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The effect of non-steroidal anti-inflammatory drugs on human colorectal cancer cells: evidence of different mechanisms of action

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit proliferation and induce apoptosis in human colorectal cancer cells *in vitro*. It remains unclear whether individual NSAIDs act by cyclooxygenase-2 (COX-2) inhibition and how NSAIDs exert their anti-proliferative effects. We investigated the effects of NS-398 (a selective COX-2 inhibitor), indomethacin (a non-selective COX inhibitor) and aspirin on four human colorectal cancer cell lines (HT29.Fu, HCA-7, SW480 and HCT116). NS-398 completely inhibited proliferation, induced G1 arrest and promoted apoptosis in COX-2-expressing cells (HT29.Fu and HCA-7). However, indomethacin had similar effects on all cells, regardless of COX-2 expression. NS-398 also had anti-proliferative activity on COX-2-negative cell lines (SW480 and HCT116). Aspirin inhibited proliferation of all cell lines but did not induce apoptosis. Indomethacin decreased β-catenin protein expression in all cells (unlike NS-398 or aspirin). NSAIDs act on human colorectal cancer cells via different mechanisms. Decreased β-catenin protein expression may mediate the anti-proliferative effects of indomethacin.

Keywords: Apoptosis; β-Catenin; Colorectal cancer; Cyclooxygenase; Non-steroidal anti-inflammatory drug

1. Introduction

Results from both epidemiological studies and animal models of colon carcinogenesis have indicated that non-steroidal anti-inflammatory drugs (NSAIDs) have anti-colorectal cancer activity [1]. However, the precise mechanism(s) by which NSAIDs exert their anti-neoplastic effects remains unclear. Currently there is intense debate about the importance of cyclooxygenase (COX) inhibition for the anti-colorectal cancer activity of NSAIDs [2].

COX is the rate-limiting enzyme for synthesis of eicosanoids such as prostaglandin (PG) E_2 and D_2 from arachidonic acid [1]. There are two isoforms of COX; a constitutive isoform, COX-1 and an inducible isoform, COX-2, which have a high degree of structural and enzymatic homology [1,3]. However, the COX isoforms are pharmacologically distinct such that they are differentially inhibited by individual NSAIDs [4].

There is direct evidence from two animal models that inhibition of COX-2 can abrogate colon carcinogenesis. In the first model, celecoxib, a selective COX-2 inhibitor, produced profound inhibition of azoxymethane-induced colonic carcinogenesis in rats [5]. In the second model, *COX-2* gene disruption and administration of MF tricyclic, another selective COX-2 inhibitor, dramatically reduced polyp formation in the murine $Apc^{\Delta 716}$ model of familial adenomatous polyposis [6]. In contrast, there is also evidence that certain NSAIDs exert their effects via non-COX-mediated mechanisms. For example, the R-enantiomer of flurbiprofen (which does not inhibit COX) has chemopreventative activity in the Min mouse model of intestinal polyposis [7] and sulindac sulphone (a metabolite of sulindac which does not inhibit either COX isoform) inhibited azoxymethane-induced colonic carcinogenesis in rats [8]. This evidence suggests that NSAIDs can act via both COX-dependent and COX-independent mechanisms.

Information regarding the molecular pathways that are regulated by the enzymatic activities of COX-1 and COX-2 is controversial at the present time, as is the importance of COX inhibition for the anti-proliferative effects of individual NSAIDs *in vitro* [9–16]. Therefore, we describe herein, the effect of three NSAIDs (NS-398, indomethacin and aspirin), which display differential COX-1/COX-2 inhibitory activity, on cell proliferation,

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cell cycle parameters and apoptosis in four human sporadic colorectal cancer cell lines which exhibit different COX-1 and COX-2 expression.

Down-regulation of β-catenin expression represents a potential mechanism whereby NSAIDs have pro-apoptotic activity in human colorectal cancer cell lines. Dysregulation of β-catenin in colorectal cancer cells leads to increased binding of β-catenin to TCF/LEF transcription factors and altered expression of genes involved in cell proliferation such as c-MYC and cyclin D1 [17,18]. Therefore, we also analysed changes in β-catenin protein expression associated with the anti-proliferative effects of NSAIDs.

2. Materials and methods

2.1. Cell culture

HT29.Fu (a gift from T. Lesuffleur, Unite de Recherches sur la Differentiation Cellulaire Intestinale, Villejuif, France), HCA-7 clone 29 (a gift from S. Kirkland, ICRF Histopathology Unit, Hammersmith Hospital, London, UK), SW480 and HCT116 cell lines (both from the European Collection of Animal Cell Cultures, Porton Down) were studied. HT29.Fu cells were originally derived from parental HT29 cells following treatment with 10 μM 5-fluorouracil (5-FU). HT29.Fu cell monolayers consist of approximately 95% differentiated cells (compared with parental HT29 cell monolayers which contain only 5% differentiated cells) and remain phenotypically stable in the continued presence of 5-FU [19]. HCA-7, SW480 and HCT116 cell lines have all been studied and characterised extensively [12,20].

All cell culture consumables were obtained from Gibco BRL, Paisley, UK, unless otherwise stated. HT29.Fu cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 μM 5-FU (Sigma, Poole, UK), SW480 cells in RPMI medium, HCA-7 cells in DMEM medium supplemented with 10% (w/v) non-essential amino acids, and HCT116 cells in McCoy's 5A medium. All media contained Glutamax™ and were supplemented with either 10% or 0.5% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin (1000 units/ml and 500 units/ml respectively). All cell lines were grown at 37°C in a humidified atmosphere of 5% CO_2. Cells were routinely subcultured using 0.25% (w/v) trypsin solution.

2.2. Reagents

We compared the selective COX-2 inhibitor, NS-398 (20–75 μM; Cayman Chemical Co., Ann Arbor, USA) [21] with the non-selective COX inhibitor, indomethacin (100–600 μM; Sigma) and the irreversible, preferential COX-1 inhibitor, aspirin (400–1500 μM; Sigma). Sodium butyrate (Sigma) was used at a final concentration of 2 mM. All compounds were prepared as 100 mM stock solutions. Control flasks contained either DMSO or 1 M Tris-HCl (for aspirin) at an equivalent concentration to that found with the highest concentration of NSAID.

Goat polyclonal anti-human COX-1 antibody (C-20) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and mouse monoclonal anti-human COX-2 antibody from Cayman Chemical Co. [22]. Mouse monoclonal anti-human β-catenin antibodies (clone nos. 6F9 and 15B8; [23]) and rabbit polyclonal anti-glucose-6-phosphate dehydrogenase (G6PDH) antibody were obtained from Sigma. Mouse monoclonal anti-human E-cadherin antibody was obtained from Transduction Laboratories (Lexington, KY, USA). Horseradish peroxidase-conjugated secondary antibodies were obtained from DAKO Ltd, Cambridge, UK (antirabbit and antimouse) and Sigma (antigoat). FITC- and TRITC-conjugated antimouse immunoglobulins were obtained from Sigma and DAKO respectively.

2.3. Western blot analysis

Confluent cell monolayers were lysed in 50 mM Tris-HCl buffer, pH 7.2 containing 0.137 M sodium chloride, 1% Brij 96, 0.2 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF), 1 mM EDTA, 20 (M leupeptin and 1 μM pepstatin (all Sigma). Cell lysates were centrifuged at 15 000 *g* for 5 min at 4°C through Quiashredder columns (Quiagen, London, UK). The resulting solution was centrifuged for 15 min as above, and the supernatant used for electrophoresis. Protein concentration was determined using a BioRad DC kit (BioRad, Hemel Hempstead, UK). SDS-PAGE was performed using 100 μg total protein aliquots together with pre-stained molecular weight standards (Novex, San Diego, USA). Proteins were transferred to polyvinylidene fluoride membranes which were then blocked with 3% (w/v) dried skimmed milk powder in 0.05% (v/v) Tween Tris-buffered saline (TTBS) for a minimum of 2 h at 20°C. Membranes were probed with primary antibodies in TTBS: anti-COX-1, 1/500; anti-COX-2, 1/500; anti-GAPDH, 1/500; anti-β-catenin antibodies, both 1/1000 for 2 h at 20°C. Horseradish peroxidase-conjugated secondary antibodies were diluted 1/5000 in TTBS and incubated with blots for 2 h at 20°C. Immunoreactive protein was detected using ECL chemiluminescence (Pierce, Chester, UK).

2.4. Indirect immunofluorescence

SW480 cells grown on glass coverslips were treated for 24 h with indomethacin (600 μM) or an equivalent dilution of DMSO, under standard culture conditions as

described above. Cells were fixed in methanol at −20°C for 5 min and washed twice in PBS. Monolayers were incubated with primary antibody (anti-β-catenin: 6F9, 1/500 or anti-E-cadherin 1/1000) in PBS + 1% dried skimmed milk powder for 2 h at 20°C. Omission of the respective primary antibody was used as a negative control. Following 4×5 min PBS washes, monolayers were incubated with secondary antibodies (both 1/100) in PBS + 1% dried skimmed milk powder for 1 h at 20°C. After further PBS washes (4×5 min), coverslips were mounted in MOWIOL® (Calbiochem, La Jolla, CA, USA). Confocal microscopy was performed using a Leica TCS SP laser scanning confocal microscope.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) for COX-1 and COX-2

Total RNA was extracted from cells using TRIzol™ (Gibco BRL). RT-PCR for COX-1, COX-2 and β-actin mRNA was performed as described [22]. Primers for β-actin were: sense 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′; antisense 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′.

2.6. Cell counting and cell cycle analysis

Cells were plated at 1×10^5 cells per 25 cm^2 flask. After 24 h, medium with or without NSAID was added and incubated with cells for up to 72 h. At 24, 48 and 72 h, floating cells were removed and adherent cells harvested by treatment with 0.25% (w/v) trypsin. Adherent and floating cell populations were combined and counted using a haemocytometer. Viability was tested by 0.4% (w/v) Trypan Blue (Sigma) exclusion. Experiments were performed in triplicate. Results are expressed as the mean times increase in total cell number (± standard error of the mean; SEM) relative to the number of cells that were originally plated.

Cell density was adjusted to $0.3–1.0\times10^7$ cells/ml. DNA content analysis was performed on propidium iodide (PI)-stained cells (DNA-Prep Reagents Kit; Coulter Ltd, Basingstoke, UK) using a Coulter Profile XL FACS scanner. A minimum of 10 000 events were counted. Signals were collected in list mode with linear amplification for PI fluorescence. Multicycle AV software was used to generate DNA content frequency histograms. Experiments were carried out in triplicate and the results are expressed as the mean percentage of cells (± SEM) in G1, G2/M and S phases of the cell cycle. The percentage of cells with a sub-diploid DNA content was also calculated.

2.7. Acridine orange staining

$5–7\times10^4$ freshly harvested floating and adherent cells were applied to glass slides by centrifugation at 500*g* for 8 min in a Cytospin (Shandon, London, UK). The cells were fixed by immersion in ice cold 100% methanol, washed with PBS and stained with 5 μg/ml acridine orange (AO; Sigma) in PBS for 10 min at 20°C. Cells were examined for morphological criteria of apoptosis (cytoplasmic/nuclear shrinkage and chromatin condensation) by UV fluorescence microscopy as previously described [13]. The mean percentage of cells which fulfilled criteria of apoptosis within a minimum of 200 cells was determined by two independent observers blinded to the origin of the cells.

2.8. Statistical analysis

Repeated measures analysis of variance (ANOVA) was used to determine the overall significance of the effect of different concentrations of NSAIDs on total cell number over time. One-way ANOVA with a Bonferroni test was used to determine whether there was a significant increase in cell number for individual concentrations of each NSAID. Statistical significance was assumed if the P value was less than 0.05. Similar statistical methods were used to determine the significance of changes in cell cycle distribution over time after NSAID treatment. Multiple linear regression was used to analyse the relationship between the percentage of apoptotic cells counted by flow cytometry and fluorescence microscopy.

3. Results

3.1. COX expression by human colorectal cancer cell lines

All four cell lines expressed COX-1 mRNA (Fig. 1, columns 1–6a). The presence of COX-1 protein was also observed in protein extracts prepared from these cell lines (Fig. 2, lanes 1–6). The anti-COX-1 antibody detected a 70 kDa doublet which has been reported previously [12] and is presumably due to differential glycosylation.

In contrast, only HT29.Fu and HCA-7 cells expressed COX-2 mRNA (Fig. 1) and the 72 kDa COX-2 protein under routine culture conditions (Fig. 2). In order to confirm that COX-2 expression was truly constitutive in HT29.Fu and HCA-7 cells, we demonstrated continued COX-2 mRNA (Fig. 1, columns 2b and 4b) and protein expression (Fig. 2, lanes 2 and 4) following culture in 0.5% FBS for 48 h.

3.2. Effect of NSAIDs on cell proliferation

In the absence of NSAIDs, there was (approximately) a 4–8-fold increase in total cell number over 72 h (Fig. 3; Table 1). Treatment with all three NSAIDs was

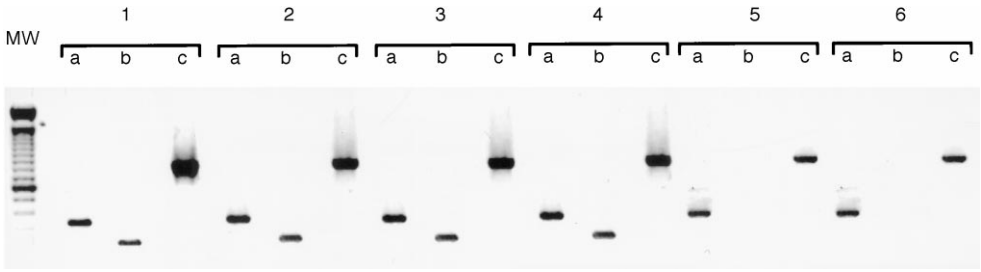

Fig. 1. RT-PCR for COX-1, COX-2 and β-actin mRNA expression. PCR products were visualised by 1% agarose gel electrophoresis. Column 1, HT29.Fu; 2, HT29.Fu (0.5% FBS); 3, HCA-7; 4, HCA-7 (0.5% FBS); 5, SW480; 6, HCT116. RT-PCR for (a) COX-1 (350 bp), (b) COX-2 (232 bp) and (c) β-actin (838 bp). A 100 bp DNA ladder (Gibco BRL) was run in the left-hand column (MW).

associated with a statistically significant, concentration-dependent decrease in cell number over time in all cell lines (repeated measures ANOVA; $P < 0.01$ in all cases; see Table 1). However, there were marked differences between the effects of the NSAIDs on individual cell lines.

NS-398, at a concentration of 75 μM, completely inhibited cell proliferation only in cell lines (HT29.Fu and HCA-7) which expressed COX-2 (Fig. 3). In COX-2-negative cell lines (SW480 and HCT116), NS-398 had a partial inhibitory effect but there was still significant cell proliferation, even in the presence of the highest concentration of NS-398 (Fig. 3; Table 1). For example, HCT116 cell proliferation was decreased by less than 50% despite the presence of 75 μM NS-398 (Fig. 3; Table 1).

At concentrations above 400 μM, indomethacin caused complete inhibition of proliferation of HT29.Fu, HCA-7 and SW480 cell lines. However, the inhibitory effect of indomethacin was not as marked on HCT116 cells which still exhibited statistically significant proliferation in the presence of 600 μM indomethacin (Table 1).

Aspirin also exhibited a dose-dependent inhibition of proliferation of all cell lines but the effect was not as marked as for NS-398 and indomethacin. In all four colorectal cancer cell lines, the highest concentration of aspirin (1500 μM) was associated with an approximate 2-fold decrease in cell proliferation rate over 72 h. However, a significant, approximately 2–4-fold increase in cell number was still observed at 72 h in all cell lines ($P < 0.05$; Table 1).

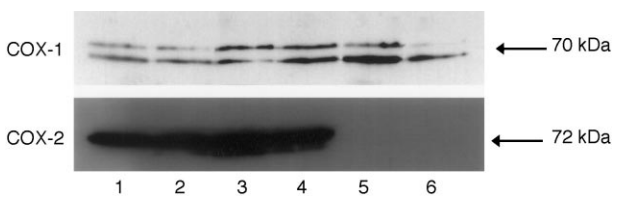

Fig. 2. Western blot analysis of COX-1 and COX-2 protein expression in human colorectal cancer cells. Lane 1, HT29.Fu; 2, HT29.Fu (0.5% FBS); 3, HCA-7; 4, HCA-7 (0.5% FBS); 5, SW480; 6, HCT116.

3.3. Effect of NSAIDs on apoptosis and cell cycle parameters

As we determined *total* cell number in the above experiments, it was likely that cells which had undergone apoptosis were included in the above data. Therefore, we also examined the effect of NSAIDs on apoptosis and cell cycle parameters under the same experimental conditions.

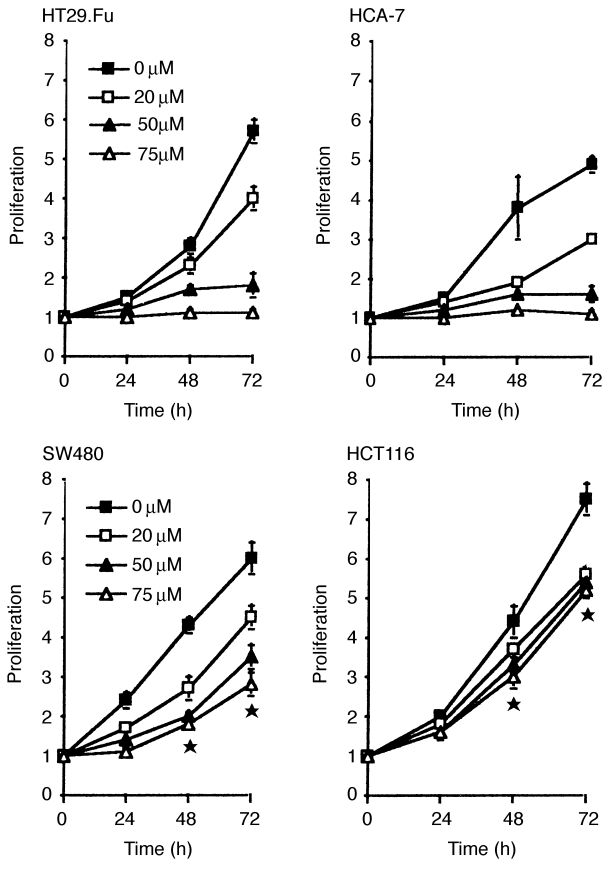

Fig. 3. The effect of NS-398 on human colorectal cancer cell proliferation (see also Table 1). Data are expressed as the mean times increase in cell number (±SEM) compared with the original cell number plated ($n = 3$). *A significant increase in cell number ($P < 0.05$, one-way ANOVA).

Table 1
The effect of NSAIDs on proliferation of human colorectal cancer cell lines

NSAID	Concentration (μM)	HT29.Fu			HCA-7			SW480			HCT116		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
NS-398	Control	1.5 ± 0.0[a]	2.8 ± 0.2	5.7 ± 0.3	1.5 ± 0.1	3.8 ± 0.8	4.9 ± 0.2	2.4 ± 0.2	4.3 ± 0.2	6.0 ± 0.4	2.0 ± 0.1	4.4 ± 0.4	7.5 ± 0.4
	20	1.4 ± 0.1	2.3 ± 0.2	4.0 ± 0.3	1.4 ± 0.1	1.9 ± 0.1	3.0 ± 0.1	1.7 ± 0.1	2.7 ± 0.3	4.5 ± 0.3	1.8 ± 0.1	3.7 ± 0.1	5.6 ± 0.2
	50	1.2 ± 0.1	1.7 ± 0.1	1.8 ± 0.3	1.2 ± 0.1	1.6 ± 0.1	1.6 ± 0.2	1.4 ± 0.0	2.0 ± 0.1	3.5 ± 0.3	1.6 ± 0.0	3.3 ± 0.2	5.4 ± 0.3
	75	1.0 ± 0.1[b]	1.1 ± 0.1[b]	1.1 ± 0.1[b]	1.0 ± 0.1[b]	1.2 ± 0.0[b]	1.1 ± 0.1[b]	1.1 ± 0.1	1.8 ± 0.1*	2.8 ± 0.3*	1.6 ± 0.2	3.0 ± 0.3*	5.2 ± 0.2*
Indomethacin	Control	1.6 ± 0.1	3.7 ± 0.2	5.9 ± 0.1	1.8 ± 0.1	3.5 ± 0.3	4.8 ± 0.2	1.8 ± 0.1	4.0 ± 0.4	7.0 ± 0.3	1.9 ± 0.1	4.1 ± 0.3	7.3 ± 0.3
	100	1.4 ± 0.1	2.7 ± 0.2	3.3 ± 0.1	1.5 ± 0.1	2.0 ± 0.1	3.1 ± 0.1	1.6 ± 0.2	2.6 ± 0.3	3.7 ± 0.3	1.8 ± 0.1	3.0 ± 0.2	5.4 ± 0.3
	400	1.2 ± 0.1[b]	1.3 ± 0.1[b]	1.3 ± 0.2[b]	1.2 ± 0.1[b]	1.6 ± 0.2[b]	1.7 ± 0.2[b]	1.4 ± 0.2	1.7 ± 0.2	1.9 ± 0.2	1.5 ± 0.1	1.7 ± 0.1	2.0 ± 0.1
	600	1.0 ± 0.1[b]	1.0 ± 0[b]	0.9 ± 0[b]	1.0 ± 0.1[b]	1.0 ± 0.1[b]	0.8 ± 0.1[b]	1.2 ± 0.2[b]	1.2 ± 0.2[b]	1.0 ± 0.2[b]	1.2 ± 0.1	1.5 ± 0.1*	1.8 ± 0.1*
Aspirin	Control	1.4 ± 0.1	3.7 ± 0.2	5.9 ± 0.3	1.8 ± 0.1	2.7 ± 0.2	4.2 ± 0.3	2.2 ± 0.1	4.4 ± 0.3	6.9 ± 0.2	2.1 ± 0.1	4.7 ± 0.3	8.4 ± 0.3
	400	1.2 ± 0.1	3.1 ± 0.2	5.1 ± 0.3	1.6 ± 0.1	2.1 ± 0.1	3.7 ± 0.1	1.7 ± 0.2	3.8 ± 0.3	4.9 ± 0.2	1.8 ± 0.1	3.9 ± 0.4	6.8 ± 0.2
	1000	1.1 ± 0	2.3 ± 0.2	4.2 ± 0.2	1.2 ± 0.1	1.9 ± 0.1	3.0 ± 0.1	1.6 ± 0.1	3.5 ± 0.3	4.4 ± 0.1	1.8 ± 0.1	3.6 ± 0.4	5.6 ± 0.3
	1500	0.9 ± 0	2.0 ± 0.1*	3.2 ± 0.2*	1.0 ± 0.1	1.7 ± 0.1*	2.3 ± 0.2*	1.4 ± 0.2	2.6 ± 0.2*	3.3 ± 0.2*	1.8 ± 0.1	2.9 ± 0.4*	4.8 ± 0.2*

[a] Data are expressed as the mean (± standard error of the mean) times increase in total cell number relative to the original number of cells plated ($n = 3$).
[b] When a particular concentration of NSAID was associated with no significant increase in cell number over a 72 h time period.
*A significant increase in cell number at the highest concentration of NSAID ($P < 0.05$; one-way ANOVA).

Table 2
The effect of NSAIDs on cell cycle parameters and apoptosis of human colorectal cancer cell lines

NSAID	Concentration (mM)	HT29.Fu				HCA-7				SW480				HTC116			
		% G1	% S	% G2/M	% Apoptotic	% G1	% S	% G2/M	% Apoptotic	% G1	% S	% G2/M	% Apoptotic	% G1	% S	% G2/M	% Apoptotic
NS-398	Control	62.6 ± 6.2[a]	31.0 ± 7.5	6.3 ± 1.4	4.0 ± 4.0	47.4 ± 3.1	35.5 ± 1.0	17.2 ± 2.6	3.0 ± 2.2	67.7 ± 0.1	29.6 ± 1.2	2.7 ± 1.3	3.2 ± 1.9	66.3 ± 0.2	11.2 ± 3.5	22.5 ± 3.7	8.8 ± 8.8
	20	67.4 ± 1.7	26.4 ± 2.3	6.2 ± 0.9	21.8 ± 8.0	49.8 ± 2.4	33.8 ± 1.3	16.4 ± 2.3	12.1 ± 3.0	70.1 ± 0.6	26.0 ± 1.6	3.8 ± 2.1	4.2 ± 2.9	60.9 ± 1.6	12.6 ± 5.3	26.4 ± 3.8	6.5 ± 6.5
	50	68.7 ± 0.9	23.0 ± 0.5	8.2 ± 1.4	24.1 ± 2.9	61.6 ± 1.4*	20.3 ± 2.1*	18.2 ± 0.9	20.3 ± 7.9	70.1 ± 0.4	24.0 ± 1.3	5.9 ± 1.2	3.7 ± 2.2	54.1 ± 0.5	17.6 ± 3.6	28.3 ± 4.1	7.0 ± 4.2
	75	76.0 ± 1.7	11.7 ± 1.6	12.3 ± 1.4	34.4 ± 3.2*	60.9 ± 2.8*	16.1 ± 3.7*	23.1 ± 2.1	27.6 ± 3.8*	71.2 ± 1.0	23.1 ± 1.6	5.8 ± 0.6	3.6 ± 3.6	56.0 ± 2.6	16.3 ± 3.5	27.7 ± 2.2	9.2 ± 5.2
Indomethacin	Control	64.3 ± 4.5	29.9 ± 4.1	5.8 ± 0.5	5.3 ± 2.7	50.9 ± 2.8	34.1 ± 1.4	15.0 ± 1.5	0.0 ± 0.0	67.4 ± 1.8	26.5 ± 1.0	6.0 ± 1.3	1.6 ± 1.6	62.5 ± 4.3	16.6 ± 6.4	20.6 ± 3.7	0.0 ± 0.0
	100	49.6 ± 6.9	45.9 ± 6.4	4.5 ± 2.7	4.4 ± 1.3	50.4 ± 3.2	36.1 ± 3.2	13.5 ± 0.2	5.7 ± 4.1	70.8 ± 1.6	23.6 ± 1.5	5.6 ± 0.9	2.3 ± 1.4	59.5 ± 3.3	16.0 ± 4.9	24.5 ± 2.8	5.5 ± 4.9
	400	73.6 ± 5.2	24.7 ± 5.5	1.7 ± 1.7	36.5 ± 8.3	68.0 ± 3.5*	16.8 ± 2.5*	15.4 ± 3.1	32.5 ± 6.9*	86.9 ± 1.0*	6.7 ± 0.9*	7.4 ± 1.1	14.9 ± 3.8*	71.5 ± 4.4*	3.7 ± 3.7	24.8 ± 4.4	27.1 ± 5.5*
	600	89.0 ± 1.9*	0.0 ± 0.0*	11.0 ± 1.9	45.0 ± 19.6	69.1 ± 3.2*	18.1 ± 5.5*	12.7 ± 8.4	21.3 ± 6.4	67.5 ± 1.1	15.8 ± 1.7	16.8 ± 1.3*	14.3 ± 1.8*	48.5 ± 3.4	0.5 ± 0.5	51.0 ± 3.8*	32.8 ± 5.2*
Aspirin	Control	63.1 ± 5.2	32.8 ± 5.3	4.1 ± 0.6	13.8 ± 5.2	48.2 ± 2.4	33.9 ± 1.3	17.8 ± 1.7	9.5 ± 4.9	68.1 ± 1.1	27.9 ± 1.7	4.1 ± 1.7	8.4 ± 4.3	67.1 ± 6.7	16.2 ± 5.2	16.8 ± 4.9	2.3 ± 0.2
	400	64.2 ± 5.8	32.1 ± 6.5	3.7 ± 1.4	10.6 ± 4.4	48.3 ± 4.2	34.8 ± 2.4	17.0 ± 2.6	11.0 ± 6.6	68.1 ± 0.4	28.7 ± 1.1	3.2 ± 1.5	9.7 ± 2.7	63.7 ± 6.0	15.8 ± 5.1	20.5 ± 3.7	8.0 ± 4.4
	1000	60.4 ± 8.3	36.3 ± 9.8	3.4 ± 2.3	18.2 ± 4.7	47.0 ± 4.0	36.4 ± 3.3	16.6 ± 1.0	8.6 ± 3.7	64.4 ± 0.5	32.0 ± 2.3	3.7 ± 1.9	11.5 ± 3.8	64.0 ± 6.0	16.6 ± 3.0	19.4 ± 4.4	9.9 ± 6.3
	1500	56.8 ± 6.0	39.2 ± 7.9	4.1 ± 2.6	16.6 ± 6.6	48.3 ± 4.1	35.2 ± 2.4	16.5 ± 1.8	16.0 ± 5.9	65.6 ± 1.0	30.8 ± 2.0	3.7 ± 1.7	11.0 ± 1.9	60.7 ± 5.1	16.5 ± 1.4	22.9 ± 4.3	6.3 ± 3.5

[a] Data are expressed as the mean (± standard error of the mean) percentage of cells in a given cell cycle phase or with sub-diploid DNA content at 72 h ($n = 3$).
*$P < 0.05$ (one-way ANOVA).

Representative DNA content frequency histograms for PI-stained HT29.Fu cells following incubation under control conditions or with 75 μM NS-398 for 72 h are depicted in Fig. 4. In order to confirm that sub-diploid DNA content was indeed indicative of apoptosis in colorectal cancer cells, we tested the effect of 2 mM sodium butyrate on production of a sub-diploid peak by all four colorectal cancer cell lines [24]. Sodium butyrate treatment was associated with a statistically significant time-dependent increase in sub-diploid DNA cell peak in all four colorectal cancer cell lines (data not shown). At 72 h, sub-diploid DNA cells made up 29, 28, 31 and 36% of the total population of butyrate-treated HT29.Fu, HCA-7, SW480 and HCT116 cells respectively. Moreover, there was a statistically significant relationship between the size of the sub-diploid cell population, which was induced by each of the NSAIDs in all four cell lines, and the percentage of apoptotic cells counted by fluorescence microscopy (multiple linear regression, $r^2 = 0.22$; $P < 0.01$). Therefore, in the following experiments, we termed the sub-diploid cell population 'apoptotic'.

There was no significant change in any cell cycle parameter or the percentage of apoptotic cells during the 72 h study period when the cell lines were grown under control conditions (data not shown). The percentage of cells in each cell cycle phase and the percentage of apoptotic cells at 72 h after addition of NSAID are presented in Table 2. Significant changes induced by NSAID treatment in a particular cell line were always time-dependent with the maximum effect demonstrated at 72 h (data not shown). In keeping with its effect on cell proliferation, NS-398 increased the proportion of cells in G1, decreased the proportion of cells in the S phase (except in HCT116 cells) and induced apoptosis in a concentration-dependent manner in HT29.Fu and HCA-7 cells but not in SW480 or HCT116 cells (Table 2). Indomethacin induced similar cell cycle changes and apoptosis in all four cell lines (Table 2). However, indomethacin did not exhibit a simple concentration-dependent relationship in SW480 and HCT116 cells. At the highest concentration (600 μM), indomethacin was associated with a reduction in the proportion of cells in the G1 phase and an increase in cells in the G2/M phase compared with lower concentrations (Table 2). Aspirin did not significantly alter cell cycle parameters or induce apoptosis, measured by DNA content analysis or acridine orange (AO) staining (Table 2).

3.4. β-catenin expression during NSAID treatment

Next, we investigated whether NSAID-induced G1 arrest and apoptosis were associated with changes in β-catenin protein expression by Western blot analysis. Monoclonal antibody 6F9 identified 92 kDa β-catenin in all four cell lines. Blots from representative COX-2-positive (HT29.Fu) and COX-2-negative (SW480) cell lines are demonstrated in Fig. 5. Similar data were obtained using HCA-7 and HCT116 cells. At 72 h, indomethacin treatment was associated with a concentration-dependent decrease in β-catenin protein expression in all four cell lines unlike G6PDH protein expression which remained unchanged (Fig. 5a). We did not detect either β-catenin cleavage products or evidence of β-catenin poly-ubiquitination during indomethacin-induced G1 arrest and apoptosis using 6F9 (Fig. 5a). We also performed Western blot analysis using an alternative monoclonal anti-β-catenin antibody

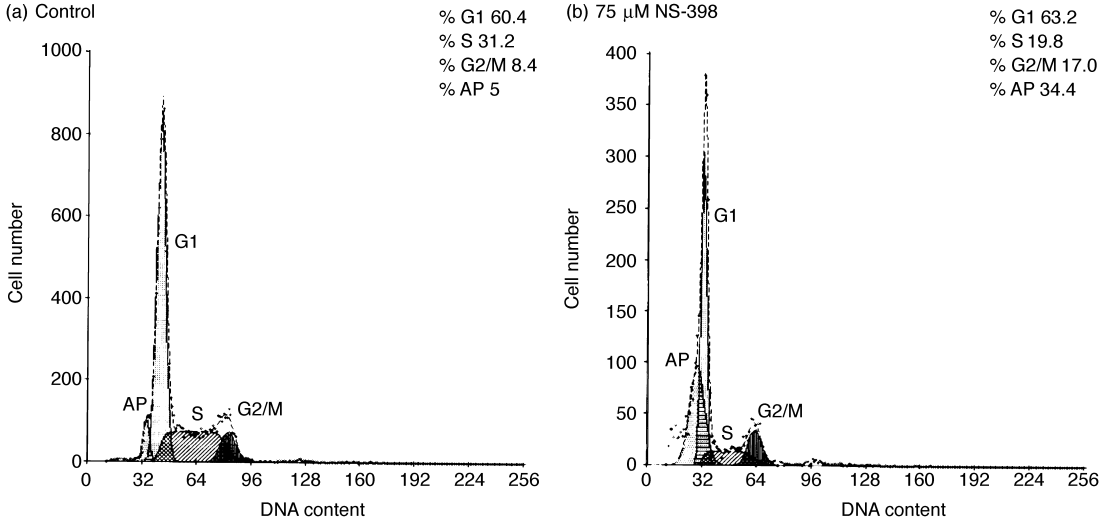

Fig. 4. Flow cytometry of HT29.Fu cells following 72 h incubation with (a) control, (b) 75 μM NS-398. The percentage cell cycle phase distribution is indicated within each panel. The (sub-diploid) peak to the left of the G1 peak represents apoptotic cells (AP).

(15B8) on indomethacin- and NS-398-treated HT29.Fu and SW480 cell lysates and obtained identical results to those obtained with 6F9 (data not shown).

Indirect immunofluorescence studies of β-catenin localisation in SW480 cells revealed that treatment with 600 μM indomethacin for 24 h was associated with decreased nuclear β-catenin in a proportion (~10–20%) of cells and a concurrent increase in β-catenin protein expression at cell-cell contacts (Fig. 6b) compared with cells treated with DMSO alone (Fig. 6a). Immunofluorescent detection of E-cadherin under the same conditions demonstrated increased cytoplasmic staining (Fig. 6d) compared with the predominantly membranous localisation in control cells (Fig. 6c).

G1 arrest and apoptosis induced by NS-398 in COX-2-positive cell lines (HT29.Fu and HCA-7) were not associated with changes in β-catenin protein expression at 72 h (Fig. 5b). Neither NS-398 administration to COX-2-negative cell lines (SW480 and HCT116), nor aspirin administration to any of the cell lines, was associated with a reduction in β-catenin protein expression (Fig. 5b, c). Similarly, butyrate-induced apoptosis was not associated with reduced β-catenin protein levels in any of the cell lines tested (Fig. 5d).

4. Discussion

Data obtained on COX expression and the effect of individual NSAIDs on each cell line are summarised in Table 3. Unlike previous reports of the effects of NSAIDs on human colorectal cancer cells *in vitro*, we studied the effects of several, diverse NSAIDs on multiple, well-characterised human colorectal cancer cell lines. Our data are consistent with previous reports of COX expression by HT29 [10,11], HCA-7 [12], HCT116 [12] and SW480 [25].

Data from this study indicate that COX-2 expression is not required for the anti-proliferative effect of the selective COX-2 inhibitor NS-398. NS-398 treatment was associated with complete inhibition of proliferation along with induction of G1 arrest and apoptosis in COX-2-expressing cell lines but still had anti-proliferative activity in the two cell lines which did not express COX-2. It is likely that NS-398 has anti-proliferative activity by both COX-2-dependent and -independent pathways. Our data are in agreement with experiments by Elder and colleagues [10] who demonstrated that induction of apoptosis by NS-398 was independent of COX-2 expression in two human

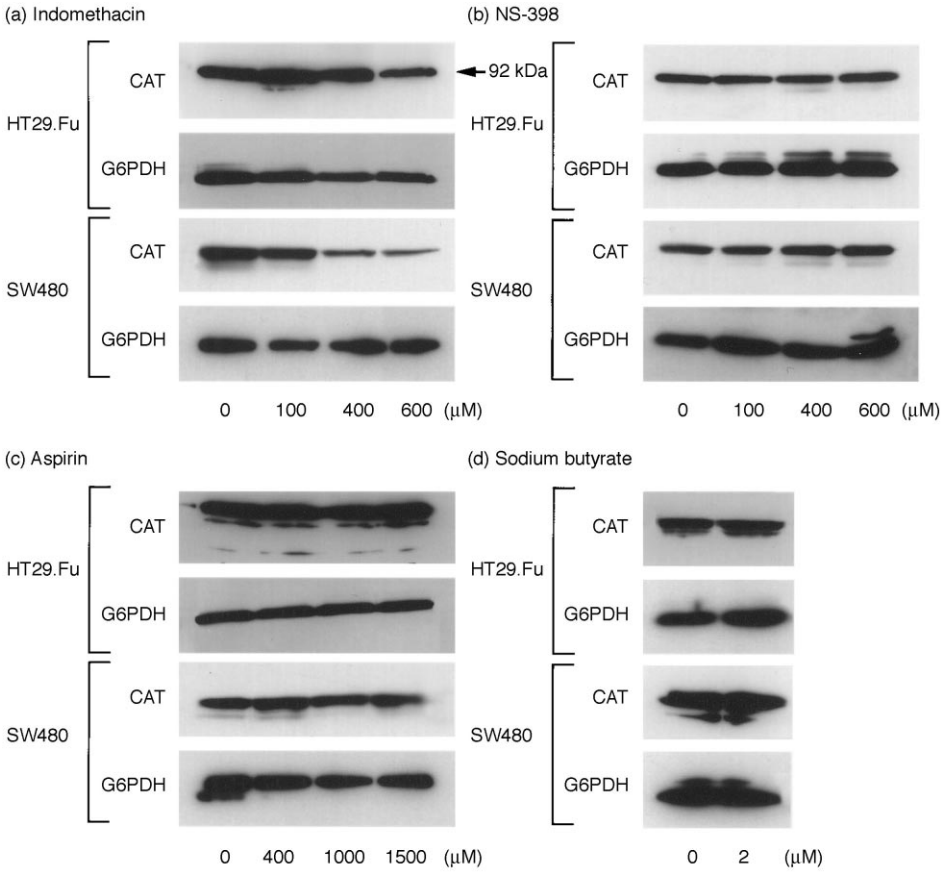

Fig. 5. Western blot analysis of β-catenin (CAT) and glucose-6-phosphate dehydrogenase (G6PDH) protein expression in HT29.Fu (COX-2-positive) cells and SW480 (COX-2-negative) cells after 72 h incubation with each NSAID or sodium butyrate.

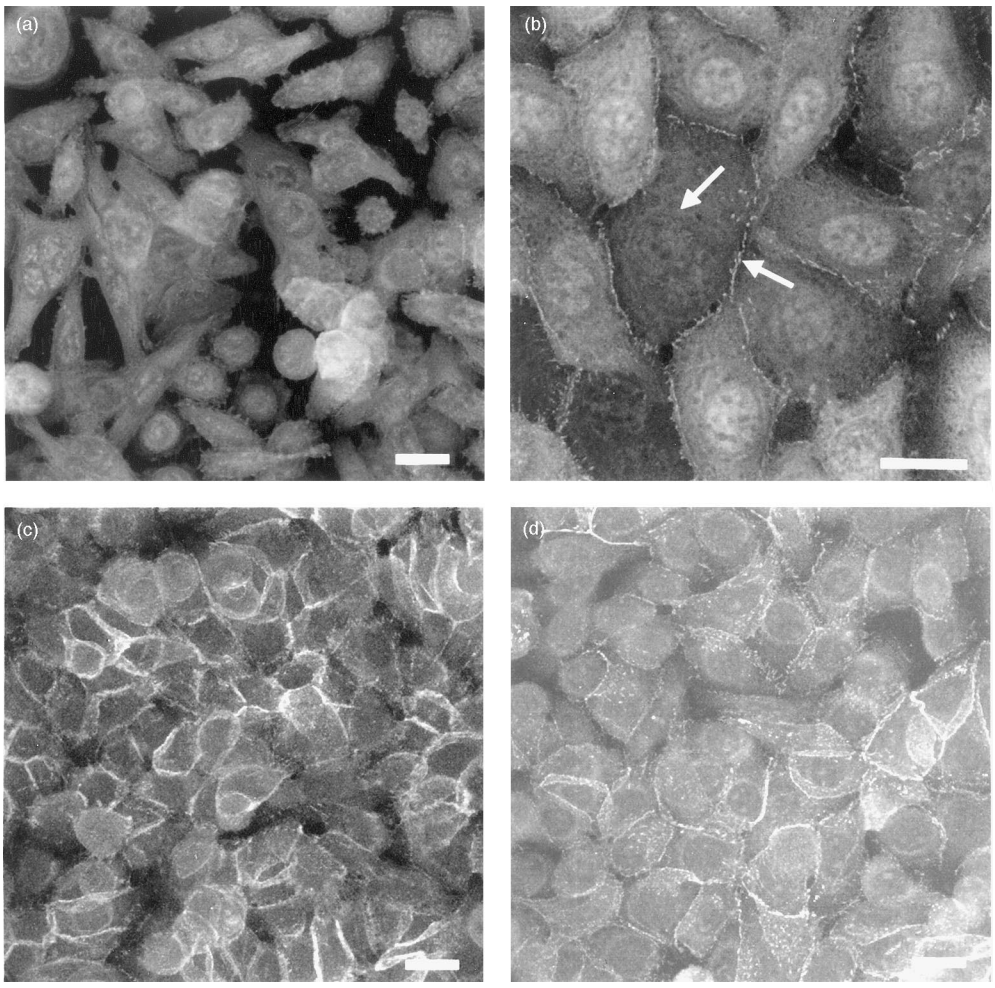

Fig. 6. Indirect immunofluorescence detection of β-catenin (a, b) and E-cadherin (c, d) in SW480 cells treated with DMSO (a, c) or 600 μM indomethacin (b, d) for 24 h. Bars = 10 μm. Indomethacin treatment was associated with diminished nuclear β-catenin staining and increased membranous staining in areas of cell-cell contact (b; arrows).

Table 3
Summary of COX expression and the effects of NSAIDs on human colorectal cancer cell lines

Cell line	COX-1[a]	COX-2[a]	NSAID	Cell proliferation[b]	G1 arrest[c]	Apoptosis[c]	β-catenin protein[c]
HT29.Fu	+	+	NS-398	↓↓	↑	↑	→
			Indomethacin	↓↓	↑	↑	↓
			Aspirin	↓	→	→	→
HCA-7	+	+	NS-398	↓↓	↑	↑	→
			Indomethacin	↓↓	↑	↑	↓
			Aspirin	↓	→	→	→
SW480	+	–	NS-398	↓	→	→	→
			Indomethacin	↓↓	↑	↑	↓
			Aspirin	↓	→	→	→
HCT116	+	–	NS-398	↓	→	→	→
			Indomethacin	↓	↑	↑	↓
			Aspirin	↓	→	→	→

[a] +, COX mRNA and protein detected; –, COX mRNA and protein not detected.
[b] ↓↓, decrease; ↓, slight decrease.
[c] ↑, increase; →, no change; ↓, decrease.

colorectal cancer cell lines. More recently, the anti-proliferative effects of NS-398 (up to 100 μM) have also been demonstrated in COX-1- and COX-2-null transformed murine embryonic fibroblasts [26], which confirms a COX-independent mechanism of action of NS-398. However, others have shown that COX-2 expression is necessary for the anti-proliferative effects of other selective COX-2 inhibitors (SC58125 and meloxicam; [12,27]). The mechanism of the COX-2-independent anti-proliferative activity of NS-398 on human colorectal cancer cells remains unknown. It is essential that the precise nature and relative importance of COX-2-dependent and independent mechanisms of action of individual selective COX-2 inhibitors is investigated, as this class of drug may be developed as chemopreventative therapy for humans, in which approximately 20–50% of human sporadic colorectal adenomas do not express COX-2 [28,29].

Indomethacin induced G1 arrest and apoptosis in COX-2-positive and COX-2-negative cell lines to a similar degree suggesting a COX-2-independent mechanism of action. Indomethacin has also been shown by Zhang and colleagues to induce apoptosis of transformed murine fibroblasts in the absence of both COX isoforms [26]. A potential COX-independent mechanism of action of indomethacin is activation of peroxisome proliferator-activated receptor γ [30], which is associated with G1 arrest and apoptosis in human colorectal cancer cell lines [31]. The effect of indomethacin on HT29.Fu cells observed in this study was similar to that reported by Shiff and colleagues [14] on HT29 cells. Otherwise, the effect of this commonly used NSAID on human colorectal cancer cells has not been examined previously.

Aspirin exhibited a consistent but small anti-proliferative effect on each of the cell lines which was not as marked as for indomethacin or NS-398. Unlike NS-398 and indomethacin, aspirin administration was not associated with significant changes in cell cycle distribution or induction of apoptosis. Qiao and colleagues [32] have recently produced evidence that aspirin does, in fact, induce 'atypical' apoptosis in HT29 cells which is not detected by traditional methods such as those used in our study. It is possible that the consistent anti-proliferative effect of aspirin on the four colorectal cancer cell lines used in this study was mediated by 'atypical' apoptosis.

We have demonstrated that G1 arrest and apoptosis induced by 72 h incubation with indomethacin was associated with decreased β-catenin protein expression in all cell lines including HCT116, which contains a heterozygous deletion mutation of *CTNNB1* which encodes β-catenin [33]. The absence of change in β-catenin protein expression associated with butyrate-induced apoptosis and the demonstration that reduced β-catenin protein levels were not explained by a global reduction in cellular protein expression during G1 arrest and apoptosis suggest that this was a specific phenomenon. Although we have not proved a causal relationship between decreased β-catenin protein expression and indomethacin-induced G1 arrest and apoptosis in these experiments, β-catenin represents a plausible target for NSAIDs. It has been reported in abstract that sulindac-induced apoptosis is associated with reduced β-catenin expression [34]. Supporting evidence *in vivo* has been provided by McEntee and colleagues who observed a reduction in intestinal epithelial cell β-catenin detected by immunohistochemistry during sulindac treatment in the Min mouse [35]. In addition, apoptosis of HT29 cells induced by TFF3 is also associated with reduced β-catenin protein expression [36]. The immunofluorescence data suggest that down-regulation of β-catenin protein measured by Western blot analysis following indomethacin treatment is associated with decreased nuclear β-catenin. The fact that this was demonstrated in only a proportion of cells may be indicative that reduction of nuclear β-catenin occurs at a precise point(s) in the apoptotic pathway. Re-distribution of E-cadherin from plasma membrane to cytoplasm has previously been reported during the early stages of apoptosis in human retinoblasts [37]. It is recognised that over-expression of E-cadherin in SW480 cells leads to decreased nuclear β-catenin and antagonism of β-catenin-LEF binding [38]. Alterations in β-catenin binding by E-cadherin and hence changes in β-catenin-TCF/LEF target gene expression may underlie the anti-proliferative effects of indomethacin and deserve further investigation.

In contrast, G1 arrest and apoptosis induced by NS-398 in COX-2-positive cell lines was not associated with a reduction in β-catenin protein expression. Decreased cell proliferation mediated by NS-398 (via COX-2-dependent and -independent pathways) does not seem to involve downregulation of β-catenin levels.

Cisplatin-induced apoptosis in other cells has been reported to be associated with caspase-mediated cleavage of β-catenin [39]. However, despite the use of anti-β-catenin clone 15B8 which has been used previously by Schmeiser and colleagues [39] to detect β-catenin cleavage products in apoptotic human retinoblasts, we were unable to detect β-catenin cleavage products during NSAID- and butyrate-induced apoptosis in human colorectal cancer cells.

In summary, we have demonstrated that the selective COX-2 inhibitor, NS-398 has anti-proliferative effects on human colorectal cancer cells, which were not entirely dependent on COX-2 expression and which were not associated with down-regulation of β-catenin protein. Indomethacin exerted anti-proliferative effects which were also independent of COX-2 expression, but which were associated with decreased β-catenin protein expression. Aspirin undoubtedly has anti-colorectal

cancer activity *in vivo* but the mechanism of action is likely to be different from both NS-398 and indomethacin as increased apoptosis (with the exception of the small increase seen in HCT116 cells) was not evident by the standard techniques used in this study and aspirin treatment was not associated with changes in β-catenin expression. Although all NSAIDs share the ability to inhibit one or both COX isoforms, it is likely that individual traditional NSAIDs and newer selective COX-2 inhibitors have anti-colorectal cancer activity via a variety of different COX-dependent and/or COX-independent mechanisms. Elucidation of the precise mechanism(s) of action and degree of anti-neoplastic activity *in vivo* of existing NSAIDs will be essential for the future development of safe, universally applicable chemopreventive therapy for colorectal cancer.

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