RESEARCH ARTICLE

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Atractylodin and β-eudesmol from *Atractylodes lancea* (Thunb.) DC. Inhibit Cholangiocarcinoma Cell Proliferation by Downregulating the Notch Signaling Pathway

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Abstract

Objective: Notch signaling pathway has been reported to be involved in the development and progression of various types of cancer, including cholangiocarcinoma (CCA). Compounds that modulate this signaling pathway could be promising candidates for CCA treatment and control. The study investigated the antiproliferative activities and modulatory effects of atractylodin and β-eudesmol, the two bioactive compounds of *Atractylodes lancea* (Thunb.) DC. , on Notch signaling and upstream molecules (Notch1 and Notch2 receptors, JAG1, mTOR, PI3K, and YAP), and downstream molecules (Snail) in HuCCT-1 (CCA cell line) and OUMS-36T-1 (normal fibroblast cell line). Gemcitabine (standard drug for CCA), and Notch inhibitors (DAPT and zebularine) were included in the experiments as positive control compounds. **Methods:** The antiproliferative activity was evaluated using MTT assay. mRNA and protein expression of Notch signaling molecules were evaluated using real-time PCR and Western blot analysis. **Results:** Atractylodin and β-eudesmol moderately inhibited HuCCT-1 cell growth with IC_{50} (concentration that inhibits cell growth by 50%) of 29.00 ± 6.44 and 16.80 ± 4.41 μg/ml (mean±SD), respectively. The direction and extent of the modulatory effects on mRNA and protein expression in the CCA cell line varied with the signaling molecules. Notch1 receptor was shown to be the most promising target of atractylodin and β-eudesmol in CCA. The level of gene expression was significantly downregulated (0.042 to 0.195 fold of control) after treating HuCC-T1 cells with both compounds at low and high concentrations. The extent and change in Notch1 gene expression correlated well with protein expression. **Conclusion:** The notch signaling pathway could be a promising target of atractylodin and β-eudesmol in CCA.

Keywords: Notch signaling pathway- cholangiocarcinoma- atractylodin- β-eudesmol- *Atractylodes lancea* (Thunb.) DC

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Introduction

Cholangiocarcinoma (CCA), an aggressive bile duct cancer, remains a significant public health problem in Thailand, particularly in the northeast region. The primary risk factor is the consumption of fermented raw cyprinoid fish, which contains the metacercariae of *Opisthorchis viverrini* (Sripa and Pairojkul, 2008). The infection induces several cellular transformations, resulting in the stimulation of cell proliferation and secretion of pro-inflammatory cytokines, which promotes chronic inflammation (Sripa et al., 2018). Most CCA patients have a late diagnosis when the disease progresses to the advanced stage. Moreover, the CCA is resistant to chemotherapy and radiotherapy. The survival rate is low, and the quality of life of the patients is poor (Mihalache et al., 2010; Mao et al., 2018a; Marin et al., 2018). Identification of molecular markers as a tool for early diagnosis and effective treatment of CCA is urgently needed.

Notch signaling pathways is one of the promising targets of CCA pathogenesis and drug research and development. It is involved in cell proliferation, differentiation, and apoptosis (Pancewicz-Wojtkiewicz, 2016). The pathway consists of four receptors (Notch1, Notch2, Notch3, and Notch4) and five ligands (Delta-like family DLL1, DLL3, DLL4, Jagged family JAG1, and JAG2). Overexpression of Notch signaling has been linked with the development and progression of several types of cancer, including ovarian, glioma, breast cancers, and CCA (Hopfer et al., 2005; Ishii et al., 2010; Stockhausen et al., 2010; Zender et al., 2013; Zhou et al., 2013; Wu et al., 2014a; Wu et al., 2014b; Li et al., 2015; Gu et al., 2016; Yamamoto et al., 2017; Kontomanolis et al., 2018; Wang et al., 2018; Li et al., 2019; Ren et al., 2019; Sheng et al., 2019; O'Rourke et al., 2020). Overexpression of NICD1

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(Notch1 intracellular domain from Notch1 signaling) has been associated with CCA development and progression through upregulation of cyclin E-associated DNA damage (Zender et al., 2013; Ding et al., 2016). Downregulation of Notch1 signaling by several interventions is proposed as a promising strategy for inhibition of CCA cell growth. These include cinobufagin (Ren et al., 2019), xanthohumol (Walden et al., 2017), verteporfin-PDT (Papoutsoglou et al., 2019), DAPT (β-secretase inhibitor) (O'Rourke et al., 2020), and PIK75 (PIK3CA specific inhibitor (Che et al., 2016). Apart from Notch1 signaling, Notch2 signaling has also been reported to play a critical role in CCA development (Wu et al., 2014a; Huntzicker et al., 2015; Li et al., 2015; Che et al., 2016; Yamamoto et al., 2017; Wang et al., 2018; Zhang et al., 2018; Xu et al., 2019)

Atractylodes lancea (Thunb.) DC. is the traditional medicine used in China, Japan and Thailand for the treatment of various diseases/health conditions, e.g., strengthening the spleen, reducing dampness, fever, cold, and sore throat (Jun et al., 2018). A series of in vitro and in vivo studies showed promising activity of A. lancea for the treatment and control of CCA (Na-Bangchang et al., 2017). Anti-CCA activities of both the crude ethanolic extract of A. lancea and its major bioactive compounds, atractylodin and β-eudesmol have been demonstrated (Plengsuriyakarn et al., 2012; Plengsuriyakarn et al., 2015; Mathema et al., 2017; Kotawong et al., 2018b; Srijiwangsa et al., 2018; Mathema et al., 2019). Several cellular signaling pathways, such as Ras/ERK, PI3K/AKT/mTOR, p38MAPK, and NF-κB pathways were reported as molecular targets of A. lancea and bioactive compounds (Kotawong et al., 2018a; Mathema et al., 2019; Acharya et al., 2021). Notch signaling pathway has been shown to be the promising target for cancer chemotherapy (Vanaroj et al., 2022). The present study investigated the involvement of Notch signaling pathways (Notch1 receptor, Notch2 receptor, JAG1 ligand, mTOR, YAP, and Snai1) and antiproliferative activities of atractylodin and β-eudesmol in a CCA cell line.

Materials and Methods

Cell lines and culture

HuCCT-1 (CCA cell line) and OUMS-36T-1(normal human cell line) were purchased from the Japanese Collection of Research Bioresources (JCRB) cell bank, Japan. HuCCT-1 and OUMS-36T-1 cell lines were cultured in RPMI-1640 medium (Gibco Co. Ltd., NY, USA) and Dulbecco's Modified Eagle Medium (DMEM: Gibco Co. Ltd., NY, USA), respectively, at 37°C under the atmosphere of 5% CO_2. Both culture media were supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Gibco Co. Ltd., NY, USA). Cells were trypsinized with 0.125% trypsin (Gibco BRL Life Technologies, Grand Island, NY, USA) and subcultured every 3-4 days.

Antiproliferative assay

HuCCT-1 and OUMS-36T-1 (8,000 cells per well) were seeded onto a 96 well-plate (Corning, NY, USA) and incubated at 37°C under 5% CO_2 for 24 hours. Atractylodin, β-eudesmol (Wako Pure Chemical Industry, Osaka, Japan), zebularine, gemcitabine and DAPT (Sigma-Aldrich, Singapore) were added to each well and incubated for 72 hours. The concentrations of each test compound were as follows: atractylodin (1.95-250 μg/ml), β-eudesmol (1.95-250 μg/ml), gemcitabine (0.78-200 μg/ml), DAPT (1.56-200 μg/ml), and zebularine (15.63-2,000 μg/ml), respectively. MTT reagent (Sigma Co. Ltd., MO, USA) (20 μl of 5 mg/ml) was added, and the plate was incubated for an additional 3.5 hours. The medium was carefully discarded, and 100 μl of DMSO (MP Biomedicals, Santa Ana, California, USA) was added to dissolve purple formazan crystals. The absorbance of cell suspension was measured at 590 nm (Varioskan microplate reader, Thermo Scientific, Rockford, USA). Dose-response analysis was performed to determine IC_{25} and IC_{50} (concentration that inhibits cell growth by 25% and 50%, respectively) values of each drug, using CalcuSyn software version 2.11, Biosoft, Cambridge, UK). The selectivity index (SI) was determined from the ratio of the IC_{50} of each test compound in HuCCT-1 and OUMS-36T-1 cells. The experiment was repeated three times, triplicate analysis each.

Gene expression analysis

Both cell lines were exposed to atractylodin (IC_{25} and IC_{50}), β-eudesmol (IC_{25} and IC_{50}), gemcitabine (IC_{25} and IC_{50}), DAPT (IC_{25}), and zebularine (IC_{25}) for 72 hours. mRNA was extracted using TRIzol reagent (Sigma, USA) and converted to cDNA using a Superscript III reverse transcriptase cDNA construction kit (Invitrogen Corporation, Carlsbad, California, USA). The cDNA was used as the template for real-time PCR (RT-PCR) analysis.

The analysis of genes involved in Notch signaling (Notch1 receptor, Notch2 receptor, JAG1 ligand, mTOR, YAP, and Snai1) was performed using RT-PCR. The PCR mixture (20 μl) consisted of 1 μl of each forward and reverse primers (Table 1), 10 μl of iTaq Universal SYBR green supermix (Bio-Rad, CA, USA), 7 μl of diethylpyrocarbonate (DEPC) water, and 1 μl of cDNA. The relative expression of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method. The experiment was repeated three times, triplicate analysis each.

Protein expression analysis

HuCCT-1 cell line was exposed to atractylodin (IC_{25} and IC_{50}), β-eudesmol (IC_{25} and IC_{50}), gemcitabine (IC_{25} and IC_{50}), DAPT (IC_{50}), and zebularine (IC_{50}) for 72 hours. Proteins were extracted with RIPA buffer and protease inhibitor, and the concentrations were measured using BCA assay. Proteins were separated onto SDS-PAGE and transferred to the nitrocellulose membrane. The membrane was incubated overnight with primary antibodies (Notch1 receptor, Notch2 receptor, JAG1 ligand, PI3K, mTOR, YAP, Snai1, and β-actin), followed by an anti-rabbit conjugated AP antibody. The intensity of protein bands was measured by using The ImageJ program (LOCI, University of Wisconsin, Wisconsin, USA).

The experiment was repeated three times, triplicate analysis each.

Results

Antiproliferative activities

The IC_{25}, IC_{50} and SI values of all test compounds are summarized in Table 2. Atractylodin and β-eudesmol inhibited the growth of the CCA cell line, HuCCT-1, with moderate potencies of activity (IC_{50} l5-30 μg/ml). Gemcitabine, on the other hand, strongly inhibited CCA cell growth (IC_{50} < 5 μg/ml). The two inhibitors of Notch signaling, DAPT (an inhibitor of the γ-secretase complex) and zebularine (a DNA methyltransferase inhibitor) weakly inhibited CCA cell growth (IC_{50}: 88.98 and 148.64 μg/ml, respectively). It was noted for the high selectivity of gemcitabine to CCA compared with the normal cell line (SI: 14.05). The selectivity of other test compounds, particularly atractylodin and β-eudesmol toward CCA cells is relatively low (SI: 0.84-4.41).

Effects on gene expression

RT-PCR analysis of the expression of genes involved in the Notch1 signaling pathway in HuCCT-1 cell line is presented in Figure 1. The pattern (direction and intensity) of gene expression in OUMS-36T-1 was inconsistent with that observed in HuCCT-1 cell line for all test compounds (data not shown). mRNA expression of Notch2 receptor and Snai1 was not observed in both cell lines. All compounds downregulated the expression of the Notch1 receptor gene in HuCCT-1 cells compared with control. However, a significant reduction of gene expression was observed with only atractylodin and β-eudesmol at IC_{25} and IC_{50} concentrations (0.042 and 0.195 fold, respectively) and gemcitabine at IC_{50} (0.318 fold). A trend of JAG1 ligand gene downregulation was found with atractylodin and β-eudesmol (IC_{25} and IC_{50}) and DAPT (0.004 to 0.192 fold). On the other hand, a trend of gene upregulation was found with gemcitabine (IC_{25} and IC_{50}) and zebularine (1.344 to 1.511 fold). It was noted for a low level of mTOR gene expression in HuCCT-1 cells. The expression of mTOR was downregulated by almost all test compounds (0.003 to 0.051 fold), except gemcitabine at both concentrations (2.14 to 33.5 fold). Similarly, the expression of PI3K was downregulated by almost all test compounds (0.008 to 0.212 fold), except gemcitabine at both concentrations (1.396 to 2.250 fold). For YAP, gene expression was significantly downregulated by atractylodin and β-eudesmol at low concentration (IC_{25}), with fold-change of expression of 0.086 and 0.280 fold, respectively. At high concentration (IC_{50}) on the other hand, both compounds upregulated the expression of this gene (2.219 and 1.978 fold, respectively). Gemcitabine and DAPT also upregulated gene expression (1.429 to 2.278 fold), while zebularine downregulated gene expression (0.647 fold).

Effects on protein expression

Western blot analysis was performed to confirm protein expression of all genes following the exposure of HuCCT-1 cells to the test compounds (Figure 2). The expression of Notch2 and Snai1 proteins was not observed in HuCCT-1 cell line. In general, there was the correlation between gene and protein expression of most genes after exposing the cells to the test compounds. Complete correlation between protein and gene expression was found with the Notch1 receptor (downregulation: 0.35 to 0.57 fold, upregulation 1.406 fold). The expression of the JAG1 ligand gene was upregulated (1.344 fold) after exposing the cells to gemcitabine at IC_{25}, while the protein expression was downregulated (0.610 fold). The expression of mTOR gene was downregulated (0.002 fold) after exposure to atractylodin at IC_{25}, while the protein expression was upregulated (1.67 fold). The expression of the PI3K gene was upregulated after exposure to gemcitabine at both concentrations (1.4 and 2.25 fold for IC_{25} and IC_{50}, respectively), while the protein expression

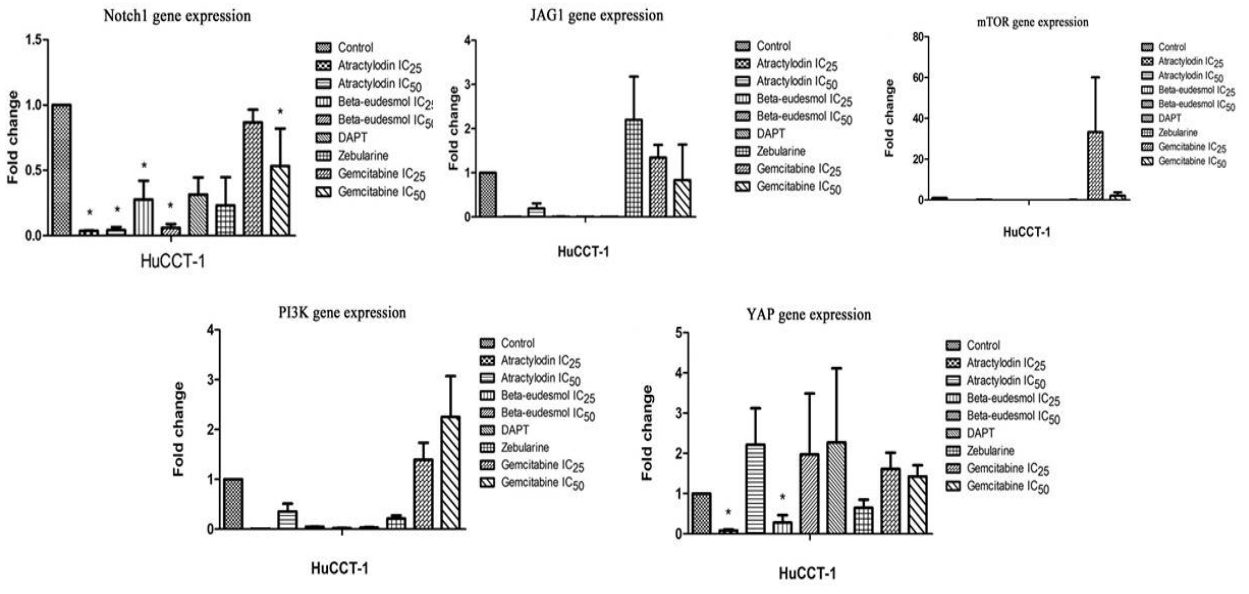

Figure 1. mRNA Expression of the Genes in Notch Signaling in HuCCT-1 Cell Line Following Exposure to Atractylodin (IC_{25} and IC_{50}), β-eudesmol (IC_{25} and IC_{50}), DAPT (IC_{50}), zebularine (IC_{50}), and gemcitabine (IC_{25} and IC_{50}).

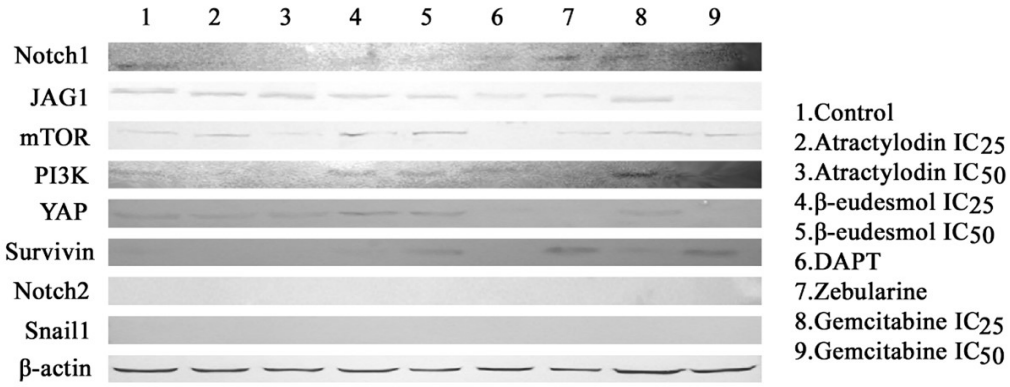

Figure 2. Expression of Key Proteins in Notch Signaling in HuCCT-1 Cell Line Following Exposure to Atractylodin (IC_{25} and IC_{50}), β-eudesmol (IC_{25} and IC_{50}), DAPT (IC_{50}), zebularine (IC_{50}), and gemcitabine (IC_{25} and IC_{50}).

Table 1. The Primers Sequences Used in RT-PCR Analysis

Gene		Primer sequence (5' to 3')	Reference
PI3K	forward	CATTTGCTCCAAACTGACCA	(Kowalczuk et al., 2022)
	reverse	GATTGGCATGCTGTCGAATA	
Snai1	forward	ACCCCACATCCTTCTCACTG	(Kim et al., 2019)
	reverse	TACAAAAACCCACGCAGACA	
YAP	forward	CCATGCTGTCCCAGATGAACGTCACA	(Karystinou et al., 2015)
	reverse	ATCCCGGGAGAAGACACTGGATTT	
mTOR	forward	TGAGACCCTTCCCATTTCCC	(Acharya et al., 2021)
	reverse	GGGCCTATGATCTCACTCCC	
JAG1 ligand	forward	GAAGCAGAACACGGGCGTT	(Ahmad et al., 2022)
	reverse	CAGGTCACGCGGATCTGAT	
Notch1 receptor	forward	ATCCTGATCCGGAACCGAG	(Ndong et al., 2018)
	reverse	CGTCGTGCCATCATGCAT	
Notch2Receptor	forward	ACAGTTGTGTCTGCTCACCAGGAT	(Poletti et al., 2021)
	reverse	GCGGAAACCATTCACACCGTTGAT	
GADPH	forward	ATTTGGTCGTATTGGGCGCCT	(Acharya et al., 2021)
	reverse	GATGATGACCCTTTTGGCTCC	

was downregulated (0.47 and 0.677 fold for IC_{25} and IC_{50}, respectively). For YAP, the correlation between gene and protein expression was found only with atractylodin at IC_{25} concentrations (0.086 and 0.819 fold for gene and protein expression, respectively).

Discussion

Results of the study confirm moderate potencies of the antiproliferative activities of atractylodin and β-eudesmol against the CCA cell line, HuCCT-1, as reported in previous studies (Mahavorasirikul et al., 2010; Kotawong et al., 2018a; Mathema et al., 2019). The activities of both inhibitors of Notch signaling, i.e., DAPT (an inhibitor of β-secretase complex) and zebularine (an inhibitor of DNA-methyltransferase) were weak, while that of the standard drug gemcitabine was strong. It was noted for about 8-16-fold higher selectivity

Table 2. The IC_{25} and IC_{50} (mean±SD) of the Test Compounds for HuCCT-1 (CCA Cells) and OUMS-36T-1 (Normal Cells) Cell Lines, Including Selectivity Index (SI)

Compounds	HuCCT-1		OUMS-36T-1		SI
	IC_{25} (μg/ml)	IC_{50} (μg/ml)	IC_{25} (μg/ml)	IC_{50} (μg/ml)	
Atractylodin	13.44 ± 6.28	29.00 ± 6.44	10.08 ± 4.27	24.47 ± 8.46	0.84
β-eudesmol	6.37 ± 2.53	16.80 ± 4.41	18.12 ± 7.43	28.30 ± 9.16	1.68
Gemcitabine	0.22 ± 0.35	4.97 ± 5.09	3.40 ± 5.78	69.85 ± 38.25	14.05
DAPT	-	89.88 ± 36.11	-	150.29 ± 23.79	1.67
Zebularine	-	148.64 ± 46.83	-	655.44 ± 448.07	4.41

of gemcitabine to CCA cells compared with atractylodin and β-eudesmol. The results suggest that HuCCT-1 is a gemcitabine-sensitive CCA cell line, and the mechanism of action of gemcitabine is selective to molecular target(s) in CCA cells (Boonsri et al., 2021). Low selectivity of both bioactive compounds could be linked to their activities on cell cycle arrest (Kotawong et al., 2018a). Furthermore, the OUMS-36T-1 cell is an immortal fibroblast cell line that is transfected with the hTRT (human telomerase reverse transcriptase) gene. The use of OUMS-36T-1 cell line to represent normal human cells may, therefore, be limited by its relatively similar characteristic with cancer cells with rapid cell cycle progression (Kouchi and Namba, 2000). Clinical use of atractylodin and β-eudesmol as pure compounds or active components of A. lancea should be careful to avoid possible toxicity. Nevertheless, results of toxicity long-term toxicity of A. lancea in rats and Phase I clinical study (Na-Bangchang et al., 2021) showed no evidence of significant toxicity.

Notch signaling pathway is an important pathway that has been linked with several types of cancer, including CCA (Ishii et al., 2010; El Khatib et al., 2013; Wu et al., 2014b; Guest et al., 2016; Kwon et al., 2017; Wang et al., 2018). In hepatocyte-specific Notch-transgenic mice, overactivation of tumor formation occurred, which led to intrahepatic CCA development (Guo et al., 2019). The present study investigated the possible role of Notch signaling as molecular targets of action of atractylodin and β-eudesmol, the two bioactive compounds of A. lancea in CCA. The key molecular targets included in the study were Notch1 receptor, Notch2 receptor, JAG1 ligand of Notch receptor, upstream signaling molecules --mTOR, PI3K, and YAP, and a downstream signaling molecule -- Snai1.

HuCCT-1 cell line expressed all genes/proteins at a relatively high level compared with OUMS-36T-1 cell line. The expression (gene/protein) of Notch2 receptor and Snai1 was not observed in both cells. Unlike in other cancer types, the role of these two molecules in CCA may be limited (Zhou et al., 2013; Che et al., 2016; Yamamoto et al., 2017; Wang et al., 2018). In CCA, Snai1 was reported to promote the CCA phenotype and activate Notch2 receptor (Xu et al., 2019).

Among the signaling molecules in Notch signaling pathways, Notch1 receptor was shown to be the most promising target of atractylodin and β-eudesmol in CCA. The level of gene expression was significantly downregulated compared with control (0.042 to 0.195 fold) after treating HuCC-T1 cells with both compounds at low and high concentrations. The extent and change in Notch1 receptor gene expression correlated well with the protein expression. In the previous studies in CCA-xenograft nude mice, loss of Notch1 receptor expression led to a reduction of HES1 (Hes Family BHLH Transcription Factor 1) and cyclin E expression, but a marked increase in the expression of p53, p27, and p21, which led to induce apoptosis (El Khatib et al., 2013; Zender et al., 2013; Zhou et al., 2013; Tschaharganeh et al., 2014; Ding et al., 2016). Furthermore, suppression of the Notch1 receptor reduced CCA tumor growth and invasiveness, inhibited blood vessel information, and thus, enhanced the chemotherapeutic efficacy (Wu et al., 2014b; Mancarella et al., 2020). The standard drug gemcitabine also inhibited the expression of the Notch1 receptor gene in a concentration-dependent manner. Gemcitabine is a deoxycytidine analogue which has been shown to inhibit DNA synthesis (S phase) and suppress cell cycle progression at the G1/S phase boundary. The mechanism of gemcitabine action on Notch1 signaling is unclear, but downregulation of Notch3 receptor was reported in pancreatic cells treated with gemcitabine. Moreover, modulation of the intrinsic apoptosis pathway was shown to be involved in Notch-induced chemoresistance to gemcitabine in pancreatic cancer cells (Du et al., 2014; Jia and Xie, 2015). The current study is the first to demonstrate the suppressive effect of gemcitabine on the Notch1 receptor gene and protein expression in gemcitabine-sensitive CCA cell line. The level of inhibitory effects of both compounds on Notch1 receptor was comparable with the two Notch inhibitors, DAPT, and zebularine.

Apart from Notch1 receptor,β-eudemol at IC_{25} concentrations also significantly downregulated YAP gene expression in HuCCT-1 cells (0.280 fold). Gemcitabine, DAPT and zebularine upregulated the expression of YAP gene, while downregulated the expression of YAP protein. Inconsistent results in the expression of some genes and proteins could be due to the modification during the post-translation process. YAP is one of the upstream molecules of the Notch signaling pathway. It was reported to play a role in the mediation of resistance of cancer cells to gemcitabine (Gujral and Kirschner, 2017; Nguyen and Yi, 2019), and gemcitabine in combination with a YAP inhibitor was shown to enhance the efficacy of gemcitabine in CCA (Kitagawa et al., 2020) Other upstream signaling molecules of the Notch signaling pathway, i.e., JAG1, mTOR and PI3K were also downregulated after exposing HuCCT-1 cells to both compounds, but with relatively lower activity compared with Notch1 receptor and YAP. JAG1 (Jagged1) is a ligand for the Notch receptor; it has an important function in both physiological and pathological conditions, including cancer (angiogenesis, cell proliferation, cancer stem cells, invasion, and metastasis) (Li et al., 2014; Xiu et al., 2020). The suppressive effect of β-eudesmol on JAG1 in HuCCT-1 cells was relatively more potent than atractylodin. The two Notch inhibitors, DAPT and zebularine showed consistent results with atractylodin and b-eudesmol. Gemcitabine also downregulated JAG1 expression, which indicates that the HuCCT-1 cell line is sensitive to this drug. JAG1 has been shown to be an important Notch ligand in CCA, especially in intrahepatic CCA (Che et al., 2016; Huang et al., 2016). However, overexpression of JAG1 alone is not sufficient for tumor development (Che et al., 2016). DAPT was shown to be a potent inhibitor of JAG1 mRNA and protein expression. DAPT treatment with Notch2 shRNA suppressed JAG1-induced cell proliferation, inhibited colony formation and promoted cell cycle arrest (Manokawinchoke et al., 2017).

mTOR is the upstream molecule of Notch signaling. It is a key regulator of cell growth and division, but abnormally activated mTOR in cancer cells stimulates cancer cell growth, metastasis, and invasion (Zou et al.,

2020). Both atractylodin and β-eudesmol as well as DAPT and zebularine, downregulated the expression of mTOR gene and protein. The current data support the results of the previous study regarding the suppression of p-AKT/AKT, p-mTOR/mTOR and p-p38MAPK/p38MAPK expression in HuCCT-1 cells after exposure to atractylodin (Acharya et al., 2021). Gemcitabine at both concentrations upregulated mTOR gene and protein expression. Interestingly, the level of upregulation was markedly seen (33.27 fold) at low concentration. Gemcitabine has been reported to inhibit cell growth, induce apoptosis, and activate autophagy via the AMP-activated protein kinase (AMPK) pathway (Zhu et al., 2018). It has also been reported as a crucial factor for gemcitabine resistance in many types of cancer (Kagawa et al., 2012; Chawsheen and Dash, 2021) and gemcitabine combined with mTOR inhibitor can sensitize the leiomyosarcoma cell line (Bobinski et al., 2020).

Phosphatidylinositol-3-kinase (PI3K)/AKT pathway plays a crucial role in the development and progression of several types of cancer. In the signaling network, PI3K is an upstream molecule of Notch signaling, which promotes cell proliferation and induces autophagy (Palomero and Ferrando, 2008). In CCA, increased activation of PI3K/AKT pathway was reported (Yothaisong et al., 2013). Atractylodin, β-eudesmol, DAPT and zebularine were shown to downregulate the PI3K gene and protein expression. The activity of atractylodin in suppressing gene and protein suppression was more potent at low compared with higher concentrations. The results support our previous studies that atractylodin and β-eudesmol downregulate PI3K to inhibit proliferation and activate autophagy in the CCA cell line (Kotawong et al., 2020; Acharya et al., 2021). Interestingly, gemcitabine at both concentrations upregulated both PI3K gene and protein expression. Inhibition of PI3K was shown to enhance the chemosensitivity of gemcitabine (Jung et al., 2014; Mao et al., 2018b). DAPT was also shown to inhibit PI3K and suppress CCA cell invasion, migration, metastasis, and epithelial-mesenchymal transition (EMT) (Peng et al., 2020).

In conclusion, Atractylodin and β-eudesmol, the two bioactive compounds of *A. lancea* (Thunb.) DC. modulate the Notch signaling pathway and its upstream/downstream molecules in the CCA cell line at the gene and protein expression levels. Notch1 receptor is the key target molecule of antiproliferative activities of both compounds against CCA. Further study is required to confirm this conclusion in other CCA cell types and in animal models.

Author Contribution Statement

KN and WC were involved in the design of the experimental study. PV performed the experiments. PV, WC, and KN performed data analysis. PV drafted the manuscript. WC and KN revised the manuscript. KN finalized the manuscript. All authors reviewed and approved the final manuscript for submission. All meet the ICMJE criteria for authorship.

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Approval of Scientific Body

The article is part of an approved thesis of Ms. Peeranate Vanaroj.

Availability of Data

The datasets generated during and/or analyzed during this study are the corresponding author on reasonable request.

Conflict of Interest

The authors declare no competing interests.

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