

The cannabinoid Δ^9 -tetrahydrocannabinol inhibits RAS-MAPK and PI3K-AKT survival signalling and induces BAD-mediated apoptosis in colorectal cancer cells

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Deregulation of cell survival pathways and resistance to apoptosis are widely accepted to be fundamental aspects of tumorigenesis. As in many tumours, the aberrant growth and survival of colorectal tumour cells is dependent upon a small number of highly activated signalling pathways, the inhibition of which elicits potent growth inhibitory or apoptotic responses in tumour cells. Accordingly, there is considerable interest in therapeutics that can modulate survival signalling pathways and target cancer cells for death. There is emerging evidence that cannabinoids, especially Δ^9 -tetrahydrocannabinol (THC), may represent novel anticancer agents, due to their ability to regulate signalling pathways critical for cell growth and survival. Here, we report that CB1 and CB2 cannabinoid receptors are expressed in human colorectal adenoma and carcinoma cells, and show for the first time that THC induces apoptosis in colorectal cancer cells. THC-induced apoptosis was rescued by pharmacological blockade of the CB1, but not CB2, cannabinoid receptor. Importantly, THC treatment resulted in CB1-mediated inhibition of both RAS-MAPK/ERK and PI3K-AKT survival signalling cascades; two key cell survival pathways frequently deregulated in colorectal tumours. The inhibition of ERK and AKT activity by THC was accompanied by activation of the proapoptotic BCL-2 family member BAD. Reduction of BAD protein expression by RNA interference rescued colorectal cancer cells from THC-induced apoptosis. These data suggest an important role for CB1 receptors and BAD in the regulation of apoptosis in colorectal cancer cells. The use of THC, or selective targeting of the CB1 receptor, may represent a novel strategy for colorectal cancer therapy.

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Key words: colorectal cancer; survival signalling; cannabinoid; apoptosis; BAD

Despite advances in treatment, colorectal cancer remains the second most common cause of cancer death in the Western world. Understandably, there is great interest in the discovery of new strategies for colorectal cancer therapy. In recent years there has been intense interest in cannabinoids (the active components of marijuana, *Cannabis sativa*) and their derivatives as novel therapeutic agents for a range of common diseases.^{1,2} The identification of the major active component of marijuana, Δ^9 -tetrahydrocannabinol (THC), led to the discovery that cannabinoids function by activating specific cell surface G-protein coupled cannabinoid receptors: CB1³ and CB2.⁴ CB1 receptors are particularly abundant in the brain,⁵ and CB2 receptors are highly expressed in cells of the immune system.⁶ Cannabinoid receptors are now known to be engaged by a family of endogenous ligands termed the endocannabinoids, which are implicated in a range of physiological processes in many tissues and organs, including the gastrointestinal system.⁷ Compelling evidence suggests that the use of cannabinoids, or manipulation of the cannabinoid receptors and endocannabinoid system, could be of benefit in many diseases, including pain,⁸ spasticity and tremor in multiple sclerosis,⁹ atherosclerosis,¹⁰ inflammatory bowel disorders,¹¹ and of particular relevance here, cancer.¹² Cannabinoids have attracted much attention in recent years as potential anticancer agents, owing to their ability to exert antitumoral effects in a number of tumour cell types.^{13–18} We and others have previously reported that the endocannabinoid anandamide exerts antitumoral effects in colorectal cancer cells^{19,20}; however, the ability of cannabinoids, either endogenous or plant-derived, to induce apoptosis in colorectal cancer cells is yet to be reported. Thus, although THC has been shown

to induce apoptosis in other tumour cell types, given the significance of colorectal cancer as a major cause of cancer death, it is important to determine whether the applicability of THC as a novel anticancer agent could potentially be extendable to colorectal cancers.

There is growing evidence that cannabinoids may be able to selectively target tumour cells, at least in part through differential regulation of signalling pathways that control cell survival and apoptosis.^{12,19} However, the mechanisms underlying the antitumoral effects of cannabinoids are incompletely understood, and evidence suggests these effects may be cell-type specific.¹² Two signalling pathways that have been reported to be either positively or negatively regulated by cannabinoids (depending on the cell type and cannabinoid studied) are the RAS-MAPK/ERK pathway and the PI3K-AKT pathway.^{21–24} The RAS-MAPK pathway is frequently deregulated in colorectal tumours, with about 50% harbouring mutations in the *KRAS* gene²⁵ and another 10–20% harbouring mutations in *BRAF*²⁶; both of which switch on a constitutively active RAS-MAPK signalling cascade. In addition, the PI3K-AKT survival signalling pathway is aberrantly activated in almost 40% of colorectal tumours,²⁷ which leads to constitutively phosphorylated (active) AKT.²⁸ Aberrant activation of these survival pathways deregulates cell growth, proliferation and apoptosis, and are thus important forces driving colorectal tumorigenesis. Drugs targeting deregulated components of the RAS-MAPK or PI3K-AKT survival signalling pathways have attracted considerable attention in recent years as potential anticancer agents.^{29,30} Given that THC has the potential to modulate RAS-MAPK and PI3K-AKT signalling pathways, often deregulated in colorectal tumours, we sought to determine whether THC exerts antitumoral and/or proapoptotic effects in colorectal cancer cells. Here, we show, for the first time, that THC induces apoptosis in colorectal cancer cells, and that this occurs through the activation of the CB1 cannabinoid receptor. THC treatment resulted in the inhibition of both RAS-MAPK and PI3K-AKT survival signalling cascades and the activation of the proapoptotic BCL-2 family member BAD via its dephosphorylation on both serine 112 and 136. Reduction of BAD protein expression by RNA interference rescued colorectal cancer cells from THC-induced apoptosis. These data highlight a potential mechanism for THC-induced apoptosis in colorectal cancer cells, and bring to light a possible use for the naturally occurring canna-

Abbreviations: THC, Δ^9 -tetrahydrocannabinol; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-3 kinase; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand; H₂O₂, hydrogen peroxide; FBS, foetal bovine serum; DMSO, dimethyl sulfoxide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; PARP, poly(ADP-ribose) polymerase; p38-MAPK, p38 mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein kinase 1.

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binoid THC, or CB1 receptor targeting, as a novel approach for colorectal cancer therapy.

Materials and methods

Cell culture and reagents

Human colorectal adenoma- and carcinoma-derived cell lines were maintained under standard conditions as described previously.^{31–35} The colorectal carcinoma cell lines SW480, HCT-15, HT29, Caco-2, HCT116, LS174T and SW620 cells were from the ATCC (Rockville, MD). The colorectal adenoma AA/C1, AN/C1, BH/C1, RG/C2; carcinoma JW2; and transformed adenoma AAC1/SB/10C (SB10C) cell lines were derived in this laboratory and are described elsewhere.^{31–35} HCA7 colony 29 (HCA7) cells were a kind gift from Dr. Susan Kirkland (Imperial College London, UK). C6.9 rat glioma cells were a kind gift from Dr. Didier Wion (Centre Hospitalier Universitaire, Angers, France). TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) was a kind gift from Dr. Marion MacFarlane (MRC Toxicology Unit, University of Leicester, UK). THC was obtained from Sigma (Poole, UK); AM251 and AM630 were from Tocris (Bristol, UK); ZVAD.fmk was obtained from Alexis (San Diego, CA); LY-294002 was obtained from Calbiochem (La Jolla, CA); U0126 was from Cell Signaling Technology (Danvers, MA); hydrogen peroxide (H₂O₂) was obtained from BDH (Poole, UK).

Cell proliferation and apoptosis assays

For experiments, cells were seeded at a density of 40,000 cells/cm² in DMEM supplemented with 10% foetal bovine serum (FBS). It is important to note that previous reports indicate that when using cannabinoids such as THC in *in vitro* cell culture models, the presence of serum (i.e., FBS) has been reported to affect experimental outcomes.^{17,36} Therefore, in all experiments, after 24 hr growth in serum-containing medium, cells were serum-starved in DMEM:F12 medium for 24 hr, prior to treatment in serum-free DMEM:F12 medium. Control cells were treated with equivalent vehicle (ethanol or DMSO). Following treatment, cell yield was determined either using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, or by harvesting the attached cells (those remaining adhered to the tissue culture flask), and floating cells (those having detached from the tissue culture flask), which were subsequently counted separately as described previously.^{34,35} Apoptotic cells in cultured colorectal tumour cell lines detach and float in the growth medium^{35,37,38}; the percentage apoptosis was determined by measuring the proportion of apoptotic floating cells as a percentage of the total (attached plus floating) cell population, as described previously.^{34,35} Floating cells were confirmed as apoptotic using a combination of biochemical and morphological analyses.^{34,35}

Western blot analysis

Cells were washed once with ice-cold PBS before being disrupted on ice for 20 min using Cell Signaling Technology Lysis Buffer supplemented with protease inhibitors (Roche Diagnostics, East Sussex, UK). After lysis, cells were scraped and the lysate was cleared by centrifugation. Protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hertfordshire, UK). Equal amounts of protein were separated by SDS-PAGE, transferred to membranes, immunoblotted with specific primary and secondary antibodies and visualised using a chemiluminescence detection kit (KPL, Gaithersburg, MD) as described previously.³² Antibodies for p-Akt (Ser473), p-ERK (Thr202/Tyr204), p-BAD (Ser112), p-BAD (Ser136), p-p38 (Thr180/Tyr182), p-JNK (Thr183/Tyr185), Akt, ERK, BAD, p38, JNK, activated (cleaved) caspase-3 (Asp175) were from Cell Signaling Technology. Antibodies to PARP (poly[ADP-ribose] polymerase) and CB1 were obtained from Alexis (San Diego, CA), α -tubulin was from Sigma (Poole, UK) and CB2 was from Cayman (Ann Arbor, MI).

RNA extraction and reverse transcriptase-polymerase chain reaction

Total RNA was isolated from cells using an RNeasy kit (Qiagen, Crawley, West Sussex, UK). RNA was treated with DNase I (Ambion, Huntingdon, Cambridgeshire, UK) and reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out as described previously.^{19,31} 405 bp CB1 transcripts were detected using the primers 5'-cgtctgaggatgggaaggta-3' (forward) and 5'-tcttgaccgtctctgatg-3' (reverse); 562 bp CB2 transcripts were detected using the primers 5'-cgctatccaccctctctac-3' (forward) and 5'-ccctcacacactctctcc-3' (reverse); 238 bp GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcripts were detected using the primers 5'-cttcaccacatggagaaggc-3' (forward) and 5'-ggcatg-gactgtggtcatgag-3' (reverse). DNA sequencing confirmed that amplified sequences were identical to those for human CB1, CB2 and GAPDH. No template and minus-RT controls confirmed the absence of contaminating DNA.

Reduction of BAD protein expression using siRNA

Small interfering RNAs (siRNAs) were obtained from Ambion (Huntingdon, Cambridgeshire, UK). Cells were transfected with 2 independent siRNA sequences targeted against human BAD, or a validated negative control siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) prior to THC treatment. A validated GAPDH siRNA was also used as a positive control for knock-down. Cells were grown in 10% FBS DMEM for 24 hr and transfected with siRNAs for 6 hr in OptiMEM (Invitrogen), after which fresh DMEM:F12 media was added to the cells. siRNA-transfected cells were treated with THC for 72 hr in DMEM:F12 media.

Statistical analyses

Statistical analyses were performed using Student's *t*-test and Dunnett's *t*-test and expressed as: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, NS: not significant.

Results

THC, the plant-derived CB1 and CB2 receptor agonist, induces apoptosis in colorectal cancer cells

Cannabinoids have been proposed as novel cancer therapeutics, primarily due to their ability to induce apoptosis in a number of different tumour cell types.¹² However, the ability of cannabinoids to induce apoptosis in colorectal cancer cells has not been reported. Therefore, we investigated whether the plant-derived cannabinoid THC would affect the growth and survival of colorectal cancer cells in culture. We treated a panel of colorectal cancer cell lines with THC; cell survival was assessed using the MTT assay. Previous reports indicate that when using *in vitro* cell culture models, the presence of serum in the cell culture medium may affect experimental outcomes when studying the effects of cannabinoids on cell survival and proliferation^{17,36}; for this reason, all experiments involving cannabinoids were carried out in serum-free medium (as described in materials and methods). THC treatment of colorectal cancer cells resulted in a dose-dependent decrease in cell survival, which was significant in all cell lines tested at concentrations of 2.5 μ M and above (Figs. 1a–1d). Lower concentrations of THC (i.e., 100 nM–1 μ M) had no discernible effect on colorectal cancer cell survival or proliferation (data not shown). To establish whether the decrease in cell survival induced by THC was as a result of apoptosis, we treated colorectal cancer cells with THC and examined the cells for evidence of apoptosis. Treatment of SW480 cells with THC led to a dose-dependent increase in the activity of caspase-3, a key effector of apoptosis, as measured by an increase in the presence of cleaved (active) caspase-3 protein products in THC-treated cells (Fig. 2a, upper panel). Furthermore, THC caused a simultaneous increase in the cleavage of the caspase-3 substrate PARP (Fig. 2a, middle panel). Notably, the presence of only cleaved PARP in the floating cell

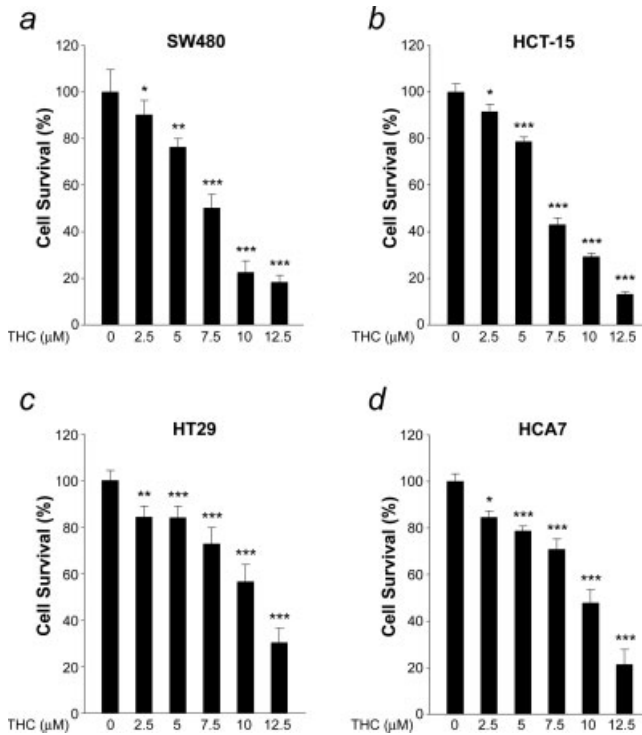


FIGURE 1 – The cannabinoid THC reduces cell survival in colorectal cancer cells: (a) SW480; (b) HCT-15; (c) HT29 and (d) HCA7 cells were treated with THC as indicated for 72 hr, after which cell survival was determined using the MTT assay. Columns represent the mean \pm SEM of 3 (a & d) or 4 (b & c) independent experiments performed in sextuplicate (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Dunnett's *t*-test).

population suggests that the majority of cell death induced by THC treatment was apoptosis (Fig. 2b). In addition, THC-treated floating cells exhibited morphological characteristics of apoptosis such as chromatin condensation and micronucleation as determined by staining with DAPI (Fig. 2c). As further evidence that THC-induced cell death was indeed apoptosis, we pretreated cells for 2 hr with the broad-spectrum caspase inhibitor ZVAD.fmk (50 μ M), prior to treatment with THC (10 μ M) and determined the proportion of apoptotic cells. ZVAD.fmk provided almost complete protection from the cell death induced by THC (Fig. 2d). Similar results were also obtained in HCT-15, HCA7 and HT29 colorectal cancer cell lines (data not shown). Taken together, these data indicate that THC reduces cell survival in colorectal cancer cells through the induction of apoptosis.

CB1 and CB2 cannabinoid receptor mRNA and protein are expressed in colorectal tumour cell lines

Given that cannabinoids such as THC are known to mediate their effects primarily through activation of the CB1 and CB2 cannabinoid receptors, we investigated whether CB1 and CB2 receptors are expressed in colorectal tumour cells. RT-PCR was carried out on 14 human colorectal cell lines derived from colorectal adenomas and carcinomas. We confirmed the specificity of the primers for CB1 and CB2 mRNA by sequencing the 405- (CB1) and 562- (CB2) base pair PCR products (data not shown). All colorectal tumour cell lines expressed detectable levels of CB1 and CB2 mRNA (Fig. 3a). Having shown that CB1 and CB2 mRNA was expressed in colorectal tumour cells, levels of CB1 and CB2 protein expression were determined using Western blot analysis. As a positive control for CB1 and CB2 expression, the glioma cell line C6.9 was also included.¹³ We detected a band of approximately 53-kDa in C6.9 cells and all of the colorectal cell lines analysed

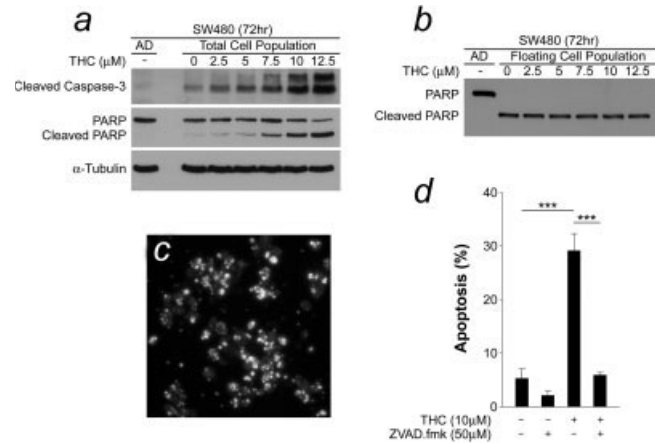


FIGURE 2 – THC induces apoptosis in colorectal cancer cells. (a) THC-induced caspase-3 cleavage (top panel) and PARP cleavage (middle panel) in SW480 cells. Cells were treated with increasing concentrations of THC (as indicated) for 72 hr. The total cell population (attached cells plus those floating in the culture medium) were harvested and lysed as described in materials and methods. Cell lysates were then subject to Western blot analysis with the indicated antibodies. As a control, untreated attached cells (AD) were included; as expected attached cells expressed full length PARP and minimal cleaved caspase-3. Equal protein loading was confirmed using an α -tubulin antibody. (b) the floating cell population from THC treated and control treated SW480 cells exhibit PARP cleavage; attached cells (AD) were included as a control. (c) THC treated SW480 cells exhibit condensed chromatin and micronucleation as determined by visualisation with DAPI staining. (d) the broad-spectrum caspase inhibitor ZVAD.fmk rescued SW480 cells from THC-induced apoptosis. SW480 cells were pretreated for 2 hr with 50 μ M ZVAD.fmk prior to THC treatment for 72 hr. The percentage of apoptotic cells was determined as described in materials and methods. Columns represent the mean \pm SEM of three independent experiments performed in triplicate (** $p < 0.001$; Student's *t*-test).

(Fig. 3b), consistent with the molecular weight of CB1. In addition, we detected a 39-kDa protein consistent with the size of the native CB2 receptor in C6.9 cells and all the colorectal cell lines analysed. These data suggest that CB1 and CB2 cannabinoid receptors are present in colorectal adenoma and carcinoma cells. Indeed, the presence of functional cannabinoid receptors in human colonic epithelial cells has recently been reported.³⁹

Activation of the CB1 cannabinoid receptor mediates the proapoptotic effects of THC in colorectal cancer cells

To address the question of whether THC reduces colorectal cancer cell survival and induces apoptosis through a cannabinoid receptor-dependent mechanism, we adopted a cannabinoid receptor pharmacological blockade approach using the CB1-selective receptor antagonist AM251, and CB2-selective receptor antagonist AM630. The concentrations of AM251 and AM630 used were based on those used in previous reports.^{8,40} Furthermore, prior to using AM251 and AM630 in conjunction with THC, we confirmed that the concentrations used minimally affected cell survival in a number of colorectal tumour cell lines (data not shown). As shown in Figure 4a, the reduction in cell survival induced by THC was rescued by dose-dependent blockade of the CB1 receptor with AM251. However, in contrast to AM251, blockade of the CB2 receptor with AM630 did not significantly rescue cells from the decreased cell survival induced by THC (Fig. 4b). When AM630 was combined together with AM251 and THC, the rescue from THC was equivalent to that with AM251 alone (data not shown). This suggests that signalling through CB1, but not CB2, is involved in the antitumour effects of THC in colorectal cancer cells. We next tested whether pharmacological blockade of CB1 with AM251 could rescue the cells from THC-induced apoptosis.

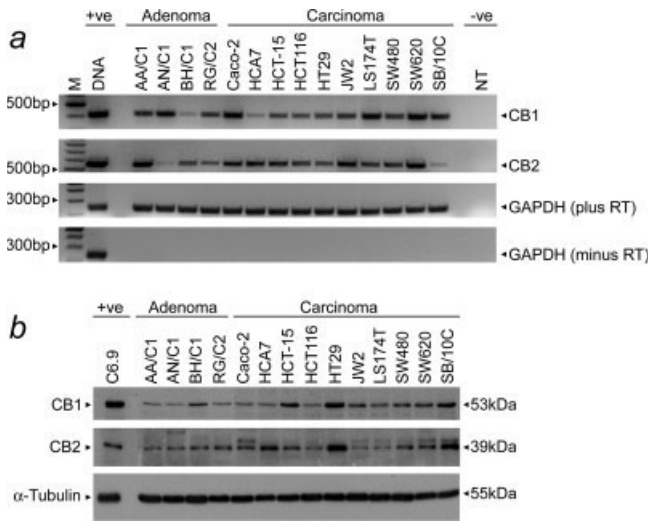


FIGURE 3 – Colorectal adenoma and carcinoma cell lines express CB1 and CB2 cannabinoid receptor mRNA and protein. (a) total cellular RNA from 14 colorectal tumour cell lines was extracted and RT-PCR performed using primers to CB1, CB2 and GAPDH; as described in materials and methods. Amplicons of expected sizes were obtained for each primer set, and genomic DNA (+ve, DNA) served as a positive control. No bands were detected in the no template control (-ve, NT) or minus reverse transcriptase reactions (minus RT), indicating the absence of genomic DNA contamination. M, DNA marker. (b) Western blot analysis of CB1 and CB2 expression from protein lysates of 14 colorectal tumour cell lines. The C6.9 glioma cell line was included as a positive control for CB1 and CB2 expression. Equal protein loading was confirmed using an α-tubulin antibody. Data shown are representative of experiments performed at least three times.

To do this, we determined the percentage of apoptotic cells in SW480 cells treated with THC in the presence or absence of the CB1-selective receptor antagonist AM251. The results in Figure 4c show that addition of AM251 significantly blocked THC induced apoptosis. These data indicate that THC reduces cell survival and induces apoptosis in colorectal cancer cells through activation of the CB1 receptor.

THC treatment results in CB1 receptor-dependent inhibition of RAS-MAPK/ERK and PI3K-AKT survival signalling pathways in colorectal cancer cells, but has no effect on p38-MAPK or JNK pathways

It has been proposed that cannabinoids can regulate cell signalling pathways which control cell fate.¹² Two key cell survival pathways regulated by cannabinoids are the RAS-MAPK and

PI3K-AKT pathways^{21–23}; both of which are often aberrantly activated in colorectal tumours. To determine whether THC regulated RAS-MAPK and PI3K-AKT survival signalling, we examined the activity of ERK and AKT during THC treatment of colorectal cancer cells using Western blotting and phosphorylation-specific antibodies. THC treatment caused a decrease in the levels of both phosphorylated ERK and AKT compared with vehicle-treated controls after 24 hr treatment (Fig. 5a). This decrease in activity

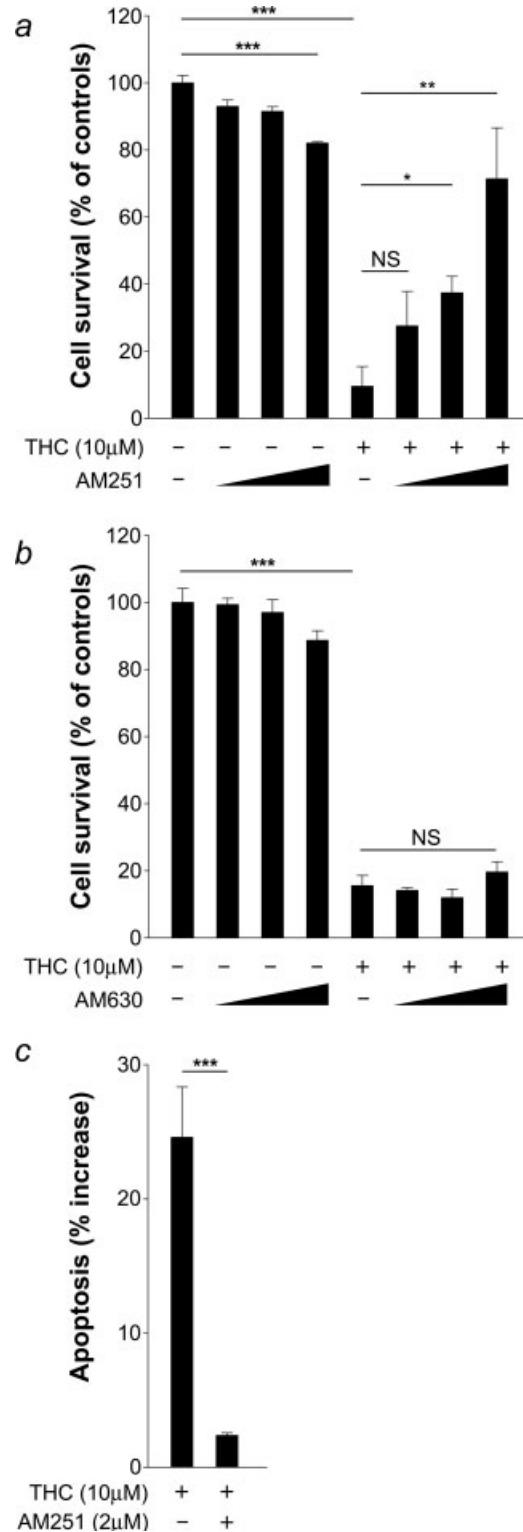


FIGURE 4 – Antitumoral effects of THC in colorectal cancer cells are mediated by signalling through the CB1 receptor. (a) SW480 cells were pre-treated for 2 hr with increasing concentrations of AM251 (0.5, 1 or 2 μM), prior to addition of equivalent vehicle or 10 μM THC, and cell survival was determined using the MTT assay. AM251 dose-dependently rescued cells from the reduction in cell survival induced by THC. Results are shown as percentage cell survival relative to controls. (b), SW480 cells were pretreated for 2 hr with increasing concentrations of AM630 (1, 2.5 or 5 μM), prior to addition of 10 μM THC or equivalent vehicle control, and cell survival was determined using the MTT assay. AM630 failed to rescue cells from the reduction in cell survival induced by THC. Results are shown as percentage cell survival relative to controls. (c) SW480 cells were pre-treated for 2 hr with 2 μM AM251, prior to addition of 10 μM THC or equivalent vehicle control. Percentage apoptosis was determined as described in materials and methods. Results are expressed as the percentage increase in apoptosis by subtracting corresponding control values. Columns show the mean ± SEM of three independent experiments (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, NS: not significant; Student’s *t*-test).

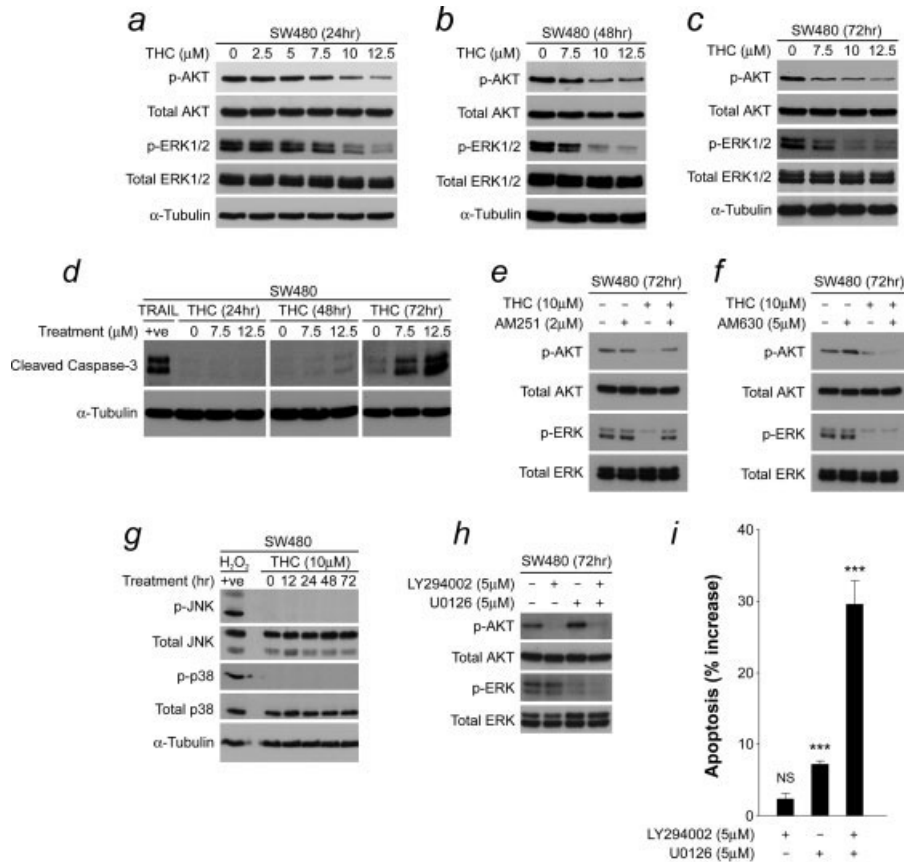


FIGURE 5 – THC treatment caused the inhibition of RAS-MAPK/ERK and PI3K-AKT survival signalling pathways in colorectal cancer cells. (a) SW480 cells were treated for 24 hr; (b) 48 hr; (c) 72 hr with increasing concentrations of THC (as indicated) and Western blotting was carried out on cell lysates with the indicated antibodies. THC caused a decrease in phosphorylated (active) ERK and AKT, whereas total ERK and AKT levels remained constant. Data shown are representative of experiments performed at least three times. (d) cleavage (activation) of caspase-3 was not detected after 24 hr THC treatment. As a positive control for caspase-3 cleavage, cells were treated with 0.1 μ g/ml TRAIL (+ve, TRAIL) as described previously.³³ Caspase-3 cleavage occurred after 48–72 hr THC treatment. (e) AM251 prevented the inhibition of ERK and AKT phosphorylation mediated by THC. (f) AM630 failed to prevent THC-mediated inhibition of ERK and AKT phosphorylation. (g) THC did not cause phosphorylation (activation) of JNK or p38 pathways; 1 mM H₂O₂ (+ve, H₂O₂) treated cells (20 min) were included as a positive control for phosphorylation of p38 and JNK. (h) the PI3K inhibitor LY294002 and MEK inhibitor U0126 inhibited AKT and ERK phosphorylation, respectively. (i) Inhibition of PI3K-AKT and RAS-MAPK signalling pathways induced synergistic apoptosis in colorectal cancer cells. Results are expressed as the increase in apoptosis by subtracting the vehicle treated controls. Columns show the mean \pm SEM of three independent experiments performed in triplicate (***) $p < 0.001$; Student's *t*-test).

was maintained at 48 and 72 hr treatment (Figs. 5b and 5c). Importantly, these events preceded significant increases in caspase-3 activation (Fig. 5d), indicating that these events might play an important role in the initiation of THC-induced apoptosis. To establish whether signalling via the CB1 receptor was responsible for the inhibition of ERK and AKT activity, colorectal cancer cells were treated with THC in the presence of the CB1 antagonist AM251, and the CB2 antagonist AM630, and levels of ERK and AKT phosphorylation were examined. Treatment of colorectal cancer cells with THC in the presence of AM251 prevented the dephosphorylation of both ERK and AKT caused by THC (Fig. 5e), whereas AM630 failed to prevent ERK and AKT dephosphorylation (Fig. 5f). This suggests that the inhibition of ERK and AKT activity by THC might play a causative role in THC-mediated apoptosis, given that AM251 prevented both THC-mediated apoptosis, and THC-mediated ERK and AKT inhibition. Finally, we examined whether THC modulated two other signalling pathways previously reported to be regulated by cannabinoids; the p38-MAPK and c-Jun N-terminal kinase (JNK) pathways.^{13,18,41} Interestingly, we saw no evidence for the regulation of either p38-MAPK or JNK pathways by THC (Fig. 5g). Taken together, these data indicate that the inhibition of RAS-MAPK and PI3K-AKT pathways, but not activation of JNK or p38 pathways, may be

involved in the induction of apoptosis by THC in colorectal cancer cells. In summary, these data suggest that THC causes a decrease in ERK and AKT activity via the activation of the CB1, but not CB2, cannabinoid receptor.

Inhibition of both RAS-MAPK and PI3K-AKT pathways induces synergistic apoptosis in colorectal cancer cells

If THC induces apoptosis in colorectal cancer cells through the inhibition of ERK and AKT signalling, one might expect a similar consequence in response to the direct inhibition of the RAS-MAPK and PI3K-AKT pathways with specific chemical inhibitors of these pathways. Therefore, to address this, we treated SW480 cells with the PI3K inhibitor LY294002 and selective MEK inhibitor U0126. As expected, LY294002 caused a decrease in phosphorylated AKT levels, whereas U0126 caused a decrease in phosphorylated ERK levels (Fig. 5h). When LY294002 and U0126 were combined, a decrease in both phosphorylated AKT and ERK levels was observed (Fig. 5h). Treatment of colorectal cancer cells with LY294002 alone did not significantly induce apoptosis, whereas U0126 alone caused a modest increase in apoptosis (Fig. 5i). However, treatment of colorectal cancer cells with a combination of both LY294002 and U0126 resulted in marked, synergistic

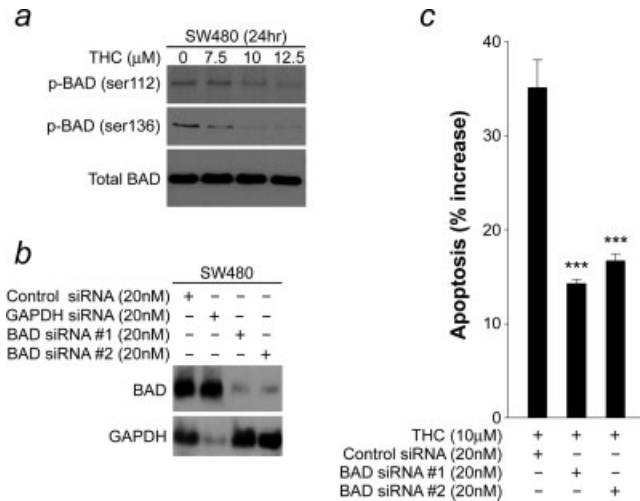


FIGURE 6 – THC treatment caused dephosphorylation of BAD, and BAD-mediated apoptosis in colorectal cancer cells. (a) SW480 cells were treated for 24 hr with increasing concentrations of THC (as indicated) and Western blotting was performed on cell lysates with the indicated antibodies. Data shown are representative of experiments performed at least three times. (b) SW480 cells were transfected with either 20 nM of negative control siRNA, 2 independent siRNA sequences targeted against human BAD, and as a positive control for knockdown, GAPDH siRNA. Knockdown was confirmed by Western blotting. (c) SW480 cells were transfected with negative or BAD siRNA prior to treatment with THC (10 μ M). Reduction of BAD protein expression with 2 independent siRNA sequences rescued SW480 cells from THC induced apoptosis. Results are expressed as the increase in apoptosis by subtracting the corresponding vehicle treated controls. Columns show the mean \pm SEM of three independent experiments performed in triplicate (***p* < 0.001; Student's *t*-test).

apoptosis (Fig. 5i). Thus, direct inhibition of both the RAS-MAPK and PI3K-AKT pathways with specific chemical inhibitors resulted in the induction of apoptosis in colorectal cancer cells. Furthermore, direct inhibition of both the RAS-MAPK and PI3K-AKT pathways was both necessary and sufficient for the induction of apoptosis to levels comparable to that induced by 10 μ M THC.

THC treatment of colorectal cancer cells results in the dephosphorylation of proapoptotic BCL-2 family member BAD

The proapoptotic BH3-only BCL-2 family member BAD plays an important role in regulating apoptosis in cells deprived of survival signals.⁴² In the presence of survival signals, BAD is phosphorylated and sequestered in the cytoplasm bound to 14-3-3 proteins; thus the proapoptotic activity of BAD is repressed by phosphorylation.⁴³ Phosphorylation of BAD occurs on a MAPK-dependent site, serine 112⁴⁴; and serine 136, a site phosphorylated directly by AKT.⁴⁵ Phosphorylation of either serine 112 or serine 136 is sufficient to render BAD inactive.^{43,46} We therefore examined whether treatment with THC led to the dephosphorylation of BAD at serine 112 and 136, which would promote apoptosis. Levels of phosphorylated BAD were examined after 24 hr THC treatment, at which time both AKT and ERK phosphorylation were reduced (Fig. 5a), but preceded significant caspase-3 activation (Fig. 5d). THC treatment of colorectal cancer cells caused dephosphorylation of BAD at both serine 112 and serine 136 residues (Fig. 6a). Therefore, these data suggest that activation of BAD may play a role in mediating THC-induced apoptosis in colorectal cancer cells.

Reduction of BAD expression by RNA interference rescues colorectal cancer cells from THC-induced apoptosis

Having shown that THC treatment led to BAD dephosphorylation (following RAS-MAPK and PI3K-AKT inhibition), we

hypothesised that reduction of BAD protein expression might render colorectal cancer cells more resistant to THC-induced apoptosis. Therefore, to test whether the mechanism of THC-induced apoptosis directly involves the activation of BAD, we used siRNA to reduce the expression of endogenous BAD protein in SW480 cells treated with THC. Two independent siRNAs targeted to BAD were used to reduce BAD protein expression; knockdown was confirmed using Western blotting (Fig. 6b). As shown in Figure 6c, reduction of BAD protein levels using two independent siRNA sequences targeted to BAD significantly attenuated the induction of apoptosis mediated by THC. These data illustrate the involvement of BAD in THC-induced apoptosis, and suggest that THC-induced inhibition of RAS-MAPK and PI3K-AKT survival signalling mediates the induction of apoptosis in colorectal cancer cells through the activation of the proapoptotic BAD protein.

Discussion

As a major cause of cancer death in much of the industrialised world, colorectal cancer continues to be a public health issue of great significance. The ability of cancer therapies to induce apoptosis in tumour cells is considered fundamental to the success of cancer treatment,^{38,47} given that disturbances to normal cell survival mechanisms which deregulate proliferation and apoptosis are widely accepted to be hallmarks of most, if not all cancers.⁴⁸ In the present study, we report for the first time that the plant-derived cannabinoid THC induces apoptosis in colorectal cancer cells, and that this is mediated by activation of the CB1 cannabinoid receptor. Importantly, we show that by signalling through the CB1 receptor, THC induces apoptosis in colorectal cancer cells via a mechanism that involves the proapoptotic BCL-2 family member BAD, which is likely to become active following THC-mediated inhibition of the RAS-MAPK and PI3K-AKT cell survival pathways.

The plant-derived cannabinoid THC is of particular interest for cancer therapy; THC has been shown to promote regression of some tumour types *in vivo*,^{12,13} and is approved for use in the clinic by the US FDA as an antiemetic and appetite stimulant.⁴⁹ Furthermore, as part of a small clinical trial, THC has been administered intratumourally in a number of patients with glioblastoma multiforme, without eliciting overt psychoactive effects.^{50,51} However, although THC is generally well tolerated, and has antitumoral activity in a number of cancer cell types, it is important to note that under certain circumstances THC may enhance tumour growth, either by suppression of the antitumour immune response,⁵² or inducing tumour cell proliferation.⁵³ Previously, cannabinoids have been reported to cause heterogeneous changes to the growth and survival of tumour cells. These changes include alterations to the cell cycle, resulting in growth arrest,^{54,55} the induction of apoptosis,^{16,56} nonapoptotic cell death,¹⁹ necrosis,⁵⁷ and in some instances the promotion of cell proliferation.⁵³ Interestingly, we found that relatively low concentrations of THC were required to cause a considerable reduction in colorectal cancer cell survival (i.e., 7.5–12.5 μ M), which is comparable with the concentrations of THC required to elicit antitumoral responses in other epithelial cells, such as breast cancer cell lines.¹⁵ It is interesting to note that in one study, low concentrations of THC (100 nM–1 μ M) have been reported to increase cancer cell proliferation.⁵³ However, in colorectal cancer cells, we found that 100 nM–1 μ M THC had no discernible effect on cell proliferation (data not shown); furthermore, concentrations as low as 2.5 μ M also significantly reduced colorectal cancer cell survival in all cell lines tested.

Until relatively recently, the expression of the CB1 and CB2 cannabinoid receptors was thought to be largely restricted to the brain and central nervous system, and cells of the immune system, respectively.⁵⁸ It is now clear that the cannabinoid receptors are expressed more widely in human tissues than originally thought. In the gastrointestinal tract, the endogenous cannabinoid system and cannabinoid receptors regulate functions such as gastric emptying, secretion, intestinal motility⁵⁹; primarily via CB1 receptors expressed in the neurons of the myenteric and submucosal plexuses. Although it has recently been reported that functional CB1 receptors are present in epithelial

cells from the human colon,³⁹ the precise role of the CB1 and/or CB2 receptors in epithelial cells of the colon remains unclear. Therefore, our finding that THC inhibited survival signalling and induced apoptosis in colorectal cancer cells via the CB1 receptor is of potential importance for two reasons. Firstly, these results suggest that the CB1 receptor may have a role in the control of cell numbers in the colon; and secondly, the CB1 receptor may represent a novel target for colorectal cancer therapy. It has been proposed that it is the differential signalling downstream of cannabinoid receptor activation that enables cannabinoids to selectively target tumour cells.¹² For this reason, it is notable that activation of the CB1 receptor by THC in colorectal cancer cells caused the inhibition of ERK and AKT survival signalling. These findings are particularly relevant to colorectal cancer given that aberrant activation of both ERK and AKT signalling occurs in the majority of colorectal tumours due to mutations in components of the RAS-MAPK and PI3K-AKT signalling pathways.^{25–28} Many tumour cells depend upon highly activated pathways such as RAS-MAPK and PI3K-AKT signalling for growth and survival; such that the inhibition of these pathways to which tumour cells have become 'addicted', causes potent growth inhibitory or apoptotic responses.^{46,60,61} This phenomenon has been illustrated previously by a report showing that inhibition of the RAS-MAPK cascade with the MEK inhibitor PD184352 results in the dramatic growth inhibition of colorectal tumour cell xenografts, without signs of toxicity; exemplifying the heavy reliance of colorectal tumour cells on RAS-MAPK signalling.⁶¹ In our study, selective inhibition of both PI3K and MEK activity with LY294002 and U0126, respectively, induced synergistic apoptosis in colorectal cancer cells, emphasising the dependence of colorectal cancer cells on ERK and AKT signalling. Another report recently noted a similar synergistic induction of apoptosis in response to PI3K and MEK inhibition in melanoma and glioblastoma cells with aberrant RAS-MAPK and PI3K-AKT signalling.⁴⁶ Therefore, the CB1-mediated inhibition of both ERK and AKT activity we observed in this study may be particularly significant for colorectal tumours, and other tumours with deregulated ERK and AKT survival signalling pathways.

Despite the fact that cannabinoids have been shown to exert antitumoural activity in other tumour cell types, the mechanisms of cannabinoid action appear to differ between discrete cell types.¹² For example, in contrast to our findings reported here, it has previously been shown that THC mediates apoptosis in glioma cells via both CB1 and CB2 activation and sustained ERK signalling,¹³ and more recently via induction of the stress-induced protein p8.¹⁶ Furthermore, in addition to promoting apoptosis, THC has also been reported to block cell cycle progression in breast cancer cells via CB2 receptor activation and downregulation of CDC2.¹⁵ Interestingly, whereas we observed no involvement of either the p38-MAPK or JNK pathways during THC-induced apoptosis in colorectal cancer cells, in other cell types activation of these stress pathways mediates the proapoptotic effects of THC.^{18,41}

Recently, it has been reported that treatment of C6 rat glioma cells with synthetic cannabinoid WIN-55,212-2 resulted in reduced BAD phosphorylation.²³ In addition, during the preparation of this manuscript, Jia *et al.* reported that THC induces BAD-dependent apoptosis in Jurkat leukaemia cells via inhibition of the MAPK pathway and activation of BAD by its dephosphorylation on Ser112.²⁴ Therefore, our data support those of Jia *et al.* and further suggest an important role for BAD in THC-induced apoptosis. Interestingly, however, there are several important differences

between the mechanism of THC-induced apoptosis in colorectal cancer cells (our findings), when compared with the mechanism of THC-induced apoptosis in Jurkat leukaemia cells. For example, Jia *et al.* report that THC promotes dephosphorylation of BAD on Ser112 only, suggesting that inhibition of the RAS-MAPK pathway (responsible for phosphorylation of BAD on Ser112), but not the PI3K-AKT pathway (responsible for phosphorylation of BAD on Ser136), is involved in THC-induced apoptosis in Jurkat cells.²⁴ We, however, show that in colorectal cancer cells, BAD becomes dephosphorylated on both Ser112 and Ser136 (MAPK- and AKT-dependent sites, respectively); suggesting that inhibition of both the RAS-MAPK and PI3K-AKT pathways may be involved in THC-induced apoptosis in colorectal cancer cells. Indeed, Jia *et al.* reported that treatment of Jurkat cells with the MEK inhibitor U0126 mimicked the proapoptotic effects of THC, indicating that inhibition of the RAS-MAPK pathway is sufficient for the robust induction of apoptosis in Jurkat cells.²⁴ Conversely, we found that inhibition of the RAS-MAPK pathway with U0126 was insufficient for the induction of robust apoptosis in colorectal cancer cells, and inhibition of both the RAS-MAPK pathway, and the PI3K-AKT pathway (with LY294002), was required to mimic THC-induced apoptosis. Finally, we show that although colorectal cancer cells express both CB1 and CB2 receptors, signalling via CB1, but not CB2, is responsible for both inhibition of AKT and ERK, and the induction of apoptosis by THC; in contrast to the findings of Jia *et al.* who reported an equal involvement of CB1 and CB2 in THC-mediated apoptosis in Jurkat cells.²⁴ Our results further the understanding of cell-type specific cannabinoid signalling by highlighting the ability of THC to induce apoptosis in colorectal cancer cells, and to inhibit ERK and AKT signalling via the CB1 receptor.

In this study, we found that BAD, a proapoptotic protein inhibited downstream of both RAS-MAPK and PI3K-AKT survival signalling, plays a critical role in the induction of apoptosis by THC. This finding, together with previous studies reporting the constitutive phosphorylation, and thus inactivation, of BAD in colorectal tumour cell lines,^{62,63} suggests an important role for suppression of BAD activity in colorectal tumour cell survival and tumorigenesis. One possible cause of the constitutive phosphorylation of BAD in colorectal tumour cells is the aberrant activation of RAS-MAPK and PI3K-AKT signalling pathways that are found frequently in these tumours. Therefore, by inhibiting ERK and AKT activity, THC may induce apoptosis in colorectal cancer cells by reactivating the BAD-mediated cell death pathway that colorectal tumour cells have become dependent on suppressing. It is interesting to note that BAD has previously been shown to be a key mediator of apoptosis in response to the inhibition of constitutive RAS-MAPK and PI3K-AKT signalling in breast cancer cells, supporting the notion that suppression of BAD activity is particularly important in tumours highly dependent upon ERK and AKT signalling.⁴⁶

In summary, we report for the first time that the plant-derived cannabinoid THC induces apoptosis in colorectal cancer cells, and that the induction of cell death is mediated through BAD activation via CB1-dependent RAS-MAPK and PI3K-AKT pathway inhibition. Given that both these survival signalling pathways are frequently deregulated in colorectal tumours, our results suggest that exploiting the antitumoural properties of the naturally occurring cannabinoid THC, or selective targeting of the CB1 receptor, may represent novel strategies for colorectal cancer therapy.

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