2023

Targeting ribonucleotide reductase for breast cancer treatment

Nahid Sultana
University of the Pacific

TARGETING RIBONUCLEOTIDE REDUCTASE FOR BREAST CANCER TREATMENT

by

Nahid Sultana

A Dissertation Submitted to the

Office of Research and Graduate Studies

In Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Thomas J. Long School of Pharmacy
Pharmaceutical and Chemical Sciences Program

University of the Pacific
Stockton, California

2023

TARGETING RIBONUCLEOTIDE REDUCTASE FOR BREAST CANCER TREATMENT

By

Nahid Sultana

APPROVED BY:

Thesis Advisor: Jesika S. Faridi Ph.D.

Committee Member: Roshanak Rahimian Ph.D

Committee Member: Atefeh Rabiee Ph.D

Committee Member: Mamoun Alhamadsheh Ph.D

Committee Member: Khyati Niral Shah Ph.D

Department Chair: Roshanak Rahimian Ph.D

Dean of Graduate Studies: Bhaskar Jasti Ph.D

Dedication

In dedication to my dear husband for supporting me all the way!!!

Acknowledgement

I am grateful to Dr. Jesika Faridi for her support, encouragement, positive criticism, and guidance in my PhD journey. I am truly thankful for her help in my academic and personal development as well as making this an excellent learning experience. I would like to thank the members of my dissertation committee for their time and patience and their intellectual contributions to my career development as a scientist. I would like to thank Dr. Shah for her continuous guidance. I would also like to thank Dr. Jasti, Dr. Alhamadsheh, Dr. Rahimian, and Dr. Rabiee for their continuous encouragement. I am sincerely grateful to all the faculty members of the PCSP program for nurturing my scientific and personal goals. I would like to thank Dr. Li and Dr. Jasti, for giving me the opportunity to work at the University of the Pacific. I would also like to acknowledge Ms. Bonnie O'Hearn, Ms. Kathy Kassab and Ms. Sonya North for their administrative support.

TARGETING RIBONUCLEOTIDE REDUCTASE FOR BREAST CANCER TREATMENT

Abstract

By Nahid Sultana

University of the Pacific
2023

Breast cancer is the second most common type of cancer in the world. Hormone receptor (HR) positive breast cancer (BC) is a prevalent disease accounting for approximately 2 million new cases globally. Almost 70-80% of breast cancer patients are women with a positive score for the estrogen receptor (ER). Triple-negative breast cancer (TNBC) which have a negative score for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type 2 (HER2) is considered an aggressive histological breast cancer subtype with limited treatment options.

Frequently, doxorubicin (DOXO)-based chemotherapy is utilized in this patient population due to the lack of available molecular targets. While DOXO is an effective chemotherapeutic agent, its efficacy is limited due to acquired drug resistance and cardiotoxicity. Therefore, the identification of other treatment options for TNBC is needed. TNBC is a heterogeneous malignancy, with 70% of cases classified as a basal subtype as they look similar to the epithelial cells of the outermost basal layer of the breast's milk ducts. This further complicates the search for an effective molecular target. Doxorubicin and other anthracycline derivatives are frequently used as part of the adjuvant chemotherapy regimen for triple- negative breast cancer (TNBC). Although effective, doxorubicin is known for its off-target and toxic side effect profile, particularly

with respect to the myocardium, often resulting in left ventricular (LV) dysfunction and congestive heart failure when used at cumulative doses exceeding 400 mg/m^2.

Ribonucleotide reductase (RR) is a rate limiting enzyme in DNA synthesis consisting of two subunits RRM1 and RRM2. Both RRM1 and RRM2 are encoded by different genes in their chromosomes. Their mRNAs are also differentially expressed throughout the cell cycle. Didox inhibits ribonucleotide reductase subunit 2 (RRM2) which ultimately blocks DNA synthesis. We have observed that the ribonucleotide reductase subunit 2 (RRM2) is significantly over-expressed in estrogen receptor (ER)–negative cells as compared with ER-positive breast cancer cells. Here, we inhibited RRM2 in ER-negative breast cancer cells as a target for therapy in this difficult-to-treat population. We observed that through the use of didox (3,4-dihydroxybenzohydroxamic acid), a ribonucleotide reductase inhibitor, the reduction in RRM2 was accompanied by reduced NFκB activity *in vitro*. When the ribonucleotide reductase inhibitor didox was used in combination with the chemotherapeutic drug doxorubicin, we observed significant downregulation of NFκB proteins in TNBC. As well, we observed that protein levels of mutant p53 were significantly reduced by didox or combination therapy *in vitro*. Xenograft studies showed that combination therapy was found to be effective *in vivo*, resulting in a significantly reduced tumor volume as compared with doxorubicin monotherapy. In addition, the use of didox was also found to ameliorate the toxic myocardial effects of doxorubicin *in vivo* as measured by heart mass, LV diameter, and serum troponin T protein levels which are released by heart during muscle damage. The data present a novel and promising approach for the treatment of TNBC that merits further clinical evaluation in humans.

Hormone receptor positive breast cancers of all stages are selectively treated with endocrine therapy targeting estrogen receptor (ER) activity. But success is limited by the development of

acquired resistance owing to long-term therapy. The cyclin D1 and cyclin dependent kinase 4/6 (CDK4/6) complex causes phosphorylation and subsequent inactivation of retinoblastoma (Rb) tumor suppressor protein which promotes progression of the cell cycle from G1 to S phase. This observation led to the development of the first CDK4/6 inhibitor Palbociclib (Ibrance; Pfizer) which induces cell cycle arrest at G1 phase in cancer cells. Intrinsic and acquired drug resistance development, have impacted the therapeutic success rate despite promising clinical outcomes. This situation necessitates the development of potential combination strategies to overcome drug resistance. The combination of didox with palbociclib is a potential strategy to target ER positive and ER negative/triple-negative breast cancer. In our recent study, we confirmed that didox in combination with palbociclib significantly lowers the growth of ER positive and ER negative breast cancer cells along with their palbociclib resistant counterparts compared to no treatment or palbociclib treatment alone. We confirmed that ER positive MCF7 and ER negative MDA-MB-468 parental breast cancer cells exhibit lower IC_{50} values of palbociclib drug as compared to their palbociclib resistant counterparts. Here, we are reporting that didox alone or in combination with palbociclib decreases cell cycle proteins in ER positive MCF7 and ER negative MDA-MB-468 parental and palbociclib resistant breast cancer cells. This finding opens a novel approach for targeting both ER positive as well as ER negative breast cancer treatment. We are also reporting that didox treatment alters cyclin D1 (CCND1) and RRM2 expression in MCF7 and MDA-MB-468 breast cancer cells along with their palbociclib resistant counterparts. Additionally, we observed that didox alone or in combination with palbociclib alters the cell cycle of MCF7 and MDA-MB-468 parental and palbociclib resistant breast cancer cells. Our data present a novel and promising approach for the treatment of ER positive and ER negative breast cancer that involves inhibition of

RRM2, NFκB, and the CDK4/6-cyclin D1/pRb axis that merits further clinical investigation in human models.

Table of Contents

List of Figures

Figure

List of Abbreviations

AI	Aromatase inhibitor
CDK	Cyclin Dependent Kinase
CI	Combination Index
DDX	Didox
DBD	DNA binding domain
DMFS	Distant metastasis free survival
dNTP	Deoxyribonucleotide triphosphate
EGFR	Epidermal growth factor receptor
ER-/ER-negative	Estrogen receptor negative cell lines or tumors
ER+/ER-positive	Estrogen receptor positive cell lines or tumors
ERE	Estrogen responsive elements
ER-α	Estrogen receptor-alpha
ER-α36	Estrogen receptor-alpha 36kDa variant
ER-α66	Estrogen receptor-alpha 66kDa wild type
ER-β	Estrogen receptor-beta
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEO	Gene expression omnibus
HKG	Housekeeping gene
HR	Hazard ratio
IGF-IR	Insulin grown factor-I receptor
IKK	Inhibitor of Kappa gene enhancer in B-cells, Kinase

IκBα	nuclear factor of kappa gene enhancer in B-cells inhibitor, alpha
MAPK	Mitogen activated protein kinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OS	Overall survival
PLB	Palbociclib
p53R2/RRM2B	p53 regulated ribonucleotide reductase M2B
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
PKB/Akt	Protein Kinase B/ v-Akt oncogene
Rb	Retinoblastoma protein
RFS	Recurrence free survival
RPL13A	Ribosomal Protein L13a
RR	Ribonucleotide reductase
RRM1	Ribonucleotide reductase M1 subunit
RRM2	Ribonucleotide reductase M2 subunit
RT-qPCR	Realtime-quantitative polymerase chain reaction
SERD	Selective estrogen receptor down-regulator
SERM	Selective estrogen receptor modulator

CHAPTER 1: INTRODUCTION

Breast cancer is recognized as the second leading cause of death for women worldwide (1). As a heterogenous disease it is categorized into different subtypes (1,2). The subtypes are luminal A, luminal B, human epidermal growth factor receptor type 2 (HER2) positive, basal-like and normal like (3,4). All these subtypes can be divided further. Luminal A and luminal B are considered as hormone receptor positive breast cancer as they have high levels of estrogen receptor (ER) and progesterone receptor (PR). Luminal B has a positive score for HER2. Luminal A has low levels of Ki67 which is a cell proliferation marker but luminal B has high levels of Ki67 (5). Normal-like breast cancer shares similar characteristics with luminal A with slight difference in genetic makeup (6). Both normal-like breast cancer and luminal A have similar classical immunohistochemistry marker expression like ER positive, PR positive, HER2 negative and low Ki67. Overall gene expression pattern of normal-like breast cancer is same as normal breast tissue. Owing to their similarity, luminal A and normal-like breast cancer are difficult to be distinguished. There is one finding which shows a difference in expression of long non-coding RNAs in normal-like and luminal breast cancer (7). However, normal-like breast cancer can be better prognosed than luminal A subtype (8,9). Basal-like breast cancer is also known as triple-negative breast cancer (TNBC) as they possess a negative score for estrogen, progesterone and HER2 receptors (2,3,10). TNBC can be further categorized into four subtypes such as basal-like 1, basal-like 2, mesenchymal and luminal androgen receptor. 80% of TNBC have similar traits as basal-like subtypes (5,11). ER overexpressing breast cancer are the most common and prevalent type as they have diverse gene mutations and they usually require multiple treatments (6). HER2 overexpressing breast cancer is 2-20% prevalent (12). Among all breast cancer subtypes, TNBC is

the most difficult to treat breast cancer due to its negative score for all three-hormone receptor and lack of specific druggable targets. Overall, heterogeneity of breast cancer makes the treatment development process quite complex, intriguing and challenging (1,10).

Except for skin cancer, breast cancer is the most common cancer in women in the United States which is about 30% or 1 in 3 of new female cancers every year (13). In 2023, the American Cancer Society estimates about 297,790 new cases of invasive breast cancer in women in the U.S. alone. Among them 55,720 new cases will be of ductal carcinoma in situ (DCIS). 1 in 8 women in the U.S. will be diagnosed with breast cancer in her lifetime and every 2 minutes one new confirmed case is added as well. Total of 43,700 women death is estimated from breast cancer (13). Even though breast cancer is rare in men, it is estimated that about 2,710 men will be diagnosed with breast cancer in the U.S. and 530 will die from breast cancer (14). Epidemiological studies coupled different factors to address risk development or progression to breast cancer (15,16). There are several risk factors of breast cancer development among them menopause, first childbirth and late age for marriage are strongly associated with breast cancer development (17, 18, 19, 20, 21). Other risk factors for breast cancer are family history, delayed puberty, lactation failure, hormone replacement therapy, use of contraceptive, obesity, alcohol consumption, smoking and environment toxicants (18).

Estrogen receptor (ER) positive breast cancer tend to grow slower than those that are ER negative. The physiological function of the estrogenic compounds such as estradiol are largely controlled by estrogen receptor subtypes alpha (ERα) and beta (ERβ) (19). ERα plays a major role in the progression of human breast cancer. ERα66 is the classical ERα of 66kDa which is often referred to as ER. Cancer cells that have high levels of ERα66 are called ER

positive whereas cells lacking ERα66 are termed ER negative. According to clinical evidence it is suggested that approximately 40% of ERα66 positive breast cancers also express ERα36. Patients expressing ERα36 are less likely to be benefited from antihormone therapy tamoxifen (20).

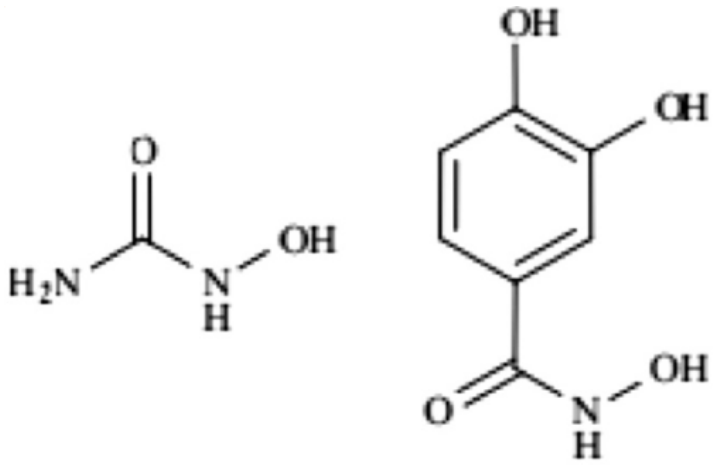
According to the National Comprehensive Cancer Network, hormone receptor positive but HER2 negative breast cancer are treated with adjuvant endocrine therapy. The principal systematic treatment for non-metastatic hormone receptor positive breast cancer e.g. tamoxifen when tumor size is less than 0.5 cm. If tumor size is equal to or more than 0.5 cm, then adjuvant chemotherapy followed by endocrine therapy will be provided. Adjuvant endocrine therapy such as tamoxifen for 5 years with or without ovarian suppression or aromatase inhibitor which lowers estrogen levels by stopping aromatase enzyme e.g. anastrozole for 5 years will be given to premenopausal breast cancer patients at diagnosis (21). Postmenopausal women are treated with tamoxifen for 2-3 years or aromatase inhibitor for 2-3 years or tamoxifen for additional 5 years (21, 22). Treatment for TNBC includes chemotherapy with doxorubicin or cyclophosphamide followed or preceded by paclitaxel every 2 weeks or weekly paclitaxel or capecitabine (21, 22). However, the preferred regimen for hormone receptor ER/PR positive but HER2 negative breast cancer is an aromatase inhibitor with CDK4/6 inhibitor such as palbociclib, ribociclib or abemaciclib as first line therapy. Second line therapy for this class of cancer is treatment with the estrogen receptor antagonist fulvestrant with an CDK4/6 inhibitor if it was not previously used (23,24).

Uncontrolled cellular growth is considered the most common hallmark of cancer. This process is dependent on the sufficient supply of the dNTPs. Ribonucleotide reductase plays the most important role in maintaining dNTP pools. It is the rate limiting enzyme required for

conversion of NTPs to dNTPs. Ribonucleotide reductase enzyme is composed of two subunits RRM1 and RRM2. Both of the subunits play a significant role in the development of cancer. There are many known ribonucleotide reductase targeted agents available for cancer treatment. The majority of them are nucleotide analogs such as gemcitabine. RRM1 targeting drugs are antimetabolites, which function by preventing cancer cell growth by interfering with DNA synthesis such as 5-flurouracil. There are also specific targeted therapies available for RRM2 such as hydroxyurea or the iron chelator triapine (23). The hydroxyl-benzohydroxamic acid derivatives didox is RR enzyme inhibitor, free radical scavenger and iron chelator which can complex with iron to inhibit RRM2. Didox has exhibited synergistic effects with approved chemotherapies. Didox worked synergistically with temozolimide to impair brain cancer cell growth (24–27). Phase 1 and phase 2 studies of didox have previously been completed (30). Even though didox showed tolerable dosing and toxicity, it did not warrant phase 3 clinical study due to its lack of efficacy due to the small sample size of these studies. In the phase 2 study 14 patients with advanced breast cancer were treated with didox 6 grams for 36 hours by intravenous infusion over 3 weeks. But no patient responded even though toxicity was minimal (31). There is still scope for this drug to be evaluated in combination studies with antimetabolite such as cytosine arabinoside using other schedules of didox treatment (30, 31).

Figure 1

Structure of hydroxyurea (left) and didox(right) (30)

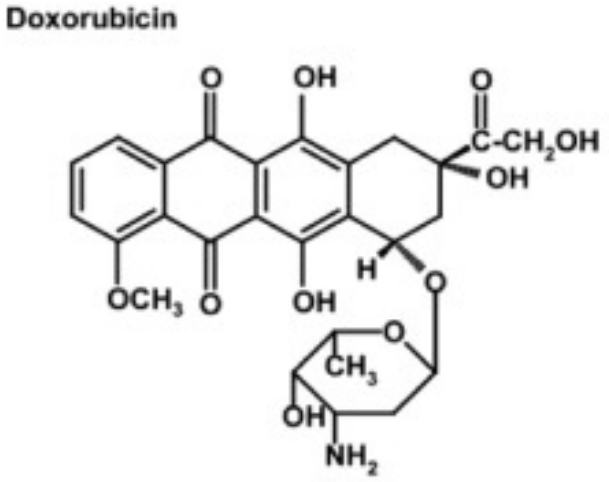


According to chemical composition and function, chemotherapeutic drugs can be divided into several groups, among which alkylating agents directly cause DNA damage to repress tumor cell division. Other chemotherapeutic drugs interfere with the synthesis of DNA and RNA by substituting for the normal building blocks while inhibiting normal DNA synthesis, replication, and transcription. Doxorubicin is an FDA approved chemotherapeutic drug, with proven ability to combat rapidly dividing cells and slow progression of cancer. The nonselective mechanism of action of doxorubicin limits its efficacy due to toxicity development. It possesses aglycone and sugar moieties where aglycone is comprised of a tetracyclic ring with quinine-hydroquinone adjacent groups with methoxy substitute short side chain and carbonyl group. Doxorubicin acts by binding to DNA-associated enzymes, as it intercalates inside the base pairs of the DNA double helix. It binds to multiple molecular targets such as topoisomerase enzymes I and II, causing cytotoxic effects as it may target ribonucleotide reductase in healthy cells, in addition with antiproliferation by DNA damage. Unfortunately, doxorubicin is not a targeted therapy for cancer as it affects the growth of many other types of cells in the body. This results in depression of immune system due to

reduction in immune cell numbers paving the path of microbial infection, fatigue, and decreased healing time. The greatest risk factor of doxorubicin treatment is cardiotoxicity as measured by increased heart mass or enlargement of the heart (31,32).

Figure 2

Structure of doxorubicin (33)

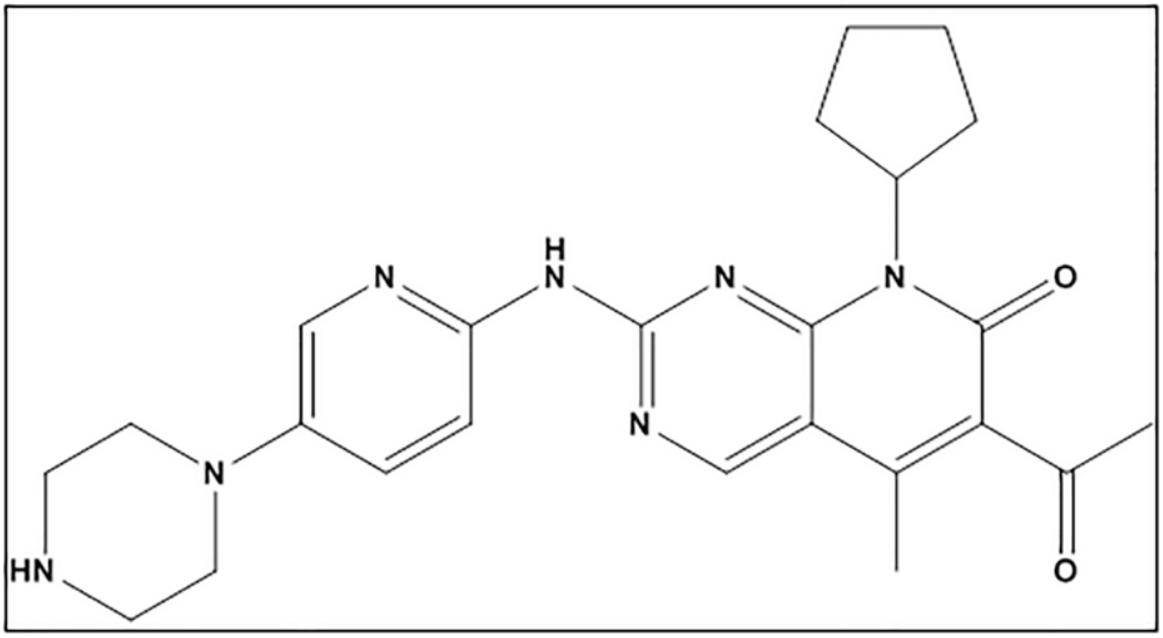


Palbociclib is an FDA approved small-molecule inhibitor of CDK4 and CDK6 cyclin dependent kinases in the cell cycle. It is indicated for the treatment of hormone receptor positive but HER2- breast cancer. CDK4/6 cyclin dependent kinases are activated by binding D type cyclins and promoting the progress of cell cycle G1-S phase. Cyclins of class D which are D1, D2 and D3 are the regulators of the cyclin dependent kinases CDK4/6 and together they form active complexes. CCND1 gene or cyclin D1 is the transcriptional target of the estrogen receptor and overexpression of D type cyclins is one the breast cancer growth pathways. Multiple oncogenic signals promote expression of cyclin D1 to form a complex with either CDK4 or CDK6 to drive cell proliferation. Selective inhibition of CDK4/6 by

palbociclib results in the loss of phosphorylation of the tumor suppressor Rb protein which retains an inhibitory effect on the E2F transcription factor for blocking progression from G1-S phase. Palbociclib works synergistically with endocrine therapies such as letrozole and fulvestrant. Palbociclib is given at a dose of 25-150 mg/day in a 3-week dose schedule to breast cancer patients. Yet, there is no strong evidence that supports the use of palbociclib in TNBC. However, one preclinical study reported that the luminal androgen receptor subgroup of triple-negative breast cancer was highly sensitive to CDK4/6 inhibition. As well, there was a study in 2017 (NCT02605486) on androgen receptor positive TNBC showing effective treatment with the combination of palbociclib and the anti-androgen bicalutamide (36, 37, 38).

Figure 3

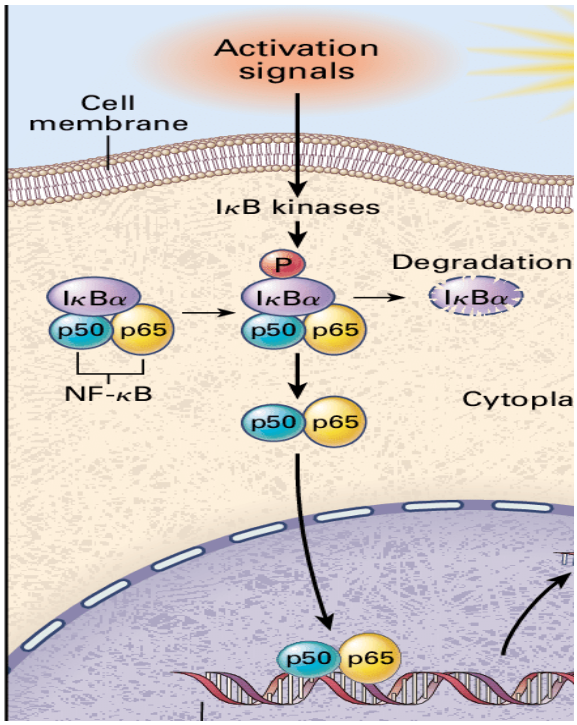
Chemical structure of palbociclib (37)



Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is a family of transcription factors regulating a large array of genes which are involved in multiple processes of the immune and inflammatory system (38). It is comprised of five structurally related members which include NFκB1 (also named p50), NFκB2 (also named p52), RelA (also named p65), RelB and c-Rel. All NFκB members are responsible for mediating transcription of target genes by binding to specific DNA element (39). The primary mechanism of NFκB activation is the degradation of inhibitory IkB proteins through site specific phosphorylation and activation of the NFκB complex (40). Previously, our lab showed that RRM2 is associated with increased NFκB activity and that didox is a potent inhibitor of NFκB activation (41).

Figure 4

NFκB pathway (38)



In our first study, we aim to show that the ribonucleotide reductase inhibitor didox, which functions as a powerful free radical scavenger and iron chelator, can alleviate the non-selective cytotoxic effects of doxorubicin on non-cancerous tissue, including cardiotoxicity and can potentiate doxorubicin's efficacy against malignant cells. Didox in combination with doxorubicin can effectively treat TNBC by enhancing tumor inhibition and minimizing doxorubicin-induced heart damage. We hypothesize that didox alone or in combination with doxorubicin can downregulate cell growth, apoptotic and NFκB signaling protein levels in TNBC. We also hypothesize that mutant p53 is a valid therapeutic target in TNBC as it can downregulate mutant p53 levels and thus enhance tumor inhibition.

In the second study, we hypothesize that the combination of didox with palbociclib is a potential strategy to target ER positive and ER negative/triple negative breast cancer. Previously, we have shown that didox can significantly halt malignant breast cancer cell division in combination with doxorubicin by targeting RRM2, mutant p53 and NFκB regulatory proteins. Here we also aim to confirm, the IC_{50} of palbociclib in palbociclib sensitive as well as resistant ER positive MCF7 and ER negative MDA-MB-468 breast cancer cells along with examining the effect of didox on cell cycle, growth signaling, apoptotic and NFκB signaling proteins alone or in combination with palbociclib. Lastly, we aim to measure the effects of didox and palbociclib treatment on the cell cycle profile of MCF7 and MDA-MB-468 parental and palbociclib resistant breast cancer cells.

References

1. Wang Y, Minden A. Current Molecular Combination Therapies Used for the Treatment of Breast Cancer. IJMS. 2022;23:11046.
2. Loibl S, Poortmans P, Morrow M, Denkert C, Curigliano G. Breast cancer. The Lancet. 2021;397:1750–69.
3. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406:747–52.
4. Gazinska P, Grigoriadis A, Brown JP, Millis RR, Mera A, Gillett CE, et al. Comparison of basal-like triple-negative breast cancer defined by morphology, immunohistochemistry and transcriptional profiles. Modern Pathology. 2013;26:955–66.
5. Yersal O. Biological subtypes of breast cancer: Prognostic and therapeutic implications. WJCO. 2014;5:412.
6. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490:61–70.
7. Mathias C, Muzzi JCD, Antunes BB, Gradia DF, Castro MAA, Carvalho de Oliveira J. Unraveling Immune-Related lncRNAs in Breast Cancer Molecular Subtypes. Front Oncol. 2021;11:692170.
8. Russnes HG, Lingjærde OC, Børresen-Dale A-L, Caldas C. Breast Cancer Molecular Stratification. The American Journal of Pathology. 2017;187:2152–62.
9. Charan M, Verma AK, Hussain S, Misri S, Mishra S, Majumder S, et al. Molecular and Cellular Factors Associated with Racial Disparity in Breast Cancer. IJMS. 2020;21:5936.

10. Garrido-Castro AC, Lin NU, Polyak K. Insights into Molecular Classifications of Triple-Negative Breast Cancer: Improving Patient Selection for Treatment. Cancer Discovery. 2019;9:176–98.
11. Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, et al. Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. Modern Pathology. 2011;24:157–67.
12. Rakha EA, Green AR. Molecular classification of breast cancer: what the pathologist needs to know. Pathology. 2017;49:111–9.
13. American Cancer Society. Key Statistics for breast cancer [Internet]. Breast Cancer. 2022. Available from: https://www.cancer.org/cancer/breast-cancer/about/how-common-is-breast-cancer.html
14. National Beast Cancer Foundation. Breast cancer facts [Internet]. Breast cancer facts. 2022. Available from: https://www.cancer.org/cancer/breast-cancer/about/how-common-is-breast-cancer.html
15. Sun Y-S, Zhao Z, Yang Z-N, Xu F, Lu H-J, Zhu Z-Y, et al. Risk Factors and Preventions of Breast Cancer. Int J Biol Sci. 2017;13:1387–97.
16. Gandhi AK, Kumar P, Bhandari M, Devnani B, Rath GK. Burden of preventable cancers in India: Time to strike the cancer epidemic. Journal of the Egyptian National Cancer Institute. 2017;29:11–8.
17. Gold EB. The Timing of the Age at Which Natural Menopause Occurs. Obstetrics and Gynecology Clinics of North America. 2011;38:425–40.

18. Kashyap D, Pal D, Sharma R, Garg VK, Goel N, Koundal D, et al. Global Increase in Breast Cancer Incidence: Risk Factors and Preventive Measures. Teekaraman Y, editor. BioMed Research International. 2022;2022:1–16.
19. Li G, Zhang J, Jin K, He K, Zheng Y, Xu X, et al. Estrogen receptor-α36 is involved in development of acquired tamoxifen resistance via regulating the growth status switch in breast cancer cells. Molecular Oncology. 2013;7:611–24.
20. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF. Identification, cloning, and expression of human estrogen receptor-α36, a novel variant of human estrogen receptor-α66. Biochemical and Biophysical Research Communications. 2005;336:1023–7.
21. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2022. CA A Cancer J Clinicians. 2022;72:7–33.
22. Cobleigh MA, Anderson SJ, Siziopikou KP, Arthur DW, Rabinovitch R, Julian TB, et al. Comparison of Radiation With or Without Concurrent Trastuzumab for HER2-Positive Ductal Carcinoma In Situ Resected by Lumpectomy: A Phase III Clinical Trial. JCO. 2021;39:2367–74.
23. Huff SE, Winter JM, Dealwis CG. Inhibitors of the Cancer Target Ribonucleotide Reductase, Past and Present. Biomolecules. 2022;12:815.
24. Elford HL. New Ribonucleotide Reductase Inhibitors with Antineoplastic Activity. Cancer Research. 1979;39:844–51.
25. Elford HL, Van't Riet B, Wampler GL, Lin AL, Elford RM. Regulation of ribonucleotide reductase in mammalian cells by chemotherapeutic agents. Advances in Enzyme Regulation. 1981;19:151–68.

26. Tihan T, Elford HL, Cory JG. Studies on the mechanisms of inhibition of L1210 cell growth by 3,4-dihydroxybenzohydroxamic acid and 3,4-dihydroxybenzamidoxime. Advances in Enzyme Regulation. 1991;31:71–83.

27. Szekeres T, Gharehbaghi K, Fritzer M, Woody M, Srivastava A, van't Riet B, et al. Biochemical and antitumor activity of trimidox, a new inhibitor of ribonucleotide reductase. Cancer Chemother Pharmacol. 1994;34:63–6.

28. Carmichael J, Cantwell BM, Mannix KA, Veale D, Elford HL, Blackie R, et al. A phase I and pharmacokinetic study of didox administered by 36 hour infusion. The Cancer Research Campaign Phase I/II Clinical Trials Committee. Br J Cancer. 1990;61:447–50.

29. on behalf of the Cancer Research Campaign Phase I/IT Clinical Trials Committee, Rubens R, Kaye S, Soukop M, Williams C, Brampton M, et al. Phase II trial of didox in advanced breast cancer. Br J Cancer. 1991;64:1187–8.

30. Bhave S, Elford H, McVoy MA. Ribonucleotide reductase inhibitors hydroxyurea, didox, and trimidox inhibit human cytomegalovirus replication in vitro and synergize with ganciclovir. Antiviral Research. 2013;100:151–8.

31. Tacar O, Sriamornsak P, Dass CR. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. Journal of Pharmacy and Pharmacology. 2012;65:157–70.

32. Ozcan M, Guo Z, Valenzuela Ripoll C, Diab A, Picataggi A, Rawnsley D, et al. Sustained alternate-day fasting potentiates doxorubicin cardiotoxicity. Cell Metabolism. 2023;S1550413123000426.

33. Mitry MA, Edwards JG. Doxorubicin induced heart failure: Phenotype and molecular mechanisms. IJC Heart & Vasculature. 2016;10:17–24.

34. Toogood PL, Harvey PJ, Repine JT, Sheehan DJ, VanderWel SN, Zhou H, et al. Discovery of a Potent and Selective Inhibitor of Cyclin-Dependent Kinase 4/6. J Med Chem. 2005;48:2388–406.

35. Turner NC, Ro J, André F, Loi S, Verma S, Iwata H, et al. Palbociclib in Hormone-Receptor–Positive Advanced Breast Cancer. N Engl J Med. 2015;373:209–19.

36. Kwapisz D. Cyclin-dependent kinase 4/6 inhibitors in breast cancer: palbociclib, ribociclib, and abemaciclib. Breast Cancer Res Treat. 2017;166:41–54.

37. Magdy G, Shaldam MA, Belal F, Elmansi H. Multi-spectroscopic, thermodynamic, and molecular docking/dynamic approaches for characterization of the binding interaction between calf thymus DNA and palbociclib. Sci Rep. 2022;12:14723.

38. Sun S-C, Chang J-H, Jin J. Regulation of nuclear factor-κB in autoimmunity. Trends in Immunology. 2013;34:282–9.

39. Oeckinghaus A, Ghosh S. The NF- B Family of Transcription Factors and Its Regulation. Cold Spring Harbor Perspectives in Biology. 2009;1:a000034–a000034.

40. Liu T, Zhang L, Joo D, Sun S-C. NF-κB signaling in inflammation. Sig Transduct Target Ther. 2017;2:17023.

41. Shah KN, Wilson EA, Malla R, Elford HL, Faridi JS. Targeting Ribonucleotide Reductase M2 and NF-κB Activation with Didox to Circumvent Tamoxifen Resistance in Breast Cancer. Molecular Cancer Therapeutics. 2015;14:2411–21.

CHAPTER 2: MOLECULAR TARGETING OF RRM2, NFκB, AND MUTANT TP53 FOR THE TREATMENT OF TRIPLE-NEGATIVE BREAST CANCER

2.1 Introduction

Falling under the basal-like classification, triple-negative breast cancer (TNBC) is defined as lacking three specific receptor types: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2). Although the HER2+ and HR+ cancer types may be effectively treated with targeted anti-HER2 e.g. trastuzumab or hormonal treatments, the basal-like and TNBC groups are much more challenging to treat due to their general lack of essential receptors for drug targeting (1).

Although definitive targets for TNBC remain elusive, there are certain immunotherapies or targeted therapies that may improve patient survival. Poly (ADP-ribose) polymerase (PARP), programmed cell death protein 1 (PD-1) and its ligand (PD-L1), receptor tyrosine kinase targets (such as VEGF, EGFR, FGF/FGFR) which function by inhibiting angiogenesis, and MEK and AKT cell growth pathways are among the most common current targets for the treatment of TNBC (2–6). However, some of these treatment modalities encompass major drawbacks and toxicities with some patients failing to respond to treatment, whereas others have a response that is short lived with resistant growth subsequently occurring, and others seem promising, but trials end with no available published data. In addition, the cost of the therapeutics for these patients is particularly high despite only minor increases in overall patient survival rates (7–10). In this study, we focus on improving the toxicity of current chemotherapy as well as enhancing the standard of care for patients with TNBC.

Specific activity of ribonucleotide reductase (RR), which is an enzyme that catalyzes

the rate-limiting step in DNA synthesis converting ribonucleotides into deoxyribonucleotides, has been previously correlated with tumor growth rates (11). RR is present as a heterodimeric tetramer consisting of two subunits, RRM1 and either RRM2 or p53R2 (12). Overexpression of RRM2 has been linked to higher proliferation and invasiveness of malignant cells (7, 13). Previously, we have shown that RRM2 is upregulated in ER-negative as well as tamoxifen-resistant ER-positive breast cancers (14, 15). Others have also shown the upregulation of RRM2 in breast cancer, as well as suggesting it to be a possible prognostic indicator (8, 16–20).

The tumor suppressor protein p53 (TP53 or p53) prevents tumorigenesis by maintaining genome integrity and preventing the proliferation of cells with a damaged genome (21). In response to cellular stresses such as DNA damage, oncogene expression, or ribosome dysfunctions, p53 becomes post translationally modified, stabilized, and activated. Once activated, p53 triggers transcription of an important number of direct target genes mainly implicated in cell-cycle arrest (such as CDKN1A/ p21), DNA repair, apoptosis, and senescence as well as to enhance metabolic changes and antioxidant responses (22). However, mutated p53 may actually contribute to tumor progression by a loss of tumor suppression as well as a gain of oncogenic activity (23). Somatic mutations in the TP53 gene occur in almost every type of cancer at rates up to 50% and are more frequent in advanced stage or in cancer subtypes with aggressive behavior (23). In particular, the TP53 gene was found to be mutated in approximately 80% of TNBC, and high levels of p53 in TNBC have been associated with poor prognosis (24). The synthetic antioxidant and potent RR inhibitor didox (3,4- dihydroxybenzohydroxamic acid) was originally developed as an antineoplastic and antiproliferative agent to improve upon the activities of hydroxyurea (25). Didox (DDX)

is a bifunctional compound that possesses both iron-chelating and free-radical scavenging functions and has proven enhanced efficacy when used in combination with DNA-targeting agents (9, 25, 26). Specifically, the use of DDX has been shown to display synergism when used in combination with the DNA agent doxorubicin (DOXO) by inactivating nuclear transcription factor NFκB, increasing intercellular DOXO concentrations, and facilitating apoptosis (27). In addition, DDX displays a favorable side effect profile when used at therapeutic concentrations (28).

We hypothesize that through the inhibition of RRM2, mutant p53, and the suppression of the NFκB pathway, DDX will work synergistically with DOXO in order to halt malignant TNBC cell division *in vivo*. We also hypothesize that this combination therapy has the potential to reduce anthracycline-associated cardiomyopathy as indicated by *in vivo* increased heart mass and left ventricle diameter. Measurement of troponin T levels which is indicative of heart muscle damage will confirm cardiac protective properties of DDX. In addition, we aim to show that DDX therapy is effective in targeting or altering expression of proteins in NFκB (IKKα, IKKβ, pIkBα, IkBα, p52, P-p65, p65, p100, p105, RelB, c-Rel), growth (pAKT, AKT, RRM2), apoptotic (BclXL, Bcl2, caspase 3, PARP, cleaved-PARP, p21, Pp53S392, Pp53 S15, p53) pathways and DNA damage (pH2AX and H2AX). Our data will present a novel and promising approach for the treatment of TNBC

2.2 Materials and Methods

2.2.1 Treatments

DDX was synthesized and kindly provided by Dr. Howard L. Elford, Molecules for Health (25). DOXO hydrochloride for cell experiments was obtained through VWR. DOXO hydrochloride (2 mg/mL) for *in vivo* experiments was obtained from APP Pharmaceuticals,

LLC. All the compounds were dissolved in 0.9% sterile saline solution, filtered through a 0.45-mm syringe filter, and stored at 4^0C in the dark for a maximum of 1 week.

2.2.2. Cell culture and treatment doses

MDA-MB-468, MDA-MB-231, BT20, MCF7, and ZR751 cells were routinely purchased from the ATCC every 6 months. Cells were maintained in advanced DMEM/F12 (Fisher) supplemented with 5% FBS, 1% L-glutamine, 1% streptomycin, and penicillin. Cells were treated with 600 mmol/L DDX, 100 nmol/L DOXO, or a combination of 600 mmol/L DDX and 100 nmol/L DOXO in phenol-red–free, serum-free, DMEM/F12 for 24 hours and compared against a control sample consisting of no treatment (NT). In the dose-dependent study, DDX concentrations between 30 and 900 mmol/L were used.

2.2.3 Western blot analysis

Cells and tumor samples were disrupted in RIPA buffer (Sigma), and lysates were clarified by centrifugation for 15 minutes at 15,000 X g. After the protein concentration was determined, an equal amount of total protein for each sample was loaded for Western blotting. RRM2 (Sigma), pH2AX, BclXL, Bcl2, caspase 3, PARP, cleaved-PARP, p21, Pp53S392, Pp53 S15, p53, IKKα, IKKβ, pIkBα, IkBα, p52, P-p65, p65, p100, p105, RelB, C-Rel, AKT, pAKT, GAPDH (Cell Signaling Technology), apoptosis sampler kit (Cell Signaling Technology), NFκB family members sampler kit (Cell Signaling Technology), NFκB pathway sampler kit (Cell Signaling Technology), and pH2AX (Millipore) were detected and visualized once incubated with secondary donkey anti-rabbit or goat anti-mouse IgG antibody conjugated with IR Dye 800CW or 680RD (LI-COR Biosciences) for 1 hours at room temperature. Membranes were subsequently washed and visualized using a LI-COR Odyssey Imaging System.

2.2.4 Xenograft studies

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of the Pacific. A total of three experiments were performed: MDA-MB-468 was injected subcutaneously into the flank of nude female mice (27–33 tumors/ group/study). As indicated, mice received vehicle (intraperitoneal, sterile water), DOXO (intravenous, 2–10 mg/kg weekly), DDX (intraperitoneal, 425 mg/kg daily), or a combination of both. For the DDX injections, solutions were made daily and injected fresh. Tumor volumes were measured using the ellipsoid formula of [4/3p(r1)2(r2)], where r1 < r2. Body weights were taken weekly with no changes in body weight observed in the DDX group. Tumors were collected and weighed at study termination, snap-frozen in liquid nitrogen, and stored at -80^{0}C for Western analysis. Whole hearts were also harvested at the end of the study, their mass measured, and either snap-frozen for Western analysis. Blood samples were also collected postmortem and centrifuged (14,000 rpm X 10 minutes, 4^{0}C) in order to obtain individual serum samples.

2.2.5 Troponin T studies

Serum samples were assayed for troponin T in duplicate using an ELISA (Enzyme–Test cTnT). Troponin T assay was from LSBIO and performed by the National Mouse Metabolic Phenotyping Center at the University of California, Davis. Data can be found at https://www. mmpc.org/shared/phenotype/showAssay.aspx?id1⁄41004.

2.2.6 Statistical analysis

Data was analyzed using unpaired two sample t-test of GraphPad Prism software. Results are presented as mean ± SEM. p-value < 0.05 was considered statistically significant.

2.3 Results

2.3.1 DDX inhibits NFκB protein expression in a dose- dependent manner

DDX has been shown to display synergism when used in combination with DOXO by downregulating the activation of the nuclear transcription factor NFκB, we wanted to determine the concentrations of DDX that would decrease NFκB–related proteins in TNBC cells. Therefore, DDX concentrations ranging from 30 to 900 mmol/L were used to treat MDA-MB-468 cells for 24 hours. DDX reduces the expression of RRM2 and the NFκB proteins p52 and p100 as well as the related proteins IKKβ and RelB. Interestingly, DDX also reduced the total p53 and S15 phosphorylated p53. Caspase 3 was also downregulated as DDX exposure increased (Fig. 5A).

MDA-MB-468 cells were exposed to varying treatment times of 600 mmol/L DDX (15 minutes–24 hours) and compared with control (24 hours of NT). Treatment with DDX reduced the expression of p53 beginning as early as 6 hours and more significantly at the 12- and 24-hour time points, compared with vehicle control. Total IkBα remains unchanged as didox-timed exposure was increased. Total H2AX expression progressively increased through the 24-hour DDX period, and phosphorylated H2AX only expressed a band at 24 hours of DDX treatment (Fig. 5C).

2.3.2 DDX downregulates cell growth signaling pathways in vitro

To evaluate the effect of DDX and DOXO combination therapy on the IKK (IKKα, IKKβ, pIkBα, IkBα), NFκB (p52, P-p65, p65, p100, p105, RelB, C-Rel), growth receptor (AKT, pAKT) signaling, and apoptosis (BclXL, Bcl2, caspase 3, PARP, cleaved-PARP, p21, Pp53S392, Pp53 S15, p53) pathways, MDA-MB-468 cells were treated with vehicle (NT), 600 mmol/L DDX, 100 nmol/L DOXO, and 600 mmol/L DDX and 100 nmol/L DOXO for 24

hours. IKK signaling proteins were all downregulated with DDX treatment in the DDX alone as well as DDX & DOXO groups (Fig. 6). Downregulation in the NFκB p52, p65, p100, p105, RelB, and C-Rel proteins was observed in the DDX and DDX & DOXO groups. Similarly, both total AKT and p53 were downregulated in the DDX and DDX & DOXO groups compared with NT and DOXO alone. Phosphorylation of AKT and total p21 expression remained consistent across all groups, whereas phosphorylated p53 (both S392 and S15) were downregulated in both the DDX and DDX & DOXO groups (Fig. 6).

2.3.3 Administration of daily DDX in combination with DOXO reduces tumor growth and ameliorates DOXO-induced cardiotoxicity in vivo

Nude mice were injected with TNBC MDA-MB-468 cells in order to determine the effects of the DDX/DOXO combination *in vivo*. Tumors were allowed to reach an average size of 64 mm3 before treatments began (day 0). In the vehicle (NT) treatment group, mice tumor volumes reached an average tumor volume of 217 mm^3 at the 31-treatment day mark, whereas DOXO-treated mice saw an average 3 tumor volume of only 68 mm. Animals treated with the DDX/DOXO combination were significantly smaller tumors that had an average tumor volume of 16 mm^3 (Fig. 7A). All animals survived until takedown at posttreatment day 31. Tumors were collected, weighed, and snap-frozen at study end. Tumors of mice that had been treated with DDX and DOXO displayed significantly smaller masses than their DOXO-treated counterparts ($P < 0.05$, Fig. 7B).

To evaluate animals for DOXO-induced cardiac damage, serum from NT, DDX, and DDX & DOXO treated animals was collected and analyzed for troponin T. Increased levels of troponin T were observed in the DOXO group as compared with that of NT and DDX & DOXO-treated animals. There were no statistically significant differences in troponin T

levels between the NT and DDX & DOXO groups ($P > 0.05$, Fig. 7C). At the end of the study, the hearts of all mice were collected, weighed, and sectioned. DOXO-treated hearts displayed a significant increase in heart mass when compared with all other treatment groups ($P < 0.05$, Fig. 7D). Left ventricular thickness also appeared enlarged in the DOXO group compared with other treatment groups (Fig. 7E). There were no significant differences between the NT and DDX & DOXO treated heart masses or left ventricle diameters ($P > 0.05$).

2.3.4 DDX reduces mutant p53 expression but not WT p53

Examination of signaling pathways revealed that total and phosphorylated p53 protein levels are decreased in MDA-MB-468 cells after DDX treatment (Figs. 5A and B, 6). To determine if this is an isolated cell type effect, a panel of breast cancer cell lines with WT p53 [MCF7, A3B5 (MCF7-overexpressing AKT), and ZR-75-1] and mutant p53 (BT20, MDA-MB-231, and MDA-MB- 468) were analyzed for expression level of total and phosphorylated p53. DDX treatment resulted in downregulation of total p53 (as well as total mutant p53) in all three TNBC cell lines with mutant p53 (Fig. 8, bottom). The effect of DDX on decreasing phosphorylated p53 was more in MDA-MB-468 and BT20 cells as compared with MDA-MB-231 cells. In contrast, in breast cancer cell lines with WT p53, total p53 remained unchanged after DDX treatment. Yet, phosphorylated p53 was reduced with DDX treatment in breast cancer cell lines with WT p53 (Fig. 8, top).

Figure 5

Didox inhibits NFκB protein expression in a dose-dependent manner. Protein expression in MDA-MB-468 cells decreases as DDX concentration increases from 30 to 900 mmol/L (A). Western blot analysis of cellular and apoptotic proteins of MDA-MB-468 cells treated with vehicle (NT) and 600 mmol/L DDX (varied treatment times from 15 minutes to 24 hours; B). Here, n=3.

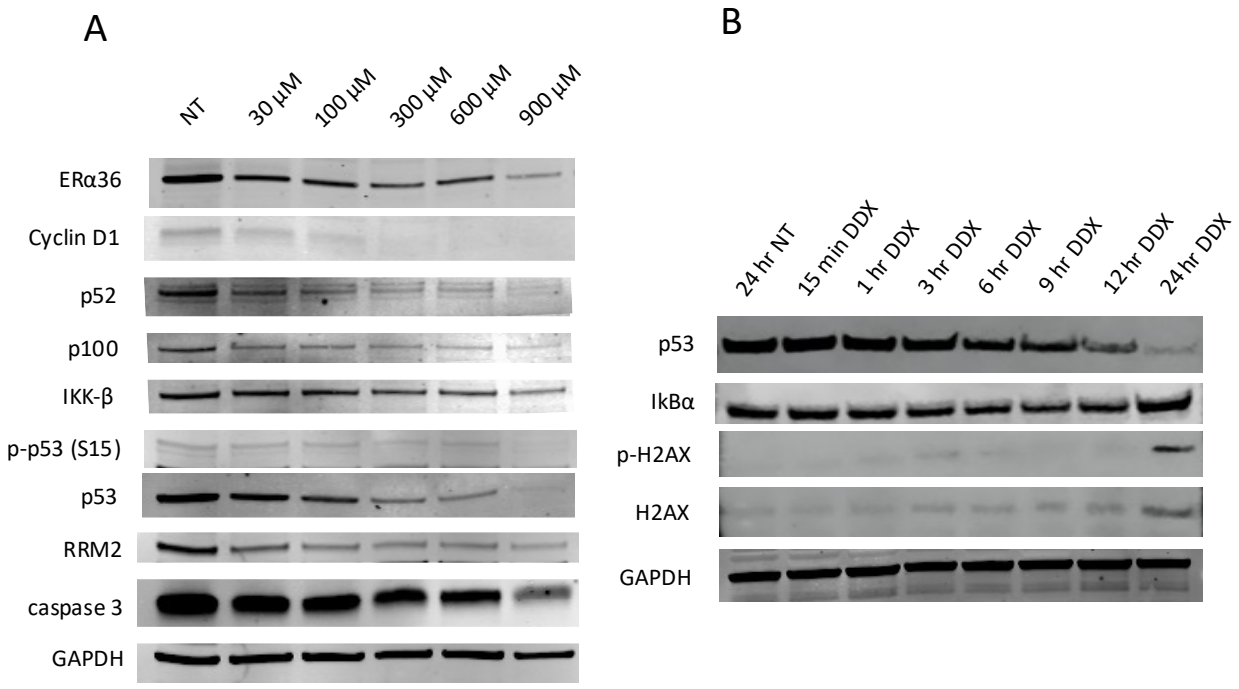


Figure 6

DDX decreases protein expression through the inhibition of NFκB in vitro. Western blot analysis of cellular and apoptotic proteins of MDA-MB-468 cells treated with vehicle (NT), 600 mmol/L DDX, 100 nmol/L DOXO, and combination of 600 mmol/L DDX & 100 nmol/L DOXO, n=3.

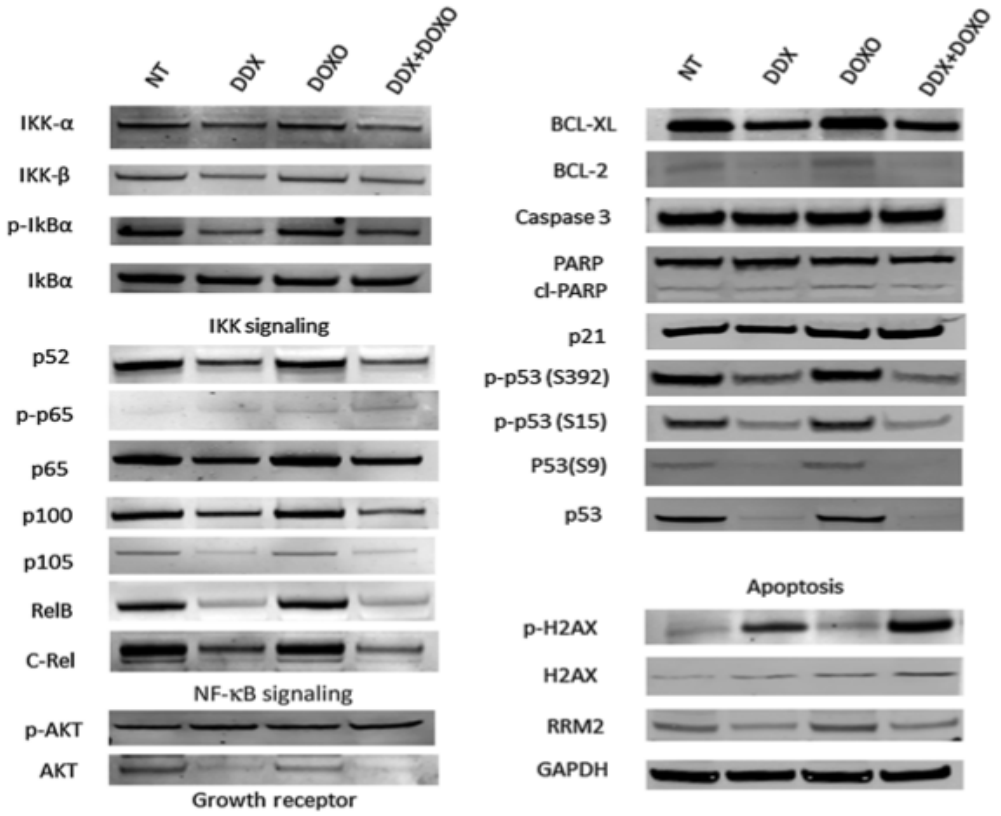
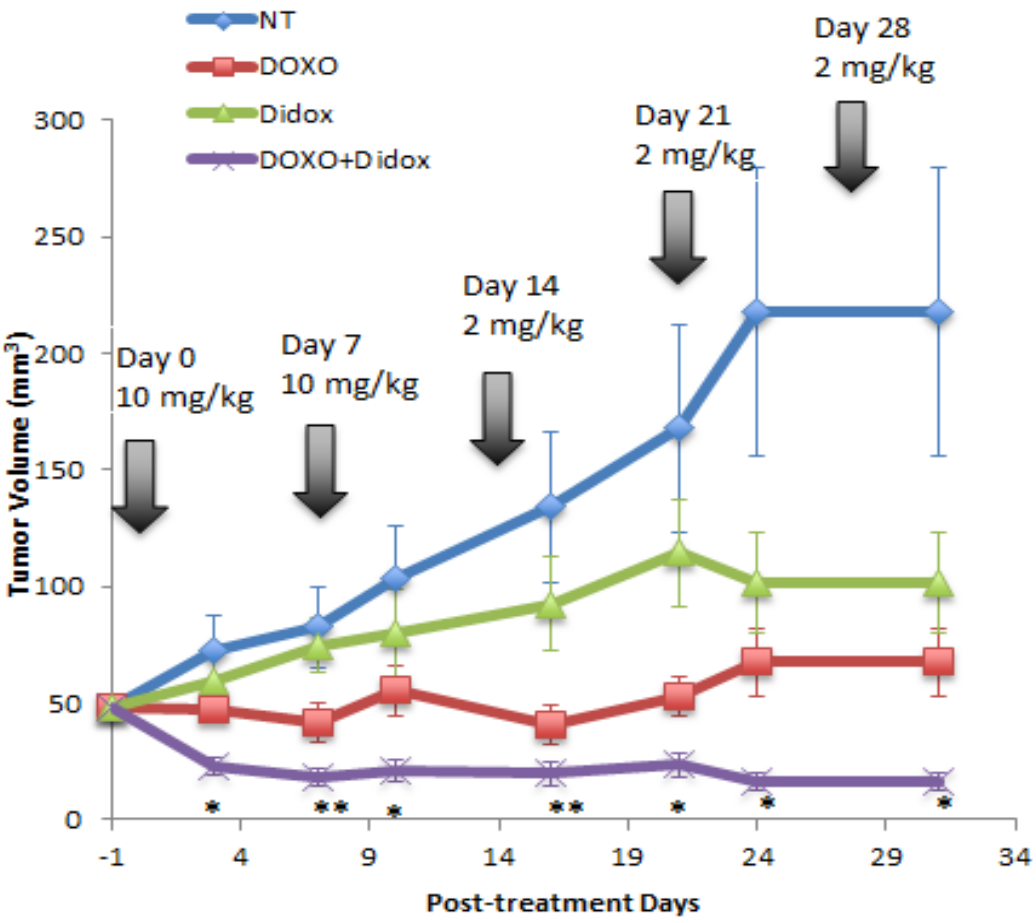


Figure 7

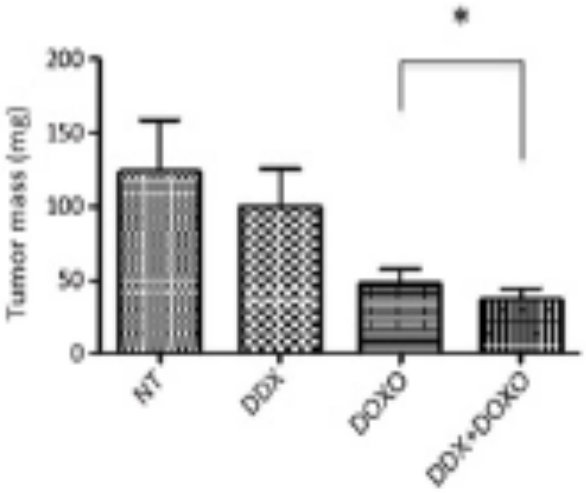
Addition of DDX reduces TNBC tumor growth and ameliorates DOXO-induced cardiotoxicity. Mice bearing MDA-MB- 468 tumors displayed reduced tumor growth with DDX and DOXO treatment when compared with doxorubicin alone (DOXO), didox alone (DDX), or NT. The experiment was performed three times independently and representative experiment shown (A). All animals survived until takedown at posttreatment day 31. Tumor weights in the DDX & DOXO group are significantly reduced when compared with the DOXO group, *, $P < 0.05$ by unpaired two-sample t test (B). Troponin T levels are significantly higher in the DOXO group, whereas the DOXO & DDX group displays significantly reduced troponin T levels, *, $P < 0.05$ by unpaired two-sample t test (C). Heart masses are reduced in the DOXO & DDX

group when compared with the DOXO group, *, P < 0.05 by unpaired two- tailed t test (D). Representative whole heart and left ventricular (LV) cross- sections displaying enlarged DOXO- treated hearts versus DDX & DOXO- treated hearts (E). Results are in averages ± SEM, n=3.

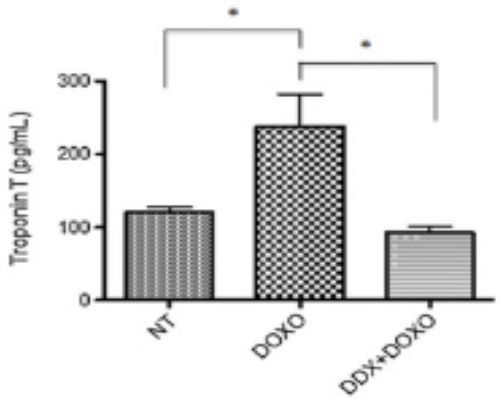
A



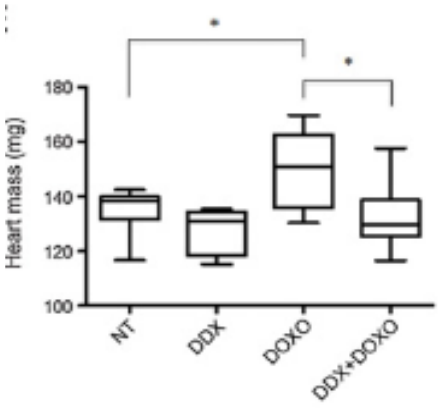
B


Tumor mass (mg)
200
150
100
50
0
*
NT
DDX
DOXO
DDX+DOXO

C


Troponin T (pg/mL)
300
200
100
0
*
*
NT
DOXO
DDX+DOXO

D


Heart mass (mg)
180
160
140
120
100
*
*
NT
DDX
DOXO
DDX+DOXO

E

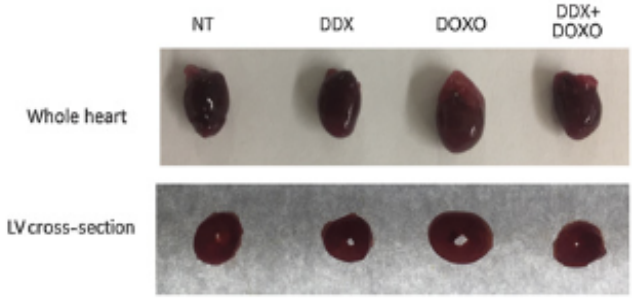
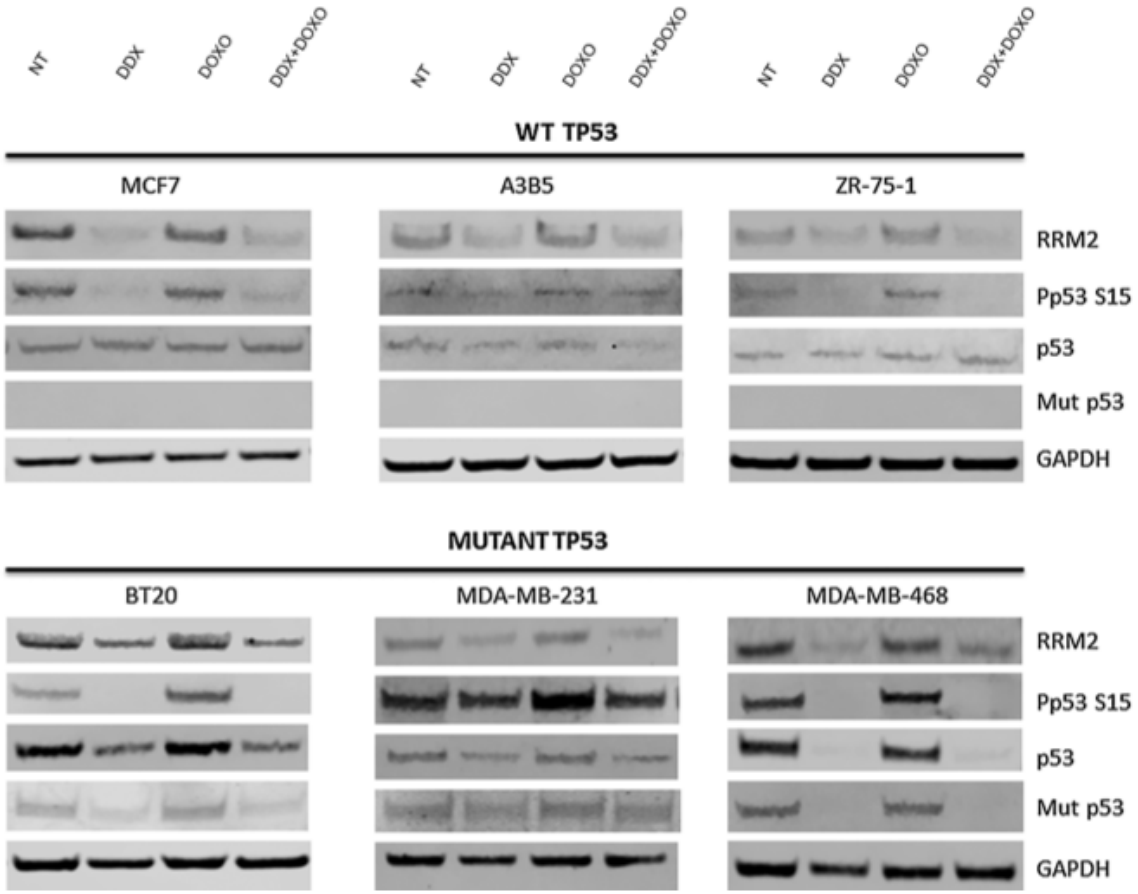

NT
DDX
DOXO
DDX+
DOXO
Whole heart
LV cross-section

Figure 8

DDX suppresses expression of mutant p53 but not WT p53 proteins. Western blot analysis of RRM2 and p53 proteins of cells which possess WT p53 such as MCF7, A3B5, and ZR-75-1 (top) and mutant p53 such as BT20, MDA-MB-231, and MDA-MB-468 (bottom). Cells were treated with vehicle (NT), 600 mmol/L DDX, 100 nmol/L DOXO, and combination of 600 mmol/L DDX & 100 nmol/L DOXO for 24 hours. All samples were analyzed in triplicate, n=3.



2.4 Discussion

Effective targets for this particularly virulent TNBC subtype are largely unknown. Here, we have demonstrated that coupling an RRM2 inhibitor with traditional anthracycline therapy is effective in the inhibition of TNBC tumors. Several studies have linked RRM2 overexpression in breast cancer to increased cell proliferation and invasiveness as well as conferring chemoresistance (8, 16, 20). We have previously observed that RRM2 is upregulated in TNBC subtypes when compared with ER-positive breast cancers, thus potentially providing a rationale for the targeting of RR.

NFκB and associated NFκB genes have been purported to be key regulators in TNBC (30–32). Responsible for cellular proliferation, survival, and apoptosis, NFκB is activated by nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IkB) kinases and tightly conserved through transcriptional regulation (33, 34). NFκB activation has been established as a poor prognostic predictor for patients with TNBC treated with adjuvant anthracycline chemotherapy in addition to playing a role in DOXO chemoresistance (30–32). Specifically, DOXO was shown to induce NFκB–dependent gene expression of migration, cell adhesion, and metastasis-related NFκB target genes (31). We have found that the RR inhibitor DDX has the potential to alter the expression of several NFκB proteins [NFκB1 (p105/p50) and NFκB2 (p100/p52)] that aid malignant cell survival and proliferation. Significantly decreased expression of RelB, NFκB1 (p105), NFκB2 (p100), and C-Rel was also present in the DDX and DOXO combination therapy tumors. Both IKKβ and total IkBα were similarly repressed in the combination therapy group (Fig. 6).

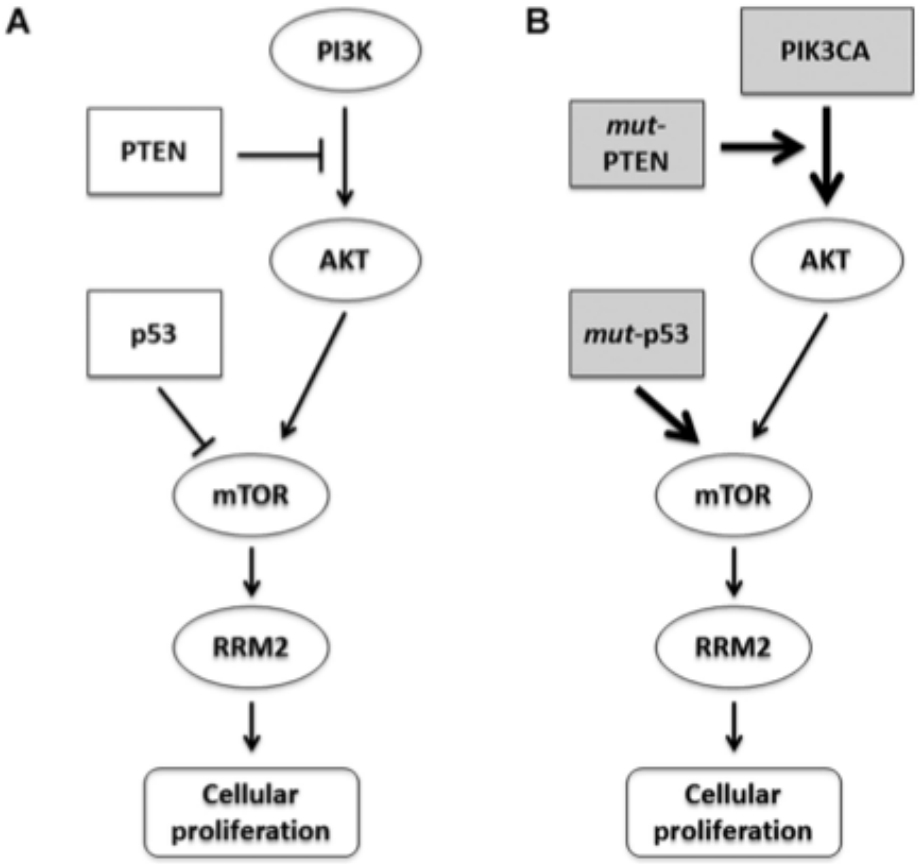
WT p53 is widely acknowledged as the “guardian of the genome” and is responsible for a variety of tumor-suppressive effects, including DNA repair, cell-cycle arrest, and

apoptosis (33–35). However, p53 is often found in a mutated form in a variety of breast cancers, including 80% of TNBC, and is linked to poor prognosis in these patients (36–39). In one study, mutated p53 mediated the DOXO induction of NFκB regulated gene transcription in TNBC (31). In addition, it has been previously noted that RRM2 levels are increased in the presence of mutant p53 (40). Therefore, the elimination of mutant p53 is of particular therapeutic interest in TNBC. Similarly, reduction in mutant p53 is observed in two additional mutant p53 cell lines, BT20 and MDA-MB-231 along with MDA-MB-468, when treated with DDX *in vitro* (Fig. 8). Interestingly, this effect is observed in a dose- and time-dependent manner *in vitro* in MDA-MB-468 cells (Fig. 5A and B). Total and phosphorylated mutant p53 displayed decreased levels of expression in the combination therapy group *in vivo*, indicating further suppression of the prosurvival pathway known to be present in MDA-MB-468 cells (Fig. 7A).

We hypothesize that MDA-MB-468 cells rely heavily on the dysregulation/activation of the PI3K pathway due to their mutated form of PTEN and p53, in order to increase cellular proliferation and oncogenesis. As given in Fig.9A, PTEN and p53 cooperate to control cellular proliferation in normal breast tissue. However, in the presence of PTEN and p53 mutations (as in the MDA-MB-468 cells; Fig. 9B), levels of AKT and RRM2 are increased, promoting cellular proliferation. Because DDX is able to inhibit both mutant p53 and RRM2 possibly by targeting NFκB activation, decreased cellular proliferation in the presence of DOXO was observed in the MDA-MB-468 cell line.

Figure 9

The interplay between PTEN, AKT, p53, and RRM2 and their roles in regulating cellular proliferation in normal breast tissue (A), aberrations in the PI3K/AKT/mTOR pathway led to downstream effects in TNBC (B). Additional components and pathways have been omitted for simplification.



Here we observed that the addition of daily DDX to DOXO therapy was superior to DOXO monotherapy in the suppression of MDA-MB-468 tumor growth *in vivo* (Fig. 7A). End tumor masses in the DDX & DOXO group were statistically smaller as compared with

DOXO monotherapy (Fig. 7B, $P < 0.05$). In a previous phase II clinical study, DDX alone was administered as an infusion once every 3 weeks to patients with advanced breast cancer, with no signs of efficacy (41). Because DDX has a half-life of less than 45 minutes, it is not surprising that the dosing interval used in the clinical study resulted in a lack of a response, as we observed favorable efficacy outcomes when administering DDX daily in xenograft models.

In addition to its chemotherapeutic synergism potential, DDX is well known for its favorable safety profile (9). Here, we have demonstrated that the addition of DDX ameliorates the detrimental off-target effects of DOXO toxic metabolites on the myocardium. Presumably, DOXO injures the heart by generating damaging free radicals through iron-catalyzed redox cycling (42). We observed that overall heart mass in the DOXO monotherapy group was statistically increased when compared with the NT and DDX monotherapy groups, whereas the addition of DDX to DOXO was sufficient to protect against this hypertrophy (Fig. 7E). Troponin T levels, which are a known indicator of cardiac damage, were elevated in the DOXO monotherapy group when compared with the NT and DDX and DOXO combination group (Fig. 7D). Total heart and left ventricle cross-sections displayed enlargement with DOXO monotherapy as compared with combination therapy (Fig. 7F). The observed cardioprotective benefits of DDX are likely due to its iron-chelating characteristic that may protect against iron perturbations likely due to the DOXO alcohol metabolite doxorubicinol as well as its free radical scavenging characteristic that may protect against the oxidant activity likely due to DOXO deoxyaglycone and DOXOl hydroxyaglycone (43). Specifically, DDX was shown to offer cardio protection against DOXO-mediated injury through its ability to scavenge free radicals (44).

In summary, our findings regarding therapeutically targeting RRM2, NFκB, and mutant p53 in TNBC complement the current breast cancer landscape. We have found evidence that increased levels of RRM2 and, as a consequence, greater NFκB activation may be a hallmark of TNBC tumors, giving rise to their aggressive and difficult- to-treat nature. Reduced RRM2 and NFκB pathway protein expression, as well as the overturn of the prosurvival mutant p53 using DDX, results in reduced tumor size. We have observed that the addition of DDX also reduces the cardiotoxicity associated with anthracycline use, as evidenced by reduced heart mass and troponin T levels. Ultimately, our data add to the rationale that inhibitors of RRM2, NFκB, and mutant p53 may be used to supplement traditional chemotherapies and offer improved efficacy and reduced toxicity.

One of the limitations of this study is that protein expression by western blotting was not quantified by normalization which would add significant difference in protein expression. In addition, cardiac function can be evaluated by echocardiography by using a Vevo 2100 high resolution imaging system. There are still many topics which needs to be explored in the future. Firstly, the effects of RRM2 on the NFκB activation and mechanism involved needs further investigation. RRM2 knockdown studies can be performed to evaluate the effect of DDX in absence of RRM2 in TNBC. Next, TNBC cell lines with RRM2 can be established to clarify whether RRM2 overexpression has a consistent impact on cell cycle. Finally, reactive oxygen species (ROS) measurement should be performed to investigate free radical scavenging properties of DDX in TNBC.

2.5 References

1. Jhan JR, Andrechek ER. Triple-negative breast cancer and the potential for targeted therapy. Pharmacogenomics 2017;18:1595–609.
2. Schmid P, Rugo HS, Adams S, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab plus nab-paclitaxel as first-line treatment for unresectable, locally advanced or metastatic triple-negative breast cancer (IMpassion130): updated efficacy results from a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 2020;21:44–59.
3. Robson ME, Tung N, Conte P, Im S-A, Senkus E, Xu B, et al. OlympiAD final overall survival and tolerability results: Olaparib versus chemotherapy treatment of physician's choice in patients with a germline BRCA mutation and HER2- negative metastatic breast cancer. Ann Oncol 2019;30:558–66.
4. Nakhjavani M, Hardingham JE, Palethorpe HM, Price TJ, Townsend AR. Druggable molecular targets for the treatment of triple-negative breast cancer. J Breast Cancer 2019;22:341–61.
5. García-Aranda M, Redondo M. Immunotherapy: a challenge of breast cancer treatment. Cancers 2019;11:1822.
6. Mehanna J, Haddad FGH, Eid R, Lambertini M, Kourie HR. Triple-negative breast cancer: current perspective on the evolving therapeutic landscape. Int J Womens Health 2019;11:431–7.
7. Duxbury MS, Whang EE. RRM2 induces NF-kappaB-dependent MMP-9 acti- vation and enhances cellular invasiveness. Biochem Biophys Res Commun 2007; 354:190–6.

8. Zhang H, Liu X, Warden CD, Huang Y, Loera S, Xue L, et al. Prognostic and therapeutic significance of ribonucleotide reductase small subunit M2 in estro- gen-negative breast cancers. BMC Cancer 2014;14:664.
9. Veale D, Carmichael J, Cantwell Bm, Elford Hl, Blackie R, Kerr Dj, et al. A phase 1 and pharmacokinetic study of didox: a ribonucleotide reductase inhibitor. Br J Cancer 1988;58:70–2.
10. Aly A, Shah R, Hill K, Botteman MF. Overall survival, costs and healthcare resource use by number of regimens received in elderly patients with newly diagnosed metastatic triple-negative breast cancer. Future Oncol 2019;15: 1007–20.
11. Elford HL, Freese M, Passamani E, Morris HP. Ribonucleotide reductase and cell proliferation. I. Variations of ribonucleotide reductase activity with tumor growth rate in a series of rat hepatomas. J Biol Chem 1970;245: 5228–33.
12. Tanaka H, Arakawa H, Yamaguchi T, Shiraishi K, Fukuda S, Matsui K, et al. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. Nature 2000;404:42–9.
13. Zhou B-S, Tsai P, Ker R, Tsai J, Ho R, Yu J, et al. Overexpression of transfected human ribonucleotide reductase M2 subunit in human cancer cells enhances their invasive potential. Clin Exp Metastasis 1998;16:43–9.
14. Shah KN, Mehta KR, Peterson D, Evangelista M, Livesey JC, Faridi JS. AKT-induced tamoxifen resistance is overturned by RRM2 inhibition. Mol Cancer Res 2014;12:394–407.
15. Shah KN, Wilson EA, Malla R, Elford HL, Faridi JS. Targeting ribonucleotide reductase M2 and NF-kappaB activation with didox to circumvent tamoxifen

resistance in breast cancer. Mol Cancer Ther 2015;14:2411–21.

16. Putluri N, Maity S, Kommagani R, Creighton CJ, Putluri V, Chen F, et al. Pathway-centric integrative analysis identifies RRM2 as a prognostic marker in breast cancer associated with poor survival and tamoxifen resistance. Neoplasia 2014;16:390–402.
17. Koleck TA, Conley YP. Identification and prioritization of candidate genes for symptom variability in breast cancer survivors based on disease characteristics at the cellular level. Breast Cancer 2016;8:29–37.
18. Li J-P, Zhang X-M, Zhang Z, Zheng Li-H, Jindal S, Liu Y-J. Association of p53 expression with poor prognosis in patients with triple-negative breast invasive ductal carcinoma. Medicine 2019;98:e15449.
19. Gong MT, Ye SD, Lv WW, He K, Li WX. Comprehensive integrated analysis of gene expression datasets identifies key anti-cancer targets in different stages of breast cancer. Exp Ther Med 2018;16:802–10.
20. Chen W-X, Yang L-G, Xu L-Y, Cheng L, Qian Qi, Sun Li, et al. Bioinformatics analysis revealing prognostic significance of RRM2 gene in breast cancer. Biosci Rep 2019;39:BSR20182062.
21. Lane DP. Cancer. p53, guardian of the genome. Nature 1992;358:15–6.
22. Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. Oncogene 2005;24:2899–908.
23. Silwal-Pandit L, Langerød A, Børresen-Dale AL. TP53 mutations in breast and ovarian cancer. Cold Spring Harb Perspect Med 2017;7:a026252.
24. Qamar S, Khokhar M, Farooq S, Ashraf S, Humayon W, Rehman A. Association of p53 overexpression with hormone receptor status and triple-negative breast

carcinoma. J Coll Physicians Surg Pak 2019;29:164–7.

25. Elford HL, Wampler GL, van't Riet B. New ribonucleotide reductase inhibitors with antineoplastic activity. Cancer Res 1979;39:844–51.
26. Elford HL, Van't Riet B, Wampler GL, Lin AL, Elford RM. Regulation of ribonucleotide reductase in mammalian cells by chemotherapeutic agents. Adv Enzyme Regul 1980;19:151–68.
27. Khaleel SA, Al-Abd AM, Ali AA, Abdel-Naim AB. Didox and resveratrol sensitize colorectal cancer cells to doxorubicin via activating apoptosis and ameliorating P-glycoprotein activity. Sci Rep 2016;6:36855.
28. Carmichael J, Cantwell B, Mannix KA, Veale D, Elford HL, Blackie R, et al. A phase I and pharmacokinetic study of didox administered by 36 hour infusion. The cancer research campaign phase I/II clinical trials committee. Br J Cancer 1990;61:447–50.
29. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006;58:621–81.
30. Ossovskaya V, Wang Y, Budoff A, Xu Q, Lituev A, Potapova O, et al. Exploring molecular pathways of triple-negative breast cancer. Genes Cancer 2011;2:870–9.
31. Dalmases A, Gonzalez I, Menendez S, Arpí O, Corominas JM, Servitja S, et al. Deficiency in p53 is required for doxorubicin induced transcriptional activation of NFkB target genes in human breast cancer. Oncotarget 2014;5: 196–210.
32. Kim J-Y, Jung HH, Ahn S, Bae S, Lee SEK, Kim SW, et al. The relationship between nuclear factor (NF)-kB family gene expression and prognosis in triple- negative breast cancer (TNBC) patients receiving adjuvant doxorubicin treatment. Sci Rep

2016;6:31804.

33. Turner N, Moretti E, Siclari O, Migliaccio I, Santarpia L, D'Incalci M, et al. Targeting triple-negative breast cancer: is p53 the answer? Cancer Treat Rev 2013; 39:541–50.
34. Yadav BS, Chanana P, Jhamb S. Biomarkers in triple-negative breast cancer: a review. World J Clin Oncol 2015;6:252–63.
35. Horigome E, Fujieda M, Handa T, Katayama A, Ito M, Ichihara A, et al. Mutant TP53 modulates metastasis of triple negative breast cancer through adenosine A2b receptor signaling. Oncotarget 2018;9:34554–66.
36. Duffy MJ, Synnott NC, Crown J. Mutant p53 in breast cancer: potential as a therapeutic target and biomarker. Breast Cancer Res Treat 2018;170: 213–9.
37. Nik-Zainal S, Davies H, Staaf J, Ramakrishna M, Glodzik D, Zou X, et al. Landscape of somatic mutations in 560 breast cancer whole-genome sequences. Nature 2016;534:47–54.
38. Bae SY, Nam SJ, Jung Y, Lee SB, Park B-W, Lim W, et al. Differences in prognosis and efficacy of chemotherapy by p53 expression in triple-negative breast cancer. Breast Cancer Res Treat 2018;172:437–44.
39. Hashmi AA, Naz S, Hashmi SK, Hussain ZF, Irfan M, Khan EY, et al. Prognostic significance of p16 & p53 immunohistochemical expression in triple negative breast cancer. BMC Clin Pathol 2018;18:9.
40. Kollareddy M, Dimitrova E, Vallabhaneni KC, Chan A, Le T, Chauhan KM, et al. Regulation of nucleotide metabolism by mutant p53 contributes to its gain-of-function activities. Nat Commun 2015;6:7389.

41. Rubens RD, Kaye SB, Soukop M, Williams CJ, Brampton MH, Harris AL. Phase II trial of didox in advanced breast cancer. Cancer research campaign phase I/II clinical trials committee. Br J Cancer 1991;64:1187–8.

42. Al-Abd AM, Al-Abbasi FA, Asaad GF, Abdel-Naim AB. Didox potentiates the cytotoxic profile of doxorubicin and protects from its cardiotoxicity. Eur J Pharmacol 2013;718:361–9.

43. Licata S, Saponiero A, Mordente A, Minotti G. Doxorubicin metabolism and toxicity in human myocardium: role of cytoplasmic deglycosidation and car- bonyl reduction. Chem Res Toxicol 2000;13:414–20.

44. Elford HL, Cardounel AJ, Zweier J, Henry J, Sumpter R, Oakley O, et al. Didox, a unique ribonucleotide reductase inhibitor and free radical scavenger, can protect against doxorubicin caused cardiotoxicity with enhanced antitumor activity. Cancer Res 2006;66:502.

45. Gruosso, T., et al., Chronic oxidative stress promotes H2AX protein degradation and enhances chemosensitivity in breast cancer patients. EMBO Mol Med, 2016. 8(5): p. 527-49.

46. Komatsu, M., et al., Molecular features of triple negative breast cancer cells by genome-wide gene expression profiling analysis. Int J Oncol, 2013. 42(2): p. 478-506.

47. Maubant, S., et al., Transcriptome analysis of Wnt3a-treated triple-negative breast cancer cells. PLoS One, 2015. 10(4): p. e0122333.

48. Barrett, T., et al., NCBI GEO: archive for functional genomics data sets--update. Nucleic Acids Res, 2013. 41(Database issue): p. D991-5.

49. Győrffy, B., et al., An online survival analysis tool to rapidly assess the effect of

22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat, 2010. 123(3): p. 725-31.

CHAPTER 3: TARGETING RRM2 AND CYCLIN D1-CDK 4/6 AXIS FOR PALLBOCICLIB RESISTANT BREAST CANCER TREATMENT

3.1 Introduction

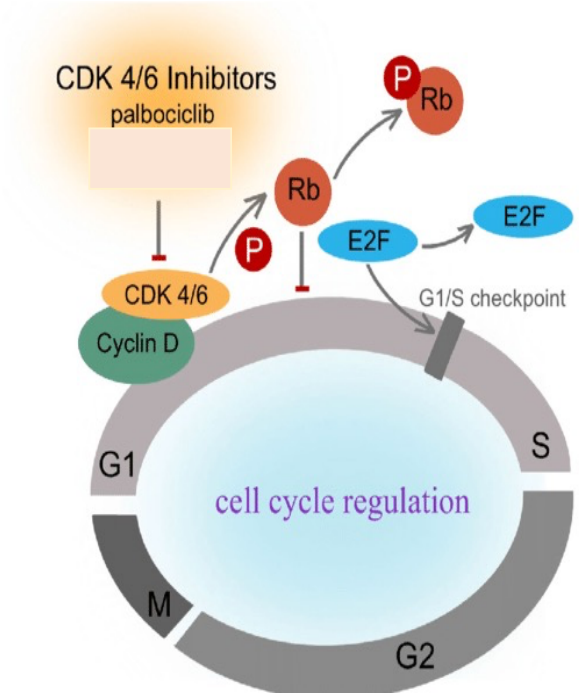
The cell cycle is a series of regulated events orchestrated by specific enzymes and proteins. Cyclins and cyclin dependent kinases (CDKs) are the key regulatory machinery known as cell cycle checkpoints that can speed, slow, or even halt the process. CDKs form a complex with cyclins during each phase of the cell cycle. During G1 phase, CDK4/6 bind with cyclin D1 to form an active kinase complex, which phosphorylates retinoblastoma protein 1 (RB1) to counteract its inhibitory effect on the transcription factor E2F causing G1-S phase shift (1, 2). Uncontrolled cell growth owing to an abnormal cell cycle is one of the hallmarks of cancer (3). In cancer cells, the CDK4/6-cyclin D1-RB1-E2F axis is regarded as the most dysregulated cell cycle pathway (1).

Endocrine treatment is an effective first-line therapy for targeting ER+, HER2- breast cancer. But success is limited by development of acquired resistance due to long-term therapy. Cyclin D and CDK4/6 complex mediated retinoblastoma (Rb) tumor suppressor protein phosphorylation and subsequent inactivation drives the cell cycle from G1 into S phase for DNA synthesis. This observation led to the development of the first CDK4/6 inhibitor palbociclib which induces cell cycle arrest at G1 phase in cancer cells (4). Palbociclib is a specific CDK4/6 inhibitor, which decreases the phosphorylation of RB and subsequently inhibits cancer cell growth (5). Additional efficacy was observed when given in combination with selective estrogen receptor degrader (SERD) fulvestrant or aromatase inhibitor (AI) letrozole (6). Unfortunately, despite the success rate of cell cycle checkpoint CDK4/6 inhibitors, 10% of breast cancer

patients develop intrinsic resistance while many later presents with acquired resistance after 24-28 months or less when used as first or second line therapy (7).

Figure 10

Cell cycle regulation (1)



Deregulation of cell signaling molecules and activation of several escape pathways could be the mechanism behind progression of resistance in breast cancer. This paves the pathway for designing novel therapeutic strategies to improve patient outcomes (8). Here we focus on circumventing palbociclib resistance by targeting estrogen receptor positive (ER+) and estrogen receptor negative (ER-) breast cancers with a unique ribonucleotide reductase subtype II (RRM2) enzyme inhibitor.

Tumor growth is dependent on the activity of ribonucleotide reductase (RR) which is the rate limiting enzyme to catalyze ribonucleotides to deoxyribonucleotides during DNA synthesis

(9). RR is a tetramer composed of two dissimilar subunits RRM1 and RRM2. Whereas RRM1 contains allosteric regulatory sites for maintenance and balancing of deoxyribonucleotide triphosphate pools, RRM2 contains a binuclear iron center and a tyrosyl free radical for the enzymatic conversion of ribonucleotides to deoxyribonucleotides. The cell cycle regulates mammalian RR where RRM2 subunit is made in the late G1 phase before DNA replication, and it disappears in late S or early G2 phase (10). Overexpression of RRM2 is associated with higher proliferation and invasiveness of malignant cancers (11). In our previous study, we have shown that RRM2 is upregulated in ER- as well as tamoxifen resistant ER+ breast cancers (12-14). Other studies have demonstrated RRM2 as a potential prognostic indicator in breast cancer treatment (15-20).

The free radical scavenger didox (3,4-dihydroxybenzohydroxamic acid) upon introduction to RRM2, chelates its iron (III) from the radical center, impeding conversion of ribonucleotides to deoxyribonucleotides (21). In addition to RR inhibition, didox also possess synthetic antioxidant properties. This drug was originally developed as an antineoplastic and antiproliferative agent improving the activities of hydroxyurea (21, 22). As well, it improves the efficacy of DNA-targeting agents when used in combination (14, 22, 23).

The NF-κB transcription factor functions as a nuclear factor which binds to the enhancer element of the immunoglobulin kappa light-chain of activated B cells (IkB) (24). Constitutive activation of NF-κB signaling can lead to various disorders as it regulates more than 500 genes responsible for proliferation, invasion, angiogenesis, metastasis, cellular transformation, and inflammation (25, 26). Upregulation of NF-κB in breast cancer primarily leads to cell proliferation, survival, metastasis and resistance to radiotherapy and chemotherapy (27, 28). Cell cycle progression and uncontrolled cell proliferation through upregulation of the expression of

the cell cycle proteins cyclin D1 and CDK with NF-κB activation has been reported in some studies (29, 30).

In this study, we hypothesize that through the inhibition of RRM2, cyclin D1 and the NFκB pathway as well as upregulation of pH2AX, a DNA damage signaling protein, DDX with PLB can significantly halt ER+ and ER- breast cancer growth along with their PLB resistant counterparts. We also want to delineate that this combination therapy has the potential to arrest the cell cycle at G1 in both ER+ and ER- breast cancer along with their PLB resistant counterparts. In addition, in ER- breast cancer cells with mutant p53, we aim to show that DDX therapy effectively targets and downregulates mutant p53 in a time dependent manner with no effect on total levels of wild type p53. Our data will present a new approach for the treatment of ER+ and ER- breast cancer and their PLB resistant counterparts.

3.2. Materials and Methods

3.2.1 Cell culture and drug treatment

MCF7 and MDA-MB-468 cells were purchased from ATCC every 6 months. Cells were maintained in advanced DMEM/F12 (Thermo Fisher Scientific) supplemented with 5% FBS, 1% L-glutamine and 1% streptomycin and penicillin. Didox (DDX) was synthesized and kindly provided by Dr. Howard L. Elford, Molecules for Health. Palbociclib (PLB) was purchased from Sigma Aldrich. Didox was dissolved in 0.9% sterile saline solution and Palbociclib was dissolved in 0.1M HCl. All compounds were filtered through a 0.2μM syringe filter and stored at -10^0C in the dark for a maximum of 1 week. Cells were treated with DDX, PLB, or a combination of DDX and PLB in phenol red free, serum free DMEM/F12 media (Thermo Fisher Scientific).

3.2.2 Establishment of the palbociclib resistant cell lines

Palbociclib drug resistant cells were developed by culturing in media mentioned above containing increasing doses of palbociclib 0.1 - 4 μmol/L. Cells were subcultured at almost 95% confluency with an increment of 25% drug concentration. The resistant cells were established after 6 months and maintained in 1 μmol/L palbociclib.

3.2.3 IC50 determination

IC_{50} values were normalized to those of their no treatment controls and analyzed in GraphPad Prism by non-linear regression to obtain the IC_{50} values.

3.2.4 Western blot analysis

Cells were lysed with the cell lysis buffer (Cell Signaling Technology) and lysates were clarified by centrifugation for 10 minutes at 14,000 rpm. After protein quantification with BCA assay, 50ug of each sample was resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were blocked with blocking buffer (LI-COR Biosciences) for 1 hour at room temperature, incubated with primary antibody (1:1000 dilution) overnight at 4^{o}C, followed by incubation with secondary goat anti-rabbit or goat anti-mouse IgG antibody (1:10000 dilution) conjugated with IR Dye 800CW or 680RD (LI-COR Biosciences) at room temperature for 60 minutes. Membranes were subsequently washed, visualized, and quantified using Li-COR Odyssey Fc and CLX imaging system and image studio software. The following antibodies were used: RRM2 (Sigma Aldrich), Cell Cycle Regulation Antibody Sampler Kit II, NFκB pathway sampler kit, NFκB Family Member Antibody Sampler Kit, Phospho-p53 Antibody Sampler Kit, Rb Antibody Sampler Kit, Akt Isoform Antibody Sampler Kit, Phospho-Akt Pathway Antibody Sampler Kit and GAPDH (Cell Signaling Technology).

3.2.5 Real time-quantitative polymeric chain reaction (RT-qPCR) analysis

Total RNA was isolated using RNeasy (QIA-GEN) according to the manufacturer's protocol. 1µg RNA was converted to cDNA using RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific). RT-qPCR reactions were performed using human predesigned CCND1 (1:20) and Tp53 (1:20) probe-based primers (Integrated DNA Technology). Fold change was determined using the comparative $2^{-\Delta\Delta Ct}$ method and RPL13A housekeeping gene (Integrated DNA Technology).

3.2.6 Cell Cycle Analysis

Cells were grown in 100 mm cell culture dishes and incubated with drugs for 24 hours. Cells were then harvested (1 X 10^6 cells) with trypsin, washed with HBSS, resuspended in 200 µl PBS, fixed in 1 ml ice cold 70% ethanol, and stored overnight at -20^{o}C. Afterwards, fixed cells were strained, centrifuged, and washed with 0.25 ml 1% PBS, resuspended with 200 µl cell cycle reagent (Luminex), and incubated in the dark for 30 mins at room temperature. Cell cycle phase analysis was done using the Guava Muse flow cytometer and cell analyzer software with cell cycle kit (MCH100106) from Luminex according to manufacturer's protocol.

3.2.7 Cellular proliferation assay

For cell proliferation studies, 1000 cells were plated in into each well of 96 well plates in phenol red free, serum free DMEM/F12 media supplemented with 2% charcoal stripped serum (CSS, Thermo Fisher Scientific) and incubated at 37^{o}C for 24 hours before treatment with DDX, PLB, or a combination of DDX and PLB in phenol red free, serum free DMEM/F12 media. Treatment media was replenished on days 2, 4 and 6. CellTiter Glo 2.0 solution (Promega) was added to each well according to the manufacturer's protocol and luminescence was measured.

3.2.8 NFκB activity assay

40,000 cells were seeded in 96 well plate and incubated overnight. Next day, NF-κB (CSS-013L Cignal reporter assay kit, QIA-GEN) was transfected using Fugene HD transfection reagent (Promega) according to the manufacturer's protocol. After 24 hours, cells were treated with DDX 30 - 900μmol/L and luminescence was measured using Dual luciferase reagent (Promega).

3.2.9 Statistical Analysis

Data was analyzed using unpaired two sample t-test of GraphPad Prism software. Results are presented as mean ± SEM. p-value < 0.05 was considered statistically significant.

3.3 Results

3.3.1 Resistance to palbociclib (PLB) alters expression of proteins involved in cell growth and cell cycle regulatory pathways

Here, we seek to evaluate the effect of palbociclib resistance on cell cycle, apoptosis, growth signaling, NFκB & IKK signaling in Rb sensitive (MCF7) and Rb deficient (MDA-MB-468) parental and palbociclib (PLB) resistant breast cancer cells. With palbociclib resistance we observed that pRb (S807), Rb, cyclin D1, p21, p105, RelB, IKKα, ERα and RRM2 levels are downregulated in untreated MCF7 cells (Rb sensitive). Interestingly, upon palbociclib treatment we observed that resistant cells expressed elevated p21, p100, p105, RelB, ERα and RRM2 levels when compared to NT group of MCF7 PR cells. Interestingly, pRb (S807) level remained unchanged with PLB treatment in MCF7 PR cells (Fig. 11A).

Next, we examined the inhibition of ribonucleotide reductase on our parental and resistant breast cancer cells by using the ribonucleotide reductase inhibitor didox alone or in combination with palbociclib. Cell cycle regulatory proteins cyclin D1, Rb and pRb S807 along

with IKK and NFκB signaling proteins p100, p105, RelB, cRel and IKKβ were downregulated with DDX and combination treatment. Yet, pIKKα/β was significantly increased with DDX combination in both parental and palbociclib resistant MCF7 cells. DDX alone or in combination with PLB also caused an increase in the DNA damage signaling protein pH2AX indicating DNA damage due to PLB (Fig. 11A, B).

Although, MDA-MB-468 ER- cells are Rb-deficient, here we have found that DDX treatment actually brings Rb levels back in MDA-MB-468 parental and palbociclib resistant breast cancer cells. We also found upregulation of cyclin D1, AKT and RRM2 with palbociclib resistance in MDA-MB-468 breast cancer cells compared parental cells. Upon DDX treatment we observed downregulation of the cell cycle regulatory proteins cyclin D1, cyclin A2, and cyclin E2 along with the NFκB signaling proteins p100, p105, and RelB and the apoptotic proteins p53, pp53 (S392), and mutant p53 in MDA-MB-468 cells along with its palbociclib resistant counterpart (Fig. 11C). DDX increased the DNA damage signaling protein pH2AX expression in MDA-MB-468 parental PLB resistant cells (Fig. 11C). DDX did not reduce wild type p53 in MCF7 parental or PLB resistant cells (Fig. 11A**)**. Yet, mutant p53 and phosphor p53 S392 went down in DDX and DDX+PLB treated group in both parental and PLB resistant MDA-MB-468 cell (Fig.11C). Upregulation of cyclin D1, AKT and RRM2 were only observed in MDA-MB-468 PLB resistant NT group but not in parental MDA-MB-468 cells (Fig. 11C).

To examine the effect of treatment on cellular growth in our parental and resistant breast cancer cells, we conducted cell proliferation assays. Here, we are reporting that DDX in combination with PLB significantly decreases cellular proliferation of ER+ MCF7 (Fig, 12A**)** and ER- MDA-MB-468 (Fig. 12B) breast cancer cells along with their palbociclib resistant

counterparts (Fig. 12C, D). IC_{50} values of PLB drug were higher for PLB resistant breast cancer cells compared to parental cells (Fig. 12E).

3.3.2 Inhibition of ribonucleotide reductase alters cell cycle regulatory and NFκB pathway expression in a dose dependent manner

Based on the proliferation assays, we determined to use the ER+ MCF7 (Rb active) and the ER- MDA-MB-468 (Rb deficient) parental and PLB resistant breast cancer cell lines. In order to determine the optimal concentration of DDX we conducted dose dependent western blot analysis on cell cycle regulatory, apoptotic and NFκB pathways using DDX concentrations varying between 30 to 600 μmol/L in MCF7 and MDA-MB-468 parental and PLB resistant cells. DDX reduced expression of cell cycle regulatory proteins cyclin D1, phosphorylated Rb S807 and NFκB regulatory RelB and IKBα but increases pH2AX level in MCF7 parental and PLB resistant cell lines (Fig. 13A). Interestingly, DDX also reduced cyclin D1, cyclin E2, NFκB regulated p100 and mutant p53 while increasing Rb, pRb S807 and cRel expression in MDA-MB-468 parental and PLB resistant cell lines (Fig. 13B). We observed the most alterations in the mentioned proteins at the highest DDX dose in both MCF7 and MDA-MB-468 parental and PLB resistant cell lines.

To determine whether palbociclib resistance leads to transcriptional changes, we assessed the mRNA expression of RRM2 and CCND1 in ER+ and ER- parental and PLB resistant breast cancers. Results show more than a 2.8-fold increase of RRM2 and 2.2-fold increase of CCND1 expression in PLB resistant as compared to parental ER+ MCF7 breast cancer cells (Fig. 13C, D). ER- MDA-MB-468 breast cancer cells exhibited approximately 4-fold greater expression of RRM2 and 19-fold higher expression of CCND1 as compared to MCF7 cells (Fig. 13C, D). As ER- MDA-MB-468 breast cancer cells already have higher levels of RRM2 and CCND1

expression, we do not observe additional increase in PLB resistant MDA-MB-468 cells (Fig. 13C, D). To determine the effect of inhibiting RR on these observed increases in gene transcription, cells were treated with DDX for six and twelve hours, RRM2 expression is downregulated in a time dependent manner in MCF7 PLB resistant as well as MDA-MB-468 and MDA-MB-468 PR cells (Fig. 13C). Reduced CCND1 expression was also observed in the ER- cells with DDX treatment both at six and twelve hours (Fig. 13D).

To examine whether NFκB is activated in PLB resistant cells, we performed an NFκB activity assay. When comparing MCF7 untreated cells to MDA-MB-468 cells, we observed 24-fold greater NFκB activity. We also observed a small increase in palbociclib resistant MCF7 cells but not MDA-MB-468 cells (Fig. 13E). RRM2 inhibition reduced NFκB activity by 60% - 80% in both ER+ and ER- cells as well as their palbociclib resistant counterparts in a dose dependent manner (Fig. 13E).

3.3.3 Inhibition of ribonucleotide reductase causes cell cycle arrest at G1 phase

To further understand whether palbociclib resistance may lead to cell cycle changes, we used flow cytometry and also evaluated the effect of DDX alone and in combination with palbociclib on the cell cycle profile of breast cancer cell lines. We are reporting that the development of palbociclib resistance in ER+ MCF7 and ER- MDA-MB-468 breast cancer cells yielded a shift in their cell cycle profile (Fig. 14A, B). As palbociclib treatment is known to induce G1 cell cycle arrest in Rb sensitive cells, we observed a significant increase in the percentage of cells in G1 phase in the parental MCF7 (Rb sensitive) but not MDA-MDB-468 (Rb deficient) breast cancer cells (Fig. 14A, B). However, MCF7 palbociclib resistant cells exhibited a reduced G1 cell cycle arrest as compared to parental MCF7 cells. Interestingly,

MDA-MB-468 PR cells exhibited a lower percentage of G1 cells both untreated as well as with PLB treatment (Fig. 14D).

Next, to determine whether RR inhibition would have an additive effect on G1 arrest, cells were treated with DDX alone or in combination with PLB. Upon treatment with DDX, we observed an increase in percentage of G1 cells from 36.8% (NT) to 46.6% (DDX alone) to 58.4% (DDX+PLB) in MCF7 cells. Similarly, in the MCF7 palbociclib resistant counterpart, the G1 cell cycle shift was 46.9% with DDX and 56.1% with combination therapy as compared with 39.6% of NT (Fig. 14A, B). Additionally, we also observed significantly higher G1 cell cycle arrest in MDA-MB-468 and MDA-MB-468 PR cells treated with DDX alone and with the combination of DDX and PLB compared to no treatment. G1 cell cycle shifted to 53.6% with DDX and 53.4% with combination therapy from 37.6% NT in MDA-MB-468 cell. On the other hand, palbociclib resistant MDA-MB-468 cells G1 phase shifted from 20.8% of NT to 34.4% with DDX alone and 30.8% with combination treatment (Fig. 14C, D). There appears to be no additive benefit of PLB treatment on G1 arrest in the parental or palbociclib resistant Rb deficient MDA-MB-468 cells.

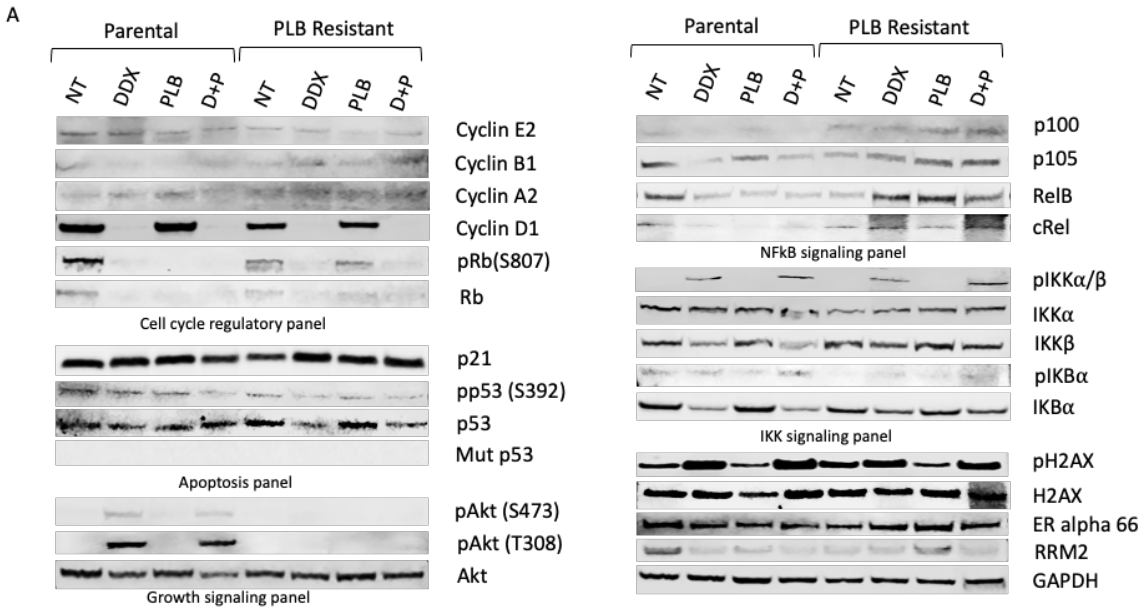
3.3.4 Inhibition of ribonucleotide reductase suppresses mutant p53 expression in a time dependent manner in ER- breast cancer with p53 mutation

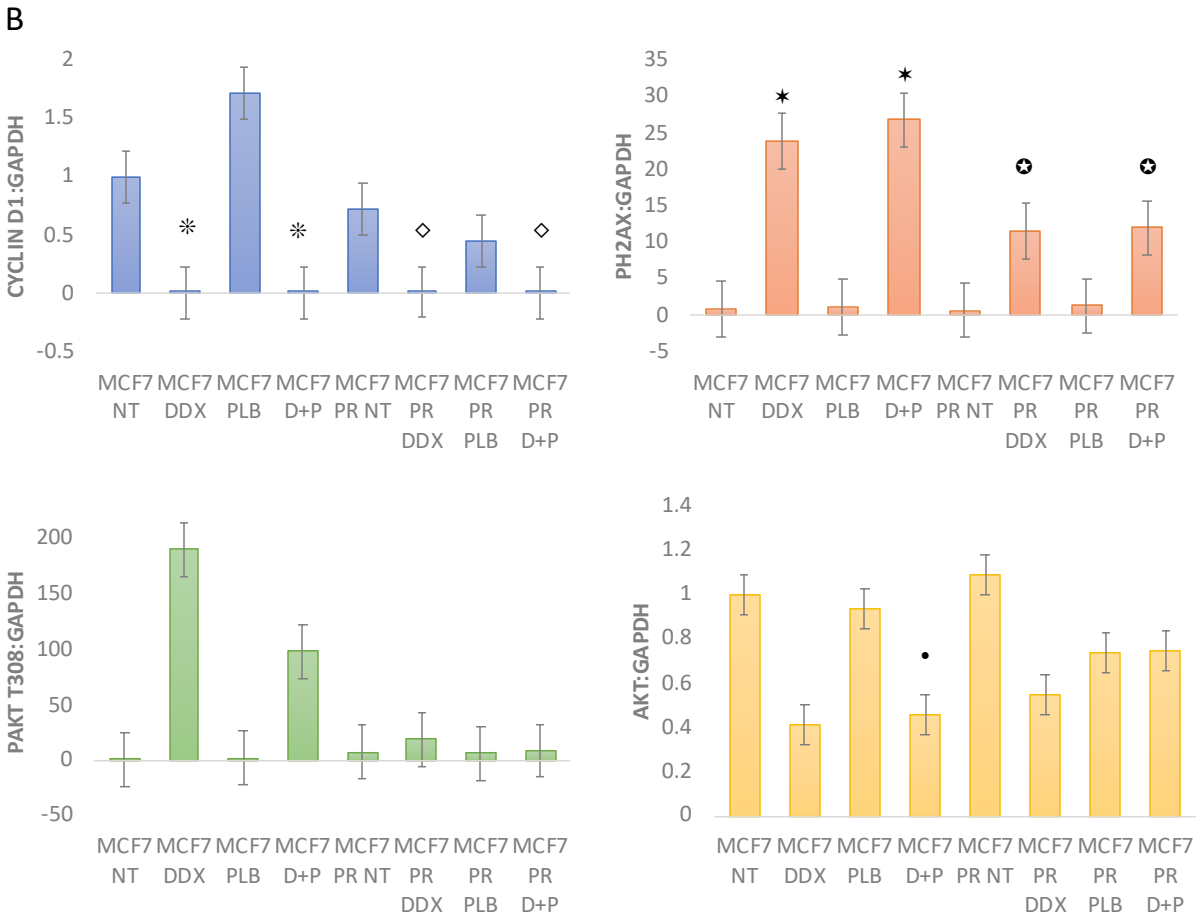
In order to further elucidate the effect of RR inhibition on p53 modulation in parental as compared to palbociclib resistant cells, we conducted protein and gene expression analysis in a time dependent manner. DDX treatment resulted in a downregulation of mutant p53 over time as compared to NT (Fig. 15A, B). This decrease in protein levels of mutant p53 was observed as early as 6 hours and was significantly lowered by 24 hours.

To determine whether the reduction in mutant p53 protein levels was due to transcriptional regulation, we conducted quantitative p53 expression analysis. We observed that ER- MDA-MB-468 PR and ER+ MCF7 cells had significant downregulation of p53 mRNA when treated with DDX for 6h, 12h and 24h (Fig. 15C, E). Alternatively, p53 mRNA levels are not significantly altered in MDA-MB-468, BT-20 and MDA-MB-231 cells treated with DDX compared to NT (Fig. 15A, E & F).

Figure 11

Resistance to palbociclib alters expression of proteins involved in cell growth and cell cycle regulatory pathways. Western blot analysis of cellular and apoptotic proteins of MCF7 and MCF7 palbociclib resistant (PR) (A), Quantification of MCF7 and MCF7 PR protein expression (B), western blot analysis of MDA-MB-468 and MDA-MB-468 PR (C) cells treated with vehicle (NT), 600µmol/L DDX (didox), 1µmol/L PLB (palbociclib), and combination of 600µmol/L DDX + 1µmol/L PLB. Results are in averages ± SEM, n=3. Comparisons between groups were made by unpaired two-sample t test. Significant differences when ✻P<0.01; MCF7 NT vs DDX & D+P ◇P<0.02; MCF7 PR NT vs DDX & D+P, ★P<0.02; MCF7 NT vs DDX & D+P, ✪P<0.04; MCF7 PR NT vs DDX & D+P, ❖P<0.05; MCF7 NT vs D+P, P<0.05; MCF7 PR NT vs D+P, ●P<0.05; MCF7 NT vs D+P treatment.


A
Parental
PLB Resistant
NT
DDX
PLB
D+P
Cyclin E2
Cyclin B1
Cyclin A2
Cyclin D1
pRb(S807)
Rb
Cell cycle regulatory panel
p21
pp53 (S392)
p53
Mut p53
Apoptosis panel
pAkt (S473)
pAkt (T308)
Akt
Growth signaling panel
p100
p105
RelB
cRel
NFkB signaling panel
pIKKα/β
IKKα
IKKβ
pIKBα
IKBα
IKK signaling panel
pH2AX
H2AX
ER alpha 66
RRM2
GAPDH


B
CYCLIN D1:GAPDH
PH2AX:GAPDH
PAKT T308:GAPDH
AKT:GAPDH
MCF7 NT
MCF7 DDX
MCF7 PLB
MCF7 D+P
MCF7 PR NT
MCF7 PR DDX
MCF7 PR PLB
MCF7 PR D+P

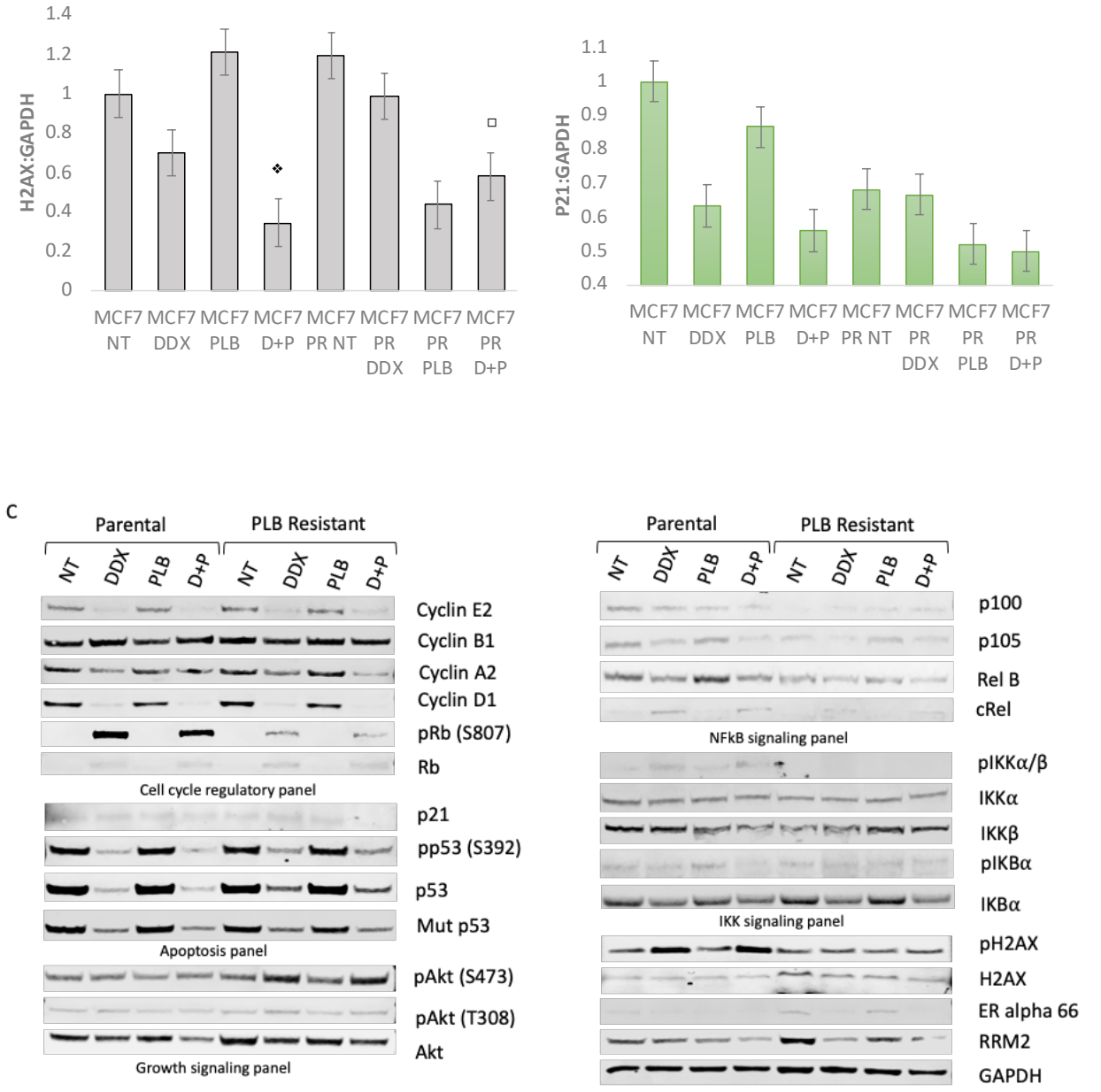
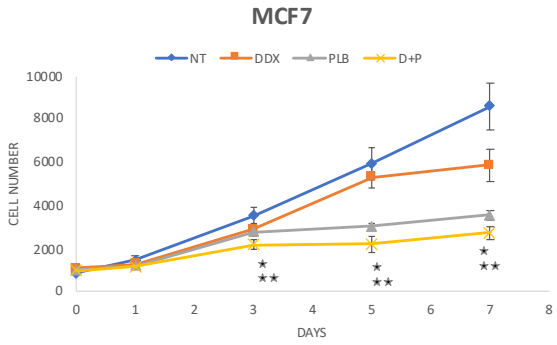


Figure 12

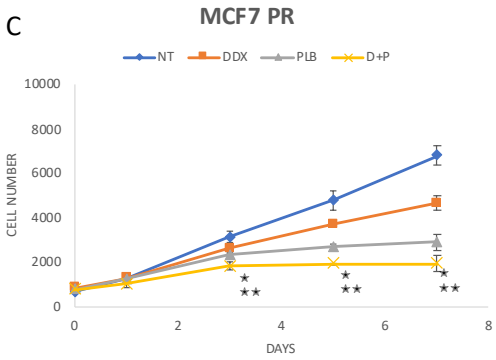
Didox decreases ER+ MCF7 and ER- MDA-MB-468 parental and palbociclib resistant cell proliferation in vitro. All proliferation experiments performed in triplicate. The combination of 30µmol/L DDX and 1µmol/L PLB decreases cellular proliferation over 1µmol/L PLB or 30µmol/L DDX or Vehicle (NT) in MCF7, MCF7 PR, MDA-MB-468 and MDA-MB-468 PR

breast cancer cells. IC_{50} values of drug palbociclib and didox in all cell lines (D). Results are in averages ± SEM, n=3. Comparisons between groups were made by unpaired two-sample t test. ★ P<0.04; significant difference between NT and D+P treated breast cancer cell lines. ★★ P<0.03; significant difference between PLB and D+P treated breast cancer cell lines.

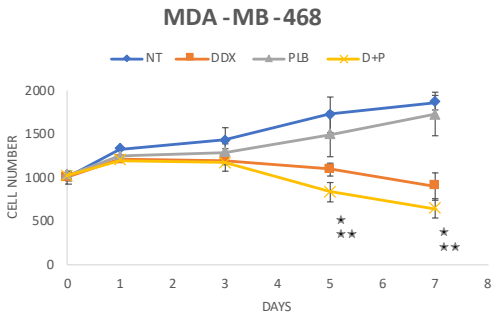
A



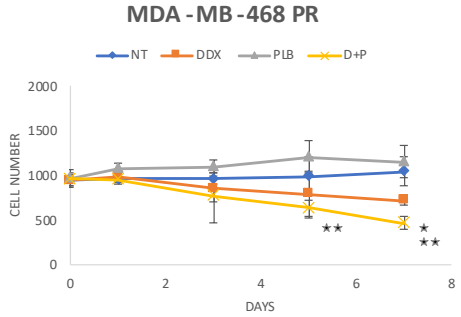
C



B



D



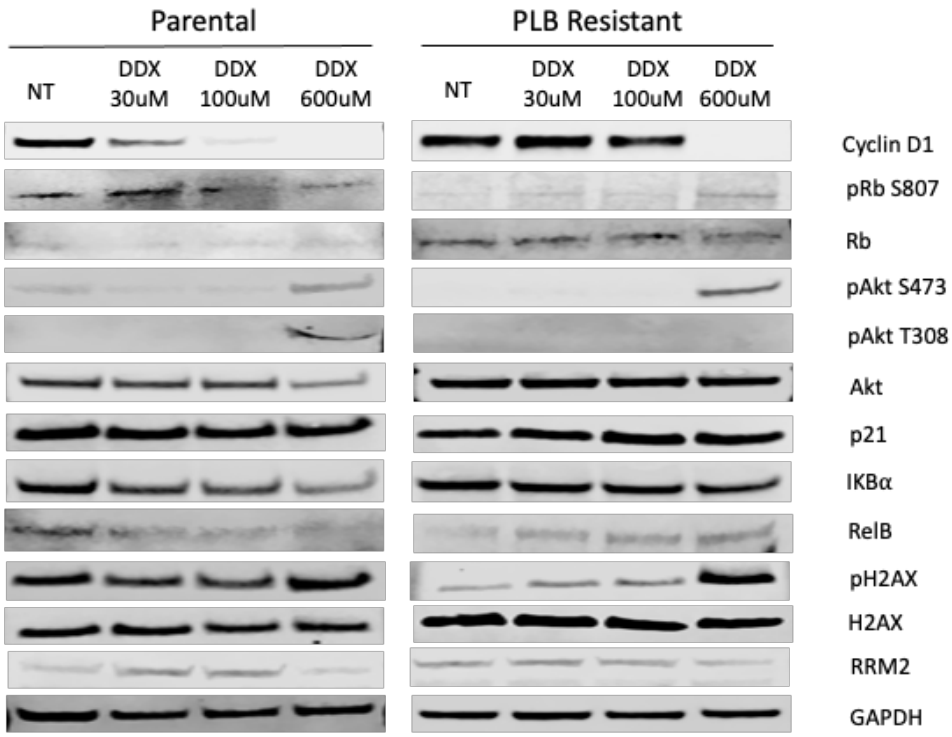
E

Cell line	IC_{50} (µM)	
	PLB	SD
MCF7	13.18	0.067
MCF7 PR	20.85	0.161
MDA-MB-468	9.98	0.011
MDA-MB-468 PR	19.23	0.025

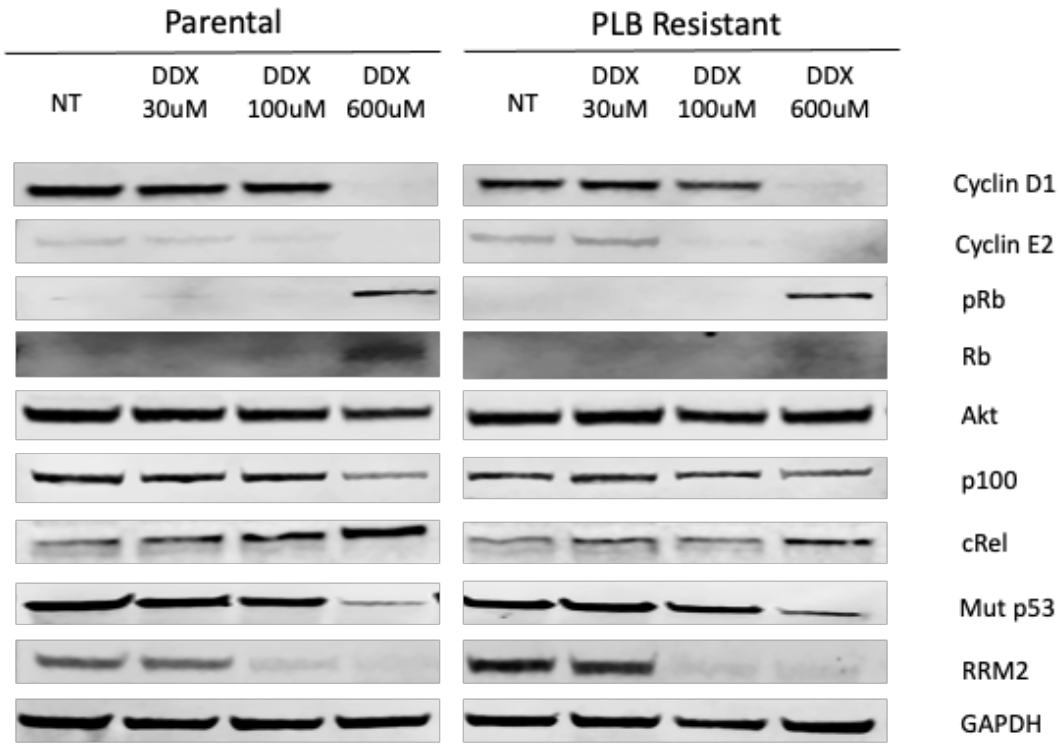
Figure 13

DDX inhibits cell cycle regulatory proteins and RRM2 in a dose dependent manner in both parental and PLB resistant ER+ and ER- breast cancer cell lines as well as CCND1 and RRM2 genes. Western blot analysis of cellular and apoptotic proteins in MCF7 (A) and MDA-MB-468 (B) cells treated with vehicle (NT), 30µmol/L DDX, 100µmol/L DDX, and 600µmol/L DDX for 24 hours. RT-qPCR of RRM2 mRNA in all cell lines (C) and CCND1 fold changes with didox 100 µmol/L for 6hr and 12hr compared to NT in ER+ and ER- breast cancer and their PLB resistant counterparts (D). Promoter analysis of NFκB in all breast cancer cells treated with DDX at a dose ranging from 30µmol/L to 1mmol/L for 6 hrs (E). All experiments are done in triplicate. Data are presented as mean ± SEM, n=3. Comparisons between groups were made by unpaired two sample t test. ✯ P=0.002; significant difference between MCF7 and MCF7 PR breast cancer cell lines. ✯✯ P=0.0001; significant difference between MCF7 and MDA-MB-468 and MDA-MB-468 PR breast cancer cell lines. ✻ P<0.01 and ✻✻ P<0.0003; significant difference between MCF7 and MCF7 PR, MDA-MB-468, MDA-MB-468 PR breast cancer cells. ◇P<0.0003 and ◉ P<0.002; significant difference between MDA-MD-468 NT vs DDX treatment 6h and 12h, MDA-MB-468 PR NT vs DDX treatment 6h and 12hr. ❖P<0.03, MCF7 PR NT vs DDX treatment 12h. ▲P<0.0003; significant difference between MDA-MD-468 NT vs DDX treatment 6h and 12h, ▩ P<0.0002, MDA-MB-468 PR NT vs DDX treatment 6h and 12hr. • P<0.05, MCF7 NT vs DDX treatment 100umol/L & 600 umol/L. ⬭ P<0.003; significant difference between MCF7 PR NT vs DDX treatment 100umol/L & 600umol/L, ▼ P<0.003, MDA-MB-468 NT vs DDX treatment 100umol/L & 600umol/L, ▽P<0.0007 MDA-MB-468 PR NT vs DDX treatment 600umol/L.

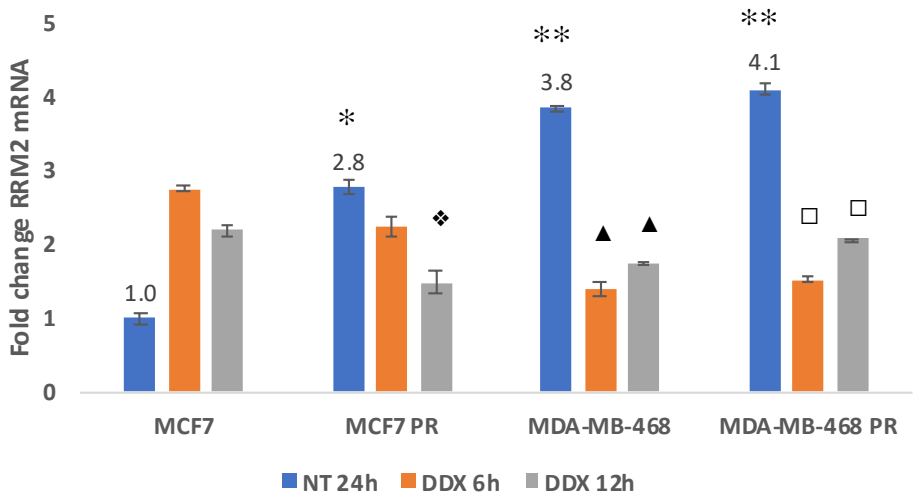
A


Parental
PLB Resistant
NT
DDX 30uM
DDX 100uM
DDX 600uM
Cyclin D1
pRb S807
Rb
pAkt S473
pAkt T308
Akt
p21
IKBα
RelB
pH2AX
H2AX
RRM2
GAPDH

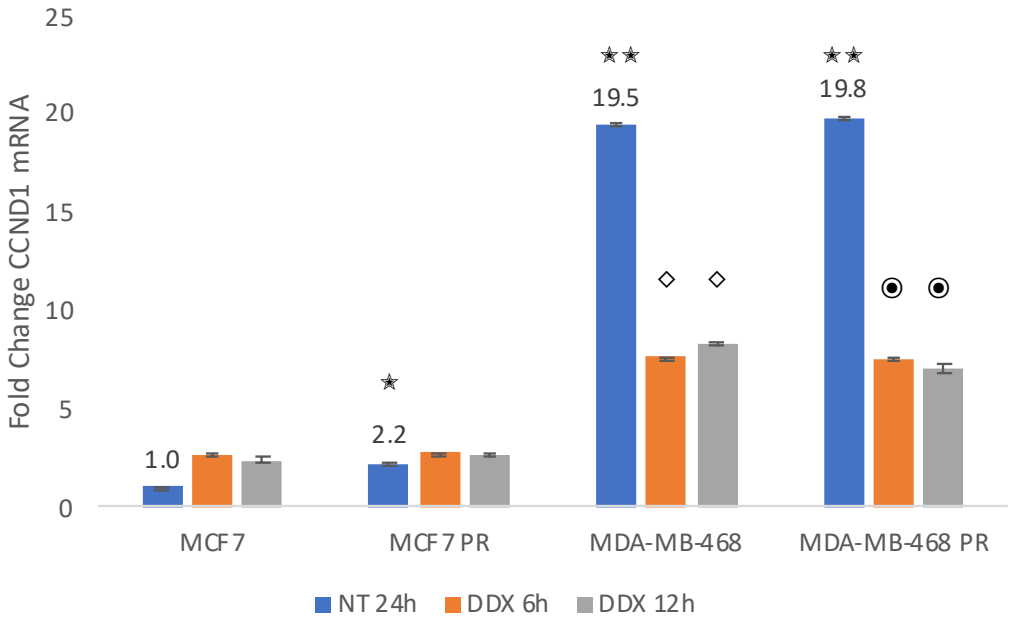
B


Parental
PLB Resistant
NT
DDX 30uM
DDX 100uM
DDX 600uM
Cyclin D1
Cyclin E2
pRb
Rb
Akt
p100
cRel
Mut p53
RRM2
GAPDH

C


Fold change RRM2 mRNA
5
4
3
2
1
0
1.0
2.8
3.8
4.1
*
**
**
❖
▲
▲
□
□
MCF7
MCF7 PR
MDA-MB-468
MDA-MB-468 PR
NT 24h
DDX 6h
DDX 12h

D


Fold Change CCND1 mRNA
25
20
15
10
5
0
1.0
2.2
19.5
19.8
★
★★
★★
◇
◇
◉
◉
MCF7
MCF7 PR
MDA-MB-468
MDA-MB-468 PR
NT 24h
DDX 6h
DDX 12h

E

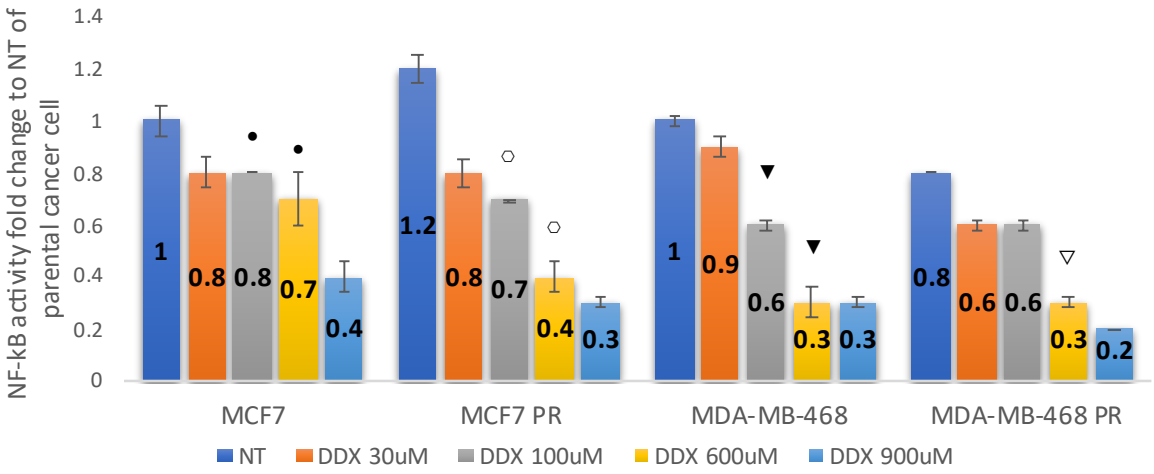
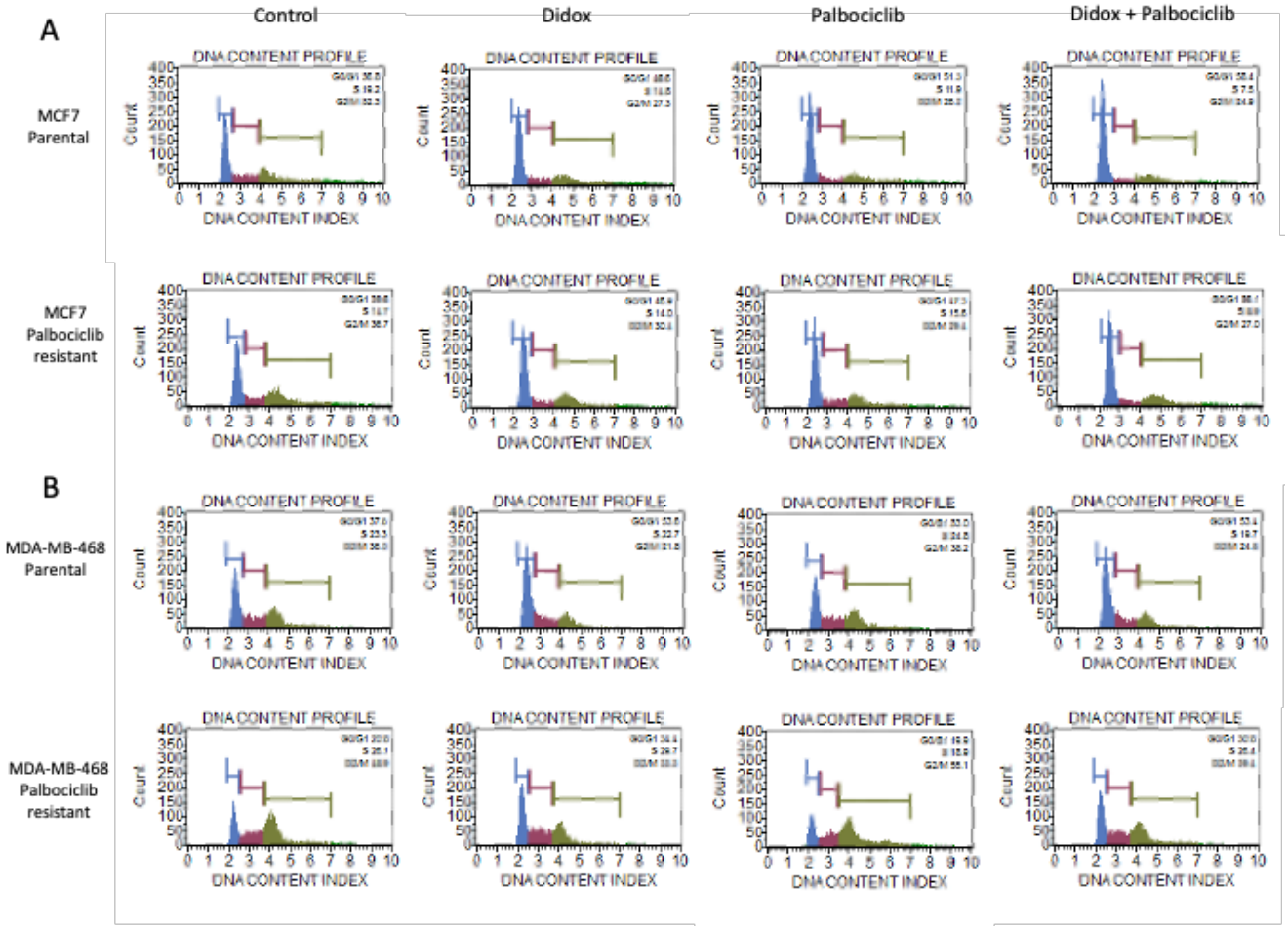
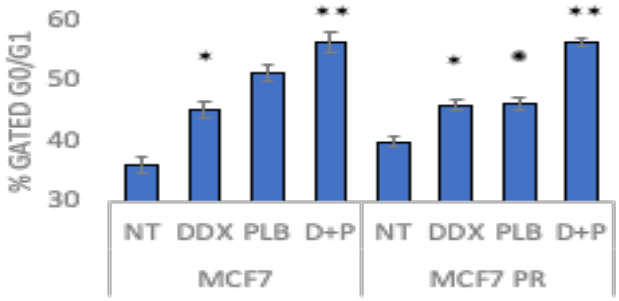


Figure 14

DDX alters the cell cycle and causes cell cycle arrest in MCF7 and MDA-MB-468 parental and palbociclib resistant breast cancer cells. Cell cycle analysis by flow cytometry of MCF7 and MCF7 PR (A), MDA-MB-468 and MDA-MB-468 PR (B) cells treated with NT, DDX 100µmol/L, PLB 1µmol/L and combination of DDX and PLB for 24 hours. Difference between percent gated at G0/G1 in MCF7 and MCF7 PR (C), MDA-MB-468 and MDA-MB-468 PR (D) with same treatment conditions as before. Data are presented as mean ± SEM, n=3. Comparisons between groups were made by unpaired two sample t test. ★ P< 0.009, ★★ P< 0.01; significant difference between NT and DDX, NT and D+P treated breast cancer cells. ✺ P< 0.02, ✺✺ P< 0.0002; significant difference between MCF7 and MCF7 PR, MDA-MB-468 and MDA-MB-468 PR breast cancer cells treated with PLB.


A
Control
Didox
Palbociclib
Didox + Palbociclib
MCF7 Parental
MCF7 Palbociclib resistant
B
MDA-MB-468 Parental
MDA-MB-468 Palbociclib resistant
DNA CONTENT PROFILE
Count
DNA CONTENT INDEX

C


% GATED G0/G1
60
50
40
30
NT DDX PLB D+P NT DDX PLB D+P
MCF7
MCF7 PR

D

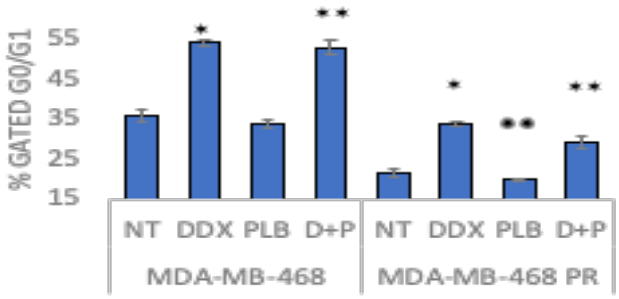
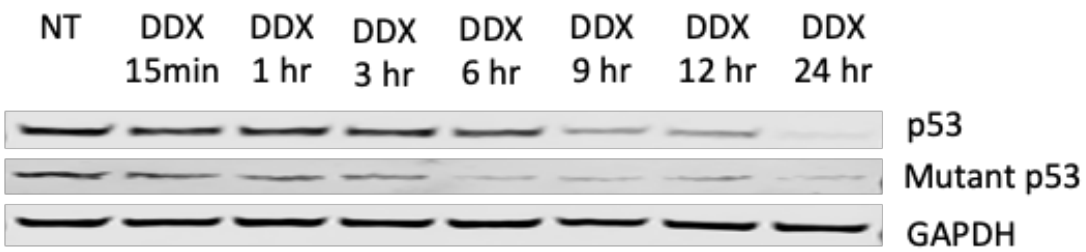

% GATED G0/G1
55
45
35
25
15
NT DDX PLB D+P NT DDX PLB D+P
MDA-MB-468
MDA-MB-468 PR

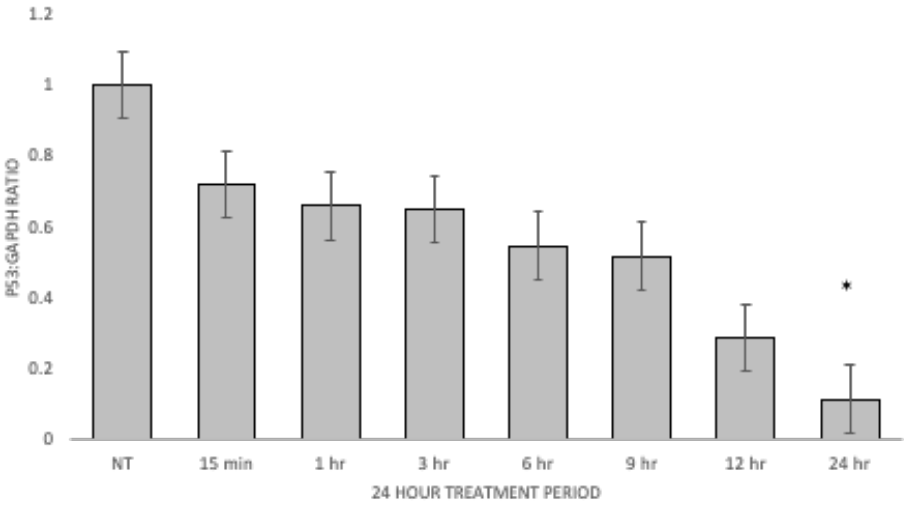
Figure 15

DDX decreases mutant p53 in a time dependent manner. (A) Western blot analysis of MDA-MB-468 parental cells treated with vehicle (NT) and 600µmol/L DDX in a time dependent manner for 24 hrs shows decrease in mutant p53 and WT p53. (B) Quantification of total p53 expression in MDA-MB-468 parental cells treated with vehicle (NT) 24 hr, DDX 600µmol/L 15 min, 1 hr, 3 hr, 6 hr, 9 hr, 12 hr and 24 hr. RT-qPCR of four different variants of Tp53 gene in parental breast cancer cell lines MDA-MB-468, MCF7, BT-20 and MDA-MB-231 treated with vehicle (NT), DDX 600µmol/L and samples were collected at 24 hr NT, 6 hr DDX, 12 hr DDX and 24 hr DDX. RT-qPCR of p53 mRNA in MDA-MB-468 (C) and MDA-MB-468 PR (D) cells treated with didox 100 µmol/L for 6hr,12hr and 24hr. Data are presented as mean ± SEM, n=3. Comparisons between groups were made by unpaired two sample t test. ✶P<0.05, Significant difference between MDA-MB-468 p53:GAPDH NT vs 24hr treated with didox 600umol/L. ✯P<0.04, ◇P<0.02, ✻P<0.05, ▲P<0.002; significant difference p53 6686 NT vs DDX treatment 12h & 24h, p53 3122 NT vs 12h & 24h, p53 3224 NT vs 12h & 24 h DDX, p53 9752 NT vs 12h & 24h DDX in MDA-MB-468 PR cells. ❖P<0.009 p53 6686 NT vs DDX 24h, ✪P<0.02 p53 3122 NT vs DDX 24h, ☐P<0.003 p53 3224 NT vs DDX 24h, ●P<0.05 p53 9752 NT vs DDX 24h.

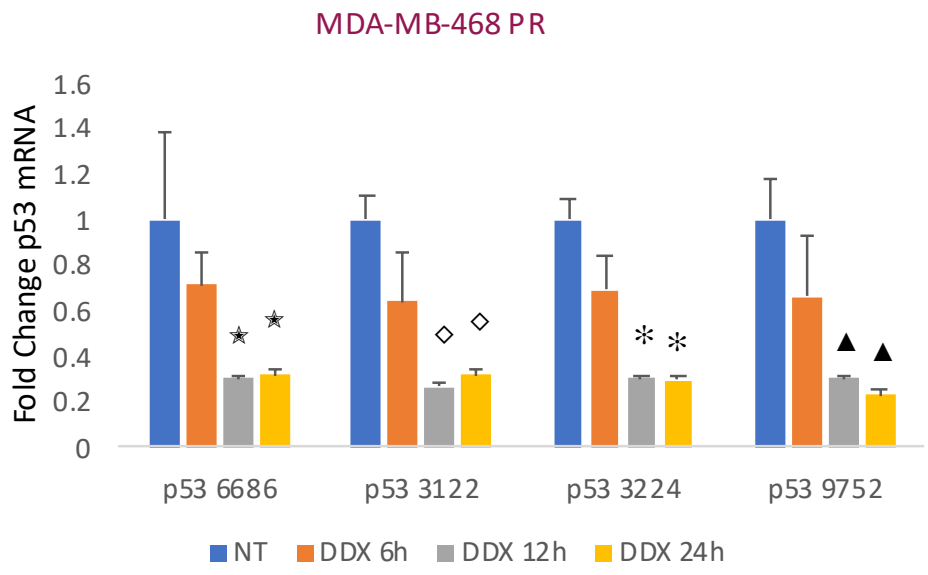
A


NT
DDX 15min
DDX 1 hr
DDX 3 hr
DDX 6 hr
DDX 9 hr
DDX 12 hr
DDX 24 hr
p53
Mutant p53
GAPDH

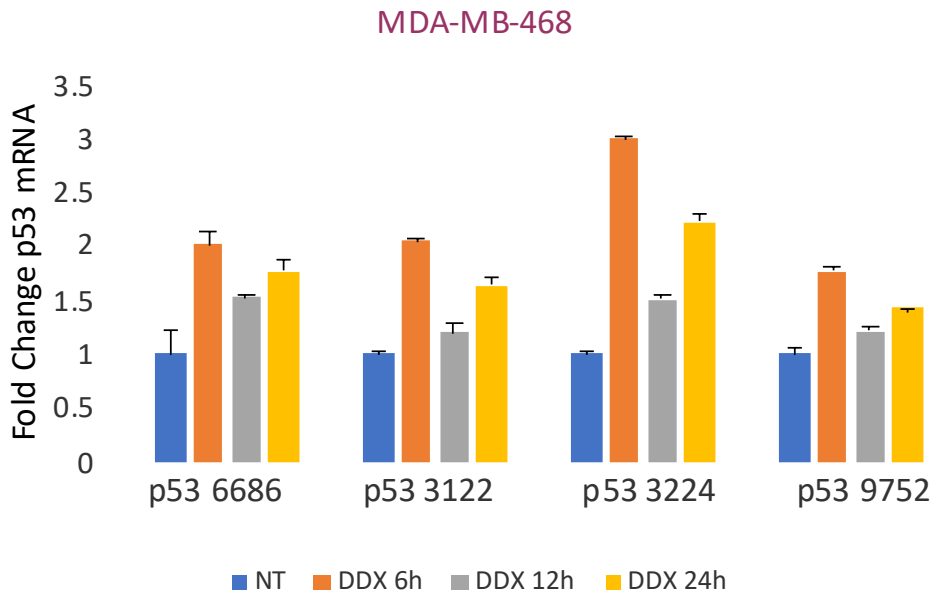
B


1.2
1
0.8
0.6
0.4
0.2
0
P53:GAPDH RATIO
NT
15 min
1 hr
3 hr
6 hr
9 hr
12 hr
24 hr
24 HOUR TREATMENT PERIOD

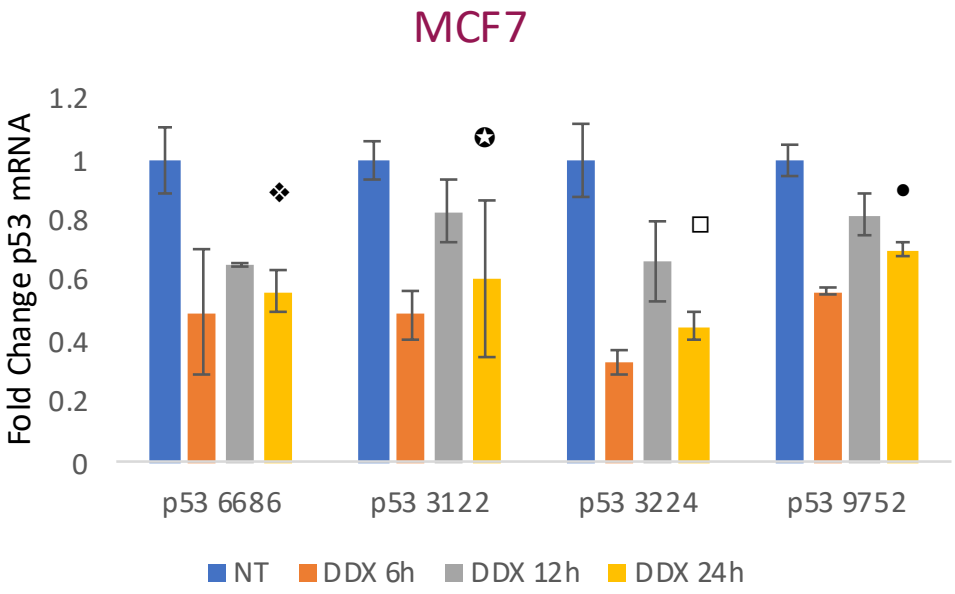
C


MDA-MB-468 PR
Fold Change p53 mRNA
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
p53 6686
p53 3122
p53 3224
p53 9752
NT
DDX 6h
DDX 12h
DDX 24h

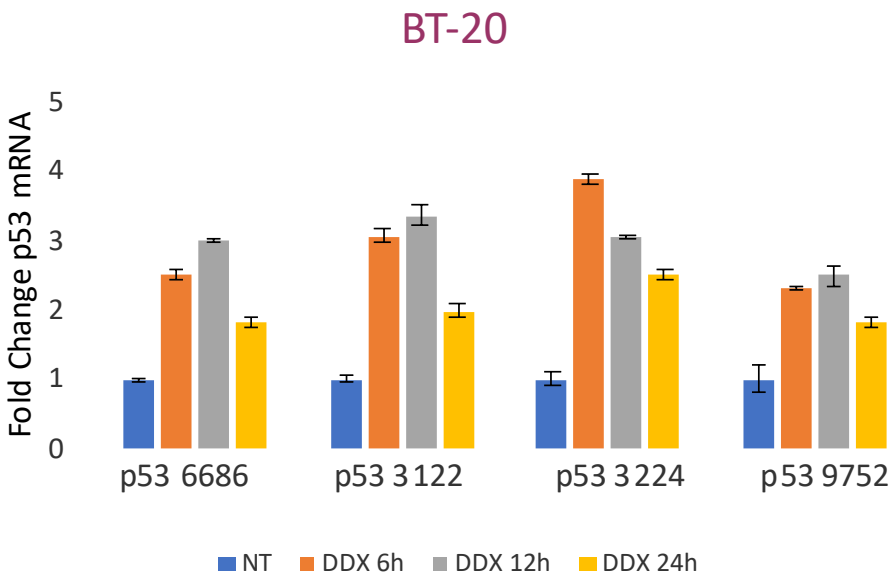
D


MDA-MB-468
Fold Change p53 mRNA
3.5
3
2.5
2
1.5
1
0.5
0
p53 6686
p53 3122
p53 3224
p53 9752
NT
DDX 6h
DDX 12h
DDX 24h

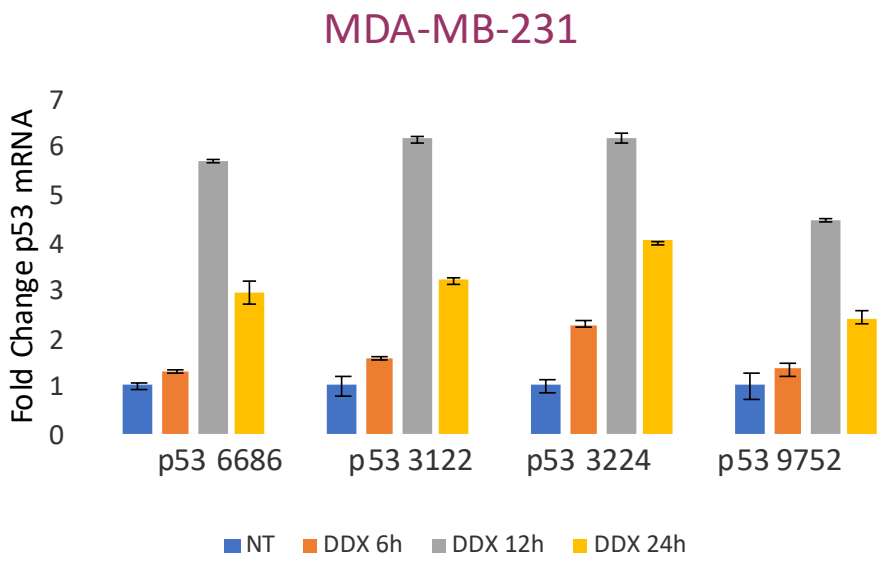
E


MCF7
Fold Change p53 mRNA
1.2
1
0.8
0.6
0.4
0.2
0
p53 6686
p53 3122
p53 3224
p53 9752
NT
DDX 6h
DDX 12h
DDX 24h

F


BT-20
Fold Change p53 mRNA
0
1
2
3
4
5
p53 6686
p53 3122
p53 3224
p53 9752
NT
DDX 6h
DDX 12h
DDX 24h

G


MDA-MB-231
Fold Change p53 mRNA
0
1
2
3
4
5
6
7
p53 6686
p53 3122
p53 3224
p53 9752
NT
DDX 6h
DDX 12h
DDX 24h

3.4 Discussion

The development of acquired and *de novo* palbociclib resistance in breast cancers remains a management challenge in clinical oncology for the treatment of hormone receptor positive and HER2 negative breast cancer. Limited data exist regarding the role of CDK4/6 and cyclin D1 interaction in the development of palbociclib resistance, and effective targets for palbociclib resistant ER+ and ER- breast cancers are largely unknown. To date, there are no studies to examine the feasibility of a combination of CDK4/6 inhibitor, palbociclib, with an RRM2 inhibitor for treating both ER+ and ER- palbociclib resistant breast cancer. In addition, this combination strategy might have the possibility of avoiding the development of palbociclib resistance.

Multiple studies have reported a direct link between overexpression of RRM2 in breast cancers to increased cell proliferation, invasiveness, as well as drug and chemotherapy resistance (12, 13, 29, 31). Previously, we found that RRM2 is upregulated in ER- and drug resistant breast cancer cells rationalizing our hypothesis of targeting ribonucleotide reductase for ER+ as well as ER- breast cancer treatment (12,13,14). Our current study shows increased expression of RRM2 and CCND1 mRNA in ER+ MCF7 PR and ER- MDA-MB-468 PR cells compared to parental cells (Fig. 13C, D). We also observe an upregulation of RRM2, cyclin D1 and total AKT protein level in ER- palbociclib resistant breast cancer cells (Fig. 11C) which suggests a possible mechanism of resistance development. Inhibition of ribonucleotide reductase by DDX alone or in combination with palbociclib decreases RRM2, cyclin D1 and pRb levels in MCF7 ER+ breast cancer cells. Although, the exact mechanism underlying palbociclib resistance is largely unknown, this study demonstrates that inhibition of RRM2 by DDX circumvents emergence of palbociclib resistance.

NFκB signaling has a unique importance in breast cancer as it regulates a myriad of key regulatory genes necessary for cell proliferation, invasion, metastasis, and angiogenesis. Some studies have reported that NFκB suppression can re-sensitize resistant breast cancer cells to treatment (32-34). We have demonstrated that DDX alters expression of various NFκB proteins p105 and p100 halting the aid of these proteins in cancer cell survival and proliferation. DDX alone or in combination with PLB significantly decreased expression of RelB, cRel, IKKβ, p105 and p100 *in vitro* (Fig. 11A, C). We have also observed that NFκB promoter activity decreases by 20-70% beginning at a concentration of 100uM DDX in ER+ and ER- breast cancer cells along with their palbociclib resistant counterparts (Fig. 13E). This supports the role of DDX not only as an inhibitor of ribonucleotide reductase but also as an inhibitor of NFκB activation perhaps at higher concentrations.

Cyclin D1 plays a crucial role in regulating the progress of cell cycle progression during transition from G1 to S phase. The cyclin D1 gene (CCND1) is amplified in approximately 20% of breast carcinoma (35). Intensive studies have been conducted to ascertain cyclin D1 as a biological marker in breast cancer (36). Dysregulation of cyclin D1 function or gene expression causes loss of normal cell cycle control during cancer development (35-38). This is the first report to examine the potential of an RR inhibitor DDX in combination with a CDK4/6 inhibitor palbociclib for the treatment of ER+ and ER- breast cancer with or without palbociclib resistance. We observed that DDX inhibited cyclin D1 expression alone or in combination with palbociclib in parental and palbociclib resistant ER+ and ER- breast cancer cells (Fig. 11A, 11C, 13A and 13B).

The function of cyclin D1 is to form a complex with CDK4/6 and activate or upregulate pRb allowing for progression of the cell cycle to next DNA synthesis phase. Downregulation of

pRb is the mechanism by which PLB functions (39). It was previously reported that PLB treatment decreases the palbociclib effectors, pRb and total Rb levels, resulting in G1 arrest in breast cancer (40, 41). Our study confirms that upon the development of PLB resistance, PLB treatment loses its effect of reducing Rb protein levels as seen in PLB resistant ER+ breast cancer MCF7 cells (Fig. 11A). Interestingly, we have observed that DDX treatment has the same inhibitory function on pRb and likely total Rb as does PLB treatment in not only MCF7 cells but also in the PLB resistant counterpart (Fig. 11A).

In the ER- parental and palbociclib resistant MDA-MB-468 (Rb deficient) breast cancer cells, DDX also downregulated cyclin E2 and cyclin A2 and unexpectedly restored the presence of the cell cycle repressor Rb which is an integral part of the cell cycle checkpoint inhibiting progression past G1 (Fig. 11C and 13B). The ability to restore Rb to Rb-deficient breast cancers may improve the efficacy of PLB treatment when given in combination with ribonucleotide reductase inhibitors such as DDX.

Here, we demonstrate a comprehensive analysis of the impact of RR inhibition by DDX in combination with the CDK4/6 inhibitor palbociclib on the cell cycle in parental and palbociclib resistant ER+ and ER- breast cancer cells. This data reveals that DDX reduces protein levels of RRM2, NFκB and cyclin D1, and Rb resulting in the significant accumulation of cells at G1 phase of the cell cycle arrest in the cell cycle (Fig. 14A-D). We also demonstrate that palbociclib treatment alone fails to cause cell cycle arrest at G1 phase in both ER+ and ER- palbociclib resistant breast cancer cells (Fig. 14C, D). These data are consistent with the inhibitory effects of DDX on the cell cycle. The unique mechanism of action of DDX with palbociclib to block the progression of the cell cycle supports a strategy for combination therapies for patients with ER+ and ER- parental and palbociclib resistant breast cancer. In

addition, we observed a significant increase in pH2AX levels in DDX treated ER+ and ER- breast cancer which confirms the DNA damaging effect of the drug (Fig. 11A, 11C).

Our previous study showed downregulation of mutant p53 with DDX treatment without any negative impact on wild type p53 in triple-negative breast cancer (14). In this study, we are showing that DDX has a similar inhibitory effect on mutant p53 in the parental ER- MDA-MB-468 breast cancer cells and the palbociclib resistant counterpart cells in a time dependent manner (Fig. 11B). This inhibitory effect was nonexistent in ER+ breast cancer with wild type p53 and its palbociclib resistant counterpart (Fig. 11A).

In summary, our findings strongly support the hypothesis that DDX in combination with palbociclib can potentially treat ER+ and ER- breast cancer and perhaps prevent palbociclib resistance by targeting RRM2, NFκB, cyclin D1 and pRb. Also, DDX successfully targets mutant p53 in ER- breast cancer with no possible inhibition of wild type p53. We also found evidence that RRM2 and cyclin D1 levels are upregulated in ER- palbociclib resistant breast cancer giving rise to a difficult to treat breast cancer population. Reduced expression of RRM2, cyclin D1, NFκB protein as well as elevated level of pH2AX results in decrease breast cancer growth and survival. We have also observed that the addition of DDX also halts cell cycle progression at G1. Ultimately, our data suggests a strategy to target RRM2, NFκB protein, and cyclin D1 to supplement traditional therapies while offering improved efficacy for breast cancer treatment.

Restoration of Rb in MDA-MB-468 (Rb deficient) ER- breast cancer cells after DDX treatment appears to re-sensitize the cancer cells to palbociclib therapy. However, the exact mechanism of this effect is still unknown. Future studies need to be performed to provide a path for the development of new drug molecules targeting restoration of Rb status in ER- Rb deficient

breast cancer. Another limitation of this study is that all western blot data were not quantified by normalization method. Differences in band intensity of the protein expression upon Western blot was used as a measurement of difference in protein expression.

Future studies showing the effects of DDX alone or combination with palbociclib in ER+ and ER- breast cancer as well their palbociclib resistance counterparts *in vivo* would add valuable insights to this study. In addition, Western blot analysis of mouse tumors with and without DDX and palbociclib treatment as well as the combination should be performed.

3.5 References

1. Tong J, Tan X, Song X, Gao M, Risnik D, Hao S, et al. CDK4/6 Inhibition Suppresses p73 Phosphorylation and Activates DR5 to Potentiate Chemotherapy and Immune Checkpoint Blockade. Cancer Research. 2022;82:1340–52.
2. Tien AH, Sadar MD. Cyclin-dependent Kinase 4/6 Inhibitor Palbociclib in Combination with Ralaniten Analogs for the Treatment of Androgen Receptor–positive Prostate and Breast Cancers. Molecular Cancer Therapeutics. 2022;21:294–309.
3. Hanahan D. Hallmarks of Cancer: New Dimensions. Cancer Discovery. 2022;12:31–46.
4. De Angelis C, Fu X, Cataldo ML, Nardone A, Pereira R, Veeraraghavan J, et al. Activation of the IFN Signaling Pathway is Associated with Resistance to CDK4/6 Inhibitors and Immune Checkpoint Activation in ER-Positive Breast Cancer. Clinical Cancer Research. 2021;27:4870–82.
5. Fry DW, Harvey PJ, Keller PR, Elliott WL, Meade M, Trachet E, et al. Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. Mol Cancer Ther. 2004;3:1427–38.
6. Chirila C, Mitra D, Colosia A, Ling C, Odom D, Iyer S, et al. Comparison of palbociclib in combination with letrozole or fulvestrant with endocrine therapies for advanced/metastatic breast cancer: network meta-analysis. Current Medical Research and Opinion. 2017;33:1457–66.
7. Papadimitriou MC, Pazaiti A, Iliakopoulos K, Markouli M, Michalaki V, Papadimitriou CA. Resistance to CDK4/6 inhibition: Mechanisms and strategies to overcome a therapeutic

problem in the treatment of hormone receptor-positive metastatic breast cancer. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2022;1869:119346.

8. Pandey K, An H, Kim SK, Lee SA, Kim S, Lim SM, et al. Molecular mechanisms of resistance to CDK4/6 inhibitors in breast cancer: A review. Int J Cancer. 2019;145:1179–88.
9. Elford HL, Freese M, Passamani E, Morris HP. Ribonucleotide Reductase and Cell Proliferation. Journal of Biological Chemistry. 1970;245:5228–33.
10. Lozano G, Elledge SJ. p53 sends nucleotides to repair DNA. Nature. 2000;404:24–5.
11. Mazzu YZ, Armenia J, Chakraborty G, Yoshikawa Y, Coggins SA, Nandakumar S, et al. A Novel Mechanism Driving Poor-Prognosis Prostate Cancer: Overexpression of the DNA Repair Gene, Ribonucleotide Reductase Small Subunit M2 (RRM2). Clinical Cancer Research. 2019;25:4480–92.
12. Shah KN, Mehta KR, Peterson D, Evangelista M, Livesey JC, Faridi JS. AKT-Induced Tamoxifen Resistance Is Overturned by RRM2 Inhibition. Molecular Cancer Research. 2014;12:394–407.
13. Shah KN, Wilson EA, Malla R, Elford HL, Faridi JS. Targeting Ribonucleotide Reductase M2 and NF-κB Activation with Didox to Circumvent Tamoxifen Resistance in Breast Cancer. Molecular Cancer Therapeutics. 2015;14:2411–21.
14. Wilson EA, Sultana N, Shah KN, Elford HL, Faridi JS. Molecular Targeting of RRM2, NF-κB, and Mutant TP53 for the Treatment of Triple-Negative Breast Cancer. Molecular Cancer Therapeutics. 2021;20:655–64.
15. Du SM. The SNHG16/miR-30a axis promotes breast cancer cell proliferation and invasion by regulating RRM2. neo. 2020;67:567–75.

16. Gandhi M, Groß M, Holler JM, Coggins SA, Patil N, Leupold JH, et al. The lncRNA lincNMR regulates nucleotide metabolism via a YBX1 - RRM2 axis in cancer. Nat Commun. 2020;11:3214.

17. Shi S, Zhang Y, Wang T. High RRM2 expression has poor prognosis in specific types of breast cancer. Batra SK, editor. PLoS ONE. 2022;17:e0265195.

18. Zhuang S, Li L, Zang Y, Li G, Wang F. RRM2 elicits the metastatic potential of breast cancer cells by regulating cell invasion, migration and VEGF expression via the PI3K/AKT signaling. Oncol Lett [Internet]. 2020 [cited 2022 Oct 17]; Available from: http://www.spandidos-publications.com/10.3892/ol.2020.11428

19. Chen W, Yang L, Xu L, Cheng L, Qian Q, Sun L, et al. Bioinformatics analysis revealing prognostic significance of *RRM2* gene in breast cancer. Bioscience Reports. 2019;39:BSR20182062.

20. Zhang H, Liu X, Warden CD, Huang Y, Loera S, Xue L, et al. Prognostic and therapeutic significance of ribonucleotide reductase small subunit M2 in estrogen-negative breast cancers. BMC Cancer. 2014;14:664.

21. Huff SE, Winter JM, Dealwis CG. Inhibitors of the Cancer Target Ribonucleotide Reductase, Past and Present. Biomolecules. 2022;12:815.

22. Elford HL, Wampler GL, van't Riet B. New ribonucleotide reductase inhibitors with antineoplastic activity. Cancer Res. 1979;39:844–51.

23. Elford HL, Van't Riet B, Wampler GL, Lin AL, Elford RM. Regulation of ribonucleotide reductase in mammalian cells by chemotherapeutic agents. Advances in Enzyme Regulation. 1981;19:151–68.

24. Ling J, Kumar R. Crosstalk between NFkB and glucocorticoid signaling: A potential target of breast cancer therapy. Cancer Letters. 2012;322:119–26.

25. Wang W, Nag S, Zhang R. Targeting the NFκB Signaling Pathways for Breast Cancer Prevention and Therapy. CMC. 2014;22:264–89.

26. Gilmore TD. Multiple Myeloma: Lusting for NF-κB. Cancer Cell. 2007;12:95–7.

27. Chaturvedi MM, Sung B, Yadav VR, Kannappan R, Aggarwal BB. NF-κB addiction and its role in cancer: 'one size does not fit all.' Oncogene. 2011;30:1615–30.

28. Karin M, Cao Y, Greten FR, Li Z-W. NF-κB in cancer: from innocent bystander to major culprit. Nat Rev Cancer. 2002;2:301–10.

29. Perkins ND, Felzien LK, Betts JC, Leung K, Beach DH, Nabel GJ. Regulation of NF-κB by Cyclin-Dependent Kinases Associated with the p300 Coactivator. Science. 1997;275:523–7.

30. Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C, Strauss M. NF-κB Function in Growth Control: Regulation of Cyclin D1 Expression and G_0/G_1-to-S-Phase Transition. Mol Cell Biol. 1999;19:2690–8.

31. He J, Wei Q, Jiang R, Luan T, He S, Lu R, et al. The Core-Targeted RRM2 Gene of Berberine Hydrochloride Promotes Breast Cancer Cell Migration and Invasion via the Epithelial–Mesenchymal Transition. Pharmaceuticals. 2022;16:42.

32. Abdin SM, Tolba MF, Zaher DM, Omar HA. Nuclear factor-κB signaling inhibitors revert multidrug-resistance in breast cancer cells. Chemico-Biological Interactions. 2021;340:109450.

33. Baldwin AS. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB. J Clin Invest. 2001;107:241–6.

34. Park YH. The nuclear factor-kappa B pathway and response to treatment in breast cancer. Pharmacogenomics. 2017;18:1697–709.

35. Barnes DM, Gillett CE. Cyclin D1 in Breast Cancer. Breast Cancer Res Treat. 1998;52:1–15.

36. Cai Z, Wang J, Li Y, Shi Q, Jin L, Li S, et al. Overexpressed Cyclin D1 and CDK4 proteins are responsible for the resistance to CDK4/6 inhibitor in breast cancer that can be reversed by PI3K/mTOR inhibitors. Sci China Life Sci. 2023;66:94–109.

37. Wang G, Gormley M, Qiao J, Zhao Q, Wang M, Di Sante G, et al. Cyclin D1-mediated microRNA expression signature predicts breast cancer outcome. Theranostics. 2018;8:2251–63.

38. Velasco-Velázquez MA, Li Z, Casimiro M, Loro E, Homsi N, Pestell RG. Examining the role of cyclin D1 in breast cancer. Future Oncology. 2011;7:753–65.

39. Vijayaraghavan S, Karakas C, Doostan I, Chen X, Bui T, Yi M, et al. CDK4/6 and autophagy inhibitors synergistically induce senescence in Rb positive cytoplasmic cyclin E negative cancers. Nat Commun. 2017;8:15916.

40. Chen X, Lowe M, Keyomarsi K. UCN-01-mediated G1 arrest in normal but not tumor breast cells is pRb-dependent and p53-independent. Oncogene. 1999;18:5691–702.

41. Gray-Bablin J, Zalvide J, Fox MP, Knickerbocker CJ, DeCaprio JA, Keyomarsi K. Cyclin E, a redundant cyclin in breast cancer. Proc Natl Acad Sci USA. 1996;93:15215–20.

CHAPTER 4: SUMMARY

Triple-negative breast cancer (TNBC) which is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type 2 (HER2) is a highly aggressive type of cancer associated with poor prognosis. TNBC accounts for 10 - 15% of all new cases of breast cancer. It is heterogenous in nature (70% basal-like) and currently does not have effective targeted therapies available. Dose-dense chemotherapeutic agents such as doxorubicin with cyclophosphamide are used in combination and are the regimen of choice for TNBC. However, doxorubicin has limited efficacy as a therapeutic agent alone owing to the development of acquired drug resistance along with its potent cardiotoxic effects.

Ribonucleotide reductase (RR) is the rate limiting enzyme required for the conversion of ribonucleotide to deoxyribonucleotide in DNA synthesis during cell proliferation. Didox (3,4 dihydroxybenzohydroxamic acid) is a unique RR inhibitor with iron chelating and free radical scavenging properties. We have previously identified that ribonucleotide reductase subtype 2 is upregulated in TNBC cells and is a key contributor to acquired drug resistance. We found that didox not only inhibits RRM2, but it also reduces mutant p53 protein levels. Through this inhibition, we found that didox works synergistically with doxorubicin to inhibit TNBC tumor growth while also minimizing doxorubicin induced cardiac damage in mice.

The mitotic cell cycle is a highly sophisticated process where cells proliferate through sequential activation of several downstream cyclin-CDK complexes known as checkpoints. Cancer cells proliferate in a deregulated state through bypassing these checkpoints. The retinoblastoma (Rb) tumor suppressor protein controls a key cell cycle checkpoint during G1-S phase transition in the absence of mitotic growth signals. Cancer cell overcome Rb growth

suppression through phosphorylation and inactivation of Rb by complexes of D-type cyclins and cyclin dependent kinase (CDK)4/6.

Palbociclib (Ibrance; Pfizer) is an FDA approved selective CDK4/6 inhibitor for the treatment of estrogen receptor (ER) positive, HER2 negative advanced breast cancer. Despite the advances of such targeted therapy, patients develop resistance with long-term treatment. This circumstance demands novel combination treatment strategies to delay cancer progression and improve patient survival. A potential strategy is the combination of a unique ribonucleotide reductase subunit 2 (RRM2) inhibitor with palbociclib.

The RR inhibitor didox in combination with palbociclib significantly lowers the growth of ER positive and ER negative breast cancer and their palbociclib resistant counterparts as compared to no treatment or palbociclib treatment. We also confirmed that ER positive MCF7 and ER negative MDA-MB-468 parental breast cancer cells exhibit IC_{50} values of palbociclib that are lower than their palbociclib resistant counterparts. Inhibition of RRM2 decreases cell cycle proteins alone or in combination with palbociclib in ER positive MCF7 and ER negative MDA-MB-468 parental and palbociclib resistant breast cancer cells. This finding opens a novel approach for targeting both ER positive as well as ER negative breast cancer treatment.

RR inhibition alters cyclin D1 (CCND1), RRM2, pRb and pH2AX expression in MCF7 and MDA-MB-468 breast cancer along with their palbociclib resistant counterparts. Additionally, DDX alone or in combination with palbociclib alters the cell cycle of MCF7 and MDA-MB-468 parental and palbociclib resistant breast cancer cells as well as G1 cell cycle arrest. However, palbociclib resistant MCF7 and MDA-MB-468 cells are less resistant to palbociclib dependent G1 cell cycle arrest. Both ER positive MCF7 and ER negative MDA-MB-468 breast cancers show elevated expression of RRM2 and CCND1 which can be decreased with

RR inhibition. As well, NFκB activity is significantly decreased with RR inhibition in both ER positive MCF7 and ER negative MDA-MB-468 breast cancer cells including their palbociclib resistant counterparts. Finally, didox targets mutant p53 in both ER negative parental and palbociclib resistant MDA-MB-468 breast cancer cells but not wild type p53 in ER positive breast cancer cells MCF7 as well as its palbociclib resistant counterpart.

Neutropenia (lowering of neutrophils in blood) is the dose-limiting toxicity in patients with metastatic breast cancer. One of the limitations of this study is the measurement of palbociclib dose limiting toxicity. By characterizing palbociclib-induced neutropenia through absolute neutrophil count in mouse model would give an insight of palbociclib toxicity. Another limitation of the study is that most Western blot data were not quantified by normalization method. A third limitation of the study is the measurement of changes in cyclin dependent kinase 4/6 protein levels during the development of the resistant cell lines which would have explained the palbociclib dose dependent effect on cancer cells during resistance development.

For future studies, measuring the combination index of palbociclib and didox would help us to better understand whether synergy occurs. Since didox functions as a free radical scavenger, it would be helpful to measure reactive oxygen species (ROS) levels in breast cancer cells treated with didox alone and in combination with palbociclib. It will give us a better understanding regarding the functions of didox as a free radical scavenger. Lastly, evaluation of protein expression of RRM2, cyclin D1 and Rb in an *in vivo* model would add valuable insights regarding palbociclib resistance and effect of didox treatment.

This study presents a novel and promising approach for the treatment of breast cancer that involves inhibition of ribonucleotide reductase that merits further clinical investigation in human models.

Appendix

List of IDT Primers

Gene name	Exon location	Product	Assay name
RPL13A	-	PrimeTime Std qPCR assay	Hs.PT.49a.21531404
TP53	1-2	PrimeTime Mini qPCR assay	Hs.PT.58.39676686
TP53	3-4b	PrimeTime Mini qPCR assay	Hs.PT.58.123122
TP53	5-6	PrimeTime Mini qPCR assay	Hs.PT.58.38763224.g
TP53	11-11	PrimeTime Mini qPCR assay	Hs.PT.58.39489752.g
CCND1	1-2	PrimeTime Mini qPCR assay	Hs.PT.56a.4930170
CCND1	4-5	PrimeTime Mini qPCR assay	Hs.PT.56a.3857509
CCND1	2-3	PrimeTime Mini qPCR assay	Hs.PT.56a.23166104
CCND1	3-3	PrimeTime Mini qPCR assay	Hs.PT.56a.39100953.g
RRM2	8-9	PrimeTime Mini qPCR assay	Hs.PT.53a.19811647

List of Primary Antibody

Product	Catalog no.	Isotype	Company	Dilution Factor
Rb (4H1)	9309	Mouse IgG2A	Cell Signaling Technology	1:1000
Phospho-Rb (S807)	8516	Rabbit IgG	Cell Signaling Technology	1:1000
RRM2	SAB2701764	Rabbit IgG	Sigma Aldrich	1:1000
p53 (7F5)	2527	Rabbit IgG	Cell Signaling Technology	1:1000
Phospho-p53 (S392)	9281	Rabbit IgG	Cell Signaling Technology	1:1000
Phospho-p53 (T81)	2676	Rabbit IgG	Cell Signaling Technology	1:1000
Phospho-p53 (S15)	9286	Mouse IgG	Cell Signaling Technology	1:1000
Phospho-p53 (S6)	9285	Rabbit IgG	Cell Signaling Technology	1:1000
Phospho-p53 (S9)	9288	Rabbit IgG	Cell Signaling Technology	1:1000
Phospho-p53 (S20)	9287	Rabbit IgG	Cell Signaling Technology	1:1000
AKT (C67E7)	4691	Rabbit IgG	Cell Signaling Technology	1:1000

Phospho-AKT (S473)	4060	Rabbit IgG	Cell Signaling Technology	1:1000
Phospho-AKT (T308)	13038	Rabbit IgG	Cell Signaling Technology	1:1000
IKKα (3G12)	11930	Mouse IgG	Cell Signaling Technology	1:1000
IKKβ (D30C6)	8943	Rabbit IgG	Cell Signaling Technology	1:1000
Phospho-IKKα/β	2697	Rabbit IgG	Cell Signaling Technology	1:1000
IκBα (L35A5)	4814	Mouse IgG	Cell Signaling Technology	1:1000
Phospho-IκBα	2859	Rabbit IgG	Cell Signaling Technology	1:1000
RelB (C1E4)	4922	Rabbit IgG	Cell Signaling Technology	1:1000
c-Rel (D4Y6M)	12707	Rabbit IgG	Cell Signaling Technology	1:1000
p105/p50	12540	Rabbit IgG	Cell Signaling Technology	1:1000
p100/p52	4882	Rabbit IgG	Cell Signaling Technology	1:1000

Cyclin E2	4132	Rabbit IgG	Cell Signaling Technology	1:1000
Cyclin B1	12231	Rabbit IgG	Cell Signaling Technology	1:1000
p21	2947	Rabbit IgG	Cell Signaling Technology	1:1000
Cyclin D1	55506	Rabbit IgG	Cell Signaling Technology	1:1000
ER	8644	Rabbit IgG	Cell Signaling Technology	1:1000
GAPDH	97166	Mouse IgG	Cell Signaling Technology	1:1000
H2AX	7631	Rabbit IgG	Cell Signaling Technology	1:1000
Phospho-H2AX	9718	Rabbit IgG	Cell Signaling Technology	1:1000

List of Secondary Antibody

Product	Catalog no.	Isotype	Company	Dilution Factor
IRDye 800CW	925-32211	Goat anti-Rabbit	LI-COR	1:10000
IRDye 680RD	925-68071	Goat anti-Rabbit	LI-COR	1:10000
IRDye 800CW	925-32210	Goat anti-Mouse	LI-COR	1:10000
IRDye 680RD	025-68070	Goat anti-Mouse	LI-COR	1:10000