

Original Research Article

Anticancer effect of a combination of cisplatin and matrine on cervical cancer U14 cells and U14 tumor-bearing mice, and possible mechanism of action involved

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Abstract

Purpose: To investigate the anticancer effects of cisplatin (DDP) in combination with matrine on cervical cancer U14 cell tumor-bearing mice.

Methods: The cell proliferation of cervical cancer U14 cells treated with DDP (25, 20, 15, 10 and 5 µg/mL); matrine (2.5, 2.0, 1.5, 1.0 and 0.5 mg/mL); or DDP (15 µg/mL) + matrine (2.5, 2.0, 1.5, 1.0 and 0.5 mg/mL) was determined with MTT assay. The anticancer effect of DDP + matrine in U14 tumor-bearing mice was also investigated, based on expression of tumor suppressor lung cancer 1 (TSLC1) using quantitative real time-polymerase chain reaction (qRT-PCR) and immunohistochemistry.

Results: The inhibition of proliferation of U14 cells ranged from 26.68–70.25, 10.20–61.73, and 51.89–89.75 % for DDP, matrine and DDP + matrine, respectively. In mice with U14 solid tumors, the DDP group had 12.3 % weight loss ($p < 0.05$). Treatment with DDP, matrine, and DDP + matrine reduced tumor growth by 64.56, 42.22–56.67, and 67.78–81.11 %, respectively ($p < 0.01$). Results from RT-qPCR and immunohistochemistry showed corresponding increases in expression levels of TSLC1.

Conclusion: These results indicate that the anticancer activity of DDP + matrine is higher than that of a single treatment with either DPP or matrine. The likely mechanism of action might be related to promotion of TSLC1 expression. This finding provides a potential strategy for the management of cervical cancer.

Keywords: Cervical cancer, Cisplatin, Matrine, Anticancer activity, Combined chemotherapy, Natural products

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INTRODUCTION

Worldwide, cervical cancer is the fourth most common cancer among women [1]. Although the incidence of cervical cancer has been slowly

decreasing due to advances in human papillomavirus (HPV) vaccination and cervical screening, it remains one of the most common cancers among women today [2]. It has been estimated that the vaccination coverage currently

achieved, mainly in high-income countries, will prevent about one million cervical cancer cases within half a century between 2020 and 2069 [3]. However, the burden of cervical cancer remains high in less developed countries, according to GLOBOCAN 2018 [4].

Currently, the primary treatment for cervical cancer at the early stage is surgery, sometimes accompanied by chemotherapy or radiation [5]. Chemotherapy is a very popular and the most suitable in the treatment of several cancers. It interferes with the cell-cycle, leading to cancer cell death by preventing tumor growth [6].

Cisplatin (DDP) (Figure 1 A) is the first-line treatment for different types of solid tumors, and it targets DNA and active DNA-damage repair systems [7]. Radical hysterectomy and adjuvant DDP-based chemotherapy have positive effects on overall survival (OS) and progression-free survival (PFS) of cervical cancer patients [8]. However, the important issue in DDP clinical use is related to its low response rate, short median PFS and toxicity. The combination of DDP and other chemotherapeutic agents (combinatory regimens) produced better results than the use of DDP alone, in terms of response and PFS [7]. Natural products have been widely investigated as anticancer drugs, based on their widespread biological activities and minimal toxic. Alkaloids are synthesized by plants for their protection. Matrine (Figure 1 B) is an alkaloid found in *Sophora flavescens Aiton* (Fabaceae), a Chinese medicinal herb, and it is traditionally used for its sedative, anti-inflammatory, immunity-regulatory, antiviral, and antitumor properties [9].

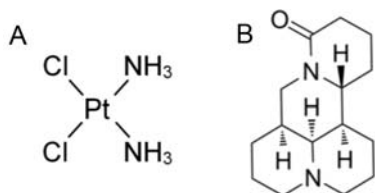


Figure 1: Chemical structures of DDP (A) and matrine (B)

It has been reported that matrine exerted antineoplastic effect by inhibiting proliferation and inducing apoptosis of cancer cells via up-regulation or down-regulation of the expressions of cancer-associated factors [9]. Thus, there is need for clinical trials on the use of novel molecules as anticancer agents. In the present study, the anticancer effect of combination of matrine and DDP was investigated in a mouse model of cervical cancer, and in a cervical cancer cell line.

EXPERIMENTAL

Drug

Matrine was purchased from China National Pharmaceutical Group Co. Ltd. (Sinopharm) (Beijing, China). Cisplatin (DDP) injection was purchased from Qilu Pharmaceutical (Jinan, China).

Patients

The cervical tissue specimens were taken from hospitalized patients of The First Hospital of Lanzhou University. Informed consent was obtained from the patients. The study was approved by the ethics committee of the First Hospital of Lanzhou University (approval no. LDYYLL2016-101). All the cervical cancer cases were new and primary cases, and had not received any treatment. There were 36 cases of cervicitis, 101 cases of cervical intraepithelial neoplasia (CIN), and 39 cases of cervical cancer.

Animals

A total of 80 healthy female Kunming mice aged 6-8 weeks, with mean weight of 20 ± 2 g, were obtained from Lanzhou University Experimental Animal Center. The animals were housed in a controlled environment at temperature of 22 ± 2 °C and humidity of 40–60 % in an environment with 12-h light/12-h dark cycle photoperiod, and were provided *ad libitum* access to water and feed. The animals were randomly divided into 8 groups, with 10 mice per group: normal saline group, DDP group (2 mg/kg), 35 mg/kg matrine group, 50 mg/kg matrine group, 75 mg/kg matrine group, DDP (2 mg/kg) + matrine (75 mg/kg) group, DDP (2 mg/kg) + matrine (50 mg/kg) group, and DDP (2 mg/kg) + matrine (35 mg/kg) group.

Cervical cancer cell line

Mouse cervical cancer U14 cell line was purchased from Institute of Basic Medicine of Chinese Academy of Medical Sciences (Beijing, China). The U14 cells were transplanted into mouse peritoneal cavity three times weekly. Ascites-fluid containing U14 cells was diluted with normal saline to a concentration of 5×10^6 living cells per 0.2 mL, and the diluted suspension (0.2 mL) was injected into the right axilla of mouse to produce solid tumor growth.

Methyl thiazolyl tetrazolium (MTT) assay

The U14 cells were seeded in a 96-well plate at a density of 2×10^4 cells per well for 24 h. Thereafter,

they were treated with DDP (25, 20, 15, 10 and 5 µg/mL); matrine (2.5, 2.0, 1.5, 1.0 and 0.5 mg/mL); and DDP (15 µg/mL) + matrine (2.5, 2.0, 1.5, 1.0 and 0.5 mg/mL), while the normal group was cultured in a serum-free medium, followed by incubation at 37 °C. Culturing was terminated at 24, 48 and 72 h. Then, 20 µL of MTT (5 mg/mL) was added to each well.

After incubation for 4 h in a cell culture incubator, the medium in each well was replaced with a known volume of DMSO to solubilize the formazan crystals formed. The absorbance (A) of each well was measured at 490 nm using a microplate reader (Molecular Devices, USA). Inhibition of cell proliferation (H) was calculated according to Eq 1.

$$H (\%) = \{(Ac - At)/Ac\} 100 \dots\dots\dots (1)$$

where Ac and At are the absorbance of treatment and control group samples, respectively.

Mouse xenograft studies

All mice received implants of U14 cervical cancer cells. The DDP group received intratumoral DDP injection at the dose of 2 mg/kg/day. The matrine groups received intratumoral matrine injection at doses of 35, 50, and 75 mg/kg/day, and DDP + matrine groups received intratumoral DDP injection (2 mg/kg/day) and matrine (35, 50 and 75 mg/kg/day), whereas normal saline group received daily intratumoral saline injection as in the model control. The experiment lasted for 10 days. Thereafter, the mice were anesthetized with 5 % isoflurane and sacrificed via decapitation on the 11th day.

The primary tumors were excised and weighed [10]. All animal experimentals were approved by the Institutional Animal Care and Use Committee of Lanzhou University (LDYLL2016-102), and were conducted in accordance with the National Institute of Health Laboratory Animal Care and Use Guidelines [11].

Immunohistochemistry

Tumor specimens were fixed with formalin. The specimens were paraffin-embedded and cut into 5-µm sections, deparaffinized and rehydrated [12]. Citrate buffer was used for antigen retrieval, and the protein expression was assessed using HRP anti-Rabbit IgG Detection System (Boster Biological Technology Co. Ltd., Wuhan, China). The primary antibody of rabbit anti-human polyclonal antibody against tumor suppressor lung cancer 1 (TSLC1) was obtained from Bioss (Beijing, China).

Quantitative real-time PCR

Total mRNA was extracted from the cervical tissue specimens using TRIzol reagent (Beyotime, Shanghai, China), and was reverse-transcribed to cDNA using a reverse transcription kit (Beyotime, Shanghai, China). The SYBR Master Mixture system (Beyotime, Shanghai, China) was used for real-time quantitative PCR (RT-qPCR). The relative gene expressions were calculated using the $2^{-\Delta\Delta C_t}$ method, after normalization to β -actin. The sequences of primers used for RT-qPCR are shown in Table 1.

Table 1: Sequences of primers used for quantitative PCR

Gene	Primer sequence
TSLC1	Forward: 5'-CCCCAGCCTGTGATGGTAA-3'
	Reverse: 5'-GGATAGTTGTGGGGGATCGT-3'
β -actin	Forward: 5'-AGCCTCGCCTTTGCCGA-3'
	Reverse: 5'-CTGGTGCCTGGGGCG-3'

Statistical analysis

Results are presented as mean \pm standard deviation (SD). Differences were determined using two-tailed Student's *t*-test or one-way ANOVA. All statistical analyses were performed using software of GraphPad Prism (version 5.0). Values of $p < 0.05$ were considered indicative of statistically significant differences.

RESULTS

TSLC1 expression levels in cervical tissue specimens

In this study, immunohistochemical analysis of 176 human cervical tissue specimens confirmed the protein expression of TSLC1. The TSLC1 showed cytolymph or cytoplasmic expression in the cervical tissue cells. Representative images showing TSLC1 expression in the human cervicitis, CIN and cervical cancer are presented in Figure 2. Significantly high TSLC1 expression was seen in cervicitis (Figure 2 A and D). In contrast, TSLC1 was significantly decreased in CIN (Figures 2 B and D) and cervical cancer (Figure 2 C and D) ($p < 0.001$). To further investigate the expression pattern of TSLC1, RT-qPCR was used to determine changes in expression of TSLC1 at mRNA level in cervicitis, CIN and cervical cancer. In line with the immunohistochemical results, the TSLC1 mRNA was significantly decreased in CIN and cervical cancer, when compared to cervicitis (Figure 2 E, $p < 0.001$). These results suggest that TSLC1 might function as a tumor suppressor in cervical cancer. Thus, TSLC1 expression was used to

evaluate the anticancer effect of DDP + matrine in subsequent experiments.

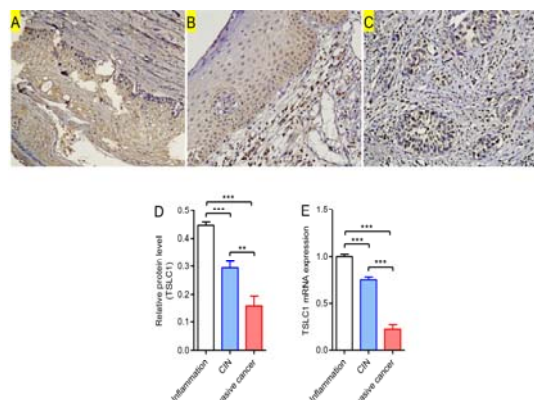


Figure 2: Expression of TSLC1 in cervical tissue specimens. Immunohistochemical analysis of TSLC1 in human cervicitis (A), CIN (B) and cervical cancer (C). The expression level of TSLC1 was significantly decreased in CIN and cervical cancer (D). The expression level of TSLC1 mRNA was significantly decreased in CIN and cervical cancer (E). ** $P < 0.01$, *** $p < 0.001$

In vitro anticancer effect of DDP + matrine

The anticancer effects of DDP, matrine and DDP + matrine on cervical cancer U14 cells were determined using MTT assay, and TSLC1 mRNA expression was used to evaluate the anticancer effect. The results indicated that the growth of U14 cells was inhibited in a concentration- and time-dependent fashion when treated with DDP at doses of 5.0–25.0 $\mu\text{g}/\text{mL}$ for 24, 48, and 72 h. The inhibition ranged from 26.68 to 70.25 % (Figure 3 A). The inhibition at 15.0 $\mu\text{g}/\text{mL}$ for 72 h reached 62.65 %, basically in the plateau period (Figure 3A). Cells incubated with matrine at concentrations of 0.5 – 2.5 mg/mL were also inhibited, with the inhibition ranging from 10.20 to 61.73 % (Figure 3 B), suggesting that matrine exerted anticancer effect on U14 cells. The growth of U14 cells was inhibited significantly on incubation with DDP (15 $\mu\text{g}/\text{mL}$) + matrine (0.5 – 2.5 mg/mL), with inhibition range of 51.89 – 89.75 %. These levels of inhibition were much higher than that produced by DDP treatment (Figure 3 C). The results indicate that the anticancer effect of the DDP + matrine were higher than that of DPP or matrine alone. Similar results were obtained from evaluation of the anticancer effect of DDP + matrine based on TSLC1 mRNA expression using RT-qPCR. The level of TSLC1 mRNA was significantly elevated when the U14 cells were incubated in DDP, matrine and DDP + matrine, especially DDP + matrine (Figure 3 D - F).

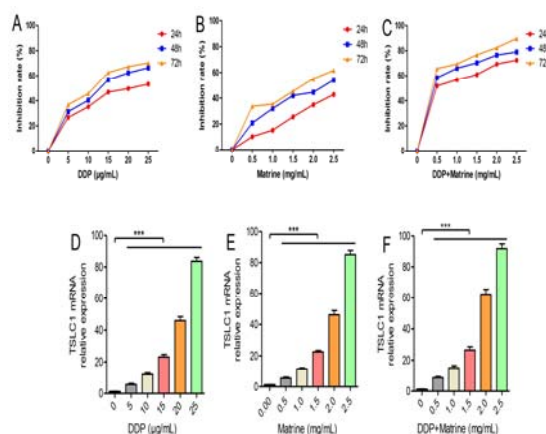


Figure 3: Anticancer effects of DPP, matrine and matrine + DPP combination, and their influence on TSLC1 mRNA expression in U14 cells. Percentage inhibition of U14 cells after treatment with DDP (A), matrine (B) and DDP + matrine (C). The expression level of TSLC1 mRNA was significantly elevated after treatment with DDP (D), matrine (E) and DDP + matrine (F). *** $P < 0.001$

In vivo anticancer effect of the combination of DDP and matrine

In order to achieve better understanding of the anticancer effects produced by DDP, matrine and DDP + matrine, the body weight and tumor weight of the cervical cancer U14 tumor-bearing mouse model were determined. There were no significant differences in body weight among all groups, except the DDP group which showed marked weight loss after 10 days ($p < 0.05$; Table 2). In addition, DDP mice had dry fur, decreased appetite, lethargy and reduced autonomic activity. The tumor weights obtained from mice treated with DDP, matrine and DDP + matrine were considerably lower than that of saline group ($p < 0.01$; Table 2). Cisplatin, at a dose of 2 mg/kg, reduced tumor growth by 64.56 % (Table 2). Matrine, at doses of 75, 50 and 35 mg/kg, reduced tumor growth by 56.67, 46.11, and 42.22 %, respectively (Table 2), while DDP (2 mg/kg) + matrine (75, 50 and 35 mg/kg) reduced tumor growth by 81.11, 73.89, and 67.78 %, respectively (Table 2). These results suggest that the DDP + matrine markedly inhibited tumor growth in cervical cancer U14 tumor-bearing mouse.

Effect of DDP + matrine on TSLC1 expression in tumors from cervical cancer U14 tumor-bearing mice

The effect of DDP + matrine on tumor suppressor gene TSLC1 was determined using immunohistochemical analysis. The results indicated that TSLC1 was expressed in

membrane or cytoplasm in the cervical tissue cells, as shown in Figure 4. With increase in matrine concentration, TSLC1 expression in tumor tissues increased gradually (Figure 4 D-F). Compared with the model control group, positive expression of TSLC1 in tumor cells of the matrine treated group was significantly increased (30 – 50 % vs 10 %, $p < 0.01$; Table 2). Significant increase in positive expression of TSLC1 was also observed in the DDP + matrine-treated groups (70–90 % vs 10 %, $p < 0.01$; Figure 4 G - I, Table 2). These results indicate that matrine up-regulated TSLC1 expression in U14 tumor tissues in a dose-dependent manner. Moreover, TSLC1 was significantly enhanced after matrine was combined with DDP, suggesting that the enhancement of TSLC1 expression may be one of the antitumor mechanisms of matrine.

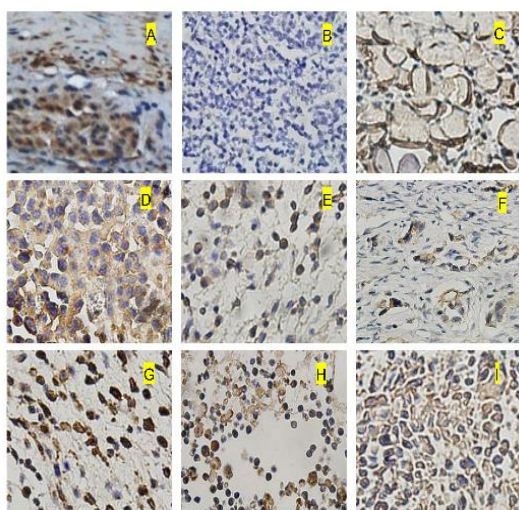


Figure 4: Immunohistochemical analysis of expressions of TSLC1 in tumors from cervical cancer U14 tumor-bearing mice (400 \times). (A) Membrane or cytoplasmic expression of TSLC1 in positive control group. (B) TSLC1 was not expressed in normal saline group. (C) TSLC1 expression was lower in DDP group. (D) High expression of TSLC1 in matrine (75 mg/kg). (E-F) TSLC1 expression increased on treatment with matrine (50, 35 mg/kg). (G-I) TSLC1 expression increased markedly and dose-dependently on treatment with DDP + matrine (75, 50, 35 mg/kg)

DISCUSSION

The prognosis of recurrent and metastatic cervical cancer treated with conventional chemotherapy is poor. Although DDP is the first-line treatment for cervical cancer, it is associated with the problem of drug resistance. Thus, there is need for development of new therapies to overcome the disadvantages of DDP. Chinese herbal medicines used for cancer management

are very common in the East, based on their widespread biological activities and minimal toxicity. *Ganoderma lucidum* has been shown to suppress migration and adhesion of invasive prostate and breast cancer cells by inhibiting constitutively active NF- κ B and AP-1 [13]. *Cordyceps sinensis* has apoptosis-inducing and immunity-modulating effects [14]. It also exerts anti-metastatic action by accelerating the secretion of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 from cancer cells, and inhibiting the activity of matrix metalloproteinases (MMP)-2 and MMP-9 [15]. Matrine is extracted from a Chinese therapeutic herb *Sophora flavescens*, and it exerts a variety of pharmacological activities. Matrine exhibits anticancer activities against lung cancer [16], hepatoma cancer [17], and gastric cancer [17]. It produces anticancer effects by inhibition of proliferation and induction of apoptosis cells [18].

In recent years, the use of combined chemotherapy has become popular. Studies have shown that DDP-based doublets (e.g., DDP + paclitaxel) are superior to DDP monotherapy in the treatment of cervical cancer [19]. Therefore, this study was carried out to investigate the anticancer effect of matrine + DDP through *in vitro* and *in vivo* studies.

In this study, MTT assay showed that the ranges of inhibition of DDP and matrine on cervical cancer U14 cells were 26.68–70.25 and 10.20–61.73 %, respectively. In contrast, there were 51.89–89.75 % inhibition by DDP + matrine, which was much higher than that due to monotherapy with matrine or DDP. In order to evaluate the mechanism involved in the anticancer effect, the expression of TSLC1 mRNA was assayed using RT-qPCR. Several studies have shown that TSLC1 is a tumor suppressor gene which inhibits cell proliferation, differentiation, migration, and invasion of cervical cancer [20]. This study showed that TSLC1 mRNA level was significantly elevated when the U14 cells were incubated in DDP, matrine and DDP + matrine, especially DDP + matrine. These results demonstrate that DDP + matrine exerted strong anticancer effect *in vitro*.

A significant and dose-dependent reduction in tumor weight was observed in U14-bearing mice following DDP + matrine treatment. In addition, the expression of TSLC1 was significantly increased in all groups treated with DDP + matrine, when compared with model control group, as determined using immunohistochemistry.

Table 2: Effect of DDP + matrine on the inhibition of tumor growth and TSLC1 expression in U14 tumor-bearing mice

Group	Body weight (g)		Tumor weight (g) (mean ± SD)	Tumor inhibition (%)	TSLC1 expression		Positive rate (TSLC1)
	Beginning (g)	Ending (g)			n (Negative)	n (Positive)	
Normal saline	21.09±0.2	20.64±0.4	1.80±0.02	-	9	1	10%
DDP (2 mg/kg)	20.44±0.2	17.93±0.7*	0.64±0.01**	64.44	3	7	70%**
Matrine (75 mg/kg)	20.11±0.1	20.10±0.3	0.78±0.04**	56.67	4	6	60%**
Matrine (50 mg/kg)	20.41±0.1	20.74±0.1	0.97±0.05**	46.11	5	5	50%**
Matrine (35 mg/kg)	20.35±0.1	20.87±0.2	1.04±0.05**	42.22	7	3	30%
DDP+matrine (75 mg/kg)	20.31±0.2	20.56±0.1	0.34±0.02***##	81.11	1	9	90%**
DDP+matrine (50 mg/kg)	20.21±0.0	20.59±0.1	0.47±0.02***##	73.89	2	8	80%**
DDP+matrine (35 mg/kg)	20.25±0.2	20.96±0.1	0.58±0.01***##	67.78	3	7	70%**

The % tumor inhibition = $(\text{Weight}_{\text{model control}} - \text{Weight}_{\text{treatment group}}) / \text{Weight}_{\text{model control}} \times 100$; *p < 0.05, **p < 0.01, compared with normal saline group; ##p < 0.01, compared with DDP and matrine groups

CONCLUSION

The results obtained in this study indicate that DDP + matrine exerts anticancer activity against cervical cancer U14 cells *in vitro* and *in vivo*, via a mechanism which might be related to TSLC1. This finding provides a potential strategy for the management of cervical cancer.

DECLARATIONS

Acknowledgement

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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. The study was designed by Aihong Yang and Yongxiu Yang. All experiments are performed by Aihong Yang, Jun Zhu, Feixue Xu, Jiao Yang, Ying Wang, Min Wei and Xuefei Bai. Jun Zhu and Feixue Xu collected data, and gave suggestions in designing this manuscript. Aihong Yang and Yongxiu Yang drafted the manuscript. All authors read and approved the final manuscript.

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