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ORIGINAL ARTICLE

Cannabinoid receptor-independent cytotoxic effects of cannabinoids in human colorectal carcinoma cells: synergism with 5-fluorouracil

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Abstract Cannabinoids (CBs) have been found to exert antiproliferative effects upon a variety of cancer cells, including colorectal carcinoma cells. However, little is known about the signalling mechanisms behind the antitumoural effect in these cells, whether the effects are shared by endogenous lipids related to endocannabinoids, or whether such effects are synergistic with treatment paradigms currently used in the clinic. The aim of this preclinical study was to investigate the effect of synthetic and endogenous CBs and their related fatty acids on the viability of human colorectal carcinoma Caco-2 cells, and to determine whether CB effects are synergistic with those seen with the pyrimidine antagonist 5-fluorouracil (5-FU). The synthetic CB HU 210, the endogenous CB anandamide, the endogenous structural analogue of anandamide, *N*-arachidonoyl glycine (NAGly), as well as the related polyunsaturated fatty acids arachidonic acid and eicosapentaenoic acid showed antiproliferative and cytotoxic effects in the Caco-2 cells, as measured by using [^{3}H]-thymidine incorporation assay, the CyQUANT proliferation assay and calcein-AM fluorescence. HU 210 was the most potent compound examined, followed by anandamide, whereas NAGly showed equal potency and efficacy as the polyunsaturated fatty acids. Furthermore, HU 210 and 5-FU produced synergistic effects in the Caco-2 cells, but not in the human colorectal carcinoma cell lines HCT116 or HT29. The compounds examined produced cytotoxic, rather than antiproliferative effects, by a mechanism not involving CB receptors, since the CB receptor antagonists AM251 and AM630 did not attenuate the effects, nor did pertussis toxin. However, α-tocopherol and the nitric oxide synthase inhibitor L-NAME attenuated the CB toxicity, suggesting involvement of oxidative stress. It is concluded that the CB system may provide new targets for the development of drugs to treat colorectal cancer.

Keywords Cannabinoids · Polyunsaturated fatty acids · 5-Fluorouracil · Colorectal cancer · Cytotoxicity

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Introduction

Colorectal cancer (CRC) is considered to be one of the major causes of cancer death in the Western world [39], and it is important to find new approaches to prevent or treat this type of cancer. The discovery of the cannabinoid (CB) signalling system, comprising CB receptors, endogenous ligands and enzymes for ligand biosynthesis and inactivation, has led to the identification of new targets for therapeutic drugs [38]. One of the most promising areas of current research in the therapeutic potential of CBs is cancer. The antiproliferative effect of CBs was first observed over 30 years ago [35], and a growing body of evidence suggests that CBs compounds have antitumoural effects by decreasing the viability, proliferation, adhesion and migration of a variety of cancer cells, as well as modulating angiogenesis and metastasis [3].

Endocannabinoids are formed in mammalian colon tissue [25] and the levels are greatly increased when normal mucosa is transformed to adenomatous tissue [28]. The endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), as well as the AEA uptake inhibitor

VDM11, have been reported to reduce cell viability of human colorectal Caco-2 carcinoma cells by a CB_1 receptor-mediated mechanism [28]. These observations suggest that targeting the endocannabinoid system may be an important therapeutic approach to treat CRC. In addition, plant-derived and synthetic CBs have also been shown to inhibit CRC cell viability [14, 28].

Cannabinoids can exert effects in the body not only as a result of their activation of the seven transmembrane CB_1 and CB_2 receptors [31, 34], but also by interaction with other targets, such as the endothelial non-CB_1, non-CB_2 receptors, for a review see [4]. The situation is even more complicated for the endogenous CBs AEA and 2-AG, which can interact with ion channels such as vanilloid TRPV1 receptors [8] and ligand-gated transcription factors such as the peroxisome proliferator-activated receptors [37], PPARα [26] and PPARγ [29]. In addition, these compounds can be metabolised to biologically active compounds by the enzymes fatty acid amide hydrolase (FAAH) [13], cyclooxygenase (COX)-2 [51], lipoxygenases (LOX) [10, 17, 49], monoacylglycerol lipase [9], and cytochrome P450 enzymes [47]. Thus, there are a variety of potential mechanisms and pathways to account for the effects of (endo)cannabinoids upon cell viability.

Since mechanisms underlying the effects of CBs in CRC cells are incompletely understood, this preclinical study was undertaken to determine whether (1) synthetic (HU 210) and endogenous (AEA) CBs and related polyunsaturated fatty acids [arachidonic acid (AA), eicosapentaenoic acid (EPA), *N*-arachidonoyl glycine (NAGly)] have similar effects on the proliferation of human CRC cells, (2) CBs produce synergistic actions with standard treatment paradigms such as 5-fluorouracil (5-FU), a pyrimidine antagonist used in the adjuvant treatment of CRC, and (3) the effects are mediated by CB receptors, the ceramide pathway or by other pathways involving FAAH-, COX-, or LOX-catalysed metabolites.

Materials and methods

Chemicals

AM251 (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl] (4-methoxyphenyl)methanone), HU 210 ((6aR)-*trans*-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol), NG-nitro-L-arginine methyl ester (L-NAME) hydrochloride, NAGly, 7-nitroindazole (7-NI) and SB366791 (4'-chloro-3-methoxycinnamanilide) were obtained from Tocris Bioscience (Bristol, UK). Anandamide (*N*-(2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; AEA), HU 211 (3-(1,1-dimethylheptyl)-6aS, 710,10aS-tetrahydro-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol), nimesulide (*N*-4-(nitro-2-phenoxyphenyl)-methanesulfonamide) and URB 597 ((3'-(aminocarbonyl [1,1'-biphenyl]-3-yl)-cyclohexylcarbamate) were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Cinnamyl-3-4-dihydroxy-α-cyanocinnamate (CDC) was obtained from Biomol International (Plymouth Meeting, PA, USA). AA, cell dissociation solution, EPA, 5-FU, forskolin (7-beta-acetoxy-8,13-epoxy-1-alpha, 6-beta, 9-alpha-trihydroxylabd-14-en-11-one), fumonisin B_1 (FB_1), IBMX (3-isobutyl-1-methylxanthine), ISP-1 ((2S,3R,4R,6E)-2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxo-6-eicosenoic acid; myriocin), PD 098,059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one), pertussis toxin (PTx) and (+)-α-tocopherol (2,5,7,8-tetramethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol) were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Calcein-AM and CyQUANT proliferation assay kits were purchased from Invitrogen Molecular Probes (Eugene, OR, USA). Ascorbic acid and Triton X-100 were obtained from MERCK (Darmstadt, Germany). Foetal bovine serum (FBS), glutamine, minimal essential medium (MEM) containing Earle's salts and L-glutamine, non essential amino acids (NEAA), and penicillin-streptomycin (PEST) were purchased from Invitrogen Life technologies (Uppsala, Sweden). [methyl-^{3}H]-thymidine (specific activity 48 Ci/mmol) was purchased from GE Healthcare (Uppsala, Sweden).

Cell cultures

Human colon adenocarcinoma Caco-2 cells (passages 81-106) were cultured in MEM, supplemented with 10% FBS, 100 U/ml penicillin + 100 U/ml streptomycin (1% PEST), 1% NEAA and 2 mM glutamine. The Caco-2 cells were a kind gift from Dr Jonas Nilsson, Department of Molecular Biology, Umeå University, Sweden. Human colon cancer HCT116 and HT29 cells, cultured in Dulbecco's MEM + GlutaMAX™-1 supplemented with 10% FBS and 1% PEST, were a kind gift from Dr Richard Palmqvist, Department of Medical Biosciences, Umeå University. The cells were grown in T-75 flasks at 37°C in humidified atmosphere with 5% CO_2 in air and the culture medium was replaced every 2–3 days and passages were performed 1–2 days per week. P19 cells and C6 were obtained from the European Collection of Cell Cultures (ECACC; Porton Down). P19 cells were cultured in MEM-α with 10% FBS, 1% PEST and 1% NEAA, whereas C6 cells were cultured in F-10 Ham with 2 mM glutamine and 10% FBS, and 1% PEST.

Cell viability assays

The cells were harvested the day before exposure and seeded (10 000 cells/well) into a 96-well microplate in

respective culture medium containing 1% FBS. The cells in the dose–response experiments were exposed to the test substances for 24 h at 37°C. The total volume in each well was 200 µl and the total solvent (DMSO and/or EtOH) concentration was kept constant at 0.5% in all assays, no solvents were added to the untreated controls. The cell viability was determined by using the CyQUANT cell proliferation assay kit, which measures the total nucleic acid content. Briefly, the medium was aspirated and the plates frozen at −80°C until used. The plates were thawed at room temperature and processed according to the manufacturer's instructions. The plates were analysed by using a FLUOstar Galaxy microplate reader (excitation 485 nm/emission 520 nm; BMG Labtechnologies, Offenburg, Germany).

Cytotoxicity was measured by using the fluorescent probe calcein-AM. The cells were washed once with phosphate buffered saline (PBS) and calcein-AM was added to a final concentration of 1 µM in PBS. After 1 h of incubation at room temperature, the plates were analysed by using the FLUOstar Galaxy microplate reader (excitation 485 nm/emission 520 nm). Triton X-100 at a final concentration of 2% was added 30 min before the calcein-AM analyses, as a control of maximal cell death.

In the combination study with 5-FU, and/or HU 210 and AEA, the cells were exposed to the test compounds for 48 h at 37°C before analyses.

When antagonists, enzyme inhibitors, and antioxidants were used, the cells were preincubated for 20–240 min as appropriate at 37°C prior to addition of HU 210, AEA or AA and incubation for a further 24 h.

[^{3}H]-Thymidine incorporation

Caco-2 cells in 96-well plates (18 000 cells/well) were incubated 24 h at 37°C followed by addition of the test substances. After 48 h, 0.04 µCi/well [^{3}H]-thymidine was added and after additional 24 h of incubation the cells were vacuum filtered through a Whatman GF/B glass microfiber filter (Skatron Instruments, Sterling, USA) using a Micro Cell Harvester (Skatron Instruments). The samples were dissolved in Ultima Gold scintillation fluid and the tritium content analysed by liquid scintillation spectroscopy with quench correction in a WinSpectral™ liquid scintillation counter (Perkin™ Elmer, Wellesley, USA).

Intracellular cyclic AMP assay

Caco-2 cells were plated as in the calcein-AM experiments. PTx was added to a final concentration of 100 ng/ml and the cells were incubated for the times shown in the figures. The cells were incubated with IBMX (0.5 mM), forskolin (3 µM) and CBs HU 210 (4 µM) and AEA (25 µM) for 15 min. After exposure the cells were lysed and the samples were immediately frozen in microplates at −80°C. The determination of intracellular cyclic AMP content was performed by using the cAMP Biotrak Enzyme immunoassay system (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. The amount of cyclic AMP was measured as absorbance at 450 nm by using ThermoMax microplate reader. Results were expressed as pmol intracellular cyclic AMP/well. A protection study with PTx was also performed by using the calcein-AM viability assay. Caco-2 cells were pre-incubated for 4 h with the toxin (100 ng/ml), and incubated with HU 210 (4 µM), AEA (25 µM) and AA (40 µM) for 24 h.

Data and statistical analyses

Data are presented as either untransformed values (fluorescence units or dpm) or normalised against standard curves or as percent of untreated controls. The IC_{50} values from the concentration-effect experiments were calculated from non-linear regression (sigmoidal dose–response curves) using GraphPad Prism 5 computer program software for Windows (GraphPad Software Inc., San Diego, CA, USA). The computer-generated curves from the built-in equation log (inhibitor) vs. response–variable slope, were constrained by setting the bottom plateau (maximal toxicity) to be zero or greater. Statistically significant differences [analysis of variance (ANOVA), two-way ANOVA, and post hoc tests] were undertaken using the GraphPad Prism software. See details of each statistical analysis used in the legends to figures. All the analyses reported were repeated at least three times.

Results

Effects of 5-FU, cannabinoids and related fatty acids upon Caco-2 cell proliferation and viability

We first examined the antiproliferative effects of the synthetic CB receptor ligand HU 210 and the endogenous CB AEA in the Caco-2 cells. The effects were compared with those produced by the chemotherapeutic agent 5-FU, a pyrimidine antagonist. After 3 days of exposure, all compounds tested produced a concentration-dependent decrease in Caco-2 cell proliferation as measured by [^{3}H]-thymidine incorporation and total nucleic acid content (Fig. 1). However, there was a great difference between the potencies and efficacies of the examined compounds. At a concentration of 100 µM, 5-FU caused a 30 ± 9% decrease in nucleic acid content compared to untreated control cultures, and a 53 ± 13% decrease in [^{3}H]-thymidine incorporation (Fig. 1a). In contrast, a concentration of 3 µM of HU

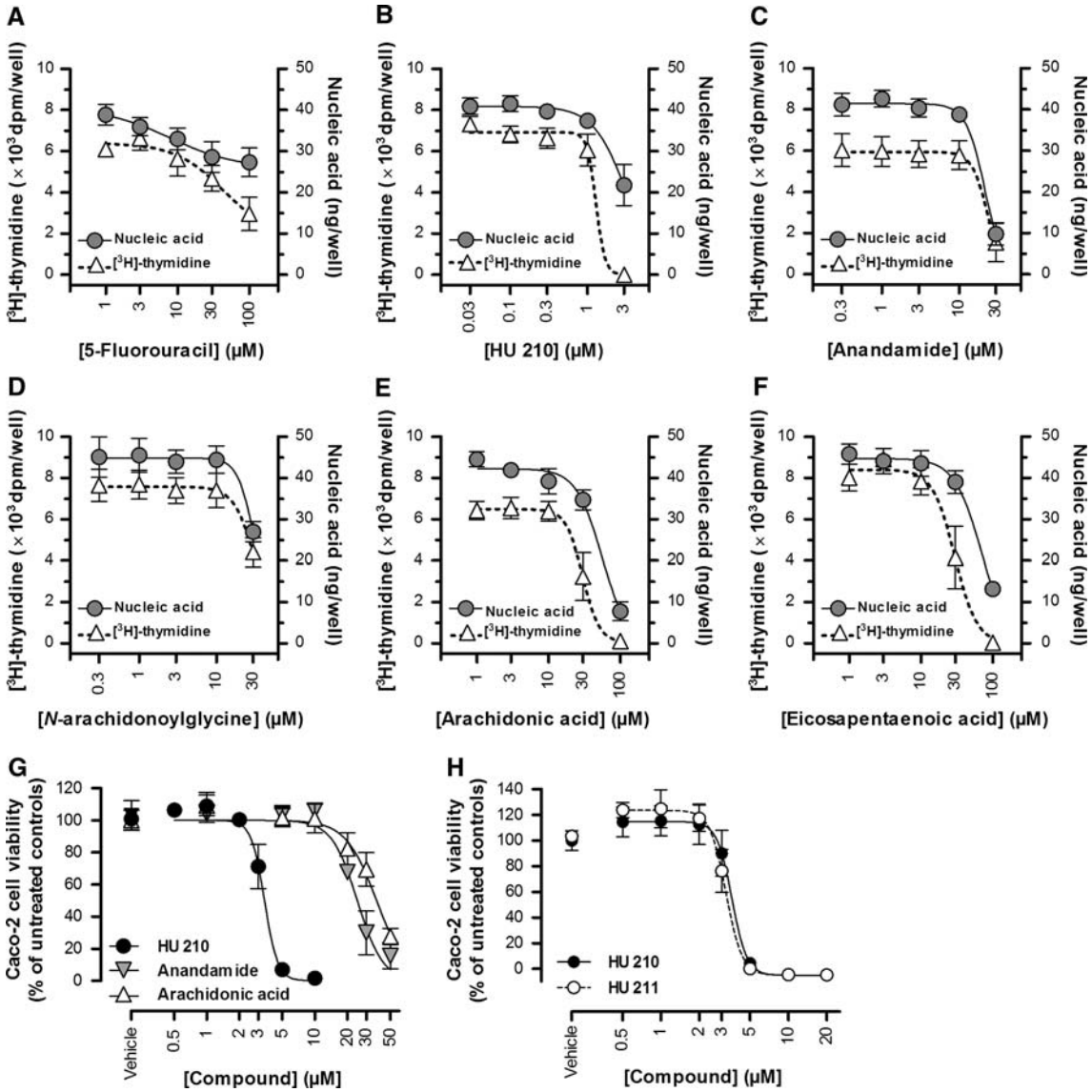

Fig. 1 Concentration-dependent effects of **a** 5-FU, **b** HU 210, **c** AEA, **d** NAGly, **e** arachidonic acid and **f** EPA upon [^{3}H]-thymidine incorporation and nucleic acid content in Caco-2 cells (10 000 cells/well) after 3 days of exposure. Concentration-dependent effects of **g** HU 210, AEA and arachidonic acid, and **h** comparison between HU 210 and its enantiomer HU 211 on calcein-AM fluorescence in the Caco-2 cells after 24 h of exposure. Data are mean ± SEM of four to six different experiments. "Vehicle" indicates cells treated with the solvent carriers for respective compound

210 totally inhibited the [^{3}H]-thymidine incorporation (IC_{50} ~1.2 μM) and the nucleic acid content was decreased to 47 ± 12% of the DMSO-treated controls (Fig. 1b). The endogenous CB receptor agonist AEA decreased the [^{3}H]-thymidine incorporation in Caco-2 cells with an IC_{50} value of ~23 μM, and at the highest concentration tested (30 μM), the [^{3}H]-thymidine incorporation was decreased by 70 ± 14% and the nucleic acid content was decreased by 77 ± 6% (Fig. 1c).

In order to investigate if the effect of AEA is related to the arachidonoyl moiety of the molecule, the effects were compared to those produced by the endogenous structure analogue of AEA, NAGly, and the related polyunsaturated fatty acids AA and EPA, all lacking affinity at the CB receptors. NAGly, AA and EPA were all less potent than AEA with IC_{50} values of ~30 μM in inhibiting [^{3}H]-thymidine incorporation. At 30 μM, NAGly produced 21 ± 6% and 40 ± 5% decrease in [^{3}H]-thymidine incorporation and nucleic acid content, respectively (Fig. 1d). AA and EPA totally blocked the [^{3}H]-thymidine incorporation at a concentration of 100 μM, and the nucleic acid content were decreased by 83 ± 5% and 71 ± 4%, respectively (Fig. 1e, f).

The results from the concentration-effect experiments were confirmed by microscopic examinations of the Caco-2 cells after 3 days of exposure, and it was found that 5-FU

decreased the density of the Caco-2 cell layer without causing any dramatic change in morphology compared to untreated control cultures (representative photomicrographs of Caco-2 cells exposed to the test compounds are available as Supplementary Figure 1). All other compounds, however, induced a significant decrease in cell density concomitant with a high number of small defragmentated floating cells with high phase-contrast, similar to what is observed during apoptotic cell death. This prompted the use of a cytotoxicity assay by using calcein-AM, a fluorescent compound accumulated only in living cells. It was found that HU 210, AEA and AA, after only 24 h of incubation, showed essentially similar IC_{50} values as those determined by using the proliferation assays after 3 days of exposure (3.4, 25 and 37 μM, respectively; Fig. 1g). This indicate that the effects of the CBs and AA are cytotoxic, rather than antiproliferative. HU 211, an enantiomer of HU 210 with no activity at CB_1 or CB_2 receptors, showed identical concentration-dependent cytotoxic effects in the Caco-2 cells as HU 210 with an IC_{50} value of 3.2 μM (Fig. 1h).

Human Caco-2 cells are less sensitive towards the cytotoxic effects of cannabinoids and arachidonic acid compared to other cell lines

In order to compare the relative sensitivity of the Caco-2 cells with other cell lines, the effect of 24 h exposure of HU 210, AEA and AA upon the cell viability of rat C6 glioma cells and mouse P19 teratocarcinoma cells was examined under identical conditions on the same microplate (Fig. 2). It was found that the human Caco-2 colorectal carcinoma cells were the least sensitive cell line of those studied. In summary, the results show that the relative orders of sensitivity are as follows: P19 = C6 > Caco-2 for HU 210; P19 > C6 = Caco-2 for AEA; and P19 > C6 > Caco-2 for AA. This pattern of sensitivity correlated with the doubling time of the cell lines. The mouse P19 cell line have a reported doubling time 8–16 hours depending on the culture conditions, the rat C6 cell line 15–30 hours, and the human Caco-2 cell line 48–72 hours.

HU 210 and 5-FU have synergistic cytotoxic effects in Caco-2 cells

To investigate whether the CBs could enhance the effects of the chemotherapeutic agent 5-FU, Caco-2 cells were exposed to threshold concentrations of HU 210 or AEA and 5-FU for 48 h. It was found that HU 210 and 5-FU acted synergistically in inducing cytotoxicity (Fig. 3a, d). Both 5-FU per se and HU 210 per se, induced a significant concentration-dependent reduction in both calcein-AM fluorescence and in total nucleic acid content ($P < 0.0001$ and $P < 0.001$, respectively). HU 210 at a concentration of 1 μM did not affect the 5-FU-induced effects, but at 3 μM a significant interaction with 5-FU on both calcein fluorescence and nucleic acid content was observed. However, no such interaction between HU 210 and 5-FU was observed in the human HTC116 or HT29 colon cancer cell lines (Fig. 3b, c, respectively). HCT116, with a doubling time of about 18 h, was more sensitive towards the cytotoxic effect of 5-FU compared to Caco-2 and HT29 cells (doubling time > 24 h). HU 210 per se was more efficacious in both HCT116 and HT29 cells compared to Caco-2 cells. At 3 μM, HU 210 produced 100% cell death in the HCT116 cells and 94 ± 2% cytotoxicity in the HT29 cells. We failed to detect any synergy between AEA and 5-FU (Fig. 3e, f).

The cytotoxic effects of the cannabinoids do not involve CB receptors

To determine if the CB-induced cytotoxicity is mediated through the CB receptors, the effects of the selective CB_1 and CB_2 receptor antagonists AM251 and AM630, respectively, and PTx were studied. The CB receptors are primarily $G_{i/o}$ protein-coupled receptors producing among other responses a decreased adenylyl cyclase activity [18]. Previous research has shown that PTx, derived from *Bordetella*

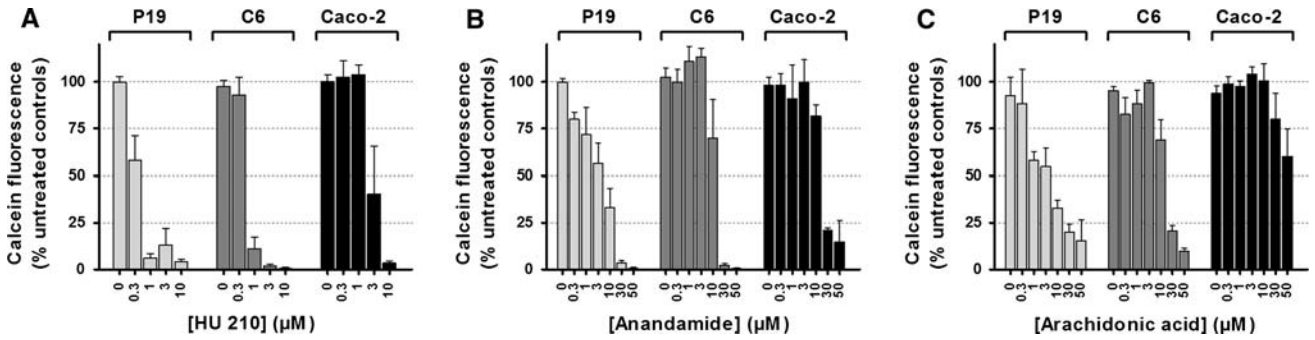

Fig. 2 Dose–response comparison of the cytotoxic effects of 24 h exposure to **a** HU 210, **b** AEA, and **c** arachidonic acid in mouse P19 teratocarcinoma cells, rat C6 glioma cells, and human Caco-2 colorectal carcinoma cells. Shown are mean ± SEM of three different experiments

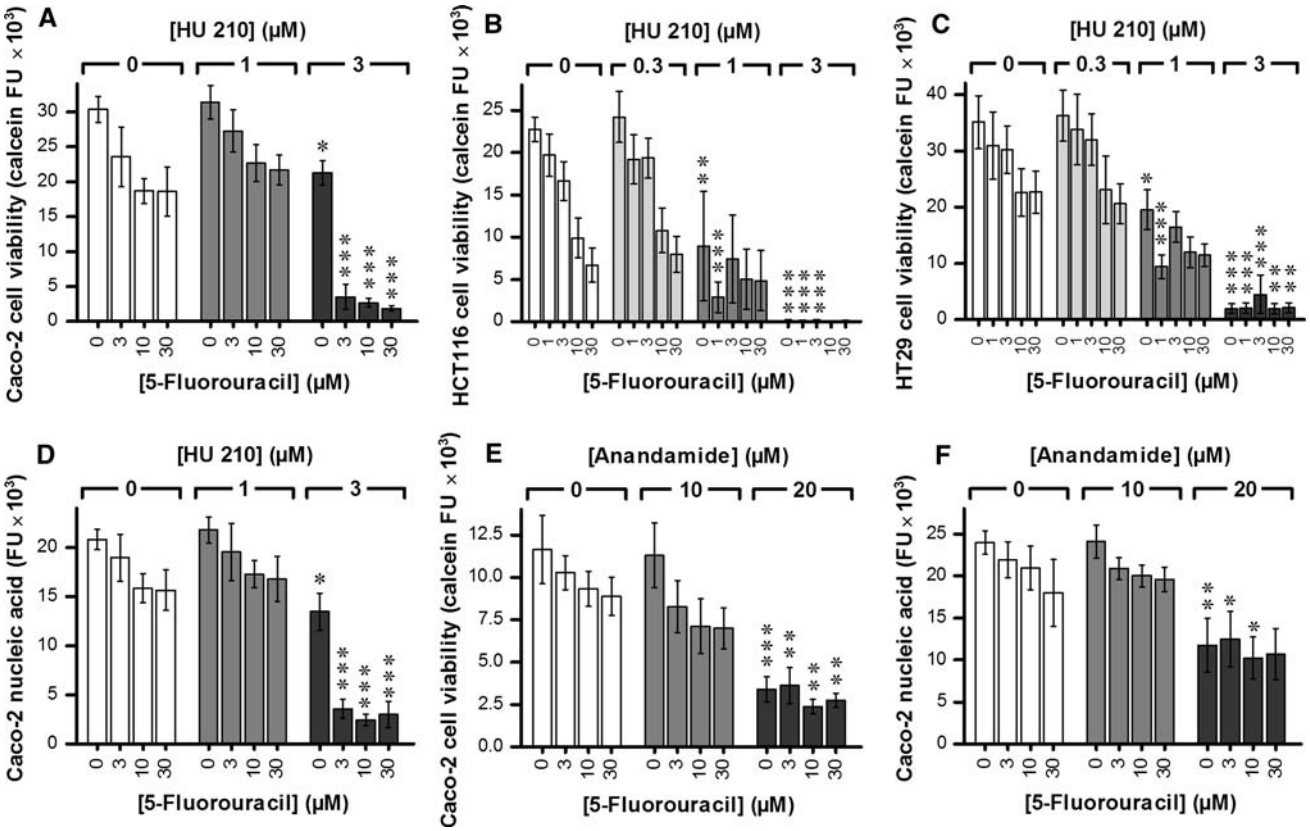

Fig. 3 Effects of 48 h exposure to either 5-FU alone or the combination of indicated concentrations of HU 210 upon **a, d** Caco-2, **b** HCT116, and **c** HT29 cell viability. Effects of **e, f** 5-FU alone or in combination with AEA upon Caco-2 cell viability. Viability was assessed by measuring **a, b, c, e** calcein-AM fluorescence or **d, f** nucleic acid content. Data are mean ± SEM of four to six different experiments. Statistical analysis (two-way ANOVA with post hoc Bonferroni's multiple comparison test) revealed significant effects of both 5-FU ($P < 0.0001$) and HU 210 [$P < 0.0001$ in panel (**a**) and $P < 0.001$ in panel (**d**)] per se and a significant interaction between 5-FU and HU 210 [$F_{6,27} = 2.79$; $P = 0.0304$ in (**a**), and $F_{6,27} = 5.34$; $P = 0.001$ in (**d**)]. Statistically significant differences between concentrations of 5-FU per se and corresponding concentrations in the presence of the cannabinoids are indicated as $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

Pertussis, can disrupt the function of $G_{i/o}$-coupled receptors in various cell types and thereby inhibit the response produced by CBs [19–21, 24, 45]. To explore the presence of functional PTx-sensitive CB receptors in the Caco-2 cells, the effect of HU 210 upon forskolin-induced intracellular cyclic AMP was studied. We found that 3 μM forskolin increased the intracellular cyclic AMP from 0.42 ± 0.2 pmol/well (mean ± SEM) to 5.7 ± 0.2 pmol/well (Supplementary Figure 2). HU 210 at a concentration of 4 μM produced a significant decrease of forskolin-induced cyclic AMP to 4.2 ± 0.3 pmol/well. This decrease was inhibited by 100 ng/ml PTx when the incubation time was four hours or longer (Supplementary Figure 2), indicating that PTx disrupts the $G_{i/o}$-coupled CB receptor.

Although the Caco-2 cells express functional CB receptors in our hands, these receptors are not involved in the toxicity produced by the CBs in this cell line, since neither AM251, AM630 nor PTx affected the toxicity produced by the HU 210 (Fig. 4a, b) or AEA (Fig. 5a, b). Consistent with this, myriosin (ISP-1), FB_1 and PD98059, compounds interfering with the ceramide and MAPK pathways downstream of CB receptors in cancer cells [6, 12, 33, 42, 44, 50] did not prevent to any large or consistent extent the antiproliferative effects of HU 210 and AEA (Figs. 4c, d, 5c, d).

The effect of the vanilloid TRPV1 receptor antagonist SB 366791 [15] was also studied, since AEA has been found to activate TRPV receptors, and it has been indicated that TRPV1 activation could be involved in antiproliferative mechanisms [30, 41]. However, the TRPV1 antagonist was not protective against AEA-induced cytotoxicity at the concentrations examined (Fig. 5a).

The cytotoxic effects of HU 210 and AEA are attenuated by the antioxidant α-tocopherol and the nitric oxide synthase (NOS) inhibitor L-NAME

It has been previously shown that the intracellular acting antioxidant α-tocopherol has an ability to protect against the antiproliferative effects of various CBs [23]. In the Caco-2 cells, 100 μM α-tocopherol produced a statistically

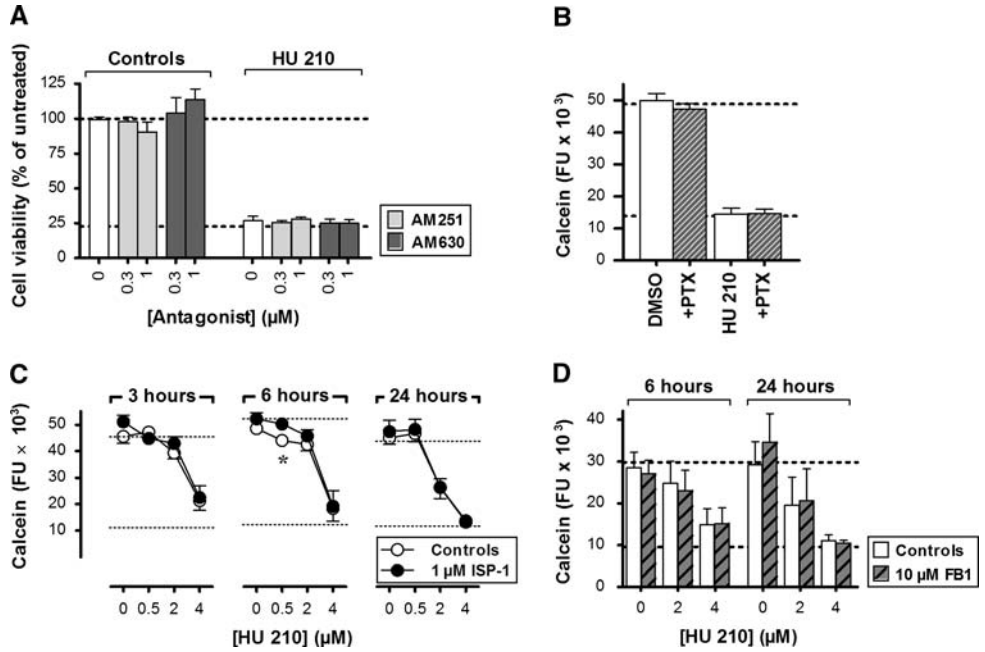

Fig. 4 The effects of **a** CB_1 and CB_2 receptor antagonists (AM251 and AM630, respectively), **b** pertussis toxin (PTx), **c** the serine palmitoyltransferase inhibitor ISP-1, and **d** the ceramide synthase inhibitor fumonisin B_1 (FB_1), upon the toxicity in Caco-2 cells after 24 h exposure to 4 μM (or indicated concentration) of HU 210. Data (mean ± SEM of 4–6 different days of experiments) are expressed as percentage of untreated controls (**a**) or a as relative calcein-AM fluorescence units (**b**, **c**, **d**). Statistical treatment of data in (**a**) and (**c**) was undertaken using one-way ANOVA with Dunnett's post hoc multiple comparison test, and in **b** and **d** two-tailed *t*-test: $^*P < 0.05$ (effect of the treatment compared with the corresponding HU 210 control concentration)

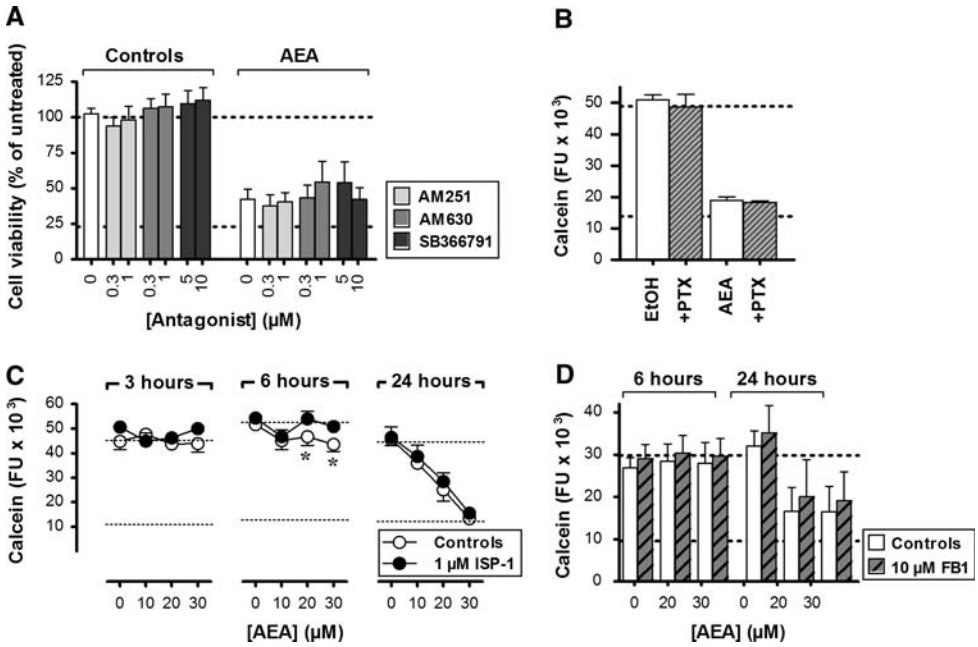

Fig. 5 The effects of **a** the receptor antagonist AM251, AM630 and SB366791, **b** pertussis toxin, **c** ISP-1, and **d** FB_1 upon the toxicity in Caco-2 cells after 24 h exposure to 25 μM (or indicated concentration) of AEA. Data (mean ± SEM of 4–6 different days of experiments) are expressed as percentage of untreated controls (**a**) or as relative calcein-AM fluorescence units (**b**, **c**, **d**). Statistical treatment of data in **a** and **c** was undertaken using one-way ANOVA with Dunnett's post hoc multiple comparison test, and in **b** and **d** two-tailed *t*-test: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (effect of the treatment compared with the corresponding AEA control concentration)

significant reduction of the cytotoxicity by HU 210 (Fig. 6a), AEA (Fig. 6b), and the combined toxicity produced by 3 μM 5-FU and 3 μM HU 210 (Fig. 6e). The extracellular acting antioxidant ascorbic acid had no effect on the CB-induced cytotoxicity. Moreover, the nitric oxide synthase inhibitor L-NAME showed a significant protection against HU 210- (Fig. 6c) and AEA-induced (Fig. 6d) toxicity, but not against the toxicity produced by 5-FU and HU 210 in combination (Fig. 6e). This protective effect of L-NAME, however, may not be related to its actions on NOS and nitric oxide production, as 7-NI, another NOS inhibitor, did not share this protective effect (Fig. 6c, d). No protective effects of α-tocopherol or L-NAME were observed against AA toxicity (Fig. 7c, d).

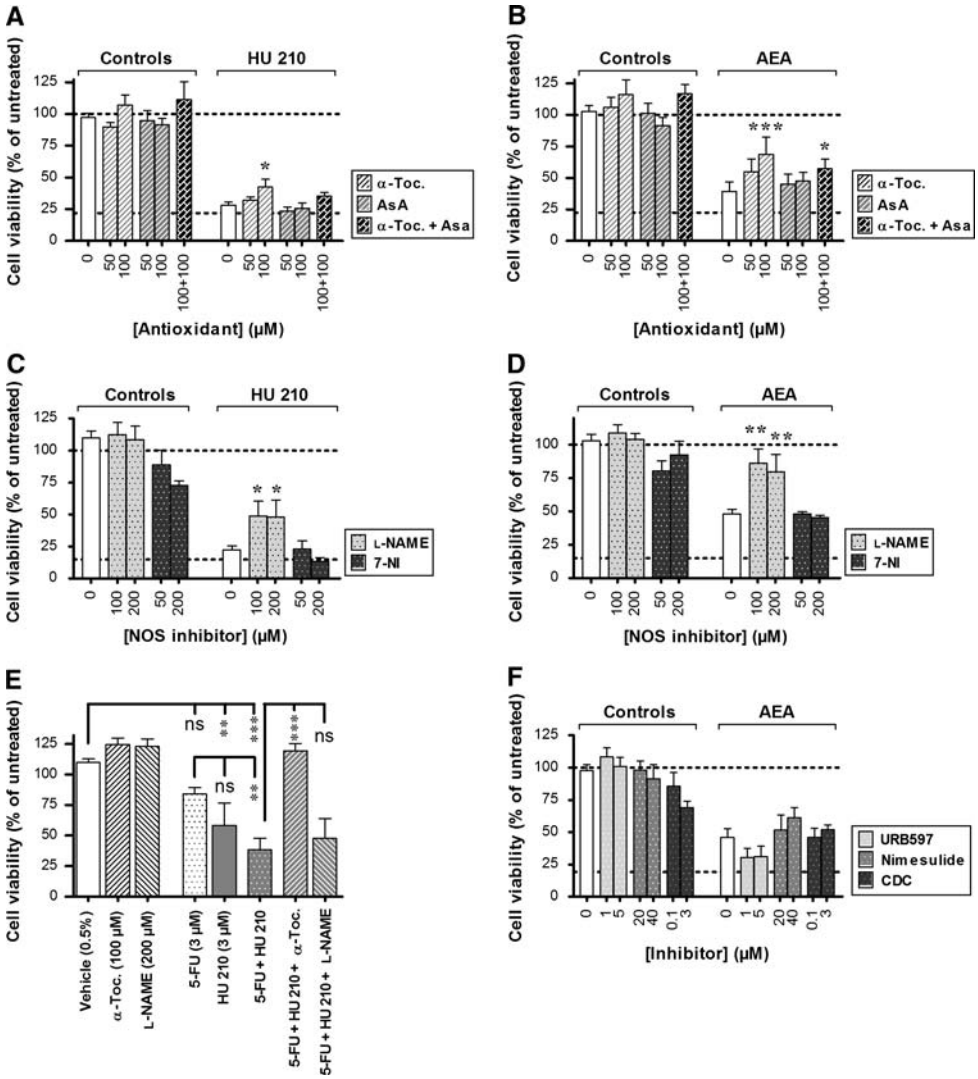

Fig. 6 The effects of **a, b** the antioxidants α-tocopherol (α-Toc.) and ascorbic acid (AsA), **c, d** the nitric oxide synthase inhibitors L-NAME and 7-NI, upon the toxicity in Caco-2 cells after 24 h exposure to **a, c** 4 μM HU 210 or **b, d, f** 25 μM AEA. The effects of **e** α-tocopherol and L-NAME upon cytotoxicity in Caco-2 cells produced by 3 μM 5-FU and 3 μM HU 210 in combination. In **f** the effects of the enzyme inhibitors URB597, nimesulide and CDC upon the toxicity produced by 25 μM AEA are shown. Data (mean ± SEM of 4–6 different days of experiments) are expressed as percentage of untreated controls. Statistical treatment of data was undertaken using one-way ANOVA with Dunnett's post hoc multiple comparison test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (effect of the treatment compared with the corresponding HU 210 or AEA control concentration)

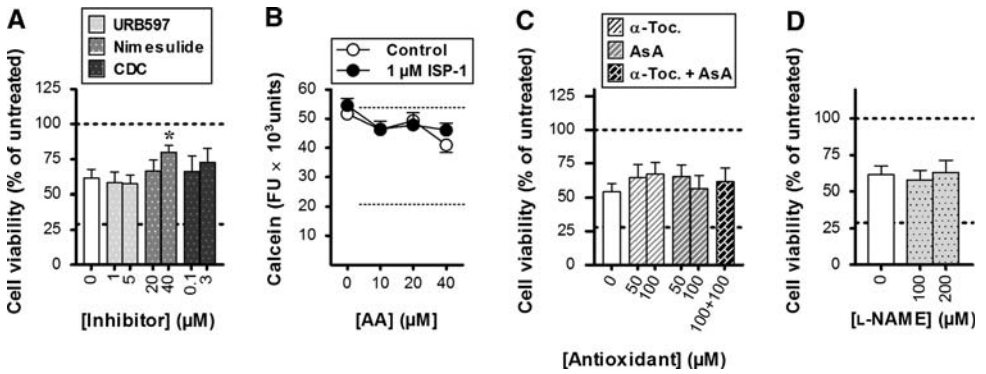

Fig. 7 The effects of **a** the enzyme inhibitors URB597, nimesulide and CDC, **b** ISP-1, **c** α-tocopherol and ascorbic acid, and **d** L-NAME upon the toxicity in Caco-2 cells after 24 h exposure to 40 μM arachidonic acid. Data (mean ± SEM of 4–5 different days of experiments) are expressed as percentage of untreated controls (**a**, **c, d**) or as relative calcein-AM fluorescence units (**b**). Statistical treatment of data in **a**, **c** and **d** was undertaken using one-way ANOVA with Dunnett's post hoc multiple comparison test, and in **b** two-tailed *t*-test: $^{*}P < 0.05$ (effect of the treatment compared with the corresponding arachidonic acid control concentration)

Effects of fatty acid metabolising enzyme inhibitors upon cannabinoid-induced cytotoxicity

To further explore alternative mechanisms whereby CBs induce cytotoxicity in the Caco-2 cells, the cultures were treated with inhibitors of intracellular enzymes involved in endocannabinoid biotransformation. The FAAH inhibitor URB597, the COX inhibitor nimesulide and the LOX inhibitor CDC did not exhibit any protective effect against the toxicity produced by HU 210 (data not shown) or AEA (Fig. 6f). In contrast, 40 μM nimesulide was protective against the toxicity produced by a 24-h incubation of 40 μM AA (Fig. 7a).

Discussion

The aims of this preclinical study were to investigate the effect of synthetic and endogenous CBs and their related fatty acids on the viability of human colorectal carcinoma Caco-2 cells, and to explore whether such effects are synergistic with a treatment paradigm currently used in the clinic. Cancer treatment with chemotherapeutic agents is complicated by the development of tumour resistance and unspecific toxic effects of the drugs. Therefore, there is an intense research to find new therapeutic agents that sensitise tumour cells to chemotherapy, without disturbing normal cell function. Cannabinoids have been described to have a favourable drug safety profile, and are currently used in some countries to palliate wasting, emesis and pain during and after treatment with chemotherapeutic drugs in cancer patients [48]. In various cell culture and animal model studies, CBs have been shown to induce cell growth inhibition and induction of apoptosis in tumour cells, including lung adenocarcinomas, gliomas, thyroid epitheliomas, various lymphomas, uterus carcinomas, breast carcinomas, prostate carcinomas and neuroblastomas, for a review see [16].

The data presented here demonstrate a synergistic interaction between 5-FU and HU 210 in the Caco-2 cells, but not in HCT116 or HT29 cells or between 5-FU and AEA. It has been shown previously that the endocannabinoid AEA and the synthetic CB HU 210 effect cell proliferation in Caco-2 cells [28], and were thus chosen for this study. A key aspect of the study has been the elucidation of the mechanism(s) involved in the cytotoxic effects of HU 210 and AEA, by using a pharmacological approach. This study presents several indications of a non-CB receptor-mediated mechanism behind the effects of HU 210 and AEA, despite the expression of functional CB receptors in the Caco-2 cells.

In particular (a) the high concentrations of HU 210 and AEA required for effect compared with the submicromolar concentrations needed for activation of CB receptors [5, 40], (b) the lack of a blockade produced either by CB receptor antagonists or by compounds interfering with downstream pathways of CB receptor activation, and (c) the toxicity produced by the potent CB receptor agonist HU 210 is mimicked by the enantiomer HU 211 that does not interact with CB receptors, but rather acts as a NMDA receptor antagonist argue strongly for a CB-receptor independent action of these compounds. This result differs from the report of Ligresti et al. [28], who found that AEA, 2-AG and HU 210 inhibited the growth of Caco-2 cells at submicromolar concentrations and in a manner modulated by the CB_2/PPARα receptor antagonist SR144528. Two possibilities for this difference can be considered. Firstly, although both studies used Caco-2 cells and both showed the presence of CB receptors, different "strains" of the same cell line may show large differences, due to differences in the expression level of key components for the response in question. This has been demonstrated for C6 glioma cells, where different subclones with markedly different sensitivities to Δ^9-tetrahydrocannabinol have been reported [12]. Secondly, the assays or experimental conditions used are likely to be important determinants of the outcome. For example, we and others have reported that the potencies of CBs are dependent upon the concentration of FBS albumin used in the assays [11, 22, 32]. With respect to the assay used to assess cytotoxicity, the use of three separate methods in the present study, all of which gave essentially the same results, would suggest that lack of CB receptor-mediated cytotoxic effects here cannot be ascribed to the use of an invalid assay.

In order to determine the mechanism(s) involved in the CB receptor-independent cytotoxic effects of AEA and HU 210, a variety of pharmacological approaches have been undertaken, including blockade of TRPV1 receptors and blockade of the enzymes involved in AEA metabolism. It could be hypothesised, for example, that AA, produced by the FAAH-catalysed hydrolysis of AEA, could explain the cytotoxic effects of this endocannabinoid. Certainly, we found that AA and other fatty acid compounds (NAGly and EPA) affected Caco-2 cell viability. However, the lack of effects of the FAAH, COX and LOX inhibitors would argue against the contribution of AEA metabolites in the cytotoxic effects of this compound, as is the finding that the cytotoxicity of AA is reduced by the COX-2 inhibitor nimesulide.

Although AEA is mainly known as an endocannabinoid acting at the CB receptors, it can exert other effects, e.g. by acting as an agonist at vanilloid TRPV1 receptors [46, 52], or being biotransformed into bioactive metabolites. We have previously showed that in rat C6 glioma cells, AEA was antiproliferative due to a combined activation of CB and vanilloid receptors, since the CB_1 receptor antagonist AM251 and the vanilloid receptor antagonist capsazepine

completely blocked the antiproliferative effect of AEA. In C6 cells, the effects of synthetic CBs were not inhibited by AM251 or the CB_2 receptor antagonist AM630, either alone or in combination [23]. Moreover, in undifferentiated mouse P19 teratocarcinoma cells, we have found that both AM251 and AM630 attenuate the toxicity of HU 210, whereas AM630, but not AM251 attenuate the toxicity of AEA (unpublished data). However, this activity profile could not be observed in human Caco-2 cells.

It has been reported earlier that α-tocopherol has the ability to protect against the antiproliferative effects of CBs, and this was also seen in our results. The NOS inhibitor L-NAME also inhibited the toxicity produced by HU 210 and AEA, but had no effect on the toxicity produced by 5-FU and HU 210 in combination. It has been shown that NO plays an important role in colon tumourigenesis [1, 7, 27, 36] and, in addition, an increased activity and expression of iNOS has been reported in human colon adenomas [43]. Furthermore, it has been reported that NOS inhibition could be useful as chemoprevention of colon cancer [43]. It is therefore logical that an inhibition of NOS would affect the proliferation of colon adenoma cells. However, the attenuating effect of L-NAME upon HU 210- and AEA-induced cytotoxicity in this study, is apparently unrelated to its actions at NOS, since another non-selective NOS inhibitor, 7-NI, was without effect. Nevertheless, the effects of the interplay between CBs on NO signalling needs to be further examined.

In conclusion, CBs can induce apoptosis in human colon cancer cells and HU 210 acts synergistically with 5-FU in the Caco-2 cell line, in a manner involving oxidative stress, but not CB receptor activation. In this respect, there is a report that CBs can exert direct effects upon mitochondrial function [2], and such a mechanism may be operative here. Whatever the explanation, this study adds to the growing body of evidence that CB compounds have actions against tumour cells and that may point to the development of novel agents for the treatment of cancer, including CRC. However, the biological response to CB compounds seem to be critically dependent upon the experimental drug concentration used and the type of cell or tissue examined. Therefore, we believe it is important further to investigate the apparently complex mechanism of actions of CBs on cell fate to be able to discriminate between versatile anticancer effects and "promiscuous" toxicological effects.

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