Involvement of Bcl-xL degradation and mitochondrial-mediated apoptotic pathway in pyrrolizidine alkaloids-induced apoptosis in hepatocytes

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are natural hepatotoxins with worldwide distribution in more than 6000 high plants including medicinal herbs or teas. The aim of this study is to investigate the signal pathway involved in PAs-induced hepatotoxicity. Our results showed that clivorine, isolated from Ligularia hodgsonii Hook, decreased cell viability and induced apoptosis in L-02 cells and mouse hepatocytes. Western-blot results showed that clivorine induced caspase-3/-9 activation, mitochondrial release of cytochrome c and decreased anti-apoptotic Bcl-xL in a time (8–48 h)- and concentration (1–100 μM)-dependent manner. Furthermore, inhibitors of pan-caspase, caspase-3 and caspase-9 significantly inhibited clivorine-induced apoptosis and rescued clivorine-decreased cell viability. Polyubiquitination of Bcl-xL was detected after incubation with 100 μM clivorine for 40 h in the presence of proteasome specific inhibitor MG132, indicating possible degradation of Bcl-xL protein. Furthermore, pretreatment with MG132 or calpain inhibitor I for 2 h significantly enhanced clivorine-induced decreased Bcl-xL level and cell viability. All the other tested PAs such as seneconine, isoline and monocrotaline decreased mouse hepatocytes viability in a concentration-dependent manner. Clivorine (10 μM) induced caspase-3 activation and decreased Bcl-xL was also confirmed in mouse hepatocytes. Meanwhile, another PA seneconine isolated from Senecio vulgaris L also induced apoptosis, caspase-3 activation and decreased Bcl-xL in mouse hepatocytes. In conclusion, our results suggest that PAs may share the same hepatotoxic signal pathway, which involves degradation of Bcl-xL protein and thus leading to the activation of mitochondrial-mediated apoptotic pathway.

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Introduction

Apoptosis is an important mode of cell death, and the characteristic changes associated with apoptosis are due to the activation of a family of intracellular cysteine proteases known as caspasases (Fraser and Evan, 1996; Boyce et al., 2004). Mitochondrial-mediated apoptotic pathway is one of major pathways involved in apoptosis, which requires the release of mitochondrial cytochrome c and activation of caspase-3/-9 signaling cascade, leading to the apoptotic destruction of the cell (Green and Reed, 1998). Bcl-2 family members function as regulators of almost all known forms of apoptosis and comprise three subfamilies, the anti-apoptotic subfamily (e.g. Bcl-xL, Bcl-2), the multi-domain pro-apoptotic subfamily (e.g. Bax, Bak) and the pro-apoptotic BH3-only subfamily (e.g. Bim, Bad) (Cory and Adams, 2002). The anti-apoptotic Bcl-2 proteins protect the mitochondria during apoptosis, while the pro-apoptotic members disrupt the mitochondria (Gross et al., 1999).

Pyrrolizidine alkaloids (PAs) are the most potential natural hepatotoxins found in a wide variety of plant species of different families worldwide (Roeder, 1995, 2000). Consumption of PAs-containing plants leads to high risk to humans and live stocks (Coulombe, 2003). Based on its possible hazards to human health, the U.S. Food and Drug Administration nominated riddelliine for genotoxicity and carcinogenicity testing conducted by the National Toxicology Program (NTP), and British Medicines Healthcare Products Regulatory Agency (MHRA) alert people to pay attention to the toxicity of seneconine-containing herbs.

There are two mainly types of toxic PAs, retronecine- and otonecine-type, of which characteristically the 8-membered heterocyclic necine base is bicyclic and monocyclic respectively (Lin et al., 1998). There are already many reports about retronecine-type PAs,
which are bioactivated in the liver to pyrrolic derivatives via the P450 system, and further react with DNA to form DNA adducting products (Mattocks et al., 1989; Huan et al., 1998; Kim et al., 1999). However there are few reports about the signal molecules involved in their hepatotoxicity, and the research on otonecine PAs, which is abundant in many plants, is fewer.

Otonecine-type PA clivorine is abundant in Ligularia hodgsonii Hook and Ligularia dentat Hara, which have been used for cough, hepatitis and inflammation traditionally in Chinese medicine (Ji et al., 2002; Kuhara et al., 1980). Retronecine-type PA senecionine is abundant in the genus of Senecio (Compositae) plants in Asia, Europe, North American and other regions (Li et al., 2008; Wiedenfeld et al., 2006; Walsh and Dingwell, 2007). In the present study, we have investigated clivorine and senecionine induced apoptosis in hepatocytes and the involved apoptotic signal pathways.

Materials and methods

Cells and reagents. The L-02 cell line was derived from adult human normal liver (Yeh et al., 1980; Zhang et al., 2007) (Cell Bank, Type Culture Collection of Chinese Academy of Sciences), and cells were cultured in RPMI1640 supplemented with 10% [v/v] fetal bovine serum. Clivorine (Fig. 1A) isolated from L. Hook with the purity ≥99.5%. Senecionine and Isoline (Fig. 1A) isolated from Senecio vulgaris L. and L. duciformis respectively both with the purity ≥95.0%. Monocrotaline was from Sigma Chemical Co. (St. Louis, MO). Ac-IETD-pNA, z-VAD-fmk, z-LEHD-fmk, z-DEVD-fmk, MG132 and calpain inhibitor I were from ALEXIS Biochemicals (San Diego, CA). Caspase-3/CPP32, Caspase-9 Colorimetric Assay Kits and Protein A-Agarose were from BioVision (Mountain View, CA).

The following antibodies for caspase-3, caspase-9, cytochrome c, Bcl-xl and Ubiquitin were from Cell Signaling Technology (Danvers, MA). The antibody for actin was from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody for Bcl-2 was from BioLegend (San Diego, CA). Peroxidase-conjugated goat anti-Rabbit IgG(H+L) and Peroxidase-conjugated goat anti-mouse IgG(H+L) were from Jackson ImmunoResearch (West Grove, PA). Nitrocellulose membranes and prestained protein marker were from Bio-Rad (Hercules, CA) and enhanced chemiluminescence detection system was from Amersham Life Science (Buckinghamshire, UK). RevertAid first strand cDNA synthesis kit was from Fermentas International Inc. (Ontario, Canada). All other reagents unless indicated were from Sigma Chemical Co.

DNA fragmentation assay. DNA fragmentation assay was performed as previously described method with minor revision (Zhang et al., 2001). Briefly, cells were lysed with buffer containing 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, 150 mM NaCl, 0.4% SDS and 100 μg/ml proteinase K and left in 37 °C overnight. The fragmented DNA in the lysate was
extracted with phenol/chloroform/isopropanol alcohol (25:24:1, v/v), and then precipitated for 5 min at liquid nitrogen with chilled 100% ethanol and 3 M sodium acetate. The DNA pellets were saved by centrifuging at 15,000 g for 15 min and then washed with 70% ethanol and resuspended in Tris-Cl (pH 8.0), with 100 μg/ml RNase A at 37 °C for 1 h. The DNA fragments were separated by 2% agarose gel electrophoresis, stained with ethidium bromide and photographed in UV light.

Caspase colorimetric assay. Activities of caspase-3 and caspase-9 were determined according to the caspase colorimetric assay kits. The cell lysates containing 80 μg of protein were incubated with 200 μM of caspase-3 and caspase-9 specific labeled substrates Ac-DEVD-pNA and Ac-LeHD-pNA at 37 °C for 2 h. Caspase-3 and caspase-9 activities were determined as the cleavage of colorimetric substrate by measuring the absorbance at 405 nm. Results are expressed as the percent change of the activity compared to the untreated control.

Preparation of mitochondrial and cytosolic fractions. Cells were lysed in lysis buffer containing 10 mM Tris (pH 7.5), 10 mM KCl, 0.15 mM MgCl2, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 150 mM sucrose for 15 min on ice, then cell was broken with 10 passages through a 26-gauge needle, and the homogenate was centrifuged at 800 g for 10 min to remove nuclei and unbroken cells. Supernatant was centrifuged at 12,000 g for 15 min to collect supernatant as cytosolic and pellet as mitochondrial fraction.

Cell viability assay. After various treatments, cells were incubated with 500 μg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h. The functional mitochondrial succinate dehydrogenases in survival cells can convert MTT to formazan that generates a blue color (Hansen et al., 1989). At last the formazan was assayed and normalized to equal protein concentration. Proteins were separated by SDS-PAGE and blots were probed with appropriate combination of primary and HRP-conjugated secondary antibodies. For repeated immunoblotting, membranes were stripped in 62.5 mM Tris (pH 6.7), 20% SDS and 1 M 2-mercaptoethanol for 30 min at 50 °C.

To identify polyubiquitinated Bcl-XL, cells were pretreated with the proteasome specific inhibitor MG132 (20 μM) for 2 h and then incubated with chlorovine (100 μM) for 40 h. Cells were lysed and protein concentrations were determined, then equal amounts of protein were subjected to immunoprecipitation with anti-Bcl-XL antibody. The immunoprecipitate was separated via SDS-12% PAGE and the conjugates were detected by Western-blot analysis, and actin was used for normalization.

Mouse hepatocyte isolation. Five or 7-week-old ICR male mice were purchased from Shanghai Laboratory Animal Center (Shanghai, China). They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Experiments were performed following the guidelines of the local Committee for Care and Use of laboratory animals. Hepatocytes were isolated from ICR mice by enzymatic digestion and centrifugation (500 rpm, 1.5 min). Freshly isolated hepatocytes were seeded in RPMI1640 medium with 10% Fetal calf serum, 1 μg/ml 3-mercaptoethanol at 37 °C with 630 nm as a reference and cell viability was normalized as a percentage of control.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from cells that had been treated with chlorovine (100 μM) or vehicle for various times using a TRIzol (Life Technologies, USA) reagent according to the manufacturer’s protocol. To synthesize single strand cDNA, reverse transcription of 2 μg total RNA was carried out using 200 U MMLV-RT and reaction mixture (2.5 mM dNTP, 100 pmol oligo-dT primer, RT buffer, 50 U RNasin) according to the manufacturer’s protocol. Transcripts of the gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an internal control. The primer sequences: Bcl-XL forward: 5′-TTCGACAACTGACTGTTG-3′, Reverse: 5′-CTGACCTGGATCATGACG-3′, 25′-GTGACGTTGATGACG-3′ (765 bp product) (Weinmann et al., 1999), GAPDH forward: 5′-CACCCACATGGAGAAGGTGG-3′, Reverse: 5′-CCCCAGTTGACTGACGACCC-3′ (200 bp product) (Hougyard et al., 2005). The PCR protocol for Bcl-XL consisted of denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 30 s and extension at 72 °C for 2 min, final extension at 72 °C for 15 min. The protocol for GAPDH was the same except for annealing at 65 °C for 1 min and amplification for 25 cycles. The PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide. The quantity of Bcl-XL and GAPDH products were automatically analyzed by GIS gel image processing system (Version 3.10, Tanon Technology Limited Company, Shanghai, China).

Statistical analysis. The results were expressed as means±SD. Differences between groups were assessed by one-way ANOVA using the SPSS software package for windows. p<0.05 was considered as statistically significant.

Results

Clivovine induced human normal liver L-2 cell apoptosis

Clivovine exposure (10–100 μM) of L-02 cells for 48 h resulted in a dramatic decrease of cell viability in the concentration-dependent manner (Fig. 1B), and the IC50 is about 40.8 μM. As shown in Fig. 1B that other PAs such as senecionine, isocnicrolone and allonine lack all have no significant toxicity on L-02 cells from 10–100 μM. The results are consistent with our previous studies that the toxicity of clivovine may not need metabolic activation in liver (Ji et al., 2002). Our previous study has showed that clivovine induced L-02 cells apoptosis as detected by FACS analysis of sub-G1 peak and Hoechst 33258 staining.
L-02 cells were treated with 100 μM clivorine for 24, 36 and 48 h, as shown in Fig. 1C, clivorine with 48 h treatment induced apoptotic DNA ladder in L-02 cells, which is considered a biochemical hallmark for apoptosis (De Maria et al., 1997). The results further confirmed clivorine-induced apoptosis in hepatocytes.

Clivorine induced caspase-3 and caspase-9 activation

Our previous study showed that clivorine induced PARP [Poly (ADP-ribose) polymerase] cleavage (Ji et al., 2005), which is a specific physiological substrate of executioner caspases (caspase 3, 6, and 7), indicating that clivorine may induce apoptosis through caspase signaling cascade. Caspases are synthesized as inactivezymogens, whose cleavage represents its activation (Boyce et al., 2004). To further investigate whether caspase signal cascade is involved in clivorine-induced apoptosis, L-02 cells were treated with 100 μM clivorine for indicated times or treated with 1–100 μM clivorine for 48 h. As shown in Figs. 2A, B, and C, clivorine decreased pro-caspase-3/-9 expression and increased the expression of cleaved caspase-3/-9 in a time and concentration-dependent manner, which indicate that clivorine can induce caspase-3/-9 activation. The caspase colorimetric assay showed that 100 μM clivorine induced the cleavage of caspase-3/-9 substrates, which confirmed clivorine-induced caspase-3/-9 activation (Fig. 2D).

Effects of caspase inhibitors on the toxicity of clivorine

To determine whether the activation of the caspases is required for the induction of apoptosis, L-02 cells are pretreated with various caspase inhibitors for 2 h prior to the addition of 100 μM clivorine. The results of DNA fragmentation and MTT assays showed that pretreatment with specific caspase-3 inhibitor z-DEVD-fmk (20 μM), specific caspase-9 inhibitor z-LEHD-fmk (20 μM) and pan-caspase inhibitor z-VAD-fmk (20 μM) significantly inhibited clivorine-induced cell apoptosis and rescued clivorine-decreased cell viability (Fig. 3), but specific caspase-8 inhibitor Ac-IETD-pNA (20 μM) had no such protective effect (Fig. 3).

Clivorine induced release of cytochrome c from mitochondria

The release of cytochrome c from mitochondria is believed to be an initiator of the caspase cascade in mitochondrial-mediated apoptotic pathway. To investigate whether the caspase-9 activation is due to the release of cytochrome c from mitochondria, cellular cytosolic and mitochondrial fractions were prepared after treated with 100 μM clivorine for indicated times or various concentrations of clivorine for 48 h. The Western-blot result of Fig. 4 showed that clivorine caused a time and concentration-dependent decrease in mitochondrial cytochrome c and a concomitant increase in cytosolic cytochrome c.

Clivorine decreased the level of cellular anti-apoptotic Bcl-xL protein

Anti-apoptotic members of the Bcl-2 family (Bcl-xl, Bcl-2 etc.) play important roles in regulating the release of cytochrome c from mitochondria. After treated with 100 μM clivorine for indicated times or various concentrations of clivorine for 48 h, the expression of Bcl-xl and Bcl-2 was detected by Western-blot. Fig. 5 showed that clivorine decreased Bcl-xl amount with a time and concentration-dependent manner, while had no effect on another anti-apoptotic Bcl-2 protein.

Effects of clivorine on Bcl-xl protein biosynthesis and degradation

To further observe how clivorine decreased the level of Bcl-xl protein, we analyzed the expression of Bcl-xl mRNAs in L-02 cells.
After treated with 100 μM clivorine for indicated times, the results of RT-PCR showed that clivorine had no effect on Bcl-xL gene expression in mRNA level (Figs. 6A, B). Cycloheximide is a well-known inhibitor of protein biosynthesis. We further used cycloheximide to inhibit the biosynthesis of protein and observed the expression of Bcl-xL in clivorine-treated cells. We found that in the presence of 200 μM cycloheximide clivorine (100 μM) still significantly decreased Bcl-xL level and cycloheximide did not inhibit the decrease of Bcl-xL (Fig. 6C), indicating that clivorine decreased Bcl-xL expression not via inhibiting protein biosynthesis.

Ubiquitin-dependent proteolysis mediated by the 26S proteasome and calpain (calcium-activated neutral protease) are implicated in proteolysis of a number of proteins not only under normal conditions, but also during apoptosis (Relnstein and Clechanover, 2006; Sorimachi et al., 1997). To observe whether clivorine-decreased Bcl-xL is due to protein degradation, L-02 cells were pretreated with 20 μM proteasome inhibitor MG132 and 50 μM calpain inhibitor I for 2 h, and then incubated with 100 μM clivorine for 40 h. As shown in Fig. 7A MG132 and calpain inhibitor I significantly rescued clivorine-decreased Bcl-xL protein.

If Bcl-xL is the target of the ubiquitin/proteasome pathway in clivorine-treated hepatocytes, inhibition of the proteasome activity by specific inhibitor MG132 shall accumulate polyubiquitinated Bcl-xL. L-02 cells were pretreated with MG132 (20 μM) for 2 h, and then incubated with 100 μM clivorine for 40 h. As shown in Fig. 7B, Bcl-xL immunoprecipitates from cells treated with clivorine in the presence of MG132 displayed increases in high molecular weight ubiquitin immunoreactive materials as compared with cells not treated with clivorine.

**Effect of proteasome and calpain inhibitors on cell viability and caspase-3/-9 activation induced by clivorine**

To further observe the roles of ubiquitin/proteasome and calpain in clivorine-induced hepatotoxicity, L-02 cells were pretreated with...
20 μM MG132 and 50 μM calpain inhibitor I for 2 h prior to the addition of 100 μM clivorine. MG132 and calpain inhibitor I significantly reversed clivorine-decreased cell viability (Fig. 8A). Western-blot results showed that MG132 and calpain inhibitor I also inhibited caspase-3/-9 activation induced by clivorine (Fig. 8B).

Effects of various PAs on fresh isolated mouse hepatocytes

We further observed the toxicity of other retronecine-type PAs and clivorine on mouse hepatocytes. As shown in Fig. 9A, all the tested PAs decreased mouse hepatocytes viability in a concentration-dependent manner after 48 h treatment, and the IC_{50} values of clivorine, senecionine, isoline and monocrotaline are about 2.1 μM, 7.3 μM, 39.6 μM and 44.1 μM respectively. Further results showed that clivorine (10 μM) and senecionine (100 μM) induced apoptotic DNA ladder, caspase-3 activation and decreased anti-apoptotic Bcl-xL in mouse hepatocytes after 48 h treatment (Figs. 9B, C). All the results suggest that clivorine and senecionine induce apoptosis in mouse hepatocytes, of which the involved toxic mechanisms are same as clivorine in L-02 cells.

Discussion

In our previous work clivorine-induced apoptosis in L-02 cells was reported (Ji et al., 2005). In this study, our results showed that clivorine induced apoptotic DNA ladder in L-02 and mouse hepatocytes (Figs. 1C, 9B), which further confirmed that the hepatotoxicity of clivorine was due to inducing apoptosis. Our results also showed that the toxicity of clivorine on mouse hepatocytes was stronger than L-02 cells (Figs. 1B, 9B), which may be due to the species difference or the enhanced toxicity of metabolic products in mouse hepatocytes.

In mammalian cells caspase-3 is an important downstream executioner in apoptosis, and several substrates such as PARP, lamin are cleaved by activated caspase-3 (Cohen, 1997; Lee et al., 2002). Our results showed that clivorine induced caspase-3 activation in L-02 and isolated mouse hepatocytes (Figs. 2, 9C). The caspase-3 inhibitor, z-DEVD-fmk significantly inhibited clivorine-induced cell apoptosis and rescued clivorine-reduced cell viability (Fig. 3). These results suggest that caspase-3 plays an important role in clivorine-induced apoptosis on hepatocytes. During apoptosis, caspase-3 can be activated by caspase-9 via mitochondrial-mediated pathway or by caspase-8 via...
death-receptor mediated pathway (Kauffman and Earnshaw, 2000). Our results in Fig. 2 showed that clivorine induced caspase-9 activation and further results in Fig. 3 showed that caspase-9 inhibitor, z-LEHD-fmk, significantly inhibited clivorine-induced cell apoptosis and rescued clivorine-decreased cell viability, indicating that clivorine may induce caspase-3 cleavage via activation of caspase-9. To further investigate whether death-receptor mediated apoptotic pathway is also involved in clivorine-induced apoptosis, we observed the activation of caspase-8 after 48 h treatment, whose activation is the hallmark of the death-receptor mediated apoptosis. The caspase colorimetric assay showed no activation of caspase-8 (data not shown), and the caspase-8 inhibitor, Ac-IETD-pNA, also had no effect on clonorine-induced cell apoptosis and clivorine-reduced cell viability (Fig. 3). All the results indicate that death-receptor mediated apoptotic signal pathway may not be involved in clivorine-induced apoptosis.

The release of cytochrome c from mitochondria plays an important role in mitochondria triggered apoptosis (Newmeyer and Ferguson-Miller, 2003; Wang, 2001), which promotes the activation of caspase-9 by forming a complex with Apaf-1 in the presence of ATP (Li et al., 1997). In the present study, both cytotoxic and mitochondrial fractions were prepared and the cytosolic translocation of cytochrome c was detected by Western-blot. Our results demonstrated that clivorine induced cytochrome c release from mitochondria to cytosol in L-02 cells (Fig. 4). These results further confirmed that mitochondria-mediated apoptotic pathway was involved in clivorine-induced apoptosis.

The anti-apoptotic Bcl-xL protein is localized on outer mitochondrial membrane, and functions to prevent cytochrome c release from mitochondria (Green and Reed, 1998; Terui et al., 1998). In our results, clivorine decreased the level of Bcl-xL protein in L-02 and isolated mouse hepatocytes (Figs. 5, 9C). These results suggest that clivorine may lead to the release of cytochrome c from mitochondria by decreasing the level of anti-apoptotic Bcl-xL.

Clovorine decreased the level of Bcl-xL may be due to the inhibition of the gene expression or the biosynthesis of Bcl-xL protein. In our results we found that clivorine had no effect on Bcl-xL gene expression and cycloheximide pretreatment did not affect clivorine-induced decrease of Bcl-xL protein (Fig. 6). These results indicate that clivorine decreased Bcl-xL protein may not via inhibiting gene expression or protein biosynthesis and probably through posttranslational modifications.

Ubiquitin is a ubiquitously expressed 76 amino acid protein that can be covalently attached to target proteins, leading to their ubiquitination, and then the ubiquitinated proteins can be degraded by the proteasome. Numerous studies have demonstrated that the ubiquitin/proteasome system plays an important role in controlling the levels of various cellular proteins involved in cell cycle progression, apoptosis and cell transformation including Bcl-2 family member such as Bcl-2, Bim, Bax and Bid (Ciechanover, 2004; Breitschopf et al., 2000; Li and Dou, 2000; Ley et al., 2003; Chanvorachote et al., 2006), however there is no related report about Bcl-xL. In our results, we showed that clivorine induced Bcl-xL polyubquitination in the presence of proteasome specific inhibitor MG132 and clivorine-decreased Bcl-xL level was enhanced by MG132 (Fig. 7A). Further results showed that MG132 also partially rescued clivorine-reduced cell viability and inhibited clivorine-induced activation of caspase-3/9 (Fig. 8). Our results suggest that ubiquitin/proteasome plays important roles in clivorine-induced degradation of Bcl-xL, and which is involved in clivorine-induced apoptosis. All these results suggest that the ubiquitin/proteasome system may change the ratio of Bcl-2 family members and therefore alter the susceptibility of cells to various apoptotic stimuli. It is hoped that in the near future to identify the enzymes responsible for ubiquitination of Bcl-xL, which could also provide valuable targets for modulating apoptosis of normal or tumor cells.

Calpain is a family of Ca2+-dependent cysteine proteases, which has been implicated in proteolysis of a number of proteins including some Bcl-2 family members (Gil-Parrado et al., 2002; Goll et al., 2003). Our results showed that clivorine-decreased Bcl-xL level was enhanced by calpain inhibitor I (Fig. 7A), meanwhile calpain inhibitor I also partially improved clivorine-decreased cell viability and inhibited clivorine-induced caspase-3/9 activation (Fig. 8). All the results indicate that calpain may play some roles in clivorine-induced degradation of Bcl-xL and hepatocytes apoptosis, however which calpain is involved in the degradation of Bcl-xL and whether Bcl-xL is the direct substrate of calpain in clivorine-treated hepatocytes needs further investigation.

It has been reported that PAs like monocrotaline has no toxic effects on cells lacking of hepatic enzymes (Thomas et al., 1998), and cultured L-02 cells may lose the hepatic enzymes that’s why the other tested PAs have no significant toxicity on L-02 cells (Fig. 1B). Next we observed that other PAs senecionine, monocrotaline and isoline also decreased mouse hepatocytes viability in a concentration-dependent manner (Fig. 9A). The further study showed that senecionine and senecionine both induced apoptotic DNA ladder, caspase-3 activation and decreased Bcl-xL (Figs. 9B,C), which indicating that senecionine induced hepatocytes apoptosis and the involved apoptotic signal pathway is the same as clivorine.

Up to now, more than 400 PAs have been reported for structures elucidation, but only few of them have been assayed for hepatotoxicity. In the present study, our results suggest that clivorine induced anti-apoptotic Bcl-xL degradation via ubiquitin/proteasome and calpain systems, thus regulating the release of cytochrome c, leading to the activation of caspase-9/caspase-3 signaling cascade and finally apoptosis, and further results indicate that the involved hepatotoxic mechanisms of senecionine may be the same as clivorine. Our results define a novel-signaling pathway for PAs-induced hepatocytes apoptosis and may also provide further approach for the detoxification of PAs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2008.05.015.

References


