Nonsteroidal anti-inflammatory drugs (NSAIDs*) have been demonstrated to reduce cancer rates in oesophagus, stomach and colon of humans and animals. Earlier, we showed that high human gastrointestinal tissue levels of glutathione S-transferase (GST), a family of detoxification enzymes consisting of class α, μ, π and θ isoforms, were inversely correlated with cancer risk. We investigated whether the NSAIDs indomethacin, ibuprofen, piroxicam, acetyl salicylic acid (ASA), and sulindac, supplemented in the diet consisting of class a, X, and colon of humans and animals. Earlier, we showed that the binding of a large variety of electrophiles to the sulphydryl group of glutathione (GSH). Since most reactive ultimate carcinogenic forms of chemical carcinogens are electrophiles, GSTs take considerable importance as a mechanism for carcinogen detoxification (43,44). Enhancement of the activity of this gen detoxification (43,44). Enhancement of GSTs in the rat upper part of the digestive tract, resulting in a more efficient detoxification, may explain in part the anticarcinogenic properties of NSAIDs.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs*) are among the most prescribed drugs worldwide. They have anti-inflammatory, analgesic, and antipyretic activities. They are used clinically for the treatment of patients with (e.g.) acute and chronic rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, gouty arthritis, bursitis, tendositis, and inflammatory arthritis (1).

In addition to their therapeutic use, there is strong epidemiological evidence that NSAIDs may have anticarcinogenic effects in humans. Sulindac caused regression of adenomatous polyps in patients with familial adenomatous polyposis (FAP) (2-4), whereas no effect on sporadic colonic polyps was found (5). Epidemiological studies suggest that regular, prolonged use of aspirin-based NSAIDs may reduce the risk of development and mortality of oesophageal, gastric, colonic, or rectal cancer (6-9), although in one prospective study no support for such an association was found (10). Several NSAIDs are currently evaluated in clinical trials. Effects of NSAIDs on neoplastic growth in the colon of animals and humans, including possible mechanisms involved, were recently reviewed (11,12).

Many animal studies have revealed significant protection against development of chemically induced cancers by treatment with NSAIDs. Ibuprofen inhibited carcinogenesis in rat colon (13), mouse forestomach and lung (14). Indomethacin inhibited tumorigenesis in rat colon (15-19), stomach (16,20), mammary gland (21), urinary bladder (22,23), and liver (24,25), as well as in mouse oesophagus (26,27). Piroxicam reduced tumour incidence in the colon (13,15,17-19,28-31), small intestine (18), and liver (25) of the rat. Dietary acetyl salicylic acid inhibited carcinogenesis in rat colon (32-34) and bladder (35), whereas sulindac reduced tumour multiplicity in the rat colon (36) and mouse forestomach (14).

A generally accepted mechanism of action of NSAIDs is the inhibition of cyclooxygenases, the rate-limiting enzymes that catalyse the formation of prostaglandin precursors from arachidonic acid (12,37). Prostaglandins play a role in the control of cell proliferation and regulation of immune functions (38-41). However, doses of NSAIDs required to suppress inflammation may exceed substantially the doses necessary to inhibit prostaglandin synthesis, suggesting that the anti-carcinogenic properties of these drugs may be achieved through additional unidentified mechanisms (42).

Inhibitors of carcinogenesis often have an enhancing effect on carcinogen detoxification systems such as glutathione S-transferases (GSTs; EC 2.5.1.18) (43-45). The soluble glutathione S-transferases are a gene family of dimeric enzymes comprised of four classes: α, μ, π and θ (43,44). They catalyse the binding of a large variety of electrophiles to the sulphhydryl group of glutathione (GSH). Since most reactive ultimate carcinogenic forms of chemical carcinogens are electrophiles, GSTs take considerable importance as a mechanism for carcinogen detoxification (43,44). Enhancement of the activity of this system may result in a more efficient elimination of carcinogens and may ultimately lead to the prevention of cancer.

The present study was designed to investigate the effects of dietary administration of indomethacin, ibuprofen, piroxicam, acetyl salicylic acid and sulindac on glutathione and glutathione S-transferases in the rat oesophagus, intestine, stomach and liver.

Materials and methods

Animal treatment

Forty-eight male Wistar rats (183±2 g; Central Laboratory Animal Centre, University of Nijmegen, The Netherlands) were housed in pairs on wooden shavings in macrolon cages, maintained at 20-25°C and 30-60% relative humidity. A ventilation rate of seven air cycles/h and a 12 h light/dark cycle.

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*Abbreviations: NSAIDS, nonsteroidal anti-inflammatory drugs; GST, glutathione S-transferase; ASA, acetyl salicylic acid; FAP, familial adenomatous polyposis.

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were used. The rats were randomly assigned to one of the dietary treatment
groups. All groups were fed powdered RMH-TM lab chow (Hope Farms,
Woerden, The Netherlands) from the same batch. After acclimatization for 7
days the animals were fed either the basal diet (control group) or one of the
five experimental diets. Food and water were available ad libitum. Food cups
were replenished every 2–3 days. Food consumption and gain in body weight
were recorded daily.

Diet
Selection of NSAIDs as well as feeding period and dose levels were based on
studies by others, showing reduction of tumour incidence in humans and
inhibition of chemically induced carcinogenesis in animal models, where
NSAIDs were adjusted 2 weeks prior to carcinogen treatment (13,18,21,32,36).
The following six diet groups (eight animals per group) were studied: (a) RMH-TM
lab chow only or supplemented with (b) 25 ppm indomethacin, (c) 400 ppm ibuprofen, (d) 400 ppm piroxicam, (e) 400 ppm acetyl salicylic acid,
or (f) 320 ppm sulindac. The NSAIDs were purchased from Sigma Chemical
Company, St Louis, MO, USA. A food processor was used to obtain a
homogenous mixture of test compound and powdered lab chow. After receiving
the diets for 2 weeks the rats were killed by decapitation. The study protocol
was approved by the local ethical committee for animal experiments of the
University of Nijmegen.

Tissue preparation
All handlings were performed on ice. After decapitation, oesophagus, stomach,
intestine (proximal, middle, and distal small intestine and colon) and liver
were excised immediately. The stomachs were slit longitudinally and the
contents were removed by washing with cold buffer A (0.25 M sucrose,
20 mM Tris, 1 mM dithiothreitol, pH 7.4). The organs were directly frozen
in liquid nitrogen and stored at -20°C until use. For preparation of the
cytoplasmic fraction the tissue was thawed quickly using cold running water.
The mucosal surface of stomach and intestine was collected by scraping with a
scalpel and was homogenized in buffer A (4 ml/g tissue) in a glass/glass
Potter-Elvehjem tube. The liver was homogenized in buffer A (4 ml/g tissue)
with 10 strokes at 1000 rpm of a motor-driven glass/Teflon homogenizer
(Braun, Germany). The homogenate was centrifuged at 9000 g (4°C) for 30
min. The resulting supernatant fraction was transferred to an ultracentrifuge
tube and spun at 150000 g (4°C) for 60 min. The oesophagus was homogenized
in 5 ml buffer A per gram tissue in a glass/glass Potter-Elvehjem tube. These
homogenates were centrifuged at 150 000 g for 60 min (4°C). Aliquots of the
150 000 g supernatant, representing the cytosolic fraction, were frozen in
liquid nitrogen and stored at -20°C.

Assay
Protein concentration was assayed in quadruplicate by the method of Lowry
et al. (46) using bovine serum albumin as the standard. GST activity was
determined in triplicate according to Habig et al. (47), using 1-chloro-2,4-
dinitrobenzene (CDNB) as substrate. GST isoenzyme levels were determined
as described before (45). In short, cytosolic fractions were subjected to sodium
dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described
before. In short, cytosolic fractions were subjected to sodium
dodecyl sulphate polyacrylamide gel electrophoresis (10%). The specific
binding of the monoclonal antibodies to the isoenzymes was demonstrated by
incubation with the secondary antibody peroxidase-conjugated rabbit anti-
mouse immunoglobulin (Dakopatts, Glostrup, Denmark) and subsequent development of the peroxidase label
with 4-chloro-1-naphthol and hydrogen peroxide. Staining intensity on the
immunoblots was quantified using a laser densitometer (Ultrascan XL, LKB,
Brumma, Sweden). Known amounts of purified GSTs were run in parallel
with the experimental samples and served as standards for the calculation of
the absolute amounts of the isoenzymes in the cytosolic fractions. Total
glutathione was quantified by high performance liquid chromatography after
reaction with monobromobimane, as described before (45). In this assay,
oxidized glutathione present is reduced by adding sodium borohydride to the
reaction mixture.

Statistical analyses
The Wilcoxon rank sum test was used to assess statistical significance of
differences between experimental and control groups; *P<0.05, **P<0.01 and 
***P<0.005.

Results
Daily food consumption, intake of NSAIDs and gain in body
weight are given in Table I. In the sulindac and piroxicam
groups food consumption was significantly reduced as com­
pared to the control group, whereas in the ibuprofen group the
food consumption was induced. In the piroxicam group the
lower food consumption was paralleled by a reduced gain in
body weight. During the course of the experiment no changes in
behavioral pattern of the animals were observed. In addition,
none of the organs studied showed any macroscopic sign of
toxicity of the dietary additive at the end of the experiment.

Table II shows the effects of the NSAIDs on GST activity
in the organs investigated. No change in activity was found
with acetyl salicylic acid (ASA). In the oesophagus and
proximal small intestine (PSI), GST activity was induced by
indomethacin (both 1.3X), ibuprofen (1.3X and 1.2X,
respectively), piroxicam (1.8X and 1.2X) and sulindac (both
1.3X). In addition, ibuprofen elevated GST activity in the
colon (1.3X).

In Tables III, IV, and V the effects of the NSAIDs on GST
class α, μ and π isoenzyme levels are given. In control animals
GST α (Table III) was undetectable in oesophagus and colon,
low in stomach (222±20 ng/mg protein) and high in liver
(1228±1227 ng/mg protein) and small intestine. In middle
small intestine (MSI), none of the diets significantly influenced
GST α expression. ASA did not influence GST α levels.
Indomethacin increased levels of GST α in PSI and DSI (1.6X
and 1.9X, respectively) as compared to controls. Ibuprofen
increased GST α levels in PSI (1.4X), piroxicam increased
levels in stomach, DSI and liver (1.8X, 2.8X, and 1.2X) and
sulindac had an inducing effect on GST α levels in DSI
(2.1X). GST μ (Table IV) was expressed at high levels in all
tissues examined. Parallel to GST activity, GST μ levels in
oesophagus and DSI were modulated by the same NSAIDs:
indomethacin (1.4X and 1.6X, respectively), ibuprofen (1.4X
and 1.3X), piroxicam (1.6X and 1.4X) and sulindac (1.5X
and 1.4X), whereas ASA did not change GST μ levels. GST
π (Table V) was undetectable in oesophagus and liver, and
low in all other organs studied, ranging from 114±21 ng/mg
protein in MSI to 568±84 ng/mg protein in PSI in control
animals. All NSAIDs tested increased GST π levels at one or
more sites: indomethacin increased GST π levels in MSI
(2.1X), ibuprofen in stomach (1.9X), piroxicam in both MSI
and DSI (2.4X and 2.8X, respectively), ASA in stomach, MSI
and DSI (1.9X, 3.2X and 1.9X, respectively) and sulindac in
stomach and MSI (2.6X and 3.6X).

Table VI shows the effect of the NSAIDs on the GSH
content in the organs studied. Gastric, colonic and hepatic
GSH contents were not influenced by any of the NSAIDs
tested. Elevation of the GSH content was seen by indomethacin
in PSI, MSI and DSI (1.2X, 2.3X and 2.3X, respectively,
by piroxicam in oesophagus (1.6X), and by ASA in MSI (1.6X).

Discussion
In the present study we have demonstrated that NSAIDs are
able to induce glutathione-S-transferases, especially in the
upper part of the rat digestive tract.

During the last decade, many studies have shown significant
protection against the development of cancer by NSAIDs.
Compelling evidence is presented in several epidemiological
studies, suggesting that NSAIDs have significant protective
activity against human oesophageal, gastric, and colonic cancer
(11,12). Regression of colon adenomas during treatment with
NSAIDs, particularly sulindac, occurred in patients with familial
adenomatous polyposis coli who are at high risk for

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Effects of NSAIDs on glutathione S-transferases

**Table I. Daily food consumption, NSAID-intake and gain in body weight of male Wistar rats receiving diets supplemented with indomethacin, ibuprofen, piroxicam, ASA or sulindac**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dose (ppm)</th>
<th>Food consumption (g/day)</th>
<th>Total NSAID-intake (mg/day/kg b.)</th>
<th>Gain in body weight (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>16.1±0.3</td>
<td>–</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>25</td>
<td>15.9±0.5</td>
<td>2.0±0.1</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>400</td>
<td>17.0±0.2ₐ</td>
<td>34.0±0.5</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>400</td>
<td>14.7±0.3ₐ</td>
<td>29.4±0.5</td>
<td>1.2±0.2ₐ</td>
</tr>
<tr>
<td>ASA</td>
<td>400</td>
<td>16.3±0.5</td>
<td>32.7±0.4</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>Sulindac</td>
<td>320</td>
<td>14.8±0.3ₐ</td>
<td>29.6±0.7</td>
<td>1.8±0.2</td>
</tr>
</tbody>
</table>

Values given are means ±SEM. The one-tailed Wilcoxon rank sum test was used to assess statistical significance of differences between control and treated groups. ₐP<0.05, and ₐₐP<0.01.

**Table II. Effects of indomethacin, ibuprofen, piroxicam, ASA or sulindac on rat alimentary tract glutathione S-transferase activity**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Glutathione S-transferase activity (nmol/min·mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oesophagus</td>
</tr>
<tr>
<td>Control</td>
<td>49±5</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>66±5ₐ</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>63±6ₐ</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>45±5</td>
</tr>
<tr>
<td>ASA</td>
<td>63±4ₐ</td>
</tr>
</tbody>
</table>

PSI, proximal small intestine; MSI, middle small intestine; DSI, distal small intestine; ₐP<0.05, ₐₐP<0.01, and ₐₐₐP<0.005.

**Table III. Effects of indomethacin, ibuprofen, piroxicam, ASA or sulindac on rat alimentary tract glutathione S-transferase α levels**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Glutathione S-transferase α level (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oesophagus</td>
</tr>
<tr>
<td>Control</td>
<td>ND</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>ND</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>ND</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>ND</td>
</tr>
<tr>
<td>ASA</td>
<td>ND</td>
</tr>
<tr>
<td>Sulindac</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not detectable; ₐP<0.05, and ₐₐP<0.01.

**Table IV. Effects of indomethacin, ibuprofen, piroxicam, ASA or sulindac on rat alimentary tract glutathione S-transferase μ levels**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Glutathione S-transferase μ level (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oesophagus</td>
</tr>
<tr>
<td>Control</td>
<td>3469±326</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>4738±343ₐ</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>4792±268ₐ</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>5342±336ₐ</td>
</tr>
<tr>
<td>ASA</td>
<td>4008±399</td>
</tr>
<tr>
<td>Sulindac</td>
<td>5223±461ₐ</td>
</tr>
</tbody>
</table>

ₐP<0.05, ₐₐP<0.01 and ₐₐₐP<0.005.

development of colonic cancer (2–4). NSAIDs such as aspirin, indomethacin, piroxicam, and sulindac were repeatedly shown to inhibit chemically induced tumours of the colon (15, 17–19, 21, 22, and 24) in laboratory animals. Several hypotheses have been proposed to explain the mechanism of chemoprevention by NSAIDs: (a) NSAIDs reduce the gastrointestinal permeability of carcinogens and their metabolites (55), (b) NSAIDs are scavengers of reactive oxygen species involved in initiation and promotion of cancer (56), (c) NSAIDs can bind to cytochrome P450 monooxygenases, thereby inhibiting P450-mediated activation of procarcinogens to reactive electrophilic intermediates (57, 58).

On the other hand, ibuprofen and indomethacin are able to induce prokaryotic cytochrome P450 BM_3 (CYP102) (59). (d) In parallel with the inhibition of tumour growth, aspirin, indomethacin,
and piroxicam, reduce the prostaglandin levels in the colon of rodents treated with carcinogens (15,17,60), by inhibition of cyclooxygenases, the rate-limiting enzymes in the synthesis of prostaglandins (61,62). On the other hand, GSTs are involved in the synthesis of prostaglandin D2, E2 and F2α (63). (e) NSAIDs can inhibit the induction of ornithine decarboxylase activity and tissue levels of putrescine, two markers of tumour promotion (64,65). In addition, NSAIDs may inhibit the activity of enzymes such as phosphodiesterases or cyclic GMP-AMP protein kinases (66), which may be central to cancer initiation and promotion.

Much of the research on NSAIDs and cancer prevention at this moment is focused on the hypothesis that prostaglandins may play a key role in the regulation of neoplasia. However, there is no direct evidence that NSAIDs prevent tumour development solely through inhibition of cyclooxygenases (67), and prevention of cancer could be due to multiple mechanisms. Another way of action of NSAIDs, in addition to the possibilities cited above, may be the enhancement of GST activities by NSAIDs, as shown in this study. A more efficient detoxification could lead to a reduction of biologically active compounds and thus prevent carcinogenesis.

No information about the possible effects of NSAIDs on GST level in the same order of magnitude. This was expected, since GST α is the most prominent of all GSTs in humans at high risk for cancer development. GST α, GST μ and GST π were undetectable in both oesophagus and liver and none of the NSAIDs had any effect on this. The increase in GST activity in oesophagus and PST by indomethacin, ibuprofen, piroxicam and sulindac was paralleled by a rise in GST μ level in the same order of magnitude. This was expected, since GST μ is the most prominent of all GSTs in the rat (Tables III–V). In two of the sites in which both GST activity and GST μ levels were induced, GST α level was increased as well. The molecular basis for these inductions is not clear yet.

Indomethacin, ibuprofen, piroxicam, and sulindac each induced the GST enzyme activity as well as GST α, GST μ or GST π levels in at least one organ. Indomethacin, ibuprofen and sulindac were equally efficient in inducing glutathione S-transferase, in seven out of 28 possibilities (25%). Piroxicam appeared to be the most active, with inductions seen in 32% of all possibilities. Acetyl salicylic acid showed an increase of glutathione S-transferase enzyme activity or isoenzyme levels in only 11% of all possibilities, which makes it the least

### Table V. Effects of indomethacin, ibuprofen, piroxicam, ASA or sulindac on rat alimentary tract glutathione S-transferase activity levels

<table>
<thead>
<tr>
<th>Treatment group (n=8)</th>
<th>Glutathione S-transferase activity (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oesophagus</td>
</tr>
<tr>
<td>Control</td>
<td>ND</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>ND</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>ND</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>ND</td>
</tr>
<tr>
<td>ASA</td>
<td>ND</td>
</tr>
<tr>
<td>Sulindac</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not detectable; aP<0.05, bP<0.01, and cP<0.005.

### Table VI. Effects of indomethacin, ibuprofen, piroxicam, ASA or sulindac on rat alimentary tract glutathione levels

<table>
<thead>
<tr>
<th>Treatment group (n=8)</th>
<th>Glutathione (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oesophagus</td>
</tr>
<tr>
<td>Control</td>
<td>18.1±1.6</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>24.5±3.4</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>20.1±1.6</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>28.2±3.1b</td>
</tr>
<tr>
<td>ASA</td>
<td>16.6±1.4</td>
</tr>
<tr>
<td>Sulindac</td>
<td>17.8±1.2</td>
</tr>
</tbody>
</table>

aP<0.05, bP<0.01, and cP<0.005.
active form of the NSAIDs tested. The amount of NSAIDs consumed by the rats in our study (2–35 mg/day/kg b.w.) matches very well with doses prescribed to patients suffering from rheumatic diseases (3–100 mg/day/kg b.w.; see Table 1), suggesting that the effects of NSAIDs on GSTs as found in rats may be achieved in humans as well. NSAIDs have side-effects in both humans as well as animals. We observed a decreased food consumption in the piroxicam and sulindac groups. In these animals, however, none of the organs showed any macroscopical sign of toxicity at the end of the experiment, suggesting that the induction of GSTs are not the consequence of toxic effects of these NSAIDs. This is further supported by the observation that ibuprofen induced food consumption as well as GST activity and isoenzymes.

No data on the effects of the NSAIDs tested on GSH levels have been reported before. GSH is an important physiological nucleophile which is coupled with reactive electrophiles such as carcinogenic nitrosamines (45), catalysed by the glutathione S-transferases. Significantly increased levels of GSH, however, were found in only five out of 35 possibilities: in small intestine by indomethacin, in oesophagus by piroxicam and in MSI by ASA. In PSI and oesophagus, this increase in GSH level paralleled an induction of GST activity.

In conclusion, our data demonstrate that NSAIDs are able to elevate the detoxification potential of tissues from the gastrointestinal tract, by increasing the expression of GSTs. This seems to be a common working mechanism in the prevention of carcinogenesis.

Acknowledgements

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