Published in final edited form as:

Neuropharmacology. 2017 September 01; 123: 477–487. doi:10.1016/j.neuropharm.2017.06.027.

Bortezomib Induces Neuropathic Pain through Protein Kinase C-Mediated Activation of Presynaptic NMDA Receptors in the Spinal Cord

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Abstract

Chemotherapeutic drugs, including bortezomib, often cause painful peripheral neuropathy, which is a severe dose-limiting adverse effect experienced by many cancer patients. The glutamate N-methyl-D-aspartate receptors (NMDARs) at the spinal cord level are critically involved in the synaptic plasticity associated with neuropathic pain. In this study, we determined whether treatment with bortezomib, a proteasome inhibitor, affects the NMDAR activity of spinal dorsal horn neurons. Systemic treatment with bortezomib in rats did not significantly affect postsynaptic NMDAR currents elicited by puff application of NMDA directly to dorsal horn neurons. Bortezomib treatment markedly increased the baseline frequency of miniature excitatory postsynaptic currents (EPSCs), which was completely normalized by the NMDAR antagonist 2-amino-5-phosphonopentanoic acid (AP5). AP5 also reduced the amplitude of monosynaptic EPSCs evoked by dorsal root stimulation in bortezomib-treated, but not vehicle-treated, rats. Furthermore, inhibition of protein kinase C (PKC) with chelerythrine fully reversed the increased frequency of miniature EPSCs and the amplitude of evoked EPSCs in bortezomib-treated rats. Intrathecal injection of AP5 and chelerythrine both profoundly attenuated mechanical allodynia and hyperalgesia induced by systemic treatment with bortezomib. In addition, treatment with bortezomib induced striking membrane translocation of PKC- β II, PKC- δ , and PKC- ϵ in the dorsal root ganglion. Our findings indicate that bortezomib treatment potentiates nociceptive input from primary afferent nerves via PKC-mediated tonic activation of presynaptic NMDARs. Targeting

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

J.-D.X., S.-R.C., and H.C. conducted experiments. J.-D.X., S.-R.C., and H.-L.P. performed data analysis. H.-L.P. conceived the project and wrote the manuscript with input from the other authors.

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presynaptic NMDARs and PKC at the spinal cord level may be an effective strategy for treating chemotherapy-induced neuropathic pain.

Keywords

synaptic plasticity; NMDA receptor; synaptic transmission; proteasome; neuropathic pain; spinal cord

Introduction

Chemotherapy-induced peripheral neuropathy is a major adverse effect associated with many cancer therapeutic agents (Grisold et al., 2012; Polomano and Bennett, 2001; Staff et al., 2017). Bortezomib, a boronic acid dipeptide that inhibits the 20S proteasome complex, is used clinically for the treatment of multiple myeloma and mantle cell non-Hodgkin's lymphoma (Jackson et al., 2005; Piperdi et al., 2011). Bortezomib-induced peripheral neuropathy occurs in about half of patients (Argyriou et al., 2008; Gilardini et al., 2008), with sensory symptoms including numbness, tingling, and a burning sensation in a stocking-and-glove distribution. These conditions are chronic and respond poorly to conventional analgesics such as opioids, anticonvulsants, and antidepressants (Sisignano et al., 2014). Persistent, severe neuropathic pain may require dose reduction or cessation of chemotherapy, which can increase cancer-related morbidity and mortality (Grisold et al., 2012; Seretny et al., 2014). Thus, development of effective mechanism-based therapies for chemotherapy-induced chronic pain is essential.

Maintaining the balance between protein synthesis and degradation is an important homeostatic function of eukaryotic cells. The ubiquitin-proteasome system is responsible for degradation of most intracellular proteins and therefore plays an essential regulatory role in critical cellular processes (Imai et al., 2003; Kornitzer and Ciechanover, 2000). Proteasomes are large multicatalytic proteinase complexes involved in maintenance of normal cellular functions, and alteration of proteasomal activity contributes to the pathological states of several clinical disorders, including inflammation and cancer. Most tumor cells are highly proliferative and have a greater requirement for protein synthesis than do normal cells, making them more vulnerable to proteasome inhibition (Yang et al., 2009). The proposed antitumor actions of proteasome inhibitors include inducing cell-cycle arrest at G2-M phase, promoting apoptosis, inhibiting NF- κ B activity, and inhibiting angiogenesis (Jackson et al., 2005; Piperdi et al., 2011; Voorhees et al., 2003). Although bortezomib treatment is well known to damage the dorsal root ganglion (DRG) and peripheral nerves (Bruna et al., 2010; Carozzi et al., 2013; Cavaletti et al., 2007; Zheng et al., 2012), the exact cellular and molecular mechanisms responsible for bortezomib-induced persistent pain remain elusive.

The glutamate N-methyl-D-aspartate receptors (NMDARs) at the spinal cord level play a critical role in central sensitization and the development of chronic pain after traumatic nerve injury (Chen et al., 2014b; Li et al., 2016; Yamamoto and Yaksh, 1992). NMDARs are commonly present at the postsynaptic densities of excitatory synapses, where they mediate the slow excitatory postsynaptic potential (Traynelis et al., 2010). Increased postsynaptic NMDAR activity in spinal dorsal horn neurons results in neuropathic pain by increasing

neuronal excitability and diminishing synaptic inhibition via K^+ - Cl^- -cotransporter-2 proteolysis (Chen et al., 2014b; Zhou et al., 2012). NMDARs are also present presynaptically, where they can enhance neurotransmitter release and be tonically activated by ambient glutamate (Brasier and Feldman, 2008; Glitsch and Marty, 1999; Ye et al., 2011). At the spinal cord level, NMDARs are expressed at the central terminals of primary afferent nerves (Liu et al., 1994). Although presynaptic NMDARs in the spinal dorsal horn are latent and not functionally active under physiological conditions, they become activated under various painful conditions (Chen et al., 2011; Chen et al., 2014a; Li et al., 2016; Xie et al., 2016; Zhao et al., 2012). Nevertheless, little is known about whether proteasome inhibition affects presynaptic or postsynaptic NMDAR activity at the spinal cord level.

Therefore, in the present study, we investigated the effect of systemic treatment with bortezomib on presynaptic and postsynaptic NMDARs of spinal dorsal horn neurons in rats. Our findings indicate that bortezomib-induced neuropathic pain is associated with potentiated activity of presynaptic, but not postsynaptic, NMDARs via protein kinase C (PKC) at the spinal cord level. This study provides new insight into the functional significance of presynaptic NMDARs in bortezomib-induced neuropathic pain and the signaling mechanism involved in NMDAR activation by proteasome inhibition.

Materials and Methods

Animal model

Experiments were carried out using adult male Sprague-Dawley rats (220–250 g; Harlan, Indianapolis, IN). A total of 78 rats was used for the entire study. All procedures and protocols were approved (approval #1186-RN00) by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and IASP Ethical Guidelines for pain studies in conscious animals (Zimmermann, 1983). To induce peripheral neuropathy, rats were intraperitoneally injected with bortezomib (0.2 mg/kg, 1 ml/kg; Millennium Pharmaceuticals, Cambridge, MA) on four alternate days (days 1, 3, 5, and 7; total cumulative dose, 0.8 mg/kg). Rats in the control group received intraperitoneal injection of a vehicle (Mannitol) on the same four alternate days. The presence of mechanical hyperalgesia and tactile allodynia in the hindpaw of all rats were confirmed after completion of bortezomib treatment. It has been shown that bortezomib-induced pain hypersensitivity in rats lasts for more than 6 weeks (Zheng et al., 2012). All final electrophysiological recordings, biochemical assays, and behavioral tests were done 7–10 days after the final bortezomib or vehicle injection.

Intrathecal injections in rats were performed using a lumbar puncture technique. In brief, the rats were anesthetized with 2% isoflurane in oxygen, and the lumbar region was shaved and prepared with Betadine solution. The lumbar intervertebral spaces were widened by placing the rats on a plexiglas tube. A 27-gauge needle was inserted at the L5-6 interspace with a deflection of the tail indicating the entry to the subarachnoid space.

Nociceptive behavioral tests

Rats were individually placed in suspended chambers on a mesh floor. To determine their tactile sensitivity, a series of calibrated von Frey filaments was applied perpendicularly to the plantar surface of both hindpaws with sufficient force to bend them for 6 s after at least 30 min of acclimation. Brisk withdrawal or flinching of the paw was considered a positive response. In the absence of a response, the filament was applied at the next greater force. If the animal had a response, the filament was applied at the next lower force. The tactile stimulus producing a 50% likelihood of withdrawal response was calculated using the “up-down” method (Chaplan et al., 1994). Each test was repeated 2 or 3 times at approximately 2-min intervals, and the mean value of withdrawal responses was used as the force to produce withdrawal responses.

To determine the mechanical nociceptive threshold in the rats, we conducted a paw pressure (Randall-Selitto) test on the hindpaw by using an analgesimeter (Ugo Basile, Varese, Italy). To activate the device, a foot pedal was pressed to initiate a motor that applied a constantly increasing force on a linear scale. When an animal displayed pain response by either withdrawing the paw or vocalizing, the pedal was released immediately, and the animal's nociceptive threshold on the scale was recorded (Chen et al., 2011; Chen et al., 2014a). A maximum of 400 g force was used as a cutoff to avoid tissue injury to the animals.

Spinal cord slice preparation and electrophysiological recordings

Rats were anesthetized with 2–3% isoflurane, and their lumbar spinal cords at the L3–L6 level were quickly removed through laminectomy. The resected spinal cords were immediately transferred into ice-cold sucrose artificial cerebrospinal fluid presaturated with 95% O₂ and 5% CO₂. The fluid contained (in mM) 234.0 sucrose, 3.6 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 1.2 NaH₂PO₄, 12.0 glucose, and 25.0 NaHCO₃. The spinal cords in artificial cerebrospinal fluid were then placed onto the stage of a vibratome (Leica Microsystems, Wetzlar, Germany) and sliced transversely into 400-μm sections that were preincubated in Krebs solution oxygenated with 95% O₂ and 5% CO₂ at 34°C for at least 1 h before being transferred to a recording chamber. The Krebs solution contained (in mM) 117.0 NaCl, 3.6 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 1.2 NaH₂PO₄, 11.0 glucose, and 25.0 NaHCO₃.

Each spinal cord slice was placed in a glass-bottomed chamber with continuous perfusion of Krebs solution at 5 ml/min and 34°C. The spinal lamina II in each slice was identified under an upright fixed-stage microscope with differential interference contrast/infrared illumination. Lamina II outer zone neurons were visualized and selected for recording because they preferentially receive nociceptive input from primary afferent nerves (Pan and Pan, 2004). Most lamina II neurons are glutamate-releasing excitatory interneurons (Santos et al., 2007) and thus play a pivotal role in transmitting nociceptive information from the primary afferents. Excitatory postsynaptic currents (EPSCs) of lamina II neurons were recorded using whole-cell voltage-clamp techniques. The impedance of the glass electrode was 4–7 MΩ when a pipette was filled with an internal solution consisting of (in mM) 135.0 potassium gluconate, 5.0 KCl, 2.0 MgCl₂, 0.5 CaCl₂, 5.0 HEPES, 5.0 EGTA, 5.0 ATP-Mg, 0.5 NaGTP, and 10.0 QX314 (adjusted to pH 7.25 with 1.0 M KOH, 280–300 mosM). Miniature EPSCs (mEPSCs) of lamina II neurons were recorded at a holding potential of

–60 mV in the presence of 2 μ M strychnine, 10 μ M bicuculline, and 1 μ M tetrodotoxin (Chen et al., 2011; Chen et al., 2014a). In addition, EPSCs were evoked from the dorsal root using a bipolar tungsten electrode linked with a stimulator (0.2 ms, 0.3–0.5 mA, and 0.1 Hz; Grass Instruments, Quincy, MA) (Li et al., 2002; Zhou et al., 2010). The input resistance was monitored, and the recording was abandoned if the resistance changed more than 15%. EPSCs were recorded using an amplifier (MultiClamp 700A, Axon Instruments, Foster City, CA), filtered at 1–2 kHz, and digitized at 10 kHz.

To record postsynaptic NMDAR activity, currents were elicited by puff application of 100 μ M NMDA directly to the recorded neuron at a holding potential of –60 mV. To minimize the magnesium block of NMDARs, the puff NMDA currents were recorded in an extracellular solution containing Mg^{2+} at a low concentration (0.1 mM), 10 μ M glycine, and 1 μ M tetrodotoxin (Chen et al., 2014a; Li et al., 2016). The pipette internal solution contained (in mM) 110.0 Cs_2SO_4 , 2.0 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10.0 HEPES, 2.0 $MgATP$, and 0.3 Na_2GTP (pH adjusted to 7.25 with 1.0 M $CsOH$ [280–300 mosM]). The puff electrode was placed about 150 μ m away from the neuron being recorded. Puff application of NMDA was performed using a Pressure System IIe (4 p.s.i., 15 ms; Toohey Co., Fairfield, NJ). Only one neuron was recorded in each spinal cord slice, and at least four rats were used in each group.

All drugs were freshly diluted in artificial cerebrospinal fluid before the experiments and delivered via syringe pumps to reach their final concentrations. 2-Amino-5-phosphonopentanoic acid (AP5) was purchased from Abcam (Cambridge, MA), and chelerythrine was obtained from Sigma-Aldrich (St. Louis, MO).

Western immunoblotting

To measure protein levels of PKC isoforms in plasma membrane and cytosolic fractions, subcellular fractionation was carried out using a membrane protein extraction kit (Cat. #89842, Thermo Fisher Scientific, Rockford, IL). Lumbar DRG tissues were rapidly removed from rats anesthetized with 3% isoflurane and cut into small pieces. The homogenate was centrifuged at 16,000 g for 15 min at 4 °C to separate the plasma membrane (pellet) and cytoplasmic (supernatant) fractions.

F11, a DRG neuronal cell line (Jow et al., 2006), was maintained in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. For all assays, cells were incubated with 10 nM bortezomib at 37 °C in 5% CO_2 for 0.5, 1, and 6 h and then quickly washed with PBS. F11 cells were harvested and homogenized in ice-cold radioimmune precipitation assay buffer containing cocktails of protease inhibitors (Sigma). The homogenate was centrifuged at 16,000 g for 15 min at 4 °C to separate the plasma membrane (pellet) and cytoplasmic (supernatant) fractions.

The protein samples extracted from DRG tissues and F11 cells were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The blots were probed with mouse monoclonal antibodies against PKC- α , PKC- β I, PKC- β II, PKC- δ , and PKC- ϵ (Cat. #sc-8393, #sc-209, #sc-13149, #sc-8402, and #sc-214, respectively; Santa Cruz Biotechnology). The specificity of these anti-PKC isoform

antibodies was validated in previous studies (Hafeez et al., 2011; He and Wang, 2015; Metzger et al., 2010). An enhanced chemiluminescence kit (GE Healthcare) was used to detect the protein band, which was visualized and quantified using an Odyssey Fc Imager (LI-COR Biosciences, Lincoln, NE). The amount of each PKC isoform was normalized by that of GAPDH in the same samples. The mean value of each PKC isoform protein in vehicle-treated samples was considered to be 1.

Statistical analysis

All data are expressed as means $\pm$ SEM. The amplitude of the evoked EPSCs was quantified by averaging 10 consecutive EPSCs using the Clampfit 10.0 software program (Molecular Devices, Sunnyvale, CA). The mEPSCs were analyzed using a peak detection program (MiniAnalysis, Synaptosoft Inc., Leonia, NJ). The cumulative probability of the amplitudes and inter-event intervals of the mEPSCs were compared using the Kolmogorov-Smirnov test, which estimates the probability that two distributions are similar. We used the two-tailed Student's t-test to determine the statistical differences between two groups. To compare more than two groups, we used one-way analysis of variance (ANOVA) followed by the Dunnett's or Tukey's post hoc test. $P < 0.05$ was considered to be statistically significant.

Results

Treatment with bortezomib causes long-lasting mechanical hypersensitivity

Systemic injection of bortezomib on four alternate days at a clinically relevant dose (0.2 mg/kg) (Meregalli et al., 2014) caused a gradual decrease in the tactile and pressure withdrawal thresholds in 7 rats (Fig. 1). Bortezomib-induced reduction in the paw withdrawal thresholds persisted for at least another week after the last injection. In another 7 rats, systemic treatment with the vehicle did not significantly alter the paw withdrawal thresholds measured using von Frey filaments and noxious pressure stimulus (Fig. 1).

Bortezomib increases presynaptic NMDAR activity at the spinal cord level

We determined whether bortezomib alters the activity of presynaptic NMDARs by recording glutamatergic mEPSCs, which reflect spontaneous quantal release of glutamate from presynaptic terminals (Zhao et al., 2012; Zhou et al., 2012). In dorsal horn neurons of vehicle-treated rats, the baseline frequency of mEPSCs was 3.88 ± 0.36 Hz ($n = 8$ neurons). Bath application of AP5 (50 μ M), a specific NMDAR antagonist, did not significantly affect the frequency or amplitude of mEPSCs in these neurons (Fig. 2, A and C). In contrast, the baseline frequency of mEPSCs in dorsal horn neurons of bortezomib-treated rats was much higher (8.50 ± 0.58 Hz, $n = 10$ neurons) than that in vehicle-treated rats. Bath application of 50 μ M AP5 largely normalized the frequency of mEPSCs in dorsal horn neurons recorded in bortezomib-treated rats (Fig. 2, B and C). These data suggest that systemic treatment with bortezomib increases the activity of presynaptic NMDARs in the spinal dorsal horn.

Bortezomib increases NMDAR activity at primary afferent terminals in the spinal cord

To determine whether bortezomib treatment increases the activity of NMDARs at primary afferent terminals, we recorded monosynaptic EPSCs of spinal dorsal horn neurons evoked

from the dorsal root, which represents induced release of glutamate from primary afferent terminals. Monosynaptic EPSCs were identified on the basis of the constant latency and absence of conduction failure of evoked EPSCs in response to a 20-Hz electrical stimulation, as we described previously (Li et al., 2002; Zhou et al., 2010). The baseline amplitude of evoked EPSCs was significantly higher in bortezomib-treated rats than in vehicle-treated rats (Fig. 3A). Because the stimulation intensity can affect the amplitude of evoked EPSCs, the amplitude of evoked EPSCs was normalized to the baseline for each recorded neuron. Bath application of 50 μ M AP5 markedly reduced the amplitude of monosynaptic EPSCs of dorsal horn neurons in bortezomib-treated rats ($n = 12$ neurons, Fig. 3A). However, AP5 had no significant effect on the amplitude of monosynaptic EPSCs of dorsal horn neurons in vehicle-treated rats ($n = 11$ neurons, Fig. 3A). These results suggest that presynaptic NMDAR activity at primary afferent terminals is increased by bortezomib treatment.

Bortezomib does not affect postsynaptic NMDAR activity of dorsal horn neurons

To determine the possible effect of systemic treatment with bortezomib on postsynaptic NMDARs in the spinal cord, we recorded currents elicited by puff application of 100 μ M NMDA directly onto the recorded spinal dorsal horn neuron (Chen et al., 2014a; Li et al., 2016). The amplitude of the inward NMDAR currents of dorsal horn neurons did not differ significantly between vehicle-treated rats ($n = 10$ neurons) and bortezomib-treated rats ($n = 12$ neurons) (Fig. 3B).

PKC mediates bortezomib-induced increases in presynaptic NMDAR activity in the spinal cord

PKC is a family of serine/threonine protein kinases that serve as major effectors of functional modulation of the neuronal signaling machinery. Increased PKC activity can promote NMDAR expression on the cell surface and regulate channel gating (Chen and Huang, 1992; Lan et al., 2001). We thus determined whether PKC plays a role in bortezomib-induced increase in presynaptic NMDAR activity in the spinal cord. Chelerythrine is a specific membrane-permeable inhibitor of the catalytic site of PKC (Herbert et al., 1990). It has been shown that treatment with 10 μ M chelerythrine inhibits PKC activity in spinal cord slices (Zhao et al., 2012). We obtained spinal cord slices from vehicle-treated rats and incubated them with 10 μ M chelerythrine for 60 min. Bath application of 50 μ M AP5 had no effect on the baseline frequency of mEPSCs (Fig. 4) or amplitude of evoked monosynaptic EPSCs in spinal dorsal horn neurons (Fig. 5). In dorsal horn neurons from bortezomib-treated rats, chelerythrine treatment completely normalized the baseline frequency of mEPSCs ($n = 12$ neurons, Fig. 4). In these neurons, bath application of 50 μ M AP5 had no effect on the frequency of mEPSCs.

Furthermore, we examined the effect of chelerythrine on the amplitude of EPSCs monosynaptically evoked from the dorsal root in spinal cord slices from rats injected with the vehicle or bortezomib. After treatment with chelerythrine (10 μ M for 60 min), bath application of AP5 had no significant effect on the amplitude of evoked EPSCs of dorsal horn neurons from rats receiving the vehicle ($n = 11$ neurons) or bortezomib ($n = 13$ neurons, Fig. 5). These findings suggest that PKC plays a critical role in bortezomib-induced increases in presynaptic NMDAR activity in the spinal cord.

Bortezomib increases PKC membrane translocation in the DRG

Increased cellular PKC activity is reflected by the translocation of PKC proteins from the cytoplasm to the plasma membrane (Shirai and Saito, 2002; van Baal et al., 2005). We thus examined the effect of bortezomib treatment on the translocation of major PKC isoforms expressed in the DRGs (He and Wang, 2015; Laumet et al., 2015). Western blotting analysis of DRG tissues showed that the immunoreactivity of PKC- β II, PKC- δ , and PKC- ϵ on the plasma membrane increased, whereas their protein levels in the cytosolic fraction were reduced by bortezomib treatment (Fig. 6). In contrast, bortezomib treatment had no effect on the distribution of PKC- α or PKC- β I in the plasma membrane or cytosolic fractions from the DRGs of bortezomib-treated rats (Fig. 6).

We next selected PKC- δ and PKC- ϵ as the two representative PKC isoforms to determine the time-course effect of bortezomib on PKC activity using F11 cells, a DRG neuronal cell line. We examined the translocation of PKC- δ and PKC- ϵ at 0.5, 1 and 6 h after incubation of F11 cells with 10 nM bortezomib. It has been shown that bortezomib at 10 nM is effective in inhibiting proteasome activity (Bil et al., 2010; Meregalli et al., 2014). Our Western blotting analysis demonstrated that the immunoreactivity of PKC- δ and PKC- ϵ was potentiated on the plasma membrane, whereas their protein levels in the cytosolic fraction were reduced by bortezomib exposure. The effect of bortezomib on PKC translocation in F11 cells occurred within 30 min and was augmented in a time-dependent manner (Fig. 7). These data suggest that inhibiting proteasome activity with bortezomib rapidly increases the activity of several PKC isoforms in the DRGs.

Spinal NMDARs contribute to pain hypersensitivity induced by bortezomib treatment

Having shown that the presynaptic NMDAR activity in the spinal cord is increased in rats treated with bortezomib, we next determined whether increased NMDAR activity contributes to bortezomib-induced pain hypersensitivity. We examined the effect of intrathecal injection of AP5 via lumbar puncture on tactile allodynia and mechanical hyperalgesia in rats 7–10 days after the last treatment with bortezomib. AP5 was dissolved in saline and administered in a volume of 10 μ l via lumbar puncture. Intrathecal injection of the vehicle did not significantly alter bortezomib-induced tactile allodynia or hyperalgesia (Fig. 8, A and B). In contrast, intrathecal administration of both 1 and 10 μ g of AP5 produced a marked reversal of tactile allodynia and mechanical hyperalgesia in bortezomib-treated rats ($n = 8$ rats per group, Fig. 8, A and B). The AP5 effect at 10 μ g was maximal at 90 min after administration and lasted for about 2.5 h. These results suggest that increased spinal NMDAR activity contributes to bortezomib-induced pain hypersensitivity.

Inhibition of PKC activity at the spinal cord level reverses pain hypersensitivity caused by bortezomib treatment

In addition, we determined whether increased PKC activity at the spinal cord level is involved in the mechanical pain hypersensitivity caused by systemic treatment with bortezomib. In bortezomib-treated rats, intrathecal injection of the vehicle had no effect on bortezomib-induced tactile allodynia or mechanical hyperalgesia (Fig. 8, C and D). In contrast, intrathecal administration of 1 or 10 μ g of chelerythrine via lumbar puncture profoundly reduced tactile allodynia and mechanical hyperalgesia in bortezomib-treated rats

(n = 8 rats per group, Fig. 8, C and D). At 10 μ g, the effect of chelerythrine was maximal at 90 min after intrathecal injection and subsided 2.5 h after injection. These data suggest that PKC at the spinal cord level plays a critical role in pain hypersensitivity induced by bortezomib.

Discussion

A major finding of our study is that bortezomib treatment induces tonic activation of presynaptic NMDARs in the spinal cord to potentiate nociceptive input from primary afferent nerves. This is supported by evidence that bortezomib treatment increased the baseline mEPSC frequency in dorsal horn neurons, which was largely normalized to the level seen in vehicle-treated rats by blocking NMDARs with AP5. We also found that AP5 reduced the amplitude of glutamatergic EPSCs monosynaptically evoked from primary afferents in bortezomib-treated, but not vehicle-treated, rats. Nevertheless, unlike increased postsynaptic NMDAR activity in the spinal dorsal horn caused by traumatic nerve injury and calcineurin inhibitors (Chen et al., 2014a; Chen et al., 2014b; Li et al., 2016), bortezomib treatment had no effect on postsynaptic NMDAR activity of dorsal horn neurons. NMDARs are present at unmyelinated (C-fibers) and some thinly myelinated (A δ -fibers) afferent nerve terminals in spinal lamina I-II (Liu et al., 1994). The evoked EPSCs in our study may be mediated mainly via A δ -fibers, because EPSCs were elicited without failure at a 20 Hz electrical stimulation. Our data suggest that presynaptic NMDARs at primary afferent terminals become tonically activated by ambient glutamate after bortezomib treatment, which can increase nociceptive input to cause persistent pain.

Balance between phosphorylation and dephosphorylation, which are controlled by kinases and phosphatases, respectively, is essential to maintaining NMDAR activity in the spinal dorsal horn (Chen et al., 2014a; Chen et al., 2014b). Dysregulation of this balance in favor of phosphorylation is likely a pivotal mechanism of potentiated presynaptic NMDAR activity under pathological pain conditions. For example, inhibition of the protein phosphatase calcineurin increases presynaptic NMDAR activity in the spinal dorsal horn (Chen et al., 2014a). On the other hand, PKC increases NMDAR phosphorylation to enhance NMDAR activity by reducing the Mg²⁺ block of NMDARs and/or promoting NMDAR trafficking to the plasma membrane (Chen and Huang, 1992; Lan et al., 2001). In the present study, we showed that inhibition of PKC activity completely reversed increased frequency of mEPSCs and NMDAR-mediated glutamate release from primary afferents in bortezomib-treated rats, indicating that increased PKC activity plays an essential role in bortezomib-induced increases in presynaptic NMDAR activity. PKC isoforms are categorized into three subfamilies: conventional (or classical), novel, and atypical (Nishizuka, 1995). Conventional PKC isozymes include the isoforms PKC- α , - β I, - β II, and - γ . They are activated by both diacylglycerol and Ca²⁺. Novel PKC isozymes include the PKC- δ , - ϵ , - η and - θ , which require diacylglycerol, but not Ca²⁺, for activation. We found that bortezomib treatment potentiated the membrane translocation of PKC- β II, PKC- δ , and PKC- ϵ in the DRGs, which can phosphorylate NMDARs at presynaptic terminals of primary afferent nerves. Increased presynaptic NMDAR phosphorylation and activity by PKC promotes glutamate release and nociceptive input to cause pain hypersensitivity (Fig. 9). The mechanism of the differential effect of bortezomib on different PKC isoforms is uncertain, although it could be due to that

proteasomes may preferentially interact with PKC- β II, PKC- δ , and PKC- ϵ , but not PKC- α and PKC- β I, in the DRG. Because currently available PKC isoform inhibitors/activators are not highly selective, future studies are warranted to identify the role of specific PKC isoforms in NMDAR activation and pain using genetic approaches such as validated siRNA and knockout mice.

Our findings provide further evidence of the causal relationship between augmented PKC and NMDAR activity at the spinal cord level and pain hypersensitivity induced by systemic treatment with bortezomib. Activation of NMDARs and the resulting Ca^{2+} influx through them are crucial for synaptic plasticity associated with neuropathic pain. The rapid and time-dependent effect of bortezomib on PKC activity in a DRG cell line may explain the development of pain hypersensitivity even after injection of the first dose of bortezomib. NMDAR antagonists are effective in treating patients with certain neuropathic pain conditions (Loy et al., 2016; Zhou et al., 2011). Systemic administration of memantine, an orally active NMDAR antagonist used clinically, can reduce chemotherapy (paclitaxel)-induced pain hypersensitivity (Xie et al., 2016). We showed in this study that blocking NMDARs at the spinal cord level with intrathecal injection of AP5 profoundly reversed pain hypersensitivity induced by bortezomib. In addition, we found that intrathecal injection of a specific PKC inhibitor effectively reduced tactile allodynia and mechanical hyperalgesia in bortezomib-treated rats. It should be noted that although our study is focused on the relationship between neuronal PKC and NMDAR activity, PKC expressed in glia may contribute to bortezomib-induced painful neuropathy. Because increased PKC activity in DRGs can increase nociceptive input through presynaptic NMDARs, PKC may serve as an upstream signaling mechanism of bortezomib-induced presynaptic NMDAR activation and pain hypersensitivity.

How proteasome inhibition leads to a sustained increase in PKC activity in the DRG remains unclear. The key function of the proteasome is to degrade unneeded or damaged proteins via proteolysis, and the proteasomal degradation pathway is essential for many cellular processes (Imai et al., 2003; Kornitzer and Ciechanover, 2000). PKC is ubiquitinated following activation and then degraded by proteasomes (Kang et al., 2000). Proteasome inhibition prevents downregulation of activated forms of PKC and cannot remove membrane surface-anchored PKC (Lee et al., 1997; Lee et al., 1996; Melnikov and Sagi-Eisenberg, 2009), which leads to prolonged activation of PKC and NMDAR phosphorylation. Paclitaxel, which primarily stabilizes microtubules, disrupts the axonal transport of proteins and subcellular organelles (Dumontet and Jordan, 2010; Jordan and Wilson, 2004). Although paclitaxel and bortezomib have distinct molecular targets, our study reveals that they have similar effects on presynaptic NMDAR activation and PKC activity in the DRG (He and Wang, 2015; Xie et al., 2016). Thus, these two drugs probably have overlapping mechanisms in causing painful neuropathy. In this regard, proteasome inhibition with bortezomib may indirectly cause tubulin polymerization and microtubule stabilization, which contribute to impaired axonal transport, resulting in cytotoxicity in primary afferent neurons and nerves (Ale et al., 2015; Meregalli et al., 2014; Staff et al., 2013). In addition, the possible relationship between changes in PKC and NF- κ B induced by bortezomib remains to be determined. Bortezomib may prevent the dissociation of I κ B α from NF- κ B so that the NF- κ B in the cytosol remains in the state of I κ B α binding (Bonvini et al., 2007).

Also, the translocation of PKC from the cytosol to membrane caused by bortezomib may decrease phosphorylation of cytosolic I κ B α , which results in the reduction of activated NF- κ B entering the nucleus to alter the cellular function.

In conclusion, our study provides substantial new evidence that treatment with the proteasome inhibitor bortezomib increases the activity of presynaptic NMDARs in the spinal cord. PKC-mediated tonic activation of presynaptic NMDARs contributes to increased glutamatergic input and pain hypersensitivity induced by bortezomib. This new information extends our understanding of the molecular mechanism of chemotherapy-induced synaptic plasticity and suggests new strategies for treating this neuropathic pain condition.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (R01 GM120844) and by the N.G. and Helen T. Hawkins Endowment (to H.-L.P.).

Abbreviations

NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
EPSCs	excitatory postsynaptic currents
mEPSCs	miniature excitatory postsynaptic currents
PKC	protein kinase C
AP5	2-amino-5-phosphonopentanoic acid
DRG	dorsal root ganglion

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Highlights

- Many chemotherapeutic drugs causes neuropathic pain in cancer patients.
- Bortezomib treatment increases presynaptic NMDA receptor activity in spinal cords.
- Bortezomib induces membrane translocation of PKC in primary sensory neurons.
- PKC inhibition reverses NMDA receptor activity and pain induced by bortezomib.
- Bortezomib causes neuropathic pain via PKC-presynaptic NMDA receptor activation.

FIGURE 1. Chronic systemic administration of bortezomib induces pain hypersensitivity in rats *A* and *B*, time course of changes in the hindpaw withdrawal thresholds of rats given bortezomib (0.2 mg/kg, i.p.; $n = 7$ rats) or a vehicle (mannitol, $n = 7$ rats) for 4 alternate days. Injections are indicated by arrows. The hindpaw withdrawal thresholds were tested using von Frey filaments (*A*) or a noxious pressure stimulus (*B*). * $P < 0.05$ compared with the respective baseline control (time 0). One-way ANOVA followed by the Dunnett's post hoc test.

FIGURE 2. Bortezomib increases presynaptic NMDAR activity in the spinal cord

A, representative recording traces and cumulative plots show the effect of bath application of 50 μ M AP5 on the frequency and amplitude of mEPSCs of a lamina II neuron from a vehicle-treated rat. *B*, original traces and cumulative plots show the effect of AP5 on the frequency and amplitude of mEPSCs of a lamina II neuron from a bortezomib-treated rat. *C*, mean changes in the frequency and amplitude of mEPSCs of lamina II neurons during baseline control and bath application of AP5 in vehicle-treated ($n = 8$ neurons) and bortezomib-treated ($n = 10$ neurons) rats. * $P < 0.05$ compared with the baseline control in bortezomib-treated rats. # $P < 0.05$ compared with the baseline control in vehicle-treated rats. One-way ANOVA followed by the Tukey's post hoc test.

FIGURE 3. Bortezomib increases the activity of NMDARs at primary afferent terminals, but not postsynaptic NMDARs, in spinal dorsal horn neurons

A, representative recordings and summary data show the effect of 50 μ M AP5 on the monosynaptic EPSCs of lamina II neurons evoked from the dorsal root in vehicle-treated ($n = 11$ neurons) and bortezomib-treated ($n = 12$ neurons) rats. * $P < 0.05$ compared with the respective baseline control. # $P < 0.05$ compared with the baseline amplitude of evoked EPSCs in vehicle-treated rats. One-way ANOVA followed by the Dunnett's post hoc test. **B**, original traces and summary data show the inward NMDAR currents of lamina II neurons elicited by puff application of NMDA in vehicle-treated ($n = 10$ neurons) and bortezomib-treated ($n = 12$ neurons) rats.

FIGURE 4. PKC mediates bortezomib-induced potentiation of presynaptic NMDAR activity in spinal dorsal horn neurons

A, B, representative recordings and cumulative plots show the lack of effect of 50 μ M AP5 on the frequency and amplitude of mEPSCs of a lamina II neuron in a vehicle-treated and a bortezomib-treated rats (the spinal cord slices were pretreated with 10 μ M chelerythrine for 60 min). *C*, mean changes in the frequency and amplitude of mEPSCs in dorsal horn neurons pretreated with chelerythrine show the lack of effect of AP5 on the frequency and amplitude of mEPSCs in spinal cord slices obtained from vehicle-treated ($n = 8$ neurons) and bortezomib-treated ($n = 12$ neurons) rats. The bortezomib group without chelerythrine treatment (i.e., data in Fig. 2) was included as the control.

FIGURE 5. PKC mediates bortezomib-induced activation of NMDARs at primary afferent terminals

A, representative current traces show a lack of effect of 50 μ M AP5 on the amplitude of monosynaptic EPSCs of a lamina II neuron from a spinal cord slice pretreated with 10 μ M chelerythrine in a vehicle-treated and a bortezomib-treated rat. *B*, summary data show the lack of effect of AP5 on the amplitude of evoked EPSCs in spinal cord slices pretreated with chelerythrine in vehicle-treated ($n = 11$ neurons) and bortezomib-treated ($n = 13$ neurons) rats.

FIGURE 6. Bortezomib treatment causes translocation of PKC isoforms in the DRGs

A and *B*, original Western blots (*A*) and quantification (*B*) of protein expression levels for various PKC isoforms in cytosolic and plasma membrane fractions of DRG tissues obtained from vehicle (Veh)-treated and bortezomib (BTZ)-treated rats (eight DRGs per rat, $n = 8$ rats per group). * $P < 0.05$ compared with the respective value in the vehicle-treated group. Two-tailed Student's t -test.

FIGURE 7. Bortezomib induces translocation of PKC in F11 cells in a time-dependent manner
A and *B*, original gel images and quantification of protein expression levels for PKC- δ (*A*) and PKC- ϵ (*B*) in cytosolic and plasma membrane fractions of F11 cells treated with bortezomib (10 nM) for 0.5, 1, and 6 h ($n = 6$ samples per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ each compared with the control group (time 0).

FIGURE 8. Inhibition of NMDAR or PKC activity at the spinal cord level reverses pain hypersensitivity caused by bortezomib

A and *B*, time course of the effect of a single injection of AP5 (1 µg and 10 µg) on the paw withdrawal thresholds assessed using von Frey filaments (*A*) and a noxious pressure stimulus (*B*) in rats given bortezomib ($n = 8$ rats per group). *C* and *D*, time course of the effect of a single injection of chelerythrine (CLT; 1 µg and 10 µg) on the tactile (*C*) and pressure (*D*) paw withdrawal thresholds in rats given bortezomib ($n = 8$ rats per group). BL, the baseline tactile and pressure thresholds before bortezomib treatment. * $P < 0.05$ compared with the respective baseline value at time 0.

FIGURE 9. Schematic drawing shows the effect of bortezomib on PKC and NMDARs at primary afferent terminals

Under the normal condition, NMDARs at the presynaptic terminals of primary afferent nerves are not functionally active. Treatment with bortezomib induces translocation of intracellular PKC to the plasma membrane, leading to phosphorylation of NMDARs at presynaptic terminals. Increased presynaptic NMDAR activity augments glutamate release to activate NMDARs and AMPA receptors (AMPA receptors) on postsynaptic neurons in the spinal dorsal horn to cause pain hypersensitivity.