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Chapter 6

Inhibiting Lactate Dehydrogenase A Enhances the Cytotoxicity of the Mitochondria Accumulating Antioxidant, Mitoquinone, in Melanoma Cells

Ali A. Alshamrani, James L. Franklin,
Aaron M. Beedle and Mandi M. Murph

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Abstract

Limited options exist for inhibitors targeted against melanoma tumors with mutation subtypes other than BRAF. We investigated the cytotoxic activity of mitoquinone (MitoQ), an antioxidant and ubiquinone derivative, on various human melanoma cell lines, alone or in combination with other agents to perturb cellular bioenergetics. This lipophilic cation crosses the cell membrane, enters and accumulates in the mitochondria where it can disrupt mitochondrial function at micromolar concentrations or act as an antioxidant to preserve membrane integrity at nanomolar concentrations. Consistent with previous studies, cells treated with 12.5 μM MitoQ show significantly reduced viability versus control treatments. Although all melanoma cells were susceptible to cytotoxicity induced by MitoQ, cells with wild-type BRAF were responsive to lower doses, compared to cells with activating mutations in BRAF. Mechanistically, the positively charged lipophilic moiety of the MitoQ induced a dose-dependent collapse of the mitochondrial membrane potential ($\Delta\psi m$) and significantly reduced the mitochondrial ATP production and reduced oxygen consumption rate, suggesting mitochondrial dysfunction. We also combined MitoQ with a glycolytic lactate dehydrogenase A inhibitor (FX-11) and observed an enhanced reduction in viability, but not other therapies examined. To summarize, the data suggest that FX-11 enhances the cytotoxic effects of MitoQ in cells with wild-type BRAF.

Keywords: MitoQ, BRAF, dTPP, melanoma, cytotoxicity

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1. Introduction

Mitoquinone (MitoQ) is a synthetic compound and functional antioxidant that enters the mitochondria and accumulates there. Low doses thwart lipid peroxidation, whereas doses above 1 μM can disrupt mitochondria membrane integrity [1, 2]. MitoQ has a ubiquinone moiety covalently connected through a 10-carbon alkyl chain to a lipophilic cation triphenylphosphonium (TPP+) moiety [3, 4]. Recently, this TPP+ moiety has also been shown to inhibit the mitochondrial electron transport chain and induce mitochondrial proton leak [5].

However, additional molecular mechanisms by which these lipophilic cations induce antitumorigenic effects likely exist. Previously, such mitochondria-targeted lipophilic cations displayed cytotoxic activity against hepatocellular carcinoma and breast cancer using cell culture and/or animal models of malignancy [6–8]. Unfortunately, controversy surrounds whether MitoQ can be utilized to prevent age-associated diseases, since some clinical trials showed a lack of efficacy in models outside of cancer [9, 10].

The mitochondria are the cell's powerhouse, responsible for the production of adenosine triphosphate (ATP), the energy required by the cell, utilizing a process called oxidative phosphorylation. Although mechanisms of aerobic cellular respiration are far more efficient in the production of ATP, many tumorigenic cells curiously switch to anaerobic metabolism (glycolysis) during malignant transformation, despite the presence of oxygen, which can be referred to as the "Warburg effect" [11]. This abnormal reprogramming of energy metabolism is therefore a hallmark of cancer [12]. However, not all cancer cells utilize glycolysis, which provides far less ATP, but at a much faster rate. At least prostate and breast cancers, as well as leukemias, likely require oxidative phosphorylation [13].

Intriguingly, studies also suggest that melanoma cells are dependent upon oxidative phosphorylation and show significantly more oxygen consumption than their normal counterparts, the melanocytes [13]. Alternatively, other studies suggest that melanoma cells may vacillate between utilizing either oxidative phosphorylation or glycolysis, depending on the environmental conditions [14]. Since cells found within tumors are highly heterogenic, it is likely that both conditions could be found at different locations when sampling the same tumor specimen.

Malignant cells reprogram or vacillate their cellular metabolism to meet the anabolic requirements for growth and proliferation while also sustaining their survival and viability amid harsh microenvironments with limited nutrients [15]. Among melanoma cells, this bioenergetic switch has been suggested to be a direct consequence of an oncogenic activating mutation in BRAF [13]. This further insinuates that melanomas expressing wild-type BRAF versus mutant BRAF proteins would respond differently to compounds that target the mitochondria. Since 2011, the armamentarium has grown tremendously for small molecule inhibitors targeting BRAF melanomas, including vemurafenib, cobimetinib, dabrafenib, and trametinib, but there is a lack of targeted therapeutics for those cancer subtypes without the BRAF mutation.

In this study, we sought to investigate whether MitoQ has cytotoxic activity against human melanoma cell lines, both wild-type and BRAF mutant melanomas, alone or in combination

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with other agents to perturb cellular bioenergetics. We observed that cells treated with MitoQ have significantly less viability than controls and display enhanced mitochondrial dysfunction due to a decrease in mitochondrial metabolism. Our results also demonstrate that the cytotoxic effect was mediated by the positively charged lipophilic moiety of the MitoQ, since (1-Decyl)triphenylphosphonium bromide (dTPP) recapitulated the reduction in cell viability. Furthermore, we found that MitoQ displayed lower IC_{50} when combined with the FX-11, a small molecule that inhibits lactate dehydrogenase A, compared to single agent treatment.

2. Materials and methods

2.1. Cell culture

BRAF wild-type (MeWo) and *BRAF* mutant (A375) human melanoma cell lines were originally purchased from the American Type Culture Collection (ATCC®, Manassas, VA). *BRAF* wild-type (SB-2) and *BRAF* mutant (SK-MEL-5) human melanoma cell lines were obtained from The University of Texas MD Anderson Cancer Center (Houston, TX) and the National Cancer Institute NCI/NIH (Frederick, MD), respectively. All cell culture materials were purchased from Life Technologies®, Thermo Fisher Scientific Inc. (Waltham, MA). SB-2 and SK-MEL-5 cells were grown in DMEM while MeWo and A375 cells were grown in Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 5% fetal bovine serum, or without for serum-free medium, and 1% penicillin/streptomycin was used to culture and maintain cell lines (Gibco® and Thermo Fisher Scientific Inc.). Cells were cultured at 37°C in an atmosphere of 95% humidity and 5% CO_2. The medium was changed every 48 h. Cells were maintained for at least three subsequent passages after thawing prior to conducting the experiments to ensure the stability of their physiochemical properties. For the no-glucose media, we used RPMI 1640 deprived of glucose and HEPES buffer (Invitrogen®, Carlsbad, CA) that contained 2 mM L-glutamine and was supplemented with 5% FBS and 1% penicillin/streptomycin. For the high-glucose media, we used no-glucose media (above) supplemented with 25 mM glucose. For the galactose media, we used no-glucose media (above) supplemented with 10 mM galactose. The 100 mM glucose and galactose stock solutions were prepared by dissolving 1.8016 g of glucose or galactose powders into a 50 mL deionized water, volume to 100 mL, and then either sterilized by autoclaving (glucose solution) or filtration (galactose solution) to make it suitable for cell culturing purposes.

2.2. Chemicals

The mitochondrial antioxidant MitoQ was kindly provided by Dr. Michael P. Murphy, Medical Research Council Mitochondrial Biology Unit, Cambridge, United Kingdom, to J.L.F. Chemotherapeutic agents cis-Diamineplatinum(II) dichloride and dacarbazine were purchased from Sigma-Aldrich® (St Louis, MO). The lipophilic cation (1-Decyl)triphenylphosphonium bromide (dTPP) was purchased from Santa Cruz Biotechnology® (Dallas, TX). The LPA1/3 receptor antagonist, Ki16425 was purchased from Selleck® Chemicals (Houston, TX). The autotaxin inhibitors HA-130 and PF-8380, along with the lactate dehydrogenase A inhibitor

FX-11 were purchased from Calbiochem®/EMD Millipore (Billerica, MA). The oxidative stress and apoptosis inducer elesclomol was purchased from ApexBio® Technology LLC (Houston, TX).

2.3. Cell viability assay

MeWo, SB-2, SK-MEL-5, and A375 cells were seeded into standard, flat-bottom, clear 96-well plates at 5000–10,000 cells per well. Twenty-four hours after seeding, cells were maintained in either high glucose or galactose media for 48 h as previously described [16]. For drug treatments, compound stock solutions were prepared in distilled water (MitoQ, dTPP, Ki16425) or dimethyl sulfoxide (DMSO – cisplatin, DTIC, Elesclomol, FX-11, HA-130, and PF-8380), and then added to the wells to give the final drug concentrations (ranging from 0.1 to 200 μM) using different conditioned media where indicated. Cells were then incubated for 24 h and cell viability was measured using the CellTiter-Blue® viability assay Promega (Madison, WI) as previously described [17–20]. For combination experiments, MeWo cells were treated with the IC_{50} of FX-11, HA-130 or PF-8380 in combination with increasing concentrations of the MitoQ (0.8–50 μM) and incubated for 24 h in serum-free medium.

2.4. Mitochondrial toxicity assay

MeWo cells were plated at 5000 cells/well on standard, flat-bottom, clear 96-well plates with a final media volume of 100 μL/well. After 24 h, cells were then maintained in either high glucose or galactose media for 48 h as previously described prior to treatment with different compounds. Cells were then treated with MitoQ at different concentrations ranging from 1 to 200 μM in different conditioned media as specified above. In addition, cells were treated with a positive control toxic compound, digitonin (200 μM) and then both groups were incubated for 3 h at 37°C in an atmosphere of 95% humidity and 5% CO_2. Cellular toxicity profiles were generated using the Mitochondrial ToxGlo™ Assay Promega (Madison, WI) following the manufacturer's protocol. Next, an ATP detection reagent that consists of luciferin, ATPase inhibitors, and thermostable Ultra-Glo™ luciferase was utilized to lyse viable cells and assess their ATP levels. This combination of reagents generates a luminescent signal proportional to the amount of ATP present.

2.5. Oxygen consumption rate assay

MeWo cells were seeded at 15,000 cells/well on standard, flat-bottom, clear 96-well plates, and incubated for 24 h. Cells were treated with increasing concentrations of MitoQ (6.25–100 μM) for 20 min prior to the assessment of cellular respiration using Oxygen Consumption Rate Assay Kit MitoXpress®-Xtra HS Method, Cayman Chemicals (Ann Arbor, MI) following the manufacturer's protocol. The phosphorescent oxygen probe provided by the kit is quenched by oxygen in the extracellular medium. Therefore, the signal intensity obtained using this kit is proportional to the increase in the oxygen consumption rate by cells.

2.6. Assessment of the mitochondrial membrane potential (Δψm)

MeWo cells were plated at 3000 cells/well in standard, flat-bottom, clear 96-well plates, and incubated for 24 h. Cells were washed twice with warm phosphate buffered saline and the nuclei were stained using NucBlue® live cell Hoechst 33342 stain following the manufacturer's protocol. Cells were then washed one time with warm PBS and then incubated in warm live cell imaging solution containing 20 nM tetramethylrhodamine methyl ester (TMRM) dye (Molecular Probes™, Thermo Fisher Scientific) for 30 min in the dark at room temperature prior to the treatment with MitoQ (12.5–100 μM) or left untreated. Fluorescent imaging was performed to visualize nuclear (Hoechst) and mitochondrial (TMRM) staining with DAPI and TRITC filters, respectively, using an X71 inverted fluorescent microscope (Olympus, Center Valley, PA).

2.7. Fluorescence images analysis

MeWo cells were viewed using an Olympus X71 inverted epifluorescent microscope (40× objective) with an ND25 neutral density filter and images were captured using a DP-72 camera with identical black balance correction and exposure time in the CellSens Software (Olympus). Fluorescence microscopy experiments were repeated three times and three random pictures per condition per experiment were used to quantify the TMRM dye fluorescence intensity ($n = 3$) using Image-Pro® Insight 8.0 (MediaCybernetics®, Rockville, MD). The TMRM corrected fluorescence intensity was calculated for each image by normalizing the total red fluorescence of each entire 40× image (total TMRM intensity) by the number of cells in the same image (determined by the number of DAPI nuclei counted by manual tag in Image-Pro® Insight) to eliminate the impact of the differences in cell numbers between wells on our interpretation of data. Cells per image ranged from 135 to 270. Average TMRM corrected intensities for each dosing condition were expressed as relative percentage of the fluorescence intensities of untreated cells.

2.8. Statistical analysis

The statistical differences in experimental data were analyzed using analysis of variance (ANOVA) test, followed by either Tukey's or Bonferroni's multiple comparisons tests between groups using GraphPad Prism (La Jolla, CA). Student's *t*-test was used when only two groups are compared. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ indicate the levels of significance.

3. Results

To study the cytotoxic effects of the mitochondria-targeted lipophilic cation MitoQ in melanoma cells, we treated BRAF wild-type melanoma cells, MeWo and SB-2, or melanoma cells with *BRAF* activating mutations, A375 or SK-MEL-5, with increasing concentrations of MitoQ (0.8–50 μM) for 24 h (white bars) or 48 h (red bars). The data suggest that incubation with MitoQ during this period significantly suppresses the viability all cell lines in a dose-depend-

ent manner (**Figure 1A**). Notably, MeWo and SB-2 cells are more sensitive to lower concentrations of MitoQ (0.8–12.5 μM at 24 h; $p < 0.001$), when compared to A375 or SK-MEL-5 cells (**Figure 1B**). We assessed cell viability 24 h posttreatment in MeWo cells with increasing concentrations (0.8–200 μM) of cisplatin, dacarbazine, Ki16425, PF-8380, and HA-130 and elesclomol to evaluate the cytotoxic potency of MitoQ in comparison with other chemotherapeutics (as negative controls) or investigational compounds (**Figure 1C**). MitoQ significantly affected cell viability at lower concentrations (3.1–50 μM) in MeWo cells when compared with other agents ($^{*}p < 0.05$).

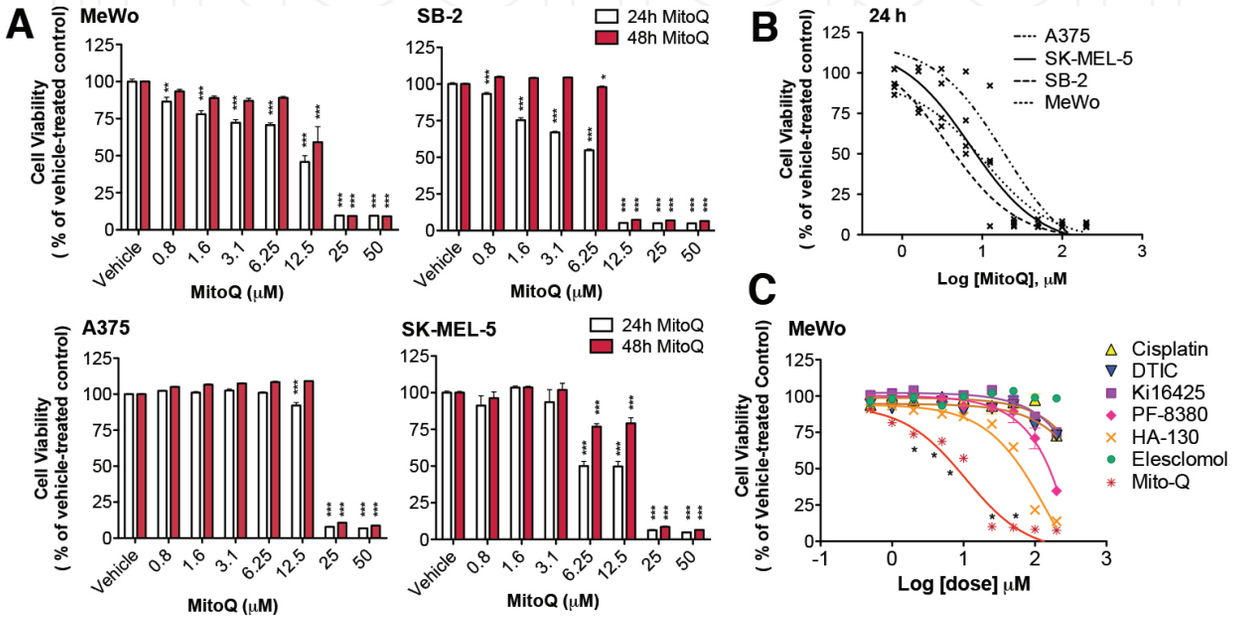


Figure 1. The viability of melanoma cells is significantly impacted after MitoQ treatment. To evaluate the potential cytotoxic effects of MitoQ in melanoma cells, (A) *BRAF* wild-type cells, MeWo and SB-2, or *BRAF* mutant cells, A375 and SK-MEL-5, were treated with increasing concentrations for 24 h (white bars) or 48 h (red bars) prior to determining cell viability. The data are expressed as the percentage of vehicle-treated controls (set at 100%) within each experiment and the mean ± SEM, $n = 3$ per treatment group ($^{**}p < 0.01$; $^{***}p < 0.001$) indicate significant differences between vehicle versus treatment conditions. (B) The 24 h treatment data are also presented in logarithmic scale as a comparison between cell lines. (C) To assess the cytotoxicity of MitoQ in comparison with other approved drugs or investigational compounds, MeWo cells were treated with increasing concentrations (0.8–50 μM) for 24 h prior to the assessment of viability.

Since MeWo cells are more sensitive to MitoQ treatment than A375 or SK-MEL-5 cells, we used MeWo cells to examine whether the MitoQ-induced cytotoxicity of melanoma cells is resultant from dysfunctional mitochondria. For this assay, cells were treated with increasing concentrations (0.8–200 μM) of MitoQ in the presence of high glucose or glucose-deprived/galactose-supplemented medium. Replacing glucose with galactose in the medium is a well-established approach to study the effect of mitochondrial toxins in cancer cells [16, 21–23]. The purpose of this switch is to augment the susceptibility of cells to the MitoQ-mediated mitochondrial toxicity. Indeed, replacing glucose with galactose significantly exacerbates the cytotoxic effects of MitoQ after 24 or 48 h of treatment (**Figure 2A**). As a correlative, we measured the intracellular ATP levels after a 3 h treatment with increasing concentrations of MitoQ. MeWo cells

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cultured in galactose-supplemented medium exhibited significant reduction (***$p < 0.001$) among intracellular ATP levels with MitoQ treatment (**Figure 2B**).

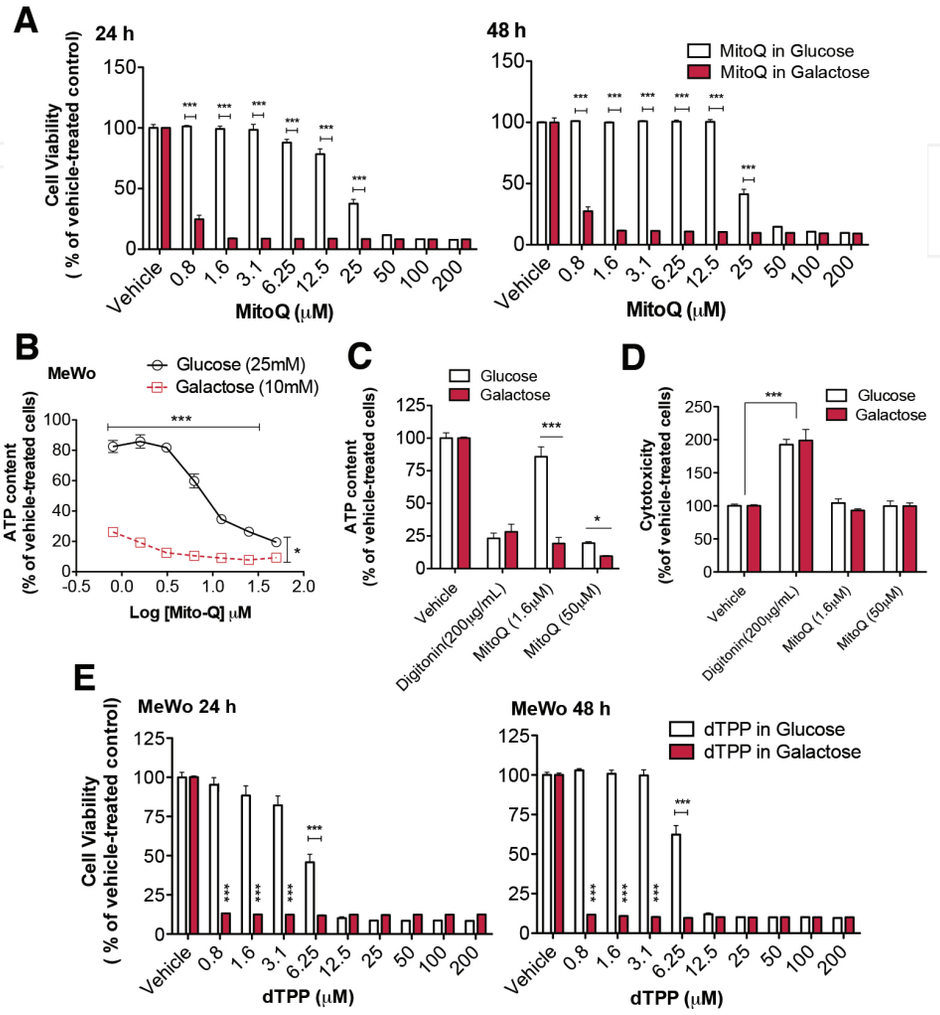


Figure 2. Replacing cell culture medium containing glucose with galactose increases susceptibility to MitoQ-mediated cytotoxicity. To determine whether the MitoQ-induced cytotoxicity is the result of dysfunctional mitochondria, we maintained MeWo cells in high glucose (25 mM) or galactose (10 mM)-supplemented medium for (A) 24 or 48 h prior to MitoQ treatment. Cells cultured in galactose-supplemented media rely on the mitochondria to generate ATP and sustain viability, which make them more suitable to mitochondrial toxicants. (B) ATP levels of MeWo cells were measured using ToxGlo™ Assay after 3 h exposure to increasing concentrations of MitoQ with cells cultured in different medium. (C) Results are also shown as the percentage of vehicle-treated controls (set at 100%) within experiments using the indicated concentrations of MitoQ or digitonin. (D) Plasma membrane cytotoxicity was assessed using the indicated concentrations of MitoQ or digitonin. (E) The viability of MeWo cells was measured in the presence of dTPP with cells cultured in either glucose (black bars) or galactose (red bars) for 24 or 48 h as indicated. Data are expressed as means ± SEM, $n = 3$ per treatment group. *$p < 0.05$ and ***$p < 0.001$ indicate significant differences between groups.

We then assessed the cell membrane integrity using a fluorogenic peptide substrate (bis-AAF-R110) that measures dead-cell protease activity. This peptide cannot cross the intact cell membranes of live cells and, therefore, the fluorescence signal is proportional to the non-live cells with compromised cell membranes. MitoQ treatment did not change cell membrane integrity in conditioned medium, unlike the cytotoxic compound digitonin, which is a

detergent that can dissolve cell membranes, block ATP production, and subsequently cause cell death. Here, the positive control digitonin caused a significant reduction in ATP (**Figure 2C**) and a twofold change in the cell membrane integrity (**Figure 2D**). Taken together, these data suggest that the cytotoxicity mediated via MitoQ potently affects mitochondria; however, it does not indicate the moiety responsible. Thus, we treated cells with dTPP, the positively charged lipophilic cation contained within the structure of MitoQ. Indeed, cells in galactose-containing medium were not viable in the presence of 0.8 μM dTPP at 24 or 48 h (**Figure 2E**), suggesting this component is responsible for the MitoQ-induced cytotoxicity.

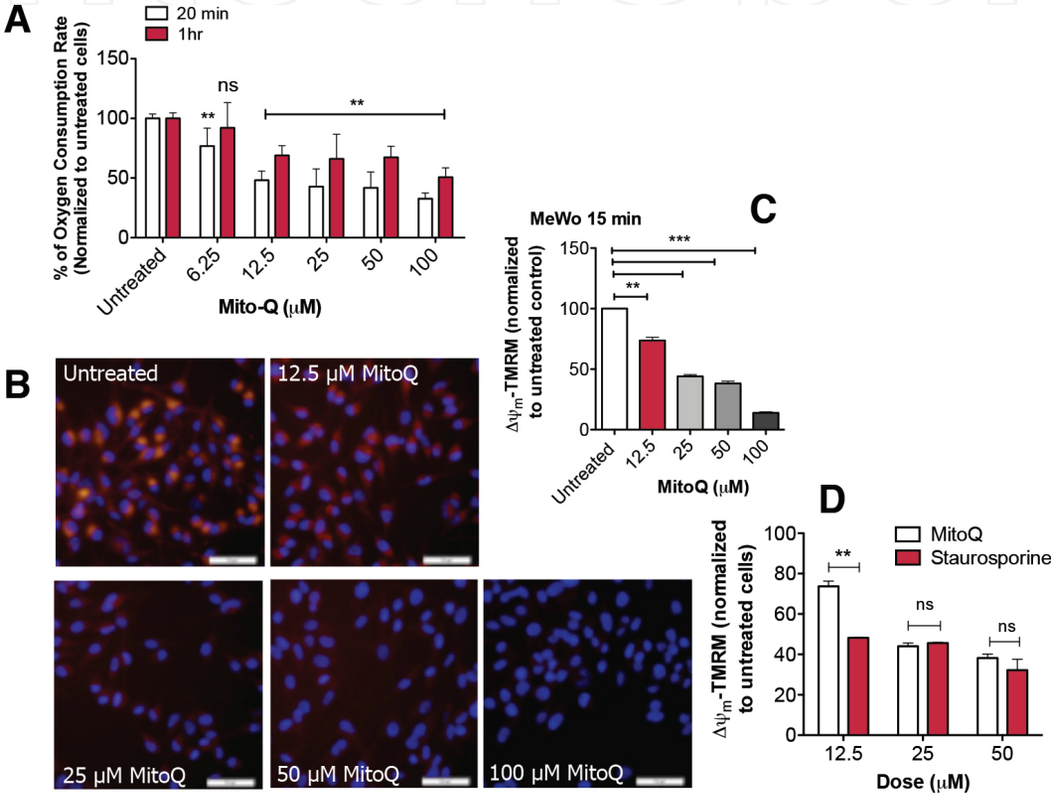


Figure 3. MitoQ induces a dose-dependent reduction in the mitochondrial transmembrane potential in melanoma cells. (A) The oxygen consumption rate was measured in untreated or MeWo cells treated with increasing concentrations of MitoQ for 20 min (white bars) or 1 h (red bars). (B) Representative fluorescence microscopic images of MeWo cells are shown after staining with TMRM (20 nM) and nuclear DAPI stain in the absence or presence of MitoQ (12.5, 25, 50, and 100 μM). (C) The bar graph shows quantification of TMRM signals after incubation for 30 min followed by 15 min treatment with MitoQ. The intensity of TMRM reflects the level of mitochondrial transmembrane potential, which indicate functional respiratory chain complexes. Treating MeWo cells with MitoQ resulted in a significant, dose-dependent reduction in the mitochondrial transmembrane potential, further suggesting mitochondrial dysfunction. (D) The bar graph shows TMRM intensity of MitoQ-treated cells is compared to staurosporine treatments. All data are expressed as mean ± SEM. Scale bar: 50 μm. $^{**}p < 0.01$, $^{***}p < 0.001$ indicate a significant difference between MitoQ treated and untreated cells.

To further confirm this mechanism, we measured the oxygen consumption rate of MeWo cells in response to acute exposure. The data show that MitoQ (20 min to 1 h) causes a significant reduction in the respiratory capacity of the mitochondria (**Figure 3A**). In addition, we assessed the impact of MitoQ on the mitochondrial membrane potential (Δψm) using fluorescent

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TMRM dye, which reflects the level of mitochondrial transmembrane potential—an indication of functional respiratory chain complexes. Data show the dose-dependent (**Figure 3B**) and rapid (15 min) collapse (**Figure 3C**) of the mitochondrial membrane potential ($\Delta\psi m$) in treated MeWo cells. Unlike staurosporine, the potent protein kinase inhibitor that is cytotoxic to mammalian tumor cell lines, which induced an apparent maximal reduction in the $\Delta\psi m$ at different concentrations (12.5–50 μM), MitoQ caused a dose-dependent collapse of the $\Delta\psi m$ (**Figure 3D**). These data show that MitoQ disrupted the mitochondrial respiratory chain and oxidative phosphorylation prior to decreases in cell viability, suggesting that these events lead to the subsequent melanoma cell cytotoxicity.

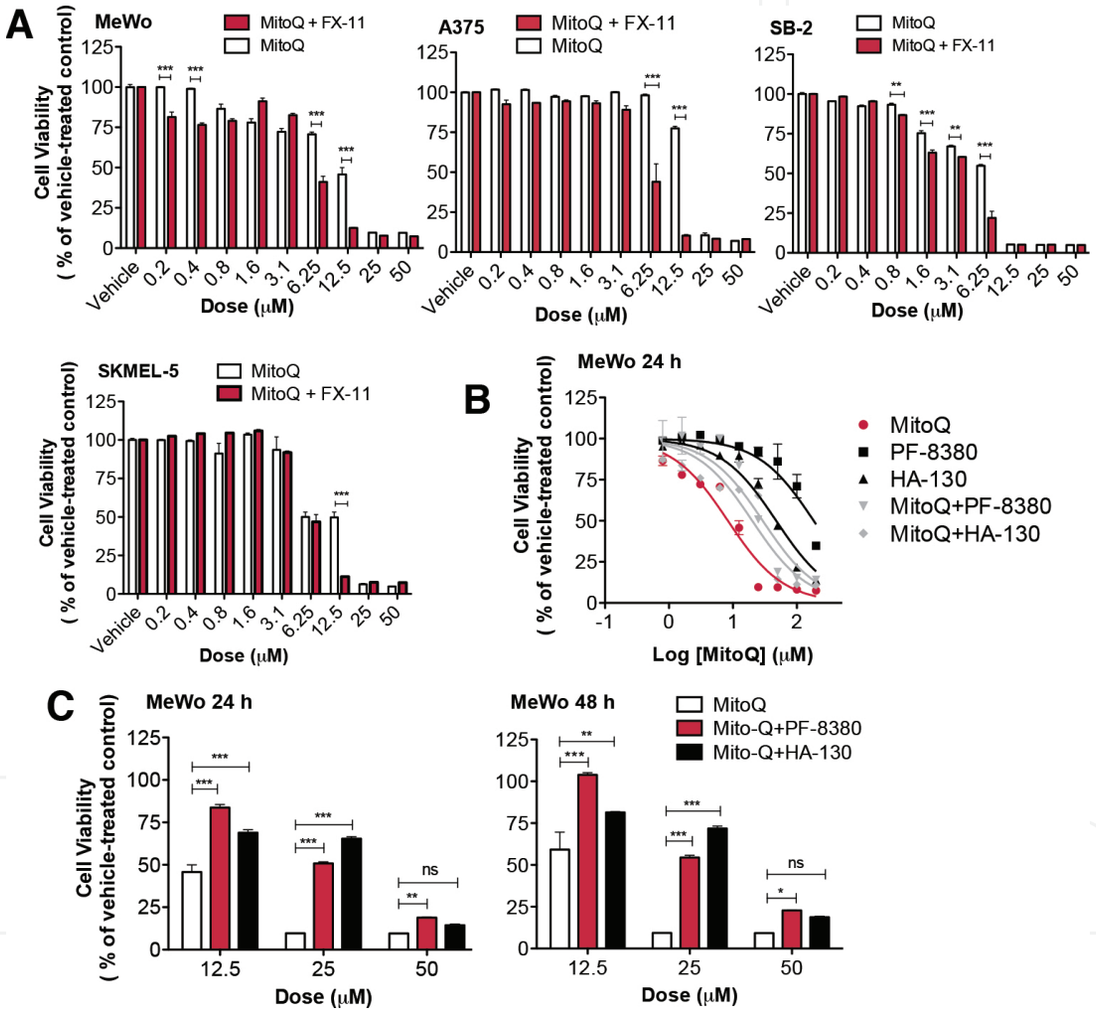


Figure 4. Inhibiting lactate dehydrogenase A enhances the cytotoxicity induced by MitoQ in melanoma cells. (A) MeWo, A375, SB-2, and SK-MEL-5 cells were treated with increasing concentrations of MitoQ for 24 h in the absence (white bars) and presence (red bars) of the lactate dehydrogenase inhibitor (FX-11, 5 μM). (B) Treatment of MeWo cells with 24 h MitoQ in combination with the autotaxin inhibitors, PF-8380 and HA-130 reduces, rather than enhances, the cytotoxic effects of MitoQ. (C) The viability of MeWo cells treated with the highest concentrations (12.5, 25, and 50 μM) of MitoQ alone or in combination with different autotaxin inhibitors for 24 and 48 h are shown. Cell viability is shown as percentage of vehicle-treated controls (set at 100%) within all experiments. Data shown represent the mean ± SEM, $n = 3$ per treatment group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant differences between single and combination therapies.

Since melanoma cells can reprogram their metabolism toward aerobic glycolysis to survive in case of mitochondrial dysfunction, we hypothesized that inhibition of the lactate dehydrogenase A (LDHA) enzyme would force the cells to rely on the mitochondria. Thus, this would increase vulnerability to MitoQ-induced cytotoxicity. Indeed, inhibition of LDHA using FX-11 enhanced the cytotoxic effects of MitoQ among MeWo, A375, SB-2, and SK-MEL-5 cells after 24 h of incubation (**Figure 4A**). Interestingly, the combination of MitoQ with investigational autotaxin inhibitors PF-8380 and HA-130 for 24 h reduced, rather than enhanced, the cytotoxic capabilities of MitoQ (**Figure 4B**). The significant difference among treated groups is clearly demonstrated at 12.5 and 25 μM (**Figure 4C**). The IC_{50} values further reflect the increase in cytotoxicity with combinations between MitoQ and FX-11 against other comparisons (**Table 1**). These data suggest that disruption of the cellular metabolic machinery serves as a potential cytotoxic strategy against melanoma *in vitro* and warrants further investigation *in vivo*.

Cell line	**MitoQ Ave IC_{50} (μM) 24 h**	**MitoQ 95% CI**	**MitoQ + FX-11 Ave IC_{50} (μM) 24 h**	**MitoQ + FX-11 95% CI**
MeWo	8.415	6.826–10.37	4.957	3.559–6.905
SB-2	5.152	2.694–9.856	2.876	2.041–4.052
A375	18.44	8.302–40.96	4.594	2.196–9.615
SK-MEL-5	10.67	4.666–24.39	1.344	0.6846–2.638
Cell line	**MitoQ Ave IC_{50} (μM) 48 h**	**MitoQ 95% CI**	**MitoQ + FX-11 Ave IC_{50} (μM) 48 h**	**MitoQ + FX-11 95% CI**
MeWo	13.08	9.371–18.02	8.009	5.252–12.21
SB-2	11.01	3.683–32.88	6.505	3.194–13.25
A375	21.41	8.534–53.70	5.284	2.359–11.83
SK-MEL-5	26.04	9.498–71.40	7.327	4.251–12.63

Table 1. Cell viability IC_{50} values after 24 or 48 h of treatment with MitoQ and FX-11.

4. Discussion

The data suggest that melanoma cells are susceptible to cytotoxicity mediated by the functional antioxidant, MitoQ, by inducing a dose-dependent reduction in the basal oxygen consumption rate and a rapid depolarization of the mitochondrial membrane potential. Culturing MeWo cells in galactose-supplemented medium significantly reduces intracellular ATP levels in response to MitoQ treatment, compared with culturing in glucose-containing medium. The data show that MitoQ did not affect the plasma membrane integrity, unlike the cell membrane permeabilizing compound, digitonin. Importantly, our study demonstrates that dual disruption of the metabolic machinery enhances the cytotoxicity of MitoQ using FX-11 (**Figure 5**).

The ability of cancer cells, melanoma cells in particular, to reprogram their metabolism has emerged as a major factor that leads to the development of resistance to many existing

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therapeutics [15, 24]. Recent studies have demonstrated that high levels of lactate dehydrogenase (LDH), an enzyme that converts the cytosolic pyruvate into lactate, could be utilized as a predictor of disease progression and chemotherapy response in addition to its involvement in the resistance of different types of cancer cells, including melanoma cells to chemotherapeutic drugs [25, 26]. Results from a recent Phase III clinical trial revealed that metastatic melanoma patients with high serum levels of LDH have shown less favorable responses to elesclomol, a promising first-in-class mitochondria-targeted compound that exerts anticancer activity by inducing oxidative stress and subsequent apoptotic cell death [27].

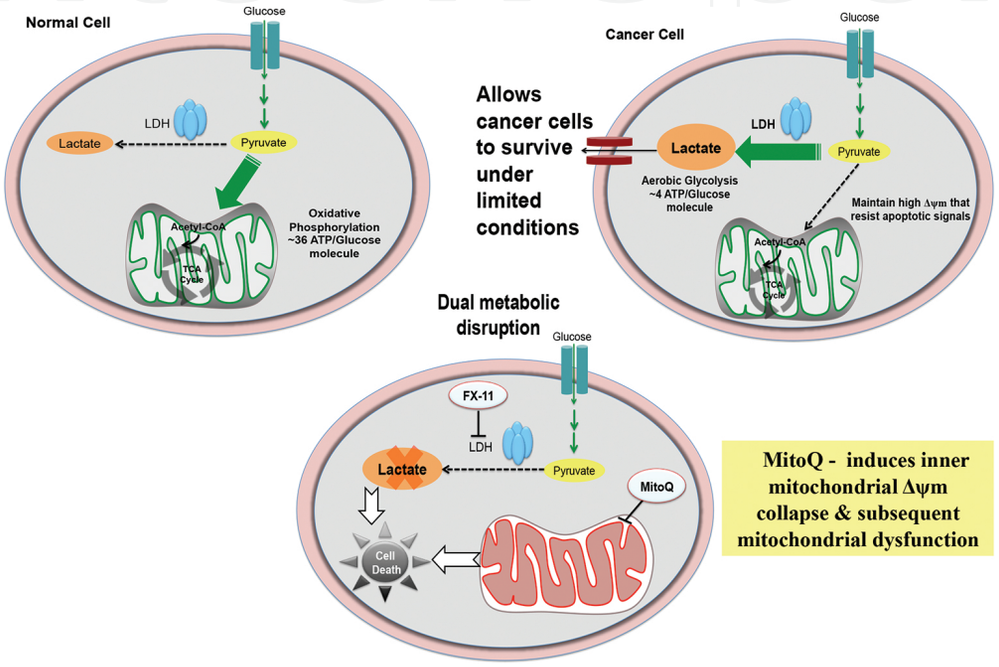


Figure 5. Working model of the observed treatment effects. This schematic illustration represents how targeting lactate metabolism enhances the cytotoxic effects of the mitochondria-targeted lipophilic cation MitoQ in melanoma cells. The normal cell depicted here is generating ATP through mitochondrial oxidative phosphorylation. During malignant transformation, cancer cells tend to strategically reprogram their metabolism toward aerobic glycolysis to produce lactate in order to acidify the surrounding tumor microenvironment and to survive in the harsh and metabolically limiting conditions, which is illustrated here by the cancer cell. In addition, the cancer cell is also maintaining functional mitochondria to resist apoptotic signals. The bottom cell shows our working model with dual disruption of metabolic machinery using a combination of MitoQ and FX-11 to counteract the melanoma cell's viability.

Therefore, we hypothesized that inhibiting cellular aerobic glycolysis would create a synergistic response to the cytotoxic effects of MitoQ, an approach conducted by several studies whereby mitochondria-targeted compounds were used in combination with glycolysis inhibitor, 2-deoxyglucose (2-DG). However, due to the high concentration of 2-DG needed to achieve the desirable synergistic cancer cell growth arrest [7, 8, 28], we were eager to find a more potent and irreversible glycolysis inhibitor that could augment MitoQ's cytotoxicity. Thus, in this study we found that the cytotoxic effects of MitoQ were synergistically enhanced when combined with a subtoxic (5 μM) concentration of FX-11, a selective suppressor of lactate dehydrogenase A. These data suggest that FX-11-treated cells were forced to rely more on

mitochondrial oxidative phosphorylation to survive, which made them more vulnerable to the effects of the lipophilic cation MitoQ.

Recently, Trnka et al. have shown that longer aliphatic chains that link the positively charged triphenylphosphonium with any biologically active compound to target mitochondria inhibited the mitochondrial electron transport chain and induced mitochondrial proton leak [5]. Herein we observed that the MitoQ-induced cytotoxicity was mediated by the lipophilic cation dTPP moiety of MitoQ, rather than the redox cycling of the antioxidant moiety (ubiquinone). If dTPP is more potent than MitoQ, this is suggestive that the ubiquinone moiety may be protecting against the toxic effect of dTPP. Lastly, our results are in agreement with other publications [3, 5] showing the massive mitochondrial accumulation of the lipophilic cation moiety disrupts cellular respiratory capacities and induces cytotoxicity.

Surprisingly, autotaxin inhibitors reduced, rather than increased, the potency of MitoQ. Since autotaxin inhibitors have shown superior activity in melanoma models [18, 20, 29], we hypothesize that this reduction in MitoQ potency could have resulted from the disruption of mitochondrial membrane potential by autotaxin inhibitors. If so, this would affect the integration and accumulation of MitoQ into the mitochondria of melanoma cells and reduce the compound's efficacy. Our observation is in agreement with previous studies in which autotaxin has been reported to protect breast cancer and melanoma cells against Taxol-induced cell death through maintaining their mitochondrial membrane potential [30].

Consistent with previous studies showing that BRAF wild-type cells, including MeWo cells, display enhanced oxidative phosphorylation capabilities and mitochondrial capacity [31], we observed that these cells are more sensitive to MitoQ treatment than A375 cells, which possess an activating BRAF mutation. Therefore, our study is relevant to developing targeted strategies against wild-type BRAF melanomas, which includes the subtypes RAS, NF1, and Triple-WT [32], with the most relevance to Triple-WT. Although the majority of melanoma patients have tumors with activating mutations in BRAF, and thus are candidates for BRAF inhibitors like vemurafenib, trametinib, dabrafenib, and cobimetinib, those patients that have tumors with wild-type BRAF lack a clear strategy for targeted therapy. BRAF status of melanoma cells has been directly linked to cellular metabolism and the bioenergetic switch between mitochondrial oxidative phosphorylation and aerobic glycolysis [13, 15]. Given the ability of MitoQ to accumulate at large concentrations in the mitochondria [3], it is not altogether surprising that MitoQ has a profound effect on the viability of cells with increased mitochondrial respiratory capacities. In summary, more research is needed to investigate molecular vulnerabilities among these subgroups.

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Author details

Ali A. Alshamrani, James L. Franklin, Aaron M. Beedle and Mandi M. Murph*

*Address all correspondence to: mmurph@uga.edu

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA, United States of America

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