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Bortezomib and metformin opposingly regulate the expression of hypoxia-inducible factor alpha and the consequent development of chemotherapy-induced painful peripheral neuropathy

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Abstract

Chemotherapy-induced painful peripheral neuropathy is a significant clinical problem that is associated with widely used chemotherapeutics. Unfortunately, the molecular mechanisms by which chemotherapy-induced painful peripheral neuropathy develops have remained elusive. The proteasome inhibitor, bortezomib, has been shown to induce aerobic glycolysis in sensory neurons. This altered metabolic phenotype leads to the extrusion of metabolites which sensitize primary afferents and cause pain. Hypoxia-inducible factor alpha is a transcription factor that is known to reprogram cellular metabolism. Furthermore, hypoxia-inducible factor 1 alpha protein is constantly synthesized and undergoes proteasomal degradation in normal conditions. However, metabolic stress or hypoxia stabilizes the expression of hypoxia-inducible factor 1 alpha leading to the transcription of genes that reprogram cellular metabolism. This study demonstrates that treatment of mice with bortezomib stabilizes the expression of hypoxia-inducible factor 1 alpha. Moreover, knockdown of hypoxia-inducible factor 1 alpha, inhibition of hypoxia-inducible factor 1 alpha binding to its response element, or limiting its translation by using metformin prevent the development of bortezomib-induced neuropathic pain. Strikingly, the blockade of hypoxia-inducible factor 1 alpha expression does not attenuate mechanical allodynia in mice with existing bortezomib-induced neuropathic pain. These results establish the stabilization of hypoxia-inducible factor 1 alpha expression as the molecular mechanism by which bortezomib initiates chemotherapy-induced painful peripheral neuropathy. Crucially, these findings reveal that the initiation and maintenance of bortezomib-induced neuropathic pain are regulated by distinct mechanisms.

Keywords

Neuropathy, chemotherapy-induced painful peripheral neuropathy, hypoxia-inducible factor 1 alpha, metformin, aerobic glycolysis, dorsal root ganglion

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Introduction

The American Cancer Society estimates that in 2019, 1,762,450 new cancer cases are projected to occur in the United States. The majority of these patients will require chemotherapy. The most common toxicity associated with many chemotherapeutics is chemotherapy-induced painful peripheral neuropathy (CIPN) which is the principle reason why many patients stop potential curative therapy, impacting their survival. Moreover,

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CIPN can persist in cancer survivors negatively impacting their quality of life.[1–3]

Rapid cell proliferation is the main pathobiological feature of cancers. Chemotherapeutics mainly target mechanisms that are crucial for cellular replication which eventually leads to cell death. Bortezomib, a proteasome inhibitor, arrests the cell cycle in the G_2-M phase by preventing the degradation of cyclin-dependent kinase inhibitors.[4,5] Paclitaxel and vincristine affect microtubule stability which interferes with the proper segregation of the chromosome during anaphase. Finally, platinum-based drugs prevent DNA replication by forming DNA adducts. However, these mechanisms are not relevant to primary afferents because neurons are post-mitotic. The incomplete elucidation of the mechanisms of action of chemotherapeutics in neurons remains a significant barrier in understanding how CIPN develops. Hence, there is a critical need for uncovering these mechanisms which might lead to the development of novel therapeutic strategies.

Mitotoxicity and changes in primary afferent metabolism have been long established as a common feature in the pathobiology of CIPN.[6–9] Moreover, bortezomib has been shown to induce aerobic glycolysis in sensory neurons by enhancing the expression of pyruvate dehydrogenase kinase 1 (PDHK1) and lactate dehydrogenase A (LDHA).[10] A key transcription factor that is known to reprogram cellular metabolism is hypoxia-inducible factor 1 alpha (HIF1A).[11–15] HIF1A regulates the expression of PDHK1 and LDHA.[11–13,16,17] The amount of HIF1A protein that is expressed within a cell is dictated by the balance between the rate of its synthesis and degradation. HIF1A is constantly synthesized within a cell, and in normal metabolic conditions, it is degraded through a highly regulated mechanism.[12] HIF1A mRNA translation has been shown to be regulated by the mTOR pathway.[18–22] As for its degradation, HIF1A requires its hydroxylation by a group of proteins known as prolyl hydroxylase domain-containing proteins (PHDs). PHDs require the Krebs cycle intermediate, alpha-ketoglutarate, and oxygen as substrates to catalyze the hydroxylation of HIF1A on proline 402 and 564. Hydroxylated HIF1A recruits the von Hippel-Lindau (VHL) complex. The VHL complex possesses ubiquitin E3 ligase activity which ubiquitinates HIF1A leading to its proteasomal degradation. However, metabolic insults or hypoxia disrupt the hydroxylation of HIF1A which prevents its ubiquitination and subsequent degradation. Stable HIF1A expression controls a transcriptional program that allows cells to cope with hypoxia by promoting the expression of genes that regulate glycolysis and sustain energy production when cellular respiration is reduced.[12–15]

The anti-diabetic drug metformin has been demonstrated to prevent cisplatin and paclitaxel-induced neuropathic pain. However, the mechanisms by which metformin prevents the development of CIPN have remained elusive. Metformin activates the AMP-activated protein kinase (AMPK), also known as the "energy sensor" of a cell that plays a crucial role in the cellular energy homeostasis. Upon activation, AMPK increases energy levels of the cell by inhibiting energy-intensive processes such as protein synthesis and stimulates alternate energy producing processes.[23–32] AMPK inhibits protein synthesis by inhibiting the mTOR pathway. mTOR exerts its effect via phosphorylation of its two key substrates, p70 S6 kinase (S6K) and eIF4E-binding proteins (4E-BPs). The phosphorylation of rS6 protein via the mTOR pathway stimulates mRNA translation.[23–29,33] These observations suggest that metformin might prevent the development of CIPN by inhibiting the expression of HIF1A because the translation of HIF1A is regulated by the mTOR pathway.[18–22]

This study examined the effect of bortezomib on the expression of HIF1A and the role of HIF1A in the development of bortezomib-induced neuropathic pain. We determined that bortezomib stabilized the expression of HIF1A in normoxic conditions, and the stabilization of HIF1A was sufficient to cause pain. Moreover, blockade of HIF1A expression either via small interfering RNA (siRNA) or metformin treatment prevented the development of bortezomib-induced neuropathic pain. Crucially, limiting the expression of HIF1A was not effective in alleviating established bortezomib-induced neuropathic pain. These findings provide insights into the basic mechanism of action through which bortezomib initiates CIPN.

Materials and methods

Experimental animals

Pathogen-free, adult male ICR mice (3–4 weeks old; Envigo) were housed in a temperature ($23^{\circ}C \pm 3^{\circ}C$) and light (12-h light/12-h dark cycle; lights on 07:00–19:00) controlled rooms with standard rodent chow and water available *ad libitum*. Animals were randomly assigned to treatment or control groups for the behavioral experiments. Animals were initially housed five per cage. All behavioral experiments were performed by experimenters who were blinded to the experimental groups and treatments. The Institutional Animal Care and Use Committee of the University of Maryland approved all experiments. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain.

Mechanical testing

Male ICR mice were placed in acrylic boxes with wire mesh floors, and baseline mechanical withdrawal thresholds of the left hindpaw were measured after habituation for 1 h using the up-down method.[34] After determining the baseline withdrawal thresholds of mice hindpaw using von Frey filaments, the mice were treated with either intraperitoneal (IP) vehicle or 0.2 mg/kg of bortezomib (Millipore Sigma, Cat # 5.04314.0001) for five consecutive days for a total dose of 1 mg/kg.[7] IP metformin (150 mg/kg, Axxora, Cat # LKT-M2076) was either co-injected with bortezomib or alone at the indicated time points. Intrathecal (IT) siRNA (1 μg), dimethyloxalylglycine (DMOG, 1 μg, Millipore Sigma, Cat # D3695), or echinomycin (1 μg, Millipore Sigma, Cat # SML0477) injections were done between the L4 and L5 vertebrae at the indicated time points under isoflurane anesthesia. Negative control (Millipore Sigma, Cat # SIC001) and HIF1A (Millipore Sigma, SASI_Mm01_00070473) high-performance liquid chromatography (HPLC) purified siRNAs were injected at a dose of 1 μg in 5 μl of i-Fect (Neuromics, Cat # NI35150). Starting on day 7, the tactile withdrawal thresholds were tested.

Dorsal root ganglion cultures

Dorsal root ganglia (DRGs) were excised aseptically and placed in Hank's Buffered Salt Solution (Thermo Fisher, Cat # 14170112) on ice. The ganglia were dissociated enzymatically with collagenase A (1 mg/ml, 25 μmin, Millipore Sigma, Cat # 10103578001) and collagenase D (1 mg/ml, Millipore Sigma, Cat # 11088858001) with papain (30 U/ml, Millipore Sigma, Cat # 10108014001) for 20 μmin at 37°C. To eliminate debris and large diameter sensory neurons, 70 μm (Thermo Fisher, Cat # 087712) cell strainers were used. The dissociated cells were resuspended in DMEM/F12 (Thermo Fisher, Cat # 10565042) containing 1X pen-strep (Thermo Fisher, Cat # 15070063) and 10% fetal bovine serum (Millipore Sigma, Cat # F2442). The cells were plated in poly-D-lysine-coated six-well dishes (Corning, Cat # 35613) or glass bottom 35-mm dishes (Mattek, Cat # P35GC-1.5-10). The primary afferent cultures were incubated overnight or five days at 37°C in a humidified 95% air/5% CO_2 incubator.

Calcium imaging

Seven days following the initiation of the treatment protocol, DRGs were dissected, dissociated, and cultured overnight. Dissociated DRG cells were loaded with Fluo4-AM (1 μM, Thermo Fisher, Cat # F14217) for 20 μmin at 37°C in DMEM (Millipore Sigma, Cat # D5030). The cells were then transferred to a recording chamber placed on an inverted microscope (Olympus IX73, Japan). Images were captured using Micro-Manager 1.4 and analyzed with Fiji/ImageJ 1.52c software (NIH). Neurons measuring between 20 and 35 μm in diameter were analyzed. The E_{max} and time to half-maximum ($t_{1/2}$) were determined using GraphPad Prism 7. After baseline measurement, glucose (10 mM) was added at the 90-s time point and measurement continued for another 310 s.

Western blotting

Protein was extracted from the L4-6 DRGs in lysis buffer (50 mM Tris HCl, 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA at pH 7.4) containing protease and phosphatase inhibitor mixtures with an ultrasonicator on ice, and cleared of cellular debris by centrifugation at 14,000 relative centrifugal force for 15 μmin at 4°C. Fifteen micrograms of protein per well were loaded and separated by standard 7.5% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P membranes (Millipore Sigma, Cat # IPVH00010) and then blocked with 5% dry milk for 3 h at room temperature. The blots were incubated with primary antibody overnight at 4°C and detected the following day with donkey anti-rabbit or goat anti-mouse antibody conjugated to horseradish peroxidase (1:10,000, Jackson Immunoresearch, Cat # 711-036-152, Cat # 115-036-062). Signal was detected by enhanced chemiluminescence on films. For assessment of phospho-proteins, membranes were stripped and reprobed for total-protein of interest for normalization. Densitometric analyses were done using UN-SCAN-IT 7.1 software (Silk Scientific Corp.). Primary antibodies include HIF1A, p-AMPK, AMPK, p-rS6, and rS6 (1:1000 Cell Signaling Technology, Cat # 14179, 2535, 2603, 2215, 2217) and beta-III-tubulin (1:50,000 Promega, Cat # G7121).

Statistical analysis and data presentation

Data are based on the means and the standard error of the means (± *SEM*). Graph plotting and statistical analysis used GraphPad Prism Version 7 (Graph Pad Software Inc., San Diego, CA, USA). When analyzing evoked pain behavior data, two-way repeated-measure analysis of variance (ANOVA) followed by post hoc pairwise comparisons with Bonferroni correction was used. Western blot data were analyzed using the unpaired *t*-test. A priori level of significance at 95% confidence level was considered at $P < 0.05$.

Results

Bortezomib stabilized HIF1A expression in DRGs and knockdown of HIF1A prevented the development of bortezomib-induced neuropathic pain

The adverse effects chemotherapeutics have on mitochondria and the metabolism of sensory neurons have been long established.[6–9] HIF1A is a key transcription factor that regulates the expression of mitochondrial and metabolic genes. Moreover, HIF1A is constantly synthesized, and in the absence of hypoxia or other metabolic stressors, it undergoes proteasomal degradation.[12,14,15] Since bortezomib is a selective inhibitor of 26S proteasome,[35] the effect bortezomib has on HIF1A levels in DRGs was measured. Lumbar 4-6 DRGs excised on day 5, from mice treated with bortezomib showed enhanced HIF1A levels relative to the vehicle-treated group (Figure 1(a), Unpaired *t*-test revealed significant ($t = 2.43$, $df = 8$, $^{*}P = 0.0412$) difference between the vehicle- and bortezomib-treated groups, five mice/group).

To determine whether HIF1A mediates the development of pain following bortezomib treatment, HIF1A expression in lumbar DRGs was knocked down using siRNA. IT delivery of 2 to 5 μg of siRNA for three to four days into the general lumbar region has been shown to knockdown a gene of interest both in lumbar DRGs and spinal cord.[36–38] Crucially, knockdown of HIF1A will not completely eliminate the target gene and it is reversible. It should be noted that the nerve roots originating from L4-6 DRGs enter the spinal cord around 17 mm rostral to the site of the injection[39] (Figure 1(b)). Hence, only 1 μg of siRNA (using i-Fect transfection reagent for two consecutive days) was administered IT between L4 and L5 vertebrae. The control group receives siRNA in i-Fect that does not target any mouse genes.[38] Western blot analysis showed a significant reduction of HIF1A in the experimental group relative to the control group (Figure 1(c), Unpaired *t*-test revealed a significant ($t = 5.419$, $df = 8$, $^{***}P = 0.0006$) difference between the two groups, five mice/group). Crucially, Western blot analysis of the spinal cord that corresponds to the entry site of the nerve root emerging from L4-6 DRGs did not show any change in HIF1A levels (Figure 1(d), five mice/group). After determining baseline withdrawal thresholds using von Frey filaments, male ICR mice received IT siRNA for two consecutive days (days –2 and –1). On day 0, mice received IP vehicle or bortezomib for five consecutive days and additional IT siRNA on days 2 and 3. The withdrawal thresholds were measured on days 7 to 14. IT HIF1A siRNA prevented the development of bortezomib-induced pain. HIF1A siRNA administration itself did not alter the tactile thresholds (two-way repeated-measure ANOVA revealed a main effect for time ($F(6, 96) = 15.96$, $P < 0.0001$) and group ($F(3, 16) = 450.1$, $P < 0.0001$). Post hoc pairwise comparisons with Bonferroni correction revealed a significant ($^{****}P < 0.0001$) difference between the IP Bort + IT Cont siRNA treated and all the other groups, five mice/group).

Blockade of HIF1A binding to its response element prevented the development of bortezomib-induced neuropathic pain and abrogated calcium responses augmented by bortezomib treatment

Stabilized HIF1A translocates to the nucleus where it binds to its response element (hypoxia response element, HRE) leading to the transcription of genes that reprogram cellular metabolism. However, HIF1A has been demonstrated to regulate cellular function via non-transcriptional mechanisms. HIF1A can directly interact with Cdc6[40] and potentiate gamma-secretase activity.[41] Hence, we sought to determine if the bortezomib-mediated neuropathic pain was due to the transcriptional activity of HIF1A.

Echinomycin is a highly potent compound that selectively binds to the HRE, preventing the binding of HIF1A thus blocking the transcription of genes under its control.[42,43] After determining baseline withdrawal thresholds, ICR mice received IP injection of vehicle or 0.2 mg/kg of bortezomib and IT vehicle or echinomycin (1 ng in 5 μl) once a day for five consecutive days. The withdrawal thresholds were measured on days 7 to 14. Echinomycin prevented the development of bortezomib-induced pain (Figure 2, two-way repeated-measure ANOVA revealed a main effect for time ($F(5, 20) = 8.18$, $P = 0.0002$) and group ($F(3, 12) = 235$, $P < 0.0001$). Post hoc pairwise comparisons with Bonferroni correction revealed a significant ($^{****}P < 0.0001$) difference between the IP Bort + IT Vehicle-treated and all the other groups, five mice/group). Echinomycin administration itself did not alter the tactile thresholds. These results suggest that the transcription of genes that are under HIF1A control are crucial for the development of bortezomib-induced neuropathic pain.

HIF1A is known to regulate the expression of LDHA and PDHK1 which are critical for the maintenance of aerobic glycolysis.[11–15] Moreover, enhanced LDHA and PDHK1 expression are known to enhance calcium responses in DRG neurons following glucose treatment.[10] Hence, L4-6 DRGs were dissected from mice that were treated with IP vehicle or 0.2 mg/kg of bortezomib and IT vehicle or echinomycin (1 ng in 5 μl) once a day for five consecutive days. On day 7 following the initiation of chemotherapy, the DRGs were dissociated and the cells were cultured overnight. On the

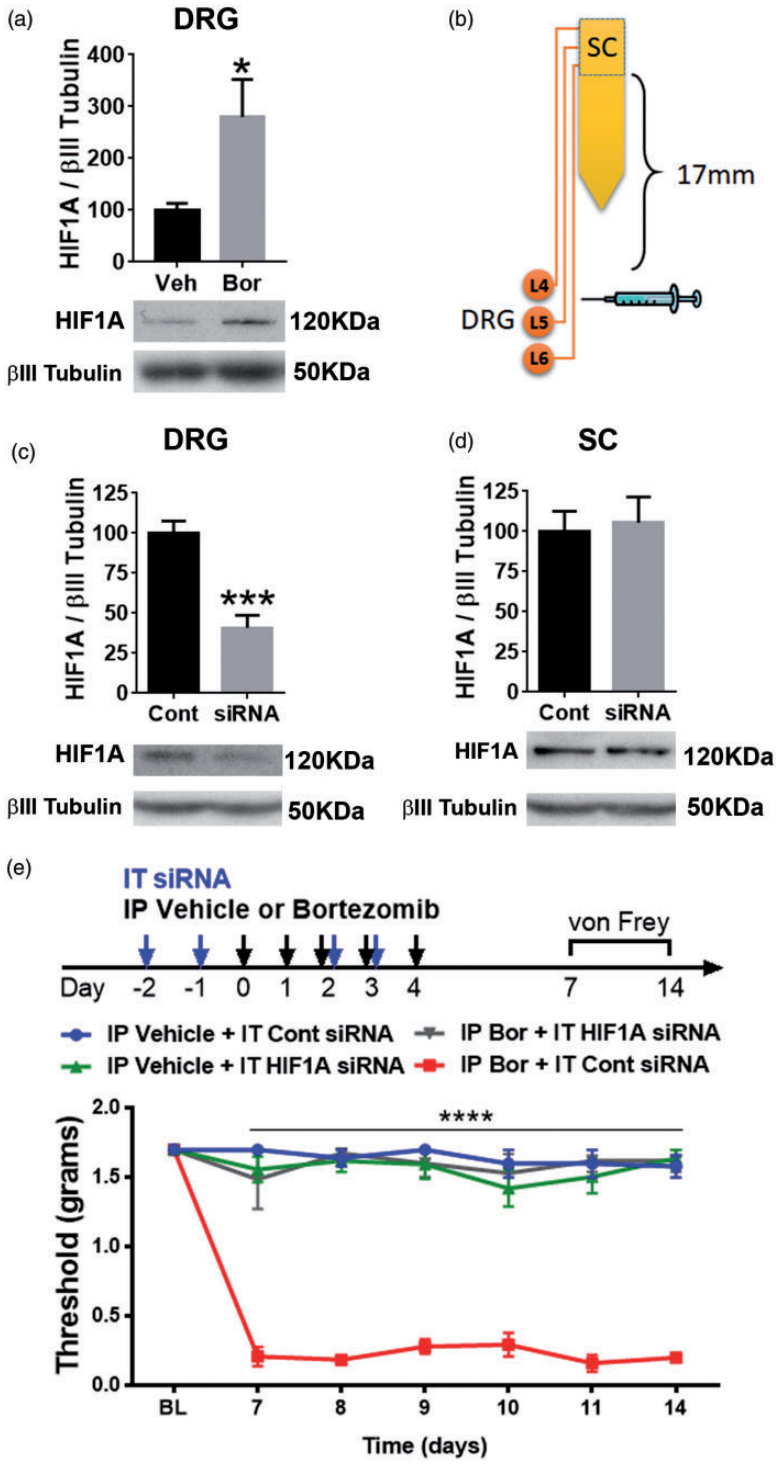

Figure 1. (a) Treatment of mice with bortezomib (Bor) for five days augmented HIF1A expression in L4-6 DRGs (*$P = 0.0412$, five mice/group) relative to the vehicle-treated group. (b) A schematic depicting the site of the intrathecal (IT) siRNA injection. The siRNA was administered between the L4 and L5 vertebrae which is around 17 mm rostral to the spinal cord (SC) section innervated by the L4-6 DRGs. (c) IT injection of siRNA (1 μg in 5 μl) that targets HIF1A (siRNA) but not control siRNA (Cont), for two consecutive days, significantly reduced the levels of HIF1A in L4-6 DRGs. (***$P = 0.0006$, five mice/group). (d) IT siRNA did not affect HIF1A levels in L4-6 spinal cord (five mice/group). (e) After determining baseline withdrawal thresholds using von Frey filaments, male ICR mice received IP injection of vehicle or bortezomib (black arrows) and IT siRNA (blue arrows). The withdrawal thresholds were measured on days 7 to 14. IT HIF1A siRNA prevented the development of bortezomib-induced neuropathic pain. (****$P < 0.0001$, five mice/group). DRG: dorsal root ganglia; HIF1A: hypoxia-inducible factor 1 alpha; IT: intrathecal; IP: intraperitoneal; siRNA: small interfering RNA.

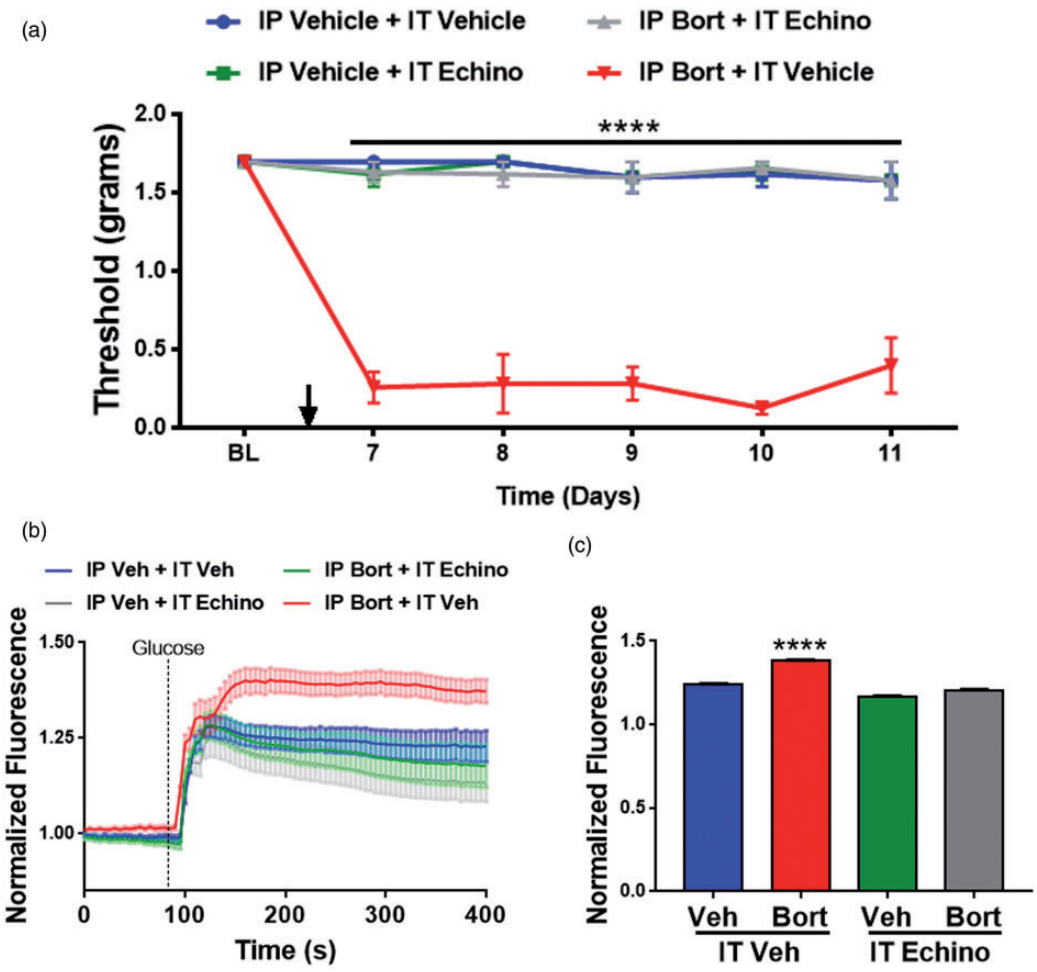

Figure 2. (a) Treatment of mice with IT of echinomycin (Echino, 1 ng in 5 μl) and IP bortezomib (0.2 mg/kg) for five consecutive days (arrow), prevented the development of bortezomib-induced allodynia (****$P<0.0001$, five mice/group). (b) Cumulative average of calcium imaging traces showing the changes in Fluo4 fluorescence in dissociated DRG neurons dissected on day 7, from mice treated with IP vehicle (Veh) or IP bortezomib (Bort) and IT vehicle or IT echinomycin (Echino) for five consecutive days. After baseline measurement was established for 90 s, glucose (10 mM) was added and the Fluo4 fluorescence was recorded for 310 s. (c) The maximum response, E_{max}, to glucose, revealed that DRG neurons dissected from mice pretreated with IP bortezomib and IT vehicle display higher E_{max} of calcium responses which were attenuated in neurons dissected from mice pretreated with IT echinomycin. (****$P<0.0001$, 10–25 neurons/group). IT: intrathecal; IP: intraperitoneal.

day of the experiment, the cells were loaded with calcium probe Fluo4-AM. Imaging of DRG neurons was performed in DMEM without glucose, pyruvate, or glutamine. Baseline measurements were made and at the 90-s mark, glucose was added and imaging of calcium transients continued for another 310 s. Addition of glucose led to a significant increase in the calcium responses of neurons excised from bortezomib-treated mice relative to other groups (Figure 2(b)). These results demonstrate that IT echinomycin pretreatment blocks bortezomib-mediated enhancement of calcium responses. Neurons with diameters measuring 20 to 35 μm were analyzed. Glucose addition revealed a significantly higher maximum response, E_{max}, in DRG neurons dissected from mice pretreated with IP bortezomib relative to the other groups (Figure 2(c), one-way ANOVA revealed a significant difference between the groups ($F(3, 59) = 635$, $P < 0.0001$). Tukey post hoc analysis revealed significant (****$P < 0.0001$) difference between the Bort + Veh and the other groups, 10–25 neurons). Analysis of the time to half-maximum ($t_{1/2}$) did not reveal any significant difference among the groups (data not shown). Collectively, these results show that echinomycin, which blocks the transcription of genes under HIF1A control, inhibits bortezomib-mediated metabolic reprogramming of DRG neurons and the consequent sensitization of primary afferents and neuropathic pain.

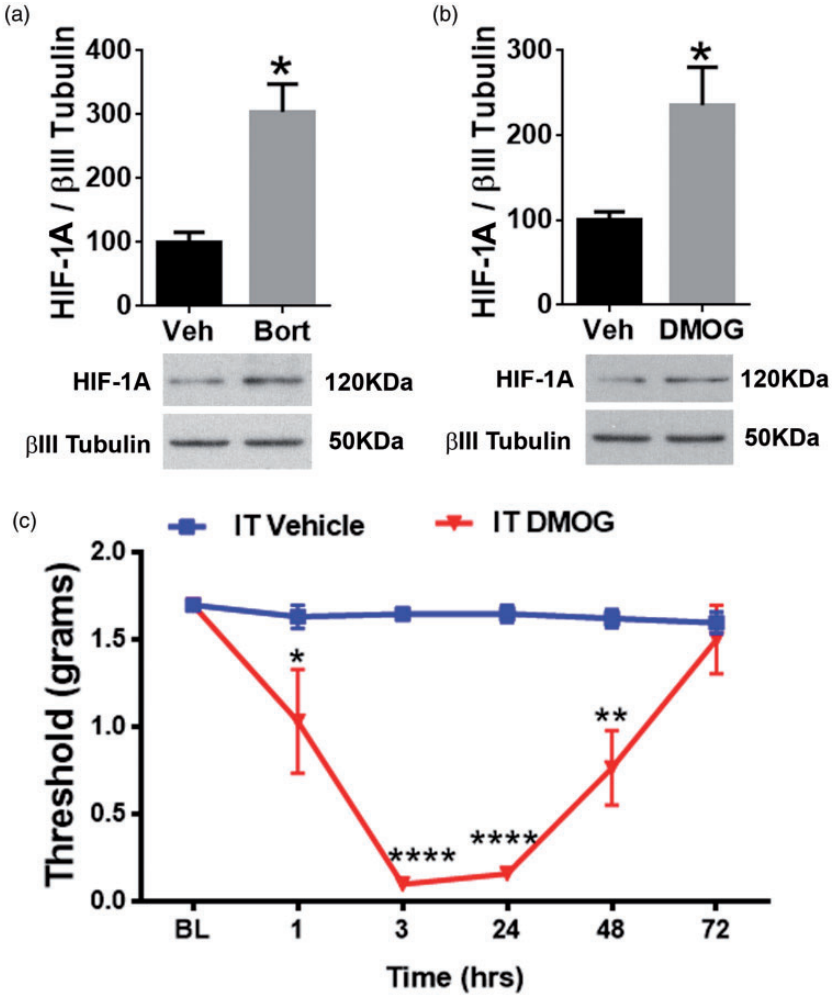

Figure 3. (a) Treatment of DRG cultures with bortezomib (1 μM) for 1 h increases HIF1A levels (*P= 0.0115, six wells/group). (b) DRG cultures exposed to PHD inhibitor DMOG (1 mM) for 1 h stabilized HIF1A expression (*P=0.0410, six wells/group). (c) A single intrathecal injection of DMOG (1 μg) produced mechanical allodynia that lasted several days (*P=0.04, **P=0.0027, and ****P<0.0001, five mice/group). IT: intrathecal; IP: intraperitoneal; HIF1A: hypoxia-inducible factor 1 alpha; DMOG: dimethyloxalylglycine.

Bortezomib stabilized HIF1A expression in normoxic conditions and the stabilization of HIF1A caused allodynia

We demonstrated that Bortezomib treatment stabilizes the expression of HIF1A (Figure 1(a)). However, it was not clear whether bortezomib stability of HIF1A expression was due to the direct effect of the drug on DRG neurons or due to inadequate oxygenation within the DRGs in vivo. Hence, we sought to determine if bortezomib can stabilize HIF1A expression in DRG neurons under normoxic conditions. This direct effect was determined by treating DRG cultures with bortezomib (1 μM) for 1 h at normal oxygen tension. Bortezomib treatment caused a threefold increase in the HIF1A levels relative to the vehicle-treated group (Figure 3(a), Unpaired t-test revealed significant ($t = 4.419$, $df = 4$, $^*P = 0.0115$) difference between the vehicle- and bortezomib-treated groups, six wells/group). This demonstrates that bortezomib can stabilize HIF1A expression in DRG neurons under normoxic conditions.

We sought to determine if the stabilization of HIF1A is sufficient to cause allodynia. DMOG is a cell-permeable, competitive inhibitor of PHD. DMOG is commonly used to stabilize HIF1A expression under normoxic conditions.[17,44–48] Hence, DRG cultures were treated with 1 mM of DMOG for 1 h. Similar to bortezomib, DMOG treatment stabilized HIF1A expression in DRG cultures (Figure 3(b), Unpaired t-test showed a significant ($t = 2.974$, $df = 4$, $^*P = 0.0410$) difference between the vehicle- and DMOG-treated groups six wells/group). Next, mice received either IT vehicle or DMOG (1 μg in 5 μl), and tactile thresholds were measured using von Frey filaments. A single IT injection of DMOG caused allodynia that peaked at around 3 h and resolved within 72 h (Figure 3(c), two-way repeated-

measure ANOVA revealed a main effect for time $F(5, 20) = 14.58$, $P < 0.0001$) and group ($F(1, 4) = 315.6$, $P < 0.0001$). Post hoc pairwise comparisons with Bonferroni correction revealed a significant ($^{*}P = 0.04$, $^{**}P = 0.0027$, and $^{****}P < 0.0001$) difference between the vehicle- and DMOG-treated group, five mice/group). Collectively, these data demonstrate that bortezomib acts directly on DRG neurons to stabilize HIF1A expression and stabilization of HIF1A is sufficient to cause nociception that can last several days.

Metformin reduced the expression of HIF1A and prevented the development of bortezomib-induced neuropathic pain

The results presented suggest that inhibition of HIF1A might serve as a viable therapeutic strategy to inhibit the development of CIPN. Inadequate tumor oxygenation often results in the activation of HIF1A in cancer cells. This has led to several clinical trials that target HIF1A. Unfortunately, many of these trials were terminated due to lack of efficacy or side effects.[49–52] Therefore, directly targeting HIF1A might not serve as a viable therapeutic option for CIPN.

The amount of a protein that is expressed within a cell is dictated by the balance between the rate of its synthesis and degradation. Bortezomib prevents the degradation of HIF1A by inhibiting the proteasome. Hence, limiting the synthesis of HIF1A might prevent bortezomib-induced neuropathic pain. Protein synthesis inhibitors such as anisomycin and rapamycin are known to cause pain by activating kinases.[26,53] Thus, we chose to activate AMPK using metformin. AMPK suppresses the translation of proteins in sensory neurons[24,26] and has been shown to inhibit a variety of pain conditions[24–27,29] including CIPN.[54] Treatment of primary sensory neurons in culture with metformin (20 mM) for 1 h reduced the HIF1A levels. The activation of AMPK was confirmed by demonstrating increased phosphorylation of AMPK on Thr172.[24,26] AMPK-mediated translational suppression was demonstrated by the reduction in phosphorylated ribosomal S6 protein (rS6) in response to metformin treatment (Figure 4(a), Unpaired *t*-test revealed a significant (HIF1A, $t = 4.378$, $df = 4$, $^{*}P = 0.0119$; AMPK, $t = 5.713$, $df = 4$, $^{**}P = 0.0046$; rS6, $t = 4.102$, $df = 4$, $^{*}P = 0.0148$) difference between the two groups (six wells/group). These results demonstrate that metformin suppresses the expression of HIF1A, suggesting that metformin treatment might prevent the development of bortezomib-induced CIPN.

Mice were treated with bortezomib (IP injection for five consecutive days) along with either vehicle or metformin (150 mg/kg). Similar to mice treated with HIF1A siRNA and echinomycin, metformin prevented the development of bortezomib-induced neuropathic pain. Metformin (IP injection for five consecutive days) alone did not lead to any changes in tactile hypersensitivity (Figure 4(a), two-way repeated-measure ANOVA revealed a main effect for time ($F(6, 24) = 26.77$, $P < 0.0001$) and group ($F(3, 12) = 746.9$, $P < 0.0001$). Post hoc pairwise comparisons with Bonferroni correction revealed a significant ($^{****}P < 0.0001$) difference between the Bort + Veh treated and all the other groups, five mice/group). Collectively, these results demonstrate that metformin suppresses the translation of HIF1A in DRGs and prevents the development of bortezomib-induced neuropathic pain.

Attenuating HIF1A expression did not alter existing bortezomib-induced neuropathic pain

Simultaneous but not delayed administration of metformin has been shown to be effective in preventing cisplatin-induced neuropathic pain.[54] Hence, we sought to determine if limiting the expression of HIF1A would reverse existing bortezomib-induced neuropathic pain. Mice were treated with IP vehicle or bortezomib (0.2 mg/kg) for five days where bortezomib treatment led to the development of neuropathic pain. Daily (day 7–10) IT injection with HIF1A siRNA did not attenuate mechanical allodynia in mice with established bortezomib-induced neuropathic pain (Figure 5(a), two-way repeated-measure ANOVA revealed a main effect for time ($F(5, 20) = 53.44$, $P < 0.0001$) and group ($F(3, 12) = 822.5$, $P < 0.0001$). Post hoc pairwise comparisons with Bonferroni correction revealed a significant ($^{****}P < 0.0001$) difference between the bortezomib and vehicle pretreated groups regardless of the siRNA treatment, five mice/group).

Similar to the knockdown experiment, treatment of mice with metformin (IP, 150 mg/kg, days 8–10) did not attenuate existing bortezomib-induced neuropathic pain (Figure 5(b), two-way repeated-measure ANOVA revealed a main effect for time ($F(6, 24) = 57.5$, $P < 0.0001$) and group ($F(3, 12) = 524.2$, $P < 0.0001$). Post hoc pairwise comparisons with Bonferroni correction revealed a significant ($^{****}P < 0.0001$) difference between the bortezomib and vehicle pretreated groups regardless of the metformin treatment, five mice/group). Limiting the expression of HIF1A either by using siRNA or metformin in mice with existing bortezomib-induced neuropathic pain did not attenuate the tactile thresholds. These results suggest that stabilization of HIF1A is critical for the development of bortezomib-induced neuropathic pain but not crucial for its maintenance.

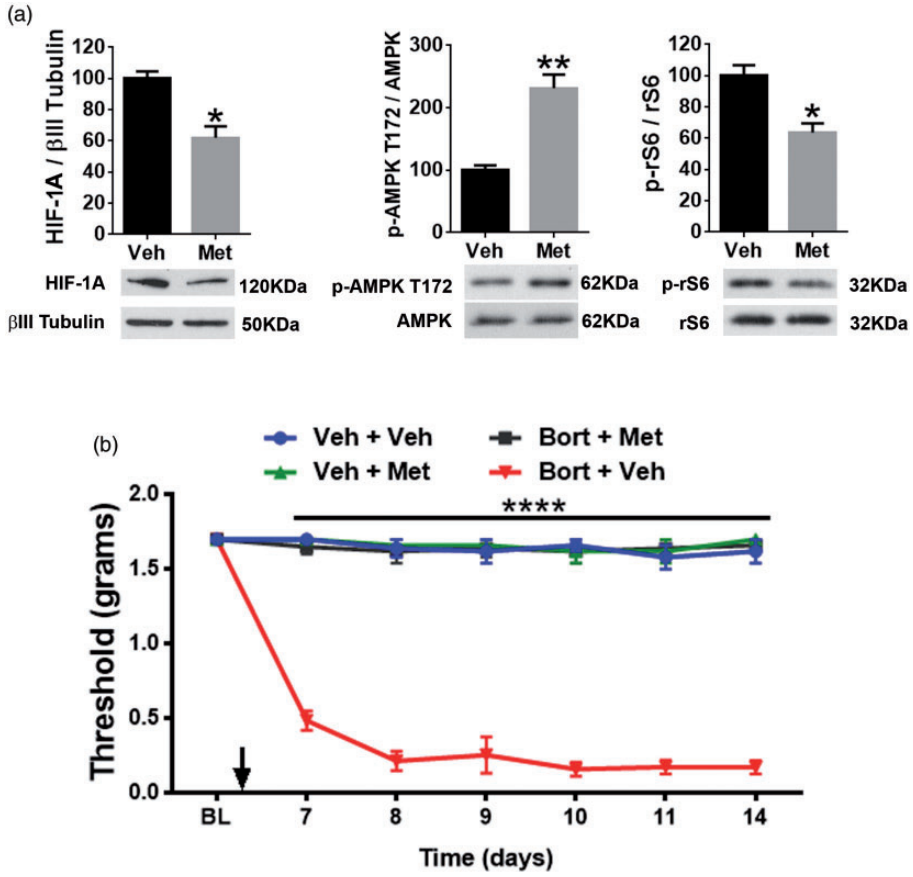

Figure 4. (a) Treatment of DRG cultures with metformin (20 mM) reduced the levels of HIF1A, activated AMPK which is confirmed by increased phosphorylation of Thr172 and suppressed translation which is validated by reduction in the phosphorylation of rS6 (HIF1A, *P= 0.0119; AMPK, **P= 0.0046; rS6, *P= 0.0148; six wells/group). (b) Co-injection of metformin (Met, 150 mg/kg) with bortezomib (Bort) for five consecutive days prevented the development of bortezomib-induced neuropathic pain (****$P<0.0001$, five mice/group). HIF1A: hypoxia-inducible factor 1 alpha; AMPK: AMP-activated protein kinase.

Discussion

Chemotherapeutics that cause CIPN mainly target mechanisms that allow cancer cells to proliferate. However, these mechanisms are not relevant to post-mitotic cells such as sensory neurons. This study uncovers the mechanism by which bortezomib initiates CIPN. Bortezomib stabilized the expression of HIF1A in sensory neurons. Moreover, bortezomib-induced neuropathic pain was prevented by (1) knockdown of HIF1A expression in DRGs, (2) inhibition of HIF1A association with its response element (HRE), and (3) inhibition of HIF1A expression using metformin. Stabilization of HIF1A expression was sufficient to cause tactile hypersensitivity for several days. However, attenuating the expression of HIF1A did not alter tactile thresholds in existing bortezomib-mediated neuropathic pain. These results establish stabilization of HIF1A expression as a crucial mechanism that initiates bortezomib-induced neuropathic pain.

The expression of HIF1A is a highly regulated process which is dependent on the oxygen tension and metabolic integrity of a cell. Metabolic insults stabilize HIF1A expression by limiting its post-translational modifications and subsequent proteasomal degradation. Stable HIF1A expression enhances the transcription of genes (e.g., PDHK1 and LDHA) that induce a metabolic phenotype known as aerobic glycolysis (Figure 6). This phenotype is characterized by increased glycolytic flux and reduced oxidative phosphorylation. Bortezomib has been demonstrated to induce aerobic glycolysis in sensory neurons by enhancing the expression of PDHK1 and LDHA.[10] Moreover, bortezomib-induced aerobic glycolysis leads to augmented extrusion of metabolites that sensitize primary afferents and cause pain.[10] Crucially, this study demonstrates that the blockade of the transcription of genes under HIF1A control prevents the sensitization of primary afferent neurons and the development of the bortezomib-induced neuropathic pain (Figure 2).

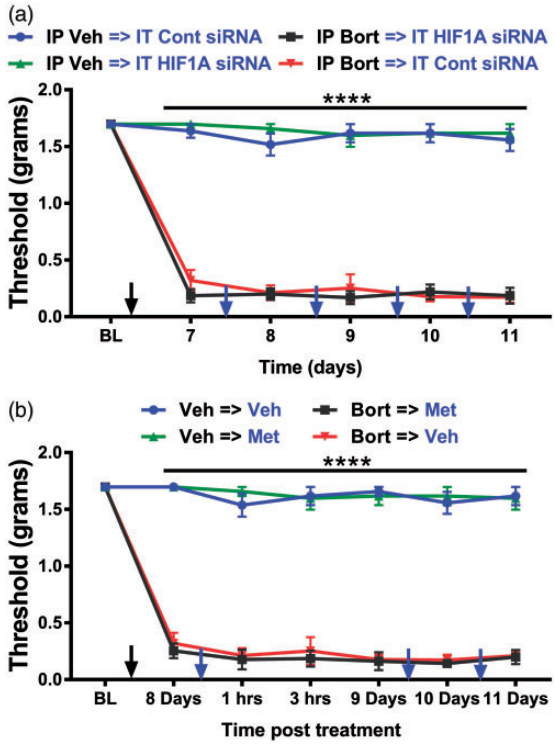

Figure 5. (a) After determining baseline withdrawal thresholds, male ICR mice received IP injection of vehicle or bortezomib (black arrows, days 0–4). IT siRNA was administered daily (blue arrows, days 7–10), and withdrawal thresholds were measured. IT HIF1A siRNA did not attenuate existing bortezomib-induced neuropathic pain. (****$P<0.0001$, five mice/group). (b) Daily metformin (150 mg/kg, blue arrows, days 8–10) treatment of mice with existing neuropathic pain did not alter the tactile thresholds (****$P<0.0001$, five mice/group). HIF1A: hypoxia-inducible factor 1 alpha; IT: intrathecal; IP: intraperitoneal.

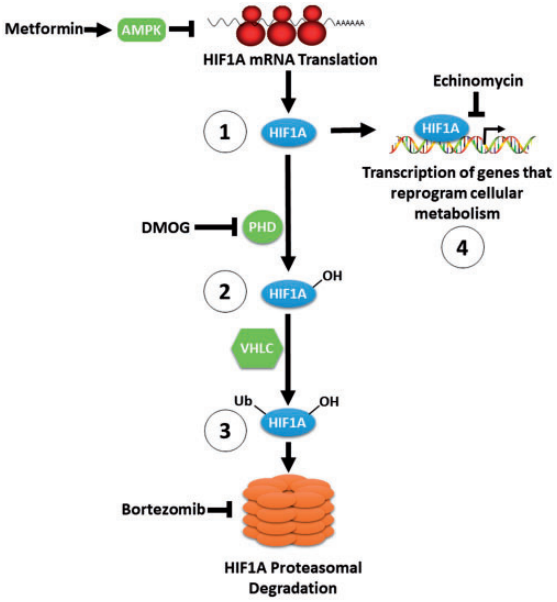

Figure 6. Schematic diagram summarizing the mechanisms through which bortezomib stabilizes HIF1A. (1) HIF1A protein is constantly synthesized and degraded in cells under normoxic and normal metabolic conditions. (2) HIF1A is hydroxylated on proline 564 and 402 via PHD. DMOG inhibits PHD, thus preventing the hydroxylation of HIF1A and stabilizing it. (3) Hydroxylated HIF1A is ubiquitinated by VHL complex (VHLC), which destines it for proteasomal degradation. Bortezomib is a proteasome inhibitor which stabilizes HIF1A expression. (4) Stabilized HIF1A translocates to the nucleus where it initiates the transcription of genes that reprogram cellular metabolism. Metformin activates AMPK which inhibits the translation of HIF1A. HIF1A: hypoxia-inducible factor 1 alpha; DMOG: dimethyloxalylglycine; PHD: prolyl hydroxylase domain-containing proteins.

Metformin can prevent the development of CIPN in response to paclitaxel and cisplatin.[54] However, the mechanisms by which metformin protects against the development of CIPN has remained elusive. This study uncovered that metformin attenuates the expression of HIF1A in DRG cultures under normoxic conditions and protects mice against bortezomib-induced neuropathic pain. These results suggest that the stabilization of HIF1A expression by chemotherapeutics might serve as a fundamental mechanism that underpins the development of CIPN.

Metformin prevents the development of CIPN in mice treated with cisplatin and bortezomib (Figure 5(b)); however, it cannot alleviate existing neuropathic pain.[54] Similarly, knockdown of HIF1A does not attenuate the tactile thresholds of mice with existing bortezomib-induced neuropathic pain (Figure 5(a)). These observations suggest that HIF1A is essential for the initiation but not the maintenance of CIPN. Bortezomib and paclitaxel are known to reprogram the metabolism of sensory neurons in a manner consistent with aerobic glycolysis.[9,10] Crucially, targeted inhibition of the enzymes that maintain the aerobic glycolysis phenotype alleviate existing neuropathic pain,[10] suggesting that aerobic glycolysis is critical for the maintenance of CIPN. However, the mechanisms that sustain the expression of the enzymes that maintain aerobic glycolysis remain elusive. Aerobic glycolysis is characterized by enhanced glycolytic flux and reduced oxidative phosphorylation. Bortezomib treatment has been shown to limit mitochondrial oxidation of pyruvate,[10] which would deplete the metabolic intermediates that are essential for chromatin remodeling and altering the epigenetic landscape.[55–59] Crucially, altered metabolic states are now recognized to produce epigenetic changes.[55–59] These changes might potentially reprogram the transcriptional networks that sustain the expression of metabolic enzymes, thus maintaining the aberrant metabolic phenotype of sensory neurons in an HIF1A-independent manner.

Metformin is a widely prescribed medication for type II diabetes, thus these findings strongly argue for its use for the treatment of CIPN. However, a potential problem that might limit the efficacy of metformin is that it increases glycolytic flux,[60–62] which has been shown to exacerbate bortezomib-induced neuropathic pain.[10] Lack of efficacy or side effects have led to the termination of clinical trials that directly inhibit HIF1A,[49–52] which precludes direct HIF1A inhibition as a strategy for prevention of CIPN. However, HIF1A expression is highly regulated by a multitude of molecular targets that might potentially protect against CIPN (Figure 6). This necessitates a detailed elucidation of the mechanisms by which chemotherapeutics stabilize the expression of HIF1A and the use of the insights gained for the development of novel therapies.

In conclusion, this study is the first to demonstrate that the stabilization of HIF1A expression underpins the development of bortezomib-induced neuropathic pain. Crucially, these findings reveal that the initiation and maintenance of bortezomib-induced neuropathic pain are regulated by distinct mechanisms.

Author Contributions

TL designed and performed research. OKM designed research, performed research, analyzed data, and wrote the paper.

Declaration of Conflicting Interests

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