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Small structural changes on a hydroquinone scaffold determine the complex I inhibition or uncoupling of tumoral oxidative phosphorylation

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ABSTRACT

Mitochondria participate in several distinctiveness of cancer cell, being a promising target for the design of anti-cancer compounds. Previously, we described that *ortho*-carbonyl hydroquinone scaffold **14** inhibits the complex I-dependent respiration with selective anti-proliferative effect on mouse mammary adenocarcinoma TA3/Ha cancer cells; however, the structural requirements of this hydroquinone scaffold to affect the oxidative phosphorylation (OXPHOS) of cancer cells have not been studied in detail. Here, we characterize the mitochondrial metabolism of TA3/Ha cancer cells, which exhibit a high oxidative metabolism, and evaluate the effect of small structural changes of the hydroquinone scaffold **14** on the respiration of this cell line. Our results indicate that these structural changes modify the effect on OXPHOS, obtaining compounds with three alternative actions: inhibitors of complex I-dependent respiration, uncoupler of OXPHOS and compounds with both actions. To confirm this, the effect of a bicyclic hydroquinone (**9**) was evaluated in isolated mitochondria. Hydroquinone **9** increased mitochondrial respiration in state 4o without effects on the ADP-stimulated respiration (state 3_{ADP}), decreasing the complexes I and II-dependent respiratory control ratio. The effect on mitochondrial respiration was reversed by 6-ketocholestanol addition, indicating that this hydroquinone is a protonophoric uncoupling agent. In intact TA3/Ha cells, hydroquinone **9** caused mitochondrial depolarization, decreasing intracellular ATP and NAD(P)H levels and GSH/GSSG ratio, and slightly increasing the ROS levels. Moreover, it exhibited selective NAD(P)H availability-dependent anti-proliferative effect on cancer cells. Therefore, our results indicate that the *ortho*-carbonyl hydroquinone scaffold offers the possibility to design compounds with specific actions on OXPHOS of cancer cells.

1. Introduction

Cancer cells exhibit different metabolic organization compared to normal cells (Jose et al., 2011), existing several predominant metabolic phenotypes, depending on the type of cancer (Obre and Rossignol, 2015). It has long been described the high glycolysis rate in aerobic conditions (Warburg effect) with reduced or damaged mitochondrial function in some cancer cells (Cuezva et al., 2002; Wu et al., 2007; Bellance et al., 2009); however, a large amount of evidence have demonstrated the existence of cancer cells with a high oxidative phenotype, having a functional mitochondria (Moreno-Sánchez et al., 2014). This phenotype is observed in some lymphomas (Jitschin et al., 2014), melanomas (Barbi de Moura et al., 2012), and breast cancer (Diers et al., 2013), showing dependence on oxidative phosphorylation (OXPHOS) for the supply of ATP and availability of intermediates of tricarboxylic acid (TCA) cycle, required for survival and growth (Zu and Guppy, 2004; Moreno-Sánchez et al., 2014).

Mitochondrial respiration occurs in the electron transport chain (ETC), which is composed by the respiratory complexes I, II, III and IV. The ETC activity depends on the availability of NADH and $FADH_2$ from TCA cycle, which are oxidized by complexes I and II, being molecular

Abbreviations: ROS, reactive oxygen species; OXPHOS, oxidative phosphorylation; ANT, adenine nucleotide translocator; CCCP, carbonyl cyanide m-chlorophenylhydrazone; RCR, respiratory control ratio; ETC, electon transport chain; $\Delta\Psi m$, mitochondrial membrane potential; OCR, oxygen consumption rate; NAC, N-acetyl L-cysteine; RRI, relative resistance index; mPTP, mitochondrial permeability transition pore; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine.

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oxygen the final acceptor (Brand and Nicholls, 2011). The energy released in the transfer of electrons is used to pump protons from the matrix into the intermembrane space by complexes I, III and IV, generating a proton-based electrochemical gradient. Dissipation of this gradient through FoF_1-ATP synthase drives ADP phosphorylation (Mitchell, 1961). Complex I has an important control in the electron transfer (Rodríguez-Enríquez et al., 2000) and contributes about 40% of the proton motive force required for mitochondrial ATP synthesis (Galkin et al., 2006; Zickermann et al., 2015). The coupling between these redox reactions and ATP synthesis varies among cell lines and in pathophysiological conditions, such as cancer, leading to different pattern of energetic metabolisms (Jose et al., 2011). The modification of this coupling through the modulation of mitochondrial respiration has emerged as a potential therapeutic target to obtain new anti-cancer compounds, especially in cancer cells with a high oxidative phenotype. Interestingly, several compounds with anti-cancer actions selectively modulate the mitochondrial respiration by inhibiting of the electron flow at complex I level (Hail and Lotan, 2004; Pereira et al., 2007; Chen et al., 2011; de Pedro et al., 2013) or by uncoupling of OXPHOS (Han et al., 2009; Pardo-Andreu et al., 2011), producing a decreased proliferation and cell death.

Previously, we have described that antioxidant *para*-hydroquinones, which incorporate a carbonyl group in the *ortho*-position to one of the phenolic hydroxyl groups, inhibit the respiration of cancer cells (Araya-Maturana et al., 2002, 2006; Rodríguez et al., 2007). One of them, the hydroquinone **14**, 9,10-dihydroxy-4,4-dimethyl-5,8-dihydroanthracen-1(4*H*)-one (Fig. 2A), affects selectively the proliferation of mammary cancer cells, inducing cell cycle arrest in the G2/M phase (Urra et al., 2013). This effect is consequence of a dysfunction in the mitochondrial bioenergetics by the electron flow inhibition through complex I, leading to ADP-stimulated oxygen consumption inhibition, transmembrane potential dissipation, and cellular ATP level decrease (Urra et al., 2013). Despite this proposed mechanism, the structural requirements of this hydroquinone scaffold to affect the OXPHOS of cancer cells have not been studied in detail. On the basis of these considerations, we explore the effect of structural modifications on *ortho* carbonyl hydroquinone scaffold, structurally related to hydroquinone **14**, on the coupled and uncoupled respiration using mouse mammary adenocarcinoma TA3/Ha cells, which exhibits a high oxidative metabolism as here we describe. Our results indicate that the effect of *ortho* carbonyl hydroquinone on OXPHOS is modified with structural changes, allowing obtaining compounds with three alternative types of actions: inhibitors of complex I dependent-respiration, uncouplers of OXPHOS and compounds with both actions (dual agents). Besides, the effect of a bicyclic hydroquinone (**9**) that affected the OXPHOS with selective anti-proliferative effects on TA3/Ha cancer cells is described.

2. Materials and methods

2.1. Compounds and reagents

All reagents were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Stock solutions of all hydroquinones were prepared in dimethyl sulfoxide (DMSO).

2.2. Chemicals

Duroquinol was synthesized by reduction from duroquinone as previously described (Plaza et al., 2008). ^1H and ^{13}C NMR spectra were obtained from a Bruker AVANCE DRX 300 spectrometer operating at either 300.13 MHz (^1H) or 75.47 MHz (^{13}C). Measurements were carried out at 300 K in $CDCl_3$. Chemical shifts are reported as ppm downfield from TMS for ^1H NMR and relative to the central $CDCl_3$ resonance (77.0 ppm) for ^{13}C NMR. All melting points are uncorrected and were determined on a Kofler hot stage apparatus. IR spectra (KBr discs) were recorded on an FT-IR Bruker IFS 55 spectrophotometer; wave numbers are reported in cm^{-1}. High resolution mass spectra were obtained on a MAT 95XP Thermo Finnigan spectrometer. Hydroquinones **8**, **9**, **11** and **14**–**21** were synthesized by published procedures (Araya-Maturana et al., 1999, 2006, 2002; Mendoza et al., 2005; Vega et al., 2009; Dobado et al., 2011). The new compounds were synthesized as described in the Supplementary Fig. 1 and in "Synthesis and Spectra Analysis" section.

2.3. Cell lines

Mouse mammary adenocarcinoma TA3/Ha cell line was kindly provided by Dr. Gabriel Jose Gasic, University of Pennsylvania, and has been used by our laboratory since 1989 (Fones et al., 1989). TA3-MTXR methotrexate-resistant cell line was generated as described previously by us (Araya-Maturana et al., 2002; Urra et al., 2013). This cell line exhibits methotrexate resistance and cross-resistance to cisplatin, doxorubicin, 5-fluorouracil and vinblastine (Plaza et al., 2008). Mouse mammary epithelial MM3MG and NMuMG cell lines, human acute lymphoblastic leukemia CCRF-CEM cell line, and its camptothecin resistant variant, CEM/C2 were purchased from American Tissue Culture Collection (ATCC). All cells were grown in DMEM high-glucose (MM3MG, TA3/Ha and TA3-MTXR), RPMI-1640 (CCRF-CEM and CEM/C2) or RPMI-1640 plus insulin (NMuMG) supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL) in a humidified atmosphere (37 °C and 5% CO_2).

2.4. Harvesting TA3/Ha and TA3-MTXR tumor cells

Mammary adenocarcinoma TA3/Ha 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. TA3-MTXR methotrexate-resistant cell line was propagated in the same mouse strain (Araya-Maturana et al., 2002; Urra et al., 2013). Mice were housed and fed under the conditions previously described (Plaza et al., 2008), in the animal facility of Facultad de Medicina, Universidad de Chile. All the experiments were approved by two local ethics committees, from this Faculty and from the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT). Tumor cells were harvested 5–7 days after i.p. inoculation of fluid from donor mice according to procedures described by us (Plaza et al., 2008).

2.5. Cellular respiration

Oxygen consumption was measured polarographically at 25 °C with a Clark electrode No. 5331 (Yellow Springs Instruments, Yellow Spring, OH, USA) using a YSI model 53 monitor, connected to a DI-148U data acquisition module with a USB interface. The data were obtained with Windaq Acquisition Waveform Recorder software (DataQ Instruments, USA). To characterize the mitochondrial metabolism of TA3/Ha cells, basal, ATP-linked, uncoupled and non-mitochondrial respiration were measured in two conditions: 1) only 4 mM glutamine and 2) 4 mM glutamine plus 5 mM glucose. TA3/Ha cells (5 mg protein/mL) were incubated in DMEM without glucose, L-glutamine and phenol red. Basal respiration was obtained from oxygen consumption rate (OCR) stimulated by addition of glutamine or glutamine plus glucose. Proton leak stimulated-respiration was obtained by 1 μM oligomycin addition and ATP synthesis-linked respiration was calculated by subtraction of proton leak stimulated respiration to basal respiration. Uncoupled and non-mitochondrial respirations were obtained by 200 nM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 1 μM antimycin A additions, respectively. All mitochondrial OCR values were calculated from non-mitochondrial OCR subtraction. To evaluate the effect of hydroquinones on cellular respiration, TA3/Ha cells (5 mg protein/mL) were incubated in RPMI-1640 without phenol red for 5 min and the respiration rates were registered in the absence (control in DMSO) or presence of hydroquinones for up to 30 min. The IC_{50} values were