Phytomedicine 21 (2014) 631–639

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Inhibition of colon carcinogenesis by a standardized *Cannabis sativa* extract with high content of cannabidiol

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ARTICLE INFO

Article history:
Received 11 October 2013
Accepted 29 November 2013

Keywords:
Cannabinoid receptors
Cannabidiol
Δ^9-Tetrahydrocannabinol
Cancer cell growth
Chemoprevention
Colorectal cancer

ABSTRACT

Purpose: Colon cancer is a major public health problem. *Cannabi*s-based medicines are useful adjunctive treatments in cancer patients. Here, we have investigated the effect of a standardized *Cannabis sativa* extract with high content of cannabidiol (CBD), here named CBD BDS, *i.e.* CBD botanical drug substance, on colorectal cancer cell proliferation and in experimental models of colon cancer *in vivo*.
Methods: Proliferation was evaluated in colorectal carcinoma (DLD-1 and HCT116) as well as in healthy colonic cells using the MTT assay. CBD BDS binding was evaluated by its ability to displace [^{3}H]CP55940 from human cannabinoid CB_1 and CB_2 receptors. *In vivo*, the effect of CBD BDS was examined on the preneoplastic lesions (aberrant crypt foci), polyps and tumours induced by the carcinogenic agent azoxymethane (AOM) as well as in a xenograft model of colon cancer in mice.
Results: CBD BDS and CBD reduced cell proliferation in tumoral, but not in healthy, cells. The effect of CBD BDS was counteracted by selective CB_1 and CB_2 receptor antagonists. Pure CBD reduced cell proliferation in a CB_1-sensitive antagonist manner only. In binding assays, CBD BDS showed greater affinity than pure CBD for both CB_1 and CB_2 receptors, with pure CBD having very little affinity. *In vivo*, CBD BDS reduced AOM-induced preneoplastic lesions and polyps as well as tumour growth in the xenograft model of colon cancer.
Conclusions: CBD BDS attenuates colon carcinogenesis and inhibits colorectal cancer cell proliferation *via* CB_1 and CB_2 receptor activation. The results may have some clinical relevance for the use of *Cannabis*-based medicines in cancer patients.

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Introduction

Cancer is a prominent health problem in the world. One in 4 deaths in the United States is due to cancer (Siegel et al. 2013). Colorectal cancer represents the third most common cancer worldwide, both in men and women, with 142,820 new cases and 50,830 deaths estimated to occur in 2013 (Siegel et al. 2013). Pharmacoeconomic studies have highlighted a trend for rising costs associated with colorectal cancer, which is linked to the increasing use of targeted biological therapies (Kriza et al. 2013). Screening strategies are utilized but have not reduced disease incidence or mortality (Derry et al. 2013). Furthermore, therapeutic intervention, which is by itself very toxic, may fail to prevent disease progression to metastatic disease (Ebos and Kerbel 2011). Therefore, there is an interest in both cancer preventive strategies – which include experimentation with safe phytochemical agents – and new curative treatments (Franceschi and Wild 2013).

Cannabis extracts and plant-derived cannabinoids (named phytocannabinoids) have demonstrated direct anti-cancer effects and are also used in cancer patients to stimulate appetite and as antiemetics (Fowler et al. 2010; Carter et al. 2011; Pertwee 2012; Velasco et al. 2012; Massi et al. 2013). Recent progress in plant biotechnology has made possible the cultivation of *Cannabis* chemotypes rich in specific phytocannabinoids, from which standardized extracts, containing known amounts of phytocannabinoids, may be obtained (Russo 2011). The best studied among these extracts is generally referred as cannabidiol (CBD) botanical drug substance (CBD BDS, that is a standardized *Cannabis* extract with high content of CBD). In several pharmacological

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; THC, Δ^9-tetrahydrocannabinol; CBD, cannabidiol; CBD BDS, cannabidiol botanical drug substance; CHO, Chinese hamster ovarian; HCEC, healthy colonic epithelial cells.

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http://dx.doi.org/10.1016/j.phymed.2013.11.006

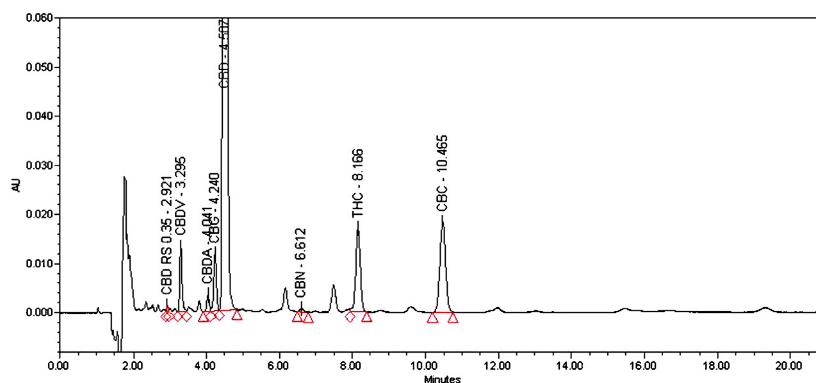

Fig. 1. HPLC chromatogram of *Cannabis sativa* CO_2 extract. Retention time for cannabidiol (CBD) and the other phytocannabinoids [cannabidivarin (CBDV), cannabidiolic acid (CBDA), cannabinol (CBN), Δ^9-tetrahydrocannabinol (THC) and cannabichromene (CBC)] are indicated.

assays, CBD BDS has been shown to be more potent or efficacious than pure CBD (Comelli et al. 2008; Capasso et al. 2011; De Petrocellis et al. 2013; Russo 2011), suggesting additive or synergistic interactions can occur between CBD and minor phytocannabinoids (or the non-cannabinoid fraction) contained in the extract, which, in turn might be useful from a therapeutic viewpoint. CBD is the most common phytocannabinoid in fibre (hemp) plants, it is non-psychotropic and, among potent and different pharmacological actions, it exerts antitumoural actions both *in vitro* and *in vivo* (Ligresti et al. 2006; Wilkinson and Williamson 2007; Sreevalsan et al. 2011; McAllister et al. 2011; Maor et al. 2012; Ramer et al. 2012; Solinas et al. 2012; Hernán Pérez de la Ossa et al. 2013). Of relevance to the present investigation, is our recent discovery that CBD exerts antiproliferative effects in colorectal carcinoma cells and chemopreventive actions in an experimental model of colon cancer (Aviello et al. 2012).

Therefore, here we extended our previous investigations of the intestinal antitumoural action of CBD (Aviello et al. 2012) by exploring the effect and the mode of action of CBD BDS in colorectal carcinoma cells and in *in vivo* murine models of colon carcinogenesis.

Materials and methods

Plant material and extraction

A *Cannabis sativa* chemotype with a controlled high amount of CBD was used (de Meijer et al. 2003). *Cannabis sativa* was grown in highly secure computer-controlled glasshouses. All aspects of the growing climate, including temperature, air change and photoperiod, were computer-controlled and the plants were grown without the use of pesticides (see details at: http://www.gwpharm.com). Cannabis dry flowers and leaves were extracted at room temperature with CO_2 to give an extract which, evaporated to dryness, was a brownish solid. A portion of the extract was dissolved in methanol for HPLC analysis (Agilent 1100) using a C18 column (150 mm × 4.6 mm, 1 ml/min flow rate). HPLC chromatogram and composition of the main cannabinoids are reported in Fig. 1, respectively.

Drugs

Cannabidiol (CBD, purity by HPLC: 99.8%) and *Cannabis* sativa extract with high content of cannabidiol (here named CBD botanical drug substance (CBD BDS), see HPLC chromatogram in Fig. 1 and composition in Table 1) were prepared as described above (see subheading "plant Material and extraction"). The concentrations (or doses) of CBD BDS reported in the present paper indicated the amount of CBD contained in the extract (*e.g.* 1 μmol of CBD BDS contained 1 μmol of CBD). Azoxymethane (AOM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (NR), were purchased from Sigma (Milan, Italy); AM251 and AM630 were obtained from Tocris Cookson (Bristol, UK). Rimonabant and SR144528 (N-[-1S-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) were provided by Sanofi-Aventis (Montpellier, France). All reagents for cell culture were obtained from Microtech (Naples, Italy). For the binding experiments, [^{3}H]CP55940 (160 Ci/mmol) was obtained from PerkinElmer Life Sciences Inc. (Boston, MA, USA). The drugs vehicles used in *in vivo* experiments (10% (v/v) ethanol, 10% (v/v) Tween-20, 80% (v/v) saline, 2 ml/kg), in the experiments with cell lines (0.01% DMSO v/v in cell media) and in the radioligand binding assays with hCB_1/hCB_2 CHO cells (0.1% DMSO, v/v) had no effect on measured response.

Table 1
Content of the main phytocannabinoids contained in cannabidiol (CBD) botanical drug substance (CBD BDS).

Phytocannabinoid	Content (% w/w)
Cannabidiol (CBD)	65.9
Δ^9-Tetrahydrocannabinol	2.4
Cannabigerol	1.0
Cannabidivarin	0.9
Cannabidiolic acid	0.3
Cannabinol	0.1

Cell culture

For proliferation experiments, human epithelial colon adenocarcinoma cells (*i.e.* HCT 116 and DLD-1) (ATCC) and healthy colonic epithelial cells (HCEC) were used. HCT 116 and DLD-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) in conformity with the manufacturer's protocols. HCEC were cultured in DMEM supplemented with 10% FBS, 100 Units/ml penicillin, 100 μg/ml streptomycin, 200 mM L-glutamine, 100 mM Na-pyruvate and 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, (HEPES). Cell viability was evaluated by trypan blue exclusion. In all the experiments, HCT 116, DLD-1 and HCEC were used at passages 10–12, 20–25 and 27–29, respectively.

For radioligand binding assays, Chinese hamster ovarian (CHO) cells, stably transfected with complementary DNA encoding human cannabinoid CB_1 receptors and human cannabinoid CB_2 receptors, were cultured in Eagle's medium nutrient mixture F-12

Ham supplemented with 1 mM L-glutamine, 10% (v/v) FBS and 0.6% penicillin–streptomycin together with geneticin (600 mg/ml). These CHO-hCB_1/hCB_2 cells were passaged twice a week using a non-enzymatic cell dissociation solution.

Animals

Male ICR mice (Harlan Italy, S. Pietro al Natisone UD, Italy) weighing 25–30 g were used after a 1 week-acclimation period (temperature 23 ± 2 °C; humidity 60%, free access to water and standard food). The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care (NIH publication no. 86-23, revised 1985) and the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC).

Neutral red uptake

The NR assay system, one of the most used and sensitive cytotoxicity test, is a means of measuring living cells *via* the uptake of the vital dye neutral red. HCT 116, DLD-1 cells and HCEC were seeded in 96-well plates (2.5×10^3 cells *per* well for tumoral cell lines, 1.0×10^4 cells *per* well for the healthy ones) and allowed to adhere for 48 h; after this period, cells were incubated with CBD or CBD BDS (both at the concentration range of 1–5 μM) for 24 h and subsequently with NR dye solution (50 μg/ml) for 3 h (Aviello et al. 2011). Cells were lysed with 1% (v/v) acetic acid, and the absorbance was read at 532 nm (iMark™ microplate absorbance reader, Bio-Rad). Dimethyl sulphoxide (DMSO, 20%, v/v) was used as a positive control. The results are expressed as percentage of cell viability (n = 3 experiments including 8–10 replicates for each treatment).

MTT assay

HCT 116, DLD-1 cells and HCEC were seeded in 96-well plates (2.5×10^3 cells *per* well for tumoral cell lines, 1.0×10^4 cells *per* well for the healthy ones) allowed to adhere (within 48 h) and starved by serum deprivation for 24 h. For the MTT assay, cells were treated with CBD or CBD BDS, (both at 0.3–5 μM) for 24 h and incubated with MTT (250 μg/ml) for 1 h at 37 °C. The mitochondrial reduction of MTT to formazan was then quantitated at 490 nm (iMark™ microplate reader, BioRad, Italy). This assay was also used to establish if the antiproliferative effect of CBD and CBD BDS was due to the activation of CB receptors (tested only in the DLD-1 cell line). For this aim the antiproliferative effect of CBD and CBD BDS, (both at 3 μM) was evaluated in the presence of rimonabant or AM251 (CB_1 receptor antagonists, 0.1 μM and 1 μM, respectively) or in presence of a CB_2 receptor antagonist: either SR144528 or AM630 (0.1 μM and 1 μM, respectively). All the antagonists used were incubated for 30 min before the addition of CBD or CBD BDS.

Membrane preparation

Binding assays were performed with membranes from CHO cells transfected with human CB_1 or CB_2 receptors (Ross et al. 1999a). The CHO cells were removed from flasks by scraping and then frozen as a pellet at −20 °C until required. Before use in a radioligand binding assay, cells were defrosted, diluted in 50 mM Tris buffer and

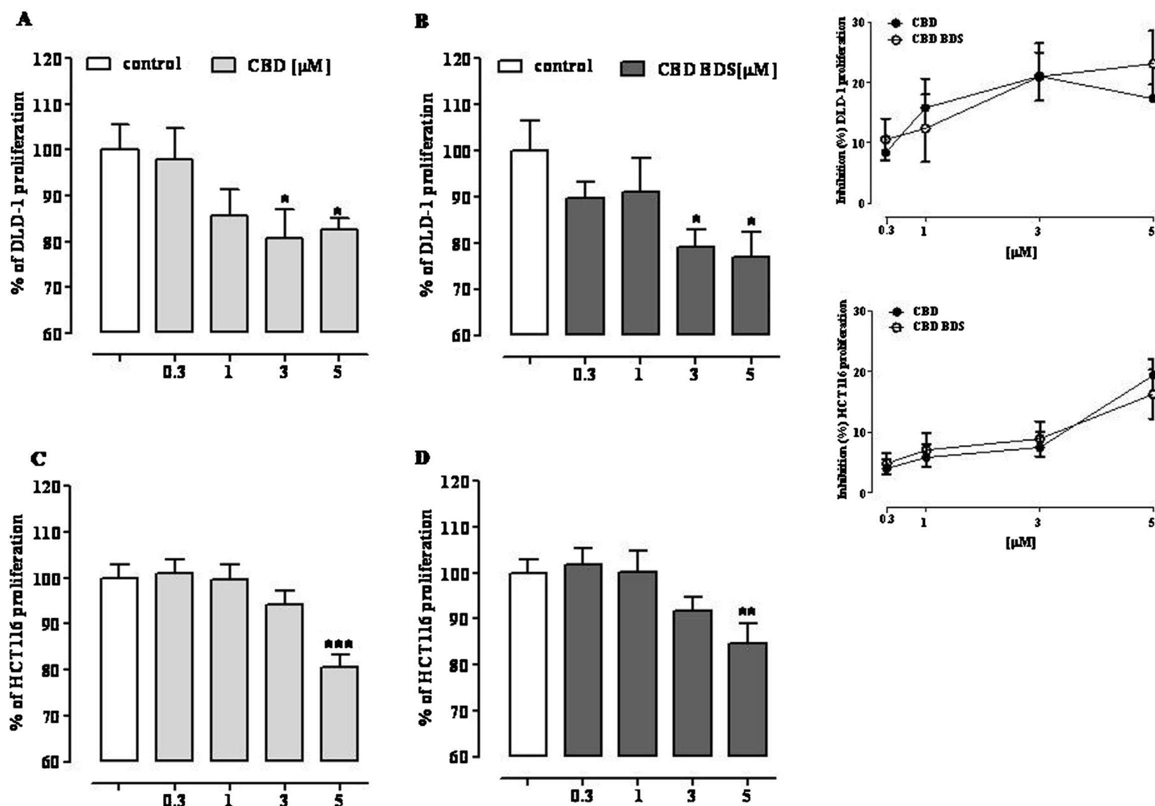

Fig. 2. Antiproliferative effects of cannabidiol (CBD, 0.3–5 μM, 24-h exposure) and a *Cannabis sativa* extract with high content of CBD (CBD BDS, 0.3–5 μM, 24-h exposure) in DLD-1 (A and B) and HCT 116 cells (C and D). Proliferation (expressed as percentage of cell proliferation) rate was studied using the MTT assay. Each bar represents the mean ± SEM of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* control. The inserts (on top of the figures) show the effect of CBD and CBD BDS (expressed as percentage of cell proliferation inhibition). No statistically significant difference was observed between the cannabinoids response curves reported in the inserts.

homogenized with a 1 ml hand-held homogenizer. Protein assays were performed using a Bio-Rad Dc kit (Bio-Rad, Hercules, CA, USA).

Radioligand displacement assay

The assays were carried out, as previously described by Ross et al. (1999b), with [^{3}H]CP55940, 50 mM Tris–HCl, 50 mM Tris Base and 1 mg/ml BSA (assay buffer), total assay volume 500 μl. Unlabeled cannabinoids and [^{3}H]CP55940 were each added in a volume of 50 μl following their dilution in assay buffer. Binding was initiated by the addition of hCB_1- or hCB_2-CHO cell membranes (25 μg protein *per* tube) and all assays were performed at 37 °C for 60 min before termination by the addition of ice-cold wash buffer (50 mM Tris buffer, 1 mg/ml BSA) and vacuum filtration using a 24-well sampling manifold (Brandel Cell Harvester) and Whatman GF/B glass-fibre filters that have been soaked in wash buffer at 4 °C for 24 h. Each reaction tube was washed three times with a 4 ml aliquot of buffer. The filters were oven-dried for 60 min and then placed in 5 ml of scintillation fluid (Ultima Gold XR, Packard). Radioactivity was quantified by liquid scintillation spectrometry. Specific binding was defined as the difference between the binding that occurs in the presence and absence of 1 μM unlabeled CP55940. The concentration of [^{3}H]CP55940 used in the displacement assays was 0.7 nM.

Azoxymethane (AOM)-induced tumours

Mice were randomly divided into the following three groups: group 1 (control) was treated with vehicle; group 2 was treated with azoxymethane (AOM) plus the vehicle used to dissolve CBD BDS; group 3 was treated with AOM plus CBD BDS (5 mg/kg). AOM (40 mg/kg in total) was administered intraperitoneally (IP) once a week, at the single dose of 10 mg/kg, at the beginning of the first, second, third and fourth week. CBD BDS was given (IP) three times *per* week for the whole duration of the experiment starting one week before the first administration of AOM (Aviello et al. 2012). All animals were euthanized by asphyxiation with CO_2 three months after the first injection of AOM. Based on our laboratory experience, this time (at the dose of AOM used) was associated with the occurrence of a significant number of aberrant crypt foci (ACF), polyps and tumours.

For ACF, polyp and tumour determination, the colons were rapidly removed after sacrifice, washed with saline, opened longitudinally, laid flat on a polystyrene board and fixed with 10% buffered formaldehyde solution before staining with 0.2% methylene blue in saline. Colons were examined as previously reported (Borrelli et al. 2002; Aviello et al. 2012) using a light microscope at 20× magnification (Leica Microsystems, Milan, Italy). Only foci with four or more crypts (which are best correlated with the final tumour incidence) were evaluated since they represent the early neoplastic lesion (Washington et al. 2013). ACF were distinguished from surrounding normal crypts by greater size, larger and elongated luminal opening, thicker lining, and compression of the surrounding epithelium. The criterion used to distinguish polyps from tumours was based on the main characteristic features of these two lesions (*i.e.* crypt distortion around a central focus and increased distance from luminal to basal surface of cells for polyps and high grade dysplasia with complete loss of crypt morphology for tumours) (Boivin et al. 2013).

Xenograft colon cancer model

Colorectal carcinoma HCT 116 cells (2.5×10^6) were injected subcutaneously into the right flank of each athymic mouse using a total volume of 200 μl per injection (50% cell suspension in PBS, 50% Matrigel™). At day 10 after the inoculation (once tumours had reached a size of 300 mm^3 approximately), mice were randomly assigned to control and CBD BDS (5 mg/kg, IP, once a day) groups, and treatment was initiated. Tumour size was measured every day by digital caliper measurements, and tumour volume was calculated according to the modified formula for ellipsoid volume (volume = $\pi/6 \times$ length $\times$ width^2).

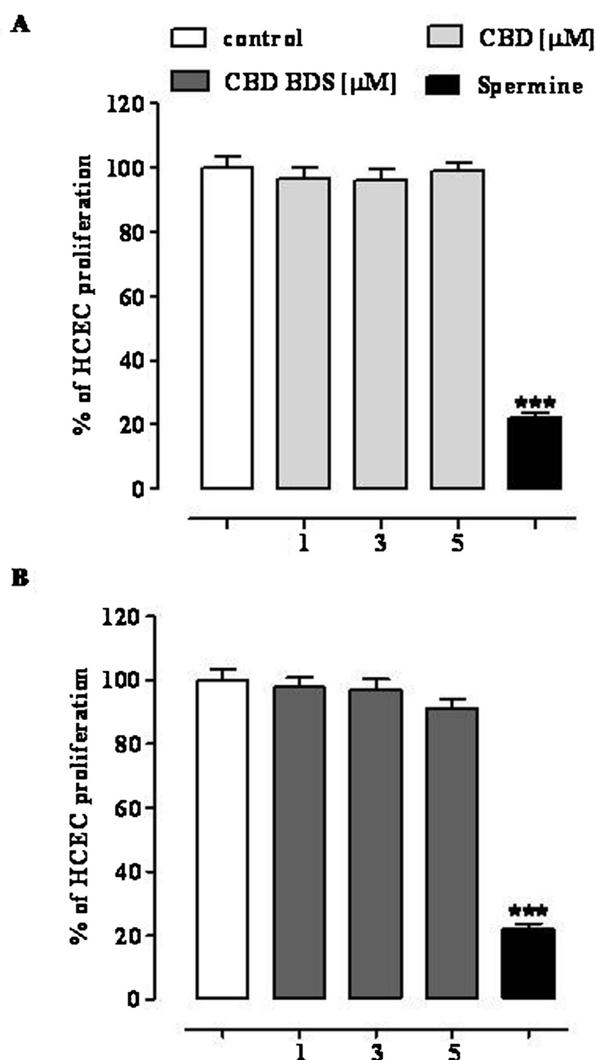

Fig. 3. Effect of cannabidiol (CBD, 1–5 μM, 24-h exposure) and a *Cannabis sativa* extract with high content of CBD (CBD BDS, 1–5 μM) on cell proliferation in healthy human colonic epithelial cells (HCEC). Proliferation rate was studied using the MTT assay. Each bar represents the mean ± SEM of two independent experiments. Spermine (300 μM) was used as a positive control. $^{***}p < 0.001$ *vs.* control.

Statistics

Data are expressed as the mean ± standard error (SEM) of *n* experiments. To determine statistical significance, Student's *t* test was used for comparing a single treatment mean with a control mean; one-way analysis of variance (ANOVA) followed by a Tukey–Kramer multiple comparisons test was used for analysis of multiple treatment means. *p* values < 0.05 were considered significant. ANOVA was used to compare different concentration-effect curves with $p < 0.05$ considered significant. For radioligand binding assays, values are expressed as means and variability as SEM or as 95% confidence limits. Log concentration–response curves are constructed by nonlinear regression analysis using the equation for a sigmoid log concentration–response curve (GraphPad

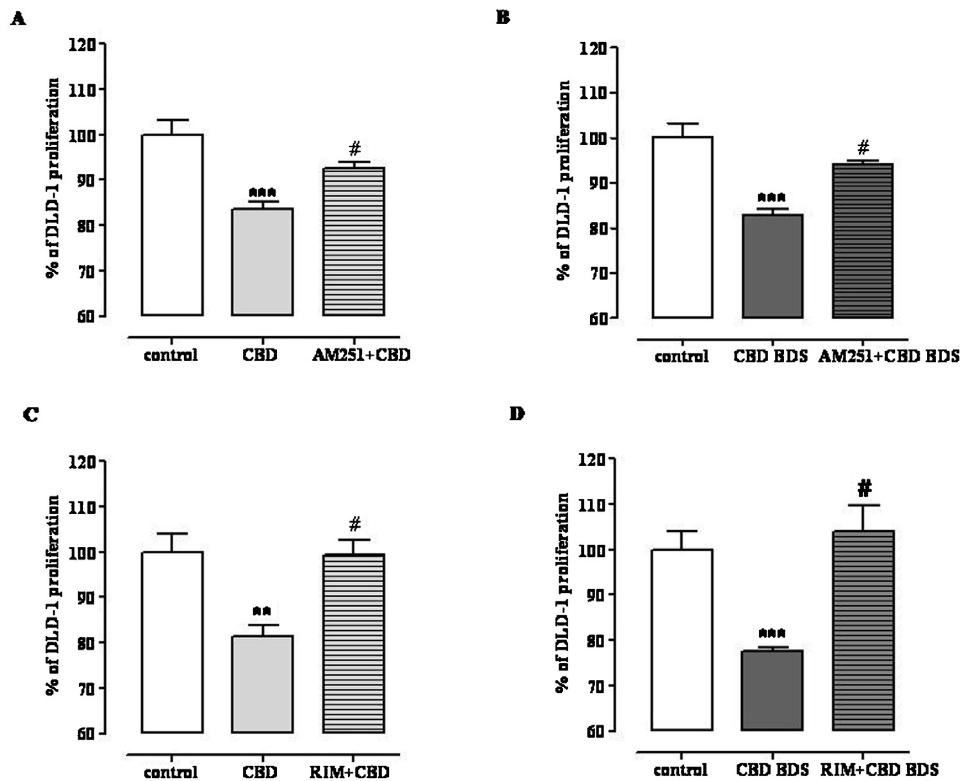

Fig. 4. Antiproliferative effect, evaluated by MTT assay, of cannabidiol (CBD) and a *Cannabis sativa* extract with high content of CBD (CBD BDS, both at 3 μM, 24 h-exposure) alone or in the presence of one or other of two selective cannabinoid CB_1 receptor antagonists, *i.e.* rimonabant (RIM, 0.1 μM) and AM251 (1 μM). The antagonists were incubated 30 min before cannabinoid drugs. Each bar represents the mean ± SEM of two independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* control; $^{\#}p < 0.001$ *vs.* CBD (or CBD BDS).

Prism 5). The concentration of a drug that produces a 50% displacement of [^{3}H]CP55940 from specific binding sites (IC_{50}) is calculated using GraphPad Prism 5. Its dissociation constant (K_i value) is calculated using the equation of Cheng and Prusoff (1973). The parameters for [^{3}H]CP55940 binding to hCB_1 and hCB_2 CHO cell membranes have been determined by fitting data from saturation binding experiments to a one-site saturation plot using GraphPad Prism 5. They are 57.00 pmol/mg and 215 pmol/mg (B_{max}), and 1.1 nM and 4.3 nM (K_d) in hCB_1 and hCB_2 CHO cell membranes, respectively.

Results

CBD BDS and CBD do not affect cell viability

The effect of CBD BDS and CBD on viability was evaluated in colorectal (DLD-1 and HCT116) cells and in healthy colonic epithelial cells (HCEC) by using the neutral red assay. CBD BDS and CBD, at concentration ranging from 1 μM to 5 μM, did not affect cell viability (expressed as percentage of viability ± SEM) after 24-h exposure (DLD-1 cells: control 100 ± 5.84; CBD BDS 1 μM: 106 ± 4; CBD BDS 3 μM: 103 ± 3.3; CBD BDS 5 μM: 99.6 ± 3.7; CBD 1 μM: 106.0 ± 5.4; CBD 3 μM: 102.8 ± 6.99; CBD 5 μM: 102.9 ± 5.18; HCT 116 cells: control 100 ± 7.05; CBD BDS 1 μM: 108.3 ± 5.11; CBD BDS 3 μM: 107 ± 4.75; CBD BDS 5 μM: 105.5 ± 5.44; CBD 1 μM: 111.4 ± 6.56; CBD 3 μM: 116.3 ± 6.49; CBD 5 μM: 110.4 ± 4.30; HCEC cells: control 100 ± 7.05; CBD BDS 1 μM: 86.74 ± 4.8; CBD BDS 3 μM: 95.19 ± 5.93; CBD BDS 5 μM: 92.81 ± 4.08; CBD 1 μM: 101.6 ± 4.99; CBD 3 μM: 101.6 ± 4.99; CBD 5 μM: 97.03 ± 5.66) (n = 3 experiments). DMSO 20% (v/v) used as positive control, significantly reduced DLD-1, HCT 116 and HCEC cell viability (data not shown).

CBD BDS and CBD exert antiproliferative effects in colorectal cancer cells

The effect of non-cytotoxic concentrations of CBD BDS and CBD were evaluated on cell proliferation in both DLD-1 and HCT116 cells using the MTT assay. Both CBD BDS and CBD exerted a significant antiproliferative effect (Fig. 2). No difference in potency and efficacy were observed between CBD BDS and pure CBD in either cell line (see inserts to Fig. 2).

CBD BDS and CBD do not affect cell proliferation in healthy colonic epithelial cells (HCEC)

In order to verify if the effect of *Cannabis*-based products was specific for cancer cells, we investigated the effect of both CBD BDS and CBD on proliferation in HCEC. Both CBD BDS and CBD, up to 5 μM, did not affect significantly proliferation in HCEC (Fig. 3). Spermine (300 μM), used as a positive control, significantly reduces HCEC proliferation (Fig. 3).

The effect of CBD BDS and CBD on colorectal cancer cell proliferation is counteracted by selective cannabinoid CB_1 and CB_2 receptor antagonists

Since CBD BDS contains many *Cannabis* constituents, it was of interest to investigate the possible involvement of cannabinoid CB_1 and CB_2 receptors. Therefore, we investigated the effect of CBD BDS and pure CBD on DLD-1 cell proliferation in the presence of selective cannabinoid CB_1 and CB_2 receptor antagonists. We found that selective cannabinoid CB_1 receptor antagonists (*i.e.* rimonabant 0.1 μM and AM251 1 μM) counteracted the effect of both CBD BDS (3 μM) and pure CBD (3 μM) on cell proliferation (Fig. 4). On

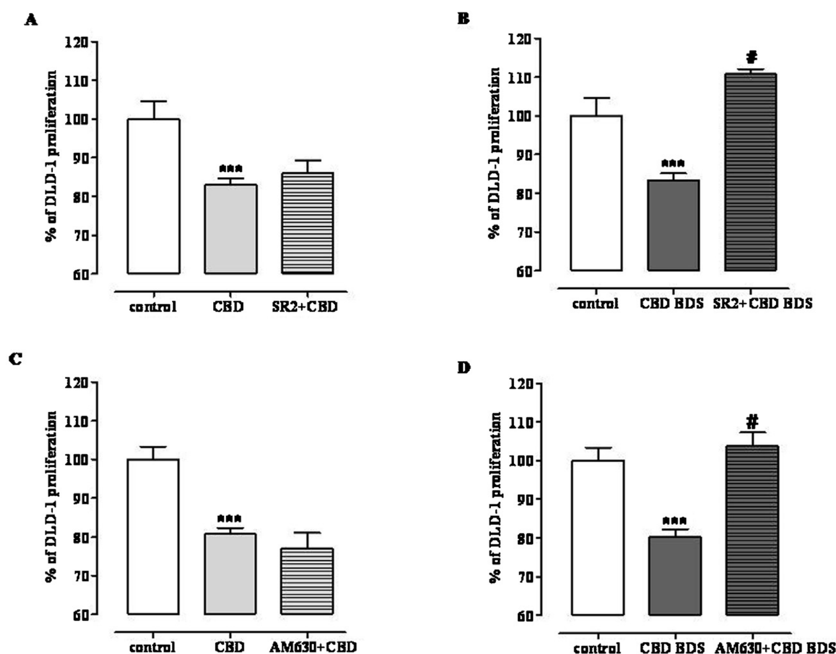

Fig. 5. Antiproliferative effect, evaluated by MTT assay, of cannabidiol (CBD) and a *Cannabis sativa* extract with high content of CBD (CBD BDS, both at 3 μM, 24 h-exposure) alone or in presence of one or other of two selective cannabinoid CB_2 receptor antagonists, *i.e.* SR144528 (SR2, 0.1 μM) and AM630 (1 μM). The antagonists were incubated 30 min before cannabinoid drugs. Each bar represents the mean ± SEM of two independent experiments. $^{***}p < 0.001$ *vs.* control; $^{\#}p < 0.001$ *vs.* CBD (or CBD BDS).

the other hand, selective cannabinoid CB_2 receptor antagonists (*i.e.* SR144528 0.1 μM and AM630 1 μM) counteracted the effect of CBD BDS (3 μM), but not the effect of pure CBD (3 μM), on cell proliferation (Fig. 5).

All the cannabinoid receptor antagonists employed in this set of experiments, at the concentrations used, did not affect, *per se*, cell viability or proliferation (data not shown).

CBD BDS and CBD have different cannabinoid receptor binding profiles

Because selective CB_1 and CB_2 receptor antagonists differently affected the response to CBD BDS and pure CBD, we performed displacement binding assays to compare the cannabinoid binding profiles of CBD BDS and pure CBD. CBD BDS showed greater affinity for cannabinoid receptors than pure CBD in both hCB_1-CHO and hCB_2-CHO cell membranes (Fig. 6). The CBD BDS K_i values for CB_1 and CB_2 receptors were 0.18 μM and 0.14 μM, respectively; pure CBD only (and partially) displaced [^{3}H]CP55940 at the highest concentration tested (10 μM) (Fig. 6).

CBD BDS inhibits carcinogenesis in the azoxymethane (AOM) murine model of colon cancer

In order to establish if CBD BDS exerted chemopreventive effects, we investigated the effect of this *Cannabis* extract in the AOM model of colon carcinogenesis. As expected, AOM, given alone, induced the appearance of ACF with 4 or more crypts (preneoplastic lesions), polyps and tumours (Fig. 7). CBD BDS (5 mg/kg, IP) significantly reduced AOM-induced ACF (86% inhibition) and polyps (79% inhibition). CBD BDS also reduced tumour formation by 40%, although a conventional statistical significance was not fully achieved (Fig. 7).

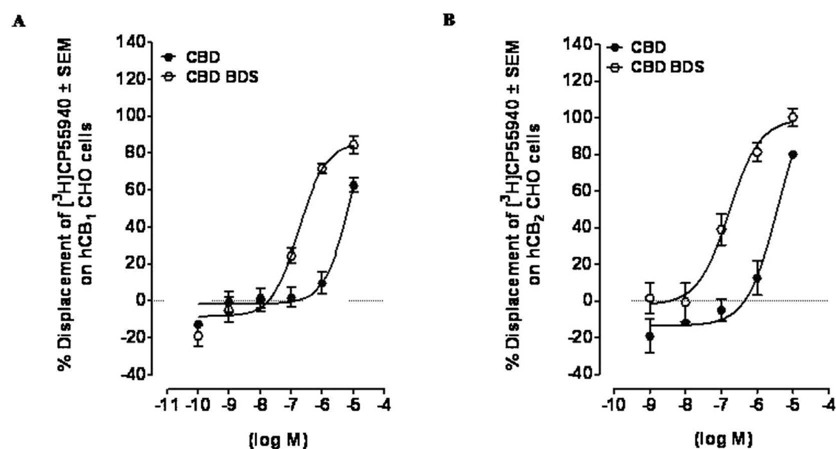

Fig. 6. Displacement of [^{3}H]CP55940 by cannabidiol (CBD) and a *Cannabis sativa* extract with high content of CBD (CBD BDS) from specific binding sites on hCB_1-CHO cell membranes (A) and hCB_2-CHO cell membranes (B). Each symbol represents the mean percent displacement ± SEM ($n = 4$).

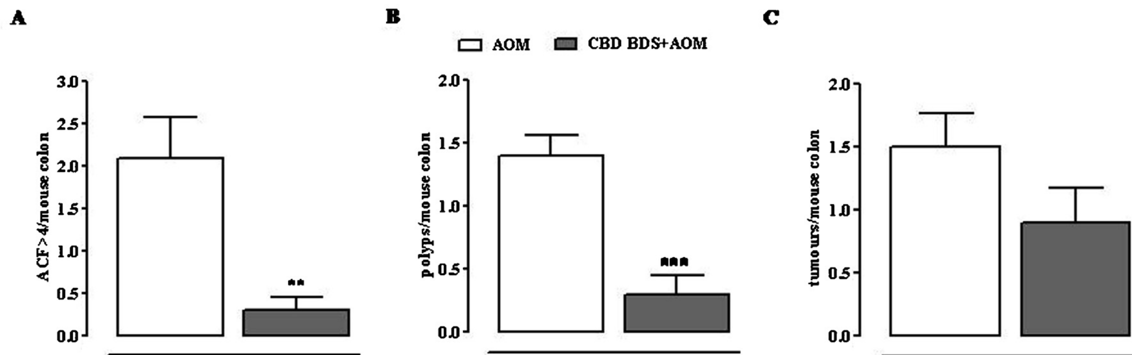

Fig. 7. Aberrant crypt foci with four or more crypts (ACF ≥ 4/mouse) (A), polyps (B) and tumours (C) induced in the mouse colon by azoxymethane (AOM): effect of a *Cannabis sativa* extract with high content of CBD (CBD BDS, 5 mg/kg, IP). AOM (40 mg/kg in total, IP) was administered, at the single dose of 10 mg/kg, at the beginning of the first, second, third and fourth week. CBD BDS was given three times a week for the whole duration of the experiment, starting 1 week before the first administration of AOM. Measurements were performed 3 months after the first injection of AOM. Each bar represents the mean ± SEM of 9–11 mice. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* AOM.

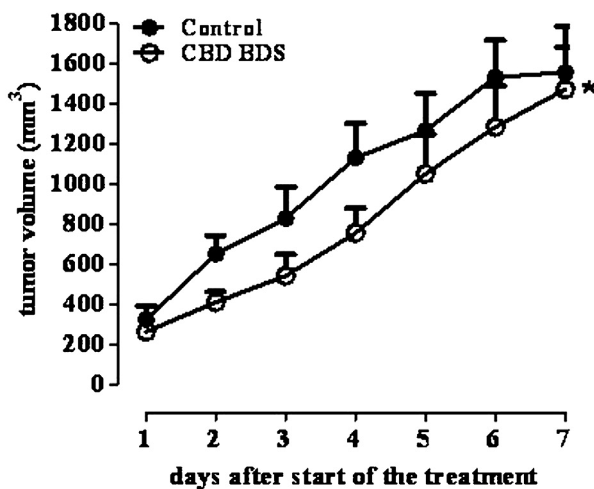

Fig. 8. Tumours generated by subcutaneous implantation of xenograft (human colorectal carcinoma HCT 116) cells in athymic mice: effect of a *Cannabis sativa* extract with high content of CBD (CBD BDS, 5 mg/kg, IP). CBD BDS treatment (once a day for seven days) started 10 days after tumour cell injection (*i.e.* when tumours reached approximately a volume of 300 mm^3). Each point represents the mean ± SEM of 8 animals for each experimental group. $^{*}p < 0.05$; ANOVA CBD BDS curve *vs.* control curve.

CBD BDS retards the growth of xenograft colorectal tumours in mice

To assess the potential curative effect of CBD BDS on colorectal cancer, athymic nude mice bearing colorectal tumour xenografts were treated daily with CBD BDS (5 mg/kg, IP). The average tumour volume in mice treated with CBD BDS was significantly lower compared with vehicle-treated control mice (Fig. 8). For example, 4 days after the commencement of CBD BDS challenge, the average tumour volume in control mice (mean ± SEM: 1130 ± 171.6 mm^3) was approximately 1.5-fold higher as compared to mice treated with 5 mg/kg CBD BDS (mean ± SEM: 755 ± 124 mm^3). However, no differences in tumour growth were observed after 7-days CBD BDS treatment.

Discussion

CBD BDS is one of the main components of Sativex (Nabiximols in the USA), a cannabinoid formulation actually used for the treatment of pain and spasticity associated with multiple sclerosis. Clinical studies have shown that Sativex may provide a protection against chemotherapy-induced nausea and vomiting (Duran et al. 2010) and may be a useful add-on analgesic for patients with opioid-refractory cancer pain (Johnson et al. 2010, 2012; Portenoy et al. 2012). In the present study we have shown, for the first time, that CBD BDS exerts antiproliferative effects in carcinoma cell lines and attenuates colon carcinogenesis *in vivo*.

It is well established that synthetic, plant and endogenous cannabinoids may inhibit colorectal cancer cells growth *via* a multitude of mechanisms, including direct activation of CB_1 and CB_2 receptors (Ligresti et al. 2003; Izzo and Coutts 2005; Cianchi et al. 2008; Izzo and Camilleri 2009). Here, we have shown that CBD BDS as well as pure CBD reduced cell proliferation in colorectal cancer (DLD-1 and HCT116) cells. In contrast to other assays (Capasso et al. 2011; Comelli et al. 2008), there was no significant difference in potency and efficacy between CBD BDS and pure CBD. Since CBD BDS is a mixture containing many cannabinoids, including the cannabinoid receptor agonist Δ^9-tetrahydrocannabinol (THC), in order to identify the receptor(s) underlying the antiproliferative action of CBD BDS (and, for comparison, pure CBD), we investigated the potential involvement of cannabinoid CB_1 and CB_2 receptors. In agreement with the results obtained in another colorectal carcinoma cell line (*i.e.* Caco-2 cells), we found that the antiproliferative effect of CBD in DLD-1 cells was counteracted by selective cannabinoid CB_1 – but not CB_2 – receptor antagonists, suggesting an involvement of CB_1 receptors. It is likely that CBD activates cannabinoid CB_1 receptors indirectly, *i.e. via* enhancement of endocannabinoids levels. This is because (1) CBD does not efficiently binds cannabinoid CB_1 receptors (Pertwee 2008; see also Fig. 4) and (2) CBD has been recently shown to increase the levels of the endocannabinoid 2-arachidonoylglycerol in colorectal carcinoma cells (Aviello et al. 2012). When we evaluated the pharmacological effect of CBD BDS, we found that its action on cell proliferation was sensitive to both CB_1 and CB_2 receptor antagonists, thus suggesting that CBD and CBD BDS have a different mode of action.

In order to give insights into the observed different mode of action, we compared the cannabinoid receptor binding of CBD BDS to that of pure CBD. In hCB_1 and hCB_2 transfected CHO cells, we found that CBD BDS showed greater affinity than pure CBD for both CB_1 and CB_2 receptors. Pure CBD had little affinity for either CB_1 or CB_2 receptors, with only the concentration of 10 μM exhibiting any significant binding. Among the other phytocannabinoids contained in CBD BDS (see Table 1 for the composition of this BDS) THC has been shown to be a potent CB_1 and CB_2 receptor agonist; cannabinol has a weak partial agonist activity at the CB_1 receptor and moderate partial agonist activity at the CB_2 receptor and cannabigerol has been shown to be a weak ligand at both CB_1 or CB_2 receptors (Pertwee 2005; Pertwee 2008; Cascio et al. 2010; Pollastro et al. 2011). Together, these binding data suggest

that the presence of both THC (contained in CBD BDS at a 2.4% concentration) and to a very less extent cannabinol (present in CBD BDS at a 0.1% concentration) could account for the ability of CBD BDS to displace [^{3}H]CP55940 with higher affinity than pure CBD.

It is also noteworthy that CBD BDS most probably shares the ability of CBD to activate cannabinoid receptors indirectly by increasing the levels of endogenously released endocannabinoids (Aviello et al. 2012). Clearly, therefore, there is a need for further research directed at establishing the manner in which CB_1 and/or CB_2 receptors contribute both to the antiproliferative effect of CBD BDS in colorectal cancer cells and to its ability to retard the growth of colorectal tumours *in vivo*.

Pure CBD is known to reduce glioma formation (Hernán Pérez de la Ossa et al. 2013), to inhibit cancer cell invasion (Ramer et al. 2010, 2012) and angiogenesis (Solinas et al. 2012) and to decrease the growth of breast carcinoma and lung metastasis in rodents (Ligresti et al. 2006; Shrivastava et al. 2011; Ramer et al. 2012). Furthermore, CBD BDS has been shown to reduce the growth of xenograft tumours obtained by injection into athymic mice of human breast (Ligresti et al. 2006) and prostate (De Petrocellis et al. 2013) carcinoma cells. We have therefore investigated the possible chemopreventive effect of CBD BDS in the AOM model of colon carcinogenesis in mice and its possible curative effects in the xenograft model of cancer induced by injection of colorectal cancer cells in athymic mice. We used the CBD BDS dose of 5 mg/kg, since CBD, at the 5 mg/kg dose, has been shown to be effective in several models of cancer (Aviello et al. 2012; Ramer et al. 2010, 2012). In the AOM model of colon carcinogenesis, it has been previously demonstrated that (1) a pharmacological enhancement of endocannabinoid levels reduces the development of precancerous lesions and, more importantly (Izzo et al. 2008), (2) CBD exerts chemopreventive effects (Aviello et al. 2012). Also, the atypical cannabinoid O-1602 has been recently shown to inhibit tumour growth in AOM-induced colitis-associated colon cancer (Kargl et al. 2013). In the xenograft model of colon cancer, semi-synthetic cannabinoids such as the cannabinoid quinone HU-331 and the hexahydrocannabinol analogue LYR-8 (Thapa et al. 2012; Kogan et al. 2007), as well as the selective CB_2 receptor agonist CB13 (Cianchi et al. 2008) exerts antitumour activity. In the present study, CBD BDS significantly reduced the formation of aberrant crypt foci and polyps. The same pharmacological treatment also reduced tumour formation by 40%, although a statistical significance was not achieved. In addition, we have shown that CBD BDS also retards the formation of tumours induced by xenograft injection in nude mice. Collectively, such results suggest that CBD BDS may attenuate experimental colon carcinogenesis *in vivo*.

In conclusion, we have shown that CBD BDS exerts beneficial actions in experimental models of colon cancer and antiproliferative CB_1 and CB_2 mediated effects in colorectal cancer cells. Such results are timely and relevant from a clinical viewpoint in the light of the proposed medical use of *Cannabis*-based medicines, including Sativex, for the symptomatic relief of cancer pain (Johnson et al. 2012; Pertwee 2012).

Conflict of interest

This investigation was partly supported by grants from GW Pharmaceuticals (Porton Down, Wiltshire, UK).

Acknowledgement

BR is grateful to the “Fondazione Enrico & Enrica Sovena”.

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