Original Research Article

Antitumor effects of candidone extracted from *Derris indica* (Lamk) Bennet in cholangiocarcinoma cells

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**Abstract**

**Purpose:** To investigate the antitumor effect of candidone extracted from *Derris indica*, against human cholangiocarcinoma (CCA) cells.

**Methods:** Candidone was purified from the hexane extract of *Derris indica* fruit. CCA cell lines, KKU-M156 and KKU-M213, were treated with candidone. Sulforhodamine B (SRB) assay and acridine orange/ethidium bromide (AO/EB) staining were used to investigate the effects of candidone on cell proliferation and induction of apoptosis, respectively. The effect on cell migration was assessed by wound healing assay. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was employed to assess the effects of candidone on the expression of genes that regulate proliferation and apoptosis.

**Results:** Candidone exerted strong anticancer effects on CCA cells. The agent suppressed CCA cell proliferation and induced apoptotic cell death. RT-qPCR assay revealed that candidone significantly increased the expression of anti-proliferative and pro-apoptotic genes, including p21 and Bax, and decreased the expression of anti-apoptotic genes, including Bcl-2 and survivin. Moreover, candidone inhibited the migration of CCA cells induced by IGF-1.

**Conclusion:** Candidone exhibits potent antitumor effect on CCA cells. These findings suggest that candidone is potentially suitable for the management of CCA and, therefore, warrants further investigation.

**Keywords:** Candidone, *Derris indica*, Cholangiocarcinoma, Cytotoxicity, Apoptosis

**INTRODUCTION**

The incidence of cholangiocarcinoma (CCA) is increasing worldwide \([1]\). The highest incidence is in Southeast Asia, especially Thailand, Laos, and Cambodia \([2]\). Cholangiocarcinogenesis results from multiple factors, particularly parasitic infestation by *Opisthorchis viverrini*. The most effective treatment of CCA for non-metastatic stage is the complete surgical removal of the tumor. However, in metastatic or locally advanced CCA, chemotherapy and radiotherapy...
are provided as palliative treatment, and show poor responsiveness [3]. To improve treatment outcome, many attempts have focused on the combination of chemotherapy [4] and molecularly targeted therapy [5]. However, drug resistance is a problem that frequently results in treatment failure [1]. For this reason, therapeutic strategies need to be improved in order to overcome this fatal malignancy.

Natural compounds from medicinal plants have gained much attention during the past decades. Phytochemicals with bio-active properties, including antioxidant and antitumor effects, have been widely studied. These may shed some light on potential candidates in preclinical study of anticancer agents [6]. Importantly, many phytochemicals have been shown to suppress cellular signaling pathways and promote cancer cell death. They behave like pro-oxidants, increasing oxidative stress in cancer cells by inhibiting ROS scavenging system, inactivating pro-survival signaling pathways, and finally triggering apoptotic cell death [7]. Bioflavonoids are a large group of bioactive compounds that mediate antitumor effects in many cancer models. For example, apigenin exhibits antiproliferation and induces apoptosis in renal carcinoma [8]. In CCA, luteolin suppresses the proliferation and migration of CCA cell lines [9].

_Derris indica_ is a medicinal plant that is widely grown in Southeast Asia, including the southern part of Thailand. The extracted compounds from _Derris indica_ exhibit various pharmacological properties such as antioxidant, antimicrobial, anti-inflammatory, and anti-diabetic activities [10]. Its cytotoxic effect on cancer cells has been demonstrated in HepG2 and CCA cell lines. Candidone is a flavanone derivative extracted from the fruit of _Derris indica_. Among 16 compounds that are extracted from _Derris indica_, candidone is one of the most potent cytotoxic compounds against CCA cells [11]. Thus, candidone represents a potential therapeutic compound that warrants further study. The present study aims to investigate antitumor effects of candidone on cell proliferation, apoptosis and migration in CCA cells.

**EXPERIMENTAL**

**Materials**

Ham F12 media, fetal bovine serum (FBS) and other cell culture reagents were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Sulforhodamine B (SRB) was obtained from Sigma-Aldrich (St Louis, MO, USA). Candidone was provided by Assoc Prof Chavi

Cell lines and cell culture conditions

The human cholangiocarcinoma cell lines including KKU-M156 and KKU-M213, were kindly provided by Prof. Banchob Sripa of the Department of Pathology, Faculty of Medicine, Khon Kaen University. These CCA cells were cultured in Ham F12 medium supplemented with 10 % fetal bovine serum, 100 U/mL penicillin G and 100 μg/mL streptomycin and maintained in 5 % CO₂ incubator at 37 °C. The cells were trypsinized with 0.25 % trypsin-EDTA and media was renewed every 2 to 3 days.

**Cell viability assay**

Briefly, KKU-M156 and KKU-M213 cell lines were plated in a 96-well plate at density of 7,500 cells/well. After exposure to candidone at various concentrations for 8 or 24 h, SRB assay was performed as previously described [12]. Briefly, the cells were fixed with trichloroacetic acid and stained with protein-bound SRB dye. The dye was dissolved with Tris base solution and measured the absorbance at 540 nm using microplate reader.

**Fluorescence-acridine orange/ethidium bromide (AO/EB) staining**

AO/EB fluorescence staining was employed to measure live and apoptotic cells as previously described [13]. After treatment with 0, 2.5, or 5 μg/mL of candidone, cultured cells were stained with AO and EB (each 1 μg/mL). The images were taken under a Nikon Eclipse TS100 inverted microscope. The percentage of live and apoptotic cells were calculated and divided by total number of cells in the same area.

**Quantitative real-time polymerase chain reaction**

KKU-M156 cells were seeded in a 6 well-plate at a density of 300,000 cells/well. After exposure to candidone at various concentrations for 6 or 24 h, total RNA was extracted using TriZol® reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer’s instructions. Total RNA (1 μg) was converted to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer’s protocol. The Fifty nanograms of cDNA served as template for real-
time PCR. CDKN1A, Bax, BCL-2, BIRC5, and β-actin mRNA expression level were analyzed with Taqman gene expression assay using TaqMan™ Fast Advanced Master Mix and Taqman probes (CDKN1A, Hs00355782_m1; Bax, Hs00180269_m1; BCL-2, Hs00608023_m1; BIRC5, Hs04194392_s1; and β-actin, Hs99999903_m1) on a QuantStudio™ 6 Flex Real-Time Refurbished PCR System (Applied Biosystems™, CA, USA). To determine the relative expression of genes, the fold changes were analyzed with a cycle threshold (Ct) in the linear range of amplification. Relative mRNA expression of the interested genes was expressed as a ratio to β-actin mRNA.

Wound healing assay

The scratch wound healing assay was employed to evaluate cell migration as previously described [9]. Briefly, KKU-M156 cells were seeded into a 24-well plate at a density of 250,000 cells/well and allowed to grow for 24 h. An opened wound was scratched using a sterile 200 μL pipette tip. After removing detached cells, cells were treated with 50 nM of insulin like growth factor-1 (IGF-1) alone or with various concentrations of candidone. The images of the opened wound were captured from 0 to 48 h. The closing of wound area was represented the capability of cell migration. The distance of closing wound was determined using Image-Pro Plus software (Media Cybernetics, LP, USA).

Statistical analysis

Statistical differences between the control and treatment groups were analyzed using Student’s t-test and one-way analysis of variance (ANOVA) using SPSS software. The results were considered to be statistically significant if p < 0.05.

RESULTS

Candidone suppresses cell proliferation in CCA cells

To determine the efficacy of candidone (Figure 1) in CCA cells, two CCA cell lines; KKU-M156 and KKU-M213 were exposed to various concentrations from 0.5 - 10 µg/mL for 8 and 24 h. After a 6-hour exposure, phase-contrast microscopy revealed an increase in dead cells as the concentration of candidone increased from 2.5 to 10 µg/mL (data not shown). The effect of candidone on cell viability was measured by SRB assay. As shown in Figure 2, candidone exhibits antiproliferative effect in a concentration- and time-dependent manner in KKU-M156 (Figure 2 A) and KKU-M213 (Figure 2 B). IC50 values at 8 and 24-h for KKU-M156 cells were 6.00 and 4.24 µg/mL, respectively, and IC50 values for KKU-M213 cells were 5.70 and 5.74 µg/mL, respectively cells (Figure 2 C). The results suggest that candidone in the micromolar range mediates anticancer effects in both CCA cells.

Candidone induces apoptosis in CCA cells

Since candidone mediated antiproliferation in CCA cells, we then determined whether candidone can induce apoptosis in CCA cells. KKU-M156 (A) and KKU-M213 (B) cells were treated with various concentrations of candidone (0 - 10 µg/mL) for 8 and 24 h. After treatment, the cell viability was determined by SRB assay. The IC50 values for KKU-M156 and KKU-M213 cells are shown as mean ± SD (C) from three independent experiments.
Candidone induced apoptosis in CCA cells.

After treatment with candidone (CD) (0, 2.5 and 5 µg/mL) for 24 h, KKU-M156 cells were stained with fluorescent dye AO/EB. The numbers of live (A) and apoptotic cells (B) were counted, and the percent of the total number of cells was calculated. Results are expressed as mean ± SD from three independent experiments; *p < 0.05 versus control group; **p < 0.05 versus each treatment group.

Candidone modulates expressions of genes involved in proliferation and apoptosis

To examine the mechanism by which candidone suppressed cell proliferation and induction of cell death, the expression of genes involved in regulation of cell proliferation (CDKN1A or p21) and apoptosis (Bax, Bcl-2, BIRC5) were measured using RT-qPCR. Candidone upregulated p21 expression in a concentration- and time-dependent manner. The increase of p21 level was detected as early as 6 h and the levels were markedly increased at 24 h (Figure 4).

Figure 4: Effect of candidone on the expression of genes involved in proliferation and apoptosis. KKU-M156 cells were treated with candidone (CD) (0, 2.5 or 5 µg/mL) for 6 and 24 h. The expressions of CDKN1A (p21), Bax, Bcl-2, and BIRC5 (Survivin) were measured using RT-qPCR. Expression of target genes was normalized to the expression of β-actin. Data are expressed as mean ± SD from three independent experiments; *p < 0.05, **p < 0.001 versus control group.
Candidone suppresses IGF-1 mediated CCA cells migration. The scratched wounds of KKU-M156 monolayer cells were treated with IGF-1 alone (50 nM) or in the presence of candidone (0, 1, 2.5 and 5 μg/mL) for 48 h. Representative images from wound healing assay taken from the same field at 0, 24, and 48 h are shown (A). The percent of wound closure after 24 and 48 h is shown in B and C. Data are expressed as mean ± SD from three independent experiments; *p < 0.05 compared to control group; #p < 0.05 compared to IGF-1 control group.

Bax expression was unchanged at 6 h, but significantly increased at 24 h (Figure 4 B). In contrast, expression of the antiapoptotic gene Bcl-2 decreased when treated with candidone for 24-h (Figure 4 C). However, the ratio of Bax/Bcl-2 was significantly increased in the 5 μg/mL candidone group (Figure 3 D). The antiapoptotic gene, BIRC5 (survivin) was also significantly suppressed by 5 μg/mL of candidone compared to untreated control in the 24 h treatment group (Figure 3 E). Taken together, these results suggest that candidone caused CCA cell death by modulating the expression of genes involved in proliferation and apoptosis.

**Candidone suppresses IGF-1 mediated CCA cells migration**

Wound healing assay was employed to examine the effects of candidone on inhibition of migration of CCA cells induced by IGF-1. IGF-1 significantly induced KKU-M156 cell migration compared to untreated cell. Treatment with candidone suppressed the migratory effect mediated by IGF-1 in a concentration-dependent manner (Figure 5 A). Moreover, 2.5 and 5 μg/mL of candidone significantly suppressed wound closing after 24 (Figure 5 B) and 48 hours (Figure 5 C). The results demonstrate that candidone significantly diminishes IGF-1 dependent CCA cell motility.

**DISCUSSION**

CCA is a biliary cancer with poor prognosis. Since there is no effective treatment for this fatal malignancy, many previous studies have investigated compounds to improve survival. *Derris indica* is widely grown in Thailand, an area in which CCA is endemic. The antitumor activity of candidone, a flavanone compound extracted from *Derris indica*, has been demonstrated in various cancers [10]. We therefore evaluated the antitumor effect of candidone in two CCA cells. The results illustrate that candidone has a cytotoxic effect against CCA cells, with IC50 values in the micromolar range. The compound mediates antiproliferation as well as apoptotic cell death in CCA cells. The effects of candidone are associated with alteration in the expression of genes involved in proliferation and apoptosis.
Furthermore, candidone also inhibits cell migration.

Flavonoids have been documented as rich source of compounds with chemopreventive effects on cancer. Compounds in the flavanone class similar to candidone, such as hesperidin and naringin, show anticancer activity against breast and lung cancer [14, 15]. They mediate anticancer effects with selective toxicity towards cancer cells [16]. The antitumor effect of flavonoids including luteolin, quercetin, and EGCG in CCA are mediated through the suppression of JAK/STAT signaling [9, 17] and induction of mitochondrial cell death pathway [18]. In the present study, candidone showed a cytotoxic effect against two CCA cells, KKU-M156 and KKU-M213, in the micromolar range. Notably, candidone mediates a rapid cytotoxic effect, which is evident as early as 8 h in both cell lines.

The effect of candidone on apoptotic cell death in CCA cells was elucidated by AO/EB fluorescence staining. Candidone at a concentration of 2.5 and 5.0 μg/mL induced apoptosis in KKU-M156 cells. It also suppressed cell proliferation, as shown by the decrease in percentage of live cells. Like other flavonoids, candidone mediates its antitumor effects through antiproliferation and induction of apoptosis.

We investigated the underlying mechanism of action of candidone by RT-qPCR. It was found that the expression of CDKN1A (p21) mRNA was upregulated. p21 is a negative regulator of cell cycle progression, and its activation leads to suppression of cell proliferation. We then examined the expression of Bax, Bcl-2, and BIRC5 (survivin). Bax and Bcl-2 are known regulators of apoptosis through the intrinsic or mitochondrial cell death pathway. Bcl-2 and survivin play an important role as inhibitors of apoptosis. Bcl-2 is localized to the outer membrane of mitochondria and inhibits the proapoptotic proteins Bax and Bak from forming oligomeric mitochondrial pore, whereby cytochrome C is released from the mitochondrial matrix into cytosol and initiates apoptotic cell death cascade [19]. Survivin acts by inhibiting caspase activation and promoting cell survival [20]. The balance of Bax and Bcl-2 regulates whether a cell will live or die [21], and the balance of Bax and Bcl-2 induced by candidone favors cell death.

Cancer progression is usually involved with invasion of surrounding tissues and metastasis into distant tissues. These processes are involved with migration capability of cancer cells. Candidone induced suppression of cell migration at low cytotoxic concentration. In our wound healing assay, cell migration was stimulated by IGF-1. Candidone strongly inhibits cell migration in wound healing assay. This suggests that the anticancer activity of candidone involves the suppression of tumor invasion and metastasis.

CONCLUSION

The results of this study demonstrate that the phytochemical, candidone, mediates antitumor effect in CCA cells. This preclinical study provides new insights into the anticancer activity of flavanone derivatives from Derris indica. The precise underlying mechanisms by which candidone suppresses CCA should be further elucidated to develop a therapeutic compound in the treatment of cholangiocarcinoma.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. SK, VK, and AP designed the study, BK and SK performed experiments, CJ provided candidone compound, SK and VK wrote manuscript, SK analyzed the results and supervised the study. All authors approved the manuscript.

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