Novel functions of aberrant cyclin D1/CDK4 activity in mantle cell lymphoma and consequences for proteasome inhibitor treatment

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Dusslingen, den 05. Mai 2022

Simon Heine

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List of abbreveations

°C	degree Celsius
3MA	3-methyladenine
ACD	accidental cell death
AIDA	advanced image data analyzer
ALP	autophagy–lysosomal pathway
AMPK	AMP-activated protein kinase
APC	allophycocyanin
ASCT	autologous stem cell transplantation
ATG	autophagy-related gene
a. u.	arbitrary unit
Bcl-2	B cell lymphoma 2
BCR	B-cell receptor
BH	Bcl-2 homology
BRCA1	breast cancer type 1 susceptibility protein
BrdU	bromodeoxyuridine/fluorodeoxyuridine
BSA	bovine serum albumin
BTK	Bruton's tyrosine kinase
CD	cluster of differentiation
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
cDNA	complementary deoxyribonucleic acid
CHIP	carboxyl-terminus of Hsc70-interacting protein
CHK	checkpoint kinase
CHX	cycloheximide
CLL	chronic lymphocytic leukemia
CMA	chaperone-mediated autophagy
cosi	control siRNA

CT	computed tomography
DDR	DNA damage response
DHAP	dexamethasone, cytarabine and cisplatin
DLBCL	diffuse large B cell lymphoma
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FASN	fatty acid synthase
FBS	fetal bovine serum
FDA	Food and Drug Administration
FDR	false discovery rate
FITC	fluorescein isothiocyanate
FOXO3	forkhead transcription factor 3
GSH	L-Glutathione
h	hour
HDAC6	histone deacetylase 6
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	human epidermal growth factor receptor 2
HIF1α	hypoxia-inducible factor 1α
HRP	horse radish peroxidase
IGH	immunoglobulin heavy chain
IGHV	immunoglobulin heavy chain variable
kDa	kilo Dalton
LIR	LC3-interacting region
mA	milliampere
MCL	mantle cell lymphoma
mg	milligram

µg	microgram
µl	microliter
µM	micromolar
min	minute
MCM	minichromosome maintenance
MIPI	mantle cell lymphoma prognostic index
ml	mililiter
mM	millimolar
MOMP	mitochondrial outer membrane permeabilization
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
nm	nanometer
nM	nanomolar
ns	not significant
PBMNC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCD	programmed cell death
PE	phycoerythrin
PEITC	phenethyl isothiocyanate
PFS	progression-free survival
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PMA	phorbol-12-myristate-13-acetate
PMAIP1	PMA-induced protein 1
pRB1	phosphorylated RB1
PTEN	phosphatase and tensin homolog

PVDF	polyvinylidene difluoride
RB1	retinoblastoma protein
R-BAC	Rituximab, Bendamustine and Cytarabine
RCD	regulated cell death
R-CHOP	rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RT-PCR	real-time polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering ribonucleic acid
SQSTM1	Sequestosome 1
SYK	spleen tyrosine kinase
TBS	TRIS-buffered saline
TBST	TRIS-buffered saline with Tween 20
TEMED	tetramethylethylenediamine
TNF	tumor necrosis factor
TRIS	tris(hydroxymethyl)aminomethane
ULK1	Unc-51–like kinase 1
UPS	ubiquitin proteasome system
UPR	unfolded protein response
UTR	untranslated region
v/v	volume/volume
VR-CAP	bortezomib, rituximab, cyclophosphamide, doxorubicine and prednisone
w/v	weight/volume
Z-VAD-FMK	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

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Summary

Mantle cell lymphoma (MCL) is an aggressive B cell-non-Hodgkin lymphoma with generally poor outcome and only transient responses to conventional chemotherapy. Cells of MCL patients are characterized by an aberrant high cyclin D1-driven cyclin-dependent kinase 4 (CDK4) activity. Recently, the ubiquitin proteasome system (UPS) inhibitor bortezomib has been approved for the treatment of relapsed/refractory MCL and has shown promising results in first-line treatment of MCL patients. However, little is known about how the aberrant cyclin D1-driven CDK4 activity does affect the treatment efficacy of UPS inhibitors in MCL. The present work aimed to identify novel functions for cyclin D1/CDK4 activity in MCL that regulate the efficacy of proteasome inhibitor treatment.

In this study, the efficacy of proteasome inhibitor treatment was found to depend on the aberrant cyclin D1/CDK4 activity in MCL. Inhibition of cyclin D1/CDK4 activity with the CDK4 inhibitor palbociclib antagonized bortezomib induced cell death in MCL cells. Moreover, the antioxidant defense protein sestrin 3 was identified to be strongly regulated by cyclin D1-driven CDK4 activity. Although, bortezomib efficacy was demonstrated to be regulated by reactive oxygen species (ROS) or antioxidants, changes in sestrin 3 expression levels are not responsible for cell death regulation after UPS blockage in MCL. Furthermore, even though targeting cyclin D1/CDK4 activity with palbociclib induces cell cycle arrest, changes in cell cycle distribution after cyclin D1/CDK4 inhibition also do not mediate the antagonizing effect on bortezomib induced cell death. Astonishingly, it could be demonstrated that cyclin D1/CDK4 inhibition antagonizes the bortezomib induced cell death through a reduction of NOXA protein accumulation. It could also be shown that this mechanism is not exclusive for the proteasome inhibitor bortezomib but also applies to other agents that depend on NOXA protein accumulation for efficient cell death induction in MCL cells.

Importantly, results of the present study revealed that this regulation of NOXA protein levels by cyclin D1/CDK4 activity is not associated with a reduction of *NOXA* transcript levels but is mediated by the regulation of NOXA protein stability. This identified novel function of cyclin D1/CDK4 to regulate NOXA protein half-life in the presence of UPS blockage is mediated by the autophagic degradation machinery. This study identified, the regulation of autophagic activity as a novel cell cycle independent function of cyclin D1/CDK4 activity in MCL. In this context, it was demonstrated that inhibition of cyclin D1/CDK4 activity by palbociclib treatment induces autophagic activity in MCL. Cyclin D1/CDK4 and autophagic activity, however, are not linked by the common autophagy regulating pathways, AMP-activated protein kinase (AMPK) or ROS.

In addition, genetic or pharmacological inhibition of autophagic activity reverses the palbociclib mediated antagonism on bortezomib induced cell death and NOXA protein accumulation. Remarkably, this study demonstrates that the NOXA protein can be targeted for proteasomal as well as autophagosomal degradation. Targeting NOXA protein for degradation through selective autophagy might be mediated through a LC3-interacting region (LIR) motif that was identified in the amino acid sequence of the NOXA protein. Interestingly, combination of bortezomib with known autophagy inhibitors potentiates cell death induction as well as NOXA protein accumulation compared to bortezomib treatment alone. Furthermore, screening for a highly efficient combinatorial blockade of the UPS and the ALP revealed that the fatty acid synthase (FASN) inhibitor orlistat can be repositioned for autophagy inhibition. Consequently, the combinatorial treatment with bortezomib and orlistat leads to a very efficient NOXA protein induction and caspase dependent cell death in MCL cells. This cell death is again dependent on the aberrant high cyclin D1/CDK4 activity. The expression levels of certain other apoptotic proteins, however, are not affected by the combinatorial treatment.

In conclusion, the present study revealed the regulation of autophagic activity as a novel function of cyclin D1/CDK4 activity in MCL. These findings are of utmost importance for the treatment of MCL patients, as combination of proteasome inhibitors with autophagy inhibitors could greatly improve therapy outcomes or overcome bortezomib resistances. In addition to known autophagy inhibitors, treatment options could include conventional drugs that are repurposed for targeting the autophagic degradation machinery. On the other hand, care must be taken when combining proteasome inhibitors with other chemotherapeutics that might impair cyclin D1/CDK4 activity or induce autophagy.

Zusammenfassung

Das Mantelzell-Lymphom (MCL) ist ein aggressives B-Zell-Non-Hodgkin-Lymphom mit sehr schlechter klinischer Prognose und nur zeitweiligem Ansprechen auf konventionelle Chemotherapie. Zellen von MCL-Patienten zeichnen sich durch eine anormal hohe Cyclin D1-vermittelte Cyclin-abhängige Kinase 4 (CDK4) Aktivität aus. Kürzlich wurde der Ubiquitin-Proteasom-System (UPS)-Inhibitor Bortezomib für die Behandlung des rezidivierten bzw. refraktären MCl zugelassen und zeigte vielversprechende Ergebnisse unter anderem in der Primärtherapie von MCL-Patienten. Es ist jedoch wenig darüber bekannt, wie die anormale Cyclin D1/CDK4-Aktivität beim MCL die Wirksamkeit der Behandlung von UPS-Inhibitoren beeinflusst. Ziel der vorliegenden Arbeit war es daher neue Funktionen für die Cyclin D1/CDK4 Aktivität im MCL zu identifizieren, die die Wirksamkeit der Behandlung von Proteasominhibitoren beeinflussen.

In dieser Arbeit konnte gezeigt werden, dass die Wirksamkeit der Proteasominhibitoren von der anormalen Cyclin D1/CDK4-Aktivität in MCL-Zellen abhängig ist. Außerdem wurde nachgewiesen, dass das Antioxidans-Protein Sestrin 3 durch die Cyclin D1-vermittelte CDK4-Aktivität reguliert wird. Es konnte dabei gezeigt werden, dass die Wirksamkeit von Bortezomib durch reaktive Sauerstoffspezies (ROS) oder Antioxidantien reguliert wird. Dennoch sind Veränderungen der Sestrin 3-Expressionslevel nicht für die Regulation des Zelltods nach UPS-Blockade im MCL verantwortlich. Obwohl die Hemmung der Cyclin D1/CDK4-Aktivität durch Palbociclib einen Zellzyklusarrest induziert, vermitteln diese Veränderungen im Zellzyklus nicht die antagonisierende Wirkung von Palbociclib auf den Bortezomib induzierten Zelltod. Erstaunlicherweise konnte gezeigt werden, dass Palbociclib den Bortezomib induzierten Zelltod durch eine reduzierte NOXA-Proteinakkumulation antagonisiert. Es konnte außerdem nachgewiesen werden, dass dieser Mechanismus nicht nur auf den Proteasominhibitor Bortezomib beschränkt ist, sondern auch für andere Wirkstoffe gilt, die für eine effiziente Zelltodinduktion im MCL auf die Akkumulation des NOXA-Proteins angewiesen sind.

Wichtig ist hierbei, dass die Cyclin D1/CDK4 vermittelte Regulierung der NOXA-Proteinexpression, nicht durch eine Reduzierung des *NOXA*-Transkripts, sondern durch eine Regulierung der NOXA-Proteinstabilität vermittelt wird. Diese Funktion von Cyclin D1/CDK4, die Halbwertszeit des NOXA-Proteins in Anwesenheit einer UPS-Blockade zu regulieren, wird hierbei durch die autophagosomalen Abbauwege vermittelt. Die Regulation der autophagosomalen Aktivität wurde im Rahmen dieser Arbeit, als eine neue zellzyklusunabhängige Funktion der Cyclin D1/CDK4 Aktivität im MCL identifiziert.

In diesem Zusammenhang wurde gezeigt, dass die Hemmung der Cyclin D1/CDK4 Aktivität durch die Behandlung mit Palbociclib, die autophagosomale Aktivität im MCL erhöht. Cyclin D1/CDK4 und autophagosomale Aktivität stehen jedoch nicht durch die Signalwege miteinander in Verbindung, die üblicherweise Autophagie regulieren, wie die AMP aktivierte Proteinkinase (AMPK)- oder ROS Signalwege.

Des Weiteren hob die genetische oder pharmakologische Hemmung der autophagosomalen Aktivität, den durch Palbociclib vermittelten Antagonismus auf den Bortezomib induzierten Zelltod und die NOXA-Proteinakkumulation auf. Erstaunlicherweise zeigt die vorliegende Arbeit, dass das NOXA-Protein sowohl proteasomal als auch autophagosomal abgebaut werden kann. Die Markierung des NOXA-Proteins für den Abbau durch selektive Autophagie, könnte durch ein LC3-interagierende Region (LIR)-Motiv vermittelt werden, das in der Aminosäuresequenz des NOXA-Proteins identifiziert werden konnte. Interessanterweise potenziert die Kombination von Bortezomib mit bekannten Autophagie-Inhibitoren die Zelltod- sowie NOXA-Induktion, im Vergleich zu der Bortezomib Behandlung allein. Zudem ergab ein Substanzscreening zur Identifikation einer hocheffizienten dualen Blockade des proteasomalen und autophagosomalen Abbaus, dass der Fettsäure-Synthase-Inhibitor Orlistat für die Autophagie-Inhibition umfunktioniert werden kann. Infolgedessen führte die Kombinationsbehandlung mit Bortezomib und Orlistat zu einer sehr effizienten NOXA-Proteininduktion und einem Caspase-abhängigen Zelltod in MCL-Zellen. Dieser Zelltod ist ebenso abhängig von der anormal hohen Cyclin-D1/CDK4-Aktivität. Die Expressionsraten bestimmter anderer apoptotischer Proteine werden jedoch durch die Kombinationsbehandlung nicht beeinflusst.

Zusammenfassend konnte in der vorliegenden Arbeit die Regulation der autophagosomalen Aktivität als neue Funktion der Cyclin D1/CDK4-Aktivität im MCL identifiziert werden. Diese Ergebnisse sind für die Behandlung von MCL-Patienten von großem Interesse, da die Kombination von Proteasominhibitoren mit Autophagie-Inhibitoren die Therapie deutlich verbessern, sowie Bortezomib-Resistenzen überwinden könnte. Außerdem besteht die Möglichkeit, dass diese Behandlungsoptionen, zusätzlich zu den bekannten Autophagie-Inhibitoren, auch konventionelle Medikamente beinhalten die hierbei als Regulatoren der autophagosomalen Abbaumechanismen eine neue Verwendung finden. Wiederum besteht das Risiko von antagonistischen Effekten bei der Kombination von Proteasominhibitoren mit Chemotherapeutika, da diese die Aktivität von Cyclin D1/CDK4 beeinträchtigen oder Autophagie induzieren könnten.

1 Introduction

1.1 Mantle cell lymphoma overview

1.2 Clinical features and subtypes

Mantle cell lymphoma (MCL) is a rare subtype of mature B cell lymphoid neoplasms, accounts for approximately 5-7 % of all cases of non-Hodgkin lymphoma, and has an annual incidence of ~1 in 100000 in the Western Europe population (Dreyling et al., 2017b). Median age at diagnosis is 65 years with a strong male to female ratio in Europe of 3:1 (Sant et al., 2010). MCL is an aggressive neoplasm with a median overall survival of 4-5 years (Vose, 2017). MCL patients typically respond to frontline therapy, but clinical course is characterized by frequent relapses and the disease still remains incurable with standard therapy approaches (Pérez-Galán et al., 2011a). The term mantle cell lymphoma was chosen because of the growth pattern in the early stages of the disease with tumor cells colonizing the mantle zone of the lymphoid follicles (Weisenburger et al., 1982). Since the introduction of the Revised European-American classification of the International Lymphoma Study Group (R.E.A.L.-classification) in 1994, MCL is regarded as a distinctive lymphoma subtype in the nowadays renowned World Health Organization classification of malignant lymphoid disorders (Swerdlow, 2008).

The initiating oncogenic event and hallmark of this cancer is the t(11;14)(q13;q32) translocation. This translocation results in juxtaposing the gene *CCND1*, which encodes the cell cycle regulating protein cyclin D1, to the strong immunoglobulin heavy chain (IGH) complex gene transcription enhancers (Bertoni et al., 2006; Jares et al., 2012). This translocation is acquired in pre-B cells and subsequently causes the constitutive overexpression of cyclin D1. MCL cells also express surface immunoglobulins (IgM/IgD), B cell associated antigens such as cluster of differentiation (CD)20, CD22, CD79, and the T-cell-associated antigen CD5 (Swerdlow, 2008). Unfortunately, most of the MCL patients are diagnosed with advanced stage disease (Ann Arbor stage III, IV). The typical clinical presentation of MCL comprises general lymphadenopathy with common involvement of extranodal sites such as bone marrow, the gastrointestinal tract, Waldeyer's ring, and liver (Argatoff et al., 1997). MCL is also frequently associated with extensive splenomegaly and a leukemic phase of the disease (Pittaluga et al., 1996; Bosch et al., 1998; Wong et al., 1999).

The diagnosis is usually made after an excisional biopsy of an enlarged lymph node or bone marrow and subsequent identification of typical monomorphic small to medium-sized lymphoid cells with irregular shaped nuclei (Swerdlow, 2008). The final diagnosis is then based on either fluorescent in situ hybridization for the detection of the t(11;14)(q13;q32)

translocation or an analysis of cyclin D1 expression by immunohistochemistry. There are rare cases of cyclin D1-negative MCL where SOX11 can serve as a biomarker for the diagnosis. In addition, upregulation of cyclin D2 or D3 may substitute for cyclin D1 in the pathogenesis of the disease (Fu et al., 2005a; Narurkar et al., 2016). SOX11 is a transcription factor that is not expressed in lymphoid progenitors, normal B cell cells, or other B cell malignancies, whereas in MCL *SOX11* mRNA and protein expression is a specific marker for both cyclin D1-positive and -negative cases (Mozos et al., 2009; Ferrando, 2013). In MCL, SOX11 is involved in the regulation of B cell differentiation, proliferation, cell cycle, apoptosis, and cell migration (Vegliante et al., 2013).

The initial translocation event is followed by different molecular pathways that result in distinct subtypes of MCL. The classical form of MCL is the most common form of this disease. In the pathogenesis of this subtype the mature B cells do not enter the germinal center of the lymph node and acquire only a limited number of somatic mutations in the immunoglobulin heavy chain variable (IGHV) genes. MCL cells of the classic form express the transcription factor SOX11 and are genetically unstable. Cells of this subtype tend to accumulate alterations in genes responsible for cell cycle regulation, DNA damage response (DDR), and cell survival, which results in a more aggressive behavior (Jares et al., 2012). Through acquisition of additional molecular or cytogenetic abnormalities this subtype can further progress to the more aggressive subtypes of blastoid or pleomorphic MCL (Swerdlow et al., 2016). The leukemic, non-nodal MCL represents a second subtype, which is less common and is characterized by higher genetic stability, low SOX11 protein expression, non-nodal presentation, and an indolent clinical course that might be managed more conservatively (Orchard et al., 2003; Fernàndez et al., 2010). Progenitor cells of this subtype enter the follicular germinal center, interact with the germinal center microenvironment and acquire IGHV somatic hypermutations (Jares et al., 2012; Royo et al., 2012; Campo and Rule, 2015). Investigations for specific phenotypic and cytogenetic markers for this more indolent subtype are still ongoing to avoid overtreatment of the patients (Gallo et al., 2016). In contrast, the blastoid variant is associated with a more aggressive clinical course. Blastoid MCL cells are characterized by neoplastic cells resembling lymphoblasts with dispersed chromatin, prominent nucleoli, and high proliferation rates (Hoster et al., 2016; Swerdlow et al., 2016). The *in situ* mantle cell neoplasia represents another rare subtype of MCL. This subtype is characterized by cyclin D1 positive cells, a very indolent clinical course, and cells located in the inner mantle zones of the lymphoid follicles (Carbone and Santoro, 2011; Carvajal-Cuenca et al., 2012).

1.2.1 Molecular basis and pathogenesis

Several studies have shown that cyclin D1 overexpression alone is not sufficient for cell transformation and the aggressive behavior of MCL (Bodrug et al., 1994; Lovec et al., 1994). A small number of cells with the characteristic t(11;14)(q13;q32) translocation can even be found in the peripheral blood of healthy individuals (Hirt et al., 2004). In addition to the translocation event, secondary genetic alterations contribute to the aggressiveness of the disease (Jares et al., 2012). The frequently detected complex karyotypes in MCL (≥3 chromosomal abnormalities) have been associated with worse clinical outcomes (Greenwell et al., 2018). Recurrent defects in DDR such as the ATM pathway and downstream targets such as checkpoint kinase 1 (CHK1) and CHK2 account for the high genomic instability in MCL (Tort et al., 2002; Tort et al., 2005; Jares and Campo, 2008; Beà et al., 2013). An unbalanced expression of the DNA licensing factors geminin, Cdt1, and Cdc6 in MCL cases with inactivation of the p53/p14ARF pathway have been shown to further contribute to the genomic instability (Pinyol et al., 2006). Mutations in genes that regulate epigenetic modifications have been discovered through exome sequencing of MCL tumors and normal tissue, providing a potential mechanism for the acquisition of somatic mutations in MCL (Zhang et al., 2014).

Several deregulated signaling pathways are contributing to the pathogenesis of MCL. Mature B cell lymphoid neoplasms repurpose the biologic features of normal B cells from which they originate for their own malignant purpose. The function of such B cell neoplasms therefore depends considerably on the differentiation state of their progenitor cells (Lenz and Staudt, 2010). Proliferation and differentiation of B cells is closely regulated by the function and activation status of the B cell receptor (BCR) and its key components, the spleen tyrosine kinase (SYK), the phosphoinositide 3-kinase (PI3K), and the Bruton's tyrosine kinase (BTK) (Seda and Mraz, 2015). Phospho-proteomic analysis of MCL cells identified many regulated proteins connected to BCR signaling, confirming a chronic BCR activation (Pighi et al., 2011). In addition, previous reports already identified the overexpression of the tyrosine kinase SYK as a possible therapeutic target in MCL (Rinaldi et al., 2006). The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway was also identified to be frequently constitutively activated in MCL and is associated with a poor clinical outcome (Pham et al., 2003; Yang et al., 2008; Camara-Clayette et al., 2014). Gene expression profiling of 38 MCL cases revealed up-regulation of genes of the NF-κB pathway, including the NF-kB activator IKBKB (Martínez et al., 2003). Chronic BCR signaling might also mediate NF-κB activation but the molecular basis of constitutive NF-κB activation in MCL cells remains not fully understood (Jares et al., 2012).

The PI3K/Akt/mammalian target of rapamycin (mTOR) pathway is also implicated in contributing to the pathogenesis as well as drug resistances in MCL (Rizzatti et al., 2005). In normal B cells, this pathway regulates various cellular functions including proliferation, differentiation, apoptosis, survival, metabolism, cytoskeletal reorganization, and membrane trafficking (Okada et al., 2000). The often deregulated PI3K/Akt/mTOR pathway therefore plays a prominent role in many types of cancers (Engelman, 2009). In MCL, several studies confirm the constitutive activation of Akt as well as mTOR, although they have distinct functional relevance in MCL (Peponi et al., 2006; Rudelius et al., 2006; Dal Col et al., 2008). In addition, the gene copy number of *PIK3CA*, coding for the gene of the PI3K catalytic subunit p110α, is frequently increased in MCL. The concomitant deletion of the phosphatase and tensin homolog (*PTEN*), a phosphatase that negatively regulates the PI3K pathway, further contributes to the activation of the PI3K/Akt/mTOR pathway (Psyrri et al., 2009). In addition, the chronic BCR activation acts upstream of the PI3K/Akt/mTOR pathway in MCL. Yet, the exact mechanisms of the chronic PI3K/Akt/mTOR pathway activation, however, still need further investigations (Rickert, 2013). Furthermore, aberrant canonical Wnt signaling might explain chemoresistance of MCL cells and represents a potential therapy target (Gelebart et al., 2008; Mathur et al., 2015). Mutations in the Notch signaling pathway receptors NOTCH1/2 are found in approximately 10 % of MCL patients, are associated with poor overall survival and might represent another therapeutic alternative for a subset of MCL patients (Kridel et al., 2012; Beà et al., 2013; Silkenstedt et al., 2019).

Because of the frequent involvement of the gastrointestinal tract in MCL, MCL pathogenesis might be associated with infectious agents affecting the gastrointestinal tract or the microbial gut flora (Romaguera et al., 2003). Other studies found an increased risk for developing MCL among patients who suffer from type 1 diabetes or have a family history of hematopoietic malignancies (Smedby et al., 2006; Wang et al., 2007). But most available published studies of MCL etiology have been hampered by a low statistical power to test MCL-specific associations due to weak associations, small numbers of participants, or lack of reproducibility (Smedby and Hjalgrim, 2011).

1.2.2 Treatment

Therapy decisions incorporate a risk stratification scheme based on the mantle cell lymphoma prognostic index (MIPI) that includes patient age, Eastern Co-operative Oncology Group performance status, lactate dehydrogenase levels, and white blood cell count. Yet, the most important prognostic marker independent of clinical features is the proliferation rate and the expression of genes related to proliferation (Hoster et al., 2016; Dreyling et al., 2017b).

In the clinical setting, immunohistochemical determination of Ki-67 expression has been prospectively confirmed as a reliable prognostic marker. In combination with the MIPI, Ki-67 expression represents a highly recommended tool to estimate the individual risk profile and to identify high-risk patients who may qualify for more aggressive therapeutic approaches (Hoster et al., 2016). Other markers such as the SOX11 protein or the tumor suppressor protein p53 further help to predict time to treatment failure and overall survival (Fernàndez et al., 2010; Aukema et al., 2018). Recently, a cell proliferation gene signature has been identified that distinguishes patient subsets that differ by more than five years in median survival (Rauert-Wunderlich et al., 2019). Available treatment options depend on careful staging of the disease, previous treatments, clinical risk factors, symptoms, and patient characteristics. Staging should contain a computed tomography (CT) scan of the neck, thorax, abdomen and pelvis, and a bone marrow biopsy (Dreyling et al., 2017b).

In young and fit patients (≤ 65 years), a dose-intensified concept containing an immunochemotherapy induction followed by a high-dose consolidation regimen, and autologous stem cell transplantation (ASCT) constitutes the current standard of care (Dreyling et al., 2017b). Rituximab maintenance after ASCT should be included in treatment regimens for younger patients with MCL based on the results of a large Phase III clinical trial showing a significant optimization of progression-free survival (PFS) and overall survival (Le Gouill et al., 2017). Addition of Rituximab to conventional chemotherapy such as the combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) improved complete response rates, overall response rates and overall survival, making immunochemotherapy regimen the standard of care in first-line treatment for patients with advanced-stage MCL. Including administration of the dexamethasone, cytarabine, and cisplatin (DHAP) regimen to the R-CHOP regimen more than doubled time to treatment failure compared to administration of R-CHOP alone (Delarue et al., 2013). An aggressive regimen, which is part of the US but not EU guidelines, consists of rituximab combined with hyperfractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone alternating with high-dose methotrexate and cytarabine (R-hyperCVAD) (Dreyling et al., 2017b). However, this regimen is hampered by significant therapy-associated toxicity, including secondary malignancies and should only be considered in young, fit patients.

Because of the median age of 65 years at first diagnosis, the majority of MCL patients does not qualify for dose-intensified regimens. Fit patients > 65 years should receive conventional immunochemotherapy followed by Rituximab maintenance. A combination of bortezomib, rituximab, cyclophosphamide, doxorubicine, and prednisone (VR-CAP) represents the new standard induction therapy for this group of patients, based on a recent Phase III clinical trial

comparing R-CHOP with VR-CAP (Robak et al., 2018). Alternatively, the combination of Rituximab, Bendamustine, and Cytarabine (R-BAC) offers another useful option for fit older patients with high-risk disease. R-bendamustine, however, is an appropriate alternative for patients not qualifying for such intensive therapy regimens.

For patients not eligible for immunotherapy, molecular targeted therapies should be applied. Novel targeted therapies should also strongly be considered in cases of early relapses or in refractory cases (Dreyling et al., 2017a). Temsirolimus targets the mTOR pathway, which is downstream of BCR signaling, and has been shown to be effective in MCL. Temsirolimus, however, should only be applied in combination with conventional chemotherapy (Hess et al., 2009). Another promising targeted therapy option is venetoclax, which is a highly selective inhibitor of the prosurvival B cell lymphoma 2 (Bcl-2) protein. Venetoclax has a significant single-agent activity in MCL, with 75 % of patients achieving responses and 21 % achieving complete response (Davids et al., 2017). The immunomodulatory drug lenalidomide in combination with rituximab has also been shown to achieve ongoing remissions for some patients (Ruan et al., 2015). Among the novel targeted therapeutics, Ibrutinib achieves the highest response rates and sometimes even long-term remissions (Dreyling et al., 2016; Wang et al., 2016). Ibrutinib targets BCR signaling by inhibition of the tyrosine kinase BTK (Wang et al., 2013). In clinical trials, treatment of MCL patients with the BTK inhibitor Ibrutinib was most effective for MCL cases with chronic activation of a BCR-driven classical NF-κB pathway (Yang et al., 2008; Rahal et al., 2014). An alternative therapy approach to the standard R-CHOP regime, includes substituting vincristine with the proteasome inhibitor bortezomib, which resulted in an almost doubled median PFS in MCL patients (Robak et al., 2015).

1.3 Cyclin D1/CDK4

1.3.1 Cell cycle function

Cell cycle progression in eukaryotes is under strict control. Cell cycle checkpoints are mainly regulated through internal DNA damage signals, external signals like mitogens or nutrients, and the expression of CDKs (Nurse et al., 1998; Dasika et al., 1999; Bohnsack and Hirschi, 2004). Successful cell division is only possible if DNA replication occurs in S-phase and if the chromosome division machinery during mitosis is correctly assembled (Nyberg et al., 2002). Cyclin D1 acts as the regulatory subunit of a holoenzyme together with the cyclin dependent kinases CDK4 and CDK6. As CDK6 is hardly expressed in MCL cells, cyclin D1 almost exclusively exerts its cell cycle regulatory function in MCL in association with CDK4 (Marzec et al., 2006). After assembly, the cyclin D1/CDK4 complex phosphorylates the retinoblastoma

protein (RB1), which has multiple phosphorylation sites (Sherr, 1994; Grosicki et al., 2014). In the unphosphorylated state, RB1 stably binds to transcription factors of the E2F family and thereby negatively regulates their function (Dyson, 1998). After phosphorylation of RB1 and subsequent release of the E2F transcription factors, the transcription of genes required for S-phase entry and DNA replication, including the cell cycle regulator *cyclin E*, is induced (Harbour, 2000). Cyclin E together with its associated kinase CDK2 further phosphorylates phosphorylated RB1 (pRB1), which leads to the inactivation of pRB1 and finally promotes cell cycle progression into S-phase (Lundberg and Weinberg, 1998; Seville et al., 2005). Cyclin D1 is generally not detected in normal B lymphocytes, the overexpression in MCL cells therefore causes a deregulated G1/S-phase cell cycle transition, facilitating cell cycle progression and tumor proliferation (Campo et al., 1999). Cyclin D1/CDK4 complexes have been demonstrated to sequester proteins of the Cip/Kip family, including p27Kip1 and p21Cip1. Since these proteins normally inhibit cyclin E/CDK2 as well as cyclin D1/CDK4 activity, this mechanism further promotes G1/S progression (Quintanilla-Martinez et al., 2003; Sherr and Roberts, 2004). In addition to the translocation event, cyclin D1 protein overexpression is further driven by other factors in MCL. *Cyclin D1* mRNA half-life is determined by destabilizing AU-rich elements in the 3′ untranslated region (UTR) (Deshpande et al., 2009). In MCL, however, transcripts with genomic deletions or point mutations in the 3′ UTR are commonly found, resulting in a more stable full-length mRNA and a worse clinical outcome (Wiestner et al., 2007). Another possible mechanism to further increase the cyclin D1 overexpression in MCL are amplification events of the *CCND1*/*IGH* fusion gene (Beà et al., 2009). Deletions and mutations of the cyclin-dependent kinase inhibitors (CDKIs) *p21Waf1* and *p16INK4a* are often detected in aggressive MCL patient samples and synergistically deregulate cell cycle and proliferation in addition to the cyclin D1 overexpression (Lukas et al., 1995; Dreyling et al., 1997; Pinyol et al., 1997). Additionally, in most MCL cells the CDKI p27Kip1 cannot be detected on protein level (Quintanilla-Martinez et al., 1998).

1.3.2 Cell cycle independent function

Apart from functioning as a cell cycle regulating protein, cyclin D1 has been implicated with various functions that might contribute to the pathogenesis of MCL. Cyclin D1 is associated with the regulation of the cellular metabolism. Functional analysis of cyclin D1 antisense transgenics in mammary epithelial demonstrated that cyclin D1 inhibits oxidative glycolysis, lipogenesis, and mitochondrial function (Sakamaki et al., 2006). Furthermore, in both, normal and malignant B cells, cyclin D1 binds to the voltage-dependent anion channel, which normally transports metabolites and ions across the outer mitochondrial membrane. This interaction

subsequently causes an inhibition of the mitochondrial metabolism and therefore might be involved in the transformation process (Tchakarska et al., 2011). In addition, cyclin D1/CDK4 complexes are able to suppress gluconeogenesis through phosphorylation of the peroxisome proliferator activated receptor coactivator 1-α. Cyclin D1 therefore might contribute to the Warburg Effect, which is characterized by a glucose metabolism predominantly via glycolysis rather than oxidative phosphorylation (Bhattacharya et al., 2016; Hosooka and Ogawa, 2016).
It has been shown that cyclin D1 is also involved in DDR via recruiting of RAD51 to damaged DNA, initiating homologous recombination-mediated DNA repair and increasing resistance of cells to radiation (Jirawatnotai et al., 2011; Chalermrujinanant et al., 2016). On the other hand, cyclin D1/CDK4 was shown to phosphorylate the breast cancer type 1 susceptibility protein (BRCA1), which is a tumor-suppressor protein that is critical for genomic stability. Cyclin D1 thereby inhibits the ability of BRCA1 to be recruited to particular promoters which are involved in DDR (Kehn et al., 2007). Cyclin D1 overexpression also triggers DNA re-replication during S-phase by stabilizing Cdt1, which subsequently can cause DNA double strand breaks and genomic instability (Aggarwal et al., 2007; Shimura et al., 2013). One the other hand, one study demonstrated that in MCL cyclin D1 depletion deregulated replication initiation, subsequently caused DNA damage and increased chemosensitivity to replication inhibitors (Mohanty et al., 2017). Cyclin D1 has also been shown to promote cellular migration through inhibiting the expression and function of the Rho-associated protein kinase and the metastasis suppressor thrombospondin 1 (Li et al., 2006b). Furthermore, a recent study showed that the contribution of cyclin D1 levels to cellular migration and invasiveness in MCL depends on the localization of cyclin D1 in the cytoplasm (Body et al., 2017).

1.3.3 Targeting cyclin D1/CDK4 activity

The first CDK inhibitors have been developed 20 years ago and since then numerous trials in various cancer types have been conducted. The first-generation of CDK inhibitors like flavopiridol are relatively unspecific, target multiple CDKs and showed only moderate activity in early clinical trials for patients with non-Hodgkin lymphoma, prostate, renal, colon, and gastric carcinoma (Kelland, 2000; Sherr et al., 2016). In the meantime, more specific inhibitors such as palbociclib (PD0332991), ribociclib (LEE011), and abemaciclib (LY2835219) were developed (Sherr et al., 2016). Among them, palbociclib achieved the most promising clinical results, especially in patients with hormone receptor-positive/human epidermal growth factor receptor 2 (HER2)-negative locally advanced or metastatic breast cancer, relapsed/refractory multiple myeloma, and relapsed MCL (Leonard et al., 2012; Niesvizky et al., 2015; Iwata et al., 2017). Palbociclib, an orally available pyridopyrimidine-derived CDK inhibitor,

specifically targets CDK4/CDK6 with a half maximal inhibitory concentration in the low nanomolar range and exhibits only little activity against other kinases like CDK1, CDK2 or CDK5 (Fry et al., 2004; Toogood et al., 2005; Asghar et al., 2015). Palbociclib binds in the cleft between a small amino-terminal lobe and a larger carboxy-terminal lobe of the CDKs, which inhibits ATP binding (Roskoski, 2016). As a result, palbociclib reversibly blocks RB1 phosphorylation on CDK4/6-specific sites, while retaining functional RB1. Subsequently, palbociclib causes cell cycle arrest in G1-phase and consequently inhibits proliferation in different human cancer cell lines (Fry et al., 2004; Finn et al., 2009; Chiron et al., 2013; Cook Sangar et al., 2017). In different *in vitro* studies, potential biomarkers such as cyclin D1, cyclin E, RB1, E2F1, and the CDKIs p15 as well as p16INK4a were identified to predict the response to palbociclib treatment (Konecny et al., 2011; Logan et al., 2013; Ettl, 2016). The gene expression signature of palbociclib resistant cells often features amplification of *CDK4* or mutation as well as loss or *RB1* (Fry et al., 2004; Cen et al., 2012; Malorni et al., 2016; Bollard et al., 2017).

Palbociclib is generally well tolerated in patients with mild to moderate adverse effects such as neutropenia, fatigue, nausea, anemia, thrombocytopenia, and leukopenia. Dose-limiting toxicities mainly result from myelosuppression (Schwartz et al., 2011; Turner et al., 2015; Finn et al., 2016). *In vitro* data of MCL cell lines and primary MCL cells demonstrated that palbociclib suppresses proliferation and cell cycle progression at low nanomolar concentrations but does not induce apoptotic cell death (Marzec et al., 2006). The antiproliferative effect of palbociclib was confirmed in a phase 1 clinical trial in patients with previously treated MCL. Palbociclib treatment efficacy was demonstrated by a marked decrease of pRB1 and Ki-67 positive cells and a reduction in tumor metabolism, assessed by 2-deoxy-2-[18F]fluoro-D-glucose and 3-deoxy-3[18F]fluorothymidine-positron emission tomography (Leonard et al., 2012). Although palbociclib is well tolerated and provides a clinical benefit for a subset of patients, the single agent activity of palbociclib is limited. Therefore, investigations for potential combination therapies with palbociclib are warranted. Prolonged CDK4/6 inhibition with palbociclib has been shown to sensitize primary MCL cells for PI3K inhibition and apoptosis induction (Chiron et al., 2013). In a small phase 1 clinical trial with previously treated MCL patients, the combination of Ibrutinib together with palbociclib resulted in 67 % overall and 37 % complete response rates, with 59 % of patients achieving an estimated 2-year progression-free survival (Martin et al., 2019a).

1.4 Apoptosis

In the past decades, multiple cell death pathways were unveiled, each characterized by unique morphological, biochemical and functional properties (Galluzzi et al., 2018). In both, physiological and pathological settings, the controlled removal of dispensable, damaged, or potentially harmful cells is ensured by a regulated cell death (RCD). RCD always relies on distinct molecular mechanisms, inferring that it can be modulated by the regulation of transduction signals (Galluzzi et al., 2015). Accidental cell death (ACD) on the other hand is the instantaneous and uncontrollable result of chemical, physical or mechanical stress (Radi et al., 2018). Completely physiological forms of RCD that occur in the absence of any exogenous environmental perturbation, as part of a developmental program or to preserve cellular and tissue homeostasis, are referred to as programmed cell death (PCD) (Conradt, 2009; Nagata and Tanaka, 2017; Galluzzi et al., 2018).

Apoptosis represents the best studied form of RCD and was originally described by Kerr, Wyllie and Currie in 1972. Kerr et al. performed ultrastructural analyses of different animal and human tissue and identified a morphologically distinct form of cell death, which was characterized by nuclear as well as cytoplasmic condensation and breaking up of the cell into preserved fragments (Kerr et al., 1972). Apoptotic cell death involves caspase activation, subsequent DNA fragmentation, membrane blebbing, degradation as well as cross-linking of proteins, formation of apoptotic bodies, and expression of ligands for the recognition by phagocytic cells (Coleman et al., 2001; Elmore, 2007). One hallmark of apoptosis, the typical “DNA laddering”, is a result of endonuclease activation by caspases. After activation, endonucleases cleave DNA, producing oligomer fragments of about 180 base pairs, which appear as a DNA ladder when run on an agarose gel (Matassov et al., 2004). Caspases are a family of cysteine proteases, are expressed as proenzymes, and are activated by proteolytic processing (Shi, 2004). Caspases degrade cellular structures, deregulate proteins by separating regulatory and catalytic domains, inactivate inhibitors of proteins that promote apoptosis, and activate other caspases (Thornberry, 1998; Shi, 2004).

The execution pathway of apoptosis is initiated by caspase 3 activation. Caspase 3 can be activated by the intrinsic mitochondrial pathway, the extrinsic “death receptor pathway”, or the perforin/granzyme pathway (Elmore, 2007; Nagata, 2018). In cytotoxic lymphocytes the perforin/granzyme pathway is regulated by granule enzymes, which are serine proteases contained in granules of immune cells. This pathway is part of the innate and adaptive immune response by controlling the survival of activated lymphocytes and the elimination of viruses and tumor cells (Chowdhury and Lieberman, 2008). The extrinsic pathway requires the

stimulation via a death receptor family member such as the tumor necrosis factor (TNF)-related apoptosis-inducing ligand-receptor, FAS, or TNF receptor (Ichim and Tait, 2016). Ligand binding induces the formation of the so called death-inducing signal complex, leading to the activation of caspase 8, which in turn triggers the execution phase of apoptosis (Li et al., 1998). The mitochondrial pathway is regulated by proteins of the Bcl-2 family and can be initiated by various stimuli, including cytokine deprivation, DNA damage, endoplasmic reticulum (ER) stress, radiation, toxins, hypoxia, hyperthermia, viral infections, or free radicals (Elmore, 2007; Tabas and Ron, 2011; Czabotar et al., 2014). Once initiated, this pathway leads to the opening of the mitochondrial permeability transition pore, mitochondrial outer membrane permeabilization (MOMP), and subsequently to the release of pro-apoptotic proteins into the cytosol such as cytochrome c, apoptosis-inducing factor, endonuclease G, and the second mitochondria-derived activator of caspases (Saelens et al., 2004; Kalkavan and Green, 2018). Cytochrome c and Apaf-1 form the so called apoptosome, which in turn recruits the procaspase 9. Afterwards, procaspase 9 undergoes autocatalytic cleavage, gets activated, and finally activates the effector caspase 3 (Yuan and Akey, 2013).

1.4.1 Bcl-2 family proteins

Primarily by regulating MOMP, different pro-apoptotic or anti-apoptotic Bcl-2 family members are responsible for controlling apoptosis induction by the mitochondrial pathway. Bcl-2 family members share one or more of the four Bcl-2 homology (BH) domains (BH1, BH2, BH3, and BH4). Based on their apoptotic function, the Bcl-2 family can be divided into groups of anti-apoptotic proteins (Bcl-2, Bcl-xl, Bcl-w, Mcl-1, Bfl-1/A1), pro-apoptotic BH3-only proteins (Bad, Bid, Bik, Bim, Bmf, Hrk, NOXA, Puma), and pro-apoptotic effectors (Bax, Bak, Bok) (Kale et al., 2018). The pro-apoptotic BH3-only proteins bind with different specificity to their anti-apoptotic counterparts. Bim, Bid, and Puma can bind to all anti-apoptotic Bcl-2 proteins, whereas Bmf, Bid, Hrk, Bik, and NOXA bind more selectively (Letai, 2017). The pro-apoptotic effectors are also selectively activated. Bak preferentially gets activated by Bid, whereas Bim preferentially activates Bax (Sarosiek et al., 2013). After oligomerization, the effector proteins Bax and Bak are able to induce MOMP, which subsequently leads to the execution of the apoptotic program (Adams and Cory, 2018). Bok shares 70–80 % sequence homology with Bax and Bak and is considered to be a potential Bcl-2 family effector. Although studies suggest that Bok is able to directly cause MOMP independently of Bax and Bak, its exact role as a Bcl-2 effector remains unclear (Kalkavan and Green, 2018).

Complex interactions between the three groups of the Bcl-2 family determine Bax/Bak activation, which can be explained by either the “direct activation”, “displacement”, “embedded

together" or "unified" model (Shamas-Din et al., 2013). In the "direct activation" model, pro-apoptotic activators Bid, Bim, and Puma can bind and directly activate Bax/Bak causing subsequent pore formation (Kim et al., 2006). The pro-apoptotic BH3-only proteins Bad, NOXA, Bik, Bmf, and Hrk, however, act as "sensitizer", by liberating BH3-only activators through binding of the anti-apoptotic proteins (Letai et al., 2002; Shamas-Din et al., 2011). The "displacement" (or indirect activation) model suggests that Bax/Bak are constitutively active and that the binding of anti-apoptotic proteins inhibits apoptosis. In this scenario, the pro-apoptotic BH3-only proteins therefore have to displace Bax/Bak from the anti-apoptotic proteins to initiate apoptosis (Willis et al., 2005). The "embedded together" model features both, the displacement and direct activation model. It suggests that anti-apoptotic proteins act as dominant-negative regulators of Bax/Bak by binding activators and effectors in membranes as "locus of action", inhibiting both the activation and the oligomerization step. Sensitizer BH3 proteins neutralize the anti-apoptotic proteins by displacing both the pro-apoptotic activators and Bax/Bak from the membrane (Leber et al., 2010; Shamas-Din et al., 2013). The unified model incorporates components of the previous models, integrating features of Bcl-2 protein interactions inherent to the regulation of the outer mitochondrial membrane integrity and mitochondrial dynamics (Llambi et al., 2011).

Evading apoptosis is one of the hallmarks of cancer, which is often associated with a deregulation of the Bcl-2 family proteins and subsequently the mitochondrial pathway (Hanahan and Weinberg, 2011; Campbell and Tait, 2018). Early studies showed that the founder member of the Bcl-2 family, Bcl-2 together with aberrant activated MYC, contributes to neoplasia in B cell precursors (Vaux et al., 1988). In line with this, amplification of the anti-apoptotic *Mcl-1* and *BCL2L1* (encoding for Bcl-xl) as well as co-amplification of *MYC* was frequently found in a large study of over 3000 cancer samples (Beroukhim et al., 2010). In chronic lymphocytic leukemia (CLL) resistance to apoptosis induction results from high *Mcl-1* mRNA and protein expression, which is driven by high c-ABL, STAT3 and NF-kB activity (Allen et al., 2011). Regulation of Mcl-1 protein stability -1 has also been shown to determine tumor resistance to anti-tubulin agents (Wertz et al., 2011). On the other hand, a decreased expression of the pro-apoptotic Bcl-2 family proteins in cancer can have a similar functional outcome as an increased expression of anti-apoptotic proteins. It was shown that *Bad*-deficient knockout mice are more likely to develop hematopoietic malignancies, which are attributable to diffuse large B cell lymphoma (DLBCL) (Ranger et al., 2003). Carcinogenesis and resistance to chemotherapeutics such as 5-Flourouracil in colorectal epithelial cells was shown to be mediated by the lack or decrease of the pro-apoptotic effector protein Bax (Manoochehriet al., 2014). In MCL, the cytotoxicity of the proteasome inhibitor bortezomib

was shown to be dependent on the induction of the pro-apoptotic NOXA protein (Pérez-Galán et al., 2006).

1.4.2 NOXA

NOXA was first described as phorbol-12-myristate-13-acetate (PMA)-responsive gene, after being identified in a screen of genes, which might be involved in the development of adult T-cell leukemia. The gene was later renamed *PMA-induced protein 1* (*PMAIP1*) and the function was finally elucidated, as the NOXA protein was responsible for the induction of apoptosis in primary mouse cells after x-ray irradiation (Oda et al., 2000). Initially, identified as a primary p53-responsive gene, *NOXA* can also be upregulated by the activity of p73, MYC, E2F1, hypoxia-inducible factor 1α (HIF1α), the PI3K/Akt/mTOR pathway via cAMP response element-binding, or the forkhead transcription factor 3 (FOXO3) (Hershko and Ginsberg, 2004; Kim et al., 2004; Mei et al., 2007; Nikiforov et al., 2007; Grande et al., 2012; Ausserlechner et al., 2013). On the other hand, by inducing histone methylation, the B-lymphoma Mo-MLV insertion region 1 homolog was reported to repress the expression of *NOXA*, thereby regulating memory Th1/Th2 cell homeostasis (Yamashita et al., 2008).

The sensitizer BH3 protein NOXA does not bind to pro-apoptotic effector proteins but binds highly selective to the anti-apoptotic Mcl-1 and, with lower affinity, to Bfl-1/A1 (Chen et al., 2005). Although much less sensitive, NOXA can also bind the anti-apoptotic proteins Bcl-xl or Bcl-2. Interestingly, Bcl-2 mutants have also been described, which bind NOXA in an extent comparable to Mcl-1 (Smith et al., 2011). Protein levels of the pro-apoptotic NOXA have been shown to determine therapy responsiveness in many different cancers. It was demonstrated that testicular germ cell tumors are hypersensitive to cisplatin treatment because of constitutively high NOXA protein levels (Gutekunst et al., 2013). In acute lymphoblastic leukemia, cell death induction after chemotherapy with betulinic acid, doxorubicin, and vincristine is mediated by NOXA (Ehrhardt et al., 2012). Moreover, expression of the NOXA protein is crucial for the effectiveness of bortezomib treatment in melanoma and multiple myeloma (Qin et al., 2005; Gomez-Bougie et al., 2007).

Although a functional regulation of NOXA protein levels seems to be important for cancer therapy, NOXA deficiency alone does not lead to spontaneous tumor development in knockout mice (Shibue et al., 2003). Proper NOXA function is particularly important for the regulation of its anti-apoptotic counterpart Mcl-1 because many tissues express Mcl-1 and frequently found *Mcl-1* gene amplifications enable many cancer genomes to ensure high levels of Mcl-1 protein (Krajewski et al., 1995; Beroukhim et al., 2010; Ertel et al., 2013). Apart from functioning as a sensitizer, the NOXA protein also contributes to the proteasomal degradation

of Mcl-1 by affecting Mcl-1 localization and ubiquitination (Gomez-Bougie et al., 2011; Nakajima et al., 2014). Apart from its role in mitochondrial apoptosis, NOXA is also implicated in the regulation of autophagy. NOXA contributes to autophagy induction by displacing Mcl-1 and Bcl-xl from the key autophagy regulator Beclin-1 (Elgendy et al., 2011; Kang et al., 2011; Tang et al., 2012; Hagenbuchner et al., 2017).

1.5 Regulation of protein stability

1.5.1 Ubiquitin-proteasome system and cancer

The UPS is the most important intracellular protein degradation machinery, which is involved in the regulation of most cellular processes such as protein homeostasis, apoptosis, cell survival, cell-cycle progression, DNA repair, and antigen presentation. Early studies of the UPS first described a process, in which certain proteins were covalently conjugated to a protein called ubiquitin and subsequently degraded by an ATP-dependent protease (Hershko et al., 1980). The core component of the UPS, the 26S proteasome complex is responsible for at least 80 % of the protein degradation in mammalian cells (Collins and Goldberg, 2017). The 26S proteasome is a multicatalytic complex of a 20S catalytic core and two 19S regulatory caps on both ends and is referred to by its Svedberg sedimentation coefficient (Di Chen and Dou, 2010; Kish-Trier and Hill, 2013). The 20S catalytic core is also known as the 20S core particle or 20S proteasome and consists of the three catalytic subunits β1, β2, and β5. The β-subunits active sites contain peptidyl-glutamyl-hydrolyzing or caspase-like, trypsin-like, and the chymotrypsin-like activity to execute their different proteolytic specificities (Livneh et al., 2016). Substrates are selectively targeted for UPS dependent degradation by polyubiquitination through specific enzymes. The tagged proteins are subsequently unfolded and cleaved into short peptides by the 26S proteasome complex (Ravid and Hochstrasser, 2008).

The process of protein ubiquitination is carried out by the concerted action of the Ub-activating (E1), Ub-conjugating (E2), and Ub-ligating (E3) enzyme (Scheffner et al., 1995). In a first step the E1 enzyme activates ubiquitin in an ATP-dependent manner by forming a thioester bond with the carboxyl terminus of ubiquitin. During the next steps, the activated ubiquitin is transferred to an E2 enzyme and is finally conjugated to one of the seven lysine residues (K6, K11, K27, K29, K33, K48 or K63) of the target substrate, with the help of a substrate specific E3 ligase (Hochstrasser, 1996). In successive rounds, E3 ligases can add additional ubiquitin to the existing chain and create a polyubiquitin chain. The E3 enzymes control both the substrate specificity and the efficiency of the ubiquitination process. The possibility to link ubiquitin to different lysine residues and to create ubiquitin chains of variable length, creates different

ubiquitination patterns to determine what downstream signals are affected (Buetow and Huang, 2016). On the other hand, the action of deubiquitinating enzymes antagonizes the action of the E3 ligase by cleaving ubiquitin or ubiquitin-like proteins from target proteins (Wilkinson, 1997).

Proteasomal activity, however, is not only determined by the rate of protein ubiquitination, but can be regulated by changes in proteasome abundance, function, and composition (Dikic, 2017). Regulation of proteasomal activity can be carried out by transcriptional regulation of proteasomal subunits or factors triggering degradation of proteasomes. Furthermore, regulation of the assembly and disassembly rate of the proteasomal subunits, as well as posttranslational modifications modulate the degradative capacity of proteasomes (Meiners et al., 2003; Livnat-Levanon et al., 2014; Hirano et al., 2016). Cancer cells are often characterized by a higher proteasomal activity than normal cells (Kumatori et al., 1990; Loda et al., 1997). Conversely, cancer stem cells, which are responsible for therapy resistance and tumor recurrence, are associated with a low proteasomal activity (Lenos and Vermeulen, 2016). Targeting proteasome activity for cancer treatment is currently under intensive investigation, as cancer cells have been shown to be more sensitive to the cytotoxic effects of proteasome inhibition than healthy cells. A high proliferative activity, which represents a cancer hallmark, might account for an increased demand for protein synthesis and render malignant cells susceptible to proteasome inhibition (Crawford et al., 2011).

The three proteasome inhibitors bortezomib, ixazomib, and carfilzomib advanced the most to the clinic to date. These proteasome inhibitors target the 20S core particle of the proteasome and are currently approved for the treatment of multiple myeloma and MCL (Manasanch and Orlowski, 2017). The first clinically approved proteasome inhibitor bortezomib (trade name Velcade) reversibly blocks the caspase-like and chymotrypsin-like proteolytic activity of the 20S proteasome, but only blocks the trypsin-like activity at high concentrations (Teicher and Tomaszewski, 2015). It has been shown that proteasome inhibitors such as bortezomib are well tolerated in normal cells (Kisselev et al., 2006). Bortezomib therapy options are under intensive investigation for the treatment of MCL patients. In patients with relapsed or refractory MCL, bortezomib showed promising single-agent activity with good responses and increased survival (Goy et al., 2009). In addition, the combination of bortezomib with fractionated cyclophosphamide and rituximab resulted in an overall response rate of 74 % and a complete response rate of 42 % in patients with relapsed or refractory MCL (Lee et al., 2017).

1.5.2 Autophagy–lysosomal pathway and cancer

In addition to the proteasomal degradation machinery, autophagy represents the second most important mechanism for removing and recycling of misfolded or aggregated proteins, intracellular pathogens, as well as damaged organelles such as mitochondria, the endoplasmic reticulum, or peroxisomes (Nedelsky et al., 2008; Boya et al., 2013). Especially after starvation, the products of this catabolic process can subsequently be used for energy supply and for the buildup of cellular components (Levine and Yuan, 2005; Glick et al., 2010). The term autophagy was first coined by Christian de Duve at the *Ciba Foundation symposium on lysosomes* in 1963 and is derived from the Greek words auto (self) and phagy (eating) (Klionsky, 2014). Early descriptions of autophagy were already made decades ago. In recent years, however, numerous studies helped to elucidate the molecular basis of this degradation machinery and thereby contributed to the understanding of the physiological significance of this process. To date, over 40 so called autophagy-related genes (ATG) were discovered, including genes responsible for autophagosome formation (referred as core machinery), as well as genes required for selective types of autophagy (Yang and Klionsky, 2010; Ohsumi, 2014; Klionsky et al., 2021). The observation that features of high autophagic activity frequently accompany cell death, led to the classification of another type of programmed cell death, the so called “autophagic cell death”. It is clear that this type of cell death somehow involves the degradative capacity of autophagy. Further research still needs to clarify, however, how this cell death pathway is initiated as well as executed and what biomarkers can be used to identify this type of cell death (Yonekawa and Thorburn, 2013; Liu and Levine, 2015).

Autophagic degradation in mammalian cells is usually subdivided into microautophagy, chaperone-mediated autophagy (CMA), or macroautophagy (generally referred as autophagy). The cargo of all three types of autophagy is delivered to the lysosome for degradation and subsequently recycled. The underlying mechanisms and associated morphological features, however, are different for each type of autophagy (Parzych and Klionsky, 2014). Soluble or particulate cellular constituents, which are targeted for microautophagy, directly enter the lysosome through invagination, protrusion or septation of the lysosomal membrane (Hayat, 2017). The CMA selectively mediates cargo to lysosomes and involves specific chaperones such as the heat shock cognate protein of 70 kDa. Chaperone-protein complexes are translocated across the lysosomal membrane and subsequently recognized by the lysosome-associated membrane protein type 2A (Kaushik et al., 2011). Macroautophagy (hereafter only referred to as autophagy) represents the best characterized autophagic pathway, which delivers cytoplasmic cargo to the lysosome. For autophagy initiation, cargo is sequestered at the

phagophore assembly site, which consists of the double membrane structure called phagophore and the autophagy core machinery proteins (Xie and Klionsky, 2007). After subsequent expansion and closure, the phagophore structure forms an autophagosome. The autophagosome gets trafficked along microtubules for subsequent fusion with the lysosome to form an autolysosome, which finally causes the degradation of the cargo (Jahreiss et al., 2008; Luo and Rubinsztein, 2010). Lysosomal degradation of different kind of substrates is carried out in an acidic environment by approximately 60 different soluble hydrolases such as sulfatases, glycosidases, peptidases, phosphatases, lipases, and nucleases (Settembre et al., 2013). Resulting degradation products are transported back into the cytosol through membrane permeases for reuse (Yang and Klionsky, 2010).

The role of autophagy in cancer remains controversial and depends on the cellular background. In different cancer types, autophagy has been shown to either promote or suppress tumor development and tumorigenesis (Takamura et al., 2011; Yang et al., 2011). In addition, the role of autophagy in cancer therapy remains also controversial, as autophagy has been shown to mediate cytotoxic effects of chemotherapy but generally has been associated with chemotherapy resistance (Amaravadi et al., 2007; Lee et al., 2007). Therefore, many groups investigate how targeting autophagy might improve therapy outcome and help overcoming therapy resistances. Different chemotherapeutics have been shown to induce autophagy such as DNA-damaging agents, antimetabolites, microtubule interfering molecules, or kinase inhibitors (Notte et al., 2011; Garbar et al., 2017).

There are various agents that are used in preclinical trials to regulate autophagy, while most of them inhibit autophagic activity or autophagosomal degradation. Autophagy inhibitors inhibit autophagy at different stages and typically target class I PI3Ks, VPS34, autophagy-activating Unc-51-like kinase 1 (ULK1/ATG1), or the lysosomal function (Pasquier, 2016). As most autophagy inhibitors lack specificity, clinical results have to be carefully interpreted by the use of multiple autophagy inhibitors and appropriate autophagy markers. Chloroquine and its derivative hydroxychloroquine have been initially approved by the Food and Drug Administration (FDA) as antimalarial drug, but were later repurposed as late step autophagy inhibitors and are currently under investigation in several clinical trials (Chude and Amaravadi, 2017). It was shown that inhibitors of autophagosome function can overcome autophagy mediated resistance to anti-estrogens such as tamoxifen, in estrogen receptor–positive breast cancer cells (Samaddar et al., 2008). Furthermore, preclinical data demonstrates that molecular targeted therapies can also benefit from combined autophagy regulation. Autophagy induction was demonstrated to protect MCL cells from agents that target Akt/mTOR activity, which could be counteracted by autophagy inhibition (Rosich et al., 2012).

1.6 Aims

MCL is still incurable to date and the identification of novel treatment options is urgently needed. Targeted treatment options such as proteasome inhibitors are on the rise, but therapy resistance and insufficient treatment efficacy have been reported. The proteasome inhibitor bortezomib is already approved for the treatment of multiple myeloma as well as MCL. Both of these cancer types, as many others, are frequently associated with cyclin D1 overexpression and aberrant cyclin D1/CDK4 activity. Several studies show that cyclin D1/CDK4 activity does not only regulate cell cycle but also various other cellular functions. Therefore, the present study aimed to investigate how cyclin D1 overexpression and subsequent aberrant cyclin D1/CDK4 activity affect the treatment efficacy of proteasome inhibitors in MCL. To address this issue, MCL cell lines and primary samples from MCL patients were used to

(I) identify functions of cyclin D1/CDK4 activity apart from cell cycle regulation in MCL.

(II) determine whether novel cyclin D1/CDK4 functions in MCL can be exploited for cell death regulation after treatment with proteasome inhibitors.

(III) elucidate underlying molecular mechanisms of identified novel cyclin D1/CDK4 functions in MCL.

(IV) identify drugs or drug combinations to enhance treatment efficacy of proteasome inhibitors in MCL.

2 Material and Methods

2.1 Cell culture

2.1.1 Cell lines

All cell lines used in this study are listed in Table 1.

Table 1: Cell lines

cell line	source	obtained from
Mino	mantle cell lymphoma	DSMZ (Braunschweig, Germany)
Jeko-1	mantle cell lymphoma	DSMZ (Braunschweig, Germany)
Rec-1	mantle cell lymphoma	DSMZ (Braunschweig, Germany)
Granta-519	mantle cell lymphoma	DSMZ (Braunschweig, Germany)
Jvm2	mantle cell lymphoma	DSMZ (Braunschweig, Germany)
NIH3T3/CD40	mouse embryonic fibroblast	kind gift from Dr. Martina Seiffert from the German Cancer Research Center, DKFZ (Heidelberg, Germany)

2.1.2 Cell culture

All cell lines, peripheral blood mononuclear cells (PBMNC) as well as primary MCL cells were cultured in supplemented RPMI-1640 as indicated below.

500 ml RPMI-1640 supplemented with:	Biochrom (Berlin, Germany)
20 % (10 % for PBMNCs) (v/v) FBS	Gibco (Carlsbad, USA)
0.1 g/l Penicillin/Streptomycin	Gibco (Carlsbad, USA)
10 mM HEPES pH 7,4	Merck (Darmstadt, Germany)
2 mM L-glutamine	Biochrom (Berlin, Germany)
0.13 mM L-asparagine	Serva (Heidelberg, Germany)
0.05 mM ß-mercaptoethanol	Merck (Darmstadt, Germany)
1 mM sodium pyruvate	Gibco (Carlsbad, USA)
3 ml 100x non-essential amino acids	Biochrom (Berlin, Germany)

Cells were maintained in a sterile laminar flow hood using sterile culture flasks and disposables. Cell cultures were grown in an incubator at 37 °C with 5 % CO_2 and subcultured every 2-3 days.

Cell culture flasks	Sarstedt (Nümbrecht, Germany)
6-, 12-, 24-, 96-well-plates	Greiner Bio-One (Frickenhausen, Germany)
Serological pipettes	Corning (Corning, USA)
15-, 50 ml tubes	Sarstedt (Nümbrecht, Germany)
CO_2-incubator	Heraeus (Hanau, Germany)
Laminar flow hood	Heraeus (Hanau, Germany)

Cell count and cell viability was assessed by trypan blue vital staining using a hemocytometer. Trypan blue vital staining is based on the principle that live cells possess intact cell membranes that are able to exclude certain dyes such as trypan blue whereas dead cells do not (Zhang et al., 2018).

Trypan blue 0.5 % (w/v) in PBS	Biochrom (Berlin, Germany)
PBS	Biochrom (Berlin, Germany)
Neubauer hemocytometer	Roth (Karlsruhe, Germany)
Microscope	Carl Zeiss AG (Oberkochen, Germany)

Maximum culture period of all cell lines was 3 months to prevent genetic changes. For cryopreservation of cells, 1-5 x 10^6 cells were centrifuged (5 min, 1400 rpm, 4 °C), collected in ice-cold dimethyl sulfoxide:FBS (1:10), and transferred to a cryotube. Cells were then gradually cooled down to -80 °C in a freezing container before long term storage at -196 °C in liquid nitrogen. To establish a new culture, cell lines were thawed in a water bath at 37 °C, transferred to prewarmed RPMI-1640 medium, centrifuged (5 min, 1400 rpm, room temperature), resuspended in fresh medium, and then placed in an incubator at 37 °C with 5 % CO_2.

1.8 ml CryoTube TM	Nalgene Nunc (Rochester, USA)
5100 Cryobox 1°C ‚Mr. Frosty‘	Nalgene Nunc (Rochester, USA)
Dimethyl sulfoxide	Merck (Darmstadt, Germany)
FBS	Gibco (Carlsbad, USA)

2.1.3 Isolation of primary MCL cells and healthy blood cells

The local ethics committee approved the collection of patient material as well as sample collection from healthy donors. The study was carried out in accordance with protocols approved by the Ethics Committee of the Robert-Bosch-Hospital (Stuttgart, Germany). All patients and donors signed an informed consent according to the Declaration of Helsinki.
Lymph node biopsy material of MCL patients was cut into small pieces using a sterile scalpel and subsequently transferred to a cell strainer (Becton Dickinson, Franklin Lakes, USA) to obtain a single cell suspension of primary MCL cells. Primary MCL cells were centrifuged (5 min, 1400 rpm, room temperature) and washed in PBS. Afterwards, cells were counted and cryopreserved or directly cultivated.
To obtain PBMNCs, blood samples from healthy donors were mixed with an equal amount of sterile PBS, slowly layered on an equal amount of Ficoll (Biochrom, Berlin, Germany) in a 50 ml Falcon tube, and centrifuged (25 min, 1400 rpm, without brake, room temperature). The PBMNC fraction that formed in the interface between both layers was transferred to a new falcon tube, diluted with PBS, and centrifuged again (5 min, 1400 rpm, room temperature). Afterwards, cells were resuspended in PBS, counted, and cryopreserved or directly cultured.

2.1.4 Culture of primary cells

Normal PBMNCs from healthy donors and primary MCL cells were cultured with supplemented RPMI-1640 (as described in 2.1.2 Cell culture) and placed in an incubator at 37 °C with 5 % CO_2. To stimulate growth of PMNCs, 2 µg/ml phytohemagglutinin-L was added during culture. Primary MCL cells were exposed to 50 ng/ml Interleukin-10, 50 ng/ml B cell activating factor, 1 ng/ml of Insulin-like growth factor-1, and 1 ng/ml Interleukin-6 and co-cultured with the CD40 ligand expressing cell line NIH3T3/CD40 (10:1 MCL:3T3 ratio). The NIH3T3/CD40 cell line was X-ray irradiated with 30 gray, one day before co-culture.

Phytohemagglutinin-L	Roche (Basel, Switzerland)
Interleukin-6	Merck (Darmstadt, Germany)
Interleukin-10	Merck (Darmstadt, Germany)
B cell activating factor	Merck (Darmstadt, Germany)
Insulin-like growth factor-1	Merck (Darmstadt, Germany)

2.2 Reagents

2.2.1 Proteasome inhibition

To study the effect of proteasome inhibition cells were treated with 5 or 7 nM of bortezomib or carfilzomib (Selleckchem, Houston, USA).

2.2.2 Cyclin D1/CDK4 inhibition

Cells were treated with either 100 nM (Mino) or 300 nM (Jeko-1, Granta-519, and primary MCL cells) palbociclib (Selleckchem, Houston, USA) to inhibit cyclin D1/CDK4 activity and investigate the role of aberrant cyclin D1 expression.

2.2.3 ROS modulating agents

The ROS modulating substances L-Glutathione (GSH) reduced, phenethyl isothiocyanate (PEITC), and hydrogen peroxide were used to study the effects of ROS. Cells were pretreated with GSH at a concentration of 2 mM. PEITC and hydrogen peroxide were used at a concentration of 8 µM and 500 µM, respectively.

GSH	Merck (Darmstadt, Germany)
PEITC	Merck (Darmstadt, Germany)
Hydrogen peroxide	Merck (Darmstadt, Germany)

2.2.4 Autophagy inhibitors

Autophagic activity was inhibited using 3-methyladenine (3MA), liensinine, spautin-1, and hydroxychloroquine. 3MA was demonstrated to inhibit autophagy at an early stage through suppression of class III PI3K activity (Petiot et al., 2000) and was used at a concentration of 2 mM and 5 mM. Spautin-1 was used at a concentration of 5 µM. The ability of spautin-1 to inhibit autophagy at an early stage was shown to be mediated by Beclin-1 downregulation (Shao et al., 2014). Liensinine on the other hand was shown to inhibit autophagy at a later stage by blocking autophagosome-lysosome fusion (Zhou et al., 2015) and was used at a concentration of 20 µM. Hydroxychloroquine, representing another late stage autophagy inhibitor, was used at 120 µM and has been demonstrated to inhibit the acidification of lysosomes (Amaravadi et al., 2011).

3MA	Merck (Darmstadt, Germany)
Liensinine	ChemFaces (Wuhan, China)
Spautin-1	Merck (Darmstadt, Germany)
Hydroxychloroquine	Merck (Darmstadt, Germany)

To screen for substances that block autophagic activity in combination with proteasome inhibition, different substances that are currently used in MCL treatment or that are under clinical investigation (Table 2) were co-treated with 5 nM bortezomib.

Table 2: Substances used for screening of autophagy inhibitors.

substance	Mode of action/target	concentration	company
Orlistat	FASN inhibitor	15 µM	Merck (Darmstadt, Germany)
MLN4924	Neddylation inhibitor	50 nM	ChemieTek (Indianapolis, USA)
Ibrutinib	BTK inhibitor	1 µM	Selleckchem (Houston, USA)
Cisplatin	DNA damage	10 µM	Robert Bosch Hospital, Stuttgart
Temsirolimus	mTOR inhibitor	10 nM	Merck (Darmstadt, Germany)
Lenalidomide	various	1 µM	Selleckchem (Houston, USA)
Dinaciclib	CDK inhibitor	9 nM	Selleckchem (Houston, USA)
Etoposide	DNA damage	10 µM	Robert Bosch Hospital, Stuttgart
Oligomycin	ATP synthase inhibitor	5 µM	Merck (Darmstadt, Germany)
ABT199	Bcl-2 inhibitor	50 nM	Selleckchem (Houston, USA)
BEZ235	PI3K/mTOR inhibitor	1 µM	Selleckchem (Houston, USA)

2.2.5 Caspase inhibition

Caspase activity in MCL cell lines was inhibited using the caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK) (Bachem, Bubendorf, Switzerland). Z-VAD-FMK is a cell-permeable pan-caspase inhibitor that irreversibly binds to the catalytic site of caspases and was used at a concentration of 50 µM.

2.2.6 AMPK inhibition

AMPK activity was targeted by treating MCL cell lines with 2 µM dorsomorphine (Selleckchem, Houston, USA). Dorsomorphine is a reversible ATP-competitive AMPK inhibitor (Zhou et al., 2001).

2.3 Cell death analysis

To assess cell death and to distinguish between vital and apoptotic cells, Annexin V- propidium iodide (PI) staining (Cornelissen et al., 2002) and flow cytometry was used. Annexin V-PI staining allows for the discrimination between cells undergoing early and late stage apoptosis. During the early apoptotic stage, cells expose the phospholipid phosphatidylserine to their cell surface. In viable cells phosphatidylserine, however, is located on the inner membrane. Annexin V is able to bind phosphatidylserine and can therefore be used in combination with a fluorophore to visualize apoptotic events. Combination of Annexin V with the fluorescent dye PI allows the identification of late stage apoptosis. During late stage apoptosis or necrosis cellular membranes become permeable and therefore allow PI to enter the cells. For cell death analysis via Annexin V-PI staining, cells were centrifuged (5 min, 1400 rpm, 4 °C), washed in ice-cold PBS, centrifuged again (5 min, 1400 rpm, 4 °C), washed in ice-cold Annexin V binding buffer (10 mM HEPES, 140 mM sodium chloride, 25 mM calcium chloride, pH 7.4), centrifuged again (5 min, 1400 rpm, 4 °C), and finally resuspended in 100 µl staining solution (93 µl Annexin V binding buffer, 5 µl Annexin V- fluorescein isothiocyanate (FITC), and 2 µl PI stock solution). After 10 min incubation time at room temperature, 300 µl Annexin V binding buffer was added to the cell suspension before flow cytometry analysis on a FACSCalibur flow cytometer. Accordingly, cell death of primary MCL cells was assessed by Annexin V-allophycocyanin (APC) staining (without PI). Prior to Annexin V-APC staining, however, cells were washed with 1 % (w/v) BSA/PBS and stained on ice for 15 min with anti CD5-phycoerythrin (PE) antibody and anti CD20-FITC antibody. CD5-CD20 positive cells were subsequently analyzed on a flow cytometer to assess Annexin V expression and cell death induction.

Annexin V-FITC	Becton Dickinson (Franklin Lakes, USA)
Annexin V-APC	Becton Dickinson (Franklin Lakes, USA)
Anti CD5-PE antibody	Miltenyi (Bergisch Gladbach, Germany)
Anti CD20-FITC antibody	Miltenyi (Bergisch Gladbach, Germany)
Propidium iodide stock solution (1 mg/ml)	Merck (Darmstadt, Germany)
Annexin V binding buffer	Becton Dickinson (Franklin Lakes, USA)
CELLQuest software	Becton Dickinson (Franklin Lakes, USA)
FACSCalibur flow cytometer: Laser: 488 nm/635 nm Detector: 530 nm/585 nm/650 nm	Becton Dickinson (Franklin Lakes, USA)

2.4 Protein expression analysis

2.4.1 Protein extraction and sample preparation

Cell suspensions were centrifuged (5 min, 1400 rpm, 4 °C), washed with ice-cold PBS, and centrifuged again (5 min, 1400 rpm, 4 °C). Cell pellets were transferred into liquid nitrogen after removal of supernatants. Frozen cell pellets were subsequently resuspended in 100 µl lysis buffer (50 mM TRIS, 250 mM sodium chloride, 0.1 % (v/v) Triton X-100, 5 mM EDTA, pH 7.6, with protease and phosphatase inhibitors) and sonicated for 20 seconds at 4 °C. After cell lysis, the cell suspensions were centrifuged (15 min, 13000 rpm, 4 °C) and the resulting supernatants were transferred to new tubes. The protein concentration was subsequently assessed by Bradford assay using bovine serum albumin (BSA) as standard protein. Protein lysates were diluted (resulting in 20-40 µg total protein amount) in Laemmli buffer (62.5 mM TRIS, 20 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol, 2 % (w/v) SDS, 1 % (w/v) bromophenol blue, pH 6.8) and boiled at 95°C for 5 min.

EDTA	Roth (Karlsruhe, Germany)
TRIS	Merck (Darmstadt, Germany)
Sodium chloride	Merck (Darmstadt, Germany)
Triton X-100	Bio-Rad (Munich, Germany)
PhosphoSTOP	Roche (Basel, Switzerland)
Protease inhibitor cocktail tablets	Roche (Basel, Switzerland)
Ultrasonic homogenizer Sonopuls HD200	Bandelin Elektronik (Berlin, Germany)
Protein Assay Dye Reagent	Bio-Rad (Munich, Germany)
BSA	Merck (Darmstadt, Germany)
Novaspec II Visible spectrophotometer	GE Healthcare (Chicago, USA)
Glycerol	Merck (Darmstadt, Germany)
Bromophenol blue	Bio-Rad (Munich, Germany)

2.4.2 Protein separation by SDS-PAGE

Separation of proteins according to their molecular weight was carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Different concentrations of acrylamide were used depending on the protein of interest. Lower acrylamide concentrations in the gel were used to separate proteins with high molecular weight, whereas high acrylamide concentrations in the gel were used to resolve proteins with lower molecular weight.

Gel polymerization was initiated by the addition of tetramethylethylenediamine (TEMED) and ammonium persulfate (APS). Gradient gels with an acrylamide concentration between 5 and 20 % were used for the separation of small proteins (molecular weight <10 kDa). To determine the molecular weights of the separated proteins, a protein marker was used. Protein separation was carried out at 6-12 mA, over 18-20 hours in electrophoresis buffer (25 mM TRIS, 0.2 M glycerol, 1 % (v/v) SDS).

APS	Bio-Rad (Munich, Germany)
TEMED	Roth (Karlsruhe, Germany)
30-, 40 % acrylamid/bis-solution (37.5:1)	Bio-Rad (Munich, Germany)
Prestained Protein Marker, Broad Range	NEB (Ipswich, USA)
Thermomixer Comfort	Eppendorf (Hamburg, Germany)
Vertical electrophoresis system, Protean II	Bio-Rad (Munich, Germany)

2.4.3 Western Blot analysis

After protein separation by SDS-PAGE, proteins were transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer cell. The SDS-PAGE gel was covered with the respective membrane and layered between three layers of Whatman filter paper, which were pre-soaked with transfer buffer (0.025 M TRIS, 0.192 M glycerol, 20 % (v/v) methanol, 1 % (w/v) SDS). Transfer of proteins was carried out at 200 mA for 90 min. Membranes were washed in TBST (137 mM sodium chloride, 2.7 mM potassium chloride, 25 mM TRIS, 0.1 % (v/v) Tween-20, pH 7.4) and blocked in 5 % (w/v) skim milk in TBST for one hour at room temperature. Membranes were incubated with primary antibodies according to Table 3 for 18-20 hours at 4 °C. Afterwards, membranes were washed four times in TBST for at least 5 min at room temperature and subsequently incubated with the corresponding secondary antibody (Table 3) according to their source species. After secondary antibody incubation, membranes were again washed in TBST four times for at least 10 min at room temperature and subsequently incubated with the SuperSignal West Dura Extended Duration Substrate. For detection of proteins of interest, membranes were analyzed using a Luminescent imager and the imaging software advanced image data analyzer (AIDA).

Skim milk powder	Merck (Darmstadt, Germany)
Amersham Protran® nitrocellulose membrane	GE Healthcare (Chicago, USA)
Amersham Hybond P PVDF membrane	GE Healthcare (Chicago, USA)
Filter paper, Gel-blotting paper	Schleicher & Schuell (Dassel, Germany)
Trans-Blot® semi-dry transfer cell	Bio-Rad (Munich, Germany)
Biometra Fastblot	Analytik Jena (Jena, Germany)
Western blocking reagent	Roche (Basel, Switzerland)
SuperSignal® West Dura Substrate	Pierce Biotechnology (Waltham, USA)
LAS-1000 Luminescent imager	FUJI Medical Systems (Stamford, USA)
AIDA software version 2.31	Elysia-raytest (Straubenhardt, Germany)

Table 3: Primary and HRP-coupled secondary antibodies.

antibody	source	dilution	company
α-tubulin	mouse	1:5000 in 5 % (w/v) skim milk/TBST	NEB (Ipswich, USA)
β-actin	mouse	1:5000 in 5 % (w/v) skim milk/TBST	NEB (Ipswich, USA)
AMPKα	rabbit	1:1000 in 5 % (w/v) BSA/TBST	NEB (Ipswich, USA)
ATG5	rabbit	1:1000 in 5 % (w/v) BSA/TBST	NEB (Ipswich, USA)
ATG7	rabbit	1:1000 in 5 % (w/v) BSA/TBST	NEB (Ipswich, USA)
Bak	rabbit	1:500 in 5 % (w/v) BSA/TBST	NEB (Ipswich, USA)
Bax	rabbit	1:1000 in 5 % (w/v) BSA/TBST	NEB (Ipswich, USA)
CDK4	rabbit	1:1000 in 5 % (w/v) BSA/TBST	NEB (Ipswich, USA)
Cyclin D1	mouse	1:500 in 5 % (w/v) skim milk/TBST	Santa Cruz (Dallas, USA)
GAPDH	rabbit	1:5000 in 5 % (w/v) skim milk/TBST	NEB (Ipswich, USA)
LC3	rabbit	1:1000 in 5 % (w/v) BSA in TBST	NEB (Ipswich, USA)
NOXA	mouse	1:500 in 1 % (v/v) Roche block/TBS	Merck (Darmstadt, Germany)
Mcl-1	rabbit	1:500 in 5 % (w/v) BSA/TBST	NEB (Ipswich, USA)
SQSTM1	rabbit	1:1000 in 5 % (w/v) BSA/TBST	NEB (Ipswich, USA)
p-AMPKα	rabbit	1:1000 in 5 % (w/v) BSA/TBST	NEB (Ipswich, USA)
pRB1	rabbit	1:1000 in 5 % (w/v) BSA/TBST	NEB (Ipswich, USA)
Puma	rabbit	1:1000 in 5 % (w/v) BSA/TBST	NEB (Ipswich, USA)
RB1	mouse	1:2000 in 5 % (w/v) skim milk/TBST	NEB (Ipswich, USA)
Sestrin 3	rabbit	1:1000 in 5 % (w/v) BSA/TBST	ProteinTech (Chicago, USA)
anti-rabbit-IgG-HRP	goat	1:2000 in 5 % (w/v) skim milk/TBST	NEB (Ipswich, USA)
anti-mouse-IgG-HRP	goat	1:2000 in 5 % (w/v) skim milk/TBST	NEB (Ipswich, USA)

2.4.4 Analysis of protein half-life

The half-life of NOXA protein was determined by cycloheximide (CHX) pulse-chase experiments. The translation inhibitor CHX has been shown to inhibit translation elongation and therefore to efficiently block *de novo* protein synthesis (Schneider-Poetsch et al., 2010). MCL cell lines were treated with 20 µg/ml CHX (Merck, Darmstadt, Germany) and harvested at different time points (0-180 min) for subsequent analysis by western blot. Densitometric quantification of NOXA protein expression was done using the imageJ v1.5 software (Schneider et al., 2012).

2.5 Cell cycle analysis

Cell cycle distribution was analyzed by bromodeoxyuridine/fluorodeoxyuridine (BrdU)-PI staining (Dolbeare et al., 1983). This method combines the flow cytometric analysis of cellular DNA content and DNA synthesis. The fluorescent dye PI intercalates into DNA, which allows for the quantification of DNA. Active DNA synthesis on the other hand can be investigated by the incorporation of the thymidine analog BrdU into recently synthesized DNA and subsequent antibody staining. G1-phase cells are BrdU negative and have one single set of chromosomes. DNA replication takes place during S-phase. S-phase cells therefore are BrdU positive and possess between one to two set of chromosomes. G2-cells are characterized by a double set of chromosomes and BrdU negativity. For BrdU-PI staining, 10 µM BrdU was added to proliferating cells for 45 min at culture conditions. Cells were subsequently harvested by centrifugation (5 min, 1400 rpm, room temperature), fixed with 70 % (v/v) ethanol at -20 °C, and stored overnight at 4 °C. Cells were then centrifuged (5 min, 1400 rpm, room temperature) and resuspended in 2 M HCl/ 5 % (v/v) Triton X-100. After centrifugation (5 min, 1400 rpm, room temperature), cells were resuspended with 0.1 M sodium borate and washed in PBS/1 % (w/v) BSA/0.5 % (v/v) Tween. Cell pellets were stained for 30 min at 4 °C with an anti-BrdU antibody (1:200 dilution in PBS/1 % (w/v) BSA/0.5 % (v/v) Tween 20). After washing cells with PBS/1 % (w/v) BSA/0.5 % (v/v) Tween, cells were stained with a goat-anti-mouse (GAM)-DyLight488 secondary antibody (1:200 dilution in PBS/1 % (w/v) BSA/0.5 % (v/v) Tween 20) for 30 min at 4 °C in the dark. After antibody staining, cells were washed in 1 % (w/v) Glucose/PBS solution and subsequently stained with PI staining solution (50 µg/ml PI, 1 mg/ml RNase A, PBS) for 10 min at room temperature. After BrdU/PI co-staining, cells were analyzed on a FACSCalibur flow cytometer.

BrdU	Merck (Darmstadt, Germany)
Anti BrdU antibody	Becton Dickinson (Franklin Lakes, USA)
GAM-DyLight488 antibody	Dianova (Hamburg, Germany)
Tween 20	Merck (Darmstadt, Germany)
Ethanol	Merck (Darmstadt, Germany)
Glucose	Merck (Darmstadt, Germany)
RNase A	Qiagen (Hilden, Germany)

2.6 RNA analysis

2.6.1 RNA isolation

Isolation of total RNA from MCL cell lines was performed using RNeasy kit Qiagen (Hilden, Germany) according to the manufacturer's instructions.

2.6.2 cDNA synthesis

Synthesis of complementary DNA (cDNA) was carried out by mixing 250 ng total RNA with 200 ng oligo(dT) primer in water (total volume 15 µl). Samples were boiled at 70 °C for 5 min and subsequently placed on ice. Samples were mixed with 10 µl reaction solution (2x first strand-buffer, 1.25 mM deoxy-nucleoside triphosphates (dNTP), 25 units RNasin, and 200 units of reverse transcriptase M-MLV) and incubated at 42 °C for 60 min with a following incubation at 70 °C for 10 min. The resulting cDNA was stored at -20 °C or directly used for analysis.

RNasin Ribunuclease inhibitor	Promega (Fitchburg, USA)
M-MLV Reverse transcriptase	Promega (Fitchburg, USA)
Oligo(dT) primer	Promega (Fitchburg, USA)
5x first strand buffer	Promega (Fitchburg, USA)
dNTPs	Thermo Fisher Scientific (Waltham, USA)

2.6.3 RNA expression analysis

Expression levels of *NOXA* mRNA were analyzed by real-time polymerase chain reaction (RT-PCR). For the analysis of *NOXA* gene expression in a 384-well-plate, 1 µl of cDNA was mixed with 2.5 µl 2x Taqman Universal PCR Master Mix, 1.25 µl water, and 0.25 µl of NOXA TaqMan assay (Hs00560402_m1). *NOXA* gene expression was normalized against the

housekeeping gene *TBP* (Hs00427620_m1). Gene expression was subsequently analyzed using a 7900HT Fast Real-Time PCR System with following thermal cycling conditions:

50 °C for 2 min

95 °C for 10 min

40 cycles of: 95 °C for 15 seconds and 60 °C for 60 seconds

2x Taqman Universal PCR Master Mix	Thermo Fisher Scientific (Waltham, USA)
TaqMan assay (*NOXA*, *TBP*)	Thermo Fisher Scientific (Waltham, USA)
Optical Adhesive Covers	Thermo Fisher Scientific (Waltham, USA)
MicroAmp ® Optical 384-well reaction plate	Thermo Fisher Scientific (Waltham, USA)
7900HT Fast Real-Time PCR System	Thermo Fisher Scientific (Waltham, USA)

2.7 Global transcriptome analysis

RNA isolation for the analysis of the global transcriptome was carried according to 2.6.1 RNA isolation. Gene expression was investigated by microarray analysis using the GeneChip Human Transcriptome Array 2.0 (Thermo Fisher Scientific, Waltham, USA). Microarray analysis was performed by a "Certified Service Provider for Gene Expression Microarrays" (IMGM Laboratories GmbH, Martinsried, Germany). Raw data was normalized and differential gene expression was analyzed using the Transcriptome Analysis Console Software 4.0 (Thermo Fisher Scientific, Waltham, USA). Microarray data were deposited in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7866.

2.8 RNA interference

For gene silencing siGENOME siRNA Reagents - Human SMARTpool siRNA (Table 4) were used (Dharmacon, Lafayette, USA). As control siRNA (cosi) non-Targeting siRNA#1 (Dharmacon, Lafayette, USA) was used. MCL cell lines were transfected with siRNA by electroporation using the Nucleofector™ II/2B and the Cell Line Nucleofector® Kit V (Lonza Group Ltd., Basel, Switzerland). Cell pellets (8×10^6 cells) were resuspended in 120 µl transfection solution. After addition of 3.6 µl siRNA (50 µM stock solution), cells were transferred to an electroporation cuvette, transfected according to program X-001 and transferred into a culture flask containing pre-warmed culture medium.

Table 4: Sequences of SMARTpool siRNAs.

gene	SMARTpool siGenome sequences	
ATG5	GGAAUAUCCUGCAGAAGAA	CAUCUGAGCUACCCGAUA
	GACAAGAAGACAUUAGUGA	CAAUUGGUUUGCUAUUUGA
ATG7	CCAAAGUUCUUGAUCAAUA	GAUCAAGGUUUUCACUAA
	GAAGAUAACAAUUGGUGUA	CAACAUCCCUGGUUACAAG
CCND1	GUUCGUGGCCUCUAAGAUG	CCGAGAAGCUGUGCAUCUA
	GAACAGAAGUGCGAGGAGG	ACAACUUCCUGUCCUACUA
CDK4	GCAGCACUCUUAUCUACAU	GGAGGAGGCCUUCCCAUCA
	UCGAAAGCCUCUCUUCUCU	GUACCGAGCUCCCGAAGUU
PMAIP	AAACUGAACUUCCGGCAGA	AAUCUGAUAUCCAAACUCU
	CUGGAAGUCGAGUGUGCUA	GCAAGAACGCUCAACCGAG
Mcl-1	GCUACGUAGUUCGGGCAAA	GGACCAACUACAAAUUAAU
	AGAAGAAUUGAUGUGUAA	CGAAGGAAGUAUCGAAUUU

2.9 Detection of autophagosomes and autophagolysosomes

Detection of autophagosomes and autophagolysosomes was carried out using CYTO-ID® Autophagy detection kit (Enzo Biochem (Farmingdale, USA). The proprietary dye Cyto-ID specifically stains autophagosomes and autophagolysosomes instead of lysosomes (Marx, 2015). For Cyto-ID staining 1×10^6 cells were centrifuged (5 min, 1400 rpm, room temperature) and washed in RPMI 1640 (5 % (v/v) FBS, without phenol red). After centrifugation, cell pellets were resuspended in 200 µl RPMI 1640 (5 % (v/v) FBS, without phenol red) containing 0.2 µl Cyto-ID Green Detection Reagent and incubated for 30 min at 37 °C in the dark. Afterwards, cells were washed with Annexin V binding buffer and stained with Annexin V-APC (according to 2.3 Cell death analysis). Cyto-ID fluorescence intensity of Annexin V-APC negative cells was detected and compared by histogram overlays for the different treatments.

2.10 Statistics

Data are expressed as standard deviation (SD) of the mean. Statistics were calculated using GraphPadPrism 4.0 software (GraphPad Software, La Jolla, CA, USA). Changes in paired samples were analyzed using two-sided paired *t*-Test and results were considered statistically significant when $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) or not significant (ns) when $P > 0.05$.

3 Results

3.1 Cyclin D1/CDK4 activity regulates sestrin 3

Changes in cell cycle distribution do have a major impact on the expression pattern of the human transcriptome (Dominguez et al., 2016). Moreover, different studies show that manipulation of the cell cycle may prevent or induce apoptosis depending on the cellular context (Pucci et al., 2000). In line with this, the efficacy of the proteasome inhibitor bortezomib has already been demonstrated to be regulated by cell cycle changes in multiple myeloma (Huang et al., 2012). In addition, downstream of cyclin D1/CDK4, the activity of the E2F family members potentially transactivates thousands of genes (Rabinovich et al., 2008) and subsequently alters various cellular processes and signaling pathways. Transcriptional changes after manipulation of cyclin D1/CDK4 activity therefore are more diffuse further downstream of the signaling cascade. In order to identify novel direct cyclin D1/CDK4 functions that are not primarily mediated by changes in cell cycle, the first part of this study aimed to investigate the dynamic of cell cycle changes and cyclin D1/CDK4 activity after palbociclib treatment.

3.1.1 Cyclin D1/CDK4 activity regulates *sestrin 3* transcript and protein levels

As previous reports indicate, palbociclib mediated inhibition of cyclin D1/CDK4 activity and concomitant cell cycle arrest in cell lines is rapid (Cretella et al., 2018). Therefore, a short-term treatment of the MCL cell line Mino with the CDK4/6 inhibitor palbociclib was carried out and several samples for cell cycle and western blot analysis were collected. As mentioned earlier, CDK6 is hardly expressed in MCL cells (Marzec et al., 2006). Consequently, palbociclib efficacy in MCL is therefore primarily mediated by the inhibition of cyclin D1/CDK4 activity. Palbociclib treatment inhibited cyclin D1/CDK4 activity, as demonstrated by reduced levels of pRB1 (Figure 1, upper panel). Furthermore, the palbociclib effect on RB1 phosphorylation was already detectable after 2 h of treatment and lasted over the whole treatment period of 16 h. Long-term palbociclib treatment of MCL cell line Mino for 16 h resulted in an almost complete G1-arrest, whereas an 8 h treatment only resulted in minor changes in cell cycle distribution (Figure 1, lower panel). These results demonstrate that after palbociclib treatment, inhibition of cyclin D1/CDK4 activity is already efficient after 2 h of drug treatment, lasts for at least 6 subsequent hours and does not result in major cell cycle changes within this period. The time period of 8 h hours palbociclib treatment was sufficient to expect changes in further experiments.

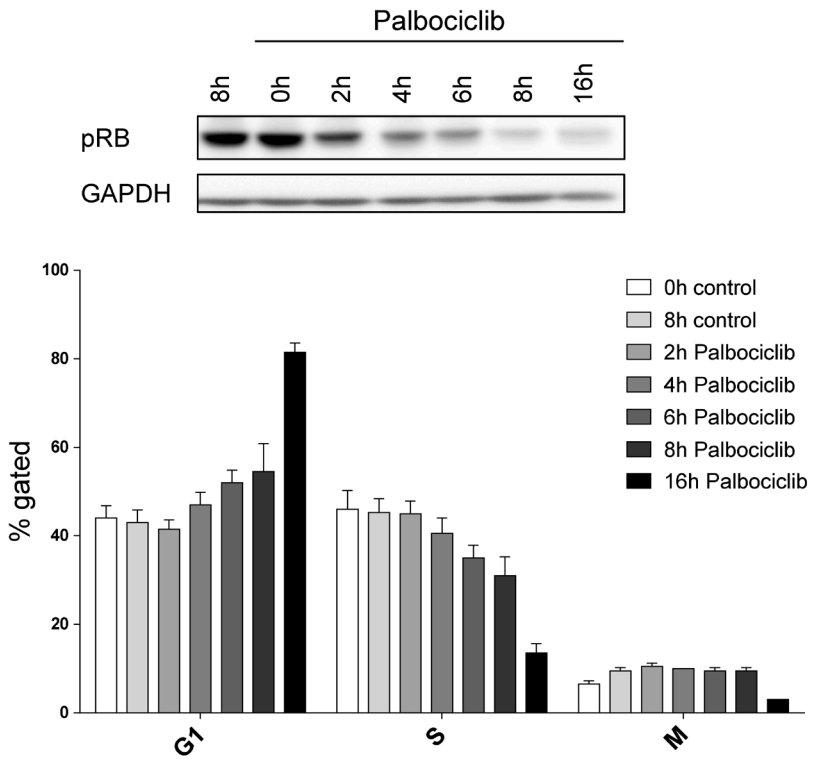
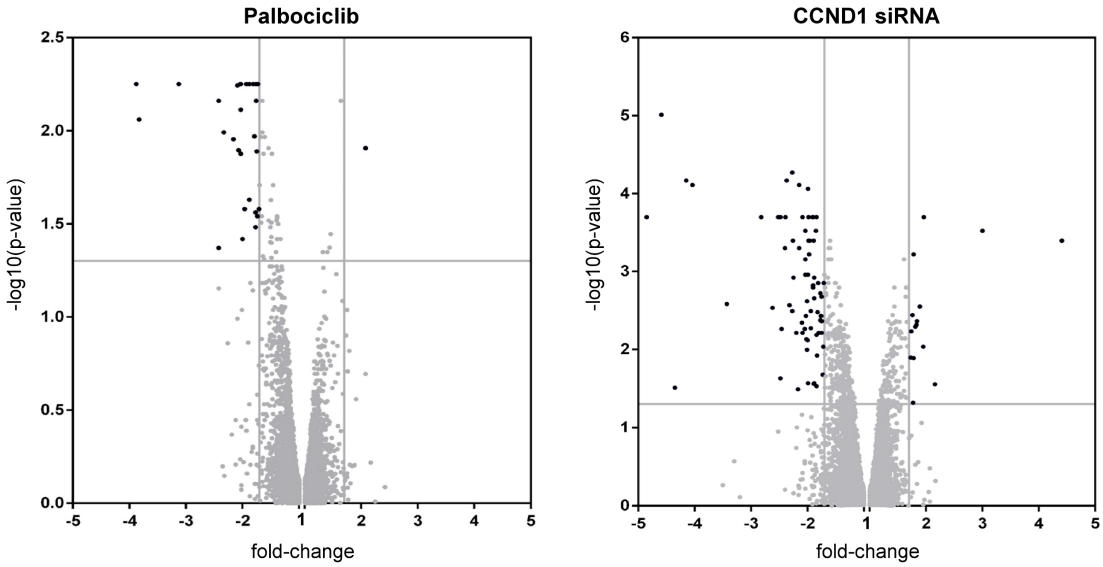


Figure 1: Short-term treatment with palbociclib inhibits cyclin D1/CDK4 activity, but hardly alters cell cycle distribution. MCL cell line Mino was treated with 100 nM palbociclib. After 0, 2, 4, 6, 8, and 16 h, samples were collected and analyzed by western blot using the indicated antibodies (upper panel) and BrdU staining (lower panel). Data represent means ± SD from two independent experiments.

After the time period for effective palbociclib treatment without major changes in cell cycle distribution was established (Figure 1), a global transcriptome analysis in the presence or absence of cyclin D1/CDK4 activity was performed. The MCL cell line Mino was treated with palbociclib according to the 8 h time period established earlier. In addition, to clarify if changes in the transcriptome after pharmacological inhibition of cyclin D1/CDK4 activity were also cyclin D1 dependent, MCL cells were treated with siRNA targeting *cyclin D1* (*CCND1*). Cyclin D1 knockdown as well as palbociclib treatment inhibited cyclin D1/CDK4 activity, as indicated by reduced pRB1 levels (Figure 2, lower right panel). After palbociclib treatment, 31 genes were differentially regulated, with more than ± 1.7-fold changes in mRNA levels and false discovery rate (FDR)-adjusted p-values < 0.05 (Figure 2, upper left and lower left panel). Downregulation of cyclin D1 expression by siRNA treatment targeting *cyclin D1* mRNA, however, led to a differential expression of 72 genes (Figure 2, upper right and lower left panel). All the 31 differentially regulated genes identified in the global transcriptome analysis after palbociclib treatment overlapped with the genes regulated after *CCND1* siRNA treatment, except for the gene *CHAF1A*. These 30 overlapping differentially regulated genes indicate that the modulation of the global transcriptome in response to palbociclib treatment, is indeed dependent on the inhibition of aberrant cyclin D1 dependent CDK4 activity.



Treatment	Number of regulated genes	Gene
Palbociclib	31	PCNA DONSON E2F8 EXO1 TYMS CDCA7 CDT1 CDC6 MCM6 ATAD2 POLD3 ORC1 MCM4 CLSPN DTL GMNN UNG FAM111B USP37 RRM2 GINS2 CDC7 MCM10 SESN3 CCNE1 WDR76 MSH6 CCNE2 ZNF367 PRIM1 CHAF1A
CCND1 siRNA	72	PCNA DONSON E2F8 EXO1 TYMS CDCA7 CDT1 CDC6 MCM6 ATAD2 POLD3 ORC1 MCM4 CLSPN DTL GMNN UNG FAM111B USP37 RRM2 GINS2 CDC7 MCM10 SESN3 CCNE1 WDR76 MSH6 CCNE2 ZNF367 PRIM1 SAMD9L CHAF1B E2F7 ASAP2 RFC5 MCM3 MXD4 MCM5 TIPIN TP53INP1 WDHD1 HILPDA POLA2 MCM2 ARL6IP6 PDZD8 NUDT19 DSCC1 UHRF1 TCP11L2 ZWINT TP63 UBE2T SLC20A1 ORC6 MIR25 FEN1 RAD51 MMS22L CDK2 ABAT HELLS CCND1 E2F1 RFC4 APLP2 FAM8A1 DDIT4 PSMC3IP E2F2 CDC25A XRCC2

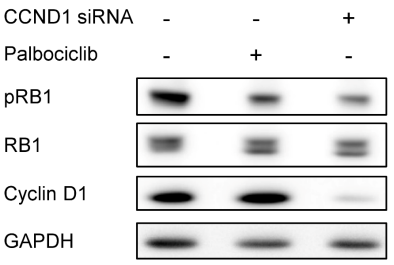


Figure 2: Inhibition of cyclin D1/CDK4 activity by palbociclib treatment or cyclin D1 knockdown causes changes in global mRNA expression. MCL cell line Mino was transfected with control siRNA and treated with 100 nM palbociclib or transfected with siRNA targeting *cyclin D1*. After 8 h, samples were collected and analyzed by global transcriptome analysis and western blot using the indicated antibodies (lower right panel). Genes with more than ± 1.7-fold changes in mRNA levels and FDR-adjusted p-values < 0.05 are marked by black dots. Data represent means from three independent experiments.

After 30 differentially regulated genes in response to the modulation of cyclin D1/CDK4 activity were identified in the global transcriptome analysis, these genes were further investigated. In order to identify candidate genes that are associated with cell death regulation or with the modulation of the cellular response to proteasome inhibitor treatment, functional gene annotation was carried out using a gene database. In line with the main function of cyclin D1 to regulate cell cycle progression, almost all of the differentially expressed genes were associated with cell cycle functions (Figure 3A), according to the Gene Ontology database (Ashburner et al., 2000; Carbon et al., 2009; The Gene Ontology Consortium, 2017) (AmiGo 2.0, www.geneontology.org, accessed January 2018). These genes were either directly associated with the regulation of cell cycle progression or were linked to cell cycle associated

processes such as DNA replication or DNA repair. The expression levels of almost all these cell cycle related genes were reduced after inhibition of cyclin D1/CDK4 activity, which is in line with the known function of cyclin D1/CDK4 to regulate cell cycle regulating proteins. *SESN3* was the only regulated gene that was not associated with cell cycle regulation (Figure 3A, marked *). Contrary to the downregulated cell cycle related genes, inhibition of cyclin D1/CDK4 activity increased the expression of *SESN3*. Interestingly, the *SESN3* gene and the corresponding protein sestrin 3 are implicated in cellular response to ROS and ROS dependent cell death (Dolado and Nebreda, 2008; Hagenbuchner et al., 2012). In addition, the identified function of cyclin D1/CDK4 to regulate of *SESN3* mRNA levels, could also be confirmed for the corresponding protein, as sestrin 3 protein levels increased in MCL cell lines, after cyclin D1/CDK4 inhibition with palbociclib (Figure 3B).

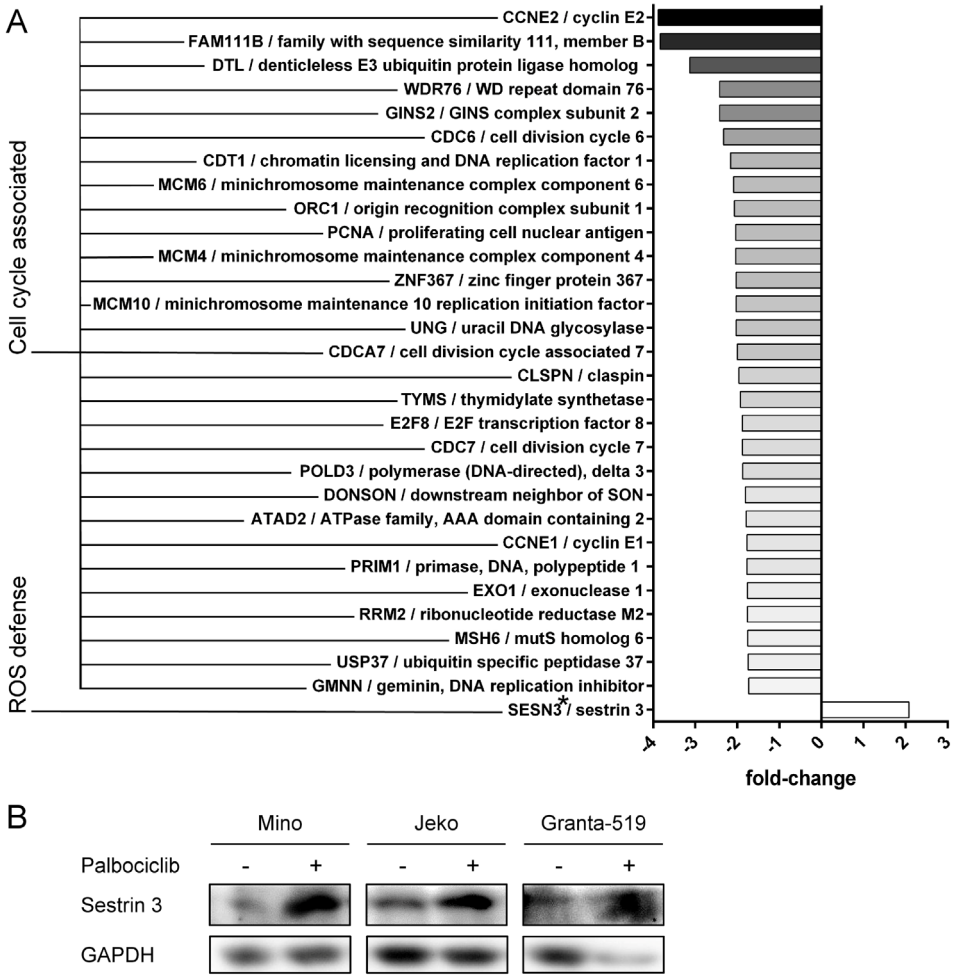


Figure 3: Regulated genes after inhibition of cyclin D1/CDK4 activity by palbociclib treatment or cyclin D1 knockdown are related to cell cycle functions or ROS defense. (A) Relative expression levels of the genes that are differentially regulated in the global transcriptome analysis after palbociclib and *CCND1* siRNA treatment and their biological function according to the Gene Ontology database AmiGo 2.0. (B) Inhibition of cyclin D1/CDK4 activity induces sestrin 3 protein expression in MCL. MCL cell lines were treated with 100 nM (Mino) or 300 nM (Jeko-1 and Granta-519) palbociclib for 16 h and analyzed by western blot using the indicated antibodies.

3.1.2 Bortezomib induced cell death can be modulated by ROS regulating substances

Sestrin 3 was identified to be highly regulated by cyclin D1/CDK4 activity in MCL (Figure 3). Moreover, expression levels of the sestrin 3 protein are associated with cellular stress response. Interestingly, bortezomib mediated cell death has been demonstrated to be antagonized by antioxidants (Halasi et al., 2013b). In addition, sestrin proteins have been shown to be upregulated in response to bortezomib treatment, potentially to cope with ER-stress and associated ROS induction (Brüning et al., 2013). Therefore, the question whether bortezomib induced cell death is ROS dependent and cell death can be regulated by ROS modulators was addressed next. Indeed, co-treatment of bortezomib with the ROS scavenger GSH rescued cell death induction in MCL cell lines Mino, Jeko-1, and Granta-519 (Figure 4A). Furthermore, co-treatment of bortezomib with a known ROS inducer PEITC (Jutooru et al., 2014) potentiated cell death induction (Figure 4B). In addition, inhibition of cyclin D1/CDK4 activity by palbociclib rescued cell death induction after treatment with the ROS inducer hydrogen peroxide (Figure 4C). These results indicate that MCL cells are susceptible to ROS dependent cell death, which can be antagonized by palbociclib treatment.

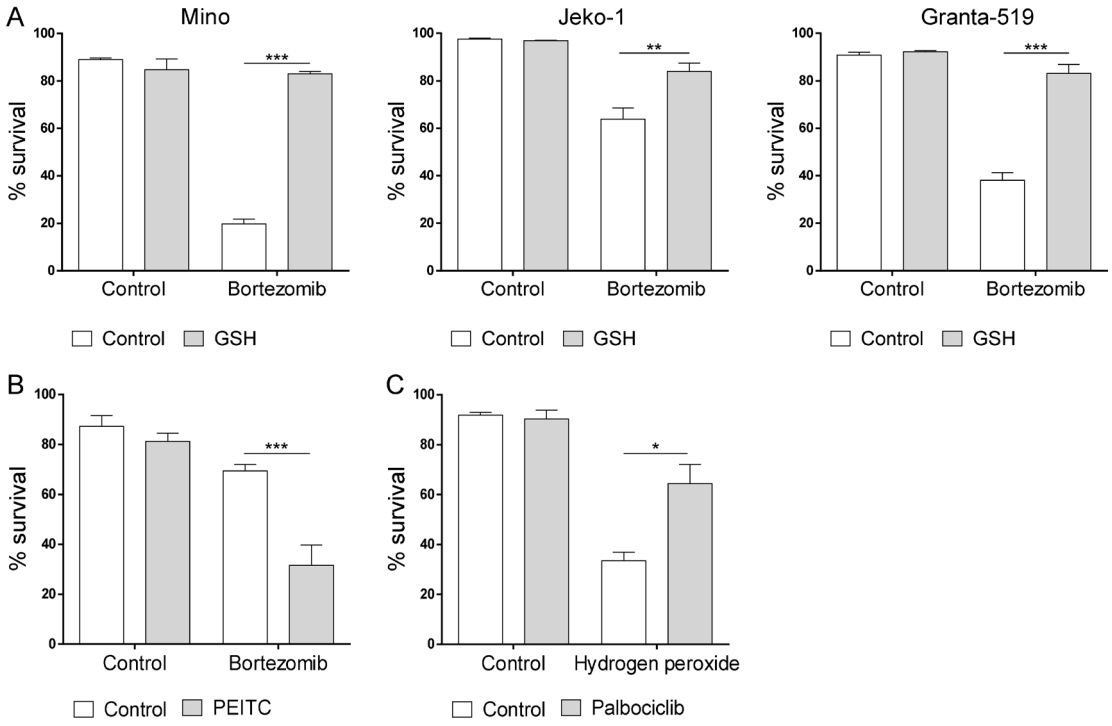


Figure 4: MCL cells are susceptible to ROS dependent cell death, which can be antagonized by inhibition of cyclin D1/CDK4 activity. (A) ROS scavenger GSH rescues bortezomib induced cell death. MCL cell lines Mino, Jeko-1, and Granta-519 were pre-treated with 2 mM GSH for 2 h and subsequently co-treated with 7 nM bortezomib. After 24 h, cell death was analyzed by Annexin V-PI staining. (B) ROS inducer PEITC potentiates bortezomib induced cell death. MCL cell line Mino was co-treated with 8 µM PEITC and 7 nM bortezomib. After 24 h, cell death was analyzed by Annexin V-PI staining. (C) Inhibition of cyclin D1/CDK4 activity by palbociclib

antagonizes hydrogen peroxide induced cell death. MCL cell line Mino was pre-treated with 100 nM palbociclib for 16 h and subsequently co-treated with 500 µM hydrogen peroxide. After 24 h, cell death was analyzed by Annexin V-PI staining. Data represent means ± SD from three independent experiments.

3.1.3 Sestrin 3 does not regulate bortezomib induced cell death

After establishing that MCL cells are susceptible to a ROS dependent cell death, the role of cyclin D1/CDK4 mediated downregulation of sestrin 3 protein levels in the regulation of this type of cell death was examined. First, MCL cell line Mino was treated with hydrogen peroxide or PEITC in combination with palbociclib to investigate if cells regulate sestrin 3 protein expression in the presence or absence of cyclin D1/CDK4 activity and ROS. In the presence of aberrant high cyclin D1/CDK4 activity MCL cell line Mino did not upregulate sestrin 3 in response to ROS stress induced by PEITC or hydrogen peroxide treatment (Figure 5A). After palbociclib treatment, however, upregulation of sestrin 3 was potentiated after hydrogen peroxide co-treatment but not after PEITC co-treatment when compared to palbociclib treatment alone. Aberrant cyclin D1/CDK4 activity therefore seems to inhibit the stress response to ROS. Next, the role of sestrin 3 in the regulation of cell death and susceptibility of MCL cells to proteasome inhibitors as well as ROS was further investigated. MCL cell line Mino was treated with bortezomib or hydrogen peroxide with or without siRNA targeting *sestrin 3* in the presence or absence of cyclin D1/CDK4 activity. Interestingly, cell death induction after bortezomib or hydrogen peroxide treatment was not altered in the presence or absence of *sestrin 3* siRNA (Figure 5B). Moreover, inhibition of cyclin D1/CDK4 activity by palbociclib efficiently antagonized cell death induction of bortezomib as well as hydrogen peroxide. This cell death antagonism, however, was the same in sestrin 3 knockdown and control cells (Figure 5B). These results demonstrate that changes in sestrin 3 protein expression levels mediated by changes in cyclin D1/CDK4 activity, do not regulate cell death induction after treatment with proteasome inhibitors or ROS. The cyclin D1/CDK4 mediated antagonism on cell death induction after proteasome inhibitor treatment therefore is not mediated by sestrin 3 levels. Consequently, the identified novel function of cyclin D1/CDK4 to regulate *SESN3* (Figure 3) cannot be exploited to increase the efficacy of proteasome inhibitor treatment in MCL.

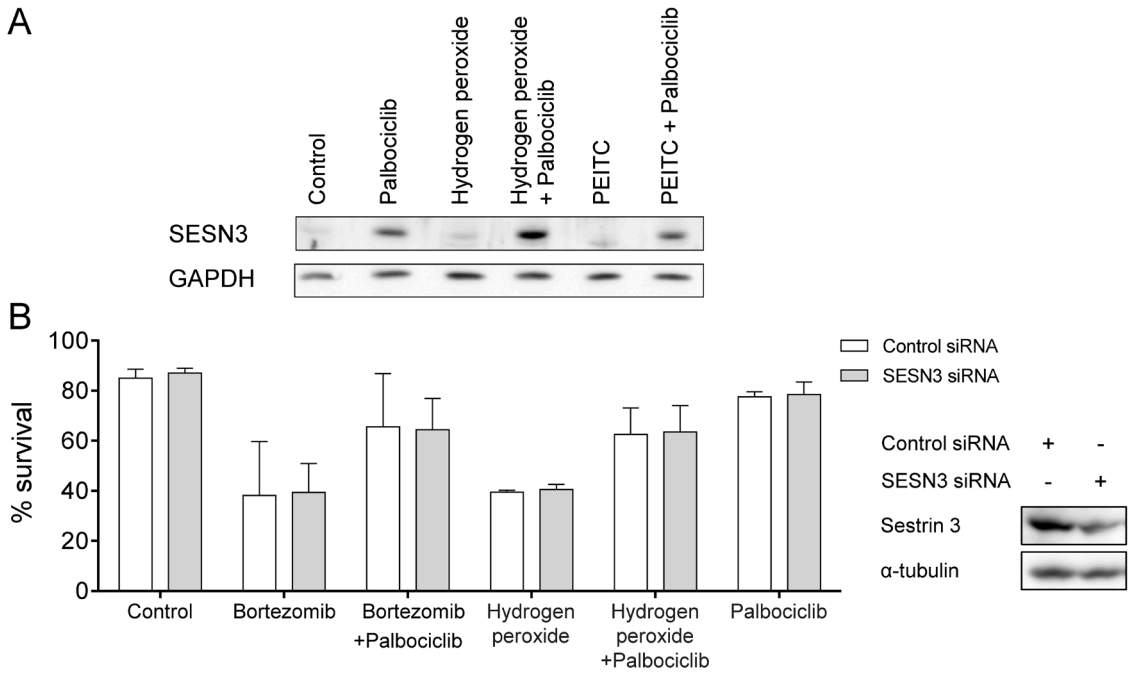


Figure 5: Cyclin D1/CDK4 inhibition antagonizes cell death by bortezomib and ROS inducers, which is not mediated by sestrin 3. (A) ROS inducing substances induce sestrin 3 protein expression in the absence of cyclin D1/CDK4 activity. MCL cell line Mino was pre-treated with 100 nM palbociclib for 16 h and subsequently co-treated with 500 µM hydrogen peroxide or 10 µM PEITC. After 24 h, protein expression was analyzed by western blot using the indicated antibodies. (B) Sestrin 3 does not regulate bortezomib or ROS induced cell death. MCL cell line Mino was transfected with control siRNA or siRNA targeting *sestrin 3*. 24 h after transfection, protein expression was analyzed by western blot using the indicated antibodies (right panel) and cells were treated with 7 nM bortezomib or 500 µM hydrogen peroxide. Cell death was assessed by Annexin V-PI staining 24 h post treatment (left panel). Data represent means ± SD from two independent experiments.

3.2 Cyclin D1/CDK4 activity is required for bortezomib efficacy in MCL

3.2.1 Cyclin D1/CDK4 inhibition antagonizes bortezomib induced cell death in MCL

Palbociclib and bortezomib are both approved drugs for the treatment of MCL patients (Goy et al., 2009; Leonard et al., 2012). Furthermore, multiple myeloma patients responded well to the combination of palbociclib with bortezomib and dexamethasone (Niesvizky et al., 2015). Despite the good single agent activity of palbociclib and bortezomib in MCL patients, combination therapy with palbociclib and bortezomib in patients with previously treated MCL did not result in a clinical outcome supporting this therapy regimen (Martin et al., 2019b). As both drugs target either MCL cell proliferation or survival, understanding potential antagonizing mechanisms between these two drugs would be of great clinical relevance. The previous results have shown that inhibition of cyclin D1/CDK4 activity antagonized Bortezomib induced cell death in one electroporated and siRNA treated MCL cell line (Figure 5B). To confirm this function of cyclin D1/CDK4 activity, three MCL cell lines were treated

with the proteasome inhibitor bortezomib in combination with palbociclib. Indeed, cyclin D1/CDK4 inhibition antagonized bortezomib induced cell death (Figure 6) and reduced levels of phosphorylated RB1 (Figure 6, lower right panel). The aberrant cyclin D1/CDK4 activity in MCL is therefore required for cell death induction after bortezomib treatment.

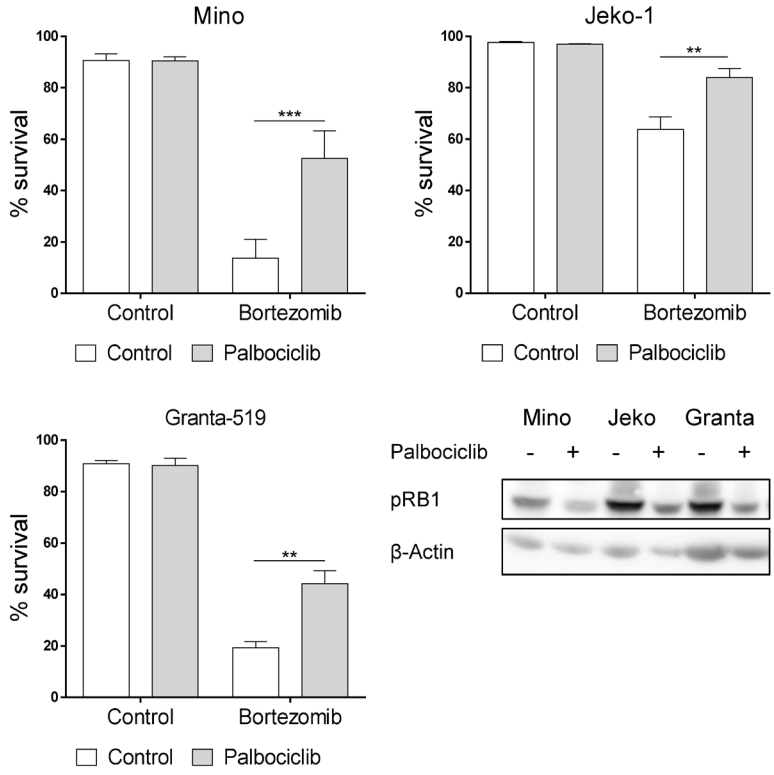


Figure 6: Cyclin D1/CDK4 inhibition antagonizes bortezomib induced cell death. MCL cell lines were treated with 100 nM (Mino) or 300 nM (Jeko-1 and Granta-519) palbociclib. After 16 h, palbociclib treatment, protein expression was analyzed by western blot using the indicated antibodies (lower right panel) and cells were subsequently co-treated with 8 nM bortezomib for 24 h. Cell death was assessed by Annexin V-PI staining. Data represent means ± SD from three independent experiments.

In order to clarify if the regulation of bortezomib induced cell death by palbociclib is a direct function of cyclin D1/CDK4 activity or if changes in cell cycle distribution are the primary cause for this antagonism, siRNA mediated knockdown of RB1 and cell cycle analysis was performed. Changes in cell cycle distribution can be associated with resistance to chemotherapy (Shah and Schwartz, 2001). In addition, loss of RB1 function has been demonstrated to be associated with higher proliferation and better response to chemotherapy (Ertel et al., 2010). Consequently, a cell cycle arrest after cyclin D1/CDK4 inhibition and a concomitant decrease in proliferation could therefore potentially impair cytotoxic effects. To address this, a knockdown of RB1 was performed in MCL cell line Mino, to allow cell cycle progression while cyclin D1/CDK4 activity was inhibited by palbociclib. Interestingly, even though palbociclib treated RB1 knockdown cells progressed to S-phase (Figure 7, upper panel), palbociclib

treatment still antagonized bortezomib induced cell death, in the same extent as in cells transfected with control siRNA (Figure 7, lower panel). Furthermore, bortezomib treatment led to a similar decrease of S-phase cells as palbociclib treatment did (Figure 7, upper panel). These results indicate that cell cycle changes are not responsible for the palbociclib mediated antagonism on bortezomib induced cell death in MCL.

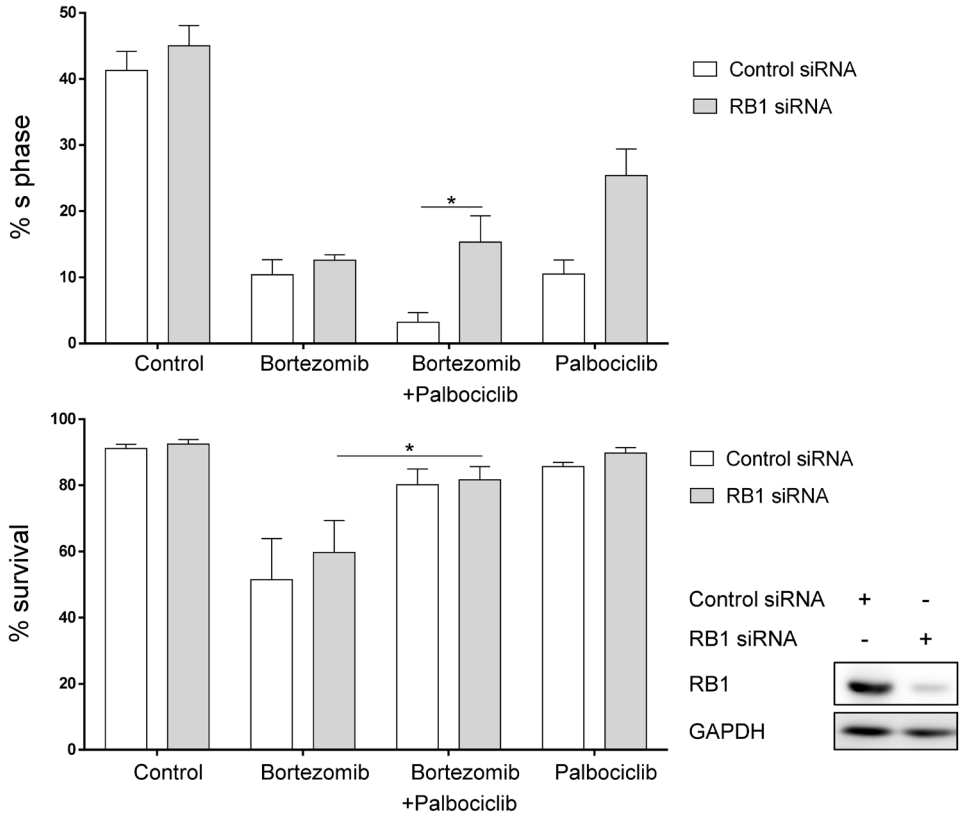


Figure 7: Changes in cell cycle distribution are not responsible for the palbociclib mediated antagonism on bortezomib induced cell death. MCL cell line Mino was transfected with siRNA targeting *RB1* and treated with 100 nM palbociclib 24 h post transfection. After 16 h, cells were treated with 8 nM bortezomib. 24 h after treatment, cell cycle distribution was measured by BrdU staining (upper panel), cell death was assessed by Annexin V-PI staining (lower panel), and protein expression was analyzed by western blot using the indicated antibodies. Data represent means ± SD from three independent experiments.

3.2.2 Cyclin D1/CDK4 activity is required for bortezomib induced NOXA accumulation in MCL

So far, this study has shown that Bortezomib induced cell death is regulated by cyclin D1/CDK4 activity which, however, is not mediated by cell cycle changes (Figure 7). Bortezomib induced cell death in MCL has been shown to be dependent on the Bcl-2 family protein NOXA (Pérez-Galán et al., 2006). Furthermore, it was shown that the susceptibility of MCL cells to bortezomib depends on extensive proteasomal degradation of NOXA protein and high *NOXA* transcript levels (Dengler et al., 2014). The results of the present study also showed that

hydrogen peroxide induced cell death is antagonized by cyclin D1/CDK4 inhibition (Figure 5B). Interestingly, it was reported that hydrogen peroxide induced cell death is associated with increased NOXA protein levels (Aikawa et al., 2010). Therefore, to investigate if the palbociclib mediated antagonism on bortezomib induced cell death (Figure 6) is associated with changes in NOXA protein levels, MCL cells were treated with bortezomib and palbociclib to subsequently analyze NOXA protein expression. Indeed, palbociclib co-treatment with bortezomib reversed the accumulation of NOXA protein in the MCL cell lines Mino, Jeko-1, and Granta-519 (Figure 8). Aberrant cyclin D1/CDK4 activity is therefore required for effective bortezomib treatment and NOXA accumulation in MCL.

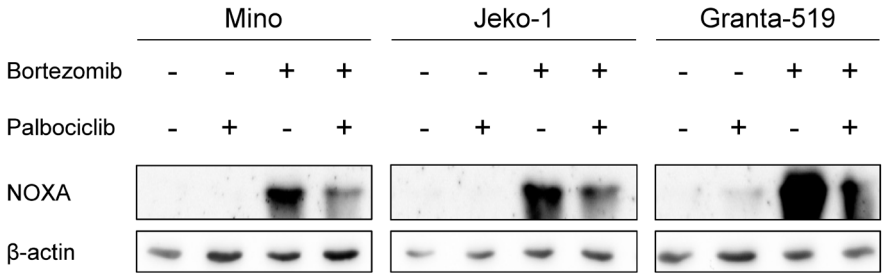


Figure 8: Cyclin D1/CDK4 inhibition antagonizes bortezomib induced NOXA accumulation in MCL cell lines. MCL cell lines were treated with 100 nM (Mino) or 300 nM (Jeko-1 and Granta-519) palbociclib for 16 h and subsequently co-treated with 8 nM bortezomib. 8 h post treatment proteins were analyzed by western blot using the indicated antibodies.

The previous results demonstrated that cyclin D1/CDK4 activity does not only regulate bortezomib induced cell death in MCL cell lines (Figure 6) but also antagonizes bortezomib induced NOXA accumulation (Figure 8). This cyclin D1/CDK4 function is of high clinical interest because NOXA functions as a critical cell death mediator in many cancer therapies (Qin et al., 2005; Gomez-Bougie et al., 2007; Ehrhardt et al., 2012; Gutekunst et al., 2013) and bortezomib therapy in MCL requires improvement regarding response rates and resistances.
As cell lines always to some degree differ genetically and phenotypically from their origin tissue (Alge et al., 2006; Pan et al., 2009), it is important to confirm this cyclin D1/CDK4 function, to regulate NOXA protein, in primary patient cells. Therefore, primary MCL cells were co-treated with bortezomib and palbociclib. To prevent excessive cell death by the extended culture duration during the co-treatment schedule, primary MCL cells were co-cultured with CD40 ligand expressing fibroblasts and treated with different cytokines. Therefore, bortezomib treatment with or without palbociclib did not result in substantial cell death (Figure 9, upper panels). The regulation of NOXA protein after inhibition of cyclin D1/CDK4 activity, however, was the same in primary MCL cells (Figure 9, lower panels), as in the MCL cell lines (Figure 8).

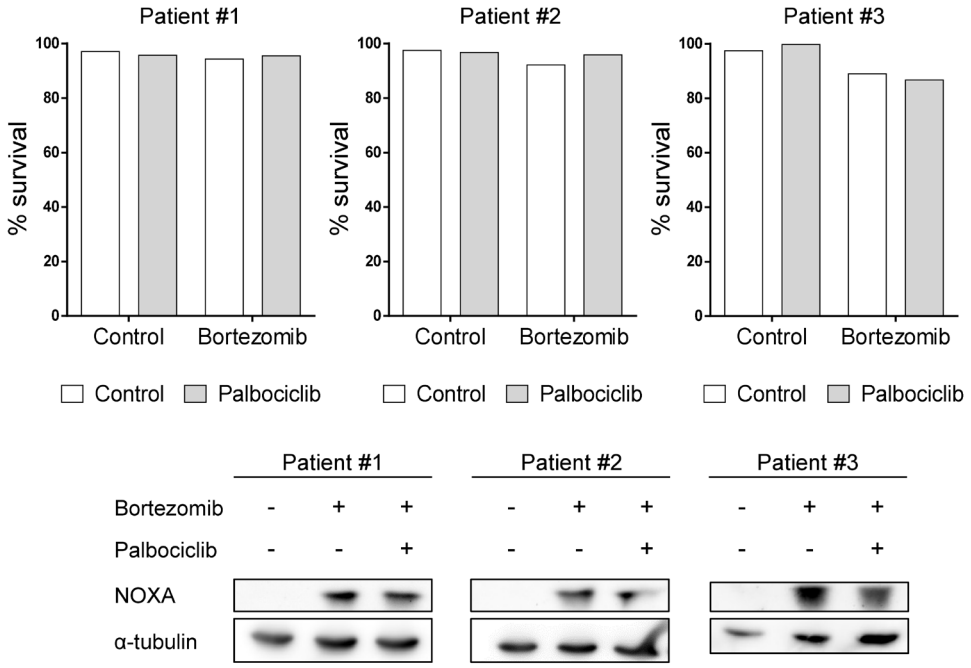


Figure 9: Cyclin D1/CDK4 inhibition antagonizes bortezomib induced NOXA accumulation in primary MCL cells. Bortezomib does not induce cell death in co-cultured primary MCL cells but induces NOXA protein, which can be antagonized by palbociclib co-treatment. Primary MCL cells were exposed to 1 ng/ml of Insulin-like growth factor-1, 50 ng/ml Interleukin-10, 50 ng/ml B cell activating factor, 1 ng/ml Interleukin-6 and co-cultured with the CD40 ligand expressing cell line NIH3T3/CD40. One day before co-culture, the NIH3T3/CD40 cell line was irradiated with 30 gray. After 8 h of co-culture, cells were treated with 300 nM palbociclib for 16 h and subsequently co-treated with 10 nM bortezomib. After 8 h, protein expression was analyzed by western blot using the indicated antibodies (lower panels) and after 24 h, cell death of CD5-CD20 positive cells was assessed by Annexin V-PI staining (upper panels).

3.2.3 Downregulation of cyclin D1 or CDK4 antagonizes bortezomib induced cell death as well as NOXA accumulation

Palbociclib has been demonstrated to specifically target CDK4/CDK6 with only little activity against other kinases such as CDK1, CDK2 or CDK5 (Fry et al., 2004; Toogood et al., 2005; Asghar et al., 2015). However, since there are over 500 protein kinases encoded by the human genome, off-target binding can always happen to some extent (Klaeger et al., 2017). Therefore, in order to analyze if the palbociclib mediated antagonism on bortezomib efficacy is dependent on specific inhibition of the cyclin D1-driven CDK4 activity, siRNA mediated knockdown of either cyclin D1 or CDK4 was performed. A comparable reduction in bortezomib induced cell death was observed after knockdown of cyclin D1 or CDK4 in MCL cell lines Mino and Jeko-1 (Figure 10A, upper and middle panels). In addition, bortezomib induced NOXA accumulation was also antagonized by knockdown of cyclin D1 or CDK4 (Figure 10A, lower panel). Double knockdown of cyclin D1 and CDK4, however, did not potentiate the antagonism on bortezomib induced cell death compared to CDK4 single knockdown (Figure 10B). These results indicate

that the palbociclib mediated antagonism on bortezomib induced cell death in MCL is indeed a function of cyclin D1/CDK4 activity.

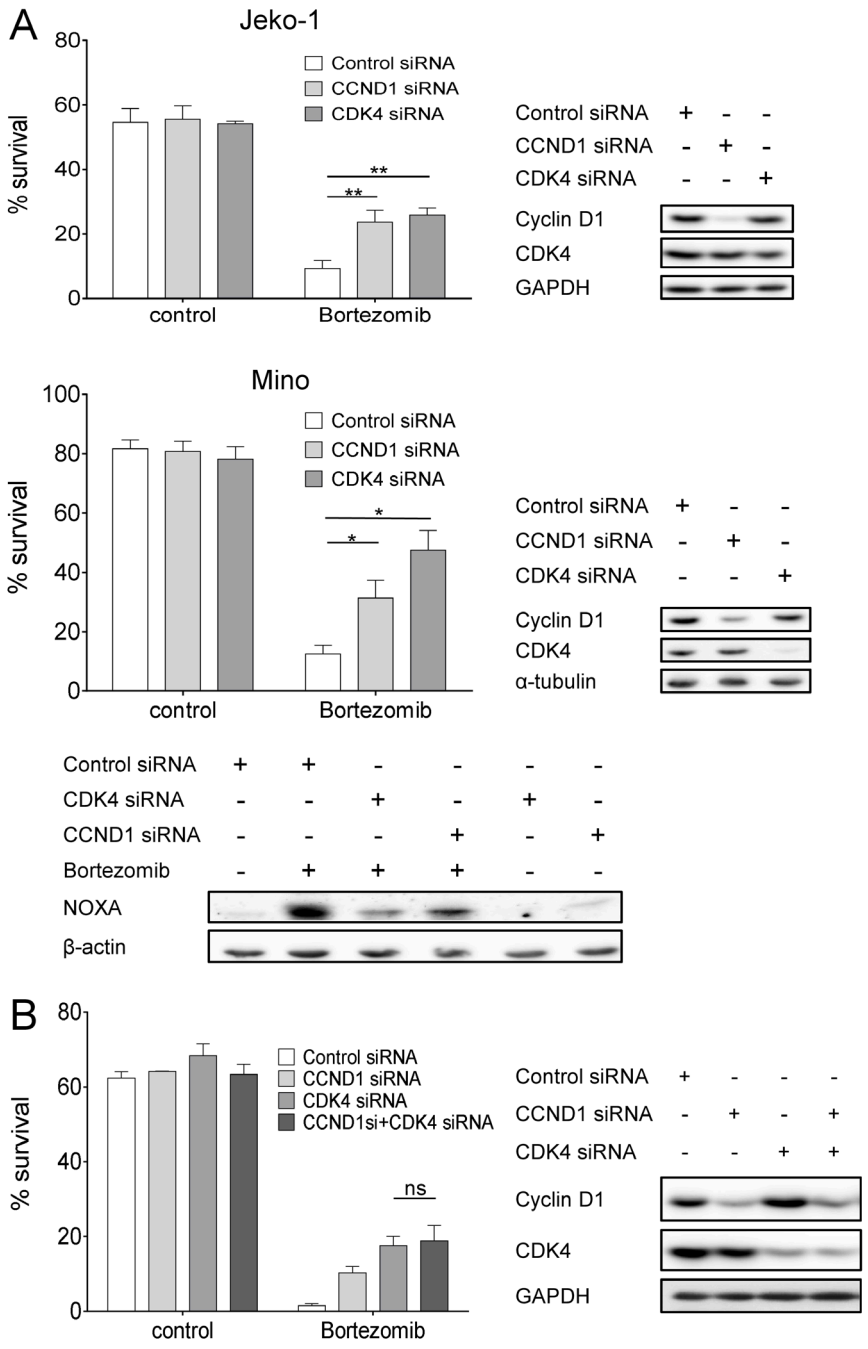


Figure 10: Knockdown of cyclin D1 or CDK4 antagonizes bortezomib induced cell death and NOXA accumulation. (A) MCL cell lines Mino and Jeko-1 were transfected with siRNA targeting *CCND1* or *CDK4*. 24 h after transfection, protein expression was analyzed by western blot using the indicated antibodies (right panel) and cells were treated with 8 nM bortezomib. After 8 h bortezomib treatment, protein expression was analyzed (Mino, lower panel). Cell death was assessed by Annexin V-PI staining (Mino and Jeko-1, upper left and left middle panels) 24 h post treatment. (B) MCL cell line Mino was transfected with siRNA targeting *CCND1*, *CDK4,* or both. 24 h after transfection, protein expression was analyzed by western blot using the indicated antibodies (right panel) and cells were treated with 8 nM bortezomib. After 24 h, cell death was assessed by Annexin V-PI staining (left panel). Data represent means ± SD from three independent experiments.

3.2.4 Cyclin D1/CDK4 inhibition antagonizes substances that depend on NOXA accumulation for cell death induction

The previously demonstrated antagonism on bortezomib induced cell death and NOXA protein expression after cyclin D1/CDK4 inhibition, could be a drug specific effect that only applies to bortezomib and not represent a general function of cyclin D1/CDK4 activity. Therefore, it was investigated if other drugs that are effective in inducing NOXA dependent cell death in MCL are regulated by the aberrant cyclin D1/CDK4 activity. In line with the previous results obtained with bortezomib (Figure 6 and Figure 8), palbociclib exerted the same antagonism on cell death and NOXA accumulation in the MCL cell lines Jeko-1 and Mino after treatment with the clinically relevant proteasome inhibitor carfilzomib (Figure 11). Importantly, this could also be demonstrated for the FASN inhibitor orlistat (Figure 11), which has been identified to be effective in MCL cells via upregulation of NOXA protein (Dengler et al., 2014).

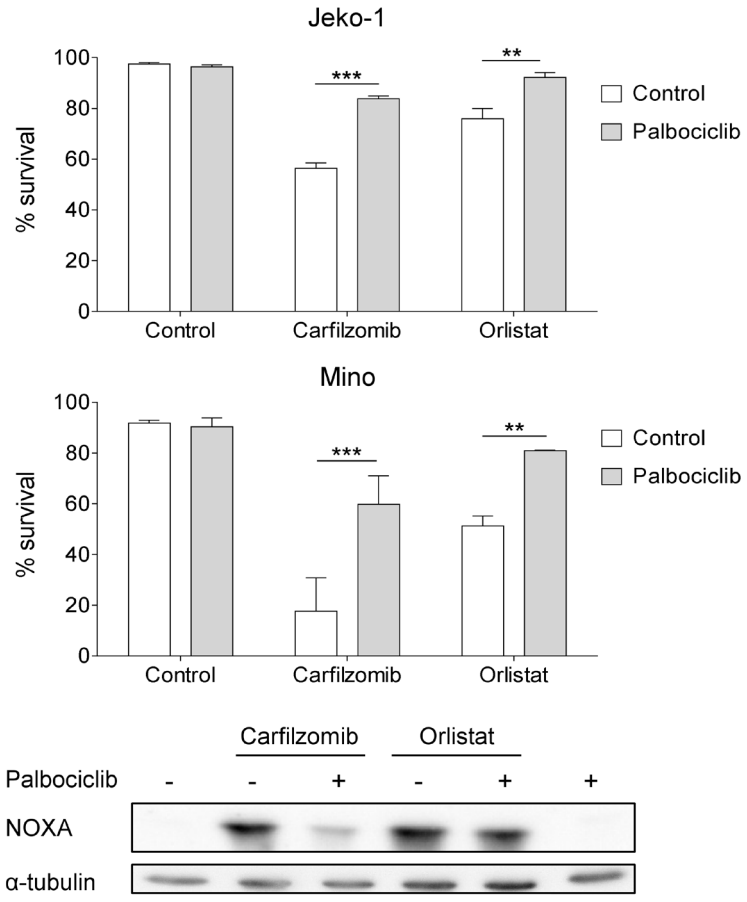


Figure 11: CDK4 inhibition antagonizes cell death of NOXA inducing substances and NOXA accumulation. MCL cell lines were treated with 100 nM (Mino) or 300 nM (Jeko-1) palbociclib for 16 h and subsequently co-treated with either 7 nM carfilzomib or 20 µM orlistat. After 8 h, protein expression was analyzed using the indicated antibodies (Mino, lower panel) and after 24 h, cell death was assessed by Annexin V-PI staining (Mino and Jeko-1, upper and middle panels). Data represent means ± SD from three independent experiments.

3.2.5 Palbociclib mediated antagonism on bortezomib induced cell death is dependent on the NOXA/Mcl-1 axis

Bortezomib induced cell death in MCL has been shown to be dependent on NOXA protein (Pérez-Galán et al., 2006). In addition, the downregulation of its main anti-apoptotic binding partner Mcl-1 has been demonstrated to potentiate NOXA dependent cell death (Höring et al., 2017). In order to confirm the previous results, that the inability of bortezomib to induce cell death after cyclin D1/CDK4 inhibition is dependent on NOXA protein levels, knockdowns of NOXA and Mcl-1 were performed in MCL cell line Mino. Knockdown of NOXA protein rescued bortezomib induced cell death (Figure 12), which confirms the NOXA dependency of bortezomib induced cell death. More importantly, knockdown of Mcl-1 reversed the palbociclib mediated antagonism on bortezomib induced cell death, whereas NOXA knockdown further increased the antagonism on cell death induction (Figure 12). These results corroborate the previous observations that palbociclib mediated antagonism on bortezomib induced cell death is NOXA dependent.

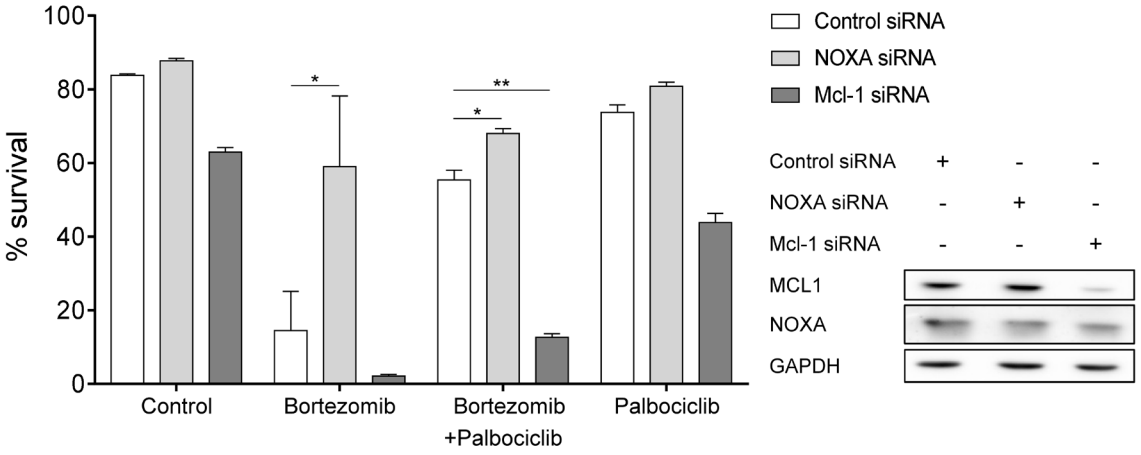


Figure 12: Knockdown of NOXA or Mcl-1 regulates palbociclib mediated antagonism on bortezomib induced cell death. MCL cell line Mino was transfected with siRNA targeting *PMAIP1* or *Mcl-1* and treated with 100 nM palbociclib. 24 h after transfection, protein expression was analyzed by western blot using the indicated antibodies (right panel) and cells were treated with 8 nM bortezomib. Cell death was assessed by Annexin V-PI staining 24 h post treatment (left panel). Data represent means ± SD from three independent experiments.

3.2.6 Palbociclib mediated antagonism on bortezomib induced cell death is mediated by regulation of NOXA protein stability

Studies have shown that inhibition of the proteasome by bortezomib treatment leads to the induction of *NOXA* transcript levels (Thaler et al., 2015). In MCL, however, the predominant mechanism of bortezomib efficacy was demonstrated to be increasing the short NOXA protein half-life, by targeting the rapid NOXA protein turnover (Dengler et al., 2014). To uncover the

underlying mechanisms of the cyclin D1/CDK4 mediated antagonism on bortezomib induced cell death and NOXA protein accumulation, *NOXA* transcript levels were quantified first. In line with previous studies, a slight increase in *NOXA* transcript levels after bortezomib treatment in MCL cell lines Mino and Granta-519 was observed (Figure 13). Co-treatment of bortezomib with palbociclib, however, only resulted in minor changes of the *NOXA* transcript levels, compared to transcript levels after bortezomib treatment alone (Figure 13). Therefore, transcriptional regulation of *NOXA* upon cyclin D1/CDK4 inhibition does not seem to be the predominant mechanism of the palbociclib mediated antagonism on bortezomib in MCL.

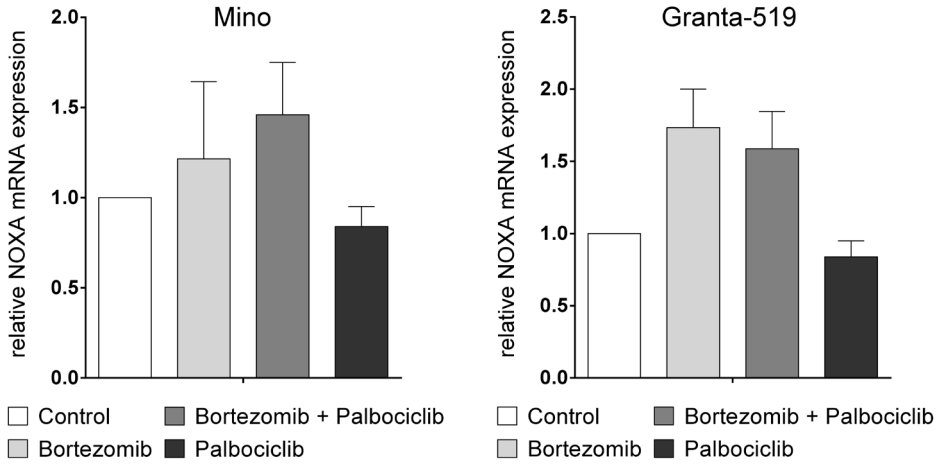


Figure 13: Inhibition of cyclin D1/CDK4 activity hardly alters *NOXA* mRNA levels after proteasome inhibition. MCL cell lines were treated with 100 nM (Mino) or 300 nM (Granta-519) palbociclib for 16 h and subsequently co-treated with 8 nM bortezomib. After 8 h co-treatment, samples were collected and analyzed by real-time PCR. *NOXA* mRNA expression was normalized to *TBP*. Data represent means ± SD from 3 experiments.

After establishing that the antagonizing effect on bortezomib efficacy by palbociclib is neither mediated by changes in cell cycle distribution (Figure 7) nor a reduction in *NOXA* transcript levels (Figure 13), NOXA protein stability was investigated using CHX pulse-chase experiments in MCL cell line Mino. As previously published, bortezomib prolonged NOXA protein half-life (Dengler et al., 2014). Interestingly, after inhibition of cyclin D1/CDK4 activity, bortezomib did no longer prolong NOXA protein half-life, whereas palbociclib treatment alone had no obvious effect on the short half-life of NOXA (Figure 14). Palbociclib co-treatment with bortezomib resulted in a protein stability comparable to the control treatment. These results indicate a possible function of cyclin D1/CDK4, which regulates a proteasome-independent degradation pathway, leading to the degradation of NOXA protein under UPS blocked conditions.

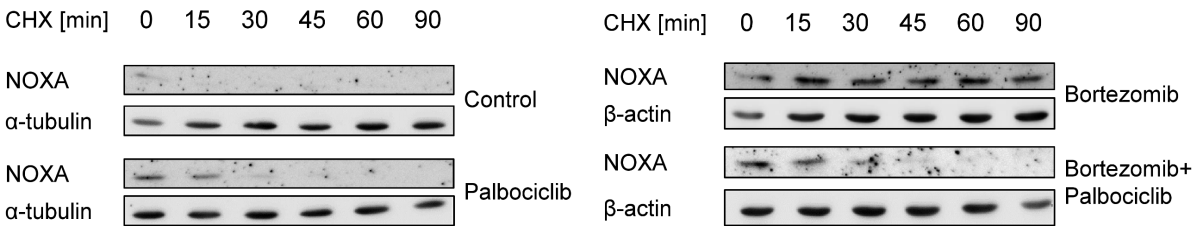


Figure 14: Increased half-life of NOXA protein after bortezomib treatment is diminished after palbociclib co-treatment. MCL cell line Mino was treated with 100 nM palbociclib for 16 h and subsequently co-treated with 8 nM bortezomib. After 8 h co-treatment, 20 µg/ml CHX was added to the cells and samples were collected 0, 15, 30, 45, 60, and 90 min after CHX exposition for western blot analysis using the indicated antibodies.

3.3 Regulation of bortezomib efficacy by cyclin D1/CDK4 depends on autophagic activity

After demonstrating that aberrant cyclin D1/CDK4 activity in MCL is required for efficient cell death induction after bortezomib treatment, a novel function of cyclin D1/CDK4 activity was identified, which is associated with the regulation of NOXA protein half-life (Figure 14). Because inhibition of cyclin D1/CDK4 activity promoted NOXA degradation even under UPS blocked conditions, the underlying mechanism of NOXA protein degradation was further investigated.

3.3.1 Cyclin D1/CDK4 inhibition increases autophagic activity

In addition to proteasomal degradation mediated by the UPS, autophagic degradation represents the second central degradation machinery. To clarify if the cyclin D1/CDK4 function to regulate bortezomib efficacy and NOXA dependent cell death was mediated by autophagy, the potential regulation of autophagic activity by cyclin D1/CDK4 activity was investigated first. During autophagy, the ATG8 family protein LC3-I is conjugated to phosphatidylethanolamine (PE) and gets subsequently cleaved during autophagosome maturation to form the autophagy marker protein LC3-II (Birgisdottir et al., 2013). Interestingly, the autophagy marker LC3-II was increased upon inhibition of cyclin D1/CDK4 with palbociclib and after co-treatment with bortezomib and palbociclib in MCL cell line Mino (Figure 15A). Bortezomib treatment also led to a minor increase in LC3-II expression. In addition to the LC3-II marker protein, autophagic activity was investigated by direct staining of autophagic vacuoles with the autophagy Cyto-ID Green dye for subsequent cytometric analysis. Again, palbociclib treatment increased autophagic activity, as indicated by an increase in green fluorescence in MCL cell lines Mino and Jeko-1 (Figure 15B and C) or after co-treatment with bortezomib in MCL cell line Mino (Figure 15B). Bortezomib single treatment led to minor increase in autophagic

vacuoles. The autophagy inhibitor hydroxychloroquine, which inhibits autophagosomal degradation further downstream of the ALP, was used as a positive control and consequently led to the strongest increase in autophagic vacuoles. These results corroborate the results that were obtained by analyzing LC3-II protein expression (Figure 15A). To investigate, if autophagy activation after cyclin D1/CDK4 inhibition is a direct effect of palbociclib treatment or requires the activation of intermediate pathways, MCL cells were treated with palbociclib for a short period of time before measuring autophagic vacuoles. In contrast to the long-term palbociclib treatment (Figure 15C), short-term treatment of MCL cell line Mino with palbociclib, however, did not increase the amount of autophagic vacuoles (Figure 15D).

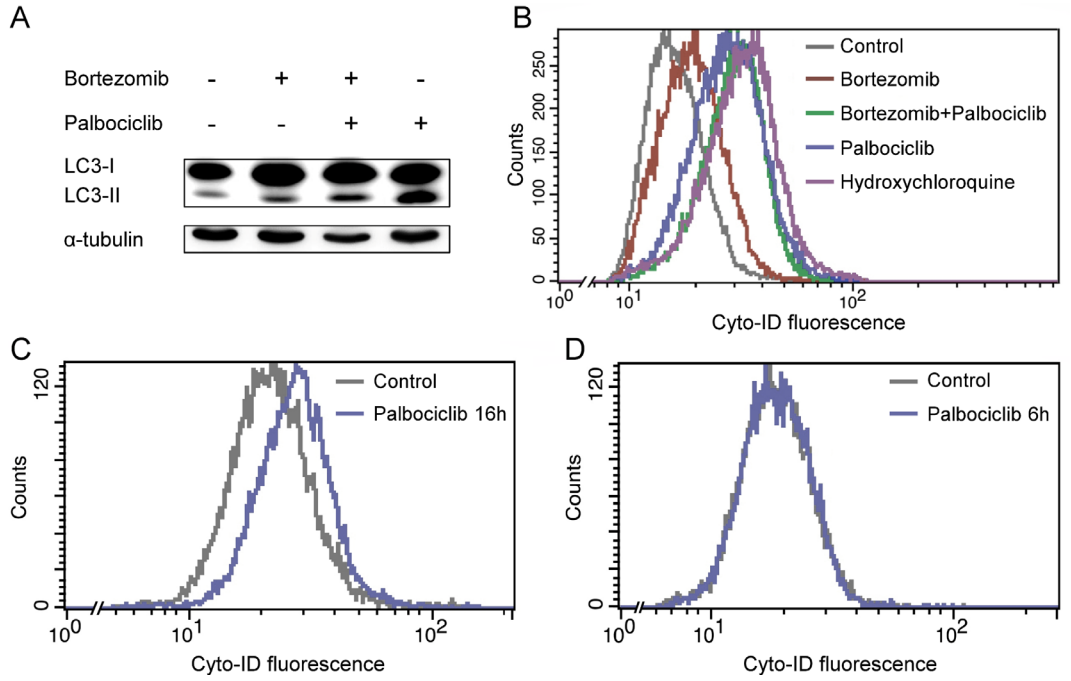


Figure 15: Palbociclib treatment induces autophagy but not after short-term treatment.MCL cell line Mino was treated with 100 nM palbociclib for 16 h and subsequently co-treated with 8 nM bortezomib. After 8 h, protein expression was analyzed by western blot using the indicated antibodies. (B) MCL cell line Mino was treated with 100 nM palbociclib for 16 h and subsequently co-treated with 8 nM bortezomib for 24 h or treated with 40 µM hydroxychloroquine for 24 h. After treatment, autophagic vacuoles were measured by Cyto-ID staining. (C) MCL cell line Jeko-1 was treated with 300 nM palbociclib for 16 h. After treatment, autophagic vacuoles were measured by Cyto-ID staining. (D) MCL cell line Mino was treated with 100 nM palbociclib for 6 h. After treatment, autophagic vacuoles were measured by Cyto-ID staining.

After identifying autophagy regulation as a function of aberrant cyclin D1/CDK4 activity, the increase in autophagic activity, which was associated with cyclin D1/CDK4 inhibition, was further investigated. The aim was to distinguish between an increase in overall autophagic activity, which includes autophagosome formation, maturation, fusion with the lysosome, breakdown of cargo, and subsequent release of macromolecules, termed “autophagic flux” and

blockage of downstream steps in autophagy such as impaired fusion or decreased lysosomal degradation (Zhang et al., 2013). According to the Guidelines for the use and interpretation of assays for monitoring autophagy (Klionsky et al., 2021), it is necessary to have an appropriate autophagy marker, an autophagy regulating substance or process, and a substance that blocks autophagic degradation at a late stage to distinguish between autophagic flux and autophagic blockage. Accordingly, any increase in expression of an autophagy marker, after addition of an autophagy inducing substance to a substance that blocks autophagic degradation, indicates an increase in autophagic flux. In line with this, MCL cell lines Mino and Jeko-1 were co-treated with palbociclib and a substance that leads to an autophagic blockage, namely hydroxychloroquine (Amaravadi et al., 2011). Bortezomib treatment did not increase the expression of the autophagy marker LC3-II under ALP blocked conditions in MCL cell line Mino (Figure 16A). Inhibition of cyclin D1/CDK4 by palbociclib treatment, however, potentiated the accumulation of LC3-II when combined with hydroxychloroquine. The function of cyclin D1/CDK4 to regulate autophagic flux, was also confirmed via Cyto-ID staining in MCL cell line Jeko-1 (Figure 16B). Again, palbociclib treatment potentiated the accumulation of autophagic vacuoles under ALP blocked conditions. In summary, inhibition of cyclin D1/CDK4 activity therefore does not lead to a blockage of autophagic degradation but results in an increased autophagic flux.

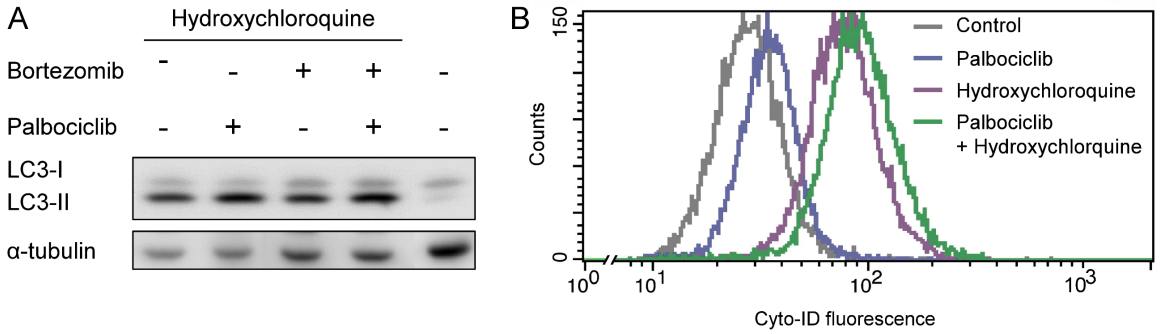
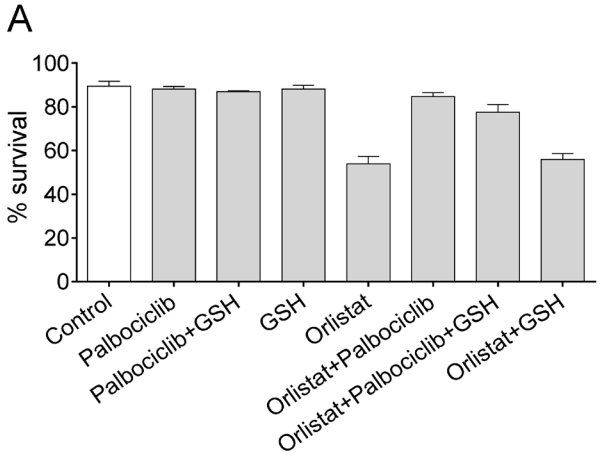


Figure 16: Palbociclib treatment induces autophagic flux. MCL cell line Mino was treated with 40 µM hydroxychloroquine and 100 nM palbociclib for 16 h and subsequently co-treated with 8 nM bortezomib. After 8 h, protein expression was analyzed by western blot using the indicated antibodies. (B) MCL cell line Jeko-1 was treated with 300 nM palbociclib for 24 h with or without 40 µM hydroxychloroquine. After treatment, autophagic vacuoles were measured via Cyto-ID staining.

3.3.2 Palbociclib mediated antagonism on bortezomib is not mediated by ROS or AMPK signaling

Previous results show that autophagic flux is induced in response to cyclin D1/CDK4 inhibition. Which intermediate pathways connect cyclin D1/CDK4 activity and autophagic flux in MCL remains to be investigated. Other groups demonstrated that cyclin D1/CDK4 inhibition mediated autophagy induction is associated with AMPK activation and/or ROS induction (Hsieh et al., 2017; Valenzuela et al., 2017). Inhibition of ROS or AMPK signaling could therefore interfere with the palbociclib mediated antagonism on bortezomib and NOXA dependent cell death. Bortezomib induced cell death was shown to be antagonized by ROS scavengers (Figure 4). Therefore, bortezomib cannot be used to investigate if inhibition of ROS can reverse the palbociclib mediated antagonism on cell death. Instead, MCL cell line Mino was treated with the FASN inhibitor and NOXA inducer, orlistat and co-treated with palbociclib and the ROS scavenger GSH. Co-treatment with GSH, however, did not reverse the palbociclib mediated antagonism on NOXA dependent cell death after orlistat treatment (Figure 17A). Similar results were obtained, when MCL cell line Mino was co-treated with the NOXA inducer bortezomib, the AMPK inhibitor dorsomorphin, and palbociclib. Co-treatment with dorsomorphin did not reverse the palbociclib mediated antagonism on bortezomib induced cell death (Figure 17B, upper panel). Moreover, no changes in p-AMPKα expression levels, after palbociclib treatment in MCL cell lines Mino and Jeko-1 were observed (Figure 17B, lower panel). These results do not confirm a possible link between ROS or AMPK signaling and the induction of autophagic flux in response to cyclin D1/CDK4 inhibition.



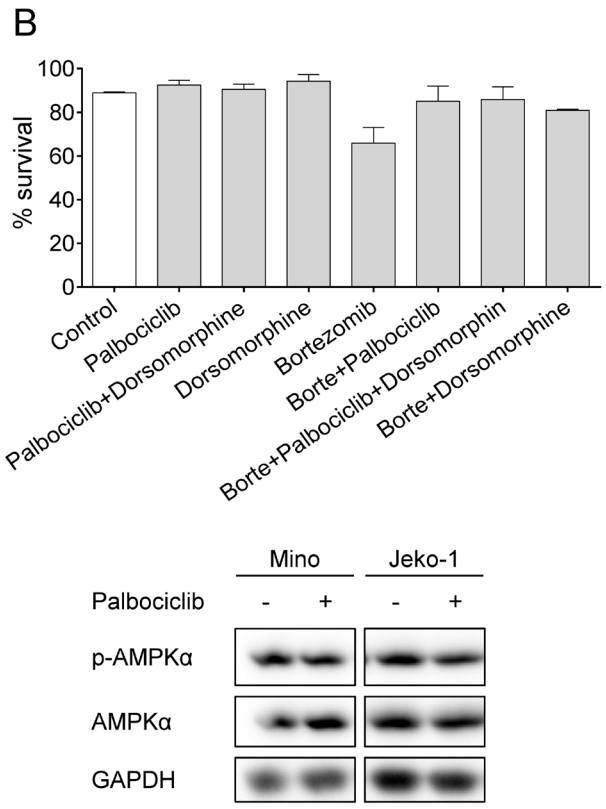
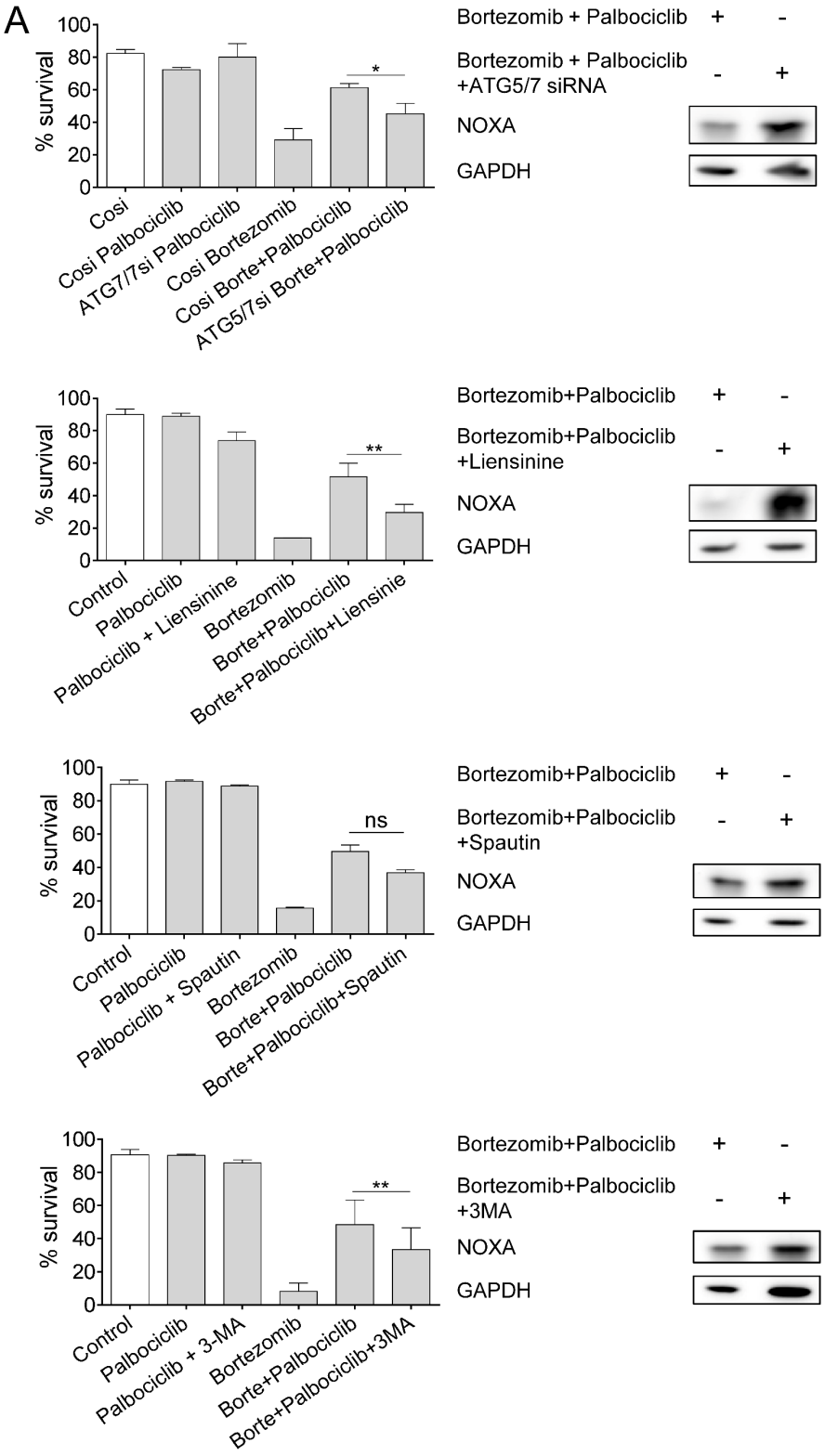


Figure 17: Palbociclib mediated antagonism on bortezomib induced cell death is neither mediated by ROS induction nor AMPK signaling. (A) MCL cell line Mino was treated with 100 nM palbociclib and 2 mM GSH for 16 h and subsequently co-treated with 20 µM orlistat. After 24 h, cell death was assessed by Annexin V-PI staining. (B) MCL cell line Mino was treated with 100 nM palbociclib and 2 µM dorsomorphin for 16 h and subsequently co-treated with 8 nM bortezomib. After 24 h, cell death was assessed by Annexin V-PI staining (upper panel). MCL cell lines were treated with 100 nM (Mino) or 300 nM (Jeko-1) palbociclib. After 16 h, proteins were analyzed by western blot using the indicated antibodies (lower panel). Data represent means ± SD from two independent experiments.

3.3.3 Autophagy inhibition restores bortezomib efficacy after palbociclib treatment

Previous results confirmed the regulation of autophagic flux as a function of cyclin D1/CDK4 activity in MCL. To confirm that this function is responsible for the palbociclib mediated antagonism on the bortezomib induced cell death and NOXA accumulation, MCL cell lines Mino and Jeko-1 were co-treated with bortezomib, palbociclib, and genetic or pharmacological autophagy inhibitors. Interestingly, genetic inhibition of autophagy by ATG5/7 knockdown (Figure 18A upper left panel) or pharmacological inhibition by spautin-1, liensinine, or 3MA treatment restored bortezomib induced cell death after palbociclib co-treatment (Figure 18A middle and lower left panels and Figure 18B). In addition, autophagy inhibition also restored bortezomib induced NOXA protein accumulation after palbociclib co-treatment in MCL cell line Mino (Figure 18A right panels). These results demonstrate that inhibition of

cyclin D1/CDK4 activity antagonizes bortezomib efficacy via the activation of autophagy and concomitant autophagosomal degradation of NOXA.



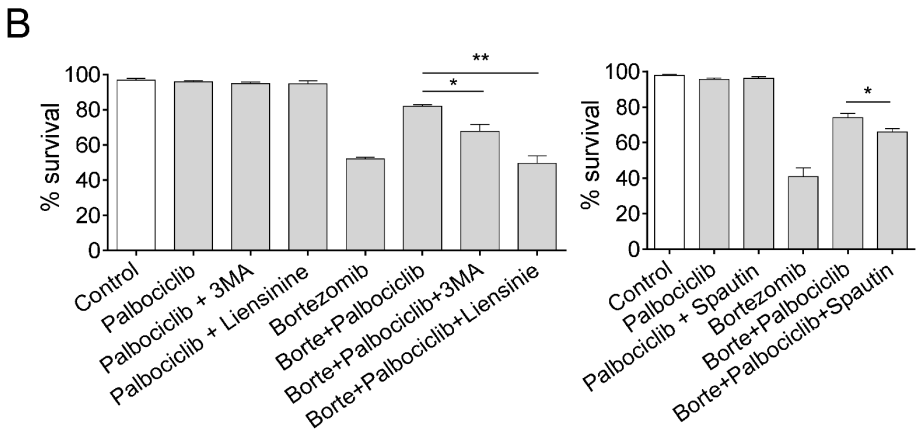


Figure 18: Knockdown of autophagy related genes as well as treatment with autophagy inhibitors counteract the palbociclib mediated antagonism on bortezomib induced cell death and NOXA induction. (A) MCL cell line Mino was transfected with siRNA targeting *ATG5* and *ATG7*. 24 h after transfection cells were treated with 100 nM palbociclib for 16 h and subsequently co-treated with 8 nM bortezomib. After 8 h, protein expression was analyzed by western blot using the indicated antibodies (upper right panel) and cell death was assessed by Annexin V-PI staining 24 h post treatment (upper left panel). MCL cell line Mino was treated with 100 nM palbociclib and 20 µM liensinine, 5 µM spautin-1, or 2 mM 3MA for 16 h and subsequently co-treated with 8 nM bortezomib. After 8 h, protein expression was analyzed by western blot using the indicated antibodies (right panels) and cell death was assessed by Annexin V-PI staining 24 h post treatment (left panels). (B) MCL cell line Jeko-1 was treated with 300 nM palbociclib and 20 µM liensinine, 2 mM 3MA (left panel), or 10 µM spautin-1 (right panel) for 16 h and subsequently co-treated with 8 nM bortezomib. Cell death was assessed by Annexin V-PI staining 24 h post treatment. Data represent means ± SD from three independent experiments.

The previous results demonstrated that inhibition of cyclin D1/CDK4 activity antagonizes bortezomib efficacy via the activation of autophagic flux and the regulation of NOXA protein levels. In order to confirm that the autophagy activation exerts its antagonizing function by direct regulation of NOXA protein stability, NOXA half-life was investigated under cyclin D1/CDK4 and UPS blocked conditions, in the presence or absence of ALP activity. Therefore, siRNA-mediated knockdown of ATG5 and ATG7 was performed in the presence of palbociclib and bortezomib and NOXA protein stability was investigated using CHX pulse-chase experiments in MCL cell line Mino. Interestingly, genetic blockade of autophagy by pre-incubation with *ATG5/7* siRNA prolonged NOXA half-life in cells co-treated with palbociclib and bortezomib, compared to control siRNA treated cell (Figure 19). In summary, under UPS blocked conditions, NOXA protein stability can be regulated in an UPS independent manner, through ALP dependent degradation.

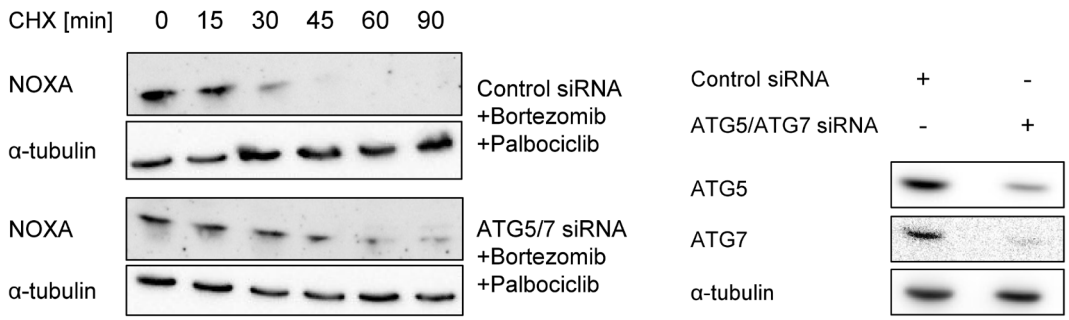


Figure 19: Knockdown of autophagy related genes reverses the palbociclib mediated antagonism on bortezomib induced stabilization of NOXA protein. MCL cell line Mino was transfected with siRNA targeting *ATG5* and *ATG7*. 24 h after transfection, cells were treated with 100 nM palbociclib for 16 h and subsequently co-treated with 8 nM bortezomib. After 8 h, cells were exposed to 20 µg/ml CHX and samples were collected 0, 15, 30, 45, 60, and 90 min after CHX addition and analyzed by western blot using the indicated antibodies.

3.3.4 NOXA protein contains a potential LIR motif

Previous results demonstrated that apart from UPS, NOXA can be degraded via ALP. How NOXA is targeted for autophagosomal degradation remains to be elucidated. It is known that selective types of autophagy involve specific amino acid motifs for correct targeting of substrates. In this context, a peptide motif biochemically related to KFERQ targets substrates for selective degradation via CMA (Kaushik et al., 2011). During selective macroautophagy/autophagy on the other hand, proteins of the ATG8 family promote the entry of adaptor proteins into the autophagy pathway through interaction with a LIR (Birgisdottir et al., 2013). The LIR motif consists of the core consensus sequence [W/F/Y]xx[L/I/V] (xx can be any amino acid) and an amino acid with an acidic residue flanking the motif. In order to narrow down the specific type of selective autophagy that mediates the cyclin D1/CDK4 function to regulate NOXA protein stability, the amino acid sequence of NOXA was investigated for motifs associated with selective autophagy. Interestingly, even though the NOXA amino acid sequence does not contain a peptide motif biochemically related to KFERQ, a potential LIR motif could be identified (Figure 20), which therefore might target NOXA for ALP dependent proteolysis.

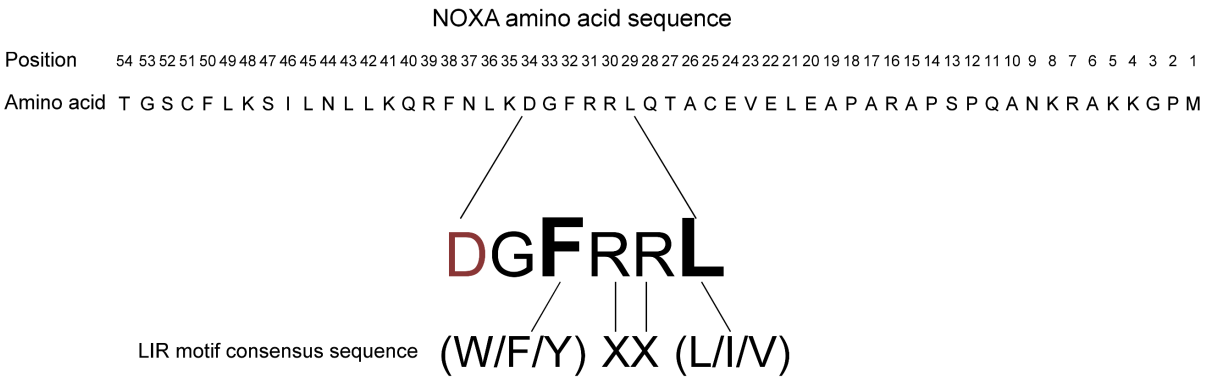


Figure 20: NOXA protein contains a potential LIR motif, which might target for selective autophagic degradation. The amino acid sequence DGFRRL at the position 29-34 in the NOXA protein represents a potential LIR motif corresponding to the core consensus sequence. The acidic amino acid is highlighted in red.

3.4 Combined inhibition of the ALP and the UPS efficiently kills MCL cells

So far, this study has demonstrated that inhibition of cyclin D1/CDK4 activity in MCL antagonizes NOXA dependent cell death and most importantly, cell death after proteasome inhibition. This antagonism was demonstrated to be mediated by the activation of autophagic flux, which causes the degradation of NOXA protein in an UPS independent manner. As autophagy inhibitors can reverse the antagonizing effect on bortezomib induced cell death after cyclin D1/CDK4 inhibition, the efficacy of proteasome inhibitor treatment should be improved by co-treatment with autophagy inhibitors.

3.4.1 Autophagy inhibitors potentiate bortezomib induced cell death and increase NOXA protein stability

To investigate, if bortezomib induced cell death can be potentiated by co-treatment with autophagy inhibitors, MCL cell lines with a reduced sensitivity to bortezomib treatment, Rec-1 and Jeko-1, were treated with the autophagy inhibitors liensinine, hydroxychloroquine, or 3MA and bortezomib. The late stage autophagy inhibitor liensinine showed the strongest effects in previous results (Figure 18). Therefore, the previously used autophagy inhibitor spautin-1 (Figure 18) was replaced by the FDA approved late stage autophagy inhibitor hydroxychloroquine. In line with the previous results, autophagy inhibition potentiated cell death after UPS blockage in MCL cell lines Rec-1 and Jeko-1 (Figure 21, upper and middle panels). In addition, potentiated cell death was accompanied by an increased NOXA protein accumulation in MCL cell line Jeko-1 (Figure 21, lower panel).

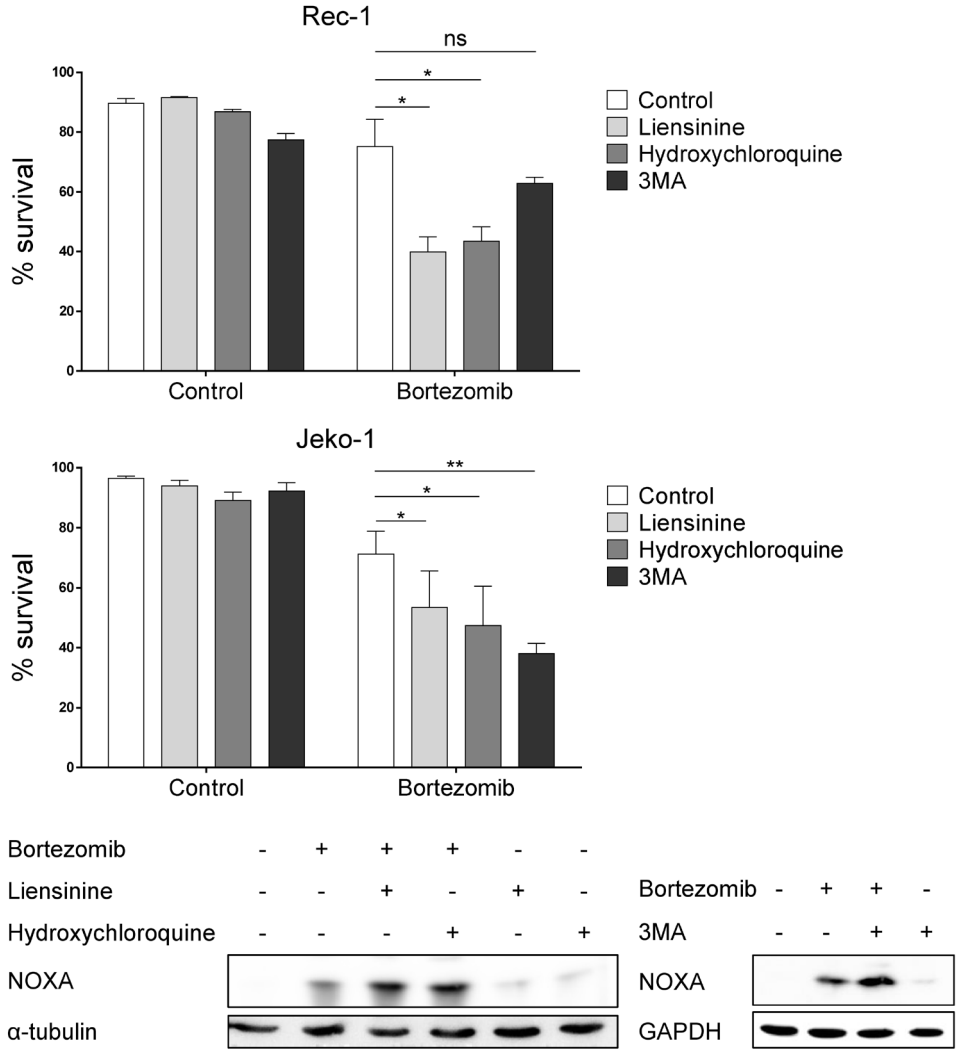


Figure 21: Autophagy inhibitors potentiate bortezomib induced cell death and NOXA accumulation. MCL cell lines were treated with 20 µM liensinine, 120 µM hydroxychloroquine, and 5 mM (Rec-1) or 2 mM (Jeko-1) 3MA for 16 h and subsequently co-treated with 8 nM bortezomib. After 8 h, protein expression was analyzed by western blot using the indicated antibodies (Jeko-1, lower panels) whereas cell death was assessed by Annexin V-PI staining 24 h post treatment (Rec-1 and Jeko-1, upper and middle panels). Data represent means ± SD from three independent experiments.

Autophagy inhibition potentiated cell death after UPS blockage in MCL cells and resulted in increased NOXA accumulation (Figure 21). The previous results suggest that this synergistic cell death and NOXA accumulation should be mediated by an increased NOXA protein stability after dual blockage of the UPS and the ALP. To confirm this underlying mechanism, NOXA protein stability was investigated using CHX pulse-chase experiments after co-treatment with bortezomib and 3MA in the MCL cell line Jeko-1. Compared to proteasome inhibition alone, combined inhibition of the UPS and the ALP led to increased NOXA protein stability as shown by western blot analysis (Figure 22, left panel) and corresponding densitometric quantification

(Figure 22, right panel). Inhibition of autophagy with 3MA alone had no effect on NOXA protein half-life in cell with active UPS (Figure 22).

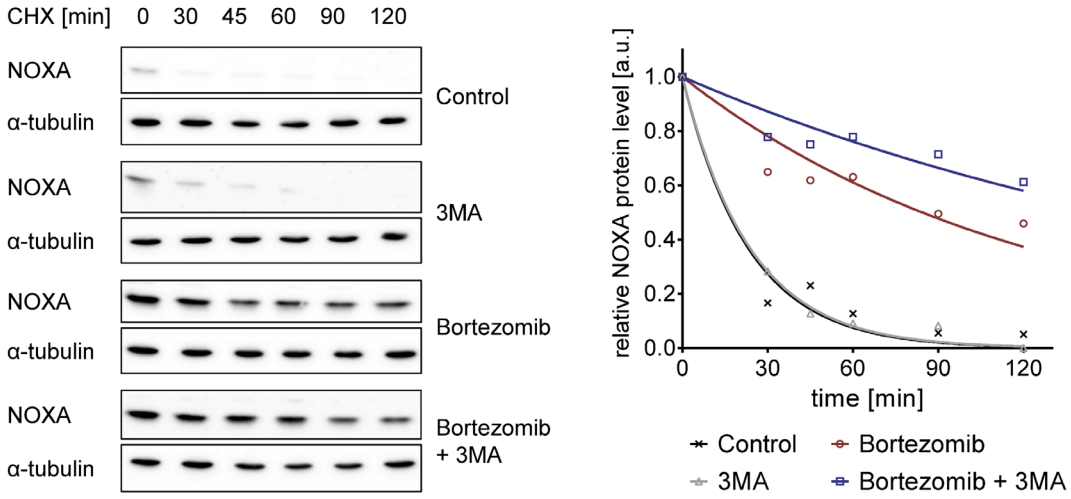


Figure 22: Autophagy inhibition improves bortezomib induced NOXA stabilization. MCL cell line Jeko-1 was treated with 2 mM 3MA for 16 h and subsequently co-treated with 7 nM bortezomib. After 14 h, 20 µg/ml CHX was added to the cells and samples were collected 0, 30, 45, 60, 90, and 120 min after CHX exposition for western blot analysis using the indicated antibodies (left panel) and corresponding densitometric analysis of NOXA protein stability (right panel).

3.4.2 FASN and neddylation inhibition synergize with bortezomib treatment

Previous results demonstrated that autophagic activity antagonizes proteasome inhibitor induced cell death in MCL and that the efficacy of proteasome inhibitors such as bortezomib, can be increased by combinatorial treatment with autophagy inhibitors. Treatment efficacy of combined UPS and ALP inhibition, however, was still limited (Figure 21). Therefore, more efficient combinations were screened, to exploit the dual UPS and ALP inhibition axis for most efficient NOXA stabilization and cell death induction. At first, different drugs were screened for their ability to potentiate bortezomib induced cell death. Consequently, the MCL cell line Jeko-1 was treated with bortezomib and different compounds that are known to be effective in MCL. Co-treatment of bortezomib with the FASN inhibitor orlistat or the neddylation inhibitor MLN4924 was most efficient and resulted in almost complete cell death (Figure 23).

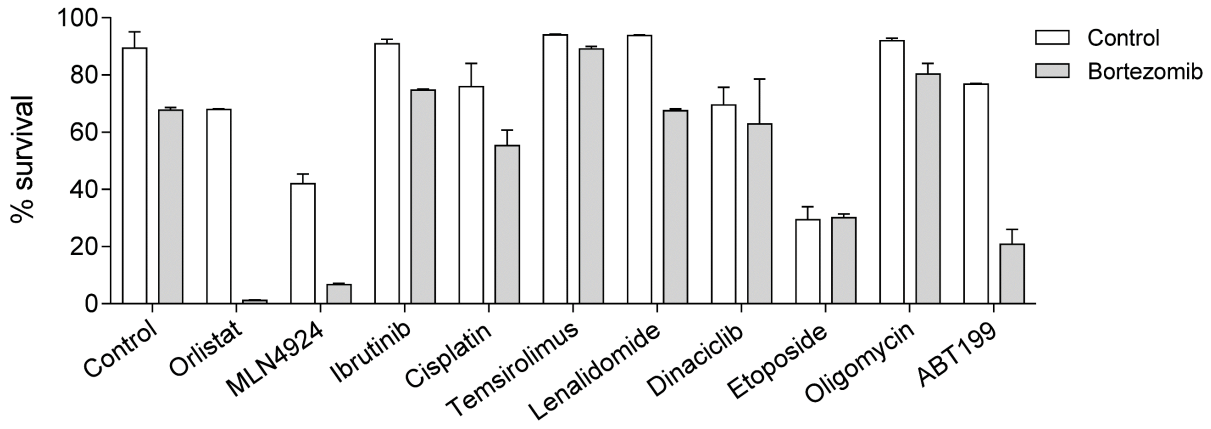


Figure 23: FASN and neddylation inhibition synergize with bortezomib treatment. MCL cell line Jeko-1 was treated with either 15 µM orlistat, 50 nM MLN4924, 1 µM ibrutinib, 10 µM cisplatin, 10 nM temsirolimus, 1 µM lenalidomide, 9 nM dinaciclib, 10 µM etoposide, 5 µM oligomycin, or 50 nM ABT199 and co-treated with 5 nM bortezomib. Cell death was assessed by Annexin V-PI staining 24 h post treatment. Data represent means ± SD from two independent experiments.

3.4.3 FASN inhibition blocks autophagic flux after proteasome inhibition

Orlistat and MLN4924 were identified to be very effective in combination with bortezomib for cell death induction in MCL. In order to elucidate the potential role of autophagy in mediating cell death after combinatorial treatment, the expression levels of the autophagy marker LC3-II and the sequestosome 1 protein (SQSTM1) (also known as p62), which is exclusively degraded by the ALP, was investigated (Komatsu and Ichimura, 2010). In addition, the combinatorial treatment was co-treated with a known autophagy inducer, the PI3K/mTOR dual inhibitor BEZ235 (Chang et al., 2013; Oh et al., 2016). BEZ235 treatment was shown to induce autophagic activity by inhibiting the mTOR mediated inhibitory phosphorylation of the ULK1 kinase (Kim et al., 2011). Interestingly, co-treatment of bortezomib and orlistat or MLN4924 with the autophagy inducer BEZ235 antagonized cell death induction in the MCL cell line Mino (Figure 24, upper panel). More importantly, co-treatment of bortezomib with orlistat led to a blockade of autophagic degradation in MCL cell line Mino (Figure 24, middle panel), which is characterized by an elevated LC3-II protein expression and a concomitant accumulation of the SQSTM1 protein (Klionsky et al., 2021). Furthermore, co-treatment of BEZ235 with bortezomib and orlistat did cause a strong reduction in SQSTM1 protein, which is indicative of autophagy induction (Figure 24, middle panel). Combined treatment of MCL cells with bortezomib and MLN4924, however, did neither cause autophagic blockade nor change expression levels of autophagy markers LC3-II or SQSTM1 (Figure 24, lower panel).

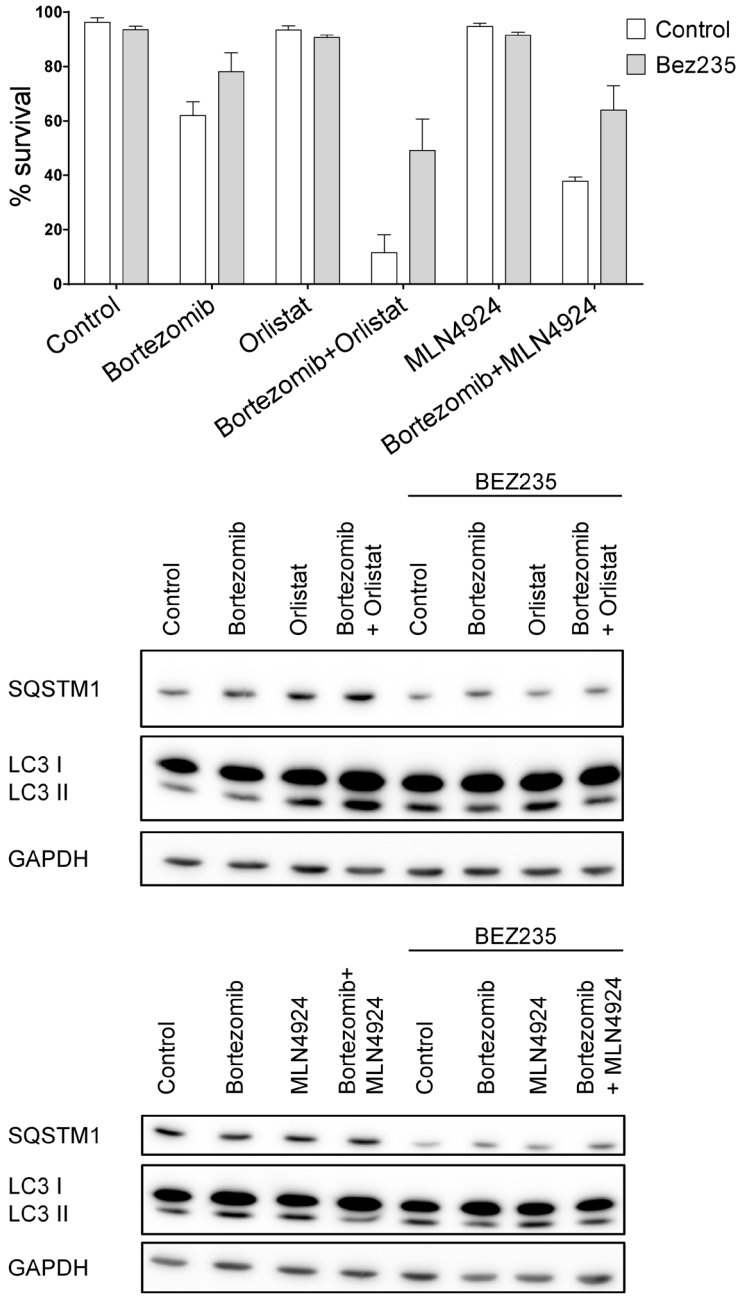


Figure 24: FASN inhibition, but not neddylation inhibition blocks autophagic degradation after proteasome inhibition. MCL cell line Mino was treated with 1 µM BEZ234 for 16 h and subsequently co-treated with either 15 µM orlistat or 300 nM MLN4924 and 7 nM bortezomib. Protein expression was analyzed by western blot using the indicated antibodies 8 h post treatment (middle and lower panels) and cell death was assessed by Annexin V-PI staining 24 h post treatment (upper panel). Data represent means ± SD from two independent experiments.

3.4.4 FASN inhibition increases NOXA protein stability after bortezomib treatment

This study demonstrated that combining autophagy inhibitors with proteasome inhibition leads to efficient cell death in MCL (Figure 21), mediated by a synergistic stabilization of NOXA protein (Figure 22). After a more efficient combination for dual targeting of the UPS and the ALP and cell death induction in MCL was discovered (Figure 23 and 24), the potential role of

a prolonged NOXA half-life as underlying mechanism needed to be confirmed. To clarify this, the NOXA protein stability after combined bortezomib and orlistat treatment was investigated using CHX pulse-chase experiments in MCL cell line Jeko-1. Compared to proteasome inhibition alone, combination treatment with orlistat led to a potentiated NOXA protein stability as shown by western blot analysis (Figure 25, left panel) and corresponding densitometric quantification (Figure 25, right panel). Orlistat treatment alone also prolonged NOXA protein half-life, compared to control treatment.

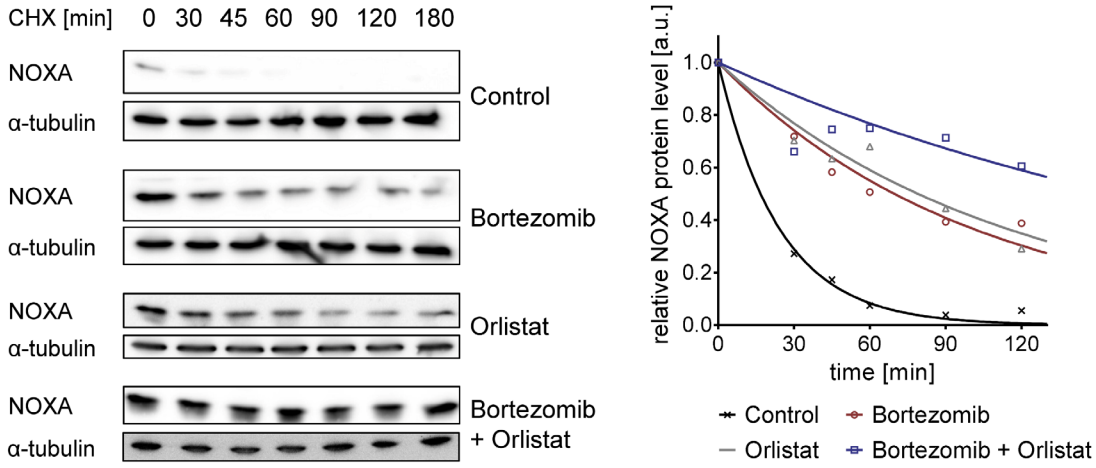


Figure 25: Inhibition of the proteasome and FASN further increases NOXA half-life. MCL cell line Jeko-1 was treated with 15 µM orlistat and 7 nM bortezomib. After 14 h, 20 µg/ml CHX was added to the cells and samples were collected 0, 30, 45, 60, 90, 120, and 180 min after CHX exposition for western blot analysis using the indicated antibodies (left panel) and quantification of NOXA protein stability (right panel).

3.4.5 Combined inhibition of FASN and the proteasome kills MCL cell lines efficiently and specifically

Combination of bortezomib with a FASN inhibitor led to dual blockage of the UPS and the ALP (Figure 24) and subsequently increased NOXA half-life (Figure 25). To confirm that this combination is efficient and specific for MCL, four MCL cell lines and PBMNCs from healthy donors were treated with bortezomib and orlistat. Interestingly, compared to bortezomib or orlistat treatment alone, combinatorial treatment potentiated cell death induction and resulted in an almost complete cell death in all MCL cell lines (Figure 26A, upper and middle panels). In line with cell death induction, combined blockage of the UPS and the ALP led to substantially potentiated accumulation of NOXA protein in MCL cell line Jeko-1, compared to UPS blockage alone (Figure 26A, lower panel). This effect was not exclusive for bortezomib, as co-treatment with the proteasome inhibitor carfilzomib resulted in similar NOXA induction. Most importantly, different concentrations of the combinatory treatment had only minor effects on PBMNC from healthy donors (Figure 26B).

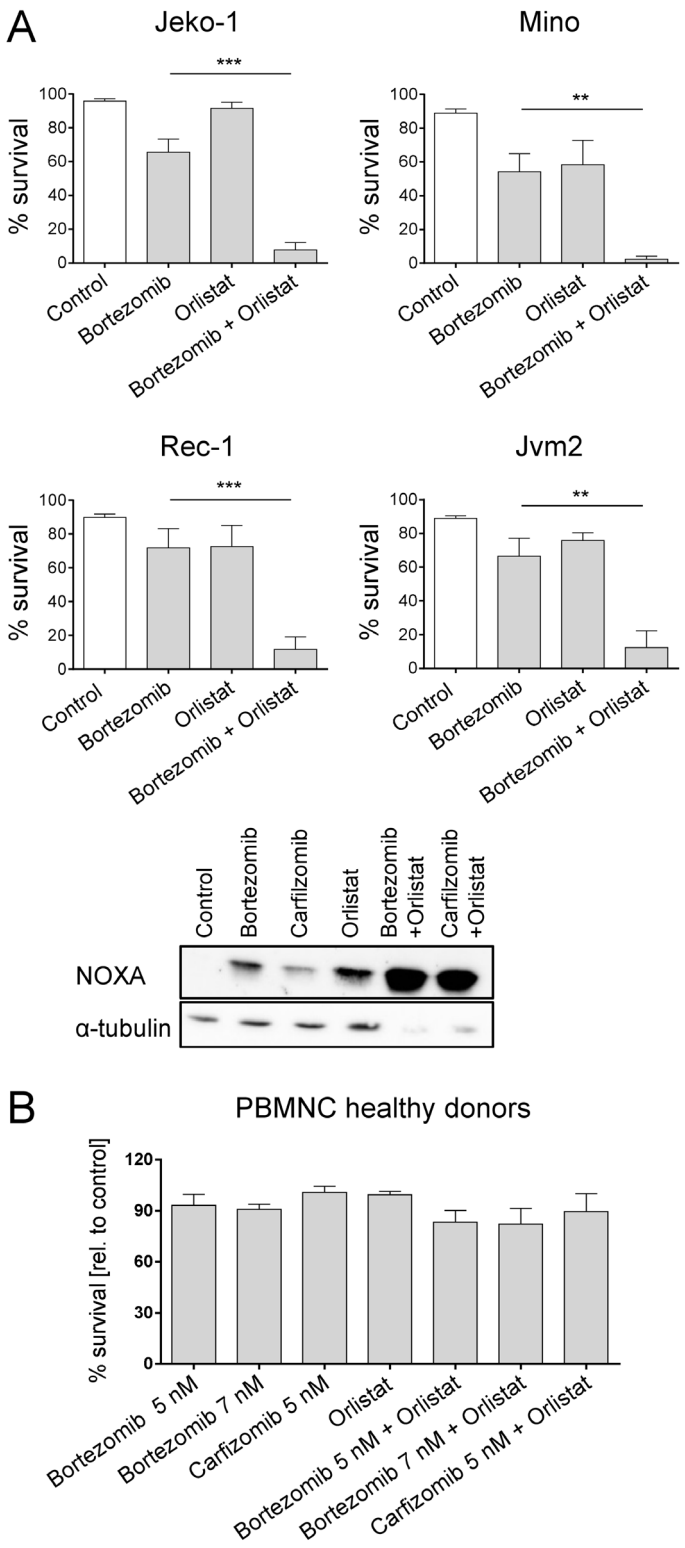


Figure 26: Co-treatment of bortezomib with orlistat induces highly efficient and specific cell death in MCL cell lines. (A) MCL cell lines were treated with 15 µM orlistat and 7 nM (Jeko-1 and Rec-1) or 5 nM (Mino and Jvm2) bortezomib or 5 nM carfilzomib (Jeko-1). After 14 h, protein expression was analyzed by western blot using the indicated antibodies (Jeko-1) and after 24 h, cell death was assessed by Annexin V-PI staining. (B) Healthy lymphocytes and monocytes are hardly affected by co-treatment of proteasome inhibitors with orlistat. PBMNCs were treated with 15 µM orlistat and 5 nM or 7 nM bortezomib or 5 nM carfilzomib. After 24 h, cell death was assessed by Annexin V-PI staining (normalized to untreated control samples). Data represent means ± SD from three independent experiments.

3.4.6 Cell death after combination treatment is cyclin D1/CDK4 dependent and NOXA specific

Previous results demonstrated that cyclin D1/CDK4 activity is important for the response of MCL cells to bortezomib (Figure 6). Consequently, it was investigated if the same dependence was present for the more effective combination of bortezomib with orlistat. Indeed, consistent with the effects observed in cells treated with bortezomib alone, genetic or pharmacological blockade of cyclin D1/CDK4 also rescued the more effective cell death after co-treatment in MCL cell line Jeko-1 (Figure 27A and B, left panels). In addition, after knockdown of cyclin D1 or CDK4 and subsequent combinatorial treatment with bortezomib and orlistat in MCL cell line Jeko-1, NOXA accumulation was blocked as effective as direct NOXA knockdown (Figure 27A, right panel). In line with this, pharmacological inhibition of cyclin D1/CDK4 by palbociclib treatment abrogated NOXA accumulation after combinatorial treatment (Figure 27B, right panel). In conclusion, these results demonstrate that the aberrant cyclin D1/CDK4 activity in MCL is required for the effective accumulation of NOXA protein and concomitant induction of cell death after combined UPS and ALP inhibition.

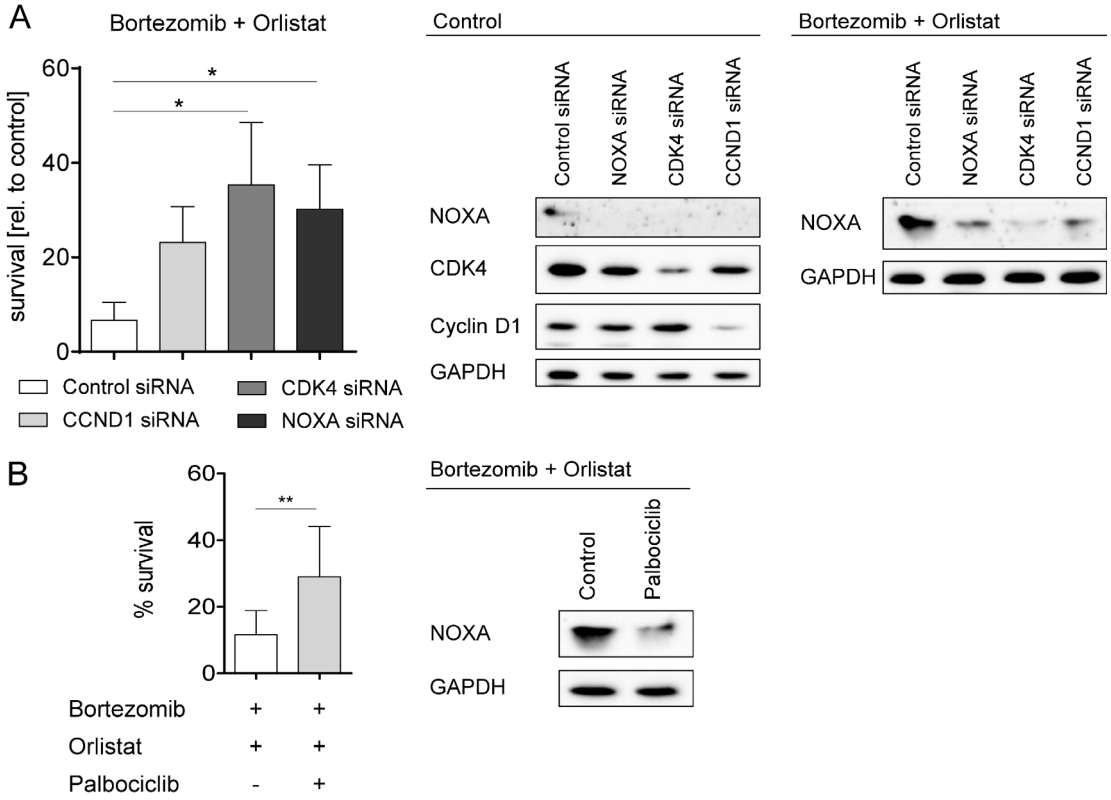


Figure 27: Cyclin D1/CDK4 activity is required for cell death induction after combined UPS and ALP inhibition. (A) Knockdown of either cyclin D1 or CDK4 antagonizes cell death as well as NOXA protein induction as efficient as knockdown of NOXA. MCL cell line Jeko-1 was transfected with siRNA targeting *NOXA*, *CCND1,* or *CDK4*. 24 h after transfection, cells were treated with 15 µM orlistat and 7 nM bortezomib. After 14 h,

protein expression was analyzed by western blot using the indicated antibodies (middle and right panels) and cell death was assessed by Annexin V-PI staining 24 h post treatment (left panel) (normalized to untreated control siRNA samples). (B) Co-treatment of bortezomib with orlistat was antagonized by palbociclib treatment. MCL cell line Jeko-1 was treated with 300 nM palbociclib and subsequently co-treated with 15 µM orlistat and 7 nM bortezomib. After 14 h, protein expression was analyzed by western blot using the indicated antibodies (right panel) and cell death was assessed by Annexin V-PI staining (left panel). Data represent means ± SD from three independent experiments.

This study so far, has shown that regulation of autophagic activity is an important function of aberrant cyclin D1/CDK4 activity in MCL. Furthermore, previous results demonstrated that after proteasome inhibition NOXA protein can be targeted for autophagosomal degradation. Because the NOXA protein can be degraded by the UPS as well as the ALP, targeting both degradation systems proved to be highly efficient, to induce cell death in MCL cells. The combination of UPS and ALP inhibition using bortezomib and orlistat resulted in a very efficient NOXA and cyclin D1/CDK4 dependent cell death (Figure 26 and 27). Changes in expression levels of other apoptotic proteins, however, might contribute to cell death induction after combinatorial treatment. Interestingly, the expression levels of the anti-apoptotic counterpart of NOXA, Mcl-1, remained the same after combined treatment with UPS inhibitors and orlistat in the MCL cell line Jeko-1, compared to bortezomib treatment alone (Figure 28). Bortezomib induced cell death in cancer cells has also been shown to be associated with the expression levels of the pro-apoptotic protein Puma (Ding et al., 2007a). However, no major changes in Puma protein levels were detected after combinatorial treatment. In line with this, the expression levels of the pro-apoptotic effector proteins Bax and Bak also remained unaffected after combinatorial treatment, confirming the prominent role of NOXA protein for cell induction after combinatorial treatment.

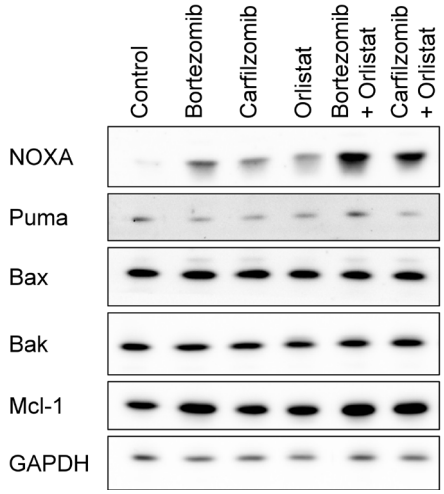


Figure 28: Combination of UPS inhibition and ALP inhibition regulates mainly NOXA protein levels and not Puma, Bax, Bak or Mcl-1. MCL cell line Jeko-1 was treated with 7 nM bortezomib or carfilzomib and co-treated with 15 µM orlistat. After 14 h, protein expression was analyzed by western Blot using the indicated antibodies.

Successful execution of NOXA dependent mitochondrial apoptosis involves the activation and activity of different caspases. Other types of cell death such as type II autophagic cell death or caspase independent apoptosis, however, do not require caspase activity (Kim et al., 2005; Kroemer and Martin, 2005; Green and Llambi, 2015). In order to further characterize the specific type of cell death after combined inhibition of the UPS and the ALP, MCL cell line Jeko-1 was treated with the pan-caspase inhibitor Z-VAD-FMK. Interestingly, cell death induced by the combinatorial treatment of either bortezomib or carfilzomib with orlistat, was dependent on caspase activity and could be blocked by pre-incubation with the caspase inhibitor Z-VAD-FMK (Figure 29).

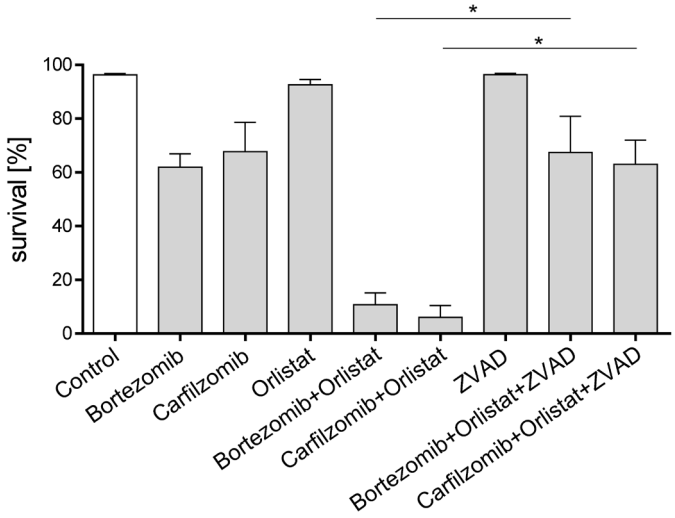


Figure 29: Synergistic cell death after UPS and ALP inhibition is caspase dependent. MCL cell line Jeko-1 was treated with 50 µM of the pan-caspase inhibitor Z-VAD-FMK for 2 h, subsequently treated with 7 nM bortezomib or carfilzomib, and co-treated with 15 µM orlistat. After 24 h, cell death was assessed by Annexin V-PI staining. Data represent means ± SD from three experiments.

4 Discussion

Mantle cell lymphoma is a rare aggressive B cell lymphoid neoplasm, with only short remission duration after standard therapy (Vose, 2017). To date, MCL still remains virtually incurable and novel therapy options are urgently needed (Pérez-Galán et al., 2011a). The cyclin D1 overexpression and the associated aberrant cyclin D1/CDK4 activity represent hallmark features of MCL and the associated molecular consequences are extensively studied. In recent years the proteasome inhibitor bortezomib provided a new targeted treatment option for MCL patients (Robak et al., 2015). In this study, novel functions of cyclin D1 and associated cyclin D1/CDK4 activity in MCL were identified. These novel cell cycle independent functions of cyclin D1 help to clarify the preferential susceptibility of MCL cells to proteasome inhibitors and provide urgently needed novel therapy options. The present work demonstrates that aberrant cyclin D1/CDK4 activity suppresses the expression of an antioxidant defense protein, namely sestrin 3. Moreover, cyclin D1/CDK4 activity was shown to be a prerequisite for efficient cell death induction after proteasome inhibition in MCL. This cell death regulating function of cyclin D1/CDK4 activity, however, is not associated with the identified function to regulate sestrin 3. Cyclin D1/CDK4 activity rather allowed for efficient stabilization of NOXA protein after proteasome inhibition. Consequently, inhibition of cyclin D1/CDK4 activity did not only antagonize bortezomib induced cell death but also cell death induced by NOXA stabilizing compounds. This antagonism was demonstrated to be mediated by a mitigated autophagy due to high cyclin D1/CDK4 activity. In the absence of cyclin D1/CDK4 activity NOXA protein was shown to be degraded by the autophagosome under UPS blocked conditions. Furthermore, combination of the proteasome inhibitor bortezomib with autophagy inhibitors resulted in highly efficient cell death induction in MCL cells. Thus, dual targeting of proteasomal and autophagosomal degradation pathways represents a novel treatment strategy in MCL. The results of this study might help to overcome resistances to proteasome inhibitors and might improve treatment efficacy in MCL patients.

4.1 Cyclin D1/CDK4 cell cycle independent functions

Although the cyclin D1 overexpression represents a hallmark feature in almost all MCL cases, observations have been made that suggest that cyclin D1 overexpression may be dispensable for the pathogenesis in MCL (Jares et al., 2012). Apart from regulating cell cycle progression, cyclin D1 has been implicated in various cellular functions such as metabolism, cellular migration, autophagy, DDR, as well as maintaining genomic stability (Sakamaki et al., 2006; Aggarwal et al., 2007; Jirawatnotai et al., 2011; Body et al., 2017; Casimiro et al., 2017).

Moreover, targeting the deregulated cell cycle progression in MCL, that is associated with aberrant cyclin D1/CDK4 activity, with kinase inhibitors such as palbociclib does not induce apoptosis in MCL cell lines (Chiron et al., 2013). In addition, single agent activity of palbociclib in MCL patients was limited and only beneficial for a subset of MCL patients (Leonard et al., 2012). For patients with relapsed or refractory MCL, proteasome inhibitors such as bortezomib have shown promising results in clinical trials (Goy et al., 2009). Studies showed that this drug exerts its function via the pro-apoptotic BH3-only protein NOXA (Pérez-Galán et al., 2006) but little is known how the aberrant cyclin D1/CDK4 activity in MCL affects the treatment efficacy of such UPS inhibitors. In this study, novel cell cycle independent functions of aberrant cyclin D1/CDK4 activity in MCL were identified that could help to improve proteasome inhibitor-based therapy of MCL patients. Apart from MCL, cyclin D1 overexpression is also a key feature of several other cancers such as head and neck squamous cell carcinoma, endometrial cancer, melanoma, breast cancer, and multiple myeloma (Moreno-Bueno et al., 2004; Arnold and Papanikolaou, 2005; Bergsagel and Kuehl, 2005; Li et al., 2006a; Smeets et al., 2006). Additionally, deregulation of cyclin D1/CDK4/6 activity is a feature of many cancers, as the CDK inhibitor p16INK4a represents the most frequently deleted locus in human cancers (Beroukhim et al., 2010). Identification of cyclin D1/CDK4/6 functions that are independent from cell cycle, are therefore highly relevant for many different cancer types and cancer patients.

4.1.1 Cyclin D1/CDK4 activity regulates *SESN3*

Because of the physiological role of the cyclin D1 in promoting cell proliferation, the oncogene cyclin D1 has been found to be amplified or overexpressed in a variety of tumors (Musgrove et al., 2011). Interestingly, different kinase independent functions of cyclin D1 were identified in several studies such as induction of chromosomal instability, stimulation of the transcriptional activity of the estrogen receptor, and interaction with histone acetyltransferases and histone deacetylases (Neuman et al., 1997; McMahon et al., 1999; Fu et al., 2005b; Casimiro et al., 2015). Cyclin D1 has even been shown to promote cell cycle progression in CDK independent manner by enhancing NDR kinase activity (Du et al., 2013). However, since specific CDK4/6 inhibitors such as palbociclib have progressed towards the clinic for the treatment of breast cancer, multiple myeloma, and MCL patients (Leonard et al., 2012; Niesvizky et al., 2015; Iwata et al., 2017), research is focused on identifying novel kinase dependent functions of cyclin D1. Further elucidating the mode of action of palbociclib and investigating the molecular consequences of inhibiting cyclin D1/CDK4/6 activity will help to improve therapy options for various cancer types. Changes in cellular functions after CDK4/6 inhibition are mainly

mediated by the RB1 pathway and are a result of transcriptional regulation by E2F family members (Markey et al., 2002; Knudsen and Witkiewicz, 2017). Apart from controlling cell cycle progression, the cyclin D1/CDK4/6-RB1-E2F pathway is also implicated in the regulation of cell cycle associated processes such as DNA damage checkpoint and repair pathways, DNA replication, chromatin assembly/condensation, chromosome segregation, and mitosis (Polager et al., 2002; Ren et al., 2002). After assembly with the corresponding cyclin dependent kinases CDK4 and CDK6, the cyclin D1/CDK4/6 complex enables S-phase entry by RB1 phosphorylation and subsequent release of transcription factors of the E2F family (Kato et al., 1993). In line with this, inhibition of cyclin D1/CDK4 activity with the kinase inhibitor palbociclib resulted in a decreased phosphorylation of RB1 and subsequently caused a G1-phase cell cycle arrest (Figure 1). The doubling times of most MCL cell lines range from 19 – 72 h (Fogli et al., 2015) and the duration of the eukaryotic cell cycle is mostly defined by the duration of the interphase (Cooper and Hausman, 2016). Major changes in cell cycle distribution should therefore not be possible after only a few hours of palbociclib treatment in MCL cell lines. Furthermore, the decrease in RB1 phosphorylation is an early event in the cyclin D1/CDK4/6-RB1-E2F cell cycle regulating pathway, whereas cell cycle changes are the downstream endpoint of this pathway. Consequently, by investigating RB1 phosphorylation it was possible to determine the efficacy of the palbociclib treatment and study associated cellular changes, before major cell cycle changes occurred (Figure 1).

In this study, after modulation of cyclin D1/CDK4 activity, the global transcriptome analysis revealed the regulation of *SESN3* as a novel kinase dependent and cell cycle independent function of aberrant cyclin D1/CDK4 activity in MCL (Figure 2 and 3). Changes in cell cycle distribution do have a major impact on the global transcription pattern (Dominguez et al., 2016). As a longer treatment period would have resulted in a G1-phase cell cycle arrest (Figure 1), modulation of cyclin D1/CDK4 activity was carried out as a short-term treatment to screen for cell cycle independent functions (Figure 2). Moreover, to identify novel cyclin D1/CDK4 functions that are associated with the response of MCL cells to proteasome inhibitors, treatment duration was chosen accordingly, as cell cycle changes are also associated with apoptosis regulation (Pucci et al., 2000). The cyclin D1/CDK4 inhibitor palbociclib has been shown to be highly specific, especially regarding the specificity towards other CDKs (Chen et al., 2016a). However, since there are over 500 protein kinases encoded by the human genome, off-target binding should always be addressed in experimental settings (Klaeger et al., 2017). In addition, previous reports have shown that transcriptional inhibition of genes with siRNA, which recognizes complementary mRNA and induces its degradation, generally suffers from nonspecific effects on mammalian gene expression (Persengiev et al., 2004).

Changes revealed in the global transcriptome analysis, that are both caused by genetic modulation with *CCND1* siRNA as well as pharmacological modulation with palbociclib are therefore more likely to be mediated by cyclin D1/CDK4 activity (Figure 2). The cyclin D1/CDK4/6-RB1-E2F pathway is known to regulate G1 to S-phase progression. In line with this, most of the regulated genes in the global transcriptome analysis were associated with cell cycle progression such as *CCNE1*, *CCNE2*, *E2F1*, and *E2F8* or cell cycle associated processes such as *GMNN, CDT1*, *CDC6, or* genes coding for the minichromosome maintenance (MCM) protein family (Figure 2). Interestingly, aberrant high cyclin D1/CDK4 activity in MCL was demonstrated to suppress the expression of a gene that is not directly associated with cell cycle regulation, namely *SESN3* (Figure 2 and 3). Transcriptional regulation of *SESN3* is controlled by the activity of FOXO transcription factors, which again is inhibited through direct phosphorylation by Akt (Hay, 2011). In normal B cells and in MCL cell lines, FOXO transcription factors are inactivated to ensure proper cell-cycle progression and survival (Yusuf et al., 2004; Obrador-Hevia et al., 2012). Induction of FOXO activity or inhibition of Akt as a consequence of cyclin D1/CDK4 inhibition might therefore contribute to *sestrin 3* transcript upregulation (Figure 3). Conversely, different studies show that inhibition of cyclin D1/CDK4 activity leads to the activation of mTOR and Akt signaling pathways (Tarrado-Castellarnau et al., 2017; Cretella et al., 2018). Therefore, it is unlikely that Akt signaling mediates the transcriptional regulation of sestrin 3 expression in MCL (Figure 2 and 3). Other studies demonstrated that cyclin D1 depletion corresponded with increased activity of FOXO proteins (Gan et al., 2009; Laphanuwat et al., 2018). This cyclin D1 effect on FOXO activity, however, was independent of kinase activity, unlike the transcriptional regulation of *sestrin 3* expression in the global transcriptome analysis (Figure 2). A number of kinases have been implicated in the regulation of FOXO transcription factors that might be responsible for the cyclin D1/CDK4 mediated regulation of *SESN3*, such as SGK, AMPK, JNK, ERK, or MST1. In addition, as FOXO transcription factors contain several lysine residues that are targeted by protein deacetylases, acetyltransferases, ubiquitin ligases, and methyltransferases (Brown and Webb, 2018), cyclin D1/CDK4 could also exert a regulatory activity through these regulatory mechanisms.

Interestingly, inhibition of cyclin D1/CDK4 was demonstrated to stimulate glycolytic and oxidative metabolism, which was associated with an increase of mitochondrial mass and ROS production (Franco et al., 2016). Increased ROS production might therefore directly lead to upregulation of sestrin 3 expression. Several studies have shown that downregulation of members of the sestrin family proteins contributes to increased ROS levels and associated DNA damage induced by oxygen species (Kopnin et al., 2007). Sestrin family proteins have been

linked to the regeneration of peroxiredoxins, which are responsible for the decomposition of peroxides (Budanov et al., 2004). Conversely, sestrin 3 expression has been shown to induce Akt signaling (Tao et al., 2015), which in turn has been associated with elevated ROS levels (Nogueira et al., 2008). To date the exact mode of action of the antioxidant activity by sestrin 3, however, is not yet fully understood. Many chemotherapeutics have shown to induce ROS, which is often critical for treatment efficacy (Conklin, 2000). Bortezomib treatment and apoptosis induction has also been associated with the production of ROS (Ling et al., 2003). In line with this, treatment efficacy of bortezomib was demonstrated to be compromised by ROS scavengers and increased by ROS inducers (Figure 4). Moreover, inhibition of cyclin D1/CDK4 activity antagonized cell death induced by ROS (Figure 4). Compared to other cancer types, MCL cells are particularly sensitive to bortezomib treatment (O'Connor, 2005; Barr et al., 2007), which could be partially associated with the ROS and antioxidant status of MCL cells. Although studies demonstrated that sensitivity of cancer cells to bortezomib treatment can be increased by increasing ROS levels (Halasi et al., 2013a), treatment efficacy of bortezomib, however, was not regulated by sestrin 3 in the presence or absence of cyclin D1/CDK4 activity in MCL (Figure 5). Interestingly, in some cancer types CDK4 inhibition and palbociclib treatment have been shown to be associated with an increase in ROS (Franco et al., 2016; Valenzuela et al., 2017). Consequently, palbociclib treatment might have caused an increase in ROS levels and therefore potentially abrogated the antioxidant effect of elevated sestrin 3 protein levels (Figure 5). In addition, the previously mentioned converse role of sestrin 3, to act as a ROS inducer through Akt signaling, might abrogate possible ROS antagonizing effects. Moreover, despite the reported role of sestrin 3 to regulate peroxidase activity (Budanov et al., 2004), apoptosis induction mediated by hydrogen peroxide was not regulated by sestrin 3 in MCL cell lines either (Figure 5). In summary, even though bortezomib induced cell death in MCL was ROS dependent and ROS induced cell death could be reversed by cyclin D1/CDK4 inhibition (Figure 4), these mechanisms were not associated with the cyclin D1/CDK4 mediated downregulation of the antioxidant defense protein sestrin 3. Nevertheless, the identified function of cyclin D1/CDK4 to regulate sestrin 3 expression might be interesting in a different cellular context and should be further investigated with other treatment options in MCL or other cancer types with or without aberrant cyclin D1/CDK4 activity. In this context, treatment with tyrosine kinase inhibitors in Bcr-Abl expressing cells has been demonstrated to induce *SESN3* mRNA and sestrin 3 protein expression, which subsequently promoted antileukemic responses (Vakana et al., 2013).

4.1.2 Cyclin D1/CDK4 activity regulates NOXA dependent cell death after proteasome inhibition

The efficacy of proteasome inhibitor treatment in cancer has been shown to be mediated by ER stress, changes in NF-κB activity, DDR pathways, cell cycle regulation, ROS production, and apoptosis induction. The exact mechanism of action, however, is not yet fully understood (Fribley et al., 2004; Crawford et al., 2011). It has been demonstrated that proteasome inhibitors target the NF-κB pathway by stabilizing the NF-κB inhibitor protein IκB-α (Traenckner et al., 1994). Furthermore, studies have shown that NOXA mediated apoptosis after bortezomib treatment is dependent on MYC levels and that *MYC* gains and/or amplifications are a common feature in MCL (Nikiforov et al., 2007; Wirth et al., 2014; Yi et al., 2015). On the other hand, various mechanisms have been described that counteract bortezomib efficacy in MCL. These resistance mechanisms include accumulation of the Bcl-2 protein, plasmacytic differentiation, mutation as well as overexpression of proteasomal subunits, elevated NF-κB activity, or increased autophagic activity (Oerlemans et al., 2008; Yang et al., 2008; Pérez-Galán et al., 2011b, 2011b; Smith et al., 2011; Chen et al., 2016b). However, combined therapy of bortezomib with substances that target the DNA methyltransferase, histone acetylation, prosurvival chaperones, PI3K/Akt signaling, or the anti-apoptotic protein Mcl-1 have been shown to improve the efficacy of bortezomib in MCL (Roué et al., 2011; Leshchenko et al., 2015; Qu et al., 2015, 2015; Zhao et al., 2015). To date, there are no standard biomarkers available to precisely predict response rates to bortezomib treatment. A recent study in multiple myeloma patients, however, has shown that the expression levels of clusterin, angiogenin, complement component C1q, albumin, beta-2-microglobulin, paraprotein, and the kappa/lambda ratio can help to predict response to treatments containing bortezomib (Ting et al., 2017). In addition, the unfolded protein response (UPR) activity and function, as measured by ATF6 expression and the size of the ER, have been shown to be useful predictors of the sensitivity to bortezomib treatment (Nikesitch et al., 2016). The results of the present study demonstrate that impaired cyclin D1/CDK4 activity after palbociclib treatment reduced the response to bortezomib treatment in different MCL cell lines (Figure 6). This antagonizing effect of palbociclib treatment is of great clinical interest since both drugs are approved for the treatment of MCL patients. Furthermore, other FDA approved CDK4/6 inhibitors such as ribociclib and abemaciclib might demonstrate the same antagonism in combination therapy. Importantly, both of these CDK4/6 inhibitors and bortezomib have all already been independently investigated in clinical trials with hormone receptor-positive metastatic breast cancer patients (Adelson et al., 2016; Lee et al., 2019; Rascon et al., 2019). Apart from MCL

or multiple myeloma, cyclin D1 overexpression is also a common feature in breast cancer (Mohammadizadeh et al., 2013). Interestingly, studies have shown that cyclin D1 overexpression correlates with the response to bortezomib treatment in breast cancer (Ishii et al., 2006). Moreover, cyclin D1 expression was also shown to sensitize cancer cells to bortezomib treatment in multiple myeloma patients, presumably through the activation of the UPR pathway and ER-stress signaling (Bustany et al., 2015). In addition, another study has demonstrated that a high expression of the CDK4/CDK6 inhibitor $p16^{INK4A}$ negatively correlates with bortezomib response (Dawson et al., 2009).

Palbociclib inhibits the cyclin D1/CDK4 activity, therefore alters cell cycle distribution and finally causes G1-phase arrest (Figure 1). Different studies demonstrate that depending on the specific type of cell cycle arrest, artificial manipulation of the cell cycle can either prevent or potentiate apoptosis (Meikrantz and Schlegel, 1995). Indeed, different studies suggest a link between proliferation and apoptosis *in vivo* (Alenzi, 2004). Interestingly, the morphology of cells undergoing normal mitosis is similar to cells undergoing apoptosis, which led to the early assumption that the underlying biochemical causes might be related (King and Cidlowski, 1995). Furthermore, several genes such as *MYC* and *p53* that are associated with cell cycle regulation, are also involved in apoptosis regulation (Pucci et al., 2000). The antagonizing effect of palbociclib on bortezomib efficacy in MCL (Figure 6) could therefore be partially mediated by changes in cell cycle. The palbociclib mediated antagonism on bortezomib induced cell death, however, was the same in MCL cells that were able to progress in cell cycle, compared to cells that were cell cycle arrested (Figure 7). In addition, the bortezomib treatment induced cell cycle arrest was comparable to the cell cycle arrest after palbociclib treatment (Figure 7). Consequently, it is therefore unlikely that changes in cell cycle distribution are predominantly responsible for the palbociclib mediated antagonism on bortezomib induced cell death.

The efficacy of bortezomib treatment in melanoma, multiple myeloma and MCL has been demonstrated to be dependent on the induction of the pro-apoptotic protein NOXA (Qin et al., 2005; Pérez-Galán et al., 2006; Gomez-Bougie et al., 2007). How the aberrant cyclin D1/CDK4 activity in MCL affects bortezomib treatment efficacy and associated NOXA induction was unknown so far. Interestingly, inhibition of cyclin D1/CDK4 activity did not only antagonize bortezomib induced cell death (Figure 6) but also reversed the accumulation of NOXA protein in MCL cell lines (Figure 8). However, it is important to note that cell lines have often been in culture for decades and are therefore well adapted to the growth in a two-dimensional culture environment. As a result, cell lines usually greatly differ genetically and phenotypically from their origin tissue (Alge et al., 2006; Pan et al., 2009). The regulation pattern of NOXA protein expression after palbociclib and bortezomib treatment in MCL cell lines, however, could be

confirmed in primary MCL cells (Figure 9). After co-culture with CD40 ligand expressing fibroblasts, primary MCL cells did not undergo spontaneous apoptosis but acquired therapy resistance to bortezomib (Figure 9). The development of therapy resistance mechanisms after co-culturing primary MCL cells with stromal cells, have been demonstrated to be associated with the activation of survival pathways and the increased expression of anti-apoptotic Bcl-2 family proteins (Medina et al., 2012; Chiron et al., 2015). The palbociclib mediated antagonism on bortezomib induced cell death that was demonstrated in MCL cell lines (Figure 6), could therefore not be confirmed in primary MCL cells (Figure 9). However, other studies show that bortezomib treatment leads to efficient NOXA dependent cell death in MCL cell lines as well as primary patient samples (Pérez-Galán et al., 2006). In addition, bortezomib has already been successfully used in different clinical trials for the treatment of MCL patients. The observation that palbociclib treatment antagonized the bortezomib induced NOXA induction (Figure 8 and 9) therefore presumably still applies to cell death induction and the situation *in vivo*.

Cyclin D1 exerts its cell cycle regulatory function with its associated kinases CDK4 and CDK6. Several studies, however, demonstrate that cyclin D1 does also have various kinase independent functions (Neuman et al., 1997; McMahon et al., 1999; Fu et al., 2005b; Du et al., 2013; Casimiro et al., 2017). In addition, CDK4 can exert its function also through binding of cyclin D2 and cyclin D3 (Day et al., 2009). Another study even demonstrated, that in some situations CDK4 can function independently of a cyclin or of its kinase activity (Sun et al., 2013). Knockdown of cyclin D1 or CDK4 reversed bortezomib induced cell death and NOXA accumulation (Figure 10) in a similar manner as palbociclib treatment did (Figure 6 and 8). It can therefore be concluded that the function of palbociclib to inhibit bortezomib efficacy is mediated by cyclin D1 and cyclin D1/CDK4 activity. Knockdown of CDK4 had a stronger impact on bortezomib induced cell death than cyclin D1 knockdown did (Figure 10), which might be explained by the very different protein half-lives and protein turnover rates of cyclin D1 and CDK4 (Diehl et al., 1997; Lee and Kay, 2003). Double knockdown of cyclin D1 and CDK4 was as effective in antagonizing bortezomib efficacy as the single knockdown of CDK4 (Figure 10). This indicates that cyclin D1 and CDK4 both exert their antagonizing function through the cyclin D1/CDK4/6-RB1-E2F pathway.

The antagonizing effect of cyclin D1/CDK4 inhibition on bortezomib efficacy might also be mediated by regulating bortezomib specific resistance mechanisms such as regulating the expression of efflux transporters (Lü and Wang, 2013). Studies have shown that after bortezomib treatment intracellular bortezomib concentration is associated with the expression levels of transporter proteins such as OATP1B1 or P-glycoprotein. However, it was also demonstrated that changes in the expression levels of single transporters do not have a strong

effect on bortezomib efficacy. Considering that the different concentrations of intracellular bortezomib mostly still resulted in a saturated proteasome inhibition (Clemens et al., 2015). Inhibition of cyclin D1/CDK4 activity resulted in a similar antagonism on cell death and NOXA induction after treatment with the proteasome inhibitor carfilzomib or the FASN orlistat (Figure 11), compared to the bortezomib treatments (Figure 6 and 9). In this context, it seems unlikely that cyclin D1/CDK4 mediated changes in expression levels of cellular transporters, subsequently reduced the intracellular concentration and efficacy of all of the three aforementioned drugs. Bortezomib, in contrast to carfilzomib, reversibly blocks the chymotrypsin- and caspase-like activity of the proteasome. Whereas carfilzomib is more specific and irreversibly binds to the N-terminal threonine active sites of the proteasome (Demo et al., 2007; Livneh et al., 2016). As both drugs target the same proteasomal subunits, inhibition of cyclin D1/CDK4 activity could potentially affect proteasomal activity by regulating the expression and assembly as well as disassembly rate of certain proteasomal subunits (Meiners et al., 2003; Livnat-Levanon et al., 2014; Hirano et al., 2016). The efficacy of the FASN inhibitor orlistat to induce cell death and NOXA protein expression, however, was also impaired by inhibition of cyclin D1/CDK4 (Figure 11). The efficacy of orlistat to induce NOXA protein and cell death in MCL was shown to be mediated by completely abrogating NOXA ubiquitination and therefore interfering with targeting NOXA for proteasomal degradation (Dengler et al., 2014). It is therefore unlikely that regulation of proteasomal subunits mediates the antagonizing function of cyclin D1/CDK4 inhibition. Furthermore, palbociclib treatment antagonized hydrogen peroxide induced cell death (Figure 4). Hydrogen peroxide induced cell death has been demonstrated to be associated with increased NOXA protein levels and decreased proteasomal degradation (Aikawa et al., 2010). The mechanism of the cyclin D1/CDK4 mediated antagonism on cell death and NOXA protein induction might therefore also apply to hydrogen peroxide treatment. These collective observations suggest a general function of cyclin D1/CDK4 activity to regulate NOXA protein levels and NOXA dependent cell death, that is not exclusive for bortezomib treatment.

In line with published data, bortezomib induced cell death was confirmed to be NOXA dependent (Figure 12). More importantly, knockdown of the anti-apoptotic binding partner of NOXA, namely Mcl-1, abrogated the antagonizing effect of cyclin D1/CDK4 inhibition on bortezomib efficacy (Figure 12). These data confirm the specific role the pro-apoptotic NOXA in mediating the cyclin D1/CDK4 antagonizing function on bortezomib induced cell death. Because inhibition of cyclin D1/CDK4 activity did not completely abrogate the bortezomib induced expression of NOXA (Figure 8), knockdown of NOXA protein further increases the antagonizing effect of palbociclib treatment on bortezomib induced cell death (Figure 12).

On the other hand, knockdown of Mcl-1 abrogates the palbociclib mediated antagonism on bortezomib induced cell death (Figure 12). Again, presumably due to the incomplete reduction of NOXA protein levels after bortezomib treatment and cyclin D1/CDK4 inhibition (Figure 8). These data provide further evidence for a general cyclin D1/CDK4 function, that is not specific for the drug bortezomib but specific for the regulation of a NOXA dependent cell death.

Interestingly, by investigating data from the Cancer Cell Line Encyclopedia (Barretina et al., 2012), a study demonstrated that *NOXA* mRNA expression levels in MCL are among the highest compared to various tumor entities (Dengler et al., 2014). The high basal *NOXA* mRNA expression levels have been shown to be critical for the response of MCL cells to bortezomib treatment. Moreover, it was demonstrated that interfering with pathways that mediate these high *NOXA* transcript levels such as the PI3K/Akt/mTOR pathway, impaired the treatment efficacy of bortezomib (Dengler et al., 2014). Although it was suggested that cyclin D1 expression might mediate the high *NOXA* transcript levels in MCL via the same pathways as the PI3K/Akt/mTOR pathway did (Dengler et al., 2014), inhibition of cyclin D1/CDK4 activity after bortezomib treatment did not result in significantly downregulated *NOXA* transcript levels (Figure 13). In addition, a recent study even demonstrated that cyclin D1/CDK4 inhibition can increase Akt activity (Cretella et al., 2018). *NOXA* was initially described to be transcriptionally activated in response to cellular stress in a p53 dependent manner (Yakovlev et al., 2004). Palbociclib treatment, however, neither reduced *NOXA* transcript levels in the *p53*-wildtype MCL cell line Granta-519 (Amin et al., 2003) nor in the *p53*-mutated MCL cell line Mino (Figure 13). These data demonstrate that palbociclib treatment does not antagonize bortezomib efficacy by transcriptional regulation of high basal *NOXA* mRNA levels.

The expression of NOXA appears to be a critical determinant for the cellular response to anticancer treatment regimens such as γ-irradiation and the efficacy of chemotherapeutics (Ploner et al., 2008). However, in different tumor entities that are characterized by high *NOXA* levels, the efficacy of chemotherapeutics that are associated with a NOXA dependent cell death varies considerably. In this context, studies show that testicular germ cell tumors are very sensitive to cisplatin treatment (Gutekunst et al., 2013), whereas MCL cells show only transient responses to standard chemotherapy (Martin et al., 2017). Tumor cells seem to adapt to a constant apoptotic signal, mediated by high *NOXA* transcript levels, through reduction of NOXA protein levels. Of note, a study investigated the correlation between RNA and protein products of 1066 genes across 23 human cell lines and only one third of the protein products correlated with the corresponding RNA expression (Gry et al., 2009). One possible mechanism by which protein levels can be kept low is the control of translation efficiency. Indeed, translational control serves as a common mechanism in cancer cells by which oncogenic

pathways promote cellular transformation and tumor development (Ruggero, 2013). Furthermore, regulation of the degradation of tumor suppressor proteins such as REST, Merlin, p53, or PTEN in cancer cells was demonstrated to be responsible for increased malignancy and tumor progression (Trotman et al., 2007; Yang et al., 2009; Morrow et al., 2011; Karlin et al., 2014). Bortezomib efficacy in MCL has first been attributed to the transcriptional regulation of *NOXA* (Pérez-Galán et al., 2006). Another study, however, demonstrated that the predominant mechanism of bortezomib efficacy in MCL is targeting the short half-life of NOXA protein (Dengler et al., 2014). A similar mechanism of keeping NOXA protein expression low, by reducing the protein stability was also demonstrated in CLL (Baou et al., 2010). In this context, the possible role of an altered NOXA protein stability after cyclin D1/CDK4 inhibition and bortezomib treatment was investigated in the present study. In line with previous studies that suggested modulation of NOXA protein half-life as main determinant of bortezomib function, inhibition of cyclin D1/CDK4 abrogated the bortezomib induced increase of NOXA protein half-life (Figure 14). These results clearly demonstrate that cyclin D1/CDK4 activity therefore does not regulate NOXA protein expression by targeting *NOXA* mRNA transcript levels (Figure 13) but by inducing or enabling the degradation of NOXA protein.

4.2 Cyclin D1/CDK4 inhibition induces autophagosomal degradation of NOXA under UPS blockage

Inhibition of cyclin D1/CDK4 activity drastically reduced NOXA protein half-life in the presence of UPS blockage (Figure 14), which suggests a proteasome independent degradation of NOXA protein in MCL. Apart from the UPS, the autophagic degradation machinery represents the second most central degradation machinery in mammalian cells, delivering cytosolic proteins, aggregates, or organelles to lysosomes (Yu et al., 2017). This study for the first time demonstrated the induction of autophagic activity after inhibition of cyclin D1/CDK4 activity in MCL. These findings have great clinical implications for MCL patients who are treated with bortezomib. The results of this study provide novel combination therapy options for proteasome inhibitors in MCL but also suggest that special care must be taken when MCL patients are treated with drugs that are associated with NOXA dependent cell death. The strong association of cyclin D1/CDK4 activity and autophagic activity might also affect therapy options for other cancer types that are associated with high cyclin D1 expression or aberrant cyclin D1/CDK4 activity.

4.2.1 Cyclin D1/CDK4 regulates autophagy

Increased autophagic activity usually represents a cellular stress response to various signals such as nutrient starvation, hypoxia, ROS, pathogen infection, radiation, or anticancer drug treatment (Yang and Klionsky, 2010). Correct regulation of autophagic activity, however, is also important under non stressed conditions for the proper function of the intracellular quality control that involves the turnover of cytoplasmic components (Mizushima, 2009). Several groups have shown that cyclin D1/CDK4 activity negatively regulates autophagic activity in different cell types (Brown et al., 2012; Acevedo et al., 2016; Okada et al., 2017; Vijayaraghavan et al., 2017). In addition, the expression levels of several CDKIs such as p16, p19, p21, and p27 have been reported to induce autophagy (Liang et al., 2007; Capparelli et al., 2012). In line with these studies, inhibition of cyclin D1/CDK4 activity induced autophagic activity under stressed or non-stressed conditions in MCL (Figure 15). Interestingly, studies suggest a potential crosstalk between cell cycle and autophagy, as autophagy is preferentially induced in the G1 and S-phase. This preferential induction was demonstrated to be independent of the type of autophagic inducer or the autophagic cargo. Underlying molecular mechanisms, however, remain to be determined (Tasdemir et al., 2007). Furthermore, another study found a strong reduction of autophagic activity in animal cells undergoing mitosis, which could not be reversed by nutrient starvation (Eskelinen et al., 2002). The antagonizing effect of cyclin D1/CDK4 inhibition, however, was independent of changes in cell cycle distribution (Figure 7). Cell cycle associated changes in autophagic activity therefore do not seem to mediate the antagonizing effects on proteasome inhibitor treatment.

Regulation of autophagy initiation and autophagosome formation is mediated by the core machinery. Especially important for the regulation of autophagy initiation are ULK1, the class III PI3K Vps34, and ATG6/Beclin1 (Mizushima, 2010; Kimmelman, 2011). Autophagy induction as response to cyclin D1/CDK4 inhibition was demonstrated to be mediated by ROS induction in breast cancer cells (Vijayaraghavan et al., 2017). Moreover, studies show that ROS can increase autophagic activity by increasing the expression of Beclin-1 or by directly inactivating the cysteine protease ATG4 (Azad et al., 2009). Co-treatment of orlistat with an antioxidant, however, had only negligible effects on the cell death antagonism after cyclin D1/CDK4 inhibition (Figure 17). Akt activity on the other hand, has been demonstrated to negatively regulate autophagic activity by activation of mTOR, which subsequently inhibits the ULK1 kinase complex through an inhibitory phosphorylation (Degtyarev et al., 2008; Kim et al., 2011). Cyclin D1/CDK4 inhibition, however, was rather implicated with increased Akt activity (Cretella et al., 2018). In contrast, the energy sensor AMPK is a well described positive

regulator of the autophagic degradation machinery, which activates the ULK1 kinase complex through phosphorylation (Hardie, 2011; Kim et al., 2011). Furthermore, studies show that after proteasome inhibition, activation of AMPK and inhibition of mTOR signaling can mediate the activation of autophagy. In this context, these pathways have been demonstrated to function as a pro-survival mechanism in cancers (Brüning et al., 2013; Jaganathan et al., 2014). Palbociclib treatment of MCL cells, however, did not induce AMPK signaling nor did co-treatment of palbociclib with an AMPK inhibitor reverse the antagonizing effect of palbociclib on bortezomib induced cell death (Figure 17). Interestingly, the RB1-E2F1 pathway has been shown to suppress autophagic activity (Jiang et al., 2010). A recent study demonstrated that E2F1 inhibits autophagy by regulating the v-ATPase activity, endosomal trafficking, and mTORC1 activation (Meo-Evoli et al., 2015). This inhibitory function of E2F1 might be mediated by the upregulation of Bcl-2 (Gomez-Manzano et al., 2001), which has been shown to inhibit Beclin-1 and subsequently autophagic activity (Pattingre et al., 2005). Palbociclib treatment in MCL causes inhibition of the aberrant cyclin D1/CDK4 activity and subsequently leads to the downregulation of the downstream target E2F1 (Alinari et al., 2012). Conversely E2F1 has also been shown to upregulate the expression of LC3, ATG1, ATG5, and the damage regulated autophagy modulator DRAM (Polager et al., 2008). However, as E2F1 potentially transactivates thousands of genes (Rabinovich et al., 2008) the exact role of E2F1 in the regulation of autophagic activity must always be carefully evaluated in the specific context. The antagonizing effect of cyclin D1/CDK4 inhibition was the same in the presence or absence of siRNA targeting *RB1* (Figure 7). Given the conflicting role of E2F1 in the regulation of autophagy, changes in E2F1 expression after cyclin D1/CDK4 inhibition could still be responsible for mediating palbociclib induced upregulation of autophagic activity in MCL (Figure 15 and 16).

4.2.2 NOXA is targeted for autophagy

Studies show that the NOXA protein is degraded via the proteasome and thereby also functions as a sensor of 26S proteasome integrity (Baou et al., 2010; Craxton et al., 2012). In line with this, NOXA protein expression has been shown to mediate bortezomib induced cell death in MCL (Pérez-Galán et al., 2006). Results of the present study demonstrate that inhibition of cyclin D1/CDK4 activity in MCL antagonizes bortezomib induced cell death as well as NOXA half-life extension (Figure 6 and 14) and induces autophagic activity (Figure 15 and 16). In line with the previous results that suggested UPS independent degradation of NOXA, genetic or pharmacological inhibition of autophagy restored bortezomib induced cell death and NOXA protein induction after cyclin D1/CDK4 inhibition (Figure 18). Moreover, autophagy inhibition

prolonged NOXA protein half-life after co-treatment with bortezomib and palbociclib (Figure 19). These results demonstrate that in addition to the proteasomal degradation, NOXA can also be degraded via the ALP, which is mediated by changes in cyclin D1/CDK4 activity. Targeting a protein, damaged organelle, or aggregate for selective autophagy can be carried out in various manners. Cargo, which is selectively degraded by the CMA pathway requires a peptide motif biochemically related to KFERQ (Kaushik et al., 2011). The NOXA amino acid sequence, however, does not contain similar motifs (Figure 20). Moreover, the observed increase of the autophagy marker LC3-II (Figure 16) is not associated with elevated CMA activity, as degradation through CMA does not require the formation of autophagosomes for the import of cargo into lysosomes (Klionsky et al., 2021). It is therefore unlikely that NOXA is target for CMA. For proteasomal targeting, NOXA contains six lysine sites and is substrate for polyubiquitination with polyubiquitin chains of the K11 or K48 linkage (Craxton et al., 2012; Brinkmann et al., 2013; Zhou et al., 2017). Which E3 ligase is responsible for NOXA ubiquitination is not definitely clarified yet. Recent reports, however, showed that the Cullin-Ring-ligase-5 E3 ligase is capable to ubiquitinate NOXA by Lysine-11 in lung cancer patients (Zhou et al., 2017). Moreover, the E3-ligase MARCH5 was demonstrated to regulate the degradation of NOXA/Mcl-1 protein complexes and to ubiquitinate both Mcl-1 and NOXA (Haschka et al., 2020). On the other hand, the action of a de-ubiquitinating enzyme called UCH-L1 counteracts the proteasomal degradation of NOXA, by removing polyubiquitin chains from NOXA (Brinkmann et al., 2013). Apart from the role in the UPS, ubiquitination is also involved in the autophagic degradation machinery. Various ATG proteins are subject to ubiquitination in order to regulate their ability to interact with autophagy regulating proteins (Xie et al., 2015). In addition, the action of E3 ligases also control autophagy induction, nucleation, maturation, or termination through ubiquitination of autophagy regulators (Kuang et al., 2013). Furthermore, ubiquitin can not only target proteins for proteasomal degradation but also for the so called selective autophagy (Kocaturk and Gozuacik, 2018). Autophagy adaptor proteins such as SQSTM1, NBR1, OPTN, NDP52, and c-Cbl represent a major component of selective autophagy and are indispensable for basal autophagy. Ubiquitinated or aggregated proteins are recognized by adaptor proteins, which in turn subsequently get bound by proteins of the ATG8-family, to bring ubiquitinated protein aggregates to the emerging autophagosome (Shaid et al., 2013; Rogov et al., 2014). The ATG8 family proteins such as LC3, are conjugated to the inner membrane of the phagophore and recognize adaptor proteins by a LIR motif (Birgisdottir et al., 2013). Interestingly, a potential LIR motif was identified in the amino acid sequence of the NOXA protein (Figure 20), which might therefore target the NOXA protein for selective autophagy after cyclin D1/CDK4 inhibition under UPS blocked

conditions. Although LIR motifs are usually associated with the recognition of autophagic adaptor proteins and not the cargo/substrate itself, direct targeting of LIR motif containing substrates for autophagosomal degradation has already been demonstrated (Petherick et al., 2013). The only reported polyubiquitin chains for NOXA are K11, K48 (Brinkmann et al., 2013; Zhou et al., 2017), or K68 linkages (Albert et al., 2020). Although K63 linkages have been shown to preferentially direct proteins towards autophagy instead of UPS, all ubiquitin linkages including K11 and K48 have also been shown to be involved in targeting proteins for autophagosomal degradation (Tan et al., 2008; Riley et al., 2010). Of note, a recent study demonstrated that in the presence of genotoxic stress, the carboxyl-terminus of Hsc70-interacting protein (CHIP) preferentially ubiquitylates NOXA via K63-linkages in HeLa cells (Albert et al., 2020). More importantly, Albert et al. showed that K63 linked ubiquitylation targets NOXA for lysosomal degradation. Even though Albert et al. did not investigate autophagic activity or degradation mechanisms, such a selective lysosomal degradation of NOXA is likely mediated by selective autophagy. In addition, a recent report demonstrated that NOXA can be bound by SQSTM1 and subsequently be degraded by autophagy (Wang et al., 2018), corroborating the results of the present study.

Interestingly, ubiquitin independent proteasomal degradation of NOXA has also been reported (Craxton et al., 2012; Pang et al., 2014). In addition, an ubiquitin independent form of selective autophagy involves the selective autophagy receptor Nix, which is responsible for the removal of damaged mitochondria (so called mitophagy) (Novak et al., 2010). In this context, NOXA degradation after cyclin D1/CDK4 inhibition under UPS blocked conditions might also be mediated by the specific elimination of mitochondria by mitophagy. In line with this, different studies demonstrated that proteasome inhibitor treatment is associated with mitochondrial damage, likely mediated by oxidative stress (Durrant et al., 2004; Maharjan et al., 2014). Moreover, during mitochondrial apoptosis NOXA has been shown to localize to mitochondria and to be subsequently inserted in the outer mitochondrial membrane (Wilfling et al., 2012). Importantly, the proteins ATG5 and ATG7 have been demonstrated to only have a marginal role in the regulation of mitophagic activity (Hirota et al., 2015). In addition, knockdown of ATG5/7 reversed the palbociclib mediated effects on bortezomib efficacy in a NOXA dependent manner (Figure 18 and 19). Therefore, it can be concluded that mitophagy is presumably not the determining mechanism by which MCL cells regulate NOXA protein half-life after cyclin D1/CDK4 inhibition. Interestingly, inhibition of autophagosomal degradation at early stages using 3MA, spautin-1 or siRNA targeting the key autophagy regulating genes *ATG5/7* (Arakawa et al., 2017) antagonized the effect of cyclin D1/CDK4 inhibition in the same manner as inhibition at later stages of autophagy using liensinine did

(Figure 18). These results imply that targeting NOXA for autophagosomal degradation can be reversed and that NOXA still exerts its pro-apoptotic function after autophagy inhibition. How NOXA escapes the autophagosome after late step autophagy inhibition with liensinine (Figure 18), which blocks the fusion of the autophagosome with the lysosome (Zhou et al., 2015) remains to be determined. Moreover, it is unclear how NOXA is still able to induce mitochondrial apoptosis after potentially being targeted by mitophagy, as whole mitochondria are engulfed in the autophagosome in this scenario. Interestingly, recent studies demonstrated how bacterial pathogens that were targeted for selective autophagy can escape from an autophagosome (Siqueira et al., 2018). It remains to be determined, if the suggested mechanisms could also apply for protein cargo.

4.3 UPS and ALP interplay and modulation

The vast majority of cellular proteins are degraded by the activity of the UPS (Rock et al., 1994). In cells that are not subjected to stress conditions proteasomal degradation is predominant. The relative contribution of autophagy to the overall cellular protein degradation, however, may vary considerably depending on the cellular context. In muscle cells, for example, autophagic activity accounts for the degradation of up to 40 % of proteins (Zhao et al., 2007). Previous studies suggested that short-lived proteins are preferentially degraded by the UPS, whereas autophagy is responsible for the degradation of long-lived, large, and heterogeneous cytoplasmic materials such as protein aggregates, organelles, or intracellular pathogens (Nedelsky et al., 2008; Kohli and Roth, 2010; Boya et al., 2013). Other reports, however, established that ubiquitination represents a signal for proteasomal as well as for autophagosomal degradation and that there is crosstalk between these two degradation systems (Shaid et al., 2013). Furthermore, activation of a so called compensatory autophagy, can be a rescue mechanism to overcome cytotoxic effects of accumulated proteins after a reduction in proteasomal activity (Shen et al., 2013). When the degradative capacity of the proteasome is exceeded misfolded proteins aggregate, form a structures called aggresomes, which are subsequently degraded by selective autophagy (Johnston et al., 1998; Lamark and Johansen, 2012). In line with this, inhibition of the UPS with bortezomib has been shown to regulate the autophagic degradation system in various, partly contradictory manners. Treatment with bortezomib has been demonstrated to induce autophagic activity in head and neck squamous-cell carcinoma, prostate cancer, melanoma, and endothelial cells (Belloni et al., 2010; Zhu et al., 2010; Li and Johnson, 2012; Selimovic et al., 2013). Moreover, autophagy activation or aggresome formation after UPS inhibition was demonstrated to be mediated by ER stress, the unfolded protein response pathway, and JNK signaling (Hideshima et al., 2005;

Ding et al., 2007b). On the other hand, studies have shown that UPS inhibition after bortezomib treatment leads to a blockade of the autophagic degradation machinery in several solid tumors (Kao et al., 2014). As previously shown, bortezomib treatment led to an increase in autophagic vacuoles in MCL cell line Mino (Figure 15). This increase in autophagic vacuoles could have been caused by a blockade of autophagosomal degradation. In line with this, bortezomib co-treatment with hydroxychloroquine did not result in an increased expression of the autophagy marker LC3-II, compared to hydroxychloroquine treatment alone (Figure 16A). In this context, a potential blockade of autophagosomal degradation after UPS inhibition would potentiate the bortezomib induced cell death, which would be in line with the higher bortezomib susceptibility of MCL cells. On the other hand, as autophagy inhibitors potentiated bortezomib induced cell death (Figure 21), bortezomib induced increase in autophagic vacuoles could also be mediated by the activation of a compensatory autophagic activity.

The role of autophagy in cancer is particularly controversial, as studies demonstrate that autophagy can function as pro-survival or pro-apoptotic pathway and therefore differently influences therapy outcome (Hollomon et al., 2013; O'Farrill and Gordon, 2014). In addition, targeting autophagic activity in cancer cells can impair immunogenic signaling after chemotherapy or radiation therapy and might therefore negatively affect therapy outcome (Michaud et al., 2011; Ko et al., 2014). This conflicting role of autophagy has also been observed for bortezomib induced cell death in different cell types (Granato et al., 2013; Zhuang et al., 2016). Autophagy induction after cyclin D1/CDK4 inhibition in MCL, however, clearly represented a pro-survival mechanism after proteasome inhibition (Figure 18). Preclinical data from multiple myeloma cell lines indicate that the balance between proteasome workload and degradative capacity determines the efficacy of proteasome inhibitor treatment (Bianchi et al., 2009). In this context, the induction of autophagic activity after proteasome inhibition was shown to alleviate ER stress and subsequently counteract cell death induction (Ding et al., 2007b). Furthermore, in a phase 1 clinical trial, patients with relapsed/refractory myeloma could benefit from the combination of bortezomib with the autophagy inhibitor hydroxychloroquine (Vogl et al., 2014). In addition, dual targeting of both main cellular degradation systems with bortezomib and autophagy inhibitors, resulted in synergistic cell death induction in hepatocellular carcinoma (Hui et al., 2012). In line with these studies, dual targeting of the autophagosomal and proteasomal degradation pathways by combining bortezomib with different autophagy inhibitors resulted in synergistic cell death and NOXA induction in MCL (Figure 21).

Importantly, autophagy inhibition was able to potentiate bortezomib induced cell death in cell lines with reduced sensitivity to bortezomib. Autophagy inhibitors therefore might help

overcoming bortezomib resistances in MCL and subsequently increase the number of eligible patients receiving bortezomib treatment. Elevated autophagic activity has already been associated with bortezomib resistance in cancer (Chen et al., 2016b; Xia et al., 2020). In the context of other bortezomib resistance mechanisms in MCL, the potential role of autophagy, however, is more controversial. Overexpression of Bcl-2 and elevated NF-κB activity have been demonstrated to increase bortezomib resistance in MCL cells (Yang et al., 2008; Smith et al., 2011) but have also been associated with Beclin-1 dependent downregulation of autophagic activity (Xu et al., 2013; Verzella et al., 2020). It is therefore rather unlikely that these bortezomib resistance mechanisms in MCL are associated with the regulation of autophagic activity, as impaired autophagy potentiated bortezomib induced cell death in MCL (Figure 21). Furthermore, combining of UPS and ALP inhibition further prolonged NOXA protein half-life compared to UPS inhibition alone (Figure 22), confirming the dual targeting of NOXA protein for both cellular degradation systems. Interestingly, inhibition of autophagy alone with 3MA had no effect on NOXA half-life (Figure 22), indicating that in the presence of aberrant cyclin D1/CDK4 activity the ALP only has a minor role for NOXA degradation in cells with active UPS. However, autophagy inhibition in combination with UPS inhibition induced NOXA dependent cell death in MCL (Figure 21), even in the presence of aberrant cyclin D1/CDK4 activity. Therefore, residual autophagic activity in MCL already seems to be partially responsible for NOXA protein degradation under UPS blocked conditions. In addition, as discussed earlier, bortezomib treatment might induce autophagic activity (Figure 15B), which would enable the compensatory autophagosomal degradation of NOXA. The histone deacetylase 6 (HDAC6) was implicated in mediating compensatory autophagy after UPS impairment by targeting substrates for autophagosomal degradation (Pandey et al., 2007). HDAC6 thereby functions as a selective autophagy receptor that is able to target substrates in an ubiquitin dependent as well as independent manner (Kaliszczak et al., 2018). In line with this, inhibition of HDAC6 with the histone deacetylase inhibitor panobinostat abrogated bortezomib induced aggresome formation in MCL. Furthermore, combined inhibition of aggresome formation and proteasomal degradation resulted in synergistic cell death in MCL cell lines (Rao et al., 2010), corroborating the results of this study (Figure 21 and 26).

Various studies show that many chemotherapeutics and novel targeted therapies, that are currently used for the treatment of MCL patients or that are under clinical investigation, have been associated with the regulation of autophagic activity (Tettamanti et al., 2006; Groth-Pedersen et al., 2007; Bellodi et al., 2009; Xie et al., 2011; Luo et al., 2012; Liu et al., 2014; Park et al., 2016; Jiang et al., 2017; Wang et al., 2017; Peng et al., 2018). Moreover, many chemotherapeutics have been reported to cause oxidative stress in cancer cells

(Yang et al., 2018), which is a known trigger of autophagic activity (Filomeni et al., 2015). In addition, the DDR as a common result of different chemotherapy treatments, is also implicated in autophagy regulation (Eliopoulos et al., 2016). In recent trials, different conventional drugs were re-positioned to target autophagy and subsequently improve clinical outcomes of chemotherapeutics (Hsieh et al., 2016; Yoshida, 2017; Wong et al., 2018). Accordingly, in the present study two candidate drugs, orlistat and MLN4924, were initially identified to potentiate bortezomib efficacy (Figure 23). Subsequently, these candidate drugs were investigated for their potential to regulate autophagic activity (Figure 24). Combination of bortezomib with MLN4924 resulted in increased cell death induction compared to bortezomib treatment alone (Figure 23). This cell death synergism, however, was not attributed to autophagy regulation (Figure 24). MLN4924 was shown to induce NOXA dependent cell death and cause downregulation of Mcl-1 in MCL (Dengler et al., 2014), which might explain synergistic cell death after bortezomib co-treatment (Figure 23). The re-positioning approach, however, could be successfully demonstrated for the FASN inhibitor orlistat. The combination treatment with bortezomib and orlistat resulted in efficient blockade of autophagic activity (Figure 24). In line with this, autophagy induction after inhibition of Akt/mTOR activity with BEZ235 antagonized cell death after combined inhibition of the UPS and the ALP (Figure 24). Autophagy markers LC3-II and SQSTM1, however, did not clearly demonstrate autophagy activation after co-treatment of bortezomib and orlistat with the known autophagy inducer BEZ235. After BEZ235 co-treatment, SQSTM1 levels decreased which indicates increased autophagic activity (Bjørkøy et al., 2009). LC3-II expression levels, however, decreased as well (Figure 24), which conversely is associated with decreased autophagy (Klionsky et al., 2021). Temporal effects of LC3-II turnover during autophagy might account for these conflicting observations. After conjugation of phosphatidylethanolamine to LC3-I, LC3-II is present on the inner and outer autophagosomal membranes. LC3-II on the inner autophagosomal membranes is degraded inside autolysosomes, whereas LC3-II on the outer membrane is deconjugated and returned to the cytosol (Mizushima and Yoshimori, 2014). High levels of autophagic activity after BEZ235 treatment, could have led to a high turnover of the LC3-II protein. Investigating different time points and using autophagy degradation inhibitors, is necessary to precisely interpret LC3-II expression and associated autophagic activity (Yoshii and Mizushima, 2017).

In line with the results obtained with known autophagy inhibitors (Figure 21 and 22), dual blockade of the UPS and the ALP after combined treatment with bortezomib and orlistat increased NOXA half-life and cell death induction, compared to bortezomib treatment alone (Figure 25 and 26). In contrast to 3MA treatment (Figure 22), Orlistat single treatment also prolonged NOXA half-life which, however, is rather attributed to the previously described

function of the FASN inhibitor to also inhibit the proteasomal degradation of the NOXA protein (Dengler et al., 2014). Orlistat inhibits the thioesterase domain of FASN (Kridel et al., 2004) and thereby blocks the synthesis of palmitate (Wakil, 1989). Accordingly, cell death induction after orlistat treatment in MCL has been demonstrated to be dependent on palmitate levels (Dengler et al., 2014). Interestingly, the expression levels of palmitate have been demonstrated to affect autophagic activity (Chen et al., 2013; Liu et al., 2015; Park et al., 2015). In addition, recent studies also demonstrated that lipid droplets and their main components triglycerides are associated with autophagy regulation (Velázquez et al., 2016). In line with this and the results obtained with orlistat (Figure 24), another FASN inhibitor, cerulenin, has been shown to downregulate lipid droplets and subsequently interfere with the autophagosomal degradation machinery (Shpilka et al., 2015).

Different tissues rely differently on autophagic activity for maintaining proper cellular function (Amaravadi et al., 2016). Study data, however, indicate that tumors are more autophagy-dependent than most normal tissues, which allows for a therapeutic window when using autophagy inhibitors in cancer therapy (Karsli-Uzunbas et al., 2014). In addition, MCL cells are susceptible to treatment options that rely on NOXA protein stabilization, as MCL cells exhibit high NOXA transcript levels and a high NOXA protein turnover rate (Dengler et al., 2014). In line with this, normal blood cells from healthy donors were unaffected by combined treatment with bortezomib and orlistat, whereas combination therapy in MCL cell lines induced apoptosis in almost all cells (Figure 26). In addition, the efficacy of combined UPS and ALP inhibition by bortezomib and orlistat co-treatment was dependent on cyclin D1/CDK4 activity and NOXA expression (Figure 27). The inhibitory effect on autophagy mediated by the aberrant high cyclin D1/CDK4 activity in MCL therefore represents a specific susceptibility to combined inhibition of the UPS and autophagy and NOXA dependent cell death.

Apart from NOXA, other pro-apoptotic proteins such as the Bcl-2 protein Puma or the effector proteins Bax/Bak have been demonstrated to regulate the efficacy of bortezomib to induce cancer cell death (Ding et al., 2007a; Busacca et al., 2013; Su et al., 2017). Furthermore, increased Puma expression after orlistat treatment has been implicated with apoptosis regulation (Kant et al., 2012). The efficacy of the combinatory treatment of bortezomib and orlistat in MCL, however, appears to be primarily mediated by the NOXA protein and not by Bax/Bak, Puma, or its anti-apoptotic counterpart Mcl-1 (Figure 28). Activation of caspases is a crucial event for the execution of mitochondrial apoptosis and other forms of programmed cell death. Caspase independent cell death mechanisms, however, have also been described such as Type II autophagic cell death or caspase independent apoptosis as a result of extensive DNA damage (Kim et al., 2005; Kroemer and Martin, 2005; Green and Llambi, 2015).

Treatment efficacy of the bortezomib and orlistat co-treatment in MCL, however, was dependent on caspase activity, as caspase inhibition antagonized cell death induction (Figure 29). Caspase independent forms of apoptosis are generally not associated with phosphatidylserine externalization (Tait and Green, 2008). Cell death after combined treatment of bortezomib and orlistat, however, was analyzed by Annexin V staining of phosphatidylserine, corroborating the results suggesting a caspase dependent cell death (Figure 29). In summary, aberrant cyclin D1/CDK4 activity renders MCL cells susceptible to NOXA dependent cell death after UPS inhibition by exerting an inhibitory effect on the alternative degradation through the ALP. On the other hand, targeting both degradative pathways leads to very efficient NOXA protein accumulation and specific cell death in MCL.

4.4 Conclusion and outlook

The present study identified novel functions of the aberrant cyclin D1/CDK4 activity in MCL that might help to improve therapy outcome in patients. New molecular targeted therapies such as the proteasome inhibitor bortezomib or the kinase inhibitor palbociclib have shown promising results in preclinical studies and MCL patients (Leonard et al., 2012; Robak et al., 2018). In the present study, the hallmark feature of MCL, cyclin D1 overexpression, was identified to represent a prerequisite for the efficacy of proteasome inhibitors such as bortezomib. Moreover, targeting cyclin D1/CDK4 activity in MCL by RNAi-mediated silencing of cyclin D1 or treatment with the CDK4 inhibitor palbociclib, was demonstrated to lead to the upregulation of the antioxidant defense protein sestrin 3. Interestingly, even though it was shown that ROS induced cell death was abrogated by inhibition of cyclin D1/CDK4 activity and bortezomib induced cell death in MCL could be regulated by ROS modulators, the expression levels of sestrin 3 protein did not correlate with bortezomib induced cell death. Targeting sestrin 3 regulation in MCL, however, might be interesting for standard R-CHOP therapy or other novel targeted therapies and therefore warrants further investigation.

Previous research revealed stabilization of the short-lived pro-apoptotic NOXA as a critical determinant for the sensitivity of MCL cells to proteasome inhibitors (Dengler et al., 2014). In this study, inhibition of cyclin D1/CDK4 activity was demonstrated to significantly reduce proteasome inhibitor mediated stabilization of NOXA protein and associated NOXA dependent cell death in MCL. Furthermore, it was shown that the underlying molecular mechanism is a mitigated autophagy due to high cyclin D1/CDK4 activity, offering novel treatment strategies in MCL and bortezomib resistant cells. Consequently, results of the present study might also help to develop novel therapy options in other cancers with cyclin D1 overexpression and cell cycle deregulation (Vermeulen et al., 2003; Musgrove et al., 2011).

When MCL cells are exposed to bortezomib, NOXA can only be efficiently induced if the aberrant cyclin D1/CDK4 activity impairs the autophagic degradation machinery and thereby prevents the concomitant degradation of NOXA via the ALP. Consequently, aberrant cyclin D1/CDK4 activity allows for the very efficient induction of NOXA protein in the presence of UPS inhibition in MCL cells. In line with this, in multiple myeloma, cyclin D1 overexpression is also frequently observed and the disease is responding well to proteasomal inhibition (Specht et al., 2004; Rajkumar and Kumar, 2016). Further experiments, however, are needed to clarify how cyclin D1/CDK4 activity regulates autophagy. Combination therapy with palbociclib and bortezomib in MCL patients did not result in a clinical outcome supporting this therapy regimen, compared to other available treatment options (Martin et al., 2019b). Targeting the intermediate pathways that connect cyclin D1/CDK4 and autophagy might help to make the combination of palbociclib and bortezomib a more viable therapy option in MCL. Identifying these intermediate pathways could also help to better identify patients that are eligible for combined proteasome and CDK4/6 inhibitor treatment.

The data of the present study demonstrate how the novel function of cyclin D1/CDK4 activity to regulate autophagic activity has great clinical implications for the treatment of MCL patients. The efficacy of drugs that depend on NOXA stabilization for efficient cell death induction such as UPS inhibitors, can be impaired by elevated autophagic activity. Therefore, particular care must be taken when MCL patients are treated with a proteasome inhibitor in combination with other chemotherapeutics or drugs. Co-treatment with other drugs could lead to a prosurvival autophagy induction due to nutrient starvation, inhibition of the PI3K/Akt/mTOR pathway, activation of the AMPK signaling pathway, ROS, or ER stress induction, among other things (Das et al., 2018). Furthermore, co-treatment regimes could impair cyclin D1/CDK4 activity, which again could potentially induce prosurvival autophagy upon proteasome inhibition in MCL. In this context, drugs could either directly interfere with pathways regulating cyclin D1 or CDK4 activity or expression or regulate the expression of CDKIs.

On the other hand, combined treatment with agents that inhibit autophagy or lysosomal degradation could potentiate the efficacy of proteasome inhibitors such as bortezomib in MCL patients. Consequently, in the present study combination treatments potentiated induction of NOXA protein and NOXA dependent cell death. Moreover, various clinical trials are investigating the potential of combining autophagy inhibition with chemotherapy in different tumor entities (Levy et al., 2017). How different therapy options for the treatment of MCL patients could benefit from autophagy inhibition, however, needs to be determined for each drug individually. Of note, there is also increasing data highlighting the importance of T-cells for disease outcome in MCL (Nygren et al., 2014; Montico et al., 2017). Targeting autophagy

in MCL might therefore elicit an unfavorable immunogenic tumor response as autophagic activity has been shown to regulate T-cell function (Merkley et al., 2018).

The present study has shown that in MCL cells with active UPS, the ALP only has a minor role for the degradation of NOXA protein. In cells with active UPS, specific targeting of the NOXA protein for the ALP and subsequently blocking autophagosomal degradation could represent an UPS independent mechanism for stabilizing NOXA protein. This ALP mediated NOXA protein stabilization could therefore potentially address bortezomib resistances in MCL. Elucidating the molecular basis for targeting NOXA protein for ALP dependent degradation, could help to make autophagy inhibitors a novel targeted treatment option in MCL. If autophagy inhibition by the aberrant cyclin D1/CDK4 activity, however, might prevent such treatment options in all MCL patients, needs to be further investigated. Nevertheless, combination of bortezomib with autophagy inhibitors did potentiate cell death induction in the present study. ALP dependent degradation of NOXA protein under UPS blocked conditions therefore is still present in cells with aberrant cyclin D1/CDK4 activity. Addressing NOXA targeting for the ALP by specific regulation or inhibition of NOXA specific ubiquitin ligases such as CHIP (Albert et al., 2020) could represent yet another therapy approach, next to direct autophagy inhibition. In addition, the finding that NOXA can be targeted for autophagosomal degradation, can help to improve the efficacy of various drugs that rely on efficient NOXA protein accumulation for cell death induction. In addition to established autophagy modulators, a FASN inhibitor, namely orlistat was identified to efficiently inhibit autophagic activity in combination with bortezomib. As the present study demonstrated with orlistat, it is a highly interesting approach to investigate established compounds for their potential to regulate autophagy. The approach of re-positioning conventional drugs to target autophagy and to induce cancer cell death was already shown for several therapeutics (Yoshida, 2017). This re-positioning approach could lead to the rediscovery of established drugs that so far were not effective in MCL patients. Common triggers of autophagy such as oxidative stress or DNA damage are often a result of conventional chemotherapy (Conklin, 2000; Eliopoulos et al., 2016). Therefore, it will also be highly interesting to investigate if MCL patients receiving standard therapy regimens like R-CHOP could benefit from autophagy inhibitors. In summary, this study not only provides further insight into the molecular mechanisms underlying the enhanced sensitivity of MCL cells to proteasome inhibitors but also suggests novel therapeutic options for improved MCL treatment. These therapy options might also improve treatment in other neoplasms and might help to overcome chemoresistances in tumors.

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7 Publications

This work was published in part in the following publications:

Original articles

Heine, Simon; Kleih, Markus; Giménez, Neus; Böpple, Kathrin; Ott, German; Colomer, Dolors; Aulitzky, Walter E; van der Kuip, Heiko; Silkenstedt, Elisabeth. (2018): Cyclin D1-CDK4 activity drives sensitivity to bortezomib in mantle cell lymphoma by blocking autophagy-mediated proteolysis of NOXA. In: Journal of hematology & oncology 11 (1), S. 112. DOI: 10.1186/s13045-018-0657-6.

Meeting abstracts

Heine, Simon; Silkenstedt, Elisabeth; Dengler, Michael; Kleih, Markus, Ott, German; Giménez, Neus; Colomer, Dolors; van der Kuip, Heiko; Aulitzky, Walter E (2017). Abstract. Cyclin D1 Driven CDK4 Activity Is Responsible for Hypersensitivity to NOXA Inducing Combination Treatment in Mantle Cell Lymphoma (MCL) Cells. In: Blood (ASH Annual Meeting Abstracts) 130 (1 Supplement), S. 2811

Heine, Simon; Silkenstedt, Elisabeth; Kleih, Markus, Dong, Meng; Aulitzky, Walter E; van der Kuip, Heiko (2017). Poster 267. Hemmung von CDK4-Cyclin D1 antagonisiert den bortezomib induzierten Zelltod in Mantelzelllymphom-Zellen durch Aufhebung der NOXA-Proteinstabilisierung. Auf: Jahrestagung der Deutschen, Österreichischen und Schweizerischen Gesellschaften für Hämatologie und Medizinische Onkologie

Other original articles

Nellinger, Svenja; Schmidt, Isabelle; **Heine, Simon**; Volz, Ann-Cathrin; Kluger, Petra J. (2020): Adipose stem cell-derived extracellular matrix represents a promising biomaterial by inducing spontaneous formation of prevascular-like structures by mvECs. In: Biotechnology and bioengineering 117 (10), S. 3160–3172. DOI: 10.1002/bit.27481.

Kleih, Markus; Böpple, Kathrin; Dong, Meng; Gaißler, Andrea; **Heine, Simon**; Olayioye, Monilola; Aulitzky, Walter E; Essman, Frank (2019): Direct impact of cisplatin on mitochondria induces ROS production that dictates cell fate of ovarian cancer cells. In: Cell death & disease 10 (11), S. 851. DOI: 10.1038/s41419-019-2081-4.

Höring, Elisabeth; Montraveta, Arnau; **Heine, Simon**; Kleih, Markus; Schaaf, Lea; Vöhringer, Matthias C; Esteve-Arenys, Anna; Roué, Gael; Colomer, Dolors; Campo, Elias; Ott, German; Aulitzky, Walter E; van der Kuip, Heiko (2017): Dual targeting of MCL1 and NOXA as effective strategy for treatment of mantle cell lymphoma. In: British journal of haematology 177 (4), S. 557–561. DOI: 10.1111/bjh.14571.

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Other meeting abstracts

Kleih, Markus; **Heine, Simon**; Böpple, Kathrin; Dong, Meng; van der Kuip, Heiko; Aulitzky, Walter E. (2018): Abstract 3522. Both mitochondrial function and composition of BCL2 family proteins determines sensitivity to Cisplatin in ovarian cancer cells and are promising targets to overcome Cisplatin resistance in ovarian cancer. In: Cancer research 78 (13 Supplement), S. 3522. DOI: 10.1158/1538-7445.AM2018-3522.

Kleih, Markus; **Heine, Simon**; Dengler, Michael; Schaaf, Lea; Hoering, Elisabeth; Horn, Heike; Ott, German; Aulitzky, Walter E; van der Kuip, Heiko (2016): Abstract 3732. Combined targeting of NOXA and GSTpi effectively kills mantle cell lymphoma cells. In: Cancer research 76 (14 Supplement), S. 3732. DOI: 10.1158/1538-7445.AM2016-3732.

8 Curriculum vitae

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Dr. Angelika Hausser
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Dußlingen, May 5, 2022