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 BclₓL 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

**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 (BclₓL), 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, 2011). 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, 5x10⁶ 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 counted 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-horseradish peroxidase (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
Bulb/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 4X10⁶ 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.
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 IC<sub>50</sub> 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 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 IC<sub>50</sub> (6 µM) of tiliacorinine for 24, 48, and 72h and whole cell
Tiliacorinine reduced tumor growth in CCA xenografted mice

To investigate the antitumor activity of tiliacorinine in animal model, KKU-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±12.52 mm$^3$) was significantly lower than those of the control group (80.22±18.75 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±0.02 g) was significantly lower than those of the control group (0.13±0.04 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 4.5-7.0 µM. Comparing to other natural compounds, tiliacorinine seems to be more potent than tannic acid and sesquiterpene but less effective when compared to caged xanthones. Tannic acid, a natural polyphenolic compound, inhibited proliferation of malignant human cholangiocarcinoma cells with IC$_{50}$ of 60 µM (Marienfeld et al., 2003) while sesquiterpene-the
The antitumor activity of tiliacorinine was obviously demonstrated in CCA cell lines and CCA xenografted mice. Tiliacorinine appeared to have a rapid antitumor activity as it was administrated 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 pharmaco-kinetic, 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.

Acknowledgements

This study was supported by Research grants from Khon Kaen University and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Health cluster (SHeP-GMS) to S. Wongkham. S. Janeklang is supported by the Royal Golden Jubilee-Commission on Higher Education PhD Program (PHD/0133/2550). A. Suksa mrarn and A. Nakaew acknowledge supports from The Thailand Research Fund and Center of Excellence for Innovation in Chemistry. We would like to thank Prof. James A. Will for the English presentation of this manuscript via the Faculty of Medicine Publication Clinic, and the Research Instrument Center, Khon Kaen University, for the service support of flow cytometer.

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Sonkid Janeklang et al

J Gastroenterol, 16, 2235-43.


