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INHIBITION OF 19S PROTEASOME DEUBIQUITINASES AS A PROMISING STRATEGY IN CANCER THERAPY

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Inhibition of 19S proteasome deubiquitinases as a promising strategy in cancer therapy

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To my mom

ABSTRACT

The ubiquitin proteasome system (UPS) is the main degradation system in the eukaryotic cell and it is involved in the regulation of many crucial cellular pathways; such as signal transduction, proliferation, DNA repair, cell death, and cell cycle regulation. In comparison to cells in healthy tissues, cancer cells proliferate faster and have increased protein synthesis rate; therefore in order to preserve cellular fitness they are more dependent on UPS than normal cells. This therapeutic window has been investigated for more than 30 years and intensive studies led to the discovery of first and second generation 20S proteasome inhibitors that have been successfully applied in the treatment of multiple myeloma and mantle cell lymphoma. However; in spite of initial positive outcomes, patients eventually gain resistance and suffer from the disease relapse. Therefore there is a strong interest in the development of new drugs targeting the UPS through alternative mechanisms.

b-AP15/VLX1570 is a novel inhibitor of proteasome, recently discovered in our group, which does not block the activity of 20S catalytic core, but instead it inhibits two 19S deubiquitinating enzymes, USP14 and UCHL5. It is a small molecule with α,β-unsaturated carbonyl units that binds reversibly to cysteine deubiquitinases via Michael reaction. Despite reversible binding of the drug, commitment to death induced by b-AP15/VLX1570 is irreversible, which is due to the rapid uptake of the drug and subsequent retention in the cellular compartment. To some extent cellular response to the treatment with b-AP15/VLX1570 and bortezomib are similar: it results in the accumulation of polyubiquitinated proteins, induction of ER stress and eventually apoptosis and cell death. Treatment with b-AP15/VLX1570 however, induces higher level of proteotoxic stress, higher level of ROS and more rapid and potent apoptotic response. We found that VLX1570, a b-AP15 analogue optimized for clinical administration, has strong anti-cancer activity against multiple myeloma and acute lymphoblastic leukemia. In addition we found that the DUB inhibitor is active on cells resistant to bortezomib. This findings suggest that VLX1570 is a promising candidate drug and is currently investigated in Phase I clinical trials for the treatment of multiple myeloma resistant to conventional proteasome inhibitors.

Application of proteasome inhibitors in the treatment of solid tumors remains limited and primary and acquired resistance continues to be a concern during the development of new therapies. Using multicellular spheroids as solid tumor model, we discovered that overexpression of PA28, a stress-induced regulatory particle of proteasome, can moderate the response of cancer cells to proteasome inhibitors.

Taken together our data shows that proteasome DUB inhibitors have potential as a treatment option for malignancies and that differences in UPS expression may have relevance for the survival of different tumor types.

LIST OF SCIENTIFIC PAPERS

I. Brnjic S, **Mazurkiewicz M**, Fryknäs M, Sun C, Zhang X, Larsson R, D'Arcy P,Linder S. Induction of tumor cell apoptosis by a proteasome deubiquitinase inhibitor is associated with oxidative stress. Antioxid Redox Signal. 2014 Dec10; 21(17): 2271-85

II. Wang X, Stafford W, **Mazurkiewicz M**, Fryknäs M, Brjnic S, Zhang X, Gullbo J, Larsson R, Arnér ES, D'Arcy P, Linder S. The 19S Deubiquitinase inhibitor b-AP15 is enriched in cells and elicits rapid commitment to cell death. Mol Pharmacol. 2014 Jun; 85(6): 932-45

III. Wang X, **Mazurkiewicz M**, Hillert EK, Olofsson MH, Pierrou S, Hillertz P, Gullbo J, Selvaraju K, Paulus A, Akhtar S, Bossler F, Khan AC, Linder S, D'Arcy P. The proteasome deubiquitinase inhibitor VLX1570 shows selectivity for ubiquitin-specific protease-14 and induces apoptosis of multiple myeloma cells. Sci Rep. 2016 Jun 6; 6:26979

IV. **Mazurkiewicz M**, Hillert EK, Wang X, Pellegrini P, Hägg-Olofsson M, Selvaraju K, D'Arcy P, Linder S. Acute lymphoblastic leukemia cells are sensitive to disturbances in protein homeostasis induced by proteasome deubiquitinase inhibition. Oncotarget, 2017 Feb DOI: 10.18632/oncotarget.15501

V. **Mazurkiewicz M**, Hägg-Olofsson M, Serviss J, Pellegrini P, DeMilito A, Linder S, D'Arcy P. Induction of PA28 in 3D culture confers resistance to proteasome inhibitors
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LIST OF ABBREVIATIONS

AAA+ATPases	ATPases associated with diverse cellular activities
ADMR1	Adhesion regulating molecule 1
ALL	Acute lymphoblastic leukemia
AMPK	5' adenosine monophosphate-activated protein kinase
AP1	Activator protein 1
APC/C	Anaphase-promoting complex/cyclosome
ATF	Activating transcription factor
ATG	Autophagy related genes
ATP	Adenosine triphosphate
Bad	Bcl-2 associated death promoter
Bag	Bcl-2 associated athanogene
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bid	BH3 interacting-domain death agonist
Bcl-2	B-cell lymphoma 2
Bip/Grp78	Binding immunoglobulin protein/78 kDa glucose-regulated protein
BRCA1	Breast cancer gene 1
BRCC3	BRCA1/BRCA2-containing complex subunit 3
CDK	Cyclin dependent kinases
CETSA	Cellular thermal shift assay
CHIP	C-terminus of Hsp70-Interacting Protein
CHOP	C/EBP homologous protein
CP	Core particle
DNA	Deoxyrybonucleic acid
DUB	Deubiquitinase
E1	Ubiquitin activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-activating enzyme
EF2K	Eukarotic elongation factor 2
EGFR	Epidermal growth factor
EIF2α	Eukaryotic translation initiation factor α
ER	Endoplasmic reticulum
ERAA	ER-activated autophagy
ERAD	ER-associated degradation
ERK	Extracellular signal-regulating kinase

FDA	Food and drug administration
GADD34	Growth arrest and DNA damage-inducible gene 34
HDAC6	Histone deacetylase 6
HER2	Human epidermal growth factor receptor 2
HMOX-1	Heme oxygenase 1
HSP	Heat shock protein
Hul5	HECTubiquitic ligase
IAPs	Inhibitor of apoptosis
IFN-γ	Interferon-γ
IRE1α	Inositol requiring enzyme 1α
ISRIB	Integrated stress response inhibitor
IϰB	Inhibitor of nuclear factor kappa B
JAMM	JAMM/MPN domain-associated metallopeptidase
JNK	c-Jun-N-terminal kinase
kDa	Kilodalton
L-Asp	L-Asparaginase
LC3	Microtubule associated protein 1A/1B-light chain 3
Lys	Lysine
mAbs	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCL1	Induced myeloid leukemia cell differentiation protein
MCPIP	Monocyte chemotactic protein-induced proteases
MCS	Multicellular spheroid
MDa	Megadalton
Mdm2	Mouse double minute 2 homolog
MHC	Major histocompatibility complex
MJD	Machado–Joseph disease protein domain proteases
MM	Multiple myeloma
MTOC	Microtubule organizing center
mTORC1	Mechanistic target or rapamycin complex 1
NADP	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa B
nM	Nanomolar
NSCLC	Non–small cell lung carcinoma
OUT	Ovarian-tumor proteases
P53	Tumor protein 53

p62/SQSTM1	Sequestome 1
p97/VCP	Valosin-containing protein
PARP	Poly (ADP-ribose) polymerase
PERK	(Protein kinase RNA)-like ER kinase
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PI3P	Phosphatidylinositol -3-phosphate
POH1	Pad one homolog-1
PUMA	p53 upregulated modulator of apoptosis
PSMB	Proteasome subunit beta type
PSMD	Proteasome non-ATPase regulatory subunit
PSME	Proteasome activator complex subunit
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
RP	Regulatory particle
Rpn	Regulatory particle non-ATPase subunit
Rpt	Regulatory particle triple-A protein
SCF	SKP1–Cullin 1–F-box
SMAD	Mothers against decapentaplegic homolog 1
SPR	Surface plasmon resonance
siRNA	Small interfering RNA
Sirt1	Sirtuin 1
TGF-β	Transforming growth factor -β
TNF-α	Tumor necrosis factor-α
TrxR	Thioredoxin reductase
Ub	Ubiquitin
UbVS	Ubiquitin vinyl sulfone
UCH	Ubiquitin carboxy-terminal hydrolase
UPR	Unfolded protein response
UPS	Ubiquitin- proteasome system
USP	Ubiquitin specific protease
VGFA	Vascular endothelial growth factor
XBP1	X-box binding protein 1
YFP	Yellow fluorescent protein
Å	Ångström

1. INTRODUCTION

1.1. CANCER AND CANCER TREATMENT

Cancer remains one of the major causes of death worldwide. According to the World Health Organization report from 2012 there were 14,1 million new cancer cases and 8,2 million deaths [1]. Despite the improvements in the field of oncology, it is expected that in the next ten years the number of diagnosed cases will rise to 20 million annually [2]. Increasing progress in early diagnostic methods and rapid development of new therapeutics have helped to decrease the mortality rate for many tumor types. In developed countries however, a transition can be observed from infection-related cancers to diseases associated with lifestyle, hormonal and dietary factors [2]. In fact around 90% of tumors are correlated to environmental factors and only the remaining 10% are due to inherited mutations [3]. Therefore a better knowledge and understanding of the mechanisms behind tumor initiation and progression is essential for prevention and development of new treatments.

Cancer is an extremely heterogeneous disease; under the single term one can distinguish 120 different types of malignancies. Traditional classification of tumors is based on histological features and the site of origin. The major drawback of this classification is that it does not account for molecular differences between cells. Tumors that share similar morphology often do not respond identically to treatment and give different clinical outcomes [4],[5]. Therefore modern classification of tumors attempts to combine histopathology and molecular analysis in order to identify the specific type of malignancy and apply appropriate treatment. Despite the heterogeneity there are several features unique for cancer cells; genetic instability, immortality, continuous proliferation, evasion from growth suppression, resistance to induced cell death, active angiogenesis, invasion and metastasis, deregulated cell metabolism, escape form immune system surveillance and prolonged inflammation [6],[7]. This set of unique characteristics enables cancer cell to survive, proliferate in uncontrolled manner and eventually metastasize.

Additional level of complexity is added by the tumor microenvironment. Constant cross talk between tumor microenvironment components and malignant cells enables them to adapt to hostile conditions and thrive [8]. Vascular cells, fibroblasts, immune cells, and extracellular matrix associated with tumors create an environment suited for tumor progression [9]. In addition abnormal vasculature of tumors results in disrupted balance of factors such as cell metabolites, available nutrients, oxygen level and local pH of the microenvironment. These changed biochemical conditions have been proposed to modulate tumor cell metabolism and promote tumor survival [10],[11]. Tumor microenvironment not only affects development of the disease but also affects the delivery of the drug and limits response to treatment; therefore several treatment regimens attempt to target the microenvironment alongside with cancer cells [12],[13] .

Treatment of cancer has remained a challenge for many decades. Despite the major advances in the field of cancer therapy, the early-developed treatment lines such as surgery and radiation are still broadly used. Surgical removal of a primary tumor is in many cases, a

sufficient and minimally invasive approach that has been successfully applied for treatment of many solid tumors such as lung, breast or colorectal cancer [14].

Another well established localized treatment is radiotherapy, which is applied to approximately 50% of diagnosed patients [15]. Under the principal of radiotherapy lies the fact that many tumor cells have impaired deoxyribonucleic acid (DNA) repair systems and therefore are more sensitive to DNA damage than normal cells [16]. Exposure of the cells to optimized does of high-energy radiation results in DNA damage and consequently inhibition of cell growth and proliferation. Radiotherapy can be successfully used as a single therapy agent for treatment of cancers such as head and neck carcinomas, prostate carcinomas or non-small cell lung carcinomas. On the other hand some cancer types are particularly radio-resistant such as melanomas or glioblastomas [15]. In addition in many cases localization of the tumor is not favorable and/or malignancy is diagnosed at the metastatic stage of disease progression, therefore systemic treatment strategies need to be applied.

Chemotherapy is a conventional treatment approach in which cytotoxic agents are exploited. Several classes of chemotherapeutics can be distinguished such as alkylating agents, anti-metabolites, antibiotics, topoisomerase inhibitors and microtubule stabilizers [17]. Typically these agents block cell proliferation by interfering with cell cycle components or by direct intercalation with DNA. This activity is not tumor specific and the effect on non-cancerous tissues can lead to severe side effects. The efficacy of the therapy relies on the features of the tumor cell such as high proliferation rate, dependency on functional repair systems and the need for continues supply of cellular building blocks [18].

Intensified studies of tumor cell phenotypes/genotypes led to identification of alterations in growth and proliferation pathways and eventually resulted in development of targeted therapy [19]. Targeted therapies aim to directly attack the ‘hallmarks of cancer’, therefore induce cell death specifically in tumor cells. Some features that allow cancer cell to thrive such as oncogene addiction, increased mutation and DNA damage rate, metabolic reprogramming, elevated proteotoxic and oxidative stress and cancer-stroma cross talk has been exploited for successful development of therapies. Diverse range of molecules such as signal transduction inhibitors, gene expression modulators, apoptosis inducers or angiogenesis inhibitors are clinically used [20].

The discovery of immunosurveilance and the ability of the host immune system to recognize and destroy malignant cells resulted in growing interest in cancer immunotherapy [21]. Two strategies are investigated: active and passive immunotherapy [22]. Passive immunotherapy relies on application of external immunogenic factors such as; tumor targeting monoclonal antibodies, which specifically bind and neutralize antigens expressed on cancer cells or patient-derived cells of immune system (tumor infiltrating lymphocytes or T-cells), that have been genetically modified to increase killing efficiency. Active immunotherapy is focused on boosting the existing immune response of the host by immunogenic cell death inducers. Many traditionally used chemotherapeutics elicit immunomodulatory effects especially when combined with other forms of immunotherapy. Cancer cell death induced by oncolytic virus infection concurrently leads to massive release of tumor-associated antigens attracting cells of the immune system. In addition immune system can be stimulated directly by anticancer

vaccines, immunostymulatory cytokines or immunmodulatory mAbs or indirectly by inhibitors of immunosuppression [22].

Despite the vast array of therapy strategies, the field of oncology is constantly struggling with resistance and remission of the disease. Cancer cells are highly dynamic and eventually acquire sets of resistance mechanisms such as mutations in drug targets, circumvention of inhibited pathway, overexpression of drug exporters, mutations in cell death pathways, reprogramming of tumor cell metabolism or escaping immunosurveillance [19]. Therefore, treatment of a complex and diverse disease such as cancer demands a systemic approach, integrating knowledge from many fields and ideally combining several lines of treatment [23],[24].

1.2. CELLULAR DEGRADATION PATHWAYS

Cancer is a dynamic disease of disrupted cellular balance. Proteins are essential building and functional blocks of living cells; therefore protein levels must remain under continuous control in order to maintain cellular homeostasis. Cellular needs for proteins are fulfilled by the tightly synchronized action of protein synthesis and the rate of destruction [25]. In eukaryotic cells there are two main pathways for protein degradation: lysosomal-mediated proteolysis and the ubiquitin-proteasome system (UPS) [26],[27]. In 2004 Aaron Ciechanover, Avram Hershko and Irwin Rose were awarded with the Nobel Prize in Chemistry for studies on Ub-mediated protein degradation and in 2016 Yoshinori Ohsumi was awarded with the Nobel Prize in Medicine for discovery of autophagy mechanism.

1.2.1. Ubiquitin proteasome system

Since the discovery of the UPS it has been shown that more than 80% of cellular proteins are targeted to this molecular shredding system, particularly short-lived proteins, including cell cycle regulators, transcription factors, DNA damage repair proteins, cell signaling molecules, oncogenes and tumor suppressors [27],[28]. In addition proteasomes are also involved in recognition and clearance of damaged and misfolded proteins from the cell. Many polypeptides from processed proteins are presented on major histocompatibility complex (MHC) class I, suggesting an important role of proteasome in immunesurveilane. Considering the diverse role of UPS function it is not a surprise that alterations in UPS pathway are correlated with many human diseases such as neurodegenerative disorders or cancer [29]. The process of UPS mediated protein degradation can be divided into two main steps; tagging the target by ubiquitination and subsequent proteolysis in proteasome (Figure1).

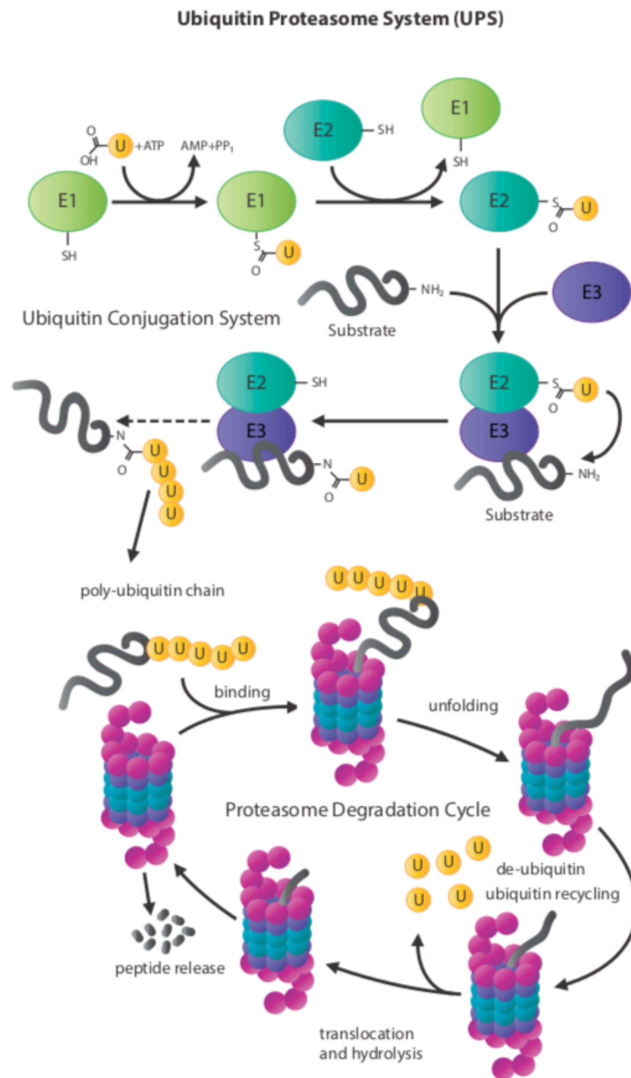

Figure 1. Structure and function of ubiquitin-proteasome system

Ubiquitination

In the first step of proteasome degradation a target protein is labeled with ubiquitin, a small, (76 amino acids) and highly protein. Ubiquitin conjugation is performed by the coordinated action of three classes of enzymes: an ubiquitin-activating enzyme (E1); ubiquitin-conjugating enzyme (E2) and an ubiquitin-ligating enzyme (E3). E1 activates ubiquitin via association of the carboxy-terminal glycine residue of ubiquitin and the active site cysteine of the E1 enzyme in the presence of ATP. Activated ubiquitin can be transferred to a cysteine residue of an E2 enzyme that is further recruited by a substrate specific E3 ligase, which coordinates the attachment of an ubiquitin moiety to lysine residues in target proteins [30]. Proteins can be modified with a single ubiquitin molecule conjugated to one or several lysine residues or with a multi-ubiquitin chain. Mono-ubiquitination is involved in histone modification, endocytosis, protein trafficking and intracellular localization and DNA damage repair [31],[32]. Self-conjugation of the ubiquitin moiety can occur at any of the seven internal lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63). The type of

linkages within a polyubiquitin chain determines the fate of a modified protein. In the canonical model, proteins tagged with four or more Lys48-linked ubiquitin subunits are considered as proteasome substrates [33]. Other modifications remain less characterized, however recent studies show that unconventional polyubiquitin chains linked through Lys6, Lys11, Lys27, Lys29 and Lys33 are abundant *in vivo* and may target specific substrates to the UPS [34]. Additionally it has been reported that Lys63-modification, typically associated with non-proteasomal functions such as protein sorting, DNA damage repair, kinase signal transduction and receptor endocytosis, can also serve as a proteasome-targeting signal [35],[36]. In human cell there are approximately 30 types of E2 enzymes, and 500-1000 E3 ligases, which allows for thousands of E2-E3 combinations and possible forms of substrate modification. Therefore it is not surprising that ubiquitinataion is emerging as one of key regulators of protein homeostasis [32].

Ubiquitination is opposed by the activity of deubiquitinases (DUBs, ubiquitin isopeptidases). DUB enzymes disassemble ubiquitin chains and remove them from target proteins by hydrolyzing the isopeptide bond between the lysine residue on target proteins and the C-terminal glycine on ubiquitin [37]. There are six different classes of DUBs: ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolases (UCHs), ovarian-tumor proteases (OTUs), Machado–Joseph disease protein domain proteases (MJDs or Josephins), JAMM/MPN domain-associated metallopeptidases (JAMMs) and monocyte chemotactic protein-induced proteases (MCPIP) [38]. There are about 100 DUBs in human cells, where the USPs are the most numerous, followed by the OTUs, JAMMs, UCHs and Josephins. The distinct linkages between ubiquitin molecules result in different chain conformations, serving as a pattern for recognition by various DUBs. Some DUBs show specificity for only one type of chain, whereas others do not discriminate between chain types [39]. DUBs can preferably cleave in the middle (endo-) or from the end (exo-) of an ubiquitin chain, which results in chain trimming or removal of monoubiquitination, respectively. DUBs and E3 ligase diversification partially explains the key role of the UPS in a multiple and various regulatory circuits.

Proteasome structure and function

Ubiquitinated substrates are hydrolyzed into small peptides by a molecular shredder called the 26S proteasome. The 26S proteasome is a large (2,5 MDa) multimeric complex, that can be furtherly devided into 20S core particle (CP) and 19S regulatory particle (19S RP). The eukaryotic 20S CP is a 730 kDa barrel-shaped structure with a central cavity, formed by axial stacking of four heteroheptameric rings. The two inner rings are composed of seven β-subunits and the two outer rings are composed of seven α-subunits giving together the general structure of the complex $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$ [40],[41]. β3, β4, β6 and β7 subunits have mainly structural function, whereas β1, β2 and β5 carry the caspase like/PGPH (peptidylglutamyl-peptide hydrolyzing), trypsin-like and chemotrypsin-like activities, respectively. This diversity in peptidase activity confers the proteasome with proteolytic activity and allows for cleavage of acidic, basic and hydrophobic amino-acid residues, respectively. Catalytically active threonine residues at the N-termini of proteolytic subunits protrude into the center of the 20S chamber where substrate proteins are hydrolyzed [42],[43]. Produced oligopeptides range in the length from 3 to 15 amino acids and can be

further degraded by cellular proteases, feeding the amino acid pool of the cell. If not digested they can serve as antigens, presented to the immune system by the MHC class I [44]. In order to reach the catalytic chamber formed by β-rings, substrate proteins need to pass through a narrow (13 Å in diameter) channel created by α-rings. Crossing the passage is preceded by recognition of polyubiquitinated substrate, removal of ubiquitin chain and unwinding of substrate protein tertiary structure, all of this performed at the 19S RP [43].

The 19S RP is a 930 kDa complex, composed of lid and base structures. The lid contains 9 non-ATPase subunits Rpn3, Rpn5-9, Rpn11, Rpn12 and Rpn15 and the base contains 10 subunits, six of which are related AAA^+ ATPases that form a heterohexameric ring [45],[46]. The ATPase ring utilizes the energy from ATP hydrolysis to unwind the tertiary and secondary structure of a substrate and to translocate it into the narrow 20S catalytic core [47]. The Rpn1 and Rpn2 subunits of the 19S base serve as binding sites for deubiquitinases (Ubp6/USP14) and E3 ligases (Hul5) indicating that ubiquitination is a highly dynamic process and that substrate fate can be changed even upon binding to the proteasome [48]. Substrate recognition is performed by the ubiquitin receptors Rpn10 and Rpn13 that bind Lys48 linked polyubiquitin chains with high affinity [49]. Three extrinsic ubiquitin receptors (Rad23, Dsk2, Ddi1) can also modulate the binding preference towards specific ubiquitin linkages and chain lengths. In addition Rpn13 also serves as a binding site for the DUB Uch37/UCHL5, providing a crosstalk site between chain recognition and disassembly [49]–[52].

Bulky ubiquitin chains interfere with the translocation of substrates into the proteasome; therefore deubiquitination is a crucial step for efficient proteolysis. In addition, DUBs clear the proteasome from substrate-free ubiquitin chains, leaving receptors available for the next interaction. Deubiquitination participates in maintaining an adequate level of ubiquitin molecules, which is essential for cell viability and the cells ability to withstand stress. Ubiquitin homeostasis is regulated by DUB activity, synthesis rate and proteasomal proteolysis, where ubiquitin can be degraded as a free monomer or as remnants attached to a targeted protein [52]. Finally deubiquitination can result in rescue of weakly ubiquitinated substrates by trimming the poly-ubiquitin chain from the distal end [53].

19S deubiquitinating enzymes

There are three DUB enzymes associated with the proteasome Rpn11/POH1, Ubp6/USP14 and Uch37/UCHl5 (yeast/human nomenclature), metalloprotease JAMM, USP and UCH family, respectively [50]. Rpn11/POH1 is a constituent part of the 19S lid, essential for cell viability, as well as 26S proteasome structure and activity [54],[55]. It is located above the entrance of the ATPase ring and makes extensive contact with Rpt3 in the base and Rpn8, Rpn9 and Rpn5 in the lid [46]. Positioning of the enzyme and the fact that Rpn11/POH1-mediated deubiquitination requires hydrolysis of ATP, indicates that the process is tightly linked to protein unfolding and substrate translocation into the 20S core. Rpn11/POH1-mediated deubiquitination is thought to promote substrate degradation [56],[57]. Concurrently Rpn11/POH1 activity is delayed until the substrate is committed to degradation. To span the distance between Rpn10 or Rpn13 ubiquitin receptors and Rpn11/POH1 the polyubiquitin chain must contain at least four ubiquitin moieties. Trimming the chain prior to degradation commitment by the two residual 19S DUBs, decreases substrate binding affinity

and promotes its dissociation from the proteasome [58]. Preferable substrates for Rpn11/POH1 are ubiquitin chains linked by Lys63 [59]. Ubp6/USP14 and Uch37/UCHL5 are cysteine proteases and unlike Rpn11/POH1, are non-essential for proteasome integrity. They associate with proteasome via Rpn1 and Rpn13 subunits, respectively. The active sites of these enzymes are inhibited by crossover loops and upon binding to the proteasome conformational changes expose the ubiquitin-binding site and allow for deubiquitination [60],[61]. In comparison to Rpn11/POH1, Ubp6/USP14 and Uch37/UCHL5 are located at a larger distance from the 20S entrance pore, therefore they presumably clip extended ubiquitin chains from the distal tip or within a chain. Ubp6/USP14 releases di and tri-ubiquitin whereas Uch37/UCHl5 cleaves off single ubiquitin moieties [60],[62]. Ubp6/USP14 cleaves preferably Lys48-linked chains, whereas Uch37/UCHL5 can cleave both Lys48- and Lys63-linked chains [63]. Proteasome associated DUBs cooperate to provide effective and controlled processing of ubiquitin chains and thus ubiquitin-tagged substrates. A mechanism proposed for this cooperation suggests that USP14/UCHL5 mediated deubiqutination prevents deubiquitination by Rpn11/POH1 and therefore delays protein degradation by proteasome [58],[64]. Additionally, Ubp6/USP14 regulates opening of the 20S gate and therefore integrates the step of substrate binding and proteolysis [65]. Single knock-down of Rpn11/POH1 or double knockdown of Ubp6/USP14 and Uch37/UCHL5 leads to a similar phenotype with inhibition of cell growth, decreased rate of protein degradation and accumulation of polyubiquitin chains [55]. Surprisingly Koulich et al. show that RNAi-mediated down-regulation of either DUB alone does not affect cell growth and it increases the rate of proteasome activity, suggesting that these enzymes may have redundant roles [53]. The detailed mechanisms of the degree of cooperation between DUBs are yet to be discovered.

1.2.2. Autophagy

Autophagy is a lysosome dependent degradation pathway of many cytoplasmic components, especially long-lived proteins and organelles. An assembly of a unique organelle called autophagosome characterizes the process [66]. The double membrane vesicle engulfs a portion of the cytoplasm and after subsequent fusion with the lysosome, mediates degradation of the cargo. Autophagy can be divided into several steps: induction, vesicle formation, vesicle maturation and degradation. Induction of autophagy is coordinated by sensors of cellular energy level, 5’ adenosine monophosphate-activated protein kinase (AMPK) and mechanistic targets of rapamycin complex 1 (mTORC1), autophagy inducer and inhibitor, respectively [67]. During phagophore formation and elongation a set of autophagy related (ATG) proteins is recruited. Coordinated action of ATG proteins results in generation of 3,4,5-triphosphate (PI3P) on membranes destined to become a phagophore and mediates elongation of the membranes until the vesicle closes and forms an autophagosome [68]. Maturation phase requires special ATG8 protein – microtubule-associated prptein1A/1B-ligth chain 3 (LC3). Formation of phosphatidylethanolamine-lipidated LC3 (LC3-II) and incorporation into phagosome membrane is coordinated by ubiquitin-like conjugation system [69].

Autophagy is executed with a different type of specificity; unselective autophagy, during which big portions of cytoplasm are processed for nutrient recycling or selective autophagy, when specific cargo is targeted for degradation [70]. In a living cell a basal level of autophagy is maintained for clearance of non-functional proteins or organelles, in order to preserve cellular homeostasis and provide continuous supply of nutrients and building blocks [71]. Interaction between membrane-bound LC3, which serves as a cargo receptor and an adaptor protein, such as sequestome 1 (p62/SQSTM1), enables recognition and sequestration of specific substrates [72]. Additional level of inducible autophagy is triggered by stress conditions such as; starvation, hypoxia or oxidative stress [73]. Nonselective engulfment of cytoplasmic material is typical for induced autophagy. The level of autophagy often decides on cell fate and extensive autophagic processes can lead to apoptosis [74].

Autophagy plays a dual role in cancer and depending on the context can be tumor inhibiting or tumor promoting [75]. Signaling pathways that regulate tumor growth and autophagy have common nodes of interaction. Activation of the PI3K-AKT-mTOR pathway, a common event in many cancers, inhibits autophagy [76]. Mutations and deletions of several ATG genes have been identified in prostate, breasts, ovarian and colon cancers. Finally, mouse models with deficient autophagy develop multiple tumors [75]. As described before, basal autophagy protects cells from metabolic and oxidative stress, however; deregulation of autophagy can result in ROS production, accumulation of cellular waste and genomic instability, common factors promoting malignant transformation [77]. On the other hand in already formed tumors, autophagy helps to preserve the fitness of malignant cells in a hostile environment. Metabolic changes of cancer cells count for elevated autophagy to reduce stress, recycle nutrients and allow cells to survive. In addition autophagy has been proposed as a resistant mechanism to many types of drugs, whose activity relies on stress induction above tolerance threshold of cancer cell. Therefore blocking autophagy is considered as a beneficial strategy for increasing the efficacy of radio- and chemotherapy [78].

1.2.3. UPS-Autophagy crosstalk

The two cellular degradation pathways were initially considered as distinct and unrelated, however there is rising evidence for an extensive crosstalk between and cooperation between them (Figure 2) [79]–[81]. As described in previous chapters UPS-based degradation is believed to be more precise and focused on degradation of soluble and short-lived proteins [28]; whereas it is suggested that autophagy is a less specific mechanism for degradation of long-lived proteins, bulky misfolded proteins, insoluble protein aggregates or whole organelles [66]. It is also speculated that with age autophagy is favorable over UPS [82]. Common denominator between these two mechanisms is the ubiquitin molecule and several adaptor molecules [83]. Autophagy is a cell contingency plan when proteasome is overloaded for example in situation of extensive ER stress or accumulation of protein aggregates [84],[85]. It is also a form of rescue after proteasome inhibition [86]. Finally 26S proteasome itself was found to be degraded by autophagy [87].

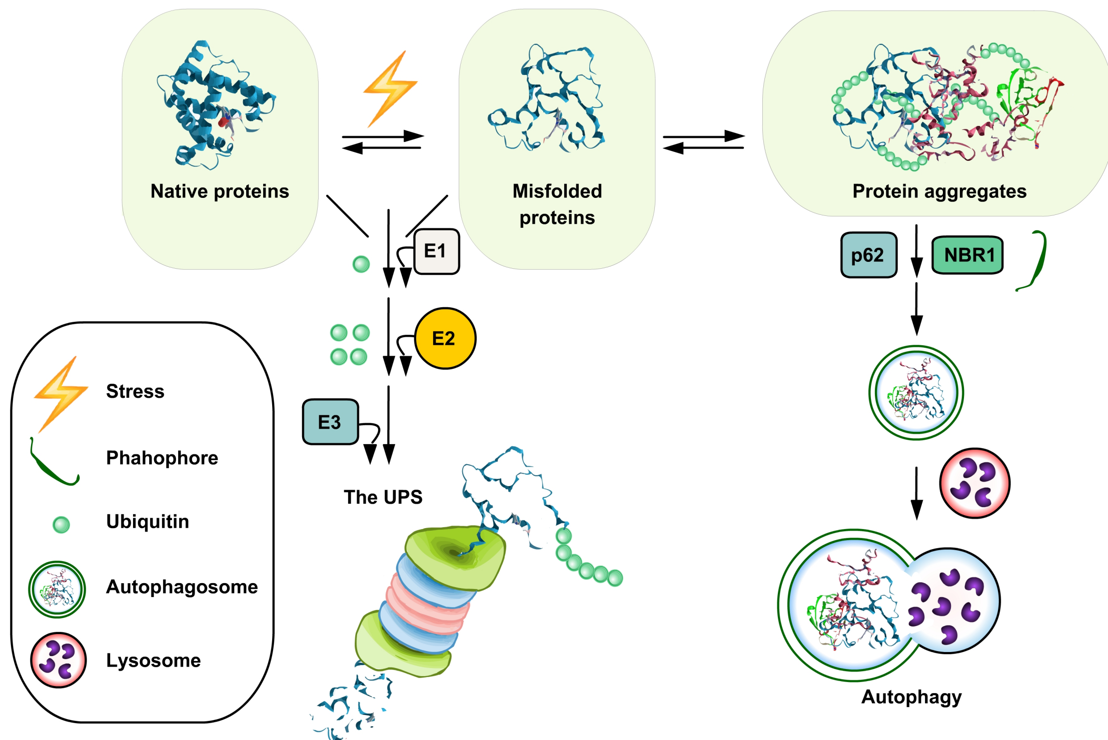

Figure 2. Crosstalk between ubiquitin-proteasome system and autophagy

1.3. UPS FUNCTION IN CANCER DEVELOPMENT

The UPS is involved in the regulation of many crucial cellular pathways; such as signal transduction, proliferation, DNA repair, cell death, and cell cycle regulation [88]. Balance between action of E3 ligases, and deubiquitinating enzymes (both cytosolic and proteasome-bound) allow for precise control of signaling molecules and their receptors. Finally UPS protects cells from toxic and damaged proteins. Taking all this function together, it is not a surprise that deregulation of the system underlies the pathogenesis of many human diseases such as Parkinson disease or cancer [89],[90].

In order to proliferate in uncontrolled manner cancer cells escape from cell cycle chekpoints, and ubiquitination and deubiquitination events have been proven to be essential for cell cycle regulation [91]. Cell cycle is regulated by periodic activation cyclin dependent kinases (CDKs) and it's key regulators: cyclins (D, E, A, B) and CDKs inhibitors ($p21^{Cip1}$ and $p27^{Kip1}$). Sufficient level of these key regulators is in turn maintained by the ubiquitin-mediated proteolysis and aberrations in the process are found in many types of cancers. There are two main E3 ubiquitin ligases involved: the SKP1–Cullin1–F-box-protein (SCF) complex and the anaphase-promoting complex/cyclosome (APC/C). Depending on co-regulators involved, SCF can act as tumor promoter or suppressor, since it participates in ubiquitination of cell cycle inhibitors: $p21^{Cip1}$ and $p27^{Kip1}$ and cell cycle promoters: cyclinE, c-myc, Notch [92]. APC/C is active mainly in anaphase and the beginning of the G1 phase, whereas the SCF complex is active from late G1 to the initiation of M phase. USP44 is a key regulator of APC activity, preventing its premature activation. USP44 is suggested to function as tumor

suppressor and cells with down regulated USP44 are prone to chromosome segregation errors [88],[93]. USP2a cleaves ubiquitin from cyclin D and cyclin A1; leading to increased protein levels and enhancement of cell proliferation in cancer cells [94],[95]. USP19 activity supports cell proliferation by promoting $p27^{Kip1}$ degradation [96]. USP28 was also demonstrated to act as tumor promoter by deubiquitinating and stabilizing Myc in colon and breast carcinoma cells [97].

P53 is an important tumor suppressor and regulator of cell proliferation, apoptosis, senescence and cell growth. In response to DNA damage or oncogene signaling p53 induces genes responsible for growth arrest and apoptosis. It is commonly mutated in many types of cancer [98]. Ubiquitination has been proposed as the main regulatory mechanism of p53 level, which is normally maintained low via the interaction with its negative regulator Mdm2 [99]. Mdm2 is an E3 ubiquitin ligase, that ubiquitinates both p53 and itself. The DUB HAUSP/USP7 has a dual role in p53 stabilization. Direct deubiquitination stabilizes p53, however HAUSP/USP7 activity also stabilizes Mdm2, creating a negative loop for p53 signaling [100]. To add an additional level of complexity, USP2a has been identified as a regulator of the Mdm2/p53 pathway. USP2a binds and deubiquitinates Mdm2/MdmX and indirectly stabilizes p53 [101]. Many additional DUBs have been implicated in p53 regulation. USP10 determines p53 localization after DNA damage, when phosphorylated USP10 is translocated into the nucleus, where it deubiquitinates p53 and prevents its nuclear export and degradation [102]. USP22 inhibits p53 transcriptional activation by deubiquitinating the class III histone deacetylase Sirt-1 and H2A and H2B histones [103],[104]. Finally USP42 directly interacts with p53 and controls its ubiquitination level during stress responses [105].

Cancer cells have the ability to evade programmed cell death by increasing the ratio between anti-apoptotic and pro-apoptotic proteins. Many proteins directly involved in the apoptotic response belong to the Bcl-2 family [106]. Pro-apoptotic Bcl-2 family members (Bax, Bad, Bid) trigger release of cytocochrom c from mitochondria, which in turn activates caspase cascade and apoptosis. On the other hand anti-apoptotic proteins such as Bcl-2, Bcl-XL and Mcl-1 promote cell survival. The balance between pro-apoptotic and anti-apoptotic proteins is maintained by UPS degradation [107]. Four different E3 ligases and USP9x deubiquitinase have been shown to regulate pro-survival Mcl-1 [108]. Mcl-1 overexpression has been correlated with poor prognosis in lymphocytic leukemia, multiple myeloma and breast cancer [109]. Uchl37/UCHL5 promotes expression of anti apoptotic Bcl-2 and UCHL5 knockout results in elevated level of pro-apoptotic Bax in a lung adenocarcinoma epithelial cell line [110]. Bax is under regulation of the IBRDC2 ligase and increased proteasomal degradation of Bax is associated with poor prognosis in chronic lymphocytic leukemia [111]. At certain threshold of induced apoptosis, IAPs (inhibitor of apoptosis) bind to caspases and sequester apoptotic signaling [112]. IAPs stability is regulated by USP19 [113] and in addition IAPs themselves carry an E3 ligase domain and can inactivate caspase via direct ubiquitination [114].

Nuclear factor kappa B (NFϰB) is a transcription factor with a well-established role in promoting cancer due to its anti-apoptotic and pro- inflammatory functions [115]. The DUB CYLD has been suggested as an important tumor suppressor, since it can promote apoptosis

by increasing the stability of IϰB, the endogenous inhibitor of NFϰB [116]. On the other hand, overexpression of the 19S DUB USP14 was shown to reduce IϰB levels in lung epithelial cells [117]. NFϰB signaling is also down-regulated by the E3 ligase domain of the A20 protein [118]. Interestingly several studies show that NFϰB can up-regulate pro-apoptotic genes such as Bax or p53 and therefore act rather as a tumor suppressor than tumor promoter [120],[121]. Another well-established mediator of apoptosis and stress related response is c-Jun-N-terminal Kinase (JNK) that belongs to mitogen-activated protein kinases (MAPK) family. JNK is activated by the AP1 (activator protein 1) transcription factor, c-Jun, which is regulated by Rpn11/POH1 [121].

Transforming growth factor-β (TGF-β) signaling is yet another signaling cascade commonly altered in human cancers [122]. Smads are mediators of TGF-β signaling and Smad4 monoubiquitination antagonizes its activity. USP9x has been shown to deubiquitinate Smad4 and restore TGF-β signaling [123]. TGF-β signaling is attenuated by inhibitory Smads that recruit the E3 ligase Smurf to the TGF-β receptor and promote its degradation. USP11 and Uch37/UCHL5 have been found to rescue the receptor from proteasomal degradation and promote cell migration and metastatic potential mediated via TGF-β signaling [124]–[126].

DNA repair pathways are often defective in cancer resulting in a mutator phenotype. POH1/Rpn11, USP11 and BRCC36 activity was suggested to regulate BRCA1- mediated DNA repair [127],[128] and USP28 was shown to regulate the Chk2–p53–PUMA-signaling pathway, important for DNA-damage-induced apoptosis [129].

Finally, activity of receptor tyrosine kinases (RTKs) is associated with many tumor types. One way of terminating the proliferative signal is receptor ubiquitination, which serves as a signal for receptor internalization and endosomal packing [130]. AMSH and USP8 are two well-established DUBs that participate in the down regulation of the epidermal growth factor receptor (EGFR) through the described mechanism [131],[132]. Depletion of either USP8 or AMSH results in deregulation of stimulated EGFR levels in several cancer types. Similarly POH1 has been shown to regulate the expression of HER2, another oncogenic RTK. Deubiquitination by POH1 rescues HER2 from proteasomal degradation and stimulates its recycling to the cell surface [133]. USP9x interacts with and stabilizes β-catenin, a member of the Wnt signaling pathway involved in the pathogenesis of colon cancer [134],[135]. A similar function was discovered for USP14 and correlated with overall survival of lung carcincoma patients [136].

1.4. TARGETING UPS IN CANCER TREATMENT

Pervasiveness of ubiquitin signaling makes it a very attractive target for therapeutic research [137]. Blocking the UPS at different nodes can be used to eliminate cancer cells via cell cycle arrest, elevated apoptosis and increased proteotoxic stress [138]. UPS can be targeted at the level of E1, E2, E3 enzymes, deubiquitinases and proteasome [139]. There are several attempts to develop E1 or E2 inhibitors, however this enzymes have very broad spectrum of action and inhibition may result in multiple side effects [139],[140]. E3 is the most specific class of enzyme in UPS system and several compounds interfering with p53 or $p27^{Kip1}$ ubiquitination are under investigation. DUB enzymes are also potentially drugable targets, however as they are cysteine kinases, abundant in cellular cytoplasm, specificity of developed

drugs is questionable. As previously described 19S DUBs undergo conformational change upon binding to proteasome and therefore are potentially better targets [60],[61]. Most of today clinically used or tested drugs are proteasome inhibitors [141]. Cytotoxicity of proteasome inhibitors is mediated by inhibition of cell cycle, induction of pro-apoptotic signaling, ER stress and oxidative stress [142].

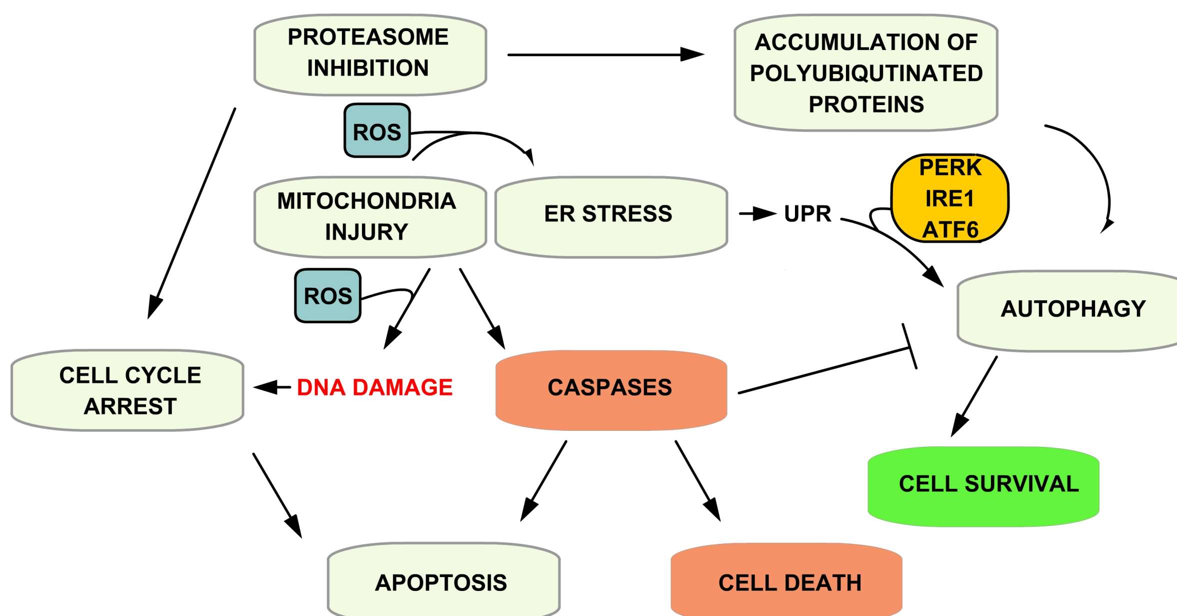

Figure 3. Effects of proteasome inhibition

1.4.1. Cell cycle inhibition and apoptosis

Levels of proteins involved in cell cycle are tightly control by UPS and inhibition of proteasome results in stabilization of negative cell cycle regulators and pro-apoptotic proteins. Accumulation of $p27^{Kip1}$ and $p21^{Cip1}$ results in cell cycle arrest in G2/M phase [143]. Prolonged cell cycle arrest triggers apoptosis, probably due to accumulation of p53 and p53induced pro-apoptotic genes, such as Bax [144]. Some evidence however, show that proteasome inhibitors can induce apoptosis independently from p53 expression [145],[146]. Furthermore, although anti-apoptotic members of Bcl-2 family are also proteasome substrates, apparently accumulation of protective signal is not able to protect cells from induced cell death [146],[111]. Pro-survival NFϰB is bound and inhibited in the cytoplasm by IϰB, a proteasome substrate. Accumulation of IϰB after the proteasome inhibition prevents maturation of NFϰB, transport of active subunits to the nucleus and induces apoptosis [147]. On the other hand, some studies show that importance of NFϰB inhibition is controversial and it is not sufficient to induce cell death [148],[149].

1.4.2. UPR and ER STRESS

Chaperones such as Hsp70 (Heat shock protein 70) assist folding of newly synthetized or misfolded proteins into their native and functional form [150]. A crosstalk between

chaperone-mediated repair and degradation is essential for protein quality control and for keeping cells free from proteotoxic stress. If the protein damage is too extensive and repair is not feasible it is targeted to degradation Chaperones and assisting co-chaperones, decide on the type of engaged E2-E3 ligases complex and as a consequence on ubiquitination pattern and protein fate. K-48-linked chains target the substrate to the proteasome, while Lys29-, Lys63- linked chaines or monoubiquitination are preferable for autophagy-mediated degradation [151],[152]. CHIP (C-terminus of Hsp70- interacting protein) and BAG (Bcl-2-associated athanogene) are co-chaperone molecules able to switch the fate of candidate proteins between two forms of degradation [82],[153]. CHIP is a co-chaperone of Hsp70 and Hsp90 and concurrently an E3 ubiquitin ligase [153],[154]. Via its functional domains it can ubiquitinate Hsp70 substrate, interact with the proteasome or autophagosome and actively switch between these two systems. In addition, BAG1 interacts with both CHIP and the proteasome and mediates recruitment of Hsp70 to the proteasome [155],[156]. On the other hand BAG3 preferentially targets for autophagy-mediated degradation and therefore BAG1/BAG3 ratio defines predominant degradation system.

Newly synthetized proteins undergo chaperone-mediated folding and postranslational modifications in endoplasmic reticulum (ER) to gain their functional structure, however mutations in protein coding genes or ER dysfunction can lead to misfolding. Such damaged proteins are transported from ER to proteasome in a process called ER-associated degradation (ERAD) [157]. P97/VCP (valosin-containing protein) chaperone is a AAA+ ATPase that is necessary for transportation of proteins from ER to the proteasome [158]. In case of ER stress or proteasome overload, global protein synthesis rate is down regulated, but a specific set of genes involved in the unfolded protein response (UPR) is up regulated: activating transcription-factor 6 (ATF6), inositol- requiring enzyme 1α (IRE1α) and protein kinase RNA-like kinase (PERK). If ERAD fails to clean ER from accumulated damaged proteins, ER-activated autophagy (ERAA) is triggered [159]. ATF6 increases expression of ER associated chaperones such as Bip/Grp78, an ER homologue of Hsp70 [160]. Upon activation, PERK phosphorylates and deactivates translation initiation factor eIF2α, therefore indirectly inhibits mRNA translation and reduces the flux of new proteins to ER. Simultaneously translation of ATF4 (activating transcription factor 4) and its target genes: transcription factor CHOP (C/EBP homologous protein) and GADD34 (growth arrest and DNA damage–inducible gene 34) is induced. CHOP controls genes involved in apoptotic pathway while GADD34 is a negative regulator of PERK activity [160]. PERK/eIF2α phosphorylation is also necessary for transformation of LC3 into membrane-bound LC3-II form and formation of mature autolysosomes [161]. IRE1α transmembrane kinase/endoribonuclease, splices and activates XBP1 (X-box binding protein 1) transcription factor, which in turn activates components of ER-mediated degradation path [160]. IRE1 kinase activity and IRE1-mediated phosphorylation of JNK and Bcl-2 is essential for autophagy and apoptosis induced by proteasome inhibitors [84],[162]. Pro-apoptotic ER residents (Bax and Bad), regulate level of Ca^{2+} released from membrane into cytoplasm and therefore control Ca^{2+}induced apoptosis [163]. Prolonged UPR induces apoptosis and cell death, however for certain types of cancer, especially the ones derived from secretory cells, such as multiple myeloma, it can have a protective function [164].

1.4.3. Accumulation of ROS

Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radicals, hydroxyl radicals, are reminders from cellular oxygen metabolism and they are constantly produced in cells utilizing aerobic processes. Leakage from mitochondrial respiratory chain is the main generator of ROS, however NAD(P)H oxidase, xanthine oxidase, uncoupled nitric oxide synthase and ER stress also contribute to accumulation of free radicals. When the level of ROS particles is properly balanced, they can act as signaling molecules in many biological processes [165]. Proper levels of ROS are maintained by the activity of various antioxidants (Vitamin C and E, gluthathione peroxidase, thioredoxin reductase) [166]. When levels of ROS exeedes antioxidant capacity of the cell it can casue damage to cellular proteins. If damage is to extensive, oxidaised proteins tend to misfold and form unsoluble aggregates. To protect the cell, oxidised proteins can be rapidly degraded in 20S proteasome in an ATP and Ub-independent manner [167]. Cancer cells have higher basal level of ROS, which was found to be essential for cell proliferation, survival and even associated with increased aggressiveness [168]. Some evidence show that elevated ROS may even confer resistance to chemotherapy [169]. On the other hand it makes tumor cells more vulnerable to disruption of redox homeostasis and it has been exploited in the development of new therapeutic strategies [170]. Interestingly, resent study questions correlation between level of accumulated ROS and cytotoxicity [171]. Nevertheless, proteasome inhibition leads to increased oxidative stress, a process that was found essential for apoptosis induction by this compunds. Since treatment with proteasome inhibitors results in a decrease of mitochondria membrane pontential, it was concluded that large amount of generated ROS upon proteasome inhibiton, comes from depolarization of mitochondirial membranes [172],[173]. As described before, p53 regulates level of pro- and anti- apoptotic genes. Accumulation of p53 and pro-apoptotic Bcl-2 family proteins affects mitochodrial membrane integrity and results in release of cyrochrome c into the cytoplasm and apoptosis [174]. Finally elevated ROS may induce apoptosis through intracellular stress-sensing kinase cascades such as the MAPK pathway, composed of apoptotic proteins (JNK and p38 MAPK) and protective extracellular signal-regulating kinase (ERK) [175]. Interestingly proteasome inhibition induces apoptosis through JNK pathway activation [176],[177].

1.4.4. Protein aggregates formation

Exposure of the cell to stress factors such as heat shock or oxidative stress can result in protein unfolding and misfolding. Some of the proteins can neither be successfully repaired nor degraded by the proteasome, due to their hydrophobic structure or simply due to their abundance and proteasome overload. These proteins tend to form potentially toxic aggregates dispersed in cell cytoplasm. To protect the cell from toxicity of insoluble protein clumps, a mechanism called aggresome formation is triggered [178]. Aggregates of insoluble proteins are transported along a microtubule network to the microtubule organizing center (MTOC). One of the key regulators of aggresome formation pathway is microtubule-associated deacetylase HDAC6, which recognizes both polyubiquitin chains and the microtubule dynein motor, therefore enabling active transport of polyubiquitinated aggregates into the perinuclear

area [179]. Another important player is P62/SQSM1, an adaptor protein that has ubiquitin and LC3 binding domains and functions as a bridge between autophagosomes and polyubiquitinated proteins clustered in aggreseomes [72]. Chaperones, UPS and autophagy components are also brought to close proximity in aggresomes in order to participate in protein refolding or degradation [180],[181]. On example is P97/VCP chaperone, which disrupts interaction between HDAC6 and ubiquitinated proteins and favors proteasomal degradation [182]. On the other hand, some reports suggest that aggresome formation may inhibit proteasome function and thus favor autophagy-dependent degradation [183]. As previously described K63-linked polyubiquitin is a signal for lysosomal degradation and the shift between K48 and K63 ubiquitination pattern modulates preferential degradation pathway. Aggresome formation is regulated by the interplay between E3 ligases such as Parkin and an aggregation-associated DUB Ataxin-3, which determines quantity and length of formed K63-linked chains on protein aggregates [184],[185]. Interestingly it was suggested that unanchored ubiquitin chains, cleaved off by Ataxin-3, are also recognized by HDAC6 [186]. P62/SQSTM1 participates in targeting ubiquitinated proteins into autophagosomes, while HDAC6 presence is essential for autophagosome maturation and aggresome clearance. It has been shown that under proteasomal stress, lysosomes are recruited to MTOC region and HDAC6 triggers F-actin remodeling, which in turn enables autophagosome-lysosome fusion and aggrephagy [187],[188]. Furthermore p62/SQSTM1 has been found to regulate HDAC6 deacetylase activity and recruitment of F-actin filaments [189]. HDAC6 plays an important role in tumorgenesis and tumor progression, protecting cells from elevated proteotoxic proteins. Aggresome pathway has been found to be crucial for survival of multiple myeloma cells that constantly overproduce proteins. HDAC6 inhibition induces apoptosis in multiple myeloma and combination of proteasome HDAC6 inhibitors results in the improved treatment outcome [190],[191].

1.4.5. Proteasome inhibitors

Bortezomib (PS-341, NSC 681239, Velcade®; Millennium Pharmaceuticals) was the first proteasome inhibitor that gained approval of US Food and Drug Administration (FDA). It is a synthetic dipeptide boronic acid and reversible inhibitor of the chymotrypsin-like and caspase-like activity of the 20S β5- and β1-subunits [192]. Bortezomib was first introduced for treatment of multiple myeloma (MM) and secondly for mantle cell lymphoma [193],[194]. Several mechanisms were proposed to explain the selective sensitivity of cell lines derived from hematological malignancies to proteasome inhibition. Extensive protein synthesis makes MM cells very dependent on proteasomal degradation as a disposal system for defective proteins. Accumulation of misfolded protein from the endoplasmic reticulum triggers an ER stress response and leads to arrest of protein synthesis [195]. Additionally, the high proliferation rate of the malignant cells is dependent on the proper level of cell cycle regulators and blocking of proteasomal degradation can lead to cell growth arrest [196]. Finally, MM cell lines derived from relapsed patients had elevated levels of pro-survival NFϰB, therefore negative regulation of NFϰB may be advantageous for bortezomib treatment [197]. 20S CP inhibition stabilizes the p53 tumor suppressor protein and cyclin kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ [198],[199]. Increase in the level or pro-apoptotic proteins such as Bid, Bax and JNK have also been observed [200],[201]. The role of NFϰB pathway is

controversial. Some studies suggest that interference with NFϰB is essential for bortezomib induced cytotoxicity, whereas other shown that inhibition of NFϰB signaling may be insufficient to induce apoptosis [202], [203].

The majority of patients treated with bortezomib suffer from toxic side effects such as peripheral neuropathy and thrombocytopenia [204]. Furthermore, patients treated with proteasome inhibitors ultimately experience relapse and disease progression. Mechanisms underlying resistance development are under investigation. Screening of bortezomib resistant cell lines revealed several mutations in PSMB5 gene, encoding the β5 subunit, however these were not identified in patients with multiple myeloma [196],[205]. Changes in protetoxicity regulators can also confer resistance. Overexpression of the ER chaperone protein Grp78/Bip protects mesothelioma cells treated with bortezomib from a typical ER stress response[206]. Similarly cells overexpressing heat shock protein 27 (HSP27), involved in control of protein folding and defense against oxidative stress, were found to be more resistant [207]. Finally, some studies have shown that over-expression of anti-apoptotic Bcl-2 proteins, can block bortezomib-induced apoptosis [208],[209] .

To overcome resistance several strategies for combinational therapy are under investigation. Bortezomib was found to act synergistically with DNA-damaging agents like doxorubicin and melphalan. Proteasome inhibition can enhance cell sensitivity to chemotherapeutic agents due to down regulation of proteins involved in DNA repair [210]. In addition histone deacetylase (HDAC) inhibitors were suggested to help in overcoming cytoprotective aggresome formation [211]. Inhibition of proteins from antiapoptotic Bcl-2 family can also have synergistic effects [212]. In addition a new generation proteasome inhibitors are under evaluation. Carfilozmib, an irreversible inhibitor of 20S chymotrypsin activity, is approved for the treatment of relapsed MM patients [213]. Oprozomib, ixazomib and delanzomib are orally bioavailable 20S inhibitors [214]. Marizomib is an orally available and irreversible proteasome inhibitor that blocks all three 20S proteolytic activities. Clinical trials have shown marizomib activity on bortezomib-resistant MM patients with no observed peripheral neuropathy [215].

Another potential target for new drugs are deubiquitinases. Inhibition of proteasome cysteine DUBs, leads to proteasome blocking and is particularly toxic to cancer cells [216]. Our group recently described b-AP15, a first in class inhibitor of the proteasome DUBs, USP14 and UCHL5 [217]. b-AP15 and its optimized lead VLX1570 generate response characteristic for proteasome blocking such as induction of $p21^{Cip1}$, $p27^{Kip1}$ and p53, massive accumulation of polyubiquitin chains, strong protetoxicity of misfolded proteins and expression of molecular chaperons (heat shock proteins) [218].

Interestingly several other DUB inhibitors have been described. WP1130 (degrasyn) is a small-molecule compound that inhibits Usp9x, USP5, USP14 and UCHL5. WP1130 induces the accumulation of polyubiquitin chains and reduces the level of anti-apoptotic protein MCL1 [219]. WP1130 has been shown to have antitumor activity in mouse xenograft models of prostate cancer. P5091 is a specific inhibitor of USP7, effective on bortezomib-resistant multiple myeloma xenografts. P5091 induces apoptosis, blocks angiogenesis, inhibits tumor growth and prolongs survival [220]. AC17 is a 4-arylidene curcumin analogue that irreversibly inhibits 19S RP DUB activity. AC17 inhibits NFϰB activity and reactivates the

p53 tumor suppressor. It had significant inhibitory effect on in a lung carcinoma xenograft model with no observable toxicity [221]. 9-ethyloxyimino-9H-indeno[1,2-b]pyrazine-2,3-dicar-bonitrile (USP8i) was shown to inhibit the activity of USP8 in non–small cell lung carcinoma (NSCLC). Inhibition of USP8 increased the down-regulation of oncogenic RTKs such as EGFR, ERBB2, ERBB3 and Met [222]. IU1 was identified as a specific inhibitor of USP14. Interestingly IU1 mediated inhibition of a single 19S DUB enhanced proteasomal degradation, shifting research interest from cancer therapeutic to neurodegenerative diseases associated with the accumulation of misfolded proteins [223]. Finally inhibition of ubiquitin receptor Rpn13 by RA-190 has been proposed as a treatment for MM [224].

1.5. IMMUNOPROTEASOME

In addition to the conventional 26S proteasomes an alternative form called the immunoproteasome (i-proteasome) has been identified. Level of i-proteasome is elevated in cells of hematopoietic origin, whereas in other cell types i-proteasome assembly is induced by oxidative stress and proinflamatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) [225]. Upon stimuli three catalytic subunits of 20S core β1, β2 and β5 are replaced by their homologs: iβ1/LMP2, iβ2/LMP10, and iβ5/LMP7, respectively [226]. The inducible counterparts have higher trypsin-like and chymotrypsin-like activities and lower levels of caspase-like activity, which results in preferential generation of oligopeptides with hydrophobic C termini [227].

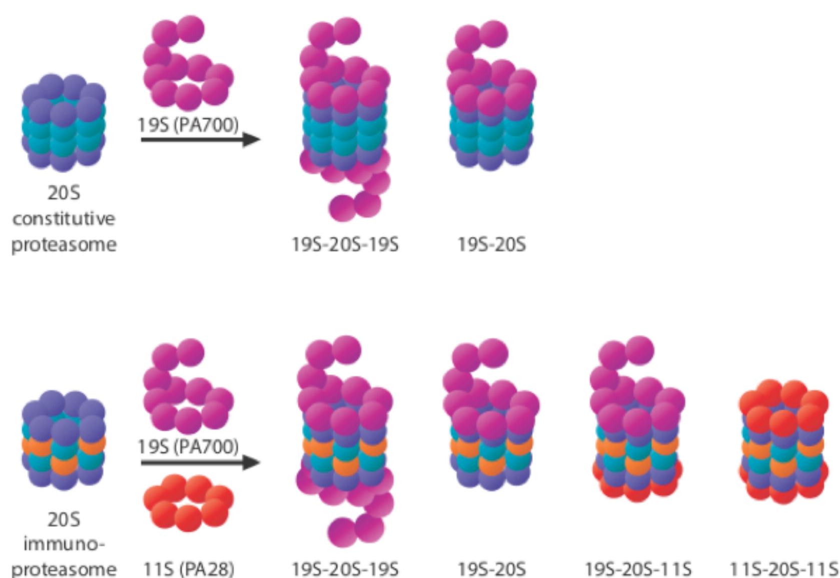

Figure 4. Formation of constitutive and immunoproteasome

Additionally, IFN-γ stimulates expression of proteasome activator 28 (PA28), which regulates access to the catalytic core [228]. Interestingly IFN-γ induced dephosphorylation of the 20S CP favors association of the catalytic core with PA28, potentially leading to enhanced i-proteasome activity [229]. The final cleavage products of i-proteasome substrates are generally 8-10 amino acid long and can be loaded onto MHC class I molecules for presentation to cytotoxic T cells as a part of immuno-surveillance. The distinct enzymatic

preferences of the i-proteasome generate more peptides with C-terminal hydrophobic anchor capable to interact with molecular cleft in MHC I [230]. Antigen processing is an essential function of i-proteasomes. Furthermore i-proteasomes have been shown to preserve cellular homeostasis upon oxidative stress and inflammatory damage. Finally, i-proteasomes are directly involved in regulation of several essential cellular pathways, such as NF-ϰB pathway components [231]. The cellular proteasome population is dynamic and contains a mixture of 20S constitutive proteasomes and i-proteasomes, with various combinations of both standard and i-subunits [232]. Additionally, different variants of catalytic core may interact with 19S or PA28 regulatory complex forming a 20S-PA28-19S hybrid and theoretically multiple combinations with distinct biological functions are possible. Large diversity of produced peptides is crucial not only for antigen production but also for the rapid cells response to the stress stimuli.

As previously mentioned, cancer cells generally display increased levels of proteasome activity, however the role of i-proteasomes in cancer is less characterized [233]. Down regulation of i-proteasomes has been observed in esophageal, renal, skin, and head and neck cancers, however the biological importance for cancer cells survival and tumor development remains unclear. On the contrary, up regulation of the i-proteasome has been reported in multiple myeloma, prostate cancer and lung cancer. The inhibition profiles of clinically used proteasome inhibitors; bortezomib and carfilzomib show similar potency towards β5 and iβ5 [234],[235]. Additionally carfilzomib has been shown to induce cell death in lymphoma cell lines with knockdown of the β5 subunit suggesting that inhibition of iβ5 solely may be sufficient to exert anticancer effect of proteasome inhibitors [235]. This finding prompted intensive search for selective inhibitors of i-proteasome subunits. The specific iβ1 inhibitor IPSI-001 has displayed promising therapeutic potential for multiple myeloma treatment. IPSI-001 leads to accumulation of ubiquitin-protein conjugates, activates caspase-mediated apoptosis and inhibits proliferation of multiple myeloma cells resistant to bortezomib [236]. However in spite of the increasing evidence the biological relevance and therapeutic potential of i-proteasome inhibitors needs further investigation.

2. AIM OF THE THESIS

The main object of this thesis is to investigate mechanism of action, binding and pharmacodynamics of a novel DUB inhibitor, b-AP15 as well as its optimized lead-VLX1570 and to define biological markers for further investigation of clinical relevance of VLX1570 in Acute Lymphoblastic Leukemia and Multiple Myeloma.

Paper I: To compare the response of cancer cells to b-AP15 and bortezomib treatment

Paper II: To study cellular uptake, metabolism and binding of b-AP15

Paper III: To characterize the response of multiple myeloma cells to VLX1570 treatment

Paper IV: To elucidate the mechanism of sensitivity of acute lymphoblastic leukemia cells to VLX1570 treatment

Paper V: To characterize changes in proteasome subpopulations in 3D cancer models and to evaluate possible influence of proteasome reorganization on cells sensitivity to proteasome inhibitors

3. RESULTS AND DISCUSSION

3.1. PAPER I: Induction Of Tumor Cell Apoptosis By A Proteasome Deubiquitinase Inhibitor Is Associated With Oxidative Stress

AIM: This study is focused on defining the differences in cellular response induced by the deubiquitinase inhibitor b-AP15 compared with bortezomib (Velcade), a clinically used 20S CP inhibitor.

RESULTS: We found that apoptotic response induced by b-AP15 is stronger and faster compared with bortezomib. We investigated the direct relationship between proteasome inhibition and cell death with a MelJuSo Ub^{G76V}-YFP, a reporter cell line constitutively expressing proteasome substrate with a fluorescent tag attached. Accumulation of the fluorescent protein after b-AP15 treatment indicated inhibition of UPS system and preceded cell commitment to death. Gene expression profiling revealed that, to some extent both b-AP15 and bortezomib induce similar stress response. Several genes associated with oxidative stress, ER stress, heat shock and the immediate early response were strongly up-regulated by both drugs. Interestingly, expression of some genes was altered more potently by b-AP15, suggesting that both drugs act in similar yet distinct way. The *HSPA6* gene, encoding HSP70B' protein was found to be induced up to 1000 fold by b-AP15 and only 60 fold by bortezomib. The HSP70B' protein is a stress induced chaperone involved in mediation of the cellular response to severe proteotoxic stress [237]. Additionally genes associated with oxidative stress (*HMOX-1, PHOX/p67*) and immediate early response (*JUN, GADD34, GADD45A* and *GADD45B*) were more strongly induced by b-AP15. Genes associated with ER stress however, were induced to similar extend by both drugs.

To further investigate the role of ER stress in b-AP15 and bortezomib mediated apoptosis, we inhibited caspase-4, which is an ER stress mediator of apoptosis [238]. Chemical or siRNA mediated inhibition of caspase-4 resulted in the reduction of apoptosis induced by both drugs. Furthermore, we found that bortezomib inhibits PKR-like ER kinase (PERK), which leads to the inhibition of eIF2 phosphorylation. It has been shown that eIF2α activation is an important cell survival mechanism, reducing misfolded proteins load on ER [239]. In contrast b-AP15 induces phosphorylation of eIF2α, yet the apoptotic response generated by b-AP15 was more rapid and potent in comparison to bortezomib. This indicates that an alternative mechanism apart from ER stress may be crucial for induction of apoptosis by 19S DUB inhibitors. We measured ROS induction with fluorescent ROS probe 2'7' dichlorofluorescin diacetate (DCFH-HA) and induction of HMOX1 protein and we found that b-AP15 induces higher level of oxidative stress compared to bortezomib. Pre-treatment with anti-oxidants resulted in reduced levels of apoptosis for both drugs. To confirm that the effect is due to ROS scavenging we ensured that the anti-oxidants used in the study do not interact with the drugs, in order to avoid formation of inactive complexes. In addition, using the previously described reporter MelJuSo cell line we analyzed the relationship between proteasome inhibition and ROS induction. Neither ROS scavengers nor ROS inducers resulted in the change of accumulation pattern of Ub^{G76V}-YFP substrate, confirming that proteasome inhibition is due to the drug activity and not due to alterations of proteasome function

mediated by oxidative stress itself.

Additionally, we found a more rapid activation of stress proteins following b-AP15 treatment; such as phosphorylation of JNK, accumulation of c-Jun and phosphorylation of p38-MAPK. We confirmed the relation between JNK activation and ROS and the contribution of JNK mediated pathway to apoptosis. Finally we show that b-AP15 analogues, generating poly-ubiquitin conjugates induce oxidative stress to the similar extend that the parent drug, whereas less active analogues were unable to induce poly-ubiquitin conjugates formation and HMOX-1 expression.

CONCLUSION: Rapid apoptotic response induced in cells by 19S DUB inhibition with b-AP15 is mediated by ER stress and oxidative stress.

3.2. PAPER II: The 19S Deubiquitinase Inhibitor b-AP15 Is Enriched In Cells And Elicits Rapid Commitment To Cell Death.

AIM: In this study we focus on mechanism of action and molecular pharmacology of b-AP15.

RESULTS: b-AP15 compound contains two different types of reactive Michael acceptors; α, β-unsaturated carbonyl units and an acrylamide residue. Toxicity investigation of analogues with substitutions at various residue sites, led to the founding that acrylamide is not required for the drug induced cytotoxicity, whereas reduced reactivity of α,β-unsaturated carbonyls is associated with lower biological activity of b-AP15. The α,β-unsaturated dienone can easily react with cysteine thiol groups in deubiquitinating enzymes.

To confirm the binding ability of the drug to USP14 and UCHL5 we performed a binding assay with Ub-VS probe. Treatment of cells with b-AP15 reduced Ub-VS labeling of both USP14 and UCHL5. Consistently inhibition of proteasome system function was confirmed in previously described MelJuSo Ub^{G76V}-YFP reporter cell line. Surprisingly, dilution of b-AP15 in cell extracts led to recovery of enzymatic function, suggesting that b-AP15 is a reversible inhibitor. We wanted to investigate if proteasome function and b-AP15 induced apoptosis can also be reversed. Cells exposed to low concentration of b-AP15 accumulate Ub^{G76V}-YFP, the proteasome substrate $p21^{Cip1}$ and polyubiquitinated proteins, however after drug removal the levels of the reporter proteins decreased. Interestingly b-AP15 induced cell death is irreversible, shown by the cleavage of caspase-3 and PARP 24h after drug removal. 1h of exposure to b-AP15 induces similar level of toxicity as continuous exposure. In contrast bortezomib requires minimum 4h of exposure to commit cells to apoptosis. We believe that the discrepancy between reversible binding and biological potency of b-AP15 can be explained by the rapid uptake and cell maintenance of the drug. 1 h incubation of the drug in the presence of cells is sufficient to observe a massive uptake of the compound from the medium. Additionally, Ligand Tracer White technique shows enrichment of the radiolabeled [^{14}C]b-AP15 in the cellular compartment 30 min after exposure and retention of approximately 50% of the compound 10 h after wash-out. These findings suggest that a significant number of active molecules remain in the cells after drug removal and are able to maintain proteasome inhibition. We confirmed that exposure of MelJuSo cells to high

concentration of b-AP15 leads to accumulation of polyubiquitinated proteins, detectable even 8 h post the drug removal. Consistently with the observations in cells, injection of b-AP15 into mice was followed by rapid clearance of the drug from plasma.

Michael acceptors easily interact with thiol groups in cysteins. Pre-treatment with N-ethylemaleimide reduces cellular level of b-AP15, suggesting that rapid uptake mechanism is thiol-dependent. Low level of a free drug in the cell after incubation indicates binding to intracellular macromolecules, possibly containing thiol groups. We found that b-AP15 binds and irreversibly inhibits thioredoxin reductase (TrxR), which regulates cellular redox homeostasis. As described in Paper I oxidative stress is considered a main mediator of apoptosis induction by b-AP15 and possibly TrxR inhibition contributes to this response. Our studies however, show that TrxR inhibition with auranoufin and the combination treatment with bortezomib and auranofin did not result in a similar degree of HMOX-1 accumulation, as did the treatment with b-AP15. In addition, we did not observe accumulation of polyubiquitinated proteins and/or Ub^{G76V}-YFP marker. Therefore, we concluded that TrxR inhibition has limited importance to overall drug toxicity and does not contribute to proteasome inhibition by b-AP15. Finally, b-AP15 did not inhibit gluthathione reductase, suggesting selectivity of the drug towards specific cellular cysteines, despite potentially reactive structure.

CONCLUSION: In conclusion the rapid b-AP15 uptake and enrichment in cells confers high cytotoxicity of the drug, despite reversible binding to proteasome deubiquitinases.

3.3. PAPER III: The Proteasome Deubiquitinase Inhibitor VLX1570 Shows Selectivity For Ubiquitin-Specific Protease-14 And Induces Apoptosis Of Multiple Myeloma Cells

AIM: In this study we examine the binding properties and cytotoxicity of an optimized lead of the 19S deubiquitinase inhibitor b-AP15 (VLX1570) and its possible clinical relevance for multiple myeloma treatment.

RESULTS: As described in study I and II, b-AP15 is a highly cytotoxic compound, however the poor solubility limits its clinical importance. An optimized lead with an improved solubility has been synthetized and proved to induced response similar to b-AP15 [240]. As b-AP15, VLX1570 contains Michael acceptor groups; therefore it is expected to bind cysteine residues in cellular enzymes. Using surface plasmon resonance (SPR), UB-VS probe labeling and Cellular Thermal Shift Assay (CETSA), we confirm binding of VLX1570 to USP14 and UCHL5 both in vitro and in vivo. Notably, we consistently report greater inhibition of USP14 in comparison to UCHL5. To confirm the relation between drug toxicity and DUB inhibition we performed siRNA knock-down experiment. Depletion of USP14 or UCHL5 resulted in decreased cell viability. The toxicity was due to the down-regulation of DUB activity, since VLX1570 compound and siRNA knock-down did not affect the proteolytic capacity of 20S proteasome. Additionally, we performed pulse and chase studies and as in study number II we observed retention of the drug in cells. This explains the strong toxicity of the compound despite reversible binding

A compound with a similar structure to b-AP15/VLX1570 was found to block ubiquitin

receptor Rpn13/ADMR1 [241], [242]. We found however, using density gradient centrifugation, co-sedimentation of accumulated polyubiquitinated proteins together with an intact proteasome structure. We therefore concluded that VLX1570 does not inhibit recognition and binding of ubiquitin conjugates to the proteasome, suggesting that ubiquitin receptor remains active.

Cytotoxicity of VLX1570 was tested on multiple myeloma cell lines and we found that the compound induces similar level of apoptosis to b-AP15. Cellular toxicity was determined by caspase-3 cleavage, accumulation of polyubiquitinated proteins, induction of chaperone Hsp70B', oxidative stress marker HMOX-1 and JNK pathway activation. Development of new proteasome inhibitors is essential in overcoming resistance developed to clinically used bortezomib. We failed to establish a cell line resistant to VLX1570, despite continuous culture of the cells with low does of the drug, however OPM2-Bz^R, a multiple myeloma cell line selected for bortezomib resistance was partially insensitive to VLX1570 treatment. We investigated further the resistance mechanism of OPM2-Bz^R cell line. While the DUB inhibition was not affected, we could observe a lower level of polyubiquitinated proteins and decreased intake of the drug. Finally we show anti multiple myeloma activity of VLX1570 *in vivo*. Reduction in tumor volume and positive survival effect was observed in xenograft multiple myeloma models in mice.

CONCLUSION: We conclude that VLX1570 is a promising candidate drug for treatment of bortezomib resistant multiple myeloma and is currently in Phase I trials in the USA (NCT02372240, clinicaltrials.gov)

3.4. PAPER IV: Acute Lymphoblastic Leukemia Cells Are Sensitive To Disturbances In Protein Homeostasis Induced By Proteasome Deubiquitinase Inhibition

AIM: In this paper we examine alterations in protein synthesis in acute lymphoblastic leukemia (ALL) cell lines in response to VLX1570 treatment.

RESULTS: We determined senistivity of 9 ALL cell lines to VLX1570 and found that most of the cells were highly sensitive with IC_{50} in the range of 50-100nM. The level of sensitivity in these cells was similar to that of multiple myeloma cells tested in study III, which exepmlifies the general vulnerability of cells with high protein turn over rate to proteasome inhibition. As previosuly shown, treatment with VLX1570 at cytotoxic doses resulted in accumulation of polyubiquitinated proteins and stress markers such as HSP70B', $p21^{Cip}$ and HMOX-1.
Suprisingly we found that increase of the compund concentration over certain level leads to decrease of the markers expression. To investigate this phenomenon further, we exposed 3 different ALL cell lines to VLX1570 and perforemd proximity antibody-based extension multiplex assay ProSeek™ to examine expression of 184 protein marekrs. Amongst 70 common markers found in all 3 ALL cell lines, HMOX-1, $p21^{Cip1}$ and VEGF-A were most strongly induced. Several proteins, however, decreased significantly after exposure to the compound. The effect was most promient for the sensitive cell lines and for high doses of VLX1570. Treatment with concentration 10 times higher than IC_{50} resulted in dimishing of

forementioned marker proteins. Comparison of expression between insoluble and soluble cell fractions did not show any differeces, therefore this discrepancy can not be simply explained by inclusion of the proteins into insoluble cell fraction but rather by changes in protein synthesis. In order to examine protein synthesis rate we measured incorporation of [^{3}H]-leucine in cells treated with VLX1570. We have observed a dose dependent decrese in [^{3}H]-leucine incorporation, already at 6h after incubation, when cells viabilty was not affected. The repression effect was stronger in sensitive SUP-B15 cells in comparison to more resistant MOLT-4 cells, suggesting that decreased protein synthesis does not provide protection mechanims to protesome inhibition. Correlation between exposure to high doeses of VLX1570 and impared protein synthesis provides, however, the explanation for vanishing of proteins such as HMOX-1, HSP70B' or p21^{Cip1}. In most of the tested cell lines however, proteasome inhibition was not correlated to decreased protein synthesis.
Treatment with L-asparaginase (L-asp) is an approved therapy for ALL. L-asp is known to affect amino acid homeostasis in ALL cells and we found that it causes a subtle but significant reduction of [^{3}H]-leucine incorporation in all tested cells lines. Inhibition of proteasome and disturbancies in amino acid synthesis could have potentially synergistic effects. We combined VLX1570 with L-asp and found a strong synergistic effect between this two compounds in sensitive SUP-B15 cell line and an additive effect in remaining cell lines. The effect of combination on protein synthesis, had however a very minor effect, suggetsing a distinct mechanism of protein synthesis reduction conferred by this two compounds.
In order to find the mechanim behind protein synthesis repression, we examined the phosphorylation pattern of two markers: EIF2α- a protein translation regulator; and eukarotic elongation factor 2 (EF2K), involved in repression of elongation phase of protein synthesis. No phosphorylation of EIF2α or EF2K was observed at pharmacologically relevant concentrations. In addition, combination of VLX1570 and L-asp did not lead to accumulation of phosphorylated EIF2α. Finally, we investigated the effect of Integrated Stress Response Inhibitor (ISRIB), a small molecule known to circumvent inhibitory pathway of EIF2α phosphorylation. Treatment with ISRIB did not overcome the effect of VLX1570 on protein synthesis, therefore we concluded that protein synthesis downregulation in ALL cells exposed to VLX1570 is uncoupled to phosphorylation of eIF2α.

CONCLUSION: VLX1570 leads to disruption of protein homestasis in ALL cells and is a promising canditate for treatment of acute lymphoblastic leukemia, as a single therapy agent or with combination with clinically used L-asp.

3.5. PAPER V: Induction Of PA28 In 3D Culture Confers Resistance To Proteasome Inhibitors

AIM: In this study we suggest upregulation of immunoproteasome as the possible mechanims of resistance to proteasome inhibitors in solid tumors.

RESULTS: We have investigated the effect of proteasome inhibitors on multicellular spheroids (MCS) and found that effectiveness of the treatment is decreased along the

diameter of spheroid. Exposure to bortezomib or b-AP15 induced response typical for proteasome inhibitors (induction of p53, $p21^{Cip1}$ and caspase 3) in the periphery of MCS, while the inner parts remained unaffected. In order to explain the mechanim behind this resistance we analysed expression pattern of proteasome subunits on a panel of cell lines cultured as MCS. We have obsereved strong up regulation of PSME1 and PSME2 proteins, the components of PA28 regulatory particle. Notheworthy this effect was significantly lower in immortalised retinal epithelial cells cultured as MCS, suggesting the response pathway typical for cancer cells. With RT-PCR analysis we have confirmed that induction of PSME1 occurred on the transcriptional level, with 10-fold induction after 7 days of growth in3D culture, while expression of 20S or i20S was less affected. Immunochistochemistry staining of MCS sections revealed that indcution of PSME1/PSME2 occurred mainly in the spheroid core, whereas PSMB5, a 20S subunit, was localised primary in the periphery. This observation suggested that previously observed resistance of MCS core to proteasome inhibitors could be partially explained by upregulation of PA28 proteasome subunit. Treatment with proteasome inhibitors of HCT116 cell lines stably overexpressing PSME1 or PSME2 confirmed that apoptotic response was decreased in those cells in comparison with HCT116 wild type. Staining of tumor biopsies from mouse model of lewis lung carcinoma showed increased level of PSME1. Interestingly tumors from b-AP15 treated animals show up regulation of PSME1 in areas unaffected by the treatment. Since i-proteasome is known to be induced under oxidative stress or inflammation we were interested if similar response can occur upon exposure to other stress condition. Solid tumors tend to have regions with poor vasculature which are subjected to low pH, therefore we investigated the expression pattern of PSME upon exposure to low pH. Transient exposure as well as adaptation to low pH lead to increased expression of PA28 particles. mRNA sequencing revealed that under conditions of low pH, induction of PA28 was correlated with reduced levels of PSMD14, a constitutive 19S DUB. Interstingly proteolytic activity of i-20S components were found lower in both cells cultured in low pH and in 3D, suggesting distinct mechanism between induction of PA28 regulatory particle and 20S catalytic core.

CONCLUSION: Induction of PA28 observed in multicellular spheroid model can partially explain the resistance mechanims to proteasome inhibitors in solid tumors.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

4.1. b-AP15 is a reversible inhibitor with an irreversible activity

b-AP15 contains two Michael acceptors, which interact easily with nucleophiles such as thiol groups. We have concluded that Michael reaction occurs between b-AP15 α,β-unsaturated carbonyl units and the cysteine located at the enzymatic catalytic triad of USP14/UCHL5. Interestingly, we did not observe a general inhibition of cytoplasmic DUBs suggesting a degree of specificity. In addition molecules that share structural features with b-AP15 such as WP1130 or AC17 seem to inhibit a different set of DUBs, suggesting specificity of interaction between these molecules and their primary targets [219],[221]. We have found that inhibition of USP14/UCHL5 with b-AP15 is reversible, both *in vitro* and in cellular assays, however commitment to death induced by the drug is irreversible. We believe that this phenomenon can be explained by the fast uptake of the drug and its subsequent retention in the cellular compartments. We have observed rapid clearance of b-AP15/VLX1570 from cell incubation media and in animal tumor models; VLX1570 disappeared from plasma shortly after the treatment. Such fast distribution can be clinically beneficial, as short exposure to the drug will result in prolonged effect.

4.2. b-AP15/ VLX1570 toxicity is due to 19S DUB inhibition

Our group has previously shown that b-AP15 inhibits the two 19S DUBs, USP14 and UCHL5. VLX1570 is an analogue of b-AP15 with a strong anticancer activity and increased solubility, optimized for clinical application. By multiple *in vitro* and cellular assays (enzymatic assays, reporter cell line, Ub-VS labeling, SPR, CETSA) we have confirmed binding of VLX1570 to USP14/UCHL5, with stronger affinity towards USP14. siRNA mediated knockout of USP14 or UCHL5 resulted in reduced viability of multiple myeloma cells, known to have high expression level of USP14. VLX1570/b-AP15 is an inhibitor of USP14/UCHL5, whereas bortezomib and other clinically available inhibitors block the activity of the 20S catalytic core. Different mechanism of action gave the possibility to use DUB inhibitors for relapsed and refractory multiple myeloma resistant to conventional proteasome inhibitors.

4.3. b-AP15 and bortezomib elicit similar yet distinct cellular response

As discussed previously b-AP15 and bortezomib interfere with proteasomal degradation, however the cellular response to 19S DUB inhibition is different to the one induced by bortezomib. Although treatment with bortezomib as well as with b-AP15 results in the accumulation of proteasome substrates (p53, $p21^{Cip}$, $p27^{Kip1}$), apoptotic response induced by b-AP15 is stronger and more rapid. In addition treatment with b-AP15 induces more potent proteotoxic response (indicated by massive accumulation of chaperone HSP70B') and higher levels of ROS. This data taken together suggests distinct mechanisms behind b-AP15 and bortezomib mediated cytotoxicity. One possible explanation to the discrepancy between the two drugs is that the inhibition of DUBs leads to accumulation of misfolded proteins with

longer polyubiquitin chains than inhibition of 20S proteolytic core. Massive accumulation of polyubiquitinated proteins may disrupt the integrity of cellular membranes and organelles and indeed treatment with VLX1570 (b-AP15 homolog) was found to increase permeability of mitochondria outer membrane [243].

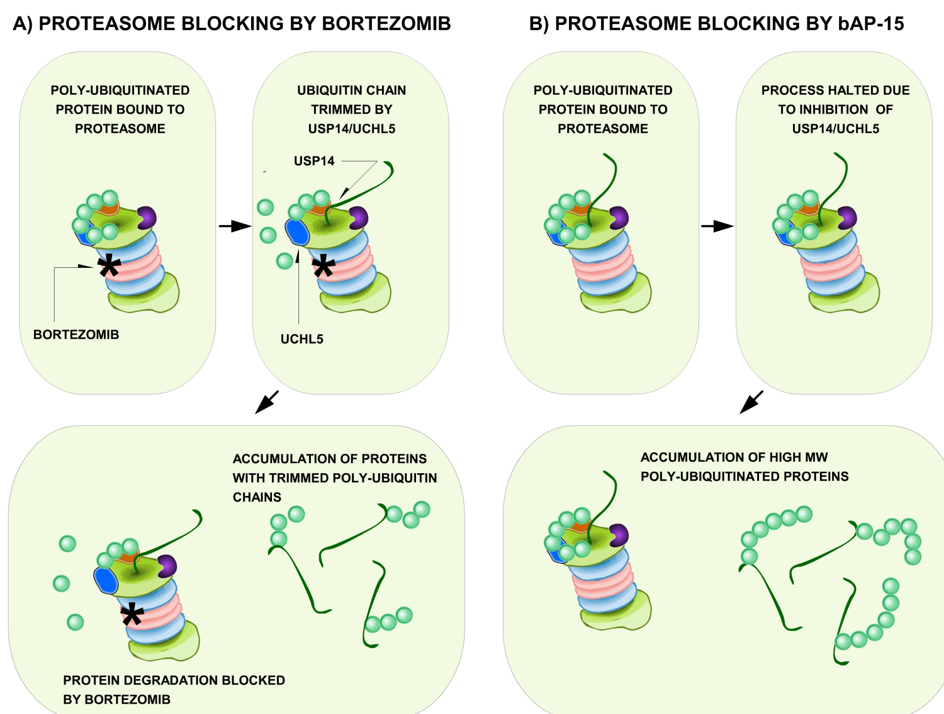

Figure 5 Mechanism of proteasome inhibition induced by bortezomib and b-AP15

Increased permeability of mitochondrial membrane may contribute to high levels of toxic ROS and we found ROS induction to be crucial for b-AP15 mediated toxicity. On the other hand we have synthetized a group of b-AP15 analogues for which induction of HMOX-1 was not correlated with apoptosis or accumulation of polyubiquitin chains. Finally, the differences in cellular response to these two compounds may be attributed to off-targets of the drug. We identified TrxR as one of b-AP15 off-targets and although TrxR inhibition contributed only mildly to overall drug toxicity it cannot be excluded that other, not yet identified targets enhance the potency of b-AP15.

4.4. ER stress is not essential for VLX1570 mediated toxicity in ALL cells

Cancer cells of hematopoetic origin such as MM are known to be sensitive to proteasome inhibition [243],[244]. Consistently we have discovered similar level of sensitivity to VLX1570 in ALL cells. Induction of ER stress (detected as phosphorylation of EIF2α) is consider as a typical response to proteasome inhibition and is associated with massive accumulation of misfolded polyubiquitinated proteins [149],[217]. In ALL cells we observed accumulation of poly-Ub chains in a dose dependent manner, however this was not related to ER stress. Furthermore the pattern of EIF2α phosphorylation did not correlate with drug induced cytotoxicity. Inhibition of protein synthesis is a cellular response to many stress

factors and EIF2α phosphorylation is an important checkpoint mediating this pathway [245]. Inhibition of protein synthesis is considered as a protective mechanism to proteotoxic stress induced by proteasome inhibitors. Surprisingly in ALL cells general protein synthesis inhibition did not correlate with cell sensitivity to drug treatment. This study shows how careful selection of relevant drug concentrations for pre clinical evaluation is crucial. In addition many assumptions considered dogmatic need evaluation when testing under new conditions.

4.5. PA28 may alter solid tumors response to proteasome inhibitors

Despite constantly growing interest in the field of UPS, clinical application of proteasome inhibitors is limited to several hematological malignancies and even though patients respond positively to treatment, they often acquire resistance and experience disease relapse. Furthermore, clinical trails performed on solid tumors were relatively unsuccessful despite several studies showing promising preclinical results [141]. It suggests that primary and acquired resistance need to be considered when new treatment based on proteasome inhibition is developed. 3D cultures of cancer cells mimic conditions of solid tumors growth; therefore we investigated the response of cells cultured as MSCs to proteasome inhibition. We found that layers of cells do not respond in the same way to the drug exposure and that outer layers are more sensitive that the spheroid core, which in fact resembles microenvironment of solid tumors more accurately [246]. Our preliminary results suggest correlation between the resistance to proteasome inhibitors and overexpression of PA28, a stress-induced regulatory particle of the 20S proteasome.

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