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Pre-clinical evaluation of cinobufotalin as a potential anti-lung cancer agent

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ABSTRACT

Lung cancer is a major cause of cancer-related mortality in the United States and around the world. Due to the pre-existing or acquired chemo-resistance, the current standard chemotherapy regimens only show moderate activity against lung cancer. In the current study, we explored the potential anti-lung cancer activity of cinobufotalin *in vivo* and *in vitro*, and studied the underlying mechanisms. We demonstrated that cinobufotalin displayed considerable cytotoxicity against lung cancer cells (A549, H460 and HTB-58 lines) without inducing significant cell apoptosis. Our data suggest that mitochondrial protein cyclophilin D (Cyp-D)-dependent mitochondrial permeability transition pore (mPTP) opening mediates cinobufotalin-induced non-apoptotic death of lung cancer cells. The Cyp-D inhibitor cyclosporine A (CsA), the mPTP blocker sanglifehrin A (SfA), and Cyp-D shRNA-silencing significantly inhibited cinobufotalin-induced mitochondrial membrane potential (MMP) reduction and A549 cell death (but not apoptosis). Using a mice xenograft model, we found that cinobufotalin inhibited A549 lung cancer cell growth *in vivo*. Thus, cinobufotalin mainly induces Cyp-D-dependent non-apoptotic death in cultured lung cancer cells. The results of this study suggest that cinobufotalin might be further investigated as a novel anti-lung cancer agent.

1. Introduction

Lung cancer, mainly non-small cell lung cancers (NSCLCs), is a major cause of cancer-related mortality in the United States and around the world [1–4]. Surgery resection is available only for the early stage of lung cancers [2,5]. Majority of NSCLC or other lung cancer patients are presenting with advanced/metastatic malignancies at time of diagnosis, and chemotherapy are important treatment options [5]. Due to the pre-existing or acquired chemo-resistance, the current standard chemotherapy regimen only showed moderate activity against lung cancers. Thus, the search more efficient agents are extremely important and urgent.

Traditional Chinese medicine has become an important source for novel chemotherapeutic compounds [6,7]. Cinobufotalin, a cardiotonic steroids or bufadienolides [8,9], is extracted from the skin secretions of the traditional Chinese medicine giant toads (*Chan Su*) [8]. Cinobufotalin has been used as a cardiotonic, diuretic and a hemostatic agent [9]. Although numerous reports have displayed anti-tumor activity and chemotherapeutic enhancing properties of many bufadienolides (i.e. bufalin) in a wide spectrum of cancer cells [10], the possible anti-tumor ability of cinobufotalin has not been extensively studied [8,9]. Its potential role in lung cancer cells is un-known. More importantly, the underlying mechanisms remain to be explored.

Mitochondrial permeability transition pore (mPTP) is a key regulator of cell death [11,12]. It is a channel complex composed of voltage-dependent anion channel (VDAC, in the outer membrane), adenine nucleotide translocator (ANT, in the inner membrane), and cyclophilin D (Cyp-D, inside the mitochondrial matrix) [11,13]. With cytotoxic stimuli, Cyp-D will associate with ANT in the inner membrane to promote mPTP opening, which leads to mitochondrial depolarization, mitochondria swelling, Ca^{2+} release, and eventually cell death [14,15]. In this study, we found that Cyp-D and mPTP are also important mediators of cinobufotalin-induced lung cancer cell death.

2. Materials and methods

2.1. Chemicals and reagents

Cinobufotalin, sanglifehrin A (SfA) and cyclosporine A (CsA) were obtained from Sigma Chemicals (St. Louis, MO); Z-VAD-fmk

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(ZVAD) was purchased from Calbiochem (Shanghai, China). Antibodies against Cyp-D and tubulin were purchased from Santa Cruz Biotech (Santa Cruz, CA).

2.2. Cell culture

A549, H460 and HTB-58 human lung cancer cell lines were purchased from Cell Bank of Chinese Academy of Medical Sciences (Shanghai, China). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, Hyclone, Shanghai, China) with antibiotics, and incubated at 37 °C in a humidified air atmosphere containing 5% CO_2.

2.3. Cell survival assay

Cell survival was evaluated by the tetrazolium dye (MTT, Sigma) assay as reported [16]. Cells were seeded into 96-well plates and then treated. MTT (5 mg/ml in PBS) was added into the wells and incubated for 3 h. After the medium was carefully aspirated, DMSO (150 μl, Sigma) was added to each well. The plates were gently agitated until the color reaction was uniform and the OD_{490} was determined using a microplate reader. Media-only treated cells served as 100% cell viability, and the relative survival was defined as absorbance of treated wells divided by that of controls. For each treatment, 5-wells were included.

2.4. Cell death assay

The number of dead cancer cells (Trypan blue positive) after treatment was counted, and the percentage (%) of dead cells was calculated by the number of the Trypan blue positive cells divided by the total number of the cells, which was automatically recorded by a cell counter (Roche, Shanghai, China).

2.5. Cell apoptosis assay

Cells were seeded into 6-well plates, and treatments were initiated when cells were 60–70% confluent. Tested reagents were added into the wells and incubated for indicated time points, and cells were then harvested by digesting with trypsin/EDTA (Gibco, NY). After fixed with cold 70% ethanol, cells were stained with propidium iodide (PI) solution (5 mg/mL, Invitrogen, CA) and Annexin V (1 mg/mL, Invitrogen, CA) for 30 min in 37 °C. Samples (10,000 cells/sample) were tested on a Becton Dickinson FACS calibur (Becton, Dickinson & Co, Mountain View, CA). Annexin V percentage was used to reflect cell apoptosis. Annexin V positive/PI negative and Annexin V positive/PI positive cells were combined as apoptotic cells, see [16].

2.6. Hoechst 33342 staining of apoptotic cells

Cells were plated at 50,000 per well in 6-well cell culture plates with glass slides (Corning Incorporated, USA) and cultured overnight. After treatment, cells were fixed with 3.7% formaldehyde in PBS for 10 min and stained with Hoechst 33342 solution (10 μg/mL, Sigma). The slides were washed twice in PBS and fixed onto the microscopic slide. The cell nuclei images were taken with a Zeiss fluorescence microscope (Zeiss, Nanjing, China), and the number of apoptotic cells was manually counted in 5 random fields for at least 500 cells from each group. The apoptotic nuclei showed characteristic condensation of the chromatin, see [16].

2.7. Western-blots

Cells were harvested with trypsin/EDTA, and PBS-washed cell pellets were treated with HEPES lysis buffer (30 mM HEPES, 1% Triton X-100, 10% glycerol, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EDTA and 10 mM NaCl). Equal amounts of protein extracts were loaded onto sodium dodecyl sulfate (SDS)–polyacrylamide gels and ran at 100 mV for 80 min, followed by transferring to PVDF membranes at 100 mV for 30 min at room temperature. The membranes were probed with indicated primary antibodies at room temperature for 180 min. As secondary antibodies, goat anti-rabbit/mouse labeled with horseradish peroxidase (HRP, Santa Cruz Biotechnology, Santa Cruz, CA) were added. Blots were developed using a chemiluminescence detection system. Blot intensity was quantified though the ImageJ software (NIH).

2.8. Cyp-D stable knockdown

Cyp-D stable knockdown in A549 cells were based on the lentiviral infection procedure as reported by Dr. Bi [16,17]. Cyp-D and equal loading (tubulin) expression in the infected cells was tested by Western blots. For Cyp-D shRNA infection assay, two non-overlapping shRNAs against Cyp-D cDNA were utilized to insure results consistency, and stable clones were selected through puromycin [16,17].

2.9. Mitochondrial membrane potential (MMP) assay

MMP was measured through JC-10 dye (Invitrogen, Carlsbad, CA) [18]. The dye forms aggregates in the mitochondrial membrane, exhibiting orange fluorescence in normally resting cells. When the membrane potential is decreasing, the monomeric JC-10 will form in the cytosol, which exhibits green fluorescence. Thus, the intensity of green fluorescence is detected as indicator of MMP reduction [19]. Briefly, after treatment, A549 cells were stained with 5.0 μg/ml of JC-10 for 15 min in the dark. Cells were then washed twice with warm PBS, and resuspended in fresh culture medium and read immediately on a microplate reader with an excitation filter of 485 nm. The OD value of treatment group was normalized to that of medium-treated control group.

2.10. Animals

Male nude mice (4–6 weeks old, BALB/c) were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Shanghai, China). All of the experiments were performed in accordance with the Guidelines for Care and Use of Experimental Animals of Experimental Animal Research Committee of authors' affiliation.

2.11. A549 tumor xenograft model in nude mice

Male nude mice were introduced to establish xenograft tumor model of A549 cells as previously described [16]. A549 cells (five million cells in 0.1 mL of culture medium) were subcutaneously injected at the right thigh of nude mice, and treatment was started when the tumors reached an average volume of 200–300 mm^3. Animals were randomized into 3 groups with 10 mice each group: (a) vehicle; (b) 1.0 mg/kg of cinobufotalin; (c) 5.0 mg/kg of cinobufotalin. Cinobufotalin was injected intraperitoneally (i.p.) twice daily for 1 weeks. The mice were examined daily for toxicity/mortality relevant to treatment, and the tumor was measured with a caliper once a week for up to 5 weeks. Mice body weight and mice survival (at week 5) were also recorded. The tumor volume (in mm^3) was calculated by the formula: volume = $(\text{width})^2 \times \text{length}/2$, and the tumor growth curve was presented.

2.12. Data analysis and statistics

The results were presented as mean ± standard deviation (SD). Comparisons between more than 2 groups were performed by analysis of variance (one-way ANOVA). $p < 0.05$ was considered statistically significant. For all the experiments in this study, concentration of drugs and experimental duration were selected based on pre-experimental results, where most significant results were obtained.

3. Results

3.1. Cinobufotalin induces cytotoxic effect in cultured lung cancer cells

We first tested the potential cytotoxicity of cinobufotalin against A549 lung cancer cells. As shown in Fig. 1A, the survival of A549 cells, detected by MTT assay, was significantly inhibited by cinobufotalin. The cell viability decreased to 99.2 ± 9.1%, 65.9 ± 6.0%, 44.8 ± 3.5%, 34.8 ± 5.0% and 23.5 ± 3.3% of control group after 0.1 μM, 0.5 μM, 1.0 μM, 5.0 μM and 10.0 μM of cinobufotalin treatment (Fig. 1A). Results in Fig. 1B demonstrated that cinobufotalin-induced anti-A549 activity was time-dependently. No cell viability reduction was seen until 72 h after cinobufotalin (1.0 μM) stimulation. Results from the Trypan blue staining assay demonstrated that cinobufotalin (0.5–10 μM) significantly induced A549 cell death, and the effect was once again concentration-dependent (Fig. 1C). In two other human lung cancer cell lines (H460 and HTB-58), cinobufotalin (1.0 μM) similarly inhibited cancer cell survival (Fig. 1D). These result together show that cinobufotalin induces cytotoxic effect against lung cancer cells.

3.2. Apoptosis is not a main contributor of cinobufotalin-induced cytotoxicity in cultured lung cancer cells

We next tested the role of cell apoptosis in cinobufotalin-induced cytotoxicity. Cell apoptosis was tested through two different assays: Annexin V FACS assay [16] and apoptotic nuclei Hoechst staining assay [16]. Results demonstrated that cinobufotalin only slightly induced A549 cell apoptosis (less than 10%) even at high doses (Fig. 2A–C). On the other hand, two apoptosis inducers ceramide (C6) [20,21] and TNFα [16,22] activated considerable apoptosis (Fig. 2A–C) in A549 cells. Correspondingly, z-VAD-fmk (ZVAD), the general caspase inhibitor, only slightly inhibited cinobufotalin-induced A549 cell viability decrease (Fig. 2D) and cell death (Fig. 2E), while ZVAD almost blocked cinobufotalin-induced apoptosis (Data not shown). Annexin V FACS assay results in Fig. 2F showed that cinobufotalin faintly activated apoptosis in two other lung cancer cell lines (H460 and HTB-58). Together, these results show that apoptosis might not be a main contributor of cinobufotalin-induced cytotoxicity in lung cancer cells.

3.3. Mitochondrial protein Cyp-D mediates cinobufotalin-induced mPTP opening and subsequent non-apoptotic death of lung cancer cells

Cyp-D, a mitochondrial protein, regulates mPTP opening and subsequent non-apoptotic cell death [23,24]. Cyp-D knockout mice were resistant to ischemia-induced brain injury [23,24]. Further, cells with Cyp-D knockout were protected from necrotic cell death triggered by Ca^{2+}-overload and oxidative stress [25]. Our previous study has shown that Cyp-D is required for doxorubicin

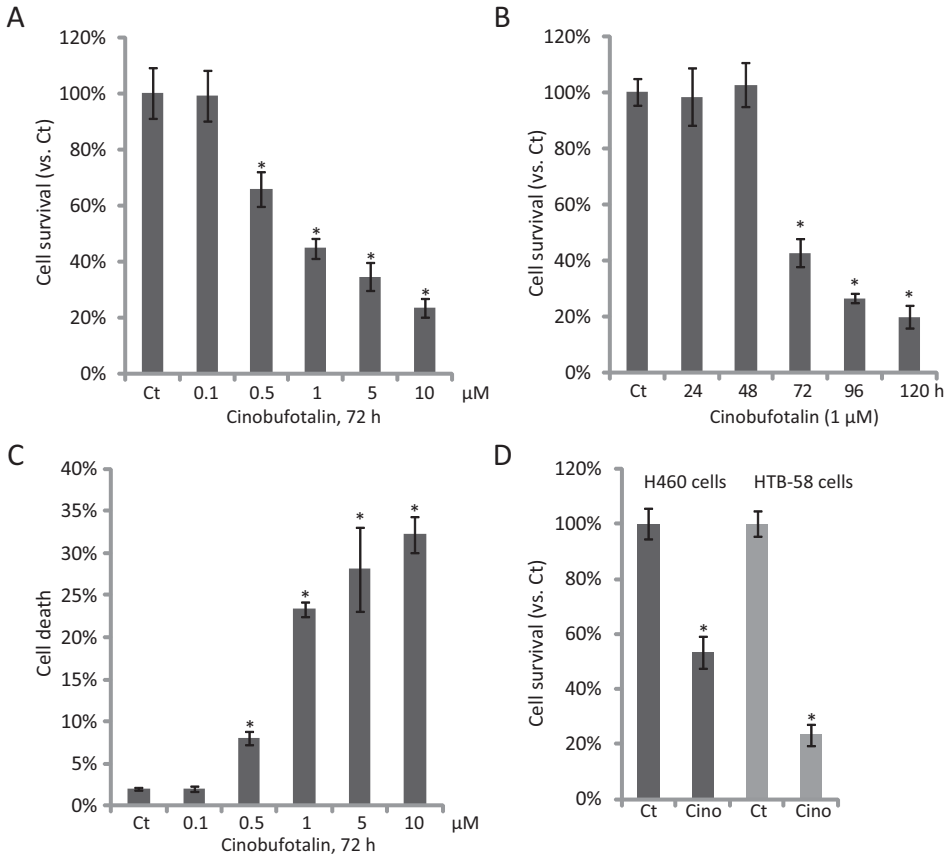

Fig. 1. Cinobufotalin induces cytotoxic effect in cultured lung cancer cells. A549 cells were treated with indicated concentration of cinobufotalin (0.1–10 μM) for 72 h, or treated with 1.0 μM of cinobufotalin for indicate h, cell survival (A and B) and cell death (C) were analyzed by MTT assay and Trypan blue staining assay, respectively. H460 cells and HTB-58 cells were treated with 1.0 μM of cinobufotalin for 72 h, MTT assay was performed to test cell survival (D). Data were expressed as mean ± SD, experiments were repeated five times. "Ct" stands for medium-treated control group. *$p < 0.05$ vs group of control (Ct).

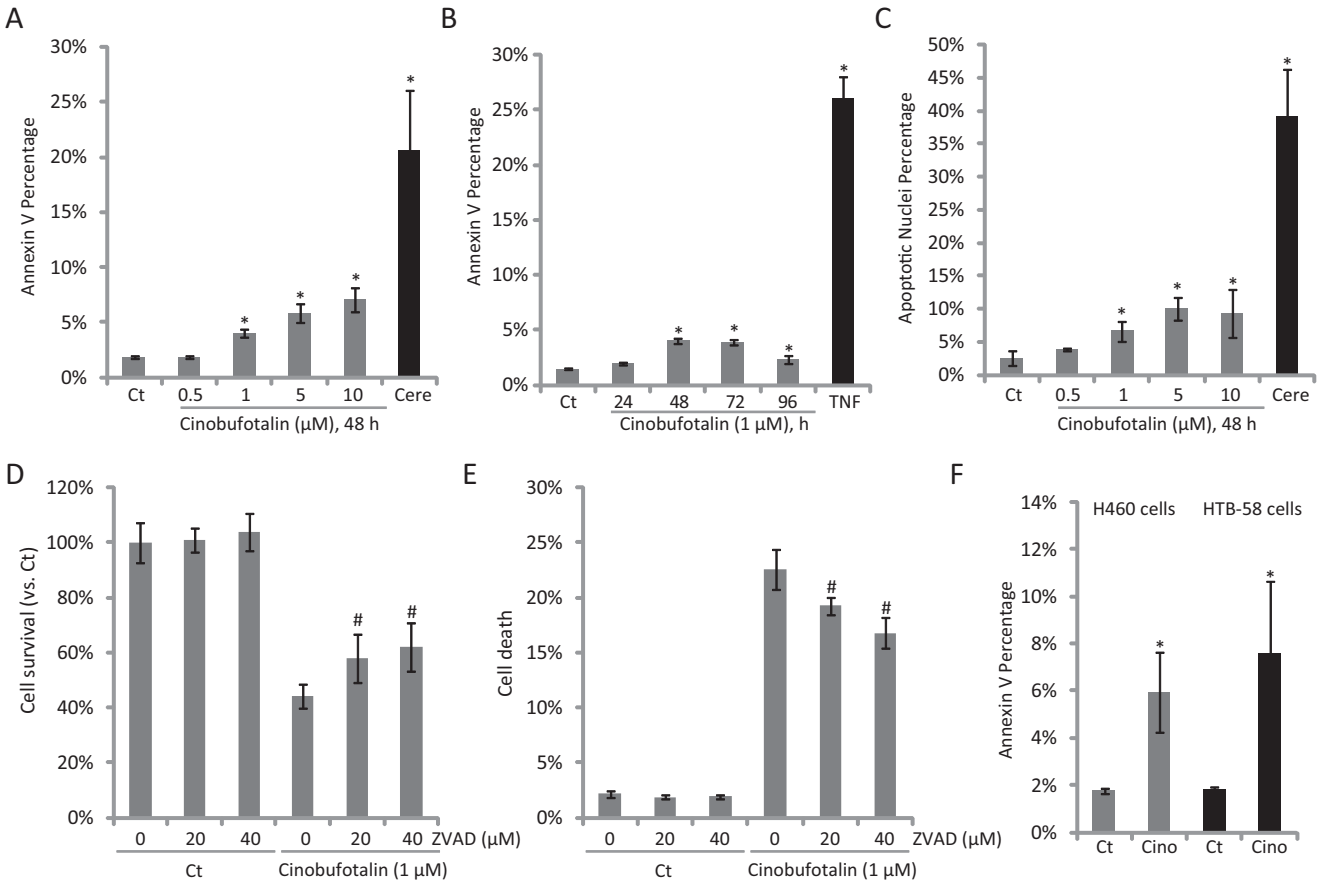

Fig. 2. Apoptosis is not a main contributor of cinobufotalin-induced cytotoxicity in cultured lung cancer cells. A549 cells were treated with indicated concentration of cinobufotalin (0.1–10 μM) for 72 h, or treated with 1.0 μM of cinobufotalin for indicate h, cell apoptosis was analyzed by Annexin V FACS assay (A and B) or apoptotic nuclei Hoechst staining assay (C). C6 Ceramide (Cere, 25 μg/ml, 48 h) or TNFα (TNF, 100 ng/ml, 48 h) was added as control (A–C). The effect of Z-VAD-fmk (ZVAD, 20/40 μM, 1 h pretreatment) on cinobufotalin (1 μM)-induced viability reduction (MTT assay, 72 h) and cell death (Trypan blue staining assay, 72 h) were shown in (D and E), respectively. H460 cells and HTB-58 cells were treated with 1.0 μM of cinobufotalin for 48 h, cell apoptosis was tested by Annexin V FACS assay (F). Data were expressed as mean ± SD, experiments were repeated four times. "Ct" stands for medium-treated control group. $^{*}p < 0.05$ vs group of Control (Ct). $^{\#}p < 0.05$ vs cinobufotalin only group.

(Dox)-induced necrotic death of A549 cells. We then tested if Cyp-D was also important for cinobufotalin-induced cytotoxicity in lung cancer cells. CsA, the Cyp-D inhibitor [26], as well as SfA, the mPTP blocker [27], significantly inhibited Dox-induced A549 cell viability reduction (Fig. 3A) and cell death (Fig. 3B). Meanwhile, Cyp-D silencing by targeted shRNAs also rescued A549 cells from cinobufotalin (Fig. 3C and D). Note that we utilized two non-overlapping shRNAs again Cyp-D, which both decreased Cyp-D expression by more than 80% (Fig. 3C, upper panel). Interestingly, cinobufotalin-induced cell apoptosis was not affected by Cyp-D silencing (Fig. 3D) or by CsA/SfA (Data not shown), suggesting that Cyp-D is only required for non-apoptotic death by cinobufotalin. Significantly, cinobufotalin decreased MMP in A549 cells, indicating mPTP opening. And the effect by cinobufotalin was prevented by CsA and SfA (Fig. 3E), or by Cyp-D shRNAs (Fig. 3F). On the other hand, C6 ceramide-induced A549 cell death and apoptosis were only slightly affected by CsA or SfA, but were largely inhibited by ZVAD (Fig. 3G). Trypan blue staining assay results in Fig. 3H showed that CsA prevented cinobufotalin-induced death of H460 cells and HTB-58 cells. Together, these results suggest that Cyp-D mediates cinobufotalin-induced mPTP opening and subsequent non-apoptotic death of lung cancer cells.

3.4. Cinobufotalin inhibits A549 cell in vivo growth

Finally, we examined the anti-cancer activity of cinobufotalin *in vivo* using mice A549 cell xenograft model [16]. Results in Fig. 4A demonstrated that cinobufotalin (1/5 mg/kg, i.p. twice daily, for 7 days) significantly inhibited A549 xenograft growth in mice. Further, same cinobufotalin administration improved mice survival at week five (Fig. 4B). Cinobufotalin administration didn't significantly affect mice body weight, indicating the relative safety of this regimen (Fig. 4C). Thus, cinobufotalin inhibits A549 xenograft growth *in vivo* and improves mice survival.

4. Discussion

Necrotic cell death has been long considered as a passive program. However, recently it is demonstrated that cell necrosis, like cell apoptosis, is also a programmed process. This programmed cell necrosis is a major contributor of cell death induced by ischemia–reperfusion (IR) injury, oxidative stresses, chemo-agents, and UV radiation [13,23,26,28–33]. Our previous study showed that doxorubicin-induced A549 cell death is also mainly due to programmed necrosis, but not apoptosis [16]. Cyp-D and it-regulated mPTP opening play a vital role in programmed necrosis. Cyp-D is shown to translocate to the inner membrane of mitochondria, where it forms a complex with local protein ANT to dictate mPTP opening [14,15], causing mitochondrial depolarization, and eventually cell necrosis.

Here we provided evidence to support that cinobufotalin mainly induced non-apoptotic cytotoxicity in lung cancer cells, and Cyp-D was required for the process This conclusion was supported by the fact that CsA, the Cyp-D inhibitor as well as Cyp-D

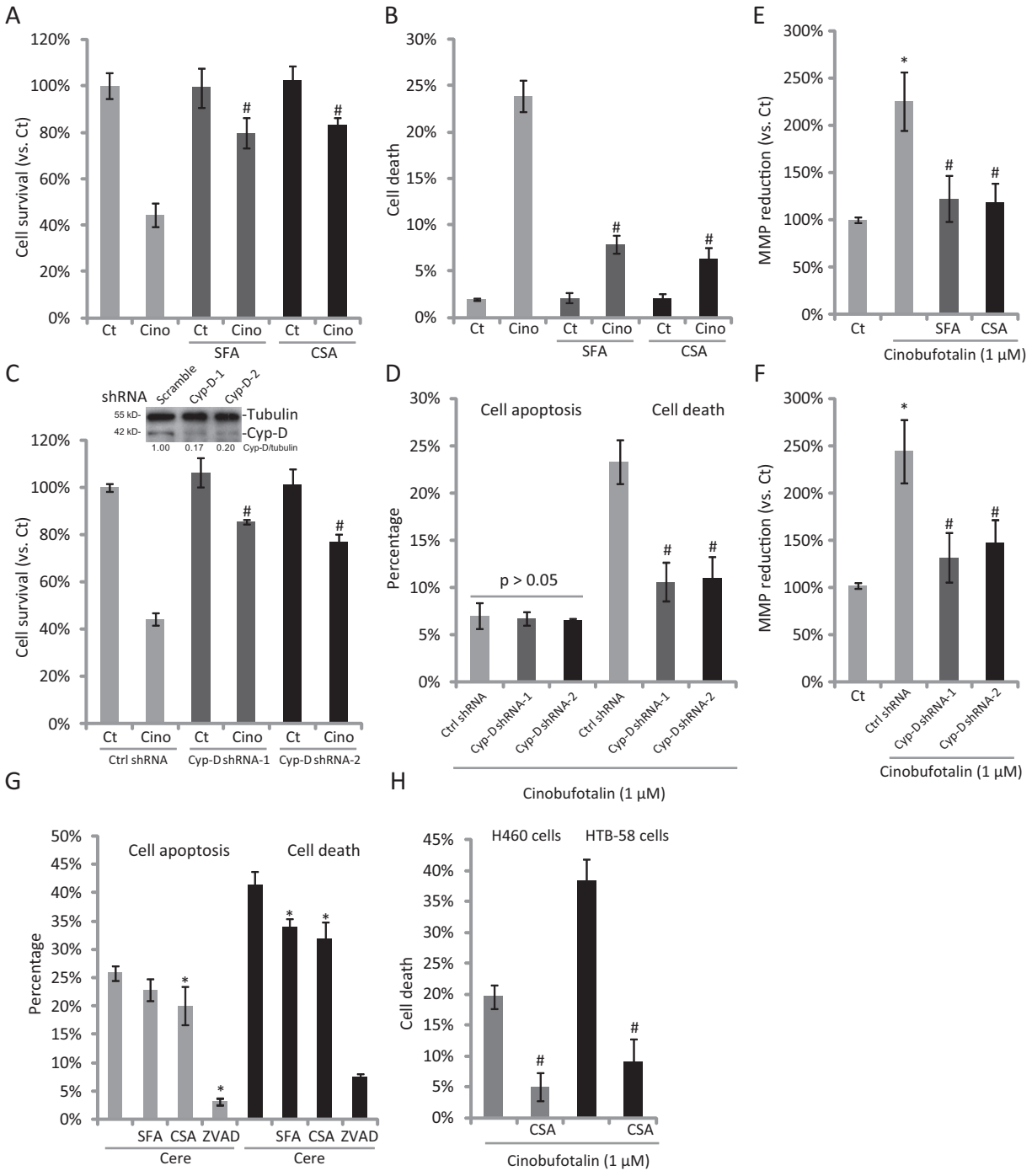

Fig. 3. Mitochondrial Cyp-D is required for cinobufotalin-induced mPTP opening and non-apoptotic death in cultured lung cancer cells. A549 cells were pre-incubated with cyclosporine A (CsA, 0.1 μM) or sanglifehrin A (SfA,1 μM) for 1 h, followed by cinobufotalin (Cino, 1 μM) stimulation, cell viability and cell apoptosis were analyzed by MTT assay (A, 72 h) and Trypan blue staining assay (B, 72 h) respectively, MPP reduction was also tested by the JC-10 staining assay (E, 24 h). Stable A549 cells expressing scramble shRNA, Cyp-D shRNA (-1 and -2) were stimulated with cinobufotalin (1 μM), cell survival (MTT, 72 h), cell apoptosis (Annexin V, 48 h) and cell death (Trypan blue, 72 h) were analyzed (C and D), MPP reduction was also tested (F, 24 h), expression of Cyp-D and tubulin in above cells was tested by Western blots (C, upper panel). The effect of CsA (0.1 μM), SfA (1 μM) or Z-VAD-fmk (ZVAD, 40 μM) on C6 Ceramide (Cere, 25 μg/ml)-induced A549 cell apoptosis (Annexin V assay, 48 h) and cell death (Trypan blue assay, 72 h) were tested (G). H460 cells and HTB-58 cells were treated with 1.0 μM of cinobufotalin or plus CsA (0.1 μM) for 72 h, Trypan blue staining assay was performed to test cell death (H). Data were expressed as mean ± SD, experiments were repeated three times. "Ct" stands for medium-treated control group. $^{\#}p < 0.05$ vs cinobufotalin only group. $^{*}p < 0.05$ vs Cere only group (G).

shRNA-silencing dramatically inhibited cinobufotalin-induced A549 cell death (but not apoptosis). Further, our data indicated that cinobufotalin promoted mPTP opening, as the MMP was decreased after cinobufotalin treatment in A549 cells. Inhibition of Cyp-D by CsA or by shRNAs prevented cinobufotalin-induced MMP reduction, suggesting that Cyp-D regulated mPTP opening after cinobufotalin stimulation. These data together with the finding that mPTP blocker SfA inhibited cinobufotalin-induced cytotoxicity suggest that cinobufotalin induces Cyp-D-regulated mPTP opening to dictate non-apoptotic death in lung cancer cells.

In summary, we suggest that cinobufotalin mainly induces Cyp-D-regulated non-apoptotic death in cultured lung cancer cells. The

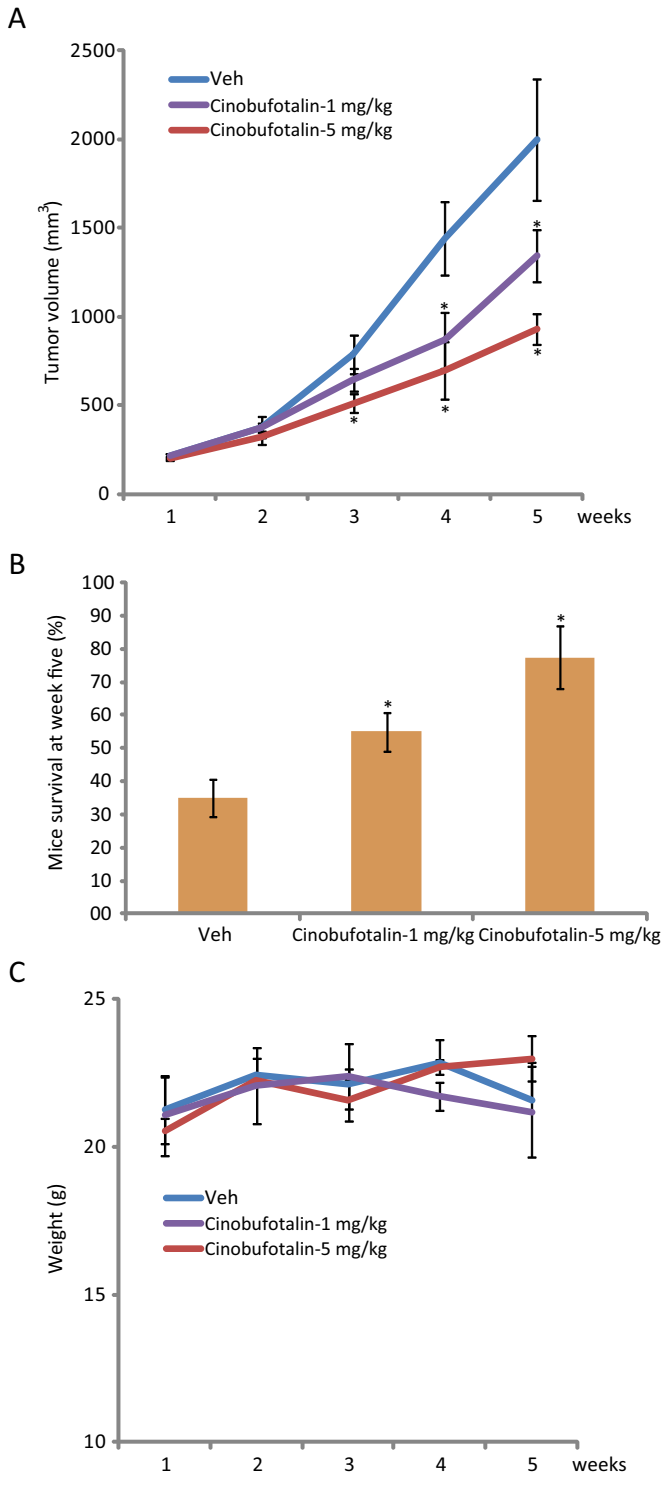

Fig. 4. Cinobufotalin inhibits A549 cell *in vivo* growth. Male nude mice, 22–25 g, 6–8 weeks old were ear tagged and randomized into 3 different groups (Vehicl/Veh, Cinobufotalin-1 mg/kg, Cinobufotalin-5 mg/kg) of 10 mice each group prior to inoculation s.c. with 2×10^6 A549 cells in a volume 0.1 ml culture medium. Treatment was started 6 days after inoculation, intraperitoneal (i.p.) injections twice per day of cinobufotalin (1–5 mg/kg) for 1 week. Tumor volume (in mm^3, recorded every week) (A), mice survival (percentage, at week 5) (B) and mice body weight (in grams, recorded every week) (C) were shown. Data were expressed as mean ± SD, experiments were repeated three times. $^*p < 0.05$ vs group of Veh (PBS administration).

results of this study indicate that cinobufotalin might be further investigated as a novel anti-lung cancer agent.

Conflict of interests

No conflict of interests was stated.

Acknowledgment

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