

RESEARCH ARTICLE

6,8-Dihydroxy-7-methoxy-1-methyl-azafluorenone Induces Caspase-8- and -9-mediated Apoptosis in Human Cancer Cells

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Abstract

6,8-Dihydroxy-7-methoxy-1-methyl-azafluorenone (DMMA), a purified compound from *Polyalthia cerasoides* roots, is cytotoxic to various cancer cell lines. The aims of this study were to demonstrate the type of cancer cell death and the mechanism(s) involved. DMMA inhibited cell growth and induced apoptotic death in human leukemic cells (HL-60, U937, MOLT-4), human breast cancer MDA-MB231 cells and human hepatocellular carcinoma HepG2 cells in a dose dependent manner, with IC₅₀ values ranging between 20-55 μ M. DMMA also decreased cell viability of human peripheral blood mononuclear cells. The morphology of cancer cells induced by the compound after staining with propidium iodide and examined under a fluorescence microscope was condensed nuclei and apoptotic bodies. Mitochondrial transmembrane potential (MTP) was decreased after 24h exposure in all five types of cancer cells. DMMA-induced caspase-3, -8, and -9 activity was strongly induced in human leukemic HL-60 and MOLT-4 cells, while in U937-, MDA-MB231- and HepG2-treated cells there was partial induction of caspase. In conclusion, DMMA-induced activation of caspase-8 and -9 resulted in execution of apoptotic cell death in human leukemic HL-60 and MOLT-4 cell lines via extrinsic and intrinsic pathways.

Keywords: Extrinsic pathway - intrinsic pathway - caspase - 6,8-dihydroxy-7-methoxy-1-methyl-azafluorenone

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Introduction

Cancer is the first cause of death in the developed countries and the second cause of death in developing countries due to the life style changes, such as smoking, lacking of exercise and taking of western food. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females (Jemal et al., 2011). Liver cancer is the fifth most frequently found cancer in male (Siegel et al., 2012). Leukemia incidence and curable rate are low compared to other kinds of cancer, which might be due to genetic information and apoptosis resistance.

Apoptosis is a programmed cell death controlled by genes to keep homeostasis and development of multicellular organisms. Loss of cell volume, plasma membrane blebbing, nuclear condensation, chromatin aggregation, and internucleosome DNA cleavage are characteristics of apoptotic cells. Inappropriate control of apoptosis is occurred in several diseases such as acute myocardial infarct and cancer (Gewies, 2003). Tumor suppressor genes, e.g., *p53* and oncogenes, such as *myc* control apoptosis on various aspects. Anti-apoptotic (e.g. Bcl-2, Bcl-xL, Mcl-1) and pro-apoptotic proteins (e.g. Bax, Bak, Bad) play pivotal roles on apoptosis induction.

Mitochondria and mitochondrial releasing proteins such as cytochrome c, apoptosis inducing factor (AIF), EndoG also control apoptosis pathway in a complicate way.

Apoptosis induction can be divided into two pathways: i) death receptor-induced extrinsic pathway via tumor necrosis factor receptor (TNFR), Fas, DR3, TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1 or DR4) and TRAIL-R2 or DR5 and ii) mitochondria-mediated intrinsic pathway via apoptosome (a complex of cytochrome c, Apaf-1 and procaspase-9). The death receptor pathway is induced by ligands such as Fas ligand, TNF, TRAIL to form a death-inducing signaling complex with the adaptor proteins and the receptors under the cell membrane and stimulates caspase-8 and then caspase-3. The mitochondrial pathway is induced by ultraviolet, gamma-ray, oxidative stress or chemotherapeutic drugs to reduce mitochondrial transmembrane potential and release several proteins such as cytochrome c to cytosol to form complex with Apaf-1 and activate caspase-9. Both caspase-8 and -9 are initiator caspases, which stimulate effector caspases, i.e., caspase-3, -6 and -7 and proteolysis of the structural proteins (e.g. actin, fodrin, lamin) ending to apoptosis (Clarke et al., 2004; Ghobrial et al., 2005; Ashkenazi, 2008).

There are several active compounds isolated from

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the roots of *Polyalthia cerasoides* with the anti-malarial and anti-mycobacterial activities against *Plasmodium falciparum* and *Mycobacterium tuberculosis*, respectively *in vitro* (Kanokmedhakul et al., 2007). *Polyalthia cerasoides* bedd. at a dose of 100 µg/kg significantly prevents the increase in serum transaminases, serum alkaline phosphatase, liver and brain lipid peroxidation and decreases total protein content in liver and brain following carbontetrachloride-induced hepatotoxicity in albino rats (Padma et al., 1999). The alcohol extract of *Polyalthia cerasoides* contains significant reactive oxygen species (ROS) scavenging activity in 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical, superoxide anion scavenging, and reducing power assays; antiproliferative effect against mouse fibroblast L929 cell line; and a high level of total phenolic content *in vitro* (Ravikumar et al., 2008). Two phytosterols (alpha-spinasterol and spinasterol) and a clerodane di-terpenoid are isolated from *Polyalthia cerasoides* seeds and contain antiproliferative and apoptotic induction effect in CACO-2 cell line (Ravikumar et al., 2010). 6,8-Dihydroxy-7-methoxy-1-methyl-azafluorenone (DMMA) is isolated from *Polyalthia cerasoides* roots and is cytotoxic to human non-small cell lung cancer A549 and human small cell lung cancer GLC4 cell lines (Pumsalid et al., 2010). The aims of this study were to determine the cytotoxic effect of DMMA and the mechanism of cell death in human cancer cell lines including leukemia, hepatocellular carcinoma and breast cancer.

Materials and Methods

Chemicals

6,8-Dihydroxy-7-methoxy-1-methyl-azafluorenone (DMMA) was isolated from *Polyalthia cerasoides* roots as previously reported (Pumsalid et al., 2010) and the structure is shown in Figure 1. Propidium iodide, histopaque, 3,3-dihexyloxacarbocyanine iodide (DiOC₆), dimethyl sulfoxide (DMSO) and 3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma/Aldrich, St. Louis, MO, USA. RPMI-1640 medium, DMEM medium, Leibovitz's L-15 medium, DEVD-pNA (Asp-Glu-Val-Asp-para-nitroaniline), LEHD-pNA (Leu-Glu-His-Asp-para-nitroaniline), and IETD-AFC (Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin) were obtained from Invitrogen, USA.

Cell culture

Human lymphoblastic leukemic MOLT-4 and monocytic U937 cells were gifts from Professor Watchara Kasinroek (Faculty of Associated Medical Sciences,

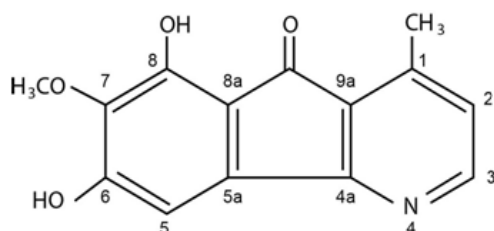


Figure 1. The Structure of 6,8-dihydroxy-7-methoxy-1-methyl-azafluorenone (DMMA) (Pumsalid et al., 2010)

Chiang Mai University). Human promyelocytic leukemic HL-60 cells were obtained from Assoc. Prof. Sukathida Ubol (Faculty of Science, Mahidol University). Human hepatocellular carcinoma HepG2 cells and breast cancer MDA-MB231 cells were gifts from Assoc. Prof. Prachya Kongtawelert, Faculty of Medicine, Chiang Mai University. Peripheral blood mononuclear cells (PBMCs) were donated from healthy volunteers. PBMCs were isolated from heparinized blood by density gradient centrifugation using histopaque according to standard protocols. The blood was obtained from adult volunteers with Institutional Review Board approval at Faculty of Medicine, Chiang Mai University. The leukemic cells were cultured in RPMI-1640 medium with 25 mM NaHCO₃, 20 mM HEPES, 100 units/mL penicillin, 100 µg/mL streptomycin and supplemented with 10% fetal bovine serum. Human hepatocellular carcinoma HepG2 cells were cultured in DMEM medium whereas human breast cancer MDA-MB231 cells were cultured in Leibovitz's L-15 medium. DMMA was dissolved in dimethyl sulfoxide (DMSO) as a vehicle and the maximal volume used did not exceed 10 µl/ml of media. The cell lines were grown at 37°C in a 5% CO₂ atmosphere. The PBMCs and human cancer cells (1x10⁶) were treated with DMMA at indicated concentrations and durations.

MTT assay for cytotoxicity

HL-60, U937 and MOLT-4 cells (3x10⁵ cells/ml), HepG2 and MDA-MB231 (7.5x10³ cells/ml) and PBMCs (1x10⁵ cells/ml) were cultured and incubated with DMMA (0, 10, 20, 40 and 80 µM) at 37°C in 5% CO₂ atmosphere for 24 h. The cell viability was determined by using MTT assay (Banjerdpongchai et al., 2010b). Briefly, MTT dye solution was added and incubated in CO₂ incubator for 4 h. Then, 100 µl of DMSO was added to dissolve the violet dye crystals. Absorbance was measured by using a microtiter plate reader (Biotek, USA) at 570 nm. The percentage of cell viability was calculated and 10, 20 and 50% inhibitory concentrations (IC₁₀, IC₂₀ and IC₅₀) were determined and used for further experiments.

Fluorescence microscopy

Human leukemic cells were treated with DMMA (IC₁₀, IC₂₀ and IC₅₀) for 24 h, and then cytospun onto glass slides, whereas HepG2 and MDA-MB231 cells were cultured on cover slip and treated with DMMA at IC₁₀, IC₂₀ and IC₅₀ for 24 h. After air drying, cells were fixed with absolute methanol for 10 min at -20°C, washed twice with phosphate-buffered saline (PBS) and air-dried. Propidium iodide (200 µg/ml) was applied to the fixed cells for 10 min at room temperature. After washing with PBS and drying, slides were mounted with 90% glycerol and examined under a fluorescence microscope (Olympus, Japan). Apoptotic cells (condensed nuclei and fragmented cells) were counted from a total of 200 cells per slide (Banjerdpongchai et al., 2011). Three independent experiments were performed in duplicate.

Measurement of mitochondrial transmembrane potential

Reduction in mitochondrial transmembrane potential (ΔΨ_m) during the induction of apoptosis were examined

with 3,3-dihexyloxacarbocyanine iodide (DiOC₆). Human cancer cells were harvested after DMMA treatment for 24 h, and DiOC₆ was added at a final concentration of 40 nM. After 20 minutes of incubation at 37°C, the leukemic cells were washed and analyzed using flow cytometry (Becton Dickinson, USA) (Banjerdpongchai et al., 2010a), whereas HepG2 and MDA-MB231 cells were analyzed by fluorescence plate reader (Biotek, USA) (Marchetti et al., 2002).

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

Cleavage of the colorimetric or fluorometric peptide substrate DEVD-pNA (Asp-Glu-Val-Asp-para-nitroaniline), Z-IETD-AFC (Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin) and LEHD-pNA (Leu-Glu-His-Asp-para-nitroaniline) are indicative of caspase-3, -8 and -9 activities, respectively. Cell lysate (from 1x10⁶ cells) and substrate (50 μM) were combined in a standard reaction buffer and added to a 96-well plate. Enzyme-catalyzed release of AFC was measured by a fluorescence plate reader (Biotek, USA) using 400 nm excitation and 505 nm emission wavelengths, whereas the absorbance of para-nitroaniline was measured by spectrophotometer at 400 nm (Faucheu et al., 1995).

Statistical analysis

Results were expressed as mean±SD (standard deviation). Statistical difference between control and treated group was determined by a nonparametric one-way ANOVA (Kruskal Wallis test) with a limit of p<0.05 in three independent experiments. For comparison between two groups, data were analyzed using Student's t-test.

Results

Cell cytotoxicity and apoptosis induction

6,8-Dihydroxy-7-methoxy-1-methyl-azafluorenone inhibited the five human cancer cell proliferation in a dose-dependent manner from MTT assay. HepG2 cells were the most sensitive to and MDA-MB231 cells were the most resistant against DMMA-induced cytotoxicity as shown by the IC₅₀ levels (Table 1). Of note, DMMA impaired normal PBMC growth with the IC₅₀ level of 31.1 μM. The mode of cell death was via apoptosis proved by the cell morphology after staining with propidium iodide and examining under a fluorescence microscope, i.e., condensed nuclei and apoptotic bodies as shown in Figure 2. The characteristic apoptotic cell morphology was found in all five cancer cell lines with the increase in percentage of apoptotic cells in the dose-response manner (Figure 3).

Reduction of mitochondrial transmembrane potential (MTP)

All five human cancer cell lines were induced by DMMA to die via apoptosis and the decrease of mitochondrial transmembrane potential occurred as shown in Figure 4. Percentage of human leukemic cells with reduced MTP increased in a concentration-response manner and the fluorescence intensity of DiOC₆ was increased as the dose increased in human HepG2 and MDA-MB231 cell lines.

Table 1. Inhibitory Concentrations at 20 and 50 percent (IC₂₀ and IC₅₀) of 6,8-Dihydroxy-7-Methoxy-1-methyl-azafluorenone (DMMA) towards Human Cancer Cells.

The cytotoxic effects of DMMA on human leukemic HL-60, U937, MOLT-4; hepatocellular carcinoma HepG2; breast cancer MDA-MB231 cells and PBMCs were measured by MTT assay

Cell types	IC ₂₀ (μM)	IC ₅₀ (μM)
HL-60	18.7	46.7
U937	11.7	29.2
MOLT-4	14.0	35.0
HepG2	7.4	20.1
MDA-MB231	16.7	55.6
PBMCs	12.4	31.1

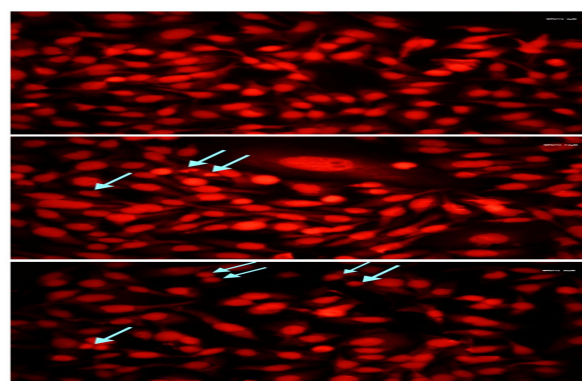


Figure 2. Representative Fluorescent Micrographs of Human Hepatocellular Carcinoma HepG2 Cells after Treatment with DMMA. HepG2 cells were untreated (A), treated with IC₂₀ (B) and IC₅₀ (C) of DMMA; and then stained with propidium iodide. The morphology of treated cancer cells were characteristic apoptotic cells, viz., condensed nuclei and apoptotic bodies as indicated by arrows

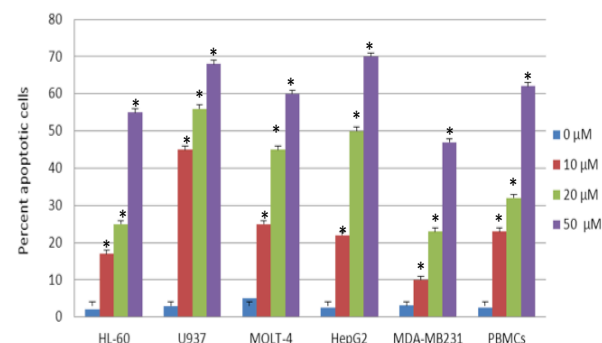


Figure 3. 6,8-Dihydroxy-7-methoxy-1-methyl-azafluorenone (DMMA)-induced Human Cancer Cell Apoptosis. Human leukemic HL-60, U937, MOLT-4; hepatocellular carcinoma HepG2 and breast cancer MDA-MB231 cells were treated with DMMA at indicated concentrations for 24 h and apoptotic cells were examined by propidium iodide staining and visualization under a fluorescence microscope. Percentage of apoptotic cells is expressed as mean and S.D. of 200 cells from 3 independent experiments conducted in duplicate. *p<0.05, compared to control

Induction of caspase-3, -8 and -9 activity

The human leukemic HL-60 and MOLT-4 cells were induced to undergo apoptosis mediated by caspase-3, -8 and -9 activities, indicating that both extrinsic and intrinsic pathways were involved. However, the other three cell

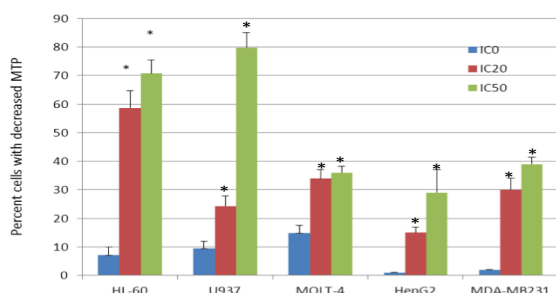


Figure 4. Reduction of Mitochondrial Transmembrane Potential of Human Cancer Cells after Treatment with DMMA. All five cancer cell lines were treated with DMMA for 24 h and mitochondrial transmembrane potential was measured using DiOC₆ and flow cytometry. Percent cells with decreased MTP of HL-60, U937, MOLT-4, HepG2 and MDA-MB231 cells are shown as mean±SD. *p<0.05, compared to control

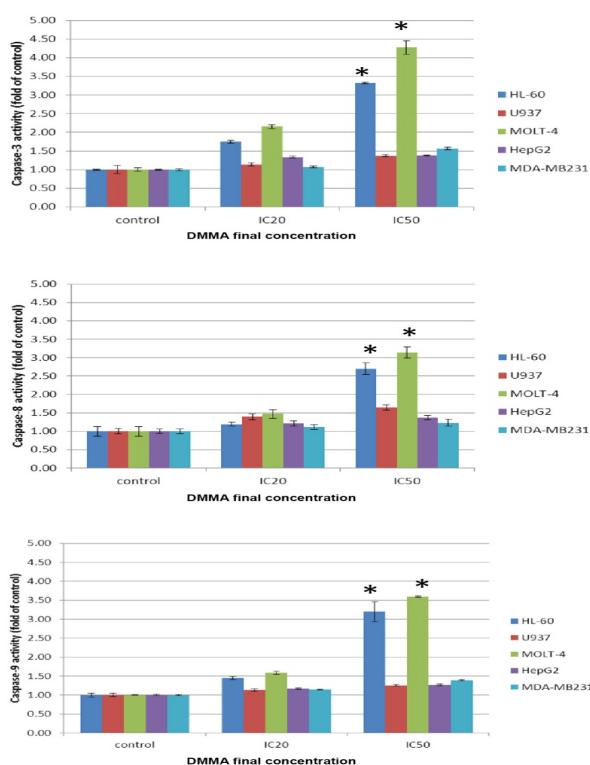


Figure 5. Caspase-3, -8 and -9 Activities of DMMA-treated Human Cancer Cells. Caspase-3 (A), caspase-8 (B) and caspase-9 (C) activities in human cancer cell lysates were determined using caspase-specific colorimetric and fluorometric substrates as described in Materials and Methods. Data are shown as mean±S.D. *p<0.05, compared to control lines had less alteration of these three caspase activities. This might be from the alternative pathways of apoptotic induction via caspase-independent pathways such as through activation of enzyme calpain, granzyme and/or cathepsins.

Discussion

This present study confirmed the previous studies of the inhibitory effects of the active compound(s) or extract from *Polyalthia cerasoides* on the human cancer cell proliferation such as CACO-2 (Ravikumar et al., 2010), L929 (Ravikumar et al., 2008), A549, GLC4 and GLC4/Adr (Pumsalid et al., 2010). 6,8-Dihydroxy-7-methoxy-1-

methyl-azafluorenone (DMMA) induced human leukemic promyelocytic HL-60, monocytic U937, lymphoblastic MOLT-4, hepatocellular carcinoma HepG2 and breast cancer MDA-MB231 cells to undergo apoptotic cell death with the effective IC₅₀ range between 20–55 μM. However, DMMA was cytotoxic to normal PBMCs with IC₅₀ level of 31.1 μM. This limits the application of DMMA to be used in the clinical level, but the lower levels than IC₁₀ of DMMA against normal cells (PBMCs) may be used. The in vivo trial in animals is needed to get the acute toxicity dose before the usage in normal human being or cancer patients. The consideration of combined treatment with TRAIL or the conventional chemotherapeutic drug(s) might lower the dose of DMMA and chemotherapeutic drug and their toxicity to normal cells (Koschny et al., 2007).

Since the mechanism of cell death induced by the active compounds of *Polyalthia cerasoides* remains elusive, the recent study reports the DMMA-induced apoptosis of two human leukemic cells via caspase-8 (extrinsic pathway) and caspase-9 (intrinsic pathway) with the alteration of mitochondrial transmembrane potential, which might lead to the release of cytochrome c (Yanase et al., 2000). Cytochrome c forms complex with Apaf-1 and induces procaspase-9 to be active caspase-9, which cleaves poly-(ADP-ribose) polymerase (PARP) and results in DNA fragmentation as shown by fragmented bodies or apoptotic bodies (Henshall et al., 2000). The apoptotic bodies were found under a fluorescence microscope after staining the five 24-h-DMMA-treated human cancer cells with propidium iodide (Figure 2).

Extrinsic pathway involved in the human DMMA-treated cancer cell apoptosis as the cell death was mediated through caspase-8. Whether the signaling via ligand binding to the death receptor in activating caspase-8 occurred as in the previous report (LaVallee et al., 2003), it needs to be further illustrated. The intrinsic and extrinsic pathways of apoptosis have been reported to mediate via the generation of reactive oxygen species (ROS) (Liu et al., 2006). This might be the same phenomenon as in DMMA-treated apoptotic cells, which required further studies. Taken together, 6,8-dihydroxy-7-methoxy-1-methyl-azafluorenone induced human cancer cell apoptosis via both the caspase-8 and -9 pathways, which indicates the involvement of extrinsic death receptor and intrinsic mitochondrial pathways.

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