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Cepharanthine triggers apoptosis in a human hepatocellular carcinoma cell line (HuH-7) through the activation of JNK1/2 and the downregulation of Akt

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Abstract **Cepharanthine (CEP), a biscoclaurine alkaloid, has been reported to induce cell death, however, the molecular mechanism of this phenomenon remains unclear. We herein report that CEP induced apoptosis in HuH-7 cells through nuclear fragmentation, DNA ladder formation, cytochrome *c* release, caspase-3 activation and poly-(ADP-ribose)-polymerase cleavage. CEP triggered the generation of reactive oxygen intermediates, the activation of mitogen activated protein kinase (MAPK) p38, JNK1/2 and p44/42, and the downregulation of protein kinase B/Akt. Antioxidants and SP600125, an inhibitor of JNK1/2, but not inhibitors of p38 MAPK and MEK1/2, significantly prevented cell death, thus implying that reactive oxygen species and JNK1/2 play crucial roles in the CEP-induced apoptosis of HuH-7 cells.**

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Keywords: Cepharanthine; Apoptosis; MAPK; PARP; SP600125

1. Introduction

Cepharanthine (CEP) is a natural alkaloid extracted from *Stephania cepharantha* Hayata [1], which has been widely used in Japan for the treatment of snake venom-induced hemolysis bronchial asthma and other types of allergic inflammation, alopecia areata, and leukopenia during radiation therapy or anti-cancer treatment without any serious side effects [2,3]. Okamoto et al. reported that CEP inhibits HIV-1 replication, suppresses PMA-induced production of inflammatory cytokine and a chemokine in monocyte/macrophage cultures, and also protects differentiated human neuroblastoma cells death induced by TNF-α and gp120 [3]. CEP has also been shown to prevent vascular injury induced by LPS [4]. Goto et al. demonstrated that CEP is beneficial for the treatment of newborn endotoxic shock [5]. Evidence is accumulating supporting the hypothesis that CEP potentiates the activity of some anti-cancer agents and restores the effect of anti-cancer drugs in multi-drug-resistance cancer cells possibly through perturbing the plasma membrane function in order to increase the intracellular accumulation of anti-cancer drugs [2,6]. Some recent reports have provided evidence that CEP itself displays both a direct anti-tumor effect, thereby inhibiting the growth of Ehrlich ascites tumors [7] and inducing apoptosis in human leukemia cell lines [8] as well as an indirect anti-tumor effect by interacting positively with human interferon [9]. Nakajima et al. recently reported that CEP co-treatment enhances the cytotoxicity of doxorubicine resistant hepatocellular carcinoma cell lines [10]. Nishikawa et al. have shown that CEP enhances adriamycin accumulation in cancer cells but lowers its accumulation in normal liver cells in both, in vitro and in vivo [11], thus suggesting that CEP plays a beneficial role in potentiating the effect of chemotherapy on the drug resistance of hepatocellular carcinoma.

The family of serine–threonine protein kinases plays an important role in apoptosis and survival signaling pathways. The major components belonging to this family are p38 mitogen activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), extracellular-signal regulating kinase1/2 (ERK1/2 or p44/p42), and protein kinase B (PKB or Akt), which are activated in response to a variety of stimuli. JNK and p38 MAPK signaling molecules are predominantly activated by the inflammatory cytokines, environmental stress, and oxidative stress, thus leading to cell differentiation and apoptosis [12–14]. p44/42 MAPK and Akt, on the other hand, are preferentially activated by mitogen through the Ras/Raf/MEK (MAP kinase kinase) and Ras/PI3-K signaling pathways, respectively; thus leading to cell growth and survival [15]. Although it has been reported that CEP causes death in hepatocellular carcinoma cell lines [10], to the best of our knowledge there have so far been no reports available on the mechanism of hepatocellular carcinoma cell death induced by CEP. The present study was undertaken to investigate the effect of CEP on human hepatocellular carcinoma using a human hepatocellular carcinoma cell line, HuH-7, and the underlying intracellular signal transduction pathways involved. We herein show evidence that CEP induced apoptosis

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Abbreviations: CEP, cepharanthine; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; ROS, reactive oxygen species; NAC, *N*-acetyl cysteine; PARP, poly-(ADP-ribose)-polymerase

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in HuH-7 cells through the generation of reactive oxygen species (ROS), the activation of stress activated kinase JNK1/2, MAPK p38, and ERK p44/42, and the downregulation of the protein kinase B (Akt).

2. Materials and methods

2.1. Reagent

CEP was purchased from Kaken Shoyaku Co. Ltd. (Tokyo, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Dojindo (Kumamoto, Japan). Hoechst 33258 (Bisbenzimide H33258) fluorochrome and phenylmethylsulfonyl fluoride were purchased from Wako Pure Chem. Inc. Ltd. (Osaka, Japan). Protease inhibitor (complete cocktail) was purchased from Boehringer–Mannheim (Germany). Hundred base pairs DNA ladder size marker was bought from Invitrogen (California, USA). *N*-Acetyl cysteine (NAC) and 5,6-carboxy-2,7-dichlorofluorescein-diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N*-*t*-Butyl-α-phenylnitrone (PBN) was purchased from ICN Biomedicals, Inc. (Ohio, USA). SB203580, SP600125 and Caspase-3 inhibitor 1 were obtained from Calbiochem (San Diego, CA, USA). U0126 was obtained from Promega (Madison, WI, USA). CPP32-like protease activity assay kit was purchased from MBL (Nagoya, Japan). Any other chemicals not specifically cited here were purchased from the Sigma Chemicals (St. Louis, MO, USA).

2.2. Antibodies

The polyclonal antibodies to p38, JNK/SAPK, and phospho-p38, phospho-JNK/SAPK, phospho-Akt (serine-473), ATF-2, phospho-ATF-2, and the monoclonal antibodies to Akt were purchased from Cell Signaling Technology (MA, USA). β-Actin and poly-(ADP-ribose)-polymerase (PARP) were purchased from Sigma (St. Louis, MO, USA) and Oncogene (Cambridge, MA, USA), respectively. Cytochrome *c* antibody was obtained from PharMingen (San Diego, CA, USA).

2.3. Cells and cell culture

Human hepatocellular carcinoma cell line, HuH-7, was obtained from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in 10% FBS-containing DMEM media (Gibco-BRL, USA) with the appropriate antibiotics at 37 °C in the presence of a humidified atmosphere of air (95%) and CO_2 (5%). All experiments were performed in the presence of a low serum (0.1% serum).

2.4. Cell viability test

MTT assays were applied to test cell viability, as described by Biswas et al. [16]. In brief, the cells were seeded at a density of $2–3 \times 10^4$ cells per well in 96-well dishes. The cells were then incubated under various conditions as indicated. Subsequently, MTT (0.5 mg/ml final conc.) was added to each well. After 3 h of additional incubation, 100 μl of a solution containing 10% SDS (pH 4.8) plus 0.01 N HCl was added to dissolve the crystals. The absorption values at 570 nm were determined with an automatic ELISA plate reader (Immuno Mini NJ-2300, Japan).

2.5. Nuclear staining with Hoechst 33258

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with Hoechst 33258 fluorochrome as described by Biswas et al. [16].

2.6. DNA fragmentation assay

To characterize the cell death pattern, DNA ladder assays were performed as previously described [16]. Briefly, 10^6 cells were lysed in 100 μl of 10 mM Tris–HCl buffer (pH 7.4) containing 10 mM EDTA and 0.5% Triton X-100. After centrifugation for 5 min at 15,000 rpm, supernatant samples were treated with RNase A and protenase K. Subsequently, 20 μl of 5 M NaCl and 120 μl isopropanol were added to the samples and kept at −20 °C for 6 h. Following centrifugation for 15 min at 15,000 rpm, the pellets were dissolved in 20 μl of TE buffer (10 mM Tris–HCl and 1 mM EDTA) as loading samples. To assay the DNA fragmentation pattern, samples were loaded onto 1.5% agarose gel and electrophoresis was carried out.

2.7. ROS assay

The formation of intracellular H_2O_2 as production was determined using dichlorofluorescein diacetate (DCFH-DA) as previously described [16].

2.8. Preparation of the cytosolic fractions, cell lysates, and Western blotting

Cellular fractions and Western blotting were carried out as described by Sarker et al. [14].

2.9. Assay of caspase-3/CPP32 enzyme activity

Caspase-3 enzyme activity was analyzed according to the manufacture's instructions as described by Sarker et al. [17].

2.10. Statistical analysis

All experiments were performed in triplicate. The results of multiple observations were presented as the means ± S.D. of at least three separate experiments. Statistical significance was determined by Student's *t*-test. A value of $p < 0.05$ was considered to be significant.

3. Results

3.1. CEP induces apoptosis in hepatocellular carcinoma cells

We evaluated for the first time the effect of CEP on the viability of hepatoma cells by stimulating HuH-7 cells with various concentrations of CEP in the presence of a low serum (0.1%) for 24 h. As shown in Fig. 1A, CEP dose dependently triggered cell death. Cell death was not significantly observed up to 10 μM CEP exposure but a marked decrease in cell viability (74 ± 8%) was observed at 15 μM CEP, which in turn sharply decreased to (60 ± 7%) when cells were exposed to 20 μM CEP. Fig. 1B shows that the cells shrank, became rounded, and the phase became bright in response to CEP treatment which was consistent with the results presented above. We then investigated the effects of CEP on the cell viability over time. A loss of cell viability was evident at 6 h (89 ± 5%), which in turn, noticeably increased to (77 ± 8%) at 12 h and a sharp increase (60 ± 7%), 24 h after 20 μM of CEP exposure (data not shown). Because chromatin condensation, nuclear fragmentation and DNA ladder formation are notable features of programmed cell death [18,19], we next sought, to examine whether CEP-induced cell death was followed by these features. In response to CEP (20 μM), both chromatin condensation and nuclear fragmentation were observed by fluorescence microscopy (Fig. 1C) and DNA ladder formation was detected by agarose gel electrophoresis (Fig. 1D), thus suggesting that CEP-induced HuH-7 cell death was due to apoptosis.

3.2. CEP induces the production of ROS

We then examined whether or not CEP-induced cell death was followed by oxidative stress. As shown in Fig. 2, CEP at a dose of 20 μM, which caused a significant degree of apoptosis, also caused a marked induction of ROS. Consistent with this result, NAC and PBN, specific scavengers of ROS, significantly attenuated CEP-induced apoptosis (cells viability increased to 85 ± 8% and 88.86 ± 7% by the pretreatment of cells with 500 μM of either NAC and PBN, respectively (data not shown), thus suggesting that CEP induced HuH-7 cell death followed by oxidative stress.

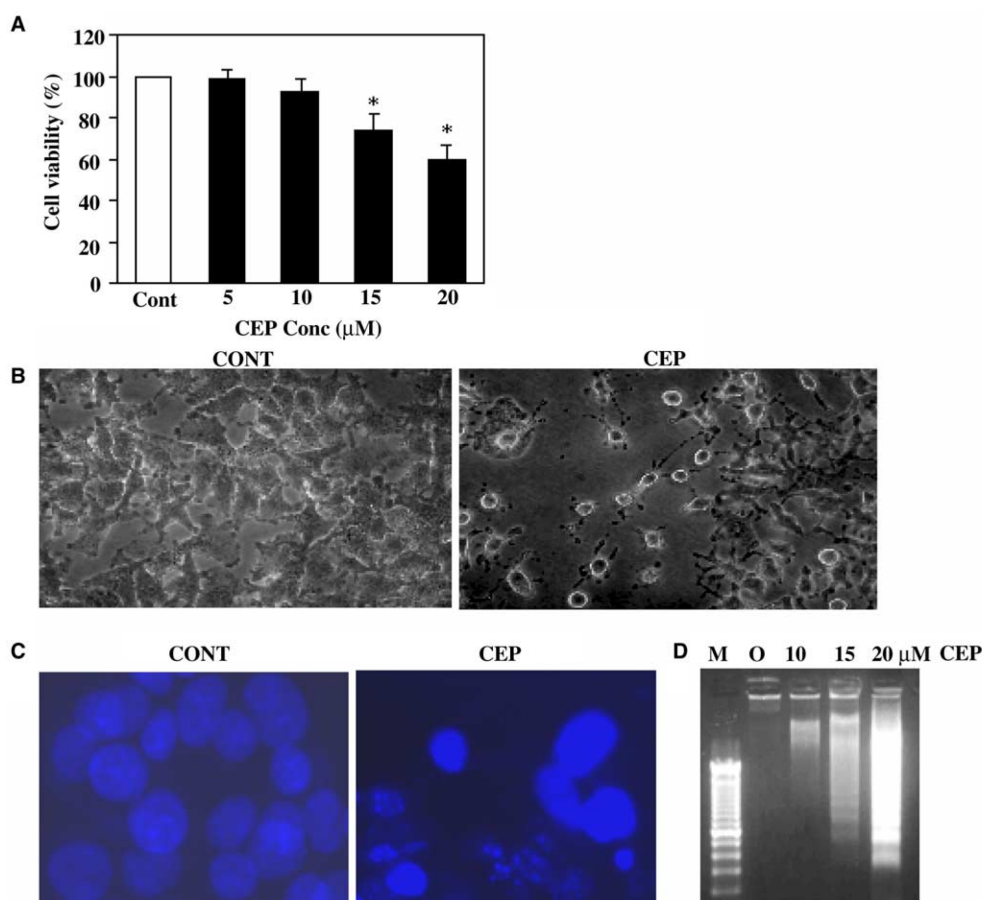

Fig. 1. CEP induces apoptosis in HuH-7 cells. The cells were subjected to various concentrations of CEP for 24 h (A). Cell viability was determined by an MTT assay. The data shown are the percentages of the control values ± S.D. ($^{*}p < 0.05$ vs. control). Microscopic observations of cell morphology (B,C) in the absence (left panels) or in the presence (right panels) of 2 μM CEP. (B) The phase contrast (original magnification 200×). (C) Hoechst staining (original magnification 400×). Note that the cells which stimulated with CEP exhibited apoptotic features characterized by condensed/fragmented nuclei. (D) DNA ladder formation. The cells were treated with the indicated concentrations of CEP for 24 h and an internucleosomal DNA fragmentation was analyzed on 1.5% agarose gel electrophoresis. M, indicates the 100-bp DNA ladder size marker. The data shown are the representatives of three independent experiments.

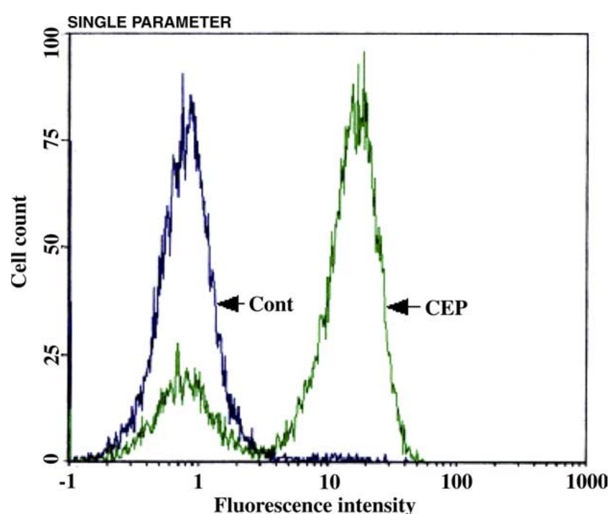

Fig. 2. CEP causes the generation of ROS. The cells were treated with 20 μM CEP for 1 h in the presence of DCFH-DA and then harvested, and the fluorescence of ROS was evaluated by FACS analyzer. Note that CEP caused a marked induction of ROS. The data shown are the representatives of three independent experiments.

3.3. CEP triggers the activation of MAPK p38, JNK1/2 and ERK1/2

Based on these findings, we sought to examine whether CEP-induced hepatoma cell death was accompanied by the activation of common pro-apoptotic signaling pathways. CEP was found to activate JNK and p38 MAPK, which mediate apoptosis in different experimental models [12–14]. The phosphorylation of both JNK and p38 MAPK was time-dependently induced by CEP (Fig. 3A). p38 MAPK and JNK activation could be detected within 7.5 min, and dramatically increased for 1 h, and thereafter was sustained for 4 h. Consistent with such JNK and p38 MAPK activation, CEP induced the activation of c-Jun (Ser 73) and ATF-2, the down-stream targets of JNK and p38. We also observed that ERK1/2 and Akt, which are generally known to promote cell survival [15] were reversely regulated in response to CEP. A marked activation of ERK1/2 was detectable at 7.5 min which thereafter maintained constant levels for 4 h, whereas protein kinase B (Akt) was downregulated in response to CEP. We next evaluated the possible roles of these activated kinases in CEP-induced cell death. As shown in Fig. 3B, a potent and a specific inhibitor of JNK, SP600125, noticeably blocked CEP-induced cell death (the cell viability increased from 60 ± 7% to 87 ± 7%), while SB203580, a specific

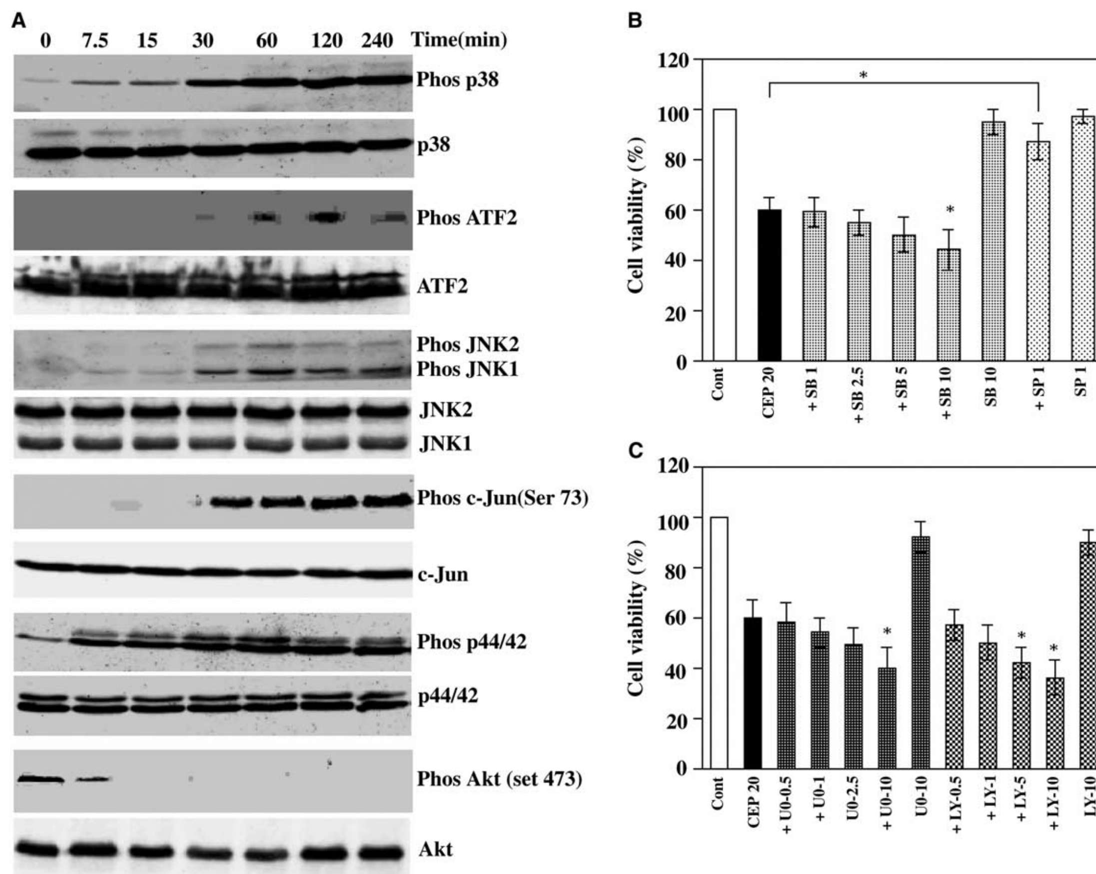

Fig. 3. CEP triggers the activation of p38, ATF-2, JNK, c-Jun, and p44/42 and downregulation of Akt. (A) The cells were stimulated with 20 μM of CEP for the indicated time periods and the activation and expression levels of different kinases and the indicated transcription factors were determined by immunoblotting as described in Section 2. (B,C) The effects of SB203580 and SP600125, and U0126 and LY290042 on CEP-induced apoptosis. The cells were pretreated with various concentrations of SB (SB203580: 1, 2.5, 5, and 10 μM) and SP 1 (SP600125: 1 μM), and U0 (U0126: 0.5, 1, 2.5, and 10 μM) and LY (LY290042: 0.5, 1, 5, and 10 μM), which are all specific and potent inhibitors of p38 and JNK & MEK1/2 and PI3-K, respectively, for 1 h. Cell viability was determined after a 24-h exposure of 20 μM CEP. The data shown are the percentages of the control values ± S.D. ($^{*}p < 0.05$ vs. CEP).

inhibitor of p38 MAPK instead of protection dose dependently enhanced CEP-induced cell death (the cell viability significantly decreased from 60 ± 7% to 44 ± 8% in the presence of 10 μM SB203580). Like SB203580, pretreatment of cells with U0126, a potent and specific inhibitor of MEK1/2, the up-stream of p44/42 signaling molecule, as well as LY29004, a specific inhibitor of PI3-K, the up-stream of Akt, could not prevent CEP-induced cell death (Fig. 3C). We also found that U0126, and SB203580, blocked activation of ERK1/2, and p38, respectively (data not shown). Data presented above indicates that ROS generation and JNK activation are positively correlated with CEP-induced apoptosis of HuH-7 cells. It is of interest to examine whether ROS act up- or down-stream of JNKs. We found that pretreatment of cells with either NAC or PBN completely blocked CEP-induced ROS accumulation, whereas failed to inhibit JNK activation (data not shown). Alternatively, SP600125 did not affect CEP-induced ROS production (data not shown), indicating that ROS production and JNKs activation represent two independent pathways.

3.4. CEP triggers cytochrome c release and PARP cleavage

Biochemical events such as the release of cytochrome *c* from mitochondria into cytosol, caspases activation, and PARP cleavage occur during mitochondrial-mediated apoptotic cell death. We therefore investigated whether CEP-induced cell death was also followed by these biochemical events. We noticed that after a 2-h exposure of CEP, cytochrome *c* was detectable and it significantly increased at 4 h in the cytoplasmic protein fraction (Fig. 4A). CEP has been shown to activate caspase-3 in jurkat cell apoptosis [8]. In addition, the release of cytochrome *c* into cytosol has been well documented to trigger caspase activation, thus resulting in cell death. As shown in Fig. 4B, CEP stimulated a 4.2 ± 0.3-fold increase in caspase-3-like activities at 12 h and the treatment of HuH-7 cells with a specific and potent inhibitor of caspase-3 significantly blocked CEP-induced apoptotic cell death (cells viability increased to 82 ± 8% in the presence of 10 μM caspase-3 inhibitor, data not shown), thus indicating the CEP-induced cell death followed by caspase-3 activation. Once caspase-3 is activated, a number of cellular proteins are cleaved, including PARP. To further confirm the activation of caspase-3 in CEP-induced cell death, we determined the PARP cleavage by Western blotting. As shown in Fig. 4C, CEP treatment markedly induced PARP cleavage, thus suggesting that mitochondrial events were also associated with the CEP-induced hepatoma cell death process. We further investigated whether or not ROS production and JNK activation occur up-stream of mitochondria. For this purpose, cells were pretreated with

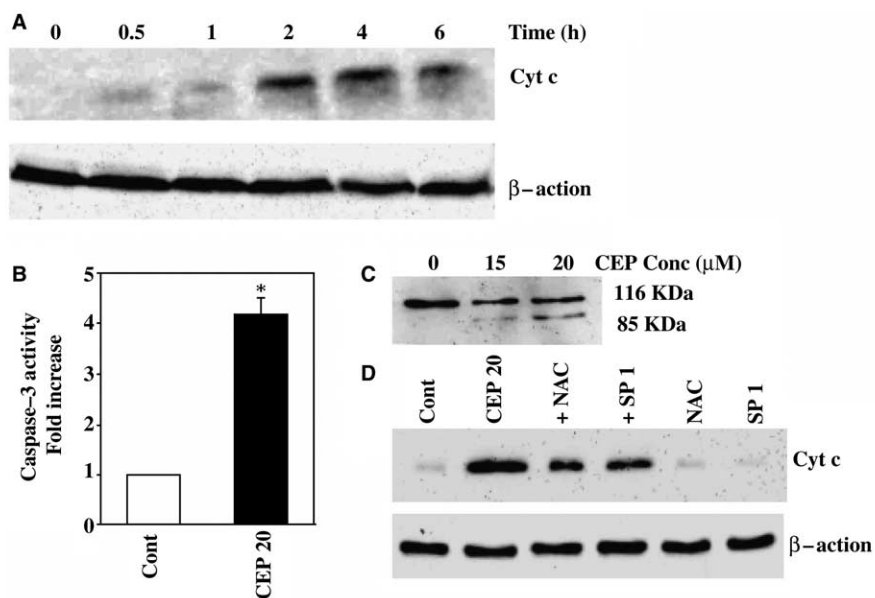

Fig. 4. CEP induces the cytochrome *c* release from the mitochondria and PARP cleavage via caspase-3 activation. (A) The cells were treated with CEP for the indicated periods of time and the cytosolic fractions were analyzed for the levels of cytochrome *c* (Cyt c, upper panel) by immunoblotting. β-Actin was used for the loading control (lower panel). Blots represent three experiments. (B) The cells were treated with 20 μM of CEP for 12 h. At the end of the treatment, caspase-3 like protease activity was assayed. The data are the means of ±S.D. ($^{*}p < 0.05$ vs. CEP) of three individual experiments. (C) PARP cleavage in CEP-treated cells was determined by immunoblotting. Blot represents four experiments. (D) The cells were pretreated with NAC (500 μM) or SP600125 (1 μM) and then exposed to 20 μM of CEP for 4 h and the cytosolic fractions were analyzed for the levels of cytochrome *c* by immunoblotting. Blot represents three experiments.

NAC or SP600125 for 1 h, followed by the 4-h exposure of CEP, and cytochrome *c* release was evaluated. As shown in Fig. 4D, both of NAC and SP600125 partially blocked CEP-induced cytochrome *c* release. On the other hand, caspase-3 inhibitor did not affect either ROS generation or JNKs activation induced by CEP (data not shown), suggesting that ROS and JNK are up-stream players in the CEP-induced HuH-7 cells death (Fig. 5).

4. Discussion

In the present study, we demonstrated that CEP induces apoptosis in human hepatocellular carcinoma, HuH-7 cells, in a dose- and time-dependent fashion. CEP induced cell death followed by chromatin condensation, nuclear fragmentation, and DNA ladder formation, which are the hallmark features of apoptosis [18,19] in HuH-7 cells. Our findings are consistent with the findings of previous reports which demonstrated that CEP induces apoptosis in human leukemia cell lines, Jurkat and K562, murine P388 doxorubicin-sensitive (P388), and resistant (P388/Dox) cells [8,20]. We also investigated the linkage between oxidative stress and cell death. Our results showed that CEP treatment conferred oxidative stress to the cells (Fig. 3A) and these results are in line with recent reports that CEP (1–10 μg/ml) induced ROS in murine p388 doxorubicin-resistance (p388/Dox) cells even though CEP did not induce ROS in murine doxorubicin-sensitive (p388/S) cells [20].

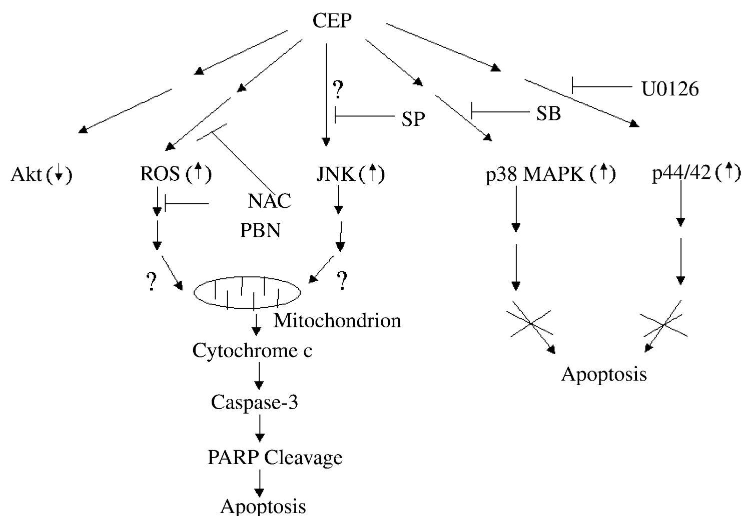

Fig. 5. A putative model for the CEP-induced apoptosis of HuH-7 cells.

However, contradictory reports regarding the generation of ROS have also been published. For example, Aakamatsu et al. reported that CEP is an effective ROS scavenger as it significantly reduces the levels of ROS generated by neutrophils [21]. These differences in their findings may be due to differences in the cell types.

The family of MAPKs play a key role in cell survival/death in many physiological and pathological settings. It is well documented that JNK or p38 MAPK activation leads to apoptosis in various experimental model systems [13,14]. A study by Ghatan et al. has shown that p38 MAPK can induce mitochondrial translocation of Bax in neurons undergoing oxidative stress-induced apoptosis [22]. Recently, we have reported that JNK and p38 MAPK are activated and positively correlated in oxidative stress-induced cell death [14,23]. However, contradictory findings have also been reported. For example, p38 MAPK was not implicated in the death of WEHI-231 and NIH3T3 cells [24]. On the other hand, the p38 MAPK is required for proliferation of leukemia, lymphoma cell lines [25]. JNK activation has been found to have no role in TNF-α-mediated apoptosis in MCF-7 cells [26]. Moreover, JNK activity has been shown to be required for growth of T98G glioblastoma [27]. A growing body of evidence shows that JNK activation plays pro-apoptotic role only if its activation is prolonged [13,28,29]. The process of cell survival occurs in conjunction with apoptosis, resulting in the inhibition or delay of apoptotic cell death. Activation of p44/42 MAPK and protein kinase B (Akt) are generally associated with cell proliferation and survival [15]. In contrast, p44/42 has been found to be activated in various apoptotic models. Recently, p44/42 MAPK activation has been demonstrated to be essential in cisplatin-induced apoptosis [30].

In the present study, we found that CEP triggered the activation of JNK, p38 MAPK, and p44/42 and the downregulation of Akt. We examined the functional relationship between the MAPK activity and the cell death process and thus observed that the JNK activity, but not the p38 or p44/42 MAPK activity, played an essential role in CEP-induced apoptosis (Fig. 3B and C) as SP600125, the pharmacological inhibitor of JNK but not U0126 or SB203580 significantly abrogated CEP-induced apoptosis, thus suggesting that p38 MAPK and p44/42 may not be involved in CEP-induced cell death. It has been reported that SB203580 can activate the Raf-1 activity in quiescent smooth-muscle cells [31]. JNK activation has also been reported to be inhibited by a p38 MAPK inhibitor [32]. To rule out any non-specific function of SB203580 and U0126 that might occur, specially, at a higher concentration, we incubated the cell with a wide range of concentrations (nanomolar to micromolar) of these inhibitors. However, we did not notice any change of JNK, p44/42 activation, or cell death by SB203580 (data not shown). Actually, the p38 MAPK subfamily which consists of p38α, p38β, p38γ, and p38δ isoforms has been shown to play different roles. Nemoto et al. reported that p38α induces apoptosis, whereas p38β inhibits apoptosis, indicating that each member of the p38 MAPK subfamily has unique role in apoptosis [33]. SB203580, the pyridinyl imidazole compound that was used in the present study for blocking the p38 MAPK, has been reported to inhibit α and β isoforms, while the γ and δ isoforms are insensitive to this compound [33]. Hence, we cannot rule out the possibility of opposite roles that may be played by other isoforms of p38 MAPK in CEP-induced HuH-7 cell apoptosis. However, our results are partly in agreement with the findings of Wu et al. who reported that p38 plays an anti-apoptotic role, while p44/42 appeared to be a pro-apoptotic player in CEP-induced Jurkat and K562 cells [8]. Wu et al. also demonstrated that CEP did not cause the activation of JNK in either Jurkat and K562 cells [8]. Interestingly, we observed a marked activation of JNK as well as its down-stream target, c-Jun (serine 73) in HuH-7 cells in a time dependent fashion and the JNK activity was found to be required for the induction of cell death. Wu et al. reported that CEP induces a marked activation of p44/42 in K562 cells but not in Jurkat cells [8]. Therefore, it appears that ROS, JNK, and p44/42 mediate CEP-induced cell death in a cell type specific manner. The generation of ROS and the activation of p38 MAPK and JNK are correlated with apoptosis in many cell types. It has been reported that stress-activated MAPKs (p38 and JNK) can be activated in a ROS-dependent as well as independent manner [29,34,35]. JNK dependent mitochondrial damage has also been shown in oxidative stress-induced cell death [36]. We observed that ROS and JNK are positively correlated with CEP-induced HuH-7 cells death. We are therefore interested in investigating the link between JNK activation and ROS production. We found that inhibition of ROS production did not prevent activation of JNK, whereas inhibition of JNK did not suppress ROS accumulation (data not shown). These observations suggest that JNK activation and ROS production represent two independent pathways in the CEP-induced cell death. The important issue that arises in this study is how JNK is activated in CEP-induced cell death. There are several lines of evidence that vanadate can trigger phosphorylation of JNK independently of ROS [35]. Clerk et al. reported that factors other than ROS are involved in the activation of SAPKs/JNKs during ischemia/reperfusion [37]. Although the exact signaling pathway for ROS-independent JNK activation in CEP-induced cell death remains to be elucidated, one possible mechanism could be that CEP treatment somehow causes tyrosine phosphorylation in JNK or inhibits phosphatases in HuH-7 cells, resulting in activation of JNK and cell death.

In addition, we found that CEP treatment caused a downregulation of PKB/Akt and pretreatment of cells with LY29004 instead of prevention, significantly enhanced CEP-induced cell death indicating that Akt might have a survival role in CEP-induced HuH-7 cell death. Our results are supported by the recent findings that FTY720, a novel anti-cancer agent, induces apoptosis in hepatoma cell lines both in vitro and in vivo by downregulating Akt [38]. As a result, our findings suggest that CEP-induced HuH-7 cell apoptosis is accompanied by the activation of JNK and the downregulation of Akt. The release of cytochrome *c* and other apoptogenic factors from the injured mitochondria has been shown to activate caspases [39]. Caspases can modulate p38 MAPK/JNK activation in TNF-α or Fas-induced apoptosis [40] and caspase inhibitor can attenuate p38/JNK activation [41], thus suggesting that these stress-activated kinases may be dependent upon caspase activation. However, JNK-mediated caspase activation has also been reported [42]. We observed that CEP-induced apoptosis was accompanied by the mitochondrial release of cytochrome *c* followed by the activation of caspase-3 and cleavage of PARP (Fig. 4A–C). Our results also show that a potent inhibitor of caspase-3 significantly abrogated cell death (data not shown), thus implying that caspase-3 protease is involved in the CEP-induced apoptosis of

HuH-7 cells. These results are consistent with the recent finding that CEP induces apoptosis in human leukemia and adenosquamous cell carcinoma cell lines through the activation of caspase-3 and the induction of PARP cleavage [8,43]. Furthermore, NAC and SP600125 partially inhibited CEP-induced cytochrome *c* release (Fig. 4D). We also observed that caspase-3 inhibitor failed to inhibit ROS generation and JNK activation (data not shown), thus implying that ROS generation and JNK activation may be up-stream of mitochondrial-associated events in CEP-induced HuH-7 cells death. Although the exact mechanism through which /JNK activation leads to cytochrome *c* release is not clear in the present study and warrants further investigation, however, it seems that, activated JNK and down-stream c-Jun play some roles in regulating expression levels of Bcl-2 and Bax which may in turn cause mitochondrial injury and release of cytochrome *c*. Lee et al. recently reported that luteolin-induced activation of JNK causes the mitochondrial translocation of Bax, cytochrome *c* release, and caspase-3 activation in human hepatoma, HepG2 cells [44]. Taken together, our results suggest that the production of ROS, the activation of JNK, and down-regulation of Akt play crucial roles in CEP-induced apoptosis of hepatocellular carcinoma, HuH 7 cells.

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References

[1] Tomita, M., Fujitani, K. and Aoyagi, Y. (1967) Synthesis of dicepharanthine. Tetrahedron Lett. 13, 1201–1206.

[2] Kato, T. and Suzumura, Y. (1987) Potentiation of antitumor activity of vincristine by biscoclaurine alkaloid cepharanthine. J. Natl. Cancer Inst. 79, 527–532.

[3] Okamoto, M., Ono, M. and Baba, M. (2001) Suppression of cytokine production and neural cell death by the anti-inflammatory alkaloid cepharanthine: a potential agent against HIV-1 encephalopathy. Biochem. Pharmacol. 62, 747–753.

[4] Murakami, K., Okajima, K. and Uchiba, M. (2000) The prevention of lipopolysaccharide-induced pulmonary vascular injury by pretreatment with cepharanthine in rats. Am. J. Respir. Crit. Care Med. 161, 57–63.

[5] Goto, M., Zeller, W.P. and Hurley, R.M. (1991) Cepharanthine (biscoclaurine alkaloid) treatment in endotoxic shock of suckling rats. J. Pharm. Pharmacol. 43, 589–591.

[6] Shiraishi, N., Akiyama, S., Nakagawa, M., Kobayashi, M. and Kuwano, M. (1987) Effect of bisbenzylisoquinoline (biscoclaurine) alkaloids on multidrug resistance in KB human cancer cells. Cancer Res. 47, 2413–2416.

[7] Asaumi, J., Nishikawa, K., Matsuoka, H., Iwata, M., Kawasaki, S., Hiraki, Y. and Nishijima, K. (1995) Direct antitumor effect of cepharanthine and combined effect with adriamycin against Ehrlich ascites tumor in mice. Anticancer Res. 15, 67–70.

[8] Wu, J., Suzuki, H., Akhand, A.A., Zhou, Y.W., Hossain, K. and Nakashima, I. (2002) Modes of activation of mitogen-activated protein kinases and their roles in cepharanthine-induced apoptosis in human leukemia cells. Cell Signal. 14, 509–515.

[9] Ono, M., Tanaka, N. and Orita, K. (1994) Positive interactions between human interferon and cepharanthine against human cancer cells in vitro and in vivo. Cancer Chemother. Pharmacol. 35, 10–16.

[10] Nakajima, A., Yamamoto, Y., Taura, K., Hata, K., Fukumoto, M., Uchinami, H., Yonezawa, K. and Yamaoka, Y. (2004) Beneficial effect of cepharanthine on overcoming drug-resistance of hepatocellular carcinoma. Int. J. Oncol. 24, 635–645.

[11] Nishikawa, K., Asaumi, J., Kawasaki, S., Kuroda, M., Takeda, Y. and Hiraki, Y. (1997) Influence of cepharanthine on the intracellular accumulation of adriamycin in normal liver cells and spleen cells of mice in vitro and in vivo. Anticancer Res. 17, 3617–3621.

[12] Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J. and Davis, R.J. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J. Biol. Chem. 270, 7420–7426.

[13] Chen, Y.R., Meyer, C.F. and Tan, T.H. (1996) Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in gamma radiation-induced apoptosis. J. Biol. Chem. 271, 631–634.

[14] Sarker, K.P., Biswas, K.K., Yamakuchi, M., Lee, K.Y., Hashiguchi, T., Kracht, M., Kitajima, I. and Maruyama, I. (2003) ASK1-p38 MAPK/JNK signaling cascade mediates anandamide-induced PC12 cell death. J. Neurochem. 85, 50–61.

[15] Kennedy, S.G., Wagner, A.J., Conzen, S.D., Jordan, J., Bellacosa, A., Tsichlis, P.N. and Hay, N. (1997) The PI3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev. 11, 701–713.

[16] Biswas, K.K., Sarker, K.P., Abeyama, K., Kawahara, K., Iino, S., Otsubo, Y., Saigo, K., Izumi, H., Hashiguchi, T., Yamakuchi, M., Yamaji, K., Endo, R., Suzuki, K., Imaizumi, H. and Maruyama, I. (2003) Membrane cholesterol but not putative receptors mediates anandamide-induced hepatocyte apoptosis. Hepatology 38, 1167–1177.

[17] Sarker, K.P., Obara, S., Nakata, M., Kitajima, I. and Maruyama, I. (2000) Anandamide induces apoptosis of PC-12 cells: involvement of superoxide and caspase-3. FEBS Lett. 472, 39–44.

[18] Wyllie, A.H., Beattie, G.J. and Hargreaves, A.D. (1981) Chromatin changes in apoptosis. Histochem. J. 13, 681–692.

[19] Shi, Y.F., Szalay, M.G., Paskar, L., Sahai, B.M., Boyer, M., Singh, B. and Green, D.R. (1990) Activation-induced cell death in T cell hybridomas is due to apoptosis. Morphologic aspects and DNA fragmentation. J. Immunol. 144, 3326–3333.

[20] Fujimura, T., Furusawa, S., Kawauchi, H., Takayanagi, Y. and Sasaki, K. (1991) Effect of cepharanthine on doxorubicin cytotoxicity in P388 murine leukemia cells in vitro and in vivo. Res. Commun. Chem. Pathol. Pharmacol. 71, 235–238.

[21] Akamatsu, H., Komura, J., Asada, Y. and Niwa, Y. (1991) Effects of cepharanthine on neutrophil chemotaxis, phagocytosis, and reactive oxygen species generation. J. Dermatol. 18, 643–648.

[22] Ghatan, S., Larner, S., Kinoshita, Y., Hetman, M., Patel, L., Xia, Z., Youle, R.J. and Morrison, R.S. (2000) p38 MAP kinase mediates bax translocation in nitric oxide-induced apoptosis in neurons. J. Cell Biol. 150, 335–347.

[23] Sarker, K.P., Biswas, K.K., Rosales, J.L., Yamaji, K., Hashiguchi, T. and Maruyama, I. (2003) Ebselen inhibits NO-induced apoptosis of differentiated PC12 cells via inhibition of ASK1-p38 MAPK-p53 and JNK signaling and activation of p44/42 MAPK and Bcl-2. J. Neurochem. 87, 1345–1353.

[24] Sutherland, C.L., Heath, A.W., Pelech, S.L., Young, P.R. and Gold, M.R. (1996) Differential activation of the ERK, JNK, and p38 mitogen-activated protein kinases by CD40 and the B cell antigen receptor. J. Immunol. 157, 3381–3390.

[25] Liu, R.Y., Fan, C., Liu, G., Olashaw, N.E. and Zuckerman, K.S. (2000) Activation of p38 mitogen-activated protein kinase is required for tumor necrosis factor-alpha-supported proliferation of leukemia and lymphoma cell lines. J. Biol. Chem. 275, 21086–21093.

[26] Ameyar, M., Atfi, A., Cai, Z., Stancou, R., Shatrov, V., Bettaieb, A. and Chouaib, S. (1998) Analysis of human breast adenocarcinoma MCF7 resistance to tumor necrosis factor-induced cell death. Lack of correlation between JNK activation and ceramide pathway. J. Biol. Chem. 273, 29002–29008.

[27] Potapova, O., Gorospe, M., Bost, F., Dean, N.M., Gaarde, W.A., Mercola, D. and Holbrook, N.J. (2000) c-Jun N-terminal kinase is essential for growth of human T98G glioblastoma cells. J. Biol. Chem. 275, 24767–24775.

[28] Javelaud, D. and Besancon, F. (2001) NF-kappa B activation results in rapid inactivation of JNK in TNF alpha-treated Ewing sarcoma cells: a mechanism for the anti-apoptotic effect of NF-kappa B. Oncogene 20, 4365–4372.

[29] Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H. and Karin, M. (2005) Reactive oxygen species promote TNFalpha-

induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell 120, 649–661.
[30] Wang, X., Martindale, J.L. and Holbrook, N.J. (2000) Requirement for ERK activation in cisplatin-induced apoptosis. J. Biol. Chem. 275, 39435–39443.
[31] Kalmes, A., Deou, J., Clowes, A.W. and Daum, G. (1999) Raf-1 is activated by the p38 mitogen-activated protein kinase inhibitor, SB203580. FEBS Lett. 444, 71–74.
[32] Harada, J. and Sugimoto, M. (1999) An inhibitor of p38 and JNK MAP kinases prevents activation of caspase and apoptosis of cultured cerebellar granule neurons. Jpn. J. Pharmacol. 79, 369–378.
[33] Nemoto, S., Xiang, J., Huang, S. and Lin, A. (1998) Induction of apoptosis by SB202190 through inhibition of p38beta mitogen-activated protein kinase. J. Biol. Chem. 273, 16415–16420.
[34] Osone, S., Hosoi, H., Kuwahara, Y., Matsumoto, Y., Iehara, T. and Sugimoto, T. (2004) Fenretinide induces sustained-activation of JNK/p38 MAPK and apoptosis in a reactive oxygen species-dependent manner in neuroblastoma cells. Int. J. Cancer 112, 219–224.
[35] Luo, J., Sun, Y., Lin, H., Qian, Y., Li, Z., Leonard, S.S., Huang, C. and Shi, X. (2003) Activation of JNK by vanadate induces a Fas-associated death domain (FADD)-dependent death of cerebellar granule progenitors in vitro. J. Biol. Chem. 278, 4542–4551.
[36] Aoki, H., Kang, P.M., Hampe, J., Yoshimura, K., Noma, T., Matsuzaki, M. and Izumo, S. (2002) Direct activation of mitochondrial apoptosis machinery by c-Jun N-terminal kinase in adult cardiac myocytes. J. Biol. Chem. 277, 10244–10250.
[37] Clerk, A., Fuller, S.J., Michael, A. and Sugden, P.H. (1998) Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. J. Biol. Chem. 273, 7228–7234.
[38] Lee, T.K., Man, K., Ho, J.W., Sun, C.K., Ng, K.T., Wang, X.H., Wong, Y.C., Ng, I.O., Xu, R. and Fan, S.T. (2004) FTY720 induces apoptosis of human hepatoma cell lines through PI3-K-mediated Akt dephosphorylation. Carcinogenesis 25, 2397–2405.
[39] Kluck, R.M., Bossy-Wetzel, E., Green, D.R. and Newmeyer, D.D. (1997) The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275, 1132–1136.
[40] Juo, P., Kuo, C.J., Reynolds, S.F., Konz, R.F., Raingeaud, J., Davis, R.J., Biemann, J. and Blenis, H.M. (1997) Fas activation of the p38 mitogen-activated protein kinase signalling pathway requires ICE/CED-3 family proteases. Mol. Cell. Biol. 17, 24–35.
[41] Ozaki, I., Tani, E., Ikemoto, H., Kitagawa, H. and Fujikawa, H. (1999) Activation of stress-activated protein kinase/c-Jun NH2-terminal kinase and p38 kinase in calphostin C-induced apoptosis requires caspase-3-like proteases but is dispensable for cell death. J. Biol. Chem. 274, 5310–5317.
[42] Seimiya, H., Mashima, T., Toho, M. and Tsuruo, T. (1997) c-Jun NH2-terminal kinase-mediated activation of interleukin-1beta converting enzyme/CED-3-like protease during anticancer drug-induced apoptosis. J. Biol. Chem. 272, 4631–4636.
[43] Harada, K., Bando, T., Yoshida, H. and Sato, M. (2001) Characteristics of antitumour activity of cepharanthine against a human adenosquamous cell carcinoma cell line. Oral Oncol. 37, 643–651.
[44] Lee, H.J., Wang, C.J., Kuo, H.C., Chou, F.P., Jean, L.F. and Tseng, T.H. (2005) Induction apoptosis of luteolin in human hepatoma HepG2 cells involving mitochondria translocation of Bax/Bak and activation of JNK. Toxicol. Appl. Pharmacol. 203, 124–128.