Effect of oxaliplatin combined with polyenephosphatidylcholine on the proliferation of human gastric cancer SGC-7901 cells

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Abstract. Oxaliplatin (L-OHP) is a platinum compound that is widely used to treat certain solid tumors, including gastric tumors. L-OHP is an effective anti-cancer treatment; however, its usage increases the probability of patients developing hepatic injury with inflammation, referred to as chemotherapy-associated steatohepatitis. The present study aimed to evaluate the outcome of L-OHP treatment combined with polyenephosphatidylcholine (PPC), a major component of essential phospholipids used to treat steatohepatitis, on SGC-7901 gastric cancer cell proliferation. This would help to determine whether combination therapy with L-OHP and PPC is clinically beneficial for patients with gastric cancer. The viability of SGC-7901 cells was verified by an MTT assay; flow cytometry was used to analyze the cell cycle and rates of cell apoptosis; oxidation-related indicators were measured by spectrophotometry, and the expression of cell cycle- and apoptosis-related proteins was determined by western blotting. The results demonstrated that L-OHP significantly inhibited SGC-7901 cell growth in a dose- and time-dependent manner (F=194.193, P<0.01 and F=12.428, P=0.01, respectively). Furthermore, PPC stimulated the growth of SGC-7901 cells and greatly promoted their apoptosis induced by L-OHP, which was supported by the upregulation of cytochrome c and the downstream activation of caspases 3 and 9. Finally, following treatment with a combination of PPC and L-OHP, the expression of cyclins D1 and E was downregulated; however, PPC did not alter the production of reactive oxygen species caused by L-OHP (P=0.88). The present study determined that the combination of L-OHP and PPC exerts a synergistic anti-tumor effect, suggesting that L-OHP and PPC combination therapy may be used as a treatment for patients with gastric cancer that reduces the side effects of L-OHP without inhibiting its efficacy.

Introduction

The incidence and mortality of gastric cancer have decreased in recent years; however, it remains the fourth most common type of cancer in the world (1,2). In the treatment of localized resectable cases, surgery is the only effective approach; however, numerous patients with gastric cancer are at a very advanced stage when they are diagnosed and do not clinically benefit from surgical resection (3). Therefore, for such patients, it is essential to combine surgical treatment with effective chemotherapy approaches.

Oxaliplatin (L-OHP) is a third-generation platinum compound that has been developed as an alternative pharmacological compound to cisplatin (4). Its usage decreases the tumor resistance, inadequate oral bioavailability and poisonous side effects associated with the use of cisplatin (5,6). LOHP is currently used in combination with other chemotherapy drugs, including 5-fluorouracil, to treat advanced gastric cancer (7,8). The response rate of patients to L-OHP is high (53-59%), and L-OHP exhibits low toxicity (4,6,9-11). However, although L-OHP is an effective anti-tumor treatment, it may cause severe adverse reactions in patients, as it enhances inflammatory activity, thus increasing the risk of hepatic injury (6,12). This phenomenon may result in the development of chemotherapy-associated steatohepatitis (CASH), which is a serious form of non-alcoholic fatty liver disease (8,13).

Polyenephosphatidylcholine (PPC) is one of the primary active components of essential phospholipids, and has high affinity and bioavailability for cellular and subcellular membranes (14). Furthermore, PPC serves a crucial role in maintaining the fluidity and function of biomembranes, and several studies have demonstrated its hepatoprotective effects (9-11,15-17).

The principal aim of the current study was to determine the effect of PPC combined with L-OHP on the growth and apoptosis of SGC-7901 cells. The present study aimed to evaluate...
whether PPC acts as a beneficial supplement, which could be used alongside L-OHP to protect against liver damage but not to compromise its anti-tumor effects.

Materials and methods

Reagents. L-OHP and PPC were purchased from Sanofi S.A (Paris, France). Fetal bovine serum (FBS) and RPMI 1640 medium were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Trypsin and MTI were obtained from Amresco, LLC. (Solon, OH, USA). The kits for glutathione peroxidase (GSH-Px), malondialdehyde (MDA) and superoxide dismutase (SOD) were all purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Cell culture plates were purchased from Corning Inc. (Corning, NY, USA), and X-ray film was obtained from Kodak (Rochester, NY, USA). Poly (ADP-ribose) polymerase and antibodies against cytochrome c (#11940), B-cell lymphoma-2 (Bcl-2) (#15071), Bcl-2 antagonist/ killer (Bak) (#12105), Bcl-2-associated X protein (Bax) (#5023), and cleaved caspase-3 (#9661), -8 (#8592), and -9 (#7273) were all purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. Cells from the human gastric adenocarcinoma cell line SGC-7901 and the human vascular endothelial cell line HMEC-1 were purchased from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium containing 10% FBS, 100 U/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified atmosphere containing 5% CO₂.

Cell viability assay. An MTT assay was used to assess cell viability. SGC-7901 and HMEC-1 cells were seeded in 96-well plates (200 µl/well containing 6,000 cells/well). After 24 h, the medium was replaced with complete culture medium supplemented with different concentrations of L-OHP and/or PPC. Following treatment for 24, 48 or 72 h, the cells were incubated with 0.5 mg/ml MTT for 4 h at 37°C. The MTT solution was discarded, and 150 µl dimethyl sulfoxide was subsequently added to each well. Absorbance was measured at 570 nm using a Sunrise™ microplate reader (Tecan, Männendorf, Switzerland) and cell viability was expressed as the ratio of absorbance of the experimental group to that of the control group. Each experiment was repeated ≥3 times.

Cell cycle analysis. The cell cycle was analyzed by flow cytometry. Cells were cultured in complete medium supplemented with 3.5 µg/ml L-OHP combined with various concentrations of PPC (0-64 µmol/l) for 48 h. For cell cycle analysis, cells were collected, washed twice with 0.01 M PBS and fixed in 70% ethanol at 4°C overnight. Subsequently, the cells were washed with PBS, digested with 200 µl trypsin (1 mg/ml) at 37°C for 30 min and stained with 800 µl propidium iodide (PI; 50 µg/ml) at room temperature for 30 min. Cells were subsequently washed with PBS and immediately analyzed via flow cytometry. The percentage of SGC-7901 cells in each phase of the cell cycle (G0/G1, S and G2/M) was calculated using the MultiCycle AV software program version 1.0 (Phoenix Flow Systems, San Diego, CA, USA).

Cell apoptosis assay. Cell apoptosis was evaluated by flow cytometry. SGC-7901 cells were treated with 3.5 µg/ml L-OHP combined with various concentrations of PPC (0-64 µmol/l) for 48 h. Cells were digested by 2.5 g/l trypsin, washed with 0.01 mol/l PBS twice, fixed with cold 95% alcohol at 4°C for 30 min, stained with PI and annexin V-fluorescein isothiocyanate, and analyzed with a FACSsort flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The apoptotic index (AI) was calculated as follows: AI = (number of apoptotic cells / total number) x 100%. Each experiment was repeated ≥3 times.

SOD assay. SOD activity in SGC-7901 cells was determined using a kit that utilizes a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine by forming a red formazan dye. The optical density of the formazan dye was then measured at 550 nm by a spectrophotometer. The enzyme activity was expressed as U/mg protein, and 1 U of enzyme was defined as the enzymatic activity inhibiting the autoxidation of pyrogallol (P0381; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) by 50%.

GSH-Px assay. GSH-Px activity in SGC-7901 cells was evaluated by a previously described method, through a coupled assay using H₂O₂ and dithiobis-nitrobenzoic acid (D8130; Sigma-Aldrich; Merck Millipore). Enzymatic activity (1 U) represented a decrease in GSH concentration of 1 mmol/l/min following subtraction of non-enzymatic mode. All measurements were performed in triplicate, and the results were normalized per mg protein.

MDA assay. Lipid peroxidation was assayed by measuring the concentration of MDA via spectrophotometry. The concentration of MDA in SGC-7901 cells was measured using thiobarbituric acid (T5500; Sigma-Aldrich; Merck Millipore) in conjunction with commercially available kits following the manufacturer’s protocol (Nanjing Jiancheng Bioengineering Institute). The samples were detected by dual wavelength in order to eliminate the influence of glycation and other lipidic aldehydes. All measurements were performed in triplicate and the results were expressed as nmol MDA per mg protein.

Western blot analysis. Following treatment with 3.5 µg/ml L-OHP and different concentrations of PPC (0-64 µmol/l) for 48 h, cells were washed twice with cold PBS at 4°C, extracted into radioimmunoprecipitation assay lysis buffer on ice for 30 min and then sonicated at 3 W for 15 sec. The cell lysates were centrifuged at 12,000 x g for 10 min at 4°C. The total protein content was determined using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Lysate aliquots were diluted with 6X SDS sample buffer and boiled for 5 min. A total of 30 µg protein from each treatment group was separated by 12% SDS-PAGE and then electrophoretically transferred onto nitrocellulose membranes from Pall Gelman Sciences (Port Washington, NY, USA). After being blocked at room temperature for 2 h with 5% non-fat milk in TBS with 0.1% Tween-20 (TBST), the membranes were incubated overnight at 4°C with antibodies against cytochrome c, Bcl-2, Bak, Bax, cleaved caspase-3, -8 and -9, cyclin D1 (#9661; Cell Signaling Technology, Inc.) and cyclin E (#9376; Cell Signaling
Technology, Inc.) at a dilution of 1:1,000, or with anti-β-actin antibody (#3700; Cell Signaling Technology, Inc.) at a dilution of 1:5,000.

After being washed with TBST, the membranes were incubated with horseradish Px-conjugated goat anti-rabbit immunoglobulin G (#7076; Cell Signaling Technology, Inc.) at a dilution of 1:5,000 at room temperature for 2 h. Following additional washes with double distilled water (5 min for three times), the membranes were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (#34094; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and exposed to X-ray film in the dark room. The densities of the bands were determined by standard scanning densitometry with normalization of the densitometry measurements to that of β-actin.

Drug combination analysis. The combination index (CI) was calculated based on the Chou-Talalay equation (12,18). CI values were calculated using the formula: CI = (D1) / (Dx)1 + (D)2 / (Dx)2 for mutually exclusive drugs, where D refers to the drug dose. In the denominator of the equation, (Dx)1 represents the D1 ‘alone’ that inhibits a system by certain percentage and (Dx)2 represents the D2 ‘alone’ that inhibits a system by certain percentage. In the numerator of the equation, (D)1 + (D)2 ‘in combination’ also inhibit a system by certain percentage. The CI values were calculated according to the different percentages of inhibition, from 0.05 to 0.95 (which represents 5-95% cell death). Briefly, CI<0.85, 0.85<CI<1.15 and CI>1.15 indicated a synergistic, additive and antagonistic effect, respectively.

Statistical analysis. Results were represented as the mean ± standard deviation. Significance was assessed by one-way analysis of the variance following appropriate transformation to normalized data and equalized variance where necessary. Differences in cell viability were compared by F-test. Statistical analysis was performed using SPSS statistical software version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Cell viability. The effect of L-OHP on SGC-7901 cell viability is presented in Fig. 1A. The growth of gastric cancer cells was inhibited by L-OHP in a time- and dose-dependent manner (F=194.193, P=0.0027 and F=12.428, P=0.01, respectively). The effect of PPC on the viability of SGC-7901 cells is presented in Fig. 1B. Low concentrations of PPC (2-16 µmol/l) increased cell viability, whereas high concentrations of PPC (32-64 µmol/l) decreased cell viability. All effects were dose-related (F=373.769, P<0.01) but not time-related (F=0.077, P=0.782). L-OHP also decreased HMEC-1 cell viability in a dose-dependent manner (F=6.23, P=0.032; Fig. 1C). However, PPC did not affect HMEC-1 cell viability (P=0.76; Fig. 1D), and therefore, it did not affect the growth of vascular endothelial cells.

Combined treatment. The half maximal inhibitory concentration (IC_{50}) of L-OHP was calculated as 5.16, 3.89 and 0.89 µg/ml

![Figure 1](image-url)
following 24, 48 and 72 h treatment, respectively. Calculations were performed by Graphpad Prism software version 6 (Graphpad Software, Inc., La Jolla, CA, USA). Therefore, for the combination groups, 3.5 µg/ml L-OHP was selected, which was slightly lower than the IC_{50} for 48 h. Combination groups were divided into three groups: i) Control group (I); ii) L-OHP group (II); and iii) L-OHP + different concentrations of PPC groups (III_{a}-III_{f}), as indicated in Table I. A low concentration of PPC combined with L-OHP inhibited cell viability in a synergistic manner, which suggested that low concentrations of PPC may enhance the anti-proliferative effect of L-OHP in SGC-7901 cells. However, high concentrations of PPC reduced this anti-proliferative effect slightly (Table I and Fig. 2).

Cell apoptosis. Treatment with L-OHP alone resulted in a slight increase in the number of apoptotic cells compared with untreated cells. Treatment with varying concentrations of PPC resulted in a slight increase in the number of apoptotic cells compared with untreated cells; however, this effect was not dose-related (P=0.07; Fig. 3A). By contrast, the combination of L-OHP and PPC treatment resulted in a significant increase in the rate of apoptosis compared with L-OHP treatment alone (Fig. 3B). These results demonstrated that PPC enhanced the apoptosis induced by L-OHP; however this effect was not dose-related (P=0.46; Fig. 3B and C).

Apoptotic proteins expression. The levels of cytochrome c, Bcl-2, Bax, caspase-9 and caspase-3 expression were analyzed by western blotting (Table II and Fig. 4) in order to characterize the signaling pathways involved in L-OHP-induced apoptosis. The release of cytochrome c from mitochondria activates downstream caspases, and is a critical step in the apoptotic cascade (19). The results indicated that there was a significant increase in cytochrome c levels in the cytosol following 48-h treatment with L-OHP. The expression of the anti-apoptotic protein Bcl-2 was decreased, whereas the expression of the pro-apoptotic protein Bax was increased, following L-OHP treatment. In addition, caspase-9 and caspase-3 activation was also examined in the present study. Upon apoptotic stimulation, caspase-9 and caspase-3 are cleaved into active fragments (20). The results of the western blot analysis indicated that levels of the cleaved activated forms of caspase-9 and caspase-3 significantly increased following L-OHP treatment and that PPC greatly promoted the apoptotic effect induced by L-OHP via a similar mechanism.

Cell cycle. FACScan (BD Biosciences) analysis of SGC-7901 cells stained with PI demonstrated that exposure to 3.5 µg/ml L-OHP for 48 h significantly induced cell cycle arrest in the G0/G1 phase compared with control cells (P=0.012), and that PPC enhanced the cell cycle arrest induced by L-OHP (P=0.035). However, treatment with different concentrations of PPC had no significant effect on cell cycle distribution (P=0.75; Fig. 5).

Cell cycle regulatory proteins. To determine the mechanism by which L-OHP induces G0/G1 arrest, the expression of the relevant regulatory proteins, cyclins D1 and E of the G0/G1 phase, was examined. As presented in Table III and Fig. 6, L-OHP downregulated the expression of cyclins D1 and E and this effect was enhanced by PPC (P=0.027). However, these effects were not dose-dependent (P=0.38).

MDA levels, SOD activity and GSH-Px activity. To assess the intracellular oxidant and antioxidant status, the MDA levels, SOD activity and GSH-Px activity were evaluated in SGC-7901 cells (Fig. 7). The MDA levels in the combined groups (treated with L-OHP and varying concentrations of PPC) were significantly higher, and the SOD and GSH-Px activities were significantly lower, compared with those in the control group. However, there were no significant differences in MDA levels, SOD activity or GSH-Px activity-between the combined groups and those treated with L-OHP alone (P=0.88).

Discussion

General improvements in health and quality of life mean that life expectancy continues to increase. However, the incidence of gastric cancer is also gradually increasing, particularly in elderly people (13,21). The majority of patients with gastric cancer are already at a very advanced stage when they are diagnosed (22). The palliative treatment accepted for
advanced gastric cancer, including recurrent and metastatic cancer, is chemotherapy, which is much better than the best supportive care in improving the survival and quality of life of patients (14,23). Furthermore, it is generally accepted

Table II. Relative intensity of Bcl-2, Bax, pro-caspase-9, cleaved caspase-9, pro-caspase-3, cleaved caspase-3 and cytochrome c.

| Protein Group | Group I | Group II | Group IIIa | Group IIIb | Group IIIc | Group IIId | Group IIIe |
|---------------|---------|----------|------------|------------|------------|------------|------------|
| Bcl-2         | 100     | 82       | 41          | 38         | 39         | 40         | 41         | 43         |
| Bax           | 100     | 132      | 178         | 180        | 182        | 179        | 177        | 177        |
| Pro-caspase-9 | 100     | 73       | 47          | 51         | 49         | 51         | 47         | 50         |
| Cleaved caspase-9 | 100    | 125      | 152         | 155        | 155        | 153        | 157        | 152        |
| Pro-caspase-3 | 100     | 93       | 73          | 70         | 69         | 70         | 69         | 72         |
| Cleaved caspase-3 | 100   | 123      | 151         | 153        | 155        | 153        | 155        | 157        |
| Cytochrome c  | 100     | 143      | 189         | 188        | 192        | 194        | 188        | 190        |

*P<0.05 compared with the control (group I); P<0.05, compared with the single-drug group (group II). Bcl-2, B-cell lymphoma-2; Bak, Bcl-2 antagonist/killer; Bax, Bcl-2-associated X protein.

Figure 3. (A) Effect of L-OHP alone on the apoptosis of SGC-7901 cells. (B) Effect of L-OHP in combination with PPC on the apoptosis of SGC-7901 cells. (C) After treatment with 3.5 µg/ml L-OHP combined with different concentrations of PPC for 48 h, cell apoptosis was evaluated by flow cytometry (P<0.05 vs. negative control; P<0.05 vs. 3.5 µg/ml L-OHP alone). L-OHP, oxaliplatin; PPC, polyenephosphatidylcholine; PI, propidium iodide.
that chemotherapy decreases the probability of relapse and improves patient survival rates, due to the high sensitivity of gastric cancer to chemotherapy (15,24). Thus, it is necessary to develop effective chemotherapy drugs to treat patients with advanced gastric cancer.

L-OHP is a new-generation platinum compound that exhibits low toxicity and lacks cross-drug resistance with cis-diamminediglycolatoplatinum, and has expanded the range of effective treatment options currently available for patients with advanced gastric cancer (16,17,25,26). It has been reported that platinum causes mitochondrial dysfunction, possibly by inhibiting the electron transfer system, resulting in the increased production of hydrogen peroxide, superoxide anions and hydroxyl radicals (18,27), which generate reactive oxygen species (ROS) (28). The majority of cells in the body contain enzymes that act as antioxidants and remove ROS (19,29). There are two primary antioxidant enzymes, GSH-Px and SOD (20,30). It is generally considered that MDA is an indicator of lipid peroxidation, as it is an oxidative degradation product of cell membrane lipids (21,31). Therefore, changes in GSH-Px and SOD activities or MDA levels may indicate production or elimination of ROS caused by anti-cancer drugs. In the present study, treatment of the gastric cancer cell line SGC-7901 with L-OHP resulted in a significant increase in MDA levels and a marked decrease in GSH-Px and SOD activities, which demonstrated its anti-tumor effect. However, several randomized studies have determined that chemotherapy treatment with L-OHP may cause liver injury followed by progressive steatohepatitis, which is associated with neurotoxicity and severe pain (22,23,32,33). Therefore, the present study assessed whether treatment with a combination of L-OHP and a liver-protective compound may reduce such side effects.

PPC is a major active ingredient in essential phospholipids, and is used in the treatment of CASH (34). It normalizes the metabolism of lipids and proteins, improves the detoxification function of cells and restores the structure of cells (35). In the current study, although PPC increased the growth and proliferation of SGC-7901 cells when used alone, it also greatly promoted their apoptosis induced by L-OHP. This suggests that PPC would not compromise the anti-tumor effects of L-OHP. In order to understand the precise molecular mechanism, the production of ROS and the activity of ROS-eliminating enzymes stimulated by L-OHP were evaluated in the current study. PPC did not influence SOD activity, GSH-Px activity or MDA levels, which were altered by L-OHP. Thus, it is likely that PPC does not alter L-OHP activity when administered.
alongside it as a combination treatment. L-OHP triggers cancer cell apoptosis by damaging the cell DNA and the main apoptotic pathway involves cytochrome c, which is present in mitochondria (12,18). Anti-cancer agents cause DNA damage and induce the transmission of death signals via the activation of the suppressor gene p53, which subsequently inhibits Bcl-2 expression in mitochondria (24,25,36,37). This inhibition leads to the release of cytochrome c, which triggers the apoptosis of cancer cells (26,38). During this process, cytochrome c activates caspases 9 and 3, with the help of
apoptotic protease-activating factor 1 (27,39), and activated caspase 3 subsequently suppresses caspase-activated DNase, inducing DNA fragmentation (28,40). The present study suggested that L-OHP may activate this apoptotic pathway in mitochondria, where it functions primarily by inducing DNA fragmentation and chromatin condensation.

Apart from stimulating the apoptosis of cancer cells, the suppressive effect of L-OHP on SGC-7901 cell growth may also be caused by cell cycle arrest at the G0/G1 phase. Cell cycle proteins, including cyclins D1 and E, which control the transition of cell cycle phases, have previously been examined (29,41). These two cell cycle proteins regulate the activity of cyclin-dependent kinases 4 and 6 by activating the transcription factor E2F and inactivating retinoblastoma protein to induce DNA synthase expression (30,42). In the present study, suppression of cyclins D1 and E was observed in SGC-7901 cells, which indicated that L-OHP may arrest the cell cycle in the G0/G1 phase.

PPC does not affect the anti-tumor activity of L-OHP; however, it may serve a synergistic role by inhibiting the growth and promoting the apoptosis of SGC-7901 cells. One possible mechanism of PPC action is that it may induce the redistribution of the primary phospholipid components in the bilayer of the membrane, particularly in cancer cells, which have a higher metabolism than healthy cells (43). This may then lead to a critical rearrangement of membrane components and even result in further architectural alterations of the membrane. These alterations could modify the diffusion properties of membranes, which are important in the development of drug resistance (31,44). Cytotoxic compounds, including L-OHP, must reach their targets inside cancer cells through their membrane. Thus, PPC may inhibit the growth and promote the apoptosis of SGC-7901 cells by boosting the accumulation of L-OHP in cancer cells.

In conclusion, the present study identified an association between PPC and L-OHP in the treatment of SGC-7901 cells. These data raise the possibility that, by combining PPC and L-OHP treatment, the dose of L-OHP administered to patients could be reduced, which would decrease the side effects associated with its use. Further studies are necessary to confirm the combinational efficacy of PPC and L-OHP. Future studies performed by the authors of the present study will focus on the fluidity and permeability of the membrane altered by PPC.
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