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7,8-Dihydroxy-4-methylcoumarin induces apoptosis of human lung adenocarcinoma cells by ROS-independent mitochondrial pathway through partial inhibition of ERK/MAPK signaling

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Abstract **Coumarins have attracted intense interest in recent years because they have been identified from natural sources, especially green plants and have diverse pharmacological properties. In this study, we investigated whether 7,8-dihydroxy-4-methylcoumarin (DHMC) caused apoptosis in A549 human non-small cell lung carcinoma cells (NSCLC) and, if so, by what mechanisms. Here, we show that, in A549 human NSCLC cells, DHMC induces apoptosis through mitochondria-mediated caspase-dependent pathway. Although an increase in the levels of reactive oxygen species (ROS) was observed, pre-treatment with antioxidant showed no protective effect against DHMC-induced apoptosis. In addition, our immunoblot data revealed that DHMC treatment led to down-regulation of Bcl-xl, Bax, p21, Cox-2, p53 and upregulation of c-Myc. Results in the present study for the first time suggest that DHMC induces apoptosis in human lung A549 cells through partial inhibition of ERK/MAPK signaling.**

Keywords: Apoptosis; Reactive oxygen species; Lung cancer; Mitochondria; ERK; Caspases

1. Introduction

Lung cancer is the leading cause of male and female cancer deaths worldwide and its incidence is increasing everyday. The conventional treatment of lung cancer includes surgery, radiation and chemotherapy. Various chemotherapy drugs, including doxorubicin, 5-fluorouracil, cisplatin, etoposide and gemcitabine, have been used to treat lung cancer [1–3]. Unfortunately, all of these anticancer drugs affect not only pathological tumour cells, but also normal cells, especially bone marrow cells or intestinal epithelia with high turnover rate, causing serious complications and toxicity. Although surgical resection together with chemotherapy offers the best hope for a cure, the 5-year survival rate remains poor (<15%), even in patients with earlier stages of the disease [4,5]. As each of these therapies have limitations, hence new strategies are needed to improve the treatment efficacy of this fatal disease. Our main thrust is on identification of those bioactive molecules which have the greatest potential for development into new therapeutic products and to understand the mechanisms of action of those bioactive molecules at the molecular and cellular levels.

Coumarin is found in a variety of plants such as tonka bean, lavender, sweet clover grass, and licorice, and also occurs in food plants such as strawberries, apricots, cherries, and cinnamon. Coumarins and related compounds have been shown to posses many biological activities such as antithrombotic [6], antimicrobial activity [7], anti-tumorigenic [8–11]. Coumarin derivatives have also been evaluated in treatment of human immuno-deficiency virus, due to their ability to inhibit human immunodeficiency virus integrase [12].

Earlier Koshy et al. [13] have studied the pro-oxidant action of 7,8-diacetoxy-4-methylcoumarin (DAMC) and DHMC on MDA-MB-468 and U-87 MG cell lines but till date, there are no reports on the cytostatic and apoptotic actions of DHMC in A549 human NSCLC cells. Thus the main focus of this study was to explore the potential of the coumarin compound, DHMC in view of apoptosis in NSCLC cells.

Apoptosis is defined as an active physiological process of cell self-destruction with specific morphological and biochemical changes in the nucleus and cytoplasm [14,15]. Agents that suppress the proliferation of malignant cells, by inducing apoptosis may represent a useful mechanistic approach to both chemoprevention and chemotherapy of cancer. In this paper, for the first time, we examined the cytotoxic effect of DHMC in A549 human NSCLC cells. Herein, we showed that DHMC induces apoptosis of A549 human NSCLC cells via mitochondria-mediated mechanism with activation of caspase-9, -3 and release of mitochondrial cytochrome *c*. In addition our findings also suggest that apoptosis induced by DHMC was reactive oxygen species (ROS) independent and occurs by downregulation of Bcl-xl, Bax, p21, p53, Cox-2, ERK/MAPK and upregulation of c-Myc. The results indicate that DHMC may provide a useful new therapeutic strategy for lung adenocarcinoma.

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Abbreviations: DHMC, 7,8-dihydroxy-4-methylcoumarin; DCFH-DA, dichlorodihydrofluorescein diacetate; HE, hydro ethidine; ROS, reactive oxygen species; Asc, ascorbic acid; DiOC6, 3,3′dihexyloxacarbocyaniniodode; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); MMP ($\Delta\psi_m$), mitochondrial membrane potential; ERK, extracellular signal-regulated kinase

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2. Materials and methods

2.1. Test compounds and reagents

DMEM, penicillin/streptomycin was obtained from GIBCO BRL Life Technologies, Rockville, MD, USA. Dimethyl sulfoxide (DMSO), MEK1/2 inhibitor (U0126), ascorbic acid, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), trypsin, carbonyl cyanide *m*-chlorophenylhydrazone, hydro ethidine (HE), 3,3′dihexyloxacarbocyaniniodode (DiOC6), dichlorodihydrofluorescein diacetate (DCFH-DA), anti-c-Myc and Histopaque were purchased from Sigma (St. Louis, MO, USA). DC protein assay was from Bio-Rad Laboratories, Hercules, CA, USA.

Fetal calf serum was purchased from Biological Industries, Kibbutz Beit Haemek, Israel. DHMC was synthesized as detailed earlier and its purity has been established on the basis of the spectral (^{1}H, ^{13}C NMR and mass) data and its melting point [16].

Annexin V-FITC kit, Apoalert Cell fractionation kit, caspase-3 and caspse-9 activity kits and anti-Cox-2 were from BD Biosciences Pharmingen, San Diego, CA, USA. DeadEnd Colorimetric TUNEL System and caspase-Glow 8 assay kit were from Promega, Madison, WI, USA. Anti-Bcl-xl, anti-Bax, anti ERK/MAPK, anti-p-ERK/MAPK, anti-p21, and anti-p53 were from Santa Cruz Biotechnology, CA, USA. Anti-GAPDH and anti-β actin were from Imgenex, San Diego, CA, USA.

2.2. Cell culture

A549 (NSCLC, lung adenocarcinoma), HepG2 (hepatocellular adenocarcinoma), Hela (cervical carcinoma) and Hep2 (laryngeal carcinoma) cells were obtained from National Center for Cell Science, Pune, India and maintained in DMEM medium containing 10% (v/v) fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere. Logarithmically growing cells were used in all experiments. DHMC stock solutions were made up in DMSO and stored at −20 °C. Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by density gradient centrifugation using Histopaque. The initial cell density for PBMC was 1×10^6 cells/ml.

Where indicated, flow cytometric analysis and Western blot analysis (described below) were done using the MEK1/2 inhibitor. U0126 (20 μM) in DMSO or an equivalent volume of DMSO as a control, was added to the culture medium 30 min prior to the treatment. The cells were then treated with DHMC (160 μg/ml) for 2, 4, 8, 12 and 24 h and taken for analysis.

2.3. Cell viability assay

A549, HepG2, Hela and Hep2 cells were grown in 96-well microtiter plates and treated with different concentrations (0–200 μg/ml) of DHMC in triplicates. As controls, 0.1% DMSO treated cells (vehicle) were included in each experiment. One hundred microliters of MTT (5 mg/ml) was added to each well followed by incubation for 4 h at 37 °C. The formazan precipitate was dissolved in 100 μl DMSO and the absorbance was measured at 570 nm using an ELISA reader [17].

2.4. Flow cytometric analysis for cell cycle and apoptosis

For analysis of cell cycle distribution, 1×10^6 cells were harvested by centrifugation, washed in phosphate-buffered saline (PBS), fixed with ice-cold 70% ethanol and treated with 1 mg/ml RNAse for 30 min. Intracellular DNA was labeled with propidium iodide (50 μg/ml) and analyzed using flow cytometer (FACS Vantage Becton Dickenson, CA, USA). Data were collected, stored and analyzed using CellQuest™ software (Becton Dickinson, CA, USA). Surface exposure of phosphatidylserine in apoptotic cells was measured by Annexin V-FITC/PI apoptosis detection kit I. FITC fluorescence was analyzed by CellQuest™ software (Becton Dickinson, CA, USA).

2.5. TUNEL assay

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

2.6. Preparation of cell lysates and immunoblot analysis

Vehicle treated and DHMC treated cells were harvested after 24 h and lysed in ice cold RIPA lysis buffer (Tris–HCl: 50 mM, pH 7.4 , NP-40: 1% , Na-deoxycholate: 0.25%, NaCl: 150 mM, EDTA: 1 mM, PMSF: 1 mM, aprotinin 1 μg/ml, leupeptin 1 μg/ml and pepstatin: 1 μg/ml, Na_3VO_4: 1 mM, NaF: 1 mM). The protein content in the lysates was measured by DC protein assay. Protein (50–60 μg) was resolved on 12–15% SDS–PAGE and electroblotted onto PVDF membrane. The membrane was then incubated in 3% BSA for 2 h followed by overnight incubation with respective antibodies. After washing, corresponding secondary antibody (horseradish peroxidase conjugate or alkaline phosphatase conjugate) was added and incubated for 2 h. After washing, the blots were developed either with NBT-BCIP or DAB. Blots were scanned by light densitometry using AlphaImager 3400 (Alpha InnoTech Corporation, San Leandro, CA). The AlphaImager was then used to calculate the band intensities in each blot from the digital picture of the membrane. Relative protein expression was quantitated by alpha imager 3400 and normalized using β actin or GAPDH [17].

2.7. Detection of cytochrome c release

Cytosolic and mitochondrial extracts were prepared as described [17]. In brief, cell pellets were homogenized in buffer (250 mM sucrose, 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride). The cytosolic and mitochondrial fraction was collected by centrifugation (15000 rpm for 30 min at 4 °C) and subjected to immunoblot analysis with a monoclonal antibody to cytochrome *c*.

2.8. Measurement of caspase-3, -8 and -9 activities

Caspase-3 activity was determined as described [17]. Briefly, after 24 h of treatment, cells were lysed in lysis buffer (10 mM Tris–HCl; 10 mM NaH_2PO_4/Na_2HPO_4 pH 7.5; 130 mM NaCl; 1% Triton®-X-100; 10 mM sodium pyrophosphate). 100 μg of total protein was incubated with a synthetic tetrapeptide fluorogenic substrate Ac-DEVD-AMC in protease assay buffer (20 mM HEPES (pH 7.5); 10% glycerol; 2 mM DTT). Fluorescence of the released AMC was measured spectrofluorometrically using an excitation wavelength of 380 nm and an emission wavelength range of 420–460 nm as described [17]. One unit is defined as the amount of enzyme required to release 1 pmol of AMC per min at 37 °C.

For caspase-9 assay, cells were lysed in the similar manner. Total protein (100 μg) was incubated with the colorimetric caspase-9 substrate LEHD-pNA in an assay buffer supplied in the kit (BD Biosciences Pharmingen, San Diego, CA, USA). The release of the chromophore *P*-nitroanilide was quantitated by spectrophotometry at a wavelength of 405 nm. The background controls (reactions where no cell lysate or where no colorimetric substrate was added) were subtracted from the experimental results before calculating the mean absorbance.

Caspase 8 activity was measured by the ''Caspase-Glow 8 assay kit'' as described by the manufacturers (Promega). The assay provides a proluminogenic caspase-8 substrate in a buffer system optimized for caspase activity, luciferase activity and cell lysis (supplied with the kit). The signal generated is proportional to the amount of caspase activity present.

2.9. Detection of intracellular reactive oxygen species generation and mitochondrial membrane potential ($\Delta\psi_m$)

It is well documented that HE and DCFH-DA have been used frequently to monitor H_2O_2 and hydroxyl radical levels in cells [18]. The cells were incubated with or without DHMC (160 μg/ml) for 24 h and were then treated with 10 μM HE or 5 μM DCFH-DA 30 min prior to harvesting. After incubation cells were washed with PBS and analyzed by flow cytometry [18].

In order to measure $\Delta\psi_m$, 4×10^5 cells were labeled with the fluorochrome DiOC6, 40 nM for 30 min prior to harvesting and incubated at 37 °C for 30 min in the dark. After incubation with the fluorochrome, cells were immediately analyzed by flow cytometry in order to measure fluorochrome incorporation.

2.10. Statistical analysis

This was performed with student's two tailed *t*-test using SPSS (windows version 7.5), values of $P < 0.05$ were considered statistically significant.

3. Results

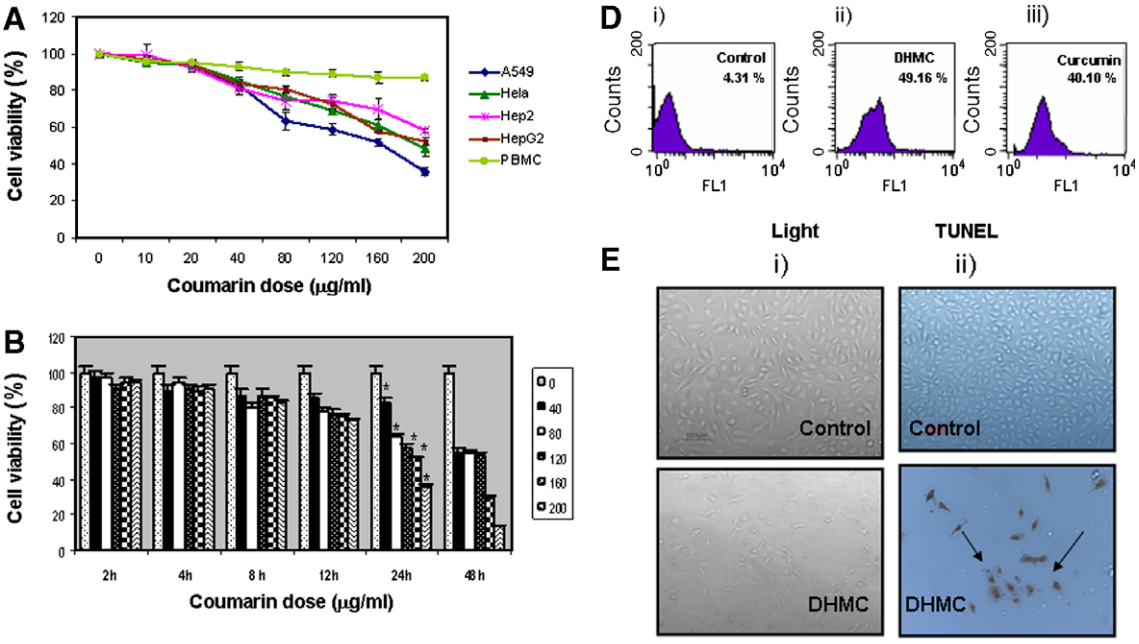
3.1. DHMC reduced cell viability of A549 cells but not of normal peripheral blood mononuclear cells

First, to examine the cytotoxic effect of DHMC on A549, HepG2, Hela and Hep2, cells were treated with different doses and cell viability was measured using MTT assay. As shown in Fig. 1A, 24 h treatment, the cytotoxic effects were dose related in each of the cell lines tested. IC_{50} value for A549 was 160 μg/ml, for Hela IC_{50} was 180 μg/ml, for Hep2 and HepG2, $IC_{50} > 200$ μg/ml. IC_{50} was lowest in A549 cells which was similar to other coumarins reported in the literature [19,20]. Interestingly, the IC_{50} concentration for A549 cells did not appear to have inhibitory effects on PBMCs. The lowest possible non-toxic dosage of 160 μg/ml was fixed for DHMC and further experiments were carried out on A549 cells exposed to 160 μg/ml.

To further assess the cytotoxic effect we did time–course experiment. In A549 cells, till 8 h exposure of DHMC, no obvious effect could be found regardless of the dose of DHMC (Fig. 1B). However, a trend of decrease of cell viability as the dose of DHMC increased was observed after 12 h exposure (Fig. 1B), a significant decrease in cell viability was observed in a dose dependent manner following 24 h exposure (Fig. 1B). According to above results, exposure of DHMC at 160 μg/ml for 24 h induced much more significant cytotoxic effect on A549 cells, thus these conditions were adopted in the following experiments to investigate the mechanism related to the DHMC induced cytotoxicity.

3.2. DHMC induces apoptotic cell death in A549 cells

Next, to investigate whether DHMC-induced cell death was due to apoptosis, FACS analysis was performed. As shown in Fig. 1C, treatment of A549 cells with DHMC resulted in marked increase in accumulation of sub-G1 phase cells, i.e., 40.76 ± 1.4% in DHMC as compared to 11.59 ± 1.7% in vehicle treated cells thereby suggesting apoptotic cell death. DHMC treatment showed that 43.41 ± 5% cells were in G0/G1 phase as compared to 57.69 ± 2% cells in vehicle treated cells. The percentage of cells in G2/M phase was decreased from 10.39 ± 2% in vehicle treated cells to 4.21 ± 5% in cells treated with DHMC (160 μg/ml) after 24 h of exposure. We also observed decrease in the proportion of cells in S phase from 4.3 ± 2% in vehicle treated cells to 2.12 ± 1% in cells



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	Percentage of cells ± S.E.M			
	Apoptotic / subG1	G0/G1	S	G2/M
Control	11.59 ± 1.7	57.69± 2	4.30 ± 2	10.39 ±2
DHMC	40.76 ± 1.4*	43.41± 5*	2.12± 1*	4.21± 5*

Fig. 1. Induction of apoptosis by DHMC. (A) Cytotoxicity evaluated by MTT assay after exposure of A549, Hela, Hep2, HepG2 and PBMCs cells with increasing concentrations of DHMC for 24 h. Each point is the mean of three replicates; bars represent the SEM. (B) Dose and time dependent response of A549 cells as evaluated by MTT assay. $^*P < 0.01$ vs vehicle control. (C) Cell cycle distribution of A549 cells with 0.1% DMSO (vehicle-treated group) or 160 μg/ml of DHMC. Each data represent means ± S.E.M. from three independent experiments. $^*P < 0.01$ vs vehicle control. (D) AnnexinV-FITC binding in A549 cells. (i) Vehicle treated control, (ii) cells treated with 160 μg/ml of DHMC for 24 h, (iii) cells treated with 60 μM curcumin for 24 h (positive control). (E) Left panel shows the representative morphology of A549 cells when exposed to 0.1% DMSO or 160 μg/ml of DHMC for 24 h under light microscope and right panel shows the TUNEL staining of A549 cells when exposed to 0.1% DMSO or 160 μg/ml of DHMC for 24 h.

treated with DHMC (160 μg/ml) after 24 h of exposure. These results demonstrate that DHMC decreases cell viability by inducing apoptosis.

For further confirmation annexin V-FITC assay was performed. As shown in Fig. 1D, the percentage of apoptotic cells increased to 49.16% in DHMC treated A549 cells after treatment with 160 μg/ml of DHMC dose as compared to 4.31 ± 1.2% in vehicle treated cells. These results show quantitatively and qualitatively that DHMC induces apoptosis in A549 cells. The results of annexin V were also validated with a positive control, curcumin (60 μM). Additionally, we also did TUNEL assay. In Fig. 1E, left panel shows the representative morphology and right panel shows the TUNEL assay of vehicle treated and DHMC treated A549 cells. Under light microscope, vehicle treated A549 cells appeared to have normal features with round and homogenous nuclei while DHMC treated cells were somewhat smaller than control cells in cellular size and exhibited the characteristic feature of apoptosis such as the appearance of the apoptotic bodies. Treatment with 160 μg/ml of DHMC for 24 h showed number of TUNEL-positive cells as compared to vehicle treated A549 cells thereby indicating that DHMC treatment induces apoptosis in A549 cells.

3.3. DHMC induces apoptosis in A549 cells through caspase-9 and caspase-3

For a direct examination of the involvement of caspases in DHMC-induced apoptosis, activity of caspase-3, -8 and -9 was examined using fluorogenic, luminogenic and colorimetric substrates. Results shown in Fig. 2 demonstrate that DHMC treatment increased the activity of caspase-3 by 3-fold ($P = 0.004$) and caspase-9 activity by 1.49-fold ($P < 0.05$) as compared to vehicle treated cells. Interestingly, caspase-8 activity in vehicle treated and DHMC treated cells remained unaffected. Increased caspase-8 activity was observed in cells treated with TNFα (positive control data not shown).

3.4. DHMC induces apoptosis in A549 cells by mitochondrial pathway

To look for the mitochondrial involvement, disruption of mitochondrial membrane potential ($\Delta\psi_m$), generation of ROS and release of mitochondrial cytochrome *c* in A549 cells was investigated by flow cytometry and immunoblotting. As indicated in Fig. 3A, as compared to controls, approximately 45% cells shifted towards left thereby indicating that the DHMC treatment induced a disruption of $\Delta\psi_m$. In contrast, administration of DHMC resulted in a right shift of the DCFH-DA and HE fluorescence curves (48% cells shifted towards right as compared to vehicle treated controls), indicating the increase in ROS generation.

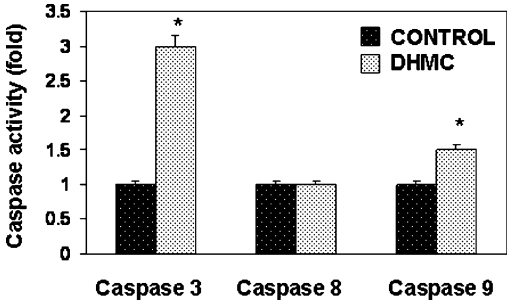

Fig. 2. Involvement of caspase activation in DHMC induced apoptosis. A549 cells were treated with 160 μg/ml DHMC for 24 h. Extracts from vehicle-treated (0.1% DMSO) or DHMC treated cells were assayed for caspase-3 activity using a synthetic tetrapeptide fluorogenic substrate Ac-DEVD-AMC; caspase-9 activity using colorimetric caspase-9 substrate LEHD-pNA; caspase-8 activity using a luminogenic substrate using LETD sequence. Each data represent means ± S.E.M. from four independent experiments. $^*P < 0.01$ compared to vehicle control.

Next cytochrome *c* was detected in the cytoplasmic and mitochondrial fraction by immunoblot analysis. As shown in Fig. 3B (left panel), the relative density of cytochrome *c* in the cytosol of the cells treated with DHMC (24 h) was increased by 1.18-fold ($P = 0.020$) as compared to the vehicle treated cells. Significant amount of cytochrome *c* was present in the mitochondria in vehicle-treated cells. Fig. 3B, right panel shows the immunoblot for COX IV (Mitochondrial Loading Control). Loading was also normalized using β-actin antibody. These data suggest that DHMC treatment causes cytochrome *c* release from mitochondria.

3.5. DHMC induced apoptosis in A549 cells cannot be reversed by antioxidants

Since we observed an increase in ROS generation during DHMC treatment of cells, we examined the cell viability and percentage apoptosis in vehicle treated and DHMC-treated cells in the presence and absence of antioxidants. As shown in Fig. 4A, pre-treatment with ascorbic acid (Asc, 300 μM) for 2 h did not show any significant change on the viability of DHMC-treated cells (compare columns 2 and 3). Similarly, no protective effect against DHMC-induced apoptosis was observed by annexin V-FITC assay (Fig. 4B, compare columns 2 and 3). Similar findings were also observed when we pre-treated the cells with *N*-acetyl cysteine (NAC, from 0.3 mM to 1 mM) (data not shown). These data suggest that DHMC-induced apoptosis is independent of ROS production in A549 cells and oxidative stress may not be playing any role in DHMC induced apoptosis.

3.6. DHMC induces apoptosis by modulating apoptosis related proteins

To gain further insights into the molecular events associated with DHMC-induced apoptosis, the expression of apoptosis-related and survival-related proteins was detected by immunoblotting. Results of immunoblot analysis (Fig. 5A and B) in this study showed that 24 h treatment with DHMC decreased the expression of Bcl-xl by 1.54-fold ($P = 0.025$), Bax by 1.72-fold ($P = 0.014$), p53 by 1.34-fold ($P = 0.020$), Cox-2 by 1.37-fold ($P = 0.025$) and p21 by 1.27-fold ($P = 0.017$) as compared to vehicle treated cells. We also observed that 24 h treatment with DHMC increased the expression of c-Myc by 1.7-fold ($P = 0.031$), as compared to vehicle treated cells.

Next, we investigated ERK/MAPK signaling in A549 cells in response to DHMC. Our time–course experiment (Fig. 5C) for immunoblot analysis of equal amounts of protein from vehicle treated and DHMC treated A549 cells shows significant decrease of p-ERK at 12 h (1.2-fold decrease, $P = 0.012$) and 24 h (1.6-fold decrease, $P = 0.018$), respectively. Equal protein amount in different lysates was ascertained by immunoblotting of parallel blot with β-actin antibody. Because the activation of the ERK/MAPK pathway has been associated with anti-

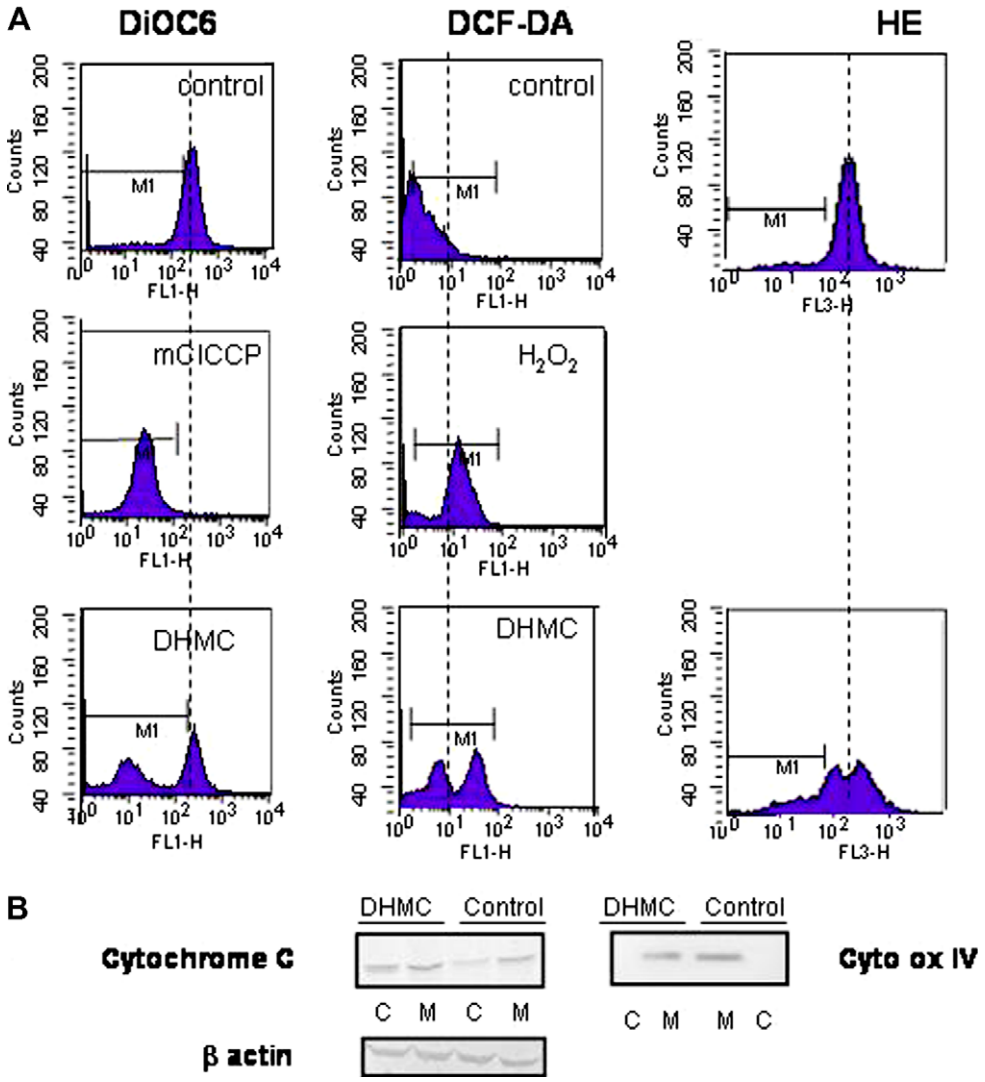

Fig. 3. A549 cells were vehicle-treated (0.1% DMSO) or treated with 160 μg/ml DHMC for 24 h. (A) $\Delta\psi_m$ and ROS generation. Thirty minutes prior to harvesting, cells were incubated with 40 nM DiOC6 or 5 μM DCFH-DA or 10 μM HE. After incubation, cells were harvested, and change in fluorescence was measured using flow cytometry. The mitochondrial uncoupler cyanide *m*-chlorophenylhydrazone (mClCCP, 50 μM) is used routinely for validation of measurements. (B) Mitochondrial cytochrome *c* release. Cytosolic fraction was isolated and the content of cytochrome *c* was examined by immunoblot analysis using anti-cytochrome *c* antibody. Cytochrome *c* oxidase subunit IV is used as a loading control and excludes mitochondrial contamination in the cytosol. The data shown are the representatives of three independent experiments. *P* value = 0.020.

apoptotic effects, we examined the possibility that inhibitors of this pathway could modulate sensitivity to the drug. To verify this hypothesis, we used U0126, a specific inhibitor of MEK1/2 [21]. U0126 caused substantial inhibition of ERK1/2 phosphorylation from 2 h (data not shown) to 24 h after treatment (Fig. 5C). Total ERK1/2 protein levels were not decreased in U0126-treated compared with control lysates (Fig. 5C). Our time–course experiment (Fig. 5D) of U0126 revealed that the U0126 treatment (20 μM) increased some basal apoptosis, and potentiated the apoptotic effect of DHMC. U0126 treatment induced 5.3% apoptosis at 2 h, 8.2% apoptosis at 4 h, 10.45% apoptosis at 8 h, 15.7% apoptosis at 12 h and 16.8% apoptosis at 24 h, respectively. DHMC alone showed 6% apoptosis at 2 h, 10% apoptosis at 4 h, 15% apoptosis at 8 h, 25% apoptosis at 12 h and 35% apoptosis at 24 h. Pretreatment of A549 cells with U0126 prior to DHMC treatment showed 70% apoptosis at 2 h and this effect was more or less consistent till 24 h (75.2%). Our results suggest that ERK1/2 inactivation by U0126 lowered the apoptotic threshold of A549 cells for DHMC as is evident by increased percentage of apoptosis (70–75.2%) even at earlier time points (2, 4, and 8 h) as compared to DHMC alone (6–15.7%).

4. Discussion

Search for new chemopreventive and antitumor agents that are more effective but less toxic has kindled great interest in phytochemicals. Coumarin is one such compound which was used in this study. No adverse effects of coumarin have been reported in humans using doses up to 7 g daily, after two weeks of continued treatment [22]. The main focus of this study was to explore the potential of coumarin compound, DHMC in relation to apoptosis.

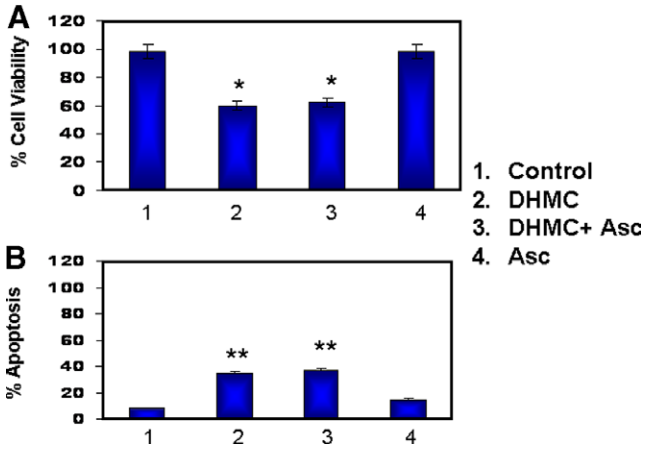

Fig. 4. A549 cells were pretreated with 300 μM of ascorbic acid for 2 h and then treated with vehicle (0.1% DMSO) or with 160 μg/ml DHMC for 24 h. (A) Cell viability was analyzed using MTT assay. (B) Annexin V-FITC assay was used to estimate percentage apoptosis. Columns: (1) Control A549 cells, (2) DHMC treated Cells, (3) DHMC + Asc (pretreated for 2 h) (4) Asc. $^{*}P < 0.01$ and $^{**}P < 0.001$ compared to vehicle control.

This study clearly and convincingly show for the first time that DHMC induced A549 cell apoptosis in a dose-dependent and time-dependent manner. More importantly, in our study PBMC showed very less sensitivity to DHMC at various concentrations (0–200 μg/ml). Since our observations with PBMC exposed to high concentrations of the coumarins compounds showed no cell death, induction of apoptosis by DHMC appears to be selective for cancer cells.

Our results indicate that DHMC-induced apoptosis in A549 human NSCLC cells was accompanied by down-regulation of Bcl-xl (anti-apoptotic), decrease of the mitochondrial membrane potential thus leading to cytochrome *c* release from the mitochondria and activating caspase-9 and -3. In conjunction to our findings, it has been noticed in many in vitro systems that apoptosis is associated with downregulation of Bcl-xl, loss of mitochondrial membrane potential, which may correspond to the opening of an outer membrane pore (permeability transition pore) and this event has been suggested to be responsible for cytochrome c release into cytosol from mitochondria [23–30]. Further in our study there appears a direct activation of the mitochondrial pathway without involvement of caspase-8. There are reports similar to our findings in the literature [31,32]. The present study also provides the evidence that DHMC induces apoptosis through ROS independent pathway since no protective effect against DHMC-induced apoptosis was observed in presence of antioxidant as assessed by annexin V-FITC assay and MTT assay. Consistent to our findings, Ko et al. [33] reported that apoptosis induced by myricetin occurs through mitochondria -dependent, ROS-independent pathway.

Apoptosis is a complex phenomenon where several genes are involved in the regulation of apoptosis. Therefore in order to better understand the molecular basis of DHMC-induced apoptosis, the expression of p53, p21, Bax, ERK/MAPK,

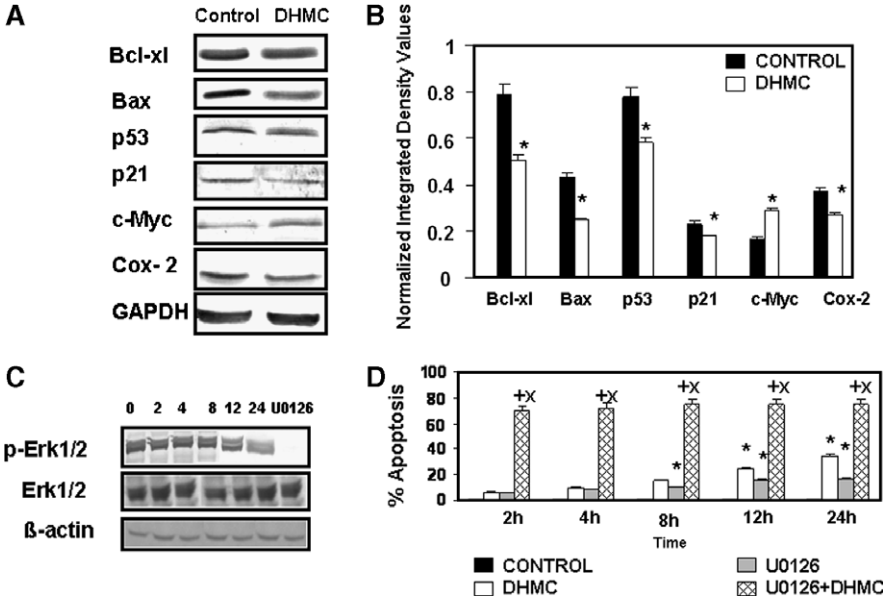

Fig. 5. (A) Effect of DHMC on the protein expression of Bcl-xl, Bax, p53, p21, c-Myc, and Cox-2 in A549 cells. 2.5×10^5 cells/well of 6-well plate were treated with vehicle (0.1% DMSO) or with 160 μg/ml DHMC for 24 h. Total cell lysates were prepared and protein (50 μg) was subjected to SDS–PAGE followed by immunoblot analysis using specific antibodies and secondary horseradish peroxidase-conjugated or alkaline phosphatase conjugated antibodies. Details are described in Section 2. GAPDH was used as internal control to ensure that equal amounts of proteins were loaded in each lane. Data are representative of a typical experiment repeated three times with similar results. (B) Densitometric analysis of immunoblot. The data shown are the representatives of three independent experiments. $^{*}P < 0.01$ compared to vehicle control. (C) Effect of treatment of A549 cells with DHMC and MEK1/2 inhibitor U0126 on ERK activation. Cells were treated with 160 μg/ml of DHMC for different time points. The cell lysates (60 μg of total protein) were separated by electrophoresis, transferred to PVDF membrane and probed with anti-p-ERK1/2 and anti-ERK1/2 antibody. Protein contents were normalized using β-actin. Data are representative of a typical experiment repeated three times with similar results. (D) Time–course for induction of apoptosis in presence or absence of DHMC and MEK1/2 inhibitor U0126. The induction of apoptosis was estimated by Annexin V-FITC/PI apoptosis detection kit I (BD Biosciences Pharmingen) and results were analyzed using CellQuest™ software (Becton Dickinson). $P < 0.05$. (*) vs vehicle control, (+) vs DHMC, (×) vs U0126.

Cox-2 and c-Myc was investigated by immunoblot analysis. p53 is a transcription factor that can induce either cell growth arrest or apoptosis. There are reports in the literature which show that apoptosis can occur in p53 dependent or independent manner by regulating its target genes through binding to DNA consensus sequence and activating the promoters of its downstream genes (p21 and Bax) [34,35]. In our study, we observed that treatment of A549 cells with DHMC leads to down regulation of p53 levels. Since in our results we also observed down regulation of Bax and p21 after DHMC treatment it seems that in this system apoptosis occurs by p53 dependent pathway. Next, we propose that in our study c-Myc might be playing a decisive role in the apoptosis induced by DHMC either by acting as sensitizer/or apoptosis inducer as our immunoblot results show that DHMC induces apoptosis by upregulating c-Myc protein. Since, we also observed cytochrome *c* release and increased c-Myc expression; this could be one of the mechanisms of DHMC induced apoptosis. There are reports similar to our findings in the literature [36]. Nevertheless, further study is needed to elucidate the precise role of c-Myc and p53 in DHMC-induced apoptosis. In the present study, we also found that DHMC induces apoptosis by ERK1/2 reduction and by down regulation of Cox-2. These results are in accordance with the well known concept that inhibition of MAPKs is likely to result in suppression of inflammatory mediators, which in turn leads to the desired therapeutic effects [37].

In conclusion, mitochondria-dependent ROS-independent apoptosis indicated by sequential events including reduction of the mitochondrial potential, inhibition of Bcl-xl expression, cytochrome *c* release from mitochondria to the cytosol, and activation of caspase-9 and -3 enzymes was identified in the present study. Results presented in this report also demonstrate that DHMC induced apoptosis of human A549 cells may be associated with partial inhibition of ERK/MAPK signaling thereby suggesting a crosstalk between the two pathways. We believe that our data should contribute to the development of DHMC or related drugs as potential cancer chemotherapeutic or chemopreventive agents for the treatment of NSCLC. However, future in vivo animal studies should help determine its therapeutic efficacy in treatment of NSCLC which are otherwise resistant to chemotherapy.

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