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Mitotoxicity and bortezomib-induced chronic painful peripheral neuropathy

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ABSTRACT

Many of the most effective anti-cancer drugs induce a dose-limiting peripheral neuropathy that compromises therapy. Evidence from animal models of chemotherapy-induced painful peripheral neuropathy produced by the taxane agent, paclitaxel, and the platinum-complex agent, oxaliplatin, indicate that they produce neuropathy via a common mechanism—a toxic effect on the mitochondria in primary afferent sensory neurons. Bortezomib is from the proteasome-inhibitor class of chemotherapeutics. It also produces a dose-limiting peripheral neuropathy, but its effects on neuronal mitochondria are unknown. To investigate this, we developed a model of bortezomib-induced painful peripheral neuropathy in the rat and assessed mitochondrial function (respiration and ATP production) in sciatic nerve samples harvested at two time points: day 7, which is three days after treatment and before pain appears, and day 35, which is one month post-treatment and the time of peak pain severity. We found significant deficits in Complex I-mediated and Complex II-mediated respiration, and in ATP production at both time points. Prophylactic treatment with acetyl-L-carnitine, which has previously been shown to prevent paclitaxel- and oxaliplatin-induced mitochondrial dysfunction and pain, completely blocked bortezomib's effects on mitochondria and pain. These results suggest that mitotoxicity may be the core pathology for all chemotherapy-induced peripheral neuropathy and that drugs that protect mitochondrial function may be useful chemotherapy adjuncts.

Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a common dose-limiting complication of cancer chemotherapy and a frequent cause of dose reduction and discontinuation of what is otherwise successful therapy (Paice, 2010; Windebank and Grisold, 2008). Aggressive therapy with doses exceeding the recommended levels greatly increases the incidence of CIPN and patients with pre-existing symptomatic or asymptomatic nerve damage, e.g., diabetes or prior chemotherapy, are at increased risk (Argyriou et al., 2008, 2012; Cavaletti and Jakubowiak, 2010; Corso et al., 2010; Lanzani et al., 2008; Quasthoff and Hartung, 2002; Windebank and Grisold, 2008). The neuropathy generally resolves within weeks to months, but it lasts for years in some patients (Boyette-Davis et al., 2011; Paice, 2010; Pietrangeli et al., 2006).

Chemotherapeutics in the taxane, platinum-complex, and proteasome-inhibitor classes are believed to kill cancer cells via distinctly different mechanisms, but clinical reports suggest that they all produce very similar chronic peripheral neuropathies For all of these drugs the chronic neuropathy is characterized by bilaterally symmetrical sensory symptoms (numbness, tingling, and pain) appearing in the feet, or in the feet and hands, after cumulative dosing (Argyriou et al., 2012; Cata et al., 2006, 2007; Dougherty et al., 2004, 2007; Quasthoff and Hartung, 2002; Windebank and Grisold, 2008). Effects on motor function have been noted, especially acutely (Argyriou et al., 2012; Loprinzi et al., 2011), but motor dysfunction appearing after chronic dosing and with a distal and symmetrical distribution that matches the sensory symptoms is absent or rare.

Work with rat CIPN models also suggests that chemotherapeutics with diverse anti-cancer mechanisms produce very similar chronic painful peripheral neuropathies. In particular, recent work indicates that for the taxane agent, paclitaxel, and the platinum-complex agent, oxaliplatin, this similarity in symptoms is due to a common pathophysiology—both drugs produce a long-lasting dysfunction in mitochondria in primary afferent sensory neurons (Xiao and Bennett, 2012; Xiao et al., 2012; Zheng et al., 2011).

These and other observations have led to the mitotoxicity hypothesis for CIPN. This proposes that a mitotoxic effect in primary afferent sensory neurons gives rise to a persistent energy deficit that results in spontaneous discharge and degeneration of the sensory afferents' terminal arbors in the epidermis (intraepidermal nerve fibers (IENF), the neuronal compartment with the highest energy requirement) (Bennett et al., 2011; Flatters and Bennett, 2006; Flatters et al., 2006; Jin et al., 2008; Siau

Abbreviations: ALCAR, acetyl-L-carnitine; ATF-3, activating transcription factor 3; CIPN, chemotherapy-induced peripheral neuropathy; CSU, citrate synthase units of activity; DRG, dorsal root ganglion; IENF, intraepidermal nerve fiber; PGP9.5, protein gene-product 9.5; VFH, von Frey hair.

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et al., 2006; Xiao and Bennett, 2008, 2012; Xiao et al., 2011, 2012; Zheng et al., 2011).

Evidence supporting the mitotoxicity hypothesis has come from work with paclitaxel and oxaliplatin, and to a lesser extent, the vinca alkaloid agent vincristine. However, it is not known if the proteasome inhibitors have similar mitotoxic effects. We examined this question in a rat model of painful peripheral neuropathy produced by bortezomib (Velcade®), the first of the proteasome inhibitor class of anti-neoplastic agents to enter clinical use (Chen et al., 2011).

Materials and methods

All experimental protocols were approved by the Animal Care Committee of the Faculty of Medicine, McGill University, in accordance with the regulations of the Canadian Council on Animal Care and conformed to the ethics guidelines of the International Association for the Study of Pain (Zimmermann, 1983), the National Institutes of Health (USA), and the Canadian Institutes of Health Research.

Animals

Adult male Sprague–Dawley rats (200–250 g, Harlan Inc., Indianapolis, IN; Frederick, MD breeding colony) were housed on sawdust bedding in plastic cages. Artificial lighting was provided on a fixed 12 h light–dark cycle with food and water available *ad libitum*.

Bortezomib administration

Bortezomib (Chemie Tek, Indianapolis, IN) was administered at 0.2 mg/ml/kg via intraperitoneal injections once per day for five consecutive days (D0–D4) for a total dose of 1.0 mg/kg. Control animals received injections of the vehicle. Animals were weighed daily during treatment and weekly thereafter.

Behavioral testing

Pain assays were conducted with the same methods used previously (Flatters and Bennett, 2006) and by an observer who was blind as to group assignment.

Mechano-allodynia and mechano-hyperalgesia were assessed with a 4 g von Frey hair (VFH), a normally innocuous stimulus, and a 15 g VFH, which is normally a noxious stimulus. Each VFH was applied to the plantar glabrous skin (distal to the calcaneus and proximal to the digital tori) five times per side; withdrawal responses were counted and expressed as an overall percentage response.

Cold-allodynia was assessed with the acetone drop method. A drop (0.05 ml) of acetone was placed against the center of the plantar hind paw and responses were graded with the following 4-point scale: 0 = no response; 1 = quick withdrawal, flick or stamp of the paw; 2 = prolonged withdrawal or repeated flicking, and 3 = repeated flicking of the paw with licking directed at the ventral side of the paw. Acetone was applied three times to each paw and cumulative scores were then generated by adding the 6 scores for each rat.

Heat hypersensitivity was assessed by the Hargreaves method (Hargreaves et al., 1988). Heat intensity was adjusted to yield normal baseline latencies of about 10 s. Scores were derived by averaging the response latencies of six trials (three per side).

Axon counts

The saphenous nerve, which is a nearly pure sensory nerve (Swett et al., 1986, 1991), was excised at mid-thigh level from bortezomib-treated (n = 4) and vehicle-treated rats (n = 3) sacrificed at the time of approximate peak pain severity (D35) and was examined with the electron microscope as described elsewhere (Jin et al., 2008; Xiao et al., 2012). The presence of allodynia and hyperalgesia in the bortezomib-treated rats was confirmed prior to sacrifice. A montage of low magnification (550×) photomicrographs was made for the area of the entire nerve and all myelinated A-fibers were counted. The number of C-fiber axons was estimated by a method that randomly samples ca. 25% of the cross-sectional area of the nerve (Xiao et al., 2012).

ATF-3 staining

Activating transcription factor-3 (ATF-3) is a nuclear marker of axotomized dorsal root ganglion neurons (Tsujino et al., 2000). ATF-3 was visualized as described previously (Jin et al., 2008; Xiao et al., 2012) in dorsal root ganglia L4 and L5 taken on D35. Anti-ATF3 primary antibody (Santa-Cruz Biotechnology; Santa Cruz, CA) was diluted 1:500. No staining was present in sections processed without exposure to the primary antibody. Cell bodies were visualized with a fluorescent Nissl stain (Invitrogen; Carlsbad, CA; dilution: 1:300). Five bortezomib-treated rats were compared to three rats whose sciatic nerves had been transected three days before sacrifice. Four non-adjacent 14 μm DRG sections were examined per rat, three non-overlapping photomicrographs (20×) were taken of each section, and all neurons sectioned through their nucleus were counted.

Mitochondria counts in primary afferent axons and their Schwann cells

Mitochondria were counted in primary afferent A-fibers and C-fibers from the animals used for the axon counts. Random samples of A-fiber and C–fiber axons and of myelinating and non-myelinating Schwann cells were obtained in cross-sections of the saphenous nerve as described previously (Jin et al., 2008; Xiao et al., 2012). The number of normal and swollen and/or vacuolated mitochondria was determined in a randomly chosen sample of at least 60 A-fibers, 60 C-fibers, and 60 myelinating Schwann cells, and in randomly chosen sections through non-myelinating Schwann cell processes (i.e., Remak bundles) that contained at least 50 mitochondria.

IENF quantification

IENFs were visualized immunocytochemically as described previously (Siau et al., 2006) in vehicle-treated and bortezomib-treated rats (n = 8/group) sacrificed at the approximate time of peak pain severity (D35). Using a 40X objective, all ascending nerve fibers that were seen to cross into the epidermis were counted. One section, 8–10 mm long, was counted per rat. The presence of allodynia and hyperalgesia in the bortezomib-treated rats was confirmed prior to sacrifice. Anti-protein gene-product 9.5 antisera (PGP9.5; Research Diagnostics; Flanders, NJ) was diluted 1:6400. IENFs in the glabrous hind paw skin were counted by an observer blind as to the animal's group assignment. IENF counts are expressed as the number per cm of epidermal border (straight line approximation). No staining was present in sections processed without exposure to the primary antibody.

Mitochondrial respiration assay

The analysis of mitochondrial respiration followed the methods described previously (Zheng et al., 2011). Rats were deeply anesthetized with isoflurane and their sciatic nerves were exposed bilaterally from the sciatic notch to the popliteal fossa. The nerves were excised and placed in ice-cold MiR05 medium (Gnaiger et al., 2000), which contains: EGTA, 0.5 mM; $MgCl_26H_2O$, 3.0 mM; K-lactobionate, 60 mM; taurine, 20 mM; KH_2PO_4, 10 mM; sucrose, 110 mM; BSA, 0.1%; and HEPES, 20 mM (all from Sigma; St. Louis, MO). The animals were then overdosed with sodium pentobarbital. The nerves were cut into 1.0–1.5 mm segments and then each segment was teased apart into microfilaments. The preparation was then transferred to the temperature controlled (37 ° C) recording chamber of a high-resolution respirometer