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Original article

Induction of cell cycle arrest by the carbazole alkaloid *Clauszoline-I* from *Clausena vestita* D. D. Tao via inhibition of the PKCδ phosphorylation

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ABSTRACT

Sixteen carbazole alkaloids from *Clausena vestita* D. D. Tao were extracted, and their anti-tumor activities were evaluated. Among the extracts, *Clauszoline-I* exhibited an obvious growth inhibitory activity against several cancer cell lines through its ability to induce cell cycle arrest in the S and G2/M phases. A dramatic morphologic change with decreased F-actin staining and RhoA activity was found in *Clauszoline-I* treated HepG2 cells, in which the phosphorylation of PKCδ (Ser643) was inhibited. Our results indicated that induction cell cycle arrest by *Clauszoline-I* might be achieved by decreasing the RhoA activity via the inhibition of PKCδ phosphorylation.

1. Introduction

The genus *Clausena* belongs to the tribe *Clauseneae* of the *Rutaceae*-subfamily *Aurantioideae*. This genus comprises 15 species and 6 varieties [1]. They are shrubs or small trees mainly distributed in South and Southeast Asia [1,2]. *Clausena* plants have been extensively subjected to phytochemical investigations [1–9]. Various biological activities have been revealed, such as antimycobacterial, antifungal [2], antiplatelet [3,4], anti-tumor promoting [5], antiplasmodial [6], antimicrobial [7], antituberculosis [8], and anti-HIV activities [9].

Clausena vestita D. D. Tao is a species endemic to China and mainly distributed in the Yunnan Province. From this species, 30 constituents were isolated, and their structures were established by comparison with data in literature [10]. Among them, 16 alkaloids (Fig. 1) were in sufficient amounts for biological activity tests. In the present study, we evaluated the anti-tumor activities of the 16 alkaloids by cell growth inhibition assay, and further explored the potential mechanism. The results revealed that *Clauszoline-I* possessed a significant anti-tumor activity, and arrested cancer cells in the S and G2/M phases of the cell cycle. This effect of *Clauszoline-I* may be attributed to the decrease of RhoA activity via the inhibition of PKCδ (Ser643) phosphorylation.

2. Chemistry

The extraction and isolation processes were performed according to Ref. [10]. Briefly, the whole-plant air-dried *C. vestita* was chopped into pieces and extracted with 95% EtOH. After evaporation of the solvent, the crude extract was then chromatographed on a silica gel column eluted with $CHCl_3$/MeOH to yield seven main fractions (F1–F7). From F1 to F7, 16 carbazole alkaloids were purified over silica gel column and Sephadex LH-20 column chromatography (Fig. 1).

3. Results and discussion

In the first set of experiments, the in vitro IC50 values of the 16 compounds on HepG2 cell were analyzed. The IC50 values in Table 1 reveal that the activity was significantly affected by structural modifications within the carbazole skeleton. Some carbazole

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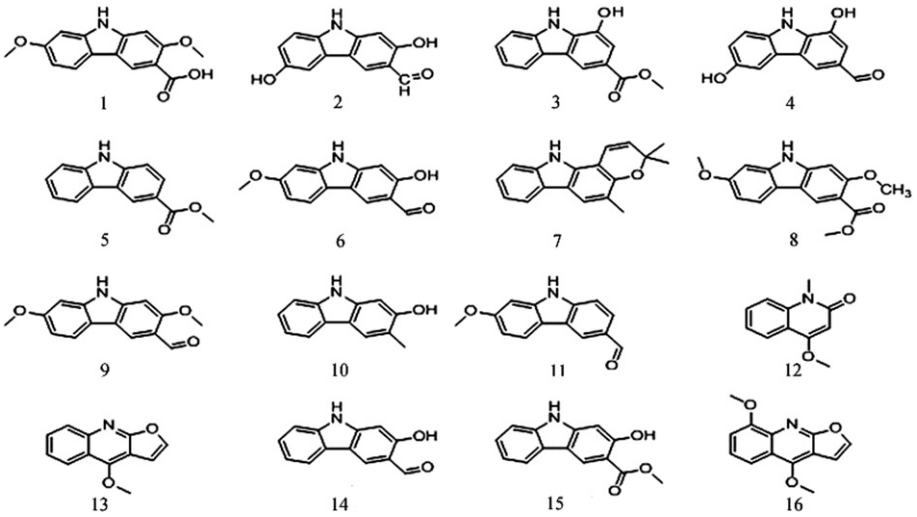

Fig. 1. Structure of the 16 alkaloids isolated from *Clausena vestita* D. D. Tao. 1: *Clauszoline-J (clausine-K)*; 2: *Clausine-O*; 3: *Clauszoline-I*; 4: *Clausine-Z*; 5: Methyl carbazole-3-carboxylate; 6: 2-*Hydroxy-3-formyl-7-methoxycarbazole*; 7: *Grinimibine*; 8: *Clauszoline-C (clausine-H)*; 9: 7-*Methoxy-O-methylmukonal*; 10: 2-*Hydroxy-3-methylcarbazole*; 11: *Clauszoline-K*; 12: *4-Methoxy-1-methylquinolin-2-one*; 13: *Dictamine*; 14: *Mukonal*; 15: *Mukonidine*; 16: 8-*Methoxy-Dictamine*.

alkaloids, especially *Clauszoline-K*, *2-hydroxy-3-methylcarbazole*, and *Clauszoline-I* had higher growth inhibitory activities than the others. The time course images of the live cells intuitionisticly demonstrated increased growth inhibition after *Clauszoline-K* or *Clauszoline-I* treatment for 24, 48, or 72 h at the IC50 concentration of 16 μM (Fig. 2).

We then estimated the cytotoxic effect of *Clauszoline-K*, *2-hydroxy-3-methylcarbazole*, and *Clauszoline-I* on the normal liver cell line LO2. Table 2 indicates that the IC50 values on LO2 cells were all much higher than those on HepG2 cells, indicating a much lower cytotoxic effect on the normal liver cell line (Table 2). Significantly, *Clauszoline-I* showed almost no effect on LO2 cells. This finding inspired us to investigate the potential anti-tumor activity and underlying mechanism of *Clauszoline-I* as a novel anti-cancer drug. Four human cancer cell lines including HeLa (cervical carcinoma), T98G (glioblastoma), CNE2 (nasopharyngeal carcinoma), and MDA-MB-231 (hormone-independent breast cancer) were used to re-evaluate the anti-cancer activity of *Clauszoline-I*. The IC50 values indicated that *Clauszoline-I* also had a certain anti-proliferative activity on the four cancer cell lines. Among them, HeLa and CNE2 were more sensitive to *Clauszoline-I* (Table 3).

Table 1
The IC50 growth inhibitory values of the sixteen alkaloids in HepG2 cells.[a]

Alkaloids	No.	IC50 (μM)
Clauszoline-J (clausine-K)	1	24.4 ± 2.17
Clausine-O	2	32.8 ± 3.3
Clauszoline-I	3	15.8 ± 1.1
Clausine-Z	4	135 ± 7.02
Methyl carbazole-3-carboxylate	5	36.9 ± 0.25
2-*Hydroxy-3-formyl-7-methoxycarbazole*	6	28.6 ± 3.75
Grinimibine	7	>160[b]
Clauszoline-C (clausine-H)	8	>160[b]
7-*Methoxy-O-methylmukonal*	9	102.2 ± 16.5
2-*Hydroxy-3-methylcarbazole*	10	14.4 ± 0.81
Clauszoline-K	11	4.23 ± 0.58
4-Methoxy-1-methylquinolin-2-one	12	>160[b]
Dictamine	13	55.2 ± 4.1
Mukonal	14	65.9 ± 10.1
Mukonidine	15	>160[b]
8-*Methoxy-dictamine*	16	>160[b]

[a] HepG2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin.
[b] The mean IC50 value could not be determined as one or more of the corresponding data points were higher than the threshold value.

During the in vitro determination of the anti-proliferative activity of *Clauszoline-I*, we found that the morphology of HepG2 cells was significantly changed (Fig. 3A). The confocal images of immune-staining for F-actin and α-tubulin revealed that HepG2 cells were normally polygonal, with few cells showing elongation. However, treatment with 16 μM *Clauszoline-I* for 24 h caused a remarkable increase in the cells with filamentous protrusions, and a decrease of F-actin staining (rhodaminated-phalloidin) compared with the control treatment (Fig. 3A).

Similar with other members of the Ras superfamily, RhoA alternates between a cytosolic GDP-bound inactive form and a GTP-bound active form. Upon activation, RhoA is translocated from the cytoplasm to the cell membrane, and involved in the formation of actin stress fibers [11]. To investigate the activity of RhoA, the membrane proteins were extracted and Western blot analysis was used to detect the changes in membrane-associated RhoA (Fig. 3B). Our results showed that the membrane-associated RhoA was significantly decreased in the HepG2 cells but not the normal liver LO2 cells treated with *Clauszoline-I*, suggesting the inhibitory effects of *Clauszoline-I* on F-actin polymerization and stress-fiber formation were achieved by repressing the activation of RhoA.

RhoA is known to play an important role in the signal transduction pathway, which regulates the reorganization of actin structures [14–16]. Recently, RhoA has gained attention as a regulator of cell cycle progression and cell transformation [17]. The activation of RhoA is necessary for cell cycle progression [18,19], whereas its inhibition blocks serum-induced cell cycle progression [20]. Over-expressed RhoA has been found in several types of cancer, including bladder, testicular, ovarian, colon, breast, and lung [21–24]. These observations suggest that elevated RhoA activity or expression is closely related to tumor development. Therefore, the inhibition of RhoA activity or expression may be a good strategy for reversing tumor cell behavior [25]. In the present study, *Clauszoline-I* significantly inhibited HepG2 cell proliferation and arrested cell cycle progression. Membrane-associated RhoA was significantly decreased in cells treated with *Clauszoline-I*. Hence, the inhibitory effects of *Clauszoline-I* on cell proliferation and cell cycle progression may be achieved via repressing the activation of RhoA.

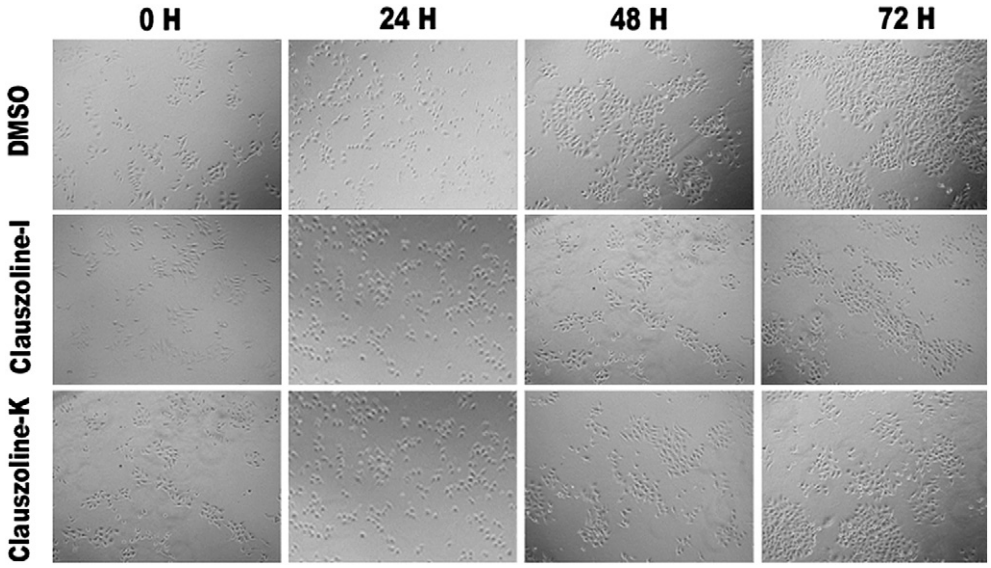

Fig. 2. Time course of the growth inhibitory effects of *Clauszoline-K* and *Clauszoline-I* on HepG2 cells. HepG2 cells were treated with 4 μM *Clauszoline-K* and 16 μM *Caluszoline-I* for 24, 48, and 72 h. The cells were photographed using inverted microscopy (200×).

Table 2
The cytotoxic effect of three alkaloids on L02 cells.[a]

Carbazole alkaloids	No.	IC50 (μM)
Caluszoline-I	3	>160
2-*Hydroxy*-3-*methylcarbazole*	10	85.2 ± 0.4
Clauszoline-K	11	72.5 ± 0.28

[a] LO2 cells were cultured in RPMI1640 with 10% FBS and 1% penicillin.

Table 3
The IC50 growth inhibitory values of *Clauszoline-I* in different cancer cell lines.[a]

Cell lines	IC50 growth inhibitory values (μM)
Hela	13.3 ± 0.9
T98G	71.6 ± 2.3
CNE2	16.9 ± 0.52
MDA-MB-231	37.1 ± 2.7

[a] Cancer cell lines were cultured as described in Table 1.

As the cell growth was inhibited, we assumed that cell cycle progression was arrested at a certain phase. To determine whether *Clauszoline-I* inhibited cell cycle progression, HepG2 cells were treated with 16 μM (IC50 concentration) of *Clauszoline-I* for 24 h after synchronization, and stained with propidium iodide. DNA contents were measured by flow cytometry. The results indicated that the cell cycle mainly was arrested in the S and G2/M phases by *Clauszoline-I* treatment. The control group treated with DMSO had approximately 71.5% cells in the G0/G1 phase, 21.6% in the S phase, and 6.9% in the G2/M phase. However, the cells in the S and G2/M phases increased to 31.1% and 13.1%, respectively, and the cells in the G0/G1 phase decreased to 55.9% after *Clauszoline-I* treatment (Fig. 4). There was no apoptotic cell induced by treatment with 16 μM *Clauszoline-I* for 24 h (Fig. 4).

Chk1, a conserved checkpoint kinase, is critical to the S phase and G2/M checkpoints [26]. Upon DNA damage, Chk1 localizes to the nucleus, where it could be activated by ATM as well as ATR kinases, thereby initiating the checkpoint response resulting in the arrest of cell cycle at S and G2/M phases [12]. To determine

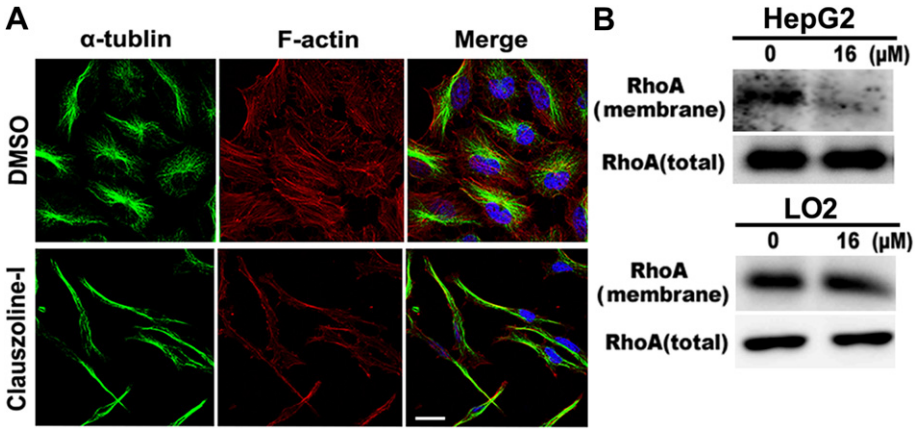

Fig. 3. Effect of *Clauszoline-I* on the morphology of HepG2 cells and RhoA activity. (A) HepG2 cells treated with 16 μM *Clauszoline-I* for 24 h were fixed and stained with rhodaminated-phalloidin (red) and anti-α-tubulin antibody (green). The images were taken using a confocal microscope (Zeiss 710). Scale bar, 20 μm. (B) The membrane proteins of HepG2 or LO2 cells treated with 16 μM *Clauszoline-I* for 24 h were extracted and separated in SDS-PAGE gel, followed by immunoblotting with antibodies against RhoA. α-Tubulin was used as the loading control (not show). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

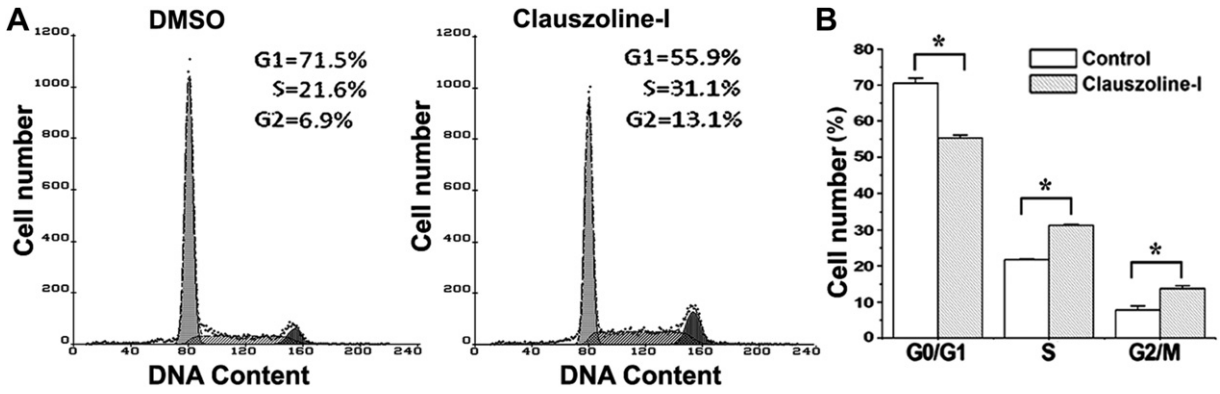

Fig. 4. Effect of *Clauszoline-I* on the cell cycle profile of HepG2 cells. Cells were synchronized by serum starvation for 24 h followed by *Clauszoline-I* treatment (16 μM, 24 h). The harvested cells were fixed in 75% ethanol, and stained with propidium iodide for 30 min at room temperature, followed by cell cycle distribution analysis (A). Data are the means ± SD of values from three independent experiments ($^{*}P < 0.05$) (B).

whether the subcellular localization of Chk1 could be altered due to *Clauszoline-I* treatment, nuclear proteins in HepG2 cells (Fig. 5A) and LO2 cells (Fig. 5B) were extracted, and Western blot analysis was used to detect the changes in Chk1 in the nucleus and cytoplasm. The results showed that Chk1 was decreased in the nucleus and increased in the cytoplasm in HepG2 cells treated with *Clauszoline-I*, but Chk2 was not altered (Fig. 5A). However, in the LO2 cells, *Clauszoline-I* treatment did not affected the expression of Chk1 and Chk2 in the nucleus and cytoplasm (Fig. 5B). It was reported that phosphorylation of Chk1 at Ser280 could induce its relocalization from the nucleus to the cytoplasm [27]; increase of phospho ser280-modified Chk1 prohibited S phase progression to G2 [28]. In the present study, Chk1 decreased in the nucleus and increased in the cytoplasm upon *Clauszoline-I* treatment. However, no alteration in Chk2 was found (Fig. 5), indicating that the arrest of the S and G2/M phases due to the decreased nuclear Chk1 may have resulted from the altered Chk1 phosphorylation.

PKCδ is a member of the protein kinase C (PKC) family, which plays critical roles in signal transduction pathways involved in proliferation, differentiation, apoptosis, and cellular senescence [29,30]. Loss of PKCδ leads to cell transformation in fibroblasts [31], whereas its overexpression results in G2/M cell cycle arrest [32]. Although most studies suggest that PKCδ suppresses proliferation, other reports have demonstrated the positive role of PKCδ in cell proliferation [33]. A clinical study has also found that PKCδ is upregulated in hepatocellular cancer [34]. PKC phosphorylation is a marker of auto-activation. To ascertain which of the PKC isoforms are affected by *Clauszoline-I*, phospho-specific antibodies were employed. The results showed that the phosphorylation of PKCδ at Ser643 was strongly inhibited by *Clauszoline-I* in a concentration-dependent manner in HepG2 cells (Fig. 6A), but did not affected in LO2 cells (Fig. 6B). However, the phosphorylation of the other PKC isoforms, such as PKCα/βII (Thr 638/641), PKCδ (Thr505), and PKCζ/λ (Thr410/403) was not affected (Fig. 6A). The further experiment found that treatment with PKCδ inhibitor rotterlin (10 μM for 1 h) also could dramatically inhibit the level of phospho-PKCδ (ser643) and the membrane-associated RhoA in HepG2 cells (Fig. 6C), suggesting the PKCδ inhibitor rotterlin could mimic the inhibitory effect of *Clauszoline-I* on HepG2 cells. Although, the assumption that Ser643 phosphorylation affects the kinase activity of PKCδ is controversial. One previous study has reported that a Ser643 to alanine mutation does not affect the kinase activity of PKCδ [35]. In contrast, another report indicates that a Ser643 mutation markedly decreases PKCδ activity. This finding suggests that Ser643 of PKCδ is an important autophosphorylation site for the enzymatic activity of PKCδ [36].

PKCδ has been implicated in RhoA-mediated actin reorganization [13]. The pharmacological inhibition of PKCδ Ser643 phosphorylation abolished the cell spreading [37], and the dominant-negative PKCδ adenovirus infection blocked actin reorganization [38]. Apparently, cytoskeleton reorganization significantly involves the induction and activation of PKCδ. Actually, a previous report has revealed that PKCδ mediates actin reorganization by inhibiting RhoA activity [39]. The chemical or molecular inhibition of PKCδ results in diminished RhoA GTPase activity, stress fiber and focal adhesion disruption, as well as endothelial barrier dysfunction in vitro [13]. The present study suggested that *Clauszoline-I* may decrease the activity of the RhoA by down-regulating the phosphorylation of PKCδ. As a result, actin depolymerization and tumor cell growth inhibition occurs.

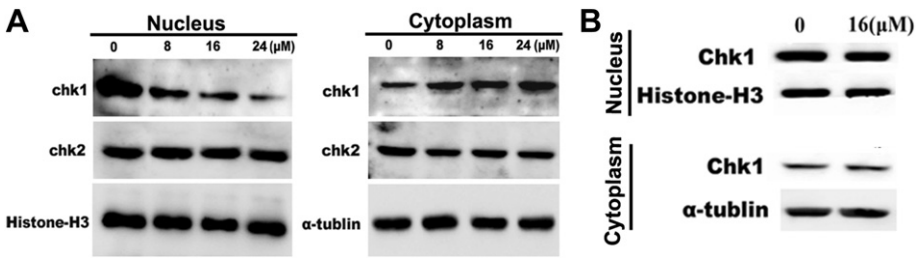

Fig. 5. Effect of *Clauszoline-I* on the translocation of Chk1 and Chk2. Proteins in the nucleus and cytoplasm of HepG2 cells (A) or LO2 cells (B) were prepared after treatment with *Clauszoline-I* at indicated concentration for 24 h. The proteins were subjected to SDS-PAGE electrophoresis before being immunoblotted with antibodies against Chk1 and Chk2. Histone-H3 and α-tubulin were used as the loading controls.

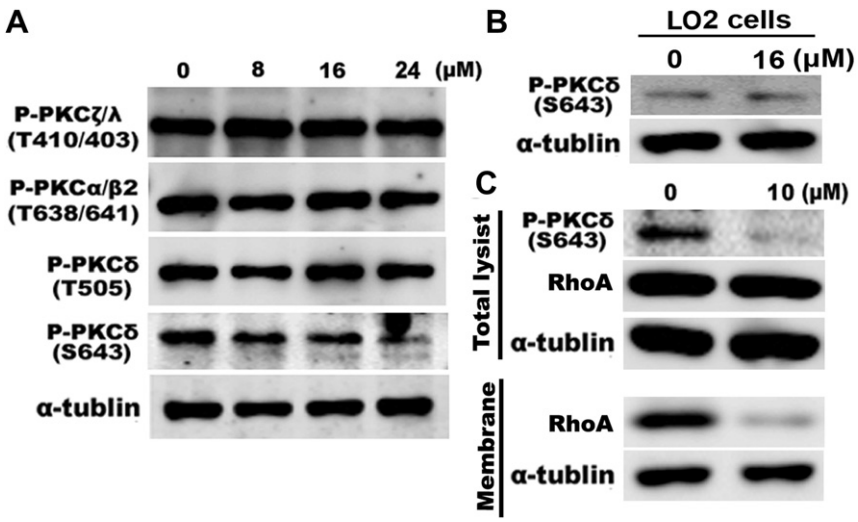

Fig. 6. Inhibition of PKCδ (Ser643) phosphorylation by *Clauszoline-I*. A. Samples of total lysates from HepG2 cells treated with 0, 8, 16, and 24 μM *Clauszoline-I* were separated in SDS-PAGE gel followed by immunoblotting with antibodies against phospho-PKCδ (ser643), phospho-PKCα/βII (Thr638/641), phospho-PKCδ (Thr505), and phospho-PKCζ/λ (Thr410/403). α-Tubulin was used as the loading control. B. Samples of total lysates from LO2 cells treated with 16 μM *Clauszoline-I* were separated in SDS-PAGE gel followed by immunoblotting with antibodies against phospho-PKCδ (ser643). α-Tubulin was used as the loading control. C. PKCδ inhibitor rotterlin mimicked the inhibitory effect of *Clauszoline-I* on RhoA. The total cell lysates and membrane proteins of HepG2 cells treated with 10 μM rotterlin for 1 h were extracted and separated in SDS-PAGE gel, followed by immunoblotting with antibodies against phospho-PKCδ (ser643), or RhoA. α-Tubulin was used as the loading control.

4. Conclusions

In conclusion, 16 alkaloids from *C. vestita* D. D. Tao were extracted, and their anti-tumor activities against HepG2 cells were evaluated. Among the extracts, *Clauszoline-I* showed an obvious growth inhibitory activity against four cancer cell lines by inhibiting PKCδ phosphorylation and decreasing RhoA activity. Consequently, F-actin depolymerization and cell cycle arrest ensued. The in vitro molecular mechanism and in vivo anti-tumor activity of *Clauszoline-I* warrant further study to assess its potential as an anti-tumor drug.

5. Material and methods

5.1. General experimental procedures

Melting points were determined using a Fisher–Johns melting point apparatus, and were reported as uncorrected values. IR spectra were recorded by a Nicolet Magna 750 FTIR (KBr) spectrophotometer. Optical rotations were measured using a Perkin–Elmer M341 polarimeter. Mass spectrometric data were obtained using a MAT-95 mass spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker instrument equipped with a 5-mm ^{1}H and ^{13}C probe operating at 300 and 100 MHz, respectively. Tetramethylsilane was used as the internal standard. ^{1}H assignments were expressed based on two-dimensional correlated nuclear overhauser effect spectroscopy (mixing time 800 ms) experiments. ^{13}C assignments were made based on heteronuclear single quantum correlation and heteronuclear multiple bond correlation experiments. The chemical shift values were reported in ppm (δ), and coupling constants (J) were given in Hz. For column chromatography, 100–200 and 200–300 mesh silica gels (Qingdao Haiyang Chemical Group Co.), Sephadex LH-20 (Pharmacia Fine Chemical Co.), as well as an RP-18 (Merck, 0.015-0.040 mm) were used. Thin layer chromatography analyses were conducted using the glass pre-coated silica gel GF_{254} (Qingdao Haiyang Chemical Group Co.) and RP-18 F_{254} plates (Merck). Spots were visualized under ultraviolet lamps (254 and 365 nm), or by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol. All reagents used were analytical grade.

5.2. Plant material

A whole-plant *C. vestita* D. D. Tao was collected in the Yunnan Province, P. R. China in July 2008, and identified by Prof. Jin-gui Shen of the Shanghai Institute of Materia Medica. A voucher specimen (SIMM 20080716) was deposited in the Herbarium of Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, P. R. China.

5.3. Cell lines

The human cancer cell lines HepG2 (liver carcinoma), HeLa (cervical carcinoma), T98G (glioblastoma), CNE2 (nasopharyngeal carcinoma), and MDA-MB-231 (hormone-independent breast cancer) were purchased from the China Center for Type Culture Collection of Chinese Academy of Sciences. The cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBIC) and 1% penicillin at 37 °C in a humidified 5% CO_2 atmosphere.

5.4. Cell growth inhibition assay

Cell viability was determined using the Cell Counting Kit-8 (CCK-8) assay based on water-soluble tetrazolium salt (WST)-8. Similar with thiazolyl blue tetrazolium bromide or MTT, WST-8 can be catalyzed by mitochondrial dehydrogenases to yield a yellow soluble Formazan dye. This dye is then used to estimate the number of viable cells. In brief, cells were seeded in 96-well culture plates at a density of 2000 per well, and were allowed to attach overnight. After serum starvation for 24 h, the cells were treated in triplicate with grade concentrations of compounds for 48 h. The cells were then incubated with 10% CCK-8 prepared in DMEM without phenol red for 1 h at 37 °C. The optical density of each well was read using a plate reader (model VERSA Max, Molecular Devices) at a wavelength of 450 nm. Wells with drug-containing medium without cells or untreated cells were used as negative and positive-controls,

respectively. The inhibitory activity was expressed as the compound concentration required for 50% growth inhibition of cancer cells (IC50). IC50 was calculated by the Logit method. The mean IC50 was determined from the results of the three independent tests.

5.5. Confocal images

HepG2 cells (2×10^4 cells/well) were grown on 12-mm glass cover slips for 24 h, followed by treatment with 16 μM *Clauszoline-I* for 24 h. The cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 min at room temperature. After incubation in blocking buffer (PBS containing 10% normal goat serum and 0.1% saponin) for 30 min at room temperature, the cells were immunoreacted with anti-α-tubulin antibodies (diluted 1:1000 in blocking-buffer) for 1 h at room temperature, followed by incubation with Alexa 488 labeled anti-mouse IgG (diluted 1:1000 in blocking buffer) and rhodaminated-phalloidin (diluted 1:500 in blocking buffer) for 1 h at room temperature. The slips were washed with PBS and mounted in Prolong Gold anti-fade reagent with DAPI (Molecular Probes), and were inspected using a confocal microscope (Zeiss 710).

5.6. Flow cytometry

The cells treated with 16 μM *Clauszoline-I* for 24 h were harvested, fixed in 75% ethanol, and stained with propidium iodide (50 μg/mL with RNase A for 30 min at room temperature). Cell cycle distribution was analyzed using FACScan (Becton-Dickinson).

5.7. Western blot analysis

After different treatments, the cells were twice washed with cold PBS, and lysed in RIPA buffer on ice. The lysates (15 μg of protein) were separated by SDS-PAGE gel electrophoresis, and transferred to nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK). The membranes were blocked with 5% nonfat milk in PBS containing 0.05% Tween 20 for 1 h, followed by incubation with the primary antibody overnight at 4 °C. The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence, and visualized with the Las4000 (GE Healthcare).

Acknowledgements

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