

β -Eudesmol induces the expression of apoptosis pathway proteins in cholangiocarcinoma cell lines

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Background: Cholangiocarcinoma (CCA) is a neglected disease prevalent in developing countries with high burden and mortality rate, and there is no effective treatment. We aimed to investigate β -eudesmol molecular target of action in human CCA cell lines using the selected key molecules of apoptotic pathways. **Materials and Methods:** Two CCA cell lines (HuH28 and HuCCT1) were assessed at different time points after β -eudesmol treatment for mRNA and protein expression profiles of *caspase-3*, *-8*, *-9*, *p53*, *p21*, *Bcl-2*, and *Bax* by real-time polymerase chain reaction and western blot, respectively. **Results:** β -eudesmol induced expressions of p21 and p53 in mRNA/protein level in HuH28 and HuCCT1 cells. These CCA cells also expressed caspase-3, -8, -9 and bax (mRNA and/or protein level) among others after β -eudesmol treatment indicating its role in both intrinsic and extrinsic caspase-dependent apoptotic pathways. **Conclusion:** The study demonstrated that β -eudesmol induced the expression of apoptosis pathway proteins, suggesting its potential role in promoting the caspase-dependent apoptotic pathway, and induction of the cell cycle arrest in CCA cell lines. β -eudesmol can be considered as a potential compound for further investigation as an anti-CCA agent.

Key words: Apoptosis, cholangiocarcinoma, mode of action, molecular targets, β -eudesmol

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INTRODUCTION

Cholangiocarcinoma (CCA) is a neglected disease most prevalent in developing countries^[1] and it has no available effective chemotherapeutics despite the high burden and mortality rate.^[2-4] This cancer arises from the abnormality of cell cycle (e.g., *p38* or mitogen activated protein kinase,^[3,5] *p53* or tumor suppressor gene *p53*,^[3,5] and *p21^{Waf1}* or cyclin-dependent kinase inhibitor 1,^[3,6] dysregulation of cell proliferation (e.g., *p14^{ARF}* or alternative reading frame protein,^[6] and the aberrant of apoptotic genes (e.g., *FasL* or Fas ligand,^[7] *Bax* or bcl2-like protein 4,^[8] and *bcl-2* or B-cell lymphoma

2.^[3,8] Thus, *caspase-3*, *-8*, *-9*, *p53*, *p21*, *Bcl-2* and *Bax*, genes among others are considered as the potential targets of CCA therapy.

Cellular apoptosis including caspase-dependent and independent pathways are substantial signaling cascades for homeostasis between cell survival and death.^[9] Apoptotic pathways (both intrinsic and extrinsic) have been identified as potential drug targets in cancer.^[9] Intrinsic pathway is activated by DNA damage, ischemia, and oxidative stress while the extrinsic pathway is triggered by death ligand binding to a death receptor, for example, tumor necrosis factor-alpha (TNF- α) to

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TNF receptor-1, etc. Caspase-dependent apoptotic pathway is a complicated process involving various molecules such as TNFR1, TNF-related apoptosis-inducing ligand receptor (TRAIL-R) Fas receptor, (FasR/CD95), Bid (BH3 interacting-domain death agonist), SMAC (second mitochondria-derived activator of caspase), BCL-2, BAX, caspase -8, -9, -6, -3, -7, and p53.^[9] Although various antagonists and agonists of apoptosis have been developed to enhance the cellular apoptosis both in preclinical and clinical studies,^[10] the efficacy of these compounds remains unsatisfactory.^[11] So far, there are only a few compounds in preclinical study focused on CCA.

Beta (β)-eudesmol (an active compound from *Atractylodes lancea* (Thunb.) DC.), elicits various pharmacological activities.^[12] It demonstrated potent anticancer activity on CCA *in vitro* and *in vivo* with low toxicity on normal cells ($IC_{50} = 24.1 \pm 3.4 \mu\text{g/mL}$, and selectivity index = 8.6).^[13] Moreover, β -eudesmol also promoted cell cycle arrest at G1 phase, with caspase-3/7 induction in CCA cell lines.^[14]

Identification of drug molecular targets in apoptosis pathway by commercial apoptotic assays are often time-consuming and cost-intensive. However, a selection of key molecules in each cascade have been commonly used and known to be effective. The objective of this study was to investigate β -eudesmol molecular target of action, using the selected key molecules of apoptosis pathways in CCA cell lines.

MATERIALS AND METHODS

Cell lines, antibodies, and compound

HuH28 (derived from a 37 years old female) and HuCCT1 (derived from a 56 years old male) CCA cell lines were maintained at Chulabhorn International College of Medicine, Thammasat University, Thailand. Cells were propagated and cultured at 37°C in a humidified atmosphere (5% CO₂) using RPMI-1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Primary antibodies were purchased from Cell Signaling Technology, MA, USA (caspase3 #9665 and caspase-8 #4790), Santa Cruz Biotechnology, Inc., TX, USA (p21 #sc-6246, caspase-9 #sc-17784 and Bax #sc-20067), and Medical and Biological Laboratories Co., Nagoya, Japan (anti- β -actin pAB-HRP-DirectT). Secondary antibodies used were from Promega, WI, USA (anti-rabbit IgG HRP) and Santa Cruz biotechnology, Inc., (anti-mouse-IgGk BP-HRP). β -eudesmol was purchased from Wako [Figure S1].

Treatment of HuCCT1 and HuH28 cells with β -eudesmol

Cells were divided into treated and control (nontreated) groups, and sampled for assessment at different time points (0, 4, 12, 24, 36, 48, and 72 h) post-treatment. Cells were seeded into 100 mm dish (3×10^6 cells/dish) and cultured

overnight. Next day, cells in the treated groups were exposed to β -eudesmol and harvested at the given time points.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from treated and untreated cells using RNeasy Mini kit (QIAGEN, CA, USA). Reverse transcription was performed with Superscript III kit (Life Technologies, CA, USA) following the manufacturer's instructions. Resulting cDNA was stored at -20°C until used.

Analysis of mRNA expression levels of apoptosis pathway proteins

The mRNA expression levels of *caspase-3*, *-8*, *-9*, *p53*, *p21*, *Bcl-2*, and *Bax* were determined by quantitative real-time polymerase chain reaction (qRT-PCR) using iTaq™ Universal SYBR Green Supermix reagents (BIO-RAD Laboratories, CA, USA) according to the manufacturer's instructions. The RT-PCR condition was set as: initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s, TM (melting temperature, varied [55°C–62°C] with primer sets) for 1 min and 95°C for 15 s. The primers and TM of each target have been provided in Table 1. Housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used to normalize the expression level.

Western blot analysis of apoptosis pathway proteins

Cells were lysed using radio-immunoprecipitation assay (RIPA) Lysis and Extraction Buffer (ThermoFisher, MT, USA). Lysate was loaded into 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVD) membrane (MilliPore, Darmstadt, Germany). After blocking with bovine serum albumin (BSA) in tris-buffered saline (TBS) with Tween20, membrane was incubated at 4°C overnight (1200 rpm) with corresponding primary antibodies according to the manufacturer's instructions. HRP-labeled secondary antibody was added and detected using Amersham™ ECL™ Start Western blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK). Protein bands were visualized by an image analyzer (LAS-4000 mid; GE healthcare).

Data analysis

All the experiments were performed 3 times. Expression levels were presented as average and error bar (standard deviation). Comparison of expression levels with the baseline was performed by Student's *t*-test. $P < 0.05$ was considered as statistically significant.

RESULTS

β -eudesmol altered the expression levels of cell cycle regulated genes (*p21* and *p53*)

As shown in the qRT-PCR results [Figure 1a], the expression levels of *p21* and *p53* were higher in treated cells lines

compared with the nontreated. In HuH28 cells, expression levels of both *p21* and *p53* increased 4 h post β -eudesmol exposure ($P = 0.036$, $P = 0.045$). In HuCCT1 cells, *p21* was expressed highest at 24 h, but expression of *p53* continuously increased with time ($P = 0.039$, $P = 0.004$) [Figure 1a].

Effect β -eudesmol on the expression levels of apoptosis pathway genes

Apoptotic genes (*caspase-3*, *-8*, *-9*, *bcl-2*, and *Bax*) expression altered with β -eudesmol treatment [Figure 1b and c]. The proapoptotic gene *Bax* showed increased expression at 4 h in HuH28 cells and HuCCT1 cells (peaked at 24 h), respectively. In HuCCT1 cells (but not in HuH28), expression of anti-apoptotic gene, i.e., *bcl-2* increased with time and peaked at 72 h posttreatment ($P = 0.0011$).

In HuH28 cells, the expression levels of *caspase-3* and *-8* were increased at 4 h and then declined, while the expression of *caspase-9* increased to peak at 72 h of β -eudesmol exposure ($P = 0.048$, $P = 0.022$, $P = 0.036$, respectively) [Figure 1c]. In contrast, HuCCT1 cells demonstrated gradual increase in *caspase-3* and *-9* expression (peaked at 72 and 48 h for *caspase-3* and *-9*, respectively) ($P = 0.003$, $P = 0.05$), while *caspase-8* increased throughout the time points ($P = 0.0019$) with a peak at 24 h after β -eudesmol treatment.

β -eudesmol induced the expression of caspase-3, -8, and -9 proteins

The results of immunoblotting showed the expression of caspase proteins in both HuH28 and HuCCT1 cell lines [Figure 2a and b]. In HuH28 cells, β -eudesmol induced caspase-9 protein expression at 4–48 h (peak at 12–36 h), whereas caspase-8 expressed between 4 and 72 h though relatively low. Caspase-3 expression started after 4 h and remained so till 48 h. β -eudesmol also induced caspase

-3, *-8*, and *-9* protein expressions in HuCCT1 cells during 4–24 h [Figure 2b].

β -eudesmol activated *p21* and *bax* protein expression

Immunoblotting revealed β -eudesmol induced *p21* protein expression particularly in HuCCT1 cells [Figure 2b]. Moreover, the *p21* expression appeared earlier in HuCCT1 cells with subsequent decline later on. Interestingly, β -eudesmol also induced persistent expression of BAX protein in HuH28 cells from 4 to 72 h, with no increased expression of *p21* protein [Figure 2b].

DISCUSSION

Results of the present study suggest that β -eudesmol may have contributed to cell cycle arrest in CCA cell lines, perhaps, through the induction of *p21* (inhibition of G1/S transition) by *p53*.^[15] Although the *p21* expression was observed at RNA and protein level in HuCCT1 cells, there was no expression at protein level in HuH28 cells. Due to slightly different expression of *p53* in mRNA and protein levels, we cannot totally rule out the potential impact of previous cell cycle especially in HuH28 cells. Besides promoting *p21* expression, *p53* modulates the intrinsic apoptotic pathway through the activation of pro-apoptotic protein (e.g., *bax*) expression and binds to antiapoptotic protein (e.g., *bcl-2*) leading to increased free proapoptotic protein. This was evidenced by the increased *bax* expression at mRNA level in both cell lines and protein level in HuH28 cell lines. In the published data, induction of pro-apoptosis pathway by anti-cancer agents was reported in CCA inhibition through *bax* expression.^[16] Although we do not have *bcl-2* protein level data, perhaps, its high expression had some role in the suppression of *bax* protein in HuCCT1 cells. The induction of *p53* expression is beneficial to cancer

Table 1: Oligonucleotide sequences used for real-time quantitative-polymerase chain reaction analysis

Target gene	Forward: Primer sequence (5'-3')	Reverse: Primer sequence (5'-3')	Temperature (°C)
Caspase-3	5'-CATACATGGAAGCGAATCAATGGACTCTGG-3'	5'-CGACATCTGTACCAGACCGAGATGTC-3'	57
Caspase-8	5'-GATATTGGGGAACAACCTGGACAGTGAAGA-3'	5'-CATTCAACCCACACCTCCAGTC-3'	57
Caspase-9	5'-GCGAACTAACAGGCAAGCAG-3'	5'-CCAATGTCCACTGGTCTGG-3'	55
p53	5'-CTTTTCGACATAGTGTGGTGGTGCCC-3'	5'-CCCATGCAGGAAGTGTACACATGTAG-3'	55
p21	5'-CCGTGAGCGATGGAACCTCGACTTT-3'	5'-CAGGTCCACATGGTCTTCTCTGCT-3'	55
Bcl-2	5'-GAGGATTGTGGCCTTCTTTGAGITCG-3'	5'-CTCCGTTATCCTGGATCCAGGTG-3'	60
Bax	5'-TCCACCAAGAAGCTGAGCGA-3'	5'-GTCCAGCCCATGATGGTTCT-3'	62
GAPDH	5'-TCAACGGATTGGTCGTATT-3'	5'-CTGTGGTCATGAGTCTTCC-3'	60

GAPDH=Glyceraldehyde 3-phosphate dehydrogenase

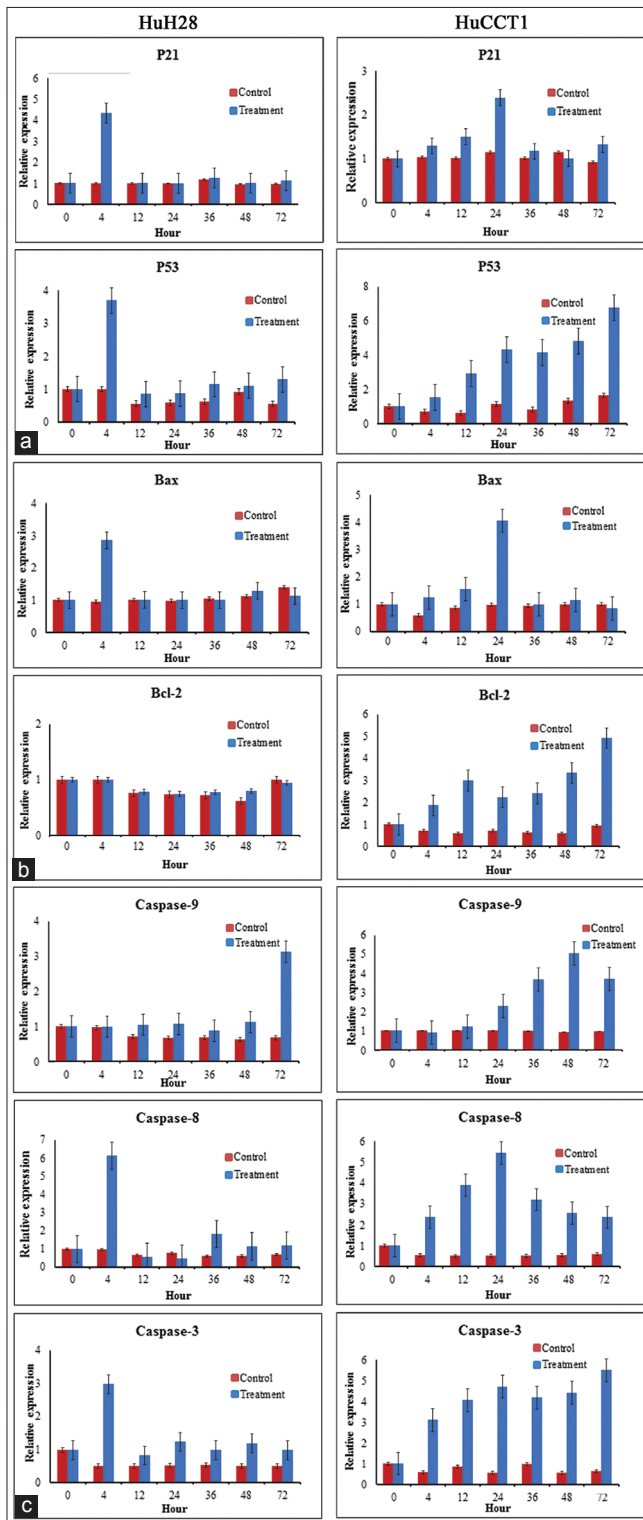


Figure 1: Expression profiles of key genes associated with cell cycle regulation and apoptosis pathway in HuH28 and HuCCT1 cell lines after treatment with β -eudesmol. (a) Expression of *p21* and *p53*; (b) *Bax* and *bcl-2*, and; (c) *caspase-9*, *-8*, and *-3* in both cell lines at different time points (h). Relative gene expression was normalized with the house-keeping gene *GAPDH*. Zero h indicates non-treated control. In HuH28 cell line, mRNA expression of *p21*, *p53*, *bax*, *caspase-3*, and *-8* were stimulated by β -eudesmol at 4 h post treatment and *caspase-9* at 72 h. In HuCCT1 cell lines, *p21* and *bax* were stimulated at 24 h, while *p53*, *bcl-2*, *caspase-3* were gradually increased from 4 to 72 h. Expression of *caspase-9* and *-8* peaked at 48 and 24 h, respectively. All the experiments were performed 3 times, and the gene expression level was presented as average with error bar (standard deviation). h = hour

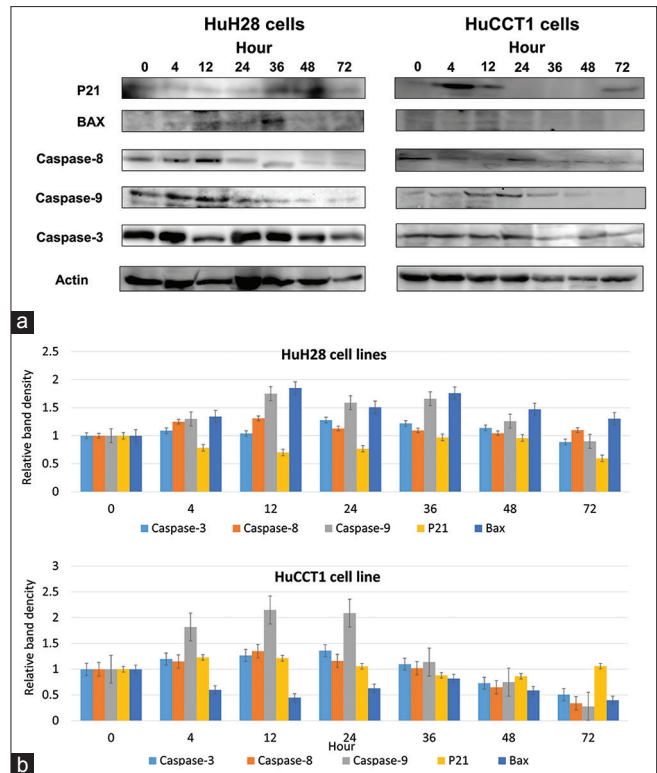


Figure 2: Effect of β -eudesmol on the expression levels of key proteins related to cell cycle regulation and apoptosis in HuH28 and HuCCT1 cell lines. Protein expression (*caspase-3*, *-8*, *-9*, *p21* and *Bax*) measured by western blot at different time points (h) in both cell lines: (a) western blot protein bands and (b) semi-quantitative measurement (densitometry plot) using band intensities. Band intensities were normalized with actin for quantification. Zero h indicates non-treated control. In HuH28 cell lines, *Caspase-3* and *-9* proteins were detected during 4–48 h, *Bax* protein was detected during 4–72 h. In HuCCT1 cell lines, *caspase-3*, *-8*, *-9* and *p21* proteins detected were during 4–36 h. All the experiments were performed 3 times, and the band intensity was expressed as average with error bar (standard deviation). h = hour

patients in terms of the enhancement of chemotherapy and radiation through promoting DNA-double strands breakdown.^[17]

Caspase-8, *-9*, and *-3* are the hallmark for the investigation of apoptotic pathway.^[9] In the previously published datasets, induction of apoptosis pathways by anticancer agents (*cucurbitacin B*, *indirubin-3'-oxime*, *allicin*, *kaempferol*, etc.) were found to suppress human CCA, where expression of *bcl-2*, *caspase-3*, *-8*, *-9* and *bax* was observed.^[18] In this study, β -eudesmol induced the caspase-dependent apoptotic activity (*caspase-3*) through both intrinsic (*caspase-9*) and extrinsic (*caspase-8*) apoptotic pathways in HuH28 and/or HuCCT1 cell lines which was evidenced by the mRNA expression and western blot profiles. Previously, in different cell lines (human CCA KCU-100 and CL-6), this compound also induced apoptosis with the activation of *caspase-3/7* pathway.^[19] Therefore, *caspase-3* may serve as an appropriate surrogate to examine drug-mediated apoptosis pathway in CCA.

Presently, a few extrinsic apoptotic modulators have been investigated for CCA therapy.^[20-22] To the best of our knowledge, the studies of drug/compound which induces extrinsic pathway in CCA cell lines (e.g. HuCCT1) are very limited. None of the available published articles showed the enhancement of extrinsic pathway. Therefore, a compound that can activate the extrinsic pathway would be of interest to drug discovery.

HuCCT1 cell line is likely to be more sensitive to β -eudesmol on induction of cell cycle regulation and activation of the intrinsic and extrinsic apoptotic pathways. This is supported by the expression of key molecules (*p53*, *Caspase -3*, *-8*, *-9*, and *bcl-2*) by real-time PCR in HuCCT1 but not in HuH28 cell lines [Figure 1a-c]. In turn, this could also explain the variation in drug response in patient treatment, for example, most effective in patients with certain clinical characteristics. Nonetheless, the different characters of the two cell lines cannot be overlooked while interpreting these findings (such as different cell morphology, doubling time, tumor specificity, etc.).

Studies revealed that caspase-3 has the widest ability to cleave substrate.^[23] Currently, there are only a few marketed drugs that promote the activation of caspase-3, particularly for CCA, e.g. sorafenib.^[24] However, it is still unsatisfactory since 75% of the patients exhibited grade 1 and 2 adverse events (AEs). It means, approved drugs for CCA therapy are still unsatisfactory in terms of survival rate, progression-free survival, and AEs. Therefore, compounds that can activate both intrinsic and extrinsic pathway on CCA cell lines should be considered priority for drug development for alleviating illness and improving the quality of life.

CONCLUSION

Taken together all the findings, β -eudesmol induced the expression of apoptosis pathway proteins, suggesting its potential role in promoting the caspase-dependent apoptotic pathway and induction of the cell cycle arrest. Although the availability of protein level data for *p53* and *bcl-2* expression would provide additional value, this is our limitation. Nevertheless, β -eudesmol can be considered as a potential compound to be further investigated as an anti-CCA agent.

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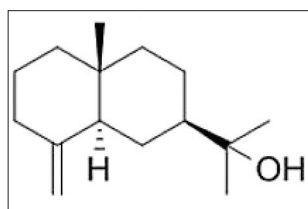
Conflicts of interest

There are no conflicts of interest.

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Supplementary Figure S1: Structure of β -eudesmol (Source: Wako, Osaka, Japan)