Sequential Combination Therapy of CDK Inhibition and Doxorubicin Is Synthetically Lethal in p53-Mutant Triple-Negative Breast Cancer

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Abstract

Triple-negative breast cancer (TNBC) is an aggressive malignancy in which the tumors lack expression of estrogen receptor, progesterone receptor, and HER2. Hence, TNBC patients cannot benefit from clinically available targeted therapies and rely on chemotherapy and surgery for treatment. While initially responding to chemotherapy, TNBC patients are at increased risk of developing distant metastasis and have decreased overall survival compared with non-TNBC patients. A majority of TNBC tumors carry p53 mutations, enabling them to bypass the G1 checkpoint and complete the cell cycle even in the presence of DNA damage. Therefore, we hypothesized that TNBC cells are sensitive to cell-cycle–targeted combination therapy, which leaves nontransformed cells unharmed. Our findings demonstrate that sequential administration of the pan-CDK inhibitor roscovitine before doxorubicin treatment is synthetically lethal explicitly in TNBC cells.

Introduction

Triple-negative breast cancer (TNBC) is a clinical diagnosis that affects 10% to 20% of breast cancer patients (1). TNBC tumors are characterized by lacking expression of estrogen receptor (ER), progesterone receptor (PR) and the growth factor receptor HER2. TNBC patients are more likely to be premenopausal, of African descent, have a poor prognosis and have greater risk of recurrence within the first 5 years of diagnosis (2). Less than one-third of metastatic TNBC patients survive 5 years (3). About 70% of TNBC are classified as basal-like breast cancer (BLBC), and share many of the same characteristics, including more likely occurring in patients with BRCA1/BRCA2 gene mutations (4, 5). Dysfunction in the DNA repair pathway, resulting from BRCA1 mutations, may contribute to TNBC patients initially responding well to chemotherapy; however, many patients’ tumors recur (6). While there are several targeted therapies being developed in clinical trials, including PARP and EGFR inhibitors, there are currently no clinically available and effective targeted therapies for TNBC patients (6–8). The majority (54%–82%) of TNBC tumors harbor p53 mutations, enabling them to bypass the G1 checkpoint and complete the cell cycle even with unrepaired DNA damage (6, 9, 10). In comparison, only 13% of hormone receptor-positive luminal A tumors have p53 mutations (11). Moreover, 50% of these breast cancers overexpress cyclin D1, inhibiting retinoblastoma (Rb) regulation of E2F (12). Notably, overexpression of cyclin E serves as a poor prognostic marker in breast cancer and correlates to negative ER and PR status (13, 14). Owing to deregulation of the cell cycle in cancer cells, cyclin-dependent kinase (CDK) inhibitors were developed to inhibit tumor cell proliferation and induce apoptosis (15). However, CDK inhibitors have not been effective clinically, despite having promising results both in vitro and in vivo (16, 17). Roscovitine, a pan CDK inhibitor with activity against CDK1, 2, 5, 7, and 9 (18, 19), became the first orally bioavailable drug from this class to go into clinical trials based on the preclinical data showing induction of apoptosis in tumor cells. However, of the 77 solid tumor patients treated with single-agent roscovitine, one partial response was seen in hepatocellular carcinoma, 2 prolonged stable disease observed in non–small cell lung cancer (14 and >18 months), while stable disease was the best response seen in the remaining solid tumors (20–22). One of the reasons that these CDK inhibitors have not been more effective...
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clinically is that they are either being used as single agents or when they are used in combination therapy, both agents were delivered concomitantly to the patient (23). In addition, there was no attempt to identify those patients most likely to respond to these agents based on their biology. In fact, very few patients with breast cancer of any subtype were accrued to these trials.

CDK1 participates in the DNA double-strand break (DSB) repair pathway homologous recombination (HR). HR faithfully repairs DNA DSBs that occur in late S, G2, and M (24). CDK activity is required for the recruitment of the endonucleases Sae2 or CipT that excise the DNA DSB to generate single strands in HR in both yeast and mammalian cells, respectively (25, 26). Moreover, CDK activity is required for the recruitment and association of BRCA1 to the MRN (Mre11-Rad50-Nbs1) complex during HR (27). Concordantly, CDK inhibition with roscovitine reduced the recruitment of HR downstream protein RPA34 in irradiated sarcoma cells due to an inability to generate single strands (28). Thus, compromising HR via CDK inhibition may provide a strategy to augment TNBC cell sensitivity to chemotherapy.

No clinically available treatment strategies target the TNBC-deregulated cell cycle to exploit TNBC-cell sensitivity to DNA-damaging agents (e.g., chemotherapy). Because TNBC cells have a deregulated G1 checkpoint, enabling them to re-enter the cell cycle while harboring DNA damage, we hypothesized that TNBC cells are sensitive to cell-cycle–targeted combination therapy, which leaves nontransformed cells unharmed. Ideally, this therapeutic strategy will be synthetically lethal against TNBC cells by inhibiting pathways that can compensate for one another during tumorigenesis. We therefore examined the effect of combining the pan-CDK inhibitor roscovitine and the chemotherapeutic doxorubicin both sequentially and concomitantly. Our data suggest that sequential treatment of roscovitine before doxorubicin is synthetically lethal explicitly in TNBC cells due to p53 pathway ablation.

Materials and Methods

The source of cell lines and culture conditions of the immortalized human mammary epithelial (HMEC) cell lines MCF-10A, 76NE6, and 76NF2V, the breast cancer cell lines MDA-MB-157, MDA-MB-231, HCC1806, MCF-7, ZR75-1, T47D, MDA-MB-468 (Supplementary Table S1A), and the isogenic colorectal cancer cell lines CT116 p53 wild-type (p53+/−) and p53 knockout (p53−/−) were previously described (29–31). HEK-293T cells for lentiviral packaging were maintained in DMEM supplemented with 10% FCS. All cells were free of mycoplasma contamination, and their identities were authenticated by karyotype and short tandem repeat analysis using the MD Anderson’s Characterized Cell Line Core Facility (http://www.mdanderson.org/characterized-cell-line-core-facility/index.html) on a routine basis (every 6 months).

Immunofluorescence to detect DNA repair foci

Following treatment of cells with roscovitine (IC50) for 24 hours, doxorubicin (IC50) for 48 hours, or both drugs sequentially as indicated, they were fixed in 4% paraformaldehyde for 20 minutes followed by permeabilization with a 0.3% triton solution (20 mol/L Hepes, 50 mmol/L NaCl, 3 mmol/L MgCl2, 300 mmol/L sucrose, and TritonX-100) for 20 minutes. Cells were then blocked with 10% FBS and 2% horse serum PBS for one hour. Antibodies were diluted 1:500 and 1:1000 for anti-yH2AX (EMD Millipore) and anti-Rad51 (32), respectively, and incubated at 4°C overnight. Secondary goat anti-mouse or goat anti-rabbit antibodies (Alexa Fluor 594 and 488, respectively, EMD Millipore) were diluted at 1:750 and incubated at room temperature at 1 hour. Nuclei were stained with DAPI (Life Technology) at 1μg/mL for 5 minutes room temperature. Cells were mounted with Dako fluorescent mounting medium. Images were captured using the Olympus FV1000 Laser Confocal Microscope. Cells with ≥5 foci were considered yH2AX positive.

Neutral comet assay

A neutral comet assay was performed according to Trevigen protocols to measure DNA DSBs in HMEC and TNBC cells in response to single and combination drug treatment. Briefly, following treatment, cells were harvested and combined with low-melting agarose, and spread onto CometSlides (Trevigen). Following cell adherence to the slides, cells were lysed with Trevigen lysis solution. The slides were then placed in an
electrophoresis chamber with neutral electrophoresis buffer. A current of 21 V was used for 21 minutes. After drying, samples were stained with SYBR Green I and allowed to dry at room temperature in the dark. Images of nuclei were captured using an Eclipse 90i microscope equipped with the NIS-Elements Br 3.10 software program (Nikon). The tail moment was measured using the CometScore software program (TriTek).

**Xenograft studies**
A total of 4.5 x 10^6 MDA-MB-468 cells in a 1:2 ratio with Matrigel (BD Bioscience) were injected into the mammary fat pad of 4- to 6-week-old female Balb/c Nu/nu mice (Taconic). Mice received injections on both the right and left flanks. Once the tumors reached a volume (L x W^2 / 2) of 100 to 150 mm^3, mice were treated with vehicle + vehicle, roscovitine (50 mg/kg) 4 days on/3 days off, doxorubicin (2mg/kg) once a week, sequentially with 4 days of roscovitine followed by 1 day of doxorubicin, or concomitantly with 4 days of roscovitine and 1 day of doxorubicin administered on day 1 for four cycles.

**Results**
Sequential combination treatment with roscovitine followed by doxorubicin synergistically inhibits TNBC cells.

To interrogate whether treatment of breast cancer cells with roscovitine would alter their cell-cycle profiles based on their different receptor and p53 status, we treated 10 different cell lines, including three HMEC, three ER/PR-positive cell lines, and four triple-negative cell lines, (Supplementary Table S1A) with 20 ?mol/L roscovitine for 24 hours followed by flow-cytometric analysis (Fig. 1A). Roscovitine induced only a 10% or less increase in G2-M phase in the p53 wild-type HMEC cells (MCF-10A and 76NF2V), p53 wild-type ER/PR-positive cells (ZR75-1 and MCF-7), and p53 heterozygous ER/PR-positive cells (T47D; 34). In contrast, treatment of the p53-muta-
ted TNBC cell lines (MDA-MB-468, MDA-MB-157, HCC1806) and p53 inactivated HMECs (76NE) resulted in a 30% and 20% increase of G2-M phase, respectively, demonstrating a significantly greater change (P < 0.05) in the G2-M phase compared with p53 wild-type cells (Fig. 1A and 1B, Supplementary Fig. S1A).

These results raised the hypothesis that sequential treat-
ment of TNBC cells by roscovitine would accumulate them in the G2-M phase, and when followed by a DNA-damaging agent, such as doxorubicin, would lead to synergistic cell killing. We tested this hypothesis by examining the schedule and duration of combination therapy with roscovitine in a panel of five cell lines, including HMEC MCF-10A cells, ER-positive ZR75-1 and TNBC MDA-MB-157, MDA-MB-231 and MDA-B-468 cell lines (Fig. 1C). A high throughput survival assay (HTSA) was used as a means for determining cell survival. This assay evaluates in vitro survival of cells in response to different treatment strategies, either as single agents or in combination (35, 36). As depicted in the diagram (Supplementary Figs. 1B and C), each cell line was treated with three different combination strategies: (i) concomitant treatment with roscovitine and doxorubicin; (ii) sequential treatment with doxorubicin followed by roscovitine; and (iii) sequential treatment with doxorubicin followed by doxorubi-
cin. At the conclusion of the experiment (12 days), cell viability was assessed based upon a combinational index (CI) algorithmic that the statistical program CalcuSyn provides. CI values of 0–0.9 are indicative of synergistic response, whereas CI values of 0.9–1.1 or >1.1 indicate an additive or antagonistic response, respectively (37). The CI values demonstrated that concomitant treatment induced an antagonistic or additive response in all cell lines (Fig. 1C, R + D). Sequential administration of doxorubicin treatment before roscovitine also induced antagonism or additivity in all cell lines (Fig. 1C and D → R). However, administration of roscovitine before doxorubicin induced a synergistic response only in TNBC cell lines, but an antagonistic response in the p53 wild-type HMEC and ER-positive cell lines (Fig. 1C, R → D) with a
significantly lower (P < 0.05) CI value in all TNBC cell lines (Fig. 1C). Furthermore, when TNBC cells were treated with roscovitine for 24 hours followed by another 48 hours concomitantly with doxorubicin, we measured synergism and additivity (Supplementary Fig. S2A). To examine the specificity of the synergistic response to antracyclines, we also performed sequential treatment of TNBC cells with roscovitine followed by the antimicrotubule chemotherapeutic taxol, which induced an additive and antagonistic response in MDA-MB-157 and MDA-MB-231 cells, respectively (Supplementary Fig. S2B). Therefore, only sequential combination treatment of roscovitine followed by doxorubicin can specifically inhibit TNBC cells, and not HMEC or ER-positive cells.

Next, we examined whether inhibition of CDK1, CDK2, or both CDKs simultaneously was required for synergism with doxorubicin treatment. MCF-10A, ZR75-1, and MDA-MB-468 cells were transiently transfected with siRNA against either CDK1 or both, with nontargeting siRNA used as a control (Fig. 1D; Supplementary Fig. S2C and S2D). Cell viability was assessed by HTSA using siCDK1or siCDK2 as single agents and in combination with doxorubicin. Although siCDK1 or siCDK1/siCDK2 reduced cell viability in MCF-10A by 50% to 60%, there was no further reduction in viability in these cells with the addition of doxorubicin. Knockdown of CDK2 did not decrease percent viability; however, the addition of doxorubicin treatment again reduced viability by 50% to 60% in MCF-10A cells (Fig. 1E). Similar results were seen with ZR75-1 cells (Supplementary Fig. S2D). Knockdown of CDK1 or both CDKs simultaneously reduced viability by about 50% in MDA-MB-468 cells, whereas siCDK2 caused only 20% cell inhibition. However, the addition of doxorubicin to either siCDK1 or siCDK1/siCDK2 knockdown cells caused 90% cell inhibition only in the TNBC MDA-MB-468 cells, but not in the MCF-10A (Fig. 1E) or ZR75-1 cells (Supplementary Fig. S2D). These results suggest that inhibition of CDK1 combined with doxorubicin treatment is sufficient to induce synergistic cell inhibition in TNBC but not in non-TNBC cells.

Combination treatment increases apoptosis specifically in TNBC cells

To examine cell death in TNBC and HMEC cells in response to single and combination treatments both during and following drug treatment, we used both dye-exclusion assay with propidium iodide (PI) and PARP-1 cleavage as readouts for apoptosis. PI positivity is detected when the cell membrane is compromised, a characteristic of apoptosis (38) as depicted by the use of staurosporine as a positive control in our assays (Fig. 2A). Roscovitine-induced PI positivity peaked at 24 hours after drug exposure with no further increase up to 9 days after treatment (Fig. 2A, left). Doxorubicin treatment steadily reduced cell viability over time; with MDA-MB-157 cells showing over 30% PI positivity at 9 days after drug removal (Fig. 2A, middle). Combination-sequential treatment of cells with roscovitine followed by doxorubicin induced only a 15% PI positivity in MCF-10A cells, while the combination treatment induced 45% and 30% PI positivity in MDA-MB-157 and MDA-MB-468 cells, respectively. Moreover, only TNBC cells continued to exhibit decreased cell viability, or PI positivity, following release from treatment with maximum PI positivity at 72 hours after treatment in MDA-MB-157 and 120 hours after treatment in MDA-MB-468 cells (Fig. 2A, right).

PARP-1 cleavage was not observed in MCF-10A cells following any of the treatment arms, or following drug removal (Fig. 2B and Supplementary Fig. S2E). However, both TNBC cell lines showed sustained levels of PARP-1 cleavage both during and following release from combination treatment (Fig. 2B). For example, MDA-MB-468 cells had the highest ratio of PARP-1 cleavage at 24 hours during the combination treatment, which was sustained up to 72 hours after release (Fig. 2B) suggesting a persistent apoptotic signal only in TNBC cells and not in MCF-10A cells (Supplementary Fig. S2E).

We also examined whether the synergism observed between CDK1 siRNA + doxorubicin (Fig 1C–1o) also resulted in higher PARP-1 cleavage. Western blot analysis revealed that MCF-10A cells, showed no change (compared with siControl) in their ability to cleave PARP-1 following either CDK1/2 siRNA or treatment with doxorubicin (Fig. 2C, top). In contrast, knockdown of CDK1, CDK2, or both increased PARP-1 cleavage in MDA-MB-468 cells compared with siControl transfected cells. Moreover, the addition of doxorubicin to CDK knockdown cells increased cleaved PARP-1 expression (Fig. 2D, top). Forty-eight hours after treatment, MCF-10A knockdown and combination treated cells showed complete recovery, expressing only full-length PARP-1 (Fig. 2C, bottom). However, MDA-MB-468 cells had persistent cleaved PARP-1 expression in both CDK transiently knockdown cells in the presence or absence of doxorubicin 48 hours after treatment (Fig. 2D, bottom). These findings suggest that TNBC cells do not readily recover from pharmacologic inhibition or molecular knockdown of CDK combined with doxorubicin, leading to an enduring apoptotic signal.

TNBC cells bypass the G1 checkpoint in response to combination treatment

The sequential combination treatment of roscovitine followed by doxorubicin resulted in an accumulation of cells in G2–M and generated a polyploid population only in TNBC cell, with 55% of MDA-MB-231 cells accumulating in G2–M (Fig. 3A and Supplementary Fig. S3A). The TNBC polyploid population

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**Figure 3.** Sequential combination treatment of roscovitine and doxorubicin induces a G2–M arrest and accumulation of polyploidy population. A, MCF-10A and MDA-MB-231 cells were treated with roscovitine (24 hours), doxorubicin (48 hours), or sequential combination treatment followed by cell-cycle analysis with flow cytometry. Drugs were administered at IC50 concentrations (see Supplementary Fig. S1B). B, HMEC (MCF-10A) and TNBC (MDA-MB-231 and MDA-MB-468) cells were transfected with siControl, siCDK1, siCDK2, or both siCDK1/siCDK2. Western blot analysis was used to confirm knockdown of CDK1 and CDK2. C, cell-cycle analysis was performed on HMEC (MCF-10A) and TNBC (MDA-MB-231 and MDA-MB-468) siRNA-transfected cells in the absence or presence of 48 hours doxorubicin (D), or combination treatment (R–D) at IC50 concentrations, harvested (using RIPA buffer), and subjected to Western blot analysis with the indicated antibodies. Actin was used for equal loading determination. D, ImageJ was used to measure relative protein expression of each antigen. Protein expression was normalized to actin.
increased by 20% following the sequential combination therapy, compared with untreated cells, whereas HMEC cells did not accumulate in G2–M or increase their polyploid population following any of the treatments (Fig. 3A). The G2–M and polyploidy population induced by concomitant treatment (45%) was less than sequential (55%) treatment for TNBC cells (Supplementary Fig. S3B).

Transient knockdown of CDK1, CDK2 of both CDKs (Fig. 3B) revealed that HMEC cells only had about a 10% increase in G2–M upon CDK knockdown, with the addition of doxorubicin either reverting cells to a G1 accumulation or causing no change in their cell-cycle profiles (Fig. 3C). Conversely, in TNBC cells, knockdown of CDK1 or CDK1/CDK2 lead to a G2–M accumulation that was augmented with the addition of doxorubicin. In MDA-MB-468 cells, 80% of the cells accumulated in the G2–M when the knockdown of CDK1 or CDK1/ CDK2 was combined with single drug treatments. There was no additional gain in the G2–M phase when both CDKs were knocked down in the presence of doxorubicin compared with CDK1 knockdown plus doxorubicin in TNBC cells (Fig. 3C).

In MCF-10A cells, the expression of both total and phospho-Rb was reduced, whereas the expression of p21 and p27 were both increased following single and combination treatment (Fig. 3D and 3E). In contrast, treatment of the TNBC MDA-MB-157 cells with single or combination treatment increased both total and phospho Rb with minimal changes in p21 or p27 expression (Fig. 3D and 3E). These results suggest that the G1 checkpoint activation is intact only in the HMEC cells. In addition, p21 was also transcriptionally activated upon combination treatment only in MCF-10A cells (data not shown), suggesting that wild-type p53 induced a G1 arrest that may protect MCF-10A cells against the toxic effects of combination therapy.

**Loss of wild-type p53 activity is required for roscovitine/doxorubicin synergistic response**

The results from the cell line panel study (Fig. 1) suggested that absence of a wild-type p53 could predict for a synergistic response of roscovitine followed by doxorubicin treatment. Since the majority of TNBC cell lines identified to date harbor p53 mutations (39), we utilized two different isogenic cell systems to test this hypothesis: breast epithelial cells (HMECs) 76NF2V and 76NE6 and colorectal cancer cells HCT116 p53+/− and HCT116 p53−/− cell lines. 76NE6 cells were immortalized from 76N primary HMEC cells via transfection with the viral oncoprotein HPV16-E6, which binds to and degrades p53, while 76NF2V cells were immortalized with the F2V-mutant HPV-16E6 gene, which renders cells immortalized but with a functional and wild-type p53 (40, 41). Sequential administration of roscovitine followed by doxorubicin resulted in an additive response in p53 wild-type cells 76NF2V, while it induced synergism in the p53 inactive 76NE6 cells (Fig. 4A), with twice as many cells with polyploid nuclei in 76NE6 compared with 76NF2V cells (Fig. 4B). Levels of phospho-and total Rb were reduced following single and combination therapy in 76NF2V cells compared with 76NE6 cells. In addition, p21 levels were induced only in 76NF2V cells, in response to combination treatments (Fig. 4C).

Because drug treatment reduced phospho-Rb expression in both MCF-10A (Fig. 3D) and 76NF2V (Fig. 4C) cells, while remaining elevated in MDA-MB-157 cells, we interrogated whether loss of Rb would render 76NF2V cells more synergistic to the combination treatment. Rb was stably knocked down in 76NF2V cells (Fig. 4D) and subjected to HTSA followed by CalcuSyn analysis (Fig. 4D). Roscovitine–doxorubicin combination treatment induced only additivity or antagonism in the Rb knocked down cells (Fig. 4D). Moreover, ablation of the Rb pathway did not cause 76NF2V cells to accumulate more in the G2–M phase compared with shScramble cells (Supplementary Fig. S3B). Therefore, Rb inactivation is not sufficient to cause roscovitine–doxorubicin-induced synthetic lethality in HMEC cells.

When HCT116 p53+/− and HCT116 p53−/− cells were subjected to HTSA and CalcuSyn analysis, p53 wild-type cells responded antagonistically to combination treatment, while deletion of p53 induced synergism in HCT116 cells (Fig. 4E, 4F). Sequential roscovitine–doxorubicin treatment reduced phospho-Rb expression in p53 wild-type cells. Moreover, both single and combination drug treatment caused increased expression of p27 and p21 only in the p53 wild-type cells (Fig. 4G). Finally, combination treatment increased G2–M accumulation and polyploidy in both p53 wild-type and knockout cells. However, combination-treated p53 knockout cells had twice the amount of sub-G1 cells compared with p53 wild-type cells both during and after treatment (Fig. 4H) suggesting that the loss of p53 is required for synergism in tumors cells.

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**Figure 4.**

Absence of p53 is required for synergism between roscovitine and doxorubicin. A, isogenic HMEC cells, 76NF2V and 76NE6, were subjected to sequential roscovitine–doxorubicin treatment via HTSA followed by CalcuSyn analysis. The concentrations used for each agent are listed in Supplementary Fig. S1B. Average combination doxorubicin (D) is shown for each cell line. B, 76NF2V and 76NE6 cells were treated with roscovitine (R) for 24 hours, doxorubicin (D) for 48 hours, or sequential combination treatment (R−D) at IC50 values (Supplementary Fig. S1B). Following fixation, samples were subjected to cell-cycle analysis via flow cytometry. Untreated (C) samples served as a control. C, cells were treated with R, D, or R−D at IC50 values (Supplementary Fig. S1B) followed by Western blot analysis of G1 checkpoint proteins. D, lentivirus was used to generate stable Rb knockdown 76NF2V cells. Western blot analysis of total Rb was used to confirm knockdown compared with parental and stably expressing nontargeting shScramble cells. Rb knockdown and shScramble cells were treated with sequential roscovitine–doxorubicin treatment via HTSA followed by CalcuSyn analysis. Average combination index (C) is shown for the shScramble, the two shRb variants of 76NF2V. E, Western blot analysis of p53 expression in HCT116 p53+/− cells compared with wild-type HCT116 p53+/− and MCF-10A cells. F, HCT116 wild-type and knockout cells were subjected to roscovitine–doxorubicin combination treatment via HTSA followed by CalcuSyn analysis. The concentrations used for each agent are listed in Supplementary Fig. S1B. Average combination index (C) is shown for each cell line. G, Western blot analysis was performed to examine the effect of single and sequential combination drug treatment on G1 checkpoint proteins of HCT116 p53 wild-type and knockout cells. Actin was used as a loading control. H, HCT116 wild-type and knockout cells treated with R−D at IC50 concentration of each drug and cells were removed either immediately after treatment or 24 hours after drug treatment (R+D+c24) was subjected to cell-cycle analysis.
Combination-based treatment increased DNA double-strand breaks and decreased DNA repair

Because CDK1 activity is required for HR, we next hypothesized that this combination treatment strategy mediates its activity through the alteration of DNA damage response. To this end, we initially examined a neutral comet assay to quantitate DNA DSB in our panel of HMEC and TNBC cells following single and combination treatments (Fig. 5A). The read-out of the neutral comet assay is the tail moment, or the amount of DNA in the distance traveled, which measures the extent of DNA DSBs. As expected, doxorubicin treatment induced DNA DSBs, indicated by an increased tail moment, in all cell lines examined (Fig. 5A). However, administering roscovitine before doxorubicin caused a significant (P < 0.05) increase in the amount of DNA DSBs only in TNBC cells. Furthermore, doxorubicin treatment induced γ-H2AX foci, a marker of DNA DSBs, in MCF-10A cells and both TNBC cell lines compared with untreated control cells (Fig. 5B–D). However, treatment with roscovitine before doxorubicin significantly increased (P < 0.05) the percentage of γ-H2AX-–positive cells by 20% explicitly in TNBC cells with no increase in MCF-10A cells (Fig. 5B–D). Moreover, Western blot analysis revealed that only combination treatment induced an increase in phospho-H2AX (γ-H2AX) in TNBC MDA-MB-157 cells, but not in HMEC MCF-10A cells (Fig. 5B).

Quantification of γ-H2AX-–positive cells with Rad51 foci was used next to examine the recruitment of downstream HR proteins. Untreated control HMEC and TNBC cells had limited DNA damage, but had over 60% γ-H2AX (Fig. 5C–F). Combination treatment decreased the recruitment of Rad51 in both MCF-10A and MDA-MB-157 cells. In addition, roscovitine treatment reduced Rad51 protein expression in both MCF-10A and MDA-MB-157 cells (Fig. 5B). However, combination treatment significantly reduced (P < 0.05) the formation of Rad51 foci compared with doxorubicin treatment only in TNBC cells even though these cells had increased γ-H2AX positivity (Fig. 5C–F). Combination and doxorubicin only treated MCF-10A cells were able to recruit Rad51 foci at a similar percentage (Fig. 5C and 5F). In parallel, although Rad51 expression decreased in combination-treated MDA-MB-157 cells, it remained elevated in MCF-10A combination-treated cells (Fig. 5B). These results suggest that treating TNBC cells with roscovitine before doxorubicin can enhance DNA damage while impairing their ability to repair DNA DSBs, regardless of increased DNA damage.

Deletion of p53 allows combination treatment to increase DNA DSBs

HCT116 p53fl/+ and HCT116 p53–/– were used to determine the effect of p53 on DNA damage and repair. Presence or absence of p53 had little effect on doxorubicin-induced DNA damage, with 41% and 48% of p53 wild-type and p53 knockout cells having γ-H2AX positivity, respectively (Supplementary Fig. S4A, B, and C). However, combination treatment significantly increased (P < 0.01) γ-H2AX–positive cells only in p53 knockout cells compared with doxorubicin-treated cells. Combination treatment did not augment γ-H2AX positivity in p53 wild-type cells. Moreover, combination-treated p53 knockout cells had significantly more (P < 0.01) γ-H2AX–positive cells (by 20%) compared with combination-treated p53 wild-type cells (Supplementary Fig. S4C).

Furthermore, upon doxorubicin treatment, p53 wild-type and p53 knockout cells recruited Rad51 to γ-H2AX sites in 63% and 59% of cells, respectively (Supplementary Fig. S4A, B, and D). Combination treatment had no effect on Rad51 recruitment in p53 wild-type cells. However, p53 knockout cells had a significant (P < 0.05) decrease in the percent of γ-H2AX–positive cells with Rad51 foci compared with doxorubicin-treated cells (Supplementary Fig. S4A, S4B, and S4D). Despite having more G2–M cells, p53 knockout cells had less recruitment of Rad51 foci that p53 wild-type cells during combination treatment (Fig. 4G; Supplementary Fig. S4A, S4B, and S4D). Collectively, these results suggest that deletion of p53 causes tumor cells to have increased DNA damage and reduces the ability of tumor cells to recruit downstream HR proteins in response to roscovitine–doxorubicin combination treatment.

Combination treatment increases overall survival and decreases proliferation in human breast cancer xenograft

MDA-MB-468 cells were used to establish human xenograft tumors in the right and left mammary fat pads of nude mice (Fig. 6A). There were five treatment arms: vehicle, roscovitine for 4 days on/3 days off, doxorubicin once a week, concomitant treatment of roscovitine with doxorubicin administered on day 4, or sequential treatment of roscovitine followed by doxorubicin for four cycles. Sequential combination-treated tumors were the only arm of the study that did not increase in volume while on treatment. Averaging at 125 mm³, sequential combination-treated tumors were significantly smaller (P < 0.01), than vehicle-treated tumors that averaged to be

Figure 5.

Sequential combination treatment of roscovitine and doxorubicin causes more DNA double-strand breaks while simultaneously reducing downstream homologous recombination proteins. A, neutral comet assay was performed on HMEC (MCF-10A) and TNBC (MDA-MB-157 and MDA-MB-468) cells that were treated with roscovitine (R) for 24 hours, doxorubicin (D) for 48 hours, or sequential combination treatment (R–D) at IC50 concentrations (Supplementary Fig. S2B). Samples were subjected to a current of 21V for 21 minutes and subsequently stained with SYBR green for visualization (right). Images were captured at >10 magnification with an Eclipse 90i microscope equipped with the NIS-Elements Br 3.10 software program. The tail moment (the amount of DNA in the distance traveled) was measured using Comet Score software and plotted as bar graphs (left). B, (A) MDA-MB-157 and MCF-10A cells were treated with roscovitine (R), doxorubicin (D), or sequential combination treatment (R–D). Untreated cells were used as a control (C). Following treatment, cells were harvested and Western blot analysis was performed to detect expression of phospho-H2AX and Rad51. C and D, MCF-10A and MDA-MB-231 cells were treated with single and combination drug treatment as in A and B. DNA repair foci γ-H2AX and Rad51 were detected with immunoﬂuorescence. DAPI was used to detect nuclei. Images were captured at >100 with an Eclipse 90i microscope. E, percent of γ-H2AX-positive was quantiﬁed in HMEC and TNBC cells in response to single and combination drug treatment. Only cells with >5 foci were considered as positive. One hundred cells per sample, per replicate were counted. F, percent of γ-H2AX-positive cells with Rad51 foci recruitment was quantiﬁed. Cells with ≥1 Rad51 foci were considered positive. Images for quantification were captured at >60 with a confocal Olympus FV100 microscope. Experiments were repeated three times; error bars, 95% conﬁdence intervals. The Student t test (two-tailed, equal variance) was used to derive the P values.
330 mm³ on day 26 (Fig. 6B and Supplementary Fig. S5A and S5C). Indeed, concomitantly treated mice had a 5-fold increase in tumor size on day 26 (Supplementary Fig. S5C). Moreover, sequential combination-treated tumors were significantly smaller (P < 0.05) than roscovitine, doxorubicin, and concomitant-treated tumors during and following treatment (Fig. 6B and Supplementary Fig. S5A and S5C). Notably, no measurable difference was observed between vehicle and roscovitine-treated mice; supporting clinical findings that roscovitine is inefficient as a single agent (Fig. 6A).

In addition, none of the sequential combination-treated mice suffered from increased toxicity or tumor burden during the 60-day experiment (Fig. 6D and Supplementary Table S2). As such, sequential combination therapy significantly increased overall survival (P < 0.05) compared with vehicle, single-agent, and concomitant-treated mice (Fig. 6C). Overall toxicity was assessed by weight loss during and following treatment. Neither the roscovitine alone arm nor the combination arm animals suffered any weight loss, while 80% and 50% of doxorubicin and concomitant-treated mice had to be sacrificed due to >20% weight loss, respectively, again revealing the limitations of doxorubicin as monotherapy and the importance of drug delivery scheduling (Fig. 6C: Supplementary Fig. SSE and Supplementary Table S2).

These results suggested that treatment with roscovitine could limit doxorubicin-induced toxicities. To test this, a dose-escalation study examining weight loss and blood count was performed on nontumor-bearing nude mice that were treated with vehicle, roscovitine (50 mg/kg), doxorubicin (5 mg/kg or 10 mg/kg), or sequential therapy. At 1 week into treatment, sequential-treated mice maintained normal weights compared with doxorubicin treatment at 5 mg/kg. However, as the amount of doxorubicin accumulated, the protective effect of roscovitine against weight loss diminished (Supplementary Fig. S6A). Following the completion of treatments, blood was collected from all mice. Treatment of roscovitine before doxorubicin was able to rescue the reduction in the white blood cell (WBC) count caused by doxorubicin alone (Supplementary Fig. S6B). In addition, doxorubicin significantly (P < 0.05) reduced the platelet count in mice. However, sequentially treated mice had twice as many platelets (Supplementary Fig. S6C). None of the treatments affected the red blood cell count (RBC; Supplementary Fig. S6D). Therefore, sequential combination therapy rescues doxorubicin-induced neutropenia and platelet loss.

To assess proliferation of tumors in each treatment arm, bromodeoxyuridine (BrdUrd) incorporation was measured at the end of treatment (day 26) and revealed that both doxorubicin and combination-treated tumors had a significant (P < 0.05) decrease in proliferation compared with vehicle-treated tumors (Fig. 6D). Western blot analysis of PARP-1 on tumors resected on day 26 revealed that combination therapy significantly increased cleaved PARP-1 expression (P < 0.05) compared with doxorubicin-treated tumors, indicative of apoptosis (Fig. 6E). On the basis of hematoxylin and eosin staining, tumors from all four treatment arms had similar histology, with combination-treated tumors having marked fibrous (Supplementary Fig. SSD). Overall, these data suggest that roscovitine–doxorubicin combination therapy is both well tolerated and efficacious against TNBC.

Discussion

Here, we describe that sequential administration of roscovitine followed by doxorubicin is a well-tolerated, synthetic lethal combination treatment strategy that explicitly targets TNBC cells, while leaving nontransformed cells unharmed. Hallmarks of cancer include limitless proliferation and genome instability (42, 43). By targeting CDK1, which has a role in both cell-cycle promotion and DNA DSB repair, followed by treatment with a chemotherapeutic, we have developed a novel synthetic lethal combination. Simultaneous drug administration or treating cells with doxorubicin prior to roscovitine was antagonistic in TNBC cells and toxic in vivo. Furthermore, roscovitine followed by doxorubicin treatment inhibited tumor growth in vivo and significantly increased overall survival compared with mice receiving concomitant treatment of the two drugs or either of the drugs as single agents.

Because TNBC cells have lost their G1–S regulation, treatment of these cells with roscovitine resulted in their accumulation in G2–M phase, while non-TNBC or HMEC cells with a regulated G1–S transition, accumulated in the G1 phase of the cell cycle. In addition, combination treatment maintained a G2–M arrest and/or induced polyplloid nuclei specifically in TNBC cells. Moreover, knockdown of CDK1, a G2–M-specific CDK, was sufficient to augment the percentage of G2–M accumulation in the presence of doxorubicin only in TNBC cells. Collectively these results suggest that a sequential treatment strategy that differentially targets the cell cycle in TNBC versus HMEC cells is more likely to produce a synergistic response only in those cells with a deregulated cell cycle (i.e., TNBC cells).

TNBC cells with p53 mutations had a diminished capacity to induce p21 transcription compared with p53 wild-type HMEC cells. Knockdown of Rb was insufficient to cause a synergistic response in HMEC cells. However, combination treatment was synthetically lethal only in p53-compromised HEMC and tumors cells, whereas the paired isotogenic p53 wild-type cells were additive or antagonistic to the combination treatment. Moreover, the cell-cycle profile of HMEC and tumor cells lacking p53 activity closely mimicked the cell-cycle profile of TNBC cells. Thus, p53 inactivation could serve as a predictor of synergistic response to sequential roscovitine-doxorubicin combination treatment.

Mechanistically, administration of roscovitine before doxorubicin caused increased DNA DSBs while simultaneously inhibiting recruitment of downstream HR proteins in TNBC cells. Combination treatment did not subject HMEC cells to increased DNA damage. HR is the preferred method of DNA DSB repair during S and G2–M phase, while non-TNBC or HMEC cells with a regulated G1–S transition, accumulated in the G1 phase of the cell cycle. Therefore, inhibiting CDK1 activity (with roscovitine) primes and sensitizes TNBC cells to doxorubicin. Nontransformed cells and tumor cells with an intact p53 pathway were less sensitive to combination treatment because they did not accumulate at the G2–M phase before doxorubicin administration.

The requirement of p53 inactivity for the sequential combination treatment strategy was validated by identifying four or six subgroups within TNBC tumors (44, 45). Thus, identifying patients that would benefit the most...
Sequential roscovitine–doxorubicin combination treatment is effective and well tolerated in vivo. A, a schematic illustrating that 4.5×10^6 MDA-MB-468 cells in a 1:2 ratio with Matrigel were injected into the right and left mammary fat pads of the four groups of 4- to 6-week-old female Balb/c Nu/nu mice. B, once tumors reached 100 to 150 mm³, they were treated with vehicle, roscovitine (50 mg/kg) 4 days on/3 days off, doxorubicin (2 mg/kg) once a week, or combination treatment for four cycles. Weekly tumor volume measurements shown. C, overall survival graphed as Kaplan–Meier curve. Mice treated with concomitant combination drug treatment (4 days of roscovitine with doxorubicin administered on day 4) included in survival analysis. Mice were euthanized if total tumor burden was 1,500 mm³ or if mice lost ≥20% of initial body weight. Statistical analysis was performed using Mantel–Cox method. D, percent BrdUrd-positive cells were averaged from three tumors resected on day 26 per each treatment arm. E, Western blot analysis performed on tumors resected on day 26. Densitometry analysis performed using ImageJ.
from combination treatment, while sparing nonresponsive patients to treatment, is crucial to successfully implementing this treatment strategy. Moreover, developing a drug treatment strategy that incorporates the clinically available agent doxorubicin may hasten the adoption of this combination in the clinic.

Although the sequential roscovitine followed by doxorubicin treatment was well tolerated in vivo (this study), a phase I clinical trial examining the MTD and efficacy of dinaciclib (the next-generation analogue of roscovitine) and the anthracycline epirubicin found that this treatment was very toxic, ending the trial before efficacy could be determined (46). Dose-limiting toxicities included grade 3 neutropenia, syncope, and vomiting (46). One solution to increase the efficacy of combination treatment while reducing toxicity is to couple the drugs with a nanocarrier delivery system. Coupling CDK inhibitors or doxorubicin with nanotechnology drug delivery system will limit dispersal of the drug to only the site of action, protecting other organs and tissues from cytotoxicity (47). There is precedence for such a strategy as anti-HER2 immunoliposomes containing doxorubicin were effectively targeted to HER2-overexpressing tumors, increasing the therapeutic benefit of doxorubicin while reducing toxicity in a xenograft mouse model (48). Alternatively, CDK inhibitors could be directly delivered to the tumor site if it was bound to a ligand-mediated active binding nanoparticle. EGFR, which is overexpressed in the majority of TNBC tumors, is a cell surface receptor that provides a putative target to deliver roscovitine to the tumor site. Indeed, EGFR-targeted polymer nanocarriers delivered paclitaxel and ionicidamine to multidrug resistant EGFR-overexpressing tumor cells, increasing drug cytotoxicity (49). Developing EGFR-targeted nanocarriers to deliver roscovitine directly to the tumor site could increase the therapeutic benefit of CDK inhibition while reducing toxicities. Moreover, liposomal–doxorubicin, which accumulates at tumor sites due to leaky vasculature, is clinically available to treat breast cancer (50). Thus, it would be clinically beneficial to consider incorporating CDK inhibitors or doxorubicin combination treatment with nanoparticle delivery system.

Although doxorubicin and combination-treated tumors had the same amount of proliferation, combination-treated tumors underwent more apoptosis, indicating increased cytotoxicity. Furthermore, doxorubicin only treated mice suffered from more weight loss compared with combination-treated mice. Thus, the combination therapy of roscovitine followed by doxorubicin can decrease tumor volume while limiting toxicity. Combining chemotherapy with a CDK inhibitor could potentially lead to a reduction in the chemotherapy dose administered to patients. Overall, roscovitine–doxorubicin sequential therapy increased the therapeutic benefit of doxorubicin while reducing toxicity, supporting future clinical studies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


CDK Inhibition Sensitizes p53-Mutated Cells to Doxorubicin


Sequential Combination Therapy of CDK Inhibition and Doxorubicin Is Synthetically Lethal in p53-Mutant Triple-Negative Breast Cancer


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