Published in final edited form as:

Pain. 2020 January ; 161(1): 177–184. doi:10.1097/j.pain.0000000000001697.

Sexually dimorphic therapeutic response in bortezomib-induced neuropathic pain reveals altered pain physiology in female rodents

Katherine Stockstill¹, Carrie Wahlman¹, Kathryn Braden¹, Zhoumou Chen¹, G. L. Yosten¹, D.K. Tosh², K.A. Jacobson², T.M. Doyle¹, W.K. Samson¹, Daniela Salvemini¹

¹Department of Pharmacology and Physiology, Saint Louis University School of Medicine, 1402 South Grand Blvd., St. Louis, MO 63104, USA

²Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-0810, USA.

Abstract

Chemotherapy-induced neuropathic pain (CINP) in both sexes compromises many current chemotherapeutics and lacks a FDA-approved therapy. We recently identified the sphingosine-1-phosphate receptor subtype 1 (S1PR1) and A₃ adenosine receptor subtype (A₃AR) as novel targets for therapeutic intervention. Our work in male rodents using paclitaxel, oxaliplatin and bortezomib showed robust inhibition of CINP with either S1PR1 antagonists or A₃AR agonists. The S1PR1 functional antagonist FTY720 (Gilenya®) is FDA approved for treating multiple sclerosis and selective A₃AR agonists are in advanced clinical trials for cancer and inflammatory disorders, underscoring the need for their expedited trials in CINP patients as chemotherapy adjuncts. Our findings reveal that S1PR1 antagonists and A₃AR agonists mitigate paclitaxel and oxaliplatin CINP in female and male rodents, but failed to block or reverse bortezomib-induced neuropathic pain (BINP) in females. Although numerous mechanisms likely underlie these differences, we focused on receptor levels. We found that BINP in male rats, but not female rats, was associated with increased expression of A₃AR in spinal cord dorsal horn, while S1PR1 levels were similar in both sexes. Thus, alternative mechanisms beyond receptor expression may account for sex differences in response to S1PR1 antagonists. Morphine and duloxetine, both clinical analgesics, reversed BINP in female mice, demonstrating that the lack of response is specific to S1PR1 and A₃AR agents. Our findings suggest that A₃AR- and S1PR1-based therapies are not viable approaches in preventing and treating BINP in females and should inform future clinical trials of these drugs as adjuncts to chemotherapy.

Summary:

Author for correspondence: Daniela Salvemini, Ph.D., Department of Pharmacology and Physiology, Saint Louis University School of Medicine, 1402 S. Grand Boulevard, Saint Louis, MO 63104. Phone: 1-314-977-6430, Fax: 1-314-977-6411, daniela.salvemini@health.slu.edu.

Katherine Stockstill and Carrie Wahlman contributed equally to this manuscript.

Conflict of interest: DS is a co-founder of BioIntervene, Inc. that licensed intellectual property from Saint Louis University related to A₃AR agonists and the inventor of U.S. patents related to S1PR1, which are assigned to and owned by Saint Louis University. All other authors declare no conflicts of interests.

We have found that there are sexually dimorphic responses to S1PR1 antagonists and A₃AR agonists for the treatment of bortezomib-induced neuropathic pain.

Keywords

chemotherapy; S1P; neuropathic pain; A₃AR

Introduction

The development of chemotherapy-induced neuropathic pain (CINP) is a major dose-limiting neurotoxicity of widely used chemotherapeutic agents, including platinum-based drugs (*e.g.* oxaliplatin) [5], taxanes (*e.g.* paclitaxel) [23] and proteasome inhibitors (*e.g.* bortezomib) [2]. CINP can persist for years and greatly impacts quality of life; treatment options are scarce and there are no FDA-approved drugs to prevent and treat CINP [16]. We have recently reported that alterations in sphingolipid metabolism and increased formation of sphingosine-1-phosphate (S1P) in the central nervous system (CNS) contribute to CINP by activating the S1P receptor subtype 1 (S1PR1) in astrocytes [38]. Moreover, we reported that the loss of adenosinergic signaling at the A₃ adenosine receptor subtype (A₃AR) in the CNS also contributes to CINP [28; 41]. Accordingly, we demonstrated that drugs that inhibit S1PR1 or activate the A₃AR can block the development of CINP caused by paclitaxel, oxaliplatin and bortezomib and reverse CINP once established [10; 27; 28; 38]. These novel targets for therapeutic intervention are poised for expedited translation. The functional S1PR1 antagonist, FTY720 (fingolimod; Gilenya®) [6] is FDA approved for multiple sclerosis and has a good safety profile, while several related second generation functional antagonists are in advanced clinical trials for various autoimmune disorders [3]. Numerous studies have also demonstrated that FTY720 has potent antitumor activity [21; 42]. A₃AR agonists are in advanced clinical trials for inflammatory disorders and cancer and also display a good safety profile [17; 25].

Historically, pain research has been performed in male rodents, although it is well known that females can respond differently to pain and therapeutics [1; 8; 37]. According to a survey by the Centers for Disease Control and Prevention, women are more likely to experience pain, including lower back, face, neck, and migraine [8]. It has also been documented that women respond differently to therapeutics [1; 32]. Even though a survey reported that 30–90% of patients can suffer from CINP [16], it did not discern the percentages of men and women. In a recent clinical analysis, there is a significantly greater incidence of peripheral neuropathies in men than women following chemotherapy [13]. Sex is not a known factor that increases the risk of developing bortezomib-induced peripheral neuropathy associated with chronic neuropathic pain (BINP) [2; 46]. However, there has been little preclinical research investigating sexually dimorphic differences in CINP among the classes of chemotherapeutics or the response to therapeutics. Woller et al. found no sex differences in the development of pain in a mouse model of cisplatin-induced neuropathy or their response to gabapentin [43]. In a rat model of paclitaxel-induced neuropathy, investigators found no sex differences in pain development or responsiveness to ketamine or morphine [24]. In contrast, both male and female rats developed hypersensitivity during

vincristine-induced neuropathy, however a PKC ϵ inhibitor reversed hyperalgesia in males but not females, but the underpinnings of such differences remain to be explored [29].

Here we used functional and competitive S1PR1 antagonists and A₃AR agonists to explore potential sex differences in therapeutic responses in mouse and rat models of CINP. Our results reveal sexually dimorphic differences in the therapeutic response among CINP models that will be critical in informing clinical trials with these therapeutic agents and chemotherapeutics.

Materials and Methods

Experimental animals:

Female or male Sprague-Dawley rats (~200 g starting weight) from Envigo Laboratories (Frederick, MD breeding colony) and female or male C57BL/6J mice (~20 g starting weight) from Jackson Laboratories (Bar Harbor, ME) were used. Rats of the same sex were housed 1–3 per cage while mice of the same sex were housed either 1–5 or 1–10 per cage with 12 hour light/dark cycles and food/water ad libitum. All experiments were conducted in accordance with the National Institutes of Health, the International Association for the Study of Pain guidelines for laboratory animal welfare, and the Saint Louis University Institutional Animal Care and Use Committee. Experimenters were blinded to treatment conditions.

Chemotherapy-induced neuropathic pain:

Rat model of bortezomib-induced neuropathic pain: Five consecutive intraperitoneal (i.p.) injections of bortezomib (0.2 mg/kg; Selleck Chemicals; Houston TX) or its vehicle (5% Tween80 and 5% EtOH in 0.9% NaCl) were given as 0.2 mL volumes on days (D) 0–4 for a cumulative dose of 1 mg/kg [47].

Mouse model of bortezomib-induced peripheral neuropathy: Three weekly i.p. injections for 4 weeks of bortezomib (0.4 mg/kg; Selleck Chemicals; Houston TX) or its vehicle (5% Tween80 and 5% EtOH in 0.9% NaCl) were given at a volume of 0.2 mL each for a final dose of 4.8 mg/kg [4].

Rat model of oxaliplatin-induced neuropathic pain: Five consecutive i.p. injections of oxaliplatin (2 mg/kg; Teva Pharmaceuticals; Petach Tikva, Israel) or its vehicle (5% dextrose) were given at 0.2 mL each on D0–4 for a cumulative dose of 10 mg/kg [28].

Rat model of paclitaxel-induced neuropathic pain: On four alternate days, i.p. injections of paclitaxel (2 mg/kg; Teva Pharmaceuticals; Petach Tikva, Israel) or its vehicle (Cremophor EL and 95% EtOH, 1:1 ratio; MilliporeSigma; Saint Louis, MO) were given as 0.2 mL each on D0, 2, 4, and 6 for a cumulative dose of 8 mg/kg [27].

Test compounds:

The functional S1PR1 antagonists FTY720 (Fingolimod, Cayman Chemical, Ann Arbor MI) and TASP0277308 (TASP, Shanghai Chempartner Co., Shanghai, China) were prepared according to a previously published protocol [18]. FTY720 and TASP0277308 were both

prepared as stocks in DMSO, for oral administration drugs were diluted to the appropriate dose in 30% DMSO in 0.5% methylcellulose; for intraperitoneal (i.p.) injections FTY720 was diluted in saline. The selective A₃AR antagonist, MRS5698 ([1S,2R,3S,4R,5S)-4-(6-((3-chlorobenzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide]) was synthesized as described previously [39] and dissolved in saline for i.p. injections. These compounds have been previously shown to be specific for their target receptors and efficacious in male rodent pain models at the doses used in this study [18–20; 38; 40]. The antidepressant, duloxetine hydrochloride, was purchased from TCI America (Portland, OR) and dissolved in DMSO as a stock solution, for i.p. injections stock was diluted in saline. Morphine sulfate was a kind gift from Mallinckrodt Pharmaceuticals (St. Louis MO, USA) for i.p. injections morphine sulfate was diluted in saline. All therapeutic test compounds were given 20–30 min prior to chemotherapy, except where stated herein as a reversal paradigm.

Estrus smears:

Rat vaginal smears were taken for two cycles prior to conducting experiments to verify normal cyclicity and then daily from D18 after first chemotherapy treatment until animals were sacrificed or until cyclicity could be determined to verify treatments did not alter cyclicity. Cells were placed on a glass slide and analyzed with a light microscope to determine their stage of estrous cycle. All rats displayed a normal 4–5 day estrous cycle. Mouse vaginal smears were taken for two cycles prior to conducting experiments to verify normal cyclicity and then daily from D21 after first chemotherapy treatment until animals were sacrificed or until cyclicity could be determined to verify treatments did not alter cyclicity. Cells were allowed to dry on a glass slide, stained with Accustain (MilliporeSigma; Saint Louis, MO) for 45 s and rinsed as adapted from a previously published protocol [7]. Fixed cells were then viewed under a light microscope to determine their stage of estrous cycle. All mice displayed a normal estrous cycle.

Behavioral testing:

Mechano-allodynia was measured using the Dixon up-and-down method [15] and calibrated Von Frey filaments (Stoelting; Wood Dale, IL). Individual animals were allowed to acclimate to behavioral chambers upon a wire mesh floor for 15–30 min before measuring their paw withdrawal thresholds (PWT, in g). Von Frey filaments for rats ranged from bending forces of 2 to 26 g, and for mice from 0.02 to 2 g. Mechano-hyperalgesia was assessed in rats using the Randall and Sellitto paw pressure test [35] and an analgesimeter (Ugo-Basile; Italy, model 37215), by which the dorsum of the rat's hind paw was stimulated to give the PWT (g). To avoid behavioral sensitization mechano-allodynia was assessed at least 15 minutes prior to mechano-hyperalgesia. Animals were assessed as showing increased mechano-hypersensitivity (or mechano-allodynia and mechano-hyperalgesia) when their PWT was significantly different ($P < 0.05$) compared to D0. Since CINP usually causes bilateral hypersensitivity, values for left and right hind paws in each animal were averaged to determine its PWT.

Western blot analysis:

Western blots were performed as previously described with minor modifications [33]. Animals were sacrificed on D25 under anesthesia via transcardiac perfusion with ice cold PBS. The dorsal lower lumbar enlargement (L4-L6) of the spinal cord was harvested and flash frozen in liquid nitrogen. Samples were homogenized, and protein concentrations were determined by BCA protein assay (Thermo-Fisher, Waltham, MA). Proteins were denatured in Laemmli buffer and boiled for 5 min. Equal proteins (5–10 µg) were loaded and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked for two hours at room temperature in 3% BSA in 1X TBS-T. Anti-A₃AR antibody (Bioss, 1:1000) [33] and anti-S1PR1 (Abcam, 1:15000) [38] were diluted in 1.5% BSA in 1X TBS-T and membranes were incubated overnight at 4°C. The bound antibodies were visualized following incubation with peroxidase-conjugated goat anti-rabbit IgG (1:5000, Jackson ImmunoResearch, PA, USA) or peroxidase-conjugated bovine anti-mouse IgG secondary antibody (1:5000, Jackson ImmunoResearch, West Grove, PA, USA) for one hour at RT. Peroxidase-conjugated antibodies were visualized by enhanced chemiluminescence (Bio-Rad, Hercules, CA). Chemiluminescence antibody signals were documented using Chemidoc XRS+ documentation system and ImageLab™ software (BioRad, Hercules, CA) using standard background subtraction and exposure settings and quantified for band densitometry. Each membrane was then probed for β-actin (1:5000, Sigma-Aldrich) as endogenous loading controls.

Values were expressed as mean ± SD for *n* animals. Data were analyzed by two-tailed, two-way ANOVA with Dunnett's multiple comparison or Student's *t*-test using GraphPad Prism (v 7.02, GraphPad Software, Inc.). The size of main effects were determined by partial eta squared (η_p^2) or Cohen's *d*-statistic corrected for low *n*-values (*n*<50). Cross-experimental mixed ANOVA analyses of time- and sex-dependent difference in the development of bortezomib-induced neuropathic pain (Fig. 1) was performed using IBM SPSS Statistics v. 24 (IBM Corp., Armonk, NY USA) with time as the within-subject variable and sex and treatment as between-subject factors and sphericity assumed based on reported Mauchly's *W* statistic. Significance was set at *p* < 0.05.

Results

BINP in females is not prevented or reversed by functional and competitive S1PR1 antagonists.

When compared to vehicle, bortezomib induced the development of neuropathic pain (mechano-hypersensitivity; mechano-allodynia and mechano-hyperalgesia) in female (Figures 1 A–F) and male rats (Figures 1 G–J). The development of BINP in these rats was dependent on time, but not the sex of the rat (Figure 1). Using a dose and schedule we previously demonstrated to prevent BINP in male rats [38], oral administration of FTY720 (0.1 mg/kg) concurrent with bortezomib (D0–4) did not prevent the development of mechano-hypersensitivity in female rats (Figures 1A, 1B). We increased the dose of FTY720 to 0.3 mg/kg in female rats to see whether they responded to higher doses and included a second functional S1PR1 antagonist (TASP) [20] to rule out drug-specific effects. Oral

administration of FTY720 (0.3 mg/kg and 3 mg/kg) or TASP (3 mg/kg and 10 mg/kg) given concurrently with bortezomib did not inhibit mechano-hypersensitivity in females (Figures 1C–F), while at these same higher doses, FTY720 and TASP prevented BINP in male rodents (Figures 1G–J). FTY720 (0.1 mg/kg) also failed to reverse established mechano-hypersensitivity in female rats when given at the time of peak neuropathic pain (D25; Figures 2A, 2B). In order to investigate whether the lack of effects were species specific, we used a mouse model of BINP in females. Bortezomib, but not vehicle treatment, led to the development of mechano-allodynia in female mice that was not blocked by FTY720 (0.3 mg/kg; Figure 3). In contrast, we previously reported that the same lot number of FTY720 at this same dose blocked BINP in male mice [38].

BINP in females is not prevented with the A₃AR agonist, MRS5698.

The development of bortezomib-induced neuropathic pain was not blocked in female rodents by concurrent administration of a highly selective A₃AR agonist, MRS5698 (0.1mg/kg; Figure 4) despite its previously reported efficacy at the same dose and using the same lot number in male rodents [41].

CINP induced by oxaliplatin and paclitaxel is prevented by FTY720 and MRS5698 in female rodents.

We have previously reported in male rats and mice that FTY720 and MRS5698 (0.1 mg/kg) can inhibit or attenuate the development of oxaliplatin and paclitaxel-induced neuropathic pain [10; 26–28; 41]. Likewise, female rats developed a similar time-dependent mechano-hypersensitivity following oxaliplatin or paclitaxel treatment as males that was prevented with concurrent treatment with previously used doses of FTY720 or MRS5698 (0.1 mg/kg; Figures 5A–5F).

A₃AR and S1PR1 pathways are differentially regulated in BINP in male and female rodents.

In order to explore mechanisms underlying potential differences in the response to A₃AR agonists and S1PR1 modulators, we investigated potential differences in the biosynthetic pathways involved or changes in receptor expression. At the time of peak mechano-hypersensitivity (D25), A₃AR expression levels were increased in the dorsal horn of the spinal cord (DH-SC) in male rats treated with bortezomib when compared to vehicle (Figure 6A); however, no changes in receptor level expression were found in female rats (Figure 6B). In contrast, S1PR1 expression in DH-SC was similar in male and female rats (Figure 6C–6D).

Opioids and antidepressants can attenuate CINP in female mice.

To confirm that female rodents respond to clinically used analgesics following bortezomib treatment, we determined whether morphine and duloxetine are able to reverse BINP. At the time of peak sensitivity (D28), both morphine (6 mg/kg) and duloxetine (30 mg/kg) reversed mechanical allodynia in a time-dependent manner in female mice (Figure 7).

Discussion

It is known that pain tolerance and threshold, as well as responses to analgesics vary widely in the human population, especially between sexes. For example, morphine, the gold standard for the treatment of pain, is reported to be more effective in male rodents [14; 34]; however, limited clinical data suggests morphine analgesia is more effective in females [12]. Here we reveal that unlike paclitaxel and oxaliplatin induced mechano-hypersensitivities, BINP in female rodents was not prevented or reversed by S1PR1 antagonists or A₃AR agonists. However, BINP was reversed by the opioid receptor agonist morphine and the serotonin reuptake inhibitor duloxetine, two analgesic classes used clinically that have been shown to reverse BINP in male rodents [45]. For comparison purposes the dosing paradigm used in this study was similar to that reported in our previous studies where S1PR1 antagonists and A₃AR agonists were administered only during treatment with the chemotherapeutic agent [26; 38; 47]. This dosing protocol was chosen in that it best reflects the clinical situation; restrict dosing only when the chemotherapeutic agent is used. Whether continued and prolonged administration of S1PR1 antagonists and A₃AR agonists may prevent BIPN in female rodents is not known.

Results suggest that in female rats, bortezomib causes the development of hypersensitivities through mechanisms that are independent of adenosine signaling at the A₃AR or through S1PR1 signaling. Although numerous mechanisms likely underlie these differences, we focused on receptor levels. We found that bortezomib treatment led to increased A₃ receptor levels in the dorsal lumbar spinal cord of male rodents, mimicking findings obtained with other chemotherapeutics (e.g., oxaliplatin) [41]. However, female rodents did not exhibit the same increase in spinal A₃AR expression, which can potentially explain the lack of response to the A₃AR agonists in females. In contrast, S1PR1 expression levels were similar in both sexes following bortezomib treatment. This suggests that the sex-dependent differences in the efficacy of S1PR1 antagonists to prevent or attenuate BINP may result from different pathways engaged by S1PR1 in males and females during bortezomib treatment. Additional studies will clarify this.

The mechanisms by which bortezomib causes neuropathic pain are not yet fully understood. Despite their different mechanisms of action in killing cancer cells, the three chemotherapeutics used here (bortezomib, oxaliplatin, and paclitaxel) result in similar pathological changes leading to pain [31]. Each of these classes of chemotherapeutics affects peripheral sensory, dorsal root ganglionic (DRG) and spinal cord nerve function through mitochondrial damage, oxidative stress and inflammation [22; 31]. The resulting neuro-excitatory and pro-inflammatory mediators contribute to hyper-excitability of sensory nerves and lead to central sensitization [31]. The level of investigation into the chemotherapeutic-specific mechanisms have varied for each class, but bortezomib may be the least researched due to its recent approval for human use in 2003 [30]. Recent evidence suggests that bortezomib increases presynaptic NMDA receptor (NMDAR) phosphorylation by protein kinase C (PKC) isoforms and thus augments glutamate release [44]. Perhaps the increased activity of atypical PKC isoforms is due to proteasome inhibition, leading to a decreased degradation of these kinases. However, PKC activity was also increased in paclitaxel-treated animals, an agent that does not inhibit proteasomes [44]. Interestingly, our research indicates

that S1PR1 antagonists decrease the frequency of miniature excitatory postsynaptic currents (mESPC) after bortezomib treatment, indicating a decrease in presynaptic glutamate release [38]. In vincristine-induced neuropathy, it was found that PKC ϵ inhibition did not reverse mechano-sensitivity in females or estrogen-supplemented ovariectomized females compared to males and ovariectomized females without estrogen [29]. Some literature suggests that estrogen regulates expression of PKC [11], and thus sex differences in such PKC-mediated NMDAR activity could explain some differences in drug responses. However, why the underlying cause of the sex differences was found only in bortezomib-treated rodents, when other chemotherapies also increase glutamate release via similar mechanisms, remains poorly understood.

We have shown previously that reactive astrocytes mediate hypersensitivity oxaliplatin and bortezomib-induced neuropathic pain [38; 41]. Additionally, the analgesic actions of both S1PR1 antagonists and A₃AR agonists are dependent on the attenuation of neuroinflammatory processes within reactive astrocytes [27; 28; 38; 41]. While there is some evidence that neuroinflammation can differ between sexes during the development of neuropathic pain, these differences were seen primarily in microglial responses [9], which have been shown to not be activated during CINP [36]. Thus, the sex differences we have observed with S1PR1 and A₃AR drug responses in BINP are not likely to be based solely on neuroinflammatory pathways. Based on the current literature and mechanistic understanding of BINP and the pharmacological action of these novel analgesic agents, our results are unexpected and more research is needed to fully elucidate the underlying mechanisms behind these sex differences. However, the results in the present study do provide critical insight as these agents move to clinical trials as adjunct to chemotherapy to prevent and treat CINP. Based on our results, clinical trials of either A₃AR agonists or S1PR1 antagonists for CINP should use caution when including females treated with bortezomib until the mechanism underlying these differences is more fully understood. Identifying and exploring these sex-dependent responses to novel therapeutics prior to clinical trials should improve the eventual success rate of translational research and therapeutic responses. This may point to future drug development efforts tailored specifically to males and females.

Acknowledgments:

K. Stockstill and C. Wahlman contributed equally to this manuscript. We would like to thank Dr. A. J. Lechner for his valuable input and careful editorial review of our work (Saint Louis University).

Grants: This study was funded by the National Institutes of Health (NIH) T32 Training grant GM008306 (K.S. & K.B.), NIH-National Cancer Institute grant R01CA169519 (D.S.), NIDDK IRP ZIA DK031117 (K.A.J.), The MayDay Fund Clinical Evaluation of Novel Biomarkers to Select and Treat Chronic Pain grant (D.S.), and Leukemia and Lymphoma Society Translational Research Award (D.S.).

References

- [1]. Bartley EJ, Fillingim RB. Sex differences in pain: a brief review of clinical and experimental findings. *British journal of anaesthesia* 2013;111(1):52–58. [PubMed: 23794645]
- [2]. Beijers AJ, Oerlemans S, Mols F, Eurelings M, Minnema MC, Vreugdenhil A, van de Poll-Franse LV. The magnitude of neurotoxicity in patients with multiple myeloma and the impact of dose modifications: results from the population-based PROFILES registry. *Annals of hematology* 2017.

- [3]. Bigaud M, Guerini D, Billich A, Bassilana F, Brinkmann V. Second generation S1P pathway modulators: research strategies and clinical developments. *Biochim Biophys Acta* 2014;1841(5): 745–758. [PubMed: 24239768]
- [4]. Boehmerle W, Huehnchen P, Peruzzaro S, Balkaya M, Endres M. Electrophysiological, behavioral and histological characterization of paclitaxel, cisplatin, vincristine and bortezomib-induced neuropathy in C57Bl/6 mice. *Sci Rep* 2014;4:6370. [PubMed: 25231679]
- [5]. Briani C, Argyriou AA, Izquierdo C, Velasco R, Campagnolo M, Alberti P, Frigeni B, Cacciavillani M, Bergamo F, Cortinovis D, Cazzaniga M, Bruna J, Cavaletti G, Kalofonos HP. Long-term course of oxaliplatin-induced polyneuropathy: a prospective 2-year follow-up study. *Journal of the peripheral nervous system : JPNS* 2014;19(4):299–306. [PubMed: 25582667]
- [6]. Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, Aradhye S, Burtin P. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nature reviews Drug discovery* 2010;9(11):883–897. [PubMed: 21031003]
- [7]. Byers SL, Wiles MV, Dunn SL, Taft RA. Mouse estrous cycle identification tool and images. *PloS one* 2012;7(4):e35538. [PubMed: 22514749]
- [8]. CDC. Summary Health Statistics for U.S. Adults: National: Health Interview Survey, 2009, Department of Health and Human Services Report. In: CDC editor, 2009 p. 7.
- [9]. Chen G, Luo X, Qadri MY, Berta T, Ji R-R. Sex-Dependent Glial Signaling in Pathological Pain: Distinct Roles of Spinal Microglia and Astrocytes. *Neuroscience bulletin* 2017;34(1):98–108. [PubMed: 28585113]
- [10]. Chen Z, Janes K, Chen C, Doyle T, Bryant L, Tosh DK, Jacobson KA, Salvemini D. Controlling murine and rat chronic pain through A3 adenosine receptor activation. *BoisFASEB J* 2012;26(5): 1855–1865.
- [11]. Cordey M, Gundimeda U, Gopalakrishna R, Pike CJ. Estrogen activates protein kinase C in neurons: role in neuroprotection. *Journal of neurochemistry* 2003;84(6):1340–1348. [PubMed: 12614334]
- [12]. Craft RM. Sex differences in opioid analgesia: “From mouse to man”. *Clinical Journal of Pain* 2003;19(3):175–186. [PubMed: 12792556]
- [13]. Cunningham D, Watkins D, Chau I, Davidson M, Starling N, Rao S, Wagner AD, Peckitt C, Kouvelakis K. 619PD_PRInfluence of sex on chemotherapy efficacy and toxicity in oesophagogastric (OG) cancer: A pooled analysis of four randomised trials. *Annals of Oncology* 2018;29(suppl_8).
- [14]. Dawson-Basoa MB, Gintzler AR. 17-Beta-estradiol and progesterone modulate an intrinsic opioid analgesic system. *Brain research* 1993;601(1–2):241–245. [PubMed: 8381698]
- [15]. Dixon WJ. Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol* 1980;20:441–462. [PubMed: 7387124]
- [16]. Farquhar-Smith P. Chemotherapy-induced neuropathic pain. *Current opinion in supportive and palliative care* 2011;5(1):1–7. [PubMed: 21192267]
- [17]. Fishman P, Bar-Yehuda S, Liang BT, Jacobson KA. Pharmacological and therapeutic effects of A3 adenosine receptor agonists. *Drug discovery today* 2012;17(7–8):359–366. [PubMed: 22033198]
- [18]. Fujii Y, Hirayama T, Ohtake H, Ono N, Inoue T, Sakurai T, Takayama T, Matsumoto K, Tsukahara N, Hidano S, Harima N, Nakazawa K, Igarashi Y, Goitsuka R. Amelioration of collagen-induced arthritis by a novel S1P1 antagonist with immunomodulatory activities. *Journal of immunology* 2012;188(1):206–215.
- [19]. Graler MH, Goetzel EJ. The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2004;18(3):551–553. [PubMed: 14715694]
- [20]. Grenald SA, Doyle TM, Zhang H, Slosky LM, Chen Z, Largent-Milnes TM, Spiegel S, Vanderah TW, Salvemini D. Targeting the S1P/S1PR1 axis mitigates cancer-induced bone pain and neuroinflammation. *Pain* 2017;158(9):1733–1742. [PubMed: 28570482]
- [21]. Hait NC, Avni D, Yamada A, Nagahashi M, Aoyagi T, Aoki H, Dumur CI, Zelenko Z, Gallagher EJ, Leroith D, Milstien S, Takabe K, Spiegel S. The phosphorylated prodrug FTY720 is a histone

deacetylase inhibitor that reactivates ERalpha expression and enhances hormonal therapy for breast cancer. *Oncogenesis* 2015;4:e156. [PubMed: 26053034]

- [22]. Han Y, Smith MT. Pathobiology of cancer chemotherapy-induced peripheral neuropathy (CIPN). *Frontiers in pharmacology* 2013;4:156. [PubMed: 24385965]
- [23]. Hershman DL, Unger JM, Crew KD, Minasian LM, Awad D, Moinpour CM, Hansen L, Lew DL, Greenlee H, Fehrenbacher L, Wade JL 3rd, Wong SF, Hortobagyi GN, Meyskens FL, Albain KS. Randomized double-blind placebo-controlled trial of acetyl-L-carnitine for the prevention of taxane-induced neuropathy in women undergoing adjuvant breast cancer therapy. *J Clin Oncol* 2013;31(20):2627–2633. [PubMed: 23733756]
- [24]. Hwang BY, Kim ES, Kim CH, Kwon JY, Kim HK. Gender differences in paclitaxel-induced neuropathic pain behavior and analgesic response in rats. *Korean journal of anesthesiology* 2012;62(1):66–72. [PubMed: 22323957]
- [25]. Jacobson KA, Klutz AM, Tosh DK, Ivanov AA, Preti D, Baraldi PG. Medicinal chemistry of the A3 adenosine receptor: agonists, antagonists, and receptor engineering. *Handbook of experimental pharmacology* 2009(193):123–159. [PubMed: 19639281]
- [26]. Janes K, Esposito E, Doyle T, Cuzzocrea S, Tosh DK, Jacobson KA, Salvemini D. A3 adenosine receptor agonist prevents the development of paclitaxel-induced neuropathic pain by modulating spinal glial-restricted redox-dependent signaling pathways. *Pain* 2014;155(12):2560–2567. [PubMed: 25242567]
- [27]. Janes K, Little JW, Li C, Bryant L, Chen C, Chen Z, Kamocki K, Doyle T, Snider A, Esposito E, Cuzzocrea S, Bieberich E, Obeid L, Petrache I, Nicol G, Neumann WL, Salvemini D. The development and maintenance of paclitaxel-induced neuropathic pain require activation of the sphingosine 1-phosphate receptor subtype 1. *The Journal of biological chemistry* 2014;289(30):21082–21097. [PubMed: 24876379]
- [28]. Janes K, Wahlman C, Little JW, Doyle T, Tosh DK, Jacobson KA, Salvemini D. Spinal neuroimmune activation is independent of T-cell infiltration and attenuated by A3 adenosine receptor agonists in a model of oxaliplatin-induced peripheral neuropathy. *Brain Behav Immun* 2015;44:91–99. [PubMed: 25220279]
- [29]. Joseph EK, Levine JD. Sexual dimorphism for protein kinase c epsilon signaling in a rat model of vincristine-induced painful peripheral neuropathy. *Neuroscience* 2003;119(3):831–838. [PubMed: 12809704]
- [30]. Kane RC, Farrell AT, Sridhara R, Pazdur R. United States Food and Drug Administration Approval Summary: Bortezomib for the Treatment of Progressive Multiple Myeloma after One Prior Therapy. *Clinical Cancer Research* 2006;12(10):2955. [PubMed: 16707588]
- [31]. Kerckhove N, Collin A, Condé S, Chaleteix C, Pezet D, Balayssac D. Long-Term Effects, Pathophysiological Mechanisms, and Risk Factors of Chemotherapy-Induced Peripheral Neuropathies: A Comprehensive Literature Review. *Frontiers in Pharmacology* 2017;8:86. [PubMed: 28286483]
- [32]. Kest B, Sarton E, Dahan A. Gender differences in opioid-mediated analgesia: animal and human studies. *Anesthesiology* 2000;93(2):539–547. [PubMed: 10910505]
- [33]. Little JW, Ford A, Symons-Liguori AM, Chen Z, Janes K, Doyle T, Xie J, Luongo L, Tosh DK, Maione S, Bannister K, Dickenson AH, Vanderah TW, Porreca F, Jacobson KA, Salvemini D. Endogenous adenosine A3 receptor activation selectively alleviates persistent pain states. *Brain* 2015;138(Pt 1):28–35. [PubMed: 25414036]
- [34]. Mogil JS, Chesler EJ, Wilson SG, Juraska JM, Sternberg WF. Sex differences in thermal nociception and morphine antinociception in rodents depend on genotype. *Neuroscience and biobehavioral reviews* 2000;24(3):375–389. [PubMed: 10781697]
- [35]. Randall LO, Selitto JJ. A method for measurement of analgesic activity on inflamed tissue. *Archives internationales de pharmacodynamie et de therapie* 1957;111(4):409–419. [PubMed: 13471093]
- [36]. Robinson CR, Zhang H, Dougherty PM. Astrocytes, but not microglia, are activated in oxaliplatin and bortezomib-induced peripheral neuropathy in the rat. *Neuroscience* 2014;274:308–317. [PubMed: 24905437]
- [37]. Sorge RE, Strath LJ. Sex differences in pain responses. *Curr Opin Physiol* 2018;6:75–81.

- [38]. Stockstill K, Doyle TM, Yan X, Chen Z, Janes K, Little JW, Braden K, Lauro F, Giancotti LA, Harada CM, Yadav R, Xiao WH, Lionberger JM, Neumann WL, Bennett GJ, Weng HR, Spiegel S, Salvemini D. Dysregulation of sphingolipid metabolism contributes to bortezomib-induced neuropathic pain. *J Exp Med* 2018;215(5):1301–1313. [PubMed: 29703731]
- [39]. Tosh DK, Deflorian F, Phan K, Gao ZG, Wan TC, Gizewski E, Auchampach JA, Jacobson KA. Structure-guided design of A(3) adenosine receptor-selective nucleosides: combination of 2-arylethynyl and bicyclo[3.1.0]hexane substitutions. *Journal of medicinal chemistry* 2012;55(10):4847–4860. [PubMed: 22559880]
- [40]. Tosh DK, Padia J, Salvemini D, Jacobson KA. Efficient, large-scale synthesis and preclinical studies of MRS5698, a highly selective A3 adenosine receptor agonist that protects against chronic neuropathic pain. *Purinergic signalling* 2015;11(3):371–387. [PubMed: 26111639]
- [41]. Wahlman C, Doyle TM, Little JW, Luongo L, Janes K, Chen Z, Esposito E, Tosh DK, Cuzzocrea S, Jacobson KA, Salvemini D. Chemotherapy-induced pain is promoted by enhanced spinal adenosine kinase levels through astrocyte-dependent mechanisms. *Pain* 2018;159(6):1025–1034. [PubMed: 29419652]
- [42]. White C, Alshaker H, Cooper C, Winkler M, Pchejetski D. The emerging role of FTY720 (Fingolimod) in cancer treatment. *Oncotarget* 2016;7(17):23106–23127. [PubMed: 27036015]
- [43]. Woller SA, Corr M, Yaksh TL. Differences in cisplatin-induced mechanical allodynia in male and female mice. *European journal of pain* 2015;19(10):1476–1485. [PubMed: 25716290]
- [44]. Xie JD, Chen SR, Chen H, Pan HL. Bortezomib induces neuropathic pain through protein kinase C-mediated activation of presynaptic NMDA receptors in the spinal cord. *Neuropharmacology* 2017;123:477–487. [PubMed: 28663117]
- [45]. Yamamoto S, Kawashiri T, Higuchi H, Tsutsumi K, Ushio S, Kaname T, Shirahama M, Egashira N. Behavioral and pharmacological characteristics of bortezomib-induced peripheral neuropathy in rats. *J Pharmacol Sci* 2015;129(1):43–50. [PubMed: 26362518]
- [46]. Zaroulis CK, Chairopoulos K, Sachanas SP, Maltezas D, Tzenou T, Pessach I, Koulieris E, Koutra E, Kilindireas K, Pangalis GA, Kyrtsonis MC. Assessment of bortezomib induced peripheral neuropathy in multiple myeloma by the reduced Total Neuropathy Score. *Leuk Lymphoma* 2014;55(10):2277–2283. [PubMed: 24552516]
- [47]. Zheng H, Xiao WH, Bennett GJ. Mitotoxicity and bortezomib-induced chronic painful peripheral neuropathy. *Experimental neurology* 2012;238(2):225–234. [PubMed: 22947198]

Figure 1. S1PR1 antagonists do not prevent bortezomib-induced neuropathic pain in female rats.

In female (A-F) and male rats (G-J), i.p. administration of bortezomib led to the development of mechano-hypersensitivity [mechano-allodynia (A, C, E, G, I) and mechano-hyperalgesia (B, D, F, H, J)]. Concurrent treatment with FTY720 [0.1 mg/kg (A,B), 0.3 mg/kg (C,D) or 3 mg/kg (E,F); p.o.] or TASP [3 mg/kg (C,D) or 10 mg/kg (E,F); p.o.] in female rats failed to prevent the development of mechano-hypersensitivity; whereas, in male rats, concurrent FTY720 [0.3 mg/kg (G,H) or 3 mg/kg (I,J); p.o.] or TASP [3 mg/kg (G,H) or 10 mg/kg (I,J); p.o.] was sufficient to prevent mechano-hypersensitivity. Y-axis has been cropped for clarity (B, D, F, H, J). Results are expressed as mean $\pm$ SD for $n=5$ /group (A,B) or $n=3$ /group (C-F) and analyzed by two-way ANOVA with Dunnett's multiple comparison with an effect size for time and treatment of (A) $\eta^2_p=0.899$, (B) $\eta^2_p=0.924$, (C) $\eta^2_p=0.892$, (D) $\eta^2_p=0.961$, (E) $\eta^2_p=0.838$, (F) $\eta^2_p=0.875$, (G) $\eta^2_p=0.837$, (H) $\eta^2_p=0.890$, (I) $\eta^2_p=0.948$ and (J) $\eta^2_p=0.809$. * $P<0.05$ compared to D0 † $P<0.05$ vs. bortezomib. Further cross-experiment mixed ANOVA analyses of Veh + Veh and Bortezomib + Veh data from C-J revealed that bortezomib-induced mechano-hypersensitivity was time-dependent [mechano-allodynia: Mauchley's $W = 0.730$; $F(3,60)=47.3$, $p=8.23 \times 10^{-16}$, $\eta^2_p=0.703$ and mechano-hyperalgesia: Mauchley's $W = 0.762$; $F(3,60)=42.8$, $p=6.50 \times 10^{-15}$, $\eta^2_p=0.681$], but not sex-

dependent [mechano-allodynia: $F(3,60)=0.336$, $p=0.800$, $\eta^2_p=0.017$ and mechano-hyperalgesia: $F(3,60)=1.37$, $p=0.260$, $\eta^2_p=0.064$].

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. The S1PR1 antagonist, FTY720), failed to reverse bortezomib-induced neuropathic pain in female rats.

When compared to D0, treatment with bortezomib but not vehicle, led to the development of mechano-hypersensitivity (A, $\eta^2_p=0.933$; B, $\eta^2_p=0.865$), which was not reversed with FTY720 (0.1 mg/kg; p.o.). Y-axis has been cropped to better display data (B). *Results are expressed as mean $\pm$ SD for n=5/group and analyzed by two-way ANOVA with Dunnett's multiple comparison. *P<0.05 compared to D0.*

Figure 3. The S1PR1 antagonist, FTY720, failed to prevent bortezomib-induced mechano-allodynia in female mice.

When compared to D0, bortezomib but not vehicle led to the development of mechano-allodynia. Prophylactic treatment with FTY720 (0.3 mg/kg; p.o.) did not prevent the development of mechano-allodynia ($\eta^2_p=0.933$). Results are expressed as mean $\pm$ SD; $n=6$ /group and analyzed by two-way ANOVA with Dunnett's multiple comparisons. * $P<0.05$ vs D0.

Figure 4. The A₃AR agonist, MRS5698, did not prevent bortezomib-induced neuropathic pain in female rats.

When compared to D0, treatment with bortezomib ($n=3$) but not vehicle ($n=5$), led to the development of mechano-hypersensitivity (A, $\eta^2_p=0.961$; B, $\eta^2_p=0.949$) which was not prevented with concurrent treatment with MRS5698 (0.1 mg/kg; i.p.; $n=5$). Y-axis has been cropped to better display data (B). Results are expressed as mean $\pm$ SD for $n=5$ and analyzed by two-way ANOVA with Dunnett's multiple comparison. * $P<0.05$ compared to D0.

Figure 5. A₃AR agonist and S1PR1 antagonists prevented oxaliplatin- and paclitaxel-induced neuropathic pain in female rats

When compared to D0, oxaliplatin or paclitaxel treatment, but not vehicle, led to the development of mechano-allodynia (A, C, E) and hyperalgesia (B, D, F). Prophylactic treatment with systemic MRS5698 (0.1 mg/kg; i.p.; C, D, E, F) and FTY720 (0.1 mg/kg; p.o.; A, B, E, F) prevented the development of mechano-hypersensitivity. Y-axis has been cropped to better display data (B, D, F). *Results are expressed as mean $\pm$ SD; n=6 (C, D) or n=5 (A,B,E,F) and analyzed by two-way ANOVA with Dunnett's multiple comparisons with an effect size for time and treatment of (A) $\eta^2_p=0.936$, (B) $\eta^2_p=0.899$, (C) $\eta^2_p=0.923$, (D) $\eta^2_p=0.940$, (E) $\eta^2_p=0.878$ and (F) $\eta^2_p=0.866$. * $P<0.05$ vs D0 and † $P<0.05$ vs. oxaliplatin or paclitaxel.*

Figure 6. A₃AR expression was increased in the spinal cord after bortezomib treatment in male rats but not female.

At time of peak mechano-hypersensitivity (D25) and when compared to vehicle, there was a significant increase in the expression of A₃AR in the spinal cord of male rats (A), but not female (B). Bortezomib had no effect on the expression of S1PR1 in either male (C) or female (D) spinal cord. Results are expressed as mean $\pm$ SD; $n=7$ /group (A,C) or $n=8$ /group (B,D) and analyzed with Student *t*-test with effect sizes of (A) $d=0.891$; (B) $d=0.439$; (C) $d=0.233$ and (D) $d=0.132$. * $P<0.05$ vs D0.

Figure 7. Morphine and duloxetine attenuated bortezomib-induced neuropathic pain in female mice.

When compared to D0, treatment with bortezomib led to the development of mechano-allodynia which was able to be attenuated with injection of morphine (6 mg/kg in saline, i.p.) or duloxetine (30 mg/kg in 2% DMSO, i.p.). Results are expressed as mean $\pm$ SD for $n=5$ /group and analyzed by two-way ANOVA with Dunnett's multiple comparison with effect size of time and treatment of $\eta^2_p=0.0.636$. * $P<0.05$ compared to D0 † $P<0.05$ compared to D28.