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Novel Therapeutic Strategy to Prevent Chemotherapy-Induced Persistent Sensory Neuropathy By TRPA1 Blockade

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Abstract

Chemotherapy-induced peripheral neuropathy (CIPN) is a severe and painful adverse reaction of cancer treatment in patients that is little understood or treated. Cytotoxic drugs that cause CIPN exert their effects by increasing oxidative stress, which activates the ion channel TRPA1 expressed by nociceptors. In this study, we evaluated whether TRPA1 acted as a critical mediator of CIPN by bortezomib or oxaliplatin in a mouse model system. Bortezomib evoked a prolonged mechanical, cold, and selective chemical hypersensitivity (the latter against the TRPA1 agonist allyl isothiocyanate). This CIPN hypersensitivity phenotype that was stably established by bortezomib could be transiently reverted by systemic or local treatment with the TRPA1 antagonist HC-030031. A similar effect was produced by the oxidative stress scavenger α -lipoic acid. Notably, the CIPN phenotype was abolished completely in mice that were genetically deficient in TRPA1, highlighting its essential role. Administration of bortezomib or oxaliplatin, which also elicits TRPA1-dependent hypersensitivity, produced a rapid, transient increase in plasma of carboxy-methyl-lysine, a by-product of oxidative stress. Short-term systemic treatment with either HC-030031 or α -lipoic acid could completely prevent hypersensitivity if administered before the cytotoxic drug. Our findings highlight a key role for early activation/sensitization of TRPA1 by oxidative stress by-products in producing CIPN. Furthermore, they suggest prevention strategies for CIPN in patients through the use of early, short-term treatments with TRPA1 antagonists. *Cancer Res*; 73(10); 3120–31. ©2013 AACR.

Introduction

Several anticancer medicines evoke sensory adverse events, collectively referred to as chemotherapy-induced peripheral neuropathy (CIPN), which are represented by sensory symptoms (from paresthesias, allodynia, and hyperalgesia to severe pain). In addition to impairing patient quality of life, CIPN may lead to dose-limitation or even discontinuation of anticancer treatment (1). No effective therapy is currently available to treat or prevent CIPN, most likely because the underlying mechanisms are poorly understood. A host of hypotheses has

been proposed to explain CIPN, including mitochondrial dysfunction, increased content of oxidative substances, and altered function of different ion channels (2–7). Nonetheless, no unified mechanism that may reconcile results of clinical investigation and findings obtained in experimental animals has been advanced so far.

Chemotherapeutic drugs, which produce CIPN, are known to increase oxidative stress and reactive oxygen, nitrogen, or carbonyl species (ROS, RNS, and RCS, respectively) and treatment with antioxidant substances has been shown to reduce sensory hypersensitivity in experimental animals and to exhibit some degree of protection in patients with CIPN (3, 7–10). The transient potential receptor ankyrin 1 (TRPA1) is a non-selective cation channel, coexpressed with TRP vanilloid 1 (TRPV1) in a subset of C-fiber nociceptors, where it functions as a multimodal sensor to noxious stimuli (11, 12). TRPA1 shows a unique sensitivity for an unprecedented number of endogenous reactive molecules produced at sites of tissue injury or inflammation, which include ROS, RNS, and RCS (13–16).

Bortezomib is a proteasome inhibitor used in different types of cancer (17). CIPN has emerged as a major complication of bortezomib therapy, which usually appears in the first courses of therapy with a number of sensory and painful symptoms, including reduced threshold to mechanical and cold stimuli (18, 19). No satisfactory explanation or effective treatment

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exists for bortezomib-evoked CIPN (20, 21). As described for other chemotherapeutics, bortezomib has been reported to increase oxidative stress (22, 23).

In the present study, first, we investigated the role of oxidative stress and TRPA1 in a mouse model of CIPN evoked by bortezomib. Biochemical, pharmacologic, and genetic findings show that TRPA1 is necessary and sufficient to develop and maintain bortezomib-evoked mechanical, cold, and chemical hypersensitivity in mice. Second, we showed that early and short-term pharmacologic TRPA1 blockade totally prevented the sensory neuropathy evoked by bortezomib and oxaliplatin (previously shown to produce a TRPA1-dependent hypersensitivity in mice; refs. 5, 6, 24), thus opening new perspectives for CIPN prevention and treatment.

Materials and Methods

Animals

Animal experiments were carried out according to Italian legislation (DL 116/92) and European Communities Council Directive (86/609/EEC). Studies were conducted under the permit (number 143/2008-B and 204/2012-B, University of Florence, Florence, Italy) approved by the Italian National Committee for Animal Research. C57BL/6 mice (male, 25–30 g; Harlan Laboratories), wild-type (*Trpa1*^{+/+}), or TRPA1-deficient mice (*Trpa1*^{-/-}; 25–30 g; Jackson Laboratories) were used. Animals were housed in a temperature- and humidity-controlled vivarium (12-hour dark/light cycle, free access to food and water). Behavioral experiments were done in a quiet, temperature-controlled room (20–22°C) between 9 a.m. and 5 p.m., and were conducted by an operator blinded to the genotype and the status of drug treatment. Animals were sacrificed with a high dose of intraperitoneal (i.p.) sodium pentobarbital (200 mg/kg).

Reagents

If not otherwise indicated, all reagents were from Sigma-Aldrich. HC-030031 [2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide; Supplementary Fig. S1A] was synthesized as previously described (15). HC-067047 (2-Methyl-1-[3-(4-morpholinyl)propyl]-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1H-pyrrole-3-carboxamide; Supplementary Fig. S1B) was obtained from Tocris Bioscience, and bortezomib was purchased from LC Laboratories.

Chemotherapy-induced painful neuropathy models

Previous studies have described rat and mouse models of peripheral neuropathy induced by repeated and prolonged administration of bortezomib (25–28). On the basis of these findings, in the first series of experiments, we explored whether a single administration of bortezomib produced mechanical and cold hypersensitivity in mice, as observed for different chemotherapeutic agents including oxaliplatin, paclitaxel, and vincristine (4, 6, 29). After habituation and baseline measurements of pain sensitivity, animals were randomized into treatment groups. C57BL/6, *Trpa1*^{+/+}, or *Trpa1*^{-/-} mice were treated with a single intraperitoneal administration of different doses of bortezomib (0.2, 0.5, and 1 mg/kg), or vehicle (dimethyl sulfoxide, DMSO 1%; ref. 27). Bortezomib, formulated at a concentration of 1 mg/mL, was first dissolved in a vehicle

containing DMSO, and the volume was adjusted to 10 mL/kg to a final concentration of 1% DMSO, then diluted in isotonic saline (NaCl 0.9%) to obtain lower doses. A different group of C57BL/6 mice was treated with a single administration of oxaliplatin (3 mg/kg, i.p.) or its vehicle (isotonic saline, NaCl 0.9%; ref. 6). No weight loss was observed in mice after bortezomib or oxaliplatin treatment throughout the duration of the experiments. Effects induced by bortezomib and oxaliplatin were tested for 14 and 30 days (starting 6 hours after drug administration), respectively. Baseline values for nociceptive tests were observed before chemotherapy treatment.

Nociceptive tests

Von frey hair test. Mechanical threshold was measured in C57/BL6, *Trpa1*^{+/+}, or *Trpa1*^{-/-} mice after a single administration of bortezomib or oxaliplatin by using the up-and-down paradigm (30). Mechanical nociceptive threshold was determined before (basal level threshold) and after different treatments. The 50% mechanical paw withdrawal threshold (in g) response was then calculated from these scores, as previously described (30, 31).

Hot plate test. The paw thermal hyperalgesia was assessed in C57/BL6, *Trpa1*^{+/+}, or *Trpa1*^{-/-} by placing animals on a hot plate (UgoBasile) with the temperature adjusted to 50 ± 0.1°C (32). The latency to the first hind paw licking or withdrawal was taken as an index of nociceptive threshold. The cut-off time was set at 30 seconds, to avoid damage to the paw. The paw-withdrawal latency to the first response was reported as mean of 2 different trials.

Cold stimulation. Cold allodynia was assessed in C57/BL6, *Trpa1*^{+/+}, or *Trpa1*^{-/-} by measuring the acute nocifensive response to the acetone-evoked evaporative cooling as previously described (29). Briefly, a droplet (50 µL) of acetone, formed on the flat-tip needle of a syringe, was gently touched to the plantar surface of the mouse hind paw, and the time spent in elevation and licking of the plantar region over a 60-second period was measured. Acetone was applied 3 times at a 10- to 15-minute intervals, and the average of elevation/licking time was calculated.

Chemical hyperalgesia. Nociceptive behavior was assessed by measuring spontaneous nociceptive response induced by intraplantar (i.pl.) injection (20 µL) of sub-threshold doses of allyl isothiocyanate (AITC; 1 nmol/paw), capsaicin (0.01 nmol/paw), hypotonic saline (NaCl, 0.45%), or prostaglandin E₂ (PGE₂, 0.3 nmol/paw) at day 7 after the administration of bortezomib or its vehicle. Immediately after the injection, mice were placed inside a Plexiglas chamber and the total time spent licking and lifting the injected hind paw was recorded for 5 minutes (AITC, capsaicin, and hypotonic saline), or 20 minutes (PGE₂). Previous experiments conducted in our laboratory and previous findings (33, 34) suggested subthreshold doses that do not cause nociception in naïve mice.

Rotarod test

Locomotor function, coordination, and sedation of animals were tested by using a rotarod apparatus (UgoBasile). The test was done as previously described (35). Briefly, 24 hours before

the experiments, the animals were trained on the rotarod apparatus, programmed at 8 rpm, until they remained without falling for 60 seconds. The day of the experiment, the latency (seconds) to the first fall and the number of falls were recorded. Cut-off time was 240 seconds.

Treatment protocols

In a first set of experiments, intragastric (i.g.) HC-030031 (300 mg/kg) or its vehicle (0.5% carboxymethyl cellulose, CMC), HC-067047 (10 mg/kg, i.p.) or its vehicle (2.5% DMSO), or α -lipoic acid (100 mg/kg, i.g.) or its vehicle (0.5% CMC), were administered at day 7 after the administration of bortezomib (1 mg/kg, i.p.) or its vehicle. In a second set of experiments, i.p. HC-030031 (100 μ g/paw, 20 μ L; ref. 36), α -lipoic acid (10 μ g/paw, 20 μ L; ref. 3), or vehicle (20 μ L/paw, 1% DMSO in isotonic saline, NaCl 0.9%) were injected at day 3 or day 7 after the administration of oxaliplatin or bortezomib (see earlier section for dosing), respectively. In a third set of experiments, HC-030031 (300 mg/kg, i.g.), α -lipoic acid (100 mg/kg, i.g.), or their respective vehicles, were administered 15 minutes before the administration of bortezomib, oxaliplatin, or their vehicles and treatment was repeated 3 times at approximately 90-minute intervals each after the administration of bortezomib or oxaliplatin. In a fourth and final set of experiments, a group of mice was treated with HC-030031 or its vehicle 15 minutes before and shortly (3 times at approximately 90-minute intervals each) after a first bortezomib (1 mg/kg, i.p.) or vehicle administration. At day 6, each group of mice received a second treatment identical to that administered at day 1, except for mice treated at day 1 with both HC-030031 and bortezomib, which were subdivided into 2 additional groups. One group was treated a second time with either HC-030031 (300 mg/kg, i.g.) and the second with its vehicle 15 minutes before and shortly after (3 times at approximately 90-minute intervals each) bortezomib administration (Fig. 6A).

Isolation of primary sensory neurons and calcium imaging experiments

Primary dorsal root ganglia (DRG) from C57/BL6 adult mice were cultured as previously described (29). Briefly, lumbosacral (L5–S2) ganglia were bilaterally excised under a dissection microscope. Ganglia were digested using 1 mg/mL of collagenase type 1A and 1 mg/mL of papain in Hank's Balanced Salt Solution (25 minutes, 37°C). Neurons were pelleted and resuspended in Ham's-F12 containing 10% FBS, 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, and 2 mmol/L glutamine, dissociated by gentle trituration, and plated on glass coverslips coated with poly-L-lysine (8.3 μ mol/L) and laminin (5 μ mol/L). Neurons were cultured for 3 to 4 days.

Cells were incubated with 5 μ mol/L Fura-2AM ester for 30 minutes at 37°C. Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured on a Nikon Eclipse TE2000U microscope. Fluorescence was measured during excitation at 340 and 380 nm, and after correction for the individual background fluorescence signals, the ratio of the fluorescence at both excitation wavelengths (Ratio_{340/380}) was monitored. Experiments were conducted using a buffer solution containing (in mmol/L): 150 NaCl, 6 KCl, 1 MgCl₂, 1.5 CaCl₂, 10 glucose, 10 HEPES and

titrated to pH 7.4 with 1N NaOH. Cells were challenged with bortezomib (10, 50, and 100 μ mol/L) or their respective vehicles (0.01, 0.5, and 1% DMSO), AITC (30 μ mol/L), and capsaicin (0.1 μ mol/L) to identify nociceptive neurons. In another series of experiments, DRG neurons were incubated with bortezomib (10 or 100 μ mol/L) or its vehicle (0.01 and 0.1% DMSO) for 2 hours and then challenged with AITC (10 or 30 μ mol/L). Results are expressed as the increase of Ratio_{340/380} over the baseline normalized to the maximum effect induced by ionomycin (5 μ mol/L) added at the end of the experiment.

Protein extraction and Western immunoblot assay

Spinal cord, DRGs, and hind paw skin were obtained from mice treated with bortezomib or its vehicle at day 7 post treatment. Tissue samples were homogenized in lysis buffer containing (in mmol/L): 50 Tris, 150 NaCl, 2 EGTA, 100 NaF, 1 Na₃VO₄, 1% Nonidet P40, pH 7.5, and complete protease inhibitor cocktail (Roche). Lysates were centrifuged at 14,000g at 4°C for 45 minutes. Protein concentration in supernatants was determined using DC protein assay (Bio-Rad). Samples with equal amounts of proteins (30 μ g) were then separated by 10% SDS-PAGE electrophoresis, and the resolved proteins were transferred to a polyvinylidene difluoride membrane (Merck Millipore Billerica). Membranes were incubated with 5% dry milk in Tris buffer containing 0.1% Tween 20 (TBST; 20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl) for 1 hour at room temperature, and incubated with rat polyclonal primary antibody for TRPA1 detection (1:200; Novus Biologicals), or mouse monoclonal primary antibody for β -actin (1:6,000; Thermo Scientific), at 4°C overnight. Membranes were then probed with goat anti-mouse or donkey anti-rabbit IgG conjugated with horseradish peroxidase (Bethyl Laboratories Inc.) for 50 minutes at room temperature. Finally, membranes were washed 3 times with TBST, and bound antibodies were detected using chemiluminescence reagents (ECL; Pierce, Thermo Scientific). The density of specific bands was measured using an image-processing program (ImageJ 1.32J, Wayne Rasband) and normalized against a loading control (β -actin).

Carboxy-methyl-lysine adducts measurement in plasma

Briefly, blood samples from C57/BL6 mice, taken 1, 3, 6, and 24 hours after the administration of bortezomib (1 mg/kg, i.p.), oxaliplatin (3 mg/kg, i.p.), or their vehicles (1% DMSO and isotonic saline, NaCl 0.9%, respectively), were centrifuged at 3,500g for 10 minutes, and plasma was used for the carboxy-methyl-lysine (CML) protein adduct ELISA assay. CML protein adducts content in plasma was measured using as ELISA kit (OxiSelect ELISA Kit, Cell Biolabs Inc. Valter Occhiena S.R.L.) according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean \pm SEM. Statistical analysis was carried out by the unpaired 2-tailed Student *t* test for comparisons between 2 groups, the ANOVA, followed by the *post hoc* Bonferroni test for comparisons of multiple groups. *P* value less than 0.05 was considered statistically significant (GraphPadPrism version 5.00). To meet ANOVA assumptions, mechanical

allodynia data were subjected to log transformation before statistical analysis.

Results

Bortezomib administration produces persistent mechanical cold and chemical hypersensitivity mediated by TRPA1

Administration of a single dose (0.2, 0.5, and 1 mg/kg, i.p.) of bortezomib induced a dose-dependent mechanical and cold hypersensitivity in C57BL/6 mice (Fig. 1A and B). Reduced mechanical threshold was observed after bortezomib (1 mg/kg, i.p.) injection as early as 6 hours and lasted until 11 days after treatment (Fig. 1A). Similar results were obtained for cold allodynia, which was evident at day 1 and persisted until day 11 after bortezomib injection (Fig. 1B). Bortezomib administration (1 mg/kg, i.p.) did not affect the heat threshold of mice at any time point, from 6 hours to 14 days after treatment. Nociception time to heat stimulus was 19.7 ± 0.8 seconds and 17.2 ± 1.0 seconds at baseline and 7 days after bortezomib treatment, respectively ($n = 8-10$ mice, $P > 0.05$, Student *t* test).

Next, we investigated whether TRPA1 activation is involved in mechanical and cold hypersensitivity induced by bortezomib. Systemic treatment with the TRPA1 selective antagonist

HC-030031 (300 mg/kg, i.g.; ref. 37) at day 7 after bortezomib treatment completely, but transiently, reverted both mechanical hyperalgesia and cold allodynia. Significant inhibition was observed from 30 to 120 minutes after HC-030031 treatment, with maximum reduction ($98 \pm 12\%$ and $90 \pm 6\%$ for mechanical hyperalgesia and cold allodynia, respectively) 60 minutes post dosing (Fig. 1C and D). Systemic treatment with HC-030031 (300 mg/kg, i.g.) at day 7 after treatment with bortezomib (0.2 or 0.5 mg/kg, i.p.) completely but transiently reverted both mechanical hyperalgesia and cold allodynia (data not shown).

Given that we, as well as others (29, 38), have found that mechanical and cold hypersensitivity evoked by paclitaxel was mediated by both TRPA1- and TRPV4-dependent mechanisms, we tested whether the TRPV4 channel contributes to bortezomib-induced sensory hypersensitivity by using a selective TRPV4 antagonist, HC-067047 (10 mg/kg, i.p.; ref. 39). HC-067047, at a dose able to reduce mechanical hyperalgesia evoked by paclitaxel (29), failed to affect bortezomib-evoked hypersensitivities (data not shown). Therefore, present pharmacologic evidence indicates an exclusive role for TRPA1 in bortezomib-induced mechanical allodynia and cold hypersensitivity in mice, whereas it rules out a contribution by TRPV4.

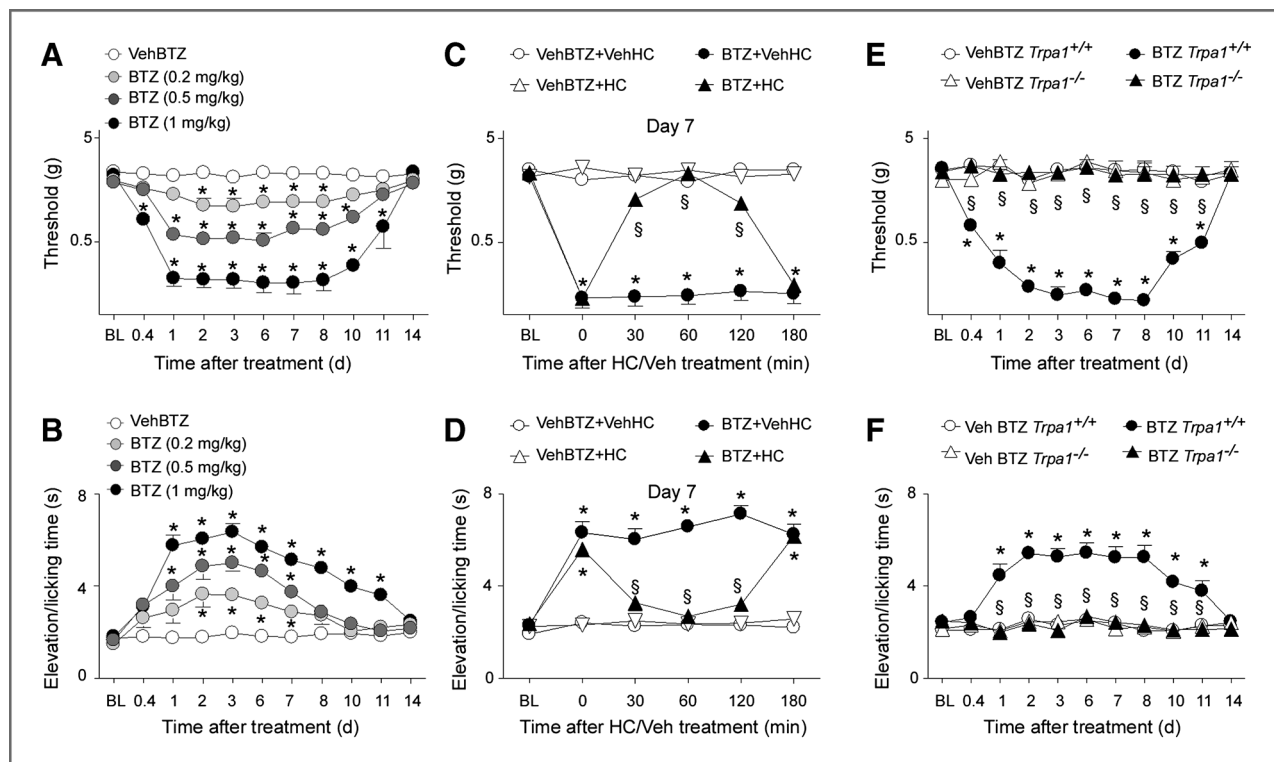


Figure 1. Bortezomib induces mechanical allodynia and cold hypersensitivity via TRPA1 activation in mice. A single dose of bortezomib (BTZ; 0.2, 0.5, and 1 mg/kg i.p.) induces in C57BL/6 mice a dose- and time-dependent mechanical (A) and cold (B) allodynia, which starts at 6 hours or day 1, respectively, and persists until day 11 after BTZ (1 mg/kg) administration. At day 7 after BTZ administration, the selective TRPA1 receptor antagonist, HC-030031 (HC; 300 mg/kg i.g.), completely reverses the mechanical (C) and cold (D) allodynia with a maximum effect, 60 minutes after dosing. BTZ treatment produces in *Trpa1*^{+/+} mice mechanical (E) and cold (F) allodynia similar to those observed in C57BL/6. These effects are completely absent in *Trpa1*^{-/-} mice (E and F). Veh, vehicle of BTZ or HC. Values are mean \pm SEM of 8 to 10 mice. #, $P < 0.05$ versus VehBTZ, Student *t* test in A and B; *, $P < 0.05$ versus VehBTZ-VehHC in C and D and VehBTZ-*Trpa1*^{+/+} in E and F; §, $P < 0.05$ versus BTZ-VehHC in C and D and BTZ-*Trpa1*^{-/-} in E and F; one-way ANOVA and Bonferroni test. BL, baseline withdrawal threshold.

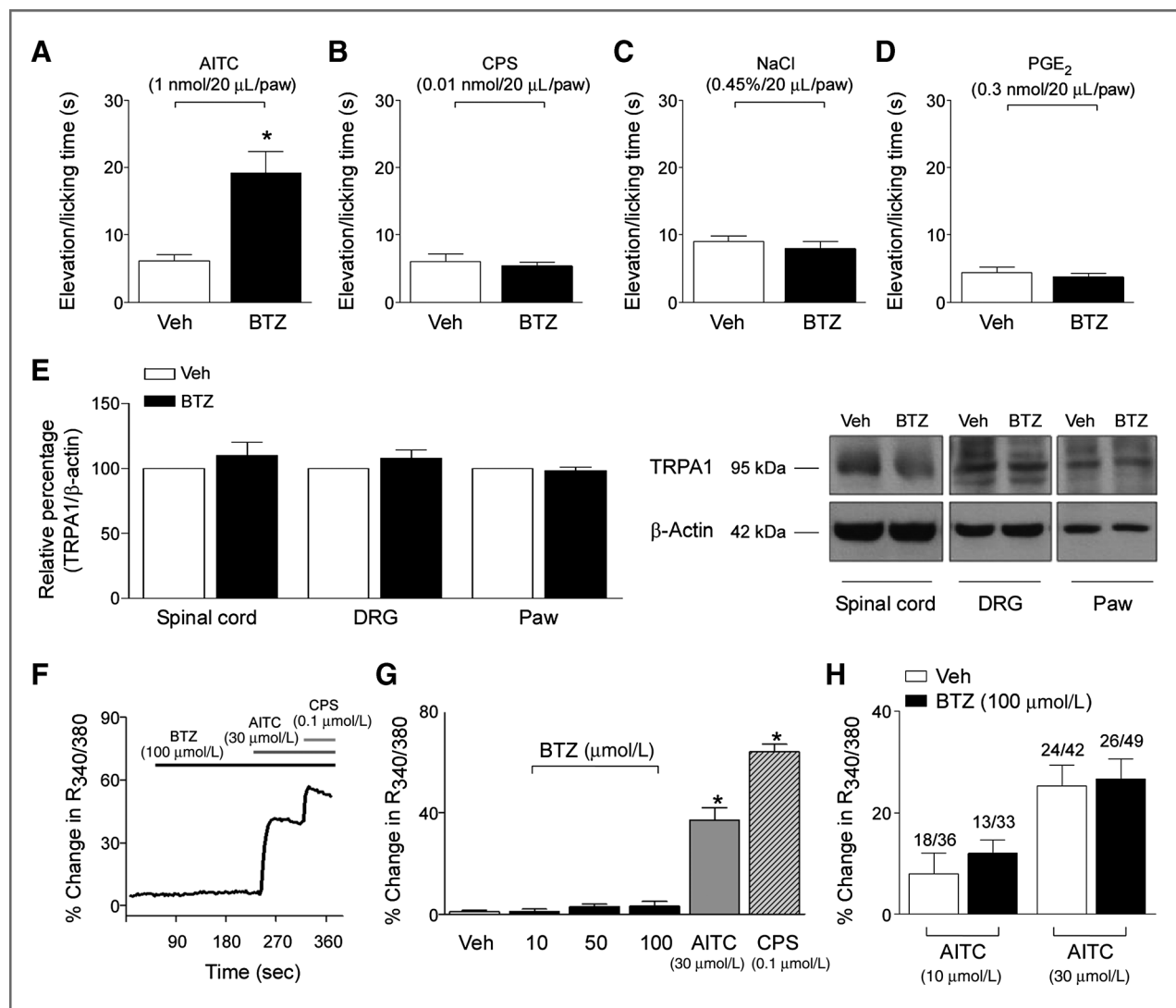


Figure 2. Bortezomib enhances allyl isothiocyanate-evoked nociceptive behavior but does not increase TRPA1 expression or directly activate TRPA1. Nociceptive behavior produced by a sub-threshold dose of intraplantar (i.pl.; 20 μ L) injection of allyl isothiocyanate (AITC; 1 nmol/paw; A) in mice is increased 7 days after BTZ (1 mg/kg i.p.). Responses to subthreshold doses of capsaicin (CPS; 0.01 nmol/paw; B), hypotonic saline (NaCl, 0.45%; C), and PGE₂ (0.3 nmol/paw; D) are not affected by BTZ. Values are mean \pm SEM of 8 to 10 mice. *, $P < 0.05$ versus VehBTZ-AITC in A; Student t test. E, TRPA1 protein content analyzed by Western blotting is not different in tissue homogenates of spinal cord, DRGs, and hind paw skin obtained from mice on day 7 after treatment with BTZ or its vehicle (Veh). Values are mean \pm SEM of 3 samples, Student t test. Equally loaded protein was checked by expression of β -actin. A representative blot is shown. BTZ (10, 50, or 100 μ mol/L) fails to evoke any visible intracellular calcium (Ca^{2+}) response in CPS (0.1 μ mol/L)-sensitive DRG neurons, which otherwise responded to AITC (F, G). Trace represents an average of 10 neurons. H, *in vitro* preexposure to BTZ (100 μ mol/L for 2 hours) does not affect the magnitude of the response, and the number of neurons responding to AITC (10 and 30 μ mol/L). Veh is the vehicle of BTZ. Values are mean \pm SEM of $n > 30$ neurons. Numbers indicate AITC-responding cells/CPS-responding cells. *, $P < 0.05$ versus Veh; one-way ANOVA and Bonferroni test.

More importantly, we found that bortezomib treatment (1 mg/kg, i.p.) produced mechanical hyperalgesia and cold allodynia in *Trpa1*^{+/+} mice with an identical time course to that observed in C57BL/6 mice, an effect that was completely absent in *Trpa1*^{-/-} mice (Fig. 1E and F).

In addition, we wondered whether bortezomib could cause selective chemical hypersensitivity to TRPA1 agonists. The study of the effects produced by sub-threshold doses of AITC (TRPA1 agonist), capsaicin (TRPV1 agonist), PGE₂ (EP₁₋₄ receptor agonist), or hypotonic saline (which can stimulate TRPV4) showed that bortezomib treatment selec-

tively increased the nociceptive behavior evoked by AITC (Fig. 2A). In fact, responses to capsaicin, PGE₂, and hypotonic saline were similar in both vehicle- and bortezomib-treated animals (Fig. 2B–D). As expected, in TRPA1-deficient mice treated with bortezomib or its vehicle, AITC failed to evoke any nociceptive response (data not shown).

Bortezomib does not affect TRPA1 receptor expression and does not directly activate TRPA1

TRPA1 expression has been found to vary in different painful conditions, including models of CIPN (9, 40). Therefore, we

evaluated, by Western blotting, the expression of TRPA1 receptor in different tissues. At day 7 after administration, when hypersensitivity was at its maximum, TRPA1 immunoreactivity in the spinal cord, DRG, and hind paw skin of mice treated with bortezomib or its vehicle, were similar (Fig. 2E).

To test the hypothesis that bortezomib directly activates the TRPA1 receptor, we studied the ability of bortezomib to evoke calcium responses in cultured mouse DRG neurons. Bortezomib (10, 50, or 100 $\mu\text{mol/L}$) failed to evoke any calcium response in capsaicin-sensitive DRG neurons (Fig. 2F and G), which otherwise responded to the TRPA1 agonist AITC (30 $\mu\text{mol/L}$). *In vitro* pre-exposure to bortezomib (100 $\mu\text{mol/L}$ for 2 hours) did not affect the magnitude or the number of neurons responding to AITC (10 and 30 $\mu\text{mol/L}$; Fig. 2H).

α -Lipoic acid transiently reverts bortezomib-evoked hypersensitivity

As reported for other anticancer drugs, such as oxaliplatin, paclitaxel, and others, there is evidence that bortezomib also produces oxidative stress (20, 21, 25, 41, 42). Therefore, we hypothesized that reactive molecules generated by the oxidative stress burst produced by bortezomib administration could be the underlying mechanism by which the anticancer drug induces TRPA1-dependent mechanical and cold hypersensitivity.

We observed that administration of α -lipoic acid (100 mg/kg, i.g.) completely abated mechanical hyperalgesia and cold allodynia evoked at day 7 after bortezomib treatment. Significant effect of α -lipoic acid was observed from 30 to 120 minutes after treatment, with maximum inhibition ($73 \pm 9\%$ and $77 \pm 6\%$ for mechanical hyperalgesia and cold allodynia, respectively) 60 minutes post dosing (Fig. 3A and B).

Local treatment with HC-030031 or α -lipoic acid transiently reverts bortezomib- or oxaliplatin-induced hypersensitivity

It has been reported that i.pl. injection of α -lipoic acid reduces oxaliplatin-elicited nociception (3). In the present study, we observed that i.pl. injection of HC-030031 (100 $\mu\text{g/paw}$) or α -lipoic acid (10 $\mu\text{g/paw}$) completely reduced bortezomib-induced mechanical and cold allodynia (Fig. 3C and D). In addition, we found that mechanical and cold allodynia elicited by oxaliplatin were markedly decreased by i.pl. injection of HC-030031 and α -lipoic acid (Fig. 3E and F). Contralateral paw thresholds to mechanical or cold stimuli were not affected by the i.pl. injection of HC-030031 or α -lipoic acid (Fig. 3C–F). Administration of HC-030031 or α -lipoic acid (i.pl.) did not produce any appreciable effect in animals treated with the vehicle of bortezomib or oxaliplatin (data not shown).

Bortezomib and oxaliplatin increase plasma level of carboxy-methyl-lysine

Systemic oxidative stress was evaluated by measuring the serum content of N ϵ -carboxy-methyl-lysine (CML) protein adducts. CML is the reaction product between lysine and glyoxal, an α -ketoaldehyde intermediate formed by ascorbate autoxidation, lipid peroxidation, and oxidative degradation of glucose and degradation of glycated proteins. Due to the fact

that CML is formed from either carbohydrates or lipids oxidation, it has been termed as an either advanced glycation or lipoxidation endproducts (EAGLE) modification. CML may be considered as a general marker of oxidative stress and, so far, it is widely used to measure oxidative stress in different pathophysiologic conditions (43). Bortezomib administration produced a transient increase in plasma CML levels. One hour after bortezomib injection, CML increased by 64% over baseline value, and returned to basal values at 3 hours after treatment (Fig. 4A). Similar to bortezomib, oxaliplatin administration produced a transient increase in plasma CML levels, which was observed at 1 hour (55% over the baseline) and 3 hours (63% over the baseline), and returned to basal levels 6 hours after treatment (Fig. 5A).

Early and short-term treatment with HC-030031 or α -lipoic acid completely prevents bortezomib- and oxaliplatin-evoked hypersensitivity

We investigated whether treatment with a TRPA1 antagonist or a ROS scavenger given shortly before and after anticancer drug administration could prevent the development of persistent mechanical, cold, and chemical hypersensitivity. To test this hypothesis HC-030031 (300 mg/kg, i.g.) or α -lipoic acid (100 mg/kg, i.g.), were given respectively, 15 minutes before and 3 times every 90 minutes after bortezomib or oxaliplatin administration. HC-030031 totally prevented the development of chemical hypersensitivity and mechanical and cold allodynia evoked by bortezomib (Fig. 4B, D, and E) and oxaliplatin (Fig. 5B, D, and E). Similarly, α -lipoic acid prevented chemical hypersensitivity and mechanical and cold allodynia evoked by bortezomib (Fig. 4C, F, and G) and oxaliplatin (Fig. 5C, F, and G). Repeated i.g. administration of TRPA1 antagonist (HC-030031, 300 mg/kg, i.g.) did not affect forced locomotion of animals, as observed by the rotarod test. HC-030031- and vehicle-treated animals did not show any fall during the test (data not shown).

Mice, protected by early and short-term treatment with HC-030031, were rechallenged 6 days after a first treatment with bortezomib with a second bortezomib administration (1 mg/kg, i.p.). In these mice, a second early and short-term treatment with HC-030031 again totally prevented the development of mechanical and cold hypersensitivity (Fig. 6B and C). In contrast, mice treated with HC-030031 vehicle did not show protection against the hypersensitivity evoked by the second administration of bortezomib. Mice treated with bortezomib and HC-030031 vehicle developed mechanical and cold hypersensitivity, a response that further increased at the second treatment with bortezomib and HC-030031 vehicle (Fig. 6B and C).

Discussion

In the present study in mice, we found that 1 single administration of bortezomib produced an early and prolonged mechanical and cold hypersensitivity that started 6 hours after and lasted for 11 days after bortezomib administration. With a slight difference in duration, the effect of bortezomib was practically identical to that previously reported for oxaliplatin (6). A number of preclinical studies and clinical investigations

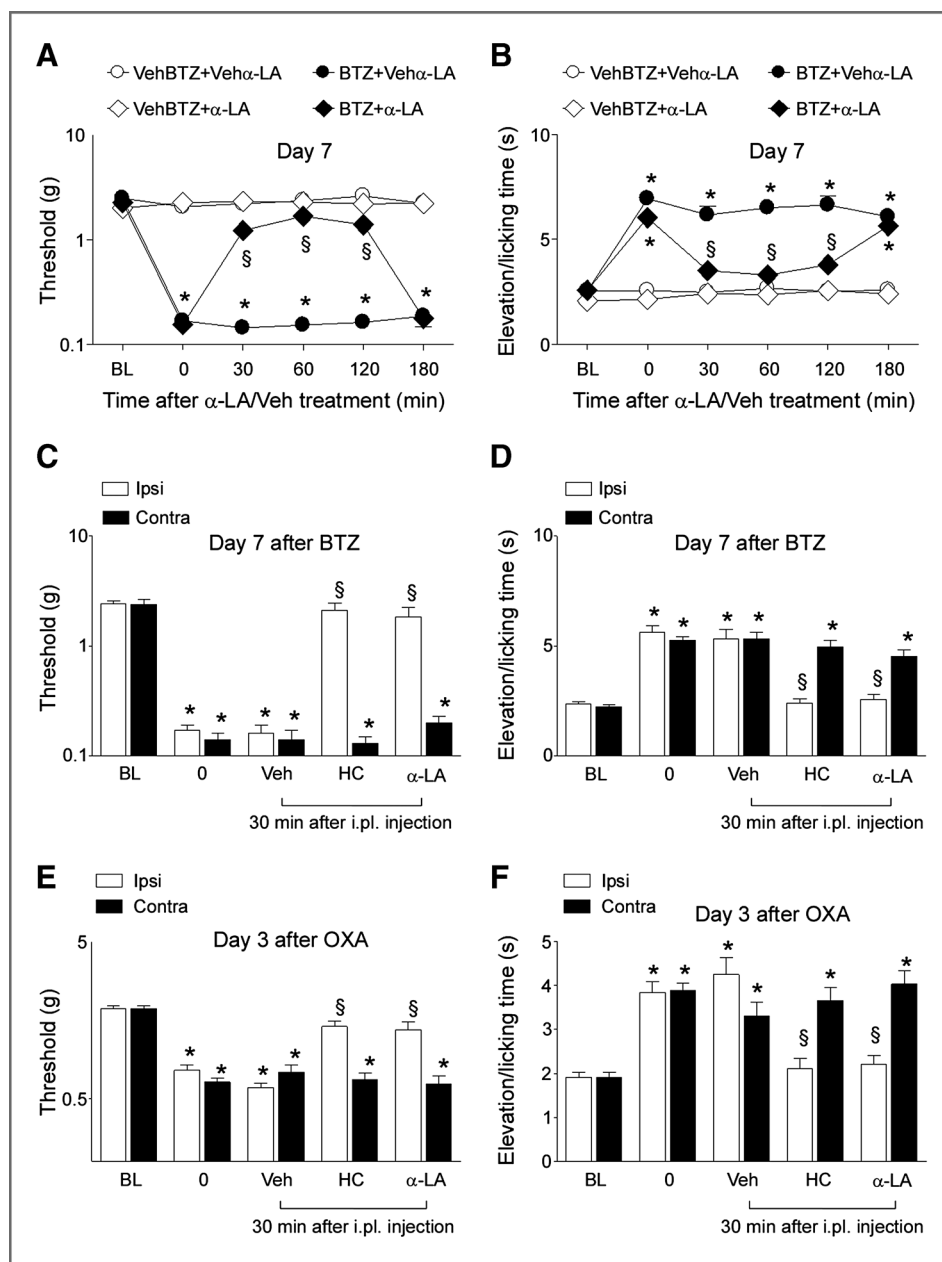


Figure 3. Systemic or local administration of α -lipoic acid (α -LA) and local administration of HC-030031 (HC) transiently reverse BTZ-evoked mechanical and cold hypersensitivity in mice. At day 7 after BTZ (1 mg/kg i.p.), α -LA (100 mg/kg i.g.) completely reverses the mechanical (A) and cold (B) allodynia with a maximum effect at 60 minutes post dosing. Veh, vehicle of BTZ or α -LA acid. C and E, HC (100 μ g/paw, i.p. 20 μ L) or α -LA (10 μ g/paw) reduce the mechanical allodynia induced by BTZ or oxaliplatin (OXA; 3 mg/kg, i.p.) in the paw ipsilateral (ipsi) to the injection. D and F, in the contralateral (contra) side, the paw threshold to mechanical stimuli is not affected by local HC or α -LA. Local HC or α -LA acid treatment produces similar findings when cold allodynia is measured. Values are mean \pm SEM of 8 to 10 mice. *, $P < 0.05$ versus Veh α -LA acid or VehBTZ or BL values; §, $P < 0.05$ versus BTZ-Veh α -LA or Vehipsi; one-way ANOVA and Bonferroni test. BL, baseline withdrawal threshold.

have shown that bortezomib, like oxaliplatin and paclitaxel, increases ROS and their by-products in plasma, cells, and tissues of treated animals or patients, and that ROS scavengers show some degree of protection against CIPN or its rodent counterpart (3, 7–10, 44). Two observations suggest that oxidative stress mediates bortezomib-evoked sensory neuropathy. First, the ROS scavenger, α -lipoic acid, completely reversed the established (at day 7 after drug administration) mechanical and cold hypersensitivity evoked by bortezomib. Second, bortezomib and oxaliplatin produced an early and transient (1–3 hours after drug administration) increase in the plasma levels of 1 major by-product of oxidative stress, CML. The finding that oxaliplatin administration also increased plasma oxidative stress by-products is consistent with the

previously reported role of oxidative stress in oxaliplatin-evoked sensory neuropathy (6).

TRPA1 has been identified as a sensor of oxidative stress, in as much as it is activated by an unprecedented series of ROS, RNS, or RCS (16, 45, 46). Thus, we hypothesized that oxidative stress by-products, generated by bortezomib, may target the TRPA1 channel in sensory nerve terminals. Indeed, both pharmacologic and genetic findings indicate that TRPA1 plays a key role in bortezomib-evoked mechanical and chemical hyperalgesia and cold allodynia, as these phenomena were completely reverted when they were at their maximum, for example, at day 7 after treatment, by a TRPA1 antagonist and were completely absent in TRPA1-deleted mice. The key contribution of TRPA1 in mechanical, chemical, and cold

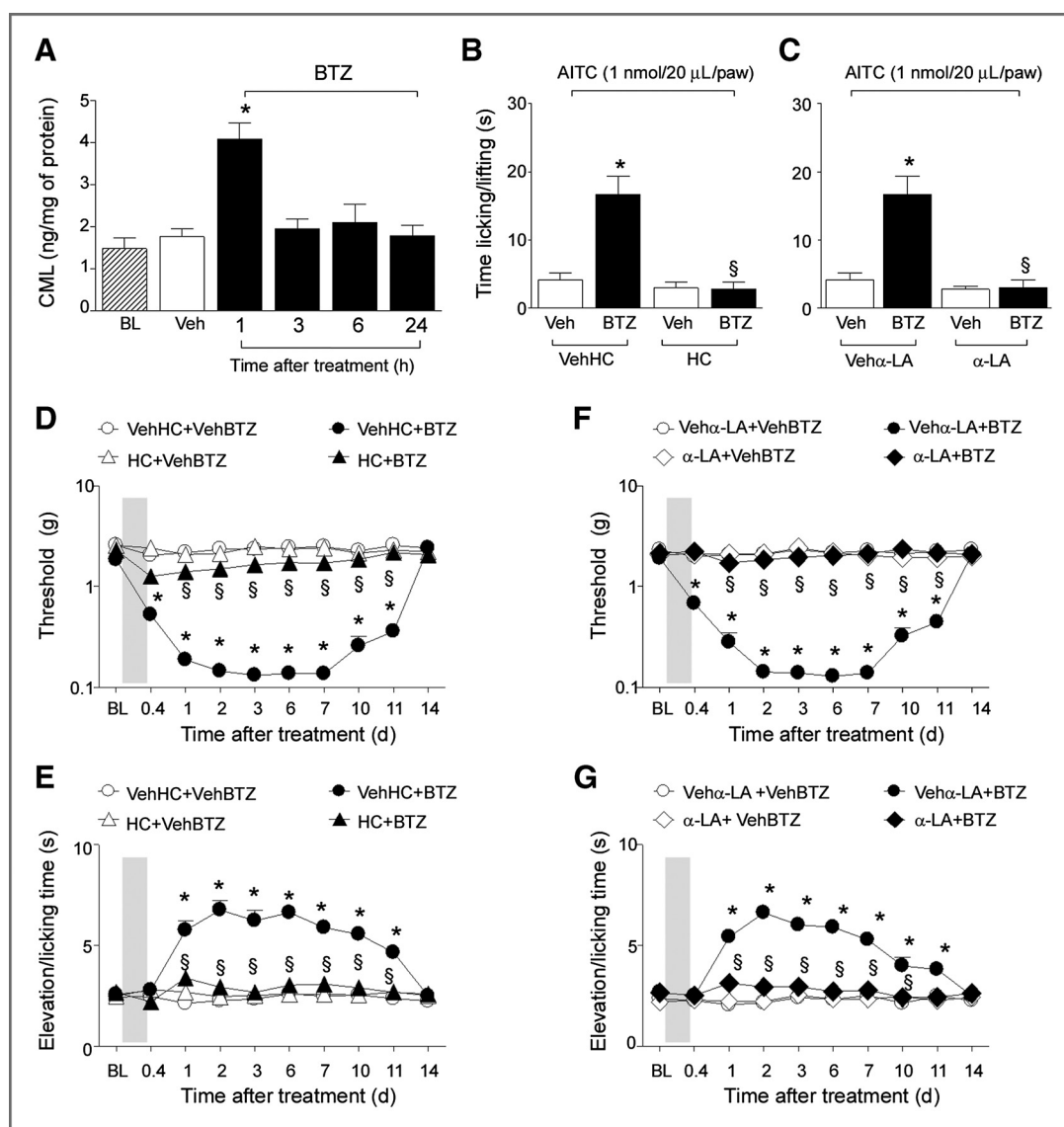


Figure 4. BTZ increases transiently oxidative stress in plasma and early and short-term treatment with HC and α -LA permanently prevents the development of mechanical, cold, and chemical hypersensitivity evoked by BTZ in mice. **A**, BTZ (1 mg/kg i.p.) transiently increases carboxy-methyl-lysine (CML) plasma levels in mice. Both HC (300 mg/kg i.g.) and α -LA (100 mg/kg i.g., 15 minutes before and 3 times at 90-minute intervals each after BTZ treatment) prevent the development and maintenance of chemical hyperalgesia (**B** and **C**) as well as mechanical (**D** and **F**) and cold (**E** and **G**) allodynia evoked by BTZ (1 mg/kg i.p.). Veh, of BTZ, HC, or α -LA. Values are mean \pm SEM of 8 to 10 mice. *, $P < 0.05$ versus BL in **A**, VehHC-VehBTZ in **B**, **D**, and **E**, or Veh α -LA-VehBTZ in **C**, **F**, and **G**; §, $P < 0.05$ versus VehHC-BTZ in **B**, **D**, and **E** or Veh α -LA-BTZ- in **C**, **F**, and **G**; one-way ANOVA and Bonferroni test. BL, basal level of CML in **A** and baseline withdrawal threshold in **D**–**G**.

allodynia does not seem confined to bortezomib model as earlier studies (5, 6, 24) showed a similar role of TRPA1 in oxaliplatin-evoked sensory neuropathy in mice. In addition, the TRPV4-resistant component (38) of the mechanical hyperalgesia evoked by paclitaxel in mice has also been ascribed to the contribution of TRPA1, whereas TRPA1 appears to be the sole channel responsible for paclitaxel-evoked cold allodynia (29). In addition, we found that an oxidative stress scavenger or a TRPA1 antagonist reversed bortezomib- or oxaliplatin-evoked hypersensitivity selectively on the treated paw, when they were given locally by i.pl. administration. This finding indicates that TRPA1 sensitization/activation occurs at the

very terminal region of nociceptive primary afferents, and that channel inhibition at this peripheral level is sufficient to revert the sensory neuropathy.

The protective effect of HC-030031 or α -lipoic acid when administered (either systemically or locally) at day 7 after bortezomib administration, although complete, was transient, lasting no longer than 120 minutes. This is probably due to the pharmacokinetic properties of the 2 drugs, as indicated by previous studies in different models of nociception or hyperalgesia/allodynia (47, 48). In contrast to the transient reversal produced by pharmacologic treatments when the hypersensitivity is already established, in TRPA1-deficient

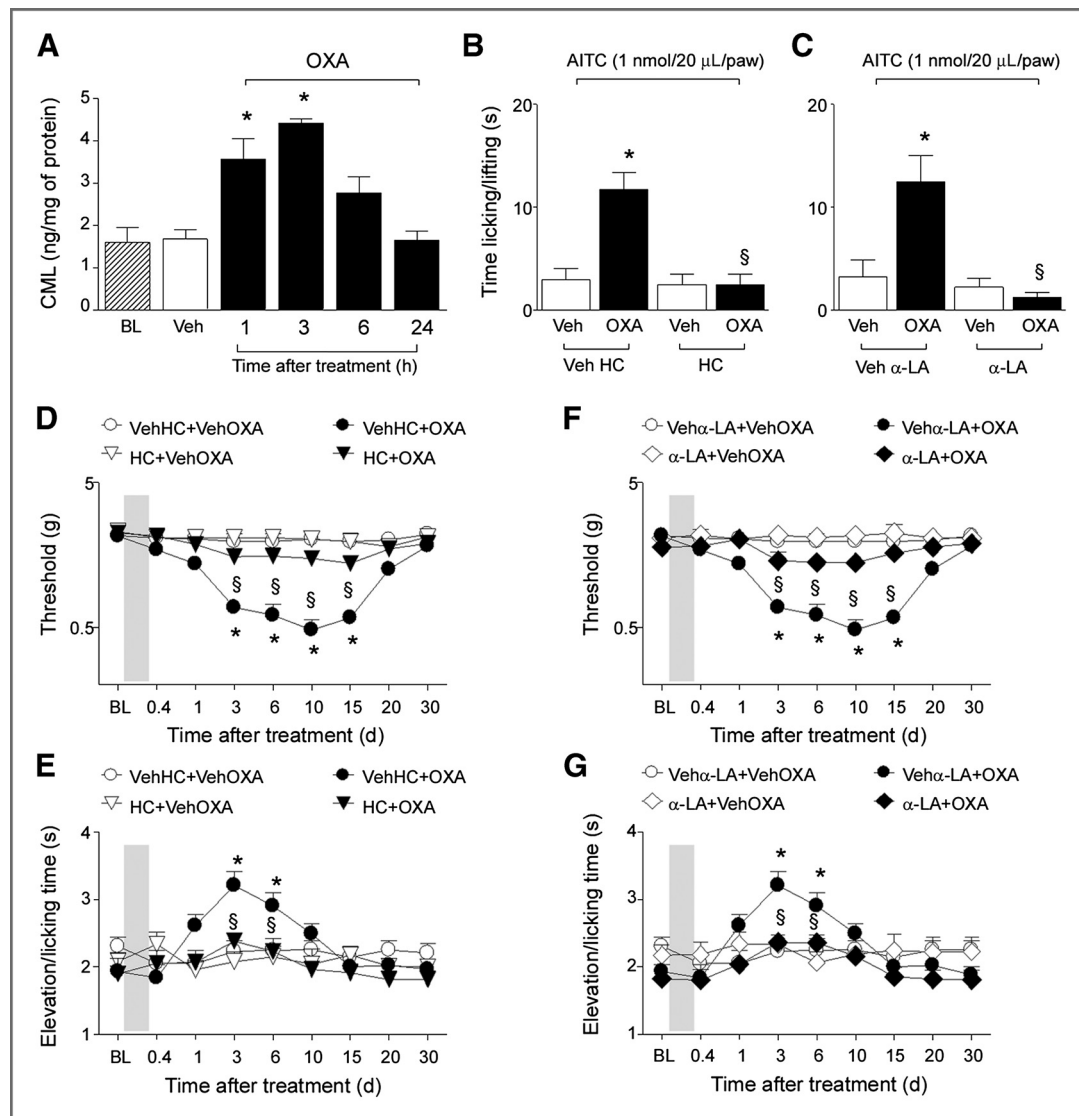


Figure 5. Oxaliplatin transiently increases oxidative stress in plasma, and early and short-term treatment with HC and α -LA permanently prevents the development of mechanical, cold, and chemical hypersensitivity evoked by BTZ in mice. **A**, oxaliplatin (OXA; 3 mg/kg i.p.) transiently increases CML plasma levels in mice. Both HC (300 mg/kg i.g.) and α -LA (100 mg/kg i.g., 15 minutes before and 3 times at 90-minute intervals each after BTZ treatment) prevent the development and maintenance of chemical hyperalgesia (**B** and **C**) as well as mechanical (**D** and **F**) and cold (**E** and **G**) allodynia evoked by OXA (3 mg/kg i.p.). Veh, vehicle of OXA, HC, or α -LA. Values are mean \pm SEM of 8 to 10 mice. *, $P < 0.05$ versus BL in **A**, VehHC-VehOXA in **B**, **D**, and **E** or Veh α -LA-VehOXA in **C**, **F**, and **G**; §, $P < 0.05$ versus VehHC-OXA in **B**, **D**, and **E** or Veh α -LA-OXA in **C**, **F**, and **G**; one-way ANOVA and Bonferroni test. BL, basal level of CML in **A** and baseline withdrawal threshold in **D**–**G**.

mice hypersensitivity to bortezomib or oxaliplatin (6) does not develop. These genetic findings and biochemical evidence of the transient increase in plasma CML suggest that early phenotypic changes of TRPA1, presumably associated with the oxidative burst, which are responsible for the development and maintenance of the hypersensitivity, occur a few hours after chemotherapeutic drug administration. To identify the critical role of these early events for the manifestation of the enduring hypersensitivity condition by anticancer drug, we designed an experiment in which HC-030031 or α -lipoic acid were given shortly before and for approximately 6 hours after bortezomib or oxaliplatin treat-

ment. These treatments not only blocked the onset of the hypersensitivity, but, rather surprisingly, completely and stably prevented its development and maintenance. Of interest for translating the present observation to the clinical perspective, the permanent protective effect by early and short-term treatment with the TRPA1 antagonist was also observed when it was repeated after a second bortezomib administration. Although it is not possible to replicate in mice the exact condition experienced by patients, these additional findings suggest a possible treatment schedule to prevent the sensory neuropathy in patients when TRPA1 antagonists are clinically available.

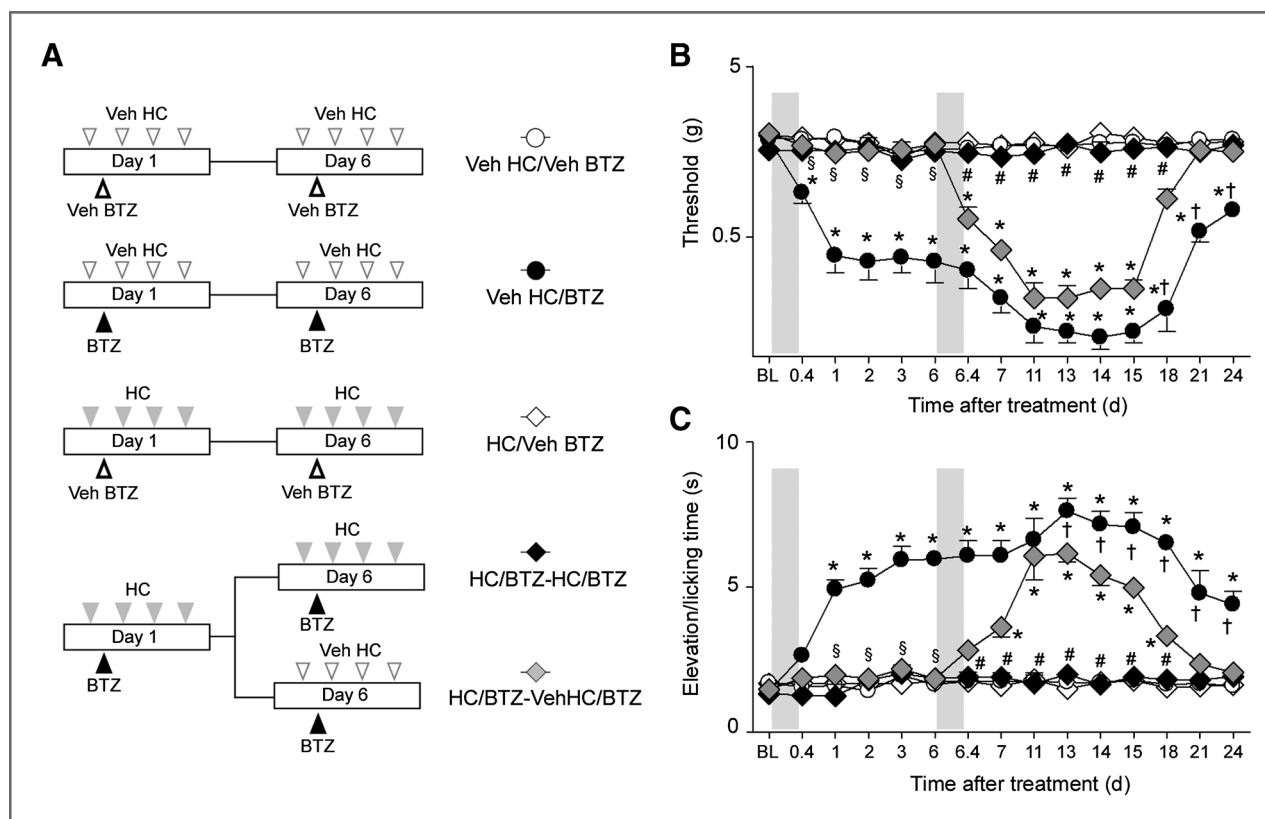


Figure 6. A repeated early and short-term treatment with HC prevents the development of mechanical and cold hypersensitivity evoked by a second BTZ treatment in mice. **A**, schematic representation of HC (300 mg/kg, i.g.) and BTZ (1 mg/kg, i.p.) treatment. A group of mice are treated with HC or its vehicle 15 minutes before and shortly after (3 times at ~90-minute intervals each) a first BTZ or vehicle administration. At day 6 after the first BTZ administration, all mice receive a second BTZ (1 mg/kg, i.p.) or vehicle administration. Mice pretreated with HC after the first BTZ dose are subdivided into 2 groups. One group is treated a second time with HC (300 mg/kg, i.g.) and a second group with its vehicle 15 minutes before and shortly after (3 times at ~90-minute intervals each) BTZ administration. The second early and short-term treatment with HC totally prevents the development of mechanical and cold hypersensitivity (**B**, **C**). **B** and **C**, mice treated with BTZ and HC vehicle develop mechanical and cold hypersensitivity, a response that is further increased by the second treatment with BTZ and HC vehicle. Values are mean \pm SEM of 8 to 10 mice. *, $P < 0.05$ versus VehHC-VehBTZ in **B** and **C**; §, $P < 0.05$ versus VehHC-BTZ in **B** and **C**; #, $P < 0.05$ versus VehHC-BTZ or HC/BTZ-VehHC/BTZ; †, $P < 0.05$ versus HC/BTZ-VehHC/BTZ one-way ANOVA and Bonferroni test. BL, baseline withdrawal threshold.

Taken together, these findings indicate that TRPA1, via its activation by oxidative stress by-products, is necessary and sufficient to produce a sensory neuropathy paradigm in mice following a single administration of different chemotherapeutics. Oxaliplatin (6), paclitaxel (29), and bortezomib failed to evoke any calcium response in cultured TRPA1-expressing neurons, thus excluding that these drugs may directly target the channel. However, *in vitro* findings support the alternative explanation, as indicated by *in vivo* results, that chemotherapeutic agents act indirectly by generating oxidative stress by-products (3, 8, 9, 23), which in turn sensitize/activate TRPA1 in sensory neurons.

TRPA1 is apparently required for those early (within 6–8 hours) phenotypic changes that eventually result in the long-term hypersensitivity to specific (AITC) and nonspecific (pressure or cold) stimuli caused by exposure to different chemotherapeutic agents in mice. Although some reports have shown changes in TRP expression in different rodent models of CIPN, under the present experimental circumstances, no change in TRPA1 protein expression in nocicep-

tive neurons was found. The molecular mechanism responsible for the TRPA1-mediated hypersensitivity phenotype, produced by chemotherapeutic agents remains unknown. Nevertheless, present experiments with bortezomib and oxaliplatin identify the early phase (a few hours) that follows chemotherapeutic drug administration as the key step when, most likely through oxidative stress by-products, TRPA1 is activated/sensitized. These early events result in a prolonged (several days) condition of hypersensitivity that markedly mimics the long-lasting duration of CIPN in patients treated with bortezomib or oxaliplatin. If ROS scavengers, most likely because of poor pharmacokinetics, could not represent a suitable and effective treatment for CIPN, the present findings suggest a novel therapeutic schedule to prevent CIPN in patients, based on TRPA1 antagonists given before and shortly after each administration of anticancer medicines.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Trevisan, S. Materazzi, C. Fusi, A. Altomare, R. Nassini
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Trevisan, S. Materazzi, C. Fusi, G. Aldini, R. Nassini
Writing, review, and/or revision of the manuscript: G. Trevisan, S. Materazzi, M. Lodovici, R. Patacchini, P. Geppetti, R. Nassini
Study supervision: G. Trevisan, P. Geppetti, R. Nassini

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