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Bortezomib alters microtubule polymerization and axonal transport in rat dorsal root ganglion neurons

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ABSTRACT

Bortezomib is part of a newer class of chemotherapeutic agents whose mechanism of action is inhibition of the proteasome-ubiquitination system. Primarily used in multiple myeloma, bortezomib causes a sensory-predominant axonal peripheral neuropathy in approximately 30% of patients. There are no established useful preventative agents for bortezomib-induced peripheral neuropathy (BIPN), and the molecular mechanisms of BIPN are unknown. We have developed an *in vitro* model of BIPN using rat dorsal root ganglia neuronal cultures. At clinically-relevant dosages, bortezomib produces a sensory axonopathy as evidenced by whole explant outgrowth and cell survival assays. This sensory axonopathy is associated with alterations in tubulin and results in accumulation of somatic tubulin without changes in microtubule ultrastructure. Furthermore, we observed an increased proportion of polymerized tubulin, but not total or acetylated tubulin, in bortezomib-treated DRG neurons. Similar findings are observed with lactacystin, an unrelated proteasome-inhibitor, which argues for a class effect of proteasome inhibition on dorsal root ganglion neurons. Finally, there is a change in axonal transport of mitochondria induced by bortezomib in a time-dependent fashion. In summary, we have developed an *in vitro* model of BIPN that recapitulates the clinical sensory axonopathy; this model demonstrates that bortezomib induces an alteration in microtubules and axonal transport. This robust model will be used in future mechanistic studies of BIPN and its prevention.

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1. Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a serious side-effect that often limits chemotherapy dosage. Pain and other quality of life impairments caused by CIPN are on the rise, as many forms of cancer become chronic conditions. Protective strategies are complicated by the possibility that preventing CIPN may reduce the primary cancer cell killing effect of a drug.

Bortezomib (Velcade®) is the first member of a new class of chemotherapeutic agents that inhibit the proteasome-ubiquitination pathway, a critical pathway for intracellular protein degradation (Adams et al., 1998). It does this by reversible binding and inhibition of the 26S subunit of the proteasome. Bortezomib is FDA-approved for use in multiple myeloma and mantle cell lymphoma, and is being investigated for use in a host of other hematological and solid tumors.

Bortezomib causes a painful axonal sensory-predominant length-dependent peripheral neuropathy in 30–40% of patients that limits the allowable dosage (Barr et al., 2009; Mauermann et al., 2012; Richardson et al., 2006). Bortezomib-induced peripheral neuropathy (BIPN) may be seen in patients with or without pre-existing peripheral neuropathy from their underlying cancer. Interestingly, approximately 2/3 of patients with BIPN have improvement or resolution of neuropathic symptoms following bortezomib discontinuation (Richardson et al., 2006). The combination of length-dependent phenotype, clinical neurophysiology that predicts axonal pathology, and clinical reversibility suggests that minimal neuronal death is occurring and that the primary pathophysiology is of a "dying back" toxic axonal neuropathy (Boyette-Davis et al., 2011).

Several mechanisms of action for bortezomib-induced cancer cell death have been proposed. The most prominent theory for its cancer-killing properties is the decreased activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) that

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leads to apoptosis (Traenckner et al., 1994). It has been demonstrated that in the presence of bortezomib, the inhibitory factor for NF-KB (IF-KB) is not degraded properly *via* the proteasome pathway and is thus available for continued inhibition of NF-KB (Traenckner et al., 1994). Other mechanisms of action for cancer cell death have been established, including effects on the Bcl-2 family members, constitutive activation of c-Jun N-terminal kinase (JNK), upregulation of p53, and increased endoplasmic reticulum stress (Voorhees and Orlowski, 2006). The relation of these cancer cell death mechanisms to BIPN has not been established.

The mechanisms of BIPN are unclear. In order to begin to mechanistically understand BIPN, we now describe an *in vitro* model of bortezomib-induced peripheral neuropathy utilizing the cell type that is affected in this condition, the dorsal root ganglion sensory neurons.

2. Materials and methods

Dorsal root ganglia (DRG) from embryonic day 15 Sprague-Dawley rat were used for all experiments in this study, as approved by the Mayo Clinic Institutional Animal Care and Use Committee. Rat DRG cultures are an established model to study various mechanisms of neurotoxicity (Gill and Windebank, 1998; Podratz et al., 2011; Scuteri et al., 2006; Ta et al., 2006; Windebank et al., 1994).

2.1. DRG explant neurite outgrowth model

Whole DRG were harvested and plated on collagen-coated plastic dishes and incubated in enhanced minimally-enriched media (EMEM) AN2 medium containing 10% calf bovine serum (Hyclone, Logan, UT), 7 mg/ml glucose (Sigma, St. Louis, MO) 1.2 mM L-glutamine (Invitrogen, Carlsbad, CA) and 10 ng/ml NGF (Bioproducts for Sciences, Indianapolis, IN). The incubation media contained varying concentrations of bortezomib (LC Laboratories, Woburn, MA) or lactacystin (Peptides International, Louisville, KY). Phase contrast low power micrographs were obtained at 24 and 48 h after culture. Measurements were made from the edge of the DRG radially to the end of the longest neurite outgrowth using Image J software (NIH).

2.2. Cell culture model

DRG were mechanically dissociated aided by trypsin digestion (0.25%) and plated on poly-lysine coated plastic dishes incubated with AN2 as previously described with 15% calf bovine serum (Ta et al., 2006). Cultures were treated with 20 μM 2,5 fluoro-2-deoxyuridine (Sigma, St. Louis, MO) and 20 μM uridine (Sigma, St. Louis, MO) for 3–5 days to decrease the numbers of supporting cells, after which neuron-enriched cultures were treated with drug and processed as outlined below.

DRG neurons were treated with bortezomib (50, 100, or 200 nM) for 48 h; cells from the same plating without bortezomib treatment were used as control. Cell survival was estimated using direct cell counting, and apoptosis was detected using morphological observation of nuclear fragmentation. For cell survival, dissociated DRG neurons were plated on plastic tissue culture dishes etched with a grid. The grids were marked and neurons for the same group were counted manually using Image J software at 0, 24, and 48 h. DRG cultures were fixed with 4% paraformaldehyde for 10 min, mounted with VECTASHIELD mounting medium (Vector Labs, Burlingame, CA) containing 4′,6-diamidino-2-phenylindole (DAPI), and observed by light microscopy for nuclear fragmentation signifying apoptosis. Phase contrast low power (20× magnification) light micrographs were obtained and manually assessed in a blinded-fashion. Only obvious nuclear fragmentation was counted as an apoptotic event.

2.3. Immunohistochemistry

Dissociated DRG neurons with and without bortezomib (100 nM) or lactacystin (10 μM) treatment were washed with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde for 10 min. Cells were treated with PBS-Triton-X (0.05%) for 5 min, washed with PBS-Tween (0.1%) and blocked with 20% goat serum in PBS-Tween. Neurons were incubated for 1 h at room temperature in 1:200 anti-Tuj-1 monoclonal antibody (Neuromics Antibodies, Edina, MN) to stain for beta-tubulin. Neurons were washed and incubated in 1:400 goat antimouse antibody conjugated to Cy3 (Jackson ImmunoResearch, Baltimore Pike, CO). Neurons were mounted on slides using VECTASHIELD mounting medium with DAPI. Images were obtained using either a Zeiss Axiovert fluorescent microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) or Zeiss LSM 500 laser scanning confocal microscope (Carl Zeiss Microimaging Inc., Thornwood, NY).

To quantify the somatic tubulin staining, low power (20× magnification) epifluorescence micrographs were obtained in control, bortezomib and lactacystin-treated cells at identical fluorescence exposure times. In order to quantify somatic tubulin ring epifluorescence in an unbiased fashion, the pixel intensity threshold was set for selected micrographs to define the somatic ring epifluorescence. This pixel intensity was then subtracted uniformly from all micrographs. This resulted in images where tubulin ringed soma were present without lower level staining in other portions of the cell culture. Micrographs were then randomized and the interpreter was blinded to the culture treatment. Ringed soma were then manually counted and presented as a fraction of all soma.

2.4. Electron microscopy

Whole DRG explants were cultured as described above, allowing neurite outgrowth for 48 h. DRG were washed with PBS and fixed for transmission electron microscopy (TEM) in Trump's fixative consisting of 4% formaldehyde, 1% glutaraldehyde in PBS (pH 7.2). Cells were postfixed in 1% osmium tetroxide and stained en bloc with 2% uranyl acetate. DRG were embedded in a mixture of Epon and araldite. All reagents were obtained from Electron Microscope Services (Ft. Washington, PA). Ultrathin sections (100 nm) were cut from the same blocks, mounted on 200 μm mesh copper grids, stained with lead citrate, examined and photographed using an FEI Technai 12 transmission electron microscope at 100 kV (Fei, Inc., Hillsboro, OR), equipped with a digital CCD camera (Advanced Microscopy Techniques, Danvers, MA).

2.5. Western blot analysis

DRG were washed with PBS, scraped from the tissue culture dish and homogenized in NP40 Cell Lysis buffer (Invitrogen, Camarillo, CA). Protein was determined using DC Protein assay (Bio-Rad, Hercules, CA). 10 μg of protein was run per lane on a 10–20% PAGE and then transferred onto PVDF membrane. For determination of acetylated tubulin, PVDF membranes were sequentially blotted with antibodies for tubulin (1:10,000; anti-Tuj-1), anti-acetylated tubulin monoclonal antibody (1:10,000; Sigma–Aldrich, St. Louis, MO) and anti-GAPDH monoclonal antibody (1:10,000; Cell Signaling, Boston, MA), stripping the membrane with guanidine (7 M) in between blots. Detection of the proteins was carried out using a BM Chemiluminescence Western Blotting kit containing the secondary antibody (Roche, Mannheim,

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