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# Apoptogenic effect of 7,8-diacetoxy-4-methylcoumarin and 7,8-diacetoxy-4-methylthiocoumarin in human lung adenocarcinoma cell line: Role of NF-κB, Akt, ROS and MAP kinase pathway

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ABSTRACT

Coumarin (1,2-benzopyrone) is a naturally occurring fragrant compound found in a variety of plants and spices. Coumarins have attracted intense interest in recent years because of their diverse pharmacological activities. This study examines the antioxidant coumarin 7,8-diacetoxy-4-methylcoumarin (DAMC) and its thiocoumarin derivative 7,8-diacetoxy-4-methylthiocoumarin (DAMTC) for their effect on human non-small cell lung cancer A549 cells. Here we show that both DAMC and DAMTC not only inhibited cell proliferation, but also induced apoptosis with an $IC_{50}$ of 160 μg/ml as confirmed by morphological examination, annexin-V assay and flow cytometric analysis. Interestingly, it was observed that these two coumarin compounds exhibited little cytotoxicity towards peripheral blood mononuclear cells but induced apoptosis in malignant cells. DAMC/DAMTC treatment also resulted in pronounced release of apoptogenic cytochrome *c* from mitochondria to cytosol, alteration of mitochondrial membrane potential ($\Delta\Psi_m$), and activation of caspase-9 and caspase-3. Although an increase in the levels of reactive oxygen species (ROS) was observed, pre-treatment with antioxidant showed no protective effect against DAMC/DAMTC-induced apoptosis. Results of present study suggest that downregulation of Bcl-xl, Cox-2 and mitogen activated protein kinase pathway and upregulation of p53, Akt and NF-κB pathway are involved in the underlying molecular mechanism of apoptosis induction by DAMC and DAMTC in A549 cells.



## 1. Introduction

Over one million new cases of lung cancer are diagnosed worldwide each year and lung cancer is the leading cause of cancer-related death in men and women, globally [1]. Despite intensive efforts to control lung cancer mortality with standard surgery, radiation and chemotherapy, the 5-year lung cancer patient survival rate of 7% in 1970 has only recently improved to <15% [2,3]. Thus lung cancers are a major clinical problem and there is high demand for new, more effective chemopreventive and therapeutic approaches.

Search for new chemopreventive and antitumor agents that are more effective but less toxic has kindled great interest in phytochemicals. Among phytochemicals, 4-methyl coumarin, having two hydroxyl (7,8-dihydroxy-4-methyl coumarin) or two acetoxy groups (7,8-diacetoxy-4-methylcoumarin) in the benzoid ring at positions ortho to each other, have been shown to have very strong antioxidant and radicals scavenging properties [4]. Several authors have reported that numerous coumarins and their derivatives show strong antiproliferative activity and induce apoptosis in various cancer cell lines [5–11]. All these studies strongly support the potential therapeutic applications of coumarins and their derivatives, making them attractive for their further evaluation as novel therapeutic agents for cancer treatment.

We have previously reported that 7,8-dihydroxy-4-methylcoumarin (DHMC) induces apoptosis of human lung adenocarcinoma cells by ROS-independent mitochondrial pathway through partial inhibition of ERK/MAPK signaling [12]. Earlier studies have shown that DHMC has a superb radical scavenging property but, it lacked the ability to inhibit P-450 linked MFO (mixed function

*Abbreviations:* DAMC, 7,8-diacetoxy-4-methylcoumarin; DAMTC, 7,8-diacetoxy-4-methyl thiocoumarin; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species; PBMC, peripheral blood mononuclear cells; DiOC6, 3,3′-dihexyloxacarbocyaniniodode; MMP ($\Delta\psi_m$), mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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oxidases) in vivo [4,13]. In another study, Raj et al. showed that 7,8-diacetoxy-4-methyl coumarin (DAMC) is more potent than 7,8-dihydroxy-4-methylcoumarin (DHMC, the deacetylated product of DAMC) as it exhibited a remarkable inhibitory potential on several liver microsomal P-450 linked MFO [14]. P-450 linked mixed function oxidases play a key role in the oxidative metabolism of xenobiotics. Previous report by Koshy et al. had investigated the pro-oxidant effect of 7,8-diacetoxy-4-methyl coumarin (DAMC) in two human tumor cell lines (MDA-MB-468, breast and U-87 MG, glioma) [15]. Although the study has shown that an increase in oxidative stress caused by the pro-oxidant action of DAMC is responsible for cell death, information on its anti-tumor properties and cellular mechanism remain limited. Since the search for new drugs requires a deep understanding of the molecular basis of drug action, we investigated the relationship between the inhibited growth of NSCLC (non-small cell lung carcinoma) cells by DAMC or DAMTC (thioderivative) and apoptotic pathways in A549 cells.

This is the first report showing that DAMC and DAMTC have antiproliferative effect on human non-small cell lung cancer A549 cells. DAMC and DAMTC were found to induce apoptosis by stimulating the release of cytochrome *c*, decreasing mitochondrial membrane potential, and by activating caspases. We also observed that these two coumarin compounds showed very little cytotoxicity towards quiescent PBMCs. In the present study, we demonstrated that DAMC/DAMTC induce mitochondria-mediated apoptosis in a ROS-independent manner through activation of Akt, NF-κB and downregulation of mitogen-activated protein kinase pathways in A549 lung cancer cells. Together, our data supports the possibility that DAMTC might be an effective cytotoxic drug as compared to DAMC and DHMC (previously reported by our group) for the treatment of lung adenocarcinoma.

## 2. Materials and methods

### 2.1. Reagents and antibodies

7,8-Diacetoxy-4-methyl coumarin (DAMC) was synthesized as described earlier [4]. The structure was established on the basis of the spectral ($^1$H, $^{13}$C NMR and mass) data and melting points. 7,8-Diacetoxy-4-methyl thiocoumarin (DAMTC) was prepared by thionation as described [16]. DAMC and DAMTC were dissolved at a concentration of 10 mg/ml in DMSO and stored at −20 °C.

DMEM, DMSO, *N*-acetyl cysteine (NAC), MTT, trypsin, carbonyl cyanide *m*-chlorophenylhydrazone (mClCCP; a mitochondrial uncoupler), dihydroethidium (DHE), 3,3′-dihexyloxacarbocyanine iodide (DiOC6), propidium iodide, TNF-α, hepes, DTT, protease inhibitor cocktail, BCIP, NBT, Ficoll-Hypaque, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), U0126 (inhibitor of MEK1/2), SP60015 (inhibitor of JNK) and SB203580 (inhibitor of p38) were purchased from Sigma (St. Louis, Missouri, USA).

BD Apoalert Cell Fractionation Kit, Caspase-3 Assay Kit and Caspase-9 Assay Kits were purchased from BD Biosciences Pharmingen, San Diego, CA, USA. DeadEnd colorimetric TUNEL assay kit, Luciferase Assay Kit and Caspase-8 Assay Kit were obtained from Promega, Madison, WI, USA. Guava Via Count, Guava Nexin Kit and Multicaspase Assay Kit was purchased from Guava Technologies (Hayward, CA, USA). Cell line nucleofactor kit V was from Amaxa Biosystems, Gaithersburg, MD, USA. Antibodies Bcl-xl, Bax, p53, Cox-2, PARP, Akt, phospho-Akt, phospho-Erk1/Erk2, phospho-p38 MAPK, phospho-JNK were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β-Actin antibody was purchased from Sigma (St. Louis, Missouri, USA).

### 2.2. Cell culture

A549 (NSCLC, lung adenocarcinoma), HepG2 (hepatocellular adenocarcinoma), HeLa (cervical carcinoma), H520 (lung carcinoma), IMR-90 (normal lung) and WI-38 (normal lung) cells were maintained in DMEM medium containing 10% (v/v) fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin in a humidified 5% $CO_2$ atmosphere. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured in our lab as described earlier [16]. Logarithmically growing cells were used for treatment with DAMC/DAMTC. Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by density gradient centrifugation using Ficoll-Hypaque. The initial density for PBMC was $1 \times 10^6$ cells/ml.

### 2.3. Cytotoxicity assay

The viability of cell lines were determined using the MTT [3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyltetrazolium bromide] assay. Cells were treated for 24 h with DAMC/DAMTC in quadruplicates at various concentrations (0–200 μg/ml). 0.1% DMSO treated cells were taken as vehicle controls. Following treatment, MTT (0.5 mg/ml) was added for 4 h at 37 °C in dark. Formazan crystals formed were dissolved with DMSO. The absorbance was measured at 570 nm in a plate reader. Cell viability was calculated by following formula: %cell viability = (mean absorbance in test wells)/(mean absorbance in control well) × 100. The concentration of DAMC/DAMTC to be used further was chosen from a dose-response experiment showing moderate toxicity to the cells. We used DAMC and DAMTC at a concentration of 160 μg/ml for our experiments. Duration of treatment was kept constant at 24 h unless otherwise stated. In some experiments, cell viability was also calculated with Guava Via Count using Guava Flow Cytometer. Guava Via Count analyzes the fluorescence of cells stained with the Via Count reagent and quantitates the number of viable and non-viable cells in each sample. The Via Count reagent contains DNA-binding dyes and stains viable and non-viable cells in a different way corresponding to their permeability to this reagent. Stained nucleated events are counted and the forward scatter properties are used to distinguish free nuclei and cellular debris from cells to determine an accurate cell count. Guava Via Count gives more realistic assessment of viability and is highly reproducible.

### 2.4. Measurement of apoptosis

#### 2.4.1. Annexin-V assay

Surface exposure of phosphatidylserine in apoptotic cells was measured by Guava Nexin Kit according to the manufacturers' protocol (Guava Technologies, Hayward, CA, USA). Annexin-PE fluorescence was analyzed by cytosoft software (Guava Technologies, Hayward, CA, USA).

In some experiments, NAC (1 mM), U0126 (20 μM) or SP60015 (10 μM) or SB203580 (10 μM) or an equivalent volume of DMSO (vehicle control) was added to the culture medium 1 h prior to the treatment. The cells were then treated with DAMC/DAMTC (160 μg/ml) for 24 h in the presence of NAC or 2, 12 and 24 h in the presence of other drugs and taken for analysis.

#### 2.4.2. TUNEL assay

Apoptotic cells were visualized by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling technique using the Dead End Colorimetric TUNEL System as described before [17].