Published in final edited form as:

J Orthop Res. 2012 July ; 30(7): 1045–1050. doi:10.1002/jor.22050.

Flavokawain B, a Kava Chalcone, Induces Apoptosis in Synovial Sarcoma Cell Lines

Toshinori Sakai¹, Ramez N. Eskander², Yi Guo¹, Kap Jung Kim¹, Jason Mefford¹, Justin Hopkins¹, Nitin N. Bhatia¹, Xiaolin Zi^{3,4}, and Bang H. Hoang¹

¹Department of Orthopaedic Surgery, University of California Irvine, 101 The City Drive South, Orange, California 92868

²Department of Obstetrics and Gynecology, University of California Irvine, 101 The City Drive South, Building 56, Suite 260, Orange, California 92868

³Department of Urology, University of California Irvine, 101 The City Drive South, Building 55, Suite 302, Orange, California 92868

⁴Department of Pharmaceutical Sciences, University of California, Irvine, 101 The City Drive South, Orange, California 92868

Abstract

Synovial sarcomas (SS) are soft tissue sarcomas with poor prognosis, displaying a lack of response to conventional cytotoxic chemotherapy. Although SS cell lines have moderate chemosensitivity to ifosfamide and doxorubicin therapy, the clinical prognosis is still poor. In this article, we showed that flavokawain B (FKB), a novel chalcone from kava extract, potently inhibits the growth of SS cell lines SYO-I and HS-SY-II through induction of apoptosis. Treatment with FKB increased caspase 8, 9, and 3/7 activity compared to vehicle-treated controls, indicating that both extrinsic and intrinsic apoptotic pathways were activated. Furthermore, FKB treatment of both cell lines resulted in increased mRNA and protein expression of death receptor-5 and the mitochondrial pro-apoptotic proteins Bim and Puma, while down-regulating the expression of an inhibitor of apoptosis, survivin in a dose-dependent manner. Our results suggest the natural compound FKB has a pro-apoptotic effect on SS cell lines. FKB may be a new chemotherapeutic strategy for patients with SS and deserves further investigation as a potential agent in the treatment of this malignancy.

Keywords

synovial sarcoma; flavokawain; apoptosis

Synovial sarcoma (SS) is an aggressive malignancy with a poor 5- and 10-year survival and a high rate of local recurrence and metastasis.^{1–4} Chemotherapy is a largely ineffective adjunct. Despite encouraging evidence that certain chemotherapeutic agents such as ifosfamide have cytotoxic effects on SS cell lines in vitro, clinical studies have failed to show any significant improvements in long-term survival.

Kava (*Piper methysticum*) is an ancient crop of the western Pacific. The root extract of kava has been a part of the Pacific Islanders' culture for thousands of years, serving medicinal purposes in socio-religious functions.⁵ Consumption of traditional aqueous kava

preparations correlates with low and uncustomary sex ratios of cancer incidences in three kava-drinking countries: Fiji, Vanuatu, and Western Samoa.⁶ Recently, flavokawain B (FKB), a kava chalcone, was shown to cause apoptosis in prostate cancer cell lines,⁷ and to inhibit growth of human squamous carcinoma cells.⁸

In this current study, we examined the effects of FKB on two SS cell lines. Our group hypothesized that this compound may act as a novel chemotherapeutic agent for SS by enhancing apoptotic mechanisms. Here, we report that FKB-induced apoptosis on SS cell lines through both extrinsic and intrinsic apoptotic pathways, associated with up-regulation of DR5, Bim and Puma expression, and down-regulation of survivin expression.

MATERIALS AND METHODS

Cell Lines, Compounds, and Reagents

The cell lines utilized in this study, SYO-I and HS-SY-II were kindly provided by M. Ladanyi (Memorial Sloan-Kettering Cancer Center). Both cell lines were cultured in MEM-Alpha supplemented with 10% fetal bovine serum (FBS). As a control cell line, the human endometrial fibroblast cell line (T-HESCs) was purchased from ATCC (Manassas, VA). All cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Medium was replaced every 2–3 days as indicated. Pure FKB was purchased from LKT laboratories (St. Paul, MN), dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at –20°C. Antibodies for DR5, Bim, Puma, survivin, Bax, and Bcl-2 were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Antibody against β-actin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Thymidine, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (Saint Louis, MO). RNAazol B was purchased from Tel-Test (Friendswood, TX). The Reverse Transcription System kit utilized was from Applied Biosystems (Carlsbad, CA).

MTT Assay

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 for 72 h, 500 μl of MTT solution was added to each well and plates were incubated at 37°C for 2 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 micro-plate reader (Bio-Rad, Hercules, CA). Dose–response curves were then created as a percentage of vehicle-treated control cells using Excel software.

Protein Isolation and Western Blot Analysis

Samples (normalized according to cell number) were treated with 0.1% DMSO or FKB at varying concentrations for 24 h. Cell extracts were then prepared in RIPA lysis buffer containing protease inhibitors (Sigma). Cell lysates were centrifuged at $12,000 \times g$ for 15 min and the supernatant was collected. The Bio-Rad DC Protein Assay was used to determine protein concentration. Volumes of clarified protein lysate containing equal amounts of protein (50 μg) were then separated on 10–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically (90 min at 100 V) transferred to a Hybond-ECL membrane (GE Healthcare, Piscataway, NJ). 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), and then incubated overnight with primary antibody at 4°C. Six microliters of primary antibody was placed in 2 ml of 5% milk-blocking buffer. 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). Immunoreactive bands were visualized using an

enhanced chemiluminescence detection system (Thermo Scientific, Rockford, IL). β -actin bands were used as housekeeping protein expression. Protein-protein expression was analyzed using densitometric calculations according to Yano et al.⁹

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Samples (normalized according to cell number) were treated with 0.1% DMSO or FKB at varying concentrations for 24 h. Following treatment with FKB, total RNA was isolated from SYO-I and HS-SY-II cell lines using the TRizol reagent (Invitrogen, Carlsbad, CA). cDNA was then synthesized from 2 μ g of total RNA using a High Capacity cDNA Reverse Transcription kit per protocol (Applied Biosystems, Foster City, CA). 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.⁷ DR5, Survivin, Bim, and Puma primers were obtained from Sigma, with primer sequences available upon request. Data were 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.

Soft Agar Colony Formation Assay

Soft agar colony formation assays were done using six-well plates. Each well contained 2 ml of 0.8% agar in complete medium as the bottom layer, 1 ml of 0.35% agar in complete medium and 6,000 cells as the feeder layer, and 1 ml complete medium as the top layer. Each well was treated with FKB at varying concentrations. Cultures were maintained under standard culture conditions. The number of colonies was determined with an inverted phase-contrast microscope at $\times 100$ magnification. A group of >10 cells was counted as a colony. The data are shown as mean number of colonies $\pm$ SEM of four independent wells at 14 days for SYO-I, 28 days for HS-SY-II after the start of cell seeding.

Caspase Activity Assay

Apoptosis was confirmed using the Caspase-Glo® 3/7, Caspase-Glo® 8, and Caspase-Glo® 9 Assay (Promega, Madison, WI) according to the manufacturer's instructions. Cells were plated in a 96-well plate and treated with 0.1% DMSO or FKB for 24 h. Then 100 μ l of Caspase-Glo® 3/7, Caspase-Glo® 8, or Caspase-Glo® 9 reagent were added to each well and the luminescence of each sample was measured in a luminometer (GloMaxVR-MultiDetection System).

Statistical Analysis

The data are presented as mean $\pm$ standard error (SE). The level of significance was set at a p -value < 0.05 . Comparisons of differences between treated and control groups were performed using Student's t -test. All statistical tests were two-sided.

RESULTS

FKB Induces Apoptotic Morphology in both SYO-I and HS-SY-II Cell Lines

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 1, treated cells exhibited typical apoptotic morphologic changes including separation from surrounding cells, cell shrinkage, cell rounding, and cell membrane blebs.¹⁰ Significant

differences in cell viability were noted between normal fibroblast and synovial cancer cell lines following FKB treatment ($<2.5 \mu\text{g/ml}$) (Student's *t*-test, $*p < 0.05$) (Fig. 2).

When anchorage-independent growth was examined in soft agar, both SYO-I and HS-SY-II cell lines formed significantly less colonies following FKB treatment ($p < 0.01$, Fig. 3).

These results suggest that treatment of both SYO-I and HS-SY-II cell lines with FKB result in a significant inhibition of growth in a dose-dependent manner.

FKB Induces Activation of Caspase-3/7, -8, and -9 Activities in both SYO-I and HS-SY-II Cell Lines

Figure 4 shows that FKB treatment increases Caspase 8, 9, and 3/7 activity compared to vehicle-treated controls, suggesting that both death receptor- and mitochondrial-mediated apoptotic pathways are activated.

FKB Upregulates Expression of Pro-Apoptotic Markers and Downregulates Anti-Apoptotic Markers Expression

Figure 5 illustrates that FKB treatment of SYO-I and HS-SY-II cell lines resulted in increased mRNA expression of death receptor (DR5) and the mitochondrial pro-apoptotic proteins Bim and Puma, while down-regulating the mRNA expression of an inhibitor of apoptosis protein (IAP), survivin. Analogously, FKB treatment of both cell lines resulted in a significant increase in protein expression of DR5 and a downregulation of survivin expression (Fig. 5B). Furthermore, Bax and Bcl-2 protein expression were significantly up- and down-regulated, respectively, at FKB dose of $7.5 \mu\text{g/ml}$. Taken together, these results imply that FKB activates both extrinsic and intrinsic apoptotic pathways, exhibiting apoptotic effects against SS.

DISCUSSION

In this study, we demonstrated that FKB-induced apoptosis in two SS cell lines (SYO-I and HS-SY-II). Previously, we reported that FKB-induced apoptosis in androgen receptor-negative, hormone-refractory, prostate cancer cells (HRPC).⁷ Recently, FKB was also reported to cause apoptosis in human squamous carcinoma cell lines.⁸ However, to the best of our knowledge, there have been no reports on the efficacy of FKB on sarcoma cell lines, including SS. Our results demonstrated that FKB promotes apoptosis by affecting both pro and anti-apoptotic proteins in a dose-dependent manner. The apoptotic mechanisms of FKB in SS are similar to those in the previous reports using HRPC.⁷ Apoptosis can be induced by the extrinsic pathway associated with death receptor stimulation on the cell surface,¹¹ and by the intrinsic pathway characterized by the involvement of mitochondrial dysfunction.¹² Although Caspase 3/7 is an effector caspase, initiator Caspases 8 and 9 are activated by death receptors and mitochondrial releasing factors, respectively.¹³ FKB-induced apoptosis via activation of caspase-3/7, -8 and -9 in both SYO-I and HS-SY-II cell lines.

In the previous studies using HRPC, FKB treatment significantly increased the protein expression of Bim and Puma without affecting the expression of other BH-3-only proteins. Expression levels of DR5 and Survivin were also influenced by FKB. In this study, FKB-induced apoptosis of both SS cell lines with an increased expression of the proapoptotic markers: death receptor-5 (DR5), Bim, Puma, and Bax, and a decreased expression of inhibitors of apoptosis: Survivin and Bcl-2.

Increased DR5 expression by FKB activates the death receptor-mediated apoptotic pathway. For example, the DR5 ligand Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL), is considered an effective anticancer agent. Although it selectively induces

apoptosis in a variety of tumor cells, it is relatively non-toxic to normal cells.¹¹ The mechanism for FKB-mediated DR5 expression remains unclear.

In addition to upregulation of DR5 expression, FKB was found to increase the expression of Puma and Bim in both SYO-I and HS-SY-II cell lines. DR5 and Puma are common p53 target genes.¹⁴ Interestingly, in comparison to other tumor types, SS display a remarkably low number of mutations in the p53 gene, implying that defects in upstream pathways may be responsible for loss of p53-mediated tumor suppression.^{15,16} We found that FKB did not change the expression of p53 in SS (data not shown). Therefore, it is less likely that FKB-mediated effects on DR5 and Puma expression are p53 mediated.

Bim directly initiates Bax-mediated mitochondrial apoptosis via binding to and stabilizing the α -helix of the Bcl-2 domains of Bax.¹⁷ Our Western blot results of Bax and Bcl-2 expression were consistent with the results detailed above.

FKB decreased the expression of an IAP, survivin, in both cell lines in a dose-dependent manner. Survivin is one of the most tumor-specific genes in the human genome. Despite discussion and investigation into the apoptotic inhibitory effects of survivin, the precise mechanism of apoptosis inhibition is still a matter of discussion.

It is important to understand that there are mainly two limitations to this article. Kava is widely used as a dietary supplement to relieve anxiety and stress in the United States. However, kava-containing products have been reported to result in liver damage, resulting in several cases of mortality or liver transplantation.^{18,19} Unfortunately, this compound is extracted in various ways, and contamination and alternate purification techniques may lead to contaminating compounds and untoward side effects. Furthermore, the hepatic toxicities illustrated in prior studies on human hepatic cell lines were obtained at FKB doses greater than five times the IC50 used on our experiments.

There have been previous reports showing that FKB has minimal effects on normal epithelial and stromal cells.⁷ In this article, we used the non-malignant human endometrium fibroblast-like (T-HESC) cell line as a control. This cell line showed significant resistance to FKB treatment as evidenced by MTT assays, protein expression of apoptotic markers (Figs. 1, 2, and 5B), and Caspase assay results (data not shown).

However, the etiology of SS is unclear at this time, and thus selection of an appropriate control cell line is limited.

Despite surgical resection with/without adjuvant radiotherapy and/or docorubicin-based chemotherapy, the long-term prognosis for patients diagnosed with SS is poor. Our data suggest that FKB may potentially have utility as a less toxic chemotherapeutic agent for reducing the recurrence of SS. To realize this possibility, further preclinical animal studies examining the in vivo anti-tumor efficacy of FKB against SS, as well as the potential side effects of this agent are required. In addition, FKB may have synergistic inhibitory effects with chemotherapeutic drugs on the growth of SS cell lines, which will be further explored in future.

Acknowledgments

This work was supported in part by NIH Grant CA116003, Department of Orthopaedic Surgery, Chao Family Comprehensive Cancer Center (to BHH) and CA122558 (to XZ).

REFERENCES

1. Bergh P, Meis-Kindblom JM, Gherlinzoni F, et al. Synovial sarcoma: identification of low and high risk groups. *Cancer*. 1999; 85:2596–2607. [PubMed: 10375108]
2. Eilber FC, Brennan MF, Eilber FR, et al. Chemotherapy is associated with improved survival in adult patients with primary extremity synovial sarcoma. *Ann Surg*. 2007; 246:105–113. [PubMed: 17592298]
3. Joyner DE, Bastar JD, Randall RL. Doxorubicin induces cell senescence preferentially over apoptosis in the FU-SY-1 synovial sarcoma cell line. *J Orthop Res*. 2006; 24:1163–1169. [PubMed: 16705698]
4. Lewis JJ, Antonescu CR, Leung DH, et al. Synovial sarcoma: a multivariate analysis of prognostic factors in 112 patients with primary localized tumors of the extremity. *J Clin Oncol*. 2000; 18:2087–2094. [PubMed: 10811674]
5. Singh YN. Kava: an overview. *J Ethnopharmacol*. 1992; 37:13–45. [PubMed: 1453702]
6. Steiner GG. The correlation between cancer incidence and kava consumption. *Hawaii Med J*. 2000; 59:420–422. [PubMed: 11149250]
7. Tang Y, Li X, Liu Z, et al. 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. [PubMed: 20112340]
8. 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*. 2011 [Epub ahead of print].
9. Yano T, Yamazaki Y, Adachi M, et al. Tara up-regulates E-cadherin transcription by binding to the Trio RhoGEF and inhibiting Rac signaling. *J Cell Biol*. 2011; 193:319–332. [PubMed: 21482718]
10. Häcker G. The morphology of apoptosis. *Cell Tissue Res*. 2000; 301:5–17. [PubMed: 10928277]
11. Finnberg N, El-Deiry WS. TRAIL death receptors as tumor suppressors and drug targets. *Cell Cycle*. 2008; 7:1525–1528. [PubMed: 18469516]
12. Kim H, Rafiuddin-Shah M, Tu HC, et al. Hierarchical regulation of mitochondrial dependent apoptosis by BCL-2 subfamilies. *Nat Cell Biol*. 2006; 8:1348–1358. [PubMed: 17115033]
13. Ho PK, Hawkins CJ. Mammalian initiator apoptotic caspases. *FEBS J*. 2005; 272:5436–5453. [PubMed: 16262685]
14. Wang W, Kim SH, El-Deiry WS. Small molecule modulators of p53 family signaling and antitumor effects in p53-deficient human colon tumor xenografts. *Proc Natl Acad Sci USA*. 2006; 103:11003–11008. [PubMed: 16835297]
15. Oda Y, Sakamoto A, Saito T, et al. Molecular abnormalities of p53, MDM2, and H-ras in synovial sarcoma. *Mod Pathol*. 2000; 13:994–1004. [PubMed: 11007040]
16. Schneider-Stock R, Onnasch D, Haeckel C, et al. Prognostic significance of p53 gene mutations and p53 protein expression in synovial sarcomas. *Virchows Arch*. 1999; 435:407–412. [PubMed: 10526004]
17. Gavathiotis E, Suzuki M, Davis ML, et al. BAX activation is initiated at a novel interaction site. *Nature*. 2008; 455:1076–1081. [PubMed: 18948948]
18. Centers for Disease Control and Prevention. Hepatic toxicity possibly associated with kava-containing products—United States, Germany, and Switzerland, 1999–2002. *JAMA*. 2003; 289:36–37. [PubMed: 12515265]
19. Centers for Disease Control and Prevention. Hepatic toxicity possibly associated with kava-containing products—United States, Germany, and Switzerland, 1999–2002. *MMWR*. 2002; 51:1065–1067. [PubMed: 12500906]

Figure 1.

FKB-induced apoptosis in SYO-I and HS-SY-II cell lines. Live cell morphology evaluated using light microscopy (magnification: $\times 4$) with image obtained from a random representative field. Representative images are shown of FKB-treated SYO-I and HS-SY-II cells: separation from surrounding cells, cell shrinkage, cell rounding, and cell membrane blebs.

Figure 2.

Cells are treated with FKB at the indicated concentrations in the figure for 72 h and cell viabilities were measured by MTT assay. Normal fibroblasts were significantly more resistant to FKB treatment in comparison to synovial cancer cell lines (FKB dose $< 2.5 \mu\text{g/ml}$) (Student's *t*-test, $*p < 0.05$).

Figure 3.

Agar assay showed that FKB is effective for anchorage-independent cells in both cell lines. Anchorage-independence assays showed a significant reduction in the number of colonies formed after treatment with FKB (Student's *t*-test, * $p < 0.05$).

Figure 4. FKB induces apoptosis and activates caspase 3/7, 8, and 9 (Student's t -test, $*p < 0.05$).

Figure 5. mRNA and protein levels of several apoptotic markers after FKB treatments of SYO-I and HS-SY-II cell lines for 24 h were analyzed using real-time PCR (A) and Western blotting (B) (Student's *t*-test, $*p < 0.05$). FKB treatment induces the expression of DR5, Bim, Puma, and Bax and decreases survivin and Bcl-2 expression in a dose-dependent manner. β -Actin was used as a loading control in these experiments. A representative blot was shown from three independent experiments.