2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone, from buds of *Cleistocalyx operculatus*, induces apoptosis in human hepatoma SMMC-7721 cells through a reactive oxygen species-dependent mechanism

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Abstract Nowadays, much effort is being devoted to detect new substances that not only significantly induce the death of tumor cells, but also have little side effect on normal cells. Our previous study showed that 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (DMC) exhibited significant cytotoxic potential with an IC_{50} value of $32.3 \pm 1.13 \mu M$ against SMMC-7721 cells and could induce SMMC-7721 cells apoptosis. In the present study, we found that DMC was almost nontoxic to human normal liver L-02 and human normal fetal lung fibroblast HFL-1 cells as their IC_{50} values (111.0 ± 4.57 and $152.0 \pm 4.83 \mu M$ for L-02 and HFL-1 cells, respectively) were much higher. To further explore the apoptotic mechanism of DMC, we investigated the role of the reactive oxygen species (ROS) in the apoptosis induced by DMC in SMMC-7721 cells. Our results suggested that the cytotoxicity and the generation of intracellular ROS were inhibited by *N*-acetylcysteine (NAC). Reversal of apoptosis in NAC pretreated cells indicated the involvement of ROS in DMC-induced apoptosis. The loss of mitochondrial membrane potential ($\Delta\Psi_m$) induced by DMC was significantly blocked by NAC. NAC also prevented the decrease of Caspase-3 and -9 activities, the increase of Bcl-2 protein expression and the

decrease of p53 and PUMA protein expressions. Together, these results indicated that ROS played a key role in the apoptosis induced by DMC in human hepatoma SMMC-7721 cells.

Keywords *Cleistocalyx operculatus* · Flavonoids · Apoptosis · NAC · Reactive oxygen species

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide, especially in regions where hepatitis B is endemic, such as southern Africa, Southeast Asia, and China (Valdameri et al. 2011). Liver cancer is the third most common cause of death from cancer. In 2008, it was estimated that 748,000 people suffered from liver cancer and the mortality was about 696,000 worldwide (Ferlay et al. 2010). Currently, the main therapy of liver cancer is surgical treatment, but chemotherapy still plays an important role in a comprehensive strategy. Numerous researches demonstrated that bioactive natural products could in theory serve as alternatives to chemically designed anticancer agents (Park et al. 2010; Yoon and Lee 2010).

Flavonoids are plant polyphenols occurring naturally in the plant kingdom, displaying a wide range of pharmacologic properties, including antipyretic, analgesic, anti-inflammatory and anticancer activities

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(Middleton et al. 2000; Zhang et al. 2013). Chalcones, abundant in edible plants, are considered as the precursor of flavonoids and isoflavonoids. There are emerging studies reported that chalcone induced cell apoptosis. Chalcone buitein and isoliquiritigenin induced apoptosis in B16 melanoma 4A5 cells (Iwashita et al. 2000) and chalcone phloretin induced apoptosis in B16 melanoma 4A5 cells and in HL-60 human leukemia cells (Kobori et al. 1999). Chalcone hydroxysafflor yellow A induced apoptosis in activated hepatic stellate cells (Yang et al. 2012).

In particular, it has been reported that chalcones have been found to possess antioxidant and prooxidant action, which may be related to cell apoptosis (Guzy et al. 2010). There are other accumulating studies reported that some chalcones, regarded as an antioxidant, have been found to induce cytotoxicity through decrease in mitochondrial membrane potential and production of reactive oxygen species (ROS) (Kuo et al. 2010; Li et al. 2010). ROS are generally derived from the normal metabolism of oxygen. At low concentrations, ROS serve as a physiological regulator of normal cell proliferation and differentiation. However, over-expression or decreased removal of intracellular ROS induces oxidative damage to cells and tissues (Klaunig et al. 2011). In addition, excessive oxidative stress especially targets mitochondria, causing the damage to lipids, proteins, and DNA and cell death (Kim et al. 2012).

Cleistocalyx operculatus (Roxb.) Merr. et Perry (Myrtaceae) is a well-known medicinal plant whose buds are commonly used as an ingredient in tonic drinks in Southern China. Our previous phytochemical studies of this plant have led to characterization of sterol, flavanones, chalcones, and triterpene acid from the buds (Ye et al. 2004a). The main compound from the buds of *C. operculatus* is the chalcone 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (DMC) (Fig. 1). It has been reported to exhibit an anti-tumor effect both in vitro and in vivo (Ye et al. 2004b, 2005; Zhu et al. 2005). According to the recent studies, DMC could reverse multi-drug resistance in resistant HCC cell line (Huang et al. 2011, 2012) and had hepatoprotective (Yu et al. 2011) and neuroprotective effects (Su et al. 2011).

Our latest results showed that DMC could induce apoptosis in SMMC-7721 cells via a mitochondria-dependent pathway involving inhibition of Bcl-2 expression leading to disintegration of the outer

mitochondrial membrane (Ye et al. 2013). Since most anticancer agents act, at least in part, by inducing ROS (Sun and Rigas 2008), to further elucidate the anticancer mechanism of DMC, the chemoprotective effects of *N*-acetylcysteine (NAC), a sulfhydryl-containing antioxidant, on DMC induced oxidative stress and apoptosis were evaluated. The present study showed the first evidence of the inhibition of DMC induced apoptosis of hepatoma cells treated with NAC.

Materials and methods

Materials

2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone was isolated from *C. operculatus* as described by Ye et al. (2004a). Previous experiments have shown that the purity of isolated DMC is more than 96 % using HPLC and spectral analysis. The structure of the compound is shown in Fig. 1. The compound was dissolved in dimethyl sulfoxide (DMSO). Control cells were treated with the same amount of vehicle alone. The final DMSO concentration never exceeded 0.1 % (v/v) in either control or treated samples. Previous experiments have shown that DMSO at this concentration does not modify the cellular activities of interest. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was obtained from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS), Rosewell Park Memorial Institute (RPMI) 1640 medium and Dulbecco's modified Eagle's medium (DMEM) medium were purchased from Life Technologies, Inc. (Gaithersburg, MD, USA). All other reagents were of analytical grade and were obtained from East China Pharmaceutical Group Co., Ltd. (Hangzhou, Zhejiang, China).

Fig. 1 Structure of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone

Cell lines and culture conditions

Human hepatoma SMMC-7721 cells, human normal liver L-02 cells and human fetal lung fibroblast HFL-1 cells were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). SMMC-7721 cells were cultured in RPMI 1640 medium with 10 % FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL). HFL-1 cells were cultured in DMEM medium with 10 % FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL). The L-02 cells were cultured in RPMI 1640 medium with 15 % FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL). All cells were incubated at 37 °C with 5 % CO₂ in an air atmosphere. Exponentially growing cells were used in all experiments.

Cytotoxicity on cells (MTT assay)

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was performed as described by Mosmann (1983). L-02, HFL-1 and SMMC-7721 cells were placed within 96-well culture plates (10⁴ cells/well), respectively, and allowed to attach for 24 h before treatment. L-02 and HFL-1 cells were treated with DMC ranging from 25 to 200 µM or vehicle (0.1 % DMSO) as a control. SMMC-7721 cells were pretreated with NAC (5 mM) for 2 h before being treated with 20 µM DMC in the presence or absence of NAC. Cytotoxicity was measured after 2 days of culture using the MTT assay. Absorbance in control and drug-treated wells was measured in an Automated Microplate Reader (Bio-Rad 550; Bio-Rad Laboratories, Hercules, CA, USA) at 550 nm. The cytotoxicity of DMC in L-02 or HFL-1 cells was expressed as IC₅₀ (concentration of 50 % cytotoxicity, which was extrapolated from linear regression analysis of experimental data). Percentage survival in SMMC-7721 cells was calculated as the fraction of the negative control. Three replicate wells were used for each data point in the experiments.

Intracellular ROS generation

The formation of ROS was determined using a fluorescein-labeled dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Xue et al. 2011). Briefly, SMMC-

7721 cells (5 × 10⁵ cells) were pretreated with NAC (5 mM) for 2 h before being treated with 20 µM DMC for 48 h in the presence or absence of NAC. After treatment, the cells were incubated with 10 µM DCF-DA at 37 °C for 30 min. The intracellular ROS mediates oxidation of DCF-DA to the fluorescent compound 2',7'-dichlorofluorescein (DCF). The fluorescence of DCF was detected at an excitation wave length of 480 nm and an emission wave length of 525 nm by flow cytometer.

Flow-cytometric analysis of nuclear DNA

Cell cycle analysis was conducted to determine the proportion of apoptotic sub-G₁ hypodiploid cells (Nicoletti et al. 1991). Briefly, SMMC-7721 cells (5 × 10⁵ cells) were pretreated with NAC (5 mM) for 2 h before being treated with 20 µM DMC for 48 h in the presence or absence of NAC. After treatment, the cells were harvested and fixed in 1 mL of 70 % ethanol for 30 min at 4 °C. The cells were washed twice with PBS and incubated in darkness in 1 mL of PBS containing 100 µg of PI and 100 µg of RNase A for 30 min at 37 °C. Flow cytometric analysis was conducted with a flow cytometer (FC500MCL; Beckman Coulter, Brea, CA, USA). The fluorescence intensity of sub-G₁ cell fraction represented the apoptotic cell population.

Annexin V-FITC/PI staining

Apoptosis was determined by staining cells with Annexin V-FITC and PI labeling according to a previous method (Wang et al. 2012). Briefly, SMMC-7721 cells (5 × 10⁵ cells) were pretreated with NAC (5 mM) for 2 h before being treated with 20 µM DMC for 48 h in the presence or absence of NAC. After treatment, cells were collected and suspended in 300 µL of 1 × binding buffer. Amounts of 5 µL of annexin V-FITC and 5 µL of propidium iodide (PI) were added to the cells. After incubation at room temperature for 15 min in the dark, cells were then analyzed for apoptosis using a flow cytometer (FC500MCL; Beckman Coulter, Brea, CA, USA) with a commercially available annexin V-FITC/propidium iodide apoptosis detection kit (KeyGen Biotech Co., Ltd., Nanjing, China) and evaluated based on the percentage of annexin V positive cells.

Measurement of the mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured by using cationic fluorescent dye rhodamine 123 according to a previous method (Cao et al. 2007). Cells were seeded at 1×10^6 cells/well in 6-well plates. After 24 h of incubation, cells were pretreated with NAC (5 mM) for 2 h before being treated with 20 μ M DMC for 48 h in the presence or absence of NAC. After treatment, cells were then resuspended in 2 mL of fresh incubation medium containing 10 μ g/mL of rhodamine 123, incubated at 37 °C in a thermostatic bath for 30 min, then washed with PBS. The cell pellet was collected and resuspended in 300 μ L of PBS. Fluorescence intensities of rhodamine 123 in cells were analyzed using flow cytometric analysis.

Measurements of caspase-3 and -9 activities

Caspase-3 and caspase-9 activity assays were measured using caspase-3 and -9 activity detection kits according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Haimen, China). Briefly, SMMC-7721 cells (5×10^5 cells) were pretreated with NAC (5 mM) for 2 h before being treated with 20 μ M DMC for 48 h in the presence or absence of NAC and whole cells were lysed in the supplied lysis buffer, followed by incubation for 10 min on ice. Supernatants were collected and total protein was quantified using the Bradford method (Bradford 1976). Assays were performed on 96 well microtitre plates by incubating 20 μ L of cell lysate protein per sample in 80 μ L of reaction buffer (1 % NP-40, 20 mM Tris-HCl at pH 7.5, 137 mM NaCl and 10 % glycerol) containing 10 μ L of 2 mM caspase-3 substrate (Ac-DEVD-pNA) and 10 μ L of 2 mM caspase-9 substrate (Ac-LEHD-pNA). Lysates were incubated at 37 °C for 2 h, then the absorbance of the lysates were measured with a microplate reader at an absorbance of 405 nm. Results are presented as the percentage change of activity compared to an untreated control.

Protein extraction and western blot analysis

SMMC-7721 cells were treated with NAC (5 mM) for 2 h before being treated with 20 μ M DMC for 48 h in the presence or absence of NAC. Cells were washed twice with cold PBS and lysed at 4 °C for 30 min in

lysis buffer (0.5 % Triton X-100, 300 mM NaCl, 50 mM Tris-Cl, 1 mM phenylmethylsulfonyl fluoride) with occasional vortexing. Insoluble material was removed by centrifugation at 4 °C for 15 min at 14,000 $\times$ g, and the total proteins extracted were quantified using a Coomassie brilliant blue G-250 assay with bovine serum albumin (BSA) as standard.

Cellular proteins (40 μ g/lane) were loaded onto 12 % polyacrylamide-SDS gel. After electrophoresis, the gels were blotted onto TotalBlot PVDF membranes (Amresco Inc., Solon, OH, USA), which were then blocked with 5 % fat-free milk and immunostained with a 1:1,000 dilution of monoclonal mouse anti-Bcl-2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), a 1:1,000 dilution of monoclonal mouse anti-bax antibody (Santa Cruz Biotechnology, Inc.), a 1:1,000 dilution of mouse anti-p53 antibody (Santa Cruz Biotechnology, Inc.) and a 1:1,000 dilution of mouse anti-PUMA antibody (Santa Cruz Biotechnology, Inc.), respectively. Immunoreactive proteins were probed with horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.), and Bcl-2, Bax, p53 and PUMA protein levels were visualized using a peroxidase reaction ECL kit (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, UK). Equal loading of extracts was confirmed using anti-Actin (Santa Cruz Biotechnology, Inc.).

Statistical analysis

Data are reported as the mean $\pm$ SD of three measurements. The scientific statistics software GraphPad Prism 3.03 (GraphPad Software, Inc., San Diego, CA, USA) was used to evaluate the significance of differences between groups. Comparisons between groups were done using the Kruskal–Wallis test followed by Dunn's post hoc test and $P < 0.05$ was regarded as significant.

Results

Cytotoxic effects of DMC in two human normal cell lines

The results of cytotoxic activities of DMC against human normal liver L-02 cells and human fetal lung fibroblast HFL-1 cells are shown in Fig. 2. The

Fig. 2 Cytotoxic effects of DMC in L-O2 (filled square) and HFL-1 (filled circle) cell. Cells were treated for 48 h in the presence of the drug in medium. Cytotoxicity was then determined by MTT assay and was expressed as mean $\pm$ SD of three separate experiments ($n = 3$ each in the three experiments). Significant differences from untreated control are indicated by * $P < 0.05$

percentages of growth inhibition of DMC at various concentrations on L-O2 cells and HFL-1 cells were determined as the percentage of viable treated cells in comparison with viable cells of untreated controls. The data demonstrated that cytotoxicity of DMC on the two tested cell lines was dose-dependent. The half maximal inhibitory concentration (IC_{50}) values on cytotoxicity were 111.0 ± 4.57 and $152.0 \pm 4.83 \mu M$ for L-O2 and HFL-1 cells, respectively.

Effect of NAC on DMC induced cytotoxicity

In order to investigate the role of ROS in DMC-induced apoptosis of SMMC-7721 cells, the effect of the antioxidant, NAC, on cell growth inhibition by DMC was measured. The cells were treated with DMC for 48 h in the presence or absence of NAC and cytotoxicity was measured by MTT assay. As shown in Fig. 3, NAC protected the SMMC-7721 from cytotoxicity by DMC. Cells preincubated with 5 mM NAC prior to 20 μM DMC for 48 h increased cell viability ratio from 68.7 ± 1.4 to $89.3 \pm 1.3 \%$. The data indicated that ROS generation plays an important role in the cytotoxic effect of DMC in SMMC-7721 cells.

NAC prevent intracellular ROS production induced by DMC

To further determine whether ROS play an essential role in the apoptosis induced by DMC in SMMC-7721

Fig. 3 NAC protects SMMC-7721 cells from DMC induced growth inhibition. SMMC-7721 cells were seeded in each well of the 96-well plates. Cells were pretreated with or without NAC (5 mM) for 2 h before being treated with DMC (20 μM) for 48 h. Cell viability was measured by MTT assay. Data are presented as mean $\pm$ SD. The viability of control group was defined as 100 %. Data are mean $\pm$ SD of three measurements. * $P < 0.05$ compared to control, # $P < 0.05$ compared to DMC (20 μM)

Fig. 4 NAC prevents ROS production induced by DMC. SMMC-7721 cells were pretreated with or without NAC for 2 h and then treated with DMC for 48 h. The generation of ROS was measured by using the fluorescent dye DCF-DA in FACSscan flowcytometry. The corresponding bar graph of FACSscan flowcytometry is shown. $n = 3$, mean $\pm$ SD * $P < 0.05$ compared to control, # $P < 0.05$ compared to DMC (20 μM)

cells, we measured intracellular ROS level by using the fluorescence dye DCF-DA. As shown in Fig. 4, the ratio of DCF-positive cells drastically increased from 3.7 ± 0.9 to $37.3 \pm 1.3 \%$ after DMC (20 μM) treatment for 48 h. To further confirm that ROS acted as initiators in DMC-induced SMMC-7721 cells apoptosis, the cells were preincubated with 5 mM NAC for 2 h prior to 20 μM DMC treatment. As expected, the ROS scavenger NAC at 5 mM markedly decreased the level of ROS to $13.5 \pm 1.2 \%$.

Fig. 5 Effect of NAC on the population of hypodiploid cells in SMMC-7721 cells. SMMC-7721 cells were pretreated with or without NAC for 2 h before being treated with DMC for 48 h. Cells were subjected to PI staining and analyzed via flow cytometry. Data are representative of one of three similar experiments

Effect of NAC on the population of hypodiploid cells

SMMC-7721 cells were pretreated with NAC (5 mM) for 2 h before being treated with DMC (20 μ M) for 48 h in the presence or absence of NAC and stained with PI. After 48 h, the sub- G_1 population increased from 4.1 ± 0.9 % (control) to 56.3 ± 1.8 % at 20 μ M DMC. On the other hand, pretreatment with NAC could markedly decrease the sub- G_1 population, with the percentage of hypodiploid cells to 15.1 ± 1.3 % (Fig. 5).

NAC against DMC induced apoptosis

SMMC-7721 cells were pretreated with NAC (5 mM) for 2 h before being treated with DMC (20 μ M) for 48 h in the presence or absence of NAC and stained with annexin V-FITC/PI. After 48 h, the number of apoptotic cells increased from 5.26 ± 0.35 (control) to 67.43 ± 2.32 % at 20 μ M DMC. On the other hand, pretreatment with NAC could markedly block DMC induced cell apoptosis, with the apoptotic rate to 9.17 ± 0.61 % (Fig. 6).

Effect of NAC on mitochondrial depolarization

Mitochondrial depolarization in SMMC-7721 cells treated with 20 μ M DMC in the presence or absence of

NAC was identified using Rho 123 stain. As shown in Fig. 7, there was a significant decreasing in mitochondrial membrane potential after treatment with DMC for 48 h. The fluorescence decreased from 98.6 ± 1.3 % of control group to 36.7 ± 1.6 % of DMC (20 μ M). The loss of mitochondrial membrane potential was almost completely blocked by pretreatment with NAC (5 mM).

The effects of NAC on DMC-induced caspase-3, -9 activation

Since caspase-3, -9 activation is predominantly triggered by the changes of mitochondrial membrane potential ($\Delta\Psi_m$). The effects of NAC on DMC-induced caspase activation were further investigated. Cells were pretreated or without NAC (5 mM) followed by 48 h treatment with DMC. Compared to the control, the activities of caspase-3 and caspase-9 in the DMC treated cells increased to 251.7 ± 5.3 and 235.3 ± 6.1 %, respectively. By pretreatment with NAC, the activities of caspase-3 and caspase-9 in the DMC treated cells decreased to 141.3 ± 4.8 and 128.1 ± 5.1 %, respectively (Fig. 8). These data demonstrated that the activities of caspase-3 and caspase-9 induced by DMC were markedly attenuated by pretreatment with NAC.

The effects of NAC on Bcl-2, Bax, p53 and PUMA protein expressions

Bcl-2 family has been associated with mitochondrial function during apoptosis, thus the level of Bcl-2 and Bax proteins were examined by western blot analysis in the presence or absence of DMC. Cells were treated with DMC for 48 h with or without pretreatment with NAC (5 mM). As shown in Fig. 9a, 20 μ M DMC largely down-regulated Bcl-2 gene expression at the protein level. No influence was observed on expression of the Bax protein. Compared to the control, the Bcl-2/Bax ratio in the DMC treated cells decreased to 27.5 ± 1.3 %. The reduction of the Bcl-2/Bax ratio is probably responsible for DMC-induced apoptosis in SMMC-7721 cells. By pretreatment with NAC, the Bcl-2/Bax ratio in the DMC treated cells increased to 89.4 ± 2.8 % (Fig. 9b).

P53 is a known tumor suppressor gene that can induce apoptosis by interfering with the normal

Fig. 6 Effect of NAC on annexin V-FITC/PI staining. SMMC-7721 cells were pretreated with or without NAC for 2 h before being treated with DMC for 48 h. Cells were subjected to annexin V-FITC/PI staining and analyzed via flow cytometry

Fig. 7 NAC prevents mitochondrial membrane potential (MMP) reduction induced by DMC. SMMC-7721 cells were pretreated with or without NAC for 2 h before being treated with

DMC for 48 h followed by incubation with rhodamine 123 dye for another 30 min. Fluorescence emission was measured via flow cytometry

Fig. 8 Effect of NAC on activities of caspase-3 (a) and caspase-9 (b). SMMC-7721 cells were pretreated with or without NAC for 2 h before being treated with DMC for 48 h. The activities of caspase-3 and caspase-9 were measured using

the substrates Ac-DEVD-pNA and Ac-LEHD-pNA, respectively. Relative caspase-3 and caspase-9 activities were calculated as a ratio of treated cells to control cells. * $P < 0.05$ compared to control, # $P < 0.05$ compared to DMC (20 μ M)

Fig. 9 Effect of NAC on Bcl-2, Bax, and Actin protein expressions. SMMC-7721 cells were pretreated with or without NAC for 2 h and then treated with DMC for 48 h. Protein (40 μ g/lane) from cell lysates was electrophoresed on SDS-PAGE gels, transferred to TotalBlot PVDF membranes, and probed with anti-Bcl-2, anti-Bax, and anti-Actin antibodies for

Bcl-2, Bax, and Actin, respectively. Intensity of each band was quantified by densitometry. The Bcl-2/Bax ratio from control group was designated as 100 %. Data are mean $\pm$ SD of three measurements. * $P < 0.05$ compared to control, # $P < 0.05$ compared to DMC (20 μ M)

apoptotic pathway and p53 upregulated modulator of apoptosis (PUMA) is a downstream target of the p53 tumor suppressor gene. Thus the levels of p53 and PUMA proteins were also examined by western blot analysis in the presence or absence of DMC. As shown in Fig. 10a, 20 μ M DMC observably up-regulated p53 and PUMA genes expressions at the protein levels. Compared to the control, the levels of p53 and PUMA in the DMC treated cells increased to 273.5 ± 5.1 and 247.3 ± 4.6 %, respectively. The increase of the p53 and PUMA genes expressions are probably responsible for DMC-induced apoptosis in SMMC-7721 cells. By pretreatment with NAC, the levels of p53 and PUMA in the DMC treated cells decreased to 149.1 ± 4.5 and 132.6 ± 4.2 %, respectively (Fig. 10b).

Discussion

Nowadays, much effort is being devoted to detect new drugs that not only significantly induce the death of tumor cells, but also have little side effects on normal cells. Our previous study demonstrated that DMC exhibited significant cytotoxic potential with an IC_{50} value 32.3 ± 1.13 μ M against SMMC-7721 cells (Ye et al. 2004b). In the present study, we found that DMC was almost nontoxic to human normal liver L-02 and human normal fetal lung fibroblast HFL-1 cells as their IC_{50} values (111.0 ± 4.57 and 152.0 ± 4.83 μ M for L-02 and HFL-1 cells, respectively) were much higher (Fig. 2). The reason why DMC is nontoxic to L-02 and HFL-1 cells but toxic to SMMC-7721 cells is that DMC probably selectively induced apoptosis of

Fig. 10 Effect of NAC on p53, PUMA, and Actin protein expressions. SMMC-7721 cells were pretreated with or without NAC for 2 h and then treated with DMC for 48 h. Protein (40 μ g/lane) from cell lysates was electrophoresed on SDS-PAGE gels, transferred to TotalBlot PVDF membranes, and

probed with anti-p53, anti-PUMA, and anti-Actin antibodies for p53, PUMA, and Actin, respectively. Intensity of each band was quantified by densitometry. Data are mean $\pm$ SD of three measurements. * $P < 0.05$ compared to control, # $P < 0.05$ compared to DMC (20 μ M)

SMMC-7721 cells, while did not induce apoptosis of L-02 and HFL-1 cells. Our results with the L-02 cell line showed that at a concentration of 50 μ M DMC (48 h of incubation) the cell viability was 90.3 ± 2.1 %. Whereas, Lu et al. (2014) demonstrated that cell viability at the concentration of DMC 50 μ M (12 h of incubation) was around 70 %. This discrepancy between our results and their results may result from the difference of the DMC preparation and time of incubation.

A large number of chalcones have been found to induce apoptosis in tumor cells (Iwashita et al. 2000; Kobori et al. 1999). Our previous studies have demonstrated that DMC could induce cancer cells apoptosis (Ye et al. 2013). Accumulated evidence has suggested that reactive ROS may act as upstream signaling regulators and trigger oxidative stress, which results in cytotoxicity enhancement of anticancer drugs to induce cancer cell apoptosis (Lo and Wang 2013). Normally, ROS can be cleared by antioxidant enzymes and non-enzymes systems in cells in order to keep the balance. Oxidative stress will occur when there is an imbalance due to excess ROS, antioxidant depletion, or both (Waris and Ahsan 2006). If the balance is broken, apoptosis will occur (Pelicano et al. 2004). Herein, we investigated the role of ROS in DMC-induced apoptosis in hepatoma cancer cells. To confirm that the apoptotic effect of DMC was mediated by ROS, antioxidant NAC was used.

NAC is an antioxidant, a free radical scavenger, an exogenous source of cysteine/precursor of glutathione (Dodd et al. 2008). It has the ability to decrease cell oxidative stress (Hur et al. 1999) and restore the glutathione content (Grinberg et al. 2005). Recent studies showed that NAC had a protective effect

against hepatic lipid accumulation in rats (Lai et al. 2012) and blocked apoptosis in human ovarian cancer cells (Rogalska et al. 2013), invasive oral cancer cells (Lee et al. 2013) and hepatocarcinoma SMMC-7721 cells (Li et al. 2013). Our study demonstrated that NAC inhibited the cytotoxicity of DMC (Fig. 3) associated with suppressed ROS generation (Fig. 4). These data suggested that DMC-induced cell death was largely mediated through the formation of intracellular ROS. We also investigated the role of ROS in apoptosis induced by DMC in human hepatoma cells. In our study, the sub- G_1 population increased by PI staining and the ratio of apoptotic cells increased by Annexin V-FITC/PI staining and the mitochondrial membrane potential decreased using Rho 123 stain when SMMC-7721 cells were treated with DMC for 48 h. NAC can markedly counteract the sub- G_1 population, the cell apoptosis and mitochondrial depolarization induced by DMC (Figs. 5, 6, 7). These results showed that ROS plays a very important role in the apoptosis induced by DMC and NAC might control the ROS pathways against DMC. This was further proven by pretreatment with NAC, which blocked the activation of caspase-3, caspase-9, down-regulation of Bcl-2 protein, up-regulation of p53 and PUMA proteins (Figs. 8, 9, 10). It was interesting that our results were consistent with previous reports, which had shown that flavonoids provoked oxidative stress and induced apoptosis of cancer cells. Apoptosis in cancer cells, induced by flavonoids, could be inhibited by pretreating cells with NAC (Lu et al. 2007; Qiao et al. 2013; Shukla and Gupta 2008).

In summary, the results of the present study showed that NAC could prevent DMC induced oxidative stress

and apoptosis in SMMC-7721 cells. The protective effects of NAC on DMC induced apoptosis were related to ROS-dependent and caspase-mediated signaling pathways. These results indicate that ROS plays an important role on DMC-induced apoptosis of SMMC-7721 cells and suggest that it may be a promising antihepatocellular carcinoma therapeutic agent.

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Conflict of interest The authors declare that there are no conflicts of interest.

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