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Induction of apoptosis in human hepatoblastoma cells by tetrandrine via caspase-dependent Bid cleavage and cytochrome *c* release

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Abstract

Tetrandrine, a bis-benzylisoquinoline alkaloid from the root of *Stephania tetrandra*, induces apoptosis in human T-cell lines, lung carcinoma and hepatoblastoma cells. However, the mechanisms by which tetrandrine inhibits tumor cell growth are poorly understood. The purpose of the present study was to investigate the intracellular signaling mechanism of tetrandrine-induced apoptosis in HepG2 cells. The induction of apoptosis was determined by morphological analysis and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. Treatment of cells with tetrandrine caused the upregulation of p53, downregulation of Bcl-X_L, cleavage of Bid and Bax, and release of cytochrome *c*, which were accompanied by activation of caspases 9, 3 and 8. The activation of caspases 9 and 3 preceded that of caspase 8. A broad-spectrum caspase inhibitor and a caspase 8-specific inhibitor completely blocked tetrandrine-induced Bid processing, cytochrome *c* release, activation of caspase 3, and cell death. These findings and data showing the early release of cytochrome *c*, cleavage of Bid and downregulation of Bcl-X_L suggest that the mitochondrial pathway is primarily involved in tetrandrine-induced apoptosis. The activation of caspase 8 after early caspases 9 and 3 activation might act as an amplification loop for activation of upstream signals such as Bid cleavage or cytochrome *c* release. These data suggest that tetrandrine may constitute a plausible therapeutic for hepatocellular carcinoma.

Keywords: Tetrandrine; Caspase; Bid; Cytochrome *c*; Apoptosis; HepG2

1. Introduction

Cytotoxic drugs cause cell death in sensitive cells, at least partly, by inducing apoptosis [1]. Two major routes have been identified through which these drugs induce apoptosis: the death receptor pathway and the mitochondrial pathway [2]. The binding of death receptors, such as Fas, by ligands or cross-linking antibodies results in receptor trimerization, which is followed by the binding of the adaptor molecule, Fas-associated death domain, to the cytoplasmic domain of the receptor. Fas-associated death domain, in turn, recruits and activates procaspase 8. Activation of the first step of the caspase cascade triggers the activation of several effector procaspases involving caspase 3 [3]. The mitochondrial pathway is dependent upon the release of cytochrome *c* from the mitochondria. The process is initiated by the interaction of the mitochondria with one or more of the proapoptotic members of the Bcl-2 family of proteins. Bcl-2 family members are major regulators of the apoptotic process, and comprise pro- as well as anti-apoptotic molecules. Among the pro-apoptotic members, Bax, Bak, and Bok share three homology domains (BH1–BH3) and contain a C-terminal membrane-anchoring domain [4,5]. In contrast, Bid, Bad, and Bik only contain the BH3 domain, which functions as a death domain in these pro-apoptotic molecules. Bid acts as one of the links between the Bcl-2 family members and the caspases. Following activation of the death receptor, full-length cytoplasmic Bid (p22) is cleaved by caspase 8 [6,7], and the tBid; p15 translocates to the mitochondria to induce the release of cytochrome *c*. Cytochrome *c*, together with Apaf-1, activates caspase 9 and then caspase 3, resulting in apoptosis [8]. Bid is an

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Abbreviations: tBid, truncated Bid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; zVAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; zIETD-fmk, *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone; PBS, phosphate buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; Ac-DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide; Ac-IETD-pNA, *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide; PARP, poly(ADP-ribose)polymerase.

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essential component of the Fas-receptor pathway, especially in hepatocytes, and Bid-deficient mice are resistant to the lethal effects of anti-Fas antibody [9].

In normal cells, signal transduction in these pathways is strictly regulated to maintain cellular homeostasis. Tumor cells, however, irrespective their origin and nature, are generally resistant to a wide variety of cell death stimuli, including virtually all classical chemotherapeutic agents, and circumvent the normal apoptotic mechanisms to avert their self-destruction. Therefore, it should be advantageous to tip the balance in favor of apoptosis over mitosis during cancer chemotherapy and prevention. Hepatocellular carcinoma is the fifth most common cancer in the world and the fourth most common cause of cancer-associated mortality [10]. Surgical resection and local treatment are frequently limited, as a result of metastasis, cirrhosis, and other pathological changes in the liver parenchyma. Therefore, the development of chemotherapeutic or chemopreventive agents for hepatocellular carcinoma is important for the reduction of the mortality caused by this disease.

Tetrandrine is a bis-benzylisoquinoline alkaloid isolated from the root of *Stephania tetrandra*, which has been used in oriental medicine for several decades to treat patients with arthritis, silicosis, and hypertension [11–13]. In addition to its anti-inflammatory effects [14], tetrandrine elicits *in vitro* cytotoxic effects on HeLa cells [15] and *in vivo* tumor suppressive effects on mouse ascites tumors [16]. Our previous study has shown that tetrandrine is a potent inducer of apoptosis in human hepatoblastoma HepG2 cells [17]. In the present investigation, we have studied pathways through which tetrandrine mediates the expression and activation of different proteins involved in the apoptotic cell death. We found that tetrandrine-induced apoptosis in HepG2 cells is associated primarily with the mitochondrial pathway involving cytochrome *c* release and caspase 9 activation, which is dependent on the activation of downstream caspases 3 and 8.

2. Materials and methods

2.1. Materials and cell culture

Tetrandrine was purchased from Aldrich chemical Co. It was dissolved in a drop of c-HCl and 0.9% NaCl and further diluted with 0.9% NaCl solution to a desired concentration. MTT was obtained from Sigma Chemical Co. Broad-spectrum caspase inhibitor (zVAD-fmk) and caspase 8 inhibitor (zIETD-fmk) were obtained from Biomol and Calbiochem, respectively. All other chemicals used were of the highest pure grade available. The human hepatoblastoma HepG2 cells were maintained in the logarithmic phase of growth in RPMI 1640 medium (GIBCO BRL) supplemented with heat inactivated 10% fetal bovine serum (GIBCO BRL), 2 mM L-glutamine (Sigma Chemical Co.) at 37° in a 5% CO_2–95% air humidified incubator.

2.2. Cell proliferation assay

HepG2 cells were treated with tetrandrine and various inhibitors and the general viability of cultured cells was determined by MTT assay [18] or trypan blue dye exclusion assay.

2.3. Morphological analysis and TUNEL assay

The cells were treated with tetrandrine for 24 hr, washed gently three times with PBS and fixed with paraformaldehyde for 30 min. The cells were washed with PBS and stained with propidium iodide solution (50 μg/mL of propidium iodide; 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. Apoptosis was detected by the TUNEL method using the Boehringer *in situ* death detection kit.

2.4. Measurement of caspase activity

The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate Ac-DEVD-pNA; caspase 3 substrate and Ac-IETD-pNA; caspase 8 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 hr at 37°. The cleavage of the peptide substrate was monitored at 405 nm. Results are expressed as the percent change of the activity compared to the untreated control.

2.5. Preparation of mitochondrial and cytosolic extracts

Cells were washed with ice-cold PBS, resuspended in ice-cold HMKEE buffer (20 mM HEPES–KOH, pH 7.0, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin) containing 250 mM sucrose and allowed to swell on ice for 20 min. Cells were homogenized by passages through a 26-gauge needle and centrifuged at 14,000 *g* for 15 min at 4°. The cytosolic supernatant was removed and the pellet containing the mitochondria was resuspended in lysis buffer and stored at −70°.

2.6. Western blot analysis

Cells were washed with PBS and lysed (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA, 50 mM β-glycerophosphate, 20 mM NaF, 1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin and 10 μg/mL aprotinin). Cell lysates were centrifuged and the protein content was determined. Equal amounts of protein were separated by SDS–polyacrylamide gel

electrophoresis (12–15%), transferred to nitrocellulose membrane and immunoblotted with antibodies as indicated. Detection was performed using enhanced chemiluminescence Western Blotting Detection Reagents (Amersham). Monoclonal human anti-cytochrome *c*, anti-Bid, anti-caspase 3 and anti-PARP antibody were purchased from PharMingen. Monoclonal human anti-caspases 8 and 9, anti-Bcl-X_L and Bax antibody were obtained from Santa Cruz Biotechnology.

3. Results

3.1. Inhibition of proliferation and induction of apoptosis by tetrandrine

The effect of tetrandrine on cell growth was examined in HepG2 cells. Cell proliferation was inhibited in HepG2 cells in a dose-dependent manner, with an IC_{50} of 23.8 ± 0.2 μM (Fig. 1A). Morphological analysis and TUNEL staining confirmed our previous results [17] that tetrandrine induces apoptosis in the HepG2 cell line (Fig. 1B).

3.2. Activation of caspases by tetrandrine

We assessed the effects of tetrandrine on caspase activity in HepG2 cells. Cells were treated with 20 μM tetrandrine for various periods, and the activation of caspases 3 and 8 was monitored by spectrophotometric and Western blot analyses. As shown in Fig. 2A, caspase 3 was proteolytically processed into active p17 fragments, and PARP was

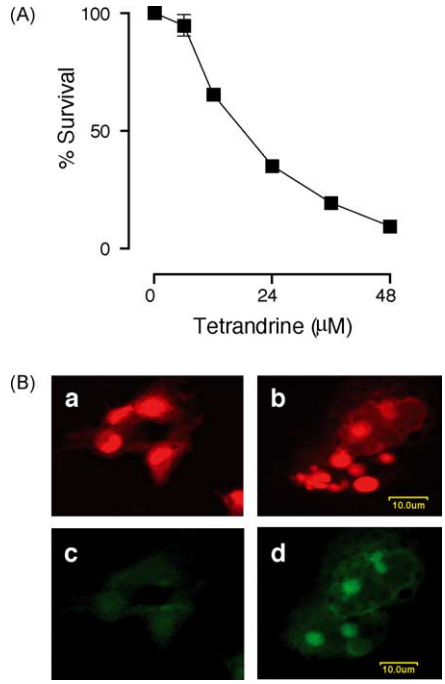

Fig. 1. Tetrandrine inhibits growth and induces apoptosis in HepG2 cells. (A) Dose-dependent inhibition of HepG2 cell growth by tetrandrine. Cells were incubated with increasing concentration of tetrandrine and cell survival was determined by MTT assay. Data are mean ± SD of three independent experiments. (B) Morphological analysis (a, b) and TUNEL assay (c, d) of HepG2 cells treated with vehicle (a, c) or tetrandrine (b, d). For morphological analysis, nuclei were stained with PI. For TUNEL assay, cells were stained with Boehringer *in situ* death detection kit and examined with confocal microscope.

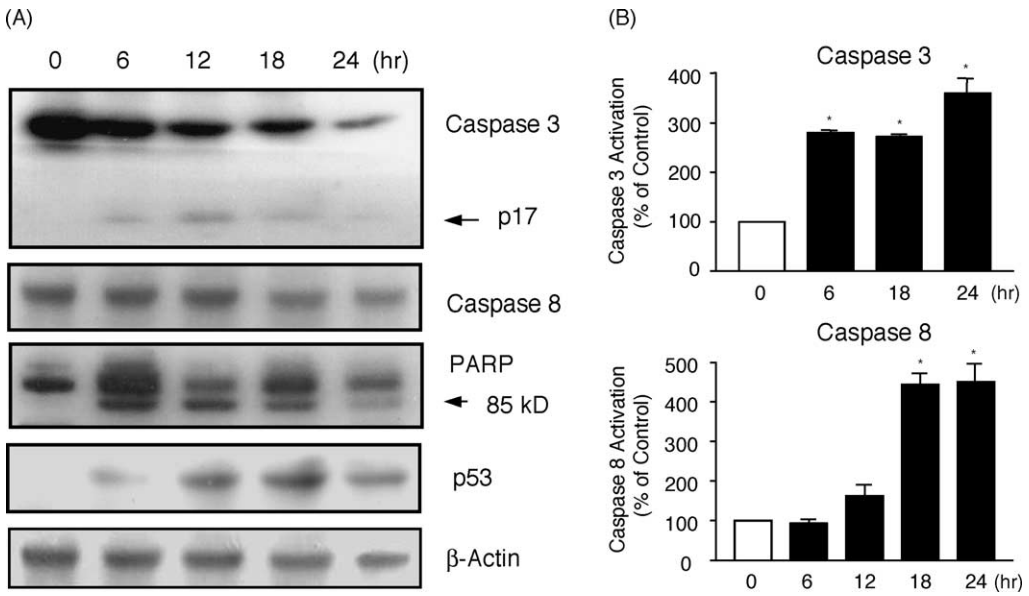

Fig. 2. Apoptosis induced by tetrandrine involves the activation of caspases 3 and 8 as well as upregulation of p53. (A) Representative Western blots show the processing of caspases 3 and 8, the cleavage of PARP and upregulation of p53 by treatment with tetrandrine. HepG2 cells were treated with tetrandrine (30 μM) 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. (B) Induction of caspases 3 and 8 activity by tetrandrine. Extracts of control cells and cells treated with tetrandrine (16 μM) were analyzed for caspase activity as a function of time. Ac-DEVD-pNA and Ac-IETD-pNA were used as the substrates for caspases 3 and 8, respectively, and the cleavage of the peptide was monitored at 405 nm. Each data represents the mean ± SD from three independent experiments ($^*P < 0.01$).

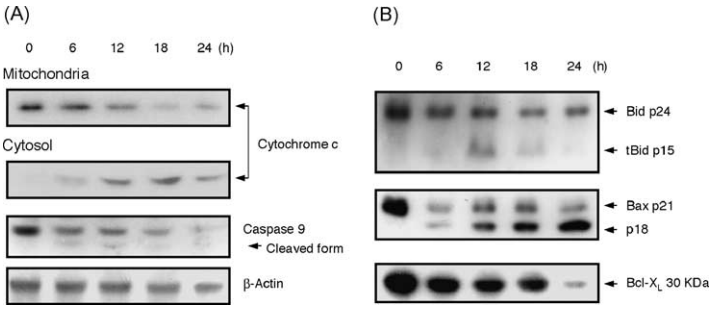

Fig. 3. Tetrandrine induces the release of cytochrome *c*, activation of caspase 9, cleavage of Bid and downregulation of Bcl-X_L. (A) The cells were treated with tetrandrine (30 μM) and cytosol and mitochondrial protein was prepared as described. They were loaded on a 12% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted with anti-cytochrome *c* antibody. For caspase 9, protein from the whole cell lysate was prepared and analyzed using anti-caspase 9 antibody. β-Actin was used for loading control. (B) HepG2 cells were treated with tetrandrine (30 μM) and the protein from the whole cell lysate was separated on a 12% SDS–polyacrylamide gel. Representative Western blots show the time-dependent cleavage of Bid to tBid (p15), Bax to cleaved Bax and downregulation of Bcl-X_L.

cleaved to its characteristic 85-kDa fragment. Procaspase 8 was also activated by treatment with tetrandrine. To confirm that the proteolytic cleavage of procaspases 3 and 8 reflected the activation of these caspases, Ac-DEVD-pNA and Ac-IETD-pNA were used as substrates for caspases 3 and 8, respectively. The activity of these two enzymes was significantly increased in HepG2 cells exposed to tetrandrine, which was in accordance with the results of the Western blot analysis (Fig. 2B). Expression of the tumor suppressor protein, p53, was increased by tetrandrine (Fig. 2A).

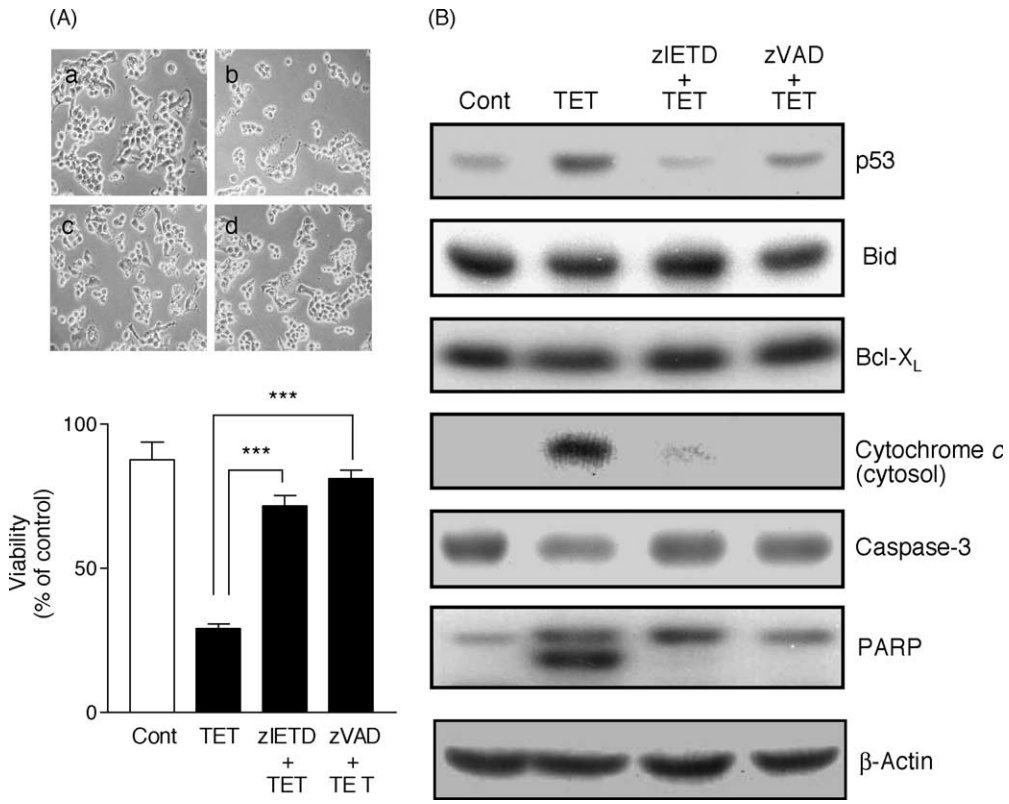

Fig. 4. Caspase inhibitors prevent tetrandrine-induced cell death and alter the expression pattern of apoptosis related proteins. (A) Inhibition of tetrandrine-induced cell death by the caspase inhibitors zIETD-fmk and zVAD-fmk. Cells were treated with vehicle (a) tetrandrine alone (b) or pretreated with zIETD-fmk (10 μM) (c) or zVAD-fmk (50 μM) (d) for 2 hr and then incubated with tetrandrine (20 μM) for 18 hr. Cell survival was monitored by trypan blue exclusion assay. Data are mean ± SD of three independent experiments ($^{***}P < 0.001$ vs. tetrandrine group). (B) Caspase inhibitors zIETD-fmk and zVAD-fmk inhibit downregulation of full-length Bid and Bcl-X_L, upregulation of p53, cytochrome *c* release, caspase 3 activation and PARP cleavage induced by tetrandrine. HepG2 cells were treated with tetrandrine alone or pretreated with zIETD-fmk (10 μM) or zVAD-fmk (50 μM) for 2 hr and then incubated with tetrandrine (20 μM) for 18 hr. Cells were then lysed, separated on a 12% SDS–polyacrylamide gel and immunoblotted with the corresponding antibodies. β-Actin was used for loading control.

3.3. Involvement of mitochondrial pathway in tetrandrine-induced apoptosis

The release of cytochrome *c* or an apoptosis-inducing factor from the mitochondria after their depolarization is believed to be an initiator of the caspase cascade. To investigate whether the pathway is also involved in the response of HepG2 cells to tetrandrine, cytochrome *c* release was monitored. Fig. 3A shows that tetrandrine treatment caused a time-dependent decrease in mitochondrial cytochrome *c* and a concomitant increase in cytosolic cytochrome *c*. Involvement of caspase 9 was also investigated in this context since it is known that mitochondrial dysfunction results in the release of cytochrome *c* and subsequent activation of one of the major initiator caspases, caspase 9. After 6 hr of treatment with tetrandrine, activation of procaspase 9 was evidenced by the decrease in the intensity of procaspase 9 and increase in the cleavage product (Fig. 3A). Activation of caspases 9 and 3 preceded activation of caspase 8 indicating that caspase 9 is apical caspase of the cascade.

3.4. Involvement of Bcl-2 family proteins in tetrandrine-induced apoptosis

Death promoting members of the Bcl-2 family such as Bax and Bid play key roles in many drug-induced cytochrome *c* release. Bid functions to receive death signals in the cytosol from upstream events and is processed to p15 subunit fragment (tBid). It induces the release of cytochrome *c*, thereby activating the mitochondrial apoptotic pathway [6,7]. The amount of full-length Bid, tBid, full-length and cleaved Bax, and Bcl-X_L in whole cell lysates of HepG2 cells treated with tetrandrine are shown in detail in Fig. 3B. Within 6 hr of treatment, the amount of full-length Bid was reduced and tBid was detected. The same is true for the level of Bax. By treatment with tetrandrine, full-length Bax was reduced with concomitant increase in the level of the cleaved form. Levels of anti-apoptotic Bcl-X_L were significantly reduced by treatment with tetrandrine.

3.5. Caspase inhibitors attenuated tetrandrine-induced apoptosis

To determine whether the activation of the caspase cascade is required for the induction of apoptosis by tetrandrine, HepG2 cells were treated with caspase inhibitors and/or tetrandrine. Incubation with tetrandrine alone markedly reduced the viability of HepG2 cells, whereas pretreatment with the caspase 8 inhibitor, zIETD-fmk, and the broad-spectrum caspase inhibitor, zVAD-fmk, significantly reduced tetrandrine-induced cell death (Fig. 4A). The caspase inhibitors also prevented the processing of Bid and the downregulation of Bcl-X_L. The release of cytochrome *c*, the activation of caspase 3, the proteolytic cleavage of PARP, and the expression of p53 were also suppressed by pretreatment with the caspase inhibitors (Fig. 4B).

4. Discussion

Tetrandrine is a cytotoxic compound capable of inducing apoptosis in many cancer cells *in vitro* [15,17,19], and exhibits remarkable antitumor activity against xenografted ascites tumors in mice [16]. To date, the molecular events necessary for tetrandrine-induced cell death have not been identified. In the present study, we describe the mechanisms via which tetrandrine induces apoptosis in human hepatoblastoma HepG2 cells. Tetrandrine caused mitochondrial cytochrome *c* release, induced the activation of caspases 9, 3 and 8, and cleaved PARP in HepG2 cells. Tetrandrine induced the cleavage of Bid, downregulation of Bcl-X_L and upregulation of p53. These responses were suppressed by pretreatment with zIETD-fmk or zVAD-fmk.

Our finding that the general caspase inhibitor, zVAD-fmk, interrupted apoptotic cell death demonstrates that caspase 3 is activated in tetrandrine-treated cells and is a necessary component of apoptosis. To figure out the upstream caspases and cytosolic factors involved, we investigated the role of mitochondrial signals. Cytochrome *c* can initiate a complex series of caspase activation events, ultimately resulting in apoptosis. Cell death initiator or repressor such as Bid, Bax and Bcl-2 have been shown to regulate this event, suggesting that this is a critical step in the death signaling cascade [6,20]. Bid is identified as a substrate of caspase 8 in the pathway of apoptosis triggered by ligation of Fas, tumor necrosis factor-α and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [6,7,21]. The active form of Bid is considered tBid, the 15 kDa C-terminal fragment resulting from caspase cleavage. It redistributes from cytosol to mitochondria and promotes the release of cytochrome *c* [22]. We found that tetrandrine-induced cleavage of Bid, which was associated with the release of cytochrome *c*. The apoptosis promoting activity of cytochrome *c* is due to its ability to interact with Apaf-1 [8]. Binding of cytochrome *c* to Apaf-1 enables this protein to recruit caspase 9 and to stimulate processing of procaspase 9 to its active form [6,23]. Once active, caspase 9 then presumably triggers a cascade of caspase activation events leading to apoptosis.

A central component of the apoptotic process is a proteolytic system involving the caspases, a highly conserved family of cysteine proteases with specific substrates. Caspase 8 represents the apical caspase in the death receptor (extrinsic) pathway and caspase 9 serves as the apical caspase of the mitochondrial (intrinsic) pathway [20,24]. Caspase 3 has been shown to play an important role in apoptosis induced by several conditions, and to be necessary in determining the nuclear alteration of apoptosis [25]. It is commonly believed that caspases with long prodomains are upstream or initiating caspases

whereas those with short prodomains are effector or executioner caspases. For example, caspase 8 is the most proximal caspase to become activated upon ligation of the Fas molecule since this caspase is directly recruited into the Fas signaling complex upon receptor aggregation [26,27]. However, recent studies have suggested that caspase 8 is not always activated early in the context of Fas signaling. This has led to the suggestion that two distinct cellular types exist with respect to Fas signaling: type I cells that activate caspase 8 early of Fas receptor aggregation and type II cells that activate caspase 8 late and in a mitochondrial-dependent fashion [28]. In type II cells, caspase 9 initiates processing of caspase 3, which in turn activates caspases 2 and 6. Caspase 6 was found to be required for the activation of downstream caspase 8 [29]. In our model, caspase 8 activation was preceded by the activation of caspases 9 and 3. This finding suggests that caspase 9 may play a role in the initiating triggering of the cleavage and activation of caspase 3 and that caspase 8 activation may represent a downstream event after the activation of caspase 9. This hypothesis is in agreement with recent evidences [30–32] suggesting that activation of caspase 8 may also occur as a consequence of the activation of caspase 9. It is conceivable that the lipophilic tetrandrine permeates the membrane and activates the intrinsic mitochondrial pathway, leading to the activation of caspase 9, thus supporting the hypothesis that caspase 9 represents the most apical caspase in chemical-induced apoptosis [33]. Recent reports suggest that caspase 8 activation, when triggered downstream of the mitochondrial pathway of apoptosis, may amplify caspase 9 activation through the cleavage of the proapoptotic protein Bid, which, in turn, elicits a further efflux of cytochrome *c* from mitochondria [6,30,31]. In agreement, we found that caspase 8-specific inhibitor abrogated some upstream events such as p53 upregulation, Bid processing, Bcl-X_L downregulation, cytochrome *c* release, caspase 3 activation. Thus, the activation of caspase 8 after early caspase 3 activation might act as an amplification loop necessary for successful apoptosis.

Tetrandrine activates various signaling molecules and apoptosis-related genes [19,34]. p53 protein is a critical mediator of cellular responses to DNA damage in mammalian cells. It exerts its function by arresting the G1 phase of the cell cycle and mediates apoptosis in cells exposed to anticancer drugs [35]. p53 exerts its effects on cells as a transcription factor for Bax, PIG genes, CD95, and DR5 [36]. In this case, an increase in p53 leads to the expression of pro-apoptotic proteins, which prompt cells to undergo apoptosis. It has recently been reported that Bid is also regulated by p53. Bid mRNA increases in a p53-dependent manner *in vitro* and *in vivo*. In addition, Bid-null mouse embryonic fibroblasts are more resistant to DNA damaging agents than are wild-type fibroblasts [37,38], indicating that Bid is a p53-responsive chemosensitivity gene that may enhance the cell-death response to chemotherapy.

In conclusion, our findings demonstrate that tetrandrine inhibits the proliferation of human hepatoblastoma cells via the early activation of caspase 9 through the mitochondrial pathway, as demonstrated by the early cytochrome *c* release from mitochondria and Bid cleavage. The activation of the mitochondrial pathway may in turn activate caspases 3 and 8, triggering a feedback amplification loop for upstream caspases. These results, together with previous findings concerning the high cytotoxic and the apoptogenic effects toward tumor cell *in vivo* as well as *in vivo* suggest a potential role of tetrandrine as a powerful chemotherapeutic agent.

Acknowledgments

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References

[1] Fisher DE. Apoptosis in cancer therapy: crossing the threshold. Cell 1994;78:539–42.
[2] Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. Exp Cell Res 2000;256:42–9.
[3] Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, Peter ME. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J 1997;16:2794–804.
[4] Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 1999;13:1899–911.
[5] Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998;281:1322–6.
[6] Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell death receptors. Cell 1998;94:481–90.
[7] Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P, Korsmeyer SJ. Caspase cleaved BID targets mitochondria and is required for cytochrome *c* release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem 1999;274:1156–63.
[8] Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell 1996;86:147–57.
[9] Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA, Korsmeyer SJ. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 1999;400:886–91.
[10] McGlynn KA, Tsao L, Hsing AW, Devesa SS, Fraumeni Jr JF. International trends and patterns of primary liver cancer. Int J Cancer 2001;94:290–6.
[11] Seow WK, Li SY, Thong YH. In vitro immunosuppressive properties of the plant alkaloid tetrandrine. Int Arch Allergy Appl Immunol 1988;85:410–5.
[12] Banks DE, Cheng YH, Weber SL, Ma JK. Strategies for the treatment of pneumoconiosis. Occup Med 1993;8:205–32.
[13] Fang DC, Jiang MX. A new calcium antagonist of Chinese medicinal origin: tetrandrine. J Hypertens 1986;4(Suppl 6):S150–2.

[14] Kondo Y, Imai Y, Hojo H, Hashimoto Y, Nozoe S. Selective inhibition of T-cell-dependent immune responses by bisbenzylisoquinoline alkaloids *in vivo*. Int J Immunopharmacol 1992;14:1181–6.
[15] Teh BS, Chen P, Lavin MF, Seow WK, Thong YH. Demonstration of the induction of apoptosis (programmed cell death) by tetrandrine, a novel anti-inflammatory agent. Int J Immunopharmacol 1991;13:1117–26.
[16] Kuroda H, Nakazawa S, Katagiri K, Shiratori O, Kozuka M. Antitumor effect of bisbenzylisoquinoline alkaloids. Chem Pharm Bull 1976;24:2413–20.
[17] Yu SM, Oh SH, Lee SJ, Lee BW, Ko WG, Moon CK, Lee BH. Inhibition of proliferaiton and induction of apoptosis by tetrandrine in HepG2 cells. J Ethnopharmacol 2002;81:225–9.
[18] Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A, Gray-Goodrich M, Campbell H, Mayo J, Boyd M. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Natl Cancer Inst 1991;83:757–66.
[19] Lai JH, Ho LJ, Lu KC, Chang DM, Shaio MF, Han SH. Western and Chinese antirheumatic drug-induced T cell apoptotic DNA damage uses different caspase cascades and is independent of Fas/Fas ligand interaction. J Immunol 2001;166:6914–24.
[20] Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh J, Schlesinger PH. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome *c*. Cell Death Differ 2000;7:1166–73.
[21] Yamada H, Tada-Oikawa ST, Uchida A, Kawanishi S. TRAIL causes cleavage of Bid by caspase-8 and loss of mitochondrial membrane potential resulting in apoptosis in BJAB cells. Biochem Biophys Res Commun 1999;265:130–3.
[22] Esposti MD. The roles of Bid. Apoptosis 2002;7:433–40.
[23] Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES. Autoactivation of procaspase 9 by Apaf-1-mediated oligomerization. Mol Cell 1998;1:949–57.
[24] Juo P, Kuo CJ, Yuan J, Blenis J. Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. Curr Biol 1998;8:1001–8.
[25] Janicke RU, Sprengart ML, Wati MR, Porter AG. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem 1998;273:9357–60.
[26] Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J 1995;14:5579–88.
[27] Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell 1996;85:817–27.
[28] Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 1998;7:1675–87.
[29] Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR, Martin SJ. Ordering the cytochrome *c*-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J Cell Biol 1999;144:281–92.
[30] Perkins CL, Fang G, Kim CN, Bhalla KN. The role of Apaf-1, caspase-9 and BID proteins in etoposide- or paclitaxel-induced mitochondrial events during apoptosis. Cancer Res 2000;60:1645–53.
[31] Viswanath V, Wu Y, Boonplueang R, Chen S, Stevenson FF, Yantiri F, Yang L, Beal MF, Andersen JK. Caspase-9 activation results in downstream caspase-8 activation and BID cleavage in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease. J Neurosci 2001;21:9519–28.
[32] Calviello G, Di Nicuolo F, Piccioni E, Marcocci ME, Serini S, Maggiano N, Jones KH, Cornwell DG, Palozza P. γ-Tocopheryl quinone induces apoptosis in cancer cells via caspase-9 activation and cytochrome *c* release. Carcinogenesis 2003;24:427–33.
[33] Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. J Biol Chem 1999;274:5053–60.
[34] Jin Q, Kang C, Soh Y, Sohn NW, Lee J, Cho YH, Baik HH, Kang I. Tetrandrine cytotoxicity and its dual effect on oxidative stress-induced apoptosis through modulating cellular redox states in Neuro 2a mouse neuroblastoma cells. Life Sci 2002;71:2053–66.
[35] Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993;74:957–67.
[36] Herr I, Debatin KM. Cellular stress response and apoptosis in cancer therapy. Blood 2001;98:2603–14.
[37] Sax JK, Fei P, Murphy ME, Bernhard E, Korsmeyer SJ, El-Deiry WS. Bid regulation by p53 contributes to chemosensitivity. Nat Cell Biol 2002;4:842–9.
[38] Fei P, Bernhard EJ, El-Deiry WS. Tissue-specific induction of p53 targets in vivo. Cancer Res 2002;62:7316–27.