

Preventive Effects of NSAIDs, NO-NSAIDs, and NSAIDs Plus Difluoromethylornithine in a Chemically Induced Urinary Bladder Cancer Model

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Abstract

Urinary bladder cancer prevention studies were performed with the nonsteroidal anti-inflammatory drugs (NSAID) naproxen (a standard NSAID with a good cardiovascular profile), sulindac, and their nitric oxide (NO) derivatives. In addition, the effects of the ornithine decarboxylase inhibitor, difluoromethylornithine (DFMO), alone or combined with a suboptimal dose of naproxen or sulindac was examined. Agents were evaluated at their human equivalent doses (HED), as well as at lower doses. In the hydroxybutyl(butyl) nitrosamine (OH-BBN) model of urinary bladder cancer, naproxen (400 or 75 ppm) and sulindac (400 ppm) reduced the incidence of large bladder cancers by 82%, 68%, and 44%, respectively, when the agents were initially given 3 months after the final dose of the carcinogen; microscopic cancers already existed. NO-naproxen was highly effective, whereas NO-sulindac was inactive. To further compare naproxen and NO-naproxen, we examined their effects on gene expression in rat livers following a 7-day exposure. Limited, but similar, gene expression changes in the liver were induced by both agents, implying that the primary effects of both are mediated by the parent NSAID. When agents were initiated 2 weeks after the last administration of OH-BBN, DFMO at 1,000 ppm had limited activity, a low dose of naproxen (75 ppm) and sulindac (150 ppm) were highly and marginally effective. Combining DFMO with suboptimal doses of naproxen had minimal effects, whereas the combination of DMFO and sulindac was more active than either agent alone. Thus, naproxen and NO-naproxen were highly effective, whereas sulindac was moderately effective in the OH-BBN model at their HEDs. *Cancer Prev Res*; 7(2); 246–54. ©2013 AACR.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAID), which exert their biologic action primarily through inhibition of COX enzymes, have been a major focus in the field of chemoprevention for more than 25 years. At first, the primary agents investigated were the standard nonselective

COX inhibitor NSAIDs, particularly piroxicam (1). Piroxicam was highly effective in preclinical models, but has infrequently been used clinically for the past 20 years. Approximately 2 decades ago, it was shown that 2 different COX enzymes exist, and were designated COX-1 and COX-2 (2). COX-1 is a constitutively expressed enzyme found in a wide variety of tissues. COX-2 is preferentially expressed in lymphoid cells, but can be readily induced by a wide variety of stimuli in many cell types. Both COX-1 and COX-2 catalyze the oxygenation of arachidonic acid to yield prostaglandin H₂, which in turn can be acted upon by different synthases to yield a variety of prostaglandins or thromboxanes depending on the site of formation. These molecules are widely considered to be proinflammatory and the measurement of urinary metabolites of prostaglandins can be used to assess COX activity or inhibition *in vivo*.

Interestingly, COX-2 proved to be a *bona fide* target for cancer intervention, as it is overexpressed in transformed cells and is increased in various tumor types (3). Specific inhibitors of COX-2 (or coxibs) were synthesized and were shown to be highly effective in preclinical studies in the prevention of various types of cancer (4–6). Clinical prevention trials of the COX-2 inhibitors demonstrated them to be highly effective against colon adenomas and skin cancer (7, 8). However, placebo-controlled trials of

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rofecoxib against colon adenomas at the standard human dose and celecoxib at doses higher than their standard dose increased the incidence of adverse cardiovascular events (9, 10). Therefore, there have been significant efforts to evaluate agents that might have the efficacy of NSAIDs, but without the gastric effects associated with standard NSAIDs or the cardiovascular risks associated with COX-2 inhibitors and some standard NSAIDs such as diclofenac.

In this study, we sought to evaluate the effect of NSAIDs in the prevention of urinary bladder cancer. Epidemiologic data indicated that certain NSAIDs, specifically naproxen, was associated with lower cardiovascular risk than other NSAIDs (11). Based on these results, we evaluated naproxen herein. The other standard NSAID used was sulindac, which has been used clinically in patients with familial adenomatous polyposis for many years (12). We also tested nitric oxide (NO) derivatives of these NSAIDs based on preclinical data that NO-NSAIDs exhibited reduced gastric toxicity when compared with standard NSAIDs. The NO-NSAIDs were also expected to release substantial levels of NO (13, 14). The NO released from NO-naproxen or NO-sulindac may have additional chemopreventive activity, and may stimulate the antioxidant response element (ARE) battery of genes and processes.

The second major area of investigation examined the efficacy of combining an NSAID with the ornithine decarboxylase inhibitor difluoromethylornithine (DFMO). DFMO has been shown to act synergistically with NSAIDs and coxibs in multiple tumor models, including colon, skin, and esophagus. In fact, the combination of sulindac and DFMO was profoundly effective in the prevention of colon adenomas, reducing total colon adenomas almost 70% and reducing advanced adenomas even more (15). We had previously demonstrated the efficacy of both naproxen and other NSAIDs against urinary bladder cancers (16). Part of the rationale for such a combined treatment was that one might be able to use lower doses of an NSAID and, thereby, reduce associated toxicities.

Although there have been a wide variety of studies examining the use of NSAIDs preclinically and in clinical trials, the mechanisms by which these agents function, besides their obvious effects on COX inhibition, remain unclear. Although *in vitro* studies have often shown apoptotic effects and inhibition of cellular proliferation, these routinely occur at doses far higher than are observed in the serum of treated animals or humans.

In this studies, we specifically examined the effects of naproxen, NO-naproxen, sulindac, NO-sulindac, and DFMO administered alone or together with one of the NSAIDs in the hydroxybutyl(butyl)nitrosamine (OH-BBN) model of urinary bladder cancers in rats. These studies showed: (i) At their standard human equivalent doses (HED) both naproxen and sulindac were effective at reducing the incidence of large bladder cancers, although sulindac was less effective ($P < 0.05$). Although NO-naproxen was highly effective, NO-sulindac was not. (ii) Both naproxen and NO-naproxen were effective in preventing the formation of large palpable cancers, even at doses less

than their HEDs. (iii) Low-dose naproxen (75 ppm) was also effective when initiated at 3 months after the last dose of OH-BBN, a time when microscopic cancers already existed. (iv) When animals received naproxen (400 ppm) or NO-naproxen (550 ppm) for 7 days, similar gene expression changes were observed in the liver. However, neither agent altered expression of certain ARE-mediated genes, which might have been expected to be altered by NO release. (v) Naproxen strikingly decreased serum levels of the major urinary metabolite of prostaglandin E (PGE)₂, implying that it inhibits both COX 1 and 2 activity. (vi) Naproxen failed to alter proliferation of bladder tumors treated short term. (vii) DFMO by itself marginally reduced urinary bladder cancer formation. Combining DFMO with NSAIDs showed no increased efficacy with naproxen, but showed some (albeit limited) synergy with a suboptimal dose of sulindac.

Materials and Methods

Reagents

Naproxen and sulindac were obtained from Sigma Chemicals, whereas NO-naproxen and NO-sulindac were provided by the Division of Cancer Prevention Repository (see supplementary materials for analytical information). The carcinogen OH-BBN was purchased from TCI America, and animals were obtained from Harlan Sprague-Dawley, Inc. Teklad mash diet was purchased from Harlan Teklad. The chemopreventive agents were incorporated into the diet using a Patterson-Kelly blender. Experimental diets were prepared weekly and stored in a cold room. Agent content in the experimental diets were determined periodically in multiple samples taken from the top, middle, and bottom portions of individual diet preparations to verify uniform distribution. Rats were allowed *ad libitum* access to the respective diets.

Rat urinary bladder cancer model

The OH-BBN-induced urinary bladder cancer studies were performed as previously described (5, 16). Female Fisher-344 (F-344) rats were obtained from Harlan Sprague-Dawley, Inc. at 4 weeks of age. Beginning at 56 days of age, the rats ($n = 30$ /group) were treated twice a week with 150 mg OH-BBN/gavage for 8 weeks. The vehicle for OH-BBN was ethanol, corn oil (10:90, v/v). The rats were weighed once per week, and palpated for bladder tumors twice per week. Diet supplementation with naproxen, NO-naproxen, sulindac, or NO-sulindac was initiated either 2 weeks or 3 months after the end of the OH-BBN treatments. The studies were terminated approximately 7 months after the end of the OH-BBN treatments. At necropsy, urinary bladders with associated lesions were excised and weighed. Percent incidence and the percent of rats with large bladder tumors (≥ 200 mg weight; an OH-BBN treated bladder with no visible lesions would weigh 130–160 mg) were selected as endpoints against which to measure cancer prevention. Statistical analysis of bladder weights were performed by the Wilcoxon-Rank test and the final incidence was analyzed by the Fischer Exact test (5, 16).

Immunohistochemical biomarkers developed in bladder cancers of rats treated short term with naproxen

Individual F-344 female rats were treated with naproxen (400 ppm), celecoxib (250 ppm), aspirin (low-dose 300 ppm or high-dose 3,000 ppm), or control diet. After 7 days of feeding, individual rats were placed in metabolic cages overnight and urine was collected into cups surrounded by dry ice. Urine was analyzed for 11- α -hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostanoic acid (PGE-M) using mass spectrometry as previously described (17, 18), without knowledge of treatment groups. PGE-M was normalized to urinary creatinine.

RNA analysis for liver isolation and amplification

Livers were rapidly obtained from rats administered naproxen (400 ppm), NO-naproxen (560 ppm), or control diet for 7 days and snap frozen in liquid nitrogen. RNA was isolated and processed using methods similar to those described previously (19). In brief, total liver RNA from untreated rats and rats treated with naproxen or NO-naproxen was isolated by Trizol (Invitrogen) and purified using the RNeasy Mini Kit and RNase-free DNase Set (Qiagen). *In vitro* transcription-based RNA amplification was then performed on the samples and cDNA for each sample was synthesized using a Superscript cDNA Synthesis Kit (Invitrogen) and a T7-(dT) 24 primer: 5'-GGCCAGT-GAATTGTAATACGACT-CACTATAGGGAGGCGG-(dT)24-3'. The cDNA was cleaned and the biotin-labeled cRNA was transcribed *in vitro* from cDNA using a BioArray High Yield RNA Transcript Labeling Kit (ENZO Biochem), and purified again using the RNeasy Mini Kit.

Affymetrix gene chip probe array and gene cluster

The labeled cRNA was applied to the Affymetrix RAE 230A Gene Chips (Affymetrix) according to the manufacturer's recommendations. Every gene or EST is represented by a probe set consisting of approximately 16 probe pairs (oligonucleotides) of 25-mer oligonucleotides. Array normalization and gene expression estimates were obtained using Affymetrix Microarray Suite 5.0 software (MAS5). The array mean intensities were scaled to 1,500. These estimates formed the basis for statistical testing. Differential expression was determined using the *t* test with adjusted $P < 0.05$. Genes meeting the criteria were called positive for differential expression. For the selected genes, expression indexes were transformed across samples to an $N(0, 1)$ distribution using a standard statistical Z-transformation. These values were input to the Gene Cluster program of Eisen (20) and genes clustered using average linkage and correlation dissimilarity.

Results

Results with naproxen and NO-naproxen

We had previously shown that higher doses of naproxen (400 ppm) and NO-naproxen (560 ppm) were effective in preventing the development of urinary bladder cancers in the OH-BBN model (13). In the current

studies, we initially examined doses approximately 66% lower (naproxen at 128 ppm and NO-naproxen at 183 ppm; Table 1 and Fig. 1A and B). We found both agents were effective, resulting in >80% decrease in the development of large palpable cancers (>200 mg combined weight of bladder plus lesions) when agents were initiated 2 weeks after the last dose of OH-BBN. The cutoff of 200 mg bladder weight was chosen as this weight serves as a surrogate for palpable lesions (16). Once the tumors are palpable they are all invasive. We examined a lower dose of naproxen (75 ppm), hoping to achieve a dose roughly 50% effective that might be used DFMO to reduce toxicity. Surprisingly, this dose was highly effective when initiated 2 weeks after the last dose of OH-BBN. Finally, we used this low dose of 75 ppm, as well as 400 ppm, beginning 12 weeks after the last OH-BBN; a time when all OH-BBN-treated rats have precancerous lesions (hyperplasias and dysplasias), and approximately 70% have microscopic cancers (16). Both doses were still effective when initiated at this later time point (Table 1).

Effects with sulindac and NO-sulindac

The NSAID sulindac and its NO derivative were also examined. Sulindac was tested at 400 ppm, its HED. Sulindac was effective, reducing the incidence of palpable tumors by 46% (Fig. 1C and D). However, sulindac was not as effective as naproxen. We also tested NO-sulindac at a molar dose equivalent to the high dose of the parent compound sulindac (520 ppm) starting 3 months after OH-BBN administration. This dose was not effective in reducing bladder weights ($P > 0.05$), although it may reflect the agent's insolubility problems. The parent compound sulindac readily went into solution and dispersed into the diet, whereas the NO derivative could not be dissolved in a vehicle and was mixed directly into the diet.

Effects of naproxen and NO-naproxen on gene expression in liver

To characterize naproxen and NO-naproxen their effects on gene expression in livers of treated rats was examined. Liver was chosen because we have previously worked with liver (19) and could therefore identify a moderate to strong ARE signature. We have previously shown that gene changes associated with a given receptor in the liver are indicative of changes in other organs for that same receptor (21). There were 2 goals to this study: (i) to determine whether naproxen and NO-naproxen yield similar gene expression profiles, which would imply that their effects are mediated by the parent NSAID; and (ii) to determine whether NO-naproxen, because of the release of NO, might cause increases in expression of genes associated with the ARE. As seen in Fig. 2 and Supplementary Figs. S1 and S2, both agents in general had strikingly similar effects, either as determined by the heat maps or by their ability to alter the same pathways. Thus, both naproxen and NO-naproxen significantly altered Gene Ontology (GO)-pathways including small

Table 1. Effects of NSAIDs, NO-NSAIDs, and NSAIDs + DFMO on bladder weights in the OH-BBN-induced urinary bladder cancer model in F-344 rats

Treatment	Final bladder weights, mg ¹	Number of rats with bladder weights \geq 200 mg at sacrifice
Agent started 2 weeks post-OH-BBN		
Naproxen, 128 ppm in diet	136 \pm 32 ^a (76%) ²	4/30 (13%)
NO-Naproxen, 183 ppm in diet	81 \pm 11 ^a (86%)	1/30 (3%)
NO-Naproxen, 550 ppm in diet	106 \pm 22 ^a (81%)	2/30 (7%)
None	560 \pm 104 ^b	19/30 (63%)
Agent started 2 weeks post-OH-BBN		
DFMO, 1,000 ppm in diet	379 \pm 65 ^a (14%)	20/30 (67%)
Naproxen, 75 ppm in diet	162 \pm 12 ^b (63%)	3/30 (10%)
Naproxen, 75 ppm in diet + DFMO, 1,000 ppm in diet	162 \pm 15 ^b (63%)	4/30 (13%)
Sulindac, 150 ppm in diet	296 \pm 41 ^a (33%)	16/30 (53%)
Sulindac, 150 ppm in diet + DFMO, 1,000 ppm in diet	198 \pm 12 ^b (55%)	11/30 (37%)
None	441 \pm 71 ^a	22/30 (73%)
Agent started 3 months post-OH-BBN		
Naproxen, 400 ppm in diet	149 \pm 8 ^a (63%)	4/30 (13%)
Naproxen, 75 ppm in diet	207 \pm 29 ^a (48%)	7/30 (23%)
None	399 \pm 40 ^b	29/37 (73%)

NOTE: Values with different subscripts (a, b) are statistically significantly different from each other ($P < 0.05$).

¹Number in parenthesis indicates percentage of decrease from controls.

²Values are means \pm SE.

GTPase-mediated signal transduction and protein transport (*Upp2*, *Gdi2*, *Pdcd4*, *Hmg2*, *Haghl*; Supplementary Fig. S1) and certain stress-related genes and genes related to stimuli (*Rad52*, *Rgs16*, *Lama5*, *Tyrp1*, *Pdcd4*; Supplementary Fig. S2). Given that the overlap of pathways was striking, the most reasonable interpretation is that both were mediated by the parent compound naproxen. The other finding is that both agents given at equimolar doses altered the same pathways and the same genes. But more interestingly, they both gave almost the same magnitude of expression differences, indirectly reflecting the reproducibility of the array response. We failed to see significant induction of ARE-inducible genes by either of these elements. The fluorescent values for glutathione S-transferase (GST)-Pi were $0.942 \times 10^3 \pm 0.036 \times 10^3$ (control), $0.936 \times 10^3 \pm 0.028 \times 10^3$ (naproxen), and $0.928 \times 10^3 \pm 0.040 \times 10^3$ (NO-naproxen). Values for aldo-keto reductase family member 3A7 (AKR 3A7) were $0.646 \times 10^3 \pm 0.027 \times 10^3$ (control), $0.653 \times 10^3 \pm 0.042 \times 10^3$ (naproxen), and $0.603 \times 10^3 \pm 0.045 \times 10^3$ (NO-naproxen). Values for quinone reductase were $0.809 \times 10^3 \pm 0.033 \times 10^3$ (control), $0.765 \times 10^3 \pm 0.038 \times 10^3$ (naproxen), and $0.838 \times 10^3 \pm 0.041 \times 10^3$ (NO-naproxen). Fluorescent values for epoxide hydrolase were $0.123 \times 10^3 \pm 0.012 \times 10^3$ (control), $0.123 \times 10^3 \pm 0.046 \times 10^3$ (naproxen), and $0.123 \times 10^3 \pm 0.018 \times 10^3$ (NO-naproxen; data not shown). None of these differences were statistically significant. In contrast, strong ARE inducers such as dithiolthione increase levels of GST-Pi 40-fold, AKR 3A7 20-fold, epoxide hydrolase 4-fold, and quinone reductase 5-fold (19).

Effects of DFMO alone or DFMO + naproxen or sulindac on OH-BBN-induced carcinogenesis

Naproxen (75 ppm) or sulindac (150 ppm) alone and in combination with DFMO (1,000 ppm) were tested (Fig. 3). DFMO alone reduced the number of rats that developed large tumors by 8% (OH-BBN only: 22/30; DFMO + OH-BBN: 20/30; Fig. 3A and B; Table 1). Naproxen at 75 ppm was highly effective (3/30 large tumors), whereas sulindac at 150 ppm, a dose used to parallel the low human dose of Meyskens and colleagues (15), was moderately effective (large tumor incidence: 16/30, $P > 0.1$). The effects of combining DFMO with naproxen or sulindac were then evaluated. There were no preventive effects of combining DFMO with naproxen versus naproxen alone. For sulindac (although there was no difference between sulindac alone and sulindac + DFMO), the combination was significantly different from control ($P < 0.05$), whereas sulindac alone was not (Fig. 3C and D and Table 1).

Effects of various NSAIDs on PGE-M production

Naïve female F-344 rats at 8 weeks of age were treated with naproxen (400 ppm), celecoxib (250 ppm), aspirin low dose (300 ppm), and aspirin high dose (3000 ppm) for a period of 7 days and then overnight urines were collected on dry ice and then stored at -80°C . Levels of PGE-M (a marker of systemic PGE₂ production) were quantified using mass spectrometry and normalized to urinary creatinine. All 4 agents decreased PGE-M levels (Supplementary Table S1). Interestingly, low-dose aspirin and low-dose celecoxib reduced levels 25% to 30%,

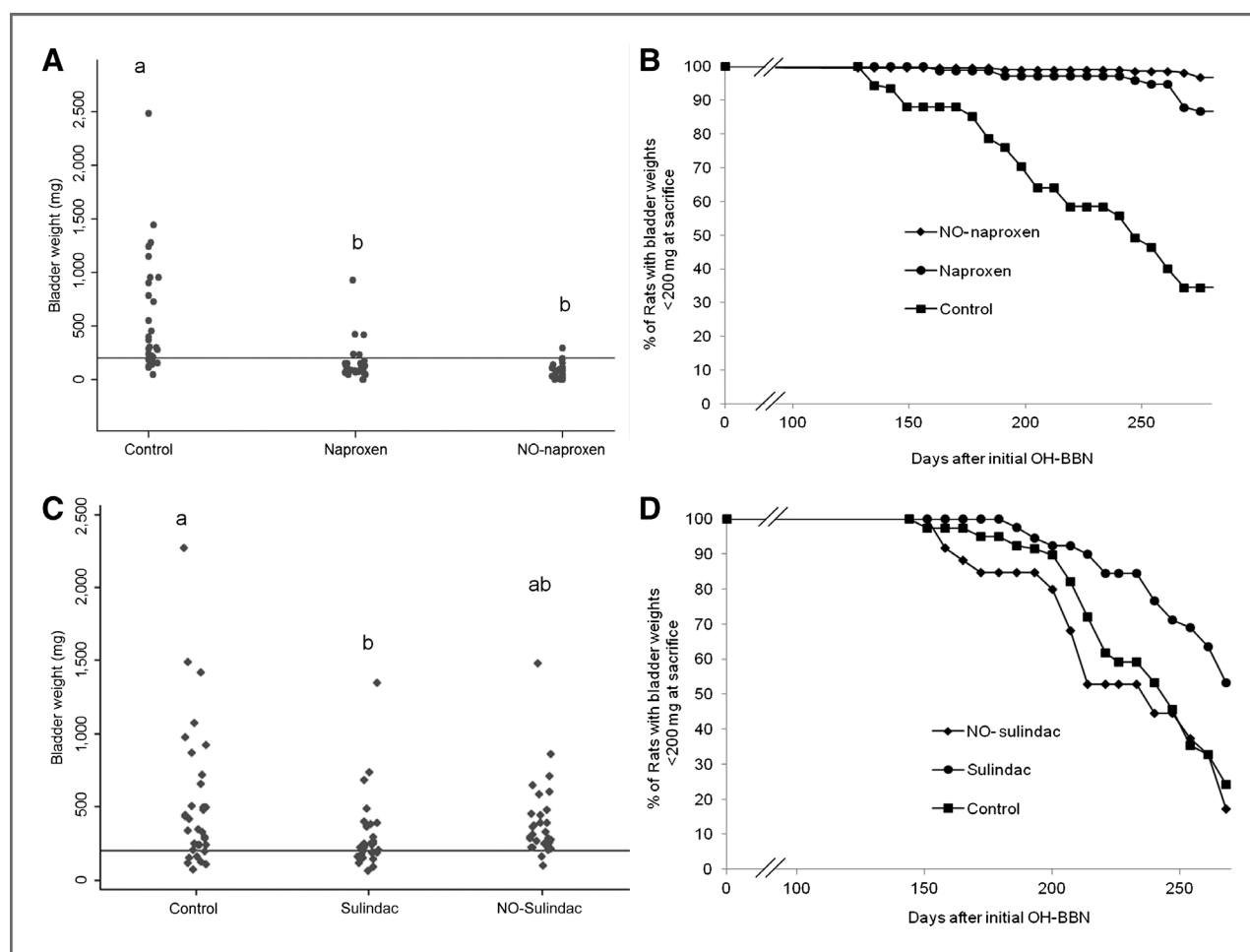


Figure 1. The effects of naproxen, NO-naproxen, sulindac, and NO-sulindac on OH-BBN-treated rats. A, final urinary bladder weights of rats administered naproxen (128 ppm), NO-naproxen (183 ppm), or no agent beginning 2 weeks after the final OH-BBN administration. B, percentage of rats without large bladder cancers at sacrifice in rats treated with naproxen (128 ppm), NO-naproxen (183 ppm), or no agent beginning 2 weeks after the final OH-BBN administration. C, final bladder weights of rats administered sulindac (400 ppm), NO-sulindac (520 ppm), or no agent beginning 2 weeks after the final OH-BBN administration. D, percentage of rats without large bladder cancers at sacrifice in rats treated with sulindac (400 ppm), NO-sulindac (520 ppm), or no agent beginning 2 weeks after the final OH-BBN administration. Superscripts with different letters are statistically significantly different from one another ($P < 0.05$).

high-dose aspirin reduced levels 55%, and naproxen reduced levels 70%. We have included in the table certain of our previous data dealing with the efficacy of the agents that showed that celecoxib and naproxen were profoundly effective in preventing bladder tumors (87% and 92% decrease, respectively), whereas high-dose aspirin was relatively effective (62% decrease) and finally low-dose aspirin was ineffective (5% decrease).

Examination of biomarkers in bladder lesions treated short term with naproxen

Animals bearing palpable bladder tumors were treated with naproxen for 5 days. We examined the effects of naproxen on Ki-67, Bub1, cdc2, TOPO2, TPX-2, and BudR proliferation-related biomarkers. We observed no significant decreases in these proteins as determined by immunohistochemistry (data not shown).

Discussion

Previously we examined the efficacy of naproxen and NO-naproxen in the OH-BBN-induced urinary bladder cancer model (13). Naproxen has perhaps the best cardiovascular profile of any NSAID other than aspirin. Aspirin, however, has uncertainty about the proper dose to administer to optimize chemopreventive activity (3). Naproxen was highly effective at the HED (400 ppm), reducing the incidence of large palpable cancers by more than 85%. We compared results with that of NO-naproxen, and found similar activity at equivalent doses. In contrast, neither agent showed preventive efficacy in a rat mammary cancer model that is routinely insensitive to NSAIDs (13). We felt the most reasonable interpretation was that although NO-naproxen may release NO, its primary effects were via its NSAID release. In the present studies, we wished to further explore the effects of naproxen and sulindac and their NO

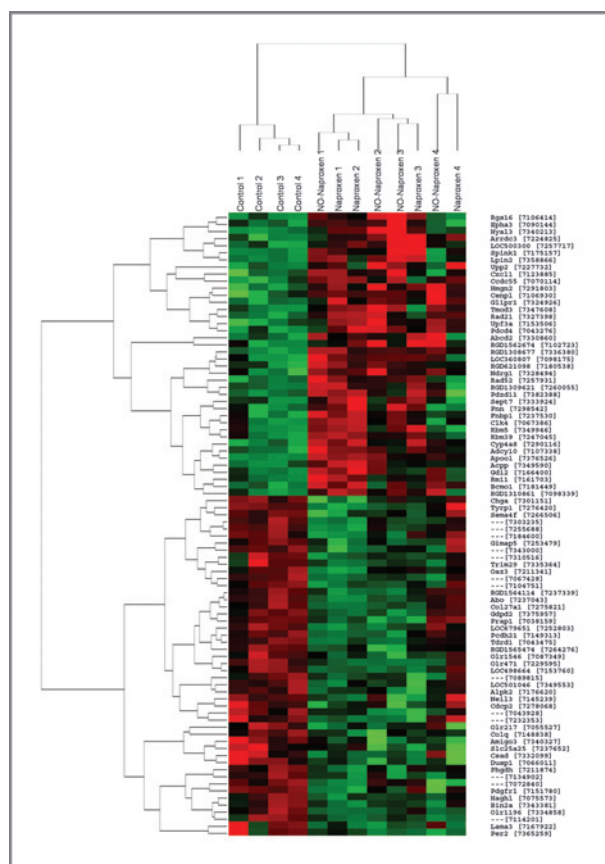


Figure 2. Heat map representation of the genes regulated by naproxen and NO-naproxen in livers. Rats were administered naproxen (400 ppm), NO-naproxen (560 ppm), or no treatment for 7 days, and total RNA liver was isolated for transcriptomics analysis by Affymetrix Gene Chip Probe Arrays. Each gene (rows) with relatively higher expression is colored with reds of increasing intensity, and genes with relatively lower expression are colored with greens of increasing intensity. Columns represent individual animals.

analogues, as well as combining the ornithine decarboxylase inhibitor DFMO with the NSAIDs. The NO-NSAIDs, by reducing gastric toxicity, and DFMO + NSAID combinations, by potentially allowing one to use lower doses of NSAIDs, may reduce toxicity.

Our initial effective dose of naproxen (400 ppm) was equivalent to the human dose. However, we observed that a dose of 75 ppm (more than 5-fold lower than the HED) was highly effective in preventing the development of large bladder cancers when initiated either 2 or 12 weeks following the last dose of OH-BBN (Table 1). Given that the serum half-life ($T_{1/2}$) of naproxen in a rat is strikingly lower than that in a human ($T_{1/2}$ in rat <5 hours; $T_{1/2}$ in human >15 hours), the efficacy of this lower dose of naproxen was surprising. Finding that these agents are effective even when administered late agrees with our previous data with an EGFR inhibitor and other NSAIDs, demonstrating that agents can be effective even when microscopic lesions already exists (16). It also supports the view that many of these agents have their effects during the latter stages of

tumor progression, which is of importance because phase III clinical prevention trials will involve a late intervention (22). The question this raises regarding the dosing of naproxen is more problematic. Does the effectiveness of a very low dose of naproxen indicate that you could use a lower dose in humans, or does it reflect species or model idiosyncrasies? Furthermore, using a lower dose of naproxen in humans, presuming that it might be effective, is the potential cardiovascular effects. Part of the rationale for using naproxen is the excellent cardiovascular profile of the agent. However, this is based on a full HED and has been attributed in part to its strong inhibition of COX-1 for an extended time period at a standard dose. However, it is not known if naproxen would have similar protective cardiovascular effects at a lower dose.

Although naproxen, NO-naproxen, and sulindac all decreased bladder weights at their HEDs, NO-sulindac did not. Two differences between NO-naproxen and NO-sulindac merit comment. First, the chemical linker between naproxen and NO-naproxen is different than that between sulindac and NO-sulindac. Thus, the 2 NO derivatives may show significant differences regarding NO release. Second, NO-sulindac was relatively insoluble (Supplementary Materials), which may make a clear interpretation of its lack of efficacy more difficult (14). However, this illustrates that different NO analogs may have substantially different chemical and pharmacokinetics (PK) properties.

We performed 2 limited studies to examine mechanistic aspects regarding the efficacy of naproxen. First, we examined the effects of NSAIDs, including naproxen, on urinary levels of PGE-M. This metabolite, which is formed during the catabolism of PGE₂, can be derived from either COX-1 or COX-2 in various tissues and has been often proposed as a potential biomarker to monitor the efficacy of NSAIDs (17,18). We treated naïve F-344 rats for 7 days with naproxen (400 ppm), celecoxib (250 ppm), high-dose aspirin (3,000 ppm), low-dose aspirin (300 ppm), or no treatment. These agents reduced PGE-M levels compared with controls by 68%, 30%, 55%, and 25%, respectively. Our interpretation of the data is that naproxen and high-dose aspirin inhibited substantially both COX-1 and COX-2. Celecoxib primarily affects COX-2 whereas the lower dose of aspirin should primarily decrease COX-1. This would be based both on effects of aspirin on purified COX enzymes and the fact that lower doses of aspirin profoundly inhibit thromboxane production, which is mediated by COX-1 but is not an effective anti-inflammatory compound at this dose. Cancer prevention data for these agents showed that naproxen, celecoxib, high-dose aspirin, and low-dose aspirin decreased tumor formation by 90%, 90%, 60%, and 10%, respectively (16). These data are compatible with the hypothesis that inhibition of COX-2 is a primary mechanism of the efficacy of these agents in this model. In a recent study, we examined the effects of NSAIDs on the development of squamous cell skin cancers in UV-exposed mice. In this model, we directly measured COX-2 inhibition by NSAIDs in the target tissue. We found when examining naproxen, NO-naproxen, sulindac, celecoxib, and aspirin (high or low doses) that there was

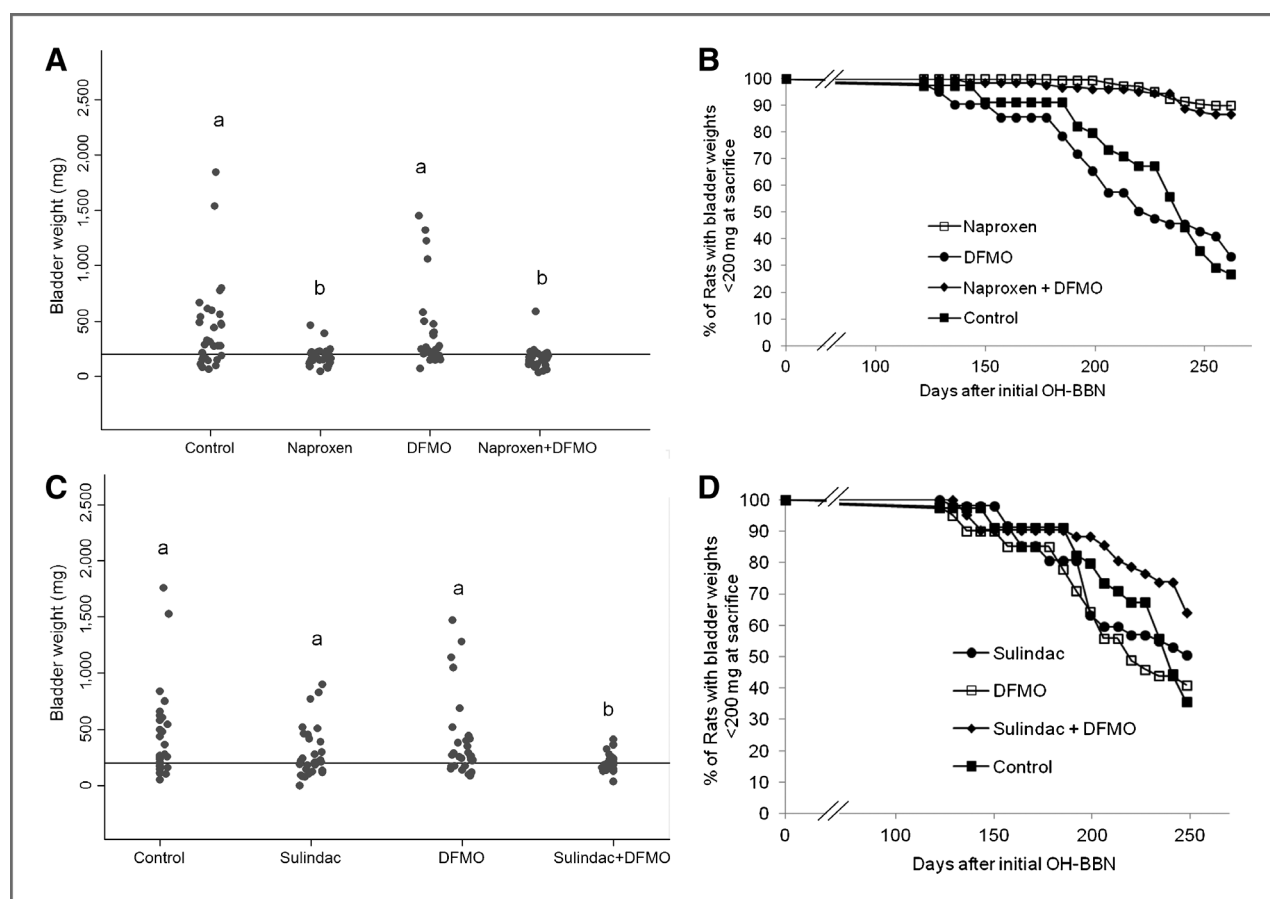


Figure 3. The effects of naproxen and sulindac, alone or in combination with DFMO, on OH-BBN-treated rats. A, final bladder weights of rats administered naproxen (75 ppm), DFMO (1,000 ppm), naproxen + DFMO, or no agent beginning 2 weeks after the final OH-BBN administration. B, percent of rats without large bladder tumors at sacrifice in rats treated with administered naproxen (75 ppm), DFMO (1,000 ppm), naproxen + DFMO, or no agent beginning 2 weeks after the final OH-BBN administration. C, final bladder weights of rats administered sulindac (150 ppm), DFMO (1,000 ppm), sulindac + DFMO, or no agent beginning 2 weeks after the final OH-BBN administration. D, percentage of rats without large bladder tumors at sacrifice in rats treated with sulindac (150 ppm), DFMO (1,000 ppm), sulindac + DFMO, or no agent beginning 2 weeks after the final OH-BBN administration. Superscripts with different letters are statistically significantly different from one another ($P < 0.05$).

a strong correlation between the ability to inhibit COX-2-mediated activity and efficacy in that model (23). This COX-2-related efficacy in the skin model would seem to agree with the correlations we found between PGE-M levels and efficacy in the bladder model.

Our second line of inquiry dealt with short-term effects of naproxen treatment of rats with palpable bladder tumors. Because naproxen was highly effective late in the bladder tumor process and prior studies with the EGFR inhibitors, which are also effective late, profoundly altered cell proliferation, we thought this was a reasonable approach (24, 25). We did not observe alterations in cell proliferation or in proliferation-related proteins. Thus, the proliferation pathway that we have previously shown is highly associated with efficacy of various agents in the breast (26) was unaffected by naproxen despite naproxen's striking efficacy. As we postulated many years back it may be that NSAIDs are working earlier on the progression from microadenocarcinoma to large lesions via angiogenesis (5), in which case examining these later lesions might not be optimal.

Although our prevention data with naproxen and NO-naproxen seemed compatible with their effects being driven by the NSAID naproxen, we took a second indirect approach to examine this. We examined the effects of naproxen and NO-naproxen on gene expression in the liver to determine whether both yielded similar array signatures and whether we observed an independent NO signal. Significant levels of NO in cell culture stimulate an antioxidant response. The antioxidant activates transcription at specific DNA sequences, designated the ARE that is associated with induction of a wide variety of genes (27) including heme oxygenase-1 (*Ho-1*), NAD(P)H:quinone oxidoreductase 1 (*Nqo1*), and glutamate cysteine ligase (*Gcl*; ref. 28). Genes that are highly induced in the livers of rats treated with agents that stimulate the ARE pathway include GST-Pi, AKR 3A7, quinone reductases, and epoxide hydrolase (19). No significant induction of these genes by either agent was observed. However, these genes were highly induced by known ARE agonists such as 1,2 dithiolthione or the triterpenoids in rat liver (19). Thus, it would seem that

substantial levels of NO were not released in the liver. In contrast, both agents did modulate expression of many of the same genes, as shown in the heat map, and the same pathways including small GTPase-mediated signal transduction, protein transport, and response to stress (Supplementary Figs. S1 and S2), implying that naproxen and NO-naproxen yield a similar signal. These data seem compatible with the hypothesis both agents function via the parent compound naproxen.

Our final line of investigation examined the effects of combining DFMO with a suboptimal dose of an NSAID. We and others have shown that the combination of an NSAID or celecoxib + DFMO was highly effective in models of colon, intestinal, squamous cell cancer of the skin, and esophageal cancer (29–31). The extensive animal data in colon supported the striking results achieved with the combination of sulindac and DFMO in a recent colon adenoma trial in humans (15). The dose of DFMO we used was low compared with the HED and was meant to parallel that of Meyskens and colleagues used in humans (15). The dose of sulindac we used also paralleled Meyskens' dose, whereas the dose of naproxen (75 ppm) was meant to yield a moderate effect but was in fact highly effective on its own. In this studies, we found that DFMO minimally affected tumor development at this low dose of 1,000 ppm. The suboptimal dose of naproxen was highly effective (63% decrease) whereas 150 ppm sulindac, although trending in the right direction (33% decrease), did not have statistically significant efficacy. Adding DFMO yielded no improvement in the strong efficacy of naproxen. However, DFMO addition slightly increased the efficacy of sulindac. In fact, sulindac, DFMO, and sulindac + DFMO yielded large cancer incidences of 67%, 53%, and 37%, respectively,

whereas the control was 77%. The combination was significantly different from controls. Given the strong efficacy of combining NSAIDs and DFMO in other organs, we anticipated an even greater decrease with this combination.

In summary, naproxen and its NO derivative were highly effective in the OH-BBN model of urinary bladder cancer, whereas sulindac had moderate efficacy and the addition of DFMO with an NSAID minimally affected efficacy.

Disclosure of Potential Conflicts of Interest

G. Milne is a consultant/advisory board member in Roche, USA. No potential conflicts of interest were disclosed by the other authors.

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