Celecoxib Inhibits N-Butyl-N-(4-hydroxybutyl)-nitrosamine-induced Urinary Bladder Cancers in Male B6D2F1 Mice and Female Fischer-344 Rats


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Abstract

Epidemiological studies have shown that nonsteroidal anti-inflammatory drugs (NSAIDs) may have a role in the prevention of human cancers. A number of preclinical studies have also suggested that inhibition of cyclooxygenase (COX) with NSAIDs has an anticancer effect in animal models of colon, urinary bladder, skin, and breast. In these studies, we evaluated the COX-2 inhibitor celecoxib in two rodent models of urinary bladder cancer. Male B6D2F1 mice treated with N-butyl-N-(4-hydroxybutyl)-nitrosamine (OH-BBN) developed transitional and squamous cell urinary bladder cancers, many of which grew rapidly and caused substantial morbidity that required sacrifice of the mice. Groups of mice received various daily doses of celecoxib in the diet (1250, 500, or 200 mg/kg of diet) beginning 7 days before the initiation of 12 weekly doses of OH-BBN. Mice were checked weekly for the presence of palpable urinary bladder masses. The study was terminated at 8 months following the initial treatment with OH-BBN. The percentage of mice with large palpable bladder lesions, which necessitated sacrifice of the mice, was 40% in the OH-BBN control group. In contrast, only 10% of all celecoxib-treated mice required sacrifice before the scheduled termination of the experiment, implying that all three doses of celecoxib inhibited the formation of large palpable lesions. Celecoxib did not significantly alter the incidence of preneoplastic bladder lesions, but did dose-dependently decrease the total number of urinary bladder cancers/mouse, palpable plus microscopic, by 77, 57, and 43% at dosages of 1250, 500, and 200 mg of celecoxib/kg of diet, respectively. In the second model, female Fischer-344 rats were administered OH-BBN twice/week for a period of 8 weeks. After 8 months, all rats developed preneoplastic lesions, whereas roughly 60% of the rats developed relatively small urinary bladder cancers. Rats were treated continually with celecoxib in the diet (500 or 1000 mg/kg of diet) beginning 1 week prior to the initial OH-BBN treatment or beginning 1 week following the last OH-BBN treatment. Neither celecoxib treatment regimen significantly altered the number of preneoplastic lesions. Whereas celecoxib treatment initiated prior to OH-BBN administration decreased cancer incidence roughly 65%, celecoxib treatment initiated beginning 1 week after the last dose of OH-BBN profoundly decreased cancer incidence (>95%). Celecoxib did not alter the body weights of the mice or rats, or cause other signs of toxicity at any of the doses studied. Taken together these results demonstrate that: (a) celecoxib effectively inhibits tumor growth and enhances survival in the mouse model of urinary bladder cancer; and (b) celecoxib profoundly inhibits development of urinary bladder cancers in the rat model even when administered following the last dose of OH-BBN. Clinical trials will be necessary to determine whether COX-2 inhibitors will provide a clinical benefit in human bladder cancer.

Introduction

Conventional NSAIDs inhibit both COX-1 and -2 and therefore inhibit the conversion of arachidonic acid to prostaglandins. COX-1 is constitutively expressed and plays an important role in gastrointestinal cytoprotection and maintenance of platelet and kidney function. In contrast to the housekeeping function of COX-1, COX-2 is primarily expressed in response to inflammatory stimuli and cellular activation (1, 2). Conventional NSAIDs have been shown to effectively inhibit inflammation. More recently, epidemiological data demonstrate that regular NSAID use can significantly reduce the risk for colon (3) and urinary bladder (4) cancers and inhibit tumor growth in animal models of cancer. Conventional NSAIDs block both forms of COX, hence their anti-inflammatory activity is linked mechanistically and intimately to gastrointestinal toxicity and altered platelet function. Molecular-based targeting for the COX-2 isoform has led to the development of COX-2 selective inhibitors such as celecoxib (5) and rofecoxib (6), which potently inhibit COX-2-dependent inflammation while avoiding typical NSAID-associated side effects (7). Although the initial premise for developing these drugs was their potential for inhibiting the inflammation and pain of adult osteo- and rheumatoid arthritis, use of these agents appears to be a promising strategy for chemoprevention and chemotherapy of various epithelial cancers.

Several laboratories have recently demonstrated that celecoxib can exert potent anticaner activity in both carcinogen-induced and genetically determined rodent models of colon (8, 9) and UV-induced skin (10) carcinogenesis. In addition, COX-2 has been shown to be overexpressed in human colon (11), breast (12), skin (13), and urinary bladder cancers (14). Taken together, these data are consistent with the hypothesis that the anticaner activity of NSAIDs observed in humans may be dependent upon their ability to inhibit COX-2.

To test this hypothesis, we examined the chemopreventive efficacy of celecoxib on the induction of urinary bladder cancer by N-butyl-N-(4-hydroxybutyl)-nitrosamine (OH-BBN) in male B6D2F1 mice and female Fischer-344 rats. Treatment of mice or rats with OH-BBN results in the development of transitional and squamous cell urinary bladder cancers (15–18) that bear significant histopathological similarities to the human disease and tend to be invasive. For these reasons, this model has been used previously to characterize the tumorigenic process for urinary bladder cancer and to assess the efficacy of potential chemopreventive agents to inhibit the development of carcinogen-induced bladder cancers (16, 17). Among the compounds which have shown promise previously in the OH-BBN urinary bladder cancer models have been a number of specific or nonspecific inhibitors of the COX isozymes (19, 20). Furthermore, the NSAID piroxicam has shown efficacy in treating spontaneous urinary bladder tumors in dogs (21). On the basis of these preclinical findings,
Materials and Methods

Male B6D2F1 (C57BL/6 × DBA/2F1) mice and female Fisher-344 rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN; virus-free colony 202) at 28 days of age and housed in polycarbonate cages (5/cage). The animals were kept in a room lighted 12 h each day and maintained at 22 ± 0.5°C. Teklad (4%) diet (Harlan Teklad, Madison, WI) and tap water were provided ad libitum. Celecoxib (SC-635) was provided by Monsanto/Searle (St. Louis, MO).

In the mouse study, celecoxib was administered at 200, 500, and 1250 mg/kg of diet when the animals were 49 days of age and was continued throughout the study. At 56 days of age, carcinogen-treated mice received the first of 12 weekly intragastric doses of OH-BBN (TCI America, Portland, OR). Each 7.5-mg dose was dissolved in 0.1 ml ethanol:water (20:80). The mice were weighed weekly and checked daily. Some animals were lost during the first two weeks of the study due to gavage errors. These mice were excluded from the final analysis. After the last dose of carcinogen, the animals were palpated weekly for urinary bladder masses. Animals which developed large palpable tumors, bloody urine, and weight loss became rapidly moribund and were sacrificed. Mice not sacrificed specifically because of the presence of large lesions were sacrificed 8 months after the initial OH-BBN treatment.

Diet supplementation of female Fischer-344 rats with celecoxib was initiated at 43 days of age (1 week prior to the initial OH-BBN treatment) or at 107 days of age (1 week after the last OH-BBN treatment). OH-BBN (150 mg/gavage, 2×/week) was started when the rats were 49 days of age and continued for 8 weeks. The carcinogen vehicle was ethanol:water (20:80); volume was 0.5 ml. The rats were observed daily, weighed weekly, and checked daily. Some animals were lost during the first two weeks of the study due to gavage errors. These mice were excluded from the final analysis. After the last dose of carcinogen, the animals were palpated weekly for urinary bladder masses. Animals which developed large palpable tumors, bloody urine, and weight loss became rapidly moribund and were sacrificed. Mice not sacrificed specifically because of the presence of large lesions were sacrificed 8 months after the initial OH-BBN treatment.

At necropsy, urinary bladders of both mice and rats were inflated with 10% buffered formalin, removed, and observed under a high-intensity light for gross lesions. After fixation, each lesion was dissected, processed for routine paraffin embedding, cut into 4-μm sections, and mounted onto polylysine-coated slides. Sections were deparaffinized in xylene, rehydrated in descending alcohols, and blocked for endogenous peroxidase (3% H₂O₂ in methanol) and avidin/biotin (Vector Blocking Kit). The sections were permeabilized in TNB-BB (0.1 M Tris (pH 7.5)/0.15 M NaCl/0.5% blocking agent/0.3% Triton-X, 0.2% saponin) and incubated with primary antibody overnight at 4°C. The isoenzyme-specific COX-2 polyclonal (PG-27; Oxford Biomedical Research) antisera were diluted to 1:500 in TNB-BB for all tissues. Control sections were incubated with antisera in the presence of 100-fold excess COX-2 protein, or with an isotype-matched IgG normal rabbit serum. Immunoreactive complexes were detected using tyramide signal and amplification (TSA-indirect; NEN Life Science) and visualized with the peroxidase substrate, AEC. Slides were then counter-stained with aqueous hematoxylin (Biomedia), mounted in crystal solution (Biomedia), and coverslipped in 50:50 xylene/Permount.

The Kaplan-Meier test (22) was used to analyze survival data. Urinary bladder cancer incidence and multiplicity were analyzed by the Fisher’s exact and Wilcoxon’s rank-sum tests, respectively.

Results and Discussion

We used two different rodent models of urinary bladder cancer induced by the organ-specific nitrosamine OH-BBN to examine the efficacy of celecoxib. In the first model, male B6D2F1 mice developed relatively aggressive bladder cancers of both transitional cell and squamous cell phenotypes. These tumors grew rapidly and, because of bleeding and obstruction of the urinary bladder, caused morbidity in a high percentage of mice (40%). The 40% incidence of palpable lesions in OH-BBN-treated mice represents almost 80% of the 51% of mice which developed urinary bladder cancers at all. Interestingly, although female Fischer-344 rats treated with OH-BBN also develop both transitional and squamous cell carcinoma, these tumors grew slowly, and virtually no rats were lost due to development of large palpable tumors. Therefore, we used these models to answer two separate questions. In the rat model, we wanted to address the efficacy of celecoxib when administration was initiated either before or after OH-BBN treatment. This was done to determine the stage in tumor development when celecoxib was effective. In the mouse model, we wished to determine whether celecoxib could substantially decrease the growth of these rapidly growing cancers when administered throughout the duration of the study.

The effect of celecoxib on survival due to bladder cancer is shown in Figure 1. Mice were sacrificed during the study if they developed large palpable tumors which caused bloody urine and weight loss (Fig. 2). The number of mice sacrificed in the vehicle-treated group (n = 27) significantly exceeded the number sacrificed in any of the celecoxib-treated groups (n ≤ 6) by Kaplan-Meier analysis (P > 0.001 for all groups). In fact, the numbers sacrificed in the control group exceeded that in all celecoxib groups combined. This result clearly demonstrates that celecoxib can profoundly suppress the

[Diagram of bladder cancer growth and survival]
growth of large tumors (Fig. 2) and reduce morbidity. In addition to improved survival, celecoxib dose-dependently inhibited tumor incidence, which reflects both palpable and microscopic tumors, by 43, 57, and 77% when dosed at 200, 500, and 1250 mg of celecoxib/kg of diet, respectively (Table 1). Celecoxib also suppressed the average number of cancers observed per mouse. These results demonstrate that celecoxib reaches a threshold level, which was achieved even at the lowest dose of celecoxib (200 mg/kg of diet), it profoundly decreases the growth of large palpable lesions.

Interestingly, celecoxib did not appear to suppress the incidence of precancerous lesions (e.g., hyperplasias) in the urinary bladders. This may, in part, reflect the technical difficulties associated with accurately quantitating tumor multiplicity, particularly in vehicle-treated animals which tend to develop large palpable tumors that nearly filled the bladder (Fig. 2). In addition, celecoxib may preferentially inhibit the growth of larger lesions, rather than some of the smaller preinvasive lesions that may not have undergone neovascularization and are therefore less likely to express COX-2.

In the rat OH-BBN model, celecoxib (500 or 1000 mg/kg of diet) was administered beginning either 1 week before the initial dose of OH-BBN or 1 week after the last dose of OH-BBN. The latter involved starting treatment roughly 30% of the way into the experiment. As can be seen in Table 2, 77% of the OH-BBN control rats (23 of 30) developed preinvasive lesions (hyperplasias or papillomas) with roughly 2.9 lesions/rat, Rats treated with either dose of celecoxib at either time point achieved a similar number of preinvasive lesions (2.7–3.6 per rat). Fifty seven percent (17 of 30) of the OH-BBN control rats developed urinary bladder cancers with roughly 0.63 cancers/rat. Many of these tumors were small and of transitional cell and/or squamous cell morphology. When rats were treated with celecoxib beginning 7 days prior to OH-BBN and continuing throughout the duration of the experiment, the incidence of cancer was 21% (6 of 29) and 14% (4 of 28) at doses of 1000 and 500 mg/kg of diet, respectively. Interestingly, when celecoxib administration was delayed until 7 days following the final dose of OH-BBN, we observed a profound decrease in tumor incidence from 57% (17 of 30) in controls to 2% (1 of 55) in celecoxib-treated rats (groups 3 plus 4). These results were obtained in the absence of any weight effects or any other signs of toxicity.

One interesting aspect of these studies is that celecoxib did not significantly decrease the incidence or multiplicity of preneoplastic lesions in either model. The results in the rat, demonstrating efficacy even after delayed administration, agrees with previous reports, in both the colon (23) and skin (24), that late treatment with this agent is highly effective. This has often been predicated on the fact that the presumed target for celecoxib is often not expressed in tumor epithelia until later in the cancer progression process. Although efficacy in the rat model was striking irrespective of the time of initiation of celecoxib treatment, the reason for the slightly greater efficacy when treatment was initiated later is not apparent.

The striking efficacy of celecoxib is consistent with previous studies in rodent bladder models showing that NSAIDs are effective chemopreventive agents (19, 20). The present results, therefore, support the hypothesis that COX-2 derived prostaglandins play a role during tumorigenesis in this model and are the effective target of the anticancer activity of conventional NSAIDs.

COX-2 has been shown to be expressed in the cancer cell per se as well as in the vasculature associated with the neoplastic lesions in a variety of human epithelial cancers (25), including human bladder carcinoma in situ and carcinomas (14). Interestingly, in the mouse OH-BBN urinary bladder cancer model, COX-2 is exclusively expressed in the vasculature adjacent to and within the tumors (Fig. 3), but not expressed in the tumor epithelia. This strong COX-2 staining in endothelial cells is consistent with previously reported in vitro studies reporting that COX-2 is expressed and can be further induced in human umbilical vein endothelial cells (26). Furthermore, in the rodent bFGF-induced corneal micropocket model of angiogenesis, COX-2 is detected in the vascular-associated cells populating the cornea, and treatment with celecoxib markedly inhibits neovascularization at doses which do not inhibit COX-1 (27). These observations suggest that COX-2 may play a functional role in tumor-induced angiogenesis, and that suppressing COX-2-derived prostaglandins in the vasculature may block neovascularization, and hence, tumor growth. The rather striking effects on the growth of larger lesions at all three doses of celecoxib in mice are consistent with the effects of celecoxib being most profound during the growth phase of the tumor process, a result which would be consistent with an antiangiogenesis hypothesis.

### Table 1. Effect of celecoxib on urinary bladder cancers in male B6D2F1 mice

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Carcinogen</th>
<th>Treatment</th>
<th>Incidence (%)</th>
<th>Average no./animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67</td>
<td>OH-BBN</td>
<td>Celecoxib, 1250 mg/kg of diet</td>
<td>120 †</td>
<td>0.13</td>
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<tr>
<td>2</td>
<td>72</td>
<td>OH-BBN</td>
<td>Celecoxib, 500 mg/kg of diet</td>
<td>240 †</td>
<td>0.24</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>OH-BBN</td>
<td>Celecoxib, 200 mg/kg of diet</td>
<td>290 †</td>
<td>0.32</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>OH-BBN</td>
<td>Celecoxib only</td>
<td>51</td>
<td>0.56</td>
</tr>
</tbody>
</table>

* Diets of male B6D2F1 mice were supplemented with celecoxib beginning when the mice were 49 days of age.
* Study was terminated 8 months after the initial administration of the carcinogen at 56 days of age.
* Significantly different from group 4.

### Table 2. Effect of celecoxib on urinary bladder lesions induced in female Fischer-344 rats

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Carcinogen</th>
<th>Treatment</th>
<th>Incidence (%)</th>
<th>Average no./animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>OH-BBN</td>
<td>Celecoxib (1000 mg/kg of diet), started 1 week prior to carcinogen</td>
<td>93</td>
<td>2.66</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>OH-BBN</td>
<td>Celecoxib (500 mg/kg of diet), started 1 week prior to carcinogen</td>
<td>100</td>
<td>3.11</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>OH-BBN</td>
<td>Celecoxib (1000 mg/kg of diet), started 1 week after carcinogen</td>
<td>90</td>
<td>3.59</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>OH-BBN</td>
<td>Celecoxib (500 mg/kg of diet), started 1 week after carcinogen</td>
<td>100</td>
<td>3.50</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>OH-BBN</td>
<td>Diet only</td>
<td>77</td>
<td>2.93</td>
</tr>
</tbody>
</table>

* OH-BBN was administered to female Fischer-344 rats for 8 weeks beginning at 49 days of age.
* Diets were supplemented with celecoxib at either 43 days of age (1 week before the initial OH-BBN treatment) or at 107 days of age (1 week after the last OH-BBN treatment).
* Significantly different from group 5.
These data demonstrate that celecoxib is highly effective in preventing OH-BBN-induced urinary bladder cancers in both mice and rats with no associated toxicity. The results in the rat, and more indirectly the results in mice, on the growth of large tumors, support venting OH-BBN-induced urinary bladder cancers in both mice and of clinical trials to determine whether COX-2 inhibitors can provide COX-2 in human bladder cancer (25), strongly supports development agent is effective when given later in the tumorigenic process is context of persons treated surgically for superficial bladder cancer tion study, in the short run, for a COX-2 inhibitor would be in the

Fig. 3. COX-2 is markedly expressed in the vasculature within and adjacent to urinary bladder tissues. Tissues were processed by standard immunohistochemical techniques and stained for COX-2.

References


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Clinton J. Grubbs, Ronald A. Lubet, Alane T. Koki, et al.

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