European Journal of Pharmacology 863 (2019) 172658

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

Flavopereirine induces cell cycle arrest and apoptosis via the AKT/p38 MAPK/ERK1/2 signaling pathway in human breast cancer cells

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ARTICLE INFO

Keywords:
Flavopereirine
Cell cycle
Apoptosis
MAPK
Breast cancer

ABSTRACT

Breast cancer, which is the most frequently diagnosed cancer, is quite heterogeneous. For breast cancer subtypes lacking targeted therapies, it is vitally essential to find novel agents that prevent chemoresistance and metastatic relapse. Flavopereirine is a β-carboline alkaloid that has antiplasmodial activity, and its antiproliferative effect in different cancers remains unclear. The effect of flavopereirine on cell cycle arrest and apoptosis signaling in breast cancer cells was analyzed by flow cytometry. An inhibitor and siRNA were used to confirm the related signaling pathways by Western blot analysis. We found that flavopereirine caused G0/G1 phase arrest in MCF-7 cells and S phase arrest in MDA-MB-231 cells. MDA-MB-231 cells were more sensitive to flavopereirine-induced apoptosis. Furthermore, we found that flavopereirine-induced apoptosis was partially reduced in MDA-MB-231 cells treated with an extracellular regulated kinase (ERK) inhibitor and p38 mitogen-activated protein kinase (MAPK) siRNA. Moreover, p38 siRNA treatment simultaneously reduced phosphorylated ERK expression levels. Conversely, the recovered phosphorylation of AKT decreased the levels of p-ERK and p-p38 MAPK. Overall, flavopereirine induces cell cycle arrest and the AKT/p38 MAPK/ERK signaling pathway, which contribute to flavopereirine-induced apoptosis in MDA-MB-231 cells.

1. Introduction

Breast cancer is the most common cancer and the leading cause of cancer-related death (accounting for 15% of all cancer deaths) in women worldwide (Ferlay et al., 2015). Based on genetic profiling and molecular features, breast cancer is considered quite heterogeneous. Each breast cancer subtype may have a different treatment response and prognosis (Heiser et al., 2012). Standard chemotherapy is the main systemic treatment for advanced breast cancer and triple negative breast cancer (TNBC), which is a specific subtype of epithelial breast cancer (Crown et al., 2012). TNBC is defined as negative for the estrogen receptor (ER) and progesterone receptor (PR) and lacks human epidermal growth factor receptor 2 (HER2) (Schneider et al., 2008). TNBC patients generally present early visceral metastases and have a poor prognosis (Dent et al., 2007; Ovcaricek et al., 2011). Metastatic relapse and chemoresistance are difficult challenges for TNBC patients due to a lack of targeted therapies.

Flavopereirine is a β-carboline alkaloid that can be extracted from the bark of trees (*Geissospermum sericeum and G. laeve*) (Steele et al., 2002). Flavopereirine has antiplasmodial activity and can selectively inhibit cancer cell DNA synthesis in vitro but not DNA synthesis in healthy tissues (Beljanski and Beljanski, 1982; Steele et al., 2002). Moreover, chemically synthesized flavopereirine has been demonstrated to selectively inhibit human glioblastoma (U251) cell proliferation but does not inhibit normal astrocyte (CRL 1656) proliferation (Beljanski, 2000). However, the antiproliferative effect of flavopereirine on other cancers remains unclear. Therefore, it is necessary to investigate the effect of flavopereirine on breast cancer, which is the most common cancer in women.

In breast cancer, tumor cell proliferation is modulated by key factors, including AKT, nuclear factor κB (NF-κB), mitogen-activated protein kinases (MAPKs), and reactive oxygen species (ROS) (Liou and

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https://doi.org/10.1016/j.ejphar.2019.172658
Received 19 February 2019; Received in revised form 28 August 2019; Accepted 9 September 2019
Available online 10 September 2019
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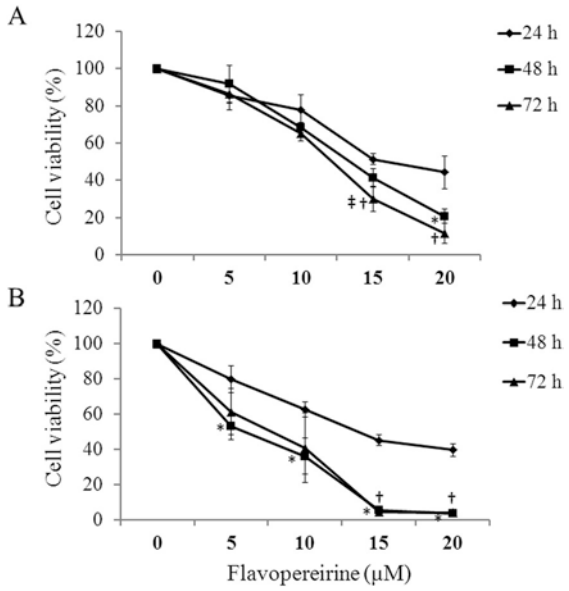

Fig. 1. Growth inhibitory effect of flavopereirine on breast cancer cells. MCF-7 (A) and MDA-MB-231 (B) cells were treated with DMSO or different doses of flavopereirine for 24, 48 and 72 h. Cell viability was determined by a CCK-8 assay. Cell viability is presented as the mean ± S.D. of three independent experiments. * and † indicate a significant difference ($P < 0.05$) comparing the effect of flavopereirine treatment at 24 h with that at 48 h and 72 h as analyzed by one-way ANOVA followed by Bonferroni test. Additionally, ‡ indicates a significant difference in cell viability between the 48 h and 72 h time points.

Storz, 2010). MAPK pathways modulate numerous proteins involved in regulating the cell cycle, migration, proliferation and apoptosis (Wada and Penninger, 2004). AKT (serine/threonine kinases) activates downstream proteins involved in promoting cellular proliferation by regulating the cell cycle and apoptosis (Xu et al., 2012). Moreover, MCF-7 is an ER-positive luminal A cell line, and MDA-MB-231 is a TNBC cell line, which are two commonly used breast cancer lines used to compare the effects of drugs on their different genotypes. To improve TNBC treatment and overcome chemoresistance, it is important to investigate new drugs with increased therapeutic efficiency. In this study, we attempted to investigate the inhibitory effects of flavopereirine on the MCF-7 and MDA-MB-231 breast cancer cell lines, and we further explored the related molecular mechanisms, including cell cycle arrest and apoptosis.

2. Materials and methods

2.1. Breast cancer cell lines and cell culture

MCF-7 and MDA-MB-231 human breast cancer cell lines were obtained from Dr. Ying-Ray Lee. The MCF-7 cell line was cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 8% fetal bovine serum (FBS). MDA-MB-231 cells were cultured in low glucose Dulbecco's modified Eagle's medium (Gibco-BRL, Carlsbad, CA, USA) containing 8% FBS. The two cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Reagents

Flavopereirine perchlorate was purchased from ChromaDex, Inc. (Irvine, CA, USA). The pan-caspase inhibitor z-VAD-fmk was obtained from Adooq Bioscience (Irvine, CA, USA). PD98059 (TargetMol, Boston, MA, USA), which is a non-ATP-competitive MEK inhibitor, was used to inhibit the phosphorylation of extracellular regulated kinase (ERK).

2.3. Viability assay

A total of 5×10^3 cells suspended in culture medium were seeded in the wells of 96-well microplates. After overnight cell adhesion, the cells were treated with medium containing 0.01% dimethyl sulfoxide (DMSO) or flavopereirine at concentrations of 5, 10, 15, and 20 μM. After 24, 48 and 72 h of incubation, a Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich, Inc., St. Louis, MO, USA) assay was used to evaluate cell viability. The cells were incubated with WST-8 solution for 2 h and analyzed using a Model 680 microplate reader (absorbance at 450 nm and reference absorbance at 655 nm) (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The percentage of metabolically active cells was calculated using the following formula: Atreated/Acontrol.

2.4. Cell cycle distribution and apoptosis assay

For cell cycle analysis, 6×10^5 cells were cultured in 10-cm dishes overnight. Then, they were in serum free medium for 24 h and further incubated with flavopereirine or 0.01% DMSO for 24 and 48 h. The cells were detached with trypsin and centrifuged at 430 × *g* for 10 min. Then, the cell pellets were suspended in 1 × phosphate-buffered saline (PBS) (1 ml) and fixed with methanol (2 ml), which was stored at 4 °C. After washing with 1 × PBS, the cells were stained with 200 μg/ml propidium iodide (PI) (Sigma-Aldrich, Inc.) containing RNase (2 mg/ml) for 30 min in the dark at room temperature. A FACSCan flow cytometer (Becton Dickinson, San Diego, CA, USA) was used to determine the cell cycle profiles. DNA content was further analyzed by Modfit LT 3.3 software. For the apoptosis analysis, 1.6×10^5 cells were cultured in 6-well plates and incubated with flavopereirine (10 and 15 μM) or 0.01% DMSO for 48 h. The cells were stained with an Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (Cat. No. AVK250, Strong Biotech Corporation, Taiwan) and analyzed by flow cytometry.

2.5. Mitochondrial membrane potential (MMP)

MMP was analyzed using rhodamine 123 (R8004) (Sigma-Aldrich, Inc.). Rhodamine 123 is a cell-permeating, cationic, mitochondria-specific fluorescent dye without cytotoxic effects. The fluorescence decay rate is proportional to the MMP (Baracca et al., 2003). Cells were seeded in 6-well plates (1×10^5 cells/well) overnight and were then treated with DMSO (control) or flavopereirine (15 μM) for 24 or 48 h. Next, the cells were stained with rhodamine 123 at a final concentration of 5 μM for 30 min at 37 °C in the dark. The cells were detached and washed with 1 × PBS, and the fluorescence intensity was measured by a flow cytometer.

2.6. Western blotting

Cells were lysed with the M-PER™ mammalian protein extraction reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) containing 0.1% protease inhibitor cocktail. Each sample (40 μg protein) was loaded and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to polyvinylidene fluoride (PVDF) membranes. Proteins regulating the cell cycle were detected by anti-cyclin A2 (GTX103042), anti-cyclin D1 (GTX112874), anti-cyclin E1 (GTX103045), anti-CDK4 (GTX102993), anti-CDK6 (GTX103992) and anti-p27 Kip1 (GTX100446) antibodies purchased from GeneTex, Inc. (Irvine, CA, USA). Anti-Cdk2 (phospho-T14, ab68265) and anti-p21 Waf1/Cip1 (#2947) antibodies were purchased from Abcam (Cambridge, MA, USA) and Cell Signaling Technology, Inc. (Beverly, MA, USA). The primary antibodies associated with apoptosis were selective for AKT (#4691), phospho-AKT (Ser 473) (#4060), caspase-3 (#9662), caspase-9 (#9502), PARP (#9542), p38 MAPK (#9212), phospho-p38 MAPK (Thr180/Tyr182) (#9211), p44/42 MAPK (ERK1/2) (#4695), phospho-p44/42 MAPK (Thr202/Tyr204)

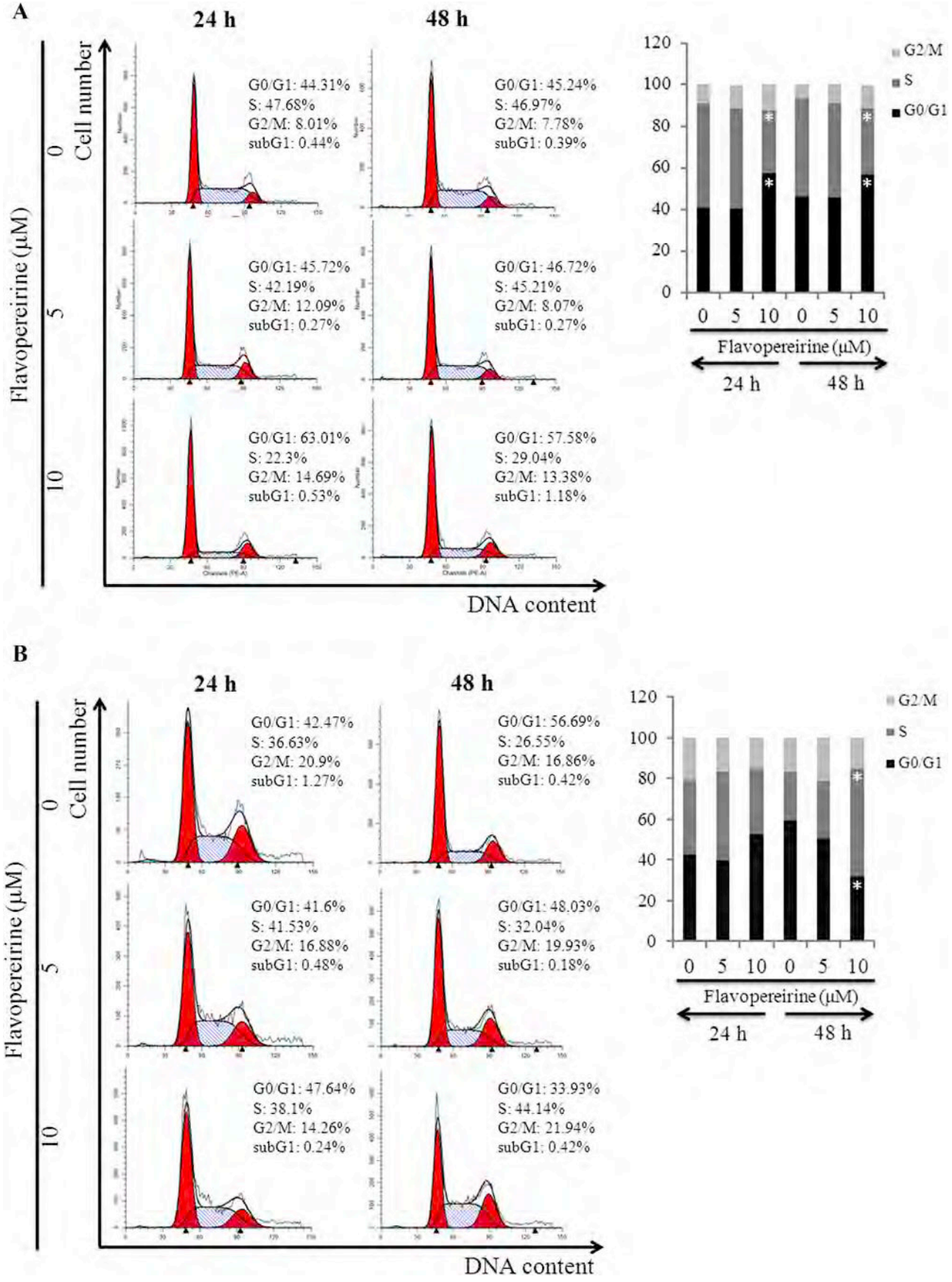

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Fig. 2. Flavopereirine caused cell cycle arrest. MCF-7 (A) and MDA-MB-231 (B) cells were treated with different doses of flavopereirine and incubated for 24 and 48 h. The cellular DNA content was stained with PI and analyzed by flow cytometry. The graphs show the cell cycle distribution from one representative experiment, and the mean percentage of cells in each phase of the cell cycle calculated from three independent experiments is shown in the histogram. * indicates a significant difference ($P < 0.05$) compared to the percentage of DMSO control in G0/G1 and S phase as analyzed by one-way ANOVA followed by Bonferroni test. (C) Cell cycle-regulated protein expression was determined by western blotting after 48 h of flavopereirine treatment. GAPDH was used as a loading control. * indicates a significant difference ($P < 0.05$) compared to the DMSO control as analyzed by one-way ANOVA followed by Bonferroni test. # indicates a significant difference between the two marked groups.

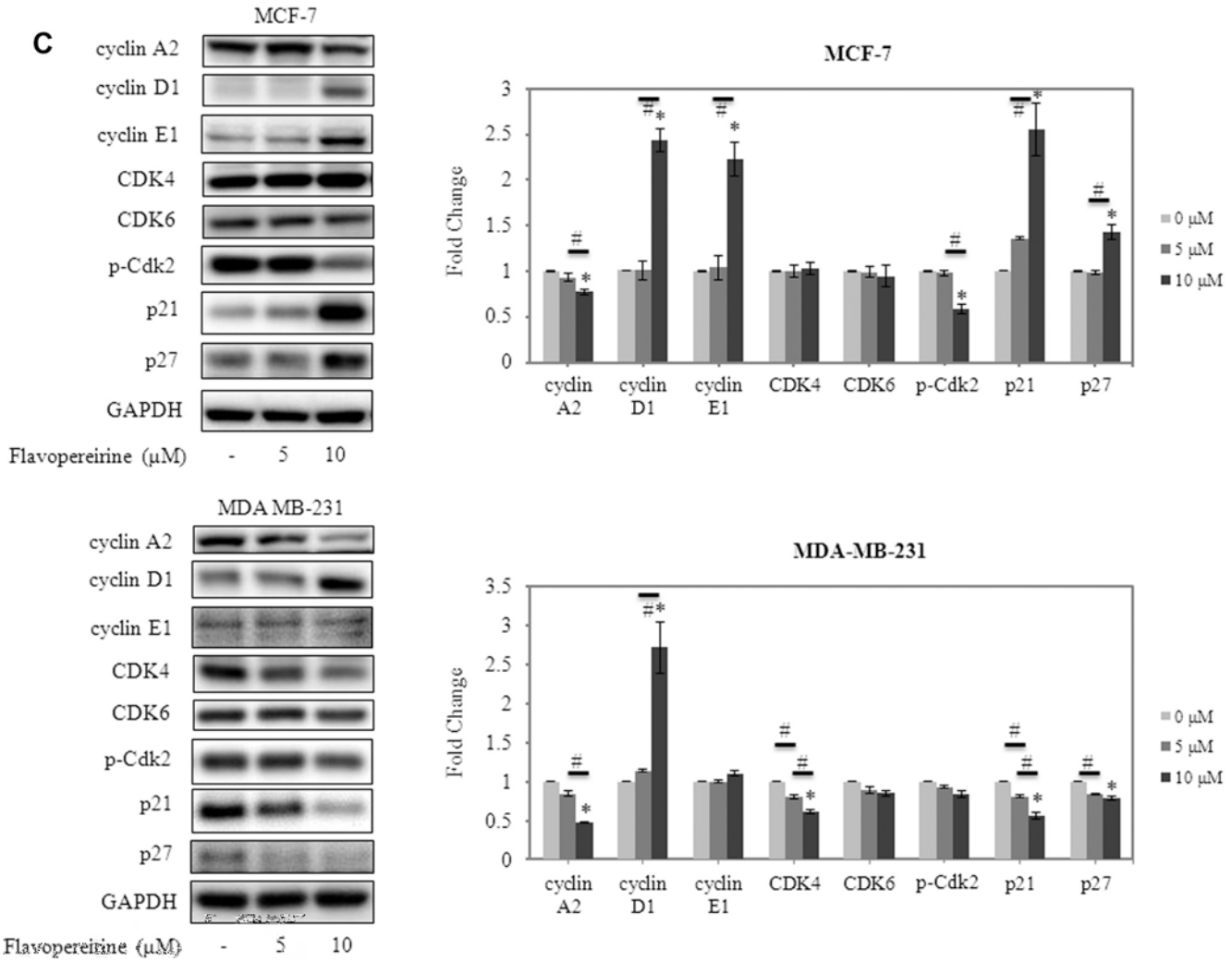
(#4377), JNK2 (#9258), and phospho-SAPK/JNK (Thr183/Tyr185) (#4668) and were obtained from Cell Signaling Technology, Inc.. An anti-caspase 8 (GTX110723) antibody was purchased from GeneTex, Inc. An anti-Bcl-XL antibody (ab32370) was supplied by Abcam. After incubation with secondary antibodies conjugated to horseradish peroxidase (HRP), Immobilon Western Chemiluminescent HRP substrate (EMD Millipore Corporation, Billerica, MA, USA) was added, and the signals were detected using a BioSpectrum Imaging System (UVP).

2.7. RNA interference

Small interfering RNAs (siRNAs) were purchased from Ambion (Life Technologies, Carlsbad, CA, USA). Knockdown of p38 MAPK expression was achieved using a mixture of Silencer Select Predesigned (ID s3585) and Validated siRNA (ID s11156) targeting human alpha p38 (MAPK 14) and beta p38 MAPK (MAPK11). MDA-MB-231 cells seeded in 6-well plates (1×10^5 cells/well) were incubated overnight and then treated with 25 pmole siRNAs using Lipofectamine™ RNAiMAX transfection reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Silencer Select Negative Control siRNA was used as the transfection baseline. After transfection for 6 h, the cell supernatants were replaced with cell medium and incubated overnight. MDA-MB-231 cells transfected with p38 MAPK siRNA were further treated with DMSO (control) or the specified concentrations of flavopereirine for 48 h. The cells were collected and harvested for apoptosis assays, western blotting.

2.8. Plasmid DNA transfection

Cells were seeded in 6-well plates (2×10^5 cells/well) overnight and transfected with Lipofectamine™ 3000 and P3000™ transfection reagent (Life Technologies, Carlsbad, CA, USA) with modified protocols based on the manufacturer's instructions. Briefly, two tubes that contained 125 µl Opti-MEM™ medium were prepared. One tube was used to dilute the Lipofectamine™ 3000 reagent (4.5 µl) and was mixed well. The other tube was used to dilute plasmid DNA (1.5 µg) and was then



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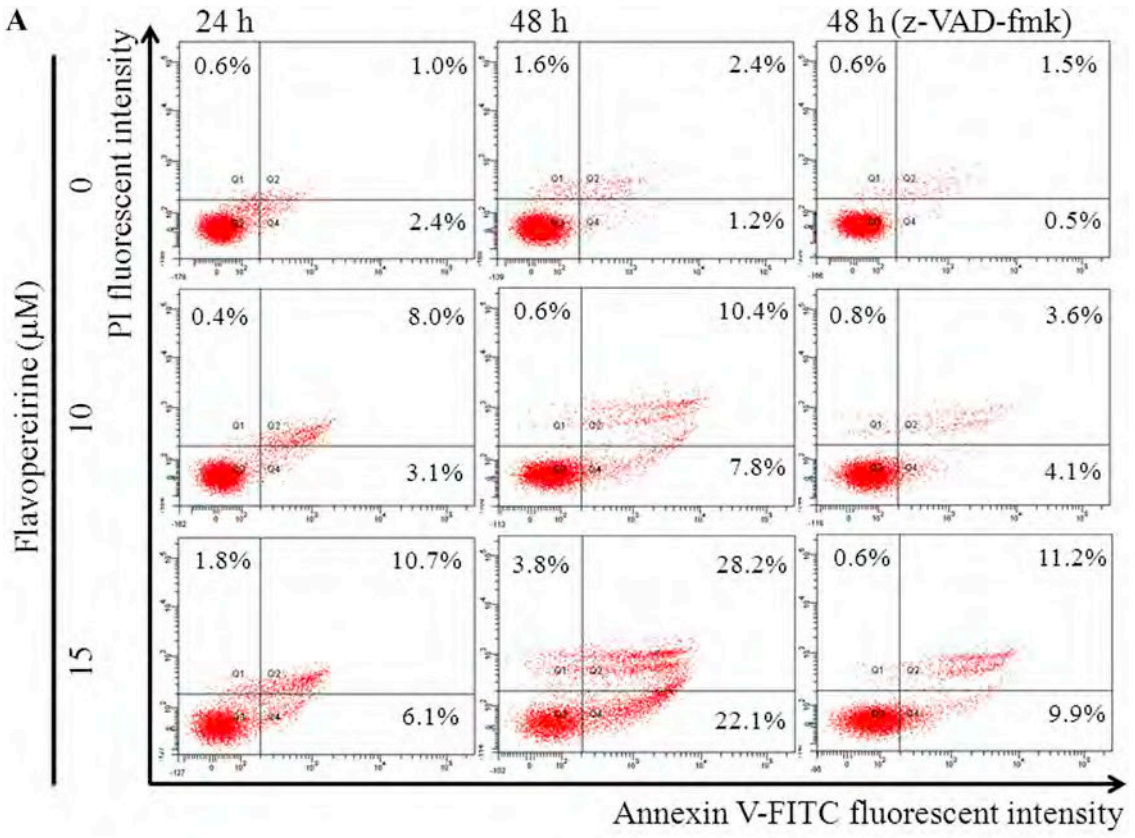

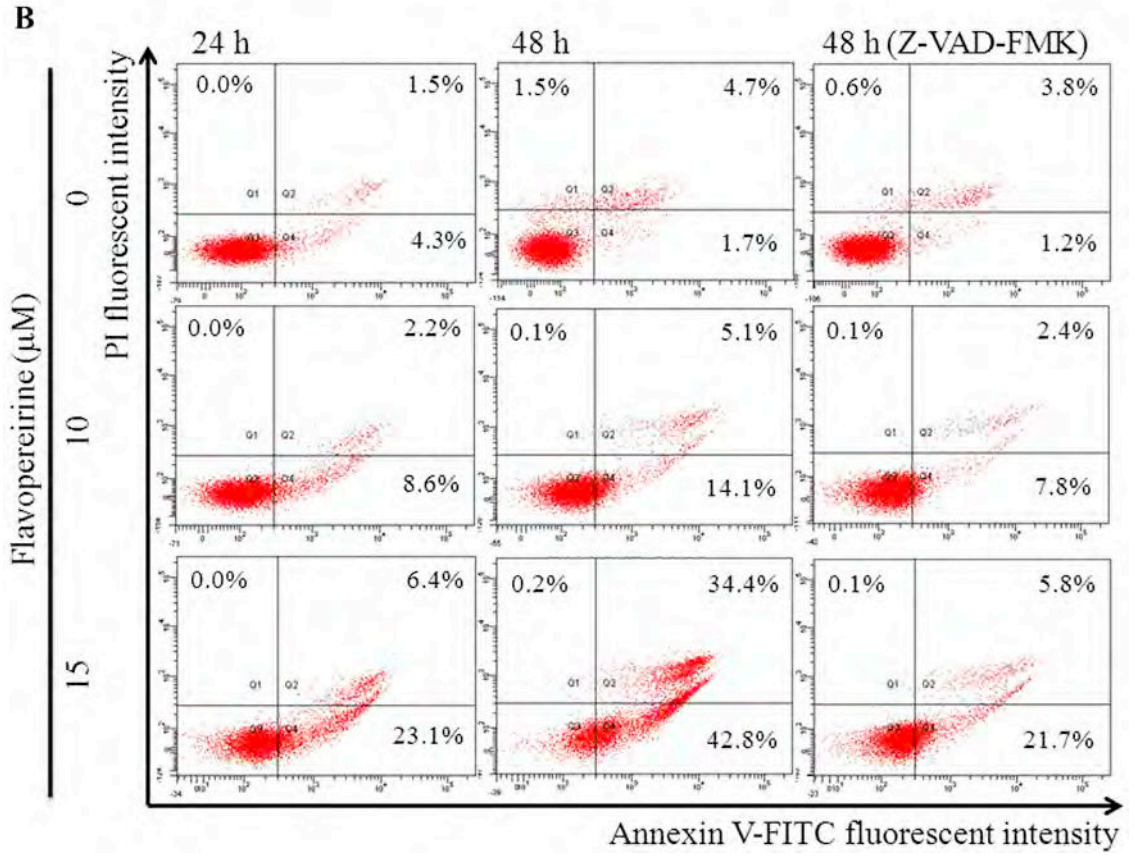

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Fig. 3. Flavopereirine induced mitochondria-dependent apoptosis. MCF-7 (A) and MDA-MB-231 (B) cells were treated with DMSO or different doses (10 and 15 μM) of flavopereirine for 24 and 48 h. Annexin V-FITC was used to stain apoptotic cells and to determine the percentage of apoptotic cells by flow cytometry. The data are representative of three independent experiments with similar results. (C) Three independent apoptosis experiments for both cell lines were quantified, and the data are presented as the mean ± S.D. (D) The activation of caspases in MDA-MB-231 cells with flavopereirine treatment for 48 h were determined by western blotting. The pan-caspase inhibitor z-VAD-fmk (Z) was used at a concentration of 20 μM to confirm the caspase-dependent apoptotic pathway. (E) The MMP of MDA-MB-231 cells treated with DMSO or flavopereirine for 24 and 48 h was analyzed with rhodamine 123 and detected by flow cytometry. The proportion of rhodamine intensity shifted to the left indicated that the cells lost MMP. Quantitative analysis based on three independent experiments is presented in the histograms. * indicates a significant difference compared to the DMSO control, and # indicates a significant difference between the two marked groups. † indicates a significant difference between 10 and 15 μM of flavopereirine treatment.

mixed with the P3000™ reagent (3 μl). The two tubes were mixed to form DNA-lipid complexes and incubated for 10 min before adding to the cells. After transfection, the procedures for medium changes and flavopereirine treatment were the same as those for p38 MAPK siRNA transfection. Then, the cells were collected and harvested for western blotting, apoptosis assays.

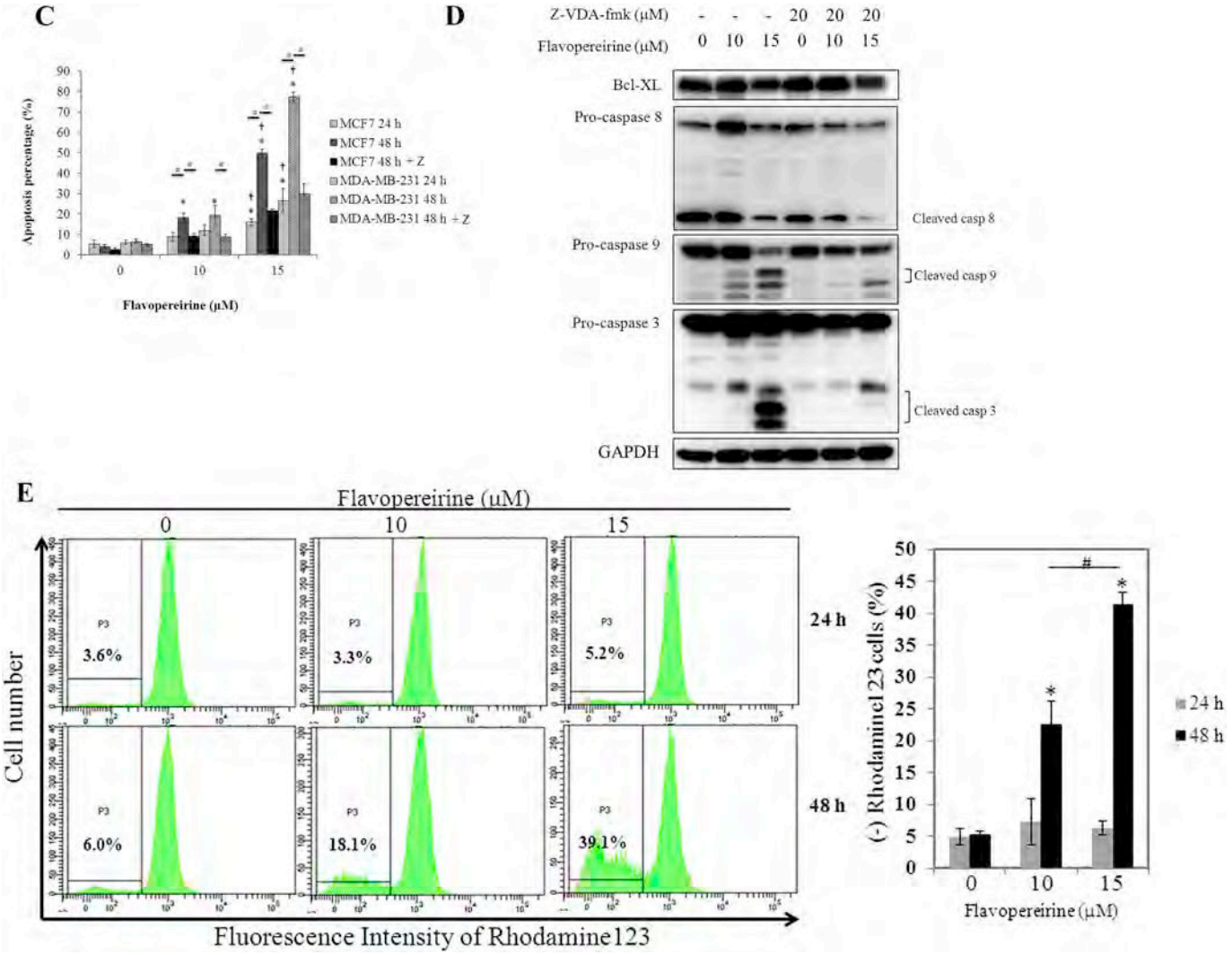
2.9. Statistical analysis

The results are presented as the mean ± standard deviation (S.D.). Statistical analysis was performed with one-way ANOVA to compare multiple groups and with an independent *t*-test to compare two groups using SPSS (Windows version 21), and P-values of < 0.05 were considered statistically significant.

3. Results

3.1. Flavopereirine decreased the viability of breast cancer cells

To assess the potential effect of flavopereirine, we used MCF-7 and MDA-MB-231 cells to investigate whether flavopereirine affects breast cancer cell growth. Flavopereirine treatment decreased the viability of MCF-7 cells in a time- and dose-dependent manner (Fig. 1A). For the high-dose (15 and 20 μM) flavopereirine treatment, MDA-MB-231 cell viability was substantially less than 10% at 48 and 72 h (Fig. 1B). The half-maximal inhibitory concentration (IC50) value for the growth inhibitory effect in MDA-MB-231 cells (5.96 μM) was half of the MCF-7 cell IC50 value (12.43 μM) at 48 h, and the IC50 values for the two cell types at 72 h were similar to those at 48 h.



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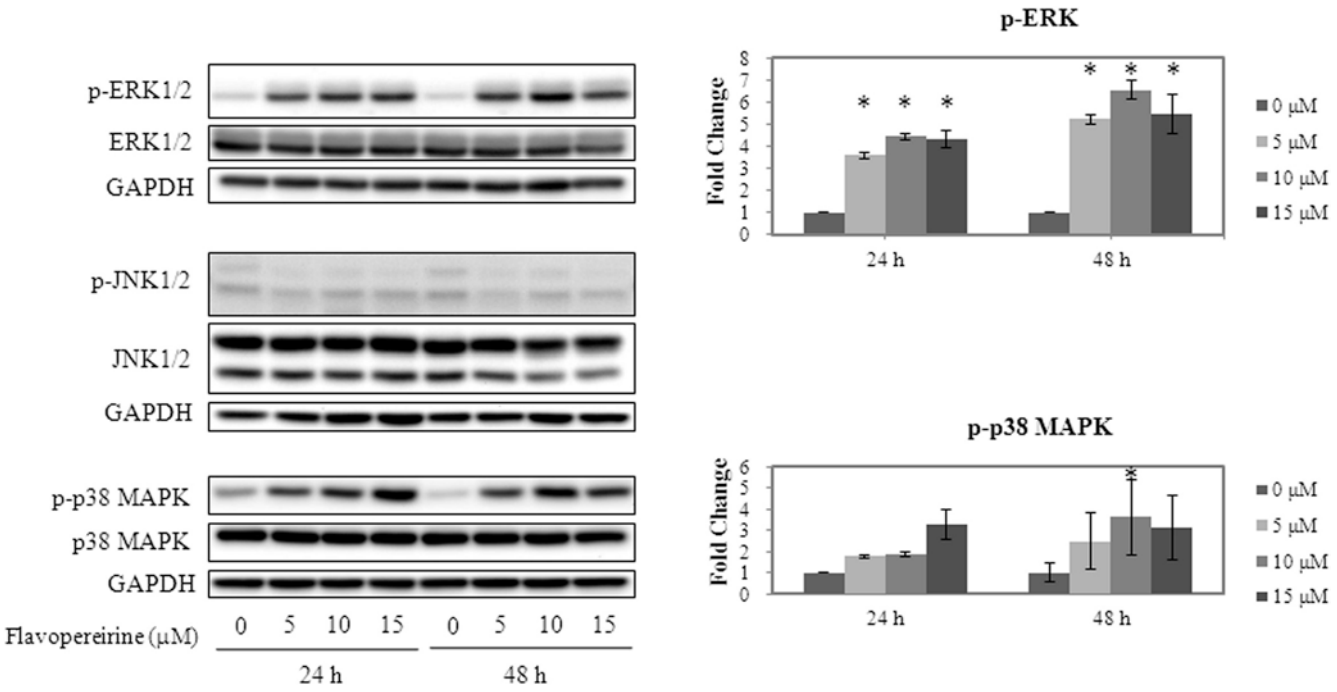

Fig. 4. ERK and p38 MAPK activation was induced by flavopereirine in MDA-MB-231 cells. MDA-MB-231 cells treated with various doses of flavopereirine for 24 and 48 h were collected to determine the expression levels of phosphorylated and total MAPKs (ERK1/2, JNK and p38) by western blotting. GAPDH was used as a loading control. * indicates a significant difference compared to the DMSO control as analyzed by one-way ANOVA followed by Bonferroni test.

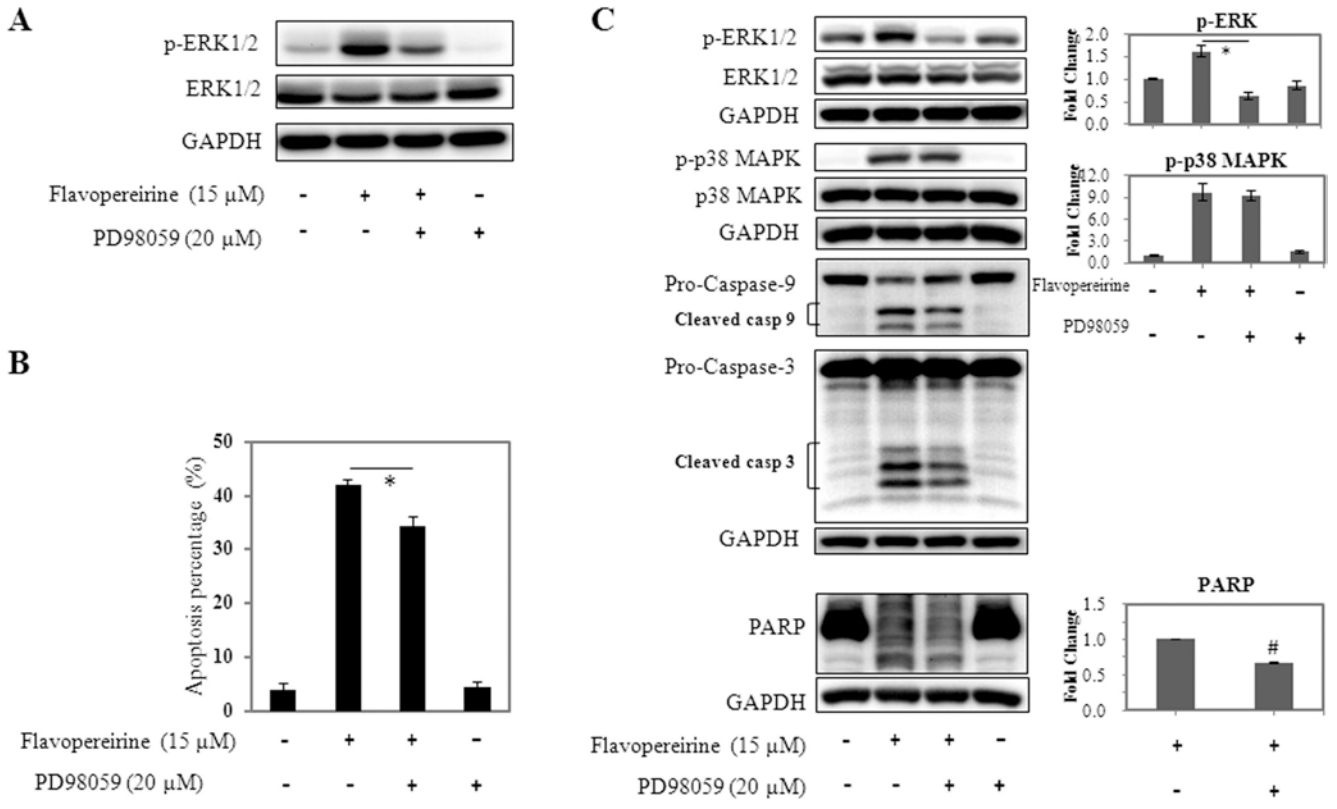

Fig. 5. Determination of the role of ERK in flavopereirine-induced apoptosis. MDA-MB-231 cells were preincubated with the ERK inhibitor PD98059 (20 μM) for 2 h and further treated with flavopereirine (15 μM) for 48 h. (A) The expression of p-ERK was detected by western blotting. (B) The percentage of apoptotic cells in the presence of flavopereirine with or without PD98059 was examined with Annexin V-FITC and analyzed by flow cytometry. The data were from three individual experiments and are shown in a histogram. (C) The levels of p-ERK, ERK, p-p38 MAPK, p38 MAPK and apoptosis-associated proteins were examined by western blotting. * indicates a significant difference ($P < 0.05$) between flavopereirine treatment with and without PD98059 treatment as analyzed by one-way ANOVA followed by Bonferroni test. # indicates a significant difference as analyzed by independent *t*-test.

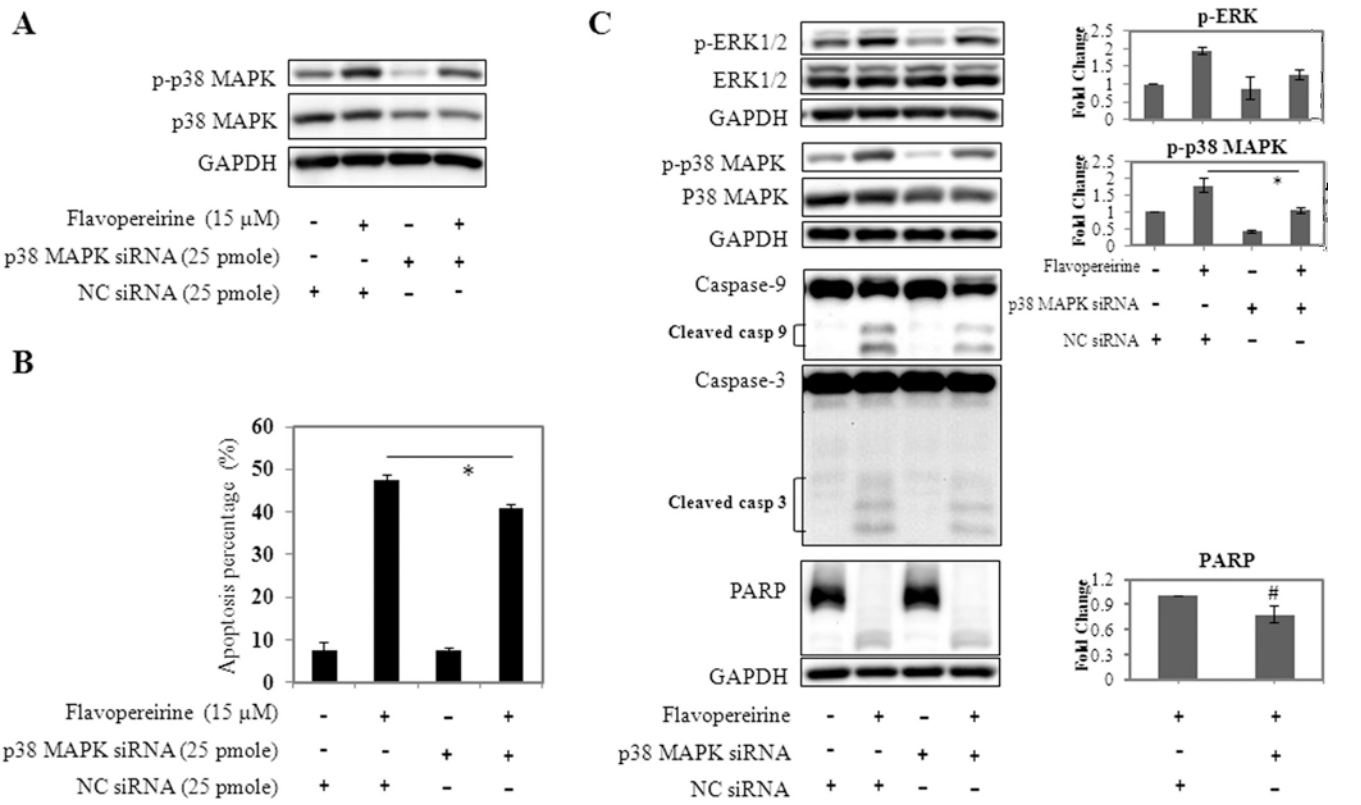

Fig. 6. Determination of the role of p38 MAPK in flavopereirine-induced apoptosis. MDA-MB-231 cells transfected with p38 or negative control (NC) siRNAs were further treated with flavopereirine (15 μM) for 48 h. (A) p-p38 MAPK and p38 MAPK knockdown were analyzed by western blotting. (B) The percentage of flavopereirine-induced apoptosis in MDA-MB-231 cells transfected with p38 siRNA or NC siRNA was examined with Annexin V-FITC and analyzed by flow cytometry. The data are from three independent experiments and are shown in a histogram. (C) The levels of p-ERK, ERK, p-p38 MAPK, p38 MAPK and apoptosis-associated proteins were measured by western blotting. * indicates a significant difference ($P < 0.05$) between MDA-MB-231 cells transfected with p38 siRNA and NC siRNA combined with flavopereirine treatment. # indicates a significant difference as analyzed by independent *t*-test.

3.2. Flavopereirine induced cell cycle arrest

Because cell cycle arrest is one of the major reasons for cell growth inhibition, we further investigated the effect of flavopereirine on cell cycle distribution by flow cytometry. As shown in Fig. 2A, treatment with a higher dose (10 μM) of flavopereirine significantly increased the number of MCF-7 cells in G0/G1 phase, which was accompanied by a significant decrease in the number of cells in S phase at 24 and 48 h ($P < 0.05$). However, there was a significant accumulation of MDA-MB-231 cells in S phase at 48 h after treatment with 10 μM flavopereirine (Fig. 2B). Upon examining the molecular mechanism of cell cycle arrest (Fig. 2C) for both cell types, we found that flavopereirine increased cyclin D1 expression levels but decreased cyclin A2 and p-Cdk2 expression levels at 48 h. Moreover, MCF-7 cells treated with flavopereirine had dose-dependently increased p21 and p27 expression levels at 48 h. However, under the same treatment conditions, the two proteins showed a dose-dependent inhibition pattern in MDA-MB-231 cells.

3.3. Flavopereirine caused mitochondria-mediated apoptosis

Cell blebbing, which is a characteristic morphological indicator of apoptosis, was observed in both MCF-7 and MDA-MB-231 cells treated with flavopereirine at 48 h (data not shown). This observation indicated that apoptosis may be the mechanism that causes flavopereirine-induced cell growth inhibition. Annexin V- FITC/PI staining demonstrated that the proportion of flavopereirine-induced apoptotic cells was dose- and time-dependent in both MCF-7 and MDA-MB-231 cell lines (Fig. 3A, B and 3C). Flavopereirine (15 μM) induced a substantially higher proportion of apoptotic cells among MDA-MB-231 cells than among MCF-7 cells at 48 h. This result suggests that MDA-MB-231 cells were more sensitive to flavopereirine; therefore, the flavopereirine-related apoptosis mechanism was investigated. Western blotting indicated that compared with the control cells, flavopereirine markedly increased the expression levels of activated caspase-3 and caspase-9 in a dose-dependent manner (Fig. 3D), while Bcl-XL and cleaved caspase-8 expression levels were decreased. This result indicates that flavopereirine induced apoptosis primarily through a caspase-9-dependent intrinsic pathway in MDA-MB-231 cells.

By using a pan-caspase inhibitor to strengthen the involvement of the caspase-dependent pathway in flavopereirine-induced apoptosis, the expression levels of activated caspase-3 and caspase-9 were rescued after treatment with z-VAD-fmk (Fig. 3D). Moreover, z-VAD-fmk significantly inhibited flavopereirine-induced apoptosis (Fig. 3A, B and 3C).

Furthermore, in confirmation of mitochondrial involvement in the caspase-9-dependent intrinsic pathway, we found that there was a significant increase in the number of cells with MMP loss (Fig. 3E) following exposure to flavopereirine for 48 h. These results suggest that flavopereirine induced mitochondria-mediated apoptosis in MDA-MB-231 cells.

3.4. Flavopereirine activated ERK and p38 MAPK during apoptosis

Previous studies have shown that three subfamilies of MAPKs, namely, ERK1/2, JNK1/2 and p38-MAPK, are involved in apoptosis (Wada and Penninger, 2004). Next, we investigated the phosphorylation level of these MAPKs by western blotting and determined the contribution of activated MAPKs to flavopereirine-induced apoptosis. Flavopereirine treatment significantly increased the p-ERK1/2 and p-

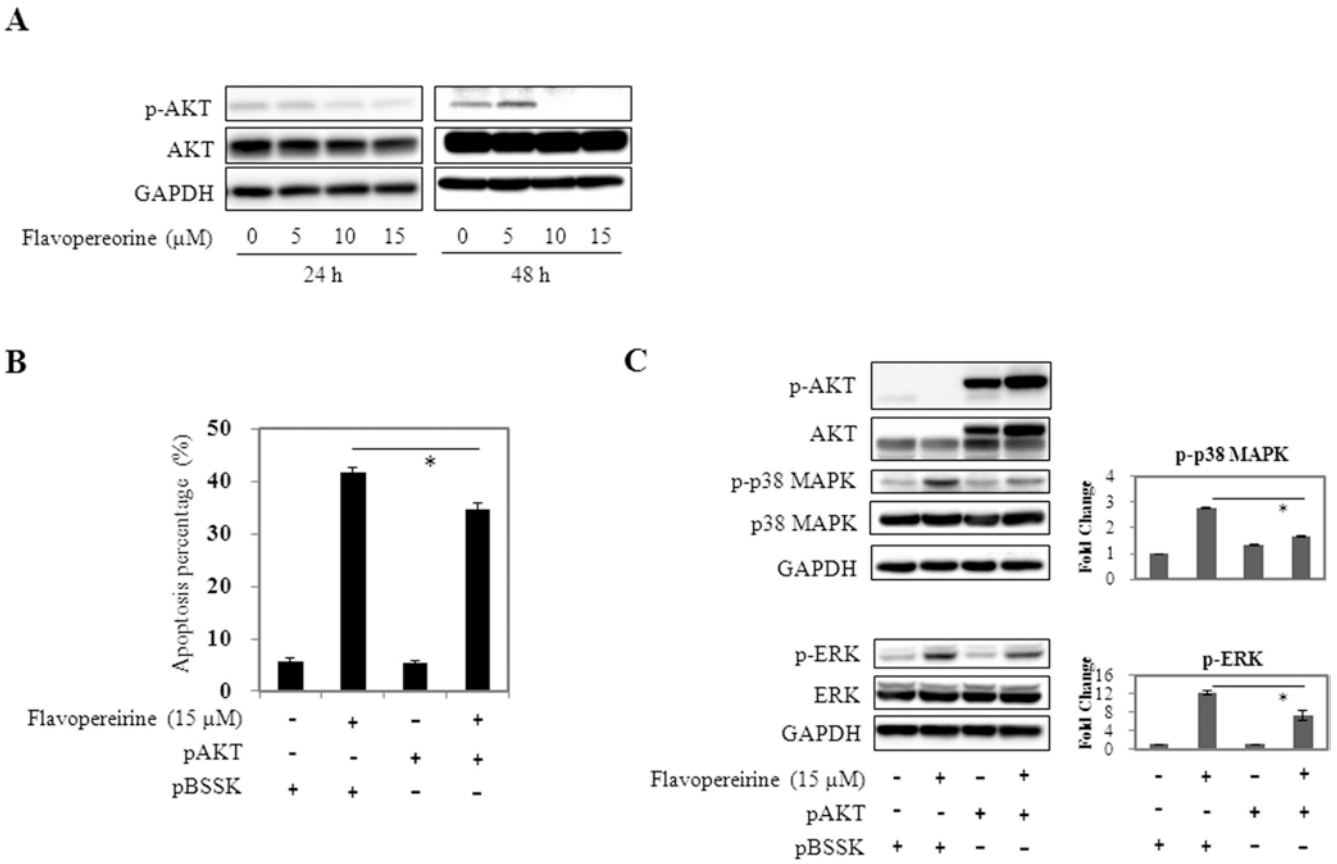

Fig. 7. AKT is an upstream inhibitor of p-p38 MAPK and p-ERK. (A) A decreased expression level of p-AKT upon flavopereirine treatment was detected by western blotting. (B) The percentage of flavopereirine-induced apoptosis in MDA-MB-231 cells transfected with plasmid constitutively expressing p-AKT (p-AKT) or control plasmid (pBSSK) was measured with Annexin V-FITC staining and flow cytometric analysis. The data are from three independent experiments and are shown in a histogram. (C) The expression of p-AKT, AKT, p-ERK, ERK, p-p38 MAPK, and p38 MAPK was examined by western blotting. * indicates a significant difference ($P < 0.05$) between MDA-MB-231 cells transfected with p-AKT or pBSSK and treated with flavopereirine.

p38 MAPK expression levels at 24 h without changing the amount of total ERK and p38 MAPK (Fig. 4). After treating cells with flavopereirine for 48 h, the p-ERK1/2 and p-p38 MAPK expression levels were also increased, and the expression levels in cells treated with 10 μM flavopereirine were substantially greater than those in the other groups. However, p-JNK and total JNK expression levels were not obviously affected by the various concentrations of flavopereirine at either time point.

To further evaluate whether ERK and p38 MAPK are involved in flavopereirine-induced apoptosis, we used the MEK inhibitor PD98059 (20 μM) to decrease the expression level of p-ERK1/2 (Fig. 5A). Compared with flavopereirine treatment alone, PD98059 significantly decreased the percentage of flavopereirine-induced apoptosis in MDA-MB-231 cells (Fig. 5B). Additionally, PD98059 inhibited the cleavage of caspase-3, caspase-9 and PARP induced by flavopereirine (Fig. 5C).

After p38 MAPK was knocked down with specific siRNA, the total protein and phosphorylation levels of p38 MAPK induced by flavopereirine were decreased (Fig. 6A). Moreover, flavopereirine-induced apoptosis was significantly inhibited (Fig. 6B), and cleavage of caspase-3, caspase-9 and PARP induced by flavopereirine was also decreased (Fig. 6C). These results suggest that both p-ERK1/2 and p-p38 MAPK participate in the signaling pathway of flavopereirine-induced apoptosis in MDA-MB-231 cells.

3.5. Molecular mechanisms related to flavopereirine-induced apoptosis

To explore whether there was crosstalk between p-ERK1/2 and p-p38 MAPK during flavopereirine-induced apoptosis, PD98059 was used to inhibit p-ERK1/2 expression, which resulted in an expression level of p-p38 MAPK similar to that with flavopereirine treatment alone (Fig. 5C). Compared with control siRNA transfection, p38 MAPK siRNA inhibited the expression of p-ERK stimulated by flavopereirine (Fig. 6C). These results revealed that p-p38 MAPK is upstream of p-ERK. We also investigated the expression level of p-AKT, which is a key regulator of cell survival, and found that the expression level of p-AKT was diminished after exposure to higher concentrations of flavopereirine (10 and 15 μM) (Fig. 7A). After MDA-MB-231 cells were transfected with a constitutively expressing p-AKT plasmid, flavopereirine-induced apoptosis was significantly rescued (Fig. 7B). Moreover, the expression levels of both p-p38 MAPK and p-ERK induced by flavopereirine were lower in MDA-MB-231 cells transfected with the p-AKT plasmid than in cells transfected with the control plasmid (Fig. 7C). These results indicate that AKT is an upstream inhibitor of p-p38 MAPK and p-ERK that regulates flavopereirine-induced apoptosis.

4. Discussion

Breast cancer is the most common cancer and the leading cause of cancer-related death in women; poor survival and distant metastasis are prominent among the heterogeneous subtypes, and developing optimal chemotherapies could result in longer metastasis-free and overall survival (Dent et al., 2007; Ferlay et al., 2015; Ovcaricek et al., 2011). The development of novel agents that can act effectively and efficiently through various anticancer mechanisms is vital. The current study investigated the effect of flavopereirine on cell cycle arrest and apoptosis signaling in breast cancer cells. We found that flavopereirine caused cell

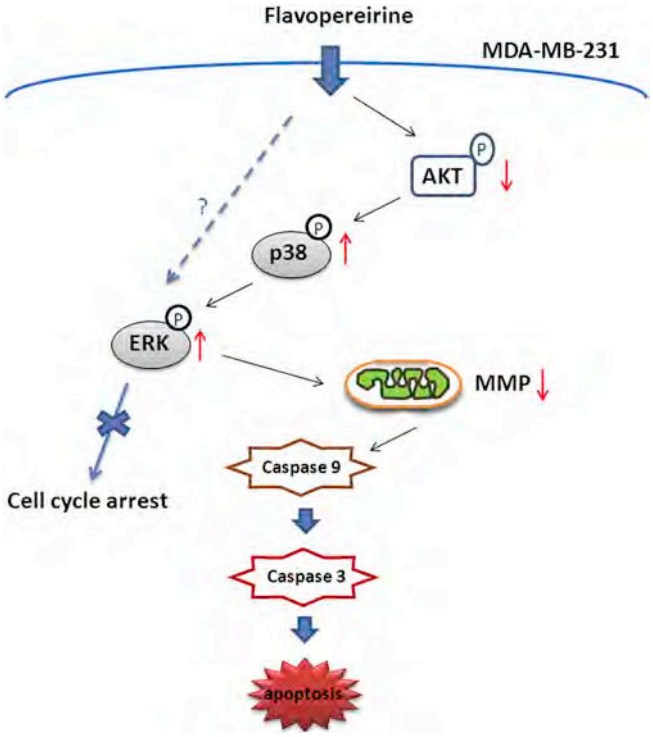

Fig. 8. The molecular mechanism underlying flavopereirine-induced apoptosis and cell cycle arrest. Flavopereirine activates p38 MAPK kinase and ERK through suppressing the upstream molecule AKT, which is partially involved in mitochondria-dependent apoptosis of MDA-MB-231 cells. Non mediation of p-ERK in flavopereirine-induced cell cycle arrest was demonstrated by using an ERK inhibitor (PD98059).

cycle arrest and apoptosis in the MCF-7 and MDA-MB-231 breast cancer cell lines. Furthermore, the AKT/p38 MAPK/ERK signaling pathway was shown to partially contribute to flavopereirine-induced apoptosis in MDA-MB-231 cells.

The activation of cyclin-Cdk complexes drives cell cycle progression. Clioquinol has been shown to induce S phase cell cycle arrest and decrease cyclin A2, cyclin D1, cyclin E1 and Cdk2 expression levels (Huang et al., 2015); however, cyclin D1 and cyclin E inhibition has also been demonstrated in honokiol or safrole induced G0/G1 cell cycle arrest (Huang et al., 2018; Yu et al., 2012). Our study revealed that flavopereirine-induced S phase arrest in MDA-MB-231 cells is associated with downregulation of cyclin A2 and upregulation of cyclin D1 (Fig. 2C). These data suggest that the expression of cyclins is not consistent at the same arrested phases and that the decrease in the expression levels of the same cyclins may be accompanied by different arrested phases. S phase arrest requires complex regulatory mechanisms, including suppressor transactivation, suppressor binding to replication machinery, and phosphorylation of critical elements of replication control (Kelly and Brown, 2000). The involvement of cyclins in cell cycle arrest can be evaluated by the up- or downregulation of the affected cyclin (Yeo et al., 2006). The Cdk inhibitors (CKIs) p21 and p27 bind to all Cdk complexes and preferentially inhibit those containing Cdk2. p27 and p21 are typically upregulated during cell cycle arrest (Huang et al., 2015; Yu et al., 2012), which was supported by the MCF-7 cell results in our study (Fig. 2C). However, p27 and p21 are downregulated in flavopereirine-induced S phase arrest in MDA-MB-231 cells (Fig. 2C). Similar to our findings, Yadav et al. showed that treatment with moxifloxacin and ciprofloxacin inhibited the expression of p21 and p27 in pancreatic cancer cell lines (Yadav et al., 2015). The molecular mechanism that resulted in flavopereirine-induced S phase arrest in MDA-MB-231 cells was demonstrated by treatment with an inhibitor (PD98059), and we found that the decrease of p-ERK did not change the percentage of cells in S phase arrest (data not shown).

MAPK pathways are involved in a variety of biological cell functions, including cell cycle progression, differentiation, migration, proliferation and apoptosis (Wada and Penninger, 2004). In our study, ERK and p38 MAPK activation was induced during flavopereirine-induced apoptosis (Fig. 4). Furthermore, we demonstrated crosstalk between ERK and p38 MAPK signaling, which revealed that activated p38 MAPK is upstream of p-ERK, as indicated by an ERK inhibitor and p38 MAPK siRNA (Fig. 5C and 6C). The exact role of p38 MAPK in the regulation of cell death remains unclear because p38 MAPK appears to mediate apoptosis or cell survival depending on the cell types and conditions (Wada and Penninger, 2004). We demonstrated that p38 MAPK is involved in flavopereirine-induced apoptosis in MDA-MB-231 cells. Many chemotherapeutic agents, such as cyclophosphamide for breast cancer, initiate the activation of p38 MAPK, resulting in cancer cell apoptosis (Olson and Hallahan, 2004; Pang et al., 2011). The ability to initiate p38 MAPK activation increases the possibility of developing new anticancer agents without side effects caused by events upstream of p38 MAPK (Olson and Hallahan, 2004).

The AKT pathway is frequently activated and promotes tumor cell survival through multiple mechanisms (Xu et al., 2012). AKT is an upstream regulator of MAPKs and can repress p38 MAPK pathways to inhibit apoptosis. In this study, flavopereirine decreased the level of AKT activation (Fig. 7A) and was upstream of p38 MAPK and ERK, as confirmed by transfection with a p-AKT overexpression plasmid (Fig. 7C). Our results were consistent with those reported by Liao and Hung (2003), who demonstrated that AKT acts upstream of p-p38 MAPK and is involved in drug-induced apoptosis in MDA-MB-231 cells. In different types of solid cancers, including breast cancer, the p-AKT expression level has been shown to be substantially high, while p38 MAPK expression is undetectable (Liao and Hung, 2003), supporting the therapeutic potential of the AKT/p38 MAPK pathway in the development of cancer cell apoptosis-inducing drugs.

Patients with the TNBC subtype have a high risk of cancer metastasis and poor prognosis. Flavopereirine has a notable effect on inducing TNBC cell apoptosis, as indicated by flavopereirine-induced apoptosis in MDA-MB-231 cells. However, flavopereirine inhibits hERG channel activity (Martin et al., 2015). In future studies, the structure of flavopereirine should be modified to decrease the inhibition of hERG channel activity and to enhance the apoptotic effect of flavopereirine on cancer cells.

5. Conclusion

This study showed that flavopereirine induces cell cycle arrest and the AKT/p38 MAPK/ERK signaling pathway, which contribute to flavopereirine-induced apoptosis in MDA-MB-231 cells (Fig. 8).

Author contributions

Conceptualization and design of the experiments: Ying-Ray Lee, Ya-Shih Tseng, and Shew-Meei Sheu. Performed the experiments: Hsuan-Te Yeh, Yi-Sheng Tsai, Yi-Chen Li, and Wen-Chun Lin. Analysis: Ming-Shan Chen, Yi-Chen Li, and Wen-Chun Lin. Drafted the manuscript: Hsuan-Te Yeh, Yi-Sheng Tsai and Shew-Meei Sheu. Revised and approved the submitted version: Shew-Meei Sheu.

Funding

This work was supported by the Ditmanson Medical Foundation of Chia-Yi Christian Hospital [grant number R106-37].

Conflicts of interest

The authors declare no conflict of interest.

Author agreement/declaration

All of the authors have thoroughly reviewed the manuscript and agreed to the submission. All the authors declare that there are no conflicts of interest associated with the submitted manuscript. The authors have no financial relationships relevant to this manuscript to disclose. This manuscript, either in part or in whole, has not been published elsewhere.

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