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Selective triggering of apoptosis of concanavalin A-activated T cells by fraxinellone for the treatment of T-cell-dependent hepatitis in mice

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ABSTRACT

Selectively inducing apoptosis of activated T cells is essential for the clearance of pathogenic injurious cells and subsequent efficient resolution of inflammation. However, few chemicals have been reported to trigger apoptosis of activated T cells in the treatment of hepatitis without affecting quiescent T cells. In the present study, we found that fraxinellone, a small natural compound isolated from the root bark of *Dictamnus dasycarpus*, selectively facilitated apoptosis of concanavalin A (Con A)-activated $CD4^+$ T cells rather than those non-activated, by disrupting the mitochondrial transmembrane potential, decreasing the ratio of Bcl-2/Bax, and increasing cytochrome *c* release from the mitochondria to the cytosol. The enhancement in Fas expression and caspase-8 activity, truncation of Bid, and down-regulation of anti-apoptotic cellular FLICE-inhibitory protein expression by fraxinellone also suggested the participation of an extrinsic apoptosis pathway. Furthermore, fraxinellone significantly alleviated Con A-induced T-cell-dependent hepatitis in mice, which was closely associated with reduced serum transaminases, pro-inflammatory cytokines, and pathologic parameters. Consistent with the in vitro results, fraxinellone dramatically induced apoptosis of activated peripheral $CD4^+$ T cells in vivo, consequently resulting in less $CD4^+$ T-cell activation and infiltration to the liver. These results strongly suggest fraxinellone might be a potential leading compound useful in treating T-cell-mediated liver disorders in humans.

1. Introduction

Apoptosis is an essential mechanism used to eliminate activated T cells during the shutdown process of excess immune responses and maintain proper immune homeostasis, while deficient apoptosis of activated T cells is associated with a wide variety of immune disorders. The activation of Fas, the cross-linkage by its natural Fas ligand (FasL) or an agonistic antibody, is known to initiate a signaling cascade leading to apoptosis [1]. Defective expression of Fas and FasL causes the development of autoimmune diseases in *lpr/lpr* and *gld/gld* mice, respectively [2]. In contrast, activated T cells can express a higher level of Fas and are more susceptible to Fas-mediated apoptosis than naive T cells [3]. Therefore, facilitating Fas-mediated apoptosis of activated T cells before they shift to inflammatory cells may be a therapeutic strategy for the treatment of T-cell-mediated autoimmune diseases. Such an effect should focus on the pathogenic T cells without affecting naïve or non-activated T cells to avoid intervention of normal immune responses to other foreign antigens. However, a chemical that selectively deletes pathogenic T cells without affecting naïve T cells is still lacking.

Under the guidance of seeking a new immunosuppressant with high selectivity, our previous studies have identified some Chinese herbs, as well as some small compounds, and confirmed the selective immunosuppressive activities of the herbs and compounds [4–6]. As a special natural small compound, fraxinellone, a lactone (limonoid) isolated from Cortex Dictamni, showed a selectively immunosuppressive feature when administered orally during the elicitation, but not the sensitization phase of various delayed-type hypersensitivity reactions [7,8]. Previously, there have been only a few reports regarding this small compound's effects other than immunosuppression, such as a feeding deterrent activity against insects, vasorelaxation, and neuroprotective effect [9–11]. Concanavalin A (Con A) is a well-known T-cell mitogen which triggers polyclonal T-cell activation in vitro, and induces a T-cell-dependent acute fulminant hepatitis in mice as an appropriate model of human autoimmune hepatitis [12]. The purpose of this study was to report a novel strategy for hepatitis therapy involving the selective triggering of apoptosis of activated T cells by means of the unique compound, fraxinellone. Our results also indicate that it might be feasible to treat T-cell-mediated liver injury using reagents like fraxinellone by facilitating apoptosis of detrimental T cells without affecting naïve T cells.

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2. Materials and methods

2.1. Mice

Specific pathogen-free, 8–10-week-old female C57BL/6 mice were purchased from Model Animal Genetics Research Center of Nanjing University (Nanjing, China). Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

2.2. Extraction and isolation

Cortex Dictamni (Bai-Xian-Pi) were purchased from Nanjing Medicinal Material Co. (Nanjing, China) and identified by Dr. Boyang Yu (Department of Chinese Medicinal Prescription, China Pharmaceutical University) as *Dictamnus dasycarpus* Turcz. Powdered root bark (5.0 kg) of *D. dasycarpus* was extracted three times with 60% EtOH under reflux. The total filtrate was evaporated under reduced pressure to remove EtOH, then the extract was suspended in distilled water and partitioned with CH_2Cl_2 and EtOAc successively, to yield CH_2Cl_2 (148 g) and EtOAc (18 g) extracts, respectively. The CH_2Cl_2 extract (100 g) was subjected to silica gel column chromatography, eluted with CH_2Cl_2/MeOH (1:1), and further purified by flash column chromatography (silica gel) using a gradient of CH_2Cl_2–MeOH to yield the compound CD01 (fraxinellone, 400 mg, 99% purity by HPLC). The structure was confirmed by comparison of MS, ^{1}H NMR and physical data with those reported in the literature [13]. In the present study, fraxinellone was dissolved at a concentration of 0.05 mol/L in 100% DMSO as a stock solution, stored at −20 °C, and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% DMSO throughout the study (all the control groups are composed of 0.1% DMSO).

2.3. HPLC analysis and structural elucidation

HPLC analysis was applied on a Shimadzu 20A series HPLC system consisting of two pumps (LC-20A Solvent Delivery Unit), a column oven (CTO-10ASVP), a SPD detector (SPD-M20AV Photodiode Array Detector) and an LC solution Work Station. CD01 was applied to YMC-pack Pro C18 column (5 μm, 150 mm × 4.6 mm, YMC Co., Ltd., Japan) and eluted with methanol–water (45:55, v/v). The effluents were detected at 225 nm. Column temperature was set up at 25 °C and the flow rate was 1 mL/min. The mobile phase was degassed by ultrasonic and filtered through a 0.22 μm membrane filter (Advantec, Tokyo Roshi Kaisha, Ltd., Japan). Before sample analysis, the column was stabilized with mobile phase for at least 30 min. NMR and ES-MS were used for structure elucidation. The ^{1}H NMR measurements were carried out in Bruker DPX-300 spectrometer operating at 300 MHz. ES-MS experiments were recorded on ABI Mariner ESI-TOF mass spectrometer.

2.4. Cells and reagents

Mouse $CD4^+$ T cells from spleen or lymph node were purified using magnetic beads (Miltenyi Biotec, Auburn, CA) with more than 95% purity. The cells were incubated in RPMI 1640 medium supplemented with 100 U mL^{-1} of penicillin, 100 μg/mL^{-1} of streptomycin and 10% fetal calf serum under a humidified 5% (v/v) CO_2 atmosphere at 37 °C. Cyclosporin A (CsA) was purchased from Sandoz Ltd. (Basel, Switzerland). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-benzimida-zolcarbocyanine iodide (JC-1) was purchased from Molecular Probes (Eugene, OR). Concanavalin A (Con A) was purchased from Sigma Chemical Co. (St. Louis, MO). Kits for determining serum alanine transaminase (ALT), aspartate transaminase (AST) and lactic dehydrogenase (LDH) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Annexin V-FITC/PI Kit was purchased from Jingmei Biotech (Nanjing, China). ELISA kits for tumor necrosis factor-α (TNF-α) and Interferon-γ (IFN-γ) were purchased from R&D Systems (Minneapolis, MN). Ac-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA), Ac-Ile-Glu-Thr-Asp-pNA (Ac-IETD-pNA) and Ac-Leu-Glu-His-Asp-pNA (Ac-LEHD-pNA) were purchased from Alexis (San Diego). FITC-anti-mouse Fas mAb and PE-anti-mouse CD4 mAb were purchased from eBioscience (San Diego, CA). FITC-anti-mouse CD69 mAb and FITC-anti-mouse CD3 mAb were purchased from Biolegend (San Diego, CA). Anti-cleaved caspase-3/8/9 and anti-poly(ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Technology (Beverly, MA). Anti-Bid, anti-Bax, anti-cytochrome *c*, anti-COX IV, anti-cellular FLICE-inhibitory protein (c-FLIP), anti-actin and anti-α tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bcl-2 was purchased from BD Pharmingen (San Diego, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

2.5. Con A-induced T-cell-dependent hepatitis

Mice were received an intravenous Con A injection (15 μg/g body weight). In the drug treatment group, fraxinellone and cyclosporin A were injected intraperitoneally twice at 8 h before and 1 h after Con A administration, respectively. The positive control animals with Con A-induced hepatitis were given intraperitoneally the same solvent (normal saline) instead of the drugs. Sera were collected at the indicated time points after Con A administration to measure the serum levels of ALT, AST, LDH and cytokines. Liver tissues were also excised for the histological assay.

2.6. Cytokine assay

Blood samples were obtained from mice at the indicated time points and immediately centrifuged at 1500 × *g* for 15 min. Serum samples were stored at −70 °C until ready for used. Serum levels of IFN-γ and TNF-α were determined using ELISA kits from R&D systems (Minneapolis, MN).

2.7. Cell apoptosis assay

The cells were staining with Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide), then cells were measured by flow cytometry as previously reported [14]. Samples were analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Annexin V^+/PI^- cells were considered as apoptotic cells.

2.8. Mitochondrial transmembrane potential assay

Mouse $CD4^+$ T cells isolated from naive mice were cultured in the presence or absence of 5 μg/ml of Con A for 24 h and then incubated with several doses of fraxinellone for 6 h. The disruption of mitochondrial transmembrane potential was measured using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimida-zolcarbocyanine iodide (JC-1) staining by flow cytometry as previously reported [15].

2.9. Flow cytometric analysis

Cells were stained with antibodies diluted in PBS containing 2% fetal calf serum and 0.1% NaN_3 then analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

2.10. Western blot

Proteins from mouse CD4$^+$ T cells were extracted in lysis buffer (30 mmol/L Tris, pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4 °C, and then incubated with a horse radish peroxidase (HRP)-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

2.11. Caspase activity assay

Cell and liver lysates were prepared by homogenizer (Homogenizer Tool SilentCrusher, Heidolph, Germany) in lysis buffer containing 10 mmol/L HEPES, 5 mmol/L dithiothreitol, 2 mmol/L EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1% CHAPS, pH 7.4. Homogenates were then centrifuged at 12,000 × *g* for 15 min. The protein concentration in the supernatant was determined by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Caspase-3, -8 and -9 activities were tested in duplicate experiments by measuring the proteolytic cleavage of specific chromogenic substrates: Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA, respectively [16].

2.12. Preparation of liver nonparenchymal cells

Liver nonparenchymal cells were isolated from liver-injured mice by the modified two-step perfusion method as previously reported [17]. In brief, the livers of the mice were first perfused in situ via the portal vein with Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS) supplemented with 0.5 mM EGTA and 25 mM HEPES at 37 °C until the blood in the organ was completely removed. Then, the buffer was replaced with 0.1% collagenase solution in HBSS (containing 4 mM $CaCl_2$ and 0.8 mM $MgSO_4$). After a few minutes of perfusion, the liver was excised rapidly from the body cavity and dispersed into cold HBSS. The cell suspension generated was filtered through a 100 gauze mesh. By differential centrifugation at 50 × *g* for 2 min, hepatocytes were recovered in the cell pellet. The supernatant obtained after isolating the above parenchymal cells was used for preparation of nonparenchymal cells by centrifugation at 300 × *g* for 10 min. The liver nonparenchymal cells were used immediately for flow cytometry assay.

2.13. Statistical analysis

All results shown represent means ± S.E.M. from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. The significance of difference is indicated as $P < 0.05$.

3. Results

3.1. Identification of fraxinellone

CD01 was subjected to HPLC analysis and structure determination. The purity of CD01 was confirmed to be 99% by HPLC (Fig. 1A). The structure of CD01 was identified as fraxinellone (Fig. 1A) by MS and NMR spectral analyses and compared with the reported data [13]. The chemical name of CD01 is (3R,3aR)-3-(furan-3-yl)-3a,7-dimethyl-3a,4,5,6-tetrahydroisobenzofuran-1(3H)-one. ESI-MS: $[M+H]^+$ 233.1253. ^{1}H NMR (300 MHz, $CDCl_3$, TMS), δ (ppm), 7.47(1H, d, 1.0), 7.44(1H, d, 1.0), 6.30(1H, s), 4.90(1H, s), 2.13(3H, s), 1.4–2.3(6H, m), 0.86(3H, s).

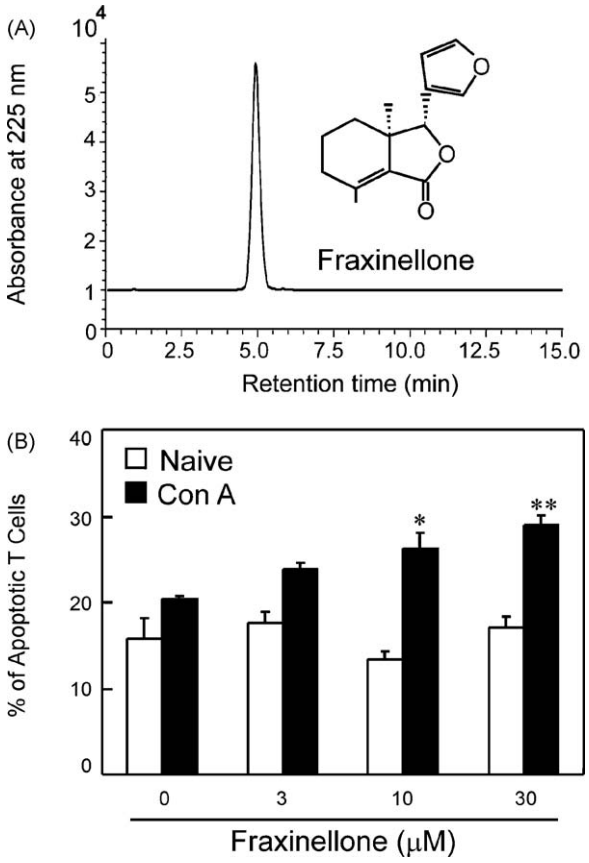

Fig. 1. Fraxinellone induced apoptosis of activated T cells in vitro. (A) The chemical structure of fraxinellone and HPLC analysis of fraxinellone isolated from *Dictamnus dasycarpus* Turcz. The purity of fraxinellone was confirmed to be 99%. (B) Splenic CD4$^+$ T cells isolated from naive mice were cultured with 5 μg/ml of Con A for 24 h and then incubated with fraxinellone for 12 h. Cells were stained by Annexin V/PI and apoptosis was analyzed by flow cytometry. Each value represents the mean ± S.E.M. of three experiments using three mice with triplicate sets in each assay. $^*P < 0.05$ and $^{**}P < 0.01$ vs. drug-untreated group.

3.2. Fraxinellone induced apoptosis of Con A-activated T cells, but did not influence naïve T cells in vitro

As shown in Fig. 1B, fraxinellone at doses of 3, 10, and 30 μM increased the percentage of apoptosis in Con A-activated CD4$^+$ T cells by Annexin V/PI staining in a concentration-dependent manner, but did not affect quiescent CD4$^+$ T cells.

3.3. Fraxinellone induced apoptosis of Con A-activated T cells through death-receptor and mitochondrial pathways

As shown in Fig. 2A, fraxinellone disrupted the mitochondrial transmembrane potential of Con A-activated CD4$^+$ T cells, but showed no effect on resting CD4$^+$ T cells. In addition, after 6 h of incubation with fraxinellone, particularly at 10 and 30 μM, the levels of the anti-apoptotic protein, Bcl-2, were down-regulated accompanying by an up-regulation in the level of the pro-apoptotic protein, Bax. The increase in cleaved caspase-3, -8, and -9, and PARP were also observed (Fig. 2B). The expression of cytochrome *c* in cytosol was greatly increased, while the expression of cytochrome *c* in the mitochondria was significantly decreased in a dose-dependent manner by fraxinellone (Fig. 2C). Interestingly, fraxinellone also increased caspase-8 activity in Con A-activated CD4$^+$ T cells in a dose-dependent fashion, as assessed

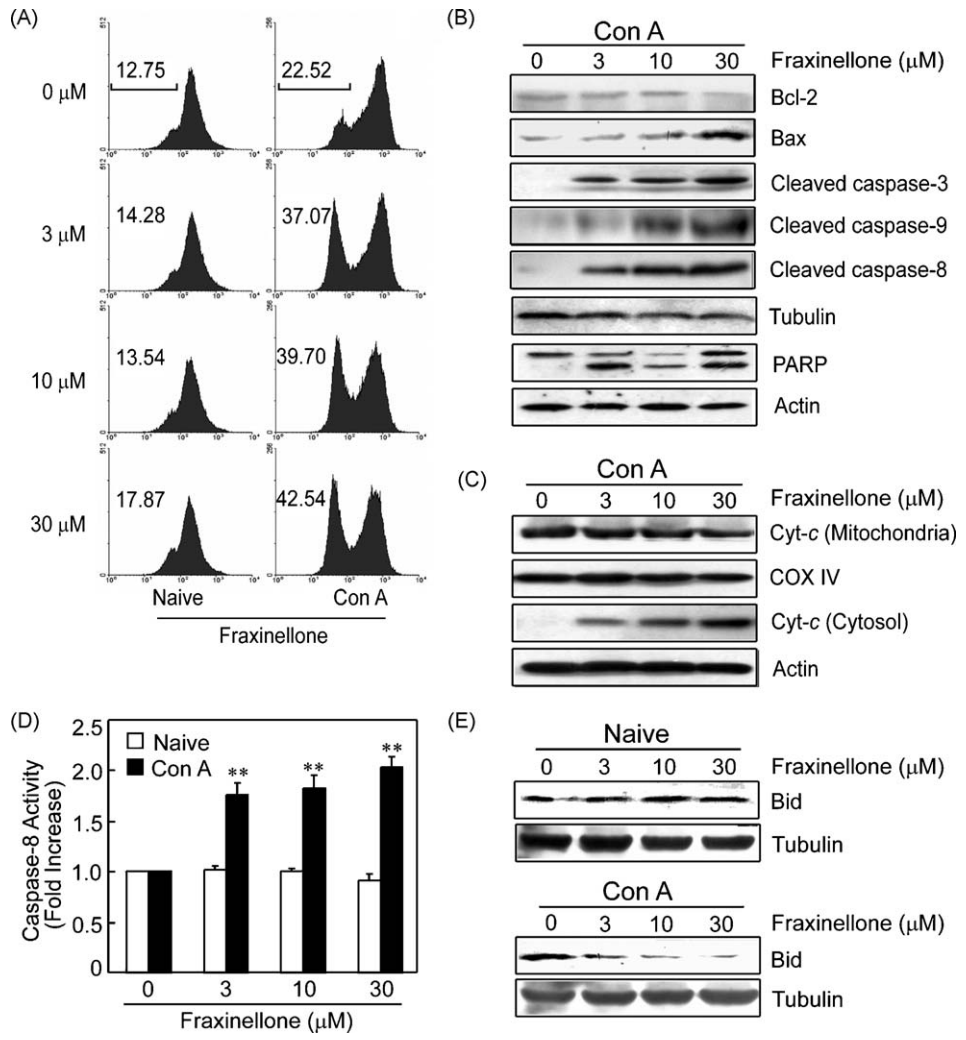

Fig. 2. Fraxinellone induced apoptosis of activated T cells through both death-receptor and mitochondrial pathways. Lymph node $CD4^+$ T cells isolated from naive mice were cultured with 5 μg/ml of Con A for 24 h and then incubated with fraxinellone for 12 h. (A) Cells were stained with JC-1 and FL-2 intensity was analyzed by flow cytometry to determine the disruption of mitochondrial transmembrane potential. The panels are representatives of three independent experiments. (B) Protein levels of Bcl-2, Bax, cleaved caspase-3, -9, and -8, and PARP were examined by Western blotting. (C) The protein level of cytochrome *c* was examined by Western blotting. These blots are the representative results of three independent experiments. (D) Activity of caspase-8 was determined by using the specific substrate. The fold increase was calculated on the basis of cells alone. Each value represents the mean ± S.E.M. of three experiments using three mice with triplicate sets in each assay. $^{**}P < 0.01$ vs. drug-untreated group. (E) Protein level of Bid was examined by Western blotting. The blot is one of three independent experiments.

using Ac-IETD-pNA peptide, but had no effect on non-activated lymph node $CD4^+$ T cells (Fig. 2D). Fraxinellone treatment consistently decreased the cytosol protein level of un-trimmed Bid in activated T cells, but not in non-activated T cells (Fig. 2E).

3.4. Fraxinellone significantly increased Fas expression and decreased c-FLIP expression in activated T cells induced by Con A

As shown in Fig. 3A, fraxinellone augmented Fas expression in Con A-activated $CD4^+$ T cells in a dose-dependent manner, but did not affect Fas expression in naïve $CD4^+$ T cells. In addition, lymph node $CD4^+$ T cells activated by Con A for 24 h had significantly increased expression of c-FLIP, which was down-regulated after fraxinellone treatment (Fig. 3B).

3.5. Fraxinellone protected mice from Con A-induced T-cell-mediated acute fulminant hepatitis by inducing apoptosis of activated T cells

To determine if fraxinellone affects T cells in vivo in the same manner it does in vitro, we used a Con A-induced T-cell-mediated acute hepatitis model and treated the mice with fraxinellone. Using a HPLC assay, we found that the drug concentration in the serum of mice treated with 7.5 mg/kg of fraxinellone twice per day reached a peak at about 10 μM, which just fell within the range of the drug concentrations (3–30 μM) in vitro. Intravenous administration of Con A resulted in a time-dependent increase in the serum ALT level. A significant elevation was detected 5 h after Con A administration and the peak was reached at 8 h. Results from Fig. 4A indicated that 7.5 mg/kg of fraxinellone significantly reduced the ALT level at 5, 8, and 12 h. Eight hours after Con A injection, fraxinellone reduced the serum levels of ALT, AST, and LDH in a dose-dependent manner with the same protective effect at 7.5 and 15 mg/kg (Fig. 4B). Fraxinellone also significantly reduced caspase-3 and -9 activities in the liver tissue at this time point (data not shown). Histologic examination of liver sections from Con A-treated mice showed massive cell death with cytoplasmic swelling of most hepatocytes, infiltration of inflammatory cells, and hyperplasia of Kupffer cells (Fig. 4C) 8 h after Con A administration. Fraxinellone, at a dose of 7.5 mg/kg, also markedly reduced the extent of liver damage with minimal inflammatory cell infiltration (Fig. 4C).

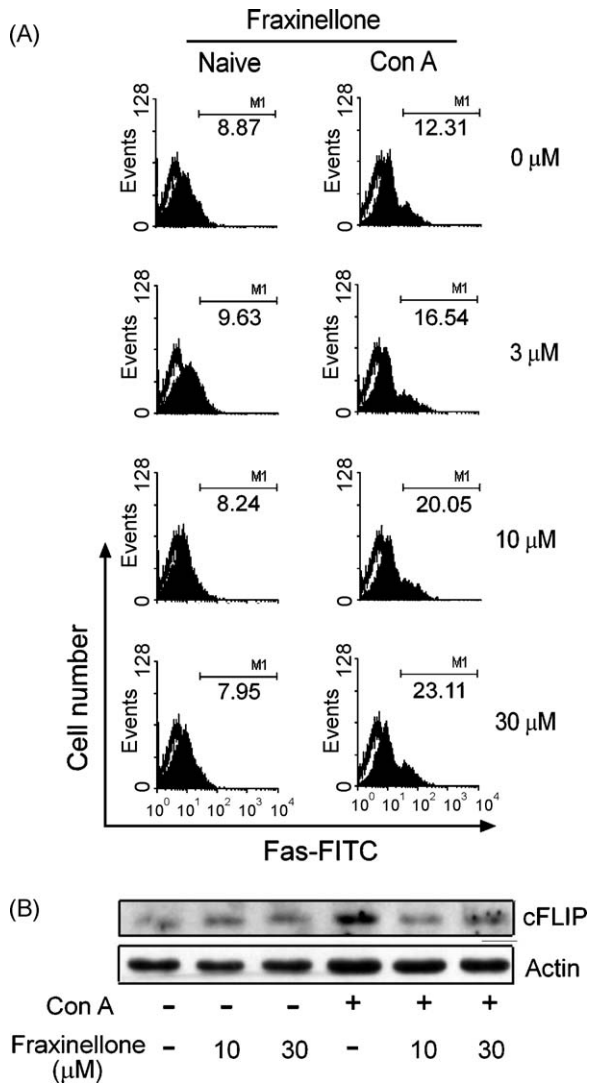

Fig. 3. Fraxinellone up-regulated Fas expression and down-regulated c-FLIP expression of activated T cells. Lymph node $CD4^+$ T cells isolated from naive mice were cultured with 5 μg/ml of Con A for 24 h and then incubated with fraxinellone for 12 h. (A) Fas expression at the surface of T cells was evaluated by flow cytometry. Open histogram, isotype antibody; filled histogram, anti-Fas-FITC. The histogram is one of three independent experiments. (B) The protein level of c-FLIP was analyzed by Western blotting. The blot is one of three independent experiments.

Compared with the significant increase in serum TNF-α and IFN-γ levels due to Con A injection, fraxinellone exhibited significant inhibition in a time- (Fig. 5A) and dose-dependent fashion (Fig. 5B). The mRNA levels of TNF-α and IFN-γ in liver tissues were also inhibited by fraxinellone (data not shown).

In freshly isolated splenic $CD4^+$ T cells from fraxinellone-treated mice, the apoptotic counts were significantly higher than those from untreated or naive mice during the in vitro incubation period (Fig. 6A). Eight hours after Con A injection, an increase in $CD3^+CD4^+$ double-positive cells was observed in liver nonparenchymal cells. Results from Fig. 6B indicated that 7.5 mg/kg of fraxinellone resulted in significant abrogation. With respect to T-cell activation, Con A remarkably enhanced the percentage of $CD4^+CD69^+$ double-positive spleen cells (upper-right quadrant). A significant increase was detected at 1, 2, and 4 h after Con A administration, and 7.5 mg/kg of fraxinellone significantly reduced the positive ratio at each time point (Fig. 6C and D).

4. Discussion

Specific induction of pathogenic T-cell apoptosis is beneficial in depressing excess immune responses and maintaining immune homeostasis, and represents a new approach for future treatment of numerous T-cell-mediated immune diseases. However, few drug candidates have been reported through such selective induction of apoptosis of activated pathogenic T cells thus far. Herein we reported that a small compound, fraxinellone, selectively induced apoptosis of Con A-activated T cells without affecting non-activated T cells in vitro. Furthermore, the facilitation of apoptosis by fraxinellone in activated T cells was remarkably effective in blocking the development of T-cell-dependent hepatitis, indicating that this small molecule might be a potential leading compound useful in treating T-cell-mediated liver disorders in humans.

Fas-mediated apoptosis can be regulated by the levels of expression of Fas. In the present study, fraxinellone up-regulated the level of expression of Fas on the surface of activated T cells, but not naïve T cells. There are two steps involved in Fas-mediated apoptosis. First, transduction of the apoptotic signal requires the oligomerization of Fas and formation of a death-inducing signal complex that involves the adapter molecules, Fas-associated death domain and procaspase-8. Next, procaspase-8 is proteolytically activated into caspase-8, which is then released from the death-inducing signal complex to the cytoplasm. It has been reported that the amount of active caspase-8 determines distinct Fas signaling pathways [18–20]. Large amounts of active caspase-8 directly catalyze the cleavage of downstream caspase-3-like caspases in a mitochondria-independent manner. Alternatively, small amounts of active caspase-8 cut Bid into a truncated form which induces the release of pro-apoptotic molecules from mitochondria. Upon examination of apoptotic pathways, we observed that both death-receptor (Fas-mediated) and mitochondrial pathways were involved in fraxinellone-induced activated-T-cell apoptosis. However, only a small proportion of apoptotic cells showed loss of the mitochondrial membrane potential, and the significant increment in caspase-8 activity and truncation of Bid were induced by fraxinellone, all of which suggest that the death-receptor pathway may play a more critical role and that the mitochondrial pathway may be activated through cross-talk via Bid. Herein we noted that fraxinellone, having little effect on non-activated T cells, selectively promoted apoptosis in activated T cells, which avoided the disadvantage of non-specific immunosuppression. This raises the possibility that fraxinellone may be especially useful for eliminating activated pathogenic T cells that contribute to T cell-related immune diseases.

Recent reports show that c-FLIP plays a crucial role in activation-induced cell death of mature T lymphocytes [21]. c-FLIP is expressed in resting T cells or induced upon initial stimulation, and appears to prevent early death-receptor-mediated T-cell apoptosis [22]. T-cell activation also leads to the induction of IL-2, which in turn down-regulates c-FLIP expression and sensitizes T cells to cell death [23]. In addition to enhancing Fas-mediated apoptosis, the decrease in c-FLIP expression by fraxinellone also results in a higher sensitivity of activated T cells to apoptosis. Collectively, fraxinellone facilitates apoptosis of activated T cells by increasing the expression of Fas and decreasing the expression of anti-apoptotic protein c-FLIP.

Increasing evidence suggests that T-cell-mediated immunity is one of the dominant causes in a variety of liver diseases involving autoimmune and viral hepatitis and T-cell activation is a critical initial step in the pathogenesis of liver damage [24]. In mice, T-cell-dependent hepatitis can be modeled using in vivo administration of Con A, a plant lectin and T-cell mitogen. This mitogen induces polyclonal T-cell activation in vitro and causes severe

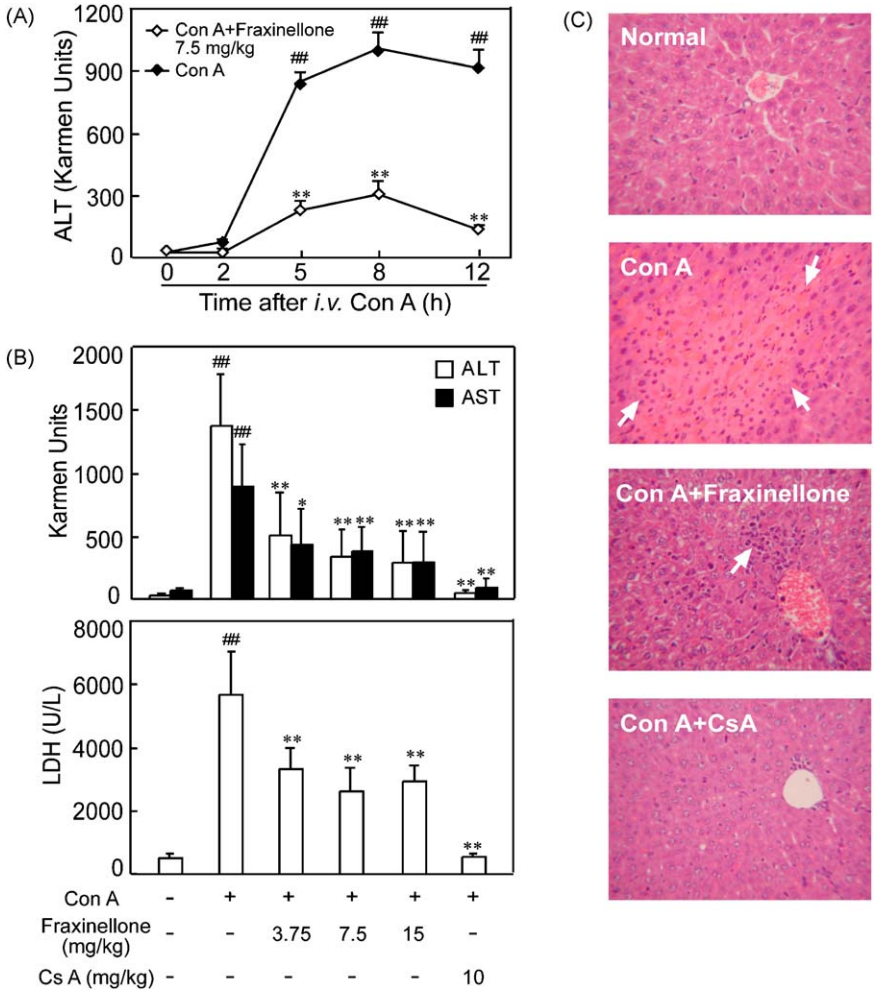

Fig. 4. Fraxinellone protected mice against Con A-induced T-cell-mediated acute fulminant hepatitis. Mice were received an intravenous Con A injection (15 μg/g body weight). In the drug treatment group, fraxinellone and cyclosporin A were injected intraperitoneally twice at 8 h before and 1 h after Con A administration, respectively. (A) Time course of serum alanine transaminase (ALT) activity. Values are shown as the mean ± S.E.M. of three mice at each time point. $^{\#\#}P < 0.01$ vs. vehicle control; $^{**}P < 0.01$ vs. Con A group at the same time point. (B) Dose-dependent inhibition on ALT, aspartate transaminase (AST) and lactic dehydrogenase (LDH) activities. Values are shown as the mean ± S.E.M. of eight mice in each group. $^{\#\#}P < 0.01$ vs. vehicle control; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Con A group. (C) Representative microphotographs showing liver histopathologic changes with hematoxylin–eosin staining (original magnification 200×). White arrows indicate massive necrosis observed in the liver.

immune-mediated hepatitis characterized by increased serum levels of transaminases and infiltration of peripheral $CD4^+$ T cells into the liver [12,25]. These findings indicate that activated $CD4^+$ T cells play a detrimental role in liver injury. Therefore, effective elimination of pathogenic effector T cells has been a therapeutic strategy for the treatment of T-cell-mediated liver diseases. Although eliminating pathogenic T cells is very important for the treatment of liver diseases, the fact is that there is still a lack of drug candidates selectively targeting pathogenic T cells in hepatitis. As a novel example, facilitation of apoptosis in activated pathogenic T cells was observed in the present study by the administration of fraxinellone in Con A-induced T-cell-dependent hepatitis in mice. In this model, fraxinellone significantly alleviated Con A-induced hepatitis with an almost complete recovery from the elevation of ALT, AST, and LDH serum levels, and a decrease in the extent of necrosis in liver. Splenic $CD4^+$ T cells isolated from mice in the fraxinellone-treated group showed a higher potential to Con A-induced apoptosis than those from vehicle-treated group, indicating a higher sensitivity of activated peripheral T cells to apoptosis by fraxinellone in vivo. It coincided with our in vitro result that activated T cells by Con A showed a higher percentage of apoptosis after fraxinellone treatment. Consequently, this effect of fraxinellone may result in less activated peripheral $CD4^+$ T cell infiltration into the liver, supported by a marked reduction in the percentage of $CD3^+CD4^+$ hepatic T cells and $CD4^+CD69^+$ splenic T cells in fraxinellone-treated mice. For the first time, this study demonstrates the feasibility of treatment of T-cell-mediated liver diseases by a small compound, like fraxinellone, that facilitates apoptosis of activated pathogenic T cells.

Until now, there are only a few reports on small compounds that induce activated T cells to apoptosis. For example, bisindolylmaleimide VIII, a protein kinase C inhibitor, reportedly potentiates apoptosis in activated T cells through the inhibition of kinases other than protein kinase C [26]. KE-298, an anti-rheumatic drug, selectively augments apoptosis of activated T cells in rheumatoid arthritis by decreasing the expression of the caspase inhibitor, X-linked inhibitor-of-apoptosis protein [27]. A recent report demonstrates that resveratrol-induced T-cell apoptosis in experimental allergic encephalomyelitis correlates with up-regulation of aryl hydrocarbon receptor expression [28]. However, demonstration of a small compound that selectively augments apoptosis of activated T cells in hepatitis was still lacking. In the present study, we showed that fraxinellone, a natural small compound, exerted a unique immunosuppressive action in hepatitis by up-regulating Fas expression and down-regulating c-FLIP expression, then

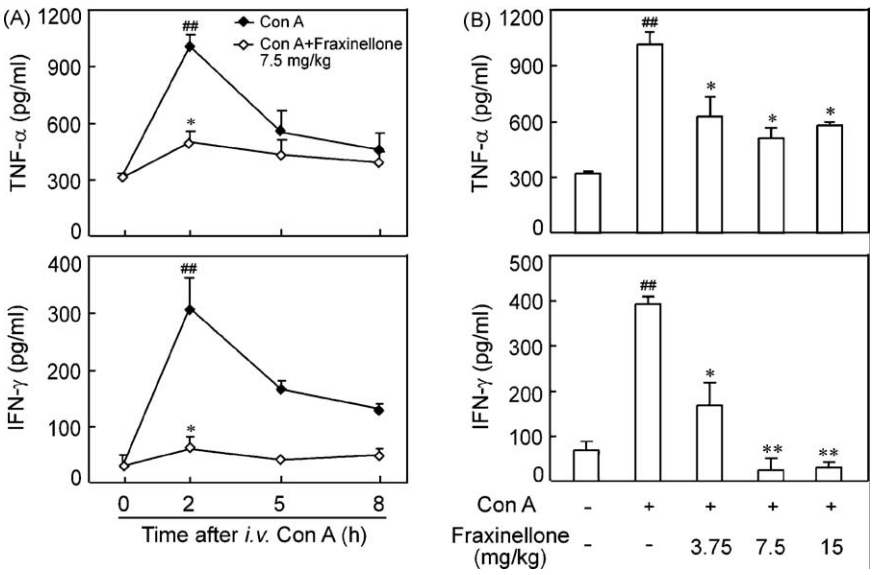

Fig. 5. Fraxinellone inhibited Con A-induced TNF-α and IFN-γ levels in serum. (A) Time course of TNF-α and IFN-γ release into serum. Cytokine levels in serum were measured by ELISA. Values are shown as the mean ± S.E.M. of three mice at each time point. $^{\#\#}P < 0.01$ vs. normal; $^{*}P < 0.05$ vs. Con A group at the same time point. (B) Dose-dependent inhibition on TNF-α and IFN-γ levels. Values are shown as the mean ± S.E.M. of eight mice in each group. $^{\#\#}P < 0.01$ vs. vehicle control; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Con A group.

subsequently enhancing the Fas-mediated apoptosis signaling pathway in Con A-activated T cells, showing a distinct mechanism from the aforementioned small compounds. Most importantly, in the present study, the activated T cells induced by Con A in vitro accounted for most of the pathogenic T cells which induced T-cell-mediated hepatitis in vivo. However, other small compounds mentioned above triggered apoptosis of activated T cell stimulated by anti-CD3, or phorbol/inomycin, which were not the actual etiopathogenesis of their in vivo animal model of immune diseases, including experimental allergic encephalitis, adjuvant arthritis, and rheumatoid arthritis. The significant innovation of fraxinellone is the selective triggering apoptosis of activated T cells involved in hepatitis, which significantly contributes to the improvement of Con A-induced liver injury. The discovery and confirmation of the selective elimination of pathogenic T cells involved in hepatitis will bring more advantage in the treatment of liver disorders.

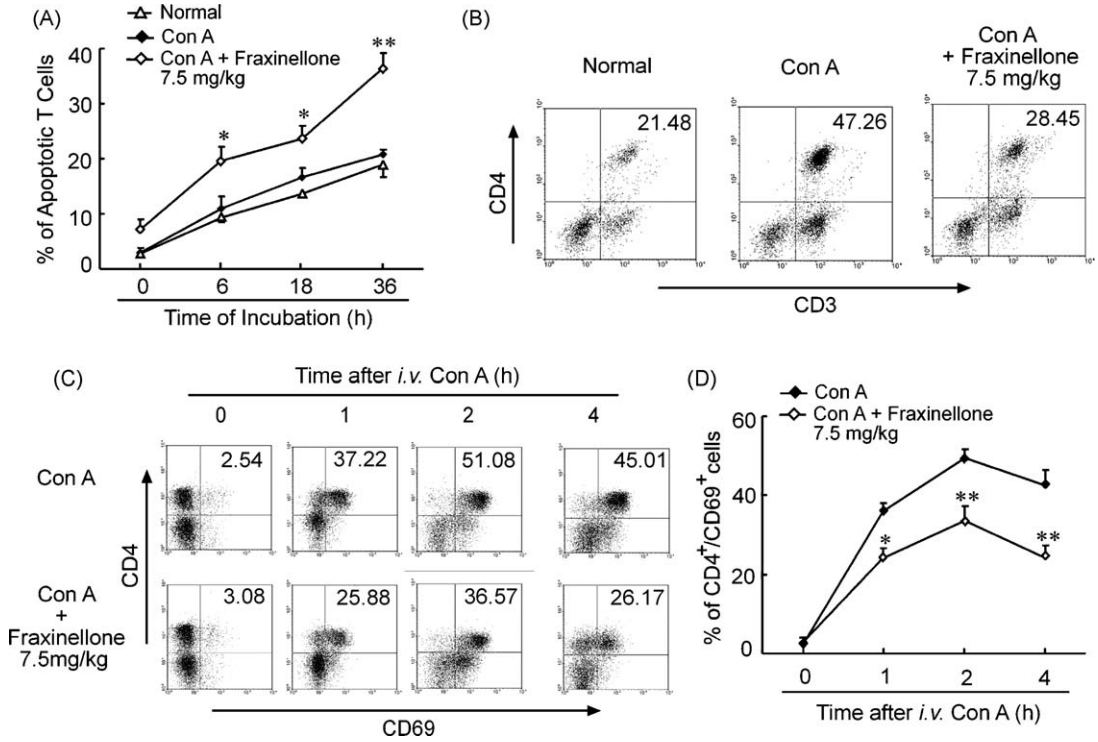

Fig. 6. Fraxinellone induced apoptosis of activated T cells in Con A-induced T-cell-mediated acute fulminant hepatitis. (A) Mice were treated and splenic $CD4^+$ T cells were prepared at 2 h after Con A administration. After culture for 0, 6, 18, and 36 h, cells were co-stained with Annexin V/PI to determine spontaneous apoptosis by flow cytometry. Values are shown as the mean ± S.E.M. from three mice at each time point. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Con A group at the same time point. (B) Hepatic lymphocytes were isolated at 8 h after Con A administration. The percentage of $CD3^+CD4^+$ T cells was analyzed by flow cytometry. The panel is one of three independent experiments. (C) Spleen cells were isolated at 0, 1, 2, and 4 h after Con A injection. The surface molecules, CD4 and CD69, were analyzed by flow cytometry. The panels are representatives of three independent experiments. (D) Values are shown as the mean ± S.E.M. from three mice at each time point. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Con A group at the same time point.

Recently, there are some reports of cases of toxic hepatitis that occurred after taking a decoction made by boiling down the root of *D. dasycarpus* [29,30], indicating the great differences between the total decoction and the purified compound. It is difficult to provide conclusive evidence of what caused hepatitis, as the decoction of the root bark of *D. dasycarpus* is a mixture that may contain various chemical constituents, including fraxinellone, dictamnine, skimmianine, gamma-fagarine, beta-sitosterol, obacunone, limonin disophenol, fraxinellonone, wogonin, rutevin, kihadinin B, dasycarine, dasycarpuside A, and dictamnosides [31–33]. The actual ingredients that lead to hepatotoxicity need to be further investigated. However, in our studies, fraxinellone was found to protect mice from Con A-induced hepatitis, together with the previous finding that fraxinellone was reported to ameliorate CCl_4-induced mouse acute liver injury by Ran et al. [34], suggesting that fraxinellone was not the chemical component that contributed to the toxic effect of the total decoction of *D. dasycarpus*. Moreover, it should be noted that there was no significant difference in the weight and cell numbers of lymphoid tissues (thymus, spleen, and lymph nodes) between the mice intraperitoneally injected with fraxinellone (15 mg/kg) and the mice treated with normal saline, suggesting the selectivity and safety of fraxinellone to some degree. Previously, fraxinellone was also demonstrated to possess feeding deterrent activity against adults and larvae of *Tribolium castaneum*, as well as adults of *Sitophilus zeamais*, in the screening for insecticidal principles [9]. Although fraxinellone (15 mg/kg) was not toxic for administration to mice, the possibility that the compound causes toxicity in humans cannot be ruled out. The safety of fraxinellone needs detailed investigation in the future.

In conclusion, fraxinellone can selectively induce apoptosis of Con A-activated T cells rather than naïve T cells both in vitro and in vivo, through a unique mechanism via up-regulating Fas expression and down-regulating c-FLIP expression of activated T cells. The observation that in vivo administration of fraxinellone alleviates Con A-induced hepatitis in mice has important therapeutic implications for the treatment of T-cell-mediated liver diseases in humans.

Conflict of interest statement

The authors have no conflicts of interest.

Acknowledgments

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