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Research Article

Keywords: Triple-negative breast cancer, Flavopiridol, FOXM1, NF- κ B, RAS/MAPK, Doxorubicin

Posted Date: April 10th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9296085/v1>

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Additional Declarations: No competing interests reported.

Flavopiridol inhibits FOXM1/ NF- κ B and KRAS/MAPK signaling and DNA repair mechanisms and Sensitizes Triple-Negative Breast Cancer Cells to Doxorubicin the

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Running Title: Flavopiridol Sensitizes TNBC to Doxorubicin

Key words: Triple-negative breast cancer; Flavopiridol; FOXM1; NF- κ B; RAS/MAPK; Doxorubicin

Abstract

Purpose

Triple-negative breast cancer (TNBC) is an aggressive subtype characterized by frequent chemoresistance and poor clinical outcomes. Doxorubicin (DOX) is a standard therapeutic agent; however, acquired resistance limits its efficacy. Forkhead box M1 (FOXM1) and nuclear factor- κ B (NF- κ B) are key regulators of pro-survival signaling pathways implicated in TNBC progression and drug resistance. This study aimed to investigate whether flavopiridol enhances DOX sensitivity in TNBC cells by targeting the FOXM1–NF- κ B signaling axis.

Methods

MDA-MB-231 and BT-549 TNBC cell lines were treated with DOX, flavopiridol, or their combination. Cell viability, clonogenic survival, apoptosis, and cell-cycle distribution were assessed. Protein expression levels of FOXM1, NF- κ B, RAS/MAPK, and PARP were analyzed by Western blot. FOXM1 function was further evaluated באמצעות siRNA-mediated gene silencing.

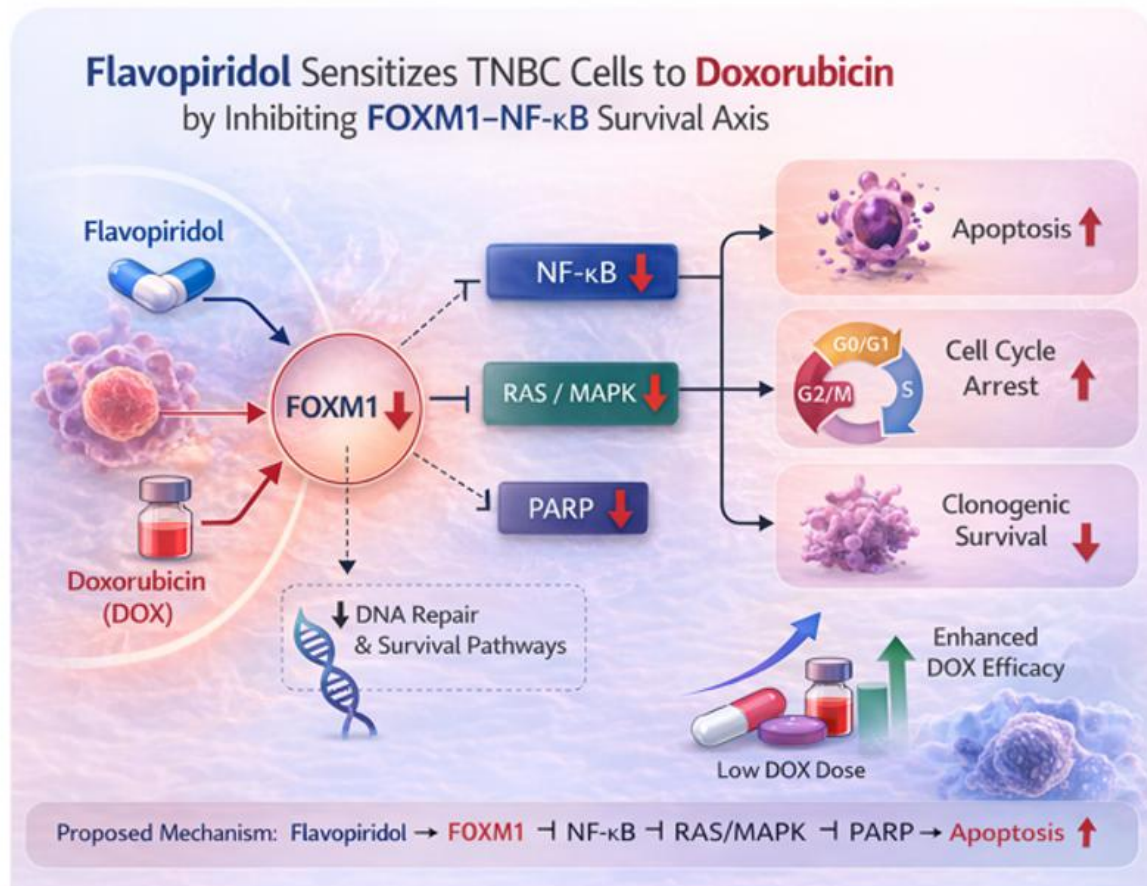
Results

Combination treatment significantly reduced cell viability and clonogenic potential compared to single-agent treatments. Flavopiridol enhanced DOX-induced apoptosis and induced marked alterations in cell-cycle progression in both TNBC cell lines. At the molecular level, combination therapy suppressed FOXM1 expression and downregulated NF- κ B and RAS/MAPK signaling pathways. FOXM1 silencing recapitulated these effects, confirming its central role in pro-survival signaling. Additionally, decreased PARP expression suggested impaired DNA repair capacity.

Conclusion

The FOXM1–NF- κ B–RAS/MAPK axis plays a critical role in DOX resistance in TNBC.

Targeting FOXM1 with flavopiridol enhances DOX sensitivity and represents a promising therapeutic strategy to overcome chemoresistance in aggressive TNBC.



Graphical Abstract. Flavopiridol enhances the anticancer efficacy of doxorubicin (DOX) in triple-negative breast cancer (TNBC) by suppressing FOXM1 signaling. Inhibition of FOXM1 attenuates NF-κB and Ras/MAPK pathways and reduces PARP expression, leading to impaired survival signaling, increased apoptosis, and decreased clonogenic survival. This mechanism sensitizes TNBC cells to DOX and suggests that targeting the FOXM1–NF-κB axis may improve therapeutic response.

Introduction

Triple-negative breast cancer (TNBC) is one of the most aggressive and therapeutically challenging subtypes of breast cancer, characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and HER2 amplification. TNBC accounts for approximately 15–20% of all breast cancers and is associated with early metastasis, high recurrence rates, and poor overall survival due to the lack of effective targeted therapies [1, 2]. The marked molecular heterogeneity of TNBC, together with its early acquisition of chemoresistance, represents a major challenge for effective clinical management [2].

Antracyclins such as Doxorubicin (DOX) is a DNA-intercalating agent inhibiting topoisomerase II and constitutes a cornerstone of first-line and standard-of-care chemotherapy as used as part of neoadjuvant (before surgery) and adjuvant (after surgery) regimens to treat early and advanced stage TNBC patients. However, DOX treatment is often associated with side effects such as cardiotoxicity and frequent resistance to DOX, limiting its therapeutic efficacy [3].

Activation of pro-survival signaling pathways and dysregulated transcriptional networks are major contributors to DOX resistance in aggressive breast cancers [3, 4]. Among these pathways, nuclear factor-kappa B (NF- κ B) signaling plays a central role in tumor progression and chemoresistance. Therefore, NF- κ B activation enhances tumor cell survival under genotoxic stress and has been strongly implicated in chemotherapy resistance [5-7].

FOXM1 (Forkhead Box M1) is a key oncogenic transcription factor that regulates genes required for G1/S and G2/M transition, DNA damage response, mitotic progression, invasion, and therapeutic resistance [8, 9]. FOXM1 is markedly overexpressed in TNBC and correlates with poor prognosis [10-12]. Given its involvement in DNA damage response and stress adaptation, FOXM1 is considered a critical mediator of chemoresistance.

Recently evidence suggests functional crosstalk between FOXM1 and NF- κ B signaling networks [13-15]. NF- κ B activation has been shown to influence FOXM1-dependent

transcriptional programs under stress conditions [16], whereas FOXM1 has been reported to enhance pro-survival and inflammatory signaling partly through regulation of NF- κ B-associated transcriptional activity [13, 15, 17]. However, the relationship between FOXM1 and NF- κ B signaling in DOX resistance remains unexplained in TNBC.

CKD4/6 inhibitors such as ribociclib and abemaciclib have been shown to be particularly effective in combination with chemo-, radio-, or immunotherapy. Flavopiridol (alvocidib), a potent pan-cyclin-dependent kinase (CDK) inhibitor, exhibits antitumor activity and cell-cycle arrest [18, 19]. Flavopiridol has been shown to suppress NF- κ B activation via inhibition of IKK activity and p65 phosphorylation [20-22] and to enhance apoptotic responses in combination therapies [22, 23]. In our previous studies in glioblastoma (GBM) cells, we found that flavopiridol significantly suppresses FOXM1 expression and downstream proliferative signaling pathways, leading to reduced GBM cell survival. Based on these observations, we hypothesized that flavopiridol-induced inhibition of NF- κ B and FOXM1-dependent oncogenic signaling [24] enhances the anticancer efficacy of DOX and sensitizes TNBC cells to DOX.

In the current study we demonstrated that flavopiridol enhanced DOX-induced suppression of KRAS/MAPK and FOXM1/NF- κ B signaling axis and enhanced DOX-induced inhibition of cell proliferation and cytotoxicity and, promoted DOX-induced apoptosis, suggesting that flavopiridol DOX.

Material and Methods

Cell Lines and Cell Culture

Human triple-negative breast cancer (TNBC) cell lines MDA-MB-231 and BT-549 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All breast cancer cell lines were maintained in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St.

Louis, MO, USA). Cells were cultured at 37°C in a humidified incubator containing 5% CO₂ [10-12, 25].

Drug Preparation and Treatment

Flavopiridol (#F3035 Sigma-Aldrich) and doxorubicin (DOX) (#D1515 Sigma-Aldrich) were prepared according to the manufacturers' instructions. Both compounds were dissolved in sterile water to prepare stock solutions. The DOX stock solution was stored at -20°C, whereas the flavopiridol stock solution was stored at +4°C. For experimental use, working concentrations were freshly prepared by diluting stock solutions in FBS-free culture medium prior to treatment.

Transfection with siRNA

To investigate the functional role of FOXM1, gene silencing experiments were performed using small interfering RNAs (siRNAs). Two different FOXM1-siRNAs (FOXM1#1 : SASI_Hs01_00052108_AS; FOXM1#2: SASI_Hs01_00243977_AS) targeting the FOXM1 gene and non-targeting control siRNA (SIC002-10NMOL Sigma Lot: WDAA1098) were purchased from Sigma-Aldrich [10-12, 25, 26]. Exponentially growing breast cancer cells were seeded and allowed to adhere for 24 hours prior to transfection. Cells were then transfected with FOXM1-specific siRNAs or non-silencing control siRNA at a final concentration of 50 nM using HiPerFect Transfection Reagent (Qiagen), according to the manufacturer's instructions. After 72 hours of transfection, cells were harvested and processed for subsequent analyses, including protein expression and signaling pathway evaluation. Cells transfected with non-silencing control siRNA used as the negative control group. The selected siRNA concentration was based on previously published studies [10-12, 25, 26].

Cell Viability Assay

Cell viability was evaluated using the MTS assay. MDA-MB-231 and BT-549 cells were seeded into 96-well plates at a density of 1,500 cells per well in 100 µL of culture medium. Cells were

then treated with increasing concentrations of flavopiridol (100, 200, 300, and 500 nM) or DOX (1, 3, 5, 8, and 10 μ M) as single agents for 24 hours. For combination experiments, cells were treated with flavopiridol (500 nM) and DOX (10 μ M), corresponding to concentrations that produced approximately 50% growth inhibition (IC_{50}) based on MTS dose–response analyses. Untreated cells used as the control group. Following treatment, a solution containing MTS reagent and phenazine methosulfate (20:1, v/v) was added to each well according to the manufacturer's protocol. After incubation for 3 hours at 37°C, cell viability was determined by measuring absorbance at 490 nm using a microplate reader (Glomax-Multi). Cell viability was expressed as a percentage relative to the untreated control group [10-12, 24, 25].

Colony Formation Assay

To evaluate the long-term proliferation following treatment, clonogenic assays were performed. MDA-MB-231 and BT-549 TNBC cells were seeded into 6-well plates at a density of 1.5×10^3 cells per well. Cells were then treated with flavopiridol, DOX, or their combination for 48 hours. Following treatment, cells were incubated for 14 days at 37°C in a humidified atmosphere containing 5% CO₂ to allow colony formation. At the end of the incubation, cells were stained with methanol-containing crystal violet solution. Colonies consisting of ≥ 50 cells were considered viable colonies and were counted under a light microscope. Colony formation efficiency was calculated relative to the untreated control group [10-12, 24, 25].

Detection of Apoptotic Cell Death

Apoptotic cell death was evaluated using Annexin V/7-AAD staining followed by flow cytometric analysis. MDA-MB-231 and BT-549 cells were seeded at a density of 1×10^4 cells per well and treated with flavopiridol, DOX, or their combination for 24 hours. Untreated cells used as controls. Following treatment, both floating and adherent cells were collected, washed twice with cold phosphate-buffered saline (PBS), and resuspended in binding buffer. Apoptosis was assessed using the Muse™ Annexin V & Dead Cell Kit (MCH100105, Merck Millipore)

according to the manufacturer's instructions using the Muse Cell Analyzer. Total apoptotic cell percentages were calculated as the early and late apoptotic cell [11, 24, 27].

Cell Cycle Analysis

Cell-cycle distribution was evaluated using the Muse™ Cell Cycle Kit (MCH100106, Merck Millipore) according to the manufacturer's instructions. MDA-MB-231 and BT-549 cells were treated with doxorubicin (10 μ M), flavopiridol (500 nM), or their combination for 24 hours. Untreated cells used as controls. Following treatment, cells were harvested, fixed in 70% ethanol at -20°C overnight, and stained with Muse™ Cell Cycle Reagent. Samples were analyzed using the Muse Cell Analyzer, and the percentages of cells in G0/G1, S, and G2/M phases were determined according to the manufacturer's protocol [28].

Western Blot Analysis

MDA-MB-231 and BT-549 cells (3.5×10^5 cells) were seeded in T-25 flasks. After 24 hours, cells were transfected with FOXM1-specific siRNAs (50 nM) or control siRNA for 72 hours and subsequently treated with doxorubicin (10 μ M), flavopiridol (500 nM), or their combination for 24 hours.

Cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and lysed in lysis buffer at 4°C . Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad). Equal amounts of protein (40 μ g per sample) were separated by 4–20% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1 hour at room temperature in TBS-T containing 5% non-fat dry milk and 0.1% Triton X-100. After washing with TBS-T, membranes were incubated overnight at 4°C with the following primary antibodies: FOXM1 (Cell Signaling Technology, cat#5436S), NF- κ B (Abcam, ab16502), RAS (Proteintech, 12063-1-AP), MAPK (Cell Signaling, 4370S), PARP (Proteintech, cat#13371-1-AP) and β -actin (Proteintech, cat#60008-1-Ig). All primary antibodies were diluted 1:1000 in TBS-T containing 5% non-fat

dry milk. After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies (Bio-Rad, #170-6515) for 1 hour at room temperature. β -actin was used as a loading control. Protein bands were detected using Clarity™ Western ECL Substrate (Bio-Rad) and visualized with a ChemiDoc MP Imaging System (Bio-Rad). Densitometric analysis was performed using Image Lab software (Bio-Rad), and relative protein expression levels were normalized to β -actin [10-12, 24-27].

Statistical Analysis

All experiments were performed in triplicate and data were presented as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). p-value of < 0.05 was considered statistically significant.

Results

Flavopiridol Enhances Doxorubicin-induced Cytotoxicity in TNBC Cells

First, we investigated dose-dependent effects of flavopiridol and DOX in TNBC cells lines such as in MDA-MB-231 and BT-549 and later evaluated their combined effects at 24 and 48 hours. In MDA-MB-231 cells, DOX induced a significant dose-dependent reduction in cell viability at 24 hours (**** $p < 0.0001$ at 5–10 μ M). Flavopiridol similarly reduced viability in a concentration-dependent manner, with significant suppression observed at 300–500 nM. At 48 hours, flavopiridol exhibited enhanced cytotoxic effects. Combination treatment significantly reduced viability compared to either monotherapy (** $p < 0.01$, *** $p < 0.001$), decreasing cell viability to approximately 30–40% (Figure 1a). In BT-549 cells, DOX treatment produced dose-dependent cytotoxicity, with significant reductions observed at concentrations ≥ 3 μ M. Flavopiridol reduced viability in a concentration-dependent manner, with stronger effects at 48 hours. Importantly, combination treatment resulted in the lowest viability (~30–35%),

significantly lower than either agent alone (Figure 1b). These findings indicate that flavopiridol increased DOX-mediated cytotoxicity in TNBC cells.

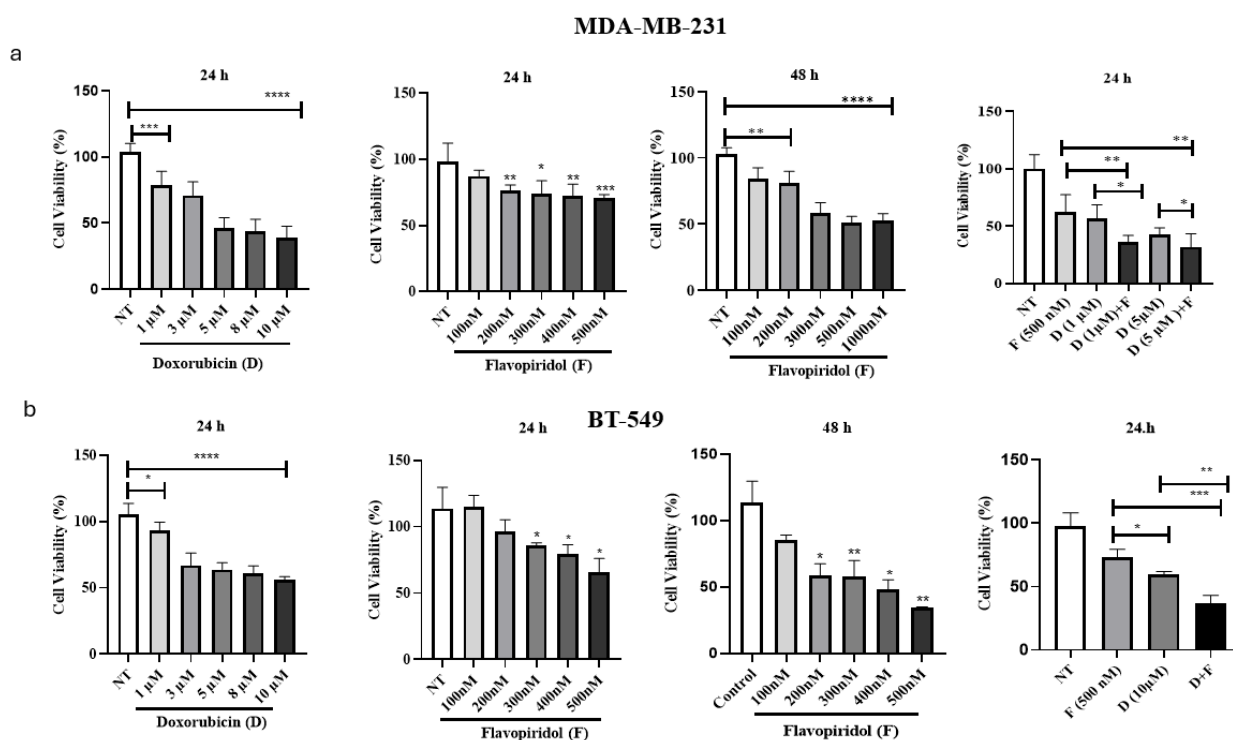


Fig 1. Flavopiridol enhances doxorubicin-induced growth inhibition in TNBC cells.

(a) MDA-MB-231 and (b) BT-549 cells were treated with increasing concentrations of doxorubicin (D) or flavopiridol (F) for 24 h and 48 h as indicated. Cell viability was assessed by MTS assay and expressed as percentage relative to untreated control (NT). For combination experiments, cells were treated with flavopiridol (500 nM) alone, doxorubicin alone, or the combination (D+F) for 24 h. Combination treatment resulted in a greater reduction in cell viability compared with single-agent treatment in both TNBC cell lines. Data are presented as mean $\pm$ SD. Statistical significance was determined (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, NT: non-treatment).

Combination Treatment Suppresses Colony Formation in TNBC cells

We also determined dose-dependent effects of flavopiridol and DOX and their combined effects in cell and colony formation.

In MDA-MB-231 cells, treatment with increasing concentrations of doxorubicin (1–30 nM) resulted in significant and dose dependent reduction in cell proliferation and colony formation. While low doses (1–5 nM) produced moderate decreases in colony numbers, higher concentrations (20–30 nM) significantly suppressed colony formation (**** $p < 0.0001$). At 30 nM, only a minimal number of colonies remained, indicating impairment of long-term survival. Similarly, flavopiridol (50–600 nM) significantly reduced colony formation in a concentration-dependent manner. Notably, treatment with 300–600 nM almost completely inhibited colony formation (**** $p < 0.0001$). Most importantly, combination treatment (DOX 10 nM + flavopiridol 100 nM) resulted in a significantly greater reduction in colony number compared to either monotherapy (** $p < 0.001$ to **** $p < 0.0001$). While DOX alone reduced colony numbers to approximately 40–50% of control and flavopiridol alone produced moderate suppression, the combination nearly abolished colony formation (Figure 2a). We observed similar findings in BT-549 cells following single and combined flavopiridol and DOX treatments.

Compared with NT controls, DOX treatment significantly impaired colony formation of BT-549 cells. Flavopiridol (50–600 nM) also reduced colony formation in a concentration-dependent manner. At 300–600 nM, colony formation was dramatically suppressed (**** $p < 0.0001$). Most importantly, co-treatment with DOX and flavopiridol resulted in significantly fewer colonies compared to each treatment alone (** $p < 0.01$ to **** $p < 0.0001$) (Figure 2b). Collectively, data indicated that marked decrease in colony formation by the combined treatments and suggesting that flavopiridol enhances the antiproliferative effects of DOX in TNBC cells.

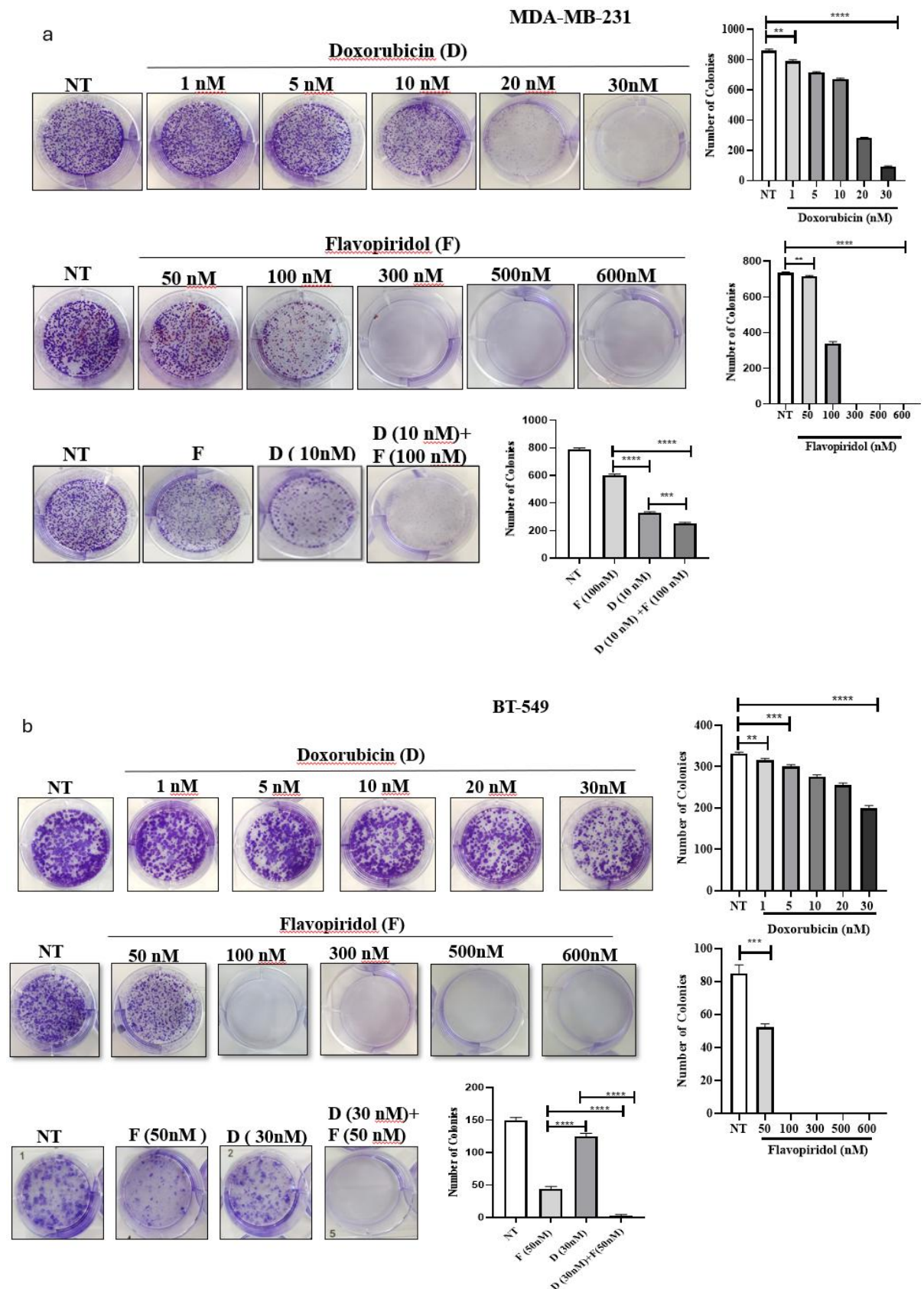


Fig 2. Flavopiridol enhances doxorubicin-mediated suppression of colony formation in TNBC cells. Representative images and quantification of colony formation in MDA-MB-231

(a) and BT-549 (b) cells following treatment with increasing concentrations of doxorubicin (DOX), flavopiridol (F), or their combination. Cells were treated for 24 h as indicated, and cells were allowed to grow for 10–14 days to assess colony formation. Combination treatment markedly decreased clonogenic survival compared with single-agent treatment in both TNBC cell lines. Data are presented as mean $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NT: non-treatment).

Flavopiridol Enhances DOX-Induced Apoptosis in TNBC Cells

To determine whether Flavopiridol enhances DOX-induced apoptotic cell death, we evaluated the combined effects of both drugs in apoptosis which was quantified by Annexin V/PI staining and flow cytometry analysis. In MDA-MB-231 cells, DOX (10 μ M) treatment significantly increased total apoptotic cell compared to NT controls (*** $p < 0.001$), reaching approximately 65–70%. Flavopiridol (500 nM) alone induced a moderate apoptotic response (~45–50%), consistent with CDK inhibition-mediated stress. Combination treatment markedly increased apoptosis (~90% total apoptotic cells), significantly higher than either monotherapy (** $p < 0.01$) (Figure 3a). In BT-549 cells, DOX treatment moderate increased apoptosis (~70–75%), while flavopiridol alone further elevated apoptotic rates (~80%). Notably, combination treatment resulted in the highest apoptotic cells (~88–90%), significantly increased single-agent treatments (* $p < 0.05$, ** $p < 0.01$) (Figure 3b).

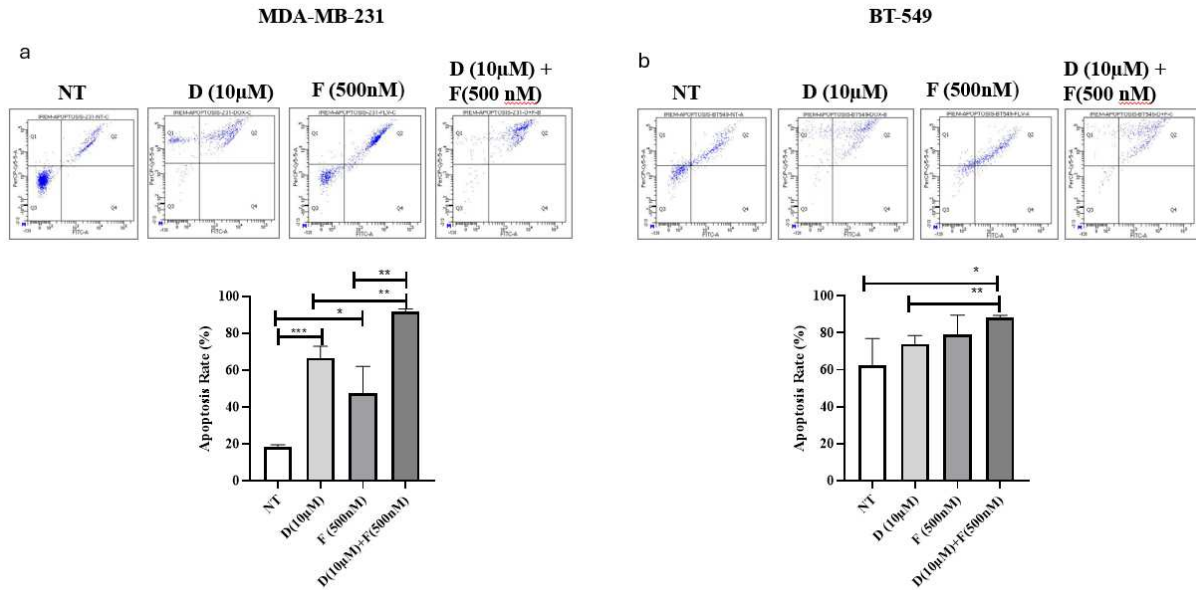


Fig 3. Flavopiridol enhances doxorubicin-induced apoptosis in TNBC cells. Representative Annexin V–FITC/propidium iodide (PI) flow cytometry plots and quantitative analysis of apoptotic cell populations in MDA-MB-231 (a) and BT-549 (b) cells following treatment with doxorubicin (DOX), flavopiridol (F), or their combination (D+F) for 24 h. Combination treatment significantly increased the percentage of apoptotic compared with single-agent treatments in both TNBC cell lines. Data are presented as mean $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NT: non-treatment).

Combination Treatment enhances DOX-induced effects in Cell-Cycle Progression in TNBC cells

To further investigate whether the enhanced cytotoxicity observed with combination treatment was associated with cell cycle disruption, cell cycle analysis was performed by flow cytometry. In MDA-MB-231 cells, DOX (10 μ M) treatment increased the G0/G1 population compared to non-treated (NT) controls (approximately 60–62% vs ~45%), indicating cell cycle arrest following DNA damage. Flavopiridol (500 nM) alone also elevated the G0/G1 to a similar extent. Importantly, the combination of DOX and flavopiridol maintained a high G0/G1, but

was associated with a significant alteration in the G2/M population. While DOX alone increased G2/M accumulation (~28–30%), combination treatment significantly reduced G2/M compared to DOX alone (** $p < 0.001$), suggesting that cells failed to complete mitotic progression. The S-phase relatively low, indicating that the treatment in MDA-MB-231 cells was G0/G1 arrest rather than replication arrest. Our findings, In MDA-MB-231 cells, DOX and flavopiridol arrest the in G0/G1 phase of cell-cycle. Combination treatment reduced G2/M accumulation compared to DOX alone (Figure 4a).

In BT-549 cells, a distinct pattern of cell cycle was observed. In NT cells, approximately 40–45% of cells were in G0/G1 phase. DOX treatment significantly reduced the G0/G1 (~35%) while increasing the G2/M cell population (~30%). Flavopiridol alone markedly decreased the S-phase cell population (~10–12% vs ~20% in NT) and arrest in G0/G1 phsae. Notably, combination treatment significantly increased G2/M accumulation compared to single treatments (**** $p < 0.0001$). S-phase cells were significantly reduced. These data demonstrated that in BT-549 cells, DOX increased G2/M accumulation, whereas flavopiridol reduced S-phase progression. Combination treatment significantly enhanced G2/M arrest and reduced S-phase (Figure 4b). Although MDA-MB-231 cells predominantly exhibited G0/G1 arrest and reduced G2/M, whereas BT-549 cells displayed G2/M accumulation. These findings indicated that flavopiridol treatment disrupted cell-cycle progression in both TNBC cell lines.

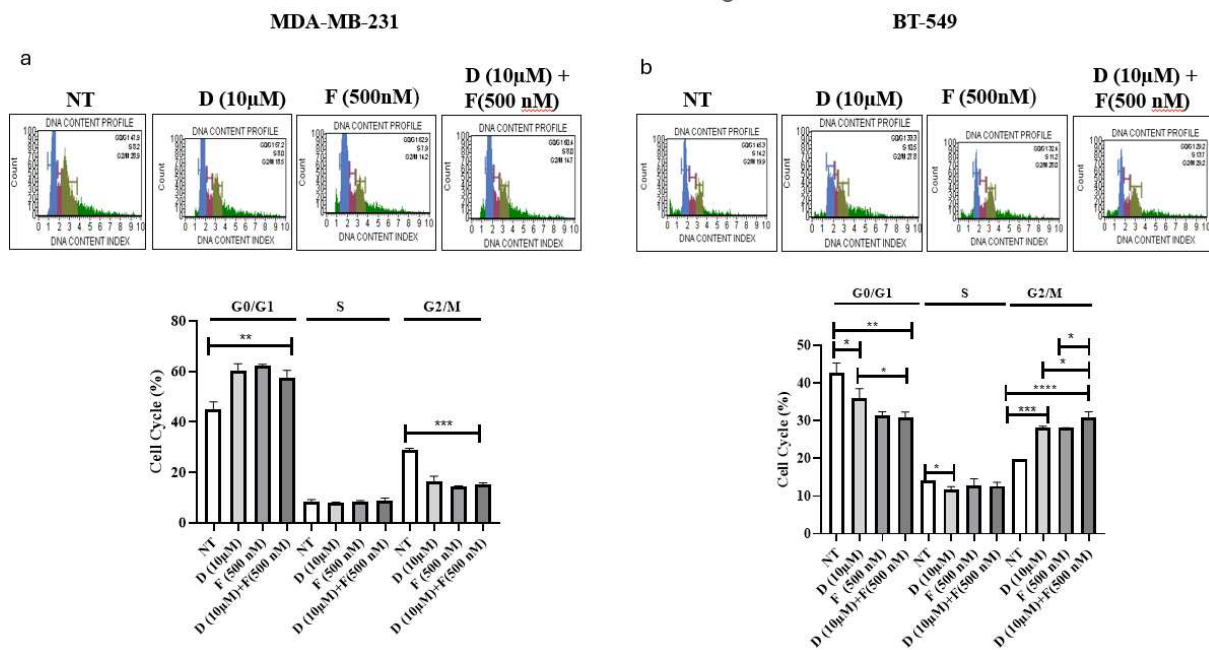


Fig 4. Flavopiridol effects cell-cycle distribution DOX-induced in TNBC cells. Cell-cycle distribution was analyzed in MDA-MB-231 (a) and BT-549 (b) cells following treatment with doxorubicin (DOX), flavopiridol (F), or their combination (D+F) for 24 h. DOX treatment altered cell-cycle progression, while flavopiridol induced additional changes in distribution. Combination treatment further modified cell-cycle compared with single-agent treatments. Bar graphs represent the percentage of cells in each phase of the cell (mean $\pm$ SD) (* p < 0.05, ** p < 0.01, *** p < 0.001, NT: non-treatment).

Flavopiridol and DOX combination therapy suppresses FOXM1/NF- κ B and RAS/MAPK-Survival pathways in TNBC Cells

To elucidate the molecular mechanisms underlying the enhanced cytotoxicity of the combination treatment, the expression levels of FOXM1 and its associated signaling molecules (NF- κ B, RAS, MAPK, and PARP1) were evaluated by Western blot analysis in both MDA-MB-231 and BT-549 cells.

In MDA-MB-231 cells, treatment with DOX (10 μ M) alone led to a moderate reduction in FOXM1 expression compared with NT controls, whereas flavopiridol (500 nM) caused a

greater decrease. Combined treatment resulted in a significantly greater reduction in FOXM1 expression compared with either treatment alone (**** $p < 0.0001$). In parallel with FOXM1 downregulation, NF- κ B, RAS, and MAPK protein levels were significantly lower in the combination group than in the monotherapy groups (Figure 5a).

Similar, dose-responsive pattern was observed in BT-549 cells. 500 nM of Flavopiridol alone reduced FOXM1 expression compared to NT controls, while DOX treatment at 1 μ M and 5 μ M suppression. However, combination treatments (1 μ M DOX + Flavopiridol and 5 μ M DOX + Flavopiridol) resulted in significantly higher FOXM1 downregulation than single-agent treatments (* $p < 0.05$ to **** $p < 0.0001$). NF- κ B expression followed a similar pattern, with the combination groups exhibiting the lowest protein levels. RAS and MAPK expression were also significantly reduced following combination therapy (Figure 5b).

Together, these findings indicate that in both TNBC cell lines, FOXM1 downregulation was associated with decreased NF- κ B and RAS/MAPK signaling following combination treatment. Given the role of FOXM1 in regulating cell-cycle progression and DNA damage response, its suppression may reduce chemoresistance by inhibiting RAS/MAPK- and NF- κ B-associated survival pathways [13, 15, 17, 30-32], impair recovery from DOX-induced DNA damage, and thereby enhance therapeutic sensitivity.

Considering the critical role of PARP1 in DNA damage repair (29), we also evaluated PARP levels and found that combination therapy resulted in a marked reduction in total PARP1 protein levels compared with single-agent treatments indicating compromised DNA repair capacity. (Figure 5-6). Together, these findings suggest that flavopiridol enhances DOX-induced effects through multiple mechanism in TNBC cells.

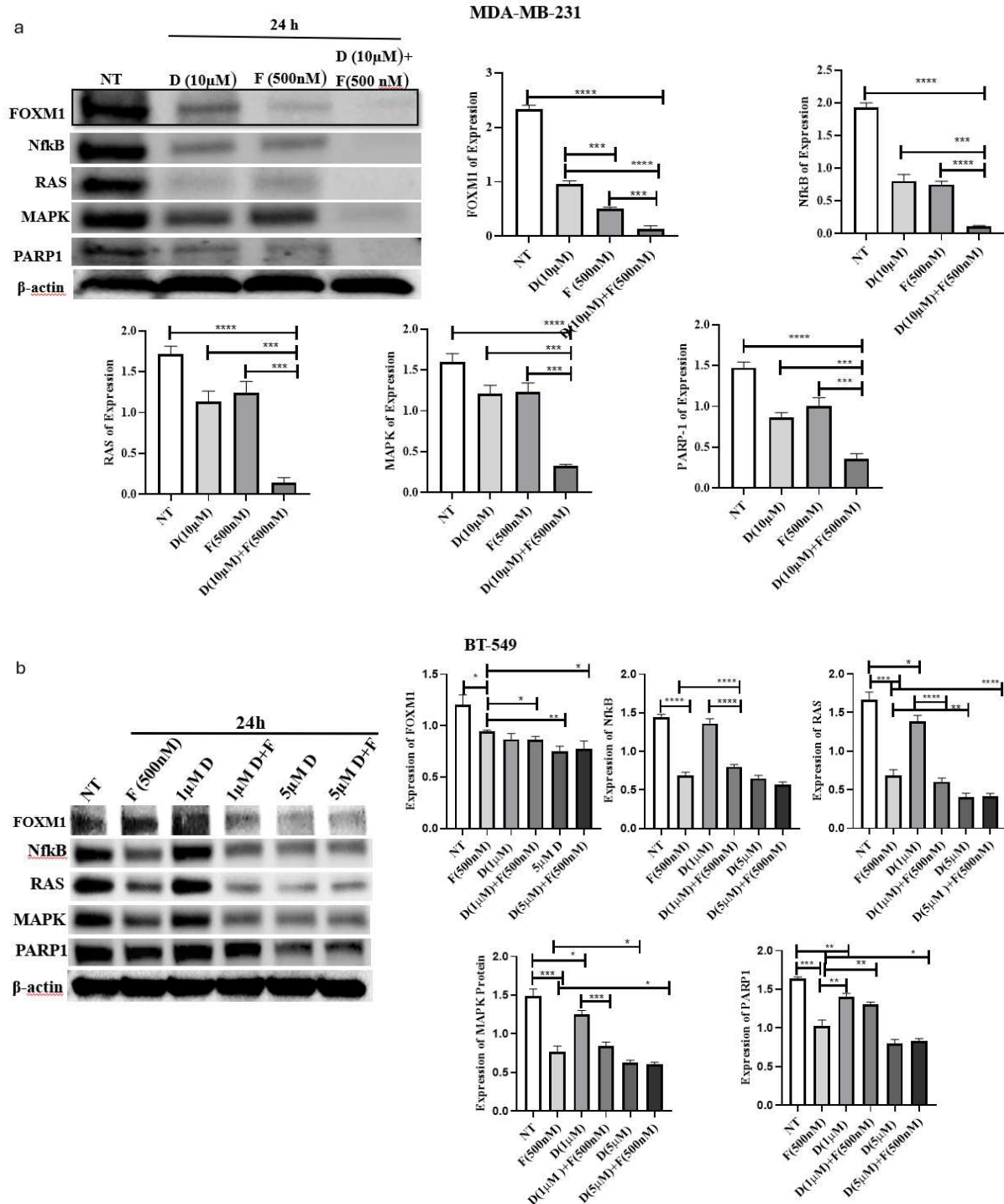


Fig 5. Combined treatment with flavopiridol and DOX suppresses FOXM1, NF- κ B, and RAS/MAPK expression in TNBC cells. FOXM1, NF- κ B, and RAS/MAPK protein expression levels were analyzed by Western blot in MDA-MB-231 (a) and BT-549 (b) cells following treatment with doxorubicin (DOX), flavopiridol (F), or their combination (D+F) for 24 h. Combination treatment further reduced FOXM1 expression and was associated with decreased

NF- κ B and RAS/MAPK levels compared with single-agent treatments. PARP expression was also reduced following combination treatment. Protein levels were normalized to β -actin (loading control) and quantified by densitometric analysis. Bar graphs represent mean $\pm$ SD (* p < 0.05, ** p < 0.01, *** p < 0.001; NT, non-treated control).

Intibition of FOXM1 Reduces RAS/MAPK- and NF- κ B-Associated Survival Signaling in TNBC Cells

To determine whether suppression of RAS/MAPK and NF- κ B signaling was directly mediated by FOXM1 inhibition, siRNA-mediated FOXM1 knockdown was performed in both TNBC cell lines to evaluate the regulatory role of FOXM1 in these pathways.

In MDA-MB-231 cells, silencing of FOXM1 using two independent siRNAs (FOXM1#1 and FOXM1#2) significantly reduced FOXM1 protein expression compared with control siRNA (**** p < 0.0001). FOXM1 knockdown led to significant reductions in NF- κ B and RAS expression levels (** p < 0.01 to **** p < 0.0001). MAPK expression was likewise significantly decreased following FOXM1 silencing (**** p < 0.0001). In addition, total PARP levels were reduced in FOXM1-silenced cells (Figure 6a).

In BT-549 cells, FOXM1 knockdown significantly decreased FOXM1 protein expression (* p < 0.05 to **** p < 0.0001). Suppression of FOXM1 resulted in significant downregulation of NF- κ B and RAS expression (*** p < 0.001 to **** p < 0.0001). In contrast to MDA-MB-231 cells, MAPK expression showed minimal change, suggesting potential cell line-specific differences. Total PARP levels were also significantly reduced following FOXM1 silencing (**** p < 0.0001) (Figure 6b).

Collectively, siRNA-mediated FOXM1 knockdown reduced FOXM1 expression in both TNBC cell lines and was accompanied by decreased NF- κ B and RAS expression, as well as reduced

MAPK levels, particularly in MDA-MB-231 cells. These molecular alterations closely paralleled those observed following flavopiridol treatment.

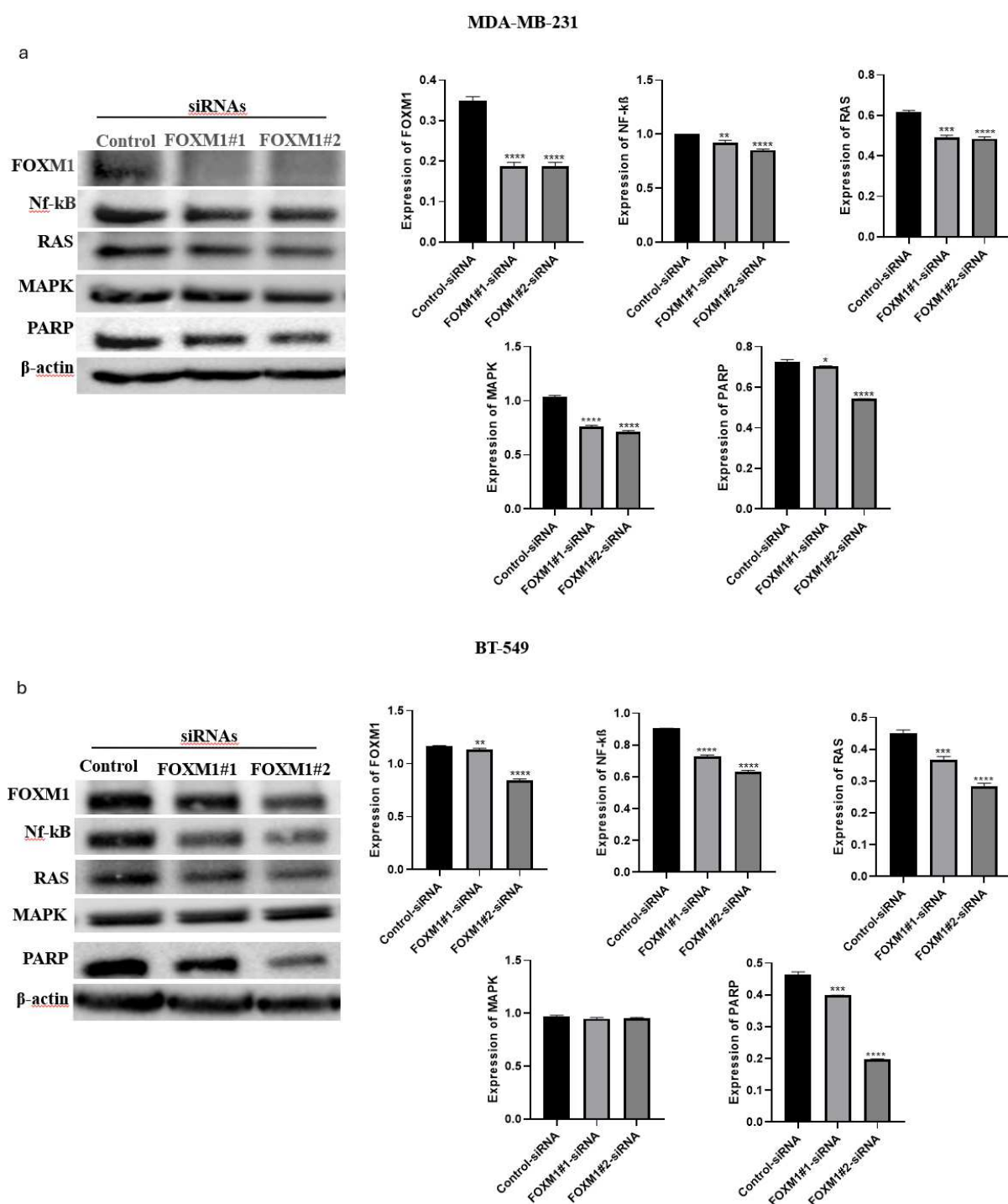


Fig 6. Knockdown of FOXM1 using FOXM1-targeting siRNAs suppresses NF-κB and RAS/MAPK signaling in TNBC cells. FOXM1, NF-κB, and RAS/MAPK protein expression levels were analyzed by Western blot in MDA-MB-231 (a) and BT-549 (b) cells following

transfection with control siRNA or two independent FOXM1-targeting siRNAs (FOXM1#1 and FOXM1#2). FOXM1 knockdown markedly reduced FOXM1 protein levels and was accompanied by decreased expression of NF- κ B and RAS/MAPK signaling components. PARP expression was also reduced following FOXM1 silencing. Protein levels were normalized to β -actin (loading control) and quantified by densitometric analysis. Bar graphs represent mean $\pm$ SD (* p < 0.05, ** p < 0.01, *** p < 0.001, NT, non-treated control).

Discussion

Triple negative breast cancer (TNBC) has high recurrence and low survival rates among breast cancer subtypes. To date, treatment options have largely been limited to several chemotherapeutic agents alone including DOX and in combination with immunotherapy; However, these therapies are associated with frequent recurrence and the development of drug resistance. Therefore, combination treatment strategies targeting resistance to doxorubicin which is one of the most commonly used agents in TNBC therapy can enhance poor patient survival and early deaths [4, 33, 34].

In the present study, we demonstrate that flavopiridol enhances DOX sensitivity in TNBC cells through multiple mechanisms including suppression of the FOXM1/NF- κ B and RAS/MAPK-survival signaling as well as inhibition of DNA-repair pathways by reducing PARP expression. Combination treatment reduced short-term viability, impaired long-term clonogenic survival, altered cell-cycle progression, and significantly increased apoptosis compared with DOX monotherapy. At the molecular level, these effects were accompanied by downregulation of FOXM1, reduced

DOX resistance remains a major challenge in TNBC therapy and has been strongly associated with activation of NF- κ B-mediated survival pathways [35-37]. NF- κ B activation is well established as a mediator of chemotherapy resistance through transcriptional regulation of anti-

apoptotic and proliferative genes [5–7, 35–37]. IKK-mediated activation of NF- κ B promotes tumor cell survival in response to DNA damage [35]. NF- κ B signaling maintains pro-survival transcriptional mechanisms and contributes to anthracycline resistance [35–37]. Previous reports demonstrated that NF- κ B inhibition sensitizes cancer cells to cytotoxic agents [21, 38, 39]. Our findings suggest that suppression of FOXM1 by flavopiridol is associated with decreased NF- κ B expression and enhanced doxorubicin-induced apoptosis in TNBC cells. Furthermore, our results indicate that FOXM1 may act upstream of NF- κ B, sustaining adaptive survival signaling in response to DOX-induced DNA damage. Consistently, siRNA-mediated FOXM1 silencing suppressed NF- κ B and RAS/MAPK signaling to a similar extent as flavopiridol treatment, supporting a regulatory role for FOXM1 in maintaining these pro-survival pathways.

FOXM1 is a key regulator of cell-cycle progression and the DNA damage response and has been closely linked to therapeutic resistance [12, 40]. It drives the transcription of genes involved in G1/S and G2/M transitions, mitotic progression, and DNA repair, thereby supporting tumor cell proliferation and adaptation to cellular stress [12, 40]. Our previous findings revealed that elevated FOXM1 expression was significantly associated with enhanced tumor aggressiveness and reduced survival in patients with TNBC [10, 11, 25, 26]. In addition, FOXM1 regulates autophagy-related genes such as LC3 and Beclin-1, contributing to cytoprotective survival mechanisms that may promote chemoresistance [12]. Together, our findings support a central role of FOXM1 in regulating stress-response pathways in aggressive breast cancer and its function as a driver of chemoresistance. Studies indicate functional crosstalk between FOXM1 and NF- κ B signaling pathways [13–17], although their interaction in TNBC chemoresistance remains incompletely understood.

Flavopiridol has been reported to inhibit NF- κ B activation through suppression of I κ B kinase (IKK) activity and p65 phosphorylation and to enhance apoptosis in combination treatments [20-23]. In glioblastoma multiforme (GBM) cells, we previously demonstrated that flavopiridol markedly reduced FOXM1 expression and downstream oncogenic signaling [24]. In this study, we found that flavopiridol similarly inhibits FOXM1-dependent survival signaling in TNBC. Furthermore, combination treatment enhanced anticancer efficacy at lower DOX concentrations compared with DOX monotherapy. Given the well-documented dose-limiting cardiotoxicity associated with DOX, strategies that enable dose reduction therapeutic effectiveness are of clear clinical importance [44, 45]. Our findings suggest that targeting FOXM1 using flavopiridol indicates a combinational approach to overcome chemoresistance in aggressive TNBC.

RAS/MAPK signaling plays an important role in cell proliferation, DNA damage response, and therapeutic resistance. Activation of this pathway supports tumor cell survival under genotoxic stress by promoting repair [41-43]. Our results show that decreased RAS/MAPK signaling following FOXM1 inhibition suggests a regulatory role for FOXM1 in multiple pro-survival pathways that facilitate tumor cell adaptation to DOX-induced genotoxic stress. The accompanying reduction in PARP expression further indicates that combination treatment may impair DNA repair capacity.

Our data extend these observations by demonstrating that FOXM1 suppression reduces NF- κ B- and RAS/MAPK-associated signaling and decreases PARP expression, changes consistent with enhanced apoptotic progression.

Collectively, our findings indicate that the FOXM1/NF- κ B and RAS/MAPK- signaling axis plays an important role in DOX resistance in TNBC and that flavopiridol-mediated FOXM1 inhibition enhances DOX sensitivity. Furthermore, combined therapy also blocks DNA-repair

mechanisms by inducing PARP1 expression, which facilitates DOX induced apoptosis. Given the dose-limiting cardiotoxicity associated with DOX, flavopiridol-based combination strategies may reduce DOX-doses in patients and may prevent or reduce DOX-induced side effects, representing a promising therapeutic option to improve treatment outcomes in aggressive TNBC.

However, our study has several limitations as our study was conducted exclusively in in vitro TNBC models. Therefore in vivo validation of flavopiridol; and DOX is needed to to further assess the therapeutic efficacy and safety of the flavopiridol–DOX combination.

Acknowledgments

The authors thank all individuals who contributed to data collection and manuscript preparation.

Author Declarations

The authors declare that they have no competing financial or non-financial interests related to this study. The results presented in this paper have not been published previously in whole or part.

Funding Information

This work was supported by the Erciyes University Research Fund (Grant No. TYL-2022-11882).

Conflicts of Interest

The authors declare that they have no competing financial or non-financial interests related to this study.

The results presented in this paper have not been published previously in whole or part.

Ethics Approval

This study was conducted using established cell lines; therefore, ethics committee approval was not required

Animal Studies: N/A.

Author contributions

Zuhal Hamurcu conceptualized and coordinated the study, performed experiments, secured funding, and drafted the manuscript. **Bulent Ozpolat** contributed to study conception and design, supervised the project, and critically revised the manuscript. **Irem Mert, Ahsen Guler, and Venhar Cinar** assisted with experimental procedures. All authors analyzed the data and approved the final version of the manuscript.

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