Neurotherapeutics (2022) 19:434–449
https://doi.org/10.1007/s13311-021-01164-w

ORIGINAL ARTICLE

The Cannabidiol Analog PECS-101 Prevents Chemotherapy-Induced Neuropathic Pain via PPARγ Receptors

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Accepted: 12 November 2021 / Published online: 13 December 2021

Abstract

Chemotherapy-induced peripheral neuropathy (CIPN) is the main dose-limiting adverse effect of chemotherapy drugs such as paclitaxel (PTX). PTX causes marked molecular and cellular damage, mainly in the peripheral nervous system, including sensory neurons in the dorsal root ganglia (DRG). Several studies have shown the therapeutic potential of cannabinoids, including cannabidiol (CBD), the major non-psychotomimetic compound found in the Cannabis plant, to treat peripheral neuropathies. Here, we investigated the efficacy of PECS-101 (former HUF-101), a CBD fluorinated analog, on PTX-induced neuropathic pain in mice. PECS-101, administered after the end of treatment with PTX, did not reverse mechanical allodynia. However, PECS-101 (1 mg/kg) administered along with PTX treatment caused a long-lasting relief of the mechanical and cold allodynia. These effects were blocked by a PPARγ, but not CB1 and CB2 receptor antagonists. Notably, the effects of PECS-101 on the relief of PTX-induced mechanical and cold allodynia were not found in macrophage-specific PPARγ-deficient mice. PECS-101 also decreased PTX-induced increase in *Tnf*, *Il6*, and *Aif1* (Iba-1) gene expression in the DRGs and the loss of intra-epidermal nerve fibers. PECS-101 did not alter motor coordination, produce tolerance, or show abuse potential. In addition, PECS-101 did not interfere with the chemotherapeutic effects of PTX. Thus, PECS-101, a new fluorinated CBD analog, could represent a novel therapeutic alternative to prevent mechanical and cold allodynia induced by PTX potentially through the activation of PPARγ in macrophages.

Keywords Neuropathy · Chemotherapy · Cannabidiol · PECS-101 · Paclitaxel · PPARγ

Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a disabling and frequent dose-limiting side effect associated with drugs used in cancer treatment such as paclitaxel (PTX) [1]. Chemotherapeutic agents can cause severe toxic damage to the peripheral nervous system, leading to a debilitating condition that may limit their clinical use. In some cases, the interruption of the treatment is necessary [2]. CIPN is partially reversible upon chemotherapy withdrawal in most patients, yet the sensory damage may be permanent [3].

PTX is widely employed alone or combined with other chemotherapeutic agents against many types of tumors [4]. PTX binds to and stabilizes microtubules, interrupting mitosis on the G2/M phase, followed by cell death and apoptosis [5]. Although the specific mechanisms underlying the development of PTX-induced peripheral neuropathic pain remain unknown, it is thought that chemotherapy-induced

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neurotoxicity activates the immune system in the peripheral nervous system, especially in the dorsal root ganglia (DRG) [6]. Multiple types of immune cells are recruited to the DRGs following chemotherapy [7, 8]. Moreover, PTX treatment can reprogram macrophages from an M2 profile to an active and inflammatory M1 phenotype [9]. These processes trigger the release of proinflammatory cytokines, leading to the sensitization of nociceptive neurons [10, 11].

The neuronal injury induced by PTX can also be the initial stimulus for developing a local inflammatory response in the DRGs [12]. Besides, PTX can disrupt axonal transport [13–15], causing a decrease in intra-epidermal nerve fibers (IENFs). These processes have been associated with CIPN symptoms such as allodynia and hyperalgesia [16–18]. However, despite its clinical relevance, the currently available treatments for CIPN are ineffective in most cases [19–21].

Cannabinoids are used clinically to treat many conditions, including neuropathic pain [22, 23]. However, several side effects caused by the central activation of cannabinoid CB1 receptors limit the widespread therapeutic use of these compounds [24]. Cannabidiol (CBD) is a non-psychotomimetic phytocannabinoid that has been investigated for the treatment of neuropathic pain [25–27]. Our previous studies found that a fluorinated CBD analog, PECS-101 (former HUF-101), induces antinociceptive effects at lower doses than CBD in acute pain models. Part of these effects involved the activation of CB1 and CB2 receptors without producing the cannabinoid tetrad, which is typically induced by CB1 agonists such as delta-9-tetrahydrocannabinol (THC) [28].

In addition to the cannabinoid receptors, CBD can exert its effects through peroxisome proliferator-activated receptors (PPARs) [21, 29–33]. PPARs, which belong to the nuclear receptor superfamily, have three isoforms: α, β, and γ. They participate in lipid and glucose metabolism and inflammatory responses [34]. PPARγ agonists have been associated with improved cancer chemotherapy efficacy and decreased neuropathic pain [35].

Here, using the PTX-induced neuropathic pain model, we assessed the effects of PECS-101 in reversing and preventing neuropathic pain, the upregulation of pro-nociceptive cytokines, and the loss of IENFs caused by PTX. The involvement of CB1, CB2, and PPARγ receptors in these effects was also tested. Finally, we evaluated whether PECS-101 interferes with the chemotherapy efficacy of PTX and causes changes in motor coordination and morphine-like rewarding effects.

Material and Methods

Animals

Most of the experiments were performed in male C57BL/6 mice ($n = 292$). Female BALB/c mice ($n = 33$) were used in the in vivo breast tumor model experiment. Male Nav1.8$^{Cre/+}$-Td-tomato$^{flox/+}$ mice (Nav1.8^{Tdtomato} mice, $n = 12$) were employed to study the drug effects on intra-epidermal nerve fibers. Male and female LysM$^{Cre/-}$-PPARγ$^{flox/flox}$ ($n = 28$) and LysM$^{-/-}$-PPARγ$^{flox/flox}$ mice ($n = 26$) were used to investigate if PECS-101 effects involve the activation of PPARγ receptors in Lysm cells (macrophages). At the time of the experiments, animals were 7–8 weeks old and weighting 20–30 g. C57BL/6 and BALB/c mice were obtained from the Central Animal House of the Ribeirao Preto Medical School, University of Sao Paulo. Nav1.8^{Tdtomato} mice were generated by breeding Nav1.8$^{Cre/Cre}$ [36] with Td-tomato$^{flox/flox}$ mice (Ai14, [37]) purchased from Jackson Laboratory. Macrophage-specific PPARγ-deficient mice were generated by crossing LysM$^{Cre/-}$ [9] and PPARγ$^{flox/flox}$ mice [38] purchased from Jackson Laboratory. The genotype of conditional mice was confirmed by standard PCR analysis. Primer pairs: LysM $^{Cre/-}$—mutant (5′-CCCAGAAATGCCAGATTACG-3′), common (5′-CTTGGGCTGCCAGAATTTCTC-3′), and WT (5′-TTACAGTCGGCCAGGCTGAC-3′); PPARγ$^{flox/flox}$—forward (5′-TGGCTTCCAGTGCATAAGTT-3′) and reverse (5′-TGTAATGGAAGGGCAAAAGG-3′). Mice were housed in groups of 5 mice/cage under a 12-h light cycle (lights on at 7 a.m.) with food and water ad libitum. All behavioral tests were performed from 8 a.m. to 6 p.m. Procedures were in accordance with the International Association for the Study of Pain (IASP) guidelines. Furthermore, they were approved by the Institutional Animal Care and Use Committee of the Ribeirão Preto Medical School (protocols #100/2016 and #005/2020–1).

Drugs

Paclitaxel (PTX, ONTAX®, Libbs), PECS-101 (synthesized by Prof. Raphael Mechoulam group, Hebrew University of Jerusalem, Israel) [39], AM251 (CB1 antagonist; Tocris), AM630 (CB2 antagonist; Tocris), GW9662 (PPARγ antagonist; Tocris), diazepam (Tocris), and morphine (Prati-Donaduzzi) were used. PTX, diazepam, and morphine were diluted in sterile isotonic saline. PECS-101 and GW9662 were dissolved in Tween 80 2% in sterile isotonic saline. AM251 and AM630 were dissolved in DMSO 2% in sterile isotonic saline.

Experimental Design

Protocols for the Prevention of Mechanical and Cold Allodynia Induced by Paclitaxel

In our first protocol, animals received vehicle or PTX (8 mg/kg; i.p.) on days 0, 2, 4, and 6 and PECS-101 injections (3, 10, or 30 mg/kg/daily; i.p.) or vehicle from the day 0 to 14.

Treatment time was based on a previous study that used CBD in the same model [26]. Based on the results of this first protocol (see below), we ran a second protocol in which, in addition to receiving vehicle or PTX (8 mg/kg; i.p.) on days 0, 2, 4, and 6, the mice were treated with PECS-101 (1 and 3 mg/kg/daily; i.p.) or vehicle for a shorter period, from days 0 to 7. On the days when the animals received the two compounds, PECS-101 was administered 30 min before PTX [40]. To evaluate the mechanical and cold allodynia induced by PTX, the von Frey and acetone tests were performed on days 0, 1, 3, 5, 7, 10, 14, and 21. On the days the animals received the treatments, the behavioral tests were performed before the drug administration. For macrophage-specific PPARγ-deficient mice, the second protocol was performed and the right hind paw withdrawal frequency using the 0.16 g von Frey filament was also evaluated [41]. Doses of PECS-101 were based on a previous study from our group [28]. The experiment was blind to the drug treatment in all behavioral tests.

Protocols for the Reversal of Mechanical and Cold Allodynia Induced by Paclitaxel

Mice received vehicle or PTX injection (8 mg/kg; i.p.) on days 0, 2, 4, and 6. From days 7 to 21, the animals received vehicle or PECS-101 (3, 10, or 30 mg/kg/daily; i.p). Mechanical allodynia was evaluated using von Frey filaments on days 0, 1, 3, 5, 7, 10, 12, 15, 18, 20, and 22. To evaluate the acute effect of PECS-101, after 24 h of the last PTX injection (day 7), animals received a single PECS-101 injection (3, 10, and 30 mg/kg; i.p.), and mechanical allodynia was evaluated 1, 3, 5, and 7 h after the treatment.

Involvement of CB1, CB2, and PPARγ Receptors in the Effects of PECS-101

AM251 (0.3 mg/kg; CB1 antagonist), AM630 (0.3 mg/kg; CB2 antagonist), or GW9662 (2 mg/kg; PPARγ antagonist) were administered (i.p.) 30 min before PECS-101 (1 mg/kg) from days 0 to 7. PTX (8 mg/kg) was administered 30 min after PECS-101, on days 0, 2, 4, and 6. The doses of AM251, AM630, and GW9662 were based on previous studies from our group [33, 42].

Mechanical Nociception Tests

Animals were placed on an elevated wire grid. After 60 min of acclimation, the plantar surface of the right hind paw was stimulated with a series of ascending force von Frey filaments using the up-down method (Stoelting, Chicago, IL, USA) with logarithmically increasing stiffness (0.008–1.0 g). The filament with the least strength capable of inducing a paw withdrawal response was recorded as the animal's mechanical threshold. The equation $\log_{10}$ (withdrawal threshold value in g × 1000) was used, and the data were expressed by mg, log. [43]. To better characterize macrophage-specific PPARγ-deficient mice, another mechanical behavioral test was carried out, in which the frequency of right hind paw withdrawal after 10 applications of the 0.16 g von Frey filament with 5 min interval in each application was evaluated [41].

Acetone Test

Evaporative cooling by the acetone drop method measured cold allodynia after the von Frey test in the same apparatus. Briefly, 50 µl of acetone was instilled on the plantar surface of the right hind paw using a syringe of 1 ml (Tuberculin slip tip, BD, Franklin Lake, NJ, USA). The animals were observed for 1 min, and nociceptive behavior was expressed as reaction time in seconds. We considered flinching, licking, biting, and shaking as positive responses to the acetone test [41].

Rotarod Test

The rotarod was carried out to evaluate the potential effects of PECS-101 on motor coordination. The apparatus consisted of a bar with a diameter of 2.5 cm subdivided into five compartments by disks of 25 cm in diameter (Ugo Basile, Model 7600). For habituation, the animals were initially kept on the bar rotating at a constant speed of 22 rotations per minute for 5 min before starting the drug treatments. On the test day, 24 h after the last injection of PECS-101 and PTX, mice were again placed on the rotarod, and the latency to fall was measured. The cutoff time was 180 s. Diazepam (10 mg/kg; i.p.) was used as a positive control 30 min after injection [44].

Conditioned Place Preference Test

The conditioned place preference (CPP) was performed to evaluate whether PECS-101 would induce morphine-like rewarding effects. CPP was carried out in an acrylic box consisting of two equal-sized chambers with different walls connected by a central compartment. One of the chambers had the walls painted with vertical stripes, and the floor consisted of a removable metal grid with parallel, equally spaced rods. The other chamber had its walls painted with horizontal stripes and a metal floor with circular holes. The light intensity was similar among the three compartments. In the pre-test (day 0), each mouse was placed in the central compartment and allowed to freely explore all compartments for 15 min. Mice that spent more than 70% of the session time in one compartment were excluded. The animals were

randomly assigned to the experimental treatments in the conditioning phase (days 1–6). They received saline (i.p.), PECS-101 (3 or 30 mg/kg; i.p.), or morphine (20 mg/kg; i.p., as positive control) on days 1, 3, and 5 and were immediately confined to one of the compartments (drug paired side) for 30 min. On alternate days (2, 4, and 6), mice were injected with saline and confined to the other compartment for 30 min. Finally, on the test day (day 7), each mouse was placed in the central compartment and freely explored all compartments for 15 min [45]. The exploration time of the compartments was evaluated with the assistance of the ANY-maze software (Stoelting). The CPP score was defined using the following equation: CPP score = time spent in the drug paired side during the test day − time spent in the drug paired side during the pre-test.

Quantitative Real-Time PCR

At 6 h or 3, 7, 10, 14, and 21 days after the treatment only with PTX and 6 h, 3, 5, and 7 days after the treatment with PECS-101 and PTX, the animals were anesthetized and then perfused with PBS. DRGs (L3–L5) were collected and homogenized in Trizol reagent (Sigma) at 4 °C. The RNA was quantified and then converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using a StepOnePlus Real-Time PCR system (Applied Biosystems). The gene expression of *Il1β*, *Tnf*, *Il6*, and *Aifi1* was determined relative to GAPDH, and the fold change was calculated using the $2^{-\Delta\Delta CT}$ threshold cycle method [46, 47]. Primer pairs for mouse *Gapdh*, *Il1β*, *Tnf*, *Il6*, and *Aif1*were as follows:

Gapdh fwd: 5′-GGGTGTGAACCACGAGAAAT-3′
Gapdh rev: 5′-CCTTCCACAATGCCAAAGTT-3′
Il1β fwd: 5′-TGACAGTGATGAGAATGACCTGTTC-3′
Il1β rev: 5′-TTGGAAGCAGCCCTTCATCT-3′
Tnf fwd: 5′-AGCAAGCAGCCAACCAGG-3′
Tnf rev: 5′-GCCACAAGGAGGAATGAGAAG-3′
Il6 fwd: 5′-AGCTGGAGTCACAGAAGGAGTGGC-3′
Il6 rev: 5′-AGGCATAACGCACTAGGTTTGCCGA-3′
Aif1 fwd: 5′-GCTTCAAGTTTGGACGGCAG-3′
Aif1 rev: 5′-TGAGGAGCCATGAGCCAAAG-3′

Immunostaining and Quantification of Intra-epidermal Nerve Fibers

For IENF analysis, Nav1.8$^{Cre/-}$-Td-tomato$^{flox/+}$ mice, which expressed the red fluorescent protein (Td-tomato) on voltage-gated sodium channel 1.8 (Nav1.8)-expressing peripheral sensory neurons, were treated with PECS-101 (1 mg/kg) from days 0 to 7 and PTX on days 0, 2, 4, and 6. Twenty-four hours after the last injection, the animals were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and then perfused with PBS and PFA 4%. The hind paw's glabrous skin was excised and post-fixed for 2 h in PFA 4% and then transferred to a 30% sucrose solution overnight at 4 °C. The samples were embedded in Tissue-Tek® O.C.T.™ (Sakura Finetek) and sectioned at 40 µm. All sections were blocked with 1% BSA in 0.1% Triton X100 for 1 h at room temperature and incubated overnight at 4 °C with antibodies against Collagen IV (Goat Anti-Type IV Collagen; SB; cat. 1340–1; 1:500) followed by AlexaFluor-488 (anti-goat; Invitrogen; cat. A11055; 1:1000). Sections were visualized using a Leica fluorescence microscope (Leica TSE-SPE), and 4 to 5 randomly chosen sections from each paw were quantified using the 40× objective. The ImageJ software measured the number of nerve fibers crossing the collagen-stained dermal/epidermal junction into the epidermis and the epidermis length within each field. IENF density was expressed as the total number of fibers/length of the epidermis (IENFs/mm) [48].

Culture of Human and Murine Cell Lines

Cells of murine and human breast cancer (4T1 and MCF-7, respectively—ATCC), and human colon cancer (HCT-116—ATCC), were cultured in DMEM medium with 10% FBS, 100 U/ml penicillin, and 2 µg/ml amphotericin B and maintained in an incubator with 5% CO_2 at 37 °C and 95% humidity. Cells ranging from 70 to 80% of confluence were detached with trypsin–EDTA 0.25% and washed in PBS twice. After four passages, all cancer cell suspensions (1×10^4 cells/well) were seeded into 96-well plates. 4T1 cells were treated with PTX (0.1, 0.3, 1, 3, and 10 µM). In a second experiment, 4T1 cells were plated in the presence of PTX (3 and 10 µM) and PECS-101 (0.1, 0.3, 1, and 3 µM). MCF-7 and HCT-116 were plated in the presence of PTX (3 or 10 µM) and PECS-101 (0.3, 1, 3, 10, or 30 µM) [49, 50]. Twenty-four hours after the treatment, the cells were incubated with 150 µl of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; Sigma) at 1 mg/ml for 4 h, at 37 °C. The medium was removed, and 150 µl of absolute alcohol was added to each well to dissolve the dark blue crystals. The optical density of the individual samples was measured at 570 nm (Spectra Max-250, Molecular Devices). Cell viability (%) was calculated as the MTT product absorbance in the treated cells/control cells × 100 [51].

In Vivo Breast Tumor Model

Female BALB/c mice were subcutaneously inoculated with 5×10^4 4T1 cells of the breast cancer cell line 4T1 [9]. After 9 days, the animals were randomly divided into four groups that received vehicle + vehicle; vehicle + PTX (8 mg/kg; i.p.) administered on days 9, 11, 13, 15, 17, and 19; PECS-101

(1 mg/kg; i.p.) administered from days 9 to 21 + vehicle; or PTX (8 m/kg; i.p) administered on days 9, 11, 13, 15, 17, and 19, 30 min after PECS-101 injection. Twenty-four hours after the last injection, animals were euthanized for tumor weight assessment.

Data Analysis

Data were presented as the mean ± SEM and analyzed through repeated measures ANOVA (rmANOVA) and one- and two-way ANOVA, followed by Newman-Keuls (S–N-K) or Dunnett's post hoc test. $p < 0.05$ was considered statistically significant.

Results

PECS-101 Prevents but Does Not Reverse PTX-Induced Neuropathic Pain Behaviors

The drugs available for preventing or treating PTX-induced neuropathic pain have limited efficacy. Therefore, there is a significant need to identify effective pharmaceutical therapies to relieve this debilitating condition [52]. Initially, we evaluate whether PECS-101 would prevent PTX-induced neuropathic pain. We observed that the chronic treatment for 14 days with PECS-101 prevented mechanical allodynia induced by PTX (treatment $F_{4,20} = 128.01$; time $F_{6,120} = 38.89$; and interaction between treatment and time $F_{24,120} = 17.84$, $p < 0.05$, rmANOVA). S–N-K post-test revealed that while the treatment PECS-101, at 3 and 10 mg/kg, prevented mechanical allodynia, at 30 mg/kg, PECS-101 only attenuated this effect (Supplementary Fig. S1). Based on these findings, we next analyzed whether the treatment for 1 week with low doses of PECS-101 (1 and 3 mg/kg) would prevent PTX-induced allodynia. PECS-101, at 1 and 3 mg/kg, caused a long-term effect, preventing PTX-induced mechanical allodynia up to 2 weeks after treatment (treatment $F_{3,24} = 36.56$; time $F_{6,144} = 4.74$; and interaction between treatment and time $F_{18,144} = 2.66$, $p < 0.05$, rmANOVA; Fig. 1a). In this treatment regimen, PECS-101 also prevented cold allodynia induced by PTX (treatment $F_{3,24} = 25.80$, $p < 0.05$; but there was no effect of time $F_{6,144} = 0.60$ and interaction between treatment and time $F_{18,144} = 0.61$, $p > 0.05$, rmANOVA; Fig. 1b). However, when PECS-101 was administered after the end of the PTX treatment (from days 7 to 21), it did not reverse PTX-induced mechanical allodynia (Supplementary Fig. S2a, b). These data indicate that PECS-101 co-administered with PTX for 1 week prevents PTX-induced mechanical and cold allodynia.

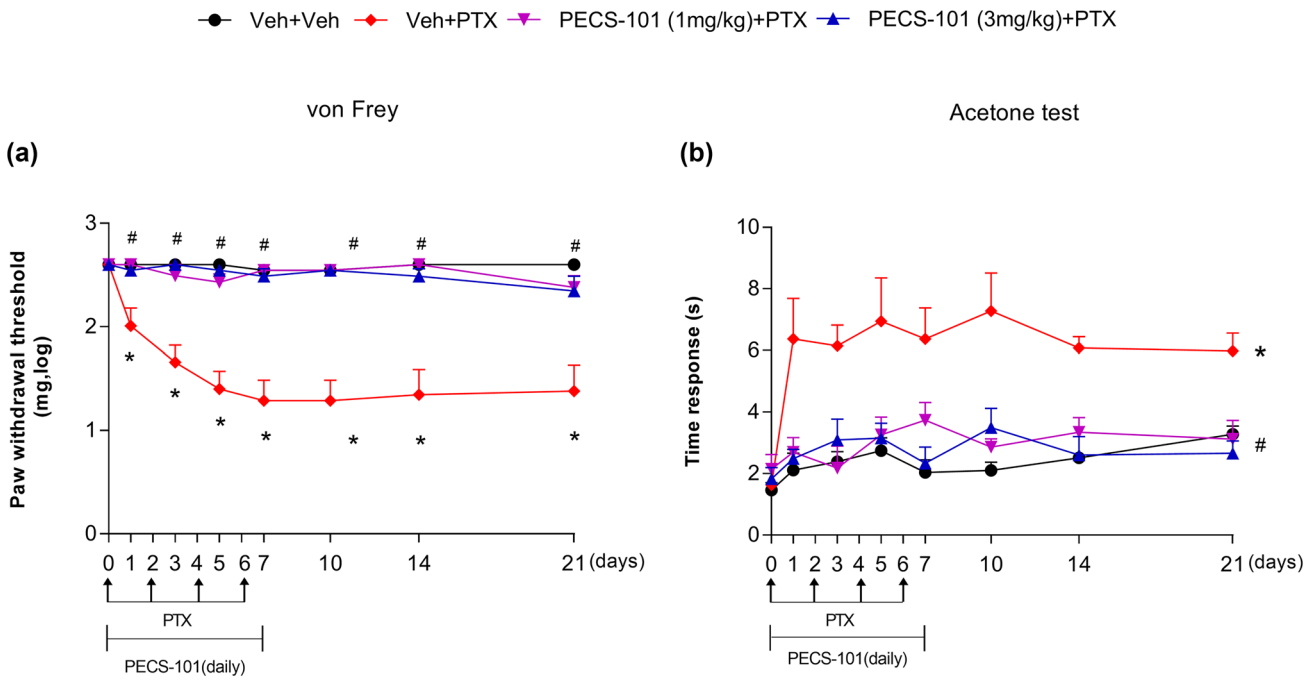
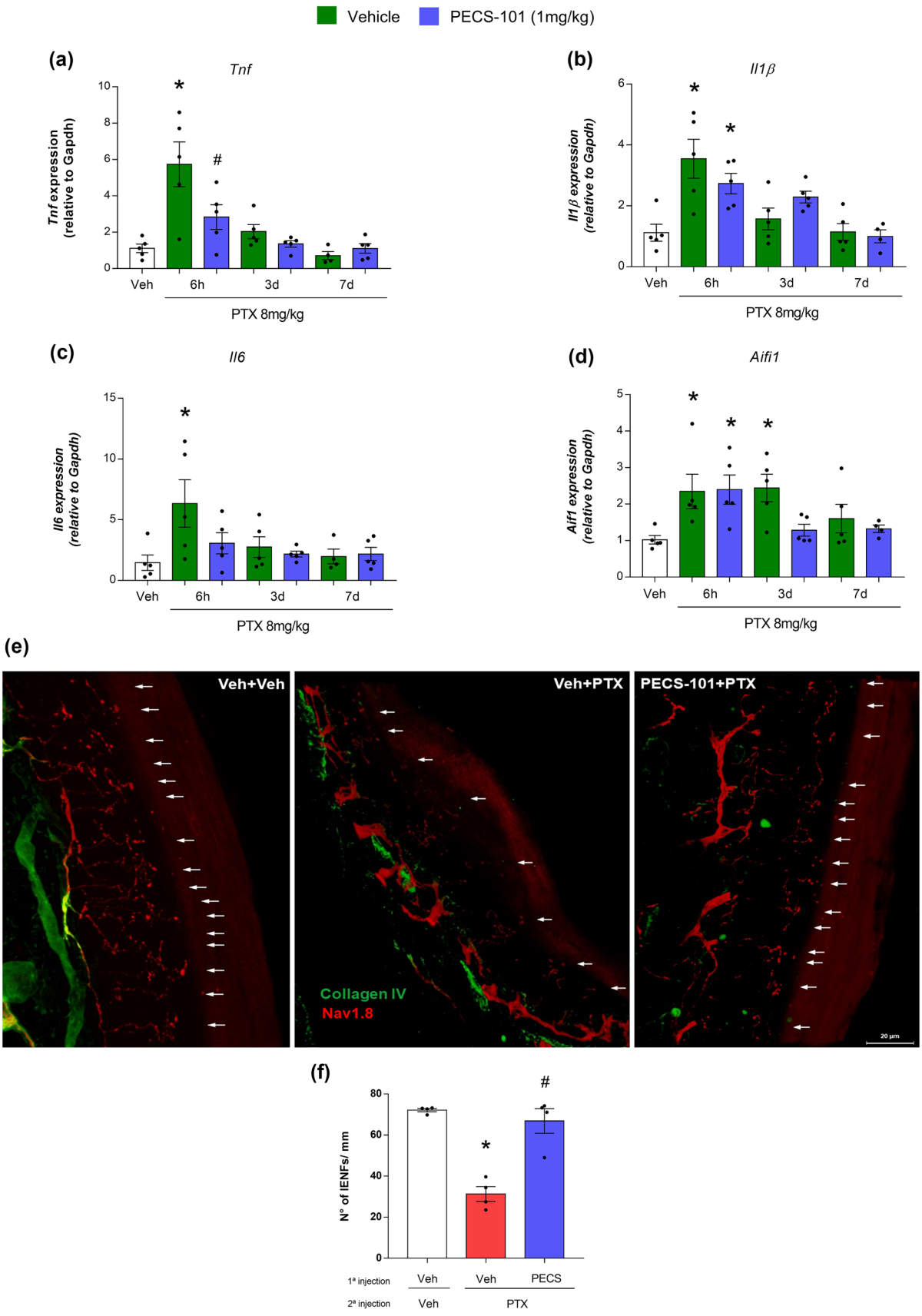


Fig. 1 PECS-101 prevents mechanical and cold allodynia induced by PTX. For mechanical allodynia evaluation, PTX (8 mg/kg) was administered on days 0, 2, 4, and 6 and (**a**) PECS-101 (1 and 3 mg/kg) was administered from day 0 to day 7. For cold allodynia evaluation, PTX (8 mg/kg) was administered on days 0, 2, 4, and 6 and (**b**) PECS-101 (1 and 3 mg/kg) was administered from day 0 to day 7. Data represent the mean ± SEM. $^{*}p < 0.05$ from Veh + Veh group, $^{\#}p < 0.05$ from Veh + PTX group; $n = 7$/group


Vehicle
PECS-101 (1mg/kg)
(a)
Tnf
Tnf expression (relative to Gapdh)
Veh
6h
3d
7d
PTX 8mg/kg
(b)
Il1β
Il1β expression (relative to Gapdh)
(c)
Il6
Il6 expression (relative to Gapdh)
(d)
Aif1
Aif1 expression (relative to Gapdh)
(e)
Veh+Veh
Veh+PTX
PECS-101+PTX
Collagen IV
Nav1.8
20 μm
(f)
N° of IENFs/ mm
1ª injection
2ª injection
Veh
PECS
PTX

◂**Fig. 2** PECS-101 attenuates the increase in gene expression of pro-inflammatory cytokines and *Aifi1* and prevents the reduction in the density of IENFs caused by PTX. After 6 h, 3, 5, and 7 days of PTX (8 mg/kg) and PECS-101 (1 mg/kg) treatment, the gene expression of (**a**) *Tnf*, (**b**) *Il1β*, (**c**) *Il6*, and (**d**) *Aifi1* was evaluated ($n = 4$–5/group). Nav1.8^{Tdtomato} mice received injections of PECS-101 (1 mg/kg) from day 0 to day 7 and PTX (8 mg/kg) on days 0, 2, 4 and 6. Twenty-four hours after the last injection, the IENFs were analyzed. (**e**) Representative images and (**f**) quantification of IENFs ($n = 4$/group). Data represent the mean ± SEM. $^{*}p < 0.05$ from Veh + Veh group, $^{\#}p < 0.05$ from Veh + PTX group

PECS-101 Treatment Reduces the Upregulation of Pro-nociceptive Cytokines in the DRGs

Evidence indicates that PTX induces neuropathic pain by stimulating the release of pro-nociceptive cytokines (e.g., TNF, IL-1β, Il-6) in the DRGs [7, 53, 54]. Thus, we investigated whether the effect of PECS-101 on PTX-induced neuropathic pain would be associated with a reduction in these cytokines. Initially, we evaluated the gene expression of these proinflammatory cytokines and *Aifi1* (a marker of macrophage activation/accumulation) in the DRGs after PTX treatment at different time points. PTX increased the gene expression of *Tnf*, *Il1b*, *Il6*, and *Aifi1* in the DRGs compared to the vehicle group. The increased expression of *Tnf*, *Il1b*, and *Il6* was observed 6 h after the first PTX injection (Supplementary Fig. S3). For *Aif1*, the increase in gene expression occurred 6 h and on day 3 after PTX (Supplementary Fig. S3, (a) *Tnf* $F_{6,27} = 8.14$; (b) *Il1b* $F_{6,28} = 4.84$; (c) *Il6* $F_{6,27} = 4.56$; and (d) *Aif1* $F_{6,28} = 3.55$, $p < 0.05$; one-way ANOVA followed by Dunnett's). Notably, PECS-101 (1 mg/kg) attenuated the PTX-induced increase in gene expression of *Tnf*, *Il6*, and *Aif1*, but not *Il1b*, 6 h after treatment (Fig. 2, (a) *Tnf* $F_{6,27} = 8.63$; (c) *Il6* $F_{6,27} = 2.80$; and (d) *Aif1* $F_{6,27} = 3.36$, $p < 0.05$; (b) *Il1b* $F_{6,27} = 7.11$, $p > 0.05$, one-way ANOVA followed by S–N-K and Dunnett's). These data indicate that PECS-101 reduces the levels of proinflammatory cytokines and a macrophage marker in DRG after PTX treatment.

PECS-101 Prevents the Loss of IENFs Caused by PTX

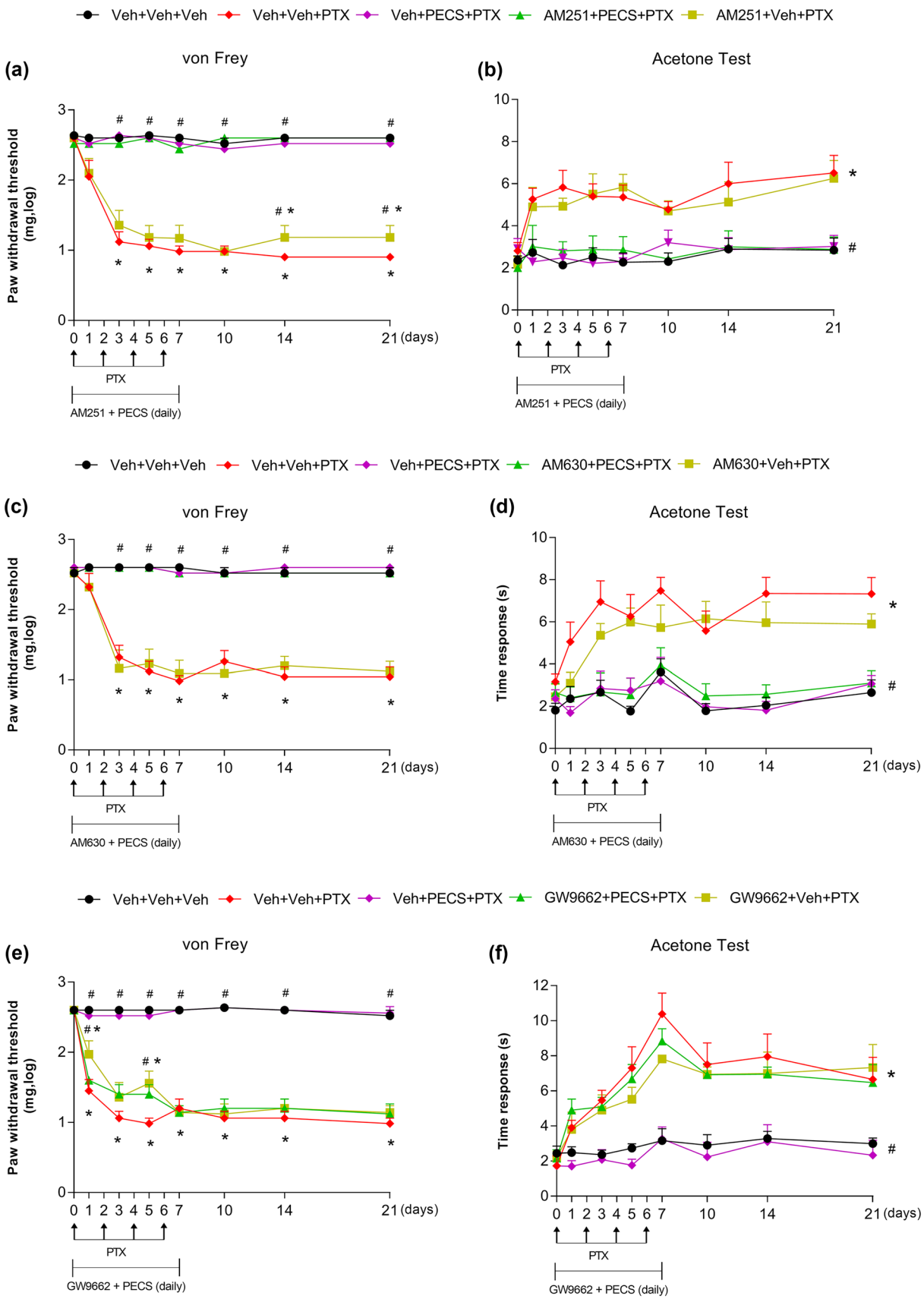
The decreased density of sensory nerve fibers in the periphery is a hallmark of CIPN [55]. Then, we investigated whether PECS-101 would affect PTX-induced loss of IENFs**.** For that, PTX-induced peripheral neuropathy was developed in Nav1.8^{Tdtomato} mice that received PECS-101 or vehicle treatment. PECS-101 (0–7 days) prevented the decrease in the density of IENFs caused by PTX ($F_{2,9} = 29.95$, $p < 0.05$, one-way ANOVA followed by S–N-K; Fig. 2e, f). These results suggest that PECS-101 effects in preventing PTX-induced neuropathic pain may be associated with preventing sensory nerve fiber loss.

PECS-101 Prevents PTX-Induced Neuropathic Pain Dependent on PPARγ Activation, but not by CB1 and CB2 Receptors

The involvement of PPARγ and cannabinoid receptors in the effects of drugs that relieve PTX-induced neuropathic pain has been described [35, 56]. Based on these findings, we investigated the involvement of CB1, CB2, and PPARγ receptors on the antinociceptive effects of PECS-101 upon PTX-induced neuropathic pain. All antagonists were administered 30 min before PECS-101 injection. The CB1 antagonist AM251 (0.3 mg/kg) and the CB2 antagonist AM630 (0.3 mg/kg) did not reverse PTX-induced mechanical and cold allodynia (Fig. 3a–d). On the other hand, the pre-treatment with the PPARγ antagonist GW9662 (2 mg/kg) blocked PECS-101 effects in preventing mechanical (treatment $F_{4,20} = 91.83$; time $F_{6,120} = 10.83$; and interaction between treatment and time $F_{24,120} = 3.60$, $p < 0.05$, rmANOVA; Fig. 3e) and cold allodynia (treatment $F_{4,20} = 27.12$; time $F_{6,120} = 11.39$, $p < 0.05$; there was no interaction between treatment and time $F_{24,120} = 1.19$, $p > 0.05$, rmANOVA; Fig. 3f) induced by PTX. Altogether, these results suggest that PECS-101 effects in mechanical and cold allodynia depend on PPARγ activation, but not on the activation of CB1 and CB2 receptors.

PPARγ Signaling in Macrophage Is Necessary to PECS-101 Effects in Preventing PTX-Induced Neuropathic Pain

PPARγ located at the macrophages is implicated in pain processing [57–59]. Thus, we investigated the effect of PECS-101 on PTX-induced neuropathic in macrophage-specific PPARγ-deficient mice (LysM$^{Cre/-}$-PPARγ$^{flox/flox}$). In addition, using these animals, we also assessed the impact of sex differences on the PTX and PECS-101 effects. No sex versus treatment interaction was found in von Frey ($F_{4,44} = 1.51$, $p = 0.213$), withdrawal frequency test ($F_{4,44} = 0.139$, $p = 0.967$), and cold allodynia ($F_{4,44} = 0.537$, $p = 0.709$) data not shown. There was, however, a significant treatment versus time interaction in mechanical and cold assessments (von Frey $F_{28,308} = 9.28$, $p < 0.05$; withdrawal frequency $F_{28,308} = 4.35$, $p < 0.05$; cold test $F_{28,308} = 3.72$, $p < 0.05$; Fig. 4). S–N-K post-test revealed that PTX induced mechanical and cold allodynia in LysM$^{Cre/-}$-PPARγ$^{flox/flox}$ and LysM$^{-/-}$-PPARγ$^{flox/flox}$ mice. At the same time, PECS-101, at 1 mg/kg, did not prevent mechanical and cold allodynia in macrophage-specific PPARγ-deficient mice (LysM$^{Cre/-}$-PPARγ$^{flox/flox}$). No significant differences in animal weight were observed (Supplementary Fig. S4). These results suggested that PPARγ in macrophages is necessary for the PECS-101 to prevent PTX-induced neuropathic pain in a sex-independent manner.


Veh+Veh+Veh
Veh+Veh+PTX
Veh+PECS+PTX
AM251+PECS+PTX
AM251+Veh+PTX
(a)
von Frey
Paw withdrawal threshold (mg,log)
PTX
AM251 + PECS (daily)
(b)
Acetone Test
AM251 + PECS (daily)
Veh+Veh+Veh
Veh+Veh+PTX
Veh+PECS+PTX
AM630+PECS+PTX
AM630+Veh+PTX
(c)
von Frey
AM630 + PECS (daily)
(d)
Acetone Test
Time response (s)
AM630 + PECS (daily)
Veh+Veh+Veh
Veh+Veh+PTX
Veh+PECS+PTX
GW9662+PECS+PTX
GW9662+Veh+PTX
(e)
von Frey
GW9662 + PECS (daily)
(f)
Acetone Test
GW9662 + PECS (daily)
21 (days)

◂**Fig. 3** A PPARγ receptor antagonist, but not CB1 or CB2 antagonists, blocks the effects of PECS-101 in preventing mechanical and cold allodynia induced by PTX. The antagonists were administered 30 min before PECS-101 (1 mg/kg) injection from days 0 to day 7. PTX (8 mg/kg) was administered on days 0, 2, 4 and 6. Effects of the CB1 antagonist AM251 (0.3 mg/kg) on (**a**) mechanical and (**b**) cold allodynia. Effects of the CB2 antagonist AM630 (0.3 mg/kg) on (**c**) mechanical and (**d**) cold allodynia. Effects of the PPARγ antagonist GW9662 (2 mg/kg) on (**e**) mechanical and (**f**) cold allodynia. Data represent the mean ± SEM. $^{*}p < 0.05$ from Veh + Veh + Veh group, $^{\#}p < 0.05$ from Veh + Veh + PTX group; $n = 5$ group

PECS-101 Does Not Alter Motor Coordination or Produce Addiction-Like Effects

Analgesic agents used in neuropathic pain conditions are associated with some unwanted side effects [20, 25, 60–62]. We analyzed whether PECS-101 would impair motor coordination and induce morphine-like rewarding effects. Unlike diazepam, PECS-101 (1 mg/kg) did not alter motor coordination in the rotarod test (Fig. 5a). In the CPP test, PECS-101 treatment did not induce morphine-like rewarding effects (Fig. 5b). Unlike classical drugs used in neuropathic

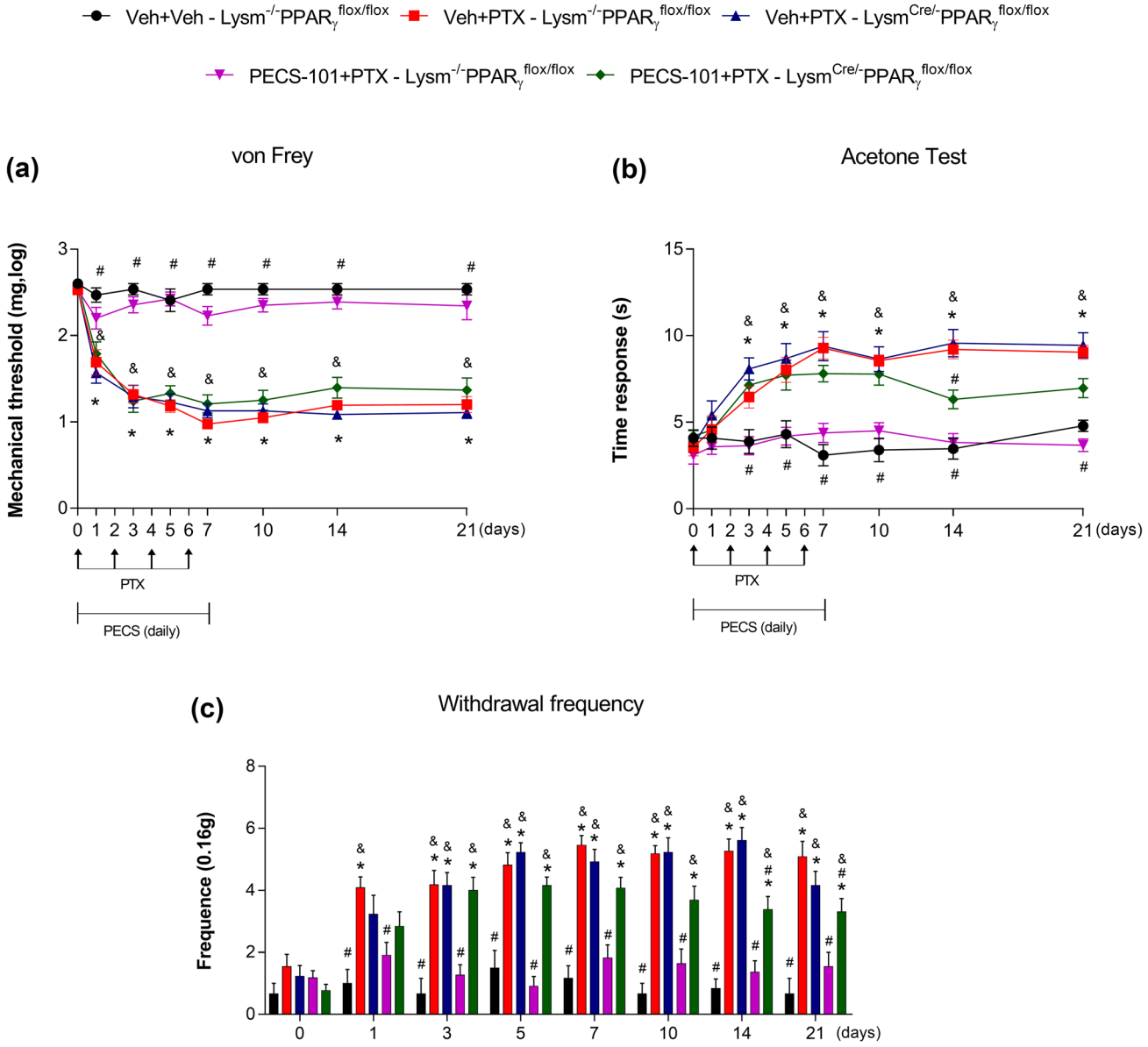


Fig. 4 PPARγ receptors in macrophages are required for the PECS-101 effects in preventing PTX-induced neuropathic pain in a sex-independent manner. PECS-101 (1 mg/kg) injection was administered from days 0 to 7 and PTX (8 mg/kg) was administered on days 0, 2, 4, and 6. For mechanical and cold evaluations, von Frey (**a**), withdrawal frequency (**c**), and acetone test (**b**) were performed in male and female knockout mice. Data represent the mean ± SEM. $^{*}p < 0.05$ from Veh + Veh-Lysm$^{-/-}$PPARγ$^{flox/flox}$, $^{\#}p < 0.05$ from Veh + PTX-Lysm$^{-/-}$PPARγ$^{flox/flox}$ and $^{\&}p < 0.05$ from PECS-101 + PTX-Lysm$^{-/-}$PPARγ$^{flox/flox}$; $n = 6$–13/group

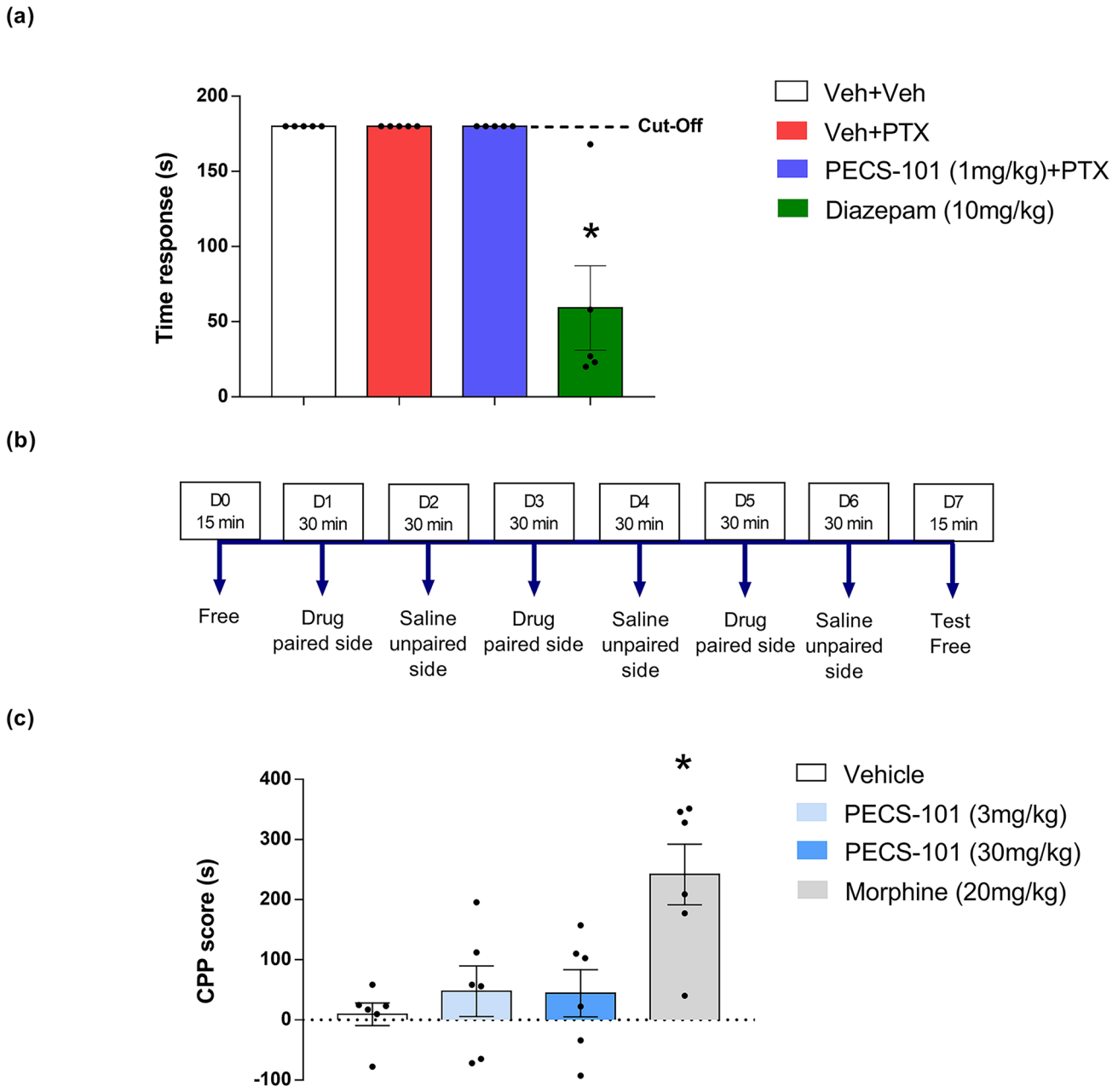


Fig. 5 PECS-101 does not impair motor coordination nor cause conditioned place preference. (**a**) Effects of PECS-101 (1 mg/kg) treatment from day 0 to day 7 alone or in association with PTX (8 mg/kg) on days 0, 2, 4, and 6 on motor coordination after 24 h of the last injection and diazepam (10 mg/kg) administered 30 min before the rotarod test ($n=5$/group). (**b**) Experimental procedures of the conditioned place preference test. (**c**) Effects of PECS-101 (3 and 30 mg/kg) and morphine (20 mg/kg) treatments in the CPP test ($n=6$/group). Data represent the mean ± SEM. *$p<0.05$ from Veh + Veh or Sal

pain, PECS-101 does not seem to produce the common adverse effects associated with these treatments.

PECS-101 Does Not Impair the Chemotherapeutic Effect of PTX

It is important that new drugs used in CINP do not interfere with the chemotherapy efficacy. Moreover, multiple studies show that PPARγ agonists and cannabinoids can also act as an antitumor agent [25, 63, 64]. We analyzed whether PECS-101 would alter the antineoplastic effects of PTX. Initially, we built a concentration-effect curve for PTX in vitro murine breast cancer cell culture (4T1 cells). PTX (0.3, 1, 3, and 10 μM) decreased the viability of 4T1 cells ($F_{5,30}=83.37$, $p<0.05$, one-way ANOVA followed by S–N-K; Fig. 6a). Then, PECS-101 was tested, alone or in

combination with PTX, in different lines of cancer cells. In the 4T1 cells, PECS-101 (0.1–3.0 μM) did not alter the PTX effect in decreasing cell viability. In addition, PECS-101, at 1 μM, decreased cell viability by itself ($F_{14,45} = 39.73$, $p < 0.05$, one-way ANOVA followed by S–N-K; Fig. 6b). In the human breast and colon tumor cells (MCF-7 and HCT-116 cell lines, respectively), PECS-101 (0.3–30 μM) also did not interfere with PTX effects and decreased the percentage of cell viability by itself at 0.3–30 μM for MCF-7 and 3–30 μM for HCT-116 cell lines. Moreover, on human cancer cells, PECS-101 potentiated the PTX effect (MCF-7: Fig. 6c, F17,54 = 24.23, $p < 0.05$ and HCT-166: Fig. 6d, F17,54 = 33.06; $p < 0.05$, one-way ANOVA followed by S–N-K).

As expected, PTX decreased the tumor weight in a mouse model of breast cancer. PECS-101 (1 mg/kg) did not alter this effect. PECS-101 alone also decreased tumor weight (second injection $F_{1,29} = 5.76$, $p < 0.05$; there was no effect of the first injection $F_{1,29} = 2.17$, $p > 0.05$; and a trend for interaction between the first and second injection $F_{1,29} = 3.12$, $p = 0.08$, two-way ANOVA following S–N-K; Fig. 6e). These findings show that PECS-101 treatment does not interfere with chemotherapy efficacy.

Discussion

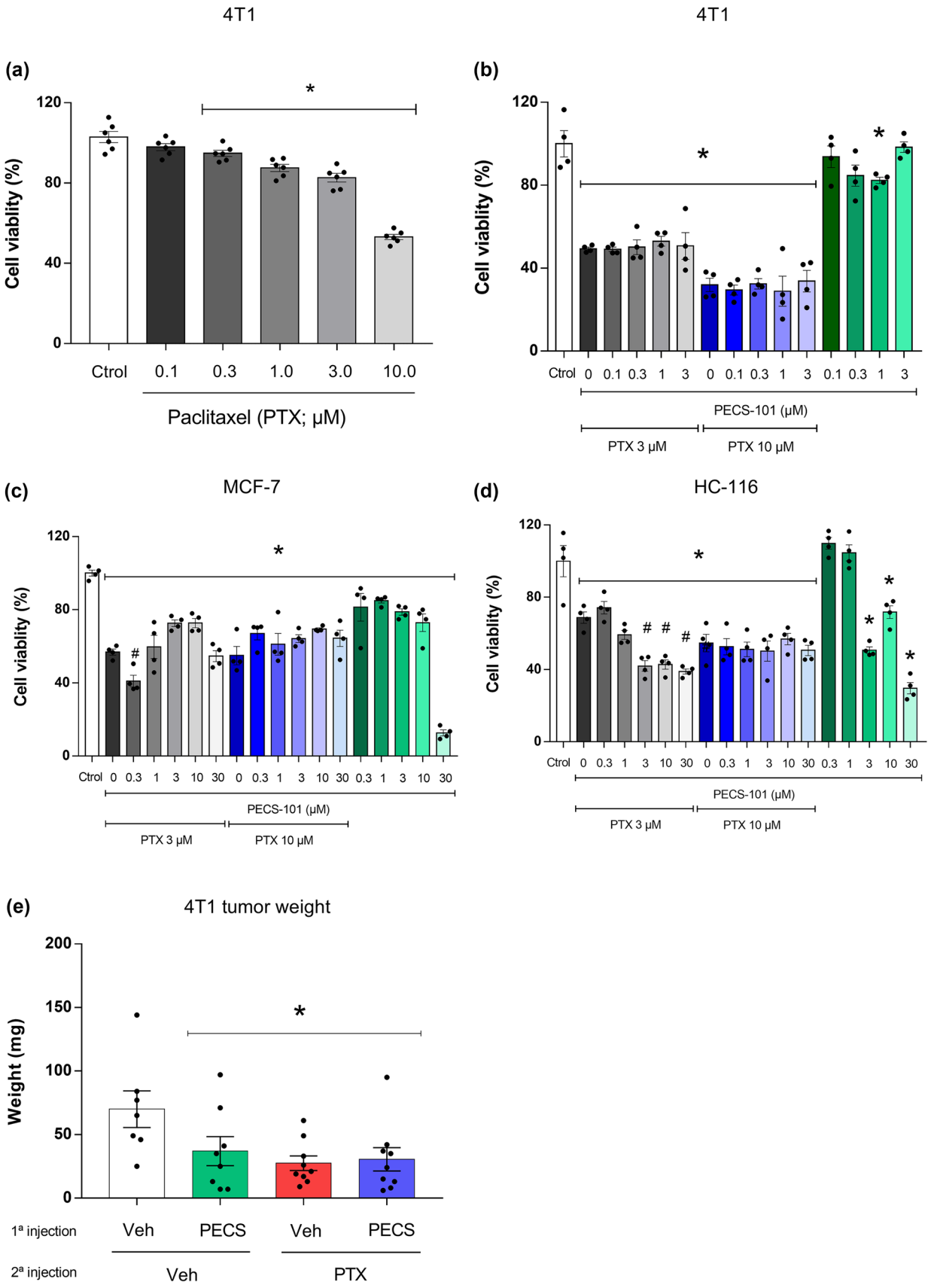
In the present study, we investigated the efficacy of PECS-101, a fluorinated CBD derivate, in a mouse CIPN model caused by PTX. Here, we showed that PECS-101 could prevent PTX-induced neuropathic pain behaviors. Furthermore, these effects of PECS-101 were associated with a prevention of the increase in pro-nociceptive cytokines expression in the DRG and the IENF loss. Mechanistically, the beneficial effect of PECS-101 on PTX-induced neuropathic pain depended on activation of PPARγ, but not CB1 and CB2 receptors. In addition, using macrophage-specific PPARγ-deficient mice, we observed that the PECS-101 effects involve the activation of PPARγ receptors in these cells. Importantly, PECS-101 did not cause tolerance, motor coordination impairment, and morphine-like rewarding effects. Finally, the dose of PECS-101 that prevented the development of PTX-induced neuropathic pain did not impair PTX chemotherapeutic activity.

Peripheral neuropathy induced by PTX is the main limiting adverse effect resulting from the use of this compound. It frequently requires a decrease in PTX dose or even the treatment interruption, leading to cancer progression [65]. The epidemiological data on the incidence of CIPN are variable, but this pathology can affect about 70% of patients who start chemotherapy treatment [1]. Despite advances in understanding the molecular mechanisms involved in CIPN, there is no effective drug for preventing and/or treating this condition. Furthermore, the compounds currently employed for its symptomatic relief are often associated with many side effects [19, 20]. Therefore, due to its higher incidence and inadequate therapeutic response to the available treatments, new drugs that could reverse or prevent the development of this pathology are needed.

Cannabinoids represent a promising therapeutic strategy for pain relief in several conditions, including CIPN [25, 66]. We had previously found that PECS-101, a CBD fluorinated analog, caused antinociceptive effects at lower doses than CBD in acute pain models [28]. In the present work, we investigated if this compound would also be useful in treating neuropathic pain behaviors (mechanical and cold allodynia) caused by repeated PTX treatment. PECS-101 failed to reverse CINP once it had been established. However, it can prevent its development when administered for 1 week at the low dose of 1 mg/kg during the PTX treatment. Cannabinoids produce analgesic effects by acting on multiple pain targets in the peripheral and central nervous systems [66]. Besides acting on the classical cannabinoid receptors, CB1 and CB2 [67], they may relieve pain by interacting with several other receptors such as GPCR55 [68], opioids [69], serotonin [70], transient receptor potential (TRP) channels [71], and PPARs [35].

In acute pain models, the antinociceptive effects of PECS-101 were at least partially mediated by the activation of CB1 and CB2 receptors [28]. However, the pre-treatment with CB1 and CB2 antagonists did not block PECS-101 effects in PTX-induced neuropathic pain behaviors in the present study. As described earlier, another important target for the anti-inflammatory effects of some cannabinoids is the PPAR receptor [72]. Unlike CB1 and CB2 antagonists, the PPARγ antagonist GW9662 blocked the mechanical and cold PECS-101 antiallodynic effects.

We also observed the involvement of PPARγ receptors in PECS-101 effects in preventing PTX-induced neuropathic pain using male and female macrophage-specific PPARγ-deficient mice. Although males presented higher values in mechanical assessments than females, no sex difference was observed in the PECS-101 and PTX effects. PPARγ receptors located in macrophages are implicated in pain processing. The activation of these receptors may alleviate neuropathic pain symptoms by switching macrophage polarization and preventing proinflammatory cytokines [57, 73–77]. Macrophages in the DRG of both male and female mice contribute to and are required for neuropathic pain initiation and maintenance [78]. PECS-101-activated PPARγ signaling caused a long-lasting relief in mechanical and cold allodynia when administered in the early phase of neuropathic pain development. Thus, through a possible PPARγ activation in macrophages, PECS-101 could prevent the sensitization of nociceptive neurons and pain persistence [10, 11].


4T1
(a)
Cell viability (%)
120
80
40
0
*
Ctrol
0.1
0.3
1.0
3.0
10.0
Paclitaxel (PTX; μM)
4T1
(b)
Cell viablity (%)
120
80
40
0
*
*
Ctrol 0 0.1 0.3 1 3 0 0.1 0.3 1 3 0.1 0.3 1 3
PECS-101 (μM)
PTX 3 μM
PTX 10 μM
(c)
MCF-7
Cell viability (%)
120
80
40
0
*
#
Ctrol 0 0.3 1 3 10 30 0 0.3 1 3 10 30 0.3 1 3 10 30
PECS-101 (μM)
PTX 3 μM
PTX 10 μM
(d)
HC-116
Cell viability (%)
120
80
40
0
*
# # #
*
*
*
Ctrol 0 0.3 1 3 10 30 0 0.3 1 3 10 30 0.3 1 3 10 30
PECS-101 (μM)
PTX 3 μM
PTX 10 μM
(e)
4T1 tumor weight
Weight (mg)
200
150
100
50
0
*
1ª injection
Veh
PECS
Veh
PECS
2ª injection
Veh
PTX

◂**Fig. 6** PECS-101 attenuates murine and human cancer cell viability and decreases the weight of breast tumors in female BALB/c mice without interfering with PTX effects. (**a**) Effects of PTX (0.1–10 μM) on murine breast cancer cell viability. (**b**) Effects of PECS-101 (0.1–3 μM) alone or associated with PTX (3 and 10 μM) on murine breast cancer cell viability. Effects of PECS-101 (0.3–30 μM) on human breast (**c**) and colon (**d**) cancer cell viability. (**e**) Effects of PECS-101 (1 mg/kg) treatment from day 9 to day 21 alone or in association with PTX (8 mg/kg) on days 9, 11, 13, 15, 17, and 19 in the weight breast tumors induced in female BALB/c mice, measured 24 h after the last injection of PECS-101 ($n = 7$–9/group). Data represent the mean ± SEM. $^{*}p < 0.05$ from control group (Ctrol), $^{\#}p < 0.05$ from PTX (3 μM) group, $^{*}p < 0.05$ from Veh + Veh

The anti-inflammatory effects of PPARγ agonists in neuropathic pain are related to suppressing the expression of inflammatory genes through a trans-repression mechanism that blocks the activity of transcription factors, such as NF-kB and AP1, that control cytokine production [79, 80]. PTX increases inflammatory cells and proinflammatory cytokines in the DGRs, spinal cord, and peripheral nerves [11, 53, 54, 81]. We observed that PECS-101 attenuated the increase in the gene expression of proinflammatory cytokines and a marker for macrophage in the DRGs caused by PTX. The inflammatory process observed in earlier stages of the PTX treatment contributes to the persistent neuropathic pain symptoms observed in PTX-induced neuropathy [11]. PTX can reprogram macrophages to an inflammatory M1 phenotype [9], and PPARγ receptors play an important role in inflammatory-associated processes [82].

Neuronal injury induced by PTX could be the initial stimulus for developing a local inflammatory response in the DRGs [12]. It has been suggested that this neuronal damage occurs mainly because PTX affects their cell bodies located in the DRGs, resulting in degeneration of nerve endings and a loss of sensory fibers [10, 83]. In the skin, IENFs are represented mainly by sensory fibers C and Aδ, which can be identified by molecular markers. Nav1.8 calcium channels are localized predominantly in small/medium nociceptive C/Aδ-type DRG neurons [17, 84–87]. Partial loss of these nerve fibers may lead to the hyperexcitability observed in peripheral neuropathies, which in turn would result in thermal and mechanical allodynia and hyperalgesia [10, 77, 88, 89]. Using the Nav1.8^{Tdtomato} mice, PECS-101 prevented the decrease in the number of IENFs caused by PTX. Similarly, neuroprotective effects of PECS-101 in spinal motor neurons and DRG sensory neurons were observed in a rat model of neonatal sciatic nerve axotomy [90].

In the treatment of CINP, it is essential that a drug does not induce tolerance, addiction, or motor impairment, as reported for other compounds [20, 60, 91, 92]. Also, it must not interfere with the chemotherapy efficacy. Tolerance and motor coordination impairment were not observed with PECS-101 treatment. Unlike morphine, PECS-101 did not induce positive rewarding effects in the CPP test, indicating that this compound has a low potential for addiction. This latter result agrees with our previous report that PECS-101 does not cause the cannabinoid tetrad, meaning that it does not act as typical CB1 agonists [28].

Regarding its antineoplastic effects, PECS-101 did not alter the decrease in cell viability of different cancer cells caused by PTX. At higher concentrations, PECS-101 by itself decreased the cell viability of all cancer cell lines tested. Moreover, in human breast and colon cancer cell lines, PECS-101 potentiated the effects of PTX. Similar results were observed in vivo, in which PECS-101 did not alter PTX effects but, by itself, decreased tumor weight. CBD has also been reported not to interfere in the antineoplastic effect of PTX on breast cancer cells in culture. It also produced a synergistic tumor inhibition effect when combined with PTX [25]. Even if preclinical and clinical studies have shown antitumoral effects of PPARγ agonists alone or in combination [93, 94], the mechanisms of these potential anticancer effects of PECS-101 remain to be investigated.

In conclusion, our findings indicate that PECS-101 could prevent PTX-induced neuropathy. This effect involves PPARγ activation in macrophages, leading to an inhibition of the inflammatory response in DRG and damage of sensory nerve fibers. Furthermore, PECS-101 does not seem to impair motor coordination or cause tolerance and addiction. Also, it does not interfere with the chemotherapeutic effect of PTX and may possess antitumor activity by itself. Taken together, these results suggest that PECS-101 could be a useful treatment for PTX-induced neuropathy.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13311-021-01164-w.

Acknowledgements We thank Ieda Regina dos Santos for her technical support.

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Funding This study was supported by grants from the National Institute of Science and Translational Medicine, CNPq (grant number 465458/2014–9), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES), Finance Code 001, and São Paulo Research Foundation (FAPESP 2017/24304–0).

Declarations

Conflict of Interest FSG is a co-inventor (Mechoulam R, JC, Guimaraes FS, AZ, JH, Breuer A) of the patent "Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023" Def. US no. Reg. 62193296; 29/07/2015; INPI on 19/08/2015 (BR1120150164927). The University of São Paulo has licensed the patent to Phytecs Pharm (USP Resolution No. 15.1.130002.1.1). The University of São Paulo has an agreement with Prati-Donaduzzi (Toledo, Brazil) to "develop a pharmaceutical product containing synthetic cannabidiol and prove its

safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson's disease, and anxiety disorders."

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