

An *ortho*-carbonyl substituted hydroquinone derivative is an anticancer agent that acts by inhibiting mitochondrial bioenergetics and by inducing G₂/M-phase arrest in mammary adenocarcinoma TA3

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ABSTRACT

Tumor cells present a known metabolic reprogramming, which makes them more susceptible for a selective cellular death by modifying its mitochondrial bioenergetics. Anticancer action of the antioxidant 9,10-dihydroxy-4,4-dimethyl-5,8-dihydroanthracen-1(4H)-one (HQ) on mouse mammary adenocarcinoma TA3, and its multiresistant variant TA3-MTXR, were evaluated. HQ decreased the viability of both tumor cells, affecting slightly mammary epithelial cells. This hydroquinone blocked the electron flow through the NADH dehydrogenase (Complex I), leading to ADP-stimulated oxygen consumption inhibition, transmembrane potential dissipation and cellular ATP level decrease, without increasing ROS production. Duroquinol, an electron donor at CoQ level, reversed the decrease of cell viability induced by HQ. Additionally, HQ selectively induced G₂/M-phase arrest. Taken together, our results suggest that the bioenergetic dysfunction provoked by HQ is implicated in its anticancer action.

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Introduction

Mitochondria are part of multiple pro-survival and pro-death signaling pathways, regulating several biochemical cascades that trigger cellular death which include the intrinsic pathways of apoptosis, autophagy and necrosis (Chen et al., 2010; Galluzzi et al., 2010; Wang and Youle, 2009). Tumor cell mitochondria have been recognized to play a role in multiple cancer landmarks (Hanahan and Weinberg, 2011) that, in contrast with their nonmalignant counterparts, may be sensitive to the induction of selective cellular death mechanisms by means of mitochondrial bioenergetic alterations (Gogvadze and Zhivotovsky, 2007). Metabolic transformations contribute to the biochemical characteristics of tumors (Tennant et al., 2010). Most tumors present a highly glycolytic phenotype in the presence of oxygen, known as the Warburg effect (Gogvadze et al., 2010). However, this feature depends primarily on mitochondria. Tumors express the hexokinase II (HK-II) isoform, which

has a hydrophobic N-terminal sequence that allows it to anchor on the voltage-dependent anion-selective channel (VDAC) in the outer mitochondrial membrane (OMM). Although this modification increases the catalytic efficiency, it also restricts ATP access to only that which is synthesized by oxidative phosphorylation (OXPHOS) to initiate glycolysis (Mathupala et al., 2010). Aerobic glycolysis leads to increased lactate production that contributes to the acidification of the cellular microenvironment, a pivotal event in invasiveness and metastasis into other tissues (Swietach et al., 2007). Moreover, it has been reported that the HK-VDAC interaction confers resistance to apoptosis through inhibition of the opening of the permeability transition pore (PTP) complex (Majewski et al., 2004). On the other hand, to respond to the high demand of energy due to increased cell proliferation, several tricarboxylic acid (TCA) cycle intermediates are used as precursors in the synthesis of fatty acids (citrate), proteins, and nucleotides (oxaloacetate and α -ketoglutarate) (Kroemer and Pouyssegur, 2008; Pathania et al., 2009). To supply these TCA cycle intermediates the tumor cells need to have high glutamine consumption (Israel and Schwartz, 2011). Moreover, the mitochondria of tumor cells are relatively small, present ultrastructural alterations, are deficient in β -F1 ATP synthase subunits, and have elevated mitochondrial membrane potential ($\Delta\Psi_m$) (Cuezva et al., 2002), indicating that the primary defects in the respiratory chain

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may contribute to aerobic glycolysis (Galluzzi et al., 2010). Based on the above, mitochondria have emerged as a target for designing new selective drugs. There are compounds that act at different levels in mitochondrial homeostasis as modulating agents of the family of Bcl-2 proteins, metabolic inhibitors, agents acting on ANT, ROS regulators, and modulators of mitochondrial respiration (Biasutto et al., 2010; Fulda et al., 2010). The latter could affect OXPHOS by uncoupling or inhibition of electron flow. It has also been described that small molecules such as polyphenols can inhibit electron transport in mitochondria and present in vitro and in vivo anti-cancer activity (Frey et al., 2007; Plaza et al., 2008, 2009). In previous papers we have described that *para*-hydroquinones which incorporate a carbonyl group in the *ortho*-position to one of the phenolic hydroxyls have antioxidant and antitumor properties (Araya-Maturana et al., 2002; Rodríguez et al., 2007). In addition, small structural changes in substituents that increase the lipophilicity and improve the stability of the active species provide better availability at the cellular level and also provide an opportunity for analogs with greater potency in their antitumor activity. However, the mechanism involved in the inhibition of respiration in cancer cells is unknown. In this article we evaluated the effect of 9,10-dihydroxy-4,4-dimethyl-1,4,5,8-tetrahydroanthracen-1-one (HQ, Fig. 1A) on the mitochondrial OXPHOS function and viability in mouse mammary adenocarcinoma TA3 and its TA3-MTXR variant multiresistant to methotrexate.

Materials and methods

Compounds and reagents. All reagents were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). HQ was synthesized according to previously described procedures (Araya-Maturana et al., 2006). Stock solutions of HQ were prepared in dimethyl sulfoxide (DMSO). Duroquinol (DUR) was prepared from duroquinone by reduction with sodium borohydride followed by recrystallization, dissolving the stock solution in DMSO (Cordano et al., 2002).

Harvesting tumor cells. Adenocarcinoma TA3 ascites tumor cell line was grown by weekly intraperitoneal (i.p.) injection of 1.0×10^6 cells into young adult male CAF 1 Jax mice. The methotrexate-resistant cell line (TA3-MTXR) was generated as described by Frey et al. (2007) and propagated in the same mouse strain. Mice were housed and fed under the conditions described by (Plaza et al., 2008) in the animal facility of the Facultad de Medicina of the Universidad de Chile. The local ethic committees of that Faculty and of CONICYT approved all the experiments. Tumor cells were harvested 5–7 days after i.p. inoculation of fluid from donor mice by centrifugation at $100 \times g$ for 5 min at 4°C , and washed twice with 150 mM NaCl, 5 mM KCl and 10 mM Tris-HCl, pH 7.4 (Moreadith and Fiskum, 1984). Then they were resuspended in the same medium at a concentration of 30–40 mg protein/mL (Cordano et al., 2002).

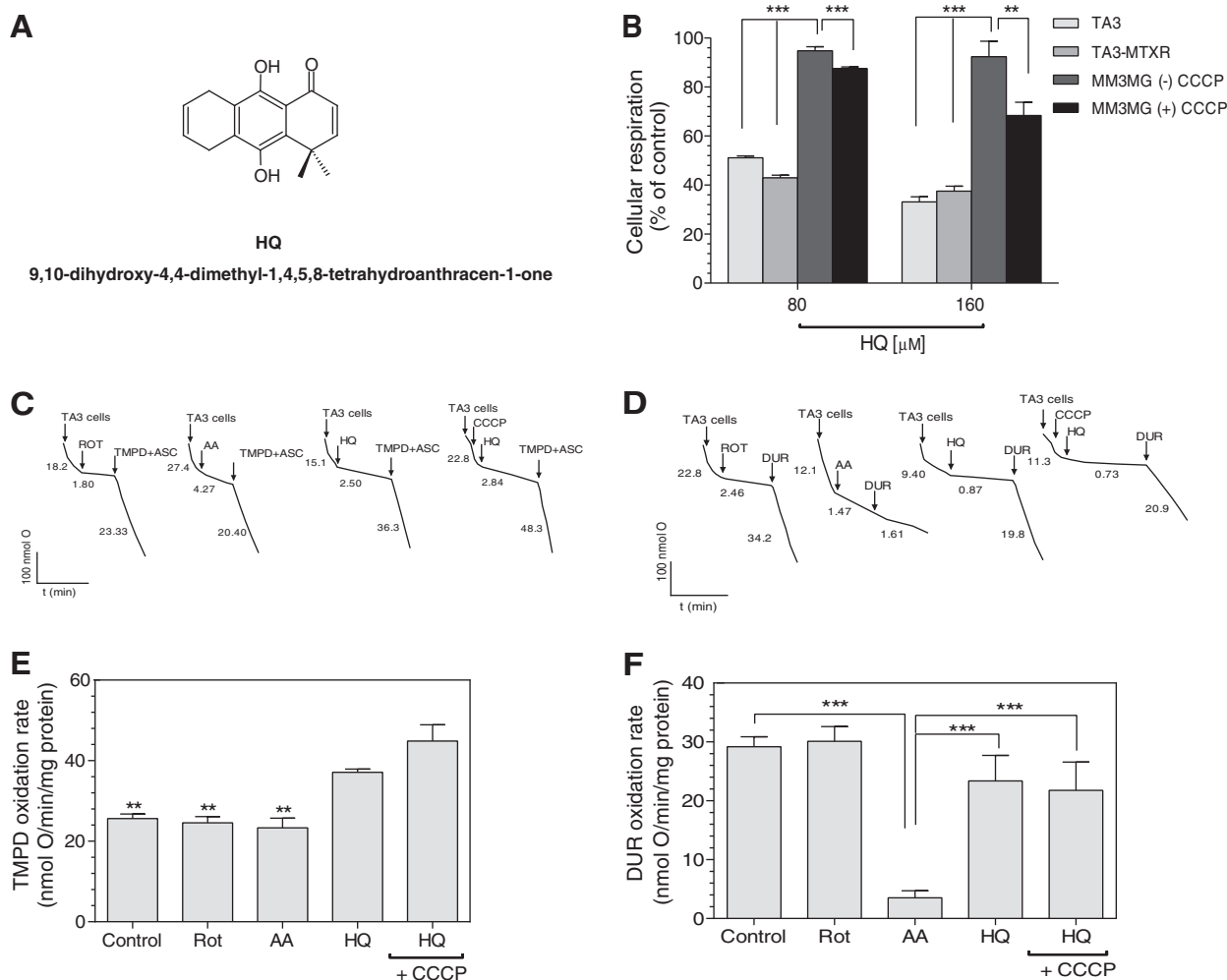


Fig. 1. HQ selectively inhibits the tumor cellular respiration. Tumor cells (1.5 mg/mL) were incubated in assay medium and oxygen consumption was measured with a Clark oxygen electrode (See Materials and methods). (A) Chemical structure of HQ. (B) Effect of HQ on cellular respiration in TA3, TA3-MTXR and MM3MG cells. Representative traces of oxygen consumption in the presence of TMPD plus ascorbate (C) and DUR (D) as electron donors. (E–F) Effect of ETC inhibitors on the TMPD and DUR oxidation rates in TA3 tumor cells. The inhibitors ROT, AA and HQ were added at their respective concentrations where the maximal inhibitory effects were observed. Inhibitors: Rot, Rotenone; AA, Antimycin A; Substrates: DUR, Duroquinol; TMPD, N,N,N',N'-tetramethyl-*p*-phenylenediamine; ASC, Ascorbate. Data shown are the means \pm SEM of three independent experiments. ** $p < 0.01$, *** $p < 0.001$ vs. MM3MG (–) CCCP, Control or AA.

Protein concentration in cells virtually free of erythrocytes and other contaminants was determined by using the Biuret method.

Cellular respiration. Oxygen consumption was measured polarographically at 25 °C with a Clark electrode No. 5331 (Yellow Springs Instruments) using a YSI model 53 monitor connected to a 100 mV single channel Goerz RE 511 recorder. The 0.6 mL reaction mixture contained 150 mM NaCl, 3 mM KCl, and 10 mM Tris–HCl, pH 7.4, plus 5 mM glutamine as substrate and 2.5 mg protein/mL of either TA3 ascites tumor cells derived from mouse mammary adenocarcinoma or their TA3-MTXR multidrug-resistant variant (Plaza et al., 2008), as described previously (Araya-Maturana et al., 2002). Oxygen consumption of MM3MG mouse mammary epithelial cells was measured in similar conditions. When indicated, 0.13 μM (TA3 and MM3MG) or 0.30 μM (TA3-MTXR) carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to attain maximum uncoupling effect. Cells were incubated for 5 min and the respiration rates were registered in absence (control in DMSO) or presence of HQ up to 30 min. The IC₅₀ values were collected from the dose–response data of at least three independent experiments.

Preparation of mitochondria and determination of mitochondrial respiration. Mitochondrial suspensions of about 0.5 mg protein/mL were prepared from tumor cells as described by Cordano et al. (2002) and Moreadith and Fiskum (1984). To determine the effect of HQ on the OXPHOS, oxygen consumption was monitored polarographically with a Clark electrode at 25 °C. The respiration medium contained 200 mM sucrose, 50 mM KCl, 3 mM K₂HPO₄, 2 mM MgCl₂, 0.5 mM EGTA, and 3 mM HEPES, pH 7.4, and the respective substrates plus 0.25 mM ADP. Mitochondria were stimulated with substrates for each respiratory chain complex: 4.2 mM glutamate + 4.2 mM malate for Complex I, 5.0 mM succinate + 0.170 μM rotenone for Complex II, and 1.5 mM ascorbate + 75 μM TMPD for Complex IV. Mitochondria were incubated with 1 μM cyclosporine A for 2 min to determine the role of the PTP complex in the inhibitory effect of respiration by HQ. 0.30 mM duroquinol (DUR) was added to reverse the inhibition of oxygen consumption stimulated by ADP.

Cell culture and viability assay. Mouse mammary epithelial MM3MG cell line was purchased from the American Type Culture Collection (ATCC, Catalog No. CRL-6376, Manassas, VA). MM3MG, TA3 and its TA3-MTXR multiresistant variant were grown in DMEM high-glucose medium supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$) in a humidified 5% CO₂ incubator at 37 °C. MM3MG (3×10^3) cells, TA3 and TA3-MTXR (1×10^4) cells/100 μL were seeded in 96-well microtiter plates and incubated for 24 h. The cells were then treated for 24, 48 and 72 h with increasing concentrations of HQ (1, 5, 10, 50, 100 and 150 μM) to obtain a dose–response curve. After treatment, the medium was removed by centrifugation and replaced with 100 μL /well of fresh medium to remove traces of HQ. Then 100 μL of MTT (final concentration 0.5 mg/mL) was added to each well and incubated at 37 °C for 3 h. After the addition of 0.01 N HCl/Triton X-100 1% to dissolve formazan crystals, OD was measured at 595 nm. Similar conditions were used to determine the effect of DUR and DUR + HQ combinations on the viability of TA3 cells at 24 h of

exposure. The IC₅₀ values were obtained from the dose–response data of at least three independent experiments.

Mitochondrial membrane potential ($\Delta\Psi_m$) assay in TA3 cells. Mitochondrial membrane potential was determined by mitochondrial incorporation of the fluorescent cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide). 1×10^6 TA3 cells/mL was seeded into a 6-well plate and treated for 12 h in the absence (control in DMSO) or presence of HQ (1–100 μM) or CCCP (10 μM). They were then washed with PBS by centrifugation at low speed. The cellular suspensions were incubated for 15 min with 1 $\mu\text{g}/\text{mL}$ JC-1, washed again with PBS, and the incorporation of JC-1 was determined for the green fluorescent monomer of JC-1 and the red fluorescent J-aggregates at 485/540 nm and 485/590 nm excitation/emission wavelength pairs, respectively, on a JASCO FP-6200 spectrofluorometer coupled to a JASCO ETC-272 stirrer and JASCO ETC-272T temperature controller.

Cell cycle analysis. To estimate cell cycle distribution, cellular DNA contents were measured by flow cytometry. TA3 and MM3MG cells were plated at a density of 1×10^5 cells on 12-well plates for 12 h and exposed to 10, 50 and 100 μM HQ for 24 h under cell culture conditions. At the end of the treatment, the cells were centrifuged at 1000 $\times g$ for 5 min, washed in 1 mL cold PBS, and the cell pellets were resuspended with 1 mL of propidium iodide (PI) staining buffer containing 4 mM sodium citrate, 0.1% Triton X-100, 50 $\mu\text{g}/\text{mL}$ PI and 200 $\mu\text{g}/\text{mL}$ RNase and incubated for 30 min at 37 °C in the dark. Similar conditions were used to determine the effect of DUR (25 μM) and DUR + HQ combinations on the distribution of cell cycle in TA3 cells at 24 h of exposure. All samples were analyzed for cell cycle distribution using a FACS Calibur flow cytometer and the Becton–Dickinson CellQuest Acquisition software (San Jose, CA, USA) as has been described (Wu et al., 2010; Yeh et al., 2011). Data were reported as the percentage of cells in each phase of the cell cycle.

Cell death assay. Cell death was assessed as has been reported (Syed et al., 2012). The Annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) labeling was performed with the Apoptosis/Necrosis Detection Kit (Abcam, UK) according to the manufacturer's instructions. After 24 h of treatment with 25, 50 and 100 μM HQ, TA3 and MM3MG cells were harvested, and Annexin V-FITC was added to a final concentration of 2.5 mg/mL. To detect necrotic cells, PI was added at a concentration of 5 mg/mL. The Annexin V-FITC and PI-labeled cells were analyzed by FACS (FACS canto, BD biosciences, San José, CA, USA). Using flow cytometry, dot plots of Annexin V-FITC on the X-axis against PI on the Y-axis were used to distinguish viable cells (which are negative for both PI and Annexin V-FITC), early apoptotic cells (which are Annexin V positive but PI negative) and late apoptotic or necrotic cells (which are positive for both PI and Annexin V-FITC staining). Unstained cells and untreated cells were used as negative controls. The data were analyzed using the Cyflog software (non-commercial version, CyFlo Ltd.).

Determination of reactive oxygen species (ROS) production. ROS production was determined by intracellular oxidation of the 2',7'-dichlorodihydrofluorescein diacetate probe (H₂DCFDA, Invitrogen, Eugene, OR, USA) to 2,7-dichlorofluorescein (DCF) through fluorescence increase. After treatment with HQ as indicated in the corresponding figures, 0.5×10^6 TA3 cells/mL was seeded into of a 96-well plate and incubated with H₂DCFDA for 15 min at 37 °C in a 5% CO₂ incubator. The suspension was then washed with PBS and resuspended in solution in the absence (control in DMSO) or presence of HQ compound (1–100 μM) or Rotenone (25 μM). Fluorescence was measured using a BioTek Synergy HT spectrofluorometer at the 488/520 nm excitation/emission wavelength pair and the readings were made for 1 h at constant temperature (37 °C).

Table 1
Inhibitory effect of HQ on respiration from TA3, TA3-MTXR and MM3MG cells.

| Compound | TA3 | | TA3-MTXR | | MM3MG | |
|----------|------------------------------------|------------------------------------|------------------------------------|------------------------------------|------------------------------------|------------------------------------|
| | IC ₅₀ [μM] | IC ₅₀ [μM] | IC ₅₀ [μM] | IC ₅₀ [μM] | IC ₅₀ [μM] | IC ₅₀ [μM] |
| HQ | (–) CCCP | (+) CCCP | (–) CCCP | (+) CCCP | (–) CCCP | (+) CCCP |
| | 81.75 \pm 1.29 | 65.60 \pm 2.70 | 70.10 \pm 1.65 | 32.90 \pm 2.16 | >150 | >150 |

Data shown are the means \pm SEM of three independent experiments.

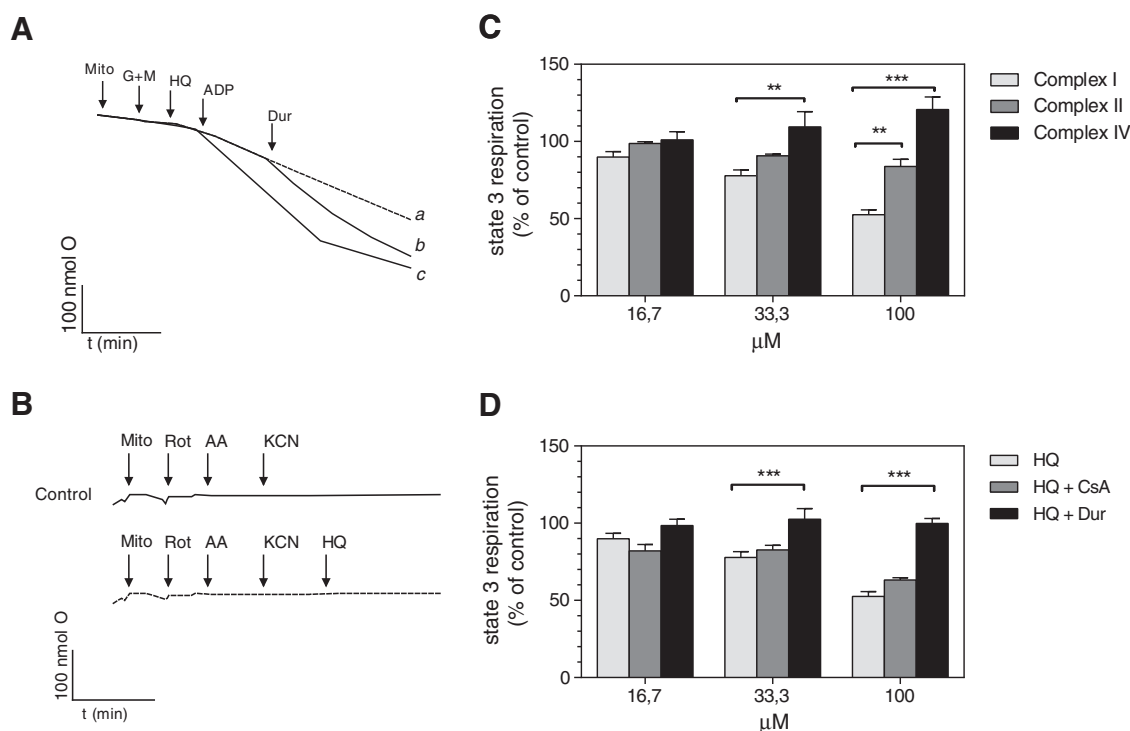


Fig. 2. Effect of HQ on mitochondrial respiration from TA3 cells. Mitochondria (0.5 mg/mL) were incubated in the medium assay supplemented with substrates for the respective respiratory complexes. Representative traces of mitochondrial oxygen consumption are shown (A). Arrows show the points where different additions were made: mitochondria (Mito), glutamate plus malate (G + M), ADP, HQ and DUR. Trace a, in the presence of HQ (100 μM). Trace b, in the presence of HQ and DUR. Trace c, in the absence of HQ and DUR (control). (B) HQ requires a functional ETC for its inhibitory effect. (C) Differential effect of HQ on ADP-stimulated respiration of Complexes I, II and IV. (D) Inhibitory mechanism of mitochondrial respiration by HQ; AA, Antimycin A; CsA, Cyclosporin A 1 μM; Rot, Rotenone. Data shown are the means ± SEM of three independent experiments. ** $p < 0.01$, *** $p < 0.001$ vs. Complex I or HQ.

ATP assay in TA3 and MM3MG cells. ATP levels were determined through the luciferin–luciferase assay system according to the specification of the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, USA). 1×10^5 TA3 cells/mL and 1×10^5 MM3MG cells/mL were seeded into a 24-well plate and incubated in DMEM in the absence (control in DMSO) or presence of HQ (25, 50 and 100 μM) for 12 h. After exposure, the bioluminescence was measured on a BioTek luminometer.

Statistical analysis. All statistical analyses were performed using Graph Pad Prism 4.03 (GraphPad Software, San Diego, California USA). The data are expressed as mean ± SEM of three independent experiments. IC_{50} values were calculated from the dose–response curves (MTT assays and inhibition of cellular respiration) obtained by nonlinear curve fitting. Statistical analysis was performed using two-way ANOVA with Bonferroni's post test for pairwise comparisons. The data were considered statistically significant when $p < 0.05$.

Results

HQ selectively affects cellular respiration, inhibiting electron transport through the NADH dehydrogenase-CoQ segment

Previously, we have reported that HQ can inhibit the cellular respiration in tumor cells (Araya-Maturana et al., 2006). To determine if HQ can interact with electron transport chain (ETC) and modulate the OXPHOS, it was added to TA3, TA3-MTXR and MM3MG cells, both in the absence and in the presence of the uncoupling agent CCCP, and the effect on oxygen consumption was evaluated at 30 min of exposure. HQ selectively inhibited cellular respiration in both tumor cell lines in a dose-dependent manner. Table 1 shows the IC_{50} inhibition values calculated for intact cells. In presence of CCCP, HQ increased the respiration inhibition in 1.24 and 2.13 folds in TA3 and its multiresistant variant

TA3-MTXR cells, respectively. These results suggest that HQ acts on electron flow through the ETC. On the other hand, HQ induced a small decrease in cellular respiration in MM3MG cells. In order to show the selective oxygen consumption decrease induced by HQ, it was graphed at two HQ concentrations: IC_{50} (80 μM) and twofolds IC_{50} (160 μM). In presence of CCCP, HQ decreased the cellular respiration at 7.28% ($p < 0.001$ vs –CCCP) and 24.02% ($p < 0.01$ vs –CCCP) with 80 μM and 160 μM, respectively in MM3MG cells.

To determine the effects of the inhibitory action of HQ on ETC in intact tumor cells, TA3 cells were also treated with the well-known inhibitors of Complex I (rotenone, ROT) and Complex III (antimycin A, AA). Both inhibitors decreased cellular respiration. However, when the electron donors at the Complex IV level N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) plus ascorbate (ASC) were added, the oxygen consumption was restored (Fig. 1C), indicating that the electron flow through the cytochrome c oxidase is not inhibited. TMPD oxidation rate in the presence of HQ is twice as great as in the presence of ROT or AA (Fig. 1E), suggesting a possible uncoupling effect as a secondary action when the terminal segment of ETC in intact cells is stimulated. Fig. 1D shows that duroquinol (DUR) restored the inhibitory effect of ROT but not of AA, because the oxidation of DUR occurs at the CoQ level. In the presence of HQ, the oxidation rate of DUR was identical to that obtained with the inhibitor of Complex I, ROT (Fig. 1F). This evidence indicates that HQ inhibited electron transport at the level of the NADH dehydrogenase-CoQ segment.

HQ inhibits mitochondrial respiration at the NADH dehydrogenase (Complex I) level

In order to evaluate the effect of HQ and the possible mechanism involved in the inhibition of mitochondrial respiration, we measured the oxygen consumption of mitochondria isolated from TA3 tumor cells. HQ caused a decrease of ADP-stimulated respiration when

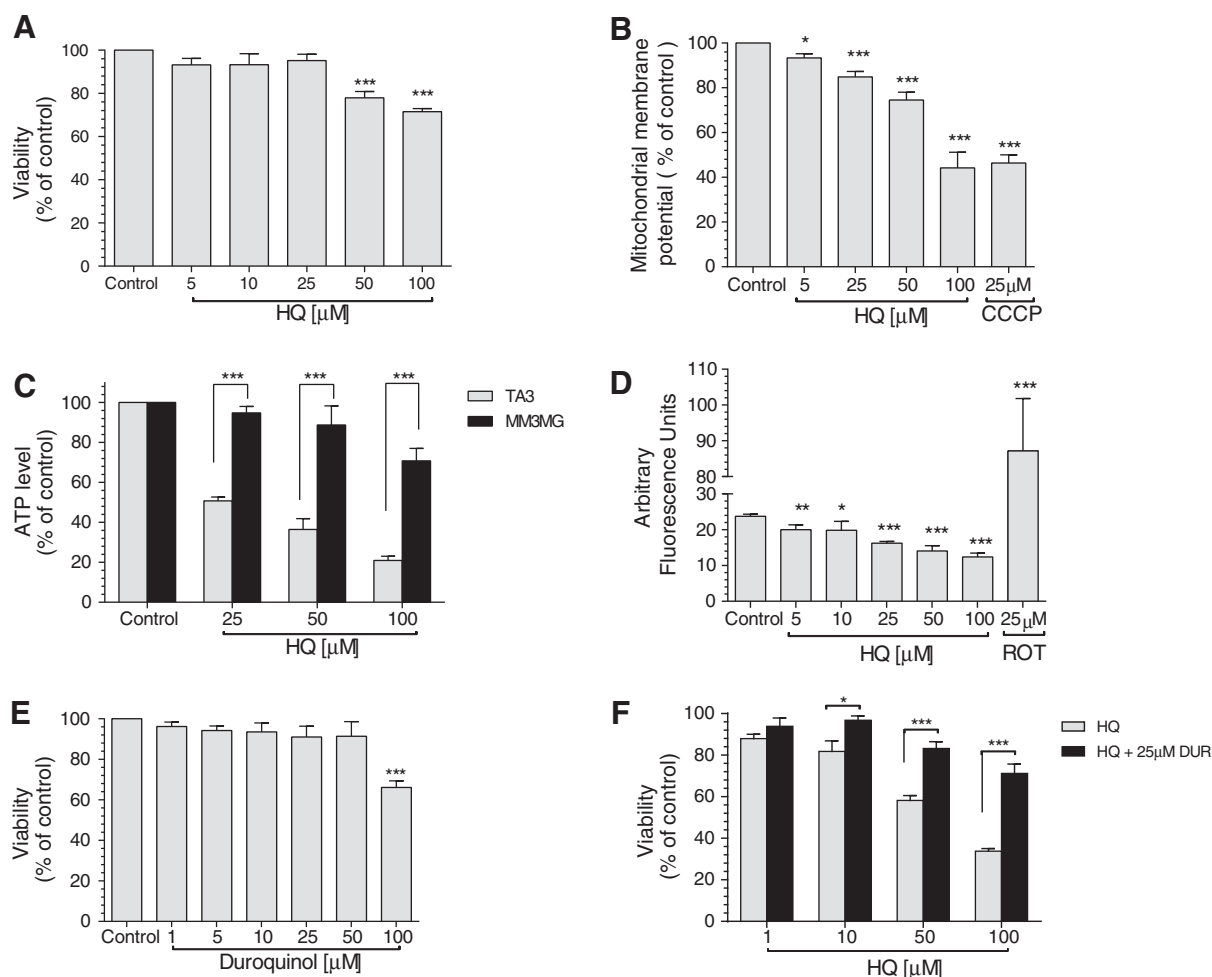


Fig. 3. Effect of HQ on viability and mitochondrial bioenergetics of TA3 tumor cells. The viability (A), mitochondrial membrane potential (B) and ATP levels (C) were evaluated at 12 h by MTT assay, JC-1 assay, luciferin–luciferase assay, respectively. (D) Intracellular ROS levels in TA3 cells were measured by oxidation of H_2DCFDA . (E) Effect of DUR on viability of TA3 cells at 24 h of exposure. (F) Effect of DUR + HQ combination on viability of TA3 cells at 24 h of exposure. The results are expressed as means \pm SEM of three independent determinations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Control or HQ + DUR.

electron donor substrates for Complex I were added (Fig. 2A, trace a versus trace c), but it did not affect the respiration when Complexes III and IV were stimulated (Fig. 2C). The decrease of Complex I-dependent mitochondrial respiration may involve several mechanisms. A possible way to evaluate this is by inducing PTP opening. PTP is a highly dynamic supra-molecular complex that in its open conformation allows the deregulated entry of small solutes into the mitochondrial matrix. The loss of pyridine nucleotides from this inner compartment due to PTP opening can cause a large decrease of mitochondrial respiration by an indirect mode of action on the electron transport chain (ETC). To find out if this is so, isolated mitochondria were incubated with cyclosporin A (CsA), a specific PTP inhibitor, and were exposed to HQ (Fantin et al., 2002). On the other hand, to evaluate if HQ acts by direct inhibition on ETC, DUR was added to restore the mitochondrial respiration at the CoQ level. As shown in Fig. 2D, CsA was unable to prevent the inhibitory effect of HQ; in contrast, DUR reversed the inhibition of the mitochondrial respiration induced by HQ. Furthermore, when a set of mitochondrial inhibitors (rotenone + antimycin A + cyanide) was added to mitochondria isolated from TA3 cells, HQ did not reverse the absence of respiration; therefore, it does not induce cyanide-insensitive respiration, indicating that HQ requires a functional ETC for its inhibitory effect (Fig. 2B). Similar results were obtained with the TA3-MTXR cell line.

These results indicate that HQ causes a decrease of mitochondrial respiration by direct inhibition of ETC, specifically on Complex I.

Effect of HQ on viability, $\Delta\Psi_m$, ATP levels, and ROS production in TA3 cells

It is well known that inhibition of ETC produces a loss in the capacity to pump protons from the mitochondrial matrix towards the intermembrane space, affecting the maintenance of $\Delta\Psi_m$ (Brand and Nicholls, 2011). We evaluated whether the electron flow inhibition caused by HQ at the Complex I level changes mitochondrial homeostasis and its consequences for tumor cell vitality. When TA3 tumor cells were treated with HQ for 12 h, a small decrease of cell viability was observed (Fig. 3A), with a notable dissipation of the $\Delta\Psi_m$ (Fig. 3B). The decrease of $\Delta\Psi_m$, which is used by ATP synthase as proton-motive force for ATP synthesis, also affected the intracellular ATP levels in a concentration-dependent manner in TA3 tumor cells (Fig. 3C). The ATP level is an indicator of OXPHOS normal function and can show the selective effect of HQ on mitochondrial bioenergetics. At 25, 50 and 100 μM , HQ induced a decrease in ATP levels of 1.86, 2.43 and 3.37 folds respectively, in TA3 cells compared with MM3MG cells at 12 h of exposure ($p < 0.001$).

At the same exposure time in which these mitochondrial parameters decrease, the effect on the tumor cell viability of HQ was lower than that evidenced at 24 h (Fig. 3F). These data indicate that the bioenergetics crisis provoked by HQ is the primary step leading to inhibition of cell viability. On the other hand, mitochondria are an important source of ROS production, which is increased during mitochondrial dysfunction and apoptosis. Interestingly, HQ did not induce increased intracellular ROS levels like other Complex I inhibitors (Li

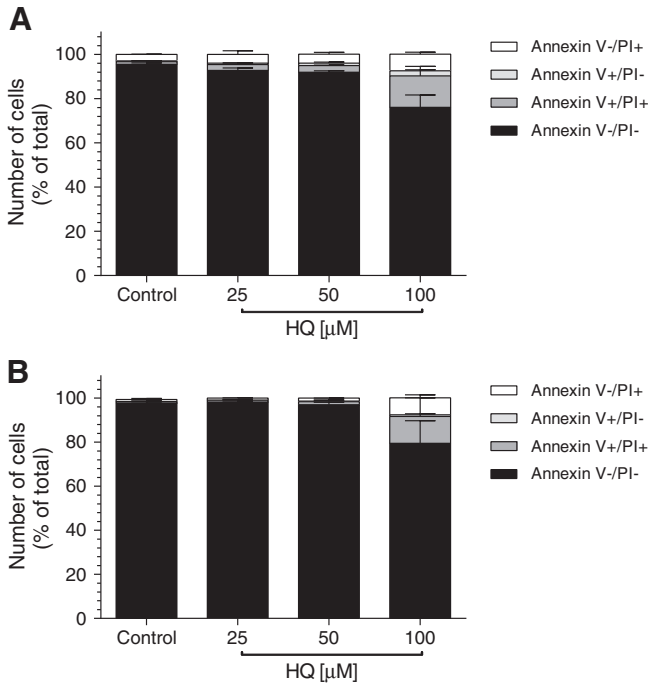


Fig. 4. Effect of HQ on phosphatidylserine exposure and PI incorporation in MM3MG (A) and TA3 (B) cells. Cells were exposed for 24 h to 25, 50 and 100 μM HQ and subpopulations were analyzed by flow cytometer.

et al., 2003): Rotenone (25 μM) increased in 367% the relative fluorescence compared with control ($p < 0.001$). In contrast, HQ decreased ROS generation in a concentration-dependent manner, suggesting certain intracellular antioxidant properties as shown in Fig. 3D.

HQ affects cell viability of TA3 tumor cells by mitochondrial dysfunction at NADH dehydrogenase (Complex I)

Alteration of mitochondrial homeostasis has been related to early phases of several cell death types, affecting cellular fate (Fulda et al., 2010). Fig. 3F shows that HQ treatment for 24 h decreased the cellular viability of TA3 cells in a concentration-dependent manner. In order to evaluate whether mitochondrial dysfunction is involved in the alteration of tumor cell viability induced by HQ, TA3 cells were exposed to DUR for 24 h and its effect on viability was determined. Values $> IC_{10}$ were considered cytotoxic. 100 μM DUR attained $66.16 \pm 3.3\%$ viability ($p < 0.001$ vs control); however, between 1 and 50 μM it did not present toxic effects (Fig. 3E). 25 μM DUR was selected to treat TA3 tumor cells in combination with HQ. Duroquinol reversed the electron flow inhibition provoked by HQ in isolated mitochondria from TA3 tumor cells (Fig. 2A, trace b). Therefore, we exposed TA3 cells to DUR + HQ for 24 h to determine its effect on viability. Interestingly, Fig. 3F shows that the decrease of viability induced by HQ was reversed by DUR, an electron donor at the CoQ level in the ETC. HQ + DUR versus HQ alone in the same concentrations increased the viability of TA3 tumor cells by 15.02%, 24.97% and 37.41%, respectively, for the following respective combinations: 10 μM HQ + 25 μM DUR, 50 μM HQ + 25 μM DUR, and 100 μM HQ + 25 μM DUR. These results indicate that the inhibition of mitochondrial respiration caused by HQ has important implications in its anticancer action.

HQ is not a cytotoxic agent for TA3 and MM3MG cells

To determine if alteration in mitochondrial bioenergetics is an event that precedes the cell death, TA3 and MM3MG cells were exposed for 24 h to 25, 50 and 100 μM HQ and subpopulations present in Annexin-V/PI flow cytometer analysis were calculated. Interestingly, both cell lines did not exhibit significant reduction of the living cell population (Annexin V^- /PI $^-$). Moreover, increase of apoptotic population

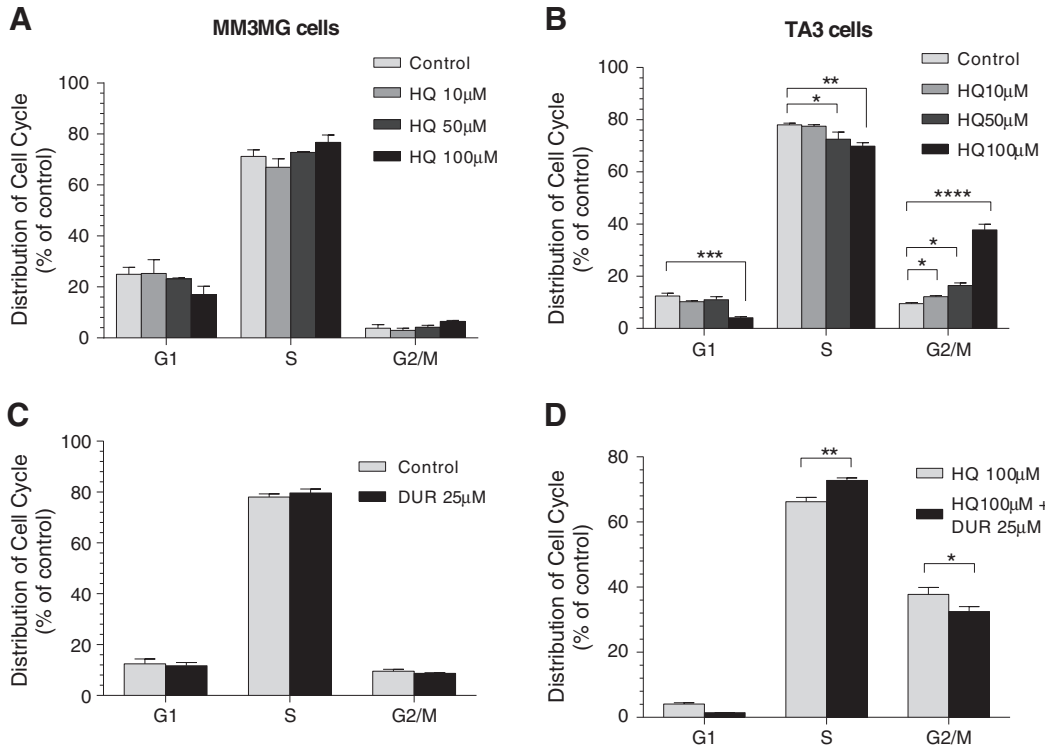


Fig. 5. HQ causes selective TA3 cell cycle arrest in G₂/M-phase. MM3MG (A) and TA3 (B) cells were treated with DMSO (Control) and 10, 50 and 100 μM HQ for 24 h. (C) Effect of 25 μM DUR on TA3 cell cycle. (D) Reversion of TA3 cell cycle arrest in G₂/M-phase by 25 μM DUR + 100 μM HQ combination. Data were expressed as mean \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs Control or 100 μM HQ.

Table 2
Effect of HQ on the proliferation of TA3, TA3-MTXR and MM3MG cells.

| IC ₅₀ [μ M] | TA3 | TA3-MTXR | Relative resistance | MM3MG |
|-----------------------------|------------------|------------------|---------------------|------------------|
| Exposure time | | | | |
| 24 h | 54.91 \pm 1.62 | 22.75 \pm 2.28 | 0.41 | > 100 |
| 48 h | 13.00 \pm 3.58 | 12.90 \pm 0.84 | 0.99 | 90.48 \pm 4.29 |
| 72 h | 6.35 \pm 2.60 | 6.70 \pm 2.35 | 1.06 | 78.91 \pm 4.35 |

Data shown are the means \pm SEM of three independent experiments.

was not detected (Fig. 4). Similar results were obtained when sub-population sub-G₁ was analyzed in experiments of cell cycle distribution: HQ does not induce significant increase in both cell lines (data not shown). Taken together, these results suggest that HQ is not a cytotoxic agent in our experimental conditions and other phenomena can be affecting the cell proliferation. Consistent with this idea, we evaluated the effect of HQ on cell cycle progression.

HQ induces TA3 cell cycle arrest in G₂/M-phase

To identify the anticancer mechanism of HQ, we evaluated the effect of HQ on the cell cycle distribution of TA3 and MM3MG cells, incubating this compound for 24 h. Interestingly, HQ did not affect cell cycle progression in the MM3MG line (Fig. 5A); in contrast, it induced a decrease of the subpopulation in the S-phase by about 5.51% and 8.17% ($p < 0.05$ and $p < 0.01$ vs Control, respectively) and accumulation of the subpopulation in the G₂/M-phase in a concentration-dependent manner in TA3 cells (Fig. 5B). Mitochondria are essential in cancer cell proliferation and several cell signaling are implicated in the control cell cycle progression (Alberghina et al., 2012; Smith et al., 2008). To determine if the alteration in mitochondrial bioenergetics is implicated in the cell cycle arrest in the G₂/M-phase, TA3 cells were exposed with 25 μ M DUR and 25 μ M DUR + 100 μ M HQ combination for 24 h. DUR had no effect on cell cycle progression (Fig. 5C); in contrast, the DUR + HQ combination decreased the subpopulation in the G₂/M-phase in 5.27% ($p < 0.05$) and increased the subpopulation in the S-phase in 6.5% ($p < 0.001$) compared with

HQ (Fig. 5D). These results indicate that the alteration in mitochondrial bioenergetics induced by HQ is implicated in the selective cell cycle arrest of TA3 cells.

HQ is a selective anticancer agent with anti-proliferative effects on TA3 and TA3-MTXR cells

In order to evaluate if cell cycle arrest induced by HQ is a crucial event in its anticancer action, adenocarcinoma TA3 and TA3-MTXR tumor cells and mammary epithelial MM3MG cells were exposed to increasing concentrations of HQ for 24 h, 48 h and 72 h (Fig. 5). This compound presented its anticancer action after 48 h of exposure with IC₅₀ values of about 10 μ M, affecting similarly the proliferation of both TA3 and TA3-MTXR tumor cell lines. The latter tumor cell line has been described as resistant to several known chemotherapeutic agents (Plaza et al., 2008). Interestingly, HQ presented a relative resistance index close to 1 (calculated as the ratio of IC₅₀TA3-MTXR-R/IC₅₀TA3 drug-sensitive cells). This pattern was maintained at 48 h and 72 h (Table 2). Additionally, the effect of HQ on MM3MG normal mammary epithelial cells was also evaluated under the same experimental conditions. HQ did not affect the cell viability of the nonmalignant cell line (Fig. 6). At 48 h and 72 h of exposure it was 7.00 and 12.43 times more active, respectively, on tumor cells than the MM3MG cell line. These results show that HQ has selective anti-proliferative effects on TA3 and TA3-MTXR tumor cells.

Discussion

In a previous paper we reported the biological activity of hydroquinones that incorporate a carbonyl substitution in the *ortho*-position to one of the phenolic hydroxyls. HQ showed the best inhibitory activity of oxygen consumption in intact tumor cells (Araya-Maturana et al., 2002, 2006). However, the mechanism of action involved in its anticancer activity remains uncertain. Therefore, the purpose of this work was to evaluate the effect of HQ on tumor bioenergetics.

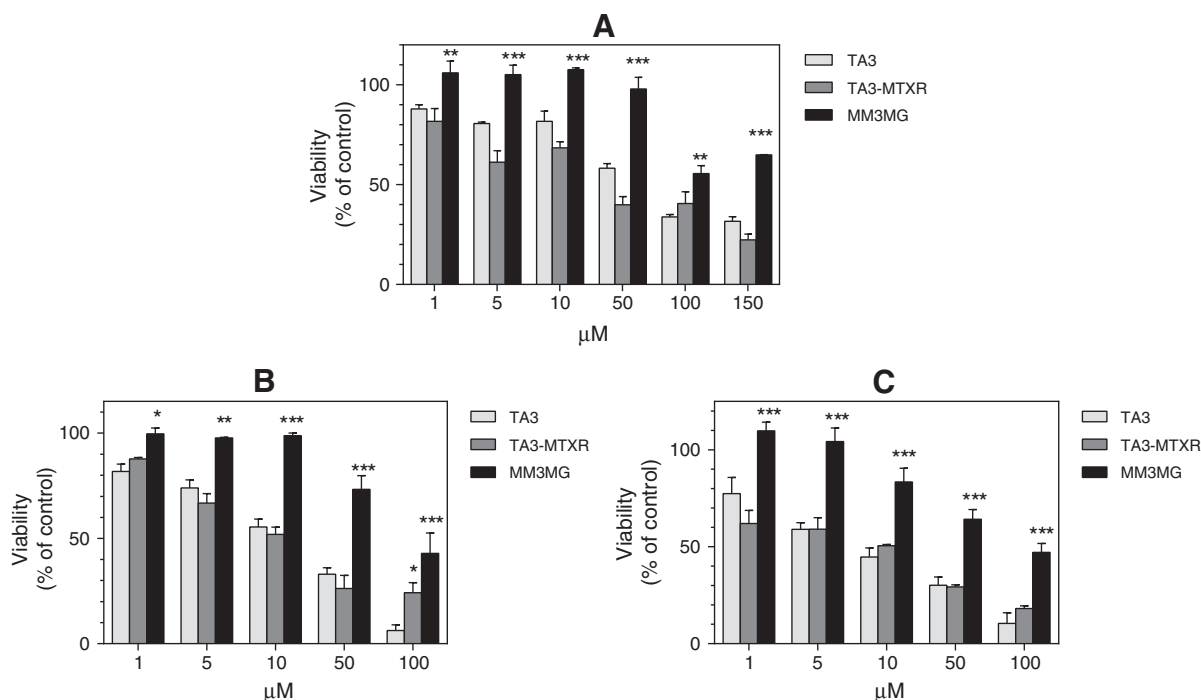


Fig. 6. HQ selectively affects the growth of TA3 and TA3-MTXR tumor cell lines at 24 h (A), 48 h (B) and 72 h (C). The viability of tumor cells (TA3 and TA3-MTXR) and normal mammary epithelial cells (MM3MG) was evaluated by MTT assay. Data shown are the means \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs TA3 cells.

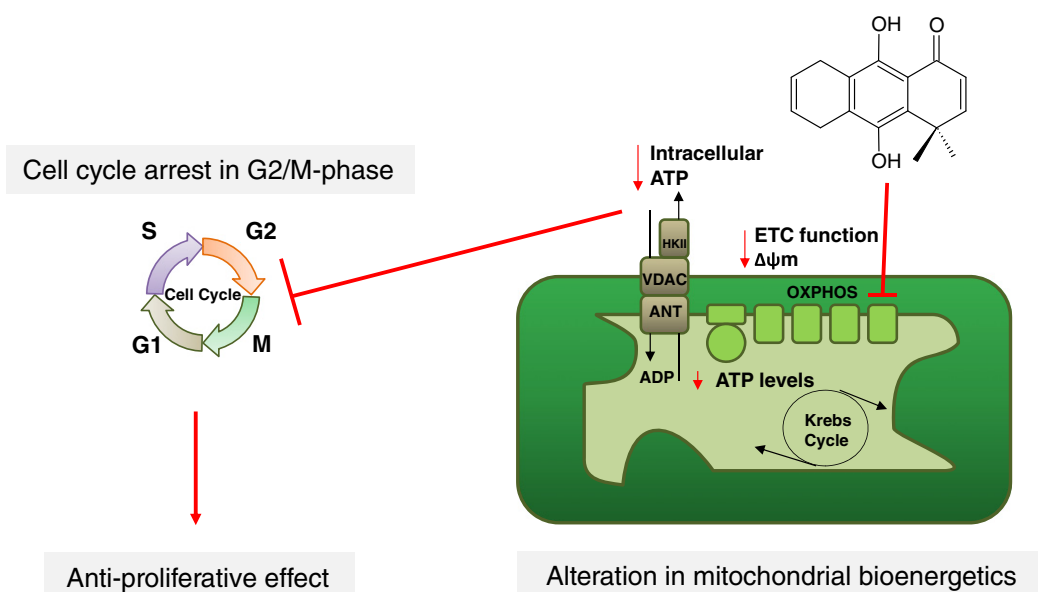


Fig. 7. Anticancer mechanism proposed for HQ in adenocarcinoma TA3 cells. HKII: hexokinase-II; VDAC: voltage-dependent anion channel; ANT: adenine nucleotide translocator; OXPHOS: oxidative phosphorylation; $\Delta\psi_m$: mitochondrial membrane potential.

It has been recognized that cancer cells show extensive metabolic reprogramming, changing the dependence of the energy-generating processes. Glycolysis under aerobic conditions has been reported as a central transformation in tumorigenesis, and is involved in the evasion of apoptosis, tissue invasion, and chemoresistance. Although about 40–75% of synthesized ATP comes from aerobic glycolysis, the remaining energy is supported by the mitochondria (Mathupala et al., 2010). In addition, the ATP produced by OXPHOS is the one preferred by HK-II to catalyze the initial step of glycolysis, increasing the efficiency of this process (Pedersen, 2007). Therefore, depletion of the intracellular ATP levels through targeting the OXPHOS system is an alternative in designing and developing anticancer agents with selectivity towards malignant cells.

Our results show that HQ inhibits the electron flow through NADH dehydrogenase, producing ADP-stimulated oxygen consumption inhibition, and mitochondrial membrane potential dissipation with consequent intracellular ATP level decrease. Both cellular respiration and ATP level are selectively affected in tumor cells, indicating that the mitochondrial bioenergetic inhibition is a selective mechanism exerted by HQ.

It has been extensively reported that alterations in mitochondrial functions produce cell death (Fulda et al., 2010; Ralph et al., 2010). However, we have showed that HQ can induce mitochondrial dysfunction producing cell cycle arrest without progression to cell death. A crucial tool to produce mitochondrial dysfunction is oxidative stress, saturating the antioxidant systems (Gogvadze et al., 2010; Kroemer et al., 2007). Several compounds that present anticancer activity by inhibiting mitochondrial bioenergetics act through an oxidative mechanism, involving the inhibition of Complex I (Chen et al., 2011; Hail and Lotan, 2004; Pramanik et al., 2011). In contrast, HQ exhibits a no-oxidative anticancer mechanism. This different mitochondrial dysfunction pathway can be implicated in its cellular selectivity. Ubiquinone analogs can act as a cytoprotective agent by antioxidant properties to mitochondrial level (Duveau et al., 2010) and as an anticancer compound, this property can provide an improved security profile in potential in vivo applications, as have been reported previously (Plaza et al., 2008, 2009). The latter is interesting if it is considered that a certain anticancer inhibitor of Complex I, such as rotenone, which induces ROS-mediated mitochondrial dysfunction, has been recognized as a

neurodegenerative agent (Li et al., 2003). This damage has limited their application in chemotherapy and it has recently suggested that it can be prevented by antioxidants (Hinenó et al., 2011; Wu et al., 2011).

We have reported that antitumor activity of some hydroquinones is correlated with antioxidant properties, such as hydrogen atom-donating ability, antioxidant capability (% of scavenging of hydroxyl radicals), and Trolox equivalent, showing similar trends as the inhibition of oxygen consumption by hydroquinones (Rodríguez et al., 2007). Moreover, the structure–activity relationships and the results found here suggest that HQ has structural features that may explain its mechanism of action. Previously, we identified that an *ortho*-carbonyl substitution in a hydroquinone system is a structural motif that increases the inhibitory activity of respiration (Araya-Maturana et al., 2002), and the greatest increase in potency compared to bicyclic hydroquinones is due to substitution at carbons C6 and C7 when a third ring is incorporated (Araya-Maturana et al., 2006). These simple structural arrangements of HQ may be responsible for the stability of the semiquinone radical by protecting the positions susceptible to enzymatic detoxification mechanisms in cells (Siegel et al., 2012). In addition, differences in pH between interstitial and cellular compartments of tumors favor the in vivo action of anti-cancer compounds with physicochemical properties of weak acids (Biasutto et al., 2010; Gerweck et al., 2006). This principle may also govern the selective accumulation of HQ in cancer cells, as has been described for an apoptogenic compound (Neuzil et al., 2002).

On the other hand, anticancer agents that affect mitochondrial respiration can also alter cell cycle progression in malignant cells (Han et al., 2008, 2009). We report here that HQ selectively induced accumulation in the G₂/M-phase in TA3 cells together with ATP level depletion by inhibition of Complex I. Interestingly, these effects are linked because are reversed by addition of duroquinol, which is oxidized at the CoQ level in the ETC, suggesting that HQ promotes a bioenergetic crisis with implications in the growth of tumor cells. A possible pathway involved in deregulation of the cell cycle checkpoint has been suggested for rotenone-like compounds. They decrease the activity of the PI3K/Akt pathway leading to apoptosis in human cancer cells (Chun et al., 2003; Murillo et al., 2002; Yi et al., 2008). This is due to alterations in tumor bioenergetics, achieved via the Complex I inhibition, causing decreased phosphorylation of essential signaling intermediates in tumor growth (Hail and Lotan,

2004). In this view, reduced ATP levels induced by HQ in tumor cells could be utilized in essential ATP-consuming processes used in the maintenance of cell viability. Other possible mechanisms that can be present associated to the anticancer action of HQ is the enzymatic inhibition of cell cycle regulators. It has been reported that anticancer “quinoid” compounds inhibit CDC25 phosphatases, inducing G₂/M arrest (Han et al., 2004). This could be a secondary involved action that will be further evaluated. Moreover, consistent with selective G₂/M arrest induced by HQ in tumor cells, a time-dependent decrease of tumor proliferation was observed. Interestingly, this effect was similar in TA3 and multiresistant TA3-MTXR cells, especially at 48 h and 72 h of exposure. Recently, new evidence indicates that metabolic flux in malignant cells is able to regulate the response to chemotherapeutic agents (Martinez-Outschoorn et al., 2011a). Moreover, changes in genetic expression of metabolic regulators cooperate in drug resistance and this phenomenon can be overcome by targeting mitochondrial function in certain cancer cells (Martinez-Outschoorn et al., 2011b).

Finally, evidences suggest that inhibition of the ETC activity enhances cellular susceptibility to apoptotic stimuli (Gogvadze and Zhivotovsky, 2007). Therefore, the antioxidant HQ that produces selective inhibition of mitochondrial bioenergetics with G₂/M arrest (Fig. 7) could sensitize cancer cells to the action of cytotoxic agents, which can be considered as a supplemental approach to anticancer therapies, especially in cancer cells arrested in G₂/M-phase, which are most affected by radiotherapy (Pawlik and Keyomarsi, 2004).

Conflict of interest statement

The authors have no conflicts of interest to declare.

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