Flavokawain A, a Novel Chalcone from Kava Extract, Induces Apoptosis in Bladder Cancer Cells by Involvement of Bax Protein-Dependent and Mitochondria-Dependent Apoptotic Pathway and Suppresses Tumor Growth in Mice

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Abstract

Consumption of the traditional kava preparation was reported to correlate with low and uncustomary gender ratios (more cancer in women than men) of cancer incidences in three kava-drinking countries: Fiji, Vanuatu, and Western Samoa. We have identified flavokawain A, B, and C but not the major kavalactone, kawain, in kava extracts as causing strong antiproliferative and apoptotic effect in human bladder cancer cells. Flavokawain A results in a significant loss of mitochondrial membrane potential and release of cytochrome *c* into the cytosol in an invasive bladder cancer cell line T24. These effects of flavokawain A are accompanied by a time-dependent decrease in Bcl-x_L, a decrease in the association of Bcl-x_L to Bax, and an increase in the active form of Bax protein. Using the primary mouse embryo fibroblasts Bax knockout and wild-type cells as well as a Bax inhibitor peptide derived from the Bax-binding domain of Ku70, we showed that Bax protein was, at least in part, required for the apoptotic effect of flavokawain A. In addition, flavokawain A down-regulates the expression of X-linked inhibitor of apoptosis and survivin. Because both X-linked inhibitor of apoptosis and survivin are main factors for apoptosis resistance and are overexpressed in bladder tumors, our data suggest that flavokawain A may have a dual efficacy in induction of apoptosis preferentially in bladder tumors. Finally, the anticarcinogenic effect of flavokawain A was evident in its inhibitory growth of bladder tumor cells in a nude mice model (57% of inhibition) and in soft agar. (Cancer Res 2005; 65(8): 3479-86)

Introduction

About 400,000 superficial bladder cancer patients in the United States are at risk of recurrence and/or progression to invasive diseases, and in addition, numerous smokers and workers exposed to industrial carcinogens are at risk of developing primary bladder tumors (1). For these people, effective preventive measures are needed.

Henderson et al. (2) noted unusually low cancer incidences in the Pacific Island nations despite a high portion of smokers in these populations and speculated possible chemopreventive agents in their diet. Steiner (3) reported that age-standardized cancer incidences for the three highest kava-drinking countries, such as Vanuatu, Fiji, and Western Samoa, were one fourth or one third the cancer incidences in non-kava-drinking countries, such as New Zealand (Maoris) and United States (Hawaii and Los Angeles), and nondrinking Polynesians. Uniquely, these three kava-drinking countries have lower incidences of cancer for men than women, which only occur in 10 of 150 cancer incidence-reporting locations in the world (3). Furthermore, more men drink kava and smoke than woman do in these kava-drinking countries (3–5). Given that smoking is a major risk factor for bladder cancer and that generally bladder cancer is three to four times more common in men than in women (6), these reports are very intriguing to us.

The traditional kava preparation has been part of the Pacific Islanders' culture for thousands of years, serving as a beverage and medication and used during socioreligious functions (5). At the beginning of the 20th century, kava extracts were employed to treat chronic inflammations of the urinary tract and bladder disorders in Europe (7). Recently, kava extracts have been used as treatment for anxiety, nervous tension, agitation, and/or insomnia (8). Kapadia et al. (9) reported that kava extract was the most effective antitumor promotion herb among the eight common herbs, including black cohosh, *Echinacea*, *Ginkgo*, goldenseal, valerian, saw palmetto, and St. John's Wort. Because tumor necrosis factor-α was considered as an endogenous tumor promoter (10), Hashimoto et al. (11) showed that kava extract effectively inhibited tumor necrosis factor-α release from okadaic acid (a tumor promoter)–treated BALB/3T3 cells as well as in lipopolysaccharide-treated mice.

Kavalactones and chalcones are two main classes of compounds identified from kava extracts (12). The major kavalactones include kawain, methysticin, desmethoxyyangonin, yangonin, dihydrokawain, and dihydromethysticin (12). The chalcones are flavokawain A, B, and C that constitute up to 0.46%, 0.015%, and 0.012% of kava extracts, respectively (12). Chalcones are the intermediate precursors for all flavonoids in the phenylpropanoid pathway in plants and are unique in the flavonoid family (13). Because of α,β-unsaturated ketones in their structures, chalcones have a preferential reactivity toward thiols in contrast to amino and hydroxyl groups (14). Therefore, chalcones is less likely to interact with nucleic acids and then avoid the problems of mutagenicity and carcinogenicity associated with certain alkylating agents in cancer chemotherapy (14). In addition, chalcones are susceptible to the Michael reaction at the ene-one (CH = CH-CO), which can cause binding to particular receptors and lead to the induction of phase II enzymes against carcinogens (15). Furthermore, chalcones possessed a variety of biological activities, including antioxidant, anti-inflammation, antimicrobial, antiprotozoal, antiulcer, as well as other activities [reviewed by Dimmock et al. (16)]. Importantly, chalcones have shown several anticancer

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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activities as inhibitors of cancer cell proliferation, carcinogenesis, and metastasis (16–20).

Taken together, these anticancer properties of chalcones combined with the epidemiologic and experimental data about kava have prompted us to characterize possible active compounds in kava extracts for bladder cancer prevention. We found that flavokawains and a crude kava extract induced apoptosis in bladder cancer cells, but kavalactones did not. Notably, flavokawain A exhibits significant anticarcinogenic effect on colony formation in soft agar and tumor growth in a xenograft mouse model.

Materials and Methods

Materials. Antibodies against Bcl-2, Bax, Bcl-x_L, cytochrome *c*, and β-actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-poly(ADP-ribose) polymerase (PARP) and FITC-conjugated Annexin V were from PharMingen (San Diego, CA). Antibodies against caspase-9, cleaved caspase-9, caspase-3, cleaved caspase-3, cleaved PARP, *survivin*, and X-linked inhibitor of apoptosis (XIAP) were from Cell Signaling Technology, Inc. (Beverly, MA). Anti-Bax 6A7 antibody, which recognizes only the open form of Bax, was from Sigma (St. Louis, MO). Chemiluminescence Western blot detection reagents were from Amersham Life Sciences, Inc. (Arlington Heights, IL). JC-1 probe was from Molecular Probes, Inc. (Eugene, OR). Cytochrome *c*–releasing apoptosis assay kit was from Biovision, Inc. (Mountain View, CA). Caspase-3 inhibitor II (z-DEVD-fmk), caspase-9 inhibitor I (z-LEHD-fmk), and caspase inhibitor I (z-VAD-fmk) were from Calbiochem-Novabiochem, Inc. (La Jolla, CA). The membrane-permeable peptide, Bax inhibitor peptide P5 (Pro-Met-Leu-Lys-Glu), and negative control (IIe-Pro-Met-IIe-Lys) were from Tocris (Ellisville, MO; ref. 21). Kava extract standardized with 70% kavalactones was a generous gift from Dr. Wang Xiping (Gaia Herbs, Inc., Brevard, NC). The human bladder transitional cell carcinoma RT4 and T24 cell lines were from the American Type Culture Collection (Manassas, VA). The EJ cell line was a generous gift from Dr. Peter A. Jones (University of Southern California, Los Angeles, CA). Primary mouse embryo fibroblasts (MEF) deficient for Bax (double knockout) and control cells were generous gifts from Dr. Stanley J. Korsmeyer (Harvard Medical School, Boston, MA). Hoechst 33258, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide, and all other chemicals used were from Sigma or Fisher Scientific (Irvine, CA).

Cell culture conditions and compounds. RT4, T24, and EJ cells were cultured in McCoy's 5A medium containing 10% fetal bovine serum (FBS) under standard culture conditions. Bax double knockout and control cells were grown in DMEM with 10% FBS. Pure flavokawain A, B, and C as well as kavalactones, including kawain, methysticin, and yangonin, were isolated from kava extracts by LKT Laboratories, Inc. (St. Paul, MN). Their chemical structures are shown in Fig. 1*A* and were confirmed by using ^{1}H and ^{13}C nuclear magnetic resonance data. They were dissolved in DMSO, aliquoted, and stored at −80°C. The DMSO in culture medium never exceeded 0.1% (v/v), a concentration known not to affect cell proliferation. Kava extract standardized with 70% kavalactones was dissolved in grain alcohol at a stocking concentration of 75 mg/mL.

Cell viability 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. T24, RT4, EJ, Bax double knockout, and control cells were plated at a density of 5×10^4 per well in 24-well culture plates in medium containing 10% FBS. After 24 hours, the medium was refreshed with fresh medium and left untreated or was treated as indicated in the figure legends. After treatment, MTT was added to the wells at a final concentration of 1 mg/mL and incubated at 37°C for 3 hours. The absorbance was determined at 570 nm.

Hoechst 33258 staining. Cells were treated with either 0.1% DMSO or flavokawain A, B, and C or kawain at the indicated doses for 24 hours. The cells were then fixed with 4% paraformaldehyde for 30 minutes at room temperature. Hoechst 33258 (50 ng/mL) dissolved in PBS was added to the

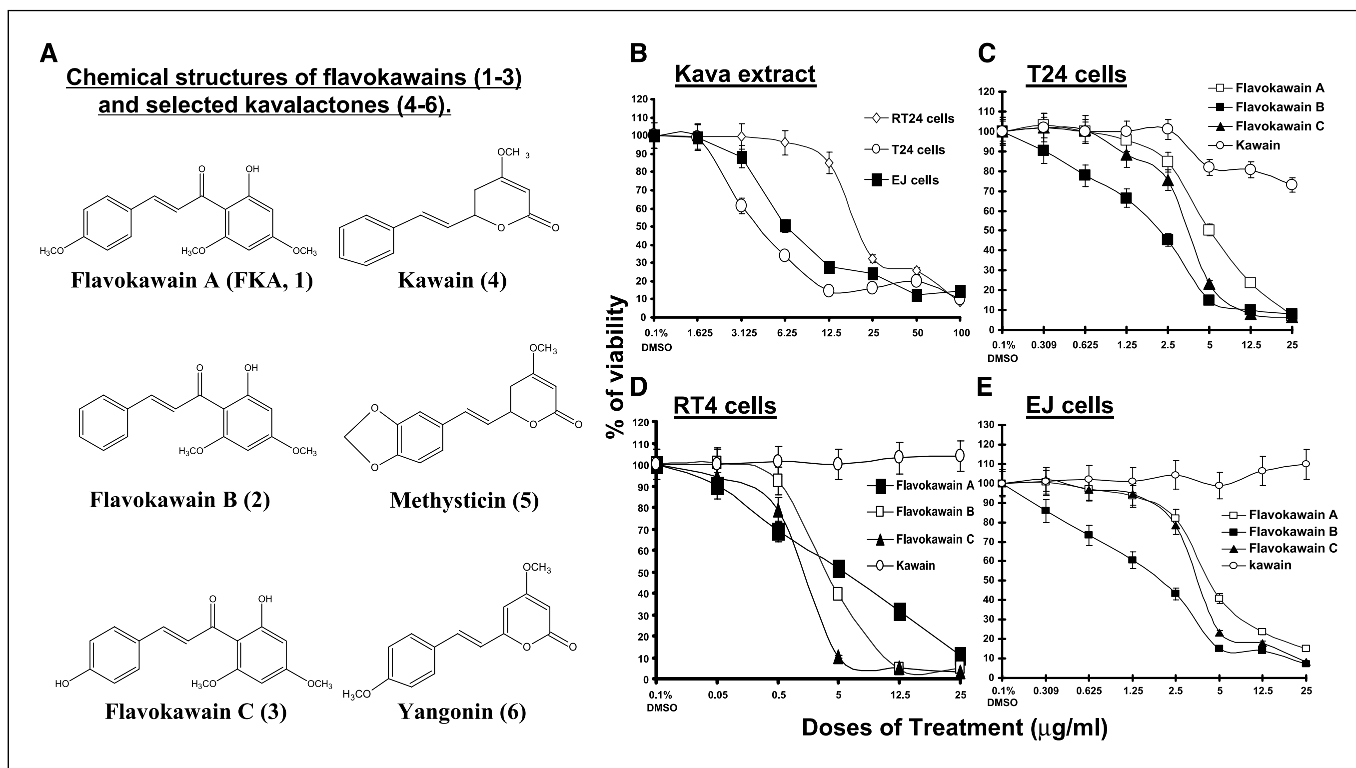

Figure 1. Antiproliferative and apoptotic effect of kava extract, flavokawain A (*FKA*), B, and C, and kawain on bladder cancer cells. *A,* chemical structures of flavokawains (*1-3*) and kavalactones (*4-6*). *B-E,* 5×10^4 RT4, T24, and EJ cells were plated in 24-well culture plates. After 24 hours, the medium was changed to fresh medium and treated with 0.1% DMSO alone or kava extract, flavokawain A, B, or C, or kawain at the indicated doses. After 48 hours of treatment, cell densities were measured by MTT assay. *Points,* mean of four independent plates; *bars,* SE. Each sample was counted in duplicate. IC_{50}s were estimated by dose-response curves.

fixed cells, incubated for 30 minutes at room temperature, and washed with PBS twice. Cells were mounted and examined by fluorescence microscopy. Apoptotic cells were identified by the condensation and fragmentation of their nuclei.

Flow cytometry assays. T24 cells at 70% to 80% confluency were treated with either 0.1% DMSO or 12.5 μg/mL flavokawain A for 8, 16, and 24 hours. After these treatments, the following were carried out: (*a*) For the studies assessing early and late apoptosis, cells were stained with FITC-conjugated Annexin V and propidium iodide in PBS according to the manufacturer's protocol (PharMingen). All analyses of cells were done using appropriate scatter gates to exclude cellular debris and aggregated cells. Ten thousand events were collected for each sample stained with Annexin V. (*b*) For studies analyzing mitochondrial membrane potential, cells were stained with 3 μmol/L JC-1 in 5 mL HBSS for 45 minutes at 37°C. Cells were then collected and washed twice with cold PBS. Cells were resuspended in PBS and analyzed by flow cytometry using the Becton Dickinson fluorescence-activated cell sorting system (San Jose, CA; ref. 22).

Measurement of cytochrome *c* release from mitochondria. T24 cells were treated with 0.1% DMSO or 12.5 μg/mL flavokawain A for 8 and 16 hours. Mitochondria and cytosol were separated using a cytochrome *c*–releasing apoptosis assay kit. Briefly, cells were suspended in cytosol extraction buffer. After incubation for 10 minutes on ice, the cell suspension in the extraction buffer was homogenized by Dounce homogenizer and centrifuged at 700 × *g* for 10 minutes. The supernatant was then collected and centrifuged at 10,000 × *g* for 30 minutes at 4°C. The resulting supernatant (cytosolic fraction) and pellet (mitochondrial fraction) were processed for Western blot analysis.

Western blotting and immunoprecipitation. After treatment under each experimental condition, cells were lysed in radioimmunoprecipitation assay buffer (23) or CHAPS buffer [150 mmol/L NaCl, 10 mmol/L HEPES (pH 7.4), 1.0% CHAPS]. Clarified protein lysates (20-80 μg) were electrophoretically resolved on denaturing SDS-polyacrylamide gel (8-16%), transferred to nitrocellulose membranes, and probed with primary antibodies. Proteins were revealed using horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibodies and visualized by the enhanced chemiluminescence detection system. For immunoprecipitation (23), lysates (200 μg/mL) were precleared with 25 μL protein G plus-agarose and then precipitated with 2 μg anti-Bax 6A7 antibody and 25 μL protein G plus-agarose overnight at 4°C. Bax/Bcl-x_L complexes were detected by immunoblotting.

Soft agar colony formation. A soft agar colony formation assay was done using six-well plates. Each well contained 2 mL of 0.5% agar in complete medium as the bottom layer, 1 mL of 0.38% agar in complete medium and 3,000 cells as the feeder layer, and 1 mL of 0.38% agar in complete medium with either vehicle 0.1% DMSO or different doses of flavokawain A indicated in the figure as the top layer. Cultures were maintained under standard culture conditions. The number of colonies was determined by counting them under an inverted phase-contrast microscope at ×100 magnification; a group of >10 cells was counted as a colony. Data are means ± SE of four independent wells at optimum time of 14 days after the start of cell seeding.

***In vivo* tumor model.** Flavokawain A was formulated in 10% grain alcohol in 0.9% saline and given by gavage. NCR-*nu/nu* (nude) mice were obtained from Taconic (Germantown, NY). EJ bladder tumor cells were concentrated to 2×10^6 per 200 μL and injected s.c. into the right flank of each mouse. Next day, the mice were randomly divided and pair matched into treatment and control groups of 18 mice each, and daily dosing was begun with vehicle or 50 mg/kg flavokawain A. Because there were no *in vivo* data regarding flavokawain A before this study, the dose of flavokawain A (50 mg/kg/d) was used according to 1:60 of 3,000 mg/kg, a LD_{50} dose for a similar chemical structure compound, isoliquiritigenin (17). Body weight, diet, and water consumption were recorded thrice weekly throughout the study. Once xenografts started growing, their sizes were measured every other day. The tumor volume was calculated by the formula: $0.5236\, L_1(L_2)^2$, where L_1 is the long axis and L_2 is the short axis of the tumor. At the end of experiment, tumors were excised, weighed, blood collected, and stored at −80°C until additional analysis.

Statistics. Comparisons of apoptosis, mitochondrial membrane potential, and cell viabilities between treatment and control were conducted using Student's *t* test. For tumor growth experiments, repeated-measures ANOVA was used to examine the differences in tumor sizes among treatments, time points, and treatment-time interactions. Additional post-test was done to examine the differences in tumor sizes between control and flavokawain A treatment at each time point by using conservative Bonferroni method. All statistical tests were two sided.

Results

Kava extract and flavokawain A, B, and C cause strong antiproliferative and apoptotic effects in human bladder cancer cells (characterized as low grade or high grade), but kawain (a major kavalactone) did not. As shown in Fig. 1*B*, the kava extract that standardized with 70% kavalactones inhibits proliferation of RT4, T24, and EJ cells in a dose-dependent manner. The effect of the kava extract on the growth of bladder cancer cells is expressed as percentage of cell viability relative to control. RT4 cell line with wild-type p53 was derived from recurrent, superficial bladder tumor (24). T24 and EJ cells with mutant p53 were derived from muscle invasive bladder tumors (25, 26). The IC_{50}s of a kava extract for RT4, T24, and EJ cells were estimated to be ∼20.1, 6.2, and 5.1 μg/mL, respectively. T24 and EJ cells are three to four times more sensitive to the treatment of the kava extract than RT4 cells. Figure 1*C* to *E* shows the differential abilities of the major kavalactone from kava extract, kawain, and three chalcones, flavokawain A, B, and C, on proliferation of bladder cancer cell lines. At a dose up to 25 μg/mL, there were no inhibitory effects on proliferation of RT4 and EJ cells, and a <27% inhibition of T24 cell proliferation was observed with kawain treatment. On the other hand, compared with 0.1% DMSO-treated controls, flavokawain A, B, and C at a dose of 25 μg/mL caused 80% to 95% inhibition of RT4, T24, and EJ cell proliferation (Ps < 0.0001, Student's *t* test). The IC_{50}s for flavokawain A, B, and C in T24 cells were estimated to be 16.7, 6.7, and 10.6 μmol/L, respectively. The IC_{50}s in EJ cells were 17.2 μmol/L for flavokawain A, 5.7 μmol/L for flavokawain B, and 14.6 μmol/L for flavokawain C. In RT4 cells, the IC_{50}s for flavokawain A, B, and C were 20.8, 15.7, and 4.6 μmol/L, respectively.

As shown in Supplementary Fig. S1, all of the cells treated by either flavokawain A, B, and C or kava extract show typical apoptotic morphologies, including cell shrinkage and rounding up, cell membrane blebbing, as well as nuclear fragmentation and condensation. However, none of the kawain-treated cells shows a difference from the 0.1% DMSO-treated cells (Supplementary Fig. S1). The apoptotic effect of flavokawain A on T24 cells was further confirmed by Annexin V and propidium iodide staining (Supplementary Fig. S2A and B). These results suggested that their inhibitory effect on cell growth might be through induction of apoptosis.

Flavokawain A results in cleavage of caspase-3/9 and poly(ADP-ribose) polymerase in T24 cells in a dose- and time-dependent manner. To see if the apoptotic effect of flavokawain A is through activation of a cascade of caspases, the cleavage of caspase-9, caspase-3, and PARP were detected by Western blot. Treatment of T24 cells with flavokawain A caused activation of caspase-9 in a dose- and time-dependent manner, as indicated by reduction in the intensity of the proenzyme, and the cleaved product of caspase-9 (Fig. 2*A*, *a* and *b*). Activation of caspase-3 was detected after flavokawain A treatment as a double band representing the p19 proteolytic fragment, and the active subunit p17, respectively (Fig. 2*A*, *c*). Similar results were observed

in PARP cleavage (Fig. 2*A*, *d* and *e*). However, the major kavalactones, including kawain, methysticin, and yangonin, at doses of 40 and 80 μg/mL did not induce a detectable cleavage of PARP and caspase-3 in T24 cells (Supplementary Fig. S2C), further indicating the specificity of the apoptotic effect of flavokawain A (versus kavalactones) in bladder cancer cells.

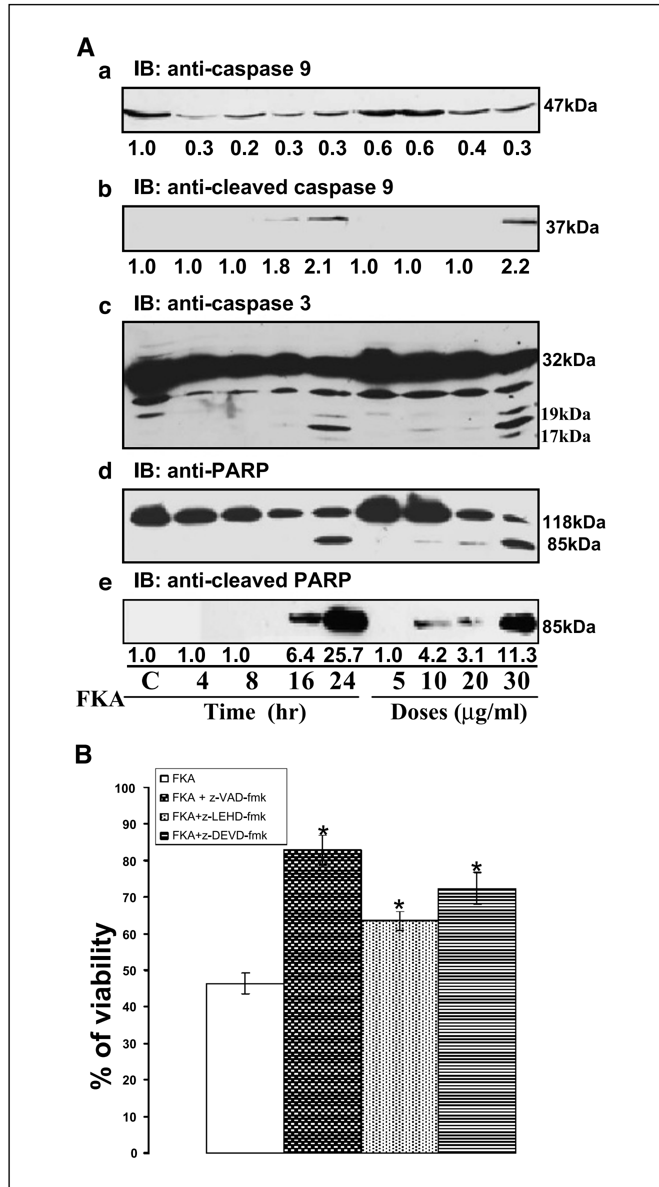

Figure 2. The apoptotic effect of flavokawain A is related to caspase-9/3-mediated pathway. *A,* T24 cells were cultured in McCoy's 5A medium containing 10% FBS. At 70% to 80% confluence, the cells were treated with either 0.1% DMSO (*C*) for 24 hours; 12.5 μg/mL flavokawain A for 8, 16, and 24 hours; or 5, 10, and 30 μg/mL flavokawain A for 12 hours. Whole cell lysates from the indicated treatments were prepared and Western blots were done as described in Materials and Methods. Representative of three independent experiments. *Numbers at the bottom,* change in protein expression of the bands normalized to β-actin. *B,* 5×10^4 T24 cells were plated in 24-well plates. After 70% to 80% confluence in the presence of 10% FBS condition, cells were pretreated with 0.1% DMSO, caspase-3 inhibitor II (z-DEVD-fmk), caspase-9 inhibitor I (z-LEHD-fmk), or caspase inhibitor I (z-VAD-fmk) each at 150 μmol/L for 1 hour followed by 0.1% DMSO or 12.5 μg/mL flavokawain A for an additional 24 hours. Viability of cells was measured by MTT assays. *Columns,* mean of four independent plates; *bars,* SE. Each sample was counted in duplicate. *, $P < 0.05$; **, $P < 0.01$, flavokawain A versus flavokawain A + inhibitors.

Pretreatment with a broad-spectrum caspase inhibitor (z-VAD-fmk), a caspase-9 specific inhibitor (z-LEHD-fmk), and a caspase-3 specific inhibitor (z-DEVD-fmk) significantly reduced the ability of flavokawain A to induce cell death in T24 cells (flavokawain A versus flavokawain A plus inhibitors; all *P*s < 0.05, Student's *t* test; Fig. 2*B*). Cell death was measured by MTT assay after 24 hours of incubation with 12.5 μg/mL flavokawain A and with or without 1-hour pretreatment with the selected caspase inhibitors each at 150 μmol/L. The caspase inhibitors alone did not cause any significant changes in cell viabilities (data not shown).

The loss of mitochondrial membrane potential and release of cytochrome *c* caused by flavokawain A are associated with an increase in Bax/Bcl-x_L ratio and Bax confirmation change in T24 cells. The cleavage of caspase-9/3 by flavokawain A suggests that its apoptotic effect involves the mitochondrial apoptotic pathway. The lipophilic cation JC-1 was used to detect the alterations of mitochondrial membrane potential in T24 cells. JC-1 is mitochondria selective and forms aggregates in normal polarized mitochondria that result in a green-orange emission of 590 nm after excitation at 490 nm (21). However, the monomeric form present in cells with depolarized mitochondrial membranes emits only green fluorescence at 527 nm (21). T24 cells were treated with 0.1% DMSO or 12.5 μg/mL flavokawain A for 8, 16, and 24 hours, stained with JC-1, and analyzed by flow cytometry. Figure 3*A* shows an increase in the percentage of cells (*bottom right quadrant*) that emitted only green fluorescence after flavokawain A treatment for 16 hours (0.1% DMSO 24 hours 0.3% versus flavokawain A 16 hours 11.8%; $P < 0.01$, Student's *t* test). This increased population represents cells with depolarized mitochondrial membranes. The number of cells with loss of mitochondrial membrane potential further increased up to 21.1% after the treatment for 24 hours (0.1% DMSO 24 hours versus flavokawain A 24 hours; $P < 0.01$, Student's *t* test). The loss of mitochondrial membrane potential will lead to the release of cytochrome *c* from mitochondria to cytosol. Consistent with the above results, a significant release of cytochrome *c* from mitochondria into cytosol in T24 cells by 12.5 μg/mL flavokawain A treatment was seen at 16 hours (Fig. 3*B*).

Bax triggers cytochrome *c* release, whereas Bcl-2 and Bcl-x_L inhibit it (27). Compared with 0.1% DMSO control, treatment of T24 cells with 12.5 μg/mL flavokawain A for 8, 16, and 24 hours results in a slight increase of Bax protein level by 54%, 64%, and 88%, respectively, but a significant decrease of Bcl-x_L protein levels by 41%, 65%, and 71%, determined by densitometry and adjusted by β-actin levels. The corresponding ratios of Bax and Bcl-x_L levels were then calculated as an increase by 59%, 166%, 431%, and 650%, respectively (Fig. 4*A*). Under the identical treatment conditions, this increase in the ratio of Bax and Bcl-x_L by flavokawain A is accompanied by a highly significant to complete inhibition of the formation of Bax and Bcl-x_L immunocomplexes (Fig. 4*B*, *Ab* denotes light and heavy chains of immunoprotein IgG). The decrease in the immunocomplexes of Bax and Bcl-x_L then leads to increased appearance of active Bax protein in bladder cancer cells as early as 8 hours after flavokawain A treatment (2- to 3.1-fold increase relative to control determined by densitometry and normalized by β-actin levels; Fig. 4*C*). It seems that Bax lost its ability to heterodimerize with Bcl-x_L and was converted to its active form that was recognized by anti-Bax 6A7 antibody during the flavokawain A treatment (23).

The antiproliferative effect of flavokawain A on primary mouse embryo fibroblasts requires Bax protein, and Bax inhibitory peptide P5 attenuates the antiproliferative effect of flavokawain A on T24 cells. We next determined if Bax protein

plays a crucial role in the antiproliferative and apoptotic effect of flavokawain A. Primary MEFs, wild-type and $Bax^{-/-}$, were treated by different doses of flavokawain A for 48 hours. Wild-type MEFs were less sensitive to antiproliferative effect of flavokawain A than bladder cancer cells (e.g., IC_{50}, 43 μmol/L for wild-type MEFs versus 16.7 μmol/L for T24 cells) but more sensitive than $Bax^{-/-}$ MEFs (Fig. 4*D*). Disruption of Bax almost completely compromised flavokawain A–inducing apoptosis in the MEFs (Fig. 4*E*).

Bax-inhibiting peptide derived from the Bax-binding domain of Ku70 was used to inhibit the mitochondrial translocation of Bax (21). We pretreated T24 cells with 100 μmol/L Bax-inhibiting P5 or negative control peptides for 1 hour and then with 12.5 μg/mL flavokawain A for 24 hours. As shown in Fig. 4*F*, the Bax-inhibiting peptide P5 significantly attenuated the antiproliferative effect of flavokawain A by 33% ($P < 0.05$, Student's t test), but the negative control peptide did not ($P > 0.05$, Student's t test). This result suggests that the effect of flavokawain A on T24 cells may be involved in the factors affecting Bax translocation.

Flavokawain A remarkably decreases the levels of X-linked inhibitor of apoptosis and survivin in T24 cells. XIAP and survivin are two factors in maintaining apoptosis resistance in cells (28–30). Compared with 0.1% DMSO-treated control, treatment of T24 cells with flavokawain A results in a significant dose- and time-dependent down-regulation of survivin and XIAP (Fig. 5). The protein expression of survivin was completely inhibited by 12.5 μg/mL flavokawain A treatment for 4 hours (Fig. 5). Complete inhibition was seen at 16 hours in terms of XIAP (Fig. 5). In addition, we attempted to examine if the effect of flavokawain A on XIAP and survivin is associated with Smac/DIABLO protein. However, expression of Smac/DIABLO in T24 cells is under the detectable limit of Western blotting (data not shown).

Flavokawain A inhibits anchorage-independent growth of EJ cells in soft agar and tumor growth in nude mice. EJ cell line was thought to be the derivative of T24 cells (31). However, EJ but not T24 cells can grow in soft agar and nude mice. Figure 6*A* shows that flavokawain A inhibits anchorage-independent growth of EJ cells in a dose-dependent manner. As many as 118.46 ± 11.2 (mean ± SE of four wells) colonies per 3,000 cells per well were counted in 0.1% DMSO-treated controls (data not shown). Compared with control, treatment with flavokawain A at 5, 12.5, and 25 μg/mL resulted in 44.9%, 75.6%, and 86.9% inhibition, respectively (all Ps < 0.05-0.01, Student's t test; Fig. 6*A*). Treatment with 50 μg/mL flavokawain A completely inhibited colony formation (Fig. 6*A*).

Finally, we examined whether flavokawain A can suppress the tumor growth in an *in vivo* bladder tumor xenograft model. We chose oral administration of 50 mg/kg flavokawain A daily for 25 days. Figure 6*B* showed a progressive tumor xenograft growth during the entire study in vehicle control group (10% grain alcohol in 0.9% saline). The flavokawain A treatment, however, resulted in a clear separation in tumor growth curves showing decreased rate of tumor growth compared with control group throughout the study. The wet tumor weights in control and flavokawain A–treated group recorded at the end of the treatment are 465 ± 217 and 199 ± 110 mg, respectively (mean ± SD; $P < 0.001$, Student's t test). Flavokawain A treatment attenuated tumor growth by 57%. The body weight gain and diet and water consumption of the flavokawain A–treated mice were similar to the control group of mice. In addition, the mice did not show any gross abnormalities on necropsy at the end of the treatment.

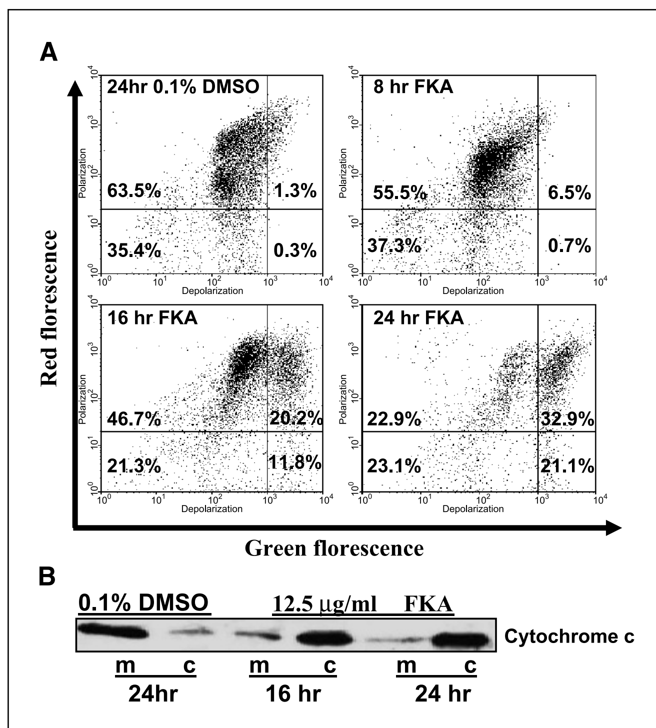

Figure 3. Flavokawain A induces loss of mitochondrial membrane potential and release of cytochrome *c*. T24 cells at 70% to 80% confluence were treated with either 0.1% DMSO for 24 hours or 12.5 μg/mL flavokawain A for 8, 16, and 24 hours under 10% FBS. *A,* cells were stained by JC-1 probe and analyzed by flow cytometry as described in Materials and Methods. Different treatments were done for each case. *Numbers in the bottom right quadrant,* percentage of cells that emit only green fluorescence attributable to depolarized mitochondrial membranes. *B,* mitochondria (*m*) and cytosolic (*c*) extracts from indicated treatments were prepared and Western blots were done as described in Materials and Methods. Representative of three independent experiments.

Discussion

Mutations or altered expression of p53, Fas, death receptor 4, Bax, CD40L, and survivin are frequently observed in human bladder cancer (32–35), suggesting that deregulation of apoptotic pathways may be important for the carcinogenesis and neoplastic progression of human urinary bladder cancer. In addition, apoptosis is considered as a physiologic process to remove DNA-damaged cells with minimal damage to surrounding normal cells or tissue. Novel apoptosis inducers, therefore, would provide more effective therapy and prevention against bladder cancer.

Flavonoids, a group of ~5,000 naturally occurring compounds, have long been known to function as defense compounds in protecting the seeds and roots of plants from insects, bacteria, fungi, nematodes, and alien plants (36, 37). Because Bcl-2 family proteins are not present in plants, certain flavonoids that directly bind to Bcl-2 family proteins may act as natural pesticides selectively to induce cell death in insects and nematodes that are harmful for plants (38). Such functionalities in induction of apoptosis might be intrinsic to certain flavonoids found in edible plants (e.g., epigallocatechin gallate) for their mechanisms of anticarcinogenesis (38, 39). Compared with other classes within the flavonoid family, there are unique α,β-unsaturated ketones with the chemical structure of chalcones (e.g., flavokawains). Therefore, our results have identified flavokawains as a new class of flavonoids for inducing apoptosis in human bladder cancer cells. Further investigation of the underlying mechanisms of flavokawain A–inducing apoptosis is warranted.

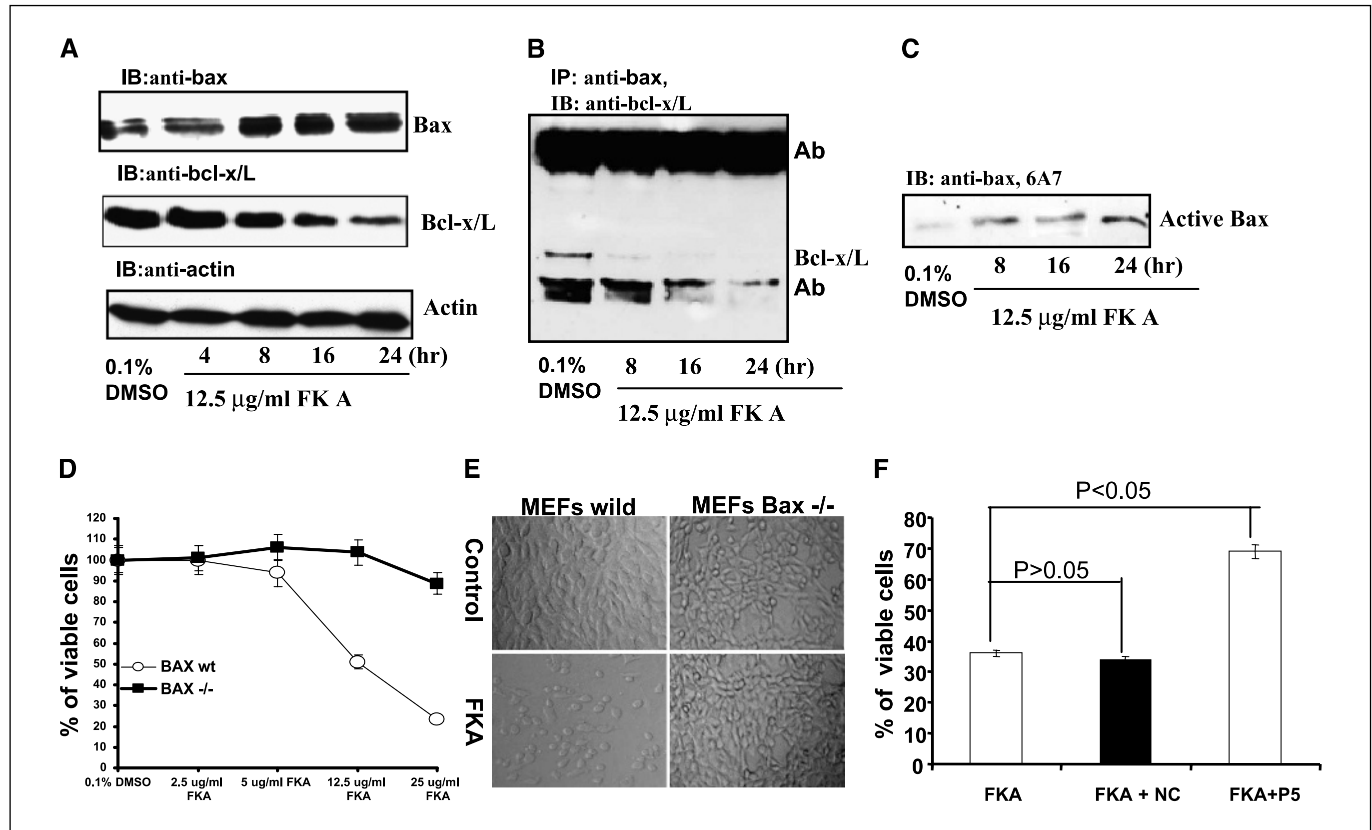

Figure 4. The apoptotic effect of flavokawain A is critically involved in change of Bax protein. T24 cells were treated as indicated and then lysed in radioimmunoprecipitation assay buffer (*A*) or CHAP lysis buffer (*B* and *C*). *A,* protein levels of Bax, Bcl-x_L, and actin were measured by Western blotting analysis. *B,* binding of Bax to Bcl-x_L was measured by immunoprecipitation of cell lysates by anti-Bax and then Western blotting analysis of immunoprecipitates by anti-Bcl-x_L. *C,* Bax activation was detected by conformation-specific anti-Bax 6A7 antibody. Representative of three independent experiments. *D,* primary MEFs, wild-type and $Bax^{-/-}$, at 80% to 90% confluency were treated with 0.1% DMSO or indicated doses of flavokawain A for 48 hours. Cell viabilities were measured by MTT assays. *Points,* mean of four independent plates; *bars,* SE. Each sample was counted in duplicate. *E,* MEFs, wild-type and $Bax^{-/-}$, at 70% to 80% confluence were treated with either 0.1% DMSO or 12.5 μg/mL flavokawain A for 24 hours. Magnification under normal light, ×100. *F,* 5×10^4 T24 cells were plated in 24-well plates. After 24 hours of attachment, cells were pretreated with 0.1% DMSO or 100 μmol/L Bax-inhibiting P5 or negative control peptides for 1 hour and then treated with or without 12.5 μg/mL flavokawain A for 24 hours. Cell viabilities were measured by MTT assays. *Columns,* mean of four independent plates; *bars,* SE. Each sample was counted in duplicate.

To this end, we searched backward to identify possible molecular steps for flavokawain A–inducing apoptosis. We have traced from PARP cleavage and activation of caspase-9/3 to loss of mitochondrial membrane potential and release of cytochrome *c*. Finally, we found that flavokawain A resulted in a decrease in formation of Bax and Bcl-x_L heterodimer and conversion of Bax protein to its active form. By using a model system consisting of primary MEFs, wild-type and $Bax^{-/-}$, we showed that Bax protein was critically involved in flavokawain A–inducing apoptosis. Bax constitutes a requisite gateway to most, if not all, the mitochondrial-dependent apoptosis (26). Bcl-x_L is the only Bcl-2 family protein specifically targeted to the mitochondrial outer membrane (40). The mitochondria permeability and release of cytochrome *c* are fine tuned by the balance between Bax and Bcl-x_L (26). However, the exact mechanisms of how cytochrome *c* are released from mitochondria remain elusive (41–43).

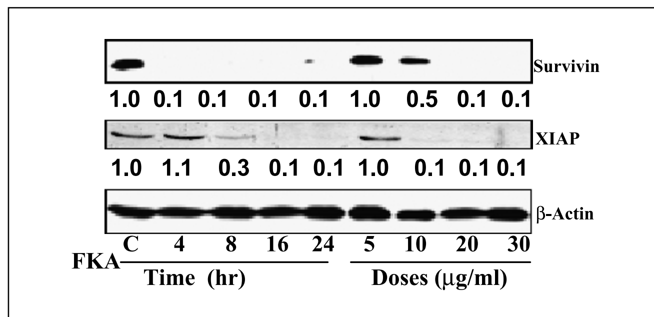

Figure 5. Flavokawain A down-regulates XIAP and survivin protein. T24 cell lysates from the identical treatments as described in Fig. 2*A* were obtained and subjected to Western blotting analyses using antibodies against XIAP and survivin. Representative of three independent experiments. *Numbers at the bottom,* change in protein expression of the bands normalized to β-actin.

Overall, Bax activation seems to play a central role in the mitochondria-dependent apoptosis. The BH3-only proteins, including Bad, Bim/Bod, Bid, Bmf, Bik/NBK, BNIP3/NIX, Blk, Hrk, NIP3, Noxa, and PUMA, are immediate upstream triggers for Bax activation either directly or indirectly by sequestration of anti-apoptotic Bcl-2 and Bcl-x_L proteins (27, 41). In addition, Bax activation is regulated by p53 via a transcription-dependent and/or transcription-independent mechanism when p53 acts similarly as BH3-only proteins (44). RT4 cells harboring wild-type p53 is less sensitive to the apoptotic effect of flavokawain A than T24 cells with p53 mutation, suggesting that the activation of Bax protein by flavokawain A in T24 cells may be p53 independent. DNA repair factor Ku70, Bax inhibitor-1, and humanin are physiologic suppressors for Bax translocation (21, 45, 46). We showed that the peptide derived from the Bax-binding domain of Ku70 (21)

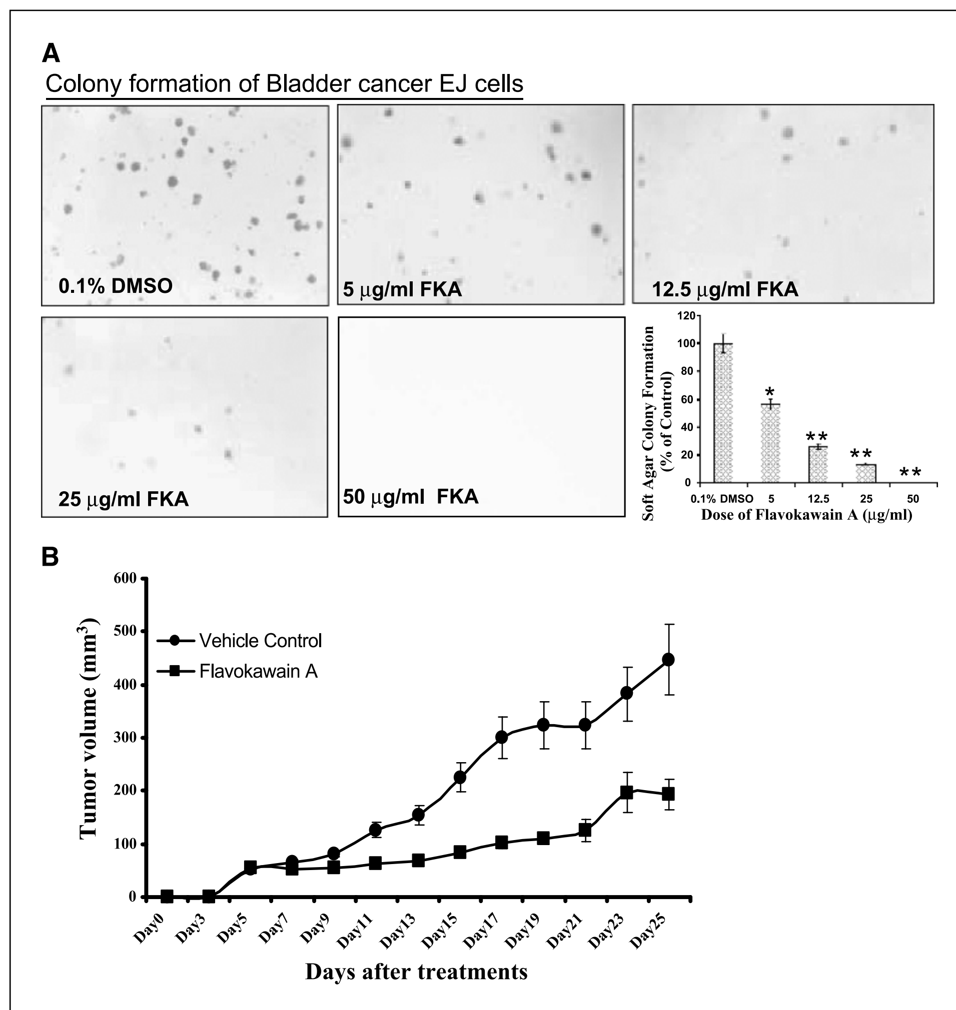

Figure 6. Flavokawain A inhibits EJ cell colony formation in soft agar and EJ tumor growth in mice. *A,* EJ cells were grown in soft agar in six-well plates as described in Materials and Methods and treated with 0.1% DMSO or flavokawain A at the indicated doses for 14 days. The number of colonies was determined by counting them under an inverted phase-contrast microscope at ×100 magnification; a group of >10 cells was counted as a colony. *Columns,* mean of four independent wells at an optimum time of 14 days after the start of cell seeding; *bars,* SE. Qualitative analysis of soft agar colony formation and quantitative analysis of dose-dependent inhibitory effect of flavokawain A against soft agar colony formation of EJ cells. *, $P < 0.05$; **, $P < 0.01$, control versus treatments. *B,* EJ tumor cells (2×10^6) were injected into the right flank of NCR-*nu/nu* (nude) mice. Next day, the mice were randomly divided and pair matched into treatment and control groups of 18 mice each, and daily dosing was begun with vehicle or 50 mg/kg flavokawain A. *Points,* mean tumor volumes; *bars,* SE.

significantly attenuated the apoptotic effect of flavokawain A on T24 cells. This result suggests that flavokawain A–inducing apoptosis may be associated with the change of the factors affecting Bax protein translocation. Therefore, further studies are needed to identify upstream triggers for Bax translocation and activation by flavokawain A.

Nevertheless, the mechanism of flavokawain A–inducing apoptosis in T24 cells may not limit to BH3-only protein-mediated Bax activation as discussed above. Whereas the activation of Bax and caspase-3/9 is critical for initiation and amplification of the mitochondria-dependent apoptosis, respectively, their activation is normally blocked by Bcl2 and Bcl-x_L at the mitochondrial checkpoint or inhibitors of apoptosis proteins (e.g., survivin and XIAP) at caspases levels. XIAP inhibits both caspase-9 and caspase-3 (28). Although the crystal structure of survivin did not reveal the similar binding sites of other inhibitors of apoptosis proteins with caspases, the antiapoptotic function of survivin is well established (29). Interestingly, recent data showed that depletion of survivin in melanoma cells initiated mitochondrial events before caspase activation and apoptosis (30). Our results showed that a depletion of survivin by flavokawain A in T24 cells occurred before loss of the mitochondria potential and caspase cleavage, as early as 4 hours after treatment. Therefore, in addition to Bax activation, depletion of survivin may also serve an initiation factor for flavokawain A–inducing apoptosis.

XIAP, survivin, and Bcl-x_L are predominant factors for apoptosis resistance and subject to transcriptional and translational controls as well as post-translational modification (29, 47, 48). It is tempting to speculate that the multilayers and complexities in regulation of these antiapoptotic proteins make them more likely to be dysregulated during carcinogenesis. Indeed, survivin is undetectable or present at low levels in normal tissues but becomes dramatically overexpressed in cancer in response to oncogene activation, loss of p53, or deregulated transcription of Wnt pathways (29). XIAP expression was minimally detected on superficial layer cells of normal urothelium but was up-regulated in bladder tumors (49). Given that many tumor cell lines and tissues are the coexistence of activated apoptotic signaling (existence of active caspases) and high levels of antiapoptotic proteins compared with normal cells, the antiapoptotic proteins may represent more important targets for cancer prevention and therapy (50). In our study, the down-regulation of antiapoptotic proteins, such as XIAP, survivin, and Bcl-x_L, by flavokawain A would change the balance between apoptotic and antiapoptotic molecules and then induce cell death in tumor cells.

In summary, we have identified novel apoptosis inducers and anticarcinogenic agents, flavokawains, from kava extracts that have been commonly used by the South Pacific Islanders for thousands of years. We have the evidence that the mechanisms of flavokawain A–inducing apoptosis are, at least in part,

through both the Bax-dependent, mitochondrial pathway and down-regulation of antiapoptotic proteins, including Bcl-x_L, XIAP, and survivin. Upstream triggers for Bax activation and/or additional initiators for flavokawain A–inducing apoptosis remain to be elucidated. Our findings should encourage not only the development of more potent chalcone derivatives for prevention and treatment of bladder cancer but also epidemiologic study of the relationship between kava consumption and cancer.

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