Liver Research 9 (2025) 49–56

Contents lists available at ScienceDirect

Liver Research

journal homepage: http://www.keaipublishing.com/en/journals/liver-research

Original Article

Combination of brefeldin A and tunicamycin induces apoptosis in HepG2 cells through the endoplasmic reticulum stress-activated PERK-eIF2α-ATF4-CHOP signaling pathway

Minghong Li [a, b, 1], Mengyi Duan [a, 1], Ying Yang [a], Xingdao Li [a], Dan Li [a], Wenting Gao [a], Xiaotong Ji [a], Jianying Bai [a, c, d, *]

[a] Department of Environmental Health, School of Public Health, Shanxi Medical University, Taiyuan, Shanxi, China
[b] Department of Occupational Disease Control, Ansteel Group General Hospital, Anshan, Liaoning, China
[c] MOE Key Laboratory of Coal Environmental Pathogenicity and Prevention, Taiyuan, Shanxi, China
[d] Center for Ecological Public Health Security of Yellow River Basin, Shanxi Medical University, Taiyuan, Shanxi, China

ARTICLE INFO

Article history:
Received 3 April 2024
Received in revised form
9 June 2024
Accepted 15 January 2025

Keywords:
Brefeldin A (BFA)
Tunicamycin (TM)
Hepatocellular carcinoma (HCC)
Apoptosis
Endoplasmic reticulum stress (ERS)
Caspase-3

ABSTRACT

Background and aims: Hepatocellular carcinoma (HCC) is a malignant tumor with a high mortality rate, but there are still no effective treatments. The aim of this study was to investigate the anticancer potential of the combined use of brefeldin A (BFA) and tunicamycin (TM) in HepG2 cells, as well as the underlying mechanisms.

Methods: HepG2 cells were treated with different concentrations of BFA (0.1−2.5 mg/L) and TM (1−5 mg/L) for 24 h. DMSO (0.1 %, v/v) was used as a vehicle control. Cell viability and cell migration were measured using MTT assay and scratch wound assay, respectively. Apoptosis was detected using flow cytometry and acridine orange (AO) staining. The protein and mRNA levels of various factors involved in apoptosis (poly (ADP-ribose) polymerase-1 (PARP-1), caspase-12, caspase-3, and stearoyl-CoA desaturase 1) and endoplasmic reticulum (ER) stress (binding immunoglobulin protein (BiP), protein kinase R-like endoplasmic reticulum kinase (PERK), p-PERK, phosphorylation of eukaryotic translation initiation factor 2alpha (p-eIF2α), activating transcription factor (ATF) 4, and C/EBP homologous protein (CHOP)) were measured using Western blotting and qRT-PCR, respectively.

Results: Both BFA and TM alone significantly reduced the viability of HepG2 cells in a dose-dependent way. The co-incubation with TM (1 mg/L) further significantly reduced the viability of HepG2 cells treated with BFA (0.25 mg/L) alone ($P < 0.05$). BFA significantly increased the protein and mRNA levels of caspase-3 and PARP-1 ($P < 0.05$) compared to control and DMSO-treated cells, indicating that BFA induced apoptosis in HepG2 cells by increasing the expression of caspase-3 and PARP-1. The induction of apoptosis by BFA could be further significantly enhanced by co-incubation with TM. In addition, BFA significantly increased the mRNA levels of *BiP*, *PERK* and *ATF4* ($P < 0.05$) compared to control and DMSO-treated cells. After co-incubation of BFA and TM, the protein levels of BiP, p-PERK, p-eIF2α and CHOP were significantly increased, indicating that TM could enhance BFA-induced ER stress in HepG2 cells through the PERK-eIF2α-ATF4-CHOP pathway.

Conclusions: BFA could induce apoptosis and ER stress, and TM could enhance the ability of BFA to induce apoptosis and ER stress in HepG2 cells through the PERK-eIF2α-ATF4-CHOP pathway. The findings highlight the therapeutic potential of the combined use of BFA and TM in treating HCC.

1. Introduction

Liver cancer has been considered a global health challenge, ranking as the sixth most common cancer and the third leading cause of cancer death globally.[1] Hepatocellular carcinoma (HCC)

* Corresponding author. Department of Environmental Health, School of Public Health, Shanxi Medical University, Taiyuan, Shanxi, China.
E-mail address: jybai66@sxmu.edu.cn (Jianying Bai).
Peer review under the responsibility of Editorial Office of Liver Research.
[1] These authors contributed equally to this work and should be considered co-first authors.

https://doi.org/10.1016/j.livres.2025.01.004

is a major type of liver cancer and accounts for 75%–85% of cases in 2020.[1] The treatment options for HCC are highly dependent on the stages of HCC at diagnosis. Generally, HCC at early and intermediate stages is commonly treated with locoregional therapies, such as surgical resection, liver transplantation, local ablation with radiofrequency, and transarterial chemoembolization. However, as for HCC at advanced stages, it is often treated with palliative therapies (i.e, systemic therapies), such as single-agent (sorafenib and lenvatinib) targeted therapy and the combination of a checkpoint inhibitor plus targeted therapy (atezolizumab plus bevacizumab).[2] Nevertheless, the effects of palliative therapies are limited, and only a small portion of patients can obtain durable therapeutic benefit. Moreover, the prognosis of HCC patients is poor and characterized by frequent intrahepatic recurrence and extrahepatic metastasis.[3] According to cancer statistics (2015–2019) published by the National Disease Registration Service (NDRS) of the National Health Service (NHS) of England,[4] more than 45% of patients with stage I liver cancer will survive their cancer for 4 years or more after they are diagnosed, while the survival rates decreased to 5% for stage IV patients. Therefore, it is urgent to develop an effective treatment strategy for HCC.

Recent studies have shown that natural compounds have antiproliferative and pro-apoptotic activities against cancer cells, without apparent side effects on normal cells.[5] Brefeldin A (BFA) is a natural fungal metabolite produced by a marine fungus *Penicillium sp.* (HS-N-29), and it has proven to have robust anti-cancer activity against various types of cancer, such as leukemia, melanoma, prostate, breast, and colon cancer.[6–8] Moreover, it is also reported that BFA inhibits colorectal prostate cancer growth by triggering binding immunoglobulin protein/protein kinase B (BiP/Akt)-regulated autophagy.[9] However, the biological responses of BFA are complex and highly dependent on cell lines. In addition, the anti-cancer activity of BFA is independent of the tumor suppressor p53, making it a promising candidate for chemotherapeutic agents.[10,11] At present, the synthesis of BFA and its derivatives for cancer therapy has received considerable attention.[12,13] Tunicamycin (TM) is also a natural product derived from *Streptomyces*, and it exerts anti-tumor efficacy by inducing endoplasmic reticulum (ER) stress, triggering apoptosis and inhibiting tumor growth.[14,15] Thus, it is of great significance to develop natural product-based drugs in cancer treatment.

ER stress plays an important role in apoptosis. BiP is an indicator of ER stress, and the overexpression of BiP has been found in tumor samples of HCC patients compared to surrounding non-tumor tissues.[16] In addition, stearoyl-CoA desaturase 1 (SCD1) is over expressed in liver cancer patients and highly associated with poor prognosis.[17] The inhibition of SCD1 can induce ER stress and elicit anti-tumor effects.[18,19] Especially, SCD1 may be a new therapeutic target in the treatment of HCC.[17] Protein kinase R-like endoplasmic reticulum kinase (PERK) is a type I transmembrane protein in the ER, and autophosphorylated PERK (p-PERK) can phosphorylate the eukaryotic translation initiation factor 2alpha (eIF2α), leading to the up-regulation of activating transcription factor (ATF) 4 and C/EBP homologous protein (CHOP).[20,21] ER stress can activate the PERK-eIF2α-ATF4-CHOP signaling pathway leading to apoptosis. In addition, ER stress can induce the dissociation of cysteine-aspartic acid protease 12 (caspase-12) from the ER, resulting in the activation of BiP and caspase-3 to induce apoptosis.[22]

In this study, we hypothesized that the combination of BFA and TM had great potential in the treatment of liver cancer. We investigated whether BFA could induce apoptosis in HepG2 cells through the ER stress signaling pathway. We further examined the synergistic effect of TM on the ability of BFA to induce apoptosis by mediating ER stress.

2. Materials and methods

2.1. Cells and materials

The human hepatocellular carcinoma cell line (HepG2) was purchased from the Stem Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HepG2 cells were cultured in high-glucose Dulbecco's minimal essential medium (DMEM, Cytiva, Hangzhou, China) supplemented with 10% fetal bovine serum (SORFA, Huzhou, China), 100 units/mL penicillin (Solarbio, Beijing, China), 100 μg/mL streptomycin (Solarbio, Beijing, China), and 2 mmol/L glutamine (Cytiva, Hangzhou, China) at 37 °C under 5% CO_2. At 80% confluence, cells were detached using 0.25% trypsin-EDTA (Solarbio, Beijing, China) and seeded in 96-well plates (6×10^5 cells/mL) for toxicity tests.

BFA (purity >98%) and TM (purity >98%) were purchased from Beyotime Biotechnology (Shanghai, China) and Shanghai Macklin Biochemical (Shanghai, China), respectively. The stock solutions of BFA (1 g/L) and TM (2 g/L) in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) were separately prepared and stored at 4 °C. Prior to use, they were diluted in DMEM at different concentrations.

2.2. Cell viability, morphological observation and cell migration

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Wuhan Boster Biological Technology, Wuhan, China). Briefly, cells were seeded in 96-well plates (6×10^5 cells/mL) and cultivated for 24 h. Then, cells were treated with different concentrations of BFA (0.1, 0.25, 0.5, 1.0, and 2.5 mg/L) and TM (1, 2, 3, 4, and 5 mg/L) for 24 h. DMSO (0.1%, v/v) was used as a vehicle control. To observe the combined effect of BFA and TM, 0.25 mg/L was selected as the concentration for BFA based on the present results and relevant research,[8,23] at which BFA had been shown to significantly inhibit the viability of HepG2 cells, and 1 mg/L was selected for TM because this dose has been clinically used in cancer treatment.[24] Cells were treated with the combination of BFA (0.25 mg/L) and TM (1 mg/L) for 24 h. After treatment, cells were washed with phosphate-buffered saline (PBS, Solarbio, Beijing, China) once and incubated in fresh DMEM containing 0.5 mg/mL MTT for another 4 h. After incubation, the media were removed and formazan was dissolved with 100 μL DMSO at 37 °C for 10 min. The absorbance was measured at 570 nm on a microplate reader (BioTek, Winooski, VT, USA). The changes in cell morphology were observed using a BX51 inverted microscope (Olympus, Japan). Cell migration was measured using scratch wound assay on 6-cell plates. Briefly, cells were seeded in 6-well plates (6×10^5 cells/mL) and cultivated overnight. The wounds were made using sterile 20 μL pipette tips. After washing, cells were treated with BFA (0.25 mg/L) and TM (1 mg/L), either individually or in combination, for 24 h. The plates were photographed at 0 h and 24 h using the BX51 inverted microscope, and the areas (S) of the wounds were quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA, https://imagej.net/ij/index.html). Wound closures (%) were calculated as $[1-(S_{24\ h}/S_{0\ h})] \times 100\%$.

2.3. Flow cytometry and acridine orange staining

The percentage of apoptotic cells was determined by flow cytometry using an annexin V-FITC/propidium iodide (PI) detection kit (Dalian Meilun Bio, China) according to the manufacturer's protocol. Briefly, HepG2 cells were treated with BFA and TM, either individually or in combination, for 24 h. After treatment, cells were digested with 0.25% trypsin for 10 min and collected by centrifugation at 800 *g* for 5 min. After washing twice with PBS, cells were

re-suspended in 1 × binding buffer at a density of 1 × 10^6 cells/mL. After adding annexin V-FITC and PI, cells were incubated at room temperature for 15 min in the dark. Cells were analyzed using a BD AccuriTM C6 flow cytometer (BD Bioscience, CA, USA). In addition, apoptotic bodies in cells were detected using acridine orange (AO) staining (CA1142, Solarbio, China). After treatment, cells were washed with PBS, digested with 0.25% trypsin, stained with AO, and observed under a BX51 inverted fluorescence microscope.

2.4. Western blot assay

Cells were seeded (6 × 10^6 cells/mL) in 6-well plates and cultivated for 24 h. Then, the cells were treated with BFA and TM, either individually or in combination. After treatment, cells were washed with PBS three times and lysed with loading buffer containing 50 mmol/L Tris-HCl (pH 6.8), 5% glycerol, 5% β-mercaptoethanol, 5% sodium dodecyl sulfate (SDS, Shanxi MiniBio Technology, Shanxi, China), and 0.25% bromophenol blue. Protein levels were determined using a bicinchoninic acid (BCA) assay kit (Boster Biological Techonology, Wuhan, China). The proteins were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P transfer membrane, Merck KGaA, Darmstadt, Germany). The membranes were blocked with 5% skim milk in PBS with 0.05% Tween-20 (G-CLONE, Beijing, China) (PBST) for 1 h at room temperature and incubated with primary antibodies (Table 1) overnight at 4 °C. The membranes were washed with PBST three times (10 min per wash), followed by incubation in horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 1) for 1 h at room temperature. The membranes were washed with PBST three times (10 min per wash), and the proteins were visualized using an enhanced chemiluminescence (ECL) detection kit (ThermoFisher, NJ, USA) and quantified using ImageJ.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

HepG2 cells were treated with BFA and/or TM for 24 h. After treatment, the cells were harvested and total RNA was extracted using RNAiso Plus reagent (TaKaRa, Japan). The purity and concentration of RNA were determined using a NanoDrop TM 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The cDNA was synthesized using a PrimeScript RT Master Mix kit (TaKaRa, Japan), and qRT-PCR was performed using a SYBR Premix Ex Taq™ II kit (TaKaRa, Japan) on a LineGene 9600 Plus real-time PCR detection system (Hangzhou Bioer Technology, China). The fluorescence emission data were acquired for 40 cycles at an annealing temperature of approximately 60 °C. The primer sequences used for PCR are listed in Table 2. The relative mRNA expression levels were normalized to the level of the internal reference gene (*β-actin*) using the $2^{-\Delta\Delta Ct}$ method.

2.6. Statistical analysis

Data are presented as means ± standard deviation (SD). Statistical analyses were performed by using GraphPad Prism 9.0 (GraphPad Software, CA, USA). Data were analyzed by one-way analysis of variance (ANOVA), with differences between groups evaluated by Tukey's post hoc tests. $P < 0.05$ was considered as significant.

3. Results

3.1. Combination of BFA and TM reduces cell survival in HepG2 cells

HepG2 cells treated with 0.1% DMSO showed no significant changes in cell viability compared to untreated cells (control). In contrast, BFA and TM significantly decreased the viability of HepG2 cells in a dose-dependent manner (Fig. 1A and B). Interestingly, the combination of BFA (0.25 mg/L) and TM (1 mg/L) significantly decreased the viability of HepG2 cells compared to BFA alone (Fig. 1C), indicating that TM synergistically enhanced the cytotoxicity of BFA to HepG2 cells. Therefore, 0.25 mg/L BFA and 1 mg/L TM were selected for subsequent experiments. In addition, HepG2 cells in the control and DMSO groups showed normal cell morphology and were evenly distributed, however, the cells treated with BFA and TM showed disrupted morphology and were sparsely distributed (Supplemental Fig. 1). Moreover, we performed the scratch wound assay and found that BFA could remarkably inhibit the migration of HepG2 cells, which could be enhanced by TM to a certain extent (Supplemental Fig. 2).

3.2. Combination of BFA and TM inhibits the proliferation capability of HepG2 cells

HepG2 cells in the control and DMSO groups showed similar protein levels of proliferating cell nuclear antigen (PCNA). Compared to the control/DMSO group, BFA or TM alone significantly decreased the protein levels of PCNA. Moreover, the

Table 1
Antibodies used for Western blots.

Antibody	Manufacturer	Cat. No.	Dilution
Mouse anti-human β-actin	Protein Tech, China	66009-1-Ig	1:2000
Rabbit anti-human ATF4	Protein Tech, China	10835-1-AP	1:1000
Rabbit anti-human BiP/GRP78	Protein Tech, China	66574-1-Ig	1:1000
Rabbit anti-human caspase-3	Protein Tech, China	19677-1-AP	1:500
Rabbit anti-human caspase-12	Protein Tech, China	55238-1-AP	1:500
Rabbit anti-human CHOP	Protein Tech, China	15204-1-AP	1:500
Rabbit anti-human eIF2α	Protein Tech, China	11170-1-AP	1:1000
Rabbit anti-human PARP-1	Protein Tech, China	66520-1-IG	1:1000
Mouse anti-human PCNA	Wuhan Boster Bio, China	BM0104	1:1000
Rabbit anti-human PERK	Protein Tech, China	24390-1-AP	1:1000
Rabbit anti-human Phospho-PERK	Beyotine, China	AF-5902	1:1000
Rabbit anti-human Phospho-eIF2α	CST, USA	3398T	1:1000
Rabbit anti-human SCD1	Protein Tech, China	28678-1-AP	1:1000
Goat anti-mouse IgG	Protein Tech, China	SA0000-1-1	1:5000
Goat anti-rabbit IgG	Protein Tech, China	SA0000-1-2	1:5000

Abbreviations: ATF4, activating transcription factor 4; BiP, binding immunoglobulin protein; CHOP, C/EBP homologous protein; eIF2α, eukaryotic translation initiation factor 2α; PARP-1, poly (ADP-ribose) polymerase-1; PCNA, proliferating cell nuclear antigen; PERK, protein kinase R-like endoplasmic reticulum kinase; SCD1, stearoyl-CoA desaturase 1.

Table 2
Primers of the genes used for qRT-PCR.

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
ATF4	TTCCGAGATTCCATCCTACG	AGGCTCACAAACGAATGGAC
β-actin	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA
BiP	TTCCCAGCCCCTCAGATAC	CAAAGAGAAGCACCAAGGAGAC
Caspase-12	CACCAGTCCTCAGACAGCACATTC	AGACTCTGGCAGTTACGGTTGTTG
Caspase-3	CTCGGTCTGGTACAGATG	GGTTAACCCGGGTAAGAATGTGCA
CHOP	GCGATATGCTGTGGTGCTTA	AGCTCCAAGTGAAACCAGGA
eIF2α	CCTGGCAAGAATGCAGTCAC	GGCGAAACCAATGTATTTCTGGA
PARP-1	AGGCTTGAAAAGCCCTAAAGG	CTGCTTGTTGAAGATGAGTAGC
PERK	ACGATGAGACAGAGTTGCGAC	ATCCAAGGCAGCAATTCTCCC
SCD1	GCGATATGCTGTGGTGCTTA	AGCTCCAAGTGAAACCAGGA

Abbreviations: ATF4, activating transcription factor 4; BIP, binding immunoglobulin protein; CHOP, C/EBP homologous protein; eIF2α, eukaryotic translation initiation factor 2α; PARP-1, poly (ADP-ribose) polymerase-1; PERK, protein kinase R-like endoplasmic reticulum kinase; qRT-PCR, quantitative real-time polymerase chain reaction; SCD1, stearoyl-CoA desaturase 1.

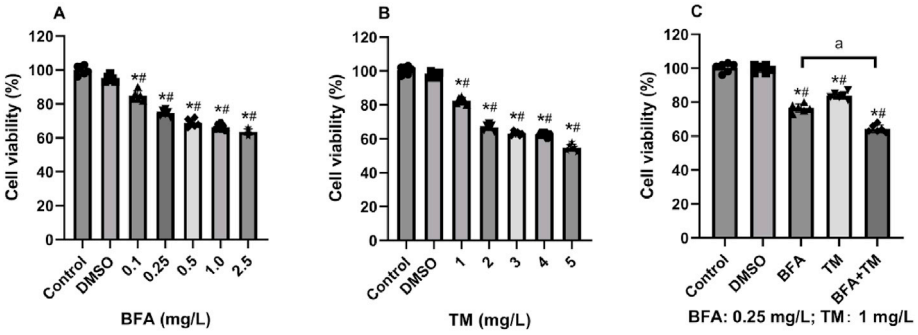

Fig. 1. Effects of BFA and TM on the viability of HepG2 cells at 24 h. (A) BFA alone, **(B)** TM alone, and **(C)** the combination of BFA and TM. Data are presented as means ± SD from six independent experiments performed in triplicate. *$P < 0.05$ *vs.* control group; #$P < 0.05$ *vs.* DMSO (0.1% v/v, vehicle control) group; a$P < 0.05$ *vs.* BFA (0.25 mg/L) group. Abbreviations: BFA, brefeldin A; DMSO, dimethyl sulfoxide; SD, standard deviation; TM, tunicamycin.

combination of BFA and TM decreased the protein level of PCNA to a greater extent compared to BFA alone, though no significant difference was found (Fig. 2).

3.3. Combination of BFA and TM induces early apoptosis of HepG2 cells

We observed the presence of apoptotic bodies in HepG2 cells treated with BFA and TM using AO staining (Supplemental Fig. 3). To further observe whether TM could enhance the ability of BFA to induce apoptosis, we measured the percentage of apoptotic cells using flow cytometry. As shown in Fig. 3, DMSO and TM did not induce apoptosis in HepG2 cells, but BFA significantly increased the percentages of early apoptotic cells and total apoptotic cells. Furthermore, the addition of TM significantly increased the percentage of early apoptotic cells induced by BFA, but it did not affect the percentages of late and total apoptotic cells. The results suggested that TM could enhance early apoptosis of HepG2 cells induced by BFA.

3.4. Combination of BFA and TM affects apoptosis-related factors in HepG2 cells

To examine whether TM could affect the expression of apoptosis-related genes induced by BFA, we measured the mRNA levels of several key genes (PARP-1, caspase-12, caspase-3, and SCD1) in HepG2 cells using qRT-PCR. Compared to the control and DMSO groups, BFA alone significantly increased the mRNA levels of PARP-1, caspase-12, caspase-3, and SCD1 in HepG2 cells (Fig. 4A–D). Though TM alone did not affect the mRNA levels of these genes, it could significantly increase the mRNA levels in combination with BFA compared to BFA alone (Fig. 4A–D).

Next, we measured the levels of these four proteins (PARP-1, caspase-12, caspase-3, and SCD1) using Western blots. Compared to the control and DMSO groups, BFA alone significantly increased the protein levels of cleaved PARP-1 and caspase-12 and decreased the expression of SCD1 (Fig. 4E, F, and H), and it also induced the activation and cleavage of caspase-3 (Fig. 4G). Compared to BFA alone, the addition of TM significantly decreased the protein level of cleaved PARP-1 (Fig. 4E), but only slightly increased the protein levels of caspase-12, cleaved caspase-3, and SCD1 (Fig. 4F–H), without a significant difference between them. TM alone did not affect the protein levels of cleaved PARP-1 and cleaved caspase-3 (Fig. 4E and G), but it significantly increased the protein levels of caspase-12 and decreased the expression of SCD1 (Fig. 4F and H).

3.5. Combination of BFA and TM induces ER stress in HepG2 cells

To understand whether apoptosis induced by BFA and TM was associated with ER stress, we studied the changes in the expression of several key proteins involved in the PERK-ATF4-CHOP signaling pathway. As shown in Fig. 5, the levels of BiP, PERK, ATF4, and CHOP proteins significantly increased ($P < 0.05$) in the BFA and BFA + TM groups, compared to those in the control and DMSO groups. Similar results were obtained when measuring the mRNA levels of the corresponding genes (*BiP, PERK, ATF4,* and *CHOP*). As shown in Fig. 6, the combination of BFA and TM significantly increased the mRNA levels of *PERK, eIF2α, ATF4,* and *CHOP* ($P < 0.05$) compared to those of BFA alone. The results suggested that BFA could activate the PERK-ATF4-CHOP signaling pathway, and TM synergistically

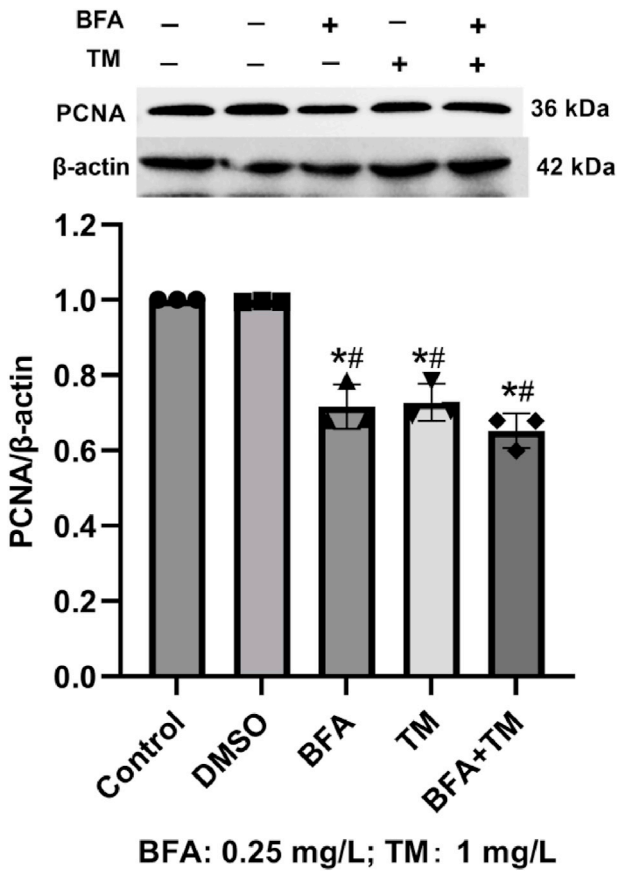

Fig. 2. Effects of BFA and TM on the expression of PCNA in HepG2 cells. HepG2 cells were treated with BFA (0.25 mg/L) and TM (1 mg/L), either individually or in combination, for 24 h. The protein levels of PCNA were determined using Western blot assay. Data are presented as means ± SD from three independent experiments performed in triplicate. *$P < 0.05$ *vs.* control group; #$P < 0.05$ *vs.* DMSO (0.1% v/v, vehicle control) group. Abbreviations: BFA, brefeldin A; DMSO, dimethyl sulfoxide; PCNA, proliferating cell nuclear antigen; SD, standard deviation; TM, tunicamycin.

enhanced this process by increasing both the gene and protein expression.

4. Discussion

This study investigated the inhibitive effects of BFA and TM, either alone or in combination, on the growth and proliferation of human liver cancer cells (HepG2). The results showed that BFA and TM alone could reduce the viability of HepG2 cells in a concentration-dependent manner, indicating that BFA and TM could suppress the proliferation of HepG2 cells. Compared with individual BFA or TM treatment, the combination of BFA and TM significantly reduced cell survival, suggesting that TM could synergistically enhance the inhibitive effects of BFA on the proliferation of HepG2 cells. We also provided molecular evidence using PCNA to confirm that BFA and TM inhibited the proliferation capability of HepG2 cells. Synthesized and expressed in the nucleus, PCNA is an essential protein that participates in a variety of processes of DNA metabolism, including DNA replication and repair, and chromatin organization. PCNA has been commonly used as a biomarker for prognostic prediction in HCC patients due to its increased expression.[25] Here, our results showed that BFA and TM, either alone or in combination, could effectively reduce the expression levels of PCNA in HepG2 cells, indicating that BFA and TM inhibited the proliferation of HepG2 cells.

Apoptosis plays a key role in maintaining homeostasis in multicellular organisms, especially under pathophysiological stresses such as developing progressive tumors. Our results demonstrated that BFA alone significantly increased the mRNA and protein levels of PARP-1, caspase-12, and caspase-3 in HepG2 cells, and the combination of BFA and TM could increase their levels to a greater extent, indicating that TM as an ER stress inducer could enhance the ability of BFA to induce apoptosis. This synergistic effect could be explained as follows: BFA blocked the transport of proteins from the ER to the Golgi apparatus,[26,27] and TM caused cell cycle arrest at the G1 and G2/M phases in HCC cells, leading to apoptosis.[28] Since current data only proved that BFA mainly induced the early stage of apoptosis in HepG2 cells, more dedicated and controlled investigations should be conducted to shed light on this possibility in tumor treatment.

Our study also demonstrated that BFA/TM-induced apoptosis was associated with excessive ER stress. The ER is involved in protein synthesis, processing, folding, and secretion. Many environmental factors can disrupt ER homeostasis and induce ER stress, leading to the activation of the unfolded protein response (UPR), which involves three different signaling pathways (inositol-requiring enzyme 1 (IRE1), PERK, and ATF6).[29] These pathways inhibit protein translation, stimulate protein degradation, and produce chaperone proteins, leading to the restoration of ER function or cell death. As a protein transport inhibitor, BFA can induce Golgi dispersal and prevent the transport of proteins from the ER to the Golgi. Our results showed that the combination of BFA and TM could significantly increase the protein and mRNA levels of several key factors (BiP, PERK, ATF4, and CHOP) in HepG2 cells compared to BFA alone, especially the levels of p-PERK and p-eIF2α.

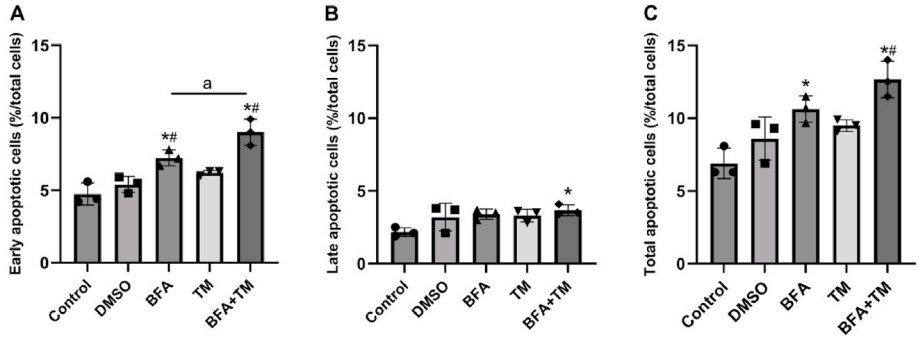

Fig. 3. Effects of BFA and TM on the percentage of apoptotic cells in HepG2 cells. (A) Early apoptotic cells, **(B)** late apoptotic cells, and **(C)** total apoptotic cells. The percentage of apoptotic cells in HepG2 cells was determined using flow cytometry. Data are presented as means ± SD from three independent experiments performed in triplicate. *$P < 0.05$ *vs.* control group; #$P < 0.05$ *vs.* DMSO (0.1% v/v, vehicle control) group; [a]$P < 0.05$ *vs.* BFA (0.25 mg/L) group. Abbreviations: BFA, brefeldin A; DMSO, dimethyl sulfoxide; SD, standard deviation; TM, tunicamycin.

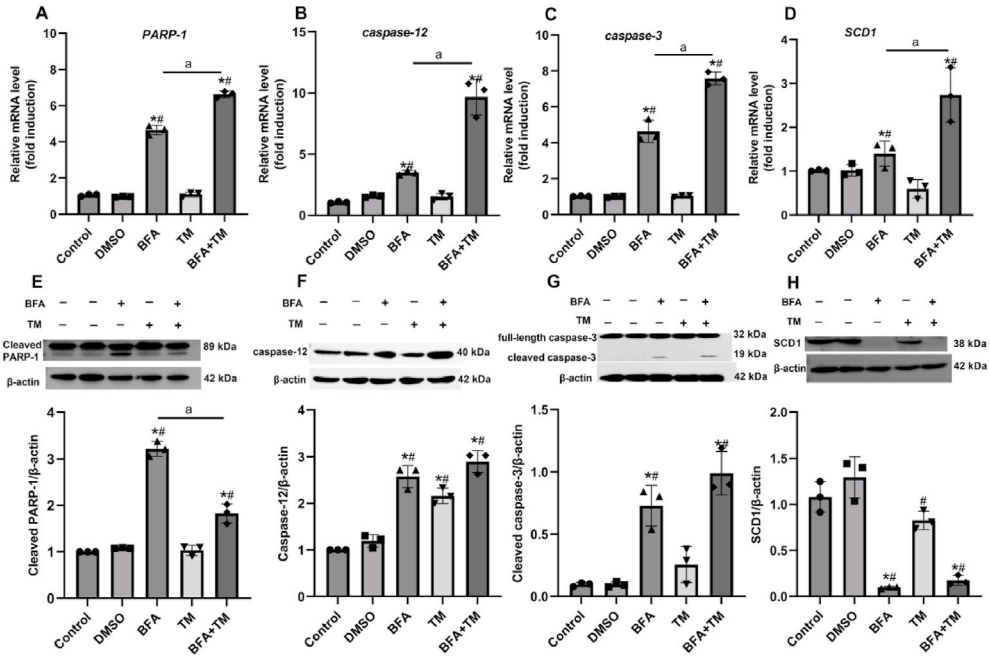

Fig. 4. Effects of BFA and TM on the mRNA and protein levels of apoptosis-related factors (PARP-1, caspase-12, caspase-3, and SCD1). HepG2 cells were treated with BFA (0.25 mg/L) and TM (1 mg/L), either individually or in combination, for 24 h. The relative mRNA levels of **(A)** *PARP-1*, **(B)** *caspase-12*, **(C)** *caspase-3*, and **(D)** *SCD1* were determined by qRT-PCR. The protein levels of **(E)** cleaved PARP-1, **(F)** caspase-12, **(G)** full-length and cleaved caspase-3, and **(H)** SCD1 were determined by Western blotting. Data are presented as means ± SD from three independent experiments performed in triplicate. $^{*}P < 0.05$ *vs.* control group; $^{\#}P < 0.05$ *vs.* DMSO (0.1% v/v, vehicle control) group; $^{a}P < 0.05$ *vs.* BFA (0.25 mg/L) group. Abbreviations: BFA, brefeldin A; DMSO, dimethyl sulfoxide; PARP-1, poly (ADP-ribose) polymerase-1; qRT-PCR, quantitative real-time polymerase chain reaction; SCD1, stearoyl-CoA desaturase 1; SD, standard deviation; TM, tunicamycin.

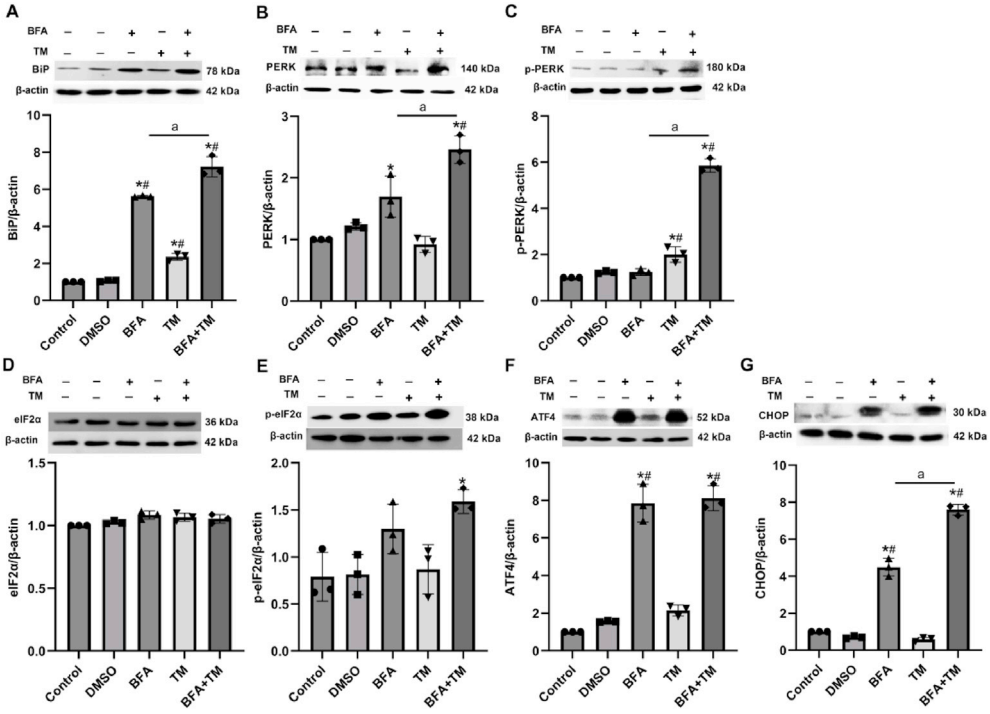

Fig. 5. Effects of BFA and TM on the levels of ER stress-related proteins (BiP, PERK, eIF2α, ATF4, and CHOP). HepG2 cells were treated with BFA (0.25 mg/L) and TM (1 mg/L), either individually or in combination, for 24 h. The protein levels of **(A)** BiP, **(B)** PERK, **(C)** phosphorylated PERK (p-PERK), **(D)** eIF2α, **(E)** phosphorylated eIF2α (p-eIF2α), **(F)** ATF4, and **(G)** CHOP were determined by Western blotting. Data are presented as means ± SD from three independent experiments performed in triplicate. $^{*}P < 0.05$, compared to the control group; $^{\#}P < 0.05$, compared to the DMSO (0.1% v/v, vehicle control) group; $^{a}P < 0.05$, compared to the BFA (0.25 mg/L) group. Abbreviations: ATF4, activating transcription factor 4; BFA, brefeldin A; BiP, binding immunoglobulin protein; CHOP, C/EBP homologous protein; DMSO, dimethyl sulfoxide; eIF2α, eukaryotic translation initiation factor 2α; PERK, protein kinase R-like endoplasmic reticulum kinase; SD, standard deviation; TM, tunicamycin.

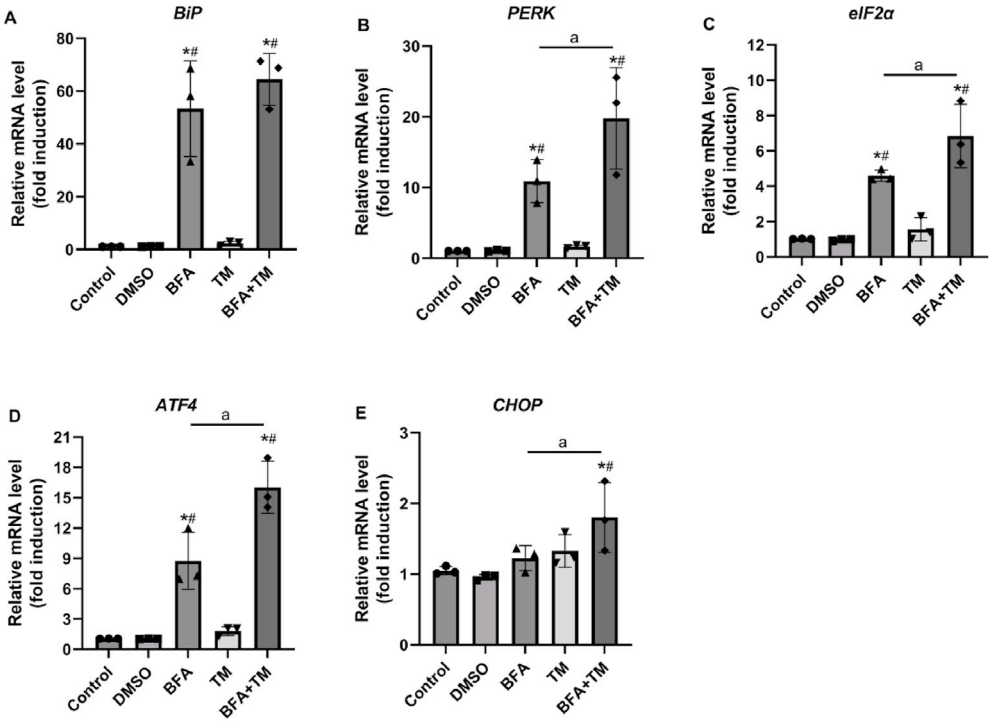

Fig. 6. Effects of BFA and TM on the mRNA levels of ER stress-related genes (*BiP, PERK, eIF2α, ATF4,* and *CHOP*). HepG2 cells were treated with BFA (0.25 mg/L) and TM (1 mg/L), either individually or in combination, for 24 h. The relative mRNA levels of **(A)** *BiP*, **(B)** *PERK*, **(C)** *eIF2α*, **(D)** *ATF4*, and **(E)** *CHOP* were determined by qRT-PCR. Data are presented as means ± SD from three independent experiments performed in triplicate. *$P < 0.05$, compared to the control group; $^{\#}P < 0.05$, compared to the DMSO (0.1% v/v, vehicle control) group; $^{a}P < 0.05$, compared to the BFA (0.25 mg/L) group. Abbreviations: ATF4, activating transcription factor 4; BFA, brefeldin A; BiP, binding immunoglobulin protein; CHOP, C/EBP homologous protein; DMSO, dimethyl sulfoxide; eIF2α, eukaryotic translation initiation factor 2α; PERK, protein kinase R-like endoplasmic reticulum kinase; SD, standard deviation; TM, tunicamycin.

Lebeaupin *et al.*[30] reported that the increase in CHOP expression in the liver of mice treated with LPS and tunicamycin is associated with the activation of both the PERK pathway and the IRE1α/spliced X box-binding protein 1 (sXBP-1)-dependent pathway. In addition, BFA and TM induce the activation of ATF4 at both transcriptional and translational levels in lung cancer cells, but they do not affect other ER stress molecules.[31] Therefore, our results suggested that BFA could activate the PERK-ATF4-CHOP signaling pathway in HepG2 cells, and TM could enhance BFA-induced ER stress.

As one of macrolide antibiotics, BFA has remarked inhibitory effects on various cancers, such as prostate cancer, breast cancer, colon cancer, and liver cancer.[6,8,10,11,32] Zhang *et al.*[33] found that nanomicelle-encapsulated BFA (M-BFA) induces caspase-dependent apoptosis in HepG2 cells through caspase-3 activation and PARP cleavage, which is similar to our results, suggesting that BFA had a potential therapeutic effect on liver cancer. We further demonstrated that BFA could induce apoptosis in HepG2 cells through the ER stress-mediated PERK-ATF4-CHOP pathway. In addition, TM is an ER-stress inducer and can activate IRE1-related ER stress signaling pathways.[34] In this study, we found that TM significantly increased the expression of BiP but did not induce the cleavage and activation of caspase-3 in HepG2 cells, and it only slightly induced the activation of the PERK-ATF4-CHOP pathway, which was much weaker than that induced by BFA. However, TM significantly enhanced BFA-induced CHOP expression, which in turn promoted the apoptosis of HepG2 cells. These results indicated that the combination of BFA and TM could synergistically inhibit the growth of tumor cells and had the potential to be used in cancer treatment.[8,33–35] In addition, we found that BFA and TM had different effects on the mRNA and protein levels of SCD1. BFA increased the mRNA expression of SCD1 but inhibited its protein expression. The results suggested that BFA decreased the protein levels of SCD1 mainly by enhancing its degradation, rather than inhibiting its gene expression at the transcriptional level. However, TM can simultaneously inhibit SCD1 expression at the transcriptional, translational, or post-translational levels. This suggests that BFA and TM have different mechanisms for their anti-cancer activities.

5. Conclusions

In summary, our results demonstrated that BFA could inhibit the proliferation and induce apoptosis in HepG2 cells through the ER stress-activated PERK-ATF4-CHOP signaling pathway. More importantly, TM could enhance the apoptosis induced by BFA. Therefore, the combined administration of BFA and TM may have great potential in the treatment of aggressive cancer like HCC. However, some limitations need to be addressed in the future: (i) additional HCC cell lines (such as Huh7 or Hep3B) could be tested to enhance the generalizability of our findings; (ii) xenograft nude mice model should be used to validate the *in vivo* therapeutic effects of the combination regimen.

Data availability statement

The data that support the findings of this work are available from the corresponding author upon reasonable request.

Authors' contributions

Minghong Li and Mengyi Duan contributed equally to this work and should be considered co-first authors. **Minghong Li:** Writing – original draft, Validation, Software, Methodology, Investigation. **Mengyi Duan:** Writing – original draft, Methodology,

Investigation. **Ying Yang:** Methodology, Investigation. **Xingdao Li:** Methodology, Investigation. **Dan Li:** Methodology, Investigation. **Wenting Gao:** Methodology, Investigation, Formal analysis. **Xiaotong Ji:** Writing – review & editing, Formal analysis. **Jianying Bai:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. All authors have read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that there is no conflicts of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 30972445) and the Natural Science Foundation of Shanxi Province of China (No. 202203021211231).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.livres.2025.01.004.

References

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71:209–249. https://doi.org/10.3322/caac.21660.
2. Song BG, Kim MJ, Sinn DH, et al. A comparison of factors associated with the temporal improvement in the overall survival of BCLC stage 0 hepatocellular carcinoma patients. *Dig Liver Dis*. 2021;53:210–215. https://doi.org/10.1016/j.dld.2020.10.030.
3. Gao X, Zhang J, He Z, et al. Targeting delivery of synergistic dual drugs with elastic PEG-modified multi-functional nanoparticles for hepatocellular carcinoma therapy. *Int J Pharm*. 2022;616:121567. https://doi.org/10.1016/j.ijpharm.2022.121567.
4. Cancer Survival in England, cancers diagnosed 2015 to 2019, followed up to 2020. https://digital.nhs.uk/data-and-information/publications/statistical/cancer-survival-in-england/cancers-diagnosed-2015-to-2019-followed-up-to-2020. Accessed December 15, 2024.
5. Huang M, Lu JJ, Ding J. Natural products in cancer therapy: past, present and future. *Nat Prod Bioprospect*. 2021;11:5–13. https://doi.org/10.1007/s13659-020-00293-7.
6. Tian K, Xu F, Gao X, et al. Nitric oxide-releasing derivatives of brefeldin A as potent and highly selective anticancer agents. *Eur J Med Chem*. 2017;136:131–143. https://doi.org/10.1016/j.ejmech.2017.05.018.
7. Zeng F, Chen C, Al Chnani AA, et al. Dibrefeldins A and B, A pair of epimers representing the first brefeldin A dimers with cytotoxic activities from Penicillium janthinellum. *Bioorg Chem*. 2019;86:176–182. https://doi.org/10.1016/j.bioorg.2019.01.042.
8. Huang H, Liu T, Guo J, et al. Brefeldin A enhances docetaxel-induced growth inhibition and apoptosis in prostate cancer cells in monolayer and 3D cultures. *Bioorg Med Chem Lett*. 2017;27:2286–2291. https://doi.org/10.1016/j.bmcl.2017.04.047.
9. Zhou L, Gao W, Wang K, et al. Brefeldin A inhibits colorectal cancer growth by triggering Bip/Akt-regulated autophagy. *FASEB J*. 2019;33:5520–5534. https://doi.org/10.1096/fj.201801983R.
10. Wallen E, Sellers RG, Peehl DM. Brefeldin A induces p53-independent apoptosis in primary cultures of human prostatic cancer cells. *J Urol*. 2000;164:836–841. https://doi.org/10.1097/00005392-200009010-00058.
11. Shao RG, Shimizu T, Pommier Y. Brefeldin A is a potent inducer of apoptosis in human cancer cells independently of p53. *Exp Cell Res*. 1996;227:190–196. https://doi.org/10.1006/excr.1996.0266.
12. Jiang YY, Gao Y, Liu JY, et al. Design and characterization of a natural arf-GEFs inhibitor prodrug CHNQD-01255 with potent anti-hepatocellular carcinoma efficacy in vivo. *J Med Chem*. 2022;65:11970–11984. https://doi.org/10.1021/acs.jmedchem.2c00532.
13. Wang M, Chen X, Qu Y, et al. Design and synthesis of brefeldin a-isothiocyanate derivatives with selectivity and their potential for cervical cancer therapy. *Molecules*. 2023;28:4284. https://doi.org/10.3390/molecules28114284.
14. Feng B, Huang X, Jiang D, et al. Endoplasmic reticulum stress inducer tunicamycin alters hepatic energy homeostasis in mice. *Int J Mol Sci*. 2017;18:1710. https://doi.org/10.3390/ijms18081710.
15. Wang Y, Zhang L, He Z, et al. Tunicamycin induces ER stress and inhibits tumorigenesis of head and neck cancer cells by inhibiting N-glycosylation. *Am J Transl Res*. 2020;12:541–550.
16. Ajoolabady A, Kaplowitz N, Lebeaupin C, et al. Endoplasmic reticulum stress in liver diseases. *Hepatology*. 2023;77:619–639. https://doi.org/10.1002/hep.32562.
17. Raeisi M, Hassanbeigi L, Khalili F, Kharrati-Shishavan H, Yousefi M, Mehdizadeh A. Stearoyl-CoA desaturase 1 as a therapeutic target for cancer: a focus on hepatocellular carcinoma. *Mol Biol Rep*. 2022;49:8871–8882. https://doi.org/10.1007/s11033-021-07094-2.
18. Wong TL, Loh JJ, Lu S, et al. ADAR1-mediated RNA editing of SCD1 drives drug resistance and self-renewal in gastric cancer. *Nat Commun*. 2023;14:2861. https://doi.org/10.1038/s41467-023-38581-8.
19. Lee J, Jang S, Im J, et al. Stearoyl-CoA desaturase 1 inhibition induces ER stress-mediated apoptosis in ovarian cancer cells. *J Ovarian Res*. 2024;17:73. https://doi.org/10.1186/s13048-024-01389-1.
20. Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol*. 2012;13:89–102. https://doi.org/10.1038/nrm3270.
21. Li X, Zheng J, Chen S, Meng FD, Ning J, Sun SL. Oleandrin, a cardiac glycoside, induces immunogenic cell death via the PERK/eIF2alpha/ATF4/CHOP pathway in breast cancer. *Cell Death Dis*. 2021;12:314. https://doi.org/10.1038/s41419-021-03605-y.
22. Hitomi J, Katayama T, Taniguchi M, et al. Apoptosis induced by endoplasmic reticulum stress depends on activation of caspase-3 via caspase-12. *Neurosci Lett*. 2004;357:127–130. https://doi.org/10.1016/j.neulet.2003.12.080.
23. Pommepuy I, Terro F, Petit B, et al. Brefeldin A induces apoptosis and cell cycle blockade in glioblastoma cell lines. *Oncology*. 2003;64:459–467. https://doi.org/10.1159/000070307.
24. Guha P, Kaptan E, Gade P, Kalvakolanu DV, Ahmed H. Tunicamycin induced endoplasmic reticulum stress promotes apoptosis of prostate cancer cells by activating mTORC1. *Oncotarget*. 2017;8:68191–68207. https://doi.org/10.18632/oncotarget.19277.
25. Xiao CL, Zhong ZP, Lu C, et al. Physical exercise suppresses hepatocellular carcinoma progression by alleviating hypoxia and attenuating cancer stemness through the Akt/GSK-3beta/beta-catenin pathway. *J Integr Med*. 2023;21:184–193. https://doi.org/10.1016/j.joim.2023.01.002.
26. Olson C, Zhang P, Ku J, Flojo R, Boyes D, Lu B. A novel dual-reporter system reveals distinct characteristics of exosome-mediated protein secretion in human cells. *Biol Proced Online*. 2023;25:25. https://doi.org/10.1186/s12575-023-00219-w.
27. Luesch H, Paavilainen VO. Natural products as modulators of eukaryotic protein secretion. *Nat Prod Rep*. 2020;37:717–736. https://doi.org/10.1039/c9np00066f.
28. Hou H, Ge C, Sun H, Li H, Li J, Tian H. Tunicamycin inhibits cell proliferation and migration in hepatocellular carcinoma through suppression of CD44s and the ERK1/2 pathway. *Cancer Sci*. 2018;109:1088–1100. https://doi.org/10.1111/cas.13518.
29. Read A, Schroder M. The unfolded protein response: an overview. *Biology (Basel)*. 2021;10:384. https://doi.org/10.3390/biology10050384.
30. Lebeaupin C, Proics E, de Bieville CH, et al. ER stress induces NLRP3 inflammasome activation and hepatocyte death. *Cell Death Dis*. 2015;6:e1879. https://doi.org/10.1038/cddis.2015.248.
31. Gao Z, Peng M, Chen L, et al. Prion protein protects cancer cells against endoplasmic reticulum stress induced apoptosis. *Virol Sin*. 2019;34:222–234. https://doi.org/10.1007/s12250-019-00107-2.
32. Zhang JM, Wang CF, Wei MY, et al. Brefeldin a induces apoptosis, inhibits BCR-ABL activation, and triggers BCRABL degradation in chronic myeloid leukemia K562 cells. *Anticancer Agents Med Chem*. 2022;22:1091–1101. https://doi.org/10.2174/1871520621666210608110435.
33. Zhang JM, Jiang YY, Huang QF, et al. Brefeldin A delivery nanomicelles in hepatocellular carcinoma therapy: characterization, cytotoxic evaluation in vitro, and antitumor efficiency in vivo. *Pharmacol Res*. 2021;172:105800. https://doi.org/10.1016/j.phrs.2021.105800.
34. Wu S, Ma S, Yin X, Yi P, Liu J. An integrated PKD1-dependent signaling network amplifies IRE1 prosurvival signaling. *J Biol Chem*. 2019;294:11119–11130. https://doi.org/10.1074/jbc.RA118.003311.
35. Jiang YY, Wu S, Wu YW, et al. New brefeldin A-cinnamic acid ester derivatives as potential antitumor agents: design, synthesis and biological evaluation. *Eur J Med Chem*. 2022;240:114598. https://doi.org/10.1016/j.ejmech.2022.114598.