Cytotoxic Effect and Its Mechanism of Dioscorealide B from *Dioscorea membranacea* against Breast Cancer Cells

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Background: *Dioscorealide B is an active compound from the rhizome of Dioscorea membranacea Pierre which locally known as "Hua-Khao-Yen". This medicinal plant has long been used in the anticancer prescription of Thai traditional medicine..*
Objective: *To examine the cytotoxic effect and mechanism of Dioscorealide B in human breast carcinoma cells.*
Material and Method: *Dioscorealide B was isolated from the rhizome of Hua KhaoYen (Dioscorea membranacea).The cytotoxicity of Dioscorealide B was evaluated in two human breast cancer cell lines, MCF-7 and MDA-MB 468 by Sulphorhodamine B (SRB) assay. RT-PCR and Caspase-Glo® assay were used to further elucidate its cytotoxic mechanism.*
Results: *Dioscorealide B showed cytotoxic effect on MCF-7 (IC_{50}=2.76 μM) and MDA-MB 468 (IC_{50}=9.93 μM). The mRNA level for p53, p21 and Bax were increased while Bcl-2 was decreased after the treatment. MCF-7 treated with Dioscorealide B showed the induction of apoptosis via the activation of caspase-9 and -7*
Conclusion: *The results suggested that the mechanisms of Dioscorealide B might be involved in p53 and the intrinsic apoptotic pathway*

Keywords*: Dioscorea membranace, Dioscorealide B, SRB assay, Apoptosis, MCF-7*

J Med Assoc Thai 2010; 93 (Suppl. 7) : S277-S282
Full text. e-Journal: *http://www.mat.or.th/journal*

Cancer is an aberrant net accumulation of atypical cells which can arise from an excess of proliferation, an insufficiency of apoptosis, or a combination of the two[1,2]. The frequency of apoptosis could conduce to cell loss in tumors and promote tumour regression. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. The life span of both normal and cancer cells is significantly affected by the rate of apoptosis. Therefore, modulating apoptosis may be useful in the management and therapy or prevention of cancer[3-5].

The *p53* tumor suppressor limits cellular proliferation by inducing cell cycle arrest and apoptosis in response to cellular stresses such as DNA damage, hypoxia, and oncogene activation. In response to oncogene activation, *p53* mediates apoptosis through an intrinsic pathway involving bax transactivation, Bax translocation from the cytosol to membranes, cytochrome c release from mitochondria, and caspase-9 activation, followed by the activation of caspase-3, -6, and -7[6-8]. Numerous studies have demonstrated that *p53* directly activates the transcription of a number of genes including cyclin-dependent kinase inhibitor p21 [WAF1/CIP1] which leads to G1 arrest[9-11].

Hua Khao Yen or *Dioscorea membranacea* Pierre is a member of Dioscoreaceae. The Thai traditional doctors generally use this plant to treat dermopathy, lymphopathy, venereal diseases, leprosy, and cancer as well as inflammatory conditions associated

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with diseases such as rheumatism, infectious diseases and other pain-causing condition[12-14]. The previous studies demonstrated that Dioscorealide B, one of isolated compounds from *D. membranacea*, serve as antiproliferative agent. It was found that this bioactive compound selectively inhibit the proliferation of lung cancer cell (CORL-23) and particularly breast cancer cell (MCF-7) without being significantly cytotoxic towards non-malignant cells (SVK)[15]. In this study, we investigated the mechanism of Dioscorealide B against breast cancer. Our results demonstrated that Dioscorealide B had cytotoxic effect on two human breast cancer cell lines: MCF-7 and MDA-MB 468 and the cytotoxic activity of Dioscorealide B in MCF-7 appeared to be mediated by the regulation of Bcl-2, Bax and p53 genes leading to activation of caspase-9 and-7, respectively.

Material and Method

Plant materials

The rhizomes of D. membranacea Pierre (Dioscoreaceae) were collected from Pa-tue, Chumporn, Thailand. Authentication of plant materials was carried out at the herbarium of the Department of Forestry, Bangkok, Thailand where the herbarium voucher (SKP A062041305) is kept. Specimens are also kept in the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla,Thailand

Isolation of Dioscorealide B

Dioscareaolide B was isolated following the method previously described and agreed in all respects as regards reported chromatographic and spectral data[15].

Table 1. PCR primers used in the gene expression studies

Gene	Sense(S) and antisense (AS) primers
p53	S: 5'GCTCTGACTGTACCACCATACC3'
	AS: 5'CTCTCGGAACATCTCGCAGCG3'
p21	S:5'CTCAGAGGAGGCGCCATG3'
	AS: 5'GGGCGGATTAGGGCTTCC3'
Bax	S:5'CACCAGCTCTGAGCAGATG3'
	AS: 5'GCGAGGCGGTGAGCACTCC3'
Bcl-2	S: 5'CTGGCATCTTCTCCTTCCAGC3'
	AS: 5'ACCTACCCAGCCTCCGTTATC3'
GAPDH	S: 5'GAAGGTGAAGGTCGGAGT3'
	AS: 5'GAAGATGGTGATGGGATTTC3'

Cell Culture Conditions:

MCF-7 human breast cancer cell line was kindly provided from Dr. P. Twentyman and Dr. P. Rabbitts of MRC Clinical Oncology & Radiotherapeutics Unit, Cambridge, UK. MDA-MB 468 was acquired from the American Type Culture Collection (HTB-132). Cells were cultured in monolayers in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 µg/mL penicillin, and 100 µg/mL streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO_2.

In vitro assay for Cytotoxic activity

In this study, the antiproliferative effect of Dioscorealide B on two human breast cancer cell lines ; MCF-7 and MDA-MB 468 was determined. Cells were treated at different concentrations of Dioscorealide B for 72 hours, the proportions of surviving cells were then estimated and IC_{50} values (concentrations leading to 50% inhibition of viability) were calculated as shown in Table 2. Cells incubated with 0.2% DMSO was used as a solvent control. The cytotoxicity assay was carried out using Sulphorhodamine B (SRB) assay[15,16]. Briefly, 3,000 cells of MCF-7 or 5,000 cells of MDA-MB 468 were plated per well in 96-well culture plates kept in the incubator at 37°C. After overnight incubation, the cells were treated without or with Dioscorealide B of 0.03, 0.15, 0.3, 1.5, 3, 15, 30, 150 µM with 6 replications. The cells were incubated for the exposure time of 72 hours and then the medium was removed and washed. The survival percentage was measured colorimetrically using SRB assay and IC_{50} values was calculated by means of Prism program. Cells incubated with regular cell culture media with 0.2% DMSO was used as a negative control.

RT-PCR

Total RNA was extracted from 1×10^7 washed cells by the Trizol reagent (Invitrogen). RT-PCR was

Table 2. Cytotoxicity of Dioscorealide B (IC_{50} (µM) ± SEM) against breast cancer cell lines, MCF-7 and MDA-MB 468

Compound	IC_{50} (µM) ± SEM	
	MCF-7	MDA-MB 468
Dioscorealide B	2.76 ± 0.18	9.93 ± 0.93

performed by using Qiagen OneStep RT-PCR kit. 0.25 µg of total RNA was subjected to one-step RT-PCR in 25 µL reaction volume containing 2.5 µL 5x Qiagen OneStep RT-PCR buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 12.5 $MgCl_2$, DTT ; pH 8.7), 0.5 µL 10 mmol/L deoxynucleoside triphosphate (dNTP), 0.5 µL Qiagen OneStep RT-PCR Enzyme Mix (Omniscript™ Reverse Transcriptase, Sensiscript™ Reverse Transcriptase and HotStarTaq® DNA polymerase), 0.75 µL of 10 µmol/L each primer, 0.25 µL of Rnase inhibitor and RNase-free water to 25 µL. The reverse transcription step was initiated at 50°C for 30 minutes, followed by PCR activation at 95°C for 15 minutes. The primer sequences and PCR conditions used in these experiments were shown in Table below. The RT-PCR products were analyzed in 2.5% agarose gel.

Caspase-7 and-9 Activity Assay

The Caspase-Glo 3/7 and -9 assays (Promega, Madison, WI) were used to measure caspase 3/7 and caspase-9 activity. Briefly, Cells were cultured in 96-well plates and treated with Dioscorealide B. After the periodic incubation, caspase-Glo reagent was added to each well according to the manufacturer's instructions. Plates were mixed on a plate shaker for 30 seconds and incubated at room temperature for 3 hours. Luminescence was measured using the luminometer. The assay was performed in triplicate.

Statistics

Data were expressed as means ± SEM. Statistical comparisons of the results were made using analysis of variance (ANOVA) and a P value less than 0.05 was considered significant.

Results

The cytotoxic effect of Dioscorealide B were found that Dioscorealide B showed the highest susceptibility against breast cancer cells depended on hormone or MCF-7 (IC_{50}= 2.76 µM), but less actice against breast cancer cells non depended on hormone or MDA-MB 468 cells (IC_{50}= 9.93 µM).

To examine whether caspases involves in dioscorealide B-induced apoptosis, the caspase-7 and-9 activity were measured. Dioscorealide B showed the induction of apoptosis via caspase-7 in a dose- and time dependent manner (Fig. 2). At 3 hours, the caspase-7 activity was significantly increased to 326.54% and 408.95% in MCF-7 treated with 6 and 12 µM of Dioscorealide B, respectively. Next, the effect of caspase-9 inhibitors on Dioscorealide B-induced apoptosis was studied. MCF-7 cells were pretreated with 50 µM of the caspase-9 inhibitor Z-LEHD-FMK for 3 hours prior to treatment with 3 µM of Dioscorealide B. Pretreatment of MCF-7cells with the caspase-9 inhibitor significantly decreased the caspase-9 activity (Fig. 3).

The result of RT-PCR for determining the molecular pathway of apotosis revealed that after the exposure time 1 h 3 µM Dioscorealide B was treated , ,the *p53*, *p21* and *Bax* showed an increase in their expressions, while *Bcl-2* expression was down-regulated in a time-dependent manner (Fig. 4).

Discussion and Conclusion

A large number of drugs for treating cancer are proapoptotic. The majority of proapoptotic cytotoxic drugs currently used to treat cancer patients take advantage of cell division itself in an attempt to achieve selective action, based on the more rapid division of cancer cells compared to their normal counterparts[17]. Nevertheless, major problems with these molecules persist because they are not sufficiently selective or

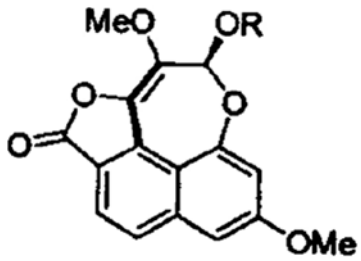

Fig. 1 Chemical structure of Dioscorelide B isolated from *Dioscorea membranacea*.

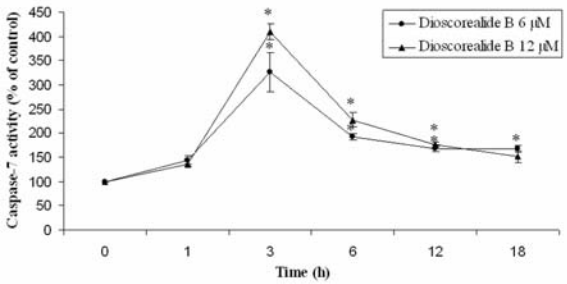

Fig. 2 The activation of caspase-7 in MCF-7 by Dioscorealide B. MCF-7 cells were incubated with 6 and 12 µM of Dioscorealide B for the indicated times. The data represented the average value of 3 replications from 2 independent experiments.

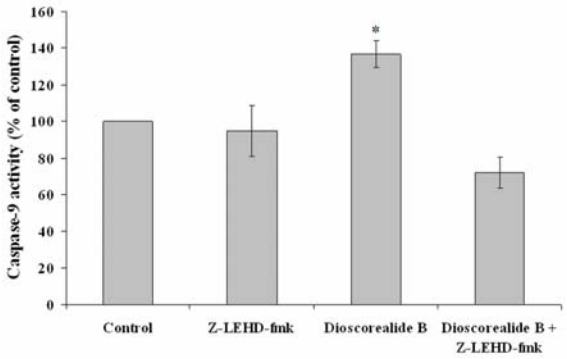

Fig. 3 The effect of caspase-9 inhibitor on Dioscorealide B-induced apoptosis. MCF-7 cells were pre-incubated with 50 µM of Z-LEHD-FMK are caspase-9 inhibitor for 3 hours before challenging with 3 µM Dioscorealide B. After 3 hours of treatment, caspase-9 activity was measured by Caspase-Glo® assay. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and Dioscoerealide B-treated cells, as analyzed by ANOVA $p < 0.05$

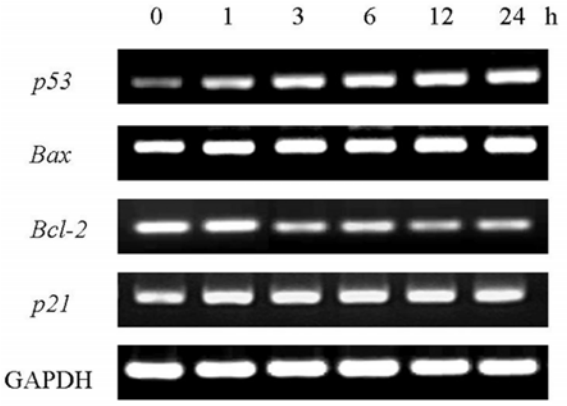

Fig. 4 mRNA expression of *p53, Bax, Bcl-2* and *p21* genes in human breast cancer cell lines, MCF-7, treated with 3 µM Dioscorealide B. mRNA expression of these genes were measured by RT-PCR. RT-PCR product was resolved in 2.5% agarose gel. GAPDH mRNA was analyzed as a control.

cancer cells, resulting in toxicity to normal cells and provoking widespread and serious consequences in patients[18]. Thus, the need to identify the chemical structure of potent anticancer agents that target tumor cells more selectively remains to be identified.

Dioscorea membranacea locally known as "Hua-Khao-Yen" have mostly been used in Thai traditional medicines. Interview of the selected traditional doctors in Southern Thailand revealed that they used Hua Khao Yen as ingredients in their remedies for cancer which accounted for about sixty percent of the list of herbal drugs used for cancer treatment[19]. In recent study, we found that Dioscorealide B isolated from the ethanolic extract of *D. membranacea* was able to inhibit *in vitro* growth of breast cancer cell lines: MCF-7 and MDA-MB 468. The data demonstrated that Dioscorealide B was much more potent in MCF-7 cells than MDA-MB 468 cells.

Various forms of cellular stress such as DNA damage, the level of *p53* ,an important tumor suppressor, appears to be increased and triggers the mitochondrial apoptotic pathway via regulating transcription *Bcl-2* family member, leading to up-regulated expression of the proapoptotic *Bax* and down-regulated expression of the antiapoptotic *Bcl-2*[20-22]. Also, the *p53* is essential for the checkpoint control which arrests human cells with damaged DNA in G1 by transactivating target gene such as cyclin -kinase inhibitor, $p21^{WAF1/CIPI\ (23)}$. To investigate the possible involvement of *p53* in the apoptotic inducing pathway of Dioscorealide B in MCF-7, the levels of mRNA expression of *p53* and its relevant target genes, including *bax, bcl-2* and *p21* were measured in the cell after treated with this compound. Intriguingly, this pure compound from *D. membranacea* was shown to upregulate *p53* expression as well as the proapoptotic *Bax*, while it induced downregulation of antiapoptotic *Bcl-2*. During the time course experiment, upregulation of *p53* expression was first detected at 1 h after treated with 3 µM of Dioscorealide B as well as the changes in the level of *Bax* and *Bcl-2* expression. The expression of the *p21*, and *p53* targeted gene, was also up-regulated after treatment with Dioscorealide B. These data suggested that the mechanism of Dioscorealide B involved in *p53* and the modulation of *Bcl-2* family expression which leads to the activation of caspase-9 and -7, respectively. In addition, the results of cytotoxic test revealed that p53 mutant cell line[24], designated MDA-MB 468 displayed the lower susceptibility to Dioscorealide B than MCF-7 which harbor a functional *p53* gene[25,26]. Taken together, these findings suggested that *p53* was likely involved in the apoptosis induction pathway initiated by Dioscorealide B. However, further study would need to refine the molecular mechanism underlying this cytotoxic effect. The specific molecular target of this compound would need to be identified to clarify the mechanism. This study has provided the foundation for further study. It would be worth to iden-

tify the mechanism for *p53* transactivation by Dioscorealide B.

Acknowledgement

We would like to thank Prince of Songkla University for financial support

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ฤทธิ์และกลไกการต้านมะเร็งเต้านมของสาร diosorealide B จาก Dioscorea membranacea

จิราพร แซ่คู, ชวบูลย์ เดชสุขุม, พจนพร ไกรดิษฐ์, อรุณพร อิฐรัตน์

ภูมิหลัง: *สาร dioscorealide B เป็นสารออกฤทธิ์ที่แยกได้จาก rhizome ของ Dioscorea membranacea หรือหัวข้าวเย็น ซึ่งเป็นสมุนไพรไทยที่นำมาใช้ในตำรับยารักษามะเร็งของหมอพื้นบ้าน*

วัตถุประสงค์: *เพื่อศึกษาฤทธิ์และกลไกการต้านมะเร็งเต้านมของสาร dioscorealide B*

วัสดุและวิธีการ: *แยกสาร dioscorealide B จากเหง้า ของหัวข้าวเย็น (Dioscorea membranacea) ศึกษาฤทธิ์ต้านมะเร็งเต้านม 2 ชนิด (MCF-7 และ MDA-MB 468) โดยใช้ SRB assay และใช้วิธี RT-PCR และ Caspase-Glo® assay ในการศึกษากลไกการออกฤทธิ์ของสาร dioscorealide B*

ผลการศึกษา: *สาร dioscorealide B มีฤทธิ์ต้านเซลล์มะเร็งทั้งชนิด MCF-7 และ MDA-MB 468 (IC_{50} เท่ากับ 2.76 และ 9.93 μM ตามลำดับ) และพบว่าภายหลังจากการ treat ด้วย dioscorealide B มีการแสดงออกของ p53, p21 และ Bax เพิ่มมากขึ้น ในขณะที่ Bcl-2 มีการแสดงออกลดลงในระดับ mRNA รวมถึงมีการกระตุ้น caspase-9 และ caspase 7*

สรุป: *กลไกการออกฤทธิ์ต้านมะเร็งเต้านมมีความเกี่ยวข้องกับการทำงานของ p53 และผ่านกลไกการเกิด apoptosis แบบ intrinsic*