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ORIGINAL LABORATORY INVESTIGATION

Effective combination treatments for breast cancer inhibition by FOXM1 inhibitors with other targeted cancer drugs

Valeria Sanabria Guillen[1] · Yvonne Ziegler[1] · Chirag Gopinath[1] · Sandeep Kumar[1] · Parama Dey[1] · Blake N. Plotner[1] · Nadia Z. Dawson[1] · Sung Hoon Kim[2] · John A. Katzenellenbogen[2,3] · Benita S. Katzenellenbogen[1,3,4]

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Abstract

Purpose Few targeted treatment options currently exist for patients with advanced, often recurrent breast cancers, both triple-negative breast cancer (TNBC) and hormone receptor-positive breast cancer. Forkhead box M1 (FOXM1) is an oncogenic transcription factor that drives all cancer hallmarks in all subtypes of breast cancer. We previously developed small-molecule inhibitors of FOXM1 and to further exploit their potential as anti-proliferative agents, we investigated combining FOXM1 inhibitors with drugs currently used in the treatment of breast and other cancers and assessed the potential for enhanced inhibition of breast cancer.

Methods FOXM1 inhibitors alone and in combination with other cancer therapy drugs were assessed for their effects on suppression of cell viability and cell cycle progression, induction of apoptosis and caspase 3/7 activity, and changes in related gene expressions. Synergistic, additive, or antagonistic interactions were evaluated using ZIP (zero interaction potency) synergy scores and the Chou–Talalay interaction combination index.

Results The FOXM1 inhibitors displayed synergistic inhibition of proliferation, enhanced G2/M cell cycle arrest, and increased apoptosis and caspase 3/7 activity and associated changes in gene expression when combined with several drugs across different pharmacological classes. We found especially strong enhanced effectiveness of FOXM1 inhibitors in combination with drugs in the proteasome inhibitor class for ER-positive and TNBC cells and with CDK4/6 inhibitors (Palbociclib, Abemaciclib, and Ribociclib) in ER-positive cells.

Conclusion The findings suggest that the combination of FOXM1 inhibitors with several other drugs might enable dose reduction in both agents and provide enhanced efficacy in treatment of breast cancer.

Keywords Synergy · Breast cancer suppression · FOXM1 inhibitors · Proteasome inhibitors · CDK4/6 · Inhibitors

Introduction

The past few decades have seen significant advancements in the treatment of breast cancer thanks to the discovery of specific, molecularly targeted therapies like estrogen receptor (ER) modulators and human epidermal growth factor 2 (HER2) inhibitors that effectively suppress tumor growth in patients with hormone receptor-positive (HR +) cancers [1]. Despite these advances, few treatment options exist for patients with hormone receptor-positive tumors that do not respond to hormone therapy, acquire resistance to targeted therapy or become metastatic [2, 3]. There are even fewer options for patients with triple-negative breast cancer (TNBC), a particularly aggressive group of breast cancers, where the lack of expression of the usual druggable molecular targets makes cytotoxic chemotherapy the standard systemic treatment option [4]. TNBCs are usually associated with worse patient clinical outcomes compared to the hormone receptor-positive (HR +) subtypes, and many with metastatic TNBC will unfortunately die of this disease [5]. TNBCs are very heterogeneous and have only recently

✉ Benita S. Katzenellenbogen
katzenel@illinois.edu

1 Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

2 Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

3 Cancer Center at Illinois, Urbana, IL 61801, USA

4 Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

been classified into 6 subtypes that possess unique biological, histological, molecular, and transcriptional profiles [4, 6]. As a result of this diversity, the development of targeted molecular therapies for TNBC as a whole has been difficult [7].

Forkhead box M1 (FOXM1) is an oncogenic transcription factor that is overexpressed in all breast cancer subtypes compared to normal tissue and its overexpression is associated with poor clinical outcomes in patients [8]. FOXM1 is most highly elevated in TNBC compared to other subtypes of breast cancer and has a distinct gene expression signature that is enriched in pathways driving cancer cell growth and cell cycle progression, making it an important marker in TNBC and a potential target for novel therapies [9, 10]. In ER + cancers, FOXM1 regulates ER expression and acts as a binding partner at genomic estrogen-responsive elements (EREs) to promote mitogenic tumor growth, metastasis, and treatment resistance [11–13]. Similarly, FOXM1 is a downstream target of HER2 that helps promote cell cycle progression and deregulation of mitotic checkpoints [14].

We recently reported on our development of small-molecule inhibitors of FOXM1 that interact directly with the FOXM1 protein and decrease its cellular mRNA and protein levels, resulting in potent suppression of breast cancer cell and tumor growth in pre-clinical models [13, 15, 16]. Furthermore, these inhibitors downregulated expression of FOXM1-controlled gene networks to induce cell cycle arrest, suppress proliferation, and stimulate apoptosis in several breast cancer subtypes [13] and also in other aggressive cancers, including some multiple myelomas [17] and high-grade serous ovarian cancer [18, 19].

To further investigate the potential of these inhibitors to suppress breast cancer, we sought to combine them with other therapies currently being used in the treatment of breast and other cancers or with compounds in early clinical trials that show promise in the treatment of breast cancer. We found that our inhibitors synergize with several cancer drugs in different pharmacological classes. We measured strong synergy when our compounds and other FOXM1 inhibitors were combined with drugs in the proteasome inhibitor class, with induction of G2/M cell cycle arrest and apoptosis. In ER-positive breast cancer cells, the combination of FOXM1 inhibitors with CDK4/6 inhibitors also resulted in synergistic suppression of growth and the expression of genes regulating cell proliferation.

Methods

Cell lines and cell culture methods, FOXM1 inhibitors, and cancer drugs studied

All breast cancer cell lines (MDA-MB-231; MDA-MB-468; MCF7; ZR75-1) were obtained from the ATCC and were maintained and cultured as described [11, 20]. Cells were STR profiled to confirm identity and routinely tested for mycoplasma using Real-Time PCR Mycoplasma Detection Kit (Akron Biotech, Boca Raton, FL).

FOXM1 inhibitors, NB55, NB73, and NB115, were synthesized in our laboratories as previously reported [13]. FDI6 was obtained from Millipore Sigma. The CDK4/6 inhibitors, Abemaciclib, Palbociclib, and Ribociclib, were obtained from Selleckchem (Houston, TX). The proteasome inhibitors, Bortezomib and TAK-243, were obtained from Selleckchem or MedChemExpress (MCE, Monmouth Junction, NJ). The PI3K inhibitor, Alpelisib, was from Selleckchem; the ALK inhibitor, Crizotinib, was from Selleckchem; and the AURKA inhibitor, Alisertib, was from Selleckchem.

Cell viability assay and assessment of synergistic efficacy of test compounds

The WST-1 assay (Roche, Basel, Switzerland) was used to quantify cell viability as described [20]. Absorbance was measured at 450 nm using a VICTOR X5 PerkinElmer 2030 Multilabel Plate Reader. All assays were performed in triplicate and analyzed using Graph Pad Prism 8.0. As detailed further in Results, assessments of synergy in the effectiveness of FOXM1 inhibitors and other cancer drugs used Zero Interaction Potency (ZIP) synergy score determinations, as well as the Bliss and Loewe model assessments [21–24], and evaluation using the Chou–Talalay combination index (CI) where a CI less than 1 implies synergy [25].

Cell cycle analysis

Cells were synchronized by double-thymidine block prior to cell cycle analysis by Propidium Iodide (PI) staining. Briefly, the cells were blocked with 2-mM thymidine for 18 h and then released with fresh media for 8 h followed by re-blocking with thymidine for another 16 h. Following the second block, the cells were released with or without treatments with the NB compounds and/or other drugs and cells were collected and fixed in 70% ethanol at the time points indicated. After alcohol fixation for 2 h, the

cells were washed with cold 1XPBS followed by staining with 50-µg/ml PI solution in PBS with addition of 5-µg/ml RNAse A for at least 4 h. The cells were then analyzed using the Flow Cytometry analyzer BD LSR II for percentage of cells in G0/G1, S, and G2/M phases of the cell cycle.

Apoptosis analysis

The cells were analyzed for percentage of apoptotic cells following vehicle or compound treatments by staining with the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher) and flow cytometry according to the manufacturer's protocol. Caspase activity in the cells after treatment with control vehicle or compounds was determined using the Caspase-Glo 3/7 Assay system (Promega) in a 96-well format following the manufacturer's instructions.

RNA isolation and real-time PCR

Total RNA was isolated using TRIzol (Invitrogen) and reverse transcribed using MMTV reverse transcriptase (New England BioLabs). Real-time PCR was performed using SYBRgreen PCR Master Mix (Quantabio) as described [13]. Relative mRNA levels of genes were normalized to the housekeeping gene 36B4 and fold change calculated relative to the vehicle-treated samples. Results are the average ± SD from at least two independent experiments carried out in triplicate. Primer sequences for the genes studied were obtained from the Harvard Primer Bank. Sequences are available on their website.

Proteasome activity analysis

Cells were exposed to 25 µM of the indicated test compounds or control vehicle prior to addition of the 20S proteasome substrate LLVY-AMC (7-amino-4-methylcoumarin) and free AMC fluorescence was monitored after 2 h at 380 nm/460 nm (excitation/emission). Values are mean ± SEM of 4 replicate determinations.

Statistical analyses

Statistics were calculated using analysis of variance (ANOVA), 2-way ANOVA with Dunnett's multiple comparisons, or Student's *t* test, as appropriate, using GraphPad Prism 8.0 software. Significance was designated as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and **** for $p < 0.0001$.

Results

Identification of potential combination candidates

In this study, we looked only at drugs that are currently being used as clinical anti-cancer agents or are in pre-clinical development for the treatment of cancer. To narrow down potential candidates, the landscape of potential cancer drugs was divided into (1) FDA-approved cancer drugs and (2) pre-clinical compounds of interest (Table 1). From the group of FDA-approved drugs, first priority was given to those already being used in the treatment of breast cancer, focusing on drugs that can inhibit either HR + breast cancer (such as the CDK4/6 inhibitors) or both HR + and TNBC. To further address the need for new molecular targeted therapies in TNBC, the next level of priority was given to FDA-approved drugs currently used to treat other cancers but shown to be highly effective in TNBC pre-clinical models, such as Crizotinib, Alpelisib, and Bortezomib. Pre-clinical compounds of interest were chosen on the basis of rational selection of targets related

Table 1 Compounds examined for enhancement of anti-cancer efficacy in combination with FOXM1 inhibitors

Candidate compounds		FDA approval
Clinical compounds	Target	
Abemaciclib Palbociclib Ribociclib	CDK4/6	Approved in combination with an aromatase inhibitor as initial endocrine-based therapy for postmenopausal women with HR-positive, HER2-negative advanced or metastatic breast cancer
Bortezomib	26S proteasome	Approved for patients with multiple myeloma and mantle cell lymphoma
Crizotinib	ALK	Approved for patients with metastatic ALK or ROS1-positive non-small cell lung cancer
Alpelisib	PI3K	Approved in combination with fulvestrant for the treatment of postmenopausal women and men, with HR-positive, HER-negative, PIK3CA-mutated, advanced, or metastatic breast cancer
Preclinical compounds		*Approved information for all drugs obtained directly from https://www.accessdata.fda.gov
Alisertib	AURKA	
TAK-243	Ubiquitin-activating enzyme (UAE)	

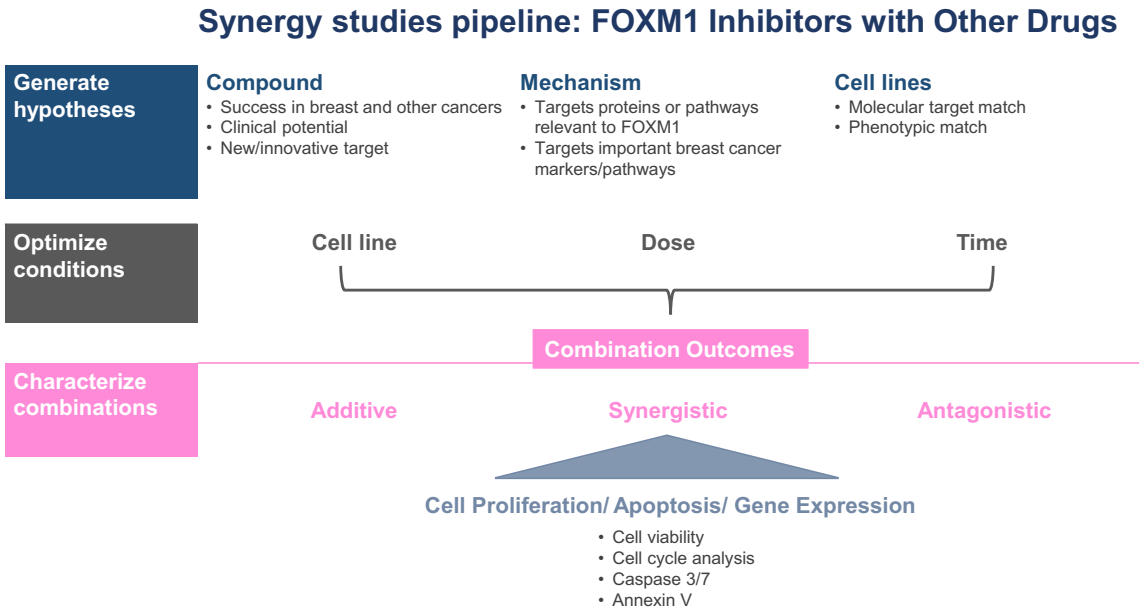


Fig. 1 Synergy studies pipeline for examination of FOXM1 inhibitors with other cancer drugs

to FOXM1, expression of the target in HR + and/or TNBC, and effectiveness in pre-clinical cancer studies. An overview of the synergy studies pipeline is presented in Fig. 1.

We used triple-negative MDA-MB-231 cells and ER-positive (MCF7, ZR75-1) breast cancer cells as our initial screening cell lines. To select doses for combination studies in the cell lines, we first characterized the dose–response curves of each compound alone in suppression of cell viability and chose NB73, which displayed good FOXM1-specific activity in our previous studies [13, 15, 16], as the first FOXM1 inhibitor to screen in combination studies. We implemented a 3 × 3 matrix design with NB73 and each combination compound (Fig. 2), estimating 3 doses for each compound that would fall at or under its IC50. Percent inhibition matrices for four of these combinations (NB73 with Bortezomib, Crizotinib, Alisertib, or Alpelisib) are shown in Fig. 2. We quantified synergy using the Zero Interaction Potency (ZIP) model with the SynergyFinder web application, which produces a delta score at each dose pair to represent the level of synergy between two compounds and allows for visualization of all delta scores in a combination matrix as an interaction landscape [23]. The magnitude of the delta score is symmetrically centered around zero, where delta scores that are more negative are more antagonistic, while delta scores that are more positive are more synergistic. Generally, a delta score ranging from 0 to 10 is representative of additivity and scores above 10 are representative of synergistic interactions [21]. Negative scores (below zero) indicate compound antagonism. In some cases, synergy was also evaluated using the Chou–Talalay method [25].

Our findings showed combinations of NB73 with Bortezomib had high delta scores, suggesting synergy (Fig. 2). Combination of Crizotinib or Alisertib with NB73 gave delta scores in the additivity range, while combinations with Alpelisib were neither additive nor synergistic (Fig. 2). Additional analysis of Crizotinib and Alisertib to quantify possible synergy confirmed that these combinations with NB73 were likely to be additive or only weakly synergistic, whereas NB73 with Bortezomib gave a high ZIP synergy score (Fig. 2), and we therefore continued with Bortezomib and also evaluated another proteasome inhibitor, TAK-243, in further studies.

FOXM1 inhibitors interact synergistically with proteasome inhibitors

The ubiquitin–proteasome pathway (UPP) is highly dysregulated and contributes to the pathobiology of many cancers, and small-molecule inhibition of 20S proteasome activity has become a successful treatment strategy in hematological malignancies, like mantle cell lymphoma and multiple myeloma [26]. Proteasome inhibitors act as potent cancer-suppressing agents through regulation of cell cycle proteins, inhibition of the NFkB pathway, induction of endoplasmic reticulum stress and the unfolded protein response (UPR) and importantly, induction of pro-apoptotic proteins [26, 27]. It has been shown that the proteasome inhibitor Bortezomib is a potent inducer of apoptosis in breast cancer cells [28]. Furthermore, proteasome addiction was identified as a vulnerability of triple-negative breast cancer cells relative to other breast cancer subtypes [29]. In previous studies, we

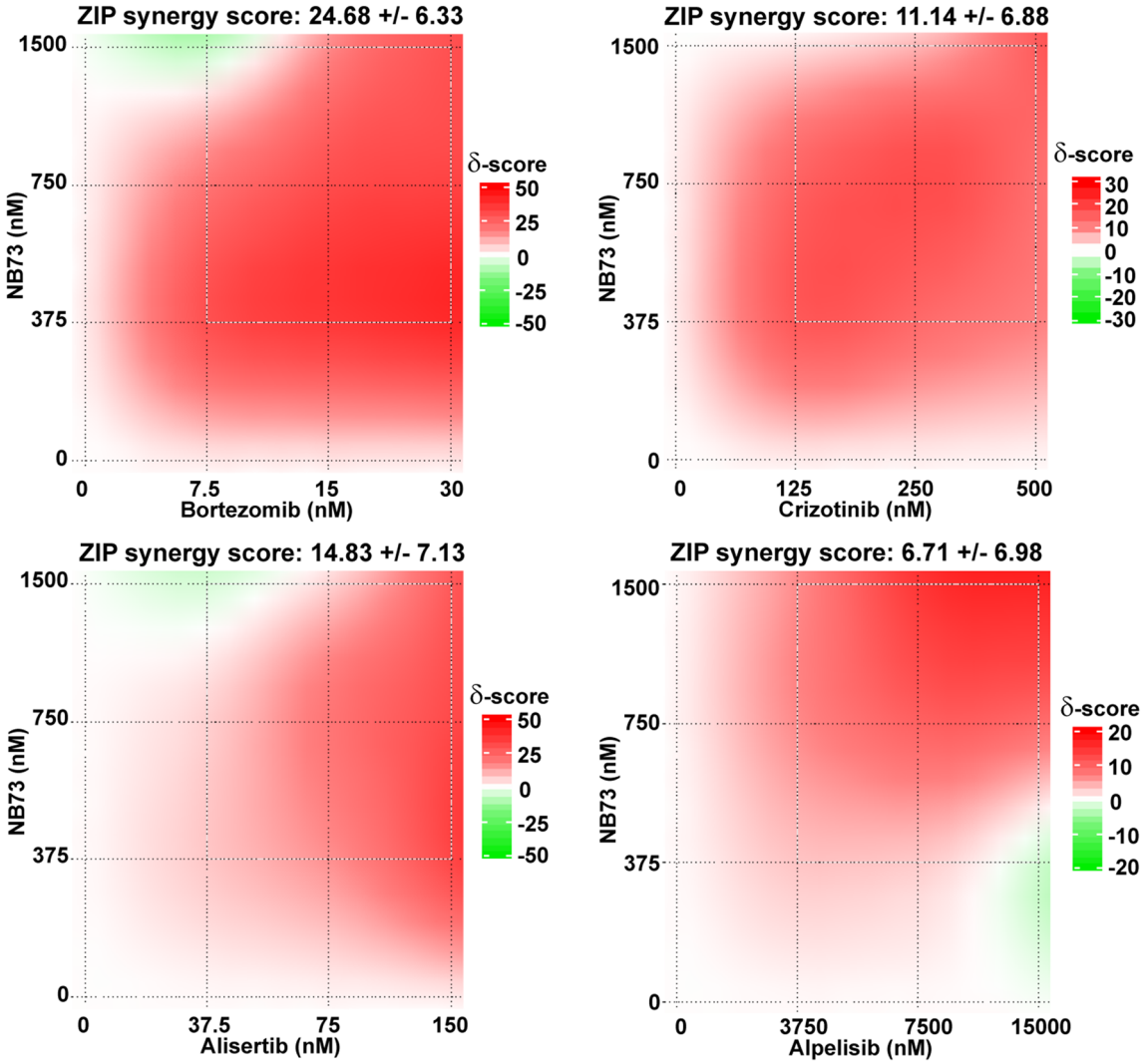


Fig. 2 ZIP synergy matrices of combinations of NB73 and candidate compounds. ZIP synergy score is average synergy score of all dose pairs in the matrix with 95% confidence interval, $n = 4$ at each treatment. MDA-MB-231 cells were treated for 72 h with the doses shown of NB73 or candidate compound alone and in combination at each dose pair

found that the FOXM1 inhibitors we developed induce cell death and apoptosis in breast cancer cells [13], leading to the hypothesis that the combination of FOXM1 inhibitors with proteasome inhibitors may enhance this process. Thus, we hypothesized that combining our FOXM1-targeted compounds with proteasome inhibitors might effectively inhibit breast cancer cells through several potential mechanisms, including enhancement of cell cycle inhibition, UPR induction, and pro-apoptotic signaling.

Effects of FOXM1 inhibitor and proteasome inhibitors alone and together on the cell cycle and on apoptosis

As seen in Fig. 3 and Supp. Fig. S1, we evaluated the combination of FOXM1 inhibitors NB73, or NB55, or NB115 with Bortezomib or with TAK-243 (MLN7243). TAK-243 is a compound entering human clinical trials that inhibits the ubiquitin proteasome system (UPS) by inhibiting ubiquitin activating enzyme (UAE) rather than the 26S proteasome [30]. Bortezimib or TAK-243 with FOXM1 inhibitors synergistically inhibited estrogen receptor-positive MCF7 cell proliferation as quantified by the Zero Interaction Potency (ZIP) and Chou–Talalay models, as did FDI6, a different inhibitor of FOXM1 [31]. To further evaluate this, we examined a broader range of proteasome inhibitor concentrations both in the ER + MCF7 cells and in TNBC MDA-MB-231 cells (Fig. 4). We observed synergy between the FOXM1 inhibitor and proteasome inhibitor in MCF7 cells (Fig. 4a and b), but notably we found that viability of the TNBC cell lines MDA-MB-231 and MDA-MB-468 (data not shown) was not effectively suppressed by cotreatment with FOXM1

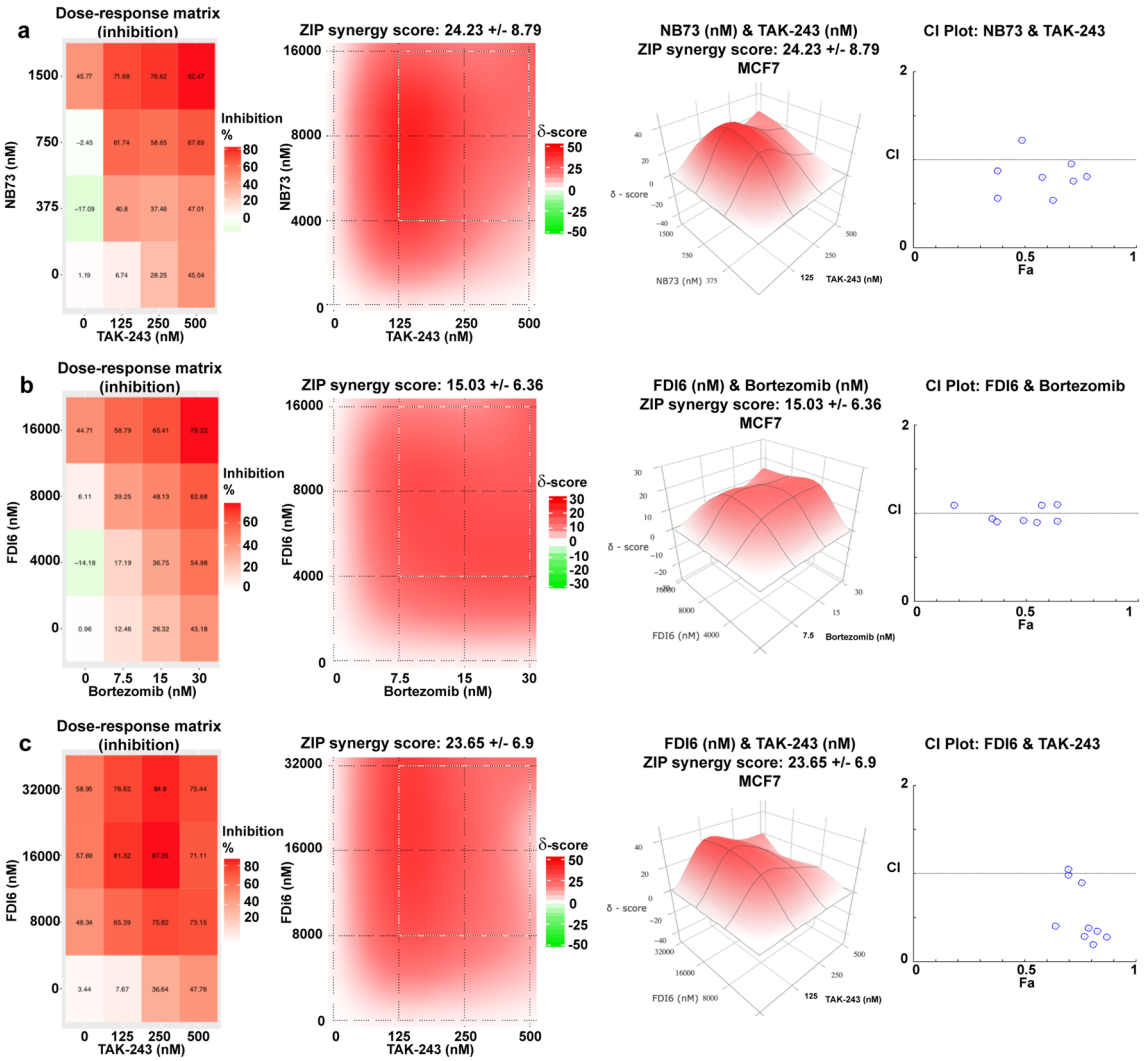


Fig. 3 Assessment of synergistic interaction in growth suppression between FOXM1 inhibitors (NB73; FDI6) and proteasome inhibitors. MCF7 cells were treated for 72 h with the doses shown of FOXM1 inhibitor and proteasome inhibitor alone or in combination at each dose pair. Inhibition matrices, ZIP synergy matrices, 3D ZIP synergy plots, and Combination Index (CI) plots are shown for all combinations. **a** NB73 + TAK-243. **b** FDI6 + Bortezomib. **c** FDI6 + TAK-243. Inhibition matrices show the average of 4 replicates. ZIP Synergy score is average synergy score of all dose pairs in the matrix with 95% confidence interval, 4 replicates per treatment. Combination Index (CI) scores were calculated using the average of 4 replicates at each dose pair using CompuSyn software

inhibitor and TAK-243 (Fig. 4c). In fact, cotreatments consistently gave negative ZIP synergy scores, indicating that the two types of inhibitors are likely acting antagonistically in these cells. This suggests that the 26S-directed more general proteasome inhibition by Bortezomib may be more broadly compatible with FOXM1 inhibition across cell types than is the novel UAE-targeting mechanism of TAK-243.

To further investigate the FOXM1 inhibitor/proteasome inhibitor combination, we performed cell cycle analysis with cells synchronized at the G1/S boundary with double-thymidine block and then treated with NB73 and Bortezomib or each compound alone. While both compounds alone arrested cells at the G0 and G1 phases, the combination of the compounds resulted in dramatic G2/M arrest of the majority of the cell population, indicating enhanced inhibition of cell cycle progression (Fig. 5a). Next, to investigate the effect of the combination on the apoptosis pathway, we tested the effect of the combination or of the compounds alone on caspase 3/7 activity (Fig. 5b). We treated MDA-MB-231 cells with doses of NB73 and Bortezomib that were expected to elicit little apoptotic activity alone. At these doses, NB73 alone did not increase caspase activity, while Bortezomib alone displayed a dose-dependent increase in caspase 3/7 activity, reaching a maximum of fourfold over Vehicle at the highest dose. When the compounds were combined, we saw a tenfold increase in caspase 3/7 activity over Vehicle at the

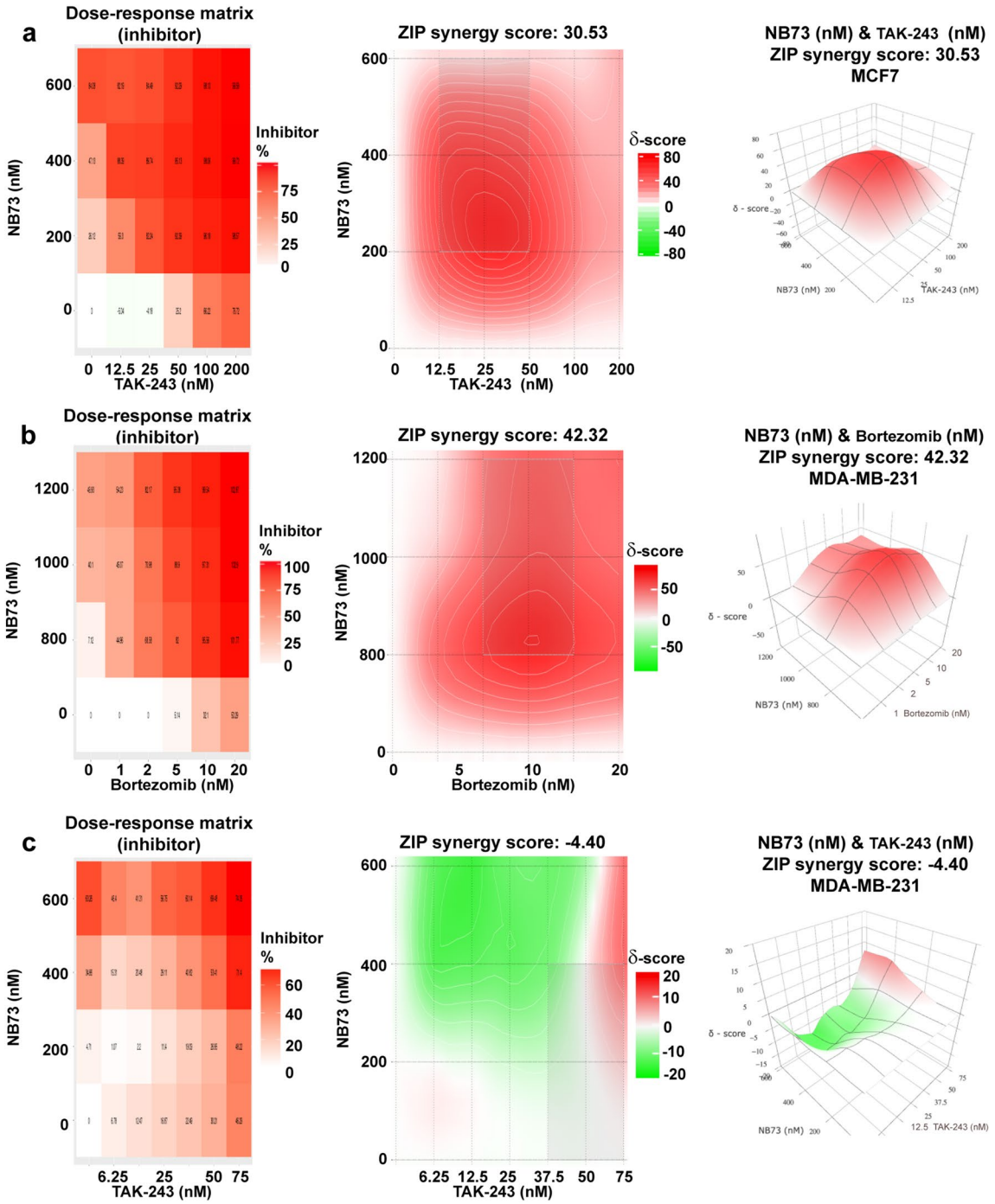


Fig. 4 Effect of FOXM1 inhibitor and proteasome inhibitor alone and together in HR + and in TNBC cells, studied over a broad range of concentrations. All cell treatments were for 72 h. **a** NB73 and TAK-243, MCF7 cells; **b** NB73 and Bortezomib, MDA-MB-231 cells; and **c** NB73 and TAK-243, MDA-MB-231 cells. Inhibition matrices show the average of 4 replicates. ZIP synergy score is average score of all dose pairs in the matrix

1.5-μM NB73 + 30-nM Bortezomib dose, which was 2.8-fold greater than the effect of Bortezomib alone at 30 nM. Thus, NB73, while having no effect on its own at the 0.375 or 1.5 μM dose, significantly enhanced the pro-apoptotic effect of Bortezomib.

We also evaluated whether there was possible proteasome-modulating activity of our NB compounds using a fluorescence-based proteasome substrate activity assay. While thiostrepton, lactacystin, and MG132 reduced proteasome activity as expected [14, 32], NB55, NB73, and

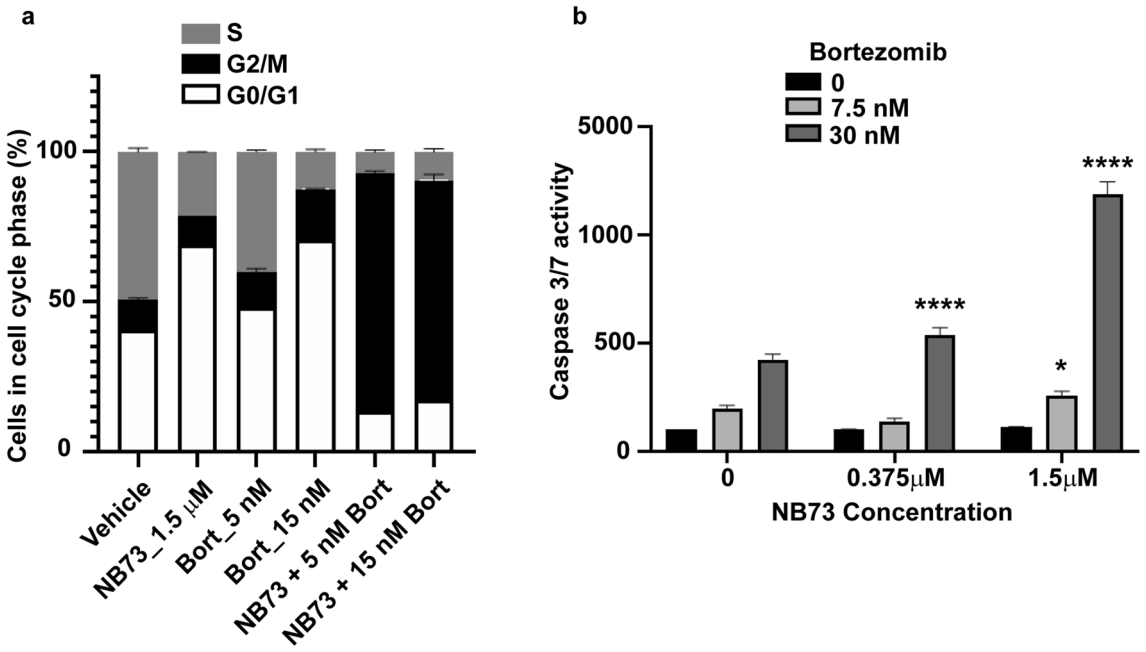


Fig. 5 Effect of NB73 and Bortezomib alone and in combination on the cell cycle and on caspase 3/7 activity. **a** MDA-MB-231 cells were synchronized by double-thymidine block and were released for 24 h with or without treatment with Vehicle, NB73 (1.5 μM), Bortezomib (5 or 15 nM) or the indicated combinations. The percent of cells in different phases of the cell cycle were monitored by flow cytometry. $n=2$ experiments, mean + SD. **b** Caspase 3/7 activity was monitored after cell treatment with Veh, NB73 (at 0.375 or 1.5uM), Bortezomib (7.5 or 30 nM), or the indicated combinations for 24 h before using the Caspase-Glo assay; $n=3$ experiments, mean + SD. $^{*}p<0.05$ and $^{****}p<0.0001$ for combinations versus Bortezomib alone

FDI6 at quite high concentrations (25 μM) did not elicit any suppression of proteasome activity (Supp. Fig. S2). Thus, the NB and FDI6 FOXM1 inhibitors do not themselves have proteasome-modulating activity.

FOXM1 inhibitors act synergistically with CDK4/6 inhibitors to inhibit ER-positive breast cancer cells

CDK4/6 inhibitors are currently used to treat HR +, HER2- breast cancers, providing a significant improvement in progression-free survival in these patients [33–35]. The cyclin-D-CDK4/6 complex is important for cell growth in response to mitogenic growth signals and is necessary for phosphorylation of checkpoint protein retinoblastoma (Rb), lifting its inhibition of entry into S phase [36, 37]. In addition, multi-site phosphorylation of FOXM1 by CDK4/6 in G1 and G1/S stabilizes FOXM1 against proteasome-mediated degradation and activates FOXM1 transcriptional activity, providing a rationale for the combination of FOXM1 inhibitors with CDK4/6 inhibitors [38]. In our extended dose studies we identified that the combination of NB73 or NB115 with Abemaciclib, Palbociclib, or Ribociclib gave very good synergy scores in ER-positive MCF7 cells (Fig. 6) and also in ER-positive ZR75-1 cells (Supp. Fig. S3).

Regulation of gene expression by combined FOXM1 and CDK4/6 or proteasome inhibition

To begin to understand how the combination of NB73 and CDK4/6 inhibitor might be enhancing suppression of cell proliferation, we conducted gene expression studies in MCF7 cells treated with different dose combinations of NB73 and Palbociclib. In these studies, we found that combining the two compounds resulted in greater suppression of the expression of genes involved in cell cycle progression (*AURKB, E2F1, PLK1, CCNB1, CENPF,* and *MCM3*) than either compound alone (Fig. 7a–f). Likewise, the combination of NB73 and Bortezomib in TNBC MDA-MB-231 cells generally elicited greatly reduced expression of proliferation-related genes (*E2F1, PCNA*), *survivin/BIRC5* gene, and of the *FOXM1* gene itself at both 24 h and 48 h of treatment (Fig. 8).

Discussion

We have identified several compounds that display synergistic effects when combined with FOXM1 inhibitors, with proteasome inhibitors and CDK4/6 inhibitors showing especially robust enhanced efficacy in suppressing breast cancer viability and related gene expressions.

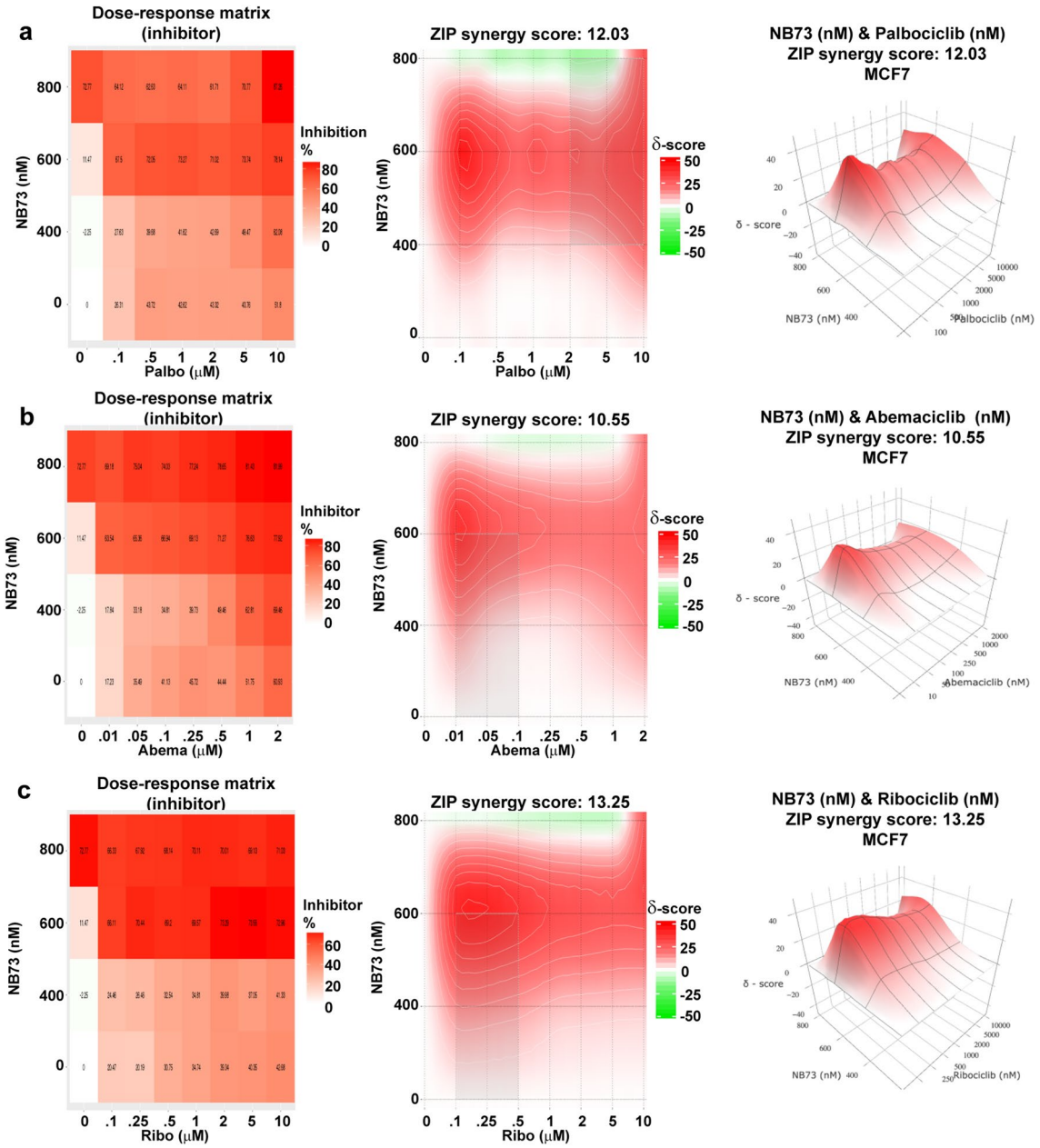
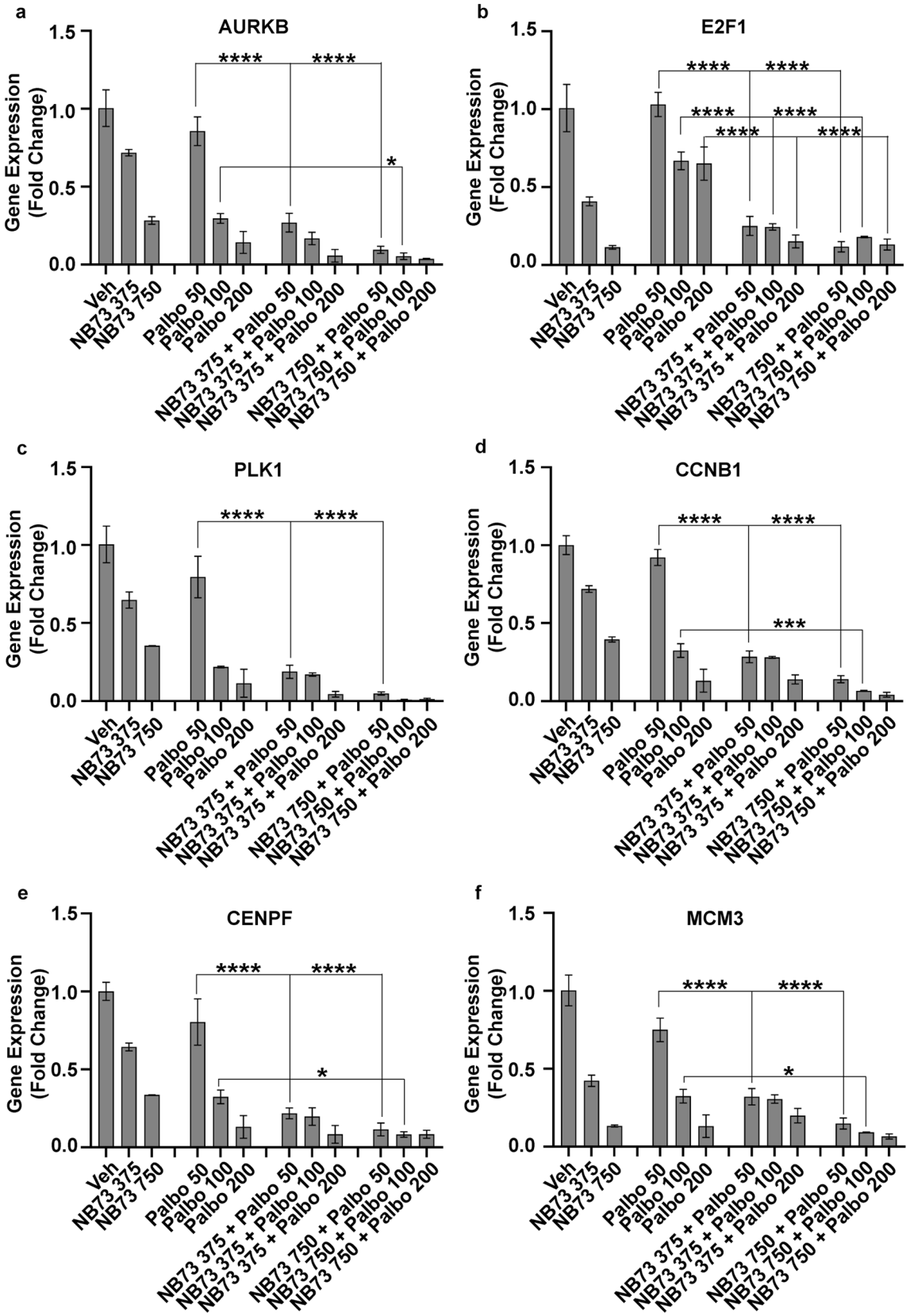


Fig. 6 Assessment of enhanced growth suppression of ER-positive breast cancer cells by FOXM1 inhibitors and CDK4/6 inhibitors. MCF7 cells were treated for 72 h with the doses shown of FOXM1 inhibitor or CDK4/6 inhibitor alone or in combination at each dose pair. Cell viability dose–response inhibition matrices, ZIP synergy matrices, and 3D ZIP synergy plots are shown for all combinations. **a** Cells treated with NB73 and Abemaciclib. **b** Cells treated with NB73 and Palbociclib. **c** Cells treated with NB73 and Ribociclib. Inhibition matrices show the average of 4 replicates. ZIP Synergy score is average synergy score of all dose pairs in each matrix

Marked synergy between FOXM1 inhibitors and proteasome inhibitors

NB73 and Bortezomib synergistically inhibited cell proliferation, as did NB55 and NB115, two other FOXM1 inhibitors we developed [13]. Importantly, the combination of Bortezomib with FDI6, a FOXM1 inhibitor of a different chemical class [31], was also highly synergistic, implicating the FOXM1-specific effect of this interaction. Furthermore, we observed that the combination of NB73 plus Bortezomib resulted in dramatic arrest of cells at G2/M and induced more robust caspase 3/7 activity than either compound alone. Since our FOXM1 inhibitors suppress the expression of genes promoting cell cycle progression and enhance apoptosis stimulating genes, as do proteasome inhibitors [13, 26, 27], it appears that combined targeting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◂**Fig. 7** Enhanced suppression of cell cycle genes by the combination of NB73 with Palbociclib. MCF7 cells were treated for 24 h with the nM doses shown of NB73 or Palbociclib alone or in combination as indicated. RNA was extracted from cells and gene expression was monitored by qRT-PCR. Assays were run in triplicate. Values are mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$ for combinations versus compounds alone

of FOXM1 and proteasome activity may effectively inhibit breast cancer cells by several possible mechanisms, including enhancement of cell cycle inhibition, induction of the unfolded protein response, and stimulation of pro-apoptotic signaling [13].

The success of proteasome inhibitors in hematological malignancies, such as malignant myeloma, has made them an important area of investigation for other cancer types, particularly those that display dependence on the ubiquitin–proteasome system [39]. Triple-negative breast cancers are an example of such malignancies, and proteasome inhibitors have been identified in several pre-clinical drug screens as agents that have cytotoxic and pro-apoptotic activity in this subtype, which is generally resistant to many other treatments [29, 40, 41]. Despite its promise in pre-clinical solid tumor animal models, however, clinical studies of Bortezomib have failed to demonstrate efficacy in solid tumors [42]. This is likely due to the non-optimal pharmacokinetic properties of Bortezomib, which appear to result in impaired distribution to solid tumors [43]. Therefore, we conducted some studies with TAK-243, a novel inhibitor of the unfolded protein system (UPS) that targets ubiquitin-activating enzyme (UAE) and thus is mechanistically and chemically distinct from other proteasome inhibitors which are targeted to the 26S proteasome [44]. TAK-243 has shown effective tumor suppression in pre-clinical solid tumor studies and thus is a potential future avenue for UPS-inhibiting therapy in cancer. In addition, by inhibiting the ubiquitin-activating enzyme (UAE), TAK-243 treatment impairs the function of multiple E2 enzymes needed for DNA damage repair, resulting in enhanced killing of lung carcinoma cells by UV irradiation due to unresolved DNA damage repair [44]. Notably, we found that ER-positive breast cancer cells (MCF7 and ZR75-1) showed synergy in suppression of cell viability with FOXM1 inhibitors and Bortezomib or TAK-243, whereas the TNBC cells examined showed synergy only with FOXM1 inhibitor and Bortezomib but not with TAK-243.

Synergy of FOXM1 inhibitors with CDK4/6 inhibitors in impeding growth of ER-containing breast cancer cells

The cyclin D-CDK4/6 complex regulates the G1/S transition through phosphorylation of Rb and FOXM1 in cells entering the cell cycle after mitogenic growth signaling [45, 46]. Hence, blockade of cyclin-dependent kinase phosphorylation and activation of FOXM1 by CDK4/6 inhibitors may well contribute in further enhancing the suppression of FOXM1 activity by FOXM1 inhibitors. Most luminal breast cancers display upregulation of the Rb pathway, and CDK4/6 inhibitors like Abemaciclib or Palbociclib in combination with hormone therapy are an important treatment avenue for advanced-stage or metastatic HR +, HER2- breast cancer [47]. Nonetheless, resistance to these treatments frequently develops. Thus, we examined the rational combination of CDK4/6 inhibition with FOXM1 inhibition in ER-positive breast cancer cell lines, which theoretically modulates three important regulators of the G1/S transition (FOXM1, CDK4/6, and Rb). Our studies with ER-positive cells (MCF7 and ZR75-1) revealed enhanced effectiveness when Abemaciclib, Palbociclib, or Ribociclib were combined with NB73 or NB115, as well as with FDI6. Indeed, combinations of CDK4/6 inhibitors with other agents, such as PI3K inhibitor or other antiestrogens, are being explored clinically [48]. Because TNBC tumors frequently exhibit functional loss of Rb, triple-negative breast cancers are currently considered poor candidates for CDK4/6 inhibition [49]. Still, precision medicine approaches may make it possible to identify if specific subsets of TNBC patients can benefit from CDK4/6-targeted therapy alone or in combination with FOXM1 inhibitors in the future. Recent studies have demonstrated the sensitivity of the luminal androgen receptor (LAR) TNBC subtype to CDK4/6 inhibition [50]. In addition, other cyclin-dependent kinase inhibitors are currently in clinical development that show promise as possible therapeutic approaches in TNBC, including the CDK1/5/9 inhibitor Dinaciclib [51].

Possible benefits from combination therapies

Maintenance of efficacy with possible reduced toxicity is a key consideration in the study of any combination therapies [52]. The toxicity profile of Bortezomib is characterized by a high incidence of polyneuropathy (80%) in multiple myeloma (MM) patients, but this finding is complicated by the fact that neuropathy is a common symptom of MM in itself, as well as from other drugs used to treat MM, like vinca alkaloids [53]. Other side effects include thrombocytopenia, neutropenia, anemia, fatigue, and diarrhea [26]. Nevertheless, Bortezomib has been combined with lenalidomide and dexamethasone in an MM treatment regimen that was well tolerated in phase II clinical studies and is frequently used as initial therapy in the USA [53]. In addition, next-generation proteasome inhibitors like Carfilzomib appear to have improved toxicity profiles compared to Bortezomib, providing an additional avenue for future investigation in combination treatments with FOXM1 inhibitors [54].

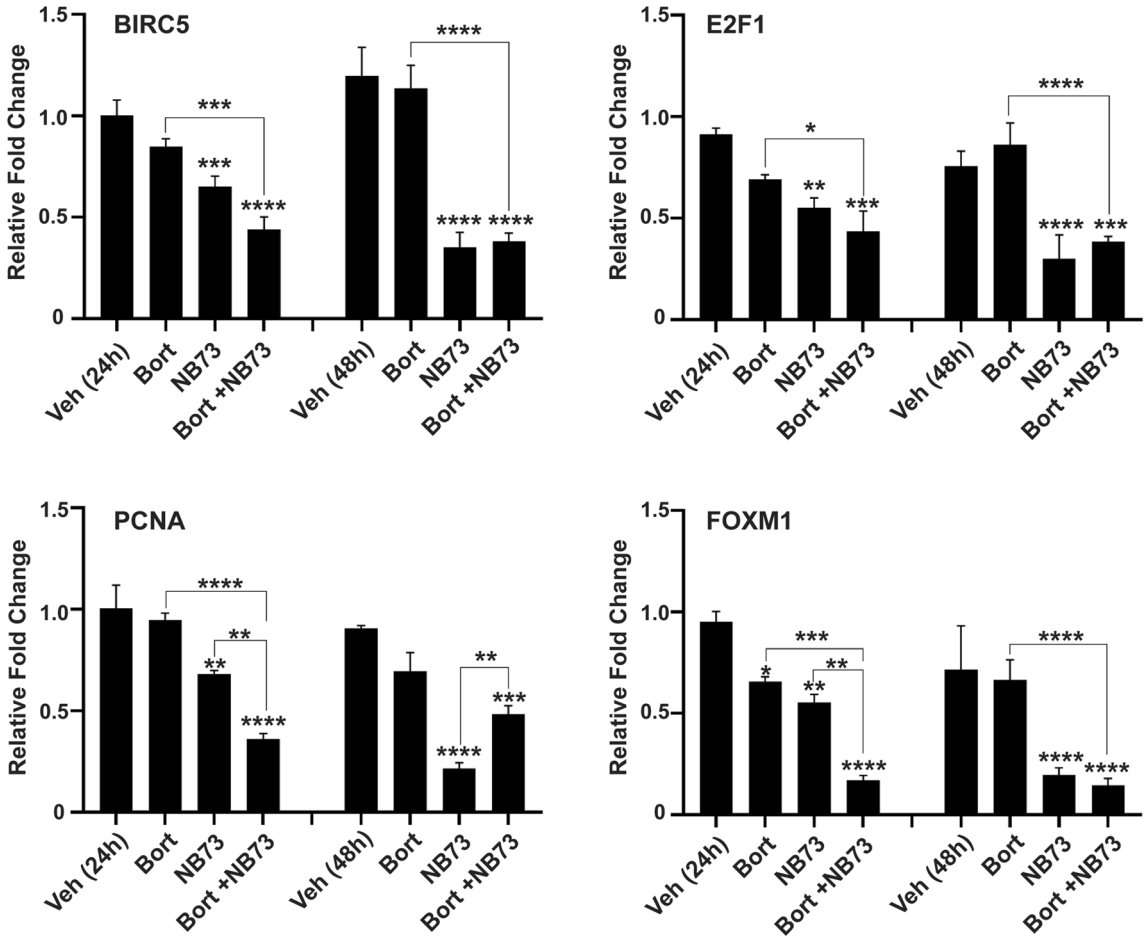

Fig. 8 Enhanced up or down regulation of key gene expressions by combined treatment with NB73 plus Bortezomib. MDA-MB-231 cells were treated for 24 h or 48 h with Bortezomib (Bort, 6 nM) alone or NB73 alone (850 nM) or with both in combination. RNA was extracted from cells and gene expression was monitored by qRT-PCR. Assays were run in triplicate. Values are mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$ for combinations versus compounds alone

As reported in the MONARCH 2 phase III clinical trial, the most common toxicities for Abemaciclib as a monotherapy are diarrhea, nausea, and fatigue, with most patients reporting no or grade 1–2 diarrhea; grade 3–4 neutropenia occurred in 10% of patients [55]. When Abemaciclib was combined with aromatase inhibitors (AI) in the MONARCH 3 phase III trial, the most increased adverse effect was grade 3 or 4 neutropenia, which is reversible and can be clinically managed without compromising treatment efficacy [56]. Although our FOXM1 inhibitors showed no obvious toxicity in pre-clinical in vivo test systems [13, 17], as we learn more about the possible potential side effects of our FOXM1 inhibitors in vivo, it will be important to select combination candidates with toxicity profiles that are not exacerbated by the addition of a FOXM1 inhibitor.

Conclusion

The present studies show that the FOXM1 inhibitors work synergistically with several different compounds to suppress breast cancer cells. From profiles of the compounds having the most effective combined activity, it appears that FOXM1 inhibitors may synergize best with compounds that directly affect the cell cycle and apoptotic pathways. More specifically, our findings suggest that FOXM1 inhibition, along with CDK4/6 or proteasome inhibition might ultimately prove to be a useful approach for breast cancer treatment because synergy is especially noticeable at low doses of each inhibitor, suggesting that good suppression of cancer cell viability might still be maintained with reduced doses of both agents with the additional benefit

of reduced side effects. Clearly, future studies will be needed to further characterize the mechanisms behind these synergistic combinations, as well as explore other novel combinations that have the potential to complement the biological effects of FOXM1 inhibition.

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Author contributions BSK, VSG, YZ, and JAK conceived the project and provided leadership for the project. VSG, YZ, CG, SK, PD, BNP, NZD, SHK, JAK, and BSK carried out experiments and/or analyzed data. VSG, BSK, YZ, and JAK wrote the manuscript. All authors discussed the results and provided input and edits on the manuscript.

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Data availability All data are available within the manuscript or may be obtained from the Corresponding Author (B.S.K) upon reasonable request.

Declarations

Conflict of interest BSK, JAK, and SHK are co-inventors on a Provisional Application filed by the University of Illinois to cover the FOXM1 inhibitor compounds described in this paper. The other authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants or involving animals performed by any of the authors.

Informed consent Not applicable.

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