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Effects of the Kava Chalcone Flavokawain A Differ in Bladder Cancer Cells with Wild-type versus Mutant p53

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Abstract

Flavokawain A is the predominant chalcone from kava extract. We have assessed the mechanisms of flavokawain A's action on cell cycle regulation. In a p53 wild-type, low-grade, and papillary bladder cancer cell line (RT4), flavokawain A increased p21/WAF1 and p27/KIP1, which resulted in a decrease in cyclin-dependent kinase-2 (CDK2) kinase activity and subsequent G₁ arrest. The increase of p21/WAF1 protein corresponded to an increased mRNA level, whereas p27/KIP1 accumulation was associated with the down-regulation of SKP2 and then increased the stability of the p27/KIP1 protein. The accumulation of p21/WAF1 and p27/KIP1 was independent of cell cycle position and thus not a result of the cell cycle arrest. In contrast, flavokawain A induced a G₂-M arrest in six p53 mutant-type, high-grade bladder cancer cell lines (T24, UMUC3, TCCSUP, 5637, HT1376, and HT1197). Flavokawain A significantly reduced the expression of CDK1-inhibitory kinases, Myt1 and Wee1, and caused cyclin B1 protein accumulation leading to CDK1 activation in T24 cells. Suppression of p53 expression by small interfering RNA in RT4 cells restored Cdc25C expression and down-regulated p21/WAF1 expression, which allowed Cdc25C and CDK1 activation and then led to a G₂-M arrest and an enhanced growth-inhibitory effect by flavokawain A. Consistently, flavokawain A also caused a pronounced CDK1 activation and G₂-M arrest in p53 knockout but not in p53 wild-type HCT116 cells. This selectivity of flavokawain A for inducing a G₂-M arrest in p53-defective cells deserves further investigation as a new mechanism for the prevention and treatment of bladder cancer.

Bladder cancer is a major public health burden. Tumor resection with possible intravesical treatments for superficial disease, and cystectomy or chemotherapy with radiation protocols for invasive bladder cancer have associated limitations. Despite improvement in treatments, patients with advanced bladder cancer still have a significant risk of mortality. Therefore, different and novel approaches need to be considered.

Carcinogens such as cigarette smoking and occupational exposure to aromatic amines contribute to >75% of all bladder cancer cases in the United States (1). However, there are many potentially protective phytochemicals contained in plant-derived food that are excreted through the urinary tract and concentrated in urine (2). For example, urinary levels of the free form of genistein can be 50 µmol/L or higher, levels at which genistein exhibits effective anticancer activities *in vitro* (3). Thus, investigation of these phytochemicals for prevention

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and therapeutic intervention against urinary bladder cancer is plausible. Moreover, prevention and therapeutic intervention by using well-studied, effective phytochemicals could be a newer approach with potentially fewer side effects and, if targeted correctly, more efficacy as compared with current chemotherapeutic agents in cancer management.

Consumption of the traditional kava tea preparation, predominately by men, has been reported to correlate with low and uncustomary gender ratios of cancer incidences (more cancer in women than men) in three kava-drinking countries: Fiji, Vanuatu, and Western Samoa, despite the presence of many smokers in these populations (4–7). We have shown that flavokawain A, the major chalcone in kava extracts, exhibits strong antiproliferative and apoptotic effects against human urinary bladder cancer cell lines derived from different stages of bladder cancer (8). In this study, we have shown that flavokawain A differentially induced G₁ and G₂-M arrests in p53 wild-type RT4 cells and p53 mutant-type T24, UMUC3, TCCSUP, 5637, HT1376, and HT1197, respectively, as the mechanisms for its cell growth-inhibitory effect. In p53 wild-type RT4 cells, flavokawain A inhibits cyclin-dependent kinase-2 (CDK2) kinase activity through the accumulation of p21/WAF1 (p21) and p27/KIP1 (p27), and down-regulation of SKP2 and CDK2. In p53 mutant-type T24 cells, flavokawain A activates CDK1 kinase activity via decreasing CDK1 inhibitory kinases Myt1 and Wee1 and increasing cyclin B1. Interestingly, flavokawain A is more effective in inhibiting the growth of p53-defective bladder cancer cells via mechanisms of down-regulation of Myt1 and Wee1 and dephosphorylation of Cdc25C leading to CDK1 activation.

Materials and Methods

Cell Lines, Compounds, and Reagents

The RT4, T24, UMUC3, HT1376, 5637, TCCSUP, and HT1197 cell lines were obtained from American Type Culture Collection. RT4 and T24 cells were maintained in McCoy's 5A medium containing 10% fetal bovine serum. UMUC3, HT1376, TCCSUP, and HT1197 cells were cultured in EMEM medium with 10% fetal bovine serum. 5637 cells were cultured in RPMI 1640 with 10% fetal bovine serum. p53 knockout colon cancer HCT116 cells and their wild-type isogenic cell line were a generous gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) and cultured in McCoy's 5A medium. Pure flavokawain A (99%) was isolated from kava extracts by LKT Laboratories, Inc. (St. Paul, MN). Flavokawain A was dissolved in DMSO, aliquoted, and stored at –80°C. The DMSO in culture medium never exceeded 0.1% (v/v), a concentration known to not affect cell proliferation. Antibodies for CDK2, Cdc25C, Cdc25A, Cdc25B, Wee1, Myt1, CHK1, CHK2, phosphorylated CHK1 at Ser³¹⁷ and phosphorylated CHK2 at Thr⁶⁸, phosphorylated Cdc25C at Ser²¹⁶ and phosphorylated CDK1 at Tyr¹⁵ were from Cell Signaling Technology, Inc. Cyclin D1, cyclin B1, CDK1, and β -actin were from Santa Cruz Biotechnology, Inc. Cyclin E antibody was from BD Biosciences, Inc. The antibody for MPM-2 was from Upstate Biotechnology. Anti-p27/Kip1 antibody was from Neomarkers. Anti-p21/WAF1 antibody and CHK2 inhibitor II were purchased from Calbiochem, Inc. Anti-SKP2 was from Zymed, Inc. Histone H1 was from Boehringer Mannheim, Corp. Protein A/G-plus agarose and protein A-plus agarose beads were from Santa Cruz Biotechnology, Inc., [γ -³²P]ATP (specific activity, 3,000 Ci/mmol) and enhanced chemiluminescence detection system were from Amersham Corporation. Thymidine, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), cycloheximide, and propidium iodide were from Sigma. RNAazol B was purchased from Tel-Test. The reverse transcription system kit was from Promega. The small interfering RNA (siRNA) suppression kit for p53 was purchased from Cell Signaling Technology, Inc.

MTT Assay

RT4, T24, UMUC3, HT1376, 5637, TCCSUP, and HT1197 cells were plated at a density of 2×10^4 cells per well in 24-well culture plates in medium containing 10% fetal bovine serum. After 24 h, fresh medium was introduced and left untreated or was treated as indicated in the figure or table legends. After treatment, MTT was added to the wells at a final concentration of 1 mg/mL and incubated at 37°C for 3 h. The absorbance was determined at 570 nm.

Fluorescence-Activated Cell Sorting Analysis of Cell Cycle Distribution

RT4, T24, UMUC3, HT1376, 5637, TCCSUP, and HT1197 cells at 70% to 80% confluency were treated with 0.1% DMSO or 4, 8, 16, and 40 μ mol/L of flavokawain A for 4, 8, 16, and 24 h or with 1 μ mol/L of paclitaxol or 50 ng/mL of nocodazole for 24 h. After treatment, cells were fixed in ice-cold 70% ethanol overnight. After fixation, cells were washed thrice with cold PBS and then stained in 500 μ L of propidium iodide solution. Samples were analyzed on a BD FACScan flow cytometer and the percentage of cells in the S, G₀-G₁, and G₂-M phases of the cell cycle was determined.

P27 and p21 Degradation Assay

RT4 cells were cultured in the presence or absence of flavokawain A for 24 h. The following day, cycloheximide was added to the culture medium (final concentration, 100 μ mol/L) to inhibit protein synthesis. At preset times following the addition of cycloheximide, cells were lysed, and p27 and p21 protein levels were determined by Western blot analysis and semiquantified by densitometry.

Real-time Reverse Transcription-PCR

Total RNA was isolated from RT4 cells using the RNAazol B method as previously described (9). Real-time quantitative PCR amplification reactions for *SKP2*, *p21*, and *p27* mRNA levels were carried out using MyiQ system (Bio-Rad) as described previously (10); the sequences of the primer sets used are available upon request. Data were analyzed by using the comparative C_t method, where C_t is the cycle number at which fluorescence first exceeds the threshold. The C_t values from each sample were obtained by subtracting the values for β -actin C_t from the *Skp2*, *p21*, and *p27* C_t values. The variation of β -actin C_t values was <0.5 among different samples. One difference of C_t value represents a 2-fold difference in the level of mRNA. The specificity of the resulting PCR products was confirmed by melting curves.

Western Blot Analysis

Cells were treated with 0.1% DMSO or flavokawain A under each experimental condition. After treatment, clarified protein lysates (20–80 μ g) were denatured at 95°C for 5 min and resolved by 8% to 16% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and probed with antibodies and visualized by an enhanced chemiluminescence detection system.

Immunoprecipitation and Kinase Assay

Total protein (200 μ g) was precleared with protein G plus-agarose and then precipitated with 2 μ g of anti-CDK2 (M2) or anti-CDK1 antibody overnight at 4°C. Agarose beads were washed four times with lysis buffer and resuspended in SDS-PAGE 2 $\times$ sample buffer. Proteins were eluted by boiling the beads and subjected to immunoblotting.

For H1 kinase assays, 100 to 300 μ g protein lysates were incubated by rotating end over end with anti-CDK2 or anti-CDK1 antibodies overnight at 4°C and incubated for 1 h at 4°C with protein G-agarose or protein A-agarose. Beads were washed twice with lysis buffer and twice with kinase buffer and incubated in kinase buffer containing 1 μ g of histone H1. The kinase

reaction was done for 30 min at 37°C in the presence of 10 µCi of [γ -³²P]ATP with 1 µg of histone H1 as substrate. Labeled proteins were resolved on 12% SDS-PAGE and subjected to autoradiography. The standard Coomassie brilliant blue assay for staining of proteins in gels was used to show equal loading of the kinase substrate histone H1.

SiRNA Suppression of p53

RT4 cells were plated in 24-well plates with complete medium. RT4 cells at 50% confluency were transfected with commercially available siRNA for control or p53 from Cell Signaling according to the manufacturer's instructions. After 24 h of transfection, the transfected RT4 cells were treated with 16 µmol/L of flavokawain A for 24 h. Cell viabilities were determined by MTT assay as described above. Expression of wild-type p53 (p53wt) and nontargeted mitogen-activated protein kinase proteins in siRNA control or siRNA p53-transfected RT4 cells was determined by Western blotting analysis.

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. Papillary RT4 bladder tumor cells were concentrated to 2×10^6 per 100 µL, mixed with an equal volume of Matrigel and injected s.c. into the right flank of each mouse. The next day, mice were randomly divided and pair-matched into treatment and control groups of 10 mice each, and daily dosing was begun with vehicle or 50 mg/kg of flavokawain A. Body weight, diet, and water consumption were recorded thrice weekly throughout the study. Once xenografts started growing, their sizes were measured every 4 days. 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 the experiment, tumors were excised, weighed, and blood was collected and stored at -80°C until additional analysis.

Statistics

Comparisons of cell cycle population 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

Flavokawain A Differentially Induced G₁ and G₂-M Arrests in Bladder Cancer Cell Lines

Table 1 showed that flavokawain A inhibited the growth of human papillary (RT4), muscle-invasive (T24, UMUC3, HT1376, 5637, and HT1197) bladder cancer cell lines, and a bone metastatic bladder cancer TCCSUP cell line. The RT4 cell line was derived from recurrent, superficial bladder tumor. T24, UMUC3, HT1376, 5637, and HT1197 cell lines were derived from different stages (T2–T4) of muscle-invasive urinary bladder tumor samples. These cell lines are characterized by different p53, pRB, and INK4A status (11). RT4 cells have wild-type p53 but a homozygous INK4A and lack pRB expression. T24 and UMUC-3 cells harbor TP53 mutations in the NH₂-terminal transactivation domain and INK4A mutations with high pRB levels. HT1376 and 5637 cells contain TP53 mutations in the core domain, undetectable pRB levels, and wild-type INK4A. TCCSUP and HT1197 cells hold TP53 mutations in the tetramerization domain, undetectable pRB levels, and wild-type INK4A. The IC₅₀s of flavokawain A treatment of RT4, UMUC-3, T24, HT1376, 5637, TCCSUP, and HT1197 cells for 48 hours were estimated to be 20.8, 17.7, 16.7, 14.7, 13.1, 10.55, and 7.9 µmol/L, respectively (Table 1). Bladder cancer cell lines with TP53 mutations and undetectable pRB

levels were more sensitive to the growth-inhibitory effect of flavokawain A. Notably, among all the tested cell lines, HT1197 and TCCSUP cell lines harboring TP53 mutations in the tetramerization domain were the most sensitive to flavokawain A treatment.

To examine whether the cell growth-inhibitory effects of flavokawain A are induced via perturbation in cell cycle progression, we performed fluorescence-activated cell sorting analysis of control (0.1% DMSO) and flavokawain A-treated cells grown in 10% fetal bovine serum. Figure 1A indicated a G₁ arrest in p53 wild-type RT4 cells treated with flavokawain A (G₁ population, 50% for control versus 74% for flavokawain A at 24 hours of treatments; Student's *t* test, *P* < 0.01). For p53 mutant bladder cancer cell lines (T24, UMUC-3, TCCSUP, and HT1197), flavokawain A at the same concentrations induced a significant G₂-M arrest (G₂-M population, 30–39% for control versus 51–82% for flavokawain A at 24 hours of treatments; Student's *t* test, *P* < 0.01). However, paclitaxol at a concentration of 1 μmol/L and 50 ng/mL of nocodazole for 24 hours caused a significant accumulation in G₂-M population in both p53 wild-type and mutant-type bladder cancer cells (Student's *t* test, *P* < 0.01). These results clearly showed that flavokawain A was a novel agent differentially inducing G₁ in p53 wild-type and G₂-M arrests in mutant-type bladder cancer cell lines. In addition, fluorescence-activated cell sorting analysis revealed that there was a cell population with >4 N in both p53 wild-type and mutant-type bladder cancer cells after flavokawain A treatment. 4',6-Diamino-2-phenylindole staining of the nuclei further showed that a portion of flavokawain A-treated bladder cancer cells became multinucleated (data not shown), suggesting that these cells exit mitosis without division.

The accumulation in G₁ population by 40 μmol/L of flavokawain A treatment over different time periods (from 4 to 24 h) was accompanied by a decrease of cells in both S and G₂-M phases (Fig. 1B, *right*). In contrast, control treatment of p53 wild-type RT4 cells caused a slight decrease of the G₁ population in cell cycle progression over a period of 24 hours (Fig. 1B, *left*). For p53 mutant T24 cells, flavokawain A at the same concentration induced a marked G₂-M arrest in cell cycle progression over a period of 24 hours (Fig. 1B). The G₁ in RT4 and G₂-M arrest in T24 cells by flavokawain A were also found at other concentrations (e.g., 4, 8, and 16 μmol/L) of flavokawain A (data not shown). Western blotting analysis by using antibody MPM-2 against mitosis-specific phosphoepitopes suggested the specificity of flavokawain A for inducing M phase arrest in T24 but not in RT4 cells (Fig. 1C, *left* versus *right*). However, paclitaxol and nocodazole, which were used as positive controls for the induction of M phase arrest, increased the expression of mitosis-specific phosphorylated proteins in both p53 wild-type RT4 and mutant-type T24 cells (Fig. 1C). The MPM-2 antibody is a specific antibody for mitotic versus interphase cells, and reacts with subsets of proteins that are phosphorylated upon entry into mitosis (12). These results further confirmed that the growth-inhibitory effects of flavokawain A on p53 wild-type RT4 and p53-mutant T24 cells were associated with a G₁ or M phase arrest, respectively.

The Effect of Flavokawain A on G₁ Cell Cycle Regulation in p53 Wild-type RT4 Cells

We next examined whether G₁ arrest by flavokawain A in RT4 cells was associated with a decreased CDK2 kinase activity (a major kinase for G₁ progression and entry into S phase). Flavokawain A treatment (40 μmol/L) caused a time-dependent decrease in CDK2 kinase activity in RT4 cells, as early as 4 hours after treatment. At 24 hours, the kinase activity was not detectable (Fig. 2A). Staining of proteins in gels by the standard Coomassie brilliant blue assay indicated equal loading of the kinase substrate histone H1 (data not shown).

The CDK2 kinase activity is negatively regulated by association with CDK kinase inhibitors: p21 and p27 (13). To examine whether flavokawain A regulates p21 and p27 expression in RT4 cells, we performed real-time PCR to quantify the mRNA levels of p21, p27, and SKP2 after 40 μmol/L flavokawain A treatment of RT4 cells for 4 and 8 hours. Figure 2B showed

that flavokawain A treatment resulted in an increase in the mRNA levels of p21 and a decrease in the mRNA levels of SKP2, but did not change the mRNA levels of p27. SKP2 is an oncogene and E3-ubiquitin ligase responsible for the regulation of p27 protein degradation (14). Western blotting analysis further revealed that flavokawain A increased the protein levels of both p21 and p27 in RT4 cells, but decreased the protein expression of SKP2 in a time-dependent manner (Fig. 2C). Furthermore, Fig. 2D showed that the increased protein levels of p21 and p27 led to an increased formation of p21 or p27 with CDK2 immunocomplex, which was detected by immunoprecipitation of CDK2 complexes with anti-CDK2 antibody, and by Western blotting analysis of p27 or p21 levels with anti-p27 or anti-p21 antibody. Figure 2D showed a doublet of p21 protein, which may represent the existence of phosphorylated p21 protein in the CDK2 complex. In addition, flavokawain A treatment resulted in a slight decrease in CDK2 expression, but did not significantly affect the protein expression of cyclin D1 and E at the same concentrations and time points (Fig. 2C). Together, these results suggest that flavokawain A induced p21 mRNA and decreased SKP2 mRNA expression, leading to the accumulation of p21 and p27 proteins and subsequent increase in binding to CDK2 in RT4 cells. Additionally, flavokawain A slightly decreased CDK2 protein expression in RT4 cells. The effects of flavokawain A caused an inhibition of CDK2 kinase activity and G₁ arrest in RT4 cells.

The Mechanisms of Flavokawain A–Induced Accumulation of p27 and p21 Proteins in p53 Wild-type RT4 Cells

To further examine whether increased protein levels of p27 and p21 by flavokawain A were due to the effect of flavokawain A on the stability of these proteins, we analyzed the degradation of p27 and p21 with protein synthesis inhibitor, cycloheximide, in the presence or absence of 40 μ mol/L of flavokawain A. Figure 3A showed that treatment of vehicle control–treated RT4 cells with cycloheximide resulted in a steady decrease of p27 protein levels. At 4 and 8 hours after cycloheximide treatment, p27 protein level decreased by 63% and 77%, respectively, measured by densitometry; whereas p27 levels declined more slowly in both flavokawain A–treated and cycloheximide-treated RT4 cells (p27 protein levels decreased by 21% and 32% at 4 and 8 hours of cycloheximide treatment, respectively). Given that flavokawain A did not affect the mRNA expression of p27 in RT4 cells (Fig. 2B), this result suggested that the accumulation of p27 by flavokawain A was due to the increased stability of the protein. In contrast, flavokawain A did not seem to enhance the stability of p21 proteins in RT4 cells. The protein levels of p21 declined by 77% and 93% in the vehicle control–treated RT cells versus a decline of 225% and 236% in the flavokawain A–treated RT cells at 4 and 8 hours of cycloheximide treatment, respectively (Fig. 3B).

To determine whether accumulation of p21 and p27 was only a consequence of the flavokawain A–induced G₁ arrest during cell cycle progression, RT4 cells were arrested in S phase using thymidine and then treated with flavokawain A. As seen in Fig. 3C and D, the induction of p21 and p27 by flavokawain A compared with controls was seen whether the majority of cell population was in S phase or in G₁ phase. This result suggested that the observed accumulation of p21 and p27 by flavokawain A treatment in RT4 cells was a specific cause for G₁ cell cycle arrest regardless of cell cycle position.

The Effect of Flavokawain A on G₂-M Cell Cycle Regulation in p53 Mutant-Type T24 Cells

Cell cycle progression through the G₂-M phase was under tight control by CDK1 kinase activity, which was held in an inactive state before mitosis by inhibitory phosphorylation of CDK1 at Thr¹⁴ and Tyr¹⁵. Additional control is achieved by the low levels and predominantly cytoplasmic localization of cyclin B1 (15). The dephosphorylation of CDK1 at Thr¹⁴ and Tyr¹⁵ is regulated by the balance between phosphatase Cdc25C and CDK1 inhibitory kinases, Wee1 and Myt1 (15,16). We therefore examined whether M phase arrest by flavokawain A in T24 cells (Fig. 1C) was associated with an increased CDK1 kinase activity. Figure 4A showed

a time-dependent increase of CDK1 kinase activities in flavokawain A-treated T24 cells. Consistently, positive controls such as paclitaxol at a concentration of 1 $\mu\text{mol/L}$ and 50 ng/mL of nocodazole also caused an increased CDK1 kinase activity. The Coomassie brilliant blue staining of proteins in gel indicated equal loading of the kinase substrate histone H1 (data not shown).

We therefore assessed the effect of flavokawain A on the major CDK1 regulators (Cdc25C, Myt1, Wee1, and cyclin B1) in T24 cells. Flavokawain A treatment decreased Cdc25C protein expression and its phosphorylation at Ser²¹⁶ in T24 cells in a time-dependent manner (Fig. 4B, *a* and *b*). Alone, the decrease in Cdc25C protein levels would be expected to lead to an increase in CDK1 phosphorylation at Tyr¹⁵ (inactive CDK1) in T24 cells. However, the protein expression of the CDK1-inhibitory kinases Wee1 and Myt1 by flavokawain A were also markedly decreased in T24 cells (Fig. 4B, *c* and *d*). As a result, Fig. 4B (*f* and *h*) showed that flavokawain A treatment (40 $\mu\text{mol/L}$) first increased the phosphorylation of CDK1 at Tyr¹⁵ at 8 hours, but then resulted in a complete dephosphorylation of CDK1 at Tyr¹⁵ at 16 and 24 hours without changes in CDK1 protein levels. In addition, there was an accumulation of cyclin B1 by 40 $\mu\text{mol/L}$ of flavokawain A treatment at 4, 8, 16, and 24 hours in T24 cells compared with control (Fig. 4B, *e*). Together, flavokawain A up-regulated cyclin B1 protein expression and caused dephosphorylation of CDK1 at Tyr¹⁵ via down-regulation of CDK1-inhibitory kinases Myt1 and Wee1 to counteract the decreased level of Cdc25C, leading to increases in CDK1 kinase activity in T24 cells (Fig. 4C). In addition, 40 $\mu\text{mol/L}$ of flavokawain A treatment of T24 cells for 24 hours decreased the protein expression of Cdc25 A and B (Fig. 4B, *h* and *i*), whereas paclitaxol and nocodazole at the same concentrations described above increased the protein expression of Cdc25B and Cdc25C without affecting Cdc25A expression (Fig. 4B, *a*, *h*, and *i*). These results suggested that flavokawain A may induce an M phase arrest in T24 cells via molecular mechanisms that were, at least in part, different from those of paclitaxol and nocodazole. Flavokawain A seems to more effectively reduce the protein expression of Myt1 and Wee1 in T24 cells.

Suppression of p53 Expression Led to Flavokawain A Activating CDK1 and the Enhanced G₂-M Arrest and Growth-Inhibitory Effect of Flavokawain A

We have shown that the growth-inhibitory effect of flavokawain A is more pronounced in p53 mutant-type T24 cells than in p53 wild-type RT4 cells (8). It is possible that flavokawain A's differential effects on these two types of cells may be related to their p53 status. We therefore transiently transfected p53 siRNA into RT4 cells. As seen in Fig. 5A (*left*), p53 siRNA specifically knocked down p53 expression in RT4 cells without affecting the expression of nontargeted protein p42 mitogen-activated protein kinase after 48 hours of transfection. As expected, siRNA suppression of p53 expression in RT4 cells potentiated the growth-inhibitory effect of flavokawain A (Fig. 5A, *left*; cell viabilities for flavokawain A treatment at a concentration of 16 $\mu\text{mol/L}$ in the presence of either control siRNA or p53 siRNA are $95 \pm 7.3\%$ versus $76 \pm 8.1\%$; $P < 0.05$, Student's *t* test).

Figure 5B (*right*) showed that 16 $\mu\text{mol/L}$ of flavokawain A treatment alone for 24 hours increased a G₁ population of RT4 cells by 24% compared with vehicle control treatment alone. In contrast, p53 siRNA and flavokawain A combination resulted in ~9% increase in the G₂-M population accompanying a decrease in both G₁ and S phase populations when compared with vehicle control treatment (Student's *t* test, $P < 0.05$). Consistently, p53 siRNA and flavokawain A act synergistically to activate CDK1, as indicated by an increased CDK1 kinase activity and dephosphorylation of CDK1 at Tyr¹⁵, without affecting the total protein levels of CDK1 (Fig. 5C, *right*, and Fig. 5D, *1* and *2*). Paclitaxol and nocodazole treatments at the same concentrations described above were used as positive controls for CDK1 kinase activity in this experiment. Consistent with the regulatory role of p53 in G₂-M transition (17–19), Fig. 5D

(3 and 4) showed that p53 siRNA suppressed p21 expression and induced Cdc25C expression in RT4 cells. In addition, Fig. 5D (4 and 5) showed that flavokawain A treatment of p53-suppressed RT4 cells leads to Cdc25C activation as indicated by the restored expression of Cdc25C and dephosphorylation of Cdc25C at Ser²¹⁶ (active Cdc25C). This concentration (16 μ mol/L) of flavokawain A did not significantly decrease Cdc25C protein levels in p53-suppressed RT4 cells (Fig. 5D, 4). The results described above suggested that suppression of p53 in RT4 cells allowed flavokawain A to activate Cdc25C via its dephosphorylation at Ser²¹⁶ and then partly contributed to CDK1 activation.

To further confirm the role of p53 status in determining flavokawain A's effect on cell cycle progression, HCT116 cells, wild-type and p53 knockout, were treated with 16 μ mol/L of flavokawain A for 24 hours. Figure 5B (*left*) showed that flavokawain A treatment resulted in an increase in G₂-M population of ~20% in p53 knockout HCT116 cells, whereas in p53 wild-type HCT116 cells, flavokawain A treatment led to an ~7% increase of the G₁ cell population. Consistently, flavokawain A increased CDK1 kinase activity only in p53 knockout HCT116 cells. The results further confirmed that p53 status plays a crucial role for flavokawain A inducing cell cycle arrest.

Flavokawain A Activated CHK1/2 Uncoupled with Dephosphorylation of Cdc25C

G₂-M cell cycle checkpoints exist to prevent cell cycle progression in response to DNA damage and replication inhibition (20,21). ATM/ATR-Chk1/2 activation has been shown to lead to inactivating phosphorylation of Cdc25C followed by the accumulation of Tyr¹⁵-phosphorylated CDK1 (20–22). Based on the observation that flavokawain A caused dephosphorylation of Cdc25C at Ser²¹⁶, we examined the effect of flavokawain A on CHK1/2 activation. Unexpectedly, flavokawain A treatment resulted in a dose-dependent and time-dependent increase in phosphorylated CHK1 at Ser³¹⁷ and phosphorylated CHK2 at Thr⁶⁸ in both RT4 and T24 cells (Supplementary Fig. S2A). Although activation of CHK1 in response to block replication and certain forms of genotoxic stress involves the phosphorylation of Ser³¹⁷ and Ser³⁴⁵ (21), phosphorylation of CHK2 at Thr⁶⁸ is considered a prerequisite for the subsequent activation of CHK2 (22). Our results suggested that flavokawain A activates CHK1/2 in bladder cancer cells. However, further studies showed that a specific CHK2 inhibitor (CHK2 inhibitor II, 2-[4-(4-chlorophenoxy) phenyl]-1H-benzimidazole-5-carboxamide) at effective concentrations (3 and 6 μ mol/L) capable of inducing G₂-M arrest did not influence the effects of flavokawain A on G₂-M cell cycle arrest and the growth of T24 cells (Supplementary Fig. S2B and C). These results suggested that flavokawain A might target downstream events of CHK1/2 for its effect on G₂-M transition.

Flavokawain A Inhibited Tumor Growth of RT4 Cells

We have shown that flavokawain A inhibits *in vivo* tumor growth of human invasive bladder cancer EJ cells, cells similar to T24 cells (8). Here, we examined whether flavokawain A can also suppress the *in vivo* tumor growth of papillary RT4 cells in a xenograft model. Nude mice were injected with RT4 cells and treated with daily oral vehicle (10% grain alcohol in 0.9% saline) or 50 mg/kg of flavokawain A starting at the day of tumor cell implantation (day 1) and ending on day 65. Flavokawain A resulted in a significant decrease in the growth rate of tumors compared with vehicle control (ANO-VA, $P < 0.01$; Fig. 6A). The wet tumor weights in control and flavokawain A-treated groups recorded at the end of the treatment were 339.5 ± 248.2 and 122.3 ± 118.5 mg, respectively (Fig. 6B; $n = 10$, mean $\pm$ SD; $P < 0.05$, Student's *t* test). Flavokawain A treatment attenuated tumor growth by 64%. The body weight gain and diet and water consumption of the flavokawain A-treated mice were similar to the control groups of mice. In addition, the mice did not show any gross abnormalities upon necropsy at the end of the treatment.

Discussion

Because the normal urothelium is a tissue of self-renewal under tight regulation (23), enhanced cell growth is a particular property of human urinary bladder cancer. Uncontrolled proliferation of premalignant and malignant cells has been commonly reported during the process of bladder carcinogenesis, through accumulation of defects in cell cycle regulation (i.e., loss of checkpoint control and/or the inappropriate activation of CDKs; refs. 24–28). Notably, several tumor suppressor genes and their products (e.g., p53, pRb, p27/KIP1, p16INK4A, p15INK4B and p14ARF) are dysfunctional as a result of chromosome deletion, gene mutation, or methylation (29–32). These tumor suppressors normally act at the G₀–G₁ checkpoint of the cell cycle to prevent loss of cell cycle control. Furthermore, the activation of the receptor tyrosine kinase-Ras pathway including overexpression of FGFR3, erbB-2, FGF, HGF, c-met, epidermal growth factor receptor, and H-ras in bladder tumors provide self-sufficiency in growth signaling for cancer cells by activating mitogen-activated protein kinase pathway and then overriding G₀–G₁ checkpoint through the increased activity of CDKs (e.g., CDK2; refs. 33–36). Many bladder cancer cells, even at the early (pre-malignant) stage of bladder carcinogenesis, have developed a defective G₀–G₁ checkpoint (26,28,30). In addition, the degree of cell cycle deregulations parallels the biological and clinical aggressiveness of human urinary bladder cancer (28,37). As such, different types of bladder cancer differ in the mechanisms leading to altered cell cycle regulation. Although loss or down-regulation of Rb, p21, and p27 is commonly observed in muscle-invasive bladder cancer (38–41), overexpression of cyclin D1 and p21 is characteristic of early stage, papillary bladder cancer (28,37). Therefore, targeting unlimited growth and deregulated cell cycle progression in bladder cancer cells may be one of the most promising strategies for bladder cancer prevention and therapy.

The cell cycle, consisting of four phases, G₁ phase, S phase (DNA replication), G₂ phase, and M phase (mitosis and cytokinesis) is remarkably conserved in yeast, animals, and plants (42). Flavonoids including chalcones that play a role in cell cycle regulation in plant cells (43–45) may serve as natural modulators for deregulated cell cycle in cancer cells, and thus, are useful for cancer prevention and therapy. In low-grade and p53 wild-type bladder cancer RT4 cells, we have shown that flavokawain A induced G₁ arrest via inhibition of CDK2 kinase activity, which was accompanied by accumulation of CDK inhibitors p21 and p27 and a slight decrease in the expression of CDK2 protein. There were no observed changes in the protein levels of major G₁ cyclins, cyclin D1 and E, by flavokawain A in RT4 cells. Further studies showed that accumulation of p21 protein by flavokawain A was due to increased expression of p21 mRNA, and that the increase in p27 was associated with down-regulation of SKP2 mRNA and protein expression which increased protein stability of p27 in RT4 cells. SKP2 is responsible for polyubiquitylation of cell cycle regulators (14,46). Its major physiologic target is p27. Accumulating evidence suggest that SKP2 is an important oncoprotein. Frequent amplification of the *SKP2* gene has been detected in primary lung cancers and in cell lines expressing high-risk human papilloma virus (47,48), and overexpression of SKP2 has been observed in many human tumors (49). In addition, double-transgenic mice that coexpress Skp2 and activated N-Ras in the T-cell lineage develop T-cell lymphoma three times more often than mice harboring the N-Ras transgene alone (50). The role of SKP2 in bladder cancer is still in its infancy, and the status of SKP2 expression in bladder cancer has not yet been reported. Given that both mRNA and protein expression of SKP2 was down-regulated by flavokawain A in bladder, prostate and breast cancer cell lines (DU145, PC-3, MCF7, and SKBR-3),¹ we speculated that SKP2 may be a potential direct target for flavokawain A. Further studies are in progress for identifying the potential transcriptional regulation of SKP2 by flavokawain A in bladder cancer cells.

¹Y. Tang, AR Simoneau, J. Xie, and X. Zi, unpublished data.

In high-grade and p53 mutant-type bladder cancer T24 cells, flavokawain A augmented the CDK1 kinase activity by decreasing the inhibitory factors Myt1 and Wee1, and increasing the activating factor cyclin B1 to offset its effect on down-regulation of Cdc25C protein expression (Fig. 4). Based on observations that cancer cells commonly develop defective G₀–G₁ checkpoints via loss of tumor suppressors (e.g., p53 and RB) and thus depended on the G₂ checkpoint more than normal cells, a “cell cycle G₂ checkpoint abrogation” strategy has been developed for cancer cell-specific medicines or as sensitizers for chemotherapy/radiotherapy (e.g., UCN01, a CHK1 inhibitor and G₂ checkpoint abrogator in phase I/II clinical trials for cancer indications; ref. 20). Phosphorylation of Cdc25C at Ser²¹⁶ is a downstream event initiated by the activation of CHK1 or CHK2 (20–22). Our data showed that flavokawain A, although it activated CHK1/2, decreased the phosphorylation of Cdc25C at Ser²¹⁶. Moreover, a specific CHK2 inhibitor (2-[4-(4-chlorophenoxy) phenyl]-1H-benzimidazole-5-carboxamide) at effective concentrations for induction of G₂-M arrest did not affect the efficacy of flavokawain A in inducing G₂-M arrest and inhibiting cell growth of T24 cells. Thus, we speculate that CHK1/2 activation may not be a primary target for flavokawain A's action in T24 cells, rather CHK1/2 activation is a consequence of flavokawain A-induced apoptosis and DNA damages. The effect of flavokawain A on induction of CDK1 kinase activity may not depend on CHK2 activation. This seemingly conflicting result of dephosphorylation of Cdc25C and activation of CHK1/2 by flavokawain A suggests that flavokawain A may act downstream of CHK1/2 activation for its effect on Cdc25C activation via its dephosphorylation. In addition, we have shown that flavokawain A decreased Wee1 and Myt1 protein expression in T24 cells. Wee1 has been shown to be an interesting target for the purpose of a “cell cycle G₂ checkpoint abrogation” strategy. Down-regulation of Wee1 by RNA interference approach did not cause significant cell death, but dramatically sensitized cancer cells, but not normal cells, to the apoptotic effect of Adriamycin (51). In addition, a novel Wee1 inhibitor (PD0166285) has been selected for sensitizing p53-defective cancer cell lines more than p53 wild-type lines to radiotherapy (52). Here, we have shown that flavokawain A exhibits a robust mechanism for abrogating the G₂ checkpoint, including down-regulation of Wee1 and Myt1 and dephosphorylation of Cdc25C. In addition, flavokawain A is more effective in inhibiting the growth of cancer cell lines with mutant p53. Therefore, flavokawain A is suggested to be a “G₂ checkpoint abrogator” in p53-defective bladder cancer cells and deserves further investigation as a sensitizer for current chemotherapy/radiotherapy.

In general, cells with defective p53 fail to undergo apoptosis in response to a variety of proapoptotic stimuli and have growth advantage over cells with wild-type p53 (53). However, our data have shown that cancer cell lines with mutant p53 (e.g., T24, UMUC3, HT1376, 5637, TCCSUP, HT1197, DU145, PC-3, and SKBR-3) were even more sensitive to the growth-inhibitory effects of flavokawain A compared with corresponding types of cell lines with wild-type p53 (RT4, 22Rv1, LNCaP, normal prostate epithelial cells, MCF-7, and MCF10A).¹ Functional p53 not only plays a major role in mediating cell cycle arrest at the G₁ checkpoint but also, in general, acts to prevent G₂-M transition. The mechanisms of repressing key regulators for the G₂-M transition by p53 are very complicated and less clear. We found that RT4 cells with wild-type p53 expressed fewer Cdc25C proteins than T24 cells harboring mutant p53 did. In addition, we did not detect any significant differences in protein expression of Cdc25A and Cdc25B between these two cell lines (data not shown). Moreover, suppression of p53 expression in RT4 cells with siRNA increased the protein expression of Cdc25C. These results are consistent with the report that functional p53 transcriptionally represses Cdc25C expression to inactivate CDK1 kinase activity (17). Furthermore, Imbriano et al. (18) showed that direct p53 suppression of Cdc25C was through an association among the *Cdc25C* promoter, nuclear factor-Y trimers, and a region close to the tetramerization domain of p53. Coincidentally, our results showed that among the bladder cancer cell lines tested, the cell lines with mutations in the tetramerization domain of p53 (TCCSUP and HT1197) are the most sensitive to the growth-inhibitory effect of flavokawain A. Further experiments are therefore

in progress to examine deep mechanisms of the association among p53 mutations, transcriptional regulation of key regulators (e.g., Cdc25B, Cdc25C, cyclin B1, Cdc2, and topoisomerase II α) for the G₂-M transition and the growth-inhibitory effect of flavokawain A in bladder cancer cells.

In summary, we have described the mechanisms of flavokawain A's action on cell cycle regulation in cell lines derived from different stages of bladder cancer. For low-grade and p53 wild-type papillary bladder cancer RT4 cells, flavokawain A induced an accumulation of p21 and p27 protein by increasing p21 mRNA and decreasing SKP-2 mRNA, which lead to increased binding of p21 and p27 to CDK2 with subsequent inhibition of CDK2 kinase activity followed by a resultant G₁ arrest. For high-grade and p53 mutant-type, muscle-invasive or metastatic bladder cancer cells, flavokawain A induced a G₂-M arrest. Flavokawain A activated CDK1 kinase activity by decreasing inhibitory kinases, Myt1 and Wee1, and increasing cyclin B1 to counteract its effect on down-regulation of Cdc25C protein expression, with subsequent M phase arrest in T24 cells. The mechanisms for the observed differential effects of flavokawain A on cell cycle regulation in bladder cancer cells were associated with their difference in p53 status. As p53 mutations occur in ~50% of cancers, flavokawain A may work as a p53 mutant cancer-specific agent and deserves further investigation as a sensitizer for current radiotherapy/chemotherapy.

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Fig. 1.

The effect of flavokawain A on cell cycle distribution in p53 wild-type and mutant-type bladder cancer cell lines. RT4, T24, UMUC-3, TCCSUP, 5637, HT1376, and HT1197 cells were treated with DMSO (control) or 40 μ mol/L of flavokawain A for 4, 8 16, and 24 h or 1 μ mol/L of paclitaxol or 50 ng/mL of nocodazole for 24 h. Cell cycle distribution was analyzed by flow cytometry as detailed in Materials and Methods.

A and B, percentage of cell cycle distribution after flavokawain A treatment for different periods of time. C, RT4 and T24 cells were treated with DMSO (control) or 1 μ mol/L of paclitaxol or 50 ng/mL of nocodazole for 16 h or 40 μ mol/L of flavokawain A for 8 and 16 h. Western blotting analysis of mitosis-specific phosphorylated proteins in RT4 and T24 cells. *Points*, mean of three samples for each treatment; *bars*, SE. These results were similar in two independent experiments.

Fig. 2.

The effect of flavokawain A on G₁ cell cycle regulation in p53 wild-type RT4 cells. **A**, cells were treated with either DMSO or 40 μ mol/L of flavokawain A in complete medium for 4, 8, 16, and 24 h. CDK2-associated H1 histone kinase activities were determined as described in Materials and Methods. **B**, RT4 cells were treated with DMSO or 40 μ mol/L of flavokawain A in complete medium for 4 and 8 h. At the end of each treatment time, total RNA were prepared and quantitative real-time PCR analysis of *SKP2*, *p21*, and *p27* mRNA was done as mentioned in Materials and Methods. Gene expression is presented as a fold increase in ΔC_t compared with DMSO control treatment. *Columns*, mean of four independent quantitative real-time PCR experiments; *bars*, SE. **C**, RT4 cells were treated as indicated. Protein lysates were used for SDS-PAGE and Western blotting. Membranes were probed with anti-p27, CDK2, SKP2, cyclin D1, cyclin E, and p21 primary antibodies followed by peroxidase-conjugated appropriate secondary antibody, and visualized by enhanced chemiluminescence detection system. The membrane was stripped and reprobed with antiactin antibody for protein loading correction. Representative blot from three independent experiments. **D**, for binding of p21 and p27 to CDK2 kinase, RT4 cells were treated as indicated. Protein lysates were immunoprecipitated by anti-CDK2 antibody and probed by anti-p21 and p27 as mentioned in Materials and Methods. Representative blot from three independent experiments.

Fig. 3.

The effect of flavokawain A on p21 and p27 protein stability in p53 wild-type RT4 cells. **A** and **B**, RT4 cells were treated in the absence or presence of 40 $\mu\text{mol/L}$ of flavokawain A (FKA). After 16 h of treatment, 100 $\mu\text{mol/L}$ of cycloheximide was added. Total cell lysates were prepared at the indicated time points and analyzed for the expression of p27 by Western blot. β -Actin was detected as a loading control. The protein levels were quantified by densitometry and plotted as a percentage of protein levels in control treatment alone for 16 h. *Points*, mean of three independent quantitative measures; *bars*, SE. **C**, RT4 cells were treated with 2 mmol/L of thymidine for 24 h to arrest the cells in S phase of the cell cycle. If indicated, 40 $\mu\text{mol/L}$ of flavokawain A was then added, and the cells were further incubated in the presence of both thymidine and flavokawain A. Fluorescence-activated cell sorting analysis of cells treated with DMSO, flavokawain A alone, thymidine alone, or thymidine plus flavokawain A. Thymidine and flavokawain A treatments showed that these cells remained arrested in S phase, whereas the DMSO-treated cells showed the normal cell cycle phase

distribution and flavokawain A alone induced a G₁ arrest. *D*, RT4 cells were treated as the same indicated in *C*. Cell lysates were prepared and expression of p27 and p21 proteins in RT4 cells was compared in the presence or absence of thymidine and flavokawain A. Representative blot from three independent experiments.

Fig. 4.

The effect of flavokawain A on G₂-M cell cycle regulation in p53 mutant-type T24 cells. T24 cells were treated with DMSO or 40 μ mol/L of flavokawain A in complete medium for 4, 8, 16, and 24 h or 1 μ mol/L of paclitaxol or 50 ng/mL of nocodazole for 24 h. At the end of each treatment time, cell lysates were prepared as mentioned in Materials and Methods. **A**, CDK1-associated H1 histone kinase activities were determined as described in Materials and Methods. **B**, protein lysates were used for SDS-PAGE and Western blotting. Membranes were probed for phosphorylated Cdc25C at Ser²¹⁶, total Cdc25C, Myt1, Wee1 cyclin B1, phosphorylated CDK1 at Tyr¹⁵ and total CDK1, and stripped for reprobing with antiactin antibody for protein loading correction. *a*, total Cdc25C; *b*, phosphorylated Cdc25C at Ser²¹⁶; *c*, Myt1; *d*, Wee1;

e, cyclin B1; *f*, phosphorylated CDK1 at Tyr¹⁵; *g*, total CDK1; *h*, Cdc25A; and *i*, Cdc25B. Representative blot from three independent experiments. *C*, hypothetical presentation of CDK1 kinase regulation by flavokawain A: the CDK1 kinase activity is determined by the balance between Cdc25C and CDK1 kinase inhibitors, Myt1 and Wee1, as well as cyclin B1 protein level. Flavokawain A increases cyclin B1, decreases Myt1 and Wee1, and dephosphorylates Cdc25C to counteract its effect on down-regulation of Cdc25C protein expression for CDK1 activation.

Fig. 5.

The effect and mechanism of flavokawain A on cell growth and cell cycle progression in p53 suppression or deficient cancer cell lines. **A**, RT4 cells were transfected with siRNA control or siRNA p53 for 48 h and then exposed to 16 $\mu\text{mol/L}$ of flavokawain A or 0.1% DMSO for an additional 24 h. Cell viabilities were determined by MTT assay. *Columns*, mean percentage of cell viability relative to control (siRNA control plus 0.1% DMSO; $n = 4$); *bars*, SE. Western blotting analysis of p53 and p42 mitogen-activated protein kinase expression. **B**, RT4 cells were transfected with siRNA control or siRNA p53 for 48 h and then exposed to 16 $\mu\text{mol/L}$ of flavokawain A or 0.1% DMSO for an additional 24 h. HCT116 cells, p53 wild-type, and p53 knockout were exposed to 16 $\mu\text{mol/L}$ of flavokawain A or 0.1% DMSO for 24 h. Cell cycle distribution was analyzed by flow cytometry as detailed in Materials and Methods. **C**,

RT4 and HCT116 (p53 wild-type and p53 knockout) cells were treated as described above. CDK1-associated H1 histone kinase activities were determined as described in Materials and Methods. *D*, Western blotting analysis of phosphorylated CDK1 at Tyr¹⁵, total CDK1, p21, Cdc25C, and phosphorylated Cdc25C at Ser²¹⁶.

Fig. 6.

The effect of flavokawain A on tumor growth of RT4 cells in a xenograft model. *A*, 100 μ L volume of RT cells (2×10^6) in serum-free medium were mixed with equal volumes of Matrigel and injected into the right flank of NCR-*nu/nu* (nude) mice. The next day, the mice were randomly divided, pair-matched into treatment and control groups of 10 mice each, and daily dosing was begun with vehicle or flavokawain A at 50 mg/kg. Tumor volumes were recorded, and presented as mean $\pm$ SE. *B*, at the termination of the study, tumors were excised from each mouse in different groups and weighed. *Columns*, mean wet weight of 10 tumors from individual mice in each group; *bars*, SE; *FKA*, flavokawain A ($P = 0.033$ at the termination of the experiments).

Table 1

The IC₅₀ of flavokawain A and tumor suppressor gene status in bladder cancer cell lines

	Cell lines							
	RT4	UMUC-3	T24	HT1376	5637	TCCSUP	HT1197	
Estimated IC ₅₀ (μmol/L)	20.8	17.7	16.7	14.7	13.1	10.55	7.9	
p53	WT	MT/Exon 4	MT/Exon 5	MT/Exon 7	MT/Exon 8	MT/Exon 10	MT/Exon 10	
RB	-	+	+	-	-	-	-	
ARF/p16	HD/HD	HD/HD	WT/MT	WT/WT	WT/WT	WT/WT	WT/WT	

NOTE: Retinoblastoma gene status implied from the expression of protein localized to the nucleus (+, presence of nuclear expression; -, absence of nuclear expression). Tumor suppressors p14 (ARF) and p16 (INK4A; p16).

Abbreviations: WT, wild-type; MT, mutant allele; RB, retinoblastoma; HD, homozygous deletion.