13-Acetoxy sarcocrassolide induces apoptosis in human hepatocellular carcinoma cells through mitochondrial dysfunction and suppression of the PI3K/AKT/mTOR/p70S6K signalling pathway

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ABSTRACT

Context: 13-Acetoxy sarcocrassolide, isolated from the Taiwanese soft coral Sarcophyton crassocaule Moser (Alcyoniidae), has biological activity and induces apoptosis in hepatocellular carcinoma cells.

Objective: To elucidate the mechanisms underlying apoptosis induced by 13-acetoxy sarcocrassolide in HA22T and HepG2 hepatocellular carcinoma cells.

Material and methods: MTT and morphology assays were employed to assess the anti-proliferative effects of 13-acetoxy sarcocrassolide (1-5 μM). TUNEL/DAPI staining and annexin V-fluorescein isothiocyanate/propidium iodide staining were used to detect apoptosis. Cells were treated with 13-acetoxy sarcocrassolide (0, 1, 2, and 4 μM) for 24 h, and the mechanism of cells apoptotic was detected by western blotting. Cells treated with DMSO were the control.

Results: Survival of the cells decreased with the addition of 13-acetoxy sarcocrassolide, and at 4 μM cell survival was inhibited by approximately 40%. After treatment of cells with 13-acetoxy sarcocrassolide, the incidence of early/late apoptosis to be 0.3%/0.5%~5.4%/22.7% for HA22T cells, in the HepG2 cells were 0.6%/2.0%~14.4%/23.7%. Western blotting analysis showed that the expression of Bax, Bad, cleaved caspase 3, caspase 9, cleaved-PARP-1, cytochrome c, and p-4EBP1 increased with an increasing concentration of 13-acetoxy sarcocrassolide (0, 1, 2, and 4 μM), whereas that of Bcl-2, Bcl-XL, Mcl-1, p-Bad, p-PI3K, p-AKT, p-mTOR, p-70S6K, p-S6, p-eIF4E, and p-eIF4G decreased.

Discussion and conclusions: Apoptosis induced by 13-acetoxy sarcocrassolide in HA22T and HepG2 cells is mediated by mitochondrial dysfunction and inactivation of the PI3K/AKT/mTOR/p70S6K pathway. The potential of 13-acetoxy sarcocrassolide as a chemotherapeutic agent should be further assessed for use in human hepatocellular carcinoma treatment.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide and the third most common cause of death due to cancer (Ferenci et al. 2010). Currently, HCC treatment methods include surgical resection, radiation therapy, chemotherapy, targeted therapy, and liver transplantation, which depend on the progression of the tumour (Lurje et al. 2019; Viveiros et al. 2019). Surgery is the main treatment regimen for early-stage HCC, but since patients are usually diagnosed at a late stage, adjuvant therapy including systemic chemotherapy is often administered (De Rosa et al. 2015). However, chemoresistance is a major obstacle to chemotherapy and often results in a poor prognosis (Lohitesh et al. 2018). Therefore, it is important to develop new drugs and understand the mechanisms of these drugs.

Apoptosis can be induced by intrinsic and/or extrinsic pathways (Denicourt and Dowdy 2004; Matthews et al. 2012). Research has shown that the mitochondria and endoplasmic reticulum, in the event of damage or stress to such organelles, can trigger the intrinsic pathway to induce apoptosis (Ron and Walter 2007; Matthews et al. 2012; Zielinski et al. 2013). The intrinsic pathway, also known as the mitochondria-mediated apoptotic pathway, is mainly mediated by the Bcl-2 family proteins, such as the apoptotic proteins Bad and Bax and antiapoptotic protein Bcl-2. Bax, translocating from the cytoplasm to the mitochondria after cell stimulation, triggers the formation of pores in the mitochondria and facilitates cytochrome c release, which is followed by the activation of caspase 9 and downstream caspase 3, thereby leading to the cleavage of poly (ADP-ribose) polymerase-1 (PARP-1) (Amarante-Mendes et al. 1998; Graham and Chen 2001; Putcha et al. 2002; Broughton et al. 2009).

There are several signalling pathways involved in the malignant progression of normal cells, including heat shock protein (Gao et al. 2019), MAPK (Bhatt et al. 2021), AMPK (Visnjic et al. 2019), and PI3K (Narayanankuty et al. 2019, 2020; Lengyel et al. 2020; Wong et al. 2021) signalling, among others, with PI3K being an important signalling pathway associated with metabolism, cell survival, motility, and cancer progression (Engelman 2009; Vanhaesebroeck et al. 2010). Previous studies have shown that PI3K is closely associated with various types of tumours,
including colorectal cancer (Papadatos-Pastos et al. 2015; Narayanankutty 2019), breast cancer (Qin et al. 2018), liver cancer (Golob-Schwarzl et al. 2017; Narayanankutty 2021), and pancreatic cancer (Murthy et al. 2018).

PI3K is the first molecule in the PI3K signalling system and can be activated via interactions with growth factor receptors that harbour phosphorylated tyrosine residues, resulting in conformational changes in the dimer, or through direct binding between Ras and p110 (Ward and Finan 2003). Activated PI3K induces AKT phosphorylation, leading to AKT activation, and activated AKT promotes cell survival by phosphorylating mTOR and its downstream molecules (Zhou et al. 2004; Rosner et al. 2008). The PI3K/Akt/mTOR signalling pathway has also been shown to inhibit apoptosis and promote cell survival and proliferation, and it is associated with angiogenesis, tumorigenesis, invasion, and metastasis after activation in several malignant tumours (Engelman 2009; Fruman and Rommel 2014; Wang et al. 2018).

Compared to conventional chemotherapeutic agents, natural products often have promising therapeutic value and fewer side effects (Stonik and Fedorov 2014; Raffa et al. 2017). In recent years, molecules from natural products have become increasingly important for the development of new drugs for the treatment of various cancers, and they also provide alternative therapeutic strategies for HCC (Lichota and Gwozdzinski 2018). Marine soft corals are rich in bioactive substances with anticancer, antiinflamatory, antiviral, and cytotoxic effects (Radhika 2006). Recent studies have shown that flavicidoxide-13-acetate, 7-acetylsinumaximol B, sinularin, 11-epi-sinulariolide acetate, and 13-acetoxysarcocrassolide, isolated from soft corals, have apoptotic and antitumor effects (Lin et al. 2014; Su et al. 2014; Wu et al. 2016, 2020; Tsai et al. 2018).

Flavicidoxide-13-acetate, isolated from marine soft coral Sinularia gibberosa Tixier-Durivault (Alcyoniidae), has been found to inhibit the activation of the FAK/PI3K/Akt/mTOR signalling pathway, as well as the expression of MMP-2 and MMP-9, leading to the inhibition of the metastasis of HA22T and HepG2 HCC cells (Wu et al. 2020). 7-Acetylsinumaximol B is a biologically active compound extracted from the cultivated soft coral Sinularia sandoensis Verseveldt (Alcyoniidae) and has been found to induce the apoptosis and autophagy of human gastric cancer cells by causing mitochondrial dysfunction and activating the PERK/eIF2α/CHOP signalling pathway (Tsai et al. 2018). Sinularin, which is an active compound isolated from the cultivated soft coral Sinularia flexibilis Quoy & Gaimard (Alcyoniidae), can induce the apoptosis of gastric cancer cells by triggering mitochondrial dysfunction and inactivating the p3K/Akt/mTOR pathway (Wu et al. 2016). Further, Lin et al. (2014) found that 11-epi-sinulariolide acetate (an active compound isolated from the cultured soft coral Sinularia flexibilis) reduces the migration and invasion of human HCC cells by downregulating the activation of ERK1/2, p38MAPK, and FAK/PI3K/AKT/mTOR signalling pathways. Moreover, Liu et al. (2020) found that 13-acetoxysarcocrassolide, isolated from marine soft coral Lobophytum crissum Von Marenzeller (Alcyoniidae), can exert its cytotoxic activity on oral cancer cells by promoting ROS generation and inhibiting antioxidant enzyme activity, and by disrupting the Keap1/Nrf2/p62/SQSTM1 pathway. Su et al. (2014) pointed out that 13-acetoxysarcocrassolide, an active compound extracted from cultivated soft coral, can induce the apoptosis of human gastric carcinoma cells by activating the p38 MAPK and JNK pathways and inhibiting the PI3K/AKT pathway.

The compound, 13-acetoxysarcocrassolide (Figure 1), extracted from the soft coral Sarcophyton crassoceae, exerts extensive cytotoxic effects against bladder female transitional carcinoma (BFTC) cells (Su et al. 2011) and human gastric adenocarcinoma (AGS) cells (Su et al. 2014). However, to our knowledge, the effects of 13-acetoxysarcocrassolide on HCC cells have not been investigated. Therefore, in this study, we used human HCC cells, namely HA22T and HepG2 lines, to observe the cytotoxic effects of 13-acetoxysarcocrassolide in vitro and investigate the mechanism through which it induces apoptosis.

Materials and methods

Reagents

13-Acetoxysarcocrassolide was purified after extracting it from the cultured Formosa soft coral S. crassoceae, according to the method described by others (Duh et al. 2000). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), Z-DEVD-FMK (caspase-3 inhibitor), Z-VAD-FMK (a cell-permeable pan-caspase inhibitor), a protease inhibitor cocktail, and rabbit anti-human β-actin antibodies were purchased from Sigma (St. Louis, MO, USA). Antibodies against pro-caspase 9, cleaved-caspase 9, pro-caspase 3, cleaved-caspase 3, cleaved-PARP-1, Bax, Bad, Bcl-xl, Mcl-1, p-Bad, PARP-1, Bel-2, PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR, p70S6K, p-p70S6K, 4EBP1, p-4EBP1, p-S6, p-eIF4B, and p-eIF4E, and goat anti-rabbit and horseradish peroxidase-conjugated immunoglobulin G were obtained from Cell Signalling Technology (Danvers, MA, USA). Dulbecco’s modified Eagle’s medium (DMEM) and foetal bovine serum (FBS) were obtained from Biowest (Nuaillé, France).

Cell culture

HA22T and HepG2 cells were procured from the Food Industry Research and Development Institute (Hsinchu, Taiwan). We selected and cultured HA22T and HepG2 HCC cells to perform the analyses. These cells were cultured in DMEM with 4 mM l-glutamine, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose, and 10% (v/v) FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 mM sodium pyruvate were added. The cells were maintained in an incubator with 5% CO2 at 37°C. Cells were treated with different concentrations of 13-acetoxysarcocrassolide (1–5 μM), with DMSO added for the control group, and all cells were incubated for 24 h for subsequent analyses. Cell morphology was observed under an inverted light microscope.
MTT assays

The cells were cultured in 24-well plates at $1 \times 10^4$ cells/well. After adherence, cells were treated with different concentrations of 13-acetoxysarcocrassolide for 24 h. After removing the culture media, the cells were washed three times with phosphate-buffered saline (PBS) and 200 $\mu$L of 1 mg/mL of MTT was added to each well. The cells were then incubated at 37°C for 15 min, and the solution was removed from each well. Next, 200 $\mu$L of DMSO was added to each well to dissolve the formazan crystals. Absorption intensities were analysed at 590 nm using an enzyme-linked immunosorbent assay plate reader, and the results were used to quantify the viability of the cells.

Cell morphology assay

HA22T and HepG2 HCC cells were inoculated in 24-well plates ($1 \times 10^5$ cells/well). After 24 h, different concentrations of 13-acetoxysarcocrassolide (final concentrations of 1, 2, and 4 $\mu$M) were added to the cells in 2 mL of serum-complete medium. Cells were scanned using a high-resolution scanner (Scan Maker 9800XL, Microtek, Hsinchu, Taiwan).

TUNEL/DAPI staining

TUNEL/DAPI staining was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Indianapolis, IN, USA), and DAPI (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured on coverslips at $1 \times 10^4$ cells/well. After adhesion, the cells were treated with different concentrations of 13-acetoxysarcocrassolide for 24 h. Next, the culture medium was removed, and the cells were washed with cold PBS three times. Next, 30 $\mu$L of 37% formaldehyde for 20 min at room temperature and then washed three times with PBS before the cell membranes were permeabilized with 0.1% Triton-X100 for 2 min on ice. Subsequently, the cells were washed with cold PBS three times. Next, 30 $\mu$L of pre-chilled TUNEL staining solution and enzyme were mixed in a 1:19 ratio before adding them to the cells; this was incubated at 37°C for 1 h. Cells were then washed three times with PBS and stained with $1 \mu$L of DAPI diluted with 1 mL of PBS for 10 s. The samples were placed on a slide and observed under a fluorescence microscope.

Annexin V/propidium iodide (PI) staining

Annexin V/PI staining was performed using the Annexin V-FITC Apoptosis Detection Kit (Strong Biotech Corporation, Taipei, Taiwan) according to the manufacturer’s instructions, the cells were inoculated in 6-well plates at a concentration of $3 \times 10^5$ cells/well. After adhesion, the cells were treated with different concentrations of 13-acetoxysarcocrassolide for 12 h and then harvested by trypsinization. Cells were centrifuged at 2000 rpm for 5 min, and the cell pellet was re-suspended in 1 mL of PBS and transferred to a 1.5 mL Eppendorf tubes. The cells were washed with PBS three times and then with 100 $\mu$L Annexin V binding buffer, followed by the addition of 5 $\mu$L of Annexin V and PI. After incubation for 10–15 min in the dark, Annexin V binding buffer was added to make up a total volume of 1 mL. The cells were then transferred to a flow cytometer tube and analysed using a flow cytometer (Cytometric FC 500, Beckman, Atlanta, GA, USA).

Protein extraction and estimation

The cells were treated with different concentrations of 13-acetoxysarcocrassolide for 24 h and incubated with Cell Extraction Buffer (BioSource International, Camarillo, CA, USA) and protease inhibitor cocktail (Sigma) for cell disruption. After centrifugation at 12,000 rpm for 10 min, the supernatant was collected, and the amount of protein in samples was quantified using the Bradford reagent (Bio-Rad).

Western blotting analysis

After protein quantification, 30 $\mu$g of each sample was used for immunostaining. Next, after performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a Transphor TE 62 (Hoeffer) was used at 400 mA for 1.5 h to electrophoretically transfer the proteins from the electrophoretic gel to the polyvinylidene fluoride (PVDF) membrane (Millipore). After transfer, different primary antibodies were added, and the blot was incubated at 4°C overnight in accordance with the dilution required for the reaction. Next, the PVDF membrane was washed three times with PBST (10 mM NaH2PO4, 130 mM NaCl, 0.05% Tween 20) for 10 min each, and the secondary antibody (with horseradish peroxidase conjugate; 1:5,000 in blocking solution) was added and the reaction was allowed to occur for 1 h. The membranes were again washed three times with PBST and finally assayed with an ECL Western Blotting Reagents (Pierce Biotechnology; Rockford, IL, USA). The western blot data were quantified with Image J software (NIH, Bethesda, MD, USA).

Data compilation and statistical analysis

The data of cell viability were obtained by aforementioned experiments. The independent t-test was using to analysis the difference by SPSS version 24.0 software (IBM, Endicott, NY, USA). The results indicate a statistically significant difference while p value < 0.05.

Results

Effect of 13-Acetoxysarcocrassolide on the viability of HA22T and HepG2 cells

The survival rate of HA22T and HepG2 HCC cells after treatment with 1–5 $\mu$M of 13-acetoxysarcocrassolide (Figure 1) for 24 h was measured using an MTT assay. The results showed that the survival decreased as the concentration of 13-acetoxysarcocrassolide increased, and at 4 $\mu$M, cell survival was inhibited by approximately 40% (Figure 2A). In addition, we analysed the cell morphology after a 24 h treatment with 1, 2, and 4 $\mu$M of 13-acetoxysarcocrassolide. The results showed that the cell survival decreased as the concentration of 13-acetoxysarcocrassolide increased (Figure 2B). These results indicated that 13-acetoxysarcocrassolide can inhibit hyperplasia in HA22T and HepG2 cells.

13-Acetoxysarcocrassolide induces apoptosis in HA22T and HepG2 cells

Since apoptosis causes changes to cell morphology and leads to cellular shrinkage, chromosome condensation and DNA fragmentation in the nucleus, we used TUNEL and DAPI fluorescence staining analysis to observe the effects of 13-acetoxysarcocrassolide on the apoptosis of HA22T and HepG2 cells.
cells. When 13-acetoxysarcocrassolide was added to HA22T and HepG2 cells and the cells were incubated for 24 h, TUNEL staining was positive in HA22T and HepG2 cells treated with 4 \( \mu \text{M} \) 13-acetoxysarcocrassolide, as observed by fluorescence microscopy (Figure 3). In addition, we used a fluorescent stain, fluorescein isothiocyanate (FITC-labeled Annexin V, green fluorescence), in combination with PI, to further investigate the phenomenon of apoptosis induced by 13-acetoxysarcocrassolide in HA22T and HepG2 cells through flow cytometry. Results showed that the rate of early apoptosis was 0.3% in untreated HA22T cells, which increased to 5.4% when these cells were treated with 4 \( \mu \text{M} \) 13-acetoxysarcocrassolide; similarly, in untreated HepG2 cells, the rate of early apoptosis was 0.6%, which increased to 14.4% after treatment with 4 \( \mu \text{M} \) 13-acetoxysarcocrassolide. Moreover, the rate of late apoptosis was 0.5% without 13-acetoxysarcocrassolide treatment in HA22T cells, which significantly increased to 22.7% after treatment with 4 \( \mu \text{M} \) 13-acetoxysarcocrassolide; this rate was 0.2% in HepG2 cells without 13-acetoxysarcocrassolide treatment, and similarly, this rate increased to 23.7% after treatment with 4 \( \mu \text{M} \) 13-acetoxysarcocrassolide (Figure 4). These results indicated that treatment with 13-acetoxysarcocrassolide can lead to apoptosis in HA22T and HepG2 cells.

### 13-Acetoxysarcocrassolide-induced apoptosis is associated with mitochondrial dysfunction in HA22T and HepG2 cells

Apoptosis can be induced by intrinsic and/or extrinsic pathways (Denicourt and Dowdy 2004; Matthews et al. 2012). The intrinsic pathway, also known as the mitochondria-mediated apoptotic pathway, is mainly induced by Bcl-2-family proteins. To investigate the mechanism underlying 13-acetoxysarcocrassolide-induced apoptosis in HA22T and HepG2 cells, we used western blotting to analyse the expression of mitochondria-associated proteins in cells that were treated with different concentrations of 13-acetoxysarcocrassolide (1, 2, and 4 \( \mu \text{M} \)). Results showed that the expression of Bax, Bad, and cytochrome c increased with an increasing concentration and time, whereas the expression of Bcl-2, Bcl-xL, Mcl-1, and p-Bad decreased (Figure 5). This result indicated that 13-acetoxysarcocrassolide triggered apoptosis in HA22T and HepG2 cells, which was associated with the expression of Bcl-2-family proteins. Furthermore, the increased expression of Bax and cytochrome c after treatment with 13-acetoxysarcocrassolide also indicated that this compound suppressed mitochondrial activation.

When the mitochondria are inactivated, cytochrome c is released into the cytoplasm, which leads to the activation of caspase 9 and caspase 3, thereby inducing apoptosis. To investigate whether 13-acetoxysarcocrassolide-induced apoptosis is associated with caspase activation, we performed western blotting to analyze the expression of caspases in association with apoptosis in 13-acetoxysarcocrassolide-treated HA22T and HepG2 cells. Our results showed that the expression of pro-caspase 3 and pro-caspase 9 proteins decreased as the concentration of 13-acetoxysarcocrassolide increased, whereas that of cleaved-caspase 3 and cleaved-caspase 9 increased (Figure 6A). In addition, the expression of cleaved PARP-1 was also elevated with an increase in the 13-acetoxysarcocrassolide concentration. We suspected that 13-acetoxysarcocrassolide-induced apoptosis was mediated by mitochondrial dysfunction. We also used Z-DEVD-FMK (caspase 3 inhibitor) and Z-VAD-FMK (a cell-permeable pan-caspase inhibitor) to further validate the role of the caspase cascade in 13-acetoxysarcocrassolide-induced apoptosis in HA22T and HepG2 cells. The results showed that cell viability increased after the addition of the inhibitors (Figure 6B), suggesting that...
13-acetoxysarcocrassolide-induced apoptosis in HA22T and HepG2 cells occurred via caspase 3 and caspase 9.

**13-Acetoxysarcocrassolide inhibits the PI3K/AKT/mTOR/P70S6 signalling pathway**

Studies have shown that over-activated PI3K/AKT/mTOR signalling pathway inhibits apoptosis in several malignant tumours, promotes cell survival and proliferation, and is associated with angiogenesis, tumorigenesis, invasion, and metastasis (Engelman 2009; Fruman and Rommel 2014; Wang et al. 2018). Therefore, we studied whether the induction of apoptosis could be achieved by suppressing PI3K/AKT/mTOR signalling in HA22T and HepG2 cells after 13-acetoxysarcocrassolide treatment. The results showed that the expression of PI3K, AKT, and mTOR in HA22T and HepG2 cells did not change significantly after 13-acetoxysarcocrassolide treatment, whereas the expression of p-PI3K, p-AKT, and p-mTOR was significantly decreased. Studies have shown that p-mTOR activates p-p70S6K, eIF4B,
p-S6, and 4EBP1, which in turn affects protein translation and proliferation (Rosner et al. 2008; Boer et al. 2010). In addition, eIF4E activity is regulated by the PI3K/mTOR pathway (Joshi and Platanias 2014; Saxton and Sabatini 2017). Our results showed that the expression of p-70S6K, p-S6, p-eIF4E, and p-eIF4B was decreased in HA22T and HepG2 cells treated with 13-acetoxysarcocrassolide but that the expression of p-4EBP1 was increased (Figure 7). Based on these results, we concluded that cytotoxicity and apoptosis can be achieved by inhibiting the PI3K/Akt/mTOR/P70S6 signalling pathway in HA22T and HepG2 cells treated with 13-acetoxysarcocrassolide.

Discussion

Chemotherapy uses drugs that directly destroy cancer cells or inhibit their growth and metastasis. However, chemoresistance is still a problem to be solved. In recent years, several researchers have screened natural active compounds of plants from terrestrial and marine environments and explored their antitumor activities (Lichota and Gwozdzinski 2018; Huang et al. 2021; Zhang et al. 2021). One such compound, 13-acetoxysarcocrassolide, extracted from the soft coral Sarcophyton crassocaule, exhibits broad-spectrum cytotoxicity against BFTC cells (Su et al. 2011) and human AGS cells (Su et al. 2014). In this study, the cytotoxic effect of 13-acetoxysarcocrassolide on HA22T and HepG2 cells was investigated, and changes in apoptotic signalling were assessed to determine the mechanism underlying apoptosis induction. The results showed that 13-acetoxysarcocrassolide inhibited the growth of HA22T and HepG2 cells, which was positively correlated with its concentration. This compound also induced early and late apoptosis in HA22T and HepG2 cells. Furthermore, the results indicated that 13-acetoxysarcocrassolide, a natural compound, has a cytotoxic effect on and induces apoptosis in HA22T and HepG2 cells.

Studies have reported that apoptosis can occur through intrinsic and/or extrinsic pathways (Wajant 2002; Denicourt and Dowdy 2004; Matthews et al. 2012). The intrinsic pathway of apoptosis is also known as the mitochondria-mediated apoptotic pathway. Mitochondria-mediated apoptosis is regulated by the Bcl-2 family of proteins, where the balance between the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2, the release of cytochrome c into the cytoplasm, and the activation of downstream caspases are all important factors responsible for triggering apoptosis (Putcha et al. 2002; Wang et al. 2006; Shankar and Srivastava 2007). The mitochondria-mediated apoptotic pathway is mainly caused by the movement of Bax, a pro-apoptotic protein of the Bcl-2 family, from the cytoplasm to the mitochondria after cell stimulation; it is binding to Bak, located in the outer mitochondrial membrane, to form a dimer; and its polymerization in the outer mitochondrial membrane to form a pore (Graham and Chen 2001; Broughton et al. 2009; Akpan and Troy 2013). After the formation of pores, apoptotic factors such as cytochrome c, apoptosis-inducing factor (AIF), and endonuclease G are released from the mitochondrial intermembrane space. Consequently, when cytochrome c is released into the cytoplasm, it binds to apoptotic protease activating factor-1 (Apaf-1), forming an apoptosome with pro-caspase 9, which then activates caspase 9. Next, caspase 3 is activated due to cleavage by caspase 9, which subsequently results in the cleavage of PARP-1, triggering apoptosis (Germain et al. 1999; Graham and Chen 2001; Broughton et al. 2009).

Our experimental results showed a reduction in the expression of the apoptosis inhibitory proteins Bcl-2, Bcl-xL, p-Bad, and Mcl-1 and an increase in the expression of the apoptosis-promoting proteins Bad, Bax, and cytochrome c after treating HA22T and HepG2 cells with 13-acetoxysarcocrassolide.

Figure 5. Western blotting for the analysis of mitochondria-associated protein expression after 13-acetoxysarcocrassolide treatment. HA22T and HepG2 cells were treated with different concentrations of 13-acetoxysarcocrassolide (1, 2, and 4 μM) for 24 h and analysed by western blotting. The results showed changes in cytochrome C, Bax, Bcl-2, Bcl-xl, Mcl-1, Bad, and p-Bad. β-Actin was used as the internal protein loading control.
In addition, there was an increase in the expression of cleaved-caspase 3, cleaved-caspase 9, and cleaved-PARP-1 and a reduction in the expression of pro-caspase 3 and pro-caspase 9. We also used Z-DEVD-FMK (caspase 3 inhibitor) and Z-VAD-FMK (a cell-permeable pan-caspase inhibitor) to further validate the role of the caspase cascade in 13-acetoxysarcocrassolide-induced apoptosis in HA22T and HepG2 cells. The results showed that cell viability was increased after addition of the inhibitors. Based on these findings, 13-acetoxysarcocrassolide triggers mitochondrial dysfunction in HA22T and HepG2 cells, leading to apoptosis.

Recently, many researchers have pointed out that the PI3K/AKT/mTOR pathway is one of the major pathways regulating cell proliferation, apoptosis, and cell migration (Street et al. 2004; Bader et al. 2005). In recent years, there have been numerous researchers investigating the effects of natural compounds on HCC. Flaccidoxide-13-acetate, which was isolated from the marine soft coral Sinularia gibberosa, can inhibit the metastasis of HA22T and HepG2 HCC cells by inhibiting the FAK/PI3K/AKT signalling pathway, as well as the expression of MMP-2 and MMP-9 (Wu et al. 2020). Further Kim et al. (2019) found that the ethanol extracts XS-5 and XS-6 of Xanthium strumarium L. (Asteraceae), a traditional herb used to treat inflammatory diseases, can significantly induce apoptosis and inhibit proliferation in HCC cells (Huh-7 and Hep3B) by inhibiting the PI3K/AKT/mTOR pathway. The findings reported by Hui et al. (2019) showed that a crude plant compound of Alpinia oxyphylla oil can inhibit the growth of HCC cells by blocking PI3K signalling.

Lin W et al. (2016) found that emodin, an active ingredient in the rhubarb root and rhizome, can induce the apoptosis of liver cancer cells via the MAPK and PI3K/AKT signalling pathways. Wu et al. (2015) pointed out that sinulariolide, an active compound extracted from cultivated soft coral, can suppress the migration and invasion of the hepatoma cell line HA22T and alter its metastatic capacity by inhibiting the MAPK, PI3K/AKT, and FAK/GRB2 signalling pathways. Lin et al. (2014) found that 11-epi-sinulariolide acetate, an active compound isolated from the cultured soft coral Sinularia flexibilis, can effectively inhibit the migration and invasion of the HCC cell line HA22T by downregulating the expression of MMP-2, MMP-9, and uPA via ERK1/2, p38MAPK, and FAK/PI3K/AKT/mTOR signalling pathways. Our results showed that there were no significant changes in the expression of PI3K, AKT, and mTOR in HA22T and HepG2 cells.
HepG2 cells treated with 13-acetoxysarcocrassolide, whereas a reduction in the expression of p-PI3K, p-AKT, and p-mTOR was observed, indicating inhibition of the PI3K/Akt/mTOR signalling pathway. Previous studies have shown that mTOR regulates the activity of p70S6K, 4EBP1, S6, and eIF4E (Raught et al. 2004; Joshi and Platanias 2014; Saxton and Sabatini 2017). Our experimental results showed that mTOR could inhibit the phosphorylation of p70S6K and the downstream factors S6 and eIF4B. Moreover, the results also showed an increase in the phosphorylation of 4EBP1, whereas the phosphorylation of eIF4E was decreased. In summary, we conclude that 13-acetoxysarcocrassolide inhibits activation of the PI3K/Akt/mTOR/p70S6K signalling pathway and further inhibits the phosphorylation of its downstream factors S6 and eIF4B to induce apoptosis. In addition, apoptosis is also induced via the 4E-BP1 and eIF4E pathways.

### Conclusions

13-Acetoxysarcocrassolide, a natural compound derived from a marine soft coral, can induce apoptosis in HA22T and HepG2 cells. According to the evidence found in the current study, 13-acetoxysarcocrassolide-induced apoptosis is mediated by mitochondrial dysfunction and PI3K/AKT/mTOR pathway inhibition in these cells. The hypothetical mechanism through which 13-acetoxysarcocrassolide exerts its effects on HA22T and HepG2 HCC cells is illustrated in Figure 8. We ultimately found that 13-acetoxysarcocrassolide has the potential to be used as a new drug for the treatment of cancer. There is a possibility that various signalling pathways may have effects on Hepatocellular Carcinoma Cells at the same time, the correlation between this pathway and other pathways can be further explored in the future, which will help to develop 13-acetoxysarcocrassolide as a new therapeutic drug.

![Figure 7. Western blotting for key proteins involved in the PI3K/Akt/mTOR signalling pathway in HA22T and HepG2 cells treated with 13-acetoxysarcocrassolide. After HA22T and HepG2 cells were treated with different concentrations of 13-acetoxysarcocrasside (1, 2, and 4 μM), the expression of AKT, p-AKT, PI3K, p-PI3K, mTOR, p-mTOR, p70S6K, p-p70S6K, p-4EBP1, p-S6, p-eIF4E, and p-eIF4B proteins was analysed. β-Actin served as a loading control.](image-url)
Author contributions
C.-I. Liu conceived and designed the experiments. J-H. Su isolated and identified the compound. Chang-Min Hsu and Y.-J. Lin were performed the experiments and analysed the data. C.-I. Liu and Chang-Min Hsu wrote the paper. All authors read and approved the final manuscript.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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