1. Introduction

The proteasome inhibitor (PI) Bortezomib is used as a first- and second-line treatment of multiple myeloma (MM) (Anderson et al., 2011). Proteasomes’ (Finley, 2009; Lander et al., 2012) main function is to degrade ubiquitinated proteins in a controlled manner (Bedford et al., 2011; Finley, 2009; Glickman and Ciechanover, 2002). Proteasomes are comprised of a cylindrical core particle (CP) capped at each end by a regulatory particle (RP) (Lander et al., 2012; He et al., 2012). The RP captures and denatures ubiquitin-marked protein substrates, and translocates their unfolded polypeptide chains towards proteolytic active-sites in the CP’s lumen (Finley, 2009). CP contains three types of active-sites, each of which comprises a peptide-docking area and an exposed catalytic threonine. PIs including Bortezomib prevent protein hydrolysis by forming covalent adducts with the catalytic threonines of the active-sites (Groll et al., 2009; Beck et al., 2012). Some proteasome activity is necessary for any cell to live (Heinemeyer et al., 1997), not just MM cells (Craxton et al., 2012; Suraweera et al., 2012).

Although the Bortezomib concentrations at which cells of different cancers die differ widely, from low nanomolar to high micromolar IC50 concentrations, cells of the (incurable) B-cell malignancy MM are exquisitely sensitive (Shabaneh et al., 2013), hence Bortezomib’s success in treatment of MM (Anderson et al., 2011). Intriguingly, Bortezomib at its low IC50 concentration causes only a small reduction in proteasomes’ ability to degrade proteins (Kisselev et al., 2006; Shabaneh et al., 2013). The reduction is small because Bortezomib preferentially inhibits the chymotrypsin-like (CT-Like) active-site, but – at IC50 – does not inhibit the caspase-like and trypsin-like active-sites (Fig. S2A) (Kisselev et al., 2006); however, protein substrates can be hydrolysed by any of the three types of active-sites (Kessler et al., 2001; Kisselev et al., 2006). Thus, the question arises why minimal inhibition of proteasome function suffices to induce apoptosis in MM but not in Bortezomib-insensitive cells. Several explanations have been proposed, including high proteasome workload in MM cells (Bianchi et al., 2009; Meister et al., 2007; Shabaneh et al., 2013).

We report that, surprisingly, a (low) IC50 Bortezomib challenge – in vitro minimally inhibits proteasomes – in living MM cells severely inhibits proteasomes’ hydrolytic activity. Our data suggest that, in living MM cells, a Bortezomib-induced structural change in the
proteasome (Pitcher et al., 2014) is responsible for this severe degree of proteasome inhibition.

2. Experimental procedures

Antibodies from Enzo Life Sciences: α-ubiquitin (FK2, PW8810), α-cleaved caspase 3 (Pab, ADI-SAces-103), α-20S α7 (MoAb LN43, BML-PW8110), α-Rpn12/S14 (Pab, BML-PW8815), α-Rpn10/55a (MoAb S5a-18, BML-PW9250), α-Rpt5/S6a (MoAb TBP1-19, PW8770), α-Rpt4/S10b (MoAb p42-23, PW8830), α-Rpt2/S4 (Pab, BML-PW8305), and α-JS1/LMP7 (Pab, PW8355). Antibodies from other sources: α-streptag (Qiagen, MoAb 34485), α-PARP (Cell Signalling, Pab, #9542) and α-procaspase 3 (Cell Signalling, Pab, #9661). CTAB-PAGE as described (Pitcher et al., 2014; Simpson, 2010). Additional Reagents: Ada-K (Biotin)-Ahx1-L3-VS, epoxomicin (Enzo Life Sciences), Bortezomib (Millennium/Takeda), Streptactin resin (Qiagen, 30004), Ni + + NTa resin (Sigma-Aldrich, His Select HF Nickel Affinity Gel, H5037 (Fig. 2c), or His Select agarose, P2266). AnnexinV/7AAD apoptosis staining (EBioscience 88-8007-74) was used to assess cell viability. Enzymes: DNase1, micrococal nuclease, RNaseA/T1, RNaseH, S1 nuclease and RNaseA1 (Fermentas/Thermo), PDE1 from Crotalus adamantius venom (Sigma, P2343-1VL). Caspase Inhibitor Set III (Enzo Life Sciences, ALX-850-227-K01), used at 1:500 dilution = 4 μM. Antibodies were diluted in PBS + 0.5% Tween20 for Western blotting, PVDF membranes were re-probed multiple times (Yeung and Stanley, 2009). Myeloma cell lines NCI-H929, KMS12-BM, RPMI-8226, OPM2, and the T-lymphocyte Jurkat cell line, were grown in RPMI (Sigma), supplemented with 10% v/v FBS, and 1% v/v Pen/strep. Cell fractionation procedure as described (Pitcher et al., 2014). For fractionation, a cystosol extraction (CE) buffer (25 mM Tris–HCl, pH 7.8, 5 mM MgCl2/EDTA, 1 mM ATP/ADP, 2 mM DTT, 150 mM NaCl, 0.1 or 0.5% NP-40/IGEPAL) and nuclear extraction (NE) buffer (Bakondi et al., 2011) (20 mM HEPES [pH 7.4], 420 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 1 tablet of inhibitor cocktail (Roche, 11-873-580-001) per 50 ml) were used. When using the NE for subsequent affinity-purification, 2 volumes of H2O were added to 1 volume NE to reduce the NaCl concentration to physiological levels. For non-fractionated lysates, cell pellets snap-frozen in LN and stored at −80 °C were resuspended and combined in 10 × volume of CE buffer and passed through a NanoDeBEE (BEE international) homogeniser at 19,000 PSI. Proteasome activity was measured basically as described (Kisselev and Goldberg, 2005; Vlchez et al., 2012), using fluorogenic protease substrates Suc–LLVY–AMC, Ac–RLR–AMC, Ac–GPLD–AMC (Enzo Life Sciences) for the three types of active-site. Cells were lysed in proteasome assay buffer (50 mM Tris–HCl, pH 7.5, 250 mM sucrose, 5 mM MgCl2, 0.5 mM EDTA, 2 mM ATP and 1 mM DTT) by passing cells through a 29 G needle ten times. Fluorescence (380 nm excitation, 460 nm emission) was monitored continuously on a microplate fluorometer for 1 h at 37 °C. To measure for the presence of free 20S proteasomes, 0.015% SDS was added to the proteasome assay buffer (Fig. 2C). For each well, a second was set up with the addition of 40 mM Bortezomib; any activity in the Bortezomib wells was subtracted from corresponding wells to compensate for unspecific protease activity. The ubiquitinated substrate (G3P) (Matyskiela et al., 2013) was kindly provided by Dr Andreas Martin, and used as described. In-vitro degradation with purified yeast (Fig. 54) and human (Fig. 3CD) proteasomes was done in PBS supplemented with 2.5 mM ATP, 2.5 mM MgCl2, 2.5 mM DTT, and 1% DMSO in a total volume of 20 μl. Reaction was performed for 1 h either at 30 °C or 37 °C (Fig. 3CD), after which the reaction was stopped by addition of 20 μl × SDS sample buffer and boiled.

Graphs were produced using Graph pad Prism 6. Data plotted were mean of replicates, with error bars plotted of the standard error of mean (SEM).

Research was supported by Leukaemia Lymphoma Research UK (LLR Grant 10016 to MFK, LLR Gordon Piller Studentship award Grant 101043 to MFK, AK, DP), by an MRC-Imperial Confidence-in-Concept (ICIC) grant to MFK, and by the NIHR Biomedical Research Centre at Imperial College NHS Trust, London.

3. Results

NCI-H929 MM cells were challenged with a lethal (10 nM) Bortezomib concentration for varying lengths of time. Cells were then lysed and an artificial fluorogenic peptide substrate was used to measure proteasomes’ hydrolytic activity (Fig. 1a). Over 0–6/8 h, CT-like activity declined continually to almost nothing. Only when it was nearly absent (~10%), after 6–8 hour incubation, was an increase in ubiquitin conjugate levels observed; intracellular accumulation of ubiquitinated proteins indicates that proteolytic workload exceeds proteasome capacity, accumulation being a phenotype which integrates other regulatory mechanisms in the cell including deubiquitinating enzyme activity (Fig. 1a; see also Fig. S1A). However, when cells were first lysed and then challenged with 10 nM Bortezomib, only a 40% reduction in proteasomal (CT-like) activity was observed (Fig. S2A: 61.5% CT-like activity left, and as previously reported (Kisselev et al., 2006)). A much stronger-than-expected in-vivo inhibitory effect was also observed for the other types of proteasomal active-sites (Figs. S2E,S2C).

Predictable explanations for Bortezomib’s severe inhibition of proteasomes’ activity in MM cells did not apply: (1) severe inhibition was not due to prolonged incubation, because exposing purified proteasomes in the test tube to 10 nM Bortezomib for longer periods of time still showed modest inhibition in line with Fig. S2A (Figs. 1B; Fig. S1B) (Bianchi et al., 2009; Kisselev et al., 2006; Shabaneh et al., 2013). Furthermore, we identified a MM cell line, RPMI-8226, in which CT-like proteasome activity dropped very rapidly, within 2 h, to <10% (Fig. 1e); this time-frame approaches that of the in-vitro test using cell lysate (Fig. S2A), but in-vitro 61.5% of activity remains. (2) Although 10 nM Bortezomib induced apoptosis, and apoptosis is known to inhibit proteasomes via caspase activation (Sun et al., 2004; Adrain et al., 2004), caspase activation was not responsible for the observed proteasome inhibition: inhibition preceded caspase activation (Fig. 1c), occurred when caspase inhibitors were present (Fig. 1d), cells were alive until 10 h post-challenge as measured by Annexin-V staining (Fig. S2B), and there was a non-Myeloma, T-lymphocyte cell line that did not die with 10 nM Bortezomib but in which proteasome activity still dropped well beyond expectation to ~20% (Figs. 1F,S2D; see also lung carcinoma A549 cells: Fig. S2D). (3) Severe inhibition was not because MM cells actively pumped in and/or retained Bortezomib to establish a 10–100 fold higher (Fig. S2A) intracellular Bortezomib concentration. We used the irreversible biotinylated inhibitor Ada-K(Biotin)-Ahx1-L3-VS (Kessler et al., 2001) to simultaneously measure inhibition of activity and the degree to which active-sites were inhibited, i.e. biotinylated (Kessler et al., 2001). We observed in-vivo a discrepancy between active-site inhibition and reduction in hydrolytic activity of those active-sites (Fig. 2A).

We now report a structural change in the proteasome which does correlate with Bortezomib-triggered early shutdown of proteasomal activity: Bortezomib-triggered early changes in posttranslational modifications on proteasomal subunits. We recently reported that human nuclear proteasomes carry a constitutive, CTAB-PAGE-detectable, posttranslational modification, exactly at Bortezomib’s IC50 concentration and above (Pitcher et al., 2014). Fig. 2b – CTAB-PAGE analysis of total cell lysate – shows that changes in these modifications of the proteasomal Rpn12 subunit occur between 1 h (RPMI-8226) and 4 h (H929) of PI challenge. To examine these Bortezomib-triggered changes in finer detail, we combined cell fractionation with affinity-purification of human proteasomes. (Purification is a technical trick that enables modified subunits to also become compatible with, and visible on, SDS-PAGE (Pitcher et al., 2014).) We generated retrovirally-transduced OPM2 MM cells, which expressed a Rpn11 subunit tagged N-terminally with...
a His6-StrepII–TEV-affinity cassette. Cell fractionation and then affinity-purification (using streptactin or Ni2+NTA) showed that subunits from specifically nuclear proteasomes are extensively modified, as we reported previously (Pitcher et al., 2014). Repeating this fractionation-then-affinity-purification procedure, but this time after OPM2 cells had been treated with 20 nM (lethal) Bortezomib for up to 5–7 h, showed that patterns of modified subunits changed (Fig. 2d,e). These changes’ characteristics depended on the particular proteasome subunit that was analysed. Generalising, these changes involved (A) a reduction in complexity for nuclear proteasomes (e.g. Rpt5, Rpn12), and/or (B) the appearance of modified subunit species in the cytosol fraction (e.g. β5i, Rpn12). (A) and (B) raise the possibility that severe inhibition of proteasome activity in total lysate (Fig. 1) may result from changes in proteasome structure which happen both in the cytosolic and nuclear compartments of the cell after cells are challenged with Bortezomib. We are currently investigating the exact nature of the proteasome modifications.

In order to test directly if changes in these proteasome modifications affect proteasome function, we first searched for commercial enzyme preparations that can digest the modifications of nuclear proteasomes. We discovered that a combination of venom phosphodiesterase-1 and S1 nuclease was efficient in trimming these modifications and collapsing modified Rpt2 species into its correct subunit-size species (Fig. 3A).
we combined this enzyme protocol with an in-vitro proteasome-mediated proteolysis assay, which uses a ubiquitinated model protein as substrate (Matyskiela et al., 2013) (Figs. 3B, S4). We scaled up cell growth and affinity-purified proteasomes from total (i.e. including nuclear) lysate. Proteasomes bound to affinity-resin were mock-treated or treated with PDE1/S1 at a sub-optimal (20 °C) temperature, after which resin was washed to remove enzymes. Next, proteasomes were eluted to generate a control and a PDE1/S1-treated proteasome.
preparation. Most subunits of PDE1/S1-treated versus untreated proteasomes were very similar, but for example Rpn12 showed pronounced differences (Fig. S3). We then incubated the proteasome preparations with a ubiquitinated model protein (Matyskiela et al., 2013) (Fig. S4) in order to assess the ability of these proteasome preparations to process a ubiquitinated substrate. At low proteasome/substrate ratio at 30 °C, the PDE1/S1-treated proteasomes were impaired in degrading ubiquitinated substrate (Fig. 3C). However, increasing the ratio overcame this defect, with both preparations degrading substrate equivalently. At even higher proteasome/substrate ratio, and at 37 °C, proteasomes processed the substrate towards deubiquitination rather than degradation, and again both preparations behaved equivalently (Fig. 3B). In addition, we found that changes in the redox state of the reaction conditions uncovered qualitative differences between enzyme-treated and untreated proteasomes (Fig. 3D): in the oxidizing conditions minus DTT, enzyme-treated proteasomes removed streptag epitope (i.e. substrate) more efficiently than untreated proteasomes but did not shift down the ubiquitin signal correspondingly, indicating that these enzyme-treated
proteasomes only partially digested the substrate protein – starting from the tagged carboxyterminus – before premature release. In contrast, under reducing conditions, untreated proteasomes were more efficient in degrading substrate than enzyme-treated proteasomes (see also Fig. 3C). In sum, our data indicate that, under certain experimental conditions, changes in proteasome modifications affect proteasome function, thereby strengthening the case that Bortezomib-triggered changes in proteasome modifications within cells also affect proteasome function.

4. Discussion

In summary, our data reveal a dramatic inhibition of proteasome activity in MM cells after a (low) IC₅₀ Bortezomib challenge, and suggest that this inhibition is the compound result of, first, inhibition of a subset of active-sites, and, second, structural changes in the proteasome which further impair hydrolytic activity (Fig. 3E). Engagement of PIs with active-sites changes proteasome conformation and stabilizes the (distant) CP–RP (Kleijnen et al., 2007; Park et al., 2008) and RP-hPLIC/ubiquitin (Kleijnen et al., 2000) interactions, thus providing a possible signalling mechanism into the cell that may enable active-site inhibition to directly trigger activation of the cellular machinery that then changes the posttranslational modifications of the proteasomes. Whereas it has been very difficult to explain why MM cells die from a nanomolar IC₅₀ Bortezomib challenge when assuming that the modest level of proteasome inhibition observed in vitro holds true in vivo (Bianchi et al., 2009; Kisselev et al., 2006; Shabanah et al., 2013), it is not surprising that a myeloma cell with over 95% CT-inhibition and observable proteasome stress (i.e. accumulation of ubiquitin conjugates, Fig. 1a) will undergo apoptosis. In addition, our data show that Bortezomib, in cancer cells which are Bortezomib-resistant, does not achieve the same degree of proteasome inhibition as in (Bortezomib-sensitive) MM cells (Figs. 1F, S2D), thus providing a molecular mechanism explaining what differentiates MM from most other cancers which Bortezomib cannot treat. Please note that our data indicate that a high proteasome workload in MM cells (Bianchi et al., 2009; Meister et al., 2007; Shabanah et al., 2013) cannot be the primary reason for MM cells’ sensitivity to Bortezomib: for this explanation to work, all proteasomes in a cell would need to be fully engaged – with no spare capacity left – in order for a minimal inhibition of proteasomes to produce proteasome stress; instead, we observed that MM cells have much spare proteasome capacity, and that reducing capacity even to 20% still did not yield proteasome stress (Fig. 1a). Understanding the cellular mechanism via which Bortezomib amplifies its effect on proteasome function may enable future intervention to re-sensitize Bortezomib-resistant cells to treatment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.05.006.

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Disclosure of conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors would like to thank Prof. Irene Roberts for helpful discussions.

Authorship contributions

DSP designed, performed research, analysed data, and wrote manuscript. KdM-S, KT designed, performed research and analysed data. HWA, AK designed research and analysed data. MFK designed, performed research, analysed data, and wrote manuscript.

D.S. Pitcher et al. / EBioMedicine 2 (2015) 642–648

647
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