Isocorydine inhibits the proliferation of human endometrial carcinoma HEC-1B cells by downregulating the Ras/MEK/ERK signaling pathway

Yu Fu1, Rui-Rui Jin1, Yi-Lin Li1, Hua Luan1, Tao Huang2, Yuan Zhao1, Lin Yang1, Fa-Hui Niu1, Qing-Mei Sun1, 2, Qing Liu1, 2, Yong-Xiu Yang2, 3

1 Gansu Provincial Maternity and Child-care Hospital, No.143 North Street, 730050 Lanzhou, Gansu, China
2 The First Hospital of Lanzhou University, No.1 Donggang West Road, 730020 Lanzhou, Gansu, China
*Correspondence: sqm20170915@163.com (Qing-Mei Sun); LQ20180116@163.com (Qing Liu); yyx20180116@163.com (Yong-Xiu Yang)
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Objective: Isocorydine (ICD), an aporphine alkaloid, plays a role in anticancer activity that is still being evaluated. However, the exact function and mechanism of ICD in endometrial carcinoma is largely unknown. Methods: MTT, flow cytometry, western blot, immunofluorescence, RT-PCR and ELISA were used in this research. Results: In our study we showed that ICD inhibited endometrial carcinoma HEC-1B cell proliferation at certain times and concentrations. Simultaneously, ICD induced HEC-1B cells apoptosis. Further investigation indicated that ICD reduced the phosphorylation protein levels of c-Raf (rapidly accelerated fibrosarcoma), MEK1/2 (MAPK/Erk kinase) and ERK1/2 (extracellular regulated protein kinase). Moreover, a cell immunofluorescence assay confirmed that ICD reduced intracellular expression of Ras and then promoted FOXO3 (forkhead box O3) nuclear localization to further increase the mRNA level of Bim and p27Kip1. Additionally, an enzyme-linked immunosorbent assay (ELISA) revealed that ICD inhibited the production of EGF (epidermal growth factor) and PDGF (Platelet-Derived Growth Factor), which indicated that ICD indirectly inhibited the activation of the Ras/MEK/ERK signaling pathway at the ligand level. Conclusion: Taken together, these data suggest for the first time that ICD inhibits cell proliferation and induces cell apoptosis in endometrial carcinoma through suppressing Ras/MEK/ERK signaling, which may provide a theoretical foundation to the application of ICD for the treatment of human endometrial carcinoma.

Keywords
Isocorydione; Endometrial carcinoma; Proliferation; Ras/MEK/ERK signaling pathway

1. Introduction
Endometrial carcinoma is one of the most prevalent gynecological malignant carcinomas of female genital system. Around the world, endometrial carcinoma is the sixth most common cancer among the women occurring at a peak age of between 50–65 years [1]. Risk factors for the development of endometrial carcinoma are obesity, estrogen replacement therapy, diabetes, hypertension, late menopause and infertility [2]. Although progress has been made with therapeutic approaches, recurrent and distant metastases frequently take place in endometrial carcinoma. Thus, it is vital to recognize more key regulators of endometrial carcinoma to develop more effective therapies [3, 4].

Isocorydine (ICD), a kind of natural aporphine tetrahydroisoquinoline alkaloid, is widely distributed in many plants in nature including menispermanceae, papaveraceae, the ananacaceae families and Dicranostigma leptopodium (Maxim). Fedde exhibits several dramatically biological activities such as central nervous system activity, antitumor and antibacterial activity [5]. It was approved as an antispasmodic agent by the China Food and Drug Administration (CFDA). It has recently been reported that ICD inhibits cell proliferation by inducing G2/M cell cycle arrest and cell apoptosis in hepatocellular carcinoma cell (HCC) lines; it also targets chemotherapeutic drug-resistant cellular side population (SP) cells by inducing tumor suppressor gene PDCD4 (programmed cell death 4)-related apoptosis [6, 7]. After this discovery, more isocorydine derivatives were designed, synthesized and shown to possess more effective anticancer properties [8]. Furthermore, an isocorydine derivative (d-ICD) inhibited IGF2BP3 expression in a time-dependent manner that was negatively correlated with d-ICD-induced growth suppression, thus, it inhibited HCC cell growth, particularly among the CD133+ subpopulation, and resulted in HCC cells being more sensitive to sorafenib treatment [9]. However, the function and molecular mechanisms of ICD in endometrial carcinoma have not been thoroughly researched.

In this study, we primarily focused on investigating the mechanism by which ICD inhibits the proliferation of endometrial carcinoma HEC-1B cells. The Ras/MEK/ERK pathway, being the central transducer of oncogenic signals in solid tumors, was considered to play an important part in the transmission of signals from growth factor receptors as well as modulating gene expression and cell apoptosis [10, 11]. Furthermore, our study suggested that ICD suppressed the activation of Ras/MEK/ERK signaling.
2. Materials and methods

2.1 Cell lines

Human endometrial carcinoma cell line HEC-1B was purchased from the Cell Bank of the typical culture preservation Committee of the China Academy of Sciences (Shanghai, China). HEC-1B was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ at 37 °C.

ICD was provided by professor Junxi Liu from Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences (Lanzhou, China). ICD was extracted from romneya dicranotigma using macroporous adsorption resin technology.

2.2 MTT assays

Exponentially growing HEC-1B cells were used to perform MTT (3-(4,5-dimethylthiazole-yl)-2,5-diphenyl tetrazolium bromide) assays. The cells were dyed and seeded in 96-well plates with 8000 cells in each well treated with 0, 4, 8, 16, 32 or 64 mg/L ICD for 24 h. After 24 h, 48 h and 72 h respectively, the cells were harvested and washed three times with PBS and then suspended in 1 × Annexin V Binding Solution at a final concentration of 1 × 106 cells/mL. Cells of 100 μL were incubated with 5 μL Annexin V FITC and 5 μL PI Solution for 30 min at room temperature. The cells were analyzed by system software.

2.3 Apoptosis analysis

Flow cytometry analysis of Annexin V/PI staining was used. Exponentially growing HEC-1B cells were incubated and treated with different concentrations of ICD for 24 h. The cells were harvested and washed three times with PBS and then suspended in 1 × Annexin V Binding Solution at a final concentration of 1 × 106 cells/mL. Cells of 100 μL were incubated with 5 μL Annexin V FITC and 5 μL PI Solution for 30 min at room temperature. The cells were analyzed by system software.

2.4 Western blot

Exponentially growing HEC-1B cells were maintained in culture dishes treated with 0, 8 or 16 mg/L ICD for 24 h. The cells were directly lysed on ice with RIPA containing protease and phosphatase inhibitors. Denaturation proteins were separated by 10% SDS-PAGE. The proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes, followed by 5% nonfat milk blocking for 1 h at room temperature. The membranes were incubated with primary antibodies anti-ERK1/2, anti-p-ERK1/2, anti-c-Raf, anti-p-c-Raf, anti-MEK1/2, anti-p-MEK1/2 and anti-β-actin overnight at 4 °C. The membranes were then incubated with an anti-rabbit IgG secondary antibody conjugated with Horse Radish Peroxidase for 1 h at room temperature. ECL detection and visualization using medical X-ray film were then carried out.

2.5 Immunofluorescence of cultured cells

Exponentially growing HEC-1B cells were maintained in a 6-well plate on the bottom of which were cover slips treated with 0, 8 or 16 mg/L ICD for 24 h and were fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with Triton X-100 for 20 min at room temperature, then they were incubated with the primary antibodies anti-Ras (1 : 50) and anti-FoxO3 (1 : 50) overnight at 4 °C. The secondary antibodies were used included fluorescence-antirabbit IgG for 30 min at 37 °C. After being washed, the cells were observed and photographed under fluorescence microscope.

2.6 RNA extraction and real-time quantitative PCR

Exponentially growing HEC-1B cells were treated with 0, 8 or 16 mg/L ICD for 24 h. Total RNA were extracted using the TRIZol Reagent. Total cDNA was used as template for PCR amplification with GAPDH as a control. Real-time quantitative PCR was performed in triplicate using an IQ5 real-time PCR Detection System. The protocol was 95 °C for 15 min, 40 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 30 seconds. The results were analyzed through the ΔΔCt method and the primers were as follows: Bim: 5′-ATA CCA TTC AGC TTC GTG ATG G-3′; 5′-CCC AAC CTC TGA CAA GTG ACC -3′, p27kip1: 5′-CAA GTA CGA GTG GCA AGA GTT G-3′; 5′-ATG CTG TCC TCA GAG TTA GCC -3′, GAPDH: 5′-AAG GCT GTG GGC AAG G-3′; 5′-TGG AGG AGT GGG TGT CG -3′.

2.7 ELISA assay

Exponentially growing HEC-1B cells were treated with 0, 8 or 16 mg/L ICD for 24 h. Collected serum-free mediums were determined using EGF and PDGF enzyme-linked immunosorbent assay (ELISA) kits in accordance with the manufacturer’s instructions.

2.8 Statistical analysis

All statistical analyses were performed using SPSS Version 21.0 (IBM, Armonk, NY, USA). Data were analyzed using one-way ANOVA. Student’s t-tests were used to analyze the significance of the differences between treated and control groups. Data were presented as mean values with standard deviation. P < 0.05 was indicated as statistical significance.

3. Results

3.1 ICD treatment inhibits the proliferation and induces cell apoptosis in endometrial carcinoma HEC-1B cells in vitro

In order to investigate the effect of ICD treatment on endometrial carcinoma HEC-1B cells, an MTT assay was applied to detect the inhibition ratio of ICD to HEC-1B cells. The result showed that with an increased concentration of ICD, the inhibition of HEC-1B cell proliferation correspondingly increased. When the concentration was between 4 mg/L and 32 mg/L, the longer the treatment time of ICD on HEC-1B cells, the greater the inhibition rate was. Thus, the inhibition of cell proliferation was time and concentration-dependent (Fig. 1A). Next to understand the mechanism by which ICD inhibited cell proliferation, we assessed the effect of ICD on cell apoptosis with an Annexin V/PI staining kit. We found that with a concentration of 0, 8 or 16 mg/L ICD the percentage of apoptosis cells increased from 6.17%, 15.00% to 34.11% (Fig. 1B and C). These results demonstrated that ICD inhibited the proliferation of and induced cell apoptosis in endometrial carcinoma HEC-1B cells in vitro.
ICD inhibits cell proliferation through downregulating Ras/MEK/ERK signaling pathway

The Ras/MEK/ERK signaling pathway is reported to play a central role in transmitting oncogenic signals. The Ras/MEK/ERK signaling is aberrantly activated in cancer promoting cell proliferation, cell survival and metastasis, particularly by upstream activation through the epidermal growth factor receptor (EGFR) [12]. Also, it is well known that phosphorylated ERK (pERK) is a vital downstream component of the signaling pathway [13]. Therefore, we examined the effect of ICD on Ras/MEK/ERK signaling. Our results showed that with the increase concentration of ICD the phosphorylated c-Raf, MEK1/2 and ERK1/2 proteins gradually decreased compared to the control. However, there were few changes in the total c-Raf, MEK1/2 and ERK1/2 protein level (P<0.05, Fig. 2). These results primarily suggested that ICD may inhibit cell proliferation and induce apoptosis through suppressing the Ras/MEK/ERK signaling pathway.

ICD reduces ras expression and promotes FOXO3 nuclear localization, and then increases the expression of Bim and p27kip1

To further verify the inhibitive effect of ICD on the Ras/MEK/ERK signaling pathway, an analysis of Ras treated with ICD and control HEC-1B cells was performed by immunofluorescence staining. The expression of Ras was gradually reduced with an increase in the concentration of ICD (Fig. 3A top). FOXO3 was shown to be related to tumor suppression and inhibition of FOXO3 promoted tumor progression and angiogenesis [14, 15]. It was reported ERK could suppress the stability of FOXO3 and induce its nuclear exclusion [16]. The results also showed that with the increase concentration of ICD the expression of FoxO3 in the HEC-1B cells was increasing and many cells localized in nucleus (Fig. 3A bottom). FoxO3 activated multiple target genes involved in tumor suppression, such as Bim [17] and p27kip1 [18]. Finally, real-time-PCR was performed to detect the mRNA levels of Bim and p27kip1. The results was that the mRNA levels of Bim and p27kip1 increased with the increased concentration of ICD (P<0.05, Fig. 3B,C). Overall, all the results suggested that ICD (in order of succession) inhibited Ras/MEK/ERK signaling pathway, in succession promoted nuclear localization of FOXO3 and, finally, activated the expression of Bim and p27kip1 to inhibit cell proliferation.

ICD inhibits HEC-1B cells secretion of EGF and PDGF

It was thought that ligand-independent activation of extracellular signal growth factors EGF and PDGF could subsequently increase in both Ras and ERK1/2 activity [19]. Therefore, we examined the levels of EGF and PDGF in the serum-free medium of HEC-1B cells after treatment with ICD for 24 h. ELISA results showed that EGF activity in the 8 and 16 mg/L concentration (313.98±68.71 and 223.67±59.37, respectively) were lower than in the control (421.57±79.33) (Table 1, P<0.05; P<0.01). Furthermore, PDGF activity in the 8 and 16 mg/L concentration (4.89±0.48 and 3.01±0.22, respectively) were lower than in the control (6.72±0.67) (Table 1, P<0.05; P<0.01). These results indicated that ICD indirectly inhibited the Ras/MEK/ERK signaling pathway at the ligand level.

<table>
<thead>
<tr>
<th>The concentration of isocorydine (mg/L)</th>
<th>EGF (pg/mL)</th>
<th>PDGF (ng/L)</th>
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<tbody>
<tr>
<td>0</td>
<td>421.57 ± 79.33</td>
<td>6.72 ± 0.67</td>
</tr>
<tr>
<td>8</td>
<td>313.98 ± 68.71*</td>
<td>4.89 ± 0.48*</td>
</tr>
<tr>
<td>16</td>
<td>223.67 ± 59.37**</td>
<td>3.01 ± 0.22**</td>
</tr>
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Data are expressed as the mean ± SD. versus control group, *P<0.05, **P<0.01.

Discussion

ICD has been found possessing antispasmodic, analgesia, antimalarial, antiarrhythmic, and antitumor properties along with other pharmacological effects [20]. Recent studies have shown that ICD could reduce the size and weight of side population cells that induce tumor masses in nude mice thus was a potential therapeutic drug for targeting side population can-

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**Fig. 1. ICD treatment inhibits the proliferation of endometrial carcinoma HEC-1B cells depending on time and concentration and induces cell apoptosis in vitro.** (A) MTT assays were performed to detect the inhibition ratio of ICD to HEC-1B cells for 24 h, 48 h and 72 h in different concentration. (B,C) Apoptotic cells were detected by Annexin V-FITC and PI double staining following the treatment of HEC-1B cells with 0, 8 or 16 mg/L ICD for 24 h. The values presented come from independent experiments performed in triplicate as the mean ± SD.
Fig. 2. ICD inhibits cell proliferation by downregulating p-c-Raf, p-MEK1/2 and p-ERK1/2 levels. (A) Western blot analysis of phosphorylated and total c-Raf, MEK1/2 and ERK1/2 were conducted in HEC-1B cells treated with 0, 8 or 16 mg/L ICD for 24 h, β-actin was used as an internal control. (B–D) Quantitative analysis of phosphorylated and total c-Raf, MEK1/2 and ERK1/2 expression. The values are presented for independent experiments performed in triplicate as the mean ± SD. *P < 0.05, **P < 0.01.

cer cells in hepatocellular carcinoma [6]. The derivatives of ICD for example 8-amino-isocorydine (NICD) were promising chemotherapeutic agents for the treatment of HCC because they target CSCs and EMT [8]. Other d-ICD was reported to inhibit HCC cell migration and invasion via down-regulating E2F1 expression; it directly upregulated the expression of ITGA1, which was associated with promoting the migration and invasion of HCC cells [21]. The results indicated that these isocorydine derivatives may potentially be used in targeted chemotherapy or be further analyzed in combination with conventional chemotherapy drugs to be an effective therapy in HCC. However, no previous results have identified a role for ICD in endometrial carcinoma.

Consistent with this finding, our study found that treatment with ICD inhibited cell proliferation and induced cell apoptosis in endometrial carcinoma HEC-1B cells in vitro (Fig. 1). The Ras/MEK/ERK pathway is known to play a pivotal role in cell proliferation, differentiation, development, apoptosis and tumor progression [13, 22, 23]. Ras/MEK/ERK pathway is activated in several human cancers including breast cancer, colorectal cancer, prostate cancer and ovarian cancer [24–26]. A previous study indicated that small molecules benzothiazole suppressed cell proliferation and invasion via the Ras/MEK/ERK-dependent pathway in highly malignant breast cancer MCF-7 cells [11]. In our study, the results suggested that ICD decreased phosphorylated c-Raf, MEK1/2 and ERK1/2 protein levels and reduced Ras expression (Fig. 2 and Fig. 3A top).
It has been documented that the expression of Bim and p27^Kip1 can be transcriptionally regulated by FOXO3, which is a downstream target protein of ERK [16, 27, 28]. In this study, we further confirmed the inhibitive effect of ICD to on the Ras/MEK/ERK signaling pathway. The decrease of Ras as it was suppressed by ICD promoted FOXO3 nuclear localization which increased the expression of Bim and p27^Kip1 (Fig. 3). Finally, our study showed ICD reduced EGF and PDGF levels to inhibit the Ras/MEK/ERK signaling pathway at the ligand level (Table 1).

In conclusion, the present results indicated that ICD inhibits the proliferation of and induces cell apoptosis in endometrial carcinoma, all of which may be associated with the suppression of Ras/MEK/ERK signaling. These findings suggest that ICD is a potential treatment for endometrial carcinoma. Further in vivo studies are warranted to confirm the role of ICD in endometrial carcinoma.

Author contributions
YF performed the experiments and wrote the manuscript; RJ participated in the experiments and interpreted the data; YL explained the data and drew the figures; HL analyzed the data and statistical analysis; TH provided the references and data declaration; YZ contributed reagents and materials; LY and FN were responsible for the guidance of experimental instruments and data collection; QS designed and revised manuscript; QL and YY conceived and supervised this research.

Ethics approval and consent to participate
Not applicable.

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Conflict of interest
The authors declare no conflict of interest.

References