Heliyon 5 (2019) e01675

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.heliyon.com

Antitumor effects of flavopiridol, a cyclin-dependent kinase inhibitor, on human cholangiocarcinoma *in vitro* and in an *in vivo* xenograft model

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ARTICLE INFO

Keywords:
Biochemistry
Cancer research
Molecular biology

ABSTRACT

Flavopiridol, a pan-cyclin-dependent kinase (CDK) inhibitor, was recently identified as an effective antitumor agent for several cancers. We investigated the antitumor effect of flavopiridol on cholangiocarcinoma (CCA), *in vitro* and *in vivo*. A methylthiotetrazole assay revealed that the proliferation of certain CCA cells was inhibited by flavopiridol, which induced the caspase-dependent apoptosis of CCA cells. Although increased cell cycle arrest was observed at the G2/M phase, caspase activation occurred earlier than 24 h, indicating that caspase-dependent apoptosis is the major pathway for the suppression of cell proliferation. Flavopiridol potently reduced the CCA tumor growth in a xenograft model without observable adverse effects. These findings indicated that flavopiridol could be a potential antitumor agent for the treatment of CCA.

1. Introduction

Cholangiocarcinoma (CCA) is a malignant tumor of biliary epithelial cells. A high incidence of CCA has been reported in northeastern Thailand, where liver fluke *Opisthorchis viverrini (Ov)* infection is endemic and presents a major risk factor for CCA [1, 2]. Surgery and chemotherapy are the treatments of choice, but these treatments can only be offered to a limited number of patients and may not be successful due to delayed diagnosis [3]. Flavopiridol (5, 7-dihydroxy-8-(4-N-methyl-2-hydroxypyridyl)-6′-chloroflavone) is a semi-synthetic flavone analog of an alkaloid derived from an Indian plant, *Dysoxylum binectariferum*. It was the first pan cyclin-dependent kinase (CDK) inhibitor to be tested in clinical trials, in 1999 [4]. Flavopiridol competitively binds to the ATP binding domain of CDKs and reduces the activity of CDKs (CDK1, 2, 4, 6 and 7) in cell cycle regulation [5, 6]. Additionally, flavopiridol decreases transcription by inhibiting phosphorylation activity of CDK9 of RNA polymerase II which is essential for both transcriptional initiation and elongation [6, 7]. Its antitumor activity has been related to cell cycle arrest and apoptosis in hematological malignancies and solid tumors [8]. Recently, CDK4/6-selective inhibitors, such as palbociclib, ribociclib and abemaciclib, have been developed and shown significant benefits in clinical studies including breast cancer, non-small cell lung cancer, melanoma and head and neck squamous cell carcinoma, ref. in [9]. In this study, we evaluated the effect of flavopiridol on cell proliferation in CCA cell lines and its antitumor activity in a CCA xenograft mouse model. We found that flavopiridol induced cell cycle arrest and caspase-dependent apoptosis. It also suppressed CCA growth in a xenograft mouse model. These results suggest that flavopiridol might be a potential drug for CCA treatment.

2. Materials and methods

2.1. Cell lines

CCA cell lines (KKU-055, KKU-100, KKU-213 and KKU-214) established from primary cultures of Thai CCA patients' tissues [10] were obtained from the Japanese Collection of Research Bioresources (JCRB) cell bank, Osaka, Japan. CCA cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (HyClone

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https://doi.org/10.1016/j.heliyon.2019.e01675
Received 17 November 2018; Received in revised form 8 March 2019; Accepted 3 May 2019

Laboratories, Inc., Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were maintained in humidified incubator at 37 °C and 5% CO_2.

2.2. Chemicals and antibodies

Flavopiridol was purchased from Sigma-Aldrich (131740-30-5) (St Louis, MO) and Cayman Chemical (146426-40-6) (Ann Arbor, MI). Antibodies to β-actin (8432), cyclin B1 (594), cdc2 (54) were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies to cleaved caspase-3 (9661), cleaved caspase-8 (9496), cleaved caspase-9 (20750), HRP-linked goat-anti-rabbit IgG (7074) and horse-anti-mouse IgG (7076) were purchased from Cell Signaling (Danvers, MA).

2.3. MTT assay

CCA cells were seeded into 96-well plate at 2.5×10^3 cells/well in triplicate and cultured overnight in a humidified incubator at 37 °C with 5% CO_2. Cells were then treated with 0, 50, 100, 200 or 300 nM flavopiridol for 24, 48 or 72 h. MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) solution (Sigma, St. Louis, MO) was added to each well and incubated for 3 h. Formazan crystals were dissolved by adding DMSO. The absorption was measured at 595 nm using a microplate reader (iMark; Bio-Rad Laboratories, Hercules, CA) and the absorption values were normalized to a vehicle control.

2.4. Annexin V/PI staining assay

The number of apoptotic cells was quantified using a Pacific Blue™ Annexin V apoptosis detection kit (BioLegend, San Diego, CA) according to the manufacturer's instructions. CCA cells were seeded into 12-well plate at 5×10^4 cells and treated with various concentrations of flavopiridol. The dead and adherent cells were harvested, incubated with Pacific Blue™ Annexin V at room temperature for 30 min in the dark and stained with 1 μg/ml propidium iodide (PI). The cells were analyzed using a BD LSR II™ flow cytometer (BD Bioscience, San Jose, CA). Data analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR).

2.5. Cell cycle analysis

KKU-055 and KKU-213 cells were treated with increasing concentrations of flavopiridol for 24 h. The treated cells were washed in PBS and fixed in 70% ethanol overnight at 4 °C. The fixed cells were stained with PBS containing 200 μg/ml of PI, incubated for 30 min and subsequently analyzed using a BD LSR II™ flow cytometer (BD Bioscience, San Jose, CA). Data analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR).

2.6. Western blotting

KKU-055 and KKU-213 CCA cells were treated with flavopiridol at different concentrations and times. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM NaF, 1 mM Na_3VO_4) containing protease inhibitor cocktail (Nacalai Tesque, Tokyo, Japan). Protein amounts were determined by the bicinchoninic acid (BCA) protein assay (Thermo Science, Rockford, IL). Ten micrograms of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a PVDF membrane (GE Healthcare Japan, Tokyo, Japan), which was probed with primary antibodies. Horseradish peroxidase conjugated secondary antibodies were further incubated. Proteins were detected using Chemi-Lumi One Super reagents (Nacalai Tesque, Kyoto, Japan) and visualized with an ImageQuant Las4000 system (GE Healthcare Japan). β-actin was used as the internal control.

2.7. Xenograft mouse model

KKU-213 cells ($1x10^5$) were subcutaneously injected into the flanks of male Balb/c RJ mice [10, 11]. The dosing schedule of flavopiridol used in this study is based on previous preclinical study [12]. Five to six mice per group were administered an intraperitoneal injection of 100 μl PBS or flavopiridol (5 and 7.5 mg/kg body weight) 3 days after tumor transplantation and then 5 days a week for 2 weeks. Tumor growth was monitored every day using a vernier caliper. The mice were sacrificed on day 17 by cervical dislocation and the tumors were removed and weighed. Body weights were recorded twice a week in order to observe the condition of the mice. The procedures and protocols of animal study were approved by the Institutional Animal Care and Use Committee of Kumamoto University.

2.8. Statistical analysis

Data are shown as the mean ± standard deviation (SD). The statistical significance of differences observed between the experimental groups and the control group was determined using the *Student's t*-test. A value of $P < 0.05$ was considered significant. SPSS software version 17 (SPSS, Inc., Chicago, IL) was used to perform the statistical analysis.

3. Results

3.1. Flavopiridol inhibited proliferation in CCA cell lines

The proliferations of four CCA cell lines (KKU-055, KKU-100, KKU-213 and KKU-214) treated with flavopiridol (0, 50, 100, 200 and 300 nM) for 24, 48 and 72 h were analyzed by MTT assay. Flavopiridol potently inhibited proliferation in all CCA cell lines in a dose- and time-dependent manner (Fig. 1A). The half-maximal inhibitory concentrations (IC_{50}) of flavopiridol at different time points were in the 40–213 nM range, as shown in Table 1.

3.2. Flavopiridol induced G2/M cell cycle arrest

To verify the causal relationship between cell proliferation inhibition and cell cycle arrest, the cell cycle distribution was analyzed. KKU-055 and KKU-213 cell lines were treated with increasing concentrations of flavopiridol for 24 h and analyzed by flow cytometry after PI staining. The cell cycle analysis showed that flavopiridol induced a tendency toward G2/M arrest in a dose-dependent manner in both cell lines (Fig. 1B). Expression of Cdc2, the regulatory protein in the G2/M phase, was not changed, but that of cyclin B1 was slightly increased in a time-dependent manner (Fig. 1C). These results suggest that flavopiridol triggered cell cycle arrest at the G2/M phase but its function of cell cycle arrest for CCA is weak.

3.3. Flavopiridol induced apoptosis via caspase-dependent pathway

Next, we examined whether the inhibition of flavopiridol on CCA cell proliferation was due to the induction of apoptosis. Flow cytometric analysis via Annexin V/PI staining was performed in KKU-055 and KKU-213 cell lines exposed to increasing concentrations of flavopiridol for 24 h. As shown in Fig. 2A, the proportions of early-stage (Annexin V-positive/PI-negative) and late-stage (Annexin V-positive/PI-positive) apoptotic cells increased in a dose-dependent manner. The sub-G1 population (apoptotic cell population) also increased upon flavopiridol treatment in a dose-dependent manner (Fig. 2B). To further determine whether or not flavopiridol-induced apoptosis was caspase dependent, we investigated the expression of cleavage caspases by western blotting. As shown in Fig. 2C, the cleavage forms of caspases in flavopiridol treated cells increased in a time-dependent fashion. KKU-055 cells were more sensitive to flavopiridol than KKU-213 cells. Flavopiridol obviously activated caspase proteins in KKU-055 at 6 h that was earlier than KKU-

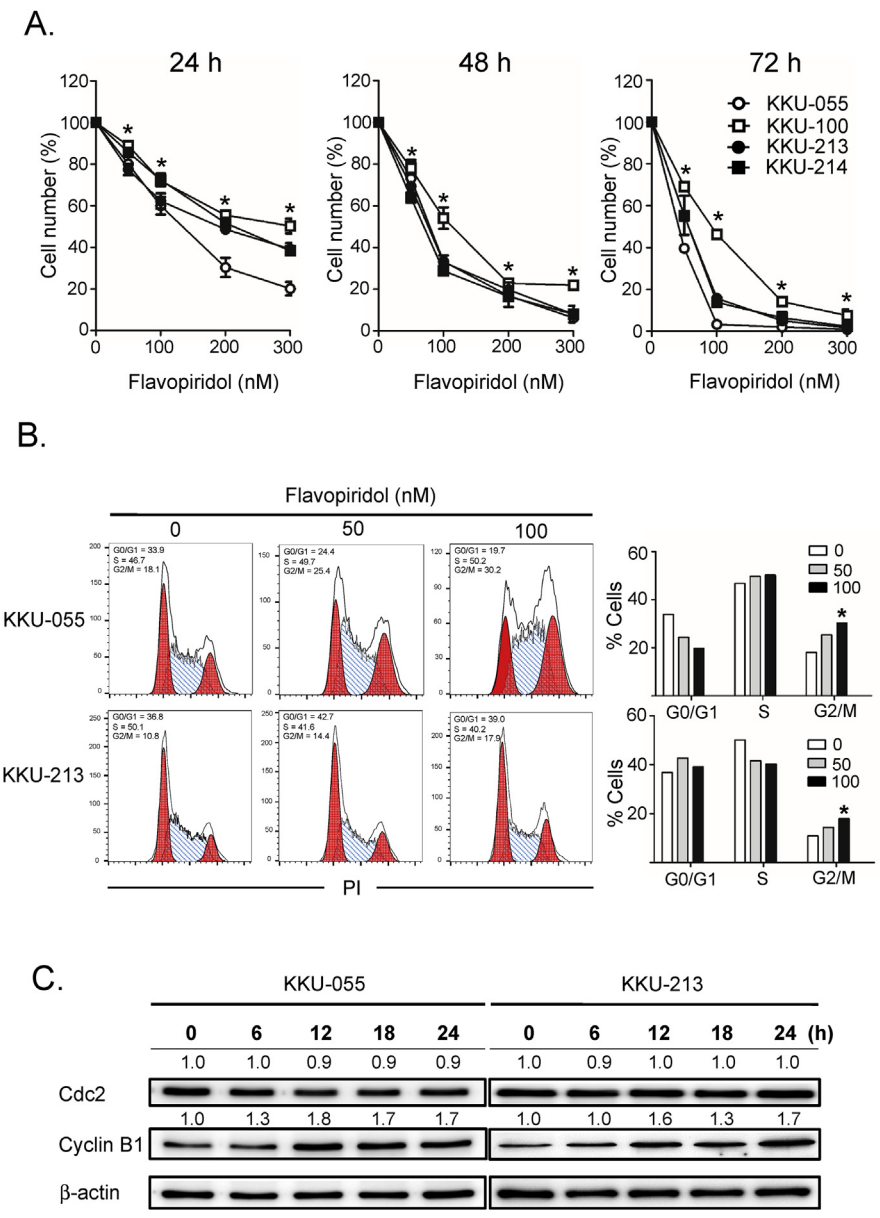

Fig. 1. (A) The antiproliferative effect of flavopiridol on CCA cell lines was determined using an MTT assay. KKU-055, KKU-100, KKU-213 and KKU-214 cells were treated with 50, 100, 200 or 300 nM of flavopiridol at 24, 48 or 72 h. The percentage of cell number in vehicle control was taken as 100%. Data are mean ± SD of three independent experiments. *P *0.05* in all CCA cell lines, significantly different for each time point compared with vehicle control. (B) Flavopiridol induced G2/M phase arrest in CCA cell lines. A) KKU-055 and KKU-213 cells were treated with the indicated concentrations of flavopiridol for 24 h. DNA content was analyzed by PI staining. Distribution of the cell cycle was assessed by flow cytometry. Representative flow cytometric profiles are shown and the percentage of cells in each phase is shown as a representative from three independent experiments. *P *0.05*, significantly different compared with vehicle control. (C) KKU-055 and KKU-213 cells were exposed to 100 nM flavopiridol for the indicated times. Expression of the regulatory protein of the G2/M phase was analyzed by western blotting. Full size images are shown in supplementary material.

Table 1
IC_{50} values of flavopiridol in four CCA cell lines. The values were calculated from three independent experiments.

Time (h)	IC_{50} (nM)			
	KKU-055	KKU-100	KKU-213	KKU-214
24	134.3 ± 11.6	>300	183.3 ± 17.8	213 ± 15.6
48	77.1 ± 8.2	111.9 ± 13.6	75.7 ± 0.8	68.8 ± 2.1
72	40.1 ± 1.8	91.9 ± 6.2	58.2 ± 4.3	56 ± 9.7

213. Annexin V/PI staining as shown in Fig. 2D showed the number of apoptotic cells after treatment with flavopiridol in KKU-055 were highest at 6 h compared to KKU-213. To confirm that the effect of flavopiridol is a caspase-dependent apoptosis, KKU-055 and KKU-213 cell lines were exposed to 300 nM flavopiridol for 24 h in the presence or absence of a pan-caspase inhibitor, Q-VD-OPh (Tonbo Bioscience, San Diego, CA) for 2 h, stained with PI, and analyzed by flow cytometry. As shown in Fig. 2E, Q-VD-OPh rescued CCA cells from flavopiridol-induced cell death. We also investigated the effect of flavopiridol on myeloid cell leukemia 1 (Mcl-1) anti-apoptotic protein which is a known target of flavopiridol action. KKU-055 and KKU-213 CCA cells were treated with 300 nM flavopiridol for 6–24 h and then determined the expression of Mcl-1 using western blotting. The result showed that flavopiridol decreased the Mcl-1 protein expression in CCA cell lines (Fig. 2F). Taken together, these data indicated that flavopiridol induced apoptosis in CCA cells via caspase-dependent pathways.

3.4. Flavopiridol suppressed CCA tumor growth in xenograft model

To examine the antitumor activity of flavopiridol in a xenograft model, the male Balb/c RJ mice were subcutaneously injected with KKU-213 cells [10] and treated with PBS or flavopiridol (5 and 7.5 mg/kg). Flavopiridol 5 and 7.5 mg/kg significantly reduced the tumor volume of CCA tumor growth (Fig. 3A) as well as tumor weight (Fig. 3B) compared with the control group, in a dose-dependent fashion ($P < 0.05$). The tumor sizes in flavopiridol-treated mice were markedly smaller than those in control mice (Fig. 3C). All mice were observed to be healthy and no apparent adverse effects. Mice in control group were significantly different in body weights on day 15 and 17 when compared with day 0. Weight loss may be due to the progression of cancer and stress (Fig. 3D).

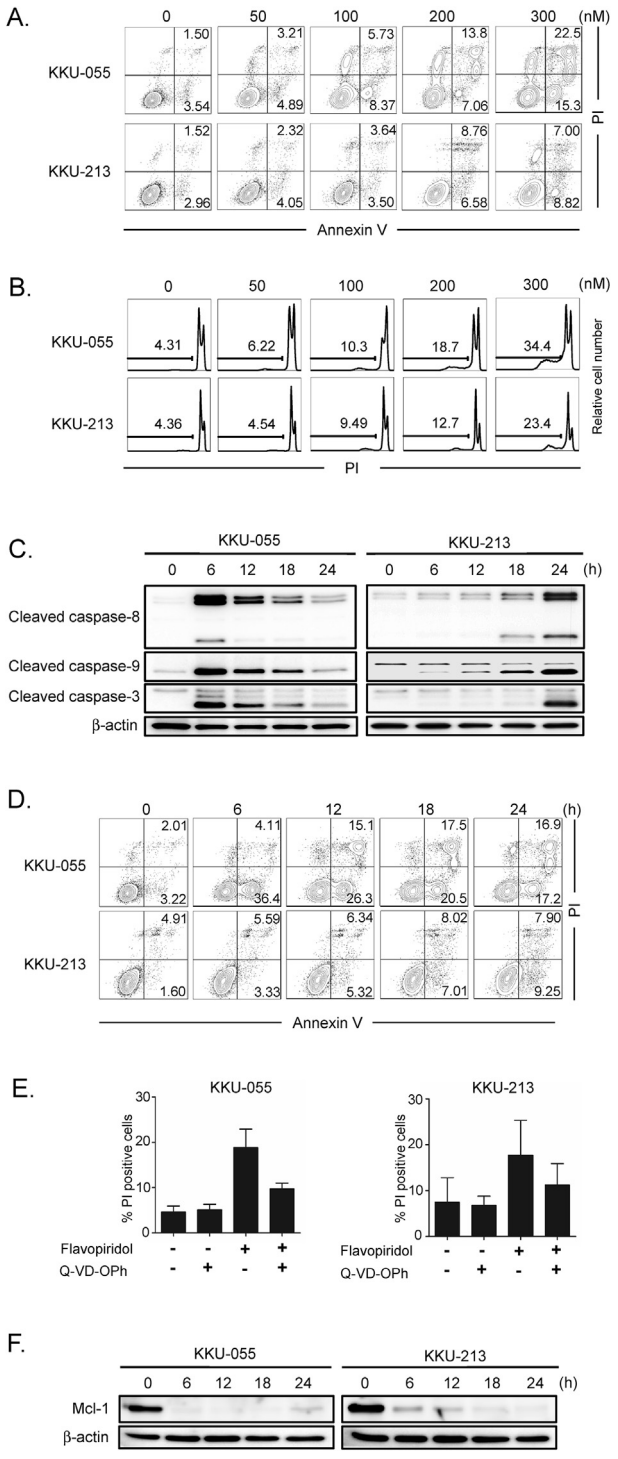

Fig. 2. Apoptosis induction by flavopiridol in CCA cell lines. KKU-055 and KKU-213 cells were treated with increasing concentrations of flavopiridol for 24 h. (A) Cells were stained with Annexin V/PI and analyzed by flow cytometry. Right upper quadrant represents Annexin V and PI double-positive cells (late apoptotic cells) and the right lower quadrant represents Annexin V positive cells (early apoptotic cells). The number of apoptotic cells increased with flavopiridol treatment in a dose-dependent fashion. (B) Sub-G1 (apoptotic) populations were analyzed by flow cytometry. A dose-dependent increase of percentages of the sub-G1 population was seen in flavopiridol treatment at 24 h. (C) Caspase activation was determined by western blotting. Flavopiridol treatment (300 nM) induced the expressions of cleaved caspase-8, -9 and -3 in a time-dependent manner. (D) Apoptosis induction of flavopiridol in CCA cell lines. KKU-055 and KKU-213 cells were treated with flavopiridol every 6 h for a total of 24 h. Cells were stained with Annexin V/PI and analyzed by flow cytometry. The numbers of apoptotic cells increased with flavopiridol treatment in time-dependent manner. (E) Cells were exposed to 300 nM flavopiridol for 24 h in the presence or absence of Q-VD-OPh. Flavopiridol triggered caspase-dependent apoptosis in CCA cells. (F) Decreasing of Mcl-1 protein expression level in flavopiridol treatment. KKU-055 and KKU-213 cells were treated with 300 nM flavopiridol for 6–24 h and then determined the expression of Mcl-1 using western blotting. Full size images are shown in supplementary material.

These results indicated that flavopiridol effectively inhibited CCA tumor growth in the xenograft model without noticeable side effects.

4. Discussion

In the present study, we demonstrated for the first time flavopiridol-mediated growth inhibition and induction of apoptosis in CCA cells both *in vitro* and *in vivo*. Flavopiridol is a potent pan-CDK inhibitor; its anti-tumor activity with cell growth inhibition, cell cycle arrest and induction of apoptosis has been shown, and it has been used in clinical trials [12, 13]. The IC_{50} values in CCA are 40.1–91.9 nM at 72 h, similar to those reported for other tumor cell types and the level achieved after systemic application of non-toxic doses [5]. Flavopiridol was also effective without adverse effects in a mouse model, indicating the possibility of clinical application in the treatment of chemotherapy-resistant CCA.

Flavopiridol is a synthetic flavonoid that inhibits a wide range of cyclin-dependent kinase (CDKs), and induces cell growth arrest at either the G1 and/or G2 phases of the cell cycle. It has been also shown to have anticancer effects through inducing apoptosis. In this study, treatment with flavopiridol induced G2/M arrest in CCA cell lines and increased the expression of cell cycle regulatory protein, cyclin B1, but not that of Cdc2 (CDK1) (Fig. 1B, 1C). In addition, the increase of cyclin B1 was weak compared with other cell types [14], and strong activation of caspases was observed earlier than the increase of cyclin B1 in the KKU-055 cell line (Fig. 1C and Fig. 2), indicating that G2/M arrest seems not to be the main pathway for the growth inhibition of CCA.

Treatment with flavopiridol clearly induced caspase-dependent apoptosis in CCA cells (Fig. 2). An increase of early and late apoptotic cells was detected in Annexin V/PI staining and sub-G1 population analysis, and this was concomitant with increases of cleaved forms of caspase-3, 8, and 9 on western blotting. We also confirmed that a pan-caspase inhibitor, Q-VD-OPh, could protect cell death against flavopiridol treatment. These data clearly demonstrated that flavopiridol induced caspase-dependent apoptosis in CCA cells. Our results are in agreement with the studies of flavopiridol treatment in numerous cancers [15, 16, 17, 18, 19]. It is of interest that, although the IC_{50} values of KKU-055 and KKU-213 CCA cell lines were similar (Fig. 1A, Table 1), the time courses of caspase activation and induction of apoptosis were different from these cell lines (Fig. 2C and D), suggesting that several different mechanisms may be responsible for the apoptosis-inducing effect of flavopiridol. Moreover, the downregulation of Mcl-1 expression which is one of known mechanism of action of flavopiridol was demonstrated in flavopiridol-treated CCA cell lines (Fig. 2F). Our data were similar to other reports in multiple myeloma cells [19], lung carcinoma cells [20] and cholangiocarcinoma cells [21].

Flavopiridol has been shown to inactivate glycogen phosphorylase, decreasing glucose availability for glycolysis [22], which can be an important target for cancer therapy [23]. It was also recently shown that flavopiridol has anti-lung cancer stem cell activity [16], and inhibited metastasis of osteosarcoma cells [24], indicating a variety of targets of this compound against cancer cells. This can represent an advantage of flavopiridol in overcoming the drug resistant nature of CCA as a single agent and combination chemotherapy.

Flavopiridol also significantly inhibited CCA tumor growth in a xenograft model in a dose-dependent manner with no observable adverse effects. Our result was consistent with previous studies in rhabdoid tumor [13] and glioma [25]. This study demonstrated that flavopiridol is

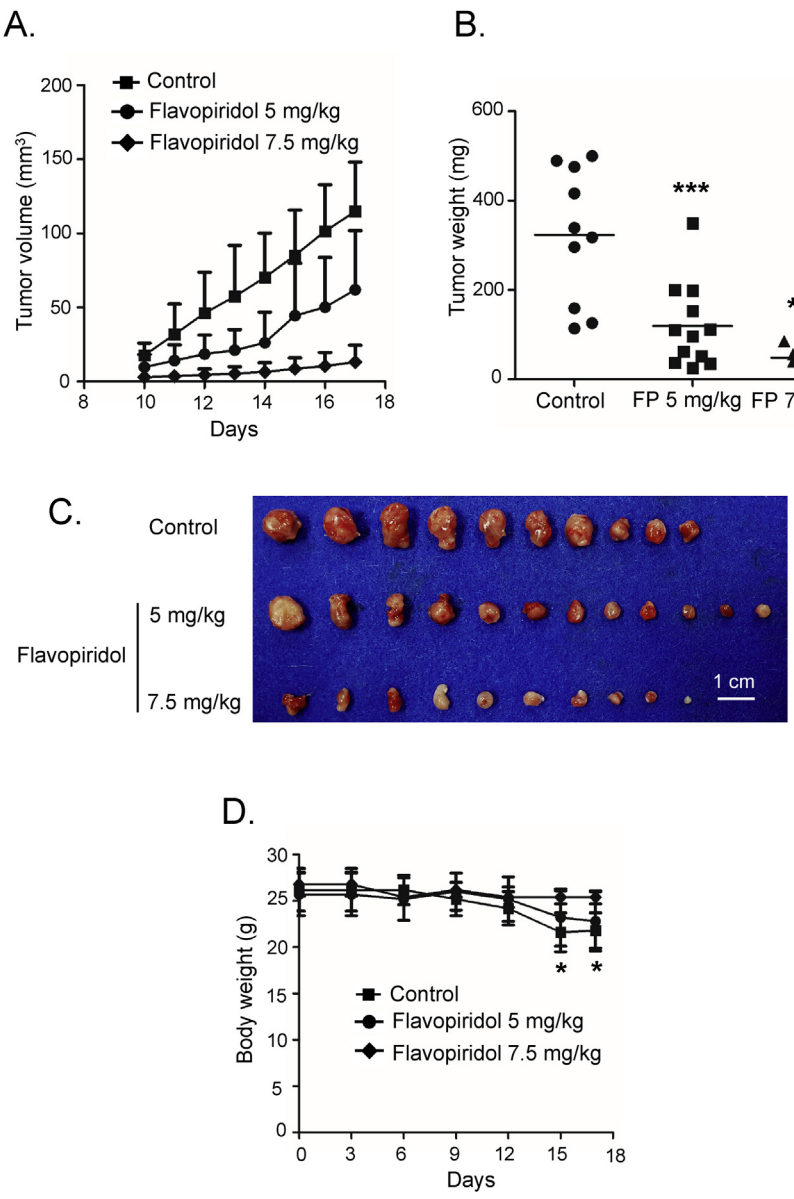

Fig. 3. Flavopiridol inhibited CCA growth in a xenograft model. KKU-213 cells were subcutaneously inoculated to the flanks of Balb/c RJ mice. The mice were administered an intraperitoneal injection of PBS or flavopiridol (5 and 7.5 mg/kg) three days after tumor transplantation, and then 5 days per week for a total of 14 days. Tumor growth was monitored every day. The mice were sacrificed on day 17. (A) Tumor volumes were recorded every day from day 10 until 17. (B) Average tumor weights of the mice in each group were compared. (C) CCA tumor tissues were obtained from control and flavopiridol-treated mice. ***P *0.001* independent sample *t*-test compared to the control group. (D) The body weights of the mice from the control and flavopiridol-treated groups were recorded twice a week. The data are presented as the mean ± standard deviation of each day. *$P < 0.05$ vs. Day 0 of each group.

effective for inhibiting CCA tumor growth by inducing apoptosis, and our findings suggest that flavopiridol can be an efficient alternative anti-cancer agent for the treatment of patients with CCA.

Declarations

Author contribution statement

Saowaluk Saisomboon: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ryusho Kariya: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Kulthida Vaeteewoottacharn: Performed the experiments; Analyzed and interpreted the data.

Sopit Wongkham: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Kanlayanee Sawanyawisuth, Seiji Okada: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by a Grant-in-Aid for Science Research (No. 16K08742) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and an invitation research grant (IN60323), Faculty of Medicine, Khon Kaen University. SS was supported by a scholarship from Japan Student Services Organization (JASSO) and Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University.

Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2019.e01675.

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