Critical role of oxidative stress and sustained JNK activation in aloe-emodin-mediated apoptotic cell death in human hepatoma cells

Guo Dong Lu1,2, Han-Ming Shen2, Maxey C.M. Chung1,3 and Choon Nam Ong2,4

1 Department of Biochemistry, 2 Department of Community, Occupational and Family Medicine, Yong Loi Lin School of Medicine, 3 Department of Biological Sciences, Faculty of Science and 4 Office of Life Sciences, National University of Singapore, Singapore, 117597

To whom correspondence should be addressed. Tel: +65 6516 4982; Fax: +65 6779 1499;
Email: cofongcn@nus.edu.sg

Aloe-emodin (AE), one of the main bioactive anthraquinones of Rheum palmatum, possesses potent antitumor properties. Our previous proteomic study revealed that AE-induced apoptosis was associated with oxidative stress and oxidation of many redox-sensitive proteins. In this study, we aimed to further dissect the cell death-signaling pathways in AE-induced apoptosis. AE was found to cause redox imbalance and deplete the intracellular-reduced glutathione (GSH). Manipulation of the intracellular GSH with buthionine-sulfoximine (a GSH synthesis inhibitor) sensitized, and with glutathione monomethyl ester (a GSH donor) protected the AE-induced apoptosis, respectively. More importantly, AE treatment led to evident and sustained activation of c-Jun N-terminal kinase (JNK), an important stress-responsive mitogen-activated protein kinase (MAPK). Over-expression of an antioxidant gene sod1 significantly reduced AE-induced JNK activation and cell death, suggesting that oxidative stress-mediated JNK is the effector molecule in AE-induced apoptosis. Such a notion was clearly supported by subsequent studies in which JNK activation was inhibited by JNK inhibitor, JNK small interfering RNA knockdown or over-expression of dominant-negative JNK. In addition, we provided evidence demonstrating the critical role of apoptosis signal-regulating kinase 1, a well-established MAPK kinase kinase, in AE-induced JNK activation and apoptotic cell death. Finally, we showed that dissociation of inactive JNK–Glutathione S-transferase pi (GST-pi) complex was also involved in JNK activation through GST-pi oxidation. Taken together, these results suggest that AE-induced apoptotic cell death is mediated via oxidative stress and sustained JNK activation.

Introduction

Aloe-emodin (AE) is an active anthraquinone found in Chinese herb Rhubarb (Rheum palmatum). This compound has been reported to induce cell-cycle arrest and apoptosis in several cancer cell lines, including human hepatoma (1), leukemia (2), glioma (3) and lung carcinoma cells (4), although the underlying mechanisms are yet to be elucidated. In our recent proteomic investigation using HepG2 cells, AE was found to alter the expression of a number of proteins involved in oxidative stress, cell-cycle arrest, anti-metastasis and apoptosis (5). Among those affected proteins, redox-sensitive proteins [e.g. peroxiredoxins (PRDXs) and DJ-1] were of the highest up-regulated. Consistently, AE was capable of altering the expression of a number of proteins including human hepatoma (1), leukemia (2), glioma (3) and lung carcinoma (8).

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ethanol for 30 min before nuclear staining with 4,6-diamidino-2-phenylindole (300 nM) and visualized under an inverted fluorescence microscope. Cells with clear condensed nuclei were considered as apoptotic cells. Ten randomly selected fields with a total of 200 cells were examined.

**Analysis of intracellular glutathione (GSH/GSSG)**

Intracellular glutathione were analyzed as described previously (22). Briefly, after treatment, same number of cells were collected and re-suspended in sodium phosphate-ethylenediaminetetraacetic acid buffer. After cell homogenization, proteins were precipitated by mixing with 25% metaphosphoric acid. The supernatant was collected and the GSH content were measured by adding o-phthalaldehyde (50 μg/ml) for 15 min before fluorescence detection at 420 nm. For GSSG, same amount of supernatant were reacted with N-ethylmaleimide (40 μM) for 20 min before o-phthalaldehyde reaction and fluorescence measurement.

**Measurement of ROS production in cells**

The production of ROS was estimated by flow cytometry using CM-H2DCFDA (Molecular Probe, Eugene, OR) as described previously with minor modifications (22). Briefly, cells were pre-stained with CM-H2DCFDA at 37°C for 30 min and then treated with AE as indicated. After treatment, cells were collected and washed with phosphate-buffered saline for three times before analyzing by flow cytometry.

**Cell subfraction**

Cell subfraction was carried out as described previously (23). In brief, cells were collected and re-suspended in isotonic homogenization buffer (250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid, 0.1 mM phenylmethylsulfonyl fluoride and 10 mM Tris). After passing through gage #27 needle for 20 times to break the cell membrane, the cell homogenates were applied to a series of centrifugation (50 g, 10 min, 300g 20 min and 15 000g 20 min) to fractionate unbroken cells, heavy nuclear fraction and mitochondria fraction, respectively.

**Transient transfection**

HepG2 cells were transiently transfected with either empty vector or indicated expression vectors using Nucleofector® Device from Amaxa (Gaithersburg, MD) according to the manufacturer’s user manual. Cells were subjected to transient transfection with AE or dimethyl sulfoxide at 36 h after transfection in gene over-expression assay or 48 h in gene knockdown study.

**Immunoprecipitation and western blot**

At the end of treatment, cells were collected by scraping and then washed twice with ice-cold phosphate-buffered saline. Cells were lysed in buffer [20 mM Tris, 0.5% NP-40, 250 mM NaCl, 3 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-bis(aminohexanethio)tetraacetic acid, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 0.5 mM sodium vanadate and 1× Complete® protease inhibitor (Roche Molecular Biochemicals, Indianapolis, IN)] for 30 min. For analysis of MAPK molecules, cells were lysed in sodium dodecyl sulfate (SDS) lysis buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS and 10% glycerol). For immunoprecipitation, each sample was added sequentially with 2 μg antibody and 25 μl protein A/G agarose beads (Roche Molecular Biochemicals) and rotated overnight at 4°C. The beads were washed five times using ice-cold lysis buffer and then eluted with SDS sample loading buffer before separation by SDS–polyacrylamide gel electrophoresis. For western blot, equal amounts of proteins were fractionated on SDS–polyacrylamide gel electrophoresis in the Mini-PROTEAN II system (Bio-Rad, Hercules, CA) and blotted onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). Analysis of oligomerization of GST-pi was done in non-reducing conditions (no dithiothreitol in lysis buffer and no β-mercaptoethanol in SDS sample loading buffer) as described previously (24). After blocking with 5% non-fat milk in Tris-buffered saline Tween-20 (TBST), the membrane was probed with various antibodies and developed with enhanced chemiluminescence (Pierce, Rockford, IL) using a Kodak Image Station (Kodak, Rochester, NY).

**Results**

**AE induces apoptotic cell death via the mitochondria-dependent intrinsic pathway**

We have recently shown that AE was capable of inducing apoptosis in human hepatoma cells (5). Here, we further investigate the cell death-signaling pathway. As shown in Figure 1A, AE (40 μM) induces time-dependent increase of apoptotic cell death in HepG2 cells, visualized by 4,6-diamidino-2-phenylindole staining as typical apoptotic nuclear condensation. Pretreatment of a pan-caspase inhibitor z-VAD-fmk (25 μM) completely blocked AE-induced cell death (Figure 1A), suggesting that AE-induced cell death is caspase dependent. It was further evidenced by the time- and dose-dependent cleavage/activation of caspase-9 and caspase-3 (Figure 1B). Consistently, AE induced cleavage of PARP, one of the caspase-3 substrates. However, we failed to detect the cleavage/activation of caspase-8 (Figure 1B), the initiator caspase for the death receptor-mediated extrinsic apoptotic pathway. Next, there was evident translocation of cytochrome c from mitochondria to cytosol, preceding activation of caspase-9 and -3 (Figure 1C). These results thus suggest that AE-induced HepG2 apoptotic cell death is mainly executed via the mitochondria-dependent intrinsic pathway. And it is comparable with neuroblastoma cells, for which mitochondrial-dependent intrinsic but not extrinsic apoptotic pathway activation was involved in AE-induced apoptosis (25). We also examined this effect in two other hepatoma cell lines (HCCM and Hep3B cells). Both of them succumbed to AE-induced apoptotic cell death, as evidenced by the time-dependent increase of nuclear condensation (Figure 1D) and the cleavage of caspase-9, caspase-3 and PARP, but not caspase-8 (data not shown). Therefore, these findings suggest that the AE-induced intrinsic apoptotic cell death is not cell line specific.

**AE induces cell death by exhausting the intracellular GSH buffer system**

Our earlier study demonstrated the increased production of ROS and the up-regulation of various redox-sensitive proteins, such as PRDXs and protein DJ-1 upon treatment of AE (5). In the present study, we further examine redox alteration of PRDX [protein antioxidant and marker for oxidative stress in liver tissue and HepG2 cells (26)] and found that AE exposure markedly increased the oxidation of PRDX with the formation of cysteine sulfonic acid at its cysteine residues (Figure 2A). Transient knockdown of antioxidant PRDXs by siRNA sensitized HepG2 cells to cell death induced by AE (Figure 2B). These findings prompt us to investigate whether AE treatment results in redox imbalance (especially the intracellular GSH redox system) and its role in apoptosis. To capture the instant production of ROS, pretreatment of ROS dye indicator CM-H2DCFDA preceding AE treatment was carried out. As shown in Figure 2C, AE increases the intracellular ROS level as early as 1 h whereas the ratio of GSH to GSSG decreased coincidently (Figure 2D). As expected, both HCCM and Hep3B cells also increased ROS levels and depleted GSH when treated with AE (data not shown).

We next examined the involvement of the GSH buffer system in AE-induced apoptosis. Pretreatment with buthionine-sulfoximine (500 μM for 24 h), a specific GSH synthesis inhibitor (27), significantly decreased the intracellular GSH level (Figure 2E) and augmented apoptosis by AE (Figure 2F). In contrast, a GSH donor glutathione monomethyl ester (5 mM for 2 h) (28) significantly enhanced the intracellular GSH level (Figure 2E) and subsequently blocked AE-induced apoptosis (Figure 2F). Collectively, the above results suggest that AE may induce apoptotic cell death by inducing oxidative stress and exhausting the intracellular GSH buffer system.

**AE induces sustained activation of JNK**

It has been well established that MAPKs such as JNK are redox sensitive and apoptosis involved (13–15). Here, we examined whether the MAPK pathways (JNK, p38 and ERK) were involved in AE-induced hepatoma cell apoptosis. As shown in Figure 3, AE treatment induces sustained phosphorylation/activation of JNK, which is also evidenced by the phosphorylation of its downstream substrate c-Jun. On the contrary, no evident JNK and c-Jun phosphorylation could be found within the first 3 h of AE treatment (data not shown). Thus, AE-induced JNK activation is a delayed and sustained, but not an early and transient process. On the other hand, phosphorylation/activation of p38 remained unchanged by AE (Figure 3), although AE has been reported to induce apoptosis in human lung non-small cell carcinoma cell line H460 via p38 pathway (16). The pro-survival ERK pathway,
however, was found to be inhibited by AE treatment, as evidenced by the decreased phosphorylation/activation of ERK. This inhibitory effect is similar to the finding of Mijatovic et al. done in glioma cells (28). To exclude the cell-specific effect of AE, we also investigated MAPK pathways in HCCM and Hep3B cells and found similar results of sustained JNK activation and ERK inhibition (data not shown); suggesting that sustained JNK activation and ERK inhibition may contribute to AE-induced cell death.

**AE-induced apoptotic cell death and JNK activation is ROS dependent**

To further investigate the association of oxidative stress with MAPK activation and apoptosis, we transiently over-expressed antioxidant sod1 (pEGFP-C3/sod1) in HepG2 cells. Over 90% cells were transfected with sod1 and the expression level was sustained as long as 72 h (data not shown). We found that over-expression of antioxidant sod1 efficiently decreased AE-induced oxidative stress, as indicated by the significant decrease of PRDX oxidation (Figure 4A). Coincidently, over-expression of sod1 decreased AE-induced JNK phosphorylation/activation (Figure 4A). As a result, sod1 over-expression protected HepG2 cells against AE-induced apoptosis, as shown by the decrease of caspase-9 activation and PARP cleavage (Figure 4A) and the percentage of cells with nuclear condensation (Figure 4B). On the contrary, AE-induced ERK inhibition was unaffected by over-expression of antioxidant sod1 (Figure 4A), indicating that ERK inhibition by AE may not be involved in AE-induced apoptosis. Taken together, the above results suggest that oxidative stress-mediated JNK may be the effector molecule in AE-induced apoptosis.

**JNK activation plays a crucial role in AE-induced apoptosis**

The role of the JNK pathway in apoptosis is both cell type and stimulus dependent. The pro-apoptotic effect of JNK also depends on the extent and duration of its activation (14). We thus decided to evaluate the role of sustained JNK activation in AE-induced apoptosis through
both pharmacological and genetic approaches. On one hand, we found that AE-induced apoptosis can be partially prevented by cell-permeable JNK inhibitor III (Figure 5A). On the other hand, knocking-down of both \textit{Jnk1} and \textit{Jnk2} genes in HepG2 cells by siRNA significantly decreased JNK expression level as well as JNK phosphorylation/activation, when compared with cells transfected with control siRNA (Figure 5B). As expected, AE-induced apoptotic cell death was also inhibited by the declined cleavage of caspase-9, PARP (Figure 5B) and the percentage of cell death (Figure 5C). Taken together, JNK activation is involved in AE-induced apoptosis. This hypothesis was further supported by the protective effect of over-expressing dominant-negative JNK1 and JNK2. Similar to the result of knocking-down assay, inhibition of JNK activity by over-expression of dominant-negative JNK1 and JNK2 protected cells from AE-induced apoptosis (Figure 5D and E). On the contrary, over-expression of consistent active JNKK2-JNK1, which maintains sustained JNK1 activation, consistently augmented AE-induced apoptotic cell death (data not shown). Taken together, the above findings indicate that sustained JNK activation is crucial for AE-induced apoptosis.

**ASK1 enhances JNK activation and AE-induced apoptosis**

It is well established that oxidative stress can induce JNK activation by activating its upstream MAPK kinase kinase such as ASK1 (14). Redox-sensitive ASK1 can be activated by oligomerization after dissociation from its inhibitory partner thioredoxin in the presence of ROS (29). Here, we examined the role of ASK1 in AE-induced apoptosis. As shown in Figure 6A, AE induced sustained ASK1 activation by phosphorylation at site thr845, a conserved site for ASK1.
This high but tolerable ROS production may help cancer cells survive inactive JNK–GST-pi complex and its upstream ASK1 activation. AE-induced JNK activation was initiated from dissociation of the oxidative stress and sustained JNK activation in hepatoma cells. In this study, we found that AE induced apoptotic cell death via the inactive JNK–GST-pi complex via oxidative stress. Therefore, AE may also initiate JNK activation by dissociation of inhibitory thioredoxin from inactive ASK1–thioredoxin complex was also found upon treatment of AE (data not shown). Collectively, the above data suggested that activation of ASK1 is crucial for AE-induced sustained JNK activation and apoptosis.

Dissociation of GST-pi from JNK through GST-pi oxidation is also involved in JNK activation

As revealed by our previous proteomic study (5), AE treatment caused oxidation in many proteins in HepG2 cells. It is probable that AE-induced oxidative stress may also affect some proteins (e.g. GST-pi and thioredoxin) and in turn initiate apoptosis, since GST-pi and thioredoxin were redox sensitive in their cysteine residues and protein oxidations of these two proteins were found to be important for JNK-mediated apoptosis (24,29). We thus questioned whether AE can induce JNK activation via dissociation of GST-pi from the inactive JNK–GST-pi complex, another redox-sensitive mechanism for JNK activation (24). As shown in Figure 7A, the inhibitory binding subunit GST-pi co-precipitated with JNK decreases as early as 3 h, at least similar to the time of JNK activation (Figure 3). We next found that the reduced monomer form of GST-pi in the non-reducing gel but not the total GST-pi in reducing gel was decreased upon treatment of AE from 3 h (Figure 7B), suggesting GST-pi aggregation mediated by cysteine oxidation occurred upon AE treatment. Similarly, dissociation of inhibitory thioredoxin from inactive ASK1–thioredoxin complex was also found upon treatment of AE (data not shown). Therefore, AE may also initiate JNK activation by dissociation of the inactive JNK–GST-pi complex via oxidative stress.

Discussion

In this study, we found that AE induced apoptotic cell death via oxidative stress and sustained JNK activation in hepatoma cells. AE-induced JNK activation was initiated from dissociation of the inactive JNK–GST-pi complex and its upstream ASK1 activation. Sustained oxidative stresses are maintained in cancer cells (30). This high but tolerable ROS production may help cancer cells survive and proliferate through activating redox-sensitive transcription factors and responsive genes (e.g. nuclear factor-kappa B (NF-kB) and activator protein-1 (AP1)). However, when intolerable high level of ROS production (e.g. induced by therapeutic agents) reaches certain threshold, such as irreversible DNA damage, cells may switch to senescence or apoptotic cell death (31). Through manipulation of the redox balance, some phytochemicals, such as anthraquinones from Rhus succedanea (32) and polyphenols from grapes (33) seem to be good candidates for a direct or combined application in cancer chemotherapeutics and/or chemopreventives. On one hand, AE and other anthraquinones can counteract the harmful oxidative injury (34–36). On the other hand, therapeutic dose of AE and other anthraquinones can selectively induce apoptotic cell death through sustained and site-directed oxidative stress (8).

Owing to its quinone structure, AE may generate ROS in cells. And this effect has been confirmed by Lee et al. (8) and our recent report (5). To exclude the possibility that AE’s cytotoxicity was derived from an artificial production of H₂O₂ in culture medium, we tested the in vitro generation of H₂O₂ in the culture medium (without cell) by ferrous oxidation-xylene orange assay (37). Less than 10 µM hydrogen peroxide can be detected (data not shown) when AE only was incubated in the culture medium as long as 24 h. Thus, the cytotoxicity of AE is unlikely to be derived from the artificial production of H₂O₂ in culture medium.

To evaluate whether AE could specifically inhibit hepatoma cells against other normal cells, trypan blue exclusion assay was carried out after indicated treatment of AE in HepG2 cells together with several normal cells, including BEAS-2B (human airway epithelial cell line), OK (opossum kidney proximal tubule cell) and HACAT (human

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Fig. 3. AE induced sustained JNK activation and ERK inhibition. HepG2 cells were treated with indicated dose of AE for a serial of time as indicated. The phosphorylation/activation of JNK, ERK and p38 were detected by western blot.

Fig. 4. Over-expression of antioxidant sod1 reduced JNK activation and apoptosis by AE. HepG2 cells were electrotransfected with EGFP-sod1. After 36 h, cells were treated with AE or dimethyl sulfoxide vector for 24 h. (A) Cleavage of caspase-9 and PARP, phosphorylation/activation of JNK and oxidation of PRDX were investigated by western blot. (B) Quantification of the cell death by counting the percentage of apoptotic cell among the transfected cells in a total of 200 randomly selected cells.
keratinocyte cell line). We found that higher concentration (up to 100 µM) of AE failed to induce a similar cytotoxicity in these normal cells as AE did in HepG2 cells (half inhibitory concentration (IC50): 20 µM, data not shown). It thus suggested that AE specifically inhibited HepG2 cell growth. The lower cytotoxicity of AE has also been reported previously in other normal cells, including human gingival fibroblasts (38), human lung fibroblast (MRC5) cells, hemopoietic progenitor cells (39) and primary astrocytes and fibroblasts (3). This anticancer specificity further suggests that AE could be a potent chemotherapeutics or chemopreventive compound. It is noteworthy that endothelial cells (bovine aortic endothelial cells and human umbilical vein endothelial cells) were recently reported to be sensitive to AE (40). This property was suggested to be useful for modulation of angiogenesis, as well as the antitumor effects (40).

Our results showed that exposure of AE interrupted the redox balance and decreased the intracellular GSH to GSSG ratio and in turn induced intrinsic apoptotic pathway. Pretreatment of the cell-permeable GSH synthesis inhibitor BSO sensitized AE-induced cell death, whereas the glutathione-donor glutathione monomethyl ester completely protected it (Figure 2E). Consistently, over-expression of antioxidant enzyme sod1 effectively protected cells from AE-induced apoptosis (Figure 4). These results clearly suggested the crucial proapoptotic role of oxidative stress in AE-induced apoptotic cell death.

On the other hand, protein oxidation, especially on the cysteine residues, may be crucial for AE-induced apoptosis. In most eukaryotes, PRDXs are highly conserved and extremely abundant antioxidant enzymes (41). Oxidation of the unique PRDX homolog Tpx1 in Schizosaccharomyces pombe is essential for peroxide-induced activation of the JNK/p38 homolog.

Fig. 5. Sustained JNK activation by AE is crucial for AE-induced cell death. (A) Cells were pretreated with cell-permeable JNK inhibitor III for 1 h and then 40 µM AE for 48 h. The percentage of cell death was counted by 4’,6-diamidino-2-phenylindole staining. (B and C) JNK1 and JNK2 (JNK1/2) were transiently knocked-down in HepG2 cells. After 48 h, transfected cells were treated with AE for 24 h. (D and E) HepG2 cells were transiently transfected with dominant-negative JNK1 and JNK2 protein vector and then treated with AE for 24 h. Cleavage of caspase-9 and PARP, oxidation of PRDX and phosphorylation/activation of JNK were investigated by western blot. Cell death was determined as above.
Stry 1 (42). Wood et al. (41) suggested that eukaryotic PRDXs evolved to act as a ‘floodgate’: PRDXs are sensitive to H$_2$O$_2$ but can be rapidly inactivated to allow increasing H$_2$O$_2$ levels to stimulate signal transduction. We found that knocking-down of PRDXs sensitized the cells to AE-induced apoptosis (Figure 2B). Although PRDXs normally function to protect cells from oxidative stress-induced cell death (43,44), the physiological association of PRDXs and JNK in mammalian cells and the proposed floodgate effect of endogenous PRDXs (41) are still yet to be further investigated.

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In addition, protein oxidation occurred in GST-pi by decreasing the reduced monomer form of GST-pi (Figure 7B), which has been reported as an essential redox-sensitive pathway for JNK activation (24). The coincidence in the decrease of GST-pi co-precipitated with JNK and JNK activation suggested that JNK activation was initiated by AE-induced GST-pi oxidation (Figure 7A). In addition, ASK1 (JNK upstream kinase) could be activated in a similar mode by dissociation from its inhibitory binding subunit thioredoxin in dependent of ROS (29). Consistently, we found that thioredoxin co-precipitated with
ASK1 was decreased from 6 h onward (data not shown), which in turn activated ASK1. Taken together, AE-induced oxidative stress may activate JNK and ASK1 by oxidizing their respective redox-sensitive inhibitory binding subunit GST-pi and thioredoxin.

It has now been well recognized that activation of JNK consists of two different modes. The earlier and transient activation is through the signaling cascade of pro-inflammatory cytokines (tumor necrosis factor-α and interleukin-1), whereas the delayed and sustained activation is mediated by ROS (14,45). On the other hand, the pro-apoptotic effect of JNK depends on the extent and duration of its activation: sustained JNK activation may lead to apoptotic cell death, whereas transient activation is not capable of inducing apoptosis and even have an anti-apoptotic effect. The sustained JNK activation is suggested to be mainly due to its upstream kinase ASK1, another redox-sensitive pro-apoptotic kinase (14). ASK1 is a ubiquitously expressed MAPK kinase kinase that activate both JNK and p38 by phosphorylating respective MAPK kinases. In the presence of ROS, ASK1 itself was activated by oligomerization after dissociation from its inhibitory binding subunit thioredoxin (18).

Induction of differentiation but not apoptosis has been observed to be accompanied with ERK inhibition (within 4 h) in rat C6 glioma cells by AE treatment, although JNK and p38 pathways were not involved (3). However, in this study, the delayed and sustained mode of JNK activation was not investigated. In the present study, we found that AE treatment resulted in sustained activation of JNK which was responsible, at least in part, for apoptosis induction in the hepatoma cells. It is clearly demonstrated by the findings that AE-induced apoptosis could be reversed by both pretreatment of pharmacological JNK inhibitor and genetic manipulation of JNK expression. This action of AE in hepatoma cells. Our findings suggest that AE may induce apoptotic cell death via marked oxidative stress and sustained JNK activation.

Acknowledgements

Conflict of Interest Statement: None declared.

References


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