Gambogic acid reverses oxaliplatin resistance in colorectal cancer by increasing intracellular platinum levels

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Abstract. Resistance to oxaliplatin (L-OHP) is a major obstacle to successful chemotherapy in colorectal cancer (CRC). In the present study, the ability of gambogic acid (GA) to reverse L-OHP resistance in CRC LoVo cells was investigated. L-OHP-resistant LoVo/L-OHP cells were established by exposing them to increasing concentrations of L-OHP. GA-reversed L-OHP-sensitive LoVo/L-OHP/GA cells were established by exposure to 0.5 μmol/l GA for 2 weeks. A Cell Counting Kit-8 assay was used to assess levels of proliferation. Flow cytometry was applied to detect apoptosis rates. Transwell assays were used to analyse invasion. Inductively coupled plasma mass spectrometry was used to determine intracellular platinum (Pt) content. Western blot analysis was used to reveal the protein levels of Human copper transporter 1 (hCTR1), Copper-transporting p-type adenosine triphosphatases 1 (ATP7A) and Copper-transporting p-type adenosine triphosphatases 2 (ATP7B). LoVo/L-OHP and LoVo/L-OHP/GA cell lines were successfully established, and it was identified that L-OHP inhibited the proliferation of LoVo, LoVo/L-OHP and LoVo/L-OHP/GA cells in a dose-dependent manner. Compared with the parent LoVo cells, the anti-apoptosis and invasion properties of LoVo/L-OHP cells were enhanced, and were reversed by GA treatment. Intracellular Pt content was highest in the LoVo cells, followed by LoVo/L-OHP/GA cells, and then lowest in the LoVo/L-OHP cells. Downregulated hCTR1 and upregulated ATP7A and ATP7B were associated with L-OHP resistance, and GA reversed the resistance by increasing levels of hCTR1 and decreasing levels of ATP7A and ATP7B. In conclusion, GA has the potential ability to reverse L-OHP resistance in CRC cells by increasing intracellular Pt content, which it achieves by increasing hCTR1 levels and decreasing ATP7A and ATP7B levels. GA may represent a promising treatment agent for L-OHP resistance.

Introduction

Colorectal cancer (CRC), the second-most diagnosed cancer and the fourth-most frequent cause of cancer-associated mortality (1), remains one of the most serious health problems worldwide. In China, it ranks fifth in the morbidity and mortality rates among all types of cancer, with 191,000 mortalities in 2015 (2).

Chemotherapy serves a vital role in the treatments of CRC, particularly for patients with advanced CRC; it lessens the number and severity of clinical symptoms, improves the quality of lives and prolongs survival (3). Drug resistance is a major obstacle in chemotherapy (4). Oxaliplatin (L-OHP), a third-generation platinum (Pt) compound, is the first-line drug for CRC chemotherapy (5). However, resistance to L-OHP leads to treatment failure and relapse in patients with CRC (4).

Reduced intracellular Pt accumulation has been identified as a major mechanism of L-OHP resistance (6). Adequate accumulation of intracellular Pt is essential for anticancer drugs to exert their cytotoxic effects (7). Copper transporters serve important roles in the cellular import and export of Pt drugs (8). Human copper transporter 1 (hCTR1) and Copper-transporting p-type adenosine triphosphatases 1 (ATP7A) and 2 (ATP7B) have been identified as key copper transporters (9). hCTR1 regulates the influx of Pt drugs, while ATP7A and ATP7B regulate their efflux (9). The upregulation of hCTR1 and downregulation of ATP7A and ATP7B may be potential mechanisms of L-OHP resistance (10).

Gambogic acid (GA), an active component of the traditional Chinese medicine *Garcinia hanburyi*, exhibits multi-target anti-tumour effects with few side effects (11). Previously, GA was identified to be able to reverse resistance
to anticancer drugs, including resistance to 5-fluorouracil in CRC (12), to doxorubicin in breast (13) and ovarian cancer (14), and to docetaxel in gastric (15) and human epithelial cancer (16).

However, to the best of our knowledge, the ability of GA to reverse L-OHP resistance in CRC cells has not been investigated. Therefore, in the present study, using a stepwise increasing concentration method, L-OHP-resistant LoVo/L-OHP and L-OHP-sensitive LoVo/L-OHP/GA cell lines were successfully established, and it was identified that GA may reverse L-OHP resistance, potentially by increasing intracellular platinum through increasing hCTP1 and decreasing ATP7A and ATP7B protein levels. GA may represent a promising treatment agent for L-OHP resistance.

Materials and methods

Materials. LoVo cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% foetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. L-OHP was purchased from Jiangsu Hengrui Pharmaceutical Co., Ltd. (cat. no. H20000337; Lianyungang, China). GA was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The Cell Counting Kit-8 (CCK-8) was obtained from Beyotime Institute of Biotechnology (Haimen, China). The Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis kit was purchased from Invitrogen; Thermo Fisher Scientific, Inc. Antibodies against hCTR1 (cat. no. ab108481; rabbit polyclonal), ATP7A (cat. no. ab42486; rabbit polyclonal), ATP7B (cat. no. ab124973; rabbit monoclonal) and GAPDH (cat. no. ab9485; rabbit polyclonal) were purchased from Abcam (Cambridge, MA, USA).

Establishment of LoVo/L-OHP and LoVo/L-OHP/GA cell lines. The L-OHP-resistant LoVo/L-OHP cell line was established by exposing LoVo cells to increasing concentrations of L-OHP (1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45 and 50 µmol/l) for 48 h at each concentration as described previously (17,18). LoVo/L-OHP cells were then cultured in complete RPMI-1640 medium with 4 µmol/l L-OHP at 37°C with 5% CO₂. After 6 months, LoVo/L-OHP cells capable of growing in 60 µmol/l L-OHP were obtained. To examine the effects of drug intervention, the culture medium was changed to complete RPMI-1640 medium without L-OHP 1 week prior to experimentation.

The GA-reversed L-OHP-sensitive LoVo/L-OHP/GA cell line was established by continuous exposure of LoVo/L-OHP cells to GA.

Briefly, LoVo/L-OHP cells were cultured in complete RPMI-1640 medium without L-OHP for 1 week, and then cultured in complete RPMI-1640 medium with 0.5 µmol/l GA at 37°C with 5% CO₂ for 2 weeks. The culture medium was changed every 24 h. The LoVo/L-OHP/GA cells were then collected and stored for subsequent experiments.

Morphological observations. The recovery established LoVo, LoVo/L-OHP or LoVo/L-OHP/GA cells were cultured to ~80% confluency. Cells were observed after 24 h using an inverted light microscope (magnification, x800) in order to observe morphological changes.

Cell viability assay. Cytotoxicity was determined by a CCK-8 assay. Briefly, LoVo, LoVo/L-OHP or LoVo/L-OHP/GA cells (4x10^3 cells/ml) were cultured in 96-well plates overnight. A total of 100 µl of different concentrations of L-OHP (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 µmol/l) were then added for at 37°C with 5% CO₂ 48 h. Next, 10 µl CCK-8 reagent was added for 2 h, and the absorbance at 450 nm was determined on a microplate reader (iMark™; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Resistance index (RI)=half maximal inhibitory concentration (IC₅₀) of drug-resistant cells/IC₅₀ of drug-sensitive cells (10).

Assessment of cell apoptosis. Cells were harvested (0.25% trypsin was added for 30 sec to digest the cells, followed by centrifugation at 560 x g at 37°C for 5 min and the supernatant was then discarded) following treatment with 20 µmol/l L-OHP for 6 h and re-suspended in Annexin-binding buffer (In vitrogen; Thermo Fisher Scientific, Inc.) to a concentration of 2x10^9/ml. Annexin V (solution in 25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, 0.1% bovine serum albumin) and propidium iodide working solutions (1 mg/ml) were then added at room temperature for 15 min. Flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was then performed, and data was analysed using FlowJo 7.6 software (FlowJo LLC, Ashland, OR, USA).

Transwell matrix penetration assay. Cells (LoVo, LoVo/L-OHP and LoVo/L-OHP/GA cells) were cultured in RPMI-1640 medium without FBS for 24 h, following which 2x10^5/ml cells suspended in 2 µmol/l L-OHP were plated in the upper chamber of a polycarbonate Transwell filter in BioCoat™ Invasion Chambers (BD Biosciences) and incubated for at 37°C with 5% CO₂ for 24 h. RPMI-1640 medium with 10% FBS was added to the lower chamber at 37°C with 5% CO₂ for 24 h. Cells that migrated to the lower membrane were fixed with 1% paraformaldehyde at 37°C for 10 min, stained with 1% haematoxylin at 37°C for 10 min and counted by microscopy in 10 fields of view using a light microscope (magnification, x400).

Intracellular accumulation of Pt. A total of 1x10⁷ cells/ml of LoVo, LoVo/L-OHP or LoVo/L-OHP/GA cells were seeded into 10 cm culture dishes for 24 h. Then, 0, 0.5, 1, 2 or 4 µmol/l L-OHP was added for 4 h, or 2 µmol/l L-OHP for 1, 4, 12 or 24 h. Cells were harvested (0.25% trypsin was added for 30 sec to digest the cells, followed by centrifugation at 560 x g at 37°C for 5 min and the supernatant was then discarded) following treatment, washed with PBS and lysed with TRizol® (Life Technologies; Thermo Fisher Scientific, Inc.). Intracellular Pt was determined by inductively coupled plasma mass spectrometry (ICP-MS; PerkinElmer, Inc., Waltham, MA, USA) as described previously (19).

Western blotting. Total protein from LoVo, LoVo/L-OHP and LoVo/L-OHP/GA cells were extracted with SDS-PAGE Sample loading buffer (cat no. P0015; Beyotime Institute of Biotechnology), and proteins were determined using the
The cytotoxicity of L-OHP to LoVo cells was 11.82 µmol/l and the RI was 1.09. The resistance was statistically significant difference post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

L-OHP inhibits the proliferation of the LoVo, LoVo/L-OHP and LoVo/L-OHP/GA cells. The cytotoxicity of L-OHP to LoVo, LoVo/L-OHP and LoVo/L-OHP/GA cells was first analysed using a CCK-8 assay. As demonstrated in Fig. 1A, as the concentration of L-OHP increased, the survival rates of cells decreased, indicating that L-OHP increased the levels of cytotoxicity in a dose-dependent manner. The survival rate of the LoVo/L-OHP cells was increased compared with those of the LoVo and LoVo/L-OHP/GA cells (P<0.05). After 24 h treatment with 40 µmol/l L-OHP, the LoVo and LoVo/L-OHP/GA cells were almost entirely killed. However, 72.53±3.06% LoVo/L-OHP cells survived.

The IC₅₀ of L-OHP was then calculated (Fig. 1B). The L-OHP IC₅₀ for LoVo cells was 11.82 µmol/l, while that for the LoVo/L-OHP cells was 53.81 µmol/l. The RI for the LoVo/L-OHP cells was 4.55. The IC₅₀ for the LoVo/L-OHP/GA cells was 12.54 µmol/l and the RI was 1.06. The results demonstrated that the LoVo/L-OHP cells were resistant to L-OHP, and that GA inhibited this resistance.

Whether the established L-OHP-resistant cells and sensitive cells were able to maintain their characteristics was also assessed. LoVo/L-OHP cells were cultured in complete RPMI-1640 medium without L-OHP for 15 days, following which the IC₅₀ for the LoVo/L-OHP cells was 46.43 µmol/l, the RI was 3.92 and resistance remained at 86.29% viable cells. Subsequent to storage in liquid nitrogen (-196°C) for 2 months, recovered LoVo/L-OHP cells were able to grow and proliferate. The IC₅₀ for the recovered LoVo/L-OHP cells was 47.97 µmol/l and the RI was 4.05, indicating that the established LoVo/L-OHP cells were able to maintain resistance. Regarding the LoVo/L-OHP/GA cells, following culture incomplete RPMI-1640 medium without GA for 15 days, the IC₅₀ was 12.93 µmol/l and the RI was 1.09. The resistance was 27.85% of all viable LoVo/L-OHP cells, which was higher compared with the first established cells (23.0% viable cells). The LoVo/L-OHP/GA cells recovered from liquid nitrogen were also able to grow and proliferate. The IC₅₀ for the recovered cells was 13.92 µmol/l and the RI was 1.18, suggesting that GA was able to reverse L-OHP resistance, and that the L-OHP-sensitive cells had been successfully established.

Morphological changes of LoVo/L-OHP and LoVo/L-OHP/GA cells. The morphological changes of the established cells were then observed through inverted light microscopy (magnification, x800). As demonstrated in Fig. 2, the parent LoVo cells were adherent, flat and polygonal, with numerous cell junctions. LoVo/L-OHP-resistant cells were rounder and bigger, and the nuclei were clearer. The LoVo/L-OHP/GA cells exhibited a similar appearance to the recovered LoVo cells.
LOVO/L-OHP cells was increased compared with the LoVo and LoVo/L-OHP/GA cells, and that GA reversed these effects.

**GA attenuates invasion in the LoVo/L-OHP cells.** LoVo, LoVo/L-OHP and LoVo/L-OHP/GA cells were treated with 2 µmol/l L-OHP for 24 h, following which the levels of invasion were determined in Transwell assays. The numbers of invasive LoVo, LoVo/L-OHP and LoVo/L-OHP/GA cells was 21±0.12, 46±0.15 and 17±0.09, respectively. As demonstrated in Fig. 4, a comparison of the numbers of invading cells revealed that the LoVo/L-OHP cells yielded an increased number of invasive cells compared with the LoVo and LoVo/L-OHP/GA cells (P<0.01). Subsequent to treatment with L-OHP, the invasive ability of the LoVo/L-OHP cells was increased compared with that of the LoVo and LoVo/L-OHP/GA cells, suggesting that the rates of invasion in LoVo/L-OHP-resistant cells increased, and that GA was able to reverse and attenuate the invasion.

**Determination of intracellular Pt content.** To explore the potential mechanisms by which GA reversed resistance to L-OHP, the intracellular content of Pt was detected. As demonstrated in Fig. 5A, it was identified that intracellular Pt accumulated as the concentration of L-OHP increased, indicating that L-OHP entered into cells in a dose-dependent manner. Following 4 h treatment with different concentrations (0, 0.5, 1, 2 or 4 µmol/l) of L-OHP, the Pt content in LoVo and LoVo/L-OHP/GA cells was increased compared with the LoVo/L-OHP cells (P<0.05). Intracellular Pt content was highest in the LoVo cells, followed by LoVo/L-OHP/GA cells, and then lowest in the LoVo/L-OHP cells.

The Pt content of cells then was detected following treatment with 2 µmol/l L-OHP for different times (1, 4, 12 and 24 h). It was identified that intracellular Pt accumulated as treatment time intervals increased, indicating a time-dependent effect. There was no difference between the LoVo, LoVo/L-OHP and LoVo/L-OHP/GA cells at 1 and 4 h (P>0.05). At 12 and 24 h, the Pt content in the LoVo and LoVo/L-OHP/GA cells was increased compared with the LoVo/L-OHP cells (P<0.05). Pt content in the LoVo cells was increased compared with the LoVo/L-OHP/GA cells at 12 and 24 h (P<0.05; Fig. 5B).

**hCTR1, ATP7A and ATP7B protein levels.** In order to determine whether the changes in intracellular Pt were associated with copper transporters, the protein expressions of hCTR1, ATP7A and ATP7B in LoVo, LoVo/L-OHP and LoVo/L-OHP/GA cells were examined. As demonstrated in Fig. 6, hCTR1 protein levels were decreased in the LoVo/L-OHP cells compared with in the LoVo and LoVo/L-OHP/GA cells (P<0.01), and decreased in the LoVo/L-OHP/GA cells compared with the LoVo cells (P<0.01). The protein levels of ATP7A and ATP7B were increased in the LoVo/L-OHP cells compared with the LoVo and LoVo/L-OHP/GA cells.
(P<0.01), with levels being increased in the LoVo/L-OHP/GA cells compared with the LoVo cells (P<0.01). These results suggest that downregulated hCTP1 and upregulated ATP7A and ATP7B were associated with L-OHP resistance, and that GA reversed the resistance by increasing the levels of hCTR1 and decreasing ATP7A and ATP7B levels.
Low expression of hCTR1 was determined to be associated with oxaliplatin and carboplatin in small-cell lung cancer cells. Ishida et al. (26) identified that the downregulation of hCTR1 resulted in the reduced accumulation of cisplatin and increased cisplatin resistance. Song et al. (27) also identified that the upregulation of hCTR1 enhanced the accumulation of oxaliplatin and carboplatin in small-cell lung cancer cells. Low expression of hCTR1 was determined to be associated with poor prognosis in patients with non-small cell lung cancer (NSCLC) and ovarian cancer treated with Pt-based chemotherapy (28,29). hCTR1 is a potential biomarker for intracellular Pt accumulation and Pt drug resistance. ATP7A serves an important role in Pt resistance by transporting Pt drugs out of cells (30). The overexpression of ATP7A was associated with Pt resistance in oesophageal squamous cell cancer (31), NSCLC (32), CRC (33) and ovarian cancer (34). Overexpressed ATP7A was also identified to predict a poor prognosis in patients with NSCLC receiving Pt-based chemotherapy (32). Similar to ATP7A, ATP7B facilitates the efflux of Pt drugs, and also affects resistance to Pt drugs (35). ATP7B silencing resulted in improved cisplatin sensitivity in cisplatin-resistant ovarian cells (36). The overexpression of ATP7B was associated with Pt resistance in patients with CRC, and predicted poor prognosis in patients following oxaliplatin-based chemotherapy (37).

Therefore, in the present study, the possibility of using GA to reverse L-OHP resistance in CRC cells was evaluated. It was identified that the LoVo/L-OHP cells were resistant to L-OHP, and that GA reversed this resistance. Compared with the parent LoVo cells, the anti-apoptosis and invasive abilities of resistant LOVO/L-OHP cells were improved, and GA was able to reverse these effects. Intracellular Pt content was highest in the LoVo cells, followed by the LoVo/L-OHP/GA cells, and then lowest in the LoVo/L-OHP cells. Decreased hCTP1 levels and increased ATP7A and ATP7B levels were associated with L-OHP resistance, and GA reversed this resistance by increasing hCTR1 and decreasing ATP7A and ATP7B levels. These results indicated that GA exhibited the ability to reverse L-OHP resistance in CRC cells, which was associated with an increase in intracellular Pt content and a regulation of the protein expression levels of copper transporters.

The cytotoxic effects of Pt drugs are directly associated with intracellular Pt content, and the majority of resistant cells exhibit decreased intracellular accumulation of these drugs (25). Adequate intracellular accumulation of Pt drugs is essential to exert their anticancer effects (7). Intracellular Pt content was directly associated with the content of L-OHP in cells, while intracellular L-OHP content is positively associated to the sensitivity of cells to L-OHP (7). In the present study, intracellular Pt content was determined by ICP-MS, and it was identified that intracellular L-OHP content increased in a dose- and time-dependent manner. Intracellular L-OHP content was highest in the LoVo cells, followed by the LoVo/L-OHP/GA cells, and then lowest in the LoVo/L-OHP cells, suggesting that parent LoVo cells were relatively sensitive to L-OHP, that LoVo/L-OHP cells were resistant to L-OHP, and that GA was able to reverse this resistance.

The process of cellular import and export of Pt drugs is primarily mediated by copper transporters (8). hCTR1, ATP7A and ATP7B are key copper transporters involved in intracellular Pt accumulation (9). hCTR1 regulates the influx of Pt drugs, while ATP7A and ATP7B regulate the efflux of these drugs (9). Previous studies have indicated that copper transporters not only regulate the influx and efflux of Pt drugs, but also affect cell cytotoxic sensitivity to Pt drugs: Ishida et al. (26) identified that the downregulation of hCTR1 resulted in the reduced accumulation of cisplatin and increased cisplatin resistance. Song et al. (27) also identified that the upregulation of hCTR1 enhanced the accumulation of oxaliplatin and carboplatin in small-cell lung cancer cells. Low expression of hCTR1 was determined to be associated with poor prognosis in patients with non-small cell lung cancer (NSCLC) and ovarian cancer treated with Pt-based chemotherapy (28,29). hCTR1 is a potential biomarker for intracellular Pt accumulation and Pt drug resistance. ATP7A serves an important role in Pt resistance by transporting Pt drugs out of cells (30). The overexpression of ATP7A was associated with Pt resistance in oesophageal squamous cell cancer (31), NSCLC (32), CRC (33) and ovarian cancer (34). Overexpressed ATP7A was also identified to predict a poor prognosis in patients with NSCLC receiving Pt-based chemotherapy (32). Similar to ATP7A, ATP7B facilitates the efflux of Pt drugs, and also affects resistance to Pt drugs (35). ATP7B silencing resulted in improved cisplatin sensitivity in cisplatin-resistant ovarian cells (36). The overexpression of ATP7B was associated with Pt resistance in patients with CRC, and predicted poor prognosis in patients following oxaliplatin-based chemotherapy (37).


