**Name of journal:** **World Journal of Gastroenterology**

**ESPS Manuscript NO:** 9864

**Columns: Research Report**

**Lobaplatin inhibits growth of gastric cancer cells by inducing apoptosis**

Yin CY *et al*. Lobaplatin and gastric cancer cells

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**Supported by** National Natural Science Foundation of China, No. 81101648

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**Received:** March 1, 2014 **Revised:** April 9, 2014

**Accepted:** July 22, 2014

**Published online:**

**Abstract**

**AIM:** To assess the anti-cancer effect of lobaplatin on human gastric cancer cells, and to explore the underlying molecular mechanisms.

**METHODS:** The human gastric cancer cell lines MKN-28, AGS and MKN-45 were used. The cytotoxicity of lobaplatin was detected using an MTS cell proliferation assay. Flow cytometry was used to detect cell apoptosis using Annexin V-FITC Apoptosis Detection kit. The expression of apoptosis-regulated genes was examined at the protein level using Western blots.

**Results:** Lobaplatin inhibited the proliferation of human gastric cancer cells and induced apoptosis, which may be associated with the up-regulation of Bax, poly ADP-ribose polymerase (PARP) cleavage, p53 expression and the reduction of Bcl-2 expression.

**Conclusion:** Thecytotoxicity of lobaplatin may be due to its ability of inducing cell apoptosis of gastric cancer cells, which would support the potential use of lobaplatin for the therapy of gastric cancer.

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**Key words:** Gastric cancer; Lobaplatin; Apoptosis

**Core tip:** Gastric cancer is one of the common malignancies and the main cause of death. Although cisplatin had become a primary therapeutic drug in advanced gastric cancer, drug resistance was the leading cause of treatment failure. To find an effective treatment is particularly urgent and important. Lobaplatin has been investigated in patients with advanced solid tumors, yet it has not been comprehensive studied in gastric cancer cells. In the study, we found the cytotoxicity and the apoptosis promoting effects of lobaplatin, which can provide new basis for the clinical application of gastric cancer.

Yin CY, Lin XL, Tian L, Ye M, Yang XY, Xiao XY. Lobaplatin inhibits growth of gastric cancer cells by inducing apoptosis. *World J Gastroenterol* 2014; In press

**INTRODUCTION**

Gastric cancer is one of the common malignancies and the main cause of death in the world. Chemotherapy is a primary method in the therapy of advanced gastric cancer. An increased understanding of the carcinogenesis processes and the response scheme of gastric cancer can contribute to progress in the treatment of gastric cancer. Research of the anticancer drug has been conducted by the success of cisplatin. Forty years after the discovery of cisplatin’s biological activity for the first time, oxaliplatin and carboplatin as routine medications are widely used in clinical today, while nedaplatin, heptaplatin and lobaplatin have only been authorized respectively in Japan, South Korea and China[1].

Lobaplatin is a representative of the third generation platinum drugs. It has been investigated in patients with advanced solid tumors, including relapsed ovarian cancer, canine appendicular osteosarcoma and hepatocellular carcinoma[2-5]. However, there are few reports about lobaplatin in the treatment of gastric cancer, and its mechanism of action has not been clearly understood[6]. The experiments in this study were performed to enhance our understanding of the pharmacological effects of lobaplatin in human gastric cancer. In this study, we found that lobaplatin was most cytotoxic in human gastric cancer cells with poor differentiation state and that lobaplatin induced cell apoptosis in human gastric cancer cells.

**Materials and Methods**

***Compounds and reagents***

Lobaplatin was purchased from Hainan Chang’an International Pharmaceutical Co., Ltd (Hainan, China). The antibodies against Poly (ADP-ribose) polymerase (PARP), p53, Bcl-2 (100), Bax (N-20), β-actin, and the secondary antibodies (horseradish peroxidase–linked anti-rabbit immunoglobulin G, anti-mouse immunoglobulin G, and anti-goat immunoglobulin G) were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc.). The other materials were sourced as follows: Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640, penicillin and streptomycin (100 ×), fetal bovine serum (FBS), trypsin-EDTA and dimethyl-sulfoxide (DMSO) (Gibco); CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega Corp. United States); Tween 20 (Promega); Annexin V-FITC Apoptosis Detection Kit (BioVision); Protein Assay Kit (Bio-Rad); ECL Plus Western Blotting Detection System (Pierce). All of the other reagents used are widely available commercially.

***Cell culture***

The human gastric cancer cell lines MKN-45 (poorly differentiated), AGS (moderately differentiated) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and maintained in an incubator with a humidified atmosphere of 5% CO2 at 37 °C. The MKN-28 (well-differentiated) cell line was cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and maintained in an incubator with a humidified atmosphere of 5% CO2 at 37 °C.

***Cell proliferation assay***

Cell proliferation assay was carried out according to the protocol for the MTS assay supplied by the manufacturer (Promega Corp. United States). In brief, 24 h after seeding, cells were exposed to lobaplatin with different concentrations (0, 1, 5, 10 and 25 μg/ml). After incubation for 24 h, 48 h or 72 h, 10 µl of the MTS solution was added to each well, and then cultures were continued to incubate for 2 h at 37 ℃. The absorbance was read at 450 nm on a microplate reader (Tecan Infinite F200, Switzerland), and the inhibitory rates were calculated as [1-(OD treated/OD control) × 100%].

***Cell apoptosis analysis***

Lobaplatin-induced apoptosis in AGS, MKN-28 and MKN-45 cells was determined by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit. Simple steps were as follows, 3 × 105 cells were plated and treated in 24 h with lobaplatin (0, 1, 5, and 10 μg/ml in MKN-45 and AGS, and 0, 25, 50, and 100 μg/ml in MKN-28). And then, cells were harvested, washed twice in PBS, and incubated with Annexin V and propidium iodide for 10 min at room temperature in the dark. And then, the gastric cancer cells were analyzed by the FACSAria Flow Cytometer.

***Western blot analysis***

Briefly, 3 × 105 cells were cultured in 6-well plates and processed with lobaplatin with different concentrations (0, 2.5, 5, and 10 μg/ml in MKN-45 and AGS, and 0, 10, 25, and 50 μg/ml in MKN-28) for 24 h. The cultured cells were trypsinized, washed with PBS, and then re-suspended in lysis buffer at 4 °C. The lysates were denatured in loading buffer at 99 °C for 10 min. Proteins were transferred onto a PVDF membrane after being separated using SDS-PAGE gel electrophoresis. The membrane was incubated with the primary antibody at 4 °C overnight. After being blocked in blocking buffer with 5% nonfat milk [20 mmol/L TBS (pH 7.5) containing 0.1% Tween 20], and then incubated at 37 °C with the appropriate secondary antibody with horseradish peroxidase-conjugated. The reactive bands were visualized by the ECL Plus Western Blotting Detection System. The level of β-actin was used as a loading control in each sample.

***Statistical analysis***

The experimental results with the mean and standard deviation were expressed with the ± SD. And the statistical significance was determined using a two-tailed Student’s *t*-test between different groups. *P*-value of less than 0.05 was defined as statistical significance.

**Results**

***Cytotoxicity of lobaplatin in gastric cancer cells***

Antitumor activity of lobaplatin was evaluated by cell proliferation MTS assay after 24 h, 48 h, and 72 h of treatment (Figure 1) in AGS, MKN-28, and MKN-45 cells. Lobaplatin inhibited the proliferation of these three human gastric cancer cell lines in a dose-dependent manner, and the IC50 values were 6.11 ± 1.44 μg/ml (AGS), 16.10 ± 0.81 μg/ml (MKN-28), and 1.78 ± 0.16 μg/ml (MKN-45), respectively. The cytotoxicity of lobaplatin varied in different cell lines by comparing with the IC50 values. Moreover, lobaplatin was less cytotoxic to normal human gastric mucous GES-1 cells (IC50 value 56.17 ± 1.57 μg/ml) than it was to cancer cells.

***Apoptosis induced by lobaplatin in gastric cancer cells***

To assess whether lobaplatin-induced growth inhibition in gastric cnacer was related to cell apoptosis, AGS, MKN-28, and MKN-45 cells were dealt with lobaplatin as described above, and cell apoptotic was evaluated by flow cytometry with the Annexin V-FITC Apoptosis Detection kit. After exposing to lobaplatin for 24 h, the number of apoptotic MKN-28 cells significantly increased in a dose-dependent manner [2.3% in 0 μg/ml, 15.6% in 25 μg/ml (*P* < 0.01), 27.6% in 50 μg/ml (*P* < 0.01), and 35.4% in 100 μg/ml (*P* < 0.01)] (Figure 2A). When the AGS and MKN-45 cells were exposed to lobaplatin for 24 h, similar effects were obtained (Figure 2B, C). These results suggest that lobaplatin can effectively induce apoptosis in three gastric cancer cell lines despite of their different differentiation states.

***Expression of apoptosis-related proteins induced by lobaplatin***

Bcl-2 family proteins are critical regulatory factors in response to apoptosis. The expression changes of Bax and Bcl-2 were analyzed in gastric cancer cells after exposure to lobaplatin. The results showed increases in the expression of Bax and dose-dependent reduction in the levels of Bcl-2 protein compared with the control cells (Figure 3).

To clarify the mechanism by which the apoptotic pathway is activated by lobaplatin, its effects on the activation of apoptosis associated protein PARP and p53 were assessed. Dose-dependent increase in the cleavage of p53 and PARP was found under the treatment by lobaplatin in three gastric cancer cells (Figure 3). This result indicates that lobaplatin might activate p53-dependent and the canonical mitochondrial apoptotic pathways in gastric cancer cells.

**Discussion**

Gastric cancer is one of the common malignancies and the second leading cause of cancer-related death[7]. Presently, surgery is the conventional strategy to the treatment of gastric cancer. However, more than half of the patients have been in the advanced stages when diagnosed and cannot undergo a surgical operation. Therefore, chemotherapy is presently the main treatment for advanced gastric cancer. However, the overall response rate is generally less than 50% in gastric cancer and concurrent with high incidence of adverse effects[8]. Therefore, it is increasingly concerned about new chemotherapy drugs to treat gastric cancer.

Platinum drugs have been widely used to treat a variety of malignant tumors. Clinically three common platinum drugs (cisplatin, carboplatin and oxaliplatin) are marketed for different malignancies. The new platinum compounds, lobaplatin, nedaplatin and heptaplatin have gained official approval for anti-cancer purposes (regionally limited)[9]. Cisplatin is the first generation platinum drug, and has become a major compound on the treatment of solid tumors, such as bronchial carcinoma, ovarian cancer, germ cell cancer, and bladder cancer, but its clinical application is limited by its side effects including gastrointestinal, renal, ototoxicity and neurological toxicity[10]. Lobaplatin (D-19466; 1,2-diamino-methyl-cyclobutane-platinum (II)- lactate) is a representative one of the third-generation platinum drugs, containing a 1,2-bis(aminomethyl) cyclobutane stable ligand with lactic acid as the leaving group. Its anti-tumor activity primarily due to the conformation of DNA-drug adducts, mainly as AG and GG intra-strand cross-links. It had been reported that lobaplatin effected the expression of the c-Myc gene, which is involved in cell proliferation, apoptosis and oncogenesis[11]. Phase II clinical trials of lobaplatin has also completed in the United States, Australia, European Union, Brazil and South Africa for the treatment of various cancers, including lung, breast, ovarian and esophageal cancers, as well as CML[12]. However, this drug has not yet been used to treat gastric cancer. Therefore, this study investigated the effects of lobaplatin in three gastric cancer cell lines and explored the underlying molecular mechanisms.

We found that lobaplatin inhibited the survival of different human gastric cancer cells in a dose-dependent manner, showing an in vitro anti-tumor effect. Interestingly, we found that lobaplatin was more effective on the less-differentiated MKN-45 cells (IC50 value 1.78 ± 0.16 μg/ml), then the moderately differentiated AGS cells (IC50 value 6.11 ± 1.44 μg/ml), while it presented less effect on the MKN-28 cells (IC50 value 16.10 ± 0.81 μg/ml) which are well-differentiated. Lobaplatin presented almost no toxicity to the normal human gastric epithelial cell line GES-1 (IC50 value 56.17 ± 1.57 μg/ml), which indicates that lobaplatin shows a therapeutic effect that is specific to human gastric cancer cells. This result is in agreement with previous reports of the work on other solid tumors, such as hepatocellular carcinoma and ovarian cancer[13,14].

Lobaplatin has been reported to induce apoptosis in cancer cells in the past few years. McKeage *et al*[15] reported that lobaplatin inhibited tumor cells by affecting the expression of the *c-Myc* gene, which involved in cell proliferation, apoptosis and oncogenesis, but the exact mechanism is not fully understood. Our research found that lobaplatin can induce gastric cancer cellapoptosis in multiple cell lines (AGS, MKN-28 and MKN-45) with different differentiation states. The results indicated a decrease in Bcl-2 expression and an increase in Bax expression in three gastric cancer cell lines. The change in the ratio of Bax to Bcl-2 promotes the release of cytochrome C from the mitochondria to the cytosol. Then cytochrome C can bind to APAF-1 in the cytosol, and lead to the activation of PARP and caspase 3[16]. Our study further indicated that lobaplatin activated PARP cleavage in a dose-dependent manner. These results showed PARP played an important role in lobaplatin induced apoptosis in gastric cancer cells with different differentiation. Lobaplatin’s induction of a p53-independent apoptotic mechanism has potential anti-tumor effects on gastric cancer. These results are similar to the results of our initial research showing that Licochalcone A induced apoptosis in gastric cancer cells[17]. It is because of the pro-apoptotic effects of lobaplatin in gastric cancer cells with different differentiation degree, we speculate lobaplatin is especially useful for the treatment of poorly differentiaed gastric cancer.

The tumor suppressor p53 is an essential regulator that plays an important role in the antitumor efficacy of platinum agents. The p53 protein can prevent cancer development, including cell cycle stagnation and the induction of apoptosis by a series of mechanisms. It has been reported that lobaplatin arrested cell cycle progression of hepatocellular carcinoma cellsin the G1 and G2/M phases in a time-dependent manner[5,18,19]. To further investigate the mechanism, p53 expression were further studied before and after lobaplatin treatment in multiple gastric cancer cell lines. The results show that p53 levels increased in a dose-dependent manner after exposing to lobaplatin, indicating that the p53-dependent apoptotic pathway is activated by lobaplatin. Whether lobaplatin influences the cell cycle in gastric cancer merits further research.

In conclusion, lobaplatin inhibited the growth of gastric cancer cell mainly by apoptosis *via* the caspase-dependent mitochondrial pathway. Therefore, lobaplatin is a promising candidate against gastric cancer. It warrants additional investigation and clinical evaluation.

**comments**

***Background***

Gastric cancer is one of the leading causes of death worldwide. Cisplatin was an important drug in the treatment of advanced gastric cancer, but drug resistance reduced the actual effect. Lobaplatin has been investigated in patients with advanced solid tumors, yet it has not been comprehensive studied in gastric cancer cells.

***Research frontiers***

Lobaplatin is a new representative compound as third-generation platinum drug. It appears to overcome tumor resistance to cisplatin and carboplatin. And it has been investigated in some solid tumors, but little focus on gastric cancer. In the study of lobaplatin’s inhibitory effects to gastric cancer cells, the research hotspot is that we found lobaplatin was most cytotoxic in human gastric cancer cells with poor differentiation states and that lobaplatin induced the apoptosis of gastric cancer cells.

***Innovations and breakthroughs***

Studies have reported that lobaplatin can inhibite some solid cancer’s proliferation, such as primary hepatic carcinoma, cholangiocarcinoma, non-small-cell lung cancer, relapsed ovarian cancer, and so on. In this study, we investigated lobaplatin’s anti-tumor effects in gastric cancer cells. We used both cancer cells and normal epithelial cell lines to perform the experiments. We found lobaplatin caused cytotoxicity and induced apoptosis via the caspase-dependent mitochondrial pathway in gastric cancer cells. Lobaplatin is a promising anti-cancer candidate drug for the treatment of gastric cancer.

***Applications***

The study results suggest that lobaplatin is a potential chemotherapy agent that it can provide a theoretical basis for the clinical treatment of gastric cancer.

***Terminology***

Apoptosis: Apoptosis also called the process of programmed cell death (PCD) that is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. Apoptosis is considered a vital component of various processes including normal cell turnover, hormone-dependent atrophy, embryonic development, *etc.* Inappropriate apoptosis is a factor in many human diseases including neurodegenerative diseases, autoimmune disorders and many types of cancer, etc. Anti-cancer drugs can lead to characteristic tumor cell changes (morphology, signaling proteins) and death.

***Peer review***

The purpose of the present study is to determine possible effects of lobaplatin on growth, viability and apoptosis of various gastric cancer cells. It is an interesting work in which the effects of the drug on gastric cancer cells in various degrees of differentiation are compared.

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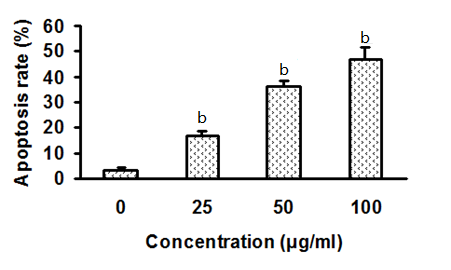
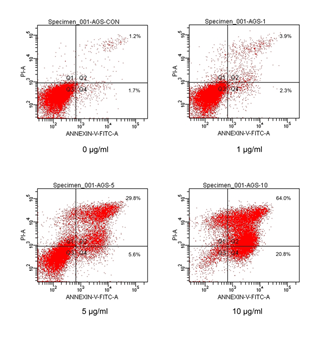
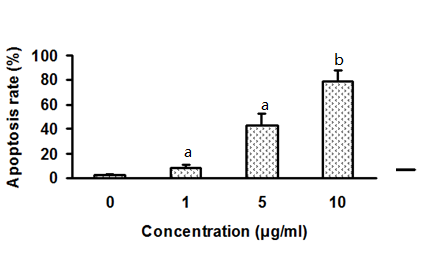
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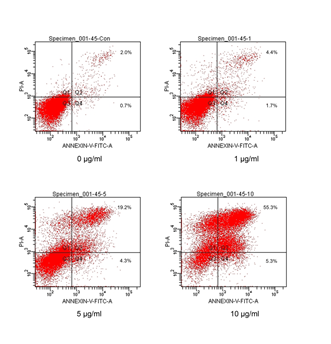
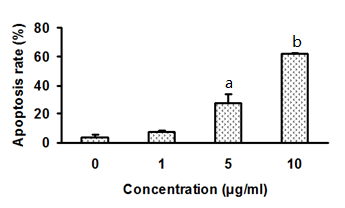
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**A B C**

**Figure 1 Cytotoxicities of lobaplatin in human gastric cancer cell lines.** Cells were exposed to various concentrations of the compound (0, 1, 5, 10 and 25 μg/ml) for 24 h, 48 h, and 72 h followed by analysis by CCK-8 assay.A: AGS; B: MKN-28; C: MKN-45. All of the assays were performed in triplicate.

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A B

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**Figure 2** **Induction of apoptosis in human gastric cancer cells by lobaplatin treatment using the Annexin V-FITC Apoptosis Detection Kit.** MKN-28 (A), AGS (B), and MKN-45 (C) cells were exposed to various concentrations of lobaplatin (0, 1, 5 and 10 μg/ml or 0, 25, 50 and 100 μg/ml) for 24 h and their apoptosis ratios were analyzed. All assays were in triplicate. a*P* < 0.05 *vs* control group; b*P* < 0.01 *vs* control group.

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A B C

**Figure 3** **Cellular expression of apoptosis-related proteins was determined by Western blot after gastric cells were exposed to various concentrations (0, 5, 25 and 50 μg/ml) of lobaplatin for 24 h.** A: MKN-28; B: AGS; C: MKN-45.