

Induction of G2M Arrest by Flavokawain A, A Kava Chalcone, Increases the Responsiveness of HER2 Overexpressing Breast Cancer Cells to Herceptin

Running title: Flavokawain A Counteracts HER2 Mediated Resistance to Apoptosis

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Abstract

HER2/neu positive breast tumors predict a high mortality and comprise 25-30% of breast cancer. We have shown that Flavokawain A (FKA) preferentially reduces the viabilities of HER2 overexpressing breast cancer cell lines (*i.e.* SKBR3 and MCF7/HER2) *versus* those with less HER2 expression (*i.e.* MCF7 and MDA-MB-468). FKA at cytotoxic concentrations to breast cancer cell lines also has minimal effect on the growth of non-malignant breast epithelial MCF10 cells. FKA induces G2M arrest in cell cycle progression of HER2 overexpressing breast cancer cell lines through inhibition of Cdc2 and Cdc25C phosphorylation and down-regulation of Myt1 and Wee1 expression leading to increased Cdc2 kinase activities. In addition, FKA induces apoptosis in SKBR3 cells by increasing the protein expression of Bim and BAX and decreasing expression of Bcl₂, Bcl_{xL}, XIAP and survivin. FKA also down-regulates the protein expression of HER-2 and inhibits AKT phosphorylation. Herceptin plus FKA treatment leads to enhanced growth inhibitory effect on HER-2 overexpressing breast cancer cell lines through down-regulation of Myt1, Wee1, Skp2, Survivin and XIAP. Our results suggest the promise of FKA as a novel apoptosis inducer and G2 checkpoint abrogating agent in combination with Herceptin for treatment of HER2 overexpressing breast cancer.

Keywords: Flavokawain A; HER2 overexpression; resistance to apoptosis

Introduction

Conventional chemo-/radio-therapies for breast cancer non-specifically cause deleterious effects to health tissues and have been associated with significant side effects [1, 2]. In this regard, some phytochemicals are either part of the human diet or consumed as dietary supplements and do not show adverse health effects even at large doses [3]. Incorporation of tumor-specific targets and use of less or non-toxic phytochemicals may therefore represent new dimensions in management of breast cancer.

Human epidermal-growth-factor receptor 2 (HER2) and estrogen receptor (ER) are two breast tumor-specific targets [4, 5]. HER2 breast tumors predict a high mortality and comprise 25-30% of breast cancer [6]. The use of trastuzumab (Herceptin, a blocking antibody for HER2 signaling) has demonstrated clinical benefits in the management of HER2 positive metastatic breast cancer [7]. However, the outcome of this therapy in metastatic breast cancer remains unsatisfying due to frequent development of resistance to trastuzumab and risks of cardiac dysfunctions [7, 8]. HER2 also could be a target for breast cancer prevention because HER2 is overexpressed in a majority of ductal carcinoma in situ of the breast, a non-obligate precursor of invasive breast cancer [9-11]. At least 50% of HER2-overexpressing breast cancer is ER-positive at baseline [12]. Tamoxifen is the most commonly used drug for the treatment of ER-positive breast cancer and has been shown to be effective in prevention [5, 13]. However, HER2-overexpressing ER-positive tumors can develop resistance to tamoxifen by the acquisition of tamoxifen-stimulated growth [14]. Thus, new therapeutic or preventive approaches for HER2-overexpressing breast cancer are urgently needed.

Flavokawain A (FKA) is the predominant kava chalcone that constitutes up to 0.46% of the kava extract and was firstly screened out by our group as a potent apoptosis inducer against the growth of many types of cancer cell lines, including bladder, breast, colon, liver, and prostate cancers, melanoma, sarcoma, etc [15-20]. FKA at concentrations which significantly inhibit the growth of cancer cell lines has minimal effect on the growth of normal cells [18]. FKA activates both death-receptor and the mitochondria-mediated apoptotic pathways through increasing the expression of pro-apoptotic proteins DR5, Bim and Bax and decreasing the expression of anti-apoptotic proteins Bcl2, Bcl_{xL}, survivin and XIAP [15, 16]. We have further demonstrated that FKA is a novel neddylation inhibitor and causes degradation of Skp2 protein [18, 20]. Dietary feeding of FKA inhibits tumorigenesis in the mouse transgenic models of bladder and prostate carcinogenesis without exhibiting any adverse effects on major organ function (including liver function) and homeostasis in mice [18, 20-22]. However, the effect of FKA on HER2 overexpressing breast cancer has not been reported yet. In this study, we have shown that FKA preferentially inhibits the

growth of HER2 overexpressing breast cancer cell lines versus those with less HER2 expression by down-regulating the expression of Myt1 and Wee1 and phosphorylation levels of Cdc2 and then leading to G2M arrest and apoptosis. In addition, FKA enhances the growth inhibitory effect of Herceptin on Her2 overexpressing breast cancer cells.

Results

FKA preferentially inhibits the anchorage-dependent and independent growth of HER2 overexpressing breast cancer cell lines.

To examine whether FKA specifically inhibit the growth of breast cancer cells or even HER2 overexpressing breast cancer cells, an immortalized normal breast epithelial cell line MCF10A, an ER positive (MCF7) and ER negative (MDA-MB-468) breast cancer cell lines with less HER2 expression, and an ER positive (MCF7/HER2) and ER negative (SKBR3) breast cancer cell lines with HER2 overexpression were used. SKBR3 was isolated from the pleural effusion of a 43-year-old female with metastatic ductal adenocarcinoma of the breast with HER2/neu amplification [23]. MCF10A was originally derived from benign breast tissue from a woman with fibrocystic disease [24]. MCF/HER2 was a MCF7 clone engineered to overexpress HER2 [25]. Fig. 1B shows that FKA inhibited the growth of MCF7, MDA-MB-468, SKBR3 and MCF7/HER2 cells in a dose-dependent manner. At the same concentrations, FKA did not cause any noticeable inhibition on the growth of the MCF10A cell line (Fig.1B). Moreover, FKA treatment more effectively inhibited the growth of SKBR3 and MCF-7/HER2 cell lines with more HER2 than those with less HER2 (Fig. 1B). The IC₅₀s of FKA on the growth of HER2 overexpressing SKBR3 and MCF-7/HER2 cells are 10 and 13.6 μ M, respectively, *versus* HER2 less MCF7 and MDA-MB-468 cells 38.4 and 45 μ M, respectively (Table 1) ($P_s < 0.05$).

Consistently in a growth condition closer to *in vivo* situations (soft agar), FKA resulted in a greater decrease in the colony formation of MCF/HER2 than that of its parental cell line MCF7 (Figs 1 C&D). It appears that FKA is more effective in inhibition of colony formation than cell growth in dishes. FKA at a concentration of 4 μ M inhibits the colony formation of MCF/HER2 and MCF7 by 80% and 54%, respectively (Fig. 1D). Together, these results suggested that FKA can specifically inhibit HER2 overexpressing breast cancer cells with minimal effect on normal breast epithelial cells.

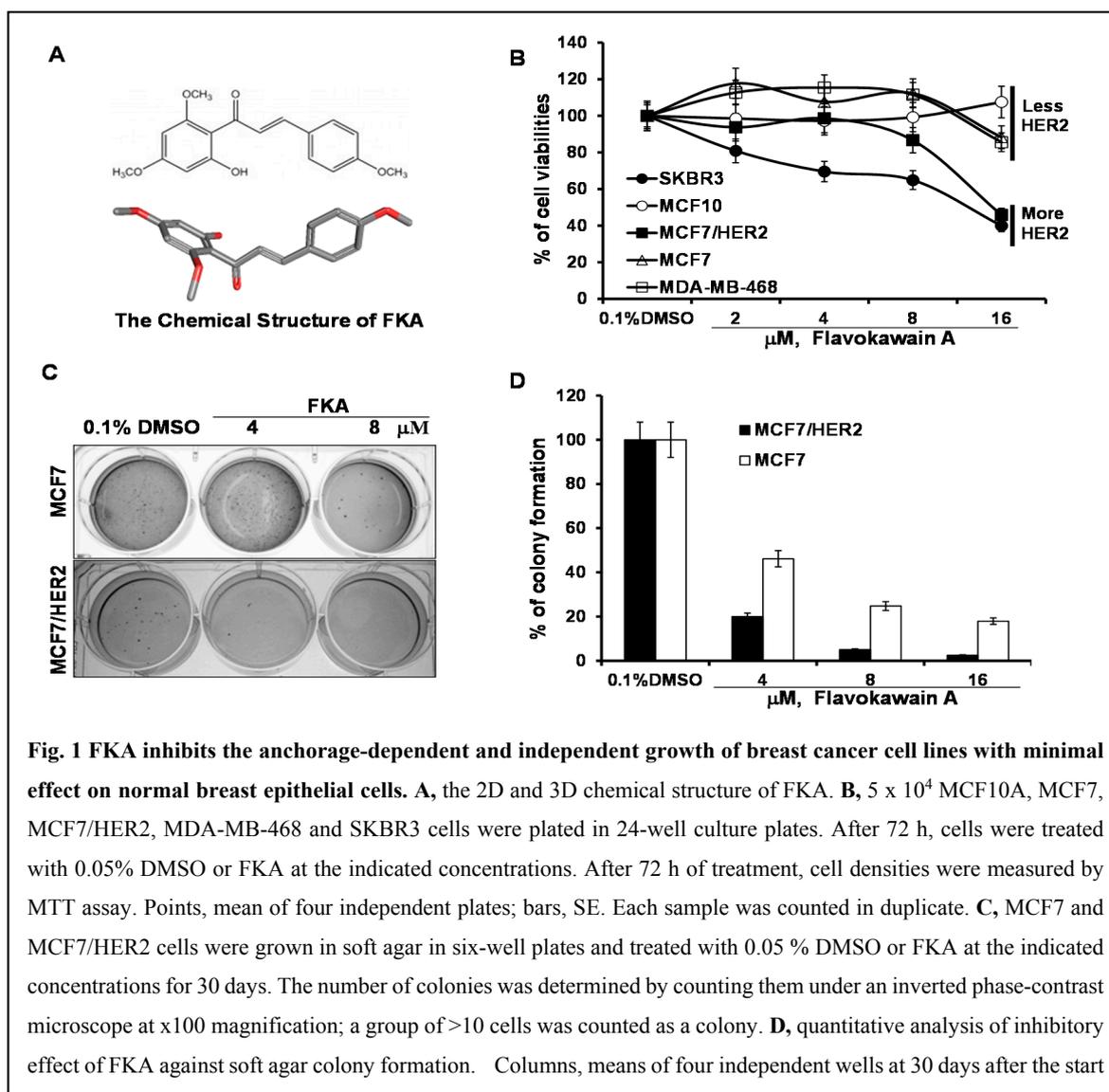
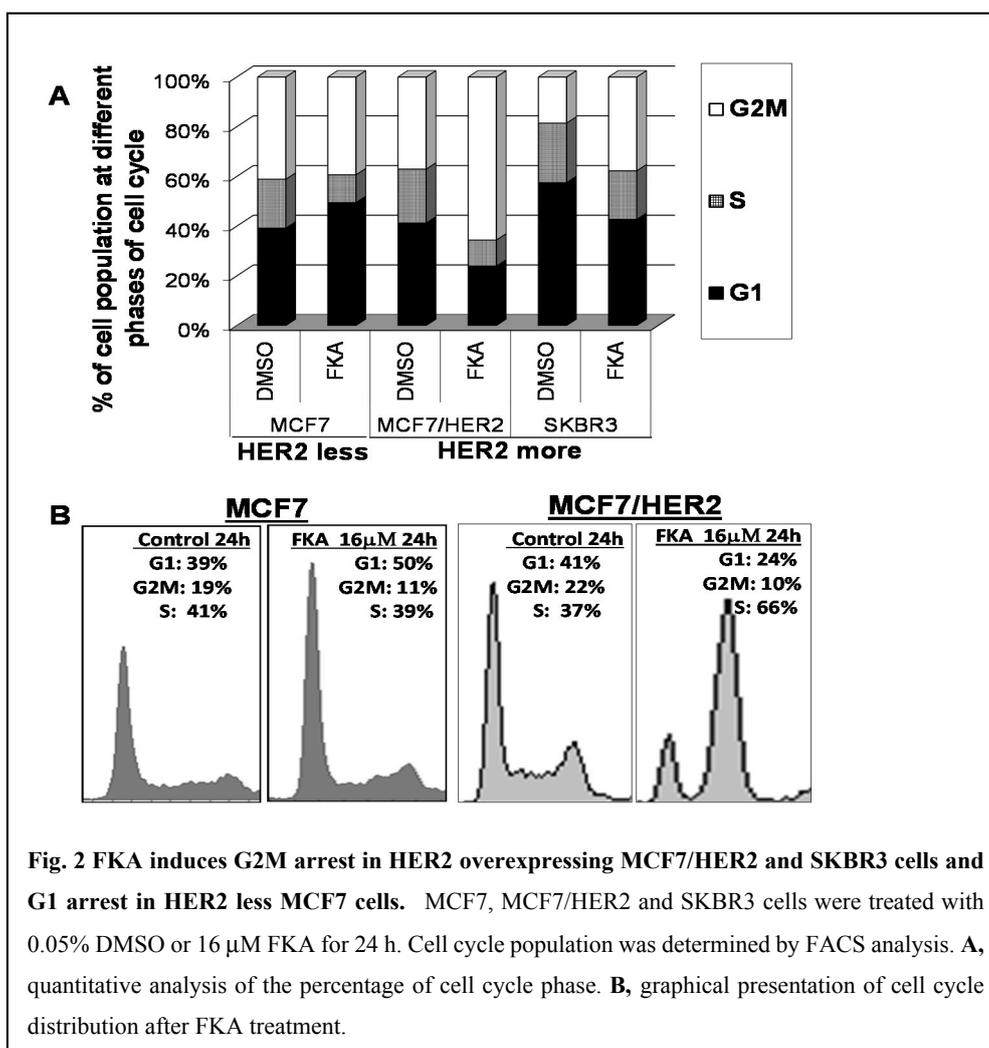


Table 1 The IC₅₀s of FKA and statuses of estrogen receptor, p53 and HER2 in breast cancer cell lines.

	MCF10A	MDA-MB468	MCF7	MCF7/HER2	SKBR3
Estrogen receptor	absent	mutant	wild-type	wild-type	mutant
P53	wild-type	mutant	wild-type	wild-type	mutant
HER2 expression	+	+	+	++++	++++
IC₅₀s	>100 μM	38.4 μM	45 μM	13.6 μM	10 μM

The effect of FKA on cell cycle progression differs between HER2 overexpressing versus HER2 less breast cancer cell lines.

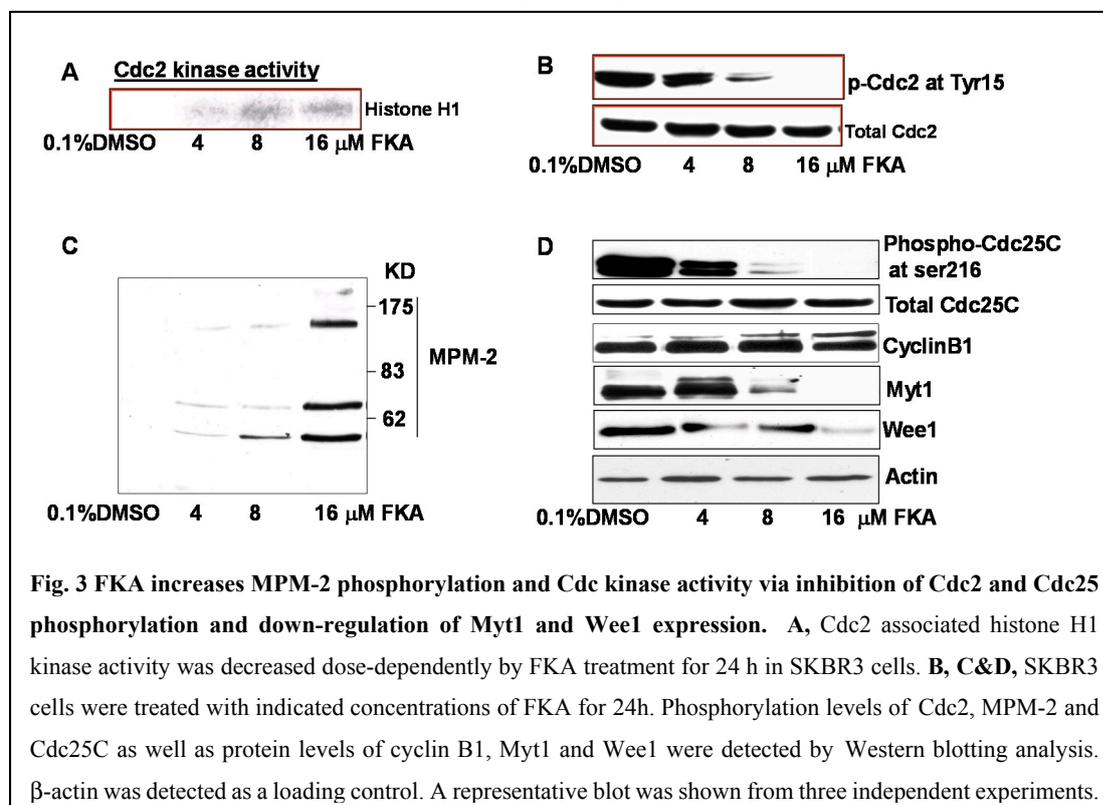
To examine whether the cell growth inhibitory effects of FKA were induced via perturbation in cell cycle progression, we performed fluorescence-activated cell sorting analysis of control (0.1% DMSO) and 16 μ M FKA-treated cells. Figs. 2A&B indicated a G1 arrest in p53 wild type and HER2 less MCF7 cells treated with FKA (G1 population, 39.2 % for control *versus* 49.5 % for FKA at 24 hours of treatments; Student's t test, $P < 0.01$), For HER2 overexpressing but p53 wild-type MCF7/HER2 cells as well as HER2 overexpressing and p53 mutant SKBR3 cells, FKA at the same concentrations induced a significant G2-M arrest (G2-M population, 36.9 and 18.5 % for control *versus* 65.5 and 37.7 % for FKA treatments of MCF7/HER2 and SKBR3 cells, respectively, for 24 hours; Student's t test, $P_s < 0.01$) (Figs. 2A and B). These results indicates that the growth-inhibitory effects of FKA on HER2 less or overexpressing breast cancer cells is associated with a G1 or M phase arrest, respectively, and that the FKA induced G2M arrest in HER2 overexpressing breast cancer cells is independent of p53 status.



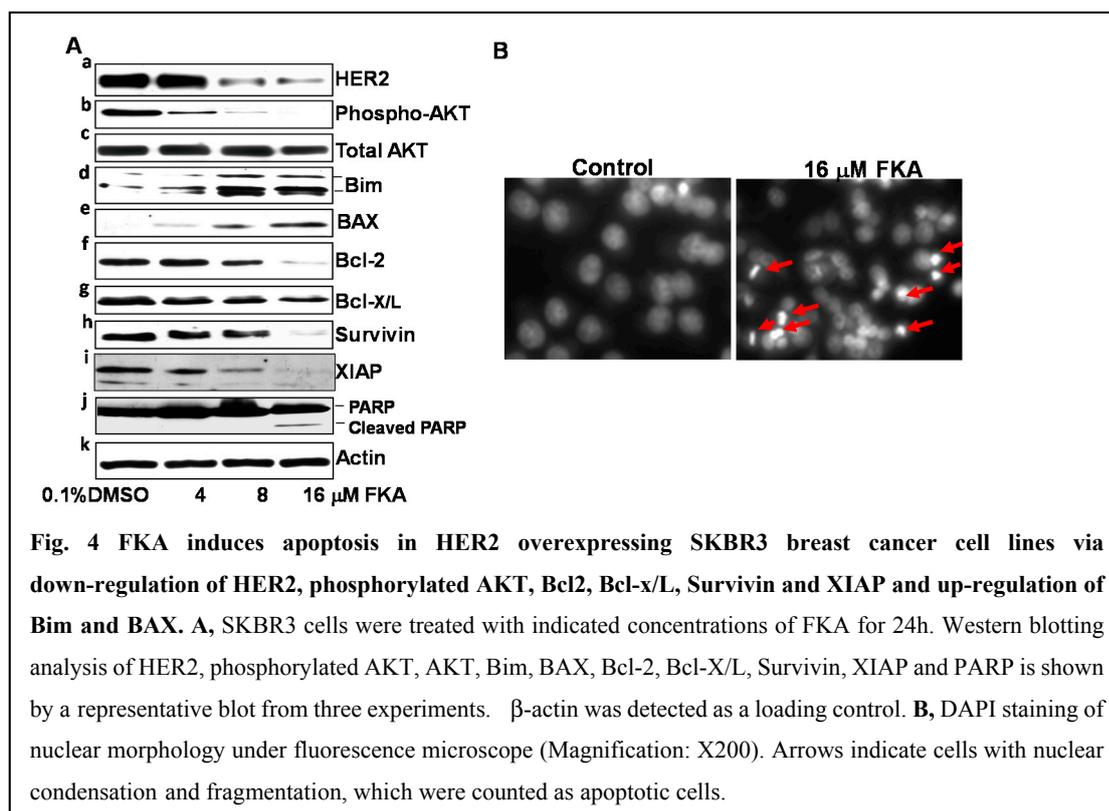
The mechanisms of FK A–induced G2M arrest in HER2 overexpressing SKBR3 cells are associated with inhibition of Cdc2 phosphorylation via down-regulation of Wyt1 and Wee1 expression and Cdc25C phosphorylation

Fig. 3A shows that FKA treatment resulted in a dose-dependent increase in Cdc2 kinase activity. Cdc2 kinase that is the driving force for G2M transition is activated by dephosphorylation of Cdc2 at Tyr15 [26]. HER2 was shown to bind to Cdc2 and phosphorylate Cdc2 at Tyr15, leading to a delay in G2M transition [26]. Fig.3B shows that FKA treatment decreased the phosphorylation levels of Cdc2 at the Tyr15 site in a dose-dependent manner without a change in Cdc2 protein expression. By using the MPM-2 antibody (an antibody specific for its preferential reactivity towards mitotic *versus* interphase cells can react with subsets of proteins that are phosphorylated upon entry into mitosis) [27], we further showed that FKA increased the expression of mitotic phosphoproteins (Fig. 3C), which confirmed an M phase arrest by FKA. The decrease in Cdc2 phosphorylation at Tyr15 after FKA treatments was accompanied by reduced expression of Cdc2 inhibitors Wee1 and Myt1 and dephosphorylation of Cdc25C. FKA treatment did not affect the expression of Cyclin B1. Taken together, these results suggest that FKA activated Cdc25C via its dephosphorylation at Ser216 and decreased the expression of Cdc2 inhibitors for promoting mitosis via dephosphorylation of Cdc2 at Tyr15 and then enhancement of Cdc2 kinase activity.

FKA induces apoptosis in HER2 overexpressing breast cancer SKBR3 cells.



We further examined the mechanisms by which FKA is more effective in inhibiting the growth of HER2 overexpressing breast cancer cell lines. We first examine whether FKA can regulate the receptor levels of HER2 in HER2 overexpressing SKBR3 cells. Fig. 4A panel a shows that flavokawain A caused a dose-dependent decrease in the protein levels of HER2. HER2 overexpression was reported to cause resistance to apoptosis by increasing the expression of antiapoptotic proteins (e.g. survivin, Bcl-2, and Bcl-X/L) and decreasing the expression of a proapoptotic protein Bim via activation of the AKT mediated survival



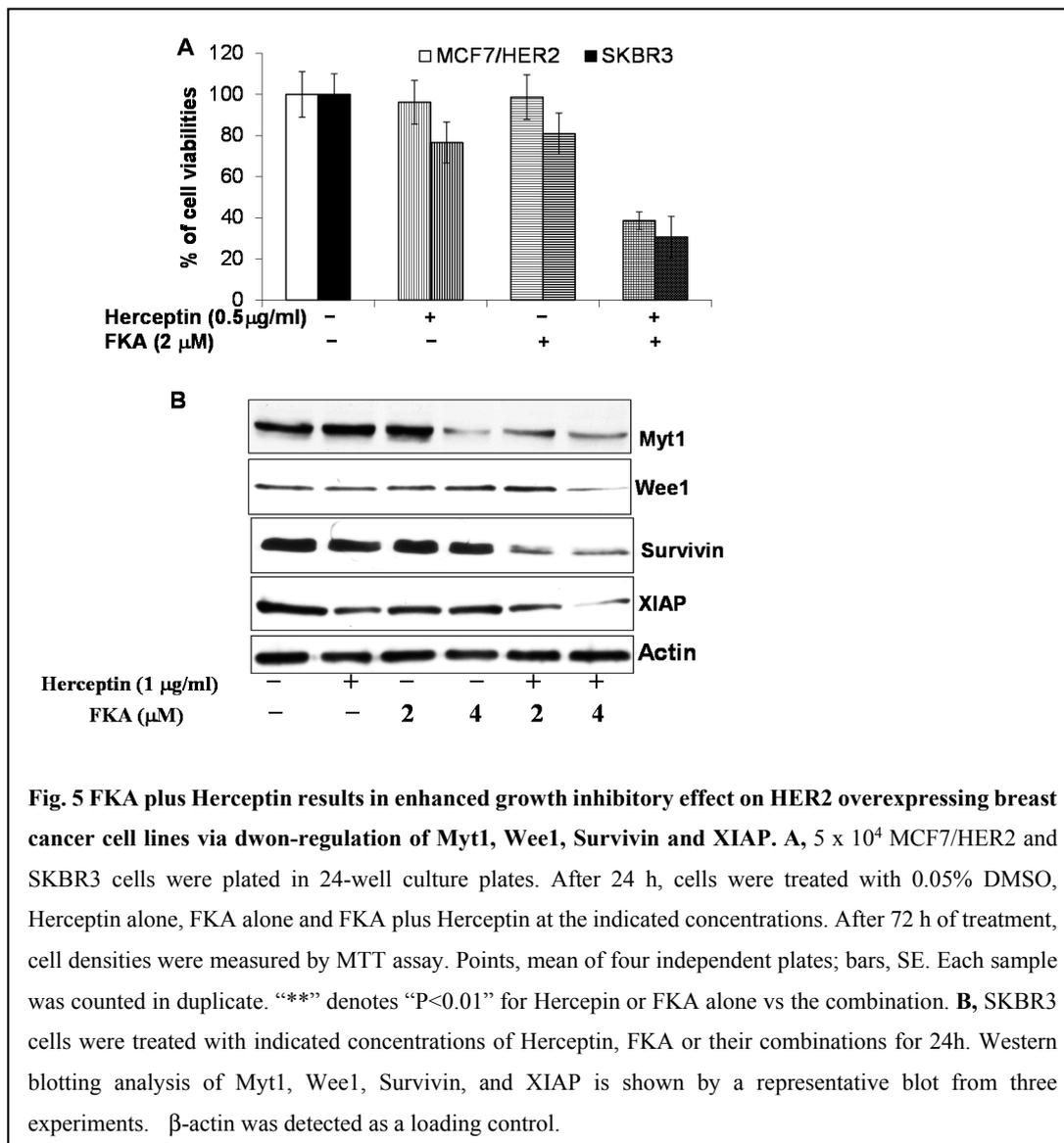
pathway [26, 28-31]. Therefore, we examine whether FKA can affect the expression of these HER2-modulated molecules in SKBR3 cells. FKA treatment resulted in decreased levels of AKT phosphorylation without change in its protein levels (Fig. 4A, panels b and c). In addition, FKA increased the expression of pro-apoptotic proteins Bim and Bax and decreased the expression of anti-apoptotic proteins survivin, XIAP, Bcl-2, and Bcl-x/L in a dose-dependent manner (Fig. 4A panels d to i). As a result of these alterations, FKA induced a cleavage of PARP protein as well as nuclear fragmentation and condensations, hallmarks for apoptosis (Figs. 4Aj and 4B). These results suggested that FKA exhibit a robust mechanism in induction of apoptosis in HER2 overexpressing SKBR3 cells.

Herceptin and FKA combination causes enhanced growth inhibitory effect on HER2 overexpressing breast cancer cells via down-regulation of Myt1, Wee1, Survivin and XIAP expression.

Herceptin is a major drug for treatment of HER2 positive and metastatic breast cancer and can improve patients' overall survival [32]. However, development of resistance to

Herceptin is common and the treatment is associated with cardiac dysfunction in 2-7% of cases [32]. Therefore, there is a need for a novel agent that can improve both the efficacy and safety of Herceptin treatment through combination. As shown in Fig.5A, Herceptin in combination with FKA reduced the viabilities of MCF7/HER2 and SKBR3 cells by 61% and 69%, respectively, whereas 2 μ M FKA alone only decreased the viabilities by 1% and 19%, respectively, and 0.5 mg/ml Herceptin by 4% and 23%, respectively (Fig. 5A). These results suggest that FKA and Herceptin may act synergistically to inhibit the growth of HER2 overexpressing breast cancer cells. Further experiment shows that FKA enhanced the inhibitory effects of Herceptin on the protein expression of Myt1, Wee1, Survivin and XIAP

(Fig.5B). The combined effect of Herceptin and FKA on down-regulation of Cdc2 inhibitors (i.e. Myt1 and Wee1) and inhibitors of apoptosis (i.e. Survivin and XIAP) is likely attributable to the enhanced growth inhibition of HER2 overexpressing breast cancer cells by these two agents.



Discussion:

The genetic and molecular heterogeneity of breast cancer represents a challenge for treatment and prevention strategies, and thus the development of innovative targeted therapies for specific tumor subtypes is crucial [33]. Overexpression of HER2 defines subtype of breast cancer associated with a poor clinical outcome [34], which has presented as a critical therapeutic target. Phase III clinical trials [35, 36] have demonstrated the efficacy of the humanized HER2 antibody Herceptin as a single agent for treatment of advanced breast cancer, while also improving survival when used as a first-line therapy in combination with chemotherapy. In addition, Herceptin has the potential for improving the outcomes among women with HER2 positive early breast cancer [37]. Nevertheless, the outcome of current therapies for HER2-positive breast cancer remains unsatisfying, as only a fraction of patients responds successfully to Herceptin therapy and risks of recurrence are still high. In addition, an increased risk of cardiac dysfunction associated with Herceptin therapy requires stringent criteria for selection of patients [38]. In this study, we have demonstrated that FKA, a kava chalcone, preferentially inhibited the growth of HER2 overexpressing breast cancer cells with a minimal effect on the growth of non-malignant breast epithelial cells. The growth inhibitory effect of FKA on HER2 overexpressing breast cancer cells is associated with G2M arrest in cell cycle progression and induction of apoptosis. In addition, Herceptin plus FKA treatment led to enhanced inhibitory effects on the growth of HER2 overexpressing breast cancer cells. These results indicates that FKA with excellent safety profile [18, 21] deserves further investigation for its potential use as an adjuvant agent in combination with Herceptin for treatment of HER2 positive breast cancer or as a preventative agent for targeting HER2 positive early breast cancer in preventing its recurrence and progression.

Overexpression of HER2 commonly causes the resistance of apoptosis by increasing anti-apoptotic proteins (e.g. Bcl-2, Bcl-x_L, Mcl-1, and Survivin) and decreasing the proapoptotic protein Bim, and thus contributes to tumor progression and the resistance to chemotherapy [9, 28-31]. The underlying mechanism(s) responsible for the antiapoptotic effects of ErbB2 overexpression may be due to the concomitant up-regulation of the PI3K-Akt/NF-κB survival pathway [29, 39]. We have shown here that FKA inhibits the PI3K/AKT pathway by down-regulation of HER2 protein expression and dephosphorylation of AKT. In addition, Kwon et al [40] reported that FKA blocked the lipopolysaccharides-induced activation of NF-κB in RAW 264.7 macrophages. Consistent with our previously published results in prostate and bladder cancer cell lines [15-18], we also have shown that FKA exhibited a robust mechanism of inducing apoptosis in HER2 overexpressing breast cancer cell lines by increasing the expression of proapoptotic proteins Bim and BAX and decreasing anti-apoptotic proteins (i.e. Bcl-2, Bcl-x_L, XIAP, and Survivin). Therefore, the accumulating evidences support that FKA can counteract the HER2 overexpression mediated apoptosis resistance by inhibiting the PI3K-Akt/NF-κB

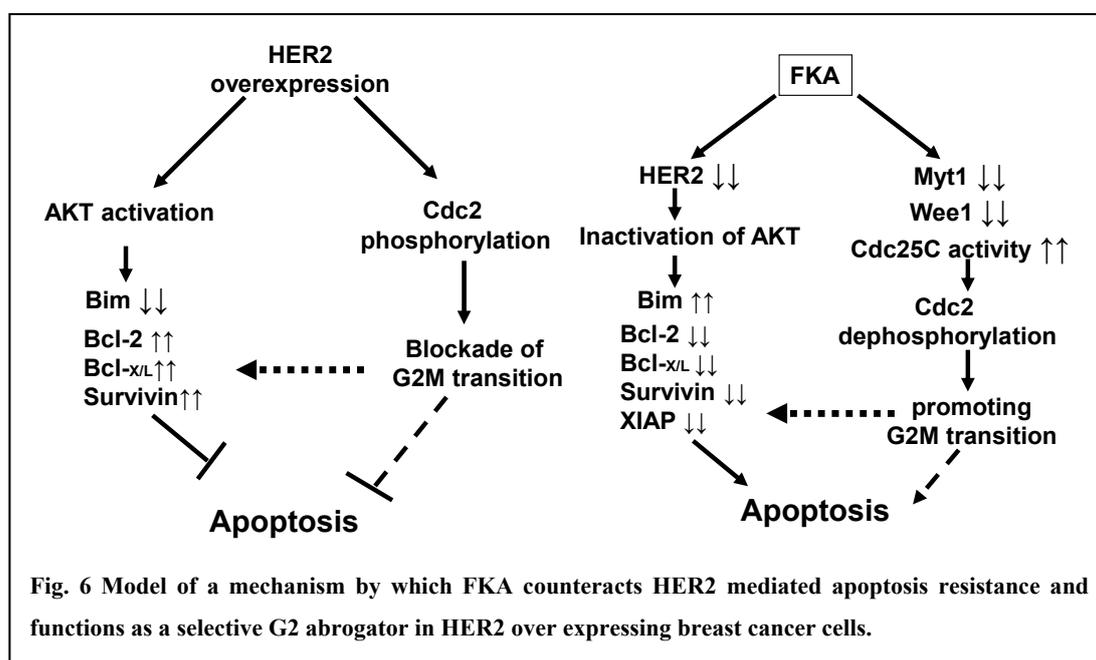
survival pathway, which leads to up-regulation of proapoptotic proteins and down-regulation of anti-apoptotic proteins.

Cell cycle progression through G1 phase, S phase (DNA replication), G2 phase, and M phase (mitosis and cytokinesis) is essential for cell growth. However, there are also two important cell cycle checkpoints at G1 and G2 phases, respectively, for controlling unlimited growth [41]. Cancer cells commonly develop defective G1 checkpoint via loss of tumor suppressors (e.g., p53, and RB), where G2 checkpoint is often intact and left to be critical in cancer cell survival [41]. HER2 overexpression or activation has been shown to activate G2 checkpoint in breast cancer cells by directly interacting with Cdc2 and then phosphorylating Cdc2 at Tyr15 and decreasing Cdc2 kinase activity [26, 42]. Therefore, HER2 overexpressing breast cancer often has a higher level of Cdc2 phosphorylation at Tyr15, which may be particularly susceptible to agents that can abrogate G2 checkpoint. We show here that FKA inhibits Cdc2 phosphorylation, increases Cdc2 kinase activity and induces G2M arrest in HER2 overexpressing breast cancer cell lines by both 1) de-phosphorylation of Cdc25C and 2) down-regulation of Cdc2 inhibitors: Myt1 and Wee1. Similarly, we previously reported that FKA selectively induces G2M arrest and growth inhibitory effect on p53 and pRb defective bladder and prostate cancer cell lines [16, 18]. In addition, FKA enhanced the growth inhibitory effect of HER2 specific antibody Herceptin on HER2 overexpressing breast cancer cell line by down-regulation of Myt1, Wee1, Survivin and XIAP.

S phase kinase-associated protein 2 (Skp2) is the F-box component of an E3 ubiquitin ligase complex, which recognizes and degrades many substrates, such as p27, MacroH2A1 and etc. for promoting cell cycle progression [43]. Skp2 depletion in melanoma cells and mouse embryonic fibroblasts (MEFs) results in G2M arrest and polyploidy accumulation [44, 45]. More importantly, Skp2 deletion in a Skp2 knockout mouse model has been shown by multiple groups to markedly restrict tumorigenesis under different conditions of tumor initiation and promotion, including PTEN, ARF, pRB inactivation as well as HER-2/Neu overexpression [46, 47]. Furthermore, Skp2 was associated with Herceptin sensitivity and suppression of Skp2 expression sensitized HER2 positive breast tumors to Herceptin treatment [48, 49]. Our recent publication also has demonstrated that FKA acts as a neddylation inhibitor to degrade Skp2 and inhibits prostate tumorigenesis in the TRAMP model [18]. In our studies, FKA down-regulated the protein expression of Skp2 in all tested cancer cell lines that were derived from prostate, breast, renal, liver, lung, colon and cervical cancers, melanoma and osteosarcoma regardless of their genetic background [18]. Therefore, the down-regulation of Skp2 expression may also contribute to the FKA induced G2M arrest and cell growth inhibition in HER2 overexpressing breast cancer. Further in vivo testing of FKA's effect on HER2 driven mammary carcinogenesis in a transgenic model and tumor growth in a xenograph model is warranted.

Our results in this study can be summarized as Fig. 6. HER-2 overexpression activates AKT, suppresses the expression of apoptotic protein Bim, and increases the expression of

antiapoptotic proteins Bcl2, Bcl-x/L and survivin, which then causes resistance to apoptosis. In addition, Her2 overexpression directly phosphorylates Cdc2 and inactivates Cdc2, which delays G2M transition and then increases the survival of cancer cells. We have shown that FKA can counteract the effect of HER2 overexpression-mediated resistance to apoptosis by inactivating AKT, with a subsequent increase of Bim expression and decrease of Bcl2, Bcl-x/L, XIAP and survivin; and by activating Cdc25C and down-regulating Myt1 and Wee1, which dephosphorylates and activates Cdc2 to promote G2M transition and premature mitosis. Therefore, FKA suggests its usefulness as a “selective G2M abrogator” aiming to target HER2 overexpressing breast cancer and may be a sensitizer for Herceptin-based therapies.



Materials and Methods

Cell lines, Compounds and Reagents

The SKBR3, MCF10A, MCF7, and MDA-MB-468 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines used in this study were within 20 passages after receipt. The cell lines were tested and authenticated by ATCC. MCF-7/HER2 cells [25] were provided by Dr. Alaoui-Jamali (McGill University). The MCF-10A cell line was grown in MEGM Bulletkit media. The MCF7, SKBR3 and MDA-MB-468 cell lines were cultured and passaged in minimum essential Eagle's medium, McCoy's 5A medium and Leibovitz's L-15 medium, respectively, supplemented with 10% fetal bovine serum (FBS). MCF-7/HER2 cells were maintained in EMEM medium with 10% FBS and 400 µg/ml G418 at 37°C and 5% CO₂. FKA (99%) were isolated from kava extracts by LKT Laboratories, Inc. (St. Paul, MN). Antibodies for HER2, phosphorylated AKT,

AKT, Bim, Survivin, XIAP, PARP, Cleaved PARP, Cdc25C, Cdc2, Wee1, Myt1, phosphorylated Cdc25C at Ser216 and phosphorylated Cdc2 at Tyr15 were from Cell Signaling Technology, Inc. Cyclin B, BAX, Bcl2, Bcl-xL and β -actin were from Santa Cruz Biotechnology, Inc. The antibody for MPM-2 was from Upstate Biotechnology. 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. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl- tetrazolium bromide (MTT) and propidium iodide was obtained from Sigma (St Louis, MO).

MTT assay

Cells were seeded onto 24 well plates at a density of 2×10^4 cells for 24 hours (h) and then treated with FKA at indicated concentrations. After 72 h incubation, 200 μ L of MTT solution was added to each well and incubated at 37°C for 2 h. The MTT solution was then aspirated and 200 μ L of dissolving buffer was added to each well. Cell viability was determined by measuring absorbance at 570 nm in a microplate reader (Bio-Rad, Hercules, CA). Dose response curves were generated as a percentage of vehicle control treated cells using Excel software, and IC₅₀ values were estimated graphically from the plot.

Soft Agar Colony Formation

A total number of 5,000 MCF7 or MCF7/HER2 cells were seeded on the top layer containing 0.35% solidified agar in complete medium in 6-well plates and the bottom layer consisted of 0.8% agar in complete medium. Vehicle control (0.05% DMSO) or indicated concentrations of FKA in complete medium were added and replaced every 3 days. After 3 weeks of cell seeding, the number of colonies was counted under an inverted phase-contrast microscope at 100 \times magnification and a group of >10 cells was indicated as a colony.

Flow Cytometric Analysis of Cell Cycle Distribution

MCF7, MCF7/HER2 and SKBR3 cells at 70% to 80% confluency were treated with 0.1% DMSO or 16 μ mol/L of FKA 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.

Western Blotting Analysis

Volumes of clarified protein lysates containing equal amounts of protein (50 μ g) were separated on 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to Hybond-ECL membranes (GE Healthcare, Piscataway, NJ). The blots were then probed with primary antibody, followed by

secondary antibodies as described previously [15]. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Thermo Scientific, Rockford, IL).

***In vitro* Kinase Assay**

Cdc2-associated H1 histone kinase activity was determined as described by Zi et al [16]. Briefly, using anti-Cdc2 antibody and protein A-agarose beads, Cdc2 was immunoprecipitated from 200 µg of protein lysate per sample as detailed above. Beads were washed three times with lysis buffer and then once with kinase assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, and 1 mM DTT). Phosphorylation of histone H1 was measured by incubating the beads with 40 µl of "hot" kinase solution [0.25 µL (2.5 µg) of histone H1, 0.5 µL of [γ -³²P] ATP, 0.5 µL of 0.1 mM ATP, and 38.75 µL of kinase buffer] for 30 min at 37 °C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS-PAGE, and the gel was dried and subjected to autoradiography.

4',6-diamidino-2-phenylindole (DAPI) Nuclear Staining

SKBR3 cells (4×10^4 cells/well) were cultured on chamber slides for 24 h. The cells were then treated with different concentrations of FKA for 24 h. After treatments, the cells were rinsed in $1 \times$ PBS for 3 times, and fixed in 4% paraformaldehyde. The fixed cells were mounted in Vector shield medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA) in a darkroom and visualized with a Nikon Eclipse TE2000-S (200 \times magnification) microscope under ultraviolet light. Apoptotic cells were identified by the nuclear condensation and fragmentation.

Statistical Analysis

Comparisons of cell density, number of colonies and relative levels of protein expression between the different treatments were conducted using Student's t test. All statistical tests were two sided. $P < 0.05$ was considered statistically significant.

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Author Contributions Statement

D.D. Jandial and L.S. Krill: Data acquisition and analysis, figures and manuscript preparation, and methodology development; L. Chen, C. Wu, Y. Ke, and J. Xie: Data acquisition; B.H. Hoang: Technical and material support; X. Zi: Conception and design, data interpretation, figures and manuscript preparation, administrative support and study supervision.

Competing Financial Interests

The authors declare no competing financial interests.

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