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Original Article

Effects and mechanisms of chloroquine alone and in combination with cisplatin in the treatment of human ovarian cancer cell SKOV3 in vitro

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Abstract: Chloroquine is widely used as an effective and safe anti-malarial agent and has been a hotspot for its potential role in cancer therapies recently. Ovarian cancer is the most lethal gynecologic malignancy worldwide with chemotherapy drug resistance as a major obstacle in the treatment. But there is still no experiment to illustrate the relationship between chloroquine and ovarian cancer currently. The study focused on the issue and used CCK-8 assay, flow cytometry analysis, inverse phase light microscope observation, fluorescent microscope observation, Western Blot assay and acridine orange (AO)-relocation test to explore the mechanisms. We found out that chloroquine inhibited the proliferation of SKOV3 cells, presenting a synergetic effect with cisplatin. Chloroquine induced apoptosis and arrested SKOV3 cells in the G0/G1 phase, with an up regulation of p27 Kip1, and down regulation of CyclinD1 and cyclin-dependent kinase 4 (CDK4). Chloroquine at lower concentration combined with cisplatin caused lysosomal volume expansion and lysosomal membrane leakage. Chloroquine at higher concentration combined with cisplatin induced apoptosis and necrosis at the same time, but no similar lysosomal changes occurred. Chloroquine enhanced the inhibitory effects of cisplatin on SKOV3 cancer cells through the implement of internal apoptotic pathway or lysosomal death pathway depending on the chloroquine concentration.

Keywords: Chloroquine, cisplatin, ovarian cancer, growth inhibition, apoptosis, lysosomal death pathway

Introduction

Ovarian cancer is the most lethal gynecologic malignancy worldwide. At present, the standard treatment of comprehensive surgical staging, satisfied cytoreductive surgery (with residual lesion less than 1 cm in size) and postoperative adjuvant chemotherapy of platinum and paclitaxel cannot effectively improve the 5-year survival rate or prolong the survival period. The lack of efficient early molecular markers for diagnosis and drug resistance are two major obstacles for ovarian cancer treatment. Chemotherapy drug resistance seriously affects the curative effect and reduces the long-term survival rate. TP program, which is platinum-based with a combination of paclitaxel, is currently the standard chemotherapy of ovarian cancer. It is effective, but also easily to develop platinum resistance, presenting with a high relapse rate of cancer. In patients with ovarian cancer, the 5-year survival rate is about 30% [1], 90% of the deaths can be attributed to drug resistance [2]. Platinum resistance is the key problem and difficulty in improving the prognosis of ovarian cancer patients. The development of new drugs and strategies for chemotherapy to further improve its efficacy in ovarian cancer treatment is the top priority.

Chloroquine (CQ) is widely used as an effective and safe anti-malarial and anti-rheumatoid agent. It is also the only drug approved usage for clinical autophagy inhibitor [3]. Chloroquine has an incomparable advantage in the application of tumor therapy research, that is a direct access to clinical tumor treatment research with no need for an animal or phase I clinical trial. Recently, in the oncological research of breast cancer, lung cancer, colon cancer, melanoma, Burkitt lymphoma and so on, accumulating lines of evidence suggest that CQ has a

direct tumor cell killing effect as well as a sensitize to that of traditional cancer treatments by ionizing radiation and chemotherapeutic agents [4-7]. Chloroquine has been a hot spot for its potential role in cancer therapies. Whether chloroquine could inhibit ovarian cancer growth or sensitize cisplatin (CIS) in ovarian cancer treatment? There is still no experiment to illustrate the relationship between chloroquine and ovarian cancer currently, which is the incentive of our study.

SKOV3 cells belongs to serous papillary cystic gland cancer cells, which derived from one of the most common ovarian tissue types. The research on the drug inhibition of SKOV3 cells plays a guidance role in the drug treatment of ovarian cancer. In our study, using SKOV3 cells as a representative, we proved for the first time that chloroquine had a synergetic effect with cisplatin in inhibiting the growth of SKOV3 cells and then, we explored the related mechanisms. Results show that this process may be related to cell cycle arrest via influence of cell cycle control factors and the activation of lysosomal death pathway via the induction of related changes in lysosomes according to the chloroquine concentration.

Materials and methods

Cell and cell culture

Human ovarian cancer cells SKOV3 were purchased from Chinese academy of sciences, and were cultured as monolayers in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA), containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin; Sigma Chemical, St Louis, MO, USA) at 37°C in a humidified 5% CO_2 incubator.

Reagents and antibodies

Chloroquine was purchased from Sigma-Aldrich (St. Louis, MO, USA), Cisplatin was purchased from Enzo life sciences (Plymouth Meeting, PA, USA); Cell Counting Kit-8 was purchased from Dojindo (Tokyo, Japan); Annexin V-FITC Appotosis Detection Kit I was purchased from BD Pharmingen (San Jose, CA, USA); Cell cycle staining solution and PI solution was purchased from Multisciences Biotech (Hangzhou, China); Cell Cycle Regulation Kit was purchased from Cell Signaling Technology (Beverly, MA, USA); Acridine orange was purchased from Sigma Aldrich (St. Louis, MO, USA).

In vitro assay for cytotoxic activity (CCK-8 assay)

The cytotoxicity effect was measured by the CCK-8 assay. Cells (1×10^4/well) were seeded in 100 μL medium per well in 96-well plates (Costar Corning, Rochester, NY) and incubated at 37°C overnight. After incubation in a humidified 5% CO_2 incubator for 1, 2, 3 days with different drug treatment, the samples were incubated for another 1.5 hours with CCK-8 agent-medium. 3 wells were included in each treatment group, wells without cells as blanks and wells with medium only as control group. The absorbance at 450 nm was measured with a scanning multi-well spectrophotometer Bio Tek (Vermont, USA). All experiments were performed in triplicate. The cell growth inhibition was calculated with the following formula: % inhibition = (A450 of control-A450 of treated cells)/(A450 of control-A450 of blanks) × 100%.

Apoptosis assay

Annexin V-FITC was used in conjunction with propidium iodide (PI), to distinguish apoptotic (Annexin V-FITC positive, PI negative) from necrotic (Annexin V-FITC positive, PI positive) cells. Cells were collected and washed twice with cold PBS and incubated with Annexin V-FITC and PI in a binding buffer for 15 minute at room temperature according to manufacturer's instructions. Analysis of apoptotic cells was performed by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA, USA). 1×10^5 cells were acquired for each sample. Analysis was carried out by triplicate determination on at least three separate experiments.

Cell cycle analysis

Cells were prepared and treated as previous described. According to manufacturer's instructions, treated cells were trypsinized, washed once with PBS, and fixed in cold methanol overnight, both attached cells and floating cells were harvested. Fixed cells were washed once with PBS and resuspended in 1 mL agent A of the cell cycle staining solution containing PI solution, RNase and so on. Samples were incubated at 4°C for 30 minutes in the dark and

analyzed in flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ) immediately. The experiment was performed three times, and the ratio of cells in the G0/G1, intra-S and G2/M phases were expressed as mean ± SD.

Morphological analysis

Collect SKOV3 cells of logarithmic phase, approximately 5 × 10^5/ml, and seed 1 ml cell solution in each well of 6-well plates. After incubation overnight, discard the original culture medium and add chloroquine of 50 and 100 μM, then incubate the cells at 37°C/5% CO_2 for 48 hours. Examine for morphological changes of the cell membrane, cell serum and cell nucleus by inverted phase contrast microscope (Olympus, Tokyo, Japan). For the identification of the nucleus in apoptotic cells, cells were stained with PI solution containing 50 μg/mL propidium iodide, 0.1% sodium citrate plus 0.1% Triton X-100, then examined with an inverted fluorescence microscope (Nikon, Tokyo, Japan).

Acridine orange assay

Acridine orange (AO) is a lysosomotropic weak base with metachromatic features. Oligomeric form of highly concentrated and protonated AO (AOH^+) exhibits red fluorescence, as is the case in intact lysosomes. Lysosomal alkalinization and translocation of lysosomal content to the cytosol during lysosomal stress results in the formation of the monomeric deprotonated form of AO with green fluorescence [8]. SKOV3 cells in a 96-well plate were labeled with AO (5 μg/ml in PBS) for 15 min at 37°C, rinsed 3 times with PBS, re-fed with phenol-red free medium, and then analyzed for fluorescence intensity using a BiotekMx microplate reader (excitation/emission of 475/620 nm for red fluorescence, excitation/emission of 475/520 nm for green fluorescence). To analyze the lysosome integrity, the fluorescence intensity of the labeled cells was tracked over a 48 hours period of time. The corresponding red/green fluorescence intensity of control group with non-drug treatment is defined as the average 100%, and the rest were converted into a percentage normalized to the control group. Each experiment was repeated three times, and the results were expressed as mean ± SD.

Western blot analysis

SKOV3 cells were treated with drugs in designed concentrations for 48 hours. Equal amounts of protein from control cells and treated cells were subjected to electrophoresis in sodium dodecyl sulfate-acrylamide gel followed by electroblot transfer to PVDF membrane (Bio-Rad, Hercules, CA). Membranes were probed with antibodies for CyclinD1 (Cell Signaling Technology, #2926), CDK4 (Cell Signaling Technology, #2906), p27 Kip1 (Cell Signaling Technology, #2552) and β-tubulin antibody (Santa-Cruz Biotechnology, Santa Cruz, CA, USA). Following the primary antibody incubation, the blots were washed and incubated with 1:10000 dilution of Goat Anti-Mouse IgG or Goat Anti-Rabbit IgG (West Grove, PA, USA). The immunoreactive bands were visualized by enhanced chemiluminescence using the ECL detectionsystem (Thermo Scientific, MA, USA).

Results

Synergetic effect of chloroquine and cisplatin in growth inhibition of SKOV3 cells

Chloroquine showed a dose- and time-dependent inhibitory effect on the growth of SKOV3 ovarian cancer cells. The results of cytotoxic activity of chloroquine and cisplatin against SKOV3 cells were shown in **Figure1A**, **1B**. When the SKOV3 cells were treated with the two drugs in combination, the inhibition rate increased significantly compared with the single use of either drug, as shown in **Figure 1C**, **1D**. Chloroquine enhanced the CIS-induced growth inhibition of SKOV3 cells. The combined treatment of two drugs showed significant synergies.

Effects of chloroquine on cell apoptosis and necrosis

Treatment with chloroquine on SKOV3 cells resulted in morphological changes consistent with apoptosis. Under phase contrast microscope, SKOV3 cells without drug treatment were in plump long fusiform or polygon shape, with high cell density and good growth state. Cell nucleus were located in the central, with clear nucleoli and a uniform PI dying under fluorescence microscope. Few apoptotic cells appeared. Compared with the control, SKOV3 cells treated with chloroquine 50 or 100 uM shriveled to round or oval shape, showing folds

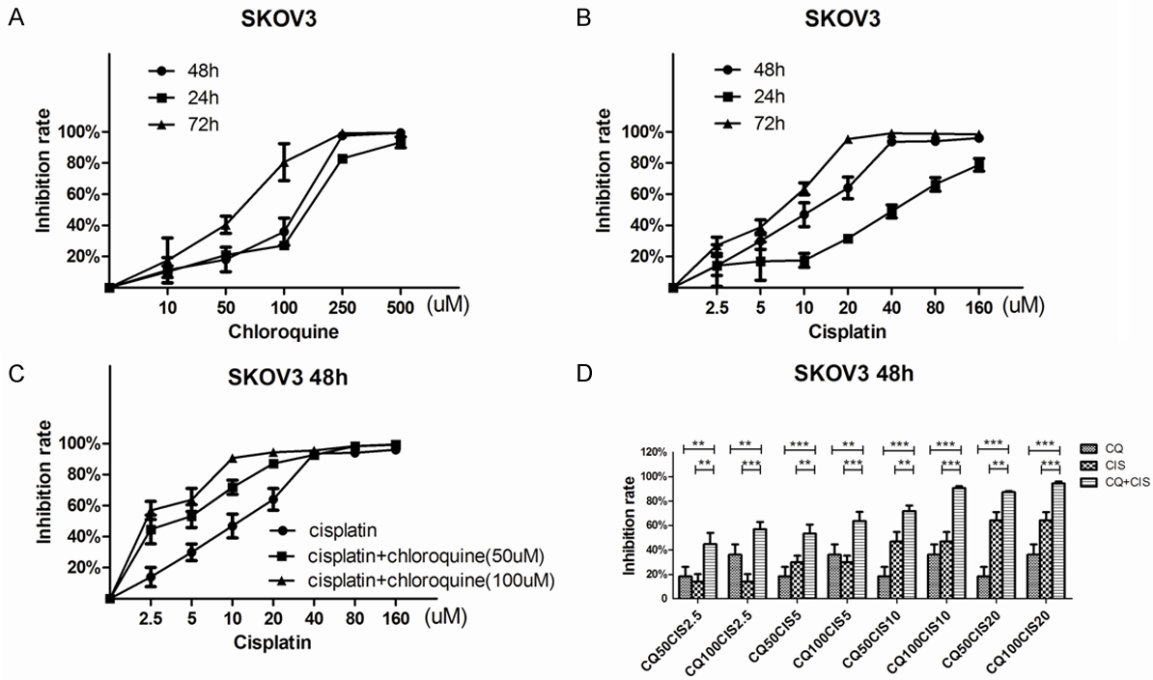

Figure 1. Effects of CQ and CIS on the proliferation of SKOV3 cells. A. Inhibition of SKOV3 growth by CQ for 24, 48, 72 hour assessed by CCK-8 method. B. Inhibition of SKOV3 growth by CIS for 24, 48, 72 hour assessed by CCK-8 method. C. Inhibition of SKOV3 growth by CIS combined with CQ 50, 100 uM for 48 hour assessed by CCK-8 method. D. Combination treatment of CQ with CIS for 48 hour resulted in decreased proliferation in SKOV3 cells assessed by CCK-8 method. The inhibitory effects of SKOV3 cells induced by CQ and CIS was time- and dose-dependently increased. CQ enhanced CIS-induced growth inhibition of SKOV3 cells. Results were expressed as means ± SD of independent experiments performed in triplicate. The asterisk indicated a significant difference between the assessed groups by ANOVA followed by Bonferroni adjustment method (*P < 0.05, **P < 0.01, ***P < 0.001).

of cell membrane and a higher density of cytoplasm. Cells presented with vacuolation and eventually detached from the surface of the tissue culture dish. Characteristics of apoptosis were also seen with PI staining, including uneven dying, chromatin concentration, nucleus pycnosis and fracture. With the increase of drug concentration, the number of exfoliated cells and apoptosis cells also increased significantly (**Figure 2A**).

We also evaluated the induction of apoptosis on SKOV3 cells by FACS analysis. After the treatment with CQ (10, 50, 100 uM) and CIS (5 uM) for 48 hours, we stained the cells with Annexin V-FITC and PI, as described above. As shown in **Figure 2B**, **2C**, we found a significant increase of apoptotic cells treated with two drugs in combination, compared to untreated cells or cells treated with a single drug. In details, we found that the treatment with CIS 5 uM alone induced apoptosis in only about 8.73% of SKOV3 population and the treatment combined with chloroquine 10, 50, 100 uM on SKOV3 cells for 48 hrs resulted in significantly higher percentage of apoptotic cells, ie. 15.35 ± 1.69%, 23.75 ± 2.75% and 21.32 ± 2.01% respectively compared to 3.63 ± 0.41% of untreated cells. On the other hand, the cell necrosis rate increased obviously only when 5 uM cisplatin combined with chloroquine of a higher concentration (100 uM), rising from 5.59 ± 0.93% of the control group to 19.79 ± 3.33%. The results prompted that cisplatin combined with chloroquine of a low concentration mainly caused cell apoptosis while with chloroquine of a high concentration instead increased cell necrosis meanwhile.

Effects of chloroquine on cell cycle regulation and cell cycle-related protein expression

Effect of chloroquine on cell cycle distribution was determined to gain insights into the mechanism of its antiproliferative activity. As shown in **Figure 3A**, **3B**, a 48 hours exposure of SKOV3 cells to chloroquine resulted in significant accumulation of cells in G0/G1 phase, accompanied

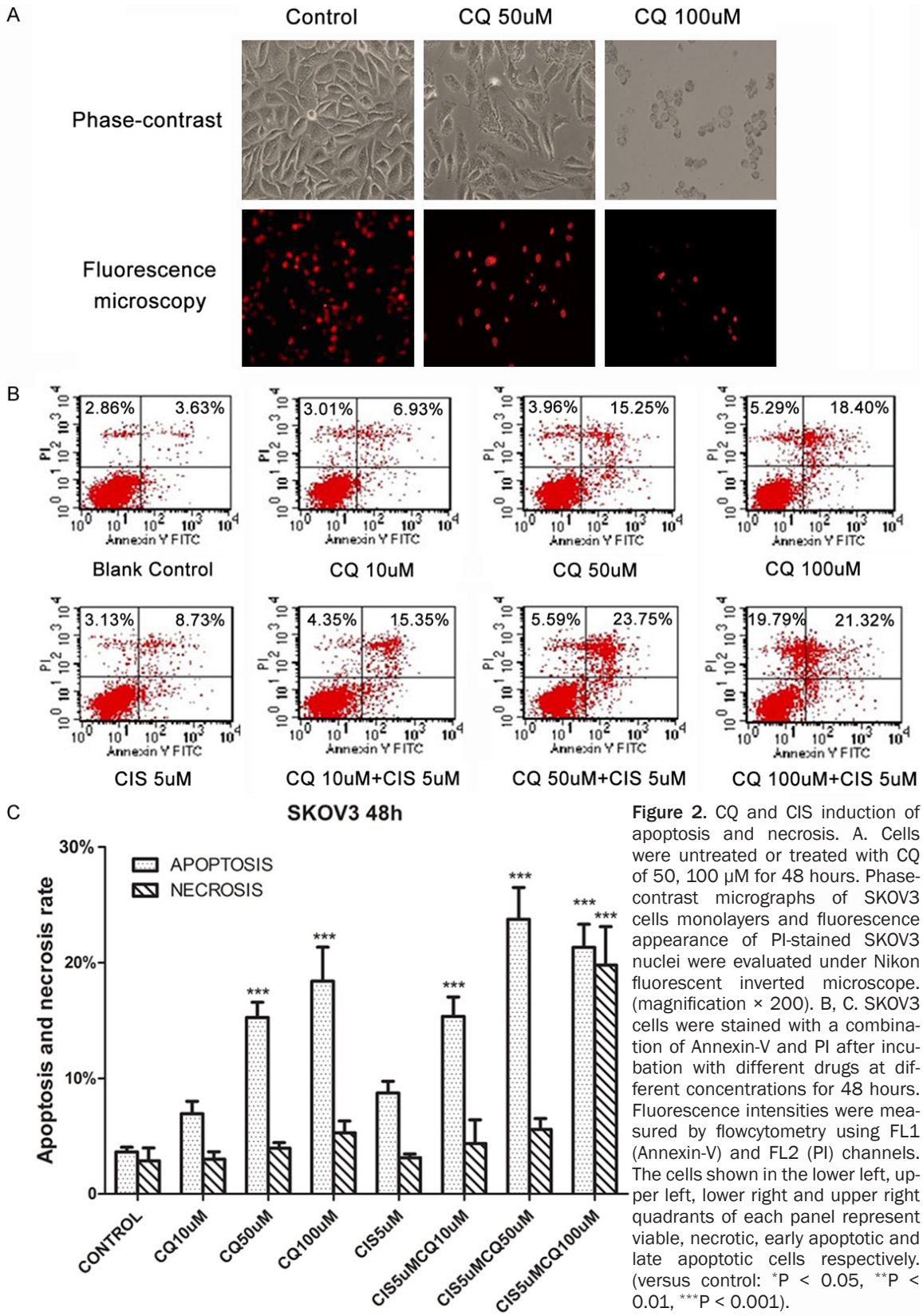

Figure 2. CQ and CIS induction of apoptosis and necrosis. A. Cells were untreated or treated with CQ of 50, 100 μM for 48 hours. Phase-contrast micrographs of SKOV3 cells monolayers and fluorescence appearance of PI-stained SKOV3 nuclei were evaluated under Nikon fluorescent inverted microscope. (magnification × 200). B, C. SKOV3 cells were stained with a combination of Annexin-V and PI after incubation with different drugs at different concentrations for 48 hours. Fluorescence intensities were measured by flowcytometry using FL1 (Annexin-V) and FL2 (PI) channels. The cells shown in the lower left, upper left, lower right and upper right quadrants of each panel represent viable, necrotic, early apoptotic and late apoptotic cells respectively. (versus control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

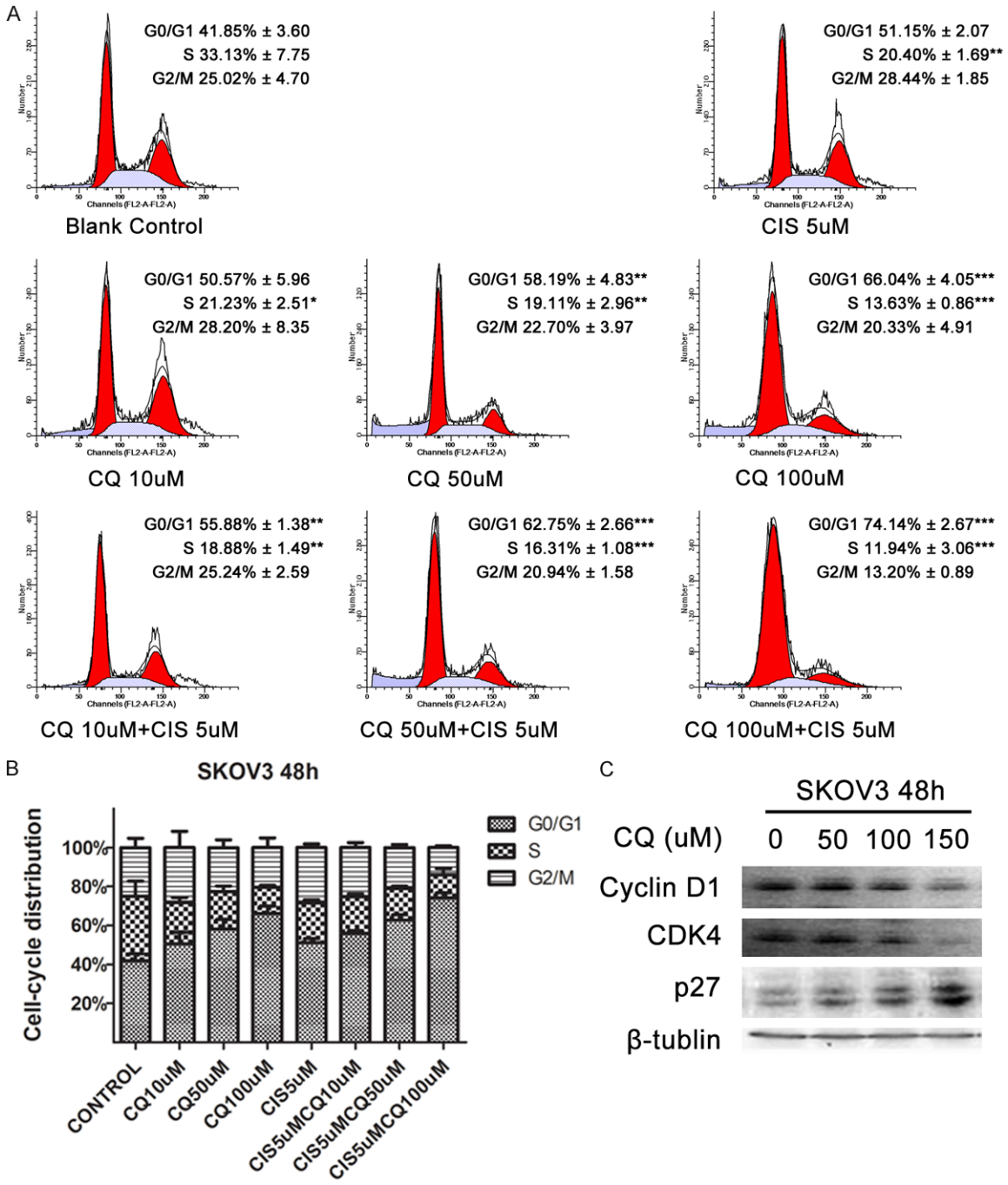

Figure 3. Inhibition of cell-cycle progression and changes of cell-cycle proteins expression in SKOV3 cells induced by CQ and CIS. A, B. The cells were treated with CQ and CIS of different concentrations, alone or in combination for 48 hours. CQ of higher concentrations caused a concomitant accumulation of cells in G0/G1 phase (versus control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). C. CQ changed expression of proteins involved in the G1/S phase transition. In vitro treatment of SKOV3 cells with CQ for 48 hours caused up-regulation of p27 Kip1 and down-regulation of CDK4 and CyclinD1.

with a decrease of cells in S phase and no significant numerical changes in cells of G2/M phase. When 5 uM cisplatin used in combination with chloroquine of 10, 50, 100 uM, the results were the same. Combined treatment of two drugs showed significant synergies. In addition, the transition of G0/G1 phase to S phase was related to CDK4/CDK6 complexes forma-

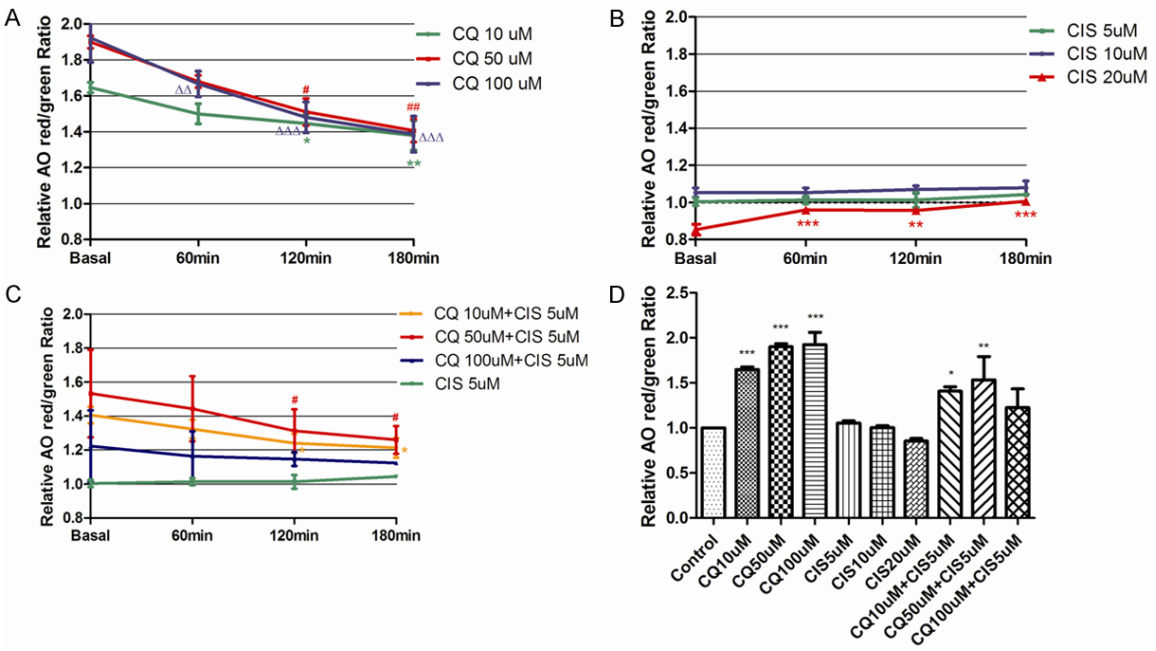

Figure 4. Alternations of the volume and membrane integrity changes of lysosomes was estimated by the AO relocation test. A-C. SKOV3 cells were treated with the indicated drugs for 3 hours, labeled with AO and measured for the cellular fluorescence at the indicated time points, and the average red/green ratio normalized to the non-treatment condition was presented with standard deviation indicated. D. The cells were treated with indicated drugs for 48 hours, labeled with AO and measured for the cellular fluorescence at the indicated time points, and the average red/green ratio normalized to the non-treatment condition was presented with standard deviation indicated. The average was normalized to the control group and presented with standard deviation indicated (Representatives of three independent experiments, $^{*,\#,\Delta}P < 0.05$, $^{**,\#\#,\Delta\Delta}P < 0.01$,$^{***,\Delta\Delta\Delta}P < 0.001$).

tion, and CyclinD1 played a positive regulatory role in the process. Chloroquine induced down-regulation of CDK4 and CyclinD1, and up-regulation of p27 Kip1 (**Figure 3C**), suggesting the antiproliferative effect of chloroquine may be due to G0/G1 cell cycle arrest.

Effects of chloroquine on lysosomal volume and lysosomal membrane permeabilization

To determine the effect of chloroquine on lysosomal volume and lysosomal membrane permeabilization, AO-relocation assay was applied. Lysosomal membrane permeability can be detected by a rapid decrease in the red/green fluorescence ratio [9, 10]. As shown in the **Figure 4D**, chloroquine increased the red/green fluorescence ratio compared with the control group, indicating increased lysosomal volume and AO content in the lysosome compared with the control group. In the **Figure 4A**, the red/green fluorescence ratio in high concentration (100 uM) chloroquine treatment group significantly decreased after adding AO for 1 hour, while in low concentration (10, 50 uM) chloroquine treatment group, there was a remarkable decrease after 2 to 3 hours. This indicated while chloroquine increased lysosomal volume it also dampened the integrity of lysosomes and the leakage of high concentration drug group was faster than that of low concentration groups. As **Figure 4B**, **4C** shown, cisplatin 5 uM induced no obvious change of lysosomal volume or lysosomal membrane integrity. But when the treatment were combined with chloroquine of low concentration (10, 50 uM) together, the results were totally different, suggesting that CQ initiates an early lysosomal destabilization pathway leading to apoptosis in the combined treatment. When 5 uM cisplatin were used in combination with chloroquine of high concentration (100 uM), there was no corresponding performance, indicating that chloroquine of high concentration combined with cisplatin induced slightly the increase of both lysosomal volume and lysosomal membrane leakage. Cell death may not rely on the same process when cisplatin combined with chloroquine of different concentrations, in accordance with the previous findings in cell apoptosis and necrosis assay.

Discussion

Chloroquine alone and combined with cisplatin had obvious inhibitory effect on the growth of human ovarian cancer cell SKOV3, presenting a time- and dose-dependent effect. Dose under simulated clinical drug concentration range of chloroquine and cisplatin had a synergetic effect on the inhibition of ovarian cancer cells, providing with the feasibility of clinical application.

The susceptibility to certain apoptosis inducement of proliferating cells often depended on the cell cycle. Some researchers put forward an opinion that apoptosis might be a suspended type of mitosis and cell cycle regulation point seemed to be the central hub connecting cell proliferation and apoptosis, a two-way switch to control cell proliferation and death [11]. Previous studies of apoptosis induced by chloroquine reported that low-dose chloroquine alone or in combination with other drugs had no obvious influence on the cell cycle distribution of non-small cell lung cancer [12], but could cause G1 phase cell block in mouse breast cancer cells [13] and human breast cancer cells MCF-7 [14], or a G2/M phase arrest in human breast cancer Bcap-37 cells [15]. So apoptosis was multipoint launched, which could happen in any phase of the cell cycle. The cell cycle specificity of apoptosis depended on the damage degree of stimulation and the damage repair ability in different cell cycle period. Cisplatin is a non-cell cycle-specific drug, inducing tumor cell apoptosis mainly by getting in combination with DNA chain to form an outra- or intra-crosslinked structure to induce tumor cell DNA damage and activate a variety of apoptosis signaling pathway. Platinum resistance mechanism involved increased damage repair function in cells after DNA damage polymer was formed. The G0/G1 phase block induced by chloroquine in combination with cisplatin had a double meaning: providing “buffer time” for the repairment of DNA damage, and also increasing the exposure of DNA and its repair system to exogenous noxious stimulus. In the contest, the result might be an increase of the frequency and extent of DNA damage, at the same time a reduction of the body’s anti-injury repair capacity, inducing apoptosis of damage cells ultimately.

There are 4 checkpoints in the cell cycle, the late G1 restriction point, the DNA damage checkpoints of G1-S phase transition and G2-M phase transition, and the mitotic metaphase checkpoint of M-G1 phase transition. The late G1 restriction point monitor the cell size of G1 stage. The G1-S checkpoint monitor DNA damage. The G2-M checkpoint monitor DNA damage and DNA copy process. Through controlling the restriction points to lead to delays in G1 and G2 phase, cells achieve time to finish repairment before cell replication and mitosis when they are damaged. The key molecules in eukaryotic cells to regulate cell cycle include cyclin-dependent kinases (CDKs), cyclins and cyclin-dependent kinase inhibitors (CKIs). Various extracellular stimuli control the cell cycle through regulating the formation of different Cyclin/CDK complex. In G1 phase, the cell cycle protein D activated under the mitotic signal, and combined to the CDK4 and CDK6, resulting in the activation of CyclinE/CDK2 compound in late G1 phase and CyclinA/CDK2 complex in S phase. CyclinD promote mitosis through regulating the checkpoint in late G1 phase. Our western blot results was in consistent with the previous view. With the increase of chloroquine concentration and treatment time, CyclinD1 and CDK4 cell cycle protein expression reduced. Therefore we conclude that chloroquine influenced the formation of CDK4/CDK6 and CyclinE/CDK2 complex through down regulating CyclinD1, CDK4 and up regulating of p27 Kip1, which finally lead to G0/G1 cell cycle phase arrest.

Chloroquine caused lysosomal dysfunction with the result of cellular debris and toxicity accumulation, which leaded to the lysosomal membrane permeabilization (LMP) change ultimately [16]. Low-dose chloroquine and cisplatin can induce apoptosis of SKOV3 cells in a dose-dependent manner in a certain range of concentrations. This process may be related to the G0/G1 cell cycle phase arrest, lysosomal volume expansion and lysosomal membrane leakage caused by chloroquine which resulted in internal and related apoptotic pathway activation. High-dose chloroquine with cisplatin obviously increased cell apoptosis and cell necrosis at the same time. The lysosomal volume expansion or lysosomal membrane leakage increase didn’t occur. Take these results into consideration together, we suspect that low concentration chloroquine combined with cisplatin induced an increase in lysosomal vol-

ume and membrane leakage, causing an occurrence of small-scale LMP to achievethe activation of apoptotic pathways. High concentration chloroquine in combination with cisplatin may cause large scale LMP or lysosomal rupture in the early stage. A large number of lysosomal enzymes in the cytoplasm are activated, leading to cell autolysis necrosis to realize the lysosome death pathways. In previous studies related to chloroquine inhibition on A549 lung cancer cell growth, Fan, etc. [17] proposed that CQ inhibit A549 lung cancer cell growth at lower concentrations by increasing the volume of lysosomes, at higher concentrations, its ability to increase the volume of lysosome gradually declined. Our experiment had the similar results. Since there are often defects in the apoptotic pathways in cancer cells [18], lysosomal targeting drugs are noteworthy since they can activate the lysosome death pathways and kill tumor cells with apoptosis defects. Specifically induction of LMP may become a new method for the treatment of cancer. This result provides a theoretical basis for the treatment combining chloroquineand cisplatin, and ultimately provide new options for drug treatment of ovarian cancer.

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Disclosure of conflict of interest

None.

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