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Amitriptyline down-regulates coenzyme Q_{10} biosynthesis in lung cancer cells

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ABSTRACT

Amitriptyline, a tricyclic antidepressant, has been proposed as an antitumoral drug in oxidative therapy. Its pro-apoptotic effects, mediated by high reactive oxygen species generation, have been already described. In this study we analysed the effect of amitriptyline on the biosynthesis of coenzyme Q_{10} (CoQ), an essential component for electron transport and a potent membrane antioxidant involved in redox signaling. We treated H460 cells, a non-small-cell lung cancer cell line, with amitriptyline and we analysed CoQ levels by HPLC and CoQ biosynthesis rate, as well as the enzymes involved in CoQ biosynthesis by real-time PCR and Western blot. Amitriptyline treatment induced a dose-dependent decrease in CoQ levels in tumor cells. CoQ decreased levels were associated with down-regulation of the expression of *COQ4* gene, as well as decreased Coq4 and Coq6 protein levels. Our findings suggest that the effect of amitriptyline on CoQ biosynthesis highlights the potential of this drug for antitumoral oxidative therapy.

1. Introduction

It has been observed that most cancer cells have altered mitochondria and increased reactive oxygen species steady state levels, making them more vulnerable to reactive oxygen species-mediated damage and resulting in increased cell death (Szatrowski and Nathan, 1991; Burdon, 1995; Pelicano et al., 2004; Tomasetti et al., 2015). Thus, manipulating reactive oxygen species levels by redox modulation could exert a remarkable antitumoral effect and potentially induce programmed cell death without causing significant toxicity to normal cells (Trachootham et al., 2009; Zhang et al., 2015). This strategy is called oxidation therapy" and it is achieved through delivering cytotoxic reactive oxygen species to solid tumors, or alternatively inhibiting the antioxidative enzyme system (Fang et al., 2009; Pathania et al., 2014; Fang et al., 2008). Recent studies have shown that tricyclic antidepressants (TCAs), such as imipramine, desipramine, nortriptyline or amitriptyline, induce cell death through mechanisms involving neurotrophin receptor (Pula et al., 2013), (JNK)/c-Jun pathway (Jahchan et al., 2013), Fas death receptor (Yuan et al., 2015); or mechanisms involving mitochondria-mediated cell death (Yuan et al., 2015; Zhang et al., 2013). Our group has proposed amitriptyline as an anticancer agent for oxidation therapy targeted to mitochondrial as it increases oxidative stress by generating high amounts of reactive oxygen species and decreasing antioxidant activity in several human cancer cell lines, leading to cell death through caspase-3-dependent apoptosis (Cordero et al., 2010). Reactive oxygen species increase seems to be due to an alteration of complex I + III of the mitochondrial respiratory chain (Cordero et al., 2009, 2010; Villanueva-Paz et al., 2016).

It is noteworthy that amitriptyline decreases the amount or activity of several antioxidants such as catalase and superoxide dismutase (Cordero et al., 2010; Moreno-Fernandez et al., 2008), and reduces levels of coenzyme Q_{10} (CoQ) (Bentinger et al., 2007). CoQ or ubiquinone is a lipid-soluble component of virtually all cell membranes and is both an essential component for electron transport in oxidative phosphorylation of mitochondria (Battino et al., 1990), and a powerful antioxidant (Turunen

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et al., 2004). Paradoxically, CoQ is also involved in superoxide production by the respiratory chain (James, Smith, and Murphy, 2004). Antioxidant and prooxidant mechanisms of CoQ are shown in Fig. 1. On the other hand, it is described that deficiency of CoQ decreases the activity of enzymes of the mitochondrial respiratory chain, the mitochondrial membrane potential, the expression of the mitochondrial proteins involved in oxidative phosphorylation and cell growth rates; and this deficiency also increases cell death, the production of reactive oxygen species, mitochondrial permeabilization and triggers mitochondria elimination by mitophagy (Rodriguez-Hernandez et al., 2009; Cotan et al., 2011). Given the critical role of CoQ in mitochondrial function, it has been suggested that CoQ levels could be a useful biological marker of mitochondrial function (Haas et al., 2008). *In vivo* studies have shown that treatment with amitriptyline induces CoQ deficiency and oxidative stress in some organs and peripheral blood cells (Bautista-Ferrufino et al., 2011; Moreno-Fernandez et al., 2012). Therefore, alterations in the amount of CoQ are somehow involved in the increase in oxidative stress induced by amitriptyline, although no study has investigated the effect of amitriptyline in the biosynthesis of CoQ at the molecular level.

The present study aimed to assess the changes provoked by amitriptyline in gene expression and protein levels of the enzymes involved in CoQ biosynthesis, as well as CoQ biosynthesis rate, in a human lung cancer cell line.

2. Materials and methods

2.1. Reagents

Amitriptyline and trypsin were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). Hoechst 3342 and MitoSOX Red were purchased from Invitrogen/Molecular Probes (Eugene, Oregon, USA). Anti-COQ proteins and Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and from Research Diagnostic Inc. (Flanders, New Jersey, USA), respectively.

2.2. Cell culture

H460 cells (from human non-small cell lung carcinoma: NSCLC) were purchased from the American Type Culture Collection (Manassas, Virginia, USA) and cultured in RPMI-1640 medium supplemented with L-glutamine, antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO, USA), and with 10% fetal bovine serum (FBS). Cells were incubated at 37 C in a 5% CO_2 atmosphere.

2.3. Dose-dependent toxicity, and viability assays

H460 cells were cultured with different concentration of amitriptyline (0 M, 20 M, 50 M, and 100 M) for 6 h, 12 h, and 24 h. After incubation, cell viability was analysed by trypan blue assay and an automated cell counter (TC10) according to the manufacturer s instructions (Bio-Rad Laboratories, Inc.). The rest of the studies were performed after treating H460 cells with 50 M amitriptyline for 24 h. To study the putative restore effects of external CoQ on cell viability of amitriptyline-treated cells, H460 cultures were supplemented with 50 M amitriptyline and 25 M CoQ for 24 h.

2.4. Biochemical determination of coenzyme Q_{10} levels

Lipid extraction from H460 cells after treatment with several doses of amitriptyline (20 M, 50 M, and 100 M) for 6 h, 12 h, and 24 h was performed as described earlier (Santos-Ocana et

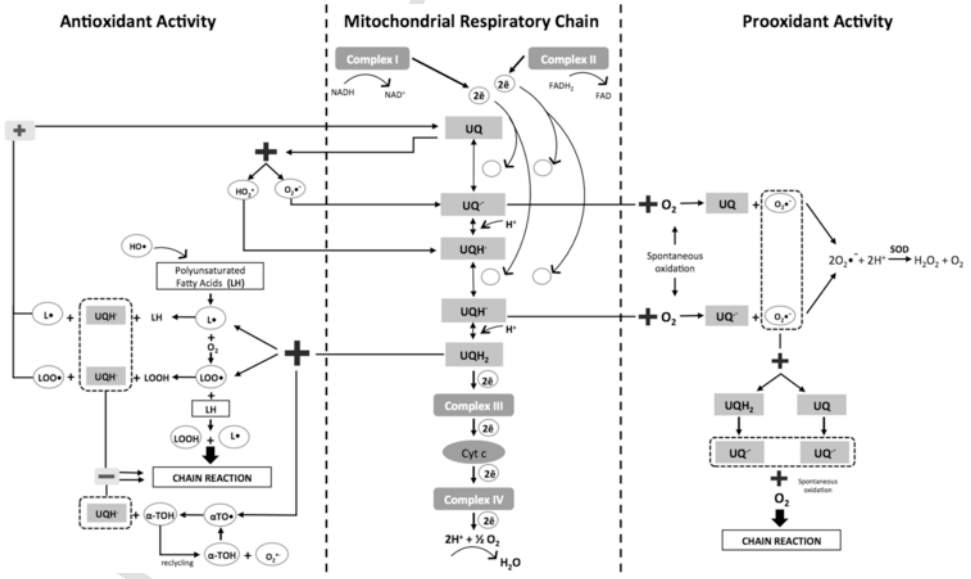

Fig. 1. Antioxidant and prooxidant mechanisms of Coenzyme Q (CoQ). Mitochondrial Respiratory Chain: Mitochondrial electron transport chain transfers electrons from NADH and FADH2 to molecular oxygen to form H_2O through multiprotein complexes, named complex I IV, and of a series of carriers such as flavoproteins, iron-sulfur proteins, CoQ and cytochromes. Antioxidant Activity: The antioxidant capacity of CoQ depends on its redox forms: fully reduced (Ubiquinol, UQH_2), semireduced (Ubisemiquinone, UQH ·) and oxidized (Ubiquinone, UQ). Antioxidants such as -tocopherol (-TOH) can donate a hydrogen ion to a superoxide radical (O2 -) to form tocopheroxyl radical (TO), which in turn can react with UQH_2 for recycle -TOH and produce UQH ·. At the same time, UQH_2 reacts with lipid-derived free radicals such as alkyl radical (L), formed by the attack of a reactive species (hydroxyl radical: HO) to a hydrogen atom (H) from unsaturated fatty acids of phospholipids (LH); and with peroxyl radical (LOO), formed by the spontaneous union of L with oxygen molecules. Finally, UQH ⁻ reacts with radicals to generate more UQ and to inhibit the chain reaction of lipid peroxidation. Prooxidant activity: Ubisemiquinone spontaneously oxidizes in the presence of oxygen. The superoxide formed produces hydrogen peroxide (H_2O_2). O2 ⁻ can react with UQH_2 or UQ to form further ubisemiquinone radicals, starting a chain reaction of autoxidation. However, this reaction occurs when there is a disorder of the phospholipid bilayer.

al., 2002). The cell samples were lysed with 1% SDS and vortexed for 1 min. A mixture of ethanol:isopropanol (95:5) was added and samples were vortexed for 1 min. To recover CoQ, 5 ml of hexane was added and samples were centrifuged at 1000*g* for 5 min at 4 C. The upper phases from three extractions were recovered and dried on a rotatory evaporator. Lipid extract was suspended in 1 ml of ethanol, dried in a speed vac and kept at −20 C. Samples were suspended in the suitable volume of ethanol prior to high-performance liquid chromatography (HPLC) injection. Lipid components were separated by a Prominence Shimadzu HPLC system (Shimadzu Scientific Instruments Columbia, MD, USA) equipped with a Shimadzu Shim-pack XR-ODS column. Coenzyme Q_9 (CoQ_9) was used as an internal standard. CoQ levels were analysed using an ultraviolet SPD-20A detector.

2.5. CoQ biosynthesis rate

Human H460 cells were cultured in 10 ml of PBS (Invitrogen) containing 1*g* of glucose/100 ml, 10% FBS, penicillin (100 units/ml), and streptomycin (100 mg/ml), with and without 50 M amitriptyline for 24 h. Then, 0.5 mCi of [3 H]mevalonate (3.25 Ci/mmol, Perkin-Elmer Life Sciences, Waltham, MA, USA) was added, and incubation continued for an additional 24 h. Finally, the cells were harvested by trypsinization and stored at −20 C for later analysis. Lipids were extracted from cells with chloroform/methanol/water (1:1:0.3) at 37 C for 1 h with magnetic stirring). The extracts thus obtained were adjusted to achieve a final chloroform/methanol/ water ratio of 3:2:1, and complete phase separation was then accomplished by centrifugation. The lower chloroform phase was removed and evaporated to dryness under a flow of nitrogen, and the residue was subsequently redissolved in chloroform. These solutions were placed onto a silica column (50 mg/1.5 ml; Extract-Clean; Alltech, Deerfield, IL, USA) and thereafter eluted with 6 ml of chloroform. After evaporation of the solvents, the neutral lipids were dissolved in chloroform/methanol (2:1). For analysis of CoQ, the samples were analysed by thin layer chromatography. The lipid extracts were supplemented with 5 g of cold CoQ and then loaded onto silica gel plates (Silica gel 60, Merck KGaA, Darmstadt, Germany). The plates were developed with hexane/ethyl ether 80:20 and bands corresponding to these metabolites were dyed with iodine vapors. The band corresponding to CoQ was scrapped off from the silica gel plates and transferred to liquid scintillation vials. Radioactivity was then measured by liquid scintillation in a Beckman Coulter LS6500 liquid scintillator.

2.6. Western blotting for Coq proteins

H460 cells were treated with 50 M amitriptyline for 24 h. Whole cellular lysates were prepared in a buffer, gentle shaking, composed of 0.9% NaCl, 20 mM Tris-HCl, pH 7.6, 0.1% triton X-100, 1 mM phenylmethyl-sulfonylfluoride, and 0.01% leupeptine. Electrophoresis was carried out in a 10 15% acrylamide SDS/PAGE. Proteins were transferred to Immobilon membranes (Amersham Pharmacia Biotech, Madrid, Spain). Mouse anti-Coq proteins and mouse antiglyceraldehyde 3-phosphate dehydrogenase antibodies were used to detect proteins by Western blotting. The proteins were electrophoresed, transferred to nitrocellulose membranes, and, after blocking overnight at 4 C, incubated with the respective antibody solution at 1:1000 dilutions. Then, membranes were probed with their respective secondary antibody labelled with fluorescence (1:2500). Immunolabelled proteins were detected by using a chemiluminiscence method (Bio-Rad Laboratories Inc., Hercules, California, USA). The protein concentration was determined by the Bradford method (Bradford, 1976).

2.7. Quantitative real-time RT-PCR

Total cellular RNA was purified from cultured H460 cells after treatment with 50 M amitriptyline for 24 h using the TRIsure method (Bioline, London, UK), according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically. In order to avoid genomic DNA contamination, one microgram of total RNA from each sample was incubated in gDNA Wipeout Buffer (Quantitect Reverse Transcription Kit, Qiagen. Hilden, Germany) at 42 C for 5 min. RNA samples were subsequently retrotranscribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen. Hilden, Germany). Quantitative RT-PCRs were performed in a Miniopticon unit (Bio-Rad, Hercules, CA) making use of the SensiMix One-Step qRT-PCR Kit (Bioline, London, UK), in accordance to the comparative 2 ^{CT} method described by Livak and Schmittgen (Livak and Schmittgen, 2001). The thermal cycling conditions used were: denaturation at 95 C for 20 s, alignment at 54 C for 20 s and elongation at 72 C for 20 s, for 40 cycles. Beta-actin was used as an internal control in each reaction. Primers utilized are listed in Table 1. All reactions were performed in duplicate. Reaction mixtures, without RNA, were used as negative controls in each run.

Table 1
Primers used for qRT-PCR analysis, with their annealing temperatures and size of PCR products.

Gene	Primer sequence	PCR product (bp)	Annealing temp.
beta-actin	F: 5 -CCAGATCATGTTTGAGACC-3 R: 5 -ATGTCACGCACGATTTCCC-3	275	55
COQ2	F: 5 -AGAACAGCCAATCGTCCAATAGC-3 R: 5 -CCCAATTAAATGTCAAGCCCAAGG-3	220	55
COQ4	F: 5 -CCCCAGACACCCGAGCAC-3 R: 5 -ACAGCCTCAAACCATTTCACCAC-3	154	55
COQ6	F: 5 -CTGCTGTTGTGGCTACTCTGC-3 R: 5 -GGACCAAACCAAGGAACTCAAGG-3	128	55
COQ7	F: 5 -GGACGCTGATGGAGGAGGAC-3 R: 5 -TGCTCTTCAGGACGGCATAGG-3	144	55

2.8. Mitochondrial reactive oxygen species production

Mitochondrial reactive oxygen species generation was assessed by MitoSOX , a red mitochondrial superoxide indicator. Approximately 1 10^6 H460 cells treated with 50 M for 24 h were incubated with 1 M MitoSOX for 30 min at 37 C, washed twice with PBS and resuspended in 500 l of PBS and analysed by flow cytometry. H460 cultures were also treated with 25 M CoQ in order to investigate the effect of supplemented CoQ on amitriptyline-induced reactive oxygen species production.

2.9. Statistical analysis

All results are expressed as means values S.D. Each experiment was repeated three times. The unpaired Student's *t*-test was used to evaluate the significance of differences between groups (treatments and control). The level of significance was set at *P* value of less than 0.05.

3. Results

3.1. Dose-dependent toxicity of amitriptyline and reactive oxygen species production

The toxicity of amitriptyline was studied on cultured H460 cells by analysing cell viability. Fig. 2 shows the percentages of living cells after administration of the drug at different concentrations (20 M, 50 M, and 100 M), and at several times (6 h, 12 h, and 24 h). The values obtained from controls at time 0 h (T_0) were very similar among groups with different drug concentrations (data not shown). After 12 h and 24 h of incubations, the average number of cells was notably decreased in the presence of amitriptyline at concentration of 50 M (61% and 36%, respectively) and 100 M (28% and 26%, respectively) as compared with control cultures (97% and 98%, respectively). In consequence of these results, the condition selected for the rest of the experiments was 50 M amitriptyline for 24 h because it was the lowest dose with high effect on cell viability.

Interestingly, when cells were treated for 24 h with 50 M amitriptyline supplemented with 25 M CoQ, the percentage of cell viability increased significantly from 55% (amitriptyline alone) to 79% (Fig. 3A). On the other hand, when we analysed reactive oxygen species production in H460 cells treated with

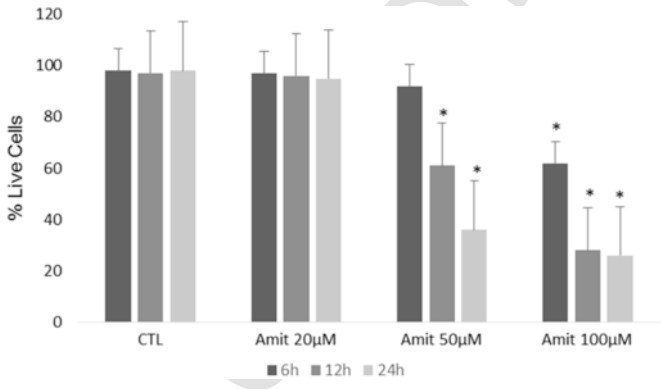

Fig. 2. Effects of amitriptyline on cell survival. Amitriptyline dose-dependent toxicity in H460 cells treated for 6 h, 12 h, and 24 h. H460 cells were treated with amitriptyline 20, 50, and 100 M, and cell counting was determined as shown in Section 2.3 of *Material and Methods*. Results are expressed as mean S.D. of three independent experiments. * $P<0.05$ between control and treated cells.

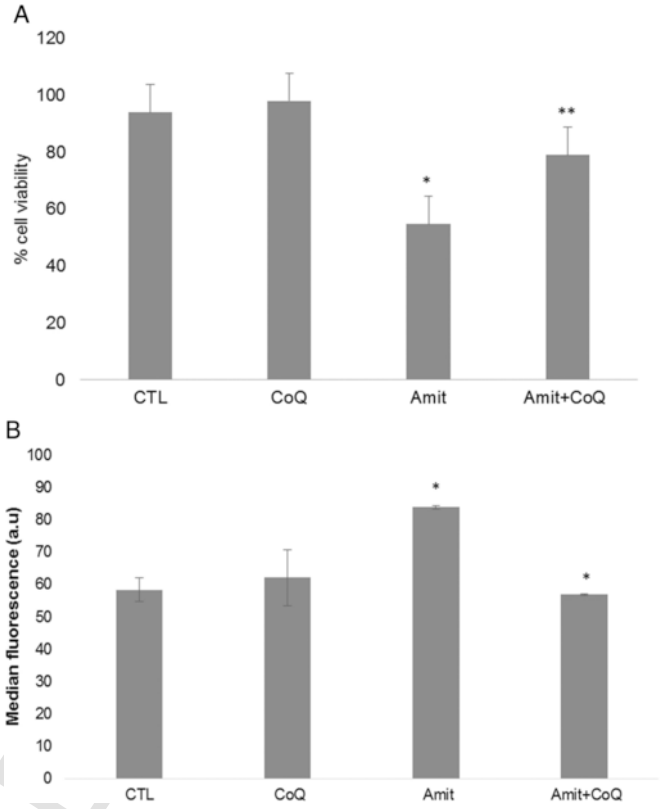

Fig. 3. Effect of supplementary CoQ on survival percentages and reactive oxygen species production in H460 cells treated with 50 M amitriptyline for 24 h. A: Cell viability for cells treated with amitriptyline and supplemented with 25 M CoQ. B: Mitochondrial reactive oxygen species generation assessed by MitoSOX in tumor cells in the presence of amitriptyline and supplementary CoQ. Results are expressed as mean S.D. of three independent experiments. * $P<0.05$ between control and treated cells; ** $P<0.05$ between amitriptyline treated cells and those complemented with CoQ.

50 M amitriptyline we found a significant increase in reactive oxygen species levels (83.92 a.u.; control: 58.36 a.u.) that was restored to normal values in the presence of supplementary CoQ (56.87 a.u.) (Fig. 3B).

A multivariate analysis showed that cells treated with 50 M amitriptyline for 24 h had a strong positive association between cell death and reactive oxygen species production in H460 cells (Coef. = 0.893), with statistical significance.

3.2. Biochemical analysis of coenzyme Q_{10} levels

We determined the levels of CoQ by HPLC in H460 cells after treatment with 20 M, 50 M, and 100 M amitriptyline for 6 h, 12 h, and 24 h. We found that amitriptyline treatment induced a significant dose-dependent decrease of CoQ levels (Fig. 4A). The data, expressed as pmol CoQ/mg protein, were 224.67 1.34 for control cells and 130.47 10.63 for H460 cells treated with 50 M amitriptyline for 24 h. To elucidate whether CoQ levels down-regulation was due to a decrease in CoQ biosynthesis rate, we examined the incorporation of the radiolabelled CoQ precursor [3H]mevalonate in 50 M amitriptyline-treated cells for 24 h. We observed that amitriptyline induced a significant decrease of CoQ biosynthesis (Fig. 4B).

Multivariate analysis showed that higher amount of CoQ was associated with a decrease in cell viability and in reactive oxygen species levels (Coef. = −0.5964 and Coef. = −0.8027, respectively); all variables being statistically significant.

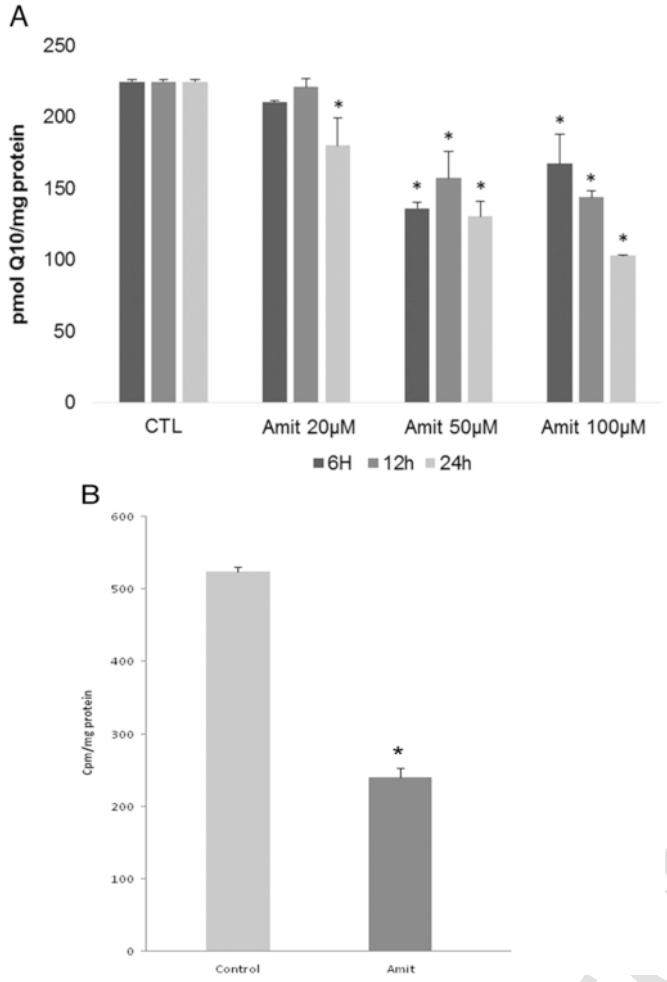

Fig. 4. Effects of amitriptyline on CoQ levels and biosynthesis rate. A: H460 cells were treated with amitriptyline at different doses (20 M, 50 M, and 100 M) for 6 h, 12 h, and 24 h; and CoQ levels were determined by HPLC as described in Section 2.4 of *Material and Methods*. B: For the analysis of CoQ biosynthesis rate, control and 50 M amitriptyline-treated cells were incubated with [3H]mevalonate for 24 h, and radiolabelled CoQ was analysed as described in Section 2.5 of *Material and Methods*. Results are expressed as mean S.D. of three independent experiments. *$P<0.05$ between control and treated cells.

3.3. Amitriptyline modulates COQ gene expression

In order to underline the effect of amitriptyline on CoQ synthesis in H460 cells, we also determined the expression levels of key genes and proteins involved in CoQ synthesis. Expression levels of *COQ2*, *COQ4*, *COQ6* and *COQ7* genes were analysed at mRNA level by real time quantitative RT-PCR and at protein level by Western blot. The relative number of gene copies within the total RNA isolated from amitriptyline-treated cells was compared to the number in their non-treated counterparts. Amitriptyline induced significant down-regulation of mRNA levels of *COQ4* gene and up-regulation of *COQ2* and *COQ7* (Fig. 5). Accordingly, Western blotting analysis revealed a decrease in the expression levels of Coq4 and Coq6 translated proteins and an increase in the expression levels of Coq7 (Fig. 6), indicating that the decrease of CoQ induced by amitriptyline can be caused by both transcription and translational alterations of key components of the CoQ biosynthesis pathway.

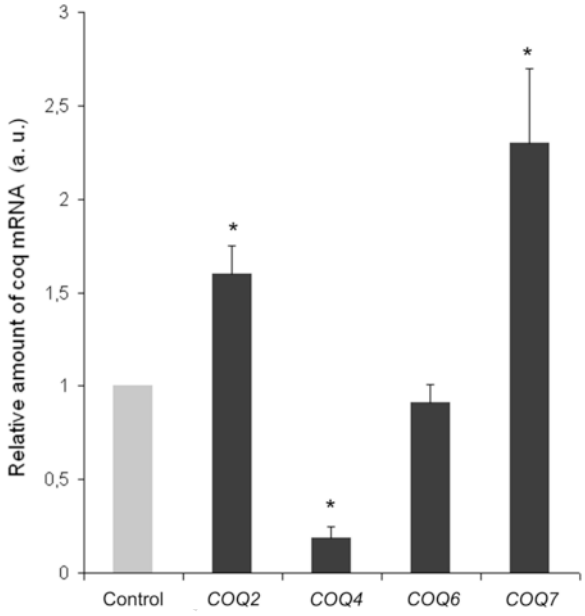

Fig. 5. qRT-PCR based analysis of the effect of amitriptyline treatment on coenzyme-Q genes *COQ2*, *COQ4*, *COQ6* and *COQ7* in H460 cell cultures treated with 50 M amitriptyline for 24 h. Graphs show the specific mRNA relative amount using appropriate primers (Table 1) and normalized to the beta-actin mRNA expression. * $P<0.05$.

4. Discussion

Mitochondria have recently emerged as novel targets for cancer therapy due to its important roles in cellular function such as programmed cell death regulation and reactive oxygen species generation (He et al., 2015c; Weinberg and Chandel, 2015). Increased generation of reactive oxygen species and an altered redox status have been observed in cancer cells, and recent studies suggest that these biochemical properties can be used for therapeutic benefits (Trachootham et al., 2009; Fang et al., 2007; Chandra et al., 2000). Oxidative therapy is presented as an antitumoral strategy based on oxidative susceptibility of most cancer cells due to their intrinsic high oxidative stress, close to the lethal threshold. The proposed mechanism consists in the use of pro-oxidant agents that generate high levels of reactive oxygen species or inhibit reactive oxygen species elimination by diminishing the antioxidant system of tumor cells, leading to oxidative damage and cell death through the activation of the caspase cascade (Chandra et al., 2000; Fang et al., 2009; Tomasetti et al., 2015; Conklin, 2004).

In recent studies, we have shown that amitriptyline, a tricyclic antidepressant commonly prescribed for depression and therapeutic treatments of several neuropathic and inflammatory illnesses, increases reactive oxygen species levels and induces apoptosis through mitochondrial dysfunction, inhibiting mitochondrial complex III activity (Cordero et al., 2009; Bentinger et al., 2007; Villanueva-Paz et al., 2016); and it has been propose to be considered as a putative new anticancer drug (Cordero et al., 2010; Villanueva-Paz et al., 2016). Amitriptyline induces high reactive oxygen species generation as a result of mitochondrial dysfunction, provoking a higher level of apoptosis of tumor cells than common chemotherapeutic drugs (Cordero et al., 2010), and reduces important antioxidants of the cell-defense machinery, dramatically limiting tumor cell response to reactive oxygen species production; although some studies have reported protective actions and increased antioxidant status by

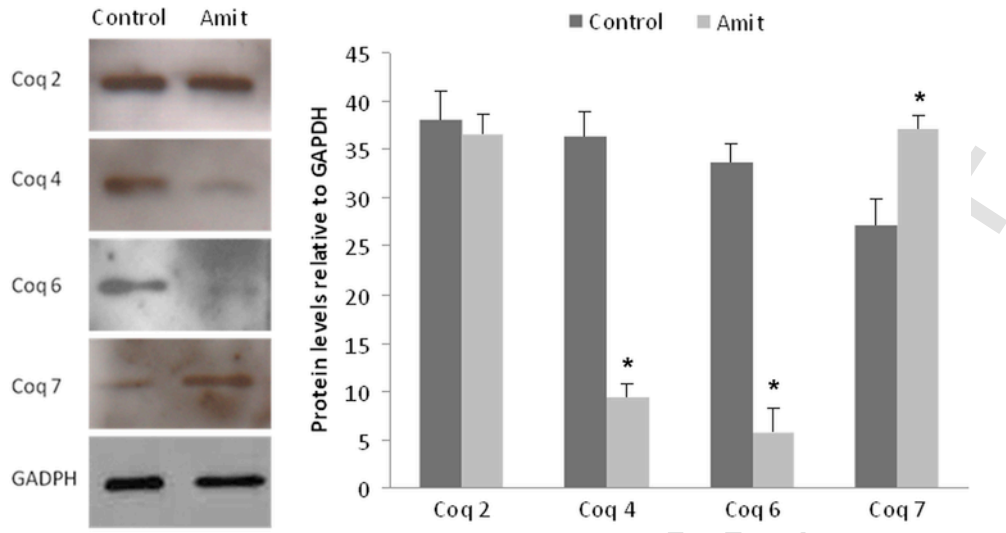

Fig. 6. Effects of amitriptyline in the content of CoQ biosynthetic protein in H460 cells treated with 50 M amitriptyline for 24 h. Western blots were performed as described in Section 2.6 of *Material and Methods* with antibodies against Coq2, Coq4, Coq6 and Coq7 and GADPH. Protein levels were determined by densitometric analysis of three different Western blots and normalized to GAPDH signal. * $P < 0.05$ between control and treated cells.

upregulation of glutathione reductase (GR) and superoxide dismutase (SOD) activity of TCAs (Kolla et al., 2005; Herbet et al., 2013). These discrepancies could be explained by differences in the concentrations of the antidepressants used that could achieve effects sub-cytotoxic (protective) or cytotoxic (deleterious). This finding is consistent with previous results found by Yuan et al. in bladder cancer cells (Yuan et al., 2015), where they have shown that other TCAs, such as nortriptyline, also induce caspase-dependent apoptosis by two different pathways: mitochondria-mediated and death receptor-mediated.

CoQ is one of the antioxidant that is significantly decreased by amitriptyline treatment, as we have previously demonstrated in both fibroblasts and tumor cells (Cordero et al., 2009, 2010), although the expression levels of enzymes involved in CoQ biosynthesis were not studied. Furthermore, when CoQ levels were decreased by amitriptyline treatment, CoQ and alpha-tocopherol supplementation ameliorated amitriptyline-induced toxicity in both cultured human primary fibroblasts and zebrafish embryos (Cordero et al., 2009).

CoQ has been shown to be a component of the structure of Complex III, contributing to both its assembly and stability; and it is one of the most important sites of reactive oxygen species production (Quinlan et al., 2013). In mammalian cells, *COQ* genes involved in the biosynthesis of ubiquinone encode at least nine Coq proteins (Coq1-Coq9), which are mainly located in the matrix side of the inner mitochondrial membrane (Tran and Clarke, 2007), although they are also present in plasma membrane, endomembranes and in serum lipoproteins, where they exert a key component in the antioxidant machinery (Fernandez-Ayala et al., 2005). CoQ is our only lipid soluble antioxidant that is synthesized endogenously. CoQ tissue content is independent on the diet, and it is effective in preventing both lipid peroxidation and protein oxidation as well as defending DNA from oxidative damage, especially important for mitochondrial DNA since it is more susceptible to oxidative damage than nuclear DNA (Bentinger et al., 2007). CoQ is also reported to be an inhibitor of apoptosis by inhibiting mitochondrial depolarization, independently of its free radical scavenging property (Papucci et al., 2003). In the yeast *Saccharomyces cerevisiae* (representative of eukaryotic cells), CoQ is synthesized by 11 known genes (*COQ1-COQ9*, *YAH1*, and *ARH1*) (Allan et al., 2015). It has been reported that the coordinated function of the eleven polypeptides (Coq1-Coq9, Arh1, and Yah1) is required for the production of CoQ (Nguyen et al., 2014; He et al., 2014). The Coq10 protein is localized to the mitochondria but it is not directly involved in its biosynthesis in *S. cerevisiae* (Barros et al., 2005). Several evidences suggest that the Coq proteins are associated in a multi-subunit complex in the inner mitochondrial membrane termed "CoQ-synthome" (He et al., 2014; Nguyen et al., 2014; He et al., 2015a; Allan et al., 2015; Kawamukai, 2015). Although the function of some of the Coq proteins remains unknown, such as Coq4, Coq8, and Coq9 (Tran and Clarke, 2007; He et al., 2014; Allan et al., 2015; Kawamukai, 2015), the presence of each Coq polypeptide is essential for the assembly of the Coq polypeptide complex (Nguyen et al., 2014), which is required for biosynthesis of ubiquinone (He et al., 2015a). Indeed, deletion of any of the *COQ* genes leads to destabilization of several other Coq polypeptides (He et al., 2014). In humans, mutations in several *COQ* genes are associated with mitochondrial, cardiovascular, kidney and neurodegenerative diseases (Nguyen et al., 2014). These results indicate the interdependence of the Coq polypeptides. It is recognized that Coq4 seems to be in the centre of the CoQ-synthome as organizer, and the Coq3, Coq5, Coq6 and Coq7 polypeptides as more peripheral members of the complex (He et al., 2014; Kawamukai, 2015). Although the molecular function of the Coq4 protein has not been elucidated, it is known that the lack of *COQ4* in yeast causes the instability of several Coq proteins, such as Coq7 and Coq3 (Kawamukai, 2015).

It has been also reported that the over-expression of Coq8, which encodes a putative regulatory kinase, stabilizes the Coq polypeptide complex in several coq null mutants (Nguyen et al., 2014; He, Black et al., 2015a; He et al., 2014), and it has been implicated in the complex assembly through the phosphorylation of Coq3, Coq5, and Coq7 (Kawamukai, 2015). Coq9 is a lipid-binding protein that associates with Coq7 to enable CoQ biosynthesis (Lohman et al., 2014). Recently, it has been identified a new protein associated with the CoQ biosynthetic complex, Coq11, which is not required for CoQ synthesis, but its deletion reduces the CoQ levels in *S. cerevisiae* (Allan et al., 2015).

As we have already demonstrated, amitriptyline induces reactive oxygen species production and apoptosis in H460 cells, as well as decreases CoQ levels in a dose-dependent manner (Cordero et al., 2010). In the present study, we have demonstrated a dysregulation of CoQ biosynthesis by amitriptyline, at both mRNA and protein levels, in a human lung cancer cell line. According to the model of mitochondrial CoQ biosynthesis complex proposed by Tran & Clarke (Tran and Clarke, 2007), we studied the Coq proteins most closely associated to Coq2 protein, which serves as an anchor to the inner mitochondrial membrane.

Most chemotherapeutic drugs, such as camptothecin, doxorubicin, and methotrexate, do not provoke any decrease of antioxidants. Instead, they frequently induce an increase of antioxidants as a protecting mechanism against reactive oxygen species generation, leading to low cell death rates (Brea-Calvo et al., 2006). The fact that amitriptyline reduces CoQ levels can be very interesting in terms of oxidative therapy since it diminishes even more the already decreased antioxidant defenses present in cancer cells, making amitriptyline-induced free radicals a more effective weapon against tumor cells.

In this study, we have observed a decrease of *COQ4* mRNA expression levels as well as a decrease of the amount of Coq4 and Coq6 protein expression levels after amitriptyline treatment. Amitriptyline-induced high reactive oxygen species levels probably trigger these alterations, as we have detected a significant correlation between increased reactive oxygen species levels and decreased amount of CoQ. Moreover, we have shown that CoQ supplementation restored reactive oxygen species levels increased by amitriptyline treatment and hence cell survival. However, the mechanism by which amitriptyline provoke these downregulations is still unknown and it should be investigated. It has been hypothesized in yeasts that Coq4 functions as the core component of the polypeptide CoQ biosynthetic complex, holding together the other *COQ* gene products, although a clear enzymatic function has not been assigned (Tran and Clarke, 2007). The human gene has probably the same function than in yeasts since it can effectively complement a yeast $COQ4^{null}$ mutant (Casarin et al., 2008). On the other hand, knockdown of *COQ4* causes CoQ deficiency in HeLa cells (Salviati et al., 2012).

Our results showed that Coq2 expression was not affected by amitriptyline treatment, but Coq7 was overexpressed, probably as an attempt to compensate the reduction in Coq4 and Coq6. Increased reactive oxygen species may cause the transcriptional effects on *COQ7* genes through the activation of NF-*k*B, which has been demonstrated to be activated by camptothecin in H460 cells (Brea-Calvo et al., 2009). However, the downregulation of Coq4 stimulated by amitriptyline could be enough to induce a decrease of CoQ levels, reducing the antioxidant capacity of tumor cells.

In conclusion, we have demonstrated that amitriptyline induces a down-regulation of CoQ levels in a lung cancer cell line. As CoQ is the main lipid soluble endogenous antioxidant, this effect can limit cell defenses against amitriptyline-induced reactive oxygen species production. Therefore, we added new evidences showing that amitriptyline could be considered a good candidate for future assays in antitumoral oxidative therapy since it induces high reactive oxygen species production and also diminishes the antioxidant machinery.

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Conflict of interest

The authors of this study declare that they have no conflict of interest.

References

Allan, C.M., Awad, A.M., Johnson, J.S., Shirasaki, D.I., Wang, C., Blaby-Haas, C.E., Merchant, S.S., Loo, J.A., Clarke, C.F., 2015. Identification of Coq11, a new coenzyme Q biosynthetic protein in the CoQ-synthome in Saccharomyces cerevisiae. J. Biol. Chem. 290 (12), 7517–7534. http://dx.doi.org/10.1074/jbc.M114.633131.

Barros, M.H., Johnson, A., Gin, P., Marbois, B.N., Clarke, C.F., Tzagoloff, A., 2005. The Saccharomyces cerevisiae COQ10 gene encodes a start domain protein required for function of coenzyme Q in respiration. J. Biol. Chem. 280 (52), 42627–42635. http://dx.doi.org/10.1074/jbc.M510768200.

Battino, M., Fato, R., Parenti-Castelli, G., Lenaz, G., 1990. Coenzyme Q can control the efficiency of oxidative phosphorylation. Int. J. Tissue React. 12 (3), 137–144.

Bautista-Ferrufino, M.R., Cordero, M.D., Sanchez-Alcazar, J.A., Illanes, M., Fernandez-Rodriguez, A., Navas, P., de Miguel, M., 2011. Amitriptyline induces coenzyme Q deficiency and oxidative damage in mouse lung and liver. Toxicol. Lett. 204 (1), 32–37. http://dx.doi.org/10.1016/j.toxlet.2011.03.033.

Bentinger, M., Brismar, K., Dallner, G., 2007. The antioxidant role of coenzyme Q. Mitochondrion 7 (Suppl:S41-Suppl:S50)http://dx.doi.org/10.1016/j.mito.2007.02.006.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem 72, 248–254.

Brea-Calvo, G., Rodriguez-Hernandez, A., Fernandez-Ayala, D.J., Navas, P., Sanchez-Alcazar, J.A., 2006. Chemotherapy induces an increase in coenzyme Q10 levels in cancer cell lines. Free Radic. Biol. Med. 40 (8), 1293–1302. http://dx.doi.org/10.1016/j.freeradbiomed.2005.11.014.

Brea-Calvo, G., Siendones, E., Sanchez-Alcazar, J.A., de Cabo, R., Navas, P., 2009. Cell survival from chemotherapy depends on NF-kappaB transcriptional up-regulation of coenzyme Q biosynthesis. PLoS One 4 (4), e5301. http://dx.doi.org/10.1371/journal.pone.0005301.

Burdon, R.H., 1995. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Radic. Biol. Med. 18 (4), 775–794.

Casarin, A., Jimenez-Ortega, J.C., Trevisson, E., Pertegato, V., Doimo, M., Ferrero-Gomez, M.L., Abbadi, S., Artuch, R., Quinzii, C., Hirano, M., Basso, G., Ocana, C.S., Navas, P., Salviati, L., 2008. Functional characterization of human COQ4, a gene required for Coenzyme Q10 biosynthesis. Biochem. Biophys. Res. Commun. 372 (1), 35–39. http://dx.doi.org/10.1016/j.bbrc.2008.04.172.

Chandra, J., Samali, A., Orrenius, S., 2000. Triggering and modulation of apoptosis by oxidative stress. Free Radic. Biol. Med. 29 (3 4), 323–333.

Conklin, K.A., 2004. Chemotherapy-associated oxidative stress: impact on chemotherapeutic effectiveness. Integr. Cancer Ther. 3 (4), 294–300. http://dx.doi.org/10.1177/1534735404270335.

Cordero, M.D., Moreno-Fernandez, A.M., Gomez-Skarmeta, J.L., de Miguel, M., Garrido-Maraver, J., Oropesa-Avila, M., Rodriguez-Hernandez, A., Navas, P., Sanchez-Alcazar, J.A., 2009. Coenzyme Q10 and alpha-tocopherol protect against amitriptyline toxicity. Toxicol. Appl. Pharm. 235 (3), 329–337.

Cordero, M.D., Sanchez-Alcazar, J.A., Bautista-Ferrufino, M.R., Carmona-Lopez, M.I., Illanes, M., Rios, M.J., Garrido-Maraver, J., Alcudia, A., Navas, P., de Miguel, M., 2010. Acute oxidant damage promoted on cancer cells by amitriptyline in comparison with some common chemotherapeutic drugs. Anticancer Drugs 21 (10), 932–944. http://dx.doi.org/10.1097/CAD.0b013e32833ed5f7.

Cotan, D., Cordero, M.D., Garrido-Maraver, J., Oropesa-Avila, M., Rodriguez-Hernandez, A., Gomez Izquierdo, L., De, M., la Mata, De Miguel, M., Lorite, J.B., Infante, E.R., Jackson, S., Navas, P., Sanchez-Alcazar, J.A., 2011. Secondary coenzyme Q10 deficiency triggers mitochondria degradation by mitophagy in MELAS fibroblasts. Faseb J. 25 (8), 2669–2687. http://dx.doi.org/10.1096/fj.10-165340.

Fang J. Nakamura H. Iyer A.K. 2007. Tumor-targeted induction of oxystress for cancer therapy J. Drug Target 15 (7 8), 475–486 http://dx.doi.org/10.1080/10611860701498286

Fang, J., Deng, D., Nakamura, H., Akuta, T., Qin, H., Iyer, A.K., Greish, K., Maeda, H., 2008. Oxystress inducing antitumor therapeutics via tumor-targeted delivery of PEG-conjugated D-amino acid oxidase. Int J. Cancer 122 (5), 1135–1144. http://dx.doi.org/10.1002/ijc.22982.

Fang, J., Seki, T., Maeda, H., 2009. Therapeutic strategies by modulating oxygen stress in cancer and inflammation. Adv. Drug Deliv. Rev. 61 (4), 290–302. http://dx.doi.org/10.1016/j.addr.2009.02.005.

Fernandez-Ayala, D.J., Brea-Calvo, G., Lopez-Lluch, G., Navas, P., 2005. Coenzyme Q distribution in HL-60 human cells depends on the endomembrane system. Biochim. Biophys. Acta 1713 (2), 129–137. http://dx.doi.org/10.1016/j.bbamem.2005.05.010.

Haas, R.H., Parikh, S., Falk, M.J., Saneto, R.P., Wolf, N.I., Darin, N., Wong, L.J., Cohen, B.H., Naviaux, R.K., 2008. The in-depth evaluation of suspected mitochondrial disease. Mol. Genet. Metab. 94 (1), 16–37. http://dx.doi.org/10.1016/j.ymgme.2007.11.018.

He, C.H., Xie, L.X., Allan, C.M., Tran, U.C., Clarke, C.F., 2014. Coenzyme Q supplementation or over-expression of the yeast Coq8 putative kinase stabilizes multi-subunit Coq polypeptide complexes in yeast Coq null mutants. Biochim. Biophys. Acta 1841 (4), 630–644. http://dx.doi.org/10.1016/j.bbalip.2013.12.017.

He C.H. Black D.S. Nguyen T.P. Wang C. Srinivasan C. Clarke C.F. 2015. Yeast Coq9 controls deamination of coenzyme Q intermediates that derive from para-aminobenzoic acid Biochim. Biophys. Acta 1851 (9), 1227–1239 http://dx.doi.org/10.1016/j.bbalip.2015.05.003

He, H., Li, D.W., Yang, L.Y., Fu, L., Zhu, X.J., Wong, W.K., Jiang, F.L., Liu, Y., 2015. A novel bifunctional mitochondria-targeted anticancer agent with high selectivity for cancer cells. Sci. Rep. 5, 13543. http://dx.doi.org/10.1038/srep13543.

Herbet, M., Gawronska-Grzywacz, M., Graca, A., Jagiello-Wojtowicz, E., 2013. Influence of combined therapy with rosuvastatin and amitriptyline on the oxidation-reduction status in rats. Acta Pol. Pharm. 70 (5), 913–917.

Jahchan, N.S., Dudley, J.T., Mazur, P.K., Flores, N., Yang, D., Palmerton, A., Zmoos, A.F., Vaka, D., Tran, K.Q., Zhou, M., Krasinska, K., Riess, J.W., Neal, J.W., Khatri, P., Park, K.S., Butte, A.J., Sage, J., 2013. A drug repositioning approach identifies tricyclic antidepressants as inhibitors of small cell lung cancer and other neuroendocrine tumors. Cancer Disco. 3 (12), 1364–1377. http://dx.doi.org/10.1158/2159-8290.cd-13-0183.

James, A.M., Smith, R.A., Murphy, M.P., 2004. Antioxidant and prooxidant properties of mitochondrial Coenzyme Q. Arch. Biochem. Biophys. 423 (1), 47–56.

Kawamukai, M., 2015. Biosynthesis of coenzyme Q in eukaryotes. Biosci. Biotechnol. Biochem. 80 (1), 23–33. http://dx.doi.org/10.1080/09168451.2015.1065172.

Kolla, N., Wei, Z., Richardson, J.S., Li, X.M., 2005. Amitriptyline and fluoxetine protect PC12 cells from cell death induced by hydrogen peroxide. J. Psychiatry Neurosci. 30 (3), 196–201.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25 (4), 402–408. http://dx.doi.org/10.1006/meth.2001.1262.

Lohman, D.C., Forouhar, F., Beebe, E.T., Stefely, M.S., Minogue, C.E., Ulbrich, A., Stefely, J.A., Sukumar, S., Luna-Sanchez, M., Jochem, A., Lew, S., Seetharaman, J., Xiao, R., Wang, H., Westphall, M.S., Wrobel, R.L., Everett, J.K., Mitchell, J.C., Lopez, L.C., Coon, J.J., Tong, L., Pagliarini, D.J., 2014. Mitochondrial COQ9 is a lipid-binding protein that associates with COQ7 to enable coenzyme Q biosynthesis. Proc. Natl. Acad. Sci. USA 111 (44), E4697–E4705. http://dx.doi.org/10.1073/pnas.1413128111.

Moreno-Fernandez, A.M., Cordero, M.D., de Miguel, M., Delgado-Rufino, M.D., Sanchez-Alcazar, J.A., Navas, P., 2008. Cytotoxic effects of amitriptyline in human fibroblasts. Toxicology 243 (1 2), 51–58. http://dx.doi.org/10.1016/j.tox.2007.09.021.

Moreno-Fernandez, A.M., Cordero, M.D., Garrido-Maraver, J., Alcocer-Gomez, E., Casas-Barquero, N., Carmona-Lopez, M.I., Sanchez-Alcazar, J.A., de Miguel, M., 2012. Oral treatment with amitriptyline induces coenzyme Q deficiency and oxidative stress in psychiatric patients. J. Psychiatr. Res. 46 (3), 341–345. http://dx.doi.org/10.1016/j.jpsychires.2011.11.002.

Nguyen, T.P., Casarin, A., Desbats, M.A., Doimo, M., Trevisson, E., Santos-Ocana, C., Navas, P., Clarke, C.F., Salviati, L., 2014. Molecular characterization of the human COQ5 C-methyltransferase in coenzyme Q10 biosynthesis. Biochim. Biophys. Acta 1841 (11), 1628–1638. http://dx.doi.org/10.1016/j.bbalip.2014.08.007.

Papucci, L., Schiavone, N., Witort, E., Donnini, M., Lapucci, A., Tempestini, A., Formigli, L., Zecchi-Orlandini, S., Orlandini, G., Carella, G., Brancato, R., Capaccioli, S., 2003. Coenzyme q10 prevents apoptosis by inhibiting mitochondrial depolarization independently of its free radical scavenging property. J. Biol. Chem. 278 (30), 28220–28228. http://dx.doi.org/10.1074/jbc.M302297200.

Pathania, D., Sechi, M., Palomba, M., Sanna, V., Berrettini, F., Sias, A., Taheri, L., Neamati, N., 2014. Design and discovery of novel quinazolinedione-based redox modulators as therapies for pancreatic cancer. Biochim. Biophys. Acta 1840 (1), 332–343. http://dx.doi.org/10.1016/j.bbagen.2013.08.005.

Pelicano, H., Carney, D., Huang, P., 2004. ROS stress in cancer cells and therapeutic implications. Drug Resist. Updat. 7 (2), 97–110. http://dx.doi.org/10.1016/j.drup.2004.01.004.

Pula, G., Pistilli, A., Montagnoli, C., Stabile, A.M., Rambotti, M.G., Rende, M., 2013. The tricyclic antidepressant amitriptyline is cytotoxic to HTB114 human leiomyosarcoma and induces p75(NTR)-dependent apoptosis. Anticancer Drugs 24 (9), 899–910. http://dx.doi.org/10.1097/CAD.0b013e328364312f.

Quinlan, C.L., Perevoshchikova, I.V., Hey-Mogensen, M., Orr, A.L., Brand, M.D., 2013. Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. Redox Biol. 1, 304–312. http://dx.doi.org/10.1016/j.redox.2013.04.005.

Rodriguez-Hernandez, A., Cordero, M.D., Salviati, L., Artuch, R., Pineda, M., Briones, P., Gomez Izquierdo, L., Cotan, D., Navas, P., Sanchez-Alcazar, J.A., 2009. Coenzyme Q deficiency triggers mitochondria degradation by mitophagy. Autophagy 5 (1), 19–32.

Salviati, L., Trevisson, E., Rodriguez Hernandez, M.A., Casarin, A., Pertegato, V., Doimo, M., Cassina, M., Agosto, C., Desbats, M.A., Sartori, G., Sacconi, S., Memo, L., Zuffardi, O., Artuch, R., Quinzii, C., Dimauro, S., Hirano, M., Santos-Ocana, C., Navas, P., 2012. Haploinsufficiency of COQ4 causes coenzyme Q10 deficiency. J. Med. Genet. 49 (3), 187–191. http://dx.doi.org/10.1136/jmedgenet-2011-100394.

Santos-Ocana, C., Do, T.Q., Padilla, S., Navas, P., Clarke, C.F., 2002. Uptake of exogenous coenzyme Q and transport to mitochondria is required for bc1 complex stability in yeast coq mutants. J. Biol. Chem. 277 (13), 10973–10981. http://dx.doi.org/10.1074/jbc.M112222200.

Szatrowski, T.P., Nathan, C.F., 1991. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res. 51 (3), 794–798.

Tomasetti, M., Santarelli, L., Alleva, R., Dong, L.F., Neuzil, J., 2015. Redox-active and redox-silent compounds: synergistic therapeutics in cancer. Curr. Med. Chem. 22 (5), 552–568.

Trachootham, D., Alexandre, J., Huang, P., 2009. Targeting cancer cells by -mediated mechanisms: a radical therapeutic approach?. Nat. Rev. Drug Disco. 8 (7), 579–591. http://dx.doi.org/10.1038/nrd2803.

Tran, U.C., Clarke, C.F., 2007. Endogenous synthesis of coenzyme Q in eukaryotes. Mitochondrion 7, Suppl:S62–Suppl:S71. http://dx.doi.org/10.1016/j.mito.2007.03.007.

Turunen, M., Olsson, J., Dallner, G., 2004. Metabolism and function of coenzyme Q. Biochim. Biophys. Acta 1660 (1 2), 171–199.

Villanueva-Paz, M., Cordero, M.D., Pavon, A.D., Vega, B.C., Cotan, D., De, M., la Mata, Oropesa-Avila, M., Alcocer-Gomez, E., de Lavera, I., Garrido-Maraver, J., Carrascosa, J., Zaderenko, A.P., Muntane, J., de Miguel, M., Sanchez-Alcazar, J.A., 2016. Amitriptyline induces mitophagy that precedes apoptosis in human HepG2 cells. Genes Cancer 7 (7 8), 260–277. http://dx.doi.org/10.18632/genesandcancer.114.

Weinberg, S.E., Chandel, N.S., 2015. Targeting mitochondria metabolism for cancer therapy. Nat. Chem. Biol. 11 (1), 9–15. http://dx.doi.org/10.1038/nchembio.1712.

Yuan, S.Y., Cheng, C.L., Ho, H.C., Wang, S.S., Chiu, K.Y., Su, C.K., Ou, Y.C., Lin, C.C., 2015. Nortriptyline induces mitochondria and death receptor-mediated apoptosis in bladder cancer cells and inhibits bladder tumor growth in vivo. Eur. J. Pharm. 761, 309–320. http://dx.doi.org/10.1016/j.ejphar.2015.06.007.

Zhang, L., Wang, K., Lei, Y., Li, Q., Nice, E.C., Huang, C., 2015. Redox signaling: potential arbitrator of autophagy and apoptosis in therapeutic response. Free Radic. Biol. Med 89, 452–465. http://dx.doi.org/10.1016/j.freeradbiomed.2015.08.030.

Zhang, Z., Du, X., Zhao, C., Cao, B., Zhao, Y., Mao, X., 2013. The antidepressant amitriptyline shows potent therapeutic activity against multiple myeloma. Anticancer Drugs 24 (8), 792–798. http://dx.doi.org/10.1097/CAD.0b013e3283628c21.