

Research Article

Overexpression of Cyclooxygenase-2 in Rat Oral Cancers and Prevention of Oral Carcinogenesis in Rats by Selective and Nonselective COX Inhibitors

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Abstract

Oral squamous cell carcinomas induced in rats by 4-nitroquinoline-1-oxide (NQO) show substantial overexpression of cyclooxygenase-2 (COX-2) when compared with adjacent phenotypically normal oral tissues. By contrast, neither 5-lipoxygenase (LOX) nor 12-LOX is overexpressed in rat oral cancers. Two chemoprevention studies were done to test the resulting hypothesis that COX-2 is a useful target for oral cancer chemoprevention in the rat. In both studies, male F344 rats received drinking water exposure to NQO (20 ppm) for 10 weeks, followed by administration of chemopreventive agents from week 10 until study termination at week 26. In the first study, groups of rats were fed basal diet (control), or basal diet supplemented with the selective COX-2 inhibitor celecoxib (500 or 1,500 mg/kg diet), the nonselective COX inhibitor piroxicam (50 or 150 mg/kg diet), or the 5-LOX inhibitor zileuton (2,000 mg/kg diet). In the second study, rats were fed basal diet (control) or basal diet supplemented with nitric oxide-naproxen (180 or 90 mg/kg diet), a nonselective COX inhibitor that shows reduced gastrointestinal toxicity. When compared with dietary controls, celecoxib decreased oral cancer incidence, cancer invasion score, and cancer-related mortality. Piroxicam decreased cancer-related mortality and cancer invasion score, whereas nitric oxide-naproxen decreased oral cancer incidence and cancer invasion score. By contrast, zileuton showed no chemopreventive activity by any parameter assessed. These data show that both selective and nonselective inhibitors of COX-2 can prevent NQO-induced oral carcinogenesis in rats. The chemopreventive activity of COX inhibitors may be linked to overexpression of their enzymatic target in incipient oral neoplasms. *Cancer Prev Res*; 3(1); 73–81. ©2010 AACR.

Introduction

As a general term, oral cancer (often grouped with esophageal and laryngeal cancers and called cancers of the head and neck) includes malignancies of the mouth, tongue, pharynx, and other sites in the oral cavity. Major risk factors for carcinoma of the oral cavity include the use of alcohol and tobacco, including “smokeless” tobacco products such as chewing tobacco and snuff (1–3). The WHO estimates that tobacco and alcohol use are responsible for ~75% of all oral cancer cases worldwide (4).

The oral cavity provides an attractive site for clinical efforts in cancer prevention, as site accessibility and the existence of grossly visible preneoplastic lesions (such as leukoplakias) facilitate the evaluation of disease progression (5). However, in spite of well-established relationships between tobacco use, alcohol use, and oral cancer

risk, efforts in primary prevention of oral carcinogenesis have met with only limited success. The overall incidence of oral cancer seems to be declining slowly, most likely as a result of efforts in tobacco control (6, 7). In spite of this progress, the American Cancer Society estimates that ~35,700 new cases of oral cancer will be diagnosed in the United States in 2009, and that ~7,600 Americans will die of the disease (8). In consideration of the significant mortality associated with oral cancer (5-year survival rate of ~60%; ref. 8) and the adverse effect of surgical interventions on the quality of life in many oral cancer patients, prevention of oral carcinogenesis through the administration of chemopreventive agents may yield substantive benefits in terms of both reductions in disease-associated mortality and improvements in the quality of life in individuals at increased risk of this family of diseases.

Inhibitors of prostaglandin endoperoxide synthase (cyclooxygenase; COX) are a group of chemically diverse compounds with a common activity in the suppression of the COX arm of the arachidonic acid cascade. Nonspecific COX inhibitors such as aspirin, ibuprofen, naproxen sodium, and piroxicam inhibit both COX-1 and COX-2 isozymes, and have been used for decades to treat arthritis and as over-the-counter pain medications (9); these agents

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are often called nonsteroidal anti-inflammatory drugs (NSAID). In consideration of our extensive knowledge of their experimental and clinical safety profiles (10), NSAIDs are an attractive class of compounds for possible development as cancer preventive agents.

Several commonly prescribed NSAIDs inhibit cancer induction in laboratory animals in sites including the colon, lung, urinary bladder, esophagus, skin, and breast (reviewed in refs. 11, 12). Epidemiologic data also show chemopreventive activity for NSAIDs in several sites. Recent meta-analyses of site-specific epidemiology data have shown that aspirin reduces the incidence of colorectal adenomas (13, 14), and other studies suggest chemopreventive activity against human esophageal cancer (15), lung cancer (16), and nonmelanotic skin cancer (17), among other tissues. Although intestinal toxicity is a common finding in individuals given high doses of NSAIDs (9, 10), extensive clinical experience shows that pharmacologically active doses of several NSAIDs can be administered chronically to many patients with minimal adverse effects.

To address the sometimes limiting intestinal toxicity associated with administration of nonspecific COX inhibitors, specific inhibitors of the COX-2 isozyme have been developed. The gastrointestinal toxicity profiles of COX-2 inhibitors such as celecoxib and rofecoxib are superior to those of traditional NSAIDs (18, 19), and these agents have been studied extensively in arthritis therapy and for possible activity in cancer prevention. In experimental animals, celecoxib inhibits carcinogenesis in several sites, including the colon, stomach, skin, mammary gland, lung, prostate, urinary bladder, and oral cavity (11, 12). However, results in the clinic have been less positive; although specific COX-2 inhibitors can prevent the development of colorectal adenomas (20), significant increases in the incidence of cardiovascular and thrombotic events have been noted in chemoprevention trials in which patients were treated with coxib drugs (21–24). Based on the serious toxicities associated with these agents, the potential value of specific COX-2 inhibitors for cancer prevention is being re-evaluated. One possible conclusion from the comparative pharmacodynamics and toxicities of specific and nonspecific COX inhibitors is that manageable gastrointestinal toxicity associated with nonspecific COX inhibition is a less important limitation to cancer prevention in high-risk populations than the significant cardiovascular effects associated with administration of specific COX-2 inhibitors.

In the present studies, we compared the chemopreventive activities of a specific COX-2 inhibitor (celecoxib), a classic nonspecific COX inhibitor (piroxicam), a new nitric oxide (NO)-releasing NSAID (NO-naproxen) that inhibits both COX-1 and COX-2 while showing reduced gastrointestinal toxicity, and a 5-lipoxygenase (5-LOX) inhibitor (zileuton) in a rat model for oral cancer. We report significant chemopreventive efficacy for all three COX inhibitors, but no chemopreventive activity for the LOX inhibitor. The chemopreventive efficacies of these two classes of agents are correlated with the differ-

ential expression of their putative enzymatic targets in normal and neoplastic rat oral tissues.

Materials and Methods

Before the initiation of *in vivo* work, study protocols were reviewed and approved by the IIT Research Institute Animal Care and Use Committee. All aspects of the program involving animal care, use, and welfare were performed in compliance with USPHS Policy on Humane Care and Use of Laboratory Animals, and in compliance with the guidelines stated in the National Research Council *Guide for the Care and Use of Laboratory Animals*.

Animal receipt, housing, and quarantine

Male F344 rats were received at 6 to 7 wk of age from virus-free barrier colonies that are maintained under contract to the National Cancer Institute, Frederick, MD. Rats were held in quarantine for a minimum of 1 wk before study initiation; before randomization into a study, each rat underwent a hand-held physical examination to ensure its suitability for use as a test subject.

Animals were housed on hardwood bedding in polycarbonate shoebox cages (two to three per cage) in a windowless room that was illuminated for 12 h each day and maintained at $22 \pm 1^\circ\text{C}$ and within the range of 30% to 70% relative humidity. Throughout all studies, rats were permitted free access to Purina 5001 Laboratory Diet (PMI Feeds) and City of Chicago drinking water (provided in water bottles). All food cups and water bottles were replaced a minimum of twice weekly.

Carcinogen administration

4-Nitroquinoline-1-oxide (NQO) was purchased from Sigma-Aldrich and was stored in the dark at -20°C until used. NQO was administered in the drinking water at 20 ppm for a period of 10 wk. After dose preparation, all formulations of NQO were stored in the dark at 4°C until used. Bottles containing NQO-supplemented drinking water were wrapped with foil to preclude possible photodegradation of the carcinogen, and were changed at 2- to 3-d intervals throughout the study. Rats received their first exposure to NQO at 7 to 9 wk of age.

Gene expression study

Quantitative reverse transcription-PCR (RT-PCR) analysis of gene expression was done using 10 paired sets of normal and neoplastic oral tissues harvested from rats at 24 to 26 wk after their first exposure to NQO. At necropsy, gross lesions were collected from the tongue of NQO-treated rats and bisected longitudinally. Half of each lesion was frozen in liquid nitrogen for analysis of gene expression. The other half of each lesion was fixed in formalin, processed using routine histologic methods, stained with H&E, and evaluated microscopically to confirm malignancy. Phenotypically normal tissue was collected from adjacent areas of the tongue of the same NQO-treated rats, and was frozen or fixed in a manner identical to that used for tumor tissues.

Total RNA was isolated from histologically confirmed squamous cell carcinomas and adjacent phenotypically normal tissues by homogenization in 4 mol/L guanidinium isothiocyanate, followed by extraction of RNA by using Trizol reagent (Invitrogen) and alcohol precipitation. Gene expression was analyzed in paired samples of normal and neoplastic oral tissues using the Taqman real-time PCR method and the Applied Biosystems 7700 Sequence Detection System; β -actin expression was used as a control. Primers and probes used for RT-PCR were as follows:

- COX-2: forward, ctcttgaacacggacttgc; reverse, tcaggga-gaagcgtttgc; probe, aagcagtggcaaaggcctccat
- 5-LOX: forward, ccatcagctcaaccaaacc; reverse, gatgtgtgcg-gagaagatgg; probe, cccaatttctcctacggactca
- 12-LOX: forward, aagcggatttctccttctgg; reverse, gttcaagctg-gatggctatgg; probe, agtacctggctgccctctcgtc

Chemoprevention studies

After release from quarantine, rats were assorted into experimental groups of 25 or 30 per group using a constrained randomization procedure that blocks for body weight. Chemopreventive agents were obtained from the Chemopreventive Agent Repository maintained by the Division of Cancer Prevention, National Cancer Institute, and were administered as dietary supplements. Dietary administration of each chemopreventive agent was initiated 1 d after the completion of NQO exposure (study week 10), and was continued until study termination at 26 wk after the first exposure to NQO. Dose levels of each agent were selected on the basis of the results of preliminary 6-wk toxicity/diet tolerance studies, and in consideration of levels of each agent that show chemopreventive activity in other rodent cancer models. Celecoxib was administered at 500 and 1,500 mg/kg diet; piroxicam was administered at 50 and 150 mg/kg diet; NO-naproxen was administered at 90 and 180 mg/kg diet; and zileuton was administered at 2,000 mg/kg diet.

Throughout the in-life period of each chemoprevention bioassay, twice daily observations were done to evaluate animal health status, and to identify possible toxic effects of the carcinogen and the agents being evaluated for chemopreventive activity. Animals were weighed at the time of randomization into experimental groups and once weekly throughout the studies. Monitoring of body weights is particularly important during latter stages of oral cancer studies in the NQO model, as body weight loss is a useful indicator of clinical progression of oral cancers induced by NQO. Weekly observations were done to monitor the appearance of oral lesions.

Necropsy, tissue collection, and pathology

All study animals underwent a gross necropsy that was focused on the tongue, oral cavity, and gastrointestinal tract. Rats found dead were necropsied immediately on discovery; rats identified as moribund before study termination were euthanized by CO₂ asphyxiation and necropsied immediately. Chemoprevention studies were terminated 26 wk after

initial exposure to NQO, at which time surviving rats were euthanized by CO₂ asphyxiation and were necropsied. At necropsy, the tongue from each animal was carefully excised, gross lesions were charted, and the tongue was fixed in 10% neutral-buffered formalin for histopathologic evaluation. All grossly visible abnormalities that were identified at necropsy were embedded in paraffin and processed by routine histologic methods. If no gross oral lesions were noted by the prosector, the histologist bisected whole tongues longitudinally, and embedded both halves in paraffin so that the longitudinal axis was cut into sections. Replicate sections from each block were cut at 5 μ m and stained with H&E for microscopic evaluation. The histopathologic classification of each lesion, its location, and the depth of invasion of each lesion were noted to quantify tumor incidence and to determine if a shift to a less invasive lesion may have occurred as a result of chemopreventive agent administration. Cancer invasion was classified using a semiquantitative grading system; lesions scored as +1 extended through the mucosal epithelial basement membrane and into the lamina propria only. Lesions scored as +2 extended through the lamina propria and into the upper muscle layers. The highest invasion score, +3, was assigned to lesions showing extensive infiltration into underlying muscle.

Statistical analysis

Evidence of chemopreventive activity was defined as a statistically significant ($P < 0.05$) reduction in oral cancer incidence, reduction in oral cancer invasion score, or increase in survival in a group treated with a chemopreventive agent versus that in the carcinogen-treated dietary control group. Intergroup comparisons of survival at study termination and the incidence of oral squamous cell carcinoma were made using χ^2 analysis. Because oral cancer invasiveness was evaluated using a semiquantitative scoring system, comparisons of invasion scores were done using nonparametric statistics (Wilcoxon rank-sum analysis). Body weights and other continuous data were compared by ANOVA, with *post hoc* comparisons made using Dunnett's test.

Results

Gene expression studies

RT-PCR analysis of gene expression in paired sets of normal and neoplastic oral tissues harvested from rats treated with NQO showed that COX-2 is significantly overexpressed in oral cancers induced by NQO. Mean expression of COX-2 in oral squamous cell carcinomas was 15.9 ± 6.7 -fold higher than was the expression of COX-2 in adjacent phenotypically normal oral tissues harvested from the same animal. COX-2 was overexpressed in 9 of 10 tissue sample pairs analyzed, with overexpression ranging approximately 12- to 23-fold in pairs of malignant and normal oral tissues collected from the same animal (Fig. 1). COX-2 was not significantly overexpressed in the malignant lesion in 1 of the 10 tissue pairs analyzed.

By contrast to the substantial and consistent overexpression of COX-2 in oral cancers, 5-LOX and 12-LOX showed

no consistent patterns of differential expression in normal and neoplastic oral tissues harvested from NQO-treated rats (data not shown). Both increases and decreases in LOX expression were seen in different pairs of neoplastic and normal oral tissues; although mean expression of 12-LOX in oral cancers was slightly elevated from that in phenotypically normal tissues (fold elevation of 1.8 ± 3.9), 12-LOX expression showed substantial interanimal variability in oral cancers and this difference was not statistically significant.

Chemoprevention studies

Comparisons of oral cancer incidence, oral cancer invasion score, survival, and body weight patterns in groups fed control diet and groups fed diets supplemented with COX inhibitors show that all three COX inhibitors conferred significant protection against oral cancer induction by NQO. In comparison with a 96% incidence of oral squamous cell carcinoma in dietary controls in the first study, rats fed the low and high doses of the selective COX-2 inhibitor, celecoxib, showed cancer incidences of 70% and 75%, respectively ($P < 0.05$ versus control for both comparisons; Table 1). The nonspecific COX inhibitor, piroxicam, was somewhat less active than was celecoxib, and achieved only a marginally significant reduction in oral cancer incidence; at study termination, incidences of oral cancer in groups fed the low and high doses of piroxicam were 82% ($0.05 < P < 0.10$) and 83%, respectively (Table 1). In the second chemoprevention study, both doses of the nonselective COX inhibitor, NO-naproxen, reduced oral cancer incidence versus controls: compared with an oral cancer incidence of 80% in dietary controls, groups fed the low and high doses of NO-naproxen both showed oral cancer incidences of 48% ($P < 0.05$ versus control; Table 2).

Depth of invasion of malignant oral lesions was a second parameter used to evaluate potential chemopreventive activity, and showed a pattern of chemopreventive efficacy that was similar to that seen in comparisons of cancer incidence. In the first study, 63% of NQO-treated rats in the dietary control group showed highly invasive oral cancers (cancers that invaded the underlying muscle, invasion score of +3; Table 3). By contrast, highly invasive cancers were seen in only 37% and 14% of rats fed the low and high doses of celecoxib, respectively ($P < 0.01$). Piroxicam also reduced oral cancer invasiveness, as 39% and 28% of rats fed the low and high doses of piroxicam showed invasion scores of +3 ($0.05 < P < 0.10$ for the low dose of piroxicam; $P < 0.01$, for the high dose of piroxicam). In the second chemoprevention study, NO-naproxen was found to provide similar protection: whereas 60% of NQO-treated rats in the dietary control group showed highly invasive cancers (Table 4), only 32% and 28% of NQO-treated rats in groups fed the low and high doses of NO-naproxen showed highly invasive lesions ($P < 0.05$ for both comparisons).

Both doses of celecoxib and both doses of piroxicam also resulted in significant reductions in tumor-associated mortality during later weeks of the study (Table 1; Fig. 2). In the first chemoprevention study, survival in the dietary control group was 50% at study termination at 26 weeks. By contrast, survival at 26 weeks in the four groups treated with celecoxib or piroxicam ranged from 80% to 90%; all differences were statistically significant at the 5% level of confidence. In the second chemoprevention study, survival at study termination was higher in both groups receiving NO-naproxen (dietary control, 88%; low-dose NO-naproxen, 96%; high-dose NO-naproxen, 100%; Table 3). However, neither of these

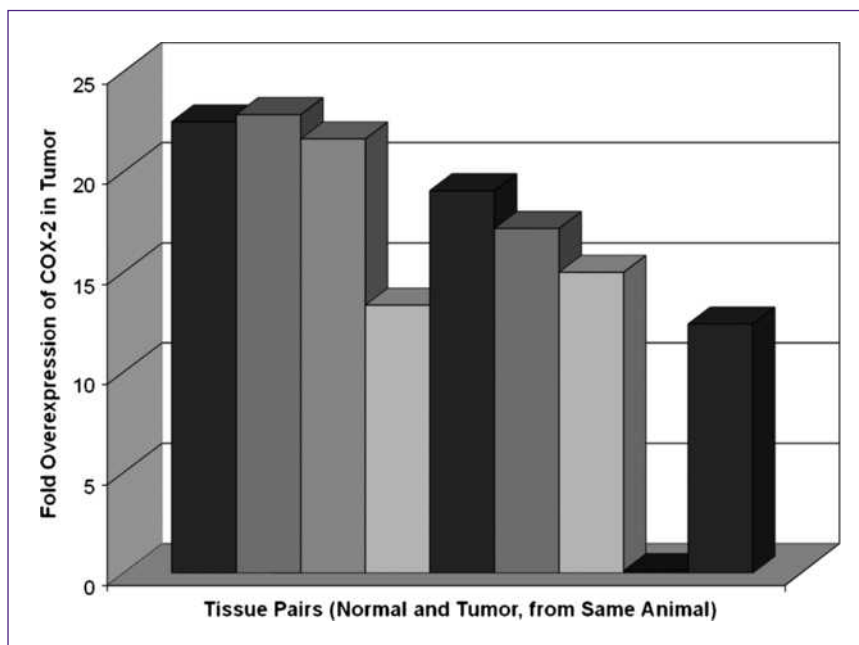


Fig. 1. Comparative expression of COX-2 in paired sets of oral tissues (squamous cell carcinomas and adjacent phenotypically normal tissue) harvested from 10 NQO-treated rats. COX-2 is overexpressed in 9 of 10 oral cancers, with level of overexpression ranging from 12- to 22-fold compared with adjacent normal tissue harvested from the same rat.

Table 1. Influence of celecoxib, piroxicam, and zileuton on survival and oral cancer incidence in rats treated with NQO

Group	Agent	Agent dose (mg/kg diet)	Survival (%)	Number (%) with lesion		
				Squamous epithelial hyperplasia	Squamous cell papilloma	Squamous cell carcinoma
1	None (control)	—	15/30 (50)	0/27 (0)	0/27 (0)	26/27 (96)
2	Celecoxib	500	24/30 (80)*	3/27 (11)†	1/27 (4)	19/27 (70)*
3	Celecoxib	1,500	27/30 (90)†	5/28 (18)*	1/28 (4)	21/28 (75)*
4	Piroxicam	50	27/30 (90)†	4/28 (14)*	0/28 (0)	23/28 (82)‡
5	Piroxicam	150	25/30 (83)†	5/29 (17)*	0/29 (0)	24/29 (83)
6	Zileuton	2,000	15/30 (50)	1/25 (4)	0/25 (0)	24/25 (96)

* $P < 0.05$ versus dietary control (group 1).

† $P < 0.01$ versus dietary control group.

‡ $0.05 < P < 0.10$ versus dietary control group.

differences from control was significant at the 5% level of confidence.

In addition to improving animal survival, COX inhibitors prevented the tumor-associated loss in body weight that was seen in dietary control animals during the final 8 weeks of both chemoprevention studies. Although percentage differences in mean terminal body weights between the dietary control group and the four groups fed diets supplemented with celecoxib or piroxicam were relatively modest (8-12%), these differences were all statistically significant ($P < 0.05$; Fig. 3). A similar pattern was seen in the second study, as mean terminal body weights in groups fed the low and high doses of NO-naproxen were 108% and 110% of control, respectively ($P < 0.01$ for high-dose NO-naproxen; data not shown).

By contrast to the significant protection against oral carcinogenesis conferred by the three COX inhibitors, the LOX inhibitor, zileuton, showed no chemopreventive activity in the NQO rat oral cancer model. At study termination, oral cancer incidence in rats receiving chronic dietary exposure to zileuton was essentially identical to that seen in the dietary control group (96% cancer inci-

dence in both groups; Table 1). Zileuton also had no effect on oral cancer invasiveness: cancers in 68% of rats treated with NQO + zileuton were highly invasive (invasion score of +3), a number that is comparable with that seen in dietary controls (63%; Table 3). Survival in the group treated with zileuton was below that seen in dietary controls throughout the majority of the study (Fig. 2), and was identical to the dietary control group at study termination. Mean body weights in the dietary control and zileuton-treated groups were comparable throughout the study (Fig. 3).

Other than effects that are attributable to carcinogen exposure, no clinical evidence of agent toxicity was identified in any animal receiving dietary exposure to celecoxib, piroxicam, NO-naproxen, or zileuton. Similarly, no evidence of gross pathology attributable to chemopreventive agent exposure was identified at the terminal necropsy. On this basis, and in consideration of the findings that survival and body weight in all groups treated with COX inhibitors were comparable with, or better than, dietary controls, it can be concluded that the dose levels of the chemopreventive agents used

Table 2. Influence of NO-naproxen on survival and oral cancer incidence in rats treated with NQO

Group	Agent	Agent dose (mg/kg diet)	Survival (%)	Number (%) with lesion		
				Squamous epithelial hyperplasia	Squamous cell papilloma	Squamous cell carcinoma
1	None (Control)	—	22/25 (88)	4/25 (16)	0/25 (0)	20/25 (80)
2	NO-naproxen	90	24/25 (96)	7/25 (28)	4/25 (16)*	12/25 (48)*
3	NO-naproxen	180	25/25 (100)†	13/25 (52)‡	0/25 (0)	12/25 (48)*

* $P < 0.05$ versus dietary control (group 1).

† $0.05 < P < 0.10$ versus dietary control group.

‡ $P < 0.01$ versus dietary control group.

Table 3. Influence of celecoxib, piroxicam, and zileuton on oral cancer invasion score in rats treated with NQO

Group	Agent	Agent dose (mg/kg diet)	Number (%) with lesion invasion score*		
			+1 invasion	+2 invasion	+3 invasion
1	None (control)	—	3/27 (11)	6/27 (22)	17/27 (63)
2	Celecoxib	500	7/27 (26)	2/27 (7)	10/27 (37) [†]
3	Celecoxib	1,500	5/28 (18)	12/28 (43)	4/28 (14) [†]
4	Piroxicam	50	4/28 (14)	8/28 (29)	11/28 (39) [‡]
5	Piroxicam	150	8/29 (28)	8/29 (28)	8/29 (28) [†]
6	Zileuton	2,000	3/25 (12)	4/25 (16)	17/25 (68)

*Invasion scores: +1, extension through the mucosal epithelial basement membrane and into lamina propria only; +2, extension into the upper muscle layers; +3, extensive infiltration into the underlying muscle.

[†] $P < 0.01$ versus dietary control group.

[‡] $0.05 < P < 0.10$ versus dietary control group.

in these studies were without gross systemic or organ-specific toxicity.

Discussion

RT-PCR analysis of gene expression in paired sets of normal and neoplastic oral tissues harvested from rats treated with NQO showed that COX-2 is significantly overexpressed in oral squamous cell carcinomas. By contrast, comparisons of 5-LOX and 12-LOX expression in the same paired sets of oral tissues failed to show significant differential expression of either LOX isozyme. Based on the overexpression of COX-2 in oral squamous cell carcinomas induced by NQO, we hypothesized that COX inhibitors would be effective chemopreventive agents in the NQO oral cancer model in rats. This hypothesis was confirmed, as a specific COX-2 inhibitor (celecoxib) and two nonspecific COX inhibitors (piroxicam and NO-naproxen) inhibited oral carcinogenesis in NQO-treated rats. The chemopreventive activity of these agents was shown by statistically significant reductions in oral cancer incidence (celecoxib and NO-naproxen), oral cancer invasiveness (celecoxib, piroxicam, and NO-naproxen), and cancer-associated mortality (celecoxib and piroxicam). In addition, all three COX

inhibitors protected against tumor-associated body weight loss during later weeks of the study.

Although cancer incidence, cancer invasiveness, and survival are common end points for efficacy evaluations of chemopreventive agents in animal models, the relationships between chemopreventive efficacy, mortality, and body weight loss in the NQO rat oral cancer model seem to be model specific. In several studies conducted in our laboratory using this model, we have found that virtually all mortality occurring after the completion of carcinogen exposure is tumor related. In essentially all cases, mortality is associated with the development of one or more large exophytic neoplasms at the base of the tongue. As a result of their strategic location, these lesions may interfere with normal feeding patterns, resulting in reduced food consumption, body weight loss, and subsequent moribund kills or intercurrent deaths. Although cancer-associated cachexia in later stages of disease progression cannot be ruled out, there seems to be a clear temporal relationship between the presence of a rapidly growing exophytic lesion at the base of the tongue, body weight loss, and death.

In the present study, dietary administration of both doses of celecoxib, both doses of piroxicam, and both

Table 4. Influence of NO-naproxen on oral cancer invasion score in rats treated with NQO

Group	Agent	Agent dose (mg/kg diet)	Number (%) with lesion invasion score*		
			+1 invasion	+2 invasion	+3 invasion
1	None (control)	0 (control)	3/25 (12)	2/25 (8)	15/25 (60)
2	NO-naproxen	90	3/25 (12)	1/25 (4)	8/25 (32) [†]
3	NO-naproxen	180	3/25 (12)	2/25 (8)	7/25 (28) [†]

*Invasion scores: +1, extension through the mucosal epithelial basement membrane and into lamina propria only; +2, extension into the upper muscle layers; +3, extensive infiltration into the underlying muscle.

[†] $P < 0.05$ versus dietary control (group 1).

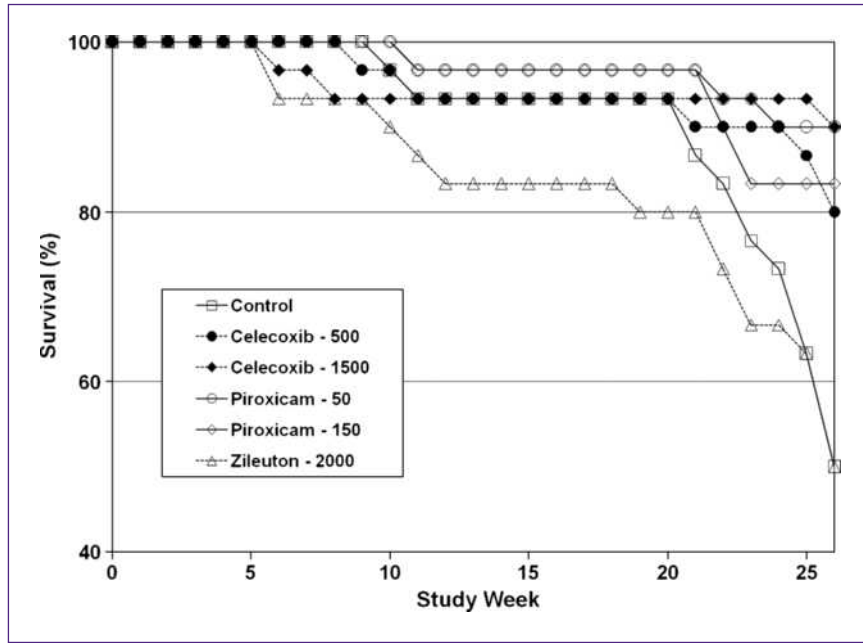


Fig. 2. Survival in carcinogen-treated rats fed control diet or diets supplemented with celecoxib, piroxicam, or zileuton.

doses of NO-naproxen decreased mortality in carcinogen-treated rats, and prevented the body weight loss that was seen in dietary controls beginning at approximately study week 18 (Fig. 3). The location of oral cancers induced by

NQO in this model greatly complicates their accurate measurement; as such, we did not attempt to measure lesion size during the in-life period. However, in consideration of the effects of COX inhibitors on tumor invasiveness,

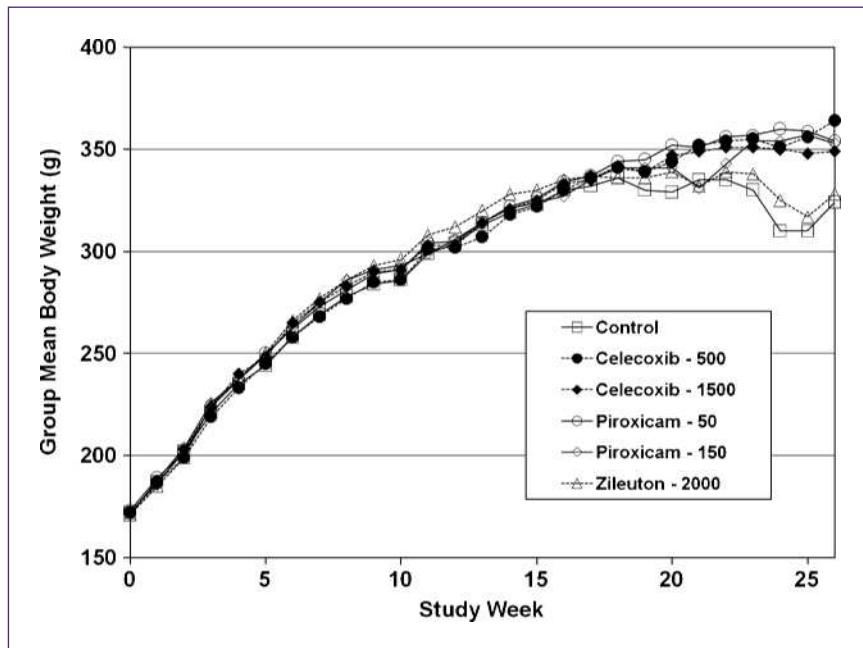


Fig. 3. Group mean body weight in carcinogen-treated rats fed control diet or diets supplemented with celecoxib, piroxicam, or zileuton.

body weight, and survival, it is not unreasonable to assume that total tumor burden in animals fed diets supplemented with COX inhibitors was less than that seen in dietary controls. As such, reductions in the size of strategically located oral cancers may underlie the improvements in survival and body weight that was seen in groups fed the two COX inhibitors.

It is considered likely that the effects of COX inhibitors on oral cancer invasiveness are linked to their effects on oral cancer incidence; we propose that both parameters are secondary to increases in cancer latency and/or delays in tumor progression that are induced by the chemopreventive agents. Restated, cancers that develop later (in animals treated with a COX inhibitor) will often present with a less invasive phenotype than do cancers (such as those in the dietary control groups) that have an earlier time of appearance and thus a longer natural history. However, it is not possible to exclude the hypothesis that COX inhibitors modulate early stages of carcinogenesis, resulting in a less invasive tumor phenotype.

The chemopreventive efficacies of celecoxib, piroxicam, and NO-naproxen in the rat oral cancer model were comparable at both dose levels evaluated for each agent. Although the reasons for this lack of a clear dose-response relationship are unclear, one or more of several mechanisms may be involved. First, the biological target(s) of the agents could become saturated at tissue concentrations that are reached following administration of both the low and high doses of each agent. Second, the chemopreventive agents may be effective in only a subset of incipient tumor cells; in such a case, insensitive cells would develop into cancers whether or not the agent was present, whereas the malignant progression of sensitive cell populations may be completely suppressed. Finally, the shape of the dose-response curve for chemopreventive efficacy may be such that both dose levels of each agent lie in a region where a comparable level of protection is conferred.

The significant efficacy of celecoxib, piroxicam, and NO-naproxen as chemopreventive agents in the rat oral cavity is in contrast to the lack of activity of the 5-LOX inhibitor, zileuton, which had no significant effects on oral cancer incidence, cancer invasion score, cancer-related mortality, or cancer-related body weight loss in NQO-treated rats. The lack of chemopreventive activity of zileuton in the rat oral cancer model system is correlated to the lack of overexpression of LOX isozymes in oral cancers harvested from NQO-treated rats.

The lack of chemopreventive efficacy of zileuton in the present study differs from the findings of Li et al. (25) who reported that celecoxib and zileuton both inhibit oral cancer induction by 7,12-dimethylbenz(a)anthracene in the hamster cheek pouch model. Although the reasons underlying the differences in zileuton efficacy in the two experiments are unclear, the designs of our studies and that of Li (25) show important differences. The specific site of carcinogenesis in the two models is different (cheek pouch versus tongue), and although tu-

mor histology seems to be comparable (squamous cell carcinomas) in both models, the molecular mechanisms underlying carcinoma induction by NQO in the F344 rat may differ from those associated with the induction of oral cancers in the Syrian golden hamster by 7,12-dimethylbenz(a)anthracene. A more probable explanation, however, may lie with agent administration: whereas zileuton was administered by dietary supplementation in our study, Li et al. (25) administered the drug by direct topical application to the oral mucosa. Direct application to the oral mucosa may result in greater local delivery to the target tissue than is achievable by systemic administration, thus resulting in a greater probability of chemopreventive activity.

Data generated in several laboratories, including our own, show that carcinogenesis in several organ sites may be suppressed by inhibiting both the COX and LOX pathways of eicosanoid metabolism (11, 12, 26–29). In the present studies using the NQO rat oral cancer model, the differential activity of COX inhibitors (piroxicam, celecoxib, and NO-naproxen) and the 5-LOX inhibitor (zileuton) suggests that the COX-2 pathway of arachidonic acid metabolism is more likely to be causally involved in rat oral carcinogenesis than is 5-LOX. This conclusion is supported by the substantial overexpression of COX-2 in rat oral cancers, and suggests that as a class, COX inhibitors merit further study as chemopreventive agents for oral cancer in high-risk populations. In this regard, overexpression of COX-2 in human oral cancers has recently been reported (30, 31). Furthermore, in a different study, patients with higher levels of COX-2 transcripts in oral premalignant lesions were found to be at an increased risk of disease progression (32).

In consideration of the shown chemopreventive activity of both specific and nonspecific COX inhibitors, and the substantial (and sometimes fatal) cardiovascular toxicities that have been observed in clinical populations receiving celecoxib and other COX-2 inhibitors, nonspecific COX inhibitors such as piroxicam and NO-naproxen merit reconsideration as agents for cancer chemoprevention. In terms of anticarcinogenic efficacy, this approach is supported by the negative results generated in a recent pilot oral cancer chemoprevention study with celecoxib (32). In this regard, NO-releasing NSAIDs such as NO-naproxen are of particular interest: as a class, NO-releasing NSAIDs induce less gastrointestinal toxicity than do their non-NO-releasing parent drugs (classic NSAIDs; ref. 33), and NO-naproxen inhibits both COX-1 and COX-2 isozymes (34). Although the gastrointestinal toxicity of nonspecific COX inhibitors may certainly be limiting in some patients, we propose that manageable, low-level gastrointestinal toxicity induced by chronic administration of a nonspecific COX inhibitor may pose a less significant limitation to cancer chemoprevention in high-risk populations than does administration of a potentially more active COX-2 inhibitor that carries with it the risk of potentially cardiovascular toxicity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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