A cyclooxygenase-2 (COX-2) selective non-steroidal anti-inflammatory drug enhances the growth inhibitory effect of butyrate in colorectal carcinoma cells expressing COX-2 protein: regulation of COX-2 by butyrate

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Epidemiological, clinical, animal and laboratory studies have all provided evidence for the protective effects of non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, against colorectal cancer. The main established target for NSAID action is cyclooxygenase (COX) and the inducible isoform, COX-2, is up-regulated in colorectal cancer. Rat intestinal epithelial cells transfected with a COX-2 expression vector have previously been found to be resistant to butyrate-induced apoptosis. Butyrate, a by-product of dietary fibre fermentation, is known to induce differentiation and apoptosis in colorectal tumour cells in vitro. In recent years there has been considerable interest in the possible role of dietary fibre/resistant starch in the prevention of colorectal cancer. In this study we investigated whether inhibition of COX-2 with a highly selective COX-2 inhibitor (NS-398) would sensitize human colorectal carcinoma cells to the growth inhibitory effect of butyrate. HT29 and S/KS colorectal carcinoma cell lines were treated for 72 h with 2 mM butyrate and/or 10 μM NS-398. Addition of 10 μM NS-398 alone (to inhibit COX-2 activity) did not result in detectable growth inhibition in either of the cell lines. NS-398 enhanced sensitivity to the growth inhibitory effect of butyrate in HT29 cells expressing COX-2 protein. In contrast, NS-398 did not sensitize S/KS cells lacking detectable COX-2 protein and function (as determined by prostaglandin E₂ production) to the growth inhibitory effect of butyrate. In addition, we report that butyrate treatment of carcinoma (HT29) and adenoma (PC/AA/C1) cells leads to up-regulation of COX-2 protein. Thus NS-398 only appears to sensitize human colorectal carcinoma cells expressing COX-2 protein to the growth inhibitory effect of butyrate. As COX-2 is up-regulated in colorectal carcinogenesis, this could have important implications for the selective inhibition of cells expressing COX-2 protein over those lacking COX-2 protein expression and for dietary modification to be considered alongside NSAIDs in the prevention, and possibly treatment, of colorectal cancer.

Introduction
In industrialized nations, colorectal cancer is a major cause of cancer death and there is increasing urgency to develop strategies to prevent this disease. With regard to prevention, diet and non-steroidal anti-inflammatory drugs (NSAIDs) have attracted considerable interest in recent years. Evidence has pointed to a diet low in fat and high in dietary fibre being protective for colorectal cancer (1,2). Dietary fibre (non-starch polysaccharides) by-passes digestion in the stomach and small intestine, as do oligosaccharides and some resistant starches (3). In the large bowel, symbiotic bacteria ferment these carbohydrates releasing butyrate, a short chain fatty acid, as a by-product (4). At physiological concentrations, butyrate has been shown to induce growth inhibition, differentiation and apoptosis in colorectal tumour cells in vitro (5–7). These observations may, in part, explain the correlation between a high fibre diet and low incidence of colorectal cancer. In animal models, butyrate, administered orally as gastroresistant, slow release pellets, was found to increase apoptosis in the colonic mucosa of rats, thus providing further evidence that it may be beneficial against the development of colon cancer (8). Butyrate enemas have been suggested to be useful in long-term therapy to reduce the risk of colon cancer in patients with ulcerative colitis (9,10). A recent study demonstrated that butyrate is able to enhance the sensitivity of colon carcinoma cell lines to Fas-mediated apoptosis (11), thus suggesting that butyrate may be an effective adjuvant for chemotherapy and immunotherapy in colorectal cancer.

Epidemiological, clinical, animal and laboratory studies have all provided evidence for the protective effects of NSAIDs, such as aspirin and sulindac, against colorectal cancer (12). Although the precise mechanisms for the protective effects of NSAIDs are unknown, the ability of these drugs to induce cell cycle arrest and apoptosis has received much attention in recent years (reviewed in ref. 13). The most recognized target for NSAIDs is COX (cyclooxygenase or prostaglandin endoperoxide synthase) (reviewed in ref. 14), which is the key enzyme in the conversion of arachidonic acid (AA) to prostaglandins and other eicosanoids (15). COX-1 is the constitutive isoform, expressed in many mammalian tissues, whereas COX-2 expression is undetectable in most tissues under physiological conditions. Cytokines, growth factors, oncogenes, serum and tumour promoters have been found to induce COX-2 expression (16–20). COX-1 expression remains unaltered in colorectal cancer, whereas COX-2 mRNA levels are increased in ~85% of human colorectal cancers and in a subset of adenomas (21). Other studies have shown an increase in COX-2 protein levels in adenomas (22) and in carcinomas (22–24). Increased prostaglandin E₂ (PGE₂) synthesis has also been associated with colorectal neoplasia (25), while various prostaglandins (including PGE₂) have been found to be growth stimulatory to colon tumour cells in vitro (26). COX-2 is suggested to play a key role in driving intestinal tumourigenesis. In vivo support for this comes from Oshima et al. (27), where inactivating the Ptgs2 gene (murine COX-2) in the ApcΔ716 mouse (a model of familial adenomatous polyposis in humans) reduced the size and number of intestinal polyps.

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; DMSO, dimethylsulphoxide; IC₅₀, 50% inhibitory concentration; NSAID, non-steroidal anti-inflammatory drug; PBS, phosphate-buffered saline; PGE₂, prostaglandin E₂; RIE, rat intestinal epithelial.
COX-2 selective inhibitors, such as NS-398 (28), are being manufactured in order to help overcome the gut-related side-effects of ‘traditional’ NSAIDs, caused largely by the latter inhibiting the production of prostaglandins by COX-1. In animal studies COX-2 selective inhibitors show considerable promise as chemopreventives for intestinal tumourigenesis. For example, Kawamori et al. (29) showed that the induction of chemically induced colon tumours was completely prevented in 93% of rats fed the COX-2 selective NSAID celecoxib (SC-58635).

The role played by COX-2 in cancer development at the cellular level was investigated by Tsuji and DuBois (30) when they transfected a COX-2 expression vector into rat intestinal epithelial (RIE) cells (which lack detectable COX-2 protein expression when quiescent; ref. 18), thus increasing COX-2 levels. Amongst the changes, that were characteristic of a transformed phenotype, seen in these cells was an increased resistance to butyrate-induced apoptosis. This increased resistance was lost upon addition of the ‘traditional’ NSAID sulindac sulfide (which selectively inhibits COX-1 over COX-2). The transfected RIE cells also demonstrated increased adhesion to extracellular matrix proteins, decreased levels of E-cadherin and transforming growth factor β2 receptor and elevated Bcl-2 protein levels (30). In this study the potential interactions between butyrate and NSAIDs were not developed further. In the past few years COX-2 selective inhibitors have come to the forefront of colorectal cancer research. Considering the potential importance of COX-2 selective NSAIDs and dietary fibre in the prevention of colorectal cancer, the aim of our study was to determine how human colorectal carcinoma cells respond to combined treatment with a highly selective COX-2 inhibitor (NS-398) and butyrate, in terms of growth inhibition and apoptosis. Specifically, would NS-398 sensitize colorectal carcinoma cells to the growth inhibitory effect of butyrate and would any sensitization only be seen in cells expressing COX-2 protein? Given the likely significance of COX-2 regulation in colorectal carcinogenesis, would butyrate affect the regulation of COX-2 protein levels in cells? Answers to these questions may help us to design protocols for using dietary modification alongside COX-2 selective NSAIDs in the prevention of colorectal cancer.

Materials and methods

Cell lines and culture conditions

The colorectal carcinoma cell lines used in this study were HT29 and S/KS, both of which have been described previously (5,31). PC3/AA/C1 is a clonogenic, non-tumourigenic adenoma-derived cell line from a patient with familial adenomatous polyposis (32,33). Cells were grown in T25 flasks (Corning Costar Europe) at 37°C in a 5% CO2 incubator. For standard growth conditions each cell line was grown in DMEM (Life Technologies, UK) supplemented with 20% fetal bovine serum (batch selected), insulin (0.2 U/ml), glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). There was no addition of hydrocortisone sodium succinate to the medium, due to its possible role in interfering with AA mobilization (the substrate for COX) (34).

Cell treatments

A stock solution of 100 mM butyrate (Sigma, UK) was prepared in tissue culture water (Life Technologies, UK) and diluted in standard growth medium to give a final concentration of 1–6 mM butyrate. A stock solution of 30 mM NS-398 (Cayman Chemical Co., MI) was prepared in dimethylsulphoxide (DMSO) (Analar BDH, UK) and diluted in standard growth medium to give a final concentration of 10–100 µM. Cell lines were seeded at a density of 1×10^4 cells/T25 flask. Cultures at 70% confluence were incubated in butyrate-supplemented medium, NS-398-supplemented medium or medium containing both agents for 3 days, unless otherwise stated. Control cultures received DMSO and tissue culture water, as appropriate. To determine the longer term effects of the agents, in some experiments the cells were treated for 7 days. For 7 day incubations, the cells were grown fresh medium with or without the addition of 2 mM butyrate and/or 10 µM NS-398 after 72 h. Cell cultures were treated in triplicate and, at the required time points, the attached cells (those remaining attached to the tissue culture plate) and floating cells (those remaining in the medium) were harvested and counted separately. IC50 (50% inhibition concentration values) for butyrate and for NS-398 were determined from dose–response curves of the concentration of the agent used versus attached cell yield. They represent the concentration of the agent required to reduce the attached cell yield of a cell line to 50% of the control level over 3 days of treatment.

In certain experiments, following butyrate and NS-398 treatment of HT29 cell cultures the medium was sampled to determine PGE2 levels. In these experiments, for HT29 cell cultures treated with butyrate for 72 h, an exogenous supply of AA (35) was added for the final 15 min of treatment. For HT29 cell cultures treated with NS-398 for 24 h, AA was present for the entire treatment time. S/KS cell cultures were treated at 70% confluence with an exogenous supply of AA for 24 h. A stock solution of 15 mM AA (Sigma, UK) was prepared in DMSO and diluted in standard growth medium to give a final concentration of 50 µM. Control cultures received DMSO alone. Maximum final volume of DMSO per flask was 0.33% (v/v) for all treatments.

Measurement of apoptosis

It has previously been shown that during the culture of colorectal tumour cells the majority of spontaneously occurring floating cells are apoptotic (5,7) and treatment with agents such as butyrate results in an increase in the proportion of cells that are floating. For agents such as butyrate, it has been found that the proportion of floating cells that are apoptotic is the same for both treated and control cultures, showing that the increase in floating cells is due to the induction of apoptosis and not necrosis (5). Therefore, the extent of apoptosis can be determined by measuring the proportion of the total cell population that has detached from the cell monolayer. In this study, after determining the proportion of cells that had detached from the cell monolayer and were floating in the medium, the fraction of these ‘floating’ cells that were apoptotic was assessed by morphology following acridine orange staining. Cells were stained for 10 min with 5 µg/ml acridine orange in phosphate-buffered saline (PBS) and were viewed immediately by fluorescence microscopy, as described by Gregory et al. (36). Cells were counted as apoptotic on the basis of well-documented morphological criteria, most obviously by their characteristic condensed bright chromatin. At least 200 cells were counted per treatment. Acridine orange stained cells were photographed using FujiChrome Provia (ISO 400) film. On some occasions cells were stained with a solution of acridine orange and ethidium bromide (each 5 µg/ml in PBS) and analysed as above. Apoptotic cells that have lost their membrane integrity appear orange due to co-staining with acridine orange and ethidium bromide.

SDS-PAGE western blotting

Cells (1×10^6) were washed in PBS and resuspended in 100 µl of gel sample buffer (10% glycerol, 5% 2-mercaptoethanol, 4% SDS, 62.5 mM Tris-HCl, pH 6.75, 0.01% bromophenol blue). After incubation in a boiling water bath for 5 min, samples were stored at −20°C. Proteins from cell lysate samples were resolved on 10 or 12.5% polyacrylamide gels and electrobotted onto Immobilon-P polyvinylidene difluoride membranes (Millipore, MA), which were probed with various primary antibodies. Polyclonal anti-COX-2 antibody (a gift from Dr S.Prescott, University of Utah, Salt Lake City, UT) was used at a 1:4000 dilution. Monoclonal anti-ε-tubulin (Sigma, UK) was used to control for loading and transfer at a 1:10 000 dilution. The secondary antibodies were goat anti–rabbit (Sigma, UK), donkey anti–goat (Santa Cruz Biotechnology, CA) and goat anti–mouse (Sigma, UK), respectively. All were conjugated with horseradish peroxidase and used at a 1:1000 dilution. Protein bands were visualized using an enhanced chemiluminescence detection system (Kirkland and Perry Laboratories, MD). Enzymatically inactive native ovine COX-2 protein (Biogenesis Ltd, suppliers for Oxford Biomedical Research, UK) was initially used as a positive control for COX-2 protein. This commercially available COX-2 protein positive control was compared with COX-2 protein expression in the HCA-7 cell line (37) (a gift from Dr S.Kirkland, Imperial College of Science, Technology and Medicine, London, UK). This cell line is known to produce high levels of COX-2 protein (38). After confirmation of the size of COX-2 protein, HCA-7 cells were also used as a COX-2 protein positive control.

PGE2 immunoassay

A Cayman Chemical Co. (MI) PGE2 competitive enzyme immunoassay was used to measure PGE2 levels in cell culture supernatants. Medium was sampled, combined from replicate flasks and particulates removed by centrifugation. Aliquots of culture medium (1 ml) were stored at −70°C until assayed.
undiluted. The immunoassay was carried out according to the manufacturer’s protocol. The assay was sensitive to 30 pg PGE2/ml. PGE2 production was normalized with respect to the number of adherent cells present in the particular culture at the time of sampling and the results are expressed as pg PGE2/10^6 cells. It was found that AA did not intrinsically affect the levels of PGE2 detected for the standards in the assay.

Statistical analysis
For both cell lines separately, cell counts for control, butyrate alone, NS-398 alone and butyrate + NS-398 were compared using analysis of variance (ANOVA) with a 5% significance level. The statistical model used included terms for experiment, treatment and the interaction by experiment interaction. In both cases, the interaction was not significant, meaning that the main effects could be interpreted. Further analysis was completed using contrasts for: (i) control versus NS-398 alone; (ii) the average of butyrate + NS-398 together and butyrate alone versus control; (iii) butyrate + NS-398 together versus butyrate alone. The level of significance was chosen as 5%.

Results

COX expression and PGE2 production in human colorectal carcinoma cells

The colorectal carcinoma cell lines used in this study were HT29 and S/KS. Western blot analysis of cell lysates prepared from these cell lines confirmed constitutive expression of COX-2 protein in HT29 cells but there was no detectable COX-2 protein in S/KS cells (Figure 1A), which is in agreement with Elder et al. (39). Two peptide bands of 72 and 74 kDa co-migrated with the COX-2 protein positive control and were identified as the COX-2 protein doublet. It is likely that these peptides represent different N-glycosylation states for the enzymatically active COX-2 molecules as described by Otto et al. (40). COX-1 expression was detectable in both cell lines, with higher levels of expression in HT29 cells. PGE2 production and its inhibition by the highly selective COX-2 inhibitor NS-398 for the HT29 cell line are shown in Figure 1B. Addition of 10 or 50 µM NS-398 to cultures of HT29 cells for 24 h completely inhibited PGE2 release into the culture medium. The inhibition of PGE2 production by NS-398 in HT29 cells indicates that NS-398 actively inhibits COX-2 under these conditions and that the PGE2 detected is COX-2 derived. The dose of 10 µM NS-398 was enough to reduce the PGE2 levels in the culture medium to control levels (≤8 pg PGE2/10^6 cells). Coffey et al. (41) also showed equivalent reductions in the levels of PGE2 in culture medium at all concentrations of COX-2 selective inhibitor tested (no dose dependency). No effects on attached cell yield were seen after 24 h treatment of the HT29 cells with 10 or 50 µM NS-398 (data not shown). Under standard growth conditions, PGE2 secretion into the culture medium for S/KS cells was undetectable and this remained the case when S/KS cells were stimulated with up to 50 µM AA for 24 h (data not shown). This dose of AA did not affect attached cell yields for S/KS cells (data not shown). The lack of PGE2 production in the S/KS carcinoma cells, even after stimulation with AA, is consistent with the lack of detectable COX-2 protein in these cells.

Butyrate and NS-398, a COX-2 selective NSAID, have dose-dependent anti-proliferative effects in colorectal carcinoma cells

Butyrate has previously been shown to induce growth inhibition, differentiation and apoptosis in a variety of colorectal tumour cell lines (5–7). In this study, the effect of butyrate on cell growth was determined after 72 h. Inhibition of growth of each cell line is indicated by the reduced attached cell yield of treated cultures versus control cultures (Figure 2A). The maximum concentration tested was 6 mM and the growth inhibitory effect of butyrate increased up to this dose in a dose-dependent manner. The effects of NS-398 were also examined after 72 h. In agreement with previous studies (39), similar dose-dependent growth inhibition was seen in both cell lines, with HT29 cells being slightly more sensitive (Figure 2B). NS-398 induces growth inhibition in S/KS cells despite the lack of detectable COX-2 protein in these cells. A low dose of 10 µM NS-398 for 72 h resulted in attached cell yields that were similar to control values in both cell lines. Despite the lack of effect on growth of the cultures, this dose inhibited COX-2 enzyme activity in HT29 cells (Figure 1B). Hence, a dose of 10 µM NS-398 was chosen for the studies detailed below.
Due to the importance of COX-2 in colorectal cancer, we investigated whether the same effect found for treatment of RIE cells with a COX-2 expression vector, resulting in elevated COX-2 protein, made these cells more resistant to butyrate-induced apoptosis than the parental cell line. In this study we asked whether, by using a highly selective COX-2 inhibitor (NS-398) to inhibit endogenous COX-2 enzyme activity, human colorectal carcinoma cells are made more sensitive to the growth inhibitory effect of butyrate. HT29 and S/KS cells were treated with 2 mM butyrate (a dose that resulted in 25–35% growth inhibition) with or without 10 mM NS-398 for 72 h. In HT29 cells, with constitutive COX-2 protein expression, the decrease in attached cell yield compared with control values resulting from combining treatment with butyrate and NS-398 was 36.8% and greater than that seen for butyrate treatment alone (26.4%, significant, \( P < 0.001 \)) (Figure 3A). Treatment with both agents together also resulted in an increase in apoptosis compared with treatment with butyrate alone, as measured by the proportion of the cell population that was floating (Figure 3B). In contrast, when examining the attached cell yields for S/KS cells that have no detectable COX-2 protein, no difference was seen between butyrate treatment with NS-398 compared with butyrate treatment alone (Figure 3C). Similarly, there was no difference in the level of apoptosis seen for treatment of S/KS cells with butyrate alone and treatment with butyrate and NS-398 combined, as measured by the proportion of the cell population that was floating (Figure 3D). NS-398 was also able to sensitize HT29 cells, but not S/KS cells, to butyrate-induced growth inhibition and apoptosis after 7 days treatment, as well as after 3 days treatment. For example, for HT29 cells the decrease in attached cell yield compared with control values resulting from 7 days combined treatment with butyrate and NS-398 was 44.6% and greater than that seen for butyrate treatment alone (27.5%, significant, \( P < 0.001 \)).

To confirm the induction of apoptosis, the morphological appearance of the floating cells was examined using fluorescence microscopy, following acridine orange staining of the cells. Previously, both butyrate and NS-398 have been shown to independently induce apoptosis in colorectal tumour cell lines (5,39). That the observed cell death was due to the induction of apoptosis was confirmed here, especially for combined treatment with butyrate and NS-398. The typically apoptotic appearance (condensed chromatin) of cells treated with both butyrate and NS-398 in the floating cell population of the HT29 cell line is illustrated in Figure 4.

**Butyrate up-regulates COX-2 protein levels**

Due to the importance of COX-2 in colorectal cancer, we examined the cell lines used in the above studies for changes in COX protein expression, when treated with butyrate alone, NS-398 alone or both agents together. Under standard growth conditions no COX-2 protein was detected in S/KS cells (Figure 1A) and this situation was unchanged by treatment with butyrate and/or NS-398 (data not shown). Treatment of HT29 cells with 2 mM butyrate for 72 h resulted in an increase in COX-2 protein levels (Figure 5). The increase in COX-2 protein upon butyrate treatment of HT29 cells was not seen at 6 h but could be detected from 24 to 48 h after treatment began and was found to be dose dependent (data not shown). The increase in COX-2 protein levels was reversible, indicating that the observed increase was not due to the selection of a subpopulation of cells expressing elevated levels of COX-2 (data not shown). To confirm the induction of apoptosis, the morphological appearance of the floating cells was examined using fluorescence microscopy, following acridine orange staining of the cells. Previously, both butyrate and NS-398 have been shown to independently induce apoptosis in colorectal tumour cell lines (5,39). That the observed cell death was due to the induction of apoptosis was confirmed here, especially for combined treatment with butyrate and NS-398. The typically apoptotic appearance (condensed chromatin) of cells treated with both butyrate and NS-398 in the floating cell population of the HT29 cell line is illustrated in Figure 4.

**NS-398 sensitizes colorectal carcinoma cells expressing COX-2 protein to the growth inhibitory effect of butyrate**

Tsujii and DuBois (30) found that permanently transfecting RIE cells with a COX-2 expression vector, resulting in elevated COX-2 protein, made these cells more resistant to butyrate-induced apoptosis than the parental cell line. In this study we asked whether, by using a highly selective COX-2 inhibitor (NS-398) to inhibit endogenous COX-2 enzyme activity, human colorectal carcinoma cells are made more sensitive to the growth inhibitory effect of butyrate. HT29 and S/KS cells were treated with 2 mM butyrate (a dose that resulted in 25–35% growth inhibition) with or without 10 mM NS-398 for 72 h. In HT29 cells, with constitutive COX-2 protein expression, the decrease in attached cell yield compared with control values resulting from combining treatment with butyrate and NS-398 was 36.8% and greater than that seen for butyrate treatment alone (26.4%, significant, \( P < 0.001 \)) (Figure 3A). Treatment with both agents together also resulted in an increase in apoptosis compared with treatment with butyrate alone, as measured by the proportion of the cell population that was floating (Figure 3B). In contrast, when examining the attached cell yields for S/KS cells that have no detectable COX-2 protein, no difference was seen between butyrate treatment with NS-398 compared with butyrate treatment alone (Figure 3C). Similarly, there was no difference in the level of apoptosis seen for treatment of S/KS cells with butyrate alone and treatment with butyrate and NS-398 combined, as measured by the proportion of the cell population that was floating (Figure 3D). NS-398 was also able to sensitize HT29 cells, but not S/KS cells, to butyrate-induced growth inhibition and apoptosis after 7 days treatment, as well as after 3 days treatment. For example, for HT29 cells the decrease in attached cell yield compared with control values resulting from 7 days combined treatment with butyrate and NS-398 was 44.6% and greater than that seen for butyrate treatment alone (27.5%, significant, \( P < 0.001 \)).

To confirm the induction of apoptosis, the morphological appearance of the floating cells was examined using fluorescence microscopy, following acridine orange staining of the cells. Previously, both butyrate and NS-398 have been shown to independently induce apoptosis in colorectal tumour cell lines (5,39). That the observed cell death was due to the induction of apoptosis was confirmed here, especially for combined treatment with butyrate and NS-398. The typically apoptotic appearance (condensed chromatin) of cells treated with both butyrate and NS-398 in the floating cell population of the HT29 cell line is illustrated in Figure 4.
Treating colon tumour cells with butyrate + NS-398

**Fig. 3.** NS-398 sensitizes colorectal carcinoma cells expressing COX-2 protein to the growth inhibitory effect of butyrate and enhances butyrate-induced apoptosis. (A and B) HT29 cells (constitutive COX-2 protein expression); (C and D) S/KS cells (undetectable COX-2 protein expression). (A) and (C) illustrate attached cell yields expressed as a percentage of control values after 72 h treatment with 2 mM butyrate and/or 10 µM NS-398. (B) and (D) represent the induction of apoptosis after 72 h treatment with 2 mM butyrate and/or 10 µM NS-398. The data indicate the quantity of floating cells, as a proportion of the total cell number (attached and floating), in relation to control (spontaneous) quantities. Actual spontaneous levels of apoptosis (means ± SEM) were 0.82 ± 0.14% for HT29 and 2.84 ± 0.25% for S/KS. Over 85% of the floating cells in control and treated cultures were apoptotic (data not shown), indicating that the increase in floating cells upon treatment was due almost entirely to the induction of apoptosis. Results are means of three or more experiments ± SEM.

**Discussion**

In this study we investigated whether treatment of colorectal carcinoma cell lines with a COX-2 selective NSAID could enhance the growth inhibitory effect of butyrate. Interest stemmed from the fact that there is evidence that a high fibre diet and the use of NSAIDs are preventive for colorectal cancer. The ability of butyrate (a short chain fatty acid released by the fermentation of dietary fibre in the large bowel) and NSAIDs to independently induce growth inhibition and apoptosis in colorectal tumour cell lines may, in part, explain their protective role *in vivo*. We have shown differences in the response of colorectal carcinoma cells to treatment with a COX-2 selective NSAID (NS-398) and butyrate depending on the COX-2 protein status of the cells. NS-398 was used at a
Fig. 5. Expression of COX proteins in HT29 cells following treatment with 2 mM butyrate, 10 µM NS-398 or both agents together for 72 h. Western blot analysis for COX-1 and COX-2 protein expression was carried out on lysates of cells taken from one of the experiments illustrated in Figure 3. Levels of COX-2 protein increased when the cells were treated for 72 h with butyrate alone or butyrate together with NS-398. The film was underexposed relative to that for COX-2 expression in Figure 1A. Levels of COX-1 protein remained constant upon treatment with butyrate and/or NS-398. Lane 1, untreated control (CON); lane 2, 2 mM butyrate-treated (BT); lane 3, 10 µM NS-398-treated (NS); lane 4, treatment with 2 mM butyrate and 10 µM NS-398 (BT+NS). Repeat probing with anti-α-tubulin controls for equal loading and transfer. The results shown are typical of the three experiments completed.

Fig. 6. Up-regulation of COX-2 protein levels by butyrate in the PC/AA/C1 adenoma cell line. Cells were treated for 72 h and then lysates of cells analysed by western blotting. Lane 1, COX-2 protein positive control (HCA-7 cells; see Figure 1A); lane 2, untreated control (0 mM butyrate); lane 3, 1 mM butyrate-treated; lane 4, 2 mM butyrate-treated; lane 5, 3 mM butyrate-treated; lane 6, 4 mM butyrate-treated; lane 7, 6 mM butyrate-treated. Repeat probing with anti-α-tubulin controls for equal loading and transfer. Note that the COX-2 protein positive control is underloaded (0.1×10^6 cells) compared with the other lanes (1×10^6). The results shown are typical of the three experiments completed.

dose which was not growth inhibitory to the cells but which inhibited COX-2 enzyme activity in COX-2 positive cells. In colorectal carcinoma cells expressing COX-2 protein, NS-398 sensitized the cells to the growth inhibitory effect of butyrate, whereas in colorectal carcinoma cells lacking detectable COX-2 protein and function (as determined by PGE_2 production), NS-398 did not sensitize cells to the effect of butyrate. We have recently examined another colorectal carcinoma cell line expressing COX-2 protein (HCA-7) for sensitization by NS-398 to butyrate-induced growth inhibition. NS-398 was also found to enhance the growth inhibitory effect of butyrate in these cells (T.E.Crew, D.J.E.Elder and C.Paraskeva, unpublished results). As with HT29 cells, NS-398 was shown to decrease the level of PGE_2 in the culture medium of HCA-7 cells. Although we have found that NS-398 sensitizes HT29 carcinoma cells to the growth inhibitory effect of butyrate, salicylate (the active metabolite of aspirin) and butyrate were only found to have additive (but not greater than additive) growth inhibitory effects upon treatment of HT29 cells (D.J.E.Elder and C.Paraskeva, unpublished results). As salicylate did not sensitize the cells to butyrate-induced growth inhibition, this may reflect the relatively poor COX-inhibitory properties of salicylate. Kamitani et al. (42) found that combining indomethacin (an NSAID selective for COX-1 over COX-2) with butyrate made no difference to the levels of apoptosis induced by butyrate treatment alone in Caco-2 cells that express COX-2 protein. Hence, the effects on butyrate-induced growth inhibition and apoptosis are highly dependent on the colorectal tumour cell line examined and whether ‘traditional’ NSAIDs are used compared with COX-2 selective NSAIDs.

Although NSAIDs are known to have effects on a large number of parameters, including cell proliferation, apoptosis, immune surveillance and angiogenesis (12), apoptosis is considered to be their main anti-proliferative effect in vitro and in vivo (13). In the current study comparing the HT29 (COX-2 positive) and S/KS (COX-2 negative) colorectal carcinoma cell lines, as well as examining growth inhibitory effects, we asked whether NS-398 was able to sensitize the cells to butyrate-induced apoptosis. NS-398 was seen to enhance butyrate-induced apoptosis in HT29 cells but not in S/KS cells.
These data imply that both growth inhibition and apoptosis may be important responses of the cells to the combined treatment with butyrate and NS-398. Even though the difference between the level of apoptosis induced by butyrate treatment alone compared with the level induced by butyrate treatment with NS-398 is small in HT29 cells, low levels of apoptosis (if not balanced by proliferation) can result in significant tissue regression over time (43). Hence, with prolonged treatment in vivo with a COX-2 selective NSAID and a high fibre/resistant starch diet (resulting in a high level of luminal butyrate), minor differences in the sensitivity of cells to undergo apoptosis may result in the relative regression of COX-2-positive tumour tissue.

Given that the majority of colorectal tumours have increased COX-2 protein (23,24), an effective high fibre diet may enable lower doses of COX-2 selective NSAIDs to be used in the prevention of colorectal cancer. This may further reduce the potential side-effects from using such drugs and thus dietary modification during drug treatment may be one way of increasing the efficacy of chemopreventive agents.

Other studies have combined two agents to investigate whether one agent can sensitize cells to the actions of another agent. For example, in human fibroblasts butyrate has been found to act in synergy with ionizing radiation, UV light and cisplatin to induce apoptosis, perhaps by lowering the level of BclX<sub>L</sub> (an apoptosis antagonist) in cells (44). Suganuma et al. (45) found that drinking green tea could potentially enhance the cancer preventive activity of sulindac (a ‘traditional’ NSAID) and Bonnotte et al. (11) showed dramatic enhancement of the sensitivity of colon carcinoma cell lines to Fas-mediated apoptosis after treatment with butyrate. The clinical toxicity of butyrate is low and the authors of the latter paper propose that butyrate may be a useful adjuvant in therapeutic trials. In many studies, butyrate has been the agent that sensitzes cells to the effects of another agent, whereas our study is the first known report of a COX-2 selective inhibitor being able to sensitize colorectal carcinoma cells expressing COX-2 protein to butyrate-induced growth inhibition. Hence, the question remains as to how a low dose of NS-398 (which does not induce growth inhibition) achieves this sensitizing effect.

Of possible direct relevance to this question is the fact that butyrate increases expression of COX-2 protein in HT29 cells. This response was also seen in an adenoma cell line (PC/AA/C1), which constitutively expresses COX-2 protein. COX-2 is considered to be an ‘immediate early’ gene, which means its induction is independent of any intervening protein synthesis. Butyrate has been shown to induce immediate early genes such as c-fos (46) and c-jun (47) with the expected time scale in vivo and in vitro. However, the response of COX-2 protein to butyrate treatment is more delayed in the HT29 and PC/AA/C1 cell lines (24–48 h), occurring concurrently with detectable growth inhibition and apoptosis. It is not, therefore, a typical immediate early gene response.

In a manner analogous to ceramide (48), butyrate induces apoptosis and in our hands has been shown to up-regulate COX-2 protein expression. It is possible that any increase in COX-2 protein is a mechanism of induced resistance to apoptosis and by up-regulating COX-2 the cells are resisting apoptosis. Therefore, it might be the case that the pro-apoptotic effects of butyrate are counteracted to some extent by its ability to up-regulate COX-2. This is supported by the data which shows that NS-398, which inhibits COX-2, potentiates butyrate-mediated apoptosis. That NS-398 does not prevent the increase in COX-2 protein by butyrate suggests that the increase in COX-2 protein is independent of prostaglandin synthesis.

These data may go some way to explain previous studies which have reported that agents such as NSAIDs and TGFβ, at doses causing growth inhibition and apoptosis, can cause an increase in COX-2 protein expression (49–52). In our hands, TGFβ treatment of an adenoma cell line that responds by growth inhibition and cell death by apoptosis results in an increase in COX-2 protein (T.E.Crew, A.Hague, D.J.E.Elder and C.Paraskeva, unpublished results). This is in agreement with Sheng et al. (52), where chronic TGFβ treatment of rat intestinal epithelial cells up-regulated COX-2 protein levels and increased the tumourigenic potential of the cells. Other ‘stresses’, such as ceramide, UV light and deoxycholate, have also been found to lead to an up-regulation of COX-2 protein (48,53,54). In contrast to the data reported in our study, it has previously been reported that butyrate down-regulates expression of COX-2 protein in Caco-2 cells, under conditions where butyrate induces apoptosis in these cells (42).

In summary, we report here for the first time that butyrate treatment of adenoma and carcinoma cells leads to the up-regulation of COX-2 protein (in association with growth inhibition and apoptosis). Furthermore, we have shown that NS-398 is able to sensitize HT29 cells (COX-2 positive) to the growth inhibitory effect of butyrate but is unable to sensitize S/KS cells (COX-2 negative) to butyrate-induced growth inhibition. For the majority of colorectal tumours that express COX-2, this may mean that a high fibre/resistant starch diet could enable lower doses of COX-2 selective NSAIDs to be used in chemoprevention. We are a long way from fully understanding the regulation of the COX-2 gene but it continues to be an established area of research, which will hopefully lead to a greater understanding of colorectal cancer in terms of the molecular pathways involved and provide potential applications for the clinic.

Acknowledgements

We would like to thank Dr Steve Prescott for the generous gift of the anti-COX-2 antibody and Dr Susan Kirkland for the HCA-7 cell line. We are also grateful to Paul Miller for his assistance with the statistical analysis. This work was supported by a program grant from the UK Cancer Research Campaign and by the BBSRC. T.E.C. is a BBSRC funded PhD student.

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Received July 12, 1999; revised October 4, 1999; accepted October 5, 1999