Dysregulation of sphingolipid metabolism contributes to bortezomib-induced neuropathic pain

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The development of chemotherapy-induced painful peripheral neuropathy is a major dose-limiting side effect of many chemotherapeutics, including bortezomib, but the mechanisms remain poorly understood. We now report that bortezomib causes the dysregulation of de novo sphingolipid metabolism in the spinal cord dorsal horn to increase the levels of sphingosine-1-phosphate (S1P) receptor 1 (S1PR1) ligands, S1P and dihydro-S1P. Accordingly, genetic and pharmacological disruption of S1PR1 with multiple S1PR1 antagonists, including FTY720, blocked and reversed neuropathic pain. Mice with astrocyte-specific alterations of S1pr1 did not develop neuropathic pain and lost their ability to respond to S1PR1 inhibition, strongly implicating astrocytes as a primary cellular substrate for S1PR1 activity. At the molecular level, S1PR1 engaged astrocyte-driven neuroinflammation and altered glutamatergic homeostasis, processes blocked by S1PR1 antagonism. Our findings establish S1PR1 as a target for therapeutic intervention and provide insight into cellular and molecular pathways. As FTY720 also shows promising anticancer potential and is FDA approved, rapid clinical translation of our findings is anticipated.

Introduction

Bortezomib (Velcade) is a dipeptide boronic acid inhibitor of the 26S proteasome widely used in the treatment of multiple myeloma and mantle cell non-Hodgkin’s lymphoma (Chen et al., 2011). More than 40% of patients receiving bortezomib develop chronic, distal, and symmetrical sensory peripheral neuropathy often accompanied by a neuropathic pain syndrome (chemotherapy-induced painful peripheral neuropathy [CIPN]) that may last for weeks, months, or even years after drug termination (Farquhar-Smith, 2011). CIPN greatly compromises the quality of life of cancer survivors and patients under treatment and can interfere with effective anticancer treatment (Farquhar-Smith, 2011). Dose reduction or treatment discontinuation is often the sole recourse for patients who develop neuropathic pain, because traditional analgesics are ineffective or have their own serious unwanted side effects (Farquhar-Smith, 2011). Advances in strategies to address this growing problem are urgently needed with increased efficacy of cancer therapy that has resulted in nearly 14 million cancer survivors in the United States (de Moor et al., 2013), many suffering from these long-term side effects (Farquhar-Smith, 2011). The mechanisms underlying CIPN remain poorly understood and differ among the chemotherapeutic agents (Han and Smith, 2013). However, neuropathological changes in the dorsal horn of the spinal cord (DHSC) have been documented (Han and Smith, 2013).

As a desirable anticancer mechanism, bortezomib activates the sphingolipid biosynthesis pathway to produce the potent proapoptotic sphingolipid ceramide (Gong et al., 2014). Ceramide is hydrolyzed by ceramidases to sphingosine that is phosphorylated by sphingosine kinases (SphK1 and 2) to produce sphingosine-1-phosphate (SIP; Spiegel and Milstien, 2011). SIP initiates signaling through a family of five cognate G protein-coupled receptors (SIPR1–5; Spiegel and Milstien, 2011) and, in contrast to ceramide, is a potent antiapoptotic sphingolipid (Ogretmen and Hannun, 2004). It is hypothesized that the ceramide/SIP rheostat plays a critical role in regulating cancer cell fate with elevated levels of ceramide inducing cell death and elevated...
levels of S1P leading to survival and proliferation (Ogretmen and Hannun, 2004). Thus, therapeutic strategies aimed at reducing S1P bioavailability or signaling are attracting attention as novel anticancer agents (Ogretmen and Hannun, 2004). Numerous studies have demonstrated that the S1PR1 functional antagonist and orally available multiple sclerosis drug FTY720 (fingolimod; Gilenya; Brinkmann et al., 2010) has potent antitumor activity (Hait et al., 2015; White et al., 2016) and can synergize with bortezomib to target multiple myeloma cells in vitro and multiple myeloma in vivo xenografts (Beider et al., 2017).

Despite having opposing effects on cancer cells, compelling evidence is emerging that ceramide and S1P share potent inflammatory and nociceptive actions (Salvemini et al., 2013). It is noteworthy that altered sphingolipid metabolism caused by mutations in serine palmitoyltransferase (SPT) contributes to neuropathic pain in humans (Dawkins et al., 2001). Whether sphingolipid dysregulation drives bortezomib-induced neuropathic pain is not known. Here, we demonstrate that S1PR1-dependent neuroinflammatory signaling pathways contribute to the development of bortezomib-induced neuropathic pain identifying S1PR1 as a molecular target for therapeutic intervention.

Results and discussion
Dysregulation of sphingolipid metabolism by bortezomib
Bioactive sphingolipid metabolites are powerful signaling molecules, and minor changes are known to have significant impact in signal transduction (Hannun and Obeid, 2008). Liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) analysis of multiple sphingolipid species (Fig. 1 A) revealed that bortezomib treatment (Zheng et al., 2012; Janes et al., 2013) caused dysregulation of sphingolipid metabolism in the DHSC. Indeed, bortezomib induced significant increases in the levels of ceramide (Figs. 1 B and S1 A) and its de novo biosynthetic pathway precursors, dihydrosphingosine and dihydroceramide (Figs. 1 B and S1 B). Bortezomib also increased the levels of sphingosine, S1P, and dihydro-S1P (DH–S1P), which are metabolites of ceramide and dihydroceramide (Fig. 1 B). In contrast, there were no observable changes in sphingomyelin (Fig. 1 B). These results suggest that dysregulation of sphingolipid metabolism from the de novo pathway within the DHSC is among the early events of bortezomib-induced neuropathic pain. Dysregulation of sphingolipid metabolism was associated with time-dependent development of neuropathic pain (mechano-allodynia and mechano-hyperalgesia; Figs. 2 and S2). Our sphingolipidomic data supporting the contribution of the de novo pathway in bortezomib-induced neuropathic pain were substantiated with studies performed with myriocin, a well-characterized inhibitor of SPT (Delgado et al., 2006), the first and rate-limiting enzyme of de novo pathway. Myriocin, when used at doses reported to block SPT (Delgado et al., 2006) and S1P levels in DHSC (Muscoli et al., 2010) significantly attenuated the development of neuropathic pain (Fig. 1, C and D). Collectively, the data strongly suggest that bortezomib alters S1P signaling by triggering SPT and de novo sphingolipid metabolism.

Genetic and pharmacological inhibition of the S1PR1 axis mitigates bortezomib-induced neuropathic pain
Because S1P and DH-S1P are potent bioactive mediators and ligands with similar affinities for S1PR1 (Strub et al., 2010), we explored the functional contribution of S1PR1 using NIBR14, a methyl ester prodrug that is rapidly hydrolyzed in vivo to the carboxylic acid, NIBR15, a potent and selective S1PR1 antagonist (Angst et al., 2012). i.th. administration of NIBR14 attenuated the development of mecanho-allodynia and mecanho-hyperalgesia in rats (Figs. 2 A and S2 A). In support of these pharmacological effects, i.th. administration of S1pr1-targeting dicer-substrate short interfering RNA (DsirNA) during bortezomib treatment recapitulated the pharmacological blockade (Figs. 2 B and S2 B) by significantly reducing S1PR1 protein levels in the DHSC (Fig. 2 C). These data provide convergent evidence that blocking S1PR1 signaling mitigates bortezomib-induced neuropathic pain.

The pharmacological efficacy and potency of oral administration of S1PR1-targeted agents, which is the preferred clinical approach, was examined using S1PR1 competitive (NIBR14) and functional (FTY720, ponesimod) antagonists (Bigaud et al., 2014). FTY720 (once phosphorylated by SphK2 to the biologically active FTY720-P) and ponesimod are S1PR1 agonists that act as “functional antagonists” by potently and irreversibly down-regulating S1PR1 (Bigaud et al., 2014). Oral administration of NIBR14 or FTY720 during bortezomib treatment dose-dependently prevented neuropathic pain from developing for the 20-d observation period after the completion of bortezomib treatment (Figs. 2, D and E; and S2, C and D; ED50 values in Table 1). Oral administration of ponesimod exhibited similar beneficial effects (Fig. S2, E and F). The beneficial effects observed with FTY720 were independently verified by another laboratory in our group using another method of behavioral assessment (Fig. 2, F and G).

Numerous studies have convincingly demonstrated the anti-tumor effects of FTY720 on many types of cancer cells in vitro and in tumor-bearing animals (Yasui et al., 2005; Alinari et al., 2011; Neviani et al., 2013; Hait et al., 2015). Therefore, S1PR1 agents are not expected to reduce the chemotherapeutic effectiveness of bortezomib. We confirmed this with in vitro tumor cell–killing studies using FTY720. Based on pharmacokinetic studies in rats (Brinkmann et al., 2010), the anticipated plasma levels of FTY720 at doses providing maximal blockade of bortezomib-induced neuropathic pain are ∼0.5–0.7 nM. When tested at a much higher dose, FTY720 (5 µM) did not significantly diminish the in vitro tumor cell toxicity of bortezomib in human multiple myeloma (RPMI 8226) cells (LC50, 34 nM; 95% confidence interval [CI], 27–42 nM; n = 5) compared with bortezomib and vehicle (LC50, 31 nM; 95% CI, 23–42 nM; n = 5).

Astrocytes are a primary cellular substrate for S1PR1 activity after bortezomib treatment
In the central nervous system (CNS), astrocytes express much higher levels of S1PR1 than microglia and neurons (Zhang et al., 2014), marking these cells as a prime target for S1PR1 signaling in the development of bortezomib-induced neuropathic pain. To test this possibility, we used mice with floxed S1pr1 recombined with a cre gene under the control of a glial fibrillary acidic protein (GFap) promoter that results in the deletion of S1pr1 in GFAP-
Figure 1. Bortezomib-induced neuropathic pain is dependent on increased de novo biosynthesis of ceramide and its metabolites in the lumbar DHSC. (A) Sphingolipid metabolic pathway. (B) Extracted lumbar DHSC lipids of rats treated with saline (day 0) or bortezomib (24 h after the last bortezomib injection; day 5) were analyzed by LC-ESI-MS/MS (n = 4 per group; representative of two separate experiments). (C and D) Mechano-allodynia (C) and mechano-hyperalgesia (D) were measured in rats (n = 6 per group) treated on days 0–4 with bortezomib or vehicle and concurrent i.th. delivery of myriocin (0.3 µM/d) or its vehicle. Data are mean ± SD for n rats; (B), *, P < 0.05 versus day 0 by Welch’s corrected, unpaired, one-tailed Student’s t test and (C) **, P < 0.05 versus time-matched bortezomib plus vehicle by one-way ANOVA with Holm-Sidak. False discovery rate was controlled by Benjamini-Hochberg procedure (B: q < 0.05; q* = 0.027).
astrocytes in the CNS (Choi et al., 2011). We confirmed the deletion of Sprr1 in the spinal cord (Fig. 3, A and B) and intact Sprr1 in dorsal root ganglion (DRG) tissues (Fig. 3 A). When compared with control littermates, male mice with astrocyte-specific deletions of Sprr1 did not develop neuropathic pain (Fig. 3 C). In animal models of multiple sclerosis, Choi et al. (2011) demonstrated that these mice develop a lesser form of the disease while losing the ability to respond to FTY720. Because in our pain model the complete deletion of Sprr1 from astrocytes resulted in no development of pain, we used mice with astrocyte-specific reductions of Sprr1 to examine the role of Sprr1 in astrocytes on the pharmacological effects of S1PR1 antagonists. Mice with astrocyte-specific reductions of Sprr1 had delayed development of neuropathic pain compared with controls and, because of reduced levels of S1PR1, they also lost their responsiveness to the beneficial effects of FTY720 (Fig. 3 D). These data, which mimic those obtained with S1PR1 antagonists and siRNA, strongly suggest that activation S1PR1 signaling in astrocytes is necessary for both bortezomib-induced neuropathic pain and the analgesic effects of FTY720, thus identifying astrocytes as a primary cellular substrate of S1PR1 activity.

S1PR1-driven neuroinflammation contributes to bortezomib-induced neuropathic pain

Bortezomib-induced dysregulation of sphingolipid metabolism in the spinal cord was associated with increased levels of the astrocyte marker GFAP (Fig. 4, A–D and G) in the superficial dorsal horn (laminae I and II), confirming a previous study (Robinson et al., 2014). The increase in GFAP suggests an increase in the number of astrocytes, their activation, or both in the DHSC in response to bortezomib. S1P triggers astrocytes to become reactive (Sorensen et al., 2003), and activation of S1PR1 can induce astrocyte migration in culture (Mullershausen et al., 2007). In other neurodegenerative diseases with a prominent astrocyte-driven inflammatory component (e.g., multiple sclerosis, Alzheimer’s disease, or Huntington’s disease), blocking the S1PR1 axis with S1PR1 antagonists attenuates astrocyte activation and inhibits increased production of inflammatory cytokines in the spinal cord; in contrast, levels of IL-10 increase (Brinkmann et al., 2010; O’Sullivan and Dev, 2017). These antiinflammatory effects of S1PR1 antagonism are further supported by a recent study showing that FTY720 can prevent endotoxin activation, migration, and ensuing release of inflammatory mediators in primary astrocyte cultures, but not microglia (Rothhammer et al., 2017). In our study, bortezomib-induced dysregulation of sphingolipid metabolism was also associated with concomitant increases in TNF and IL-1β (Fig. 4, H and I). In contrast, antiinflammatory cytokines (IL-10 and IL-4) decreased compared with those in vehicle-treated rats (Fig. 4, J and K). Concurrent administration of FTY720 attenuated increased GFAP immunolabeling (Fig. 4, E–G) and production of TNF and IL-1β (Fig. 4, H and I) associated with bortezomib treatment while preventing reductions in IL10 and IL4 (Fig. 4, J and K). Similar effects on cytokine expression were obtained with ponesimod (Fig. 4, H–K). This S1PR1-driven neuroinflammation in the spinal cord may, in turn, establish “feed-forward” mechanisms that can sustain dysregulation of sphingolipid metabolism. For example, TNF and IL-1β can activate the major enzymes involved in the biosynthesis of ceramide and S1P pathways: SphK1, sphingomyelinases, SPT, and ceramidases (Snider et al., 2010). Interruption of this cross talk early in the bortezomib regimen may explain how neuropathic pain is prevented with S1PR1-targeted agents from developing for many days after bortezomib discontinuation (Figs. 2 and S2).

S1PR1-driven alterations in glutamatergic signaling

Spinal synaptic mechanisms underlying the development of bortezomib-induced neuropathic pain have not been identified. We investigated whether glutamatergic synaptic activities in the DHSC of rats with bortezomib-induced neuropathic pain were altered using whole-cell patch-clamp recording techniques. Miniature excitatory postsynaptic currents (mEPSCs) were recorded from neurons in the spinal outer lamina II receiving monosynaptic input from the primary afferents (Yoshimura and Jessell, 1989; Weng et al., 2006). Alterations in mEPSC frequency indicate changes in the presynaptic transmitter release probability, whereas alteration in mEPSC amplitude indicates changes in the postsynaptic glutamate receptor function (Lissin et al., 1999; Yan et al., 2013; Yan and Weng, 2013). We found that

Table 1. Analgesic ED50 values for oral administration of NIBR14 or FTY720 in bortezomib-induced neuropathic pain (day 25)

| Treatment | Pain behavior | ED50  | 95% CI | n   |
|-----------|--------------|------|--------|-----|
|           |              | μmol/kg |       |     |
| NIBR14    | Allodynia    | 2    | 1–3    | 6   |
| Hyperalgesia |           | 2    | 1–3    | 6   |
| FTY720    | Allodynia    | 0.09 | 0.06–0.2 | 3–11 |
| Hyperalgesia |           | 0.09 | 0.06–0.1 | 3–11 |
both the frequency (6.10 ± 0.44 Hz) and amplitude (26.37 ± 0.52 pA) of mEPSCs in neurons from rats with bortezomib-induced neuropathic pain were significantly higher (P < 0.0001, Welch's uncorrected t test) than in vehicle-treated rats (frequency , 3.14 ± 0.33 Hz; amplitude, 22.87 ± 0.77 pA), suggesting that glutamate release from presynaptic terminals and ligand-gated glutamate receptor function in postsynaptic neurons are enhanced in bortezomib-treated rats (Fig. 4, L–O).

Previous studies by others and us have shown that spinal glutamatergic synaptic activities are enhanced by inflammatory mediators (e.g., TNF, IL-1β, and MCP1) under pathological pain conditions (Kawasaki et al., 2008; Yan and Weng, 2013). However, their regulation by the S1PR1 axis is not known. NIBR15 treatment significantly reduced the mEPSC frequency, but not amplitude, from 6.10 ± 0.44 Hz to 4.09 ± 0.23 Hz (Fig. 4, L and N) in DHSC neurons from rats with bortezomib-induced neuropathic pain. Furthermore, perfusion of the selective S1PR1 agonist SEW2871 in normal rats significantly increased mEPSC frequency with no significant change in amplitude (Fig. 4, M and O). Collectively, these data suggest that S1P in the DHSC of bortezomib-treated rats enhances glutamate release from presynaptic terminals, which is blocked by S1PR1-targeted agents.

These findings demonstrate the dysregulation of excitatory glutamatergic signaling as a second factor contributing to neuropathic pain induced by chemotherapeutics (Boyette-Davis et al., 2015). More importantly, our study demonstrates for the first
time that S1PR1 signaling is responsible for increased presynaptic glutamate release and the genesis of bortezomib-induced neuropathic pain. Because we found that the development of neuropathic pain is lost when S1PR1 is deleted in astrocytes (Fig. 3C) and FTY720 lost its effectiveness when S1PR1 is reduced in astrocytes (Fig. 3D), it is likely that S1PR1-targeted agents suppress astrocyte activity and ensuing neuroinflammation (Rothhammer et al., 2017), attenuating the ability of proinflammatory cytokines to enhance glutamatergic synaptic activity (Clark et al., 2013).

**S1PR1-targeted agents reverse bortezomib-induced neuropathic pain**

The increased efficacy of cancer therapy has led to nearly 14 million cancer survivors in the United States (de Moor et al., 2013). Many of these survivors continue to suffer from long-term neuropathic pain states that are poorly managed. To address this major unmet medical need, we examined the effectiveness of targeting S1PR1 in models of established bortezomib-induced neuropathic pain. At the time of peak pain (day 25), subcutaneous
minipump infusions of FTY720, ponesimod, or NIBR14 over 6 d (Janes et al., 2014) completely reversed neuropathic pain (Fig. S3) throughout the entire period of drug infusion.

The mechanisms of bortezomib dysregulation of sphingolipid metabolism are not known, but several possibilities are envisioned (Fig. 4 P). The limited ability of bortezomib to cross the blood–brain barrier (Adams et al., 1999) suggests that sphingolipid metabolism in the CNS may be triggered by peripheral inflammatory changes (Seruga et al., 2008). Accordingly, bortezomib increases levels of various proinflammatory cytokines, such as TNF and IL-1β (Liang et al., 2014), in plasma that gain access to the CNS (Yarlagadda et al., 2009) and activate de novo biosynthesis to increase DH-SIP and SIP (Snider et al., 2010; Maceyka and Spiegel, 2014). Alternatively, bortezomib-induced events in the DRG (Carozzi et al., 2013) may provoke production of reactive oxygen and nitrogen species (Duggert and Flatters, 2017) and release of glutamate (Ghelardini et al., 2014) from presynaptic nerve endings that could activate glial sphingolipid metabolism (Kim et al., 2012). The resulting increased S1PR1 signaling in astrocytes promotes their activation, migration, and ensuing release of inflammatory mediators (Rothhammer et al., 2017) to reinforce neuroinflammation and drive the establishment and maintenance of the neuronal sensitization in the CNS. In turn, enhanced reactive oxygen and nitrogen species or glutamatergic signaling may perpetuate the cycle and transition the DHSC toward a chronic hypersensitized state.

In conclusion, by using a multidisciplinary approach, our studies provide new insight into the underlying S1PR1-driven mechanisms engaged during bortezomib-induced neuropathic pain and establish the foundation for proof-of-concept studies with S1PR1-targeted agents that are already in clinical use (FTY720; Brinkmann et al., 2010) or advanced clinical trials for other indications (ponesimod; Bigaud et al., 2014). Our studies provide a compelling case for the consideration of repurposing (FTY720) or advanced clinical trials for bortezomib as an adjuvant to bortezomib for the prevention and establishment and maintenance of the chronic hypersensitized state.

Materials and methods

Experimental animals
Male Sprague Dawley rats (200–220 g starting weight) were from Harlan Laboratories. For S1pr1 knockout and knockdown mice, transgenic mouse colonies were descendants of original homozygous S1pr1fl/fl;GFAP-Cre breeder mice gifted to us by J. Chun (The Scripps Research Institute, La Jolla, CA; Choi et al., 2011) and interbred to maintain their homozygosity for the floxed S1pr1 gene with regular backcrosses with C57BL/6J wild-type mice (Harlan Laboratories) to reduce inbred genetic defects. Prior characterizations of these conditional mutants showed the removal of S1pr1 from GFAP+ astrocytes in the CNS (Choi et al., 2011). Mice with astrocyte-specific deletion of S1pr1 (S1pr1fl/fl;GFAP-Cre) and their control littermates (S1pr1fl/+;GFAP-Cre) were generated from mating male S1pr1fl/fl;GFAP-Cre mice. Mice with astrocyte-specific reductions in S1pr1 (S1pr1fl/a;GFAP-Cre) and their control littermates (S1pr1fl/a;GFAP-Cre) were generated from mating male C57BL/6j wild-type mice with female S1pr1fl/a;GFAP-Cre mice. All mice were genotyped before breeding or use in experiments by endpoint PCR of ear-punch DNA. The primers used to detect floxed and deleted S1pr1 were spb1 (Invitrogen; 5′-TTC TAGCAAGCGATGTCCA-3′), Edg1M/Sp1r1 (IDT; 5′-CCTCAG AGTCTGGTTAAATTGT-3′), and bttc (Invitrogen; 5′-CACAACCTC TTCTCGATGAA-3′) primers. The primers used to detect Gfap-cre were gfp-cre forward (5′-ATGGTCGGTCTCCAGGACC-3′) and cre reverse (5′-CACGCTGCTGTCACTCTTGTC-3′). All mice were ear-tagged for identification, and sex- and age-matched littermate controls were used in all experiments.

Animals were housed two to four per cage (for rats), five per cage (for mice), or single housed after surgery in a controlled environment (12-h light/dark cycle) with food and water available ad libitum. All experiments were conducted with the experimenters blinded to treatment conditions. All experiments were performed in accordance with the guidelines of the International Association for the Study of Pain and the National Institutes of Health and approvals from the Saint Louis University, University of Georgia, and the University of California, San Diego, Institutional Animal Care and Use Committees.

Osmotic minipump
Rodents were lightly anesthetized with 3% isoflurane and maintained on 2% isoflurane in 100% O2. An incision was made in the interscapular region for subcutaneous implantation of primed 7-d osmotic minipumps (Alzet 2001; Alza) that infused 1 µl/h as previously described (Janes et al., 2014). Minipumps were filled according to the manufacturer’s specifications with test compounds or their vehicle. Immediately after surgery, i.p. loading doses of test compounds or their vehicle were administered, and minipumps were allowed to deliver test agents for 6 d. After implantation, the animals were singly housed for the remainder of the experiment.

Bortezomib-induced neuropathic pain model
In rats, bortezomib (S1013; Selleck Chemicals) or its vehicle (5% Tween 80, P1754 [Sigma-Aldrich]; 5% ethanol, 493546 [Sigma-Aldrich]; and saline) was administered by i.p. injection in rats on 5 consecutive days (day 0 to day 4; 0.2 mg/kg) at an injection volume of 0.2 ml for a final cumulative dose of 1 mg/kg (Janes et al., 2013). In mice, bortezomib (0.4 mg/kg i.p.) was administered every other day three times per week for 4 wk (day 0 to day 25).

Test compounds
All compounds in the prevention models were administered concurrent with bortezomib at the doses and by the routes described in the text after behavior measurements. For the reversal studies, compounds were administered via osmotic minipumps implanted on D24 or D25. FTY720 (fingolimod) was purchased from Cayman Chemical (162359-56-8). Ponesimod

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C31H38ClN3O5S; m/z = 600 [M + H+]).

An Agilent Eclipse (XDB-C18; 4.6 × 150 mm, 5 μm) column (retention time = 7.15 min; TFA over 6 min) performed by using a Waters Alliance SQ 3100.

Sphingolipids and S1PR1 in bortezomib-induced pain

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For synthesis of NIBR14, the methyl ester pro-drug, the Novartis competitive antagonist NIBR14 (mol wt = 600.17) was prepared as described in the literature (Angst et al., 2012). Molecular identity was confirmed by LCMS (50–95% acetonirole in 0.05% TFA over 6 min) performed by using a Waters Alliance SQ 3100 system (positive ion electrospray mode) with an Agilent Eclipse (XDB-C18; 4.6 × 150 mm, 5 μm) column (retention time = 7.15 min; C31H38ClN3O5S; m/z = 600 [M + H+]).

For synthesis of NIBR15, the methyl ester prodrgu NIBR14 (90 mg, 0.15 μmol) was dissolved in 6 ml acetic acid and treated with 1.5 ml of concentrated HCl. This mixture was stirred at 50°C for 12 h and concentrated. Recrystallization from acetonirole afforded 45 mg (48% yield) NIBR15 HCl salt (mol wt = 622.60), confirmed by LCMS (15–95% acetonirole in 0.05% TFA over 6 min; retention time = 6.97 min; C30H37Cl2N3O5S; m/z = 586 ([M + H+ – Cl]–); Angst et al., 2012).

For myriocin, [25,3R,4R,6E]-2-Amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxo-6-eicosenoic acid] from Mycelia sterilia was purchased from Sigma-Aldrich (35891-70-4).
**S1PR1 knockdown PCR**

Ear snip, spinal cord, or DRG DNA was isolated by using a QIAamp DNA mini isolation kit (Qiagen) and quantitated by NanoDrop (NanoDrop Products) for PCR as previously described (Choi et al., 2011). In brief, DNA (20–25 ng) was subjected to PCR (25 µl) in duplicate by using two separate PCR reactions: (1) Edg1M (5′-CTCTAGCTGTGTTAAATTTG-3′; Integrated DNA Technologies), SPB (5′-TTCAGCGACCTTGCCCAAC-3′; Invitrogen), and BITIC (5′-CACAACCTCTCTCTATGGA-3′; Invitrogen) sites by using the following amplification cycle conditions: 94°C, 5 min; 35 cycles (94°C, 30 s; 59°C, 45 s; 72°C, 1 min), and 72°C, 7 min and (2) GFAP-cre by using forward GFAP-cre primers (5′-ATGGCTTGCCCTCAGATCC-3′; Integrated DNA Technologies) and reverse cre primers (5′-CAGCATTGCTGTCACTGTC-3′; Integrated DNA Technologies) by using the following amplification cycle conditions: 94°C, 3 min; 35 cycles (94°C, 25 s; 59°C, 35 s; 72°C, 45 s); and 72°C, 7 min. Blue/orange 6× loading dye (2 µl; Promega) was added, and 10 ng PCR product was loaded for 1.8% agarose gel electrophoresis in 1× Tris-borate-EDTA buffer. When measured against a 100-bp DNA ladder (Promega), PCR yielded intensive bands: 177 bp (wild-type S1pr1), 450 bp (floxed S1pr1), 450 bp (deletion product), and 625 bp (Gâp-cre). Animals with a 450-bp band in ear snip DNA samples indicated germline deletions of S1PR1 and were excluded from breeding and experiments. For each experiment, the heterozygous S1pr1fl/+;Gfap-Cre mice were paired with their heterozygous S1pr1fl/+ littermates, and their genotypes were blinded to the experimenters.

**Immunofluorescence**

Immunofluorescence and image analysis was performed by using modifications to previously reported methods (Little et al., 2012; Janes et al., 2014). The spinal cord (L4–6) was harvested, transferred to optimum cutting temperature compound, and rapidly frozen in an isopropanol/dry ice bath. Transverse sections (20 µm) were cut by using a cryostat, collected on gelatin-coated glass microscope slides, and stored at −20°C. Spinal cord sections were fixed in 10% buffered neutral formalin for 10 min (SF100; Sigma-Aldrich), after a series of PBS rinses, sections were incubated in PBS (BP39920; Thermo Fisher Scientific) for 1 h and then immunolabeled by using an 18-h incubation (4°C) with mouse monoclonal anti–GFAP antibody, clone G-A-5 (1:200, G3893; Sigma-Aldrich). After a series of PBS rinses, sections were incubated for 2 h with a goat anti–mouse Alexa Fluor 488 antibody (1:250, A-11001; Invitrogen). The coverslips were mounted with Fluorogel II containing DAPI (17985-5; Electron Microscopy Sciences) and photographed with an Olympus FV1000 MPE confocal microscope (multiline argon lasers with excitation at 405 nm and 488 nm) using a 10× objective (UPLSAPO; 0.4 numerical aperture) for regional fluorescence intensity image analysis and with a 60× oil-immersion objective (PLAPONI, 1.42 numerical aperture) and 2.4× optical zoom (0.1-µm pixel dimensions in the X-Y plane and the pinhole set at 1 Airy unit) for higher magnification images. Images were acquired within the dynamic range of the microscope (i.e., no pixel intensity values of 0 or 255 in an 8-bit image). Sections treated with type control (X0931; Agilent-Dako) at concentrations equivalent to the primary antibody yielded only nonspecific background fluorescence. Image analysis of GFAP immunolabeling was performed by using the National Institutes of Health freeware program ImageJ (version 1.43; Schneider et al., 2012). The bilateral superficial DHSC (laminae I and II) at the L4, L5, and L6 levels was outlined on images using the ImageJ region of interest tool. The superficial DHSC was determined and confirmed by using adjacent cresyl violet–stained sections and an atlas (Paxinos and Watson, 1998). Images received automated background threshold corrections (histogram-based threshold) before analysis. The mean fluorescence intensity (MFI) of the superficial DHSC was determined as previously reported (Little et al., 2012; Janes et al., 2014). MFI was calculated using the following equation: \[ \text{MFI} = \frac{i(p^2 + p^2)}{i(p^2 + p^2)} \], where i is the mean gray value, \( p^2 \) is the positive pixel area, and \( p^2 \) is total pixel area. There were no significant differences bilaterally within each group, so MFI was calculated as a combined value for each animal and reported as fold change compared with the vehicle group.

**Cytokine assays**

Cytokine levels were assessed in DHSC (L4–6) homogenates using a commercially available multiplex cytokine kit (Bio-Rad Laboratories) as previously described (Janes et al., 2014). In vitro whole-cell recordings and data analysis

Male Sprague Dawley rats (170–180 g) were administered bortezomib (0.2 mg/kg) or its vehicle (5% Tween 80, 5% ethanol in saline) on days 0–4. This resulted in significantly reduced (40.47 ± 8.13%; \( P < 0.001 \)) mechanical PWTs by day 7 with bortezomib treatment. On day 7, traverse L4–5 spinal cord slices were prepared as previously described (Weng et al., 2007; Nie and Weng, 2009). Surgery was performed in deeply isoflurane-anesthetized rats to expose and remove the spinal lumbar enlargement segments. The section was placed in ice-cold sucrose artificial cerebrospinal fluid (234 mM sucrose, 3.6 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 1.2 mM NaH₂PO₄, 12.0 mM glucose, and 25.0 mM NaHCO₃) presaturated with 95% O₂ and 5% CO₂. The pia-arachnoid membrane was removed from the section before it was attached with cyanoacrylate glue to a cutting support and then glued onto the stage of a vibratome (Series 1000; Technical Products International). Transverse spinal cord slices (400 µm) were cut in the ice-cold sucrose artificial cerebrospinal fluid and presoaked in Krebs solution (117.0 mM NaCl, 3.6 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 1.2 mM NaH₂PO₄, 11.0 mM glucose, and 25.0 mM NaHCO₃) at 35°C) oxygenated with 95% O₂ and 5% CO₂ at 35°C. Each single slice was placed in the recording chamber (volume, 1.5 ml), perfused with Krebs solution at 35°C, and saturated with 95% O₂ and 5% CO₂. Borosilicate glass recording electrodes (resistance, 3–5 MΩ) were pulled and filled with an internal solution containing 135 mM potassium-glucurate, 5.0 mM KCl, 2.0 mM MgCl₂, 0.5 mM CaCl₂, 5.0 mM Hepes, 5.0 mM EGTA, 5.0 mM ATP-Mg, 0.5 mM Na-GTP, and 10 mM QX-314. Live dorsal horn neurons in the spinal outer lamina II were visualized by microscopy and approached by using a three-dimensional motorized manipulator (Sutter Instrument), and whole-cell configurations were established by applying

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moderate negative pressure after electrode contact (Nakatsuka et al., 2003). Recordings of mEPSCs were made from neurons receiving monosynaptic input from the primary afferents by using the criteria established previously (Yoshimura and Jessell, 1989; Weng et al., 2006). mEPSCs were recorded at a membrane potential at −70 mV in the presence of 1 µM tetrodotoxin (T8024; Sigma-Aldrich), 10 µM bicuculline (14340; Sigma-Aldrich), and 5 µM strychnine (S0532; Sigma-Aldrich) in the external solution to block voltage-gated sodium channels, GABA<sub>B</sub>, and glycine receptors. Data were recorded using Axopatch 700B amplifiers, digitized at 10 kHz, and analyzed off-line. The frequency and amplitude of mEPSCs in 3 min before and during perfusion of tested drugs were analyzed and averaged using a peak detection program (MiniAnalysis; Synaptosoft Inc.).

**Tumor cell–killing assays**

The effects of FTY720 on the tumor cell toxicity of bortezomib on human multiple myeloma cells (RPMI 8226) was assessed by using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, M5655; Sigma-Aldrich) assay as previously described (Janes et al., 2013).

**Statistical analysis**

The data are expressed as mean ± SD or mean ± SEM for n animals and analyzed by paired or unpaired t test or one-way or two-way repeated-measures ANOVA with Holm-Sidak corrected comparisons. Significant differences were defined as P < 0.05, and the false discovery rate for sphingolipid analysis was controlled by Benjamini-Hochberg procedure at q < 0.05 using SPSS software (v21; IBM). All other statistical analyses were performed using GraphPad Prism.

**Online supplemental material**

Fig. S1 shows bortezomib-induced ceramide and dihydroceramide acyl chain species in the spinal cord. Fig. S2 shows the effects of i.th. or oral administration S1PR1-targeted agents on bortezomib-induced mecano-hyperalgesia. Fig. S3 shows the effects of S1PR1-targeted agents on the reversal of established bortezomib-induced mecano-hyperalgesia.

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**References**

Adams, J., V.J. Palombella, E.A. Sausville, J. Johnson, A. Destree, D.D. Lazarus, J. Maas, C.S. Pien, S. Prakash, and P.J. Elliott. 1999. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res. 59:2615–2622.

Ahninari, L., E. Mahoney, J. Pattou, X. Zhang, L. Huynh, C.T. Earl, R. Mani, Y. Mao, B. Yu, C. Trainor, et al. 2011. FTY720 increases CD4<sup>+</sup> expression and sensitizes mantle cell lymphoma cells to mitoluzumab-mediated cell death. Blood. 118:6893–6903. https://doi.org/10.1182/blood-2011-06-363879

Anget, D., P. Janser, J. Quancard, P. Buelhmayer, F. Berst, L. Oberer, C. Beerli, M. Streiff, C. Pally, R. Hersperger, et al. 2012. An oral sphingosine 1-phosphate receptor 1 (S1P(1)) antagonist prodrug with efficacy in vivo: discovery, synthesis, and evaluation. J. Med. Chem. 55:9722–9724. https://doi.org/10.1021/jm3009508

Beider, K., E. Rosenberg, H. Bitner, A. Shimon, M. Leiba, M. Koren-Michowitz, E. Rubakovsky, S. Klein, D. Olam, L. Weiss, et al. 2017. The sphingosine-1-phosphate modulator FTY720 targets multiple myeloma via the CXCR4/CXCL12 pathway. Clin. Cancer Res. 23:1733–1747. https://doi.org/10.1158/1078-0432.CCR-15-2618

Bigaud, M., D. Guerini, A. Billich, F. Bassilana, and V. Brinkmann. 2014. Sec14 promotes the formation of the stimulatory complex involved in the development of chemotherapy-induced neuropathy. Pain. 155:285–296. https://doi.org/10.1016/j.pain.2015.19

Brinkmann, V., A. Billich, T. Baumrucker, P. Heinig, R. Schmouder, G. Francis, S. Aradhye, and P. Burtin. 2010. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. Nat. Rev. Drug Discov. 9:883–897. https://doi.org/10.1038/nrd3248

Carozzi, V., A.L. Renn, M. Bardini, G. Fazio, A. Chiorazzi, C. Meregalli, N. Oggioni, K. Shanks, M. Quartu, M.P. Serra, et al. 2013. Bortezomib-induced painful peripheral neuropathy: an electrophysiological, behavioral, morphological and mechanistic study in the mouse. PLoS One. 8:e72995. https://doi.org/10.1371/journal.pone.0072995

Chen, D., M. Frezza, S. Schmitt, J. Kanwar, and P.Q. Dou. 2011. Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. Curr. Cancer Drug Targets. 11:239–253. https://doi.org/10.2174/1568026110435159752

Chi, X.X., and G.D. Nicol. 2010. The sphingosine 1-phosphate receptor, S1PR<sub>3</sub>, plays a prominent but not exclusive role in enhancing the excitability of sensory neurons. J. Neurophysiol. 104:2741–2748. https://doi.org/10.1152/jn.00709.2010

Choi, J.W., S.E. Gardell, D.R. Herr, R. Rivera, C.W. Lee, K. Noguchi, S.T. Teo, Y.C. Yung, M. Lu, G. Kennedy, and J. Chun. 2011. FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation. Proc. Natl. Acad. Sci. USA. 108:751–756. https://doi.org/10.1073/pnas.1014154108
Little, J.W., Z. Chen, T. Doyle, F. Porreca, M. Ghaffari, L. Bryant, W.L. Neu-
mann, and D. Salvenmii. 2012. Supraspinal peroxynitrite modulates pain
signaling by suppressing the endogenous opioid pathway. J. Neu-
roscil. 32:10979–10980. https://doi.org/10.1523/JNEUROSCI.6345-11.2012
Maceyka, M., and S. Spiegel. 2014. Sphinilipid metabolites in inflammatory
nature. 510:58–67. https://doi.org/10.1038/nature13475
Mullershausen, F., L.M. Craveiro, Y. Shin, M. Cortes-Coss, F. Bassilana, M.
Osinde, W.L. Wishart, D. Guerini, M. Thallmair, M.E. Schwab, et al. 2007.
Phosphorylated FTY720 promotes astrocyte migration through sphin-
golip-1-phosphate receptors. J. Neurochem. 102:1151–1161. https://doi.
gg/10.1118/j.phb.2016.14063
Farquhar-Smith, P. 2011. Chemotherapy-induced neuropathic pain. Curr. Opin.
Support. Palliat. Care. 5:204–212. https://doi.org/10.1016/j.copsyn.2011.07.005
Hait, N.C., J. Allegood, M. Maceyka, G.M. Strub, K.B. Harikumar, S.K. Singh,
L. Maceyka, and S. Spiegel, 2009. Regulation of histone acetylation in the
nucleus by sphingosine-1-phosphate. Science 325:1254–1257. https://doi.
gg/10.1126/science.1176709
Hait, N.C., D. Avni, A. Yamada, M. Nagahashi, T. Aoyagi, H. Aoki, C.I. Dumur,
Z. Zelenko, E.J. Gallagher, D. Leroith, et al. 2015. The phosphorylated prodrug
FTT720 is a histone deacetylase inhibitor that reactivates ERα expression
and enhances hormonal therapy for breast cancer. Oncogene 34:135–143.
https://doi.org/10.1038/onc.2015.16
Hann, Y., M.T. Smith. 2013. Pathobiology of cancer chemotherapy-induced
peripheral neuropathy (CIPN). Front. Pharmacol. 4:1165. https://doi.org/
gg/10.3389/fphar.2013.00116
Hannun, Y.A., and L.M. Obeid. 2008. Principles of bioactive lipid signalling:
lessons from sphingolipids. Front. Pharmacol. 10:1038/s00280-011-00156-
9
ạnh, A.J., K.A. Orr Gandy, and L.M. Obeid. 2010. Sphingosine kinase: Role
in regulation of bioactive sphingolipid mediators in inflammation.
J. Immunol. 185:1263–1272. https://doi.org/10.4049/jimmunol.1001373
Kim, S., A.J. Steelman, Y. Zhang, H.C. Kinney, and j. Li. 2012. Aberrant upreg-
ulation of astroglial ceramide potentiates oligodendrocyte injury. Brain Pathol.
gg/10.1169/j.pain.2013.07.032
Janes, K., J.W. Little, C. Li, L. Bryant, C. Chen, Z. Chen, K. Kamocki, T. Doyle,
A. Snider, E. Esposito, et al. 2014. The development and maintenance of
paclitaxel-induced neuropathic pain require activation of the sphing-
golip-1-phosphate receptor subtype 1. J. Biol. Chem. 289:20102–20109.
https://doi.org/10.1074/jbc.M114.569574
Kawasaki, Y., Z. Zhang, J.K. Cheng, and R.R. Ji. 2008. Cytokine mechanisms of
central sensitization: distinct and overlapping role of interleukin-1β,
interleukin-6, and tumor necrosis factor-alpha in regulating synaptic
and neuronal activity in the superficial spinal cord. J. Neurosci. 28:5189–
5194. https://doi.org/10.1523/JNEUROSCI.0876-08.2008
Kim, S., A.J. Steelman, Y. Zhang, H.C. Kinney, and j. Li. 2012. Aberrant upreg-
ulation of astroglial ceramide potentiates oligodendrocyte injury. Brain Pathol.
gg/10.1169/j.pain.2013.07.032
Liang, Y., S. Ma, Y. Zhang, Y. Wang, Q. Cheng, Y. Wu, Y. Jin, D. Zheng, D. Wu,
and H. Liu. 2014. IL-1β and TLR4 signaling are involved in the aggravated
murine acute graft-versus-host disease caused by delayed bortezomib
administration. J. Immunol. 192:1277–1285. https://doi.org/10.4049/
jimmunol.1203428
Lisset, D.V., R.C. Carroll, R.A. Nicoll, R.C. Malenka, and M. von Zastrow. 1999.
Rapid, activation-induced redistribution of ionotropic glutamate recep-
tors in cultured hippocampal neurons. J. Neurosci. 19:1263–1272. https://
doi.org/10.1523/JNEUROSCI.09-02-1999
Strub, G.M., M. Maceyka, N.C. Hait, S. Milstien, and S. Spiegel. 2010. Extracellular and intracellular actions of sphingosine-1-phosphate. Adv. Exp. Med. Biol. 688:141-155. https://doi.org/10.1007/978-1-4419-6741-1_10

Walk, R.M., S.T. Elliott, F.C. Blanco, J.A. Snyder, A.M. Jacob, S.D. Rose, M.A. Behlke, A.K. Salem, S. Vukmanovic, and A.D. Sandler. 2012. T-cell activation is enhanced by targeting IL-10 cytokine production in toll-like receptor-stimulated macrophages. ImmunoTargets Ther. 1:13–23.

Walk, R.M., S.T. Elliott, F.C. Blanco, J.A. Snyder, A.M. Jacobi, S.D. Rose, M.A. Behlke, A.K. Salem, S. Vukmanovic, and A.D. Sandler. 2012. T-cell activation is enhanced by targeting IL-10 cytokine production in toll-like receptor-stimulated macrophages. ImmunoTargets Ther. 1:13–23.

Weng, H.R., J.H. Chen, and J.P. Cata. 2006. Inhibition of glutamate uptake in the spinal cord induces hyperalgesia and increased responses of spinal dorsal horn neurons to peripheral afferent stimulation. Neuroscience. 138:1351–1360. https://doi.org/10.1016/j.neuroscience.2005.11.061

Weng, H.R., J.H. Chen, Z.Z. Pan, and H. Nie. 2007. Glial glutamate transporter 1 regulates the spatial and temporal coding of glutamatergic synaptic transmission in spinal lamina II neurons. Neuroscience. 149:898–907. https://doi.org/10.1016/j.neuroscience.2007.07.063

White, C., H. Alshaker, C. Cooper, M. Winkler, and D. Pchejetski. 2016. The emerging role of FTY720 (Fingolimod) in cancer treatment. Oncotarget. 7:23106–23127. https://doi.org/10.18632/oncotarget.7145

Yan, X., and H.R. Weng. 2013. Endogenous interleukin-1β in neuropathic rats enhances glutamate release from the primary afferents in the spinal dorsal horn through coupling with presynaptic N-methyl-D-aspartic acid receptors. J. Biol. Chem. 288:30544–30557. https://doi.org/10.1074/jbc.M113.495465

Yoshimura, M., and T.M. Jessell. 1989. Primary afferent-evoked synaptic responses and slow potential generation in rat substantia gelatinosa neurons in vitro. J. Neurophysiol. 62:96–108. https://doi.org/10.1152/jn.1989.62.1.96

Zhang, Y., K. Chen, S.A. Sloan, M.L. Bennett, A.R. Scholze, S. O’Keeffe, H.P. Phatnani, P. Guarnieri, C. Caneda, N. Ruderisch, et al. 2014. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J. Neurosci. 34:11929–11947. https://doi.org/10.1523/JNEUROSCI.1860-14.2014

Zheng, H., W.H. Xiao, and G.J. Bennett. 2012. Mitotoxicity and bortezomib-induced chronic painful peripheral neuropathy. Exp. Neurol. 238:225–234. https://doi.org/10.1016/j.expneurol.2012.08.023