RESEARCH ARTICLE

Heptaphylline Induces Apoptosis in Human Colon Adenocarcinoma Cells through Bid and Akt/NF-κB (p65) Pathways

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Abstract

Heptaphylline derivatives are carbazoles in *Clausena harmandiana*, a medicinal plant that is utilized for headache, stomach ache, and other treatments of illness. The present study examined the effects of heptaphylline and 7-methoxyheptaphylline on apoptosis of human colon adenocarcinoma cells (HT-29 cell line). Quantification of cell viability was performed using cell proliferation assay (MTT assay) and of protein expression through immunoblotting. The results showed that only heptaphylline, but not 7-methoxyheptaphylline, significantly significantly activated cleaved of caspase-3 and poly (ADP-ribose) polymerase (PARP-1) which resulted in HT-29 cell death. We found that heptaphylline activated BH3 interacting-domain death agonist (Bid) and Bak, proapoptotic proteins. In contrast, it suppressed X-linked inhibitor-of-apoptosis protein (XIAP), Bcl-xL and survivin, inhibitors of apoptosis. In addition, heptaphylline inhibited activation of NF-κB/p65 (rel), a regulator of apoptotic regulating proteins by suppressing the activation of Akt and IKKα, upstream regulators of p65. The findings suggested that heptaphylline induces apoptosis in human colon adenocarcinoma cells .

Keywords: Heptaphylline - colon adenocarcinoma cells - Bid - XIAP - Bcl-xL - Akt - p65

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Introduction

Cancer is a disease of deregulated cell proliferation and inhibition of apoptosis. The deregulated growth found in cancer cells is frequently attributed to the loss of control in proliferation and program cell death processing (Evan and Vousden, 2001). The treatment of cancers has broadly been unsuccessful because of its uncontrolled growth. Therefore, it is highly required for the improvement of novel and effective chemotherapeutic agents, which contains a capacity to target various signaling pathways to interfere cell growth signaling, to promote the death of cancer cells.

Apoptosis is a programmed cell death that occurs during multiple important physiological conditions, for example, embryonic development, tissue remodeling, the maintenance of homeostasis, and immune repertoires. The deregulation of apoptosis is the cause of diseases, including cancers, autoimmune diseases and neurodegenerative disorders (Okada and Mak, 2004; Sankari et al., 2012). Apoptosis is also considered the basis for cancer treatment to target cancerous cells but not normal cells in order to limit the cytotoxicity from chemotherapy (Debatin et al., 2004; Irene et al., 2005; Millimouno et al., 2014). Induction of apoptosis causes the activation of Bid protein and Bid can activate mitochondria via direct interaction with Bax or Bak. Cytosolic Bid is cleaved at the amino terminus to generate a truncated form of Bid (tBid) that mediates cytochrome C release from mitochondria, which serves as an amplification signal by activating downstream effectors, including caspase-3 lead to cleaving of cellular proteins, such as poly (ADP-ribose) polymerase-1 (PARP-1) (Perez and White, 2000; Chen et al., 2006; Stevenson et al., 2007; Pei et al., 2007; Alenzi et al., 2010; Kantari and Walczak, 2011; Alshammariiet al., 2014). Caspase cascade can be blocked by antiapoptotic proteins, for instance, cIAP1/2, Bcl-xl, Mcl-1, survivin and XIAP (Thorburn et al., 2008). One mechanism of cancer cells to escape apoptosis is overexpression of antiapoptotic proteins. The efficacy of many chemotherapeutics is due to their capacity to induce the death of tumor cells by apoptosis. Apoptosis-inducing compounds are commonly regarded as candidate anticancer agents (Hanahan and Weinberg, 2000; Debatin and Krammer, 2004; Hanahan and Weinberg, 2011; Meiyanto et al., 2012; Pan et al., 2014).

Clausena harmandiana, known as "Song Fa" in Thai, belongs to the Rutaceae family widely distributed in Southeast Asia. In Thailand, especially in the northeastern

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part, the young leaves are accounted as a vegetable in Thai traditional foods and as ruminants feed. C. harmandiana is regarded as the herb for health and it is utilized for the headache, stomachache, and illness treatment. It had been reported that the roots of this plant included plenty of carbazole alkaloids together with coumarins (Aouacheria et al., 2002; Thongthoom et al., 2010). Carbazoles and coumarins have been isolated and evaluated for antimalarial, antifungal, and anti-TB. Carbazole alkaloids were a main component of the Clausena genus, especially Clausena excavate, Clausena anisata and Clausena harmandiana. It had been found that carbazole alkaloids showed various pharmacological properties, consisting of anticancer, antiplatelet aggregation and vasorelaxing, antimycobacterial, anti HIV-1, antiplasmodial, antimycobial, anti-inflamatory, and cytotoxicity against the leukemia cell line , through inhibiting topoisomerase II and antidiabetes activity (Ito et al., 2000, Yenjai et al., 2000; Songsiang et al., 2011). The major carbazole components in C. harmandiana were heptaphylline and 7-methoxyheptaphylline (Figure 1A), which showed cytotoxicity against the NCI-H187 cell line (Thongthoom et al., 2001). This study investigates the apoptotic effects of heptaphylline and 7-methoxyheptaphylline from C. harmandiana on HT29 colorectal adenocarcinoma cells.

Materials and Methods

Cell culture

HT-29 cells are maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100µg/ml streptomycin at 37°C in 5% CO_2. The quantification of cell viability is performed by using the cell proliferation reagent MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide). HT-29 cells are plated in 96-well microplates at $6x10^3$ cells/wells, and then incubated for 24h.

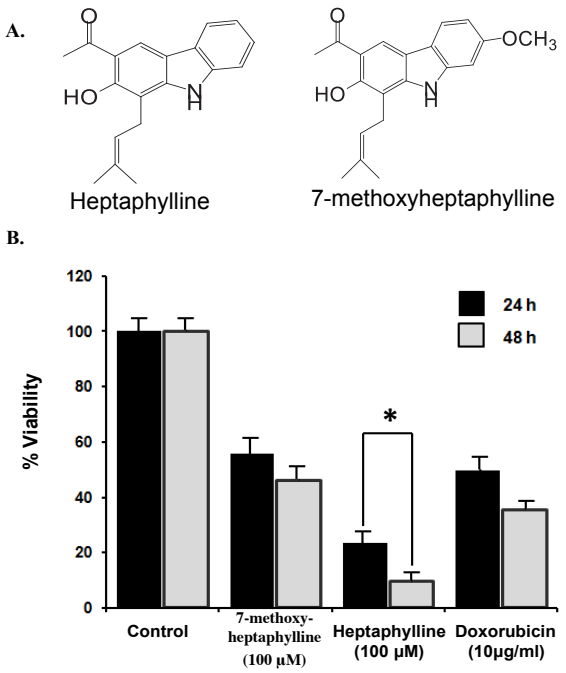

Figure 1. Effects of Hetaphylline and 7-methoxyheptaphylline on Viability of HT-29 Cells. A) Chemical structure of hetaphylline and 7-methoxyheptaphylline. **B)** HT-29 cells were treated with 100µM, heptaphylline 7-methoxyheptaphylline and 10µg/ml doxorubicin (reference compound) for 24 and 48h. Cell proliferation was determined by MTT assay. *p<0.001

Cell cytotoxicity assay

Cells are treated with heptaphylline, 7 -methoxyheptaphylline (Yenjai, 2000) at different concentrations and reference compound (doxorubicin) for 24 and 48h. Add 10µl MTT Reagent (5mg/ml). They are incubated for 2 to 4 hours until purple precipitate is visible. The absorbance at 570 nm is measured. Percentage calculation of cell viability uses the following formula;

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbace of control (untreated cells)}} \times 100$$

Cell morphology is examined by Phase contrast microscope.

Preparation of cell extracts

In order to investigate the mechanism of the compounds on apoptotic pathway in HT29 cells, cells are treated with the test compounds at different concentrations for 4h. Whole cell lysates are prepared with lysis buffer (25mM HEPES pH7.7, 0.3mM $MgCl_2$, 0.2mM EDTA, 10% Triton X-100, 20mM β-glycerophosphate, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM dithiothreitol (DTT), 10µg/ml aprotinin, and 10µg/ml leupeptin). Cell lysate is collected from supernatant after centrifugation at 14,000 rpm for 10min.

Immunoblotting

Cell lysate is resolved by SDS-PAGE and transferred to an Immobilon-P-nylon membrane (Millipore). The membrane is treated with BlockAce (Dainippon Pharmaceutical Co. Ltd, Suita, Japan) and probed with primary antibodies (anti-caspase-3, PARP-1, phosphor-P65, P65, Bcl-2, BID, survivin, XIAP, and anti-actin antibodies). The antibodies are detected by the use of horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat IgG (DAKO, Glostrup, Denmark), and visualized by the enhanced chemiluminescence system (Amersham Biosciences).

Results

Effects of hetaphylline and 7-methoxyheptaphylline on viability of HT-29 cells

To examine whether carbazole extract of C. harmandiana inhibited viability of HT-29 cells, the cells were treated with 100 μM hetaphylline, 7-methoxyheptaphylline and 10 μg/ml doxorubicin (reference compound) for 24 and 48h then cell viabilities were determined by MTT assay. Both hetaphylline and 7-methoxyheptaphylline showed cytotoxicity on HT-29 cells by hetaphylline showed more stronger than 7-methoxyheptaphylline. Cell viability at 24h was decreased to 59.92% with 7-methoxyheptaphylline and 23.37% with heptaphylline, In addition, heptaphylline caused significantly decreased

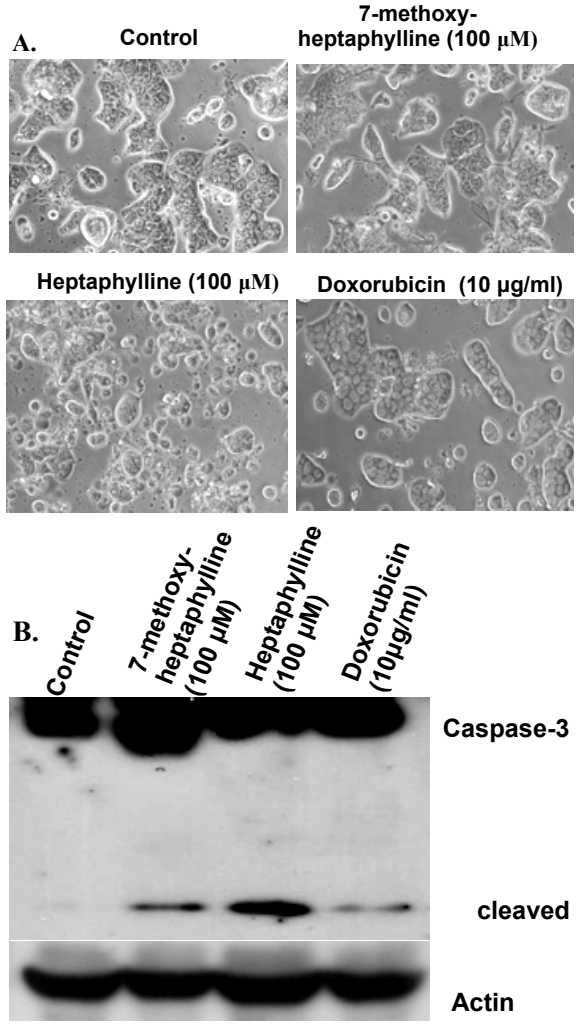

Figure 2. Effects of Hetaphylline and 7-methoxyheptaphylline on HT-29 Cell Apoptosis. **A)** Cells were treated with 100 μM, heptaphylline 7-methoxyheptaphylline and 10μg/ml doxorubicin (reference compound) for 24 h. Cell morphology was investigated by phase contrast microscope. **B)** Whole cell extract was prepared, and analyzed by Western blotting using anti-caspase-3 and β-actin antibodies. Arrows indicate cleaved forms of caspase-3

in cell number at 48h (Figure 1B). This result indicated that hepaphylline strongly induced death of HT-29 cancer cells.

Effects of hetaphylline and 7-methoxyheptaphylline on apoptotic responses of HT-29 cells.

To test the effect of the compounds on HT-29 cell morphology, cells were treated with 100μM of hetaphylline and 7-methoxyheptaphylline or doxorubicin (positive control) for 24h and morphological changes were observed by phase contrast microscopy. The result showed that hetaphylline, 7-methoxyheptaphylline and doxorubicin induced morphology change of cell death consisting of rounding and shrinkage of cells by heptaphylline exhibited strongest effect on HT-29 cells (Figure 2A). The result indicated that heptaphylline markedly induced cell death morphological changes. As shown in Figure 2B, heptaphylline, 7-methoxyheptaphylline and doxorubicin induced cleavage of caspase-3, cellular pro-apoptotic responses by heptaphylline showed strongest apoptosis-promoting activity in HT-29 cells. To confirm the effects of heptaphylline on HT-29 cell proliferation, cells were treated with various concentrations of heptaphylline for 24 and 48h and the cell viability were examined by MTT assay. Heptaphylline significantly inhibited cell growth at concentrations 25, 50 and 100μM in a dose- and time-dependent manner with 50 % growth inhibitory concentration (IC_{50}) value of 60.67 μM at 24h and 46.72μM at 48h respectively (Figure 3A). The apoptotic effects of heptaphylline were investigated by determining the cleavage of caspase-3 and PARP-1 using Western blot analysis. The result in Figure 3B showed that heptaphylline markedly induced the cleavage of caspase-3 and PARP-1 in a concentration-dependent manner. The results indicated that hepaphylline has apoptotic effect to HT-29 cells.

Heptaphylline induced bid activation and inhibited phosphorylation of Akt/NF-κB(p65) pathway

To understand the mechanism by which heptaphylline exerts its apoptotic effects, we next examined the effect of heptaphylline on the expression of key proteins involved in apoptosis regulation including Bid, Bak, Bcl-2, Bcl-xL, Mcl-1, XIAP, and survivin by using immunoblotting assay. The results in Figure 4A showed that hepataphylline at concentration 25μM markedly induced cleavage of Bid correlation with increasing in Bak protein. Incontrast, the expression of XIAP, Bcl-xL and survivin, but not Bcl-2 and Mcl-1 was inhibited. It has been reported that the nuclear transcription factor, NF-κB/p65 (rel) has important role to control expression of many anti-apoptotic proteins consisting of Bcl-xL, survivin and XIAP. Therefore, we further observed the proteins involved in p65 signaling

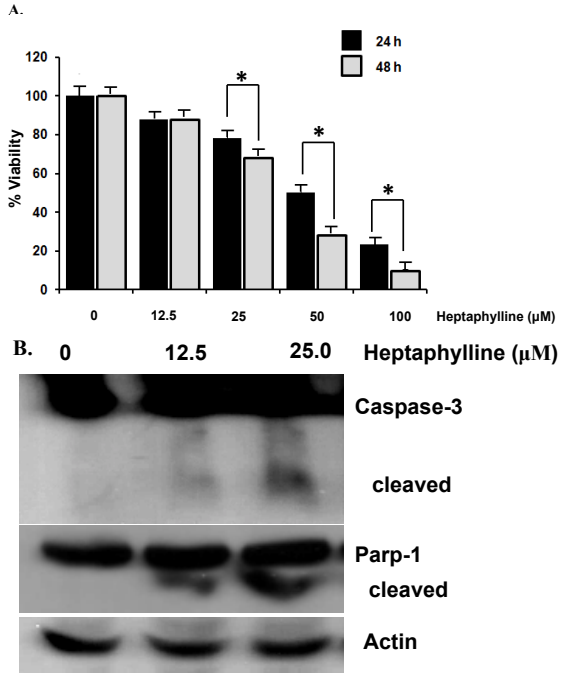

Figure 3. Apoptotic Effect of Hetaphylline in HT-29 Cells. **A)** HT-29 cells were treated with various concentrations of heptaphylline for 24 and 48h. Cell proliferation was determined by MTT assay. **B)** Cells were treated with various concentrations of heptaphylline for 4 h. Whole cell extract was prepared, and analyzed by Western blotting using anti-caspase-3, PARP-1 and β-actin antibodies. Arrows indicate cleaved forms of caspase-3 and PARP. *p<0.01

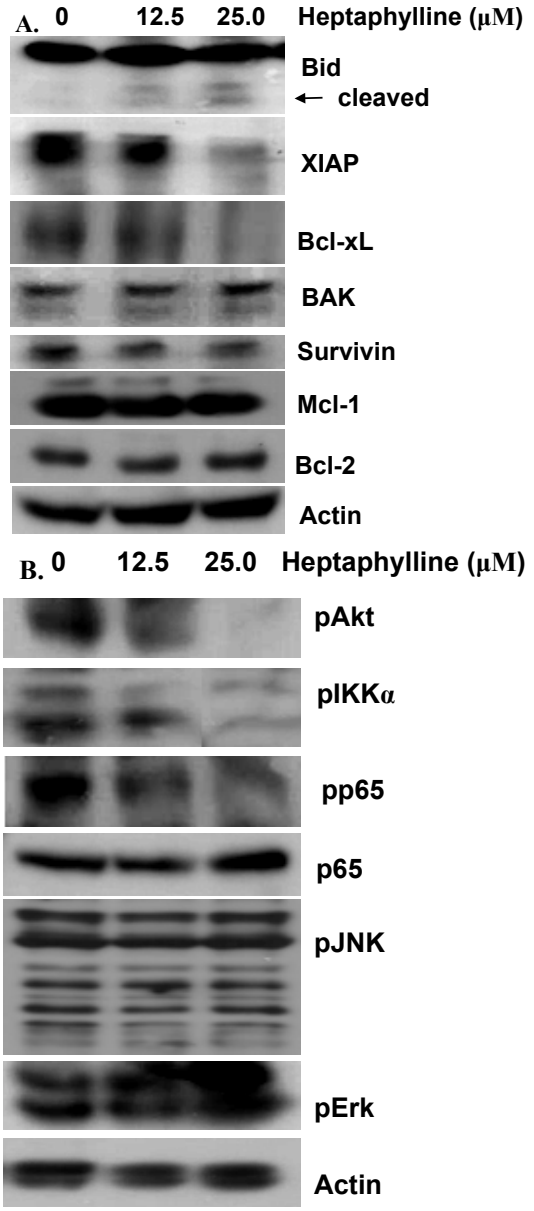

Figure 4. Heptaphylline induced Bid Activation and Inhibited Akt/NF-ϰB (p65) Pathway in HT-29 Cells. Cells were treated with various concentrations of heptaphylline for 4h. Whole cell extract was prepared, and analyzed by Western blotting utilizing Bid, Bak, Mcl-1, Bcl-xL, survivin, XIAP, Bcl-2, phospho-p65, IKKα, Akt, JNK, Erk and β-actin antibodies. **A)** Effect of heptaphylline on apoptotic regulating proteins. **B)** Effect of heptaphylline on Akt, IKKα, p65, JNK and Erk phosphorylation

pathways and found that hepataphylline suppressed Akt, IKKα and p65 phosphorylation in the dose dependent manner but it has no effect to JNK and Erk phosphorylation (Figure 4B). This result indicated that heptaphylline induced apoptosis by induced Bid activation and inhibited Akt/NF-ϰB (p65) pathway.

Discussion

Heptaphylline and 7-methoxyheptaphylline are a carbarzole isolated from the roots of Clausena harmandiana which exhibited cytotoxicity against NCI-H187 (human small cell lung cancer cells) and KB cells (human epidermoid carcinoma of oralcavity cell lines) (Ito et al., 2000; Yenjai et al., 2000; Thongthoom et al., 2011; Wangboonskul and Yenjai., 2012). In this study we test the apoptotic effects of heptaphylline and 7-methoxyheptaphylline on human colon adenocarcinoma cell line (HT-29). Heptaphylline showed more stronger apoptotic effects than 7-methoxyheptaphylline by induced cell death morphology change of HT-29 colon adenocarcinoma cells. In order to control cell proliferation or in response to stimuli, cells undergo death through a process called apoptosis or programmed cell death. The understanding of apoptosis has provided the basis for new targeted therapies that can induce death in cancer cells. Most of the cytotoxic anticancer agents in present use have been shown to induce apoptosis in cancer cells such as etoposide, capsaicin, berberine, gomisin N and doxorubicin (Ghobrial et al., 2005; Wong, 2011). The anti-cancer drug etoposide is able to induce caspase-8 processing and apoptosis in cancer cells, capsaicin induces apoptosis through cellular stress, involving mitochondria and endoplasmic reticulum in several human cancer cells, berberine, an isoquinoline alkaloid, has several pharmacological properties and can affect in different levels the mitochondrial functions, gomisin N , a dibenzocyclooctadiene lignan, isolated from Schizandra chinensis Baill induced apoptosis of human leukemia U937 cells and doxorubicin also induces apoptosis in many cancer cells (Kim et al., 2010; Liu et al., 2011; IP et al., 2012). In apoptotic pathways, the Bcl-2 family of proteins are significant regulators of apoptosis and include both anti-apoptotic members (such as Bcl-2, Mcl-1, survivin and Bcl-xL) and pro-apoptotic members (for example, Bax, Bak, Bad, Bid, and Bik) (Wong, 2011). Antiapoptotic proteins acts as suppressors of apoptosis by blocking the activation of cytochrome-C and caspase-3, whereas proapoptotic members act as apoptosis promoters and are overexpressed in massive malignancies. In response to apoptotic stimuli, proapoptotic proteins undergo posttranslational modifications that include dephosphorylation and cleavage resulting in activation of Bid, which oligomerizes Bak or Bax into pores that result in the release of cytochrome C. Cytochrome C, once released in the cytosol, interacts with Apaf-1, leading to the activation of caspase-9 proenzymes. Active caspase-9 then activates caspase-3, which subsequently activates the cleavage of PARP-1 and leads to apoptosis. Increased expression of antiapoptotic proteins causes resistance to chemotherapeutic drugs, while decreasing antiapoptotic proteins expression may promote apoptotic responses to anticancer drugs. In this study, heptaphylline activated cleavage of caspase-3, PARP-1 and proapoptotic proteins, Bid, Bak. In contrast, it inhibits antiapoptotic proteins including Bcl-xL, XIAP and survivin (Korsmeyer et al., 2000; Thorburn, Behbakht and Ford, 2008). It has been reported that the extrinsic and intrinsic apoptotic pathways are regulated by proteins, for instance, the p53 and NF-ϰB pathways. NF-ϰB is a nuclear transcription factor that regulates expression of many genes involved in the regulation of apoptosis, the activation of NF-ϰB induces resistance to apoptotic stimuli through the activation of a number of complex proteins including Bcl-xL, survivin, and X-linked IAP (XIAP). Moreover, previous reports

have shown that NF-κB also suppressed Bid activation through incresase TRAF resulting in inhibition of Bid activation via TRAF expression induction (Shukla, et al., 2004; Dai, et al., 2005; Mendoza, et al., 2005; Namba et al., 2007). To investigate the role of NF-κB in heptaphylline-mediated apoptotic regulators regulation, we treated cells with heptaphylline in various concentrations and assessed Akt, IKKα and NF-κB/p65 (rel) phosphorylation. We found that heptaphylline acts as Akt/NF-κB (p65) pathway inhibitor in concentration-dependent manner. According to all of above, heptaphylline activates proapoptotic proteins (Bid and Bak) and suppresses expression of antiapoptotic proteins including Bcl-xL, XIAP and survivin that lead to apoptosis of HT-29 cancer cells and the regulation may involve in Akt/NF-κB (p65) pathway. Indicating that hepataphylline acts as apoptotic inducing agent which could be developed to be a potential anticancer agent.

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