

ORIGINAL ARTICLE

Gentiopicroside exerts convincing antitumor effects in human ovarian carcinoma cells (SKOV3) by inducing cell cycle arrest, mitochondrial mediated apoptosis and inhibition of cell migration

Xiaohao Li¹, Chenglin Yang², Hao Shen³

¹Department of Gynaecology, The People's Hospital of Tongzhou District, Nantong City, Nantong, Jiangsu, 226300, China;

²Department of Gynaecology, The People's Hospital of Yining County, Yining, Xinjiang, 835100, China; ³Department of Doppler Ultrasonography, Traditional Chinese Medicine of Nantong City, Nantong, Jiangsu, 226001 China.

Summary

Purpose: Gentiopicroside is an important plant secondary metabolite and has been reported to exhibit tremendous pharmacological potential. In the present study we investigated the anticancer effects of gentiopicroside against ovarian SKOV3 cancer cells.

Methods: Anticancer effects were determined by MTT assay. Apoptosis was investigated by DAPI and annexin V/propidium iodide (PI) double staining. Mitochondrial membrane potential (MMP) determination and cell cycle analysis were carried out by flow cytometry. Protein expression was examined by western blotting.

Results: The results showed that gentiopicroside exerted anticancer effects on SKOV3 cancer cells in a dose-dependent

manner. The IC_{50} of gentiopicroside was 20 μ M against the SKOV3 cancer cells. The anticancer effects were mainly found to be due to loss of MMP and induction of apoptosis. The Bax/Bcl-2 expression ratio was also altered upon gentiopicroside treatment. Furthermore, gentiopicroside could arrest the SKOV3 cells in G2/M phase of the cell cycle and prevent their migration and invasion.

Conclusions: These results indicate that gentiopicroside could prove a potential lead molecule in the treatment and management of ovarian cancer.

Key words: apoptosis, cell cycle arrest, cell migration, gentiopicroside, ovarian cancer

Introduction

Natural products of plant origin have long been considered as source of anticancer agents and are being evaluated against several types of cancers. Gentiopicroside (Figure 1) is an important plant secondary metabolite that has been shown to exhibit anticancer effects [1]. Ovarian cancer represents one of the most fatal gynecological malignancies. It accounts for about 3% of all cancers in females with 0.24 million new cases being diagnosed every year. Moreover, 0.15 mil-

lion deaths are due to ovarian cancer annually across the globe [2]. In the United States alone, around 22,000 ovarian cancer cases are diagnosed and about 14,000 deaths are reported annually [3]. Ovarian cancer is often referred to as 'silent killer' as 80% of the patients show no symptoms of the disease until they reach advanced stage [4]. It has been reported that in the last few decades or so, 20% of patients diagnosed with ovarian cancer at early stage exhibit over 90% survival rate [5]. Ef-

Correspondence to: Hao Shen, MD. Department of Doppler Ultrasonography, Traditional Chinese Medicine of Nantong City, Jianshe Rd no. 41, Nantong, Jiangsu, 226001 China.

Tel/Fax: +86 0513 85126061, E-mail: haoshen12@hotmail.com

Received: 02/06/2018; Accepted: 21/06/2018

efficient surgical interventions and use of different combinations of anticancer drugs have shown decent results, but the overall long term complete remission rate still remains at 30% only [6]. Owing to the distinct biology of ovarian cancers, the selection of treatment options and effective drug combinations still remain subtle. Thus, there is a pressing need to explore new and more effective drugs for the treatment of this disease. Consistently, natural products have displayed a diversity of positive human health promoting properties since times immemorial. Natural products are mainly the secondary metabolites synthesized by plants to adapt to and survive under extreme environmental conditions. Owing to the tendency of these secondary metabolites to interact with enzymes, receptors and other cellular entities, they exhibit appropriate drug-like properties [7]. In the present study we observed that gentiopicroside exhibited significant anticancer activity against SKOV3 ovarian cancer cells. The anticancer effects were found to be due to mitochondrial apoptosis, cell cycle arrest and inhibition of cell migration and invasion, indicating the potential of gentiopicroside as lead molecule for the treatment of ovarian cancer.

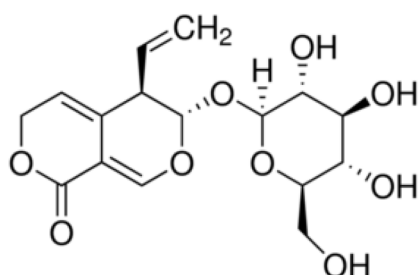


Figure 1. Chemical structure of gentiopicroside.

Methods

Chemicals, reagents and cell cultures

Gentiopicroside and other chemicals were of reagent grade and purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Non-small ovarian cancer cell line cell (SKOV3) were procured from Cancer Research Institute of Beijing, China, and were cultured continuously in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics (streptomycin 100 µg/ml and penicillin G 100 U/ml) and maintained at 37°C.

Antiproliferative assay

The anti-proliferative effect of gentiopicroside against SKOV3 cells was investigated by MTT assay. All cells were cultured at the density of 1×10^6 cells/well in 96 well plates for 12 hrs. The cells were then exposed to 0-80 µM gentiopicroside for 24 hrs. Thereafter, 20 µl of MTT solution were added to each well. Prior to

the addition of 500 µl of DMSO, the cells were separated from the medium. For solubilizing the MTT formazan crystals, we added dimethylsulfoxide (500 µl). Thereafter, absorbance was taken by ELISA plate reader at 570 nm. Since gentiopicroside exhibited lowest IC_{50} against SKOV3 cells, further experiments were performed only at 0, 10, 20 and 40 µM concentrations.

Apoptosis assay

SKOV3 cells were cultured at the density of 1×10^6 cells per well in 6-well plates and then were exposed to 0, 7.5, 15 and 30 µM gentiopicroside for 24 hrs. This was immediately followed by DAPI staining. Then, the cell samples were examined and photographs were taken with a fluorescence microscope. To estimate the apoptotic cell populations, SKOV3 cells were plated at a density of 1×10^6 cells/well in 6-well plates and treated with varied concentrations (0, 7.5, 15 and 30 µM) of gentiopicroside for 24 hrs. Thereafter, the cells were collected and washed with phosphate buffered saline (PBS). The cells were then incubated with Annexin V/FITC and PI for 15 min and the apoptotic cell populations were estimated by a flow cytometry (BDBiosciences, San Jose, CA, USA).

Cell cycle analysis

To investigate the distribution of SKOV3 cells in different phases of the cell cycle, approximately 1×10^5 cells in each well of 6-well plates were kept at 37°C overnight to allow the cells to adhere. This was followed by treatment with various concentrations of gentiopicroside (0, 7.5, 15 and 30 µM) and then were incubated at 37°C for 24 hrs. Thereafter, the cells were trypsinized and were resuspended in ice-cold PBS. Finally, the cells were treated with ethanol (70%) at -20°C and allowed to fix overnight. Following fixation with ethanol, the cells treated with ice-cold PBS were centrifuged (1000 rpm) for 10 min at 4°C. Afterwards, the cells were resuspended in 1 ml PI/Triton-X 100 solution for 30 min in the dark. Finally, the distribution of the cells at each phase was examined from 10,000 cells in a FacScan flow cytometer (BD Biosciences). The estimation of the percentage of cells in each phase of the cell cycle was carried out by WinMDI software.

Boyden chamber assay for cell migration and invasion

Cell migration assay was carried out by Boyden chamber assay with some modifications. Cells at the density of 5×10^4 cells/well were suspended in 2% FBS medium and placed in the upper chamber of 8 µm pore size transwells. Afterwards, medium supplemented with 10% FBS was added to the lower chamber. This was followed by 24-h incubation. On the upper surface of the membrane, unmigrated cells were removed while on the lower surface of the membrane the migrated cells were fixed with methanol (100%) and Giemsa-stained. The cell migration was assessed by counting the number of migrated cells under a microscope.

Determination of protein expression by western blotting

Protein expression was determined by western blot analysis. Briefly, proteins present in the cell extracts

were resolved by SDS-PAGE. This was followed by transference of the blots on nitrocellulose membrane. Following this, the membrane was treated with non-fat milk (5%) in PBS and then incubated with suitable primary antibody for 2 hrs at room temperature followed by secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hr at room temperature. The western blot results were then observed in an enhanced chemiluminescence (ECL) western blot analysis system (Amersham Biosciences).

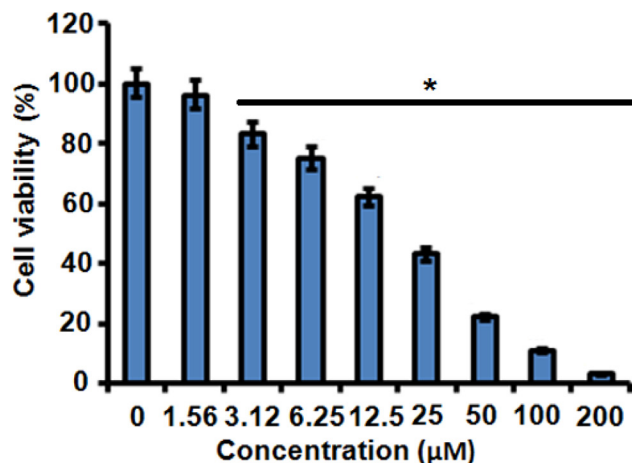


Figure 2. Effect of indicated doses of gentiopicroside on cell viability of SKOV3 cells. The results are mean \pm SD. The values were considered significant at * $p < 0.01$.

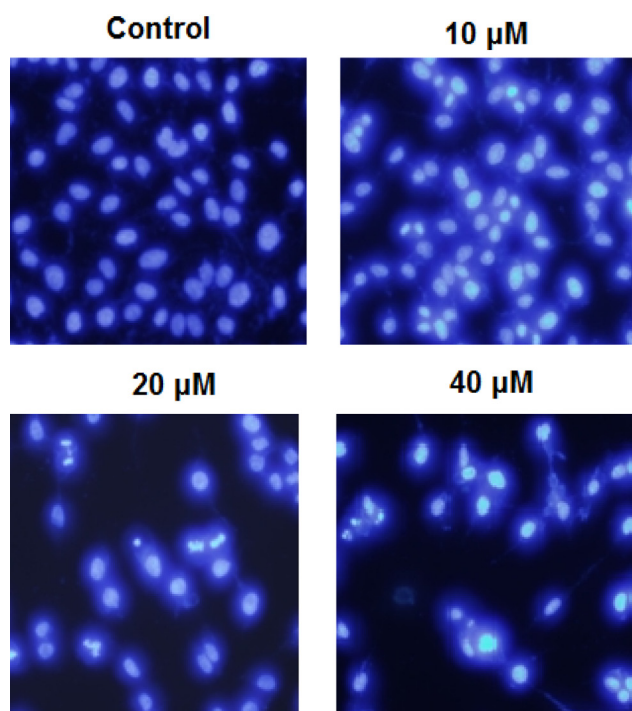


Figure 3. Effect of indicated doses of gentiopicroside on the cell apoptosis as indicated by DAPI staining. The experiments were carried out in triplicate. The Figure shows that gentiopicroside exerts dose-dependent apoptotic effects on the SKOV3 cells.

Statistics

The experiments were performed in triplicate and presented as means \pm standard deviation (SD). Student's t-test was used and Graphpad prism 7 software was used for statistical analyses. Results were considered significant at $p < 0.01$.

Results

Gentiopicroside exerts anti-proliferative effects on SKOV3 cells

Gentiopicroside displayed significant anticancer effects against SKOV3 cells with an observed IC_{50} 20 μ M. The effect of gentiopicroside on the cell viability showed a dose-dependent trend (Figure 2).

Gentiopicroside causes apoptosis in SKOV3 cells

Gentiopicroside caused apoptosis in SKOV3 cells in a concentration-dependent manner as it was obvious from the increased number of cells with white color nuclei (Figure 3). Estimation of apoptotic cell population was determined by flow cytometry as shown in Figure 4. The percentage of apoptotic cells from 2.6% in the control increased to 39.6% at 30 μ M concentration of gentiopicroside. To examine if the apoptosis followed the mitochondrial pathway we determined the protein expression of Bax and Bcl-2. The results showed that the protein expression of Bax was upregulated in a concentration-dependent manner and that of Bcl-2 was decreased (Figure 5).

Gentiopicroside induces G2/M cell cycle arrest of SKOV3 cells

To assess the effect of gentiopicroside on the cell cycle phase distribution of SKOV3 cells, the

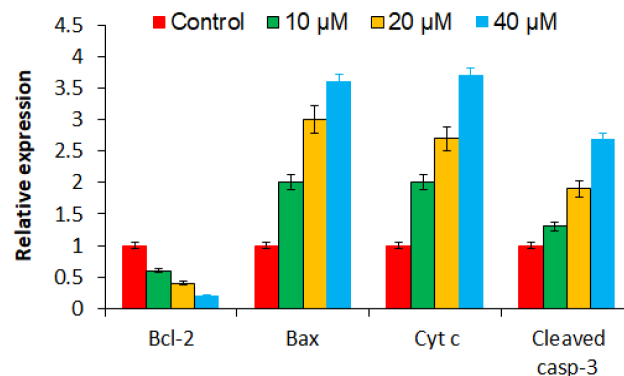


Figure 4. The percentage of cell apoptosis induction (various stages of apoptotic cells) and the cell viability was quantified from Annexin V FITC and PI staining by flow staining analysis (** $p < 0.001$, *** $p < 0.0001$).

cells were administered 0, 10, 20 and 40 μM of gentiopicroside for 24 hrs. What happened was that the number of cells at G2 phase increased in a dose-dependent manner causing cell cycle arrest (Figure 6). At 40 μM there was a marked increase in G2 phase cells.

Gentiopicroside suppresses cell migration of SKOV3 cells

The results of cell migration assay after gentiopicroside treatment at 20 μM concentration for 24 hrs, indicated that gentiopicroside reduced the motility and migration of the of SKOV3 cells in a dose-dependent manner (Figure 7).

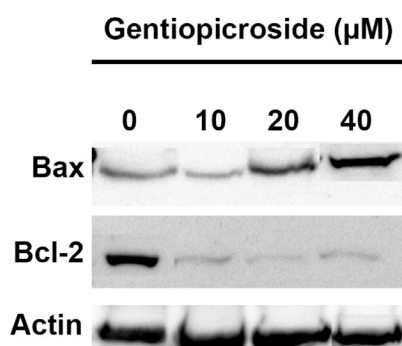


Figure 5. The effect of indicated doses of gentiopicroside on Bax and Bcl-2 expression as indicated by western blotting. The experiments were carried out in triplicate. The results of this Figure show that gentiopicroside increased Bax and decreased Bcl-2 in SKOV3 cells in a dose-dependent manner.

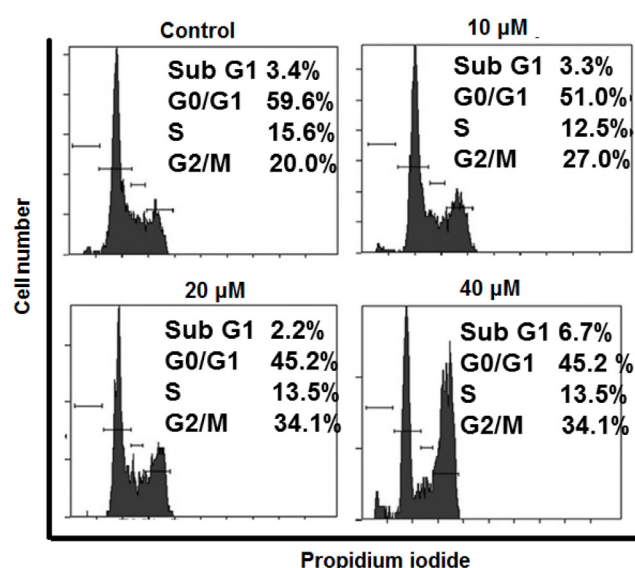


Figure 6. The effect of indicated doses of gentiopicroside on cell cycle distribution as indicated by flow cytometry. The experiments were carried out in triplicate. The Figure proves that G2/M phase cells increased in a concentration-dependent manner.

Discussion

Ovarian cancer, being one of the fatal cancers, demands immediate development of novel treatment strategies that could prove beneficial in decreasing its incidence. Natural products could prove crucial source for identifying lead molecules for the treatment of ovarian cancer [8]. In the current study we investigated the anticancer effects of gentiopicroside against ovarian cancer cell line SKOV3. The results showed that gentiopicroside could significantly inhibit the growth of SKOV3 cancer cells. The IC_{50} of gentiopicroside was 20 μM and its anticancer effects were dose-dependent. These results are also consistent with previous studies. For instance, gentiopicroside was found to exhibit significant anticancer effects on ovarian cancer cell line OVACAR-3 through induction of apoptosis [9]. Keeping this in mind, we also investigated if gentiopicroside could trigger apoptosis in SKOV3 ovarian cancer cells. The results of DAPI staining revealed that gentiopicroside could trigger apoptosis in SKOV3 in a dose-dependent manner as evidenced from the DNA fragmented white color nuclei. We also estimated the apoptotic cell populations at different doses by annexin V/PI double staining and observed that the apoptotic cell populations increased from 2.6% in the control to 39.6% at 40 μM concentrations. Our results are also justified by the fact that gentiopicroside and other related natural products exert anticancer effects on cancer cells by induction of apoptosis [10,11]. Apoptosis was found to be mediated via the mitochon-

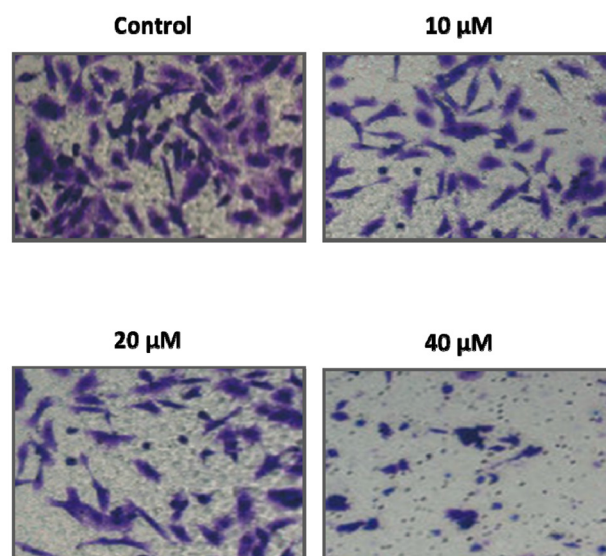


Figure 7. The effect of indicated doses of gentiopicroside on cell migration as evidenced from migration assay.

drial pathway as evidenced from the Bax/Bcl-2 ratio. Apoptosis is an important mechanism by which anticancer agents eliminate the cancer cells from the body, indicative of the fact that gentiopicroside could prove a potential anticancer agent [12,13]. In addition, cell cycle arrest is another mechanism by which anticancer drugs cells inhibit the growth of cancer cells [14,15]. In the present study we observed that gentiopicroside induced G2/M cell cycle arrest in SKOV3 cells. Gentiopicroside also inhibited the cell migration of SKOV3 cancer cells, suggesting that it could potentially inhibit metastasis of cancer cells *in vivo*.

Conclusion

Taken together, these results indicate that gentiopicroside exerts anticancer effects on ovarian SKOV3 cancer cells by induction of apoptosis and G2/M cell cycle arrest. Besides, it could also inhibit cell migration of cancer cells *in vivo*. Therefore, gentiopicroside is a potent lead molecule for the management of ovarian cancer and deserves further research endeavours.

Conflict of interests

The authors declare no conflict of interests.

References

- Huang Xh, Luo Mz, Qi H, Wang ZZ. Proliferation effects of gentiopicroside and other five Traditional Chinese Medicines on SMMC-7721 Human Hepatocarcinoma Cell Line Northwest Pharma 2004;4:014.
- Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA: Cancer J Clin 2014;64:9-29.
- Bast Jr RC, Urban N, Shridhar V et al. [Eds): Early detection of ovarian cancer: promise and reality. In Ovarian Cancer 2002, pp 61-97, Springer, USA.
- Berek JS. Ovarian cancer. In: Berek JS, Hacker NF (Eds): Practical Gynecologic Oncology, Lippincott Williams and Wilkins, Philadelphia, PA, USA, 2005, pp 443e511.
- Bast RC, Hennessy B, Mills GB. The biology of ovarian cancer: new opportunities for translation. Nat Rev Cancer 2009;9:415-28.
- Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 2012;75:311-35.
- Over B, Wetzel S, Grütter C et al. Natural-product-derived fragments for fragment-based ligand discovery. Nat Chem 2013;5:21-26.
- Mann J. Natural products in cancer chemotherapy: past, present and future. Nat Rev Cancer 2002;2:143-7.
- Tian H, Liu L, Yang TJ, Wang YQ. Gentiopicroside inhibits cancer cell growth in OVCAR-3 ovary cancer cells through the mediation of apoptosis, loss of mitochondrial transmembrane potential and NF-kB signalling pathway. Biomedical Research 2016;27:5-9.
- Wani BA, Ramamoorthy D, Rather MA et al. Induction of apoptosis in human pancreatic MiaPaCa-2 cells through the loss of mitochondrial membrane potential ($\Delta\Psi_m$) by Gentiana kurroo root extract and LC-ESI-MS analysis of its principal constituents. Phytomedicine 2013;20:723-33.
- Fulda S. Modulation of apoptosis by natural products for cancer therapy. Planta Medica 2010;76:1075-9.
- Gamet-Payraastre L, Li P, Lumeau S et al. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res 2000;60:1426-33.
- Mantena SK, Sharma SD, Katiyar SK. Berberine, a natural product, induces G1-phase cell cycle arrest and caspase-3-dependent apoptosis in human prostate carcinoma cells. Mol Cancer Ther 2006;5:296-308.
- Da Rocha AB, Lopes RM, Schwartzmann G. Natural products in anticancer therapy. Curr Opin Pharmacol 2001;1:364-9.
- Hung DT, Jamison TF, Schreiber SL. Understanding and controlling the cell cycle with natural products. Chem Biol 1996;3:623-39.