

# Effects of the Cyclooxygenase Inhibitor, Piroxicam, in Combination with Chemotherapy 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 objectives of this study were: (a) to determine the antitumor activity and toxicity of a cyclooxygenase inhibitor (piroxicam) combined with cisplatin chemotherapy in dogs with naturally-occurring, invasive transitional cell carcinoma (TCC) of the urinary bladder; and (b) to determine the effects of this treatment on prostaglandin E<sub>2</sub> concentration, tumor cell proliferation and apoptosis, and angiogenesis. Pet dogs with naturally-occurring invasive TCC underwent complete tumor staging before and after 10 weeks of piroxicam/cisplatin treatment. Prostaglandin E<sub>2</sub> concentrations were determined by immunoassay in snap-frozen tumor tissues. Apoptosis (terminal deoxynucleotidyl transferase-mediated nick end labeling assay), proliferation (proliferating cell nuclear antigen), and microvessel density were determined in formalin-fixed tissues. Urine basic fibroblast growth factor and vascular endothelial cell growth factor concentrations were determined by immunoassay. Partial remission ( $\geq 50\%$  reduction in tumor volume) was noted in 6 of 12 dogs treated with piroxicam/cisplatin. Renal toxicity was dose-limiting. Apoptotic index doubled with treatment in 11 of 12 dogs but was not associated with tumor response. Proliferative index decreased in five dogs, and tumor decreased in size in three of the five dogs. Change in urine basic fibroblast growth factor and vascular

endothelial cell growth factor was associated with tumor response. microvessel density was not associated with tumor response. In conclusion, piroxicam/cisplatin had antitumor activity against canine TCC, a disease that closely mimics human invasive urinary bladder cancer. Strategies to prevent renal toxicity of this protocol are needed. Induction of tumor apoptosis and reduction in angiogenic factor concentrations were observed, but additional studies are needed to further define the mechanisms of the antitumor activity of piroxicam/cisplatin.

## Introduction

cox<sup>3</sup> inhibitors have been found to have chemopreventive and antitumor activity and to potentiate the effects of chemotherapy in a variety of tumors (1–3). In naturally-occurring canine invasive urinary bladder cancer, the cox inhibitor, piroxicam, induced remission in 18% of dogs and resulted in stable tumor size in 50% of dogs. In canine urinary bladder cancer, cisplatin combined with piroxicam, resulted in a significant increase in remission rate (Fisher's exact test,  $P < 0.004$ ) compared with cisplatin alone in a randomized study (4). Unfortunately, the renal toxicity of piroxicam/cisplatin was frequent and dose-limiting. One purpose of this pilot study was to determine the renal toxicity of piroxicam/cisplatin when given with more prolonged saline administration.

The mechanisms by which cox inhibitors have antitumor activity in bladder cancer and in other forms of cancer are not completely defined. In our laboratory, piroxicam and other cox inhibitors had no direct *in vitro* cytotoxic activity against canine and human bladder cancer cell lines at concentrations that could be attained *in vivo* (5). The currently proposed mechanisms of cox inhibitor antitumor activity include: reduction in cell proliferation, induction of apoptosis, and inhibition of angiogenesis (6–7). Results of several studies have demonstrated that cox inhibitors induce apoptosis (8–9). Cisplatin and other chemotherapeutic agents depend on apoptosis for cell death after DNA damage. Obtaining further understanding of the mechanisms of cox inhibitor (alone and with chemotherapy) antitumor activity could lead to therapy that is more effective.

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

The purpose of this study was to determine the antitumor activity and renal toxicity of piroxicam/cisplatin when given with a 21-h saline administration protocol and to determine the effects of piroxicam/cisplatin on tumor cell proliferation, apoptosis, angiogenesis, and cox products (PGE<sub>2</sub>). In this study, piroxicam was given alone for 4 weeks followed by piroxicam/cisplatin for 6 weeks. This was done in an attempt to activate apoptotic pathways to prepare the cells for apoptotic death when exposed to cisplatin (7).

## Materials and Methods

**Subject Eligibility.** Pet dogs with histopathologically confirmed, measurable TCC of the urinary bladder were enrolled in a clinical trial at the Purdue University Veterinary Teaching Hospital following guidelines and approval of the Purdue Animal Care and Use Committee. Entry requirements for the dogs in this study included: no prior cisplatin or piroxicam therapy, normal blood urea nitrogen (BUN) and creatinine concentrations, expected minimum survival of 6 weeks, and informed pet owner consent. With the exception of days when dogs were undergoing clinical evaluation and cisplatin treatment, the dogs lived at home with their owners.

**Tumor Staging and Dog Evaluation.** Dogs were evaluated with physical examination, thoracic and abdominal radiography, bladder ultrasonography, and cystography as described previously by Chun *et al.* (10) before and after 10 weeks of treatment. Further monitoring included a complete blood count (CBC), platelet count, serum biochemical profile, and urinalysis before each cisplatin treatment and CBC and platelet count 7 to 10 days after each cisplatin treatment. Tissue samples were collected by cystoscopy before and after 10 weeks of treatment. Tissue samples were immediately frozen in liquid nitrogen for PGE<sub>2</sub> analysis or immersed in neutral buffered formalin for immunohistochemical analysis. Urine samples were collected before and after 10 weeks of treatment. Samples were centrifuged, and the supernatants were aliquoted and stored at -80°C until analysis.

**Treatment.** Dogs were given piroxicam (Pfizer, New York, NY) at a dosage of 0.3 mg/kg every 24 h p.o., alone for 4 weeks, followed by piroxicam combined with cisplatin (60 mg/m<sup>2</sup> i.v. every 21 days). Cisplatin was provided by Bristol Laboratories, Bristol-Myers Squibb Co., Princeton, N.Y. Diuresis was induced by administering 0.9% saline i.v. at a rate of 18 ml/kg/h for 4 h before and 2 h after cisplatin administration. Then, saline was given at a rate of 5 ml/kg/h for 15 h. Cisplatin was administered i.v. over a 20-min period. Butorphanol (Torbugestic; 0.4 mg/kg i.v.; Fort Dodge Laboratories, Fort Dodge, IA) was given 30 min before cisplatin to decrease vomiting. Signs of gastrointestinal and renal toxicity were recorded and were categorized as mild, moderate, or severe as described previously (4). Treatment was scheduled to continue until two doses of cisplatin had been given after complete remission or until progressive disease or unacceptable toxicity occurred.

**Tumor Response.** Tumor size was measured before and after 10 weeks of piroxicam/cisplatin treatment with ultrasonography and contrast cystography (10). Tumor responses were reported as the percentage change in tumor volume and were categorized as: (a) CR, complete remission,

complete resolution of all evidence of tumor; (b) PR, partial remission, ≥50% decrease in tumor volume and no new tumor lesions; (c) StD, stable disease, <50% change in tumor volume and no new tumor lesions; and (d) PD, progressive disease, ≥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 EIA (Amersham Pharmacia Biotech, Piscataway, NJ) in snap-frozen tumor tissue samples. Briefly, frozen tumor tissue samples were weighed and homogenized in PBS containing 5 μg/ml indomethacin. After homogenization and extraction of PGE<sub>2</sub> with methanol, samples were passed through activated Amprep C18 reverse-phase columns. 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's instructions (Amersham Pharmacia Biotech, Piscataway, NJ).

**Apoptosis.** Apoptosis was measured in formalin-fixed tissues by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay using Apop tag peroxidase *in situ* apoptosis detection kit, according to the manufacturer's directions (Intergen Company, Purchase, NY). Briefly, 5-μm sections were cut from paraffin-embedded tissues and placed on Superfrost plus® slides. The slides were deparaffinized and hydrated through xylene and graded alcohol. The sections were immersed 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 section. Cells undergoing apoptosis were visualized with anti-digoxigenin peroxide and chromogen substrate. The average number of stained cells was recorded. The average was determined for five fields counted by two scorers (P. W. S., D. W. K.).

**Immunohistochemistry.** Immunohistochemical analyses of dog tissues were performed as described by Khan *et al.* (11) with some modification. Briefly, 5-μm sections were cut from paraffin-embedded tissues and placed on Superfrost plus® slides. The slides were deparaffinized and hydrated through xylene and graded alcohol. The sections were 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 of the tissues were preblocked in TBS-BB [0.3% triton/0.2% saponin/0.5% blocking agent (NEN Life, Boston, MA) in Tris-buffered saline] and then were incubated in primary antibody rabbit anti-human von Willebrand Factor VIII-related antigen or proliferating nuclear cell antigen (Dako Corporation, Carpinteria, CA) overnight at 4°C for MVD or proliferating cell nuclear antigen, respectively. The antibody was 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 (TSA-indirect; NEN Life Sciences), and visualized with the peroxidase substrate, aminoethylcarbazole (Zymed Laboratories, San Francisco, CA). Slides were counterstained briefly in hematoxylin-1 (Richard-Allan Scientific, Kalamazoo, MI). In control slides, the primary antibody was omitted and

**Table 1** Subject characteristics of 14 dogs with naturally-occurring transitional cell carcinoma of the urinary bladder

Characteristic	Value	No. of dogs
Gender (no. of dogs)	SF <sup>a</sup>	7
	NM	6
	IM	1
Age (yr)	Mean	10.7
	Median	10.5
	Range	8–14
Weight (kg)	Mean	18.0
	Median	12.5
	Range	5–36
Tumor volume (cm <sup>3</sup> )	Mean	18.5
	Median	7.1
	Range	0.42–78.9
Stage <sup>b</sup> (no. of dogs)	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub>	10
	T <sub>2</sub> N <sub>1</sub> M <sub>0</sub>	2
	T <sub>2</sub> N <sub>1</sub> M <sub>1</sub>	2

<sup>a</sup> SF, female spayed; NM, neutered male; IM, intact male.

<sup>b</sup> WHO TNM staging of canine urinary bladder cancer: T<sub>2</sub>, tumor invading the bladder wall with induration; N<sub>0</sub>, no evidence of regional lymph node (RLN) involvement; N<sub>1</sub>, RLN involvement; M<sub>0</sub>, no evidence of distant metastasis; M<sub>1</sub>, distant metastasis (29).

the slides were then incubated with biotinylated goat anti-rabbit IgG (Dako, Carpinteria, CA) at the appropriate dilution to accompany the positive slides. The percentage positive proliferating cells was determined by two scorers (P. W. S., D. W. K.), and the scores were averaged. Tumor angiogenesis was quantified using the “hot spot” method described by Weidner (12). Areas with the highest number of positive Factor VIII staining vessels were identified at lower magnification. The immunoreactive vessels in five different hot spots were counted under higher magnification (×20–40× objectives). Two scorers (P. W. S., D. W. K.) counted each slide, and the scores were averaged.

**Determination of Urine bFGF.** Urine bFGF concentrations were determined according to ELISA validated for dog urine by Allen *et al.* (13) using a commercially available ELISA kit (Quantikine HS; R&D Systems, Minneapolis, MN). The urine bFGF concentration was normalized to the urine creatinine and expressed as nanograms bFGF per gram creatinine.

**Determination of VEGF.** Urine VEGF concentrations were determined using an ELISA kit (Quantikine HS; R&D Systems) as validated and described by Lazarous *et al.* (14) and Gu *et al.* (15), respectively. The protein amino acid sequence of dog VEGF is highly homologous to human VEGF<sup>165</sup> protein (16) used in the kit. Urine VEGF concentrations were normalized to the urine creatinine and expressed as nanograms VEGF per gram 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  $P < 0.05$ . A sample size of 14 dogs was selected so that there was a high probability (at least 95%) of detecting antitumor activity in at least 1 dog if the true remission rate were 20% or greater. Tumor response (remission versus stable and/or progressive disease, or actual change in the tumor volume) with piroxicam/cisplatin treatment was compared with respect to cox-2 expression, PGE<sub>2</sub> concentration, MVD, proliferative index, and apoptotic

**Table 2** Toxicity associated with piroxicam/cisplatin treatment in dogs with TCC of the urinary bladder and reason for discontinuing therapy

Toxicity	No. of dogs
Renal toxicity <sup>a</sup>	
None	3
Mild	1
Moderate	9
Severe	1
GI toxicity <sup>b</sup>	
None	2
Mild	3
Moderate	2
Severe	7
Reason piroxicam/cisplatin discontinued	
Tumor progression	1
Renal toxicity	6
Renal toxicity and tumor progression	4
GI toxicity	2
Renal and GI toxicity	1

<sup>a</sup> Renal toxicity was categorized as: mild, blood urea nitrogen (BUN) 33–40 mg/dl, creatinine 1.6–2.0 mg/dl; moderate, BUN 41–70 mg/dl, creatinine 2.1–3.5 mg/dl; severe, BUN >70 mg/dl, creatinine >3.5 mg/dl.

<sup>b</sup> Gastrointestinal (GI) toxicity was categorized as: mild, <1 day anorexia, 1–2 episodes vomiting, no melena, 1–2 episodes of diarrhea, and no supportive care needed; moderate, 2–3 days anorexia, >2 episodes vomiting, melena, >2 episodes diarrhea, supportive care needed; or severe, >3 days anorexia, uncontrolled vomiting, melena, uncontrolled diarrhea, supportive care and hospitalization needed.

index. Categorical variables were compared using a Fisher’s exact test or  $\chi^2$  analysis. Continuous variables were compared using Wilcoxon two-sample test. Pearson correlation coefficients were computed to test for an association between pretreatment 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 induction of apoptosis with piroxicam/cisplatin treatment and change in the following variables: cox-2 expression, PGE<sub>2</sub> concentration, MVD, bFGF concentration, and VEGF concentration.

## Results

### Antitumor Activity and Toxicity of Piroxicam/Cisplatin.

Subject characteristics are summarized in Table 1. Fourteen privately owned pet dogs were enrolled in this study. Dogs had no other major co-morbid diseases. Two dogs received three doses of cisplatin, five dogs received two doses of cisplatin, and seven dogs received one dose of cisplatin. Two dogs were not evaluated for piroxicam/cisplatin response because of early withdrawal from the protocol caused by toxicity. The toxicity associated with piroxicam/cisplatin is summarized in Table 2. Tumor response, apoptotic index, proliferative index, and bFGF and VEGF concentrations are summarized in Table 3.

**Survival.** The median survival for 14 dogs was 329 days (range, 97–1000 days), with one dog still alive at 973 days. Seven dogs survived more than 1 year.

**Induction of Apoptosis.** The apoptotic index was determined before and after piroxicam/cisplatin treatment in 12 dogs. Previous studies and reports in the literature have

Table 3 Tumor volume (TV), tumor response (TR), and change of proliferative index (PCNA), apoptotic index, bFGF (ng/g creatinine), and VEGF (ng/g creatinine) before (0) and after (1) piroxicam/cisplatin treatment

Case no.	TV (cm <sup>3</sup> )	PCNA-0	PCNA-1	Apoptotic index-0	Apoptotic index-1	bFGF-0	bFGF-1	VEGF-0	VEGF-1	TR
1	13.5	2.0	8.0	0.5	5.0	4.3	NA <sup>a</sup>	2996.0	NA	PR
2	7.1	6.0	1.0	2.0	50.0	15.9	10.3	1251.9	694.5	PR
3	15.8	1.0	5.0	0.5	7.0	7.3	11.6	2010.1	2005.8	PR
4	2.9	16.0	5.0	5.0	40.0	NA	NA	NA	NA	PD
5	78.9	1.0	1.0	0.5	1.3	4.7	0.2	1239.5	885.4	PR
6	65.7	NA	NA	1.0	2.0	0.1	0.5	2036.0	2998.0	StD
7	14.9	9.0	13.0	1.0	5.3	1.1	7.8	1040.3	2660.7	PD
8	0.4	70.0	1.0	1.0	1.0	4.4	4.0	1062.4	1448.0	PR
9	4.3	10.0	NA	NA	1.5	NA	NA	NA	NA	NA <sup>b</sup>
10	3.6	30.0	1.0	0.5	1.0	14.4	5.0	1870.0	594.4	PR
11	1.7	5.0	5.0	0.5	1.5	2.6	8.0	858.8	1046.2	PD
12	5.8	15.0	1.0	0.5	1.0	NA	NA	NA	NA	SD
13	26.9	8.0	15.0	0.5	20.0	4.3	NA	2118.2	NA	PD
14	0.5	18.0	NA	0.1	NA	3.0	NA	1078.5	NA	NA <sup>b</sup>

<sup>a</sup> NA, not available; PR, partial remission ( $\geq 50\%$  decrease in TV and no new lesions; StD, stable disease ( $< 50\%$  change in TV and no new lesions); PD, progressive disease ( $\geq 50\%$  increase in TV or development of new tumor lesions).

<sup>b</sup> Tumor response not determined because of toxicity and early withdrawal in 2 dogs.

indicated that a meaningful change in apoptotic index is a doubling of the index (17). Therefore, we categorized change in the apoptotic index based on at least a doubling of the index. The apoptotic index doubled in 11 of the 12 dogs (Table 3). In 3 of these 12 dogs, the apoptotic index increased 10-fold with treatment. There was no association between change in apoptotic index and reduction in tumor size.

**Proliferative Index.** Immunostaining for proliferating cell nuclear antigen was performed on 11 tumor samples pre- and post-piroxicam/cisplatin therapy. The mean proliferative indices (percentage positive-staining tumor cells,  $n = 11$ ) were  $14.8 \pm 20\%$  (range, 1–70%) and  $5.1\% \pm 5\%$  (range, 1–15%) before and after piroxicam/cisplatin treatment, respectively. When assessing change in individual cases, however, the proliferative index decreased in five dogs, increased in four dogs, and did not change in two dogs (Table 3). No significant relationship between proliferative index and change in tumor volume was noted.

**PGE<sub>2</sub> Concentration.** PGE<sub>2</sub> concentration in tumor tissues was measured before and after piroxicam/cisplatin therapy in 12 dogs. The mean concentration of PGE<sub>2</sub> before treatment was  $780 \pm 558$  ng/g tissue (range, 125–1624 ng/g tissue). For comparison, the mean PGE<sub>2</sub> concentration in 10 samples of normal bladder mucosa previously measured in our laboratory was  $46 \pm 32$  ng/g tissue (18). After piroxicam/cisplatin treatment, the concentration of PGE<sub>2</sub> decreased (by  $\geq 20\%$ ) in six dogs (average decrease of 78%) and increased ( $\geq 20\%$  increase) in six dogs. There was no significant association between the change in PGE<sub>2</sub> concentration and tumor response to piroxicam/cisplatin treatment.

**MVD.** MVD was measured in the tumor tissue in nine dogs before and after treatment, and a mean of  $63.9 \pm 41.5$  and  $92.4 \pm 48.9$  microvessels in “hot spots” per high power field were counted before and after treatment, respectively. There was no association between initial MVD or change in MVD and tumor response to therapy.

**Urine bFGF Concentration.** The mean concentration of bFGF in urine before piroxicam/cisplatin treatment was  $6.3 \pm$

$5.9$  ng/g creatinine ( $n = 8$ ), significantly higher ( $P < 0.05$ ) than that of eight normal dogs ( $0.31 + 0.61$  ng/g creatinine). Urine bFGF concentration decreased with piroxicam/cisplatin treatment in four (50%) of eight dogs. Tumors in these four dogs with decreasing urine bFGF concentration decreased in tumor volume by 59–95%. Urine bFGF concentration increased in four dogs, and the tumors increased in size in two of these four dogs (Table 3). There was no significant association between change in urine bFGF concentration and change in tumor size in eight dogs.

**Urine VEGF Concentration.** VEGF concentrations were analyzed in eight dogs. The mean concentration of VEGF in urine before piroxicam/cisplatin treatment was  $1421.1 \pm 474.8$  ng/g creatinine (mean urine VEGF in 11 normal dogs was  $494.32 \pm 233.95$  ng/g creatinine). After piroxicam/cisplatin treatment, VEGF concentration decreased (0.2–68% decrease) in four of eight dogs and increased in four of eight dogs (Table 3). The three dogs with decreased VEGF had decrease in tumor size. There was no significant association between change in VEGF concentration and change in tumor size in eight dogs.

## Discussion

Piroxicam/cisplatin had marked antitumor activity in canine invasive TCC, a disease that closely mimics human invasive bladder cancer, and is usually resistant to chemotherapy (19). Unfortunately, renal toxicity remains a concern even with more prolonged diuresis. Currently, there are no published *in vivo* studies of the results of combination therapy using cisplatin and cox inhibitors in human bladder cancer patients, but such studies are being considered. Several approaches have been used to lessen cisplatin-induced renal toxicity in humans including Amifostine, sodium thiosulfate, and Mesna (20). These may be evaluated in the future in canine studies. A correlation has been found between the length of diureses and cisplatin nephrotoxicity in dogs (21). Therefore, an initial approach in our study was to increase the length of saline diuresis. Unfortunately, renal toxicity was

still frequent and was dose limiting. Piroxicam/cisplatin was withdrawn in seven dogs because of toxicity even when the tumor appeared to be responding well to treatment. We acknowledge that a conservative approach was taken in withdrawing piroxicam/cisplatin in the presence of even mild azotemia. This approach was used to minimize the risk of inducing clinically relevant renal dysfunction. It is worth noting, however, that even with withdrawal of therapy in some dogs, the survival times in this study compared favorably with those reported previously (4, 22, 23).

Strategies to prevent renal toxicity of cox inhibitor/cisplatin therapy are crucial. Another approach to lessen renal toxicity is to substitute a selective cox-2 inhibitor for piroxicam. Piroxicam blocks the activity of both cox-1 and cox-2. The renal toxicity observed was most likely caused by the direct tubular damage by cisplatin and impaired renal blood flow attributable to cox-1 inhibition in the kidney (11). A nonselective cox inhibitor (piroxicam) was used in our study because, at that time, a cox-2 inhibitor was not available for use in dogs. Celebrex and Vioxx do not consistently yield appropriate drug concentrations *in vivo*. A cox-2 inhibitor, Deracoxib, has been recently approved for use in dogs. Until studies with cox-2 inhibitors are complete, it will not be known whether cox-2 inhibitor/cisplatin will be devoid of renal toxicity. Although cox-1 predominates in the renal vasculature, cox-2 is present in the maculae densa and thick ascending limb in dogs, and in glomerular podocytes and small renal blood vessels in humans (11). Studies of cox-2 inhibitors combined with cisplatin are clearly warranted. Another approach under investigation is to combine piroxicam with low-dose chemotherapy (metronomic approach).

Another part of this pilot study was to study cellular processes that could be involved in the mechanisms of antitumor activity of piroxicam/cisplatin. The mechanisms by which piroxicam and other cox inhibitors exert antitumor activity are not well understood but are thought to involve the reduction of cox products such as PGE<sub>2</sub>. PGE<sub>2</sub> has been implicated in tumor cell resistance to apoptosis, enhanced proliferation, and induction of angiogenesis (24). In this study, the apoptotic index doubled with piroxicam/cisplatin treatment in tissues of 11 of the 12 dogs evaluated. Two dogs were withdrawn from the study early because of toxicity, and posttreatment samples were not collected. Induction of apoptosis by piroxicam given as a single agent has also been reported and correlated with tumor remission (7). It is not known why change in apoptosis did not also correlate with tumor response to piroxicam/cisplatin. Cisplatin cell killing depends on apoptotic death after DNA damage, and the failure of cells to undergo apoptosis is a major mechanism of cisplatin resistance (25). The finding that the apoptotic index decreased, even in dogs that failed to respond to piroxicam/cisplatin treatment, suggests that other mechanisms of cisplatin resistance were involved. Other mechanisms of cisplatin resistance include a decrease in drug accumulation in the cell, enhanced DNA repair, and increase in cellular glutathione (26).

Piroxicam/cisplatin therapy resulted in more than an 80% decrease in proliferative index in four dogs and correlated

with reduction in tumor volume in these cases. However, there was not a consistent relationship between change in proliferative index and tumor response. Some tumors were fairly large, and, although attempts were made to obtain biopsies from a similar location during each cystoscopy, this was difficult with gross change in tumor shape and size with treatment. It is also likely that tumor heterogeneity was present, and this could have prevented detection of changes with treatment. A study of a larger number of dogs is needed to further define the relationship between change in proliferative index and tumor response.

Accumulating experimental evidence demonstrates that cancer growth and lethality is dependent on angiogenesis (27). PGE<sub>2</sub> is one of several factors that induce angiogenesis. It was postulated that reducing PGE<sub>2</sub> with piroxicam treatment would have an antiangiogenic effect. We found no association between initial MVD or change in MVD and response to therapy. This lack of association, however, does not exclude the possibility that piroxicam/cisplatin had an antiangiogenic effect. It has recently been recognized that MVD may not be an accurate measure of drug effects on angiogenesis (28). Measurement of angiogenic factors in urine is thought to be more meaningful. Measurement of angiogenic factors such as bFGF and VEGF is not only valuable in measuring angiogenesis, but may be studied in the future as a possible noninvasive predictor of response to therapy. Urine bFGF and VEGF concentrations before therapy were significantly higher than urine bFGF and VEGF concentrations in normal dogs. Piroxicam/cisplatin therapy resulted in a decrease in both factors, in dogs whose tumors were shrinking. This was not a statistically significant association, most likely because of small sample size. Further study is needed to determine whether the reduction in bFGF and VEGF concentration is a reflection of simply a change in tumor mass (and the number of viable cells remaining to produce bFGF and VEGF) or whether piroxicam causes a change in bFGF concentration independent of tumor size.

It was expected that PGE<sub>2</sub> concentrations would decrease in all dogs with piroxicam treatment. PGE<sub>2</sub> concentration however, decreased in only 6 of 12 dogs. To date, only two isoenzymes of cox (cox-1 and cox-2) have been identified, and piroxicam blocks both of these. Pet owners reported giving piroxicam as directed. It is recognized that a great heterogeneity of cox expression exist in the same TCC specimens. It is possible that heterogeneity of PGE<sub>2</sub> production precluded detection of falling PGE<sub>2</sub> concentration overall. Heterogeneity in drug delivery throughout the tumor is also possible.

In conclusion, piroxicam/cisplatin induced remission in dogs with invasive TCC of the urinary bladder, but renal toxicity was frequent and dose-limiting. Biological changes observed during treatment included induction of apoptosis and, in some cases, inhibition of proliferation and reduction in urine angiogenic factors. Additional studies are needed to define the specific cellular and molecular processes involved in the antitumor activity and to address strategies to prevent the renal toxicity of this treatment.

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