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In Vitro Evaluation of Mitochondrial Function and Estrogen Signaling in Cell Lines Exposed to the Antiseptic Cetylpyridinium Chloride

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Background: Quaternary ammonium salts (QUATS), such as cetylpyridinium chloride (CPC) and benzalkonium chloride (BAK), are frequently used in antiseptic formulations, including toothpastes, mouthwashes, lozenges, throat and nasal sprays, and as biocides. Although in a recent ruling, the U.S. Food and Drug Administration (FDA) banned CPC from certain products and requested more data on BAK's efficacy and safety profile, QUATS, in general, and CPC and BAK, in particular, continue to be used in personal health care, food, and pharmaceutical and cleaning industries.

Objectives: We aimed to assess CPC's effects on mitochondrial toxicity and endocrine disruption *in vitro*.

Method: Mitochondrial O_2 consumption and adenosine triphosphate (ATP) synthesis rates of osteosarcoma cybrid cells were measured before and after CPC and BAK treatment. Antiestrogenic effects of the compounds were measured by a luciferase-based assay using recombinant human breast carcinoma cells (VM7Luc4E2, ERalpha-positive).

Results: CPC inhibited both mitochondrial O_2 consumption [half maximal inhibitory concentration (IC_{50}): 3.8 μM] and ATP synthesis (IC_{50}: 0.9 μM), and additional findings supported inhibition of mitochondrial complex 1 as the underlying mechanism for these effects. In addition, CPC showed concentration-dependent antiestrogenic activity half maximal effective concentration [(EC_{50}): 4.5 μM)]. BAK, another antimicrobial QUATS that is structurally similar to CPC, and the pesticide rotenone, a known complex 1 inhibitor, also showed mitochondrial inhibitory and antiestrogenic effects. In all three cases, there was overlap of the antiestrogenic activity with the mitochondrial inhibitory activity.

Conclusions: Mitochondrial inhibition *in vitro* occurred at a CPC concentration that may be relevant to human exposures. The antiestrogenic activity of CPC, BAK, rotenone, and triclosan may be related to their mitochondrial inhibitory activity. Our findings support the need for additional research on the mitochondrial inhibitory and antiestrogenic effects of QUATS, including CPC and BAK. https://doi.org/10.1289/EHP1404

Introduction

Antimicrobial quaternary ammonium salts (QUATS) compounds, such as cetylpyridinium chloride (CPC) and benzalkonium chloride (BAK), have been used in personal care products, such as soaps and body washes, until the recent U.S. Food and Drug Administration (FDA) ruling (FDA 2016), and are currently being used in hand lotions, toothpastes, mouthwashes, nasal sprays, lozenges, deodorants, intravaginal sponges, and in multidose pharmaceutical formulations, such as eye drops (Lang et al. 2013; Tan et al. 2002). The antimicrobial properties of QUATS were first discovered in the 1930s, and since then, they have been widely used as topical antiseptics and disinfectants (Tischer et al. 2012). In general, the QUATS are a group of compounds that contains a positively charged nitrogen atom in their otherwise lipophilic chemical structures, and these structural characteristics (lipophilic cations) make them favorable to be taken up by mitochondria (Murphy and Smith 2007). Some of the QUATS, such as dequalinium chloride (Gamboa-Vujicic et al. 1993) and *n*-decyl trimethylammonium bromide (Inácio et al. 2013), have been previously reported to inhibit mitochondrial oxidative phosphorylation at low concentrations. Although the recent FDA ruling (FDA 2016) revoked the generally recognized as safe (GRAS) status of CPC in certain products and requested more evidence supporting GRAS status for BAK due to concerns about their potential to promote antimicrobial resistance and other potential safety issues, including possible hormonal effects (FDA 2016), their usage in numerous other formulations in health care, food, and pharmaceutical and cleaning industries are being continued.

Mitochondria are the critical cellular organelles responsible for energy generation and cellular homeostasis. In the last decade, mitochondrial dysfunction has emerged as a potential contributing pathologic mechanism for several health problems, including cardiac diseases (Schwarz et al. 2014), diabetes (Szendroedi et al. 2011), obesity (Heinonen et al. 2015), Alzheimer's disease, Parkinson's disease (Yan et al. 2013), and cancer (Rogalinska 2016). Since initial steps of testosterone, progesterone, and estrogen biosynthesis take place in the mitochondrial matrix (Felty and Roy 2005; Ramalho-Santos and Amaral 2013), a disruption in mitochondrial integrity or function could possibly lead to endocrine disruption. In addition, several studies have shown that mitochondrial gene expression (Chen et al. 2009; Sanchez et al. 2015), structure, function (Arnold et al. 2012), and morphology (Hara et al. 2014) can be regulated by estrogen, and mitochondrial effects of estrogen are thought to work through the mitochondrially localized estrogen receptor β (ERβ) (Liao et al. 2015). The mitochondrial regulation of estrogen signaling at the cellular level, however, has not been well studied. Several endocrine disrupting chemicals, such as triclosan (TCS) (Newton et al. 2005; Weatherly et al. 2016), bisphenol A (BPA) (Jiang et al. 2014; Xia et al. 2014) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Aly and Domènech 2009; Chen et al. 2010; Shertzer et al. 2006), have also been shown to cause mitochondrial dysfunction. A mechanism through which mitochondrial dysfunction might cause endocrine disruption is not known.

For the present study, we evaluated the *in vitro* effects of CPC as a representative QUATS compound on mitochondrial function and mitochondrial complex 1, and evaluated its antiestrogenic activity.

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Materials and Methods

Cell Lines and Cell Culture

The Leber's Hereditary Optic Neuropathy (LHON) osteosarcoma cytoplasmic hybrids (cybrids) were kindly gifted from Drs. Valerio Carelli and Andrea Martinuzzi, and the retinal ganglion cell line (RGC-5) was purchased from American Type Culture Collection. The RGC-5 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine and 100 mM of sodium pyruvate (DMEM; Corning) and 10% fetal bovine serum (FBS; Corning). For the osteosarcoma cells, the DMEM was further supplemented with 50 μg/mL uridine (Sigma), and antibiotics (50 units/mL) of penicillin/50 μg/mL of streptomycin (Gibco). The cells were maintained under 5% carbon dioxide at 37°C.

The human breast carcinoma MCF-7-derived VM7Luc4E2, ERalpha-positive cells were grown and maintained in alpha Minimum Essential Medium (Gibco-BRL) containing 10% fetal bovine serum and maintained at 37°C under 5% CO_2 and 85% humidity.

Chemicals and Chemical Library

The Pharmakon collection containing 1,600 FDA-approved/clinically evaluated drugs [10 mM dimethylsulfoxide (DMSO)] was purchased from Microsource Discovery Systems Inc. CPC (Cat# C0732; CAS No. 6,004-24-6) and all other chemicals were purchased from Sigma-Aldrich unless otherwise specified. The adenosine Triphosphate (ATP) Bioluminescence Assay Kit CLS II was purchased from Roche Life Science. The ATP-free Adenosine diphosphate (ADP) was purchased from Cell Technology. BAK (Cat# B6295; CAS No. 63,449-41-2) stock solutions were prepared assuming a molecular weight of 375 as determined by perchloric acid titration by the manufacturer. Rotenone (Cat# 45,656; CAS No. 83-79-4) and DMSO (Cat# D8418; CAS No. 67-68-5) were obtained from Sigma-Aldrich and used as a positive control and vehicle control, respectively.

Mitochondrial Complex I–Driven ATP Synthesis Measurement Assay

Mitochondrial complex 1-driven ATP synthesis assays (mtCIDAS) of vehicle- and CPC-treated LHON mutation (11,778) carrying osteosarcoma cybrid cells were performed as previously described with slight modification (Datta et al. 2016). In the previous high-throughput screen, 2 h of rotenone treatment was done after 22 h of drug treatment; however, in the current study, the cells were treated for 24 h with either vehicle or CPC at the specified concentrations (without any rotenone treatment). Subsequently, the conditioned media was removed, and the cells were permeabilized with streptolysin O. Permeabilized cells were incubated with a buffer containing complex I substrates for 30 min, and the mitochondrial ATP production was measured by using the ATP Bioluminescence Assay Kit CLS II following manufacturer's instruction.

Oxygen Consumption Assay by BD Biosensor Plates

Oxygen consumption was measured after 2 h of incubation with the compounds of interest or controls during the high-throughput screening in RGC-5 cells, as previously described (Sahdeo et al. 2014). Briefly, RGC-5 cells were grown in the media specified above and were aliquoted (70,000 cells, 90 μL media/well) into 384 well oxygen biosensor plates (BD Biosciences) and allowed to equilibrate for 20–30 min. The compounds were diluted in phosphate-buffered saline (PBS) (100 μM). The compounds (10 μL) DMSO or [2-([4-(trifluoromethoxy)phenyl] hydrazinylidene)propanedinitrile (FCCP) were added in their respective wells, and fluorescence was monitored using a POLARstar Omega Plate Reader (BMG Labtech) set at 37°C. Fluorescence was monitored at 0 and 2 h postaddition, and plates were incubated at 37°C under 5% CO_2 between readings. The final concentration of DMSO was 0.1%, and DMSO and FCCP (5 μM) were used as negative and positive controls, respectively (Sahdeo et al. 2014). Each compound was evaluated in triplicate ($n = 3$).

Oxygen Consumption Assay by Clarke Electrode and Seahorse XF24 Flux Analyzer

Mitochondrial O_2 consumption rates of osteosarcoma cybrid cells carrying healthy (control) or 11,778 LHON mutant mitochondrial DNA were measured with a Seahorse XF-24 system (Seahorse Biosciences, currently Agilent Inc.) (Danielson et al. 2002; Tomilov et al. 2014) and an Oxytherm Clark electrode system (Hansatech) (Liu et al. 2009). For oxygen consumption assay by Seahorse XF24 flux analyzer, 50,000 control osteosarcoma cybrids cells in 200-μL medium/well was plated on 24-well plates and incubated overnight. Media was changed to unbuffered DMEM, 20% FBS, 200 mM200 mM Glutomax (Thermofisher Scientific Cat# 35050061), 100 mm sodium pyruvate, 25 mm glucose, and pH 7.4. Cells were pre-equilibrated for 20 min; oxygen consumption rates (OCR) were recorded with Seahorse XF-24 before and after addition of CPC.

Cell-Based ER-Mediated Bioassay

Recombinant human breast carcinoma cells (VM7Luc4E2, ERalpha-positive) were grown and maintained as previously described (Rogers and Denison 2000). These cells contain a stably integrated, ER-responsive firefly luciferase reporter plasmid, pGudLuc7ERE. Cells were maintained in estrogen-stripped media for 5 d before they were plated into white, clear-bottomed 96-well tissue culture dishes at 75,000 cells/well and allowed to attach for 24 h. Cells were then incubated with carrier solvent (DMSO; 1% final solvent concentration), E_2 (17β-estradiol, 1 nM), the indicated concentration of compound (for measurement of agonist activity), or the indicated concentration of compound plus 1nM E_2 (for measurement of antagonist activity) for 24 h at 37°C with triplicate wells per chemical or control. After incubation, cells were rinsed twice with PBS, lysed with Promega cell lysis buffer, and shaken for 20 min at room temperature to allow complete cell lysis. Luciferase activity in each well was measured using an Orion microplate luminometer as previously described (Baston and Denison 2011).

Assessment of Cytotoxicity

Cytotoxicity of CPC- and BAK-treated cells were assessed after 24 h of incubation under a brightfield microscope. The cells were examined for any gross morphological changes (such as rounding or detachment), and the concentrations of CPC and BAK at which cells did not show any gross morphological changes were considered as nontoxic concentrations.

Data Analysis and Statistics

Fluorescence readings representing oxygen consumption were recorded at 2 h post-chemical library treatment, and luminescence readings for ATP content were collected after 24 h of chemical treatment. The fold change from baseline (FCB) was calculated as previously reported (Datta et al. 2016; Sahdeo et al. 2014). Briefly, for oxygen consumption, fluorescence was measured immediately after drug addition (t0) and after 2 h incubation. The FCB was calculated by normalizing postincubation readings to the t0 reading. FCB responses for drug-treated wells were then

normalized to the average FCB for the 16 vehicle-treated wells producing the fold change from vehicle value for each well. For ATP synthesis, cells were drug treated for 22 h and then treated with rotenone (0.1 μM) for 2 h in 96-well plates. The plate median was determined and fold change of ATP synthesis rate over the plate median for each drug-treated well was calculated. The concentration–response curves were generated, and the half maximal inhibitory concentration IC_{50} values were determined by nonlinear regression curve fit analysis using Graphpad Prism 5.0 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

Results

Evaluation of Mitochondrial Function of Cells Exposed to Quaternary Ammonium Salts

We previously reported two high-throughput screens of a library of clinically evaluated additives, disinfectants, and drugs to identify their effects on two of the mitochondrial functional parameters, i.e., mitochondrial O_2 consumption (Sahdeo et al. 2014) and complex 1-driven ATP synthesis (CIDAS) (Datta et al. 2016). Mitochondrial O_2 consumption was measured using BD biosensor plates in RGC-5 cells after 2 h of compound treatment. MtCIDAS was measured in permeabilized compound and rotenone-treated (22 h + 2 h) cybrid cells containing LHON mutation (11,778) using a bioluminescence method. In order to identify the compounds that affect both mitochondrial O_2 consumption and mtCIDAS, we plotted the results of the O_2 consumption screen (Sahdeo et al. 2014) against the mtCIDAS screen (Datta et al. 2016) (Figure 1). Of the 11 compounds in the bottom left quadrant (indicative of simultaneous inhibition of mitochondrial O_2 consumption and mtCIDAS), 6 were found to be QUATS, making QUATS the single largest mitochondrial inhibitory structural class. QUATS are lipophilic cations, which is a structure that has previously been shown to be preferentially taken up into mitochondria (Murphy and Smith 2007). There were 10 QUATS compounds common in both the screens, and 6 out of 10 QUATS compounds showed inhibition of mtCIDAS (24 h) and mitochondrial O_2 consumption (2 h) when tested at 10 μM concentration (Figure 1). The QUATS compounds that did not show mitochondrial inhibition are: clidinium bromide, hexamethonium bromide, tolonium chloride, and cefalonium. In the high-throughput screen, CPC was identified as the most potent of the QUATS tested with regard to mitochondrial O_2 consumption and mtCIDAS (Figure 1).

In Vitro *Effects of CPC on Mitochondrial O_2 Consumption and Complex 1-Driven ATP Synthesis*

Mitochondrial O_2 consumption was inhibited after 30 min of incubation with CPC (IC_{50}: 3.8 μM, Figure 2A). MtCIDAS, which was measured after 24 h (to allow more complete penetration of the chemical into mitochondria) also decreased in a concentration-dependent manner (IC_{50}: 0.9 μM, Figure 2B). QUATS, such as dequalinium chloride (Gamboa-Vujicic et al. 1993) and *n*-decyl-N,N,N-trimethylammonium (C_{10}TAB) (Inácio et al. 2013), are already known to inhibit complex 1 reduced nicotinamide adenine dinucleotide [(NADH)-ubiquinone oxidoreductase]; hence, we hypothesized that CPC might inhibit mitochondrial function by targeting complex 1 (NADH-ubiquinone oxidoreductase) in the mitochondrial electron transport chain. The mitochondrial electron carrier ubiquinone carries electrons deposited at mitochondrial complex 2 (succinate dehydrogenase) to complex 3 (ubiquinol-cytochrome c oxidoreductase), independent of complex 1 inhibition (Nicholls and Ferguson 2013). Therefore, addition of a complex 2 substrate (succinate) subsequent to a complex 1 inhibitor in permeabilized cells should allow mitochondrial O_2 consumption to resume. Hence, rescue of CPC-induced mitochondrial O_2 consumption inhibition by a complex 2 substrate (succinate) suggests that the target of CPC is complex 1. CPC (100 μM) was added to digitonin-permeabilized osteosarcoma cells respiring on complex 1 substrates malate (5 mM) and pyruvate (5 mM). Within 3 min of CPC addition, the mitochondrial O_2 consumption rate decreased from the baseline

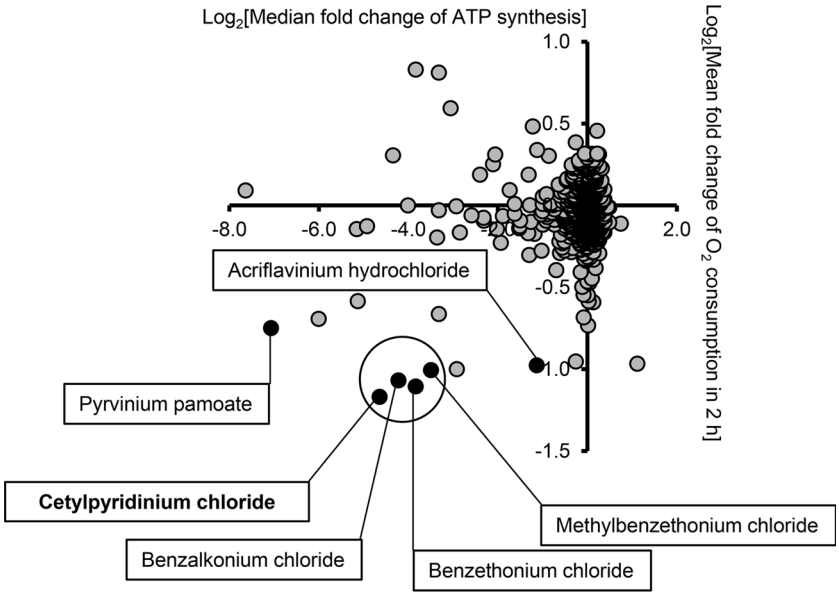

Figure 1. Effects of quaternary ammonium salts on mitochondrial O_2 consumption and complex 1-driven adenosine triphosphate (ATP) synthesis (CIDAS) *in vitro*. We evaluated 1,600 drugs and preservatives from the Pharmakon collection for mitochondrial activity. For CIDAS, 11,778 mutant osteosarcoma cybrids were treated with the drugs (10 μM) for 22 h followed by 2 h incubation with rotenone (0.03 μM). Subsequently, the cells were permeabilized, and mitochondrial CIDAS (mtCIDAS) was measured. For oxygen consumption assay, the RGC-5 cells were incubated with the compounds for 2 h, and the fluorescence was measured. The data are presented as $\log_2$(median fold change of ATP synthesis rate) vs $\log_2$(mean fold change of O_2 consumption in 2 h) from three independent observations. Six quaternary ammonium salts (QUATS) that inhibit mitochondrial O_2 consumption and mtCIDAS are highlighted here.

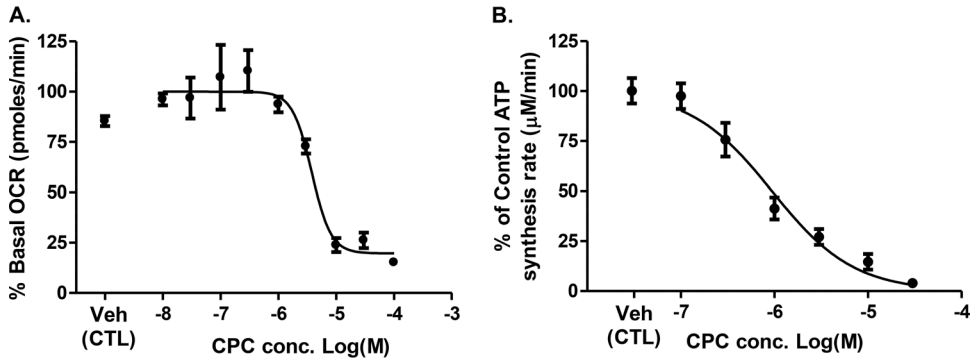

Figure 2. Mitochondriotoxic effects of cetylpyridinium chloride (CPC). (*A*) Inhibition of mitochondrial O_2 consumption by CPC. The osteosarcoma cells were treated with CPC at specified concentrations (0.01–100 μM), and cellular oxygen consumption was measured after 10 min of initial addition and for 4 times total at 10-min intervals. The data are presented as average percentage of basal oxygen consumption rates (OCR) ± standard deviation from three independent observations. (*B*) Inhibition of mitochondrial complex 1-driven adenosine triphosphate (ATP) synthesis (mtCIDAS) by CPC. The osteosarcoma cells were treated with CPC at specified concentrations (0.1–10 μM) for 22 h followed by 2-h incubation with rotenone (0.03 μM). The mtCIDAS was measured in permeabilized cells. Data are presented as average fold change of ATP synthesis rate ± standard deviation from three independent observations. The IC_{50} values for mitochondrial O_2 consumption and mtCIDAS inhibition are 3.8 μM and 0.9 μM, respectively. The IC_{50} values were determined by nonlinear regression curve fit analysis using Graphpad Prism 5.0.

rate of 11.0 nmol/ml/min to 3.5 nmol/ml/min, an ~70% decrease (Figure 3). After addition of succinate (5 mM), the mitochondrial O_2 consumption rate increased to 8.3 nmol/ml/min, which is consistent with a direct effect of CPC on complex 1 that inhibited mitochondrial O_2 consumption.

In Vitro *Effects of CPC and BAK on Estrogenic Signaling*

Mitochondria are essential for proper steroidogenesis (Ramalho-Santos and Amaral 2013). Multiple endocrine disruptors, such as TCS (Weatherly et al. 2016), BPA (Kaur et al. 2014; Lin et al. 2013), and TCDD (Chen et al. 2010), have shown to adversely affect mitochondrial function, suggesting a possible correlation between mitochondrial function and endocrine disruption. We hypothesized that mitochondrial inhibitors might disrupt endocrine signaling, and tested QUATS for antiestrogenic and proestrogenic effects. At its highest nontoxic concentration (10 μM), CPC showed 86% inhibition of estrogen activity in VM7Luc4E2, ERalpha-positive human breast carcinoma cells after 24 h of incubation (Figure 4A). The concentration–response curve for antiestrogenic activity overlapped with the concentration–response curve for mitochondrial O_2 consumption inhibition in osteosarcoma cybrids after 10 min of incubation with CPC (Figure 4A). The similarity in the dose–response curves for the two assays suggests that CPC's antiestrogenic activity might be a consequence of CPC-mediated inhibition of the mitochondrial electron transport chain. In previous studies, triclosan was shown to disrupt mitochondrial function (Weatherly et al. 2016) and inhibit estrogen signaling (EC_{50}: 1 μM) (Ahn et al. 2008), similar to CPC (EC_{50}: 4.5 μM). In addition, we also tested another QUATS, BAK, for its mitoinhibitory and antiestrogenic effect. BAK was a comparatively weaker mitochondrial complex 1 inhibitor and showed weaker antiestrogenic effects (Figure 4B). CPC and BAK did not show any estrogen-stimulating effects (up to 10 μM) in VM7Luc4E2, ERalpha-positive human breast carcinoma cells after 24 h of incubation (Figure 4C).

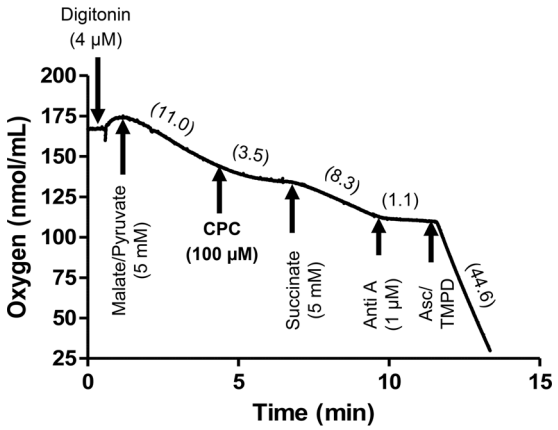

Figure 3. The cetylpyridinium chloride (CPC)–dependent respiration defect was overcome by complex II substrate succinate, but not complex I substrate. Osteosarcoma cybrids were permeabilized with digitonin (4 μM), and respiration was initiated with complex I substrate (malate/pyruvate, 5 mM). Then the cells were treated with CPC (100 μM), followed by the complex II substrate (succinate, 5 mM), a complex III inhibitor, antimycin A (Anti A, 1 μM), and the complex IV substrate ascorbate (Asc, 5 mM) and N,N,N′, N′-tetramethyl-p-phenylenediamine (TMPD) (0.2 mM) mixture. Oxygen consumption rates (OCR, nmol/ml/min) of the cybrids were measured for 1 min after each addition. The OCRs after each addition are indicated in the parentheses from one representative experiment repeated two times. The CPC-dependent respiration defect was overcome by complex II substrate succinate, but not complex I substrate.

Mitochondrial Disruption as Basis of in Vitro *Antiestrogenic Activity: CPC and Rotenone*

From the above data, we hypothesized that the antiestrogenic activity of the QUATS was dependent on their mitochondrial complex 1 inhibitory activity. To test this hypothesis, we evaluated rotenone (0.1 μM), a standard mitochondrial complex 1 inhibitor and a known environmental pollutant, for its antiestrogenic activity. Rotenone (0.1 μM) showed 75% inhibition of estrogen activity in VM7Luc4E2 human breast carcinoma cells after 24 h, and reduced mitochondrial oxygen consumption in osteosarcoma cybrids by 81% after 10 min (Figure 4A). Rotenone does not interfere with estrogen binding to estrogen receptors (Olson and Sheehan 1979); therefore, we hypothesize, as we do for CPC and BAK, that its antiestrogenic effects may be related to mitochondrial inhibition. It seems possible that mitochondrial disruption underlies the inhibition of estrogenic signaling observed in response to CPC, BAK, and rotenone, but further investigation is necessary to fully substantiate this potential mechanism.

Discussion and Conclusion

QUATS, such as CPC and BAK, are antimicrobial agents occur in toothpaste, mouthwash, lozenges, throat and nasal sprays,

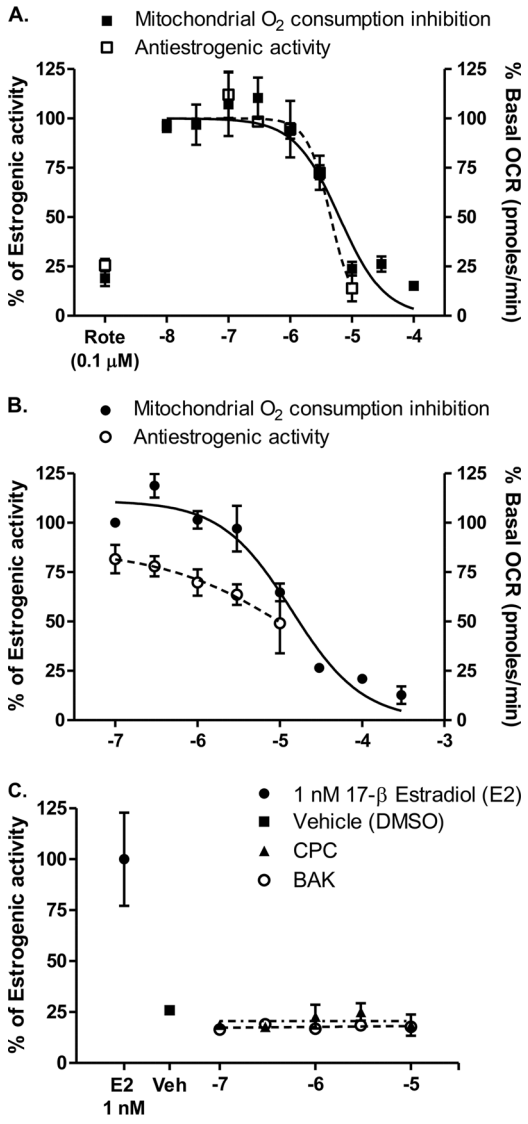

Figure 4. Comparison of cetylpyridinium chloride (CPC)'s and benzalkonium chloride (BAK)'s antiestrogenic activities with mitochondrial O_2 consumption inhibitory activities. The cells (osteosarcoma cybrids for mitochondrial O_2 consumption experiment and VM7Luc4E2, ERα-positive cells for estrogenic activity measurement experiments) were treated with (*A*) CPC and (*B*) BAK at specified concentrations for 10 min for the O_2 consumption experiment and 24 h (in the presence of E_2) for the antiestrogenic effect. (*C*) VM7Luc4E2, ERα-positive cells were treated with CPC or BAK at the specified concentrations or 17β estradiol (E_2, 1 nM) for 24 h, and the estrogenic activity was measured. Rotenone (Rote), a standard complex 1 inhibitor, was used as a positive control at a single concentration (0.1 μM). The IC_{50} values for CPC are 3.8 μM for mitochondrial O_2 consumption inhibition and 4.5 μM for its antiestrogenic activity. The IC_{50} values for BAK are 13.9 μM for mitochondrial O_2 consumption and 17.3 μM for its antiestrogenic activity. The EC_{50} value for estrogenic activity of CPC could not be determined. The solid lines represent the nonlinear regression curve fit for mitochondrial O_2 inhibitory activity, and the dotted lines () represent the nonlinear regression curve fit for estrogenic activity of the compounds. The data are presented as percentage of estrogen activity ± standard deviation and percentage of basal oxygen consumption rates (OCR) ± standard deviation from three independent observations. The IC_{50} values were determined by nonlinear regression curve fit analysis using Graphpad Prism 5.0.

shampoos, hand lotions, creams, eye drops, biocides, intravaginal sponges, consumer antiseptic rubs, and other products that come into contact with epithelial cells. In 2016, the FDA ruled that some ingredients used in consumer antiseptic wash products, including CPC as well as TCS and other antiseptics, were not GRAS or generally recognized as effective (GRAE), and that wash products including these ingredients could not be sold after September 2017 (FDA 2016). Additional data on BAK are being requested by the FDA to establish its GRAS/GRAE status. However, CPC, BAK, and other antiseptics continue to be used in other products in consumer first-aid, food, personal hygiene product, and cleaning industries.

We performed a high-throughput screen of 1,600 antiseptics, additives, and drugs, and found that, of the numerous structural classes of compounds included in the screen, the QUATS were the most mitochondrially toxic class, both in terms of inhibition of ATP synthesis and mitochondrial O_2 consumption. QUATS have the structure of lipophilic cations, which are known to be preferentially taken up by mitochondria (Murphy and Smith 2007). Lipophilic cations, including tetramethylrhodamine methyl ester (Floryk and Houštêk 1999), 1-methyl-4-phenylpyridinium (Davey et al. 1992), and triphenylphosphonium ions (Ross et al. 2005) are known to dose-dependently accumulate in the mitochondrial matrix. Six out of ten QUATS showed inhibition of mitochondrial O_2 consumption as well as mtCIDAS. This indicates a possible structure activity relationship in context to the mitochondrial effects of QUATS. A detailed comparison of the antimicrobial efficacy and mitochondrial inhibitory effects of the QUATS needs to be performed in order to identify the QUATS with high antimicrobial efficacy with minimum mitochondrial effects. Among the mitochondrial inhibitory QUATS, CPC was the most potent in the preliminary screen and was used as the representative of the mitochondrial inhibitory QUATS for further studies. Mechanistic investigation of CPC, the representative QUATS, established that CPC inhibits mitochondrial complex 1 (Figure 3) and therefore impedes mitochondrial O_2 consumption and CIDAS in a concentration-dependent manner. Furthermore, we recently demonstrated that the QUATS BAK is also a mitochondrial complex 1 inhibitor (Datta et al. 2017). Although antimicrobial agents, including antibiotics and antiseptics, are generally conceived as nontoxic to human mitochondria, in a recent study, antibiotic-induced functional impairment of host mitochondria have shown to cause serious adverse effects in eukaryotic model systems (Moullan et al. 2015). In addition, induction of mitochondrial dysfunction has been proposed as potential mechanisms for adverse effects observed in clinical settings during therapeutic use of antibiotics (Kalghatgi et al. 2013) and antiretrovirals (Kohler and Lewis 2007).

We hypothesize that CPC's antiestrogenic effects may be mediated through effects on mitochondrial complex 1 inhibition. Rotenone, an established mitochondrial complex 1 inhibitor (Heinz et al. 2017), was used as a positive control and also showed antiestrogenic effects at the single dose tested. However, additional research is needed to confirm whether effects on mitochondrial function may contribute to effects on estrogen signaling.

Although CPC continues to be used in mouthwash, toothpaste, lozenges, throat sprays, and nasal sprays, and BAK is used in intravaginal spermicidal sponges, body and hand washes, and eyedrops, pharmacokinetic studies on these QUATS are scarce, and tissue-level exposures resulting from the use of personal care products that contain these compounds are unknown. A pharmacokinetic study performed in rats indicated that BAK is absorbed after single oral administration and distributed in tissues at low micromolar concentrations (0.13–26.7 μM) (Xue et al. 2004). In the same study, aspiration of BAK through the lungs markedly increased absorption and tissue distribution of BAK (Xue et al. 2004). This is particularly important in the context of BAK and other QUATS exposure. BAK is often used as a household

disinfectant and biocide in the form of sprays and aerosols. From the above-mentioned study, it seems that inhalation of aerosols containing BAK could be a potential route for tissue exposure. According to the report by European Union's Scientific Committee on Consumer Safety (SCCS 2015), predicted aggregate absorption of CPC through various cosmetic formulations, such as mouth rinse, toothpaste, denture adhesive, denture cleaner, body lotion, face cream, hand cream, and deodorant spray, for adult humans is approximately 0.021–0.08 mg/kg body weight/day, with an estimated/day, with an estimated absorption of 50% from oral administration and 10% from dermal administration. Due to the scarcity of pharmacokinetic studies on long-term or short-term exposure of CPC and BAK by various routes, exact determination of physiologically relevant tissue concentrations of these compounds is not possible at this time.

A recent study demonstrated that exposure to QUATS mixture, including benzalkonium chloride, caused reproductive toxicity and reduced fertility in mice (Melin et al. 2014; Melin et al. 2016). It is our hypothesis that inhibition of mitochondrial function and subsequent disruption of estrogenic signaling is possibly the mechanistic basis of the toxicity observed in these studies. Further mechanistic investigation is needed to conclusively prove if that is indeed true.

In summary, our findings suggest that the QUATS CPC and BAK, which are used as disinfectants in consumer products, inhibit mitochondrial complex 1 and show antiestrogenic activity *in vitro* at low (micromolar) concentrations that may be physiologically relevant. We hypothesize a mechanistic relationship between these outcomes, whereby the antiestrogenic activity of these compounds is mediated by mitochondrial inhibition. Our observations that some QUATS do not inhibit mitochondria suggest structure-activity relationship (SAR) to explore antimicrobial QUATS without antimitochondrial activity. Overall, our findings strongly support the need for further investigation of the underlying mechanisms and potential consequences of chronic exposure to CPC and BAK in consumer products.

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