


Cetylpyridinium chloride inhibits human breast tumor cells growth in a no-selective way

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Abstract

Objective: Analyze the antitumor capacity of cetylpyridinium chloride (CPC) on human breast tumor cells, and the possible action mechanism.

Material and methods: The human breast tumor cells MCF-7 and no-tumor breast cells MCF-10A were exposed to CPC under various condition (concentration and duration). Cell viability was measured with MTT assay, the LIVE/DEAD assay, and fluorescence microscopy. Membrane permeability after CPC exposure was evaluated by Calcein AM assay, mitochondrial morphology with a MitoView staining, and genotoxicity with the comet assay and fluorescence microscopy.

Results: CPC was cytotoxic to both MCF-7 and MCF-10A as of a 24-h exposure to 0.1 μM . Cytotoxicity was dose-dependent and reached 91% for MCF-7 and 78% for MCF-10A after a 24-h exposure to 100 μM CPC, which outperformed the positive control doxorubicin in effectiveness and selectivity. The LD50 of CPC on was 6 μM for MCF-7 and 8 μM for MCF-10A, yielding a selectivity index of 1.41. A time response analysis revealed 64% dead cells after only 5 min of exposure to 100 μM CPC. With respect to the action mechanisms, the comet assay did not reveal genome fragmentation. On the other hand, membrane damage was dose-dependent and may also affect mitochondrial morphology.

Conclusion: Cetylpyridinium chloride inhibits MCF-7 cell growing in a non-selective way as of 5 min of exposure. The action mechanism of CPC on tumor cells involves cell membrane damage without change neither mitochondrial morphology nor genotoxicity.

Keywords

Antitumor activity, cetylpyridinium chloride, human breast cancer, chemotherapy, quaternary ammonium salts, LD50 assay

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Introduction

Breast cancer constitutes the main type of cancer in women worldwide; about 1/9 women in the world will suffer breast cancer during their life time. Although the mortality from breast cancer has dropped in the United States and Europe, it is still among the main diseases associated to high rates of women mortality in USA.^{1,2} Breast cancer is typically diagnosed by screening or because of detection of a symptom (palpable mass) leading to a clinical test.³ A detailed diagnostic test is achieved by using the “triple test”: breast exploration, mammography, and histological test. The employment of this protocol allows confirming the

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diagnostic of breast cancer in 95% of cases.⁴ Young healthy woman tend to have smaller, non-metastasized tumors that can be surgically removed with breast conservation.⁵ After diagnosis, surgery constitutes the first alternative of treatment, followed of radiation, or chemotherapy; depending of the stage and localization of the tumor.⁶ Over the last decades, medical developments have shown that breast cancer is a huge challenge to overcome. The employment of cytotoxic chemotherapy in advanced and early-stage breast cancer has made important progress in the last 10 years.⁷ However, antitumor agents like doxorubicin, cisplatin, and docetaxel have adverse side effects due to cytotoxicity to non-malignant tissues.^{8–10} This is an important reason for breast cancer patients to stop chemotherapy.

1-hexadecylpyridinium (Cetylpyridinium) chloride is a cationic surfactant composed by quaternary nitrogen bind to hydrophobic side chains.^{11,12} It is employed to remove emulsified oil from waste water and their bactericidal potential is associated with the side chain and is more effective when the alkyl chain contains more than 12 carbon atoms.¹³ Recently, it was shown that CPC induce AMP-activated kinase and exhibited antitumor activity on glioblastoma cells.¹⁴ Although CPC has been consumed through mouthwashes for a long time, its antitumor and cytotoxic potential has not yet been extensively studied. Therefore, we studied the antitumor activity of CPC and verified whether the action mechanism involved genotoxicity or damage to membranes and/or mitochondria.

Material and methods

CPC preparation and dilutions

CPC (Sigma-Aldrich Corporation, St. Louis, MO, USA) was freshly pass into solution in sterile ultra-pure water, obtaining a concentrated suspension of 1 mM and it was stored at room temperature. Before each experiment, the CPC stock was diluted and it has pH 7.9 in all concentrations analyzed.

Cell culture and drug exposure

The MCF-7 cell line (ATCC; HTB-22; Rockville, MD, USA) and the non-tumorigenic cell line MCF-10A (ATCC; CRL-10317; Rockville, MD, USA) were cultured as previously reported.¹⁵ Briefly, the cells were grown in DMEM culture media added with 10% fetal bovine serum (Gibco-Invitrogen, Carlsbad, CA, USA), and antibiotics at 37°C with 5% CO₂. MCF-7 or MCF-10A cells were seeded in 96-well plates (1 × 10⁵ cells in 100 μL DMEM per well) and were incubated at 37°C with 5% CO₂ for 24 h to let form a monolayer on the bottom of well. After that, the culture media is changed by one including the testing drug; CPC (concentration range, 0.1–100 μM; concentrations as indicated in results) and incubated at 37°C, 5% CO₂ for different time periods (5 min. or 1, 2, 4, and 24 h), as

previously described.^{15,16} Doxorubicin (DOX; Doxolem, Teva Lab, Madrid, Spain) at 500 μM was employed as cytotoxicity control, while a drug-free culture served as a growth control.

MTT assay, LD50 assay, and time response analysis

To assess cell viability, MTT test (Biotium, Hayward, CA, USA) was used to measure CPC antitumor potential. OD₅₇₀ were determined to obtain the reduced MTT after CPC treatment. The average of triplicates were showed as cell viability percentage.

Following the above methodology, the LD50 of CPC on MCF-7 and MCF-10A cells was obtained by MTT cell viability assays after incubation with CPC (range: 1–10 μM; duration 24 h). The mean of three independent experiments was obtained to assess the veracity of results.

For time response analysis, MTT test was employed to measure cell viability/mortality rate of cells 24 h-exposed to 100 μM CPC or 500 μM DOX (positive control) for 5, 60, 120, and 240 min. Drug-free cells served as growth control. The experiment was performed in triplicate and average of data was showed as percentage of cell viability.

LIVE/DEAD assay

Cell viability and morphology of MCF-7 cells were analyzed after drug exposure using the LIVE/DEAD assay (Biotium Hayward, CA, USA) and fluorescence microscopy (FLM).^{17,18} After a 24-h drug exposure (0, 10, and 100 μM CPC, 500 μM DOX) and a triple wash with PBS (pH 7.4), cells were incubated in the dark for 0.5 h at 37°C with 100 μL live/dead staining suspension. Finally, cells were observed with an EVOS Cell Imaging System (Thermo Fisher Scientific, CA, USA) using FITC and rhodamine filters.

Cell membrane assays

The cell membrane integrity of tumor cells was analyzed by Calcein AM assays (Biotium Hayward, CA, USA) and fluorescence microscopy.^{19,20} CPC at a final concentration of 0–100 μM were added to MCF-7 cultures for 24 h. After drug exposure, cells were washed with PBS and stained for 0.5 h in the dark at 37°C with 2 μM calcein AM (final concentration). Cells were observed with an EVOS Cell Imaging System (Thermo Fisher Scientific, CA, USA) using a FITC filter. Only living cells with an integral cell membrane retain the fluorescent signal in the cytoplasm.

MitoView assay

The MitoView assay was used to verify whether CPC damages MCF-7 cells. After a 24 h exposition to 0 (negative control), 5, or 100 μM CPC, or 500 μM docetaxel (DTX); MCF-7 mitochondria were stained with 100 nM

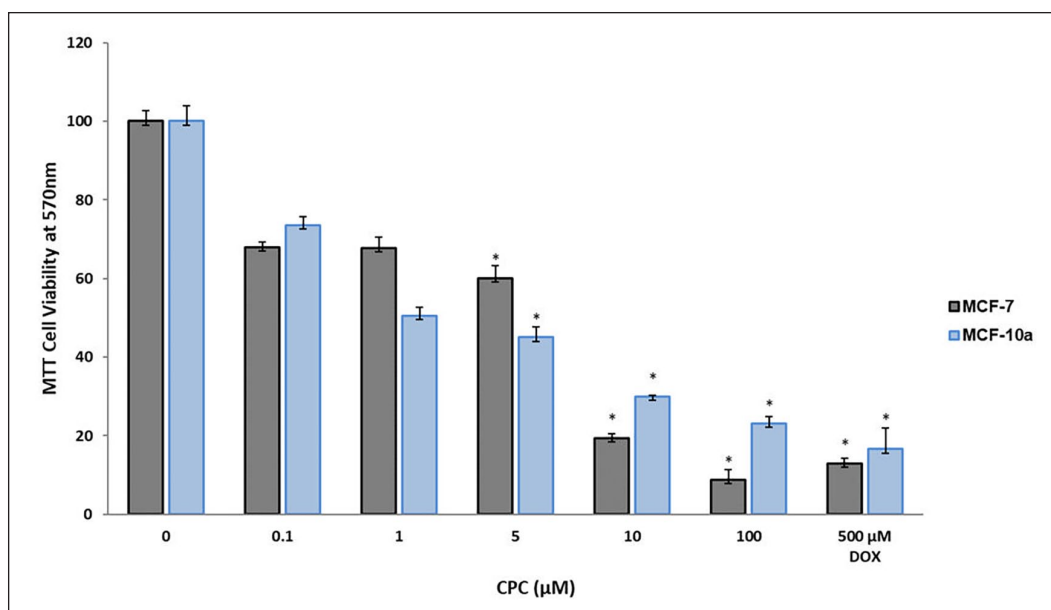


Figure 1. Antitumor activity of CPC on human breast tumor cells. MCF-7 and MCF-10A cells were 24-h exposed to 0.1–100 μM CPC. Growth control, a drug-free culture (0 μM CPC). As inhibition control 500 μM DOX were employed. SD error bars show mean ± SD ($n=3$), asterisk signifying statistical significance ($\alpha=0.05$) ($p < 0.0001$).

MitoView dye (Biotium Hayward, CA, USA) for 1 h at 37°C with 5% of CO₂. MCF-7 mitochondria were observed with an EVOS microscope (Thermo Fisher Scientific, CA, USA) using a FITC filter.

Comet assay

The OxiSelect™ Comet Assay Kit (Cell Biolabs, INC, San Diego, CA, USA) was used to verify whether CPC is genotoxic on MCF-7 cells.²¹ Briefly, after an overnight drug exposure to 0, 5, or 100 μM CPC or 100 μM etoposide (Sigma-Aldrich Corporation, St. Louis, MO, USA) employed as positive control, MCF-7 were collected and washed with PBS. DNA was stained with DAPI (1 μL/mL, final concentration; 100 μL/well). The slides were observed with an EVOS microscope (Thermo Fisher Scientific, CA, USA) using a DAPI filter.

Statistical analysis

A two-way analysis of variance (ANOVA) was employed to analyze data among groups. A significance level of $\alpha=0.05$ was considered. The selectivity index (SI) was measured with the formula: $SI = IC_{50} \text{ no cancer cells} / IC_{50} \text{ cancer cells}$.²²

Results

Antitumor activity of CPC

CPC was cytotoxic to both MCF-7 and MCF10A at all concentrations tested in a dose-dependent way (Figure 1).

At the lowest concentration tested (0.1 μM), cytotoxicity was over 20% f; at the highest concentration (100 μM) the cytotoxicity was 92% for MCF-7 and 77% for MCF-10A. In comparison, the cytotoxicity of the positive control, 500 μM DOX, was 86% for MCF-7 and 82% for MCF-10A. Thus, CPC had a five times higher antitumor activity than DOX. The effect of CPC on both kinds of breast human cells was dose-dependent.

LIVE/DEAD assay

To corroborate CPC cytotoxicity on breast cancer cells, LIVE/DEAD assays were carried out to verify cell death due to CPC. A 24-h exposition to 100 μM CPC depleted MCF-7 cells, while exposure to 10 μM CPC was less severe and similar to the effect of the positive cytotoxicity control of 500 μM DOX (Figure 2). There was no cell death in drug-free MCF-7 cultures (Figure 2; MCF-7). Altogether, these results confirm the results of the MTT assays.

Time response analysis

So far, survival or cytotoxicity assays were performed after a 24-h drug exposure. To evaluate whether CPC acts faster on MCF-7 cells, a time-response analysis with the MTT assay was carried out. Exposure to 100 μM CPC for 5 min reduced MCF-7 survival by 60% (Figure 3). Longer exposures increased toxicity to 70% after an hour and to over 80% after the longest exposure time of 4 h (Figure 3). Thus, it seems that 100 μM CPC acts fast and requires only 5 min to kill over 50% of the cells.

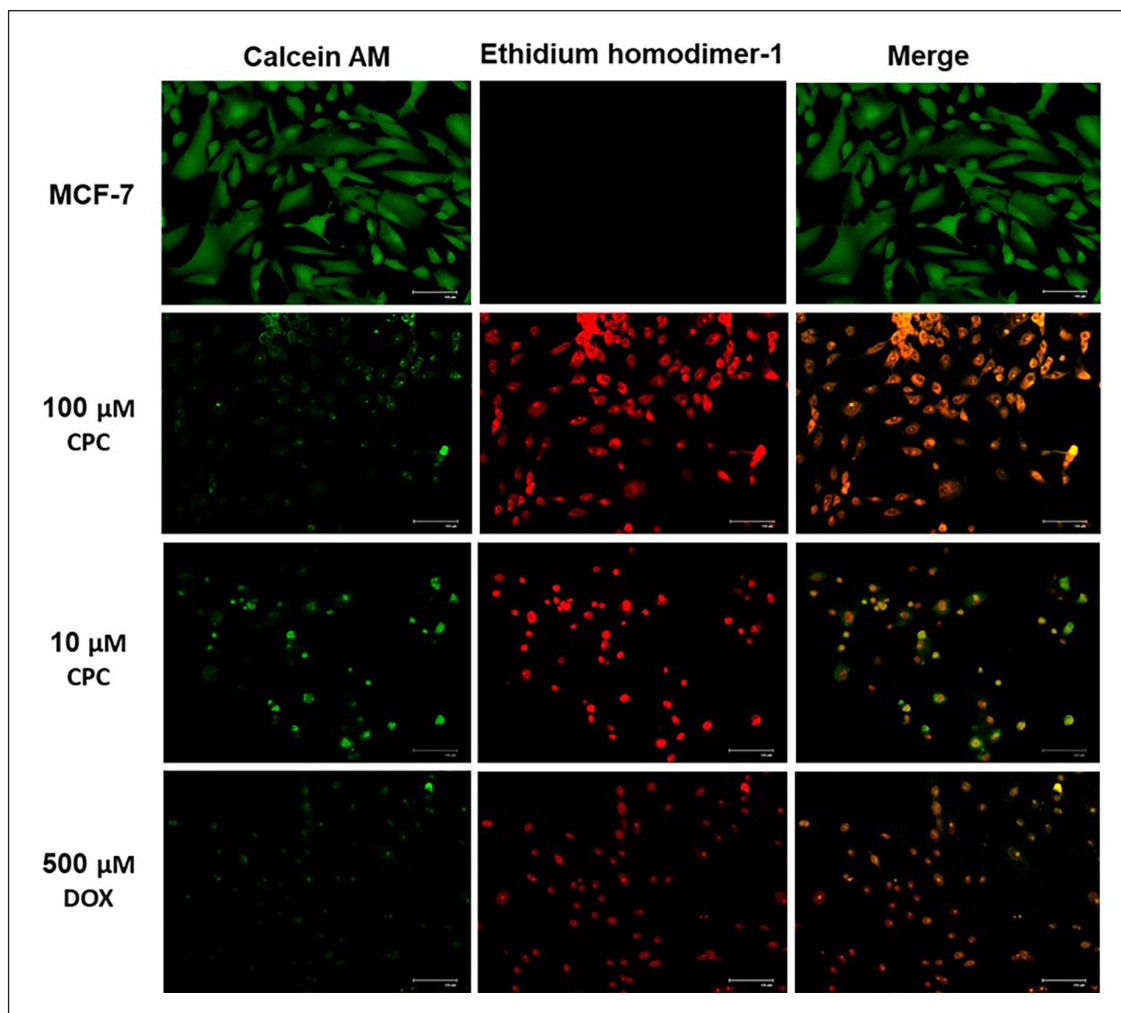


Figure 2. LIVE/DEAD assays of CPC on MCF-7 cells. Cell survival and morphology of MCF-7 were determined by LIVE/DEAD test and FLM after a 24 h-exposition to 10 and 100 μM CPC or 500 μM DOX (inhibition control). A drug-free culture served as growth control. Bar, 100 μm .

LD50 assays

Next, MTT assays were applied after a 1-h exposure to different concentrations of CPC to determine the LD50 value. The LD50 was 6 μM for MCF-7 and 8 μM for MCF-10A (Figure 4). These data confirm a dose-dependent phenomenon and a 80% toxicity at 10 μM CPC (Figure 4).

Selectivity index

The selectivity index (SI) constitutes the proportion between cytotoxic amount of a molecule versus its beneficial bioactive concentration. The SI of CPC, determined in vitro with MCF-10A and MCF-7 cells, was 1.41 (Figure 4). This low SI indicates that CPC is only slightly selective for tumor cells in comparison to healthy cells and a relevant aspect with for the possible clinical application of CPC.

Effect of CPC on cell membranes of tumor cells

To verify possible action mechanisms of CPC its effect of CPC on cell membrane of MCF-7 cells was analyzed by the calcein AM assay and fluorescence microscopy. Increasing quantities of CPC promote the exit of fluorescent calcein from MCF-7 cells after 24 h-exposition in a dose-response way (Figure 5). These results suggest that CPC alter the cell membrane integrity of MCF-7 in a dose-response phenomenon.

Effect of CPC on MCF-7 mitochondrial morphology

Another possible action mechanism that was assessed was mitochondrial damage. Hereto, mitochondria were visualized with MitoView after a 24-h exposure to toxic concentrations (10 or 100 μM CPC, and 500 μM DTX). Compared to a drug-free control, the positive control DTX modified

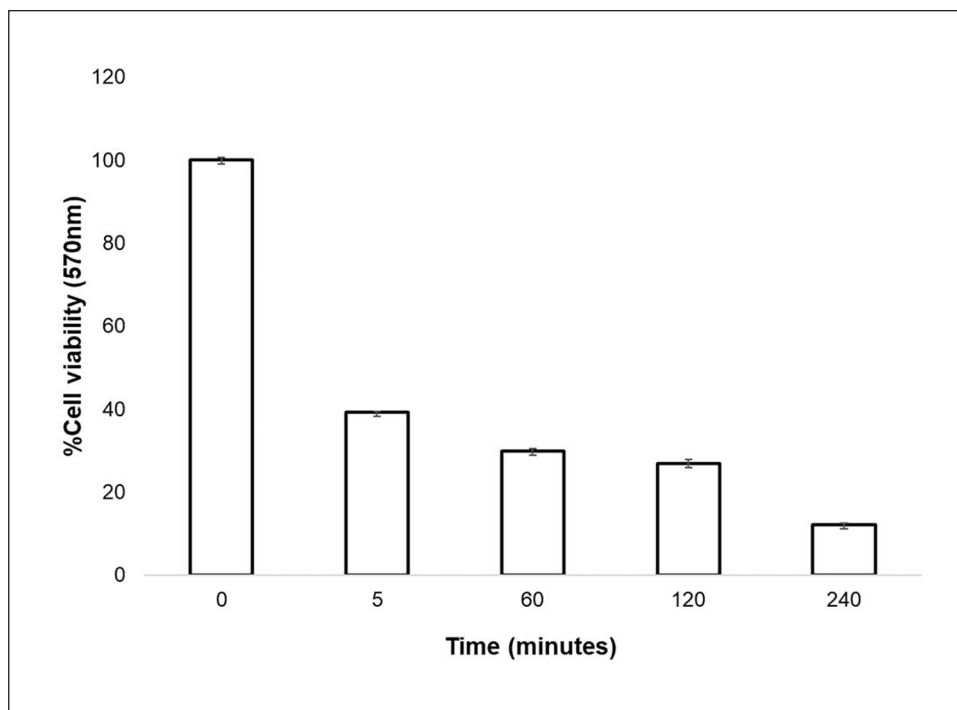


Figure 3. Time response of CPC on MCF-7 cells. Cell survival of MCF-7 was analyzed by the MTT test post exposition to 100 μ M CPC for 5, 60, 120, or 240 min. Error bars indicating mean \pm SD ($n=3$).

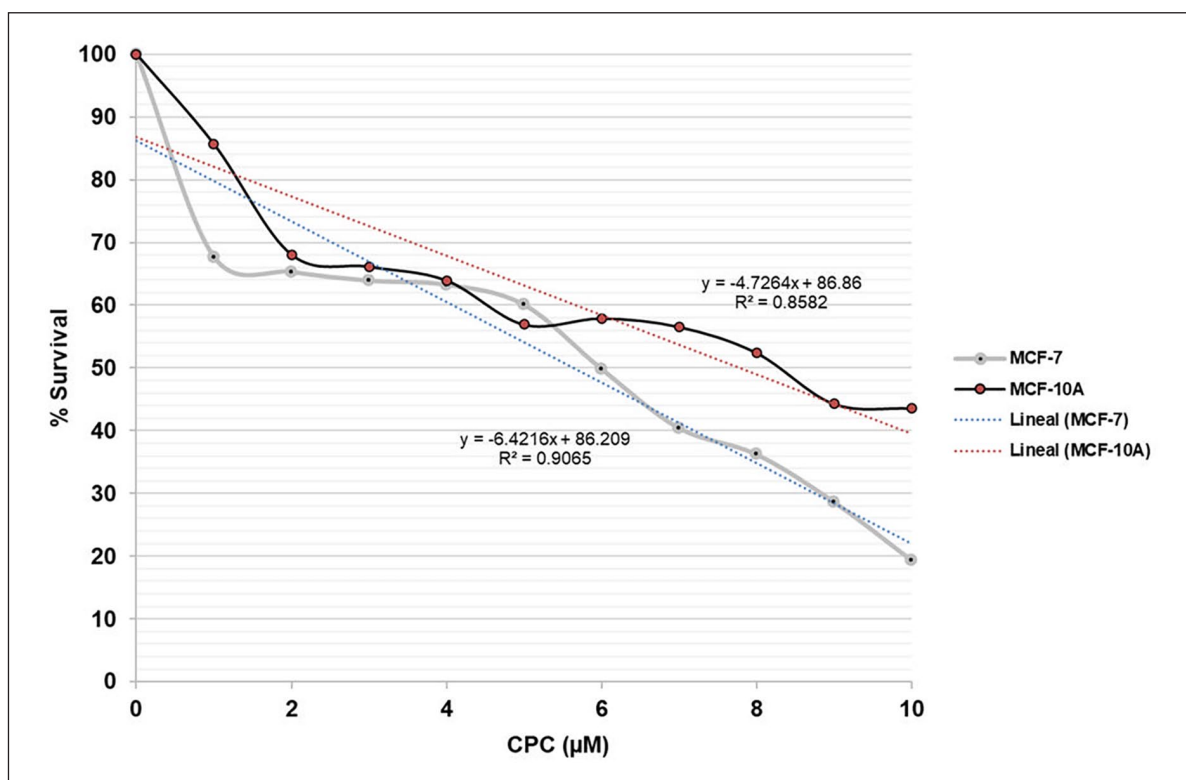


Figure 4. Determination of LD50 of CPC on human breast cancer cell line MCF-7. The effect of 24-h exposition to 1–10 μ M CPC on MCF-7 cell survival. Error bars indicating mean \pm SD ($n=3$).

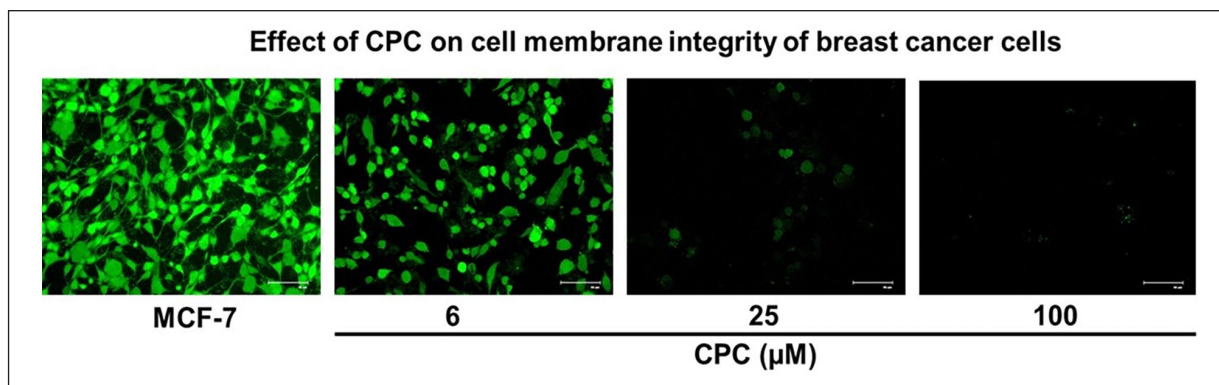


Figure 5. Influence of CPC on MCF-7 cell membranes. After a 24-h exposure to CPC at the indicated concentrations, cell membrane damage was analyzed with calcein AM test and fluorescence microscopy. Negative control: drug-free culture. Bar, 75 μm .

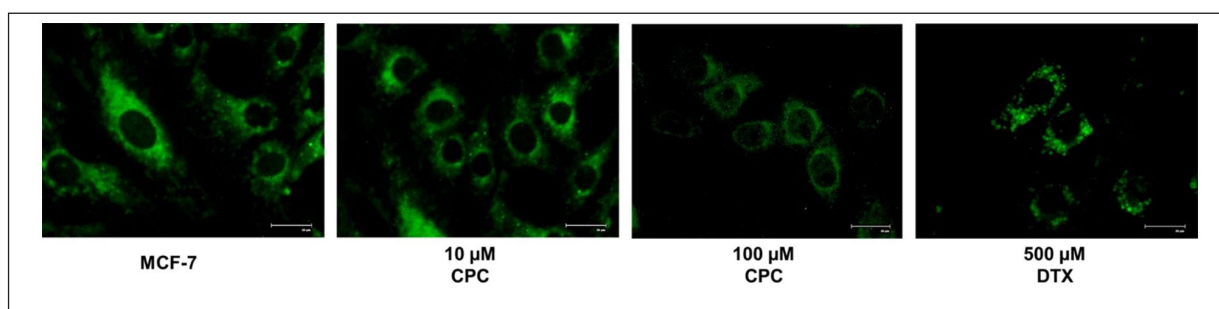


Figure 6. Mitochondrial morphology of MCF-7 after CPC exposure. MCF-7 cells were exposed for 24h to 10 or 100 μM CPC and stained with MitoView dye. As positive control of mitochondrial impact, 500 μM docetaxel (DTX) were used and a drug-free culture was taken as negative control (top row). Bar, 75 μm .

both the morphology and distribution of MCF-7 mitochondria. In contrast, exposure to CPC changed neither the distribution nor the morphology of mitochondria (Figure 6).

CPC genotoxicity

Genotoxicity was a third action mechanism that was explored. Comet assays with etoposide as a positive control of genotoxicity showed the classic stela that reveal the fragmentation of genomic DNA of tumor cells (Figure 7). In contrast, no such stela were observed after exposure to toxic concentrations of CPC (Figure 7). The drug-free control MCF-7 cells presented healthy nuclei with their typical circular shape (Figure 7). When fluorescence intensity was measured the results showed that all CPC concentrations analyzed (6–100 μM) promote 25%, 35%, or 50% of fluoresce signal in comparison with etoposide (positive control of genotoxicity). Altogether these results suggest that genomic DNA is not the main target of CPC in their antitumor effect, but it can be a synergic phenomenon to promote dead cell.

Discussion

CPC is a cationic surfactant with a quaternary amine that is employed in personal hygiene products such as toothpaste

and mouthwash.²³ There is ample evidence about the beneficial effects of CPC on gingival inflammation and plaque reduction.^{24–27} Besides, CPC seems to have antiviral properties. CPC blocked the replication of herpes simplex virus in gingival fibroblasts²⁸ and inhibited the assembly of capsid proteins of hepatitis B virus.²⁹ However, it has been reported that CPC is cytotoxic to human epithelial cells by prompting cellular killing through apoptosis activated by caspase-3. Furthermore, CPC interfered with alveolar surfactant activity due to their alkyl chain.³⁰ CPC also causes skin irritation and decreases cell survival exponentially post CPC skin exposition.³¹ CPC treatment in animal models promote the secretion of proinflammatory cytokines, mainly TNF- α , leading to pulmonary inflammation.²³ Also, it has been reported that CPC inhibited osteoclast differentiation in a dose-response way.³² All these adverse effects of CPC must be considered in clinical applications to treat chronic diseases.

In this work, we analyzed the antitumor properties of CPC on MCF-7 cells. Tumor cell growth was reduced as of a 24-h exposition to 0.1 μM CPC. Cytotoxicity of CPC on MCF-7 cells was corroborated by LIVE/DEAD assays where death cells stained red. The LD50 value of CPC on MCF-7 was 6 μM , which is nearly identical to the LD50 of doxorubicin.³³ Previously, it has been reported that CPC

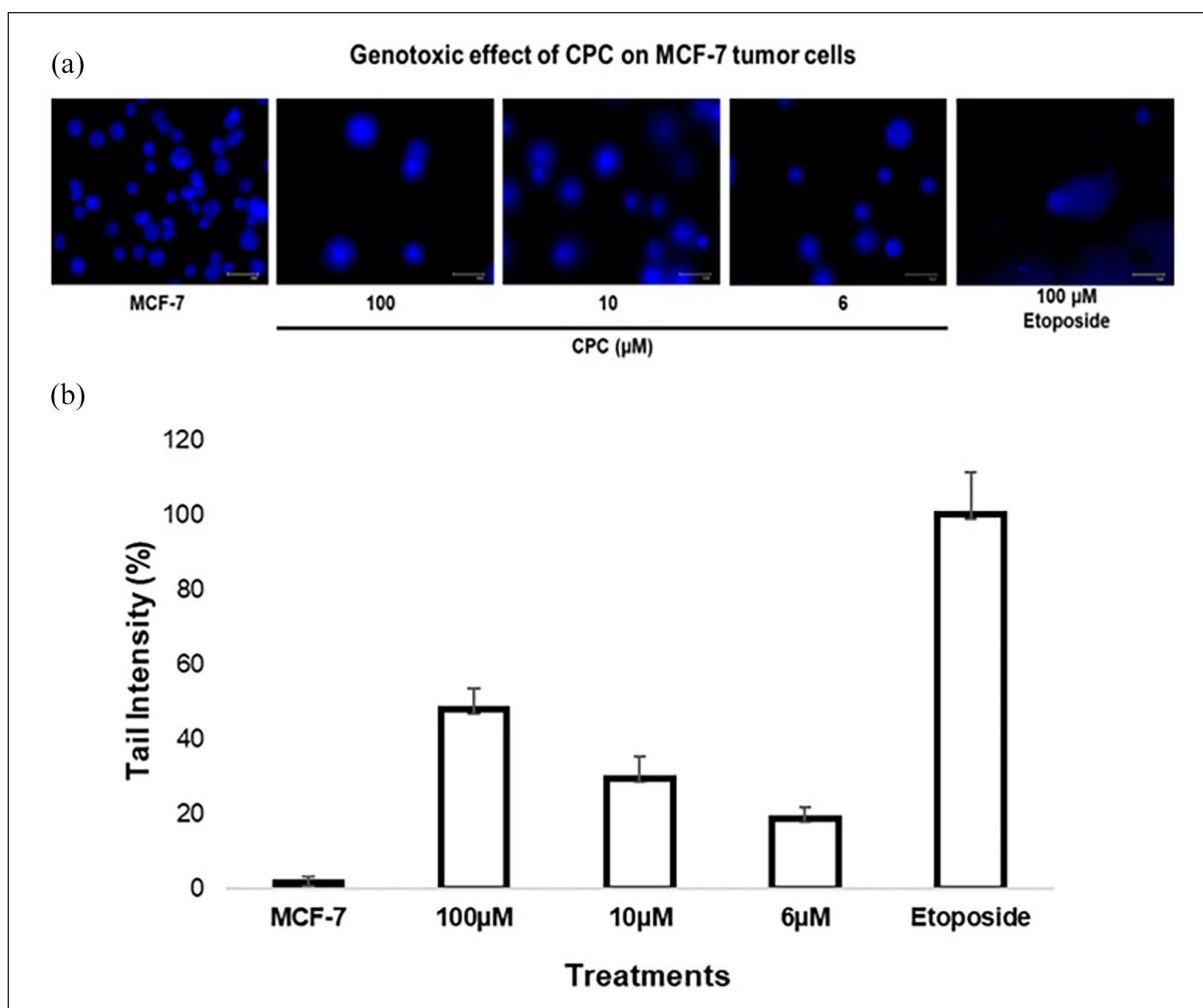


Figure 7. Genotoxicity of CPC on MCF-7 tumor cells by comet assay and fluorescence microscopy: (a) MCF-7 cells (1×10^5) were 24-h-exposed to 100, 10, and 6 μM CPC. As positive control of genotoxicity 100 μM etoposide were used, and as negative control a drug-free culture (MCF-7). Nuclei were stained with DAPI. Bar, 50 μm and (b) fluoresce intensity of tails was measured to get quantitative data of the possible genotoxic effect of CPC on MCF-7 cells.

kills glioblastoma cells with a higher efficacy than current standard temozollomide.¹⁴ Our data confirmed a recent report on dose-dependent CPC cytotoxicity on MCF-7.¹⁴ Unfortunately, this study did not evaluate the effect of CPC on non-tumorigenic cells. We found that the LD50 of CPC for non-tumorigenic MCF-10A cells was 8 μM . An SI of 1.41 suggests that CPC is only slightly more selective for tumor cells than non-tumorigenic cells. It has been reported that the conjugation of DOX to α -linolenic and docosahexaenoic acids reduces its cytotoxic effect and increases its SI.³⁴ A conjugation of CPC to hyaluronic acid present in a mouthrinse did not lead to significant differences in plaque reduction as compared to a group using a mouth supplemented with chlorhexidine.³⁵

Based on time-response experiments, more than 60% of the cytotoxicity occurred within a 5 min-exposure to 100 μM CPC. Importantly, we found that CPC decreased the cell growth of non-tumorigenic human breast

MCF-10A cells. This result suggests that CPC has, like DOX, DTX, and cisplatin, a low selectivity for tumor cells.^{9,10,36} This finding is consistent with extensive literature on the cytotoxicity of CPC.^{37,38}

Previously, it has been reported that 0.05% CPC is effective to get antiplaque and antigingivitis effects.³⁹ 0.05% CPC equals to 1400 μM . In this study the range of final concentrations 0.1–100 μM CPC were analyzed to determine its antitumor effect. If we take the higher concentration used in this work (100 μM), it means that 14 \times time-less has been employed to inhibit the MCF-7 cell growth in comparison with antiplaque and antigingivitis effects.

Based on calcein AM assays, the action mechanism of CPC involves cell membrane damage. A 24-h exposure to 10–100 μM CPC, allowed the loss of fluorescent calcein AM, which suggests a violation of cell membrane integrity. In contrast, mitochondrial morphology and distribution of

MCF-7 were not altered after a 24-h exposure to 10–100 μM CPC. Others reported that CPC decreases ATP synthesis in a dose-response manner with higher effect at a final concentration of 10 μM .¹⁴ In our study, a 24-h exposure to 10 μM CPC reduced the cell viability of MCF-7 with 81% and the one of MCF-10A with 57%. Fewer cells after CPC treatment could explain lower ATP levels. CPC was not genotoxic to human breast cancer cells. A 24-h exposure to 6–100 μM CPC did not promote DNA fragmentation as compared to the positive genotoxic control etoposide. This finding supports previous findings by Grabińska-Sota,⁴⁰ who did not found genotoxicity among different quaternary ammonium salts. Altogether, our results suggest that CPC has antitumor activity in a fast and dose-dependent way. Cell damage may be the action mechanism. On the other hand, the cytotoxicity to non-tumorigenic cells must be taken in account for a future clinical application. As a perspective, the conjugation of CPC to other molecules may reduce its cytotoxic effect and could be an interesting way to increase its biocompatibility.

Conclusions

Cetylpyridinium chloride inhibits the survival of human breast tumor cells in a fast, dose-response and non-selective manner. The cytotoxic action mechanism of CPC involves cell membrane damage without alterations in mitochondrial distribution and morphology nor genotoxicity.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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