EUROPEAN JOURNAL OF CANCER 43 (2007) 1251–1256

available at www.sciencedirect.com

journal homepage: www.ejconline.com

Sulindac treatment in hereditary non-polyposis colorectal cancer

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ARTICLE INFO

Article history:
Received 27 June 2006
Received in revised form
20 February 2007
Accepted 5 March 2007
Available online 16 April 2007

Keywords:
HNPCC
Chemoprevention
Sulindac
Proliferation
Apoptosis

ABSTRACT

Non-steroidal anti-inflammatory drugs, e.g. sulindac have been extensively studied for chemoprevention in familial adenomatous polyposis, but not in hereditary non-polyposis colorectal cancer (HNPCC). We evaluated these effects in HNPCC using surrogate end-points for cancer risk. In a randomised double-blind cross-over study, 22 subjects (9 female; age 30–66 years, mean 44), all ascertained or probable mutation carriers for HNPCC, were included. Sulindac 150 mg b.i.d. and placebo were given for 4 weeks each, with 4 weeks in between, with biopsies taken from ascending, transverse and sigmoid colon and rectum by colonoscopy after both periods. Proliferation was determined by Ki-67 staining and apoptosis by staining of cytokeratin 18 cleavage products. Expression of cyclins B1, D3 and E and p21, p27, bax, bcl2 and cox-2 was studied immunohistochemically. Proliferation was higher during sulindac treatment than drug placebo treatment in ascending and transverse colon, but not in sigmoid and rectum. Apoptosis was not affected. Besides an increase in cyclin D3, no differences were found in expression of regulating proteins in the proximal colon.
Conclusion: Sulindac induces an increase in epithelial cell proliferation in the proximal colon of subjects with HNPCC. Since colorectal cancer predominantly arises in the proximal colon in HNPCC, these results cast doubts on the potential chemopreventive effects of sulindac in HNPCC.

1. Introduction

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominantly inherited disorder, predisposing affected individuals to the development of cancer, in particular colorectal cancer. It is germline mutations in DNA mismatch repair (MMR) genes, predominantly MLH1, MSH2 and MSH6 that predispose an individual to HNPCC. Mutation carriers have an up to 80% lifetime risk to develop colorectal cancer, most prominently in the proximal colon.[1] The cancers arise at a relatively early age and often develop faster than sporadic colorectal cancers. The carcinogenic process in HNPCC is

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2007.03.001

characterised by the adenoma-to-carcinoma sequence similar to that described in familial adenomatous polyposis (FAP) or sporadic cancer, but several of the clinical manifestations, as well as the molecular pathobiology underlying them, are distinctive.[2] Preventive measures, e.g. chemoprevention, for risk reduction would be welcome aspects in the treatment of patients at high inherited risk for cancer. Broadly studied in patients with FAP, the efficacy of chemoprevention remains largely unexplored in HNPCC.

Increasing evidence from cell line, animal and human studies reveals that the administration of non-steroidal anti-inflammatory drugs (NSAIDs) represents a viable option for the chemoprevention of FAP-associated and sporadic colorectal cancer.[3–6] The use of NSAIDs is associated with a lower risk of colorectal adenoma and cancer development, a lower risk of recurrent colorectal adenomas and carcinomas and these effects are independent of gender, age and site in the colorectum.[6–10]

The molecular basis for the chemoprotective action of NSAIDs has not yet been fully elucidated. The NSAID sulindac has been reported to display profound anti-proliferative effects, to alter the cell cycle distribution and to induce apoptosis in cell lines and *in vivo*.[11–13] In particular, cyclooxygenase-2 (cox-2), which is inhibited by NSAIDs, is an important target of these drugs,[14] consistent with the fact that colorectal neoplasms often exhibit up-regulation of this enzyme. Also, other mechanisms may be involved in the chemopreventive effects of NSAIDs. Sulindac sulfide, the active metabolite of sulindac, has been shown to decrease the expression of cyclin B1 and E and increase the expression of cyclin D3.[15] Several studies demonstrated markedly induced expression of p21, a cell cycle inhibitor, and bax, a pro-apoptosis protein, after sulindac therapy, but others failed to find these changes.[16–18] Also, p27 and bcl-2 have been associated with the possible mechanism of colorectal cancer prevention by NSAIDs.

In order to explore the potential role of sulindac in HNPCC chemoprevention, we evaluated the effects of sulindac in HNPCC patients using surrogate end-points for cancer risk, including epithelial cell proliferation rate, apoptosis and expression in normal colonic epithelium of proliferation-, apoptosis- and cell-cycle-involved genes.

2. Patients and methods

The present study was a randomised, double-blinded, placebo-controlled cross-over study in ascertained MMR gene mutation carriers and subjects with more than a 50% risk to be MMR gene mutation carriers. Participants were recruited from a cohort of families with an established pathogenic mutation in MLH1, MSH2 or MSH6 and/or fulfilling the Amsterdam criteria,[19,20] and who are under regular colonoscopic surveillance at the Department of Gastroenterology of the University Medical Center Groningen. Subjects from families that meet the Amsterdam criteria were considered to have more than 50% risk to be a mutation carrier if they had previously been diagnosed with an HNPCC-related cancer, or with a high risk colorectal adenoma (larger than 1 cm and/or villous component and/or high grade dysplasia) before the age of 50, or with any adenoma before the age of 40 years. Written informed consent was obtained from each participant. All participants were older than 18 years of age and able to consume a normal diet. Prior colorectal surgery was allowed, but the subject had to have >50% of the colorectum left. Subjects with any disease of the colon apart from adenomatous polyps and diverticulosis were excluded. Further exclusion criteria consisted of a history of hypersensitivity to NSAIDs, a history of peptic ulcer disease, the inability to abstain from use of other NSAIDs and the use of oral adrenocorticosteroids and cholestyramine during the study period. The study protocol was approved by the Medical Ethical Committee of the University Medical Center Groningen, and all subjects gave written informed consent.

After randomisation to one of both sequences of treatment, subjects were given sulindac 150 mg b.i.d. for four weeks with a cross-over to twice daily placebo for four weeks, or vice versa. The first treatment period was followed by a washout period of 4 weeks. During treatment periods, the physician regularly contacted the subject to monitor possible side-effects and to increase compliance. At the end of the first four weeks treatment period a regularly planned surveillance colonoscopy was performed after lavage with a PEG-containing solution. Colonoscopy was repeated at the end of the second four weeks of treatment.

During each colonoscopy biopsies were taken from four predefined sites in the colorectum, namely from (1) the ascending colon, (2) halfway the transverse colon, (3) the sigmoid and (4) the rectum. Three biopsies of each location were fixed in buffered formalin (pH 7.47) and embedded in paraffin. Subsequently, consecutively numbered sections of 3 μm were cut from the biopsies and fixed onto 3-aminopropyl-triethoxysilane (APES, Sigma–Aldrich, Diesenhofen, Germany) coated slides, stretched for 30 min at 60 °C and dried overnight at 37 °C. Paired samples of each participant were stained in the same batch.

2.1. *Proliferation*

Proliferation was assessed by immunohistochemical staining of Ki67 using MIB1 antibody. Antigen retrieval was performed using a high-pressure cooker. Immersed in 200 μl of 2% blocking reagent and 0.2% SDS in maleic acid, pH 6.0 (Boehringer Mannheim, Germany)) the section underwent three sessions of 5 min at 115 °C in a high-pressure cooker alternating with incubation in a humid environment. The endogenous peroxidase activity was quenched by incubation with 0.3% H_2O_2 in PBS for 30 min. The sections were immersed for 1 h with the MIB1 antibody in PBS with 1% bovine serum albumin (BSA), at a dilution of 1:400. Subsequently, the sections were consecutively incubated for 30 min periods with rabbit antimouse peroxidase (RAMPO; DAKO, Glostrop, Denmark) and goat antirabbit peroxidase (GARPO; DAKO) both diluted (1:50) in PBS-1% BSA. The sections were submerged for 10 min in a solution of 25 mg 3,3′-diaminobenzidine (DAB) in PBS and 50 mg of imidazol with 50 μl 30% H_2O_2. After rinsing with demi water, the sections were counterstained with haematoxylin, washed with running water, dehydrated with graded alcohol, dried and covered with a slide.

2.2. Apoptosis

Apoptosis was assessed by immunohistochemical staining of cleavage products of cytokeratin 18 (CK18) using Mab M30. CK18 cleavage by activated caspase-3 is an early marker of apoptosis.[21] Antigen retrieval was performed by submersion of the deparaffinised, rehydrated sections in preheated 10 mM citrate buffer (pH 6.0) and heated for 8 min at 700 W in a microwave. After cooling at room temperature for 15 min the sections were thoroughly rinsed with PBS for 5 min and a 1:50 solution of Mab M30 was applied for 1 h at room temperature. Subsequently, the same steps as for Ki-67 staining were performed, starting with the incubation of the sections with RAMPO.

2.3. Proliferation- and apoptosis associated proteins

The optimal antibody–antigen reaction was determined for proliferation-regulating proteins cyclin B1, D3 and E, CDK inhibitors p21 and p27, and apoptosis regulators bcl-2, bax and cox-2. Table 1 summarises the primary antibodies that were used in immunohistochemical studies, the companies from which they were purchased, the dilution at which they were used and the corresponding technique for antigen retrieval. The high-pressure cooker antigen retrieval method is as described above for Ki-67 immunostaining and the microwave method as described for Mab M30 using either 10 mM citrate buffer (pH 6.0) or EDTA.

Optimal dilutions and optimal antigen retrieval were tested on a multi tissue block containing an array of epithelial malignancies (colon, breast and endometrium) and normal tissue (placenta, lymphnode, skin, breast, endometrium, colon). Controls consisted of omission of the first antibody.

2.4. Evaluation of staining

Without any knowledge of the treatment, three authors (F.R., T.v.d.S. and W.B.v.E.) scored all stained slides. Each set of stains was entirely scored by one author. A second author randomly chose 20 slides to confirm reproducibility of the scoring of each immunostaining. The immunoreactivity for every antibody in each biopsy was analysed and quantified in at least five entire crypts. If immunohistochemical staining was inadequate or failed to produce sufficient quantifiable crypts, consecutive slides were stained with the inclusion of a representative slide from the prior staining session.

Proliferative activity was scored as MIB1 labelling index, where positively stained nuclei were divided by the total number of counted nuclei ×100 (%). To verify the effect of sulindac on compartmentalisation of proliferation the MIB1 labelling index was quantified of the upper one-third of the crypt in a similar manner as the entire crypt. For apoptosis, the number of apoptotic cells was too low to investigate compartmentalisation.

Cyclins B1, D3 and E, p21, p27 and cox-2 were also scored as labelling index. The intensity of bax and bcl-2 expression was scored as (1) absent, (2) weak, (3) moderate, or (4) strong. Two slides from the first staining sessions were used in every consecutive staining session as reference for the intensity scoring. The intensity of bax demonstrated a gradient from the bottom to the surface of the crypt. The crypt was therefore divided into three compartments. A weighed score, intensity multiplied by the percentage of cells staining positively, was calculated for each compartment.

2.5. Statistics

Statistical analyses were performed using SPSS 12.0.1 for Windows software (Statistical Package for the Social Sciences Inc., Chigago, IL). For statistical assessment of changes in proliferative, apoptotic, cyclins B1, D3 and E, p21, p27 and cox-2 labelling indices following sulindac treatment, the paired-samples *t*-test with a 95% confidence interval was used. To evaluate the difference in bax and bcl-2 expression, Wilcoxon's non-parametric two-related-sample test was used. To determine differences in various colonic regions in HNPCC patients, the non-parametric Mann–Whitney *U* test was conducted. Reported *p*-values are two-tailed, and significance was assumed if $p < 0.05$.

3. Results

3.1. Research population

In total, 22 subjects, 9 women and 13 men were initially included in the study. Thirteen were proven mutation carriers (5 MLH1 and 8 MSH2). The remaining nine were at high risk of being a carrier, as defined above. Mutation analysis in this

Table 1 – Primary antibodies used in immunohistochemical studies

Protein	Antigen retrieval method	Clone	Company	Dilution
Ki-67	High-pressure cooker	MIB1	Immunotech, Marseille, France	1:400
Cyclin B1	Microwave EDTA	7A9	Novocastra, Newcastle, United Kingdom	1:50
Cyclin D3	Microwave EDTA	DCS-22	Novocastra, Newcastle, UK	1:10
Cyclin E	Microwave EDTA	13A3	Novocastra, Newcastle, UK	1:10
P21	High-pressure cooker	WAF1(Ab-1)	Oncogene, Darmstadt, Germany	1:50
P27	High-pressure cooker	1B4	Novocastra, Newcastle, UK	1:50
cCK 18[a]	Microwave citrate	MAb M30	Boehringer, Mannheim, Germany	1:50
Bax	Microwave citrate	B-9	Santa Cruz Biotechnology, Santa Cruz, CA	1:200
Bcl-2	High-pressure cooker	MAb 124	DAKO, Glostrup, Denmark	1:50
Cox-2	Microwave EDTA	33	Transduction Laboratories, Lexington, KY	1:50

a cCK 18: cleaved cytokeratin 18.

group of patients had not been performed, was inconclusive or was still in process. Six patients had previously been diagnosed with an HNPCC-related cancer (ovarian, rectal, endometrial and jejunal cancer). The average age of the subjects at inclusion was 44 years (range: 30–66 years). Four subjects did not undergo a second colonoscopy. Two subjects, one male (MSH2 mutation carrier) and one female (mutation status unknown), left the study due to side-effects. These patients complained of gastrointestinal discomfort, stomach cramps and diarrhoea. The other two subjects (two females, an MLH1 and an MSH2 mutation carrier) were not motivated for a second colonoscopy. So, the analysis of data is based on the remaining 18 subjects.

3.2. *Proliferative and apoptotic indices*

On average, seven full crypts (mean of 1070 cells) were evaluated per biopsy. After placebo, the mean MIB1 labelling index in the biopsies of the proximal colon was significantly higher than in the biopsies of the distal colon of the same subjects (48.4 ± 1.3% (±SEM) versus 35.4 ± 1.3%, respectively, $p < 0.001$). The MIB1 labelling index in the entire colon was higher after sulindac treatment than after placebo (46.3 ± 1.5% versus 42.6 ± 1.3%, $p = 0.004$, Table 2). This increase in the MIB1 labelling index was due to a significant increase in the proximal i.e., ascending and transverse, colon, which was not evident in the sigmoid and rectum (Fig. 1). The MIB1 labelling index in the upper crypt compartment in the proximal colon was significantly higher after sulindac treatment than after placebo (4.2 ± 0.7% versus 2.5 ± 0.2%, $p = 0.021$), whereas this was not the case in the sigmoid and rectum. Crypt length did not alter after sulindac treatment, neither in the proximal colon nor in the distal colon. Apoptotic activity was similar in each of the four locations in the colon both during sulindac and placebo use, and was not measurably affected by sulindac.

3.3. *Proliferation- and apoptosis associated proteins*

In the 'untreated' proximal colon, significantly more epithelial cells expressed cyclin B1 and less expressed cyclin D3 in comparison to those in the distal colon. Cox-2 expression was significantly higher in the proximal colon in comparison to the distal colon. No significant changes in expression of the regulating proteins were observed after sulindac treatment. A tendency towards more cyclin D3 expression was observed in the proximal colon after sulindac treatment (35.6 ± 1.6% versus 31.4 ± 1.4%, $p = 0.055$). In the distal colon, less cells expressed bax after sulindac (2.1 ± 0.1% versus 2.4 ± 0.1%, $p = 0.032$).

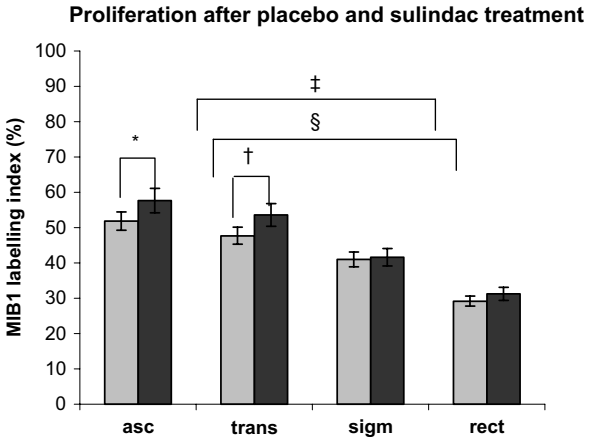

Fig. 1 – Epithelial cell proliferative activity in the colorectum after placebo and sulindac treatment. Asc, ascending colon; trans, transverse colon; sigm, sigmoid colon; rect, rectum; (□) after placebo treatment. (■) after sulindac treatment; (*) labelling index after placebo versus sulindac in the ascending colon, $p = 0.048$; (†) labelling index after placebo versus sulindac in the transverse colon, $p = 0.021$; (‡) labelling index after placebo in the proximal versus distal colon, $p < 0.001$; (§) labelling index after sulindac in the proximal versus distal colon, $p = 0.004$.

4. Discussion

Experimental and human studies have clearly demonstrated a chemopreventive effect of NSAIDs, including sulindac, on colorectal carcinogenesis. So far, however, mechanisms by which this is mediated have not been fully elucidated. Hereditary non-polyposis colorectal cancer with its high incidence of colorectal cancer at a young age represents an ideal target

Table 2 – Immunoreactivity of the proteins in the entire colon: after treatment with placebo and with sulindac

Protein	Placebo	Sulindac	*p*-Value
Ki67 (%)	42.6 ± 1.3	46.3 ± 1.5	**0.004**
Ki67 top (%)	1.5 ± 0.2	2.5 ± 0.4	**0.011**
Cyclin B1 (%)	6.6 ± 0.2	7.0 ± 0.3	0.165
Cyclin D3 (%)	35.1 ± 1.1	36.7 ± 1.0	0.279
Cyclin E (%)	3.6 ± 0.2	3.3 ± 0.2	0.176
P21 (%)	38.1 ± 1.0	39.5 ± 0.9	0.323
P27 (%)	74.3 ± 1.4	75.5 ± 1.4	0.519
cCK18[a] (%)	0.68 ± 0.10	0.70 ± 0.08	0.906
cCK18:Ki67	1.7 ± 0.2	1.5 ± 0.2	0.660
Bax (I)	2.0	2.0	0.183
Bcl2 (%)	70.4 ± 2.7	74.5 ± 2.2	0.285
Cox2 (%)	2.7 ± 0.3	2.8 ± 0.2	0.666

a cCK18: cleaved cytokeratin 18.

for chemoprevention; however, the effects of sulindac on colorectal epithelium of subjects with HNPCC have not been studied before. In the present study, the effects of sulindac on normal-appearing mucosa from HNPCC patients in four regions of the colon were evaluated using biomarkers for proliferation and apoptosis as endpoints. To identify possible mechanisms of action of chemoprevention with sulindac, proliferation and apoptosis regulating proteins were identified from previous studies and were evaluated using immunohistochemistry. Sulindac proved to induce an increase of epithelial cell proliferative activity in the proximal colon, without affecting this activity in the sigmoid and rectum, and without affecting apoptosis in any of the regions of the colorectum. Although the clinical value of the biomarkers used is disputable, the results cast doubts on the chemopreventive effects of sulindac, and NSAIDs in general, in HNPCC.

As carcinogenesis is associated with a loss of tissue homeostasis by disturbance of both cell proliferation and apoptotic cell death, both are considered potential biomarkers for activity of candidate chemopreventive agents. An increase of colorectal epithelial cell proliferative activity has been considered for a long time now as a marker for an increased susceptibility of colorectal cancer. Biologically it is thought to increase the risk of spontaneous and exogenously induced mutations, thus rendering some cells neoplastic properties. Reduction of proliferative activity by candidate chemopreventive agents has been considered for many years as an important indication for the potentially beneficial effect of such agents and as a probable mechanistic explanation for that effect. Therefore, also the effect of NSAIDs on cell proliferative activity has been studied extensively. In cell culture studies, sulindac and sulindac sulphide indeed inhibited proliferation by inducing cell quiescence, with reduction of the expression of classical biomarkers of proliferation, such as Ki-67, independent of their ability to inhibit prostaglandin synthesis.[3,13,15] However, these results were not consistently reproducible in *in vivo* studies, and conflicting data concerning effects of NSAIDs on the colorectal epithelial proliferation have been reported.[11,21–25] A majority of the studies performed on biopsies from the rectosigmoid of patients with FAP failed to demonstrate an anti-proliferative effect of sulindac in normal colorectal mucosa.[11,23,24]

A limitation of all previous studies is that only the effects on the epithelium of the rectum and sigmoid were studied. In the present study, no effect of sulindac on proliferative activity in rectum and sigmoid was observed either. However, proliferation in both transverse and ascending colon increased. The facts that proliferation increased in both regions and that this was not only apparent in the whole crypts but also in the upper third of the crypts strongly indicate that these results are not due to chance but represent a true biological effect of sulindac. Besides a tendency to an increased cyclin D3 expression in the proximal colon upon sulindac treatment, the present study cannot explain the side specific effect of sulindac on proliferation in the proximal colon of HNPCC patients. Previously, it has been suggested that increased proliferation in sulindac-treated mice could be a compensatory phenomenon occurring secondary to a loss of crypt epithelial cells by apoptosis induced by sulindac.[26] Possibly, this compensatory effect is more pronounced in the proximal than distal colon in HNPCC due to the significantly higher proliferative activity in the untreated proximal colon.

It is unknown whether similar effects occur in the proximal colon of subjects without a predisposition for HNPCC, but when found in an HNPCC population it has implications by its own. The preference for the development of cancers in the proximal colon in HNPCC makes one reluctant to use agents that affect proliferation in that part of the colon in a way that is generally considered to mark an increased cancer risk.

Currently, the chemopreventive effects of NSAIDs in the colorectum are thought to be largely due to induction of apoptosis. This view is supported by the observation of apoptosis induction in cell lines, including mismatch repair deficient cells, in the intestinal epithelium of Apc^{Min} mice and in human colorectal epithelium.[3,13,24,27] The apoptotic effect is not only partially mediated by cox-2 inhibition but it also occurs in cells lacking cox-2.[3] A variety of targets of chemoprevention as well as preconditions for NSAID-induced apoptosis have been suggested. Bax mutations may be involved in sulindac resistance while Apc^{1638N} mice with inactivation of p21 did not respond to sulindac.[17,18] In the present study, despite expression of bax, p21 and cox-2 in all biopsies, apoptosis did not alter upon sulindac treatment, neither in the proximal nor in the distal colon of HNPCC patients. Expression of bax, cox-2 as well as expression of p21 did not alter after treatment with sulindac. Previously, sulindac treatment demonstrated no effect on rectal epithelial apoptosis in young FAP patients without adenomas,[28] whereas in symptomatic FAP patients, regression of adenomas was accompanied by alteration of the rectal epithelial apoptotic ratio with a relative increase in apoptosis in surface cells compared with the deeper crypt. Our results, in normally appearing mucosa in HNPCC patients, are in line with the data of phenotypically unaffected FAP patients.[27]

In summary, in a group of HNPCC-subjects, sulindac induced an increase of epithelial cell proliferative activity, both in the whole crypt and in the upper crypt compartment, in the proximal colon without affecting proliferation in the distal colon and without affecting apoptosis anywhere in the colorectum. These results cast doubt on the chemopreventive potency of sulindac, and NSAIDs in general, in HNPCC.

Conflict of interest statement

None to be disclosed by any of the authors.

Acknowledgement

This study was made possible by a grant from the Dutch Cancer Society, RUG 98-1660.

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