

Synergistic anticancer effects of ruxolitinib and calcitriol in estrogen receptor-positive, human epidermal growth factor receptor 2-positive breast cancer cells

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Abstract. The Janus kinase (JAK)1 and JAK2 inhibitor, ruxolitinib, and the active form of vitamin D (calcitriol) were previously reported to possess anticancer effects in breast cancer. The present study investigated the combined effects of ruxolitinib and calcitriol on an estrogen receptor (ER)-positive, human epidermal growth factor receptor 2 (HER2)-positive, breast cancer cell line. The ER and HER2-positive MCF7-HER18 breast cancer cell line was used to investigate the combination effect of ruxolitinib and calcitriol. A bromodeoxyuridine (BrdU) assay was used to investigate cell growth inhibition. The synergism of this combination therapy was examined using the Chou-Talalay method. Cell cycle analysis was performed by flow cytometry, and apoptosis was evaluated by flow cytometry following Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining. Alterations in protein expression levels were analyzed by western blotting. The BrdU assay indicated that combination treatment using ruxolitinib and calcitriol produced a synergistic anti-proliferative effect in MCF7-HER18 breast cancer cells. Annexin V-FITC/PI staining and cell cycle analysis identified a synergistic increase in apoptosis and sub-G1 arrest in the presence of ruxolitinib and calcitriol. Western blot analysis revealed that these synergistic effects of ruxolitinib and calcitriol were associated with reduced

protein levels of JAK2, phosphorylated JAK2, c-Myc proto oncogene protein, cyclin-D1, apoptosis regulator Bcl-2 and Bcl-2-like protein 1, and with increased levels of caspase-3 and Bcl-2-associated agonist of cell death proteins. The results of the present study demonstrated the synergistic anticancer effects of ruxolitinib and calcitriol in ER and HER2-positive MCF7-HER18 breast cancer cells. Based on these findings, ruxolitinib and calcitriol may have potential as a combination therapy for patients with ER and HER2-positive breast cancer.

Introduction

In 2016, breast cancer was the most common type of cancer and the second leading cause of cancer mortality in women worldwide (1). To facilitate the selection of an appropriate therapeutic strategy, patients with breast cancer may be subdivided into four molecular subtypes, which are determined by hormone receptor and human epidermal growth factor receptor 2 (HER2) status (2,3). The luminal B subtype includes 10-15% of breast cancer cases and is defined as estrogen receptor (ER)-positive and HER2-positive (4,5). Clinically, this subtype has an aggressive course and the current therapeutic strategy for metastatic or locally advanced luminal B breast cancer includes cytotoxic chemotherapy, hormone therapy and targeted monoclonal antibody therapy (6). However, novel therapeutic approaches are required to overcome the limitations of the current therapeutic strategy; these include a relatively low chemosensitivity, compared with the triple negative or HER2 subtypes (7,8), and a lack of responsiveness or resistance to monoclonal antibody treatment (9,10).

The Janus kinase (JAK) pathway serves multiple roles in regulating oncogenesis and cancer cell progression, and has represented a potentially attractive therapeutic target in various solid tumors (11-13). Numerous JAK pathway inhibitors have been developed, including ruxolitinib, a JAK1 and JAK2 inhibitor that has been approved for use in primary myelofibrosis and polycythemia vera (14,15). In breast cancer, previous *in vitro* studies have demonstrated the anticancer effects of JAK inhibitors, including ruxolitinib (16-18), and the potential application of ruxolitinib for the treatment of patients with metastatic or locally advanced breast cancer is under evaluation in ongoing clinical trials.

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Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; BrdU, bromodeoxyuridine; FITC, fluorescein isothiocyanate; PI, propidium iodide; JAK, Janus kinase; VDR, vitamin D receptor; CI, combination index

Key words: breast neoplasm, ruxolitinib, calcitriol, synergism, apoptosis

In addition to its conventional role in the regulation of calcium homeostasis, vitamin D has important biological functions in cell differentiation, apoptosis and cell cycle modulation; these activities are mediated by its binding to the vitamin D receptor (VDR) (19,20). Clinically, a number of reports have suggested an association between the vitamin D status of a patient and their breast cancer prognosis (21,22). Calcitriol (1,25-dihydroxyvitamin D) is the biologically active form of vitamin D and previous *in vitro* studies have identified anticancer effects of calcitriol in various malignancies, including breast cancer (23-28).

Despite the number of previous preclinical studies into the anticancer effects of ruxolitinib and calcitriol in breast cancer, the therapeutic benefits of these treatments for breast cancer have not been established in clinical settings. Therefore, based on the reported anticancer effects of ruxolitinib and calcitriol individually, it was hypothesized that there may be a synergistic anticancer effect of combination therapy using ruxolitinib and calcitriol in breast cancer. The present study aimed to investigate the combined treatment effects of ruxolitinib and calcitriol in the ER-positive HER2-positive luminal B subtype of breast cancer.

Materials and methods

Materials. Ruxolitinib and calcitriol were purchased from Selleck Chemicals (Houston, TX, USA). The final concentrations were achieved by diluting the stock solutions with RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and all solutions were prepared immediately prior to use. The antibody raised against VDR (cat. no. SC-1008) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against caspase-3 (cat. no. 9662S), apoptosis regulator Bcl-2 (Bcl-2; cat. no. 2876S), Bcl-2-like protein 1 (Bcl-xL; cat. no. 2762S), Bcl-2-associated agonist of cell death (BAD; cat. no. 9292S), cyclin-D1 (cat. no. 2922S), JAK2 (cat. no. 3230S), phosphorylated JAK2 (p-JAK2; cat. no. 3776S), c-Myc proto-oncogene protein (c-Myc; cat. no. 13987S) and β -actin (cat. no. 4967S) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cells and cell culture. ER and HER2-positive MCF7-HER18 human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Corning Life Sciences B.V., Amsterdam, Netherlands), 100 units/ml penicillin and 100 μ g/ml streptomycin. All cells were cultured in a humidified incubator containing 5% CO₂ at 37°C.

Bromodeoxyuridine (BrdU) assay of cell proliferation. The quantification of cell proliferation was based on the measurement of BrdU incorporation during DNA synthesis. This assay was performed according to the manufacturer's protocol (Cell Proliferation ELISA BrdU, cat. no. 11647229001, Roche Diagnostics GmbH, Mannheim Germany). In brief, MCF7-HER18 cells (1x10⁴/well) were seeded in triplicate into 96-well plates and allowed to grow overnight, prior to treatment with various concentrations of

ruxolitinib alone (5, 10, 15, 20, 25 and 30 μ M), calcitriol alone (3, 6, 9, 12, 15 and 18 μ M), or a combination of ruxolitinib and calcitriol (ratio 5:3) for 72 h at room temperature. The cells were subsequently treated with BrdU labeling solution for 2 h. The culture medium was removed, the cells were fixed using a fixative solution (3.7% formaldehyde in PBS) for 30 min at room temperature and the DNA was denatured. Cells were incubated with the Anti-BrdU-POD solution for 90 min and antibody conjugates were removed in three washing cycles with 1x PBS. Following washing, the tetra-methyl-benzidine substrate was added and incubated in the dark for 15 min at room temperature. Absorbance was quantified within 30 min at dual wavelengths of 450 and 540 nm using microplate reader (VersaMax, Molecular Devices, LLC, Sunnyvale, CA, USA).

Isobologram analysis of the interaction between ruxolitinib and calcitriol. The synergistic effects of ruxolitinib and calcitriol were examined using the Chou-Talalay combination index (CI) method (29,30). The resultant CI values reflect the potential interactions between two drugs, where a CI <1 indicates a synergistic effect, CI=1 indicates an additive effect and a CI >1 indicates antagonism. The mean values of three independent experiments were used. The combination index analysis was performed using CompuSyn software version 2.0.1 (ComboSyn, Inc., Paramus, NJ, USA).

Cell cycle analysis. MCF7-HER18 cells were seeded in six-well plates (1x10⁵ cells/well). Following incubation for 24 h, the cells were treated with the test reagents (20 μ M ruxolitinib, 12 μ M calcitriol, or a combination of 20 μ M ruxolitinib and 12 μ M calcitriol). Standard growth medium was used for the negative control. At 72 h, the cells were harvested, washed with PBS, and fixed in 70% ethanol at 4°C for 24 h. The cells were incubated with PBS containing 100 μ g/ml RNase A and 100 μ g/ml propidium iodide (PI) for 30 min at room temperature in the dark. Cell cycle analysis was performed using a Navios flow cytometer and Kaluza software version 1.3 (Beckman Coulter, Inc., Brea, CA, USA).

Apoptosis assay. The apoptotic status of MCF7-HER18 cells was evaluated by flow cytometry using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (SouthernBiotech, Birmingham, AL, USA) and PI staining, according to the manufacturer's protocol. A total of 1x10⁶ cells/ml were incubated with the test reagents (20 μ M ruxolitinib, 12 μ M calcitriol, or a combination of 20 μ M ruxolitinib and 12 μ M calcitriol). Standard growth medium was used as the negative control. At 72 h, the cells were washed with PBS and suspended in binding buffer containing 0.01 M HEPES, 140 mM NaCl and 2.5 mM CaCl₂ at a final concentration of 1x10⁶ cells/ml. The cell suspension (100 μ l containing 10⁵ cells) was incubated with 5 μ l Annexin-FITC and 5 μ l PI at room temperature for 15 min in the dark. Following this incubation, 400 μ l binding buffer was added and the cells were analyzed by flow cytometry using a Navios flow cytometer with Kaluza software version 1.3 (Beckman Coulter, Inc.).

Western blot analysis. MCF7-HER18 cells were seeded in six-well plates (1x10⁶ cells/well). Following incubation for 24 h, the cells were treated with the test reagents (20 μ M

ruxolitinib, 12 μM calcitriol, or a combination of 20 μM ruxolitinib and 12 μM calcitriol). Standard growth medium was used for the negative control. At 72 h, the cells were lysed using ionic detergent protein extraction buffer (PRO-PREP™, iNtrON Biotechnology, Suwon, Korea) containing phosphatase (1/100 ml lysis buffer, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and protease inhibitors (1/100 ml lysis buffer, Sigma-Aldrich; Merck KGaA). The protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). A total of 20 μg of proteins were resolved by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked for 1 h at room temperature using 5% skim milk in TBS-Tween-20. Membranes were probed overnight at 4°C with the indicated 1:1,000 diluted primary antibodies (All primary antibodies obtained from Cell Signaling Technology, Inc.), prior to washing and incubating with the anti-rabbit immunoglobulin G horseradish peroxidase-linked antibody (1:1,000; cat. no. 7074, Cell Signaling Technology, Inc.) for 1 h at room temperature. The membranes were developed and the protein signals were detected using enhanced chemiluminescence western blotting detection reagents (GE Healthcare Life Sciences, Little Chalfont, UK). β -actin was used as a loading control in all western blotting analyses and the results were obtained using densitometry by MultiGauge software version 2.0 (FUJIFILM Corporation, Tokyo, Japan).

Statistical analysis. All data are expressed as the mean \pm standard deviation of at least three separate experiments. The statistical comparisons between multiple groups were performed using one-way analysis of variance, followed by a Tukey's post hoc test. For all tests, $P < 0.05$ was considered to indicate a statistically significant difference. The data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS version 12.0 (SPSS Inc., Chicago, IL, USA).

Results

VDR and JAK2 are expressed in MCF7-HER18 cell lines. Prior to evaluating the effects of ruxolitinib and calcitriol on MCF7-HER18 cells, the expression of VDR and JAK2 in this cell line was examined using western blotting. MCF7-HER18 cells expressed VDR and JAK2 proteins (Fig. 1).

Synergistic inhibitory effects of ruxolitinib and calcitriol on MCF7-HER18 cells. BrdU assays were performed to evaluate the inhibitory effects of ruxolitinib and calcitriol on MCF7-HER18 cell growth (Fig. 2). Compared with control cells, exposure to 5-30 μM ruxolitinib produced significant concentration-dependent cell growth inhibition in MCF-HER18 cells, with a 20% inhibitory concentration (IC_{20}) of $14.77 \pm 1.30 \mu\text{M}$ ($P = 0.0359$ for 5 μM ; $P < 0.001$ for 10, 15, 20, 25 and 30 μM). Additionally, MCF-HER18 cells exposed to 3-18 μM calcitriol demonstrated significant concentration-dependent growth inhibition, with an IC_{20} of $7.65 \pm 1.00 \mu\text{M}$ ($P < 0.001$ in the presence of 3, 6, 9, 12, 15 or 18 μM). Investigation of the combined effects of a 5:3 ratio of ruxolitinib and calcitriol was conducted, based on these IC_{20} values. Combination treatment with ruxolitinib and

Table I. Combination indices for ruxolitinib and calcitriol.

Ruxolitinib (μM)	Calcitriol (μM)	Combination index (5:3)
5	3	0.533
10	6	0.605
15	9	0.761
20	12	0.552
25	15	0.642
30	18	0.64

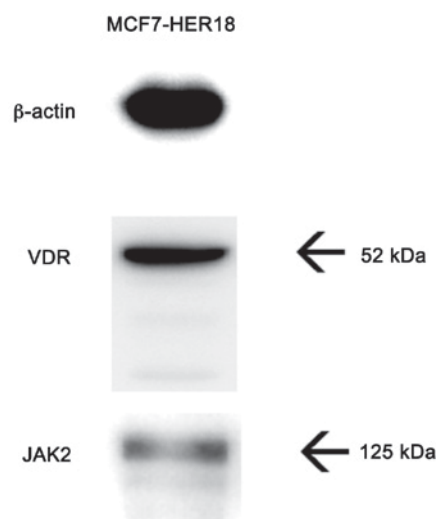


Figure 1. The expression of VDR and JAK2 in MCF7-HER18 breast cancer cells was demonstrated by western blot analysis. VDR, vitamin D receptor; JAK, Janus kinase; HER, human epidermal growth factor receptor.

calcitriol produced a synergistic growth inhibition effect on MCF7-HER18 at all concentrations tested and the combination index data are in Table I.

Ruxolitinib and calcitriol synergistically increases the number of cells in the sub-G1 phase in MCF7-HER18 cells. Cell cycle analysis was performed to evaluate the effects of treatment with ruxolitinib and/or calcitriol on MCF7-HER18 cell cycle distribution (Fig. 3). The results demonstrated that the proportion of cells in the sub-G1 phase was significantly increased in the presence of ruxolitinib alone ($7.21 \pm 1.41\%$, $P = 0.0125$) or calcitriol alone ($11.5 \pm 0.46\%$, $P < 0.001$), compared with control cells ($3.66 \pm 1.34\%$). This effect was associated with a significant decrease in the percentage of cells in the G0/G1 phase in the presence of ruxolitinib alone ($P < 0.001$), in the percentage of cells in the S phase in the presence of ruxolitinib ($P = 0.0038$) or calcitriol ($P = 0.0038$) alone, and a significant increase in the percentage of cells in G2/M phase in the presence of ruxolitinib alone ($P < 0.001$). Furthermore, combination treatment with ruxolitinib and calcitriol led to a further accumulation of cells in the sub-G1 phase, compared with control cells ($P < 0.0001$), with cells treated with ruxolitinib only ($P < 0.001$) and with cells treated with calcitriol only ($P < 0.001$).

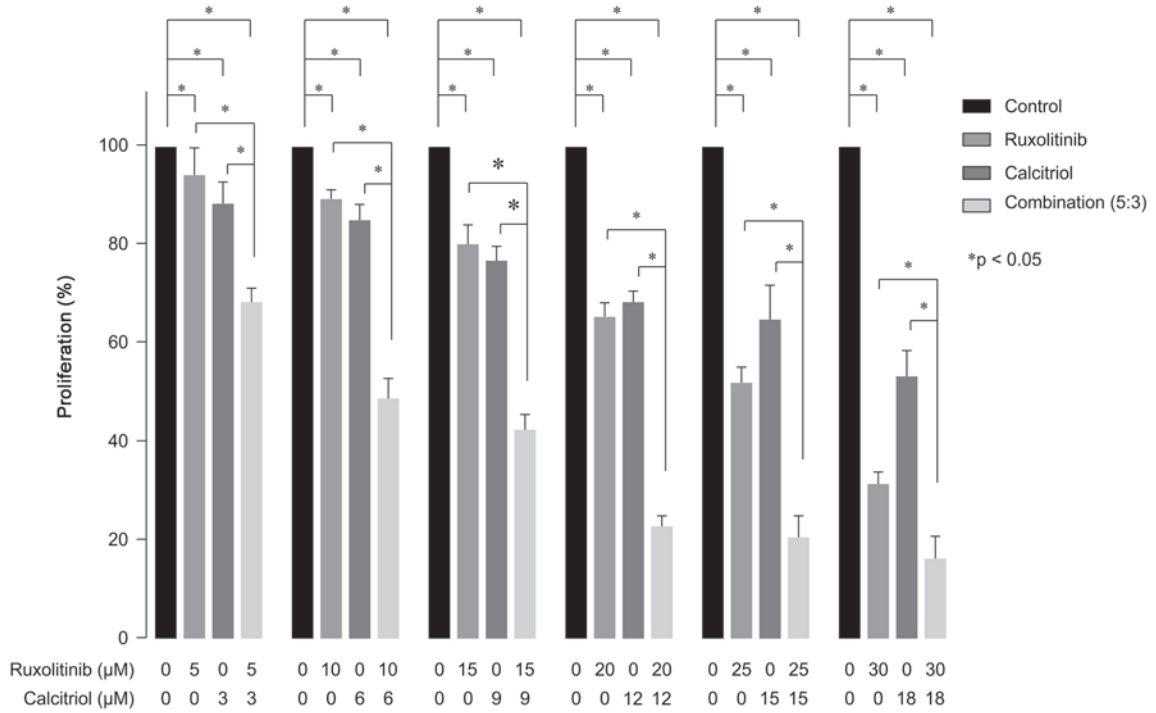


Figure 2. Cell proliferation evaluation using the BrdU assay in the presence of the indicated concentrations of ruxolitinib and/or calcitriol in MCF7-HER18 cells for 72 h. The data are presented as the mean \pm standard deviation. *P<0.05. BrdU, bromodeoxyuridine; HER, human epidermal growth factor receptor.

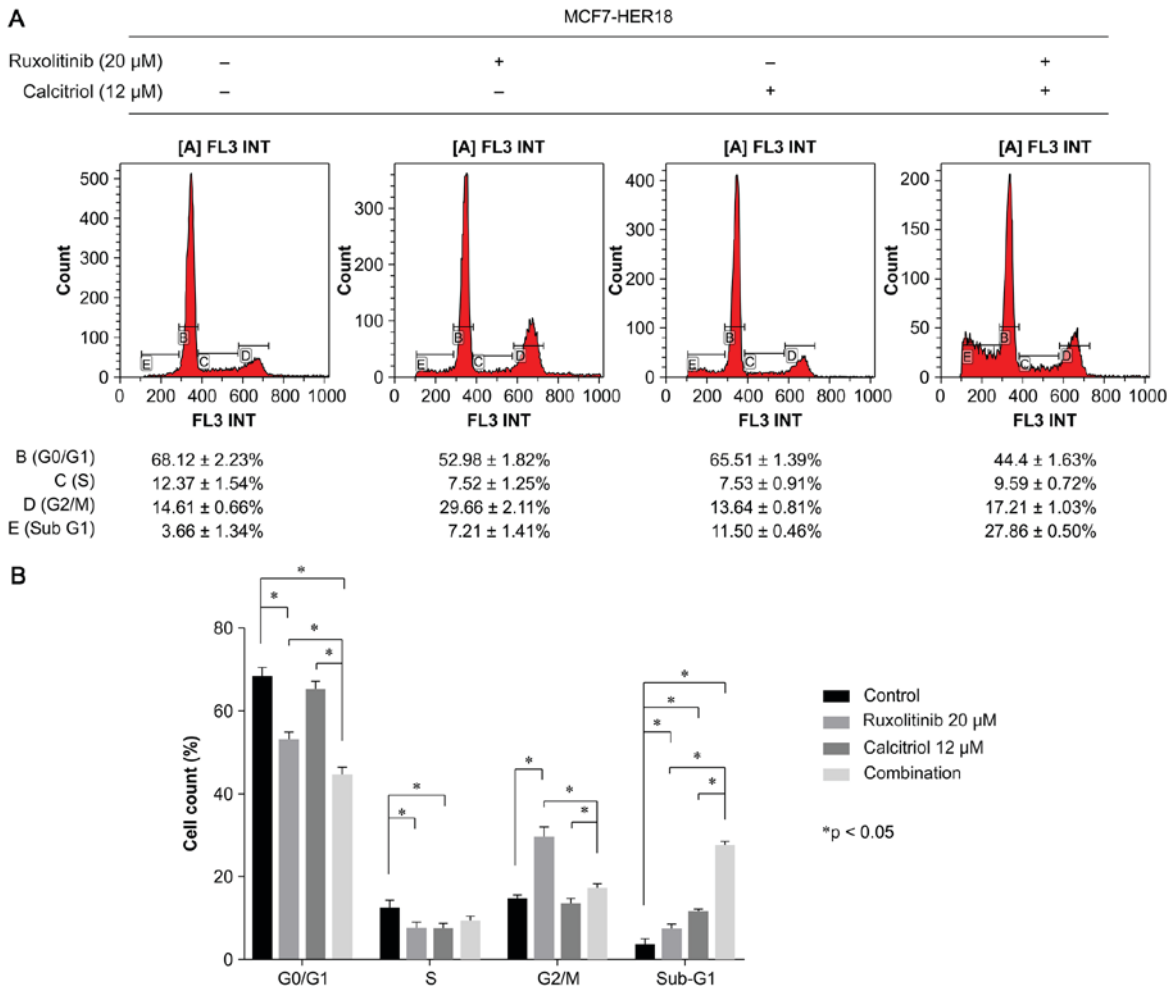


Figure 3. Cell cycle distribution of MCF7-HER18 cells treated as indicated for 72 h. (A) Cell cycle distribution was investigated using flow cytometry and (B) the data were quantified. The data are presented as the mean \pm standard deviation. *P<0.05; HER, human epidermal growth factor receptor.

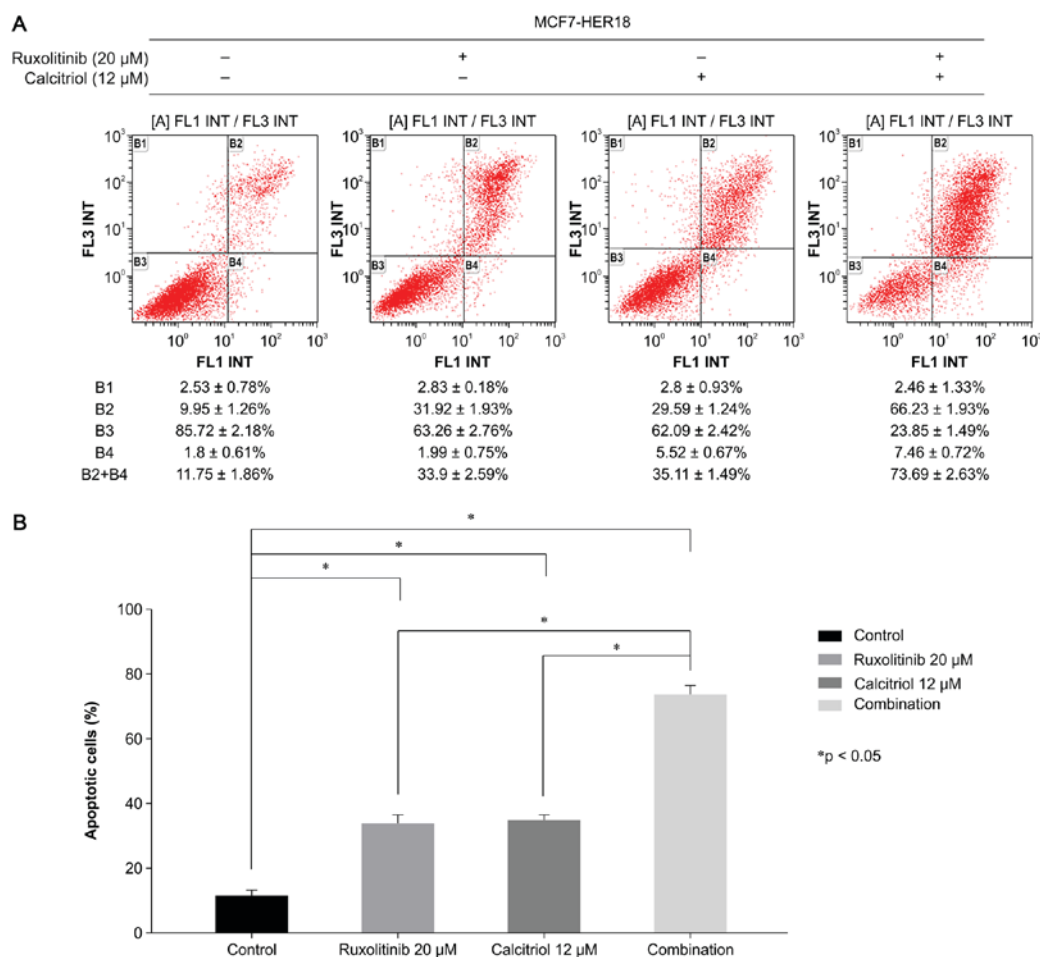


Figure 4. Flow cytometry analysis of Annexin-FITC/PI stained MCF7-HER18 cells was performed. Following exposure to the indicated treatments for 72 h. (A) B1 quadrant (Annexin⁻, PI⁺) represents necrotic cells, B2 quadrant (Annexin⁺, PI⁺) represents late apoptotic cells, B3 quadrant (Annexin⁻, PI⁻) represents viable cells, and B4 quadrant (Annexin⁺, PI⁻) represents early apoptotic cells. (B) The graph shows the proportions of early and late apoptotic cells following 72 h of treatment as indicated. The data are presented as the mean \pm standard deviation. *P<0.05. FITC, fluorescein isothiocyanate; PI, propidium iodide; HER, human epidermal growth factor receptor.

Ruxolitinib and calcitriol synergistically induce apoptosis in MCF7-HER18 cells. Flow cytometric analysis of Annexin V-FITC and PI double-stained cells was used to investigate the effects of ruxolitinib and calcitriol on apoptosis in MCF7-HER18 cells (Fig. 4). Compared with control cells (11.54 \pm 1.89%), those exposed to ruxolitinib or calcitriol demonstrated a significant induction of apoptosis (ruxolitinib, 33.90 \pm 1.89%, P<0.001; calcitriol, 35.11 \pm 1.49%, P<0.001). Furthermore, combination treatment with ruxolitinib and calcitriol induced significantly more apoptosis (73.69 \pm 2.63%, P<0.001) compared with what was observed in control or single agent-treated cells. These data suggested that ruxolitinib and calcitriol synergistically inhibited cell growth and promoted apoptosis in MCF7-HER18 cells.

Ruxolitinib and calcitriol inhibit cell growth by downregulating the expression of proteins within JAK2 and apoptosis-associated pathways. c-Myc and cyclin-D1 are known downstream targets of the JAK2 pathway, which is an important regulator of cell growth, proliferation and apoptosis (31,32). To investigate the molecular mechanism underlying the effects of combined ruxolitinib and calcitriol, western blotting was performed to evaluate the expression

levels of proteins associated with downstream JAK2 and apoptosis-associated pathways in MCF7-HER18 cells; these included JAK2, p-JAK2, c-Myc, cyclin-D1, caspase-3, Bcl-2, Bcl-xL and BAD. These results demonstrated that combination treatment with ruxolitinib and calcitriol significantly decreased the expression of JAK2, p-JAK2, c-Myc, cyclin-D1, Bcl-2 and Bcl-xL, while increasing the expression of caspase-3 and BAD, compared with control cells or those exposed to ruxolitinib or calcitriol alone (Fig. 5).

Discussion

A number of malignant diseases, including breast cancer, may be associated with two or more oncogenic signal transduction pathways, rather than with one specific oncogenic pathway. Therefore, recent studies have focused on the development of combination therapies using anticancer agents in order to inhibit multiple oncogenic pathways more effectively, compared with conventional single-agent treatment. In breast cancer, previous preclinical studies have reported the anticancer effects of ruxolitinib and calcitriol, used individually. The present study investigated whether these compounds produced synergistic effects in a breast cancer cell line.

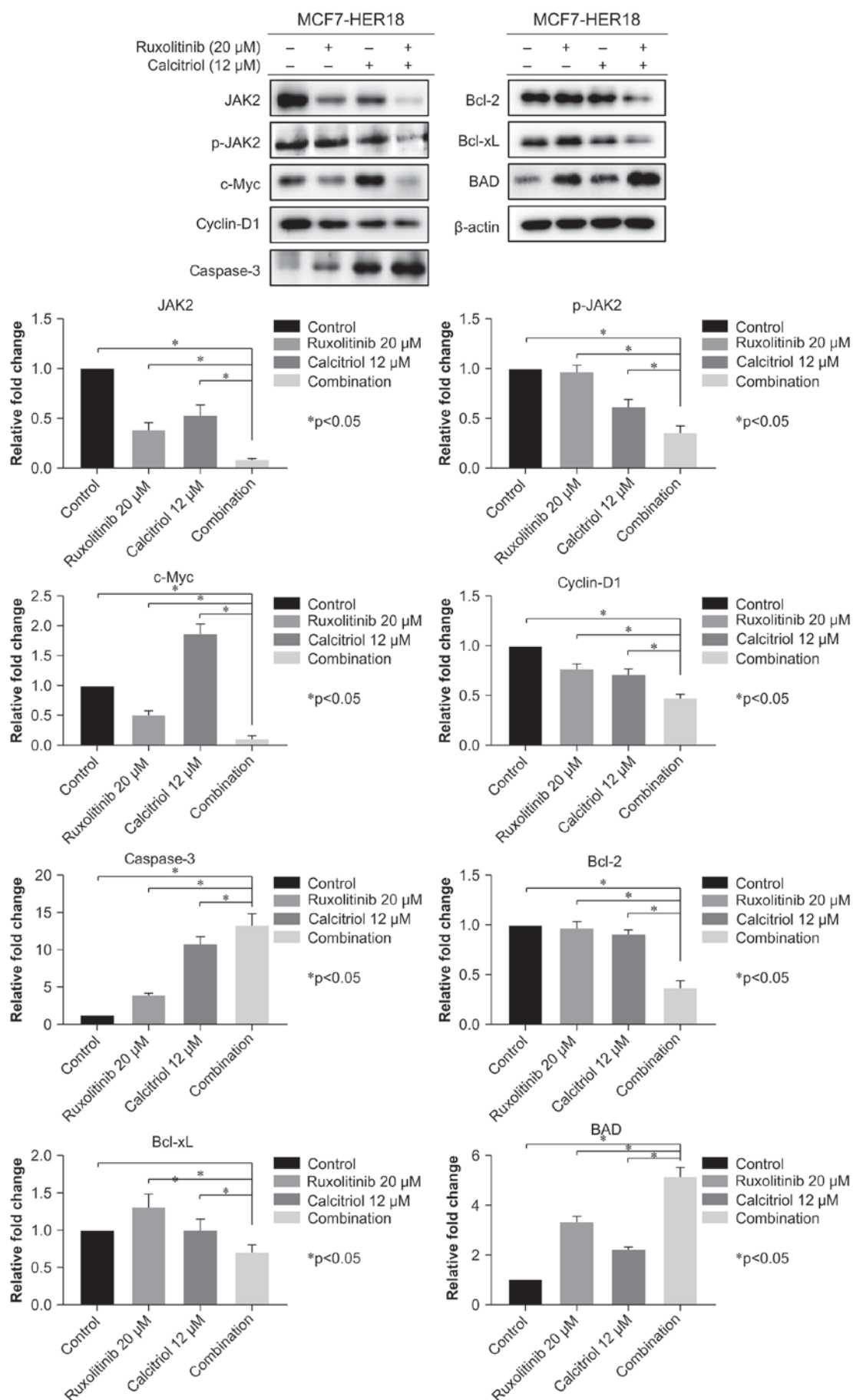


Figure 5. Levels of JAK2, p-JAK2, c-Myc, cyclin-D1, caspase 3, Bcl-2, Bcl-xL, BAD and β -actin proteins in MCF7-HER18 cells were determined by western blot analysis following the indicated 72 h treatments. The data are presented as the mean \pm standard deviation. $P < 0.05$. JAK, Janus kinase; Bcl-2, apoptosis regulator Bcl-2; Bcl-xL, Bcl-2-like protein 1; BAD, Bcl-2-associated agonist of cell death; Myc, Myc proto oncogene protein HER, human epidermal growth factor receptor.

The results of the present study demonstrated significant concentration-dependent antiproliferative effects of ruxolitinib or calcitriol in the ER and HER2-positive MCF7-HER18 breast cancer cell line. Notably, whereas the effective concentration of ruxolitinib that produced a significant antiproliferative effect was relatively consistent with previously published studies, the effective concentration of calcitriol was relatively high in the present study, compared with previous studies. In a study by Tavallai *et al* (17), the effective treatment dose of ruxolitinib in triple negative breast cancer cells (SUM149) and luminal B breast cancer cells (BT474) was in the range 0.5-2.5 μM , which was similar to the 5-30 μM identified in the present study. However, the study of Yuan *et al* (33) reported an effective calcitriol treatment dose of 1-100 nM in a luminal A breast cancer cell line (MCF7); this was decreased compared with the 3-18 μM identified in the present study. This difference may reflect the different ER and HER2 status of the breast cancer cell lines employed in these studies. Segovia-Mendoza *et al* (34) reported that VDR expression, which is an important determinant of responsiveness to calcitriol, was relatively lower in HER2-positive breast cancer cells compared with HER2-negative breast cancer cells. Compared with the study of Yuan *et al* (33), which used a HER2-negative breast cancer cell line (MCF7), the higher effective treatment concentration of calcitriol in the present study was possibly associated with the HER2-positive status of the MCF7-HER18 breast cancer cell line.

In the present study, the synergistic inhibition of breast cancer cell growth was primarily due to increased apoptosis. The JAK pathway transmits information from extracellular chemical signals to the nucleus, resulting in DNA transcription and the expression of genes involved in proliferation, differentiation and apoptosis (13). Previous reports have indicated that, when combined with other anticancer drugs, ruxolitinib and calcitriol produce synergistic antiproliferative and anticancer effects via the JAK2-associated downstream pathway. Tavallai *et al* (17) reported that combination treatment with ruxolitinib and mono-methyl fumarate decreased the expression of the anti-apoptotic signaling protein Bcl-xL, and increased that of the pro-apoptotic signaling protein BAD. Furthermore, Ju *et al* (35) reported that a combination of ruxolitinib and navitoclax produced synergistic anti-proliferative and pro-apoptotic effects that were associated with decreased expression of c-Myc and Bcl-xL, and increased expression of apoptosis regulator BAX. Additionally, Segovia-Mendoza *et al* (36) reported that a combination of calcitriol and lapatinib produced a synergistic increase in apoptosis in triple negative breast cancer cells via increased activity of caspase-3. The results of the present study demonstrated synergistic antiproliferative and pro-apoptotic effects of ruxolitinib and calcitriol in MCF7-HER18 breast cancer cells via decreased expression of c-Myc, cyclin-D1, Bcl-2 and Bcl-xL, and increased expression of caspase-3 and BAD.

No studies, to the best of the authors' knowledge, have reported definite anticancer effects of ruxolitinib or calcitriol in patients with breast cancer in a clinical setting. To date, a number of clinical trials (<https://clinicaltrials.gov/ct2/results?cond=BREAST+CANCER&term=RUXOLITINIB&cntry=&>

<https://clinicaltrials.gov/ct2/show/NCT01562873?term=RUXOLITINIB&cond=BREAST+CANCER&rank=2>); (NCT02120417) (<https://clinicaltrials.gov/ct2/show/NCT02120417?term=RUXOLITINIB&cond=BREAST+CANCER&rank=3>), and other studies are in progress. Based on the results of the present study, it is suggested that a future clinical trial be conducted to explore the possible therapeutic potential of combination treatment with ruxolitinib and calcitriol in breast cancer.

In conclusion, the present study demonstrated the synergistic anticancer effects of ruxolitinib and calcitriol in ER and HER2-positive MCF7-HER18 breast cancer cells. This indicated that combination therapy with ruxolitinib and calcitriol may provide a novel and effective treatment option for patients with ER and HER2-positive breast cancer. Further animal studies are required prior to confirmation of this result in a clinical setting.

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