

Original Research Article

Sparteine exerts anticancer effect on human cervical cancer cells via induction of apoptosis, G₀/G₁ cell cycle arrest and inhibition of VEGFR2 signalling pathway

Songnian Liang^{1*}, Linlin Liu²

¹Department of Radiology, The First Hospital of China Medical University, ²Mental health Center for Student, China Medical University, Shenyang, Liaoning110001, China

*For correspondence: **Email:** sophia.hill20@yahoo.com; **Tel/Fax:** 0086-024-83282730

Sent for review: 28 January 2019

Revised accepted: 20 June 2019

Abstract

Purpose: To investigate the anticancer effects of sparteine against human cervical cancer.

Methods: Cell viability was determined by CCK8 assay, while 4', 6-diamidino-2-phenylindole (DAPI) staining was used for determination of apoptosis. Cell cycle analysis was done with flow cytometry, while cell invasion was monitored using Transwell invasion assays. Protein expressions were determined using Western blotting.

Results: The results revealed that sparteine inhibited the viability of cervical cancer cells with half-maximal inhibitory concentration (IC₅₀) ranging from 10 to 25 µM. Sparteine exerted more profound anti-proliferative effects on DoTc2 cells, with IC₅₀ of 10 µM. However, minimal cytotoxicity was observed in normal cervical cells, as evident from the IC₅₀ of 80 µM. Sparteine triggered the generation of ROS and apoptotic cell death in DoTc2 cells. The induction of apoptosis was accompanied by upregulation of Bax expression and downregulation of Bcl-2 expression. Sparteine caused arrest of DoTc2 cells at the G₀/G₁ phase of the cell cycle, and suppressed the expressions of cyclin A and cyclin B1. Transwell assay data showed that sparteine decreased the invasion ability of DoTc2 cells. Sparteine also inhibited the phosphorylation of VEGFR2 in a concentration-dependent manner.

Conclusion: Sparteine exhibits significant anticancer activity and may prove beneficial in cervical cancer chemotherapy.

Keywords: Cervical cancer, Apoptosis, Cell cycle arrest, Sparteine

This is an Open Access article that uses a fund-ing model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>) and the Budapest Open Access Initiative (<http://www.budapestopenaccessinitiative.org/read>), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Cervical cancer imposes huge disease burden on all nations across the globe. Approximately, 260,000 cervical cancer mortalities and 530,000 new cases of cervical cancer were reported in 2012 alone [1]. Owing to disproportionate incidence of cervical cancer in low and high-

income nations, it is often referred to as the 'disease of disparity' [2]. Cervical cancer is ranked the fourth major type of cancer in the world [3]. The treatments for cervical cancer involve radical hysterectomy, chemotherapy and/or radiotherapy [4].

However, the currently available chemotherapeutic agents have adverse effects on the quality of life of patients [5]. This has fuelled the search for novel and effective anticancer agents for combating this disease. One of the major concerns of researchers is identification of natural anticancer principles for the development of chemotherapy for cervical cancer [6]. Among natural products, alkaloids constitute a large and diverse group of metabolites in the plant kingdom. The potential of alkaloids as anticancer agents has increased tremendously with the discovery of the alkaloidal anticancer agents vinblastine and vincristine [7].

Sparteine is an important alkaloid isolated from *Lupinus* species. It belongs to the group of quinolizidine alkaloids and has been reported to exhibit analgesic activity [8]. Nonetheless, there is no single report in the literature on the anticancer activity of sparteine. Therefore, this study was undertaken to investigate the anticancer effects of sparteine against a panel of human cervical cancer cell lines and one normal cell line. Moreover, the effect of sparteine on the therapeutically important VEGFR2 pathway was investigated [9].

EXPERIMENTAL

Determination of cell viability

CCK-8 assay was used for the determination of cell viability of DoTc2 cervical cancer cells. Transfected DoTc2 cells were seeded in 96-well plates and subjected to treatment with varied concentrations of sparteine at 37 °C for 24 h. Thereafter, 10 µL of CCK-8 reagent was added to the cell culture and incubated for 2 h at 37 °C in a humidified atmosphere containing 5 % CO₂ and 95 % O₂. Optical density was read at 450 nm in a microplate reader, as an index of cell viability.

DAPI staining assay

Cervical cancer DoTc2 cells (0.6×10^6) were cultured in 6-well plates. They were treated with varied concentrations of sparteine, and incubated for 24 h at 37 °C. As the cells sloughed off, 10 µL cell culture was put onto a glass slide and subjected to staining with DAPI. The slides were covered with a cover slip and examined under a fluorescent microscope.

Transwell assay

The invasion abilities of the DoTc2 cells were determined with Transwell chamber invasion assay. The cells (1×10^4) were seeded in upper

chamber of the transwell (8 µm pore size polycarbonate filters). This was followed by the incubation of the chambers at 37 °C for 24 h. The inserts were coated with extracellular matrix gel (50 µL) (ECM, Sigma, USA). Swabbing was performed to remove the non-invaded cells from the upper surface. However, the invaded cells on the lower surface were fixed with methanol and then stained with crystal violet (0.5%) for about 50 min. The cells were then washed with PBS, and finally counted under the light microscope (5 fields).

Cell cycle analysis

DoTc2 cells were treated with varied concentrations of sparteine and incubated for 24 at 37 °C. Thereafter, the cells were washed with phosphate buffered saline (PBS), and the KGN cells were stained with propidium iodide (PI). The distribution of cells in cell cycle phases was assessed using FACS flow cytometer.

Cell invasion assay

Western blot analysis

Sparteine-treated DoTc2 cells were harvested using centrifugation and then lysed in lysis buffer containing protease inhibitor. About 45 µg of protein from each sample was subjected to separation on 10 % SDS-PAGE. The protein bands were transferred to polyvinylidene difluoride (PVDF) membrane, and the membrane was then blocked with fat-free milk at room temperature for 1 h. The membrane was treated with appropriate primary antibodies at 4 °C overnight, and was subsequently incubated with secondary antibody. Finally, the signals were detected using Odyssey Infrared Imaging System (LI-COR, USA). Actin was used as control for normalisation.

Statistical analysis

All assays were performed in triplicate and the values are shown as mean ± standard deviation (SD, n = 3). Student's *t*-test was employed for statistical analysis using GraphPad Prism 7 software. Values of *p* < 0.05 were considered statistically significant.

RESULTS

Sparteine exerted growth inhibitory effects on cervical cancer cells

The effects of sparteine were evaluated against a panel of four cervical cancer cell lines (CaSki, DoTc2, SiHA, C-33A) and one normal cell line

(HCvEpC) (Figure 1 A). The results indicated that sparteine suppressed the proliferation of all the cervical cancer cells, with IC_{50} values ranging from 10 to 25 μ M (Table 1). It was observed that sparteine exerted more significant anticancer effects on the DoTc2 cells, with an IC_{50} of 10 μ M (Figure 1 B). Evaluation of the anti-proliferative effects of sparteine on normal HCvEpC cells showed that sparteine exerted minimal growth inhibitory effects on these cells (IC_{50} = 80 μ M). The growth inhibitory effects of sparteine on the cervical cancer cells exhibited a dose-dependent pattern.

Table 1: Anticancer effect (IC_{50}) of sparteine on cervical cancer and normal cell lines

Cell line	IC_{50} (μ M)
DoTc2	10
SiHa	25
HeLa	7.5
C33A	25
HCvEpC	80

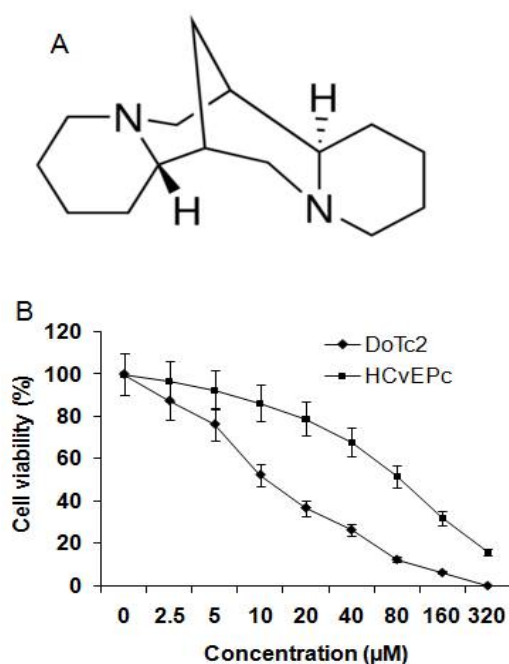


Figure 1: (A) Chemical structure of sparteine; (B) CCK-8 assay showing the effect of sparteine on the viability of cervical cancer DoTc2 and normal HCvEpC cells. Data are expressed as mean \pm SD (n = 3). * p < 0.05

Sparteine promoted generation of ROS in DoTc2 cells

The effects of different concentrations of sparteine on ROS levels in DoTc2 cells were investigated. It was revealed that sparteine caused dose-dependent increases in the ROS levels of the DoTc2 cells (Figure 2). The ROS level increased to 205 %, relative to 100 % in

control. Moreover, the sparteine-induced increases in ROS levels were concentration-dependent.

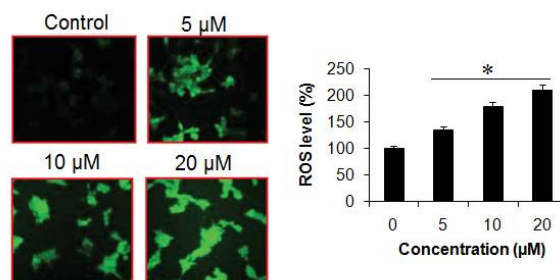


Figure 2: Effect of sparteine on ROS levels in DoTc2 cells at indicated concentrations. Data are expressed as mean \pm SD (n = 3); * p < 0.05

Sparteine caused apoptotic cell death of DoTc2 cells

To ascertain if sparteine inhibited DoTc2 cell proliferation via induction of apoptotic cell death, DAPI staining of the sparteine-treated DoTc2 cells was performed. The results showed that sparteine caused nuclear fragmentation in DoTc2 cells in a dose-dependent manner (Figure 3 A). These alterations in the nuclear morphology of the DoTc2 cells were characteristic of apoptosis. Therefore, western blot analysis was performed to determine the expressions of Bax and Bcl-2 (Figure 3 B). The expression of Bax was found to increase while as that of bcl-2 decreased in the DoTc2 cells, upon sparteine treatment.

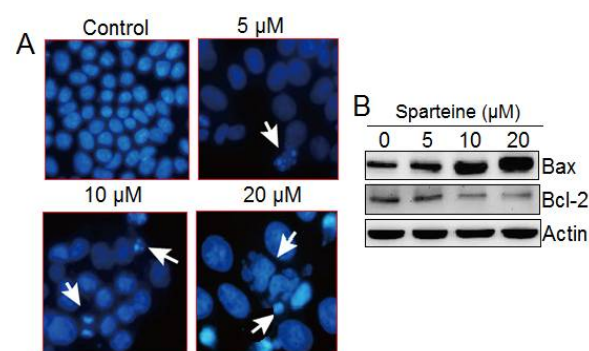


Figure 3: (A) DAPI staining showing induction of apoptosis in the sparteine treated DoTc2 cells (Arrows depict apoptotic cells) (B) Effect of sparteine on the expressions of Bax and Bcl-2 in DoTc2 cells at indicated concentrations. The experiments were performed in triplicate

Sparteine caused arrest of DoTc2 cells at G₀/G₁ checkpoint

The effect of sparteine on the distribution of the DoTc2 cells in different cell cycle phases was also studied. The results indicated that sparteine

treatment caused a significant increase in the G₀/G₁ phase of the cell cycle, which indicated G₀/G₁ arrest (Figure 4 A). This was also accompanied by downregulation of cyclin A and cyclin B1 expressions in a dose-dependent manner (Figure 4 B).

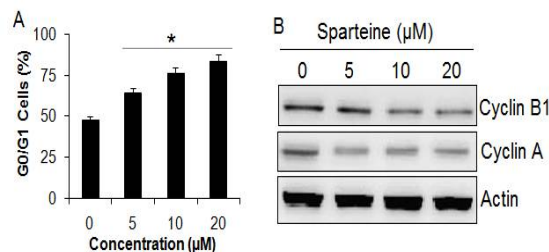


Figure 4: (A) Flow cytometric analysis showing that sparteine induced G₀/G₁ arrest of the DoTc2 cells. (B) Effect of sparteine on the expressions of cyclin A and cyclin B1. Data are expressed as mean ± SD (n = 3). **p* < 0.05

Sparteine inhibited invasion of the DoTc2 cells

Transwell assay was used to monitor the effect of 10 µM (IC₅₀) of sparteine on the invasion of the DoTc2 cervical cancer cells. The results showed that sparteine caused significant decrease in the invasion of the DoTc2 cells (Figure 5). Invasion of DoTc2 cervical cancer cells decreased to 38 %, when compared to 100 % in untreated control.

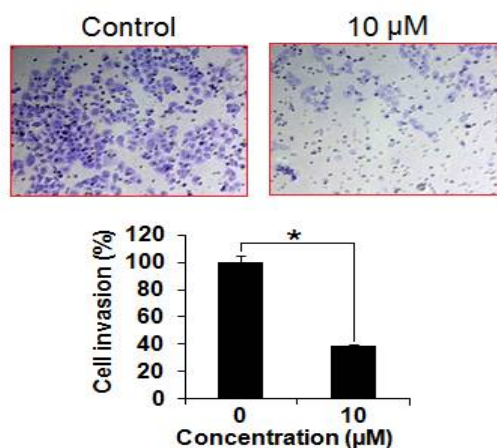


Figure 5: Effect of sparteine at IC₅₀ on invasion of DoTc2 cells as depicted by transwell assay. Data are expressed as mean ± SD (n = 3). **p* < 0.05

Sparteine deactivated VEGFR2 pathway in DoTc2 cells

The effect of sparteine was also investigated on the phosphorylation status of VEGFR2 in the DoTc2 cells (Figure 6). The results showed that

the phosphorylation of VEGFR2 cells decreased concentration-dependently upon treatment with sparteine.

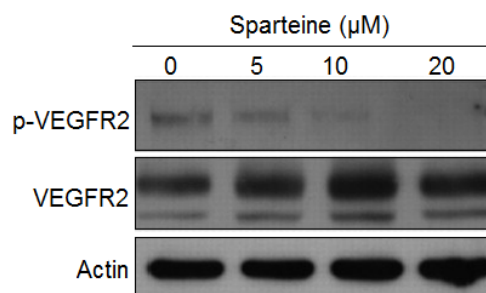


Figure 6: Effect of sparteine on the phosphorylation of p-VEGFR as depicted by western blot analysis. Experiments were performed in triplicate

DISCUSSION

Owing to drawbacks in cervical cancer chemotherapy, the development of new treatment strategies is of utmost priority for cancer researchers across the globe. Huge research efforts are regularly devoted to exploring effective treatments, as well as minimising the toxicities of the identified drugs [10]. Many of the anticancer drugs exhibit narrow therapeutic windows because of their low selectivity against cancer cells. Another goal of the researchers is to develop drugs that selectively target cancer cells or reduce their malignancy without any effect on normal cells [11].

One of the approaches is to explore chemical scaffolds isolated from terrestrial plants [12]. In the present study, the anticancer effect of the alkaloid sparteine against a panel of cervical cancer cells, was investigated. The results showed that sparteine selectively killed the cervical cancer cells, with greater profound effects on the DoTc2 cells. These observations are also supported by other studies carried on the quinolizidine alkaloids. For example, quinolizidine alkaloids from *Sophora flavescens* have been shown to inhibit the growth of cancer cells [13]. Studies have shown that many of the plant-derived alkaloids increase the generation of ROS in cancer cells [14]. For instance, it has been reported that the plant derived alkaloid berberine induced accretion of ROS in prostate cancer cells [15].

The effect of sparteine on ROS levels in the DoTc2 cells was also studied. Interestingly, it was revealed that sparteine caused accretion of significant amounts of ROS in DoTc2 cells.

Studies have shown that ROS generation is linked to induction of apoptosis in cancer cells [16]. Indeed, DAPI staining of the sparteine-treated DoTc2 cervical cancer cells revealed that sparteine caused nuclear fragmentation of the DoTc2 cells. The apoptosis was further confirmed with the expression patterns of Bax and Bcl-2 in the DoTc2 cells. It is known that Bax and Bcl-2 are important biomarker proteins of apoptosis [17]. The results showed that sparteine upregulated Bax and downregulated Bcl-2. Previous studies have shown that the alkaloid sanguinarine induced apoptosis in breast cancer cells [18]. Alkaloids have also been reported to cause cell cycle arrest in cancer cells [19]. Neferine, an alkaloid, has been shown to cause cell cycle arrest in lung cancer cells [19]. In the present study, it was found that sparteine caused arrest of the DoTc2 cells in the G₀/G₁ phase of the cell cycle. Sparteine also inhibited invasion of the DoTc2 cells, indicating its anti-metastatic potential. Vascular endothelial growth factor-2 (VEGFR2) is an important therapeutic target for cancer treatment [20]. The results obtained in this study showed that sparteine inhibited the phosphorylation of VEGFR2. This is suggestive of the anti-cancer potential of sparteine.

CONCLUSION

The findings of this study show that sparteine exerts growth-inhibitory effects on DoTc2 cells via ROS-mediated apoptosis and G₀/G₁ arrest. It also suppresses the invasion of DoTc2 cells via inhibition of VEGFR2 phosphorylation. Therefore, sparteine is a potential lead molecule for the development of cervical cancer chemotherapy.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

Open Access

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/>

4.0) and the Budapest Open Access Initiative (<http://www.budapestopenaccessinitiative.org/read>), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

REFERENCES

1. Schiffman M. Cervical cancer screening: epidemiology as the necessary but not sufficient basis of public health practice. *Prev Med* 2017; 8: 3-6.
2. Arbyn M, Castle PE. Offering self-sampling kits for HPV testing to reach women who do not attend in the regular cervical cancer screening program. *Cancer Epidemiol Biomarkers Prev* 2015; 1: 1414-1417.
3. Di Felice E, Caroli S, Paterlini L, Campari C, Prandi S, Rossi PG. Cervical cancer epidemiology in foreign women in Northern Italy: role of human papillomavirus prevalence in country of origin. *Euron J Cancer Prev* 2015; 24(3): 223-230.
4. Motoki Y, Mizushima S, Taguri M, Takahashi K, Asano R, Kato H, Asai-Sato M, Katayama K, Okamoto N, Hirahara F, et al. Increasing trends in cervical cancer mortality among young Japanese women below the age of 50 years: an analysis using the Kanagawa population-based Cancer Registry, 1975–2012. *Cancer Epidemiol* 2015; 39(5): 700-706.
5. Muñoz N, Bosch FX, De Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003; 348(6): 518-527.
6. Felice MR, Giuffre L, El LA, Hafidi M, Criseo G, Romeo O, Scordino F. Looking for new antifungal drugs from flavonoids: impact of the genetic diversity of *Candida albicans* on the in-vitro response. *Curr Med Chem* 2017; 21: 2-6.
7. Owellen RJ, Owens Jr AH, Donigian DW. The binding of vincristine, vinblastine and colchicine to tubulin. *Biochem. Biophys Res Commun* 1972; 47(4):685-691.
8. De Pinto MC, Barceló AR. Superoxide anion scavenger properties of sparteine, a quinolizidine alkaloid from *Lupinus*. *Biochem Biophys Res Commun* 1997; 150(1-2): 5-8.
9. Shi L, Zhang S, Wu H, Zhang L, Dai X, Hu J, Xue J, Liu T, Liang Y, Wu G. MiR-200c increases the radiosensitivity of non-small-cell lung cancer cell line A549 by targeting VEGF-VEGFR2 pathway. *PloS One* 2013; 30; 8(10):e78344.
10. Piver MS, Rutledge F, Smith JP. Five classes of extended hysterectomy for women with cervical cancer. *Obstetrics Gynecol* 1974; 44(2):265-272.
11. Rates SM. Plants as source of drugs. *Toxicon*. 2001; 39(5): 603-613.
12. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. *J Ethnopharmacol* 2005; 100(1-2):72-9.
13. Lin Z, Huang CF, Liu XS, Jiang J. In Vitro Anti-Tumour Activities of Quinolizidine Alkaloids Derived from *Trop J Pharm Res*, July 2019; 18(7): 1459

- Sophora Flavescens* Ait. *Basic Clin Pharmacol Toxicol* 2011;108(5):304-9.
14. Efferth T, Giais M, Merling A, Krammer PH, Li-Weber M. Artesunate induces ROS-mediated apoptosis in doxorubicin-resistant T leukemia cells. *PLoS One*. 2007; 2(8):e693.
 15. Meeran SM, Katiyar S, Katiyar SK. Berberine-induced apoptosis in human prostate cancer cells is initiated by reactive oxygen species generation. *Toxicol App Pharmacol* 2008; 229(1):33-43.
 16. Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis*. 2000; 5(5): 415-418.
 17. Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B, Reed JC. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene*. 1994;9(6):1799-1805.
 18. Choi WY, Kim GY, Lee WH, Choi YH. Sanguinarine, a benzophenanthridine alkaloid, induces apoptosis in MDA-MB-231 human breast carcinoma cells through a reactive oxygen species-mediated mitochondrial pathway. *Chemotherapy* 2008; 54(4): 279-287.
 19. Poornima P, Weng CF, Padma VV. Neferine, an alkaloid from lotus seed embryo, inhibits human lung cancer cell growth by MAPK activation and cell cycle arrest. *Biofactors* 2014; 40(1): 121-131.
 20. Choueiri TK, Vaishampayan U, Rosenberg JE, Logan TF, Harzstark AL, Bukowski RM, Rini BI, Srinivas S, Stein MN, Adams LM, et al., Phase II and biomarker study of the dual MET/VEGFR2 inhibitor foretinib in patients with papillary renal cell carcinoma. *J Clin Oncol* 2013; 31(2):181.