

Combined treatment with ruxolitinib and MK-2206 inhibits the JAK2/STAT5 and PI3K/AKT pathways via apoptosis in MDA-MB-231 cells

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Abstract

Background: Due to deficiencies in the expression of hormone receptors, such as PR, ER and HER2, it is challenging to treat triple-negative breast cancer (TNBC), which does not respond to single targeted therapy. Ruxolitinib is a Janus kinase (JAK)1/JAK2 inhibitor. MK-2206 is an allosteric AKT inhibitor. Due to the limited activities of ruxolitinib and MK-2206 for monotherapy, the need for cotreatment with other drugs has emerged. This study is the first to examine the effects of ruxolitinib and MK-2206 cotreatment on apoptosis and JAK2/STAT5 and PI3K/AKT signaling in MDA-MB-231 breast cancer cells. Additionally, this work aimed to decrease the side effects of ruxolitinib and increase its anticancer effects with MK-2206 cotreatment.

Methods and Results: Cell viability was reduced in a dose- and time-dependent manner after exposure to ruxolitinib, MK-2206 or both for 48 h, as shown by MTT assay. Ruxolitinib had a synergistic antiproliferative effect, as demonstrated by colony formation and wound healing assays. The effects of ruxolitinib, MK-2206 and their combination on apoptosis, as well as PI3K/AKT and JAK/STAT signaling, were examined by western blot analyses. Cotreatment with ruxolitinib and MK-2206 reduced proliferation with the dual inhibition of JAK2/STAT5 and PI3K/AKT signaling by decreasing PI3K, AKT, JAK2, STAT5, Caspase-9, Caspase-7, PARP, c-Myc, and Bcl-2 and increasing P53 and PTEN protein expression.

Conclusions: Our results revealed the roles of P53 and PTEN in the regulation of apoptosis and the PI3K/AKT and JAK2/STAT5 signaling pathways. The dual inhibition of JAK2/STAT5 and PI3K/AKT may reduce metastasis by decreasing tumor cell survival.

Introduction

Triple-negative breast cancer (TNBC) is known to involve a deficiency in the expression of hormone receptors, such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth Factor 2 receptor (HER2), or HER2 amplification [1, 2]. Therefore, performing TNBC therapy is difficult, and novel therapeutic approaches that target receptor tyrosine kinases (RTKs), PI3K/AKT/mTOR (PAM), RAS/MAPK, poly(ADP-ribose) polymerase (PARP), and JAK/STAT for TNBCs need to be developed [3–8]. Recent studies have shown that monotargeted therapies are not effective for TNBC because of intrinsic or acquired resistance.

A therapeutic strategy for TNBC is to inhibit the signaling pathways that have a role in tumor formation and metastatic progression [7]. Janus kinase (JAK) and its related signal transducer and activator of transcription (STAT) signaling play major roles in cancer progression; activation of JAK/STAT by cytokines and growth factors induces cell proliferation, survival, migration and differentiation [9–11]. STAT3 and STAT5 have a major role in tumor development, cell cycle regulation and apoptosis and have been found to be activated in breast, lung, prostate, and pancreatic cancers and other hematological malignancies [12–14]. Studies have shown that activation of JAK2/STAT5 causes resistance to PI3K/mTOR suppression in breast cancer. Ruxolitinib is an RTK inhibitor that suppresses JAK/STAT

signaling targeting JAK1/JAK2 and was approved to treat patients with myelofibrosis and polycythemia vera [15].

PI3-kinase (PI3K) signaling has a major role in RTK signaling in breast cancer to modulate cell proliferation and has a crucial role in breast tumor formation [16, 17]. This pathway interacts with other signaling pathways to promote tumor formation and resistance to therapies [18]. Downstream of PI3K are protein kinase B (AKT) and mammalian target of rapamycin (mTOR). This signaling is modulated by multiple phosphatases that include inositol polyphosphate-4- phosphatase type II B (INPP4B) and phosphatase and tensin homolog (PTEN). MK-2206 is a potent allosteric AKT inhibitor that is currently the subject of clinical experiments, in which the cytotoxicity against different cancer types is being examined [19]. With the development of new drug targets and combined therapy, multiple oncogenic signaling pathways can be targeted, and drug resistance can be eliminated.

Combination therapy is a treatment type that uses a combination of multiple therapeutic agents in cancer treatment [20]. When used together with anticancer drugs, compared with the monotherapy approach, the combined therapy improves the efficacy of drugs by targeting key pathways synergistically or additively. Combination therapy possibly decreases drug resistance and provides some advantages, such as decreasing tumor growth, arresting mitotically active cells, and triggering apoptosis [21, 22]. Recently, studies have shown that JAK/STAT pathway activation is related to other signaling pathways, especially PI3K/AKT signaling [23]. Due to the advantages of combined therapy and the systemic side effects of ruxolitinib in the treatment of myelofibrosis, we treated both to decrease the cytotoxicity of ruxolitinib and to increase the anticancer effect of ruxolitinib with ruxolitinib and MK-2206 together in MDA-MB-231 cells, which are TNBC cells. In this study, we aimed to prevent drug resistance and reduce metastasis by especially affecting the survival of tumor-initiating cells using dual inhibition of JAK2/STAT5 and PI3K/AKT.

Materials And Methods

Drugs, chemical and antibodies

Ruxolitinib and MK-2206 were purchased from MedChemExpress (USA). Hoechst 33342 nuclear stain (ENZ-51031-0050) was purchased from Enzo Life Sciences. β -actin (CST-4970), PARP (CST-9532), Caspase-9 (CST-9508), Caspase-7 (CST-12827), and Caspase-3 (CST-9662) (1:1000 dilution) rabbit antibodies were purchased from Cell Signaling Technology (CST, Danvers, MA, USA). JAK2 (sc-390539), STAT5 (sc-74442), STAT3 (sc-8019), Caspase-8 (sc-56070), Bax (sc-20067), Bcl-2 (sc-7382), c-Myc (sc-40), PI3K (sc-1637), AKT1 (sc-5298), PTEN (sc-7974), and P53 (sc-126) (1:500 dilution) mouse antibodies were purchased from Santa Cruz Biotechnology (sc, Oregon, USA). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (1:5000 dilution) were purchased from CST.

Cell culture

MDA-MB-231 cells were cultured in DMEM (Gibco) containing 10% fetal calf serum (Gibco) and 100 U/100 mg/ml penicillin/streptomycin (Gibco) at 5% CO₂ in humidified air at 37°C (Memmert CO₂ Incubator, INCO153med, Germany).

Viability assay

Cells were seeded at 1×10^4 density in 96-well plates and exposed to different concentrations of ruxolitinib (0–50 µM) and MK-2206 (0–7.5 µM) for 48 hours (h). Then, 10 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) dye (5 mg/ml in 1X PBS, Cayman Chemical, Michigan, USA) was added. After incubation at 37°C for 4 h, the medium was removed, and then 200 µl of 10% SDS dissolved in 0.01 M HCl was added. The absorbance of the suspensions was measured at 570 nm with a microplate reader (Multiskan™ FC Microplate Photometer, ThermoFisher Scientific, Multiskan FC China).

Growth inhibition assay

Cells were seeded at 1×10^6 density in 6-well plates and exposed to ruxolitinib (22,5 µM), MK-2206 (7,5 µM) and their combination (18 µM ruxolitinib/5 µM MK-2206) at 24, 48, 72 and 96 h. The cells were stained with 50 µl trypan blue (0.4% w/v). The unstained cells were counted with a Neubauer hemocytometer under inverted microscopy.

Hoescht staining

Cells were seeded at 2.5×10^2 density in 6-well plates. The cells were treated with ruxolitinib, MK-2206, and their combination. The cells were washed twice with 1X assay buffer. Seventy microliters of Microscopy Dual Detection Reagent (including 1 µL of Hoechst 33342 and 2 µL of CYTO-ID® Green Detection Reagent in 1 mL of 1X assay buffer) was added to each sample. After incubation for 15 minutes at 37°C, the cells were washed with 100 µl of 1X Assay Buffer and fixed with 4% formaldehyde for 20 minutes. The formaldehyde was removed by washing the cells 3 times with 1X assay buffer. The stained cells were analyzed by fluorescence microscopy.

Colony formation assay

Cells were seeded at 4×10^3 density in 6-well plates and incubated for 2 weeks. To remove the dead cells, 1X PBS solution was added to the cells. Colonies were fixed with acetic acid:methanol (1:3) for 20 min at room temperature and washed with distilled water. The cells were stained with crystal violet in methanol (0.5% w/v) for 15 min, and morphological images were taken under an inverted microscope.

Wound healing assay

A total of 5×10^5 cells/well on 6-well plates were seeded and incubated to adhere at 5% CO₂ and 37°C overnight. The cells were scraped with a 200 µl sterile pipette tip to create wounds. Wound widths were measured with an inverted microscope at 0, 24, 48, 72, and 96 hours.

Protein extraction and immunoblotting

Cells were exposed to ruxolitinib, MK-2206 and their combination for 48 h. Every sample was washed, and 1X PBS was collected for 2 minutes at 13200 rpm. Radioimmunoprecipitation assay (RIPA) solution (CST- 9806) was added to lyse the cells for 5 minutes on ice. The lysed cells were shaken for 20 minutes at room temperature. The cells were centrifuged to remove cell debris for 15 minutes at 13200 rpm, and the supernatant was collected. Total protein lysates (50 µg), which were measured by the Bradford protein assay, were loaded onto a 12% SDS-PAGE gel and transferred to PVDF membranes (Merck Millipore) with a semidry transfer system (Hoefer). The membranes were blocked with 5% nonfat milk in 1XTBS-Tween 20 for 1 hour at room temperature and incubated with primary and secondary antibodies at 4°C overnight. The membranes were washed with 1X TBS for 10 minutes three times and were washed with 1X TBS-Tween 20 for 10 minutes one time. The protein expression of PARP, Caspase 9, β-actin, Caspase 8, Bax, Caspase 3, Bcl-2, Caspase 7, c-Myc, PI3K, AKT1, PTEN, P53, JAK2, STAT5 and STAT3 was analyzed with enhanced chemiluminescence (ECL, Thermo Fisher Scientific) in a Syngene G:Box Chemi XRQ system.

Statistical analysis

Statistical analysis for every experiment was performed by one-way analysis of variance (ANOVA) using GraphPad Prism 9.0.0. Standard deviation (SD) values used for determining error bars in the graphs. The intensities of the protein bands obtained by western blot analysis were measured by using the ImageJ program. Each band of proteins was measured three times to use the mean value of three measurements. For the calculation of relative expression levels, each value was divided by the value of β-actin. P values were considered statistically significant as * P < 0.05; ** P < 0.01; ***P < 0.001; **** p < 0.0001.

Results

The combination of ruxolitinib with MK-2206 reduced cell viability

To clarify the cytotoxic effect of ruxolitinib and MK-2206 on cells, an MTT assay was used. The cells were exposed to different concentrations of ruxolitinib (0–40 µM), MK-2206 (0–10 µM) and their combination at different ratios (1:1, 1:1.5, 1:1.75, 1:4) for 48 h. The cell viability decreased with only ruxolitinib and only MK-2206 treatment in a dose-dependent manner (Fig. 1a, 1b). The most appropriate dose concentrations of ruxolitinib, MK-2206, and their combination were determined to be 22,5 µM, 7,5 µM, and 18 µM + 5 µM, respectively (Fig. 1a, 1b, 1c). When the cells were treated with ruxolitinib, MK-2206 and their combination, cell proliferation was reduced in a time-dependent manner. The cytotoxic effect was reduced with the combined treatment (Fig. 1d). Ruxolitinib was synergized with MK-2206.

The combination of ruxolitinib with MK-2206 inhibits metastasis

To understand the metastatic effect of ruxolitinib and MK-2206 on cells, a colony formation assay was used. No changes in cell proliferation were observed for the cells in the untreated control group treated with 0.1% DMSO. While there was a time-dependent decrease in cell proliferation for the cells treated with ruxolitinib compared to the untreated control group, no significant reduction in cell proliferation was detected in the cells exposed to MK-2206. Exposure to cotreated ruxolitinib and MK-2206 cells significantly reduced colony formation compared to that of the control group, DMSO-treated cells and the single drug-treated cells (Fig. 2a).

The effects of ruxolitinib and MK-2206 on lateral cell movement in MDA-MB-231 cells were examined by wound healing analysis (Fig. 2b). It was found that the width of the wound opened in the untreated control and DMSO-treated groups of MDA-MB-231 cells gradually closed over time. It was found that the width of the wound opened in MDA-MB-231 cells exposed to ruxolitinib and MK-2206 and together could not close in time. The results showed that combined treatment with ruxolitinib and MK-2206 increased the closure rate of wound widths that were opened in the cells compared to that of a single application of drugs (Fig. 2b).

The combination of ruxolitinib with MK-2206 induces apoptosis

The effect of ruxolitinib, MK-2206, and their combination on apoptosis was examined by western blot analyses (Fig. 3a). The cells were exposed to 22.5 μ M ruxolitinib, 7.5 μ M MK-2206 and 18 μ M ruxolitinib + 5 μ M MK-2206 for 48 hours. As shown in Fig. 3a, the expression of PARP, Caspase-9, Caspase-3, and Caspase-7 decreased in the cells treated with the combination of ruxolitinib + MK-2206 for 48 h. No significant change was observed in the expression of Caspase-8 in the cells treated with ruxolitinib, MK-2206, and ruxolitinib + MK-2206. As shown in Fig. 3b, proapoptotic Bax expression increased in the cells treated with ruxolitinib and MK-2206, while antiapoptotic Bcl-2 expression was reduced. Additionally, c-Myc expression was decreased in the cells treated with both ruxolitinib and MK-2206 (Fig. 3b).

The cells were stained with Hoechst 33342 nuclear stain to observe morphologic changes, such as nuclear condensation and DNA fragmentation, in the apoptotic cells (Fig. 3c). While DNA fragmentation was not detected in the untreated control cells or DMSO-treated cells, DNA fragmentation increased in the cells exposed to only MK-2206 and ruxolitinib + MK-2206. According to our findings, cotreatment with ruxolitinib and MK-2206 in MDA-MB-231 cells can trigger apoptosis by decreasing the expression of Caspase-9 and Caspase-7, playing a role in the intrinsic pathway.

The combination of ruxolitinib with MK-2206 inhibits PI3K/AKT and JAK2/STAT5 signaling

The effects of ruxolitinib, MK-2206, and their combination on PI3K/AKT and signaling were observed by western blot analyses (Fig. 4a, 4b). Only the MK-2206 combined with ruxolitinib treatment stimulated PTEN and P53 upregulation. PI3K expression decreased after only ruxolitinib and ruxolitinib + MK-2206

treatment. However, AKT expression decreased with the combined treatment (Fig. 4a). While JAK2 was upregulated after single doses of ruxolitinib and MK-2206, JAK2 was downregulated after combined treatment with ruxolitinib and MK-2206. Although STAT5 expression increased with only ruxolitinib treatment, STAT5 decreased with only MK-2206 treatment. However, STAT5 expression completely disappeared after cotreatment with ruxolitinib and MK-2206. STAT3 expression increased at the same rate in the MDA-MB-231 cells treated with ruxolitinib or MK-2206 and cotreated with ruxolitinib and MK-2206. According to these results, cotreatment with ruxolitinib and MK-2206 may inhibit PI3K/AKT and JAK2/STAT5 signaling in cells by increasing P53 and PTEN expression and decreasing PI3K, AKT, JAK2 and STAT5 expression.

Discussion

TNBC exhibits aggressive features that are characterized by poor prognosis and, consequently, a high risk of metastasis. Due to deficiencies in PR, ER, and HER2, TNBC therapy becomes difficult to perform [3]. Single-targeted therapy is not effective for TNBC because of genetic heterogeneity and acquired resistance. Combined targeted therapy approaches aimed at inhibiting oncogenic signaling networks have been investigated to achieve efficient therapy of the intractable TNBC subtype [4, 24]. Therefore, the development of targeted anticancer drugs that target signaling pathways and regulate tumor formation and metastatic progression is necessary.

The ruxolitinib drug has been approved drug for treating cancer patients who have a risk of myelofibrosis and patients who have an insufficient response to hydroxyurea or are intolerance of hydroxyurea [15]. Because of the toxicities of ruxolitinib, it is used together with other drugs [15, 25–27]. In this study, we aimed both to decrease the side effects of ruxolitinib and increase the anticancer effects of ruxolitinib using MK-2206 together in MDA-MB-231 cells. In this work, we first studied the effects of ruxolitinib in combination with MK-2206 in MDA-MB-231 cells and then evaluated the cytotoxic and growth inhibitory effects of ruxolitinib, MK-2206, and ruxolitinib + MK-2206 in MDA-MB-231 cells. As shown in our findings, the viability of cells treated with ruxolitinib, MK-2206, and their combination was reduced in a dose- and time-dependent manner. The determined dose concentrations of ruxolitinib, MK-2206, and their combination were found to be 22,5 μM , 5 μM , and 18 μM + 5 μM for 48 hr and decreased cell viability to 50%, 56%, and 55%, respectively.

Tavalli et al. [27] demonstrated that ruxolitinib has a synergistic effect with lapatinib to eliminate SUM149 mammary tumor cells in transitory drug exposure to lapatinib and ruxolitinib colony formation assays. Khan et al. [28] illustrated that peripheral blood CD34⁺ cells from primary myelofibrosis (PMF) patients that were treated with MK-2206 inhibited colony formation in a dose-dependent manner. Additionally, the researchers showed that MK-2206 had a synergistic effect with ruxolitinib in subduing the growth of JAK2V617F-mutant SET2 cells [28]. Similar to these results, we found that ruxolitinib synergizes with MK-2206, inhibiting the formation of colonies in MDA-MB-231 cells (Fig. 2a). We determined that the combination of ruxolitinib and MK-2206 inhibits cell migration (Fig. 2b). According to our results, ruxolitinib has a synergistic antiproliferative effect with MK-2206 in MDA-MB-231 cells.

Apoptosis is organized by a chain of events [29]. Studies have shown that ruxolitinib and MK-2206, when used together with other drugs, inhibit the growth of cancer cells synergistically by triggering apoptosis, but there are no studies on the combination of these two drugs with each other [25, 30, 31]. Pro- and anti-apoptotic members of the Bcl-2 family act as regulators of apoptosis [25, 29]. Due to the activation of anti-apoptotic proteins that belong to the Bcl-2 family, which contains Bcl-2, Mcl-1, Bcl-w and Bcl-XL, the resistance of several cancer cells to chemotherapy has increased [32]. In many malignancies, such as aggressive TNBCs, the MYC oncogene is overexpressed [32]. After cotreatment with ruxolitinib and MK2206, pro-apoptotic Bax expression was increased significantly, while Caspase-9, Caspase-7, PARP, c-Myc, and Bcl-2 expression were decreased (Fig. 3a, 3b). Nuclear condensation and DNA fragmentation are the main characteristics of apoptotic cells [33]. Exposing the cells to ruxolitinib + MK-2206 increased DNA fragmentation (Fig. 3c). According to our findings, the combination of ruxolitinib and MK-2206 in MDA-MB-231 cells induced the intrinsic pathway of apoptosis by decreasing the expression of Caspase-9 and Caspase-7 proteins, and compared to single treatments, the combination was more effective in triggering apoptosis.

PI3K/AKT signaling is usually activated in breast cancers and has an important role in tumorigenesis, apoptosis, and autophagy [34]. PTEN functions as a tumor suppressor and negative regulator of this pathway. In many cancers, the PTEN-inactivated form genetically concludes with the upregulation of PI3K signals. Additionally, mutations in P85, which is a regulatory subunit of PI3K, have been identified in tumors in human. The activities of P53 and c-Myc, which are transcriptional regulators, are affected by the PI3K–AKT pathway. These proteins have all been linked to oncogenic transformation, but their exact roles during PI3K-mediated oncogenesis are still unknown. AKT phosphorylates and suppresses P53 activity; however, the activity of c-Myc is increased by AKT. The effects of AKT and PI3K on transcriptional regulators also play a major role in tumor progression. Recently, studies have shown that stimulating the phosphorylation of proapoptotic proteins and Caspase-9 by AKT triggers the inactivation of these proteins [35]. According to our findings, cotreatment with ruxolitinib and MK-2206 inhibits PI3K/AKT signaling in MDA-MB-231 cells by increasing P53 and PTEN expression and decreasing PI3K and AKT expression (Fig. 4a).

JAK2 has an important role in regulating the apoptosis and proliferation of cancer cells by activating different signaling pathways, such as the STAT5/STAT3 and PI3K/AKT signaling pathways [26]. STAT5 and STAT3 are STAT family members and regulate the expression of genes that have roles in angiogenesis, survival and cell growth [7]. Therefore, resistance to therapy and recurrence may occur. Pro- and antiapoptotic proteins, such as Bak/Bax and Bcl-2, are also related to JAK/STAT signaling. The PI3K/AKT and JAK/STAT pathways control the expression of proteins that regulate mitochondrial apoptosis. Girardot et al. [36] showed that P53 interacts with tyrosine-phosphorylated and unphosphorylated (U) STAT5 by coimmunoprecipitation. Moreover, they showed that WT P53 might suppress STAT5 transcriptional activity in myeloid neoplasms. Recent studies have focused on JAK2/STAT3 signaling in breast cancer models. Britschgi et al. [9] determined that dual inhibition of PI3K/mTOR and JAK2/STAT5, which were treated with a combination of BEZ235 and NVP-BSK805 in RAS-mutated MDA-MB-231 LM2 and PTEN-deficient MDA-MB-468 breast cancer cells and in the mouse

breast cancer cell Line 4T1, caused the activation of Bim and inhibition of Mcl-1. Additionally, they showed that inhibiting JAK2 prevents the resistance to PI3K/mTOR inhibition and that the combination therapy of PI3K/mTOR and JAK2 negatively affects tumor growth, cancer cell number and metastasis [7, 9]. According to our study, the combined treatment of ruxolitinib and MK-2206 can similarly inhibit the JAK/STAT signaling pathway in MDA-MB-231 cells by decreasing JAK2 expression and STAT5 expression. In addition, our study showed for the first time that the increased expression of P53 and PTEN tumor suppressor genes at the protein level after combined treatment with ruxolitinib and MK-2206 may be associated with the inhibition of STAT5 expression in breast cancer cells (Fig. 4b).

Conclusions

In conclusion, the JAK2 inhibitor ruxolitinib has a synergistic antiproliferative effect with the AKT inhibitor MK-2206. Additionally, compared to single use of drugs, the combined treatment with ruxolitinib and MK-2206 is more effective. The increased expression of P53 and PTEN tumor suppressor genes at the protein level with combined treatment with ruxolitinib and MK-2206 may have an important role in triggering apoptosis in MDA-MB-231 cells by dual inhibition of JAK2/STAT5 and PI3K/AKT signaling. The dual inhibition of JAK2/STAT5 and PI3K/AKT may reduce metastasis by decreasing the survival of tumor cells.

Declarations

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The authors of this paper state that they have no conflicts of interest.

Competing Interests

The authors have no relevant financial or nonfinancial interests to disclose.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Esin Guvenir Celik. The draft of the manuscript was written by Esin Guvenir Celik and Onur Eroglu, and all authors approved the version to be published. All authors read and approved the final manuscript.

Ethics approval

The research performed in article used a commercially available cell line, and we declare that it does not include any studies with human participants or animals by any of the authors.

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Figures

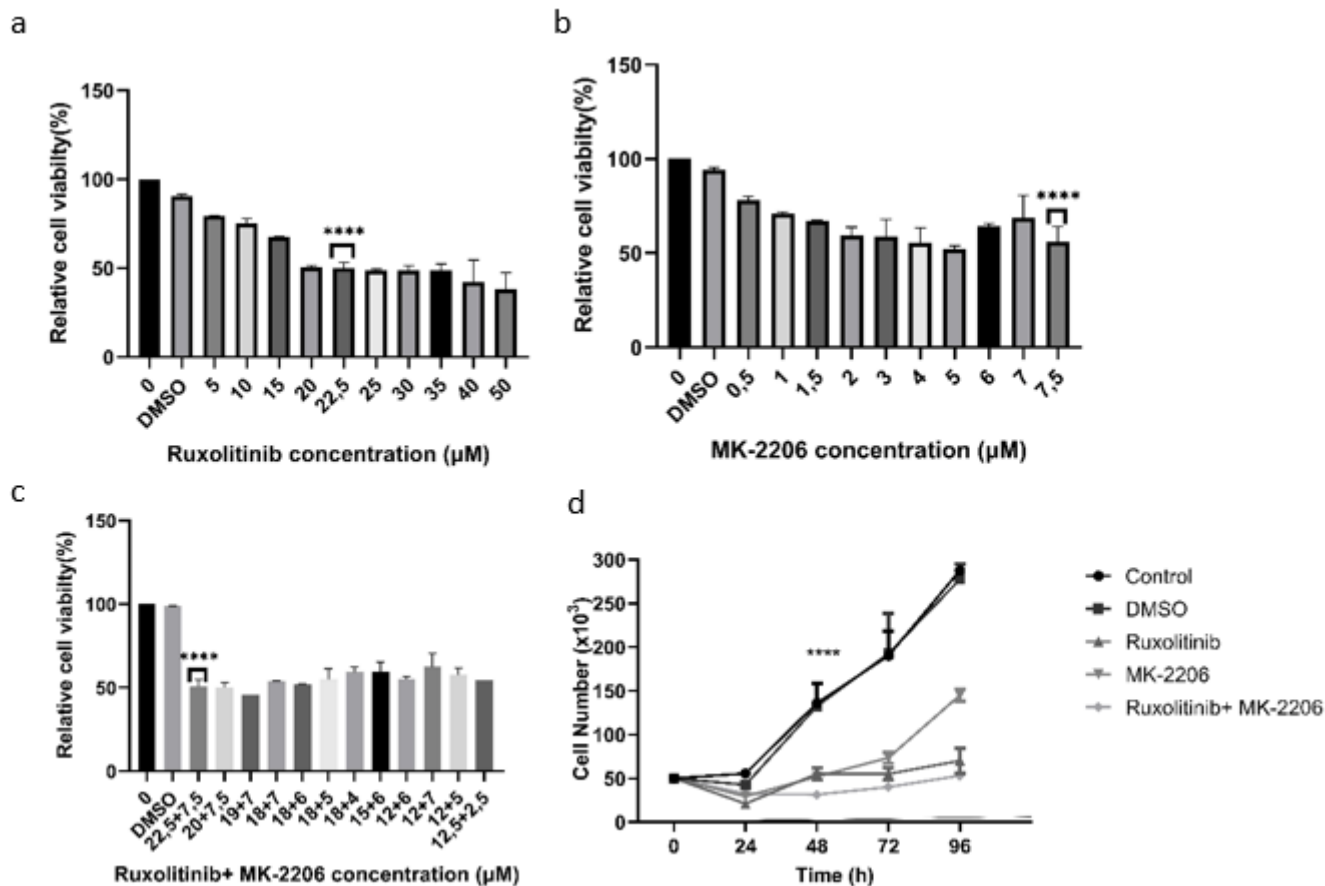


Figure 1

Effect of ruxolitinib, MK-2206, and their combination on MDA-MB-231 cell viability. Cell viability was measured by MTT assay after ruxolitinib (a), MK-2206 (b), and their combination (c) treatment. The Trypan blue assay was used to determine the time-dependent effect of ruxolitinib, MK-2206, and their combination on cell viability (d). \pm S. D values indicate three independent experiments with at least 3 replicates. Statistical differences were analyzed using one-way ANOVA and two-way ANOVA. *, **, *** and **** indicate $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

Figure 2

Determination of the metastatic effect of ruxolitinib and MK-2206 on MDA-MB-231 cells (a). Determination of the effects of ruxolitinib and MK-2206 on lateral cell movement in MDA-MB-231 breast cancer cells (b). \pm S. D values indicate three independent experiments with at least 3 replicates. Every group was compared with the untreated group. Statistical differences were analyzed using two-way ANOVA. *, **, *** and **** indicate $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

Figure 3

Effects of ruxolitinib, MK-2206, and ruxolitinib+MK-2206 on apoptosis. The effects of ruxolitinib, MK-2206, and ruxolitinib+MK-2206 on apoptosis (a) and Bcl-2 family members (b) were investigated using β -actin as a loading control by western blot. Nuclear condensation and DNA fragmentation were observed in the cells exposed to ruxolitinib, MK-2206, and ruxolitinib+MK-2206 by Hoechst 33342 staining (c). The error bars represent the mean \pm S.D. from three independent experiments. *, **, *** and **** indicate $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

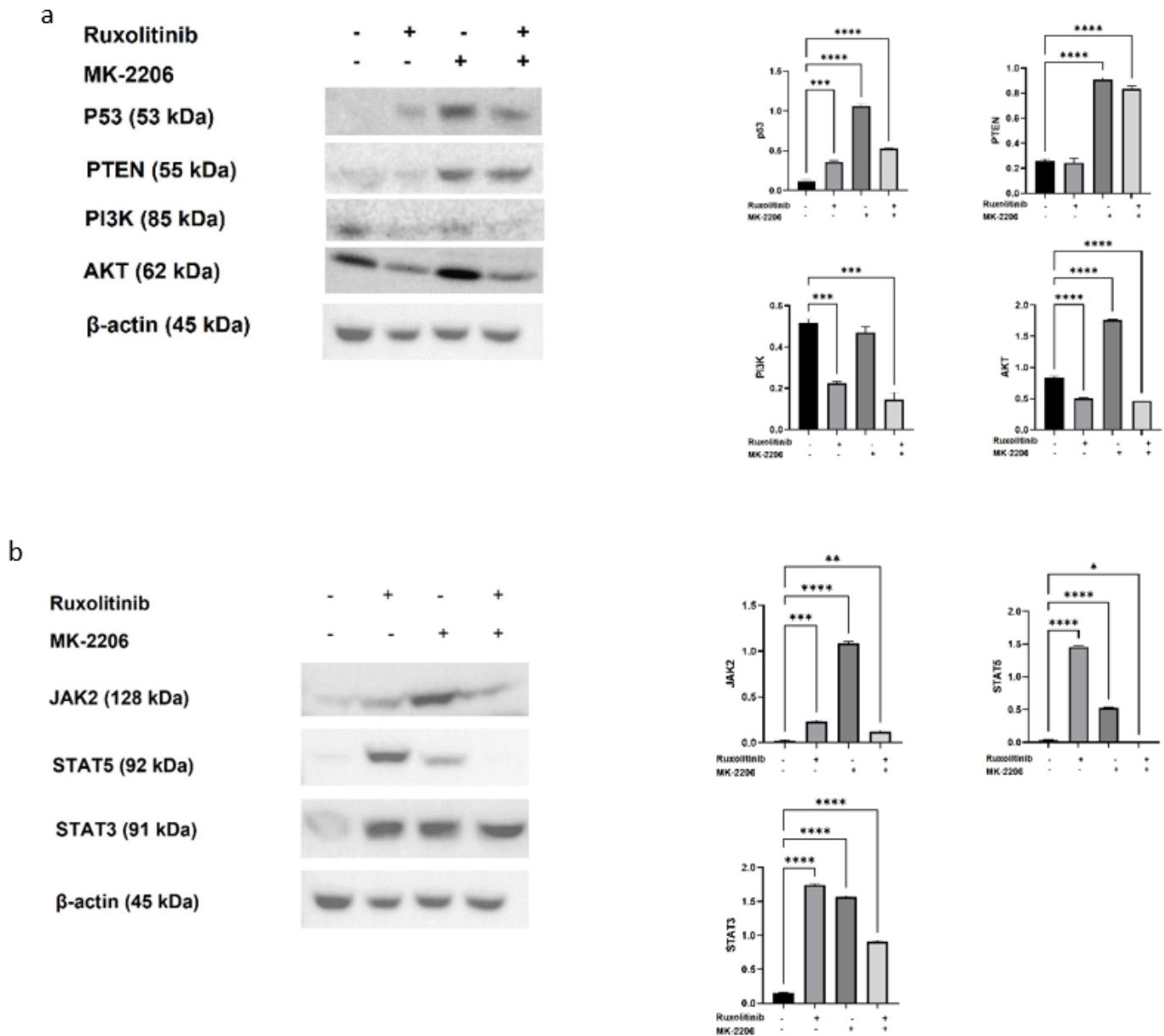


Figure 4

Effects of ruxolitinib, MK-2206, and ruxolitinib+MK-2206 on the PI3K/AKT signaling pathway (a) and JAK/STAT signaling (b). β -Actin was used as a loading control. Error bars represent the mean \pm S.D. from three independent experiments. *, **, *** and **** indicate $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.