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Synthesis of Flavokawain B and its Anti-proliferative Activity Against Gefitinib-resistant Non-small Cell Lung Cancer (NSCLC)

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Non-small cell lung cancer (NSCLC) is the most common type of lung cancer and that accounts for 85% of lung cancer patients. Although several EGFR-targeted drugs have been developed in the treatment of NSCLC, the clinical efficacy of EGFR-targeted drugs in NSCLC is limited by the occurrence of drug resistance. In this regard, Hsp90 represents great promise as a therapeutic target of cancer due to its potential to simultaneously disable multiple signaling pathways. In this study, we discovered that a natural product, flavokawain B disrupted Hsp90 chaperoning function and impaired the growth of gefitinib-resistant non-small cell lung cancer (H1975). The result suggested that flavokawain B could serve as a potential lead compound to overcome the drug resistance in cancer chemotherapy.

Key Words : Flavokawain B, Hsp90, Gefitinib resistance, Natural product, Cancer

Introduction

Lung cancer is the leading cause of cancer-related mortality in the world and there are two main forms of lung cancer, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is the most common type of lung cancer and that accounts for 85% of lung cancer patients. The major cause of lung cancer is long-term exposure to tobacco smoke, which is responsible for 80-90% of lung cancer patients.[1] Despite recent advancements in the diagnosis and conventional treatment of NSCLC, the prognosis remains unsatisfactory, with 15% of 5-year survival.[2] The epidermal growth factor receptor (EGFR) is a cell membrane receptor tyrosine kinase and regarded as a molecular target of particular relevance to lung cancer. EGFR plays a major role in regulating cancer cell proliferation and progression. The overexpression of EGFR has been observed in 40-80% of NSCLC patients.[3] For this reason, several anti-EGFR agents, including monoclonal antibodies of cetuximab (trade name; Erbitux) and small molecule inhibitors of gefitinib (trade name; Iressa) and erlotinib (trade name; Tarceva) were introduced for the treatment of NSCLC and the progress has been made using these EGFR-targeted therapies. However, the clinical efficacy of these EGFR-targeted therapies is being challenged by the occurrence of drug resistance. NSCLC cells bypass EGFR blockage by mutating EGFR, and amplifying an alternative signaling pathway of Met tyrosine kinase receptor.[4,5] Therefore, there is a pressing need to discover a new therapeutic agent to defeat drug resistance in the war on lung cancer.

Hsp90 is an ubiquitous molecular chaperone protein, which accounts for 1-2% of the cytosolic protein under non-stressed conditions.[6] Hsp90 plays an important role in regulating maturation and stabilization of many oncogenic proteins in six essential hallmarks of cancer, including EGFR, Her2, Met, Akt, Raf, HIF-1and MMP2.[1,3,7] To survive in the hostile environment of oncogenic stressors, such as hypoxia, low pH and poor nutrition, cancer cells tend to depend on Hsp90 chaperoning function, which explains 2-10 fold higher expression level of Hsp90 in tumor cells than normal cells.[8-10]

Hsp90 has received significant attention in that, the inhibition of single Hsp90 protein induces simultaneous blockage of multiple signaling pathways in tumor and potentially overcomes the inevitable drug resistance of single-targeted therapies. In this regard, Hsp90 has been extensively pursued as a drug target for overcoming drug resistance. Besides, accumulating evidence in recent studies has demonstrated

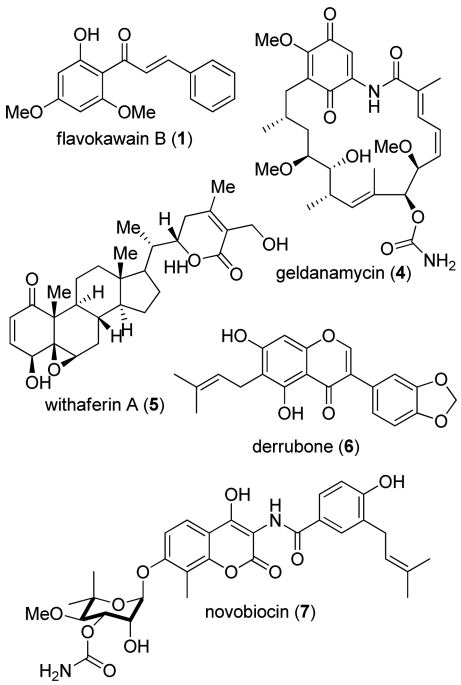


Figure 1. Structure of flavokawain B and natural product inhibitors of Hsp90.

that Hsp90 is a potential therapeutic target for neurodegenerative diseases, including Alzheimer's, Parkinson's, Prion and Hodgkin's diseases.[11-13] The rational has been that inhibition of Hsp90 activates heat shock factor-1 (HSF-1) to induce production of Hsp70 which in turn, promote disaggregation of neuronal toxic proteins, which are implicated in the development of neurodegenerative diseases. Therefore, the potential therapeutic benefits associated with Hsp90 modulation emphasize the importance of identifying novel Hsp90 inhibitors.

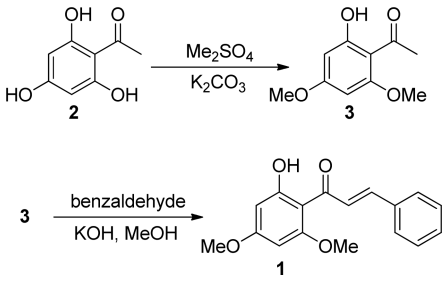
Historically, the discovery of new drugs from natural products proved to be the single most successful strategy and natural products still provide ample sources of chemical diversity for the discovery of pharmaceutical agents.[14] Several natural products targeting Hsp90 are discovered for the treatment of cancers, which includes geldanamycin[15] (**4**), withaferin A[16] (**5**), derrubone[17] (**6**), and novobiocin[18] (**7**), illustrated in Figure 1.

Kava (Piper methylsticum) is a native plant traditionally used as a medicine and social drink in the South Pacific islands. Interestingly, epidemiological studies demonstrated that kava-drinking countries in the South Pacific islands, including Fiji, Vanuatu and Western Samoa have low incidences of cancer, compared to non-kava-drinking countries of New Zealand (Maoris), United States (Hawaii and Los Angeles) and non-kava drinking Polynesians.[19,20] Furthermore, More men drink kava than women in these kava-drinking countries and cancer incidences in male population are lower than female, despite of a high smoking prevalence of men. Flavokawain B, one of chalcone components isolated from kava extracts, has been reported to exhibit strong anti-proliferative effects against several cancer cell lines.[21-23]

In our ongoing effort to discover Hsp90 inhibitors from natural sources, we found that flavokawain B inhibited Hsp90 protein folding machinery. Here, we describe anti-proliferative effect of flavokawain B against gefitinib-resistant non-small cell lung cancer cells (H1975) and its inhibitory activity against Hsp90.

Results and Discussion

Flavokawain B was synthesized following the literature procedure with slight modifications.[24] Briefly, 2'-hydroxy-4',6'-dimethoxyacetophenone (**3**) was quantitatively obtained from the reaction of compound **2** with dimethyl sulfate in the presence of K_2CO_3 in acetone.



Scheme 1. Synthesis of flavokawain B.

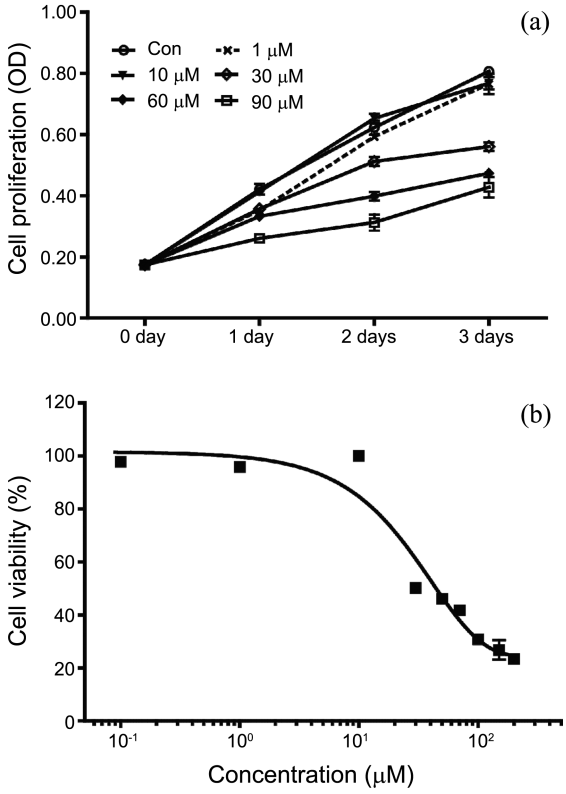


Figure 2. Flavokawain B overcomes the gefitinib resistance in non-small cell lung cancer cell line, H1975. (a) Anti-proliferative effect of flavokawain B on H1975 cells. Cell proliferation was determined at 1, 2, and 3 days using MTS assay at the indicated concentration of flavokawain B. Data are presented as mean ± SD (n = 4). (b) Cell death was induced by flavokawain B. Cells were treated for 72 hours at various concentrations of flavokawain B (0.1, 1, 10, 30, 50, 70, 100, 150, and 200 μM) and cell viability was measured by MTS assay. Data are presented as mean ± SD (n = 4).

The corresponding acetophenone **3** was further subjected to the classical base-catalyzed Claisen-Schmidt condensation reaction with benzaldehyde in methanol, to furnish flavokawain B (**1**) in 50% yield.

To evaluate the effect of flavokawain B on overcoming gefitinib resistance in NSCLC, we first measured anti-proliferative activity of flavokawain B against *in vitro* model of gefitinib-resistant non-small cell lung cancer, H1975 (Figure 2). H1975 cells were incubated with flavokawain B at various concentrations (0, 1, 10, 30, 60, and 90 μM) for 24, 48, and 72 h using MTS colorimetric assay. The assay demonstrated that flavokawain B inhibited cell proliferation in a concentration and time dependent manner. Cell viability study demonstrated that flavokawain B effectively impaired the growth of H1975 cells with IC_{50} value of 33.5 μM. (Figure 2(b)).

To investigate the molecular mechanism of the observed anti-proliferative activity, we next assessed the effect of flavokawain B on cellular hallmarks of Hsp90 inhibition (Figure 3). Since the proteasomal degradation of Hsp90's client proteins and the transcriptional up-regulation of Hsp70 are molecular hallmarks of Hsp90 inhibition,[25] we

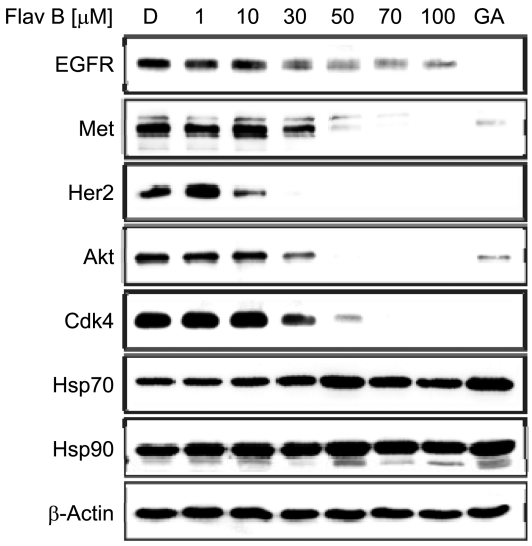


Figure 3. Effect of flavokawain B on the expression of EGFR, Her2, Met, Akt, Cdk4, Hsp70, and Hsp90. Flavokawain B degraded Hsp90 client proteins (EGFR, Her2, Met, Akt, and Cdk4) and upregulated Hsp70. Geldanamycin (GA, 1 μM) and DMSO (D) were employed as positive and negative controls, respectively.

analyzed the cellular levels of Hsp90's client proteins, along with Hsp70. H1975 cells were incubated with flavokawain B (0, 1, 10, 30, 50, 70, and 100 μM) and geldanamycin (GA, 1 mM) as a positive control, and measured the expression levels of EGFR, Met, Her2, Akt, Cdk4, Hsp70, and Hsp90, along with β-actin as an internal standard. Western blot analysis revealed that flavokawain B induced a robust degradation of Hsp90's client proteins including EGFR, Met, Her2, Akt, and Cdk4 in a concentration-dependent manner. Interestingly, the expression level of Her2 was most sensitively depleted upon the administration of flavokawain B. Even 10 μM of flavokawain B effectively degraded the expression of Her2. The expression levels of Akt and Cdk4 appeared to significantly decrease at 30 μM concentration, and almost completely depleted at 50 μM concentration of flavokawain B. In contrast, the cellular level of Hsp70 was concentration-dependently up-regulated when exposed to flavokawain B, which was consistent with other known Hsp90 inhibitors. The expression level of β-actin remained unchanged since β-actin was not a client protein of Hsp90 folding machinery.

H1975 is a gefitinib-resistant non-small cell lung cancer cell line and its resistance is mediated by 'gatekeeper' the mutation T790M-EGFR in combination with L858R.[4,5,26] The resistance is also related to Met amplification, compensating for the loss of EGFR signals.[27] Regarding that EGFR, Met, and their downstream signaling proteins are client proteins of Hsp90, the inhibition of Hsp90 protein folding machinery represents a promising strategy to overcome the resistance from EGFR mutation and Met amplification.

To quantitatively analyze protein levels of EGFR and Met, the intensity of the individual bands was quantified using ImageJ densitometry software, and expressed relative to β-actin signal. As seen in Figure 4, flavokawain B led to

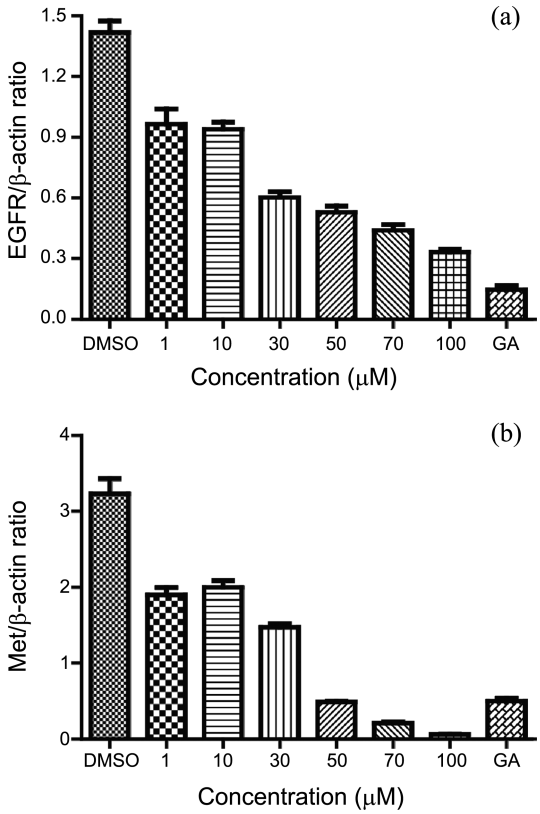


Figure 4. (a) Densitometry analysis of EGFR and (b) Met. The expression of EGFR and Met were normalized to β-actin ratio.

simultaneous degradation of EGFR and Met. 30 μM of flavokawain B induced the proteasomal degradation of EGFR and Met by more than 50%. Upon the administration of 50 μM flavokawain B, Met protein appeared to be more sensitively degraded than EGFR, and the expression level of

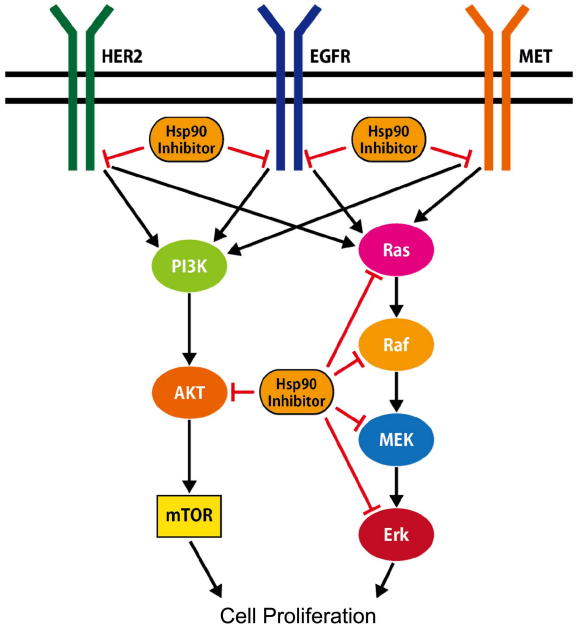


Figure 5. Molecular signaling pathways modulated by Hsp90 inhibitors in non-small cell lung cancer (NSCLC).

Met decreased by almost 85%. Overall, EGFR and Met levels responded in a concentration dependent manner to flavokawain B and these results suggested that flavokawain B circumvented the drug resistance from EGFR mutation and Met amplification by disrupting the Hsp90 folding machinery.

Conclusion

The degradation of vulnerable client proteins that associated with NSCLC such as EGFR, Met, Her2, Akt, Ras, Raf, Mek, and Erk could block several key signaling pathways including PI3K-Akt-mTOR and Ras-Raf-Mek-Erk pathways. (Figure 5) In this regard, the inhibition of Hsp90 chaperoning function has a potential to suppress multiple oncogenic signaling pathways simultaneously, thus reducing the possibility of molecular feedback loops and mutation leading to tumor resistance in NSCLC.

In summary, we discovered for the first time that a natural product, flavokawain B impaired the growth of gefitinib-resistant non-small cell lung cancer (H1975) and manifests significant degradation of Hsp90's client proteins such as EGFR, Met, Her2, Akt and Cdk4, and induction of Hsp70's expression level in a concentration dependent manner. The result suggested that flavokawain B disrupted the Hsp90 chaperone machinery and could serve as a potential lead compound to overcome the drug resistance in cancer chemotherapy. Currently efforts are directed toward synthesizing analogues of flavokawain B and its structure-activity relationship exploration is underway to improve the efficacy of the compound. The result will be reported in due course.

Experimental

General Methods. Unless otherwise noted, all reactions were performed under an argon atmosphere in oven-dried glassware. All purchased materials were used without further purification. Thin layer chromatography (TLC) was carried out using Merck silica gel 60 F_{254} plates. TLC plates were visualized using a combination of UV, *p*-anisaldehyde, ceric ammonium molybdate, ninhydrin, and potassium permanganate staining. NMR spectra were obtained on a Bruker 400 (400 MHz for ^{1}H; 100 MHz for ^{13}C) spectrometer. ^{1}H and ^{13}C NMR chemical shifts are reported in parts per million (ppm) relative to TMS, with the residual solvent peak used as an internal reference. Signals are reported as m (multiplet), s (singlet), d (doublet), t (triplet), q (quartet), bs (broad singlet), bd (broad doublet), dd (doublet of doublets), dt (doublet of triplets), or dq (doublet of quartets); the coupling constants are reported in hertz (Hz). Final products were purified by MPLC (Biotage Isolera One instrument) on a silica column (Biotage SNAP HP-Sil). On the basis of NMR and analytical HPLC data (Shimadzu prominence, VP-ODS C18 column), purity for all the tested compounds was found to be > 95%.

Procedure for the Synthesis of Flavokawain B. The synthesis of flavokawain B followed the literature procedure with slight modifications.[24] A mixture of ketone **4** (0.30 g, 1.5 mmol), benzaldehyde (0.18 g, 1.7 mmol), KOH (0.19 g, 3.4 mmol) in 5 mL of methanol was stirred at rt for 20 h. The mixture was neutralized with 3 N HCl to pH 6 and then extracted with ethyl acetate. The organic layer was dried over Na_2SO_4, and concentrated under reduced pressure, and purified by column chromatography (10% ethyl acetate in hexane) to afford compound 1 in 50% as yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (d, J = 15.6 Hz, 1H), 7.77 (d, J = 15.6 Hz, 1H), 7.61-7.57 (m, 2H), 7.42-7.36 (m, 3H), 6.09 (d, J = 2.4 Hz, 1H), 5.95 (d, J = 2.4 Hz, 1H), 3.90 (s, 3H), 3.82 (s, 3H). ESI MS (m/e) = 285 $[M+1]^+$.

Materials. Antibodies specific for EGFR, Her2, Met, Akt, Cdk4, Hsp90, Hsp70, and β-actin were purchased from Cell Signaling Technology. Goat anti-rabit IgG horseradish peroxidase conjugate was purchased from Santa Cruz Biotechnology. Cell Titer 96 Aqueous One Solution cell proliferation assay kit was purchased from Promega.

Cell Culture. H1975 cells were grown in RPMI 1640 with L-glutamine supplemented with streptomycin (500 mg/mL), penicillin (100 units/mL), and 10% fetal bovine serum (FBS). Cells were grown to confluence in a humidified atmosphere (37 °C, 5% CO_2).

Effect of Flavokawain B on Cell Proliferation. Cells were seeded at 3000 cells per well in a clear 96-well plate, the medium volume was brought to 100 μL, and the cells were allowed to attach overnight. The next day, varying concentrations of flavokawain B or 1% DMSO vehicle control was added to the wells. Cells were then incubated at 37 °C for 1, 2, and 3 days. Cell viability was determined using the Promega Cell Titer 96 Aqueous One Solution cell proliferation assay. After incubation with compounds, 20 μL of the assay substrate solution was added to the wells, and the plate was incubated at 37 °C for an additional 1 h. Absorbance at 490 nm was then read on Tecan Infinite F200 Pro plate reader, and values were expressed as percent of absorbance from cells incubated in DMSO alone.

Western Blot. Cells were seeded in 60 mm culture dishes (5×10^5/dish), and allowed to attach overnight. Flavokawain B was added at the concentrations indicated in Figure 3, and the cells were incubated for an additional 24 h. For comparison, cells were also incubated with DMSO (1%) or geldanamycin (1 μM) for 24 h. Cells were harvested in ice-cold lysis buffer (23 mM Tris-HCl pH 7.6, 130 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS), and 20 μg of lysate per lane was separated by SDS-PAGE and followed by transferring to a PVDF membrane (Bio-Rad). The membrane was blocked with 5% skim milk in TBST, and then incubated with the corresponding antibody (EGFR, Her2, Met, Akt, Cdk4, Hsp90, Hsp70, or β-Actin). After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by ECL chemiluminescence according to the instructions of the manufacturer (Thermo Scientific, USA).

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