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Targeted inhibition of the deubiquitinating enzymes, USP14 and UCHL5, induces proteotoxic stress and apoptosis in Waldenström macroglobulinaemia tumour cells

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Summary

Deubiquitinase enzymes (DUBs) of the proteasomal 19S regulatory particle are emerging as important therapeutic targets in several malignancies. Here we demonstrate that inhibition of two proteasome-associated DUBs (USP14 and UCHL5) with the small molecule DUB inhibitor b-AP15, results in apoptosis of human Waldenström macroglobulinaemia (WM) cell lines and primary patient-derived WM tumour cells. Importantly, b-AP15 produced proteotoxic stress and apoptosis in WM cells that have acquired resistance to the proteasome inhibitor bortezomib. *In silico* modelling identified protein residues that were critical for the binding of b-AP15 with USP14 or UCHL5 and proteasome enzyme activity assays confirmed that b-AP15 does not affect the proteolytic capabilities of the 20S proteasome β -subunits. *In vitro* toxicity from b-AP15

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Conflicts of interest

The authors report no conflict of interest.

Author contributions

KC designed and performed the research, analysed the data and wrote the paper and approved final version of the manuscript submitted. AP designed and performed the research, analysed the data and wrote the paper and approved final version of the manuscript submitted. SA performed the research, analysed the data and approved final version of the manuscript submitted. MKK performed the research, analysed the data and approved final version of the manuscript submitted. TRC performed the research, analysed the data, wrote the paper and approved final version of the manuscript submitted. AJN performed and analysed the research, critically revised the paper and approved final version of the manuscript submitted. SMA analysed the data provided critical materials for conduct of the research, provided critical review of the paper and approved final version of the submitted manuscript. PA analysed the data, provided critical revision of the paper and approved final version of the manuscript submitted. SA provided critical revision of the paper and approved final version of the manuscript submitted. TS provided critical revision of the paper and approved final version of the manuscript submitted. SL analysed the data, provided critical revision of the paper and approved final version of the manuscript submitted. ACK designed the research study, analysed the data, provided critical revision of the paper and approved final version of the manuscript submitted.

appeared to result from a build-up of ubiquitinated proteins and activation of the endoplasmic reticulum stress response in WM cells, an effect that also disrupted the mitochondria. Focused transcriptome profiling of b-AP15-treated WM cells revealed modulation of several genes regulating cell stress and NF- κ B signalling, the latter whose protein translocation and downstream target activation was reduced by b-AP15 *in vitro*. This is the first report to define the effects and underlying mechanisms associated with inhibition of USP14 and UCHL5 DUB activity in WM tumour cells.

Keywords

proteasome; deubiquitinase enzymes; Waldenströms macroglobulinaemia; transcriptome; preclinical

Waldenström macroglobulinaemia (WM) is an indolent and incurable Non-Hodgkin lymphoma (NHL) in which the tumour cell compartment is comprised of malignant plasmacytoid lymphocytes, plasma cells or small B-lymphocytes (Owen *et al*, 2003; Konoplev *et al*, 2005; Kaye *et al*, 2012). The unique clinical manifestations of this haematological cancer result not only from malignant cell infiltration of the bone marrow and lymphoid organs (lymph nodes, spleen), but also from abnormally high serum immunoglobulin M (IgM) levels (Ansell *et al*, 2010). WM is a rare clinicopathological entity (annual incidence ~1500 cases/year in the United States), which presents significant challenges in the development of new therapeutics (Groves *et al*, 1998). Currently, there are no US Food and Drug Administration-approved drugs for WM. Management of patients with symptomatic disease is typically conducted based upon principles established in other B-cell cancers, and this includes use of purine analogs (fludarabine and cladribine), alkylating agents (cyclophosphamide and chlorambucil), anti-CD20 monoclonal antibody therapy (rituximab) and proteasome inhibitor (PI) therapy, either alone or in various combinations with one another (Ansell *et al*, 2010; Dimopoulos *et al*, 2014).

Biologically, sustained B-cell receptor and toll-like receptor signalling propels the oncogenic drive of malignant WM clones, which results in downstream induction of powerful prosurvival elements, such as nuclear transcription factor kappa B (NF- κ B) and the Signal Transducers and Activators of Transcription (STAT) family of factors (Treon *et al*, 2012). Complementarily, upregulation of the ubiquitin-proteasome system (UPS) is induced to facilitate the production, degradation and recycling of the numerous proteins required for neoplastic cell growth (Sacco *et al*, 2011). Therefore, it is not surprising that therapeutic interference of UPS function through 20S proteasome β 5-targeting PI, such as bortezomib or carfilzomib, is catastrophic to WM cells, which rely on NF- κ B as well as optimal proteasomal functionality for cellular homeostasis (Roccaro *et al*, 2008; Sacco *et al*, 2011). Indeed, successful implementation of PI-based therapeutic strategies in WM has significantly benefited patients with the disease (Chen *et al*, 2007; Ghobrial *et al*, 2010; Dimopoulos *et al*, 2013). Despite this clinical benefit, chronic exposure to bortezomib often leads to the outgrowth of WM tumour clones that are either genetically predisposed or have acquired mechanisms to resist PI-induced cytotoxicity (Chitta *et al*, 2009). Together, these biological and clinical observations highlight the need for continued development of

therapeutics that build on the success of bortezomib in WM; yet which are able to circumvent resistance mechanisms that are acquired from its continuous use.

Ultrastructurally, the 26S proteasome is a barrel-shaped structure containing the 20S core particle, which is flanked on both sides with two 19S regulatory particles (Adams, 2003). The mammalian 19S cap contains three DUBs that unfold and deubiquitinate proteins prior to their entry into the proteasomal core (D'Arcy & Linder, 2012). Of the three, ubiquitin-specific protease 14 (USP14) and ubiquitin carboxyl-terminal hydrolase isozyme L5 (UCHL5) reversibly associate with the proteasome, whereas the third DUB, RPN11/POH1, is an intrinsic component of the lid subcomplex of the 19S cap (Pathare *et al*, 2014). Collectively, the dysregulated activity of USP14 and UCHL5 has been linked to tumour cell survival (Ramalingam *et al*, 2011), metastasis (Mines *et al*, 2009), and poor clinical outcome (Rolen *et al*, 2006; Shinji *et al*, 2006). Suppression of either DUB individually via RNA interference has been shown to upregulate proteasomal catalytic activity; however, the combined inhibition of both UCHL5 and USP14 results in lethality, indicates their non-redundancy and suggest their role in maintaining cancer cell survival (D'Arcy *et al*, 2011).

Recently, the small molecule DUB inhibitor b-API5, which selectively disrupts both USP14 and UCHL5 activity, was shown to significantly decrease leukaemic cell infiltration of normal tissues in an aggressive leukaemic murine model as well as demonstrating robust antitumour activity in multiple myeloma MM cells (Feng *et al*, 2014; Tian *et al*, 2014), and in models of different solid tumour cancers (D'Arcy *et al*, 2011). Additionally, the effects of b-API5 were shown to be independent of tumour suppressor TP53 (p53) status as well as the expression of B-cell lymphoma 2 (BCL2), both of which can influence response to bortezomib therapy (Chanan-Khan, 2004; Patel *et al*, 2009; D'Arcy *et al*, 2011; Paulus *et al*, 2014). Thus, these encouraging data prompted us to investigate the antineoplastic activity of b-API5 in paired isogenic bortezomib-sensitive (wild-type, WT) and bortezomibresistant (BR) WM cells and in patient-derived malignant WM cells. Our studies affirm that the DUBs USP14 and UCHL5 are therapeutic targets in WM, which can be disrupted by b-API5 to elicit an anti-tumour response that is independent of 20S proteasomal core activity. Moreover, we found that b-API5 induced significant cell death in BR models, suggesting its ability to circumvent mechanisms that support resistance to β 5-targeting PI.

Materials and methods

Cell lines, cell culture and reagents

Waldenströms macroglobulinaemia cell lines (BCWM.1, MWCL-1 and RPCI-WM1) and their corresponding bortezomib resistant (BR) clones [BCWM.1/BR, MWCL-1/BR and RPCI-WM1/BR, resistance of representative model shown in Fig S1 and 50% inhibitory concentration (IC₅₀) of others in Table S1.] were used in experiments, as previously described (Chitta *et al*, 2009; Paulus *et al*, 2014). CD19⁺/CD138⁺ sorted tumour cells obtained from consenting WM patients were acquired from the Predolin Biobank (Mayo Clinic, Rochester, MN, USA) following approval by the Mayo Clinic Institutional Review Board. Heparinized peripheral blood was obtained from healthy human donors. Peripheral blood mononuclear cells (PBMCs) from healthy human donors were isolated as previously described (Chitta *et al*, 2014). b-API5 was provided as a gift from Vivolux AB, (Uppsala,

Sweden). Bortezomib and carfilzomib were purchased from Sellekhem (Houston, TX, USA). RPMI medium, penicillin, streptomycin, tetramethylrhodamine, methyl ester (TMRM) and fetal bovine serum (FBS) were purchased from Life Technologies (Carlsbad, CA, USA). All antibodies were purchased from Santa Cruz biotechnology (Dallas, TX, USA) or Cell Signaling Technology (Danvers, MA, USA). Annexin-V and propidium iodide apoptosis staining kit was purchased from BD Biosciences (San Jose, CA, USA).

Proteasomal activity assay

Cells were lysed at 4×10^6 cells/ml in proteasomal activity assay buffer [assay buffer; 25 mmol/l HEPES buffer, pH7.5 containing 0.5 mmol/l EDTA, 0.05% Nonidet P-40, 0.01% sodium dodecyl sulfate (SDS)] and immediately used in the assay. Enzyme reactions were performed in 96-well plates with 100 μ l of final volume containing 5 mmol/l fluorogenic peptide substrates. The substrates used were LLVY-AMC for chymotrypsin like activity, LLE-AMC for caspase-like activity and LRR-AMC for trypsin-like activity. Reactions were incubated at 37°C for 1 h and the fluorescence measured at 360/ 460 using a BioTek synergy HT plate reader (BioTek, Winooski, VT, USA).

Viability assay

Twenty thousand cells/200 μ l in quadruplicates were incubated with serially diluted b-API5 (1–1000 nmol/l) in 96-well plates at 37°C for 72 h. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagent (Molecular Probes, Eugene, OR, USA) was added at 40 μ l/well and the plates were further incubated at 37°C for 3 h and the developed colour was read at 490 nm using a BioTek synergy HT plate reader against blanks with no cells.

Apoptosis assay

Apoptosis was measured using the Annexin V binding assay kit from BD Biosciences according to the manufacturer's instructions. Briefly, at the end of the treatment, cells were washed with PBS and 1×10^6 cells were re-suspended in 100 μ l of binding buffer. Fluorescein isothiocyanate (FITC)-labelled Annexin V (5 μ l) and propidium iodide (10 μ l) were added to each sample and incubated in the dark for 15 min at room temperature. The cells were subsequently analysed by flow cytometry using BD Accuri, the C6 flow cytometer and its software. Data from 10 000 events per sample were collected and analysed.

Determination of mitochondrial outer membrane permeability

Cells were treated with different agents for 48 h and assessed for MOMP using tetramethylrhodamine methyl ester [TMRM] (Life Technologies). TMRM was directly added to the cell cultures at 100 nmol/l concentrations and incubated at 37°C in the dark for 15 min. At the end of the incubation, cells were washed twice with cold PBS containing 2% FBS and analysed. The cells were washed for fluorescence (FL2) and analysed by BD Accuri, the C6 flow cytometer and its software. Data from at least 20 000 events per sample was collected and analysed. TMRM-negative (%) cells were calculated to determine % MOMP.

Immunoblot analysis

Total protein extracts were made using radioimmunoprecipitation assay lysis buffer (50 mmol/l Tris containing 150 mmol/l NaCl, 0.1% SDS, 1% TritonX-100, 1% sodium deoxycholate, pH 7.2) with 0.2% protease and phosphatase inhibitor cocktail (Sigma, St. Louis, MO, USA) on ice for 40 min, vortexing for 5 s every 10 min. Following centrifugation at 18 400 g for 20 min, the supernatant was collected and used for Western blot analyses. Protein content in the extracts was measured by bicinchoninic acid protein assay reagent. Aliquots of 20 µg of total protein were boiled in Laemmli sample buffer, loaded onto 10% SDS-PAGE gels and transferred onto a nitrocellulose membrane. Membranes were blocked for 1 h in TBS/Tween 20 [TTBS] containing 1% nonfat dried milk and 1% BSA. Incubation with primary antibodies was done overnight at 4°C, followed by washing 3× with TTBS and incubation for 1 h with HRP-conjugated secondary antibody. The blots were developed using chemiluminescence (Thermo Scientific, Rockford, IL, USA).

NF-κB reporter assay

HEK293 cells expressing *MYD88*L265P were generated as previously described (Ansell *et al*, 2014). Cells were transiently transfected with 0.5 ng Renilla and 0.25 µg of a pNF-κB-luciferase reporter plasmid/ 1.0×10^6 cells. b-AP15 was added to each well at the indicated doses; after 24 h, luciferase activity was measured in cell extracts and normalized against Renilla with the Dual Luciferase Kit (Promega, Madison, WI, USA).

Description of computational techniques and targeted transcriptome analysis are contained in the Supplementary Methods.

Results

In silico docking of b-AP15 with the 19S proteasome associated deubiquitinating enzymes (DUBs), UCHL5 and USP14

Given that UCHL5 and USP14 are the two established targets of b-AP15, we sought to first model their structures *in silico* and determine the residues that are critical for their binding to b-AP15. We first modelled a 3-dimensional protein structure for UCHL5 and found that it contains a Cys88 residue that may be attacked by b-AP15 via a 1,4-Michael addition reaction. The additional reaction occurs at the thiol group (-SH) from Cys88 with the aldehyde from b-AP15 (green coloured ligand, Fig 1A, B). The nitro-groups from b-AP15 participate in electrostatic interactions with the Asn/Gln residues, and transient π -cloud interactions occur with the phenyl-substituted rings from b-AP15. His164 and carbonyl oxygen from b-AP15 have stabilizing interactions. Next, we modelled USP14 and, similar to UCHL5, USP14 covalently binds b-AP15 via a 1,4-Michael addition reaction at the thiol group of the Cys114 residue (covalent linkage) with the aldehyde from the small molecule DUB inhibitor (Fig 1C–E). We found that the binding pocket is highly mobile during molecular dynamics simulations (MDS) and that b-AP15 binding occurs with cooperative changes in the pocket shape. b-AP15 shifts orientation preceding the covalent binding event at residue Cys114 (Movie S1). Importantly, b-AP15 engagement blocks access of the C-terminal of ubiquitin from binding with USP14, which is visible in the X-ray structure of

2AYO (Hu *et al*, 2005) (Fig 1D). As with UCHL5, Asn/Gln interactions stabilize the nitro-substituted phenyl rings, while the His435 does not face the carbonyl in this insertion pose for b-AP15. b-AP15 can potentially insert in a 180°-rotated orientation, such that the DUB inhibitor faces the His435 residue similarly to UCHL5; however, molecular modelling and mechanics suggests that it has a covalent interaction with Cys411, resulting in the most optimal docking orientation (Fig 1E).

Proteolytic activity of the 20S proteasome is not compromised by b-AP15

To experimentally affirm that the (19S proteasome cap) targets of b-AP15 are distinct from those of PIs, such as bortezomib or carfilzomib, we assessed the enzymatic activity of the 20S proteasome $\beta 5$ subunit after treatment with b-AP15+/- 20S targeting PI (bortezomib or carfilzomib). Using a fluorogenic peptide (Suc-LLVY-AMC), which is a chymotryptic substrate, we observed no loss of the chymotrypsin-like activity (LLVY) of the $\beta 5$ subunit in either bortezomib sensitive (WT) or BR WM tumour cells treated with b-AP15 (Fig 2A, B). In contrast, LLVY activity was significantly diminished in both WT and BR WM cells treated with bortezomib or carfilzomib, which served as comparators for b-AP15. Notably, addition of b-AP15 to either bortezomib or carfilzomib did not abrogate the $\beta 5$ inhibitory actions of the 20S-targeting PI. No change was observed in either caspase-like ($\beta 1$ subunit) or trypsin-like ($\beta 2$ subunit) proteasomal activity in b-AP15-treated WM cells (Fig S2). This important observation affirms that b-AP15 and established PIs target different locations (19S vs. 20S, respectively) of the proteasome, and their activity may potentially be complementary to one another. Altogether, these results demonstrate that b-AP15 does not inhibit proteasome β -catalytic function nor does it interfere with β -catalytic activities when combined with 20S-targeting PI.

USP14 and UCHL5 are consistently expressed in WM cells and their enzymatic inhibition with b-AP15 is associated with an increase in ubiquitinated proteins and loss of viability

Next, we sought to examine the expression of USP14 and UCHL5 proteins across WM cells. We first examined USP14 and UCHL5 protein levels in primary CD19+/CD138+ malignant WM cells from previously treated WM patients by immunoblot analysis and observed notable baseline expression of the DUBs, which did not change after exposure to b-AP15 (Fig 3A). Next, we examined this phenomenon in WM cell lines (WT and BR clones) and found that USP14 and UCHL5 were consistently expressed across all WM cells, with no observable shift after b-AP15 treatment (Fig 3B). Given that b-AP15 targets USP14/UCHL5 deubiquitinating activity, it would stand to reason that b-AP15 treatment of cells would result in build-up of ubiquitinated protein. Consistent with this, we looked at the total ubiquitinated cellular protein content and found increasing amounts of high molecular weight poly-ubiquitinated conjugates in b-AP15-treated cells in a dose-dependent manner (Fig 3C). One of the primary mechanisms whereby PIs exert their antitumour effect is through the buildup of ubiquitinated proteins in the lumen of the endoplasmic reticulum (ER), causing ER stress beyond the threshold of what the cell can compensate for, eventually leading to apoptotic cell death (Kim *et al*, 2006). To determine if increase in polyubiquitinated conjugates in b-AP15-treated WM cells coincided with loss of tumour cell viability, we conducted a 72-h MTS assay to assess WM cell viability following treatment with increasing concentrations of b-AP15 (0–1 μ mol/l). All WM cells were noted to be

exquisitely sensitive to b-AP15 with the highest sensitivity observed in MWCL-1 cells [50% inhibitory concentration (IC₅₀) 7 nmol/l] followed by BCWM.1 (IC₅₀ 13 nmol/l) and RPCI-WM1 (IC₅₀ 16 nmol/l) (Fig 3D). We next assessed the effects of b-AP15 in the corresponding BR WM cells and observed loss of viability in a similar order (MWCL-1B/BR $\gg$ BCWM.1/BR > RPCI-WM1/BR) (Fig 3E). These results affirm USP14 and UCHL5 as valid and functional targets in WM and whose inhibition with b-AP15 results in accumulation of ubiquitinated proteins, loss of tumour cell viability, despite acquired resistance to the 20S targeting PI, bortezomib.

b-AP15 induces tumour-specific apoptosis in WM cell lines *in vitro* and patient-derived WM cells *ex vivo*

We already noted loss of WM cell viability in presence of b-AP15, and therefore investigated if this was due to apoptotic mechanisms. All WM cell lines were treated with increasing concentrations of b-AP15 and induction of apoptosis was examined at different time points by annexin-V staining followed by flow cytometry. Amongst the WM models, we observed that b-AP15 treatment caused programmed cell death as early as 6 h and most significantly by 12 h in a dose-dependent manner with approximately 50% of WM cells experiencing significant apoptosis at a concentration of 0.64 μ mol/l ($P < 0.005$) (Fig 4A, 12-h time-point shown). Heat density plots from two representative (1 WT and 1 BR) WM models are shown in Fig S3. Using two concentrations of b-AP15, derived from the titration (cell line-based) experiment, we examined for b-AP15-mediated apoptosis in primary patient-derived WM cells as well as in PBMCs from healthy donors. We noted significant annexin-V positivity in primary malignant cells treated with b-AP15 (0.5 μ mol/l) by 12 h (Fig 4B), with >90% undergoing total loss of viability at a concentration of 1 μ mol/l (data not shown). Contrastingly, minimal apoptosis was observed in PBMCs cultured in b-AP15 for up to 48 h and indicate the rapid effects of the DUB inhibitor to be tumour-cell specific. Lastly, confirmation of apoptosis in WM cell lines and patient-derived WM cells was observed by immunoblotting for PARP1 cleavage (Fig 4C).

A loss of mitochondrial transmembrane potential is provoked by b-AP15 in WM cells

Disruption of the mitochondrial transmembrane potential (ψ_m) through an increase in mitochondrial membrane permeability (MOMP) is a hallmark of death receptor-independent apoptosis and engagement of the intrinsic apoptotic cascade (Kroemer *et al*, 2007). Previous reports showed the ability of b-AP15 to induce caspase-3 cleavage; (D'Arcy *et al*, 2011) as such, we sought to determine whether the intrinsic apoptotic pathway was activated by measuring MOMP and looking for caspase-9 and -3 cleavage. MOMP was measured in relation to TMRM fluorescence in all WM cell lines and TMRM-negative cells were calculated to represent (%) MOMP. MOMP was significantly induced in b-AP15-treated WT and BR WM cells, and this coincided with PARP1 cleavage as well as cleavage of executor caspase-3 (Fig 5A, B and Fig S4). To determine if b-AP15-mediated toxicity was caspase-dependent, we treated all WM cells with the pan-caspase inhibitor z.VAD.fmk +/- b-AP15. We observed that pre-treatment with z-VAD.fmk significantly reduced MOMP ($P < 0.01$) in b-AP15 co-treated WT and BR WM cells. These results suggest that WM cell mitochondria are targeted by b-AP15, which disrupts the ψ_m . Overall, this results in caspase-3 mediated tumour cell death, which is partially relieved by inhibition of caspase activity.

b-AP15 modulates genes involved in cellular stress and Nuclear factor kappa B (NFKB1) signalling

We probed for the effects of b-AP15 at the transcriptional level in WM models by looking at specific cancer-related genes. The Nanostring nCounter mRNA quantification assay was used, which has a high sensitivity for direct measurement of mRNA abundance and has been demonstrated to be an equivalent alternative to quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) or Open Array real-time PCR (Prokopec *et al*, 2013). BCWM.1 and RPCI-WM1 cells were treated with a 50 nmol/l concentration of b-AP15, whereas their respective BR clones were treated with a 100 nmol/l concentration for 24 h followed by collection of RNA for profiling. b-AP15 treatment elicited notable changes in cancer-associated genes associated with ER/cell stress response and NFKB1 signalling mechanisms, reflected by differential expression of mRNA in each of the cell lines (Fig 6A). To evaluate which genes were altered in the same orientation across all four cell lines, we performed an intersect analysis (Fig 6B), identifying 36 genes that were commonly modulated, many of whose expression are associated with NFKB1 signalling (Table S2). The relationship between the 36 genes was also explored by Ingenuity Pathway Analysis (IPA) network analysis (Fig 6C and Fig S5) and illustrates the interaction and relative expression (denoted by colour) of these genes. This analysis sheds light on the protein interactions that remain critical for WM cell survival, irrespective of resistance to β 5-targeting PI, and which can be modulated by b-AP15 therapy.

Activation and nuclear translocation of RELA (p65) is attenuated by b-AP15

Abnormalities in the NFKB1 signalling pathway have been implicated in WM cell growth and survival (Leleu *et al*, 2008; Braggio *et al*, 2009; Ansell *et al*, 2014). Moreover, nearly all WM cases (~97%) carry a mutant *MYD88* gene (*MYD88*_{L265P}), which hyperactivates NFKB1 by constitutively associating itself with IRAK4 and TRAF6 (Treon *et al*, 2012). With this in mind, we interrogated the impact of b-AP15 on mutant *MYD88*-directed activation of NFKB1 through use of a NFKB1 luciferase reporter assay in *MYD88*_{L265P} expressing HEK293T cells (Ansell *et al*, 2014). Transfected 293T cells were treated with b-AP15 for 24 h and untreated cells were used as a control for comparison. As expected, treatment with b-AP15 (0.5 μ mol/l) significantly reduced NFKB1 luciferase activity, indicating a decrease in *NFKB1* gene activation ($P < 0.004$, Fig 7A). Further, we confirmed a marked reduction in nuclear RELA (p-p65) availability directly at the protein level in b-AP15 treated WM cells (Fig 7B, results from one representative model shown). Indeed, these observations were supported by NanoString data analysis, which showed downregulation of NFKB1 target genes (Table S2). This was experimentally confirmed by examining the NFKB1 target, *MYC*, in BCWM.1 cells, which showed reduced total and nuclear expression after treatment with b-AP15. Altogether, these data reveal that b-AP15 decreases the nuclear translocation and activation potential of NFKB1 and target genes, specifically *MYC*, in *MYD88*_{L265P} WM cells.

b-AP15 causes a shift in the ER and cell-stress response proteins in WM cells

b-AP15 has a clear effect on ER stress, unfolded protein response (UPR) and cell stress-associated elements (Brnjic *et al*, 2014; Tian *et al*, 2014). In line with these prior

observations, (D'Arcy *et al*, 2011; Brnjic *et al*, 2014) we noted that ER stress machinery, such as HSPA1A was consistently present in both WT and BR cell lines, and notably more so after b-AP15 treatment (Fig 8). We also found XBP1 and its spliced active form, XBP1s, which are primary effectors of the UPR (Ron & Walter, 2007), to be significantly induced by b-AP15 across all cell lines. Another UPR effector, EIF2AK3 (PERK) was notably present in all untreated WM cells. In BR models, b-AP15 decreased EIF2AK3 levels; however, this effect was not concordantly seen in WT cell lines. Expression of the EIF2AK3 target, p-EIF2 α , did not appear to change following b-AP15 treatment. In addition to ER stress, we and others (Brnjic *et al*, 2014) have noted that b-AP15 activates cell stress-related kinases, as evidenced by modulation of MAPK proteins and downstream activation of their target transcription factors (see Table S2, Jun/Fos upregulation). We also observed an increase in phosphorylated-MAPK3/MAPK1 (ERK1/2) in WT cell lines after b-AP15 treatment; however, this was not observed in BR models. In addition, we noticed a significant increase of phosphorylated MAPK14 (p38) protein after b-AP15 (6-h treatment). MAPK14 is galvanized in response to DNA damage or cell stress and can act as either a compensatory pro-survival protein or facilitate cell death, depending on the cellular context (Wagner & Nebreda, 2009). To determine its significance, we used the MAPK14 inhibitors (SB202190 or SB580190) alone and in combination with b-AP15. Although greater loss of tumour cell viability was observed when a MAPK14 inhibitor was combined with a low concentration of b-AP15 (100 nmol/l), this effect was not observed with higher concentrations of b-AP15+ MAPK14 inhibitor (Fig S6). Lastly we examined the protein levels of TP53 and BCL2, whose dysregulated activity is implicated in bortezomib-resistance (D'Arcy *et al*, 2011; Paulus *et al*, 2014). We observed no change in BCL2, but a marginal increase in TP53 across b-AP15-treated WT and BR WM cells was noted. This is in line with previous reports, which show similar findings and demonstrate that although TP53 is induced, b-AP15 anti-tumour activity is not TP53-dependent (D'Arcy *et al*, 2011).

Discussion

We report on the functional sequelae of USP14 and UCHL5 inhibition with the small molecule DUB inhibitor, b-AP15, in established WM cell lines (including their bortezomib-resistant subclones) and in patient-derived WM cells. Our analysis demonstrates that USP14 and UCHL5 are bonafide targets in malignant WM cells and their disruption with b-AP15 elicits a strong cellular stress response, culminating in rapid apoptotic cell death. The role of DUBs in haematological malignancies has been previously reported, (Honma *et al*, 2009; Novak *et al*, 2009; Schmitz *et al*, 2009; Hussain *et al*, 2010; Chauhan *et al*, 2012) with a recent analysis specifically expositing on the significance and inhibition of USP14 and UCHL5 by b-AP15 in (bortezomib-sensitive and -resistant) MM cells (Tian *et al*, 2014). Nevertheless, there is limited data on the role of these two DUBs in other B-cell neoplasms, with our report being the first to examine the therapeutic relevance of USP14 and UCHL5 in WM.

The b-AP15 compound preferentially targets USP14 and UCHL5 and, using *in silico* techniques, we have modelled b-AP15 and USP14/UCHL5 binding, revealing important molecular interactions that allow for this specific engagement to occur. It is important to note that in the potential instance where b-AP15 is covalently bound, the 1,4-Michael type

addition reaction need not necessarily be considered ‘irreversible,’ and in some instances, when the microenvironment is conducive, these reactions can reverse and allow the small molecule to break the covalent bond. Previous studies have shown that reversibility is possible in these scenarios (Kuck *et al*, 2010; Caulfield & Medina-Franco, 2011), and pharmacological analysis of b-AP15 demonstrates that its binding with USP14 is partially reversible (Wang *et al*, 2014). It is interesting that both USP14 and UCHL5, which belong to distinct DUB families with differences in homology, (Komander *et al*, 2009) exhibit a highly similar capacity to accommodate b-AP15 docking. However, with USP14, b-AP15 has tyrosine and lysine available for stabilization at the charged nitrogens from –NO₂ groups, allowing for long-term stabilization. This observation echoes biochemical studies where b-AP15 was suggested to have a higher affinity for USP14 and was more effective in inhibiting its activity compared with UCHL5 (Wang *et al*, 2014).

b-AP15 was initially assumed to be a 20S PI due to induction of similar gene expression signatures as those shared by several well established PI (D’Arcy *et al*, 2011). Indeed, our focused gene expression analysis revealed that genes involved in cell stress and NF-κB signalling were altered in a manner consistent with those found in 20S PI-treated tumour cells (Annunziata *et al*, 2007; D’Arcy *et al*, 2011). Mounting evidence suggests that MYD88_{L265P} signalling in WM cells is a powerful driver of NF-κB and STAT signalling (Treon *et al*, 2012). When activated, MYD88_{L265P} associates with IRAK1/4 and TRAF6 leading to phosphorylation of TAK1, which acts as a critical effector for activation of NF-κB (Ansell *et al*, 2014). Gene expression data already suggested that *NFKB1* and its targets, including *MYC*, were suppressed by b-AP15, findings we observed in WM cells *in vitro* by further experimental analysis.

The strong antitumour response seen with b-AP15 treatment has been mechanistically linked to induction of ER stress and oxidative stress (Brnjic *et al*, 2014; Wang *et al*, 2014). ER stress activates the UPR network, whose members induce heatshock chaperones and numerous other cellular constituents to either repair the misfolded proteins or to activate signalling programmes that lead to apoptosis (Kim *et al*, 2006). On immunoblot analysis, we observed that chaperone machinery and ER/UPR stress proteins (XBP1), which are induced by 20S-targeting PI, were also induced by b-AP15. ER stress is physiologically countered by activation of the UPR-associated transmembrane proteins, ERN1 (IRE1), EIF2AK3 and ATF6. These function collectively to upregulate degradation (ERN1 and ATF6) of overaccumulated proteins while simultaneously suppressing global mRNA translation (EIF2AK3) to reduce the burden of protein build-up (Ron & Walter, 2007). Upon sensing ER stress, the RNase domain of ERN1 becomes activated through autophosphorylation and induces unconventional splicing of *XBPI* mRNA. The spliced *XPBIs* mRNA encodes for a transcription factor, which targets a host of other UPR genes for activation of ER degradation machinery (Ron & Walter, 2007). Whereas XBP1s is considered more stable and active, the unspliced form (XBP1u) functions to inhibit XBP1 signalling, fine-tuning the UPR system (Yoshida *et al*, 2006). Following b-AP15 treatment, we observed a significant increase of both XBP1u and XBP1s expression. Although the current data does not clarify the significance of this change in the induction of WM cell death by b-AP15, downstream increase in HSPA6/7 chaperone protein indicates that the ERN1-mediated protein degradation arm of the UPR is biochemically engaged in both WT and BR WM cells. It is

worth mentioning that, amongst haematological malignancies, such as WM, chronic lymphocytic leukaemia and MM, the exact role and function of ERN1-XBP1 signalling appear to be disease and cell-type specific (Leleu *et al*, 2009a,b) (Kriss *et al*, 2012; Leung-Hagesteijn *et al*, 2013; Tang *et al*, 2014). Given our current findings, we surmise that, despite this upregulation leading to UPR activation, XBP1s, HSPA1A and other UPR-associated elements are not able to rescue WM cells from the rapid and immense proteotoxic effects delivered by b-AP15. The significance of this finding as well as the contribution of ATF6 in b-AP15-treated WM UPR indeed warrants further investigation.

The UPS remains a viable but largely unexplored therapeutic target in lymphoid malignancies, with only β -catalytic targeting PIs having been developed for clinical use. Fortuitously, and as detailed in this investigational report, the DUBs of the 19S proteasome cap offer an attractive 'alternative' target within the UPS, and whose inhibition by b-AP15 is able to overcome resistance to traditional (β 5 subunit-targeting) PIs, such as bortezomib, in WM. Using *in silico* modelling, we have provided a unique insight into the potential molecular interactions that facilitate b-AP15-USP14/UHL5 binding; that when engaged result in significant WM tumour cell death. Thus, the studies undertaken in this report, coupled with ongoing mechanistic and efficacy analyses, provides a sound rationale for the clinical development of DUB inhibitors, such as b-AP15 (or its clinical analogs), for use in WM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1. Molecular modelling and *in silico* docking of b-AP15 with UCHL5 and USP14. (A) Molecular structure for UCHL5 with electrostatic surface, modelled from X-ray structure 3IHR. Green-coloured ligand is b-AP15 bound with UCHL5. The deubiquitinase enzyme (DUB) inhibitor fits deep into a wedge-like crevice inside UCHL5 that includes the following residues within 4Å: Leu10, Trp58, Gln82, Asn85, Cys88, Ala162, Phe163, His164, Phe165, and Leu181. (B) Magnified view of the covalent linkage formed between b-AP15 and the Cys88 residue of the UCHL5 protein. (C) Molecular structure for USP14 modelled from X-ray structure 2AYO, shown with electrostatic surface. Green-coloured ligand is b-AP15 and shown bound to USP14. The crevice where b-AP15 binds USP14 is deeper as compared to UCHL5 and includes the following residues within 4Å of the binding interaction: Asn109, Asn112, Cys114, Tyr115, Gln197, Gln198, Asp199, Ser431, Ser432, Ser433, Gly434, His435, Tyr436, and Lys454. Arrows indicate two scenarios for USP14 binding. (D) Ubiquitin (Ub) is shown as a red ribbon co-crystallizing with USP14 (Protein Data Bank code: 2AYO). b-AP15 overlay demonstrates blocking of Ub C-terminus from binding. (E) Docked model for b-AP15 covalently linked to Cys114 in a tight and narrow crevice of the USP14 protein.

Fig 2.

b-AP15 treatment does not inhibit 20S proteasome, $\beta 5$ -subunit (chymotrypsin-like) catalytic activity in WT or BR WM cells. Effect of bortezomib (Bort, 10 nmol/l), carfilzomib (Carf, 10 nmol/l) and/or b-AP15 (10 nmol/l) on the proteasomal activity of WM cell lines was measured *in vitro* using fluorogenic substrates (chymotryptic activity, LLVY shown). b-AP15 did not alter chymotryptic activity or abrogate bortezomib or carfilzomib's ability to disrupt the chymotrypsin-like activity in either (A) Wild type (WT) BCWM.1, MWCL-1 or RPCI-WM1 cells, nor (B) their bortezomib-resistant (BR) subclones.

Fig 3.

USP14 and UCHL5 are expressed in WM cells and their inhibition with b-AP15 results in accumulation of high molecular weight ubiquitinated protein and loss of cell viability. Western blot analysis shows protein expression of USP14 and UCHL5 deubiquitinase enzymes (DUBs) in (A) primary patient-derived WM cells ($n = 2$, WM1; bortezomib-refractory and WM2; previously treated but bortezomib-naïve) +/- b-AP15 (0.5 $\mu\text{mol/l}$) and in (B) wild type (WT) and bortezomib-resistant (BR) WM cell lines +/- b-AP15 (0.5 $\mu\text{mol/l}$ and 1 $\mu\text{mol/l}$). (C) Immunoblotting shows the effect of b-AP15 on cellular content of ubiquitinated proteins. (D) A 72-h MTS assay was conducted to assess WM cell viability after treatment with increasing concentrations of b-AP15 (0–1 $\mu\text{mol/l}$). MWCL-1 cells were more sensitive (IC_{50} 7 nmol/l) as compared to BCWM.1 (IC_{50} 9 nmol/l) and RPCI-WM1 (IC_{50} 16 nmol/l). BR tumour cell viability was observed in a similar order. IC_{50} of MWCL-1B/BR was lowest, at 3 nmol/l, followed by BCWM.1/BR (IC_{50} 16 nmol/l) and finally RPCI-WM1/BR (IC_{50} 57 nmol/l).

Fig 4.

b-API5 induces tumour-specific apoptosis in WM cell lines and primary patient-derived WM cells. (A) All available WM cell lines ($n = 6$, WT and BR derivatives) were treated with b-API5 at indicated concentrations and stained with annexin-V and propidium iodide followed by flow cytometry to examine apoptosis. Annexin-V positivity (apoptosis) was significantly observed in b-API5-treated WM cells by 12 h in a dose-dependent manner (** $P < 0.005$). Each experiment was conducted a minimum of three times with control cells (no drug treatment) showing a viability (annexin-V positive and propidium iodide-negative population) of 85%. Following treatment, the percentage of cells affected by b-API5 was calculated by normalizing data from treated cells relative to the control (untreated) cells. (B) Malignant CD19+/CD138+ WM cells from human patients (WM1 and WM2) and peripheral blood mononuclear cells (PBMCs, $n = 2$) were similarly stained with annexin-V and propidium iodide to assess apoptosis. Robust apoptotic cell death was noted in patient-derived WM cells after 12 h exposure to b-API5 0.5 $\mu\text{mol/l}$. Contrastingly, minimal apoptosis (~13%) was noted in b-API5-treated PBMCs exposed to the deubiquitinase enzyme inhibitor for 48 h. (C and D) Immunoblotting for PARP1 cleavage confirmed execution of apoptosis in both WM tumour cell lines and primary WM tumour cells. BR, bortezomib-resistant.

Fig 5.

b-AP15 alters mitochondrial membrane permeability (MOMP) in WM cells. (A) MOMP was measured in relation to TMRM fluorescence in all WM cell lines and TMRM-negative cells were calculated to represent (%) MOMP (four representative cell lines shown). MOMP was significantly induced in b-AP15-treated wild type (WT) and bortezomib-resistant (BR) WM cells and correlated with PARP1 cleavage as well as cleavage of executor caspase-3. (B) To determine if b-AP15 mediated toxicity was caspase dependent, WM cell lines (2 WT with respective BR subclones) were treated with the pancaspase inhibitor z.VAD.fmk +/- b-AP15. Pre-treatment with z-VAD.fmk, of b-AP15 containing WM cells, significantly reduced MOMP (** $P < 0.01$) indicating that b-AP15 associated MOMP is partially caspase-dependent in WM cells.

Fig 6.

Genes altered in b-AP15 treated WM cells. BCWM.1 and RPCI-WM1 were treated with b-AP15 (50 nmol/l) and bortezomib-resistant (BR) clones were treated with 100 nmol/l of the deubiquitinase enzyme (DUB) inhibitor for 24 h followed by collection of RNA for profiling using the NanoString nCounter assay. (A) Unsupervised, hierarchical, bi-dimensional clustering analysis of the aforementioned WM cells showed differential expression (≥ 2 fold change) and behavioral pattern of genes in response to b-AP15 treatment. Compared to untreated cells, b-AP15 treatment upregulated 12 genes and downregulated 66 genes in BCWM.1 cells. In bortezomib-resistant BCWM.1/BR cells, b-AP15 upregulated 16 genes and decreased expression of 56 genes (*versus* untreated BCWM.1/BR cells). In RPCI-WM1 cells, eight genes were differentially increased by b-AP15, whereas expression of 74 genes was reduced. In RPCI-WM/BR cells, upregulation of seven genes and downregulation of 50 genes was observed. (B) Intersect analysis, where treated (Tx) cell lines were first compared to their untreated counterparts and then against one another, was performed to delineate which genes were altered in the same orientation across all four cell lines tested. 36 genes were found and are shown in Table S2. (C) Relationship between the 36 genes was also explored by IPA network analysis and illustrates the interaction and relative expression

(denoted by colour) of these genes. Intensity of the node colour denotes degree of differential gene expression as compared to baseline, with nodes coloured in the red spectrum indicating upregulation and nodes coloured within the green spectrum indicating downregulation of the gene.

Fig 7.

Nuclear translocation of RELA (NF-κB p65) and its downstream target MYC are reduced by b-AP15 in WM cells. (A) HEK293 cells expressing *MYD88*_{L265P} were generated as previously described (Ansell *et al*, 2014). b-AP15 was added to each well at the indicated doses. After 24-h, luciferase activity was measured in cell extracts and normalized against Renilla. b-AP15 treatment resulted in significant reduction of NF-κB reporter activity (** $P < 0.004$) in these cells. Results are from two independent experiments done in triplicate. Cytoplasmic and nuclear RELA as well as total and nuclear MYC protein expression was determined by Western blot analysis in untreated and b-AP15 treated (6-h) WM cells (BCWM.1 shown). (B) Following b-AP15 treatment, RELA nuclear protein levels were markedly reduced including (C) those of its direct target, MYC.

Fig 8.

b-AP15 induces a shift in the protein profiles of WM cells. The protein profile of WM tumour cells was assessed by Western blot after treatment with b-AP15 (6 h), focusing mainly on markers of endoplasmic reticulum (ER) and cell stress associated signalling. (A) The ER stress associated protein HSPA1A was present in all cell lines and was further induced by b-AP15. Likewise, ERN1a, XBP1u (unspliced) as well as XBP1s (spliced) were significantly induced by b-AP15 across all models tested. (B) Cell stress kinases were also modulated as noted by an increase in p-MAPK3/ MAPK1 (ERK1/2) in wild type cell lines after b-AP15. No change in BCL2, but a marginal increase in TP53 was observed in b-AP15 treated BCWM.1 and BCWM.1/BR WM cells.