

Identification of aspirin analogues that repress NF- κ B signalling and demonstrate anti-proliferative activity towards colorectal cancer *in vitro* and *in vivo*

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Abstract. Substantial evidence indicates that aspirin and related non-steroidal anti-inflammatory drugs (NSAIDs) have potential as chemopreventative/therapeutic agents. However, these agents cannot be universally recommended for prevention purposes due to their potential side-effect profiles. Here, we compared the growth inhibitory and mechanistic activity of aspirin to two novel analogues, diaspirin (DiA) and fumaryl diaspirin (F-DiA). We found that the aspirin analogues inhibited cell proliferation and induced apoptosis of colorectal cancer cells at significantly lower doses than aspirin. Similar to aspirin, we found that an early response to the analogues was a reduction in levels of cyclin D1 and stimulation of the NF- κ B pathway. This stimulation was associated with a significant reduction in basal levels of NF- κ B transcriptional activity, in keeping with previous data for aspirin. However, in contrast to aspirin, DiA and F-DiA activity was not associated with nucleolar accumulation of RelA. For all assays, F-DiA had a more rapid and significant effect than DiA, identifying this agent as particularly active against colorectal cancer. Using a syngeneic colorectal tumour model in mice, we found that, while both agents significantly inhibited tumour growth

in vivo, this effect was particularly pronounced for F-DiA. These data identify two compounds that are active against colorectal cancer *in vitro* and *in vivo*. They also identify a potential mechanism of action of these agents and shed light on the chemical structures that may be important for the anti-tumour effects of aspirin.

Introduction

In Europe, colorectal cancer (CRC) is responsible for 13.6% of all cancers (1). Despite continuing research, the five-year age-standardised survival rate in the UK remains approximately 46% (CRUK). Present treatment imparts considerable morbidity. Therefore, a number of strategies are being pursued to reduce this disease burden. Of these strategies, early detection (including enhanced population screening and prophylactic surveillance in high risk groups) and prevention are considered the most promising.

Abundant evidence indicates that non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, are anti-tumorigenic and can prevent CRC (2-6 and refs. therein). For instance, in chemical and genetic animal models, NSAIDs reduce the initiation and progression of CRC (or aberrant crypt foci) (7-11). Recent meta-analysis of a large number of patient studies indicates that treatment with daily aspirin for 5 years or longer reduces the risk of developing multiple cancer types, including CRC (12). Furthermore, in a randomised controlled trial of carriers with Lynch syndrome (CAPP2 study), ingestion of a 600 mg daily dose of aspirin for ~2 years was capable of substantially reducing CRC incidence (13). Aspirin has also been proposed to suppress metastasis (14), and regular aspirin use after diagnosis of non-metastatic CRC is associated with a reduced CRC-specific mortality, an association that was strongest when the primary tumour overexpressed cyclooxygenase-2 (COX-2) (15). Despite these promising data, the use of aspirin for chemopreventative purposes universally is restricted by the potential gastropathy associated with long-term use (16). Thus, there is an overwhelming rationale to identify aspirin-related

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Abbreviations: ASA, acetylsalicylic acid; ASP, aspirin; CRC, colorectal cancer; DiA, diaspirin [bis(2-carboxyphenyl) succinate]; COX, cyclooxygenase; F-DiA, fumaryl diaspirin; mpt, melting point; MTT reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSAIDs, non-steroidal anti-inflammatory drugs

Key words: diaspirin, fumaryl diaspirin, anti-proliferative activity, implantable colorectal cancer model, cyclin D1, tumour cell lines, NF- κ B

compounds that retain the specific toxicity to CRC but are safer and more effective than aspirin itself.

A number of derivatives of the salicylate/aspirin molecule have been tested for toxicity to cancer cells. For example, 5-aminosalicylic acid (mesalazine) improves replication fidelity in HCT-116 CRC cells (17,18). Moreover, NCX-4040 (an NO releasing aspirin, 2-(acetyloxy)benzoic acid 4-[(nitrooxy)methyl]phenyl ester) can also suppress microsatellite instability (19) and sensitise human colon cancer cells, cultured *in vitro* and transplanted *in vivo* in mice, to oxaliplatin (20). Another nitro-derivative of aspirin, NCX-4016 (2-(acetyloxy)benzoic acid 3-[(nitrooxy)methyl]phenyl ester), inhibits epidermal growth factor signalling in human ovarian carcinoma and inhibits tumour growth in ovarian cancer xenografts (21). MDC-43, a para-positional isomer of phosphoaspirin (4-[(diethoxyphosphoryloxy)methyl]phenyl 2-acetoxybenzoate) reportedly inhibited the growth of cells derived from colon, lung, liver, pancreas and breast cancer (22).

The mechanism(s) by which NSAIDs protect against CRC are a matter of debate. As NSAIDs inhibit cyclooxygenase activity and prostaglandins have been implicated in neoplastic growth (23), a significant focus of research has understandably been into the development of agents that can reduce specific COX activity (24,25). However, there is a growing consensus that NSAIDs can exert their anti-proliferative activities via COX-independent effects (26,27). Other mechanisms that have been invoked for the anti-proliferative effects of these agents include: inhibition of the transcription factor NF- κ B (28); activation of the p38 MAP kinase pathway with subsequent cyclin D1 degradation (29); downregulation of Bcl-2 expression (30); activation of p53 and p21 dependent on the ATM checkpoint kinase (31); upregulation of 15-lipoxygenase-1 (32); the induction of expression of pro-apoptotic DNA repair proteins (33); inhibition of epidermal growth factor activation (34,35) and a prophylactic action wherein selection for microsatellite stability occurs (36). Studying variants of aspirin and related compounds may not only reveal more active/specific alternatives, but may also allow insight into the chemical components of the agents that are associated with these differing mechanistic activities.

We recently generated a panel of variants of salicylate in order to identify molecules that show the specific effects of aspirin against CRC, but with increased anti-proliferative activity. Of the compounds tested, 'diaspirins' [e.g. bis(2-carboxyphenyl) succinate] were noted to be significantly more toxic to CRC cells than aspirin yet appeared to retain some specificity to CRC cells (37).

Here, we extended this investigation. We generated novel compounds that are based on the 'diaspirins' and demonstrated that these compounds had potent antitumour activity against CRC cells *in vitro* and *in vivo*, in a well characterised implantable mouse model of CRC. Investigation of the mechanism of action of these agents revealed that both agents stimulated the NF- κ B pathway and repressed NF- κ B-driven transcription. However, it also suggested that the mechanism underlying this repression differs between aspirin and the diaspirin compounds. These data identify novel anticancer compounds and shed light on the structure-activity relationships important for the pro-apoptotic response to aspirin.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from PAA (Somerset, UK) or Sigma-Aldrich (Dorset, UK). Precision Plus Protein Colour standards and nitrocellulose were from Bio-Rad. RPMI-1640 medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and propidium iodide were from Sigma-Aldrich. All other reagents were obtained from Sigma-Aldrich unless otherwise stated.

Compound synthesis. Diaspirin [bis(2-carboxyphenyl) succinate; PN508] and fumaryl diaspirin [bis(2-carboxyphenyl) fumarate; PN517] were prepared as previously described (37). Aspirin is commercially available as acetylsalicylic acid, and the synthesis of the other compounds is as described in supplementary information (provided upon request). The compounds tested are shown in Fig. 1.

Preparation of PN511 and PN512. These were prepared using the method described for PN508/PN517 (37) using sebacoyl chloride and terephthaloyl chloride, respectively. Both were recrystallised from 90/10 toluene ethanol mixture. PN511 is a white solid [mpt 135-136°C; literature mpt 139-142°C [see e.g. (45)]; IR (cm⁻¹): 2917, 2854, 1754, 1683, 1604]. PN512 is a white solid [mpt 295-300°C, IR (cm⁻¹): 1736, 1698, 1605]. The molar masses of the products were confirmed as 442 and 406 respectively by titration with standardised sodium hydroxide using the method described previously (37). Samples submitted for accurate mass spectrometry returned the following data: PN511 m/z (CI) 460.1964 (M + NH₄⁺, C₂₄H₂₆O₈NH₄ requires 460.1966); PN512 m/z (CI) 424.1025 (M + NH₄⁺, C₂₂H₁₄O₈NH₄ requires 424.1027).

Preparation of PN524 and PN525. PN524 was prepared by reaction of 3-bromobenzoyl chloride with salicylic acid in the presence of 1 mole-equivalent of pyridine. 3-Bromobenzoyl chloride is available commercially but can also be produced from 3-bromobenzoic acid by the action of excess thionyl chloride (SOCl₂). Refluxing for 2-3 h (with trapping of the evolved gases) until all of the solid material had dissolved, followed by removal of any excess thionyl chloride by distillation, gave an almost quantitative yield that can be used immediately in the subsequent step. Salicylic acid (0.1 mol, 13.81 g) was dissolved in THF (50 ml) and stirred. Pyridine (0.1 mol, 8.2 ml) was then added and the mixture was stirred magnetically while 3-bromobenzoyl chloride (~13.25 ml) was added slowly over 2-3 min. The temperature rose to 35-40°C during the addition. Pyridinium chloride was precipitated and stirring was continued for a further 12-24 h. Ice-cold water (200 ml) was then added and the mixture was stirred thoroughly. The mixture was extracted with diethyl ether (150 ml) by vigorous magnetic stirring. The ether layer was then separated and extracted with 10% Na₂CO₃ solution (150 ml) to remove the PN524 from unwanted by-products. The carbonate layer was then acidified with concentrated HCl until a precipitate was seen (pH 2.0-3.0). The precipitate was taken up in diethyl ether, separated and dried with anhydrous MgSO₄ before rotary evaporation of the ether. A clean white solid (17.0 g,

53%) was obtained. Recrystallisation was achieved from boiling hexane with some acetone added, by cooling to -15°C for 24-36 h. White crystals were obtained [mpt $140-142^{\circ}\text{C}$; IR (cm^{-1}): 3068, 1738, 1682, 1605]. The molar mass of the product was confirmed as 321 by titration with standardised sodium hydroxide using the method described previously (37).

PN525 was prepared in an analogous way using 4-methylbenzoyl chloride in place of 3-bromobenzoyl chloride. The product is a white solid and was recrystallized from toluene [mpt $148-150^{\circ}\text{C}$, IR (cm^{-1}): 1734, 1674, 1605]. The molar mass of the product was confirmed as 256 by titration with standardised sodium hydroxide using the method described previously (37).

Preparation of PN528. Methyl salicylate (13.0 ml) was placed in a 100-ml conical flask. Pyridine (8.1 ml) was then added and the mixture was stirred magnetically while benzoyl chloride (11.6 ml) was added slowly over 2-3 min. The temperature rose to $48-50^{\circ}\text{C}$ during the addition. Crystals of pyridinium chloride precipitated and stirring was continued for a further 2 h. Hexane (50 ml) was then added and the mixture was stirred thoroughly. The resulting heavy white precipitate (~ 35 g) was filtered and allowed to air dry before being shaken vigorously with water (100 ml) to dissolve the pyridinium chloride by-product. The remaining solid was filtered under vacuum and dried at 60°C . The yield was 15-16 g ($\sim 65\%$). This crude product had a mpt of $82-85^{\circ}\text{C}$. The product was recrystallised by dissolving in the minimum volume of hot acetone and then adding hot hexane until the solution just remained clear. The solution was then cooled to -15°C for a minimum of 24 h. A white solid [12.0 g, mpt $84-86^{\circ}\text{C}$; IR (cm^{-1}): 1737, 1715, 1605] was obtained. The literature mpt is $83.8-84^{\circ}\text{C}$ (51).

Preparation of PN529. Isopropyl salicylate was reacted with a slight excess of 3-bromobenzoyl chloride in the presence of pyridine with no additional solvent. Isopropyl salicylate is available commercially but we also prepared small quantities from 2-propanol and salicylic acid by refluxing them with a catalytic quantity of concentrated H_2SO_4 . A ratio of 25 g salicylic acid to 100 ml 2-propanol was used with 1-2 ml of concentrated H_2SO_4 . After a minimum of 12 h refluxing, the excess 2-propanol was removed by rotary evaporation. The residue of oily solid was shaken with 10% NaHCO_3 , with more solid bicarbonate added until all the unreacted salicylic acid had dissolved. This mixture was then extracted well with diethyl ether before separating the ether layer, drying over anhydrous MgSO_4 and removing the ether by rotary evaporation. A low yield (3-5 g; 9-15%) of almost pure isopropyl salicylate (tlc) was obtained and used without further purification. 3-Bromobenzoyl chloride was obtained as described for the preparation of PN524 above. Isopropyl salicylate (1.90 g) was mixed with pyridine (1.6 ml) and was then added carefully to 3-bromobenzoyl chloride (2.38 g). An immediate reaction produced a near-solid product. The reaction vessel was protected with a silica gel guard tube and left at room temperature for 2-3 days to complete the reaction. Water (20 ml) was added to the tube which was stoppered and shaken vigorously. A sticky oil was released which was taken up in diethyl ether, washed with 10% NaHCO_3 (20 ml) and then water (20 ml). The ether layer was dried over anhydrous MgSO_4 and rotary

evaporated to give a clear, pale yellow oil (3.4 g; 87%). A solid product was obtained by dissolving the oil in a little warm diethyl ether and then diluting with boiling hexane or light petroleum ether until a cloudy solution was observed. Storing this mixture at -15°C for 2-3 days gave white crystals [mpt $49-51^{\circ}\text{C}$, IR (cm^{-1}): 2979, 1735, 1703, 1606].

Cell culture. The human colorectal adenocarcinoma cell line SW480 (supplied by ECACC) was cultured in Leibovitz's L-15 medium containing 10% (v/v) FBS supplemented with 1% L-glutamine-penicillin-streptomycin (Sigma-Aldrich). The human colorectal adenocarcinoma cell lines LoVo and HCT-116, the human breast carcinoma cell line MCF-7 and the human endothelial cell line EA.hy926 were all kindly provided by Dr Wang (University of Wolverhampton) and maintained in DMEM containing 10% (v/v) FBS supplemented with 1% L-glutamine-penicillin-streptomycin (Sigma-Aldrich). The A549 human lung adenocarcinoma cell line was provided by Dr Safrany (University of Wolverhampton) and maintained in RPMI-1640 medium supplemented with 10% FBS with 1% L-glutamine-penicillin-streptomycin. In cytotoxicity experiments for IC_{50} determinations, to minimize medium-specific variation, cells were cultured and tested in DMEM containing 10% (v/v) FBS supplemented with 1% L-glutamine-penicillin-streptomycin. The murine adenocarcinoma cell line MAC13 [supplied by Professor Double, University of Bradford (38)] was maintained in RPMI-1640 medium supplemented with 10% FBS/1% pen/strep solution/1% glutamine. The SW480 cell line was maintained in L-15 medium, was cultured at 37°C and regularly passaged at $\sim 80\%$ confluency. All other cells were cultured at 37°C in a humidified incubator with 5% CO_2 and regularly passaged at $\sim 80\%$ confluency.

Evaluation of the anti-proliferative effect. Compounds were prepared as a stock (0.5 M) in DMSO, prior to addition to cells. The cytotoxic effect of aspirin and the other novel compounds was evaluated using the MTT assay (39) with modification (40). Briefly, 10^4 cells/well were cultured overnight in 96-well microtitre plates. Twenty-four hours after the initial seeding, the culture medium was discarded, and the cells were incubated with medium containing drugs at the requisite concentration and incubated for the tested time at 37°C , then replaced with medium containing $300\ \mu\text{l}$ of 0.5 mg/ml MTT per well and incubated for 3 h at 37°C , then replaced with $200\ \mu\text{l}$ of DMSO and incubated for 10 min at 37°C to develop a coloured formazan complex. Absorbance was recorded at 540 nm using a visible plate reader (LabSystems Multiskan MS). Viability is reported as the percentage of treated cells relative to the cells in control wells. Compound anti-proliferative activity, stated as IC_{50} values, was determined by interpolation or using non-linear regression analysis using GraphPad Prism Statistics Software package (ver. 6.0; San Diego, CA, USA).

For the evaluation of toxicity (*in vitro*) to the MAC13 cell line, when plated cells reached 90% confluency they were treated with either PBS, aspirin, DiA or F-DiA dissolved in PBS. After 48 h at 37°C the cells were washed in PBS twice before 0.5 ml trypsin-EDTA (10%) was added, and the cell numbers were determined using a Coulter Counter.

Preparation of drugs. For evaluation of the activity described in Figs. 3-5, the compounds were prepared as a 1 M stock solution in DMSO and sonicated to achieve solubility. DMSO concentrations never exceeded 0.5% of the medium volume. The medium containing drugs was sonicated until all visible particles were dissolved. The pH was subsequently adjusted to 7.6 with NaOH. Aspirin (Sigma, USA) was solubilized in water using 10 M NaOH and the pH was adjusted to 7.0.

Apoptosis assays. Staining for cell surface phosphatidylserine residues was carried out using an Annexin V-FITC apoptosis detection kit (Oncogene Research Products) according to the manufacturer's instructions. The percentage of apoptotic cells was measured using BD FACS Aria II (BD Biosciences, Franklin Lakes, NJ, USA). A negative control without the Annexin V-FITC antibody and DAPI were used to assess the background signal.

Cell cycle analysis. After drug treatment, SW480 cells were trypsinized and pelleted for 5 min at 1,600 rpm. The supernatant was removed. The cells were suspended in 100 μ l citrate buffer and 450 μ l of solution A [10 mg trypsin type IX-S in 500 ml stock solution (2 g trisodium citrate, 121 mg Tris-base, 1044 mg spermine tetrahydrochloride, 2 ml Nonidet NP-40 in 2 l dH₂O, pH 7.6)] was added to the samples and vortexed for 2 min. Subsequently, 375 μ l solution B (250 mg trypsin inhibitor, 50 mg RNase A in 500 ml stock solution, pH 7.6) was added to the samples, vortexed briefly and incubated for 10 min at room temperature. Next, 250 μ l solution C (208 mg propidium iodide, 500 mg spermine tetrahydrochloride in 500 ml stock solution, pH 7.6) was added to the samples and vortexed briefly and incubated on ice in the dark for 10 min. Distribution of cells in the different phases of the cell cycle was analysed using BD FACS Aria II.

Transfections and reporter assays. For the analysis of NF- κ B-driven transcription, cells were transfected with the 3' enhancer CONA (3x κ B ConA-Luc) (supplied by Professor R.T. Hay, University of Dundee) and pCMV- β -galactosidase (Promega) plasmids as described previously (41). The relative luciferase activity was calculated as units of luciferase activity per unit of β -galactosidase activity.

Western blot analysis. Following treatment, cytoplasmic and nuclear extracts were prepared as previously described (42). The protein concentration was determined using a Bradford assay (Bio-Rad). Protein extracts (20-50 μ g) were resolved using discontinuous SDS-PAGE with a 10% gel, and immunoblotting was conducted using standard procedures. Primary antibodies used were: rabbit anti-cyclin D1 (Thermo Scientific, USA; 1:1000), sheep anti-I κ B (a kind gift from R.T. Hay) and mouse anti-actin as a loading control. Proteins were detected using western blotting luminol reagent (Santa Cruz, USA). ImageJ software (NIH) was employed for densitometric analysis of the western blots.

Implantable mouse CRC model. NMRI nude mice from the Aston colony were treated with MAC13 cells (5x10⁶) sub-cut into their flanks. Once the tumour was established, it was dissected and small fragments were re-transplanted (subcuta-

neously) into more mice. Mice (~20 g in weight) were selected at random and administered PBS or DiA or F-DiA, dissolved in PBS (1 mg/kg) intravenously. Animals were randomly divided (6 animals/group). Medication was administered intravenously daily. The mice were monitored daily for weight, food and water consumption. Tumour size was recorded using callipers. Prior to the animal model experiment, as much as 5 mg/kg compound was given to several control mice and no toxicity was observed (data available upon request). The experiment ended when the control mouse tumours started to ulcerate. All animal experiments were conducted under Home Office Licence in accordance with the UK Animals (Scientific Procedures) Act 1986.

Statistical analysis. Values are expressed as means \pm SD or SEM. To evaluate the difference in means between groups in the animal study, one-way ANOVA followed by Tukey's post-hoc test was undertaken.

Results

Aspirin analogues demonstrate potent activity against CRC cells. To understand the molecular basis for the antitumour effects of aspirin and identify more effective alternatives, we previously synthesised a series of derivatives of the aspirin molecule. These studies revealed that diaspirin (DiA) and fumaryl diaspirin (F-DiA) inhibit proliferation of CRC cell lines at significantly lower concentrations than aspirin (37). To extend these studies and identify further lead molecules, we synthesised an additional series of aspirin derivatives (Fig. 1). Cytotoxicity (MTT) assays demonstrated that at 0.5 mM (pharmacologically relevant dose for aspirin), DiA (PN508) and F-DiA (PN517) and to an even greater extent, isopropyl m-bromobenzoylsalicylate (PN529) reduced the viability of SW480 CRC cells (Fig. 2A). To investigate the specificity of compound toxicity in more detail, we tested the capacity of DiA, F-DiA and PN529 to affect proliferation of a number of established cell lines *in vitro* (Fig. 2B and Table I), controlling for any variability that could arise from cell culture conditions through culturing all cells with DMEM as the basal medium. While we noted that culturing SW480 cells in their non-native medium (DMEM rather than L-15 medium) reduced the sensitivity of the cells to the compounds tested, DiA and F-DiA in this assay system arguably showed a modicum of specificity towards the other CRC cell lines tested (HCT116 and LoVo), and given our finding that PN529 can induce necrosis (37), we focused our attention on the anti-proliferative activity of DiA and F-DiA in more detail *in vitro* and *in vivo*.

The major anti-proliferative effect of aspirin is recognised to be the induction of apoptosis. However, the mechanism by which DiA and F-DiA act against CRC cells is as yet unknown. To further understand this mechanism, we used Annexin V assays to investigate the effects of aspirin derivatives on apoptotic cell death. We found that both compounds (3 mM) induced a significant increase in the percentage of SW480 CRC cells undergoing apoptosis, compared to the carrier (DMSO)-treated controls (Fig. 3). This increase in apoptosis was paralleled by a decrease in the percentage of viable cells, confirming the death-inducing capacities of the agents (data not shown). Time course studies revealed that 3 mM F-DiA

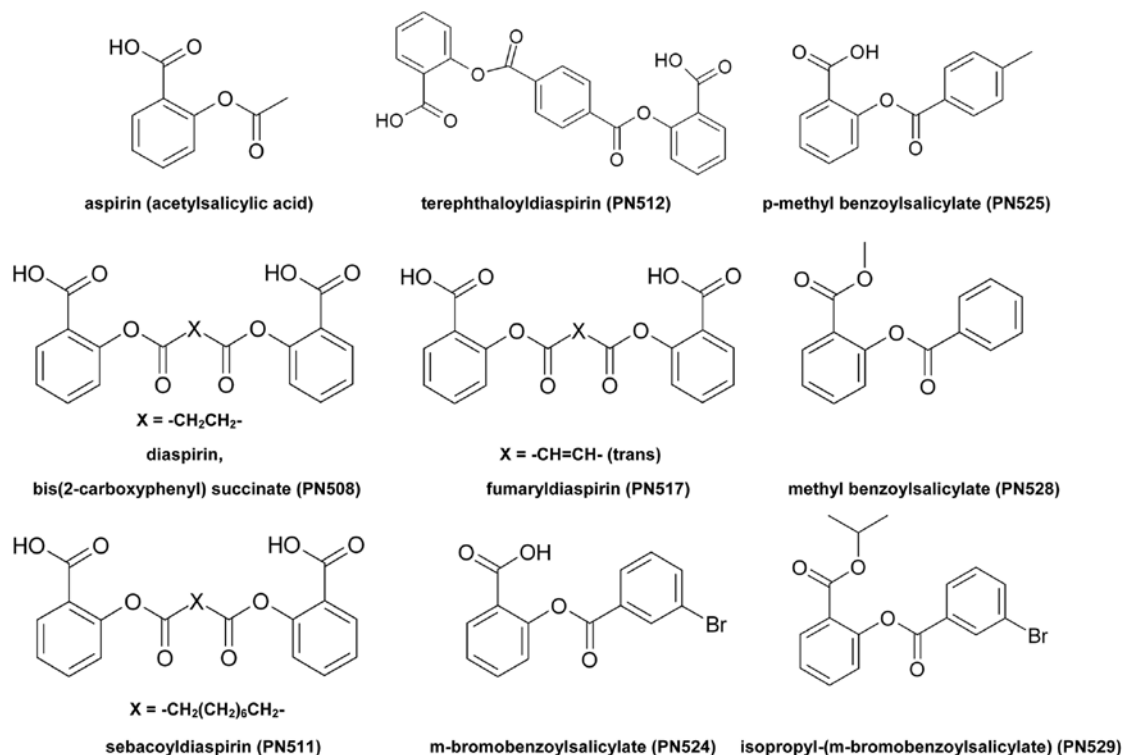


Figure 1. Structure of the compounds examined in the present study.

mediated a significant increase in apoptosis within 3 h of treatment (Fig. 3), while a more prolonged (>5 h) exposure was required before DiA-mediated apoptosis was evident (data not shown). In contrast to the diaspirin compounds, aspirin at 3 mM had minimal effect on cell growth/death after an 18-h exposure (data not shown), which is in keeping with previous studies showing that 5 mM aspirin is required to induce detectable apoptosis of these cells within this time frame (41). Taken together, these data indicate that the diaspirin compounds induce apoptosis of CRC cells and that this occurs at a lower concentration than for aspirin and that F-DiA is the more active of the compounds.

Aspirin analogues induce the degradation of cyclin D1, but do not affect cell cycle distribution. Having established that the aspirin analogues induce apoptosis, we next aimed to determine whether the underlying mechanism is similar to that of aspirin. Understanding the mechanism of action of these agents may not only identify novel apoptotic pathways, but may also reveal structural aspects of aspirin that are important for its antitumour activity. Degradation of cyclin D1 is an early response to aspirin and is critical for its apoptotic activity (29). Therefore, we firstly examined the effects of the agents on cellular levels of this protein. We found that both DiA (3 mM, 18 h) and F-DiA (3 mM, 18 h) induced a marked decrease in cyclin D1 levels (Fig. 4A). Furthermore, this decrease was time-dependent and for both agents, preceded the induction of apoptosis (Fig. 4B). However, unlike the G2/M cell cycle arrest observed in response to aspirin, DiA- and F-DiA-mediated depletion of cyclin D1 was not associated with significant changes in the cell cycle profile (Fig. 4C).

Table I. IC₅₀ values (mM) for compounds using the MTT assay to selected colorectal and non-colorectal cancer cell lines.

	Compound (mM)		
	PN508	PN517	PN529
Cell lines			
SW480	2.43±0.1	0.87±0.05	0.24±0.06
HCT116	1.08±0.2	1.47±0.1	0.15±0.01
LoVo	1.59±0.2	1.17±0.08	0.14±0.01
A549	2.3±0.1	1.47±0.08	0.13±0.001
MCF-7	2.4±0.2	1.62±0.2	0.15±0.04
EA.hy926	6.2 ^a	1.68±0.09	0.24±0.2

IC₅₀ values were obtained by interpolation and are expressed as the mean ± SEM of the data from three independent experiments. ^aObtained by extrapolation using non-linear regression.

Aspirin analogues stimulate the NF-κB pathway. NF-κB is a ubiquitously expressed transcription factor that plays a critical role in cell growth and death. It generally resides in the cytoplasm as a heterodimer of the RelA/P50 subunits, bound to the inhibitor protein, IκB. In response to a variety of agents, IκB is degraded allowing NF-κB to translocate to the nucleus and regulate expression of target genes. Thoms *et al* previously demonstrated that aspirin-induced degradation of cyclin D1 is linked to the induction of apoptosis through stimulation of this pathway (29). Moreover, cyclin D1 is transcriptionally activated by NF-κB. To determine whether DiA- and F-DiA-mediated depletion of cyclin D1 and induction of apoptosis are also

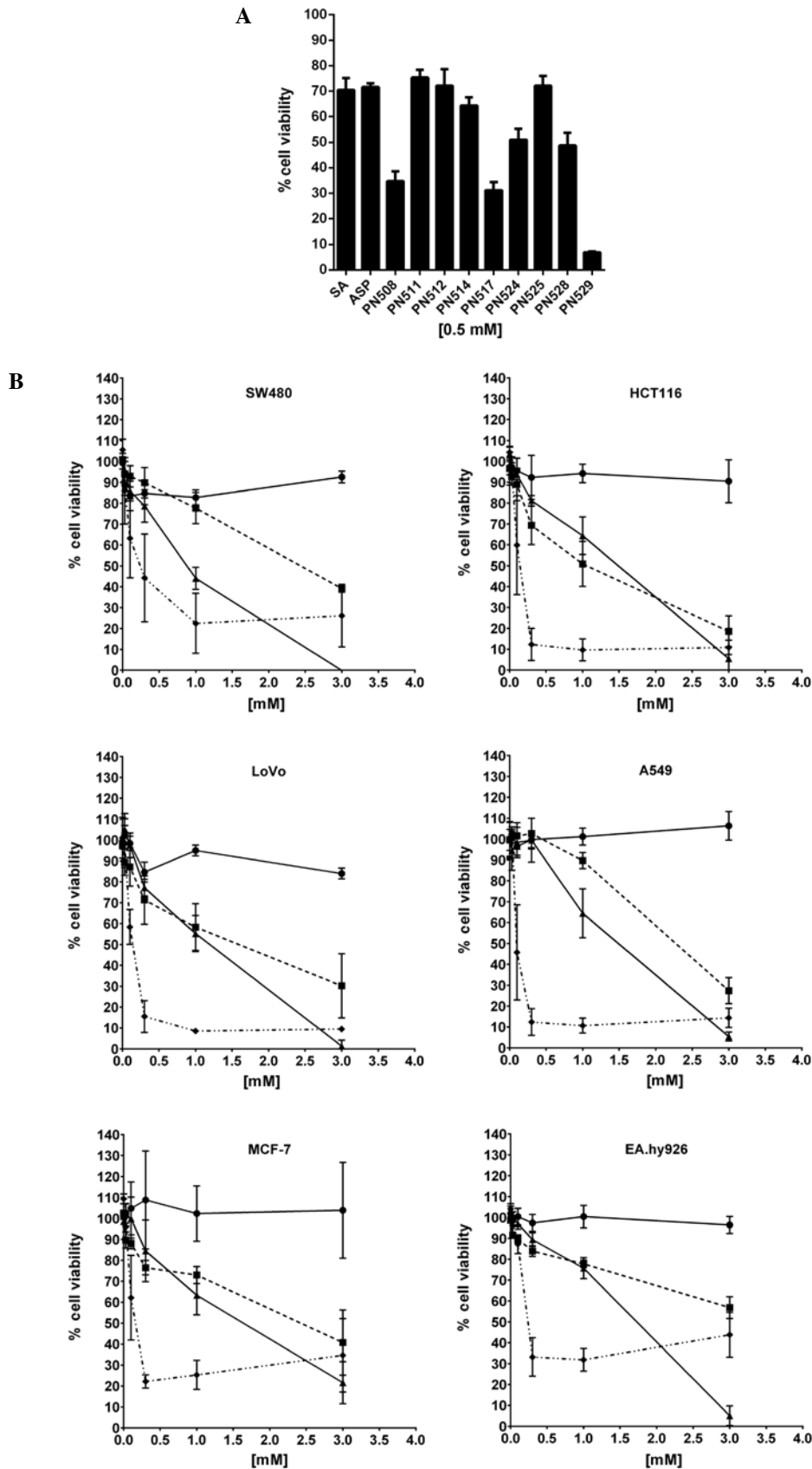


Figure 2. Cytotoxicity of aspirin analogues to selected cancer cell lines *in vitro*. (A) SW480 cells were plated at a density of 10^4 cells/ml in 96-well microtitre plates ($200 \mu\text{l}$ /well) in a L-15-based medium. Twenty-four hours after seeding, the cells were treated with compound-containing culture medium for a further 72 h. The anti-proliferative effects were measured in an MTT assay. All compounds were dissolved in DMSO. Mean \pm SEM is shown (N=3). The effect of salicylic acid (SA) and aspirin (ASP) is included for comparison. (B) Sensitivity of selected colorectal (SW480, HCT116, LoVo) and non-colorectal (A549, MCF7, EA.hy926) cancer cell lines cultured in DMEM-based culture medium, to diaspirin (PN508), fumaryl diaspirin (PN517) and isopropyl m-bromobenzoyl salicylate (PN529). Twenty-four hours after seeding, the cells were treated with compound-containing culture medium for a further 72 h. The anti-proliferative effects were measured in an MTT assay. All compounds were dissolved in DMSO. Each point represents the mean \pm SD (N=3). Circle, carrier molecule (DMSO); square, PN508 (DiA); triangle, PN517 (F-DiA); diamond, PN529.

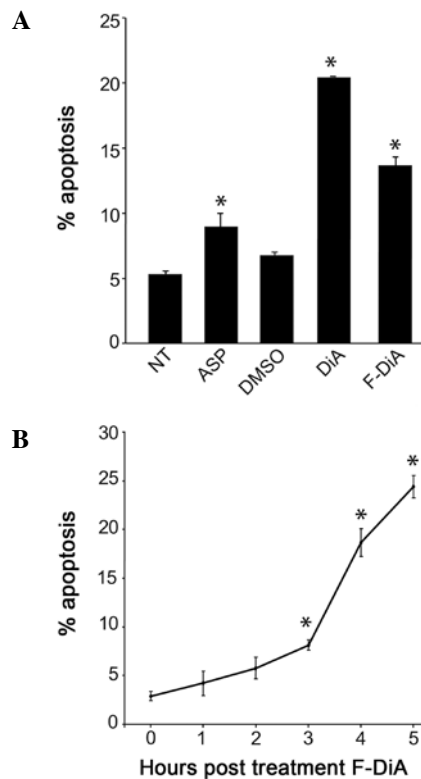


Figure 3. Aspirin analogues mediate apoptosis. SW480 cells were either (A) left untreated (NT) or treated with DMSO (final concentration as for DiA and F-DiA), 3 mM aspirin, 3 mM DiA or 3 mM F-DiA for 16 h or, (B) treated with 3 mM F-DiA for the times specified. (A and B) The percentage of cells in the population undergoing apoptosis was determined using Annexin V-FITC apoptosis assays. Data presented are the minimum of three independent experiments (\pm SEM). (A) The percentage of cells undergoing apoptosis was compared for aspirin vs. NT and DiA and F-DiA vs. DMSO. (B) The percentage of cell undergoing apoptosis at each time point was compared to time 0. * $P < 0.05$ using a Student's t-test.

associated with modulation of NF- κ B signalling, we firstly examined the effects of these agents on I κ B α . Fig. 5A demonstrates that both DiA and F-DiA induced a marked decrease in cytoplasmic levels of this protein, suggestive of proteasome-mediated degradation.

Following aspirin-mediated degradation of I κ B, RelA translocates from the cytoplasm to the nucleoplasm and then to a nuclear compartment, the nucleolus (41,43). This nucleolar compartmentalisation of RelA causes a reduction in basal levels of NF- κ B transcriptional activity, which is critical for the apoptotic effects of the agent. Immunocytochemical analysis demonstrated that DiA and F-DiA induced a substantial increase in nucleoplasmic RelA, confirming that these agents stimulated the NF- κ B pathway. However, in contrast to aspirin, the protein did not accumulate specifically in nucleoli (Fig. 5B and C). Time-course studies demonstrated that for both compounds, translocation of RelA to the nucleoplasm was subsequent to degradation of cyclin D1, but preceded the induction of apoptosis (Fig. 5C). They also demonstrated that F-DiA-mediated nucleoplasmic accumulation of RelA was more rapid and significant than that induced by DiA, in keeping with the apoptotic effects of the agents. Indeed, nucleoplasmic RelA was evident within 1 h of F-DiA treatment and cells appeared dead within 3 h.

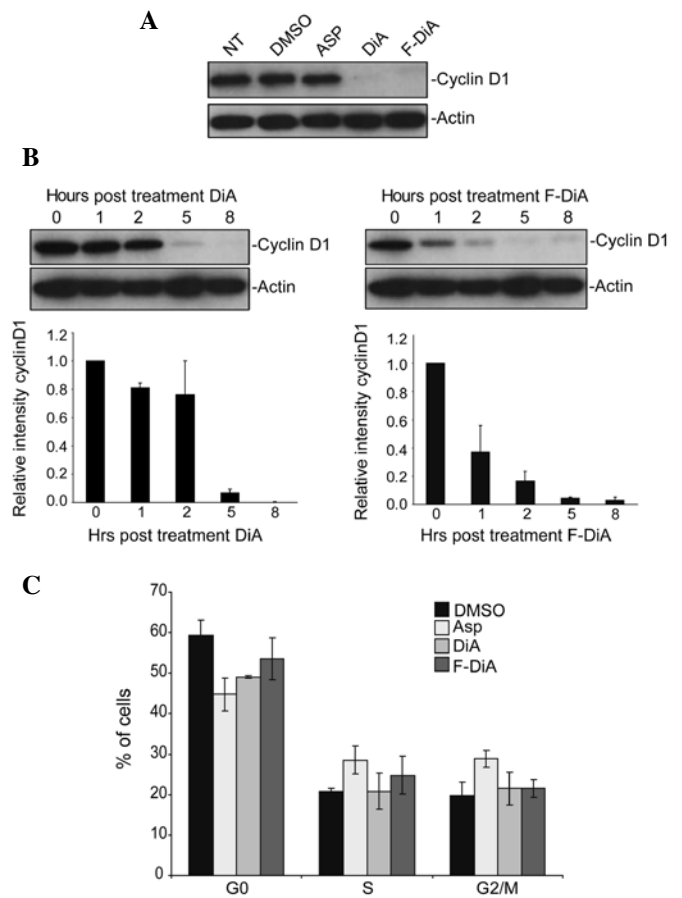


Figure 4. Aspirin analogues induce degradation of cyclin D1. SW480 cells were either (A) left untreated (NT) or treated with DMSO (final concentration as for DiA and F-DiA), 3 mM aspirin, 3 mM DiA or 3 mM F-DiA for 16 h, or (B) treated with 3 mM DiA or 3 mM F-DiA for the times specified. (A and B) Western blot analysis was performed on cytoplasmic extracts with antibodies to cyclin D1. Actin was used as a loading control. (B) Band intensities were quantified and cyclin D1 levels calculated relative to actin. Data presented are the mean of three individual experiments \pm SEM. (C) SW480 cells were treated as for A, and then the proportion of cells in each phase of the cell cycle was analysed. The graph shows the mean of 3 experiments \pm the SEM. Aspirin (Asp) influenced the proportion of cells in each phase of the cell cycle after treatment. However, this was not observed for DiA or F-DiA.

In response to specific stresses, nucleoplasmic NF- κ B/RelA complexes have been shown to mediate apoptosis by repressing basal NF- κ B-driven transcription (44). To examine this as a possible mechanism for the apoptotic effects of the diaspirin compounds, we next performed NF- κ B reporter assays. We found that both DiA and F-DiA induced a significant decrease in basal levels of NF- κ B transcriptional activity. This decrease was more pronounced than for aspirin, occurred in a time-dependent manner and was subsequent to nucleoplasmic accumulation of RelA (Fig. 5C and D). Furthermore, similar to depletion of cyclin D1, nucleoplasmic accumulation of RelA and the induction of apoptosis, we found that F-DiA had a more rapid and profound effect on NF- κ B-driven transcription than DiA (Fig. 5E). These data suggest that, similar to aspirin, the diaspirin compounds stimulate the NF- κ B pathway and mediate apoptosis through repression of NF- κ B-driven transcription. However, they also suggest that the mechanism underlying this repression differs between aspirin and the related agents.

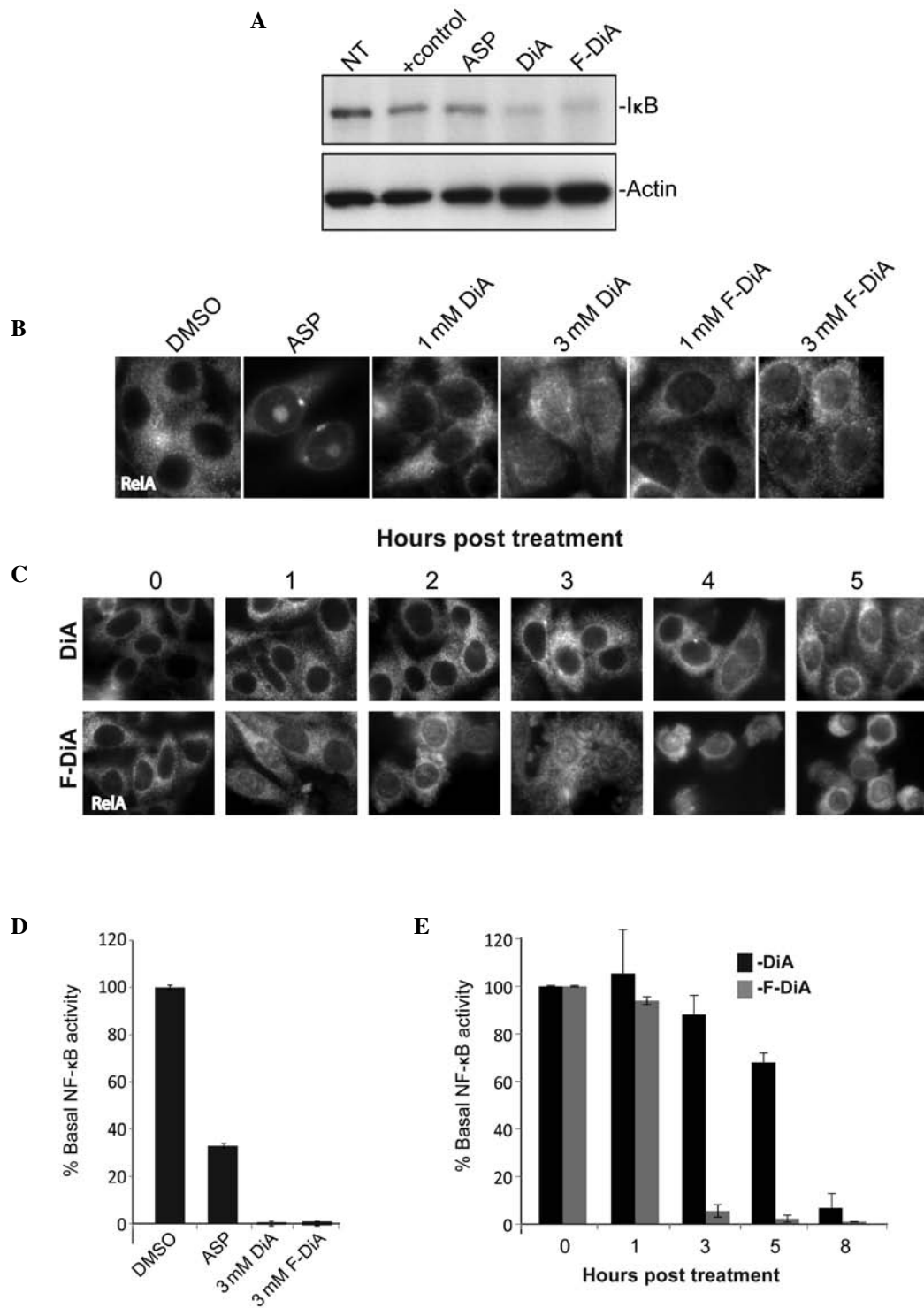


Figure 5. Aspirin analogues stimulate the NF- κ B pathway and cause repression of NF- κ B-driven transcription. (A) SW480 cells were either left untreated (NT) or treated with 3 mM aspirin, DiA or F-DiA for 16 h. TNF- α (30 min) was used as a positive control for I κ B degradation. Western blot analysis was performed on cytoplasmic extracts with antibodies to I κ B α . Actin was used as a control for protein loading. DiA and F-DiA induced degradation of I κ B. (B and C) Anti-RelA immunocytochemistry was performed on cells treated with (B) DMSO, aspirin (5 mM) or the specified concentration of DiA or F-DiA for 16 h, or (C) 3 mM DiA or F-DiA for the times specified. DiA and F-DiA induced nuclear translocation of RelA. (D and E) SW480 cells were transfected with 6 μ g of NF- κ B-dependent luciferase reporter construct (3 x κ B ConA) along with 3 μ g of pCMV β control plasmid. Twenty-four hours after transfection, cells were treated with (D) DMSO, aspirin (3 mM), DiA or F-DiA for a further 16 h, or (E) 3 mM DiA or F-DiA for the times specified. Results were normalised using β -galactosidase activity and are presented as the percentage of relative luciferase activity compared to basal levels (DMSO controls). The average of at least three independent experiments is shown \pm SEM.

Inhibition of tumour growth by diaspirins in an implantable CRC mouse model. Taken together, the above data suggest that the diaspirin compounds, particularly F-Dia, have potent activity

against CRC cells. To determine whether these effects translate into *in vivo* potency, we utilised the well characterised MAC13 syngeneic mouse model of CRC (38). In the cytotoxicity assays

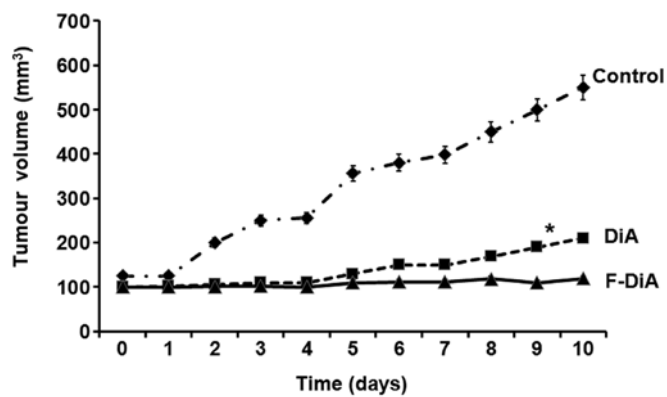


Figure 6. Effect of diaspirin and fumaryl diaspirin on an implantable colorectal mouse tumour model. MAC13 cells were implanted subcutaneously into the flank of three groups (N=6) of NMRI mice. Twelve days after implantation, mice were intravenously administered 1 mg/kg DiA or F-DiA daily, while the control group received PBS. The results demonstrate a robust inhibition of growth in the drug-treated groups, which were significantly different from the control group (* $P < 0.001$ from PBS control by one-way ANOVA followed by Tukey's post-hoc test).

MAC13 cells cultured *in vitro* were more sensitive to DiA and F-DiA than aspirin (data not shown); *in vivo*, the mouse colon carcinoma cells were implanted subcutaneously into NMRI mice and treatment commenced 12 days after implantation. Low dose (1 mg/kg) DiA or F-DiA was administered every day for 10 days by intravenous injection. No overt side-effect was observed between the treatment and vehicle-treated control group, and food and water intake was unaffected. As shown in Fig. 6, treatment with DiA and F-DiA significantly inhibited the growth of the tumours, and almost complete suppression of growth was apparent in the mice treated with F-DiA.

Discussion

Colon cancer is a major cause of cancer-related mortality worldwide, particularly in Western societies. Present therapies are associated with significant morbidity and generally provide marginal benefit. Hence, there is an overwhelming rationale for novel developments that would lead to a reduction in incidence and/or in cancer-related morbidity and mortality. Herein, we demonstrated that two analogues of aspirin had greater anti-proliferative activity against CRC cells *in vitro* than aspirin and salicylate and that these compounds exhibited a modicum of specificity for CRC cell lines *in vitro* [Fig. 2, Table I and ref. (37)]. Furthermore, we also demonstrated that DiA and F-DiA had potent antitumour activity *in vivo*, in an implantable mouse model of CRC (Fig. 6) and showed no obvious side-effects when introduced to mice intravenously over a 10-day period. Taken together, these data identify DiA and F-DiA as potential novel therapeutic agents for CRC. Although a collection of related structures termed 'diaspirins' [in particular bis(3,5-dibromosalicyl) fumarate] have long been known to cross link haemoglobin (45), as far as we are aware, this is the first report of the antitumour properties of these agents.

The phenotypic studies indicated that the diaspirin compounds were capable of inducing apoptosis (Fig. 3). On analysis of the mechanisms underlying this apoptosis,

we found that similar to aspirin, the diaspirin compounds sequentially induced depletion of cyclin D1, degradation of I κ B, nuclear translocation of NF- κ B/RelA complexes and repression of NF- κ B activity. However, in contrast to aspirin, the aspirin analogues did not induce nucleolar translocation of RelA or cell cycle arrest. Aspirin consists of acetyl and salicylate moieties, both of which have their own individual targets (46). The aspirin analogues we generated retained the salicylate part of the molecule but lost the acetyl component. Indeed, western blot analysis with antibodies against acetylated lysine indicated that aspirin induced a dose-dependent increase in protein acetylation, but this was not observed in response to DiA and F-DiA (data not shown). Therefore, one possibility is that the ability of aspirin to stimulate NF- κ B signalling is associated with the salicylate component of the compound while nucleolar translocation of RelA and cell cycle arrest are associated with its acetylating potential. In keeping with this suggestion, salicylate has previously been shown to activate p38 kinase activity (47), which we have previously shown lies upstream of aspirin effects on cyclin D1 and the NF- κ B pathway (29).

Constitutive activity of NF- κ B has been shown to contribute to carcinogenesis in a number of cancer types, including CRC (48). We previously demonstrated that aspirin-mediated nucleolar sequestration of RelA induces apoptosis, at least in part, by causing repression of this basal NF- κ B transcriptional activity (41). Here, we found that the aspirin analogues also mediated a reduction in basal levels of NF- κ B-driven transcription. Furthermore, we found that this reduction preceded the induction of apoptosis. The effects of the diaspirin compounds on NF- κ B transcriptional activity were more pronounced than for aspirin, in keeping with the enhanced apoptotic activity of these agents. Taken together, these data suggest that similar to aspirin, the analogues mediate apoptosis by repressing NF- κ B-driven transcription. We note that the NSAID ibuprofen can induce I κ B α degradation, NF- κ B nuclear localization and suppression of cyclin D1 expression (49).

As outlined above, we did not observe nucleolar sequestration of RelA in response to the diaspirin compounds, suggesting that an alternative mechanism is responsible for the effects of these agents on NF- κ B transcriptional activity. Campbell *et al* (44) previously demonstrated that stimulation of the NF- κ B pathway by specific pro-apoptotic stimuli causes nucleoplasmic accumulation of repressive RelA/NF- κ B complexes that mediate apoptosis by recruiting histone deacetylases (HDACs) to the chromatin of NF- κ B-regulated anti-apoptotic genes. We previously demonstrated that aspirin induces the ubiquitination of RelA, which targets the protein to the nucleolus (50). Therefore, it may be that in the absence of the acetylating component of aspirin, nucleoplasmic RelA cannot be ubiquitinated, allowing the recruitment of alternative repressive factors such as HDACs. Our finding that, of the two compounds, F-DiA had the most rapid effect in all of the mechanistic assays and the most significant activity against the growth of implanted tumour adds weight to our suggestion that the antitumour activity of this agent is caused by modulation of NF- κ B signalling. It also identified F-DiA as particularly significant for therapy development. From the range of analogues studied to date it would seem that increased activity is at least, in part, associated with the presence of two (or

perhaps more) suitably spaced aromatic rings and indicates the significance of two salicylate moieties in the molecule separated by ~8-10Å. We speculate that F-DiA may demonstrate greater toxicity as a consequence of its greater symmetry and reduced number of conformers.

To conclude, we identified diaspirins that have potent anti-proliferative activity against CRC cells *in vitro* and *in vivo*. The *in vitro* mechanistic studies provide compelling evidence that these agents exert their anti-proliferative effects against SW480 cells by causing repression of NF-κB-driven transcription. The mechanistic studies also helped to elucidate structure-function relationships for the aspirin molecule. In particular, these findings provide insight into the mechanisms of action of F-DiA and support further studies into the potential of F-DiA as a chemotherapeutic agent. We wish to test these agents in models of cachexia and in genetically defined mouse models [e.g. Apc(Min/+) and Apc(Min/+), Msh2(-/-)] of CRC. We acknowledge, however, that formal dose ranging and toxicity studies with, for example, clinical scoring of gastrointestinal toxicity does need to be undertaken.

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