

Reactive oxygen species-mediated induction of apoptosis by a plant alkaloid 6-methoxydihydrosanguinarine in HepG2 cells

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Abstract

We have found in the previous study that 6-methoxydihydrosanguinarine (6ME), a benzophenanthridine alkaloid isolated from *Hylomecon* species, may have potential as a chemotherapeutic agent. However, the mechanisms of 6ME-induced cell death have not been investigated. The purpose of the present study was to determine the apoptosis-inducing potential of 6ME in human hepatocarcinoma HepG2 cells and the role of reactive oxygen species in 6ME-induced apoptosis. It can be concluded from the results that 6ME inhibits the growth of HepG2 cells in a concentration- and time-dependent manner ($IC_{50}=3.8 \pm 0.2 \mu\text{M}$ following 6 h incubation). Treatment of HepG2 cells with 6ME resulted in the release of mitochondrial cytochrome *c* followed by the activation of caspase proteases, and subsequent proteolytic cleavage of poly(ADP-ribose) polymerase. 6ME increased the expression of p53 and bax and decreased the expression of bcl-2. The cytotoxic effect of 6ME is mediated by the time-dependent generation of reactive oxygen species. Our results also show that preincubation of HepG2 cells with vitamin C decreased the expression of p53 and bax and inhibited the release of cytochrome *c*, activation of downstream caspase and the cleavage of poly(ADP-ribose) polymerase, thus inhibiting the apoptosis inducing effect of 6ME.

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1. Introduction

Sanguinarine, derived from the root of *Sanguinaria canadensis* and other poppy *fumaria* species, has been used for years as a dental product for the treatment of gingivitis and plaque [1]. It has also been the subject of great interest as an antimicrobial, anti-inflammatory, antioxidant as well as anticancer drug [2–4]. Apoptosis-inducing activity and anti-

angiogenic effect were observed in vitro and in vivo at low concentration of sanguinarine, which allows the possibility to be developed as an anticancer drug [4,5]. However, the genotoxicity of sanguinarine alkaloid limits the potential use of the compound [6,7]. 6-Methoxydihydrosanguinarine (6ME) is a benzophenanthridine alkaloid derived from the methanol extracts of *Hylomecon hylomeconoides*. The anti-platelet aggregation activity of 6ME has been reported [8]; however, there has been limited research on the biological properties of 6ME with regard to the antiproliferative effects and the molecular mechanisms of action of the therapeutic effects of 6ME [9].

A large number of different chemicals are capable of inducing apoptosis by evoking oxidative stress and it has been shown that pretreatment with antioxidants abolishes such effect [10]. For example, peroxisome proliferators are not free radicals themselves, but may cause hydrogen

Abbreviations: 6ME, 6-methoxydihydrosanguinarine; ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; Ac-IETD-AMC, *N*-acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin; Ac-LEHD-AMC, *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin; PI, propidium iodide; PARP, poly(ADP-ribose) polymerase; DCFH-DA, 2',7'-dichlorofluorescein diacetate

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peroxide production and/or formation of reactive oxygen species (ROS), rendering cells more susceptible to apoptosis [11]. ROS can cause DNA strand breaks, base modification, lipid peroxidation and protein modification, resulting in oxidative stress. Various signal pathways are involved in the mechanism of ROS-induced oxidative stress, including activation of nuclear transcription factors p53 [12].

In the present study, we examined the effects of 6ME on the inhibition of proliferation and the induction of apoptosis in human hepatocellular carcinoma HepG2 cells. We investigated the biochemical pathway involved in 6ME-induced cell death. Our results indicate that 6ME-induced inhibition of cell growth involves activation of apoptosis cascade, involving mitochondrial cell death pathway. Pre-treatment of these cells with vitamin C offered a complete protection from apoptosis-inducing properties of 6ME. Therefore, our results suggest that 6ME induces apoptosis through the generation of ROS, which then serves as a signal for the release of mitochondrial cytochrome *c* and subsequent activation of caspases.

2. Materials and methods

2.1. Materials and cell culture

6ME was isolated from the methanol extracts of *H. hylomeconoides* (Fig. 1) according to Kang et al. [13]. The human hepatoblastoma cell line, HepG2, was maintained in the logarithmic phase of growth in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with heat-inactivated 10% fetal bovine serum (Gibco BRL), 2 mM L-glutamine (Sigma Chemical Co., St. Louis, MO) at 37 °C in a 5% CO₂–95% air humidified incubator. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and vitamin C were obtained from Sigma Chemical Co. All the other chemicals used were of the highest pure grade available.

2.2. Cell proliferation assay

HepG2 cells were seeded at 1×10^4 cells/well in a 96-well plate and treated with the appropriate concentra-

tions of 6ME. General viability of cultured cells was determined by reduction of MTT to formazan [14].

2.3. DNA fragmentation analysis

HepG2 cells grown at a density of 2×10^6 cells/ml were exposed to 6ME as described in the figure legends. Genomic DNA was prepared with Wizard Genomic DNA Purification Kit (Promega, Madison, WI). DNA was precipitated with isopropanol, separated in 1.5% agarose gel and visualized by UV illumination after ethidium bromide staining.

2.4. Morphological analysis

The cells were placed onto sterile microscope slides. After treatment with drugs, cells were washed with PBS and fixed with paraformaldehyde for 30 min. The cells were then stained with PI solution (50 µg/ml of PI, 100 µg/ml of RNase A). The morphology of the cells was examined using the Leitz phase-contrast microscope or Olympus Fluoview laser scanning confocal microscope.

2.5. Measurement of caspase activity

The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC; caspase-3 substrate), *N*-acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin (Ac-IETD-AMC; caspase-8 substrate) and *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (Ac-LEHD-AMC; caspase-9 substrate). Each substrate was added to the cell lysates in assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, pH 7.4) and incubated for 3 h at 37 °C. The cleavage of the peptide substrate was monitored at excitation wavelength at 380 nm and emission at 460 nm.

2.6. Western blot analysis

Cells were solubilized by suspension in a 50 mM HEPES buffer containing 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EGTA, 20 mM NaF, 50 mM β-glycerophosphate, 2 mM phenylmethylsulfonylfluoride, 1 mM Na₃VO₄, 10 mg/ml of leupeptin, 10 mg/ml of aprotinin and incubated on ice for 1 h. Cell lysates were centrifuged and the protein content was determined. After SDS-polyacrylamide gel electrophoresis, proteins were transferred onto immobilon nitrocellulose membrane (Millipore) at 200 mA for 3 h at 4 °C. Blots were probed with mouse monoclonal anti-poly(ADP-ribose) polymerase (PARP), polyclonal rabbit cleaved caspase-3, polyclonal rabbit caspase-9, polyclonal rabbit p53 (Cell Signaling Tech., Beverly, MA), monoclonal mouse anti-human bcl-2 and monoclonal mouse anti-human bax (Santa Cruz Biotech. Inc., Santa Cruz, CA). Immune complexes were detected using

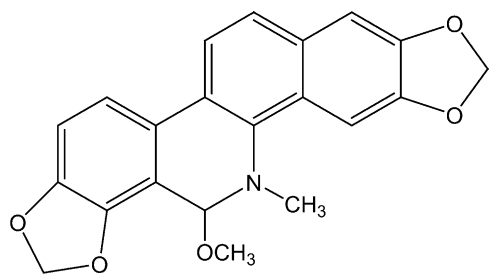


Fig. 1. Structure of 6-methoxydihydrosanguinarine (6ME).

anti-mouse peroxidase-conjugated secondary immunoglobulin G antibody (Boehringer Mannheim, Mannheim, Germany) and visualized by electrochemiluminescence Western blotting detection reagents (Amersham, Piscataway, NJ).

2.7. Determination of ROS

HepG2 cells were cultured on 96-well microplate to 1×10^4 cells/well. After treatment with 6ME for 9 h, the cells were incubated with 20 μ M DCFH-DA (Molecular Probes) in HBSS for 30 min. Fluorescence intensity was analyzed with an excitation wavelength at 485 nm and emission at 530 nm [15].

2.8. Statistical analysis

Significant differences were estimated by Student's *t*-test using a statistics software program GraphPad prism 2.0 (GraphPad software Inc., USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. 6ME inhibited the proliferation of HepG2 cells

Inhibition of cell proliferation was tested by MTT assay following treatment of HepG2 cells with 6ME. The HepG2 cells were exposed to 2–8 μ M of 6ME for up to 24 h and the number of viable cells were determined. They decreased cell proliferation in a concentration- and time-dependent manner. The concentrations required for 50% inhibition of the growth (IC_{50}) was 3.8 ± 0.2 μ M in 6 h of incubation (Fig. 2A).

3.2. Induction of apoptosis by 6ME

To clarify the mode of cell death caused by 6ME, we examined the effects on the internucleosomal DNA fragmentation. As shown by the agarose gel electrophoresis, the DNA ladder was clearly observed after treatment with 6ME (Fig. 2B). Morphological analysis of PI-stained cells also indicated that the cells showed clear apoptotic characteristics, such as nuclear fragmentation (Fig. 2C).

3.3. Activation of caspases and cleavage of PARP by 6ME

To demonstrate that, caspase activation is involved in 6ME-induced cell death, the proteolytic activity of caspase-3 and -8 was measured in cell lysates by quantitative detection of fluorometric tetrapeptide substrate. Caspase-3 and -8 activity increased in a time-dependent manner in HepG2 cells (Fig. 3A). To further confirm the involvement

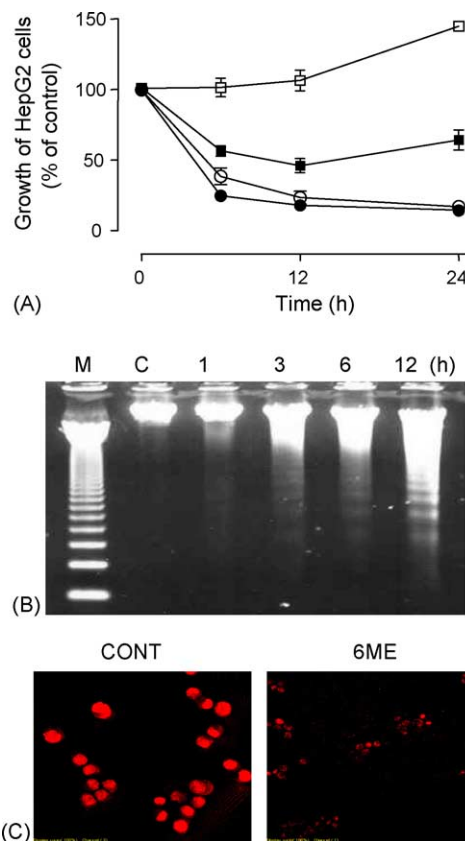


Fig. 2. 6ME inhibits growth and induces apoptosis in HepG2 cells. (A) Time- and dose-dependent inhibition of HepG2 cell growth by 6ME. Cells were incubated with 6ME for the indicated time and cell viability was determined by MTT assay: (□) control; (■) 2 μ M; (○) 4 μ M; (●) 8 μ M. Data are mean $\pm$ S.D. of three independent experiments and presented as percent changes compared to the vehicle treated control at time 0. (B) DNA fragmentation after exposure of HepG2 cells to 4 μ M 6ME. Genomic DNA was prepared and separated in 1.0% agarose gel and visualized by UV illumination after ethidium bromide staining (M, marker; C, control). (C) Morphological analysis of HepG2 cells treated with 4 μ M 6ME. Nuclei were stained with PI (50 μ g/ml of PI, 100 μ g/ml of RNase A) and examined with confocal microscope.

of caspase in 6ME-induced apoptosis, we examined the specific cleavage of PARP by caspase during apoptosis. We observed that 6ME induced cleavage of PARP (Fig. 3B). Activation of caspase-3 was further verified by Western blot analysis (Fig. 3B).

3.4. Involvement of the mitochondrial pathway in 6ME-induced apoptosis

The release of cytochrome *c* or an apoptosis-inducing factor from the mitochondria after depolarization is believed to initiate the caspase cascade. To investigate whether this pathway is also involved in the response of HepG2 cells to 6ME, we monitored cytochrome *c* release from the mitochondria. Fig. 4A shows that 6ME treatment caused a time-dependent increase in the cytosolic cytochrome *c*. We also investigated the involvement of caspase-9, one of the major initiator caspases, because mitochon-

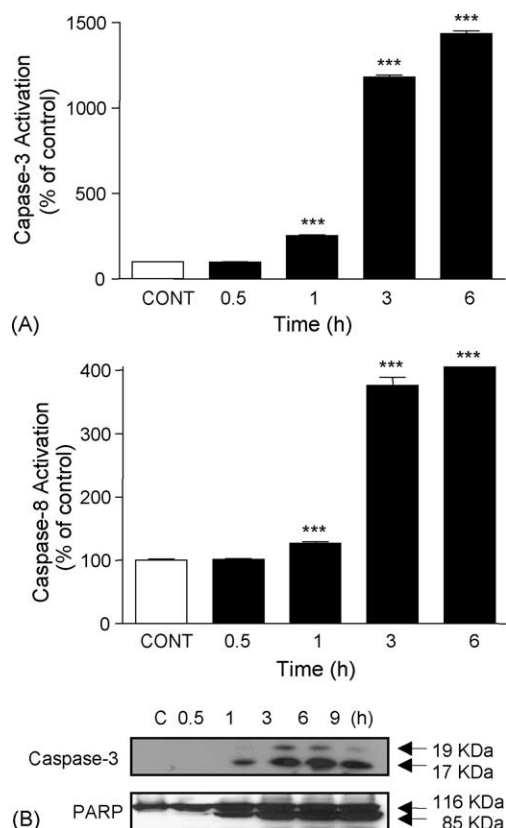


Fig. 3. 6ME induces time-dependent activation of caspase-3 and -8. (A) Extracts of control cells and cells treated with 6ME (4 μ M) were analyzed for caspase activity as a function of time. Ac-DEVD-AMC and Ac-IETD-AMC were used as the substrates for caspase-3 and -8, respectively, and the cleavage of each peptide was monitored with excitation wavelength at 380 nm and emission at 460 nm. Each data point represents the mean $\pm$ S.D. from three independent experiments (***) $P < 0.001$. (B) Representative Western blots show the cleavage of caspase-3 and PARP by treatment with 6ME. HepG2 cells were treated with 6ME (4 μ M) for indicated time and total cellular protein was separated on a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted with the corresponding antibodies.

drial dysfunction results in the release of cytochrome *c* and subsequent activation of this caspase. 6ME treatment was found to increase caspase-9 activity (Fig. 4B).

3.5. Involvement of *bcl-2* family proteins in 6ME-induced apoptosis

Death-promoting members of the *bcl-2* family, such as bax and bid, play roles in the chemical-induced release of cytochrome *c*. Bid and bax in the cytosol receive death signals from upstream events and induce the release of cytochrome *c*, thereby activating the mitochondrial apoptotic pathway. Fig. 4C shows details of the levels of bax and *bcl-2* in whole-cell lysates of HepG2 cells treated with 6ME. The levels of bax increased in a time-dependent manner, whereas the expression of *bcl-2* decreased slightly. p53, a transcription factor of bax was also upregulated in 6ME-treated cells (Fig. 4C).

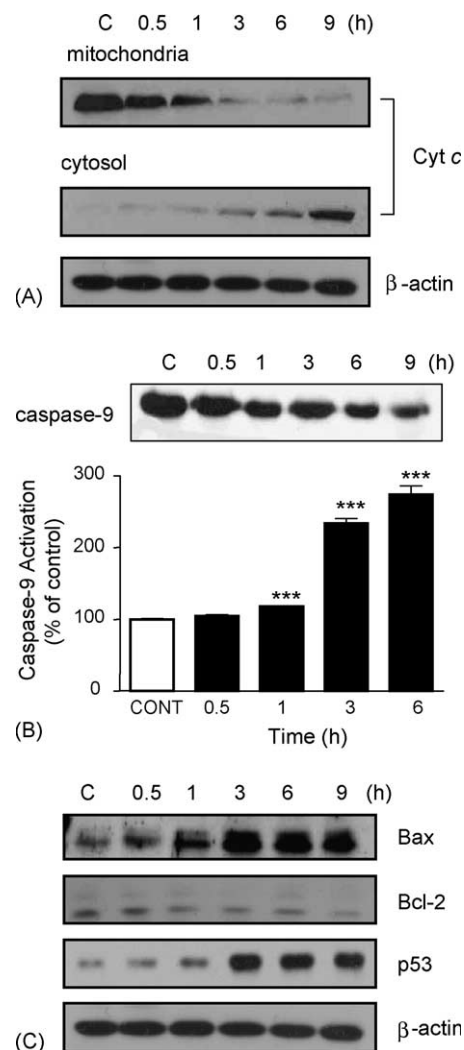


Fig. 4. Involvement of mitochondrial pathway in 6ME-induced apoptosis. (A) HepG2 cells were treated with 6ME (4 μ M) for indicated time and cytosol and mitochondrial protein was prepared as described. They were separated on a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted. (B) Activation of caspase-9 was determined by Western blot analysis and by fluorometric determination of the cleavage of the enzyme substrate Ac-LEHD-AMC. (C) For the detection of *bcl-2*, bax and p53, the protein from whole cell lysate was analyzed by Western blot analysis.

3.6. 6ME increased the production of ROS and antioxidants blocked 6ME-induced apoptosis

An increased generation of reactive oxygen species can induce apoptosis [16]. To determine whether 6ME-induced apoptosis is mediated by oxidative stress, the intracellular level of ROS was measured. As shown in Fig. 5A, treatment of cells with 6ME increased the production of ROS significantly. Furthermore, to determine whether ROS generation caused by 6ME treatment could be responsible for the loss of cell viability, effects of three structurally unrelated antioxidants, *N*-acetyl-L-cysteine (NAC), vitamin C and Trolox, on the cell survival were investigated. The viability of HepG2 cells was not altered by supple-

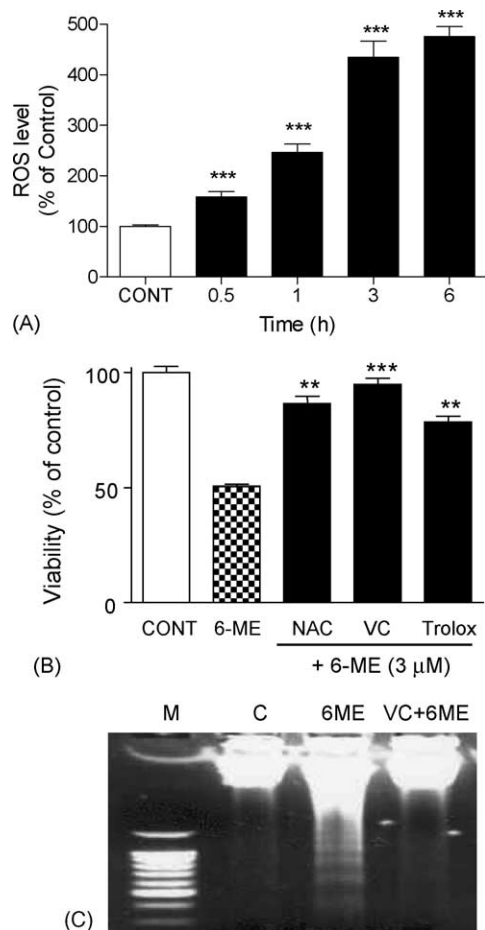


Fig. 5. 6ME increased the production of ROS and antioxidants blocked 6ME-induced apoptosis. (A) After treatment with 6ME (4 μ M) for indicated time, the cells were incubated with 20 μ M DCFH-DA for 30 min and the fluorescence was analyzed with an excitation wavelength at 485 nm and emission at 530 nm. (B) Cells were preincubated with NAC, vitamin C or Trolox (0.1 mM) for 1 h followed by treatment with 6ME for 4.5 h. Viability of the cells was determined by MTT assay. (** p < 0.01; *** p < 0.001). (C) DNA was isolated and electrophoresed by agarose gel as indicated in Fig. 2.

mentation of the medium with NAC, vitamin C, or Trolox alone at any of the tested concentrations (data not shown). As indicated in Fig. 5B, pretreatment with the antioxidants inhibited cytotoxicity, and vitamin C inhibited the DNA fragmentation by 6ME (Fig. 5C). Moreover, preincubation of the cells with vitamin C inhibited the upregulation of p53 and bax. Cytochrome *c* release was blocked completely by vitamin C. Subsequently, caspase-9 activation and PARP cleavage remained inhibited in cells pretreated with vitamin C (Fig. 6).

4. Discussion

Natural benzophenanthridine alkaloids, such as chelerythrine, sanguinarine and 6ME, are capable of inhibiting the growth of variety of human cancer cells via the induction of apoptosis [9,17,18]. Although the mechanism

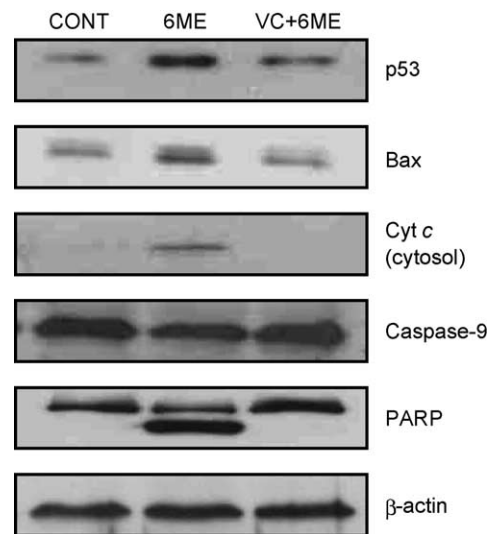


Fig. 6. Effect of vitamin C on 6ME-induced expression of apoptosis-related proteins. HepG2 cells were treated with or without vitamin C for 1 h before treatment with 6ME (3 μ M). Total cellular protein was separated on a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted with the corresponding antibodies. For cytochrome *c*, cytosolic fraction was separated as indicated in Fig. 4.

of cell death by chelerythrine and sanguinarine is partially elucidated, precise mechanism by which 6ME induces cell death remains completely unknown. Results of the present study show that apoptosis induced by 6ME occurred in a ROS- and mitochondria-dependent manner. Activation of caspase-8 and -3 was involved in 6ME-induced apoptosis.

It has been suggested that the generation of ROS is a common mechanism in one of the representative pathways of apoptosis. ROS are produced in all mammalian cells due to normal cellular metabolism and also when cells are exposed to certain exogenous agents [19]. Because of their high reactivity, ROS affect various cellular molecules, such as fatty acids, carbohydrates, proteins and nucleic acids. An excess of ROS may lead to cell death when their level overwhelms the cellular antioxidant capacity, which is linked to the antioxidant level [20–23]. Our results show that 6ME increased the level of ROS in a time-dependent manner. In the model of ROS-mediated apoptosis, the generation of ROS has been suggested as a primary regulatory component followed by the activation of caspases [24,25]. Consequently, this form of cell death could be prevented by antioxidants. In our study, the antioxidant vitamin C could decrease the 6ME-induced cell death and DNA fragmentation via inhibition of mitochondrial cytochrome *c* release, caspase activation and PARP cleavage in HepG2 cells. The data suggest that 6ME-induced ROS promotes the production of proapoptotic markers from the cell in response to some as yet undefined process.

One of the explanations of the process is the ROS-induced DNA damage and subsequent expression of p53, which is also reported in HepG2 cells [26]. It is well known that oxidants block entry into S phase by modulating the

G1 regulators such as p53, p21^{waf1}, and pRb [27,28]. In molecular level, ROS activate p53, p53 activation induces p21^{waf1}, higher p21^{waf1} level causes pRb hypo-phosphorylation, and the unphosphorylated pRb thereby blocks the G1/S transition [27]. Among the several target genes regulated, p53 is the proapoptotic gene bax [29,30]. As shown in Figs. 4 and 6, 6ME induced a time-dependent increase in the expression of p53 that paralleled that of bax and pretreatment with vitamin C decreased the expression of them.

Mitochondria play essential roles in apoptosis through the redistribution of intermembranous mitochondrial proteins, such as cytochrome *c*. The significance of cytochrome *c* to the apoptotic process was revealed by the finding that mitochondrially released cytochrome *c* combines with apoptosis protease activating factor-1, procaspase-9 and dATP in the cytosol, producing active caspase-9 [31,32]. The activation of this initiator caspase then leads to the proteolytic activation of caspase-3, the primary effector caspase of the cell. This pathway is referred to as the mitochondrial pathway of caspase activation. Our results indicate that 6ME-induced apoptosis involves the production of ROS and is mediated by mitochondrial cell death pathway. Upregulation of bax following p53 expression releases mitochondrial cytochrome *c* and activates caspase-9 and -3. The ROS-initiated apoptotic stimuli seem critical in 6ME-induced apoptosis, because the antioxidant vitamin C prevented the p53 and bax expression, cytochrome *c* release and caspase activation.

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