Denbinobin induces apoptosis in human lung adenocarcinoma cells via Akt inactivation, Bad activation, and mitochondrial dysfunction

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Abstract

Increasing evidence demonstrated that denbinobin, isolated from \textit{Ephemerantha lonchophylla}, exert cytotoxic effects in cancer cells. The purpose of this study was to investigate whether denbinobin induces apoptosis and the apoptotic mechanism of denbinobin in human lung adenocarcinoma cells (A549). Denbinobin (1–20 μM) caused cell death in a concentration-dependent manner. Flow cytometric analysis and annexin V labeling demonstrated that denbinobin increased the percentage of apoptotic cells. A549 cells treated with denbinobin showed typical characteristics of apoptosis including morphological changes and DNA fragmentation. Denbinobin induced caspase 3 activation, and \textit{N}-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), a broad-spectrum caspase inhibitor, prevented denbinobin-induced cell death. Denbinobin induced the loss of the mitochondrial membrane potential and the release of mitochondrial apoptotic proteins including cytochrome \textit{c}, second mitochondria derived activator of caspase (Smac), and apoptosis-inducing factor (AIF). In addition, denbinobin-induced Bad activation was accompanied by the dissociation of Bad with 14-3-3 and the association of Bad with Bcl-xL. Furthermore, denbinobin induced Akt inactivation in a time-dependent manner. Transfection of A549 cells with both wild-type and constitutively active Akt significantly suppressed denbinobin-induced Bad activation and cell apoptosis. These results suggest that Akt inactivation, followed by Bad activation, mitochondrial dysfunction, caspase 3 activation, and AIF release, contributes to denbinobin-induced cell apoptosis.

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1. Introduction

Lung cancer is a major cause of cancer-related death worldwide. Adenocarcinoma is the most common type, making up 30–40% of lung cancer. The curative treatments for lung cancer include surgery, radiation therapy or chemotherapy depending on the cancer stage. However, the therapeutic outcome, including survival and relapse rate, is disappointing. Thus, numerous researchers devoted their efforts to the development of novel therapeutic agents inducing lung cancer cell death.

Denbinobin (5-hydroxy-3,7-dimethoxy-1,4-phenanthraquinone), a compound extracted and purified from several \textit{Dendrobium} or \textit{Ephemerantha} (Orchidaceae) species, such as \textit{D. nobile} (Lee et al., 1995), \textit{D. moniliforme} (Lin et al., 2001), and \textit{E. lonchophylla} (Chen et al., 1999, 2000), was recently demonstrated
to induce cell death in several cancer cell lines including lung adenocarcinoma cells (A549), ovary adenocarcinoma cells (SK-OV-3), promyelocytic leukemia cells (HL60), human leukemia cells (K562), and colon adenocarcinoma cells (COLO 205) (Lee et al., 1995; Huang et al., 2005; Yang et al., 2005). Denbinobin has also been reported to have other biological effects such as antiplatelet aggregation (Chen et al., 2000), antioxidation (Chen et al., 1999), and anti-inflammation (Lin et al., 2001). Alterations of tubulin polymerization and Bcr–Abi activity have been shown to contribute to denbinobin-induced cell death in human K562 leukemia cells (Huang et al., 2005). However, the precise molecular mechanism of denbinobin-induced cell death and whether denbinobin induces cell apoptosis has not been fully delineated.

Cell death by apoptosis plays a critical role in both the normal development and pathology of a wide variety of tissues (Jacobson et al., 1997; Nagata, 1997). Initiation of apoptosis is controlled by regulation of the balance between the death and survival signals perceived by a cell (Musci et al., 1997; Wang et al., 1999). The characterization of survival signal transduction pathways stimulated by various growth factors has revealed that phosphoinositide-3-OH kinase (PI3K) is involved in protecting cells from undergoing apoptotic cell death (Jung et al., 2000; Mathieu et al., 2001). The prominent target of PI3K is the serine/threonine kinase, Akt, also termed protein kinase B. Akt mediates many PI3K-regulated biological responses including glucose uptake, protein synthesis, and inhibition of apoptosis (Cong et al., 1997; Cichy et al., 1998; Mathieu et al., 2001; Dijkers et al., 2002). Ectopic expression of Akt, especially constitutively active Akt, induces cell survival and malignant transformation, whereas inhibition of Akt activity stimulates apoptosis in a range of mammalian cells (Goswami et al., 1999).

With stimulation by growth factors or cytokines, Akt is phosphorylated at two key regulatory sites, threonine 308 (Thr308) and serine 473 (Ser473) (Alessi et al., 1996). Fully activated Akt, in turn, functions to promote cell survival by phosphorylating several downstream targets including the Bcl-2 family member Bad, IκB kinase, caspase family member caspase-9, and forkhead family transcription factor FKHL1 (Datta et al., 1997; del Peso et al., 1997; Brunet et al., 1999; Fujita et al., 1999; Ozes et al., 1999; Pastorino et al., 1999; Dijkers et al., 2002).

Bad is a member of BH3-only proteins, a subgroup of Bcl-2 apoptotic regulators which contain only one of the bcl-2-homologous regions (BH3). In response to apoptotic stimuli, BH3-only proteins are translocated to the mitochondria from other cellular compartments, leading to cell death by apoptosis (Pastorino et al., 1999; Huang and Strasser, 2000). Bad is capable of forming heterodimers with the antiapoptotic proteins, Bcl-XL and Bcl-2, and antagonizes their antiapoptotic activity resulting in mitochondrial permeabilization (Zha et al., 1996, 1997; Kelekari et al., 1997). Subsequently, mitochondrial apoptogenic proteins, including cytochrome c, the second mitochondria-derived activator of caspase (Smac), and apoptosis-inducing factor (AIF), are released into the cytosol, leading to caspase activation and eventual cell death (Kluck et al., 1997; Lorenzo et al., 1999; Du et al., 2000). Cytochrome c triggers caspase activation through interactions with apoptosis-activating factor 1 (Apaf1) (Hu et al., 1998). Smac relieves inhibitors of apoptosis (IAPs) from inhibiting caspases (Du et al., 2000; Wu et al., 2000), thus ensuring the caspase-dependent pathway. However, the mitochondrial release of AIF causes nuclear chromatin condensation and fragmentation in a caspase-independent manner (Lorenzo et al., 1999). The preferential release of select proapoptotic proteins from mitochondria might be subject to differential upstream regulation. The Akt cascade, via its control of Bad activity, has emerged as an important regulatory mechanism upstream of mitochondria in the maintenance of cell viability (Datta et al., 1997).

In the present study, we explored the roles of Akt and Bad in denbinobin-induced mitochondrial dysfunction and cell death in human lung adenocarcinoma cells (A549). We demonstrated that the Akt inactivation, followed by Bad activation, mitochondrial dysfunction, mitochondrial molecules such as cytochrome c, smac, and AIF release, and caspase 3 activation contributes to denbinobin-induced cell apoptosis.

2. Materials and methods

2.1. Materials

Denbinobin was kindly provided by Dr. Chien-Chih Chen (National Research Institute of Chinese Medicine, Taipei, Taiwan). The methods of extraction and isolation of denbinobin were reported previously (Chen et al., 2000), and the purity is over 98% based on the HPLC analysis. Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12, fetal calf serum (FCS), penicillin/streptomycin, OptiMEM, and lipofectamin plus™ reagent were purchased from Invitrogen (Carlsbad, CA). Antibodies specific for Bcl-2, Bax, and procaspase 3 were purchased from Transduction Laboratories (Lexington, KY). Protein A/G beads, antibodies specific for cytochrome c, Smac, AIF, Bcl-XL, poly (ADP-ribose) polymerase (PARP), Bad, and phospho-Bad (Ser147) were purchased from New England Biolabs (Beverly, MA). Annexin V-FITC apoptosis detection kit and caspase 3 activity kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specific for phospho-Akt (Ser473) was purchased from Cell Signaling Technology (Danvers, MA). All other reagents were obtained from Sigma (St. Louis, MO).

2.2. Cell culture

A549 cells, a human pulmonary type II epithelial adenocarcinoma cell line, were obtained from the American Type Culture Collection and cultured in DMEM/Ham’s F-12 nutrient mixture with 10% FCS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were cultured at 37°C in a humidified 5% CO2 atmosphere.
2.3. Cell viability assay

Cell viability was measured by a previously described colorimetric MTT assay (Goswami et al., 1999; Chen et al., 2002). Briefly, cells (2 × 10^5 cells/well) were cultured in 24-well plates and incubated with vehicle or various concentrations (1, 3, 10, 20 μM) of denbinobin for 24 h. After various treatments, 1 mg/ml MTT was added to the culture plates and incubated at 37 °C for an additional 4 h. Then cells were lysed in 500 μl dimethyl sulfoxide. The absorbance at 550 nm was measured on a microplate reader. Each experiment was performed in triplicate and repeated at least three times.

2.4. Flow cytometric analysis

A549 cells were cultured in 10-cm Petri dishes. After reaching confluence, cells were treated with vehicle or 20 μM denbinobin for 12, 24, 36, or 48 h. After treatment, cells were harvested and washed twice with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.5 mM KH2PO4; pH 7.4), and re-suspended in ice-cold 70% ethanol at −20 °C overnight. Cells were washed for 5 min with 0.4 ml phosphate-citric acid buffer (pH 7.8) containing 50 mM Na2HPO4, 25 mM citric acid, and 0.1% Triton X-100 and subsequently stained with 1.5 ml PI staining buffer containing 0.5% Triton X-100, 10 mM PIPES, 100 mM NaCl, 2 mM MgCl2, 0.1 U/ml RNase A, and 25 μg/ml PI for 30 min in the dark before the flow cytometric analysis. Samples were analyzed by FACSscan and the Cellquest program (Becton Dickinson, San Jose, CA).

2.5. Annexin V-propidium iodide staining

Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues which are exposed on the cell surface of apoptotic cells, as previously described (Dijkers et al., 2002). Cells were treated with vehicle or 20 μM denbinobin for 12, 24, 36, or 48 h. After treatment, A549 cells were washed twice with PBS (pH 7.4), and resuspended in staining buffer containing 1 μg/ml PI and 0.025 μg/ml annexin V-FITC. Double-labeling was performed at room temperature for 10 min in the dark before the flow cytometric analysis. Samples were analyzed using FACSscan and the Cellquest program (Becton Dickinson, San Jose, CA).

2.6. DNA fragmentation

Genomic DNA was isolated as described previously (Chen et al., 2002). Briefly, cells were treated with vehicle, denbinobin (1, 3, or 20 μM) or tumor necrosis factor-α (TNF-α, 100 ng/ml) for 24 h or 48 h. After treatment, cells were washed with PBS and lysed in cell lysis buffer containing 50 mM Tris–HCl (pH 7.4), 10 mM EDTA, 0.2% Triton X-100, 1 mM PMSF, 0.1 mM aprotinin, 1 mM leupeptin, and 0.5 mg/ml proteinase K. The supernatant was incubated with RNase A (0.2 mg/ml) at 37 °C for 2 h. An equal volume of phenol/chloroform was then added with intermittent gentle agitation for 10 min. Following centrifugation at 12,000 × g for 20 min, DNA in the upper phase was collected. Then the genomic DNA was precipitated by adding sodium acetate (0.3 M, pH 5.4) and 99.9% ethanol. The DNA pellet was washed with 70% (v/v) ethanol and dissolved in Tris–EDTA buffer. The DNA concentration was determined by spectrophotometry. DNA sample, of ~0.2 mg each, were electrophoresed on a 2% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide. DNA fragmentation bands were photographed under UV light.

2.7. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay

A549 cells were plated on glass cover slides. Cells were then treated with vehicle, 20 μM denbinobin or 100 ng/ml TNF-α for 24 h. After treatment, cells were fixed with freshly prepared paraformaldehyde (4% in PBS, pH 7.4). The slides were rehydrated in PBS and incubated in 0.3% (v/v) H2O2 in methanol for 30 min to block endogenous peroxidase. DNA nicks were determined using an in situ TdT-FragEL DNA fragmentation detection kit (Oncogene Research, Cambridge, MA). Briefly, the specimens were covered for 1.5 h at 37 °C with the TUNEL reaction mixture containing TdT enzyme solution and label solution (modified nucleotide mixture in reaction buffer). The reaction was terminated by washing with PBS, and the slides covered with Converter-POD (streptavidin conjugated with horseradish peroxidase, POD) for 30 min and visualized using 3,3′-diaminobenzidine for 10 min. Counterstaining was performed with 5% (w/v) methyl green in a 0.1 M sodium acetate solution (pH 4.0) for 2 min, after which the stained cells were analyzed by light microscopy.

2.8. Determination of the mitochondrial membrane potential

The mitochondrial membrane potential was assessed using a fluorometric probe, DiOC6 (Molecular Probes), with a positive charge of a mitochondrial-specific fluorophore, as previously described (Susin et al., 1997; Ye et al., 1999). Briefly, A549 cells were plated in 6-well culture dishes. After reaching confluence, cells were treated with vehicle or 20 μM denbinobin for 12 h or 24 h. After incubation, cells were stained with DiOC6 (40 nM) for 15 min at 37 °C. Cells were collected, washed twice in PBS, and analyzed by FACSscan flow cytometry. The probes were excited with a laser at 488 nm, and emission was monitored through a 530-nm bandpass filter. At least 10,000 cells were analyzed per sample.

2.9. Immunoblot analysis

To determine the levels of procaspase 3, PARP, Bcl-xL, Bad, phospho-Bad (Ser136) Bax, α-tubulin, phospho-Akt (Ser473), and Akt in A549 cells, the proteins were extracted as previously described (Chang et al., 2002), with modifications. Briefly, A549 cells were cultured in 6-cm dishes. After reaching confluence, cells were treated with vehicle or 20 μM denbinobin for 12, 24, 36, or 48 h. After incubation, cells were washed twice with ice-cold PBS and solubilized in extraction buffer containing 10 mM Tris (pH 7.0), 140 mM NaCl, 3 mM MgCl2, 2 mM PMSF, 5 mM DTT, 0.5% NP-40, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin, 1 mM benzamidine, and 1 mM Na3VO4. Protein concentrations of cell lysates were determined by the Bradford protein assay (Bio-Rad). Equal amount of protein (60 μg) in each sample was boiled in SDS sample loading buffer, and then fractionated on SDS-PAGE before blotting onto a polyvinylidene difluoride (PVDF) membrane. Blots were then incubated in 150 mM NaCl, 20 mM Tris, and 0.02% Tween (pH 7.4) containing 5% non-fat milk. Proteins were visualized by specific primary antibodies and then incubated with alkaline phosphatase- or horseradish peroxidase-conjugated second antibodies. After washing with PBS, blots were developed using NBT/BCIP or an enhanced chemiluminescence kit according to the vendor’s instruction before exposure to photographic film.

2.10. Isolation of the cytosolic fraction

The procedure for isolation of the cytosolic fraction was previously described (Pastorino et al., 1999). Briefly, cells were lysed with 40 strokes of a Wheaton Dounce glass homogenizer (type B pestle) in ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1.5 mM MgCl2, 10 mM KCl, 20 mM HEPES (pH 7.4), 0.4 μM aprotinin, and 10 μM leupeptin). The lysate was centrifuged at 600 × g for 10 min at 4 °C to remove nuclei and unbroken cells. The supernatant was removed and centrifuged at 14,000 × g for 15 min at 4 °C to eliminate mitochondria. The supernatant was then re-centrifuged at 100,000 × g for 1 h at 4 °C. The protein concentration of the resulting supernatant, which represented the cytosolic fraction, was assayed by the Bradford protein assay (Bio-Rad). Equal amount of total protein (20 μg) in each sample was boiled in SDS sample loading buffer and then fractionated on SDS-PAGE. The protein levels of cytochrome c, Smac, AIP, and α-tubulin in the cytosolic fraction were then analyzed by immunoblotting.

2.11. Caspase 3 activity assay

Caspase 3 activity was determined using caspase 3/CPP32 colorimetric assay kit (BioVision). Briefly, A549 cells were cultured in 6-cm dishes. After reaching confluence, cells were treated with vehicle or 20 μM denbinobin for 24 h or 48 h.
Fig. 1. Denbinobin-induced A549 cell death. (A) Cells were treated with vehicle or denbinobin (1–20 μM) for 24 h, and cell viability was determined by the MTT assay. Each column represents the mean ± S.E.M. of at least three independent experiments performed in duplicate. *p < 0.05, compared with the control group. The percentage of apoptotic cells was also analyzed by flow cytometric analysis of annexin V/PI double staining (B) and PI staining (C) as described in Section 2. Data are representative of three independent experiments with similar results. Compiled results are shown in the graph. Apo, apoptotic region.

Cells were harvested, and then cell lysates (100 μg) were incubated with DEVD-pNA (50 μM) for 2 h at 37 °C. The absorbance at 405 nm was measured on a microplate reader.

2.12. Immunofluorescence microscopy

A549 cells were grown on glass coverslips. After incubation in the absence or presence of 20 μM denbinobin for 24 h, cells were washed twice with PBS. Subsequently, cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were then permeabilized for 2 min in 0.02% (v/v) Triton X-100, and incubated with 5% (v/v) bovine serum albumin (BSA) in PBS for 1 h before staining. To observe the distribution of AIF, cells were stained with an anti-AIF antibody for 2 h at room temperature. After washing with PBS containing 0.02% Tween-20, cells were incubated with FITC-labeled secondary antiserum for an additional 1 h at room temperature. Cells were then washed with PBS containing 0.02% Tween-20 and incubated with 20 nM PI for another 15 min at room temperature. After staining, cells were observed under inverted laser scanning confocal microscopy (Olympus).

2.13. Co-immunoprecipitation (Co-IP)

A549 cells were grown in 6-cm dishes. After reaching confluence, cells were treated with 20 μM denbinobin for 6, 12, or 24 h. After incubation, cells were washed twice with ice-cold phosphate-buffered saline, lysed with lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM MgCl₂, 125 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 mM β-glycerophosphate, 50 mM NaF, and 100 μM Na₂VO₄, and centrifuged at 4 °C and 12,000 × g for 30 min. The supernatant was then immunoprecipitated with a polyclonal antibody against Akt in the presence of A/G-agarose beads overnight. The beads were washed three times with lysis buffer and two times with kinase buffer containing 20 mM HEPES (pH 7.4), 20 mM MgCl₂, and 2 mM DTT. The kinase reactions were performed by incubating immunoprecipitated beads with 20 μl of kinase buffer supplemented with 50 μg/ml of histone 2B (H2B), 20 μM ATP, and 3 μCi of [γ-32P] ATP at 30 °C for 30 min. The reaction mixtures were analyzed by 15% SDS-PAGE followed by autoradiography.
2.15. Plasmid DNA transfection

A549 cells were seeded at a density of 2 × 10^5 cells/ml into 12-well plates. Cells were transfected on the following day with the Lipofectamine plus™ reagent containing 1 μg/well of pUSEamp (mock), pUSEamp-Akt1 (wt-Akt), or pUSEamp-myr-Akt1 (myr-Akt) cDNA for 6 h. At the end of transfection, the medium was aspirated and replaced with fresh culture medium for 24 h. Cells were treated with 20 μM denbinobin for another 24 h before harvesting.

2.16. Statistical analysis

Results are presented as the mean ± S.E.M. from at least three independent experiments. One-way analysis of variance, followed by Bonferroni’s multiple-range tests when appropriate, was used to determine the statistical significance of the difference between the means. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Induction of cell apoptosis by denbinobin

Treatment of A549 cells for 24 h with denbinobin (1–20 μM) decreased cell viability in a concentration-dependent manner. Denbinobin at 10 μM and 20 μM significantly decreased the cell viability by 48.4 ± 13.8% and 56.1 ± 11.0%, respectively (n = 4) (Fig. 1A). We next investigated whether denbinobin induces cell death through an apoptotic mechanism. Annexin V-PI double-labeling was used for the detection of PS externalization, a hallmark of early phase of apoptosis. As compared to vehicle-treated cells, a high proportion of annexin V+ labeling was detected in cells treated with 20 μM denbinobin for 12, 24, 36, or 48 h (Fig. 1B), and two levels of labeling were observed: annexin V+ cells which remained PI− (lower right quadrant), corresponding to early apoptotic cells, and annexin V+/PI+ cells, corresponding to advanced apoptotic cells. In addition, cells were unaffected by the treatment with vehicle for 12, 24, 36 or 48 h (data not shown). Flowcytometry analysis of PI-stained cells was also used to further confirm that denbinobin induces cell death by apoptosis. Similar to Fig. 1B, the percentage of PI-stained cells in the apoptotic region (Apo, sub-G0/G1 peak) time-dependently increased following 20 μM denbinobin treatment. The ratio of apoptotic cells was significantly increased by 16.3 ± 2.4%, 32.1 ± 2.0%, and 38.5 ± 2.2% after denbinobin exposure for 24, 36, and 48 h, respectively (Fig. 1C). It appears that cells treated with vehicle for 12, 24, 36 or 48 h show no difference (data not shown).

Degradation of DNA into a specific fragmentation pattern (consisting of DNA ladders) is a characteristic feature of apoptosis. After 24 h or 48 h of exposure to denbinobin (1, 3, or 20 μM), the genomic DNA from A549 cells was subjected to agarose gel electrophoresis. As shown in Fig. 2A, clear DNA fragmentation ladders were detected in samples from cells treated with denbinobin (Fig. 2A, upper panel, lanes 5, and 7–9) as compared to vehicle-treated cells (Fig. 2A, upper panel, Lanes 2 and 6). DNA fragmentation ladders were also shown in cells treated with TNF-α (100 ng/ml) for 24 h (Fig. 2A, bottom panel, Lane 3) as compared to vehicle-treated cells (Fig. 2A, bottom panel, Lane 2). DNA laddering marker (M) was also shown in Fig. 2A. The TUNEL assay was also used to stain nuclei that contained nick-ended DNA, a characteristic exhibited by apoptotic cells. Vehicle-treated cells were almost completely negative for TUNEL staining (Fig. 2B, a). In contrast, following exposure to 20 μM denbinobin for 24 h, a large number of TUNEL-positive cells were observed (Fig. 2B, b). TUNEL-positive cells were also seen following 100 ng/ml TNF-α (c) for 24 h; TUNEL-positive cells were visualized using a peroxidase–substrate system as having condensed nuclei indicated by arrow. Data are representative of three independent experiments with similar results.

3.2. Denbinobin induced caspase activation and PARP cleavage

We next attempted to determine whether caspase activation contributes to denbinobin-induced A549 cell apoptosis. As shown in Fig. 3A, zVAD-fmk (100 μM), a broad-spectrum
Fig. 3. Involvement of caspase activation in denbinobin-induced cell death in A549 cells. (A) Cells were pretreated with vehicle or zVAD-fmk (100 \( \mu M \)) for 4 h before the addition with denbinobin (20 \( \mu M \)) for an additional 24 h. Cell viability was then determined by the MTT assay. Each column represents the mean \( \pm \) S.E.M. of at least three independent experiments performed in triplicate. \( *p < 0.05 \), compared with the group treated with denbinobin alone. (B) Cells were treated with vehicle or denbinobin (20 \( \mu M \)) for the indicated time intervals. Procaspase-3 and PARP levels were then determined by immunoblotting as described in Section 2. Typical traces representative of three independent experiments with similar results are shown. (C) Cells were treated with vehicle or 20 \( \mu M \) denbinobin for 24 h and 48 h. After treatment, the caspase 3 activity was assessed as described in Section 2. Each column represents the mean \( \pm \) S.E.M. of at least three independent experiments. \( *p < 0.05 \), compared with the control group.

caspase inhibitor, significantly inhibited the decrease of cell viability after exposure to 20 \( \mu M \) denbinobin for 24 h. Caspase 3 has been shown to lay downstream of the apoptotic signaling pathway regardless of whether intrinsic- or extrinsic-mediated apoptotic signaling occurs (Cohen, 1997; Susin et al., 1997). We then examined whether denbinobin is able to induce procaspase 3 degradation leading to caspase 3 activation. Denbinobin (20 \( \mu M \)) time-dependently induced procaspase 3 degradation (Fig. 3B, upper panel). A specific caspase 3 substrate, PARP, was then used to confirm that denbinobin activates caspase 3 resulting in PARP cleavage (Cohen, 1997; Nagata, 1997; Boulares et al., 1999). As shown in Fig. 3B (bottom panel), denbinobin induced the cleavage of PARP from 115 to 85-kDa fragment. In addition, caspase 3 activity is markedly increased in cells after exposure to denbinobin (20 \( \mu M \)) for 24 h or 48 h (Fig. 3C). These results suggested that caspase 3 is involved, at least in part, in denbinobin-induced A549 cell death.

3.3. Denbinobin caused mitochondrial dysfunction and release of cytochrome c, Smac, and AIF

To explore whether denbinobin-induced cell apoptosis is mediated through mitochondrial dysfunction, we determined the mitochondrial membrane potential with the mitochondria-sensitive dye, DiOC6, using flow cytometry. As shown in Fig. 4A, treatment of A549 cells with 20 \( \mu M \) denbinobin for 12 h and 24 h induced the loss of the mitochondrial membrane potential in a time-dependent manner. Several molecules...
including cytochrome c, Smac, and AIF are known to be liberated from mitochondria and to induce apoptosis via caspase-dependent and caspase-independent pathways (Saleh et al., 1999; Daugas et al., 2000; Du et al., 2000; Murahashi et al., 2003). To establish whether denbinobin induces cytochrome c, Smac, or AIF protein release from mitochondria to the cytosol, the levels of these proteins in the cytosolic fraction were determined using Western blotting analysis. As shown in Fig. 4B, denbinobin caused a marked increase in the cytosolic levels of cytochrome c, Smac, and AIF in A549 cells. Cytochrome c and Smac have been reported to induce apoptosis via caspase-dependent pathways (Du et al., 2000). In contrast, the mitochondrial death-executing molecule, AIF, can translocate to the nucleus and induce caspase-independent cell death upon apoptotic stimuli (Lorenzo et al., 1999). We thus investigated whether AIF was translocated to the nucleus after denbinobin exposure in A549 cells. Results from a confocal microscopic analysis indicated that AIF immunoreactivity (shown in green fluorescence) and nuclear staining (PI staining, shown in red fluorescence) were co-localized in denbinobin-treated A549 cells, whereas those of untreated A549 cells were mutually exclusively localized (Fig. 4C). These findings suggest that AIF is also involved in denbinobin-induced cell apoptosis in A549 cells.

3.4. Denbinobin caused Bad activation and the association of Bad and Bcl-xL

Bcl-2 family proteins regulate mitochondria-dependent apoptosis with the balance of the anti- and pro-apoptotic members arbitrating life-or-death decisions (Tsujimoto and Shimizu, 2000; Tsujimoto, 2002). Thus, we examined whether the protein levels of Bcl-2 family proteins are altered after denbinobin exposure. Treatment of A549 cells with 20 μM denbinobin for 12, 24, 36, or 48 h did not alter the protein levels of Bad, Bax, or Bcl-xL (data not shown). Next, we examined the effects of denbinobin on the phosphorylation status of Bad, which is crucial for activation of caspase 3 through a mitochondria-dependent pathway (Kennedy et al., 1999). Bad was highly phosphorylated at Ser136 in untreated A549 cells, but it was dephosphorylated in a time-dependent manner after exposure to 20 μM denbinobin (Fig. 5, upper panel). The compiled data are shown in the bottom of Fig. 5. Under normal physiological conditions, Bad, in both its phosphorylated and inactive form, is known to exist almost exclusively in the cytoplasm (Cross et al., 1995). The interaction of Bad phosphorylated at Ser136 and 14-3-3 avoided the proapoptotic function of Bad by translocation from the cytosol to the mitochondrial outer membrane (Zha et al., 1996, 1997). Activated Bad, recognized as an essential initiator of the apoptotic cascade, is capable of forming heterodimers with the anti-apoptotic mitochondrial protein, Bcl-xL, and antagonizes its antiapoptotic activity (Kelekar et al., 1997; Kelekar and Thompson, 1998). Therefore, a coimmunoprecipitation analysis was employed to confirm the hypothesis that denbinobin-induced Bad Ser136 dephosphorylation was accompanied by dissociation of the Bad-14-3-3 complex. As shown in Fig. 6, denbinobin (20 μM) induced the dissociation of Bad from 14-3-3. In addition, Bad-14-3-3 complex dissociation was accompanied by the association of Bad and Bcl-xL (Fig. 5). The compiled data are shown in the bottom of Fig. 5. The maximal response was seen after 24 h exposure to denbinobin. These results suggest that Bad dephosphorylation followed by the dissociation of Bad from 14-3-3 and the subsequent association of Bad and Bcl-xL is involved in denbinobin-induced mitochondrial dysfunction.

3.5. Akt inactivation is involved in denbinobin-induced Bad activation and subsequent cell apoptosis

We thus attempted to elucidate the signaling cascade leading to Bad dephosphorylation. It has been reported that Bad phosphorylation at Ser136 is induced by the PI3K/Akt pathway (Pastorino et al., 1999). Since serine phosphorylation of residue 473 in Akt causes enzymatic activation (Alessi et al., 1996), the antibody specific against phosphorylated Akt (Ser473) was used to examine Akt phosphorylation, an index of kinase activation. Denbinobin (20 μM) caused significant Akt Ser473 dephosphorylation after 12 h of treatment (Fig. 6A, upper panel). The protein level of Akt was not affected by the presence of denbinobin. The compiled data are shown in the bottom of Fig. 6A. In parallel, using histone H2B as an Akt substrate, a time-dependent decrease in Akt kinase activity was observed.
in 20 μM denbinobin-treated A549 cells (Fig. 6B). The compiled data are shown in the bottom of Fig. 6B. To further confirm whether Akt inactivation contributes to denbinobin-induced cell apoptosis, A549 cells were transiently transfected with empty (mock), wild-type Akt (wt-Akt) or constitutively active Akt (myr-Akt) prior to denbinobin (20 μM) treatment for 24 h. As compared to the control group (mock-transfected cells), cells transfected with wt-Akt or myr-Akt significantly inhibited denbinobin-decreased cell viability by 33.3 ± 3.1% or 18.5 ± 4.0%, respectively (Fig. 7A). In addition, flowcytometric analysis demonstrated that transfaction with wt-Akt or myr-Akt attenuated cell apoptosis after exposure to denbinobin for 24 h with the extent of cell apoptosis reduced from 48.9 ± 4.1% (denbinobin plus mock) to 28.4 ± 8.1% (denbinobin plus wt-Akt) and 30.4 ± 5.0% (denbinobin plus myr-Akt), respectively (Fig. 7B). To ascertain the linkage between Akt inactivation and Bad activation caused by denbinobin, we examined the effects of wt-Akt and myr-Akt on denbinobin (20 μM)-induced Bad Ser136 dephosphorylation. After transfection, Bad was immunoprecipitated (IP) and the status of Bad phosphorylation at Ser136 was determined by immunoblotting using specific antibodies against Bad Ser136. Immunoprecipitation using normal IgG is served as negative control. As shown in Fig. 7C, transfection with either wt-Akt or myr-Akt remarkably reversed the Bad Ser136 dephosphorylation after exposure to denbinobin for 12 h. Results of these experiments further imply a causal role of Akt in regulating denbinobin-induced Bad inactivation and A549 cell apoptosis.
4. Discussion

Apoptosis, a form of programmed cell death involved in tissue morphogenesis and homeostasis, is characterized by cytoplasmic shrinkage, nuclear condensation and DNA fragmentation (Jacobson et al., 1997; Nagata, 1997). Specific therapeutic policy designed to improve or decrease the susceptibility of individual cell types for undergoing apoptosis could form the basis for treatments of a variety of human diseases, such as cancer (Thompson, 1995).

Denbinobin is a newly bioactive extract purified from Chinese herbal plant, Shi-Hu, which was used in oriental medicine to cure lung disease. Thus, denbinobin was suggested to affect lung adenocarcinoma cell viability. In the present study, we demonstrated that denbinobin induced apoptosis in lung adenocarcinoma A549 cell. The apoptotic mechanism of denbinobin shown in this study involves the inactivation of Akt and Bad dephosphorylation, resulting in mitochondrial dysfunction.

Mitochondrial dysfunction has been implicated as being a key mechanism in apoptosis in various cell death paradigms (Susin et al., 1997). Two major events have been noted in apoptosis involving mitochondrial dysfunction. One event is the change in the membrane permeability and subsequent loss of membrane potential (Zamzami et al., 1996, 1998). The other is the release of apoptotic proteins including AIF, Smac, and cytochrome c from the intermembrane space of mitochondria into the cytosol (Liu et al., 1996; Green and Reed, 1998; Zamzami et al., 1998; Susin et al., 1999; Daugas et al., 2000; Du et al., 2000). In agreement of these observations, we noted that the denbinobin-induced loss of mitochondrial membrane potential was accompanied by the release of AIF, Smac, and cytochrome c. Thus, it is plausible that mitochondrial dysfunction may be involved in denbinobin-induced A549 cell apoptosis.

Downstream events after cytochrome c release involve a cytosolic protein known as Apaf1. Apaf1, which functions as a scaffold protein, may form a complex with cytochrome c and pro-caspase 9 in the presence of dATP. This complex formation may result in caspase 9 activation, which in turn cleaves and activates caspase 3 (Liu et al., 1996; Hu et al., 1998; Saleh et al., 1999; Zou et al., 1999). Caspase 3 plays a pivotal role in numerous apoptotic cascades and is responsible for the proteolytic cleavage of many key proteins involved in apoptosis (Cohen, 1997). In addition, the released Smac, which binds to a class of antiapoptotic proteins known as IAPs, thereby neutralizes IAP activity to promote caspase activation and apoptotic cell death (Du et al., 2000). In this study, we found that treatment of A549 cells with denbinobin caused the release of cytochrome c and Smac, and the activation of caspase 3, and that zVAD-fmk, a broad-spectrum caspase inhibitor, partially prevented denbinobin-induced cell death. These results suggest that denbinobin might induce, at least in part, the release of cytochrome c and Smac, which in turn mediates caspase 3 activation, and ultimately leads to A549 cell apoptosis. In addition to cytochrome c and Smac, AIF is also reported to induce the degradation of nuclear DNA and subsequently cause cell death (Susin et al., 1999). AIF-induced apoptosis was shown to occur in a caspase-independent manner. Several reports further indicated that translocation of AIF from mitochondria to the nucleus occurs during apoptosis (Daugas et al., 2000; Murahashi et al., 2003). In the present study, we noted that denbinobin-induced A549 cell death could not completely be abolished by the zVAD-fmk. Moreover, denbinobin induced mitochondrial AIF release and translocation from the cytosol to nuclei of A549 cells. These findings suggest that denbinobin-induced A549 cell apoptosis may occur through both caspase-dependent and -independent pathways downstream of mitochondrial dysfunction. However, the link between AIF nuclear translocation and cell apoptotic pathways remains to be established. Additional work is needed to characterize the interrelationships between AIF translocation and apoptotic pathways in denbinobin-induced A549 cell death.

Bcl-2 family proteins regulate mitochondria-dependent apoptosis with the balance of anti- and pro-apoptotic members arbitrating life-and-death decisions (Tsujimoto and Shimizu, 2000; Adams and Cory, 2001). However, denbinobin did not affect the protein levels of Bad, Bax, or Bcl-XL. There is growing evidence that a number of apoptotic stimuli cause Akt inactivation (Luo et al., 2003) Akt phosphorylates many proteins which are known to regulate apoptosis (Cross et al., 1995; Datta et al., 1997; Brunet et al., 1999; Fujita et al., 1999; Ozes et al., 1999). Among these proteins, Bad, the BH3-only member of Bcl-2 family proteins, is prominent (Datta et al., 1997; del Peso et al., 1997; Pastorino et al., 1999). In the present study, we found that denbinobin induced Akt inactivation and Bad dephosphorylation and that both the wild-type Akt and constitutively active Akt diminished denbinobin-induced Bad dephosphorylation and cell apoptosis. Furthermore, we also noted that the denbinobin-induced dissociation of Bad from 14-3-3 was accompanied by an association of Bad with Bcl-XL. The results suggest that Akt inactivation, followed by Bad activation, the dissociation of Bad from 14-3-3, and the subsequent association of Bad and Bcl-XL, is involved in denbinobin-induced mitochondrial dysfunction and cell apoptosis. Moreover, transfection of wild-type Akt and constitutively active Akt only partially inhibited denbinobin-induced cell apoptosis. Recent reports have indicated that in addition to Bad, other BH3-only proteins, such as Bid, also participate in denbinobin-induced cell death (Yang et al., 2005). Therefore, the Akt-Bad signaling cascade may partially contribute to the signaling cascade of denbinobin-induced A549 cell death. In fact, we recently found that apoptosis signal-regulating kinase 1 (ASK1), a pivotal mechanism in a broad range of cell death paradigms (Hatai et al., 2000; Matsuzawa and Ichijo, 2001), is also involved in the signaling pathway of denbinobin-induced cell death (unpublished observations).

Together, these results establish a denbinobin-mediated death signaling cascade in A549 cells involving Akt inactivation, resulting in a series of cellular events. The downstream pathway involves Bad dephosphorylation, dissociation of 14-3-3 and Bad, and binding to Bcl-XL, resulting in disruption of the mitochondrial membrane and release of cytochrome c, Smac, and AIF. Furthermore, the mechanism of denbinobin-induced A549 cell apoptosis may occur through both caspase-dependent and -independent (AIF) pathways downstream of mitochondrial dysfunction (Fig. 8). Thus, denbinobin may be useful as a potential
Denbinobin

\[
\begin{align*}
\text{Akt} & \downarrow \\
\text{Bad} \rightarrow_{\text{14-3-3}} & \\
\text{Bcl-xL} & \\
\text{Mitochondrial dysfunction} \\
\text{Caspase-independent} & \\
\text{Caspase-dependent} & \\
\text{AIF} & \\
\text{Cytochrome c, Smac} & \\
\text{Caspace 3} & \\
\text{Apoptosis}
\end{align*}
\]

Fig. 8. Schematic summary of apoptotic pathway involved in denbinobin-induced A549 cell apoptosis. Denbinobin inactivation of the Akt, leads to Bad dephosphorylation, mitochondrial dysfunction and subsequent in cell apoptosis. Approaches applied in the present studies are shown to support the causal role of each step in the cascade.

template for the development of better chemopreventive and/or chemotherapeutic agents against lung cancer.

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References


