

ORIGINAL ARTICLE
Cellular and Molecular Biology

The Dual Effects of Δ^9 -Tetrahydrocannabinol on Cholangiocarcinoma Cells: Anti-Invasion Activity at Low Concentration and Apoptosis Induction at High Concentration

Surang Leelawat,¹ Kawin Leelawat,^{1,2} Siriluck Narong,² and Oraphan Matangkasombut¹Faculty of Pharmacy, Rangsit University, Patumthani, Thailand,¹ Department of Surgery, Rajavithi Hospital, Bangkok, Thailand²

ABSTRACT

Currently, only gemcitabine plus platinum demonstrates the considerable activity for cholangiocarcinoma. The anticancer effect of Δ^9 -tetrahydrocannabinol (THC), the principal active component of cannabinoids has been demonstrated in various kinds of cancers. We therefore evaluate the antitumor effects of THC on cholangiocarcinoma cells. Both cholangiocarcinoma cell lines and surgical specimens from cholangiocarcinoma patients expressed cannabinoid receptors. THC inhibited cell proliferation, migration and invasion, and induced cell apoptosis. THC also decreased actin polymerization and reduced tumor cell survival in anoikis assay. pMEK1/2 and pAkt demonstrated the lower extent than untreated cells. Consequently, THC is potentially used to retard cholangiocarcinoma cell growth and metastasis.

BACKGROUND

Cholangiocarcinoma is a dismal disease. Patients with cholangiocarcinoma are often diagnosed at an advanced stage. A disappointing 35–50% 3-year survival rate is achieved only when the histological margins are negative after surgery (1–4). The causes of death are due not only its rapid growth but also its tendency to invade adjacent organs and metastasize (1, 4, 5). Cholangiocarcinoma cells frequently metastasize to lymph nodes and distant organs (2). It is believed that the invasiveness and resistance to anoikis are major mechanisms of cell metastasis (6, 7). Cancer cell motility and degradation of the basement membrane are the main components of cancer's invasive properties. Anoikis is a form of apoptosis caused by the lack of cell survival signals generated from interaction with the extracellu-

lar matrix (ECM) in epithelial cells. Anoikis resistance affords cancer cells increased survival times in the absence of matrix attachment, facilitating their metastasis to secondary sites (8). The inhibition of cancer cell invasion and anoikis resistance in cholangiocarcinoma cells could reduce their ability to metastasize. Previous reports have demonstrated the anticancer effects of THC by inhibiting the phosphorylation of ERK1/2, JNK1/2, and Akt in human nonsmall cell lung cancer cell lines (9). Furthermore, our previous studies reported that activation of the Akt and ERK1/2 signaling pathways are important for cholangiocarcinoma cell proliferation and invasion (10, 11). To date, only gemcitabine plus platinum regimens demonstrate the considerable activity for this type of cancer (12). In this regard, the identification of a new therapeutic drug for treatment of cholangiocarcinoma is urgently required.

Cannabinoids, the active component of marijuana (*Cannabis sativa*), have been demonstrated to produce a wide spectrum of central and peripheral effects. The principle psychoactive active component is Δ^9 -tetrahydrocannabinol (THC). THC exerts a wide variety of biological effects by mimicking endogenous substances (endocannabinoids), such as anandamide and 2-arachidonoylglycerol, that activate specific cannabinoid receptors [cannabinoid 1 (CB1) and cannabinoid 2 (CB2) receptors] (13). In addition, THC has been shown to inhibit the

Keywords: Cholangiocarcinoma, Δ^9 -Tetrahydrocannabinol, Invasion, *Cannabis sativa*

Correspondence to:

Surang Leelawat

Faculty of Pharmacy

Rangsit University

Patumthani 12000, Thailand

email: surangleelawat@gmail.com

growth of tumor cells in culture and animal models by modulating the MEK1/2 signaling pathways (13, 14). DeMorrow et al. reported opposing actions of endocannabinoids on cholangiocarcinoma cells; anandamide was antiproliferative and proapoptotic, and 2-arachidonylglycerol stimulated cholangiocarcinoma cell growth (15). However, there is no study on the effects of THC in cholangiocarcinoma cells. Therefore, the present study investigated the antitumor activities (inhibition of proliferation, invasion, and anoikis resistance) of THC in cholangiocarcinoma cell lines.

MATERIAL AND METHODS

Materials

HAM's F12 medium and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). THC was kindly provided by the molecular laboratory of the department of surgery of Rajavithi Hospital. The preparation contained more than 95% THC. Polyclonal antibodies to MEK-1/2 (phosphorylated at Ser217/221 and total), Akt (phosphorylated at Ser473 and total), and JNK (phosphorylated at Thr183/Try185 and total) were purchased from Cell Signaling Technology (Beverly, MA, USA). 24-well Biocoat Matrigel invasion chambers (8 μ m) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

Cholangiocarcinoma specimens

Cholangiocarcinoma specimens were obtained from patients that underwent surgical resection at Rajavithi Hospital from January 2005 to December 2006. These tissues were separated into two parts: normal bile duct tissue and cholangiocarcinoma tissue, and the samples were kept in RNAlater solution at -20°C until isolation of the RNA. This study protocol was approved by the Rajavithi Hospital ethics committee.

Cell cultures

The human cholangiocarcinoma cell lines, RMCCA1 (derived from a peripheral cholangiocarcinoma patient (16)) and HuCCA1, were grown in HAM's F12 medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 humidified atmosphere. For signal transduction experiments, cells were starved overnight in serum-free medium.

Detection of cannabinoid receptor expression

Total RNA was isolated from cells and specimens using the RNeasy kit (Qiagen, Inc., Valencia, CA). The amplification primers were: (a) CB1 receptor: forward primer 5'-GTGTTCCACCGCAAAGAT-3' and reverse primer 5'-GCAGTTTCTCGCAGTTCC-3'; (b) CB2 receptor: forward primer 5'-TGGCAGCGTGACTATGAC-3' and reverse primer 5'-AAAGAGGAAGGCGATGAA-3'; and (c) pro-phobilinogen deaminase (PBGD) mRNA: forward primer 5'-ACACAGCCTACTTTCCAAGCGGAGCCAT-3' and reverse primer 5'-TCTTGTCCCCTGTGGTGGACATAGCAAT-3'. Amplification and detection were performed in a BIO-RAD

iCycler iQ system (Bio-Rad, Hercules, CA). The reaction conditions were reverse transcription at 60°C for 10 min, followed by amplification by initial denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s. The PCR products were detected with 1.5% agarose gel electrophoresis.

Cell proliferation assay

Cells were seeded in 96-well culture plates at a density of 10,000 cells/well followed by the addition of THC in various concentrations or vehicle (DMSO). The cells were then incubated for 24 hr before applying the WST-1 cell proliferation assay reagent (Roche Diagnostics, Laval, Quebec) according to the recommendation of the manufacturer. The percentage of proliferation was calculated based on the untreated cells.

Detection of DNA fragmentation

Cells were seeded in a 6-cm culture dish at a density of 250,000 cells. After 24 hr, cells were treated with THC in various concentrations or vehicle for 24 hr. Cells were then collected and DNA was extracted using the QIAmp DNA minikit (Qiagen, Inc., Valencia, CA) according to the recommendation of the manufacturer. DNA fragmentation was evaluated using 1.0% agarose gel electrophoresis.

TUNEL assay

Apoptotic cells were determined by TUNEL assays using the Apo-BrdU TUNEL assay kit (Invitrogen Co., Carlsbad, CA) following the manufacturer's instructions. Briefly, cells were fixed with 1% paraformaldehyde and ice-cold 70% ethanol for 30 min. Fixed cells were then labeled with BrdUTP using Terminal deoxynucleotide transferase (TdT) at 37°C for 60 min and stained with Alexa Fluor 488-labeled anti-BrdU antibody for 30 min at room temperature. To score for apoptosis, cells were counterstained with DAPI and at least 200 cells were counted under a fluorescent microscope at $400\times$ magnification to determine the percentage of apoptotic cells per experimental condition.

Cell migration assay and cell invasion assay

The migration of cholangiocarcinoma cells was assayed using a chamber with 8 μ m pore filters (Transwell, 24-well cell culture, Costar, Boston, MA). Cholangiocarcinoma cells were pretreated with vehicle or various concentrations of THC for 24 hr at 37°C , and then 50,000 THC-pretreated cells were added to the upper chamber. Serum-free media with various concentrations of THC was added to the lower chamber. The chambers were incubated for 12 hr at 37°C . After incubation, the filters were fixed, stained with hematoxylin, and counted in five random high-power fields under a light microscope as previously described (10). The invasion of cholangiocarcinoma cells was assayed in a 24-well Biocoat matrigel invasion chamber (8 μ m; Becton Dickinson, Franklin Lakes, NJ). Similar to the migration assays, 50,000 THC-pretreated cells were seeded in the upper

chamber while the bottom chamber contained THC in various concentrations or vehicle.

Detection of actin polymerization

Cholangiocarcinoma cells were treated with vehicle or THC in various concentrations, seeded on coverslips and incubated for 24 hr. The cells were fixed with 4% paraformaldehyde, permeabilized in 1% Triton X-100 for 15 min, and blocked with 1% BSA. The cells were then exposed to Alexa Fluor 488 Phalloidin (Molecular Probes, Eugene, OR) for 30 min and washed with PBS. Then, coverslips were mounted on the slide-glass using glycerol in TTBS. The cells were examined under a confocal laser scanning microscope (CLSM) (Olympus SV1000).

Anoikis assay

Cholangiocarcinoma cells were seeded in polyhydroxyethyl methacrylate (PolyHEMA)-coated 24-well culture plates at a density of 100,000 cells per well using Ham's F12 medium with 5% fetal bovine serum and THC in various concentrations. After 6 and 24 hr, the cells were collected and 1,000 cells/well were seeded into 96-well culture plates. The cells were incubated for 4 hr and were then evaluated with WST-1 reagent for the proliferation assay as previously described.

Western blotting analyses

For western blot analysis, 500,000 cells were seeded in a six-well culture plate, followed by treatment with THC in various concentrations. Cells were collected and then western blot analyses were performed as previously described (11). The blots were probed with antibody against phosphorylated Akt, MEK1/2, and JNK, and reprobed with antibody against unphosphorylated Akt, MEK1/2, and JNK. Chemiluminescent detection of antibody-antigen complexes revealed the target proteins on X-ray film.

Statistical analysis

The experiments were all performed in triplicate and the data were described as means with SD. Data between three or more groups were compared using the one way analysis of variance (ANOVA), followed by the Dunnett's *post hoc* test. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Expression of cannabinoid receptors in cholangiocarcinoma specimens and cell lines

In order to study the influence of THC on cholangiocarcinoma cells, the expression of cannabinoid receptors (CB1 and CB2 receptors) was investigated. RT-PCR demonstrated expression of CB1 in both cholangiocarcinoma cell lines (RMCCA1 and HuCCA1), 2 of 3 of the cholangiocarcinoma specimens and very weak expression in normal bile duct tissue. CB2 was found in both cholangiocarcinoma cell lines, 3 of 3 of cholangiocarcinoma specimens, and 2 of 3 normal bile duct tissues (Figure 1).

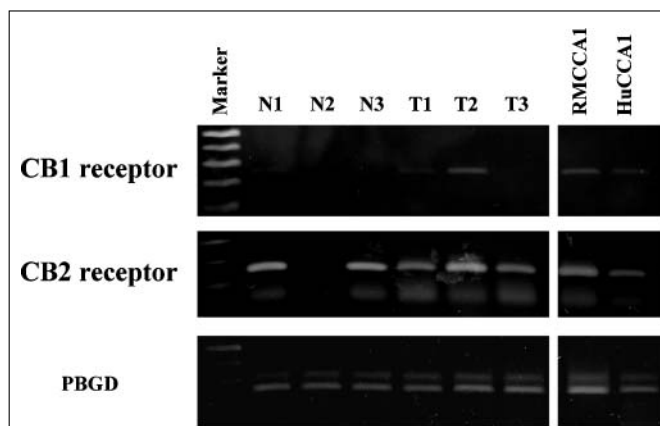


Figure 1. The expression of cannabinoid receptor mRNA in cholangiocarcinoma specimens and cell lines. Total RNA was isolated from the corresponding cholangiocarcinoma cell lines and specimens. The mRNA was reverse transcribed and amplified by PCR with selective primers for human CB1, CB2, or PBGD. Normal bile duct tissue (N) and cholangiocarcinoma tissue (T) were obtained from the same individual.

The effect of THC on cholangiocarcinoma cell proliferation

Cell proliferation assays were performed in cholangiocarcinoma cells treated with THC at concentrations of 5, 10, 20, 40, 80, and 100 μ M or vehicle (DMSO). After 24 hr of incubation, the results showed that THC at low concentrations (5–20 μ M) had no significant effect on the inhibition of cholangiocarcinoma cell proliferation when compared with vehicle-treated cells. However, at high concentrations of THC (40–100 μ M), cholangiocarcinoma cells were significantly inhibited in a dose dependent manner (Figure 2(a)).

The effect of THC on cholangiocarcinoma cell apoptosis

We next evaluated whether the THC-induced inhibition of proliferation was associated with cell apoptosis. DNA fragmentation was used to screen for cell apoptosis. We found that THC at concentrations of 40–100 μ M induced the formation of DNA fragmentation, whereas low concentrations of THC (5–20 μ M) had no effect (Figure 2(b)).

In addition, a TUNEL assay was performed to confirm the mechanism by which THC induced cholangiocarcinoma cell apoptosis. THC or control vehicle (DMSO) was added to cholangiocarcinoma cells and the cells were incubated for 24 hr. The number of apoptotic cells after exposure to 20 μ M THC was not significantly different from the controls. However, when cells were exposed to 40–100 μ M THC, the number of apoptotic cells significantly increased (RMCCA1: from $6.2 \pm 3.11\%$ at 0 μ M THC (vehicle) to $48.0 \pm 12.00\%$ at 40 μ M THC ($p < .001$) and to $83.0 \pm 9.46\%$ at 100 μ M THC ($p < 0.001$); HuCCA1: from $2.8 \pm 3.32\%$ at 0 μ M THC (vehicle) to $38.8 \pm 12.53\%$ at 40 μ M THC ($p < 0.001$) and to $70.3 \pm 20.00\%$ at 100 μ M THC ($p < 0.001$)) (Figures 2(c) and (d)).

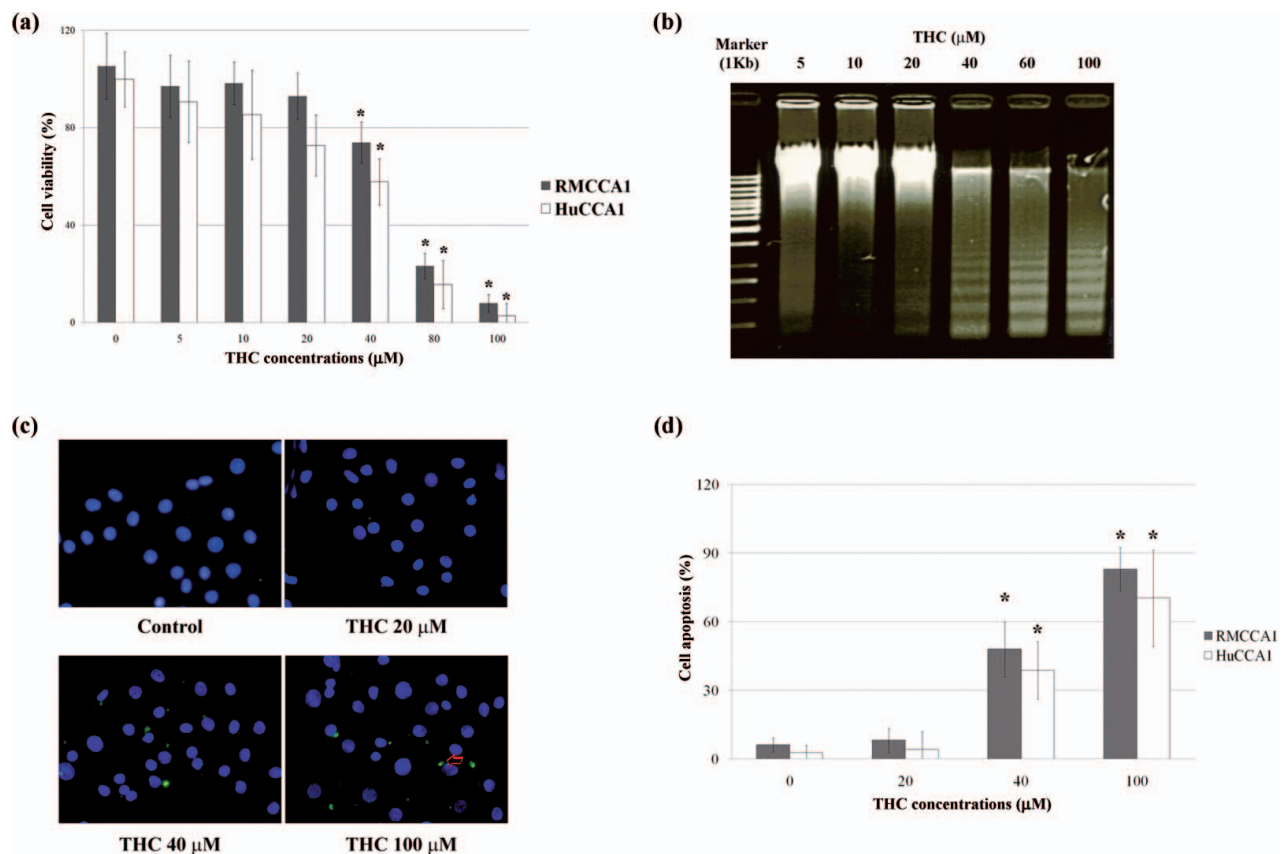


Figure 2. THC inhibits proliferation and induces apoptosis in cholangiocarcinoma cells. RMCCA1 and HuCCA1 cells were treated with THC at various concentrations (0–100 μM) for 24 hr. (a) Effect on cell proliferation was measured by WST-1 and analyzed by spectrophotometric analysis (absorbance = 450 nm). Results are represented by the mean \pm SE of three independent experiments, where the optical density value from vehicle-treated cells was set as 100% of proliferation. The black bar represents the value of cell proliferation from RMCCA1 cells, and the white bar represents the value of cell proliferation from HuCCA1 cells. (*, $p < .05$ versus the vehicle-treated cells). (b) DNA was extracted from vehicle or THC-treated HuCCA1 cells and DNA fragmentation was analyzed by gel electrophoresis. (c) Apoptotic cells (green spots) were detected with an ApopTag Staining kit and counterstained with DAPI (blue). RMCCA1 cells were treated with Vehicle (control), 20, 40, and 100 μM THC. Arrow indicates the apoptotic cells. (d) Cholangiocarcinoma cells were treated with 20–100- μM THC or vehicle. The TUNEL assay was done as described in the Section 2. The percentage of apoptotic cells in each group of treatments was demonstrated. The black bar represents the value of percent cell apoptosis from RMCCA1 cells, while the white bar represents the value of percent cell apoptosis from HuCCA1 cells. (Values shown as mean \pm SD, * $p < 0.05$ versus the vehicle treated cells).

The effect of THC on cholangiocarcinoma cell migration and invasion

The cholangiocarcinoma cells were treated with THC or vehicle for 24 hr and then migration and invasion assays were performed. The value from vehicle-treated cells was set as 100% of cell migration or invasion. Interestingly, a low concentration THC (20 μM) significantly diminished cholangiocarcinoma cell migration and invasion compared to vehicle-treated cells (cell migration: $33.6 \pm 11.04\%$ at 20 μM THC ($p < 0.001$); cell invasion: $25.6 \pm 7.92\%$ at 20 μM THC ($p < 0.001$)), (Figure 3(a)).

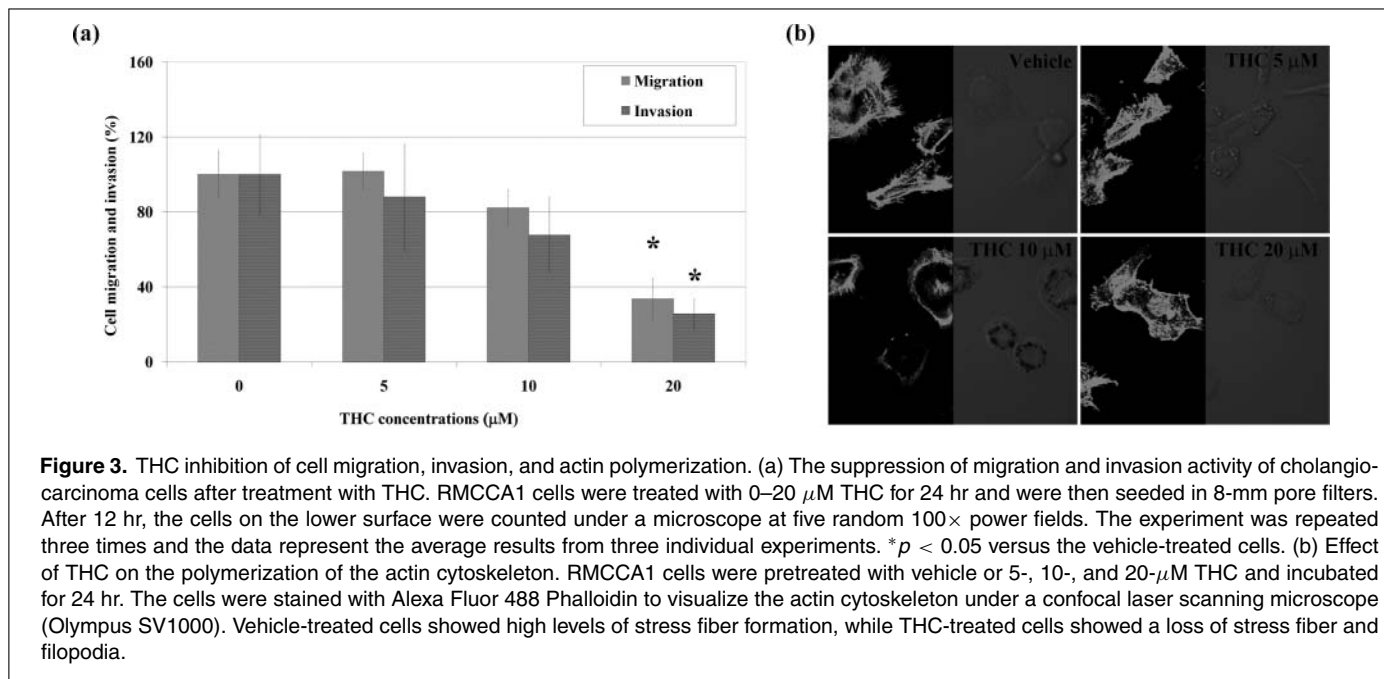
The effect of THC on the actin cytoskeleton of cholangiocarcinoma cells

We next investigated actin polymerization, which is implicated in cell motility mechanisms. Cholangiocarcinoma cells

were stained with phalloidin to detect actin polymerization. As shown in Figure 3(b), vehicle-treated cells displayed high levels of actin polymerization and distinct pseudopodia formation. After treating the cells with 5–20 μM of THC, actin polymerization and pseudopodia formation were decreased.

The effect of THC on inhibition of cholangiocarcinoma cell resistance to anoikis

In order to investigate the ability of THC to inhibit anoikis resistance in cholangiocarcinoma cells, RMCCA1 was seeded on polyHEMA plates and treated with 20 and 40 μM THC or vehicle. At 0, 6, and 12 hr after seeding, cell viability was determined by the WST-1 assay. The percentage of cell viability was set at 100% when cells were seeded on polyHEMA plates at 0 hr. At 6 and 12 hr after seeding on polyHEMA plates, 89.8 ± 3.95 and $54.9 \pm 7.91\%$ cell viability of the vehicle-treated cells



were observed, respectively. Cell viability of RMCCA1 treated with 20 μM THC was significantly decreased 12 hr after seeding compared with vehicle-treated cells. In addition, cell viability of RMCCA1 treated with 40 μM THC was significantly decreased at 6 and 12 hr after seeding compared with vehicle-treated cells (Figure 4).

The effect of THC on the phosphorylation of MEK1/2 and Akt in cholangiocarcinoma cells

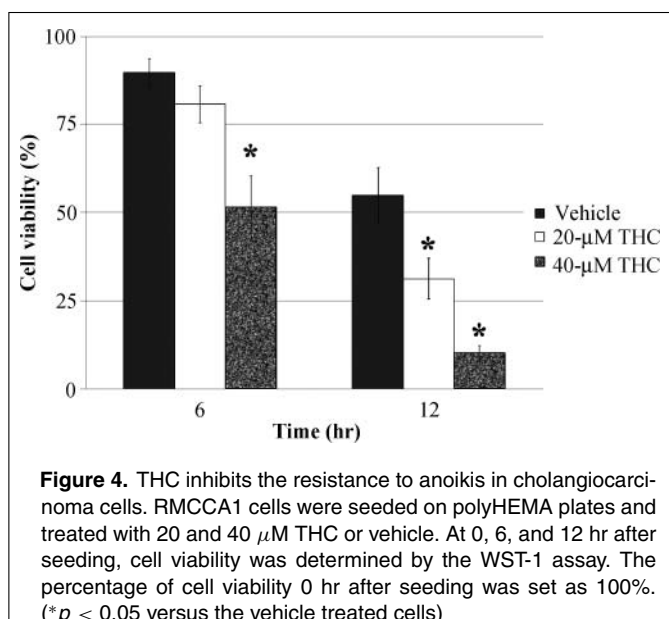
We evaluated the signaling pathways relevant to THC inhibition of cholangiocarcinoma cell progression. The

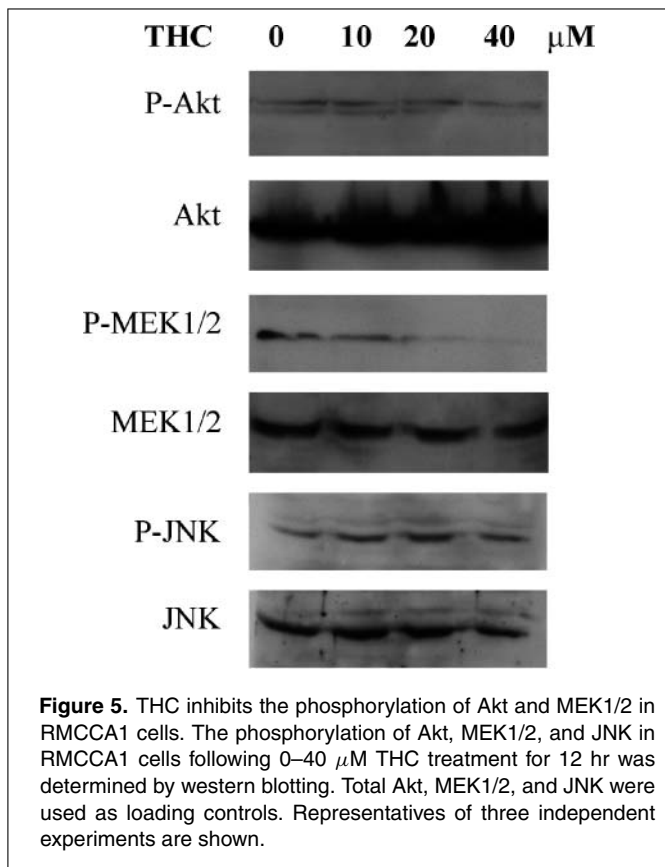
phosphorylation of Akt, MEK1/2, and JNK, which were previously demonstrated to be THC-mediated signaling molecules, was assayed (9). Cholangiocarcinoma cells were treated with THC at concentrations of 0–40 μM , and the cell lysates were collected for western blot analysis. With 40 μM THC, RMCCA1 demonstrated a lesser extent of phosphorylated Akt than vehicle-treated cells. Phosphorylated MEK1/2 was markedly decreased with 20–40 μM THC treatment. However, THC had no effect on the phosphorylation of JNK (Figure 5).

DISCUSSION

The antitumor effects of cannabinoids in cholangiocarcinoma cell lines were tested in this study. The findings are consistent with the previous literature describing the antitumor activity of cannabinoids in Jurkat leukemia T cells and colorectal cancer cells (14, 17). Previous studies have demonstrated that activation of the Raf/MEK1/2/ERK1/2 and PI3K/Akt cascades play important roles in cell survival (18, 19). From the results of western blots, the phosphorylation of MEK1/2 was decreased when the cells were treated with 20–40 μM THC, while the phosphorylation of Akt was decreased when the cells were treated with 40 μM THC. Our studies suggested that THC inhibits cancer cell proliferation and induces cancer cell apoptosis events that may involve a decrease in the phosphorylation of both MEK1/2 and Akt.

In cancer cells, high levels of actin polymerization at the periphery of the cells is key for the formation of pseudopodia, which are implicated in the enhancement of cancer cell migration and invasion (20). Treatment of cholangiocarcinoma cells with low doses of THC (10–20 μM) resulted in a dramatic decrease in actin polymerization. Our previous studies demonstrated that reduction of the phosphorylation of MEK1/2





caused a decrease in actin polymerization in the periphery of the cholangiocarcinoma cells, resulting in inhibition of cancer cell migration and invasion (10). In this study, we also found that the phosphorylation of MEK1/2 was decreased in RMCCA1 treated with 20 μM THC. Therefore, we suggested that the mechanism by which low dose THC inhibits cholangiocarcinoma cell invasion involved in a decrease of the phosphorylation of MEK1/2. A previous study reported that THC inhibited MMP-2 expression and cell invasion in cultured glioma cells (21). However, we could not detect the effect of THC on the activity of MMP2 and MMP9 (data not shown). We suggested that the mechanism of THC on cancer cell invasion might depend on the type of cancer cells. Resistance to anoikis is one of the metastatic mechanisms of cancer cells (7, 8, 22). In this study, THC decreased the resistance to anoikis in cholangiocarcinoma cells. Previous studies demonstrated that the combination of phosphorylation of Akt and MEK1/2 was necessary for anoikis resistance in cancer cells (7, 8). Therefore, we suggested that THC inhibits anoikis resistance by modulating these signal transduction pathways. In the present work, we report the expression of cannabinoid receptors (CB1 and CB2) in human cholangiocarcinoma specimens. Although previous reports suggested that activation of CB2 receptors modulates cell migration and apoptosis in breast cancer cell lines (23), other reports made exactly the opposite conclusion (15). The receptors that mediate THC-induced cholangiocarcinoma cell apoptosis and inhibit migration and invasion should be investigated further. Previous literature studied the safety

profiles of THC in patients with recurrent glioblastoma multiforme and found that 0.80–3.29 mg THC delivery in this study was safe and could be achieved without overt psychoactive effects (24). In comparison with our *in vitro* study, we used the concentration of 0–100 μM (0–31.4 $\mu\text{g}/\text{mL}$) THC. However, the appropriate dose for use as chemotherapy in cholangiocarcinoma patients should be further evaluated.

To the best of our knowledge, the present study is the first report demonstrating the anticancer effects of THC in cholangiocarcinoma cells. We suggest that it may represent one potential approach to cholangiocarcinoma therapy.

ACKNOWLEDGMENTS

The authors wish to thank Miss Yuvadee Siriyasub, Center of Nanoimaging (CNI), Faculty of Science, Mahidol University for her excellent technical support in the Confocal laser scanning microscopy analysis. This study was supported by the Thailand Research Fund (MRG4980124) and Rajavithi Hospital.

DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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