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

INTRODUCTION

Cholangiocarcinoma (CCA) is a neglected disease most prevalent in developing countries and it has no available effective chemotherapeutics despite the high burden and mortality rate. This cancer arises from the abnormality of cell cycle (e.g., p38 or mitogen activated protein kinase, p53 or tumor suppressor gene p53, or p21 (WAF) or cyclin-dependent kinase inhibitor 1. 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. Apoptotic pathways (both intrinsic and extrinsic) have been identified as potential drug targets in cancer. 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. Although various antagonists and agonists of apoptosis have been developed to enhance the cellular apoptosis both in preclinical and clinical studies, the efficacy of these compounds remains unsatisfactory. 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. It demonstrated potent anticancer activity on CCA in vitro and in vivo with low toxicity on normal cells (IC50 = 24.1 ± 3.4 µg/mL, and selectivity index = 8.6). Moreover, β-eudesmol also promoted cell cycle arrest at G1 phase, with caspase-3/7 induction in CCA cell lines.

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) with 10% fetal bovine serum and 1% penicillin/streptomycin. 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 (PVDF) 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.
Figure 8 continuously and — Although we do not have bcl-2 protein increased expression at mRNA level in both cell lines 1b and c]. 9 = 0.0011).

Figure 5'‑CTTTTCGACATAGTGTGGTGGTGCCC‑3' Forward: Primer sequence (5'‑3') expression was increased with time (P = 0.003, P = 0.004) [Figure 1b].

Although the p21 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. 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. 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

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward: Primer sequence (5'-3')</th>
<th>Reverse: Primer sequence (5'-3')</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3</td>
<td>5'-CATACATGGAAGCGATCAATGGAATCTGGG-3'</td>
<td>5'-CGACATCTGATACCAGACAGGAGATGCTG-3'</td>
<td>57</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>5'-GATATTGGGGAACAACTGGACAGTGAAGA-3'</td>
<td>5'-TCCACCAAGAAGCTGAGCGA-3'</td>
<td>57</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>5'-CTGTGGTCATGAGTCCTTCC-3'</td>
<td>5'-GTCCAGCCCATGATGGTTCT-3'</td>
<td>57</td>
</tr>
<tr>
<td>p53</td>
<td>5'-TTTTCCGCGATATGTTGCTGTTGCCC-3'</td>
<td>5'-CGGAACTAAGGCAAGCAGGAG-3'</td>
<td>55</td>
</tr>
<tr>
<td>p21</td>
<td>5'-CCGATACGAGGAAACTTGCTACATGATGAGTG-3'</td>
<td>5'-GCAATGCCATGTGGTCTGG-3'</td>
<td>55</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>5'-GAGGATTTGTGGCCCTTCTTGATGC-3'</td>
<td>5'-CTCCGTATCTGCTGATCAGGGT-3'</td>
<td>60</td>
</tr>
<tr>
<td>Bax</td>
<td>5'-CCACCAAGAAGCTGAGCGA-3'</td>
<td>5'-GTCCAGCCCATGATGGTTCT-3'</td>
<td>62</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TCAGCAGAATGTGCTATT-3'</td>
<td>5'-CTCCGTATCTGCTGATCAGGGT-3'</td>
<td>60</td>
</tr>
</tbody>
</table>

GAPDH=Glyceraldehyde 3-phosphate dehydrogenase
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 KIU-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. 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. Currently, there are only a few marketed drugs that promote the activation of caspase-3, particularly for CCA, e.g. sorafenib. 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)