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Anthracyclines Attenuate the NRF1-Mediated Bounce-Back Response

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Anthracyclines Attenuate the NRF1-Mediated Bounce-Back Response

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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ARE	Antioxidant response elements
BTZ	Bortezomib
CFZ	Carfilzomib
CHX	Cycloheximide
CK2	Casein kinase 2
Dau	Daunorubicin
DDI2	DNA damage inducible 1 homolog 2
DDI2	DNA damage inducible 1 homolog 2
DMEM	Dulbecco’s modified Eagle’s medium
Dox	Doxorubicin
Epi	Epirubicin
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERAD	Estrogen receptor-associated degradation
FDA	Food and Drug Administration
HIF-1	Hypoxia-Inducible Factor 1
Ida	Idarubicin
IXZ	Ixazomib
NGLY1	N-glycanase 1
NRF1	Nuclear factor erythroid-derived 2-related factor 1
PACE	Proteasome Associated Control Elements
PSM	Proteasome subunit
qPCR	Quantitative reverse transcription PCR
TMD	Transmembrane domain

TOP2	Topoisomerase 2
UBA	Ubiquitin-associated
UBL	Ubiquitin-like
UPS	Ubiquitin Proteasome System
WT	Wild-type

ABSTRACT

Proteasome inhibitors, such as carfilzomib, are FDA-approved to treat multiple myeloma and mantle cell lymphoma. Unfortunately, proteasome inhibitors have only produced clinically significant results in patients with hematologic cancers, despite their predicted pan-cancer utility, and even hematologic cancer types frequently show intrinsic and acquired resistance.

One proposed mechanism responsible for the proteasome inhibitors' shortcomings is the NRF1-mediated bounce-back response. Identification of drugs that can potentiate the action of proteasome inhibitors could overcome resistance in patients with hematologic cancers and expand proteasome inhibitors' use to treat solid tumors. Our previous studies have identified anthracyclines as potential compounds that interfere with the bounce-back response. Here, we found the mechanistic basics of which anthracyclines inhibit the bounce-back response. Anthracyclines were found to disrupt NRF1 binding to the antioxidant response element (the sequence where NRF1 binds to the DNA) of its targets, proteasome subunit genes. This disruption attenuated NRF1's ability to activate proteasome genes in response to proteasome inhibition, impeding the bounce-back response and increasing the duration of proteasome inhibition experienced by cells. Finally, our work provides a mechanistic explanation behind the NRF1 and anthracyclines interaction in *vitro* and could prompt future preclinical and clinical studies to further investigate CFZ and anthracyclines as combinational therapy.

CHAPTER 1: Introduction

1.1The Ubiquitin Proteasome System

1.1.1 Functions of the ubiquitin proteasome system

In eukaryotes, the majority of intracellular proteins are continually being hydrolyzed into their basic amino acids and replaced by newly synthesized proteins (Lecker et al., 2006). Removal of damaged, mutant, misfolded, or unwanted proteins is essential for the maintenance of cellular proteostasis, health, and survival (Hershko & Ciechanover, 1998; Kleiger & Mayor, 2014; Lecker et al., 2006). The highly selective and tightly regulated ubiquitin proteasome system (UPS) is responsible for the degradation of proteins that are destined for destruction (Hershko & Ciechanover, 1998). Functions of the UPS are involved in essentially every cellular process, including cell cycle progression, regulation of transcription factors and the transcription of their target genes, immune response, and apoptosis (Hershko & Ciechanover, 1998; Kleiger & Mayor, 2014; Lecker et al., 2006; Thibaudeau & Smith, 2019). Given the role of UPS in maintaining the balance in the rate of degrading unneeded proteins and preventing the untimely degradation of needed proteins, the UPS is critical in all cellular processes due to its ability in removing or protecting proteins that function as positive or negative regulators of cellular processes and pathways (Kleiger & Mayor, 2014). The overall rates of protein synthesis and degradation must be maintained because any unnecessary decrease or increase of the two functions can cause cell death and, ultimately, tissue and body mass loss (Mitch & Goldberg, 1996).

1.1.2 The 26S proteasome

The 26S proteasome is a multi-catalytic protease complex composed of about 33 subunits assembled into a barrel-like structure with two major cores: the 20S catalytic core and the 19S regulatory core (**Figure 1**). The 20S is the catalytic part where protein substrates are degraded and contains two outer alpha rings and two inner beta rings. Each ring comprises

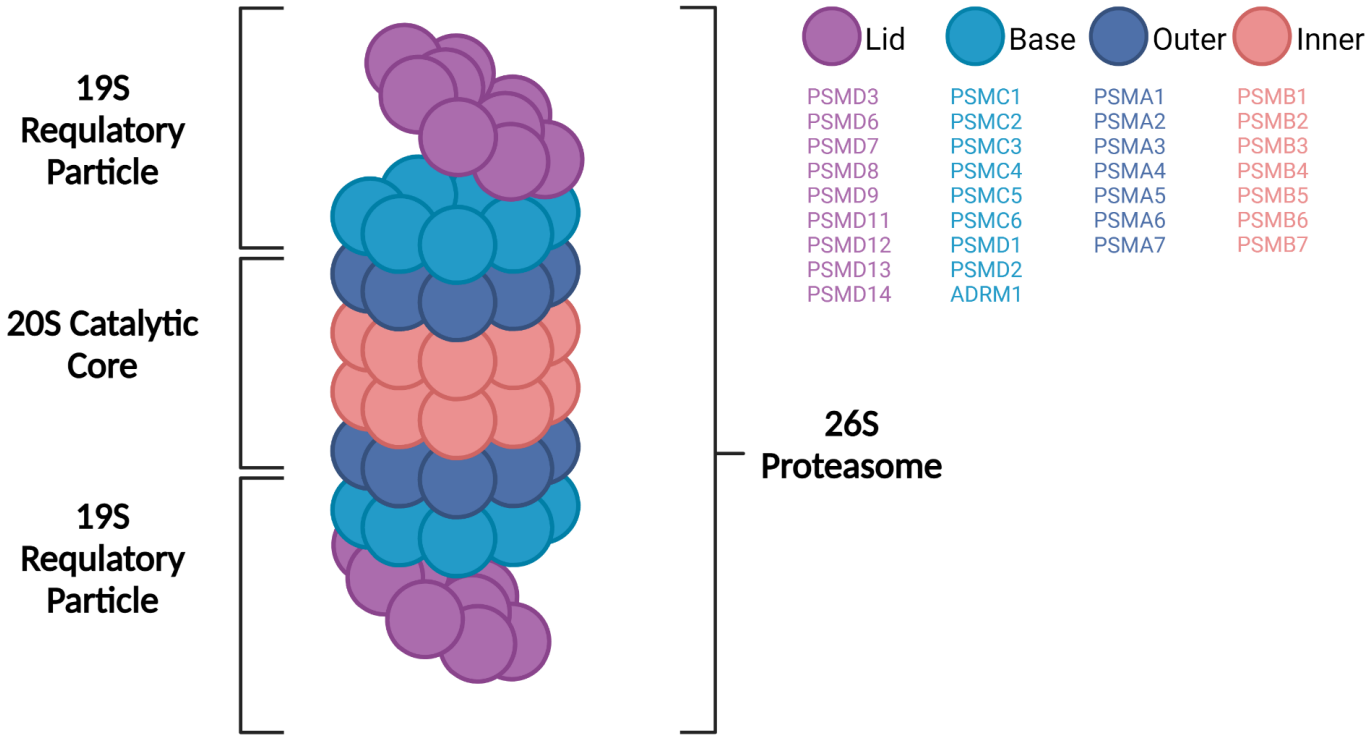

Figure 1: The 26S proteasome. Schematic of the 26S proteasome subunits and associated gene signatures subdivided into the inner and outer rings of the 20S catalytic core and the base and lid of the 19S regulatory particle.

seven protein subunits encoded by PSMA1-7 for alpha and PSMB1-7 for beta (Tomko & Hochstrasser, 2013). The Alpha rings act as gates where proteins enter or exit the catalytic sites within beta rings. Each of the two Beta rings contains three protease subunits, β1, β2, and β5, encoded by the PSMB1, PSMB2, and PSMB5 genes, respectively. In addition, the three catalytically active β-subunits are responsible for the caspase-like, trypsin-like, and chymotrypsin-like, respectively (Ben-Nissan & Sharon, 2014; Tomko & Hochstrasser, 2013).

The 19S regulatory core caps both ends of the 20S proteasome and controls the binding, deubiquitylation, and translocation of substrates into the 20S core (Tomko & Hochstrasser, 2013). The 19S comprises at least 19 subunits forming two sub-complexes: the lid and base. The lid consists of eight structural subunits encoded by PSMD3, PSMD6-9, and PSMD11-13, in addition to one deubiquitinating subunit encoded by PSMD14 (Bard et al., 2018; Tomko & Hochstrasser, 2013). The base includes six ATPase and three non-ATPase structural

subunits. PSMC1-6 encodes the ATPase subunits, and the non-ATPase subunits are encoded by ADRM1 and PSMD1-2 [8].

Adding polyubiquitin chains to proteins promotes recognition and degradation by the 26S proteasome. Many ubiquitinated proteins can interact with the proteasome through ubiquitin shuttle proteins. Both ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains allow shuttle proteins to bind to ubiquitinated proteins and deliver them to the ubiquitin-interacting subunit of the 19S regulatory core to facilitate their destruction (Bard et al., 2018). Sometimes, loosely folded proteins can be recognized and degraded by the 20S catalytic core without needing the 19S regulatory core in a ubiquitin-independent manner. Typical targets of ubiquitin-independent degradation are proteins with unstructured regions due to oxidation damage or mutations (Ben-Nissan & Sharon, 2014).

1.2 Proteasome inhibition as a cancer therapeutic

1.2.1 The ubiquitin proteasome system in cancer and proteasome inhibitors

Cancer cells' genomes tend to accumulate many genetic abnormalities, including point mutations, duplications, and deletions. It is estimated that more than 90% of solid tumors have cells with more than two copies of some chromosomes, leading to increased protein levels in these cells. These abnormalities would also increase the total load of mutant, misfolded proteins in cancer cells, making them more dependent on the UPS to degrade the increased load to maintain proteostasis and survival of cancer cells (Deshaies, 2014). This idea of increased dependency on proteasome function led to abundant work conducted to target the UPS, using proteasome inhibitors, as a potential cancer therapy (Dou & Zonder, 2014). Up to date, there are three proteasome inhibitors approved by the Food and Drug Administration (FDA): Bortezomib (BTZ) approved in 2003, Carfilzomib (CFZ) approved in 2012, and Ixazomib (IXZ) approved in 2015.

1.2.2 Bortezomib

Bortezomib is a small dipeptide boronic acid that binds reversibly to the chymotrypsin-like β5 subunit of the 20S proteasome. Compared to earlier generations of proteasome inhibitors, bortezomib showed high specificity to the 20S proteasome and less binding to other cellular proteases. After only seven years of its synthesis, bortezomib was approved by the FDA for treating multiple myeloma patients (Chen et al., 2011). Bortezomib is administered intravenously and quickly distributed into tissues, not including adipose and brain tissues, within 10 minutes (Chen et al., 2011). It operates as a reversible inhibitor of the 26S proteasome, where its maximum inhibition of 20S activity occurs within 1 hour after bortezomib administration and is undetectable within 72 to 96 hours (Schwartz & Davidson, 2004). Although bortezomib has shown promising antitumor activity in multiple myeloma, significant clinical responses were only observed in less than half of the patients, with no one having been cured of the disease using bortezomib (McConkey & Zhu, 2008). Depending on the cancer type, stage, and treatment history, bortezomib is usually administered once or twice weekly.

1.2.3 Carfilzomib

Carfilzomib is an epoxyketone-based proteasome inhibitor that binds irreversibly to the β5 subunit within the inner ring of the 20S catalytic core (Kuhn et al., 2007). A phase III clinical trial in patients with relapsed or refractory multiple myeloma found that treatment with carfilzomib and dexamethasone increased the median overall survival to 47.6 months compared to 40.0 months the bortezomib and dexamethasone treatment, yet, serious adverse events were higher in the carfilzomib group 59% compared to 40% of the bortezomib group (Dimopoulos et al., 2017). Carfilzomib still did not overcome the shortcomings of bortezomib, and both have not shown positive results in solid tumors (Demo et al., 2007). In 2012, the FDA approved the use of carfilzomib, as a single agent or in combination with lenalidomide and dexamethasone, for the treatment of adult patients with relapsed or refractory multiple myeloma who have received

one or more lines of therapy.

1.2.3 Ixazomib

Ixazomib, the third and latest FDA-approved proteasome inhibitor, also binds to the β5 subunit within the 20S catalytic core of the 26S proteasome. Ixazomib reversibly binds to its target and has the advantage of being the first orally administrated proteasome inhibitor (Dou & Zonder, 2014). Ixazomib showed improved pharmacokinetics, pharmacodynamics, and antitumor activity in preclinical models of human cancer compared with bortezomib (Dou & Zonder, 2014; Kupperman et al., 2010). Ixazomib combined with dexamethasone and lenalidomide is currently FDA-approved for patients with multiple myeloma who have previously received other treatments.

1.2.4 Next-generational proteasome inhibitors

Marizomib, oprozomib, and delanzomib are three other proteasome inhibitors that have previously or are currently being tested in clinical trials. According to clinicaltrials.gov, marizomib and delanzomib have been tested in clinical trials for both hematologic and solid tumors. Marizomib, also known as salinosporamide A, is a naturally-occurring, irreversible inhibitor of all the three protease subunits in the 20S catalytic core, and it is administered orally and able to cross the blood-brain barrier (Di et al., 2016; Feling et al., 2003). It is more effective than other inhibitors in treating relapsed, refractory multiple myeloma as a combination or monotherapy with fewer side effects (Spencer et al., 2018). Oprozomib has been investigated in solid tumors and newly diagnosed and relapsed multiple myeloma. It is the first orally available irreversible proteasome inhibitor (Chauhan et al., 2010; Zhou et al., 2009). Delanzomib is a reversible proteasome inhibitor that has increased binding to the chymotrypsin-like β5 and trypsin-like β1 subunits compared to bortezomib (Dorsey et al., 2008).

1.3 The proteasome bounce-back response

1.3.1 The history of the proteasome bounce-back response

One of the shortcomings of proteasome inhibitors is that they are only FDA-approved for treating multiple myeloma and mantle cell lymphoma (Dou & Zonder, 2014; McConkey & Zhu, 2008; Thibaudeau & Smith, 2019). Even when proteasome inhibitors are used in these cancer types, intrinsic and acquired resistance are common (Sherman & Li, 2020). Combination therapy comprising a proteasome inhibitor and a drug targeting a resistance mechanism could potentially enhance proteasome inhibitors' efficiency in hematologic cancers and expand their profile to treat solid tumors (Sherman & Li, 2020).

One potential mechanism for proteasome inhibition resistance is the highly conserved proteasome bounce-back response. Studies using yeast, *Drosophila,* and human cells revealed that the essential genes encoding proteasomal subunits are under coordinated transcriptional control (Dohmen et al., 2007). This idea was first observed in *Saccharomyces cerevisiae,* where a specific sequence element term PACE (Proteasome Associated Control Elements) was found in the promoter of almost all the genes of proteasomal subunits (Dohmen et al., 2007; Xie & Varshavsky, 2001). Rpn4 was identified as the transcriptional activator that binds to the PACE sequence and facilitates the transcription of proteasome subunits (Xie & Varshavsky, 2001). Interestingly, Rpn4 is also a short-lived substrate of the 26S proteasome (Dohmen et al., 2007). Inhibition of proteasome activity leads to stabilizing Rpn4, de novo proteasome assembly, and rescuing proteasome activity (Xie & Varshavsky, 2001). This pathway yields a negative feedback loop in which the same protein (Rpn4) up-regulates the proteasome production and is degraded by the assembled active proteasome (Xie & Varshavsky, 2001).

Like Rpn4 in yeast, the Cnc-C protein mediates the proteasome bounce-back response in *Drosophila* (Grimberg et al., 2011). The bounce-back response is also conserved in mammals and is mediated by the transcription factor nuclear factor erythroid-derived 2-related factor 1 (NRF1) of the cap ‘n’ collar basic leucine zipper family (Radhakrishnan et al., 2010). NRF1

resides in the endoplasmic reticulum (ER) membrane and translocates to the nucleus to function as a transcription factor via the activation of the NRF1 pathway (**Figure 2**).

1.3.2 NRF1 in the endoplasmic reticulum

Under unperturbed conditions, the full-length precursor form of NRF1 (p120) is inserted into the ER membrane via the classical Sec61-dependent pathway (Steffen et al., 2010). The majority of the ER-embedded NRF1 C-terminus polypeptides reside in the ER lumen, and a small portion of the N-terminus hangs in the cytosol (Radhakrishnan et al., 2014). The $C_{lumen}/N_{cytosol}$ orientation, type II membrane orientation, is facilitated by the N-terminal homology box-1 transmembrane domain (TMD) of NRF1 that is enriched with hydrophobic residues. Under unperturbed conditions, where proteasomes are active, NRF1 is degraded by the ER-associated degradation (ERAD), a pathway that specializes in the turnover of misfolded ER proteins. The ERAD pathway includes the ubiquitination of NRF1 and retrotranslocation of the C-terminus by homo-hexameric AAA ATPase p97 to the cytosol, where it will be degraded by the proteasome (Radhakrishnan et al., 2014).

1.3.3 NRF1 in the cytosol

In the cytosol, NRF1 is either degraded by active proteasomes just like any typical ERAD substrate or, when proteasomes are inhibited, NRF1 is cleaved to its active form and translocated to the nucleus. In the case of proteasome inhibition and NRF1 not being degraded in the cytosol, NRF1 N-terminus gets deglycosylated by the enzyme N-glycanase 1 (NGLY1), a co-factor of p97 (Tomlin et al., 2017). Then, an essential step of NRF1 activation is the cleavage between Trp103 and Leu104 by the protease DNA damage inducible 1 homolog 2 (DDI2) (Koizumi et al., 2016). This cleavage converts the precursor p120 NRF1 to the active p110 form

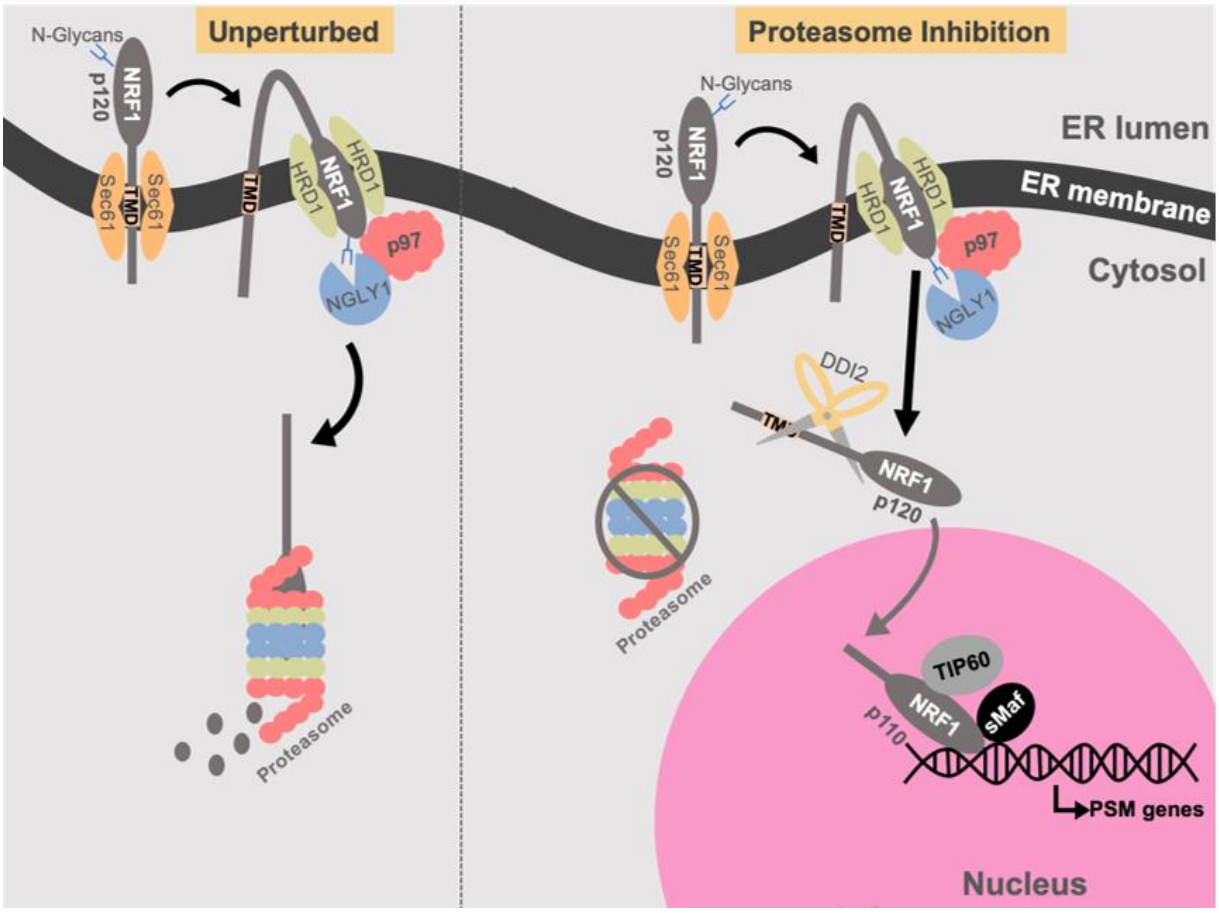

Figure 2: The NRF1 pathway. Modified from (Northrop, Byers, et al., 2020).

that can translocate to the nucleus.

1.3.4 NRF1 in the nucleus

In the nucleus, many factors regulate the activity of the p110 form of NRF1. Some of these regulators are the small-Maf proteins (MafF, MafG, and MafK) (Johnsen et al., 1996). The bZIP domain of NRF1 allows it to heterodimerize with one of the small-Mafs. This heterodimer will then be able to bind the antioxidant response elements (ARE) found on the promotor region of PSM genes and other target genes.

The activity of NRF1 in the nucleus is also regulated by glycogen synthase kinase 3 (GSK3), which interacts with and phosphorylates the Cdc4 phosphodegron domain (CPD) in

NRF1 (Biswas et al., 2013). Phosphorylated CPD enables the F-box protein Fbw7 binding, which promotes the ubiquitination and degradation of NRF1 via the proteasome (Biswas et al., 2011). Casein kinase 2 (CK2) has also been shown to phosphorylate NRF1 at residue Ser497 to decrease the transcriptional activity of NRF1 (Tsuchiya et al., 2013). More recently, TIP60 chromatin-regulatory complex has been found to be an essential regulator for the NRF1 transcriptional activation of PSM genes after proteasome inhibition (Vangala & Radhakrishnan, 2019).

1.3.5 Targeting the NRF1-mediated proteasome bounce-back response

Proteasome inhibitors have not been able to show clinically significant outcomes in solid tumors. Even when used to treat multiple myeloma and mantle cell lymphoma, some patients fail to respond and relapse. One way to increase the efficiency of proteasome inhibitors and overcome their shortcomings is by understanding their intrinsic and acquired resistance mechanisms and targeting them. One important resistance mechanism is the NRF1-mediated proteasome bounce-back response.

Since many proteins are involved in the NRF1 pathway, theoretically, inhibition of any of these proteins could decrease NRF1 activity and increase the efficiency of proteasome inhibitors. Indeed, chemical or genetic inhibition of p97, NGLY1, TIP60, or DDI2 blocked the NRF1-mediated bounce-back response (Koizumi et al., 2016; Radhakrishnan et al., 2014; Tomlin et al., 2017; Vangala & Radhakrishnan, 2019). NYGLY1 and P97 chemical inhibitors were used in chronic myelogenous leukemia and cervical cancer cells, respectively, and both have increased the efficiency of proteasome inhibitors in killing cancer cells (Auner et al., 2013; Tomlin et al., 2017). However, both have failed FDA approval as a single agent or in combination with proteasome inhibitors for cancer treatment.

Following the notion of combinational therapy, where the proteasome and the NRF1-mediated bounce-back response are inhibited simultaneously, the binding mechanism of

proteasome inhibitors is important to consider. Reversible inhibition would only inhibit the proteasomes for a short time, and then, cells can recover their proteasome activity via the disassociation of inhibited proteasome from proteasome inhibitors or via the bounce-back response (Radhakrishnan et al., 2010). However, in the case of the irreversible binding mechanism, the only way to recover proteasome activity is via the bounce-back response (Radhakrishnan et al., 2010). The only FDA-approved irreversible proteasome inhibitor, carfilzomib, has been shown to be cleared within an hour of administration from patient blood, preventing inhibition of newly synthesized proteasome from the bounce-back response, thus limiting the overall duration of proteasome inhibition experienced by cells (Radhakrishnan et al., 2010; Wang et al., 2013). Combining carfilzomib with genetic or chemical inhibition of the bounce-back response in *vitro* has shown an increase in the duration of proteasome inhibition and cell death in triple-negative breast cancer (TNBC), osteosarcoma, and mouse fibroblasts cell lines (Radhakrishnan et al., 2010; Vangala & Radhakrishnan, 2019).

1.4 Anthracycline

1.4.1 The history of Anthracyclines

In the 1960s, daunorubicin, belongs to a class of compounds with similar structures called anthracyclines (**Figure *2***), was obtained from a soil bacterium called *Streptomyces peucetius*. Daunorubicin has shown great preclinical antitumor activity against animal tumors, and clinical activities were soon observed in pediatric solid tumors, acute lymphoblastic, and myeloid leukemia (Booser & Hortobagyi, 1994). A few years after the discovery of daunorubicin, doxorubicin, also called adriamycin, was isolated from a mutant *S. peucetius* and has shown better preclinical results than daunorubicin (Arcamone et al., 1969; Wilkinson & Mawer, 1974). In the 70s and 80s, several anthracyclines have developed, such as epirubicin and idarubicin. Nowadays, anthracyclines are widely used as a single agent or in combinational therapy to treat many types of cancers, including acute lymphoblastic leukemia, metastatic neuroblastoma,

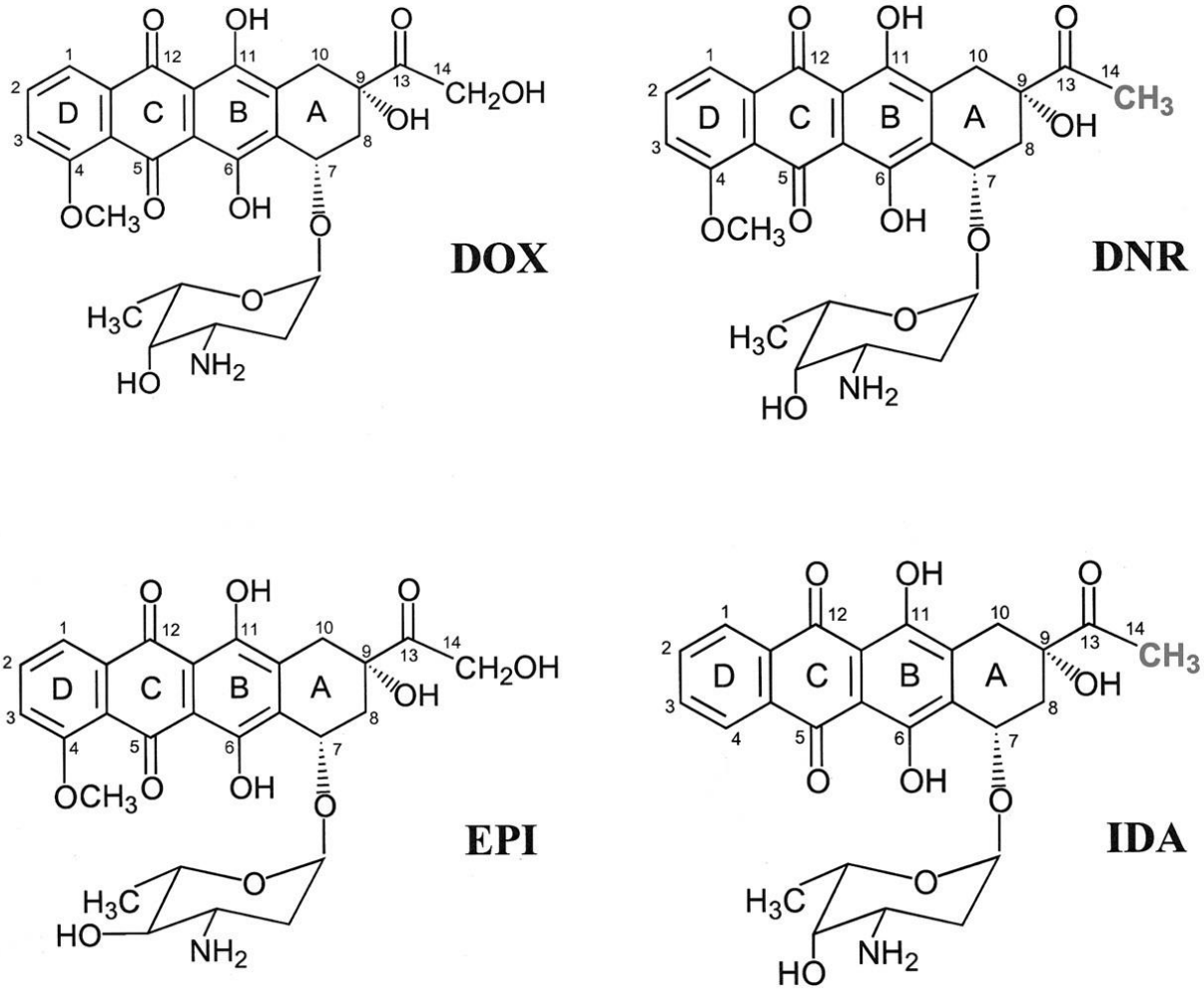

Figure 3: Chemical structures of commonly used Anthracycline Antibiotics.

ovarian carcinoma, and metastatic breast cancer.

1.4.2 Mechanisms of Action

Despite the wide clinical usage of anthracyclines such as doxorubicin, the molecular mechanism of how they induce cell death is still unclear. Several mechanisms have been proposed to explain the doxorubicin mechanism of action. Some proposed mechanisms, such as inhibition of DNA and RNA synthesis, were only seen at doses higher than recommended clinical dose (40 to 75 mg/m2) (Gewirtz, 1999). The most common proposed mechanisms for

doxorubicin antitumor activity are Topoisomerase II poisoning and the generation of free radical species (Pommier et al., 2016). Topoisomerases are highly conserved ATP-dependent enzymes that play critical roles in DNA replication, transcription, and genomic stability (Pommier et al., 2016). Human topoisomerases are classified based on their structure and mechanism into two classes: monomeric type I enzymes (TOP1), which cleave a single strand of DNA, and dimeric type II enzymes (TOP2), both strands (Pommier et al., 2010). TOP2 is further subdivided into TOP2α and TOP2β. Both are essential to relax negatively supercoiled DNA, but whereas TOP2α is needed in chromosome segregation, TOP2β is critical for transcription in non-dividing cells (Pommier et al., 2016). Recent work suggests that anthracyclines target topoisomerases, especially TOP2, causing cell death (Nitiss, 2009; Pommier et al., 2016). The general pathway of TOP2 is that they bind to supercoiled and entangled DNA, break both strands of one DNA duplex, pass the other duplex through the resulting gap, and re-ligate the break (Pommier et al., 2010). This process results in the release of the torsional stress of DNA formed by replication and transcription. Drugs that target TOP2, such as anthracyclines, preferentially intercalate into the DNA and inhibit TOP2 from re-ligating the DNA, increasing the stability of TOP2:DNA complexes and thus inducing DNA damage to therapeutically kill cancer cells (Pommier et al., 2010).

1.4.3 Combination therapy of proteasome inhibitors and anthracyclines

Currently, proteasome inhibitors are only FDA-approved for multiple myeloma and mantle cell lymphoma treatment and have not shown consistent antitumor activity in solid tumors, such as breast cancer. As discussed earlier, the NRF1-mediated bounce-back response is one of the main reasons people with multiple myeloma and mantle cell lymphoma show intrinsic and acquired resistance. Finding a way to block the NRF1 pathway could increase the response rates and positive outcomes of proteasome inhibitors treatment in patients with FDA-indicated tumors, as well as expand the range of tumors to be treated with

proteasome inhibitors, including solid tumors such as breast cancer.

A screen of a library of compounds was done in the lab to find drugs that can inhibit the bounce-back response, which might be an excellent candidate for a combinational therapy with carfilzomib to increase the duration of proteasome inhibition experienced by cancer cells and overcome carfilzomib shortcomings. The screen has shown that anthracycline compounds, such as doxorubicin, epirubicin, and idarubicin, were able to inhibit the NRF1-mediated bounce-back response. A drug that can inhibit the bounce-back response might be a good candidate for a combinational therapy with carfilzomib to increase the duration of proteasome inhibition experienced by cancer cells.

1.5 Overview of Thesis

1.5.1 Goals of this research

The research presented in this thesis aimed to evaluate the carfilzomib and anthracyclines as a potential combinational therapy to increase the duration of proteasome inhibition in preclinical models, where anthracyclines would inhibit the NRF1-mediated bounce-back response during proteasome inhibition. Inhibiting the bounce-back response could overcome resistance to proteasome inhibitors in patients with multiple myeloma and mantle cell lymphoma, as well as expand the utility of proteasome inhibitors in treating solid tumors, such as breast cancer. Therefore, in this thesis, we investigated the pathway of NRF1 during proteasome inhibition in the presence of anthracyclines to gain an understanding of the mechanistic basics of how anthracyclines attenuate the NRF1-mediated bounce-back response.

Chapter 2: Materials and methods

2.1 Cell lines and culture conditions

NIH-3T3 and MDA-MB-231 cell lines and their derivatives were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and penicillin and streptomycin (Invitrogen) at 37 °C in a humidified incubator with 5% CO2.

2.2 Quantitative reverse transcription PCR

RNeasy kit with DNase treatment (Qiagen, Germantown, MD, USA) was used to isolate RNA from cell pellets. The iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) was used to convert 1µg of RNA to cDNA. Quantitative reverse PCR (qPCR) was performed with iTaq universal SYBR green supermix (Bio-Rad, Hercules, CA, USA) in the C1000 Touch Thermal cycler (Bio-Rad, Hercules, CA, USA). Data were analyzed using CFX manager 3.1 (BioRad, Hercules, CA, USA). Levels of 18S expression were used for normalization. The forward and reverse primers used for the qPCR reactions are listed in **Table 1**.

2.3 Western blot analysis

Cells were washed with ice-cold PBS, and pellets were collected with radioimmune precipitation assay lysis buffer (50 mm Tris-HCl (pH 8.0), 150 mm NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease and phosphatase inhibitor mixture (Thermo Fisher Pierce). After that, cells were incubated on ice for 10 minutes, followed by high-speed centrifugation at 14000 rpm for 20 minutes. Total protein was quantified by Bradford reagent. Typically, 20 µg of protein was used for SDS-PAGE, followed by electrotransfer onto polyvinylidene difluoride membranes. The membranes were then blocked for 1 h with 5% nonfat dry milk powder in TBS with Tween and then incubated with the

Table 1: Forward and reverse primers used for the qPCR.

Gene	Froward Primer (5'-3')	Reverse Primer (5'-3')
Human PSMA7	CTGTGCTTTGGATGACAACG	CGATGTAGCGGGTGATGTACT
Human PSMB7	TGCAAAGAGGGGATACAAGC	GCAACAACCATCCCTTCAGT
Human PSMC4	GGAAGACCATGTTGGCAAAG	AAGATGATGGCAGGTGCATT
Human PSMD12	GTGCGCGACTGACTAAAACA	TAGGCAGAGCCTCATTTGCT
Human 18S rRNA	ATGGCCGTTCTTAGTTGGTG	CGCTGAGCCAGTCAGTGTAG
Mouse PSMA7	AACGTCTGTATGGCCTTTGC	GTCACTGGGTCCTCCACTGT
Mouse PSMB7	CTGTCTTGGAAGCGGATTTC	GCAACAACCATCCCTTCAGT
Mouse PSMC4	TGGTCATCGGTCAGTTCTTG	CGGTCGATGGTACTCAGGAT
Mouse PSMD12	TCACAGACCTGCCAGTCAAG	AGGTTTTAGTCAGCCGAGCA
Mouse 18S rRNA	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC

appropriate primary antibodies overnight at 4 °C, followed by a secondary antibody at room temperature for an hour. The antibodies used were specific for NRF1 (1:1000), P-CHK1 (1:1000), GAPDH (1:10,000), Lamin A/C (1:1000) (all from Cell Signaling Technology, Danvers, MA, USA); Calnexin (1:10,000, Santa Cruz Biotechnology, Dallas, TX, USA). The secondary antibodies used were rabbit IgG horseradish peroxidase and mouse IgG horseradish peroxidase (1:10,000, both from Bio-Rad). Band signal intensities were quantified using Image Studio Lite.

2.4 Proteasome activity recovery assay

About 90% of chymotrypsin-like proteasome activity of cells was inhibited by treatment with 40 nM carfilzomib for 1h, followed by three times washing with PBS to remove residual CFZ. Then, cells were allowed to recover in a fresh medium with or without Anthracyclines. At 0, 4, 8, 12, and 24h time points, cells were washed and frozen at -80 °C in TE buffer (20 mM Tris pH 8.0 and 5 mM EDTA). To measure chymotrypsin-like proteasome activity, the lysates obtained by freeze-thaw lysis were incubated with succinyl-Leu-Leu-Val-Tyr-amino-4-methylcoumarin (Suc-LLVY-AMC), and the resulting fluorescence was measured at 360/460 nm excitation/emission. Then, the fluorescence values were normalized by cell number, quantified using a Cell-Titer Glo kit (Promega, Madison, WI, USA), which measures the ATP levels in the cell.

2.5 Subcellular fractionation

The Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific, Waltham, MA, USA) was used to isolate membrane-bound, cytosolic, and nuclear proteins in separate fractions. The protocol was followed per the manufacturer's instructions with added washes of the cell pellet with CEB after the cytosolic protein isolation and MEB after the membrane-bound protein isolation. After the MEB wash, NEB was used to lyse the remaining pellet containing the

nuclear proteins. CEB and MEB were used at equal volumes, while NEB was used at half of the CEB and MEB volumes. Calnexin, Lamin A/C, and GAPDH were used as fractionation controls for membrane-bound, nuclear, and cytosolic proteins, respectively, in the western blots. Band signal intensities were quantified using Image Studio Lite.

2.6 Chromatin immunoprecipitation

NIH-3T3 mouse fibroblast cells stably expressing NRF1 tagged with HA at the N-terminus and Flag at C-terminus (NIH-3T3-HA-NRF1-Flag). Cells were then grown in 15 cm plates and collected using Trypsin-EDTA (0.25%). Cells were pelleted and washed twice with PBS. Five million cells were then fixed with freshly prepared formaldehyde solution (1% final volume) and incubated at room temperature for 7 min, followed by adding 0.125M glycine to quench the formaldehyde. Pellets were washed twice with ice-cold PBS and collected in PBS supplemented with a protease inhibitor mixture. Pellets were collected after centrifugation at 3000 × g at 4 °C. The Zymo-spin ChIP kit D5210 (Zymo Research) was used for further steps according to the manufacturer's protocol. Briefly, cell pellets were resuspended in Nuclei Prep Buffer, centrifuged, and the supernatant discarded. Then, cells were resuspended in Chromatin Shearing Buffer and used for chromatin shearing with Covaris M220 (10% duty factor for 10 min). Sheared chromatin was centrifuged at 12,000 × g for 5 min, and the supernatant was collected. The chromatin equivalent of 1 million cells was used for each pulldown. The ab used for overnight pulldown were DYKDDDDK Tag, IgG, and Tri-Methyl-Histone H350 (All from Cell Signaling). 15 µl ZymoMag Protein A was added to each ChIP reaction and incubated for 1h at 4 °C while rotating. Beads were then washed in order with Chromatin Wash Buffers I, II, and III. Reverse cross-linking of ChiP DNA was done at 65 for 30 min, followed by DNA purification using the Zymo-spin ChIP kit. Quantitative PCR was used to analyze the chromatin. Primers used for analysis are shown in **Table 2**.

Table 2: Forward and reverse primers used for the ChIP-qPCR

Gene	Froward Primer (5'-3')	Reverse Primer (5'-3')
Mouse PSMC4	GATCTCTGAAGTGTTTGAAG	GAAATAAGCTTTATTTAGTC
Mouse PSMD1	GCATAAAAGACACAGCGTGG	ATCCACTCGTCCACAGTCCT
Mouse PSMD12	GCCCATTCTTGGGCCTGCC	ATCGGCCCGCTCCGAGCCA

Chapter 3: Results

3.1 Anthracyclines as potential inhibitors of the bounce-back response

Previous work in the lab utilized a cell-based screening system using the WT NIH-3T3 mouse fibroblasts (Vangala & Radhakrishnan, 2019). The cell line was engineered to stably express firefly luciferase under the control of 8x ARE (the DNA sequence where NRF1 is known to bind), and as a control, the cells also express *Renilla* luciferase driven by the human phosphoglycerate kinase (hPGK) promoter (**Figure 4A**). The resulting system is referred to as WT 8xARE-luc. As expected, treating these cells with the proteasome inhibitor CFZ resulted in a dose-dependent increase in luciferase activity. However, when CFZ was combined with an inhibitor of the NRF1 pathway, NMS-873 (p97 inhibitor, also known to inhibit the bounce-back response), the CFZ-induced increased luciferase activity was attenuated (Vangala & Radhakrishnan, 2019). The WT 8xARE-luc system was then used to screen the NIH Clinical Collection and Natural Products Set libraries looking for compounds that can interfere with the NRF1 pathway and attenuate the CFZ-induced increased luciferase activity (Vangala & Radhakrishnan, 2019). Anthracycline compounds like doxorubicin, epirubicin, and daunorubicin were found to inhibit the CFZ-induced increase in luciferase activity, which means that this class of compounds can interfere with the NRF1 pathway and could be used as combinational therapy along with CFZ to inhibit the NRF1-mediated bounce-back response (**Figure 4B**).

3.2 Anthracyclines attenuate CFZ-Mediated Nrf1-Dependent PSMs transcription

Our experiments used the NIH-3T3 mouse fibroblasts and MDA-MB-231 Triple-negative breast cancer (TNBC) cell lines. We chose the NIH-3T3 cell line for the mechanistic experimentation because NRF1 migrates as discrete p120 and p110 bands by immunoblot, making it easier for interpretation. In contrast, in human cells, there is an additional presence of TCF11 (an isoform of NRF1 with additional 30 amino acids), which complicates the visualization of the two bands of NRF1 (Radhakrishnan et al., 2014). In general, breast cancer data show

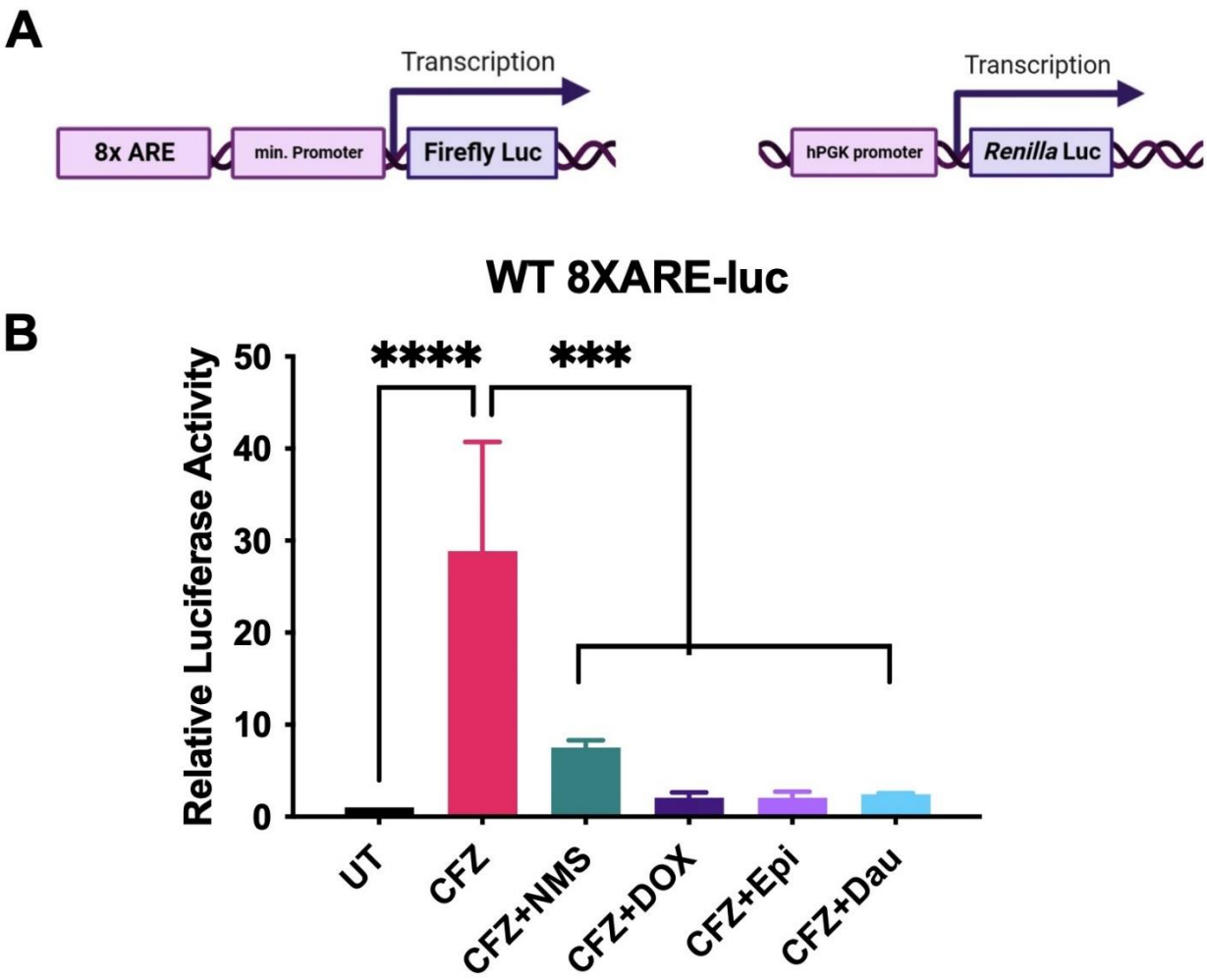

Figure 4: Anthracyclines attenuate the NRF1-mediated bounce-back response. (**A**) Schematic of the reporter construct used in the cell-based screening system. This lentiviral reporter construct expressed firefly luciferase under the control of 8xARE upstream of a minimal promoter. along with Renilla luciferase driven by the hPGK promoter. (**B**) WT 8xARE-Luc cells were treated with 200 nm CFZ alone or in combination with 10μm of NMS-873, Dox, Epi, or Dau and compared with the DMSO-treated control for 16 h. The cell lysates were then used for luciferase assays. Normalized luciferase activity is shown. The experiments were performed three independent times and error bars denote S.D. (n = 3). One-Way Anova test followed by Dunnett's multiple comparisons was used to compare relative luciferase activity.

higher proteasome activity correlated with increased levels of PSM subunits compared to other cancer types. More specifically, TNBC show increased sensitivity to proteasome inhibitors compared to other breast cancer subtypes (Berger et al., 2018). For this reason, the MDA-MB-231 cell line was used in some experiments.

To explore the mechanism of how anthracyclines attenuate the bounce-back response, we first sought to confirm that anthracyclines interfere with NRF1 transcriptional activity in the NIH-3T3 and MDA-MB-231 cell lines. Therefore, we investigated the changes in proteasome subunits (PSM) genes transcription during proteasome inhibition with CFZ in the presence of anthracyclines such as doxorubicin (Dox), epirubicin (Epi), and idarubicin (Ida), as well as the control NMS-873. We found that in both NIH-3T3 and MDA-MB-231 cell lines, CFZ treatment alone resulted in robust induction of representative PSM genes, PSMA7, PSMB7, PSMC4, and PSMD12. Additionally, confirming the screening results, the CFZ-induced increase in PSMs levels was completely abolished when either NMS-873, Dox, Epi, or Ida was added along with CFZ (**Figure *5***). These results suggest again that anthracyclines inhibit the NRF1-mediated transcription of PSM genes.

3.3 Anthracyclines do not affect the protein levels or the spatiotemporal aspects of NRF1

To further our investigation, we wanted to see if the anthracyclines affect the protein levels of the p120 (precursor) and p110 (proteolytically-processed active form) of NRF1. Therefore, we treated NIH-3T3 cells with CFZ alone and CFZ combined with NMS-873, Dox, Epi, or Ida for 8h hours. We found a robust increase in the p110 protein levels in the CFZ alone treatment. NMS-873 inhibits the retrotranslocation of NRF1, preventing it from being cleaved to its active form, and as expected, we only saw an accumulation of the p120 band, not the p110. CFZ combined with Dox, Epi, or Ida showed no significant changes in both NRF1 forms compared to the CFZ alone treatment (**Figure 6**). These results indicate that although

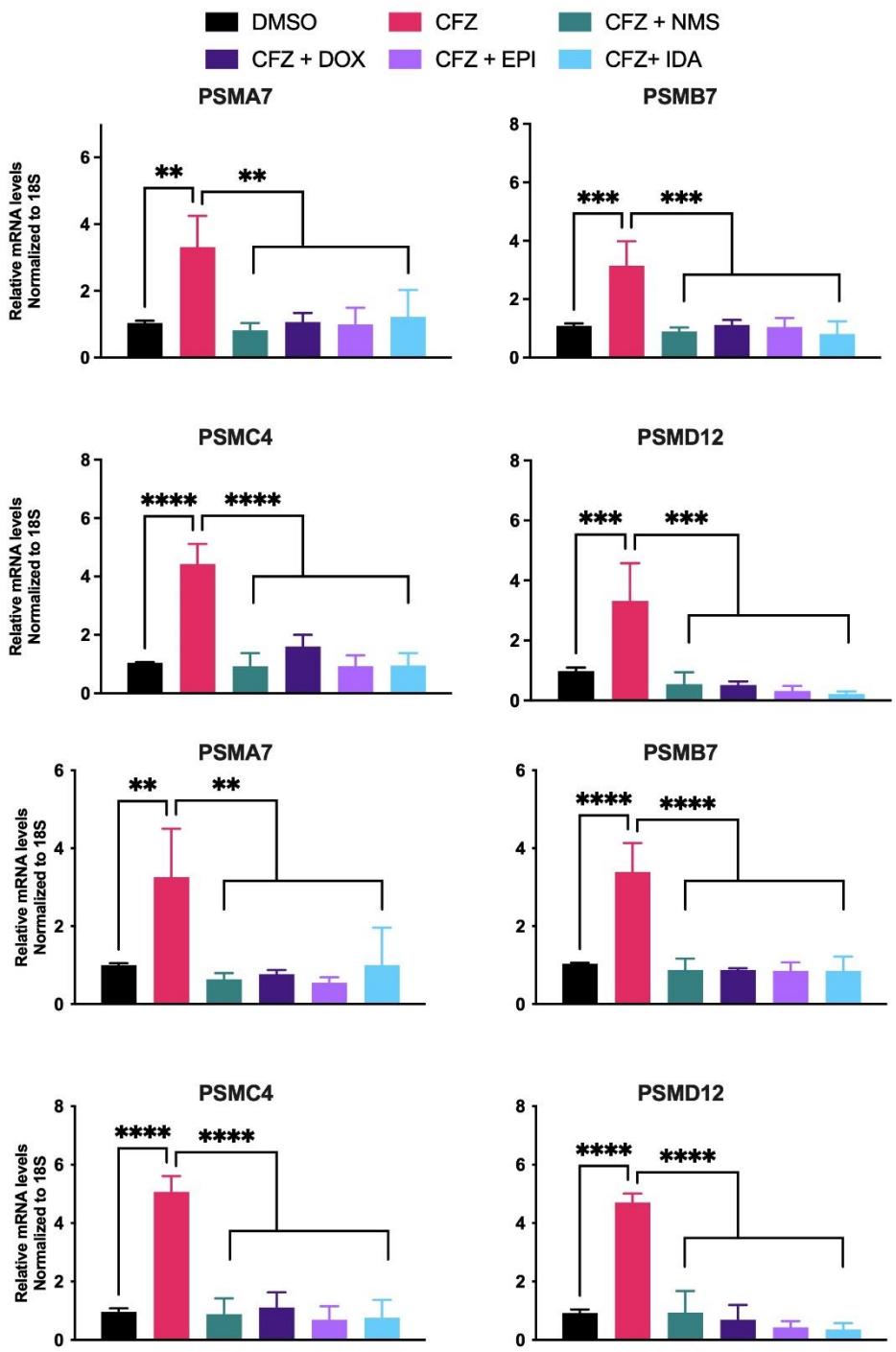

Figure 5: Anthracyclines attenuate CFZ-Mediated Nrf1-Dependent PSMs transcription. NIH-3T3 (top 4 panels) and MDA-MB-231 (bottom 4 panels) cells were treated with 200nM CFZ alone or in combination with 10μm of NMS-873, and 5uM of Dox, Epi, or Ida for 8 h. RNA extracted from the cells was converted to cDNA, and then used for qRT-PCR with primers for the genes indicated. mRNA levels of 18s were used for normalization. Error bars denote standard deviation (n=3). One-way ANOVA test followed by Dunnett's multiple comparisons was used to compare relative mRNA levels.

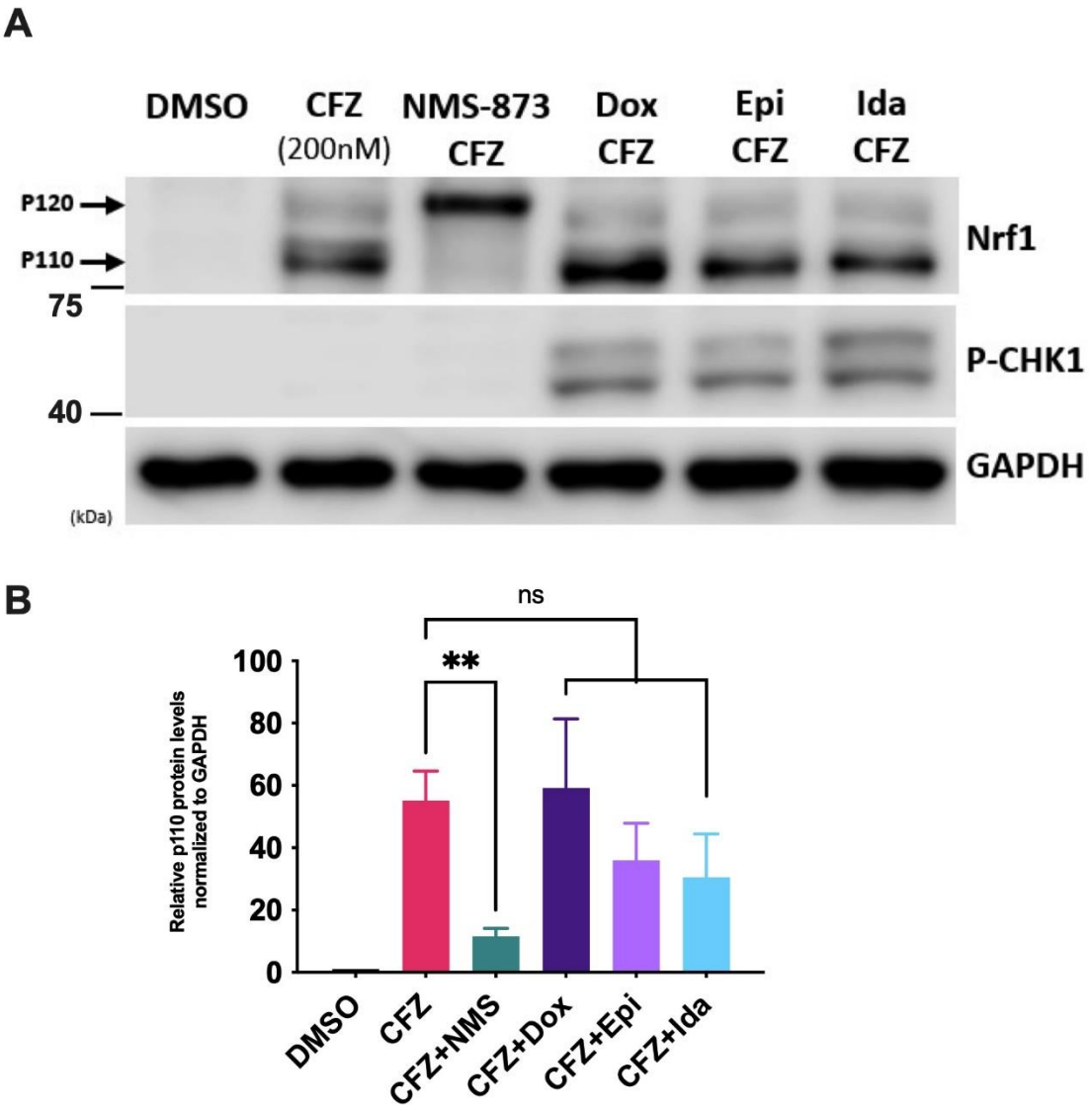

Figure 6: Anthracyclines do not affect the protein levels of NRF1. (**A**) NIH-3T3 cells were treated with 200nM CFZ alone or in combination with 10μM of NMS-873, Dox, Epi, or Ida for 6h, and then analyzed by western blot using the antibodies indicated. P-CHk1 was used as a positive control for anthracyclines. GAPDH was used as a loading control. (**B**) Quantification of A. Blots shown are representative of three independent experiments. One-way ANOVA test followed by Dunnett's multiple comparisons was used to compare p110 protein levels.

anthracyclines attenuate the NRF1-mediated transcription of PSM genes, these changes do not result from anthracycline affecting the protein levels of NRF1. Given that the p120 NRF1 is inserted into the ER in an $C_{lumen}/N_{cytosol}$ orientation, where only a small portion of the N-terminus overhangs into the cytosol, the action of the ATPase p97/VCP is required to re-position the C-terminus of NRF1 into the cytosol via retrotranslocation. The NRF1 will then be proteolytically processed into the p110 active form. To better understand the spatiotemporal aspect of the NRF1, we performed pulse-chase experiments followed by subcellular fractionation. First, NIH-3T3 cells were pulsed for two hours with the p97 inhibitor, NMS-873, to prevent NRF1 from being retrotranslocated to the cytosol. This step would lead to the accumulation of unprocessed p120. Cells were then washed to remove NMS-873 and treated either CFZ+cycloheximide (CHX) or with CFZ+CHX+Dox for 0, 30, 60, and 120min (**Figure 7A**). CFZ would inhibit the degradation of NRF1, and CHX would inhibit the synthesis of new NRF1, allowing us to track a single pool of NRF1 as it leaves the ER to enter the nucleus. After cells were collected, 10% of the cells were lysed in 2x Laemmli and used with P-CHK1 antibody as a positive control of Dox (**Figure 7B**). The remaining 90% of cells were then subjected to subcellular fractionation to isolate cytosolic, membrane-bond, and nuclear proteins followed by western blot.

In the control CFZ-CHX, as expected, there was an abundance of p120 in the membrane after NMS-873 washout. We noticed that the p120 form could enter the nucleus without being cleaved, but not as an active transcription factor. We also found that as the levels of p120 decrease over time in the membrane, the p110 levels increase in the cytosol and nucleus, indicating the NRF1 in being retrotranslocated into the cytosol, cleaved, and moved to the nucleus. The experiment treatment CFZ+CHX+Dox did not show any significant changes in p120 and p110 levels compared to the control (**Figure 7C-D**). These results indicate that NRF1 can still traverse into the nucleus in the presence of dox, and the step of the NRF1 pathway that is being compromised might be inside the nucleus.

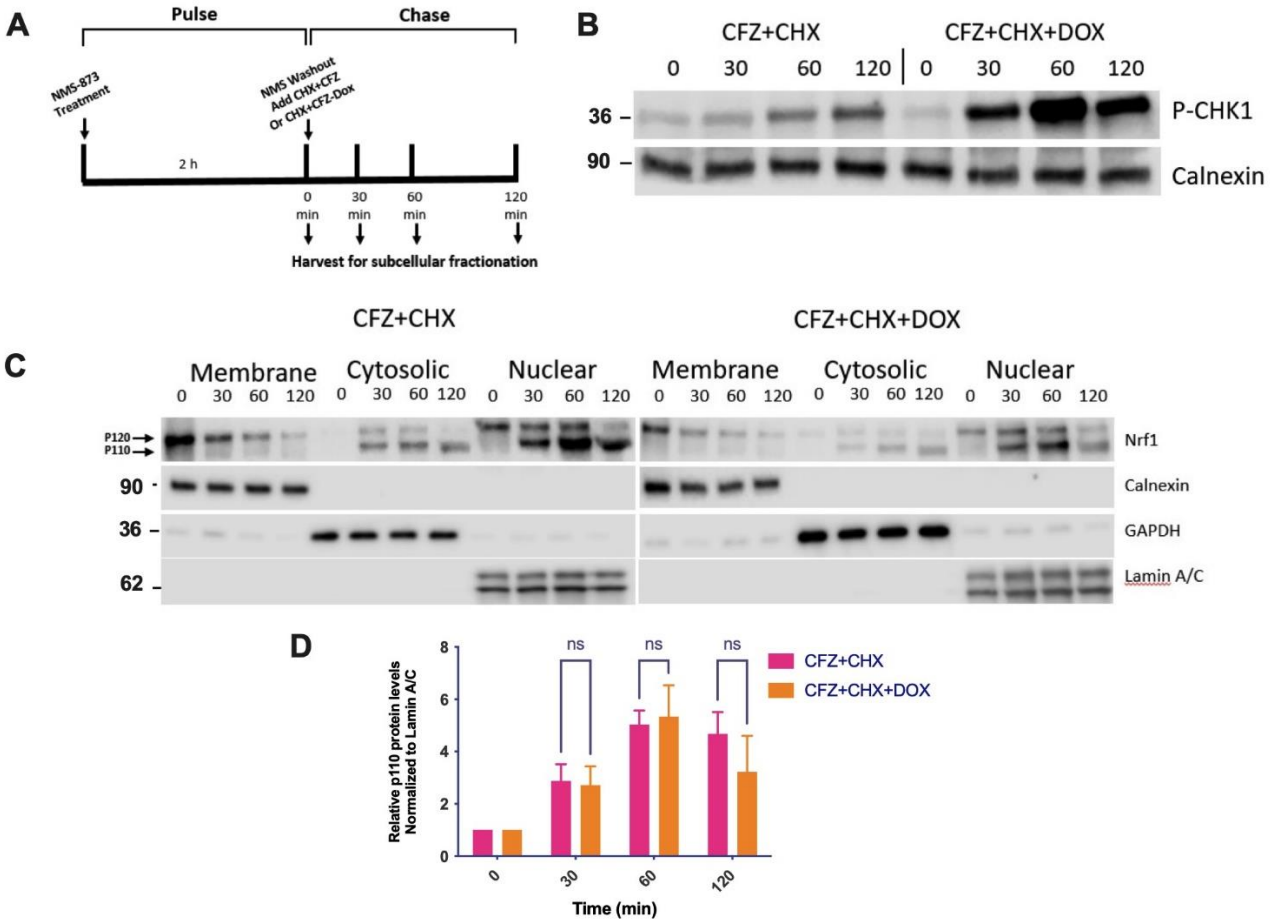

Figure 7: Anthracyclines do not affect the spatiotemporal aspects of NRF1. (**A**) A schematic representation of the pulse-chase assay is shown. (**C**) NIH-3T3 cells were pulsed with 10 μM NMS-873 for 2 h, then chased with 5uM CFZ+ 50 μg/mL cycloheximide (CHX) or with CFZ+ CHX+ 10uM Dox for 0, 30, 60, or 120min. Subcellular fractionation of membrane-bound, cytosolic, and nuclear proteins was analyzed by western blot using the antibodies indicated. Calnexin, Lamin A/C, and GAPDH are fractionation controls for membrane-bound, nuclear, and cytosolic proteins, respectively. (**D**) Quantification of p110 levels in the nuclear fractionation normalized to Lamin A/C. (**B**) 10% of the total lysate was taken aside, and protein extracted using 2× Laemmli sample buffer and P-CHK1 was used as a positive control for anthracyclines. Blots shown are representative of three independent experiments. Two-way ANOVA test followed by Dunnett's multiple comparisons was used to compare p110 protein levels.

3.4 Doxorubicin disrupts binding of NRF1 to DNA

Based on the proposed mechanism of dox, where it prefers to intercalate with DNA and inhibits TOP2, it is passable that it also inhibits NRF1 from binding its target genes. To verify this hypothesis, we used the NIH-3T3 cells stably expressing C-terminally tagged NRF1-flag. The ability of NRF1 to bind to ARE-containing promoter regions of the NRF1 target genes PSMC4, PSMD1, and PSMD12 was tested using ChIP assay (**Figure 8**). We observed that NRF1 was able to bind to its target genes in the CFZ alone treatment. However, the NRF1-dependent recruitment of its target genes promoters was abolished in the CFZ+Dox. Taking together, our results suggest that anthracyclines hinder NRF1 ability to bind the DNA of its target genes, attenuating the NRF1-mediated bounce-back response, and increasing the duration of proteasome inhibition experienced by cells.

3.5 anthracyclines impair proteasome recovery after proteasome inhibition

Finally, to characterize the anthracyclines' attenuation on the bounce-back response at a functional level, we performed a proteasome recovery assay that we have optimized in our previous studies (Northrop, Vangala, et al., 2020; Radhakrishnan et al., 2010; Vangala et al., 2020). The assay measures the ability of cells to bounce back from proteasome inhibition. It is important to note that CFZ binds irreversibly to the catalytic active site β5 of the proteasome. So, in cells pulse treated with CFZ, the proteasome recovery almost exclusively relies on the NRF1 pathway (Radhakrishnan et al., 2010). Here, we treated MDA-MB-231 with a dose of CFZ (40nM) that is sufficient to inhibit the proteasome activity by ~90% as compared to DMSO-treated cells. Cells were then washed multiple times to remove the access CFZ, and the proteasome activity was measured at 0, 4, 8, 12, and 24h in the absence or presence of Dox, Epi, or Ida (**Figure 9A**). We found that the proteasome steadily regained its activity when allowed to recover in DMSO-treated media. However, the recovery was significantly impaired in the presence of dox, epi, and Ida (**Figure 9B**). Our results indicate that anthracyclines hindered

the proteasomes' ability to recover from proteasome inhibition by blocking the bounce-back response.

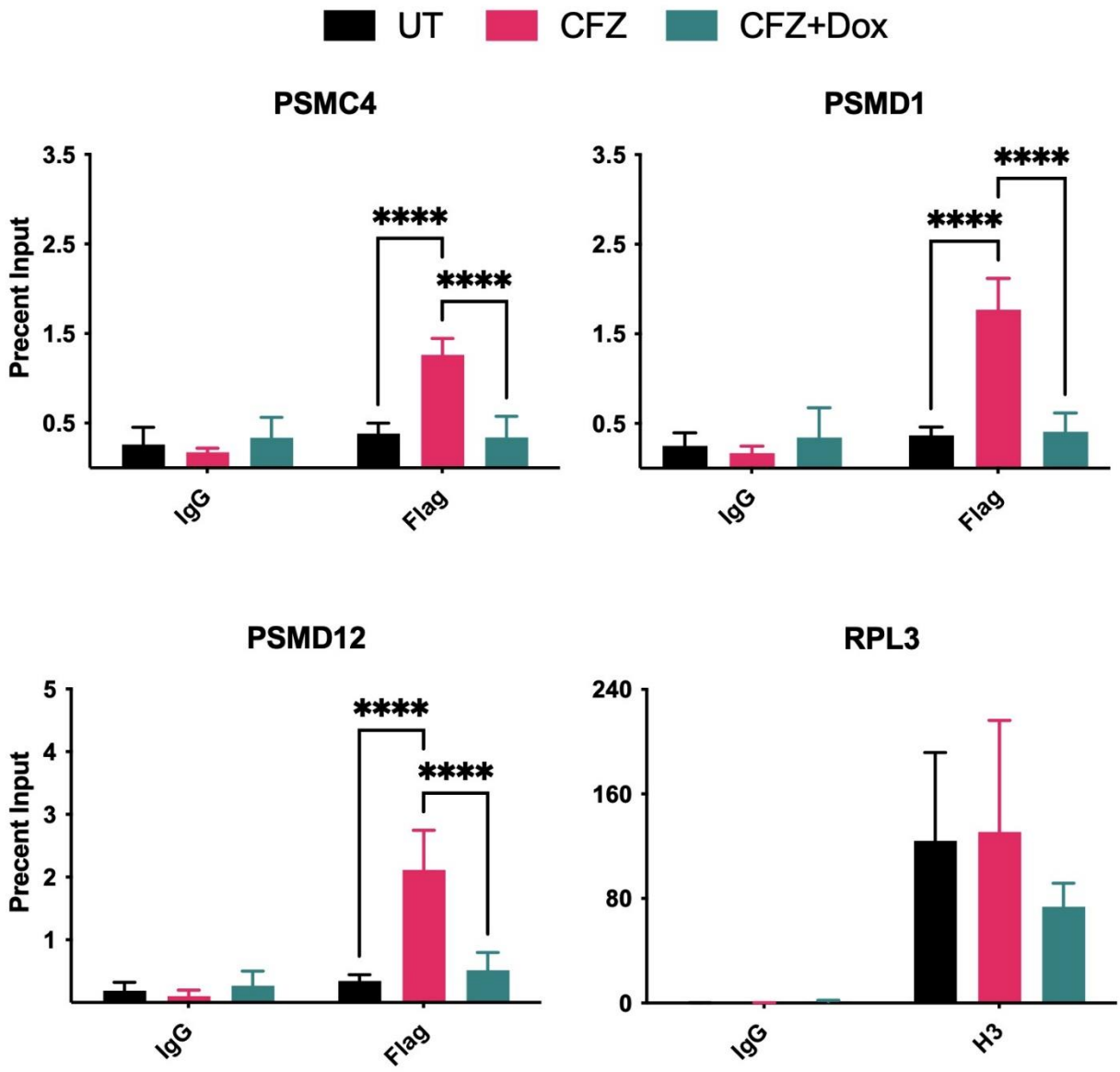

Figure 8: Doxorubicin disrupts the binding of NRF1 to DNA. NIH-3T3-HA-NRF1-Flag cells were treated with 200 nM CFZ for 8 h. The cells were then subjected to ChIP with IgG or Flag antibodies. These samples were then analyzed by quantitative PCR with primers specific for ARE-containing promoter regions of the proteasome genes PSMC4, PSMD1, and PSMD12. RPL3 was used as a positive control. Error bars denote S.D. (n = 3) Two-way ANOVA test followed by Dunnett's multiple comparisons was used to compare percent inputs.

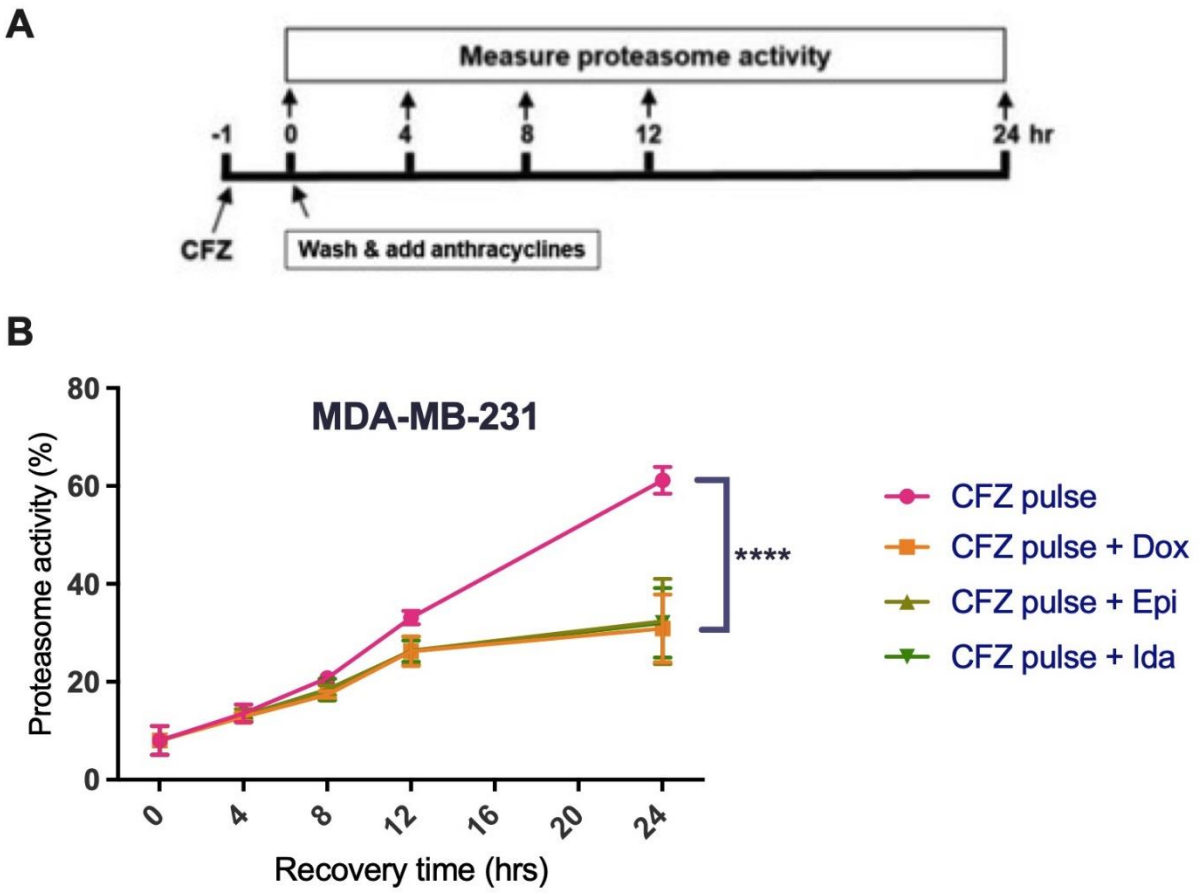

Figure 9: Anthracyclines impair proteasome recovery after proteasome inhibition. (**A**) A schematic representation of the proteasome recovery assays. (**B**) MDA-MB-231 cells were treated with 40nM CFZ for 1 h (pulse treatment). The drugs were, then, washed out and the cells were allowed to recover in the presence or absence of 5uM of Dox, Epi, or Ida for 0, 4, 8, 12, and 24h. Proteasome activity was measured at indicated time points and normalized to DMSO treated control cells. Error bars denote SD (n = 3). Two-way ANOVA test followed by Dunnett's multiple comparisons was used to compare percent inputs.

Chapter 4: Discussion and Conclusions

Proteasome inhibitors have emerged as effective therapeutic drugs to treat multiple myeloma and mantle cell lymphoma. However, their main shortcomings include resistance in patients with multiple myeloma and mantle cell lymphoma, and failure to show clinically significant positive outcomes in solid tumors. One well-established mechanism responsible for these shortcomings is the NRF1-mediated bounce-back response to proteasome inhibition (Radhakrishnan et al., 2010). Many proteins are involved in the NRF1 activation, such as NGLY1, P97, DDI2, or TIP60 (Koizumi et al., 2016; Radhakrishnan et al., 2014; Tomlin et al., 2017; Vangala & Radhakrishnan, 2019). Chemical or genetic inhibition of any of these proteins could attenuate the bounce-back response, thus increasing cell killing by proteasome inhibitors. Earlier work in the lab found that anthracyclines can attenuate the bounce-back response; however, the mechanism behind that was unclear. Anthracyclines, a class of drugs known for their great antitumor activity, preferentially intercalate with DNA and inhibit the re-ligating of TOP2-induced double-stranded DNA breaks, which initiate cell apoptosis pathways and increase cancer cell death. Here, we investigated steps of the NRF1 pathway to find out which step is being compromised by anthracyclines in the presence of CFZ. We found that NRF1 protein levels are unaffected by the presence of anthracyclines, and NRF1 can retro-translocate to the cytosol, cleaved by DDI2, and transverse to the nucleus. We also found that the last step of the NRF1 pathway, where it will bind to the DNA and initiate transcription of PSM genes, is the step being compromised by anthracyclines. We also found at a functional level that anthracyclines impaired the proteasome recovery after cells were pulse treated with CFZ.

To further support our proposed mechanism, anthracyclines are well-known as agents that bind to the DNA with optimal binding sites including 5′-(A/T)CG-3′ and 5′-(A/T)GC-3′ (Chaires et al., 1990; Lee et al., 2009). In addition, NRF1 binds to DNA at the antioxidant response elements (ARE) that include the sequence (5′-TGACNNNGC-3′) (Radhakrishnan et

al., 2010; Tonelli et al., 2018), which interestingly overlaps with anthracyclines DNA-binding sites. Previous studies have shown that anthracyclines inhibit multiple transcription factors in a comparable manner. For example, anthracyclines were shown to disrupt hypoxia-inducible factor 1 (HIF-1), which regulates the expression of genes that encode essential proteins in cancer biology, from binding to the DNA (Lee et al., 2009). GATA-4 transcription factor was also shown to be inhibited from binding to the DNA in the presence of anthracyclines (Kim et al., 2003). To show that not all transcription factors are turned off by anthracyclines, it has been found that dox stimulates proteolytic cleavage of CREB3L1, a transcription factor synthesized as a membrane-bound precursor, allowing it to enter the nucleus where it activates transcription of its target genes (Denard et al., 2012). Overall, our findings suggest that anthracyclines can target the NRF1-mediated bounce-back response to potentiate the CFZ-induced apoptosis in hematologic cancers that already respond well to proteasome inhibitors and may even increase the sensitivity of solid tumors to proteasome inhibitors.

In addition to establishing the molecular mechanism by which anthracyclines impair the bounce-back response to the proteasome inhibitor CFZ, our results have important implications for the future use of these drugs in the clinic. Anthracyclines are effective cancer chemotherapeutic agents; however, they can cause severe dose-dependent cardiotoxicity. One proposed mechanism behind dox-induced cardiotoxicity is that several studies have detected increased proteasome activity in response to dox treatment in cultured mouse cardiomyocytes and intact animals (Kumarapeli et al., 2005; Liu et al., 2008). This increased activity mediates the degradation of key transcription factors and cell survival factors in cardiac cells, which results in elevated cardiotoxicity (Ito et al., 2007; Poizat et al., 2005). Combining CFZ and dox may be advantageous for both drugs; dox would aid CFZ by limiting the bounce-back response and increasing proteasome inhibition duration. At the same time, CFZ would inhibit the dox-induced increase in proteasome activity in the heart, reducing cardiotoxicity. Further Experiments in cardiac muscle cells and in vivo are necessary to evaluate these hypotheses

and to find a good dose combination that would have the advantageous effect of both drugs. More studies should also incorporate the effect of this combinational therapy across other cancer types.

Finally, we hope that the findings presented in this thesis will contribute to the use of CFZ and anthracyclines combinational therapy to overcome shortcomings of proteasome inhibitors as a single agent in FDA-approved cancer types, as well as patients with triple-negative breast cancer and possibly other solid cancer types.

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