의학석사 학위논문

Effects and Mechanisms of Flavokawain B in Cholangiocarcinoma Cells

담관암 세포에서 Flavokawain B의 효과 및 기전

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Abstract

Background: Cholangiocarcinoma is a devastating malignancy with limited treatment options and poor prognosis. Natural products have gained considerable attention for showing antitumor effects with less toxicities. Flavokawain B (FKB), such a natural product, has been studied for its antitumor effects in various cancers. However, the antitumor effect of FKB in cholangiocarcinoma has not been determined yet. This study was designed to investigate the therapeutic efficacy of FKB in cholangiocarcinoma.

Methods: We investigated the effect of FKB on cell proliferation and apoptosis in a cholangiocarcinoma cell line, SNU-478. We examined whether the combination of FKB and cisplatin had an additional therapeutic effect. We also examined the mechanisms responsible for the effect of FKB. And we investigated the effect of FKB in xenograft model using nude mice.

Results: FKB was found to inhibit cell proliferation of cholangiocarcinoma cells in a dose and time-dependent manner. FKB also induced cellular apoptosis additively in combination with cisplatin. Expressions of Akt and P-Akt were down-regulated by FKB. In the

xenograft model, FKB treatment in combination with cisplatin/gemcitabine significantly inhibited tumor growth of SNU-478 cells.

Conclusion: FKB showed antitumor effect through apoptosis induction by suppressing Akt and P-Akt activity in cholangiocarcinoma, but the synergistic effect of FKB and cisplatin was not definite.

Key words: cholangiocarcinoma, flavokawain, cisplatin, gemcitabine, apoptosis, Akt, P-Akt

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Introduction

Cholangiocarcinoma, also known as biliary tract cancer refers to all tumors that arise from the biliary tract or the biliary drainage system, including the intra- and extra-hepatic bile ducts as well as the gallbladder. According to the 2016 Korea Central Cancer Registry data, the overall incidence of cholangiocarcinoma was 13.1 per 100,000 people per year.[1] Cholangiocarcinoma is the 6th leading cause of cancer-related deaths in Korea. Surgical treatments are the only potentially curative therapeutic options for cholangiocarcinoma, but this is applicable in fewer than 50% of cases because the majority of patients are diagnosed at a late stage.[2] Even after curative resection most patients develop recurrence. Therefore, systemic chemotherapy is a very important treatment modality for cholangiocarcinoma. Gemcitabine plus cisplatin is considered as a standard option for advanced cholangiocarcinoma. The median overall survival with gemcitabine plus cisplatin was found to be significantly greater compared with that using gemcitabine alone (11.7 vs. 8.1 months), however it is only palliative and survival benefit is modest at about 3 months.[3] Many target-oriented agents have been attempted for improving the outcomes of the disease, however no or

only marginal benefits were shown from many clinical trials.[4] The development of effective targeted therapies in cholangiocarcinoma is challenging because of the underlying genetic variability of the disease and the remarkable resistance of cholangiocarcinoma cells to drug cytotoxicity.[5]

Novel strategies for sensitizing tumor cells with naturally occurring dietary chemopreventive compounds have gained considerable attention because of their beneficial effects in overcoming tumor cells resistance and inducing apoptosis with less toxicities.[6] Flavokawain B (FKB), (E)-1-(2-Hydroxy-4,6-dimethoxy-phenyl)-3-phenyl-propenone, is a natural chalcone that can either be isolated from the kava-kava plant or synthesized via the reaction of 4′,6′-dimethoxy-2′-hydroxyacetophenon and benzaldehyde.[7] FKB is known to have anti-inflammatory, antinociceptive, and anticancer properties.[8] FKB has been found to be cytotoxic towards several cancer cell lines.[9-12] However, the anticancer effect and its molecular mechanism of FKB on cholangiocarcinoma cells are not well studied.

In this study, we investigated the effect of FKB on cell proliferation and apoptosis in a human cholangiocarcinoma cell line, SNU-478. We

also explored the mechanisms responsible for the effect of FKB. And, we investigated the effect of FKB in xenograft model using nude mice.

Materials and methods

Cell culture and reagents

SNU-478, a human cholangiocarcinoma cell line, was obtained from Korean Cell Line Bank (Seoul, Korea). SNU-478 cells were grown in Roswell Park Memorial Institute 1640 (RPMI-1640; Welgene, Gyeongsan, Korea) with 1% streptomycin and penicillin (Corning, Corning, NY, USA), 5 mM sodium pyruvate (PAN BIOTECH, Aidenbach, Germany) and 10% fetal bovine serum (FBS; Tissue Culture Biologicals, Tulare, CA, USA). Cell lines were incubated under standard culture condition (in incubator with 5% CO_2 and 37°C temperature). Antibodies against AKR mouse thymoma kinase (Akt), phospho-Akt (P-Akt), cleaved Poly (ADP-ribose) polymerase (PARP), and beta-actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). FKB was obtained from Abcam (Cambridge, MA, USA). It was dissolved in dimethyl sulfoxide as stock solutions and stored at −20°C. The test solutions of FKB were freshly prepared for different experiments; they were obtained by diluting the stock solution with Dulbecco modified Eagle medium to final concentrations. Cisplatin (Dong-A ST, Seoul, Korea) and

gemcitabine (Yuhan, Seoul, Korea) were dissolved in sterile phosphate-buffered saline to make 100-μmol/L stock solutions.

Cell growth inhibition assay

Cells were seeded on 96-well plates containing a final volume of 100 μl/well at a density of 4 x 10^3 cells/well, incubated at 37°C for 24 hours and then treated with FKB and/or cisplatin for 24, 48 and 72 hours. In order to determine the live cell numbers, 10 μl of 5 mg/ml 3(4,5-Dimethylthiazol-2yl)-2,5diphenyltetrazoliumbromide (MTT) (Promega, Madison, WI, USA) in phosphate-buffered saline (PBS) was added to the cells and allowed to develop for 1 h at 37°C. To dissolve formazan crystals, 100 μl of 10% sodium dodecyl sulfate (SDS) solution was added to cells and they were incubated overnight at room temperature. The solution was mixed to ensure complete solubilization. Colorimetric measurements were taken at 570 nm by a Sunrise™ reader (Tecan, Mannedorf, Switzerland) and the ratio of optical density of the cells to that of control was calculated.

Apoptosis detection

Apoptosis was examined using a double-staining method with allophycocyanin (APC)-labeled annexin-V (AV) and propidium iodide (PI) Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). For PI and AV staining, cells (1×10^5) were suspended with 100 μl of Annexin-binding buffer (BD Biosciences). Cell suspension was stained with 5 μl of APC-conjugated AV and 5 μl of PI for 15 min at room temperature and then 400 μl of annexin-binding buffer was added. Apoptotic and necrotic cells were analyzed with a flow cytometer (FACScanto II; BD Biosciences). The percentages of stained cells in each quadrant were quantified using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Protein extraction and Western blot analysis

Cells were harvested after treatment of FKB or cisplatin for 16 h, washed twice in PBS and then suspended in 20μl of lysis buffer (50 mM Tris-HCl at pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% NP-40,1 mM phenylmethylsulfonyl fluoride, 0.1% SDS, Protease Inhibitor

cocktails (Roche, Basel, Switzerland), 5 mM NaF, 2 mM Na_3VO_4). Cell suspensions were kept on ice for 15 min and then centrifuged at 13,500 rpm for 15 min at 4°C. Protein concentrations were determined by the BCA Protein Assay kit (Thermo Fisher Scientific,Waltham, MA, USA) following the manufacturers' instructions. An equal amount (30 μg) of each protein sample was loaded into each lane, separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gradient, and then transferred onto polyvinylidene difluoride membrane (Amersham Biosciences, Amersham, UK). Membranes were blocked with 3% bovine serum albumin in PBS containing 1% Tween-20 (PBS-T) for 30 min at room temperature and followed by incubation with primary antibodies overnight at 4 °C. Membranes were washed three times in PBS-T and incubated for 1h at room temperature with horseradish peroxidase-conjugated secondary antibody. Bands were visualized using an enhanced chemiluminescence detection system (ImageQuant LAS 4000; GE Healthcare, Uppsala, Sweden).

Experimental animals

Four-week-old male BALB/c nude mice were used for the experiment (Orient Co, Ltd, Gyeonggi-do, Korea). All mice were permitted to be acclimated for 1 week before experiments under the condition of a 12-hour light/dark cycle and room temperature. An x-ray irradiated laboratory rodent diet (Purina Korea, Gyeonggi-do, Korea) and autoclaved water were provided to these immunocompromised mice. The use and care of animals were reviewed and approved by the Institutional Animal Care and Use Committee at the Seoul National University Hospital, and all animal procedures were in accord with the "Guide for the Care and Use of Laboratory Animals" issued by the Institute of Laboratory Animal Resources Commission on Life Sciences, US National Research Council.

Tumor cell transplantation and experimental protocols

To create the subcutaneous xenograft model, mice were subcutaneously inoculated with SNU-478 cells ($\times 10^6$ cells/mouse) in 1 mL of Matrigel (BD Biosciences, Bedford, Mass). Tumor-

bearing animals were divided into the following 4 treatment groups: (1) vehicle alone (control), (2) FKB (25 mg/kg, 4 times/wk for 2 weeks), (3) cisplatin and gemcitabine (5 mg/kg, 1 time/wk and 100 mg/kg, 1 time/wk for 2 weeks), and (4) cisplatin, gemcitabine and FKB (5mg/kg, 1 time/wk, 100 mg/kg, 1 time/wk, and 25 mg/kg 4 times/wk for 2 weeks). Each group consisted of 5 animals. FKB, cisplatin, and gemcitabine were administered intraperitoneally and treatment was started when subcutaneous tumors reached a minimum 100 mm^3 in size. Tumor size was measured twice per week with calipers, and the volume was calculated by the following formula: tumor volume = (length × $width^2$) × $\pi/6$. At the end of the experiment, subcutaneous xenografts were excised and volume was measured. Some parts of the xenograft tissues were stocked in 4% formaldehyde and embedded in paraffin for pathologic assessment, and the rest were processed for protein extraction and immunoblot.

Statistical Analysis

Data are expressed as mean±SEM. Statistical analysis was performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

Differences between mean values of groups were determined by 2-tailed Student *t* test or analysis of variance. $P < 0.05$ was considered statistically significant.

Results

FKB reduced cell viability and induced apoptosis of cholangiocarcinoma cells

The effects of FKB on the proliferation of SNU-478 cholangiocarcinoma cell line was examined by the MTT assay at different concentrations (0-100 μmol/L) for 72 hours. FKB inhibited the growth of SNU-478 cell line in a dose-dependent manner and the in vitro half maximal inhibitory concentration (IC_{50}) of FKB towards SNU-478 cells was estimated to be 69.4 μmol/L (Figure 1).

The effect of FKB alone and in combination with cisplatin on survival and proliferation of SNU-478 cells was examined by the MTT assay at different incubation times (24, 48 and 72 hours). The concentration of each agent was selected to produce moderate cell death. Representative data are shown in Figure 2. Combination treatment significantly reduced cell viability compared with vehicle control (DMSO) cells at 24, 48 and 72 h ($p < 0.05$). Survival of the cells treated with FKB and cisplatin in combination was reduced by 52.4%, 34.7%, and 23.6% at 24, 48 and 72 h compared with vehicle

control, respectively. However significant differences on cell viability among FKB, cisplatin, and cisplatin with FKB treatment at 24, 48 and 72 h, respectively, were not observed.

To further explore the effect of cisplatin and FKB on cellular apoptosis, cells were stained with annexin V and PI and then were analyzed by flow cytometry. FKB and cisplatin alone significantly increased the proportion of early apoptotic cells (PI^-/AV^+) and late apoptotic/necrotic cells (PI^+/AV^+) compared with control. Cisplatin induced early apoptosis more than late apoptosis. FKB induced late apoptosis more than early apoptosis. The combination treatment of FKB and cisplatin induced apoptosis more efficiently than cisplatin ($p < 0.05$) or FKB ($p=0.626$) alone. The mean rates of apoptosis in SNU-478 cells treated with DMSO, cisplatin, FKB and combination treatment were 5.0%, 13.3%, 20.6%, and 21.8%, respectively at 24 hour (Figure 3).

To determine apoptosis in a different way, immunoblotting was performed to detect cleaved PARP. The expression of cleaved PARP was markedly increased by cisplatin treatment ($p < 0.05$, compared to control) (Figure 4). FKB treatment also increased the expression

of cleaved PARP, but not more than cisplatin treatment ($p=0.060$, compared to control).

FKB down-regulated the expression of Akt and P-Akt

In order to determine which signaling pathways were affected by FKB, immunoblotting was performed to detect Akt and P-Akt. The expressions of Akt were significantly suppressed by FKB ($p < 0.05$) and cisplatin treatment ($p < 0.05$) (Figure 5). It showed more significant suppression of Akt by FKB treatment compared with cisplatin treatment ($p < 0.05$). Similarly the expressions P-Akt were suppressed by FKB and cisplatin and it was more suppressed by FKB treatment than cisplatin treatment. FKB and cisplatin reduced phosphorylation of Akt significantly compared with control and it was more marked by FKB treatment than cisplatin treatment ($p < 0.05$).

FKB showed antitumor effect in xenograft model of cholangiocarcinoma

To determine the in vivo effect of FKB, SUN-478 subcutaneous xenograft model was established. At the end of the experiment, subcutaneous xenografts were excised and tumor volume and weight were measured (Figure 6A). The FKB treatment alone group showed tumor growth inhibition (Figure 6B). The mean final tumor volume of the FKB treatment group was 347.5 mm^3, while the mean tumor volume of the untreated group was 522.1 mm^3, and there was no significant difference between the two groups ($p=0.405$). The cisplatin/gemcitabine treatment group showed significantly reduced final tumor volume compared with the untreated group (191 mm3 vs. 522.1 mm3, $p < 0.05$). The combination treatment of cisplatin/gemcitabine with FKB exhibited noteworthy tumor growth inhibition and resulted in a significant difference of the mean final tumor volume compared with that of the untreated group (159.5 mm^3 vs. 522.1 mm^3, $p < 0.05$). However, significant difference in the mean final tumor volume between the cisplatin/gemcitabine treatment

group and the combination treatment of cisplatin/gemcitabine with FKB group was not observed (191.7 mm3 vs. 159.5 mm3, p=0.324).

The mean weight of the tumor in the cisplatin/gemcitabine treatment group and the combination treatment of cisplatin/gemcitabine with FKB group were significantly lower than that of the untreated group ($p < 0.05$) (Figure 6C). However, significant difference in the mean weight of the tumor between the cisplatin/gemcitabine treatment group and the combination treatment of cisplatin/gemcitabine with FKB group was not observed (p=0.170).

Figure 1. FKB inhibited the growth of SNU-478 cell line in a dose-dependent manner as shown in MTT assay. The graph represents the means ± SEM of three independent experiments.

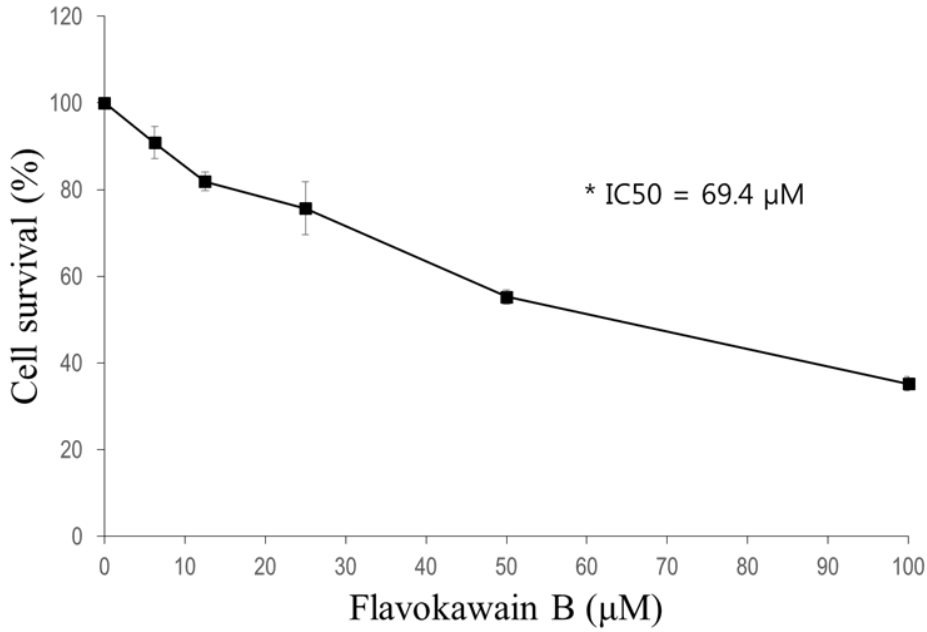


Figure 2. FKB and/or cisplatin inhibited the growth of SNU-478 cells in a time-dependent manner. The viability was calculated as the percentage viability relative to those of vehicle control (dimethyl sulfoxide; DMSO)-treated cells. The graph represents the means ± SEM of three independent experiments. *P < 0.05 compared with DMSO treatment.

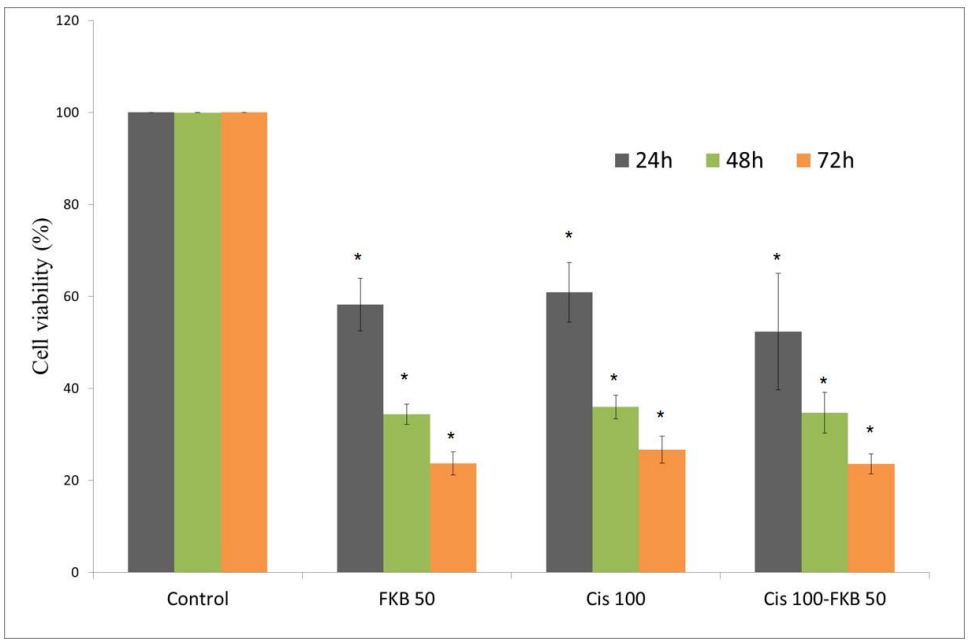
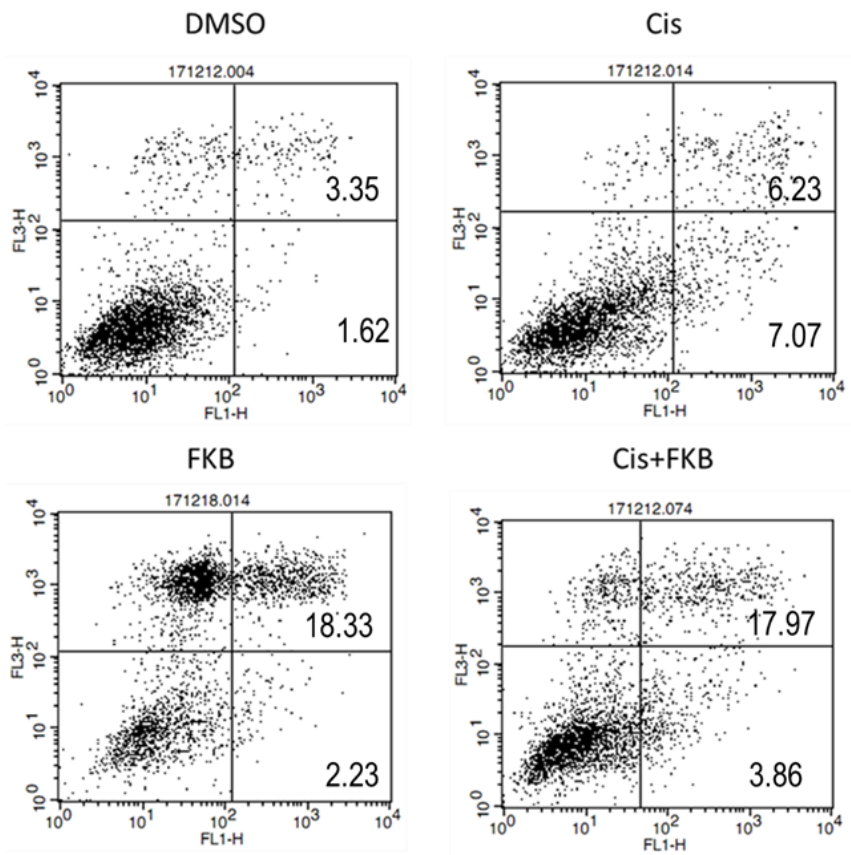


Figure 3. FKB treatment caused apoptotic cell death in SNU-478 cells. (A) SNU-478 cells were stained with allophycocyanin-conjugated annexin V and propidium iodide (PI) and analyzed by flow cytometry after FKB/cisplatin treatment for 24 h. The results are representative of three independent experiments. (B) Percentage of early apoptotic cells (PI-/annexin V+), late apoptotic/necrotic cells (PI+/annexin V+), and total apoptotic cells are displayed in the graph. Data represent the means ± SEM of three independent experiments. *P < 0.05 compared with DMSO treatment.

(A)



(B)

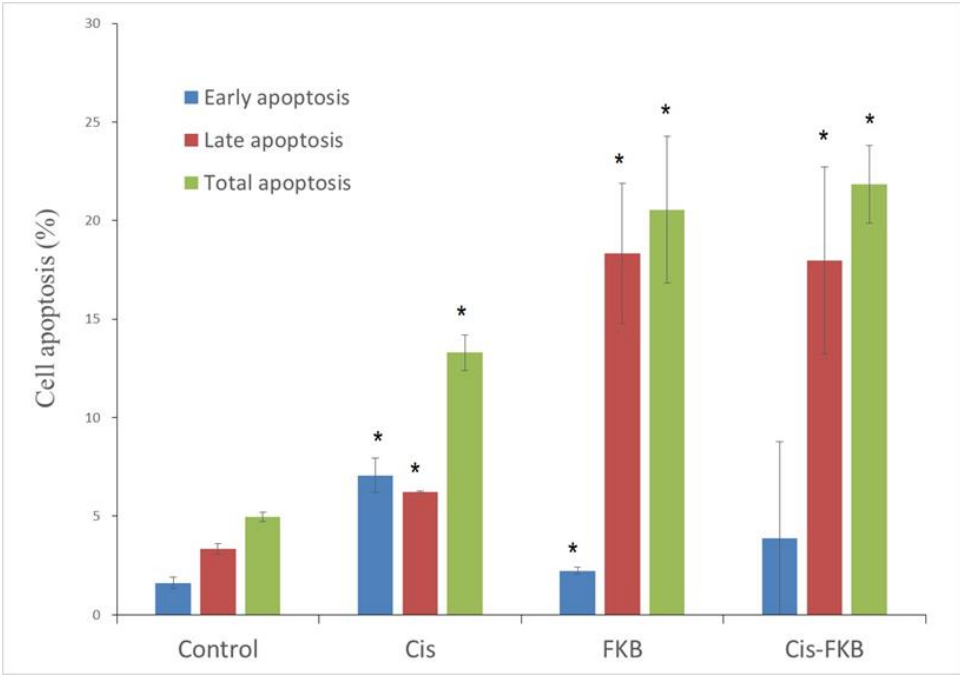
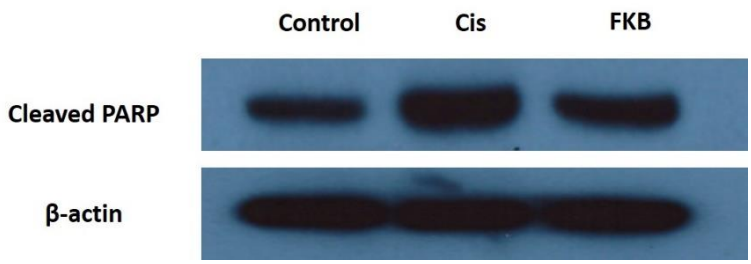

Early apoptosis
Late apoptosis
Total apoptosis
Cell apoptosis (%)
30
25
20
15
10
5
0
Control
Cis
FKB
Cis-FKB
*

Figure 4. FKB increased the expression of cleaved PARP. (A) Cells were treated with cisplatin or FKB as described in Materials and Methods, and cell signaling–associated proteins were measured by western blot analysis. The protein levels of cleaved PARP was examined using sodium dodecyl sulfatepolyacrylamide gel electrophoresis and western blot analysis. The results are representative of three independent experiments. (B) Quantification of band intensities shown in (A). Data represent the mean ± SEM of three independent experiments. *P < 0.05 compared with DMSO treatment.

(A)



(B)

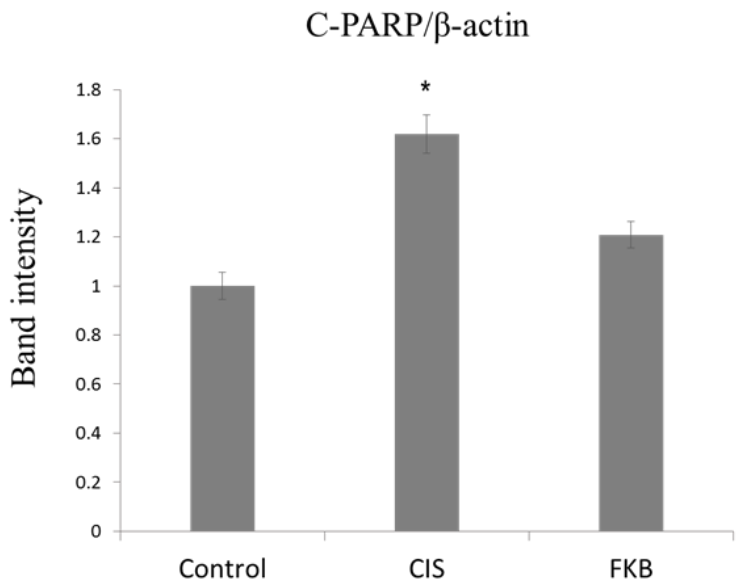
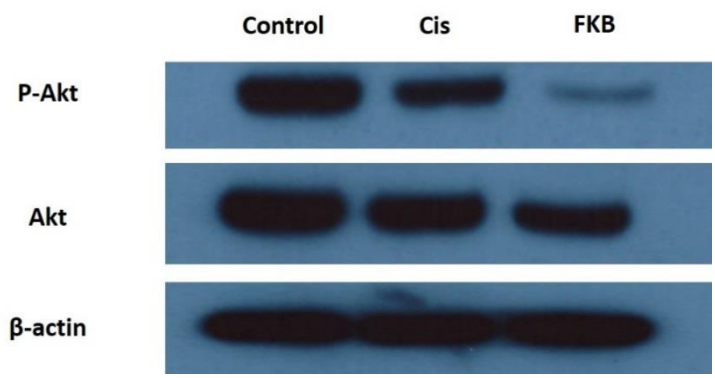
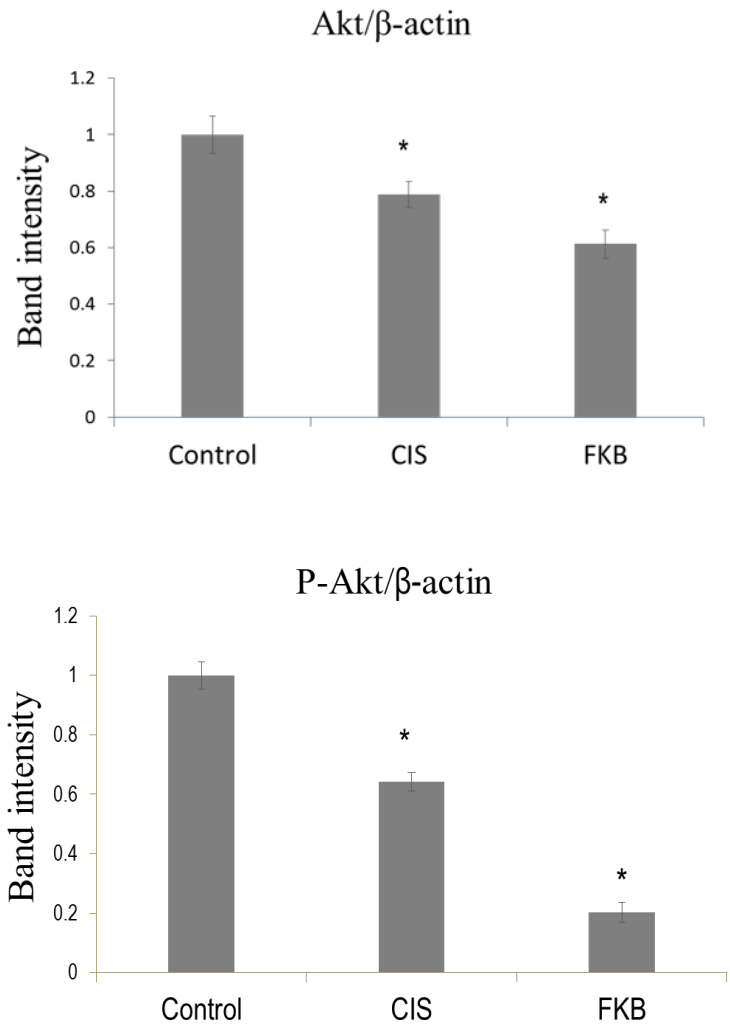

C-PARP/β-actin
Band intensity
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
CIS
FKB

Figure 5. FKB down-regulated the expression of Akt and P-Akt. (A) Cells were treated with cisplatin or FKB as described in Materials and Methods, and cell signaling-associated proteins were measured by western blot analysis. The protein levels of Akt and P-Akt were examined using sodium dodecyl sulfatepolyacrylamide gel electrophoresis and western blot analysis. The results are representative of three independent experiments. (B) Quantification of band intensities shown in (A). (C) Relative intensity of bands of phosphorylated protein to total protein is displayed in the graph. Data represent the mean ± SEM of three independent experiments. *P < 0.05 compared with DMSO treatment.

(A)



(B)


Akt/β-actin
Band intensity
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
CIS
FKB
P-Akt/β-actin
Band intensity
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
CIS
FKB

(C)

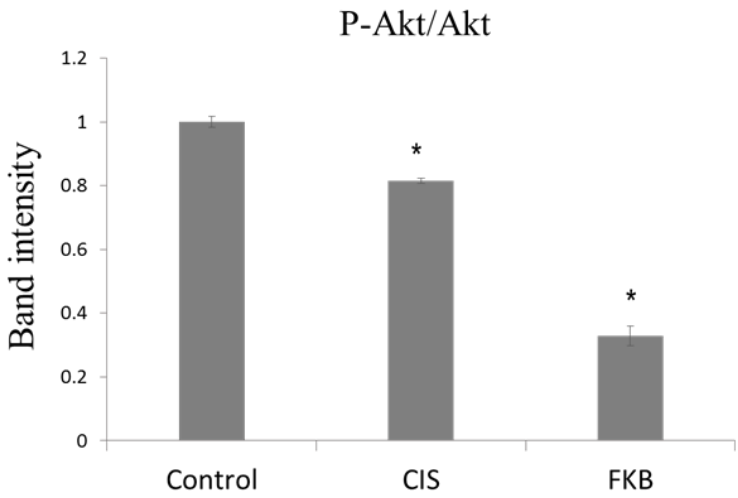
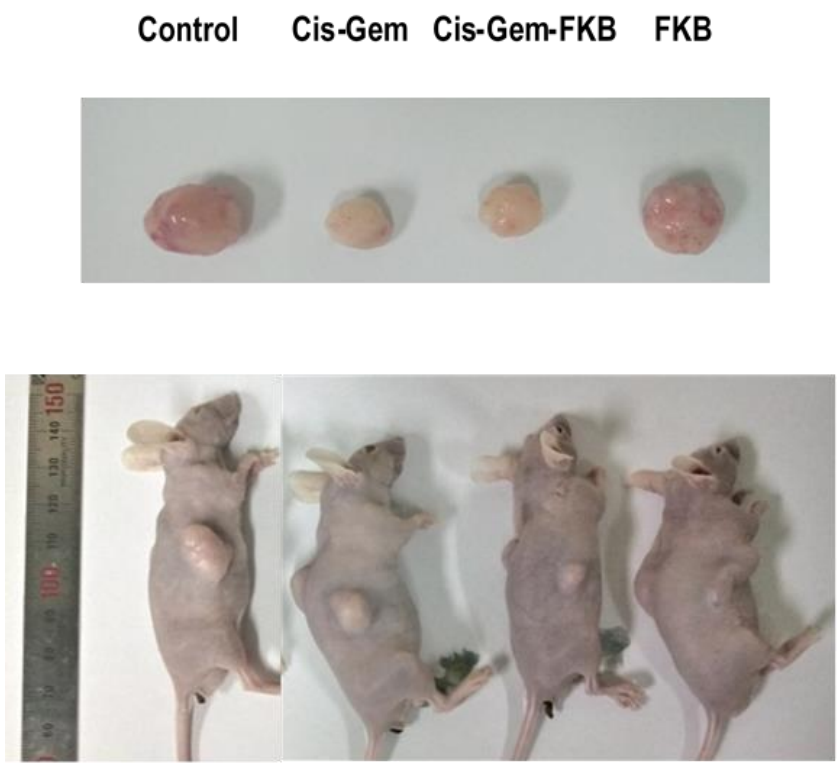
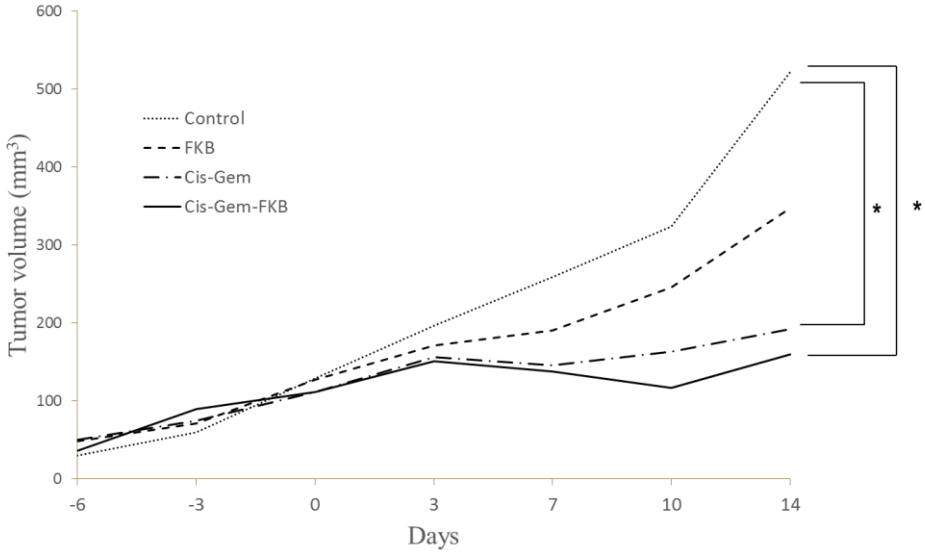


Figure 6. The combination treatment of cisplatin/gemcitabine with FKB showed significant tumor growth inhibition in the cholangiocarcinoma xenograft model. (A) At the end of the experiment, subcutaneous xenografts were excised and volume and weight were measured. The mean tumor volume over time (B) and the final mean tumor weight (C) are displayed in the graphs. Statistically significant difference ($P < 0.05$) of the combination treatment of cisplatin/gemcitabine with/without FKB compared with the control is illustrated as asterisk (*).

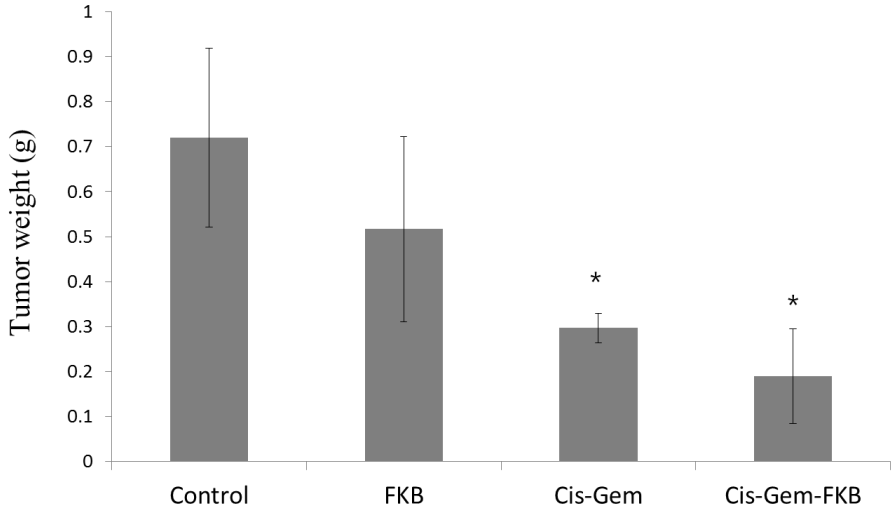
(A)



(B)


600
500
400
300
200
100
0
Tumor volume (mm3)
Control
FKB
Cis-Gem
Cis-Gem-FKB
*
*
-6
-3
0
3
7
10
14
Days

(C)


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Tumor weight (g)
*
*
Control
FKB
Cis-Gem
Cis-Gem-FKB

Discussion

Although gemcitabine plus cisplatin is known to be the current standard therapy for advanced cholangiocarcinoma, the response rate and the effect on survival is poor. The reason for the poor response of cholangiocarcinoma to anticancer agents is based on the existence of complex mechanisms of chemoresistance that usually act synergistically to help cancer cells escape from the deleterious effects of cytostatic drugs.[13] The number of clinical trials with targeted therapy has been attempted to overcome chemoresistance, however most have failed to prolong overall survival significantly.[4,13] Furthermore, considering high toxicity of chemotherapeutic agents, the use of nontoxic naturally-occurring dietary chemopreventive compounds have gained considerable attention.[6,14] FKB is one of this naturally-occurring compound, and it has been reported to have anticancer properties in several cancer cells, such as colon cancer,[9] gastric cancer,[15] breast cancer,[16] prostate cancer,[10,17] lung cancer,[18,19] squamous cell carcinoma,[20] uterine leiomyosarcoma,[21] cervical cancer,[22] synovial sarcoma,[11] oral carcinoma,[23] osteosarcoma,[12] and leukemia.[24,25] FKB is regarded to act on multiple cellular pathways and to suppress the growth of cancer cells. In this

regard, we evaluated the effect of FKB in a cholangiocarcinoma cell line, SNU-478 in vitro and in vivo in the current study.

For advanced cholangiocarcinoma, gemcitabine plus cisplatin is considered as a standard option.[26,27] In our preliminary study, we tried gemcitabine and fluorouracil (5-FU) in SNU-308, SNU-478 and SNU-1196 cholangiocarcinoma cell lines and those agents did not make appropriate cell death on MTT assay, while cisplatin made moderate cell death. Therefore, we selected cisplatin in this study. In this study, FKB inhibited cholangiocarcinoma cell proliferation and induced apoptosis. However significant differences in cell viability among cisplatin, FKB, and the combination of cisplatin and FKB treatment were not observed. We used 50 μmol/L of FKB in this study, though IC_{50} of FKB towards SNU-478 cells was about 70 μmol/L. The low concentration of FKB used in our study might have resulted in unclear synergistic effect of cisplatin with FKB towards SNU-478 cells. In the apoptosis assay, more apoptosis was occurred with the combination of cisplatin and FKB treatment compared with cisplatin or FKB treatment alone. FKB mainly induced late apoptosis while cisplatin induced early apoptosis. Therefore, it could be hypothesized that the sensitization of cancer cells was achieved by FKB during

cisplatin-induced killing, as demonstrated by more cell death compared with single-agent treatment.

To understand the mechanisms underlying the effect of FKB, we investigated the mechanism of apoptosis induction by FKB. In this study, we found that Akt and P-Akt were substantially down-regulated by the treatment of FKB. Akt plays critical roles in cell cycle progression and anti-apoptosis in various cancers, including cholangiocarcinoma, via the PI3K/Akt signaling pathway.[28] Previous studies have proved the effect of the treatment targeting Akt on tumor growth inhibition and apoptosis induction in cholangiocarcinoma.[29-32] In the current study, FKB showed tumor growth inhibition and apoptosis induction by modulating Akt signaling pathway which is consistent with the results of previous studies.

The PI3K/Akt pathway is an intracellular signaling pathway important in regulating the cell cycle.[33] The dysregulation of this pathway has been implicated in the pathogenesis of many cancers including cholangiocarcinoma by promoting cell proliferation, tumorigenesis and metastasis.[34] Studies have demonstrated that increased expression of P-Akt is present in >80% of extrahepatic

cholangiocarcinoma[30] and >60% of intrahepatic cholangiocarcinoma.[29,35] Therefore PI3K-Akt pathway has been believed to be an attractive therapeutic target. In the current study, Akt and P-Akt were suppressed by FKB treatment and it could be inferred that tumor growth inhibition and apoptosis induction were induced by inhibiting Akt signaling pathway. FKB have shown to inhibit PI3K/Akt pathway in oral carcinoma,[23] and gastric cancer cell lines.[15] To the best of our knowledge, this is the first report that shows the antitumor effect of FKB in cholangiocarcinoma cell line via modulating Akt pathway.

We subsequently performed in vivo study to verify the antitumor activities of FKB using SNU-478 subcutaneous xenograft model. The FKB treatment alone group showed tendency to inhibit tumor growth compared to the control group, but there was no significant difference in the mean final volume and weight of tumor between the two groups. The combination treatment of cisplatin/gemcitabine with FKB group showed significant tumor growth inhibition and resulted in a significant reduction of tumor volume and weight compared with those of the control group. However, no significant difference in the mean final volume and weight of tumor between the

cisplatin/gemcitabine treatment group and the cisplatin/gemcitabine with FKB treatment group. Putting these results together, it could be inferred that FKB showed tendency to inhibit tumor growth in the SNU-478 xenograft model, but did not show definite antitumor effect. It might be that the dose of FKB (25 mg/kg, 4 times/wk for 2 weeks) used in this study may have been low. There have been few in vivo studies using FKB. In those studies FKB was given orally or via intraperitoneal route. For the intraperitoneal route, FKB was injected up to 200 mg/kg daily for 28 days.[36] Thus the dose of FKB used in our study could have been low and it could be related with the slight antitumor effect of FKB shown in our study. Furthermore, we sacrificed the mice at 2 weeks after the initiation of the treatment. In the previous in vivo studies used FKB, treatment was continued for about 3 – 4 weeks.[10,20,36] Therefore our treatment duration may have been short. Meanwhile, we performed in vivo study using cisplatin/gemcitabine with FKB as combination treatment though gemcitabine was not used in our in vitro study. This might have caused the inconsistent results of the effect of FKB between our in vitro and in vivo study. Although we did not perform mechanism study in the in vivo study, previous several studies demonstrated tumor

growth inhibition in various cancer cell xenograft models with FKB treatment via various molecular mechanisms.[7,10,15,20,37]

In conclusion, the data of the current study suggest that FKB showed antitumor effect through the induction of apoptosis, which is mediated by the suppression of Akt and P-Akt activity in cholangiocarcinoma. However, the synergistic effect of FKB and cisplatin was not definite. Further research is needed to verify the antitumor effect and the mechanism of FKB in cholangiocarcinoma.

References

1. Jung KW, Won YJ, Kong HJ, Lee ES. Cancer Statistics in Korea: Incidence, Mortality, Survival, and Prevalence in 2016. Cancer Res Treat 2019;51:417-430.

2. Blechacz B. Cholangiocarcinoma: Current Knowledge and New Developments. Gut Liver 2017;11:13-26.

3. Valle J, Wasan H, Palmer DH, et al. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. N Engl J Med 2010;362:1273-1281.

4. Sahu S, Sun W. Targeted therapy in biliary tract cancers-current limitations and potentials in the future. J Gastrointest Oncol 2017;8:324-336.

5. Zabron A, Edwards RJ, Khan SA. The challenge of cholangiocarcinoma: dissecting the molecular mechanisms of an insidious cancer. Dis Model Mech 2013;6:281-292.

6. Bharti AC, Aggarwal BB. Chemopreventive agents induce suppression of nuclear factor-kappaB leading to chemosensitization. Ann N Y Acad Sci 2002;973:392-395.

7. Abu N, Mohamed NE, Yeap SK, et al. In vivo antitumor and antimetastatic effects of flavokawain B in 4T1 breast cancer cell-challenged mice. Drug Des Devel Ther 2015;9:1401-1417.

8. Abu N, Ho WY, Yeap SK, et al. The flavokawains: uprising medicinal chalcones. Cancer Cell Int 2013;13:102.

9. Kuo YF, Su YZ, Tseng YH, Wang SY, Wang HM, Chueh PJ. Flavokawain B, a novel chalcone from Alpinia pricei Hayata with potent apoptotic activity: Involvement of ROS and GADD153 upstream of mitochondria-dependent apoptosis in HCT116 cells. Free Radic Biol Med 2010;49:214-226.

10. Tang Y, Li X, Liu Z, Simoneau AR, Xie J, Zi X. Flavokawain B, a kava chalcone, induces apoptosis via up-regulation of death-receptor 5 and Bim expression in androgen receptor negative, hormonal refractory prostate cancer cell lines and reduces tumor growth. Int J Cancer 2010;127:1758-1768.

11. Sakai T, Eskander RN, Guo Y, et al. Flavokawain B, a kava chalcone, induces apoptosis in synovial sarcoma cell lines. J Orthop Res 2012;30:1045-1050.

12. Ji T, Lin C, Krill LS, et al. Flavokawain B, a kava chalcone,

inhibits growth of human osteosarcoma cells through G2/M cell cycle arrest and apoptosis. Mol Cancer 2013;12:55.

13. Marin JJG, Lozano E, Herraez E, et al. Chemoresistance and chemosensitization in cholangiocarcinoma. Biochim Biophys Acta Mol Basis Dis 2018;1864:1444-1453.

14. Yen GC, Tsai CM, Lu CC, Weng CJ. Recent progress in natural dietary non-phenolic bioactives on cancers metastasis. J Food Drug Anal 2018;26:940-964.

15. Chang CT, Hseu YC, Thiyagarajan V, et al. Chalcone flavokawain B induces autophagic-cell death via reactive oxygen species-mediated signaling pathways in human gastric carcinoma and suppresses tumor growth in nude mice. Arch Toxicol 2017;91:3341-3364.

16. Abu N, Akhtar MN, Yeap SK, et al. Flavokawain B induced cytotoxicity in two breast cancer cell lines, MCF-7 and MDA-MB231 and inhibited the metastatic potential of MDA-MB231 via the regulation of several tyrosine kinases In vitro. BMC Complement Altern Med 2016;16:86.

17. Li X, Pham V, Tippin M, et al. Flavokawain B targets protein

neddylation for enhancing the anti-prostate cancer effect of Bortezomib via Skp2 degradation. 2019;17:25.

18. An J, Gao Y, Wang J, et al. Flavokawain B induces apoptosis of non-small cell lung cancer H460 cells via Bax-initiated mitochondrial and JNK pathway. Biotechnol Lett 2012;34:1781-1788.

19. Hseu YC, Huang YC, Thiyagarajan V, Mathew DC. Anticancer activities of chalcone flavokawain B from Alpinia pricei Hayata in human lung adenocarcinoma (A549) cells via induction of reactive oxygen species-mediated apoptotic and autophagic cell death. 2019;234:17514-17526.

20. Lin E, Lin WH, Wang SY, et al. Flavokawain B inhibits growth of human squamous carcinoma cells: Involvement of apoptosis and cell cycle dysregulation in vitro and in vivo. J Nutr Biochem 2012;23:368-378.

21. Eskander RN, Randall LM, Sakai T, Guo Y, Hoang B, Zi X. Flavokawain B, a novel, naturally occurring chalcone, exhibits robust apoptotic effects and induces G2/M arrest of a uterine leiomyosarcoma cell line. J Obstet Gynaecol Res 2012;38:1086-

1094.

22. Yeap SK, Abu N, Akthar N, et al. Gene Expression Analysis Reveals the Concurrent Activation of Proapoptotic and Antioxidant-Defensive Mechanisms in Flavokawain B-Treated Cervical Cancer HeLa Cells. Integr Cancer Ther 2017;16:373-384.

23. Hseu YC, Lee MS, Wu CR, et al. The chalcone flavokawain B induces G2/M cell-cycle arrest and apoptosis in human oral carcinoma HSC-3 cells through the intracellular ROS generation and downregulation of the Akt/p38 MAPK signaling pathway. J Agric Food Chem 2012;60:2385-2397.

24. Tang YL, Huang LB, Tian Y, et al. Flavokawain B inhibits the growth of acute lymphoblastic leukemia cells via p53 and caspase-dependent mechanisms. Leuk Lymphoma 2015;56:2398-2407.

25. Lee JJ, Koh KN, Park CJ, Jang S, Im HJ, Kim N. The Combination of Flavokawain B and Daunorubicin Induces Apoptosis in Human Myeloid Leukemic Cells by Modifying NF-kappaB. Anticancer Res 2018;38:2771-2778.

26. Chen L, Chen C, Yen Y, Tam KW. Chemotherapy for advanced biliary tract carcinoma: A meta-analysis of randomized controlled

trials. Medicine (Baltimore) 2016;95:e4584.

27. Ghidini M, Pizzo C, Botticelli A, et al. Biliary tract cancer: current challenges and future prospects. Cancer Manag Res 2019;11:379-388.

28. Labib PL, Goodchild G, Pereira SP. Molecular Pathogenesis of Cholangiocarcinoma. BMC Cancer 2019;19:185.

29. Schmitz KJ, Lang H, Wohlschlaeger J, et al. AKT and ERK1/2 signaling in intrahepatic cholangiocarcinoma. World J Gastroenterol 2007;13:6470-6477.

30. Chung JY, Hong SM, Choi BY, Cho H, Yu E, Hewitt SM. The expression of phospho-AKT, phospho-mTOR, and PTEN in extrahepatic cholangiocarcinoma. Clin Cancer Res 2009;15:660-667.

31. Ewald F, Grabinski N, Grottke A, et al. Combined targeting of AKT and mTOR using MK-2206 and RAD001 is synergistic in the treatment of cholangiocarcinoma. Int J Cancer 2013;133:2065-2076.

32. Wilson JM, Kunnimalaiyaan S, Kunnimalaiyaan M, Gamblin TC. Inhibition of the AKT pathway in cholangiocarcinoma by MK2206 reduces cellular viability via induction of apoptosis. Cancer Cell Int

2015;15:13.

33. Sever R, Brugge JS. Signal transduction in cancer. Cold Spring Harb Perspect Med 2015;5.

34. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer 2009;9:550-562.

35. Javle MM, Yu J, Khoury T, et al. Akt expression may predict favorable prognosis in cholangiocarcinoma. J Gastroenterol Hepatol 2006;21:1744-1751.

36. Li X, Liu Z, Xu X, et al. Kava components down-regulate expression of AR and AR splice variants and reduce growth in patient-derived prostate cancer xenografts in mice. PLoS One 2012;7:e31213.

37. Rossette MC, Moraes DC, Sacramento EK, et al. The In Vitro and In Vivo Antiangiogenic Effects of Flavokawain B. 2017;31:1607-1613.

국문초록

배경: 담관암은 치료 방법이 제한적이며 예후가 매우 좋지 않은 치명적인 악성종양이다. 자연에 존재하는 천연물 중 암 예방 효과 및 항암 효과가 있다고 알려진 물질들은 상대적으로 독성이 적으며, 기존의 항암제와 병합 사용시 항암 효과를 증대 시킬 수 있다고 알려져 있어 주목을 받고 있다. Flavokawain B 는 천연 화합물로 다양한 종양에서 항암 효과를 보인다고 알려져 있다. 하지만 아직까지 담관암에서 flavokawain B 의 항암 효과에 대해서는 잘 알려진 바가 없다. 본 연구는 담관암에서 flavokawain B 가 항암 효과에 미치는 영향에 대해서 연구해 보고자 하였다.

방법: 담관암 세포주 (SNU-478)에 flavokawain B 를 투여하여 세포 증식 및 세포자멸사에 미치는 영향을 측정하였다. Cisplatin 에 flavokawain B 를 병합 투여하였을 때 세포 증식 및 세포자멸사에 미치는 영향에 대해 알아보았다. 또한 flavokawain B 의 항암 효과를 일으키는 기전을 살펴보기 위하여 관련된 단백질의 발현의 변화를 측정하였다. 담관암 동물 모델에서도 flavokawain B 의 종양 억제 효과와 기전을 측정하였다.

결과: 담관암 세포주에서 flavokawain B 의 투여는 용량 및 시간 의존적 방식으로 담관암 세포 증식을 억제하는 효과를 보였다. Flavokawain B

의 투여에 의해 Akt 와 P-Akt 의 발현이 저하되었다. 담관암 동물 모델에서 flavokawain B 와 cisplatin / gemcitabine 의 병합 투여는 SNU-478 세포의 종양 성장을 유의하게 억제하였다.

결론: 담관암에서 Flavokawain B 는 Akt 와 P-Akt 를 억제함으로써 항암효과를 보였다. 하지만 flavokawain B 와 cisplatin 의 병합 요법은 뚜렷한 항암효과의 증진을 보이지는 못하였다.

주요어: 담관암, flavokawain, cisplatin, gemcitabine, 세포자멸사, Akt, P-Akt

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