RESEARCH COMMUNICATION

Anti-Proliferative and Apoptotic Effects of Etoricoxib, a Selective COX-2 Inhibitor, on 1,2-Dimethylhydrazine Dihydrochloride-Induced Colon Carcinogenesis

Lalita Tanwar, Honit Piplani, SN Sanyal*

Abstract

In the present study, we assessed effects of etoricoxib, a non steroidal anti-inflammatory drug, on proliferation and apoptosis in 1,2-dimethylhydrazine dihydrochloride (DMH) induced colon lesion development. Male SD rats were divided into four groups: Group 1 controls receiving the vehicle treatment; Group 2 administered DMH weekly (30 mg/kg body weight, subcutaneously) alone; Group 3, DMH weekly plus etoricoxib (0.64 mg/kg body weight, orally) daily; and Group 4, etoricoxib alone. After six weeks of treatment, animals were sacrificed and colons were analysed for morphological and histopathological features. Well characterized pre-neoplastic aberrations such as multiple plaque lesions, hyperplasia and dysplasia were found in the DMH treated group whereas these features were reduced with co-administration of etoricoxib. To study apoptosis, colonocytes were isolated by metal chelation from colonic sacs and studied by fluorescent staining and further confirmed by DNA fragmentation. The DMH treated animals had fewer apoptotic nuclei as compared to the controls, but numbers were higher with DMH+etoricoxib as well as etoricoxib alone. Expression of proliferative cell nuclear antigen (PCNA), assessed by Western blot analysis and immunohistochemistry, was found to be elevated by DMH treatment group and again reduced by etoricoxib. Results for bromodeoxyuridine incorporation (BrdU) were in agreement. It may be concluded that the drug, etoricoxib, has the potential to act as an anti-apoptotic and anti- proliferative agent in the colon.

Keywords: Etoricoxib - PCNA - BrdU - apoptosis - proliferation

Asian Pacific J Cancer Prev, **11**, 1329-1333

Introduction

One of the molecular targets in cancer chemoprevention is cycloxygenase (COX), since eicosanoids increase the proliferation rate in colon cancer cells (Qiao et al., 1995) and the levels of prostaglandins and COX-2 gene expression are elevated in colon cancer (Marnett and DuBois, 2002; Taketo, 1998). One of the most remarkable advances in the prevention of colon cancer is the appreciation of the prophylactic effect of non-steroidal anti-inflammatory drugs (NSAIDs), which seem to reduce the risk of colorectal cancer due to their anti-proliferative and anti-inflammatory effects (Hanif et al., 1996; Chan, 2002; Stark, 2007). Both proliferation and apoptosis are critical determinants of growth of a tumour (Lowe and Lin, 2000) and has been reported that the selective COX-2 inhibitor, celecoxib reduces the proliferation and induces apoptosis in angiogenic endothelial cells in vivo (Leahy et al., 2002). Etoricoxib, a much improved COX-2 inhibitor provides a wide range of effects that could be useful in preventing colon carcinogenesis. Earlier studies from our laboratory had shown that Etoricoxib was found to effectively intervene 1,2- dimethylhydrazine dihydrochloride-induced colon carcinogenesis in rat model (Saini et al., 2009; Sharma et al., 2010).

Proliferative cell nuclear antigen (PCNA) is an intranuclear 36 kDa polypeptide whose expression and synthesis is linked with cell proliferation (Kelman, 1997). It is an auxiliary protein to DNA polymerase delta and functions as a co-factor in DNA synthesis. The synthesis and expression of PCNA are enhanced in proliferating cells (Biasco et al., 1994). BrdU, a synthetic nucleoside that is an analogue of thymidine, can also be incorporated into the newly synthesised DNA of replicating cells. Both PCNA and BrdU incorporation have been considered as the most reliable methods to evaluate proliferation in colonic tissues (Bolten et al., 1992).

Apoptosis plays an important role in the regulation of normal and cancer cells (Wyllie et al., 1972). Characteristic features which distinguish it from necrosis are cell shrinkage, cytoplasmic blebbing, loss of membrane architecture, chromatin condensation, fragmentation of DNA into oligonucleosome-sized fragments, and formation of apoptotic bodies (Kerr et al., 1994).

In the present investigation therefore, the mechanism of the apoptotic and anti-proliferative effects of Etoricoxib have been studied, which is a selective COX-2 inhibitor in the DMH induced rat colon cancer.

*Department of Biophysics, Panjab University, Chandigarh, India *For correspondence: sanyalpu@pu.ac.in*

Materials and Methods

Chemicals

DMH, Ethidium bromide, Acridine Orange and Bradford reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibody against PCNA, BrdU and anti-mouse β-actin were purchased from Santa Cruz Biotechnology Inc., CA (USA). Alkaline phosphatase-conjugated secondary antibody and BCIP-NBT were purchased from Genei, Bangalore (India). Etoricoxib was obtained from Ranbaxy Research Lab (Gurgaon, India). All other chemicals and reagents used in the present study were of the analytical grade and procured from the reputed Indian manufacturers.

Animals

Male adult SD rats of body weight between 150-200g were obtained from the inbred population of Central Animal House, Panjab University, Chandigarh. These were acclimatized to the control diet (rodent chow) and water ad libitum for at least 1 week. Animals were maintained as per the principles and guidelines of the Ethics Committee of the Animal Care of Panjab University in accordance with the Indian national law on animal care and use. The animals were housed 4 per cage in polypropylene cages with a wire mesh top and a hygienic bed of husk (regularly changed) in a well ventilated animal room till the end of the experimental period. The animals were also maintained under a 12hr photoperiod of light and darkness, respectively.

Treatment Schedule

Animals were assorted into the following groups with four to six animals in each group: Control Group, Animals were administrated the vehicle (1mM EDTA-saline subcutaneously) in weekly injection and 0.5% carboxymethyl cellulose per oral daily; DMH Group, animals were administrated with DMH weekly at a dose of 30 mg/kg body weight subcutaneously, as had been established in our laboratory earlier (Kanwar et al., 2008) - DMH was freshly prepared in 1mM EDTA-saline, pH adjusted to 7.0 using dilute NaOH solution; DMH + etoricoxib Group, Etoricoxib was given daily per oral at its therapeutic anti-inflammatory dose (ED_{50} for rats, 0.64 mg/kg body weight) to the animals along with the weekly administration of DMH (Riendeau et al., 2001); and Etoricoxib Group: Etoricoxib alone was administered orally daily (0.64 mg/kg body weight). The anti-inflammatory dose was established earlier in a model of carragenan induced oedema in rat hind paw (Sharma et al., 2010).

After six weeks, animals were kept on overnight fasting with drinking water ad libitum and sacrificed the next day. The animal body weights in all the groups were recorded once in a week till the termination.

Tissue pathology

The colons were removed and flushed clear with ice-cold physiological saline (0.9% NaCl solution). These were opened longitudinally along the median and laid flat to examine the incidence of macroscopic lesions/plaques called multiple plaque lesions (MPLs). The colons were divided into proximal, medial and distal segments for the examination.

Histopathological analysis

Colonic pieces were cut from the sacrificed rats and immediately fixed in 10% buffered formalin for 24 hrs. The tissues were dehydrated in ascending series of alcohol and kept in 1:1 mixture of absolute alcohol and benzene for 1 hr. For embedding the tissues in wax, they were kept in benzene for 40-45 min and transferred sequentially to 1:1 benzene and wax mixture at 60°C for 1hr and then pure wax for 6 hr at 60°C with two changes. The tissues were embedded in wax and five micron sections were cut using a hand driven microtome and transferred to egg albumin coated slides. Sections were then dewaxed in xylene, stained in haematoxylin and eosin, mounted in DPX and viewed under a light microscope and photographed at 200X with a Leica Optiphot microscope to which was attached a Leica Digital Camera.

Western Blot Analysis

Protein samples (100 μg) from each treatment group were separated on 10% SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membrane (Genei, Bangalore, India). Immunoblot was prepared using primary antibody (PCNA-1:1000) from Santa Cruz Biotechnology Inc., CA (USA) and alkaline phosphatase-conjugated secondary antibody at a dilution of 1:10,000 (Genei, Bangalore, India). BCIP-NBT detection system was used to develop the blots. Bands obtained were densitometrically analysed using Image J software as gray values.

For preparation of protein samples, colons were removed and rinsed from the different treatment groups after completion of 6 weeks. Total lysates were prepared in fresh ice-cold protein lysis buffer (10mM Tris HCl, 5mM EDTA, 1% Triton X-100, 1mM PMSF and 2mM DTT (pH 8.0)). The extracts were cleared by centrifugation at 10, 000 rpm for 10 min at 4°C. The supernatants were collected as the total lysate. Protein concentrations were determined by the method of Bradford (1996).

Immunohistochemistry

5μm thick paraffin sections of rat colon were deparaffinised in two changes of xylene for 10 min each. The sections were then gradually hydrated to water. The non-specific staining was blocked by incubating the sections with 2% BSA in phosphate buffered saline (10mM, pH 7.2). The sections were then incubated with polyclonal antibodies against PCNA- 1:1000 and BrdU-1:1000 in a moist chamber for 2hr at 37°C.

After incubation, the sections were then incubated with alkaline phosphatase-labelled secondary antibody for 2hr. Sections were washed again and the reaction product developed using BCIP-NBT. Reaction was terminated by washing with distilled water after which sections were counterstained with eosin and mounted in DPX.

Isolation of Colonocytes

Colonic epithelial cells (colonocytes) were obtained

from the freshly isolated colons by the method of Mouille et al., (2004), as originally described by Roediger and Truelove (1979). The colonic segments were removed and flushed with chilled physiological saline (NaCl solution, 9g/l) and then with a Ca^{2+}- and Mg^{2+}- free Krebs-Hanseleit (K-H) bicarbonate buffer (pH 7.4). The K-H buffer was equilibrated against a mixture of O_2 and CO_2 (19:1, v/v). Then, each colon was everted, distally ligated, and distended as much as possible by means of a syringe containing calcium free K-H saline with 0.25% w/v BSA. The proximal end of the colon was now ligated and placed in a plastic flask containing 100ml calcium-free K-H buffer, 5mM EDTA, and 0.25% BSA. The flask was gassed with O_2 - CO_2 (19:1 v/v) and incubated at 37°C in a shaking water bath at 60-70 oscillations per min for 30 min. Thereafter, the colons were rinsed in fresh calcium-free saline to remove the excess EDTA and placed in a plastic beaker with 50ml K-H buffer with 0.25% w/v BSA. Manual stirring with a plastic stirrer for two min readily disaggregated the colonocytes, which were then separated by centrifugation at 500g for 60 sec. The pellet was resuspended in 2 ml of the phosphate buffered saline by being taken up several times into a 1 ml polypropylene pipette.

*Fluorescence Microscopy*Acridine orange dye is used to stain the DNA where the staining procedure was performed by the method of Baker et al., (1994). Briefly, to 10 μl of the harvested cells, 10μl of 1mg/ml Ethidium bromide and acridine orange mixture (Sigma, St. Louis, USA) was added. Suspension was placed on a clean glass slide and examined under a fluorescence microscope. The percentage of apoptotic cells was calculated by counting 100 cells on separate slides as above for individual animals.

DNA Fragmentation

The apoptotic study was also confirmed by DNA fragmentation assay. DNA was isolated from the colon tissue using a phenol: chloroform extraction method (Sambrook et al., 1998). It was quantitated at 260 nm; 10 ug DNA sample was loaded in each well; electrophoresed on 1.5% agarose gel in TAE buffer and visualized after Ethidium bromide staining under UV light. Photograph was taken using a GelDoc machine (Upland, CA, USA).

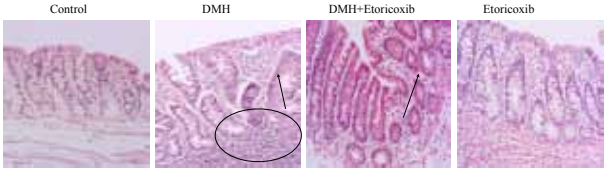

Figure 1. Morphological Appearance

Statistical Analysis

The data expressed as means ± SD (standard analysis) were analysed by One way analysis of variance (ANOVA) and post hoc comparison between the means were made by the method of least significant difference (LSD) using the statistical software package SPSS 12.0 for Windows. The values were considered significant if the *p* value was found to be 0.05 or less.

Results

Figure 1 shows the morphological features of the colons after a period of six weeks showing the mucosal surface depicting the occurrence of MPLs, recognized as either raised or non-raised stretches in the form of identifiable tissue growth, often appearing singly or in multiple forms throughout the length of the colon. A few MPLs were observed in control and etoricoxib animals. Maximum numbers of MPLs however, were observed in DMH treated animals but the number was found much less in DMH+etoricoxib treated animals (Table 1).

DMH treated animals show severe dysplasia and hyperplasia of crypt cells. The nuclei were also deeply stained and oval in shape in DMH treated animals, whereas in control animals no such features were observed. The simultaneous administration of DMH and Etoricoxib reduced these features of dysplasia and hyperplasia. No such features were observed in the Etoricoxib only group.

The control group showed maximum number of apoptotic cells whereas the number of such cells was highly reduced in DMH treated animals, this being reversed in DMH + Etoricoxib group (Table 2). Similarly, in the DMH treated group, the intensity of the bands of fragmented DNA which is indicative of the apoptotic process, was the lowest. With Etoricoxib treatment however, the intensity had increased indicating the restoration of apoptosis.

The expression of PCNA was found to be higher in DMH treated group as compared to the Control. The expression was found to be low in Etoricoxib only group. In DMH + Etoricoxib group also, the expression was found to be low as compared to control, whereas it was much less than in the DMH group (Figure 2).

The expression of both PCNA and BrdU was found to be higher in the paraffin tissue sections in DMH treated group as compared to the Control. The expression was found to be low in Etoricoxib only group. In DMH +

Table 1. Multiple Plaque Lesions (MPLs)

Animal groups	No. of MPLs in different region of colon			Total No. of MPLs	No. of rats with MPL/MPL total no. of rats	Incidence (%)	MPL burden	MPL multiplicity
	Proximal	Middle	Distal					
Control	2	1	1	4	2/5	40	0.8	2.0
DMH	7	10	9	26	5/5	100	5.2	5.2
DMH + Etoricoxib	3	5	4	12	3/5	60	2.4	4.0
Etoricoxib	1	2	1	4	2/5	40	0.8	2.0

MPL incidence= the percentage of animals having MPLs; MPL burden= total number of MPLs counted/ total number of rats; MPL multiplicity= total number of MPLs counted / number of MPLs bearing rats.

Table 2. The Percent Apoptotic Nuclei in Control and Treated Groups Studied by Ethidium Bromide/ Acridine Orange Staining by Fluorescence Microscopy

Groups	Apoptotic nuclei/100 nuclei
Control	32±5
DMH	11±3[a]
DMH + ETO	21±3[b]
ETO	29±4

Data is expressed as Mean ±S.D (n=4-6). a: p<0.001 compared to the Controls; b: p<0.001 when compared to DMH

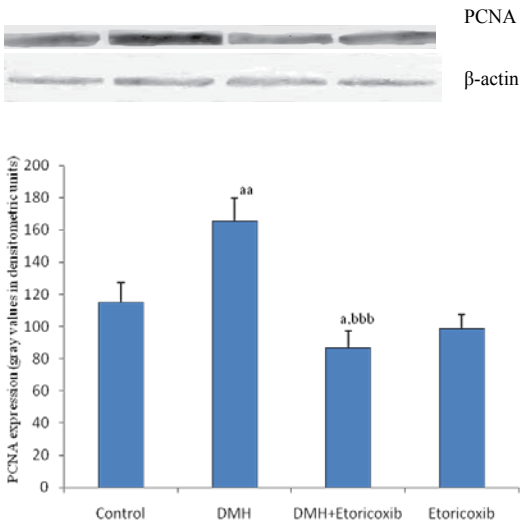

Figure 2. Expression of PCNA

Etoricoxib group also, the expression was found to be high as compared to control, whereas it was much less than the DMH group.

Discussion

In recent years an intimate linkage was established among oncogenes, anti-oncogenes, and malignancy in the context of cell growth, differentiation, proliferation and apoptosis (Evan and Vousdan, 2001). In the present study we attempted to assess the effect of Etoricoxib which is a specific COX-2 inhibitor on the status of genomic DNA, proliferation markers (PCNA and BrdU) and apoptosis in DMH induced colon cancer in rat model.

Histopathologically, there was seen a marked dysplasia as well as hyperplasia in DMH treated rats as also the morphologically identified neoplastic growth on MPLs. Oral administration of Etoricoxib was able to weaken these features prominently indicating its efficiency as a chemopreventive agent at the present dose for a period of six weeks, which can be considered as the early stage of carcinogenesis (Tanwar et al., 2009; Sharma et al., 2010).

As proliferation is a key event in the development and normal functioning of intestine, several reports from animal studies showed that experimental colonic tumors induced by DMH are of epithelial origin and results in increased colonic crypt cellularity and colonic crypt cell proliferation (Richards, 1977; Heitman et al., 1983).

Expression of PCNA by cells during the S and G2 phases of the cell cycle makes the protein a good cell proliferation marker. It also actively participates in a number of the molecular pathways responsible for the life and death of the mammalian cell (Paunesku et al., 2001). In addition to decreasing the incidence of MPLs and histopathological changes, the results from PCNA immunohistochemistry and fluorescent staining indicate that Etoricoxib reduced the proliferation and apoptosis in cancer cells, respectively. The data suggest that the cells don't undergo apoptosis in DMH treated animals as compared to control whereas simultaneous administration of Etoricoxib increased the number of apoptotic cells. It had been reported that there ought to be a balance between the antiproliferative and apoptotic effect of the NSAID such as sulindac sulphide in cultured cells and indicate that apoptotic cells may strongly express the proliferation biomarkers Ki-67 and PCNA (Qias et al., 1997). NS-398, which is a specific COX-2 inhibitor, was also described to reduce cell proliferation of MC-26 cell line (Yao et al., 2004). This effect was associated with a reduction of PCNA, thus increasing the chaperoning of DNA polymerase. Interestingly, meloxicam was also able to downregulate PCNA and cyclin A in HepG2 cell line (hepatocellular carcinoma cells) leading to an inhibition of the cell proliferation (Li et al., 2006).

BrdU is a thymidine analogue which, after incorporation into normal and malignant cells during S-phase of the cell cycle, can be detected using a monoclonal antibody and has several advantages over thymidine autoradography (Ma et al., 2002). Immunohistochemistry of BrdU demonstrates that cell proliferation to be maximum in DMH treated animals alone. As BrdU is thought to be incorporated in the cells during S-phase of cell division, therefore by allowing the knowledge of the percentage of BrdU positive cells the proliferation index can be easily deduced. Moreover, the number of BrdU positive cells was found to be maximum in DMH treated cells followed by Etoricoxib treated cells. Increased number of apoptotic cells and fragmentation of DNA after Etoricoxib co-treatment reveal proapoptotic efficacy of NSAID in colon cancer.

In conclusion, our data suggest that the proliferation marker PCNA and BrdU as found in DMH treated animals strongly indicating the proliferating events in an early stage of carcinogenesis in 6 weeks. However, the Etoricoxib showed the anti-proliferative effect on DMH and Etoricoxib co-treated animals by dominantly downregulating these antigens. Similarly, apoptosis was found to be reduced in DMH-treated animals and Etoricoxib showed the revival of such effect.

Acknowledgement

Financial assistance from the University Grants Commission, Major Research Project [32-614/2006 (SR)] is gratefully acknowledged.

References

Baker AJ, Mooney A, Hughes J, et al (1994). Mesengial cell apoptosis: The major mechanism for resolution of glomerular hypercellularity in experimental mesengial proliferative nephritis. *J Clin Invest*, **94**, 2105-16.

Biasco G, Paganelli GM, Santucci R, et al (1994). Methodological problems in the use of rectal cell proliferation as a biomarker of colorectal cancer risk. *J Cell Biochem*, **19**, 55-60.

Bolton WE, Mikulka WR, Healy CG, et al (1992). Expression of proliferation associated antigens in the cell cycle of synchronized mammalian cells. *Cytometry*, **13**, 117-26.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the priniciple of protein-dye binding. *Anal Biochem*, **72**, 248-54.

Chan TA (2002). Nonsteroidal anti-inflammatory drugs, apoptosis, and colon-cancer chemoprevention. *Lancet Oncol*, **3**, 166-74.

Evan GI, Vousdan HK (2001). Proliferation, cell cycle and apoptosis in cancer. *Nature*, **411**, 342-8.

Hanif R, Pittas A, Feng Y, et al (1996). Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem Pharmacol*, **52**, 237-45.

Heitman DW, Grubbs BG, Heitman TO, et al (1983). Effects of 1,2-dymethylhydrazine treatment and feeding regimen on rat colonic epithelial cell proliferation. *Cancer Res*, **43**, 1153-62.

Kanwar SS, Vaiphei K, Nehru B, et al (2008). Antioxidative effects of non-steroidal anti-inflammatory drugs during the initiation stages of experimental colon carcinogenesis in rats. *J Environ Pathol Toxicol Oncol*, **27**, 89-100.

Kelman Z (1997). PCNA: Structure, functions and interactions. *Oncogene*, **14**, 2796-805.

Kerr JFR, Winterford CM, Harmon BV (1994). Apoptosis. Its significance in cancer and cancer therapy. *Cancer*, **73**, 2013-26.

Leahy KM, Ornberg RL, Wang Y, et al (2002). Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells in vivo. *Cancer Res*, **62**, 625-31.

Li J, Chen X, Dong X, et al (2006). Specific COX-2 inhibitor, meloxicam, suppresses proliferation and induces apoptosis in human HepG2 hepatocellular carcinoma cells. *J Gastroenterol Hepatol*, **21**, 1814-20.

Lowe SW, Lin AW (2000). Apoptosis in cancer. *Carcinogenesis*, **21**, 485-95.

Ma QY, Williamson KE, Rowlands BJ (2002). Variability of cell proliferation in the proximal and distal colon of normal rats and rats with dimethylhydrazine induced carcinogenesis, *World J Gastroenterol*, **8**, 847-52.

Marnett LJ, DuBois RN (2002). COX-2: A target for colon cancer prevention. *Annu Rev Pharmacol Toxicol*, **42**, 55-80.

Mouille B, Robert V, Blachier F (2004). Adaptive increase of ornithine production and decrease of ammonia metabolism in rat colonocytes and hyperproteic diet ingestion. *Am J Physiol*, **287**, 344-51.

Paunesku T, Mittal S, Protic M, et al (2001). Proliferating cell nuclear antigen (PCNA): Ringmaster of the genome. *Int J Radiat Biol*, **77**, 1007-21.

Qiao L, Kozoni V, Koutsos MI, et al (1995). Eicosanoids increase the proliferation rate in human colon carcinomas cell lines and mouse colon cells. *Biochim Biophys Acta*, **1258**, 215-23.

Qiao L, Shiff SJ, Rigas B (1997). Sulindac sulfide induces several subpopulations of colon cancer cells, defined by PCNA/Ki-67 and DNA strand breaks. *Biochim Biophys Acta*, **1359**, 222-32.

Richards TC (1977). Early changes in the dynamics of crypt cell populations in mouse colon following administration of 12,-dimethylhydazine. *Cancer Res*, **37**, 1680-5.

Riendeau D, Percival MD, Brideau C, et al (2001). Etoricoxib (MK-0663): Preclinical profile and comparison with other agents that selectively inhibit cyclooxygenase-2. *J Pharmacol Exp Ther*, **296**, 558-66.

Roediger WEW, Truelove SC (1979). Method of preparing isolated colonic epithelia cells (colonocytes) for metabolic studies. *Gut*, **20**, 484-8.

Saini MK, Sharma P, Kaur J, et al (2009). The cycloxygenase-2 inhibitor Etoricoxib is a potent chemopreventive agent of colon carcinogenesis in the rat model. *J Environ Pathol Toxicol Oncol*, **28**, 39-46.

Sambrook J, et al (1998). Molecular Cloning: A laboratory manual, 2nd ed. Cold spring harber laboratory press, cold spring harber, New York. 192 pp.

Sharma P, Kaur J, Sanyal SN (2010). Effect of etoricoxib, a Cyclooxygenase-2 selective inhibitor on aberrant crypt formation and apoptosis in 1,2 dimethylhydrazine induced colon carcinogenesis in rat model. *Nutr Hosp*, **25**, 39-48.

Stark LA, Reid K, Sansom OJ (2007). Aspirin activates the NF-kappaB signalling pathway and induces apoptosis in intestinal neoplasia in two in vivo models of human colorectal cancer. *Carcinogenesis*, **28**, 968-78.

Taketo MM (1998). Cox-2 and colon cancer. *Inflamm Res*, **47**, 112-6.

Tanwar L, Vaish V, Sanyal SN (2009). Chemoprevention of 1,2-dimethylhydrazine-induced colon carcinogenesis by a non-steroidal anti-inflammatory drug, Etoricoxib, in rats: inhibition of nuclear factor kB. *Asian Pac J Cancer Prev*, **10**, 1141-6.

Wyllie AH, Kerr JFR, Currie Arm (1972). Cell death: The significance of apoptosis. *Int Rev Cytol*, **68**, 251-306.

Yao M (2004). Cyclooxygenase-2 selective inhibition with NS-398 suppresses proliferation and invasiveness and delays liver metastasis in colorectal cancer. *Br J Cancer*, **90**, 712-9.