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

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

Running title: Cannabigerol inhibits colon carcinogenesis

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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; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulphoxide; EMEM, minimum essential medium; ER, endoplasmatic reticulum; EshV, cell electroporated by the “empty”-shRNA vector; FBS, foetal bovine serum; HCEC, human healthy colonic epithelial cell line; MTT, 3-(4,5-dimethylthiazol-2-
yl)-2,5-diphenyltetrazolium bromide; NR, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride; ROS, reactive oxygen species; RR, ruthenium red; Δ⁹-THC, Δ⁹-tetrahydrocannabinol; TRP, transient receptor potential; TshV, cell electroporated by a shRNA-vector targeted to TRPM8.
Cannabigerol (CBG) is a safe non-psychotropic Cannabis-derived cannabinoid 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-HT_{1A} receptors and inhibits the reuptake of endocannabinoids. Here, we investigated whether CBG protects against colon tumorigenesis. Cell growth was evaluated in colorectal cancer cells using the MTT and NR assays; apoptosis was examined by histology and by assessing caspase 3/7 activity; ROS production by a fluorescent probe; cannabinoid (CB) receptors, TRP and CHOP mRNA expression were quantified by RT-PCR; shRNA-vector silencing of TRPM8 was performed by electroporation. The _in vivo_ antineoplastic effect of CBG was assessed using mouse models of colon cancer. Colorectal cancer cells expressed TRPM8, CB_{1}, CB_{2}, 5HT_{1A} receptors, TRPA1, TRPV1 and TRPV2 mRNA. CBG promoted apoptosis, stimulated ROS production, up-regulated CHOP mRNA and reduced cell growth in colorectal cancer 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 tumors as well as chemically-induced colon carcinogenesis. CBG hampers colon cancer progression _in vivo_ and selectively inhibits the growth of colorectal cancer cells, an effect shared by other TRPM8 antagonists. CBG should be considered translationally in colorectal cancer prevention and cure.

**Summary** The non-psychotropic _Cannabis_ ingredient cannabigerol – as well as other TRPM8 antagonists - inhibits the growth of colorectal cancer cells mainly via a pro-apoptotic mechanism and hinders the development and the growth of colon carcinogenesis _in vivo_.

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 approximately 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 United States and many other parts of the world. Accordingly, novel therapeutic approaches, including chemopreventive measures, are urgently needed (5).

The plant Cannabis sativa contains over 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 Δ⁹-tetrahydrocannabinol (Δ⁹-THC) 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, anti-inflammatory, antibacterial, antidepressant and anti-hypertensive 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 colorectal cancer 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 CB2 receptors (20), inhibits the reuptake of endocannabinoids (21), is a potent 5-HT1A 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 1) investigated the effect and the mode of action of CBG on colorectal carcinoma cells growth, 2) evaluated its possible chemopreventive action in the azoxymethane model of colon cancer and 3) assessed its possible curative effect in the xenograft model of colon cancer.

Materials and methods

Chemicals

Azoxymethane (AOM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-amino-7(dimethylamino)-2-methylphenazine hydrochloride (neutral red solution, NR), ruthenium red, icilin, 2',7'-dichlorofluorescin diacetate (DCF-HA) and ethidium bromide solution were purchase from Sigma (Milan, Italy); AM251, AM630, capsazepine, GW9662 and AMTB hydrochloride were obtained from Tocris Cookson (Bristol, UK). Matrigel™ was obtained from BD Biosciences (Buccinasco, Milan, Italy). All reagents for cell cultures were obtained from Sigma (Milan, Italy), Bio-Rad Laboratories (Milan, Italy) and Microtech Srl (Naples, Italy). The vehicles for in vitro experiments [(0.1% dimethyl sulphoxide (DMSO) v/v 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 cannabigerol-predominant Cannabis sativa plants

A Cannabis sativa chemotype cloned so to have originated from the same plant - and thus with a controlled high amount of CBG - was used. The mechanism that is responsible for the accumulation of CBG in certain phenotypes of Cannabis 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 HPLC analysis (Agilent 1100) using a C18 column (150 x 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%) was assessed by various chromatographic techniques (i.e. HPLC, gas chromatography, melting point, IR). Similarly, cannabidiol (CBD, purity by high-performance liquid chromatography, 99.3 %), cannabidivarin (CBDV, purity by high-performance liquid chromatography, 95.0 %) and cannabichromene (CBC, ethanol solution with 95.0 % of purity by high-performance liquid chromatography) 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, Milan, Italy) 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 (DMEM, for Caco-2, HCT 116 and HCEC) or in minimum essential medium (EMEM, for HEK-293). For Caco-2, HCT 116 cells and HEK-293, media (DMEM or EMEM) 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, DMEM was supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 mM Hepes [4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid], 2mM L-glutamine and 1mM 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 D.L. 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 elettroporation buffer (Bio-Rad) containing 1.5 x 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, LabOomics S.A., Belgium). Transfection efficiency (over 50% at 30 hrs from electroporation) was evaluated by fluorescence microscopy in HEK-293 cells transfected by a GFP plasmid in a parallel experiment. Transcriptional expression of TRPM8 in transfected cells, as evaluated by qRT-PCR (see below), was about 1800 fold higher then the control. After 36 hours from electroporation, CBG antagonism versus human TRPM8 was evaluated in HEK-293 transfected cells, as previously described (21).

**shRNA Transfections**

Caco-2 cells, growth to about 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 3x10^6 cell/ml and 25 µg/ml of a shRNA vector targeted to human TRPM8 (HSH018887-CH GeneCopoeia, LabOomics S.A., Belgium). Replicate samples and controls were growth in medium containing 10 % FBS in 1.6 cm²/ well plates. Transfection efficiency was evaluated by fluorescence microscopy by monitoring GFP expression. After about 36 hrs of culture, adherent cells were treated (or not) by CBG 10 µM and cultured in medium containing 1% FBS. This procedure yielded to about 65 % of TRPM8 mRNA
silencing as assessed by qRT-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 previously described (28). For the comparison of mRNA expression in different cell lines a semi-quantitative absolute determination was performed (for more details see supplementary data).

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).

**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 $1 \times 10^4$ cells per well (Caco-2 cells and HCEC) or $2.5 \times 10^3$ 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 hours. 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, BioRad, Italy). 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 hours. Subsequently, cell 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, BioRad, Italy).
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, 5HT1A receptor antagonist) on cell viability (in Caco-2 cells with 1% FBS medium for 24h) 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, CB₁ receptor antagonist), AM630 (1 µM, CB₂ receptor antagonist) and ruthenium red (10 and 25µM, 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 above described.

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 5x10⁴ cell/disk, for 48 hours 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 analyzed and the histological images were captured with the aid of a light microscope (at 200 X 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, USA) following the manufacturer’s protocol (for more details see supplementary data). 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 Excell-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 Ø 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 PBS and centrifuged at 145 x 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 EDTA, 1 % SDS, 0.5 mg/ml proteinase K) and incubated overnight at 55°C. The suspension was centrifuged (1000 x g for 5 min) in the presence of 5 M NaCl and then the DNA was precipitated in 99.8% v/v ethanol. The isolated DNA was resolved on a 1.5% agarose gel containing ethidium bromide in 40 mM TAE buffer with electrophoresis at 80 V for 25 min. DNA fragments were visualized and photographed under ultraviolet light (ImageQuont 400, GE Heathcare).

Detection of reactive oxygen species (ROS) 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 1x10^4 cells/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 hour with 100 µM DCFH-DA in Hanks’ Balanced Salt Solution containing 1% FBS. The Fenton’s reagent (H₂O₂/Fe²⁺ 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.

Colorectal cancer xenograft model

Colorectal carcinoma HCT 116 cells (2.5x10^6) were injected subcutaneously into the right flank of each athymic mice for a total volume of 200 µl per injection (50% cell suspension in PBS, 50% Matrigel™). At 10 days after inoculation (once tumours had reached a size of 550-650 mm³), 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 = \( \pi/6 \times \text{length} \times \text{width}^2 \)). 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 cannabidiol, a related non-psychotropic cannabinoid, in the xenograft model of cancer (16,30).

**Colorectal cancer azoxymethane (AOM) model**

Mice were randomly divided into the following 4 groups (10 animals/group): group 1 (control) was treated with vehicles; group 2 was treated with azoxymethane (AOM) plus the vehicle used to dissolve cannabigerol (CBG) and groups 3-4 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 cannabidiol, a related non-psychotropic cannabinoid, 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 one week before the first administration of AOM. 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 sacrifice, processed and quantified as previously reported (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, USA) and Excel–windows (for linear regression calculation). Data are expressed as the mean ± standard error (S.E.M.) 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 nonlinear regression analysis using the equation for a sigmoid concentration–
response curve (GraphPad Prism). $P$ values < 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 healthy human colonic epithelial cells
(HCEC)

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 5HT$_{1A}$ more expressed than $CB_1$ receptors,
TRPM8, TRPV2 and TRPA1 (Table I, supplementary data). In HCT 116 all the targets were
expressed, with the exception of $CB_1$ receptors. (Table I, supplementary data). The rank order of
expression was TRPV1> TRPV2> TRPA1, with TRPM8, $CB_2$, 5HT$_{1A}$ receptors very faintly
expressed (expression values very close to background values) (Table I, supplementary data). In
HCEC, TRPV1 channels were highly expressed, $CB_1$, TRPA1 and TRPV2 displayed a low
expression, while TRPM8, $CB_2$ and 5-HT$_{1A}$ receptors were very weakly expressed (Table I,
supplementary data).

TRPM8 protein expression in colorectal carcinoma (Caco-2 and HCT 116) cells and healthy
human colonic epithelial cells (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 (Figure 1, supplementary data).

**CBG antagonism at human TRPM8 channels**

CBG has been shown to antagonise 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 while 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$_{50}$ (±S.E.M.) (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 colorectal cancer (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 hours) either with 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 vs 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; DMSO 3% 95.3±7.5; DMSO 5.5% 54.6±5.8*; DMSO 10% 35.9 ±3.7*; DMSO 20% 29.2±4.1*. Cell viability (%) in presence of 10% FBS: control 100±4.8; DMSO 1% 93.7 ±6.3; DMSO 3% 83.0±8.3; DMSO 5.5% 58.1±3.4*; DMSO 10% 34.8 ±3.1*; DMSO 20% 25.2±2.5*. *P<0.001 vs 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, three hours after its incubation, CBG exerted a significant cytotoxic effect only at the highest concentration tested (30 µM), while 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 hours incubation [IC$_{50}$±S.E.M.: 3.8±2.1 µM (24 h incubation); 1.3±2.2 µM (48 h incubation)]. Considering the above results and since 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; CBG 3 µM 97.5±3.7; CBG 10 µM 75.4±3.5*; CBG 30 µM 72.2±2.9*. *P<0.001 vs control; n=3 experiments including 8–10 replicates for each treatment. [IC$_{50}$±S.E.M.: 5.97±3.2 µM].

CBG reduces viability in another colorectal cancer cell line, with a very little effect in healthy human colonic epithelial cells (HCEC)

CBG (1-30 µM) also reduced viability in another colorectal cancer (i.e. HCT 116) cell line, with a significant inhibitory effect starting from the 3 µM concentration (Figure 2A, supplementary data).

To investigate the selectivity of CBG effect in tumoural vs non-tumoural cells, various concentrations (from 1-30 µM) of CBG were tested in HCEC. CBG, at a concentration similar to its IC$_{50}$ values in colorectal cancer cells (3.8±2.1 µM), did not affect the vitality of HCEC (Figure 2B, supplementary data). 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 colorectal cancer (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 cannabidiol (CBD) and cannabidivarin (CBDV) (two Cannabis-derived TRPM8 antagonists) inhibited, in a concentration-dependent manner, Caco-2 cells viability [IC$_{50}$ (µM)±S.E.M.: AMTB 9.82±3.9; CBD 3.73±2.3; CBDV 10.09±1.32] (Figures 2A-C). Cannabichromene, 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 colorectal cancer (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 colorectal cancer (Caco-2) cells viability is not mimicked by a 5HT$_{1A}$ antagonist

CBG is a moderately potent 5-HT$_{1A}$ antagonist (20). In contrast to 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; WAY100635 1 µM 95.9±6], thus suggesting the lack of involvement of such receptor.

The effect of CBG on colorectal cancer (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 (Figure 3A, supplementary data). 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 (Figure 3A, supplementary data).

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

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 death by eosin-haematoxylin staining. As shown in Figure 4A, compared to 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 to vehicle (slopes 239.0 vs 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 (CHOP) mRNA expression in Caco-2 cells but not in Caco-2 TRPM8 siRNA cells

CCAAT/Enhancer-binding protein homologous protein (CHOP) is an activating protein of apoptosis and it is induced by endoplasmic reticulum stress (35). To further confirm the pro-apoptotic effect of CBG – and the involvement of TRPM8 in CBG action - we evaluated the effect of this non-psychotropic phytocannabinoid on CHOP mRNA expression. Treatment of cells with
CBG (10 µM) caused a dramatic (about 16 fold) increase in CHOP mRNA expression (Figure 5A) in Caco-2 cells and, to a less extent (about 4 fold increase), in cell electroporated by the “empty”-shRNA vector (EshV) (Figure 5B). By contrast, CBG did not change CHOP mRNA expression in cell electroporated by a shRNA-vector targeted to TRPM8 (TshV) (Figure 5C).

**CBG stimulates reactive oxygen species (ROS) production in colorectal cancer (Caco-2) cells, but not in healthy human colonic epithelial cells (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 CBG 10 µM significantly increased ROS production in Caco-2 cells (Fluorescence intensity: control 1.11±0.05; CGB 10 µM 1.34±0.04***; ***P<0.001, n=6 experiments) but not in HCEC (Fluorescence intensity: control 0.89±0.11; CGB 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 colorectal cancer cells**

We determined the potential *in vivo* anti-tumoural curative effect of CBG by inoculating subcutaneously colorectal cancer 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 s.c. tumours, with a mean volume (±S.E.M.) of 604±39 mm^3_. Following i.p. 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 mg/kg and 10 mg/kg doses (Figure 6A). The differences in tumour volumes between the vehicle and the 3 mg/kg 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 azoxymethane (AOM)

AOM treatment resulted in the formation of aberrant crypt foci (ACF), polyps and tumours (Figures 6B-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 (Figures 6C and D).

Discussion

Phytocannabinoids are currently discussed as potential new anticancer drugs (10). Besides the robust experimental evidence pointing to a direct anti-tumour action, the lack of severe adverse side effects of many phytocannabinoids as compared to conventional chemotherapeutic drugs strongly support their use. In the present study, we have shown that CBG, a safe non-psychotropic phytocannabinoid able to block TRPM8 channels, exerts pro-apoptotic effects in colorectal cancer 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 ∆⁹-THC as well as synthetic and endogenous cannabinoid receptor agonists target key signaling pathways involved in carcinogenesis (36). However, the clinical use of ∆⁹-THC and other cannabinoid 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 cannabinoid receptors, has substantially increased in recent years (37). The most studied among non-psychotropic phytocannabinoids is cannabidiol, which has been shown to induce apoptosis in human leukemia 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 the present study, we have shown that this phytocannabinoid reduced viability in two colorectal carcinoma cell lines, i.e. Caco-2 and HCT 116 cells. The higher potency of CBG in HCT 116 cells compared to Caco-2 cells remains to be explained and cannot be attributed to the different expression of TRPM8 between the two cell lines (see 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 since 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 healthy human colonic epithelial cells.

Because CBG is an antagonist of TRPM8 (21), we firstly 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 colorectal cancer tissues (45). We have here reported, for the first time, that TRPM8 mRNA and protein are expressed in colorectal cancer cells, with higher expression of TRPM8 in Caco-2 cells compared to 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 cannabidiol and
cannabidivarin (two phytocannabinoids 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. By contrast, cannabichromene, 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 colorectal cancer cell growth. Finally, we have demonstrated that CBG exerted a very weak cytotoxic effect in healthy human colonic epithelial cells.

To further explore the mode of CBG action, we considered the other receptors (i.e. cannabinoid receptors, TRPA1, TRPV1 and TRPV2 channels, and 5HT1A 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 CB1 or CB2 receptor activation results in inhibition of colorectal cell growth (46-48). CBG has been shown to behave as a weak partial agonist of CB1 and CB2 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 cannabinoid receptors. We have here observed that the inhibitory effect of CBG on cell viability was unaffected by the selective CB1 receptor antagonist AM251 and further increased by the CB2 receptor antagonist AM630. Such results negate the possibility that CBG acts via direct or indirect activation of cannabinoid receptors and rather suggest that an endogenous CB2 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 CB2 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 since 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), since 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 (34,53,54). 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 siRNA 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 the present study, we have shown that CBG, at the same concentration able to exert pro-apoptotic effects (see above) selectively increased ROS production in colorectal cancer 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 endoplasmatic reticulum (ER) lumen (59) and because ER stress induces the production of ROS and of the pro-apoptotic protein CHOP (60,61), which is up-regulated by CBG (present results), we hypothesise, although we are unable to prove it, that ER might be one of the sources of ROS.

In view of our colorectal cancer 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 colorectal cancer 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 cannabidiol, 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 preneoplastic 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 cannabigerol inhibits the growth of colorectal cancer 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 overproduction of ROS 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 hypothesise that CBG may be a promising
anti-colorectal cancer therapeutic agent, both for prevention and as a curative medicine.

Conflict of interest: This study was partly supported by GW Pharmaceuticals (Porton Down,
Wiltshire, UK).

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**Figure legends**

**Fig. 1.** Cannabigerol (CBG) reduces cell viability, evaluated by the MTT assay, in human colorectal cancer (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 hours in a medium containing 1% FBS (A) or 10% FBS (B).

Figure 1C shows the difference between the curves representing the inhibitory effect of CBG in the presence of 1% FBS or 10% FBS, at various incubation times (3-48 h). Each bar represents the mean ± S.E.M. of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs control (untreated cells).
Fig. 2. Effect of AMTB (5-50 μM) (A), cannabidiol (CBD, 1-30 μM) (B), cannabidivarin (CBDV, 1-30 μM) (C) and cannabichromene (CBC, 1-30 μM) (D) on cell viability, evaluated by the MTT assay, in colorectal cancer (Caco-2) cells. Cells were incubated with increasing concentration of compounds (24 h exposure in a 1% FBS medium). Each bar represents the mean ± S.E.M. of three independent experiments. **P<0.01 and ***P<0.001 vs control (untreated cells).

Fig. 3 Inhibitory effect of cannabigerol (CBG), evaluated by the MTT assay, on cell viability in human colorectal cancer (Caco-2) cells electroporated by the “empty”-shRNA vector (EshV) and in cell electroporated by a shRNA-vector targeted to TRPM8 (TshV). Cells were incubated with CBG (10 μM, 24 h exposure) in a medium containing 1% FBS. Each bar represents the mean±S.E.M. of three experiments. ***P<0.001 vs control (untreated cells) and °°°P<0.001 vs CBG treated cells transfected with empty plasmid (not silenced cells).

Fig. 4. Cannabigerol (CBG) induces apoptosis in colorectal cancer (Caco-2) cells. (A) Morphological assessment of colorectal cancer (Caco-2) cells evaluated by eosin-haematoxylin staining revealed the absence of death in untreated cells (upper pannel) 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 pannel). Original magnification x 200. The figure is representative of 3 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 standard error 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 hours; lane 3, marker. Cellular DNA was extracted and visualized on agarose gel as described in the materials and methods section.
**Fig. 5.** Effect of cannabigerol (CBG) on CHOP mRNA expression in human colorectal cancer (Caco-2) cells (A), in Caco-2 cells electroporated by the “empty”-shRNA vector (EshV) (B) and in cell electroporated by a shRNA-vector targeted to TRPM8 (TshV) (C). Cells were incubated with CBG (10 µM, 24 h exposure) in a medium containing 1% FBS. Each bar represents the mean±S.E.M. of three experiments. ***P<0.001 vs control (untreated cells).

**Fig. 6.** Cannabigerol (CBG) reduces colon carcinogenesis *in vivo*. Figure 6A reports the 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 mm³). 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. Figure 6B-D reports the inhibitory effect of CBG (1 and 5 mg/kg) on aberrant crypt foci with four or more crypts (ACF≥4/mouse) (B), polyps (C) and tumours (D) induced in the mouse colon by azoxymethane (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 wk. 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 ± S.E.M. of 9-11 mice. ***P<0.001 vs AOM alone. *P<0.001 vs control.
Fig. 1. Cannabigerol (CBG) reduces cell viability, evaluated by the MTT assay, in human colorectal cancer (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 hours in a medium containing 1% FBS (A) or 10% FBS (B).

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190x142mm (300 x 300 DPI)
**Figure 3**

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Fig. 6. Cannabigerol (CBG) reduces colon carcinogenesis in vivo. Figure 6A reports the 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 mm³). 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. Figure 6B-D reports the inhibitory effect of CBG (1 and 5 mg/kg) on aberrant crypt foci with four or more crypts (ACF≥4/mouse) (B), polyps (C) and tumours (D) induced in the mouse colon by azoxymethane (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 wk. 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 ± S.E.M. of 9-11 mice. ***P<0.001 vs AOM alone. #P<0.001 vs control.
TRPM8 channel calcium assays

The effect of CBG on intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was determined by using Fluo-4, a selective intracellular fluorescent probe for Ca\(^{2+}\). On the day of the experiment, cells were loaded for 1 h at room temperature with the methyl ester Fluo-4-AM (4 µM in dimethyl sulfoxide containing 0.02% Pluronic F-127, Invitrogen) in EMEM without foetal bovine serum, then were washed twice in Tyrode’s buffer (145 mM NaCl, 2.5 mM KCl, 1.5 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 10 mM D-Glucose, and 10 mM HEPES, pH 7.4), resuspended in the same buffer, and transferred to the quartz cuvette of the spectrofluorimeter at 22 °C (Perkin-Elmer LS50B equipped with PTP-1 Fluorescence Peltier System; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) under continuous stirring. The changes in [Ca\(^{2+}\)]\(_i\) were determined before and after the addition of various concentrations of test compounds by measuring cell fluorescence (\(\lambda_{\text{EX}} = 488\) nm, \(\lambda_{\text{EM}} = 516\) nm) at 25 °C. Potency was expressed as the concentration of test substances exerting a half-maximal agonist effect (i.e., half-maximal increases in [Ca\(^{2+}\)]\(_i\)) (EC\(_{50}\)). The efficacy of the agonists was first determined by normalizing their effect to the maximum Ca\(^{2+}\) influx effect on [Ca\(^{2+}\)]\(_i\), observed with application of 4 µM ionomycin (Alexis). Antagonist/desensitizing behavior was evaluated by adding CBG in the quartz cuvette 5 min before stimulation of cells with icilin (0.25 µM). Data are expressed as the concentration exerting a half-maximal inhibition of agonist-induced [Ca\(^{2+}\)]\(_i\) elevation (IC\(_{50}\)). which was calculated again using GraphPad Prism® software. The effect on [Ca\(^{2+}\)]\(_i\) exerted by icilin alone was taken as 100%. Dose–response curves were fitted by a sigmoidal regression with variable slope. All determinations were performed at least in triplicate.

Western blot analysis

Caco-2, HCT 116 and HCEC cytosolic lysates were obtained as previously described (1). Briefly, the medium was removed and cells were washed with ice cold PBS. The cells were collected by scraping for 10 min at 4°C with lysis buffer [50mM Tris-HCl pH=7.4, 0.25 % sodium deoxycholate, 150mM NaCl, 1mM EGTA, 1mM NaF, 1 % NP-40, 1mM PMSF, 1mM Na3VO4
containing complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)]. After centrifugation at 16,200 x g for 15 min at 4°C, the supernatants were collected and protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad, Milan, Italy). For western blot analysis, lysate aliquots containing 70 µg of proteins were separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Protran®, Protran Nitrocellulose Transfer Membrane Schleicher & Schuell Bioscience, Dassel, Germany) using a Bio-Rad Transblot (350 mA, 3 hours). Proteins were visualized on the filters by reversible staining with Ponceau-S solution (Sigma) and de-stained in PBS containing 0.1 % Tween 20. Membranes were blocked at 4°C in milk buffer (5% non-fat dry milk in PBS/Tween 0.1 %) and then incubated overnight at 4°C with rabbit polyclonal antibodies for TRPM8 (ENZO Life Sciences, Vinci Biochem S.r.l, Italy) used at 1:500 dilution in milk buffer (5% non-fat dry milk in PBS/Tween 0.1 %). Subsequently, the membranes were incubated for 1 hour at room temperature with 1:3000-dilution of anti-rabbit IgG-Horseradish peroxidase-conjugated secondary antibody (Bio Rad). After washing in PBS/Tween 0.1 %, the membranes were analyzed by enhanced chemiluminescence’s (ECL; Amersham Biosciences (UK). The optical density of the bands on autoradiographic films was determined by an image analysis system (GS 700 Imaging Densitometer, Bio-Rad) equipped with a software Molecular Analyst (IBM). The membranes were probed with an anti β-actin antibody to normalize the results, which were expressed as a ratio of densitometric analysis of TRPM8/β-actin.

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

Caco-2 cells were seeded in 12-well plates at a density of 5x10^4 cells/well. After 48 h, the cells were incubated with medium containing 1% FBS in presence or absence of CBG (10 µM, for 24-h). After incubation, cells were trypsinized, washed with PBS and processed. The assay was performed in 96-well white walled plates, adding to each well 100 µl of Caspase-Glo® 3/7 reagent to100 µl of culture medium containing 5-40 µl of cells suspension (about 1000 cells/µl) in culture medium. The cell suspension concentration was evaluated by a cell counter (Biorad TC10TM) and confirmed by
a DNA assay (Quant-it DNA assay kit, Invitrogen) considering 4 pg DNA/cell. After 1 h incubation in the dark at room temperature, chemiluminescence was measured by a VersaDoc MP System (Bio-Rad) equipped by the Quantity One® version 4.6 software.

**RT-PCR Primer design and comparison of mRNA levels in different cell lines**

Optimized primers for SYBR®-Green analysis (and relative TaOpt) were designed by the AlleleID r® software 7.0 version (Biosoft International, Palo Alto, CA). For each target, in the presence of alternative mRNA splicing, all the validated sequences were aligned and common primer pairs were designed. The primers were synthesized (high-performance liquid chromatography purification grade) by Eurofins-MWG-Synthesis Ebersberg Germany (Table II). A semi-quantitative arbitrary scale to compare the level of mRNA expression in our experimental conditions, was as follows: high expression (HE) from 20 to 25 Cq; middle expression (ME) from 25 to 30 Cq; low expression (LE) from 30 to 33Cq, very low expression (VLE) over 33Cq. Furthermore two quality parameters have been utilized in evaluating expression data: i) the maximum acceptable standard deviation for replicate samples was put ≤ 0.500 (note that at high Cq the standard deviation normally draws to increase); ii) the expression data are significant if Δ (Cqmean-Cqbackground) ≥ 5. Assays were performed in quadruplicate in two independent experiments, by using 20 ng of cDNA (as evaluated from the input RNA used for reverse-transcription).

**Reference**

Figure legends

Fig. 1. TRPM8 protein expression in colorectal carcinoma (Caco-2 and HCT 116) cells and healthy human colonic epithelial cells (HCEC) evaluated, by western blot analysis, in cytosolic lysates. Each bar represents the mean ± S.E.M. of three independent experiments. On the top a representative example of Western blot analysis is reported. ***p< 0.001 vs Caco-2 cells.

Fig. 2. Inhibitory effect of cannabigerol (CBG), evaluated by the MTT assay, on cell viability in human colorectal cancer (HCT116) cells (A) and in healthy human colonic epithelial cells (B). Both cell lines were incubated with increasing concentration of CBG (1-30 µM, 24 h exposure) in a medium containing 1% FBS. Each bar represents the mean±S.E.M. of three independent experiments. ***P<0.001 vs control (untreated cells).

Fig. 3. Cytotoxic effect of cannabigerol (CBG, 10 µM in a 1% FBS medium, 24 h exposure), evaluated by the MTT assay, alone or in the presence of (A) AM251 (1 µM, selective CB1 receptor antagonist), AM630 (1 µM, selective CB2 receptor antagonist) and (B) ruthenium red (RR, 10 and 25 µM, a non-selective TRP channels antagonist) in colorectal cancer (Caco-2) cells. The antagonists were incubated 30 min before vehicle or CBG. Each bar represents the mean ± S.E.M. of three independent experiments. #P<0.05 vs control; *P<0.05 vs CBG alone.
Table I. Detection of CB1, CB2, TRPA1, TRPV1, TRPV2, TRPM8 and 5-HT1A mRNA by quantitative (real-time) RT-PCR analysis in human colorectal carcinoma (Caco-2 and HCT 116) cells and in healthy human colonic epithelial cells (HCEC). Targets were assayed as described in the “Methods” section.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Target Acronymous</th>
<th>HCEC Cq mean (SD)</th>
<th>Caco-2 Cq mean (SD)</th>
<th>HCT 116 Cq mean (SD)</th>
<th>Background Cq NTC (SD)</th>
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<tbody>
<tr>
<td>NM_016083</td>
<td>CB1</td>
<td>33.12 (0.267) VLE</td>
<td>30.86 (0.217) LE</td>
<td>N/A</td>
<td>N/A (N/A)</td>
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<td>NM_001841</td>
<td>CB2</td>
<td>31.71 (0.136) CtB</td>
<td>29.89 (0.388) ME</td>
<td>33.97 (0.177) CtB</td>
<td>36.50 (0.154)</td>
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<td>NM_007332</td>
<td>TRPA1</td>
<td>34.37 (0.259) VLE</td>
<td>32.29 (0.227) LE</td>
<td>34.62 (0.443) VLE</td>
<td>N/A (N/A)</td>
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<td>AF196175</td>
<td>TRPV1</td>
<td>28.05 (0.091) ME</td>
<td>25.86 (0.100) ME</td>
<td>29.67 (0.197) ME</td>
<td>35.88 (0.483)</td>
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<td>NM_016113</td>
<td>TRPV2</td>
<td>34.00 (0.500) VLE</td>
<td>30.19 (0.158) LE</td>
<td>31.10 (0.383) LE</td>
<td>N/A (N/A)</td>
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<td>NM_024080</td>
<td>TRPM8</td>
<td>33.05 (0.519) CtB</td>
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<td>33.10 (0.344) CtB</td>
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<td>NM_000524</td>
<td>5HT1A</td>
<td>31.64 (0.180) CtB</td>
<td>29.25 (0.149) ME</td>
<td>34.62 (0.443) CtB</td>
<td>35.90 (0.310)</td>
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</table>

Cq, quantitative cycles; SD, standard deviation of quantitative cycles; NTC, negative control; minus template; N/A, not applicable, no quantitative cycles detected within 40 repeats. High expression (HE) from 20 to 25 Cq; middle expression (ME) from 25 to 30 Cq; low expression (LE) from 30 to 33Cq, very low expression (VLE) over 33Cq, CtB, close to background. Quality-significance parameters: Δ (Cqmean-Cqbackground) ≥ 5; replicate samples Cq Std dev ≤ 0.500.
Table II. Sequences of primers

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<th>TARGETED GENE</th>
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<th>REVERSE PRIMER SEQUENCE (5'-3')</th>
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<td>5-HT₁A</td>
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<td>TAGCAAAACGTCCTCAAT</td>
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<td>RNA POL</td>
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<td>H. Sapiens gene for RNA polymerase II subunit 14.4 kD</td>
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