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Inhibition of proteasome deubiquitinase activity: a strategy to overcome resistance to conventional proteasome inhibitors?

Karthik Selvaraju^a, Magdalena Mazurkiewicz^b, Xin Wang^a, Joachim Gullbo^c, Stig Linder^{a,b}, and Pádraig D'Arcy^{a}*

^a *Department of Medical and Health Sciences, Linköping University, Linköping, SE-581 85, Sweden.*

^b *Department of Oncology and Pathology, Karolinska Institute, SE-171 76, Stockholm, Sweden.*

^c *Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, SE-751 85, Sweden.*

* Corresponding author. Tel +46 10 1036362

E-mail address: Padraig.Darcy@liu.se

Abstract

The ubiquitin-proteasome system (UPS) is the primary mechanism controlling the degradation of damaged, unwanted or short-lived proteins in eukaryotic cells. In addition to protein homeostasis, the UPS has also emerged as a critical node in the regulation of signalling pathways implicated in the growth and survival of cancer cells. The absolute dependency of cancer cells on a functioning UPS has been exploited in the development of anti-cancer therapies as exemplified by development of proteasome inhibitors for the treatment of certain leukemic malignancies. Deubiquitinases (DUBs) are enzyme components of the UPS that catalyse re-editing of poly ubiquitin chains and/or removal of ubiquitin *en bloc* from target substrates leading to alterations in protein stability and/or downstream signalling. There is a growing recognition that targeting DUB activity may be a feasible option for the development of novel anti-cancer therapies. In particular inhibition of proteasomal cysteine DUBs (i.e. USP14 and UCHL5) has been shown to be particularly cytotoxic to cancer cells leading to the accumulation of ubiquitinated proteins and proteotoxic stress. In this review we focus on the mechanisms of action of proteasome DUB inhibitors as well as the potential of such compounds to circumvent acquired drug resistance in cancer patients.

Keywords: cancer therapeutics, small molecule inhibitors, proteasome, deubiquitinase, DUB, α,β -unsaturated ketones, apoptosis.

1. Introduction

1.1 The ubiquitin-proteasome system

Cellular protein degradation is a highly controlled event that ensures that only proteins that are damaged, mis-folded or temporally regulated are removed and destroyed. The ubiquitin proteasome system (UPS) is the main non-lysosomal pathway for protein degradation. At its most basic level, the UPS consists of a selectivity tagging factor in the form of the small molecule ubiquitin, and a multi-subunit proteolytic complex, the proteasome, that functions as the cells' molecular shredder by breaking down unwanted proteins into smaller peptides for subsequent use in cellular metabolism or for presentation in complex with MHC class I to the immune system (Goldberg, 2007; Hershko and Ciechanover, 1998) (**Fig. 1**). Ubiquitin is a 76 amino acid protein that is highly conserved between all eukaryotes. As part of its function as a destruction tag, it is covalently attached via an isopeptide bond between the carboxy glycine residue of ubiquitin and the ϵ -amino groups of lysine residues present in proteins destined for degradation. The presence of highly specific ubiquitin receptors on the proteasome ensures that only those proteins displaying the ubiquitin destruction tag are allowed to enter the proteolytic chamber. This degree of selectivity provided by ubiquitin ensures that degradation is a highly controlled event and prevents the unintended proteolysis of cellular proteins. The UPS controls the stability of a diverse range of cellular proteins. Certain substrates such as mis-folded or damaged proteins are targeted to the UPS as a part of a cellular quality control mechanism whose main function is to prevent proteotoxicity by rapidly eliminating the build-up of potentially harmful or toxic proteins. Other UPS regulated proteins are those whose regulation and function is inherently linked to their expression and stability, e.g. cell cycle mediators, oncogene and

tumour suppressor proteins (Coux et al., 1996; Hershko and Ciechanover, 1998).

The molecular shredder of the UPS is the 26S proteasome, a large multi-subunit protease complex of at least 50 subunits found in the cytosol and the nucleus of eukaryotic cells (Bochtler et al., 1999; Goldberg, 2003; Tanaka et al., 1983). The 26S proteasome can be further sub-divided into two functionally distinct complexes: the cylindrical 20S core particle (CP) that contains the active sites necessary for proteolysis and the 19S regulatory particle(s) (19S RP) that functions as a selective gatekeeper and facilitator of trafficking into the 20S CP. The 20S CP consists of two stacked heptameric inner β -rings capped by two heptameric α -subunits giving the complex a barrel-like cylinder structure (Groll et al., 1997; Unno et al., 2002). The proteolytic activity of the 20S CP is mediated by the $\beta 1$, $\beta 2$ and $\beta 5$ subunits, associated with caspase-like, trypsin-like and chymotrypsin-like activity respectively, based on the proteolytic preference of the individual β subunits for amino acid sequences in target proteins. The hydrolysis of proteins by these subunits generally results in the generation of small peptides of about 3 - 15 amino-acid residues that are expelled from the proteasome for further use in subsequent cellular processes (Groll et al., 1997; Lowe et al., 1995; Puhler et al., 1992; Zwickl et al., 1992). Control of protein translocation to the 20S CP is mediated by the 19S RP, a large complex that caps one or both ends of the 20S CP. The 19S CP contains at least 19 different subunits that are further sub-divided into the lid and base complexes (Glickman et al., 1998; Lander et al., 2012). The base of the 19S RP consists of ten subunits, six of which are related AAA⁺ ATPases, in direct contact with the α subunits of the 20S CP (Tomko et al., 2010). The ATPase activity of the 19S RP is an absolute requirement in facilitating substrate unwinding and opening of the translocation gate of the 20S CP. The other four base subunits are the scaffolding

proteins Rpn1 and Rpn2 (Bohn et al., 2010), which link the 19S RP to the 20S CP and the ubiquitin receptors S5a/Rpn10 and Rpn13, which recognize and tether ubiquitinated proteins to the proteasome for subsequent processing and degradation (Deveraux et al., 1994; Husnjak et al., 2008).

The attachment of multiple ubiquitin units to proteins provides the functional signal that induces trafficking to the proteasome. However not all ubiquitin signals are equal, the nature of the linkages between ubiquitin molecules on any particular protein ultimately determines the outcome. A ubiquitin code exists whereby proteins tagged with poly-ubiquitin linked via internal K48 or K33 lysine residues are targeted to the proteasome (Kulathu and Komander, 2012). Other linkage types such as K63 are generally involved in non-proteolytic roles such as the regulation of DNA repair and endocytosis pathways. In addition to the nature of lysine linkage types between ubiquitin in a poly-ubiquitin chain, the number of ubiquitin moieties is also an important factor. At least four ubiquitin moieties are the minimum requirement to signal trafficking to the proteasome for degradation. This appears to be a spatial requirement since 4 ubiquitin molecules is the minimum number needed to span the distance between the Rpn10/Rpn13 ubiquitin receptors and the deubiquitinase (DUB) Rpn11/POH1, which plays a key role in the coupling of ubiquitin chain removal to proteasome degradation (see below) (Lander et al., 2012; Piotrowski et al., 1997; Verma et al., 2002; Yao and Cohen, 2002).

The ubiquitination of protein substrates is regulated by an apex cascade of three enzymes, Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3) enzymes that catalyse the activation and conjugation of ubiquitin to target proteins (Hershko et al., 1983). Specificity is conferred by

the E3 ligases that bind target substrates and co-ordinate the covalent attachment of ubiquitin to lysine residues in the polypeptide chain (**Fig. 1**). There are > 500 identified E3 ligases in cells, making these enzymes the main specificity factor in the UPS (Hershko et al., 1983; Pickart and Eddins, 2004; Voges et al., 1999). The process of ubiquitination is not a one-way street however; the reverse reaction, that is the removal of ubiquitin also occurs in a highly regulated manner. Specialized enzymes, known as deubiquitinases (DUBs), cleave the isopeptide bond between lysine residues on target proteins and the C-terminal glycine residue of ubiquitin thus effectively reversing the activity of the E3 ligases. The human genome encodes ~80 functional DUBs (Komander et al., 2009), broadly classified into six groups based on sequence homologies in the catalytic domain: ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolases (UCHs), ovarian-tumour proteases (OTUs), Machado–Joseph disease protein domain proteases (MJD), JAMM/MPN domain-associated metalloproteases (JAMMs) and monocyte chemotactic protein-induced protein (MCPiP) (Amerik and Hochstrasser, 2004; Fortelny et al., 2014; Fraile et al., 2012; Nijman et al., 2005). The biological roles of many of these DUBs is a rapidly expanding area of current research; however one thing that is apparent from recent reports is that many of these DUBs appear to play roles in the genesis, maintenance and treatment response of multiple cancer types. As such there is a growing recognition of the therapeutic potential of targeting DUBs as a viable anti-cancer targets. Considering the vast number of DUBs identified we will focus the remainder of the review on proteasome associated DUBs, their potential as drug targets as well as their use as novel therapies to overcome acquired drug resistance.

1.2. The role of proteasome associated deubiquitinases (DUBs)

In order to facilitate degradation, specialized DUBs located within the 19S RP function by removing the bulky polyubiquitin chains on target proteins following their arrival and docking at the proteasome (Verma et al., 2002; Yao and Cohen, 2002). Failure to remove the bulky ubiquitin chains may otherwise sterically hinder the translocation of substrates into the 20S for proteolytic degradation. Other roles include a ubiquitin editing and rescue function, preventing degradation of improperly or insufficiently tagged substrates, a ‘toll booth’ analogous regulation of ATPase and gate activity and the recycling of ubiquitin chains for further use within the UPS (Finley, 2009; Peth et al., 2009; Peth et al., 2013). In mammalian cells three distinct DUBs are associated with the proteasome: Rpn11/POH1, Ubp6/USP14 and Uch37/UCHL5 (yeast/human nomenclature). POH1 is a metalloprotease belonging to the JAMM domain family and is an integral part of the 19S RP lid (Verma et al., 2002; Yao and Cohen, 2002). POH1 is essential for the viability of both yeast and metazoan cells, with siRNA depletion resulting in the destabilization of the 19S RP lid complex (Gallery et al., 2007; Rinaldi et al., 1998). POH1 contains a JAMM/MPN⁺ motif sequence where an aspartic acid and two histidine residues coordinate a zinc ion that is essential for DUB activity (Ambroggio et al., 2004; Maytal-Kivity et al., 2002). The activity of POH1 is thought to be tightly controlled and only becomes activated once the proteasome is committed to substrate degradation (Lee et al., 2011b; Verma et al., 2002; Yao and Cohen, 2002). POH1 cleaves the proximal end of the poly-ubiquitin chain from the substrate resulting in the release of a free ubiquitin chain and further translocation of the newly de-ubiquitinated substrate to the 20S CP for degradation (Yao and Cohen, 2002). Two other DUBs, UCHL5 and USP14 are cysteine proteases that are also physically associated with

the base complex of the 19S RP. Interestingly their intrinsic DUB activity is only stimulated upon incorporation into the proteasome, with unbound enzymes displaying significantly reduced levels of DUB activity (Leggett et al., 2002; Yao et al., 2006). Analysis of the structure of USP14 and UCHL5 has revealed the basis of their proteasome dependent activation. In USP14 the ubiquitin binding pocket is obscured by two loops that must be restructured in order to allow access to the active site and activation of deubiquitination activity (Hu et al., 2005). The binding of USP14 to the base of the 19S RP is believed to induce conformational changes in the two loops making the active site more accessible for ubiquitin (Hu et al., 2005), and resulting in ~1000 fold increase in intrinsic DUB activity (Borodovsky et al., 2001). In the case of UCHL5, the UCH-domain contains a crossover loop that precludes the active site and prevents substrate access. Interaction of UCHL5 with the ubiquitin receptor Rpn13/Admr1 reverses this auto-inhibitory function leading to restoration of UCHL5 DUB activity (Hamazaki et al., 2006; Qiu et al., 2006; Yao et al., 2006).

In contrast to POH1, USP14 and UCHL5 share a different mode of action and preferentially cleave ubiquitin from the distal ends of ubiquitin chains leading to progressive chain shortening the recycling of ubiquitin back into the UPS. USP14 preferentially cleaves Lys48-linked polyubiquitin chains (Hanna et al., 2006; Hu et al., 2005), whereas UCHL5 appears to be more promiscuous, cleaving both Lys48- and Lys63-linkage types (Jacobson et al., 2009). Interestingly USP14 appears to hinder proteasome degradation by actively trimming ubiquitin chains on target proteins resulting in prolonged occupancy at the 19S RP and potentially favouring release back to the cytosol (Hanna et al., 2006; Lee et al., 2010; Lee et al., 2011a). It would also appear that USP14 is functionally redundant in mammalian cells since knock down

produced no discernable effects on proteasome structure or polyubiquitin levels (Koulich et al., 2008). Consistent with this, the recently developed small molecule USP14 inhibitor IU1 reduces chain trimming and stimulates the proteasomal degradation of several reporter substrates (Lee et al., 2010). Although non-essential for general cell viability, USP14 does appear to be important for cell survival under certain conditions including metabolic stress and neuronal development (Chen et al., 2009; Chernova et al., 2003; Walters et al., 2008). In particular USP14 appears to play a key role in neuronal function. Mice deficient for USP14 display progressive ataxia and die by 6–10 weeks of age (Wilson et al., 2002), possibly due to defective ubiquitin recycling at synapses leading to altered UPS function (Chen et al., 2009).

In a manner similar to USP14, UCHL5 has also been shown to inhibit protein degradation by shortening the chains of inappropriately or poorly modified substrates under certain conditions (Koulich et al., 2008; Lam et al., 1997). Interestingly an orthologue of human UCHL5 has not been found in *S. cerevisiae* whereas the *S. pombe* orthologue Uch2 is nonessential for viability (Li et al., 2000) possibly suggesting a degree of redundancy between UCHL5 and USP14.

2. Targeting the UPS in cancer

Cancer cells are particularly sensitive to fluxuations in the UPS. The exact biological mechanism to why cancer, but not normal cells are sensitive to reduced UPS capacity is likely due to higher rates of protein synthesis and thus greater requirement for UPS mediated protein turnover to maintain homeostasis. This along with a susceptibility to apoptosis induced by endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) following decreased proteasome activity likely explains the cytotoxic effects of proteasome inhibitors on cancer cells (**Fig. 2**).

Comparisons of the sensitivity of different cancer sub-types towards proteasome inhibitors does appear to support this, since those cancers with high protein turnover rates also tend to be the most sensitive to proteasome inhibition. For example the plasma cell malignancy, multiple myeloma, hyper-synthesizes defunct immunoglobulins and is generally the most sensitive cancer type towards UPS inhibitors. Such cells with high protein turnover rates can be thought of as in a near constant state of proteotoxic stress caused by the tremendous strain on the protein translation machinery and predisposition to accumulate defective proteins. Thus even small changes in UPS activity are sufficient to result in a catastrophic accumulation of mis-folded immunoglobulin triggering the unfolded protein response (UPR), ER stress and apoptosis (Meister et al., 2007). The clinical development of bortezomib as a treatment for multiple myeloma has validated targeting the UPS as a viable strategy for cancer drug development (Richardson and Anderson, 2003). Bortezomib is peptide boronate derivative that primarily inhibits the chymotrypsin-like activity of the $\beta 5$ subunit, and to a lesser extent the caspase-like activity of the $\beta 1$ subunit of the 20S CP by a covalent but slowly reversible mechanism. The remarkable success of bortezomib has generated much interest in identifying the next generation proteasome inhibitors with different mechanisms of action, altered binding properties or activities on different tumour types (Mitsiades et al., 2009). The epoxyketone carfilzomib (PR-171) displays a similar mechanism of action to bortezomib by primarily targeting the chymotryptic activity of the 20S CP (Demo et al., 2007). However; a major difference between the two compounds is drug occupancy, with carfilzomib displaying an irreversible covalent binding mechanism to sites in the $\beta 5$ subunits. Since carfilzomib displays differential binding properties compared to bortezomib it has been approved by the FDA for treatment of multiple myeloma patients that have previously received two prior therapies, including bortezomib and

who subsequently relapsed. Marizomib (NPI-0052), a natural product derived from the fermentation of the marine actinobacterium *Salinospora* (Chauhan et al., 2005), also inhibits the chymotryptic-like activity of the 20S proteasome via a similar mechanism of action to bortezomib and carfilzomib (Herndon et al., 2013; Obaidat et al., 2011; Singh et al., 2010). Marizomib recently received orphan drug status for the treatment of multiple myeloma and is currently undergoing clinical evaluation (Ma and Diao, 2014).

3. Proteasomal DUBs as Drug Targets for Cancer Therapy

The development of proteasome inhibitors has also led to interest in identifying alternative targets that function upstream of the proteasome degradation machinery. Based on their diversity DUBs represent a potential wealth of untapped targets for drug development. DUBs involved in regulating p53 stability or the cell-surface expression of oncogenic receptors have already been mooted as tempting targets for therapeutic intervention with several candidate drugs already in various stages of pre-clinical development (Byun et al., 2013; Chauhan et al., 2012). In particular targeting proteasome-associated deubiquitination has the potential for increasing the levels of ubiquitin conjugated proteins triggering proteotoxic stress and apoptosis similar to that observed with inhibitors of proteolytic activity. The metalloprotease POH1 is an obvious target since it is absolutely required for cell viability and is overexpressed in a variety of tumours (Gallery et al., 2007). Although neither USP14 nor UCHL5 appears to be essential for cell survival alone, dual knockdown using RNA interference has been shown to lead to the accumulation of poly-ubiquitinated substrates and loss of cell viability (Koulich et al., 2008; Tian et al., 2013; Wang et al., 2014). Interestingly several reports have shown that USP14 is consistently overexpressed in solid tumours of lung and ovarian origin and associated with metastases in colorectal carcinoma

suggesting a potential role in oncogenesis (Shinji et al., 2006; Wang et al., 2015; Wu et al., 2013). Considering that USP14 and UCHL5 belong to different classes of DUBs, dual inhibition of these enzymes by small molecules would *a priori* be expected to be possible only in the context of broad-spectrum DUB inhibition. Recently however, a number of compounds have been described that preferentially inhibit proteasomal DUB activity compared to other cytosolic DUBs. The chalcone-derivative b-AP15 (NSC687852) inhibits the activity of USP14 and UCHL5 at concentrations of $\sim 10 \mu\text{M}$ *in vitro* and $\sim 1 \mu\text{M}$ *in vivo* ((D'Arcy et al., 2011), Wang *et al.*, submitted) (**Fig. 3**). The comparatively higher potency observed *in vivo* is associated with rapid uptake and enrichment in cells (Wang et al., 2014). The cytotoxicity of b-AP15 is tightly linked with functional proteasome inhibition, with the accumulation of the proteasome reporter substrate Ub^{G76V}-YFP (Menendez-Benito et al., 2005) preceding and correlating with cell death (Brnjic et al., 2013; Wang et al., 2014b). Examination of the cellular stress response to b-AP15 using gene expression profiling and CMAP analysis (Lamb et al., 2006) showed a similar stress response to that observed following treatment with several well characterized proteasome inhibitors (D'Arcy et al., 2011). In particular genes such as those regulating the chaperone response, ER stress and oxidative stress are typically up regulated following treatment with b-AP15 or 20S CP proteasome inhibitors. RA-9 is a small molecule structurally related to b-AP15 that also inhibits proteasomal deubiquitinase activity resulting in a similar stress response and cytotoxicity profile (Brnjic et al., 2014; Coughlin et al., 2014). Another pair of structurally related compounds, G5 and F6, initially identified in a screen for agents that induce apoptosis Bcl-2 over-expressing cells; were subsequently shown to be potent inhibitors of general DUB activity leading to acute proteotoxic stress and apoptosis (Aleo et al., 2006). Finally the 4-arylidene curcumin analog AC17 has been demonstrated to irreversibly inhibit the deubiquitinase

activity of the 19S RP leading to cell death in *in vitro* and *in vivo* models of lung cancer (Zhou et al., 2013). Although these compounds are chemically diverse, a shared similarity is the presence of α , β -unsaturated ketones and accessible β -carbons in their chemical structure (**Fig. 3**). The DUB inhibitory activity is likely to be derived from the electrophilic nature of these compounds and coupled with the fact that the vast majority of DUBs are cysteine proteases that are sensitive to electrophilic attack. Generally cysteine residues in proteins are nucleophilic and tend to show high levels of variation with regard to their reactivity to electrophiles (Codreanu et al., 2014; Weerapana et al., 2010). α , β -unsaturated ketones are generally considered as relatively soft electrophiles and are speculated to display selective reactivity towards a specific subset of cysteine thiolates in proteins (Aldini et al., 2006). The relative specificity of the various DUB inhibitors described above could potentially be due to the relatively strong reactivity of the cysteine residues located in the active sites of a limited number of DUBs. Interestingly the specificities of α , β -unsaturated ketone-containing compounds towards various DUBs have been reported to vary considerably. For example whereas b-AP15 was reported to be specific to proteasomal deubiquitinases (D'Arcy et al., 2011), the structurally related compound RA-9 was reported to display more broad range activity and shown to inhibit UCHL1, UCHL3, USP2 and USP8 in addition to the proteasomal DUBs (Anchoori et al., 2011; Coughlin et al., 2014; Issaenko and Amerik, 2012). The chalcone-derivative G5 was also reported to display a broad spectrum of inhibition of DUB activity (Aleo et al., 2006; Nicholson et al., 2008). The curcumin-analog AC17 was found to be a specific irreversible proteasomal DUBs inhibitor producing a cellular phenotype similar to that observed with b-AP15 (D'Arcy et al., 2011; Zhou et al., 2013). The mechanism(s) underlying these variations in target specificity are unclear, however, it does appear that not all cysteine enzymes are general targets of α , β -unsaturated ketone-containing

compounds, since neither glutathione reductase nor caspase-3, both of which contain critical cysteine residues in their active sites, are inhibited by b-AP15 (Wang et al., 2014b) (X.W., unpublished information). However it is likely that some of these differences may reflect the differential binding to target DUBs based on the 3-D conformation of the inhibitors. Considering the similar molecular structures of these drugs, it is surprising however that G5/F6 are broad specificity DUB inhibitors, whereas b-AP15 is specific to proteasomal DUBs. Further work will hopefully clarify these issues. Irrespective of the degree of specificity of different compounds to proteasomal DUB activity, it is likely that inhibition of proteasomal DUB (USP14/UCHL5) activity is of key importance in producing the cytotoxic effects observed when cells are exposed to α,β -unsaturated ketone-containing compounds.

4. Overcoming bortezomib resistance using deubiquitinase inhibitors

4.1. Mechanisms of resistance to bortezomib and other inhibitors of 20S proteasome activity

In spite of the clinical success of bortezomib in the treatment multiple myeloma, the majority of patients eventually acquire resistance and relapse (Kale and Moore, 2012; Kumar and Rajkumar, 2008; Niewerth et al., 2015). The molecular mechanisms underlying the development of resistance to bortezomib are becoming increasingly understood (for recent reviews, see (Kale and Moore, 2012; Niewerth et al., 2015)). Mutations in the genes encoding the catalytic β subunits appear to be a key mechanism in the development of bortezomib resistance during selection *in vitro*. Mutation hot spots clustered around the drug binding site of the $\beta 5$ subunit have been described in numerous cell lines (de Wilt et al., 2012; Franke et al., 2011; Lu et al., 2008; Oerlemans et al., 2008; Ri et al., 2010; Suzuki et al., 2011). In spite of the overwhelming

data obtained from the study of cell lines, the clinical picture is less clear since $\beta 5$ mutations are generally absent from biopsies obtained from patients displaying bortezomib-resistance (Niewerth et al., 2015). In addition to mutation, the overexpression of $\beta 5$ subunits has also been frequently observed in cell lines with acquired bortezomib resistance and is generally paralleled by down-regulation of immunoproteasome subunits (Franke et al., 2011; Niewerth et al., 2014; Oerlemans et al., 2008).

The altered activity of ABC-drug efflux pumps has also been implicated in conferring bortezomib resistance. Stable knockdown of MDR-1 (encoding the P-glycoprotein, P-gp) in cell lines was observed to lead to a 5-fold increase in sensitivity to bortezomib (Rumpold et al., 2007). However, a number of studies have subsequently shown that bortezomib is generally a poor substrate for P-gp, thus the overall significance of drug efflux pumps in conferring bortezomib resistance is unknown (Minderman et al., 2007; Verbrugge et al., 2012; Wiberg et al., 2009). In contrast to bortezomib, epoxyketone proteasome inhibitors such as ONX 0912 do appear to be P-gp substrates (Verbrugge et al., 2012).

Another potential molecular mechanism of bortezomib resistance is the overexpression of members of the Bcl-2 family of anti-apoptotic mediators such as the Bcl-2 and Bcl-X_L proteins (Hagenbuchner et al., 2010; Smith et al., 2011). Both Bcl-2 and Bcl-X_L function to dampen the apoptotic response by hindering Bax-Bak mediated perforation of the mitochondrial outer membrane, thus preventing cytochrome-c release and activation of the caspase cascade. Several groups have reported that the anti-tumour effect of bortezomib is greatly enhanced by antagonists of Bcl-2 function (Johnson-Farley et al., 2015; Kunami et al., 2014; Paoluzzi et al., 2008; Reuland et al., 2012). The Bcl-2 inhibitors ABT-737 and ABT-199 respectively enhance

the cytotoxicity of bortezomib in adult T-cell leukaemia/lymphoma (Kunami et al., 2014) and "double hit" lymphoma cells (Johnson-Farley et al., 2015). The BH3 mimetic AT-101, a pan-inhibitor of Bcl-2, Bcl-X_L and Mcl-1 was also reported to enhance apoptosis when combined with carfilzomib, but not with bortezomib in mantle cell lymphoma (Paoluzzi et al., 2008). This difference in synergy observed is presumably due to the irreversible binding of carfilzomib to the proteasome compared to the slowly reversible nature of bortezomib.

4.2. Proteasome DUB inhibitors may be used to treat cells with acquired resistance to bortezomib

Considering the various mechanisms by which cancer cells develop resistance to proteasome inhibitors, targeting alternative components of the UPS may be a logical therapeutic strategy. Mutations or alterations in the expression of 20S β -subunits would not be expected to affect the sensitivity to drugs that target the UPS at the level proteasomal DUBs since these enzymes function upstream of the proteolytic activity of the 20S CP. Interestingly the wiring of cell death signalling appears to differ between inhibitors of proteasomal degradation and inhibitors of proteasome DUB activity suggesting a degree of independence in death signalling. For example, apoptosis induced by the proteasome DUB inhibitor b-AP15 is refractory to the overexpression of the anti-apoptotic Bcl-2 protein (D'Arcy et al., 2011) and the expression levels of the pro-apoptotic Bax and Bak proteins. In contrast Bcl-2 overexpression or loss of Bax and Bak was sufficient to perturb bortezomib-induced apoptosis (Brnjic et al., 2014; D'Arcy et al., 2011). Consistent with these differences in mechanism of action and apoptosis signalling it has been shown that that bortezomib-resistant multiple myeloma cells retain sensitivity to b-AP15

suggesting a potential treatment to over-come bortezomib resistance (Tian et al., 2013). A b-AP15 analogue with improved biological activity (VLX1570; Wang et al., under revision) has recently been approved by the United States Food and Drug Administration for clinical trials and will hopefully aid in the treatment of bortezomib-resistant multiple myeloma.

4.3. Potential mechanisms of resistance to proteasome DUB inhibitors

Considering that the development of drug-resistance tends to be a universal problem in the fight against cancer, is it possible to predict what mechanism that may confer resistance to proteasome DUB inhibitors? Mutation of the catalytic cysteines of USP14/UCHL5 could be expected to lead to the loss of b-AP15 binding capacity, however such mutations have to our knowledge not been described and may not be compatible with cell viability. In order to examine potential drug resistance mechanisms, we have attempted to generate b-AP15-resistant cell lines by culturing cells in the presence of b-AP15 for extended periods. However, in spite of 9 months of continuous drug selection, only a small degree of resistance (~2-fold) was achieved, suggesting that the development of acquired resistance is a slow process (Sun *et al.*, unpublished data).

One mechanism that could theoretically be speculated to contribute to b-AP15 resistance is the increased activity of cellular detoxification systems, in particular glutathionylation processes that have the potential to react with and soak up free drug from the intracellular environment. However since glutathione has a pK_a of ~9.4 (Tajc et al., 2004) and is present in its thiol-form (i.e. not thiolate-form) at neutral pH it will only show weak spontaneous reaction with soft electrophiles such as b-AP15. Consistent with this we have found that addition of 2 mM

glutathione does not significantly inhibit the ability of b-AP15 to inhibit proteasomal DUB activity (D'Arcy et al., 2011). Cross-conjugated dienones are known to undergo reversible Michael addition reactions with glutathione, generating an equilibrium shift to the free form of the electrophile (Suzuki et al., 1997). However since the levels of glutathione in the NCI-60 cell line panel have been determined (Tew et al., 1996) it is possible to investigate the relationship between cellular IC₅₀ values to b-AP15 and cytosolic glutathione levels. No correlation between the sensitivity of the NCI -60 cell lines to b-AP15 and glutathione levels was observed (**Fig. 4**). Since glutathione is enzymatically conjugated to electrophiles by the action of glutathione-S-transferases (GSTs) such as GST- π , over expression of such enzymes could be predicted to have a detoxifying role. b-AP15 qualifies as a GST substrate since these are generally hydrophobic and have carbon at the electrophilic centres (Townsend and Tew, 2003). However similar to the expression of glutathione, the expression of GST- π in the NCI-60 cell line panel (Tew et al., 1996) showed no correlation to the anti-proliferative activity of b-AP15 (**Fig. 4**). Another mechanism that could confer resistance to b-AP15 is the increased activity or expression of ABC-drug pumps. However even this appears to be unlikely since available data suggests that b-AP15 is not a substrate for either MDR1 or MRP drug pumps as overexpression of the corresponding cDNAs in cancer cell lines does not significantly increase drug resistance (Paola Perego, Fondazione IRCCS, Istituto Nazionale dei Tumori, personal communication). Taken together both the theoretical considerations and experimental findings seem to suggest that development of drug resistance to proteasome DUB inhibitors may be difficult.

5. Perspectives

The proteasome is the only target in the UPS for which a drug has been developed and subsequently approved for clinical use. The clinical success of bortezomib and other second-generation proteasome inhibitors has raised the possibility of targeting alternative upstream components of the UPS. Since proteasomal DUB activity is a pre-requisite for proper proteasome function these DUBs represent attractive targets for the development of novel UPS inhibitors. The differences in the molecular mechanism of action of 20S proteasome inhibitors and proteasome DUB inhibitors make the development of drug cross-resistance unlikely. Based on the available data proteasome DUB inhibition may provide a novel mechanism in overcoming drug resistance to conventional proteasome inhibitors and may add to the arsenal of drugs available for the treatment of cancer.

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Conflicts of interest

Two of the authors (P.D and S.L.) are shareholders in Vivolux AB, a company that develops DUB inhibitors to be used as cancer therapeutics.

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Figure 1

Figure 1. Controlled protein degradation is mediated by the ubiquitin-proteasome system (UPS). Cascades of ligases work sequentially to covalently modify lysine residues on target proteins with the small molecule ubiquitin. The attachment of multiple ubiquitin residues signals trafficking to the proteasome and recognition by the ubiquitin receptors located at the 19S RP. Substrates are partially unfolded and stripped of their polyubiquitin chains by proteasome-associated DUBs prior to translocation into the 20S particle for proteolysis.

Figure 2 **Figure 2.** Mechanism of proteasome inhibitor induced cytotoxicity.

Inhibition of UPS activity can lead to a build up of cytotoxic proteins that accumulate within the cell. The build-up of polyubiquitinated proteins can induce a proteotoxic stress response characterized by endoplasmic reticulum (ER) stress, production of reactive oxygen species (ROS), activation of apoptosis and cell death.

Figure 3**b-AP15** **RA-9** **F6** **G5** **AC17**

Figure 3. Chemical structure of proteasome DUB inhibitors sharing α , β -unsaturated ketones and accessible β -carbons. Several characterized proteasome DUB inhibitors share a similar chemical structure consisting of sterically accessible β -carbons and α , β -unsaturated ketones. Such a pharmacophore is presumed to react with the cysteine residues in the active sites of DUBs.

Figure 4 **Figure 4.** GSH and GST- π levels do not correlate to b-AP15 sensitivity.

Glutathione (GSH) and Glutathione S Transferase- π (GST- π) are components of the cells detoxification system that could potentially lead to b-AP15 resistance. Analysis of the expression levels of GSH and GST- π in the NCI60 cell line panel shows no correlation with expression levels and b-AP15 sensitivity.