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Differential effects of repeated low dose treatment with the cannabinoid agonist WIN 55,212-2 in experimental models of bone cancer pain and neuropathic pain

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ABSTRACT

Pain due to bone malignancies is one of the most difficult types of cancer pain to fully control and may further decrease the patients' quality of life. Animal models of chronic pain conditions resulting from peripheral inflammatory reactions or nerve injuries are responsive to treatment with cannabinoid agonists. However, the use of cannabinoid agonists in humans may be hampered by CNS related side effects and development of tolerance. In the present study, we investigated the effect of repeated low dose administration of the synthetic cannabinoid agonist WIN 55,212-2 on bone cancer pain and neuropathic pain in mice. In addition, we investigated the development of CNS related side effects and tolerance. We found that 0.5 mg/kg/day for 18 days reduced pain related behavior and expression of spinal glial fibrillary acidic protein in the bone cancer pain model but not in the neuropathic pain model. Furthermore, this treatment strategy was not found to induce measurable CNS related side effects or tolerance. Cancer cell viability assays and bone volume fraction assessed by micro computed tomography (μCT) demonstrated that these effects were not due to changes in cancer progression. The difference in WIN 55,212-2 efficacy between the bone cancer and neuropathic pain models may reflect the different pain generating mechanisms, which may be utilized in designing new therapeutic drugs.

1. Introduction

Metastasizing cancers frequently spread to bone tissue where they induce bone resorption and fractures. Pain is the most common symptom of osteolytic bone metastases and increase in severity during the disease course (Mercedante, 1997). The mechanism responsible for generating the high pain symptoms observed in bone cancer patients has not yet been elucidated. However, osteoclast activity is speculated to be a prerequisite for establishing bone cancer pain (Honore et al., 2000a; Roudier et al., 2006), while factors secreted from stromal or cancer cells as well as released from degrading extracellular matrix structures or infiltrating macrophages may trigger neurons or rendering them hypersensitive (al Sarireh and Eremin, 2000; Menendez et al., 2006; Peters et al., 2004; Sevcik et al., 2005). Furthermore, considerable changes in the spinal cord, such as massive astrogliosis, dynorphin and Fos upregulation are also evident in animal models of bone cancer pain (Schwei et al., 1999) and some degree of central sensitization likely occurs (Sevcik et al., 2005).

Cannabinoid agonists have gained increasing interest as possible drug candidates in chronic pain management and has showed promising acute analgesic effects following single dose administrations in both neuropathic, inflammatory and bone cancer pain models (Bridges et al., 2001; Mitchell et al., 2005; Kehl et al., 2003). They induce their effects through two G-protein coupled receptors, CB1 and CB2. CB1 is mostly expressed by neurons, while CB2 is highly expressed in immune cells. However, other cell types such as adipocytes, osteoclasts and tumor cells also express significant amounts of CB1 and CB2 receptors (Hart et al., 2004; Ofek et al., 2006; Roche et al., 2006). Both receptors have been shown to be implicated in modulation of pain sensation and connected with the alleviating effects of cannabinoid treatment in animal models of pathological pain (Agarwal et al., 2007; Fox et al., 2001; Hohmann and Suplita, 2006; Ibrahim et al., 2003). Their analgesic qualities results in part from a direct effect on nociceptors and wide dynamic range neurons, while low threshold fibers are unaffected. In chronic pain studies, suppression of windup and spontaneous neuronal firing is also observed following cannabinoid treatment (Walker and Huang, 2002). Furthermore, descending inhibition of spinal nociceptive neurons is facilitated by cannabinoids through activation of the same brainstem circuit responsible for opioid analgesia (Meng et al., 1998), and cannabinoids have indeed been found to enhance the

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analgesic effect of opioids in a partially opioid receptor dependent fashion, thus supporting the notion of related analgesic pathways (Cichewicz, 2004).

Studies in humans have also shown that cannabinoids are effective in treating postoperative pain, cancer induced pain and pain following nerve injury (Pertwee, 2001). However, the beneficial effect of cannabinoid treatment is hampered by CNS related site effects and its link to psychosis (Henquet et al., 2005). Systemic treatment with low doses incapable of inflicting measurable adverse CNS effects but also lacking a pain relieving effect at single administration, has been shown to result in a cumulative pain relieving effect in neuropathic rats when given repeatedly (Costa et al., 2004). Development of tolerance is another question of major importance, as it has been shown to occur after prolonged treatment, thus possibly limiting the usefulness of cannabinoids in chronic disease treatment strategies (Tappe-Theodor et al., 2007). The mechanism of cannabinoid tolerance has been shown to involve CB1 receptor internalization and degradation as well as uncoupling of the G-protein and the receptor (Martini et al., 2007; Sim et al., 1996), though tissue specific changes in gene expression levels has also been reported (Romero et al., 1999; Rubino et al., 1994; Zhuang et al., 1998). However, as the experimental doses used in tolerance studies are large and often escalating compared to the doses needed to induce analgesia by repeated administration of cannabinoid agonists such as WIN 55,212-2 (WIN) (Costa et al., 2004; Hampson et al., 2003; Lever et al., 2007; Sim-Selley and Martin, 2002) the possibility for absent or decreased tolerance remains after administration of a series of small doses. Finally, cannabinoids have been proposed to have both mitogenic and toxic effects on cultured brain cells and cell lines in a concentration dependant manner (Hart et al., 2004) as well as affecting bone resorption and formation (Idris et al., 2005). Thereby making it necessary to investigate the effect of cannabinoid agonists on tumor progression and bone turnover when working with bone cancer pain models.

Repeated low dose treatment with the synthetic cannabinoid agonist WIN has been shown to induce cumulative analgesia in neuropathic rats (Costa et al., 2004). Therefore we investigated if a similar treatment strategy would lead to cumulative analgesia in mice suffering from bone cancer pain, without the induction of measurable side effects and tolerance. The studies were conducted with a dose of 0.5 mg/kg/day WIN as this dose was not associated with CNS related side effects determined by rotarod performance or able to induce tolerance or acute analgesia which would conceal any effect resulting from the repeated treatment therapy. Low dose WIN treatment reduced pain related behavior and pain related spinal glial fibrillary acidic protein (GFAP) expression in the bone cancer pain model; however, not to the extent which has previously been described for rats suffering from neuropathic pain (Costa et al., 2004). To investigate this discrepancy, mice suffering from neuropathic pain was treated with the same low dose of 0.5 mg/kg/day.

2. Methods

2.1. Animals

Animal experiments were performed on 6 weeks old male C3H/HeN mice weighing 20–25 g (M-B Taconic, Tornbjerg, DK). Animals were kept in a room with a 12 hour light/dark cycle with free access to standard diet and tap water. All experiments were approved by the Danish Committee for Experiments on Animals and conducted according to the ethical guidelines of the International Association for the Study of Pain.

2.2. Cell line

The NCTC-2472 sarcoma cell line (ATCC, CCL-11) was used throughout all experiments. Before use, 80% confluent cultures were rinsed in PBS and treated with 0.5% trypsin in 1 mM EDTA for three min, harvested and finally resuspended in α-Minimum Essential Medium (α-MEM) (Gibco, 22571-020). For tumor inoculation in mice, the cells were diluted to a final concentration of 10×10^6 cells/ml and kept on ice until use. For use in MTT assays, cells were diluted to a final concentration of 1.25×10^5 cells/ml.

2.3. Drug administration

WIN 55,212-2 (Sigma, W102), was dissolved in a mixture of ethanol, cremophore (Sigma, C5135) and water in the ratio 1:1:18 v/v/v by thorough whirl mixing and stored at 4 °C until use. For experiments with repeated administrations of WIN, fresh stock solutions of 75 μg/ml were prepared every 5th day. The mice were randomly stratified into the various treatment groups according to weight, so that light and heavy mice were evenly distributed between the groups. Mice were injected subcutaneous (s.c.) from the day following operation until day 19 post surgery with volumes never exceeding 200 μl.

2.4. Animal surgeries

Induction of tumors in the femoral bone cavity was carried out by a modified form of the technique described by Schwei et al. (1999). The animals were deeply anaesthetized with Zoletil mix-50 (zolazepam 25 mg/ml, tiletamin 25 mg/ml, xylazin 20 mg/ml, butorphanol 10 mg/ml), 2 μl/g (KVL, Denmark) and a small incision was made in the skin covering the knee. The medial side of the patella tendon was loosened from the surrounding tissue and the tendon was pushed lateral to expose the distal end of the femoral bone. A hole was drilled between the condyles to the marrow cavity with a 30 gauge needle and 100,000 NCTC-2472 cells in 10 μl α-MEM were injected. Finally, the hole was blocked with bonewax (Johnson & Johnson Intl, W810), the area was irrigated with saline and the skin was sutured. Sham animals were exposed to a similar operation but were injected with 10 μl α-MEM without NCTC-2472 cells. In the bone cancer pain WIN study one group of 15 mice were excluded from the study. The reason being that the NCTC-2472 cell preparation used for these mice were found to be contaminated. As a result, the mice did not develop cancer.

The Spared Nerve Injury (SNI) model of neuropathic pain was performed according to Rode et al. (2005). Briefly, mice were anaesthetized with Zoletil mix-50, 2 μl/g and the skin covering the right thigh was incised and the sciatic nerve's three terminal branches were exposed. The tibial and common peroneal nerves were ligated with 5/0 silk sutures and sectioned distal to the ligations while the sural nerve was left intact.

2.5. Behavioral tests

Von Frey tests were performed similar to Honore et al. (2000b). Briefly, we used a range of monofilaments supplying pressure from 0.008 to 2 g (North Coast Medical Inc., Morgan Hill, Ca, USA). Each mouse was placed in small plastic cage on a metal grid with 4×6 mm holes. After a one hour adaptation period in the test room, mice were exposed to five stimulations with each monofilament. More than three paw withdrawals from one monofilament were defined as a positive response.

Open field limb use tests were performed as described by Honore et al. (2000a) with some modifications. Briefly, all mice were acclimatized in a standard mice cage (42.5×26.6 cm) for 10 min and then each mouse was placed alone in the cage for 3 min. Thereafter the use of the tumor bearing limb was evaluated on a linear and continuous scale from 0–4, where 0 designates no use of the tumor bearing limb and 4 normal limb use.

Weight bearing tests were performed using the Power Meter for Small Laboratory Animals (TSE Systems). Mice were placed in a

Table 1
Sequences of primers and probes used for QPCR analyses of CB1/CB2 expression levels

Sequences of primers and probes	
FmrhYWHAZ	AGACGGAAGGTGCTGAGAAA
RmrhYWHAZ	GAAGCATTGGGGATCAAGAA
PmrhYWHAZ	(HEX)AGA+TG+GCH–TC+GA+GA+AT+ACA(BHQ 1)
FmrCB I	TGCTTGCGATCATGGTGTAT
RmrCB I	TGTCTCAGGTCCTTGCTCCT
PmrCl3I	(FAM)ATGATGGGG TTC ACG+GTG+GAG(BHQ1)
FmrCB2	ATATGCTGGTTCCCTGCACT
RmrCB2	GTTAACAAGGCACAGCATGG
PmrCB2	(FAM)AGAAGG+CG+AAGG+CC TCCTT(BHQ 1)

F: forward, R: reverse, P: probe, m: mouse, r: rat, h: human, LNA moieties are indicated by before the nucleotide.

customized holder made from 50 ml standard centrifuge tubes and placed on the Power Meter so that only the hind paws were in contact with the weights. Three non-consecutive measurements for 10 s each were performed and the average weight distribution as % of weight on right paw was calculated.

Rotarod tests were performed on an ENV-575M Five Station Rota-Rod Treadmill USB-Mouse (Med Associates inc., USA), at non-accelerating 16 rpm. Each mouse was tested three times for 120 s. The amount of time where a mouse was passively rotating with the rod was subtracted. The average amount of time spent on the rod was divided with 120 s and data is shown as a percent of maximum possible time on the rotarod.

Animal motility was determined with the Actimott MoTil System (TSE Systems). To avoid uneven adaptation between test groups, two independent set of mice were used to determine the effect of WIN at 30 and 140 min. Briefly, mice were injected and at the time of measurement, they were moved to the motility system cages and general motility was measured for 30 min. The motility system cages consisted of standard mice cages (42.5×26.6 cm) covered with a 5×9 infrared light beam grid. Light beam interruptions due to animal motility were counted and analyzed by the Actimot software (TSE systems).

All behavioral tests were performed by observers that were blinded to both the type of operation (sham/cancer) and treatment (vehicle/WIN). Separate groups of mice were used for rotarod and motility tests. In the repeated treatment study for bone cancer pain, weight bearing tests were performed prior to open field limb use tests on days 14 and 19. For SNI mice, tests were performed before surgery and at day 7 and 14.

2.6. Tissue preparation

For QPCR analyses, mice were decapitated, rapidly bled and placed on ice. The dorsal root ganglia (DRG) (L1–3), lumbar spinal cord (L1–3), spleen and brain were quickly removed and placed at −80 °C. As for the tumor bearing mice, the spinal cord was split in the left and right side before removal from the spine.

For histological examinations, animals were anaesthetized with Zoletil mix-50 (2 μl/g) and perfused through the left ventricle with 10 ml cold PBS followed by 40 ml 4% PFA (7 ml/min). Tumor bearing femurs and Lumbal (L3–L1) spinal cord segments were removed and post fixed in 4% PFA for 24 h. Spinal cords were cryoprotected for 24 h in 30% sucrose and fast frozen in OCT compound on a bath of ethanol and dry ice and stored at −80 °C until sectioning. Bones were stored in PBS+0.1% PFA until μCT-scannings had been performed.

2.7. RNA purification and QPCR

QPCR was performed using the Mx3000P (Stratagene). The SYBR® Premix Ex Taq™ (Takara) was used for standard SYBR green-based QPCR and for evaluation of the primers. Premix Ex Taq™ (Takara) and dual-labeled locked nucleic acid (LNA) containing fluorogenic probes (Sigma Genosys) were used for TaqMan assays where a target gene and a reference gene were assayed in a duplex reaction. All primers and probes are listed in Table 1. Cycle threshold values were obtained using Stratagene Mx3000P software and the Delta Ct method was used to calculate the relative fold change of RNA levels. "Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta isoform" (YWHAZ) was used as a reference gene and the expression level was further normalized to a calibrator sample arbitrary set equal 1. For CB1 assays, a brain sample was used as calibrator whilst a spleen sample was used for the CB2 assays. In the assays performed on the NCTC-2472 cell line the relative level of CB1 and CB2 is given as a direct ratio to YWHAZ. RNA was extracted using the RNeasy® Lipid Tissue Mini kit (Qiagen) followed by cDNA synthesis using the ImProm-II™ Reverse Transcriptase (Promega).

2.8. Micro-computer tomography (μCT) scanning

Following euthanization, the distal femur from each mouse was scanned with a high resolution μCT system (vivaCT 40, Scanco Medical AG., Bassersdorf, Switzerland), resulting in three-dimensional (3D) reconstruction of cubic voxel sizes 25*25*25 μm^3. For the distal femur, each 3D image dataset consisted of approximately 210 micro-CT slide images and 100 slice images (2500 μm) was used for analysis of subchondral bone tissues (1024×1024 pixels) with 16-bit-gray-levels.

Bone volume fraction (BV/TV) was computed based on the bone voxel size and the number of segmented voxels in the 3D image, i.e. bone voxel per total specimen voxel (Ding et al., 1999).

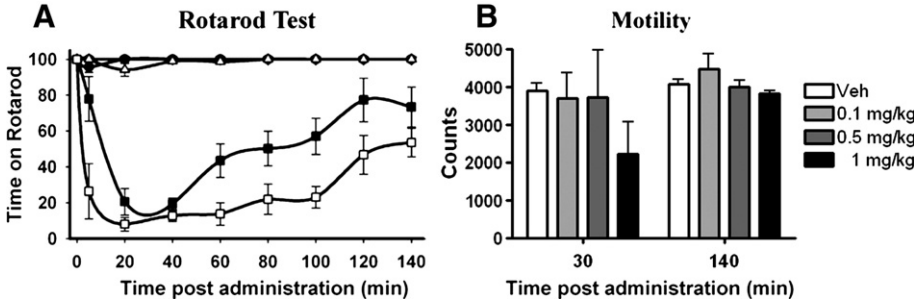

Fig. 1. Dose-effect of WIN on rotarod performance and general motility of naïve mice. Rotarod. Naïve mice were treated with vehicle (open triangles) or WIN (0.1, filled triangles; 0.5, open circles; 1.0, filled circles; 5.0, filled squares; 10.0, open squares; mg/kg) and rotarod performance was determined before injection and at 5, 20, 40, 60, 80, 100, 120, 140 min thereafter ($n=4$ for each dose). Cutoff time was set to 120 s. Each mouse was tested three times and the scores were averaged. The time spend on the rotarod is shown as the% of 120 s 5 and 10 mg/kg WIN induced a significant effect on rotarod performance ($p<0.001$) (A). Motility. Naïve mice were treated with vehicle or WIN (0.1, 0.5, 1.0 mg/kg) and general motility was determined after 30 and 140 min as the numbers of infrared beams blocked in a 5×9 beam grid in a 42.5×26.6 cm mouse cage (at least 3 mice where used in each group). Mice were tested only once and for 30 min. Motility is presented as the count of blocked beams (A). Data was analyzed with a two way ANOVA with a Bonferroni post test.

2.9. Immunohistochemistry

Spinal cords were cut at 30 µm on a cryomicrotome and stained as free floating sections. Briefly, sections were collected in PBS and OCT compound was removed by thorough washing with 0.1% Triton X-100/PBS (TPBS) for 1 h at room temperature (RT). Sections were blocked in 2% BSA/TPBS for 2 h at RT and then incubated with rabbit antibodies against GFAP (Dako, Z0334 1:500 in 2% BSA/TPBS) over night at 4 °C. Next they were washed for 3×5 min in TPBS and incubated in either fluorescein isothiocyanate (FITC)-conjugated anti rabbit IgG (Santa Cruz, SC-9020) or Alexa 594 anti rabbit IgG (Invitrogen, A31632), 1:300 in 2% BSA/TPBS for 2 h at RT. Finally the sections were washed 3×5 min in PBS and mounted with PermaFluor (Thermo, 434990) on precoated slides (Menzel-Glaser, SuperFrost plus). GFAP staining intensity was manually evaluated and scored on a linear and continuous scale from 0–4 with a Zeiss Axioskop 2 by a trained blinded observer using at least 10 sections from each mouse. Pictures were taken with a Hamamatsu C4742 camera using a 10 X Achromat objective.

2.10. MTT assay

NCTC-2472 cells were seeded at a density of 25,000 cells in 200 µl medium per well in 96 well plates. After 3 h, half the medium was exchanged with medium containing WIN and then incubated for 24 h. Cultures were then exposed to 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Fluka, 88415) for 90 min and the supernatants were removed and the formazan crystals were dissolved in 100 µl 0.04N HCl in isopropanol. MTT reduction was determined by measuring light absorption at 560 nm. Values are presented as percent of untreated control cultures.

2.11. Statistics

Data were either analyzed using one way ANOVA tests followed by Newman Keuls post tests, two way ANOVA with a Bonferroni post test or by Wilcoxon matched pairs test. $p<0.05$ were set as the threshold of significance. All data are presented as means +/−SEM.

3. Results

3.1. Dose dependent effect of WIN on naïve animal behavior

To determine a dose of WIN that was unlikely to induce CNS related side effects in the daily treatment study, naïve C3H mice were treated with a single s.c. dose of vehicle or WIN from 0.1 to 10 mg/kg and tested by rotarod or automated motility analysis system. Neither vehicle nor 0.1, 0.5, 1 mg/kg WIN affected the performance in the rotarod test when tested for up to 140 min post WIN administration. In contrast 5 and 10 mg/kg WIN significantly decreased the animals' ability to perform in the rotarod test (Fig. 1A). The effect of WIN on spontaneous locomotion was investigated using the automated motility analysis system 30 and 140 min after the administration of vehicle or 0.1, 0.5, 1.0 mg/kg WIN. No influence on animal motility was found, though a small tendency of decreased motility was observed after 30 min in animals receiving 1.0 mg/kg (Fig. 1B). These results suggest that a dose of 0.5 mg/kg is not associated with measurable CNS related effects in mice.

Table 2
Average weight of sham, cancer/vehicle or cancer/WIN treated mice (grams)

Group	Weight	SEM
Sham	24.13	0.53
Cancer Vehicle	25.09	0.54
Cancer WIN	23.73	0.58

3.2. Acute analgesic effect of WIN on bone cancer pain

Before evaluating the effect of daily WIN treatment on bone cancer bearing mice, weight bearing tests were performed to investigate if a single dose of 0.5 mg/kg WIN induced an acute analgesic effect that would obscure the cumulative effect of repeated WIN administration during testing. Furthermore, we tested the acute effect of 5 mg/kg WIN on bone cancer bearing mice to validate the ability of the weight bearing test to reveal WIN induced analgesia in these mice. At day 19 cancer bearing mice showed a significant decrease in right paw weight bearing compared to sham operated controls. Acute treatment with 5.0 mg/kg WIN significantly increased right paw weight bearing

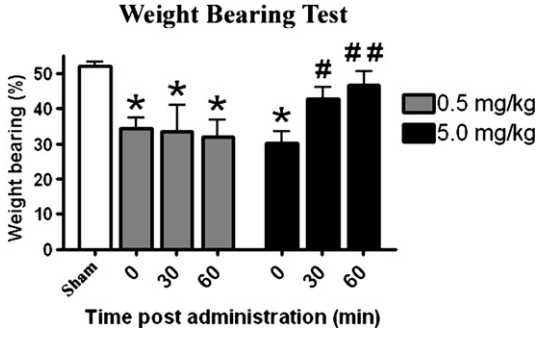

Fig. 2. Acute analgesic effect of WIN on bone cancer pain. 19 days after cancer inoculation, the acute effect of WIN on cancer bearing mice were evaluated. Weight bearing tests were performed just before (0) and 30 and 60 min post administration (s.c.). 0.5 or 5.0 mg/kg WIN were injected and mice were tested for 10 s three non-consecutive times for each time point and the average score was calculated as the weight on right hind paw as percentage of the weight on both hind paws. Data represent mean±SEM. Compared to sham *, $p<0.05$. Compared to 0 min #, $p<0.05$. # #, $p<0.01$. Data was analyzed with a standard or repeated measures one way ANOVA with a Newman Keuls post test. $n=6$ in each group.

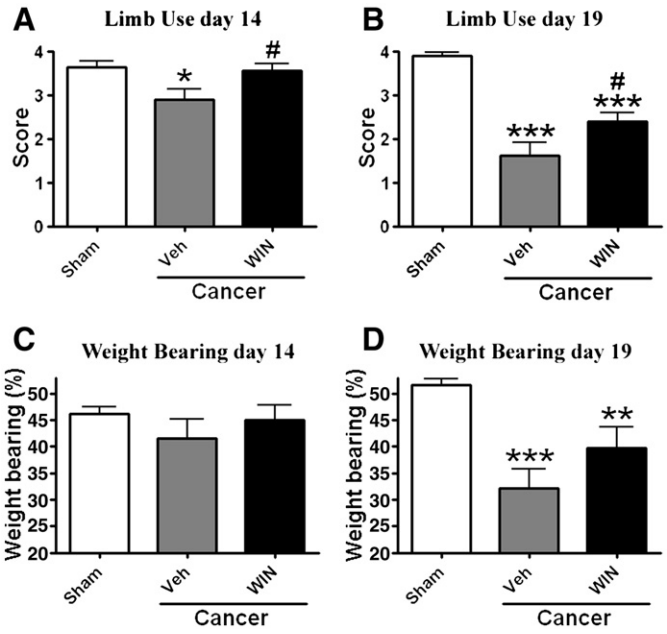

Fig. 3. Daily treatment with WIN attenuates pain behavior. Limb use. 14 and 19 days after cancer inoculation, the use of the cancer bearing limb was determined on a linear and continuous scale from 0–4 in mice treated daily with either vehicle or 0.5 mg/kg WIN (s.c.) and compared to sham animals (A and B). Weight bearing. The percentage of weight on right hind paw was likewise determined on day 14 and 19 (C and D). Data represent mean±SEM. Compared to sham *, $p<0.05$. **, $p<0.01$. ***, $p<0.001$. Compared to vehicle #, $p<0.05$. One way ANOVA with a Newman Keuls post test. $n=14$, 9, 8 for sham, vehicle and WIN respectively.

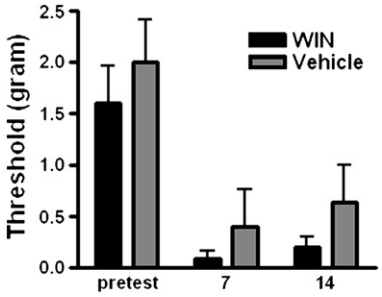

Fig. 4. Daily WIN treatment does not reduce tactile hypersensitivity in SNI operated mice. Daily treatment with 0.5 mg/kg WIN (s.c.) did not rescue SNI mice from tactile hypersensitivity as shown by von Frey tests on day 7 and 14 post injury. Data represents mean ± SEM. n = 13, 11 for WIN and vehicle treated SNI mice respectively.

30 and 60 min after administration confirming the use of the weight bearing test to measure WIN induced analgesia. In contrast, 0.5 mg/kg WIN did not induce any change in right paw weight bearing (Fig. 2). Based on these results and data obtained from the rotarod and motility tests, a dose of 0.5 mg/kg was chosen to investigate the cumulative effects of chronic low dose WIN treatment on bone cancer pain in mice.

3.3. Effect of repeated administration of WIN on bone cancer and SNI mice

Bone cancer mice were treated with either vehicle or 0.5 mg/kg WIN from day 1 to 18 and sacrificed on day 19 for histological, bone destruction and QPCR analyses. No change in weight was observed among the different groups; sham and tumor bearing, WIN or vehicle treated mice (Table 2). Limb use and weight bearing tests were performed on day 14 and 19 before administration of WIN and at least 24 h after the last treatment. At day 14, cancer bearing mice showed a 21% decrease in limb use, which at day 19 reached a 59% decrease compared to sham operated controls. WIN treatment resulted in a significant increase in limb use at both day 14 and 19 of 23% and 48% respectively when compared to vehicle treated controls (Fig. 3A,B). Furthermore, right paw weight bearing was decreased by 10% on day 14 and by 38% on day 19 compared to sham animals. Daily WIN treatment resulted in an insignificant 8% increase in right paw weight bearing on day 14 and 23% on day 19 (Fig. 3C,D).

SNI operated mice, which underwent the same treatment strategy as the bone cancer mice, were tested for tactile hypersensitivity with von Frey monofilaments. Compared to pre-surgery values a significant decrease in pain threshold was observed both at day 7 and day 14; however, in contrast to the bone cancer mice, no effect of WIN treatment was found (Fig. 4).

3.4. Spinal GFAP expression in bone cancer mice following repeated WIN treatment

To confirm a decreased pain state in cancer bearing mice following daily WIN treatment, spinal cord sections obtained from sham operated, vehicle and WIN treated cancer mice, 19 days post surgery, were stained for the astrocytic marker GFAP, which has previously been shown to correlate with pain intensity in this model (Schwei et al., 1999). Vehicle treated cancer mice displayed an increased expression of GFAP in all spinal lamina ipsilateral to the cancer bearing limb compared to the contralateral side and compared to the ipsilateral side in sham operated controls. WIN treated mice also displayed an increase in GFAP expression this was however not as pronounced as in vehicle treated cancer mice and furthermore restricted to the dorsal horn (Fig. 5A–C). Quantification of GFAP

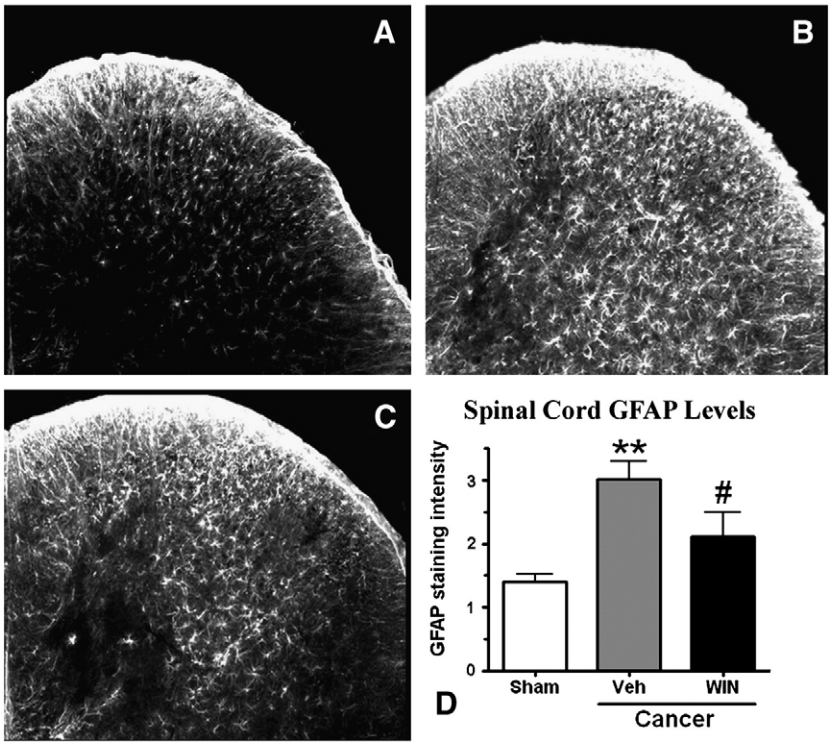

Fig. 5. Daily treatment with 0.5 mg/kg/day WIN reduces bone cancer induced astrogliosis. 19 days post surgery, spinal cord segments L1–L3 were isolated from mice receiving WIN or vehicle treatment (s.c.), and stained for GFAP. Representative GFAP stainings of the dorsal horn are shown for; sham operated mice (A), cancer bearing mice treated with either vehicle (B) or with WIN (C). The GFAP staining intensity ipsilateral to the cancer bearing leg was determined using at least 10 sections from each animal (D) Data represents mean ± SEM. Compared to sham **, $p<0.01$. Compared to vehicle #, $p<0.05$. Data was analyzed with one way ANOVA with a Newman Keuls post test. n = 8, 6, 8 for sham, vehicle and WIN respectively.

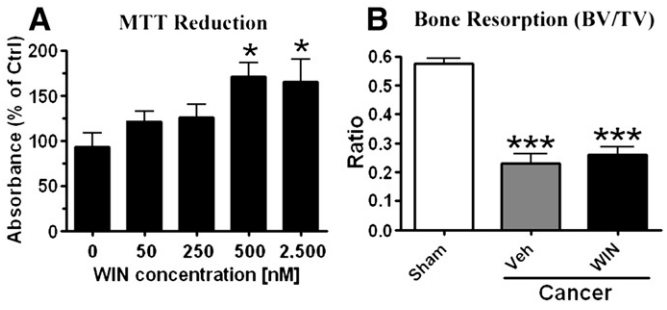

Fig. 6. WIN does not affect cancer cell growth or bone degradation. MTT assay. The effect of WIN on cancer cell viability was tested with an MTT assay. Cell cultures were either untreated (Ctrl) or treated for 24 h with vehicle or WIN (50, 250, 500, 2500 nM) and MTT reduction was assayed. WIN induced a gradual increase in MTT reduction capacity which was significant from 500 nM (A). Data represents mean±SEM. Compared to vehicle *, $p<0.05$. Data was analyzed with one way ANOVA with a Newman Keuls post test. $n=6$ in each group. Bone volume fraction. μCT scannings were performed to investigate bone volume fraction in the vehicle and WIN treated cancer bearing mice. Cancer inoculated femurs showed a 60% decrease in bone volume fraction compared to sham operated mice but no difference between vehicle and WIN treated cancer bearing mice was observed (B). Data represents mean±SEM. Compared to sham ***, $p<0.001$. Data was analyzed with one way ANOVA with a Newman Keuls post test. $n=7$, 9, 8 for sham, vehicle and WIN respectively.

expression revealed a two-fold increase in cancer bearing mice compared to sham. This increase was reduced by 35% following daily WIN treatment (Fig. 5D).

3.5. Lack of tumor suppressing and bone protective effects of WIN treatment

The fact that cannabinoids at high concentrations can induce cell death in certain cell lines (Guzman et al., 2001) and that we found cannabinoid receptors to be expressed by NCTC-2472 cells (Fig. 7C), prompted us to investigate if WIN could induce apoptosis in NCTC-2472 cells thus indirectly decreasing the pain state of the cancer bearing mice. NCTC-2472 cell cultures were treated with WIN in a concentration range expected to encompass the concentrations of WIN in the bones in vivo assuming that WIN is not concentrated in the bones. We found that WIN induced an increase in MTT reduction after 24 h, which became significant at 500 nM (Fig. 6A). At concentrations above 25 μM WIN led to a decrease in MTT reduction and cell number (data not shown). These data suggest that the viability of NCTC-2472 cells in our *in vivo* model is not compromised by 0.5 mg/kg/day WIN administration.

As cannabinoid receptors are expressed in bone tissue (Ofek et al., 2006), we analyzed the effect of daily WIN treatment on bone degradation in cancer bearing mice, as any decrease in bone degradation would be expected to decrease pain levels and the associated increase in spinal GFAP expression (Schwei et al., 1999). The distal right femurs of mice treated with 0.5 mg/kg/day WIN for 18 days were μCT-scanned and bone volume fraction was calculated. In cancer bearing mice, the bone volume fraction was decreased by 60% and WIN treatment showed no effect on bone volume fraction when compared to vehicle treated cancer mice (Fig. 6B), thus confirming that the effect on pain behavior did not results from any alterations in bone degradation due to WIN treatment.

3.6. DRG and spinal cord expression of CB1/CB2 in bone cancer bearing mice

The relative expression of CB1 and CB2 in the L1–L3 DRG and spinal cord was investigated ipsi- and contralateral to the tumor bearing limb to investigate changes due to cancer growth that might affect WIN induced analgesia. Mice were sacrificed 19 days post cancer inoculation and DRG and spinal cord sections were isolated and CB1 and CB2 mRNA expression was analyzed. No difference was found in CB1 expression levels between the ipsi- and contralateral side. However, in cancer bearing mice, a significant 75% increase in DRG CB2 mRNA expression level was found in the ipsi- compared to the contralateral side, while no change was observed in the spinal cord (Fig. 7A,B). Analyzes of cultured NCTC-2472 cells revealed similar expression levels of both CB1 and CB2 mRNA in these cells (Fig. 7C).

3.7. Lack of tolerance after daily treatment with WIN

As development of tolerance is of great concern regarding the use of cannabinoids in chronic pain management, we tested whether daily WIN treatment led to cannabinoid receptor downregulation or functional tolerance behavior. DRG and spinal cords were obtained from naïve mice receiving 0.5 mg/kg/day WIN or vehicle for 19 days and CB1 and CB2 mRNA expression was analyzed. No difference in CB1 or CB2 mRNA expression levels between the two groups was observed (Fig. 8A,B). It is well known, that surface expression levels and sensitivities of G-protein coupled receptors may vary while the receptors mRNA expression levels remain constant. To ascertain that this was not the case, we tested if 0.5 mg/kg/day WIN for 19 days would affect the animals' performance in a rotarod test after a challenge with 5.0 mg/kg WIN, which normally results in substantial loss of motor function. No significant difference between mice treated

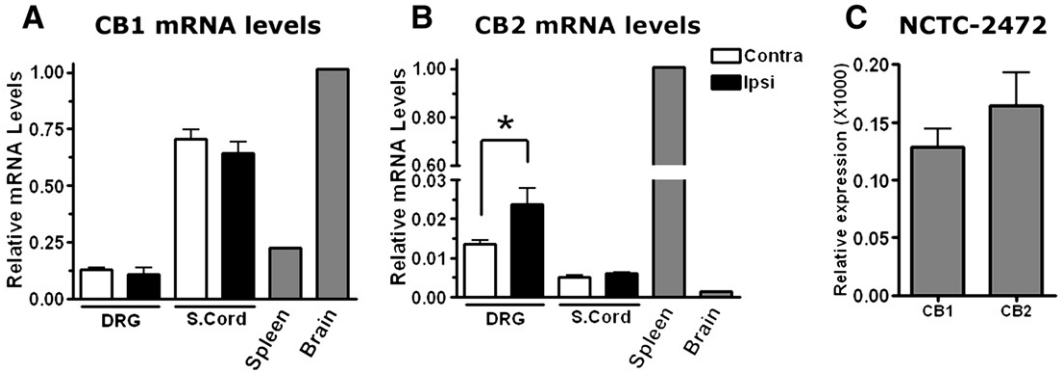

Fig. 7. Bone caner induce CB2 mRNA upregulation in DRG. The levels of CB1 and CB2 gene transcripts were determined in the DRG and spinal cord both ipsi- and contralateral to the cancer bearing limb 19 days post surgery by QPCR. The relative expression of CB1 and CB2 was measured relative to YWHAZ and normalized to Brain for CB1 and spleen for CB2, which were set arbitrary to one (A and B). Bone cancer did not lead to changed expression levels of CB1 while CB2 levels were significantly increase by 75% in the DRG ipsilateral to the cancer bearing limb. Cultured NCTC-2472 cells express equivalent levels of CB1 and CB2 mRNA but at levels 10,000 lower than in brain and spleen, respectively (C). Data represents mean±SEM. Compared to contralateral side *, $p<0.05$. Data was analyzed with Wilcoxon matched pairs test for non-parametric analyses. $n=8$.

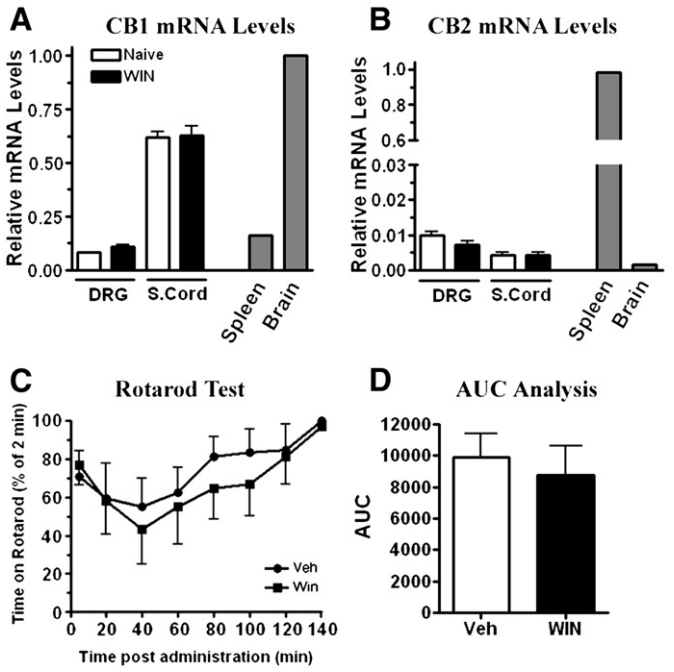

Fig. 8. No tolerance after repeated administration of 0.5 mg/kg WIN in naïve mice for 19 days. CB1 and CB2 mRNA levels following daily treatment with 0.5 mg/kg WIN (s.c.) for 19 days in naïve mice was quantified by QPCR using YWHAZ as a reference gene and normalized to brain and spleen respectively, which were set arbitrary to one. No effect of WIN treatment was observed for CB1 or CB2 expression in DRG or spinal cords (A and B). To rule out transcription independent development of tolerance, mice treated with 0.5 mg/kg/day WIN or vehicle for 19 days were challenged with 5 mg/kg WIN and their rotarod performance tested for a period of 140 min (C). AUC analysis showed no difference in rotarod performance between the two groups, thus emphasizing the absence of tolerance development (D). Data represents mean ± SEM. Data was analyzed with Mann–Whitney test for non-parametric analyses or student's *t*-test. $n = 4$ and 6 for QPCR and tolerance tests, respectively.

with vehicle or with 0.5 mg/kg WIN for 19 days (Fig. 8C,D) was found thus indicating that the mice did not develop tolerance in terms of their ability to perform on a rotarod.

4. Discussion

In the present study, we showed that chronic low dose treatment with the non-selective cannabinoid agonist WIN was capable of reducing pain related behavior and spinal changes in mice suffering from bone cancer pain. The chosen dose of 0.5 mg/kg did not induce any detectable CNS related adverse effects or tolerance over a period of 19 days. The lack of changed bone degradation or toxicity on NCTC-2472 cells following WIN treatment supports the notion that the observed changes in pain related behavior and spinal GFAP expression were due purely to decreased pain levels and did not involve a decrease in tumor progression. The results suggest, that the beneficial effects were more likely related to modulation of inflammatory aspects of bone cancer pain than an effect on damaged neurons, as no effect of the same treatment protocol was found on SNI operated mice.

The observed effect of WIN treatment on rotarod performance is in agreement with previously published results, where no effect on rotarod performance was found at doses below 3 mg/kg, while 5 mg/kg led to a decreased performance which peaked 60 min post administration (Liang et al., 2007). However, as a higher time resolution was used for this study, the effect of WIN treatment was observed to be already fully present just 5 to 20 min post administration. It has been reported that 30 mg/kg WIN does not affect the time to first fall in rotarod tests though the animals were cataleptic but they tended to rotate passively with the rotarod apparatus (Kehl et al., 2003). To ascertain that we would not get a false negative effect of WIN on rotarod performance, the time in which the animals passively rotated with the apparatus was subtracted from the total time spend on the rotarod. Together with the data from automated voluntary movement tests, these data show that a dose of 0.5 mg/kg WIN has minimal or no CNS related effects in terms of motor function.

In context of tolerance, treatment with 0.5 mg/kg WIN for 19 days had no effect on either CB1 or CB2 mRNA expression levels, though it should be noted that cannabinoid treatment on several occasions have been reported to lead to tolerance by mechanism independent of CB1/CB2 mRNA expression levels (Sim et al., 1996; Tappe-Theodor et al., 2007). It is however unlikely that tolerance had developed in this model, as a functional tolerance test on rotarod showed no effect of repeated treatment with 0.5 mg/kg WIN for 19 days.

Limb use scores in this bone cancer model have on several occasions been shown to correlate well with pain intensity and to be normalized by morphine or gabapentin treatment (El Mouedden and Meert, 2005; Luger et al., 2002; Peters et al., 2005). The use of weight bearing tests in the current bone cancer pain model has to our knowledge not previously been published but the data shows that weight bearing reflects disease progression as measured by limb use days 14 and 19 and bone loss at day 19. Furthermore, the use of the weight bearing tests on bone cancer mice was validated by acute administration of morphine (data not shown). The alleviating effect of WIN treatment on pain levels is supported by the decreased spinal GFAP expression levels, which has previously been shown in various pain models to correlate with thermal and mechanical hypersensitivity (Coyle, 1998; Garrison et al., 1991; Hashizume et al., 2000).

CB1 and CB2 expression was readily detected, though a low mRNA level was observed in NCTC-2472 cells compared to brain and spleen. However, WIN was capable of inducing increased mitochondrial activity seen as increased MTT reduction in cultures in the nanomolar range. In this regard, it cannot be ruled out that WIN may exert some of this effect through CB1/CB2 independent pathways, as have been suggested previously (Hajos et al., 2001). Though bone resorption is known to be modulated by cannabinoid agonists (Idris et al., 2005; Ofek et al., 2006) and that NCTC-2472 cells respond to WIN treatment *in vitro*, the mechanism behind the analgesic effect of repeated low dose treatment with WIN is not expected to result from a toxic effect on NCTC-2472 cells or by modulating bone degradation. This is based on the MTT assays showing that the estimated *in vivo* concentration of WIN lead to increased cell viability rather than toxicity and the μCT-scannings showing no effect of WIN treatment on bone volume fraction in mice with bone cancers.

The absent effect of repeated low dose WIN treatment on SNI mice compared to bone cancer mice suggests that the mechanism behind the development of pain behavior in these two models may differ. One possible aspect is the degree of peripheral inflammation, which is thought to be a key component of bone cancer induced pain (Mantyh, 2006) while having a limited impact in the SNI model (Broom et al., 2004). This notion is supported by the insensitivity of SNI mice to COX-2 inhibitors, which is in contrast to findings in inflammatory pain models and the present bone cancer pain model, where COX-2 inhibitors induce pain relieve (Broom et al., 2004; Sabino et al., 2002).

In contrast to Costa et al. (2004) we were not able to show any alleviating effects of repeated low dose WIN treatment in our neuropathic pain model. This could result from differences between species and also the choice of model as we investigated SNI mice while Costa et al. performed chronic constriction injury (CCI) on rats. In CCI rats, pain behavior is associated with increased plasma levels of PGE_2, a key end product of COX-2 activity (Costa et al., 2004). Daily administration of 0.1 mg/kg WIN not only induced almost complete analgesia but also decreased plasma PGE_2 levels to baseline values indicating an antiinflammatory effect (Costa et al., 2004). This is supported by results from several groups showing that, in contrast to SNI rats, CCI rats respond to COX-2 inhibitors (Schafers et al., 2004; Suyama et al., 2004; Broom et al., 2004). The discrepancies between

the effect of repeated low dose WIN treatment and treatment with COX-2 inhibitors in the SNI and the CCI model may be explained by different degrees of inflammatory impact on pain behavior due to the differences in surgery sites and types. This may also explain our data obtained from a more aggressive bone cancer pain model, where the number of cells injected into the femoral cavity was increased to 150% and no significant effect was observed following 0.5 mg/kg/day WIN treatment on limb use. The rapid progressing cancer will in this case lead to a rapid loss of nerve terminals in the bones (Peters et al., 2005), which will alter the way in which inflammatory mediators modulate the truncated nerves. Thus the complex pathology of bone cancer pain involving both inflammatory reactions and nerve injury (Mantyh, 2006), may account for the subtle alleviating effect of WIN treatment when compared to the studies performed by Costa et al. (2004).

Animal models of neuropathic pain exhibit an increased spinal expression of CB2 most likely accounted for by the increased number of microglia (Wotherspoon et al., 2005; Zhang et al., 2003). The bone cancer model used in the present report does not exhibit an increased presence of spinal microglia (Honore et al., 2000b), which corroborate our findings showing stable spinal CB2 mRNA levels. However, DRG CB2 mRNA levels were significantly increased in response to bone cancer, which is in agreement with the increased presence of macrophages localized to DRG in this model (Peters et al., 2005). Macrophages are known to be involved in generation of hypersensitivity and furthermore to be inhibited by cannabinoid agonists (Ma and Quirion, 2006; Zheng et al., 1992). Increased expression levels of CB2 in DRG involved in mediating chronic pain may enhance the impact of cannabinoid treatment in hypersensitive subjects compared to healthy controls with normal CB2 receptor levels.

In conclusion, the results showed that a low daily dose of the cannabinoid agonist WIN, which did not induce CNS related side effects measured as decreased motor function, has a subtle alleviating effect on bone cancer pain without the development of tolerance as determined by rotarod. The behavioral experiments suggested that WIN administration only affected the animals for few hours. It is possible that continuous infusion or multiple administrations of low doses of WIN during the day may lead to a more pronounced effect on pain behavior in the tested bone cancer pain model.

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