SUPPLEMENTARY MATERIAL

Prisconnatanones A, a cytotoxic naphthoquinone from *Prismatomeris connata*, suppresses the proliferation of human laryngocarcinoma HEP-2 cells *in vitro*

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Prisconnatanones A, a cytotoxic naphthoquinone from *Prismatomeris connata*, suppresses the proliferation of human laryngocarcinoma HEp-2 cells *in vitro*

Prisconnatanones A is a rare tetrahydroanthraquinone isolated from herbal *Prismatomeris connata*. In this study, we examine its anti-tumor activity on human laryngocarcinoma HEp-2 cells *in vitro*. The CCK-8 assay was performed to evaluate its cytotoxicity. Cell cycle and apoptosis were analysed using flow cytometric analysis. Here, we showed Priscon-A inhibited the proliferation of HEp-2 cells in a dose-dependent manner, and at 5 μM it almost completely inhibited cell growth. Its cytotoxicity was associated with the cell cycle arrest at G2/M phase. The Annexin V-FITC/PI binding assay showed that the cell death induced by Priscon-A was associated with apoptosis. And the levels of the apoptosis protein, cleaved caspase-3, PARP, p21 and Bax protein were increased with the anti-apoptosis protein Bcl-2 decreased, were analysed by western blot. These data demonstrated that Priscon-A significantly inhibited HEp-2 cell growth, induced the cell cycle arrest at the G2/M phase and efficiently induced cell apoptosis.

Keywords: Prisconnatanones A; HEp-2 cells; apoptosis

**Experimental**

**General**

Optical rotations were measured with a Perkin-Elmer 341 polarimeter with MeOH as solvent. The UV spectra were performed on a Thermo Scientific Evolution 300 UV-visible spectrophotometer in MeOH. The $^1$H (500 MHz), $^{13}$C (125 MHz) and 2D NMR spectra were recorded on a Bruker DRX-500 instrument using TMS as internal standard. ESI-MS were collected on a MDS SCIEX API 2000 LC/GC/MS instrument. HR-ESI-MS were carried out on a Bruker Bio-TOF-IIIQ mass spectrometer. HPLC was run with a Shimadzu LC-20A using an Inertsil ODS column (5 μm, 4.6×250
Extraction and isolation

The air-dried roots of P. connata (5.0 kg) were powdered and extracted with 95% EtOH (3×8 L) at r.t. The extract (536.0 g) was suspended in H₂O (120 mL) and partitioned with CHCl₃ (500 mL). The CHCl₃-soluble extract (85.0 g) was subjected to silica gel column chromatography (CC) eluting with CHCl₃-MeOH (from 100:1 to 9:1) in gradient to yield ten sub-fractions Frs.1-10 based on TLC. Fr. 5 (13.0 g) was applied to Sephadex LH-20 CC with the eluent of MeOH to yield four sub-fractions Frs.5a-5d. Fr.5c (1.6 g) was purified by semi-preparative RP-C18 HPLC with the eluent of 65% MeOH-H₂O (254 nm, flow rate: 3 mL/min) to give compound 1 (98 mg, t_R = 45 min).

Cell viability assay

The cell viability of prisconnatanones A was tested against human laryngocarcinoma HEp-2 cells by CCK-8 assay, with DMSO as negative control and culture medium as blank control. Cell lines were maintained in DMEM (Dulbecco's Modified Eagle Medium) medium containing 10% fetal bovine serum and were cultivated in humidified incubator at 5% CO₂ and 37 °C. The human cancer cells in the log phase of their growth cycle (5000 cells/mL) were added to each well of the 96-well plates (100 µl/well) and were cultivated until attachment. The various doses (0.5 μM, 1 μM, 2 μM, 5 μM, 10 μM, 20 μM) of prisconnatanones A were then added and the cells were further incubated for 48 h. After, 10 μL CCK-8 solution per well was added and the plate was incubated for 1 h at 37 °C. The absorbance of each well was measured on an M200pro Multimode Plate Reader (Tecan, Switzerland) at 450 nm and 650 nm. Each treatment was performed in triplicate and experiments were repeated over 3 times (Zhao et al. 2013).

Cell proliferation assay

1.5×10⁴ HEp-2 cells per well were plated in 24-wells plates and were cultivated until attachment. Then cells were treated with various doses (2.5 μM, 5 μM, 10 μM) of
prisconnatanones A, DMSO was used as negative control. Cells were trypsinized and stained with trypan blue dye, and viable cells were counted using cell counting chamber every 24 h for a total of 5 days. Viable cell numbers of each group were collected and used to plot the cell growth curves (Zhao et al. 2013).

Apoptosis assay

HEp-2 cells in 6-well plates were grown overnight, followed by incubation in the absence or presence of various concentrations (2.5 μM, 5 μM, 10 μM) of prisconnatanones A for 24 h, DMSO was used as negative control. After, stained with Annexin V (AV) conjugated with FITC and propidium iodide (PI) using the Annexin V-FITC Apoptosis Assay Kit following the manufacturer's instructions. Stained cells were analyzed with Cyflow Cube flow cytometer (PARTEC, Germany). Data were analyzed using FlowJO 7.6.5 software (Zhao et al. 2013).

Western blot analysis

HEp-2 cells in 6-well plates were incubated overnight, followed by treated with various concentrations (2.5 μM, 5 μM, 10 μM) of prisconnatanones A for 24 h, DMSO was used as negative control. After, whole cell lysates were homogenized in RIPA lysis buffer containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors cocktail. The protein concentration of cell samples was analyzed by using BCA method. Equal amount proteins of each sample were electrophoresed on 8% to 15% SDS-PAGE gel and electrotransferred onto NC membrane. After incubation with appropriate primary and secondary antibodies, protein blots were detected by using ECL solution and ChemiDoc XRS+ imaging system (Bio-Rad, USA). β-actin was used as loading control (Zhao et al. 2013).

Statistical analysis

Data were presented as mean ± S.D. of three independent determinants. Statistical analysis was performed by using Student’s t-test. Differences were considered statistically significant with p<0.05.
The HPLC result

The HPLC result of prisconnatanones A analysis is displayed in Figure S1, it was appeared at 17.922 min.

The NMR data

Yellow amorphous powder, mp = 260 ~ 262 ºC; $[\alpha]_D^{25}$-90° (c 0.6, MeOH); UV $\lambda_{\text{max}}$ (MeOH) nm (log $\varepsilon$): 217.5 (4.39), 265.0 (4.24), 290.9 (3.96), 413.9 (3.84). IR $\nu_{\text{max}}$ (KBr): 3488, 2975, 1637, 1610, 1589, 1498, 1457, 1421, 1319, 1286, 1141, 1062 cm$^{-1}$; $^1$H NMR and $^{13}$C NMR data see Table S1; ESI-MS: m/z 341 [M + Na]$^+$, 659 [2M + Na]$^+$, 317 [M − H]$^-$ and 352.3 [M + Cl]$^-$; HR-ESI-MS: m/z 341.0995 [M + Na]$^+$ (Calcd for C$_{17}$H$_{18}$NaO$_6$: $\Delta$=-0.116 ppm).

References

Zhao F., Huang W W, Ousman T, Zhang B, Han Y Y, Clotaire D Z J, Wang C, Chang H H, Luo H N, Ren X Y, Lei M. 2013. Triptolide induces growth inhibition and apoptosis of human laryngocarcinoma cells by enhancing p53 activities and suppressing E6-mediated p53 degradation. PloS one. 8(11): e80784.
### Table S1. $^1$H and $^{13}$C NMR Data of Prisconnatanones A. ($\delta$ in ppm, $J$ in Hz)

| Position | Pris-A |  
|----------|--------|
|          | $\delta_H$ | $\delta_C$ |
| 1eq      | 2.94 dd (19.0, 5.6) | 24.1 |
| 1ax      | 2.30 dd (19.0, 7.9) |  |
| 2        | 1.92 m H-a | 28.9 |
| 3        | 3.74 brq (7.3, 6.2, 6.6) | 65.8 |
| 4eq      | 3.03 dd (19.0, 4.8) | 26.2 |
| 4ax      | 2.53 dd (19.0, 8.1) |  |
| 5        |  | 150.8 |
| 6        |  | 136.5 |
| 7        |  | 152.9 |
| 8        | 7.25, s | 99.0 |
| 9        |  | 178.5 |
| 10       |  | 184.1 |
| 11       |  | 106.1 |
| 12       |  | 123.0 |
| 13       |  | 138.7 |
| 14       |  | 138.5 |
| 2-CH$_3$ | 1.12 d (6.8) | 12.6 |
| 5-OCH$_3$ |  |  |
| 6-OCH$_3$ | 3.99 s | 56.3 |
| 7-OCH$_3$ | 4.00 s | 51.7 |
| 5-OH     | 12.26 s |  |
Figures

Figure S1. The HPLC analysis result of prisconnatanones A.

Figure S2. The $^1$H NMR of prisconnatanones A.
Figure S3. The $^{13}$C NMR of prisconnatanones A.

Figure S4. The HR-ESI-MS of prisconnatanones A.
Figure S5. Western blot analysis of apoptosis proteins in Priscon-A-treated HEp-2 cells.