Possible Endocannabinoid Control of Colorectal Cancer Growth

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Background & Aims: **The endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) inhibit cancer cell proliferation by acting at cannabinoid receptors (CBRs). We studied (1) the levels of endocannabinoids, cannabinoid CB_1 and CB_2 receptors, and fatty acid amide hydrolase (FAAH, which catalyzes endocannabinoid hydrolysis) in colorectal carcinomas (CRC), adenomatous polyps, and neighboring healthy mucosa; and (2) the effects of endocannabinoids, and of inhibitors of their inactivation, on human CRC cell proliferation.** ***Methods:*** **Tissues were obtained from 21 patients by biopsy during colonoscopy. Endocannabinoids were measured by liquid chromatography-mass spectrometry (LC-MS). CB_1, CB_2, and FAAH expression were analyzed by RT-PCR and Western immunoblotting. CRC cell lines (CaCo-2 and DLD-1) were used to test antiproliferative effects.** ***Results:*** **All tissues and cells analyzed contain anandamide, 2-AG, CBRs, and FAAH. The levels of the endocannabinoids are 3- and 2-fold higher in adenomas and CRCs than normal mucosa. Anandamide, 2-AG, and the CBR agonist HU-210 potently inhibit CaCo-2 cell proliferation. This effect is blocked by the CB_1 antagonist SR141716A, but not by the CB_2 antagonist SR144528, and is mimicked by CB_1-selective, but not CB_2-selective, agonists. In DLD-1 cells, both CB_1 and CB_2 receptors mediate inhibition of proliferation. Inhibitors of endocannabinoid inactivation enhance CaCo-2 cell endocannabinoid levels and block cell proliferation, this effect being antagonized by SR141716A. CaCo-2 cell differentiation into noninvasive cells results in increased FAAH expression, lower endocannabinoid levels, and no responsiveness to cannabinoids.** ***Conclusions:*** **Endocannabinoid levels are enhanced in transformed colon mucosa cells possibly to counteract proliferation via CBRs. Inhibitors of endocannabinoid inactivation may prove useful anticancer agents.**

Numerous experimental data indicate that the activation of the endogenous cannabinoid system might represent a potential strategy for the development of new anticancer drugs.[1,2] First, the psychoactive principle of *Cannabis sativa* and marijuana, Δ^9-tetrahydrocannabinol,[3] is known to act mostly by stimulating 2 specific receptors subtypes, the cannabinoid CB_1 and CB_2 receptors[4], and was reported in the past[5] and more recently[6] to have antineoplastic activity in vivo and in vitro.[1,2] Second, endogenous agonists of the cannabinoid receptors (CBRs), i.e., *N*-arachidonoyl-ethanolamine (AEA; anandamide),[7] 2-arachidonoyl-glycerol (2-AG),[8,9] and noladin ether,[10] or their metabolically stable synthetic analogs, were found to inhibit, mostly via CB_1 receptors, the proliferation of breast and prostate cancer cells in vitro[11,12] and of rat thyroid cells transformed by the product of the K-*ras* oncogene in vivo.[13] Finally, stimulation of the 2 CBR subtypes has been found to influence the expression of various genes involved in cell survival, proliferation, and apoptosis via interference with cAMP- and ceramide-mediated signalling, mitogen-activated protein kinases and phosphatidyl-inositol-3-kinase (see Guzman et al.[14] for review).

Apart from the proposed role of CBRs in the control of cancer cell growth, transformation, and death,[2,14] there are at least 3 reasons why the endocannabinoids might be involved in the control of colorectal cancer cell proliferation. First, both AEA and 2-AG are good substrates for cyclooxygenase 2 (COX-2), which seems to play a major role in the development of colorectal carcinoma (CRC).[15,16] Because no molecular target has been reported to date for endocannabinoid COX-2 metabolites, it is possible that AEA, and particularly the more abundant 2-AG, might exert some of their biologic effects, including inhibition of CRC growth, via inhibition, by

Abbreviations used in this paper: AEA, arachidonoylethanolamide (anandamide); 2-AG, 2-arachidonoylglycerol; CBR, cannabinoid receptors; CRC, colorectal carcinoma; COX-2, cyclooxygenase 2; FAAH, fatty acid amide hydrolase; LC-MS, liquid chromatography-mass spectrometry.

0016-5085/03/$30.00
doi:10.1016/S0016-5085(03)00881-3

substrate competition, of COX-2–mediated formation of prostaglandins.[16] Second, it has been reported recently[17] that a particular class of lysophosphatidic acids, molecules known to play a major role in the development of cancer and to be overproduced in several types of tumors, including CRC,[18,19] might serve as biosynthetic precursors for 2-AG. Therefore, this possibly antiproliferative compound[11,12] is likely to be overproduced in CRC as compared with normal colon tissue. Finally, the *ras* family of oncogenes plays a crucial role in the onset and growth of CRC,[20] and we have recently reported that an endocannabinoid analogue inhibits the proliferation of v-K-*ras*–transformed thyroid cells by blocking the activity of p21*ras*, the protein encoded by *ras*.[13]

Based on this background, we have decided to investigate whether endocannabinoids, their receptors, and one of the enzymes deputed to their inactivation, the fatty acid amide hydrolase (FAAH), are present, and with what possible biologic function, in CRCs as well as in colorectal adenomatous polyps that are known to progress into CRC. We compared the levels of AEA, 2-AG, CB_1, CB_2, and FAAH in normal colon mucosa to those in transformed mucosa (adenomas and carcinomas). Furthermore, we studied the effect of the endocannabinoids, of selective CB_1 and CB_2 receptor stimulation, and of selective inhibitors of endocannabinoid inactivation (to augment AEA and 2-AG endogenous levels) on the proliferation of two human CRC cell lines: (1) the CaCo-2 cells, which are widely used for studies on this type of cancer and undergo differentiation when in culture; and (2) DLD-1 cells, which, unlike CaCo-2 cells, do not differentiate in culture. We report data pointing to a tonic limiting action by endocannabinoids and CBRs on the growth of CRC.

Materials and Methods

Drugs

AEA and 2-AG were purchased from Cayman Chemicals, and ACEA, Met-Fluoro-anandamide, and BML-190 from Tocris. HU-210 was a kind gift from Prof. R. Mechoulam, Hebrew University of Jerusalem, and SR14176A and SR144528 were donated by Sanofi Recherche. Indomethacin *N*-methyl-ester was obtained from Sigma. VDM-11, VDM-13, and arachidonoyl-serotonin were synthesized from the corresponding amines and arachidonoyl-chloride, as described previously.[21]

Biopsy

Biopsy specimens were obtained in agreement with current Italian health care rules, by means of biopsy forceps during colonoscopy on both healthy and cancer tissue in 9 patients affected with left-sided colon carcinoma (average age 64.5 ± 10 years, 3 males, 6 females; mitosis = 1.35 ± 0.33; grading = 2.44 ± 0.52; nuclear pleiomorphism 2.55 ± 0.52; means ± SD) and on healthy tissue and adenomatous polyps in 12 patients affected with colonic adenomas (average age 59.9 ± 14 years, 10 males, 2 females). A small piece (15–20 mg wet weight) from the head of each polyp removed by snare polipectomy and aliquots of each biopsy sample were kept at −80°C until processing. Adenomatous polyps and aliquots of all samples were stored in formalin for histology to evaluate tumor grading, mitoses for high-power field, and nuclear pleiomorphism.

Cell Culture and Proliferation and Differentiation Assays

Caco-2 cells were grown in Dulbecco's modified Eagle medium supplemented with 2 mmol/L L-glutamine, 1% nonessential amino acids, and 10% fetal calf serum (FCS). DLD-1 cells were grown in RPMI-1640 medium supplemented with 2 mmol/L L-glutamine and 10% FCS. Sucrase activity was assessed by the method of Dahlquist[22] by measuring the glucose released from saccharose by the enzyme under standardized condition. The sucrase activity was expressed as 1 unit = 1 μmol of glucose released/min at 37°C at pH 7. Cell proliferation assays were carried out in 6-well dishes containing subconfluent cells (5×10^4 cells). Three hours after cell seeding, test substances were added in medium and then daily at each change of medium. After 4 days, cells were treated with trypsin and counted by a hemocytometer. Cell viability was assessed by trypan blue, and no significant decrease was observed with up to 10 μmol/L anandamide. DNA fragmentation of CaCo-2 cells treated for 72 hours with met-fluoro-anandamide (Tocris) was analyzed by flow cytometry using FACScan (Becton Dickinson).[11]

Endocannabinoid Measurement

Tissues or cells were dounce-homogenized with chloroform/methanol/Tris-HCl 50 mmol/L, pH 7.4 (1/1/1 by volume), containing 5 pmol of d_8-anandamide and 50 pmol of d_8-2-AG (Cayman Chemicals) as internal standards. Lipid-containing organic phase was dried down, weighed, and prepurified by open-bed chromatography on silica gel and analyzed by liquid chromatography (LC)-atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS) (LC-APCI-MS) using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole MS via a Shimadzu APCI interface. MS analyses were carried out in the selected ion-monitoring (SIM) mode as described previously.[23] Anandamide and 2-AG quasimolecular ions were quantified by isotope dilution with the above-mentioned deuterated standards and their amounts in pmols normalized per milligram of lipid extract.[23] Data were statistically evaluated by ANOVA followed by the Bonferroni's test (as per StatMost).

Reverse-Transcriptase Polymerase Chain Reaction

The expression of messenger RNA (mRNA) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), COX-2, FAAH, CB_1, and CB_2 receptors was examined by reverse transcription coupled to the polymerase chain reaction (RT-PCR). The procedures have been widely described in our previous studies.[11–13] The PCR cycles were 35 for CB_1, CB_2, FAAH, and COX-2 and 28 for GAPDH, which were observed to be optimal and in the linear portion of the amplification curve. The specific human oligonucleotides were synthesized on the basis of cloned human cDNA sequences of GAPDH, COX-2, FAAH, CB_1, and CB_2. For GAPDH, the primer sequences were 5′-CCCTTCATTGACCTCAACTACATGGT-3′ (nt 208-233; sense) and 5′-GAGGGCCATCCACAGTCTTCTG-3′ (nt 655-677; antisense). The COX-2 sense and antisense primers were 5′-TGGGAAGCCTTCTCTAACCTCTCCT-3′ (nt 125-132) and 5′-CTTTGACTGTGGGAGGATACATCTC-3′ (nt 246-254), respectively. The FAAH sense and antisense primers were 5′-GTGGTGCT(G/A)ACCCCCATGCTGG-3′ (nt 469-475) and 5′-TCCACCTCCCGCATGAACCGCAGACA-3′ (nt 561-569), respectively. The CB_1 sense and antisense primers were 5′-GATGTCTTTGGGAAGATGAACAAGC-3′ (nt 365-373) and 5′-AGACGTGTCTGTGGACACAGACATGG-3′ (nt 460-468), respectively. For CB_2, the primer sequences were 5′-TTTCCCACTGATCCCCAATG-3′ (nt 182-188; sense) and 5′-AGTTGATGAGGCACAGCATG-3′ (nt 285-291; antisense). The expected sizes of the amplicons were 470 bp for GAPDH, 388 bp for COX-2, 300 bp for FAAH, 309 bp for CB_1, and 329 bp for CB_2. In the presence of contaminant genomic DNA, the expected size of the amplicons would be 1062 bp for GAPDH, 1668 bp for COX-2, and 1335 bp for FAAH, respectively. The GAPDH housekeeping gene expression was used to evaluate any variation in the RNA content and cDNA synthesis in the different preparations. No PCR products were detected when the reverse transcriptase step was omitted (data not shown).

Western Immunoblotting

Western immunoblotting analysis was used to determine the presence of the CB_1 and CB_2 proteins and carried out as described in detail previously.[11–13] Antibodies (both from Cayman Chemicals, Ann Arbor, MI) were used at a dilution of 1:333 for CB_1 and 1:250 for CB_2. Control of specificity was performed by preadsorbing the antibody with the homologous antigen at a concentration of 8 μg/mL of antibody solution.

Anandamide Uptake Assays

Confluent Caco-2 cells (plated in 6-well dishes, 150,000 cells per dish) take up [^{14}C]AEA (5.0 μmol/L, 20,000 cpm) from serum-free medium in a time- and temperature-dependent manner ($t_{1/2}$ = 3.5 minutes, uptake at 37°C − uptake at 4°C = 48.3 ± 4.1% of total uptake). The effect of compounds on [^{14}C]AEA uptake was studied as described previously.[21] Cells were incubated with [^{14}C]AEA for 5 minutes at 37°C, in the presence or absence of varying concentrations of the inhibitors. Residual [^{14}C]AEA in the incubation media after extraction with $CHCl_3$/CH_3OH 2:1 (by volume), determined by scintillation counting of the lyophilized organic phase, was used as a measure of the AEA that was taken up by cells.

Anandamide Hydrolysis Assays

The effect of compounds on the enzymatic hydrolysis of AEA was studied as described previously,[21] using membranes prepared from cells incubated with the test compounds and [^{14}C]AEA (10 μmol/L, 40,000 cpm) in 50 mmol/L Tris-HCl, pH 9, for 30 minutes at 37°C. [^{14}C]Ethanolamine produced from [^{14}C]AEA hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3$/CH_3OH 2:1 (by volume).

Binding Assays

Displacement assays for CB_1 receptors were carried out by using [^{3}H]SR141716A (0.4 nmol/L, 55 Ci/mmol; Amersham) as the high-affinity ligand on membrane preparations (0.4 mg/tube) from frozen male CD rat brains (Charles River, Wilmington, MA) and in the presence of 100 μmol/L PMSF.[21] Specific binding was calculated with 1 μmol/L SR141716A and was 84%. Data are expressed as the K_i, calculated using the Cheng-Prusoff equation from the concentration exerting 50% inhibition of AEA uptake (IC_{50}).

Results

Endocannabinoid Levels and CB_1, CB_2, and FAAH Expression in Human Colorectal Tissues

We found that human colon mucosa tissues contain both AEA and 2-AG (Figure 1*A*), as determined by using an ultrasensitive LC-MS technique, and express mRNA transcripts of the size expected for CB_1 and CB_2 receptors as well as FAAH (Figure 1*B*), as determined by RT-PCR. The finding of CB_1 receptors was also confirmed by Western immunoblot of proteins from biopsy specimens of normal colon mucosa (not shown). The levels of both AEA and 2-AG increased when passing from normal mucosa to transformed mucosa (Figure 1*A*). Although the levels of endocannabinoids in colorectal adenomas and carcinomas could not be determined in the same set of patients, it was possible to observe a stronger increase of the amounts of 2-AG and, particularly, AEA (3-fold vs. 2-fold, respectively) in adenomatous polyps than in CRC tissue as compared with healthy mucosa. Tissues from both normal and transformed mucosa yielded similar amounts of extracted lipids per gram of wet weight, with normal mucosa, adenomas, and CRC

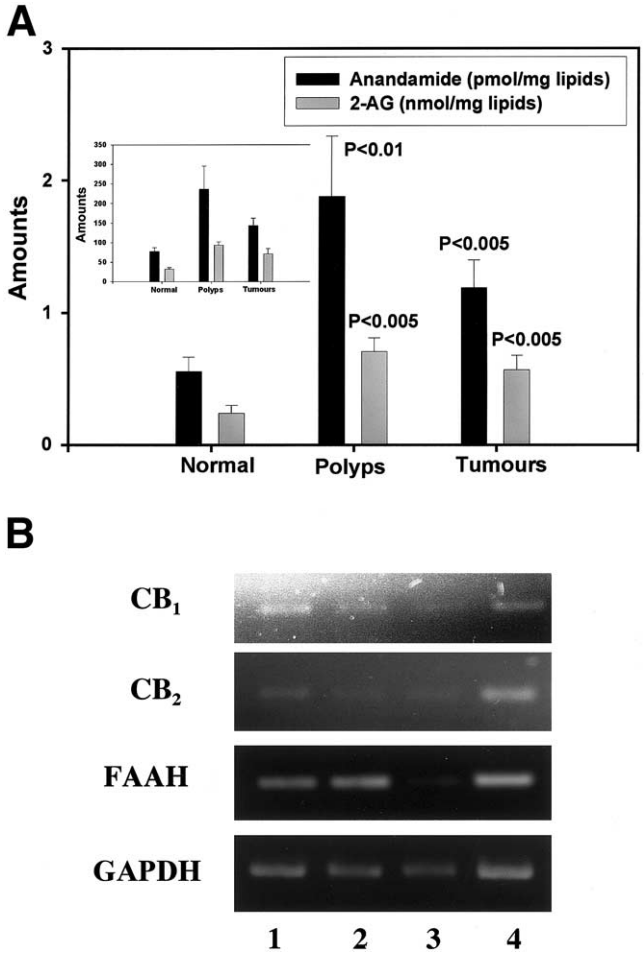

Figure 1. Endocannabinoid levels (*A*) and CB_1, CB_2, and FAAH mRNA expression (*B*) in human colorectal normal mucosa, adenomatous polyps, and carcinomas. (*A*) Anandamide and 2-arachidonoylglycerol (2-AG) levels are expressed as pmol or nmol per mg extracted lipids, respectively, and are means ± SEM of n = 12 samples for adenomas and n = 9 samples for tumors. Data for control mucosa were pooled from the 21 samples. Means were compared by 1-way ANOVA followed by the Bonferroni's test, and the level of significant difference from the respective controls is shown for each histogram. *Inset* shows the same data expressed as pmol or nmol/g wet tissue weight. (*B*) Expression of mRNA transcripts for CB_1 (*upper panel*), CB_2 (*second panel*), FAAH (*third panel*), and the housekeeping gene (GAPDH; *lower panel*) in samples from colorectal carcinomas (*lanes 1* and *2*) and healthy mucosa (*lanes 3* and *4*) from representative patients A (*lanes 1* and *4*) and B (*lanes 2* and *3*). RT-PCR was performed on tissues from 4 more patients with similar results. Amplicons were of the size expected from the type of oligoprimers used (see Materials and Methods section).

tissue containing 130 ± 7, 124 ± 5, and 119 ± 4 mg lipids/g wet tissue weight (means ± SEM, n = 21, 12, and 9, respectively), respectively. Therefore, the ranking of endocannabinoid content in the 3 types of tissues (adenomas>CRC>normal mucosa) did not change when the amounts were expressed as pmol/g wet tissue weight (Figure 1*A*, inset). No consistent differences between the levels of the expression of CB_1, CB_2, and FAAH (the former as assessed by both RT-PCR and immunoblot) were found between normal and CRC tissue (Figure 1*B* and data not shown).

CRC Cell Differentiation and/or Proliferation In Vitro

CaCo-2 cells reached confluence after about 7 days from cell seeding and, starting from the 12th day in culture, started differentiating as assessed by the progressive synthesis of sucrase (Figure 2*A*). Both AEA and 2-AG significantly inhibited the growth of undifferentiated CaCo-2 cells with IC_{50} in the submicromolar range (Figure 2*B*). No toxicity to cells, as assessed by the trypan blue method, and no effect on apoptosis, as assessed by FACScan, was observed up to 5 μmol/L AEA (not shown). The CBR agonist HU-210 inhibited CaCo-2 cell proliferation more potently than the 2 endocannabinoids. Two agonists selective for CB_1 vs. CB_2 receptors, i.e., arachidonoyl-chloro-anandamide (ACEA) and *N*-arachidonoyl-dopamine (NADA), also inhibited CaCo-2 cell proliferation, whereas the CB_2-selective agonist BML-190 was inactive (Figure 2*C*). Importantly, the effect of AEA, HU-210, and NADA was antagonized by the selective CB_1 receptor antagonist SR141716A (0.2 μmol/L) but not by the selective CB_2 receptor antagonist SR144528 (0.2 μmol/L, Figure 2*D* and data not shown). During the course of differentiation, the responsiveness of CaCo-2 cells to AEA and HU-210 changed dramatically, the proliferation of differentiated cells being almost insensitive to treatment with these 2 compounds (Table 1).

We also assessed the effect of AEA, 2-AG, HU-210, and BML-190 on the nondifferentiating DLD-1 cells. In this case, stimulation of both CB_1 and CB_2 receptors led to inhibition of cell growth, although with lower efficacy as compared with CaCo-2 cells (Table 1). The effect of HU-210 was counteracted by both SR141716A and SR144528 (0.2 μmol/L, Table 1).

The selective COX-2 inhibitor indomethacin *N*-methyl ester (0.1-25 μmol/L) inhibited undifferentiated CaCo-2 cell proliferation (Table 1). This effect of was not additive with that exerted by 2-AG, which instead occluded the antiproliferative effect indomethacin *N*-methyl ester (Table 1). The COX-2 inhibitor also blocked the proliferation of DLD-1 cells, and this effect was antagonized by SR141716A (Table 1).

Presence of CBRs in CRC Cells in Culture

By using RT-PCR, we found that CaCo-2 cells express the CB_1 receptor, whereas no evidence for the presence of CB_2 mRNA transcripts was found (Figure 3*A* and data not shown). Western immunoblotting con-

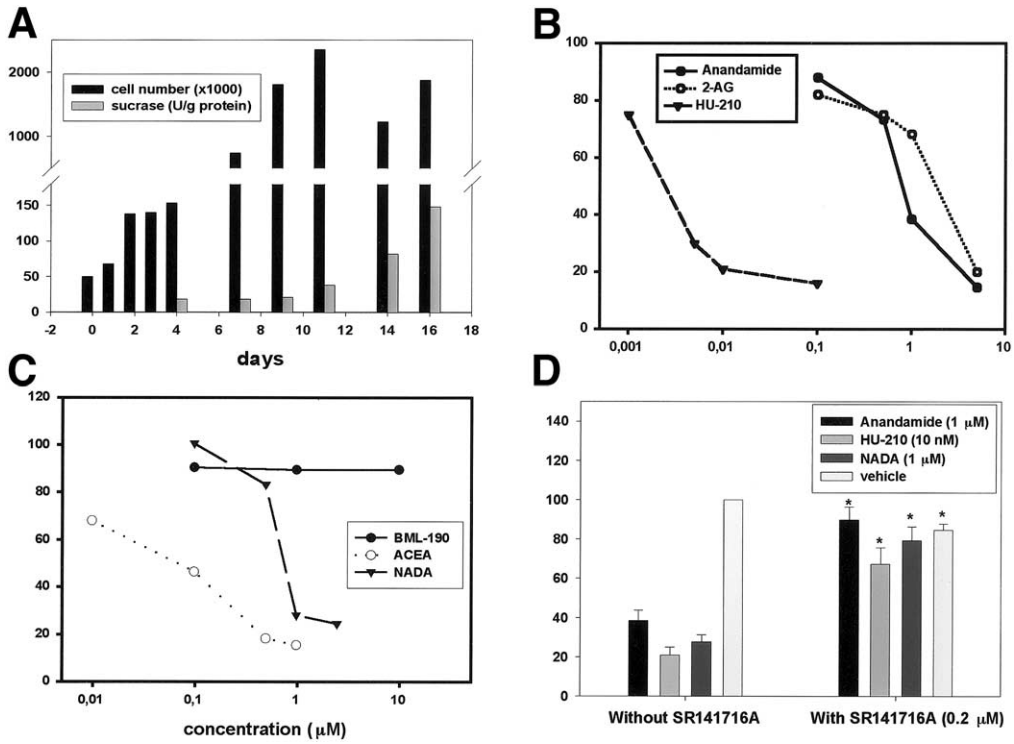

Figure 2. CaCo-2 cell proliferation and differentiation and the effect of cannabimimetics. (*A*) Growth of CaCo-2 cells in culture and sucrase production as an index of differentiation. (*B*) Effect of anandamide, 2-arachidonoyl-glycerol (2-AG), and the potent CB_1/CB_2 agonist HU-210 on the proliferation of undifferentiated (days 0–5) CaCo-2 cells. (*C*) Effect of the selective CB_1 agonists arachidonoylchloroethanolamide (ACEA) and *N*-arachidonoyl-dopamine (NADA) and of the selective CB_2 agonist BML-190 on the proliferation of undifferentiated (days 0–5) CaCo-2 cells. (*D*) Effect of the selective CB_1 antagonist SR141716A on the antiproliferative action of HU-210, anandamide, and NADA. $*P < 0.01$ vs. control, by ANOVA. In *B–D*, data are expressed as percentage of control cell proliferation (100× final treated cell number − initial cell number/final control cell number) and are means of n = 3 experiments carried out in duplicate. In *B* and *C*, SEM bars are not shown for the sake of clarity and were never higher than 5% of the means.

firmed that CB_1 is expressed in these cells, in that 3 immunoreactive bands, sensitive to saturation with the blocking peptide, were found with molecular weight very similar to those found in rat brain homogenates (Figure 3*C*) and corresponding to the truncated, native, and glycosylated forms of the CB_1 receptor. Overall, the levels of CB_1 receptors in CaCo-2 cells appeared to remain constant upon differentiation of the cells, although the amounts of the native form (~53 kilodaltons) of the receptor were significantly lower than in undifferentiated cells (Figure 3*C*). DLD-1 cells were found to express both CB_1 and CB_2 mRNA and protein, although CB_1 receptors appeared to be less abundant in these cells than in CaCo-2 cells (Figure 3*A* and *C*).

Endocannabinoids and Their Inactivation in CRC Cells

By using again our sensitive LC-MS analytical method, we found that undifferentiated CaCo-2 cells contain measurable levels of both AEA (Table 2). We also found that these cells exhibit FAAH activity (93.6 ± 11.4 pmol mg protein^{-1} minute^{-1}, mean ± SD, n = 3) and, like DLD-1 cells, express FAAH mRNA (Figure 3*B*). Intact, undifferentiated CaCo-2 cells also clear [^{14}C]AEA from the incubation medium in a temperature-dependent manner (67.0 ± 3.2 pmol minutes^{-1} per 10^6 cells, corresponding to 111.7 ± 5.3 pmol minutes^{-1} mg protein^{-1}, means ± SD, n = 3). The level of expression of FAAH mRNA in CaCo-2 cells appeared to increase upon differentiation of the cells (Figure 3*B*), thus explaining why the amounts of endocannabinoids in differentiated cells were significantly lower than in undifferentiated cells (Table 2).

The effect of the 2 selective AEA uptake inhibitors VDM11 and VDM13 and of the selective FAAH inhibitor arachidonoyl-serotonin were examined on the uptake and hydrolysis, respectively, of [^{14}C]AEA by CaCo-2 cells. VDM11 and VDM13 efficiently inhibited [^{14}C]AEA uptake with similar IC_{50} values around 3 μmol/L (Figure 4*A* and data not shown). Arachidonoyl-serotonin instead inhibited [^{14}C]AEA hydrolysis with a IC_{50} ~9 μmol/L (Figure 4*B*).

Table 1. Effect of Various Cannabimimetic Agents and of Indomethacin *N*-Methyl Ester on the Proliferation of CRC Cells in Culture

	DLD-1	CaCo-2 (days 1–5)	CaCo-2 (days 14–16)
AEA (2.5 μmol/L)	59.8 ± 5.4	27.0 ± 3.1	96.0 ± 2.2
2-AG (1 μmol/L)	75.0 ± 3.1	41.6 ± 1.5	NT
2-AG (2.5 μmol/L)	59.3 ± 3.8	23.8 ± 0.1	NT
HU-210 (0.1 μmol/L)	100.2 ± 1.5	18.3 ± 1.9	90.6 ± 2.8
HU-210 (1 μmol/L)	57.1 ± 3.5	NT	NT
HU-210 (1 μmol/L) + SR141716A (0.2 μmol/L)	79.8 ± 2.2[a]	NT	NT
HU-210 (1 μmol/L) + SR144528 (0.2 μmol/L)	88.7 ± 4.2[a]	NT	NT
BML-190 (1 μmol/L)	66.5 ± 3.1	90.7 ± 4.5	NT
BML-190 (1 μmol/L) + SR144528 (0.2 μmol/L)	90.5 ± 4.1[b]	NT	NT
INDO (0.1 μmol/L)	100.0 ± 5.2	74.0 ± 0.1	NT
INDO (1 μmol/L)	99.0 ± 3.5	74.2 ± 1.5	NT
INDO (10 μmol/L)	63.0 ± 4.2	62.6 ± 3.3	NT
INDO (25 μmol/L)	22.0 ± 0.9	10.3 ± 2.8	NT
INDO (25 μmol/L) + 2-AG (1 μmol/L)	NT	29.6 ± 3.8[c]	NT
INDO (25 μmol/L) + 2-AG (2.5 μmol/L)	NT	17.7 ± 2.1[c]	NT
INDO (25 μmol/L) + SR141716A (0.2 μmol/L)	61.5 ± 3.1[c]	NT	NT

NOTE. Data are expressed as percentage of control cell proliferation (100×; final treated cell number − initial cell number/final control cell number) and are means of n = 3 experiments carried out in duplicates. Both undifferentiated (days 1–5) and differentiated (days 14–16) CaCo-2 cells were used.

[a,b,c]$P < 0.05$ vs. agonist only (i.e., HU-210, BML-190, or INDO), by ANOVA followed by the Bonferroni test.

AEA, anandamide; 2-AG, 2-aracidonoylglycerol; NT, not tested; INDO, indomethacin *N*-methyl ester.

Effect of Selective Inhibitors of Endocannabinoid Inactivation on CaCo-2 Cell Proliferation

VDM11, VDM13, and arachidonoyl-serotonin were also evaluated for their effect on undifferentiated CaCo-2 cell proliferation. We found that the 3 compounds inhibited proliferation with IC_{50} values almost identical to those observed for the inhibition of [^{14}C]AEA uptake and hydrolysis, respectively (Figure 4*A* and *B* and data not shown). Importantly, the effect on cell proliferation of a 10 μmol/L concentration of the 3 inhibitors was antagonized by SR141716A (0.2 μmol/L; Figure 4*A* and *B*). Accordingly, we found that 24-hour incubation of undifferentiated CaCo-2 cells with either VDM-11 (10 μmol/L) or arachidonoyl-serotonin (10 μmol/L) led to a significant increase of endocannabinoid levels (Table 2).

COX-2 Expression in CRC Cell Lines

A messenger RNA transcript for COX-2 was detected both in undifferentiated CaCo-2 cells and, to a much smaller extent, DLD-1 cells (Figure 5). The level of expression of COX-2 decreased significantly when CaCo-2 cells underwent differentiation (Figure 5).

Discussion

We found that human colon mucosa tissues contain both AEA and 2-AG and express CB_1 and CB_2 receptors as well as FAAH. The endocannabinoids and FAAH previously have been described to occur in mouse and rat whole colon,[24,25] but we found here that the levels of both AEA and 2-AG increase dramatically when passing from normal mucosa to adenomatous polyps and then slightly decrease in CRC tissue. These changes are likely to result in corresponding changes in endocannabinoid tissue concentrations. In fact, considering that, on average, 1 g (and, hence, ~1 mL) of wet tissue weight yields ~125 mg of extracted lipids, it can be calculated that AEA concentrations augment from around 75 pmol/g (i.e., ~75 nmol/L) to 143 and 236 pmol/g (i.e., ~143 nmol/L and ~236 nmol/L) when passing from healthy mucosa to CRC and adenomatous polyps, respectively. Therefore, AEA concentrations are increased to concentrations well above the threshold of CB_1 activation by this lipid (the reported K_i values of AEA for CB_1 receptors are in the 40–200 nmol/L range[4]). Regarding 2-AG, the concentration of this compound augments from about 32 μmol/L, in normal tissue, to 71 and 93 μmol/L, respectively, in CRC and adenomas. Although the levels of 2-AG in healthy mucosa appear to be already sufficient to tonically activate CB_1 receptors (the reported K_i values of 2-AG for CB_1 receptors are in the 250–1200 nmol/L range[4]), it must be kept in account that only a part of 2-AG found in tissues might be used as an endocannabinoid. In fact, this compound, unlike AEA, is also an intracellular intermediate of (phospho)glyceride metabolism and is released outside cells only in part to activate CBRs.[29]

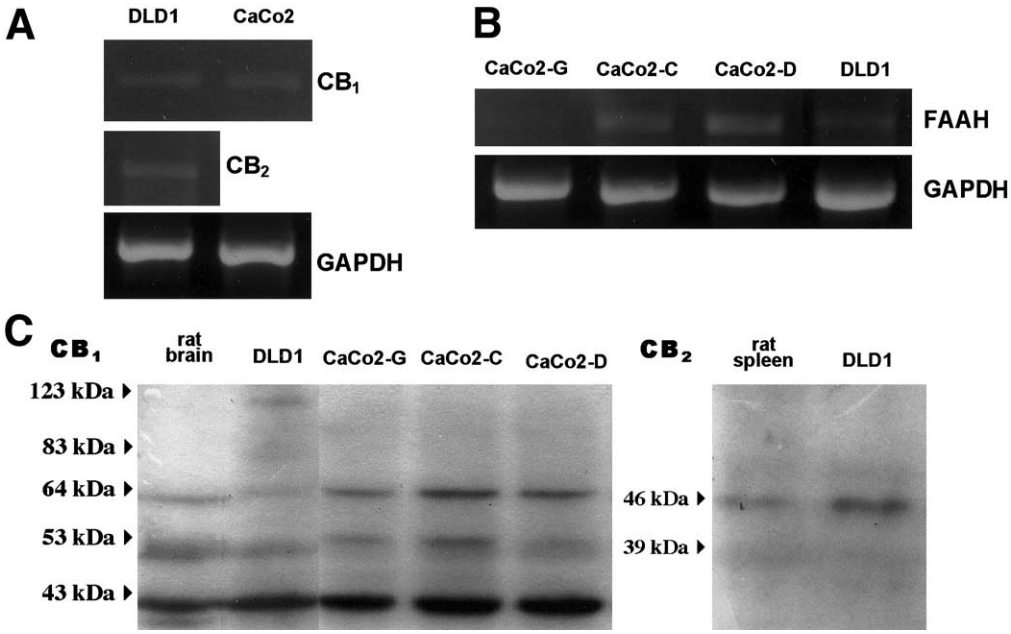

Figure 3. CB_1, CB_2, and FAAH expression in DLD-1 and CRC cells. (*A*) Expression in DLD-1 and CaCo-2 (growing phase, days 0–7) cells of mRNA transcripts with the expected sizes for CB_1 (309 bp) and CB_2 (329 bp). (*B*) Fatty acid amide hydrolase (FAAH) mRNA expression in CaCo-2 (growing phase, *G*, days 0–7; confluent phase, *C*, days 7–12; differentiated phase, *D*, days 14–16) and DLD-1 cells. The expected size of the amplicon was 300 bp. In both *A* and *B*, GAPDH (amplicon size 470 bp) was used as the housekeeping gene. (*C*) Western immunoblotting of protein homogenates from DLD-1 and CaCo-2 cells. Proteins (50 μg/lane) from lysates of rat brain (used as positive control), DLD-1 cells, and CaCo-2 cells (growing phase, *G*, days 0–7; confluent phase, *C*, days 7–12; differentiated phase, *D*, days 14–16), reacted with anti-CB_1 antibody, exhibited 3 immunoreactive bands at ~42 kilodaltons, ~53 kilodaltons, and ~63 kilodaltons, corresponding to the truncated, the native, and the glycosylated forms of the CB_1 receptor. In DLD-1 cells, 2 faint bands at ~83 kilodaltons and ~123 kilodaltons, corresponding to a further glycosylated and dimeric CB_1 receptor protein, respectively, were observed. Proteins (50 μg/lane) from lysates of rat spleen (used as positive control) and DLD-1 cells, reacted with the anti-CB_2 antibody, show 2 immunoreactive bands at ~46 kilodaltons and ~39 kilodaltons, corresponding to the glycosylated and the native forms of the CB_2 receptor, respectively. None of the immunoreactive bands was observed when the antibodies were preadsorbed with the immunizing peptide.

Several biochemical mechanisms might explain the enhancement of endocannabinoid levels in transformed human colon mucosa. First, an up-regulation of 1 of the enzymes responsible for arachidonate mobilization and phospholipid remodelling, the secretory phospholipase A_2, has been described to occur in colorectal adenomas from familial adenomatous polyposis patients.[26] Second, elevated levels of lysophosphatidic acids, possibly also including the *sn*-2-arachidonate-containing species that serve as biosynthetic precursors for 2-AG,[17] have been reported in patients with CRC.[19] These 2 events, albeit suggested to play a causative role in the onset and growth of tumors, via enhanced formation of the precursor for COX-2-catalyzed production of prostaglandins[15] or stimulation of LPA receptors,[18] respectively, might lead at the same time to elevated levels of endocannabinoids with tumor-inhibitory activity.

Table 2. Amounts of Endocannabinoids in Undifferentiated and Differentiated CaCo-2 Cells in Culture

	Anandamide	2-arachidonoyl-glycerol
Differentiated cells + vehicle	10.8 ± 3.0[a]	107.1 ± 3.5[a]
Undifferentiated cells + vehicle	41.0 ± 7.6	208.0 ± 45.1
+VDM11	70.0 ± 2.6[a]	400.0 ± 121.0[a]
+arachidonoyl-serotonin	107.6 ± 36.9[a]	405.0 ± 130.5[a]

NOTE. Table shows the effect of 24-hour cell treatment with vehicle (methanol, 0.1%) and VDM11 (10 μmol/L), and arachidonoyl-serotonin (10 μmol/L) of undifferentiated cells. Data are expressed as pmol/g wet cell weight and are means ± SD of n = 3 determinations.
[a]$P < 0.05$ vs. undifferentiated cells + vehicle, as assessed by the Student *t* test.

To test the above hypothesis, we undertook a series of experiments aimed at investigating whether (1) endocannabinoids do inhibit colon cancer cell growth in vitro; (2) substances that inhibit endocannabinoid inactivation, and hence enhance the amounts of endocannabinoids produced by CRC cells in culture, also inhibit the proliferation of these cells; and (3) changes in endocannabinoid signaling occur in CRC cells also during their differentiation in culture. We used a widely employed cell line for the study of CRC, the CaCo-2 cells, which have the special feature of being capable of differentiating in culture after having reached confluence, thus becoming more similar to enterocytes.[27] We found that

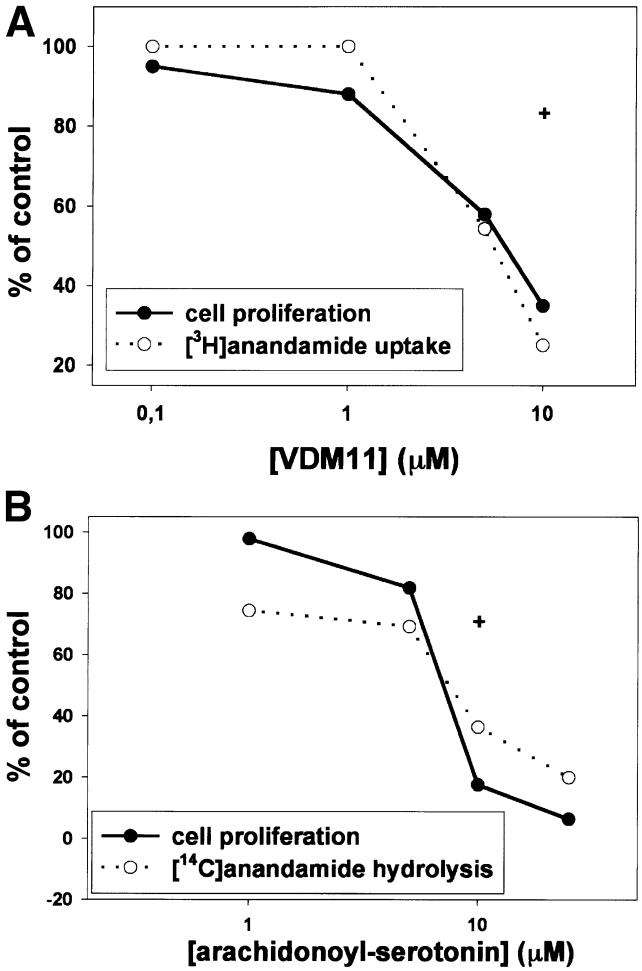

Figure 4. Effect of inhibitors of endocannabinoid metabolism on anandamide uptake and hydrolysis, and on proliferation, in undifferentiated (days 0–4) CaCo-2 cells. (*A*) Effect of the anandamide cellular uptake inhibitor VDM11 on the uptake of [^{14}C]anandamide by intact cells (○) and on cell proliferation (●). Similar data were obtained with VDM13, another anandamide cellular uptake inhibitor. (*B*) Effect of the anandamide enzymatic hydrolysis inhibitor arachidonoyl-serotonin on the hydrolysis of [^{14}C]anandamide by cell membranes (○), and on cell proliferation (●). Data for the effect on cell proliferation are expressed as percentage of control cell proliferation (see Figure 2 legend) and are means of n = 3 experiments carried out in duplicate. The effect of SR141716A (0.2 μmol/L) on the antiproliferative effect of VDM-11 (10 μmol/L) and arachidonoyl-serotonin (10 μmol/L) is shown with a *cross* and was statistically significant ($P < 0.01$ by ANOVA). Data for the inhibition of [^{14}C]anandamide uptake and hydrolysis are means of n = 3 experiments carried out in duplicate. SEM bars are not shown for the sake of clarity and were never higher than 5% of the means.

AEA, 2-AG, and the ultra-potent CBR agonist HU-210 inhibited CaCo-2 cell proliferation with IC_{50} values in the submicromolar range and with relative potencies (HU-210>>AEA≥2-AG) that reflect their relative potencies at cannabinoid CB_1 receptors.[4] Three further observations strongly supported the involvement of this receptor subtype in endocannabinoid antiproliferative effects. First, 2 agonists selective for CB_1 vs. CB_2 receptors, i.e., arachidonoyl-chloro-anandamide (ACEA) and *N*-arachidonoyl-dopamine (NADA), the latter of which is much more stable to enzymatic hydrolysis than AEA, but less potent on CB_1 receptors than AEA,[28] inhibited CaCo-2 cell proliferation with the rank of potency expected from their relative affinity for CB_1 receptors (ACEA>NADA), whereas the CB_2-selective agonist BML-190 was inactive. Second, the effect of AEA, HU-210, and NADA (which is also an agonist for the VR1 receptor for capsaicin[28]) was entirely antagonized by the selective CB_1 receptor antagonist SR141716A but not by the selective CB_2 receptor antagonist SR144528. Finally, we found that CaCo-2 cells express the CB_1 receptor, whereas no evidence for the presence of CB_2 was found. We also assessed the effect of the endocannabinoids and of HU-210 and BML-190 in another CRC cell line, the nondifferentiating DLD-1 cells. In this case, stimulation of both CB_1 and CB_2 receptors led to inhibition of cell growth, in agreement with the presence of both receptor subtypes in these cells. However, CB_1 receptors appeared to be less expressed in DLD-1 cells than in CaCo-2 cells, and this finding, together with the observation that DLD-1 cells were clearly less responsive to cannabimimetics than CaCo-2 cells, might suggest that CB_1 receptors are more important than CB_2 receptors in causing blockade of CRC cell proliferation. In summary, we found that, depending on the CRC cell line, either selective CB_1 receptor stimulation (as previously found for breast and prostate cancer cells[11,12]) or activation of both CB_1 and CB_2 receptors causes inhibition of proliferation. These findings are in agreement with the presence of both CBR subtypes in colon normal mucosa and CRC (Figure 1*B*) and suggest that endocannabinoids, present in high amounts in CRCs and, particularly, colorectal adenomas, might function as endogenous inhibitors of cancer growth.

To further challenge this hypothesis, we started a series of experiments aimed at manipulating pharmacologically the endogenous levels of endocannabinoids in CRC cells without directly activating the CBRs and at evaluating whether these treatments lead to inhibition of

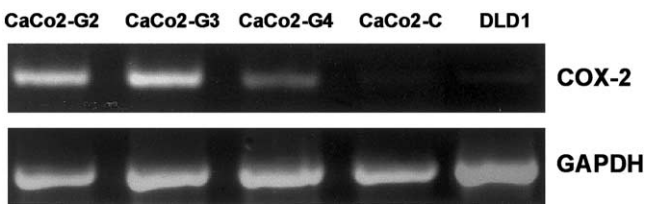

Figure 5. Cyclooxygenase 2 (COX-2) mRNA expression in CaCo-2 cells (growing phase, days 2, 3, and 4; confluent phase, days 7–12) and in DLD-1 cells. The expected sizes of the amplicons were 388 bp for COX-2 and 470 bp for GAPDH, used as the housekeeping gene.

CRC cell proliferation. We found that undifferentiated CaCo-2 cells contain AEA and 2-AG in concentrations (~40 and ~200 nmol/L, respectively) that, although smaller than those found in CRC tissue, are still compatible with a possible tonic submaximal activation of CBRs. Unlike CRC tissue, the amounts of 2-AG in these cells were only about 5-fold higher than those of AEA, in agreement with the hypothesis (see above) that only a part of the 2-AG detected in CRC tissue might derive from cancer cells and be used to activate their CBRs and to inhibit their proliferation. However, if the endocannabinoids do exert a tonic submaximal inhibition of cancer cell growth, agents that inhibit their inactivation should inhibit cancer cell proliferation. Endocannabinoids are inactivated through a 2-step process including cellular reuptake, which is facilitated by the AEA membrane transporter (AMT, a yet-to-be characterized protein facilitating the cellular reuptake not only of AEA but also of 2-AG and other endocannabinoids[28,29]), and intracellular hydrolysis by FAAH, an amidase that, in cell cultures, can also catalyze the hydrolysis of fatty acid esters such as 2-AG (see Di Marzo et al.[29] for a review). We found that undifferentiated CaCo-2 cells express FAAH mRNA and exhibit FAAH activity and that they take up anandamide in a temperature-dependent manner. We, therefore, could examine the effect of 2 selective AMT inhibitors, VDM11 and VDM13,[21] and of a selective FAAH inhibitor, arachidonoyl-serotonin,[30] on the uptake and hydrolysis, respectively, of [^{14}C]AEA by CaCo-2 cells and on CaCo-2 cell proliferation. We found that the 3 compounds inhibited with the same potency the 2 inactivation processes and CaCo-2 cell proliferation and that their antiproliferative effect was antagonized by SR141716A. Because none of these compounds is capable of directly stimulating CB_1 receptors at concentrations lower than 10 μmol/L,[21,30] this finding suggests that VDM-11, VDM-13, and arachidonoyl-serotonin inhibit CaCo-2 cell proliferation by enhancing the endogenous amounts of AEA and/or 2-AG available for CB_1 stimulation. This suggestion was strongly supported by our finding that 24-hour incubation of undifferentiated CaCo-2 cells with either VDM-11 or arachidonoyl-serotonin leads to a significant increase of endocannabinoid levels up to concentrations likely to cause a full activation of CB_1 receptors. These findings strengthen our hypothesis that endocannabinoids tonically inhibit CRC cell proliferation and suggest that inhibitors of endocannabinoid inactivation might represent useful anticancer drugs, although FAAH inhibitors might be less efficacious in vivo than was shown here in vitro, because of the presence of more than 1 hydrolyzing enzyme for 2-AG.[29]

Another approach that we used to gain further support to this hypothesis was to see whether, in CRC cells, responsiveness to (endo)cannabinoids and the extent of endocannabinoid signalling change during cell differentiation. To this purpose, we exploited the capability of CaCo-2 cells to differentiate in culture into enterocytes with a much lower degree of malignancy and invasiveness.[27,31,32] First, we observed that AEA was only efficacious against undifferentiated cells. This finding might have been caused by the fact that cell differentiation was accompanied by an increase of FAAH expression, which is likely to lead to an enhanced AEA degradation in differentiated cells. However, because HU-210, which is not a substrate for FAAH, was inactive on differentiated cells, the increase of FAAH expression might not be the sole cause of the lack of activity of AEA on differentiated cells. We also analyzed the expression of CB_1 receptors in differentiated CaCo-2 cells and found overall CB_1 levels similar to those observed in undifferentiated cells. However, the amounts of the native form (~53 kilodaltons) of the receptor appeared to be significantly lower than in undifferentiated cells. It is, therefore, possible that the smaller antiproliferative effect of HU-210 in differentiated cells is due to a decrease of the levels of functionally active CB_1 or, alternatively, to changes in CB_1-coupled intracellular signalling events during CaCo-2 cell differentiation. We also measured the endocannabinoids in differentiated cells and found significantly lower amounts of both AEA and 2-AG, in agreement with the higher FAAH expression in differentiated cells and with our data obtained with arachidonoyl-serotonin (Table 2). These findings, as well as preliminary data obtained in our laboratory, indicating no significant change in the expression of monoacylglycerol lipase (another 2-AG metobolizing enzyme[29]) during CaCo-2 cell proliferation, suggest that FAAH plays an important role in limiting the levels of both AEA and 2-AG in CaCo-2 cells. It is worthwhile noting that, in agreement with our findings, in human breast cancer cells, whose proliferation is blocked by endocannabinoids via CB_1 receptors,[11] FAAH has been shown very recently to be expressed in up to 30-fold higher levels in those cell lines with a lower degree of invasiveness and malignancy.[33] At any rate, the overall reduction of both the levels and the antiproliferative effects of endocannabinoids in differentiated (and less malignant) vs. undifferentiated (and more malignant) CaCo-2 cells suggests that, also in vitro, endocannabinoid signalling is regulated depending on colorectal cell differentiation and supports our hypothesis that AEA and 2-AG might act as endogenous CRC growth inhibitors.

The higher sensitivity to AEA and 2-AG of undifferentiated CaCo-2 cells as compared with DLD-1 and differentiated CaCo-2 cells might be also explained by the additional action of the 2 endocannabinoids, only in the former cells, on a target different from CB_1. This target might be COX-2 because (1) AEA and 2-AG compete efficaciously with arachidonic acid as COX-2 substrates[16] and might occlude the formation of protumoral prostaglandins, and (2) COX-2 is virtually absent in differentiated CaCo-2 and DLD-1 cells (Figure 5).[27,34] We found that the selective COX-2 inhibitor indomethacin *N*-methyl ester[35] inhibited undifferentiated CaCo-2 cell proliferation at concentrations ($IC_{50} \sim 14$ μmol/L) much higher than those required to inhibit the enzyme in vitro ($IC_{50} = 0.04$ μmol/L[35]). This antiproliferative effect was occluded by 2-AG, thus suggesting that the 2 substances share, at least in part, a similar mechanism of action. However, the COX-2 inhibitor (1) was also active on DLD-1 cells (which express little COX-2) where its effect was antagonized by SR141716A and (2) acted as a CB_1 receptor ligand in binding assays carried out with rat brain membranes ($K_i = 2.8 \pm 0.3$ μmol/L, mean ± SD, n = 3). This indicates that, like 2-AG, indomethacin *N*-methyl ester inhibits CRC cell proliferation by stimulating CBRs. Hence, the reason why (endo)cannabinoids are less potent in DLD-1 cells is not because they cannot act by inhibiting COX-2 expression, as suggested by the observation that HU-210, which does not inhibit COX-2, is also much less efficacious in the same cells (Table 1).

In conclusion, we have shown that endocannabinoids (1) are overproduced in cancerous and, particularly, precancerous (i.e., adenomas) colon tissue and (2) exert a growth-inhibitory effect on CRC cells in culture, in which the extent of their action and levels seems to depend on the degree of differentiation (and malignancy/invasiveness) of these cells. The antiproliferative effects of the endocannabinoids are exerted in a large part by stimulation of CBRs, which are expressed in both colorectal mucosa and CRC cells. However, these compounds might act also by inhibiting COX-2, a possibility that, although not supported by our present data, deserves further investigation. Whatever their mechanism of action, endocannabinoids can be regarded as potential endogenous tumor growth inhibitors as well as possible markers for cancer cells. This hypothesis is strengthened by a recent preliminary study[36] showing that AEA levels are increased in other tumors, including those whose growth was previously shown to be inhibited by endocannabinoids.[11,12] Metabolically stable substances that act by stimulating CBRs directly might exert anticancer actions in this as well as other type of tumors.[1,2] However, in view of (1) the possible multiple mechanisms of action of endocannabinoids, (2) the potential undesired psychotropic effect of CB_1 receptor agonists, and (3) the tonic inhibition on cancer growth suggested here for endocannabinoids, substances that act selectively by enhancing further the tumor levels of AEA and 2-AG, such as inhibitors of their cellular uptake and enzymatic hydrolysis, might provide for a more efficacious and tolerable therapeutic strategy against not only CRC but also other types of cancer.[2,36] In support of this possibility, we have found in a separate study (V. Di Marzo, G. Portella and M. Bifulco, manuscript in preparation) that the growth of transformed thyroid cells in athymic mice[13] is inhibited efficaciously by the AMT inhibitor VDM11 and by the FAAH inhibitor arachidonyl-serotonin via enhancement of tumoral endocannabinoid levels.

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Received January 8, 2003. Accepted April 24, 2003.

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Supported by funds from the Italian National Research Council, MURST (3933 to V.D.M.) and the Associazione Italiana per la Ricerca sul Cancro (to M.B.).

The authors thank Drs. Filomena Fezza and Pierangelo Orlando for valuable assistance and Dr. Gabriella Caruso (I.R.C.C.S. "S. de Bellis," Bari, Italy) for the kind gift of DLD-1 cells.

A.L. and T.B. contributed equally to this work.