Cell Growth Inhibition, G₂/M Cell Cycle Arrest, and Apoptosis Induced by Chloroquine in Human Breast Cancer Cell Line Bcap-37


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Key Words
Chloroquine • Bcap-37 breast cancer cells • Apoptosis • Cell cycle • G₂/M arrest

Abstract
Chloroquine is an antimalarial drug that has been used in the treatment and prophylaxis of malaria since the 1950s. The present study was undertaken to examine the effects of chloroquine on Bcap-37 human breast cancer cells' growth, cell cycle modulation, apoptosis induction, and associated molecular alterations in vitro. The chloroquine treatment decreased the viability of Bcap-37 cells in a concentration- and time-dependent manner, which correlated with G₂/M phase cell cycle arrest. The chloroquine-mediated cell cycle arrest was associated with a decrease in protein levels/activity of polo-like kinase 1 (Plk1), phosphorylated cell division cycle 25C (Cdc25C), phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), phosphorylated Akt. The chloroquine-treated Bcap-37 cells exhibited a marked decrease in the level of mitochondrial transmembrane potential (ΔΨm), which was accompanied by the activation of caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP).

Introduction
Chloroquine was originally synthesized in 1934 by Bayer in Germany (Resochin) through the condensation of 4-7-dichloroquinoline with 1-diethylamino-4-aminopentane [1]. As its oral lowest published lethal dose of man is 86 mg/kg (registry of toxic effects of chemical substances, RTECS), it can be well-tolerated in humans. So it has been widely used as a potent antimalarial and amebicidal drug. Recently, several studies have demonstrated that it has extensive biological effects such as inhibiting cell growth and/or inducing cell death in human lung cancer A549 cells, human ductal pancreatic
adenocarcinoma cells, human malignant lymphoreticular cells, melanoma cells, C6 glioma cells, neuronal cells, and human umbilical vascular endothelial cells, mediating radiosensitization in MDA-MB-231 mammary carcinoma cells [2-10]. These results indicated that chloroquine might cause distinct cell death in different kinds of cells.

Breast cancer is the most common malignancy in women with approximately one in nine women developing it in her lifetime [11]. Modalities available now such as surgery, radiotherapy, traditional chemotherapy or endocrinotherapy have some curative effects on breast cancer, especially in its early stages; however, more effective agents against breast cancer are still in urgent demand to better combat this malignancy. Therefore several experiments were done in which the antimalarial drug chloroquine was used to treat experimental mouse mammary carcinoma [12, 13]. However, the mechanisms underlying its cancer therapeutic activity are poorly understood at present. So we explored potential molecular targets and pathways concerning breast cancer therapy with chloroquine.

In general, the progression of cell cycle in eukaryotes is a complex process involving resting G0 phase, and cell growth involving G1, S and G2/M phases in a step-wise manner. Reversible protein phosphorylation is an important regulatory mechanism in the control of cell cycle progression [14]. Both p44/42 mitogen-activated protein kinase (MAPK) and Akt (also referred to as protein kinase B or Rac) are known to play a critical role in cell growth and survival, and they are upregulated in breast cancer cells [15, 16]. To determine the potential involvement of these protein kinases in cell cycle progression and apoptosis in breast cancer cells which have high-levels of MAPKs and Akt activity, we surveyed their phosphorylation status after 48 h exposure in dose-response experiments.

To gain insights into the mechanism of apoptosis induction caused by chloroquine, we also investigated the roles of mitochondrial transmembrane potential (internal signal pathway) and spindle assembly in this process by the fluorescence dissipation of cationic lipophilic fluorochrome rhodamine 123 and immunostaining of α-tubulin.

**Materials and Methods**

**Reagents and antibodies**

Chloroquine, paclitaxel, aphidicolin, methylthioazolyldiphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), RNase A, rhodamine 123, monoclonal anti-α-tubulin (clone B-5-1-2) were purchased from Sigma (St. Louis, MO). DNA marker, protease K were purchased from TaKaRa (Dalian, China). The primary antibodies against GAPDH, caspase-3, cleaved poly(ADP-ribose) polymerase (PARP), Akt, phosphorylated Akt (Ser473), p44/42 MAPK, phosphorylated p44/42 MAPK (Thr202/Tyr204), Cdc25C, phosphorylated Cdc25C (Ser198), and polo-like kinase 1 were acquired from Cell Signaling Technology (Beverly, MA). Secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ECL detection system was from Amersham (Arlington Heights, IL). Chloroquine was prepared as a 1×10⁻¹ M stock in DMSO. Drug stock was diluted in RPMI 1640 (containing 10% FBS) when it was required for assays.

**Tumor cell line and culture**

The human breast cancer cell line Bcap-37 (ER−, p53 mutated) purchased from Shanghai Cell Collection, CAS (Shanghai, China). The human breast cancer cell line MCF-7 (ATCC Accession No. HTB-22, ER+, wild p53), MDA-MB-231 (ATCC Accession No. HTB-26, ER-, p53 mutated), MDA-MB-435S (ATCC Accession No. HTB-129, ER-, p53 mutated), T47D (ATCC Accession No. HTB-133, ER+, p53 mutated) and the mouse mammary carcinoma cell line 4T1 (ATCC Accession No. CRL-2935) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and were grown as monolayers in Dulbecco’s modified Eagle medium or RPMI 1640 medium (Gibco BRL, Grand Island, N.Y.). These cells were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Auckland, N.Z.), 100 units/mL penicillin, 100 units/mL streptomycin, at 37°C, 95% relative humidity, under 5% CO₂.

**In vitro assay for cytotoxic activity (MTT assay)**

The cytotoxicity of chloroquine on breast cancer cells was determined by the MTT assay [17]. Cells (3-5 × 10³/well) were plated in 200 μL of medium/well in 96-well plates (Costar Corning, Rochester, NY). After incubation overnight, chloroquine was added in various concentrations (1, 2, 4, 8, 16, 32, 64 μM), paclitaxel (100 nM) used as a positive control; 6 wells were included in each concentration. After treatment with chloroquine for 1, 2, 3, 4 days, the absorbance at 570 nm was measured with SpectraMax M5 ( Molecular Devices), using wells without cells as blanks. All experiments were performed in triplicate. The effect that chloroquine on the proliferation of breast cancer cells was expressed as the % cell growth inhibition, using the following formula: % inhibition = (A₅₇₀ of control - A₅₇₀ of treated cells)/A₅₇₀ of control cells × 100%.

**Clonogenic survival determination**

Bcap-37 cells were assayed for colony-forming ability by replating them in specified numbers (300-400/well) in 6-well plates and treated with 2, 4, 8, 16, 32 and 64 μM of chloroquine, respectively. After 12 days of incubation, the cells were stained with 0.5% crystal violet in absolute ethanol and colonies with > 50 cells were counted under dissection microscope [18].

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Analysis of cell cycle progression and apoptosis by Flow Cytometry

Bcap-37 cells were synchronized at the G1/S boundary by aphidicolin block as described before [19]. Briefly, exponentially growing cells were treated with aphidicolin (4 µg/mL, Sigma) for 14 h followed by washing thrice with normal growth medium and incubation in the presence or absence of chloroquine. At the indicated intervals, both attached cells and floating cells were harvested, sediments were resuspended in 1 mL hypotonic fluorochrome solution containing 50 µg/mL propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100 and the cell cycle distribution was analyzed by flow cytometry (ESP Elite, Beckman Coulter, Fullerton, CA) immediately [20]. Apoptotic and dead cells appeared in the cell cycle distribution as cells with a DNA content of less than that of G1 cells, and was estimated with Listmode software.

Morphological analysis

According to the methods reported previously [21], approximately 2×10^6 Bcap-37 cells were seeded in each well of 6-well plates. After incubation overnight, chloroquine (16, 32 µM) was added, and cells then incubated at 37°C/5% CO2 for 48 h. Untreated or chloroquine-treated Bcap-37 cells were examined for morphological changes by inverted phase contrast microscope (Olympus, Tokyo, Japan). For the identification of the apoptotic cells, cells were stained with hypotonic propidium iodide solution containing 50 µg/mL propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100 and examined with an inverted fluorescence microscope (Zeiss, Axiosvert 200, Germany).

Agarose gel DNA electrophoresis

The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described previously [21]. Briefly, cells (5×10^6) were lysed with 0.5 mL lysis buffer containing 5 mM Tris/HCl (pH 8.0), 0.25% Nonidet P-40, and 1 mM EDTA, followed by the addition of RNase A (Sigma) at a final concentration of 200 µg/mL, and incubated for 1 h at 37°C. Cells were then treated with 400 µg/mL proteinase K (TaKaRa) for another one hour at 37°C. After addition of 4 µL loading buffer, 20 µL samples in each lane were subjected to electrophoresis on 1.5% agarose in 0.1% sodium citrate plus 0.1% Triton X-100 and examined with an inverted fluorescence microscope (Zeiss, Axiovert 200, Germany).

Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential (ΔΨm) of cells treated with chloroquine or medium alone was measured as previously described [22]. Briefly, on day 1, 2×10^6 Bcap-37 cells were plated in each well of a 6-well plate. After 48 h treatment of chloroquine (8, 16, 32 µM), ΔΨm was determined by the retention of the dye rhodamine 123 in Bcap-37 cells. About one million cells per group were harvested, washed with PBS, and then incubated with rhodamine 123 (5 µg/mL) at 37°C for 15 min in the dark. After washing twice, the cells were incubated again in PBS at 37°C for 30 min in the dark and fluorescence was then measured as described above.

Chloroquine Effects in Bcap-37 Breast Cancer Cells

Immunostaining of α-tubulin

2×10^5 Bcap-37 cells were seeded on to coverslips in a 6-well plate. Chloroquine was added at 16, 32 µM after incubation overnight. And after 48 h treatment, cells grown on glass coverslips were washed with PBS and fixed in 4% paraformaldehyde. The fixed cells were then treated with PBS containing 10% goat serum/0.1% Triton X-100 for 1 h at room temperature. After washing, cells were incubated with monoclonal anti α-tubulin antibody (clone B-5-1-2, 1:1000 dilution, Sigma) for 1 h at 37°C followed by the FITC-conjugated goat-anti-mouse antibody (1:100 dilution, Sigma) for 1 h at 37°C. The coverslips were washed, and mounted on glass slides with prolong anti-fade solution (Sigma) [19]. And they were visualized using a UV fluorescence microscope (Nikon, Japan).

Western blot analysis

Bcap-37 cells were treated with chloroquine in designed concentrations for 48 h. Equal amounts of protein from control cells and treated cells were subjected to electrophoresis in a 12% sodium dodecyl sulfate-acrylamide gel followed by electroblot transfer to PVDF membrane (Bio-Rad, Hercules, CA). For immunodetection, membranes were blocked with 5% nonfat milk in Tris-Buffered Saline Tween-20 (TBST) before incubation with mouse monoclonal anti-polo like kinase 1, anti-Akt, anti-phosphorylated Akt (Ser473) or rabbit polyclonal anti-caspase-3, anti-PARP, anti-Cdc25C, anti-phosphorylated Cdc25C (Ser198) , anti-p44/42 MAPK, anti-phosphorylated p44/42 MAPK (Thr202/Tyr204) and anti-GAPDH (Cell Signaling Technology, Beverly, MA). Primary antibodies were detected using either a goat-anti-mouse or goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The reactive band was identified using an enhanced chemiluminescenet substrate to horseradish peroxidase (Amersham).

Statistical Analysis

Data values were expressed as means ± SD. Differences were compared by chi-square test, one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison tests. P values were considered to be statistically significant when P < 0.05.

Results

Chloroquine inhibited proliferation of breast cancer cells

Initially, a small panel of six breast cancer cell lines (4T1, MCF-7, MDA-MB-231, MDA-MB-435S, T47D and Bcap-37) was used to investigate the growth inhibitory properties of chloroquine. A comparison between the six cell lines examined showed that Bcap-37 cells were by far the most sensitive to chloroquine treatment. After 48 h of chloroquine treatment, the IC50s of MCF-7, MDA-MB-231, MDA-MB-435S, T47D and Bcap-37 were 63.98, 30.18, 35.84, 132.87, 21.68 µM (Table 1).


Chloroquine Effects in Bcap-37 Breast Cancer Cells
Chloroquine showed a dose- and time-dependent inhibitory effect on the growth of Bcap-37 breast cancer cells \((P < 0.05)\). The result of cytotoxic activity of chloroquine against Bcap-37 cells is shown in Fig. 1A. The effect of chloroquine on Bcap-37 cells clonogenicity was determined in clonogenic assays (Fig. 1B). When the Bcap-37 cells were treated with chloroquine in different concentrations, their potential to form colonies was inhibited in a dose-dependent form \((P < 0.05)\).

### Table 1

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Tumor type</th>
<th>IC(_{50}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Breast (ER+, wild p53)</td>
<td>63.98</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Breast (ER−, mutated p53)</td>
<td>30.18</td>
</tr>
<tr>
<td>MDA-MB-435S</td>
<td>Breast (ER−, mutated p53)</td>
<td>35.84</td>
</tr>
<tr>
<td>T47D</td>
<td>Breast (ER+, mutated p53)</td>
<td>132.87</td>
</tr>
<tr>
<td>Bcap-37</td>
<td>Breast (ER−, mutated p53)</td>
<td>21.68</td>
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</tbody>
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**Fig. 1.** Proliferative inhibition of chloroquine on Bcap-37 human breast cancer cells. (A) inhibition of Bcap-37 growth by chloroquine 1-64 µM. Results were expressed as means ± SD of independent experiments performed in triplicate. The asterisk indicated a significant difference between control group and chloroquine-treated groups by ANOVA followed by Dunnett’s multiple comparison tests \((^* P < 0.05\) vs control group, \(^{**} P < 0.01\) vs control group, \(n = 6)\). (B) results of colony-forming assays presented as surviving colonies (percentage of untreated control). Columns represented different concentrations respectively; Shown were the average values of at least three independent experiments in duplicate, bars represented standard deviation \((^* P < 0.05\) vs control group, \(^{**} P < 0.01\) vs control group).

**Fig. 2.** Effects of chloroquine on cell cycle distribution and apoptosis induction. (A) concentration-dependent effects of chloroquine on the cell cycle. Higher concentrations of chloroquine caused a concomitant increase in the percentage of cells in the G2/M phase \((P < 0.05)\). (B) chloroquine induced apoptosis in Bcap-37 cells. Cells were treated with medium control or 4, 8, 16 µM doses of chloroquine for 48 h. Hypodiploid cells were determined by DNA fluorescence histogram. Results were expressed as means of triplicate samples; bars, SD. \((^* P < 0.05\) vs control group, \(^{**} P < 0.01\) vs control group, \(n = 3)\). (C) agarose gel electrophoretic patterns of DNA isolated from chloroquine-treated and untreated tumor cells.
Chloroquine induced cell cycle arrest and apoptosis

Effect of chloroquine on cell cycle distribution was determined to gain insights into the mechanism of its anti-proliferative activity. As can be seen in Fig. 2A, a 24 h exposure of Bcap-37 cells to growth suppressive concentrations of chloroquine (8 and 16 µM) resulted in significant accumulation of cells in G2/M phases that was accompanied by a decrease in cells with G0/G1 and S DNA content. For example, as compared with control, the percentage of cells in G2/M phases was increased by 2.7-fold upon treatment with 16 µM chloroquine for 24 h (Fig. 2A). Chloroquine also induced a distinct sub-G1 peak, which represents the population of apoptotic and dead cells. As shown in Fig. 2B, there was a marked increase in the sub-G1 peak from 5.1 ± 2.4% in untreated cells to 16.7 ± 4.3%, 32.8 ± 3.2%, 51.6 ± 5.4% respectively in 4 µM, 8 µM, 16 µM Chloroquine-treated cells for 48 h. These results suggested that chloroquine could block the cell cycle and induce apoptosis and death in Bcap-37 cells in a dose-dependent manner in vitro.

Agarose gel electrophoresis of chloroquine-treated cells demonstrated a ladder-like pattern of DNA fragments consisting of multiples of approximately 180-200 base pairs, consistent with internucleosomal DNA fragmentation (Fig. 2C).

Treatment with chloroquine of Bcap-37 cells also resulted in morphological changes consistent with apoptosis. When cultured in the control medium, Bcap-37 cells retained their normal size and shape, kept proliferating with time and became over-crowded by 48 h (Fig. 3A). Compared with the control, cells treated with chloroquine vacuolated gradually as the concentration increased (Fig. 3A). As the concentration increased to
higher than 16 µM, in addition to vacuolating, treated cells shrank and rounded up, showed membrane blebbing and eventually detached from the surface of the tissue culture dish. The morphological changes were also characteristic of apoptosis after PI-staining: a brightly red, condensed nuclei (intact or fragmented) shown by fluorescence microscopy of PI-stained nuclei (Fig. 3B). In contrast, the untreated cells showed red, diffusely stained intact nuclei (Fig. 3B).

Regulation of apoptosis and cell cycle-related protein expression

Using immunoblot analysis, we observed the effects of chloroquine treatment on the protein levels of the Akt, phosphorylated Akt (Ser473), p44/42 MAPK and phosphorylated p44/42 MAPK (Thr202/Tyr204). The data (Fig. 4) indicated that during the 48 h time period, chloroquine decreased the expression levels of phosphorylated Akt (Ser473) and phosphorylated p44/42 MAPK (Thr202/Tyr204). But total p44/42 MAPK and Akt were unaffected by the chloroquine treatment as judged by comparisons with GAPDH as a loading control.

Since chloroquine was observed to cause an arrest of cells in G2/M phases, we next assessed its effect on G2/M cell cycle regulators including Cdc25C, phosphorylated Cdc25C, and polo-like kinase 1. The results of SDS-PAGE were presented in Fig. 4. The results demonstrated that chloroquine increased Cdc25C level and inhibited polo like kinase 1, phosphorylated cdc25C (Ser198) levels.
Mitochondrial transmembrane potential changes and activation of caspase-3

$\Delta \Psi m$ was measured by flow cytometry using the cationic lipophilic green fluorochrome rhodamine 123 (Sigma). Disruption of $\Delta \Psi m$ is associated with a lack of rhodamine 123 retention and a decrease in fluorescence [22]. Therefore, we examined the effect of chloroquine on $\Delta \Psi m$ by rhodamine 123 retention. Fig. 5 showed that chloroquine treatment statistically significantly reduced rhodamine 123 fluorescence in Bcap-37 cells compared with control ($P < 0.05$, determined by chi-square test).

In our study, treatment of Bcap-37 cells with chloroquine for 48 h also resulted in the activation of caspase-3 and PARP cleavage, as was apparent by the appearance of its cleaved products at 19 and 17 kDa (activated form, caspase-3) and 89 kDa (PARP) (Fig. 4).

Effect of chloroquine on spindle assembly

To test whether chloroquine induced spindle abnormalities, Bcap-37 cells were treated with chloroquine (16, 32 $\mu$M) for 48 h, and microtubules were visualized by indirect immunofluorescence techniques. The stained cells (Fig. 6) showed that while untreated cells went through various phases of mitosis without any abnormality, chloroquine-treated cells exhibited profound abnormalities in spindle formation, resulting in the appearances of multipolar spindles.

Discussion

In the present study, several observations have been made concerning the cell growth inhibition, G$_2$/M cell cycle arrest, and apoptosis-inducing effects of chloroquine and its possible mechanism.

The response of cancer cells to chloroquine varied significantly from cell line to cell line: human lung cancer A549 cells (48 h IC$_{50}$ > 32 $\mu$M) [10]; human leukemic K562 cells (48 h IC$_{50}$ 20.59 $\mu$M); mouse colon cancer CT26 cells (48 h IC$_{50}$ > 50 $\mu$M) (unpublished work). In our study, the inhibitory effects of chloroquine on the proliferation of Bcap-37, MDA-MB-231, and MDA-MB-435S, three estrogen-receptor-negative cell lines, were greater than on MCF-7, T47D cells that are estrogen receptor positive. Estrogen receptors (ER) are expressed in ~65% of human breast cancer. Cumulative data from clinical trials, retrospective analyses and in vitro results suggested that some chemotherapeutic agents like paclitaxel might be less effective in ER-positive tumors than those ER-negative tumors [23-26]. These findings above suggested that estrogen receptor and estrogen dependency of the cancer cells appeared to be the most prominent factor influencing their response to chloroquine.

Apart from the ER, other factors might also affect chloroquine response of breast cancer cells such as p53 status, caspase-3 status, c-Myc overexpression and so on [27-29]. Chloroquine has also recently been shown to induce proliferation in the breast cancer cell line MCF-7 [30]. The discrepancy between above result and ours may derive from the different concentrations and time of treatment.

To determine the mechanisms of cell growth inhibition activity of chloroquine, we examined its effect on expression and phosphorylation of key signal transduction pathways. It is well known that p44/42 MAPK and Akt play critical roles in cell survival by RAS-RAF-MEK-MAPK pathway and inhibiting apoptosis through phosphorylating or inactivating the targets like Bad, c-Raf, caspase-9 and so on individually. As the MAPK and Akt signalling pathways not only promote cell proliferation, but also mediate cell survival and are upregulated in cancer cells, they seem to be good therapeutic targets [15, 16]. In our study, activation of both MAPK and Akt (the phosphorylated form) after chloroquine treatment was inhibited, indicating that a mechanism of activity was probably upstream of these pathways and possibly at the level of Src [31].

Some studies reported the role of p53 in chloroquine-induced cell cycle arrest and apoptosis. Loehberg et al showed that chloroquine activated the tumor-suppressor p53 and p53 downstream target gene p21, resulting in G$_1$ cell cycle arrest in mouse mammary epithelial cells [27]. The effects have also been described in the breast cancer cell line MCF-7 by Zhou et al [32]. In our study, chloroquine appeared to induce G$_2$/M arrest in Bcap-37 cells as early as 24 h after treatment. Events of the cell cycle, the stages at which the cell proliferates and divides, are facilitated and controlled by multiple signaling pathways [14]. Among the many regulatory enzymes that contribute to these processes, the polo-like kinase 1 is regarded to be critical. Plk1 has been identified to be a key player for G$_2$/M transition and mitotic progression in both normal and tumor cells. It has been reported to mediate multiple mitotic processes including bipolar spindle formation, activation of Cdc25C (phosphorylation of Cdc25C at Ser198), actin ring formation, centrosome maturation, and activation of the anaphase-promoting complex [33, 34].

As shown by Western blot analysis, the targeted
Akt activity (Fig. 4, ERK/Akt inhibition). Studies have shown that phosphorylation of the protein phosphatase Cdc25C at Ser198 by Plk1 promotes nuclear localization of Cdc25C during prophase [35]. Our data clearly demonstrated an accumulation of Cdc25C in the interphase after chloroquine treatment, and indicated that Cdc25C had not been translocated to the nucleus and thus had not been phosphorylated. Phosphorylated Cdc25C can activate the cdc2/cyclin B1 complex by dephosphorylating cdc2, resulting in the initiation of mitotic events [36]. Our data further supported the hypothesis that the mechanism by which down-regulation of Plk1 induced mitotic arrest followed by apoptosis was via the Cdc25C/cdc2/cyclin B1 feedback loop [37].

In addition to its role in growth and survival processes, Akt is involved in cell cycle regulation by preventing glycogen synthase kinase 3α/β (GSK-3α/β) mediated phosphorylation and degradation of cyclin D1 and by negatively regulating the cyclin dependent kinase inhibitors p27 Kip and p21 Waf1 [16, 38]. Wright [39] and Hayne [40] have also established that the MAPK pathway plays an important role in the G2/M transition. ERK activation is required for cyclin D1 expression, and the intensity and duration of the signalling through this pathway ultimately determines whether a cell undergoes differentiation, proliferation or cell-cycle arrest [15]. Thus the effects of chloroquine on the cell cycle might also correlate with the decreasing phosphorylated Akt activity (Fig. 4, ERK/Akt inhibition).

Chloroquine’s derivant hydroxychloroquine has been shown to induce apoptosis via destabilization of lysosomal membranes, leading to mitochondrial membrane depolarization and activation of caspases [41]. The internal signal pathway of apoptosis has been recognized as sequential event of the mitochondria changes, involving the decrease of ΔΨm and the activation of caspase cascade [42, 43]. So mitochondria can be used as a novel target for anticancer chemotherapy. A key step in the mitochondria-dependent apoptotic pathway is the disruption of the mitochondrial membrane, leading to loss of ΔΨm [44]. In our experiment, chloroquine decreased ΔΨm after 48 h of treatment, possibly triggering the apoptosis.

Caspase-3, an ICE-like protease, is an effecter caspase that plays a central role in the mitochondrial-mediated cell death pathway and is responsible for the breakdown of several cellular components involved in DNA repair and regulation. They belong to a group of enzymes known as cysteine proteases and exist within the cell as inactive pro-forms of zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis [45, 46]. PARP is a zine-dependent, eukaryotic, DNA-binding protein that specifically recognizes DNA strand breaks produced by various genotoxic agents and is cleaved into 89- and 24-kDa fragments during apoptosis. It is a substrate of caspase-3, and serves as a marker for caspase activation [47]. Caspase activity and PARP cleavage are intracellular signs of activation of the apoptotic machinery. So, in our results, the mitochondrial membrane depolarization led to the activation of caspase-3 and PARP cleavage. Taken together, our results showed that chloroquine induced apoptosis in Bcap-37 cells through the internal apoptotic pathway.

Studies suggest that G2/M arrest by anticancer drugs might participate in inhibition of microtubule dynamics [48, 49]. So we checked whether chloroquine induced spindle abnormalities. Our results showed that chloroquine induced significant abnormalities in spindle organization.

Microtubules are involved in many important cellular functions, such as morphogenesis, migration, intracellular transport, and cell division. Bipolar spindle formation and maintenance involves the remodeling of the microtubule nucleating and organizing capacities of the spindle poles, as well as proper kinetochore-microtubule attachment [48, 49]. Polo-like kinase 1 is involved in all above processes, and may be important to help coordinate these events. Loss-of-function studies on polo-like kinase 1 in a range of organisms, from yeast to mammals, showed severe mitotic spindle defects [34, 35]. spindle abnormalities can activate the mitotic-damage checkpoint and block cell cycle progression [50, 51].

Reszka et al. have presented that MAPK regulates microtubule dynamics and other cytoskeletal changes that accompany growth through phosphorylation of microtubule-associated proteins. And MAPK has also been reported to be critical for the microtubule spindle assembly checkpoint [52-54]. In our study, chloroquine-treated cells exhibited MAPK inhibition, decreased polo-like kinase 1 expression level (Fig. 4), and profound abnormalities in spindle formation, resulting in the appearance of multipolar spindle (Fig. 6), which led to misalignment of chromosomes and complete loss of coordination in mitotic spindle assembly. Chloroquine induced mitotic arrest in Bcap-37 cells, characterized by spindle abnormalities leading to their apoptotic death.

To our knowledge there is no published data...
concerning the effect of chloroquine on Bcap-37 human breast cancer cells. Our results demonstrate that chloroquine inhibits Bcap-37 cells growth in vitro through G$_2$/M phase cell cycle arrest and apoptosis associated with decreased polo-like kinase 1, ERK1/2, Akt, Cdc25C activity. Its action is also probably related to its ability to induce spindle abnormalities and down-regulate mitochondrial transmembrane potential ($\Delta$Ψm). The nature of chloroquine in mediating the aforesaid responses in breast cancer cells could make it a potentially effective therapeutic agent against breast cancer. However, further in vivo studies are needed to establish the role of chloroquine as a therapeutic agent against breast cancer.

Abbreviations

PLK1 (polo-like kinase 1); Cdc25C (cell division cycle 25C); ERK1/2 (extracellular signal-regulated kinase 1/2); $\Delta$Ψm (mitochondrial transmembrane potential); PARP (poly(ADP-ribose) polymerase); MAPKs (mitogen-activated protein kinases); PKB (protein kinase B); PI-3K (phosphoinositide 3-kinase); MTT (methylthiazolylldiphenyl-tetrazolium bromide); DMSO (dimethyl sulfoxide); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); PI (propidium iodide); ECL (enhanced chemiluminescence); ICE (interleukin-converting enzyme); SDS (sodium dodecyl sulfate); IC$_{50}$ (50% inhibiting concentration); PAGE (polyacrylamide gel electrophoresis); PVDF (polyvinylidene difluoride); GSK (glycogen synthase kinase); FITC (fluorescein isothiocyanate).

Acknowledgements

This work was supported by National Key Basic Research Program of China (2004CB518800), Project of National Natural Sciences Foundation of China (20505006 and 30701027), National 863 projects (2006AA03Z356). The authors thank Dr. Ping Cheng and Dr. Ke Jin (State Key Laboratory of Biotherapy, Huaxi hospital, Sichuan University) for excellent technical assistance in the Western blot analysis.

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