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Cannabidiol exhibits potent anti-cancer activity against gemcitabine-resistant cholangiocarcinoma via ER-stress induction in vitro and in vivo

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Abstract

Background Failure of treatment with gemcitabine in most cholangiocarcinoma (CCA) patients is due to drug resistance. The therapeutic potential of natural plant secondary compounds with minimal toxicity, such as cannabidiol (CBD), is a promising line of investigation in gemcitabine-resistant CCA. We aim to investigate the effects of CBD on gemcitabine-resistant CCA (KKU-213B^{GemR}) cells in vitro and in vivo.

Materials In vitro, cell proliferation, colony formation, apoptosis and cell cycle arrest were assessed using MTT assay, clonogenicity assay and flow cytometry. The effect of CBD on ROS production was evaluated using the DCFH-DA fluorescent probe. The mechanism exerted by CBD on ER stress-associated apoptosis was investigated by western blot analysis. A gemcitabine-resistant CCA xenograft model was also used and the expression of PCNA and CHOP were evaluated by immunohistochemical analysis.

Results The IC_{50} values of CBD for KKU-213B^{GemR} cells ranged from 19.66 to 21.05 µM. For a non-cancerous immortalized fibroblast cell line, relevant values were 18.29 to 19.21 µM. CBD suppressed colony formation by KKU-213B^{GemR} cells in a dose-dependent manner in the range of 10 to 30 µM. CBD at 30 µM significantly increased apoptosis at early (16.37%) (*P* = 0.0024) and late (1.8%) stages (*P* < 0.0001), for a total of 18.17% apoptosis (*P* = 0.0017), in part by increasing ROS production (*P* < 0.0001). Multiphase cell cycle arrest significantly increased at G0/G1 with CBD 10 and 20 µM (*P* = 0.004 and *P* = 0.017), and at G2/M with CBD 30 µM (*P* = 0.005). CBD treatment resulted in increased expression of ER stress-associated apoptosis proteins, including p-PERK, BiP, ATF4, CHOP, BAX, and cytochrome c. In xenografted mouse, CBD significantly suppressed tumors at 10 and 40 mg/kg·Bw (*P* = 0.0007 and *P* = 0.0278, respectively), which was supported by an increase in CHOP, but a decrease in PCNA expression in tumor tissues (*P* < 0.0001).

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Conclusion The results suggest that CBD exhibits potent anti-cancer activity against gemcitabine-resistant CCA in vitro and in vivo, in part via ER stress-mediated mechanisms. These results indicate that clinical explorative use of CBD on gemcitabine-resistant CCA patients is warranted.

Keywords Bile duct cancer, CBD, Cannabis, Chemoresistance, Reactive oxygen species, Cell death

Introduction

Cholangiocarcinoma (CCA) is one of the most common malignancies in northeastern Thailand with an incidence rate of 96 cases per 100,000 in men and 38 cases per 100,000 in women, being the highest rate worldwide [1]. CCA is a heterogeneous group of bile-duct cancers and has many risk factors [2, 3]. In Thailand, these include *Opisthorchis viverrini* infection, biliary cysts, Caroli disease, primary sclerosing cholangitis, hepatolithiasis, cholelithiasis, cirrhosis, and chronic viral hepatitis [3]. Surgical resection remains the only curative option for early-stage CCA patients and significantly improves survival. Unfortunately, the prognosis for CCA in northeastern Thailand remains dismal, primarily due to late-stage diagnoses that preclude surgical intervention [4, 5].

Several chemotherapeutic agents, including gemcitabine (GEM), 5-fluorouracil (5- FU), and cisplatin (CIS), have been used to treat CCA [6]. Gemcitabine, a pyrimidine anti-metabolite, is widely used to treat solid tumors, including CCA [7, 8]. As a deoxycytidine analog, it inhibits DNA synthesis and effectively incorporates into elongating DNA [9]. In addition, GEM interferes with ribonucleotide reductase, resulting in a decrease in deoxynucleoside triphosphate (dNTP) pools, which are critical for DNA repair and synthesis [10]. These effects culminate in the induction of cell apoptosis [11]. However, the initial success of chemotherapy is often followed by a decline in efficacy due to adaptive mechanisms of the cancer. In addition, the use of GEM is associated with adverse side effects, including hepatotoxicity, nausea, immunosuppression, diarrhea, and sensory neuropathy [12]. In almost all cancers, including CCA, there is evidence of the development of resistance to chemotherapeutic agents [13, 14]. The causes of resistance are multifaceted and include factors such as impaired drug uptake, activation of alternative DNA repair pathways, and patient heterogeneity [15]. To improve the efficacy of GEM chemotherapy, it is imperative to explore strategies to overcome GEM resistance. Recently, there has been an increased interest in the use of natural products as chemotherapeutic agents, especially phytochemicals, particularly to combat drug resistance in cancer treatment.

Cannabidiol (CBD), derived from extracts of *Cannabis sativa* L., stands out for its lack of psychoactive constituents, positioning it as a promising therapeutic agent in contrast to its psychotropic counterpart, tetrahydrocannabinol [16, 17]. The exploration of CBD's anti-cancer activities has been comprehensive, covering a spectrum of cancer types both in vitro and in vivo, such as hepatocellular carcinoma [18], lung cancer [19], breast cancer [20], gastric cancer [21] and head and neck squamous-cell carcinoma [22]. Additionally, CBD exerts anti-cancer effects in chemo-resistant non-small cell lung cancer, both in vitro and in vivo, through the regulation of the oxidant and anti-oxidant systems [23]. Despite an existing study highlighting the potential effects of CBD on CCA cell lines [24], its roles in chemoresistant CCA need to be elucidated before translation into clinical study.

The multiple anti-tumor properties of CBD primarily involve the induction of endoplasmic-reticulum stress (ER-stress), which leads to cell apoptosis and activation of autophagy metabolism [25, 26]. The induction of apoptosis by CBD depends on its ability to disrupt the structure of ER, thereby initiating a complex signaling network that includes the protein kinase R-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and the inositol-requiring ER-to-nucleus signal kinase-1 (IRE1) [25]. In addition, CBD has the potential to trigger pro-apoptotic signaling pathways, including IRE1, apoptosis signal-regulating kinase 1(ASK1), and c-Jun N-terminal kinase (JNKs), which ultimately orchestrate apoptosis in stellate liver cells [25]. Therefore, we hypothesize that phytochemicals such as CBD act against gemcitabine-resistant CCA via the ER stress-induced apoptosis pathway.

In this study, we employed KKU-213B-gemcitabine-resistant (KKU-213B^{GemR}) CCA cells as the focus of analysis both in vitro and in vivo. The underlying mechanism of CBD activity against KKU-213B^{GemR} cells was investigated in vitro. The anti-cancer effect of CBD on the proliferation and growth of KKU-213B^{GemR} cells was also demonstrated in a mouse xenograft model. An overview of the study is illustrated in Fig. 1. Our results provide a compelling argument for the potential utility of CBD as a therapeutic candidate for the treatment of CCA, particularly in patients who experience gemcitabine treatment failure.

Materials and methods

Human gemcitabine-resistant CCA and non-cancerous fibroblast cell lines

The CCA cell line, KKU-213B, was obtained from Thai CCA patients. Informed consent from each patient was documented in writing, following the protocol established by Prof. Banchob Sripa at Khon Kaen University [27]. To create the KKU-213B^{GemR} cell line, KKU-213B

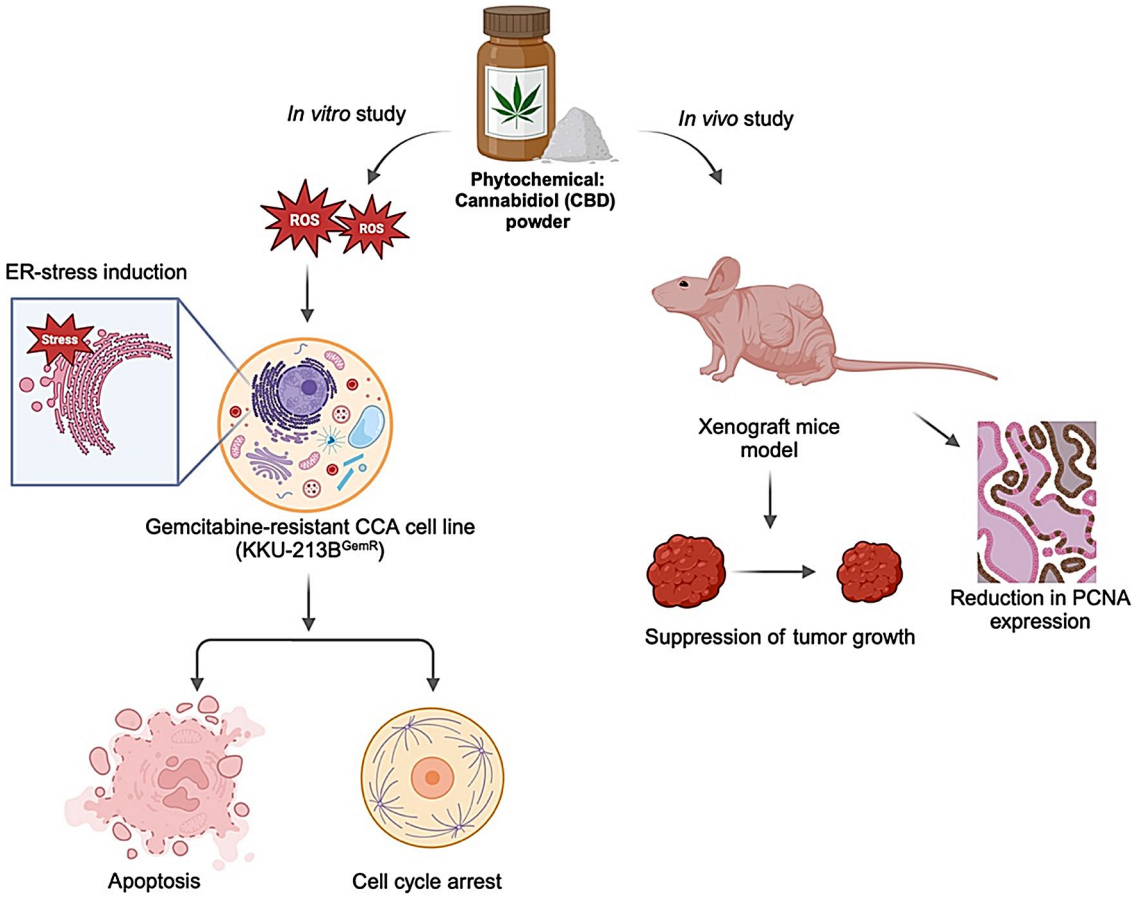

Fig. 1 Concept of experimental study. The graphical representation was created using BioRender.com (license number: NS26RT6OV6)

cells were grown using a stepwise dose-escalation process with gemcitabine, as previously described [28]. OUMS-24/P6X, transformed fibroblast cells originating from a human embryo, which are available from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan), were included for toxicity testing. The cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37 °C and 10% CO_2 in a humidified incubator. Cells were maintained in the presence of gemcitabine and then in a drug-free medium for one passage immediately before use in an experiment.

Cell proliferation detection by MTT assay

Cells (3,000 cells/well) were seeded in flat-bottomed 96-well plates (Costar, Corning, NY, USA) and treated for 24, 48, or 72 h with either 0.5% dimethyl sulfoxide (DMSO) (Sigma Aldrich, Saint Louis, MO, USA), serving as a vehicle control, or various concentrations of CBD (2.5 to 30 µM) (DMSc reference standard was purchased from the Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand) dissolved in DMSO and diluted with culture media. Then, 24 µL of 2.5 mg/mL MTT (Invitrogen™, Thermo Fisher Scientific Inc., Waltham, MA, USA) was added to the cells and incubated for 2 h at 37 °C and 5% CO_2 in a humidified incubator. DMSO was then added to dissolve the formazan, and absorbance was measured at 540 nm using a Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, MA, USA). Data derived from MTT assay was used to determine the half-maximal inhibitory concentration (IC_{50}) of CBD.

Clonogenicity assay

Approximately 2,000 cells per well of KKU-213B^{GemR} were grown in 6-well plates and treated with different concentrations of CBD (10, 20, and 30 µM). The culture medium was changed every 2 days, and the cells were cultured for approximately 2 weeks. Cells were then fixed with 4% paraformaldehyde, stained with 0.5% crystal violet, and dissolved with 33% acetic acid. Absorbance at 620 nm was measured using a Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific Inc.).

Apoptosis detection by flow cytometry

Apoptotic cells were detected by flow cytometry and a dual staining technique using Annexin V-FITC (BioLegend, San Diego, CA, USA) and propidium iodide (PI), following the manufacturer's recommendations. In brief, 3×10^5 KKU-213B^{GemR} cells were cultured for 24 h in six-well plates. Subsequently, these cells were treated with either 0.5% DMSO or different concentrations of CBD (10, 20, and 30 μM) for 24 h. After the treatment interval, cell pellets were resuspended in Annexin-V binding buffer and then subjected to dual staining with Annexin-V-FITC and PI in a light-protected environment, allowing 15 min incubation at room temperature. A BD FACSCanto II flow cytometer (BD biosciences, San Jose, CA, USA) was used to enumerate apoptotic cells, with subsequent data analysis performed using FlowJo™ software version 10.8.1 (BD biosciences, Ashland, OR, USA).

Cell cycle arrest

KKU-213B^{GemR} cells (1×10^5 cells/well in 6-well plates) were treated for 24 h with either 0.5% DMSO (control) or CBD at concentrations of 10 to 30 μM. Cells were then fixed with 70% ethanol at 4 °C for 1 h. After fixation, cells were resuspended in FxCycle™ PI /RNase staining solution (Molecular Probes, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) and incubated for 45 min in the dark at room temperature. Detection and analysis of stained cells were performed using a BD FACSCanto II flow cytometer, together with FlowJo™ software version 10.8.1 and FCS Express 7 (De Novo Software, Los Angeles, CA, USA).

ROS measurement

KKU-213B^{GemR} cells were seeded in 12-well plates at a density of 2.5×10^4 cells per well. In brief, cells were exposed to either 0.5% DMSO or CBD 30 μM for 6 h. Subsequently, cells were incubated with dichlorodihydro-fluorescein diacetate (DCFH-DA, #35845, Sigma-Aldrich, Saint Louis, MO, USA) at a concentration of 10 μM in DMEM serum-free medium at 37 °C and 10% CO_2 in a humidified incubator for 30 min. Thereafter, the supernatant was aspirated, and washed once with DMEM serum-free media followed by two washes with 1X PBS. Each well was then treated with 500 μL of 1X PBS. Images of ten visual fields from each experimental group were acquired at a magnification of 10x using a fluorescence imaging microscope (ECLIPSE Ti-U, Nikon Instruments Inc., Japan). Fluorescence integrated intensity (fluorescence intensity normalized to the area) was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Western blot

KKU-213B^{GemR} cells were treated with 0.5% DMSO and CBD at a concentration of 30 μM for different time periods (0, 2, 4, and 6 h). Protein extraction was performed using 1X RIPA reagent (#9806S, Cell Signaling Technology, Danvers, MA, USA) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail l (Thermo Fisher Scientific Inc., Rockford, IL, USA) and protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc). Protein (20–25 μg) was separated using SDS-PAGE. In brief, extracted proteins were separated by electrophoresis on an 8–12% gel based on their molecular weight at 90–110 volts for 1.30 h. Following electrophoresis, proteins were transferred from the gel onto a polyvinylidene fluoride (PVDF) membrane (#10600029, Cytiva, Dreieich, Germany) at 90 volts for 1.30 h. After separation, PVDF membrane was incubated with primary antibodies at a dilution of 1:1,000 for all. Primary antibodies used included PERK (#5683) purchased from Cell Signaling Technology, phospho-PERK (#AP0886), BiP (#A0241), ATF4 (#A0201), and CHOP (#A0221) from ABclonal in Wuhan, China. Additionally, BAX (#ab7977), Bcl-2 (#ab32124), and beta-actin (#ab3280) were purchased from Abcam, Cambridge Biomedical Campus in Cambridge, UK, while cytochrome c (#sc-7159) was sourced from Santa Cruz Biotechnology in Dallas, TX, USA. Next, the membrane was incubated with secondary antibodies, specifically HRP-conjugated goat anti-rabbit IgG (dilution 1:2,000, #111035003, Jackson Immuno Research Inc., West Grove, PA, USA) and HRP-conjugated horse anti-mouse IgG (dilution 1:3,000, #7076P2, Cell Signaling Technology).

CBD-induced CCA in xenograft mouse model

Experimental animals

Female nude mouse (BALB/CAJcl-Nu/Nu) aged 6–7 weeks weighing 22–25 g at the beginning of the experiment were obtained from Nomura Siam International, Bangkok, Thailand. The mouse was housed in individual ventilated cages under specific pathogen-free conditions at a room temperature of 23±2 °C and a 12 h light and 12 h dark cycle with a light intensity of 350–400 lx. During the experimental period, the mouse had *ad libitum* access to water enriched with choline at a concentration of 3 to 4 ppm and their dietary requirements were met with commercially available pellets. This standardized housing and diet was designed to ensure the welfare of the mouse and provide consistent environmental conditions to minimize potential sources of variability in the experimental results. We made deliberate efforts to reduce animal utilization and alleviate any pain or discomfort.

Tumor xenograft and CBD treatment in a mouse model

After a 7-day acclimatization period, 16 mice were injected subcutaneously with KKU-213B^{GemR} cells (1×10^6 cells in 50 µM serum-free medium mixed with Matrigel from Corning, Tewksbury, MA, USA) into the right axilla. The subsequent determination of the tumor volume was performed with a digital caliper and was calculated according to the following formula: Tumor volume = (length × width^2)/2. When a tumor volume of 70 mm^3 was reached, the animals were randomly assigned to one of three groups: Sesame oil (vehicle control) (#S3547, Sigma-Aldrich, Saint Louis, MO, USA) (n=4), CBD 10 mg/kg·Bw (n=6), or CBD 40 mg/kg·Bw (n=6). Equal amounts of CBD or sesame oil were administered by oral gavage every other day for a period of 9 days. The tumor suppression rate (TSR %) was then calculated using a formula previously established in the study by Liu et al. (2012) [29].

Sample collection

At the end of the treatment period, the animals were sacrificed directly. Prior to sacrifice, the mouse was rendered unconscious in a plastic chamber containing 1% isoflurane (Attane, Minrad, NY, USA). Blood was then taken directly from the animals' hearts. To ensure effective killing, the heart poles of the mouse was cut with scissors. Following this procedure, various tissues were removed from the mouse, including liver, kidney, intestine, tumor mass, feces and urine. The removed organs and tissues were weighed and immediately fixed in 10% formalin. The tumor masses were carefully arranged, photographed and safely stored for subsequent histological examination.

Immunohistochemistry study

Antigens were retrieved from tissue sections using citrate buffer followed by blocking with 5% FBS. Then tissues were incubated overnight with primary antibodies, proliferating cell nuclear antigen (PCNA) (diluted 1:750, #Ab2426, Abcam) and CHOP (diluted 1:30, #A0221, ABclonal). The resulting signal was visualized using diaminobenzidine substrate and counterstained with Mayer's hematoxylin. Positive cells were identified by the presence of brown staining. Image analysis was performed on ten fields, each at 200× magnification, using ImageJ software for precise quantification and evaluation.

Statistical analysis

Data are expressed as mean±SD. Student's t-test was used to test for differences between experimental groups. Statistical comparisons involved one-way ANOVA followed by Tukey's test for multiple group comparisons. A value of $P<0.05$ was considered statistically significant. Nonlinear regression analysis was performed to calculate the IC_{50}. All statistical analyses were performed using Graphpad Prism 9.0 for Mac (GraphPad Software, Inc., CA, USA).

Results

CBD suppresses proliferation and clonogenicity

KKU-213B^{GemR} cells were exposed to DMSO and different concentrations of CBD for up to 72 h. The MTT assay showed a dose- and time-dependent decrease in cell proliferation (Fig. 2a). Subsequently, these results were used to determine the half-maximal inhibitory concentration (IC_{50}) of CBD. IC_{50} concentrations of CBD in KKU-213B^{GemR} cells were 21.05±1.99 µM at 24 h, 19.66±2.58 µM at 48 h, and 20.67±1.43 µM at 72 h. In addition, the cytotoxicity of CBD in a fibroblast cell line (OUMS-24/P6X) was assessed (Fig. 2b). IC_{50} values of the non-cancerous cell line was 19.21±0.62, 18.46±1.12, and 18.29±1.23 µM for 24, 48, and 72 h, respectively. A clonogenic assay was performed to evaluate ability of CBD to inhibit cell division and colony formation in CCA cell lines. Consistent with the results of the MTT assay, CBD at concentrations of 20 µM ($P<0.0001$) and 30 µM ($P<0.0001$) significantly inhibited colony formation in KKU-213B^{GemR} cells compared to the control group treated with 0.5% DMSO, as shown in Fig. 2c-d.

CBD induces apoptosis and cell cycle arrest

Compared to the DMSO-treated control, 30 µM CBD significantly increased both early (16.37%) ($P=0.0024$) and late (1.8%) stages of apoptosis ($P<0.0001$), with an overall apoptosis rate of 18.17% ($P=0.0017$), as shown in Fig. 3a-b. CBD at 10 µM and 20 µM had no effect on apoptosis. We also examined cell cycle arrest (Fig. 3c-d). CBD treatment significantly increased cell cycle arrest in the G0/G1 phase at concentrations of 10 µM ($P=0.004$) and 20 µM ($P=0.017$), while 30 µM CBD significantly decreased the proportion at G0/G1 ($P=0.009$). Moreover, the population of cells in the S phase was significantly reduced at doses 10 µM ($P<0.0001$) and 20 µM ($P<0.0001$). In particular, cell cycle arrest in the G2/M phase was significantly increased by 30 µM CBD treatment ($P=0.005$).

CBD induces ROS and ER-stress-mediated signaling pathways

To investigate ROS generation in CBD-treated KKU-213B^{GemR} cells, we treated KKU-213B^{GemR} cells with 30 µM CBD for 6 h. As demonstrated in Fig. 4, the green fluorescence indicated a high level of intracellular ROS production. Compared to treatment with 0.5% DMSO, the number of ROS-positive cells (Fig. 4a) and the integral green fluorescence intensity significantly increased in KKU-213B^{GemR} cells treated with 30 µM CBD ($P<0.0001$, Fig. 4b).

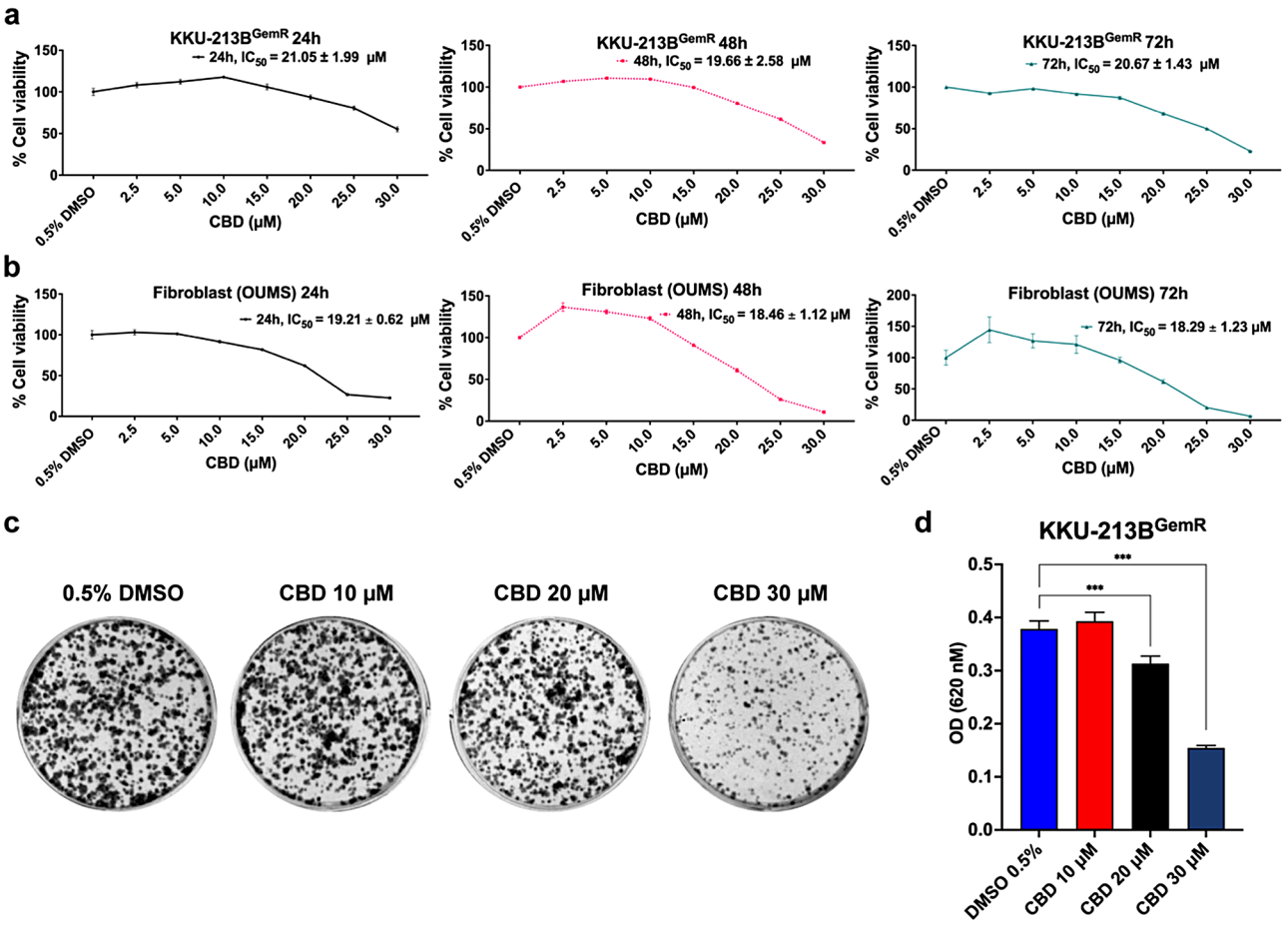

Fig. 2 CBD treatment suppresses cell proliferation and colony formation in gemcitabine-resistant CCA cells (KKU-213B^{GemR}). The anti-proliferative potential of CBD was evaluated using the MTT assay, in which the CCA cell line, KKU-213B^{GemR} (**a**), and a non-cancerous fibroblast cell line, OUMS-24/P6X (**b**) were exposed to different concentrations of CBD (2.5, 5, 10, 15, 20, 25, and 30 μM) for 24 h, 48 h, and 72 h. A parallel control group was treated with 0.5% DMSO. The effect of CBD on colony formation in the KKU-213B^{GemR} cells was examined using a clonogenic assay (**c**), in which absorbance was measured at an optical density of 620 nm to detect differences between cells treated with 0.5% DMSO or CBD (10, 20, and 30 μM) (**d**). Each experiment was performed in triplicate, and significant differences relative to controls are indicated as follows: ***$P < 0.0001$.

KKU-213B^{GemR} cells were treated with 30 μM CBD for 0, 2, 4, and 6 h, and protein expression was examined using western blot analysis. As shown in Fig. 5a, CBD treatment did not result in statistically significant changes in the expression of basal PERK at any time point compared to the 0.5% DMSO treatment control. However, phosphorylation of PERK increased significantly at 2 ($P=0.001$), 4 ($P=0.007$), and 6 h ($P=0.003$) (Fig. 5b-c). CBD treatment also significantly activated BiP expression at 6 h ($P=0.0058$) (Fig. 5d) and tended to increase the expression of ATF4 at 2 h, leading to a significant increase at 6 h ($P=0.043$) (Fig. 5e). Interestingly, the transcription factor protein CHOP showed a significant increase at 4 h ($P=0.0042$) and 6 h ($P=0.0474$) (Fig. 5f). These results suggest that CBD induces ER-stress in the gemcitabine-resistant CCA cell line. In addition, we examined the expression of apoptosis-associated proteins. Western blot analysis revealed that 30 μM CBD treatment did not alter the expression of the anti-apoptotic Bcl-2 protein at any time point (Fig. 5g). In contrast, the expression of the pro-apoptotic BAX protein increased significantly at 4 h ($P=0.0394$) and at 6 h ($P=0.0006$) (Fig. 5h). Similarly, there was a significant increase in cytochrome c at 2 h ($P=0.018$) and at 4 h ($P=0.041$) (Fig. 5i).

CBD induce ER-stress, suppresses tumor growth and cell proliferation in a xenograft mouse model

We subcutaneously injected nude mouse with 1×10^6 KKU-213B^{GemR} cells, and treated them daily with either CBD or sesame oil (vehicle control) for 9 days (Fig. 6a). As shown in the relative volume-change graph (Fig. 6b), tumor mass significantly decreased in mouse treated with CBD at doses of 10 and 40 mg/kg·Bw by three days after treatment compared to the vehicle control, with P-values of 0.0159 and 0.0186. By day 6 after treatment, tumor mass was significantly lower in mouse treated with

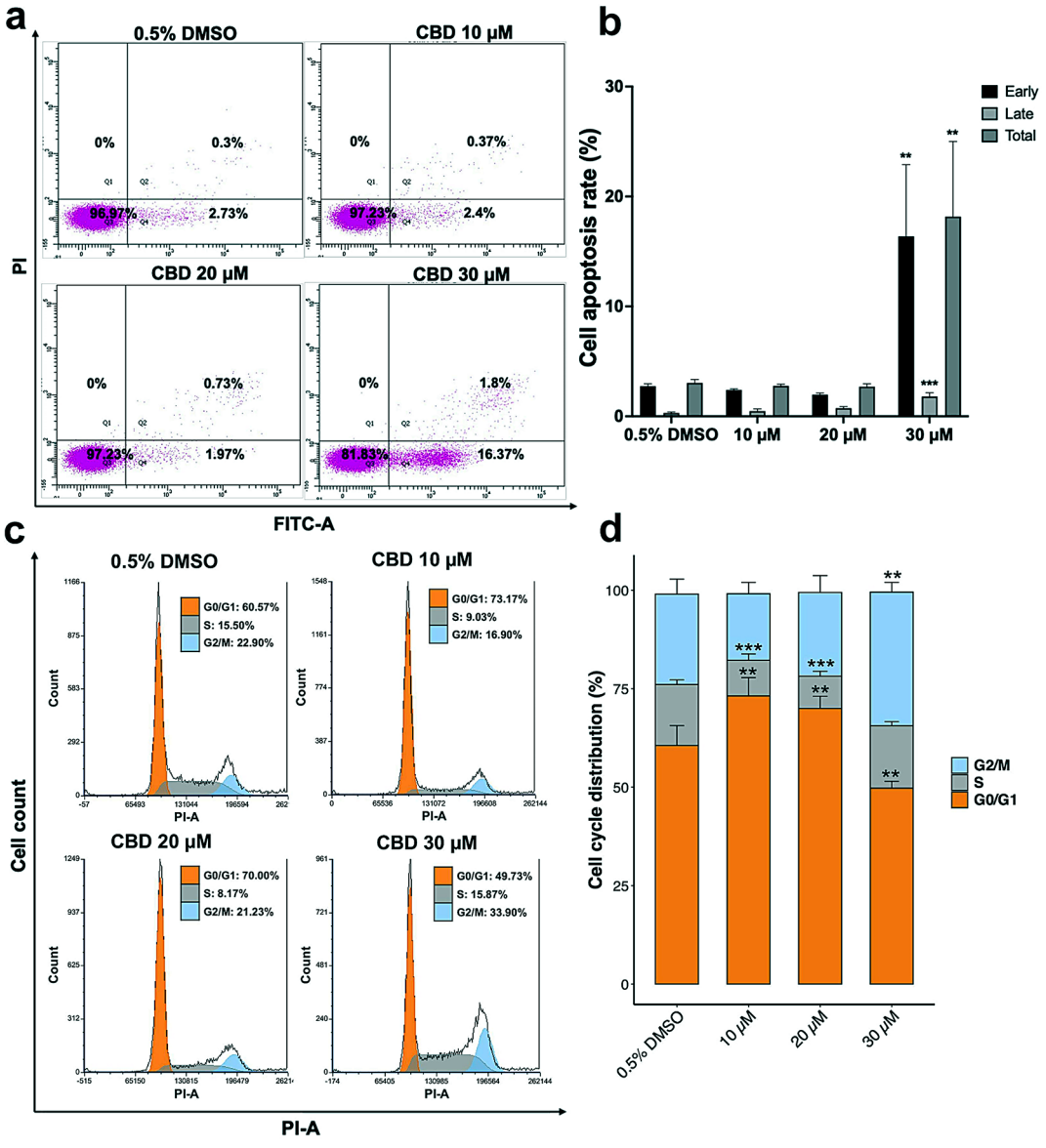

Fig. 3 Flow cytometry was used to investigate the effect of CBD on apoptosis (**a** - **b**) and cell cycle arrest (**c** - **d**) in gemcitabine-resistant CCA cells (KKU-213B^{GemR}). CBD was administered at concentrations of 10 μM, 20 μM, and 30 μM over a 24 h period, compared to a control group treated with 0.5% DMSO (**a**). To illustrate the apoptotic response, a bar graph was constructed (**b**) depicting apoptosis levels quantified by fluorescence-activated cell sorting (FACS) followed by labeling with annexin V-FITC and propidium iodide (PI). In addition, a stacked bar graph was constructed to show the distribution of cells across the different phases of the cell cycle as quantified by FACS analysis with PI labeling (**d**). Each experiment was performed in triplicate, and significant differences relative to controls are indicated as follows: * $P<0.05$, ** $P<0.01$, and *** $P<0.0001$, respectively

10 mg/kg·Bw ($P=0.0243$) and 40 mg/kg·Bw ($P=0.0446$) compared to vehicle controls. One day before the end of treatment, the volume of tumors was significantly decreased in the 10 mg/kg·Bw group ($P=0.0435$), but not in the 40 mg/kg·Bw group compared to the sesame oil-treated mouse. To confirm this, the tumor suppression rate (%) was also calculated and showed a significant increase in tumor suppression rate in mouse treated with CBD at concentrations of 10 mg/kg·Bw ($P=0.0007$) and 40 mg/kg bw ($P=0.0278$) compared to mouse receiving sesame oil (Fig. 6c). Tissues from all experimental groups were examined by immunohistochemistry. To confirm the effect of CBD on ER-stress induction in the animal model, the ER stress-associated protein marker, CHOP, was assessed in tumor tissues. CHOP expression was elevated in the tumor tissue of mouse treated with CBD at doses of 10 mg/kg·Bw ($P<0.0001$) and 40 mg/kg·Bw ($P<0.0001$) compared to the vehicle control, as shown in Fig. 7a-b. PCNA—a marker of cell proliferation—showed staining mainly in the nucleus with some in the cytoplasm. Reduction of PCNA expression was observed in the tumor tissues of mouse treated with CBD at doses of

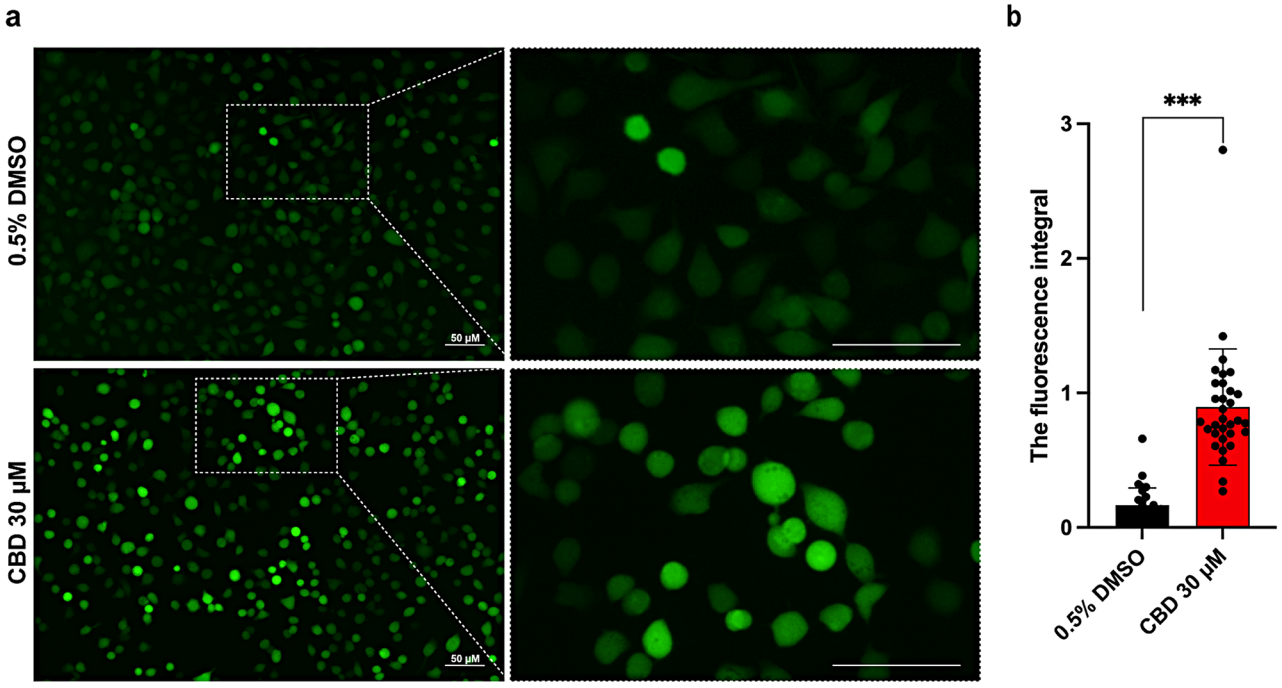

Fig. 4 Detection of ROS generation by DCFH-DA in KKU-213B^{GemR} after incubation with either 0.5% DMSO or CBD 30 µM for 6 h. The intracellular green fluorescence indicates overproduction of ROS, as ROS oxidize DCFH-DA to DCF-DA (**a**). Bar charts depict fluorescence integral values, normalized to areas using ImageJ software (**b**). The experiment was performed in technical triplicate. Statistical significance is indicated as * $P<0.05$, ** $P<0.01$, and *** $P<0.0001$

10 mg/kg·Bw ($P<0.0001$) and 40 mg/kg·Bw ($P<0.0001$) compared to vehicle controls, as shown in Fig. 7a and c.

Discussion

Drug resistance is a major challenge in treatment of various cancers, including cholangiocarcinoma (CCA) [14]. Our study pioneers the exploration of CBD to overcome gemcitabine resistance in CCA. CBD showed robust anti-cancer effects both in vitro and in vivo. CBD effectively reduced cell proliferation, suppressed colony formation, induced ROS overproduction leading to ER stress-associated apoptosis and cell cycle arrest in gemcitabine-resistant CCA cells. The possible mechanism of its action against gemcitabine resistance in CCA via the ROS-induced ER stress-mediated apoptosis signaling pathway is shown in Fig. 8.

Because CBD is safe and nonaddictive [30], it is useful in the treatment of various diseases, including end-stage cancer [31], and has a number of potential therapeutic properties, including anti-inflammatory, anti-oxidant, immunomodulatory, and anti-cancer effects [32, 33]. CBD inhibits tumor growth not only in solid tumors, including CCA [24], but also in chemoresistant cancers such as exemestane-resistant breast-cancer cells [34] and cisplatin-resistant non-small cell lung cancer [23, 35]. Thus, CBD may participate in inhibiting CCA progression. Our results showed that IC_{50} values in immortalized fibroblast cells (18.29 to 19.21 µM) were slightly lower than in CCA gemcitabine-resistant cells (19.66 to 21.05 µM), indicating that the cytotoxic effect of CBD is not limited only to CCA gemcitabine-resistant cells but is also exerted in non-cancerous cells. Similar findings were also observed in a model of prostate-cancer cells and in non-cancerous prostate epithelial cells [36], cholangiocarcinoma and immortalized cholangiocyte cells [24], and also in glioblastoma cells and non-cancerous neural progenitor cells [37]. The non-cancerous cell lines used in all these studies were immortalized cell lines, which were transformed to proliferate indefinitely. The cytotoxic effect of CBD observed in immortalized non-cancerous cells might not necessarily be observed in normal and healthy cells. This speculation is supported by a number of studies which have reported that CBD doses up to 1500 mg/day are safe and well-tolerated in humans [38]. Hence, more in-depth research is required to investigate whether CBD exerts cytotoxic effects against non-transformed healthy cells. In addition, further investigation applying nanotechnology for targeted delivery of CBD is warranted to delineate and compare CBD's actions on cancerous cells and non-cancerous cells.

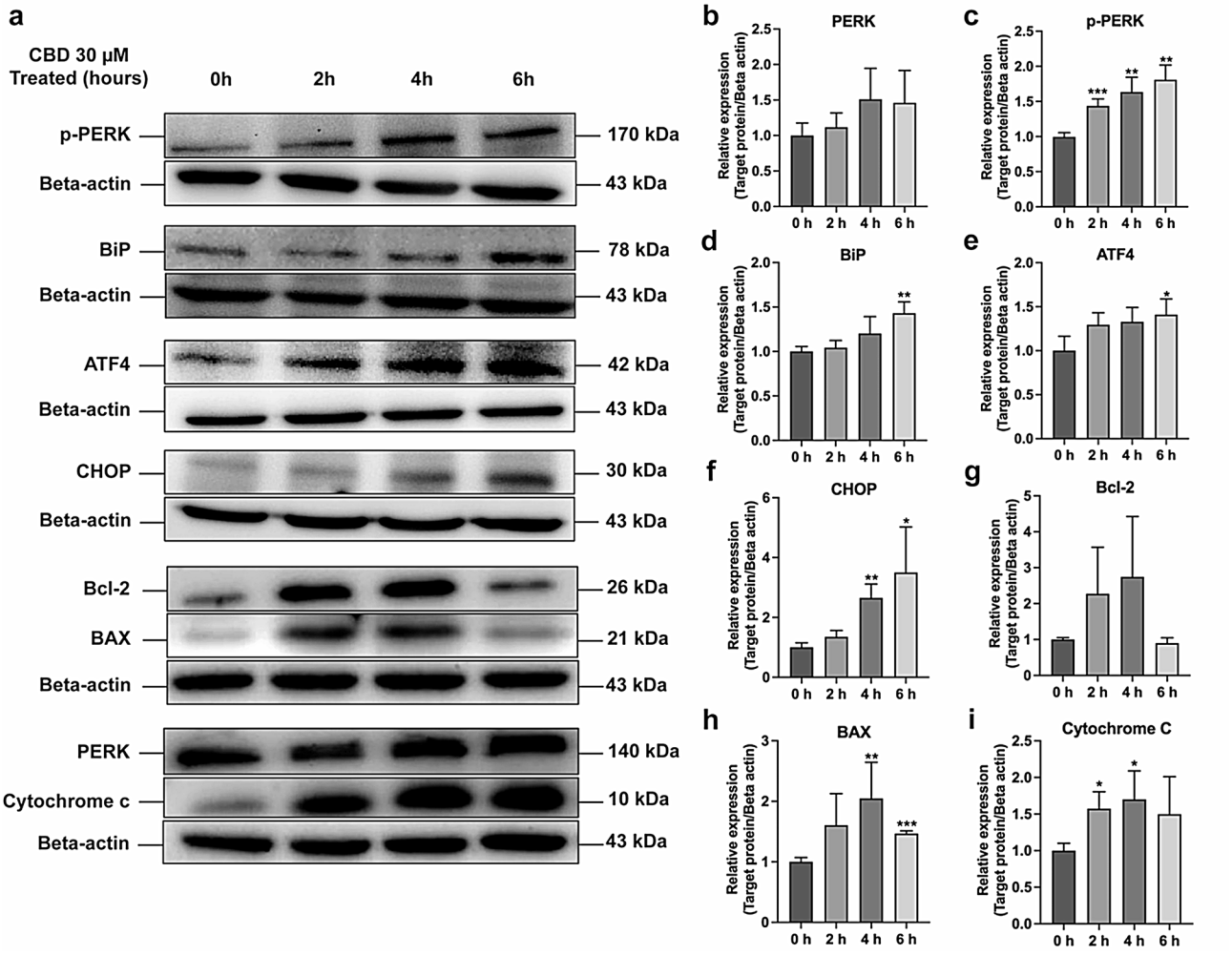

Fig. 5 A comprehensive study was conducted to investigate the effects of CBD on protein expression of markers associated with cellular apoptosis mediated by ER - stress. This study involved the relative quantification of PERK, phosphorylated PERK (p-PERK), BiP, ATF4, CHOP, Bcl-2, BAX, and cytochrome c in the KKU-213B^{GemR} cell line. This analysis was performed after treatment with CBD at a concentration of 30 μM for a period of 0 (lane 1), 2 (lane 2), 4 (lane 3), and 6 h (lane 4) (**a**). Data are presented as a bar plot of mean values + SD for biological triplicates. The level of statistical significance is indicated by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$) to denote the level of significance within the results (**b to i**). The target proteins, including p-PERK, BiP, ATF4 and CHOP, were cropped from different blots. Each target protein was paired with the corresponding beta-actin as a loading control. In addition, Bcl-2, BAX, PERK and cytochrome c proteins were obtained from different regions of the same blot. Specifically, PERK and cytochrome c were from the first biological replication experiment, while Bcl-2 and BAX were from the second biological replication experiment. Consequently, blots probed for PERK and cytochrome c used the same beta-actin as a loading control, and a similar situation applies for Bcl-2 and BAX. The original blots are shown in Supplementary Figs. S1-S11

Although there is evidence that CBD induces apoptosis in various tumor types, its specific mechanisms in drug-resistant cells, particularly gemcitabine-resistant CCA cells, remain unclear [19, 39, 40]. In chemosensitive cancers, CBD similarly induces apoptosis through ROS-mediated ER stress-associated pathways [40, 41]. The effect of CBD is reflected in overproduction of ROS leading to activation of ER-stress markers, including phosphorylated PERK, BiP, ATF4, and CHOP, as well as modulation of the pro-apoptotic protein BAX and the anti-apoptotic protein Bcl-2 [25, 41, 42]. Remarkably, Bcl-2 levels remained unchanged in this study, suggesting a shift toward a pro-apoptotic cellular environment, as previously reported in CBD-treated CCA cells [24]. In our study, a relatively modest level of cellular apoptosis was observed, a finding consistent with previous reports [43]. This suggests that the effect of CBD on cellular proliferation may be mediated by other mechanisms. To explore this hypothesis, we investigated the potential of CBD to induce cell cycle arrest and found pronounced arrest occurring at both G0/G1 and G2/M phases, results consistent with literature on colorectal cancer [43, 44].

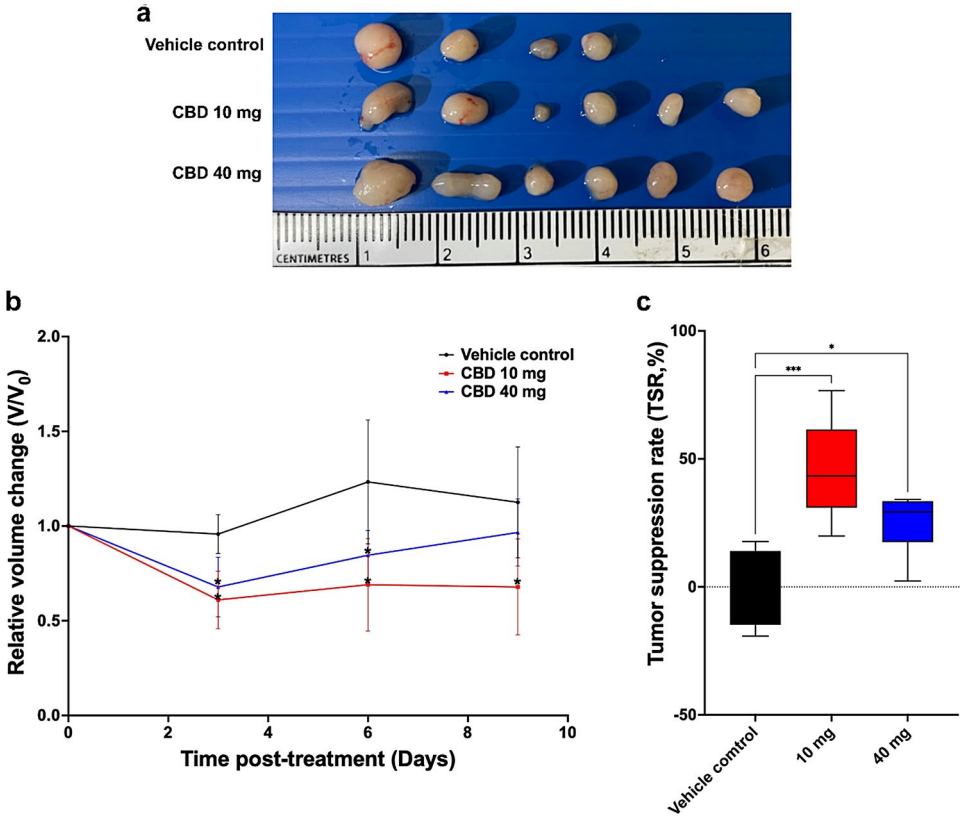

Fig. 6 The anti-tumor effect of CBD in a gemcitabine-resistant CCA cell (KKU-213B^{GemR}) xenograft mouse model was investigated. KKU-213B^{GemR} cells (1×10^6) mixed with Matrigel matrix were injected subcutaneously into the animals and allowed to grow for 21 days. Xenograft mouse was fed with CBD in sesame oil (10 and 40 mg/kg·Bw) for 9 days. Thereafter, animals were sacrificed and the image showed the total number of tumors per mouse (**a**). The mean relative change in tumor volume in KKU-213B^{GemR} is shown at days 0, 3, 6, and 9 (**b**). The tumor suppression rate is graphically depicted at day 9 (**c**). Statistically significant differences were identified as follows: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$ compared to sesame oil or vehicle treatment as the control group

Of note, we demonstrated G0/G1 phase arrest at lower CBD concentrations (10 to 20 μM) and G2/M phase arrest at higher CBD concentrations. To our knowledge, our study is the first to describe such differential effects of CBD on cell cycle arrest. This phenomenon confirms previous reports suggesting that CBD has the ability to induce multiphasic cell cycle arrest similar to the effect of hydrogen peroxide (H_2O_2) [45]. These discoveries underscore the complex nature of CBD's effects on cell cycle regulation and cellular apoptosis, necessitating further thorough investigation of the precise mechanisms underlying these effects in CCA cells. In addition, our results offer the intriguing possibility that CBD has the potential to target gemcitabine-resistant cancers. This is consistent with previous research that has underscored the ability of CBD to induce apoptosis, arrest the cell cycle, and increase chemosensitivity in resistant pancreatic cancer cells by inducing endoplasmic-reticulum stress [46].

Cannabinoids have previously shown strong anti-tumor effects in a subcutaneous xenograft tumor model with nude mouse by inducing apoptosis and inhibiting proliferation [47, 48]. Our research investigates the potential of CBD against gemcitabine-resistant CCA in an animal model. This approach provides a more holistic understanding of the scientific basis for medical applications, emphasizing efficacy and safety as critical steps in establishing a solid foundation for future clinical trials. These findings provide critical information for the potential application of CBD in the treatment of other chemoresistant cancers. Previous studies have demonstrated the ability of CBD to suppress tumor growth in cisplatin-resistant non-small cell lung cancer [23]. However, it's important to note that our current investigation didn't reveal a clear relationship dependent on dosage, which underscores the need for further validation. The lack of such a relationship could be due to various possible factors, such as hormesis or a U-shaped response curve [49], well-documented in both medical research and plant sciences [50]. CBD might exhibit a biphasic effect, where lower doses

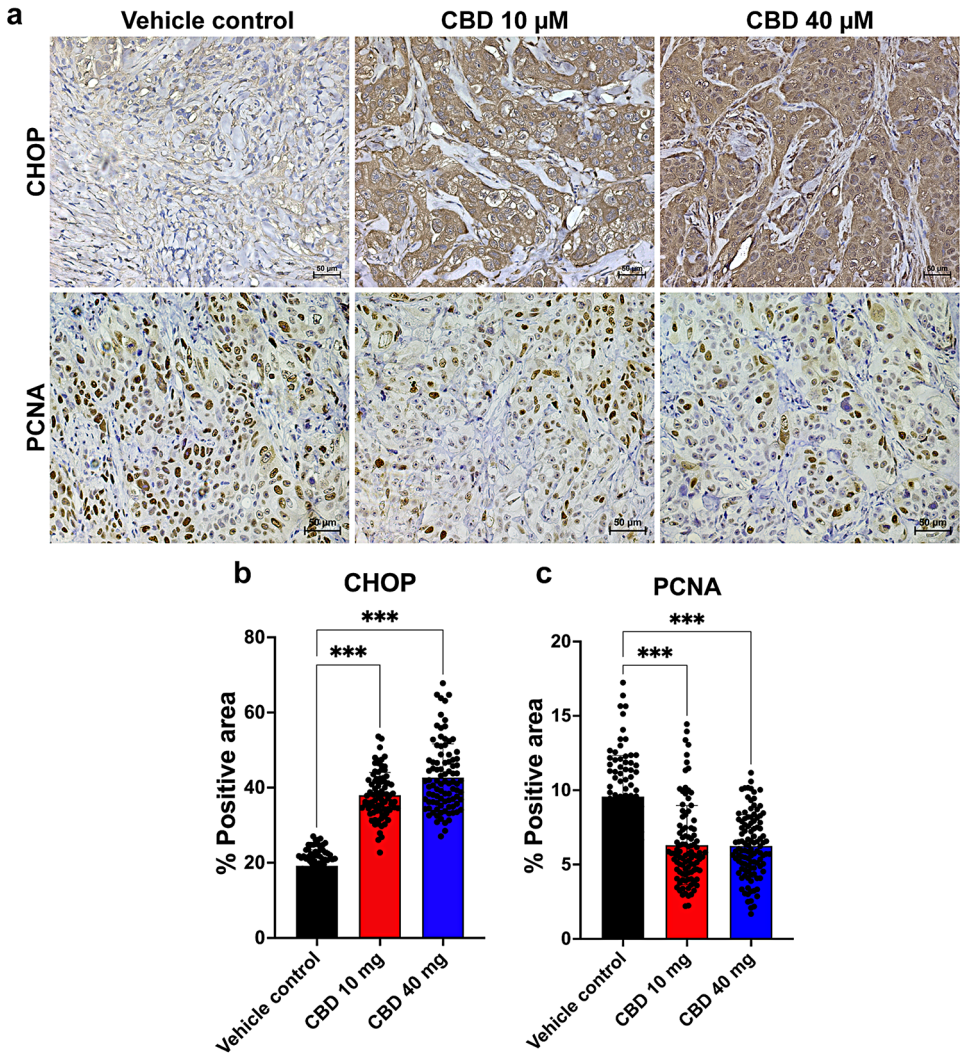

Fig. 7 The effect of CBD on the expression of CHOP and PCNA in tumor tissues. Mouse was treated with either sesame oil (vehicle control) or CBD at doses of 10 and 40 mg/kg·Bw for 9 days. Positive staining within the nucleus and cytoplasm produced a characteristic brown-yellow color with a fine-grained appearance (**a**). Image was visualized under ×200 magnification. The grading of CHOP and PCNA positive cells in the tumor mass is presented as the mean value along with the standard deviation (**b-c**). Significance values are indicated as follows: * $P<0.05$, ** $P<0.01$, and *** $P<0.0001$

stimulate anti-cancer pathways, while higher doses result in diminishing returns. Additionally, receptor saturation could occur [51], meaning all available receptors are filled with CBD, preventing further increases in CBD concentration from producing additional inhibition of cancer-cell proliferation or induction of apoptosis. This could lead to a plateau or even a decrease in the anti-cancer effects of CBD at higher doses. In the context of safety considerations, the doses of 10 and 40 mg CBD used in the xenograft mouse model were in line with many previous animal studies [18, 29]. We observed no obvious signs of toxicity or adverse effects associated with CBD administration in our experimental animals, similar to many previous reports [52, 53]. Nevertheless, the most effective dose of CBD and mode of administration of the drug for the potential therapeutic applications in the treatment of CCA patients require further investigation.

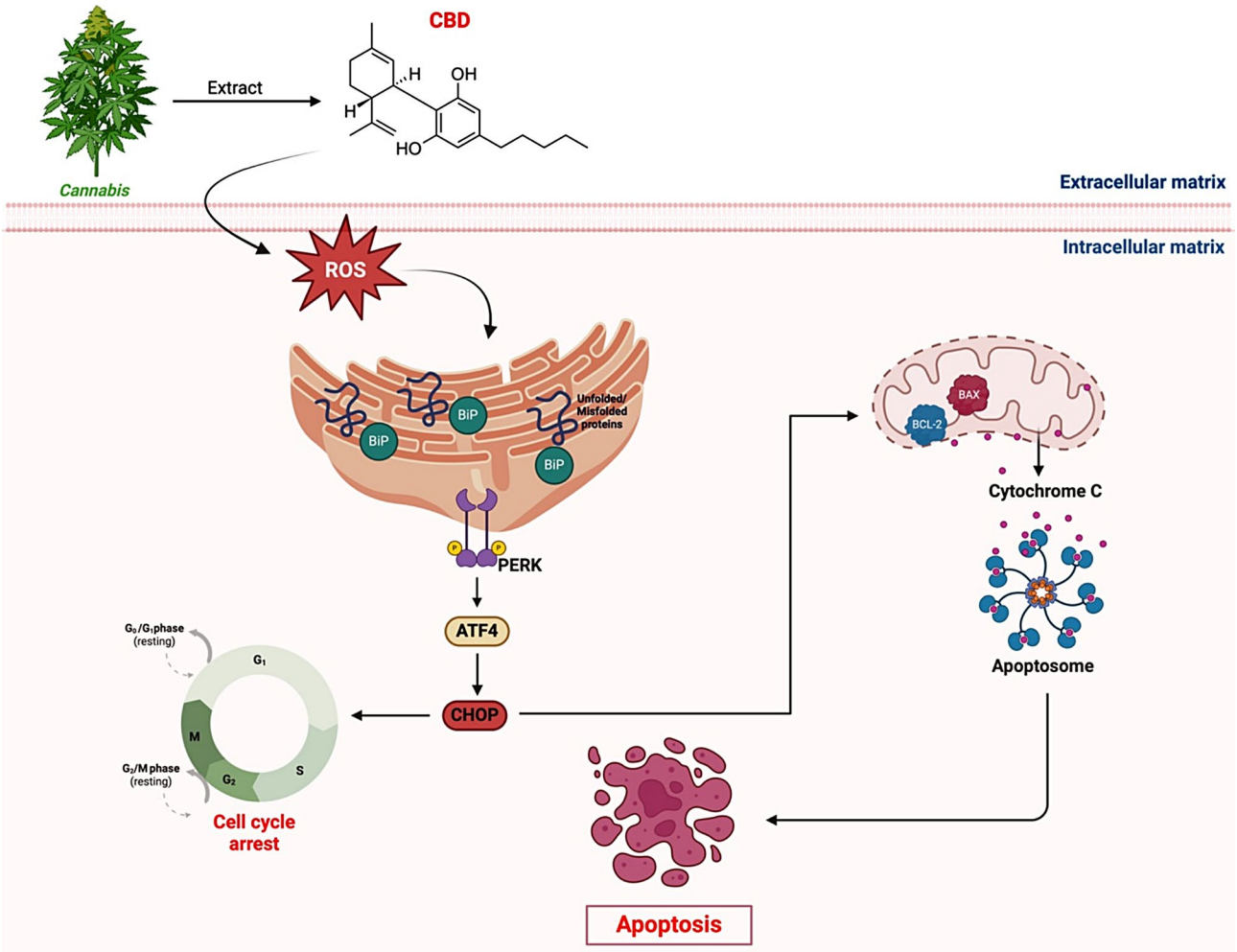

Fig. 8 The postulated mechanism underlying the effects of cannabinoid-induced apoptosis and cell cycle arrest revolves around the activation of endoplasmic-reticulum (ER) stress in gemcitabine-resistant CCA cells by CBD. The graphical representation of this proposed mechanism was created using BioRender.com (license number: YT26R9UJZP)

Conclusions

This study suggests that CBD may be a valuable therapeutic option for gemcitabine-resistant CCA, as it inhibits the growth of these resistant cells, induces apoptosis and disrupts the cell cycle. These results are in line with established oncology research and emphasize the potential of CBD as a multifaceted therapeutic agent against gemcitabine resistance in CCA. The promising results of our in vitro and in vivo experiments not only prompt further research to elucidate the underlying mechanisms, but also emphasize the necessity for rigorous clinical investigation to comprehensively evaluate the safety and efficacy profile of CBD in the context of combating gemcitabine resistance in CCA. This dual focus on safety and efficacy is critical for the potential translation of CBD into clinical applications not only in CCA but also in other cancers.

Abbreviations

CCA	Cholangiocarcinoma
CBD	Cannabidiol
GEM	Gemcitabine
CIS	Cisplatin
dNTP	Deoxynucleoside triphosphate
ER stress	Endoplasmic-reticulum stress
PERK	Protein kinase-like ER kinase
ATF6	Transcription factor 6
IRE1	Inositol-requiring ER-to-nucleus signal kinase-1
KKU-213B^{GemR}	Gemcitabine-resistant KKU-213B
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal bovine serum
DMSO	Dimethyl sulfoxide
PI	Propidium iodide
TSR %	Tumor suppression rate
PCNA	Proliferating cell nuclear antigen
H_2O_2	Hydrogen peroxide
DCFH-DA	Diacetyldichlorofluorescein

Supplementary Information

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Supplementary Material 1

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Author contributions
The collaborative efforts of the team resulted in a comprehensive and successful research project. T.P. led the way with contributions to conceptualization, methodology, validation, investigation, and formal analysis, while also playing a key role in drafting the original manuscript. P.T. and K.I. collaborated on various aspects, including conceptualization, investigation, formal analysis, and pathology analysis. Si.K. focused on investigation, validation, and formal analysis, providing valuable insights into the data. R.T., A.C., C.S., and K.V. actively participated in the project, contributing to the investigation and formal analysis phases. N.C. and Su.K. played crucial roles in methodological aspects and investigation throughout the study. S.P. demonstrated leadership through conceptualization, project administration, and successful acquisition of funding, in addition to substantial contributions to writing and editing. P.P. was instrumental in shaping the project, taking on responsibilities in conceptualization, project administration, funding acquisition, and making significant contributions to writing and editing.

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Data availability
The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate
The protocol of study in CCA cell lines was approved by Khon Kaen University Ethics Committee for Human Research based on the declaration of Helsinki and the ICH good clinical practice guidelines (HE661473). Animal experiments were conducted following the Animal Ethics Committee of Khon Kaen University and the National Research Council of Thailand's guidelines (No. IACUC-KKU-62/65). All methods are reported in accordance with ARRIVE guidelines for the reporting of animal experiments.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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