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RESEARCH PAPER

Spinal and peripheral analgesic effects of the CB_2 cannabinoid receptor agonist AM1241 in two models of bone cancer-induced pain

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Background and purpose: The activation of CB_2 receptors induces analgesia in experimental models of chronic pain. The present experiments were designed to study whether the activation of peripheral or spinal CB_2 receptors relieves thermal hyperalgesia and mechanical allodynia in two models of bone cancer pain.
Experimental approach: NCTC 2472 osteosarcoma or B16-F10 melanoma cells were intratibially inoculated to C3H/He and C57BL/6 mice. Thermal hyperalgesia was assessed by the unilateral hot plate test and mechanical allodynia by the von Frey test. AM1241 (CB_2 receptor agonist), AM251 (CB_1 receptor antagonist), SR144528 (CB_2 receptor antagonist) and naloxone were used. CB_2 receptor expression was measured by Western blot.
Key results: AM1241 (0.3–10 $mg \cdot kg^{-1}$) abolished thermal hyperalgesia and mechanical allodynia in both tumour models. The antihyperalgesic effect was antagonized by subcutaneous, intrathecal or peri-tumour administration of SR144528. In contrast, the antiallodynic effect was inhibited by systemic or intrathecal, but not peri-tumour, injection of SR144528. The effects of AM1241 were unchanged by AM251 but were prevented by naloxone. No change in CB_2 receptor expression was found in spinal cord or dorsal root ganglia.
Conclusions and implications: Spinal CB_2 receptors are involved in the antiallodynic effect induced by AM1241 in two neoplastic models while peripheral and spinal receptors participate in the antihyperalgesic effects. Both effects were mediated by endogenous opiates. The use of drugs that activate CB_2 receptors could be a useful strategy to counteract bone cancer-induced pain symptoms.
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Keywords: bone cancer-induced pain; hyperalgesia; allodynia; NCTC 2472 cells; B16-F10 cells; mice; CB_2 receptors; AM1241

Abbreviations: AM1241, (1-(methylpiperidin-2-ylmethyl)-3-(2-iodo-5-nitrobenzoyl)indole); AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; CB_1, type 1 cannabinoid receptor; CB_2, type 2 cannabinoid receptor; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; DRG, dorsal root ganglia; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; i.t., intrathecal; NP40, nonylphenylpolyethylene glycol; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; SR144528, (N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide); TBST, Tris buffered saline-Tween® 20 0.1%

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Introduction

The difficulties of relieving painful symptoms associated with different pathological entities, such as bone cancer pain, justify the continuous experimental effort addressed to define new analgesic targets. Opiates, especially morphine, are

effective drugs for the management of cancer pain in clinical settings although certain patients do not respond adequately to the treatment with these analgesics (Delaney *et al.*, 2008). Agonists of cannabinoid CB_1 or CB_2 receptors (nomenclature follows Alexander *et al.*, 2008) constitute a group of drugs often considered potentially interesting approaches to achieve this goal (Huskey, 2006). The analgesic potential derived from the selective stimulation of CB_1 receptors is widely known, although the psychological alterations that occur in response to the activation of central CB_1 receptors (Ledent *et al.*, 1999) have probably limited the clinical development of this approach. Besides CB_1 receptor stimulation, more recent evidence shows that the administration of selective agonists of CB_2 receptors reduces nociception in a variety of preclinical models without producing tolerance (Romero-Sandoval *et al.*, 2008; Yao *et al.*, 2008; Leichsenring *et al.*, 2009) or central side-effects (Hanus *et al.*, 1999; Malan *et al.*, 2001). Both properties make the stimulation of CB_2 receptors an attractive target for potential analgesics.

Whereas a great body of evidence demonstrates the analgesic efficacy of systemically administered CB_2 agonists in acute and chronic experimental pain (Jhaveri *et al.*, 2007; Cheng and Hitchcock, 2007; Guindon and Hohmann, 2008; Beltramo, 2009), there have been few reports detailing the participation of peripheral or spinal CB_2 receptors in these effects.

Thus, although some reports found that CB_2 receptor protein (Ross *et al.*, 2001; Walczak *et al.*, 2005) or its mRNA (Beltramo *et al.*, 2006) are expressed in rodent sensory ganglia, other authors did not detect the presence of either the protein (Wotherspoon *et al.*, 2005) or the corresponding mRNA (Hohmann and Herkenham, 1999; Price *et al.*, 2003). Interestingly, a more recent paper has demonstrated the expression of functional CB_2 receptors in human small-diameter dorsal root ganglion (DRG) sensory neurons (Anand *et al.*, 2008), supporting the participation of peripheral, neuronal CB_2 receptors in the analgesic effects produced by CB_2 receptor agonists. In addition, CB_2 receptor agonists can evoke peripheral analgesia by triggering the release of beta-endorphin in response to the stimulation of CB_2 receptors expressed in murine and human keratinocytes (Ibrahim *et al.*, 2005). The analgesic efficacy of peripheral CB_2 receptor stimulation has been demonstrated in inflammatory models (Nackley *et al.*, 2003; Quartilho *et al.*, 2003; Sokal *et al.*, 2003; Elmes *et al.*, 2004) and, in some (Elmes *et al.*, 2004), but not other (Yamamoto *et al.*, 2008) studies related to neuropathic pain. Moreover, increases in peripheral CB_2 receptor protein or mRNA in inflamed tissues (Richardson *et al.*, 2008) or in DRG in neuropathic states (Walczak *et al.*, 2005; Wotherspoon *et al.*, 2005; Anand *et al.*, 2008) have been reported.

CB_2 receptor expression has been also demonstrated in the spinal cord (Walczak *et al.*, 2005; Wotherspoon *et al.*, 2005; Beltramo *et al.*, 2006), as well as in other brain regions particularly relevant for nociceptive integration (Van Sickle *et al.*, 2005; Gong *et al.*, 2006; Jhaveri *et al.*, 2008). However, the CB_2 receptor mRNA or CB_2 receptor protein detected in the spinal cord of rodents may be expressed in glial cells, in particular microglia (Zhang *et al.*, 2003; Romero-Sandoval *et al.*, 2008), in neurons (Wotherspoon *et al.*, 2005) or in both cell types (Beltramo *et al.*, 2006). Spinal analgesia evoked through CB_2 receptors has been described in models of neuropathic (Sagar *et al.*, 2005; Romero-Sandoval *et al.*, 2008; Yamamoto *et al.*, 2008) and postoperative pain (Romero-Sandoval and Eisenach, 2007). Whereas increased spinal expression of CB_2 receptor mRNA and of CB_2 receptor protein has been demonstrated in neuropathic pain models (Zhang *et al.*, 2003; Walczak *et al.*, 2005; Wotherspoon *et al.*, 2005; Romero-Sandoval *et al.*, 2008), no such changes have been detected during inflammation (Zhang *et al.*, 2003; Cox *et al.*, 2007).

Apart from data showing the ability of CB_2 receptor agonists to relieve pain from neuropathic or inflammatory origin, recent reports have started to delineate their efficacy in experimental models of cancer pain. Experiments using selective cannabinoid antagonists have demonstrated that the antihyperalgesic effects induced by mixed CB_1/CB_2 agonists were mediated through the activation of CB_1, but not CB_2, receptors when grip force was measured in mice with NCTC 2472 osteosarcoma cells inoculated into the humerus (Kehl *et al.*, 2003), or mechanical hyperalgesia assessed following inoculation of cells into the calcaneus (Hamamoto *et al.*, 2007). Accordingly, the analgesic effects induced by endogenous cannabinoids in mice receiving intraosteal NCTC 2472 osteosarcoma cells are also mediated by the activation of peripheral CB_1 receptors (Khasabova *et al.*, 2008). Even if these initial data pointed towards a preferential involvement of CB_1 cannabinoid receptors, the reversal of the antihyperalgesic effect induced by WIN55,212-2 in mice inoculated with NCTC 2472 osteosarcoma cells into the calcaneus by the local administration of CB_1 as well as CB_2 receptor antagonists revealed that, at the peripheral level, the activation of both CB_1 and CB_2 receptors can alleviate the hyperalgesia produced by these osteolytic cells (Potenzieri *et al.*, 2008). A probable involvement of peripheral CB_2 receptors in antiallodynic responses has also been described in a cancer model unrelated to bone, based on the inoculation of human oral squamous carcinoma cells into the hind paw of mice (Guerrero *et al.*, 2008).

The present experiments were designed to specifically assess the efficacy of CB_2 receptor stimulation to inhibit behavioural nociceptive symptoms derived from murine bone cancer. With this aim, we have used, as pharmacological tools, AM1241 (Malan *et al.*, 2001), the selective CB_2 receptor agonist most extensively reported in pre-clinical studies (Beltramo, 2009), and the selective CB_1 and CB_2 receptor antagonists AM251 and SR144528 respectively. As bone cancer pain symptoms are not exclusively associated with a particular type of neoplastic bone injury, but can be due to different pathological features (Mantyh, 2006) we considered that the study of this hypothesis in two models of painful bone cancer with different characteristics, would give us a more complete view about the efficacy of CB_2 receptor stimulation in bone cancer-induced pain. With this aim, we have intratibially inoculated NCTC 2472 osteosarcoma and B16-F10 melanoma tumour cells in syngenetic strains of mice which further develop nociceptive symptoms due to tumour progression. As initially described, the intrafemoral inoculation of NCTC 2472 osteosarcoma cells (Schwei *et al.*, 1999) induces a tumour process accompanied by a prevailing osteolytic activity markedly greater than that secondary to the inoculation of B16-F10 melanoma cells (Sabino *et al.*, 2003). Inoculation of

NCTC 2472 osteosarcoma cells into the tibia leads to augmented osteoclast activity and osteolysis (Menéndez *et al.*, 2003), whereas the intratibial inoculation of B16-F10 melanoma cells also provokes a marked osteoblastic activity (Curto-Reyes *et al.*, 2008). We initially assessed, in these two models, whether the systemic administration of AM1241 inhibited tumour-induced thermal hyperalgesia and mechanical allodynia and next, if this effect was mediated through the stimulation of CB_2 receptors expressed peripherally, at the tumour level, or at the spinal cord. Finally, CB_2 protein expression in DRG and spinal cord of tumour-bearing mice was measured by performing Western blot assays.

Methods

Animals

All animal care and experimental procedures were approved by the Comité Ético de Experimentación Animal de la Universidad de Oviedo (Asturias, Spain). The experiments were performed with C3H/He and C57BL/6 mice (26–33 g) bred in the Animalario of the Universidad de Oviedo (Reg. 33044 13A), maintained on a 12-h dark–light cycle with free access to food and water ($n = 814$).

Cell culture and cell inoculation

NCTC 2472 osteosarcoma cells (American Type Culture Collection, ATCC) were cultured in NCTC 135 medium (Sigma) containing 10% horse serum (Sigma) and passaged weekly according to ATCC guidelines. Cells were detached by scraping and centrifuged at 400× *g*. The obtained pellet was suspended in phosphate buffered saline (PBS) and then used for intratibial injections (Menéndez *et al.*, 2003).

B16-F10 melanoma cells (American Type Culture Collection, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) enriched with 10% foetal calf serum (Gibco). When cells were preconfluent, they were detached by treatment with trypsin/EDTA (0.05%/0.02%). The trypsin/EDTA solution containing the detached cells was collected, neutralized with DMEM supplemented with 10% FCS and centrifuged at 400× *g* for 10 min. The pellet was resuspended in PBS and used for intratibial injections (Curto-reyes *et al.*, 2008).

For cell inoculation, anesthesia was induced by spontaneous inhalation of 3% isoflurane (Isoflo®, Esteve) in an induction chamber and maintained by administering 1.5% isoflurane in oxygen through a breathing mask. A minimal skin incision was made in the right leg exposing the tibial plateau and a 22 gauge needle coupled to a Hamilton syringe with 10^5 NCTC 2472 or B16-F10 cells suspended in 5 μL of PBS was used to inject the cells into the medullar cavity. Finally, acrylic glue (Hystoacril©, Braun) was applied on the incised area of the tibial plateau and the surgical procedure was completed with a stitch of the knee skin. Control groups were injected with 5 μL of PBS containing 10^5 NCTC 2472 osteosarcoma or B16-F10 melanoma cells killed by quickly freezing and thawing them three times without cryoprotection. Mice were used at the particular times at which the measured nociceptive symptoms are detected. Thus, thermal hyperalgesia was studied 4 weeks after the inoculation of NCTC 2472 osteosarcoma cells and 1 week after B16-F10 melanoma cells inoculation whereas mechanical allodynia was assessed at week 2 and 1 respectively (Menéndez *et al.*, 2003; Baamonde *et al.*, 2006; Curto-Reyes *et al.*, 2008).

Drug treatments

The CB_2 receptor agonist (R,S)-AM1241 (Sigma) was dissolved in 2% Cremophor (Sigma), 10% ethanol and distilled water. The CB_1 receptor antagonist AM251 (Tocris) was dissolved in 10% DMSO and distilled water for its administration. The CB_2 receptor antagonist SR144528 (kindly donated by Sanofi-Aventis) was diluted in 2.5% DMSO (Sigma) and distilled water for peri-tumour and systemic administration and in 2% DMSO, 6% ethanol and distilled water for intrathecal (i.t.) administration. The opioid receptor antagonist naloxone (Sigma) was dissolved in saline. In all cases, control animals received the corresponding solvent.

Intraperitoneal (i.p.) and subcutaneous (s.c.) administration of drugs were given in a volume of 10 mL·kg^{-1}. When drugs were administered in the vicinity of the tumour, they were dissolved in 0.2 mL of saline and injected subcutaneously over the tibial tumour mass (peri-tumour administration). When drugs were administered in the left, contralateral paws, injections were performed in the same region of the limb which, in this case, was free of tumour (Baamonde *et al.*, 2005).

Intrathecal injections were performed following a slight modification of the method described by Hylden and Wilcox (1980). A lumbar cut was made in mice under light ether anesthesia, then the tip of a 26 gauge needle inserted in a Hamilton syringe was introduced at the level of L5–L6 and finally, a volume of 5 μL was injected. In no case did the mice exhibit signs of neurological or motor alteration after intrathecal injections.

Nociceptive testing

To perform unilateral hot plate (UHP) test, mice were gently restrained and the plantar side of the tested paw was placed on a hot plate surface as previously described (Menéndez *et al.*, 2002). The latency for paw withdrawal from the heated surface was manually recorded with a chronometer. The mean of two measurements of the withdrawal latencies of each hind paw separately and alternately performed at 2-min intervals was calculated. A cut-off of 30 s was established in order to prevent tissue damage. In order to obtain basal withdrawal latencies of about 14 s in both strains of mice, plate temperature was adjusted at 51°C for C3H/He and 49.5°C for C57BL/6 mice.

Mechanical allodynia was assessed by applying von Frey filaments (Stoelting) to the plantar side of the paws as previously reported (Baamonde *et al.*, 2007). Mice were placed on a wire mesh platform, covered with transparent plastic containers and a 25 min period was allowed for habituation. The von Frey filaments 2.44, 2.83, 3.22, 3.61, 4.08, 4.56 were used and, starting with the 3.61 filament, six measurements were taken in each animal randomly starting in the left or right paw.

Based on the previously described 'up and down' method (Chaplan *et al*., 1994), the observation of a positive response (lifting, shaking or licking of the paw) after a 3 s application of a filament was followed by the application of the next thinner filament or the next thicker one if the response was negative. The 50% response threshold was calculated using the following formula: 50% g threshold = $(10^{Xf + \kappa\delta})/10\,000$; where Xf is the value of the last von Frey filament applied; κ is a correction factor based on pattern of responses (from Dixon's calibration table); δ is the mean distance in log units between stimuli (here, 0.4).

Western blot assays

Western blot experiments to detect CB_2 protein were performed using lumbar segments of the spinal cord and DRG of mice inoculated with NCTC 2472 osteosarcoma cells (2 and 4 weeks before) or 1 week before with B16-F10 melanoma cells.

In order to check the specificity of the CB_2 receptor antibody used, CB_2 receptor expression was initially measured in skin, a tissue where the presence of these receptors has been previously described (Casanova *et al*., 2003) and in Chinese hamster ovary (CHO) cells, a cell line which does not express CB_2 receptors (Carayon *et al*., 1998). Also, experiments with antigen preabsorption with a blocking peptide were performed in spinal homogenates.

For tissue harvesting, mice were exposed to a CO_2 atmosphere and then decapitated. The vertebral column was sectioned at thoracic and sacral levels and the lumbar cord was extracted by flushing about 3–5 mL of ice-cold saline through the spinal cavity with a syringe. L2-L6 lumbar spinal segments were selected, frozen in liquid nitrogen and conserved at –80°C. As previous studies in rodents bearing tibial fractures or hindlimb muscle injury have reported changes in L4-L6 dorsal root ganglia (DRG) (Djouhri *et al*., 2006; Smith *et al*., 2006; Sluka *et al*., 2007; Wei *et al*., 2009), L4-L6 DRG ipsilateral and contralateral to the inoculated tibia were isolated, frozen in liquid nitrogen and kept separately at –80°C. Each sample came from a single animal in experiments with spinal tissue, whereas a pool of 9 DRG from three mice was necessary for each Western blot. In each experiment with plantar glabrous skin tissue, pooled samples from different mice were used.

Spinal cord and DRG samples were homogenized in ice-cold buffer containing 10% glycerol, 60 mM Tris-HCl (pH 7.4), 80 mM sodium dodecyl sulphate (SDS) and a protease inhibitor (1 tablet per 50 mL buffer, Roche Diagnostics) in a volume of 6 $\mu L \cdot mg^{-1}$ of tissue and then centrifuged (120× *g*, 10 min, 4°C). The supernatant obtained was centrifuged again (26 000× *g*, 20 min, 4°C), collected and conserved at –80°C until its use.

Plantar tissue was ground with liquid nitrogen in a mortar and homogenized in buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 25% glycerol, 1 mM EDTA, 1 mM EGTA, 1% NP40 and protease inhibitor. Homogenized samples were exposed to five freezing/thawing/mixing cycles, then continuously mixed for 15 min at 4°C, centrifuged at 10 000× *g* for 20 min at 4°C and, finally, the supernatant was collected and kept at –80°C until use. In order to obtain CHO lysates, cells were centrifuged at 400× *g* for 10 min and the final pellet suspended in the same buffer used for spinal and DRG samples.

In all cases, protein concentrations were determined by a BCA protein assay (Pierce), according to the manufacturer's protocol. Next, the volume of homogenate corresponding to 100 µg of spinal cord protein, 40 µg of DRG protein and 60 µg of plantar tissue and of CHO lysate protein was vigorously mixed with the volume of sample buffer (0.02% bromophenol blue, 8% mercaptoethanol, 40% glycerol, 8% SDS, 200 mM Tris-HCl at pH 6.8) necessary to obtain a final volume of 30 µL, placed in an Eppendorf tube and heated at 100°C for 5 min. After this, samples were run on a 10% SDS-PAGE gel at 90 V during 90 min. Samples were then transferred onto nitrocellulose (Bio-Rad) at 4°C during 90 min using 100 V. The nitrocellulose membrane was blocked in Tris buffered saline-Tween (TBST, Tris 10 mM, NaCl 150 mM, Tween® 20 0.1%; pH = 7.6) with 5% non-fat milk for 90 min at room temperature, washed with TBST and incubated overnight at 4°C with goat polyclonal anti-CB_2 (1:200; Santa Cruz Biotechnology). After incubation, the membrane was washed with TBST and incubated with the secondary antibody (donkey anti-goat IgG-HRP, 1:20 000 Santa Cruz Biotechnology; 0.1% Tween 20; 0.1% non-fat milk) for 90 min. After final washes, labelled CB_2 receptor protein was detected at 45 kDa by enhanced chemiluminescence detection autoradiography using Supersignal West Pico Chemiluminiscent Substrate kit (Pierce), according to the manufacturer's protocol. Immune reaction intensity was determined by computer-assisted densitometry (ImageJ, NIH) on exposed Kodak X-Omat LS film.

For antigen preabsorption experiments, 2 µg of the anti-CB_2 antibody was preincubated (37°C, 1 h) with 10 µg of the corresponding immune peptide (Santa Cruz Biotechnology) in 100 µL PBS and the Western blots were subsequently performed, as described.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a constitutively expressed protein of 35 kDa, was also measured by Western blotting using a polyclonal rabbit anti-mouse GAPDH antibody (1:30 000, Sigma). Results are reported as the ratio of optical densities of CB_2 cannabinoid receptor and GAPDH by normalizing the amount of CB_2 receptor to the immunoreactivity of GAPDH.

Statistical analysis

The mean values and the corresponding standard errors were calculated for each behavioural assay or Western blot measurement. When thermal withdrawal latencies were compared, an initial one-way analysis of variance (ANOVA) was followed by either Dunnett's *t*-test when groups received different doses of a drug or by the Newman–Keuls test when groups received different drug treatments. In order to compare the mechanical threshold values obtained by the von Frey test, an initial Kruskal–Wallis test followed by the Mann–Whitney *U*-test was performed. The values of the ratios CB_2 receptor/GAPDH expression obtained in Western blot assays were compared by Student's *t*-test. In all cases, the level of significance was set at $P < 0.05$.

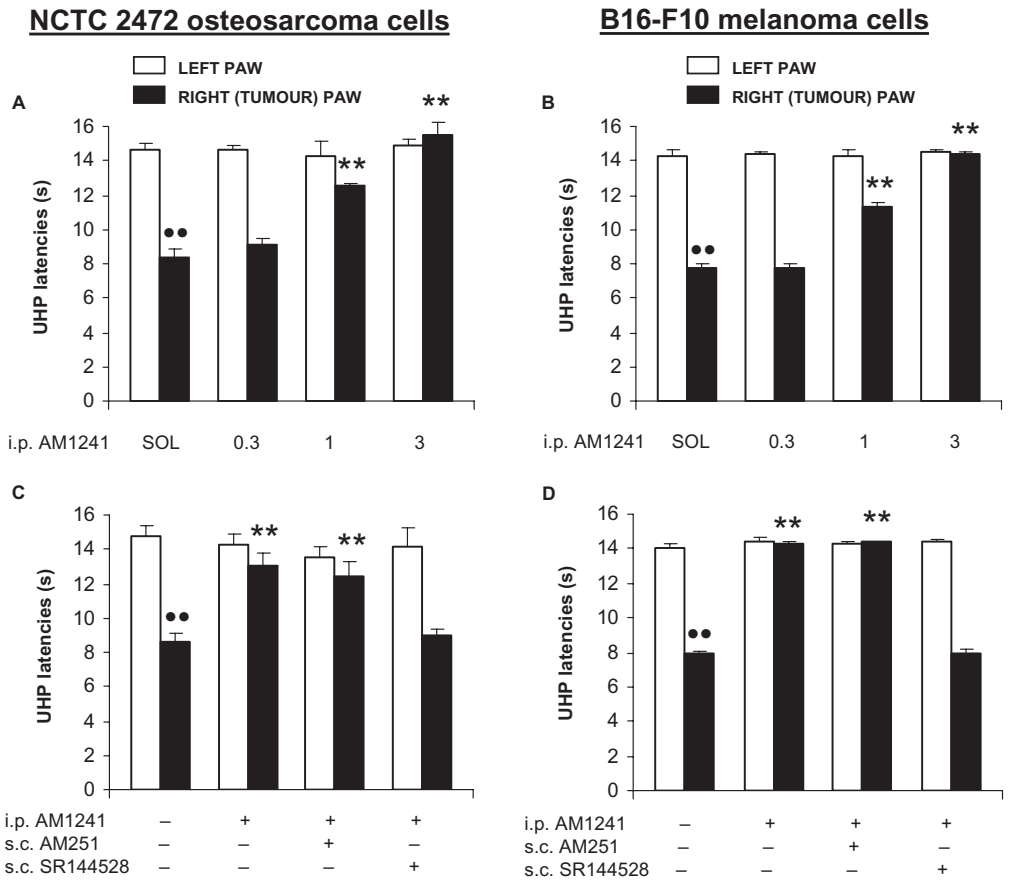

Figure 1 Antihyperalgesic effect induced by the systemic administration of AM1241 (0.3–3 mg·kg^{-1}, i.p.) or its corresponding solvent (SOL) in mice inoculated with NCTC 2472 osteosarcoma (A) or B16-F10 melanoma cells (B) measured by the unilateral hot plate test. Effect of s.c. AM251 (5 mg·kg^{-1}) or SR144528 (1 mg·kg^{-1}) on the antihyperalgesic effect induced by the i.p. administration of AM1241 (3 mg·kg^{-1}) in mice inoculated with NCTC 2472 osteosarcoma (C) or B16-F10 melanoma cells (D). Each bar represents the mean ± SEM (n = 6–8 mice). **P < 0.01 compared with the right paw of the solvent-treated group, Dunnett's t-test (A, B) or Newman-Keuls test (C, D). ••P < 0.01 compared with the corresponding left paw, Newman-Keuls test. UHP, unilateral hot plate.

Results

AM1241 inhibits tumour-derived thermal hyperalgesia by activating peripheral and spinal CB_2 receptors

Hyperalgesia was measured 4 weeks after the intratibial administration of NCTC 2472 osteosarcoma cells in C3H/He mice and 1 week after the intratibial inoculation of B16-F10 melanoma cells to C57BL/6 mice. The i.p. administration of AM1241 (0.3–3 mg·kg^{-1}, 30 min before testing) produced a dose-dependent inhibition of thermal hyperalgesia evoked either by the inoculation of NCTC 2472 osteosarcoma (Figure 1A) or B16-F10 melanoma (Figure 1B) cells. In both tumour models, the 1 mg·kg^{-1} dose produced a significant effect with the maximal antihyperalgesic effect seen when 3 mg·kg^{-1} of AM1241 was injected. The progressive increase of the withdrawal latencies measured in the injured paw in response to AM1241 was not accompanied by any modification of the values obtained in the contralateral paws. The administration of 3 mg·kg^{-1} of AM1241 to mice intratibially implanted with killed tumour cells did not modify thermal latencies (data not shown).

The antihyperalgesic effect induced by the i.p. administration of 3 mg·kg^{-1} of AM1241 in mice inoculated either with NCTC 2472 osteosarcoma (Figure 1C) or B16-F10 melanoma (Figure 1D) cells was completely prevented by the s.c. administration of the selective CB_2 receptor antagonist SR144528 (1 mg·kg^{-1}, 30 min before testing). In contrast, the s.c. administration of the CB_1 receptor antagonist AM251 (5 mg·kg^{-1}, 30 min before testing) did not modify the antihyperalgesic effect induced by systemic AM1241 in mice inoculated with either NCTC 2472 osteosarcoma (Figure 1C) or B16-F10 melanoma (Figure 1D) cells. Cannabinoid antagonists did not modify withdrawal latencies when administered alone (data not shown).

The antihyperalgesic effect induced by the systemic administration of AM1241 (3 mg·kg^{-1}, i.p.) to mice intratibially injected with either NCTC 2472 osteosarcoma (Figure 2A) or B16-F10 melanoma (Figure 2B) cells was abolished when 5 μg of the CB_2 receptor antagonist SR144528 was administered i.t. The spinal administration of SR144528 alone did not modify basal latencies. Furthermore, peripheral administration of SR144528 (10 μg) also antagonized the antihyperalgesic effect induced by 3 mg·kg^{-1} of AM1241 in mice inoculated with either osteosarcoma (Figure 2C) or melanoma cells (Figure 2D). In contrast, the antihyperalgesic effect induced by 3 mg·kg^{-1} of AM1241 was not affected by injection of 10 μg

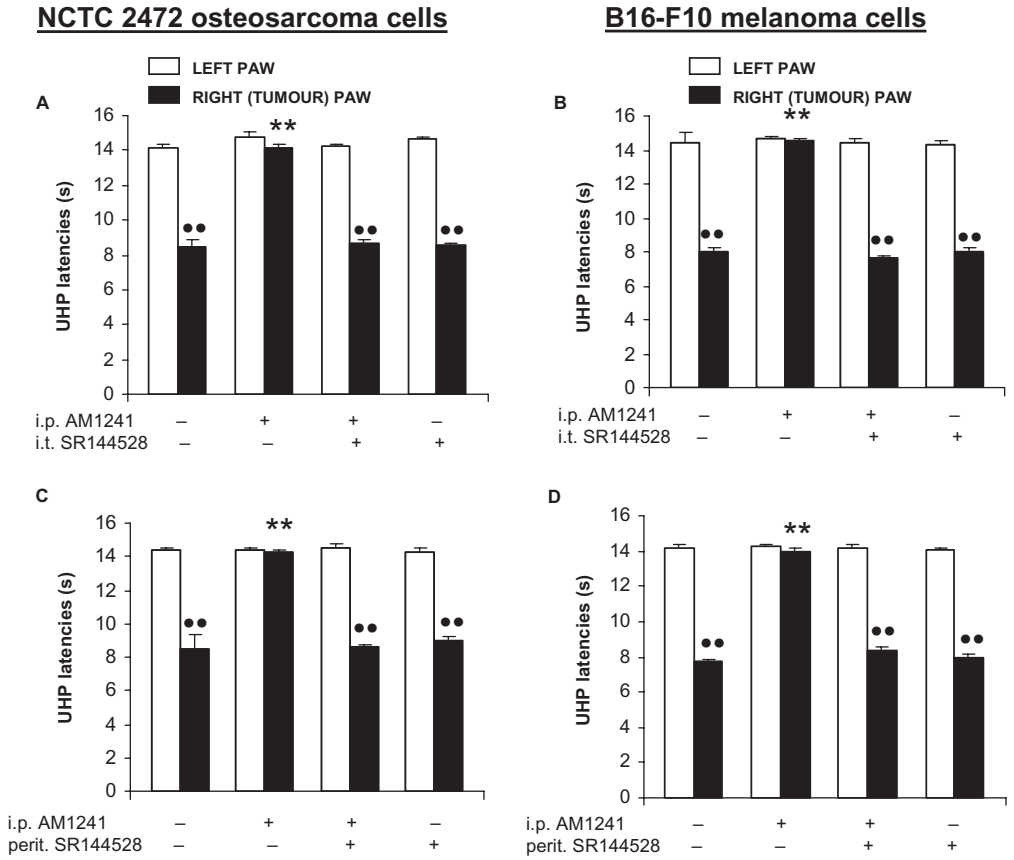

Figure 2 (A–B) Effect of the intrathecal administration of SR144528 (5 μg) on the antihyperalgesic effect induced by AM1241 (3 mg·kg^{-1}; i.p.) measured by the unilateral hot plate test in mice inoculated with NCTC 2472 osteosarcoma (A) or B16-F10 melanoma (B) cells. (C–D) Effect of the peri-tumour administration of SR144528 (10 μg) on the antihyperalgesic effect induced by AM1241 (3 mg·kg^{-1}; i.p.) in mice inoculated with NCTC 2472 osteosarcoma (C) or B16-F10 melanoma cells (D). Each bar represents the mean ± SEM (n = 7–9). **P < 0.01 compared with the right paw of the solvent-treated group, ••P < 0.01 compared with the corresponding left paw, Newman-Keuls test. UHP, unilateral hot plate.

of SR144528 in the limb contralateral to that inoculated with tumour cells (withdrawal latencies obtained in the right paws 14.1 ± 0.39 s in mice inoculated with NCTC 2472 osteosarcoma cells and 14.05 ± 0.24 s in those receiving B16-F10 melanoma cells).

Neither the i.t. nor the peri-tumour administration of SR144528 alone modified thermal withdrawal latencies when administered to mice inoculated with killed cells (data not shown).

AM1241 inhibits tumour-derived mechanical allodynia by activating spinal CB_2 receptors

A marked decrease in the mechanical threshold measured in the von Frey test occurred in C3H/He mice intratibially inoculated with NCTC 2472 osteosarcoma cells 2 weeks before (Figure 3A). Systemic administration of AM1241 (1–10 mg·kg^{-1}, i.p., 30 min before testing) dose-dependently abolished this tumour-induced mechanical allodynia. A significant antiallodynic effect was measured after the administration of 3 mg·kg^{-1} of AM1241, and mechanical allodynia was completely inhibited in the presence of 10 mg·kg^{-1}. C57BL/6 mice show unilateral mechanical allodynia 1 week after intratibial B16-F10 melanoma cells inoculation (Figure 3B). Systemic administration of AM1241 (1–10 mg·kg^{-1}, i.p., 30 min before testing) dose-dependently abolished this tumour-derived mechanical allodynia, with the maximal effect achieved with a dose of 10 mg·kg^{-1} (Figure 3B). The progressive analgesic effect induced by AM1241 in the paw inoculated with NCTC 2472 osteosarcoma or B16-F10 melanoma cells was not accompanied by a parallel increase in the scores obtained in the contralateral paws. The highest dose of AM1241 tested (10 mg·kg^{-1}) produced a modest increase in the mechanical threshold of the contralateral paws in mice inoculated with NCTC 2472 osteosarcoma cells, but this was not seen in mice inoculated with B16-F10 melanoma cells. Administration of 10 mg·kg^{-1} AM1241 to mice implanted with killed NCTC 2472 osteosarcoma or B16-F10 melanoma cells did not modify mechanical thresholds in C3H/He or C57BL/6 mice respectively (data not shown).

The antiallodynic effects produced by the i.p. administration of AM1241 (10 mg·kg^{-1}) to mice intratibially inoculated with NCTC 2472 osteosarcoma (Figure 3C) or B16-F10 melanoma (Figure 3D) cells were completely prevented by the administration of the selective CB_2 receptor antagonist SR144528 (1 mg·kg^{-1}, s.c., 30 min before testing). In contrast, the administration of the selective CB_1 receptor antagonist AM251 (5 mg·kg^{-1}, s.c., 30 min before testing) together with

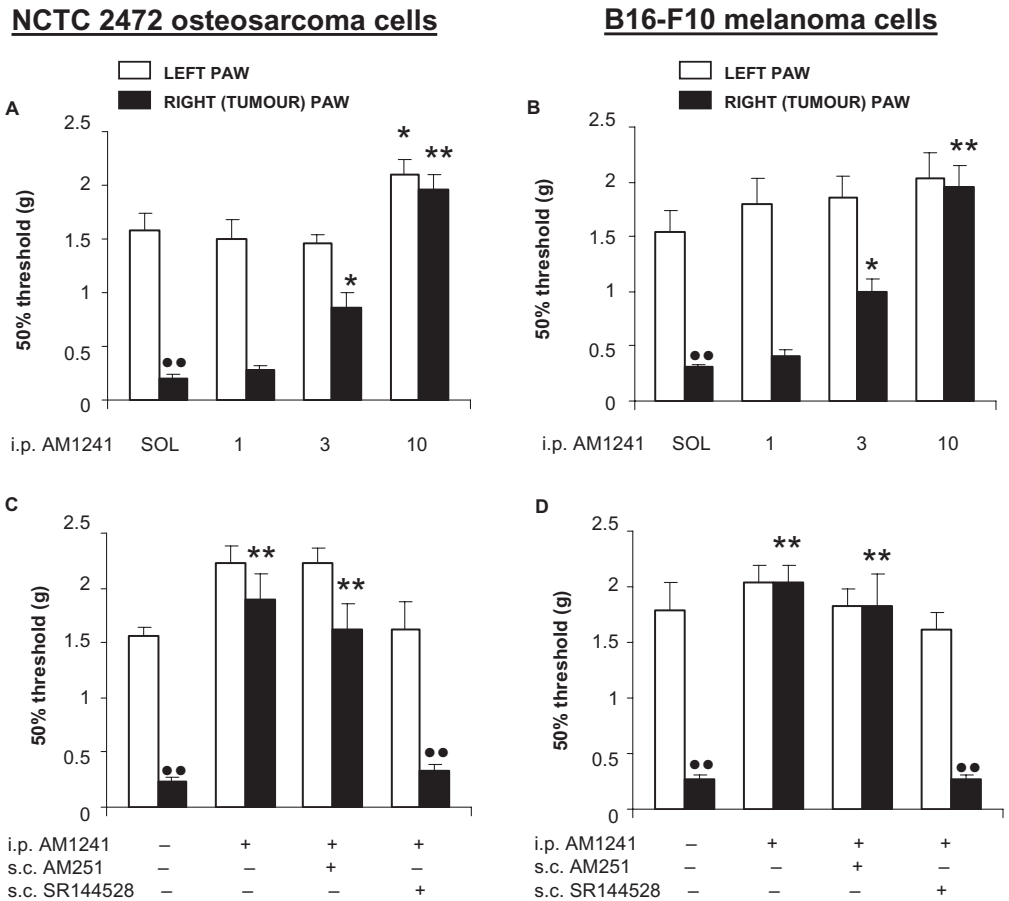

Figure 3 Antiallodynic effect induced by the systemic administration of AM1241 (1–10 mg·kg^{-1}, i.p.) or its corresponding solvent (SOL) in mice inoculated with NCTC 2472 osteosarcoma (A) or B16-F10 melanoma cells (B) measured in the von Frey test. Effect of s.c. AM251 (5 mg·kg^{-1}) or SR144528 (1 mg·kg^{-1}) on the antiallodynic effect induced by the i.p. administration of AM1241 (10 mg·kg^{-1}) in mice inoculated with NCTC 2472 osteosarcoma (C) or B16-F10 melanoma cells (D). Each bar represents the mean ± SEM (n = 8–10). *P < 0.05, **P < 0.01 compared with the right or left paw of the solvent-treated group, •P < 0.05, ••P < 0.05 compared with its corresponding left paw, Mann–Whitney U-test.

AM1241 did not modify the antiallodynic effect induced by AM1241 in mice inoculated with NCTC 2472 osteosarcoma (Figure 3C) or B16-F10 melanoma cells (Figure 3D). No modification of mechanical thresholds was obtained when these cannabinoid receptor antagonists were administered alone (data not shown).

Intrathecal administration of the CB_2 receptor antagonist SR144528 (5 μg) completely blocked the antiallodynic effect produced by the systemic administration of AM1241 (10 mg·kg^{-1}) in mice inoculated with NCTC 2472 osteosarcoma (Figure 4A) or B16-F10 melanoma (Figure 4B) cells. In contrast, the inhibition of the allodynia induced by the administration of 10 mg·kg^{-1} of AM1241 observed in mice intratibially inoculated with NCTC 2472 osteosarcoma (Figure 4C) or B16-F10 melanoma (Figure 4D) cells remained unaltered after the peri-tumour administration of SR144528 (10 μg, 30 min before testing).

The i.t. administration of AM1241 (0.03–0.3 μg, 30 min before testing) dose-dependently abolished osteosarcoma-induced thermal hyperalgesia (Figure 5A). A significant antihyperalgesic effect was detected after the administration of 0.1 μg and a complete blockade of thermal hyperalgesia was induced by 0.3 μg of this CB_2 receptor agonist. Mechanical allodynia in mice inoculated with NCTC 2472 osteosarcoma cells was also dose-dependently abolished by the i.t. administration of AM1241 (0.1–1 μg, 30 min before testing) (Figure 5B).

The inhibition of tumour-evoked hyperalgesia and allodynia induced by AM1241 is mediated by endogenous opioids

In order to elucidate the possible participation of endogenous opioids in the antihyperalgesic and antiallodynic effects induced by AM1241, experiments were performed in which 3 mg·kg^{-1} of the opioid receptor antagonist naloxone were given s.c., 20 min before testing in both tumour models. The administration of this dose of the opioid receptor antagonist inhibited the antihyperalgesic effect produced by 3 mg·kg^{-1} of AM1241 (s.c., 30 min before testing) in mice intratibially inoculated 4 weeks before with NCTC 2472 osteosarcoma cells (Figure 6A) or 1 week before with B16-F10 melanoma cells (Figure 6B). In a similar way, the antiallodynic effect induced by AM1241 (10 mg·kg^{-1} s.c., 30 min before testing) in mice inoculated with NCTC 2472 osteosarcoma or B16-F10

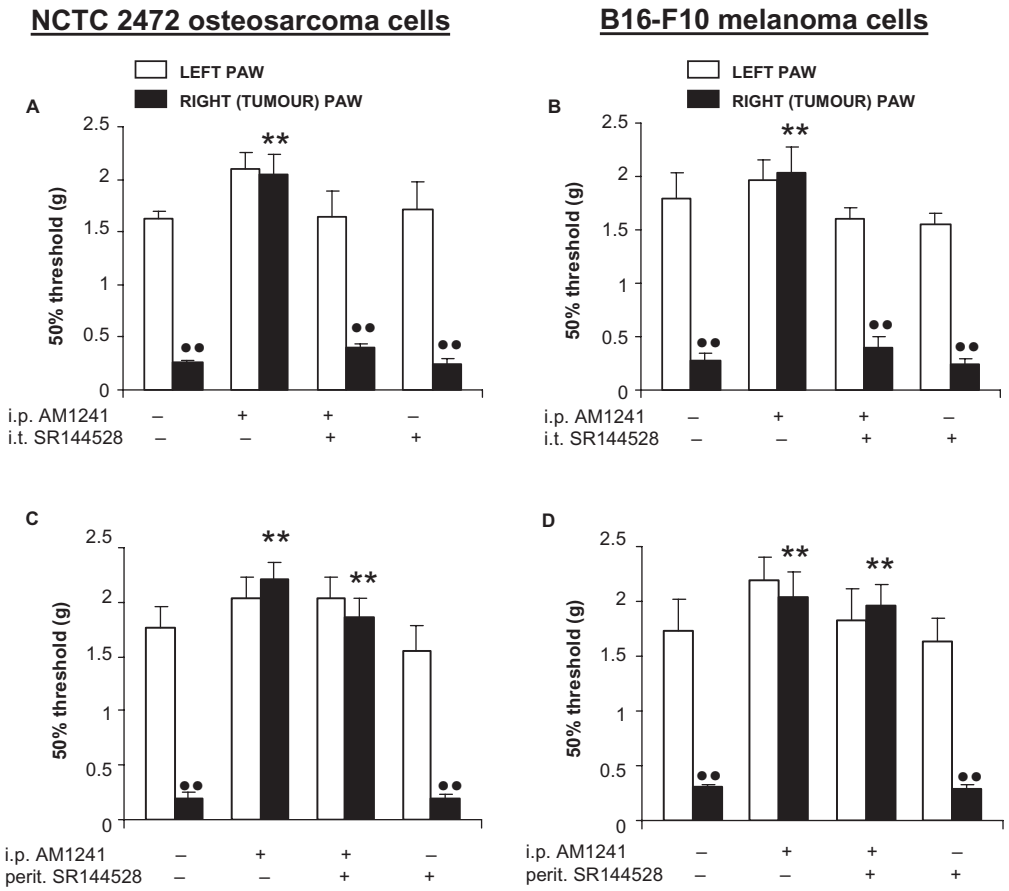

Figure 4 (A–B) Effect of the intrathecal administration of SR144528 (5 μg) on the antiallodynic effect induced by AM1241 (10 mg·kg^{-1}; i.p.) measured in the von Frey test in mice inoculated with NCTC 2472 osteosarcoma (A) or B16-F10 melanoma (B) cells. (C–D) Effect of the peri-tumour administration of SR144528 (10 μg) on the antiallodynic effect induced by AM1241 (10 mg·kg^{-1}; i.p.) in mice inoculated with NCTC 2472 osteosarcoma (C) or B16-F10 melanoma cells (D). Each bar represents the mean ± SEM (n = 6–9). **P < 0.01 compared with the right paw of the solvent-treated group, ••P < 0.01 compared with the corresponding left paw, Mann–Whitney U-test.

melanoma cells, 2 and 1 week before, respectively, was abolished by the administration of naloxone (Figure 6C,D).

The expression of CB_2 receptors in spinal cord or DRG is not modified by the presence of tumour cells

Western blot experiments with spinal cord homogenates revealed a band of approximately 45 kDa that was labelled by the CB_2 receptor antibody. Labelling was confirmed by the finding of a band of the same molecular mass in blots of samples of skin homogenates, used as a positive control and the absence of labelling with CHO cell lysates, used as a negative control. In addition, no band was detected in spinal cord homogenates when the antibody was pre-incubated with the blocking peptide (Figure 7A).

The level of CB_2 receptor expression in the spinal cord at times when mechanical allodynia and thermal hyperalgesia were measured (2 or 4 weeks) was indistinguishable from that in mice inoculated with either live or killed NCTC 2472 osteosarcoma cells (Figure 7B). The density of spinal CB_2 receptors in mice inoculated with B16-F10 melanoma cells was also similar to that measured in mice inoculated with killed cells (Figure 7C).

CB_2 receptor protein expression was measured in DRG 4 weeks after inoculation with NCTC 2472 osteosarcoma cells and 1 week after inoculation with B16-F10 melanoma cells, the times at which the involvement of peripheral CB_2 receptors in thermal hyperalgesia was detected in behavioural studies. In all cases a band of approximately 45 kDa was detected with no change in CB_2 receptor density produced by the intratibial inoculation of NCTC 2472 osteosarcoma (Figure 7B) or B16-F10 melanoma cells (Figure 7C).

Discussion

Our results demonstrate that the stimulation of CB_2 receptors effectively counteracted mechanical allodynia and thermal hyperalgesia evoked by the development of two different tumours in mice. Bone cancer-evoked mechanical allodynia was abolished through the exclusive activation of spinal CB_2 receptors, whereas tumour-derived thermal hyperalgesia was counteracted by the activation of peripheral and spinal CB_2 receptors. Both CB_2 receptor-mediated responses were inhibited by naloxone, thus demonstrating the involvement of opioid receptors probably stimulated by endogenous opioid

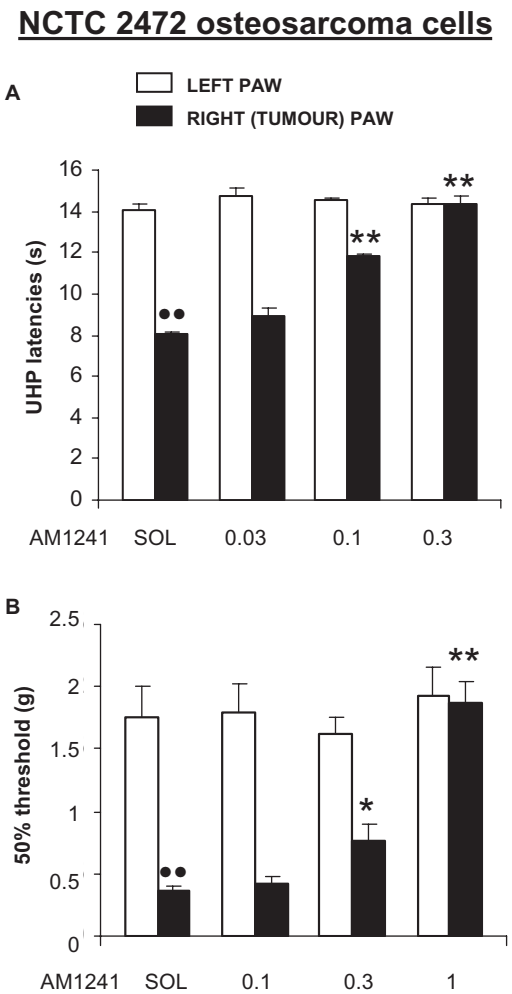

Figure 5 (A) Antihyperalgesic effect induced by the intrathecal administration of AM1241 (0.03–0.3 μg) or its corresponding solvent (SOL) measured by the unilateral hot plate test in mice intratibially inoculated with NCTC 2472 osteosarcoma cells. Each bar represents the mean ± SEM ($n = 6$). $^{**}P < 0.01$ compared with the right paw of the solvent-treated group, $P < 0.01$ compared with the left paw in the solvent-treated group, Dunnett's *t*-test. (B) Antiallodynic effect induced by the intrathecal administration of AM1241 (0.1–1 μg) or its corresponding solvent (SOL) measured by the von Frey test in mice intratibially inoculated with NCTC 2472 osteosarcoma cells. Each bar represents the mean ± SEM ($n = 7$–9). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the right paw of the solvent-treated group, $^{••}P < 0.05$ compared with its corresponding left paw, Mann–Whitney *U*-test. UHP, unilateral hot plate.

agonists. Measurement of DRG and spinal CB_2 receptor density by Western blot revealed no tumour-induced changes.

We have previously observed that some analgesic drugs, such as peripherally acting opioids (Baamonde *et al.*, 2006; Curto-Reyes *et al.*, 2008) or the antagonist of the type I interleukin-1 receptor anakinra (Baamonde *et al.*, 2007), can inhibit tumoural thermal hyperalgesia without affecting mechanical allodynia. For this reason, in order to obtain a more complete view of the analgesic profile of a CB_2 receptor agonist in experimental bone cancer-induced pain, we have tested the effect of AM1241 on both parameters. The systemic administration of the CB_2 receptor agonist, AM1241, blocked both tumour-induced thermal hyperalgesia and mechanical allodynia through the selective stimulation of CB_2, and not CB_1, receptors, as only the systemic administration of the CB_2 receptor antagonist abolished analgesic effects of AM 1241. Bearing in mind the limitations in comparing models of pathological pain when a variety of tests have been used, our data indicate that the systemic doses of AM1241 needed to antagonize hyperalgesia or allodynia due to bone cancer are similar to those effective in neuropathic pain (Ibrahim *et al.*, 2003; Rahn *et al.*, 2008) and greater than those necessary in inflamed animals (Nackley *et al.*, 2003; Gutierrez *et al.*, 2007). We further show that the blockade of these hypernociceptive symptoms in bone cancer models is related to the stimulation of CB_2 receptors located at different sites.

In order to elucidate if CB_2 receptors involved in the analgesic effect induced by AM1241 were peripherally or spinally located, we have performed experiments in which this agonist was systemically injected and the selective CB_2 receptor antagonist SR144528 was administered either at the site of the tumour or intrathecally. For thermal hyperalgesia, the finding that peri-tumour as well as i.t. injection of SR144528 antagonized the effect induced by systemic AM1241, supports the involvement of CB_2 receptors expressed both in the periphery and in the spinal cord in the antihyperalgesic effect mediated by this CB_2 receptor agonist. The local nature of the blockade induced by the peri-tumour administration of the CB_2 receptor antagonist was confirmed by the lack of effect of this drug when administered in the paw contralateral to the one bearing the tumour. The inhibition of thermal hyperalgesia by the activation of peripheral CB_2 receptors is in accordance with previous reports describing that mechanical hyperalgesia induced either by the inoculation of NCTC 2472 osteosarcoma cells in the calcaneus (Potenzieri *et al.*, 2008) or the intraplantar inoculation of human oral squamous carcinoma cells (Guerrero *et al.*, 2008) can be blocked through the stimulation of peripheral CB_2 receptors. Our results further indicate that, apart from peripheral CB_2 receptors, spinal receptors could also participate in the inhibition of thermal hyperalgesia induced by AM1241. Moreover, only the i.t. administration of SR144528 blocked the antiallodynic effect produced by systemic AM1241 in both neoplastic models, thus demonstrating that the inhibition of tumour-evoked allodynia, a hypernociceptive symptom whose modulation by CB_2 receptors has not been studied so far in bone cancer models, exclusively occurs through the stimulation of CB_2 receptors located in the spinal cord. Further supporting this idea, mechanical allodynia was totally abolished by the i.t. administration of AM1241 to mice inoculated with NCTC 2472 osteosarcoma cells.

It is not easy to explain why tumour-induced thermal hyperalgesia and mechanical allodynia are differently affected by the activation of peripheral CB_2 receptors, although it could be considered that the different neurophysiological mechanisms underlying both symptoms could help to understand this result. Thus, the inhibition of thermal hyperalgesia could be related with the ability of AM1241 to inhibit the firing of C fibres through the stimulation of peripheral CB_2 receptors (Nackley *et al.*, 2004). In this sense, it has also been demonstrated that the activation of CB_2 receptors coexpressed with the thermal transducer receptor TRPV1 in small diameter DRG neurons inhibits responses mediated by TRPV1 (Anand

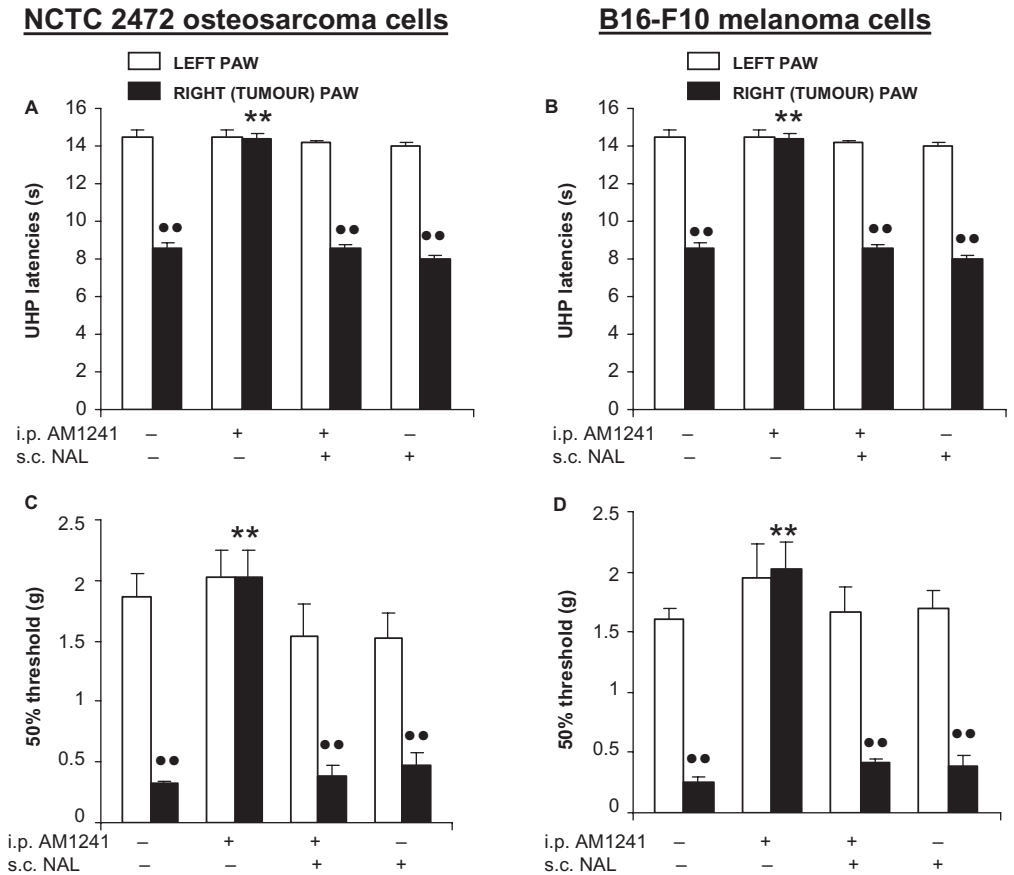

Figure 6 (A–B) Effect of the systemic administration of naloxone (3 mg·kg^{-1}; s.c.) on the antihyperalgesic effect induced by AM1241 (3 mg·kg^{-1}; i.p.) measured in the unilateral hot plate test in mice inoculated with NCTC 2472 osteosarcoma (A) or B16-F10 melanoma (B) cells. Each bar represents the mean ± SEM ($n = 5$). **$P < 0.01$ compared with the right paw of the solvent-treated group, $P < 0.01$ compared with the corresponding left paw, Newman-Keuls test. (C–D) Effect of the systemic administration of naloxone (3 mg·kg^{-1}; s.c.) on the antiallodynic effect induced by AM1241 (10 mg·kg^{-1}; i.p.) in mice inoculated with NCTC 2472 osteosarcoma (C) or B16-F10 melanoma cells (D). Each bar represents the mean ± SEM ($n = 5$). **$P < 0.01$ compared with the right paw of the solvent-treated group, ••$P < 0.01$ compared with the corresponding left paw, Mann–Whitney *U*-test. UHP, unilateral hot plate.

et al., 2008), whose involvement in osteosarcoma-induced thermal hyperalgesia has been previously established (Menéndez *et al.*, 2006). It has been reported that allodynia could be mainly triggered and maintained by the activity of myelinated Aβ fibres (Neumann *et al.*, 1996) and the fact that AM1241 when acting peripherally does not modify Aβ fibre-mediated responses (Nackley *et al.*, 2004) could explain the absence of peripheral antiallodynic effect of AM1241 in both bone cancer models. However, the possibility of producing antiallodynic effects derived from the stimulation of peripheral CB_2 receptors seems to depend on the particular underlying pathology. In inflammatory conditions the efficacy of stimulating CB_2 receptors has been proven (Nackley *et al.*, 2003; Sokal *et al.*, 2003; Elmes *et al.*, 2004; Gutierrez *et al.*, 2007), whereas controversial results have been obtained for neuropathic pain. In one report mechanical allodynia was inhibited by stimulating peripheral CB_2 receptors (Elmes *et al.*, 2004), while other authors reported that, as in our experiments, spinal but not the peripheral administration of a CB_2 receptor agonist, blocks neuropathic mechanical allodynia (Yamamoto *et al.*, 2008).

Some antinociceptive effects induced by AM1241 have been shown to be mediated through the release of endogenous opioid peptides (Ibrahim *et al.*, 2005; Yao *et al.*, 2008). Thus, we tested whether the antihyperalgesic and antiallodynic effects here described could be blocked by an opioid antagonist. The systemic administration of naloxone antagonized the antihyperalgesic and the antiallodynic effects exerted by AM1241 in both cancer models, demonstrating that this opioid-mediated antinociception derived from the activation of CB_2 receptors is also functional in neoplastic situations, as previously shown during inflammation (Yao *et al.*, 2008). These results raise the questions as to where are opioids released from and which of them are ultimately responsible for the analgesic effect. Although it has been shown that peripherally, the stimulation by AM1241 of CB_2 receptors located in keratinocytes leads to the release of beta-endorphin (Ibrahim *et al.*, 2005), the possibility that the activation of CB_2 receptors could induce the release of endogenous opioids at the spinal cord has not been previously explored. Thus, it would be interesting to determine whether beta-endorphin or other peptides are the opioids responsible for the analgesic effects produced through the stimulation of CB_2 receptors by AM1241 either at peripheral or spinal level in these particular models of cancer pain.

We investigated whether the behavioural effects we had observed were accompanied by modifications in the expres-

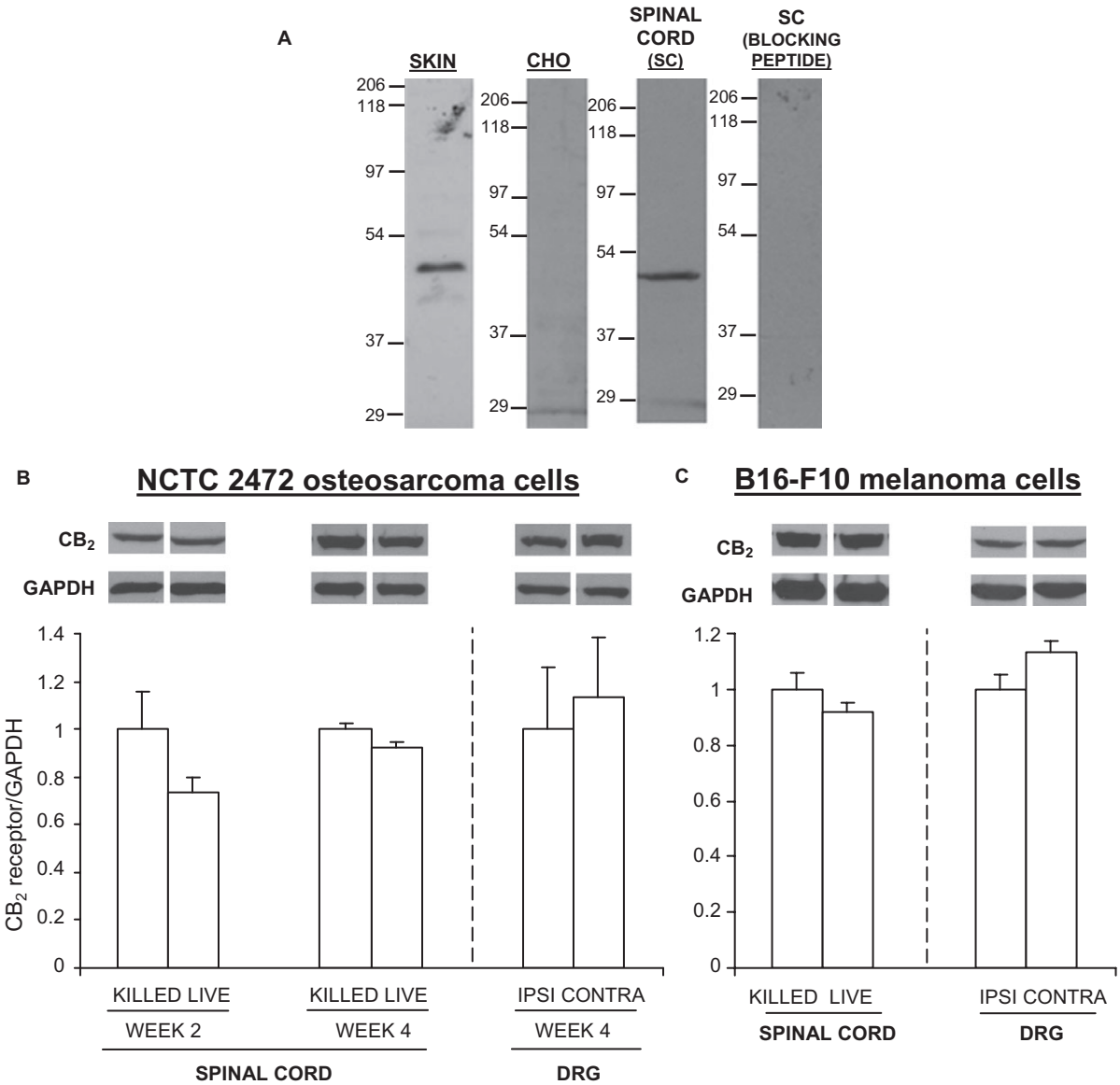

Figure 7 (A) Representative lanes of Western blots for CB_2 receptors, from plantar skin tissue (SKIN), Chinese hamster ovarian cells (CHO), lumbar spinal cord (SC) and lumbar spinal cord incubated with anti-CB_2 receptor and blocking peptide. Molecular weight markers are indicated at the left side of the corresponding lane. (B, C) CB_2 receptor expression measured by Western blot in L2-L6 lumbar segments of the spinal cord or L4-L6 DRG of mice, 2 or 4 weeks after implantation with NCTC 2472 osteosarcoma cells (B) or 1 week after implantation with B16-F10 melanoma cells (C). CB_2 receptor expression was measured in spinal cords of mice inoculated with live or killed tumour cells and in DRG contralateral or ipsilateral to the presence of tumour cells. The fold change in CB_2 receptor protein was estimated by using its corresponding GAPDH as endogenous control. Data are the mean ± SEM ($n = 4$). DRG, dorsal root ganglia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

sion of CB_2 receptors. An increase in the number of spinal CB_2 receptors has been described in neuropathic (Zhang *et al.*, 2003; Walczak *et al.*, 2005; Beltramo *et al.*, 2006; Romero-Sandoval *et al.*, 2008) but not in inflammatory (Zhang *et al.*, 2003; Cox *et al.*, 2007) pain models. However, although the up-regulation of CB_1 receptors expressed in DRG has been shown in mice inoculated with NCTC 2472 cells (Khasabova *et al.*, 2008) or with human oral squamous carcinoma cells (Guerrero *et al.*, 2008), no previous report has dealt with the possibility that the development of a painful bone tumour could provoke an increase in the expression of CB_2 receptors. When we assessed whether the population of CB_2 receptors is modified in response to tumour injuries, no changes appeared in DRG or lumbar spinal cord of mice inoculated with NCTC 2472 osteosarcoma or B16-F10 melanoma cells at the times at which behavioural tests were performed. These results indicate that the activation of the constitutive population of CB_2 receptors is sufficient to inhibit these bone cancer-induced hypernociceptive symptoms.

In conclusion, the present results indicated that the expression of CB_2 receptors in DRG and spinal cord remained unaltered during the growth of two different types of painful bone tumours and demonstrated the analgesic efficacy derived from the stimulation of peripheral and spinal CB_2 receptors by

AM1241. These data suggest activation of spinal CB_2 receptors as an effective strategy for the management of neoplastic pain.

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Conflicts of interest

The authors state no conflict of interest.

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