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Cynaropicrin Induces Reactive Oxygen Species-Dependent Paraptosis-Like Cell Death in Human Liver Cancer Cells

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Abstract

Cynaropicrin, a sesquiterpene lactone found in artichoke leaves exerts diverse pharmacological effects. This study investigated whether cynaropicrin has a paraptosis-like cell death effect in human hepatocellular carcinoma Hep3B cells in addition to the apoptotic effects reported in several cancer cell lines. Cynaropicrin-induced cytotoxicity and cytoplasmic vacuolation, a key characteristic of paraptosis, were not ameliorated by inhibitors of necroptosis, autophagy, or pan caspase inhibitors in Hep3B cells. Our study showed that cynaropicrin-induced cytotoxicity was accompanied by mitochondrial dysfunction and endoplasmic reticulum stress along with increased cellular calcium ion levels. These effects were significantly mitigated by endoplasmic reticulum stress inhibitor or protein synthesis inhibitor. Moreover, cynaropicrin treatment in Hep3B cells increased reactive oxygen species generation and downregulated apoptosis-linked gene 2-interacting protein X (Alix), a protein that inhibits paraptosis. The addition of the reactive oxygen species scavenger *N*-acetyl-L-cysteine (NAC) neutralized cynaropicrin-induced changes in Alix expression and endoplasmic reticulum stress marker proteins counteracting endoplasmic reticulum stress and mitochondrial impairment. This demonstrates a close relationship between endoplasmic reticulum stress and reactive oxygen species generation. Additionally, cynaropicrin activated p38 mitogen activated protein kinase and a selective p38 mitogen activated protein kinase blocker alleviated the biological phenomena induced by cynaropicrin. NAC pretreatment showed the best reversal of cynaropicrin induced vacuolation and cellular inactivity. Our findings suggest that cynaropicrin induced oxidative stress in Hep3B cells contributes to paraptotic events including endoplasmic reticulum stress and mitochondrial damage.

Key Words: Cynaropicrin, Paraptosis, Reactive oxygen species, Endoplasmic reticulum stress, Mitochondrial dysfunction

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INTRODUCTION

The incidence of liver cancer, a very fatal disease frequently diagnosed mainly in developing countries, has continued to increase across several countries, including some European countries and the United States (Sagnelli *et al*., 2020; Younossi *et al*., 2023). Hepatocellular carcinoma (HCC), the most frequent subtype of primary liver cancer, usually arises in chronically damaged liver tissues and carries high mortality and morbidity rates (Samant *et al*., 2021; Chakraborty and Sarkar, 2022). The major etiological factors in the development of HCC include viral hepatitis, alcoholic and non-alcoholic liver disease, hepatic steatohepatitis, and other toxic chemicals. Surgical resection, radiotherapy, and chemotherapy have been the primary clinical treatments for HCC; however, studies have shown low response rates, drug resistance, and serious side effects with chemotherapy (Kulik and El-Serag, 2019; Chidambaranathan-Reghupaty *et al*., 2021). Therefore, new strategies that can control the occurrence, progression, and prognosis of HCC are urgently needed. In line with this, several researchers have sought to identify effective anticancer drugs natural compounds for the treatment of HCC.

Sesquiterpene lactones, which belong to sesquiterpenoids containing a lactone ring, have excellent structural diversity and a wide range of biological effects, including anticancer activity (Aliarab *et al*., 2018; Fateh *et al*., 2022). Classified as a guaianolide-type sesquiterpene lactone, cynaropicrin, first isolated from the leaves of the artichoke plant (*Cynara scolymus* L.) (Suchý *et al*., 1960), has been found to have multitudinous bioactivities and health benefits (Cai *et al*., 2021; Spennato *et al*., 2022; Jin and Leng, 2023). Interestingly, this compound has promising anti-hepatitis C viral activity (Elsebai *et al*., 2016) and has recently been attracting attention as a potential chemotherapeutic agent that acts through various signaling pathways. Several studies have demonstrated that cynaropicrin exerts its anticancer activity by mainly acting directly on cancer cells, inhibiting their growth and inducing apoptosis, a programmed cell death (PCD), along with cell cycle arrest (Liu *et al*., 2019; De Cicco *et al*., 2021; Ding *et al*., 2021; Villarini *et al*., 2021; Rotondo *et al*., 2022; Yang *et al*., 2022). Recently, Yang *et al*. reported that cynaropicrin induced endoplasmic reticulum (ER) stress-dependent protective autophagy against apoptosis in neuroblastoma (Yang *et al*., 2022). Although cynaropicrin-induced autophagy has also been observed in glioblastoma cells (Rotondo *et al*., 2022), its role in cell death remains unclear. In addition, it has been suggested that unlike necrosis and apoptosis, cynaropicrin-induced cell death in multiple myeloma cells was caused by parthanatos, a form of PCD characterized by the hyperactivation of poly (ADP-ribose) polymerase 1 and nuclear translocation of apoptosis-inducing factor from mitochondria (Boulos *et al*., 2023). Although cynaropicrin-induced cancer cell death involves mitochondrial damage and excessive production of reactive oxygen species (ROS) due to oxidative stress, these results suggest that cynaropicrin may induce non-canonical PCD rather than typical apoptosis. Recently, it was demonstrated that sesquiterpene lactones, including cynaropicrin, isolated from *Cynara cardunculus* L., another species of artichoke plants, can initiate apoptosis in HCC HepG2 cells, suggesting their potential as a source of antitumor agents for HCC (Hamza *et al*., 2023). However, little research has revealed the mechanism by which cynaropicrin induces cell death in HCC cells, and limited evidence has been available on which type of PCD may be involved. For this reason, the current study explored additional types of cell death and signaling mechanisms involved in the cytotoxicity of cynaropicrin in Hep3B cells, an HCC cell line.

MATERIALS AND METHODS

Reagents and antibodies

Cynaropicrin (Fig. 1A), dimethyl sulfoxide (DMSO), carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD-fmk), necrostatin-1, bafilomycin A1, 4-phenylbutyric acid (4-PBA), cycloheximide (CHX), 2′,7′-dichlorofluorescin diacetate (DCF-DA), 4′,6-diamidino-2-phenylindole (DAPI), and N-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). All materials required for cell culture were provided by WelGENE Inc. (Gyeongsan, Korea). Annexin V-Fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) Apoptosis Detection Kit, ER-Tracker™ Red, Fluo-4 AM, and enhanced chemiluminescence (ECL) substrates were obtained from Thermo Fisher Scientific (Waltham, MA, USA). MitoTracker® Green and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole (SB203580) were provided by Cell Signaling Technology, Inc. (Danvers, MA, USA). Immobilon®-P polyvinylidene difluoride membranes and the Bio-Rad protein assay kit were purchased from Merck Millipore (Billerica, MA, USA) and Bio-Rad Laboratories, Inc. (Hercules, CA, USA) respectively. Cell Counting Kit 8 (CCK-8) and peroxidase-labeled immunoglobulins were obtained from Abcam, Inc. (Cambridge, UK). Antibodies against poly(ADP-ribose) polymerase (PARP), apoptosis-linked gene 2-interacting protein X (Alix), p38 mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (de Ridder *et al*., 2023), c-Jun N-terminal kinases (JNK), phosphor (p)-JNK, and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies for caspase-3, inositol-requiring enzyme 1α (IRE1α), C/EBP homologous protein (Chop), protein disulfide isomerase (PDI), ER oxidoreductin 1-Lα (Ero1-Lα), p-ERK, p-p38 MAPK, and JNK were supplied by Cell Signaling Technology, Inc. Cell Counting Kit 8 (CCK-8) and peroxidase-labeled immunoglobulins were obtained from Abcam, Inc. All remaining reagents used were analytical grade.

Cell culture, treatment and cell viability assay

Hep3B cells were grown in Eagle's minimum essential medium with 10% (v/v) fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C according to previously described methods (di Vito *et al*., 2023). To examine the cytotoxicity of cynaropicrin in Hep3B cells, cells were cultured in medium containing various concentrations of cynaropicrin for 24 h. Additionally, to evaluate the role of cynaropicrin in cellular signaling pathways, cells were pretreated with inhibitors specific for each signaling cascade for 1 h before cynaropicrin treatment for the indicated times. The viability of the cells cultured under different conditions using CCK-8 assay supplies was then calculated by measuring absorbance at 460 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturers' protocol. Changes in the morphology of the cells were observed using a light microscope (Carl Zeiss, Oberkochen, Germany).

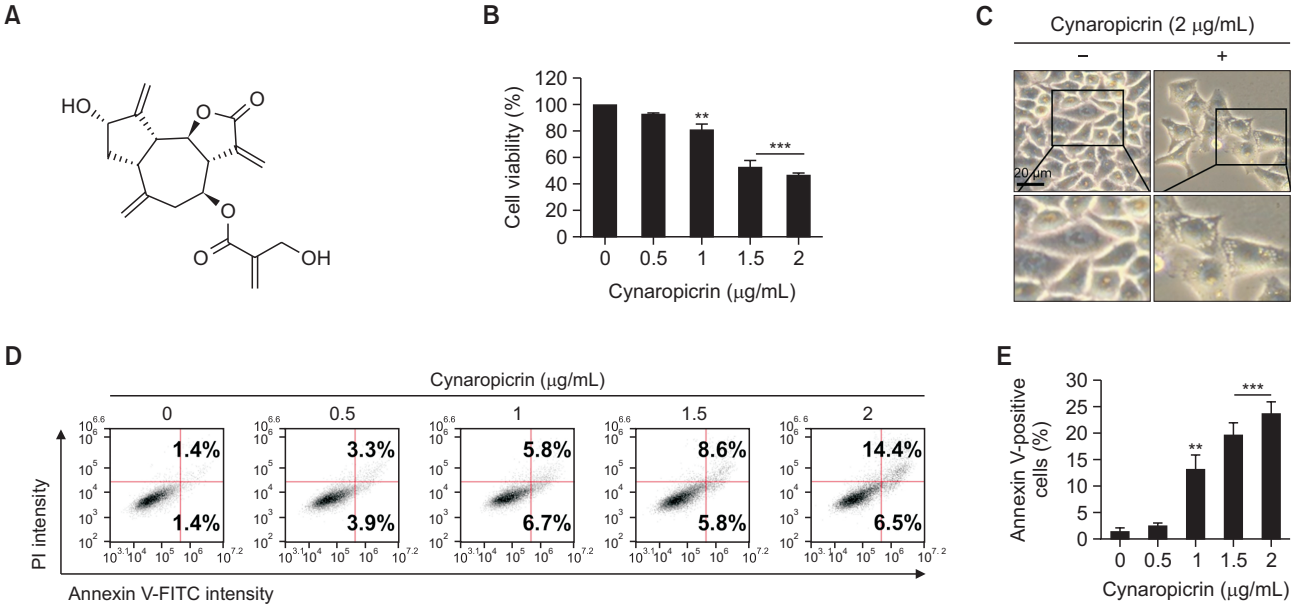

Fig. 1. Cynaropicrin-induced suppression of cell viability and cell death in Hep3B cells. (A) Chemical structure of cynaropicrin. Cells were cultured for 24 h in media containing different concentrations of cynaropicrin (B, D, E) or treated with 20 mg/mL cynaropicrin and cultured for 24 h (C). (B) After cynaropicrin treatment, cell viability was analyzed using the CCK-8 assay. (C) Changes in cell morphology after cynaropicrin treatment were examined under a light microscope. Scale bar, 20 μm. (D, E) After Annexin V/PI staining, the degree of apoptosis induced by cynaropicrin was analyzed through flow cytometry. The proportion of the cell populations in late (upper right) and early (low right) apoptosis is indicated in the dot plots (D), and the proportion of the annexin V-positive cells are shown as histograms (E). B and E Numerical data are presented as mean ± SD (n=3). ***p*<0.01 and ****p*<0.001 compared to untreated group.

Flow cytometry

To determine whether cell death and ER stress were induced, cells were stained using the Annexin V-FITC/PI or ER-Tracker according to the manufacturer's recommendations. Briefly, cells cultured under various conditions were washed with phosphate-buffered saline (PBS), and then stained with Annexin V/PI or ER-Tracker according to the manufacturer's protocol (Kim *et al*., 2023; Lee *et al*., 2023a). To quantitatively evaluate intracellular calcium ion (Ca^{2+}) levels, mitochondrial dysfunction, and ROS generation, Fluo-4 AM, a cell-permeable Ca^{2+} indicator; MitoTracker® Green, a dye that measures mitochondrial mass; and DCF-DA, a cell-permeant indicator of ROS, were used. A series of experiments were performed using a flow cytometer (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) installed at TRCORE, Dong-eui University (Busan, Korea) after reacting the treated cells with each reagent according to the instructions provided with the kits.

Immunoblotting

Hep3B cells stimulated with cynaropicrin for the appropriate time with or without 4-PBA, CHX, NAC, or SB203580 were then suspended in lysis buffer and incubated for 30 min at 4°C to extract total proteins as previously described (Lee *et al*., 2023b; Manigandan and Yun, 2023). To measure the protein concentrations of each cell pellet, a Bio-Rad protein assay kit was used, and equal amounts of protein were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred onto membranes. The membranes were then blocked with 5% skim milk for 30 min, reacted with primary antibodies overnight at 4°C, washed with PBS-T (PBS with Tween 20) for 10 min, and then incubated with secondary antibodies for 1 h at room temperature. The expression levels of proteins corresponding to each antibody were visualized using ECL solution and the Fusion Solo S system (Vilber Lourmat, Collégien, France).

Fluorescence microscope observation

To confirm the induction of ER stress and mitochondrial damage using fluorescence microscopy, cells were exposed to 2 μg/mL of cynaropicrin for 24 h in the presence or absence of NAC for 1 h. After treatment, the cells were stained with ER-Tracker and MitoTracker, and nuclei were subsequently stained with DAPI. After washing the cells with PBS, the fluorescence intensity was observed using a fluorescence microscope (EVOS FS Auto; Thermo Fisher Scientific) (Duan *et al*., 2023). To observe the accumulation of Ca^{2+} or evaluate the effects of NAC on ROS generation, cells were treated with various concentrations of cynaropicrin for 24 h or pretreated with NAC for 1 h and then treated with cynaropicrin for 24 h or 1 h. Afterwards, the cells were reacted with Fluo-4 AM or DCF-DA according to the manufacturers' instructions, followed by fluorescence image acquisition (Lu *et al*., 2023).

Statistical analysis

Statistical results were presented as mean ± standard deviation (SD). All statistical analyses were performed using one-way ANOVA and Tukey's post-hoc test in GraphPad Prism version 8.4.2 (GraphPad Software Inc., San Diego, CA, USA) (Lee *et al*., 2024), with a *p* value of <0.05 indicating statistical significance.

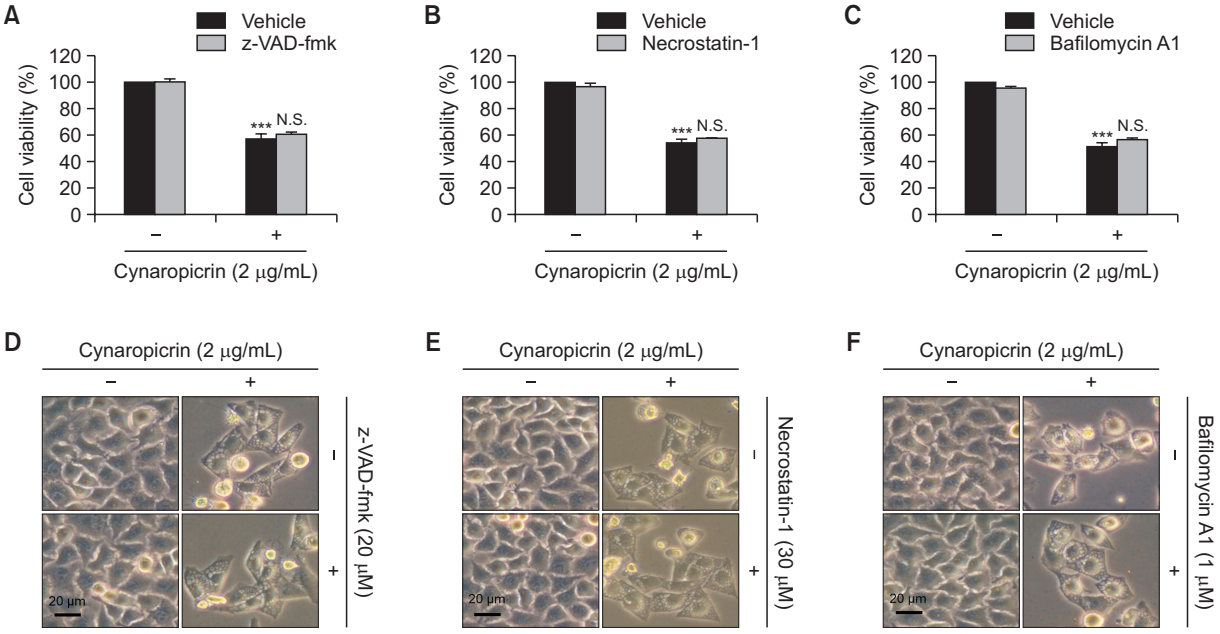

Fig. 2. Effects of a pan-caspase inhibitor, necroptosis inhibitor, and autophagy inhibitor on the cynaropicrin-induced decrease in the viability and morphological changes of Hep3B cells. Cells were pretreated with z-VAD-fmk, necrostatin-1, and bafilomycin A1 for 1 h and then treated with cynaropicrin and cultured for 24 h. The cell survival rate in each treatment group was analyzed through the CCK-8 assay (A-C), whereas changes in cell morphology were examined under a light microscope (D-F). (A-C) Numerical data are presented as mean ± SD (n=3). ***p<0.001 compared to untreated group; N.S., not significant.

RESULTS

Cynaropicrin inhibited cell survival and induced cell death in Hep3B cells

The inhibitory effects of cynaropicrin on Hep3B cell viability was investigated using the CCK-8 assay. Notably, our findings showed that an increase in cynaropicrin concentration significantly suppressed cell survival rates (Fig. 1B). Furthermore, we found that cynaropicrin-treated cells exhibited morphological modifications, including extensive cytoplasmic vacuolation around the nucleus (Fig. 1C), and that the cytotoxicity of cynaropicrin was associated with increased cell death (Fig. 1D, 1E). The proportion of the cells undergoing apoptosis increased from 1.49% to 3.07%, 13.21%, 19.11%, and 22.92% with cynaropicrin treatment at 0.5, 1, 1,5, and 2 μg/mL. However, cynaropicrin-induced cytotoxicity and morphological alterations were not significantly affected by the presence of a pan-caspase inhibitor (z-VAD-fmk), a necroptosis-selective blocker (necrostatin-1), and an autophagy inhibitor (bafilomycin A1) (Fig. 2A-2F), implying that the cytotoxicity of cynaropicrin in Hep3B cells may be associated with non-apoptotic programmed cell death.

Cynaropicrin induced mitochondrial damage while increasing ER stress in Hep3B cells

After investigating whether cynaropicrin-induced cytotoxicity in Hep3B cells was associated with ER stress induction, we found that cynaropicrin treatment gradually increased the expression of well-identified markers of ER stress, such as IRE1α, PDI, Chop, and Ero1-Lα (Barez *et al*., 2020; Pavlović and Heindryckx, 2022; Ye *et al*., 2023) in a dose-dependent manner (Fig. 3A). Flow cytometry and fluorescence microscopy analyses confirmed that cynaropicrin treatment also enhanced the frequency of ER-Tracker-positive cells (Fig. 3B-3D), which was associated with an increase in intracellular Ca^{2+} levels (Fig. 3E-3G). Additionally, MitoTracker staining showed that cynaropicrin treatment significantly decreased mitochondrial mass per cell in a concentration-dependent manner (Fig. 4A, 4B) and that cynaropicrin-treated cells had considerably increased MitoTracker fluorescence intensity (Fig. 4C). Furthermore, the expression of Alix, which is involved in paraptosis inhibition (Sperandio *et al*., 2004; Wang *et al*., 2023), gradually decreased with increasing cynaropicrin treatment concentration (Fig. 4D). Given that paraptosis is caspase-independent, morphologically different from apoptosis and autophagy, and characterized by cytoplasmic vacuolization (Broker *et al*., 2005; Park *et al*., 2023), our findings suggest that the cynaropicrin-induced formation of cytoplasmic vacuolation was attributed to the induction of paraptosis-like cell death.

Cynaropicrin-induced cytotoxicity of Hep3B cells was ER stress-dependent

A study by Yang *et al*. revealed that cynaropicrin-induced autophagy enhanced the induction of ER stress-dependent apoptosis (Yang *et al*., 2022), with other studies showing that paraptosis was closely associated with ER stress (Hanson *et al*., 2023; Chen *et al*., 2024b). To analyze the role of ER stress in cynaropicrin-induced cytotoxicity in Hep3B cells, 4-PBA, which inhibits ER stress, was used. As demonstrated in Fig. 5A and 5B, cells pretreated with 4-PBA showed a significant suppression in the cynaropicrin-induced decrease in cell survival and formation of cytoplasmic vacuolation. In addition, pretreatment with 4-PBA maintained the cynaropicrin-induced

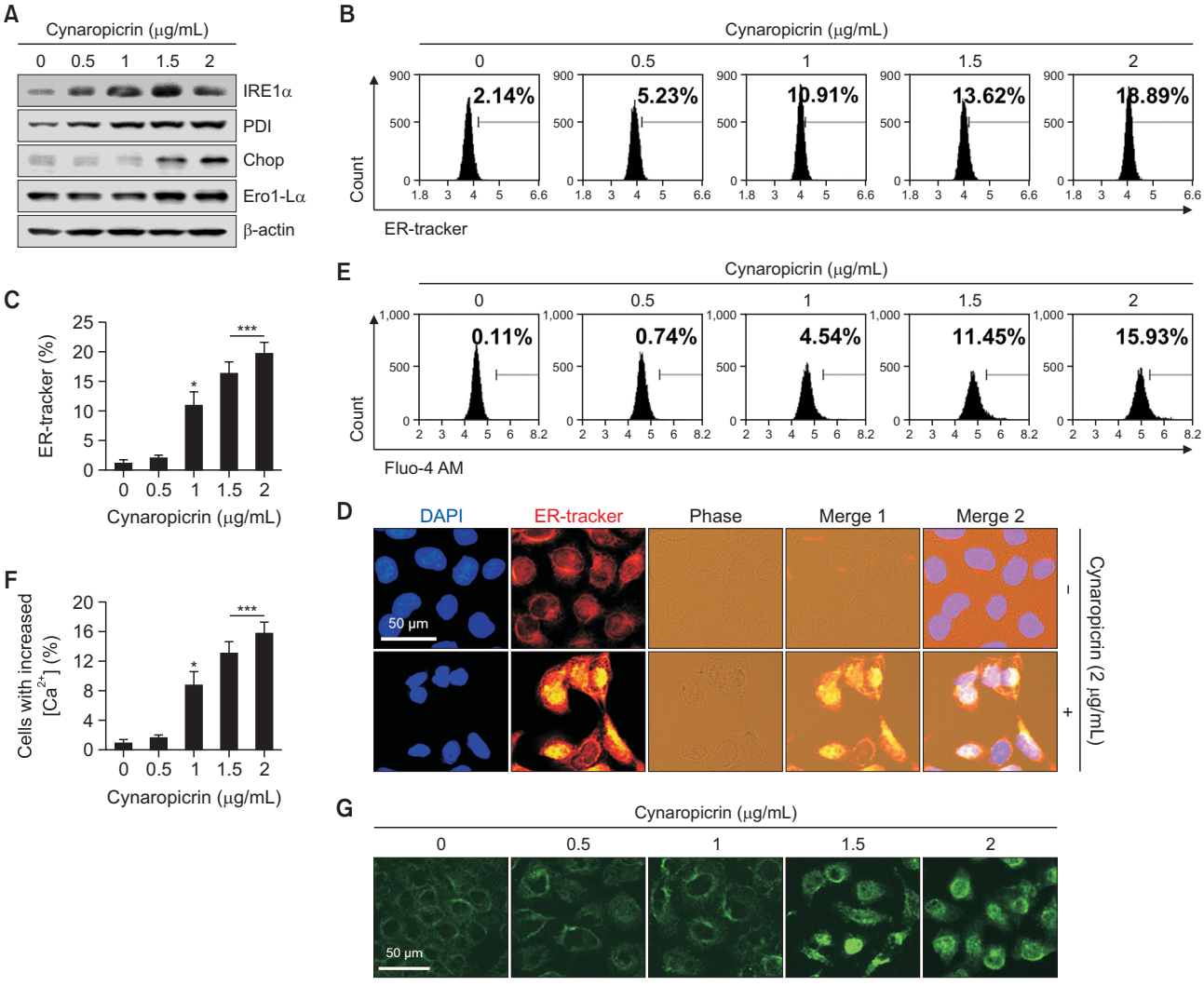

Fig. 3. Cynaropicrin-induced ER stress and increase in intracellular Ca^{2+} levels in Hep3B cells. Cells were cultured for 24 h after treatment with different concentrations of cynaropicrin (A-C, E-G). (A) After separating total proteins, changes in the expression of the representative ER stress marker proteins (IRE1α, PDI, Chop, and Ero1-Lα) were analyzed using the corresponding antibodies. (B, C) After cynaropicrin treatment, the frequency of ER-Tracker-positive cells was examined through flow cytometry. (D) Cells exposed to 2 μg/mL of cynaropicrin for 24 h were stained with ER-Tracker, after which fluorescence images were captured under a fluorescence microscope. Nuclei were counterstained using DAPI. (E-G) Changes in intracellular Ca^{2+} concentration in cells treated with cynaropicrin were calculated via flow cytometry using Fluo-4 (E, F), whereas Fluo-4 fluorescence intensity was determined using fluorescence microscopy (G). (C, F) Numerical data are presented as mean ± SD (n=3). *p<0.05 and ***p<0.001 compared to untreated group.

increase in ER stress marker proteins at control levels (Fig. 5C). Furthermore, 4-PBA pretreatment reduced ER stress while offsetting cynaropicrin-mediated intracellular Ca^{2+} increase (Fig. 5D-5G), which was associated with a reduction in the frequency of MitoTracker-positive cells (Fig. 5H, 5I). This demonstrates that ER stress-related disruption of Ca^{2+} homeostasis may be crucial for inducing the paraptosis process.

Translation inhibition blocked cynaropicrin-mediated ER stress and mitochondrial damage in Hep3B cells

Considering that PCD induction, including paraptosis, requires *de novo* protein synthesis (Saelens *et al*., 2005; Seo *et al*., 2021), we subsequently examined whether the loss of translational control was essential for cynaropicrin-induced cytotoxicity using the translation inhibitor CHX. As shown in Fig. 6A and 6B, CHX inhibited the morphological deformation and reduction in viability observed in cells exposed to cynaropicrin, similar to 4-PBA pretreatment, and prevented the accumulation of ER stress marker proteins (Fig. 6C). Moreover, CHX protected against cynaropicrin-induced Ca^{2+} accumulation and ER stress (Fig. 6D-6G) and consequently weakened mitochondrial damage (Fig. 6H, 6I), indicating that *de novo* protein synthesis contributed critically to cynaropicrin-induced paraptosis.

ROS generation served as an upstream regulator of cynaropicrin-mediated ER stress and mitochondrial damage in Hep3B cells

Recent research has placed considerable emphasis on the importance of oxidative stress in paraptosis (Kainat *et al*.,

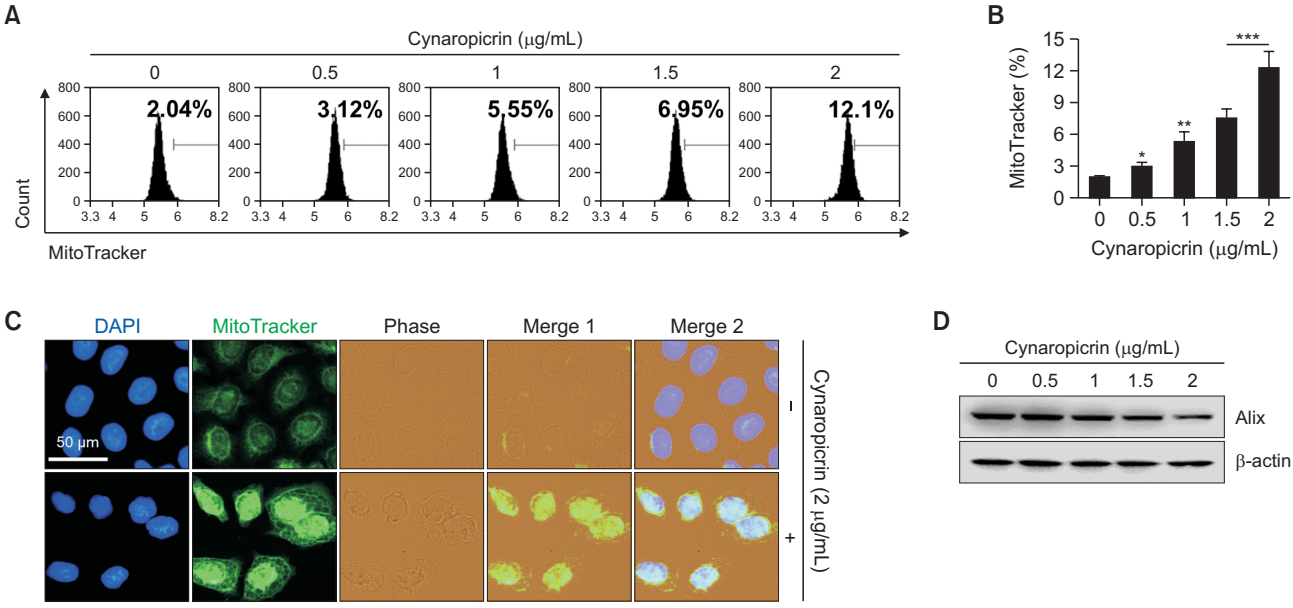

Fig. 4. Cynaropicrin-induced increase in mitochondrial dysfunction in Hep3B cells. (A, B) After exposing the cells to cynaropicrin for 24 h, they were stained with MitoTracker and analyzed for mitochondrial mass per cell using flow cytometry. (C) Cells cultured for 24 h in medium with or without cynaropicrin were stained with MitoTracker, nuclei were further stained using DAPI, and fluorescence images were obtained via fluorescence microscopy. (D) Alix expression in cells treated with various concentrations of cynaropicrin for 24 h was examined through Western blot analysis. (B) Numerical data are presented as mean ± SD (n=3). *p<0.05, **p<0.01 and ***p<0.001 compared to untreated group.

2023; Chen *et al*., 2024a), with several previous reports having showed that the cytotoxicity of cynaropicrin in cancer cells was ROS-dependent (Liu *et al*., 2019; De Cicco *et al*., 2021; Rotondo *et al*., 2022). Therefore, we further evaluated whether ROS was involved in cynaropicrin-induced cytotoxicity in Hep3B cells. Notably, flow cytometry results using DCF-DA dye found that cynaropicrin greatly increased the production of ROS, which was also observed through fluorescence microscopy (Fig. 7A-7C). Moreover, blocking ROS generation using NAC, a ROS scavenger, completely maintained the cynaropicrin-induced morphological changes of cells at control levels (Fig. 7D) and considerably attenuated cynaropicrin-induced expression of ER stress marker proteins and ER stress (Fig. 7E, 7F). In addition, NAC pretreatment significantly abolished the cynaropicrin-induced decrease in Alix expression and increase in MitoTracker fluorescence intensity (Fig. 7E, 7F) while also maintaining the cynaropicrin-induced decrease in cell viability at near control levels (Fig. 7G). These results suggest that ROS acted as upstream regulators of cynaropicrin-triggered mitochondrial dysfunction and ER stress.

p38 MAPK was involved in cynaropicrin-induced ER stress and mitochondrial impairment in Hep3B cells

MAPKs pathway activation, together with ER stress, has been recognized as a major intracellular signaling cascade involved in the regulation of PCD, including paraptosis (Wang *et al*., 2021; Li *et al*., 2022; Wang *et al*., 2023). Therefore, we finally investigated whether MAPKs were involved in cynaropicrin-induced cytotoxicity in Hep3B cells. Notably, our results showed that p38 MAPK phosphorylation rapidly increased 2 h after cynaropicrin treatment and then decreased after 12 h. We also found that ERK phosphorylation increased slightly, although the effect was minimal. However, JNK phosphorylation was not induced (Fig. 8A). Therefore, we examined whether p38 MAPK activation was involved in cynaropicrin-induced cytotoxicity. Accordingly, we found that cynaropicrin-induced formation of cytoplasmic vacuolation and decrease in cell viability were weakened in cells pretreated with SB203580, a p38 MAPK inhibitor (Fig. 8B, 8C). SB203580 pretreatment also attenuated cynaropicrin-induced decrease in Alix expression and increase in the expression of ER stress marker proteins (Fig. 8D). Furthermore, SB203580 significantly suppressed the cynaropicrin-induced increase in the frequency of ER-Tracker- and MitoTracker-positive cells (Fig. 9C-9F) while also partially alleviating the increase in Ca^{2+} levels (Fig. 9A, 9B).

DISCUSSION

The pharmacological mechanism by which cynaropicrin induces apoptosis in various human cancer cells remains unclear yet. In the current study, it was observed that cynaropicrin-treated HCC Hep3B cells exhibited extensive cytoplasmic vacuoles around the nucleus, which was not mitigated by an autophagy blocker bafilomycin A1. This implies that cynaropicrin-induced cell death may be non-autophagic. Additionally, the cynaropicrin-induced cytotoxicity was not prevented by a pan-caspase inhibitor or a necrosis inhibitor, indicating that cynaropicrin-induced cytotoxicity in Hep3B cells was caspase-independent and non-apoptotic.

Preserving the functional homeostasis of the ER is essential for cell survival (Kumar and Maity, 2021; de Ridder *et al*., 2023). Within cells, Ca^{2+} acts as an essential messenger that playing a key role in regulating multiple forms of regulated cell

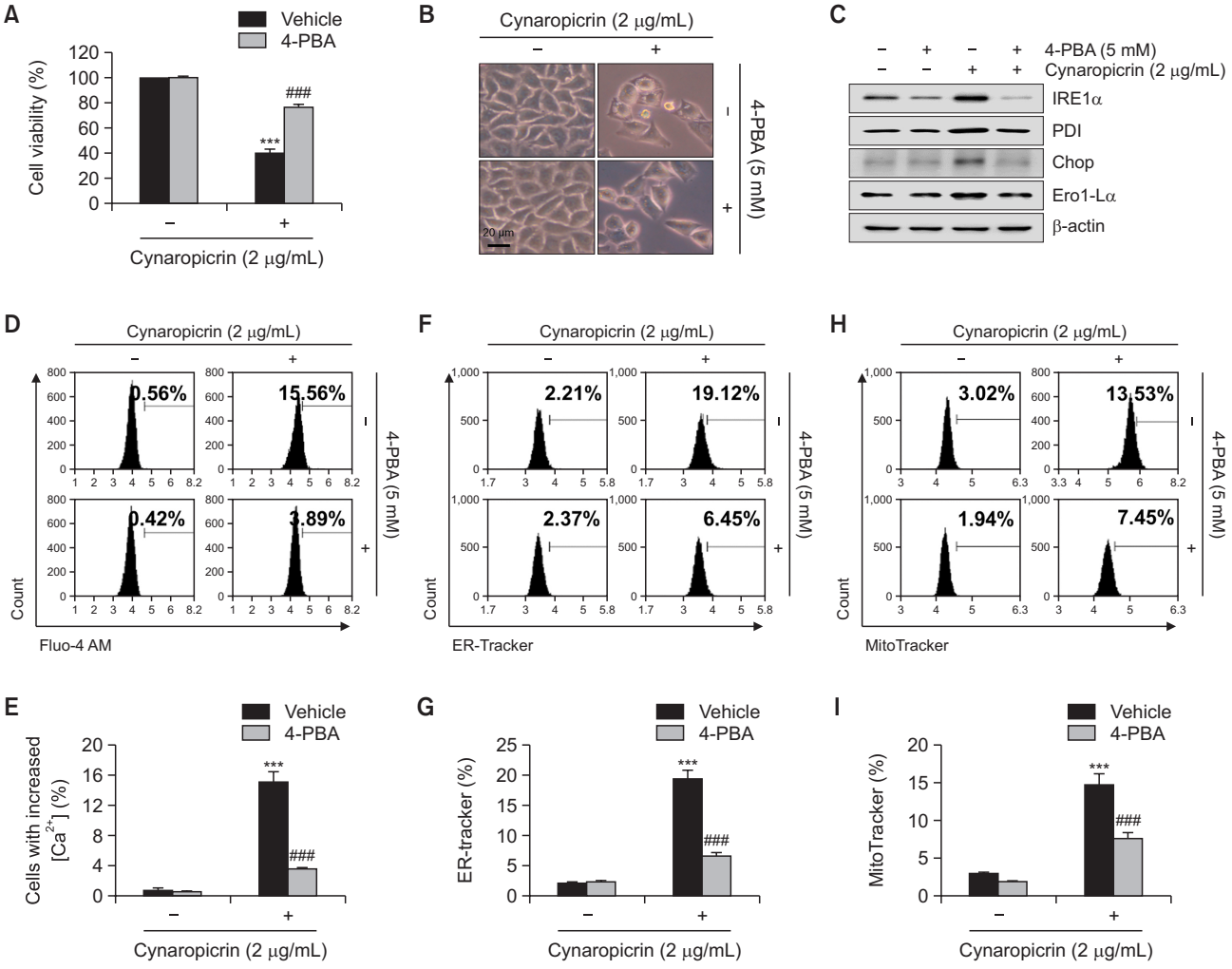

Fig. 5. Attenuation of cynaropicrin-induced mitochondrial dysfunction by an ER stress inhibitor in Hep3B cells. Cells were cultured for 1 h in the presence or absence of the ER stress inhibitor 4-PBA and then exposed to cynaropicrin for 24 h. (A-C) After treatment, cell viability and morphological changes were examined (A, B), and the expression changes in ER stress-related proteins were evaluated (C). (D-I) The effects of 4-PBA on the cynaropicrin-induced increase in intracellular Ca^{2+} concentration (D, E), ER stress (F, G), and mitochondrial dysfunction (H, I) were investigated through flow cytometry. (A, E, G, I) Numerical data are presented as mean ± SD (n=3). ***p<0.001 compared to un- treated group; ###p<0.001 compared to cynaropicrin-treated group.

death through (Kumar and Maity, 2021; Moon, 2023). Intracellular Ca^{2+} accumulation and Ca^{2+} influx from the ER to mitochondria have been associated with the initiation of several types of PCD (Michela *et al*., 2021; Dhaouadi *et al*., 2023). Paraptosis is an alternative cell death pathway characterized by cytoplasmic vacuolation and ER and/or mitochondrial swelling and is distinct from necrosis and apoptosis. Extensive vacuolation, a characteristic of paraptosis, is caused by the dilatation of the ER and mitochondria due to mitochondrial Ca^{2+} overload (Hanson *et al*., 2023; Xu *et al*., 2024). Recently, it was proposed that the inhibition of ER stress-dependent autophagy can enhance cynaropicrin-induced cell death (Yang *et al*., 2022). Previous studies implied that the anticancer activity of cynaropicrin was closely associated with the induction of ER stress. It was observed that cynaropicrin effectively increased the expression levels of ER stress regulatory proteins, such as IRE1α (one of the well-identified ER stress markers that serve as a quality regulator for protein synthesis within the ER), PDI (a molecular chaperone protein of the ER), Chop (a protein involved in inducing cell death due to ER stress), and Ero1-Lα (a representative upstream signaling molecule of ER stress) (Barez *et al*., 2020; Pavlović and Heindryckx, 2022; Ye *et al*., 2023). Flow cytometry and fluorescence microscopy also revealed that the induction of ER stress in cynaropicrin-treated cells was closely associated with the increase in Ca^{2+}. Furthermore, experiments with MitoTracker showed that cynaropicrin-treated cells exhibited mitochondrial fragmentation, suggesting that cynaropicrin can severely alter the ER and mitochondrial homeostasis of Hep3B cells. Moreover, Alix protein expression gradually decreased as cynaropicrin treatment concentrations increased, which is a typical characteristic of paraptosis (Sperandio *et al*., 2004; Wang *et al*., 2023). However, 4-PBA pretreatment markedly reversed cynaropicrin-induced Ca^{2+} accumulation, cytoplasmic vacuole formation, and changes in ER stress biomarkers and significantly blocked cynaropicrin-induced mitochondrial fragmenta-

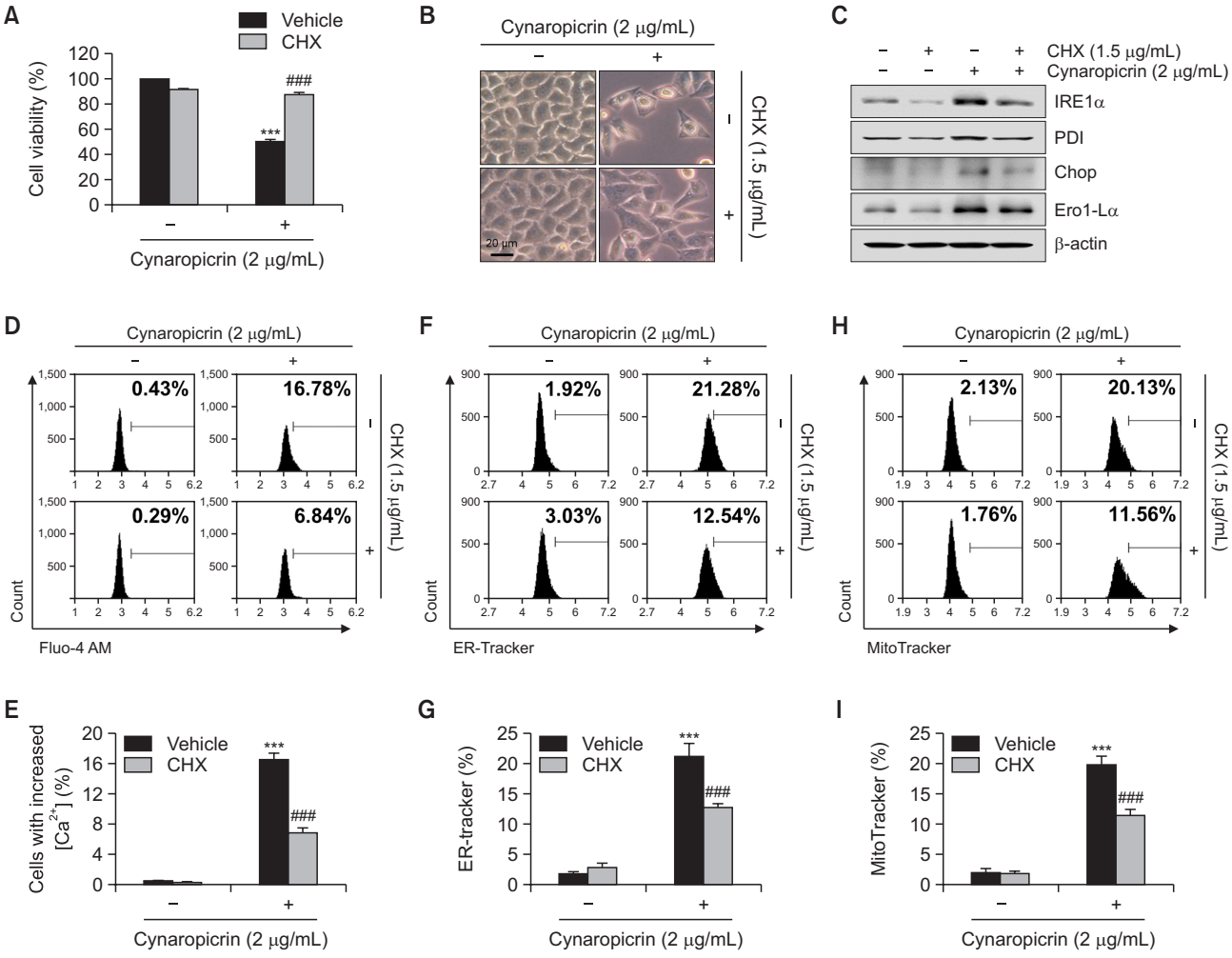

Fig. 6. Protection of cynaropicrin-induced ER stress and mitochondrial impairment by translation inhibition in Hep3B cells. After culturing cells for 1 h in the presence or absence of CHX, a protein synthesis inhibitor, they were treated with cynaropicrin for 24 h. (A-C) After treatment, cell viability and morphological changes were investigated (A, B), and the expression changes in ER stress-related proteins were examined (C). (D-I) The effects of CHX on cynaropicrin-induced increase in intracellular Ca^{2+} concentration (D and E), ER stress (F and G), and mitochondrial dysfunction (H, I) were investigated through flow cytometry. (A, E, G, I) Numerical data are presented as mean ± SD (n=3). ***p<0.001 compared to untreated group; ###p<0.001 compared to cynaropicrin-treated group.

tion while also attenuating cytotoxicity. These findings suggest that cynaropicrin-induced cytoplasmic vacuolation may occur through the accumulation of misfolded proteins and expansion of the ER lumen following ER stress. They could also suggest that ER stress may regulate cynaropicrin-induced mitochondrial dysfunction.

PCD, including paraptosis, do require the synthesis of new RNA or proteins while necroptosis does not (Saelens *et al*., 2005; Seo *et al*., 2021; Hanson *et al*., 2023). To see whether mitochondria and ER communicate with each other in the anticancer activity of cynaropicrin in Hep3B cells, CHX was used. In addition, CHX may attenuate the protein load in the ER lumen, consequently ameliorating ER stress and cell death (Szegezdi *et al*., 2006; Chevet *et al*., 2015). Notably, our results showed that CHX exposure alleviated the cynaropicrin-induced reduction in cellular activity and mitochondrial damage while reducing the size and number of cytoplasmic vacuoles and the expression levels of cynaropicrin-mediated ER stress signaling-related proteins. These results suggest that protein synthesis coordinates with cynaropicrin-induced ER stress and paraptosis. In particular, given that mitochondria serve as a major hub for intracellular Ca^{2+} signaling (Sterea and El Hiani, 2020; Dhaouadi *et al*., 2023), blocking of the cynaropicrin-induced Ca^{2+} increase using CHX and 4-PBA may have been involved in mitochondrial damage repair. Meanwhile, studies have shown that abnormal intracellular Ca^{2+} homeostasis could result in the ROS generation ultimately leading to cell death (Ji *et al*., 2022; Yang *et al*., 2023). Several mechanisms, including ER stress, Ca^{2+} influx into mitochondria, and alterations in redox homeostasis, are involved in the induction of paraptosis. As observed in other types of cancer cell lines (Liu *et al*., 2019; De Cicco *et al*., 2021; Rotondo *et al*., 2022), cynaropicrin significantly increased ROS production in Hep3B cells, whereas blocking ROS production through NAC pretreatment completely abolished the occurrence of cynaropicrin-induced vacuoles. Additionally, NAC pretreatment

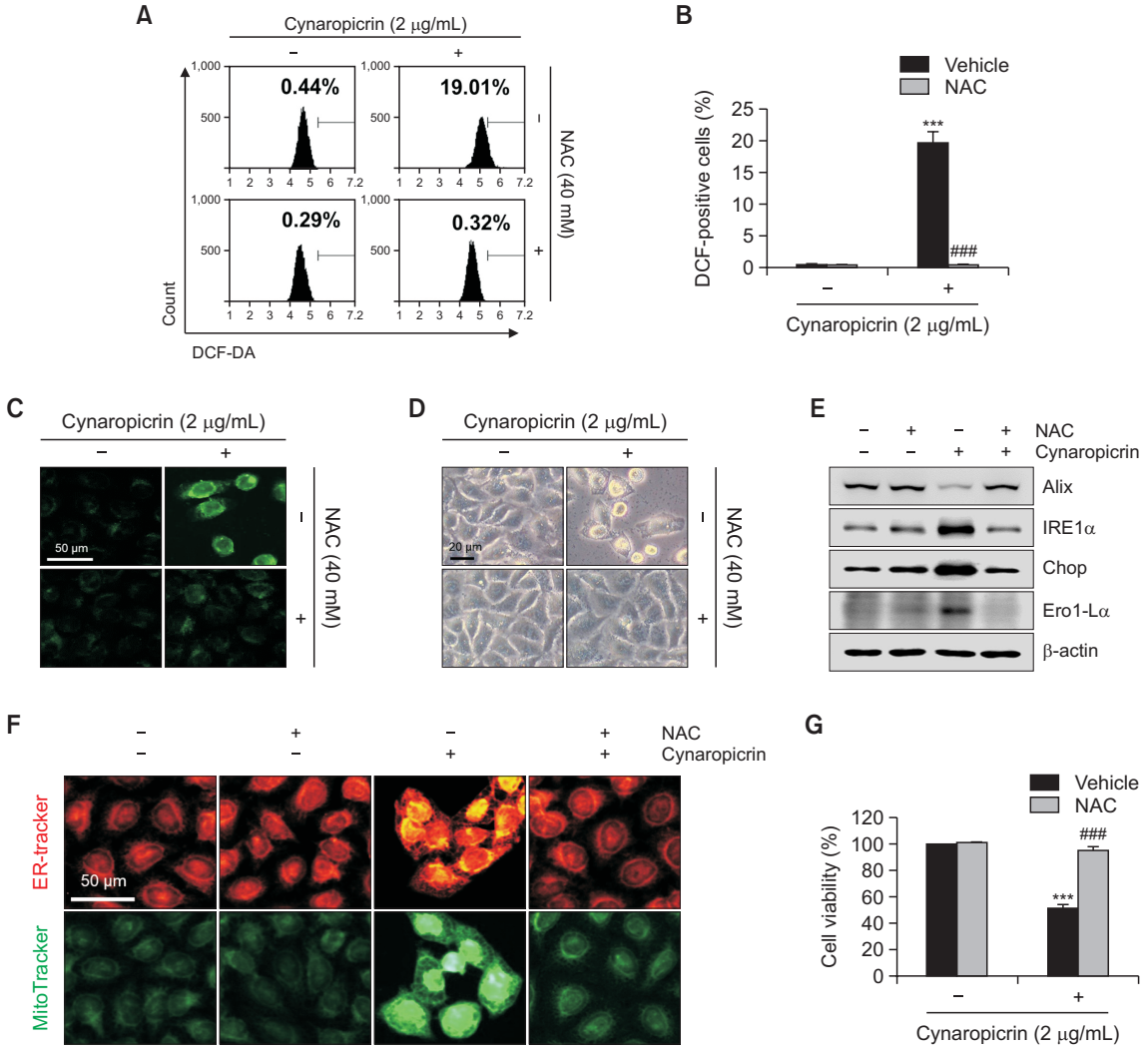

Fig. 7. Role of ROS in cynaropicrin-induced ER stress and mitochondrial impairment in Hep3B cells. Cells treated with or without NAC, a ROS generation blocker, for 1 h were treated with cynaropicrin for an additional 1 h (A-C) or 24 h (D-G). (A-C) After DCF-DA staining, ROS production was analyzed through flow cytometry (A, B) or observed under a fluorescence microscope (C). (D, E) Morphological changes in cells treated with cynaropicrin in the presence or absence of NAC were observed (D), and the expression changes in the indicated proteins were investigated using the corresponding antibodies (E). (F, G) Fluorescence intensities according to ER-Tracker and MitoTracker staining were evaluated under a fluorescence microscope (F), and cell viability was examined using the CCK-8 assay (G). (B, G) Numerical data are presented as mean ± SD (n=3). ***p<0.001 compared to untreated group; ###p<0.001 compared to cynaropicrin-treated group.

maintained the increase in the fluorescence intensities of ER-Tracker and MitoTracker in cynaropicrin-treated cells at control levels. NAC-mediated ROS scavenging also neutralized the cynaropicrin-induced downregulation of Alix expression and upregulation of ER stress regulatory proteins and significantly altered the decrease in cell viability, demonstrating that oxidative stress was closely implicated in the pro-paraptotic effects of cynaropicrin.

The MAPKs pathway is a common cascade involved in the induction of various PCDs, including paraptosis (Wang *et al*., 2021; Li *et al*., 2022; Wang *et al*., 2023), and may be ROS-dependent (Michela *et al*., 2021; Sang *et al*., 2021; Yang *et al*., 2023). We monitored the phosphorylation of three distinct MAPKs. With cynaropicrin treatment, the phosphorylation of p38 MAPK was significantly greater than that of ERK and JNK in Hep3B cells. To see the involvement of p38 MAPK pathway, the p38 MAPK inhibitor was administered before cynaropicrin treatment. SB203580 increased the expression of the paraptosis inhibitor Alix, which was downregulated by cynaropicrin to control levels while reversing the increased expression of ER stress marker proteins. Furthermore, the use of SB203580 protected the cynaropicrin-induced increase in cytoplasmic vacuolation and inhibition of cell viability to some extent, indicating that cynaropicrin-induced paraptosis in Hep3B cells was mediated by p38 MAPK. The involvement of MAPKs in inducing paraptosis varies depending on the type of phytochemical and cancer cell line (Sang *et al*., 2021; Yang *et al*., 2023). In Hep3B cells treated with cynaropicrin, recovery of cellular activity was relatively lower with p38 MAPK inhibitor than with an ROS scavenger. These findings indicate that cynaropicrin exerts its anticancer activity partially through the p38 MAPK-mediated ER stress–paraptosis axis. Our results also

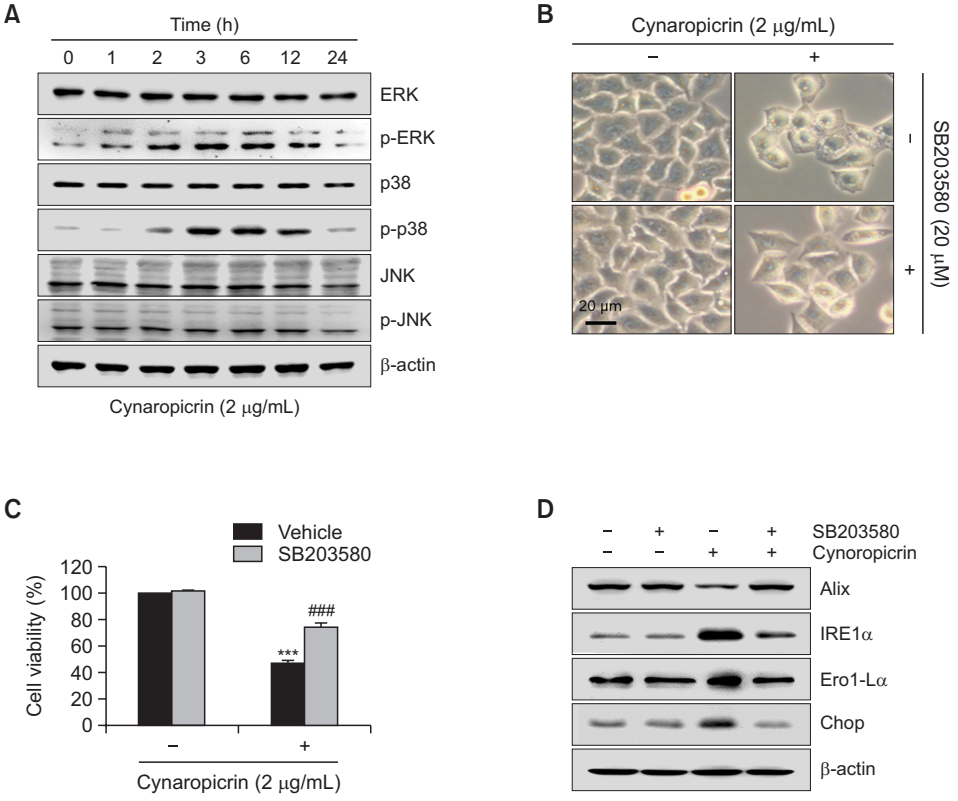

Fig. 8. Alleviation of cynaropicrin-induced cytotoxicity through a p38 MAPK inhibitor in Hep3B cells. (A) After treating cells with cynaropicrin for the indicated duration, changes in the expression of the three types of MAPKs were examined via Western blot analysis. (B-D) Cells were pretreated with the p38 MAPK inhibitor SB203580 for 1 h and then treated with cynaropicrin for 24 h. Thereafter, changes in morphology (B), cell viability (C), and expression of the indicated proteins (D) were examined. (C) Numerical data are presented as mean ± SD (n=3). ***p<0.001 compared to untreated group; ###p<0.001 compared to cynaropicrin-treated group.

highlight the involvement of oxidative stress in cynaropicrin-induced paraptosis, with ROS acting as upstream signaling factors in this process. Consequently, ROS-mediated p38 MAPK activation in Hep3B cells may serve as a potential approach for inducing cell death, implying the importance of oxidative stress in cynaropicrin-induced paraptosis.

Several natural compounds exhibit anticancer activity by causing paraptosis through Ca^{2+} homeostasis disorders. For example, the paraptotic death of HepG2 cells induced by hesperidin, a flavanone glycoside, was attributed to mitochondrial dysfunction and swelling caused by ROS-mediated Ca^{2+} influx from the ER into mitochondria (Yumnam *et al*., 2016), suggesting that ROS act as upstream regulators of Ca^{2+} influx into mitochondria. The role of MAPKs in relation to the Ca^{2+}/ROS axis in paraptotic cell death has to be revealed further. The roles of ion channels and their upstream regulators in cynaropicrin-induced Ca^{2+} influx from the ER to mitochondria need to be further elucidated too.

In conclusion, we showed that cynaropicrin induced paraptosis-like cell death, a non-canonical PCD, in Hep3B cells. Cynaropicrin increased the expression of ER stress marker proteins but decreased the expression of paraptosis inhibitor while promoting cytoplasmic vacuolization. Furthermore, we demonstrated that these changes were associated with Ca^{2+} overload, impaired mitochondrial function, and p38 MAPK activation and proved that ROS overproduction acts a pivotal upstream signal in the initiation of cynaropicrin-induced paraptosis (Fig. 10). Importantly, paraptosis represents a potential alternative target for cancer treatment, as it operates independently of apoptotic signaling, which is often associated with resistance (Kunst *et al*., 2024). Collectively, our findings underscore the importance of oxidative stress-dependent paraptosis induction through cynaropicrin as a novel anticancer strategy for HCC.

CONFLICT OF INTEREST

The authors have no conflicts of interest relevant to this study to disclose.

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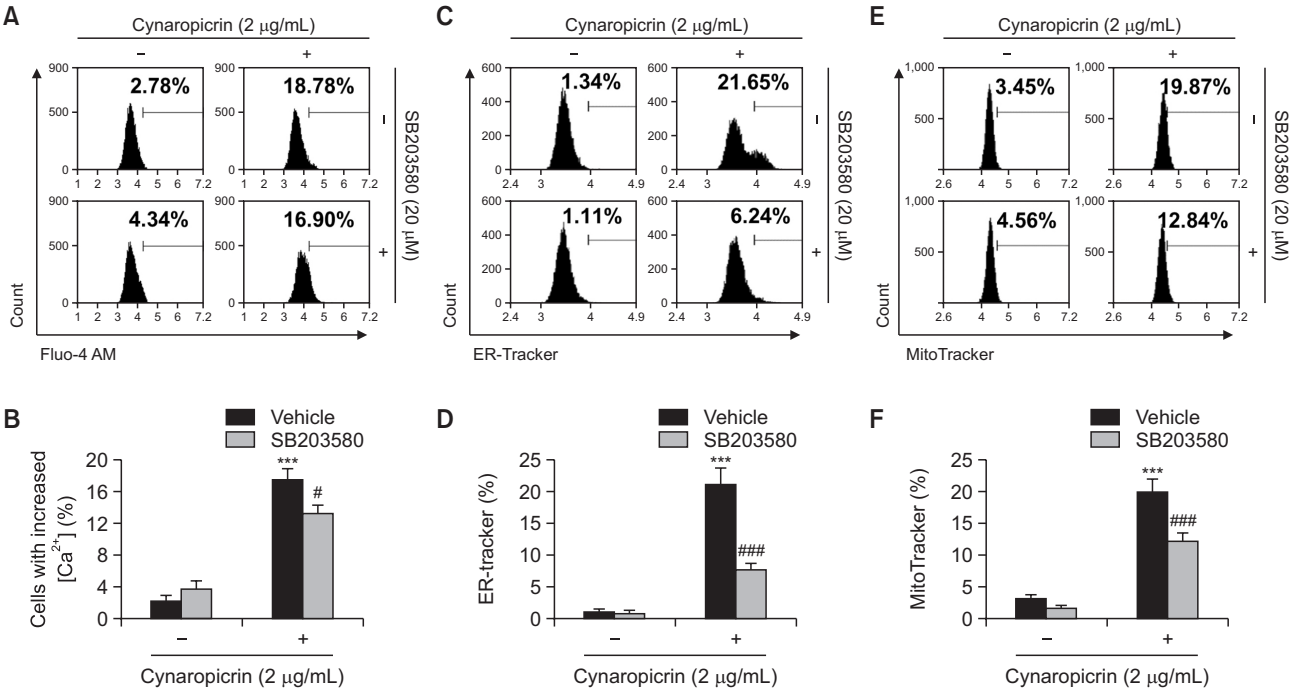

Fig. 9. Effects of the p38 MAPK inhibitor on cynaropicrin-induced ER stress and mitochondrial impairment in Hep3B cells. The effects of SB203580 on the cynaropicrin-induced increase in intracellular Ca^{2+} concentration (A, B), ER stress (C, D), and mitochondrial dysfunction (E, F) were evaluated via flow cytometry. (B, D, F) Numerical data are presented as mean ± SD (n=3). ***p<0.001 compared to untreated group; $^{\#}p$<0.05 and $^{\#\#\#}p$<0.001 compared to cynaropicrin-treated group.

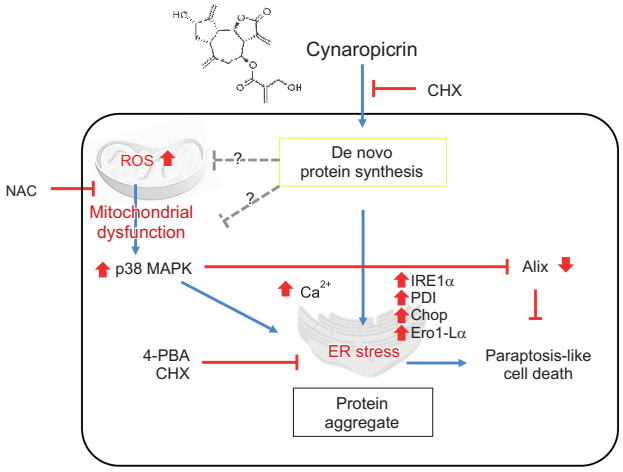

Fig. 10. A schematic diagram illustrating the mechanisms by which cynaropicrin induces cell death in Hep3B cells, including the promotion of mitochondrial ROS production, activation of the p38 MAPK pathway, inhibition of Alix protein, and enhancement of ER stress.

AUTHOR CONTRIBUTIONS

Min Yeong Kim: Writing of manuscript, design of the work, and analysis of data. Hee-Jae Cha: Conceptualization, Writing & Revision of manuscript, and Acquisition of data. Su Hyun Hong: Visualization and Data curation. Sung-Kwon Moon: Analysis of data. Taeg Kyu Kwon: Conceptualization. Young-Chae Chang: Investigation. Gi Young Kim: Visualization and Methodology. Jin Won Hyun: Data curation and Resources. A-Young Nam: Review & Editing of manuscript. Jung-Hyun Shim: Project administration, Supervision, and Review & Editing of manuscript. Yung Hyun Choi: Resources, and Review & Editing of manuscript.

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