Anti-inflammatory Properties of Cannabidiol, a Nonpsychotropic Cannabinoid, in Experimental Allergic Contact Dermatitis

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ABSTRACT
Phytocannabinoids modulate inflammatory responses by regulating the production of cytokines in several experimental models of inflammation. Cannabinoid type-2 (CB₂) receptor activation was shown to reduce the production of the monocyte chemotactic protein-2 (MCP-2) chemokine in polyinosinic-polycytidylic acid [poly-(I:C)]-stimulated human keratinocyte (HaCaT) cells, an in vitro model of allergic contact dermatitis (ACD). We investigated if nonpsychotropic cannabinoids, such as cannabidiol (CBD), produced similar effects in this experimental model of ACD. HaCaT cells were stimulated with poly-(I:C), and the release of chemokines and cytokines was measured in the presence of CBD or other phytocannabinoids (such as cannabidiol acid, cannabidivarin, cannabidivaric acid, cannabichromene, cannabinol, cannabinolic acid, cannabinore, tetrahydrocannabinol, and tetrahydrocannabinolic acid) and antagonists of CB₁, CB₂, or transient receptor potential vanilloid type-1 (TRPV1) receptors. HaCaT cell viability following phytocannabinoid treatment was also measured. The cellular levels of endocannabinoids [anandamide (AEA), 2-arachidonoylglycerol] and related molecules (palmitoylethanolamide, oleoylethanolamide) were quantified in poly-(I:C)-stimulated HaCaT cells treated with CBD. We showed that in poly-(I:C)-stimulated HaCaT cells, CBD elevates the levels of AEA and dose-dependently inhibits poly-(I:C)-induced release of MCP-2, interleukin-6 (IL-6), IL-8, and tumor necrosis factor-α. In a manner reversed by CBD and TRPV1 antagonists 6-iodoavridoline (AM630) and 5′-iodo-resiniferatoxin (5′-iRTX), respectively, with no cytotoxic effect. This is the first demonstration of the anti-inflammatory properties of CBD in an experimental model of ACD.

Introduction
Allergic contact dermatitis (ACD), a form of delayed-type hypersensitivity, is a typical T-cell-mediated skin inflammatory response that occurs after cutaneous exposure to an allergen. In particular, following first application to the skin, epidermal Langerhans cells (LCs) take up the allergen, process it, and migrate toward the regional lymph nodes, where the antigen is presented to naive T cells, which, once activated, migrate toward peripheral tissues. During this process, known as the “sensitization phase,” LCs convert from a "quiescent" into an "activated" functional state. This activation of LCs is initiated by keratinocytes, which secrete inflammatory cytokines such as interleukin (IL)-6, IL-2, tumor necrosis factor-α (TNF-α), and granulocyte-macrophage colony-stimulating factor (GM-CSF), which in turn contribute to LC activation and migration (Engk and Katz, 1992; Becker and Knop, 1993). The subsequent allergen application induces "the elicitation phase" of ACD that involves the degranulation of mast cells, vasodilatation and influx of neutrophils, followed by substantial leukocyte infiltration into tissue and edema formation peaking between 24 and 48 hours. This late-phase response has the same direct effects on the skin as the first allergen contact during sensitization (i.e., proinflammatory effects, LC activation), but TC cell activation is subjected to the release of cytokines produced by T lymphocytes, which amplify the inflammatory response by generating a process that leads to further accumulation of infiltrating cells and resulting...
in clinically manifested ACD (van Loveren et al., 1983; Watanabe et al., 2002). However, the production of cytokines (such as IL-1, IL-6, and IL-8) from keratinocytes, as well as the induction of adhesion molecules (such as intercellular adhesion molecule) in keratinocytes, is also required for T cell activation, chemotactic activity, and adhesion in the epidermis, indicating that these cells have a crucial role in ACD (Barker, 1992).

Although many studies reported anti-inflammatory properties for two major cannabinoids present in marijuana, such as the psychoactive compound Δ⁹-tetrahydrocannabinol (THC) and the nonpsychotropic compound cannabidiol (CBD) (Burstein and Zurier, 2009; Burstein, 2015), the first evidence of the anti-inflammatory effects of cannabinoids in an animal model of ACD was reported by Oka et al. (2006), and soon thereafter by Karsak et al. (2007) in collaboration with our group (Karsak et al., 2007). In particular, it was demonstrated that both subcutaneous and topical application of THC attenuated ACD in 2,4-dinitrofluorobenzene (DNFB)–treated wild-type mice (Karsak et al., 2007). THC significantly decreased ear swelling and reduced the recruitment of Gr-positive granulocytes in comparison with untreated mice (Karsak et al., 2007). Intriguingly, the cannabinoid type-2 (CB₂) receptor antagonist SR144528 was reported, on the one hand, to counteract DNFB-induced (Ueda et al., 2005) and oxazolone-induced (Oka et al., 2006) ACD in mice, and on the other hand, to inhibit the anti-inflammatory effect of THC on this condition (Karsak et al., 2007). Nevertheless, Karsak et al. (2007) also demonstrated that the synthetic cannabinoid agonist HU-210 was able to reduce the production of the monocyte chemotactic protein-2 (MCP-2) chemokine in polymorphonuclear polycytidyl acid [poly-(I:C)]–stimulated human keratinocytes (HaCaT) cells, an in vitro model of the first phase of ACD. More recently, THC was suggested to inhibit DNFB-induced dermatitis also via non-CB₁, non-CB₂–mediated pathways (Gaffal et al., 2013). Indeed, we reported that the anti-inflammatory compound palmitoylethanolamide (PEA), which belongs to the same chemical class as the endocannabinoid anandamide (AEA) but is unable to directly activate cannabinoid receptors (Petrosino and Di Marzo, 2017), also reduced the production of MCP-2 in poly-(I:C)–stimulated HaCaT cells, as well as the DNFB-induced ear skin edema in mice (Petrosino et al., 2010). Moreover, we also demonstrated that, although PEA is known to directly activate the peroxisome proliferator-activated receptor-α (Lo Verme et al., 2005), only the selective antagonism of transient receptor potential vanilloid type-1 (TRPV1) channels reversed the effects of PEA on MCP-2 production in poly-(I:C)–stimulated HaCaT cells and on the first, keratinocyte-mediated stage of DNFB-induced ear skin edema in mice (Petrosino et al., 2010), whereas CB₂ receptors were involved in PEA effects in the late, mast cell–mediated stage of this in vivo model of ACD (Vaia et al., 2016). These previous data are in agreement with the indirect stimulatory actions of PEA on TRPV1 and CB₂ and with the role of TRPV1 and CB₂ in ACD (De Petrocellis et al., 2001; Petsinosio et al., 2016).

On the basis of this background, and in view of the fact that it has been demonstrated that CBD also stimulates and desensitizes TRPV1 channels (De Petrocellis et al., 2011; Iannotti et al., 2014), the aim of the present study was to investigate the pharmacological effects of this and other

**Fig. 1.** CBD reduces MCP-2 levels in poly-(I:C)–stimulated HaCaT cells. Enzyme-linked immunosorbent assay for MCP-2 release in the supernatants of poly-(I:C)–stimulated HaCaT cells (100 μg/ml) in the presence of vehicle or CBD (1, 5, 10, and 20 μM) for 6 (A), 12 (B), and 24 hours (C) at 37°C in 5% CO₂. Data represent the mean ± S.E.M. of three independent experiments performed in triplicate. **#P < 0.001 vs. vehicle; ***P < 0.001 vs. poly-(I:C).** Assay range for MCP-2, 0.8–200 pg/ml.
nonpsychotropic phytocannabinoids in an in vitro model of ACD, as well as to identify the molecular target(s) for its actions in the in vitro model.

Materials and Methods

Drugs and Reagents. Cell culture media, antibiotics, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Milano, Italy). Poly-(I:C) was purchased from InvivoGen (Aurigena Srl, Roma, Italy). Botanical CBD, cannabidiol acid (CBDA), cannabidiolin (CBVD), cannabidivarinic acid (CBDVA), cannabichromene (CBC), cannabigerol (CBG), cannabigerolic acid (CBGA), cannabivarin (CBGV), tetrahydrocannabivarin (THCV), and tetrahydrocannabivarinic acid (THCVA) (>99.9% purity) were provided by CW Research Ltd. (Cambridge, UK). 1-(3,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl) pyrazole-3-carboxamide (AM251), 6-iodopravadoline (AM630), 3'-iodo-resiniferatoxin (I-RTX), AEA, and 3-(3-Cardamomolphyl)phenyl N-cyclohexycarbamate (URB927) were purchased from Toecis Bioscience (Space Import-Export Srl, Milano, Italy). The human MCP-2 enzyme-linked immunosorbent assay kit was purchased from RayBiotech, Inc. (Tebu-Bio Srl, Milano, Italy). The Bio-Plex Pro human cytokine assay was purchased from Bio-Rad (Life Science, Segrate, Milano, Italy). Deuterated standards—[3H]AEA, [3H]2-arachidonoylglycerol (2-AG), [3H]PGE, and [3H]oleoylthanolamine (OTA)—were purchased from Cayman Chemical (Cabrus SAS, Arezzo, Italy).

Cell Culture. The HaCaT cell line (cell number: 300493; mycoplasma-specific polymerase chain reaction: negative) was purchased from CLS Cell Lines Service (Eppelheim, Germany) and

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Fig. 2. CBG, CBG, THCV, and CBGV reduce MCP-2 levels in poly-(I:C)-stimulated HaCaT cells. Enzyme-linked immunosorbent assay for MCP-2 release in the supernatants of poly-(I:C)-stimulated HaCaT cells (150 μg/ml, 6 hours, 37°C) in the presence of vehicle or CBG (A), CBG (B), THCV (C), and CBGV (D) (all tested at 5, 10, and 20 μM). Data represent the mean ± S.E.M. of three independent experiments performed in triplicate. **P < 0.01 vs. vehicle; ***P < 0.001 vs. poly-(I:C). Assay range for MCP-2, 0.8–200 pg/ml.
cultured in Dulbecco's modified Eagle's medium supplemented with glutamine (2 mM), penicillin (400 U/mL), streptomycin (50 mg/mL), and 10% fetal bovine serum at 37°C in humidified 5% CO₂.

**Poly-(L)-Induced ACD in HaCaT Cells.** HaCaT cells were plated into 24-well culture plates at a cell density of 2 × 10⁴ cells per well, and after 1 day were stimulated with 100 µg/mL poly-(L)-leucine (Petroso et al., 2010) or vehicle (water) and incubated for 6, 12, and 24 hours at 37°C in 5% CO₂. To study the effect of CBD, poly-(L)-leucine-stimulated HaCaT cells were treated with CBD (1, 5, 10, and 20 µM) or vehicle (methanol) for the indicated times. To study the effect of CB₂, CB₅, and TRPV₁ antagonists, poly-(L)-leucine-stimulated HaCaT cells were treated with AM251 (1, 2.5, and 5 µM), AM630 (0.1, 0.5, and 1 µM), and I-RTX (0.01, 0.1, and 1 µM), respectively, in the presence or absence of CBD (20 µM) for the indicated times. AM251 was dissolved in methanol, AM630 and I-RTX in dimethylsulfoxide (DMSO). The effects of the other phytoconstituents (all dissolved in DMSO), such as CBDA, CBVD, CBDA, CBC, CBG, CBGA, CBG, THCV, and THCV (all tested at 5, 10, and 20 µM), on MCP-2 production in poly-(L)-leucine-stimulated HaCaT cells were also investigated. Finally, the effects of the endocannabinoid AEA (dissolved in methanol) and of URB597 (dissolved in methanol) (a selective inhibitor of the major enzyme responsible for AEA inactivation, fatty acid amide hydrolase) on MCP-2 production in poly-(L)-leucine-stimulated HaCaT cells were also studied. After 6, 12, and 24 hours, the supernatants were used for MCP-2 enzyme-linked immunosorbent assay and for Bio-Plex Pro assay (IL-8, IL-1α, IL-1β, IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), GM-CSF, TNF-α), according to the manufacturer's instructions. Results are expressed as picograms per milliliter of released MCP-2 and cytokines.

**Cell Viability.** Cell viability was measured after 6, 12, and 24 hours in HaCaT cells treated with CBD, CBG, CBG, THCV, CBGA (all tested at 10 and 20 µM), or vehicle by using the MTT colorimetric assay. In brief, after 6, 12, and 24 hours, HaCaT cells were incubated with MTT (5 mg/mL) for 3 hours at 37°C in 5% CO₂. After 3 hours, HaCaT cells were lysed with DMSO, and absorbance was measured at 630 nm. Results are expressed as percentage of cell viability, where optical density values from vehicle-treated cells were defined as 100% of cell viability.

**Analysis of Endocannabinoids and Related N-Acylethanolamines.** HaCaT cells were plated into six-well culture plates at a cell density of 9 × 10⁴ cells per well, and after 1 day were stimulated with poly-(L)-leucine (100 µg/mL) and treated with CBD (20 µM) or vehicle, and incubated for 6, 12, and 24 hours at 37°C in 5% CO₂. After the indicated times, the resulting cells and supernatants were subjected to measurement of endocannabinoids such as AEA and 2-AG, and N-acylethanolamines related to AEA, i.e., PEA and OEA.

Cells and supernatants were homogenized in a solution of chloroform/methanol/methanol (2:1:1 by vol.), containing 10 pmol of [3H]AEA and 5 pmol of [3H]2-AG, [3H]PEA, and [3H]OEA as internal deuterated standards. The lipid-containing organic phase was prepared by open-bed chromatography on silica gel (Bisogni et al., 1997; Di Marzo et al., 2001), and fractions obtained by eluting the column with a solution of chloroform/methanol (90:10 by vol.) were analyzed by liquid chromatography–atmospheric pressure chemical ionization (APCI)-mass spectrometry by using a Shimadzu high-performance liquid chromatography apparatus (LC10ADVP) coupled to a Shimadzu (LCMS-2020) quadrupole mass spectrometer via a Shimadzu APCI interface. Liquid chromatography–APCI–mass spectrometry analyses of AEA, 2-AG, PEA, and OEA were carried out in the selected ion monitoring mode (Marruzzo et al., 2002), using m/z values of molecular ions +1 for deuterated and undeuterated compounds, respectively, as follows: 356 and 348 (AEA), 384,383, and 379,378 (2-AG), 304 and 300 (PEA), and 328 and 326 (OEA). AEA, 2-AG, PEA, and OEA levels were calculated on the basis of ratio of their area with the internal deuterated standard signal areas, and their amounts (picomoles) were normalized per milliliter of volume.

**Statistics.** Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). The data

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**Fig. 3.** CBD reduces IL-6, IL-8, and TNF-α levels in poly-(L)-leucine-stimulated HaCaT cells after 6 hours. Bio-Plex Pro assay for IL-6 (A), IL-8 (B), and TNF-α (C) release in the supernatants of poly-(L)-leucine-stimulated HaCaT cells (100 µg/mL, 6 hours, 37°C) in the presence of vehicle or CBD (1, 5, 10, and 20 µM). Data represent the mean ± S.E.M. of one experiment performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle. Data from three independent experiments performed in triplicate.
are expressed as means ± S.E.M. Student's t test or one-way analysis of variance followed by Newman-Keuls multiple comparison test were used for analysis. P values <0.05 were considered statistically significant.

Results

CBD Reduces MCP-2 Protein Levels in Poly-(I:C)-Stimulated HaCaT Cells. We investigated the effects of CBD, CBDA, CBDV, CBDA, CBC, CBG, CBGA, CBGV, THCV, and THCV on MCP-2 protein levels in poly-(I:C)-stimulated HaCaT cells. HaCaT cells stimulated for 6, 12, and 24 hours with poly-(I:C) (100 µg/ml) and treated with the vehicle of the phytocannabinoids produced significantly higher levels of the MCP-2 chemokine as compared with vehicle-stimulated HaCaT cells (Fig. 1). When HaCaT cells were costimulated with poly-(I:C) and CBD (1, 5, 10, and 20 µM) for 6 hours, we observed a strong concentration-dependent reduction of MCP-2 protein levels as compared with poly-(I:C)-stimulated HaCaT cells treated with the vehicle of CBD (Fig. 1A). The maximum effect was observed at the highest concentration of CBD tested (20 µM), as compared with poly-(I:C)-stimulated HaCaT cells treated with the vehicle of CBD (Fig. 1A). Likewise, CBD (1, 5, 10, and 20 µM), in a concentration-dependent manner, was also able to strongly reduce MCP-2 production in poly-(I:C)-stimulated HaCaT cells after 12 and 24 hours, and the maximum effect was also observed with 20 µM CBD (Fig. 1, B and C). On the contrary, when HaCaT cells were costimulated with poly-(I:C) and CBC or CBG, no effect was observed at low concentrations (5 and 10 µM), although at the highest concentration tested (20 µM), these two phytocannabinoids were able to reduce MCP-2 production (Fig. 2, A and B). Likewise, when HaCaT cells were costimulated with poly-(I:C), THCV had no effect at the lowest concentration tested (5 µM), but at 10 and 20 µM, it was able to reduce MCP-2 production (Fig. 2C). CBGV was able to reduce MCP-2 production only at 10 µM (Fig. 2D). No effect was observed on MCP-2 protein levels after treatment of poly-(I:C)-stimulated HaCaT cells with CBDA, CBDV, CBDA, CBG, and THCVV as compared with poly-(I:C)-stimulated HaCaT cells treated with the respective vehicles (data not shown). Likewise, no significant variation was observed on MCP-2 protein levels after HaCaT cells were treated with CBD or the other phytocannabinoids alone (at highest concentration tested, 20 µM), i.e., in the absence of poly-(I:C), as compared with vehicle-treated HaCaT cells (data not shown), indicating that this concentration of CBD was not cytotoxic.

CBD Reduces IL-6, IL-8, and TNF-α Protein Levels in Poly-(I:C)-Stimulated HaCaT Cells. We also investigated the effects of CBD, CBC, CBG, THCV, and CBGV on the production of different cytokines (IL-16, IL-2, IL-6, IL-8, G-CSF, GM-CSF, TNF-α) in poly-(I:C)-stimulated HaCaT cells. HaCaT cells stimulated for 6 hours with poly-(I:C) (100 µg/ml) and treated with the vehicle of the phytocannabinoids produced significantly higher levels of only IL-6, IL-8, and TNF-α, as compared with vehicle-stimulated HaCaT cells (Fig. 3). CBD

three independent experiments performed in triplicates. *P < 0.05 vs. vehicle; ***P < 0.001 vs. poly-(I:C). Assay range for IL-6, 37.68 pg/ml; and for TNF-α, 64.80 pg/ml.
(1, 5, 10, and 20 μM), in a concentration-dependent manner, was able to strongly reduce IL-6 and TNF-α protein levels in poly-(I:C)-stimulated HaCaT cells, as compared with poly-(I:C)-stimulated HaCaT cells treated with the vehicle of CBD (Fig. 3, A and C). IL-8 protein levels were strongly reduced only by the two highest concentrations of CBD tested (10 and 20 μM) (Fig. 3B). When HaCaT cells were stimulated for 12 hours with poly-(I:C) and treated with the vehicle of the phytocannabinoids, we only

Fig. 5. CBC, CBD, and THCV reduce IL-6 and IL-8 levels in poly-(I:C)-stimulated HaCaT cells after 6 hours. Bio-Plex Pro assay for IL-6 (A, C, and E) and IL-8 (B and D) release in the supernatants of poly-(I:C)-stimulated HaCaT cells (100 μg/ml, 6 hours, 37°C) in the presence of vehicle or CBC, CBG, and THCV (all tested at 5, 10, and 20 μM). Data represent the mean ± S.E.M. of three independent experiments performed in triplicate. **P < 0.001 vs. vehicle; *P < 0.05; **P < 0.01; ***P < 0.001 vs. poly-(I:C). Assay range for IL-6, 37.63 pg/ml; and for IL-8, 48.15 pg/ml.
observed a strong increase of TNF-α protein levels (Fig. 4A), which was significantly reduced by the highest concentration of CBD tested (20 μM) (Fig. 4A). When HaCaT cells were stimulated for 24 hours with poly-(I:C) and treated with the vehicle of the phytoannabinoids, we observed a strong increase in both IL-6 (Fig. 4B) and TNF-α (Fig. 4C) protein levels, which were again strongly reduced by treatment with CBD 20 μM (Fig. 4, B and C). On the contrary, the other phytoannabinoids found here to produce anti-inflammatory effects on MCP-2 levels were able to downregulate only some of these cytokines after 6 hours, i.e., 1) CBC (20 μM) was able to reduce only IL-6 and IL-8 levels (Fig. 5, A and B), 2) CBG (10 μM) was able to reduce only IL-6 and IL-8 levels (Fig. 5, B and C), and 3) THCV (20 μM) was able to reduce only IL-6 levels (Fig. 5D). No effect was observed on the levels of other cytokines, i.e., IL-1β, IL-2, G-CSF, and GM-CSF, after stimulation of HaCaT cells with poly-(I:C) in the presence or absence of the other phytoannabinoids (data not shown).

CBD and Other Phytoannabinoids Are Not Cytotoxic to HaCaT Cells. No cytotoxicity was observed after treatment of HaCaT cells for 6 hours with CBD, CBC, CBG, THCV, or CBGV at the highest concentrations tested (10 and 20 μM) (Fig. 6A). Likewise, no cytotoxicity was observed after treatment of HaCaT cells for 12 and 24 hours with 10 and 20 μM CBD (Fig. 6B). These results indicate that the decreased MCP-2 and/or cytokine levels in poly-(I:C)-stimulated HaCaT cells were due to the anti-inflammatory effects of these compounds.

CB2 Receptors Do Not Mediate the Action of CBD in Poly-(I:C)-Stimulated HaCaT Cells. We investigated the effect of a CB2 receptor antagonist (AM251; 1, 2.5, and 5 μM) on MCP-3 protein levels in poly-(I:C)-stimulated HaCaT cells, in the presence or absence of CBD (20 μM). Our results show that when HaCaT cells were costimulated for 6 hours with poly-(I:C) and low concentrations of AM251 (1 or 2.5 μM), MCP-2 protein levels were comparable to those found in the absence of the antagonist (Fig. 7A). On the contrary, when HaCaT cells were costimulated with poly-(I:C) and the highest concentration of AM251 tested (5 μM), MCP-2 protein levels were comparable to those observed in the presence of 20 μM CBD (Fig. 7A). In addition, when HaCaT cells were costimulated with poly-(I:C), 20 μM CBD and a suboptimal concentration of AM251 (2.5 μM), MCP-2 production was comparable to that observed in poly-(I:C)-stimulated HaCaT cells treated with 20 μM CBD alone (Fig. 7A). No effect was observed on MCP-2 protein levels after treatment of HaCaT cells with the antagonist AM251 alone (at the highest concentration tested, 5 μM), i.e., in the absence of poly-(I:C), as compared with vehicle-treated HaCaT cells (data not shown). Likewise, no additive effect was found on MCP-2 protein levels after that poly-(I:C)-stimulated HaCaT cells were treated with 20 μM CBD and the highest concentration of AM251 tested (5 μM), as compared with poly-(I:C)-stimulated HaCaT cells treated with 20 μM CBD only (data not shown).

CB2 and TRPV1 Receptors Mediate the Action of CBD in Poly-(I:C)-Stimulated HaCaT Cells. We investigated the effect of a CB2 receptor antagonist (AM630; 0.01, 0.1, and 1 μM) and a TRPV1 receptor antagonist (I-RTX; 0.01, 0.1, and 1 μM) on MCP-2 protein levels in poly-(I:C)-stimulated HaCaT cells in the presence or absence of CBD (20 μM). Our results show that when HaCaT cells were costimulated for 6 hours with poly-(I:C) and low concentrations of AM630 (0.01 or 0.1 μM) or high concentration of I-RTX (1 μM), MCP-2 protein levels were comparable to those observed in the absence of the antagonists (Fig. 7B). On the contrary, when HaCaT cells were costimulated with poly-(I:C) and a higher concentration of AM630 (1 μM) or lower concentrations of I-RTX (0.01 or 0.1 μM), MCP-2 protein levels were comparable to those observed in the presence of CBD 20 μM (Fig. 7B). Importantly, when HaCaT cells were costimulated with poly-(I:C), 20 μM CBD, and the highest per se inactive concentrations of AM630 or I-RTX (0.1 and 1 μM, respectively), MCP-2 chemokine production was comparable to that observed in poly-(I:C)-stimulated HaCaT cells treated with the vehicle (Fig. 7B). However, no effects of AM630 or I-RTX (0.1 and 1 μM, respectively) on MCP-2 protein levels were observed after 12 and 24 hours in poly-(I:C)-stimulated HaCaT cells treated with 20 μM CBD, as compared with poly-(I:C)-stimulated HaCaT cells treated with 20 μM CBD alone (data not shown). In addition, no effect was observed on MCP-2 protein levels after that HaCaT cells were treated with the antagonists, AM630 or I-RTX, alone (at highest concentrations tested, 1 μM), i.e., in the absence of poly-(I:C), as compared with vehicle-treated HaCaT cells (data not shown). Likewise, no additive effects were found on MCP-2 protein levels.

Fig. 6. CBD and other phytoannabinoids are not cytotoxic to HaCaT cells. (A) MTT assay in HaCaT cells treated with vehicle (white histogram) or CBD, CBC, CBG, THCV, and CBGV (all tested at 10 and 20 μM) for 6 hours at 37°C in 5% CO2. (B) MTT assay in HaCaT cells treated with vehicle or CBD (10 and 20 μM) for 12 and 24 hours at 37°C in 5% CO2. Data represent the mean ± S.E.M. of three independent experiments performed in triplicate.
tested (0.01 and 0.1 μM), as compared with poly-(I:C)-stimulated HaCaT cells treated with 20 μM CBD (data not shown). On the basis of these results, we also investigated the effects of AM630 (0.1 μM) and I-RTX (1 μM) on the production of cytokines, such as IL-6, IL-8, and TNF-α, in poly-(I:C)-stimulated HaCaT cells in the presence or absence of CBD (20 μM). Our results show that no effect was found with AM630 (0.1 μM) or I-RTX (1 μM) on the inhibitory action of CBD (20 μM) on the levels of the other three cytokines that were elevated after poly-(I:C) stimulation of HaCaT cells (data not shown).

CBD Elevates AEA Levels in Poly-(I:C)-Stimulated HaCaT Cells. We measured the effect of CBD (20 μM) on AEA, 2-AG, PEA, and OEA levels in poly-(I:C)-stimulated HaCaT cells. We observed that when HaCaT cells were stimulated for 6 hours with poly-(I:C), AEA levels were significantly increased by 3-fold compared with vehicle-treated HaCaT cells, and a nearly statistically significant trend toward elevation of PEA levels (P = 0.0639) was also observed (Fig. 8A and C). When poly-(I:C)-stimulated HaCaT cells were treated with CBD (20 μM), AEA levels were increased by 8-fold compared with vehicle-treated HaCaT cells, and by 2.7-fold compared with poly-(I:C)-stimulated HaCaT cells (Fig. 8A). These increases were only observed during the early sensitization phase of ACD, i.e., after 6 hours (Fig. 8A). No consistent effect of CBD was observed on 2-AG and OEA levels after 6 hours (Fig. 8B and D), as well as on AEA levels after 12 and 24 hours (data not shown), in poly-(I:C)-stimulated HaCaT cells.

AEA and URB597 Reduce MCP-2, IL-6, and IL-8 Protein Levels in Poly-(I:C)-Stimulated HaCaT Cells. We investigated the effects of AEA and a selective fatty acid amide hydrolase inhibitor, URB597, on the production of MCP-2 and other cytokines (IL-6, IL-8, and TNF-α) in poly-(I:C)-stimulated HaCaT cells. Our results show that when HaCaT cells were costimulated with poly-(I:C) and AEA (10 μM), the levels of MCP-2 and IL-6 proteins were reduced as compared with poly-(I:C)-stimulated HaCaT cells treated with the vehicle (Fig. 9A and B). When HaCaT cells were costimulated with poly-(I:C) and URB597 (10 μM), the levels of MCP-2, IL-6, and IL-8 proteins were also reduced as compared with poly-(I:C)-stimulated HaCaT cells treated with the vehicle (Fig. 9B). No effect was observed on IL-6 levels after treatment of poly-(I:C)-stimulated HaCaT cells with AEA (10 μM) (Fig. 9B). Likewise, no effect was observed on TNF-α production after treatment of poly-(I:C)-stimulated HaCaT cells with AEA (10 μM) or URB597 (10 μM) (data not shown). Thus, the effects of AEA and URB597 in poly-(I:C)-stimulated HaCaT cells were similar, although not identical, to those observed with CBD (20 μM) (Fig. 9).

Discussion

In this study, we demonstrated for the first time that a nonpsychotropic plant cannabinoid, CBD, inhibits the production of the MCP-2 chemokine and other proinflammatory cytokines (i.e., IL-6, IL-8, and TNF-α) in poly-(I:C)-stimulated keratinocytes more potently and dose-dependently than other nonpsychotropic phytocannabinoids tested here (CBDα, CBDβ, CBDVA, CBC, CBG, CBGA, CBGV, THCV,
and THCV). Importantly, we also identified the molecular targets for some of the actions of CBD on keratinocytes.

First, we demonstrated that when HaCaT cells are stimulated for 6 hours and up to 24 hours with an agonist of the toll-like receptor 3, i.e., poly-(I:C), they produce higher levels of MCP-2. This chemokine is a proinflammatory mediator involved in the recruitment of macrophages and mast cells into inflammatory sites (Taub et al., 1995; de Paulis et al., 2001), and is downregulated by CB1/CB2 agonists, such as THC and HU-210, in ear keratinocytes of mice with DNF-beta-induced ACD, as well as in poly-(I:C)-stimulated HaCaT cells in vitro (Karsak et al., 2007). We previously demonstrated that this chemokine can also be downregulated by an endogenous lipid mediator, namely PEA, which is known for its important anti-inflammatory properties and is currently used in the clinic against neuropathic and inflammatory pain but, unlike AEA, is not capable of directly activating CB1 or CB2 receptors (Iannotti et al., 2016). In particular, we showed that PEA at a concentration of 10 μM was able to reduce, through a TRPV1-mediated mechanism, both the expression and the production of MCP-2 in poly-(I:C)-stimulated HaCaT cells in vitro (Petrosino et al., 2010). On the contrary, neither the antagonism of CB2 receptors nor the antagonism of peroxisome proliferator-activated receptor-α receptors reversed the anti-inflammatory effect of PEA in poly-(I:C)-stimulated HaCaT cells (Petrosino et al., 2010). Therefore, here we investigated 1) whether CBD, which, like PEA and unlike THC, is unable to directly activate CB1/CB2 receptors, can also nevertheless reduce MCP-2 protein levels in poly-(I:C)-stimulated HaCaT cell; and 2) the molecular target(s) through which CBD exerts this putative anti-inflammatory action. We found that CBD, in a concentration-dependent manner, strongly reduces MCP-2 protein levels in HaCaT cells stimulated for 6 hours and up to 24 hours with poly-(I:C), with the maximum effect being observed at the highest concentration tested (20 μM), and no significant differences in the extent of the effect being noted at different time points. This concentration is 2-fold higher than the efficacious (and maximally soluble in the same vehicle) concentration of PEA (10 μM), in the same in vitro model of ACD (Petrosino et al., 2010). Here, we also demonstrated for the first time that when HaCaT cells are stimulated for 6 hours and up to 24 hours with poly-(I:C), they produce higher levels of other proinflammatory cytokines (i.e., IL-6, IL-8, and TNF-α), which, similar to MCP-2, are produced from keratinocytes during the sensitization phase (Barker, 1992; Enk and Katz, 1992; Becker and Knop, 1993), and that CBD also reduces these effects of poly-(I:C). However, of these three cytokines, only TNF-α exhibited significant increases at all time points, suggesting their potentially different roles in different phases of inflammation, at least in this in vitro model.

Since it is well known that 1) the pharmacological blockade of CB2 receptor attenuates oxazoline-induced contact dermatitis in mice (Oka et al., 2006); 2) the activation of the CB2 receptor, but not the CB1 receptor, is involved in THC-induced
Fig. 9. AEA and URB597 reduce MCP-2, IL-6, and IL-8 levels in poly(I:C)-stimulated HaCaT cells after 6 hours. Enzyme-linked immunosorbent assay for MCP-2 (A) and Bio-Plex Pro assay for IL-6 (B) and IL-8 (C) release in the supernatants of poly(I:C)-stimulated HaCaT cells (100 μg/ml, 6 hours, 37°C) in the presence of vehicle or CBD (20 μM), AEA (10 μM), or URB597 (10 μM). Data represent the mean ± S.E.M. of three independent experiments performed in triplicate. **p < 0.001 vs. vehicle; *p < 0.05, **p < 0.01, ***p < 0.001 vs. poly(I:C). Assay range for MCP-2, 0.8–200 pg/ml; for IL-6, 37.68 pg/ml; and for IL-8, 42.15 pg/ml.
with a Sentry Symmetry® guard column (10 × 2.1 mm, 3.5 μm) was utilized with the LC/MS system. The ZQ detector was used with an electrospray ionization probe set for single ion monitoring for CBD quantification. Analysis was performed in negative mode for m/z 313 [CBD-H]+, (dwell time: 0.3 s). Capillary and cone voltage were set at 35 kV and 40 V, respectively. Source block temperature was set at 120 °C, and desolvation temperature to 250 °C. Nitrogen was used as nebulization (flow rate: 50 l/h) and drying gas (flow rate: 450 l/h). The mobile phase was comprised of 75:25 ACN:2 mM ammonium acetate buffer w/5% ACN and used at a flow rate of 0.25 mL/min resulting in a mean CBD retention time of 5.6–5.7 min. Standard curves were linear within the range 2–300 ng/mL and concentrations of samples were determined by comparison.

2.8 Immunohistochemistry

Spinal cord, dorsal root ganglia (DRG) and knee joint synovial joint capsule membranes were excised after transcardial perfusion, post-fixed with 4% buffered paraformaldehyde, cryoprotected overnight with 30% sucrose, embedded in OCT compound (Electron Microscopy Sciences, Hatfield, PA, USA) and stored at −80 °C. Tissue was sectioned (14–20 μm) with a cryostat at −20°C, sections collected on microscopic slides (Superfrost Plus, VWR, Radnor, PA) and stained. Synovial joint capsule membranes were stained with haematoxylin and eosin (H&E; Ricca Chemical Company, Arlington, TX, USA) and cover slipped using Permount (Fisher Scientific).

Spinal cord sections were stained using monoclonal mouse anti-OX-42 (1:1,000, CD11b/c; Abcam, Cambridge, MA, USA) and rabbit anti-CGRP (1:2000; Bachem, Torrance, CA, USA) antibodies. DRG sections were immunostained with mouse anti-TNFα (1:1000, #52B83; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Primary antibodies were detected using the appropriate Alexa Fluor conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Slides were cover-slipped using Vectashield Hard Set mounting medium with DAPI (VectorLabs, Burlingame, CA, USA). Controls included the absence of staining upon omission of the primary antibody and the side to side differences between the ipsilateral and contralateral sides as internal controls.

Images of haematoxylin and eosin (H&E) staining were acquired using a Nikon Eclipse E1000 microscope equipped with a Nikon DXM 1200F digital camera and ACT-1 software. Synovial membrane thickness was measured by drawing a perpendicular line from the outer surface to the inner margin of the intimal layer of the synovial membrane in two places within each section and averaging their length (Cunnane et al., 1999).

Immunostaining of fluorescent labelled tissue was visualized using a Nikon Eclipse E1000 microscope equipped with a Cool Snap photometric Camera ES and analysed off-line with MetaMorph software. Camera settings were kept constant within an antibody staining to quantify and compare immunoreactivity. Images of the dorsal horn were analysed by outlining the substantia gelatinsosa, determining and averaging the mean intensity of staining in this region. Background fluorescent intensity was measured outside of this area and subtracted. DRG sections were sampled by drawing regions of interest over five randomly selected sensory neuron somas, measuring the mean intensity and averaging for each animal sampled. Histological analysis was conducted in the four experimental groups: naive
(uninjected control animals), CFA + VEH (monoathritis treated with vehicle gel), CFA + low-dose CBD (monoathritis treated with 0.62 or 3.1 mg/day CBD) and CFA + high-dose CBD (monoathritis treated with 6.2 or 62.3 mg/day CBD). Mean intensities were averaged and compared across treatment groups.

2.9 Data analysis

GraphPad Prism version 6.1 for Windows (GraphPad Software, La Jolla, CA, USA) was used for all data statistical analyses. Data are presented as the mean ± standard error except where mentioned. Normally distributed data were analysed with one-way ANOVA followed by Bonferroni post hoc analysis. Pain rating score data were shown as medians and were compared using Kruskal–Wallis nonparametric analyses with Dunnett’s post hoc test. For statistical analysis of the joint circumference and behavioural results, the naive and naïve rats treated with CBD were combined for comparisons to CFA + VEH (vehicle), CFA + low-dose CBD (0.62 and 3.1 mg/day) and CFA + high-dose CBD (6.2 and 62 mg/day). Values of \( p \leq 0.05 \) were considered significant.

3. Results

3.1 CBD plasma concentrations

Animals were treated with CBD gel in four different doses: 0.62, 3.1, 6.2 and 62.3 mg/day. After four consecutive days of treatment, plasma CBD concentrations in all rats were 3.8 ± 1.4 ng/mL \((n = 9)\), 17.5 ± 4.4 ng/mL \((n = 8)\), 33.3 ± 9.7 ng/mL \((n = 8)\) and 1629.9 ± 379.0 ng/mL \((n = 4)\), respectively (Table 1). The three lower doses displayed excellent linear pharmacokinetic correlation (slope = 1.0, \( R^2 = 0.999 \)). However, CBD plasma concentrations after application of the 62.3 mg/day dose did not follow the linear pharmacokinetic profile.

3.2 Knee joint inflammation

In naïve animals the left and right joint circumferences were not different (mean ipsilateral = 58.0 ± 0.4 mm; mean contralateral = 57.7 ± 0.4 mm; \( n = 21 \)). Three days after adjuvant induction of monoathritis, significant swelling was noted on the side ipsilateral to the injection (ipsilateral = 73.6 ± 0.6 mm; contralateral = 55.8 ± 0.4 mm; \( n = 21 \), \( p < 0.001 \); one-way ANOVA, Bonferroni post hoc test) resulting in a 32.0 ± 1.1% circumference increase as shown in Fig. 1A. Daily applications of 6.2 mg CBD for four consecutive days significantly reduced the knee joint circumference from 72.0 ± 0.2 mm measured on day 3 after CFA injection to 65.6 ± 1.0 mm on day 7 (\( p < 0.05 \)). Use of 62.3 mg/day CBD gel similarly reduced the ipsilateral joint circumference increase from 30.9 ± 0.3% to 16.7 ± 2.8% (ipsilateral circumference on day 3 = 72.0 ± 0.2 mm; day 7 = 65.6 ± 0.7 mm; \( p < 0.01 \); one-way ANOVA, Bonferroni post hoc test). Lower doses of CBD had no effect on CFA-induced oedema (Fig. 1A).

At experiment end histological analysis after H&E staining determined that the synovial membrane was thickened 7 days after intra-articular CFA injection (Fig. 1C and D). Transdermal application of 6.2 mg/day CBD for 4 days reduced this pathological change (Fig. 1E). In naïve animals the synovial membrane measured an average of 201 ± 17 μm (\( n =
4) from outer surface to its inner margin (Fig. 1F). This increased almost 9-fold after adjuvant-induced monoarthritis to a width of 1788 ± 128 μm (p < 0.001, one-way ANOVA, Bonferroni post hoc test, n = 5). Application of the high doses of CBD (high dose = 6.2 or 62.3 mg) significantly reduced the membrane thickness by more than 50% to 767 ± 111 μm (n = 7, p < 0.001, one-way ANOVA, Bonferroni post hoc test). Treatment with the two lower doses of CBD (low dose = 0.62 or 3.1 mg) did not alter synovial membrane thickness (1631 ± 159 μm, n = 4).

3.3 Spontaneous pain rating scores

Limb posture scores as a rating of spontaneous pain were high on day 3 (median score 4) in all animals with adjuvant-induced monoarthritis. On day 7, after 4 days of transcutaneous treatment with 6.2 and 62.3 mg/day CBD gel, pain scores were significantly improved (median 1.5) compared to animals in the vehicle control group (median 3.5; p < 0.05; Kruskal–Wallis test, Dunnett's post hoc test) (Fig. 1B). Pain scores of animals that received 0.6 and 3.1 mg/day CBD were not different from vehicle controls. All naive rats scored 0 in this test.

3.4 Secondary hindpaw heat hypersensitivity

Baseline paw withdrawal latencies were similar in all experimental animals (10–12 s). Hypersensitivity to noxious heat was detected in all animals with adjuvant-induced monoarthritis. Average paw withdrawal latency (PWL) in response to radiant heat applied to the plantar surface of the same side hindpaw, was significantly decreased on day 3 from 11.2 ± 0.2 s to 7.2 ± 0.1 s (p < 0.005, one-way ANOVA, Bonferroni post hoc test). The PWL on the contralateral side did not change (day 0 = 11.1 ± 0.2 s; day 3 = 11.1 ± 0.2 s; p > 0.05, one-way ANOVA, Bonferroni post hoc test). After 2 days of treatment with 6.2 or 62 mg/day transdermal CBD, a significant improvement of heat hypersensitivity was noted on day 5 which persisted throughout the experiment (Fig. 2A). The 6.2 mg/day doses increased PWL of the inflamed leg from 7.5 ± 0.1 s on day 3 after CFA injection to 9.4 ± 0.5 s on day 5 (p < 0.05; one-way ANOVA, Bonferroni post hoc test). The 62 mg/day increased PWL from 6.7 ± 0 ± 0.1 on day 5 (p < 0.05, one-way ANOVA, Bonferroni post hoc test).

Reduction in monoarthritis-induced heat hypersensitivity was maximal after 2 days of transdermal application of 6.2 or 62 mg/day CBD, and no further improvement was evident through the remaining experimental time course. Adjuvant-induced heat hypersensitivity was not changed by transdermal application of vehicle, nor by 0.6 or 3.1 mg/day CBD (Fig. 2A). Daily application of CBD gel on naive animals did not alter heat sensitivity irrespective of the concentration used (Fig. 2B).

3.5 Activity level in open field

Potential for adverse side-effects on activity levels or motor abilities stemming from CBD gel application were assessed by monitoring open-field exploratory behaviour of naive animals for 45 min prior to and directly after treatment. Irrespective of the amount of CBD applied onto the back of animals, no changes were detected for total time spent in either exploratory activity or resting (Fig. 2C and D). Two other specific exploratory activities acquired in the open-field test but not affected by CBD were rearing events in which the animals rise on their hindlimbs to explore the environment and the total distance travelled.
during the 45 min test (data not shown). This indicates that CBD did not alter the animals' activity levels or motor abilities.

3.6 Immunohistochemical analysis of pro-inflammatory biomarkers

Pro-inflammatory biomarkers calcitonin gene-related protein (CGRP) (Fig. 3A) and OX42 (Fig. 3B) were immunostained in the lumbar spinal cord dorsal horn. Tumour necrosis factor alpha (TNFα) (Fig. 3C) was identified in DRG. After batch staining immunohistochemical methods, the intensity of immunofluorescence was determined using computer assisted quantification. The immunoreactivity for peptide CGRP was significantly increased in the superficial dorsal horn of the spinal cord in the monoarthritis group. CGRP increased from an average fluorescent intensity of 114 ± 32 (naive) to 340 ± 81 (CFA + VEH, p < 0.05, one-way ANOVA, Bonferroni post hoc test) (Fig. 4A). Treatment with high doses of CBD (6.2 and 62 mg/day) reduced immunoreactivity to the levels in naive animals (CFA + low dose CBD = 272 ± 46, p > 0.05 vs. CFA + VEH; CFA + high-dose CBD = 144 ± 28, p < 0.05 vs. CFA + VEH; one-way ANOVA, Bonferroni post hoc test).

Naive animals had low levels of OX42 expression in the spinal cord, a marker for activated microglia (Fig. 4B). Immunoreactivity for OX42 was significantly increased around the spinal cord central canal in lamina X in samples collected from animals 7 days after induction of monoarthritis (CFA + VEH = 1072 ± 80) compared to fluorescent intensities in naive rats = 762 ± 38, p < 0.01, one-way ANOVA, Bonferroni post hoc test). Treatment with low doses of CBD was effective in decreasing OX42 immunoreactivity (891 ± 48, p > 0.05 vs. CFA + VEH one-way ANOVA, Bonferroni post hoc test). Treatment with high doses of CBD significantly decreased fluorescent intensity of OX42 immunoreactivity to levels below those in the naive animals (549 ± 13, p < 0.001 vs. CFA + VEH, one-way ANOVA, Bonferroni post hoc test).

In DRG, immunoreactivity for the pro-inflammatory cytokine TNFα was significantly increased in the monoarthritis group (naive = 579 ± 33, CFA + VEH = 900 ± 63, p < 0.001; one-way ANOVA, Bonferroni post hoc test) (Fig. 4C). Treatment with high doses of CBD also reduced TNFα immunoreactivity which was equivalent to levels of naive animals (CFA + low dose CBD = 837 ± 49, p > 0.05 vs. CFA + VEH; CFA + high-dose CBD = 620 ± 23, p < 0.001 vs. CFA + VEH; one-way ANOVA, Bonferroni post hoc test).

4. Discussion

4.1 Transdermal CBD bioactivity

Outcomes of this study indicate that topical application of CBD gel is an effective treatment for reduction in inflammation and hypersensitivity associated with the rodent adjuvant-induced monoarthritis model. Transdermal administration of CBD provided good blood absorption due to avoidance of first pass metabolism encountered by orally administered drugs. Malfait et al. (2000) saw a bell-shaped dose-dependency curve for CBD given orally or intraperitoneally to attenuate nociceptive behaviour in a collagen/CFA-induced arthritis model in mice. Similarly in this study, CBD plasma concentrations for rats dosed with 0.6, 3.1 and 6.2 mg/day exhibited an excellent linear correlation. However, the highest dose, 62.3
mg per day, did not fit into the linear pharmacokinetic profile. Dosing was increased by massaging the total amount of CBD gel into a larger skin area on the back while gel concentration (1%) remained identical. A 10% gel formulation was used for the highest transdermal CBD treatment since skin area could not be appropriately increased. The 10% gel was close to solubility saturation and may have an increased absorption rate compared to 1% formulations. The lack of increased outcome for this highest CBD concentration was potentially due to maximally activated CBD effects or capacity-limit absorption and metabolism. This would account for the flattened linear pharmacokinetic profile effect of the 62.3 mg/day dose. Spreading large quantities of gel directly on the skin over the joint itself was not feasible in this transdermal dosing paradigm and would provide opportunity for oral ingestion by the rats. Application of 10% CBD gel on skin directly overlaying an inflamed joint in patients would potentially increase local CBD concentrations and enhance effectiveness with less systemic involvement.

4.2 Efficacy of transdermal CBD

Efficacy of transdermal CBD for reduction in inflammation-associated symptoms in adjuvant-induced monoarthritis animals was determined comparing knee joint circumference and other features. The 6.2 mg/day dose optimally reduced swelling and increasing the CBD dose (62.3 mg/day) did not yield additional improvement. Likewise, increased synovial membrane thickness was reduced by the 6.2 mg/day CBD treatment. These results concur with previous studies showing orally administered CBD decreased inflammation (Malfait et al., 2000; Costa et al., 2004a,b, 2007). Decreased inflammation and reduction in secretions of pro-inflammatory and matrix-degrading effector molecules by the synovial cell connective tissue membrane lining the joints are important for symptomatic treatment of patients with rheumatoid arthritis. Pro-inflammatory and matrix-degrading effector molecules produced in excess are primary contributors to cartilage degradation over time (Ospelt et al., 2004; Neumann et al., 2010).

The improvement of pain scores provided by transdermal CBD is an indirect measure of joint inflammation and direct measure of function. The PWL in response to noxious heat stimuli was optimal with both the 6.2 and 62.3 mg/day doses. These concentrations are similar to optimal doses of intraperitoneally injected or orally administered CBD used to decrease hypersensitivity and inflammation in a collagen/CFA-induced arthritis model in mice (Malfait et al., 2000). Analogous to the results presented here, the highest dose of CBD administered in that study also did not perform as well as the next lower dose. Malfait et al. (2000) found that intraperitoneal treatments using 5 mg/kg was optimal for relief of arthritis symptoms and 25 mg/kg for orally administered treatments. Orally administered CBD at concentrations of 10–40 mg/kg were also able to inhibit thermal hypersensitivity at 3 h post carrageenan-induced inflammation of the hindpaw (Costa et al., 2004a). In the same study at 6 h post inflammation, CBD treatment with two orally administered lower doses, 5 and 7.5 mg/kg, was also effective.

4.3 Mechanisms of action

Peripheral inflammation and hypersensitivity are reversed by pharmacological inactivation of both central and peripheral neurons and central microglia (Sluka et al., 1993, 1994; Guo
and Schluesener, 2006; Roberts et al., 2009). Although CBD is described as an attenuator of both mechanical and heat hypersensitivity induced by inflammatory and neuropathic pain models, the exact mechanism of action is as yet unknown (Mechoulam and Hanus, 2002; Kress and Kuner, 2009). Unlike THC and related cannabinoids, phytocannabinoid-CBD, an important bioactive component of Cannabis sativa without psychotropic effect, is an antagonist of orphan G protein-coupled receptor 55 (GPR55, a potential third metabotropic cannabinoid receptor) without binding to CB1 and CB2 receptors (Begg et al., 2005; Brown, 2007). CBD interacts with GPR55 resulting in inactivation of its pronociceptive signalling (Ryberg et al., 2007; Staton et al., 2008; Godlewski et al., 2009).

CBD also acts as an agonist for ionotropic cannabinoid receptors including chemo- and thermosensitive members of the TRP channel superfamily (TRPV1, TRPA1, TRPV2, TRPV3, TRPV4) (Fioravanti et al., 2008; Kress and Kuner, 2009). A particular focus has been on TRPA1 and TRPV1, two widely co-expressed ion channels found in CGRP expressing peptidergic nociceptors essential for neurogenic inflammation, oedema formation and inflammation-induced mechanical and thermal hypersensitivity (Davis et al., 2000; Szabo et al., 2005; Petrus et al., 2007; Kerstein et al., 2009; Akopian, 2011). Their activation by CBD in vitro results in desensitized responses following noxious stimulation with capsaicin or mustard oil, their respective agonists. This mechanism potentially decreases neuropeptide expression (Bisogno et al., 2001; Costa et al., 2004b; Akopian et al., 2008, 2009). In vivo absence or inhibition of TRPA1 results in reduced mechanical hypersensitivity in animal models of inflammation (Petrus et al., 2007; McGarvaughy et al., 2010; Akopian, 2011). Absence of TRPV1 in vivo reduces inflammation-induced swelling, thermal hypersensitivity and nociceptive behaviour in various pain models (Caterina et al., 2000; Davis et al., 2000; Keeble et al., 2005; Szabo et al., 2005). In naive animals, TRPV1 immunoreactivity is localized in nociceptive primary afferents innervating the knee joint. After inflammation, TRPV1 expression increases not only in primary afferents, but is detected in synoviocytes which secrete lubricating fluid into the synovial space and function as local immune cells (Kochukov et al., 2009). Once activated, synoviocytes release pro-inflammatory cytokines and chemokines such as IL1-α, IL-6, TNFα and RANTES, as well as COX-2 (Guerne et al., 1989; Berckmans et al., 2005; Westlund et al., 2010). Released TNFα not only sensitizes primary afferents (Sorkin and Doom, 2000; Schafer and Sorkin, 2008), but also acts in an autocrine fashion, increasing the expression of TRPV1 in synoviocytes (Kochukov et al., 2009). Primary afferents are thus not only sensitized by peripheral release of pro-inflammatory cytokines, but are surrounded by cells that produce and release these molecules themselves. Release of TNFα from cultured synoviocytes from arthritic animals is reduced by application of CBD (Malfait et al., 2000) as well as by TRPV1 antagonists (Kochukov et al., 2009). Desensitization of TRPV1 by CBD could thus interrupt further progression of this cycle. Further studies are needed to identify specific receptors and mechanisms underlying the anti-inflammatory and anti-hyperalgesic effects of CBD.

The pro-inflammatory/pro-pain spiral is further driven by similar mechanisms which induce the expression of pro-inflammatory biomarkers in the DRG of the afferent nerves and centrally in the spinal cord. Transdermal CBD application was successful in decreasing monoarthritis-associated increases of pro-inflammatory biomarkers in neuronal tissues.
Here, adjuvant-induced monoarthritis led to significant increases of TNFα expression in DRG which was decreased to baseline levels by transdermal CBD application.

In this study, the expression of CGRP in spinal cord was increased after peripheral inflammation as previously reported (Stuka and Westlund, 1993), and was decreased by high doses (6.2 and 62 mg/day) of transdermal CBD. Like other neuropeptides, CGRP is rapidly transported to nerve terminals for release centrally as well as peripherally where as a potent vasodilator it contributes to neurogenic inflammation (Kawasaki et al., 1988; Zhang et al., 2001). Although increases in CGRP are described in DRG after hind paw inflammation (Nadin and Byers, 1994), no significant changes in DRG expression were observed here (data not shown), possibly due to the small number of sensory neurons innervating the joint. It is also plausible that by 7 days post monoarthritis induction, neuropeptide content has stabilized.

In monoarthritisic animals, spinal cord OX42 expression is increased in activated microglia, the immune cells specific to the central nervous system, as previously described for CFA-induced ankle inflammation and trinitrobenzene sulfonic acid (TNBS)-induced pancreatitis (Shan et al., 2007; Liu et al., 2012). Treatment with high doses of transdermal CBD in this study effectively reduced OX42 expression below baseline levels, indicating reduced microglial activation. CBD also reduced microglial/macrophage spinal cord expression in a mouse encephalopathy model (Kozela et al., 2011).

4.4 Absence of psychoactive behaviours

CBD is a known non-psychoactive cannabinoid, and due to its low affinity for the CB1 receptor it would be expected that exploratory behavioural activity would be similar among treatment groups compared to negative side-effects associated with THC (Croxford, 2003; Malone et al., 2009). This was demonstrated in this study by lack of CBD-induced changes in open-field exploration among naive treatment groups. Combinatorial with psychoactivity, side-effects such as hypothermia and hypomobility induced by THC are avoided with use of CBD (Zimmer et al., 1999).

5. Conclusion

These studies demonstrate transdermal administration of CBD has long-lasting therapeutic effects without psychoactive side-effects. Thus, use of topical CBD has potential as effective treatment of arthritic symptomatology. At present, one in five (21%) adults worldwide are diagnosed with some form of arthritis by their physicians (Helmick et al., 2008). The data presented suggest transdermal CBD is a good candidate for developing improved therapies for these debilitating disease.

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References


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What's already known about this topic?

- Cannabinoids and cannabinoid receptors have been studied as potential targets for reducing pain and inflammation associated with osteoarthritis and rheumatoid arthritis. Cannabinoid side-effects vary and depend on several factors like administrated dose, route of administration, etc.
What does this study add?

- Transdermal cannabidiol (CBD) gel application has therapeutic potential for relief of arthritis pain-related behaviours and exerts an anti-inflammation property without evident high brain centre psychoactive effects.
Figure 1.
Assessment of knee joint inflammation. (A) Ipsilateral knee joint circumference was significantly increased in rats with adjuvant-induced monoarthritis and significantly decreased after four consecutive days of transdermal cannabidiol (CBD) treatment using 6.2 or 62.3 mg/day doses (*p < 0.01; one-way ANOVA). (B) Pain scores (median) were maximal 3 days after adjuvant-induced monoarthritis and were significantly reduced by 6.2 and 62.3 mg/day CBD (*p < 0.05; Kruskal–Wallis test). (C–E) H&E staining of the synovial membrane from (C) a naïve rat, (D) after adjuvant-induced monoarthritis and (E) after adjuvant-induced monoarthritis treated with 6.2 mg/day transdermal CBD. (F) Bar graph shows high doses of CBD (combined 6.2 and 62.3 mg/day doses) reduced synovial membrane thickening more than 50%. (n = 4–7 rats per group) (*p < 0.001; one-way ANOVA).
Figure 2.
Transdermal cannabidiol (CBD) reduced monoarthritis-induced hind paw heat hypersensitivity. (A) Transdermal CBD significantly increased heat paw withdrawal latency in rats with monoarthritis, (B) but had no effect in naïve rats (*p < 0.01 vs. day 3, one-way ANOVA). (C, D) In the open-field test, transdermal CBD exerts no detectable effect on times spent in spontaneous exploratory activity or resting. (n = 5 per group and CBD dose)
Figure 3.
Immunocytochemical localization of inflammatory biomarkers. (A) CGRP and (B) OX42 are shown in ipsilateral spinal cord dorsal horn for naïve, complete Freud's adjuvant (CFA) arthritic and cannabidiol (CBD) treated CFA arthritic rats. (C) TNFα is shown in the ipsilateral dorsal root ganglia of naïve, CFA arthritic and CBD treated CFA arthritic rats. Bar = 100 μm.
Figure 4.
Quantification of pro-inflammatory biomarkers in the lumbar spinal cord and dorsal root ganglia (DRG). Increases in spinal cord (A) CGRP and (B) OX42 and (C) DRG TNFα after adjuvant-induced monoarthritis are reduced to baseline levels by high doses of transdermal cannabidiol. (n = 4–6 animals per group) (*p < 0.05; one-way ANOVA).
Table 1

Plasma concentrations (±SD) of CBD in rats with and without complete Freud’s adjuvant (CFA) induction after 4 days treatment with transdermal CBD gel.

<table>
<thead>
<tr>
<th>Dose applied</th>
<th>Dose per unit area</th>
<th>CFA+CBD group (ng/mL)</th>
<th>All CBD treated (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6 mg/day</td>
<td>0.18 mg/cm²</td>
<td>4.3 ± 2.5</td>
<td>3.8 ± 1.4</td>
</tr>
<tr>
<td>3.1 mg/day</td>
<td>0.18 mg/cm²</td>
<td>18.8 ± 2.8</td>
<td>17.5 ± 4.4</td>
</tr>
<tr>
<td>6.2 mg/day</td>
<td>0.18 mg/cm²</td>
<td>34.6 ± 11.0</td>
<td>33.3 ± 8.7</td>
</tr>
<tr>
<td>62.3 mg/day</td>
<td>1.8 mg/cm²</td>
<td>1470.1 ± 260.7</td>
<td>1626.9 ± 370.0</td>
</tr>
</tbody>
</table>
Single oral dose of cannabinoid derivate loaded PLGA nanocarriers relieves neuropathic pain for eleven days

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Abstract

Neuropathic pain, resistant to opiates and other drugs, is a chronic/persistent state with a complex treatment and often poor efficacy. In this scenario, cannabinoids are increasingly regarded as a genuine alternative. In this paper, and in an experimental animal model of neuropathic pain, we studied the efficacy of three kinds of PLGA nanoparticles containing synthetic cannabinoid CB13: (i) plain nanoparticles (PLGA); (ii) particles coated with PEG chains (PLGA + PEG) and (iii) particles possessing hydrophilic surfaces obtained by covalently binding PEG chains (PLGA–PEG). The optimized formulation, CB13–PLGA–PEG, showed high drug loading (13%) and small size (<300 nm) with a narrow distribution and controlled surface properties (near-neutral zeta potential and stable PEG corona). Animal nociceptive behavioral studies were conducted by paw pressure and acetone tests. Versus the free CB13, CB13–PLGA–PEG nanoparticles showed a very noticeable analgesic efficacy with the longest sustained pain-relieving effect, lasting up to eleven days after one oral dose.

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Key words: PLGA nanoparticles; Synthetic cannabinoid; Oral administration; Neuropathic pain

Neuropathic pain is a chronic/persistent state resulting from injury to the nervous system due to trauma, chronic inflammation, viral infection, or metabolic disturbances, i.e., diabetes.\textsuperscript{1} Drug associations in the routine clinical treatment of neuropathic pain are frequently used, including the common use of tricyclic antidepressants. Other antidepressants, such as duloxetine or venlafaxine, anticonvulsants and opioids, are also commonly used.

Nevertheless, neuropathic pain treatment is complicated and often poorly efficacious in the majority of patients. It is resistant to opiate analgesics\textsuperscript{2,3}; antidepressants, particularly tricyclic antidepressants, contribute to a poor side-effect profile and limited patient tolerance\textsuperscript{4} due to their anticholinergic, antihistaminergic and antiadrenergic properties. The efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) in treating neuropathic pain has been questioned by many clinicians and indeed, such drugs are not recommended for treating neuropathies.

It is evident that new, effective chronic pain management drug therapy is required and that there is a drive to find more effective treatments.

In recent years there has been growing clinical evidence on the efficacy of cannabis and synthetic cannabinoid agonists in chronic pain states. Most recently, Science published a special issue dealing with pain research. The section: "The future of pain research"\textsuperscript{4} indicates that hints are emerging that cannabis could be one of today's pain management alternatives.

Cannabinoids are moderately effective in treating neuropathic pain and new synthetic cannabinoids presenting fewer side effects have been developed. This includes the potent cannabinoid...
receptor CB1/CB2 agonist 13 (CRA13 or CB13), which reverses neuropathic mechanical hyperalgesia. Like other cannabinoid compounds, CB13 is a highly lipophilic drug and belongs to class II compounds (low solubility and high permeability) of the Biopharmaceutics Classification System. In fact, in preclinical studies this drug was orally administered dissolved in a non-aqueous solvent or dispersed in an aqueous phase by the aid of dispersing agents such as Cremophor®. In addition, due to CB13’s high lipophilic nature, Trevaskis et al. studied the influence of food on its oral bioavailability. They demonstrated that the quantity and composition of food can enhance CB13 oral bioavailability by stimulating lymphatic transport.

The development of new techniques enabling lipophilic drugs to be administered orally is still a major challenge. In the search for solutions to these problems, nanotechnology-based drug delivery systems are promising tools. Moreover, the use of nanocarriers in the management of pain is a novel and exciting area of research, with a great potential for growth and clinical benefit.

We have recently developed surface-modified PLGA nanoparticles and solid lipid nanoparticles intended for oral CB13 administration. In vitro and ex vivo mucoadhesive properties were enhanced using several mucoadhesive actives such as chitosan or Eudragit®. Nevertheless, in vivo biodistribution assays revealed that most nanoparticles accumulated in the liver and spleen, indicating that nanoparticles did not prevent the opsonization process.

In this respect, polyethyleneglycol (PEG)-coated nanoparticles appear to be the most promising strategy for several reasons as they: (i) promote nanoparticles mucoadhesion; (ii) stabilize the nanoparticles in digestive fluids; (iii) avoid plasma protein adsorption; (iv) minimize the interaction with phagocytic cells; and (v) increase the blood circulating time.

The objective of the present work is, therefore, to undertake practical research into the potential of PLGA nanoparticles as oral delivery systems for CB13. For this purpose, free and nanoparticle-encapsulated CB13 was orally administered at different doses to an animal model of neuropathic pain.

We prepared three kinds of PLGA nanoparticles that possessed either unmodified hydrophobic surfaces or hydrophilic surfaces. These were obtained by coating them with PEG chains that were either adsorbed or covalently attached.

Methods

Synthesis of the PLGA-based nanoparticles

PLGA-based nanoparticles were prepared by a modified nanoprecipitation (NPF) method. Briefly, a weighed amount of PLGA or PLGA–PEG was co-dissolved with Span® 60 in acetone. 5 mL of this aceton solution was then added dropwise under magnetic stirring to 15 mL of a Pluronic® F-68 aqueous solution (0.5% w/v). The acetone content was evaporated at room temperature (r.t.) for 4 h. Finally, the nanoparticles suspension was collected by ultracentrifugation. To prepare PEG coated-PLGA (PLGA + PEG) nanoparticles, PLGA nanoparticles were incubated in a PEG (4.5% w/v) (see Supplementary Materials).

CB13 vehiculization capabilities

Drug content into the nanoparticles was determined by a previous validated and verified HPLC method. Briefly, 5 mg of lyophilized nanoparticles was accurately weighed. 1 mL of acetonitrile was then added to dissolve the particles. After this, 10 μL of the previously-filtered solution was injected into the HPLC system for CB13 detection (see Supplementary Materials).

CB13 release experiments were performed using the three PLGA-based nanoparticles. 4 mg of nanoparticle samples was suspended in 15 mL of a release medium: hydrochloric acid media, pH = 2.0 ± 0.1 or phosphate buffered saline, PBS, pH = 7.4 ± 0.1 to simulate gastric and intestinal conditions, respectively (see Supplementary Materials).

In vivo studies of CB13-loaded nanoparticles in an animal neuropathic pain model

Animals

Adult male Harlan Sprague–Dawley rats weighing 250-300 g were provided by the Experimental Unit of the University of Cádiz (registration number ES110120002210). All the experimental protocols were approved by the Committee for Animal Experimentation at the University of Cádiz (Spain) and they complied with the International Association for the Study of Pain ethical guidelines. All procedures relating to animal care and use conformed to European Ethical Standards (86/609-EEC) and Spanish Law (RD 1201/2005). All efforts were made to minimize animal suffering.

Drugs

All formulations were administered orally by gavage in a volume of 2 mL/kg. Control animals received DMSO or blank nanoparticles (with no drug) in the same amounts of loaded nanoparticles corresponding to a CB13-equivalent dose of 1.7, 3.4 and 6.8 mg/kg, respectively.

Neuropathic pain model

Chronic constriction injury (CCI) was used as a model of neuropathic pain because it induces clinical signs of hypersensitivity that mimic human conditions of neuropathic origin. CCI was produced as previously described with an intraperitoneal injection of 100 mg/kg ketamine and 20 mg/kg xylazine. The left sciatic nerve was exposed at the mid-thigh level proximal to the sciatic trifurcation, and four chronic gut (4/0) ligatures were tied loosely around the nerve, 1.0 to 1.5 mm apart, so that the vascular supply was not compromised. Sham operations were performed in the same manner but with no nerve ligation.

Nociceptive behavioral assessment

Paw Pressure Test. Mechanical threshold was determined using the paw pressure test. Briefly, increasing pressure was gradually applied to the dorsal side of the paw using a graded motor-driven device (Ugo Basile, Comerio, Italy) with an initial 30 g of pressure. Two measurements were taken for each paw at 5-min intervals and the average value was determined, with a 250 g cut-off applied to prevent damage to the paw. Mechanical hypersensitivity is indicated by a reduction in the pressure provoking withdrawal. Nociceptive behavior was assessed in both
the ipsi- and contralateral paws. The test was performed by an experimenter who was unaware of the treatment condition.

Acetone test. To assess the sensitivity to non-nociceptive thermal stimuli, a drop of acetone (100 μL) was placed gently on the plantar surface of the ipsilateral hind paw. Acetone was applied alternately five times, with a 5 min delay between each successive application. Responses were monitored for 1 min after acetone application and they were graded on a 4-point scale as described previously: 0, no response; 1, quick withdrawal, flick or stamp of the paw; 2, prolonged withdrawal or repeated licking of the paw; 3, repeated licking of the paw with persistent licking directed at the ventral side of the paw. The cumulative scores were then obtained by summing the four scores for each rat and dividing by 5 (the number of assays). The responses were scored by an observer who was unaware of the treatment condition.

Experimental protocols

Experiment 1: Evaluation of the effectiveness of PLGA nanoparticles (plain) in the CCI model. On day 7 after surgery, PLGA (1.7–6.8 mg/kg) was orally administered once. Mechanical pain hypersensitivity was evaluated using the paw pressure test at 0.5, 3, 9 and 24 h after administration. Sensory threshold was evaluated once a day on days 3 and 5. The same protocol was used for free CB13 (3.4 mg/kg, p.o.). This time schedule was designed based upon pilot experiment results.

Experiment 2: Evaluation of the effectiveness of PLGA + PEG nanoparticles (PEG adsorbed) in the CCI model. To study the effect of PLGA + PEG, the same protocol as that used for PLGA was employed. The selection of this time schedule was also based upon pilot experiment results.

Experiment 3: Evaluation of the effectiveness of PLGA nanoparticles (PEG covalently bind) in the CCI model. 7 days after surgery, PLGA–PEG (1.7–6.8 mg/kg) was orally administered once. Mechanical pain hypersensitivity was evaluated using the paw pressure test at 0.5, 3, 9 and 24 h after administration. Sensory threshold was also assessed once a day on days 3, 5, 7, 9 and 11. The same protocol was used for free CB13 (3.4 mg/kg, p.o.). This time schedule was also performed 2 days after surgery in both groups.

Results

Preparation of the CB13-loaded PLGA, PLGA + PEG and PLGA–PEG nanoparticles

The nanoparticles containing CB13 were successfully obtained by the nanoprecipitation method. The choice of a nanocapsulation method is based on the drug solubility. It is based on interfacial deposition of a polymer after displacing a water-miscible semipolar solvent from a lipophilic solution containing CB13 and PLGA. This method provides high encapsulation efficiency for drugs presenting low water solubility.

Table 1 illustrates the size characteristics of the nanoparticles obtained. The three formulations: PLGA; PLGA + PEG; and PLGA–PEG nanoparticles containing CB13 presented monodisperse profiles and narrow size distributions.

PLGA + PEG nanoparticles presented the largest size, which may be attributed to the adsorption of the PEG chains onto the PLGA surface in the form of one or more layers. This PEG adsorption could, perhaps, be attributed to van der Waals forces, hydrogen bonding, apolar interactions, etc., and not by an electrostatic interaction. The other two formulations present a smaller diameter (≈200 nm).

ζ value was found to be lowest (−31.4 ± 5.9 mV) in the case of plain PLGA nanoparticles due to their carboxyl end groups. PLGA + PEG nanoparticles have shown the highest ζ values (≈+ 0.5 mV), perhaps due to the displacement of the diffusible ionic layer onto the particle surface to greater distances. The change in ζ values is a simple means for estimating the extent of surface shielding provided by PEG. These almost neutral ζ values suggested the highest PEG chain density on particle surface.

Finally, the geometry and surface of the particles and the quality of the PLGA suspensions did not vary significantly when they were loaded with CB13. No presence of aggregates or bulky sediments was observed in any of the formulations. Figure 1, A-D) show SEM images of different PLGA nanoparticles assayed. As it can be seen, particles kept their size and spherical morphology. Nevertheless, we observed some capillary bridging between PLGA + PEG individual particles. It is probably due to an incomplete washing process before collecting nanoparticles by ultracentrifugation (PEG bridges).

Encapsulation efficiency (EE %) and drug loading (DL %) were determined by directly dissolving nanoparticles in acetonitrile (Table 1). We found values of around 80% for EE and 13% for DL, respectively. This high drug incorporation efficiency may be attributed to the organically soluble nature of CB13 that prevents partitioning into the aqueous phase, thus increasing drug entrapment in nanoparticles during polymer deposition. The high DL (%) values that were found enabled the same amount of drug to be delivered with less polymer. The method used for nanocapsulation resulted in a significant enclosure of CB13 and the process was found to be highly reproducible.

As the values between batches were not significantly different (P > 0.05), PEG coating did not influence the encapsulation, probably due to the process being performed in an aqueous solution where the drug is not soluble. Similar results were
obtained for PLGA–PEG. Indeed, the hydrophilic character of PEG kept it oriented toward the aqueous phase, while the hydrophobic core of PLGA can entrap the hydrophobic drugs.

Since EE% of three formulations was similar, we investigated a possible interaction between drug to PLGA or PEG or both. For this purpose a spectroscopy analysis using Fourier transform infrared (FT-IR) was carried out (see Supplementary Materials). Results revealed no significant differences in the frequencies of the bands in the three nanoparticle formulations compared to the single substances (CB13 and the components of the polymeric matrix) that could indicate the presence of any kind of interaction such as hydrogen bond, for example. In addition, CB13 cannot act as hydrogen donor in order to form hydrogen bond with PLGA or PEG.

The in vitro release of CB13 from nanoparticles was evaluated. CB13 release profiles were obtained by graphing the cumulative percentage of the drug released with respect to the amount of CB13 encapsulated as a function of time. The experiment was performed over 15 days.

Figure 2 collects the in vitro CB13 release profiles at pH 2.0 (Figure 2, A) and at pH 7.4 (Figure 2, B) from: PLGA, PLGA + PEG and PLGA–PEG nanoparticles. Independently of the release medium pH, a similar release profile can be observed. Remarkably, at pH 2.0 the drug release kinetics can be considered slower. Drug release was markedly inhibited in acidic environments, e.g., < 10% drug release after 2 h. The lowest release profile was obtained for plain PLGA nanoparticles. The presence of PEG chains determined a faster drug release. For PLGA + PEG and PLGA–PEG ≈ 6% drug release was obtained after 30 min.

At pH 7.4 CB13 release from both PLGA–PEG and PLGA + PEG nanoparticles was faster than from PLGA nanoparticles. A biphasic release pattern of CB13 was observed from PLGA–PEG and PLGA + PEG nanoparticles, where the initial 24 h period released 43% and 23% of drug followed by a sustained release to a total of 50% and 82%, respectively, over 15 days of assay.

CB13 release from PLGA nanoparticles was the slowest. In this case drug release was progressive, to a total of 58% after 15 days. It is possible that CB13 strongly interacts with the PLGA matrix, thus retarding the release capability, and that the PEG can increase the wettability of the polymeric surface and matrix, contributing to the increase in drug release.

These differences may be attributed to the hydrophilic character of the PEGylated nanoparticles thanks to the existence of the PEG chains on the nanoparticle surface which accelerated the degradation (hydrolysis) rate by improving buffer penetration into the nanoparticle matrix.10.25-28 Thus, surface modification of PLGA nanoparticles with PEG could be advantageously used in order to modulate drug release (nanoparticle erosion) kinetics.

In vivo studies of CB13 loaded PLGA nanocarriers in an animal neuropathic pain model

Results for in vivo assays are collected in Figures 3-6. In all cases asterisks indicate a significant difference compared to saline (*P < 0.05 vs. free CB13 control group, **P < 0.05 vs. PLGA + PEG control group, ***P < 0.05 vs. free CB13 (3.4 mg/kg)). (0 h: pre-drug administration) (see Supplementary Materials).

Experiment 1: CB13-loaded PLGA nanoparticles

The effect of three doses of CB13-loaded PLGA nanoparticles (1.7, 3.4 and 6.8 mg/kg CB13) was explored in the paw pressure test in the ipsi- and contralateral hindpaw. This effect was compared with those from free CB13 (3.4 mg/kg) (Figure 3).
Experiment 2: CB13-loaded PLGA + PEG nanoparticles

The effect of three different doses of CB13-loaded PLGA + PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg CB13) was explored in the paw pressure test in the ipsi- and contralateral hind paw over time (Figure 3, C and D). This effect was compared with those from free CB13 (3.4 mg/kg) (Figure 4). We first assessed the effect in the ipsilateral hind paw. Free CB13 showed a similar effect to that previously described (Figure 4, A). PLGA + PEG nanoparticles at 3.4 mg/kg CB13 significantly increased the pain threshold 3 h after administration (Figure 4, B). The significant analgesic effect was maintained for 5 days. At 6.8 mg/kg CB13 PLGA + PEG nanoparticles showed a significant analgesic effect from 9 h and continued for 5 days. The analgesic profile effect of PLGA + PEG nanoparticles at 3.4 and 6.8 mg/kg CB13 was very similar. The peak effect was found between 9 h and 3 days. No significant analgesic effect was found for the PLGA + PEG nanoparticles at a dose of 1.7 mg/kg CB13 (Figure 4, B).

Free CB13 showed an effect similar to that previously described. Its effect was superior to all doses of PLGA + PEG nanoparticles 0.5 and 3 h after administration. Free CB13 also showed a higher analgesic effect than 1.7 mg/kg of PLGA + PEG nanoparticles after 9 h (Figure 4, C). PLGA + PEG nanoparticles (6.8 mg/kg CB13) showed a significant anti-inflammatory effect compared with free CB13 after 9 h. As before, PLGA + PEG nanoparticles (3.4 and 6.8 mg/kg CB13) were more effective at reducing the pain threshold than free CB13 24 h, and 3 and 5 days after administration (Figure 4, C).

This indicates that CB13-loaded PLGA + PEG nanoparticles have a mechanical antihyperalgesic effect that lasts for up to 5 days.

No effect with regard to the contralateral hind paw was found in any group (Figure 4, D-F).

Experiment 3: CB13-loaded PLGA–PEG nanoparticles

The effect of three doses of CB13-loaded PLGA–PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg CB13) was explored in the paw pressure test in the ipsi- and contralateral hind paw over time. This effect was compared with those from free CB13 (3.4 mg/kg) (Figure 5). Free CB13 showed a similar effect to that previously found in the ipsilateral hind paw (Figure 5, A). PLGA–PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg CB13) showed a dose-dependent analgesic effect over time when exploring the contralateral hind paw (Figure 5, B). PLGA–PEG nanoparticles (1.7 mg/kg CB13) showed a significant analgesic effect 9 and 24 h as well as at days 5 and 9 after administration. 3.4 mg/kg CB13 PLGA–PEG nanoparticles showed a significant effect from 3 h until 9 days. The highest PLGA–PEG nanoparticles dose (6.8 mg/kg CB13) showed an analgesic effect from 0.5 up to 11 days. The greatest effects were found between 24 h and 3 days (Figure 5, B).

Free CB13 showed an effect similar to that previously found. It displayed a superior analgesic effect to PLGA–PEG nanoparticles at the beginning (0.5 h for all the doses of PLGA–PEG nanoparticles and after 3 h for PLGA–PEG nanoparticles 1.7 and 3.4 mg/kg CB13) (Figure 5, C). Free CB13 also had a significant effect versus PLGA–PEG nanoparticles (1.7 mg/kg CB13) after 9 h. After this point, the profile was the contrary. PLGA–PEG nanoparticles (1.7 mg/kg CB13) had a significant effect compared with free CB13 after 3 and 5 days. Similarly, the
highest doses of PLGA–PEG nanoparticles (3.4 and 6.8 mg/kg CB13) showed a superior effect 24 h–7 days after administration. The 6.8 mg/kg dose of CB13 PLGA–PEG nanoparticles was significantly more effective than free CB13 9–11 days after oral administration (Figure 5, C).

When exploring the effect of these CB13-nanosystems in the contralateral hind paw, we found a significant effect of PLGA–PEG nanoparticles (3.4 mg/kg nanoparticles) 24 h after administration versus control group and versus free CB13 (Figure 5, D and F). Similarly, the highest dose of PLGA–PEG nanoparticles (6.8 mg/kg CB13) had a significant analgesic effect from 3 h to 5 days versus control group (Figure 5, D). Furthermore, 6.8 mg/kg of CB13 PLGA–PEG nanoparticles displayed a significant effect versus free CB13 24 h and 3–7 days after administration (Figure 5, F).

Furthermore, we explored the effect of PLGA–PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg CB13) in the acetone test (ipsilateral hind paw) 2 days after oral administration (Figure 6). As before, this effect was compared with that from free nanoparticles (3.4 mg/kg).

The highest doses of PLGA–PEG nanoparticles (3.4 and 6.8 mg/kg CB13) showed a clear reduction in the acetone score versus control group and versus free CB13. Compared with its corresponding control group, no effect was found in the dose of free CB13 (3.4 mg/kg) explored.

Overall, CB13-loaded PLGA–PEG nanoparticles show the longest mechanical antihyperalgesic efficacy of the three preparations of nanoparticles evaluated. That is, PLGA–PEG nanoparticles have a consistent analgesic effect for up to 11 days. Furthermore, PLGA–PEG nanoparticles are also effective in thermal hypersensitivity in the neuropathic animal model studied. Finally, the highest dose of PLGA–PEG nanoparticles evaluated (6.8 mg/kg CB13) seems to increase the pain threshold in the non-injured (contralateral) paw.

**Discussion**

At this point, it can be concluded that neuropathic pain relief with CB13 can be clearly modulated using PLGA nanoformulations. It is quite noticeable that both free CB13 and the three nanosystems assayed showed the same analgesic potency while therapeutic response exists. The main difference lies in the fact that CB13-loaded nanoparticles with an optimal design maintain the analgesic effect for up to 11 days, while free CB13 exerts pain control for 9 h. It could, therefore, be expected that the longest analgesic effect (up to 11 days) of CB13–PLGA–PEG could make this formulation a good candidate for chronic pain management.

In the present work, we have used PEG for nanoparticle surface modification. Versus the free drug, the huge difference observed for CB13–PLGA–PEG on the behavioral and pharmacological effect of CB13 suggests an increased oral bioavailability. The enhanced access to the intestinal lymphatics after oral administration, as well as the capacity of PLGA matrices to control drug delivery, may explain this extremely long therapeutic response.
It is known that CB13 physicochemical profile and lipoprotein affinity (80% of CB13 plasma protein binding is due to association with plasma lipoproteins) promote lymphatic transport. Accessing lymphatics, first-pass CB13 metabolism is circumvented and its oral bioavailability substantially enhanced.7

A variety of nanoparticle delivery systems targeting intestinal lymphatic include various fatty acid mono-, di- and triglycerides in their composition in order to promote association with lymph lipoproteins. Recently, Attili-Qasr et al39 reported a significantly enhanced effect of orally-administered docetaxel when this latter is contained within nanocapsules containing a mixture of glyceryl trinitrate, oleoyl polyoxyglycerides and PLGA. In this case, the authors hypothesized that nanocapsules were transported into the intestinal lymphatics after receiving a surface coating of apoproteins and phospholipids during their passage across the enterocytes (in effect, the nanocapsules became "lipoproteinated").

The PLGA nanoparticles assayed in the present work did not include any kind of fatty acid in their composition. We suggest that, due to its affinity to lipoproteins, the CB13 being released from nanoparticles from the start can itself "lipoproteinate" the nanosystems. It is also important to notice that PLGA nanoparticles assayed contain a significant amount of Span®60 (33% w/w related to PLGA). The presence of this non-ionic lipophilic surfactant can also provide a suitable micro-environment for improving oral bioavailability by reducing interfacial surface tension, enhancing the penetration of hydrophobic drugs and promoting intestinal lymphatic transport after oral administration.35-32

Nonetheless, other factors concerning nanoparticles must also be taken into account. The particles' size and surface properties suggest that a large proportion of an absorbed dose might be expected to drain into intestinal lymphatic capillaries.33 Recent literature33-35 points out the main factors for the passive lymphatic targeting of nanoparticles: biocompatible and biodegradable components, carrier and drug stability, zeta potential and hydrophobicity. For targeting lymphatic vessels, size and hydrophobicity seem to be the most important nanocarrier design criteria.

In the case of anionic plain CB13-PLGA, it is expected that nanoparticles will be partially degraded by gastric pH11 and that they will encounter high electrostatic repulsive forces from the negatively-charged intestinal mucus, leading to faster clearance.36-38 A sufficient amount of nanoparticles was, however, viable as the therapeutic response increased from 9 h to 3 days, as compared to free CB13.

PLGA + PEG neutral nanoparticles are expected to show a high PEG density on the nanoparticles' surface, protecting them from gastric pH and enzymes. When compared to plain nanoparticles, the presence of a dense PEG coating was translated into a threefold increase in diameter. This increase would, however, be less evident through the GI tract due to a partial loss of PEG chains. A higher mucus penetration of these nanoparticles
Figure 5. Effects in the paw pressure test in the CCI model in rat of CB13–PLGA–PEG nanoparticles and free CB13 (p.o.). (A) Free CB13 (3.4 mg/kg) vs. control group; (B) CB13–PLGA–PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg) vs. control group; and (C) free CB13 (3.4 mg/kg) compared with CB13–PLGA–PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg) in the ipsilateral hind paw (n = 8–10). (D) Free CB13 (3.4 mg/kg) vs. control group; (E) CB13–PLGA–PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg) vs. control group and; (F) free CB13 (3.4 mg/kg) compared with CB13–PLGA–PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg) in the contralateral hind paw (n = 8–10).

Figure 6. Effect of CB13–PLGA–PEG nanoparticles (1.7, 3.4 and 6.8 mg/kg, p.o.) and free CB13 (3.4 mg/kg, p.o.) in the ipsilateral hind paw in the acetic test in the CCI model in rat. Results are expressed as the mean ± S.D. (n = 7–9).

is expected, followed by a smoother passage through enterocytes and finally a facilitated access to intestinal lymphatics, which would promote CB13 activity for 5 days.

Curiously, CB13–PLGA–PEG (1.7 mg/kg) nanoparticles did not show a significant analgesic effect at any time. This could be due to a combination of circumstances: (i) a small CB13 amount released from nanoparticles, not enough to achieve similar blood levels to free CB13; (ii) bigger particle size than plain or PLGA–PEG nanoparticles (600 nm versus 200 nm); (iii) near-neutral pI-potential values; (iv) a possible PEG loss preventing nanoparticles diffusion across mucus and then; (v) a higher or faster nanoparticle clearance.

Finally, moderate anionic PLGA–PEG nanoparticles showed an analgesic effect that lasted for as long as 11 days.

Difference in activity of PLGA + PEG and PLGA–PEG nanoparticles can be explained by the PEGylation procedure. For PLGA + PEG nanoparticles, PEG chains are expected to be non-strongly anchored on the surface of nanoparticles. This way, it is more than likely that PEG chains go away from nanoparticles surface in the GI tract. Once in the blood stream, a progressive PEG loss can be followed. This can be translated into a higher opsonization process and a fast clearance from the body. In short, PLGA–PEG nanoparticles with stable PEG chains anchored onto the surface of the nanoparticles presented a half-life higher than PLGA + PEG nanoparticles.

As explained in Trevaskis et al,32 nanoparticle size and surface properties suggest that a large proportion of the absorbed dose might be expected to drain into intestinal lymphatic capillaries. Trevaskis et al33 state that: "In general, few studies report nanoparticle bioavailability using detailed pharmacokinetic analyses of exposure after oral administration and the extent of lymphatic transport of nanoparticles has rarely been quantified directly. Whether sufficient quantities of nanoparticles
are absorbed to deliver a typical therapeutic load is therefore less clear. Moreover, Lammas et al39 suggest that, beyond targeting and beyond numbers, nanomedicine works and what counts is patient benefit.

In this sense, we have focused our investigation on the therapeutic effect of PLGA nanosystems in pain relief management using an animal model. Results clearly showed an ultra-long analgesic effect by means of using oral CB13–PLGA–PEG nanoparticles.

Conclusions

We have developed biodegradable nanosystems with different surface properties varying PEG surface density and anchoring. The three examples of PLGA nanosystems presented here performed the following tasks: sustained and prolonged neuropathic pain relief after just one oral dose of PLGA nanoparticles containing CB13. CB13–PLGA–PEG nanoparticles presenting small size, near-neutral ζ-potential values and stable PEG coating exert an analgesic effect for 11 days after administering just one oral dose. This noticeable difference in therapeutic effect duration suggests an enhanced passive lymphatic targeting followed by systemic drug delivery. Therapeutic effect was clearly dose-dependent after orally administering nanoparticles. Furthermore, CB13–PLGA–PEG nanoparticles presented a delayed analgesic effect of up to 0.5 h, 3 h or 9 h as a function of CB13 doses (6.8, 3.4 or 1.7 mg/kg, respectively).

The overall results may advocate the feasibility of reducing the dose and suggest that CB13–PLGA–PEG nanoparticles may be an exciting new therapeutic option for the treatment of neuropathic pain.

Moreover, these results can open up a new perspective for the future of therapeutic cannabinoid use, not only with regard to its use in pain management, but also with regard to its wide therapeutic spectra.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2017.07.010.

References


