Sulfone Metabolite of Sulindac Inhibits Mammary Carcinogenesis

Henry J. Thompson, Cheng Jiang, Junxuan Lu, Rajendra G. Mehta, Gary A. Piazza, Nancy S. Paranka, Rifat Pamukcu, and Dennis J. Ahnen

AMC Cancer Research Center, Lakewood, Colorado 80214; Cell Pathways, Aurora, Colorado 80012; Department of Surgical Oncology, University of Illinois, Chicago, Illinois 60612; and Department of Veterans Affairs Medical Center, Denver, Colorado 80220.

ABSTRACT

Sulindac sulfoxide, a commonly prescribed anti-inflammatory drug, has cancer chemopreventive activity. During its metabolism, the inactive prodrug sulindac sulfoxide undergoes either reduction to the active anti-inflammatory metabolite sulindac sulfide or irreversible oxidation to sulindac sulfone, which lacks prostaglandin synthetase inhibitory activity. Interestingly, sulindac sulfone has been reported to have cancer chemopreventive activity. The objective of the experiments reported here was to investigate the chemopreventive activity of sulindac sulfone against mammary carcinogenesis and to study its mechanism. Rats were injected with either 12.5 or 37.5 mg of 1-methyl-1-nitrosourea (MNU)/kg body weight at 50 days of age. Sulindac sulfone was incorporated into a purified diet at a concentration of either 0.03 or 0.06% (w/w) and fed to rats beginning 7 days after the injection of MNU. Sulindac sulfone at a level of 0.06% (w/w) was fed as a reference for comparison. Thirty rats were assigned to each dietary group treated with the high dose of MNU, and 44 rats were assigned to each dietary group treated with the low dose of MNU. The sulfone reduced cancer incidence and the number of cancers per rat irrespective of the dose of MNU injected, and its chemopreventive activity was comparable to that of sulindac sulfone. Cancer latency was also prolonged significantly by sulindac sulfone; the effect was particularly notable at the low dose of carcinogen, at which the prolongation of latency was >8 weeks. The sulfone inhibited the occurrence of mammary carcinomas that were classified as having either a wild-type or a mutant codon 12 in the Ha-ras gene; however, the inhibitory effect was greater against carcinomas with a mutant Ha-ras genotype. Using a mammary gland organ culture transformation assay, it was observed that sulindac sulfone also inhibited the formation of 7,12-dimethylbenz(a)anthracene-induced hyperplastic alveolar nodules and that the inhibitory activity of the sulfone was comparable to that of the sulfone. These data indicate that the observed effect of the sulfone on mammary carcinogenesis in vivo is likely to be due to a tissue-specific effect rather than to other systemic effects. The findings that both the prodrug and the sulfone inhibited carcinogenesis in vivo and nodule formation in organ culture and that the sulfone inhibited chemically induced mammary and colon carcinogenesis (6, 7). This finding is significant relative to the use of NSAIDs for cancer chemoprevention because one factor limiting their use is gastrointestinal toxicity, an effect attributed to the inhibition of prostaglandin synthetase activity, inhibited chemically induced mammary and colon carcinogenesis (6, 7).

INTRODUCTION

Sulindac sulfoxide and other NSAIDs3 have been reported to inhibit tumor formation in several models of experimentally induced cancer (1–3). The cancer-preventive activity has generally been attributed to the inhibition of prostaglandin synthesis. Sulindac sulfone is a prodrug that is either reversibly reduced to sulindac sulfide, which has antiprostaglandin synthetase activity due to its ability to inhibit cyclooxygenase I and II, or is irreversibly oxidized to the sulfone, which has been considered an inactive metabolite that is eliminated from the body (4, 5). Nonetheless, in several publications it has been reported that feeding the sulfone metabolite of sulindac, which has been shown not to affect prostaglandin synthetase activity, inhibited chemically induced mammary and colon carcinogenesis (6, 7). This finding is significant relative to the use of NSAIDs for cancer chemoprevention because one factor limiting their use is gastrointestinal toxicity, an effect attributed to the inhibition of prostaglandin synthetase activity, and implies that this aspect of toxicity may be avoidable. This issue gains increased importance in view of a recent clinical report that use of NSAIDs was associated with a reduction in human breast cancer risk (odds ratio, 0.66; 95% confidence interval, 0.52–0.83) and that breast cancer risk declined with increasing NSAID exposure (10).

In the experiments reported in this study, the effect of two dietary concentrations of sulindac sulfone on the occurrence of mammary carcinogenesis induced by a low or high dose of MNU was evaluated. The hypothesis that the sulfone exerts a selective inhibitory effect on the clonal expansion of MNU-initiated cells harboring a mutated ras gene was also tested. Such a selective effect has been attributed to another NSAID, piroxicam, in an azoxymethane-induced colon carcinogenesis model (11). In chemical carcinogenesis models, specific ras mutations are induced and are believed to be involved in the early stages of tumor development (12–16). Depending on the dose of carcinogen administered, a proportion of the mammary adenocarcinomas induced by MNU in the rat contain a G→A transition mutation in codon 12 of the Ha-ras gene (17, 18). Therefore, selective inhibition of the clonal expansion of subpopulations of initiated cells identified by this marker is expected to result in an altered proportion of tumors harboring the mutation. This hypothesis was evaluated by determining the proportion of induced tumors in each treatment group that had the codon 12 mutation in the Ha-ras gene. The hypothesis that sulindac sulfone exerts selective effects on the mammary gland independent of systemic drug metabolism was investigated by determining the effect of sulindac sulfoxide and sulfone on the formation of DMBA-induced hyperplastic alveolar nodules in a mammary gland organ culture transformation assay. This procedure is widely used in the evaluation of agents for chemopreventive activity and identifies agents effective in inhibiting mammary carcinogenesis in the rat model that was used (19). Furthermore, the effect of these metabolites on the growth of a mammary carcinoma cell line also was studied to determine whether these metabolites affect cell growth by inducing...
apoptosis. Apoptosis is a physiological cell death pathway involved in the regulation of tissue size and has been reported to be dysregulated during carcinogenesis (20, 21). NSAIDs have recently been reported to induce apoptosis in colon carcinoma cells (22), but effects on epithelial cells derived from the mammary gland have not been reported. The rationale for assessing the ability of sulfindac metabolites to induce apoptosis also took into account reports that altered regulation of the ras signal transduction pathway can induce apoptosis (23) and that azomethane-initiated colon cells harboring mutated ras genes were selectively inhibited by the NSAID piroxicam (11). As reviewed in Ref. 24, an increase in the rate of apoptosis in a cell population could inhibit that population of cells from developing.

MATERIALS AND METHODS

Drug Synthesis. The sulfone metabolite [cis-5-fluoro-2-methyl-1(p-meth-ylsulfonylbenzylidene)-3-indenylacetic acid] of sulfindac sulfoxide (purchased from Therapicon, Milan, Italy) was prepared as described in Ref. 22. Chemical analyses by high-performance liquid chromatography, nuclear magnetic resonance, infrared, and elemental analysis demonstrated the lot of sulfone used in this study to be 97.8% sulfindac sulfoxide, 1.4% sulfindac sulfoxide, and 0.8% sulfindac sulfoxide epoxide.

Animal Studies. Female Sprague Dawley rats were obtained from Taconic Farms, Germantown, NY, at 21 days of age, housed three per cage, and maintained in an environmentally controlled room at 22°C with 50% relative humidity and a 12-h-light, 12-h-dark cycle. At 50 days of age, rats were injected i.p. with either 12.5 or 37.5 mg of MNU/kg of body weight (25). Following carcinogen treatment, rats were randomized into one of four dietary groups at each dose of carcinogen. Forty-four rats were assigned to each dietary group that received the low dose of carcinogen, and 30 rats were assigned to each dietary group that received the high dose of MNU. The sulfindac sulfoxide or sulfoxide was administered in a purified high-fat (24.6%, w/w) diet formulated with corn oil (diet composition given in Ref. 26) beginning 7 days after the injection of MNU. Thus, the effects of the sulfone and sulfoxide were limited to the postinitiation events in mammary carcinogenesis. Sulfindac sulfoxide was incorporated into the diet at 0.03 or 0.06% (w/w); sulfindac sulfoxide served as a positive control and was incorporated at 0.06% (w/w). A purified diet formulation was used to eliminate the variability in diet composition associated with the use of rodent chow diets. Furthermore, a high-fat diet formulation more closely models the typical Western diet consumed by individuals to whom this intervention may someday be applied. High-performance liquid chromatography analyses showed the sulfone to be stable in this diet for a period in excess of 30 days.

Rats were weighed weekly and palpated twice each week for detectable mammary tumors. Rats injected with the high dose of MNU were killed at 24 weeks postcarcinogen. Animals injected with the low dose of MNU were killed at 37 weeks postcarcinogen. At necropsy, animals were euthanized with gaseous CO₂ and then skinned and their pelts examined under incandescent light. All tumors and suspicious areas were excised and processed for histopathological classification (27).

Haras Mutation Assay. The mutational status of Haras codon 12 (GGA-GAA) in mammary adenocarcinomas was assessed by modified PCR-generated restricted fragment length polymorphism method on paraffin-embedded tissue blocks (28). Briefly, DNA extracts from the paraffin-embedded tumors were used as templates for PCR amplification of the rat codon 12 region. The downstream PCR primer was designed with two mismatches so that a codon 12 GGA-GAA mutation would introduce a XmnI site in the PCR product. Upon digestion of the products with XmnI and separation by electrophoresis on a 6% polyacrylamide gel, a 96-bp band was generated if the mutation was present, whereas the normal PCR product was not sensitive to this enzyme and remained as a 118-bp band. To increase detection sensitivity, PCR products were labeled by incorporation of [α-32P]dCTP and detected by autoradiography.

Drug Preparation for Organ Culture and Cell Culture Experiments. Stock solutions of sulfindac sulfoxide or sulfoxide were made at 1000X concentrations in DMSO and diluted with medium to the final working concentrations. The final concentration of DMSO for all treatments was maintained at 0.1%. All drug solutions were prepared fresh on the day of testing.

Organ Culture Experiments. Virgin female BALB/c mice, 3-4 weeks of age, were obtained from Charles River, Wilmington, MA. Upon arrival the mice were treated daily with subcutaneous injections of 1 μg of estradiol 17β + 1 mg progesterone for 9 days. This treatment is a prerequisite inasmuch as animals not pretreated with steroids fail to respond to hormones in vitro (29). The entire culture procedure has been described in detail (30).Briefly, the animals were killed by cervical dislocation and the thoracic pair of mammary glands was dissected and spread out on a silk raft. These tissue preparations were incubated for 10 days in Waymouth MB752/1 medium (5 glands/5 ml of medium/dish). The medium was supplemented with 2 mm glutamine, antibiotics (penicillin and streptomycin, 100 units/ml each), and growth-promoting hormones, 5 μg of insulin, 1 μg of prolactin, 1 μg of aldosterone, and 1 μg of hydrocortisone per ml of medium. The carcinogen DMBA (2 μg/ml) was added to the medium for 24 h between days 3 and 4. For the present study, DMBA was dissolved in DMSO at a final concentration of 4 mg/ml, and 50 μl were added to 100 ml of medium, resulting in 2 μg/ml final concentration. The control dishes contained DMSO as vehicle. On day 4, DMBA was removed from the medium by rinsing the glands in fresh medium and transferring them to new dishes containing fresh medium without DMBA. After 10 days of incubation, the glands were maintained for another 14 days in the medium containing only insulin (5 μg/ml). During the entire culture period, the glands were maintained at 37°C in a 5% C0₂ and 95% environment.

Sulindac sulfoxide and sulfindac sulfoxide were added to the medium on the day the culture was initiated, and drug treatment was continued throughout the experiment. Carcinogen-treated glands without the test agent served as a positive control. At the end of the experiment, glands were fixed and stained for microscopic evaluation and lesion identification as described in Ref. 30.

Cell Culture Experiments. MCF-7 cells were obtained from American Type Culture Collection (Rockville, MD) and were grown in RPMI (Celox, Hopkins, MN) supplemented with 5% FCS (Gemini Bioproducts, Inc., Calabasas, CA), and 2 mm glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.25 μg/ml amphotericin (Life Technologies, Inc., Grand Island, NY). Cultures were maintained in a humidified atmosphere of 95% air and 5% C0₂ at 37°C. The cultures were passaged at preconfluent densities with the use of a solution of 0.05% trypsin and 0.53 mm EDTA (Celox). Cells were plated at 1 × 10⁵ cells/25-cm² flask.

Apoptosis and Necrosis. Drug effects on apoptosis and necrosis of cultured MCF-7 cells were determined morphologically by fluorescent microscopy after labeling with acridine orange and ethidium bromide as described by Duke and Cohen (31). Floating and attached cells were collected by trypsinization and washed three times in PBS. One ml aliquots of 1 × 10⁶ cells were centrifuged (300 × g). The pellet was gently resuspended in 25 μl of media and 1 μl of dye mixture containing 100 μg/ml acridine orange and 100 μg/ml ethidium bromide prepared in PBS. Ten μl of mixture were placed on a microscope slide and covered with a 22-mm coverslip and examined under × 40 dry objectives with the use of epilumination and filter combination. An observer blinded to the identity of treatments scored at least 100 cells/sample. Live cells were determined by the exclusion of ethidium bromide stain. Live and dead apoptotic cells were identified by nuclear condensation of chromatin stained by the acridine orange and the ethidium bromide, respectively. Necrotic cells were identified by uniform labeling of the cell with ethidium bromide.

Statistical Analyses. Statistical differences in cancer incidence were determined by χ² analysis (32). Tumor count data were analyzed by factorial ANOVA following square root transformation (32). Differences among groups in the proportion of tumors with mutant or wild-type ras status were evaluated by the Fisher exact test (32). Differences in cancer latency were assessed by a life table procedure (33).

RESULTS

Mammary Carcinogenesis Experiments

Low Dose of MNU. The effect of feeding sulindac sulfoxide on mammary carcinogenesis induced by the low dose of MNU is shown in Table 1. In comparison to animals that received no drug, both the sulfoxide and the sulfoxide reduced cancer incidence and reduced the total number of carcinomas detected. The inhibitory activity of both compounds was statistically significant at P < 0.05 for all comparisons with the untreated group; however, the effects of the three drug
SULINDAC SULFONE INHIBITS MAMMARY CARCINOGENESIS

Table 1 Effect of sulindac sulfone and sulfoxide on mammary carcinogenesis induced by a low dose of MNU

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Mammary carcinomas, incidence (%)</th>
<th>Mammary carcinomas, total no.</th>
<th>Final body weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>44/43</td>
<td>29.6 (100)</td>
<td>16 (100)</td>
<td>399 ± 8 (100)</td>
</tr>
<tr>
<td>0.03% sulfone</td>
<td>44/42</td>
<td>11.6 (39)</td>
<td>9 (56)</td>
<td>386 ± 6 (97)</td>
</tr>
<tr>
<td>0.06% sulfone</td>
<td>44/43</td>
<td>16.3 (55)</td>
<td>8 (50)</td>
<td>354 ± 8 (89)</td>
</tr>
<tr>
<td>0.06% sulfoxide</td>
<td>44/44</td>
<td>9.1 (31)</td>
<td>6 (38)</td>
<td>367 ± 8 (92)</td>
</tr>
</tbody>
</table>

a All rats were injected with 12.5 mg MNU/kg body weight at 50 days of age as described in “Materials and Methods.” The study was terminated 37 weeks post-carcinogen treatment.
b Number of rats randomized to an experimental group/number of rats surviving to the end of the experiment.
c Incidence of mammary carcinomas found at the end of the experiment. The number in parentheses is the percent of the response observed in the control group.
d Total number of mammary carcinomas found at the end of the experiment. The number in parentheses is the percent of the response observed in the control group.
e Body weight of the animals at the end of the study. The number in parentheses is the percent of the response observed in the control group. Values are means ± SE.
f Values with different superscripts are statistically different (P < 0.05).

Fig. 1. The effect of treatment with sulindac sulfone or sulindac sulfoxide on the induction of mammary carcinomas by a low dose (12.5 mg/kg of body weight) of MNU. The incidence of palpable mammary carcinomas as a function of time post-carcinogen treatment is shown. The effect of sulfoxide and sulfoxide on cancer latency were statistically significant by life table analyses (P < 0.05).

Organ Culture Experiments

Experiments were performed to evaluate the effects of sulindac sulfone and sulfoxide on the development of DMBA-induced mammary lesions in organ culture. A total of five organ culture experi-
DISCUSSION

The results reported in Tables 1 and 2 and Figs. 1 and 2 confirm and expand our preliminary report that sulindac sulfone, which has a minimal effect on either COX I or COX II activity in the prostaglandin biosynthetic pathway, significantly inhibits mammary carcinogenesis induced by either a low or a high dose of 1-methyl-1-nitrosurea (6). The data presented on body weights (Table 1) indicate that these treatments were well tolerated. In fact, the levels of either sulindac sulfoxide or sulfone required for chemopreventive activity against mammary carcinogenesis in the rat were lower than other laboratories have reported for the chemoprevention of colon carcinogenesis in the same species (1, 2). An additional hypothesis that was evaluated in the carcinogenesis experiment was formulated based on a report that piroxicam, another NSAID, inhibited the selective amplification of initiated cells harboring mutated ras genes in the azoxymethane-induced colon carcinogenesis model (11). As shown in Tables 1 and 2, sulindac sulfone and sulfone reduced carcinoma number; however, the data in Table 2 indicate that both compounds had greater inhibitory activity against carcinomas harboring mutant Ha-ras genes. These data indicate a selective inhibition of the clonal expansion of initiated cells harboring mutated Ha-ras genes. Of interest in this regard is a recent review (34) in which dysregulation of downstream components of the Ras signal transduction pathway was reported to play a role in human breast carcinogenesis.

The organ culture model used in the experiments reported has been used to evaluate the chemopreventive efficacy of many agents (19). Our observations indicate that both sulindac sulfoxide and sulfone inhibit DMBA-induced alveolar nodulogenesis in this mouse mammary gland organ culture transformation assay. These findings in the organ culture system are also consistent with those in the MNU model in that both sulindac sulfone and sulfoxide were inhibitory and that in both models the inhibitory effect of the sulfone was dose-dependent. This finding provides strong evidence that these sulindac metabolites exert a specific effect on the mammary gland and tumorigenesis therein that is apart from any systemic effects that either compound may exert. This observation

Cell Culture Experiments

A concentration-dependent reduction in cell number was observed in response to sulindac sulfone treatment (Fig. 3A), and the effect of the sulfone was somewhat greater than that of the sulfoxide at comparable concentrations (Fig. 3C). Accompanying the observed reductions in cell number was a proportionate increase in the percentage of cells undergoing apoptosis induced by both compounds (Fig. 3, B and D). These compounds had no significant effect on necrosis irrespective of the concentration of drug treatment (Fig. 3, B and D).

Fig. 2. The effect of sulindac sulfone and sulindac sulfoxide on the occurrence of mammary gland nodular lesions induced in mouse mammary gland organ culture by treatment with DMBA. The inhibitory activity of the sulfone on nodule occurrence was significant by logistic regression analysis (P < 0.05).

Fig. 3. The effect of sulindac sulfone and sulindac sulfoxide on cell number, apoptosis, and necrosis as assessed in MCF-7 cells. Results shown are from a representative experiment. Data points, mean; bars, SE. Experiments were done at least three times to confirm that the same patterns of response were consistently observed.
is counter to reports that the effects of NSAIDs on lung carcinogenesis include systemic mediated effects on prostaglandins (35). These data support the observation that inhibition of prostaglandin biosynthesis, an activity attributed to sulindac sulfide, appears unnecessary for chemopreventive activity in the organ culture assay or in the vivo mammary carcinogenesis model.

It has been reported in cell culture systems and in vivo colon polypys that metabolites of sulindac inhibit cell growth by inducing apoptosis (7, 22). Because one mechanism by which selection against particular transformed cell populations might be affected is induction of apoptosis and because, as noted above, sulindac sulfone and sulfoxide appear to preferentially inhibit the clonal expansion of MNU-initiated cells harboring mutated Ha-ras genes, we asked whether these metabolites of sulindac would induce apoptosis in a well-studied mammary carcinoma cell line, MCF-7. As shown in Fig. 3, both sulindac metabolites inhibited the growth of this cell line. It also was noted that each compound induced a comparable level of apoptotic cell death at the drug concentration that inhibited growth by 50%. It is also noteworthy that the sulfone and the sulfoxide induced increasing levels of apoptosis in the absence of a concomitant increase of necrosis, which supports the hypothesis that sulfone acts by triggering physiological rather than pathological cell death.

In their totality, the data presented indicate that sulindac sulfone, which lacks significant prostaglandin synthetase inhibitory activity (6), inhibits MNU-induced mammary carcinogenesis in a dose-dependent manner. Carcinogenesis induced by a low or high dose of carcinogen was inhibited to a comparable degree. The observed effects of the sulfone and the sulfoxide on the occurrence of Ha-ras codon 12 mutant carcinomas are consistent with the idea that clones of transformed cells with a specific pathogenetic characteristic were preferentially inhibited. Moreover, the data indicate that the protective effect is exerted on the target tissue and is not dependent on systemic effects that NSAIDs may exert. These effects were observed at concentrations of the sulfone that were well tolerated by the animal, and the cell culture data indicate that apoptosis can be induced by the sulfone without necrosis. These data provide important leads about the mechanisms by which sulindac sulfone works, which merit further investigation.

The data presented in this study illustrate an emerging concept for cancer prevention and control that may be applied to carcinogenesis at many organ sites. Namely, with the expanding ability to identify the pathogenetic characteristics of clones of cells that progress through the carcinogenic process, it will be possible to target specific pathogenetic cascades to prevent and control the development of cancer. By determining an individual’s genetic predisposition for a specific cancer, it may be possible to assign that individual to a treatment regime that specifically blocks the processes of clonal expansion and selection of populations of cells with those pathogenetic characteristics that are associated with that individual’s cancer risk. From such an approach, it is likely that highly specific and effective methods for the prevention and control of cancer will be developed.

ACKNOWLEDGMENTS

We thank Dr. Meenaksi Singh for her diagnosis of mammary tumors and Kim Rothammer, John McGinley, and Stephanie Briggs for their excellent technical assistance.

REFERENCES


Sulfone Metabolite of Sulindac Inhibits Mammary Carcinogenesis


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/57/2/267

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.