



RAPID COMMUNICATION

## 5-aminosalicylic acid in combination with nimesulide inhibits proliferation of colon carcinoma cells *in vitro*

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dependent and time-dependent manner ( $t = 5.448$ ,  $P < 0.05$ ;  $t = 4.428$ ,  $P < 0.05$ , respectively).

**CONCLUSION:** 5-ASA and nimesulide may inhibit the proliferation of HT-29 colon carcinoma cells and coadministration of these agents may have additional chemopreventive potential.

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**Key words:** Colorectal cancer; 5-aminosalicylic acid; Nimesulide

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### Abstract

**AIM:** To investigate the effects of 5-aminosalicylic acid (5-ASA) in combination with nimesulide on the proliferation of HT-29 colon carcinoma cells and its potential mechanisms.

**METHODS:** Inhibitory effects of drugs (5-ASA, nimesulide and their combination) on HT-29 colon carcinoma cells were investigated by thiazolyl blue tetrazolium bromide (MTT) assay. Cellular apoptosis and proliferation were detected by TUNEL assay and immunocytochemical staining, respectively.

**RESULTS:** Pretreatment with 5-ASA or nimesulide at the concentration of 10-1000  $\mu\text{mol/L}$  inhibited proliferation of HT-29 colon carcinoma cells in a dose-dependent manner *in vitro* ( $t = 5.122$ ,  $P < 0.05$ ;  $t = 3.086$ ,  $P < 0.05$ , respectively). The inhibition rate of HT-29 colon carcinoma cell proliferation was also increased when pretreated with 5-ASA (100  $\mu\text{mol/L}$ ) or nimesulide (100  $\mu\text{mol/L}$ ) for 12-96 h, which showed an obvious time-effect relationship ( $t = 6.149$ ,  $P < 0.05$ ;  $t = 4.159$ ,  $P < 0.05$ , respectively). At the concentration of 10-500  $\mu\text{mol/L}$ , the apoptotic rate of HT-29 colon carcinoma cells significantly increased ( $t = 18.156$ ,  $P < 0.001$ ;  $t = 19.983$ ,  $P < 0.001$ , respectively), while expression of proliferating cell nuclear antigen (PCNA) was remarkably decreased ( $t = 6.828$ ,  $P < 0.05$ ;  $t = 14.024$ ,  $P < 0.05$ , respectively). 5-ASA in combination with nimesulide suppressed the proliferation of HT-29 colon carcinoma cells more than either of these agents in a dose-

### INTRODUCTION

Inflammatory bowel disease (IBD) is associated with an increased risk of developing colorectal cancer (CRC)<sup>[1-3]</sup>. Primary prevention of CRC in IBD has received more attention in recent years. Epidemiological, experimental and preliminary clinical work strongly suggests that 5-aminosalicylic acid (5-ASA) may have potentially chemopreventive properties against CRC<sup>[6-10]</sup>. On the other hand, aspirin, sulindac and other non-steroid anti-inflammatory drugs (NSAIDs) are also known to reduce the risk of developing colon cancer<sup>[6,11-13]</sup>. However, the potential mechanisms of either 5-ASA or NSAIDs underlying chemoprevention of CRC in IBD remain largely unexplored.

Inhibition of inflammatory cascades involved in cell growth is the popular putative actions of 5-ASA. Arachidonic acid (AA) metabolism has obtained popular attention and can be metabolized in two major pathways including cyclooxygenase (COX) pathway and lipoxygenase (LOX) pathway. Metabolites of the former are prostaglandins which regulate cell proliferation and those of the latter are responsible for the subsequent expression of pro-inflammatory molecules<sup>[14,16]</sup>. 5-LOX and COX-2 activities have recently attracted considerable interest in CRC and data demonstrate that 5-LOX and COX-2 are both up-regulated in CRC<sup>[17,18]</sup>. It was reported that

COX-2 inhibitors or 5-LOX inhibitors may suppress CRC development<sup>[13,17]</sup>. Lots of studies<sup>[13,15,19-21]</sup> indicate that the chemopreventive effect of 5-ASA on CRC is correlated with inhibiting 5-LOX activity and COX-2 is the main target of NSAIDs to suppress CRC development. 5-LOX and COX-2 are the key enzymes for AA metabolism. Since inhibition of one pathway alone, especially the 5-LOX pathway, may change the metabolism of the other, just inhibiting the activity of 5-LOX or COX-2 may not suppress CRC development effectively. However, few studies<sup>[22]</sup> are available on the chemopreventive effect of coadministration of these agents. It makes this method an interesting new alternative to CRC chemoprevention. This study was performed to investigate the effect of 5-ASA and a selective COX-2 inhibitor, nimesulide, on the proliferation of human colon carcinoma cells (HT-29) *in vitro* and its potential mechanisms.

## MATERIALS AND METHODS

### Cell culture

HT-29 colon carcinoma cells were obtained from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences. The cells were maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 U/mL penicillin, 50 µg/mL streptomycin, in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub> at 37°C and routinely trypsinized with 0.25% trypsin solution.

### Reagents

5-ASA, nimesulide, MTT were purchased from Sigma Corp (Steinheim, Germany). FCS was purchased from Hangzhou Sijiqing Biological Corp (Hangzhou, China). Dimethylcarbinol was purchased from Bengbu Chemical Corp (Bengbu, China). TUNEL assay kits for apoptosis were purchased from Nanjing Keygen Corp (Nanjing, China). PCNA polyclonal antibody was purchased from Santa Cruz Biotechnology Corp (California, USA). S-P immunocytochemical assay kits were purchased from Fuzhou Maixin Reagent Corp (Fuzhou, China). Other reagents used in the present study were of analytical grade.

### Cell proliferation assay

Cytostatic effects were measured by MTT assay. The cells were detached with a 0.25% trypsin solution for 5 min. Subsequently, the cells were seeded onto 96-well plates ( $1 \times 10^6$  cells/well), supplemented with 10% FCS and allowed to attach for 24 h before the addition of test compounds (5-ASA, nimesulide, and their combination). Test compounds were diluted in serum-free culture medium. Then the cells were incubated in a medium or at different concentrations of drugs for 48 h, 20 µL of MTT solution (5 g/L) in PBS was added. Four hours later, the medium in each well was removed, and 120 µL of 0.04 mmol/L muriatic isopropanol was added, slightly concussed for 10 min. Dye uptake was measured at 490 nm with an ELISA reader. Five wells were used for each concentration or as a control group. On the other hand, the cells were seeded onto 96-well plates ( $1 \times 10^6$

cells/well) according to the method described above and allowed to attach for 24 h, then treated with test compounds (5-ASA, nimesulide, and their combination). The final concentration was 100 µmol/L. The same medium was added into the control group and dye uptake was then measured according to the method described above. Five wells were used for each test compound or control group.

### Immunocytochemical determination of cellular proliferation and apoptosis in cells

The procedure for the determination of cell proliferation has been described elsewhere<sup>[23]</sup>. In brief, exponentially growing cells ( $3 \times 10^5$ /mL) were trypsinized and seeded onto 24-well plates with a 10 mm × 10 mm sterile slide, supplemented with 10% FCS, allowed to attach for 24 h. The cells were then treated with different doses of drugs (5-ASA, nimesulide, and their combination). After 48-h incubation, the slides were washed twice with PBS. After blocked with normal serum, polyclonal PCNA antibody (1:200) was applied to the slides overnight at 4°C. The following steps were performed in kits following the manufacturer's introductions. Negatively expressed cells were manifested as blue-stained nuclei and the positive cells had brown-yellow cytoplasm or nuclear membrane. The expression of target protein was further semi-quantitated according to the percentage of positively-stained cells and evaluated by two blinded investigators. Negative cells were scored as 0, positive cells less than 25% were scored as 1, 25%-50% as 2, 51%-75% as 3, and higher than 75% as 4, respectively.

Apoptotic cells were visualized with the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP biotin nick end labeling (TUNEL) method<sup>[24]</sup>. In brief, cell slides were subjected to digestion by proteinase K. TdT buffer solution was added to the slides together with 50 U TdT and 5 nmol/L dUTP and incubated at 37°C for 90 min. The reaction was then terminated by adding 30 mmol/L sodium citrate buffer. Peroxidase-conjugated streptavidin from the DAKO kit was added, followed by the addition of DAB. The number of apoptotic cells was counted under a microscope (× 400) and negatively expressed cells were manifested as blue-stained nuclei and the positive cells had brown-yellow cytoplasm or nuclear membrane. Apoptotic index was determined by counting the number of positive cells among 200 cells and indicated as percentages, less than 25% was graded as 1, 26%-50% as 2, 51%-75% as 3, and higher than 76% as 4, respectively.

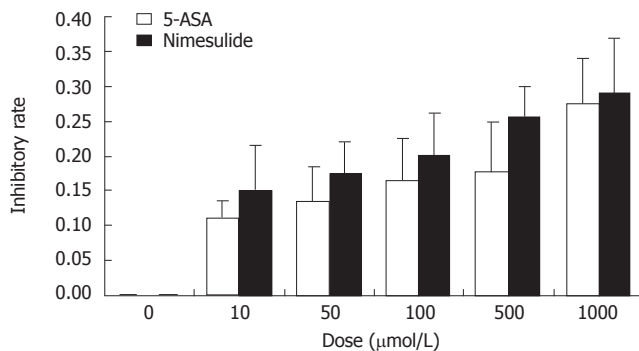
### Statistical analysis

Experimental results were analyzed by ANOVA and *t*-test for multiple comparisons between groups. Data were expressed as mean ± SD. *P* < 0.05 was considered statistically significant.

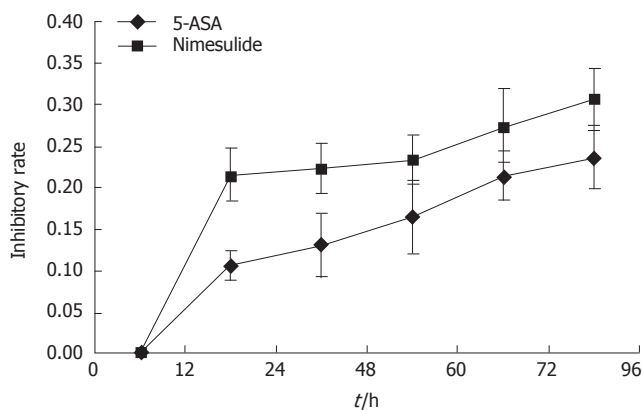
## RESULTS

### Effect of 5-ASA and nimesulide on growth of HT-29 colon carcinoma cells

Pretreatment with 5-ASA or nimesulide at different



**Figure 1** Dose-dependent inhibitory effect of 5-ASA and nimesulide on proliferation of HT-29 colon carcinoma cells. The cells were pretreated with different doses of 5-ASA or nimesulide respectively (10, 50, 100, 500, 1000 μmol/L) for 48 h, then dye uptake was measured at 490 nm using an ELISA reader. Data were expressed as mean ± SE of five independent samples. Compared with the control group, the inhibitory rate was markedly increased in a dose-dependent manner ( $P < 0.05$ ).

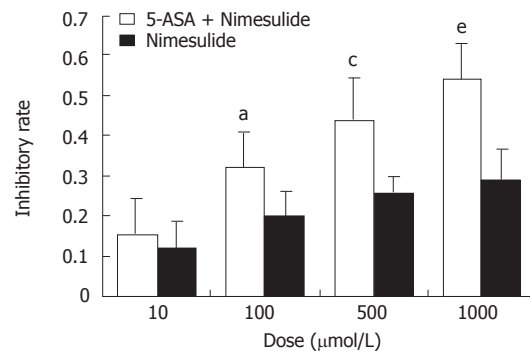


**Figure 2** Time-dependent inhibitory effect of 5-ASA or nimesulide on proliferation of HT-29 colon carcinoma cells. The cells were pretreated with 100 μmol/L 5-ASA or 100 μmol/L Nimesulide respectively for 12, 24, 48, 72, 96 h, then dye uptake was measured at 490 nm using an ELISA reader. Data were expressed as mean ± SE of five independent samples. The inhibitory rate was markedly increased in a time-dependent manner.

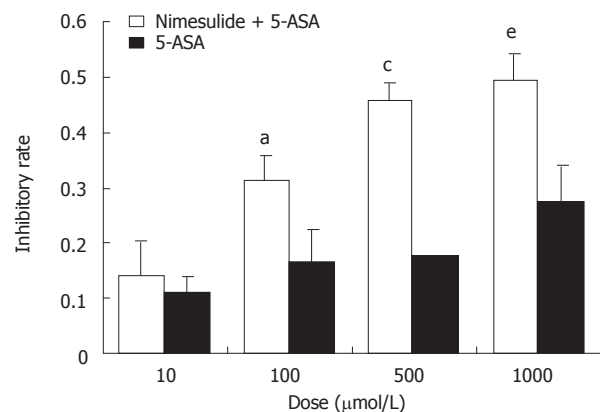
concentration (10-1000 μmol/L) for 12-96 h, inhibited the growth of HT-29 colon carcinoma cells in a dose and time-dependent manner (Figures 1 and 2). However, the suppression of 5-ASA or nimesulide had no statistical significance.

#### Effect of combined 5-ASA and nimesulide on the growth of HT-29 colon carcinoma cells

Interestingly, the growth of HT-29 colon carcinoma cells was inhibited dose-dependently when pretreated with different doses of combined 5-ASA and nimesulide. Combined 5-ASA (final concentration 100 μmol/L) and nimesulide (final concentration 10-1000 μmol/L) inhibited the proliferation of HT-29 colon carcinoma cells in a dose-dependent manner, being more potent than corresponding dose of nimesulide (Figure 3). Similarly, combined nimesulide (final concentration 100 μmol/L) and 5-ASA (final concentration 10-1000 μmol/L) also inhibited the proliferation of these cells dose-dependently, being more



**Figure 3** Inhibitory effect of 5-ASA in combination with nimesulide on proliferation of HT-29 colon carcinoma cells. The cells were pretreated with 100 μmol/L 5-ASA (final concentration) in combination with different doses of nimesulide (10, 100, 500, 1000 μmol/L) for 48 h, then dye uptake was measured at 490 nm using an ELISA reader. The inhibitory rate was markedly increased in a dose-dependent manner. Data were expressed as mean ± SE of three independent samples. <sup>a,c,e</sup> $P < 0.05$  vs the corresponding concentration of nimesulide.



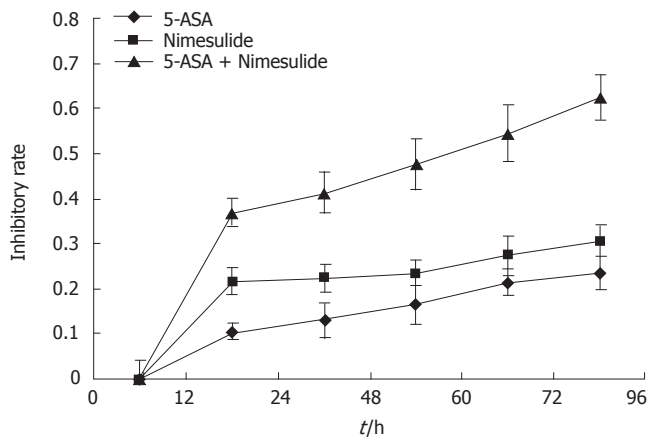
**Figure 4** Dose-dependent inhibitory effect of nimesulide in combination with 5-ASA on proliferation of HT-29 colon carcinoma cells. The cells were pretreated with 100 μmol/L nimesulide (final concentration) in combination with different doses of 5-ASA (10, 100, 500, 1000 μmol/L) for 48 h, then dye uptake was measured at 490 nm using an ELISA reader. The inhibitory rate was markedly increased in a dose-dependent manner. Data were expressed as mean ± SE of three independent samples. <sup>a,c,e</sup> $P < 0.05$  vs the corresponding concentration of 5-ASA.

potent than corresponding dose of 5-ASA (Figure 4).

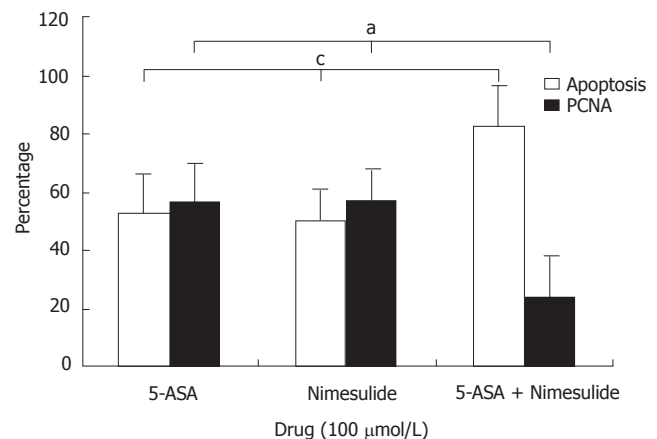
Pretreatment with combined 5-ASA and nimesulide (final concentration 100 μmol/L) for 12-96 h, inhibited the growth of HT-29 colon carcinoma cells in a time-dependent manner (Figure 5).

#### Effect of 5-ASA or nimesulide alone or their combination on proliferation and apoptosis of HT-29 colon carcinoma cells

The proliferation of HT-29 carcinoma cells was significantly inhibited when pretreated with different doses of 5-ASA. The expression of PCNA was remarkably down-regulated while cell apoptosis was remarkably up-regulated in a dose-dependent manner. Similarly, nimesulide inhibited the proliferation of HT-29 carcinoma cells and promoted cell apoptosis significantly (Table 1). Co-administration of 5-ASA and nimesulide inhibited the proliferation of HT-29 colon carcinoma cells and promoted cell apoptosis



**Figure 5** Time-dependent inhibitory effect of 5-ASA in combination with nimesulide on proliferation of HT-29 colon carcinoma cells. The cells were pretreated with 5-ASA in combination with nimesulide at the final dose of 100  $\mu\text{mol/L}$  for different times (0, 12, 24, 48, 72, 96 h), then dye uptake was measured at 490 nm using an ELISA reader. Data were expressed as mean  $\pm$  SE of five independent samples.



**Figure 6** Inhibitory effect of 5-ASA in combination with nimesulide on proliferation and apoptosis of HT-29 colon carcinoma cells. The cells were pretreated with 5-ASA in combination with nimesulide (the final concentration was 100  $\mu\text{mol/L}$ ) for 48 h, then the expression of PCNA and apoptosis was detected with kits according to the manufacturer's introductions. Data were expressed as mean  $\pm$  SE of three independent samples. <sup>a</sup> $P < 0.05$ , <sup>c</sup> $P < 0.05$ , vs 5-ASA or nimesulide alone.

**Table 1** Effect of 5-ASA and nimesulide administrated alone on apoptosis and proliferation of HT-29 colon carcinoma cells

Group	Dose ( $\mu\text{mol/L}$ )	Percentage of apoptosis (%)	PCNA label index (%)
Control	0	22.12 $\pm$ 3.61	75.13 $\pm$ 2.55
5-ASA	10	31.82 $\pm$ 2.61	61.73 $\pm$ 7.51
	100	52.96 $\pm$ 3.73 <sup>a</sup>	56.47 $\pm$ 3.21 <sup>a</sup>
	500	75.76 $\pm$ 3.28 <sup>a</sup>	31.13 $\pm$ 1.96 <sup>a</sup>
Nimesulide	10	29.13 $\pm$ 2.73	63.37 $\pm$ 3.23
	100	50.46 $\pm$ 1.83 <sup>a</sup>	57.42 $\pm$ 2.14 <sup>a</sup>
	500	70.54 $\pm$ 2.33 <sup>a</sup>	33.15 $\pm$ 1.87 <sup>a</sup>

The cells were pretreated with different doses of 5-ASA and nimesulide respectively (10–500  $\mu\text{mol/L}$ ) for 48 h, then the expression of PCNA and apoptosis were detected with kits according to the manufacturer's introductions. Data were expressed as mean  $\pm$  SE of three independent samples. <sup>a</sup> $P < 0.001$  vs control group.

significantly, being more potent than the corresponding dose of 5-ASA and nimesulide (Figure 6).

## DISCUSSION

IBD is associated with an increased risk of developing CRC at sites of chronic colon inflammation<sup>[1–5]</sup>. Primary prevention of CRC in IBD has received more attention in recent years. A body of evidence now suggests that 5-ASA can protect against development of CRC in individuals with IBD<sup>[6–10]</sup>. Eaden *et al.*<sup>[8]</sup> performed a case-control study comparing 102 cases of CRC in ulcerative colitis (UC) with matched IBD controls, and found that a history of regular 5-ASA use significantly reduces the risk of CRC by 75%. When the results were adjusted for other variables, the protective effect of 5-ASA reduced the risk of CRC by 81% at a dose of 1.2 g/d or greater. In the present study, 5-ASA inhibited the proliferation of HT-29 carcinoma cells and promoted cell apoptosis *in vitro* in a dose- and time- dependent manner, indicating 5-ASA has significant chemopreventive properties against CRC, which is also

consistent with previous reports<sup>[18,25,26]</sup>.

5-ASA is the most commonly prescribed anti-inflammatory agents for IBD and exerts its action on IBD by inhibiting 5-LOX activity. Wang *et al.*<sup>[18]</sup> performed a follow-up study of 99 patients with CRC, and found that 5-LOX expression is significantly higher in colon carcinoma cells than in normal colon mucosa, and correlates with Duke's staging, diversion and infiltration of CRC. The biological functions of 5-LOX in cancer cells have been examined using pharmacological inhibitors and/or antisense technology<sup>[15,17]</sup>. It was reported that 5-LOX and its metabolites, especially LTB<sub>4</sub> are over-expressed in colon cancer, the inhibitors of 5-LOX activity may remarkably inhibit the proliferation of colon carcinoma cells<sup>[17]</sup>. It also showed in our other study that 5-ASA may down-regulate 5-LOX expression in HT-29 carcinoma cells in a dose-depend manner (data not shown), indicating that 5-ASA anticancer property may be correlated with inhibiting 5-LOX activity.

On the other hand, many studies over the past decade have demonstrated that NSAIDs, including indomethacin, piroxicam and sulindac as well as aspirin, have a potent inhibitory effect on the growth of colorectal cancer<sup>[6,11–13]</sup>. It is postulated that this antitumor effect is mediated by inhibiting the COX-2 activity<sup>[20,21,27]</sup>. Over-expression of COX-2 in CRC may activate epidermal growth factor receptor, up-regulate the bcl-2 gene to promote cell proliferation, and inhibit cell apoptosis<sup>[28]</sup>. Being consistent with these effects, nimesulide, a selective COX-2 inhibitor, could down-regulate COX-2 expression in a dose-depend manner (data not shown) and significantly inhibit the proliferation of HT-29 carcinoma cells and promote cell apoptosis *in vitro* in a dose- and time- dependent manner.

In the present study, 5-ASA or nimesulide suppressed the proliferation of HT-29 carcinoma cells and promoted cell apoptosis *in vitro* remarkably, the possible mechanism may correlate with the inhibition of 5-LOX or COX-2 activity when pretreated with 5-ASA or nimesulide alone.



However, both 5-LOX and COX-2 were up-regulated in colon cancer cells. Ye *et al*<sup>[22]</sup> demonstrated that inhibition of COX-2 or 5-LOX reduces the tumor size. Treatment with a COX-2 inhibitor could decrease the PGE2 level and increase the LTB4 level. In contrast, treatment with a 5-LOX-inhibitor reduced the LTB4 level and did not change the PGE2 level, suggesting that just inhibiting 5-LOX or COX-2 activity alone may not suppress CRC development effectively<sup>[22]</sup>. However, treatment with COX-2 and 5-LOX inhibitors further could inhibit the tumor growth<sup>[29-32]</sup>, which was accompanied with the down regulation of PGE2 and LTB4 simultaneously<sup>[22]</sup>. Both 5-ASA and nimesulide, which are the most commonly prescribed anti-inflammatory agents in clinic, could suppress colon cancer proliferation by inhibiting 5-LOX or COX-2 activity. Can we hypothesize that co-administration of 5-ASA and nimesulide may inhibit colon cancer cell proliferation more effectively? Co-administration of these agents has more chemopreventive properties against the proliferation of HT-29 carcinoma cells *in vitro* than administration of 5-ASA or nimesulide alone, showing a dose and time dependent correlation.

In conclusion, co-administration of 5-ASA and NSAIDs, especially at a low dose may have chemopreventive effect on CRC, which may reduce the side effect of these agents when administrated alone. This approach should present a superior anticancer profile, and a new therapy for cancers associated with IBD.

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