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Neurophysiological, histological and immunohistochemical characterization of bortezomib-induced neuropathy in mice

Jordi Bruna [a,b,c], Esther Udina [a,b], Albert Alé [a], Jorge J. Vilches [a], Ann Vynckier [d], Johan Monbaliu [d], Lee Silverman [e], Xavier Navarro [a,b,*]

[a] Group of Neuroplasticity and Regeneration, Institute of Neurosciences and Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Spain
[b] Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Spain
[c] Unit of Neuro-Oncology, Department of Neurology, Hospital Universitari de Bellvitge, Hospitalet de Llobregat, Spain
[d] Johnson & Johnson, GPCD, Beerse, Belgium
[e] Millennium, Takeda Oncology Company, Cambridge, MA, USA

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ABSTRACT

Bortezomib, a proteasome inhibitor, is an antineoplastic drug to treat multiple myeloma and mantle cell lymphoma. Its most clinically significant adverse event is peripheral sensory neuropathy. Our objective was to characterize the neuropathy induced by bortezomib in a mouse model. Two groups were used; one group received vehicle solution and another bortezomib (1 mg/kg/twice/week) for 6 weeks (total dose as human schedule). Tests were performed during treatment and for 4 weeks post dosing to evaluate electrophysiological, autonomic, pain sensibility and sensory-motor function changes. At the end of treatment and after washout, sciatic and tibial nerves, dorsal ganglia and intraepidermal innervation were analyzed. Bortezomib induced progressive significant decrease of sensory action potential amplitude, mild reduction of sensory velocities without effect in motor conductions. Moreover, it significantly increased pain threshold and sensory-motor impairment at 6 weeks. According to these data, histopathological findings shown a mild reduction of myelinated (−10%; $p=0.001$) and unmyelinated fibers (−27%; $p=0.04$), mostly involving large and C fibers, with abnormal vesicular inclusion body in unmyelinated axons. Neurons were also involved as shown by immunohistochemical phenotypic switch. After washout, partial recovery was observed in functional, electrophysiological and histological analyses. These results suggest that axon and myelin changes might be secondary to an initial dysfunctional neuronopathy.

Introduction

Bortezomib (BTZ) is the first of a new class of anticancer agents known as proteasome inhibitors. BTZ is a reversible inhibitor of 20S ubiquitin-dependent proteasome complex, the major extralysosomal pathway responsible for nuclear and cytoplasmic protein degradation (Nandi et al., 2006). Proteasome inhibition is an interesting approach to cancer treatment because it acts by disruption of various critical cell signaling pathways, such as cell cycle regulation, cell adhesion and gene transcription. Thereby it leads cancerous cells to cell cycle arrest, inhibits angiogenesis and induces apoptosis (Voorhees et al., 2003; Jackson et al., 2005; Ludwig et al., 2005). BTZ is effective in the treatment of recurrent and newly diagnosed multiple myeloma (Richardson et al., 2003; San Miguel et al., 2008), and of recurrent mantle cell lymphoma (Kane et al., 2007). It is also under investigation as treatment for many common solid and hematological neoplasms, alone or in combination with other chemotherapeutic drugs (Awada et al., 2008; Caponigro et al., 2009; Davies et al., 2009).

However, one of most clinically significant adverse events and dose-limiting toxicity in BTZ therapy is the peripheral neuropathy, mainly characterized by hypoesthesia and sometimes painful paraesthesia (Richardson et al., 2006). The incidence of neuropathy reported in several phase II and III clinical trials using BTZ both in combination and as a single agent ranged from 31% to 55% (Richardson et al., 2003; Jagannath et al., 2004; Richardson et al., 2005; Harousseau et al., 2006; Bang et al., 2006; Badros et al., 2007; Min et al., 2007; Mateos et al., 2008). The neuropathy becomes severe, to grades 3 and 4 of the National Cancer Institute Common Toxicity Criteria (NCI-CTC) score (Trotti et al., 2003) in 13–17% and 1–7% of the patients respectively (Richardson et al., 2003; Jagannath et al., 2004; Richardson et al., 2005; Mateos et al., 2008). Moreover, neuropathy leads to a dose reduction in 12% and discontinuation of treatment in 5% of patients (Richardson et al., 2006) with the corresponding repercussion on patients' quality of life and survival (Jagannath et al., 2008). The

* Corresponding author. Unitat Fisiologia Mèdica, Facultat de Medicina, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain. Fax: +34 935812986.
E-mail address: xavier.navarro@uab.cat (X. Navarro).

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overall incidence of neuropathy is similar between patients without or with baseline neuropathy prior to the start BTZ therapy, a feature not unusual in myeloma patients (Richardson et al., 2006; Richardson et al., 2009). However, severe BTZ induced neuropathy is more frequent in the patients with baseline neuropathy (Richardson et al., 2006).

Current knowledge on the mechanisms underlying the BTZ induced peripheral neuropathy (BIPN) is scarce and contradictory. Only one group has described experimental studies of BIPN in a rat model (Cavaletti et al., 2007; Meregalli et al., 2009), in the first work, the electrophysiological findings were not in accordance with the main neurographic abnormalities found in BIPN patients. The study suggested that BTZ induces damage to satellite cells of dorsal root ganglia (DRG) (Cavaletti et al., 2007), whereas their second and most recent work proposes that BTZ induces painful unmyelinated axonopathy (Meregalli et al., 2009). On the other hand, in vitro studies identified the cell bodies of DRG neurons as the primary target for proteasome inhibitor peripheral neuropathy (Silverman et al., 2006). A recent in vitro study also supports the view that BTZ provokes dysfunction in sensory neurons by interfering with transcription and mRNA processing (Casafont et al., 2010). Thus, it is important to have an in vivo model available to further elucidate the mechanisms involved in BIPN, and to help in improving the clinical management of patients with BIPN. Moreover, it may be a useful model to evaluate neuroprotective agents designed to ameliorate or restore BIPN. The aim of our study was to develop and characterize an adequate model of BIPN in mice, assessed by electrophysiology, functional tests, morphology and immunohistochemistry.

Materials and methods

Animals, treatment and dose schedule

In a preliminary pilot study 20 Swiss OF1 female mice aged 2.5 months were used to assess different doses of BTZ and verify the safety and the development of neuropathic findings in this mouse strain. BTZ (provided by Millennium Pharmaceuticals Inc., and Johnson & Johnson Pharmaceutical Research & Development, L.L.C.) was dissolved in sterile saline solution and administered at doses of 0.8 mg/kg twice per week ($n=5$), 1 mg/kg twice per week (2pw) ($n=5$) or 1 mg/kg three times per week (3pw) ($n=5$). A fourth group ($n=5$) received only vehicle solution (control group). Treatment was administered subcutaneously during 6 weeks.

After determining the adequate dose to achieve a clinically relevant BIPN, a larger group of Swiss OF1 female mice aged 2.5 months ($n=20$) received BTZ treatment at the dose selected (e.g. 1 mg/kg) in the pilot study during 6 weeks, on days 1-4-8-11-15-18-22-25-29-32-36-39. At the end of the treatment period, half of the mice were sacrificed while the remaining animals were left untreated and followed-up for an additional 4-week period. In parallel, a second group of 10 mice receiving vehicle solution on the same days was used as control (CTROL).

The animals were housed under standard conditions in cages with soft bedding. Artificial lighting was provided on a fixed 12-h light–dark cycle with food and water available ad libitum. The general condition of the animals was assessed daily and body weight was recorded before each BTZ administration. The experimental procedures were approved by the Ethical Committee of our institution, and followed the rules of the European Communities Council Directive 86/609/EEC.

Functional tests

Functional evaluation was performed at baseline, before starting BTZ administration, and then every 2 weeks during treatment, and 4 weeks after finalization of treatment. Tests performed were aimed at quantitatively evaluating motor and sensory nerve conduction, autonomic sudomotor and heart function, pain sensibility, and integration of sensory-motor function.

Nerve conduction studies

For nerve conduction studies, the sciatic nerve was stimulated percutaneously through a pair of needle electrodes placed at the sciatic notch (proximal site) and at the ankle (distal site). Rectangular electrical pulses (Grass S88 stimulator) of 0.01 ms duration were applied up to 25% above the voltage that gave a maximal response (Navarro et al., 1994; Verdu et al., 1999). The compound muscle action potentials (CMAPs) were recorded from the tibialis anterior and the third interosseus muscle with microneedle electrodes. Similarly, the sensory compound nerve action potential (SNAP) was recorded by electrodes placed at the fourth toe near the digital nerves. Latencies and amplitudes of the action potentials were measured and nerve conduction velocities of motor and sensory nerve fibers were estimated. During electrophysiological tests, the animals were anesthetized (pentobarbital 40 mg/kg i.p.) and placed over a warm flat steamer controlled by a hot water circulating pump to maintain the body temperature constant.

Autonomic function tests

Sympathetic sudomotor function was evaluated by the silastic imprint technique. Sweating was stimulated by subcutaneous injection of pilocarpine (5 mg/kg), and 10 min later a silicone material (Silasoft Normal, Detax GmbH & Co., Ettlingen, Germany) was spread over the plantar surface of the hindpaw. Sweat droplet impressions made in the silicone mold were counted under a dissecting microscope (Navarro et al., 1994; Vilches and Navarro, 2002).

Heart rate variability was analyzed using an electrocardiographic recording for 3 min, acquired through a PowerLab system (ADInstruments) and stored by Chart software. Heart R-R periods were evaluated using Pearson Variation Coefficient.

Pain sensibility tests

The algesimetry technique was used to evaluate the functional status of nociceptive C fibers (Hargreaves et al., 1988). Mice were placed into a plastic box with an elevated glass floor (Plantar Algesimeter, Ugo Basile). The light of a projection lamp was focused directly onto the plantar surface of one hindpaw and the time to elevation of the heated paw was obtained from a time-meter coupled with infrared detectors. The value for a test was the mean of three trials separated by 10 min resting periods.

Sensory-motor function

A rotarod apparatus for small rodents (LIAP) was used. Mice were placed on the rod, turning at 8 rpm, and the time that each animal remained on it before falling was measured. The value for a test was the mean of three trials separated by 10 min resting intervals. Before treatment, mice were trained for 5 days. The ability to remain on the rotarod for 120 s was taken as an index of normal sensory-motor function (Navarro et al., 1993; Verdu et al., 1999).

Histological methods

At the end of the sixth week treatment period, half of the animals belonging to BTZ treated and control groups were anaesthetized and perfused with paraformaldehyde (4% in PBS 0.1 M, pH 7.4). The other half of animals was perfused after 4 weeks of washout period. A segment of the sciatic nerve at mid-thigh and a segment of the tibial nerve at the ankle level were removed. The nerve samples were fixed in glutaraldehyde–paraformaldehyde (3%:3%), washed in cacodylate buffer (0.1 M, pH 7.4), then post-fixed overnight with acetate uranile in 70% alcohol and 2% osmium tetroxide during 2 h at 4 °C, dehydrated in graded concentrations of ethanol and embedded in epon. Light