Cepharanthine exerts antitumor activity on choroidal melanoma by reactive oxygen species production and c-Jun N-terminal kinase activation

QI ZHU1,2, BAOFENG GUO3, LINLIN CHEN4, QIUYE JI3, HANG LIANG1, NAIYAN WEN1 and LING ZHANG1

1Department of Pathophysiology, College of Basic Medicine Sciences, Jilin University, Changchun, Jilin 130021; 2Department of Ophthalmology, Armed Police Force Hospital of Jilin, Changchun, Jilin 130058; 3Department of Plastic Surgery, The China-Japan Union Hospital, Jilin University, Changchun, Jilin 130021; 4Department of Ophthalmology, The Fourth Hospital of Shenyang, Shenyang, Liaoning 110031, P.R. China

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Abstract. Choroidal melanoma is the most common primary intraocular tumor in adults. Cepharanthine (CEP), a natural alkaloid extracted from the roots of Stephania cepharantha Hayata, has been demonstrated to inhibit the proliferation of various cancer cells. However, its potential anticancer effect in choroidal melanoma has not been clarified yet. In the present study, it was identified that CEP may potently inhibit the proliferation of human choroidal melanoma cells, induce cell death and cell cycle arrest, and activate cellular apoptotic proteins, including Bcl-2-associated X protein, caspase and poly(ADP-ribose) polymerase. Results also revealed that CEP induced the cellular production of reactive oxygen species (ROS) and led to cytochrome c release, whereas concurrent treatment with N-acetyl-L-cysteine (a ROS scavenger) attenuated the situation. In addition, CEP was also revealed to activate c-Jun N-terminal kinase (JNK) 1 and 2, whereas inhibition of JNK1/2 partially abrogated the proliferation inhibitory effect of CEP, indicating that JNK1 and JNK2 were involved in CEP-triggered cellular apoptosis. In addition, the anticancer effects of CEP were also observed in a choroidal melanoma xenograft model. In summary, the results of the present study demonstrated that CEP is effective in suppressing human choroidal melanoma cell and tumor cell proliferation, and that CEP may therefore represent a potentially novel therapeutic agent for the treatment of choroidal melanoma.

Introduction

Choroidal melanoma is the most common primary intraocular tumor in adults (1,2). The mean incidence of choroidal melanoma in the USA is ~6 novel cases/106 people every year (3). Currently, there are several treatment protocols for primary choroidal melanoma, including surgery, radiation therapy, chemotherapy and external beam proton therapy (4-7). The 5-year relative survival rate of choroidal melanoma is ~80% when the tumors are confined to the eyes (3,8). However, patients with choroidal melanoma have a high risk of developing metastasis (typically to the liver), which is usually fatal with a median survival time of 6-9 months subsequent to the detection of liver metastasis (3,8). Therefore, development of novel effective therapies for choroidal melanoma is required urgently.

Cepharanthine(CEP; 6',12'-dimethoxy-2,2'-dimethyl-6,7-[methylenebis(oxy)]oxyacanthan) is a biscoclaurine alkaloid extracted from the roots of the plant Stephania cepharantha Hayata, which has been broadly used in Japan for chemoprevention and treatment of numerous diseases by virtue of its anti-inflammatory and immunomodulatory activities (9,10). CEP has been reported to exert antitumor effects in numerous cancers by inhibiting cancer cell proliferation (11), cell cycle progression (12), tumor invasion (13), generating reactive oxygen species (ROS) (11,14), inducing cell apoptosis (11,14,15), regulating cell survival signaling pathways (16,17) and increasing the competence of the host (18,19). It was also identified to potentiate the anticancer effects of other chemotherapeutic agents (20-22), and was able to circumvent the multidrug resistance of doxorubicin, vincristine and other anticancer agents (21,23-26). However, little is known about the effect and molecular mechanism of CEP on choroidal melanoma.

The present study investigated the effects of CEP on choroidal melanoma cell proliferation and survival, and on a choroidal melanoma xenograft tumor. In addition, the potential underlying molecular mechanisms for CEP-induced choroidal melanoma cell apoptosis were explored.

Correspondence to: Dr Ling Zhang, Department of Pathophysiology, College of Basic Medicine Sciences, Jilin University, 828 Xinmin Street, Changchun, Jilin 130021, P.R. China
E-mail: lingzhangjlu@gmail.com

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Materials and methods

Reagents. CEP, propidium iodide (PI) and crystal violet dye were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). CellTiter 96® AQueous One Solution reagent (MTS) was purchased from Promega Corporation (Madison, WI, USA). N-acetyl-L-cysteine (NAC) and SP600125 were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cells and cell culture. The human choroidal melanoma MEL15-1 cell line was derived from a primary choroidal melanoma patient (age 60 years; female) with metastatic outcome. The human subject studies were approved by the ethical standards committee of Jilin University (Jilin, China). Written informed consent for isolating cancer cells was obtained from the patient involved in the present study. Cells were maintained in minimum essential medium supplemented with 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% sodium pyruvate, 1% nonessential amino acids and 10% fetal bovine serum (all Corning Incoroporated, Corning, NY, USA) at 37°C in a 5% CO₂ atmosphere.

Cell viability assay. MEL15-1 cells (1x10⁵ cells/well) were maintained in minimum essential medium (Cellgro; Corning Incorporated) supplemented with 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% sodium pyruvate, 1% nonessential amino acids and 10% fetal bovine serum at 37°C, and were placed in 96-well plates overnight. CEP at various concentrations (0, 20, 40, 60 and 80 µM), NAC, SP600125 or identical volumes of control (dimethylsulfoxide (DMSO)) was added to the appropriate wells. The cells were treated for 24 or 48 h prior to the addition of 20 µl CellTiter 96 Aqueous One Solution reagent. Following incubation for 4 h at 37°C, the number of cells in each well was determined by measuring the optical densities at 490 nm. The results were expressed as the percentages of the control cultures.

Cell death assay. A cell death assay was performed using the Cell Death Detection ELISAPLUS kit (Roche Applied Science, Penzberg, Germany), according to the manufacturer’s protocol. This photometric enzyme immunoassay was used for the quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) following induced cell death. MEL15-1 cells were treated with indicated concentrations (0, 40 and 60 µM) of CEP at 37°C for 24 h. The cell lysates were then placed into a streptavidin-coated microplate and incubated with a mixture of biotin-conjugated anti-histone and horse radish peroxidase (HRP)-conjugated anti-DNA (provided in the kit) at 15-25°C for 2 h. The amount of peroxidase retained in the immunocomplex was photometrically determined with 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt as the substrate. Absorbance was measured at 405 nm. The intensity of absorbance at 405 nm was proportional to the amount of cell death.

Colony formation assay. Colony formation of MEL15-1 cells following drug treatment was performed as follows. Briefly, cells were seeded onto 6-well plates (1,000 cells/well), incubated at 37°C overnight and exposed to CEP at different concentrations (0, 40 and 60 µM) for 48 h. The cells were then incubated at 37°C in fresh medium for another 12-14 days. Subsequent to washing with PBS, the cells were then fixed with 10% neutral buffered formalin for 5 min, and stained with 0.05% crystal violet solution for 30 min.

Cell cycle analysis. Cells (1x10⁶/well) maintained in complete medium at 37°C were placed in 6-well plates overnight. CEP (40 and 60 µM), or 2 µl of vehicle control (DMSO), was added to the appropriate wells. The control and treated cells were cultured for an additional 24 h at 37°C, and the cells were stained with PI. Living cells were gated for cell cycle analysis. Cell cycle data were acquired using the Cell Lab Quanta™ SC system (Beckman Coulter, Inc., Brea, CA, USA) and analyzed using FlowJo software (version 9.9.5; Tree Star, Inc., Ashland, OR, USA).

Western blot analysis. Whole cell lysates were prepared by using radioimmunoprecipitation assay buffer (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS) supplemented with 100X protease inhibitor cocktail (Roche Applied Science) and phosphatase inhibitor cocktail (Cell Signaling Technology, Inc., Danvers, MA, USA). The mitochondrial and cytosolic fractions were separated using the digitonin method (27). Briefly, 5x10⁵ cells (200 µl) were permeabilized on ice with 21.33 µg digitonin (Sigma-Aldrich; Merck KGaA) in 80 µl buffer containing 75 mM NaCl, 8 mM Na₂HPO₄, 1 mM NaH₂PO₄ and 250 mM sucrose, supplemented with 100X protease inhibitor cocktail and phosphatase inhibitors. Following incubation for 30 sec in ice cold buffer, cells were centrifuged at 4°C for 2 min at 13,000 x g. The supernatant was removed as the cytosolic fraction, and the remaining pellet was resuspended in the same volume of buffer not containing digitonin. A total of 20 µl of Laemmli sample buffer (Sigma-Aldrich; Merck KGaA) supplemented with 10% dithiothreitol and 10% SDS was added to all samples. Protein samples were analyzed by SDS-PAGE (12% gels) and blotted onto the polyvinylidene fluoride membrane (GE Healthcare Life Sciences, Chalfont, UK). Primary antibodies used were specific to: B-cell lymphoma 2 (Bcl-2; catalog no. 2872), Bcl-2-associated X protein (Bax; catalog no. 2774), caspase-3 (catalog no. 9662) cleaved caspase-3 (catalog no. 9661), caspase-9 (catalog no. 9502), cleaved caspase-9 (catalog no. 9505), cleaved poly (ADP-ribose) polymerase(PARP) (catalog no. 9542), phosphorylated c-Jun N-terminal kinase (JNK; Thr¹⁸³/Tyr¹⁸⁵) (catalog no. 9251) (1:1,000; all from Cell Signaling Technology, Inc., Danvers, MA, USA), cytochrome c (cyt c) (1:2,000; catalog no. ab13575), JNK1 (1:2,000; catalog no. ab10724), JNK2 (1:2,000; catalog no. ab76125) (all from Abcam, Cambridge, UK) and β-actin (1:8,000; catalog no. SAB550001; Sigma-Aldrich; Merck KGaA). The HRP-conjugated anti-mouse (1:4,000; catalog no. sc-2371) and anti-rabbit secondary antibodies (1:4,000; catalog no. sc-2357) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Signals were detected using the Immun-Star HRP peroxide luminol/enhancer kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Quantification of the western blots was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).
Luminescence assay/detection of ROS. The detection of ROS was performed using the ROS-Glo™ assay (Promega Corporation) according to the manufacturer's protocol. Cells treated with CEP in combination with or without NAC for 24 h were incubated with ROS-Glo™ H₂O₂ substrate for 6 h at 37˚C. The ROS-Glo™ detection solution was then added and the plate was incubated for 20 min at room temperature. The luminescence was measured using a SpectraMax L microplate reader (Molecular Devices, LCC, Sunnyvale, CA, USA). The intensity of luminescence was proportional to the amount of ROS.

Nude mice and tumor inoculations. The protocol was approved by the Committee on the Ethics of Animal Experiments of Jilin University. Mice were sacrificed by CO₂ asphyxiation followed by cervical dislocation when they became moribund and when they reached defined study end points. The present study was performed in accordance with the recommendations from the Institutional Animal Care and Use Committee of Jilin University. A total of 24 male athymic nude mice (Charles River Laboratories, Inc., Wilmington, MA, USA) purchased at 4 weeks of age with ~20 g weight were kept under sterile conditions in a pathogen-free environment. The mice were provided with sterile water and food ad libitum. All manipulations were carried out aseptically inside a laminar flow hood. The xenograft model was established in the nude mice using cells. Briefly, tumor cells (1x10⁶) were suspended in 0.1 ml serum-free medium and injected into the subcutaneous tissue of 6-week-old nude mice using a 27-gauge needle. Tumors were allowed to grow for 10 days prior to treatment. The mice were then divided into 4 groups, each of 6 mice with similar mean tumor volumes (between 100 and 150 mm³).

In vivo treatment protocol. When solid tumors grew to 100-150 mm³, mice were treated with vehicle control or CEP (25 mg/kg) for 4 weeks (5 times/week) via peritumoral injection. The tumors were measured every 3 days and the relative tumor volumes were calculated. At 27 days, mice were sacrificed by CO₂ asphyxiation followed by cervical dislocation.

Statistical analysis. All quantitative data are presented as the mean ± standard deviation. Statistical tests were performed with the SPSS software package (version 19.0; IBM SPSS, Armonk, NY, USA). Differences between two groups were tested using paired Student's t-tests. P<0.05 was considered to indicate a statistically significant difference.

Results

CEP inhibits MEL15-1 cell proliferation and induces MEL15-1 cell death. A large body of evidence demonstrated that CEP effectively inhibits various cancer cell lines (11,12,14-17). To test whether CEP may also inhibit MEL15-1 cell proliferation, MEL15-1 cells were treated with CEP at various concentrations (0, 20, 40, 60 and 80 µM) for 24 or 48 h, and then subjected to an MTS assay. Results demonstrated that CEP was able to dose-dependently inhibit cell proliferation with a half-maximal inhibitory concentration of 40-60 µM (Fig. 1A). Treatment with CEP (40 and 60 µM) for 24 h was also revealed to markedly suppress colony formation and induce cell death of MEL15-1 cells compared with the vehicle control (Fig. 1B and C). To investigate how CEP affects MEL15-1 cell proliferation, cell cycle analysis was performed following MEL15-1 cell exposure to 24 h treatment with CEP (40 and 60 µM). Results demonstrated that treatment with CEP at 40 and 60 µM induced G2 arrest (31.7 and 35.5%, respectively) compared with the vehicle control (14.0%) (Fig. 1D).

CEP induces apoptosis in MEL15-1 cells via the caspase signaling pathway. To analyze whether CEP induces MEL15-1 cell cytotoxicity by triggering the apoptotic process, the expression levels of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 were examined. The results revealed that, following CEP treatment for 24 h, the expression levels of Bax in MEL15-1 cells were markedly increased, whereas the levels of Bcl-2 were significantly decreased (Fig. 2). Since enhanced Bax/Bcl-2 ratio is known as an activator of caspase-3 (28,29), the effects of CEP on caspase-associated proteins were analyzed. The results revealed that procaspase-3, procaspase-9 and PARP were decreased dose-dependently in MEL15-1 cells treated with CEP (Fig. 2). By contrast, increased levels of active (cleaved) caspase-3, caspase-9 and PARP were observed in cells treated with CEP (Fig. 2) compared with control cells.

CEP induces cellular ROS production and cyt c release from mitochondria. Previous studies have demonstrated that CEP inhibits cancer cell proliferation by inducing ROS production (11,14). Therefore, in the present study, it was hypothesized that CEP induces MEL15-1 cells via affecting cellular ROS production. ROS production was measured in MEL15-1 cells following 24 h treatment with CEP (0-60 µM) in the presence or the absence of 5 mM NAC (a commonly used antioxidant). Results demonstrated that treatment with CEP significantly induced cellular ROS production compared with the vehicle control, whereas concurrent treatment with NAC attenuated this effect (Fig. 3A).

Considering the aforementioned observation and the evidence that ROS production is associated with mitochondria function, it was investigated whether CEP-induced cell death resulted from the effect of CEP on mitochondria function. It was revealed that cyt c was released from mitochondria in MEL15-1 cells following between 2 and 4 h treatment with 40 µM CEP, whereas concurrent treatment with 5 mM NAC prevented this event (Fig. 3B and C). These results indicated that CEP triggers MEL15-1 cell apoptosis through the mitochondrial signaling pathway by inducing ROS production.

Proliferation inhibitory effects of CEP on MEL15-1 cells are mediated by activation of the JNK signaling pathway. The JNK signaling pathway is known to be a pro-apoptotic signaling pathway and has been identified to be implicated in CEP-induced apoptosis in other tumor cells (14,30). Aiming at dissection of the molecular mechanism of the antitumor effects of CEP in MEL, the regulation of CEP on the JNK signaling pathway was examined using western blot analysis. Activation (phosphorylation) of JNK1 and JNK2 was observed following CEP treatment (Fig. 4A and B). Such activation was abolished by the co-treatment with CEP and SP600125, a novel and...
selective inhibitor of JNK (31) (Fig. 4A and B). In addition, the ROS scavenger NAC significantly decreased the CEP-induced phosphorylation of JNK1/2 (Fig. 4A and B), indicating that the activation of the JNK signaling pathway is dependent on ROS production.

To further analyze whether CEP exerts the inhibitory effects on MEL15-1 cells, dependent on ROS production and the JNK signaling pathway, MEL15-1 cells were treated with CEP in combination with or without the ROS scavenger NAC and the JNK inhibitor SP600125. The results demonstrated that NAC and SP600125 reversed the CEP-induced inhibition on MEL15-1 cell viability (Fig. 4C).

**Inhibitory effects of CEP on tumor growth.** Mice were treated with 25 mg/kg doses of CEP for 4 weeks (5 times/week) and tumor growth was measured during the treatment period. Statistically significant growth inhibition was seen with CEP treatment when compared with the control (Fig. 5A). No significant loss in body weight was observed in mice treated with CEP (Fig. 5B). To examine whether CEP induces MEL15-1 cell apoptosis and regulates the JNK signaling pathway in vivo, the expression of proteins in the caspase signaling pathway and JNK signaling pathway in tumor tissues treated with or without CEP was analyzed. Consistent with the in vitro results of the present study, increased levels of active (cleaved)
caspase-3, caspase-9 and PARP were observed in tumors treated with CEP compared with those treated with vehicle control. Activation (phosphorylation) of JNK1 and JNK2 was also observed upon CEP treatment (Fig. 5C).

**Discussion**

The results of the present study demonstrated that CEP, a natural alkaloid extracted from the roots of *S. cepharantha*...
Hayata, potently suppresses the proliferation of choroidal melanoma cells, induces cell cycle arrest and activates cellular apoptotic proteins, including Bax, caspase and PARP. The results of the present study also revealed that CEP induced the production of ROS and led to cyt c release, whereas the ROS scavenger NAC was able to attenuate the situation. In addition, consistent with a previous study (14), CEP was also revealed to activate JNK1 and JNK2 (members of the mitogen-activated protein kinase pathway), which may serve an important role in CEP induced apoptosis.

In the present study, it was demonstrated that CEP induces MEL15-1 cell apoptosis in a dose-dependent manner. The Bcl-2 protein family is known to control the commitment of cells to apoptosis (32). The increased expression level of Bax and a reduced level of Bcl-2, as well as increased levels of cleaved caspase-3 and caspase-9, induced by CEP treatment in MEL15-1 cells indicated that CEP-induced apoptosis occurs via the activation of caspase-3 and caspase-9 and the mitochondrial pathways. In addition, consistent with previous studies of CEP in other cancer cells (11,14), the results demonstrated that the production of ROS increased in CEP-treated MEL15-1 cells. The excessive production of ROS is likely due to stimuli that act on the Bcl-2 family protein, subsequently altering the mitochondrial membrane integrity. This was substantiated by the observation that CEP induced the release of cyt c from mitochondria.

JNK activation has been revealed to lead to apoptosis and correlate positively with oxidative stress-induced apoptosis (33-35). The activation of JNK was also revealed to be implicated in CEP-triggered apoptosis in human hepatocellular carcinoma cells (14). In the present study, it was observed that ROS and JNK activation are positively associated with the anticancer effects of CEP in MEL15-1 cells. Notably, the results of the present study revealed that treatment with the ROS scavenger NAC may abolish the activation of JNK in MEL15-1 cells and the inhibitory effects of CEP on cell viability, indicating that CEP-induced JNK activation is ROS-dependent. Other functional consequences for CEP-induced ROS, as well as JNK activation, are not yet established and require additional investigation.

CEP, as a natural alkaloid extract, has been demonstrated to effectively inhibit the proliferation of various cancer cell lines (9-11,14-17). Notably, it was also revealed to possess anti-metastasis activity against different types of cancer cells (13,36). CEP was demonstrated to suppress invasion and migration of cholangiocarcinoma cells, possibly by suppressing intercellular adhesion molecule-1 and matrix metalloproteinase-2 (13). Intraperitoneal injection of CEP was reported to inhibit the metastasis of Lewis lung carcinoma in a mouse model (37), and CEP inhibited metastasis of human colon cancer cells in a mouse model (36). In addition, the combination of CEP and other chemotherapeutic agents, including 5-fluourouracil, or recombinant human interferon-β or -γ, were demonstrated to be even more effective to prevent metastasis (36,37). In the present study, the inhibitory effects of CEP on primary choroidal melanoma tumor growth were observed. Since metastasis remains a life-threatening risk for choroidal melanoma (38), future studies will focus on the effect of CEP (alone or in combination with other agents) on metastasis of choroidal melanoma using a metastatic choroidal melanoma model to develop a safe and effective regimen for choroidal melanoma therapy.

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References


