

ORIGINAL ARTICLE

Inhibition of cancer cell growth by Tangeretin flavone in drug-resistant MDA-MB-231 human breast carcinoma cells is facilitated via targeting cell apoptosis, cell cycle phase distribution, cell invasion and activation of numerous Caspases

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Summary

Purpose: In this study, the anticancer effects of a natural flavonoid-Tangeretin, were examined against the drug-resistant MDA-MB-231 breast cancer (BC) cell line and the normal breast cell line Hs 841.T.

Methods: The MTT assay was employed for cell viability determination. Apoptosis was demonstrated by DAPI and Annexin V/propidium iodide (PI) staining. Flow cytometric analyses were performed to gain insights about cell cycle distribution. Western blot assay was used for protein expression determination.

Results: Tangeretin inhibited the growth of the drug-resistant MDA-MB-231 cells concentration-dependently and its IC₅₀ was 9 μM, whereas the IC₅₀ was >100 μM against the normal cells. The anti-proliferative effects were due to in-

duction of apoptotic cell death. The apoptotic cell percentage was increased from 5.7% to around 69% as the concentration of Tangeretin was increased. Tangeretin also caused an increase in the Bax/Bcl-2 ratio and activation of the Caspase 3, 8 and 9. In addition, Tangeretin led to arrest of the cells at G2/M phase which was accompanied by depletion of cyclin B1 and D. Transwell assay showed that Tangeretin also reduced the invasion of the MDA-MB-231 cells.

Conclusion: The findings of this study suggest that Tangeretin exerts potent anticancer effects on the MDA-MB-231 cells and may therefore prove a beneficial lead molecule in BC research.

Key words: Tangeretin, breast cancer, anti-proliferative effect, cell cycle, caspases

Introduction

Over the years, flavonoids have gained tremendous pharmacological potential as anti-cancer agents. They have been reported to exert anti-proliferative effects on the cancer cells via different mechanisms [1]. These molecules interact with diverse cellular molecules such as enzymes and nucleic acids, to name a few. Flavonoids are found in almost all plant species and have been shown to be safe for consumptions [2]. The human diet contains

huge amounts of flavonoids and the intake of these metabolites has been shown to be associated with lower risk of diseases such as cancer [3]. Flavonoids have been shown to destroy the growth of cancer cells via cell cycle arrest, apoptosis, and necrosis [4]. Tangeretin is an important flavonoid with very important medicinal value [5]. It has been shown to exhibit anti-proliferative effects; for example, Tangeretin has been reported to cause apoptosis

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and cell cycle arrest of colon cancer cells [5]. In this study the anticancer effects of Tangeretin were examined against the drug-resistant human breast cancer (BC) cell line MDA-MB-231. Being one of the most devastating and prevalent types of cancer in women, BC causes considerable morbidity and mortality among patients [6]. It has been reported that BC causes 0.4 million deaths annually, accounting for 14% of all the cancer-related mortality worldwide [7]. Although, early breast cancers are treated with surgery followed by chemotherapy, the overall survival rate for advanced-stage metastatic cancers is still unsatisfactory. In addition, late diagnosis, development of chemo-resistance, and lack of safe and effective drug options are obstacles to the treatment of BC [8].

The major purpose of the current study was to investigate the anticancer activity of Tangeretin in MDA-MB-231 human breast cancer line along with examining its effects on apoptosis induction, cell cycle phase distribution and cell invasion.

Methods

Cell viability assay

The viability of the BC cells was measured by MTT assay. In brief, as the confluence of the MDA-MB-231 and normal human breast cells reached around 70%, they were seeded in 96-well plates in DMEM medium and treated with 0–200 μ M of Tangeretin. After a 24-h incubation, the cells were exposed to MTT for 4 h. Afterwards, the DMEM medium was removed and the colored formazan crystals were dissolved in 200 μ l of dimethyl sulfoxide. The viability of the MDA-MB-231 and the normal human BC cells was then determined by taking absorbance at 570 nm using a spectrophotometer.

DAPI and annexin V/PI staining

The MDA-MB-231 cells were grown in 6-well plates (0.6×10^6 cells/well) and incubated for 12 h. The MDA-MB-231 cells were exposed to Tangeretin (0, 6, 12 and 24 μ M) treatment for 24 h at 37°C. As the cells grew, 10 μ l cell cultures were put onto glass slides and subjected to staining with DAPI. The slides were then covered with a coverslip and examined with a fluorescent microscope. Annexin V/PI staining was performed as described previously [9].

Cell cycle distribution analysis

After incubating the MDA-MB-231 BC cells with varied concentrations of Tangeretin (0, 4.5, 9 and 18 μ M) for 24 h, the cells were washed with phosphate buffered saline (PBS). Afterwards, the MDA-MB-231 cells were stained with PI and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Cell invasion assay

The effects of Tangeretin were also examined by Transwell assay. In brief, the MDA-MB-231 cells were

treated with Tangeretin at 0, 4.5, 9, and 18 μ M concentrations and then subjected to transwell invasion assay as described previously [9].

Western blotting

The MDA-MB-231 cells were harvested and subjected to washing with ice-cold PBS. The pellet was then suspended in a lysis buffer at 4°C and which then shifted to 95°C. Thereafter, the protein content of each cell extract was checked by Bradford assay. About 40 μ g of protein were loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then treated with tris-buffered saline (TBS) and exposed to primary antibodies (for Bcl-2 and Bax) at 4°C. Then, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

Statistics

The experiments were performed in triplicate and data are shown as mean \pm SD. SPSS 17 was used for statistical analyses using Student's t-test and one-way ANOVA. $P < 0.05$ showed statistical significance.

Results

Tangeretin inhibits the proliferation of MDA-MB-231 breast cancer cells

The effects of Tangeretin (Figure 1A) on the viability of MDA-MB-231 and normal Hs841.T human breast cells were studied by MTT assay at

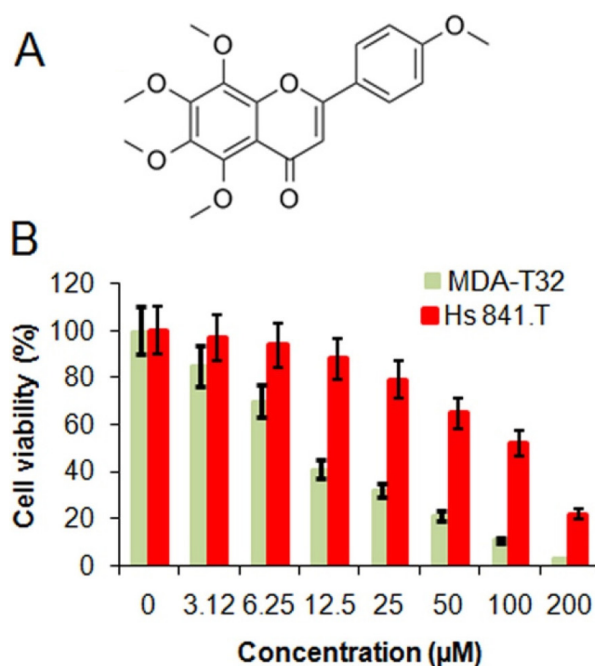


Figure 1. A: Structure of Tangeretin. **B:** Viability of Tangeretin on the MDA-MB-231 cells as indicated by MTT assay. The experiments were performed in triplicate and shown as mean \pm SD ($p < 0.05$).

concentration ranging from 0 to 200 μM . The IC_{50} was calculated by absorbance at 570 nm using a spectrophotometer. It was observed that Tangeretin suppressed the growth of the MDA-MB-231 cells in a dose-dependent manner (Figure 1B). The IC_{50} of Tangeretin against the MDA-MB-231 cells

was found to be 9 μM . Nonetheless, the effects of Tangeretin on the viability of the normal human Hs841.T breast cells were less pronounced. The IC_{50} of Tangeretin against the normal human breast cells was >100 μM (Figure 1B).

Tangeretin induces apoptotic cell death of MDA-MB-231

To understand the mechanism behind the anti-proliferative effects of Tangeretin on the MDA-MB-231 cells, the Tangeretin-treated MDA-MB-231 cells were stained with DAPI. The DAPI staining indicated that the cells with white coloured nuclei or DAPI-positive cells increased, which was indicative of apoptosis (Figure 2). The annexin V/PI staining showed that the percentage of apoptotic cells in the untreated cell samples was 5.3%. As the concentration of Tangeretin was increased to 18 μM , the apoptotic cell percentage increased to around 69%, indicative of the dose-dependent apoptotic effects of Tangeretin (Figure 3).

Effects of Tangeretin on Bax and Bcl-2 expression and caspase activation in MDA-MB-231 cells

The effects of Tangeretin were examined for the Bax and Bcl-2 expression of MDA-MB-231 cells, which are considered as important biomarker proteins of apoptosis. The results of the western blot analysis showed that Bax expression was increased dose-dependently and Bcl-2 was decreased,

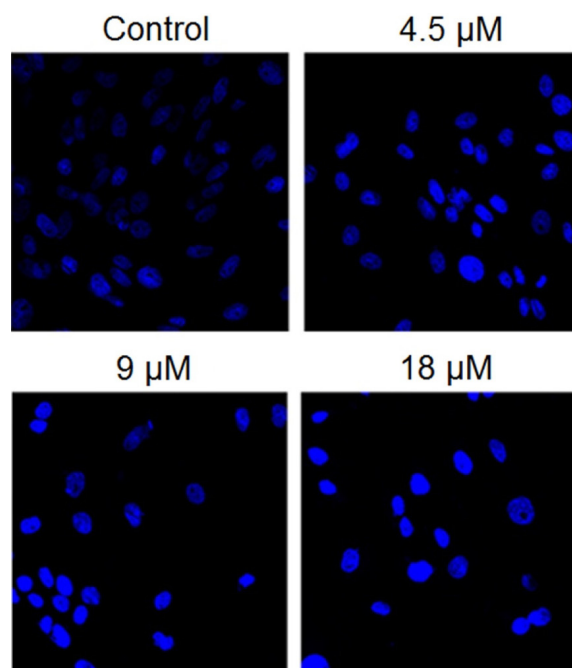


Figure 2. DAPI staining, showing concentration-dependent Tangeretin-induced apoptosis in the MDA-MB-231 cells. The experiments were performed in triplicate.

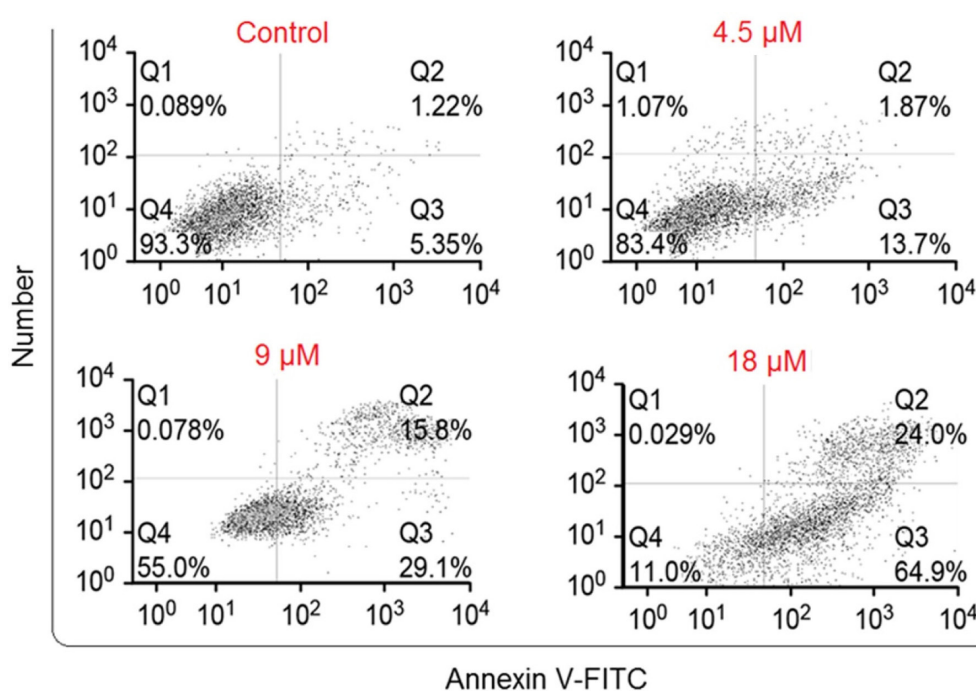


Figure 3. Flow cytometry after Annexin V/PI staining, showing the percentage of the apoptotic MDA-MB-231 cells. The percentage of both early and late apoptotic cells increased significantly with increasing doses of Tangeretin. The experiments were performed in triplicate.

indicative of apoptosis (Figure 4). The effects of Tangeretin were also investigated on the expression of caspase, 3, 9, and 8. The results showed that Tangeretin caused significant enhancement in the expression of all the three caspases, i.e. Caspase 3, 8 and 9 (Figure 5).

Tangeretin causes G2/M arrest of the MDA-MB-231 cells

The effects of Tangeretin were also investigated on the distribution of MDA-MB-231 in differ-

ent phases of the cell cycle at 0, 4.5, 9, and 18 μ M concentrations. The results showed that Tangeretin caused dose-dependently increase in the G2 phase of the cell cycle, suggesting that Tangeretin triggers the arrest of the cells at the G2/M check point of the cell cycle. The G2/M phase cells increased from 12.78% to 21.86% at 18 μ M concentration of Tangeretin (Figure 6). The G2/M arrest of the cell cycle was also accompanied with deletion of the cyclin B1 and D protein expression in a concentration-dependent manner (Figure 7).

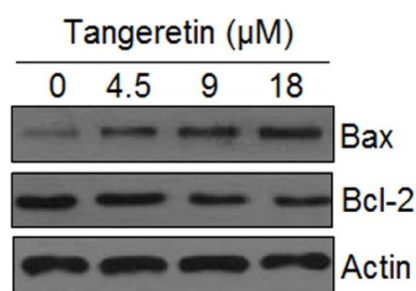


Figure 4. Effect of Tangeretin on the expression of Bax and Bcl-2 in MDA-MB-231 cells as indicated by western blot analysis. The expression of Bax protein increased while the expression of Bcl-2 decreased. The experiments were performed in triplicate.

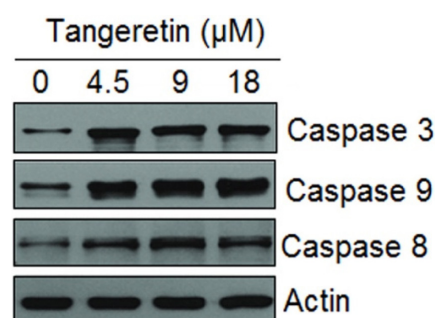


Figure 5. Effect of Tangeretin on the expression of caspases in MDA-MB-231 cells as indicated by western blot analysis. Tangeretin treatment led to increase in the expression of caspase-3, 9 and 8. The experiments were performed in triplicate.

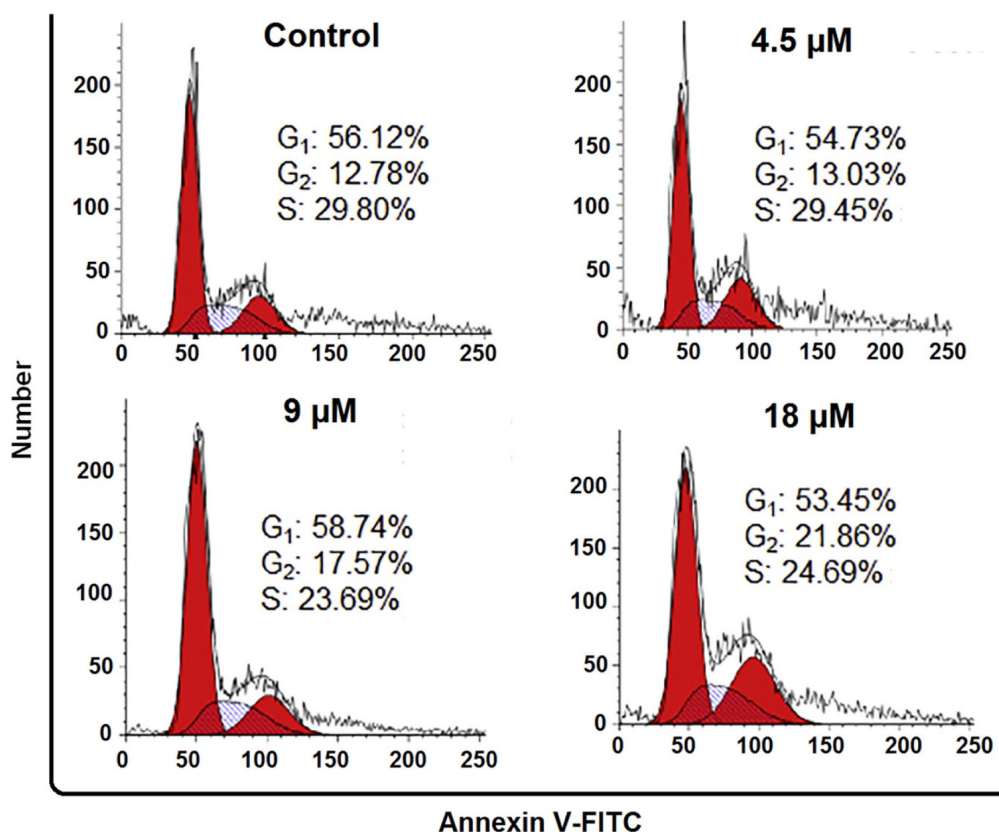


Figure 6. Effect of Tangeretin on the cell cycle distribution of MDA-MB-231 cells as indicated by flow cytometry. Tangeretin treatment led to G2/M cell phase arrest. The experiments were performed in triplicate.

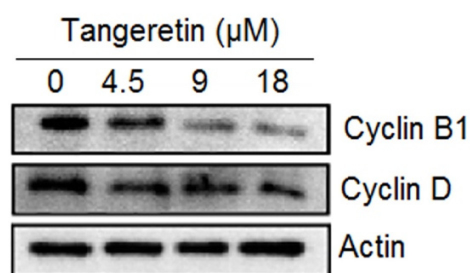


Figure 7. Effect of Tangeretin on the expression of cyclin B1 and D in MDA-MB-231 cells as indicated by western blot analysis. Tangeretin led to decrease in the expression of both cyclin-B1 and cyclin-D. The experiments were performed in triplicate.

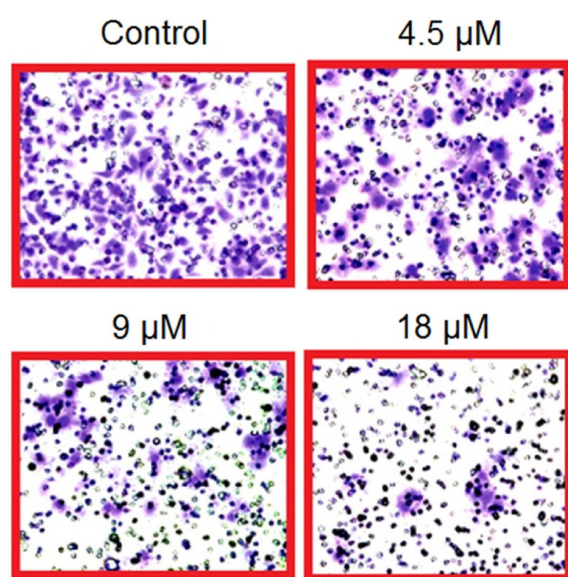


Figure 8. Effect of Tangeretin on the invasion in MDA-MB-231 cells as indicated by transwell invasion assay. Tangeretin treatment led to inhibition of cell migration. The experiments were performed in triplicate.

Tangeretin inhibits the invasion of the MDA-MB-231 cells

The transwell assay was employed to investigate the effects of Tangeretin on the invasion of the MDA-MB-231 drug-resistant BC cells at 0, 4.5, 9, and 18 μM concentrations. The results revealed that Tangeretin decreased the invasion of the MDA-MB-231 cells. These effects of Tangeretin were found to be dose-dependent (Figure 8).

Discussion

BC is one of the most common and lethal types of cancers [10]. The treatment of BC is chal-

lenged by late diagnosis, presence of metastasis, and the adverse effects of chemotherapeutic drugs [11]. Plants serve as an amazing repository of molecules with potential anticancer effects, which may prove beneficial in the development of the systemic therapy of BC [12]. In this study, the anticancer effects of Tangeretin - a plant derived flavonoid - were examined against the drug-resistant MDA-MB-231 cells and normal breast cells. What we found was that Tangeretin showed dose-dependent anti-proliferative effects on the MDA-MB-231 cells. However, Tangeretin exhibited comparatively less toxic effects on the normal human breast cells. These results are in agreement with investigations wherein Tangeretin has been reported to inhibit the growth of human gastric cancer cells through both extrinsic and intrinsic apoptotic pathways [13]. The metabolites of plant origin have been shown to suppress the proliferation of cancer cells via several different mechanisms such as apoptosis, cell cycle arrest and autophagy [2]. Herein, we observed that Tangeretin decreased the viability of the MDA-MB-231 cells by inducing apoptotic cell death. Apoptosis of cancer cells is essential to prevent the proliferation, metastasis, and development of chemoresistance among cancers [14]. The Tangeretin-induced apoptosis was also accompanied by increase in the expression of Bax and downregulation of Bcl-2 expression. Tangeretin also caused activation of caspases 3, 8, and 9, which is hallmark of apoptosis [15]. This molecule has been shown to induce the G2/M arrest of colorectal cancer cells via upregulation of p21 and p27. Herein, we also found that Tangeretin caused arrest of the MDA-MB-231 cells at the G2/M check point, which was accompanied with suppression of cyclin B1 and D expression. The effects of Tangeretin were also examined for invasion of the MDA-MB-231 cells, which is one of the important mechanisms for metastasis of cancer cells [16]. Herein, we found that Tangeretin could inhibit concentration-dependently the invasion of cancer cells. In conclusion, Tangeretin is an important flavonoid which suppresses the growth of drug-resistant BC cells via induction of apoptosis and cell cycle arrest. Tangeretin could also suppress the invasion of the cancer cells and may therefore prove important for the treatment of BC.

Conflict of interests

The authors declare no conflict of interests.

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