Induction of spermidine/spermine *N*¹-acetyltransferase (SSAT) by aspirin in Caco-2 colon cancer cells

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Epidemiological, experimental and clinical results suggest that aspirin and other NSAIDs (non-steroidal anti-inflammatory drugs) inhibit the development of colon cancer. It has been shown that the NSAID sulindac induces apoptosis and suppresses carcinogenesis, in part, by a mechanism leading to the transcriptional activation of the gene encoding SSAT (spermidine/spermine *N*¹-acetyltransferase), a rate-limiting enzyme in polyamine catabolism. In the present study, we show that a variety of NSAIDs, including aspirin, sulindac, ibuprofen and indomethacin, can induce SSAT gene expression in Caco-2 cells. Aspirin, at physiological concentrations, can induce SSAT mRNA via transcriptional initiation mechanisms. This induction leads to increased SSAT protein levels and enzyme activity. Promoter deletion analysis of the 5' SSAT promoter-flanking region led to the identification of two NF- κ B (nuclear factor κ B) response elements.

Electrophoretic mobility-shift assays showed binding of NF- κ B complexes at these sequences after aspirin treatment. Aspirin treatment led to the activation of NF- κ B signalling and increased binding at these NF- κ B sites in the SSAT promoter, hence providing a potential mechanism for the induction of SSAT by aspirin in these cells. Aspirin-induced SSAT ultimately leads to a decrease in cellular polyamine content, which has been associated with decreased carcinogenesis. These results suggest that activation of SSAT by aspirin and different NSAIDs may be a common property of NSAIDs that plays an important role in their chemopreventive actions in colorectal cancer.

Key words: aspirin, Caco-2 colon cancer cell, nuclear factor κ B (NF- κ B), non-steroidal anti-inflammatory drugs (NSAIDs), polyamine, spermidine/spermine *N*¹-acetyltransferase.

INTRODUCTION

Many epidemiological studies have revealed that the use of aspirin or other NSAIDs (non-steroidal anti-inflammatory drugs) can reduce the risk of colon cancer. The mechanism of action that defines NSAIDs as a class is their ability to inhibit the COX (cyclooxygenase) activity of the enzyme prostaglandin G/H synthase (also known as COX) and thereby block the biosynthesis of prostaglandins [1]. NSAIDs structurally consist of an acidic moiety (carboxylic acid, enols) attached to a planar, aromatic functionality. Some analgesics also contain a polar linking group, which attaches the planar moiety to an additional lipophilic group. The acidic group in these compounds serves as a major binding group (ionic binding) for plasma proteins. Thus all NSAIDs are highly bound by plasma proteins. The NSAIDs can be subclassified on the basis of chemical structure into salicylates (aspirin), propionic acid derivatives (ibuprofen), aryl and heteroarylacetic acids (sulindac and indomethacin), oxicams (piroxicam), anilides (acetaminophen) and COX-2 selective inhibitors (celecoxib). Since the well-documented pharmacological action of NSAIDs is the inhibition of COX enzymes, it could be inferred that the beneficial effect of NSAIDs may be mediated through the inhibition of prostaglandin biosynthesis. However, NSAIDs such as sodium salicylate, aspirin, sulindac, ibuprofen and indomethacin cause anti-inflammatory and anti-proliferative effects independent of COX activity and prostaglandin inhibition [2–7].

We have shown that aspirin use decreased the risk of adenoma recurrence by activating catabolism of colonic mucosal polyamines [7]. The polyamines putrescine, spermidine and spermine are abundant polycations in eukaryotic cells, which are often elevated in neoplastic cells when compared with normal cells and tissues [8]. The polyamine levels are tightly regulated by the biosynthetic enzyme, ODC (ornithine decarboxylase), and the catabolic enzyme, SSAT (spermidine/spermine *N*¹-acetyltransferase), in cells. High levels of polyamines are associated with cellular proliferation [9], while lower polyamine levels have been shown to promote apoptosis [10,11] and inhibit cell growth [12]. It has been shown that many other NSAIDs, besides aspirin, can regulate polyamine metabolism [7,13,14]. Indomethacin impaired the growth of human colon cancer cells, resulting in decreased ODC activity, increased intracellular SSAT enzyme activity and enhanced polyamine acetylation and efflux from colon cancer cells [13]. Aspirin, on the other hand, had no effects on ODC but induced SSAT expression and decreased polyamine levels at physiological concentrations in HT-29 colon cancer cells [7]. Sulindac sulphone can lead to the activation of PPAR γ (peroxisome-proliferator-activated receptor γ) in colon cancer cells, which can then induce SSAT expression, thereby leading to decreased intracellular polyamines and increased cell death [14]. Further, it was shown that the toxicity induced by sulindac, salicylate and naproxen can be prevented by polyamines, suggesting an integral role

Abbreviations used: AP-1, activator protein-1; COX, cyclo-oxygenase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEM, minimum essential α -medium; NF- κ B, nuclear factor κ B; NSAIDs, non-steroidal anti-inflammatory drugs; ODC, ornithine decarboxylase; PGE₂, prostaglandin E₂; PPAR, peroxisome-proliferator-activated receptor; PPRE, PPAR response element; PRE, polyamine response element; qPCR, quantitative PCR; RT, reverse transcriptase; SSAT, spermidine/spermine *N*¹-acetyltransferase; TESS, transcription element search system; TNF α , tumour necrosis factor α .

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of polyamines in the chemopreventive action of the NSAIDs [15].

Based on the evidence above, our interest was in examining the mechanism of aspirin- and other NSAID-induced SSAT expression in Caco-2 colon cancer cells.

EXPERIMENTAL

Materials

All cell culture reagents, DNA modifying enzymes, TRIzol[®] reagent and Lipofectamine[™] reagent were purchased from Life Technologies (Rockville, MD, U.S.A.). All primers and oligonucleotides were custom made by Invitrogen (Carlsbad, CA, U.S.A.). Indomethacin and (*S*)-ibuprofen were purchased from Biomol Research Laboratories (Plymouth, PA, U.S.A.), while sulindac and sulindac sulphone were purchased from ICN Biomedicals (Solon, OH, U.S.A.). Acetylsalicylic acid (aspirin) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Cell culture

The Caco-2 colon cancer cell line was purchased from A.T.C.C. (Rockville, MD, U.S.A.) at passage 12 and was maintained in MEM (minimum essential α -medium; Life Technologies) supplemented with 10% (v/v) FBS (fetal bovine serum) and 50 units/ml each of penicillin and streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂.

Plasmids

Full-SSAT-luc was made by cloning 3.493 kb of SSAT sequence 5' of the transcription start site into a promoterless pGL2-basic vector (Promega, Madison, WI, U.S.A.) as reported previously [16]. A series of smaller SSAT promoter elements was made from Full-SSAT-luc, using PCR. 197-SSAT-luc having SSAT sequences from +83 to -197, 358-SSAT-luc having SSAT sequences from +83 to -358, 659-SSAT-luc having SSAT sequences from +83 to -659 and 791-SSAT-luc having SSAT sequences from +83 to -791 were made from Full-SSAT-luc and subcloned into pGL2-basic vector (Figure 1B). PPRE₃-tk-luc reporter construct, having three tandem repeats of PPREs (PPAR response elements) 5' to the luciferase gene, was a gift from Dr Ronald Evans (The Salk Institute, La Jolla, CA, U.S.A.). NF- κ B₂-luc reporter, having two NF- κ B (nuclear factor κ B) response elements, and Δ NF- κ B₂-luc reporter, in which both the NF- κ B response elements have been deleted, were obtained from Dr Nancy Davidson (Johns Hopkins University, Baltimore, MD, U.S.A.).

Transient transfections

Transient transfections were performed using Lipofectamine[™] reagent according to the manufacturer's instructions. Briefly, 5×10^5 cells were seeded in a 6-well plate and cultured in normal medium [MEM with 10% FBS and 1% (v/v) penicillin/streptomycin] for 24 h. Each well was transfected with 1 μ g of firefly luciferase reporter construct along with 0.2 μ g of pCMV- β -galactosidase expression plasmid, used as a transfection efficiency control. After a 6 h incubation with Lipofectamine[™]-DNA complex, cells were supplemented with complete medium containing 20% FBS and 2% penicillin/streptomycin and grown overnight. On the following day, the transfection media were removed and cells were given fresh media with the indicated concentrations of aspirin or vehicle and treated for 48 h. All transfections were performed in triplicate. Transfected cells were washed once with PBS and lysed, and luciferase activities were measured using 10 μ l of cell extract and 50 μ l

Figure 1 Various SSAT promoter-reporter constructs

(A) Putative transcription factor binding sites in the SSAT 5' promoter flanking region as determined by TESS analysis. NF- κ B, PPRE and PRE are labelled. (B) Schematic representation of the various SSAT promoter-reporter constructs cloned into the pGL2-basic luciferase reporter plasmid, which were used in the present study, with three putative NF- κ B sites (*w*, *w*₂ and *w*₃) and the +1 site marked.

of luciferase reagent (Promega). β -Galactosidase activity was measured using the β -gal assay kit (Invitrogen) according to the manufacturer's instructions.

RNA isolation and analysis

Total cellular RNA was extracted using TRIzol[®] reagent and RNA isolation according to the method developed by Chomczynski and Sacchi [17]. Total RNA (20 μ g) was separated on 1% agarose/formaldehyde gel and transferred to a nylon membrane (Hybond-N; Amersham Biosciences). The cDNA probes for Northern blots were prepared using [α -³²P]dCTP and the RTS RadPrime DNA labelling system (Life Technologies). The probe was purified with G-50 Sepharose columns (Boehringer Mannheim, Roche, Indianapolis, IN, U.S.A.) and quantified using a scintillation counter. Membranes were hybridized overnight at 42 °C with ³²P-labelled cDNA encoding human SSAT (EcoRI-EcoRI fragment), and then washed twice in 2 $\times$ SSC/0.1% SDS at room temperature (25 °C), for 5 and 20 min respectively, and once in 0.5 $\times$ SSC/0.1% SDS for 20 min at 65 °C (1 $\times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7). GAPDH (glyceraldehyde-3-phosphate dehydrogenase; 0.75 kb PstI-XbaI fragment) was used as a control for RNA loading. Northern blot autoradiograms were quantified by densitometric analysis (ImageQuant; Molecular Dynamics, Sunnyvale, CA, U.S.A.). Results are expressed as the ratio of the integrated densities of ³²P-labelled hybridization bands for the SSAT and GAPDH mRNAs.

Gel electrophoretic mobility-shift assays

Nuclear extracts were prepared from Caco-2 cells as described previously [18]. To study the binding of NF- κ B complexes to the putative NF- κ B sites, double-stranded oligonucleotides for each of the three putative NF- κ B sites in the SSAT 5' sequence were ³²P-labelled with polynucleotide kinase (Promega). The oligonucleotide containing the first putative NF- κ B site [NF- κ B-wild-1 (*w*)] spanned from -304 to -280 of the SSAT 5' sequence and had the sequence 5'-GCTGCAGAGGGAATTACCTTCTT-3', whereas the corresponding mutant NF- κ B-mut-1 (*m*) had

the sequence 5'-GCTGCAGAtaGAATgcCCTTCTTT-3'. The oligonucleotide spanning the second putative NF- κ B site [NF- κ B-wild-2 (w_2)] spanned from -608 to -585 of the SSAT 5' sequence and had the sequence 5'-TACCTTAGGAAATTCTAAA-AGAA-3'. The oligonucleotide spanning the third putative NF- κ B site [NF- κ B-wild-3 (w_3)] spanned from -1751 to -1728 of the SSAT 5' sequence and had the sequence 5'-CC-TAGGGGATTCCACGGATCCT-3', whereas the corresponding mutant NF- κ B-mut-3 (m_3) had the sequence 5'-CCTAGG-ttGATTcagCGGATCCT-3'. The putative NF- κ B sequence is underlined and the mutated bases are shown in lower-case letters. A 10 μ l reaction containing 10 μ g of nuclear extract was incubated for 15 min at room temperature in a buffer containing 50 mM Tris/HCl (pH 7.5), 5 mM MgCl₂, 2.5 mM EDTA, 250 mM NaCl, 2.5 mM dithiothreitol, 20% glycerol and 1 μ g of poly(dI-dC) · (dI-dC). Following this, 30 000 c.p.m. of the labelled probe was added and the reaction mixture was incubated for 30 min at room temperature. The DNA-protein complexes were resolved from the free probe by electrophoresis at 4°C on a 5% (w/v) polyacrylamide gel in 0.75 × TBE buffer (10 mM Tris base, 18 mM boric acid and 0.4 mM EDTA, pH 8.0). Band density was quantified using the Typhoon 8600 PhosphorImager and the ImageQuant software (Molecular Dynamics).

qPCR (quantitative PCR)

Total RNA (3 μ g) was treated with RNase-free DNase 1 (Roche) for 30 min at 37°C before reverse transcription with Moloney-murine-leukaemia virus RT (reverse transcriptase; Promega) as described by the manufacturer. The forward and reverse primers were used at a concentration of 10 μ M. The resulting cDNA was subjected to qPCR. The DNA-intercalating SyBr Green reagent (Quantitect, Qiagen) was used for detection of the RT-PCR product. Agarose-gel electrophoresis indicated the presence of a single PCR product. To account for differences in starting material, the expression of GAPDH was determined. The experimental and GAPDH PCRs were done in separate tubes in triplicate in the MyiQ single colour real-time PCR machine (Bio-Rad, Hercules, CA, U.S.A.), and the average threshold cycle (C_T) for the triplicate was used in subsequent calculations. SSAT cDNA (180 bp) was amplified using the following primers (Invitrogen): forward, 5'-GGATCAGAAATTCTGAAGAAT-3' and reverse, 5'-ACCCTCTTCACTGGACAGATC-3'. As a loading control, GAPDH cDNA (188 bp) was also amplified using the following primers: forward, 5'-GAAGGTGAAGGTCTGGAGTC-3' and reverse, 5'-GAAGATGGTGATGGGATTTC-3'.

Immunoblotting

Caco-2 cells were seeded at 1×10^6 cells per 100 mm culture plate, grown overnight and then treated with various concentrations of aspirin or its vehicle for 48 h. Cell extracts were obtained by lysing cells on ice in RIPA buffer (PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 30 μ g/ml aprotinin, 100 μ M sodium orthovanadate and 10 μ g/ml PMSF) and centrifuging for 20 min at 4°C. Then, 50 μ g of protein was loaded per lane and run on SDS/10% PAGE gels for SSAT immunoblotting. The proteins were transferred electrophoretically to Hybond-C (Amersham, Biosciences) nitrocellulose membrane for 1 h. Blots were blocked in Blotto A [5% (w/v) non-fat dry milk in TTBS (Tris/Tween 20-buffered saline)] and immunoblotting for SSAT was carried out using a rabbit antiserum as described previously [19]. Blots were stripped and redetected with anti- β -actin (Sigma) antibody as a loading control. All Western blots were repeated three times and a representative blot was chosen for presentation.

SSAT enzyme activity determination

For enzyme activities, 1×10^6 cells were grown overnight and then treated with various concentrations of aspirin or its vehicle. Cells were harvested after 48 h of treatment and washed in cold PBS. The radiochemical assay of the SSAT activity was performed by estimation of labelled AcSpd (N^1 -acetylspermidine) synthesized from [¹⁴C]acetyl-CoA and unlabelled spermidine, as described elsewhere [20]. Fold change was calculated by dividing the enzyme activity for the sample by that for the vehicle. The enzyme assays were performed in triplicate.

Polyamine analysis

Cell extracts were prepared in 0.1 M HCl (4×10^7 cells/900 μ l). After sonication, the preparation was adjusted to 0.2 M HClO₄, and the supernatant was analysed by reverse-phase HPLC with 1,7-diaminoheptane as an internal standard [21]. Protein was determined by BCA (bicinchoninic acid) assay [22].

Statistical analysis

Northern and Western blot analyses were performed at least three times and a representative blot is shown. All transient transfection experiments were performed in triplicate and were repeated at least three times. Representative experiments or means $\pm$ S.D. are shown. Statistical differences were determined by Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Regulation of SSAT expression by various NSAIDs in colon cancer cells

Regulation of SSAT was studied with an array of NSAIDs in the Caco-2 colon cancer cells. NSAIDs used in the study were aspirin (acetylsalicylic acid), ibuprofen, indomethacin and sulindac, all of which have been shown to have some chemopreventive effects on colon cancer [6,13,23,24]. All the NSAIDs were used at concentrations that reduced intracellular PGE₂ (prostaglandin E₂) levels and have been shown previously to exert chemopreventive actions in various colon cancer cells [23,25,26]. Expression of SSAT mRNA was determined after 48 h of treatment, except in the case of 600 μ M indomethacin where treatment was for 72 h in the Caco-2 cells. This was used because indomethacin has been shown to induce SSAT mRNA in these cells after 72 h of treatment [13]. As shown in Figure 2, most of the NSAIDs were able to induce SSAT mRNA in the Caco-2 cells. Aspirin was able to induce SSAT mRNA at a lower dose of 500 μ M but not at 1 mM. In order to determine whether NSAIDs are working at the level of transcription, transient transfections with the Full-SSAT-luc reporter-promoter construct were performed in the Caco-2 cells. The transfected cells were treated with the same array of NSAIDs at the same concentrations. The NSAID, which induced SSAT mRNA also induced the SSAT full-length promoter construct in the Caco-2 cells (results not shown).

Aspirin induces SSAT expression and decreases polyamines in colon cancer cells

Most of the *in vitro* research with aspirin in colon cancer has been with concentrations ranging from 1 to 10 mM, which are relatively high as compared with the *in vivo* concentrations achievable in humans. Initial experiments on SSAT expression were with doses that cannot be achieved *in vivo*. Therefore, to determine whether physiologically relevant doses of aspirin have any effects on the SSAT expression in colon cancer cells, lower concentrations were chosen. Transient transfections using the Full-SSAT-luc

Figure 2 Various NSAIDs induce SSAT RNA expression in Caco-2 cells

Caco-2 cells were treated with different concentrations of NSAIDs or their vehicle (V; open bar) for 48 h, harvested, and total RNA was extracted and used for Northern blotting. SSAT expression was normalized to the GAPDH expression in these treatments. Fold induction was calculated by dividing normalized values of the sample with the control value. The result shown is an average from three different experiments. NSAIDs used were Sul (sulindac), So (sulindac sulphone), Indo (indomethacin), acetylsalicylic acid (ASA) and Ibu (ibuprofen). * $P < 0.05$ compared with the vehicle-treated control.

reporter–promoter construct showed that aspirin at 20 and 100 μ M led to a significant induction in the SSAT promoter activity in Caco-2 cells (Figure 3A). The same doses of aspirin were able to induce SSAT mRNA in the Caco-2 cells after 48 h incubation (Figure 3B), as measured by quantitative RT–PCR.

Next, it was important to determine whether this induction of SSAT promoter activity and SSAT mRNA had any effect on SSAT protein levels and resulting enzyme activity. Since SSAT is a rate-limiting enzyme in polyamine catabolism, polyamine analysis was done to measure any effects on cellular polyamine levels in aspirin-treated cells. As shown in Figure 3(C), 100 μ M aspirin, but not 1 mM aspirin, increased SSAT protein levels. Aspirin at 20 μ M led to approx. 3.6 ± 0.9 -fold induction in the SSAT enzyme activity in Caco-2 cells, while aspirin at 100 μ M only induced SSAT enzyme activity by approx. 2 ± 0.5 -fold (Figure 3D). Based on the effects of aspirin on SSAT expression, we hypothesized that aspirin treatment would lead to a decrease in the intracellular polyamine levels in these cells. We found that in Caco-2 cells, aspirin at 20 μ M reduced all the intracellular polyamine levels but aspirin at 100 μ M had no significant effect on the intracellular polyamine levels (Table 1). Intracellular polyamine contents can also be regulated by the expression of the biosynthetic enzyme, ODC. Hence, the reduction in intracellular polyamine content observed at 20 μ M but not at 100 μ M could be due to an inhibition of ODC in these cells. Aspirin treatment had no effect on ODC expression at 20 μ M, but led to a slight increase in ODC mRNA expression at 100 μ M (results not shown).

Mapping of aspirin-responsive elements in the SSAT promoter

Based on the result that SSAT transcription is being modulated by aspirin in the Caco-2 cells, an attempt was made to map the aspirin responsive sequence(s) in the SSAT 5' promoter flanking sequences. In the first set of experiments, transient transfection experiments were done using two SSAT reporter–promoter constructs, Full-SSAT-luc and 197-SSAT-luc, in the Caco-2 cells. Transfected Caco-2 cells were treated with 10, 50 or 100 μ M aspirin for 48 h. Aspirin at 50 and 100 μ M was able to induce Full-SSAT-luc in the Caco-2 cells but these concentrations of aspirin had no effect on the 197-SSAT-luc reporter–promoter construct (Figure 4A).

Figure 3 Aspirin induces SSAT expression in Caco-2 cells

(A) Effect on SSAT promoter activity. Caco-2 cells were grown overnight, transfected with the Full-SSAT-luc reporter–promoter construct and then treated for 48 h with either vehicle (open bar) or aspirin (20 or 100 μ M; all closed bars). Relative luciferase units (RLU) were calculated after normalizing to the protein and β -galactosidase activities in the cell lysates. Fold induction was calculated after dividing the RLU of the sample by RLU of vehicle. (B) Effect on SSAT mRNA expression. Caco-2 cells were grown overnight and then treated for 48 h with either vehicle (open bar) or aspirin (20 or 100 μ M; all closed bars). Cells were harvested, total RNA extracted and used for quantitative RT–PCR. SSAT expression was normalized to the GAPDH expression in these treatments. Fold induction was calculated by dividing normalized values of the sample with the control value. The result is an average from three different experiments. (C) Effect on SSAT protein. Immunoblotting for SSAT protein in Caco-2 cells grown overnight and then treated with either vehicle (V) or different concentrations of aspirin (ASA; 100 μ M, 1 mM and 4 mM) for 48 h. BENS (10 μ M)-treated A549 lung cancer cell protein extract [BENS, N^1,N^{11} -bis(ethyl)norspermine] was used as a positive control. (D) Effect on SSAT enzyme activity. Caco-2 cells were grown overnight and then treated with either vehicle (open bar) or 20 or 100 μ M aspirin (all closed bars) for 48 h. Cells were then harvested and SSAT enzyme activity was measured. The result shown is an average from three different experiments. * $P < 0.05$ compared with the vehicle control.

In the second set of experiments, more SSAT deletion promoter constructs were used to narrow down the aspirin responsive sequence(s) (Figures 1B and 4B). The SSAT deletion promoter constructs were transfected along with a β -galactosidase promoter

Table 1 Effect of aspirin treatment on intracellular polyamine content in human colorectal cancer cells

Caco-2 cells were grown overnight after which they were treated with either vehicle or 20 or 100 μ M aspirin for 48 h. Polyamine content was quantified by HPLC analysis of the acid-soluble fraction. The polyamine levels were normalized to the protein in the samples. The result is an average from four different experiments. Analysis was by Student's *t* test; **P* < 0.05 compared with the untreated control values.

	Intracellular polyamine content (nmol/mg of protein)		
	Putrescine	Spermidine	Spermine
Vehicle	1.24 $\pm$ 0.2	3.78 $\pm$ 0.1	4.45 $\pm$ 0.33
Aspirin (20 μ M)	0.35 $\pm$ 0.1*	1.31 $\pm$ 0.1*	2.16 $\pm$ 0.06*
Aspirin (100 μ M)	0.90 $\pm$ 0.15	3.67 $\pm$ 0.25	4.12 $\pm$ 0.2

Figure 4 Mapping of a putative aspirin responsive element in the SSAT promoter

(A) Caco-2 cells were grown overnight, transfected with either Full-SSAT-luc or 197-SSAT-luc and then treated with either vehicle (V; open bars) or various concentrations of aspirin (ASA; all closed bars) for 48 h. (B) Caco-2 cells were grown overnight, transfected with a SSAT deletion reporter construct and then treated for 48 h with either vehicle (open bars) or 100 μ M aspirin (closed bars). Relative luciferase units (RLU) were calculated after normalizing to the protein and β -galactosidase activities, and fold induction was calculated. Normalized luciferase activities are shown as mean $\pm$ S.D. (*n* = 3) and are expressed as fold inductions relative to the activity in the presence of vehicle alone; **P* < 0.05 compared with vehicle.

construct into the Caco-2 cells. Following transfection, cells were treated with 100 μ M aspirin or its vehicle for 48 h. Aspirin treatment activated transcription of these constructs, except with 197-SSAT-luc. There was a 2-fold induction with 358-SSAT-luc, approx. 3-fold induction with 659-SSAT-luc and 791-SSAT-luc and approx. 5-fold induction with Full-SSAT-luc (Figure 4B). These different levels of induction suggested that there are at least two aspirin responsive sequences in the region of the SSAT 5' promoter flanking region. One or more of the elements are in the region of the SSAT promoter which is in 358-SSAT-luc but not in 197-SSAT-luc, and another one or more are in the region

Figure 5 NF- κ B, but not PPAR, is induced by aspirin in Caco-2 cells

Caco-2 cells were grown overnight and transfected with either the NF- κ B₂ Luc reporter construct or the control NF- κ B₂-luc reporter construct along with the β -gal plasmid. The cells were then treated for 48 h with either vehicle (V; open bars) or 100 μ M aspirin (ASA; closed bars). Inset: NF- κ B₂-luc reporter construct. Relative luciferase units (RLU) were calculated after normalizing to the protein and β -galactosidase activities in the cell lysates. Normalized luciferase activities are shown as mean $\pm$ S.D. (*n* = 3) and are expressed as fold inductions relative to the activity in the presence of vehicle alone; **P* < 0.05 compared with vehicle.

of the SSAT promoter which is in Full-SSAT-luc but not in 791-SSAT-luc reporter constructs.

Aspirin can induce NF- κ B, but not PPARs, in Caco-2 colon cancer cells

In order to find potential aspirin responsive elements, a TESS (transcription element search system) database analysis on the SSAT 5' promoter flanking sequence was done. It was found that there are many putative AP-1 (activator protein-1), Sp-1 and NF- κ B sites in the SSAT promoter, in addition to the known sites, PRE (polyamine response element) and PPRE (Figure 1A). Aspirin has been shown to inhibit NF- κ B, but it has also been shown to induce NF- κ B in colon cancer cells. Sulindac sulphone induces SSAT by activating PPAR γ , which can bind to one of the PPREs in the SSAT promoter [14]. Aspirin can act as a ligand for PPARs and can also regulate the synthesis of prostaglandins, which can also act as ligands for PPARs [27]. Based on the presence of putative NF- κ B and PPAR sites in the SSAT promoter, we wanted to determine the mechanism of induction of SSAT by aspirin. Transient transfection experiments using the PPRE₃-tk-luc plasmid, which has three PPREs cloned in front of a luciferase gene, were performed in the Caco-2 cells. The transfected cells were treated with either 20 or 100 μ M aspirin for 48 h. Aspirin treatment did not lead to an increased expression of this plasmid, indicating that aspirin is not acting via PPARs in Caco-2 cells to induce SSAT expression (results not shown).

To test for the role of NF- κ B in induction of SSAT by aspirin, Caco-2 cells were transfected with either the NF- κ B₂-luc reporter plasmid or its control Δ NF- κ B₂-luc reporter construct. The transfected cells were treated with 100 μ M aspirin for 48 h. Aspirin at 100 μ M was able to induce the NF- κ B₂-luc promoter but not the control vector (Figure 5), indicating that NF- κ B is functional and that aspirin is influencing the activity/binding of NF- κ B protein complexes in the Caco-2 cells. This result is in contrast with the results that aspirin mostly inhibits NF- κ B activity, but more recently, it has been shown that in HCT-116, another colon cancer cell line, aspirin can induce NF- κ B and lead to apoptosis [28].

Figure 6 Increased binding of NF- κ B complexes to the SSAT promoter

(A) Oligonucleotides containing the NF- κ B-wild-1 (w) or NF- κ B-mut-1 (m) sequence were 32 P-labelled ('Hot Probe') and incubated with 10 μ g of nuclear extract, isolated from Caco-2 cells treated with different concentrations of aspirin (ASA) for 48 h. The unlabelled wild probe ('Cold Probe') was used at 100-fold molar excess for the competition reaction. Anti-p50 antibody was used for the supershift reactions. The specific-band shift and the supershifted bands are indicated. The specific-band shift intensity was quantified and then normalized to the vehicle-treated specific-band shift and shown as specific-band density. (B) Oligonucleotides containing the NF- κ B-wild-3 (w₃) or NF- κ B-mut-3 (m₃) sequence were 32 P-labelled and incubated with 10 μ g of nuclear extract, isolated from Caco-2 cells treated with different concentrations of aspirin for 48 h. The unlabelled wild probe was used at 100-fold molar excess for the competition reaction. Anti-p50 antibody was used for the supershift reactions. The specific-band shift and the supershifted bands are indicated. The specific-band shift intensity was quantified and then normalized to the vehicle-treated specific-band shift and shown as specific-band density. Each experiment was performed four times, the specific-band intensities were quantified and statistics were done using Student's *t* test; **P* < 0.05 compared with vehicle.

Aspirin can induce SSAT by increasing NF- κ B binding to putative NF- κ B sites in the Caco-2 cells

There are three putative NF- κ B sites in the 5'-flanking sequences of the SSAT promoter, which are present at -286 (w), -594 (w₂) and -1735 (w₃) relative to the SSAT transcription start site (Figure 1B). To determine whether aspirin can regulate the binding of NF- κ B complexes to these putative NF- κ B response elements in the SSAT promoter, which could explain the increased SSAT promoter activity, electrophoretic mobility-shift assays were performed using the nuclear extract from the Caco-2 cells, which had been treated with different concentrations of aspirin for 48 h and double-stranded oligonucleotides containing the putative wild-type NF- κ B sequences. As shown in Figure 6(A), we observed binding of nuclear proteins to the NF- κ B-wild-1 (w) probe. Aspirin treatment led to an increased binding to the w probe, with the highest for 100 μ M aspirin (1.7-fold) and then 1 and 5 mM aspirin (1.2-fold each), above the vehicle treatment. This binding was specific, as it could be abolished by NF- κ B-mutant-1 (m) sequence and could be competed by unlabelled wild probe (w). Further, there is generation of a supershifted band

with the use of p50 antibody, suggesting the presence of a p50 protein in the NF- κ B complex binding at the w probe. Using the same amount of the p50 antibody, we found a stronger intensity supershift with 100 μ M and 1 mM aspirin, but not with 5 mM aspirin, indicating that aspirin is working predominantly at lower concentrations to induce NF- κ B binding to the NF- κ B-wild-1 site in the SSAT promoter.

Using other putative NF- κ B sequences, we found no significant binding of the aspirin-treated Caco-2 nuclear extract with the w₂ NF- κ B sequence (results not shown), but found that there was binding of NF- κ B complexes to the w₃ sequence (Figure 6B). This binding was increased by 100 μ M aspirin treatment (1.3-fold as compared with the vehicle treatment), but not by 1 mM aspirin treatment. This binding was specific, as it could be abolished by NF- κ B-mutant-3 (m₃) sequence and could be competed by unlabelled wild probe (w₃). Further, there is generation of a supershifted band with the use of p50 antibody, suggesting the presence of a p50 protein in the NF- κ B complex binding to the w₃ probe.

DISCUSSION

A number of independent lines of evidence suggest that NSAIDs can prevent colorectal cancer. Aspirin is the most widely studied NSAID for the chemoprevention of colorectal cancer [29–34] and several epidemiological studies have reported an inverse association between colorectal cancer risk and aspirin intake. Recent clinical trials have established the use of lower doses of aspirin in chemopreventive effects on adenoma recurrence [33]. In our previous work, we showed that aspirin was able to induce SSAT expression and decrease intracellular polyamine levels in HT-29 colon adenocarcinoma cells [7]. Because polyamines are essential for neoplastic cell growth, the aim of the present paper was to evaluate the effects of various NSAIDs, in particular aspirin, on polyamine metabolism in Caco-2 colon cancer cells and to determine the mechanism by which aspirin is able to induce SSAT in these cells.

It was found that the NSAIDs aspirin, ibuprofen, sulindac and indomethacin are all able to induce SSAT expression in Caco-2 cells at concentrations which are sufficient to decrease intracellular PGE₂ levels. Most of the *in vitro* research with aspirin in colon cancer has been with concentrations ranging from 1 to 10 mM [3,23,26]. These concentrations are relatively high as compared with the *in vivo* concentrations achievable in humans. We used lower doses of aspirin that are more physiologically relevant and then studied their effects on SSAT expression. We found that aspirin, at these concentrations, is able to induce SSAT promoter activity and SSAT mRNA levels in Caco-2 cells. Aspirin led to a higher fold induction in the Full-length-SSAT promoter construct (7-fold) as compared with the SSAT mRNA (2-fold) in Caco-2 cells. This difference could be due to the presence of other *cis*- and *trans*-acting elements in the SSAT gene, which are not included in the promoter expression studies, and can be influenced by aspirin treatment. Further, aspirin at 20 μ M led to a 1.3 ± 0.1 -fold induction in SSAT mRNA but a 3.6 ± 0.9 -fold induction in the SSAT enzyme activity, whereas aspirin at 100 μ M induced both the SSAT mRNA and enzyme activity to approx. 2-fold. This 2-fold difference between the SSAT mRNA and enzyme activity obtained at lower concentrations of aspirin suggests a role for aspirin in the post-transcriptional regulation of SSAT. However, any post-transcriptional role for aspirin in SSAT regulation appears to be considerably less than that observed with the polyamine analogues.

SSAT leads to the production of acetylated spermidine and spermine, which can then be either excreted from the cells, or

by the action of another enzyme, polyamine oxidase, can be converted back into putrescine in the cells. Therefore an induction in the SSAT activity can lead to lower intracellular levels of spermidine and spermine, but an increase or no effect on the intracellular putrescine levels. Our results indicate that aspirin at 20 μ M led to a decrease in all three polyamines after 48 h of treatment as has been shown previously with many drugs like aspirin [7], sulindac [14], indomethacin [13] and polyamine analogues [35,36], all of which induce SSAT, and led to lower levels of all the polyamines, including putrescine, in colon and other cancer cells. One possible explanation could be that the reduction in putrescine levels could be due to an inhibition of ODC expression by aspirin in Caco-2 cells. Aspirin slightly increases ODC mRNA at 100 μ M, but has no effect on ODC mRNA at 20 μ M in the Caco-2 cells. Further, reduction in polyamines could be explained by the fact that aspirin (acetylsalicylic acid) may compete with acetyl-CoA (natural co-substrate) for SSAT enzyme activity or may act as a direct inhibitor of SSAT enzyme, leading to inhibition of enzyme activity at higher aspirin concentrations. Additional experiments are required to characterize the exact role of aspirin as an inhibitor of SSAT enzyme activity. However, it is important to note that patients given a lower dose of aspirin had a significantly greater reduction in adenoma recurrence as compared with patients given a higher dose of aspirin [37]. Alternatively, 100 μ M aspirin could lead to the inhibition of the diamine exporter [38] in these cells, which could explain the lack of change in the intracellular polyamine levels.

Due to the effects of aspirin on SSAT mRNA and promoter activation, we propose that aspirin is inducing SSAT by its effect on transcription. We identified two regions, one between 0.28 and 0.44 kb and the other between 0.88 and 3.53 kb in the SSAT 5' promoter flanking sequence, which were responsive to aspirin treatment. TESS analysis identified several potential aspirin responsive elements in this region, including three NF- κ B sites at -286, -594 and -1735 relative to the transcription start site. Aspirin has been shown to inhibit NF- κ B [39–41], but it has also been shown to induce NF- κ B in HCT-116 cells [28,42]. Further, aspirin can lead to the inhibition of AP-1 activity [43,44], and can act as a ligand for PPARs [45]. Transient transfection experiments using the NF- κ B reporter constructs demonstrated that aspirin is able to induce this promoter in Caco-2 colon cancer cells. The involvement of NF- κ B in the aspirin-induced expression of SSAT was further implicated by electrophoretic mobility-shift assays, demonstrating that nuclear extracts from Caco-2 cells treated with 100 μ M aspirin result in an increased binding at two of the three potential NF- κ B response elements in the SSAT promoter. NF- κ B complexes are present as homo- or hetero-dimers of p50 and p65 proteins. Supershift experiments demonstrated the presence of the p50 protein in the nuclear proteins bound to both the NF- κ B-wild-1 and wild-3 sequences in the SSAT promoter. In preliminary experiments, treatment of Caco-2 cells with TNF α (tumour necrosis factor α), which is known to exert its biological actions by inducing NF- κ B, led to an activation of NF- κ B signalling in the Caco-2 cells with a concomitant increase in the SSAT mRNA expression. Although observations with TNF α do not directly confirm the relationship between aspirin induction of SSAT by NF- κ B, they do strengthen the point that NF- κ B activation can lead to the induction of SSAT by increased binding at the NF- κ B response elements in the SSAT 5' promoter flanking sequence. Interestingly, unlike sulindac sulphone, which induces SSAT by activating PPAR γ , which can then bind to the PPRE in the SSAT 5' promoter flanking region, aspirin does not appear to induce SSAT by this pathway as it was not able to induce the PPRE reporter constructs.

These results appear to contradict published reports indicating that aspirin and its metabolites can bind to and directly inhibit IKK β (inhibitor of κ B kinase β), the kinase responsible for phosphorylating I κ B α (inhibitory κ B), thereby inhibiting translocation of p65 to the nucleus [46,47] and preventing apoptosis. However, more recent reports indicate that aspirin can induce the NF- κ B signalling pathway and this can lead to apoptosis in colon cancer cells [28]. Further, this apoptosis is independent of p53 status [48]. The differential effects of NF- κ B may be explained by the DNA-binding specificity and selective transcriptional activation of downstream NF- κ B target genes, which is in turn determined by the dimeric composition of the NF- κ B complex and by the transcriptional cofactors recruited to the complex [49]. Results presented here demonstrate that one of the target genes induced by NF- κ B with aspirin treatment in Caco-2 colon cancer cells is SSAT. Induction of SSAT can lead to lower intracellular polyamine levels in these cells with aspirin treatment. A decrease in polyamines has been shown to lead to increased cell death [14] and/or increased apoptosis [10,11].

In summary, the experimental results presented here demonstrate that induction of polyamine catabolism leading to a reduction in intracellular polyamines could be one of the general mechanisms for the chemopreventive actions of different NSAIDs in colon cancer. Further, the mechanism of induction of SSAT by physiologically relevant doses of aspirin involves NF- κ B, but not PPAR, transcription factor complexes, which can bind to their response elements in the SSAT 5' promoter region in Caco-2 colon cancer cells.

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REFERENCES

- Vane, J. R., Flower, R. J. and Botting, R. M. (1990) History of aspirin and its mechanism of action. *Stroke* **21**, IV12–IV23
- Piazza, G. A., Alberts, D. S., Hixson, L. J., Paranka, N. S., Li, H., Finn, T., Bogert, C., Guillen, J. M., Brendel, K., Gross, P. H. et al. (1997) Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. *Cancer Res.* **57**, 2909–2915
- Shiff, S. J., Koutsos, M. I., Qiao, L. and Rigas, B. (1996) Nonsteroidal antiinflammatory drugs inhibit the proliferation of colon adenocarcinoma cells: effects on cell cycle and apoptosis. *Exp. Cell Res.* **222**, 179–188
- Thompson, W. J., Piazza, G. A., Li, H., Liu, L., Fetter, J., Zhu, B., Sperl, G., Ahnen, D. and Pamukcu, R. (2000) Exisulind induction of apoptosis involves guanosine 3',5'-cyclic monophosphate phosphodiesterase inhibition, protein kinase G activation, and attenuated beta-catenin. *Cancer Res.* **60**, 3338–3342
- Turchanowa, L., Dauletbaev, N., Milovic, V. and Stein, J. (2001) Nonsteroidal anti-inflammatory drugs stimulate spermidine/spermine acetyltransferase and deplete polyamine content in colon cancer cells. *Eur. J. Clin. Invest.* **31**, 887–893
- Carbone, P. P., Douglas, J. A., Larson, P. O., Verma, A. K., Blair, I. A., Pomplun, M. and Tutsch, K. D. (1998) Phase I chemoprevention study of piroxicam and alpha-difluoromethylornithine. *Cancer Epidemiol. Biomarkers Prev.* **7**, 907–912
- Martinez, M. E., O'Brien, T. G., Fultz, K. E., Babbar, N., Yerushalmi, H., Qu, N., Guo, Y., Boorman, D., Einspahr, J., Alberts, D. S. et al. (2003) Pronounced reduction in adenoma recurrence associated with aspirin use and a polymorphism in the ornithine decarboxylase gene. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 7859–7864
- Pegg, A. E. (1988) Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. *Cancer Res.* **48**, 759–774
- Meyskens, Jr, F. L. and Gerner, E. W. (1995) Development of difluoromethylornithine as a chemoprevention agent for the management of colon cancer. *J. Cell. Biochem.* **22** (Suppl.), 126–131
- Scorcioni, F., Corti, A., Davalli, P., Astancolle, S. and Bettuzzi, S. (2001) Manipulation of the expression of regulatory genes of polyamine metabolism results in specific alterations of the cell-cycle progression. *Biochem. J.* **354**, 217–223

- 11 Li, L., Rao, J. N., Bass, B. L. and Wang, J. Y. (2001) NF-kappaB activation and susceptibility to apoptosis after polyamine depletion in intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **280**, G992–G1004
- 12 Vujcic, S., Halmekyto, M., Diegelman, P., Gan, G., Kramer, D. L., Janne, J. and Porter, C. W. (2000) Effects of conditional overexpression of spermidine/spermine N1-acetyltransferase on polyamine pool dynamics, cell growth, and sensitivity to polyamine analogs. *J. Biol. Chem.* **275**, 38319–38328
- 13 Turchanowa, L., Dauletbayev, N., Milovic, V. and Stein, J. (2001) Nonsteroidal anti-inflammatory drugs stimulate spermidine/spermine acetyltransferase and deplete polyamine content in colon cancer cells. *Eur. J. Clin. Invest.* **31**, 887–893
- 14 Babbar, N., Ignatenko, N. A., Casero, Jr, R. A. and Gerner, E. W. (2003) Cyclooxygenase-independent induction of apoptosis by sulindac sulfone is mediated by polyamines in colon cancer. *J. Biol. Chem.* **278**, 47762–47775
- 15 Hughes, A., Smith, N. I. and Wallace, H. M. (2003) Polyamines reverse non-steroidal anti-inflammatory drug-induced toxicity in human colorectal cancer cells. *Biochem. J.* **374**, 481–488
- 16 Xiao, L., Celano, P., Mank, A. R., Griffin, C., Jabs, E. W., Hawkins, A. L. and Casero, Jr, R. A. (1992) Structure of the human spermidine/spermine N1-acetyltransferase gene (exon/intron gene organization and localization to Xp22.1). *Biochem. Biophys. Res. Commun.* **187**, 1493–1502
- 17 Chromczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159
- 18 Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475–1489
- 19 Casero, Jr, R. A., Gabrielson, E. W. and Pegg, A. E. (1994) Immunohistochemical staining of human spermidine/spermine N1-acetyltransferase superinduced in response to treatment with antitumor polyamine analogues. *Cancer Res.* **54**, 3955–3958
- 20 Ignatenko, N. A. and Gerner, E. W. (1996) Growth arrest- and polyamine-dependent expression of spermidine/spermine N1-acetyltransferase in human tumor cells. *Cell Growth Differ.* **7**, 481–486
- 21 Seiler, N. and Knodgen, B. (1980) High-performance liquid chromatographic procedure for the simultaneous determination of the natural polyamines and their monoacetyl derivatives. *J. Chromatogr.* **221**, 227–235
- 22 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85
- 23 Qiao, L., Hanif, R., Sphicas, E., Shiff, S. J. and Rigas, B. (1998) Effect of aspirin on induction of apoptosis in HT-29 human colon adenocarcinoma cells. *Biochem. Pharmacol.* **55**, 53–64
- 24 Piazza, G. A., Rahm, A. K., Finn, T. S., Fryer, B. H., Li, H., Stoumen, A. L., Pamukcu, R. and Ahnen, D. J. (1997) Apoptosis primarily accounts for the growth-inhibitory properties of sulindac metabolites and involves a mechanism that is independent of cyclooxygenase inhibition, cell cycle arrest, and p53 induction. *Cancer Res.* **57**, 2452–2459
- 25 Din, F. V., Stark, L. A. and Dunlop, M. G. (2005) Aspirin-induced nuclear translocation of NFkappaB and apoptosis in colorectal cancer is independent of p53 status and DNA mismatch repair proficiency. *Br. J. Cancer* **92**, 1137–1143
- 26 Ricchi, P., Pignata, S., Di Popolo, A., Memoli, A., Apicella, A., Zarrilli, R. and Acquaviva, A. M. (1997) Effect of aspirin on cell proliferation and differentiation of colon adenocarcinoma Caco-2 cells. *Int. J. Cancer* **73**, 880–884
- 27 Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M. and Evans, R. M. (1995) 15-Deoxy-delta 12,14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell (Cambridge, Mass.)* **83**, 803–812
- 28 Stark, L. A., Din, F. V., Zwacka, R. M. and Dunlop, M. G. (2001) Aspirin-induced activation of the NF-kappaB signaling pathway: a novel mechanism for aspirin-mediated apoptosis in colon cancer cells. *FASEB J.* **15**, 1273–1275
- 29 Greenberg, E. R. and Baron, J. A. (1996) Aspirin and other nonsteroid anti-inflammatory drugs as cancer-preventive agents. *IARC Sci. Publ.* **139**, 91–98
- 30 Greenberg, E. R., Baron, J. A., Freeman, Jr, D. H., Mandel, J. S. and Haile, R. (1993) Reduced risk of large-bowel adenomas among aspirin users. The Polyp Prevention Study Group. *J. Natl. Cancer Inst.* **85**, 912–916
- 31 Baron, J. A., Cole, B. F., Sandler, R. S., Haile, R. W., Ahnen, D., Bresalier, R., McKeown-Eyssen, G., Summers, R. W., Rothstein, R., Burke, C. A. et al. (2003) A randomized trial of aspirin to prevent colorectal adenomas. *N. Engl. J. Med.* **348**, 891–899
- 32 Thun, M. J., Namboodiri, M. M. and Heath, Jr, C. W. (1991) Aspirin use and reduced risk of fatal colon cancer. *N. Engl. J. Med.* **325**, 1593–1596
- 33 Sandler, R. S., Halabi, S., Baron, J. A., Budinger, S., Paskett, E., Keresztes, R., Petrelli, N., Pipas, J. M., Karp, D. D., Loprinzi, C. L. et al. (2003) A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *N. Engl. J. Med.* **348**, 883–890
- 34 Thun, M. J. and Heath, Jr, C. W. (1995) Aspirin use and reduced risk of gastrointestinal tract cancers in the American Cancer Society prospective studies. *Prev. Med.* **24**, 116–118
- 35 Nairn, L. M., Lindsay, G. S., Woster, P. M. and Wallace, H. M. (2000) Cytotoxicity of novel unsymmetrically substituted inhibitors of polyamine biosynthesis in human cancer cells. *J. Cell. Physiol.* **182**, 209–213
- 36 Fraser, A. V., Woster, P. M. and Wallace, H. M. (2002) Induction of apoptosis in human leukaemic cells by IPENSpm, a novel polyamine analogue and anti-metabolite. *Biochem. J.* **367**, 307–312
- 37 Baron, J. A., Cole, B. F., Sandler, R. S., Haile, R. W., Ahnen, D., Bresalier, R., McKeown-Eyssen, G., Summers, R. W., Rothstein, R., Burke, C. A. et al. (2003) A randomized trial of aspirin to prevent colorectal adenomas. *N. Engl. J. Med.* **348**, 891–899
- 38 Xie, X., Gillies, R. J. and Gerner, E. W. (1997) Characterization of a diamine exporter in Chinese hamster ovary cells and identification of specific polyamine substrates. *J. Biol. Chem.* **272**, 20484–20489
- 39 Picado, C., Bioque, G., Roca-Ferrer, J., Pujols, L., Mullol, J., Benitez, P. and Bulbena, O. (2003) Nuclear factor-kappaB activity is down-regulated in nasal polyps from aspirin-sensitive asthmatics. *Allergy* **58**, 122–126
- 40 Muller, D. N., Heissmeyer, V., Dechend, R., Hampich, F., Park, J. K., Fiebeler, A., Shagdarsuren, E., Theuer, J., Elger, M., Pilz, B. et al. (2001) Aspirin inhibits NF-kappaB and protects from angiotensin II-induced organ damage. *FASEB J.* **15**, 1822–1824
- 41 Kopp, E. and Ghosh, S. (1994) Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* **265**, 956–959
- 42 Niederberger, E., Tegeder, I., Vetter, G., Schmidt, A., Schmidt, H., Euchenhofer, C., Brautigam, L., Grosch, S. and Geisslinger, G. (2001) Celecoxib loses its anti-inflammatory efficacy at high doses through activation of NF-kappaB. *FASEB J.* **15**, 1622–1624
- 43 Ma, W. Y., Huang, C. and Dong, Z. (1998) Inhibition of ultraviolet C irradiation-induced AP-1 activity by aspirin is through inhibition of JNKs but not ERKs or P38 MAP kinase. *Int. J. Oncol.* **12**, 565–568
- 44 Dong, Z., Huang, C., Brown, R. E. and Ma, W. Y. (1997) Inhibition of activator protein 1 activity and neoplastic transformation by aspirin. *J. Biol. Chem.* **272**, 9962–9970
- 45 Lehmann, J. M., Lenhard, J. M., Oliver, B. B., Ringold, G. M. and Klier, S. A. (1997) Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* **272**, 3406–3410
- 46 Yin, M. J., Yamamoto, Y. and Gaynor, R. B. (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature (London)* **396**, 77–80
- 47 Yamamoto, Y., Yin, M. J., Lin, K. M. and Gaynor, R. B. (1999) Sulindac inhibits activation of the NF-kappaB pathway. *J. Biol. Chem.* **274**, 27307–27314
- 48 Din, F. V., Stark, L. A. and Dunlop, M. G. (2005) Aspirin-induced nuclear translocation of NFkappaB and apoptosis in colorectal cancer is independent of p53 status and DNA mismatch repair proficiency. *Br. J. Cancer* **92**, 1137–1143
- 49 Sheppard, K. A., Rose, D. W., Haque, Z. K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M. G., Glass, C. K. and Collins, T. (1999) Transcriptional activation by NF-kappaB requires multiple coactivators. *Mol. Cell. Biol.* **19**, 6367–6378

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