Inhibition of human tumour prostate PC-3 cell growth by cannabinoids R(+)-Methanandamide and JWH-015: Involvement of CB2

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BACKGROUND: We have previously shown that cannabinoids induce growth inhibition and apoptosis in prostate cancer PC-3 cells, which express high levels of cannabinoid receptor types 1 and 2 (CB1 and CB2). In this study, we investigated the role of CB2 receptor in the anti-proliferative action of cannabinoids and the signal transduction triggered by receptor ligation.

METHODS: The human prostate cancer cell lines, namely PC-3, DU-145 and LNCaP, were used for this study. Cell proliferation was measured using MTT proliferation assay, [3H]-thymidine incorporation assay and cell-cycle study by flow cytometry. Ceramide quantification was performed using the DAG kinase method. The CB2 receptor was silenced with specific small interfering RNA, and was blocked pharmacologically with SR-144528. In vivo studies were conducted by the induction of prostate xenograft tumours in nude mice.

RESULTS: We found that the anandamide analogue, R(+)-Methanandamide (MET), as well as JWH-015, a synthetic CB2 agonist, exerted anti-proliferative effects in PC-3 cells. R(+)-Methanandamide- and JWH-015-induced cell death was rescued by treatment with the CB2 receptor antagonist, SR-144528. Downregulation of CB2 expression reversed the effects of JWH-015, confirming the involvement of CB2 in the pro-apoptotic effect of cannabinoids. Further analysing the mechanism of JWH-015-induced cell growth inhibition, we found that JWH-015 triggered a de novo synthesis of ceramide, which was involved in cannabinoid-induced cell death, insofar as blocking ceramide synthesis with Fumonisin B1 reduced cell death. Signalling pathways activated by JWH-015 included JNK (c-Jun N-terminal kinase) activation and Akt inhibition. In vivo treatment with JWH-015 caused a significant reduction in tumour growth in mice.

CONCLUSIONS: This study defines the involvement of CB2-mediated signalling in the in vivo and in vitro growth inhibition of prostate cancer cells and suggests that CB2 agonists have potential therapeutic interest and deserve to be explored in the management of prostate cancer.

Keywords: cannabinoids; CB2 receptor; ceramide; PC-3 cells; prostate cancer

Prostate cancer has become the most common cancer diagnosed in men and is one of the major life-threatening diseases in Western countries (Ukraitseva et al, 2008). Despite recent advances in its diagnosis and treatment, current therapies are unable to completely eliminate the androgen-independent prostate cancer cells that remain after androgen ablation therapy (Bahnson, 2007). Thus, understanding the mechanisms involved in the control of tumour growth and the development of chemopreventive agents are major goals of basic research in oncology.

Cannabinoids, the active components of Cannabis sativa and their derivatives, exert a wide spectrum of modulatory actions and pharmacological activities in the brain as well as in the periphery, and therefore, the therapeutic potential of cannabinoids has gained much attention during the past few years (Kogan and Mechoulam, 2007). One of the most exciting areas of current research in the therapeutic potential of cannabinoids is cancer. Recent evidence suggests that cannabinoids are powerful regulators of cell growth and differentiation. They have been shown to exert anti-tumoural effects by decreasing viability, proliferation, adhesion and migration on various cancer cells, thereby suggesting the potential use of cannabinoids in the treatment of gliomas, prostate and breast cancers and malignancies of immune origin (Bifulco et al, 2006; Flygare and Sander, 2008; Sarfaraz et al, 2007) and by an activation of the reticulum stress pathway (Carracedo et al, 2006b; Salazar et al, 2009a). Moreover, synthetic as well as naturally
occurring cannabinoids inhibit the growth of endocrine-related cancer cells, such as thyroid, breast and prostate cancer cells (revised in Lopez-Rodriguez et al. (2005) and Bifulco et al. (2008)). The antitumour effect of cannabinoids has been demonstrated both in vitro and in vivo, affecting multiple signalling pathways and biological processes that have been implicated in the development of the malignant phenotype (Bifulco et al. 2008). Cannabinoids may target tumour cells by binding to cannabinoid receptors or by a receptor-independent mechanism. Two cannabinoid receptors have been identified by molecular cloning to date: the CB1 receptor, which is highly expressed in the brain and also present in peripheral tissues, and the CB2 receptor, previously believed to be expressed primarily in immune and haematopoietic cells, although more recent studies have also identified CB2 receptors in the brain and in endothelial cells of various origins (Pertwee, 2006; Mackie, 2008). The CB2 receptor is unrelated to cannabinoid psychoactivity, and therefore targeting this receptor is one of the major challenges in cannabinoid therapeutic research (Ashton et al., 2008). After an interaction with cannabinoid receptors, cannabinoids trigger several signalling pathways depending on cell type, which may be involved in their anti-proliferative effects such as p38 MAPK and c-Jun N-terminal kinase (JNK) activation, increased synthesis of the pro-apoptotic sphingolipid ceramide and several downstream stress-related genes expressed in the endoplasmic reticulum (Diaz-Laviada and Ruiz-Llorente, 2005; Demuth and Molleman, 2006). Ceramide, the central molecule of sphingolipid metabolism, generally mediates anti-proliferative responses, such as inhibition of cell growth, induction of apoptosis and/or modulation of senescence (Saddoughi et al., 2008). Several therapeutic agents that induce ceramide-dependent apoptosis in cancerous cells currently exist, and a number of enzymes involved in ceramide metabolism are beginning to be recognised as potential targets for cancer therapy (Savtchouk et al., 2005; Carpinteiro et al., 2008). Recent research has shown that cannabinoids induce ceramide accumulation in cancer cells, which is related to the pro-apoptotic effect of cannabinoids in cancer cells (Guzman et al., 2001; Velasco et al., 2005).

Prostate cancer cells express both CB1 and CB2 receptors (Ruiz-Llorente et al., 2003; Sanchez et al., 2003), the expression of which is significantly higher than that in normal prostate epithelial cells (Sarfaraz et al., 2005). The impact of cannabinoids on prostate cancer cell viability is variable and depends on the dose used. Low doses below the micromolar range induce androgen receptor-dependent apoptosis (Ruiz et al., 1999; Mimeault et al., 2003) or cell-cycle arrest (Sarfaraz et al., 2006). The involvement of cannabinoid receptors in such effects is uncertain as CB1 antagonists blocked cytotoxic effects at short incubation times (Mimeault et al., 2003; Sarfaraz et al., 2005), although there was a lack of effect at a longer duration (Ruiz et al., 1999; Mimeault et al., 2003).

In this study, we analysed the role of CB2 receptor in the androgen-resistant prostate cell line, PC-3, which represents the androgen-refractory phase of advanced prostate cancer. We used the anandamide analogue, R( + )-Methanandamide (MET), for comparison with previous results, and a potent and selective CB2 receptor agonist, JWH-015 (JWH), as well as CB2 antagonists and RNA silencing to show the role of CB2 in PC-3 cells.

MATERIALS AND METHODS

Reagents
R( + )-Methanandamide, JWH-015 [(2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone] and Fumonisin B1 were purchased from Sigma (St Louis, MO, USA). SR 144528 (SR2) was kindly provided by Sanofi Recherche (Montpellier, France). Antibody anti-CB2 receptor was obtained from Affinity BioReagents (Golden, CO, USA). We purchased anti-tubulin, anti-phospho Akt at Ser473, anti-phospho p38, anti-total p38, anti-phospho JNK, anti-total JNK, anti-phosphor eIF2x, anti-caspase 8 and anti-caspase 9 and anti-citochrome c antibodies from Cell Signaling Technology (St Louis, MO, USA). The inhibitor D609 and the enzyme diacylglycerol kinase were supplied by Calbiochem (La Jolla, CA, USA).

Cell cultures
Human prostate epithelial PC-3, DU-145 and LNCaP cells were purchased from American Type Culture Collection (Rockville, MD, USA) and were cultivated in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U ml⁻¹ penicillin G, 100 µg ml⁻¹ streptomycin and 0.25 µg ml⁻¹ amphotericin B (Invitrogen, Paisley, UK). Low cell passages (between 10 and 20) were used for this study. Cells were seeded sub-confluently and, 1 day before the experiment, the serum was removed to work with quiescent cells.

Cell viability assays
Cells were set up 2 × 10⁴ cells per well of a 24-well plate and were cultured in the RPMI 1640 medium supplemented with 10% FCS. After treatments according to figure legends, cell viability was assayed by MTT as previously described (Sanchez et al., 2003).

Flow cytometry
Flow cytometry was used to detect apoptotic cells and the distribution of cell cycle. After being cultivated with medium alone or medium containing the indicated stimuli, 10⁵ cells in a 35-mm culture dish were harvested in 0.1% Nonidet P-40 and re-suspended in the binding buffer with 0.6 µg ml⁻¹ Annexin V-FITC for 15 min. The cells were then washed in PBS and re-suspended in the binding buffer with 0.6 µg ml⁻¹ IP (Calbiochem). In all, 20,000 cells of each sample were analysed by flow cytometry in a FACScan (Beckton Dickinson).

Measurement of [³H]-thymidine incorporation into DNA
Cells were treated with different concentrations of MET or JWH-015 according to the experiment. DNA synthesis was determined by pulsing the cells with [³H]-thymidine (1 µCi per well) during the last 16h of the culture period as previously described (Sanchez et al., 2003).

siRNA transfections
Cells were transfected in 1 ml OPTIMEN containing 4 µg lipofecta
mine 2000 (Invitrogen Co., Carlsbad, CA, USA), with 100 nM human cannabinoid receptor-2-specific small interfering RNA (siRNA) duplexes (5'-GGCCUCUUCCCAAUUAAAtt-3', Applied Biosystems, Foster City, CA, USA) or control scrambled RNA for 12 h according to the manufacturer’s protocols (Ambion, Applied Biosystems). At 24 h after transfection, the medium was removed and replaced with RPMI 1640 with 10% FCS medium. At dedicated time points after transfection, the cells were used for flow cytometry assays or western blot.
Inhibition of prostate cell growth by cannabinoids through CB2

N Olea-Herrero et al.

Measurement of ceramide levels

Ceramide quantification in cell lipid extracts was performed according to the method described by Perry et al for ceramide quantification in cultured cells (Perry et al, 2000). Briefly, cell pellets were suspended in 0.6 ml distilled water, and disrupted at 4 °C by sonication. Lipids were extracted with chloroform/methanol, and ceramide content was determined by phosphorylation using Escherichia coli diacylglycerol kinase and [32P]ATP (6000 Ci mmol-1; Perkin-Elmer, Barcelona, Spain). Products of this reaction were purified by TLC using chloroform–aceton–methanol–acetic acid–water (50 : 20 : 15 : 10 : 5, by volume) as the developing solvent. Products of this reaction were quantified and expressed as a percentage of the value observed earlier (Sanchez et al, 2007).

Western blot analysis

Cultured cells were lysed into a lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol) containing 5 µg ml-1 leupeptin, 5 µg ml-1 aprotinin and 1 mM phenylmethylsulfonyl fluoride, and were disrupted by sonication. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Western blotting was carried out as previously described (Sanchez et al, 2006).

In vivo anti-tumour activity

All animal studies were conducted in accordance with the Spanish institutional regulation for the housing, care and use of experimental animals, have been carried out with ethical committee approval and met the European Community directives regulating animal research. Recommendations made by the UKCCCR have been adhered to carefully. Athymic nude (nu/nu) 6-week-old male mice were purchased from Harlan Iberica (Barcelona, Spain) and were housed in a laminar airflow cabinet under pathogen-free conditions on a 12-h light–dark schedule. Mice were injected subcutaneously (s.c.) in the right flank with 2 ml saline (controls) or cannabinoids at the doses indicated. Lipids were extracted with chloroform/methanol, and ceramide content was determined by phosphorylation using diacylglycerol kinase and [32P]ATP (6000 Ci mmol-1; Perkin-Elmer, Barcelona, Spain). Products of this reaction were purified by TLC using chloroform–aceton–methanol–acetic acid–water (50 : 20 : 15 : 10 : 5, by volume) as the developing solvent. Products of this reaction were quantified and expressed as a percentage of the value observed earlier (Sanchez et al, 2007).

RESULTS

The cannabinoids, MET and JWH-015, inhibited cell growth of prostate cancer cells

We first examined the anti-proliferative effects of the stable anandamidine analogue, MET, and the synthetic CB2 ligand, JWH-015, on prostate PC-3 cells. The kinetics of MET and JWH-015 treatment showed that cannabinoid-induced cell death was evident from 12 h, although maximal effect was reached at 48–72 h (Figure 1A). Thus, we decided to follow all the studies at 48 h.

Cells were incubated in the presence of increasing concentrations of MET or JWH-015 for 48 h, after which cell viability was evaluated by MTT assay, [3H]-thymidine incorporation assay or by flow cytometry. As shown in Figure 1B, both MET and JWH-015 caused a dose-dependent decrease in cell viability, which was significantly different from control (doses over 5 µM). To assess the suppressive effects of R (+)-Methanandamide and JWH-015 on the proliferation of PC-3 cells, DNA synthesis was measured by [3H]-thymidine incorporation. Results shown in Figure 1C indicate that both cannabinoids inhibited the proliferation of PC-3 cells, which was totally blocked from doses over 5 µM. The cell-cycle analysis demonstrated that cannabinoid treatment resulted in a small, although significant, accumulation of cells in the sub-G1 phase of the cell cycle (Figure 1D). These results suggest that the compounds used induced a small percentage of apoptosis and growth arrest in prostate cells. To investigate whether the anti-proliferative effect of cannabinoids on prostate cancer cells was generalised, we used the androgen-refractory prostate cancer DU-145 cells and the less tumourigenic androgen-dependent prostate LNCaP cells. Results shown in Figure 2 showed that both MET and JWH-015 inhibited the growth of the three cancer prostate lines studied, although the effect was less pronounced in the androgen-sensitive LNCaP cells. As shown in Figure 2A, low doses (sub-micromolar) of MET induced a slight increase in LNCaP cell viability, as previously reported by our group (Sanchez et al, 2003).

To quantify the percentage of apoptotic cells after drug treatments, PC-3 cells were stained with Annexin V-FITC/IP. Results show that the rate of late apoptotic cells (Annexin V-FITC positive/IP positive, upper right quadrant) in MET- and JWH-015-treated cells was statistically increased compared with that in control cells (Figure 3). R (+)-Methanandamide and JWH-15 treatments also induced cell necrosis, as inferred from IP-positive cells (upper left quadrant). Early apoptotic cells (Annexin V-FITC positive/IP negative, lower right quadrant) were <5% in all cases. These findings indicate that both MET and JWH-015 promoted a low, although significant, percentage of apoptosis in prostate cancer cells, but other processes such as mitotic catastrophe, cytotoxicity or necrosis could also collaborate in the observed growth inhibition.

Involvement of CB2 in the anti-proliferative effect of cannabinoids

As we have previously shown, prostate PC-3 cells express both CB1 and CB2 cannabinoid receptors (Sanchez et al, 2003). We then investigated the role of CB1 and CB2 in cannabinoid-induced prostate cell death. Pharmacological blockade of CB1 with its antagonist Rimonabant (SR1) did not reduce the effect of MET on cell cycle or apoptosis (Figure 4A). However, the CB2 antagonist, SR 144528 (SR2), reduced the number of apoptotic cells and the number of sub-G1 cells induced by MET treatment (Figure 4A). As MET is a weak ligand for CB2, we confirmed this result with the CB2-selective agonist JWH-015. The JWH-015-induced cell death effect was reverted by SR2, suggesting a role for CB2 in the apoptotic mechanisms of cannabinoids in PC-3 cells.

To confirm the involvement of CB2, we silenced its expression with siRNA. PC-3 cells were transfected with CB2-selective siRNA or control scrambled RNA for 48 h, after which the expression of CB2 was notably reduced as it was corroborated by western blotting (Figure 5). Under these conditions, apoptosis induced by 10 µM JWH-015 was almost totally blocked in cells transfected with CB2 siRNA when compared with scrambled siRNA-transfected cells (Figure 5). These results confirm the involvement of CB2 receptor in the pro-apoptotic effect of cannabinoids in prostate cells. Therefore, we conducted the rest of the experiments with JWH-015, which is a potent and selective ligand for CB2, and which exhibits more efficacy than MET for CB2 activation.

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Figure 1  The anti-proliferative effect of the cannabinoids, R( + )-Methanandamide and JWH-015, on prostate PC-3 cells. (A) Time course of cannabinoid effect on prostate PC-3 cells viability. PC-3 cells were incubated with 10 μM MET or 10 μM JWH-015 for different times and cell viability was assayed by MTT. (B) Cells were incubated in the presence of increasing concentrations of MET or JWH-015 for 48 h and cell viability was assayed by MTT. (C) Cells were incubated in the presence of increasing concentrations of MET or JWH-015 for 48 h and cell proliferation was measured by [ 3H]-thymidine incorporation. (D) Cells were incubated in the presence of increasing concentrations of MET or JWH-015 for 48 h and cell cycle was assayed by flow cytometry. Data are the means ± s.e. of three different experiments, each performed in triplicate. *P < 0.05 and **P < 0.01 using Student’s t-test for the comparison between vehicle-treated and cannabinoid-treated cells.
Ceramide synthesis mediates the anti-proliferative effect induced by CB2 activation

Ceramide is a sphingolipid messenger that has a relevant role in the regulation of tumour cell fate (Velasco et al, 2005; Carpinteiro et al, 2008). Recent studies have suggested that apoptosis induced by cannabinoids can be preceded by ceramide accumulation (Gomez del Pulgar et al, 2002; Gustafsson et al, 2006). To further analyse the apoptotic mechanism of JWH-015, we measured intracellular ceramide concentration in PC-3 cells. As shown in Figure 6A, incubation with JWH-015 for 48 h led to a dose-dependent increase in intracellular ceramide accumulation. This effect was prevented by the CB2 antagonist SR2, which indicates that ceramide accumulation was mediated by the activation of CB2 (Figure 6B). Ceramide is formed in cellular membranes by de novo synthesis through a pathway involving the ceramide synthase or by hydrolysis of sphingomyelin catalysed by acid, neutral and alkaline sphingomyelinas. To gain insight into the origin of JWH-015-induced ceramide increase, cells were incubated in the presence of the ceramide synthase inhibitor, Fumonisin B1, or the sphingomyelinase inhibitor, D609. As shown in Figure 6B, treatment of cells with 10 μM Fumonisin B1, but not with D609, prevented the increase in ceramide concentration, a fact that suggests that ceramide came from de novo biosynthesis.

Moreover, treatment with Fumonisin B1 prevented the inhibition of cell growth induced by JWH-015 (Figure 6C), indicating that ceramide biosynthesis was involved in the action of the cannabinoid.

Signal mechanisms involved in JWH-015-induced prostate PC-3 cell death

To further explore the signalling pathways in which the CB2 agonist exerted its effect in prostate PC-3 cells, we studied stress-related MAP kinase cascades activation by western blot. PC-3 cells were treated for different times with 10 μM JWH-015 and then

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**Figure 2** Anti-proliferative effect of cannabinoids on different prostate cancer cell lines. (A) Prostate cancer LNCaP, PC-3 or DU-145 cells were incubated with different doses of MET for 48 h and cell viability was assayed by MTT. (B) Prostate cancer LNCaP, PC-3 or DU-145 cells were incubated with different doses of JWH-015 for 48 h and cell viability was assayed by MTT. Data are means ± s.e. of two different experiments, each performed in triplicate. *P<0.05 and **P<0.01 using Student’s t-test for the comparison between vehicle-treated and cannabinoid-treated cells.

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**Figure 3** Evaluation of apoptosis by Annexin V-FITC/IP staining, followed by flow cytometry analysis. Representative plots of Annexin V-FITC/IP staining of PC-3 cells cultured in the presence of increasing concentrations of MET or JWH-015 are shown. Data showing the percentage of late apoptotic cells (upper right quadrant) are the mean ± s.e. of three different experiments, each performed in duplicate. *P<0.05 and **P<0.01 using Student’s t-test for the comparison between vehicle-treated and cannabinoid-treated cells.
**Figure 4**  Inhibition of cannabinoid-induced cell death by the CB2 antagonist, SR 144528 (SR2). PC-3 cells were incubated with 10 μM MET or 10 μM JWH-015 for 48 h in the presence or absence of 0.5 μM Rimonabant (SR1) or 2 μM SR2. Apoptosis was assayed by Annexin V-FITC/IP staining (panels A and C) and cell cycle was measured by IP staining (panels B and D). Representative plots are shown in the figure and data are the mean ± s.e. of three different experiments, each performed in duplicate. *P < 0.05 and **P < 0.01 using Student’s t-test for the comparison between vehicle-treated and cannabinoid-treated cells, and *P < 0.05 and **P < 0.01 for the comparison between cannabinoid-treated and antagonist-treated cells.
phosphorylated forms of JNK and p-38 kinases, indicative for activated kinases, were detected by western blot. Results in Figure 7A show that JWH-015 activates the stress-signal-related kinase JNK as phosphorylated JNK is increased at 30 min and 1 h of treatment.

Eukaryotic cells respond to stress in their endoplasmic reticulum by phosphorylating the α-subunit of translation initiation factor 2 (eIF2α). This adaptation inhibits general protein synthesis while promoting translation and expression transcriptional regulators that induce gene expression important for cellular remediation and apoptosis. It has recently been described that cannabinoids promote endoplasmic reticulum stress and autophagy-mediated cell death in glioma cells through the Akt/mammalian target of rapamycin (mTOR) pathway inhibition and eIF2α activation (Salazar et al., 2009b). To investigate whether JWH-015 regulated this pathway in prostate cells, we treated the cells with the CB2 agonist and measured the phosphorylation of Akt in Ser473, which is negatively involved in the autophagy pathway (Todde et al., 2009). PC-3 treatment with 10 μM JWH-015 resulted in a long-term AKT phosphorylation decrease that was evident from 6 h of treatment (Figure 7A). This was in concordance with the increase in phosphorylated eIF2α (Figure 7A), suggesting that endoplasmic reticulum stress signals were activated by JWH-015 in prostate cells. We further investigated whether JWH-015 induced caspase activation, which can be detected after the decrease in the pro-caspase form and the increase in the proteolytic active form by western blot (Hoffmann et al., 2009). Results in Figure 7 show that JWH-015 induced activation of caspase 9, which has been described as an initiator caspase essential in the intrinsic or mitochondrially gated pathway of apoptosis (Johnson and Jarvis, 2004; Denault and Salvesen, 2008). To further confirm the activation of the intrinsic apoptotic...
pathway, we determined the cytosolic levels of cytochrome c, which is released into the cytosol when the cell receives an apoptotic stimulus to trigger programmed death through caspase 9 activation (Ow et al., 2008).

JWH-015 caused regression of prostate cancer xenografts in nude mice

The above observations of the anti-proliferative effects of JWH-015 were examined in a xenograft model of prostate cancer. Xenograft human prostate tumours were established in nu/nu mice by a s.c. injection. Tumour-bearing animals (~70 mm2) were treated daily with vehicle, 1.5 mg ml−1 JWH-015 or 1.5 mg ml−1 JWH-015 plus 1.5 mg kg−1 SR2. Animals were treated for 15 days and tumour volume was calculated every day. At the end of the experiment, tumours were dissected and weighed. As shown in Figure 8, JWH-015-treated animals had a rapid and dramatic reduction in tumour growth, whereas uncontrolled growth was observed in the control group. The final tumour volume as well as the final tumour weight was significantly lower in the JWH-015-treated group compared with that in the control group (Table 1). Treatment with JWH-015 plus SR2 resulted in a similar growth compared with that in the control group, suggesting that the in vivo effect of JWH-015 is also mediated through CB2 activation (Figure 8 and Table 1).

DISCUSSION

Deregulation of cell survival pathways and resistance to apoptosis are widely accepted to be fundamental aspects of tumourigenesis, as evasion of apoptosis may contribute to carcinogenesis, tumour progression and also to treatment resistance. Most current anticancer therapies act by activating cell death pathways in cancer. A number of studies have recently shed light on the role of cannabinoid family members as anticancer agents, allowing the potential development of anti-neoplastic-efficient treatments.

In this study, we demonstrated that the stable anandamide analogue, MET, as well as JWH-015, a cannabinoid receptor type 2-selective agonist, inhibited the proliferation of human prostate cancer PC-3, DU-145 and LNCaP cells. The fact that the anti-proliferative effect of cannabinoids was less pronounced in LNCaP cancer PC-3, DU-145 and LNCaP cells. A number of studies have recently shed light on the role of cannabinoids in prostate cancer. A number of studies have recently shed light on the role of cannabinoids in prostate cancer.
cannabinoid receptor type 2 is linked to various responses related to the proliferation, differentiation and survival of many cell types (Fernandez-Ruiz et al., 2007; Svizenska et al., 2008). Our observations are consistent with previous reports showing that CB2 receptor stimulation is involved in cannabinoid anti-tumour activity in vitro and in mice inoculated with tumour xenografts in vivo (Caffarel et al., 2006; Carracedo et al., 2006a; Fernandez-Ruiz et al., 2007). As previously proposed, this receptor may function as a signal favouring a non-differentiated, proliferate state of cells (Fernandez-Ruiz et al., 2007). In keeping with this notion, increased levels of CB2 have been shown in prostate cancer cells compared with normal prostate (Sarfaraz et al., 2008), and a correlation between CB2 expression and the histological grade of breast tumours has also been observed (Caffarel et al., 2006). Activation of the CB2 receptor induces apoptosis and reduces tumour growth of glioma (Sanchez et al., 2001; Blazquez et al., 2008), pancreatic carcinoma (Carracedo et al., 2006a) or breast cancer (Caffarel et al., 2006). Moreover, recent research shows that the anti-tumour action of cannabinoid receptor agonists in colon cancer cells may be exerted through the CB2 receptor more efficiently that through the CB1 receptor (Gianchi et al., 2008), which is consistent with our results in prostate cells.

Our data show that CB2 receptor activation by JWH-015 in prostate PC-3 cells induces de novo synthesis of ceramide, which mediates the apoptotic effect of JWH-015 in that the addition of the ceramide synthase inhibitor Fumonisin B1 prevented the induction of apoptosis. Ceramide is a second messenger that has been shown to act as a pro-apoptotic lipid mediator of cannabinoid action (Guzman et al., 2001). It has been previously described that, although CB1 receptor activation induces acute ceramide increase through sphingomyelin hydrolysis (Sanchez et al., 1998), sustained ceramide accumulation through enhanced de novo synthesis seems to exert a major effect in CB2-induced apoptosis (Gomez del Pulgar et al., 2002; Herrera et al., 2006; Carracedo et al., 2006a; Gianchi et al., 2008), which is in good agreement with our findings.

Figure 7 Signalling mechanisms activated by JWH-015 in prostate PC-3 cells. Cells were incubated with 10 μM JWH-015 for different times. (A) Phosphorylation levels of p38, JNK, Akt and eIF2α were measured by western blot. (B) Levels of pro-caspase 8, pro-caspase 9 and cytochrome c in the cell cytosol were detected by western blot. Figure shows a representative image of the other three experiments. Tubulin levels are shown as loading control.

Table 1 Effect of JWH-015 on PC-3 xenograft tumour growth

| Initial tumour volume (mm³) | Final tumour volume (mm³) | % tumour growth | Tumour weight (μg) |
|-----------------------------|---------------------------|-----------------|-------------------|
| Control                     | 77.61 ± 25.82             |                 | 221.28 ± 28.08    |
| JWH-015                     | 77.36 ± 13.66             |                 | 148.85 ± 29.15    |
| JWH-015 + SR2               | 71.33 ± 14.00             |                 | 236.16 ± 47.00    |

Figure 8 In vivo anti-tumoural properties of JWH-015. Athymic nude mice were injected s.c. in the right flank with PC-3 cells and 4 weeks later (day 0) were treated for 15 days with vehicle (control), 1.5 mg kg⁻¹ JWH-015 or 1.5 mg kg⁻¹ JWH-015 plus 1.5 mg kg⁻¹ SR2. Treatments were carried out by injections in the peritumoral area every day. Tumour volumes were measured daily. (A) The dorsal side of representative mice and dissected tumours after treatment. (B) Tumour growth curve after administration of vehicle (diamonds), JWH-015 (squares) or JWH-015 + SR2 (triangles). Results represent the mean ± s.e. of eight mice in each group. *P < 0.01 vs control and #P < 0.01 vs JWH-015, compared by Student’s t-test.
agreement with our data. Many anticancer drugs also induce death by stimulating the generation of ceramide (Claria, 2006; Lin et al., 2006). Ceramide mediates the apoptosis of radiation therapy (Moeller et al., 2009), and attenuation of ceramide levels confers resistance to radiation in prostate cancer cells (Mahdy et al., 2009). Ceramide has been shown to activate a number of enzymes involved in stress signalling cascades, including stress-activated protein kinases such as the Jun kinases JNKs, which are involved in ceramide-mediated apoptosis induction (Ruvolo, 2003). Our data demonstrate that JWH-015 activates JNK in prostate cells, which is in agreement with previous results showing a CB2-dependent activation of JNK in microglial cells (Correa et al., 2009) and in lung epithelial cells (Sarafian et al., 2008). This study also demonstrated that JWH-015 inhibits the Akt–mTOR pathway and activates eIF2α, which are involved in autophagy regulation and endoplasmic reticulum stress response, suggesting that these pathways are involved in JWH-015-induced prostate cell death. The release of pro-apoptotic factors such as cytochrome c from the mitochondria leads to the formation of a multimeric complex known as apoposome and initiates caspase activation cascades. The fact that JWH-015 induced cytochrome c release into cytosol and activated caspase 9 in prostate PC-3 cells confirms the involvement of apoptosis and points to an activation of the intrinsic apoptotic pathway. An improved understanding of the downstream mechanisms of prostate cell death induced by cannabinoids in prostate cells would offer new opportunities for the development of a combination therapy in the treatment of prostate cancer. Overall, our data show a role for the cannabinoid receptor CB2 in the anti-tumour effect of cannabinoids on prostate cells in vitro and in vivo. There is considerable interest in the application of selective CB2 receptor agonists, which are devoid of typical marijuana-like psychoactive properties of CB1 agonists, for future cannabinoid-based anticancer therapies. Therefore, our findings point to the potential application of cannabinoid receptor type 2 ligands as anti-tumour agents in prostate cancer.

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