Inhibition of rat colon tumors by sulindac and sulindac sulfone is independent of K-ras (codon 12) mutation

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Am. J. Physiol. Gastrointest. Liver Physiol. 278: G266–G272, 2000.—Nonsteroidal anti-inflammatory drug (NSAID) use reduces the risk of colorectal cancer by 40–50%. Previous studies suggest that effective inhibition of colorectal cancer by NSAIDs may be dependent on the presence or absence of a K-ras mutation. This study was aimed at determining the relationship between inhibition of colorectal cancer by sulindac and sulindac sulfone and the presence of activating K-ras mutations in the 1,2-dimethylhydrazine dihydrochloride rat model. Sulindac (20 mg·kg⁻¹·day⁻¹), sulindac sulfone (40 mg·kg⁻¹·day⁻¹), or vehicle was administered orally to male Sprague-Dawley rats for a 4-wk period beginning 20 wk after tumor induction. Tumor number and volume were measured before treatment by laparotomy and colonoscopy and again after treatment. Sulindac and sulindac sulfone treatment significantly reduced the number and volume of colorectal tumors compared with control rats. For K-ras (codon 12) mutation detection, frozen tumor tissue was collected at the endpoint. We found K-ras codon 12 mutations in 11 of 21 (52%) control tumors. The proportion of tumors with K-ras mutations in the sulindac-treated group (5 of 8 (62%); odds ratio = 1.51 (95% confidence interval = 0.29, 8.33)) and the proportion of sulindac sulfone-treated tumors (9 of 14 (64%); odds ratio = 1.63 (95% confidence interval = 0.41, 6.66)) were not significantly different from controls. Tumor inhibition did not correlate with K-ras (codon 12) mutation status, which suggests that the mechanism of inhibition of rat colorectal cancer by sulindac and sulindac sulfone is independent of K-ras mutation.

K-ras is one of the oncogenes most frequently mutated in human cancers. The K-ras gene is found on human chromosome 12p and encodes an ~21-kDa G protein that is involved in growth factor signal transduction (12). Activated K-ras initiates a signal transduction cascade associated with increased or uncontrolled cell proliferation. Additionally, a recent study (45) showed that the presence of an activated K-ras is associated with a reduction in the frequency of apoptotic cells in human colorectal cancer. Thus activated K-ras may exert a dual effect on both cell proliferation and apoptosis in colorectal carcinogenesis.

Chemically induced ACF and carcinomas in rodents have been shown to frequently contain K-ras mutations (15, 20, 40, 43). Constitutive activation of K-ras by point mutation occurs with a frequency of ~40–60% in DMH-induced rat colorectal cancer. The majority of K-ras point mutations occur as G-to-A transitions, and ~70% of these occur in codon 12 (15, 43). Generation of the K-ras oncogene appears to be an early event in the progression of colorectal carcinogenesis (44). K-ras mutation is an early event in human sporadic polyoid adenoma formation with a reported K-ras codon 12, 13, and 61 point mutation frequency of 40–70% in carcinomas and 15–75% in adenomas (23). Codon 12 K-ras mutations have also been found in human sporadic microadenomas (33).

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To date, only one in vivo study has examined the relationship between sulindac and K-ras activation in colorectal cancer. It was shown in an AOM model of colorectal rat cancer that sulindac selectively suppressed amplification of cells with a K-ras codon 12 mutation (37). However, cell proliferation was not measured. An in vitro study showed that an activated K-ras oncogene increases resistance to sulindac-induced apoptosis in rat enterocytes (1). This suggests that sulindac-induced apoptosis may be resisted in tumors with an activated K-ras. However, the exact mechanism of the chemopreventive action of sulindac has not yet been elucidated. It is not known whether sulindac mediates its chemopreventive effect via induction of apoptosis, via suppression of cell proliferation, or via an alternative mechanism, e.g., inhibition of ras signaling (14). The aim of our study was to determine whether, in the DMH rat model, sulindac and sulindac sulfone inhibition was selective for colorectal tumors with a K-ras (codon 12) mutation.

**METHODS**

**DMH-induced colorectal tumor model**

Animals. Six-week-old male Sprague-Dawley rats were obtained from Monash University Central Animal Services (Clayton, Australia). They were housed in a temperature-controlled environment with a 12-h light-dark cycle. Rats had access to standard rat chow (GR2+, Clark/King/Barastock) and tap water ad libitum throughout the course of study. All studies were conducted in accordance with National Health and Medical Research Council (Australia) guidelines and approved by the Monash University Standing Committee on Ethics in Animal Experimentation.

Induction of tumors, laparotomy, and measurement. Primary colorectal tumors were induced in rats (n = 30) with DMH using a protocol previously established in our laboratory (8). DMH (Fluka Chemical, Castle Hill, Australia) was dissolved in isotonic saline, and pH was adjusted to 7 with NaOH. Each animal received 5 × 30 mg/kg doses of DMH at weekly intervals by orogastric gavage. At 20 wk after the initial dose of DMH, rats were anesthetized with pentobarbital sodium (60 mg/kg; Boehringer Ingelheim, Sydney, Australia). The colon was removed via a midline laparotomy, opened along the mesenteric border, rinsed with isotonic saline, and pinned out flat. Tumors were counted macroscopically, volume measurements (height × width × length) were taken, and position (distance from cecum and rectum) was recorded.

**DMH-induced colorectal tumor model**

Posttreatment tumor measurement and histological analysis. At the end of the 4-wk treatment period, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg; Boehringer Ingelheim, Sydney, Australia). The colon was removed via a midline laparotomy, opened along the mesenteric border, rinsed with isotonic saline, and pinned out flat. Tumors were counted macroscopically, volume measurements (height × width × length) were taken, and position (distance from cecum and rectum) was recorded. The change in total tumor volume over the treatment period was calculated as the summation of all tumor volumes within a group after treatment minus the summation before treatment. A sample from each tumor was snap-frozen in liquid nitrogen and stored at −80°C for subsequent DNA extraction.

The remaining colon and tumors were fixed in 10% neutral buffered formalin (Sigma, St. Louis, MO) for 1–2 wk, and then stored in 50% (vol/vol) EtOH. Tumor tissues were processed and embedded in paraffin. Four-micrometer sections were then stained with hematoxylin and eosin (H & E). Two independent observers, blinded to treatment group, assessed standard H&E sections using an Olympus light microscope under ×40 magnification. Only those tissues confirmed as adenoma or carcinoma on histology were included in the analysis and results of the study.

**DNA extraction and detection of K-ras codon 12 G-to-A mutations.** RNAse-free DNA was extracted from rat colon (tumor and normal) tissue using the Qiagen Qiamp tissue kit according to the manufacturer’s instructions. A capillary spectrophotometer was used to measure optical density at 260 nm to determine the concentration of DNA in the samples. Extracted DNA was amplified by PCR using a technique similar to that described by Kumar and Dunn (17) to detect specific K-ras mutations. This uses a restriction fragment length polymorphism (RFLP) strategy: PCR is performed using a mismatched primer pair that generates a 75-bp product (for both wild type and mutant) with a HphI restriction site generated in the PCR product only if a G-to-A substitution is present in the second base of codon 12 in the K-ras gene. Thus after digestion with HphI, wild-type K-ras products are still 75 bp in size, whereas mutant K-ras products are cleaved into 46 bp and 29 bp, respectively. In brief, the following PCR protocol was used. DNA (200 ng) was amplified in a 100-µl reaction volume, using 0.5 μM of each primer [K-ras 1, 5′-ACTTTGTGGTAGTTGGAG; 2, 5′-CTTCACCAAGTGTTCTGAAT-3′ (mismatch in bold); and K-ras 2, 5′-ATTTGGGGGACGTCGAT-3′], 1.5 mM Mg2+, and 200 μM dNTPs. AmpliTaq Gold Taq polymerase (Perkin Elmer) was used, and cycling parameters were as follows: 94°C for 8 min (to activate AmpliTaq Gold) and then 50 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C with a further 7 min at 72°C in the final cycle only. PCR products were visualized on 3% agarose gels stained with ethidium bromide. For each PCR, a control with no DNA and a positive mutant control were included. Any DNA samples that were negative for K-ras were subjected to PCR a second time for confirmation.

Ten-microliter aliquots of K-ras PCR product were then digested with HphI (New England Biolabs, Beverly, MA) in a total volume of twenty microliters (NEB4 buffer) using one microliter (restriction enzyme supplied at 5 U/µl) of HphI per
Results of treatments with sulindac and sulindac sulfone were analyzed using RFLP-PCR and native PAGE gel analysis for the K-ras mutation status of colorectal rat tumors. DNA extracted from 43 rat colorectal tumor samples (33 carcinomas and 10 adenomas) was analyzed by RFLP-PCR and native PAGE gel analysis for the presence of K-ras codon 12 G-to-A point mutations. Figure 1 shows a representative analysis. K-ras wild-type samples and tumor samples with the K-ras mutation are depicted. Mutant K-ras 46-bp and 29-bp fragments were clearly visualized in those samples with the K-ras mutation. For statistical analysis of the difference in the proportion of K-ras mutations between treatment groups, comparisons were done using a test for equal proportions. To take into account the small sample size for the adenoma population, a two-tailed Fischer's exact test was also used.

### Table 1. Number of colon tumors per group

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Control (Vehicle)</th>
<th>Sulindac (n = 10)</th>
<th>Sulindac Sulfone (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td>20</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>After treatment</td>
<td>24</td>
<td>8*</td>
<td>14†</td>
</tr>
</tbody>
</table>

Rats received sulindac, sulindac sulfone, or vehicle for 4-wk period as described in methods, and no of tumors from all rats was determined. Histological confirmation of before-treatment values was not possible before treatment. *P = 0.0037, †P = 0.0607 vs. vehicle group.

### Table 2. Effect of sulindac and sulindac sulfone on total tumor volume

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sulindac</th>
<th>Sulindac Sulfone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td>3,384</td>
<td>1,592</td>
<td>1,653</td>
</tr>
<tr>
<td>After treatment</td>
<td>12,511</td>
<td>871</td>
<td>2,018</td>
</tr>
<tr>
<td>Change in volume</td>
<td>9,127</td>
<td>−721</td>
<td>+365†</td>
</tr>
</tbody>
</table>

The number of tumors before treatment refers to all tumors present at laparotomy, including those that disappeared during the treatment period and for which there is no histological confirmation of tumor status. "Tumors" that were identified at laparotomy but were not examined after histological analysis at the end point (after treatment) are excluded from all analysis. The number of tumors after treatment refers to the total number at the endpoint of the experiment, for each of the three groups. All of these tumors were examined by histological analysis and confirmed as adenomas or carcinomas, except for three in the vehicle group for which there was no tissue available. Only these tumors were used to compare the change in tumor volumes for each of the groups over the treatment period and for all analyses of K-ras mutation status. For statistical analysis of the difference in the proportion of K-ras mutations between treatment groups, comparisons were done using a test for equal proportions. To take into account the small sample size for the adenoma population, a two-tailed Fischer's exact test was also used.

### Statistical Analysis

Statistical analysis was performed using the SAS (SAS Institute, Cary, NC) system. The outcome variables of tumor volume and tumor number were not normally distributed; thus statistical significance was determined using nonparametric techniques (Kruskal-Wallis and Wilcoxon 2-sample test). A P < 0.05 was considered to be significant, and, to compensate for multiple comparisons where necessary, a P < 0.02 was considered to be significant.

The number of tumors before treatment refers to all tumors present at laparotomy, including those that disappeared during the treatment period and for which there is no histological confirmation of tumor status. "Tumors" that were identified at laparotomy but were not examined normal after histological analysis at the end point (after treatment) are excluded from all analysis. The number of tumors after treatment refers to the total number at the endpoint of the experiment, for each of the three groups. All of these tumors were examined by histological analysis and confirmed as adenomas or carcinomas, except for three in the vehicle group for which there was no tissue available. Only these tumors were used to compare the change in tumor volumes for each of the groups over the treatment period and for all analyses of K-ras mutation status. For statistical analysis of the difference in the proportion of K-ras mutations between treatment groups, comparisons were done using a test for equal proportions. To take into account the small sample size for the adenoma population, a two-tailed Fisher’s exact test was also used.
tumors with K-ras mutations in the sulindac-treated group (5 of 8; odds ratio (OR) = 1.51; 95% confidence interval (CI) = 0.29, 8.33) was slightly increased compared with controls as was the proportion of sulindac sulfone-treated tumors (9 of 14; OR = 1.63; 95% CI = 0.41, 6.66).

Moreover, the change in tumor volume over the 4-wk treatment period was independent of K-ras mutation status (Table 3). In the sulindac-treated group, seven of eight tumors present after treatment had decreased in size over the treatment period and five of eight had K-ras mutations. Only one tumor increased in size during the sulindac treatment, and this tumor was K-ras wild type. All three new tumors that appeared during the sulindac treatment were K-ras mutations. Only samples that have K-ras codon 12 G-to-A mutations were cut by HpaI and show 46-bp and 29-bp mutant fragments (in addition to 75-bp K-ras PCR product). Fragment size is indicated. All uncut K-ras PCR products and K-ras wild-type samples show 75-bp product.

Table 3. K-ras status of tumors after treatment  

<table>
<thead>
<tr>
<th>Tumors, new or increased in size</th>
<th>Control</th>
<th>Sulindac</th>
<th>Sulindac Sulfone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>10</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Mutant</td>
<td>11</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Tumors decreased in size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mutant</td>
<td>0</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>All tumors</td>
<td>10 (48%)</td>
<td>3 (38%)</td>
<td>5 (36%)</td>
</tr>
<tr>
<td>Wild type</td>
<td>11 (52%)</td>
<td>5 (62%)</td>
<td>9 (64%)</td>
</tr>
</tbody>
</table>

Experimental design described in Table 1. Increase/decrease in size over 4-wk treatment period is indicated. New tumors are those not present at laparotomy that appeared during treatment period. Restriction fragment length polymorphism-PCR strategy was used to determine K-ras (codon 12) mutation status in rat colon tumor DNA as described in METHODS. Mutant, G-to-A point mutation at K-ras codon 12.

during 4 wk of sulindac sulfone treatment, the proportion with a K-ras mutation was ~50% (Table 3).

**DISCUSSION**

Sulindac and its sulfone derivative inhibit tumor number and volume in DMH-induced colorectal rat tumors (8, 24, 25, 29, 38). These findings were confirmed in the present study. Our results indicated that both sulindac and sulindac sulfone (over a 4-wk treatment period) caused a significant decrease in rat colorectal tumor volume and that sulindac showed a greater inhibitory effect than sulindac sulfone.

DMH and its metabolite AOM cause colorectal cancer in rodents with a distribution and histology similar to those observed in human colorectal cancer (5) and are thus widely used as a model for human colorectal carcinogenesis. Chemically induced colorectal tumors in rodents have been shown to contain K-ras mutations (15, 20, 43) with an incidence similar to that observed in humans. In this study we have found activating K-ras (codon 12) point mutations in ~60% of tumors, which correlates well with the reported values ranging between 40 and 65% (15, 43). It has been demonstrated that all of the K-ras point mutations observed in the DMH model are G-to-A transitions, and >95% occur in codons 12 and 13, with ~70% occurring in codon 12 (43). Codon 12 mutations are considered the mutations most relevant to progression of colorectal cancer, because activating codon 12 point mutations, but not codon 13 mutations, have been found in all of the stages of colorectal tumor progression including ACFs, microadenomas, and carcinomas. The techniques (17, 43) used in our study detect these K-ras G-to-A point mutations in codon 12.

Activated K-ras has been shown to stimulate cell proliferation in cancers, and a recent study (45) showed that an activated K-ras was associated with a reduction in the frequency of apoptotic cells in human colorectal cancer. The balance between cell proliferation and cell death is critical in normal tissues. Increased cell proliferation and decreased frequency of apoptosis shift this balance in favor of development of cancer.

A recent in vitro study (14) has demonstrated that sulindac sulfide, a metabolite of sulindac, specifically inhibits signaling by the ras gene product p21ras by binding to the p21ras protein. It also blocks activation of p21ras GTPase by activation of p120GAP. The authors of that study suggest that sulindac sulfide will bind and inhibit the signaling of both wild-type and mutant p21ras (14). Given that oncogenic p21ras has unregulated GTPase activity, sulindac sulfide may have a greater effect on wild-type p21ras (where its binding also regulates GTPase activity) than on mutant p21ras protein. Thus sulindac (converted to sulindac sulfide in vivo) may have an inhibitory effect on both wild-type and mutant p21ras protein in colon tumors in the rat.

There are conflicting suggestions as to whether tumors with a K-ras mutation are more, or less, susceptible to sulindac treatment. A recent study (45) in human colorectal cancer samples showed that an activated K-ras is associated with a reduction in the
frequency of apoptotic cells. An in vitro study showed
that a K-ras oncogene increases resistance to sulindac-
induced apoptosis in rat enterocytes (1). This suggests
that an activated K-ras oncogene could confer resist-
ance to sulindac-mediated apoptosis, and thus sulin-
dac would be a more effective inhibitor of colorectal
tumors that do not have an activated K-ras, if sulindac
mediates its chemopreventive effect via apoptosis. To
date, only one in vivo study has examined a relation-
ship between sulindac and K-ras activation in colorec-
tal cancer (37). In an AOM-induced model of colorec-
tal rat cancer, sulindac caused a reduction in the number
of K-ras codon 12 mutations found in colon tumor and
stool samples. The authors of that study suggested that
sulindac may be specifically suppressing cells with a
K-ras codon 12 mutation and that sulindac mediates its
inhibitory effect in rat colorectal cancer via suppression
of cell proliferation. However, they did not measure cell
proliferation in their study (37).

Our results do not support either a specific suppres-
sion of cells with a K-ras codon 12 mutation by sulindac
or selective inhibition of tumors without an activated
K-ras. In the sulindac-treated group, seven of eight
tumors present after treatment had decreased in size
over the treatment period, and five of eight had K-ras
mutations. Only one tumor increased in size during the
sulindac treatment, and this tumor was K-ras wild
type. Our study differs from the in vivo study described
above (37) in that the duration of sulindac treatment
was for 4 wk rather than 52 wk, and in our study
sulindac was not administered before initiation of
carcinogenesis. Data from our laboratory indicate that
there is no difference in the inhibitory effect of sulindac
whether administration is for 4 wk at the end or
continuously for the 23-wk duration of the experiment
(unpublished data).

No studies to date have examined the relationship
between K-ras activation and sulindac sulfone inhibi-
tion in colorectal cancer, in vivo or in vitro. One study
(41) examined the relationship between H-ras activa-
tion and sulindac sulfone inhibition of rat mammary
carcinogenesis. This study showed effective reduction
of incidence and number of cancers by sulindac sulfone.
Interestingly, it demonstrated inhibition by sulindac
sulfone of mammary carcinomas with wild-type or
mutant codon 12 H-ras, with a greater inhibitory effect
against carcinomas with a mutant H-ras genotype.
Related in vitro studies suggested that induction of
apoptosis may be the chemopreventive mechanism.

Specific carcinogens have been shown to induce
unique mutations, and ras oncogenes present in chemi-
cally induced animal models have a narrow mutation
spectrum. Several studies indicated that H-ras muta-
tions are not detected in DMH-induced colorectal car-
cinogenesis (15, 20). It could be postulated that sulindac
sulfone may exert its inhibitory effect with regard to
K-ras codon 12 mutations in DMH-induced colorectal
carcinogenesis in a manner similar to that for H-ras
codon 12 mutations in mammary carcinogenesis. Our
results do not support this, because carcinomas with
both wild-type and mutated K-ras (codon 12) were
inhibited by treatment with sulindac sulfone (Table 3).
There was no greater inhibitory effect observed for
colorectal tumors bearing an activated K-ras in our
study. Overall, there is no apparent correlation be-
tween sulindac sulfone effect and K-ras mutation sta-
tus in our model.

Given the relatively small number of tumors in the
study, it is possible that there may be a difference in
K-ras mutation frequency between the treated (sulin-
dac group and sulfone group) and control (vehicle)
groups that was unable to be detected. The magnitude
of the difference in proportion of K-ras mutations
between the groups is ~10% (52% in controls, 62% in
sulindac-treated tumors, and 64% in sulindac sulfone-
treated tumors). The number of tumors per group in
our study ranged from 8 to 21. Because of the limited
number of tumors available, this study did not have the
power to detect a statistically significant change of this
magnitude. However, if either sulindac or sulindac
sulfone were selectively inhibiting a particular K-ras
genotype, we would expect to see a skewing of these
data such that the majority of tumors in a treatment
group were of a single K-ras genotype. In our study,
neither sulindac nor sulindac sulfone demonstrated selective inhibition of a particular K-ras genotype.

The exact mechanisms of the chemopreventive action
of sulindac and sulindac sulfone have not yet been
elucidated. Additionally, it has not been determined
whether sulindac and its sulfone metabolite, with no
known cyclooxygenase (COX) inhibitory activity, medi-
ate their inhibitory effects via different or similar
pathways. Previously, the majority of NSAIDs effective
in inhibition of colorectal cancer were thought to work
via inhibition of COX, and more specifically via inhibi-
tion of COX-2 (3, 16, 39). The ability of sulindac sulfone
to inhibit colorectal cancer in experimental systems
was unexpected given that it has no effect on either
COX-1 or COX-2. Recently, we measured prostaglandin
levels in tumor tissue after treatment with sulindac (8).
We found that, although tumor growth is significantly
inhibited by sulindac treatment, prostaglandin levels
are unaffected. Currently, a growing body of evidence
suggests that both sulindac and sulindac sulfone are
mediating their inhibitory effects independently of
COX activity (4, 13, 14, 30). There is evidence to
suggest that restoration of apoptosis (22, 30, 31, 35) or
inhibition of cell proliferation (11, 28, 36) are possible
common mechanisms of action of sulindac and sulindac
sulfone in mediating tumor inhibition.

In conclusion, the results of our study do not support
selective inhibition by either sulindac or sulindac sul-
one of colorectal rat tumors on the basis of K-ras
mutation status. This suggests that the mechanism of
effect of sulindac and of sulindac sulfone is independent
of the K-ras status of the tumor.

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