

RESEARCH ARTICLE

In vitro and *In vivo* Antitumor Activity of Tiliacorinine in Human Cholangiocarcinoma

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Abstract

Cholangiocarcinoma (CCA) is a fatal cancer with poor prognosis and less than 10% of CCA patients can be offered surgical cure. Conventional chemotherapy results in unfavorable outcomes. At present, plant-derived compounds are gaining interest as potential cancer therapeutics, particularly for treatment-refractory cancers. In this study, antitumor activity of tiliacorinine, the major alkaloid isolated from a tropical plant, on CCA was first demonstrated. Antiproliferative effects of tiliacorinine on human CCA cell lines were investigated using SRB assays. Acridine orange/ethidium bromide staining, flow cytometric analysis and DNA laddering assays were used for apoptotic determination. Apoptosis-related proteins were verified by Western blotting and antitumor activity of tiliacorinine *in vivo* was demonstrated in CCA xenografted mice. Tiliacorinine significantly inhibited proliferation of human CCA cell lines with IC₅₀ 4.5-7 μM by inducing apoptosis through caspase activation, up-regulation of BAX, and down-regulation of Bcl_{xL} and XIAP. Tiliacorinine considerably reduced tumor growth in CCA xenografted mice. These results demonstrated antitumor effects of tiliacorinine on human CCA *in vitro* and *in vivo*. Tiliacorinine may be an effective agent for CCA treatment.

Keywords: Antitumor activity - apoptosis - growth inhibition - bile duct cancer - alkaloid

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Introduction

Cholangiocarcinoma (CCA), a malignant cancer arising from bile duct epithelium, is a rare liver cancer but a serious public health problem in the northeast of Thailand as it has the highest incidence and mortality rate in the world (Sripa and Pairojkul, 2008). Generally, only 10% of patients present with early-stage disease are considered surgical candidates (Han et al., 2005) and chemotherapy is the option left for these inoperable patients (Chou and Talalay, 1984). However, the outcome of the chemo-drug treatment is unfavorable with the five year survival lesser than 10% (Butthongkomvong et al., 2013; Rizvi and Gores, 2013; Thunyaharn et al., 2013). To reduce the mortality rate of CCA, new effective treatment strategies are needed.

Plant-derived compounds are gaining interest as potential cancer therapeutics (Shukla, 2007; Aras et al., 2014), particularly for treatment-refractory cancers such as CCA (Naus et al., 2007). In this study, tiliacorinine (Figure. 1A), the bisbenzylisoquinoline alkaloid isolated

from a tropical medicinal plant, *Tiliacora triandra* (Colebr.) Diels, was investigated for antitumor activity in CCA cell lines *in vitro* and *in vivo*. The molecular mechanism by which tiliacorinine induces apoptosis of CCA was also determined. Our findings suggested tiliacorinine to be a new promising compound for effective treatment against human CCA.

Materials and Methods

Chemicals and reagents

Cell culture reagents were purchased from Gibco/Invitrogen (Carlsbad, CA). Antibodies were obtained from companies as indicated: Bcl-2-associated X protein (BAX), B-cell leukemia protein xL (Bcl_{xL}), X-linked inhibitor of apoptosis protein (XIAP), and poly-adenosine diphosphate ribose polymerase (PARP) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-caspase-3 and-9, goat anti-rabbit IgG- and goat anti-mouse IgG-conjugated to horseradish peroxidase (HRP) from Cell Signaling Technology (Beverly, MA), and β-actin antibody from

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Cell lines and cell culture

Four human CCA cell lines-KKU-M055, KKU-100-KKU-M213 and KKU-M214-were established from primary tumors of CCA patients as described previously (Sripa et al., 2005; Seubwai et al., 2010). All cells were cultured at 37°C with 5% CO₂ in Ham's F-12 containing 1% antibiotics-antimycotics solution and 10% FBS.

Isolation of tiliacorinine

Tiliacorinine was isolated from *Tiliacora triandra* as described previously (Pachaly and Khosravian, 1988) with modification. Briefly, 10.0 kg of pulverized dried roots and stems were macerated successively with n-hexane and ethyl acetate to give the hexane (535.20 g) and ethyl acetate (519.86 g) extracts. The plant material was then extracted with methanol-chloroform-ammonium hydroxide (15:5:1). After solvent evaporation, glacial acetic acid was added followed by ammonium hydroxide. The insoluble polymeric material was removed from the aqueous suspension and the latter was extracted with chloroform. The crude alkaloid extract was chromatographed to give tiliacorinine (1.053 g) and a mixture of minor alkaloids (300 mg). More tiliacorinine (96mg) was obtained from the ethyl acetate extract by column chromatographic separation. The spectroscopic (proton and carbon-13 NMR, and mass spectra) data were consistent with the literature values (Wiriyachitra, 1981; Pachaly and Khosravian, 1988). Tiliacorinine was dissolved in DMSO and diluted with completed media to the indicated concentrations.

In vitro cytotoxicity test

The effects of tiliacorinine on the proliferation of CCA cells were determined using SRB assay (Skehan et al., 1990). Briefly, CCA cells (3,000 cells/well) were incubated with tiliacorinine in a 96-well plate. At indicated time points, cells were treated with 10% ice-cold trichloroacetic acid and stained with 0.4% SRB in 1% acetic acid. The stained proteins were solubilized and the absorbance at 540nm was measured (Vichai and Kirtikara, 2006). Dose-response curves were plotted, and the concentration of drug required to inhibit cell proliferation by 50% (IC₅₀) was calculated using the Calcsyn software (Biosoft, Oxford, UK).

DNA fragmentation assay

The isolation of fragmented DNA was carried out as previously described (Herrmann et al., 1994). Briefly, 5×10⁵ cells were lysed in 100 μL of 10 mM Tris-HCl buffer (pH 7.4), 10 mM EDTA and 0.5% Triton X-100. Final samples were dissolved in 40 μL of Tris-EDTA buffer, pH 8.0, subjected to agarose gel electrophoresis and stained with ethidium bromide.

Acridine orange/ethidium bromide double staining

Cells (3,000 cells/100 μL) were treated with 0.01% DMSO or various concentrations of tiliacorinine for 72h and subjected to acridine orange/ethidium bromide (AO/EB) staining as previously described (Petit et al., 1995).

The stained cells were visualized under a fluorescent microscope (Nikon Eclipse TS100, Nikon Corporation, Tokyo, Japan). Multiple photos (9 fields/sample) were taken at randomly-selected areas and apoptotic cells were count using Image ProPlus 7.0 (Media Cybernetics, Inc., Bethesda, MD). A minimum of 100 total cells were count and expressed as a percentage. Tests were done in triplicate.

Western blotting

Cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40) supplemented with cocktail proteinase inhibitors (Roche, Mannheim, Germany). Proteins in cell lysates were separated by a 10% SDS-polyacrylamide gel electrophoresis according to Laemmli (Laemmli, 1970) and electro-transferred onto a polyvinylidene difluoride membrane according to Bolt and Mahoney (Bolt and Mahoney, 1997). The membrane was blocked with 1% (w/v) skim milk, 0.1% tween (v/v) in Tris-buffered saline, pH 7.4 for 30 min and incubated with specific primary antibodies at 4°C overnight and secondary antibody conjugated-HRP at room temperature for 2h. The expression level of proteins were detected using Amersham ECL Plus Western blotting detection reagents (GE Health care, Piscataway, NJ) and captured with ImageQuant™ 400 and analyzed with ImageQuant™ TL software.

Flow cytometric analysis of apoptotic cells

Flow cytometric analysis of cell cycle distribution was performed using a FACSCalibur flow cytometer (BD-Biosciences, San Jose, CA) as previously described (Seubwai et al., 2010). Briefly, cells were fixed with 70% ethanol at -20°C overnight and stained with 10 μg/mL propidium iodide (PI; Sigma, St Louis, MO) in phosphate buffer saline for 30 min in the dark. A total of 10,000 cells were analyzed by flow cytometry. Sub-G1 peak was analyzed using BD FACSDiva software (BD Biosciences, San Jose, CA).

In vivo assay

Balb/c Rag-2 Jak3 double knock-out mice (Ono et al., 2011) aged 8-10 week old were housed and monitored in the animal research facility according to the institutional guidelines. All experimental protocols were approved by the Institutional Animal Care and Use Committee, Kumamoto University (Kumamoto, Japan). Mice were subcutaneously injected with 4×10⁶ of KKU-M213 cells at both flank sides. Three days after CCA cell-injection, mice were intraperitoneally injected with 0.01% DMSO (control group; n=5) or tiliacorinine (10mg/kg body weight; n=5) once daily for 3 consecutive days. Body weights and tumor volumes were measured every 3 days. Tumors were removed and weighed 9 days after inoculation.

Statistical analysis

Experimental data were analyzed using SPSS 16.0 Windows Evaluation software (SPSS Inc., Chicago, IL). The results are presented as the mean±standard deviation of at least 3 separated experiments. Statistical significance

was determined with the Student t test. p values <0.05 were considered significant.

Results

Tiliacorinine inhibits growth of human CCA cells

In order to determine the growth inhibitory effect of tiliacorinine on human CCA cells-KKU-M055, KKU-100, KKU-M213, and KKU-M214-cells were treated with various concentrations of tiliacorinine for 72h and investigated by SRB assay. Growth inhibitory effects of tiliacorinine on the tested cells were KKU-M055>KKU-M213>KKU-M214>KKU-100 with IC₅₀ values of 4.5±0.3, 5.7±0.2, 6.1±0.3, and 7.0±0.6 respectively (Figure 1B). Treated cells exhibited dose-dependent sensitivity to tiliacorinine from 1.7-8.7 μM. As KKU-M214 and KKU-100 were less sensitive to tiliacorinine, they were selected as representative of CCA cells for the subsequent studies.

Tiliacorinine suppresses growth of human CCA cells by inducing apoptosis

We further investigated whether tiliacorinine inhibited growth of human CCA cells by induction of apoptosis. Apoptotic indices were determined by 3 different approaches. AO/EB double staining was applied to discriminate the live, apoptotic and necrotic cells. Exposure of KKU-M214 and KKU-100 cells to 4-7 μM of tiliacorinine significantly induced apoptotic cells with typical apoptotic features e.g., cell shrinkage, membrane

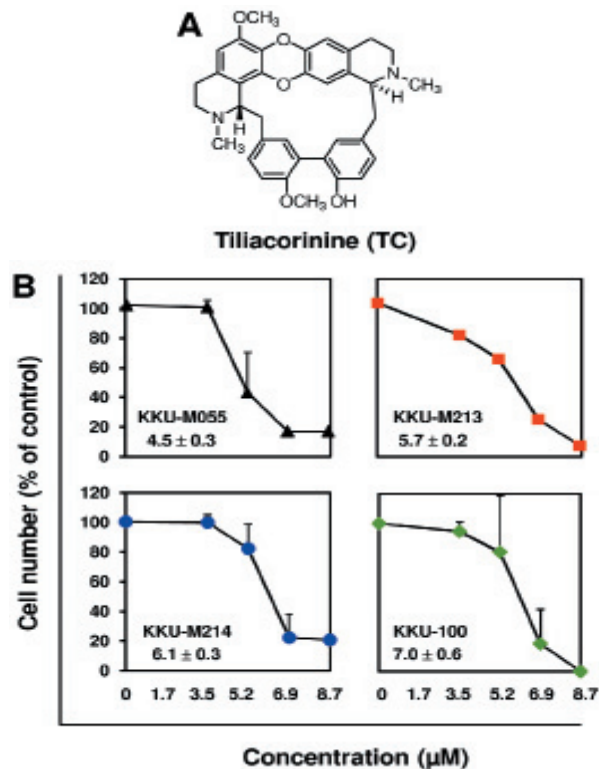


Figure 1. Chemical Structure and Antiproliferative Effect of Tiliacorinine. (A) The chemical structure of tiliacorinine (C₃₆H₃₆N₂O₅) with molecular weight of 576.26. (B) Tiliacorinine suppressed proliferation of CCA cell lines. Cells were treated with the indicated concentrations of tiliacorinine for 72h. The numbers indicate IC₅₀ of tiliacorinine

blebbing, and chromatin condensation (Figure 2A; left panel) in a dose-dependent manner (Figure 2A; right panel). Number of dead cells stimulated by tiliacorinine was next validated using flow cytometric analysis with PI staining. Cells treated with tiliacorinine for 48-72h exhibited 45-60% apoptotic cells in the sub G1 peaks which were significantly higher than the controls (p<0.05; Figure 2B). Degradation of nuclear DNA, the hallmark of apoptotic cells, was determined by DNA fragmentation assay. Cells treated with various concentrations of tiliacorinine for 24, 48, and 72h showed a gradual increase of DNA ladders, in both dose- and time-dependent manners (Figure 2C).

Tiliacorinine induces apoptosis through caspase activation

To determine whether tiliacorinine induced apoptosis via caspase activation, KKU-M214 was treated with IC₅₀ (6 μM) of tiliacorinine for 24, 48, and 72h and whole cell

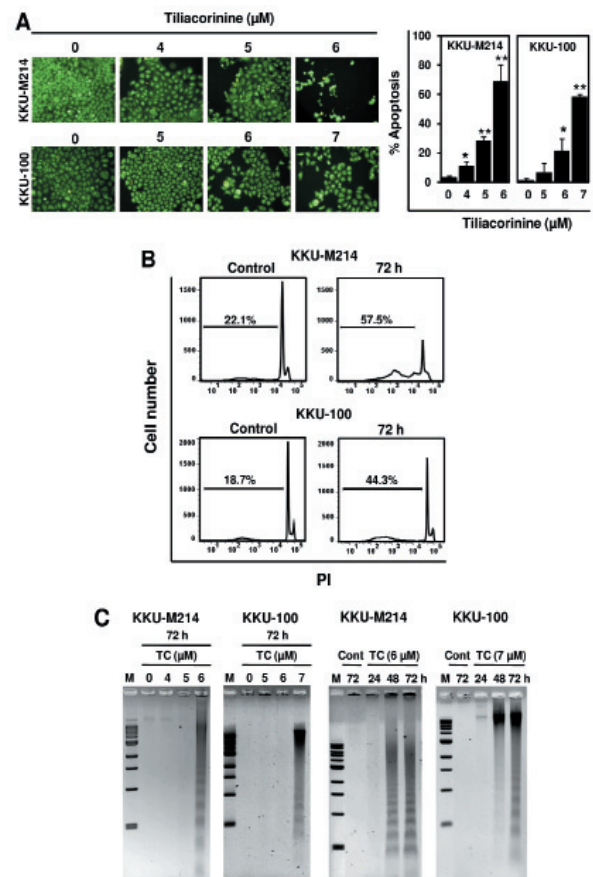


Figure 2. Tiliacorinine Induced Apoptosis in Human CCA Cells. (A) Left panel: Apoptotic cells were determined using orange/ethidium bromide staining. KKU-M214 and KKU-100 cells were treated with 0.01% DMSO or tiliacorinine at the indicated concentrations for 72h. Cells were captured at 200x magnification; right panel: apoptotic cells expressed as a percentage from each treatment were compared. Results are mean±SE and *p<0.05, **p<0.01. (B) The degree of apoptosis induced by tiliacorinine was quantified by a flow cytometry with PI staining. Sub-G1 DNA content represented the fractions undergone apoptotic DNA degradation, was evaluated. (C) DNA fragmentations of tiliacorinine treated cells were shown as time and dose dependent manners. M = 1Kb DNA ladder marker. The figures are representative results of three independent experiments

lysates were subjected to western blotting. Tiliacorinine activated caspase-3, -9 and PARP cleavages in a time-dependent fashion (Figure 3A). In addition, tiliacorinine significantly increased expression of a proapoptotic protein, BAX, and decreased expression of antiapoptotic proteins, XIAP and Bcl_{xL}, in a time-dependent manner (Figure 3B).

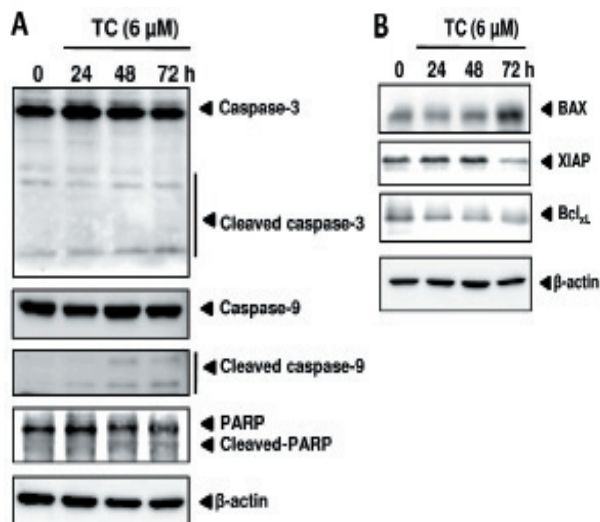


Figure 3. Tiliacorinine Induced Apoptosis Via Caspase Activation. Western blot analysis of tiliacorinine treated KKKU-M214 cells at indicated time points were determined. β -actin was used as an internal control. (A) Expression of proteins in the caspase related pathway; (B) Proapoptotic and antiapoptotic proteins. TC= tiliacorinine

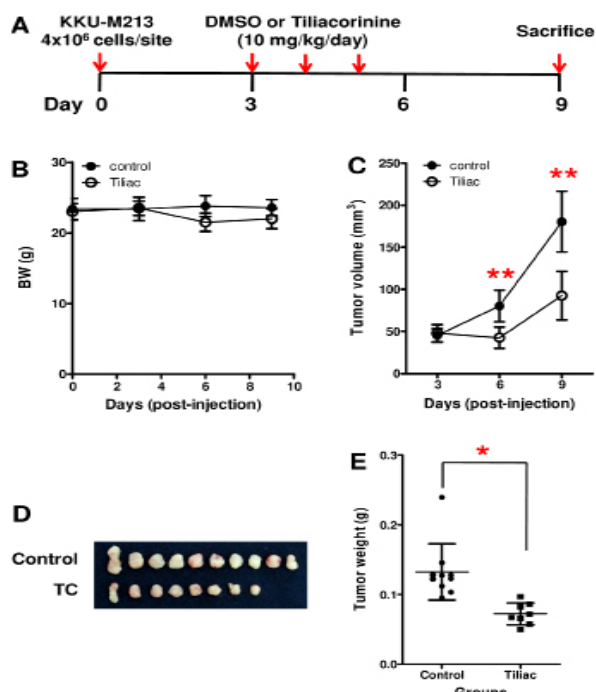


Figure 4. Antitumor Activity of Tiliacorinine in CCA Xenografted Mice. (A) Treatment protocol. Mice were subcutaneously injected with KKKU-M213 in both flanks and intraperitoneally injected with DMSO or tiliacorinine once daily for 3 consecutive days (red arrows). (B) Average body weights and (C) tumor volumes of mice in each group were evaluated. (D) Tumor tissues and (E) tumor weights from DMSO and tiliacorinine-treated mice were compared. * $p < 0.05$, ** $p < 0.001$

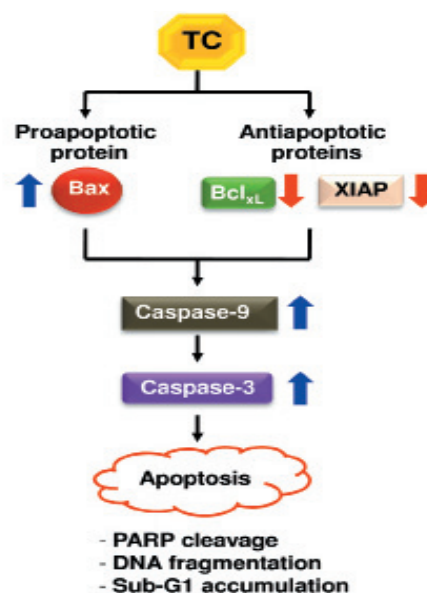


Figure 5. Proposed Mechanism of Tiliacorinine on Apoptotic Induction in Human CCA Cells

Tiliacorinine reduced tumor growth in CCA xenografted mice

To investigate the antitumor activity of tiliacorinine in animal model, KKKU-M213 cells were subcutaneously injected into both flanks of mice and tiliacorinine or DMSO (control group) was intraperitoneally injected once daily for 3 consecutive days, 3 days post-CCA cell-injection (Figure 4A). Body weights of mice from both groups were not significantly different (Figure 4B). One mouse in tiliacorinine treated group died on day 8 and hence all mice were sacrificed on day 9. Mean tumor volumes from tiliacorinine treated group ($45.16 \pm 12.52 \text{ mm}^3$) was significantly lower than those of the control group ($80.22 \pm 18.75 \text{ mm}^3$) instantly on day 3 of treatment (Figure 4C; $p < 0.001$). On day 9, mean tumor weights from tiliacorinine treated group ($0.07 \pm 0.02 \text{ g}$) was significantly lower than those of the control group ($0.13 \pm 0.04 \text{ g}$) (Figure 4E; $p < 0.05$).

Discussion

Tiliacorinine, the major alkaloid isolated from the medicinal plant-Tiliacora triandra has been proved for anti-malarial activity (Dechatiwongse et al., 1987) and antimycobacterial activity (Sureram et al., 2012). In this study, the anticancer activity of tiliacorinine was first demonstrated *in vitro* and *in vivo*. Tiliacorinine effectively inhibited proliferation of CCA cells via induction of apoptosis and significantly reduced tumor growth in CCA xenografted mice model. These results indicate the therapeutic potential of tiliacorinine against human CCA.

Tiliacorinine inhibited growth of four human CCA cell lines with IC_{50} ranging from $4.5\text{--}7.0 \mu\text{M}$. Comparing to other natural compounds, tiliacorinine seems to be more potent than tannic acid and sesquiterpene but less effective when compared to caged xanthenes. Tannic acid, a natural polyphenolic compound, inhibited proliferation of malignant human cholangiocytes with IC_{50} of $60 \mu\text{M}$ (Marienfeld et al., 2003) while sesquiterpene-the

derivative of zerumbone showed antiproliferative activity against KKKU-100 cell line with an IC_{50} of $16.44 \mu M$ (Songsiang et al., 2010). The growth inhibitory effect of four caged xanthenes-isomorellin, isomorellinol, forbesione and gambogic acid on KKKU-100 showed IC_{50} ranging from $0.11-2.64 \mu M$ (Hahnvajjanawong et al., 2010).

Tiliacorinine exhibited antiproliferative activity on CCA cell lines as time- and dose-dependent manners, with a narrow range of $4.0-7.5 \mu M$ at 72h. Comparing to other CCA cell lines, KKKU-100 was the less sensitive cell line toward most of conventional chemotherapeutic agent tested (Tepsiri et al., 2005). KKKU-100 exhibited the IC_{50} of 5-fluorouracil, mitomycin-C, paclitaxel and cisplatin = $1,018 \mu M$, $45 \mu M$, $39 \mu M$ and $37 \mu M$, respectively. KKKU-100, however, was sensitive to tiliacorinine with the IC_{50} of $7 \mu M$ which was similar to other CCA cell lines. This highlights tiliacorinine as a potent agent for CCA treatment.

Tiliacorinine inhibited CCA cell growth by inducing apoptosis as evidenced by the results from three different assays. First, tiliacorinine treatment increased number of apoptotic cells as determined by AO/EB staining. Second, DNA ladder, the hallmark of apoptosis revealed by DNA fragmentation assay was clearly demonstrated in cells treated with tiliacorinine. Third, tiliacorinine treated cells significantly increased apoptotic cells in sub G1 peak as demonstrated by flow cytometry. Induction of apoptosis has been considered to be the major mechanism of anticancer drug discovery (Reed, 2001).

The molecular mechanism by which tiliacorinine induced apoptosis was shown to be via activation of caspase-3, -9, and consequently PARP cleavage. Tiliacorinine also up-regulated BAX, a proapoptotic protein, and down-regulated XIAP and Bcl_{xL} in human CCA cells. Induction of apoptosis via caspase-activation pathways seems to be the general mechanisms of anticancer agents from natural compounds, such as cepharanthine, a biscochlorine alkaloid from roots of *Stephania cepharantha* Hayata (Wu et al., 2001; Seubwai et al., 2010), emodin, an anthraquinone derivative (Yaoxian et al., 2013) and berberine, an isoquinoline alkaloid (Yip and Ho, 2013).

Several attempts have been made to find new agents that effectively inhibited growth and metastasis of CCA, however, most of them demonstrated the effects *in vitro*, e.g., diethyldithiocarbamate (Srikoon et al., 2013), histone deacetylase inhibitors (Sriraksa and Limpaboon, 2013). The antitumor activity of tiliacorinine was obviously demonstrated in CCA cell lines and CCA xenografted mice. Tumor volumes and tumor weights of tiliacorinine treated group were 2 folds reduced compared to the control group. Tiliacorinine appeared to have a rapid antitumor activity as it was administered only 3 consecutive days after CCA cell-injection and the tumor volume was significantly reduced instantly. As this study was the first report on the antitumor activity of tiliacorinine in the xenografted mouse model, the pharmacokinetic, drug safety and efficacy of tiliacorinine have to be investigated cautiously.

The abundance of literature suggests that defects along

apoptotic pathways play a crucial role in carcinogenesis and that many new treatment strategies targeting apoptosis are feasible for the treatment of various cancers (Wong, 2011; Sankari et al., 2012). Additionally, almost clinically used anticancer drugs are aimed to activate apoptosis of tumor cells (Hadi et al., 2000). In this study, tiliacorinine showed apoptotic effect on human CCA cells and suppressed tumor growth in CCA xenografted mice, suggesting this auspicious alkaloid an effective agent for CCA treatment. Nevertheless, further intensive study in safety and efficacy of tiliacorinine is highly recommended. Furthermore, to overcome cancer by achieving synergistic therapeutic effect, reducing toxicity, and minimizing the drug resistance (Chou, 2010), further study on drug combination between tiliacorinine and the conventional chemotherapeutic drugs is encouraged.

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