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Peripheral cannabinoids attenuate carcinoma-induced nociception in mice

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Abstract

We investigated the cannabinoid receptor (CBr) agonists Win55,212-2 (non-selective) and AM1241 (CBr2 selective) and the peripheral receptor (CBr1) in carcinoma-induced pain using a mouse model. Tumors were induced in the hind paw of female mice by local injection of a human oral squamous cell carcinoma (SCC). Significant pain, as indicated by reduction in withdrawal thresholds in response to mechanical stimulation, began at 4 days after SCC inoculation and lasted to 18 days. Local administration of Win55,212–2 (10 mg/kg) and AM1241 (10 mg/kg) significantly elevated withdrawal thresholds, indicating an antinociceptive effect. Ipsilateral expression of CBr1 protein in L5 DRG was significantly upregulated compared to ipsilateral L4 DRG and in normal tissue. These findings support the suggestion that cannabinoids are capable of producing antinociception in carcinoma-induced pain.

Keywords: Cannabinoids; Cancer; Pain; Cancer mouse model; Oral cancer

Cancer pain remains poorly understood and there are no effective therapies. Mechanical hyperalgesia secondary to carcinoma, due to its intensity and impairment of function, is debilitating. Seventy-five to ninety percent of terminal cancer patients cope with opiate-resistant pain related to tumor progression [28,29,36,39]. Eighty-five percent of cancer patients experience severe pain in their final days [43].

Cancer pain is classified into three syndromes: somatic, visceral and neuropathic. Somatic cancer pain is caused by tumor invasion of connective tissues, bones and muscles. Visceral cancer pain is caused by invasion into visceral organs. Neuropathic cancer pain is caused by peripheral or central nervous system damage due to released inflammatory cytokines that sensitize neurons [37]. Carcinoma-induced pain is not related to tumor size and small carcinomas produce severe pain [6]. These observations suggest that carcinoma pain is primarily of neuropathic origin and is characterized by mechanical hyperalgesia.

Mechanical hyperalgesia secondary to carcinoma is poorly responsive to opioids, and tolerance rapidly develops [25,26,33]. Cannabinoids are analgesic in patients with neuropathic pain [12,13,20,24,35] and show promise in cancer pain [32]. Cannabinoids activate two receptors types: cannabinoid receptor 1 and 2 (CBr1 and CBr2, respectively) [27,31]. CBr1 and CBr2 contribute to analgesia. CBr1s are localized in the spinal dorsal horn, periaqueductal grey [9,11] and dorsal root ganglion (DRG) [24,40]. In neuropathic pain, cannabinoids act at central and peripheral nerve CBr1s [20,35], and at CBr2s on keratinocytes [18,20]. Cannabinoid's analgesic action in cancer pain is less clear [2,10,19]. In a murine bone sarcoma pain model, systemic cannabinoids act through CBr1 [15,21]. However, the role of peripheral CBr1 and CBr2 receptors in soft tissue carcinoma pain is not known. We hypothesize that cannabinoid agonists are analgesic with carcinoma-induced pain and that the site of action is within the tumor microenvironment. To study soft tissue carcinoma pain, we produce a mouse model by injecting human oral squamous cell carcinoma (SCC) into the hindpaws which leads to mechanical hyperalgesia [42]. Oral SCC repro-

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ducibly produces mechanical hyperalgesia in mice and humans. The mouse model can be used to test for analgesics [6,42]. We sought to determine whether peripheral cannabinoid agonists attenuate mechanical hyperalgesia in a carcinoma mouse model.

A human oral SCC cell line (ATCC, Manassas, VA) was cultured in Dulbeco's modified Eagle's medium (DMEH-21), 10% fetal bovine serum, fungizone (0.5×), penicillin–streptomycin (1×), non-essential amino acids (1×), and sodium pyruvate (1×).

The cancer pain mouse model was produced using adult (4–5 weeks old, 20–25 g) female *Foxn1*^{nu}, athymic mice as previously described [42]. Mice were housed in a temperature-controlled room on a 12:12 h light cycle (06:00–18:00 h light), with unrestricted access to food and water; estrous cycles were not monitored. All procedures were approved by UCSF Committee on Animal Research. Researchers were trained under the Animal Welfare Assurance Program. Mice were injected either with squamous carcinoma cells (SCC group) or cell culture media (sham operated). Both groups were anesthetized by intraperitoneal injection of Avertin® (0.015 ml of a 2.5% solution/g body wt). SCC injections consisted of 1.0×10^6 tumor cells in 50 μl of Dulbeco's modified Eagle's medium (DMEM) into the plantar surface of the right hind paw. The sham-operated group received injections of the cell culture media.

Behavioral testing was performed between 14:00 and 16:00 h (during the light phase) and quantitative assay guidelines were used as described previously [42]. Mice were placed in a plastic cage with a wire mesh floor which allowed access to the paws. Fifteen minutes were allowed for cage exploration prior to testing. The mid-plantar right hind paw, or the tumor-front on the hind paw toward the later stages of tumor development was tested. Paw withdrawal thresholds were determined in response to pressure from an electronic von Frey anesthesiometer (2390 series, IITC Instruments, Woodland Hills, CA). The amount of pressure (g) needed to produce a paw withdrawal response was measured three times on each paw separated by 3 min intervals. The three tests were averaged for each paw for that day. The SCC and sham injected groups were tested at 4, 7, 9, 11, 14, 16, and 18 days post-injection.

A non-selective (Win55,212-2) or a selective (AM1241) cannabinoid agonist was administered prior to paw withdrawal testing. Testing was performed at 20 days following oral SCC hindpaw inoculation. The cannabinoid agonist was injected directly into the mid-plantar hind paw at the site of greatest tumor development with a 30 gauge beveled needle. 10 mg/kg of either Win55,212-2 or AM1241 was diluted in 15 μl DMSO. A control group of mice with SCC paw tumors received 15 μl of DMSO (vehicle) injection in the same manner. Paw withdrawal testing was performed: (1) 15 min before drug or control injection and (2) 15, 30, 60, 90, 180 and 1440 min post-drug or control injection.

Mice received a lethal dose of pentobarbital (100 mg/kg, intraperitoneal), and were fixed with intracardiac PBS (10 ml) perfusion, pH 7.4, room temperature followed by an ice-cold fixative (20 ml, 4% paraformaldehyde and 0.14% picric acid in 0.1 M phosphate buffer, pH 7.4). The DRG and lumbar spinal cord were extracted. Tissue was postfixed and cryoprotected in 30% sucrose. Ten micrometer sections were cut after embedding in Tissue-Tek (Fisher Scientific, Inc., Hampton, NH) and plated on superfrost plus slides (Fisher Scientific, Inc., Hampton, NH). Sections were washed three times with PBS and incubated with an affinity purified rabbit CBr1 C-terminal antibody (1:1000) in PBS/Triton X-100 with 1% normal donkey serum (NDS) at 4 °C overnight (14–16 h). Sections were incubated with anti-rabbit Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS/Triton with 1% NDS for 2 h. Sections from ipsilateral L4 and L5 DRG were processed simultaneously. The slides were visualized on a Nikon Eclipse E600 microscope using epifluorescence. The images were captured with a RT Spot Camera and Software (Diagnostics Instruments, Inc., Sterling Heights, MI).

The colored fluorescent images of ipsilateral L4 and L5 DRG were converted to grayscale using RT Spot Software (Diagnostics Instruments, Inc., Sterling Heights, MI). The fluorescence emitted by each DRG cell body was quantified by Scion Image software as the average gray value per pixel in the selected DRG cell body (Alpha version 4.0.3.2, Scion Corporation, Frederick, MD). The gray value per pixel ranges between 0 and 256, with higher values indicating higher intensities of fluorescence. A value of 256 indicates that all of the pixels in the selected image are expressing maximum gray value. Therefore, to prevent the skewing of data by using absolute values, we calculated the fluorescence values as a percentage of 256. Only DRG neurons that did not overlap with other cells and had a visible nucleus were used for image analysis.

A one-way analysis of variance (ANOVA) with a Bonferroni Multiple Comparisons post-test was used to compare the withdrawal threshold of the SCC and sham mice over 18 days. The same test was used to compare the percent change of withdrawal threshold of the SCC inoculated mice before and after drug or control injection. A paired two-tailed *t*-test was used to compare the intensity of immunofluorescence of L4 and L5 in the SCC inoculated to the sham control.

The withdrawal thresholds for the SCC and sham group were compared. Mean paw withdrawal thresholds were significantly reduced in the SCC mice on all days of behavioral testing (Fig. 1). The mean paw withdrawal thresholds of the SCC inoculated mice and the sham group prior to inoculation were 4.21 ± 0.22 and 4.48 ± 0.45 g, respectively. The mean paw withdrawal thresholds of the SCC inoculated and sham group 14 days after inoculation were 1.84 ± 0.5 and 4.94 ± 0.85 g.

We tested the effect of peripheral administration of the non-selective CBr agonist Win55,212-2 and CBr2 selective agonist AM1241 on paw withdrawal thresholds. Win55,212-2 significantly elevated paw withdrawal thresholds of SCC-inoculated paws at 15, 30, 60, 90 and 180 min after inoculation relative to vehicle control (Fig. 2). Thirty minutes after injection of Win55,212-2 the mean paw withdrawal thresholds was 3.43 ± 1.36 g. AM1241 (10 mg/kg) significantly elevated paw withdrawal thresholds of SCC-inoculated paws at 15 min after inoculation relative to vehicle control (Fig. 2). Thirty minutes after injection of AM1241 the mean paw withdrawal thresholds was 3.02 ± 1.1 g. Recovery to baseline was observed by 90 min