

ORIGINAL ARTICLE

Antitumor effects of flavokawain-B flavonoid in gemcitabine-resistant lung cancer cells are mediated via mitochondrial-mediated apoptosis, ROS production, cell migration and cell invasion inhibition and blocking of PI3K/AKT Signaling pathway

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Summary

Purpose: Drug resistance in lung cancer is a growing and challenging problem affecting the overall treatment and quality of the patient's life. The main purpose of the current study was to investigate the anticancer effects of flavokawain-B in gemcitabine-resistant non-small lung cancer cells (NSCLC) along with evaluating its mode of action by studying its effects on programmed cell death, ROS production, cell migration and invasion and PI3K/AKT signalling pathway.

Methods: Cell proliferation rate was studied using MTS cell viability assay while apoptosis induction by flavokawain-B was studied by fluorescence microscopy using DAPI staining as well as flow cytometry using Annexin V-FITC/propidium iodide (PI). Effects on mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) were studied by flow cytometry using Rh-123 and DCH-DA dyes respectively. Effects on cell migration and cell invasion were examined by in vitro wound healing assay and transwell assay respectively. Changes in PI3K/AKT protein expressions were evaluated by western blot.

Results: Flavokawain-B selectively inhibited the viability of the human NSCLC cell line A549, indicating lower toxic-

ity compared with normal lung cancer (NLC) CCL-151 cells and both showed dose-dependent inhibition. DAPI and annexin V-FITC/PI staining showed that flavokawain-B led to a dose-dependent onset of apoptosis in lung cancer cells characterized by shrunken cells, fragmented nuclei and chromatin condensation. Western blot showed that flavokawain-B resulted in downregulation of Bcl-2 and upregulation of Bax in a dose dependent manner. Flavokawain-B treatment led to increase of intracellular ROS concentration and decrease of mitochondrial membrane potential (MMP) both showing dose-dependence. It also led to suppression of cell migration and invasion along with blocking PI3K/AKT signalling pathway.

Conclusions: Flavokawain-B targets gemcitabine-resistant NSCLC cells selectively without inducing any significant toxicity in normal cells and these effects are mediated via apoptosis induction, ROS production, loss of MMP, suppression of cell migration and invasion and blocking PI3K/AKT signalling pathway.

Key words: flavokawain-B, lung cancer, apoptosis, cell migration, cell invasion

Introduction

Lung carcinoma is a major cause of cancer-related deaths in both males and females worldwide [1]. Alone in 2013, 1.6 million deaths and approxi-

mately 1.8 million new cases of this malignant disorder were registered globally [2]. Furthermore, it is expected that lung carcinoma deaths will touch

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3 million upto 2035 [3]. There are generally two differently growing histological types of lung carcinoma i.e., non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). Among other lung cancer, i.e large cell carcinoma, squamous cell carcinoma and adenocarcinoma, NSCLC accounts for maximum (nearly about 85%) of lung carcinoma cases [4]. The most common histological subtype adenocarcinoma which accounts for around 40% of overall lung carcinoma cases. Though men are more vulnerable to this malignancy than women, with 1/18 in men and 1/51 in women, globally there is an increase of incidence of this malignancy in women. Differences between male/female regarding different aspects of lung cancer have been demonstrated, including higher treatment responses, survival, and better prognosis in women [5-8]. However, the 5-year survival rate is only less than 18% assessed for lung carcinoma, indicative of urgency for more effective drugs to tackle this lethal malignancy.

Natural products like plants have demonstrated to be a good source of anticancer drugs. Due to their huge diversity, it is believed that they can provide more molecules to tackle cancer [9]. As revealed by pharmacological studies, flavonoids have shown potential towards various biological disorders including cancer [10]. Some potential flavonoids were shown to hamper the proliferation of carcinoma cells thus can be proved as vital for development of systemic therapy for lung cancer [11]. A novel chalcone flavokawain B isolated from a spicy herb *Alpinia pricei* Hayata (native to Taiwan) and Kava root extracts can prove a potential molecule to fight lung cancer. Despite lung cancer being higher in smokers, there is a lower risk of its incidence, in the people of Pacific Islands due to their daily or routine consumption of kava root extracts [12]. Flavokawain B has been reported to exhibit both *in vitro* as well as *in vivo* anticancer effects against colon, prostate and bladder cancer cells, and also resulted in induction of apoptosis [13-15]. In the current study we examined whether the antitumor effects of flavokawain-B flavonoid in gemcitabine-resistant NSCLC cells are mediated via mitochondrial-mediated apoptosis, ROS production, cell migration and cell invasion inhibition and blocking of PI3K/AKT signalling pathway.

Methods

Cell lines and culture conditions

The NSCLC cell line A549 and NLC CCL-151 cell line were purchased from American Type Culture Collection. The cells were preserved in Dulbecco's modified Eagle's medium (DMEM) in CO₂ Thermo Scientific incubator at 37°C with 5% CO₂ and 98% humidity.

Cell viability assay

MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was used to evaluate cell viability of NSCLC A549 and NLC cells. Each well of the 96-well plate were seeded both with NSCLC A549 cells and NLC CCL-151 cells separately at 2×10^5 cells per well. Prior to treatment of cells with flavokawain-B at varying doses (ranging between 0-100 μ M) for 24 h, the cells were first incubated overnight at 37°C. After the cells were subjected to the treatment with flavokawain-B, they were incubated and then MTS solution was added. Then using ELISA plate reader (ELX 800; Bio-Tek Instruments, Inc., Winooski, VT, USA) absorbance was measured at 490 nm.

Apoptosis investigation by fluorescence microscopy and flow cytometry (DAPI and annexin V-FITC/PI assay)

The human lung carcinoma NSCLC A549 cell line was seeded in 6-well plates for apoptosis detection. Afterwards, the cells were incubated for 12 h and then treated with different concentrations (0, 7, 14, and 28 μ M) of flavokawain-B at 37°C for 24 h. About 20 μ l cell cultures were drawn out and put onto glass slides and DAPI was used for staining. The slides were then cover-slipped and examined under fluorescent microscope. The mode of cell death was also determined by Annexin V-FITC/propidium iodide (PI) assay (following manufacturer's protocol, BD Pharmingen™, USA). The flavokawain-B-treated cells (A549) were washed with phosphate buffered saline (PBS) and by adding 300 μ L of trypsin-EDTA the cell were harvested. After harvesting cells were suspended again in 1x binding buffer (i.e., 0.1M HEPES, 0.1M NaOH pH 7.4, 1.4M NaCl, 25mM CaCl₂ at a concentration of 1×10^5 cells/ml). To the suspensions 5 μ L of Annexin V-FITC and PI were consequently added. Later vortexing of the suspensions were done gently, and after addition of 400 μ L of 1x binding buffer, cells were incubated for 15 min in the dark at room temperature. The flow cytometer Beckman-Coulter Co., USA) was used to determine the number of cells that had undergone apoptosis at 488 nm with argon ion laser.

MMP and ROS determination

MMP was determined by using the fluorescent dye Rh123 (a cell permeable cationic dye) and flow cytometer (Beckman-Coulter Co., USA). The fluorescent dye Rh123 having a high MMP enters mitochondria preferentially. Loss of Rh123 from mitochondria and decrease in intracellular fluorescence is observed only when depolarization of MMP takes place. The fluorescent dye was added to cell culture at a concentration of 100 Ag/L and at 37°C for 45 min. The cells were then pipetted out and subjected to washing by PBS. Flow cytometry was used to analyse 10,000 cells per sample.

In case of ROS, the cells were pipetted out and subjected to washing with PBS. Then, for ROS estimation all the media were decanted and cells were subjected to treatment with 5 μ M DCH-DA and the data was collected through flow cytometry by placing 10,000 cells per sample.

Wound-healing assay

The culturing of flavokawain-B-treated A549 lung carcinoma cells continued till 80% confluence. Afterwards, the whole medium was removed and cells were washed with PBS. Later, a sterile pipette tip was used to scratch a wound and a picture was captured. After scratching a wound, A549 cells were incubated at 37°C for about 24h and then again a picture was captured under an inverted microscope.

Cell invasion assay

Cell invasion assay was used to determine the cell invasion ability of A549 cell line by transwell chambers with Matrigel. The upper chambers were filled with around 200 ml of cell culture and the bottom wells were filled with medium only. The cells were then drawn out of the upper chambers and incubated for 24h. After incubation, the invaded cells were fixed with methyl alcohol and then stained with crystal violet. The number of invaded cells was then calculated under inverted microscope with a magnification of 200×.

Western blotting

Western blotting technique was used to evaluate the protein expression in flavokawain-B-treated A549 lung carcinoma cells at different doses 0, 7, 14 and 28 μM . These cells were then lysed with RIPA buffer. After loading samples on the SDS-PAGE the gels were shifted to nitrocellulose membranes and then were treated with primary antibody for 24 h at 4°C. After primary antibody treatment of the membranes, the membranes were subjected to HRP-conjugated secondary antibody treat-

ment at 25°C for 50 min. To visualise the protein bands advanced Chemi-luminescence reagent was used.

Statistics

All the data were described as mean \pm standard deviation (SD) from at least three independent experiments. One-way ANOVA with graphpad prism 7 software were used to analyze the differences between groups. Statistically significant difference was taken at $p < 0.05$.

Results

Flavokawain-B selectively inhibits cell viability in A-549 NSCLC cells

To explore the effects of flavokawain-B on the NSCLC A549 cells and NLC CCL-151 cells, the viability of cells was analysed by a MTS assay. After 24 h exposure, all treated cells indicated a significant decrease in cell viability. The IC_{50} of the flavokawain-B was found to be 14 μM in A549 cells (Figure 1A) and 65 μM in CCL-151 normal lung cells cells after 24 h treatment (Figure 1B). The MTS assay indicated that flavokawain-B selectively inhibited the viability of NSCLC A549, indicating lower toxicity in NLC CCL-151, and both showed dose-dependent inhibition.

Flavokawain-B induces apoptosis in lung cancer cells

DAPI staining indicated apoptotic cells to have condensed, shrunken, and fragmented nuclei after

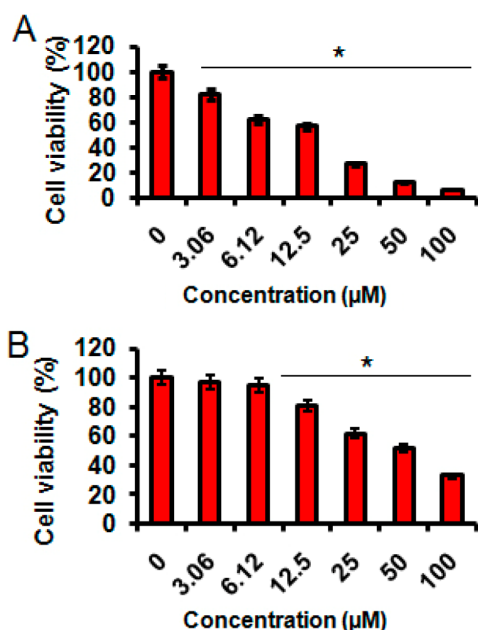


Figure 1. MTS-1 assay showing cytotoxic effects of flavokawain-B on the viability of gemcitabine-resistant A-549 human non-small cell lung cancer cells (A) and normal lung cells (B). The experiments were performed in triplicate and shown as mean \pm SD (* $p < 0.05$).

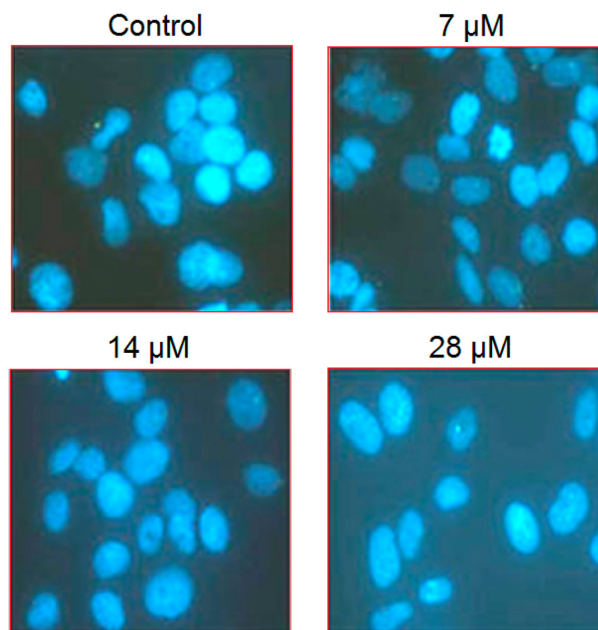


Figure 2. DAPI staining showing flavokawain-B induces apoptosis in A-549 human non-small cell lung cancer cells. The experiments were performed in triplicate. The tested molecule led to dose-dependent morphological alterations reminiscent of apoptosis.

treatment with flavokawain-B for 24 h (Figure 2). It was totally opposite in the case of untreated non-apoptotic cells, which exhibited a smooth, flattened, normal nuclei and low fluorescence as well as the chromatin was uniformly dispersed (Figure 2). Further, the effect of inducing apoptosis of flavokawain-B on human lung cancer cells A549 was evaluated by Annexin V-FITC/PI assay using flow cytometry. Flavokawain-B-treated cells revealed that apoptosis was induced in a dose-dependent manner. The percentage of apoptotic cells exposed to different concentrations of flavokawain-B i.e., 0, 7, 14, and 28 μM increased significantly with increasing doses (Figure 3). This data indicated that following flavokawain-B treatment the cell death primarily occurred via apoptosis. Western blotting was used to determine apoptosis-related protein expressions and it was found that flavokawain-B resulted in downregulation of Bcl-2 and upregula-

tion of Bax in a dose-dependent manner (0, 7, 14, and 28 μM) (Figure 4).

Flavokawain-B raised ROS and declined MMP in A549 lung cancer cell

The effect of flavokawain-B molecule on ROS and MMP of NSCLC A549 cells was evaluated by flow cytometry. After these cells were subjected to varied doses of flavokawain-B, they showed a significant rise in ROS percentage almost touching 250 (Figure 5A), revealing that flavokawain-B caused an increase in intracellular ROS concentrations in dose-dependent manner. Subsequently, MMP of NSCLC A549 cells was determined. The results indicated that the MMP percentage in flavokawain-B-treated cells decreased significantly (from 100% to around 20%) with increasing concentrations (0, 7, 14 and 28) of the molecule (Figure 5B).

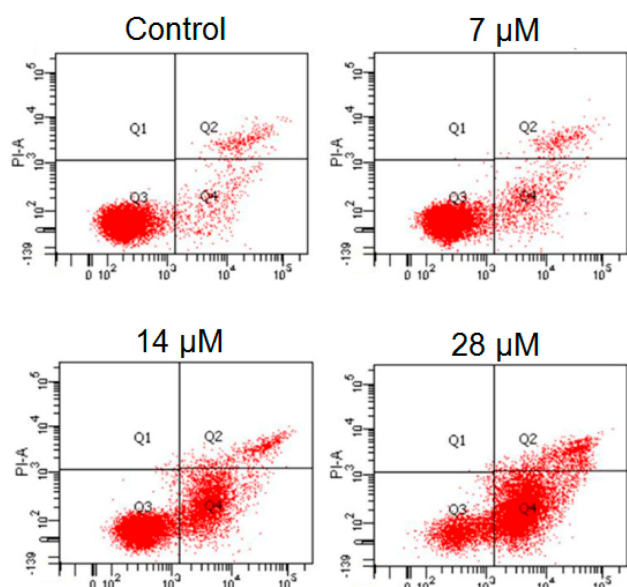


Figure 3. Annexin V/PI assay showing the percentage of A-549 apoptotic cells at various concentrations of flavokawain-B. The experiments were performed in triplicate.

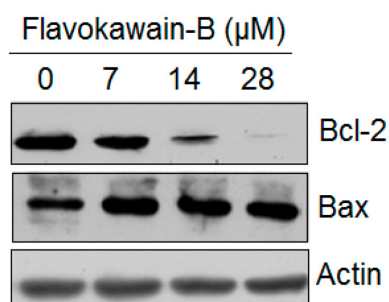


Figure 4. Effect of indicated concentrations of flavokawain-B on the expression of apoptosis-related proteins by western blot analysis. The experiments were performed in triplicate. Bcl-2 expression was decreased while as Bax expression was increased after treatment.

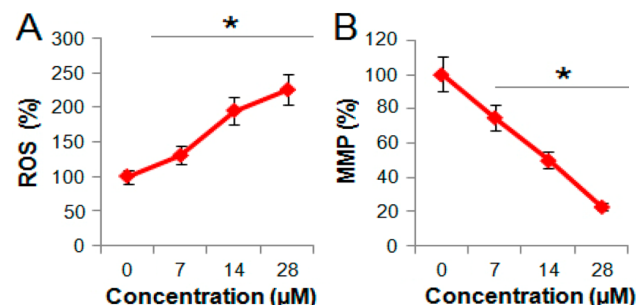


Figure 5. Effect of flavokawain-B on the reactive oxygen species (ROS) and mitochondrial membrane potential (MMP). Flavokawain-B treatment led to increase in ROS (A) and decrease in MMP (B) (* $p < 0.05$). The experiments were performed in triplicate.

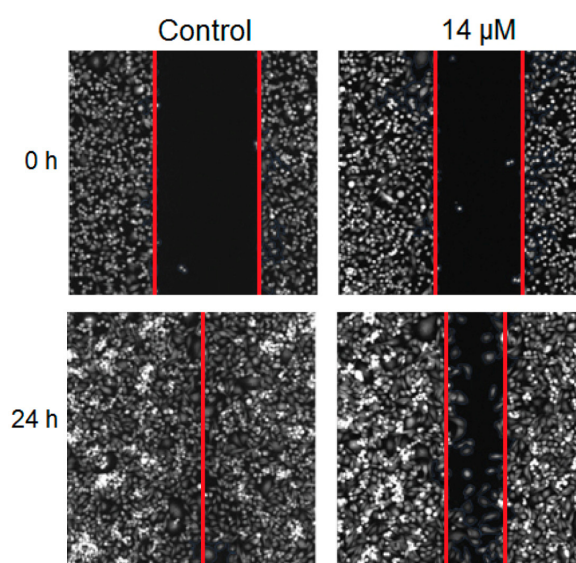


Figure 6. Effect of flavokawain-B on the cell migration of A-549 human non-small cell lung cancer cells at 14 μM dose, indicating a significant suppression of cell migration. The experiments were performed in triplicate.

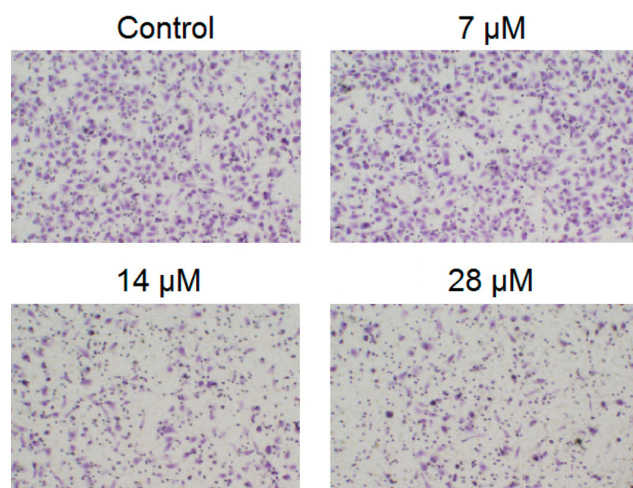


Figure 7. Effect of flavokawain-B on the cell invasion of A-549 human non-small cell lung cancer cells at various doses, indicating a dose-dependent significant suppression of cell invasion. The experiments were performed in triplicate.

Flavokawain-B flavonoid results in suppression of cell migration and cell invasion of NSCLC A549 cells

Cell migration and invasion were assessed by *in vitro* wound healing assay and transwell assay, respectively. It was found that the molecule resulted in substantial decrease in cell migration of A549 human lung carcinoma cells at a dose of 14 μ M (Figure 6). Furthermore, transwell assay revealed that flavokawain-B resulted in reduction of cell invasion of these lung cancer cells in a concentration-dependent manner (Figure 7).

Flavokawain-B blocked PI3K/AKT signaling pathway in lung carcinoma cells

The PI3K/AKT signalling cascade is believed to be a therapeutic target for the treatment of different cancers. Herein, western blotting technique was used to determine the effects of flavokawain-B on PI3K/AKT signaling pathway. The results of the western blot analysis showed that flavokawain-B inhibited the phosphorylation of PI3K and AKT in dose-dependent manner. Nonetheless, the total protein levels of PI3K and AKT remained apparently unaltered. This clearly indicated that flavokawain-B is a potential blocking molecule of PI3K/AKT signalling pathway in lung carcinoma cells.

Discussion

Despite the huge economic costs, poor prognosis and high mortality rate, lower interest has been paid towards lung cancer till 2013 [2]. To further improve the survival rate and develop-

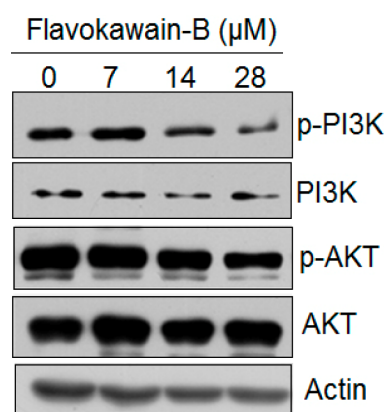


Figure 8. Effect at indicated concentrations of flavokawain-B on the expression of phosphorylated PI3K/AKT signalling pathway proteins. The Figure shows that flavokawain-B inhibits the phosphorylation of PI3K and AKT dose-dependently. The experiments were performed in triplicate.

ment of novel treatment strategies *in vitro* and *in vivo* investigations by means of diverse lung cancer models are needed to tackle this lethal malignancy. Herein, this study was carried out to unveil the antitumor effects of flavokawain-B flavonoid in gemcitabine-resistant NSCLC cells. The results revealed that flavokawain-B flavonoid caused significantly higher retardation/decline of cell proliferation of A549 NSCLC cells in a dose-dependent manner, which was in contrast to the NLC CCL-151 normal cells wherein it was significantly lower. Further, apoptotic potential of flavokawain-B was revealed by DAPI staining and Annexin V-FITC+PI assay. The results revealed that cells exposed to the given drug showed a significant suppression of Bcl-2 and increased expression of Bax, which indicated that flavokawain-B molecule induced apoptosis in NSCLC cell line A549. Further, the percentage of ROS and MMP was determined by flow cytometry, which revealed that flavokawain-B increased ROS percentage and decreased MMP percentage in a dose-dependent manner. Oncogenic rearrangement of ROS has become a well-known therapeutic target in lung cancer since around 1-2% of NSCLC patients are identified with this type of rearrangement [16,17]. Despite this prevalence may seem low at first glance, it means 2,000 to 4,500 patients will be newly diagnosed with ROS-rearranged NSCLC each year [18,19]. The current study also revealed that flavokawain-B inhibited cell migration and invasion of NSCLC A549 cells in a dose-dependent manner, thus further showing up its anticancer potential. Western blotting analysis also showed that this molecule resulted in blocking of PI3K/AKT signaling pathway by controlling the expressions of PI3K and AKT in a concentration-dependent manner.

Conclusion

This study concluded that flavokawain-B flavonoid is a potent anticancer molecule which induces selective cytotoxicity in lung cancer cells without showing significant effects on normal lung cells and these effects were mediated via apoptosis induction, ROS generation, MMP loss, inhibition of cell migration and invasion and blocking of PI3K/AKT signalling pathway.

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Conflict of interests

The authors declare no conflict of interests.

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