RESEARCH ARTICLE

Stigmalactam from *Orophea Enterocarpa* Induces Human Cancer Cell Apoptosis Via a Mitochondrial Pathway

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Abstract

Stigmalactam, an aristolactam-type alkaloid extracted from *Orophea enterocarpa*, exerts cytotoxicity against several human and murine cancer cell lines, but the molecular mechanisms remain elusive. The aims of this study were to identify the mode and mechanisms of human cancer cell death induced by stigmalactam employing human hepatocellular carcinoma HepG2 and human invasive breast cancer MDA-MB-231 cells as models, compared to normal murine fibroblasts. It was found that stigmalactam was toxic to HepG2 and MDA-MB-231 cells with IC_{50} levels of 23.0±2.67 μM and 33.2±4.54 μM, respectively, using MTT assays. At the same time the IC_{50} level towards murine normal fibroblast NIH3T3 cells was 24.4±6.75 μM. Reactive oxygen species (ROS) production was reduced in stigmalactam-treated cells dose dependently after 4 h of incubation, indicating antioxidant activity, measured by using 2',7',-dichlorohydrofluorescein diacetate and flow cytometry. Caspase-3 and caspase-9 activities were increased in a dose response manner, while stigmalactam decreased the mitochondrial transmembrane potential dose-dependently in HepG2 cells, using 3,3'-dihexyloxacarbocyanine iodide and flow cytometry, indicating mitochondrial pathway-mediated apoptosis. In conclusion, stigmalactam from *O. enterocarpa* was toxic to both HepG2 and MDA-MB-231 cells and induced human cancer HepG2 cells to undergo apoptosis via the intrinsic (mitochondrial) pathway.

Keywords: Stigmalactam - *Orophea enterocarpa* - apoptosis - hepG2 cells - MDA-MB-231 cells - mitochondrial pathway

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Introduction

Orophea enterocarpa is in Annonaceae family, found endemically in southern and eastern parts of Thailand. For other species of *Orophea*, *O. uniflora* is endemic in India whereas *O. palawanensis* is found in Philippines. Various bioactive alkaloids are extracted from medicinal herbs such as nitidine chloride from *Zanthoxylum nitidum* with anticancer effect against breast cancer cells (Sun et al., 2014). N-norchelerythrine and dihydrosanguinarine, from *Broussonetia papyrifera* (L.) Vent. (Moraceae), a traditional Chinese medicinal herb, are cytotoxic to human cervical cancer HeLa and hepatocellular carcinoma BEL-7402 cells (Pang et al., 2014). Camptothecin, a potent alkaloid drug with an anticancer effect, is extracted from the bark and stem of *Camptotheca acuminata*, which contains the mechanism of action to inhibit topoisomerase I activity (Efferth et al., 2007; Ulukan and Swaan, 2002). There has been a report of aqueous extract of *C. acuminata* fruit containing antitumor effects on human endometrial carcinoma cells via the accumulation of cyclin-A2 and -B1, then activation of caspase-3 and -7, similar to the effect of camptothecin (Lin et al., 2014).

There are various attempts to search for a new source of camptothecin from other kinds of plants. There has been a first report of camptothecin alkaloids from Meliaceae family, i.e., *Dysoxylum binectariferum* bark, to be a source for camptothecin production. Rohitukine, camptothecin and 9-methoxy-camptothecin can be isolated from *D. binectariferum* (Jain et al., 2014).

As mentioned, some alkaloids from *Broussonetia papyrifera* fruits containing isoquinonline alkaloids, viz., N-norchelerythrine and dihydrosanguinarine; are cytotoxic to human cancer cells, whereas nitidine, broussonpapyrine and chelerythrine from the same plant are also toxic to non-cancer cells. This leads to further modification of structure-activity relationship to achieve anticancer effect without cytotoxicity to normal cells (Pang et al., 2014).

Stigmalactam, a known alkaloid isolated from *Orophea enterocarpa*, is cytotoxic to human colon adenocarcinoma (HCT15) cell line with IC_{50} concentration of 1.32 μM (Nayyatip et al., 2012). However, the mechanism of cancer cell cytotoxicity remains elusive. The aims of this study were to investigate the mode of cancer cell death and the mechanism involved. The human hepatocellular carcinoma HepG2 and human breast cancer MDA-MB-231 cell lines were used in the present study as models for anticancer study. It was found that stigmalactam was cytotoxic to both cancer cell lines and

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induced HepG2 to undergo apoptosis with the reduction of mitochondrial transmembrane potential and the induction of caspase-9 and -3 activities. However, the compound acted as anti-oxidant in the HepG2 cells with the evidence of decreased 2',7'-dichlorofluorescein fluorescence intensity employing flow cytometric technique, implying the response to apoptotic death induction.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), Leibovitz's L-15 Medium, fetal bovine serum, penicillin G sodium and streptomycin were obtained from Gibco BRL, Thermo Fisher Scientific Inc., Waltham, MA, USA. Dimethyl sulfoxide (DMSO), 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$), 2',7'-dichlorohydrofluorescein diacetate (DCFH-DA) and 3-(4,5-dimethyl)-2,5-diphenyltetrazolium bromide (MTT) dye were obtained from Sigma/Aldrich, St. Louis, MO, USA.

Plant materials

The twigs and leaves of *Orophea enterocarpa* were collected from Prajeenburi Province, Thailand, in March 2009. The plant was identified by Narong Nuntasaen, with the voucher specimen (BKF no. 151499), deposited at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand.

Stigmalactam was obtained from the following procedure. Air-dried twigs and leaves (2.7 kg) were ground and extracted according to the protocol (Nayyatip et al., 2012). Briefly, the extraction was performed with hexane and subsequently with methanol-dichloromethane (3:1) successively. The crude methanol-dichloromethane extract (211.18 g) was separated by using silica gel chromatography technique. The gradient elution was conducted with hexane, enriched with ethyl acetate, followed by gradually increasing amount of methanol in ethyl acetate and finally with methanol to obtain eleven fractions, F1-F11. Then fraction F4 (16.6 g) was rechromatographed by silica gel with the gradient system using hexane, ethyl acetate and methanol to afford 4 subfractions, A1-A4. The subfraction A3 (0.38 g) underwent the purification process to obtain four subfractions, B1-B4. The yellowish precipitate in subfraction B2 was crystallized from ethanol to give a purified compound, stigmalactam (0.07 g), and the structure is shown in Figure 1.

Cell lines

Human hepatocellular carcinoma HepG2 and human breast cancer MDA-MB-231 cells were gifts from Assoc. Prof. Dr. Prachya Kongtawelert at Excellent Center of Tissue Engineering and Stem Cells, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, whereas murine fibroblast NIH3T3 cell line was from Prof. Dr. Usanee Vinitketkumneun at Department of Biochemistry, Faculty of Medicine, Chiang Mai University. HepG2 and NIH3T3 cells were cultured

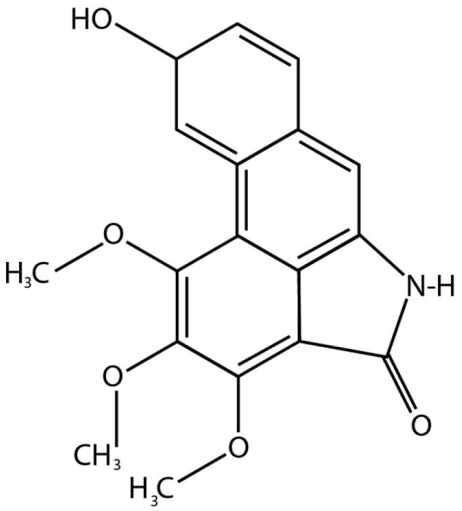

Figure 1. Molecular Structure of Stigmalactam

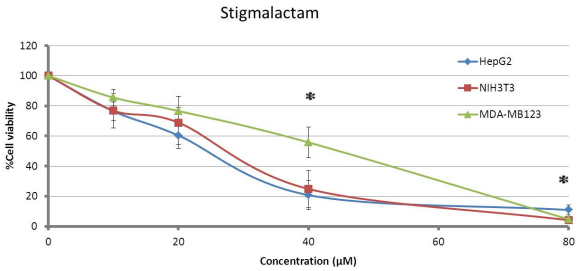

Figure 2. Cytotoxicity of Stigmalactam on Human Liver HepG2, Breast Cancer MDA-MB-231 Cells and Normal Murine Fibroblast NIH3T3 cells. Percent cell viability of each type of cells was determined by MTT assay. The significant value compared to control (without) treatment is marked with asterisk, $p<0.05$

Table 1. Cytotoxicity of Stigmalactam on Human Hepatocellular Carcinoma HepG2, Breast Cancer MDA-MB-231 Cells and Murine Fibroblast NIH3T3 Cells with IC_{10}, IC_{20} and IC_{50} of Each Cell Line

Cell lines	Stigmalactam (μM)					
	IC10		IC20		IC50	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
HepG2	3.98	0.62	7.79	0.52	22.98	2.67
MDA-MB-231	5.12	2.58	10.1	2.66	33.23	4.54
NIH3T3	3.54	1.11	7.96	2.76	24.44	6.75

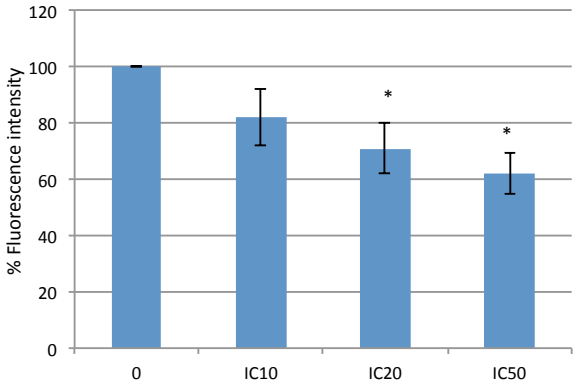

Figure 3. Reduction of Reactive Oxygen Species (ROS) in Human Liver Cancer HepG2 Cells. Stigmalactam-induced human cancer cell death decreased ROS production in a concentration dependent manner. The value is considered significant compared to control and marked with asterisk, $p<0.05$

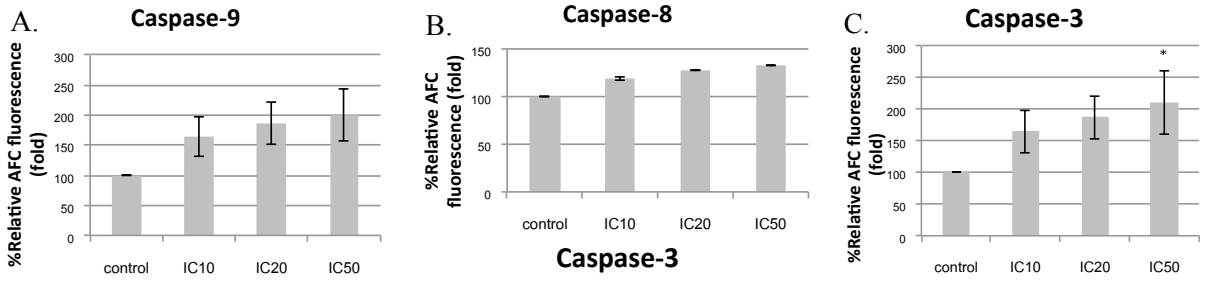

Figure 4. Caspase-9, -8 and -3 Activities Induced by Stigmalactam in HepG2 cells. The caspase activities were determined as described in Materials and Methods. (A) Caspase-9, (B) caspase-8 and (C) caspase-3 activities were shown as relative fluorescence intensity compared to control (fold). The significant enhanced activities of caspase-9 and -3 were induced in HepG2 cells compared to control, with asterisk, $p<0.05$

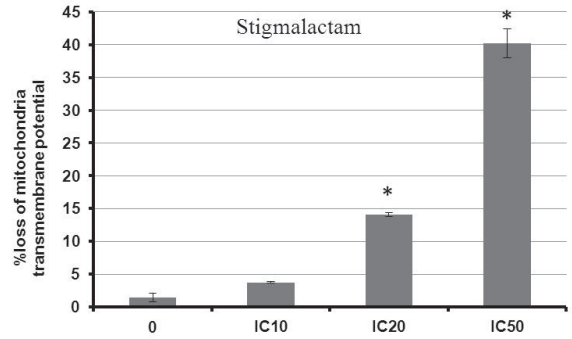

Figure 5. Loss of Mitochondrial Transmembrane Potential (MTP) of Stigmalactam-treated Human Hepatocellular Carcinoma HepG2 Cells. Percentage of treated-cells with loss of MTP was increased in a dose dependent manner. The value compared to control is marked with asterisk, $p<0.05$

in DMEM and MDA-MB-231cells were cultured in Leibovitz's L-15 Medium with 25 mM $NaHCO_3$, 20 mM HEPES, 100 units/mL penicillin, 100μg/mL streptomycin and supplemented with 10% fetal bovine serum.

Cell culture and treatment

HepG2, MDA-MB-231 and NIH3T3 cells (50,000 cells/well) were cultured in the absence or presence of stigmalactam at various concentrations, incubated at 37°C in 5% CO_2 atmosphere for 24 h. The cell viability was determined by employing MTT assay (Wudtiwai et al., 2011). Briefly, sterile MTT solution (stock solution of 5 mg/ml) was added to cell suspension at the final concentration of 100 µg/ml and incubated for 4h at 37°C in a humidified 5% CO_2 atmosphere. The medium was then removed and the crystal was dissolved with DMSO for 30 min. The absorbance of the cell lysate was measured at 540 nm with reference wavelength of 630 nm using microtiter plate reader (Biotek, Winooski, VT, USA) at 570 nm. The percentage of cell viability was calculated and 10, 20 and 50% inhibitory concentrations (IC_{10}, IC_{20} and IC_{50}) were determined and used for further experiments.

Determination of reactive oxygen species (ROS) production

HepG2 cells were treated with stigmalactam at C_{10}, IC_{20} and IC_{50} for 4 h, then the cells were washed with phosphate-buffered saline twice before 2',7'-dichlorohydrofluorescein diacetate (DCFH-DA) was added to the cell suspension with the final concentration of 5 µM. ROS production was determined and analyzed by flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) (Banjerdpongchai and Khaw-On, 2013).

Determination of caspase-3, -8 and -9 activities

Specific tetrapeptides tagged with fluorescence (7-amino-4-trifluoromethylcoumarin, AFC), viz., DEVD-AFC, IETD-AFC and LEHD-AFC were substrates for caspase-3, -8 and -9, respectively. HepG2 cells ($1x10^6$) were treated with stigmalactam at C_{10}, IC_{20} and IC_{50} for 24 h and the cell lysate was prepared. Specific fluorogenic substrate (50 μM) for each caspase was dissolved in standard reaction buffer, added into the cell suspension and incubated at 37°C for an hour. Enzymatic catalytic cleavage and the release of fluorescence were measured for its intensity by fluorescence plate reader (Biotek, Winooski, VT, USA) (Banjerdpongchai et al., 2013).

Determination of mitochondrial transmembrane potential

After HepG2 cell treatment, 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) was added to the media to the final concentration of 40 nM for mitochondrial transmembrane potential (MTP) determination. Cells were analyzed by a FACScan equipped with a 488 nm argon laser using CellQuest software (Becton-Dickinson, Franklin Lakes, NJ, USA). Data were depicted as histograms and analyzed for percentage of cells with loss of MTP (Banjerdpongchai and Wilairat, 2005).

Statistical analysis

All experiments were performed in triplicates and thrice as a representative for the three with the same results. The data were presented with mean±S.D. analyzed with One Way ANOVA and comparison between two groups of data were considered significant when $p<0.05$, by using Student's t-test.

Results and Discussion

There has been a report of other six aristolactams extracted from roots of *Ottonia anisum* (Piperaceae), including aristolactam BII, piperolactam C, goniothalactam, stigmalactam, aristolactam AII and aristolactam BIII (Marques et al., 2011).

Purification of extracts from *Fissistigma balansae*

and *Fissistigma oldhamii* results in the isolation of 11 aristolactams: stigmalactam, piperolactam A, piperolactam C, aristolactam AII, aristolactam AIIIa, aristolactam BII, aristolactam BIII, aristolactam FII, goniothalactam, enterocarpam I, and velutinam; as well as two dioxoaporphines: noraristolodione and norcepharadione B. The new compound (stigmalactam) has been firstly identified by spectral data interpretation. Almost all of 13 compounds contain the antiplatelet aggregation activity (Chia et al., 2000).

It has been reported of the cytotoxicity of stigmalactam against colon cancer cell lines (HCT15) (Nayyatip et al., 2012). However, the bioactivities on mode of cell death and death-inducing mechanisms of the compound remain unknown. This study involves the apoptotic inducing activity in liver cancer HepG2 cells of stigmalactam, which is an aristolactam-type alkaloid extracted from *Orophea enterocarpa*.

Stigmalactam was cytotoxic to human hepatocellular carcinoma HepG2, breast cancer MDA-MB-231 and murine fibroblast NIH3T cells dose-dependently with the IC50 levels of 22.98±2.67, 33.23±4.54 and 24.44±6.75 μM, respectively as shown in Figure 2 and Table 1. The compound is toxic to HepG2 > NIH3T3 > MDA-MB-231 with the range of IC_{50} levels between 23-33 μM. Stigmalactam needs to be modified in its structure to make it less toxic to normal cells but still very toxic to cancer cells. It also suggests that the metabolizing enzymes or detoxifying enzymes in the hepatocellular carcinoma HepG2 cells may metabolize the compound (stigmalactam) to be a more active metabolite and make it cytotoxic to liver cancer HepG2 cells more than MDA-MB-231 cells which do not contain metabolizing enzymes.

Due to the cell sensitivity, HepG2 cells were selected for further study of the mechanism of cell death. Stigmalactam could decrease the peroxide radical production in HepG2 cells as shown in Figure 3 significantly. However, apoptotic cells usually generate ROS. The antioxidant activity of the stigmalactam may be induced to respond to the ROS production, which might be a compensatory effect to the stigmalactam treatment or it might be indicated that stigmalactam acted as an antioxidant. However, the decreased ROS might be the response to cell death-inducing effect.

Stigmalactam induced HepG2 cells to undergo apoptosis with the evidence of significant increase in caspase-9 and -3 activities (Figure 4A, 4C), whereas the capase-8 activity did not change statistically significantly (Figure 4B). There was also a reduction of mitochondrial transmembrane potential (MTP) and percentage of cells with MTP loss significantly increased in a dose dependent manner as shown in Figure 5.

In conclusion, stigmalactam was cytotoxic to hepatocellular carcinoma HepG2 and breast cancer MDA-MB-231 cells together with normal fibroblast NIHT3T cells, with HepG2 > NIH3T3 > MDA-MB-231 cells. Stigmalactam induced human hepatocellular carcinoma HepG2 cell apoptosis via the mitochondrial pathway, with the activation of caspase-9 and -3, and a decrease in MTP. The ROS production was decreased in apoptotic cells, indicating the antioxidant activity or cell death effect of stigmalactam.

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