenylaminopyrimido(4,5-c)-isoquinolinequinones. *Bioorg. Med. Chem. Letter*, **2009**, *19*, 5060-5062.

[31] Vasquez, D.R.; Verrax, J.; Valderrama, J.A.; Buc Calderon, P. Aminopyrimidoisoquinolinequinone (APIQ) redox cycling is potentiated by ascorbate and induces oxidative stress leading to necrotic-like cancer cell death. *Invest. New Drugs*, **2012**, *30*, 1003-1011.

[32] Kviecinski, M.; Pedrosa, RC.; Felipe Bettega K.; Farias, M.; Glorieux, Chr.; Valenzuela, M.; Sid, B.; Benites, J.; Valderrama, J.A.; Verrax, J.; Buc Calderon, P. Inhibition of cell proliferation and migration by oxidative stress from ascorbate-driven juglone

redox cycling in human bladder-derived T24 cells. *Biochem. Biophys. Res. Comm.*, **2012**, *421*, 268-273.

[33] Verrax, J.; Beck, R.; Dejeans, N.; Glorieux, C.; Sid, B.; Curi Pedrosa, R.; Benites, J.; Vásquez, D.; Valderrama, J.A.; Buc Calderon, P. Redox-active quinones and ascorbate: an innovative cancer therapy that exploits the vulnerability of cancer cells to oxidative stress. *Anticancer Agents Med. Chem.*, **2011**, *11*, 213-221.

[34] Sawyers, C.L.; McLaughlin, J.; Witte, O.N. Genetic requirement for ras in the transformation of fibroblasts and hematopoietic cells-by the Bcr-Abl oncogene. *J. Exp. Med.*, **1995**, *181*, 307-313.

[35] Skorski, T.; Bellacosa, A.; Nieborowska-Skorska, M.; Majewski, M.; Martinez, R.; Choi, J.K.; Trotta, R.; Wlodarski, P.; Perrotti, D.; Chan, T.O.; Wasik, M.A.; Tsichlis, P.N.; Calabretta, B. Transformation of hematopoietic cells by Bcr-Abl requires activation of a PI3K/Akt-dependent pathway. *EMBO J.*, **1997**, *16*, 6151-6161.

[36] Carlesso, N.; Frank, D.A.; Griffin, J.D. Tyrosyl phosphorylation and DNA binding activity of signal transducers and activators of transcription (STAT) proteins in hematopoietic cell lines transformed by Bcr-Abl. *J. Exp. Med.*, **1996**, *183*, 811-820.

[37] Reuther, J.Y.; Reuther, G.W.; Cortez, D.; Pendergast, A.M.; Baldwin, A.S. A requirement for NF-kB activation in Bcr-Abl-mediated transformation. *Genes Dev.*, **1998**, *12*, 968-981.

[38] Ann, W.G.; Schulte, T.W.; Neckers, L.M. The heat shock protein 90 antagonist geldanamycin alters chaperone association with p210bcr-abl and v-src proteins before their degradation by the proteasome. *Cell Growth Differ.*, **2000**, *11*, 355-360.

[39] Peng, C.; Chen, Y.; Yang, Z.; Zhang, H.; Osterby, L.; Rosmarin, A.G.; Li, S. PTEN is a tumor suppressor in CML stem cells and Bcr-Abl-induced leukemias in mice. *Blood*, **2010**, *115*, 626-635.

[40] Marcu, M.G.; Schulte, T.W.; Neckers, L. Novobiocin and related coumarins and depletion of heat shock protein 90-dependent signaling proteins. *J. Natl. Cancer Inst.*, **2000**, *92*, 242-248.

Received: July 05, 2012 **Accepted: October 11, 2012**