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

Ratana Banjerdpongchai¹*, Benjawon Wudtiwai¹, Wilart Pompimon²

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 IC₅₀ levels of 23.0±2.67 µM and 33.2±4.54 µM, respectively, using MTT assays. At the same time the IC₅₀ 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'-dichloro-5-ethylidene fluorescein 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

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-norcherethryrine 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 isoquinoline alkaloids, viz., N-norcherethryrine and dihydrodiosanguinarine; are cytotoxic to human cancer cells, whereas nitidine, broussonpyprine 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 IC₅₀ 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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DOI:http://dx.doi.org/10.7314/APJCP.2014.15.23.10397

Stigmatactam Induces Human Cancer Cell Apoptosis in HepG2 Cells

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Abstract

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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<sub>6</sub>), 2’,7’-dichlorofluorescein 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...
potential (MTP) of Stigmalactam-treated Human Hepatocellular Carcinoma HepG2 Cells. 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'-dichlorodihydrofluorescein 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).

**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.
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, goniolactam, enterocarpam I, and velutinum; as well as two dioxoaporphines: noraristolodione and norepharadione 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 NIH3T3 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 IC50 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 caspase-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 NIH3T3 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.

**Acknowledgements**

The authors thank Faculty of Medicine Research Fund, Chiang Mai University, Grant No. 016/2557; National Research Council of Thailand (NRCT), Thailand Research Fund (TRF) and the Commission of Higher Education (CHE), Grant No. RMU5080003 for the financial support.

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