



Characterization of Cancer-Induced Nociception in a Murine Model of Breast Carcinoma

Amanda Spring de Almeida¹ · Flávia Karine Rigo² · Samira Dal-Toé De Prá² · Alessandra Marccone Milioli² · Diéssica Padilha Dalenogare¹ · Gabriele Cheiran Pereira¹ · Camila dos Santos Ritter¹ · Diulle Spat Peres¹ · Caren Tatiane de David Antoniazzi¹ · Carolina Stein³ · Rafael Noal Moresco³ · Sara Marchesan Oliveira⁴ · Gabriela Trevisan^{1,2,5}

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Abstract

Severe and poorly treated pain often accompanies breast cancer. Thus, novel mechanisms involved in breast cancer-induced pain should be investigated. Then, it is necessary to characterize animal models that are reliable with the symptoms and progression of the disease as observed in humans. Explaining cancer-induced nociception in a murine model of breast carcinoma was the aim of this study. 4T1 (10⁴) lineage cells were inoculated in the right fourth mammary fat pad of female BALB/c mice; after this, mechanical and cold allodynia, or mouse grimace scale (MGS) were observed for 30 days. To determine the presence of bone metastasis, we performed the metastatic clonogenic test and measure calcium serum levels. At 20 days after tumor induction, the antinociceptive effect of analgesics used to relieve pain in cancer patients (acetaminophen, naproxen, codeine or morphine) or a cannabinoid agonist (WIN 55,212-2) was tested. Mice inoculated with 4T1 cells developed mechanical and cold allodynia and increased MGS. Bone metastasis was confirmed using the clonogenic assay, and hypercalcemia was observed 20 days after cells inoculation. All analgesic drugs reduced the mechanical and cold allodynia, while the MGS was decreased only by the administration of naproxen, codeine, or morphine. Also, WIN 55,212-2 improved all nociceptive measures. This pain model could be a reliable form to observe the mechanisms of breast cancer-induced pain or to observe the efficacy of novel analgesic compounds.

Keywords Allodynia · Opioids · NSAIDs · Cannabinoid · Bone metastasis · WIN 55,212-2

Introduction

Breast cancer is the type of bone metastasis that is the most common among women worldwide (Zhu et al. 2015; Shenoy et al. 2016; Braun et al. 2017; Sarabia-estrada et al. 2017). These metastases can develop in various bones of the body, as well as in the spine up to the foot bone (Carlesimo et al. 2009; Adler et al. 2018). According to estimates made by the International Association for the Study of Pain (IASP), the prevalence of pain in breast cancer patients ranges from 40 to 89% (Bokhari and Sawatzky 2009; Satija et al. 2014). The management of cancer-induced pain involves different medicines following the World Health Organization (WHO) analgesic scale. However, the continuous use of these drugs for the chronic cancer-induced pain treatment can lead to the appearance of several adverse effects that limit their use (Bokhari and Sawatzky 2009; Plante and Vanitallie 2010; Satija et al. 2014; Zhu et al. 2015). Furthermore, 45% of

✉ Gabriela Trevisan
gabriela.trevisan-santos@ufsm.br

¹ Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS 97105-900, Brazil
² Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense (Unesc), Criciúma, SC 88006-000, Brazil
³ Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS 97105-900, Brazil
⁴ Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS 97105-900, Brazil
⁵ Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria (UFSM), Avenida Roraima, 1000, Building 21, Room 5207, Santa Maria, RS 97105-900, Brazil

cancer patients are accompanied by pain that cannot be adequately controlled (Zhu et al. 2015). Also, the analgesic effect of cannabinoid agonists has been described recently in models of cancer pain, but it is necessary more research to include these compounds in the clinical setting (Craft et al. 2017).

Thus, the search for new treatments and the development of drugs to treat breast cancer pain are urgently necessary. For this, it is essential to investigate the mechanisms involved in this symptom (Wright et al. 2016). However, for this to occur, it is required to characterize animal models reliable to the signs and progression of the disease, as clinically observed for breast cancer with bone metastasis. Thus, it is interesting to use models in which the cancer cells can develop bone metastasis naturally, without direct injection into the bone. Lelekakis et al. (1999) observed for the first time that 4T1 tumor cells cause bone metastasis when inoculated in the mice's mammary gland. Also there is the presence of hypercalcemia in this model, a well-known measure detected in human breast tumors (Lelekakis et al. 1999). Thus, this model is unique because the pattern of metastatic spread closely resembles that observed in human breast cancer.

Although Monteiro et al. (2013) showed the 4T1 cells natural bone metastasis capacity, to date, this murine model of metastatic breast carcinoma has not yet been used to verify the presence of nociception, or to test new mechanisms that could be involved in metastatic cancer pain (Monteiro et al. 2013). In this view, we aimed to validate a new model of pain associated with breast cancer with bone metastasis and test different analgesic drugs used in the clinical setting to treat the cancer pain. Moreover, we observed the antinociceptive effect of a cannabinoid agonist in this model of cancer pain in mice.

Materials and Methods

Animals

Female BALB/c mice (20–30 g) were used and kept at controlled temperature (22 ± 1 °C) in a light/dark cycle of 12 h (lights on 6:00 a.m. to 6 p.m.). It was provided the laboratory standard animal's food (Puro Lab 22 PB pelleted form, Puro trato, Rio Grande do Sul, Brazil) and tap water *ad libitum*, in ventilated cages (5 animals per cage) with wood shaving bedding and nesting material. A total of 154 animals were used in this study. The experiments reported in this study were performed according to the ethical guidelines to investigate pain in conscious animals (Zimmermann, 1983). The experiments were started after approval by the Institutional Committee of Animal Care and Use of the Federal University of Santa Maria (CEUA, protocol #7536250417/2017),

and by the Institutional Committee of Animal Care and Use of the University of the Extreme South Catarinense (CEUA, protocol #042-2014-02).

Induction of Breast Cancer Pain

The breast cancer pain model with metastasis was obtained through the 4T1 cells injection (Lelekakis et al. 1999; Monteiro et al. 2013). The tumor 4T1 cells were kindly provided by Dr. Adriana Bonomo (Cancer Institute, Rio de Janeiro, Brazil) (Monteiro et al. 2013). Using DMEM medium cells were cultured in monolayer supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. For cancer induction, the cells were resuspended in phosphate buffer and 50 μ l of the cell suspension or vehicle (phosphate buffer) were injected into the right fourth mammary fat pad of female BALB/c mice (10^4 cells).

Designing the Model of Breast Cancer Pain

A baseline measurement of the animals' threshold to the mechanical stimulus (von Frey test), cold thermal stimulus (acetone test), or mouse grimace scale (MGS) were performed. After cells or vehicle injection, behavioral parameters such as the development of mechanical allodynia, cold allodynia, and spontaneous pain (MGS determination) on days 5, 10, 15, 20, 25, and 30 were observed. These parameters were initially evaluated to determinate the maximum nociceptive effect caused by 4T1 cells inoculation. Then, the antinociceptive effect of different compounds was tested. The same animal was used to perform the experimental measures (mechanical threshold, nociceptive time to acetone test, and MGS).

Nociceptive Tests

Mechanical Allodynia

The mechanical sensitivity of animals was evaluated using von Frey filaments of increasing intensity (0.07–2 g) as previously described (Trevisan et al. 2012). Briefly, the animals were set in the experimental site, consisting of elevated acrylic chambers with wire mesh floor, for 1 h. After this period, the right hind paw stimulation of each animal with von Frey filaments was performed by the up-and-down method. The first filament used promoted a pressure of 0.6 g, in case of the paw withdrawal, a filament with a lower pressure was applied. If no withdrawal occurred, a filament with a higher pressure was used. In total, six simulations were performed, using filaments of 0.07, 0.16, 0.40, 0.6, 1.0, 1.4, or 2.0 g. With the results obtained, the value corresponding to 50% of the threshold, in grams, that each animal supports (threshold 50%) was calculated. A decrease in this value was

considered as mechanical allodynia and a reversal in this fall as an antinociceptive (anti-allodynic) effect.

Acetone Test (Cold Allodynia)

Cold thermal allodynia was measured using the modified acetone drop method. The acetone drop (20 μ l) was placed on the plantar surface of the animal's paw skin. The behavioral responses to acetone drop were timed using a chronometer (Trevisan et al. 2013).

Spontaneous Nociception Determination Using MGS

To evaluate the mouse grimace scale, the animals were placed in transparent acrylic boxes, and they were filmed using a high-resolution digital camera positioned on the front and back of the box for 30 min. The animals were then evaluated before (baseline measurement), after the administration of cancer cells, or at 1 h after administration of the analgesic drugs. The images were analyzed through pre-defined scores of 0 to 2, depending on the intensity of the pain (0—without modifications, 1—moderate, and 2—evident). For this, it was observed the changes in the eyes and periorbital region, ears, nose, and vibrissae of the animal (Langford et al. 2010; Miller and Leach 2015).

Locomotor Function

The rotarod test was done as previously described (Trevisan et al. 2012; Wang et al. 2015). On the day of the experiment, the latency (in seconds) to the first fall and the number of falls were recorded for 4 min. In the open field test, the mice were introduced to individual activity chambers (50 \times 50 \times 25 cm), which they had not been previously exposed. Locomotor activity was considered as the number of rearing (vertical movements) and the number of crossings (horizontal movements), which were analyzed for 5 min (Trevisan et al. 2012).

Clonogenic Metastatic Assay and Calcium Serum Measurement

Clonogenic metastatic test supplemented with 6-thioguanine was performed with cells obtained from the iliac bone marrow of BALB/c mice orthotopically injected with 4T1 tumor cells or vehicle (PBS) 20 days after inoculation. The number of metastatic cells in the iliac bone marrow was determined as described before (Pulaski et al. 2000; DuPré et al. 2007; Monteiro et al. 2013).

Animals were deeply anesthetized, and whole blood was collected by transcardial puncture 20 days after the tumor cells or the vehicle injection. Within one hour after coagulating, the blood samples were centrifuged at 2500xg for

10 min to separate the serum. Until measurements, samples were stored at -80°C . The quantitative determination of total calcium content in serum was performed using the colorimetric end-point arsenazo III assay (Bioclin, Belo Horizonte, Brazil) in the automated system BS 380 (Mindray, Shenzhen, China).

Treatment Protocols

Evaluation of the Antinociceptive Effect of Analgesics Available in the Clinic and a Cannabinoid Agonist

The animals were inoculated with 4T1 cells in the fourth mammary gland, and 20 days (time that animals presented a maximum nociceptive response) after, the analgesic effect of acetaminophen, codeine, naproxen, or morphine was tested. Animals received intragastric administration of vehicle of acetaminophen (30 mg/kg, intragastric, i.g.), naproxen (10 mg/kg, i.g.), codeine (10 mg/kg, i.g.), morphine (10 mg/kg, i.g.), or their vehicle (1% DMSO, 10 ml/kg, i.g.). The choice of the treatment doses was based on previous research (Whiteside et al. 2004; Minville et al. 2011; Rigo et al. 2013; Raskovic et al. 2015). We also tested the antinociceptive effect of a cannabinoid agonist, WIN 55,212-2 (3 mg/kg, i.g.) in this model (Herzberg et al. 1997). After drugs or vehicle administration, the development of mechanical and cold allodynia was verified after 0.5, 1, 2, and 4 h. MGS determination of animals was assessed 1 h after treatment administration. Administration of treatments was done using an intragastric gavage.

Statistical Analysis

Mean \pm SEM was used to express all values and analyzed by Student's *t* test, one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test when appropriate. GraphPad 5.0 software (San Diego, CA, USA) was used for all tests. The $P < 0.05$ values denote significant difference among groups.

Results

Breast Cancer Induced Mechanical and Cold Allodynia and Altered the MGS in Mice

Tumor cells inoculation caused mechanical allodynia from 10 up to 30 days after its injection (Fig. 1a). On days 15, 20, 25, and 30 after 4T1 cells inoculation, animals showed cold hypersensitivity. Significant increases in MGS were observed from 10 to 30 days when compared to control animals or basal values (Fig. 1c). Considering the animals

Fig. 1 4T1 cells inoculation in the mammary gland in mice induced mechanical and cold allodynia and increased MGS. The behavioral testing was performed for 30 days after tumor cells injection (10^4 4T1 cells), and baseline values were measured before tumor inoculation. Control group received PBS injection. **a** Change in mechanical threshold determined using von Frey filaments or **b** nociceptive time reaction to acetone test. **c** Nociception facial score observed after tumor inoculation using the facial grimace scale (MGS). Data are expressed as mean+SEM ($n=7$) in **a** and **b** graphs, or as median+interquartile in **C** graph. $*P<0.05$, $***P<0.001$, when compared to control group or baseline values [Two-way ANOVA, followed by Bonferroni's post hoc test (in **a**, **b**) or nonparametric Student's *t* test for each time point evaluated (in **c**)]

presented a maximum nociceptive response 20 days after 4T1 cells inoculation, we choose the day 20 to determine the presence of bone metastasis and to measure the analgesic effect of the drugs.

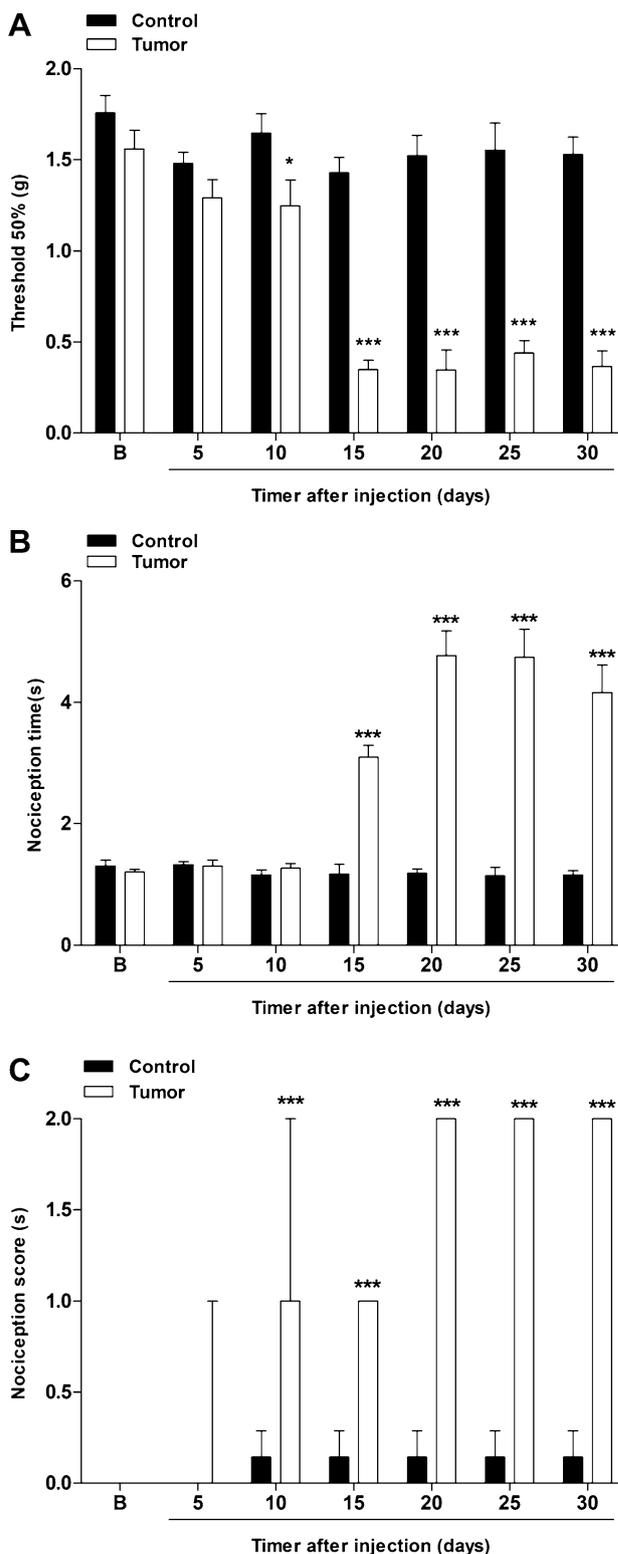
Regarding body weight gain from 5 up to 30 days after tumor cells injection, there was no difference between tumor and control group (data not shown). Also, the animals inoculated with tumor cells showed no change in spontaneous or forced locomotion measured by the open field and rotarod tests, respectively, when compared to the control group. These measurements were also taken 20 days after tumor cell injection (Table 1).

Development of Bone Metastasis in a Cancer-Induced Pain Model and Determination of Calcium Serum Levels

It was observed the presence of colonies of metastatic cells, indicating a process of metastasis in the mice bone. The number of metastatic cells in iliac bone marrow was determined as 18 ± 2 of 4T1 clones/ 10^7 cells in tumor group ($n=5$), and no metastatic clones were detected in control animals 20 days after injection of PBS. The development of bone metastasis after the injection of breast cancer tumor cells induced an increase in blood calcium levels (Table 2).

Antinociceptive Effect of Clinically Available Analgesics in Cancer-Induced Pain

The oral administration of analgesics available in the clinic to treat cancer pain, acetaminophen (30 mg/kg), naproxen (10 mg/kg), codeine (10 mg/kg) or morphine (10 mg/kg) induced an antinociceptive effect in this model of cancer pain (Figs. 2, 3). All drugs presented antiallodynic effect, concerning mechanical or cold allodynia; the antinociceptive effect was determined from 0.5 to 2 h after their administration. Acetaminophen caused a maximum inhibition (I_{max}) on the mechanical and cold allodynia of $50 \pm 6\%$ and $72 \pm 4\%$ at 1 h and 0.5 h after its administration, respectively (Fig. 2a, c). The I_{max} caused by naproxen on the mechanical and cold



hypersensitivity was $85 \pm 5\%$ and $95 \pm 2\%$ at 1 h and 0.5 h after its injection, respectively (Fig. 2b, d).

The codeine administration displayed I_{max} of $90 \pm 12\%$ to the mechanical allodynia after 0.5 h (Fig. 3a) and 100%

Table 1 Assessment of locomotor activity. The spontaneous locomotor activity (open field) was observed for 5 min and the forced locomotor activity (rotarod) observed for 4 min

Group	Open field		Rotarod	
	Crossings	Rearings	Number of falls	Latency for the 1st fall (s)
Control	159.9 ± 10	18.71 ± 2.7	1.71 ± 0.35	72.43 ± 20
Tumor	200 ± 24	30.14 ± 5	1.28 ± 0.52	35.86 ± 20

No significant differences were observed between the groups. The results were expressed as mean ± SEM ($n=7$). Student's *t* test

Table 2 Calcium serum levels after 20 days of 4T1 cells inoculation in the mammary fat pad of mice

Group	Calcium levels (mM)
Control	1.46 ± 0.12
Tumor	1.91 ± 0.08*

Data are presented as mean ± SEM. Student *t* test ($n=6$)

* $P < 0.05$ when compared to vehicle group

to the cold allodynia 1 h after its administration (Fig. 3c). The I_{\max} caused by morphine on the mechanical and cold allodynia was 100% at 1 h after injection (Fig. 3b, d). Moreover, treatment with naproxen, codeine, and morphine decreased MGS in this model of cancer pain at 1 h after its administration (Figs. 2f, 3e, f). The inhibition percentage observed for the MGS was $50 \pm 10\%$, $62 \pm 16\%$ or $78 \pm 15\%$ for naproxen, codeine, or morphine, respectively. Animals treated with acetaminophen had no significant decrease in this pain-like behavior (Fig. 2e).

A cannabinoid agonist has antinociceptive effect in a breast cancer pain model

The oral administration of the cannabinoid agonist WIN 55,212-2 showed an antinociceptive effect in this model of cancer pain (Fig. 4). The cannabinoid agonist presented antiallodynic effect, concerning mechanical and cold allodynia, from 0.5 to 2 h after its administration and decreased pain-like behavior at 1 h after the treatment. The I_{\max} to the mechanical threshold was 100%, after 0.5 h of WIN 55,212-2 administration (Fig. 4a) and the I_{\max} to the cold allodynia was $65 \pm 8\%$, after 0.5 h (Fig. 4b). Moreover, the cannabinoid agonist decreased pain-like behavior in this new pain model with bone metastasis, showing an antinociceptive effect at 1 h after its administration. The I_{\max} for MGS was $62 \pm 16\%$, after 1 h (Fig. 4c).

Discussion

Cancer is a disease that affects a large part of the world population (Desantis et al. 2014; Torre et al. 2015), and breast cancer is the leading cause of cancer-related mortality in women worldwide (Goddard et al. 2016; Cintolo-Gonzalez et al. 2017). However, the available opioid analgesics to pain control have adverse effects, such as dependence and constipation (Plante and Vanitallie 2010; Zhu et al. 2015). Thus, potential novel analgesics should be tested using animal models of cancer-induced pain (Mantyh 2013; Slosky et al. 2015; Hiasa et al. 2017). In this way, we investigate cancer-induced hypersensitivity in a murine model of breast carcinoma. Animals inoculated directly into the mammary gland showed bone metastasis, mechanical and cold allodynia, and alteration in the mouse grimace scale (as indicative of spontaneous nociception). The hypersensitivity and spontaneous nociception detected in this model were diminished by the acute administration of AINEs or opioids, and by a cannabinoid agonist. Therefore, this mouse model of breast cancer could be used to test different compounds for antinociceptive effect and to study the mechanisms involved in breast cancer-induced pain.

Cancer-induced pain is characterized by pathological symptoms such as mechanical and cold hypersensitivity (Peuckmann et al. 2009; Sarabia-estrada et al. 2017). Here, we detected that mice inoculated with tumor cells develop mechanical and cold allodynia, measured using von Frey test and acetone drop test, respectively. Mechanical and cold allodynia were also presented in other models of cancer-induced pain in mice and rats (Mantyh 2013; Slosky et al. 2015; Hiasa et al. 2017). In fact, after femoral implantation of 4T1 breast carcinoma cells, it was observed the occurrence of pain-related behaviors, cold, and mechanical allodynia (Falk et al. 2013; Zhao et al. 2013; Abdelaziz et al. 2015). Moreover, here we measured spontaneous pain caused by cancer cells inoculation using MGS, which is a relevant data because both mechanical and cold allodynia are measured using reflexive tests. MGS has been emerged as a useful resource to detect spontaneous nociception in models of pain, by measuring changes in the facial expression of mice (Matsumiya et al. 2012; Thomas et al. 2016). The MGS has already been used to assess nociception in different models of pain in mice, such as neuropathic pain (Akintola et al. 2017) and postoperative pain (Matsumiya et al. 2012; Leach et al. 2012).

In the model used in this study, the breast tumor volume usually starts to increase after 7–10 days of 4T1 inoculation; thus, primary tumor volume is often detected in studies that may observe the antitumor activity of treatments

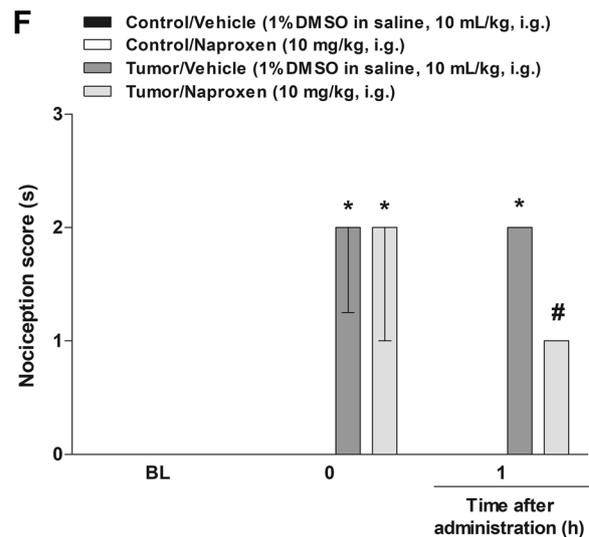
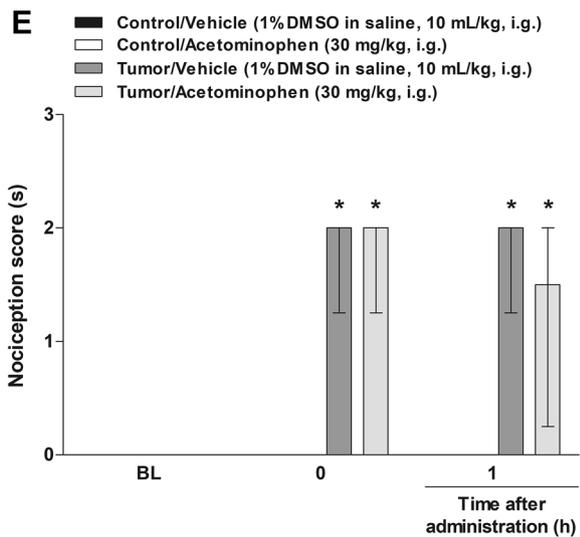
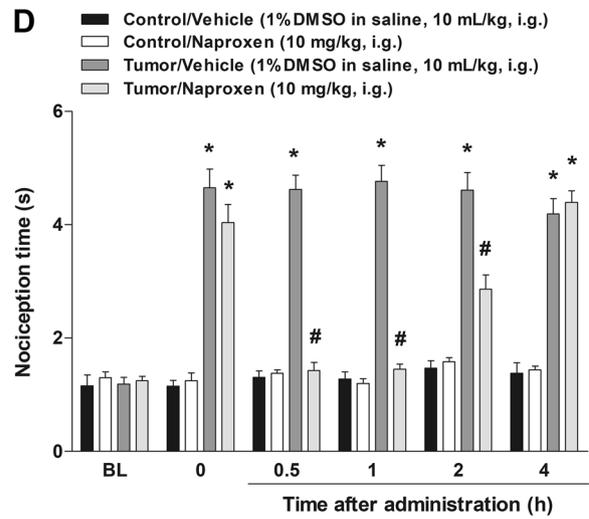
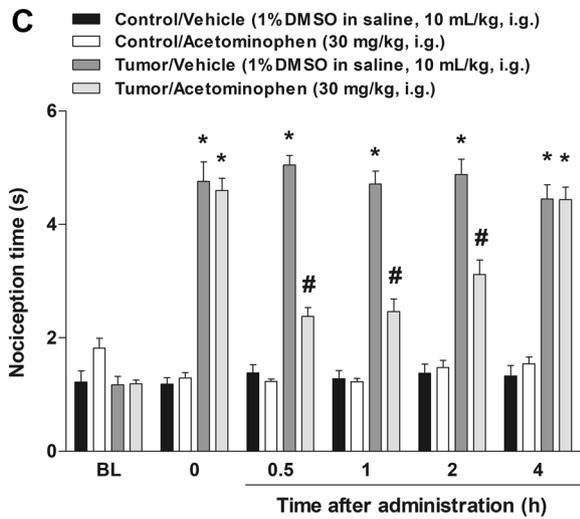
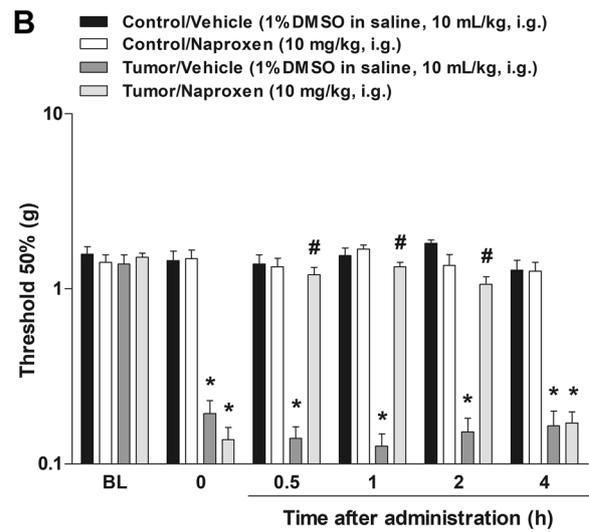
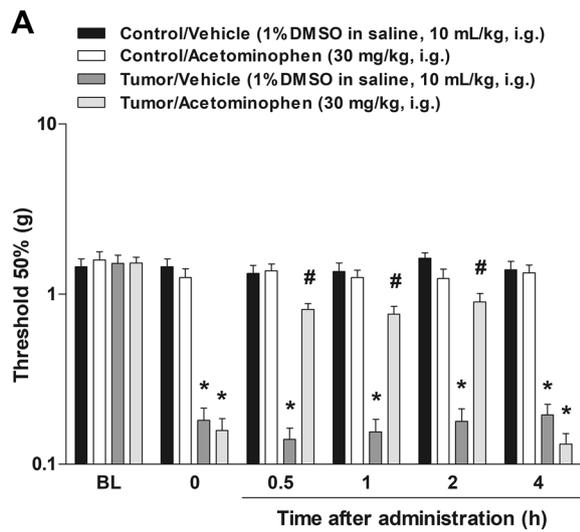


Fig. 2 Acetaminophen and naproxen administration reduced mechanical and cold allodynia, and naproxen diminished the mouse grimace scale (MSG) in a metastatic cancer pain model in mice. **a, b** Mechanical and **c, d** cold allodynia, or **e, f** facial grimace criteria alteration detected 20 days after tumor cells inoculation or vehicle (control group). Treatment with acetaminophen (30 mg/kg) and naproxen (10 mg/kg) was evaluated on a time curve of 0.5, 1, 2, and 4 h to mechanical threshold and cold allodynia and during 1 h to MGS at day 20. Data are expressed as mean \pm SEM ($n=7$) in **a, b** and **d, e** graphs, or as median \pm interquartile in **c, f** graphs. * $P < 0.05$, *** $P < 0.001$, when compared to control group or baseline values and # $P < 0.05$ when compared to tumor/vehicle group [Two-way ANOVA, followed by Bonferroni's post hoc test (in **a–d**) or nonparametric Student's *t* test for each time point evaluated (in **e, f**)]

(Withana et al. 2012; Bouchlaka et al. 2012; Luo et al. 2015; Pereira et al. 2016; Yue et al. 2018). Here we used animals for the time course of nociception tests until 30 days of tumor injection, because after this period primary tumor volume may increase, impairing the detection of the nociception tests that used reflexive measures (such as von Frey and acetone test, data not shown). Therefore, it is interesting to detect the antinociceptive effect of treatments before tumor volume may reduce locomotor activity of animals. Here we observed that 20 days after 4T1 injection into the mammary fat pad there is no alteration on locomotor activity of mice.

Previously, Monteiro et al. (2013) showed that bone loss and osteoclastogenesis occurred before metastatic bone colonization when 4T1 cells were injected into the mammary gland (Monteiro et al. 2013). Thus, the progression of the disease is well comparable to the process which occurs in the human mammary cancer (Pulaski and Ostrand-Rosenberg 2001; Monteiro et al. 2013; Takahashi et al. 2015). Thus, the adaptation of metastatic cells infiltration to the bone medium allows its growth and invasiveness (Bednarz-Knoll et al. 2011; Panis and Pavanelli 2015), resulting in bone loss and pain (Panis and Pavanelli 2015). In this model, the detection of mechanical and spontaneous pain at day 10 after tumor cell implantation precede the development of bone metastasis; this may be caused by initial bone loss and osteoclastogenesis that come first in this model and are mediated by pro-osteoclastogenic cytokines (Monteiro et al. 2013). Also, it was described that the levels of IL-17 and RANKL were already increased in iliac and tibia samples at day 11 after 4T1 inoculation (Monteiro et al. 2013). Some of these cytokines, such as IL-17 and RANKL, have already been described to mediate pain in arthritis and mouse models of cancer pain (Honore et al. 2000; Lipton and Goessl 2011; Panis and Pavanelli 2015; Takahashi et al. 2015; McCarty and DiNicolantonio 2016; Dai et al. 2018; Remeniuk et al. 2018). Also, a different study showed that 4T1 orthotopic model caused after two weeks of tumor inoculation into the mammary fat pad the presence of osteolytic lesion and

cortical bone loss in tibia samples, and these focal pre-metastatic lesions occurred before bone metastases (Cox et al. 2015).

Different models could be used to understand the mechanisms involved in bone metastatic cancer-induced pain, as the injection of tumor cells into bones, such as tibia, femur, or calcaneus (Schwei et al. 1999; Medhurst et al. 2002; Goblirsch et al. 2005; Mantyh et al. 2010; Zhao et al. 2013; Slosky et al. 2015, 2016; Hiasa et al. 2017). However, bone implantation of cells results in a significant number of tumor cells inoculated directly into bone, which is a different situation from that observed in breast cancer patients. In this view, tibial injection of 4T1 cells in mice induced nociception after three to five weeks of tumor inoculation in bone, showing that nociception using this model depends on tumor grown into bone and cancer-induced osteolysis (Abdelaziz et al. 2014).

The breast carcinoma model used in this study induces spontaneous bone metastasis after orthotopic primary tumor implantation, and this is a well-known feature of this model. Thus, this breast cancer model had been used to understand the mechanism of bone metastasis or to test novel chemotherapeutic drugs (Monteiro et al. 2013; Cox et al. 2015; Luo et al. 2015; Bottos et al. 2016; Mall et al. 2016; Pereira et al. 2016). After 2–3 weeks of tumor cells injection in the mammary pad, it is expected to observe the presence of bone metastasis, because 4T1 cells possess an invasive behavior (Lelekakis et al. 1999; Pulaski and Ostrand-Rosenberg 2001; Monteiro et al. 2013; Cox et al. 2015; Bottos et al. 2016; Mall et al. 2016; Pereira et al. 2016). Considering this, we choose the 20th day after tumor cell injection to describe the presence of bone metastasis. Importantly, on this day the animals also presented maximum nociception. Using a clonogenic metastatic assay, we detected metastatic clones in the iliac bone marrow, these data are in accordance with the literature (Monteiro et al. 2013; Pereira et al. 2016) where the metastatic cells proliferation was observed in different regions of mice (DuPré et al. 2007; Abu et al. 2015) showing the metastatic potential of this cell line. Also, at day 20 after 4T1 injection, it is also described the presence of femur and tibia metastasis (Monteiro et al. 2013). Besides, as bone metastasis was detected in iliac bone after 16 days of 4T1 injection, and at day 20 femur and tibia metastasis can detect we used the day 20 to study the antinociceptive effect of treatments. Furthermore, it was observed increased plasma calcium levels in animals that presented bone metastasis. It has already been shown that breast cancer cells that were implanted in mice were related to the development of hypercalcemia (Lelekakis et al. 1999). This increases in calcium, which is consistent with what occurs with breast cancer patients (Yong et al. 2011).

As the metastatic bone cancer grows, it brings nociceptive and inflammatory responses due to nerve compression by the

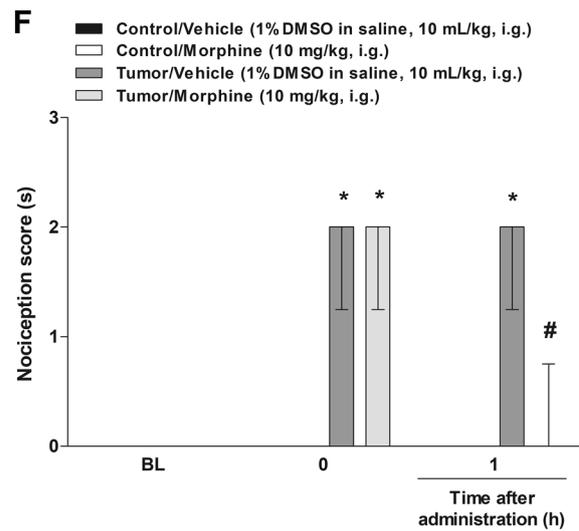
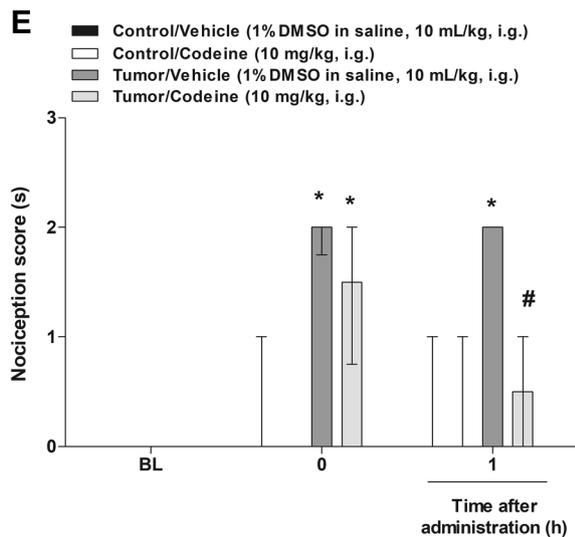
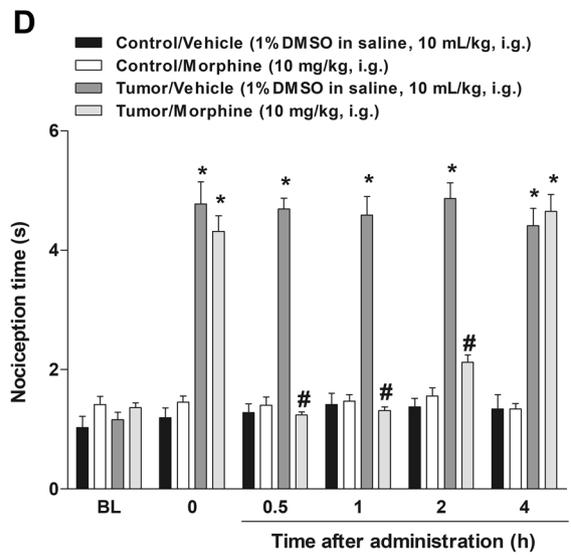
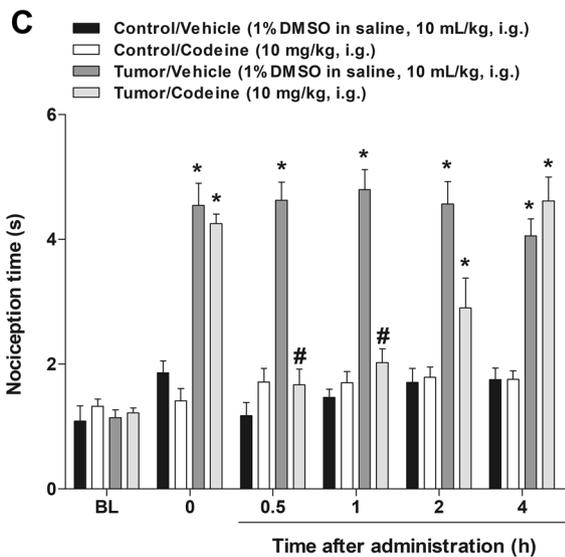
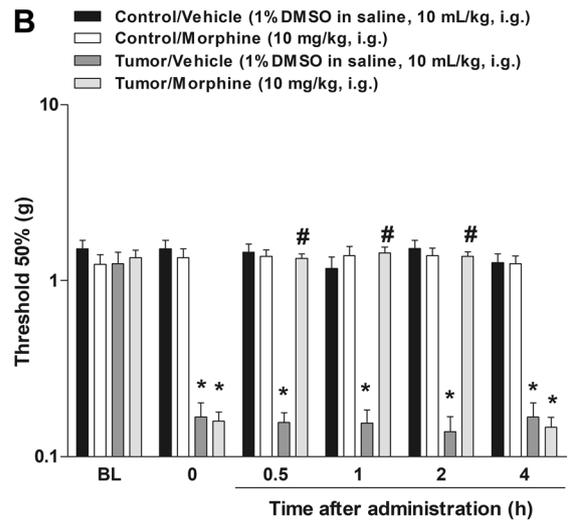
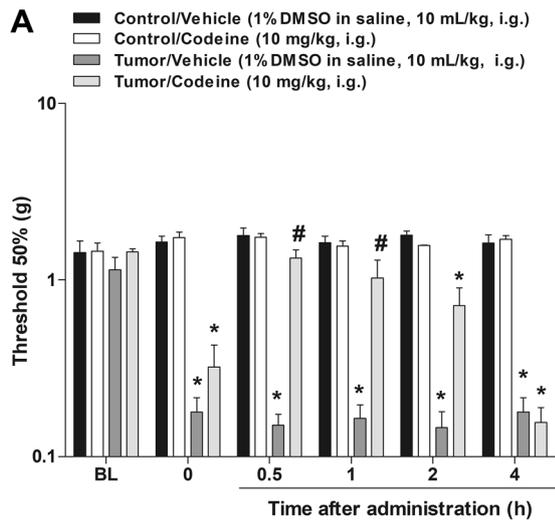


Fig. 3 Codeine and morphine attenuate evoked pain and pain-related behavior in a metastatic cancer pain model in mice. **a, b** Mechanical and **c, d** cold allodynia, or **e, f** facial grimace scale (MGS) alteration detected 20 days after tumor cells inoculation or vehicle (control). Antinociceptive effect of codeine (10 mg/kg) or morphine (10 mg/kg) was evaluated on a time curve of 0.5, 1, 2, and 4 h to mechanical and cold allodynia, or after 1 h to MGS. Data are expressed as mean + SEM ($n = 7$) in **a, b** and **d, e** graphs, or as median + interquartile in **c, f** graphs. * $P < 0.05$, *** $P < 0.001$, when compared to control group or baseline values and # $P < 0.05$ when compared to tumor/vehicle group [Two-way ANOVA, followed by Bonferroni's post hoc test (in **a–d**) or nonparametric Student's t test for each time point evaluated (in **e, f**)]

tumor, ischemia, and release of several mediators that can act on the afferent fibers. Thus, bone cells and cancer cells initiate a cascade of mechanisms, including activation and sensitization of nociceptors, which help in the pain process and central sensitization (Mantyh et al. 2002; Doré-Savard et al. 2010; Zhu et al. 2015). The World Health Organization (WHO) indicates the use of an analgesic scale describing step by step, through a hierarchy of drugs, which analgesic drugs should be used for each stage of cancer pain in general, associated with the use of an analgesic adjuvant (World Health Organization 1986; Currie et al. 2014; Ballantyne et al. 2016; Derry et al. 2017; Varrassi et al. 2018). Thus, we choose to test different analgesics on this model of cancer pain, using drugs present on the analgesic scale (NSAIDs and opioids drugs). All drugs tested had antinociceptive effect in a murine model of metastatic breast carcinoma, but acetaminophen did not reduce MGS alteration. As expected, morphine treatment showed the best antinociceptive profile when compared to acetaminophen, naproxen, or codeine. Morphine antinociceptive effect in cancer-induced nociception models is well described (Rigo et al. 2013; Xu et al. 2016), and this opioid agonist is frequently used in the clinic to treat cancer-mediated severe pain (Swarm et al. 2007). However, with the disease progression is common to observe patients with intractable pain or to fear the opioids adverse effects, such as opioid-induced hyperalgesia and constipation (Vanderah et al. 2000; Varrassi et al. 2018).

The different mechanisms involved in cancer pain are not adequately described, but the role of cannabinoid receptors

has been related to different cancer-pain models (Lozano et al. 2011; Lozano-Ondoua et al. 2013; Uhelski et al. 2013). Cannabinoid receptors are expressed in afferent neuron fibers, immune cells (as monocytes, T cells, B cells), and bone cells (osteoblast and osteoclasts) and when activated could cause the antinociceptive effect, attenuating bone cancer pain and diminish bone metastasis (Lozano-Ondoua et al. 2010; Uhelski et al. 2013). It has been reported that the attenuation of mechanical hyperalgesia by the WIN 55,212-2 agonist seems to be related to the reduction in spontaneous activity in C-fiber nociceptors that may decrease the conduction of stimuli to maintain the sensitization of nociceptive neurons of the dorsal horn, and a decrease in responses of C fibers evoked by mechanical stimuli (Uhelski et al. 2013). In this way, we also tested in this model of cancer pain a cannabinoid agonist (WIN 55,212-2) that already showed antinociceptive effect in a model of cancer pain caused by injection of 66.1 cells or CCL-11 sarcoma cells into the bone (Lozano-Ondoua et al. 2010, 2013). In the current study, the cannabinoid agonist showed positive results against mechanical and cold allodynia and on spontaneous pain in a murine model of breast carcinoma. As already demonstrated in a murine model of cancer pain, it is likely that CB receptor activation attenuated the responses of C-fiber nociceptors and reduced bone cancer pain by inhibiting the release of immune cell substances that excite and sensitize nociceptors (Uhelski et al. 2013).

In conclusion, the assessment of nociception in a murine model of metastatic breast carcinoma showed the presence of mechanical and cold hypersensitivity and alteration in the MGS measures that could be used to test the antinociceptive effect of tested compounds. Also, in this cancer-induced pain model, we detected the antinociceptive effect of clinically used analgesics, following the WHO analgesic scale. Besides, a cannabinoid agonist also reduced nociception in this murine model of metastatic breast carcinoma, showing the importance of cannabinoid receptors for cancer-mediated sensitization. Finally, this breast cancer model could be used in future studies to measure the antinociceptive and anti-metastatic effect of compounds or to study the mechanisms involved in these processes.

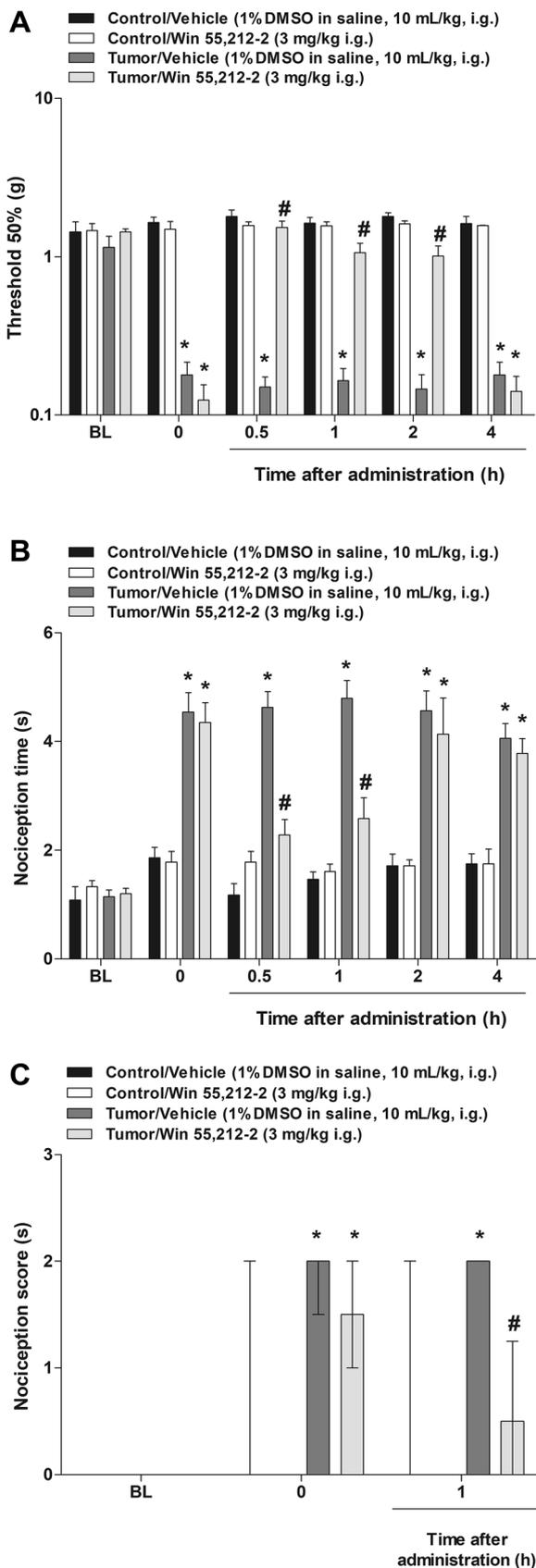


Fig. 4 Effect of WIN 55,212-2, a cannabinoid agonist, in a metastatic cancer pain model in mice. **a** Mechanical and **b** cold allodynia, and **c** facial grimace scale (MGS) alteration detected 20 days after tumor cells inoculation or vehicle (control). Antinociceptive effect of WIN 55,212-2 (30 mg/kg) was evaluated on a time curve of 0.5, 1, 2, and 4 h to mechanical and cold allodynia, or after 1 h to MGS. Data are expressed as mean + SEM ($n=7$) in **a**, **b** graphs, or as median + interquartile in **c** graph. * $P<0.05$, *** $P<0.001$, when compared to control group or baseline values and # $P<0.05$ when compared to tumor/vehicle group [Two-way ANOVA, followed by Bonferroni's post hoc test (in **a**, **b**) or nonparametric Student's *t* test for each time point evaluated (in **c**)

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Compliance with Ethical Standards

The experiments of this manuscript comply with the current laws of the country in which they were performed.

Conflict of interest The authors declare no potential conflicts of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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