Schwann cell autophagy induced by SAHA, 17-AAG, or clonazepam can reduce bortezomib-induced peripheral neuropathy

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The development of novel agents, such as proteasome inhibitors and immunomodulatory drugs has improved the survival outcome for multiple myeloma (MM) patients (Kumar et al, 2008). However, the incidence of peripheral neuropathy (PN) has emerged as a significant problem in the new therapeutic era for MM (Richardson et al, 2006, 2009b; Argyriou et al, 2008). In younger patients with MM, primary treatments have included vincristine (VCR), doxorubicin, and dexamethasone, and also high-dose therapy with melphalan supported by autologous stem cell transplantation. However, in some of these patients, VCR treatments have caused PN. Moreover, bortezomib was the first proteasome inhibitor to be approved for the treatment of relapsed/refractory as well as newly diagnosed MM patients (Richardson et al, 2003; San Miguel et al, 2008). However, this treatment can cause peripheral nerve damage leading to the development of bortezomib-induced peripheral neuropathy (BiPN). Owing to these adverse effect, bortezomib will be discontinued even in patients that respond well to this drug. Not surprisingly, bortezomib has recently become one of the mainstays in ongoing clinical trials of combination therapies for MM.

A couple of recent studies have reported neurophysiological and pathological findings for bortezomib administration in animal models (Cavaletti et al, 2007; Bruna et al, 2010; Meregalli et al, 2010). Another histopathological study in rats reported that bortezomib did not affect neurons but did cause damage to Schwann cells (Cavaletti et al, 2007). Another report has however shown that alterations to Schwann cells might be a secondary effect of bortezomib (Bruna et al, 2010). At present, treatments for BiPN are lacking, although anticonvulsants have been administered to MM patients with this disorder (Richardson et al, 2006; Argyriou et al, 2008). In addition, although a dose-modification guideline for BiPN has been published (Richardson et al, 2009b), it is difficult to accurately evaluate neurotoxicity in patients during bortezomib therapy and thus determine when treatment should discontinue. Hence, combination bortezomib treatments for MM involving agents that function as prophylactics against BiPN, rather than drugs that treat BiPN, are highly desirable. However, there are currently few (if any) investigative tools available to develop such therapies as the molecular mechanisms underlying BiPN remain to be elucidated.

To elucidate the molecular mechanisms underpinning the onset of BiPN in our current study, we first reviewed previous reports on neurodegenerative diseases in which protein aggregates are responsible for the cellular toxicity. When the activity of proteasome is inhibited, misfolded proteins will form aggregates...
known as aggresomes (Johnston et al., 1998). Aggresomes were initially described as inclusion bodies in the cells of patients with neurodegenerative diseases (Kopito, 2000) such as amyotrophic lateral sclerosis (Brujin et al., 1998; Mezey et al., 1998), Parkinson’s disease (Mezey et al., 1998), and Huntington’s disease (Bennett et al., 2007). In our present experiments, we employed a schwannoma cell system to monitor aggresome formation after treatment with bortezomib. Furthermore, we examined whether additional treatments could reduce the number and size of these aggregates and thus potentially suppress the onset of BiPN.

MATERIALS AND METHODS

Schwann cell pretreatment and bortezomib treatment

A rat schwannoma cell line RT4-D6P2T (purchased from ATCC, Manassas, VA, USA, on 28 May 2007) was cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St Louis, MO, USA) containing 10% FBS (Bioserum, Victoria, Australia). RT4-D6P2T cells were cultured for less than 2 months after reconstitution from stocks, which were frozen upon receipt from the ATCC. The cells had been validated by the supplier using DNA fingerprinting and no additional authentication was performed in our laboratory. The morphology of the RT4-D6P2T cells showed no changes over the course of the study.

At 1 day before pretreatment, the RT4-D6PT2 cells were plated at a density of $5 \times 10^4$ cells per well on four-well chamber slides. They were then either untreated or pretreated with 40 nM VCR (Sigma-Aldrich) for 1 h or pretreated for 24 h with either 5 µM suberoylanilide hydroxamic acid (SAHA; Merck & Co. Inc., Whitehouse Station, NJ, USA), 0.5 µM 17-allylamino-17-demethoxy-geldanamycin (17-AAG; Sigma-Aldrich), 50 nM clonazepam (CZP; Sigma-Aldrich), or 6 mM valproic acid (VPA; Sigma-Aldrich). The dose of each reagent was determined by its half maximal inhibitory value (IC50). For VCR pretreatments, the cells were washed twice with PBS: 2.68 mM KCl, 1.47 mM KH2PO4, 13.69 mM NaCl, and 8.10 mM Na2HPO4 (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan) before the addition of 40 nM bortezomib (Millennium Pharmaceuticals, Cambridge, MA, USA) for 3 h. Following pretreatment with other reagents, the cells were not washed before the 3-h treatment with 40 nM bortezomib. As a final step, the cells were washed twice with PBS, incubated for a further 24 h, and then fixed.

Figure 1 Bortezomib induces aggresome formation at the microtubule-organising centres (MTOCs) of Schwann cells. (A) In untreated RT4-D6P2T cells, γ-tubulin is distributed homogeneously throughout the cytoplasm (left panel). In bortezomib (Bzb)-treated cells, aggresomes form as distinct pericentriolar structures (arrows) with weak staining in the cytoplasm (right panel). Insets in the right panel show the juxtanuclear rounded structures evident at higher magnification. (B) Untreated RT4-D6P2T cells contain dynein (red), which is distributed homogeneously in the cytoplasm with predominant localisation in the perinuclear region, and vimentin (green), which is distributed diffusely throughout the cytoplasm and above the nuclei. (C) In bortezomib-treated cells, dynein (red) and vimentin (green) appear as rounded structures at the MTOC (arrows) and are colocalised in the region adjacent to the nuclei (yellow signals in the merged image of both fluorochrome channels. Bar, 20 µm. −Bzb, untreated; +Bzb, bortezomib treated.
Immunohistochemical analysis

The RT4-D6P2T cells were fixed with PBS containing 4% paraformaldehyde for 10 min at 4°C, washed with TBS (20 mM Tris and 500 mM NaCl (pH 7.4)) with 0.1% IGEPAL CA-630 (Fluka, Buchs, Switzerland) for 3 × 5 min, fixed in methanol for 10 min at 4°C, and blocked with PBS containing 4% BSA (Sigma-Aldrich) for 30 min at room temperature. The cells were then incubated overnight at 4°C with primary antibodies diluted at a ratio of 1 : 50 in PBS with 4% BSA (γ-tubulin (Sigma-Aldrich), dynein (Sigma-Aldrich), vimentin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), heat-shock protein 70 (HSP70; Santa Cruz Biotechnology), peripheral myelin protein 22 (PMP22; Millipore, Bedford, MA, USA), and lysosome-associated membrane protein type 2A (LAMP-2A; Abcam, Cambridge, MA, USA)). The cells were then washed 3 × 5 min in TBS with 0.1% IGEPAL CA-630 and incubated with secondary antibodies diluted at a ratio of 1 : 100 in PBS with 4% BSA, for 1 h at room temperature (Alexa Fluor 488-conjugated chicken anti-rabbit IgG and Alexa Fluor 555-conjugated goat antimouse IgG (Molecular Probes, Eugene, OR, USA)). After a further washing for 3 × 5 min in TBS with 0.1% IGEPAL CA-630, the cells were mounted on slides with VECTASHIELD (Vector Laboratories, Burlingame, CA, USA). We note that all washes were performed at room temperature. Images of the cells were captured on a laser scanning confocal microscope BZ-8000 (Keyence, Osaka, Japan) and analysed by BX-Analyzer software (Keyence). The thickness of the optical sections analysed was 0.4 μm.

Quantification of aggresomes and round structures outside of the Schwann cells

Aggresomes and round structures outside of the cells were identified by the colocalisation of PMP22 and γ-tubulin, counted

Figure 2  Vincristine (VCR) abrogates aggresome formation and in a combination treatment with bortezomib augments the exocytosis of endogenous misfolded proteins. (A) Peripheral myelin protein 22 (PMP22) is homogeneously distributed throughout the cytoplasm of RT4-D6P2T cells before treatment with bortezomib (left panel). After treatment with bortezomib (Bzb), PMP22 appears to undergo retrograde transport towards the MTOC where it forms perinuclear aggresomes (arrows, middle panel, green signals) and colocalises with γ-tubulin (arrows, middle panel, red signals). A merged image of both fluorophores is shown in the far right panel (yellow signal). (B) Following pretreatment with VCR, a microtubule depolymerisation agent, PMP22 signals are evident at multiple sites in a granular pattern of aggregates throughout the cytoplasm, most notably in the perikaryon. Cells pretreated with (C) suberoylanilide hydroxamic acid (SAHA), a known histone deacetylase inhibitor (HDACi), or (D) clonazepam (CZP), an anticonvulsant, and (E) 17-allylamino-17-demethoxy-geldanamycin (17-AAG), a HSP90 inhibitor, fail to form aggresomes, but instead form rounded structures outside of the cell (arrowheads), which are smaller than the perinuclear aggresomes. (F) Pretreatment with valproic acid (VPA) causes the appearance of similar rounded structures outside of the cells (arrowheads) in addition to juxtanuclear aggresomes (arrows).
in triplicate from 200 cells, and expressed as a percentage of the total cells.

**Growth inhibition assay of MM cells**

The human MM cell lines, MM.1S, RPMI8226 (purchased from ATCC), and KMS-18 (kindly provided by Dr T Otsuki, Department of Hygiene, Kawasaki Medical School, Kurashiki, Japan) were maintained in RPMI1640 (Sigma-Aldrich) containing 10% FBS. The growth-inhibitory effects upon MM cells were determined using a 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Sigma-Aldrich). At 1 day before treatment, 9.0 × 10^4 cells per 90 μl aliquot were cultured in 96-well plates (Sumitomo Bakelite, Higashikangawa, Japan) in triplicate at 37°C. Cells were either untreated or pretreated for 24 h with the same concentration of each reagent used with the RT4-D6P2T cells except for VCR. The cells were then cultured further with varying concentrations (from 0.5 to 3 nM) of bortezomib for 48 h. Optical densities (ODs) at 570 and 630 nm were measured using a multiplate reader. Stock MTT was added to each of the wells in the assay, and the plates were further incubated at 37°C for 1 h. Dimethyl sulphoxide (Sigma-Aldrich) was added to all wells and mixed thoroughly. After a few minutes at room temperature to ensure that all formazan crystals were dissolved, the plates were read on a SpectroMax 340PC VersaMax (Molecular Devices, Sunnyvale, CA, USA), using a test wavelength of 570 nm and a reference wavelength of 630 nm. Cell growth (%) was calculated as follows: (OD_{630} – OD_{570} of the samples/OD_{630} – OD_{570} of the control) × 100.

**RESULTS**

**Aggresomes form at MTOC following proteasome inhibition in Schwann cells**

A diffuse expression pattern of γ-tubulin, a protein that adheres to the centrosome (Dietzeng et al, 1998), was observed in the cytoplasm of RT4-D6P2T cells. Following a 3-h treatment with 40 nM bortezomib, however, γ-tubulin staining in the cytoplasm became weak and coalesced to form round structures in the juxtanuclear area (Figure 1A). Similarly, the dynein and vimentin proteins became rounded and colocalised in region adjacent to the nucleus after exposure to bortezomib (Figure 1C).

**Vincristine abrogates bortezomib-induced aggresome formation and combination treatments augment the exocytosis of endogenous misfolded proteins from Schwann cells**

We next examined whether endogenous misfolded proteins destined to be processed by the ubiquitin-proteasome system could be induced to aggregate and undergo retrograde transport towards the microtubule-organising centre (MTOC) upon proteasome inhibition. To accomplish this, we employed the cellular marker PMP22, a short-lived glycoprotein present in Schwann cells (Fortun et al, 2003). Following bortezomib treatment, PMP22 showed a distinct juxtanuclear and rounded appearance and colocalised with γ-tubulin to form aggresomes (Figure 2A), as previously reported (Fortun et al, 2003). Interestingly, treatments with VCR completely abrogated the bortezomib-induced accumulation of PMP22, which was instead observed as numerous spots in the perikaryon (Figure 2B).

We next analysed whether treatments with a combination of reagents could reduce aggresome formation. Intriguingly, pre-treatment with the histone deacetylase inhibitor (HDACi) SAHA (Figure 2C), the anticonvulsant CZP (Figure 2D), or the HSP90 inhibitor 17-AAG (Figure 2E) caused the appearance of round structures, which were smaller than aggresomes, outside of the nucleus (Figure 2F).

**Autophagy reduces bortezomib-induced neuropathy**

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**Chaperone-mediated autophagy is responsible for the enhanced exocytosis of misfolded proteins in Schwann cells during proteasome inhibition**

To analyse the molecular mechanisms underlying the enhanced exocytosis of misfolded proteins in Schwann cells, we used an antibodies against the HSP70 chaperone protein and the receptor for chaperone-mediated autophagy (CMA) at the lysosomal membrane (which is a unique isoform of LAMP-2, LAMP-2A) (Cuervo and Dice, 2000; Kaushik et al, 2006). After treatment with SAHA, 17-AAG, or CZP (Figure 4A, B, or C, respectively) followed by bortezomib, HSP70 and LAMP-2A were found to colocalise in structures outside of the cells.

**Drugs that protect Schwann cells from aggresome formation due to bortezomib treatment do not disrupt the growth inhibitory effects of bortezomib in myeloma cells**

Pretreatments of MM cells with the same drugs used in the RT4-D6P2T cell experiments had few negative effects on the profound growth inhibitory effects of bortezomib in myeloma cells.

**DISCUSSION**

The findings of our present study using a schwannoma cell model system suggest that aggresome formations caused by proteasome inhibition and the excretion pathways of intracellular misfolded proteins are targets for combination drug candidates that will alleviate the onset of BiPN during bortezomib treatment. A recent study of skin biopsies has revealed that BiPN manifests as predominantly large fibres (Chaudhry et al, 2008). On the other hand, in some BiPN patients who develop treatment-emergent neuropathy, the underlying cause has been attributed to the impairment of small fibres (Richardson et al, 2006), even though such fibres comprise myelinated Aδ and unmyelinated C fibres. In contrast, it has been proposed that 68–85% of BiPN cases are reversible (Richardson et al, 2009a, b).
Figure 4  Combination treatments with bortezomib can augment the exocytosis of misfolded proteins through the chaperone-mediated autophagy of Schwann cells. The distributions of HSP70/HSC70 (red), a chaperone protein, and LAMP-2A (green), a lysosomal membrane protein with a specific role in chaperone-mediated autophagy, are shown in response to combination treatments with (A) SAHA, (B) 17-AAG, and (C) CZP. The colocalisation of both proteins is evidenced by the small rounded structures outside of the cells that appear as an orange signal (arrowheads). Bar, 20 μM.

Figure 5  The same combinations used in Figures 2 and 3 do not suppress the growth inhibition of multiple myeloma (MM) cells induced by bortezomib. In MM cells (MM.1S, KMS-18, and RPMI8226) were treated with bortezomib alone or in combination with SAHA, CZP, 17-AAG, or VPA. The proportion of viable cells after pretreatment with each drug followed by bortezomib treatment is indicated as a percentage of the untreated cells. These numbers were measured in triplicate and are expressed as the means ± s.d.
Although it has already been demonstrated that the behaviour of cells of neoplastic origin can differ markedly from normal cells (Scuteri et al., 2006), cell lines are usually more tractable for experimental purpose than primary culture cells. In addition, because BiPN is predominantly sensory (Richardson et al., 2006; Richardson et al., 2009a, b), it would have been desirable to use cell lines that would somewhat mimic the peripheral sensory nerves.

No such cells are currently available however and we thus employed schwannoma cells for analysis, which are benign and differentiated tumour cells, rather than neuroblastoma cells used in previous reports (Scuteri et al., 2006; Csizmadia et al., 2008).

Our present data are consistent with previous observations that misfolded proteins form aggregates throughout the cell if they are not degraded by the proteasome (Figure 2B). Furthermore, such aggregates are then transported in a microtubule (MT)-dependent manner to the MTOC on the dynein motor complex (Figure 1C, red) (Johnston et al., 1998; Kopito, 2000; García-Mata et al., 2002). After treatment with bortezomib, it has been shown that vimentin, the most common component of the intermediate filament cytoskeleton (Franke et al., 1978), collapses to form a ‘cage’ surrounding the aggresome, which then adopts a ‘rounded’ morphology (Figure 1C, green) (Johnston et al., 1998; García-Mata et al., 1999). Moreover, our observations of aggresome formations with a distinct juxtanuclear spherical appearance that colocalise with γ-tubulin (Figure 1A, right) after treatment with proteasome inhibitor in Schwann cells corroborate those of a previous study (Fortun et al., 2003). Moreover, our results demonstrating that the fate of intracellular ubiquitinated aggregation-prone proteins may be relevant to the development of BiPN support previous findings for the gene expression profiles of bone marrow cells in MM patients with treatment-emergent BiPN (Richardson et al., 2009b).

These authors identified distinct classes of gene transcripts, namely those involved in the initiation and regulation of protein translation, and their results indicated that enriched proteins that are released from MM cells may be toxic to the peripheral nervous system (Richardson et al., 2009b).

PMP22 is associated with a demyelinating PN, Charcot–Marie– Tooth disease type 1A (Patel et al., 1992), and VCR is contra-indicated in patients with this disease. In our present study, we observed that VCR treatment resulted in the dispersion of aggregates in the cytoplasm and no formation of juxtanuclear aggresomes (Figure 2B). In other words, because VCR is an MT-disrupting drug, our result suggests that pretreatment with this agent might increase BiPN by hindering the movement of unfolded proteins along the MTs with dynein motor complexes (Figure 6A).

Indeed, other investigators have suggested that the neuropathy produced by VCR treatment may compromise the ability of the patients to receive bortezomib (Kyle and Rajkumar, 2009).

The central aim of our current study was to develop a clinically relevant in vitro system to test drugs that could be combined with bortezomib to reduce the incidence of BiPN. One of the tested candidates was the anticonvulsant VPA, which has been used previously to alleviate the symptoms of painful diabetic neuropathy (Koch et al., 2004). However, the 6 mM concentration of VPA used in our experiments is more than 4000-fold higher than the previously reported clinical dosage (Munster et al., 2009). Furthermore, our results suggest that VPA may be less effective in

Figure 6 Schematic representation of the disruption of aggresome formation (A) and chaperone-mediated autophagy (CMA) (B). (A) Small peripherally formed aggregates are transported along the microtubule (MT) tracks by retrograde motors (i.e., dyneins) to a juxtanuclear pericentriolar location, the MT organisation centre (MTOC). ‘Xvcr’ indicates that VCR pretreatment before the administration of bortezomib (Bzb) hinders aggresome formation. This is likely because VCR is an MT-disrupting drug and the aggregates would be unable to move along the MTs on the dyneins. (B) SAHA, CZP, 17-AAG, and VPA have the potential to enhance the expression of HSP70/heat-shock cognate protein of 70 Kd (HSC70), which recognises the specific motif targeted by CMA in its substrate proteins. Lysosome-associated membrane protein type 2A (LAMP-2A) is a unique receptor for CMA. Aggregated proteins are delivered from the cytoplasm out of the cells through lysosomes (L) by CMA.
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Reducing BiPN other than HDACi’s such as SAHA or anticonvulsants like CZP. Indeed, pretreatment with VPA followed by bortezomib was found to elicit juxtanuclear aggresome formation in addition to the formation of rounded structures outside of the cells (Figures 2F and 3). On the other hand, SAHA has been shown previously to disrupt bortezomib-induced aggresome formation in MM cells (Nawrocki et al, 2008) as a result of the destruction of HDAC6, which promotes aggresome inclusion of misfolded polyubiquitylated proteins on the dynein motor complexes along the MTs (Kawaguchi et al, 2003). The 5 μM concentration of SAHA used in this study was two- to five-fold higher than the clinically usable dose in our previous pharmacokinetic analyses of phase I trials of oral SAHA (Watanabe et al, 2010). However, the 40 nM quantity of bortezomib used in this study is equivalent to that observed in our earlier study (Ogawa et al, 2008), and the 40 nM of VCR, 50 nM of CZP, and 0.5 μM of 17-AAG used in our analyses are equivalent to the doses for these compounds reported in other studies (Goetz et al, 2005; Corona et al, 2008; dos Santos et al, 2009, respectively).

The results of our current analyses shown in Figure 4 suggest that following pretreatment with the candidate drugs, the aggregated proteins are discarded outside of the cells by CMA (Kauhisk et al, 2006). The evidence in support of CMA as the mechanism of disposal in this case is that the antibody used in our experiments does not distinguish between HSP70 and the heat-shock cognate protein of 70 Kd (HSC70) (Shen et al, 2009), which recognises the CMA-targeting motif in the substrate protein (Agarraberes et al, 1997). To our knowledge, the role of CMA either under conditions of proteasome inhibition or in the nervous system has never been previously reported. However, HSP70 and LAMP-2A, a specific receptor for CMA, were found in our analysis to be colocalised in the rounded structures including misfolded proteins (Figure 4). By inducing the chaperone protein, we speculate that these agents may promote an additional degradation pathway via lysosomes to excrete aggregated proteins from Schwann cells. This is different from the retrograde transport of aggregated proteins to form aggresomes along MTs from the periphery in the cytoplasm to the MTOC, thus aiding cells in the disposal of aggregated proteins (Figure 6B).

The overexpression of HSP70, which could be induced by SAHA alone in our experiments (Figure 4A, data not shown), is a well-described consequence of HSP90 inhibition by 17-AAG (Guo et al, 2005). This finding is consistent with the results from series of previous reports, which showed that a pan-HDACi similar to SAHA inhibits the HSP90 deacetylase HDAC6 (Bali et al, 2005), and that acetylation of HSP90 releases heat-shock factor-1 from HSP90 (Zou et al, 1998) and consequently induces HSP70 expression (Morimoto, 1998). Furthermore, our present in vitro data may corroborate the results of a clinical trial with bortezomib and tanespimycin (a cremophor-based formulation of 17-AAG) in which BiPN was reduced (Mitsiades et al, 2009; Richardson et al, 2010). In the case of SAHA, a multicentre phase I trial in combination with bortezomib for relapsed or refractory MM patients has been performed and only mild PN was reported (Badros et al, 2009). Another case series has reported gastrointestinal tract events only without discontinuation or dose adjustments of either agent (Mazummer et al, 2010). Interestingly, HSP70 has also been show to have a major role in the cellular defence against the toxic effects of misfolded proteins in neurodegenerative diseases such as amyotrophic lateral sclerosis (Gifondorwa et al, 2007), Parkinson’s disease (Roodveldt et al, 2009), and Huntington’s disease (Wacker et al, 2009).

As the binding of substrates, that is, misfolded proteins, to LAMP-2A is the limiting step for degradation via CMA (Cuervo and Dice, 1996), the induction of LAMP-2A as well as HSP70/HSC70 may be a promising marker for screening drugs that may reduce BiPN.

In summary, although the results of our present study are preliminary and in vitro only, our data suggest that the combination of bortezomib and SAHA, 17-AAG, or CZP has the potential to reduce BiPN. As bortezomib is currently an important component of combination treatment for MM, our in vitro system may allow MM patients to continue to benefit from bortezomib in the future.

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