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# Sulindac enhances cell proliferation in DMH-treated mouse colonic mucosa

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**Abstract.** In a previous study we reported that the NSAID sulindac had a marked inhibitory effect on the development of colonic tumours in mice treated with the carcinogen 1,2-dimethylhydrazine (DMH). In this study we examined the effects of sulindac in respect of cell-kinetic changes in mouse colonic mucosa as determined by flash labelling with the thymidine analogue bromodeoxyuridine (BrdUrd) at varying intervals during the process of colonic carcinogenesis. We also investigated the possibility that these changes may be modulated by misoprostol a prostaglandin $E_1$ analogue. Four groups of 36 mice each were treated for 18 weeks with the following drug/s respectively: (1) DMH; (2) DMH and sulindac; (3) DMH, sulindac and misoprostol; and (4) DMH and misoprostol. Three animals from each group were killed each week between the sixth week and the eighteenth week after the start of the experiment. A 1-h flash label technique was employed and paraffin sections of colonic mucosa were examined. For each animal a total of 50 perfect axially cut crypts were chosen and the following parameters determined: crypt length, labelling index and labelling index distribution: the data were analysed using the computer program GLIM. For each of the four groups, crypt lengths increased significantly with the duration of treatment with no significant difference between the groups. In sulindac-treated animals the labelling index for all positions increased with duration of treatment whereas for animals not treated with sulindac there was no significant difference in labelling index with respect to duration of treatment. The administration of misoprostol did not appear to significantly alter the effects of sulindac. It is postulated that the observed increase in cell proliferation could be a compensatory phenomenon occurring secondary to loss of crypt epithelial cells by apoptosis induced by sulindac. Also the finding of an increase in labelling index mediated by a chemopreventive agent indirectly questions the rationale behind the therapeutic manipulation of crypt cell proliferation in order to reduce the risk of colon cancer.

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There is currently a lot of interest in the use of non-steroidal anti-inflammatory drugs (NSAIDs) as possible chemopreventive agents in relation to colorectal carcinoma. NSAIDs inhibit the synthesis of prostaglandins which have long been known to be of relevance in carcinogenesis (Marnett *et al*. 1992); but the more recent interest in NSAIDs in respect of colorectal neoplasia dates to the clinical case reports of Waddell and colleagues (1983, 1989) who were first to describe the regression of tumours in patients with adenomatous polyposis after treatment with the NSAID sulindac. In a previous study we investigated this phenomenon in a mouse model of chemical colonic carcinogenesis (Moorghen *et al*. 1988). In these experiments evidence of tumour regression was lacking but sulindac had a marked inhibitory effect on the development of adenomatous polyps. The formation of micro-adenomas which preceded the appearance of macroscopic tumours was also impaired suggesting that sulindac acted early in the multistep process of colonic carcinogenesis.

Following the chronic administration of 1,2-dimethylhydrazine (DMH) there is an initial hyperplastic response in the mouse colonic mucosa which is characterized by a progressive increase in crypt length together with dilatation and tortuosity of the crypts. The number of labelled cells in the crypt also increases due to expansion of the proliferative compartment which is associated with upwards movement of the cut-off position (Chang 1978). Foci of dysplasia later arise within this setting of generalized hyperplasia and take the form of microadenomata which are fan-shaped structures composed of atypical glandular elements situated initially near the mucosal surface. These lesions grow into sessile polyps which become macroscopically evident and are later succeeded by the development of invasive adenocarcinoma in a small proportion of cases. These changes bear many similarities with the morphological changes observed in human adenomatous poloyposis. As regards the effects of NSAIDs on the human intestine, inflammatory changes, ulceration and perforation are well recognized complications (Price 1992). These changes are partly related to reduced prostaglandin production which normally protects the epithelium. The drug misoprostol is a prostaglandin $E_1$ analogue with known muco-protective properties; this drug is therefore useful clinically in protecting gastric and duodenal mucosa against NSAID-induced damage (Shield 1995). With respect to the tumour-inhibitory effects of sulindac it is envisaged that this is related to prostaglandin-mediated inhibition of cell proliferation which may possibly therefore be modulated by misoprostol.

The aims of this study were to examine the effects of sulindac in respect of cell-kinetic changes as determined by bromodeoxyuridine labelling in mouse colonic mucosa at varying intervals during the process of DMH-induced carcinogenesis. The possibility that these changes may be altered by the administration of misoprostol was also investigated.

## MATERIALS AND METHODS

### Animals

A total of 196 female Balb/C mice aged between 5 and 8 weeks at the start of the experiment were used in this study.

### Treatment schedule

Four groups of 36 animals each were used in the main experiment. These four groups of mice were treated with the following drug/s respectively (1) DMH, (2) DMH and sulindac, (3) DMH, sulindac and misoprostol, and (4) DMH and misoprostol. Three animals from each of the four groups were killed each week between the sixth week and the 18th week

after the start of the experiment. Any animal that became distressed prior to completion of the treatment period was killed immediately and excluded from the study. A further 52 animals were used in a separate fraction of labelled mitoses (FLM) experiment which was designed for the purpose of determining the duration of S-phase (Ts). In this experiment all 52 animals were treated with DMH for a period of 15 weeks. The control group comprised 26 mice all of which were allowed free access to tap drinking water. The remaining 26 animals received sulindac dissolved in drinking water.

**Carcinogen**

1,2-Dimethylhydrazine hydrochloride (Aldrich Chemical Co. Ltd, Gillingham, Dorset, UK) was dissolved in EDTA (0.4%) and normal saline (0.9%) and brought to pH 6.5 with sodium hydroxide. This solution was injected subcutaneously (25 mg DMH base/kg body weight) once weekly for up to 18 weeks.

**Sulindac**

Sulindac (Merck Sharp and Dohme Ltd, Hoddesdon, Herts, UK) was administered orally throughout the 18-week experimental period. The daily intake of water was monitored at regular intervals and the concentration of sulindac in drinking water was calculated so as to correspond to an average daily dose of 5 mg per kg per animal.

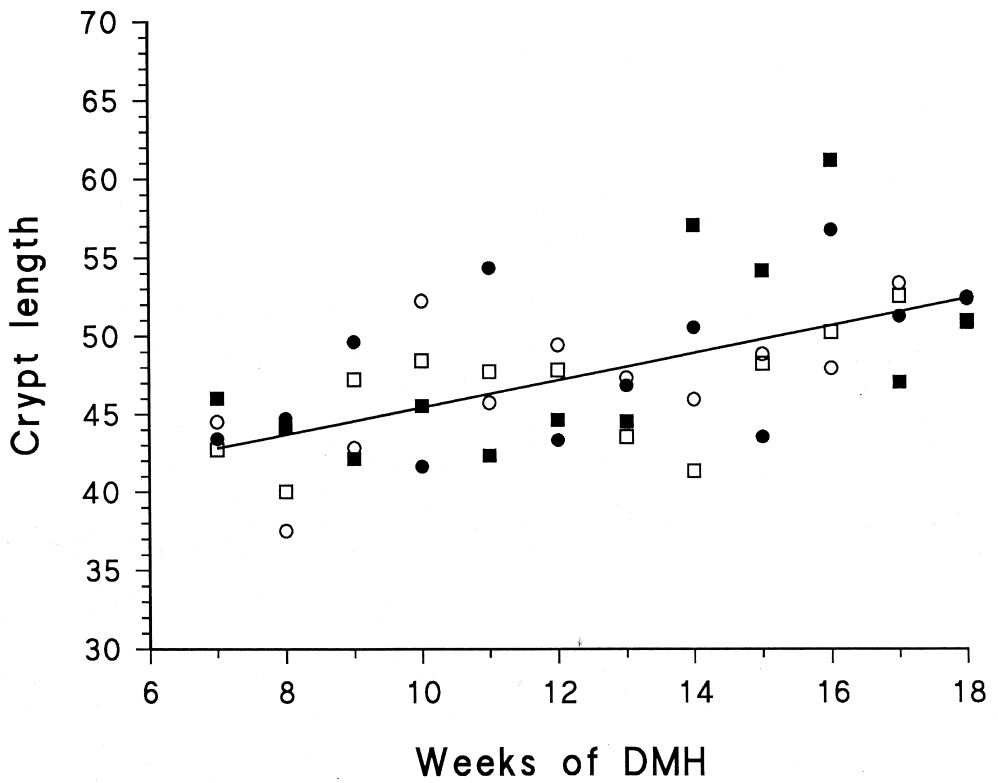


**Figure 1.** Crypt length (mean number of cells per crypt column) plotted against duration of treatment with DMH in weeks with a fitted line representing data from all four treatment groups. ○, control; □, misoprostol; ●, sulindac; ■, both.

**Misoprostol**

Misoprostol (Searle Co. Ltd, High Wycombe, Bucks, UK) was administered daily as a suspension in sterile water by gavage at a dose corresponding to 15 $\mu$g per kg per day throughout the entire duration of the experiment concurrently with DMH with or without sulindac.

**Bromodeoxyuridine**

Bromodeoxyuridine (Sigma, Poole, Dorset, UK) dissolved in sterile water was administered by intraperitoneal injections at a dose corresponding to 50 mg per kg animal weight. In the main experiment each animal was killed 1 h after the administration of BrdUrd. In the FLM experiment animals were killed at $1\frac{1}{2}$, 2, $2\frac{1}{2}$, 3, 4, $6\frac{1}{2}$, 9, 10, 11, 12, 13, 14 and 16 h. Two animals were used at each time-point.

**Tissue handling**

Animals were killed by neck dislocation. The entire intestinal tract was resected and opened longitudinally. A transverse block was taken from the mid-point of the distal quarter of the large intestine and placed in buffered formalin.

**Immunohistochemistry**

Samples were embedded in paraffin and 4 $\mu$m sections cut. The first section was stained with haematoxylin and eosin; every subsequent fifth section was placed on slides which were treated with 1M HCl at 60°C for 10 min. A standard immunoperoxidase technique was

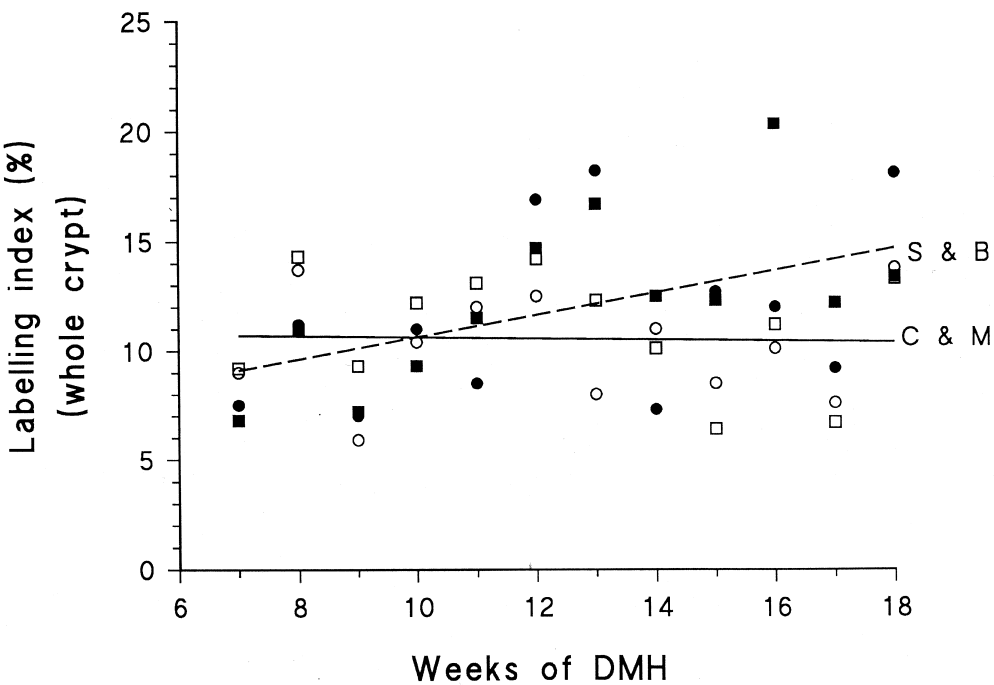


**Figure 2.** Labelling index (%) for the whole crypt plotted against duration of treatment with DMH in weeks. The two fitted lines represent combined data for animals treated with (S&B) or without (C&M) sulindac. ○, control; □, misoprostol; ●, sulindac; ■, both.

employed using an anti-bromodeoxyuridine antibody (Sera-labs, Crawley Down, Sussex, UK) and the colour reaction developed using diaminobenzidine.

**Counting**

In the main experiment, for each animal used a total of 50 perfect axially-cut crypts were chosen where the base was at a maximum depth in comparison with other crypts and in close apposition to the muscularis mucosae. The left-hand crypt column was analysed for each crypt. The lowermost cell nucleus in each column was designated as position 1. The total number of cells in each column and the position of each immunostained cell nucleus were recorded. In the FLM experiment a total of 100 mitoses were examined for each animal and the fraction of labelled mitoses was calculated.

**Statistics**

The changes in crypt lengths and labelling indices with respect to duration of treatment were analysed using the computer program GLIM (Baker & Nedler 1978). For the FLM experiment the fraction of labelled mitoses was plotted against time. The duration of the S-phase was estimated as the distance between the two half-maximum positions on a fitted quadratic curve.

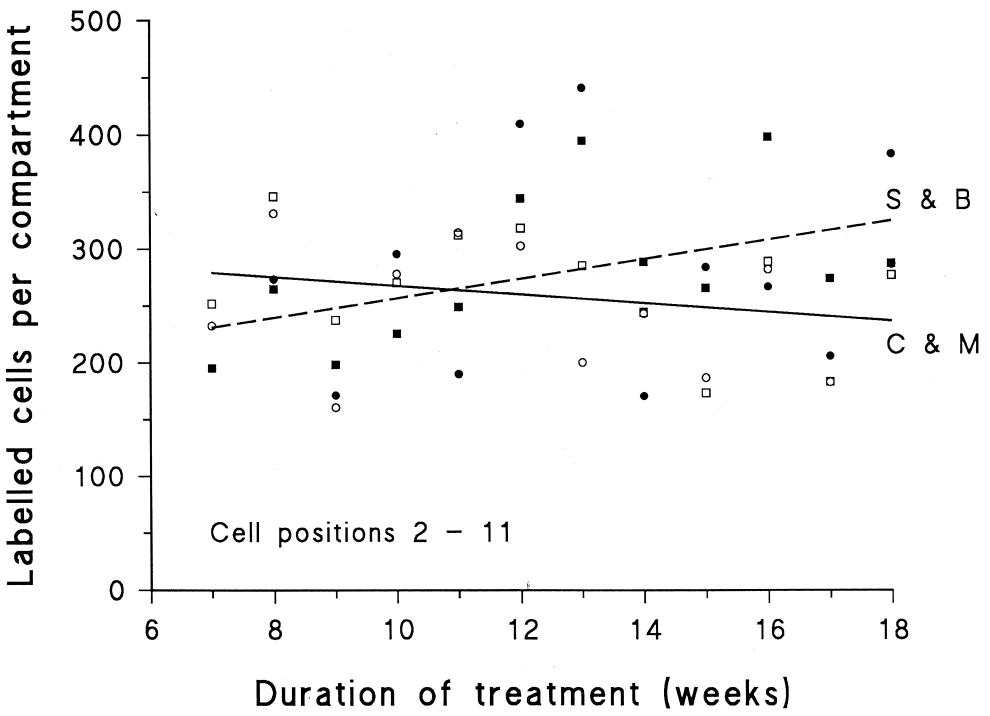


**Figure 3.** Labelled cells at positions 2–11. The two fitted lines represent combined data for animals treated with (S&B) or without sulindac (C&M). ○, control; □, misoprostol; ●, sulindac; ■, both.

## RESULTS

### Specimens examined

In the main experiment 30 animals did not survive until the end of the treatment periods and were therefore excluded from the study. At each time-point tissues from at least two animals were available except for animals killed at week 11 where only one animal was available for each of the following treatment combinations: (a) DMH and misoprostol, (b) DMH and sulindac, and (c) DMH, sulindac and misoprostol.

### Crypt lengths

For each of the four treatment groups crypt lengths increased significantly with duration of treatment (Figure 1), with no significant difference between the groups. The equation representing all four treatment groups from the fitted line is as follows: crypt length $=36.7+0.87\,T$; here and throughout T denotes time in weeks. The 95% confidence interval is 0.6–1.1 for the increase in the number of cells per week.

### Labelling data

In the following section the effect of misoprostol was never significant, nor was there an interaction with the effect of sulindac. The labelling index for the whole crypt increased significantly with duration of treatment in both groups of animals receiving sulindac (labelling index $=4.7+0.59\,T$) whereas for those animals not receiving sulindac (labelling

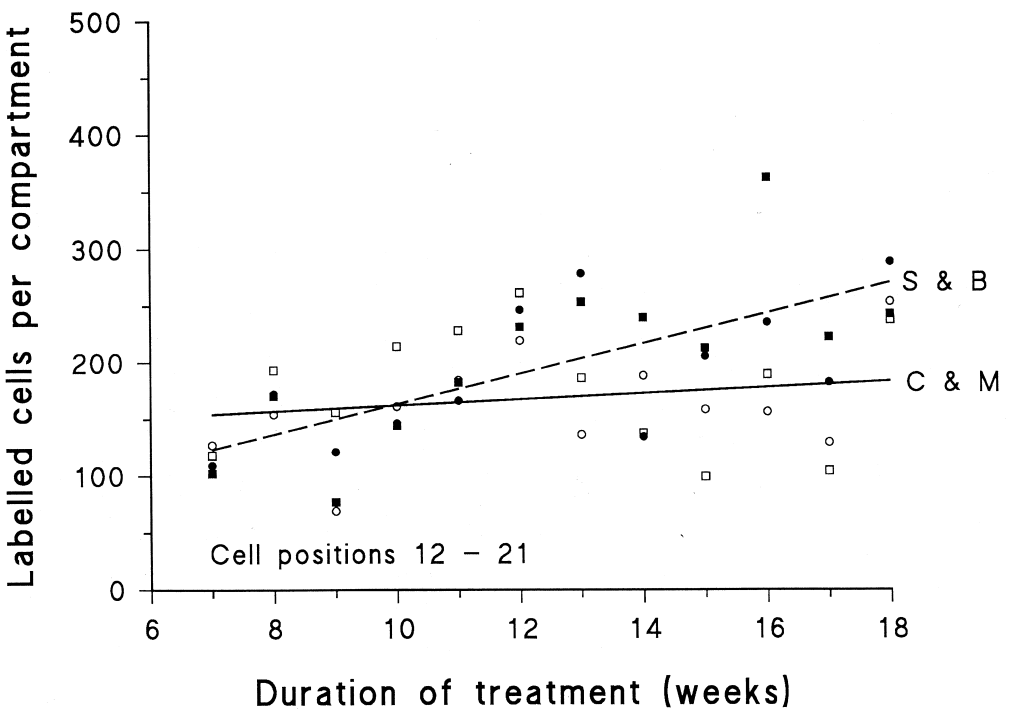


**Figure 4.** Labelled cells at positions 12–21. The two fitted lines represent combined data for animals treated with (S&B) or without sulindac (C&M). ○, control; □, misoprostol; ●, sulindac; ■, both.

index=11.5−0.07 T) there was no significant change in overall labelling index between the sixth and eighteenth week of treatment (Figure 2). The difference between the slopes of the two graphs in Figure 2 was significant (t=2.44, $P<0.05$). The data were also analysed in terms of number of labelled cells per crypt. Thus the number of labelled cells in the sulindac-treated groups increased with time (number of labelled cells=4.65+0.4 T); whereas the number of labelled cells in the non-sulindac treated groups (number of labelled cells=4.65+0.06 T) did not. The difference between the slopes for these two equations were also significant (t=2.66, $P<0.05$). Thus treatment with sulindac was associated with an increase in labelling index over the duration of the experiment. The number of labelled cells between positions 2 and 11, 12 and 21 and above 21, respectively, were also analysed. In sulindac-treated animals the labelling index for all positions increased with duration of treatment as shown by the fitted lines in Figures 3–5 whereas for animals not receiving sulindac there was no significant increase in labelling index with respect to time. The lines fitted in Figures 3, 4 and 5 are represented by the following equations:

Positions 2–11 (Figure 3): number of labelled cells=0.306–0.038 T for non-sulindac-treated animals and 1.71–0.86 T for sulindac-treated animals; comparison between the two fitted lines revealed a significant difference (t=2.27, $P<0.05$).

Positions 12–21 (Figure 4): number of labelled cells=1.36+0.382 T for non-sulindac treated animals and 0.303+0.133 T for sulindac-treated animals; comparison between the two fitted lines revealed a significant difference (t=2.56, $P<0.05$).

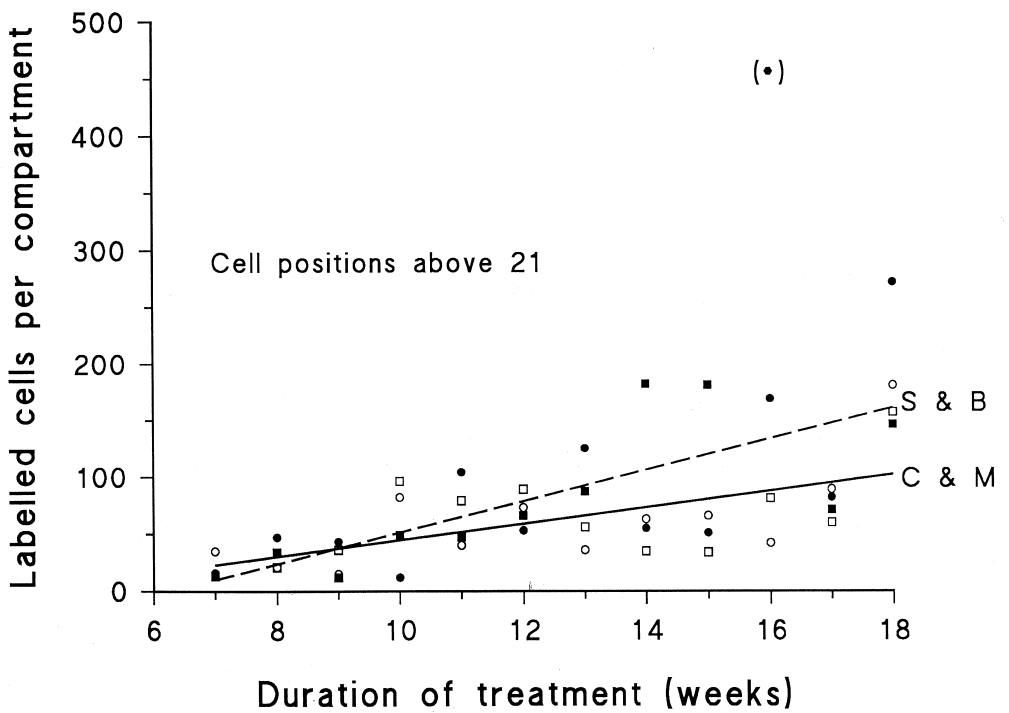


**Figure 5.** Labelled cells above positions 21. The two fitted lines represent combined data for animals treated with (S&B) or without sulindac (C&M). ○, control; □, misoprostol; ●, sulindac; ■, both.

Above position 21 (Figure 5): number of labelled cells $= -0.28 + 0.0726$ T for non-sulindac-treated animals and $-1.22 + 0.0176$ T for sulindac-treated animals; comparison between the two fitted lines revealed a significant difference ($t = 2.02$, $P < 0.05$).

For each of the four treatment groups data relating to weeks 14 to 18 were pooled and four labelling index distribution curves were derived. The positions corresponding to 50% maximum label were also determined (Figure 6) and no change was detected to indicate movement of the cut-off position. From the cumulative labelling index for the pooled data it is possible to estimate the migration rates at different cell positions in the crypt assuming $t_S$ is constant and equal to 12.4 h (see Wright & Alison). These reach maximum values of about 0.4 positions per hour in the two control groups and 0.52 and 0.62 in the sulindac-treated groups (Figure 7).

**FLM data**

The durations of S-phase for the sulindac-treated and non-sulindac-treated animals were estimated as $12.3 \pm 0.3$ and $12.4 \pm 0.5$ h, respectively (Figure 8).

## DISCUSSION

In these experiments we undertook an analysis of cell-kinetic changes which take place in mouse colonic mucosa following the administration of DMH. We investigated whether these

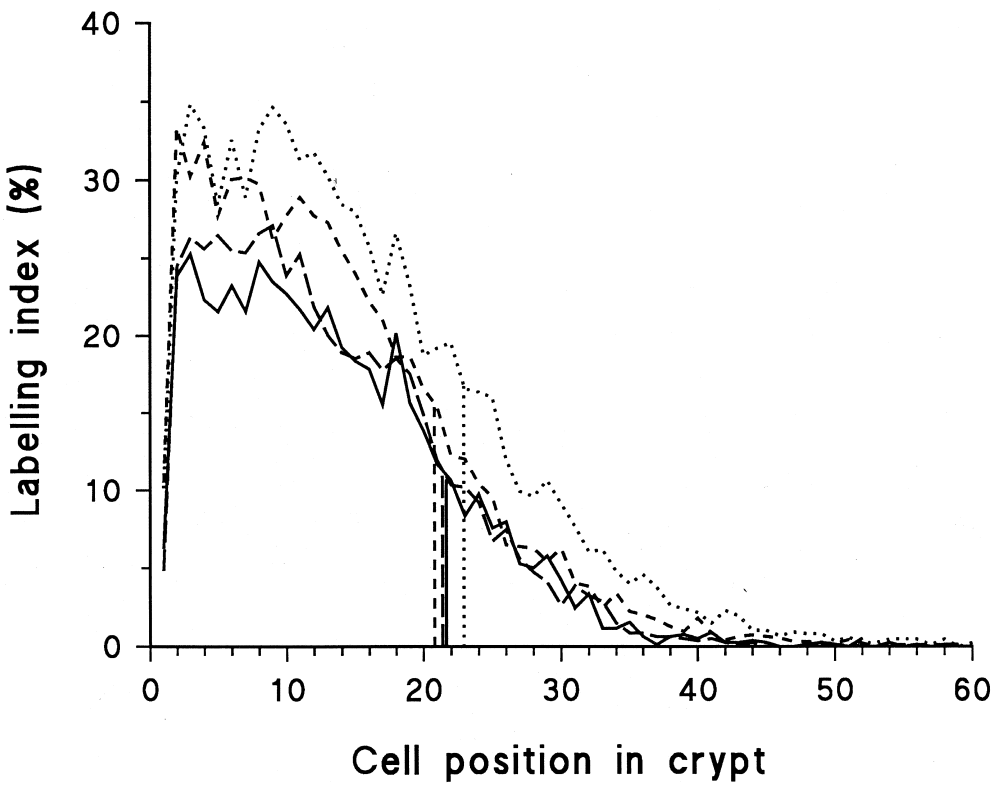


**Figure 6.** Labelling index distribution for the four treatment groups derived from pooled data for animals treated for between 14 and 18 weeks.

changes could be modulated by the NSAID sulindac and the $PGE_1$ analogue misoprostol. The administration of DMH resulted in crypt hyperplasia with a progressive increase in crypt length and increased numbers of BrdUrd-labelled cells within the crypt. These findings are consistent with the detailed studies of Chang (1978) and Chang *et al.* (1979). The drug sulindac is known to possess chemopreventive properties with respect to colonic neoplasia in both humans and rodents (Moorghen *et al.* 1988, Rigau *et al.* 1991, Labayle *et al.* 1991). In our previous study we used a similar dose of sulindac as in the present experiments; incidentally this corresponded to mean serum levels of 2.8, 0.7 and 0.6 mg/ml of sulindac sulphone, sulindac and desoxysulindac, respectively (Moorghen 1992). Bacterial flora in the colon plays an important role in reducing sulindac to its active metabolite which would reach higher local concentrations in the bowel lumen than in the bloodstream (Strong *et al.* 1985). In the previous experiments the administration of sulindac had a marked inhibitory effect on the formation of both macroscopic colonic tumours and microscopic adenomatous foci, but tumour regression was not demonstrable (Moorghen *et al.* 1988). However, other workers using twice our dose of sulindac have managed to provide evidence of tumour regression in the rat DMH model of colon carcinogenesis (Skinner *et al.* 1991, Fishbach *et al.* 1994). Other NSAIDs such as piroxicam and aspirin have also been shown to inhibit the development of chemically induced tumours in rodents (Reddy *et al.* 1992, 1993). The results of our previous study led us to postulate that sulindac exerted its effect at an early stage in the process of

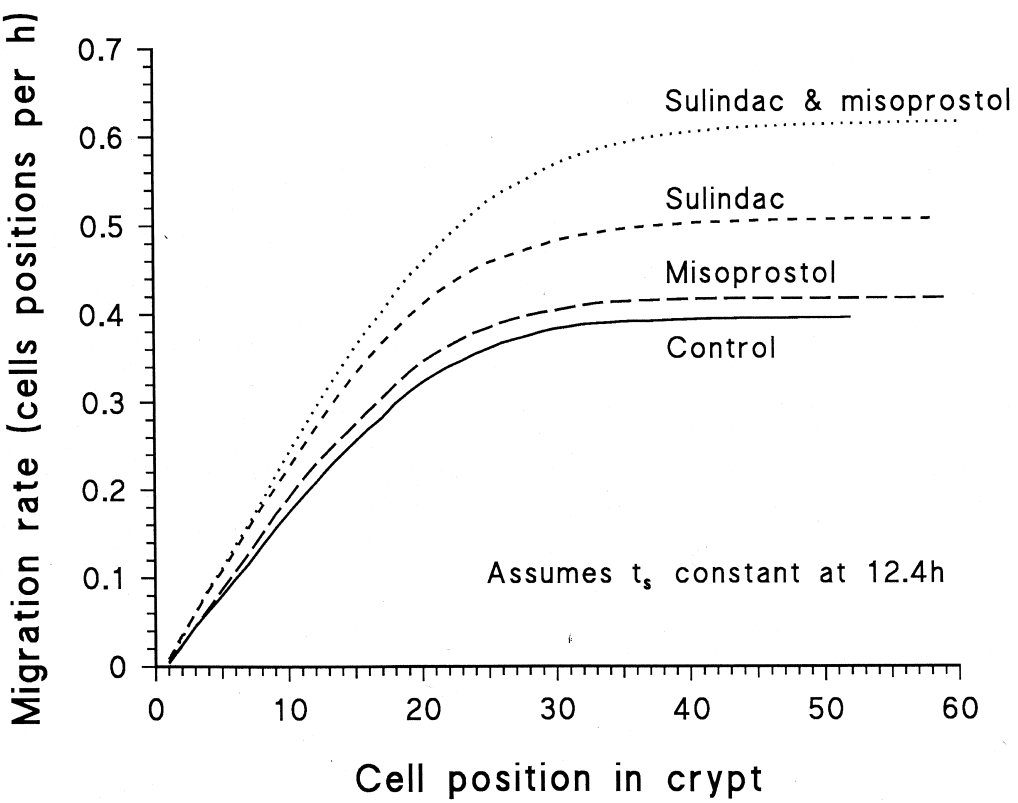


**Figure 7.** Migration rate (derived from cumulative labelling index for animals treated between 14 and 18 weeks) plotted against cell position.

colonic carcinogenesis prior to microadenoma formation possibly by inhibiting the initial phase of crypt hyperplasia. We now find that sulindac failed to reduce the progressive increase in crypt length (Figure 1). Furthermore sulindac had the unexpected effect of increasing the number of labelled cells in all compartments of the crypt without any corresponding alteration in $t_s$. Even if there were to be a change in $t_s$, our FLM results preclude it being large enough to account for the magnitude of the differences in labelling index. There is thus definite evidence of enhanced cell proliferation under conditions where sulindac is expected to inhibit tumour development. The mechanisms involved in the tumour-inhibitory effects of sulindac are unclear. Sulindac and other NSAIDs are well characterized in terms of the inhibition of cyclooxygenases which are key enzymes in prostaglandin synthesis. Whether alterations in cellular levels of prostaglandins are directly related with the suppression of tumour growth remains unproven (Marnett 1992). In our experiments we also investigated whether any sulindac-mediated effects could be modulated by the $PGE_1$ analogue misoprostol. Sulindac and other NSAIDs are commonly associated with the development of ulceration in the upper alimentary tract in human subjects. The use of misoprostol provides a means of inhibiting this troublesome side-effect. The mechanisms involved relate to the inhibition of a variety of cytokines involved in inflammation and repair (Shield 1995). In our experiments misoprostol failed to modulate the effects of sulindac in respect of crypt cell proliferation in the DMH-treated mouse colon. This suggests that local

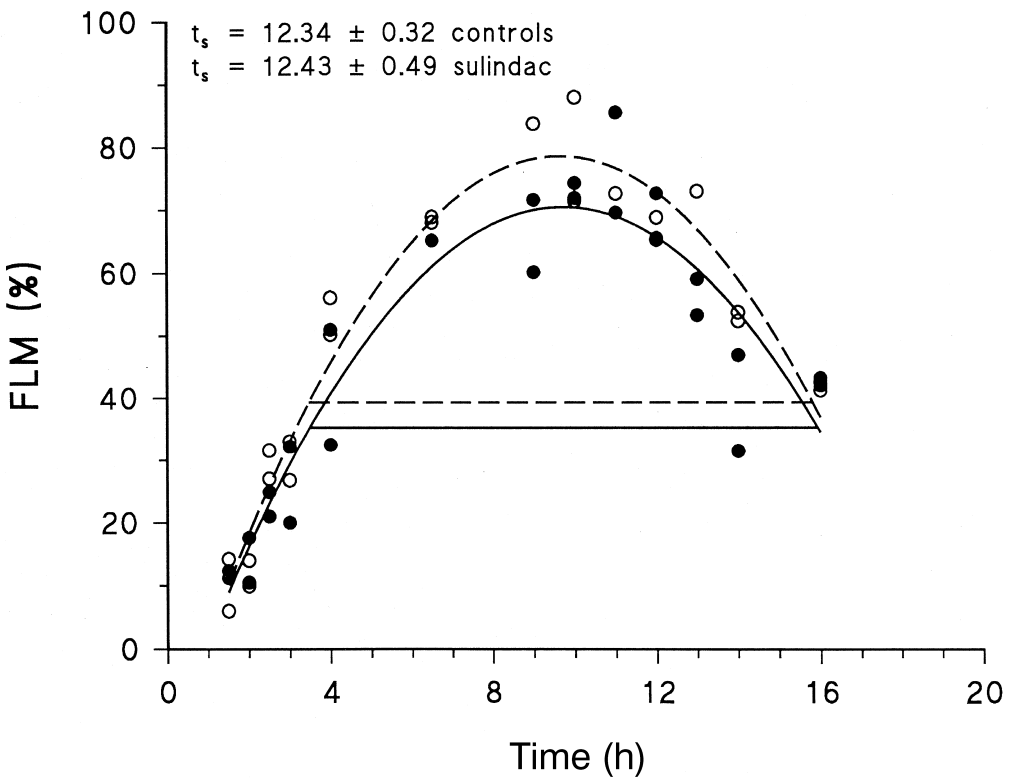


**Figure 8.** Fraction of labelled mitoses curves for animals treated with DMH with and without sulindac. A total of 100 mitoses were examined for each time-point. ○, – – –, controls; ●, ——, sulindac.

levels of $PGE_1$ are perhaps not relevant to the tumour inhibitory properties of sulindac. Other workers have indeed shown that misoprostol failed to alter the incidence of DMH-induced colonic tumours in the rat (Lawson *et al.* 1994). In the human large bowel NSAID-associated changes include the development of a lymphocytic infiltrate in the mucosa with focal epithelial cell death by apoptosis (Lee *et al.* 1993). As regards an effect on cell proliferation *per se* it would appear that NSAIDs are inhibitory to proliferative activity in regenerative epithelium adjacent to ulcerated areas in the small bowel (Levi *et al.* 1992) but in the rodent large bowel the NSAID aspirin increases cell proliferation. In the studies of Craven *et al.* (1988, 1992) both indomethacin and aspirin were associated with raised labelling indices and extension of the proliferative zone; these findings published at a time before it was realized that aspirin is of potential benefit in respect of colon cancer led the authors to postulate that aspirin may indeed be associated with an increased risk of malignancy. With respect to neoplastic colonic epithelial cells sulindac inhibits growth by inducing apoptosis (Shiff *et al.* 1995, Piazza *et al.* 1995). In this study a direct effect on apoptosis *per se* was not investigated. However, our findings could still be interpreted in terms of increased apoptotic cell loss mediated by sulindac. From the cumulative labelling index the migration rate along the crypt axis can be derived (Figure 7). An enhanced migration rate for the sulindac-treated groups in the presence of a constant rate of increase in crypt length for the different groups (Figure 1) implies increased cell loss from the crypt in sulindac-treated animals as compared with non-sulindac treated animals if we assume that sulindac does not cause an increase in the girth of DMH-treated crypts. This inferred increase in cell loss induced by sulindac presumably occurring by a process of apoptosis would then result in compensatory crypt hyperplasia as reflected by an increase in labelling index. In the microadenomatous foci there is disruption of the cytokinetic organization of the lesion such that NSAID-induced cell death does not result in compensatory hyperplasia and there is a net tumour-inhibitory effect. Within the large well-established adenomas, at the dose of sulindac employed the degree of cell loss would be insufficient to account for net tumour regression. Thus the findings from this study are in keeping with the apoptosis-inducing properties of NSAIDs in respect of colonic epithelial cells. However, our hypothesis remains speculative and would require confirmation in further studies. The alternative consideration is that sulindac has a direct trophic influence on the hyperplastic DMH-treated mouse colonic crypt which now increases in girth but not height. It now becomes difficult to reconcile the known chemo-preventive actions of sulindac in respect of colonic neoplasia with a hyperplastic response which is generally regarded as a marker of increased risk of colon cancer. Also in planning chemopreventive therapies the tendency has been to consider manoeuvres which would reduce cell proliferation. Irrespective of whether sulindac causes crypt cell apoptosis or not, our data would suggest that any preventive measure directed at modulating crypt cell proliferation must be applied with caution in view of the fact that chemoprevention is possible in the face of enhanced crypt cell proliferation.

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