

# Effects of the Cyclooxygenase Inhibitor, Piroxicam, on Tumor Response, Apoptosis, and Angiogenesis in a Canine Model of Human Invasive Urinary Bladder Cancer<sup>1</sup>

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## Abstract

The mechanisms by which cyclooxygenase inhibitors exert antitumor effects are not completely defined but are postulated to involve antiangiogenic effects and induction of apoptosis. In this study, we determined the effects of the cox inhibitor, piroxicam, on tumor response, apoptotic index, proliferative index, cyclooxygenase-2 expression, prostaglandin E<sub>2</sub> concentration, tumor microvessel density, and urine basic fibroblast growth factor and vascular endothelial growth factor concentrations in pet dogs with naturally occurring invasive transitional cell carcinoma of the urinary bladder. Piroxicam caused reduction in tumor volume in 12 of 18 dogs, and this was strongly associated with induction of apoptosis (Fisher's exact test  $P < 0.015$ ) and reduction in urine basic fibroblast growth factor concentration.

## Introduction

Cox-2,<sup>3</sup> an inducible enzyme which catalyzes the formation of prostaglandins from arachidonic acid, is up-regulated in multiple types of solid tumors, including carcinomas of the lung, breast, colon, prostate, and urinary bladder in humans and in animals (1–4). Recent epidemiological studies in humans and studies in spontaneous canine tumors and experimentally induced rodent tumors have shown that cox-2 or cox-1/cox-2 inhibitors have antitumor and chemopreventive effects in several different forms of cancer (5–7). In urinary bladder cancer, the cox inhibitor, piroxicam, has induced remission of naturally occurring invasive TCC in dogs (8). Naturally occurring canine invasive TCC is very similar to human invasive TCC in histopathological characteristics, biological behavior, and response to chemotherapy (9). The mechanisms by which cox inhibitors exert their antitumor effects are not completely understood, but studies have shown that cox-2-derived prostaglandins contribute to tumor cell resistance to apoptosis, new blood vessel formation, and tumor cell proliferation (10). PGE<sub>2</sub> has been incriminated as a major cox product involved in tumor development and progression. The purpose of this study was to confirm the antitumor effects of cox inhibitors and determine the effects of the cox inhibitor, piroxicam, on apoptosis, proliferation, PGE<sub>2</sub> concentration, cox-2 expression, angiogenesis (MVD) in the tumor, and concentrations of angiogenic factors in the urine of dogs with naturally occurring invasive urinary bladder cancer.

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<sup>3</sup>The abbreviations used are: cox, cyclooxygenase; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; TBS, Tris-buffered saline; TCC, transitional cell carcinoma; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; MVD, microvessel density; PD, progressive disease; EIA, enzyme immunoassay.

## Materials and Methods

The Purdue University Animal Care and Use Committee approved this study. Pet dogs with histopathologically confirmed invasive TCC of the urinary bladder were enrolled in a clinical trial at the Purdue University Veterinary Teaching Hospital. Dogs underwent tumor staging, including thoracic and abdominal radiography, cystography, ultrasonography, and cystoscopy (with collection of tissue samples) before treatment and after 4 weeks of piroxicam (0.3 mg/kg qd 24-h p. o.) treatment. Dogs received no other cancer treatment during the 4 weeks of piroxicam treatment. Tissue samples were immediately frozen in liquid nitrogen for PGE<sub>2</sub> analysis or fixed in 10% neutral buffered formalin for immunohistochemical examination. Urine was also collected before and after piroxicam treatment, aliquoted, and then stored at –80°C until analyzed.

**Tumor Response.** Tumor size was measured before and after piroxicam treatment with ultrasonography and contrast cystography as described previously by Chun *et al.* (11). Tumor staging was completed with thoracic radiography and physical examination. Cystoscopy was performed, and bladder and urethral lesions were noted and biopsied. Tumor responses were reported as the percentage of change in tumor volume and were categorized as: (a) complete remission, complete resolution of all evidence of cancer; (b) partial remission,  $\geq 50\%$  decrease in tumor volume and no new tumor lesions; (c) stable disease,  $< 50\%$  change in tumor volume and no new tumor lesions; or (d) progressive disease,  $\geq 50\%$  increase in tumor volume or development of new tumor lesions.

**Measurement of PGE<sub>2</sub> Concentrations.** PGE<sub>2</sub> concentrations were determined by PGE<sub>2</sub> EIA system (Amersham Pharmacia Biotech, Piscataway, NJ) in snap frozen tumor tissue samples. Briefly, frozen tumor tissue samples were weighed and homogenized in PBS containing 5  $\mu\text{g/ml}$  indomethacin. After homogenization and extraction of PGE<sub>2</sub> with methanol, samples were passed through an activated Amprep C18 reverse phase column. The PGE<sub>2</sub> was eluted using ethyl acetate. Samples were dried under nitrogen gas and resuspended in EIA buffer. PGE<sub>2</sub> concentrations were determined by EIA according to manufacturer instructions.

**Apoptosis.** Apoptosis was measured on tissue sections by the terminal deoxynucleotidyl transferase-mediated nick end labeling assay using Apop tag peroxidase *in situ* apoptosis detection kit (Intergen Co., Purchase, NY) according to manufacturer instructions. Briefly, 5- $\mu\text{m}$  sections were cut from paraffin-embedded tissues and mounted on Superfrost slides. The slides were deparaffinized and hydrated through xylene and graded alcohol. The sections were immersed first in 3% hydrogen peroxide in PBS to block the endogenous peroxidase. A reaction buffer containing digoxigenin-labeled and -unlabeled nucleotides and terminal deoxynucleotidyl transferase was added to the sections. Cells undergoing apoptosis were visualized with antidigoxigenin peroxidase and chromogen substrate. The numbers of stained tumor cells were recorded in a minimum of five fields at  $\times 400$  magnification. Two observers examined each section, and the results were averaged. Previous studies and reports in the literature have indicated that a meaningful change in apoptotic index is doubling or more of the index (12). Therefore, we categorized the change in apoptotic index as doubling or more of the index or not doubling of the index.

**Immunohistochemistry.** Immunohistochemical analyses of dog tissues were performed as described by Khan *et al.* (13) with some modification.

Briefly, 5- $\mu$ m sections were cut from paraffin-embedded tissues and placed on Superfrost slides. The slides were deparaffinized and hydrated through xylene and graded alcohol. The sections were first immersed in 3% hydrogen peroxide in methanol to block the endogenous peroxidase and then blocked for avidin and biotin (Vector Laboratories, Inc., Burlingame, CA). All tissues were preblocked in TBS-BB [0.3% triton/0.2% saponin/0.5% blocking agent (NEN Life, Boston, MA) in TBS] and then incubated in primary antibody overnight at 4°C. The antisera specific for Von Willebrand Factor VIII-related antigen, proliferating nuclear antigen (DAKO Corp., Carpinteria, CA), or cox-2 (Oxford Biomedical Research, Oxford, MI) were diluted 1:100 in TBS-BB. These antibodies are reported by the manufacturer to cross-react with canine antigens. Immunoreactive complexes were detected using tyramide signal amplification (indirect; NEN Life Sciences) and visualized with the peroxidase substrate AEC (Zymed Laboratories, San Francisco, CA). Slides were counter stained in hematoxylin-1 (Richard-Allan Scientific, Kalamazoo, MI). Appropriate controls were performed for each staining assay.

**Assessment of Staining.** MVD was quantified using the “hot spot” method described by Weidner (14). Areas of tumor on the slide with the highest number of Factor VIII related antigen-staining vessels were identified at low magnification. The immunoreactive vessels in hot spots were then counted under high magnification ( $\times 400$ ). Two scorers counted each slide independently, and the scores were averaged. Similarly, two scorers determined the percentage of positive stained cancer cells for cox-2 and proliferating nuclear antigen, and the scores were averaged.

**Determination of Urine bFGF Concentration.** Urine bFGF concentrations were determined according to ELISA validated for dog urine by Allen *et al.* (15) using a commercially available ELISA kit (Quantikine HS; R&D Systems, Inc., Minneapolis, MN), and the urine bFGF concentration was normalized to the urine creatinine and expressed as ng of bFGF per gram of creatinine.

**Determination of VEGF Concentrations.** Urine VEGF concentrations were determined using an ELISA kit (Quantikine HS; R&D Systems, Inc.) as validated and described by Lazarous *et al.* (16) and Gu *et al.* (17), respectively. The protein amino acid sequences of dog VEGF are highly homologous to human VEGF<sup>165</sup> protein (18) used in the kit. Urine VEGF concentrations were normalized to the urine creatinine and expressed as ng of VEGF per gram of creatinine.

**Statistical Analysis.** Data were analyzed using standard statistical software (SAS System Version 8.1; SAS Institute, Inc, Cary, NC, 1999). Differences were considered to be statistically significant at  $P < 0.05$ . Tumor response (remission *versus* stable and/or progressive disease or actual change in the tumor volume) with piroxicam treatment was compared with respect to cox-2 expression, PGE<sub>2</sub> concentration, MVD, proliferative index, and apoptotic index. Categorical variables were compared using a Fisher's exact test or  $\chi^2$  analysis. Continuous variables were compared using Wilcoxon's two-sample test. Pearson correlation coefficients were computed to test for an association between pre-piroxicam PGE<sub>2</sub> concentration and the following variables: cox-2 expression (percentage of positive staining tumor cells and staining intensity), MVD, apoptotic index, and proliferative index. A Wilcoxon two-sample test was used to determine whether there was an association between the induction of apoptosis with piroxicam treatment and change in the following variables: cox-2 expression, PGE<sub>2</sub> concentration, MVD, bFGF concentration, and VEGF concentration. A sample size of 14 dogs was selected so that there was a high probability ( $\geq 95\%$ ) of detecting antitumor activity in at least 1 dog if the true remission rate was  $\geq 20\%$ .

Table 1 Summary of response of canine transitional cell carcinoma of the urinary bladder to piroxicam

Response <sup>a</sup> after 4 weeks of piroxicam	No. of dogs	%
CR	0	0%
PR	6	33%
SD	9	50%
PD	3	17%
Total	18	

<sup>a</sup> Tumor response defined as: CR, complete remission; PR, partial remission,  $\geq 50\%$  decrease in tumor volume and no new lesions; SD, stable disease,  $< 50\%$  change in tumor volume and no new lesions;  $\geq 50\%$  increase in tumor volume or development of new tumor lesions.

Table 2 Apoptotic index (percentage of apoptotic tumor cells) and proliferative index (percentage of proliferating cells) in canine transitional cell carcinoma before (A) and after (B) piroxicam treatment and change in tumor size with treatment

Dog no.	Apoptotic index		Proliferative index		Tumor response
	A	B	A	B	
1	0.5	4.0	2.0	13.0	70% decrease
2	2.0	8.0	6.0	2.0	13% decrease
3	0.5	6.0	1.0	6.0	59% decrease
4	5.0	1.0	16.0	5.0	11% increase
5	0.5	2.5	1.0	1.0	75% decrease
6	1.0	1.5	9.0	12.0	30% increase
7	1.5	80.0	ND <sup>a</sup>	ND	68% decrease
8	0.5	31.0	30.0	20.0	56% decrease
9	0.5	0.5	15.0	28.0	92% increase
10	0.5	4.0	15.0	0.0	33% decrease
11	0.5	4.0	8.0	22.0	40% decrease
12	0.5	0.0	ND	ND	75% decrease
13	0.5	0.5	5.0	2.0	No change
14	ND	ND	1.0	1.0	50% increase
15	ND	ND	6.0	4.0	09% decrease

<sup>a</sup> ND, not determined because of the small size of the biopsy tissues.

## Results and Discussion

Privately owned pet dogs (18) participated in this study, including 9 spayed females, 1 intact female, and 8 neutered male dogs. Dogs ( $> 14$ ) were enrolled to obtain adequate tissue samples for tissue analyses. Mean and median age at diagnosis was 11 years (range: 8–14 years). Mean weight of the 18 dogs was 17 kg (range: 5–36 kg). Tumor invasion into the bladder wall was present in all cases. Regional lymph node metastasis was present in 2 dogs at the time of diagnosis. Distant metastasis was not detected in any of the cases. The tumor involved the urethra as well as the urinary bladder in 9 dogs. The prostate was involved (in addition to the bladder) with the TCC in 5 dogs.

**Tumor Response.** This study confirmed the antitumor effects of cox inhibitors in TCC. The tumor responses in 18 dogs are summarized in Table 1.

**PGE<sub>2</sub> Concentration.** PGE<sub>2</sub> concentration in tumor tissue was measured before piroxicam therapy and after therapy in 18 dogs. The mean concentration of PGE<sub>2</sub> before treatment was  $794 \pm 558$  ng/gram tissue (range: 57–1618 ng/gram tissue). For comparison, the mean PGE<sub>2</sub> concentration in 10 samples of normal bladder mucosa studied previously in our laboratory was  $46 \pm 32$  ng/gram tissue (19). After piroxicam treatment, the concentration of PGE<sub>2</sub> decreased (by  $\geq 20\%$ ), as expected, in 8 dogs (average decrease: 78%). PGE<sub>2</sub> concentration stayed the same ( $< 20\%$  change) in 2 dogs and actually increased in 8 dogs. These results were not anticipated, but it is important to note that these 10 dogs had relatively low concentrations of PGE<sub>2</sub> before treatment. There was a significant correlation ( $P < 0.05$ ) between decreasing PGE<sub>2</sub> concentration and increasing apoptotic index with piroxicam treatment. In addition, there was a significant correlation ( $P < 0.05$ ) between high PGE<sub>2</sub> concentrations and high urine bFGF levels before piroxicam treatment. However, there was no significant correlation between the change in PGE<sub>2</sub> concentration and tumor response to piroxicam.

**Cox-2 Expression.** We confirmed the expression of cox-2 protein in TCC samples from all dogs in this study. The percentage of cox-2 positively stained cells ranged from 1 to 22% in tumor tissue before piroxicam treatment. Cox-2 staining intensity as described by Mohammed *et al.* (3) was 1 in 6 dogs, 2 in 5 dogs, and 3 in 4 dogs. No significant association between cox-2 stained staining of tumor cells at diagnosis and tumor remission with piroxicam was found. Additionally, no consistent changes were noted in the percentage of positive cells or staining intensity after treatment.

**Induction of Apoptosis.** The apoptotic index was determined before and after piroxicam treatment in 13 dogs. Unfortunately, the

amount of tumor tissue obtained with a small diameter cystoscope was not sufficient to accurately determine apoptosis in 5 cases. There was a strong association between doubling of the apoptotic index and reduction in tumor size (Table 2; Fisher's exact test  $P < 0.015$ ). Additionally of interest was an inverse correlation between the doubling of the apoptotic index and change in PGE<sub>2</sub> concentration, *i.e.*, there was an association between doubling of the apoptotic index and declining PGE<sub>2</sub> concentrations with piroxicam treatment ( $\chi^2$ ,  $P < 0.05$ ). In 8 dogs in which the apoptotic index doubled, the mean PGE<sub>2</sub> concentration decreased by 601 ng/gram tissue. In 5 dogs in which the apoptotic index did not double, the mean PGE<sub>2</sub> concentration increased by 448 ng/gram tissue.

**Cell Proliferation.** The proliferative index was determined before and after piroxicam treatment in 13 dogs and ranged from 1 to 30% and 1 to 28%, respectively. The proliferative index decreased ( $\geq 20\%$  decrease) in 6 dogs, stayed the same ( $< 20\%$  change) in 2 dogs, and increased ( $\geq 20\%$  increase) in 5 dogs with piroxicam treatment. There was no association between initial proliferative index or change in proliferation with treatment and tumor response to piroxicam.

**MVD.** MVD was measured in 13 dogs, and a mean of  $52 \pm 39$  microvessels in hot spots per high power field were found. After piroxicam treatment, MVD decreased ( $\geq 20\%$  decrease) in 7 dogs and increased ( $\geq 20\%$  increase) in 6 cases. No association between initial MVD or change in MVD and response to therapy was noted. This lack of association, however, does not exclude the possibility that piroxicam had an antiangiogenic effect. It has been recognized recently that MVD may not be a good measure of drug effects on angiogenesis (20). Measurement of angiogenic factors in urine is postulated to be more meaningful.

**Urinary Basic FGF.** The mean concentration of bFGF in urine before piroxicam treatment was  $7 \pm 8.2$  ng/gram creatinine ( $n = 14$ ), significantly higher ( $P < .05$ ) than that of 8 normal dogs ( $0.31 \pm 0.61$  ng/gram creatinine). Urine bFGF concentration decreased with piroxicam treatment in 11 of 14 dogs (77%). Tumors in 10 dogs with decreasing urine bFGF concentration decreased in tumor volume by 9–75%. Overall, there was a positive correlation between the change in urine bFGF concentration and the change in tumor size, *i.e.*, when tumors decreased in size, the bFGF concentration decreased; when tumors increased in size, bFGF concentration increased (Table 3). Additional study is needed, however, to determine whether the reduction in bFGF concentration is a reflection of simply a change in tumor mass (and the number of viable cells remaining to produce bFGF) or if piroxicam causes a change in bFGF concentration independent of tumor size.

**Urine VEGF.** VEGF concentrations were analyzed in 13 dogs. The mean concentration of VEGF in urine before piroxicam treatment was  $1509.5 \pm 657.6$  ng/gram creatinine, which is 3-fold higher than urine VEGF in 11 normal dogs  $494.32 \pm 233.95$  ng/gram creatinine. After piroxicam treatment, VEGF concentration decreased (13–74% decrease) in 7 of 13 dogs and increased in 6 of 13 dogs (Table 3).

There was no association between change in VEGF concentration and change in tumor size.

In conclusion, piroxicam had antitumor activity against canine TCC, a disease that closely mimics human invasive urinary bladder cancer. This antitumor activity was closely associated with induction of apoptosis. Additional study is needed to determine the specific part of the apoptosis process affected, the extent to which antiangiogenic effects are involved, and what role PGE<sub>2</sub> plays in these processes. It is of interest to note the association between high concentration of PGE<sub>2</sub> and high urine bFGF level before piroxicam treatment and the inverse correlation between doubling of the apoptotic index and change in PGE<sub>2</sub> concentration with treatment. Urinary bladder cancer results in  $> 12,000$  deaths each year in the United States. Cox may be a new target for improved treatment of this disease.

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Table 3 Tumor response and change in urine bFGF and VEGF with piroxicam treatment

Tumor response	No. of dogs			
	bFGF decrease <sup>a</sup>	bFGF increase <sup>b</sup>	VEGF decrease <sup>c</sup>	VEGF increase <sup>d</sup>
>10% decrease	10	0	6	5
>10% increase	0	3	1	0
Total dogs	10	3	7	5

<sup>a</sup> bFGF decrease  $> 10\%$ .

<sup>b</sup> bFGF increase  $> 10\%$ .

<sup>c</sup> VEGF decrease  $> 10\%$ .

<sup>d</sup> VEGF increase  $> 10\%$ .