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Dacarbazine alone or associated with melanoma-bearing cancer pain model induces painful hypersensitivity by TRPA1 activation in mice

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Antineoplastic therapy has been associated with pain syndrome development characterized by acute and chronic pain. The chemotherapeutic agent dacarbazine, used mainly to treat metastatic melanoma, is reported to cause painful symptoms, compromising patient quality of life. Evidence has proposed that transient receptor potential ankyrin 1 (TRPA1) plays a critical role in chemotherapy-induced pain syndrome. Here, we investigated whether dacarbazine causes painful hypersensitivity in naive or melanoma-bearing mice and the involvement of TRPA1 in these models. Mouse dorsal root ganglion (DRG) neurons and human TRPA1-transfected HEK293 (hTRPA1-HEK293) cells were used to evaluate the TRPA1-mediated calcium response evoked by dacarbazine. Mechanical and cold allodynia were evaluated after acute or repeated dacarbazine administration in naive mice or after inoculation of B16-F10 melanoma cells in C57BL/6 mice. TRPA1 involvement was investigated by using pharmacological and genetic tools (selective antagonist or antisense oligonucleotide treatment and *Trpa1* knockout mice). Dacarbazine directly activated TRPA1 in hTRPA1-HEK293 cells and mouse DRG neurons and appears to sensitize TRPA1 indirectly by generating oxidative stress products. Moreover, dacarbazine caused mechanical and cold allodynia in naive but

Additional Supporting Information may be found in the online version of this article.
Key words: chemotherapy, nociception, paclitaxel, allodynia, melanoma, HC-030031

Abbreviations: AITC: allyl isothiocyanate; ANOVA: analysis of variance; ARRIVE: Animal Research: Reporting *in vivo* Experiments; AS ODN: antisense oligonucleotide; CPS: capsaicin; DMSO: dimethyl sulfoxide; DRG: dorsal root ganglion; DTIC: dacarbazine; EC_{50}: effective concentration 50%; FBS: fetal bovine serum; HBSS: Hank's balanced salt solution; HEK: human embryonic kidney; hPAR2: human proteinase-activated receptor 2; hPAR2-AP: activating peptide for human proteinase-activated receptor 2; HRPO: phenol red-horseradish peroxidase; KRP: Krebs-Ringer phosphate; MM ODN: mismatch oligonucleotide; MTT: 3(4-5-dimethyl)-2-5-diphenyl tetrazolium bromide; NAC: N-acetylcysteine; NGF: neuron growth factor; PAC: paclitaxel; PBN: phenyl N-tert-butyl nitrone; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; ROS: reactive oxygen species; SEM: standard error of the mean; TG: thapsigargin; TRP: transient receptor potential; TRPA1: transient receptor potential ankyrin 1; TRPV1: transient receptor potential vanilloid 1; UFSM: Federal University of Santa Maria; UNIFI: University of Florence

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not *Trpa1* knockout mice. Also, dacarbazine-induced nociception was reduced by the pharmacological TRPA1 blockade (antagonism), antioxidants, and by ablation of TRPA1 expression. TRPA1 pharmacological blockade also reduced dacarbazine-induced nociception in a tumor-associated pain model. Thus, dacarbazine causes nociception by TRPA1 activation, indicating that this receptor may represent a pharmacological target for treating chemotherapy-induced pain syndrome in cancer patients submitted to antineoplastic treatment with dacarbazine.

What's new?

Acute and chronic pain syndromes, such as chemotherapy-induced peripheral neuropathy, are extremely common among cancer patients, and can limit the use of chemotherapy. Several studies have indicated that the nerve receptor TRPA1 may be involved in this kind of pain. In this study, the authors used antisense, knockout, and pharmacological methods to conclude that the antineoplastic drug dacarbazine does induce pain signals *via* TRPA1 activation. This receptor may thus represent a valuable therapeutic target for treating chemotherapy-induced pain.

Introduction

Dacarbazine is an antineoplastic drug used to treat various types of cancers, such as Hodgkin's lymphoma, soft-tissue sarcomas, and some carcinomas, and it is the main chemotherapeutic agent used in the treatment of metastatic melanomas.[1,2] Due to its high metastatic potential, melanoma is the most aggressive and deadly form of skin cancer affecting melanocytes[1,3,4] and is among the most prevalent types of cancer, the incidence of which continues to increase.[5] Although pain is not a significant symptom of primary melanoma, metastatic melanoma patients frequently develop excruciating pain.[6,7]

In addition to cancer pain, some chemotherapeutic agents used in the treatment of melanoma have been associated with the development of pain.[8,9] It is known that products of dacarbazine photodegradation cause local venous pain when injected intravenously, as well as inducing painful symptoms after intraperitoneal administration in mice.[10] Moreover, pain, along with other symptoms, such as fatigue, nausea, dyspnea and insomnia, are reported by patients as the primary adverse effects associated with treatment with dacarbazine.[3]

It is already well established that the use of some chemotherapeutic agents can cause a pain syndrome characterized by acute and chronic pain.[11–13] Chemotherapy-induced chronic pain is prevalent in 50–70% of patients who are subjected to treatment with antineoplastic drugs.[14] Its main symptoms include paresthesia, mechanical and cold allodynia (pain caused by a normally non-painful stimulus).[9,15] Chemotherapy-induced pain syndrome is the major limitation of antineoplastic treatment, because it may require dose modification or even discontinuation, compromising the survival and life quality of oncological patients.[8] In this context, a preclinical investigation on the pathogenesis of chemotherapy-induced pain syndrome is necessary to identify targets for its pharmacological intervention.[8]

Mechanisms have been proposed to explain this syndrome, including an altered function of different receptors and ion channels, such as transient potential receptor ankyrin 1 (TRPA1).[16,17] TRPA1 is a nonselective cation channel expressed at the nerve endings and along the axons of nociceptors, where it functions as a sensor to noxious stimuli. TRPA1 is activated by natural pungent products (e.g., allyl isothiocyanate, allicin and cinnamaldehyde), pollutants (e.g., acrolein and formalin) and endogenous activators (e.g., 4-hydroxynonenal and hydrogen peroxide), leading to acute pain. TRPA1 mediates mechanical and cold allodynia after inflammatory conditions, cancer or nerve injury, contributing to the development of pathological pain.[16,18–20] Furthermore, oxidative factors also contribute to the pain syndrome evoked by chemotherapeutic agents, which may occur by oxidative stress–dependent TRPA1 activation.[13,21,22]

Because TRPA1 is involved in the pathogenesis of chemotherapy-induced pain syndrome,[13,21,22] we investigated whether dacarbazine causes cold and mechanical hypersensitivity after acute or repeated administration in naive or in melanoma-bearing mice and the involvement of TRPA1 in these models.

Materials and Methods

Animals

The experiments were conducted using adult male and female wild-type C57BL/6 mice (male, 20–25 g, 5–7 weeks), littermate wild-type (*Trpa1*$^{+/+}$), and TRPA1-deficient (*Trpa1*$^{-/-}$; B6129P-Trpa1tm1Kykw/J) mice (25–30 g, 6–8 weeks) backcrossed with C57BL/6 mice (*Trpa1*$^{-/-}$) for at least 10 generations.[20] The animals were maintained in a temperature-controlled room (22 ± 1°C) under a 12-hr light/dark cycle and with free access to food and water. All experiments were carried out between 08:00 a.m. and 5:00 p.m., and the animals were acclimatized to the laboratory room for at least 1 hr before the experiments. Experiments were performed blind to the genotype of the animals and the drug administration. The protocols employed in our study were approved by the Institutional Committee for Animal Care and Use of the Federal University of Santa Maria (UFSM; protocol #7658240417/2017) and the University of Florence (UNIFI; protocol #579/2017-PR) and

followed the Animal Research: Reporting *in vivo* Experiments (ARRIVE) guidelines.[23] The number of animals and intensity of the noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the treatments, in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals.[24]

Reagents and drugs

The description of all reagents is given in the Supporting Information. The doses of the drugs used in our study were based on previous studies.[12,13,22,25]

Cellular recordings

Cell culture. Naive untransfected HEK293 cells (RRID: CVCL_0045; American Type Culture Collection, Manassas, VA, USA; ATCC® CRL-1573™) were cultured according to the manufacturer's instructions and transfected with cDNA for human TRPA1 (hTRPA1-HEK293) as previously described.[26] Both naive HEK293 and transfected hTRPA1-HEK293 cells were authenticated using short tandem repeat profiling within the last three years. All the experiments were performed with mycoplasma-free cells. For more information, please see the Supporting Information.

Isolation of primary sensory neurons. Primary dorsal root ganglion (DRG) neurons were isolated from C57BL/6 mice and *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice and cultured as previously described.[21] Detailed information are described in the Supporting Information.

Cellular recording procedure. Cells on coated coverslips were loaded with 5 μM Fura-2 AM-ester added directly in a buffer solution. Changes in intracellular calcium, $[Ca^{2+}]_i$, were monitored by sequential dual excitation, 340 and 380 nm (emission 510 nm), and recorded with a dynamic image analysis system (XCellence Imaging software; Olympus Srl, Milan, Italy).[21] Detailed information are described in the Supporting Information.

HEK293 naive, hTRPA1-HEK293 cells and DRGs neurons were challenged with dacarbazine (0.01–300 μM) or allyl isothiocyanate (AITC, 10 or 30 μM - TRPA1 agonist). Capsaicin (1 μM—TRPV1 agonist) was used to induce a TRPV1-selective response to identify capsaicin-sensitive neurons. The activating peptide for human proteinase-activated receptor 2 (hPAR2-AP; 100 μM) was used to elicit a TRP-independent cellular response in HEK293 naive and hTRPA1-HEK293 cells. KCl (40 mM) was used to identify neurons. Some experiments were performed in the presence of the TRPA1 selective antagonist, HC-030031 (30 or 50 μM), or its vehicle (0.3 or 0.5% DMSO) and the free radical scavenger, phenyl N-tert-butyl nitrone (PBN, 1 mM) or its vehicle (buffer solution). HC-030031 and PBN were applied for the duration of the experiment after a 15-min preincubation. Other experiments were performed in a calcium-free buffer solution that contained no added $CaCl_2$ and 1.5 mM EGTA, or after exposure to thapsigargin (1 μM) diluted in calcium-free buffer solution, to induce intracellular calcium store depletion. Ionomycin was dissolved in DMSO and diluted with buffer solution or with calcium-free buffer solution added with 5 mM $CaCl_2$ before application.

Nociceptive parameters

Mechanical allodynia. Mechanical allodynia was evaluated with von Frey filaments of increasing stiffness (0.02-2 g) using the up-and-down method.[13,27] The mechanical paw withdrawal threshold was expressed in grams (g). Mechanical allodynia was considered a decrease in the withdrawal threshold when compared to the baseline values.

Cold allodynia. Cold allodynia was assessed by measuring the acute nocifensive response to the acetone-evoked evaporative cooling.[22] A droplet (20 μl) of acetone was gently applied to the plantar surface of the mouse hind paw, and the time spent in elevation and licking of the plantar region was measured for 60 sec. Acetone was applied three times at a 10- to 15-min interval, and the average of elevation/licking time was calculated. Cold allodynia was considered as an increase in the nociceptive time observed after exposure to acetone when compared to basal values.

Dacarbazine-induced pain model. Dacarbazine was administered in a similar way to that previously described for paclitaxel administration.[12] For acute treatment, mice received only one intraperitoneal (i.p.) injection of dacarbazine (1 mg/kg, i.p.). To induce chronic pain, the animals received a repeated treatment of dacarbazine (1 mg/kg, i.p.; 1, 3, 5 and 7 days) resulting in a cumulative dose of 4 mg/kg. Acute (1 mg/kg, i.p.) or repeated (1 mg/kg/4 days, i.p.) injections of paclitaxel were used as control.[12]

Mechanical and cold allodynia were measured before (baseline values; B) and at 6, 24 and 48 hr after acute treatment with dacarbazine (1 mg/kg, i.p.) or paclitaxel (1 mg/kg, i.p.) or at 7, 14, 21 and 28 days after the first injection of repeated treatment with dacarbazine (1 mg/kg/4 days, i.p.) or paclitaxel (1 mg/kg/4 days, i.p.). Mechanical and cold allodynia also were evaluated in C57BL/6, *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ mice after acute or repeated dacarbazine administration.

Effect of antisense oligonucleotide for the TRPA1 receptor on dacarbazine-induced nociception. The antisense oligonucleotide for the TRPA1 receptor to evaluate the involvement of this receptor in dacarbazine-induced nociception was also used. The animals were treated intrathecally (5 μl/site) with antisense oligonucleotide for TRPA1 (AS TRPA1 ODN, 30 μg) or with mismatch oligonucleotide (MM TRPA1 ODN; 30 μg) three times a day for three consecutive days (Days 11, 12 and 13) after the first dacarbazine administration.[20] Then, mechanical and cold allodynia were evaluated on the 14th day after the first dacarbazine (1 mg/kg/4 days, i.p.) administration.

To confirm that intrathecal treatment with the antisense oligonucleotide for TRPA1 reduced TRPA1 mRNA expression, a real-time quantitative PCR was performed. For more information, check the Supporting Information.

TRPA1 receptor protein expression. Expression of the TRPA1 receptor protein was assessed by western blotting in sciatic nerve samples after the vehicle (10 ml/kg, i.p.) or dacarbazine (1 mg/kg/4 days, i.p.) administration.[20,28] Detailed information are described in the Supporting Information.

Effect of TRPA1 antagonist and antioxidant administration on dacarbazine-induced nociception. Animals were orally treated with vehicle (10 ml/kg) or TRPA1 antagonists HC-030031 (300 mg/kg) or A-967079 (60 mg/kg) on the 14th day after the first dacarbazine (1 mg/kg/4 days, i.p.) administration. The antinociceptive potential of antioxidant compounds, such as NAC (200 mg/kg, i.p.) and α-lipoic acid (100 mg/kg, p.o.), was also evaluated in the dacarbazine-induced nociception model. First, on Day 14 after the first dacarbazine administration, the mechanical threshold (time 0) or nociception time (time 0) of mice were evaluated. Next, mice received treatment with TRPA1 antagonists or antioxidants, and the mechanical threshold and the nociception time were again evaluated from 1 to 3 hr after treatments.

Dacarbazine-induced nociception in a tumor-associated pain model. We also evaluated the effect of TRPA1 receptor antagonists on the dacarbazine-induced hypersensitivity in animals with melanoma. For tumor inoculation, 20 μl of B16-F10 melanoma cells (2×10^5 cells suspended in PBS) were injected subcutaneously into the plantar region of the right hind paw.[7,20] On the fifth day after tumor inoculation, the animals received vehicle (10 ml/kg, i.p.), dacarbazine (1 mg/kg/4 days, i.p.) or paclitaxel (1 mg/kg/4 days, i.p.). HC-030031 (300 mg/kg, p.o.—a TRPA1 antagonist) and NAC (200 mg/kg, i.p.—an antioxidant) were then administrated at Day 7 after the first administration of vehicle, dacarbazine or paclitaxel. Another group of animals received only dacarbazine (1 mg/kg/4 days, i.p.) or paclitaxel (1 mg/kg/4 days, i.p.) administrations as control. The mechanical and cold allodynia were then evaluated 1, 2 and 3 hr after treatments.

Measurement of H_2O_2 released from cells. H_2O_2 was determined in hTRPA1-HEK293 or naive untransfected HEK293 cells. For more information, see Supporting Information.

Measurement of H_2O_2 levels in tissue. The H_2O_2 levels were determined in sciatic nerve tissue collected on day 14 after the first vehicle (10 ml/kg, i.p.) or dacarbazine (1 mg/kg/4 days, i.p.) administration.[29] For more information, see Supporting Information.

Cell viability measurement by 3(4-5-dimethyl)-2-5-diphenyl tetrazolium bromide (MTT) salt reduction assay

The possible cytotoxic effect of HC-030031 (TRPA1 antagonist), dacarbazine and paclitaxel was evaluated through cell viability assay by MTT.[30] Detailed information are described in the Supporting Information.

Statistical analysis

Results were expressed as the mean and standard error of the mean (SEM). Statistical analyses were performed using Graph Pad Prism 6.0 software. The significance of difference among groups was evaluated with the Mann–Whitney test, one-way or two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test or Bonferroni *post hoc* test. To meet parametric assumptions, the data of mechanical threshold were log transformed before analyses. p values less than 0.05 were considered significant. Proportional Venn diagram was drawn using BioVinci 1.1.5 software (https://vinci.bioturing.com).

Data availability

Data will be made available upon reasonable request.

Results

Dacarbazine selectively activates the human TRPA1 channel and excites TRPA1 in rodent sensory neurons

Dacarbazine evoked a concentration-dependent calcium response in hTRPA1-HEK293 cells (EC_{50} 23 ± 0.5 μM) (Fig. 1*a*). The calcium response evoked by dacarbazine (30 μM) was abolished by TRPA1 antagonist HC-030031 which, as expected, also abolished the calcium response to TRPA1 selective agonist AITC and failed to attenuate calcium responses evoked by the PAR2 agonist, SLIGKV-NH2, thus indicating selectivity (Fig. 1*a*). Dacarbazine, as well as the selective TRPA1 agonist AITC, did not evoke a calcium response in naive HEK293 cells that responded to hPAR2-AP (Fig. 1*b*).

To determine if the calcium signals originate from intracellular stores, hTRPA1-HEK293 cells were preincubated in the absence of extracellular calcium, which completely abolished dacarbazine-evoked calcium responses (Fig. 1*c*), thus suggesting that the elevation in intracellular calcium is the result of TRPA1-mediated calcium influx. Furthermore, hTRPA1-HEK293 cells were preincubated with the intracellular calcium store depletion agent, thapsigargin (TG, 1 μM), in the absence of extracellular calcium, which again completely abolished dacarbazine calcium responses, confirming previous observations (Fig. 1*c*). In addition, the exposure of cells to the free radical scavenger phenyl N-tert-butyl nitrone (PBN) indicated that TRPA1 is directly gated by dacarbazine and not by ROS production (Fig. 1*c*). PBN also did not affect the calcium response to the PAR2 agonist (Fig. 1*c*).

Exposure of cultured mouse DRG neurons to dacarbazine evoked a concentration-dependent calcium response in a subset of cells, identified as neurons due to their ability to respond to KCl, and as nociceptors for their ability to respond to capsaicin (TRPV1 agonist) and AITC (TRPA1 agonist) (Figs. 1*d* and 1*e*). In mouse DRG neurons, the maximum calcium response to dacarbazine was 24 ± 2% of ionomycin, and EC_{50} was 16 ± 0.5 μM (Fig. 1*d*). Calcium responses elicited by dacarbazine or AITC were blocked by exposure to TRPA1 antagonist HC-030031, which did not affect the response evoked by capsaicin and KCl, indicating selectivity (Fig. 1*d*). Notably, dacarbazine and AITC produced calcium responses in capsaicin-sensitive DRG neurons isolated from *Trpa1*$^{+/+}$ mice, an effect that was absent in neurons obtained from *Trpa1*$^{-/-}$ mice (Fig. 1*e*). The calcium responses to capsaicin and KCl were unchanged in both mouse strains (Fig. 1*e*). The percentage of dacarbazine-responding and AITC-responding

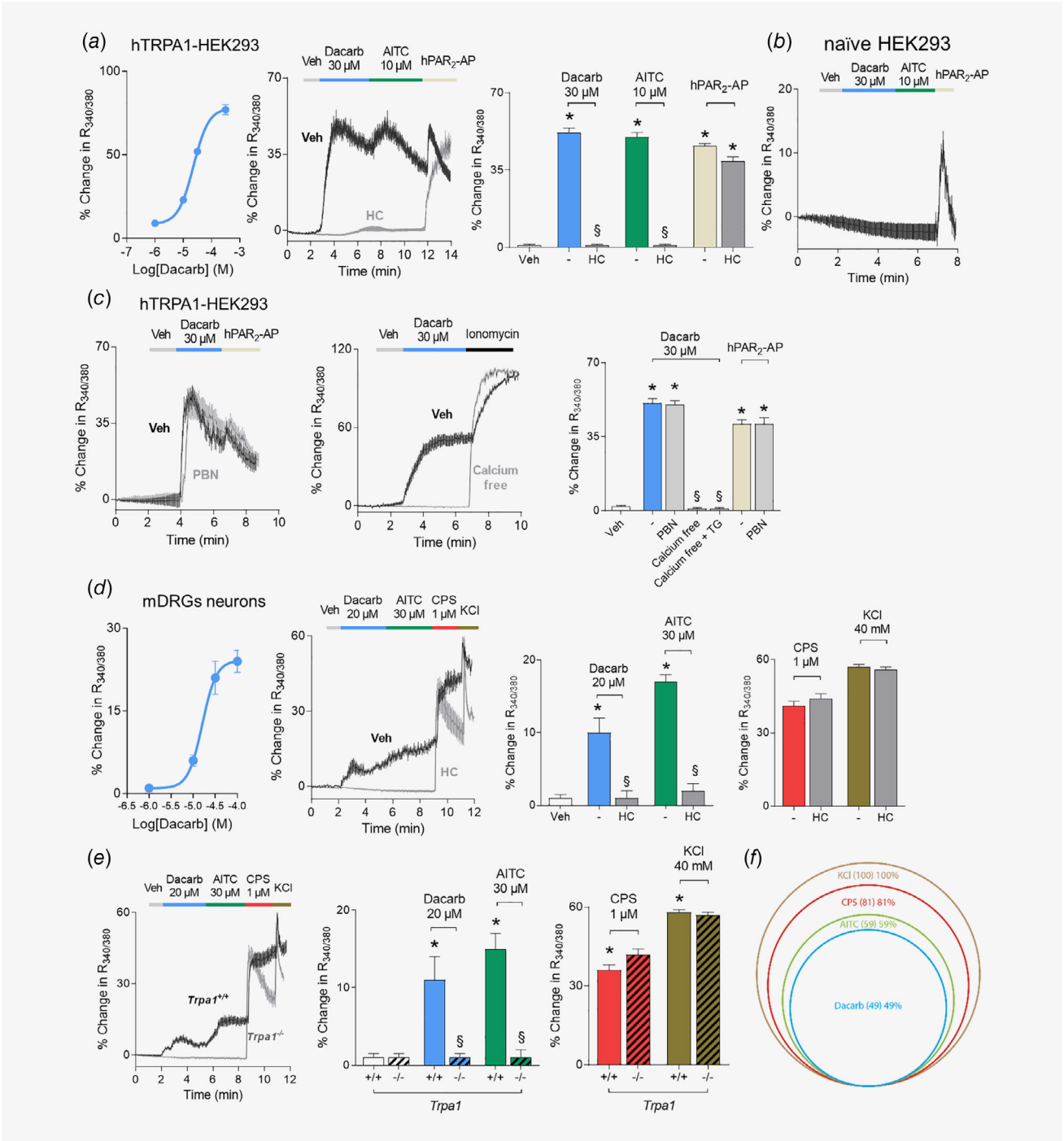

Figure 1. Dacarbazine selectively targets human and rodent TRPA1. *(a)* Concentration-response curve, typical traces and cumulative data of the calcium responses evoked by dacarbazine and AITC (TRPA1 agonist) in hTRPA1 HEK293 cells exposed to the TRPA1 selective antagonist, HC-030031 (HC, 30 µM) or its vehicle (–). *(b)* Typical trace and cumulative data of calcium responses evoked by dacarbazine and AITC in naive HEK293 cells that responded to hPAR2-AP (100 µM). Veh is the vehicle of dacarbazine, dash (–) indicates the vehicle of HC03. Data are mean + SEM of $n > 20$ cells from four independent experiments. $^{\#}p < 0.05$ when compared to the vehicle group; $^{*}p < 0.05$ when compared to the dacarbazine or AITC group; one-way ANOVA followed by Bonferroni *post hoc* test. *(c)* Typical traces and cumulative data of calcium responses evoked by dacarbazine in the absence of extracellular calcium (calcium free) or in the presence of intracellular calcium store depletion agent, thapsigargin (TG, 1 µM) and in the presence of the free radical scavenger phenyl N-tert-butyl nitrone (PBN, 1 mM). *(d)* Concentration–response curve, typical traces and cumulative data of calcium responses evoked by dacarbazine, AITC, capsaicin (CPS; TRPV1 agonist) and KCl in mouse DRG neurons exposed to HC (50 µM), or its vehicle (–). *(e)* Typical traces and cumulative data of calcium responses evoked by dacarbazine, AITC, CPS and KCl in DRG neurons isolated from *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice. Veh is the vehicle of dacarbazine. *(f)* Venn diagram showing proportionally the percentage of dacarbazine and AITC-responding neurons out of the KCl-responding neurons. Data are mean + SEM of $n > 20$ cells from four independent. $^{*}p < 0.05$ when compared to the veh; $^{\S}p < 0.05$ when compared to the dacarbazine or AITC; one-way ANOVA followed by Bonferroni *post hoc* test.

neurons out of the KCl-responding neurons was similar in mouse DRG. In 100 neurons (KCl^+) analyzed, 81%, 59% and 49% neurons showed responses to capsaicin, AITC and dacarbazine, respectively (Fig. 1*f*).

Dacarbazine administration induces mechanical and cold allodynia in mice

Dacarbazine or paclitaxel caused mechanical allodynia in C57BL/6 mice, characterized by the reduction of the paw withdrawal threshold in response to von Frey filaments, and induced cold allodynia resulting in an increased nociception time in the acetone test when compared to the vehicle. Mechanical allodynia was observed 48 hr after dacarbazine administration (46 ± 7% of threshold reduction) and at 24 and 48 hr after paclitaxel administration (71 ± 5% of threshold reduction at 24 hr). Cold allodynia was observed at 24 and 48 hr after dacarbazine and paclitaxel acute administrations (71 ± 21% and 100% of increased nociception time at 48 hr, respectively; Figs. 2*a* and 2*b*).

Repeated administration of dacarbazine, but not vehicle, also induced mechanical and cold allodynia in mice at 7, 14 and 21 days after the first administration, characterizing the development of pain hypersensitivity. The maximum reduction of the mechanical threshold observed was 60 ± 3% at Day 14, and the nociception time increase in the cold allodynia test was 100% at all days. The paclitaxel repeated administration caused mechanical and cold allodynia at 7, 14, 21 and 28 days after its first administration with a maximum reduction of the mechanical threshold of 73 ± 2% at day 7 and increased the nociception time in the cold allodynia test by 100% at all days (Figs. 2*c* and 2*d*).

TRPA1-deficient mice and antisense oligonucleotide for the TRPA1 receptor reduces dacarbazine-induced nociception

Dacarbazine acute administration induced mechanical allodynia in *Trpa1*$^{+/+}$ mice from 24 to 48 hr and cold allodynia at 48 hr after administration, when compared to the vehicle group. Repeated administration of dacarbazine also induced mechanical and cold

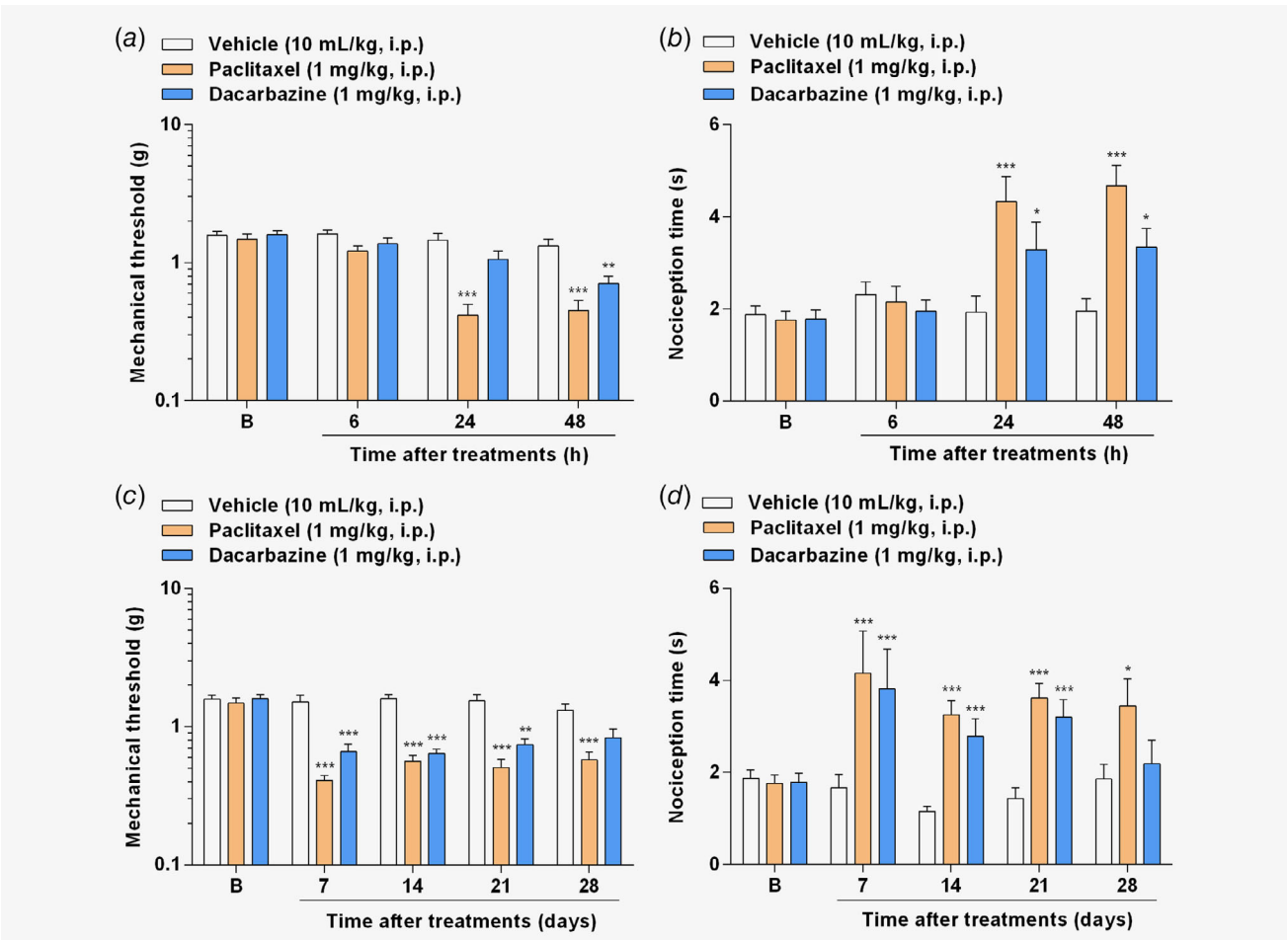

Figure 2. Dacarbazine and paclitaxel acute or repeated administration cause mechanical allodynia (*a*, *c*) and cold allodynia (*b*, *d*) in mice. The nociceptive tests were evaluated at 6, 24 and 48 hr after vehicle (10 ml/kg, i.p.), dacarbazine (1 mg/kg, i.p.) or paclitaxel (1 mg/kg, i.p.) acute administration (*a*, *b*); or at 7, 14, 21 and 28 days after first vehicle (10 ml/kg, i.p.), dacarbazine (1 mg/kg/4 days, i.p.) or paclitaxel (1 mg/kg/4 days, i.p.) repeated administration (*c*, *d*). (B) denotes baseline mechanical threshold/nociception time before administration. Data are expressed as the mean + SEM ($n = 8$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, when compared to the vehicle group (two-way ANOVA followed by Tukey's *post hoc* test).

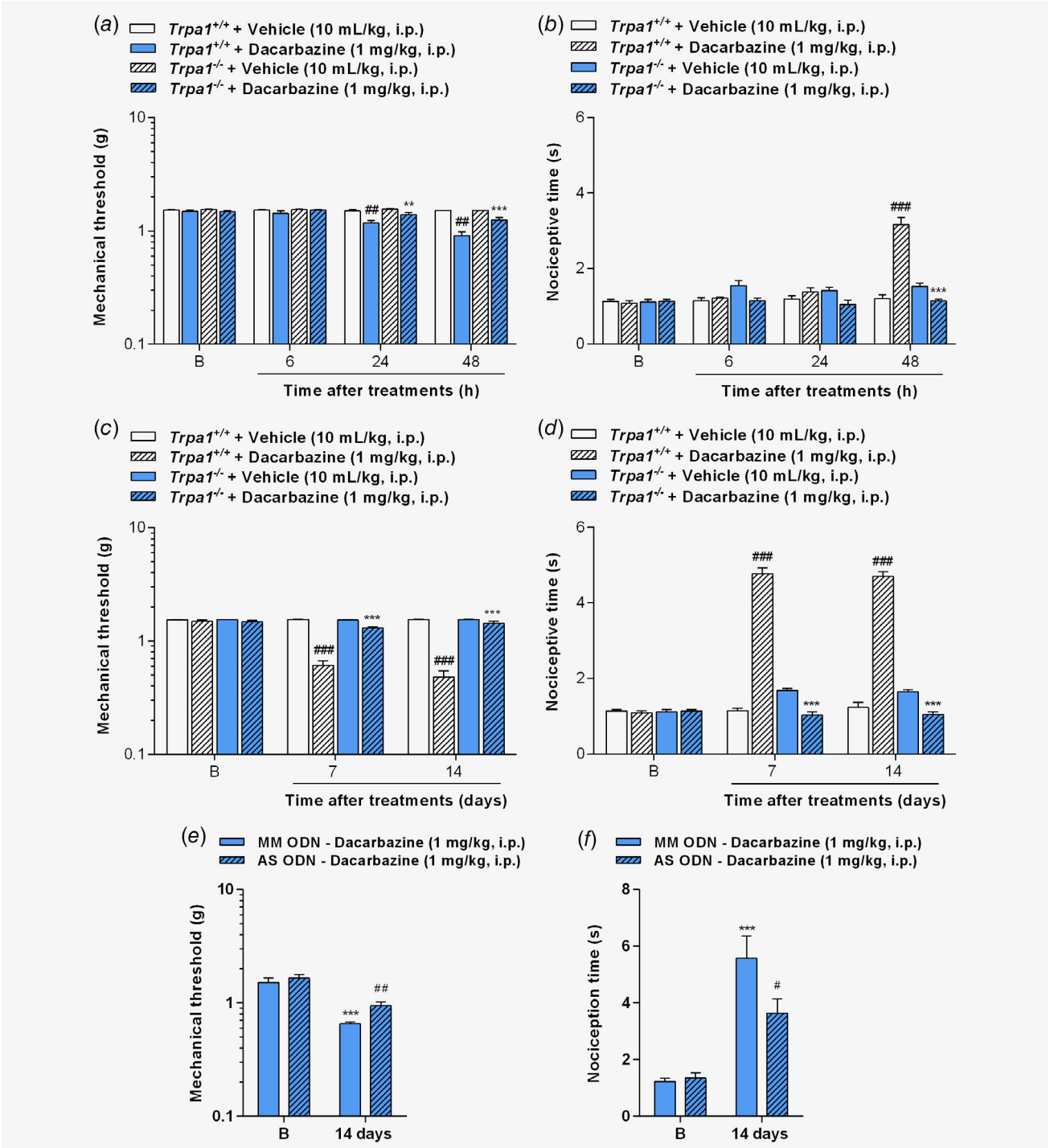

Figure 3. Gene deletion and antisense oligonucleotide for TRPA1 reduces dacarbazine-induced nociception in mice. The nociceptive tests were evaluated in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ at 6, 24 and 48 hr after vehicle (10 ml/kg, i.p.) or dacarbazine (1 mg/kg, i.p.) acute administration (*a*, *b*); or at 7 and 14 days after the first vehicle (10 ml/kg, i.p.) or dacarbazine (1 mg/kg/4 days, i.p.) repeated administration (*c*, *d*). Mechanical allodynia (*e*) and cold allodynia (*f*) were evaluated after injections of antisense oligonucleotide (AS ODN) for TRPA1 or control oligonucleotide (mismatch, MM ODN) on Day 14 after the first dacarbazine (1 mg/kg/4 days, i.p.) repeated administration. (B) denotes the baseline mechanical threshold/nociception time before dacarbazine administration. Data are expressed as mean + SEM ($n = 8$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ when compared to the wild-type mice/vehicle group or when compared to the nonsense group; $^{**}p < 0.01$; $^{***}p < 0.001$ when compared to the wild-type mice/dacarbazine group or when compared to basal values (two-way ANOVA followed by Tukey's *post hoc* test).

allodynia from Days 7–14 after the first administration when compared to the vehicle group (Fig. 3). *Trpa1*$^{-/-}$ did not develop mechanical and cold allodynia after dacarbazine acute administration when compared to *Trpa1*$^{+/+}$ mice. Maximal inhibitions were 60 ± 10% and 92 ± 4% to mechanical and cold allodynia, respectively, at 48 hr after dacarbazine administration (Figs. 3*a* and 3*b*). *Trpa1*$^{-/-}$ did not develop mechanical and cold allodynia induced by dacarbazine repeated administration. Maximal inhibitions were 95 ± 5% and 71 ± 1% to mechanical and cold allodynia, respectively, on Day 14 after the first dacarbazine administration (Figs. 3*c* and 3*d*).

Because the maximum nociceptive effect of dacarbazine was observed on Day 14 after its first administration, and TRPA1-deficient mice prevented the dacarbazine-induced chronic nociception, we chose Day 14 to evaluate the effect of the antisense oligonucleotide and TRPA1 antagonists in dacarbazine-induced pain hypersensitivity.

Intrathecal injection of TRPA1 AS ODN, which significantly reduced TRPA1 mRNA expression in L5-L6 DRGs (value of mRNA expression relative to β-actin TRPA1 MM ODN $1.24 \times 10^{-7} \pm 0.25 \times 10^{-7}$ and TRPA1 AS ODN $0.68 \times 10^{-7} \pm 0.11 \times 10^{-7}$; $n = 4$ mice), caused an anti-allodynic effect on the dacarbazine-induced chronic nociception. TRPA1 AS ODN reduced dacarbazine-induced mechanical and cold allodynia on Day 14 after the first dacarbazine administration with maximal inhibitions of 23 ± 5% and 57 ± 9%, respectively. However, the control oligonucleotide injection (TRPA1 MM ODN) did not alter the dacarbazine-induced mechanical and cold allodynia (Figs. 3*e*

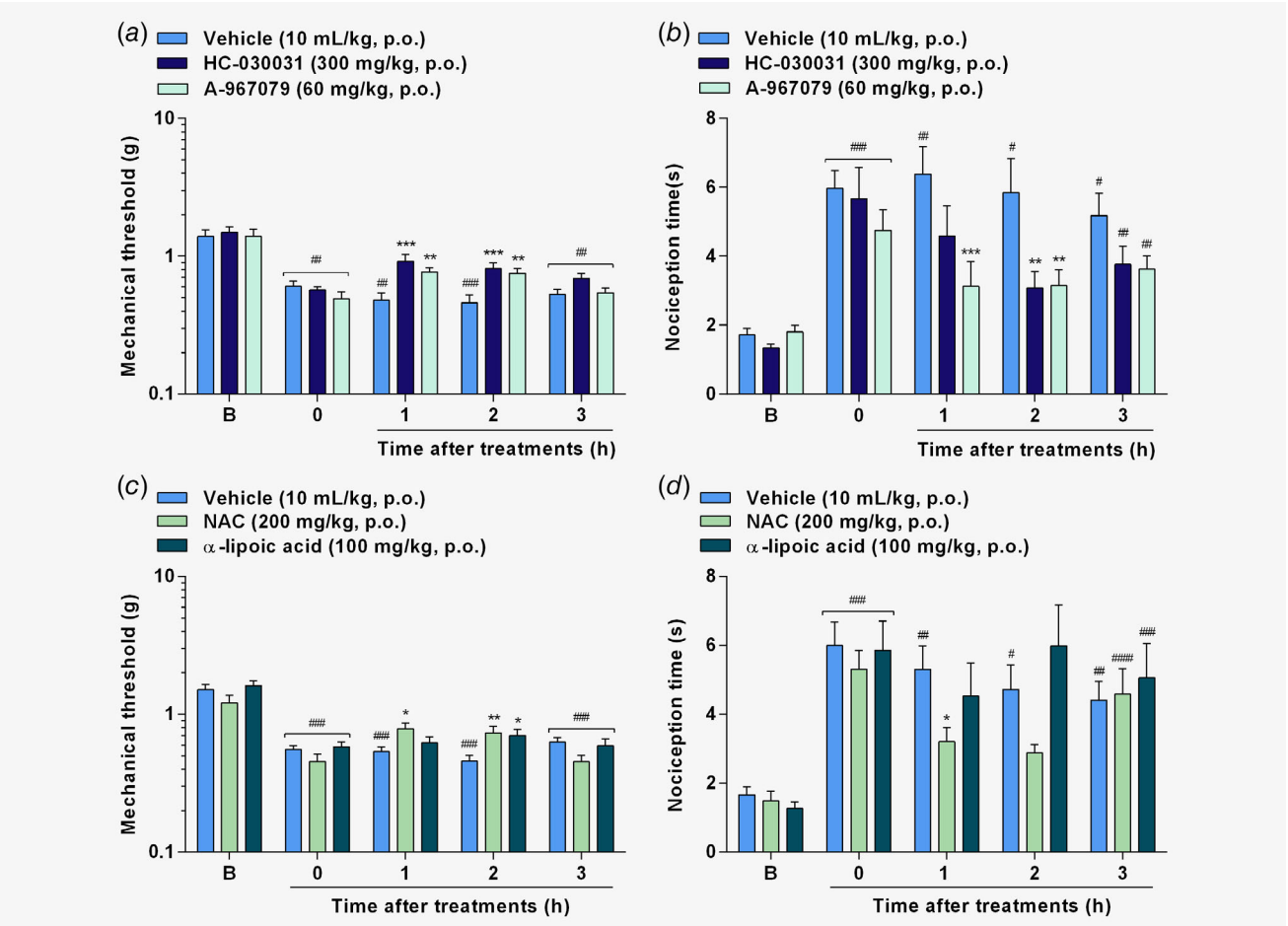

Figure 4. TRPA1 antagonists (HC-030031 and A-967079) and antioxidants (n-acetylcysteine—NAC and α-lipoic acid) cause anti-allodynic effect on the dacarbazine-induced nociception in mice. Time–response curves for mechanical allodynia (*a*) and cold allodynia (*b*) after treatment with vehicle (10 ml/kg, p.o.), HC-030031 (300 mg/kg, p.o.) or A-967079 (60 mg/kg, p.o.) in animals that received dacarbazine (1 mg/kg/4 days, i.p.) repeated administrations. Time–response curves for mechanical allodynia (*c*) and cold allodynia (*d*) after treatment with vehicle (10 ml/kg, p.o.), NAC (200 mg/kg, i.p.) or α-lipoic acid (100 mg/kg, p.o.) in animals that received dacarbazine (1 mg/kg/4 days, i.p.) administrations. (B) denotes the baseline mechanical threshold/nociception time before dacarbazine administration, while 0 indicates the basal at 14 days after first dacarbazine administration. Data are expressed as the mean + SEM ($n = 8$). $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$, when compared to the baseline (B) (One-way ANOVA followed by Tukey's *post hoc* test). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, when compared to the vehicle group (two-way ANOVA followed by Tukey's *post hoc* test).

and 3*f*). Moreover, dacarbazine did not alter the TRPA1 expression in the sciatic nerve at Day 14 after its first administration when compared to the vehicle group (data not shown).

Selective TRPA1 receptor antagonists and antioxidants reduce dacarbazine-induced chronic nociception

Oral administration of TRPA1 antagonists HC-030031 (300 mg/kg) or A-967079 (60 mg/kg) promoted anti-allodynic effects on the dacarbazine-induced chronic nociception when compared to the vehicle group. HC-030031 reduced the dacarbazine-induced mechanical and cold allodynia at 1 and 2 hr or at 2 hr after its administration, respectively. Maximal inhibitions were 48 ± 12% and 100% for mechanical and cold allodynia at 1 and 2 hr, respectively. A-967079 also reduced the dacarbazine-induced mechanical and cold allodynia at 1 and 2 hr after its administration, with maximal inhibitions of 32 ± 5% and 78 ± 10%, respectively, observed at 1 hr after treatment (Figs. 4*a* and 4*b*).

Antioxidants NAC (200 mg/kg, i.p.) or α-lipoic acid (100 mg/kg, p.o.) caused an anti-allodynic effect on dacarbazine-induced chronic nociception when compared to the vehicle. NAC reduced dacarbazine-induced mechanical and cold allodynia at 1 and 2 hr after its administration. Maximal inhibitions were 27 ± 7% and 71 ± 8% for mechanical and cold allodynia at 2 and 1 hr, respectively. However, α-lipoic acid was able to

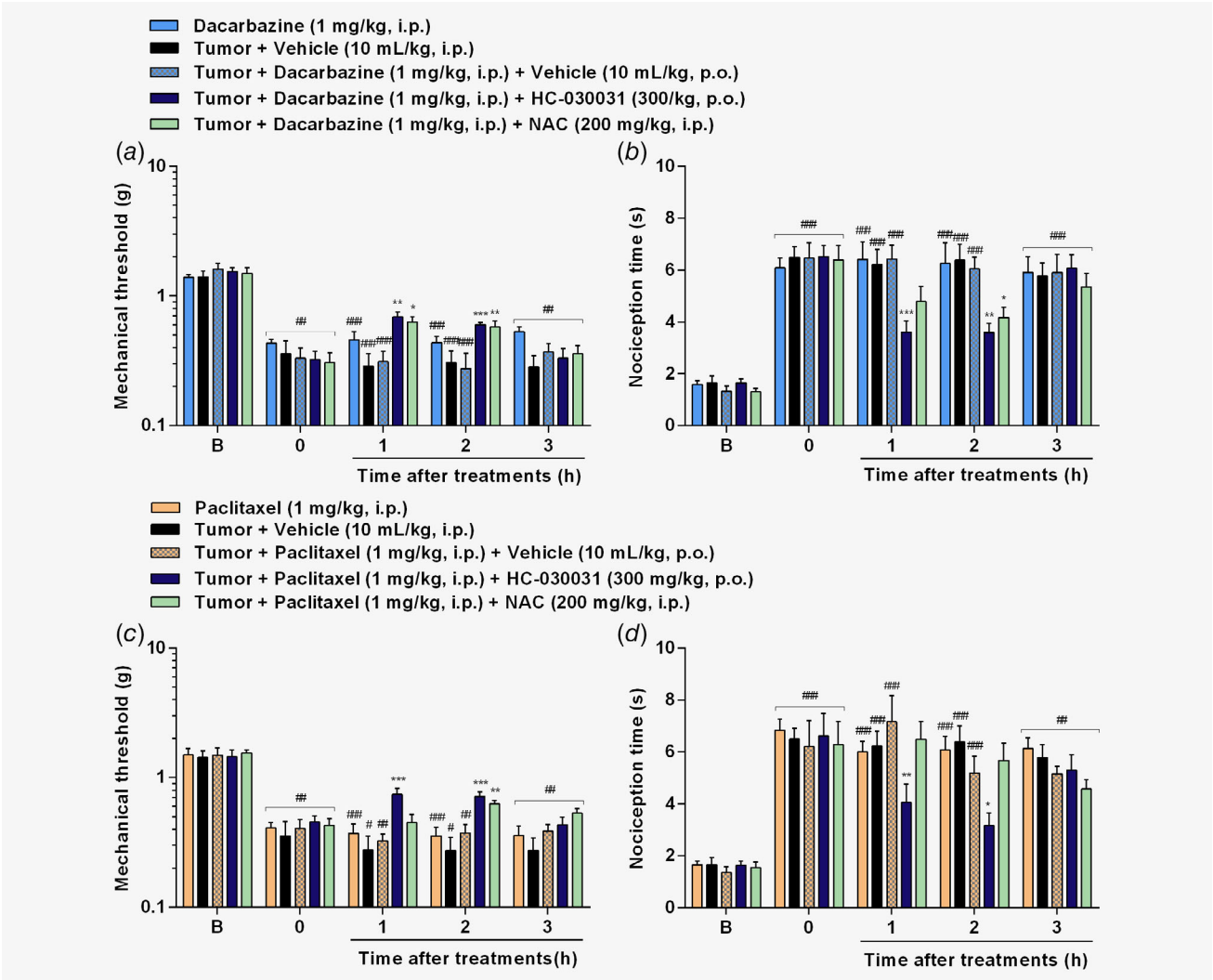

Figure 5. TRPA1 antagonist (HC-030031) and antioxidant (n-acetylcysteine, NAC) caused anti-allodynic effect on the dacarbazine- or paclitaxel-induced nociception in a tumor-associated pain model in mice. Time-response curves for mechanical allodynia (*a*, *c*) and cold allodynia (*b*, *d*) after treatment with vehicle (10 ml/kg, p.o.), HC-030031 (300 mg/kg, p.o.) or NAC (200 mg/kg, i.p.) at 12 days after tumor inoculation and at 7 days after first dacarbazine (1 mg/kg/4 days, i.p., *a*, *b*) or paclitaxel (1 mg/kg/4 days, i.p., *c*, *d*) repeated administration. (B) denotes the baseline mechanical threshold/nociception time before induction of the model, while 0 indicates the basal after induction of the model. Data are expressed as the mean + SEM ($n = 6–9$). $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$, when compared to the baseline (B) (One-way ANOVA followed by Tukey's *post hoc* test). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, when compared to the tumor plus dacarbazine or plus paclitaxel-treated with vehicle group (two-way ANOVA followed by Tukey's *post hoc* test).

reduce just the dacarbazine-induced mechanical allodynia at 2 hr after its administration with a maximum inhibition of 23 ± 7% (Figs. 4*c* and 4*d*).

We also observed that dacarbazine increased H_2O_2 levels (61 ± 5%) in the sciatic nerve at Day 14 of its first administration when compared to the vehicle group. The values observed were 19 ± 3 and 31 ± 5 nmol of H_2O_2/mg protein for the vehicle or dacarbazine group, respectively ($p < 0.05$ sec Mann–Whitney test). In cellular systems, prolonged exposure to dacarbazine resulted in H_2O_2 production *via* a TRPA1-dependent mechanism. Dacarbazine (10, 30 and 100 μM) increased the H_2O_2 levels by 2 (to 10 and 30 μM) or 3.2 times (100 μM) in hTRPA1-HEK293 cells when compared to naive untransfected HEK293 cells (data not shown; $p < 0.05$ one-way ANOVA followed by Bonferroni *post hoc* test).

TRPA1 antagonist or an antioxidant cause antinociceptive effects on dacarbazine- or paclitaxel-induced nociception in a tumor-associated cancer pain model

The tumor, or dacarbazine and paclitaxel repeated treatment, as well as tumor plus dacarbazine or plus paclitaxel repeated administration, induced mechanical and cold allodynia when compared to its baseline threshold (B) (Fig. 5). TRPA1 antagonist HC-030031 (300 mg/kg, p.o.) or the antioxidant NAC (200 mg/kg, i.p.) promoted anti-allodynic effects on the dacarbazine- or paclitaxel-induced nociception in a tumor-associated pain model. HC-030031 and NAC reduced the dacarbazine-induced mechanical allodynia at 1 and 2 hr after its administration with maximal inhibitions of 24 ± 1% and 22 ± 4% at 2 hr, respectively. HC-030031 also reduced the dacarbazine-induced cold allodynia at 1 and 2 hr after its administration with maximal inhibition of 55 ± 9% at 2 hr. NAC caused an anti-allodynic effect on cold allodynia only at 2 hr after its administration with maximal inhibition of 38 ± 10% (Figs. 5*a* and 5*b*).

HC-030031 and NAC also promoted anti-allodynic effects in animals with tumors that received repeated paclitaxel administrations. The anti-allodynic effect of HC-030031 on mechanical and cold allodynia occurred at 1 and 2 hr after its administration with maximal inhibition of 36 ± 7% and 62 ± 9% at 1 hr, respectively. NAC presented anti-allodynic effects only on the mechanical allodynia with a maximal inhibition of 23 ± 4% at 2 hr after its administration (Figs. 5*c* and 5*d*).

On the other hand, dacarbazine and paclitaxel did not reduce cell growth *in vivo* (data not shown) in the time evaluated, because the goal was to show the nociception induced by chemotherapy plus tumor inoculation. A longer time than 14 days after tumor inoculation, it would be difficult to measure the mechanical and cold allodynia because the paw edema would be large, therefore making it impossible to measure nociception.

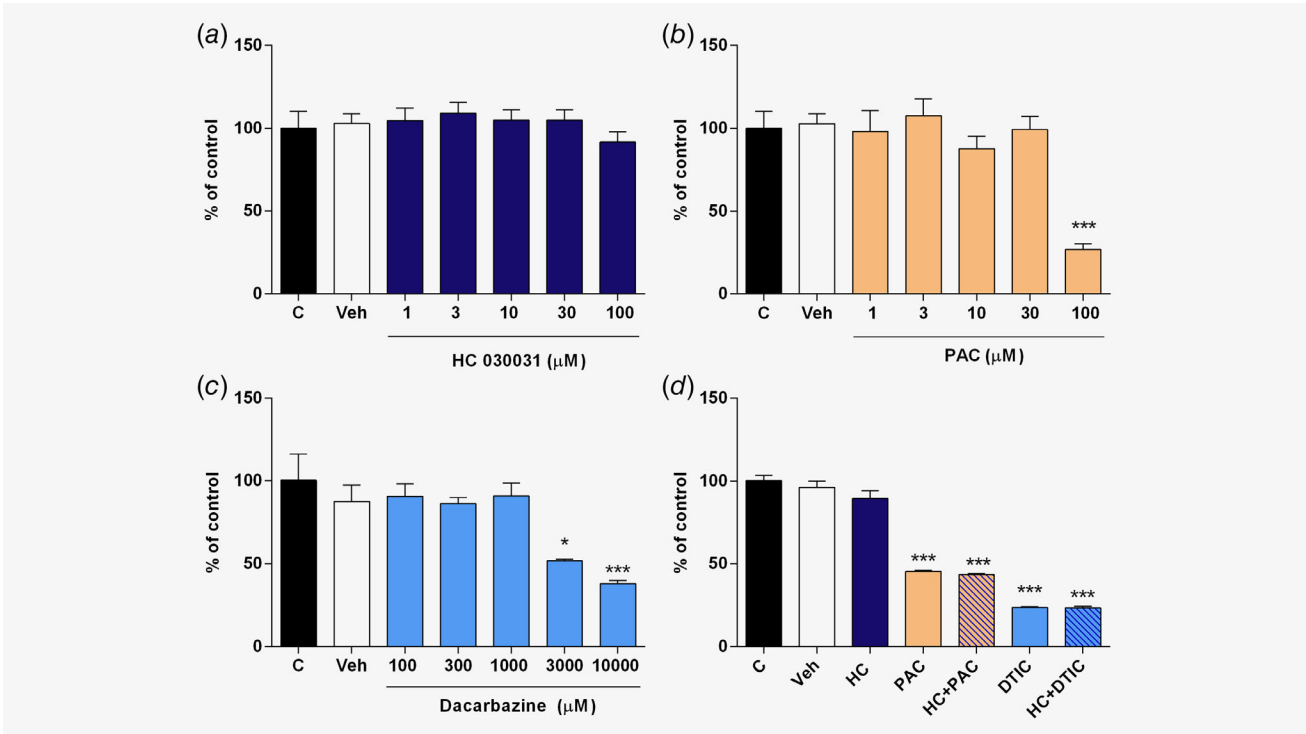

Figure 6. B16-F10 cell viability assessed *in vitro* by MTT assay. *(a)* TRPA1 antagonist HC-03003 (1–100 μM), *(b)* paclitaxel (PAC, 1–100 μM), *(c)* dacarbazine (DTIC, 100–10,000 μM). *(d)* HC-030031 (100 μM) associated with PAC (100 μM) or DTIC (10,000 μM). Control samples denote only DMEM culture medium. Control plus DMSO samples indicate DMEM culture medium plus DMSO 0.05%. ***$p < 0.001$, when compared to control samples (two-way ANOVA followed by Bonferroni's *post hoc* test).

HC-030031 did not alter the paclitaxel or dacarbazine cytotoxic action on B16-F10 melanoma cells

The toxicity development of TRPA1 antagonist HC-030031 was assessed *in vitro*, using B16-F10 melanoma cell lines through MTT assay, which revealed that HC-030031 did not alter cell viability, regardless of the concentrations tested in comparison with the control group (Fig. 6*a*). On the contrary, B16-F10 incubation with paclitaxel showed a cell viability decrease in a concentration dependent-manner at paclitaxel highest concentration (100 μM), with an inhibition percentage of 73 ± 6% after 24 hr of incubation when compared to control samples (Fig. 6*b*). Also, dacarbazine reduced cell viability to 3,000 and 10,000 μM, with an inhibition percentage of 62 ± 2% after 24 hr of incubation (Fig. 6*c*).

In the second set of experiments, we evaluated whether the association of HC-030031 might interfere with the paclitaxel or dacarbazine chemotherapeutic effect. MTT assay showed that HC-030031 in the highest concentration (100 μM), when associated with paclitaxel or dacarbazine highest concentrations (100 or 10,000 μM), did not interfere with the chemotherapeutic action of these drugs (Fig. 6*d*). The B16-F10 incubation with paclitaxel, paclitaxel plus HC-030031, dacarbazine and dacarbazine plus HC-030031 showed an inhibition percentage of 54 ± 1, 56 ± 1, 76 ± 1 and 77 ± 1%, respectively, after 24 hr of incubation, compared to control samples (Fig. 6*d*).

Discussion

Antineoplastic therapy has been associated with the development of pain syndrome, which puts limits to its use and compromises the patient's quality of life.[8] Among these therapies, dacarbazine is reported to cause pain symptoms in animal models[10] and oncological patients.[3,31] In the present study, dacarbazine stimulated TRPA1 in cultured mouse DRG neurons and in hTRPA1-HEK293 cells, effects that were abolished by a TRPA1 antagonist. Moreover, we demonstrated that dacarbazine caused mechanical and cold allodynia in mice by TRPA1 activation and sensitization, which was reduced by TRPA1 pharmacological blockade, antioxidants and by TRPA1 gene deletion or silencing. Thus, our results indicate that the acute and chronic nociception caused by dacarbazine occurs due to the TRPA1 activation or sensitization.

It is known that mechanical and cold allodynia are among the predominant symptoms of chemotherapy-induced pain syndrome.[9] Here, we observed that acute or repeated dacarbazine administration caused mechanical and cold allodynia starting at 24 hr after acute administration to Day 21 after repeated administration, thus characterizing the chronic pain development. Such effects of dacarbazine were similar to those of paclitaxel, whose pain syndrome is already well described.[9,12,32] Our findings are in accordance with preclinical and clinical data showing that chemotherapeutic agents promote acute pain starting early from 1 to 2 days after administration, and persistent pain, which is more prominent if preceded by acute pain.[8,11,12,16] Thus, we are showing for the first time that chemotherapeutic dacarbazine induces pain syndrome in mice.

Several mechanisms have been proposed to explain chemotherapy-induced pain syndrome.[8,9] TRP channels have been the subject of intense investigation, mainly due to their prevalent localization in a subset of nociceptive sensory neurons.[16,33] Among them, TRPA1 channel is an essential pain transducer of noxious stimulus (mechanical or thermal), playing a critical role in the perception and transmission of acute and persistent pain conditions, such as inflammation, cancer and neuropathies.[16,19,20,34] Many studies reported TRPA1 involvement in chemotherapy-induced pain syndrome, including the chemotherapeutic agents, oxaliplatin and cisplatin, bortezomib and paclitaxel.[13,21,22]

Different from some chemotherapeutic agents that do not appear to directly stimulate TRPA1,[13,21,22] we showed that dacarbazine selectively activates TRPA1 when evaluated in human TRPA1-HEK293 cells and cultured mouse DRG neurons, a response abolished by TRPA1 antagonist HC-030031. The dacarbazine action is like that observed for aromatase inhibitors, exemestane, letrozole and anastrozole; and the aromatase substrate androstenedione,[35,36] which were able to directly activate the TRPA1 causing pain conditions. This evidence is derived from the observation that dacarbazine evoked calcium response in hTRPA1-HEK293 cells but not in naive cells, in which it was abolished by the selective TRPA1 antagonist HC-030031. The increase of intracellular calcium evoked by dacarbazine resulted from TRPA1-mediated calcium influx and not from calcium release of intracellular stores. Moreover, exposure to a free radical scavenger (PBN) did not affect the response to calcium evoked by dacarbazine, in agreement with previous reports for the selective TRPA1 agonist AITC.[19] Likewise, mouse DRG neurons, which constitutively express the channel, when exposed to dacarbazine evoked calcium response in a subset of cells, identified as nociceptors by their ability to respond to capsaicin (TRPV1 agonist) and AITC (TRPA1 agonist). Moreover, HC-030031 blocked calcium responses elicited by dacarbazine in DRG neurons and neurons isolated from *Trpa1*$^{-/-}$ mice.

In vivo data recapitulate the *in vitro* findings. Administration of dacarbazine in mice induced mechanical and cold allodynia. To confirm the involvement of TRPA1 in dacarbazine-induced pain syndrome, we tested TRPA1 deficiency or silencing. As reported in other chemotherapy-induced pain models,[13,21] TRPA1-deficient mice prevented the development of mechanical and cold allodynia induced by dacarbazine. Likewise, TRPA1 silencing attenuated the dacarbazine-induced nociceptive responses, corroborating with previous data[19,20] that reported a selective reduction of nociceptive signals in neuropathic or cancer pain models after TRPA1 silencing. These observations indicate that the TRPA1 is necessary to trigger the nociceptive responses evoked by dacarbazine. On the other hand, dacarbazine did not affect TRPA1 expression in the sciatic nerve of the mice, confirming previous findings from our research group on the bortezomib chemotherapeutic.[13]

In addition to the genetic manipulation, we observed that the pharmacological blockade of TRPA1 using the antagonists HC-030031 and A-967079 reversed the dacarbazine-induced mechanical and cold allodynia. The ability of such antagonists

to reduce nociceptive parameters induced by chemotherapeutic agents as oxaliplatin, paclitaxel and bortezomib was previously reported.[16] In contrast to the data observed in TRPA1-deficient mice, in which dacarbazine did not induce painful hypersensitivity, the pharmacological TRPA1 blockade produced a transient reversal of nociceptive parameters when hypersensitivity was already established, corroborating with previous findings using different chemotherapy agents, such as oxaliplatin and bortezomib.[13,21] This transient antinociceptive effect has been associated with the pharmacokinetic properties of both TRPA1 antagonists.[37,38]

The temporary effect of the antagonists also suggests that other factors produced by dacarbazine may be collaborating to sustain the nociception induced by it. Such factors could be associated with oxidative mechanisms because we observed that dacarbazine increased the H_2O_2 levels in hTRPA1-HEK293 cells and in the sciatic nerve of animals. Indeed, the dacarbazine cytotoxicity effect was previously associated with reactive species generation, such as H_2O_2 and superoxide anion.[39] We also observed that the antioxidants, NAC and α-lipoic acid reduced the dacarbazine-induced mechanical and cold allodynia. Similar to our findings, antioxidant substances have been reported to reduce painful hypersensitivity in animal models[13,22] and patients with the chemotherapy-induced pain syndrome.[40,41]

Our data expand on previous findings of the oxidative role in pain syndrome evoked by chemotherapeutic agents, which seem to induce mechanical and cold allodynia by oxidative stress-dependent TRPA1 activation.[16] TRPA1 has been referred to as a significant oxidant sensor, because many by-products of oxidative stress, such as reactive oxygen and nitrogen species, including H_2O_2, selectively activate this channel.[42,43] In our study, dacarbazine increased both tissue and cellular H_2O_2 levels. Thus, we hypothesized that besides the direct TRPA1 activation and sensitization by dacarbazine, as observed by the evoked response to calcium, this chemotherapy also indirectly sensitizes/activates TRPA1 in sensory neurons by generating oxidative stress products.

Besides the involvement of TRPA1 in chemotherapy-induced pain, a recent study showed its involvement in melanoma-induced cancer pain.[20] Because dacarbazine is the main chemotherapeutic agent used to treat metastatic melanoma,[1,2,44] we performed a cancer pain protocol by associating the B16-F10 melanoma tumor cell inoculation with dacarbazine administration. The association of tumor cells and dacarbazine did not produce additional nociceptive behavior because maximum nociception was achieved after a single dacarbazine administration. However, dacarbazine treatment in melanoma cells-injected mice caused mechanical and cold allodynia in mice which was reversed by the TRPA1 antagonist HC-030031 and by the antioxidant NAC. Like some chemotherapeutic agents,[13,22] melanoma tumor cells may also increase oxidative stress products which activate TRPA1.[20,42] Moreover, both TRPA1 antagonists and antioxidants have attenuated nociceptive responses in a melanoma cancer pain model.[20]

Considering that it is difficult to evaluate if the patient's pain is caused by a tumor or by the chemotherapeutic treatment, our results can reinforce the findings of Antoniazzi et al. (2018) on the TRPA1 role in the painful hypersensitivity followed by the melanoma cell inoculation. This is because we verified that TRPA1 antagonists and antioxidants could block the pain caused by chemotherapy, as well as the dacarbazine- and paclitaxel-induced nociception in a tumor-associated pain model in mice. Similar to dacarbazine, paclitaxel is also used to treat metastatic melanoma[45,46] and has been reported to cause pain syndrome by the TRPA1 activation.[22] Thus, the TRPA1 pharmacological blockade is also expected to reduce paclitaxel-induced nociception in melanoma-inoculated mice.

Despite intensive efforts, chemotherapy-induced pain syndrome remains the major limitation of antineoplastic therapy, and no treatment is available, making it necessary to search for pharmacological intervention targets.[8] Extending previous findings on the involvement of TRPA1 in chemotherapy-induced pain,[16] our results showed that dacarbazine causes acute and chronic pain by TRPA1 activation and sensitization. It is important to note that TRPA1 antagonists have recently advanced into clinical studies for the treatment of neuropathic pain. In human Phase 2 clinical trials, the antagonist GRC 17536 was effective in reducing the pain of patients with diabetic neuropathy without presenting notable adverse effects.[47] Therefore, because blocking TRPA1 also reduced chemotherapy-induced nociception in the tumor-associated pain model, our results reinforce previous studies,[16] indicating that TRPA1 may represent a unique pharmacological target for treating cancer patients who, while undergoing chemotherapeutic treatment, develop chemotherapy-induced pain syndrome.

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