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Colon carcinogenesis is inhibited by the TRPM8 antagonist cannabigerol, a *Cannabis*-derived non-psychotropic cannabinoid

Francesca Borrelli[†,¶], Ester Pagano[†,¶], Barbara Romano[¶], Stefania Panzera, Francesco Maiello[1], Diana Coppola[1], Luciano De Petrocellis[2,¶], Lorena Buono[2,¶], Pierangelo Orlando[3,¶,§] and Angelo A.Izzo[*,¶]

Department of Pharmacy, University of Naples Federico II, Via D. Montesano 49, 80131 Naples, Italy, [1]Department of Diagnostic Services (Anatomy and Pathologic Histology Service), Ospedale dei Pellegrini, ASL 1, 80135 Naples, Italy, [2]Institute of Biomolecular Chemistry, National Research Council, Via Campi Flegrei 34, 80078 Pozzuoli, Naples, Italy and [3]Institute of Protein Biochemistry, National Research Council, Via P. Castellino 111, 80131 Naples, Italy

*To whom correspondence should be addressed. Tel: +39 081 678439/665; Fax: +39 081 678403;
Email: aaizzo@unina.it
Correspondence may also be addressed to Francesca Borrelli.
Tel: +39 081 678439/665; Fax: +39 081 678403;
Email: franborr@unina.it

Cannabigerol (CBG) is a safe non-psychotropic *Cannabis*-derived cannabinoid (CB), which interacts with specific targets involved in carcinogenesis. Specifically, CBG potently blocks transient receptor potential (TRP) M8 (TRPM8), activates TRPA1, TRPV1 and TRPV2 channels, blocks 5-hydroxytryptamine receptor 1A (5-HT_{1A}) receptors and inhibits the reuptake of endocannabinoids. Here, we investigated whether CBG protects against colon tumourigenesis. Cell growth was evaluated in colorectal cancer (CRC) cells using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide and 3-amino-7-dimethylamino-2-methylphenazine hydrochloride assays; apoptosis was examined by histology and by assessing caspase 3/7 activity; reactive oxygen species (ROS) production by a fluorescent probe; CB receptors, TRP and CCAAT/enhancer-binding protein homologous protein (CHOP) messenger RNA (mRNA) expression were quantified by reverse transcription–polymerase chain reaction; small hairpin RNA-vector silencing of TRPM8 was performed by electroporation. The *in vivo* antineoplastic effect of CBG was assessed using mouse models of colon cancer. CRC cells expressed TRPM8, CB_1, CB_2, 5-HT_{1A} receptors, TRPA1, TRPV1 and TRPV2 mRNA. CBG promoted apoptosis, stimulated ROS production, upregulated CHOP mRNA and reduced cell growth in CRC cells. CBG effect on cell growth was independent from TRPA1, TRPV1 and TRPV2 channels activation, was further increased by a CB_2 receptor antagonist, and mimicked by other TRPM8 channel blockers but not by a 5-HT_{1A} antagonist. Furthermore, the effect of CBG on cell growth and on CHOP mRNA expression was reduced in TRPM8 silenced cells. *In vivo*, CBG inhibited the growth of xenograft tumours as well as chemically induced colon carcinogenesis. CBG hampers colon cancer progression *in vivo* and selectively inhibits the growth of CRC cells, an effect shared by other TRPM8 antagonists. CBG should be considered translationally in CRC prevention and cure.

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; CB, cannabinoid; CBC, cannabichromene; CBD, cannabidiol; CBDV, cannabidivarin; CBG, cannabigerol; CHOP, CCAAT/enhancer-binding protein homologous protein; CRC, colorectal cancer; DCF, dichlorofluorescein; DCFH-DA, 7′-dichlorofluorescin diacetate; DMSO, dimethyl sulphoxide; ER, endoplasmic reticulum; EshV, cell electroporated by the "empty"-shRNA vector; FBS, foetal bovine serum; HCEC, healthy human colonic epithelial cell line; HPLC, high-performance liquid chromatography; mRNA, messenger RNA; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; NR, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride; ROS, reactive oxygen species; RR, ruthenium red; shRNA, small hairpin RNA; TRP, transient receptor potential; TshV, cell electroporated by a shRNA-vector targeted to TRPM8.

[†]These authors contributed equally to this work.
[¶]These authors belong to the Endocannabinoid Research Group.
[§]This author is a guest researcher at National Institute of Optics-CNR, Via C. Flegrei 34, Pozzuoli, Italy.

Introduction

It is estimated that by 2030, the number of new cancer cases will increase by 70% worldwide mainly due to adoption of western lifestyle habits (1–3). Globally, colorectal cancer (CRC) is a major life-threatening disease representing the third most common cancer in men and the second most common cancer in women worldwide (1). The American cancer society in the USA estimates that the probability to develop CRC during the life is 5.17% for men and 4.78% for women and predicts that this type of cancer will cause ~50 830 deaths in 2013 (3,4). Although significant progress has been made in understanding CRC development through epidemiological, laboratory and clinical studies, this type of cancer continues to be a major public health problem in the USA and many other parts of the world. Accordingly, novel therapeutic approaches, including chemopreventive measures, are urgently needed (5).

The plant *Cannabis sativa* contains >100 phytocannabinoids that have been used for years for both recreational and medicinal purposes (6,7) and, at least some of them, are now candidates for new anticancer therapies (8). Beside a direct anticancer action, phytocannabinoids have demonstrated to attenuate several important side effects induced by chemotherapeutics (9–11). Phytocannabinoids include psychotropic compounds such as Δ^9-tetrahydrocannabinol and many other non-psychotropic compounds of therapeutic interest, such as cannabigerol (CBG). CBG appears as a relatively low concentration intermediate in the plant, although recent breeding works have yielded *Cannabis* chemotypes expressing 100% of their phytocannabinoid content as CBG (12,13). Older and recent studies support analgesic, antierythemic, antibacterial, antidepressant and antihypertensive actions for CBG (8,14). Relevant to the present investigation, CBG has been proved to be cytotoxic in high dosage on human epithelioid carcinoma cells (15), to be effective against breast cancer (16) and to inhibit keratinocyte proliferation (17). Furthermore, CBG reduced experimental intestinal inflammation, which is relevant in view of the observation that the risk of developing neoplasia leading to CRC is significantly increased in ulcerative colitis patients (18,19). Pharmacodynamic studies have shown that CBG interacts with receptors/enzymes involved in carcinogenesis. Specifically, CBG is a weak partial agonist of cannabinoid $(CB)_1$ and CB_2 receptors (20), inhibits the reuptake of endocannabinoids (21), is a potent 5-HT_{1A} antagonist (20) and may interact with transient receptor potential (TRP) channels. Among the TRP channels, CBG has been shown to be a TRPA1, TRPV1 and TRPV2 agonist and, importantly, a potent TRPM8 antagonist (21), a TRP channel known to be involved in the growth of tumoural cells (22–25). Here, we have (i) investigated the effect and the mode of action of CBG on colorectal carcinoma cells growth, (ii) evaluated its possible chemopreventive action in the azoxymethane (AOM) model of colon cancer and (iii) assessed its possible curative effect in the xenograft model of colon cancer.

Materials and methods

Chemicals

AOM, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red solution, NR), ruthenium red, icilin, DCFH-DA and ethidium bromide solution were purchased from Sigma (Milan, Italy); AM251, AM630, capsazepine, GW9662 and AMTB hydrochloride were obtained from Tocris Cookson (Bristol, UK). Matrigel™ was obtained from BD Biosciences (Milan, Italy). All reagents for cell cultures were obtained from Sigma, Bio-Rad Laboratories

(Milan, Italy) and Microtech Srl (Naples, Italy). The vehicles for *in vitro* experiments [(0.1% dimethyl sulphoxide (DMSO) vol/vol in cell media] and *in vivo* experiments (10% ethanol, 10% Tween-20, 80% saline, 2 ml/kg) had no effect on the response under study.

CBG extraction from CBG-predominant C.sativa plants

A *C.sativa* chemotype, cloned from the same plant to have a controlled high amount of CBG, was used. The mechanism that is responsible for the accumulation of CBG in certain phenotypes of *C.sativa* is described in detail elsewhere (12). *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. *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 high-performance liquid chromatography (HPLC) analysis (Agilent 1100) using a C18 column (150 × 4.6 mm, 1 ml/min flow rate). CBG was crystallized from CBG extracts using alkanes as solvents. The identity and purity of CBG (purity: 95.0%) were assessed by various chromatographic techniques (i.e. HPLC, gas chromatography, melting point, infrared spectroscopy). Similarly, cannabidiol (CBD, purity by HPLC, 99.3%), cannabidivarin (CBDV, purity by HPLC, 95.0%) and cannabichromene (CBC, ethanol solution with 95.0% of purity by HPLC) were extracted by the corresponding phytocannabinoids-predominant plants (26,27).

Cell cultures

Two human colon adenocarcinoma cell lines (Caco-2 and HCT 116, ATCC from LGC Standards, Milan, Italy), a healthy human colonic epithelial cell line (HCEC, from Fondazione Callerio Onlus, Trieste, Italy) and a human embryonic kidney (HEK-293, ATCC from LGC Standards) cell line were used. The cells were routinely maintained at 37°C in a 5% CO_2 atmosphere in 75 cm^2 polystyrene flasks in Dulbecco's modified Eagle's medium (for Caco-2, HCT 116 and HCEC) or in minimum essential medium (for HEK-293). For Caco-2, HCT 116 cells and HEK-293, media (Dulbecco's modified Eagle's medium or minimum essential medium) were supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin, 1% non-essential amino acids, and 2 mM L-glutamine. For HCEC, Dulbecco's modified Eagle's medium was supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid], 2 mM L-glutamine and 1 mM Na pyruvate. The media were changed every 48 h in conformity with the manufacturer's protocols.

Animals

Male ICR mice (weighting 25–30 g) and athymic nude female 4-weeks-old mice (Harlan Italy, S. Pietro al Natisone UD, Italy) were used after 1 week acclimation period (temperature 23 ± 2°C; humidity 60%, free access to water and food). Athymic female mice, fed *ad libitum* with sterile mouse food, were maintained under pathogen-free conditions. All animal procedures complied with the Italian DL no.116 of 27 January 1992 and associated guidelines in the European Communities Council (86/609/ECC and 2010/63/UE).

TRPM8 channel calcium assay

HEK-293 cells were transfected by electroporation by a GenePulser X-cell (Bio-Rad), electroporator, following the manufacturer's standard protocol, in a 0.2 cm gap cuvette by using 200 µl of electroporation buffer (Bio-Rad) containing 1.5 × 10^6 cell/ml and 25 µg/ml of a vector containing the full sequence of human TRPM8, NM_024080 (EX-E2213-M02 ORF expression clone GeneCopoeia, LabOmics S.A., Belgium). Transfection efficiency (>50% at 30 h from electroporation) was evaluated by fluorescence microscopy in HEK-293 cells transfected by a green fluorescent protein plasmid in a parallel experiment. Transcriptional expression of TRPM8 in transfected cells, as evaluated by quantitative reverse transcription–polymerase chain reaction (RT–PCR) (see below), was ~1800-fold higher than the control. After 36 h from electroporation, CBG antagonism versus human TRPM8 was evaluated in HEK-293 transfected cells, as described previously (21).

Small hairpin RNA transfections

Caco-2 cells, growth to ~50% of confluence, were transfected by electroporation (exponential decay protocol, 150 V, capacitor: 500 µF, resistor: none) in 0.2 cm gap electroporation cuvette by using 200 µl of electroporation buffer (Bio-Rad) containing 3 × 10^6 cell/ml and 25 µg/ml of a small hairpin RNA (shRNA) vector targeted to human TRPM8 (HSH018887-CH GeneCopoeia). Replicate samples and controls were grown in medium containing 10% FBS in 1.6 cm^2/well plates. Transfection efficiency was evaluated by fluorescence microscopy by monitoring green fluorescent protein expression. After ~36 h of culture, adherent cells were treated (or not) by 10 µM of CBG and cultured in medium containing 1% FBS. This procedure yielded to ~65% of TRPM8 messenger RNA (mRNA) silencing as assessed by quantitative RT–PCR. Because electroporation by a vector affects, *per se,* cell viability (cell viability reduced by 60%), we compared the effect of CBG in cell electroporated by the "empty"-shRNA vector (EshV) and in cell electroporated by a shRNA-vector targeted to TRPM8 (TshV).

RT–PCR and western blot analyses

Quantitative-relative mRNA expression was evaluated in HCEC, Caco-2 and HCT 116 cells, treated or not by CBG, as described previously (28). For the comparison of mRNA expression in different cell lines, a semi-quantitative absolute determination was performed (for more details, see Supplementary data and Supplementary Table II, available at *Carcinogenesis* Online).

TRPM8 protein expression in HCEC, Caco-2 and HCT 116 cells was evaluated in cytosolic lysates by western blot analysis (for more details, see Supplementary data, available at *Carcinogenesis* Online).

Cytotoxicity studies: MTT and NR assays

Cell viability was evaluated by measuring the mitochondrial reductase activity (MTT assay) and the neutral red uptake (NR assay). Cells were seeded in presence of 10% FBS in 96-well plates at a density of 1 × 10^4 cells per well (Caco-2 cells and HCEC) or 2.5 × 10^3 cells per well (HCT 116) and allowed to adhere for 48 h. After this period, for the MTT assay, cells were incubated with medium containing 1% or 10% FBS in presence or absence of increasing concentrations of CBG (1–30 µM), for 3, 6, 12, 24 and 48 h. Subsequently, at each end point, the treatment medium was replaced with fresh 1% or 10% FBS medium containing MTT (250 µg/ml, for 1 h at 37°C). After solubilization in DMSO, the mitochondrial reduction of MTT to formazan was quantitated at 490 nm (iMarkTM microplate reader, Bio-Rad). For the NR assay, Caco-2 cells were incubated with medium containing 1% FBS in presence or absence of increased concentrations of CBG (1–30 µM) for 24 h. Subsequently, cells were incubated with NR dye solution (50 µg/ml in 1% FBS) for 3 h at 37°C and then lysed with 1% acetic acid. The absorbance was read at 532 nm (iMarkTM microplate reader, Bio-Rad).

In another set of experiments, the effects of CBD (1–30 µM), CBDV (1–30 µM), CBC (1–30 µM), AMTB (5–50 µM, TRPM8 channel antagonist), and WAY100635 (0.2 and 1 µM, 5-HT_{1A} receptor antagonist) on cell viability (in Caco-2 cells with 1% FBS medium for 24 h) using the MTT assay were also evaluated.

Moreover, using the MTT assay, the cytotoxic effect of CBG (10 µM) was evaluated (in Caco-2 cells with 1% FBS medium) in the presence of AM251 (1 µM, CB_1 receptor antagonist), AM630 (1 µM, CB_2 receptor antagonist) and ruthenium red (10 and 25 µM, a non-selective TRP antagonist], all incubated 30 min before CBG.

Finally, the cytotoxic effect of CBG (10 µM) was also evaluated in Caco-2 cells silenced for TRPM8 (1% FBS medium) using the MTT assay, as described previously.

All results are expressed as percentage of cell viability (n = 3 experiments including 8–10 replicates for each treatment).

Morphological assessment of apoptotic and necrotic cells

Cells were seeded on glass disk (1.3 cm in diameter) placed into wells of a 24-well plate, at a density of 5 × 10^4 cells per disk, for 48 h and then treated with medium containing 1% FBS in presence or absence of CBG (10 µM, for 24 h). After incubation, the culture medium was removed, the glass disks were collected and pasted on slides. Subsequently, cells on slides were fixed and stained by the standard haematoxylin–eosin method. The slides were analysed and the histological images were captured with the aid of a light microscope (at ×200 magnification). The number of apoptotic and necrotic cells was quantified using at least 100 cells per slide (n = 3 independent experiments).

Measurement of caspase 3/7 activity in Caco-2 cells

Apoptosis was evaluated by means of the Caspase-Glo® 3/7 Chemiluminescence Assay Kit (Promega Corporation, Madison, WI) following the manufacturer's protocol (for more details, see Supplementary data, available at *Carcinogenesis* Online). All samples were assayed in triplicate. Chemiluminescence mean values were plotted versus the cell number in the assay and the linear regression curve fit was calculated by the software Excel-Windows. The increase of caspase 3/7 enzymatic activity was calculated by the ratio of the curve slopes.

DNA fragmentation (ladder) assay

Caco-2 cells were seeded in 10 cm culture dishes at a density of 4x10^5 and treated or not with CBG (10 µM). After 24 h, the cells were detached, suspended in phosphate-buffered saline and centrifuged at 145*g* for 3 min. The cell pellet was then suspended in DNA-lysis Buffer (50 mM Tris pH 7.5, 100 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% sodium dodecyl sulphate, 0.5 mg/ml proteinase K) and incubated overnight at 55°C. The suspension was centrifuged (1000*g* for 5 min) in the presence of 5 M NaCl and then the DNA was precipitated in 99.8% vol/vol ethanol. The isolated DNA was resolved on a 1.5% agarose gel containing ethidium bromide in 40 mM Tris-acetate-EDTA buffer with electrophoresis at 80 V for 25 min. DNA fragments were visualized and photographed under ultraviolet light (ImageQuont 400, GE Healthcare).

Detection of reactive oxygen species generation

Generation of intracellular reactive oxygen species (ROS) was estimated by the fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (29). For experiments, Caco-2 cells and HCEC were plated in 96-well black plates at the density of 1×10^4 cells per well After 48 h, the cells were incubated in a medium containing 1% FBS in presence or absence of CBG (10 µM, for 24 h). Then, the cells were rinsed and incubated for 1 h with 100 µM DCFH-DA in Hanks' balanced salt solution containing 1% FBS. The Fenton's reagent (H_2O_2/Fe^{2+} 2 mM), used as a positive control, was added 3 h before fluorescence detection. The DCF fluorescence intensity was detected using a fluorescent microplate reader (Perkin-Elmer Instruments), with the excitation wavelength of 485 nm and the emission wavelength of 538 nm.

CRC xenograft model

Colorectal carcinoma HCT 116 cells (2.5 x 10^6) were injected subcutaneously into the right flank of each athymic mice for a total volume of 200 µl per injection (50% cell suspension in phosphate-buffered saline, 50% Matrigel™). At 10 days after inoculation (once tumours had reached a size of 550–650 mm^3), mice were randomly assigned to control and treated 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). CBG (1–10 mg/kg, intraperitoneally) was given every day for the whole duration of the experiment. The doses of CBG were selected on the basis of previous work showing the efficacy of CBD, a related non-psychotropic CB, in the xenograft model of cancer (16,30).

CRC AOM model

Mice were randomly divided into the following four groups (10 animals per group): group 1 (control) was treated with vehicles; group 2 was treated with AOM plus the vehicle used to dissolve CBG and groups 3 and 4 were treated with AOM plus CBG (1 and 5 mg/kg). The doses of CBG were selected on the basis of our previous work showing the efficacy of CBD, a related non-psychotropic CB, in the AOM model of colon cancer (30,31).

AOM (40 mg/kg in total, intraperitoneally) was administered, at the single dose of 10 mg/kg, at the beginning of the first, second, third and fourth week. CBG was given (intraperitoneally) three times a week starting 1 week before the first AOM administration. All animals were euthanized by asphyxiation with CO_2 3 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, which are considered pre-neoplastic lesions), polyps and tumours (31). For ACF, polyps and tumours determination, the colons were rapidly removed after killing, processed and quantified as reported previously (31). Only *foci* containing four or more aberrant crypts (which are best correlated with the final tumour incidence) were evaluated.

Statistical analysis

Statistical analysis has been carried out using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) and Excel-Windows (for linear regression calculation). Data are expressed as the mean ± standard error of the mean (SEM) or standard deviation (SD) of *n* experiments. To determine statistical significance, Student's *t*-test was used for comparing a single treatment mean with a control mean, and an one-way analysis of variance followed by the Tukey–Kramer multiple comparisons test or by the Bonferroni's test was used for analysis of multiple treatment means. The IC_{50} and EC_{50} (concentration that produced 50% inhibition of cell viability or 50% of efficacy) values were calculated by non-linear regression analysis using the equation for a sigmoid concentration–response curve (GraphPad Prism). $P < 0.05$ were considered significant.

Results

CB_1, CB_2, TRPA1, TRPV1, TRPV2, TRPM8 and 5-HT_{1A} mRNA are differently expressed in colorectal carcinoma cell lines (Caco-2 and HCT 116) and HCECs.

CBG has been shown to behave as a weak partial agonist at CB_1 and CB_2 receptors, a relatively potent and highly effective TRPA1 agonist, a weak agonist at TRPV1 and TRPV2, and a potent TRPM8 and 5-HT_{1A} receptor antagonist. Thus, we analysed, by RT–PCR, the possible presence of such potential targets in Caco-2 and HCT 116 cells as well as in HCEC. All the investigated targets were expressed in Caco-2 cells, being TRPV1, CB_2 and 5-HT_{1A} more expressed than CB_1 receptors, TRPM8, TRPV2 and TRPA1 (Supplementary Table I, available at *Carcinogenesis* Online). In HCT 116, all the targets were expressed, with the exception of CB_1 receptors (Supplementary Table I, available at *Carcinogenesis* Online). The rank order of expression was TRPV1 > TRPV2 > TRPA1, with TRPM8, CB_2, 5-HT_{1A} receptors very faintly expressed (expression values very close to background values) (Supplementary Table I, available at *Carcinogenesis* Online). In HCEC, TRPV1 channels were highly expressed, CB_1, TRPA1 and TRPV2 displayed a low expression, whereas TRPM8, CB_2 and 5-HT_{1A} receptors were very weakly expressed (Supplementary Table I, available at *Carcinogenesis* Online).

TRPM8 protein expression in colorectal carcinoma (Caco-2 and HCT 116) cells and HCEC

Western blot analysis was used to measure the expression of TRPM8 protein in Caco-2, HCT 116 and HCEC cells. TRPM8 protein was more expressed in Caco-2 than in HCT 116 cells; no significant differences between Caco-2 cells and HCEC were observed (Supplementary Figure 1, available at *Carcinogenesis* Online).

CBG antagonism at human TRPM8 channels

CBG has been shown to antagonize TRPM8 in HEK-193 cells over-expressing recombinant rat TRPM8 (rat TRPM8-HEK-293 cells) (21). Here, we verified if this phytocannabinoid behaves as TRPM8 antagonist in HEK-193 cells over-expressing recombinant human TRPM8 (human TRPM8-HEK-293 cells) too. The TRPM8 agonist icilin is known to elevate intracellular Ca^{2+} in human TRPM8-HEK-293 cells, with an EC_{50} of 1.4 µM, whereas it has no effect on HEK-293 cells transfected with the empty plasmid (32). In our experiments, when CBG was given to human TRPM8-HEK-293 cells 5 min before icilin (0.25 µM), it antagonized the Ca^{2+} elevation response. CBG, *per se,* exerted no significant TRPM8-mediated effects on intracellular calcium until the 10 µM concentration. The IC_{50} (±SEM; against icilin 0.25 µM) value of CBG was 0.11 ± 0.02 µM, which is in good agreement with the data in rat TRPM8-HEK-293 cells (21).

The inhibitory effect of CBG on CRC (Caco-2) cells viability is time- and serum protein concentration-dependent

Because the effect of phytocannabinoids on tumoural cells viability is known to be increased with a low serum proteins concentration (33), in the first series of experiments we evaluated the effect of CBG in cells incubated (3–48 h) with either 1% or 10% FBS. By using the MTT assay, we found that CBG (1–30 µM) preferentially inhibited cell viability incubated with 1% FBS rather than in cells incubated with 10% FBS during all the time points considered, with the exception of 1 µM CBG concentration after the 48 h incubation (Figure 1). The different serum concentrations (1% FBS versus 10% FBS) did not affect the cytotoxic action of DMSO [cell viability (%) in presence of 1% FBS: control: 100 ± 5.2; DMSO: 1%, 103.5 ± 6.6; 3%, 95.3 ± 7.5; 5.5%, 54.6 ± 5.8*; 10%, 35.9 ± 3.7*; 20%, 29.2 ± 4.1* and cell viability (%) in presence of 10% FBS: control: 100 ± 4.8; DMSO: 1%, 93.7 ± 6.3; 3%, 83.0 ± 8.3; 5.5%, 58.1 ± 3.4*; 10%, 34.8 ± 3.1*; 20%, 25.2 ± 2.5*. *$P < 0.001$ versus control]. We also found that the effect of CBG on cell viability increased with the time of its incubation. Thus, in the presence of 1% FBS, 3 h after its incubation, CBG exerted a significant cytotoxic effect only at the highest concentration tested (30 µM), whereas after 48 h, a significant inhibitory effect was achieved starting from the 3 µM concentration (Figure 1). A maximal inhibitory effect was achieved after 24–48 h incubation [IC_{50} ± SEM: 3.8 ± 2.1 µM (24 h incubation); 1.3 ± 2.2 µM (48 h incubation)]. Considering the above results and because (i) CBG displayed a well-defined concentration-related effect, (ii) a maximal difference in CBG inhibitory effect between the experiments with 1% FBS and the experiments with 10% FBS was observed (Figure 1C) and (iii) CBG displayed a submaximal IC_{50} value, further experiments were performed at the 24 h time point.

The effect of CBG (1–30 µM, in the presence of 1% FBS) on cell viability was confirmed by using the NR assay in Caco-2 cells. Twenty-four hours after its incubation, CBG reduced cell viability, with a significant effect starting from the 10 µM concentration [cell viability (%): control, 100 ± 4.6; CBG: 1 µM, 99.6 ± 4.7; 3 µM, 97.5 ± 3.7; 10 µM, 75.4 ± 3.5*; 30 µM, 72.2 ± 2.9*. *$P < 0.001$ versus

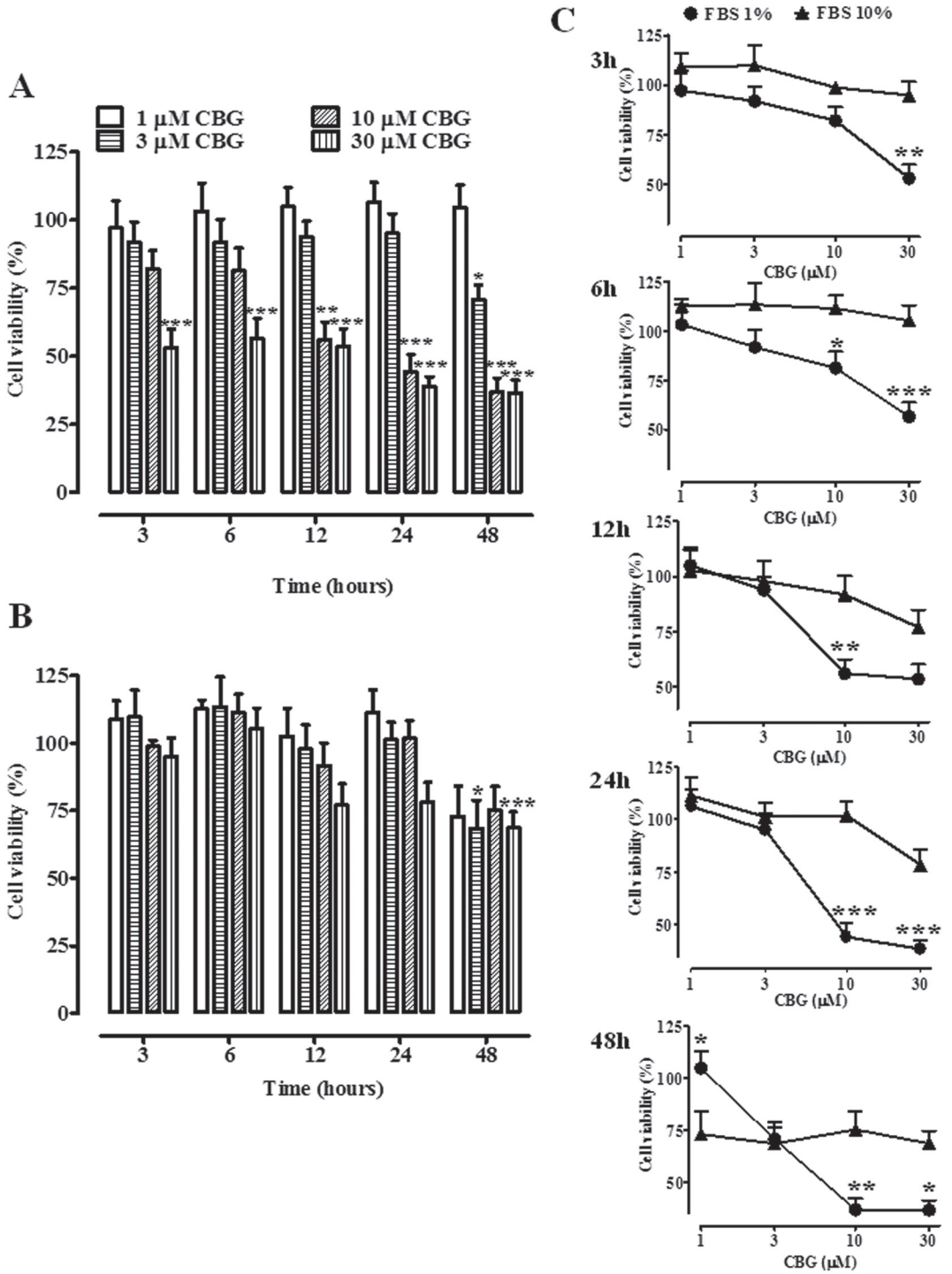

Fig. 1. CBG reduces cell viability, evaluated by the MTT assay, in human CRC (Caco-2) cells in a time- and serum protein concentration-dependent manner. Caco-2 cells were incubated with increasing concentration of CBG (1–30 µM) for 3, 6, 12, 24 and 48 h in a medium containing 1% FBS (**A**) or 10% FBS (**B**). (**C**) The difference between the curves representing the inhibitory effect of CBG in the presence of 1% or 10% FBS, at various incubation times (3–48 h) is shown. Each bar represents the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus control (untreated cells).

control; $n = 3$ experiments including 8–10 replicates for each treatment (IC_{50} ± SEM: 5.97 ± 3.2 µM)].

CBG reduces viability in another CRC cell line, with a very little effect in HCEC

CBG (1–30 µM) also reduced viability in another CRC (i.e. HCT 116) cell line, with a significant inhibitory effect starting from the 3 µM concentration (Supplementary Figure 2A, available at *Carcinogenesis* Online).

To investigate the selectivity of CBG effect in tumoural versus non-tumoural cells, various concentrations (from 1 to 30 µM) of CBG were tested in HCEC. CBG, at a concentration similar to its IC_{50} values in CRC cells (3.8 ± 2.1 µM), did not affect the vitality of HCEC (Supplementary Figure 2B, available at *Carcinogenesis* Online). Only at a concentration of 30 µM (i.e. a concentration that was 7.8-fold higher than the IC_{50} value), CBG exhibited a cytotoxic effect in these non-tumoural cells.

The effect of CBG on CRC (Caco-2) cells viability is mimicked by TRPM8 antagonists

Because CBG is a potent TRPM8 antagonist (21) in this series of experiments, we verified if the effect of CBG was shared by well-established TRPM8 antagonists. We found that, similarly to CBG, the synthetic TRPM8 antagonist AMTB as well as CBD and CBDV (two *Cannabis*-derived TRPM8 antagonists) inhibited, in a concentration-dependent manner, Caco-2 cells viability [IC_{50} (μM) ± SEM: AMTB 9.82 ± 3.9; CBD 3.73 ± 2.3; CBDV 10.09 ± 1.32] (Figure 2A–C). CBC, another phytocannabinoid without activity at the TRPM8 channel (21), inhibited cell growth only at the highest concentration (30 μM) tested (Figure 2D).

The effect of CBG on CRC (Caco-2) cells viability is reduced in TRPM8 silenced cells

To further assess the possible involvement of TRPM8 in CBG action, we performed experiments in Caco-2 cells silenced for the TRPM8. In Caco-2 cells silenced for such channel, the inhibitory effect of CBG on cell viability was significantly reduced in comparison with non-silenced cells (Figure 3).

The effect of CBG on CRC (Caco-2) cells viability is not mimicked by a 5-HT_{1A} antagonist

CBG is a moderately potent 5-HT_{1A} antagonist (20). In contrast with TRPM8 antagonists, the effect of CBG was not mimicked by the 5-HT_{1A} antagonist WAY100635 (up to 1 μM) [cell viability (%): vehicle 100 ± 6.3; WAY100635: 0.2 μM, 97.2 ± 6.2; 1 μM, 95.9 ± 6], thus suggesting the lack of involvement of such receptor.

The effect of CBG on CRC (Caco-2) cells viability is modulated by a CB_2 receptor antagonist and does not involve TRPA1, TRPV1 and TRPV2 channels

Since CBG is a constituent of *Cannabis*, we verified if its effect on Caco-2 cell viability was affected by selective CB_1 and CB_2 receptor antagonists. We found that the CB_1 receptor antagonist AM251 (1 μM) did not modify CBG (10 μM)-induced changes in cell viability (Supplementary Figure 3A, available at *Carcinogenesis* Online). By contrast, the CB_2 receptor antagonist AM630 (1 μM) not only did not counteract but, instead, significantly enhanced the inhibitory effect of CBG (10 μM) on cell viability (Supplementary Figure 3A, available at *Carcinogenesis* Online).

Ruthenium red is a non-selective TRP channel antagonists. Specifically, it blocks TRPA1 (IC_{50}: <1–3 μM), TRPV1 (IC_{50}: 0.09–0.22 μM) and TRPV2 (IC_{50}: 0.6 μM), being the TRPM8 insensitive to its action (34). We found that ruthenium red, at concentrations (10 and 25 μM) several fold higher than the IC_{50} able to block TRPA1, TRPV1 and TRPV2 channels (34), did not modify significantly the inhibitory effect of CBG on cell viability (Supplementary Figure 3B, available at *Carcinogenesis* Online).

The cytotoxic effect of CBG is due to apoptosis rather than necrosis induction

To investigate whether the growth inhibitory effect of CBG was due to induction of apoptosis or necrosis, we examined Caco-2 cell

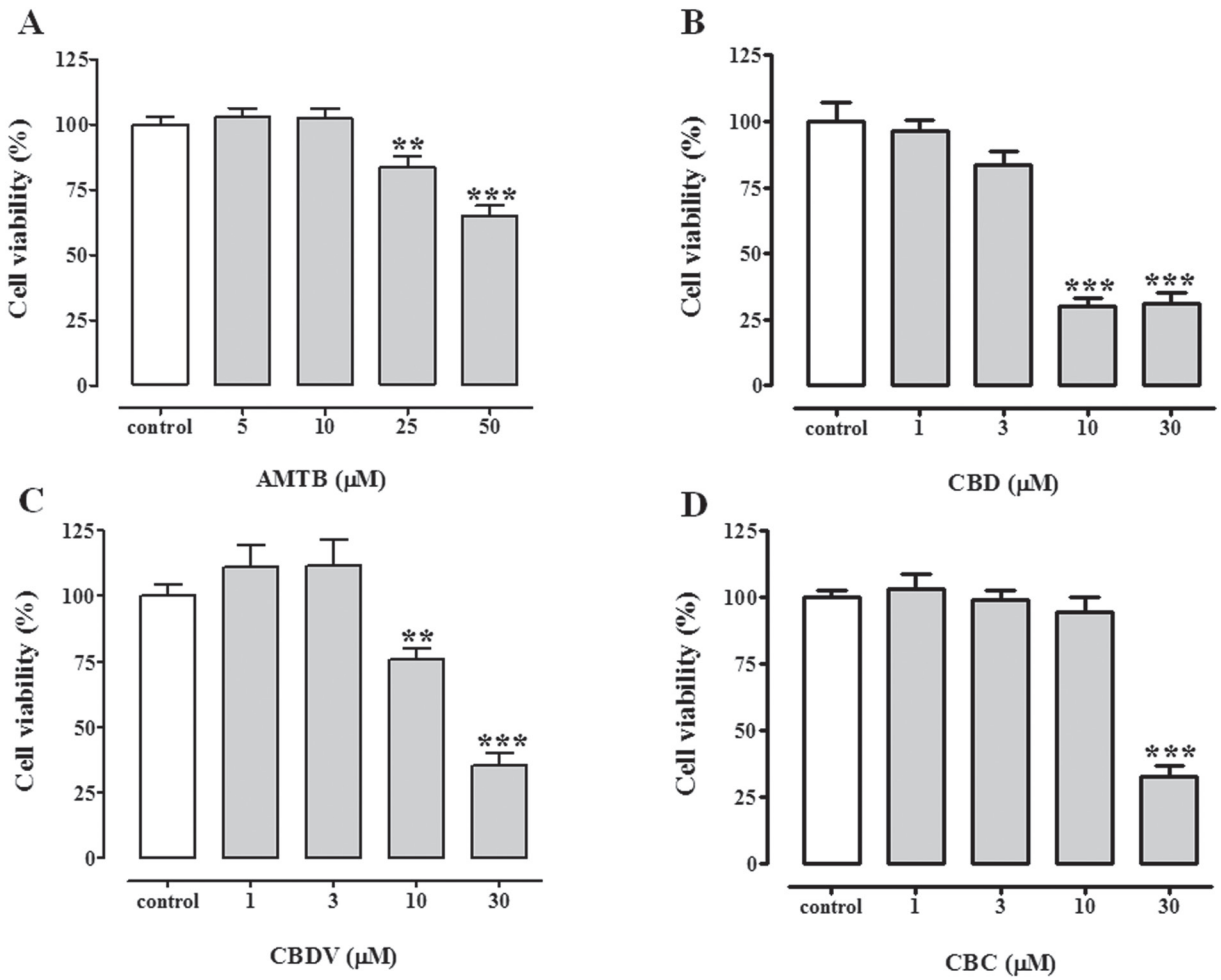

Fig. 2. Effect of AMTB (5–50 μM) (**A**), CBD (1–30 μM) (**B**), CBDV (1–30 μM) (**C**) and CBC (1–30 μM) (**D**) on cell viability, evaluated by the MTT assay, in CRC (Caco-2) cells. Cells were incubated with increasing concentration of compounds (24 h exposure in a 1% FBS medium). Each bar represents the mean ± SEM of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control (untreated cells).

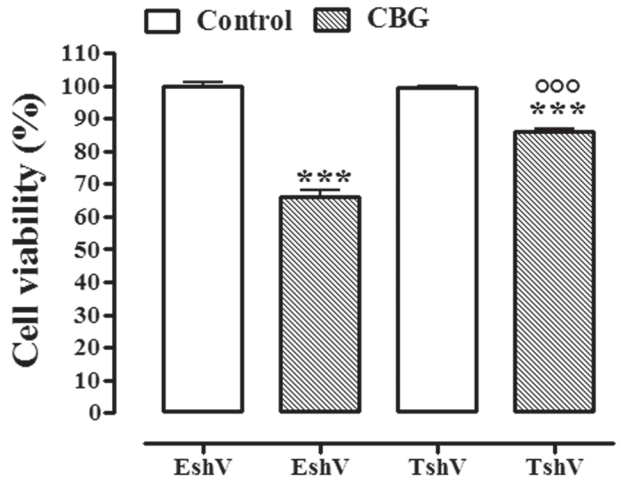

Fig. 3. Inhibitory effect of CBG, evaluated by the MTT assay, on cell viability in human CRC (Caco-2) EshV and in TshV. Cells were incubated with CBG (10 μM, 24 h exposure) in a medium containing 1% FBS. Each bar represents the mean ± SEM of three experiments. ***$P < 0.001$ versus control (untreated cells) and °°°$P < 0.001$ versus CBG-treated cells transfected with empty plasmid (not silenced cells).

death by eosin–haematoxylin staining. As shown in Figure 4A, compared with necrotic cells, the number of apoptotic cells was elevated after CBG treatment (CBG 10 μM: 72 ± 11.0% of apoptotic cells; 17.7 ± 7.2% of necrotic cells; $n = 3$). Morphological assessment revealed absence of death in untreated cells and the presence of cells with a typical apoptotic morphology (i.e. reduced size, hypereosinophilic cytoplasm, hyperchromic nucleus, irregular nuclear membrane and nuclear material outside the nucleus) in cells incubated with CBG.

The induction of apoptosis by CBG was confirmed by caspase 3/7 enzymatic assay, which indicated a 2.43-fold increase of caspase 3/7 activity in CBG-treated Caco-2 cells compared with vehicle (slopes 239.0 versus 98.41, respectively) (Figure 4B) and by the DNA fragmentation assay, which revealed the presence of DNA fragments in CBG-treated, but not in control, cells (Figure 4C).

CBG increases CCAAT/enhancer-binding protein homologous protein mRNA expression in Caco-2 cells but not in Caco-2 TRPM8 small interfering RNA cells

CCAAT/enhancer-binding protein homologous protein (CHOP) is an activating protein of apoptosis and it is induced by endoplasmic reticulum (ER) stress (35). To further confirm the pro-apoptotic effect of CBG—and the involvement of TRPM8 in CBG action—we

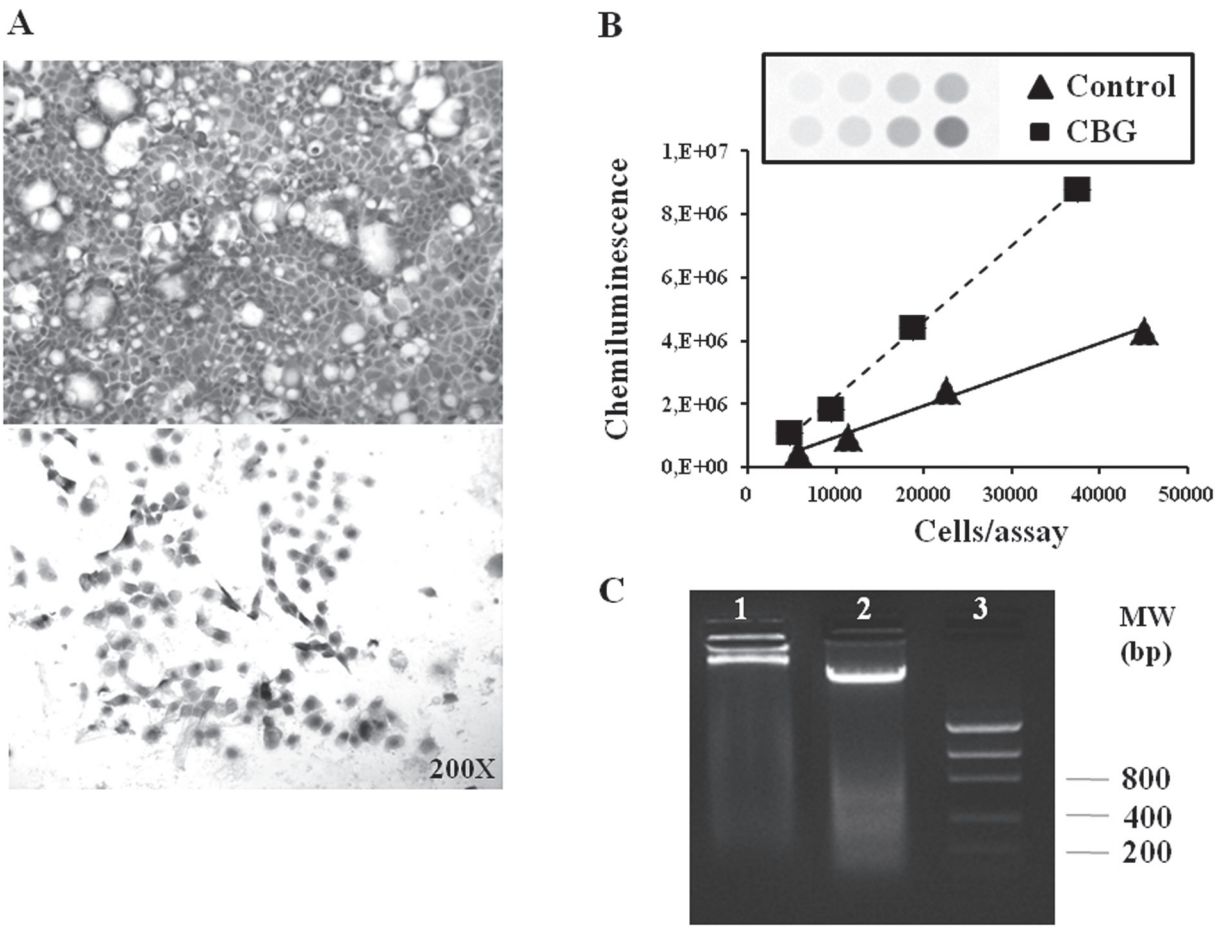

Fig. 4. CBG induces apoptosis in CRC (Caco-2) cells. (**A**) Morphological assessment of CRC (Caco-2) cells evaluated by eosin–haematoxylin staining revealed the absence of death in untreated cells (upper panel) and the presence of cells with a reduced size, showing an hypereosinophilic cytoplasm, hyperchromic nucleus, irregular nuclear membrane and nuclear material outside the nucleus in CBG-treated cells (10 μM, 24 h incubation in a 1% FBS, down panel). Original magnification ×200. The figure is representative of three experiments. (**B**) Increase of caspase 3/7 enzymatic activity evaluated by Caspase-Glo® 3/7 assay. In the plot, each point represents the mean of three independent determination (the mean SEM was not greater of 10% of the graphed value). In the insert panel, a picture of part of the plate is shown. The cell amount in each dot increases from left to right as reported in the plot abscissa. The increase of caspase 3/7 enzymatic activity (2.43-fold) was calculated by the *ratio* of the curve slopes: 239.0 and 98.41 for CBG and vehicle treated cells, respectively. (**C**) Electrophoresis of cellular DNA isolated from untreated cells (control, line 1) and cells exposed to 10 μM CBG (line 2) for 24 h; lane 3, marker. Cellular DNA was extracted and visualized on agarose gel as described in the Materials and methods.

evaluated the effect of this non-psychotropic phytocannabinoid on CHOP mRNA expression. Treatment of cells with CBG (10 µM) caused a dramatic (~16-fold) increase in CHOP mRNA expression (Figure 5A) in Caco-2 cells and, to a less extent (~4-fold increase), in EshV (Figure 5B). In contrast, CBG did not change CHOP mRNA expression in TshV (Figure 5C).

CBG stimulates ROS production in CRC (Caco-2) cells, but not in HCEC

To determine if the apoptotic action of CBG was associated to ROS production, we measured the levels of ROS generation by using the fluorescence-sensitive probe DCFH-DA. We found that 10 µM of CBG significantly increased ROS production in Caco-2 cells

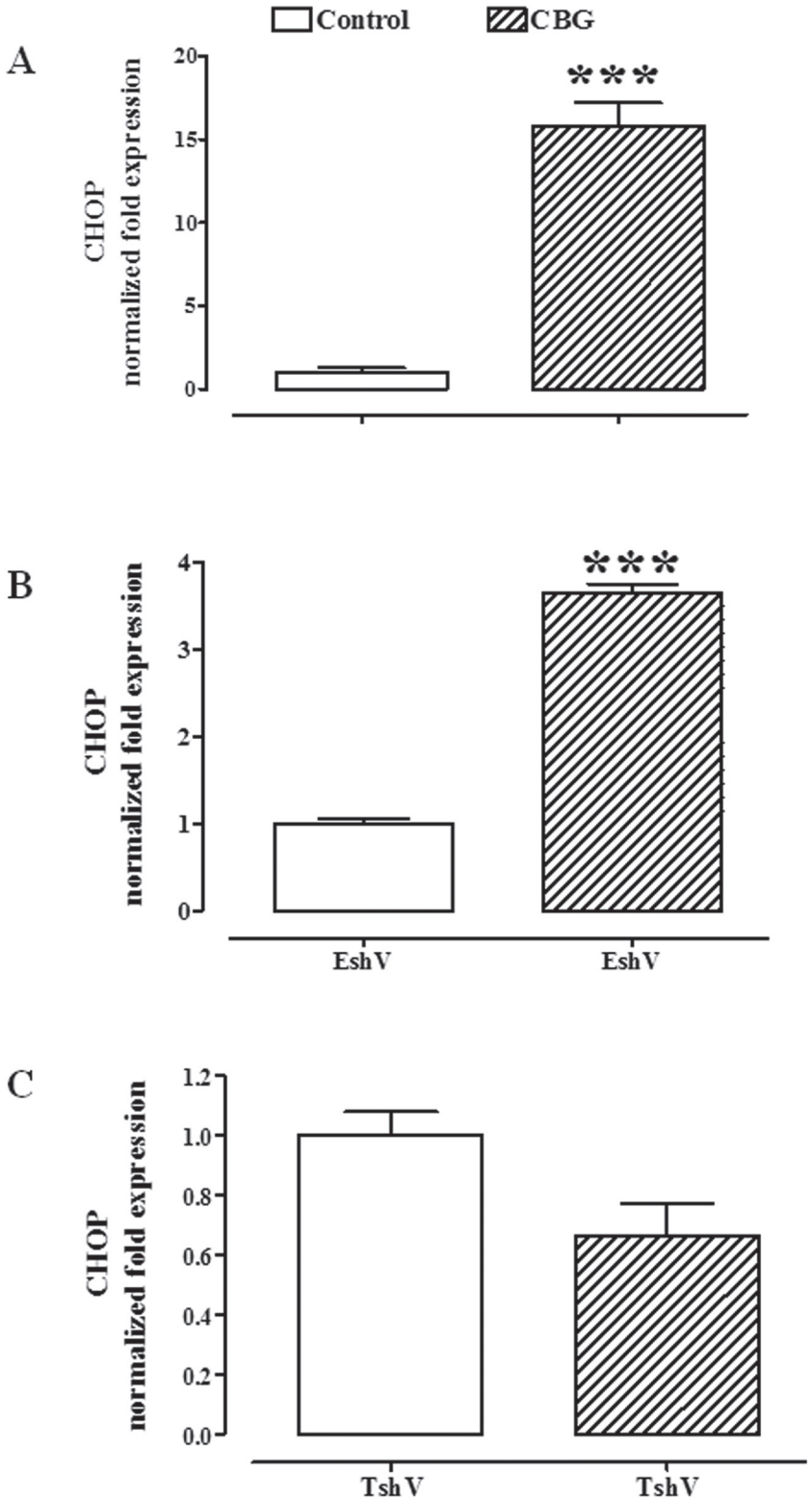

Fig. 5. Effect of CBG on CHOP mRNA expression in human CRC (Caco-2) cells (**A**), in Caco-2 EshV (**B**) and in TshV (**C**). Cells were incubated with CBG (10 µM, 24 h exposure) in a medium containing 1% FBS. Each bar represents the mean ± SEM of three experiments. ***$P < 0.001$ versus control (untreated cells).

(fluorescence intensity: control: 1.11 ± 0.05; CBG: 10 µM, 1.34 ± 0.04***; ***$P < 0.001$, $n = 6$ experiments) but not in HCEC (fluorescence intensity: control: 0.89 ± 0.11; CBG: 10 µM, 0.93 ± 0.13; $n = 6$). Fenton's reagent (2 mM of H_2O_2/Fe^{+2}), used as a positive control, increased ROS production both in Caco-2 cells and in HCEC (data not shown).

CBG reduces tumour growth induced by xenograft injection of CRC cells

We determined the potential *in vivo* antitumoural curative effect of CBG by inoculating subcutaneously CRC cells in athymic nude mice. When the tumour volumes were assessed on day 10 after inoculation, all group of animals were found to have developed subcutaneous tumours, with a mean volume (±SEM) of 604 ± 39 mm^3. Following intraperitoneal injection with CBG (1–10 mg/kg), a marked inhibition of the growth of the xenografted tumours was observed, the effect being significant for the 3 and 10 mg/kg doses (Figure 6A). The differences in tumour volumes between the vehicle and the 3 or 10 mg/kg CBG treatment group were statistically significant from day 3 of treatment to the end of the experiment. After 5 days of drug administration, the average tumour volume in the control group was 2500 ± 414 mm^3, whereas the average tumour volume in the 3 mg/kg CBG-treated group was 1367 ± 243, exhibiting a 45.3% inhibition of tumour growth (Figure 6A).

CBG exerts chemopreventive effects in the murine model of colon cancer generated by AOM

AOM treatment resulted in the formation of ACF, polyps and tumours (Figures 6B and D). It has been suggested that larger ACF (containing four or more crypts per focus) have higher risk for malignant tumour progression. Thus, only *foci* with four or more crypts were analysed. Compared with the AOM group, CBG (1 and 5 mg/kg)-treated animals showed a reduced number of ACF (Figure 6B). Notably, at the 5 mg/kg dose, CBG completely suppressed the formation of ACF. CBG did not affect significantly polyp formation, but, at least at the 5 mg/kg dose, it reduced by one half the number of tumours (Figure 6C and D).

Discussion

Phytocannabinoids are currently discussed as potential new anticancer drugs (10). Besides the robust experimental evidence pointing to a direct antitumour action, the lack of severe adverse side effects of many phytocannabinoids as compared with conventional chemotherapeutic drugs strongly support their use. In this study, we have shown that CBG, a safe non-psychotropic phytocannabinoid able to block TRPM8 channels, exerts pro-apoptotic effects in CRC cells as well as chemopreventive (AOM model) and curative (xenograft model) actions in experimental models of colon cancer *in vivo*.

It is well established that Δ^9-tetrahydrocannabinol as well as synthetic and endogenous CB receptor agonists target key signalling

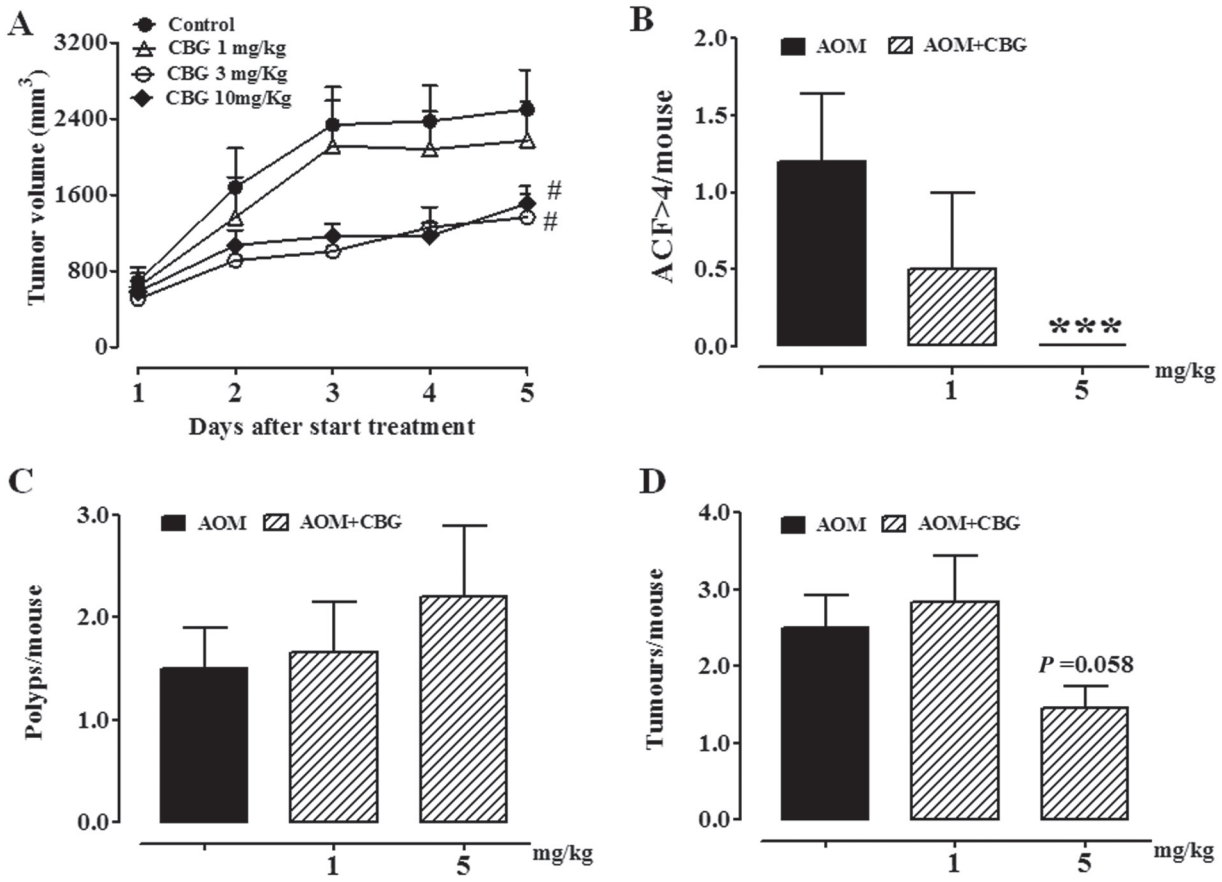

Fig. 6. CBG reduces colon carcinogenesis *in vivo*. (**A**) Inhibitory effect of CBG (1–10 mg/kg) on xenograft formation induced by subcutaneous injection of HCT 116 cells into the right flank of athymic female mice. Treatment started 10 days after cell inoculation (i.e. once tumours had reached a size of 550–650 mm^3). Tumour size was measured every day by digital caliper measurements, and tumour volume was calculated. CBG (1–10 mg/kg, intraperitoneally) was given every day for the whole duration of the experiment. (**B**–**D**) Inhibitory effect of CBG (1 and 5 mg/kg) on ACF with four or more crypts (ACF ≥ 4 per mouse) (B), polyps (C) and tumours (D) induced in the mouse colon by AOM. AOM (40 mg/kg in total, intraperitoneally) was administered, at the single dose of 10 mg/kg, at the beginning of the first, second, third and fourth week. CBG was given (intraperitoneally) 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. Results represent the mean ± SEM of 9–11 mice.***$P < 0.001$ versus AOM alone. $^{\#}P < 0.001$ versus control.

pathways involved in carcinogenesis (36). However, the clinical use of Δ^9-tetrahydrocannabinol and other CB agonists is often limited by their unwanted psychoactive side effects. For this reason, interest in non-psychoactive phytocannabinoids, that are plant-derived cannabinoids with low affinity for CB receptors, has substantially increased in recent years (37). The most studied among non-psychotropic phytocannabinoids is CBD, which has been shown to induce apoptosis in human leukaemia cells (38), to decrease the growth of breast carcinoma and lung metastasis in rodents (16,39,40), to reduce the formation of glioma (41) and the viability of bladder cancer cells (42), and to synergize with cytotoxic agents in glioblastoma cells (43).

The other non-psychotropic phytocannabinoids have been poorly investigated to date. Concerning CBG, previous investigators have shown that this phytocannabinoid inhibited keratinocyte proliferation (17) and induced cell death in high dosage in human epithelioid carcinoma cells (15). In this study, we have shown that this phytocannabinoid reduced viability in two colorectal carcinoma cell lines, that is, Caco-2 and HCT 116 cells. The higher potency of CBG in HCT 116 cells compared with Caco-2 cells remains to be explained and cannot be attributed to the different expression of TRPM8 between the two cell lines (see Discussion below). Furthermore, CBG displayed higher potency and efficacy when tested in the presence of low serum concentrations (1% concentration that does not affect, *per se*, cell viability), suggesting that the presence of the serum proteins in the medium counteracts the inhibitory effect of the phytocannabinoid on cells viability. Such observation is in agreement with previous investigations of cannabinoids in glioma and prostate cells (44,45). Moreover, we exclude that the higher cytotoxic effect of CBG, in the presence of low serum concentrations, is due to an increased sensibility of cells because the effect of the cytotoxic substance DMSO was not modified in presence of low (1%) or high (10%) FBS concentrations. Importantly, the effect of CBG was rather selective for colorectal carcinoma cells, showing the phytocannabinoid a very low inhibitory action on HCEC.

Because CBG is an antagonist of TRPM8 (21), we first investigated the possible involvement of such channels in CBG mode of action. TRPM8 is involved in the regulation of cell proliferation/apoptosis (22) and it is now considered as a promising target for cancer, particularly for prostate cancer. TRPM8 mRNA has been detected in a number of primary tumours, including CRC tissues (45). We have here reported, for the first time, that TRPM8 mRNA and protein are expressed in CRC cells, with higher expression of TRPM8 in Caco-2 cells compared with HTC 116 cells. More importantly, we have found that the effect of CBG on cell viability was mimicked by the synthetic TRPM8 antagonist AMTB, by CBD and CBDV (two phytocannabinoid, which share the ability of CBG to block the TRPM8). CBG was as potent as CBD and both phytocannabinoids were more potent than CBDV or AMTB. In contrast, CBC, a phytocannabinoid, which does not block the TRPM8 (21), had a negligible effect on colorectal cell viability. Furthermore, silencing of TRPM8 mRNA resulted in a reduced cytotoxic effect of CBG in Caco-2 cells. Collectively, such results suggest that TRPM8 might be involved in CBG-induced inhibition of CRC cell growth. Finally, we have demonstrated that CBG exerted a very weak cytotoxic effect in HCECs.

To further explore the mode of CBG action, we considered the other receptors (i.e. CB receptors, TRPA1, TRPV1 and TRPV2 channels, and 5-HT_{1A} receptors), which have been shown, based on pharmacodynamic studies, to be targeted by CBG. The results of such experiments are discussed below.

It is well established that CB_1 or CB_2 receptor activation results in inhibition of colorectal cell growth (46–48). CBG has been shown to behave as a weak partial agonist of CB_1 and CB_2 receptors (20). Furthermore, CBG inhibits the reuptake of endocannabinoids, which have been detected in Caco-2 cells (32) and thus might indirectly activate—*via* increased extracellular endocannabinoid levels—the CB receptors. We have here observed that the inhibitory effect of CBG on cell viability was unaffected by the selective CB_1 receptor antagonist AM251 and further increased by the CB_2 receptor antagonist AM630. Such results negate the possibility that CBG acts *via* direct or indirect activation of CB receptors and rather suggest that an endogenous CB_2 tone exists, which may couple negatively to the CBG signalling pathway leading to the inhibition of cell viability. A similar result has been recently observed in peritoneal macrophages, where the inhibitory effect of CBG on LPS-stimulated nitrite production was further augmented by SR144528, another CB_2 receptor antagonist (49).

CBG has been shown to behave as a relatively potent and highly effective TRPA1 agonist and a weak agonist at TRPV1 and TRPV2 channels (21,50). However, it is unlikely that CBG acts *via* activation of TRPA1, TRPV1 and/or TRPV2 channels as ruthenium red, a non-selective TRP channel antagonist, at concentrations which were several fold higher than the IC_{50} able to block TRPA1, TRPV1 and TRPV2 channels, did not modify the effect of CBG on cell viability. Finally, it is very unlikely that the effect of CBG is due to the block of 5-HT_{1A}, a receptor involved in carcinogenesis (51), as CBG effect was not mimicked by a well-established selective 5-HT_{1A} antagonist.

Apoptosis and necrosis are the two major processes leading to cell death (52). Previous investigators have shown that endogenous and plant-derived cannabinoids can induce apoptosis in cancer cells (33,53,54). However, to date, no information for CBG exists. By using eosin–haematoxylin staining, we have shown that the inhibitory effect of CBG on cell growth was due to apoptosis induction rather than necrosis. The pro-apoptotic effect of CBG was confirmed by the increased activity of caspase 3/7 (two cysteine proteases specifically involved in apoptosis) (55), by cleavage of DNA into fragments and by the increased mRNA expression of CHOP (an activating protein of apoptosis). Interestingly, the effect of CBG on CHOP mRNA expression was abolished in TRPM8 small interfering RNA Caco-2 cells, which is suggestive of an involvement of such channel in the pro-apoptotic action of this phytocannabinoid.

ROS are highly reactive molecules, generally derived from the normal metabolism of oxygen, that are produced primarily in mitochondria. Although basal ROS levels are considered to be physiological regulators of cell proliferation and differentiation, in balance with biochemical antioxidants, high levels of ROS trigger a series of mitochondria-associated events leading to apoptosis (56,57). The relationship between ROS and cancer has been also emphasized by the observation that many chemopreventive agents may be selectively toxic to tumour cells because they increase oxidant stress and enhance ROS generation, which in turn, causes apoptosis of cancer cells (58). In this study, we have shown that CBG, at the same concentration, able to exert pro-apoptotic effects (see above) selectively increased ROS production in CRC cells but not in healthy colonic cells, thus suggesting that ROS overproduction might be implicated in CBG-induced apoptosis. Because TRPM8 has been detected on the ER lumen (59) and because ER stress induces the production of ROS and of the pro-apoptotic protein CHOP (60,61), which is upregulated by CBG (present results), we hypothesize, although we are unable to prove it, that ER might be one of the sources of ROS.

In view of our CRC cell data demonstrating pro-apoptotic effects of CBG, we further evaluated its antineoplastic effect in preclinical models of colon carcinogenesis *in vivo*. We observed that mice daily injected with 3 and 10 mg/kg CBG showed a reduced growth of xenografts induced by inoculation of CRC cells. Although xenograft models have a long history in drug discovery, xenograft tumours do not evolve *in situ* and, thus, lack the appropriate cellular interactions with the host microenvironment. This prompted us to confirm the antineoplastic effects of CBG in the AOM model of colon carcinogenesis, in which the tumour grows within the colonic tissue. By using this experimental model of colon cancer, we have recently shown that a pharmacological enhancement of endocannabinoid levels reduces the development of precancerous lesions (62) and that CBD, another phytocannabinoid, exerts chemopreventive effects (32). We found that CBG, at the 5 mg/kg dose, completely abrogated the formation of ACF, had no effect on polyp formation and reduced by one half the number of tumours induced by AOM in mice. At the same doses, CBD was able to reduce significantly pre-neoplastic lesions, polyps and tumours, although the effect was not related to the doses used. Interestingly, CBG, at the 5 mg/kg dose, has been recently shown to

reduce experimental colitis in mice (49), which is relevant in the light of the well-established association existing between intestinal inflammation and colon cancer development.

In conclusion, our data show that the non-psychotropic *Cannabis* ingredient CBG inhibits the growth of CRC cells mainly *via* a pro-apoptotic mechanism and hinders the development and the growth of colon carcinogenesis *in vivo*. The inhibitory effect of CBG on tumoural cell growth is associated to ROS overproduction and is mimicked by other TRPM8 antagonists, thus suggesting that such receptor might be, at least in part, involved in its actions. In view of the safety of *Cannabis*-derived cannabinoids, we hypothesize that CBG may be a promising anti-CRC therapeutic agent, both for prevention and as a curative medicine.

Supplementary material

Supplementary data, Tables I and II and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

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