RESEARCH ARTICLE

Enterocarpam-III Induces Human Liver and Breast Cancer Cell Apoptosis via Mitochondrial and Caspase-9 Activation

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Abstract

An aristolactam-type alkaloid, isolated from Orophea enterocarpa, is enterocarpam-III (10-amino-2,3,4,6-tetramethoxyphenanthrene-1-carboxylic acid lactam). It is cytotoxic to various human and murine cancer cell lines; however, the molecular mechanisms remain unclear. The aims of this study were to investigate cytotoxic effects on and mechanism(s) of human cancer cell death in human hepatocellular carcinoma HepG2 and human invasive breast cancer MDA-MB-231 cells compared to normal murine fibroblast NIH3T3 cells. Cell viability was determined by MTT assay to determine IC_{50}, IC_{20} and IC_{10} levels, reactive oxygen species (ROS) production with 2',7'-dichlorofluorescein diacetate and the caspase-3, -8 and -9 activities using specific chromogenic (p-nitroaniline) tetrapeptide substrates, viz., DEVD-NA, IETD-NA and LEHD-NA and employing a microplate reader. Mitochondrial transmembrane potential (MTP) was measured by staining with 3,3'-dihexyloxycarbocyanine iodide (DiOC_{6}) and using flow cytometry. The compound was cytotoxic to HepG2 and MDA-MB-231 cells with the IC_{50} levels of 26.0±4.45 and 51.3±2.05 µM, respectively. For murine normal fibroblast NIH3T3 cells, the IC_{50} concentration was 81.3±10.1 µM. ROS production was reduced in a dose-response manner in HepG2 cells. The caspase-9 and -3 activities increased in a concentration-dependent manner, whereas caspase-8 activity did not alter, indicating the intrinsic pathway activation. Enterocarpam-III decreased the mitochondrial transmembrane potential (MTP) dose-dependently in HepG2 cells, suggesting that the compound induced HepG2 cell apoptosis via the mitochondrial pathway. In conclusion, enterocarpam-III inhibited HepG2 and MDA-MB-231 cell proliferation and induced human HepG2 cells to undergo apoptosis via the intrinsic (mitochondrial) pathway and induction of caspase-9 activity.

Keywords: Enterocarpam-III - Orophea enterocarpa - apoptosis - HepG2 cells - MDA-MB-231 cells - mitochondria

Introduction

There has been a report of enterocarpam-I and –II isolated from Orophea enterocarpa (Kamaliah et al., 1986). The structure of enterocarpam-I (10-amino-3-hydroxy-4,8,9-trimethoxyphenanthrene-1-carboxylic acid lactam) and enterocarpam-II (10-amino-3-hydroxy-4,8-dimethoxyphenanthrene-1-carboxylic acid lactam) are different from that of enterocarpam-III (10-amino-2,3,4,6-tetramethoxyphenanthrene-1-carboxylic acid lactam). Their core structures are classified as aristolactam-type alkaloids. Orophea enterocarpa is in Annonaceae plant family.

Two alkaloids from Pseuduvaria rugosa (Blume) Merr. (Annonaceae) are isolated and identified as 1,2,3-trimethoxy-5-oxygenoraporphine and ouregidione. Both ouregidione and 1,2,3-trimethoxy-5-oxygenoraporphine induce cytotoxicity against human leukemia HL-60, U937, and K562 cells whereas 1,2,3-trimethoxy-5-oxygenoraporphine is more active than ouregidione. Both compounds increase significantly the proportion of the cells in S phase, whereas 1,2,3-trimethoxy-5-oxygenoraporphine induces HL-60 cell line arrest in G2/M and ouregidione does the G1 phase arrest (Uadkla et al., 2013).

Alkaloids from Chinese medicinal plant Tripterygium hypoglaucum (levl.) Hutch (Celastraceae) (THH) roots are tested for cytotoxicity against HL-60 and determined for the molecular mechanism involved in the induction of HL-60 cell apoptosis by employing cDNA microarrays derived from a human leukocyte cDNA library. Sixteen genes are demonstrated to be significantly and differentially expressed in HL-60 cells upon THH treatment. Various genes relate to the NF-κB signaling pathway, such as nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta (NFKBIB); proteoglycan 1, secretory granule (PRG1); and beta-2-microglobulin (B2M) are up-regulated. Moreover, c-myc binding protein and apoptosis-related cysteine proteases caspase-3 and caspase-8 are also significantly regulated. Conclusively,
THH alkaloid mixture can induce HL-60 cell apoptosis via c-myc and NF-κB signaling pathways (Zhuang et al., 2004). Tylophora alkaloids (from Tylophora indica), which are herb products containing anti-asthmatic and antiproliferative activities. These alkaloids induce human erythroleukemic K562 cell apoptosis with characteristic apoptosis morphologies, viz., nuclear condensation, apoptotic body formation, externalization of phosphatidylserine in cell membrane, caspase-3 activation and mitochondrial cytochrome c release into cytosol. Hence, Tylophora alkaloids could be useful agents for the antiproliferative activity and for induction of apoptosis in human leukemic cells and cancer cells (Ganguly and Khar, 2002).

Pyrrolizidine alkaloids are classified as natural hepatotoxins with the distribution in more than 6,000 herbs including medicinal herbs and teas worldwide. Clivorine, isolated from Ligularia hodgsonii Hook, decreases cell viability and induces apoptosis in human embryo liver L-02 cells and mouse hepatocytes. Clivorine induces caspase-3 and -9 activation, mitochondrial cytochrome c release and decreases anti-apoptotic Bcl-xL protein level. Polyubiquitination of Bcl-xL protein and decreases cytochrome c release and increases anti-apoptotic Bcl-xL protein level. Polyubiquitination of Bcl-xL protein and decreases cytochrome c release and decreases anti-apoptotic Bcl-xL protein level. It is suggested that pyrrolizidine alkaloids activate hepatotoxic signaling pathway involving degradation of Bcl-xL protein and activation of mitochondria-mediated apoptotic pathway (Ji et al., 2008).

Enterocarpam-III contains the anti-cancer activity against human colon adenocarcinoma HCT15 cell line (Nayyatip et al., 2012). However, the mode and mechanism of cancer cell death remain unclear. The aims of this study were to identify its cytotoxic effect and mechanistic pathway of cancer cell death on human hepatocellular carcinoma HepG2 and human breast cancer MDA-MB-231 cells compared with normal murine fibroblast NIH3T3 cells as models for the study.

Materials and Methods

Materials
Leibovitz’s L-15 Medium, Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum, streptomycin and penicillin G sodium were obtained from Gibco BRL, Thermo Fisher Scientific Inc., Waltham, MA, USA. Dimethyl sulfoxide (DMSO), 3, 3’-dihexyloxycarbocyanine iodide (DiOC3), 2’,7’-dichlorohydrofluorescein diacetate (DCFH-DA) and 3-(4, 5-dimethyl)-2,5-diphenytetrazolium bromide (MTT) dye were obtained from Sigma/Aldrich, St. Louis, MO, USA. The substrates of caspase-9 (LEHD-7-para-nitroaniline, LEHD-p-NA), caspase-8 (IETD- p-NA) and caspase-3 (DEVD- p-NA) were obtained from Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA.

Figure 1. Chemical Structure of Enterocarpam-III.
(10-amino-2,3,4,6-tetramethoxyphenanthrene-1-carboxylic acid lactam)
of Medicine, Chiang Mai University. MDA-MB-231 cells were cultured in Leibovitz’s L-15 Medium whereas HepG2 and NIH3T3 cells were cultured in DMEM with 25 mM NaHCO₃, 20 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin and supplement with 10% fetal bovine serum.

**Cytotoxicity test**

HepG2 and MDA-MB-231 cells (5x10⁵ cells/ml) were treated with the compound at various concentrations for 24 hours. The cell viability was determined by MTT assay (Banjerdpongchai et al., 2013b). Briefly, MTT dye was added to cell suspension at the final concentration of 100 µg/ml and incubated for 4 h in a humidified 5% CO₂ atmosphere. The medium was removed and the crystal was dissolved with dimethyl sulfoxide for 30 min. The absorbance was determined at 540 nm with the reference wavelength of 630 nm using microplate reader (Biotek, Winooski, VT, USA). The inhibitory concentrations at 10, 20 and 50% were determined to apply for further experiments.

**Determination of ROS production**

HepG2 cells were treated with enterocarpam-III at IC₁₀, IC₂₀ and IC₅₀ for 4 h and then washed with phosphate buffered-saline before 2',7'-dichlorohydrofluorescein diacetate was added to the final concentration of 5 µM. The fluorescence intensity was measured by fluorescence microplate reader (Biotek, Winooski, VT, USA) (Wudtiwai et al., 2011).

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

After HepG2 cells were incubated with the enterolactam-III at various concentrations for 24 h, the chromogenic substrate of each caspase, i.e., Leu-Glu-His-Asp- p-NA (LEHD-p-NA), Ile-Glu-Thr-Asp (IETD-p-NA) and Asp-Glu-Val-Asp-p-NA (DEVD-p-NA), was added to the reaction buffer of the cell lysate. The mixture was incubated for an hour and the caspase-9, -8 and -3 activities were measured as their absorbance by using microplate reader (Biotek, Winooski, VT, USA) (Banjerdpongchai et al., 2013a).

**Measurement for mitochondrial transmembrane potential (MTP)**

HepG2 cells were incubated with the compounds at the concentrations of IC₁₀, IC₂₀ and IC₅₀, then DiOC₅ was added to the final concentration of 40 nM. The fluorescence intensity of each condition was determined.

**Figure 2. Cytotoxicity of Enterocarpam-III Towards HepG2, MDA-MB-231 and NIH3T3 Cell Lines.** Percent cell viability is shown as mean±S.D. *p<0.05 compared to control

**Figure 3. Reactive Oxygen Species (ROS) Production of HepG2 Cells when Incubated with Enterocarpam-III for 4 Hours.** Percent relative p-NA absorbance is shown as mean±S.D., representing activities of aspase-9 (A), -8 (B) and -3 (C) activities. *, p<0.05 compared to control

**Figure 4. Caspase-3, -8 and -9 Activities of HepG2 Cells after Treatment with Enterocarpam-III for 24 Hours.** Percent relative p-NA absorbance is shown as mean±S.D., representing activities of aspase-9 (A), -8 (B) and -3 (C) activities. *, p<0.05 compared to control
Mitochondrial transmembrane potential (MTP) Reduction

To confirm the involvement of mitochondria, HepG2 cells after incubation with various concentrations of enterocarpam-III for 24 h, the cells were stained with DiOC<sub>6</sub> and determined for MTP by using flow cytometry technique. The DiOC<sub>6</sub> dye is a cationic fluorochrome that can permeate into intermembranous mitochondrial space and accumulates in the mitochondria in live healthy cells. But in apoptotic cells, the ionic fluorochrome leaks and MTP decreases significantly (Saleh et al., 2014). Percentage of cells with the loss of MTP significantly increased in a concentration-dependent manner (Figure 5).

The increased caspase-3 activity confirmed the apoptotic cell death mode occurred in HepG2 cells when induced by enterocarpam-III (Figure 4C). The mechanism of enterocarpam-III-induced HepG2 cell apoptosis involved the intrinsic pathway (as due to increased caspase-9 activity and loss of mitochondrial transmembrane potential) without activation of extrinsic pathway (due to no significant alteration of caspase-8 activity).

Enterocarpam-III, a natural alkaloid, can be of a high potential and can play a pivotal role as anticancer agent, which will improve the patients’ quality of life and efficiency of cancer treatment by using alone or together with the conventional chemotherapy to lessen the side effects. This finding may also increase the economic cost of Oropeha enterocarpa, the Thai medicinal plant. The toxicity of the compounds towards animals and human beings should also be investigated before application. The scientists and medical doctors may consider both enterocarpam-III and stigmalactam for further study of their significant roles as anticancer drugs both in animal models, in normal human-beings and finally in clinical trials to be approved and used for cancer treatment.

In conclusion, enterocarpam-III inhibited both human liver HepG2 and breast MDA-MB-231 cell viability and induced HepG2 cells to undergo apoptosis via the intrinsic pathway by induction of caspase-9 and -3 activities with the loss of MTP. Meanwhile there was no significant alteration in the caspase-8 activity, indicating the absence of death receptor pathway involvement. This is the first report of enterocarpam-III molecular mechanism in inducing human liver cancer apoptotic death via the intrinsic pathway. Human hepatocellular HepG2 cell line, which is an important in vitro model, can represent as a human cancer disease of in vivo hepatoma, a leading cause of cancer death worldwide and in Thailand. Either is an MDA-MB-231 cell model as human breast cancer, which is also an important cause of mortality and morbidity in women.

Results and Discussion

Cytotoxicity of enterocarpam-III

Enterocarpam-III was cytotoxic to both HepG2 and MDA-MB-231 cells but less toxic to normal murine fibroblasts with the IC<sub>50</sub> of 26.0±4.45, 51.3±2.05 and 81.3±10.1 µM, respectively. Enterocarpam-III inhibited both cancer cell lines and normal fibroblast cell growth dose dependently as shown in Figure 2. The sensitivity of the cells towards the compound was as follows: HepG2 > MDA-MB-231 > NIH3T3 cells as determined by IC<sub>50</sub> levels shown in Figure 2.

Production of reactive oxygen species (ROS)

ROS production was reduced when concentrations of the compound increased (Figure 3), suggesting the antioxidant property of the enterocarpam-III or the cytotoxic effect or apoptosis-inducing response towards the compound of the HepG2 cells leading to the smaller amount of healthy viable cells left.

Caspase-9, -8 and -3 activities

After HepG2 cells were treated with various concentrations of enterocarpam-III for 24 h, caspase-9 and -3 activities were increased, especially at the concentrations of IC<sub>50</sub> (Figure 4A, 4C), whereas the caspase-8 activity did not change. It suggests that enterocarpam-III induced HepG2 apoptosis via mitochondrial or intrinsic pathway with the activation of caspase-9 activity (Alfredsson et al., 2014).

Figure 5. Reduction of Mitochondrial Transmembrane Potential in Enterocarpam-III-treated HepG2 Cells. Percentage of HepG2 cells with loss of MTP increased dose dependently. *, p<0.05 compared to control by employing flow cytometry technique and analyzing by CellQuest software (Becton-Dickinson, Franklin Lakes, NJ, USA). Data were depicted as histograms and analyzed for percentage of cells with loss of MTP (Banjerdpongchai et al., 2011).

Statistical analysis

The data are shown as mean±S.D. from three independent experiments performed in triplicate. The data were analyzed by one way ANOVA and two groups independent experiments performed in triplicate. The data were analyzed by one way ANOVA and two groups independent experiments performed in triplicate. The data were analyzed by one way ANOVA and two groups independent experiments performed in triplicate. The data were analyzed by one way ANOVA and two groups independent experiments performed in triplicate. The data were analyzed by one way ANOVA and two groups independent experiments performed in triplicate. The data were analyzed by one way ANOVA and two groups independent experiments performed in triplicate. The data were analyzed by one way ANOVA and two groups independent experiments performed in triplicate. The data were analyzed by one way ANOVA and two groups independent experiments performed in triplicate. The data were analyzed by one way ANOVA and two groups independent experiments performed in triplicate. The data were analyzed by one way ANOVA and two groups independent experiments performed in triplicate. The data were analyzed by one way ANOVA and two groups independent experiments performed in triplicate. The data were analyzed by one way ANOVA and two groups independent experiments performed in triplicate.
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