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Flavokawain B, a novel, naturally occurring chalcone, exhibits robust apoptotic effects and induces G2/M arrest of a uterine leiomyosarcoma cell line

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Abstract

Aim: To examine the effects of flavokawain B (FKB), a novel kava chalcone, on the growth of uterine leiomyosarcoma (LMS) cells and investigated its utility in the treatment of uterine LMS.

Material and Methods: Uterine leiomyosarcoma (SK-LMS-1), endometrial adenocarcinoma (ECC-1) and the non-malignant, human endometrium fibroblast-like (T-HESC) cell lines were cultured and treated with different concentrations of FKB. Cell viability was determined by MTT assays and the IC_{50} was estimated. Fluorescent-activated cell sorting (FACS) analysis of apoptosis and cell cycle was performed. Real-time reverse-transcription polymerase chain reaction and western blot analysis were utilized to evaluate differences in the expression of apoptotic markers.

Results: FKB preferentially inhibited the growth of SK-LMS-1 and ECC-1 cells compared to T-HESC control cells. FKB significantly increased both early and late apoptosis in SK-LMS-1 and ECC-1 cells relative to control. Cell cycle analysis illustrated an increase in the G2/M fraction in treated cell lines relative to control. Furthermore, FKB induced the expression of pro-apoptotic death receptor 5 (DR5), Bim, and Puma, and decreased expression of an inhibitor of apoptosis, survivin. FKB also acted synergistically when combined with docetaxel and gemcitabine (combination index = 0.260).

Conclusion: FKB treatment results in cell cycle arrest and a robust induction of apoptosis in SK-LMS-1 and ECC-1 cell lines. This natural product deserved further investigation as a potential therapeutic agent in the treatment of uterine LMS.

Key words: apoptosis, cell-cycle arrest, flavokawain B, leiomyosarcoma, uterine cancer.

Introduction

Uterine leiomyosarcoma (LMS) comprises 1.3% of all uterine malignancies, and represent the second most common subtype of uterine sarcoma, with mean age at diagnosis of 53 years. Presenting symptoms are similar to those associated with benign uterine fibroids, and include vaginal bleeding, abdominal pain and pelvic mass, making preoperative distinction difficult, with only 15% of patients diagnosed prior to surgery. Unfortunately, uterine leiomyosarcomas have a poor prognosis even after early stage diagnosis, with recurrence rates of 53–71%.[1] Recurrent disease often presents with distant metastasis involving the lungs in up to 40% of patients.

Traditional cytotoxic chemotherapy regimens have shown limited response rates. The Gynecologic Oncology Group (GOG) investigated response rates to single

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agent cisplatin, topotecan, paclitaxel, and oral etoposide showing minimal activity.[2–6] Furthermore, Omura *et al*. in a GOG study, demonstrated a 41% recurrence rate in patients treated with adriamycin in comparison to 53% in the untreated control group.[7] Hensley *et al*. investigated the use of combination gemcitabine and docetaxel in patients with unresectable leiomyosarcoma.[8] Among 34 patients, an objective response rate of 53% was noted. These trials were followed by two phase II studies by the GOG, showing a 35.8% objective response to gemcitabine and docetaxel in chemotherapy naïve patients, with 26.2% of patients having stable disease.[9,10] Furthermore, the mean overall survival was reported as 16 months, emphasizing the importance of investigating novel agents in the treatment of this aggressive disease.

Natural products have played a major role in new drug discovery for centuries, with over 47% of approved anticancer agents being of natural origin.[11] Two well-known success stories in the field of naturally derived Food and Drug Administration-approved anticancer agents are taxol and vinblastine. However, compared to common malignancies, such as lung, breast and prostate cancer, there is little new drug discovery amongst rare malignancies, such as uterine LMS. Flavokawain B (FKB) is a novel chalcone that was isolated from kava root extracts and *Alpinia pricei* Hayata (a spicy herb indigenous to Taiwan) and is composed of two benzene rings joined by a three carbon chain (Fig. 1).[12] The consumption of kava root extracts in the Pacific Islands has been associated with a lower incidence of cancer despite a higher percentage of smokers in their populations.

We, and others, recently demonstrated that FKB induced apoptosis and exhibited both *in vitro* and *in vivo* anticancer activity against bladder, prostate and colon cancer cells.[13–15] However, there is currently no data exploring the anti-tumor efficacy of FKB in the treatment of mesenchymal tumors, including uterine leiomyosarcoma.

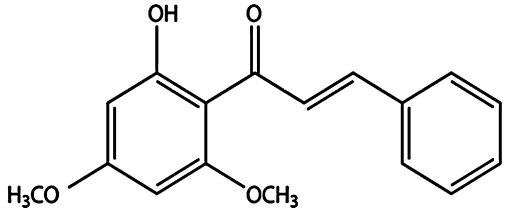

Figure 1 Molecular structure of flavokawain B (FKB).

Material and Methods

Institutional review board approval was obtained from the University of California, Irvine prior to the initiation of research.

Cell lines, compounds and reagents

The cell lines utilized in this study, SK-LMS-1, ECC-1 (endometrial adenocarcinoma) and T-HESC (normal endometrial fibroblasts) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The SK-LMS-1 cell line was cultured in MEM-alpha supplemented with 10% fetal bovine serum (FBS); ECC-1 was grown in RPMI-1640 medium supplemented with 5% FBS; T-HESC was cultured in a phenol-free DMEM-F12 1:1 mixture supplemented with 1% ITS + premix, and 10% charcoal treated FBS. All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Medium was replaced every two to three days as indicated. Pure flavokawain B (FKB) was purchased from LKT laboratories (St Paul, MN, USA), dissolved in DMSO, aliquoted and stored at –20°C. Docetaxel (Sanofi-Aventis, Bridgewater, NJ, USA) and gemcitabine (Eli Lilly and Company, Indianapolis, IN USA) were obtained from the institutional pharmacy. Antibodies for DR5, Bim, Puma and survivin were from Cell Signaling Technology (Danvers, MA, USA). Antibody against β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Thymidine, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (St Louis, MO, USA). RNAazol B was purchased from Tel-Test (Friendswood, TX, USA). The Reverse Transcription System kit utilized was from Applied Biosystems (Carlsbad, CA, USA).

MTT assay[15]

Briefly, cells were plated onto 24 well plates at a density of 2×10^4 cells in 500 μL of growth medium 24 h prior to treatment. Following treatment with FKB at doses of 0.625 μg/mL (1.1 μM), 1.25 μg/mL (2.2 μM), 2.5 μg/mL (4.4 μM) and 5 μg/mL (8.8 μM) for 72 h, 500 μL of MTT solution was added to each well and plates were incubated at 37°C for 3 h. The MTT solution was then extracted and 500 μL of dissolving buffer was added to each well. Cell viability was assessed by measuring absorbance at 570 nm in a microplate reader (Bio-Rad, Hercules, CA, USA). Dose response curves were then created as a percentage of vehicle treated control cells using Excel software, and IC_{50} values were determined graphically from the plot. In addition, a

combination index (CI) was calculated in order to determine if FKB exhibited additive, synergistic or antagonistic effects when combined with docetaxel and gemcitabine. After the half maximal inhibitory concentration (IC_{50}) was determined for both docetaxel and gemcitabine in SK-LMS-1 cells, varying concentrations of FKB were added to treated cells. Cells were treated with the desired drug or drug combination for 72 h. The type of interaction between drug activities was determined by the median effect principle according to the method of Chou and Talalay using CalcuSyn software (Biosoft, Cambridge, UK).[16] The interaction amongst drugs was then quantified by determining a CI at increasing levels of cell kill. A CI lower to, equal to, or higher than 1 indicated synergy, additivity or antagonism respectively. Each compound combination experiment was performed in triplicate.

Fluorescence-activated cell sorting (FACS) analysis of apoptosis

FACS analysis of apoptosis was performed utilizing the Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA) as detailed in the package insert. 2×10^5 SK-LMS-1 and ECC-1 cells were seeded into 60-mm dishes, and allowed to adhere to the plate base over 24 h. Cells were then treated with 0.1% DMSO or different concentrations of FKB for 24 h. Following treatment, the cells were washed with cold phosphate buffered saline (PBS) ×2, and stained with FITC annexin-V/propidium iodide (PI) solution at room temperature, in the dark, for 15 min. Treated samples were then analyzed immediately in a FACSAria flow cytometer (BD Biosciences, San Jose, CA, USA). The percentage of cells undergoing apoptosis was determined using Multicycle (Phoenix, USA). At least 50 000 events were recorded for each sample. Dual parametric dot plots were then used to calculate the percentage of non-apoptotic viable cells (annexin V-negative/PI-negative), early apoptotic cells (annexin V-positive/PI-negative), late apoptotic cells (annexin V-positive/PI-positive) and mechanically injured cells (annexin V-negative/PI-positive).

FACS analysis of cell cycle

Following the desired treatments with varying concentrations of FKB, cells were trypsinized and washed three times with cold PBS solution, and re-suspended in 900 μL of 95% ethanol. Cells were then fixed at 4°C overnight. Subsequently, the fixed cells were pelleted and re-suspended in 1 mL of staining solution containing PI, RNase and 1× PBS. Cells were then analyzed by a fluorescence-activated sorter (FACSort), with cell cycle profiles analyzed using WinMDI 2.8, a publicly available software. For each sample, at least 50 000 events were recorded. Samples were run in triplicate and each experiment was repeated three times.

Protein isolation and western blot analysis

Samples (normalized according to cell number) were treated with FKB at varying concentrations over 24 h. Cell extracts were then prepared in RIPA lysis buffer containing protease inhibitors (Sigma, St Louis, MO, USA). Cell lysates were centrifuged at 12 000 *g* for 15 min and the supernatant was collected. The BCA assay was used to determine protein concentration.[17] Volumes of clarified protein lysate containing equal amounts of protein (30 μg) were then separated on 10–12% sodium deodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically (90 min at 100 V) transferred to a Hybond-ECL membrane (GE Healthcare, Piscataway, NJ, USA). Blots were then blocked for 1 h in TBST (10 mM Tris-HCL, pH 8.0, 150 mM NaCL, and 0.05% Tween-20) containing 5% blocking grade non-fat dry milk (Bio-Rad, Hercules, CA, USA), and then incubated overnight with primary antibody at 4°C. Blots were then washed three times in TBST and incubated for 1.5 h at room temperature with HRP-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Thermo Scientific, Rockford, IL, USA).

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Following treatment with FKB, total RNA was isolated from SK-LMS-1 and ECC-1 cell lines using the TRizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was then synthesized from 2 μg of total RNA using a High Capacity cDNA Reverse Transcription kit per protocol (Applied Biosystems, Foster City, CA, USA). Real-time PCR amplification reactions for DR5, Survivin, Bim and Puma were then carried out using the MyiQ system (Bio-Rad) as previously described by Tang *et al*.[18] DR5, Survivin, Bim and Puma primers were obtained from Sigma, with primer sequences available upon request. Data was then analyzed using the C_t method, where C_t is the cycle number at which fluorescence first exceeds the threshold value. The C_t value for each primer sample was obtained by subtracting the

β-actin C_t value from the respective C_t value from each primer. A onefold change in C_t value represents a twofold difference in mRNA expression. Specificity of the PCR product was confirmed by melting curves. Each experiment was carried out in triplicate.

Statistical analysis

The data are presented as means ± standard errors (SE). The level of significance was set at a $P < 0.05$. Comparison of differences between treated and control groups in cell cycle and apoptosis assays was performed using student's *t*-test. All statistical tests were two-sided.

Results

Flavokawain B (FKB) more effectively inhibits the growth of SK-LMS-1 and ECC-1 cell lines compared to the non-malignant, immortalized, uterine fibroblast cell line T-HESC

Figure 2 shows that the IC_{50} of FKB for the uterine leiomyosarcoma cell line, SK-LMS-1, was approximately 1.25 μg/mL. At a dose of 5 μg/mL (8.8 μM), FKB inhibited the growth of SK-LMS-1 and ECC-1 cell lines by approximately 80%, but resulted in only a 10% growth reduction for the non-malignant uterine fibroblast cell line, T-HESC (Student's *t*-test, $P < 0.01$). This result indicates a certain degree of specificity in FKB's ability to inhibit the growth of uterine LMS.

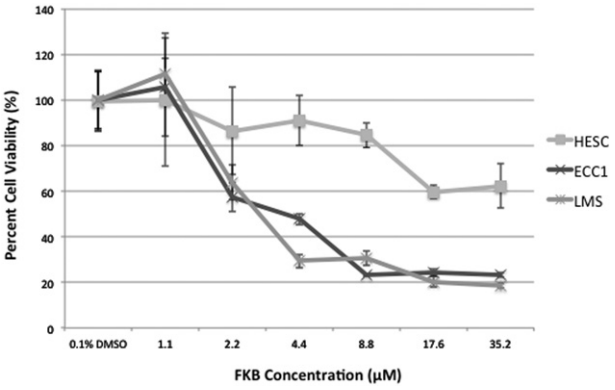

Figure 2 Flavokawain B (FKB) treatment exhibited differential effects on uterine leiomyosarcoma cell lines and non-malignant uterine fibroblasts. Cells were treated with the indicated FKB concentration for 72 h and subsequent cell densities were measured via MTT assay. The points are a mean of four independent plates. Bars, SE. The growth inhibitory effect of FKB on SK-LMS-1 and ECC-1 cells, with relative sparing of normal uterine stromal cells (T-HESC) is shown.

FKB induces apoptosis and G2/M arrest in SK-LMS-1 and ECC-1 cells

To evaluate the mechanism for the cell growth inhibitory effect of FKB, the morphology of control and FKB treated cells was examined using light microscopy. As evidenced in Figure 3a, treated cells exhibited typical apoptotic morphologic changes including separation from surrounding cells, cell shrinkage and cell rounding.[19] FACS analysis showed that FKB treatment at doses of 2.5 and 5 μg/mL (4.4 and 8.8 μM, respectively) resulted in an increase in both early (lower right, Annexin V-positive/PI-negative) and late (upper right, Annexin V-positive/PI-positive) apoptotic cell fractions as compared to control treated cells in SK-LMS-1 and ECC-1 cell lines (35.4 ± 3.1% and 29.1 ± 1.3% versus 0.02 ± 0.009 and 0.008 ± 0.004, respectively; $P < 0.0001$) (Fig. 3b).

We also examined whether the growth inhibitory effect of FKB was associated with cell cycle arrest.[14] As shown in Figure 4, FKB treatment resulted in a marked increase in the number of cells arrested at the G2/M phase in both SK-LMS-1 and ECC-1 cell lines [19.2 ± 0.34 to 35.6 ± 1.3% in SK-LMS-1 and 40.5 ± 1.1 to 54.9 ± 1.2% in ECC-1, $P < 0.001$ at an FKB dose of 2.5 μg/mL (4.4 μM)].

FKB up-regulates the expression of pro-apoptotic proteins and down-regulates survivin expression

Apoptosis can be induced via the extrinsic pathway, through cell surface death receptor stimulation, or through the intrinsic pathway mediated by mitochondrial dysfunction.[20,21] Figure 5 illustrates that FKB treatment of SK-LMS-1 cells resulted in increased expression of death receptor (DR5) and the mitochondrial pro-apoptotic proteins Bim and Puma, while down-regulating the expression of an IAP, survivin. Analogously, FKB treatment of ECC-1 cells resulted in a significant increase in mRNA and protein expression for DR5 and a down regulation of survivin expression. Taken together, this data implies that FKB activates both DR5, and mitochondrial-mediated apoptotic pathways and decreases survivin expression, exhibiting a robust apoptotic mechanism against uterine LMS.

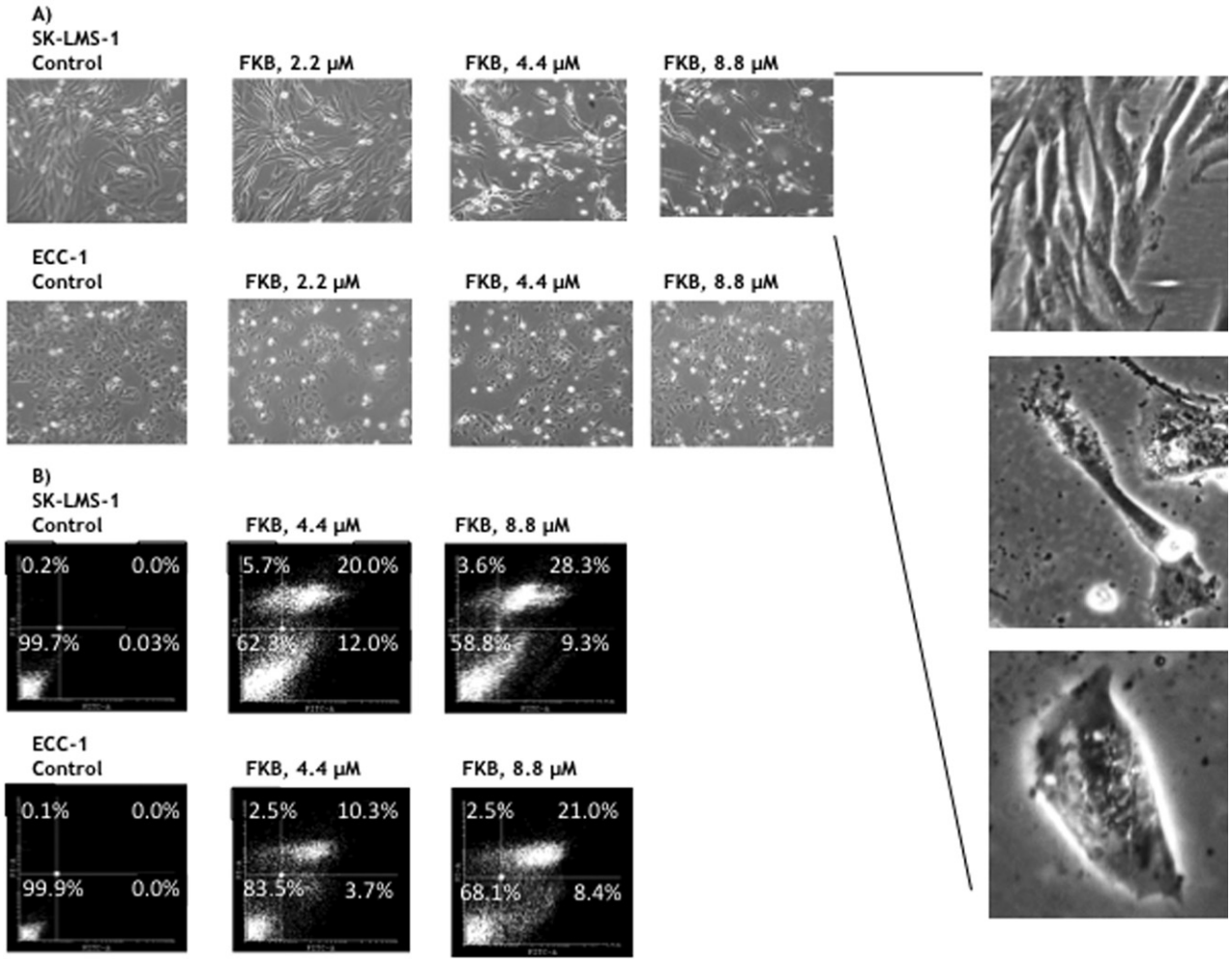

Figure 3 Flavokawain B (FKB) induced apoptosis in SK-LMS-1 and ECC-1 cell lines. (a) Live cell morphology evaluated using light microscopy (magnification: ×100) with image obtained from a random representative field. Representative images are shown of FKB-treated SK-LMS-1 cells: separation from surrounding cells, cell shrinkage and cell rounding. (b) Cells were stained with annexin V and propidium iodide (PI) and analyzed by flowcytometry. Data illustrate the mean from three separate experiments.

FKB acts synergistically with gemcitabine and docetaxel in inhibiting the growth of SK-LMS-1 cells

Currently, adjuvant treatment of uterine leiomyosarcoma utilizes gemcitabine-docetaxel combination chemotherapy. Thus, we performed synergy assays utilizing different concentrations of FKB alone or with gemcitabine and docetaxel at their respective IC_{50} for SK-LMS-1. Combination indexes (CI) of 0.260 and 0.546 at FKB doses of 1.25 and 2.5 μg/mL (2.2 and 4.4 μM), respectively were shown, indicating a strong synergistic effect amongst FKB, docetaxel and gemcitabine (Fig. 6).

Discussion

In this study, FKB preferentially inhibited the growth of uterine LMS and endometrial adenocarcinoma cells compared to non-malignant human endometrium fibroblast–like cells. This growth inhibitory effect of FKB is associated with G2/M arrest and a robust induction of apoptosis via up-regulation of the pro-apoptotic proteins DR5, Puma and Bim and down-regulation of an inhibitor of apoptosis protein (IAP), survivin.

The Gynecologic Oncology Group (GOG) has investigated greater than 10 cytotoxic chemotherapeutic agents for the treatment of uterine leiomyosarcoma.[22–24]

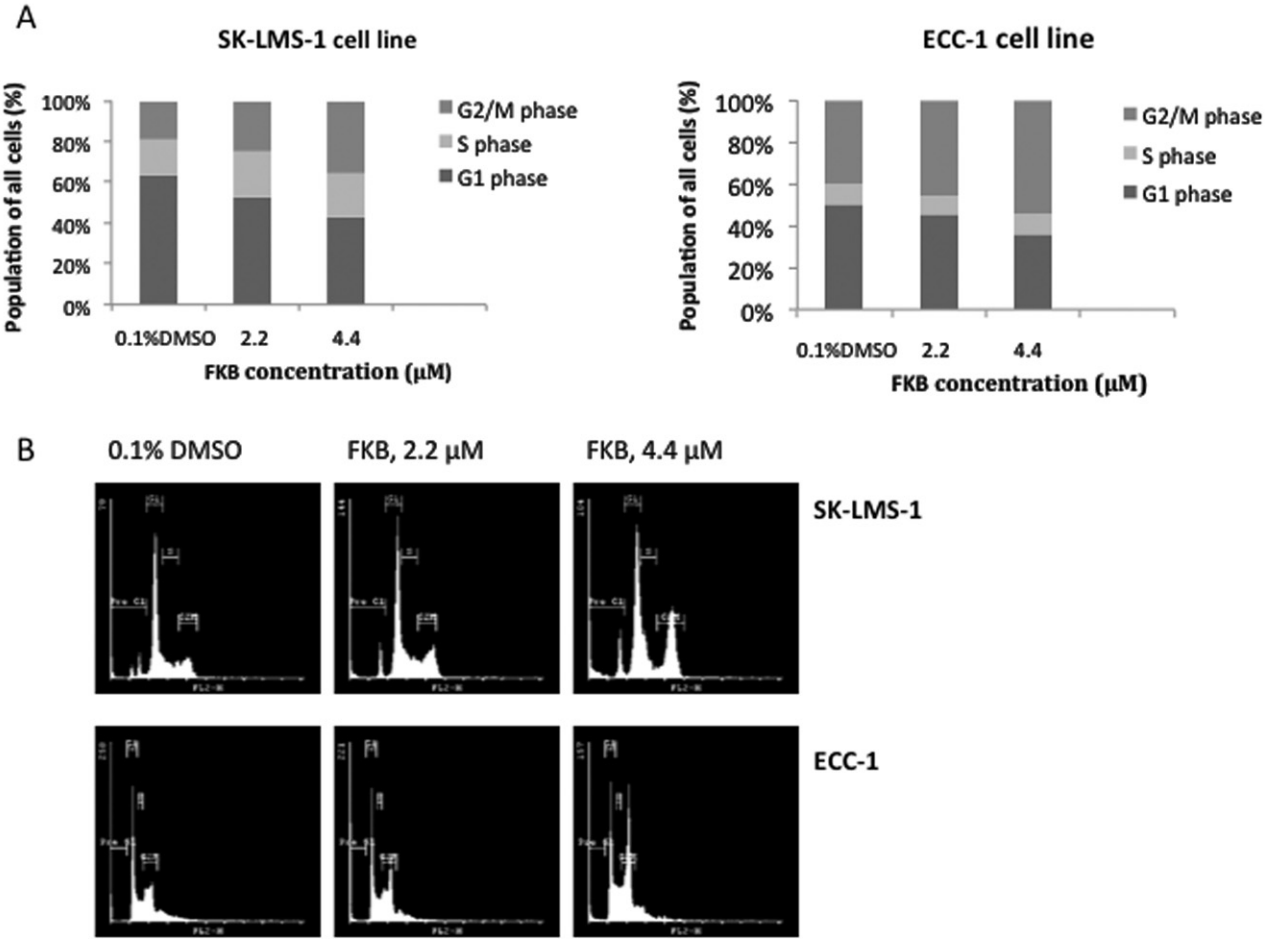

Figure 4 Flavokawain B (FKB) induced G2/M phase accumulation. SK-LMS-1 and ECC-1 cells were treated with vehicle control or FKB for 24 h and assayed for cell cycle phase. Cell cycle assays illustrate an increase in the G2/M fraction in treated SK-LMS-1 (a) and ECC-1 (b) cells relative to control (19.2 ± 0.34 to 35.6 ± 1.3% in SK-LMS-1 and 40.5 ± 1.1 to 54.9 ± 1.2% in ECC-1, $P < 0.001$ at an FKB dose of 2.5 μg/mL). Graph is representative of three separate experiments.

Despite advances in traditional chemotherapeutic techniques, including combination drug regimens, response rates have remained relatively constant.[10] Furthermore, no standard second-line chemotherapeutic agent has been identified in this disease.

Compared to cytotoxic chemotherapeutic agents, FKB exhibited selectivity against uterine LMS versus non-malignant cells. This result suggests that FKB may have a potential use as a less toxic, novel agent for the treatment of uterine LMS, where there is a need for new therapies. In addition, FKB exhibited marked synergistic and inhibitory effects on the growth of uterine LMS cells when combined with the currently used clinical chemotherapeutic agents, docetaxel and gemcitabine. These findings indicate that FKB may allow a lower dose of docetaxel and gemcitabine to be used, or potentially enhance their efficacy as therapeutic agents in the treatment this aggressive disease.

Interestingly, previous studies have shown that TRAIL, a DR5 ligand, induces apoptosis in a variety of tumor cells, while being relatively non-toxic to normal cells.[20] Similar to these reports, our result showed that non-malignant uterine fibroblasts appeared to be spared from the cytotoxic affects of FKB. Further studies are currently in progress investigating the detailed mechanisms underlying FKB-mediated regulation of DR5 and its signaling pathways, as well as the potential impact of FKB on the RAF/mitogen-activated

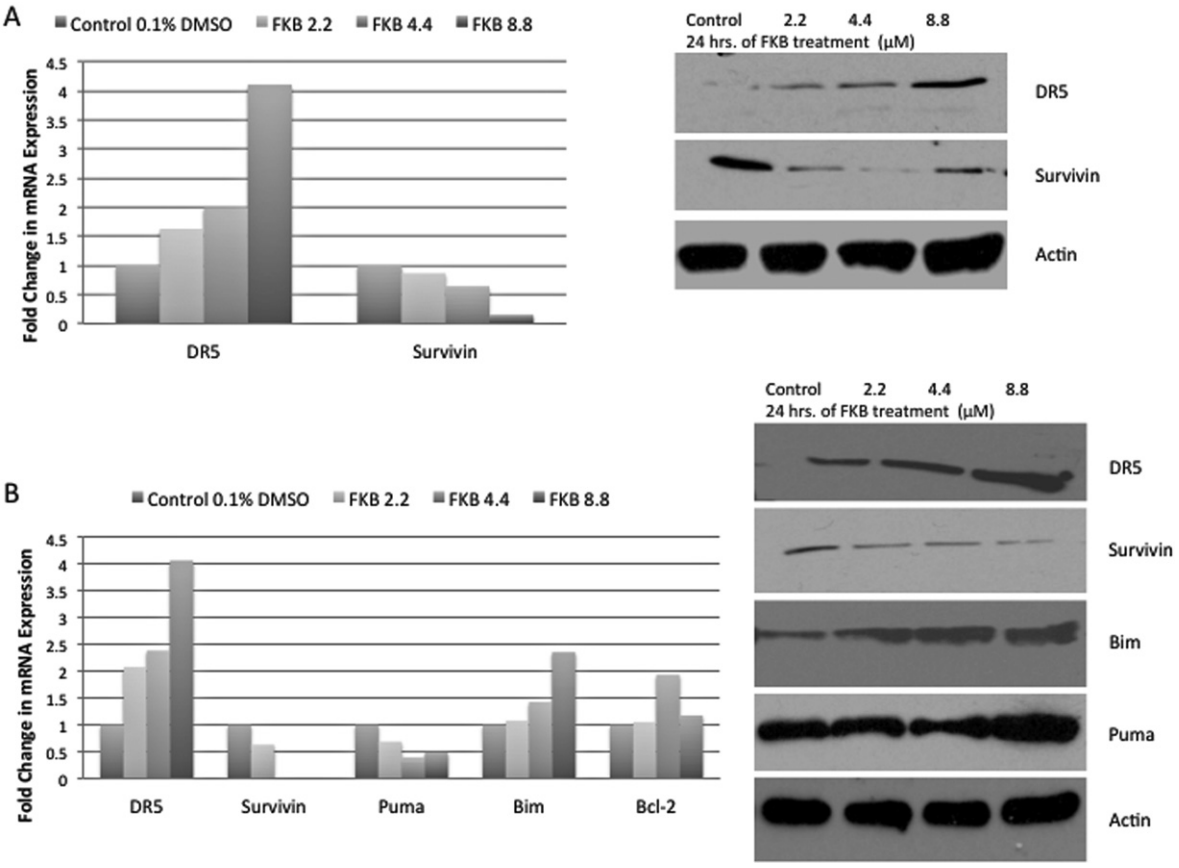

Figure 5 Flavokawain B (FKB) treatment induces the expression of DR5, Bim and Puma, and decreases survivin expression. (a) mRNA and protein levels of DR5 and survivin after indicated FKB treatments of ECC-1 cells for 24 h were analyzed using real-time polymerase chain reaction and western blotting. (b) mRNA and protein levels of DR5, Bim, Puma and survivin after indicated FKB treatments of SK-LMS-1 for 24 h were analyzed via real-time PCR and western blotting. β-Actin was used as a loading control in these experiments. A representative blot was shown from three independent experiments.

protein/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK cascade. In addition, it has been reported that the expression of both DR5 and Bim is regulated by a transcriptional factor GADD153. We will therefore examine whether FKB can induce expression of GADD153 leading to up-regulation of DR5 and Bim in our uterine LMS cells.

Any discussion surrounding novel therapeutics must include concerns regarding untoward side-effects. Recently, Zhou *et al*. investigated the potential hepatotoxic effects of kava root constituents.[25] In their studies, FKB was shown to induce cell death in two normal liver cell lines, HepG2 and L-02. However, the FKB dose used was 10-fold greater than the IC50 identified in our experiments, possibly explaining these effects. Furthermore, Zhou *et al*. independently isolated and purified FKB, introducing the possibility that other constituents included in the isolation/purification of the compound may have resulted in the hepatotoxicity. Nonetheless, investigation into the possible adverse effects of FKB is warranted.

The limitations of our study include lack of a xenograft animal model, as well as incomplete mechanistic explanations on the cellular function of FKB. It would be important to determine the specific targets of this compound, which result in the previously described growth arrest and cell death. Furthermore, exploration of FKB use on alternate uterine leiomyosarcoma cell lines would be needed. Lastly, demonstration that *in vitro* concentrations resulting in cell kill would be achievable *in vivo* would be an integral part of future animal models.

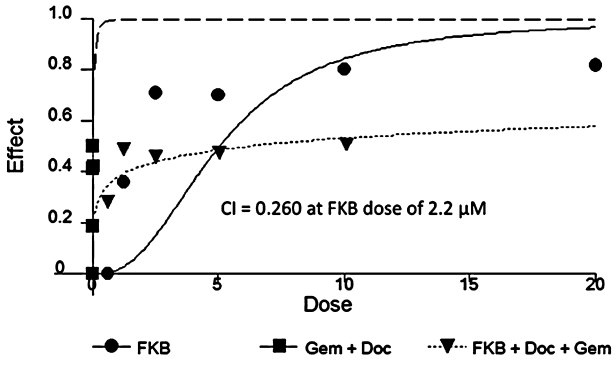

Figure 6 Graphical representation showing the type of drug interaction after the addition of flavokawain B (FKB) to the cytotoxic agents docetaxel + gemcitabine. A CI of 0.260 at FKB dose of 1.25 µg/mL (2.2 µM) is shown, representing a synergistic affect amongst the agents. Experiments were performed in triplicate. CI, combination index; Doc, docetaxel; Gem, gemcitabine.

In summary, FKB deserves further study as a novel apoptosis inducer of uterine LMS cell lines and for its potential use in enhancing docetaxel and gemcitabine's antitumor efficacy in the treatment of this aggressive malignancy.

Acknowledgment

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Disclosure

None of the above by-lined authors have any financial interests in the information contained above, or affiliations with companies that may have a financial interest.

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