Polyamines reverse non-steroidal anti-inflammatory drug-induced toxicity in human colorectal cancer cells

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Naproxen, sulindac and salicylate, three NSAIDs (non-steroidal anti-inflammatory drugs), were cytotoxic to human colorectal cancer cells in culture. Toxicity was accompanied by significant depletion of intracellular polyamine content. Inhibition of ornithine decarboxylase (the first enzyme of the polyamine biosynthetic pathway), induction of polyamine oxidase and spermidine/spermine N^1-acetyltransferase (the enzymes responsible for polyamine catabolism) and induction of polyamine export all contributed to the decreased intracellular polyamine content. Morphological examination of the cells showed typical signs of apoptosis, and this was confirmed by DNA fragmentation and measurement of caspase-3-like activity. Re-addition of spermidine to the cells partially prevented apoptosis and recovered the cell number. Thus polyamines appear to be an integral part of the signalling pathway mediating NSAID toxicity in human colorectal cancer cells, and may therefore also be important in cancer chemoprevention in humans.

Key words: apoptosis, colorectal cancer, chemoprevention, non-steroidal anti-inflammatory drug, polyamine, spermidine, spermine.

INTRODUCTION

Colorectal cancer is a major health problem in the U.K., with approximately 20 000 deaths each year, and a projected 5 year survival rate of 40 % [1]. To date, the only effective treatment is surgery and chemotherapy with 5-fluorouracil [2]. However, epidemiological studies have shown that frequent use of NSAIDs (non-steroidal anti-inflammatory drugs) can lower the incidence of, and mortality from, colorectal cancer by as much as 50 % [3].

The proposed mechanism of NSAID chemoprevention is through inhibition of the inducible isoform of cyclo-oxygenase (COX) enzyme 2 (COX-2). COX is the enzyme responsible for the metabolism of arachidonic acid to the prostaglandins (PGs), which are known to be involved in inflammation [4], tissue homoeostasis and cellular repair [5]. The PGs have also been viewed as potential co-carcinogens and tumour promoters [6].

Chemoprevention is thought to occur by inhibiting the production of the PGs, and thereby: (i) suppressing their tumour-promoting activity [7]; (ii) reversing the PG-induced immunosuppression [7]; and (iii) preventing the activation of carcinogens by the peroxidase component of COX enzymes [8].

However, there is accumulating evidence that COX-2 and the PGs are not essential for the chemopreventative effects induced by the NSAIDs, as originally suggested. Hanif et al. [9] demonstrated that the toxicity of the NSAIDs, piroxicam and sulindac sulphide (a potent metabolite of sulindac), in the HT29 human colon cancer cell line could not be reversed by re-addition of PGI_2, PGE_2 or $PGF_{2\alpha}$. Furthermore, the same group showed that both of these NSAIDs were equally toxic to another cell line, HCT-15, which lacks all COX transcripts and does not produce any PGs. Further support for a COX-independent mechanism comes from Piazza et al. [10], who similarly reported no reversal of toxicity induced by either sulindac or sulindac sulphide on the addition of a PGE_2 analogue.

In light of these observations, it seems that another mechanism of NSAID-induced toxicity must exist, independent of COX activity or the presence of PGs. One possibility is inhibition of polyamine metabolism. The polyamines are known to be essential for cell growth and to be present in increased concentrations in colorectal cancer cells [11]. A common, early event in carcinogenesis is an increase in the enzyme ornithine decarboxylase (ODC), and the subsequent increase in intracellular polyamine concentrations [12]. Indeed, in colorectal cancer the polyamine content and ODC activity of the cancer tissue is increased significantly compared with that of the equivalent normal tissue [11]. It has been suggested that the increased activity of ODC and increased production of polyamines lead to and support malignant growth, and therefore also provide a potential target for therapeutic intervention [13]. Using measurement of polyamine concentrations and/or ODC activity as a marker of risk for colorectal cancer has been proposed many times, but is rarely used clinically [14,15]. Prevention of the increase of both polyamine concentrations and ODC activity has proved sufficiently successful to warrant clinical trials of DFMO (α-difluoromethylornithine), a suicide or enzyme-activated inhibitor of ODC, as a potential chemopreventative agent [16]. These trials have been ongoing since the mid-1990s [17–19]. It is possible therefore that other effective chemopreventative agents, such as the NSAIDs, may also act through modulation of polyamine metabolism.

The aim of the present study was therefore to determine the role of the polyamines in the cytotoxicity induced in human colorectal cancer cells by NSAIDs. Treatment with NSAIDs resulted in a dose-dependent cytotoxicity and a decrease in polyamine content. Concomitant with this decrease was inhibition of ODC and induction of polyamine catabolism and export. Addition of polyamines to the treated cells resulted in reversal of apoptosis, suggesting that modulating the polyamine pathway may be an

Abbreviations used: COX(-2), cyclo-oxygenase(-2); DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; ODC, ornithine decarboxylase; NSAID, non-steroidal anti-inflammatory drug; PAO, polyamine oxidase; PARP, poly(ADP-ribose) polymerase; PG(I_2/E_2/$F_{2\alpha}$), prostaglandin I_2, E_2 or $F_{2\alpha}$ respectively; PTP, permeability-transition pore; SSAT, spermidine/spermine acetyltransferase.

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important factor in the action of NSAIDs in colorectal cancer cells.

EXPERIMENTAL

Reagents

Sulindac, naproxen, salicylate, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] and DAPI (4,6-diamidino-2-phenylindole) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Cell-culture plastics, DMEM (Dulbecco's modified Eagle's medium) growth media, penicillin/streptomycin, fetal bovine serum and trypsin were from Life Technologies (Paisley, Scotland, U.K.). The DLD-1 cell line was obtained from the European Collection of Cell Cultures (Salisbury, Wilts., U.K.).

Radioisotopes

[^{3}H]Acetyl-CoA (37.0–370.0 GBq/mmol) was from DuPont NEN, Boston, MA, U.S.A. [^{14}C]Ornithine dihydrochloride (1.85–2.22 GBq/mmol) was from American Radiolabeled Chemicals Inc., St Louis, MO, U.S.A. [1,4(n)-^{3}H]Putrescine dihydrochloride (0.18–1.48 TBq/mmol) was from Amersham Life Sciences, Bucks., U.K.

Cell culture

The human colon cancer line (DLD-1) was grown in DMEM, supplemented with 10 % (v/v) fetal bovine serum, 50 units/ml penicillin and 50 units/ml streptomycin. Cultures were maintained in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C. A seeding density of 2.0×10^4 cells/cm^2 was used for all experiments, and cells were allowed at attach for 4 h and grown for 24 h, after which the appropriate treatments were started.

Cytotoxicity assay

Cytotoxicity was quantified by the method of Mosmann [20], as modified by Denizot and Lang [21]. Cells were grown on 96-well microtitre plates and exposed to the appropriate concentration of drug. At the desired time, 10 μl of a 5 mg/ml sterile solution of MTT in DMEM was added to the cells, which were incubated for 4 h at 37 °C in a humidified incubator (Gallenkamp) containing 5 % CO_2 and 95 % air. Viable cells metabolize MTT using the mitochondrial enzyme succinate dehydrogenase, and the formazan salt can be detected at 570 and 690 nm.

Cell growth determination

Determination of cell number and viability was assessed by the exclusion of Trypan Blue. Cells were grown on 50 mm diameter plates, with appropriate treatments. Cells were harvested using trypsin/EDTA solution and counted on a Neubauer haemocytometer. Cells stained blue were counted as non-viable, whereas those excluding the dye were viable. Results were expressed as total viable cell number and as percentage viability.

Protein determination

Total cellular protein content was determined using a modification of the method described by Lowry et al. [22]. Standards were prepared from a stock solution of BSA (500 μg/ml) by serial dilution with 0.3 M NaOH to give standards within the range 0 to 250 μg/ml BSA.

Intracellular NSAID content determination

Cellular uptake of NSAIDs was measured by HPLC determination using a modification of the method previously described by Clark et al. [23]. NSAIDs were separated on a Hichrom Ltd. HIRPB C_{18}–2524 column through a 65 % 25 mM sodium actetate (pH 4.0)/35 % acetonitrile mobile phase (1 ml/min). Compounds were detected at 230 nm.

Polyamine analysis

Cells for polyamine analysis were harvested in perchloric acid as described by Wallace et al. [24], and the acid fraction containing the polyamines was stored at -20 °C until analysis by HPLC. Polyamines and their monoacetyl derivatives were separated and quantified by a modification of the HPLC method of Seiler and Knodgen [25], as described by Wallace et al. [24]

ODC enzyme activity

ODC activity was measured by the method of Coleman and Pegg [26]. Cells were lysed in 0.5 ml of Tris buffer [19.3 mg of dithiothreitol and 2 ml of 0.1 mM EDTA solution in 50 ml of 0.1 M Tris/HCl (pH 7.4 at 4 °C)]. Samples were sonicated and aliquots were taken for protein determination, before being ultracentrifuged at 40 000 ***g*** at 40 °C for 20 min. Supernatant (100 μl) was mixed with 100 μl of PDP buffer (2.5 mg of pyridoxal 5′-phosphate in 10 ml of Tris buffer) and 50 μl of [^{14}C]ornithine (3.8 nCi/nmol in 2.5 mM L-ornithine), and the amount of radiolabelled CO_2 released in 1 h was measured by collection in benzethonium hydroxide.

Polyamine oxidase (PAO) activity

Cells were lysed in 0.5 ml of Tris cell-homogenizing buffer [1 M Tris, pH 7.2, with 1 M HCl and 0.1 % (w/v) Triton X-100]. Samples (100 μl) were placed in an assay tube with 50 μl of distilled H_2O and 240 μl of assay mix (5 mM aminoguanidine, 2.5 mM pargyline and 90 units of horseradish peroxidase in 200 mM sodium borate buffer). Hydrogen peroxide generation caused by the oxidation of the substrate N^1-acetylspermine (50 μl of 2 mM stock per tube) was measured fluorimetrically by reaction with homovanillic acid (8.4 mg dissolved in 0.5 ml of ethanol, diluted to 5 ml with sodium borate buffer; 60 μl was added to each tube) for 30 min and compared with a standard curve of H_2O_2. Fluorescence was measured on a PerkinElmer LS50 luminescence spectrophotometer at an excitation wavelength of 323 nm (slit-width 10 nm) and an emission wavelength of 426 nm (slit-width 9 nm).

Spermidine/spermine acetyltransferase (SSAT) activity

SSAT activity was measured as described by Wallace and Evans [27]. Cells were harvested and lysed in Tris buffer [10 mM Tris/HCl (pH 7.5) at 4 °C with 1 mM EDTA and 2.5 mM dithiothreitol], and then ultracentrifuged at 100 000 ***g*** for 70 min at 4 °C. The supernatant was transferred to Eppendorf tubes containing 10 μl of 30 mM spermidine and 10 μl of 1 M Tris/HCl, pH 7.8, at 37 °C, with 10 μl of 250 μM acetyl-CoA and 10 μl of [^{3}H]acetyl-CoA (0.33 μCi). The reaction was terminated after

10 min with 20 μl of 1 M hydroxylamine. Aliquots (30 μl) of each supernatant were dried on Whatman P81 paper discs, counted in a scintillation counter, and results were calculated as pmol of *N*-acetylspermidine formed/min per mg of protein.

Polyamine export

Efflux of polyamines from cells was measured by incorporation of radiolabelled [^{3}H]putrescine into the intracellular polyamine pools, by the method of Wallace and Mackarel [28]. In short, plates were treated with 0.5 μCi of [^{3}H]putrescine/ml of plate-medium volume. The radioactivity present in either the extracellular medium or extracted from the cell pellet was measured in a scintillation counter and calculated as the percentage of total radioactivity from the intra- or extra-cellular environment.

Morphological characterization of cell death

The morphological features of cell death, in particular those of apoptosis, were examined by fluorescence staining of the cell nuclei. Cells detached into the medium were carefully retained, and those attached to the plates were harvested with trypsin/EDTA solution. Cells were washed with PBS, and pellets were resuspended in 4 % (v/v) formaldehyde in PBS. Cytospins of total cells (cells attached, pooled with cells in the medium) were prepared on a Shandon Cytospin at 500 rev./min for 5 min. Cell nuclei were stained with 1 μg/ml DAPI in complete PBS. Samples were counted 'blind', with 100 cells counted in five different fields per slide. The percentage apoptosis was scored on the morphological features of apoptosis, including chromatin condensation and cell shrinkage, and expressed as a percentage.

Caspase-3-like activity

Caspase-3-like activity was determined from cells first harvested by the trypsin/EDTA method. Cells were washed in PBS, and centrifuged at 10 000 ***g*** for 10 min at 4 °C, before being lysed in 200 μl of caspase lysis buffer {0.5 % (w/v) CHAPS/1 mM PMSF/0.1 mM 1,10-phenanthroline in 10 ml of caspase buffer [50 mM Tris/HCl (pH 7.4)/1 mM EDTA/10 mM EGTA]}. Lysed samples were centrifuged at 18 000 ***g*** for 10 min at 4 °C to remove cell debris. Aliquots (50 μl) of each sample were warmed to 37 °C for 5 min with 1444 μl of caspase assay buffer [0.5 % (w/v) CHAPS and 5 mM L-cysteine in assay buffer]. The fluorogenic substrate Ac-DEVD-AMC (acetyl-Asp-Glu-Val-Asp 7-amino-4-methylcoumarin; 10 mM in 100 % DMSO) (6 μl) was added to each sample, and caspase-3-like activity was measured by the change in fluorescence (excitation and emission wavelengths of 380 and 460 nm respectively; slit width of 5 nm) over 1 h per mg of protein.

RESULTS

All three NSAIDs decreased the viable cell number in the DLD-1 human colorectal cancer cell line in a dose- and time-dependent manner (Figure 1 and Table 1). The IC_{50} values were calculated, and the order of potency was determined as sulindac > naproxen > salicylate. Cytotoxicity was confirmed by the significant induction of apoptosis by both sulindac and naproxen (Table 1), and the complete loss of MTT activity by salicylate at 10 mM (Figure 1). No decrease in cell viability was observed in response to high-dose NSAID treatment, suggesting apoptotic, rather than necrotic, cell death (Table 1). No significant levels

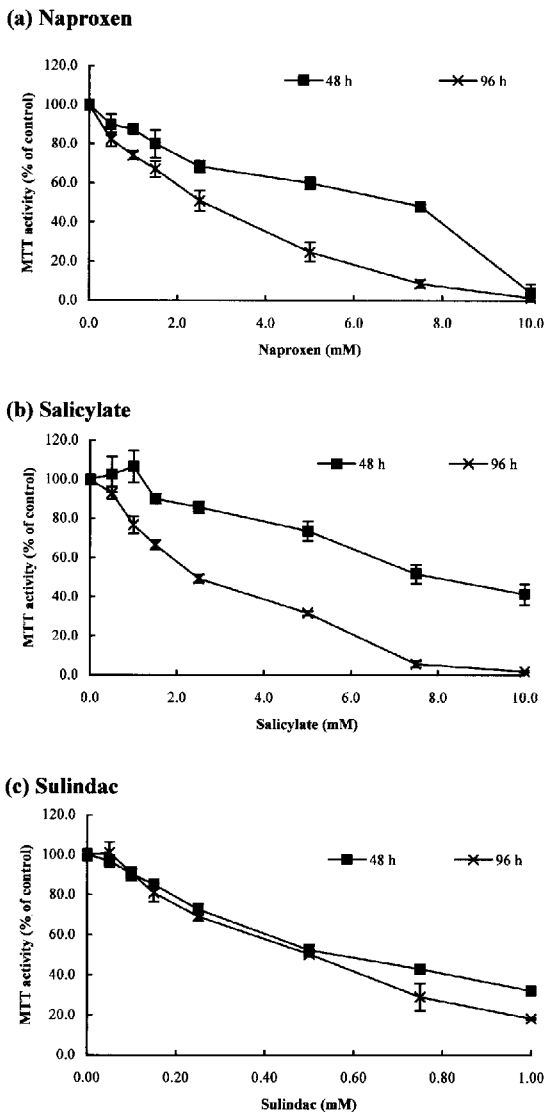

Figure 1 Effect of NSAID treatment on human colorectal cancer cell number

DLD-1 cells were seeded at a density of 2.0×10^4 cells/cm^2 on 96-well microtitre plates and attachment was allowed over 4 h. After 24 h growth, medium was replaced with medium containing drug [0–10 mM naproxen (**a**); 0–10 mM salicylate (**b**); 0–1 mM sulindac (**c**)] or vehicle. Plates were incubated and assayed as per the MTT protocol after 48 and 96 h. Results are shown as the means $\pm$ S.E.M. ($n = 3$, with six replicates per experiment).

of apoptosis were observed at IC_{50} concentrations, suggesting a growth inhibitory as well as a cytotoxic effect. The calculated IC_{50} and IC_{90} (concentration giving 90 % inhibition) values of NSAIDs were used in further experiments to evaluate the changes in polyamine metabolism during growth inhibition, and to observe the effects of polyamines upon the apoptosis induced by sulindac and naproxen.

The total polyamine pool of DLD-1 cells exposed to the NSAIDs decreased 40–55 % (Table 2). This was due mainly to a significant decrease in intracellular spermidine and spermine. Putrescine content was, however, maintained at control levels during drug treatment. The polyamine biosynthetic capacity of the DLD-1 cells was also decreased in response to NSAID treatment (Figure 2a). ODC was inhibited by approximately 50 % by both naproxen and sulindac. Salicylate, on the other hand, did not inhibit ODC activity, despite being as effective as the other

Table 1 Effect of NSAID treatment on viable cell number, membrane viability and apoptosis in human colorectal cancer cells

DLD-1 cells were seeded at a density of 2.0×10^4 cells/cm^2 and allowed 4 h for attachment. After 24 h growth, plates were treated with IC_{50} or IC_{90} doses of NSAIDs. Plates were harvested after 96 h incubation. Cell number and viability was determined by Trypan Blue exclusion and apoptosis was measured by DAPI staining. Results are shown as means ± S.E.M ($n = 3$, with two replicates per experiment). Analysis was by one-way ANOVA and Dunnett's post t test. *$P < 0.001$ compared with untreated control values.

NSAID	Concn (mM)	Viable cell number ($\times 10^6$)	Cell viability (%)	Apoptosis (%)
Control		3.61 ± 0.41*	97.9 ± 1.1	4.0 ± 1.1
Naproxen	2.5	1.49 ± 0.21*	97.8 ± 0.7	4.4 ± 1.7
	7.5	0.38 ± 0.18*	87.3 ± 7.1	55.9 ± 3.4*
Salicylate	6.0	1.89 ± 0.22*	96.1 ± 0.7	5.3 ± 1.2
	10.0	0.77 ± 0.05*	98.0 ± 0.8	5.1 ± 0.8
Sulindac	0.5	2.15 ± 0.26*	95.5 ± 1.6	7.7 ± 0.6
	1.0	1.03 ± 0.13*	95.0 ± 1.5	14.7 ± 1.7*

Table 2 Effect of NSAID treatment on intracellular polyamine content in human colorectal cancer cells

DLD-1 cells were seeded at a density of 2.0×10^4 cells/cm^2 and allowed 4 h for attachment. After 24 h growth, plates were treated with IC_{50} doses of NSAIDs (2.5 mM naproxen, 6.0 mM salicylate or 0.5 mM sulindac). Plates were harvested after 48 h incubation. Polyamine content was quantified by HPLC analysis of the acid-soluble fraction. Results are shown as means ± S.E.M ($n = 3$, with two replicates per experiment). Analysis was by one-way ANOVA and Dunnett's post t test. *$P < 0.05$; †$P < 0.001$ compared with untreated control values.

	Intracellular polyamine content (pmol/mg of protein)			
NSAID	Putrescine	Spermidine	Spermine	Total
Control	3.54 ± 0.87	8.87 ± 1.00	21.12 ± 2.38	33.47 ± 3.34
Naproxen	4.45 ± 1.20	3.05 ± 0.58†	12.17 ± 1.27†	19.76 ± 2.24†
Salicylate	4.73 ± 1.07	3.71 ± 0.62†	11.64 ± 0.76†	20.57 ± 2.13*
Sulindac	3.80 ± 0.35	3.42 ± 0.53†	8.14 ± 0.98†	15.36 ± 1.11†

NSAIDs at diminishing intracellular polyamine concentrations (Table 2).

PAO and SSAT act in concert to break down and recycle the higher polyamines, spermine and spermidine. Both enzymes were affected by the NSAIDs. Naproxen and salicylate increased PAO activity significantly after 48 h exposure, with salicylate causing over a 4-fold increase in activity, and naproxen inducing more than twice the activity of control cells (Figure 2b). Sulindac did not significantly alter PAO activity. All three NSAIDs caused increased SSAT activity after 48 h exposure with a greater-than-3-fold increase over control cells (Figure 2c). Acetylation of the polyamines renders them suitable for export and, indeed, increased export was observed. By 96 h, naproxen-treated cells had excreted 72% of their radiolabelled polyamines [67% excretion after salicylate treatment, and 61.2% excretion after sulindac treatment, compared with control excretion of 49% (Figure 3)]. The absence of any loss of cell viability in response to NSAID treatment (Table 1) indicates that the increase in export is not due to cell lysis, and therefore the radioactivity detected in the medium reflects specific export. This was confirmed by HPLC analysis of polyamines in the medium, revealing predominantly putrescine and acetylated derivatives of spermidine and spermine (results not shown). This does not reflect the intracellular distribution of polyamines, where spermine is the major amine. Similar selective export of acetylated polyamines has been shown to be a direct result of increased active export [29].

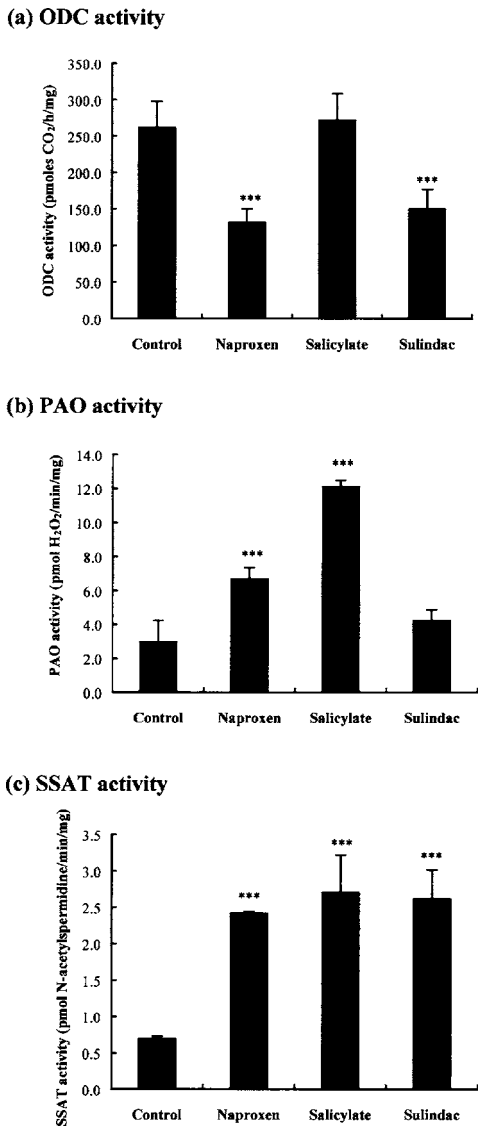

Figure 2 Polyamine metabolism after 48 h NSAID exposure

DLD-1 cells were seeded at a density of 2.0×10^4 cells/cm^2 and allowed 4 h for attachment. After 24 h growth, medium was replaced with medium containing IC_{50} concentrations of NSAIDs (2.5 mM naproxen, 6.0 mM salicylate or 0.5 mM sulindac). Plates were incubated and harvested after 48 h drug exposure. ODC enzyme activity (**a**) was measured by release of ^{14}C-radiolabelled CO_2 from ornithine. PAO enzyme activity (**b**) was measured fluorimetrically by the formation of hydrogen peroxide. SSAT enzyme activity (**c**) was measured by the formation of *N*-acetylspermidine. Results are shown as the means ± S.E.M. ($n = 3$, with three replicates per experiment). Analysis was by one-way ANOVA and Dunnett's post t test. ***$P < 0.001$ compared with control.

The type of cell death observed in response to NSAID treatment was examined. Despite the effects observed on the enzyme activities of ODC, PAO and SSAT at low concentrations of NSAIDs, there was no significant evidence of cell death until higher concentrations of NSAIDs were used (Table 1). Similarly, there was little evidence of apoptotic cell death at 48 h (results not shown), indicating both a dose- and time-dependent effect of the apoptosis induced. High-dose naproxen treatment induced caspase-3-like activity, which is often associated with apoptosis (Figure 4). Salicylate, on the other hand, significantly

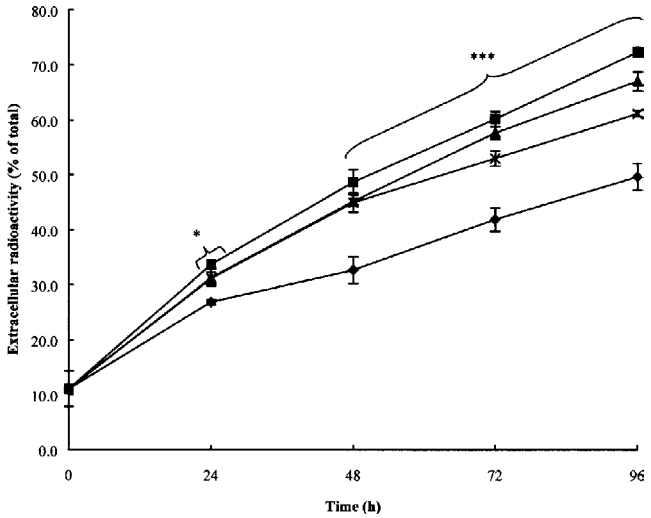

Figure 3 Increased polyamine export in response to NSAID treatment

DLD-1 cells were seeded at a density of 2.0×10^4 cells/cm^2 and allowed 4 h for attachment, before addition of 0.5 μCi of [^{3}H]putrescine per ml of medium. After 36 h incubation, medium was removed, and cell layers were washed repeatedly before medium containing IC_{50} concentrations of NSAIDs (●, control; ■, naproxen; ▲, salicylate; ×, sulindac) was added. Plates were incubated and harvested every 24 h. Export was measured by the percentage of total radiolabel detected in the medium (see the Experimental section). Results are shown as the means ± S.E.M. ($n = 3$, with two duplicates per experiment). Analysis was by one-way ANOVA and Dunnett's post t test. $^*P < 0.05$; $^{***}P < 0.001$ compared with control.

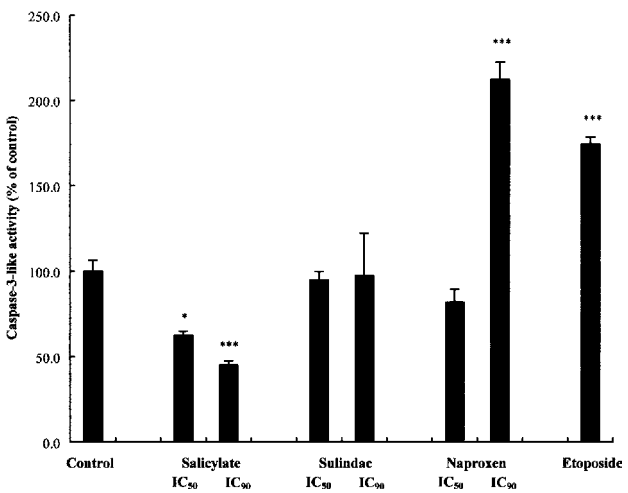

Figure 4 Induction of caspase-3-like activity by NSAID treatment

DLD-1 cells were seeded at 2.0×10^4 cells/cm^2 and allowed 4 h for attachment. After 24 h growth, medium was replaced with medium containing either the IC_{50} or IC_{90} concentrations of the drugs. Plates were incubated and harvested after 48 h of drug exposure. Caspase-3-like enzyme activity was measured as Δfluorescent units/h per mg of protein, but expressed in the Figure as the percentages of the control value for ease of comparison. Results are shown as the means ± S.E.M. ($n = 3$, with two duplicates per experiment). Analysis was by one-way ANOVA and Dunnett's post t test. $^*P < 0.05$; $^{***}P < 0.001$ compared with control.

decreased caspase-3 activity at both low and high doses, whereas sulindac had little effect. DNA fragmentation, measured by ELISA, showed no leakage of fragments into the extracellular medium, which would be indicative of necrotic cell death (results not shown). However, both naproxen and sulindac did cause a

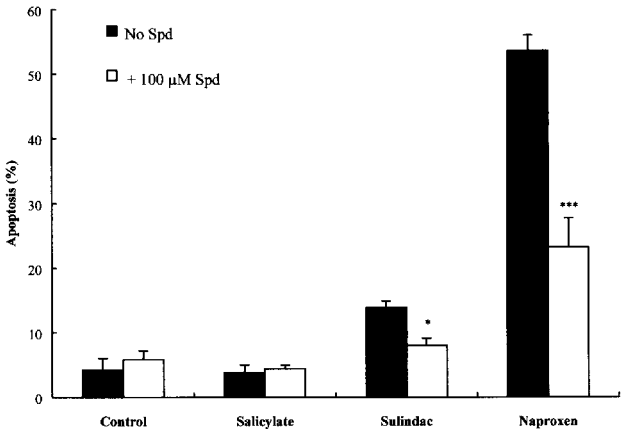

Figure 5 Inhibition of NSAID-induced apoptosis by spermidine

DLD-1 cells were seeded at a density of 2.0×10^4 cells/cm^2 and allowed 4 h for attachment. After 24 h growth, medium was replaced with medium containing IC_{90} concentrations of drugs ± 100 μM spermidine (Spd). Aminoguanidine (1 mM) was added to each plate. Plates were incubated and harvested after 96 h of drug exposure. Cytospins of the total-cell fraction (attached cells and those resuspended in medium) were stained with DAPI and counted by microscopy. Results are shown as the means ± S.E.M. ($n = 3$, with two duplicates per experiment). Analysis was by one-way ANOVA and Dunnett's post t test. $^*P < 0.05$; $^{***}P < 0.001$ compared with control.

significant increase in the amount of intracellular apoptotic DNA fragments (results not shown), despite sulindac not activating caspase-3. Morphological evidence of apoptosis was measured using the fluorescent dye, DAPI. Apoptosis was scored using established criteria, namely shrunken nuclear envelope, formation of highly stained condensed chromatin and 'ball-shaped' separated regions of chromatin. Salicylate did not induce morphological signs of apoptosis, but sulindac and naproxen did (Figure 5). Sulindac treatment caused 15% of cells to display apoptotic characteristics, whereas naproxen induced 54% apoptosis.

If polyamines are part of the pathway leading to apoptosis, then addition of exogenous polyamines should attenuate the effects of the NSAID. The addition of 100 μM spermidine to the extracellular medium at the time of NSAID addition significantly inhibited the amount of apoptosis induced by both sulindac and naproxen, with decreases of 43% and 30% respectively. This reproducible effect was also observed in two other colorectal cancer cell lines (results not shown). The effect of polyamine readdition upon caspase-3-like activity could not be determined because of interference in the assay technique caused by the polyamines (H. M. Wallace and A. V. Fraser, unpublished work). Spermidine also resulted in an increase in cell number in naproxen-treated cells from 55% of control to 70% of control (Table 3). Re-addition of spermidine also inhibited apoptosis in sulindac-treated cells (Figure 5). Spermidine alone did not cause any difference in levels of apoptosis or viable cell number (Figure 5 and Table 3). It is essential to note that aminoguanidine (1 mM) was present in the cell-culture medium of all treatments when exogenous spermidine was added. Aminoguanidine prevents the formation of reactive metabolites caused by the oxidation of spermidine by serum amine oxides; hence the effects observed in Table 3 and Figure 5 can be attributed to spermidine. Aminoguanidine affected neither the induction of apoptosis nor the viable cell number on its own (results not shown). Similarly, a decreased uptake of drug as a result of exogenous spermidine can be ruled out, since HPLC analysis of intracellular NSAID concentrations revealed no difference between spermidine-treated

Table 3 Effect of spermidine on the number of naproxen-treated cells

DLD-1 cells were seeded at a density of 2.0×10^4 cells/cm^2 on 50-mm-diam. plates and allowed to attach for 4 h. After 24 h growth, medium was replaced with medium containing either drug or drug plus 100 μM spermidine (Spd). All plates were treated with 1 mM aminoguanidine at the time of drug addition. Plates were incubated, and the number of viable cells was determined. Cell number was obtained by Trypan Blue exclusion after 96 h exposure. Results are shown as means $\pm$ S.E.M. ($n = 3$, with two replicates per experiment). Analysis was by one-way ANOVA and Dunnett's post t test. *$P < 0.05$ compared with control; †$P < 0.05$ compared with naproxen treatment.

Treatment	Total cell number ($\times 10^6$)
Control	1.93 ± 0.13
100 μM Spd	1.91 ± 0.11
IC_{50} concn. naproxen	0.86 ± 0.05*
IC_{50} concn. naproxen + 100 μM Spd	1.35 ± 0.11*†

and untreated cells (1.39 ± 0.01 μmol/mg of naproxen compared with 1.52 ± 0.08 μmol/mg in the presence of spermidine, and 0.40 ± 0.08 μmol/ mg sulindac compared with 0.53 ± 0.04 μmol/mg in the presence of spermidine).

DISCUSSION

The increasing evidence that the NSAIDs exert their chemopreventative effects in a COX-independent manner means that alternative mechanisms must exist. A logical target for the type of toxicity observed would be a pathway essential for cell growth, and of particular importance in cancer. The polyamines and their metabolic pathways are ideal candidates, because they are involved in most major steps of cell growth, acting as growth factors and facilitating protein synthesis [30]. They also interact with nucleic acids, leading to protection of DNA against denaturation, stimulation of DNA and RNA synthesis and the stabilization of both newly synthesized and folded DNA [31]. In addition, increases in ODC activity and intracellular polyamine concentrations are early events in carcinogenesis [12]. It has been suggested that these increases may support malignant growth [13]. Therefore it is our hypothesis that the polyamine-biosynthetic pathway may provide an alternative pathway for the cytotoxic, and possibly also chemopreventative, effects of the NSAIDs.

Cytotoxicity *in vitro* was measured as a surrogate end-point for chemoprevention in this investigation. The requirement for millimolar doses of drug to elicit changes in cell growth and long exposure compares favourably with the doses and exposure times used in other *in vitro* studies [32–36]. The need for prolonged exposures *in vitro* at high doses of drug appears to be universally required across the range of NSAIDs, with only the novel COX-2 inhibitors and NO-moiety-containing NSAIDs producing effects at low micromolar levels [37–39].

Both sulindac and naproxen were found to induce apoptotic cell death. It was evident from the morphology of the cells that sulindac induced classic signs of apoptosis, including the condensation and compartmentalization of chromatin, confirming observations made by Chan et al. [40] and Huang et al. [41]. Despite the morphologically distinct apoptosis, sulindac did not induce caspase-3 activation (Figure 4). It could be that sulindac is inducing structural changes within the nucleus that are not linked to apoptosis. Initially, it seemed unlikely that the morphological features of apoptosis could be induced without the activation of the main effector caspase pathway. However, sulindac has previously been shown to induce DNA fragmentation and morphological signs of apoptosis in the human breast-cancer-cell line MCF-7 [42], and MCF-7 cells have been characterized as lacking caspase-3 [43], indicating that sulindac can induce apoptosis independently of caspase-3 activation.

Salicylate has proven to be a contentious agent in terms of cell death. Salicylate induced caspase-dependent apoptosis by DNA fragmentation and PARP [poly(ADP-ribose) polymerase] cleavage in human B-cell chronic lymphocytic leukaemia cells [44], and by caspase-3 activation and PARP cleavage in myeloid leukaemia cells [45]. In the present study, no change in morphology was observed in response to salicylate treatment (Figure 5), but a significant inhibition in activity of caspase-3 at 48 h was noted (Figure 4). The reasons behind this inhibition are unknown, but salicylate, like the other two NSAIDs, does deplete polyamine content, a factor linked to cell death and growth inhibition.

Despite the discrepancies in the type of cell death observed, all three drugs tested decreased the growth of cells *in vitro*. NSAID-induced depletion of polyamine content could account for the inhibition of cell growth caused by all three drugs. To verify this hypothesis, we attempted to reverse the toxicity by the re-addition of polyamines. Only partial recovery occurred, perhaps because we were unable to restore fully the polyamine pools to control values, possibly due to the elevated levels of polyamine catabolism we observed within the cells. Elevated SSAT activity within cultured cells has recently been shown to prevent the restoration of intracellular polyamines by exogenous administration [46]. Despite this, the apoptosis induced by sulindac and naproxen was inhibited by spermidine in a reproducible manner (Figure 5). This demonstrates that polyamines can inhibit the apoptosis caused by the NSAIDs, and can also prevent their growth-inhibitory effects. This strongly suggests the NSAIDs are affecting a pathway that is dependent on polyamine content and metabolism, and provides evidence that the polyamines may be implicated in the chemopreventative effects observed *in vivo*. It is plausible that the addition of spermidine may be preventing the opening of the mitochondrial PTP (permeability-transition pore) [47,48], a key step in the mitochondrial route of apoptosis that is known to be induced by a variety of NSAIDs [49]. However, this effect is predominantly observed in response to spermine treatment, and may be more closely associated with Ca^{2+} uptake than gating of PTP [50]. Further experimentation would need to be carried out in order to establish whether the PTP is opened in response to the NSAIDs used in this study, and whether the uptake of exogenous polyamines would exert an effect on any disregulation of mitochondrial function.

Increased ODC activity has been shown to have a pivotal role in the development of carcinogenesis [51–54], and may provide a useful target for chemopreventative strategies. Inhibiting ODC activity also decreases the growth of tumours [55]. In the present study, we have demonstrated a decrease in ODC activity with NSAID treatment that may be a possible cause of the growth inhibition observed. ODC activity is not directly affected by the NSAIDs (results not shown). This suggests that modulation of ODC activity must be dependent upon the interaction of NSAIDs with some regulatory process of polyamine synthesis. One possibility is that the NSAIDs may be regulating antizyme, the sequestering protein that targets ODC for degradation by the 26 S proteasome [56].

This study suggests that the cytotoxicity induced by the NSAIDs is, at least in part, acting through a polyamine-dependent pathway. There seems to be a distinction *in vitro* between growth-inhibitory and cytotoxic effects of the NSAIDs, and this study implicates the polyamines as being essential for the completion of the apoptotic process. The involvement of the polyamines as regulators of cell death *in vitro* suggests that a more direct and

efficient chemopreventative strategy may be the use of agents directly affecting polyamine metabolism.

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