Molecular Imaging of Cyclooxygenase-2 in Canine Transitional Cell Carcinomas In Vitro and In Vivo

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Abstract

The enzyme COX-2 is induced at high levels in tumors but not in surrounding normal tissues, which makes it an attractive target for molecular imaging of cancer. We evaluated the ability of novel optical imaging agent, fluorocoxib A to detect urinary bladder canine transitional cell carcinomas (K9TCC). Here, we show that fluorocoxib A uptake overlapped with COX-2 expression in primary K9TCC cells in vitro. Using subcutaneously implanted primary K9TCC in athymic mice, we show specific uptake of fluorocoxib A by COX-2–expressing K9TCC xenograft tumors in vivo. Fluorocoxib A uptake by COX-2–expressing xenograft tumors was blocked by 70% (P < 0.005) when pretreated with the COX-2 selective inhibitor, celecoxib (10 mg/kg), 4 hours before intravenous administration of fluorocoxib A (1 mg/kg). Fluorocoxib A was taken up by COX-2–expressing tumors but not by COX-2–negative human UMUC-3 xenograft tumors. UMUC-3 xenograft tumors with no expression of COX-2 showed no uptake of fluorocoxib A. In addition, fluorocoxib A was evaluated in five dogs diagnosed with TCC. Fluorocoxib A specifically detected COX-2–expressing K9TCC during cystoscopy in vivo but was not detected in normal urothelium. Taken together, our findings show that fluorocoxib A selectively bound to COX-2–expressing primary K9TCC cells in vitro, COX-2–expressing K9TCC xenografts tumors in nude mice, and heterogeneous canine TCC during cystoscopy in vivo. Spontaneous cancers in companion animals offer a unique translational model for evaluation of novel imaging and therapeutic agents using primary cancer cells in vitro and in heterogeneous cancers in vivo.

Introduction

Bladder cancer is the fourth most common cancer in men and the eighth most common malignancy in women in the United States according to the American Cancer Society (1). In general, bladder cancer is curable when detected and treated early; therefore, a more accurate early diagnosis of bladder cancer would be advantageous. Bladder cancer in dogs is the most common tumor of the urinary tract and comprises about 1% of all canine cancers (2). Canine transitional cell carcinoma (K9TCC) closely resembles human invasive urinary bladder cancer (3). Urinary bladder tumors in dogs are usually malignant with only 3% of tumors being benign.

Sensitivity and specificity of conventional cystoscopy of bladder tumors can be significantly improved by using tumor-specific agents (4). In contrast to COX-1, COX-2 is not expressed in most normal tissues but rather is induced in inflamed tissues and in many carcinomas (5–11). COX-2 expression significantly increases with human urinary bladder tumor stage, comparing muscle-invasive tumors with superficially invasive tumors (12, 13). COX-2 is undetectable in human normal urinary bladder (0/10), but COX-2 is expressed in 29 of 29 of bladder squamous cell carcinomas (SCC) and in 12 of 35 (34%) of TCC (12, 14). Genetically modified Cox-2−/− mice exhibit decreased incidence of intestinal and skin tumors (15, 16). Numerous pharmacologic studies validate COX-2 as a therapeutic target to control the inflammation and tumorigenesis (5, 17, 18). Thus, newly synthesized nonsteroidal anti-inflammatory drugs (NSAID) are attractive not only as pharmacologic and chemopreventive agents but also as a new family of imaging agents (19, 20).

The use of the COX-2–selective inhibitors as a new class of imaging agents is based on the selective uptake by COX-2–expressing neoplastic lesions. The first synthesis and characterization of radioactively labeled COXIBs as cancer imaging agents have been reported (21, 22).

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As we published previously, promising results were obtained using a derivative of celecoxib as a single-photon emission computed tomography (SPECT) radiotracer to identify Cox-2–expressing, carcinogen-induced lung and pancreatic lesions in hamsters (22). Additional novel positron emission tomography (PET) and SPECT imaging radiotracers were later synthesized and evaluated in cancers and inflammation rodent models (23, 24). Novel synthesized derivatives of NSAIDs labeled with rhodamine dyes have been evaluated as optical imaging agents for detection of COX-2–expressing inflammation and cancer in rodents (25, 26). One of the most promising optical imaging agent that selectively binds to COX-2 called fluorocoxib A, \( \text{N}-(5\text{-carboxyl-X-rhodaminyloxy})\text{but-4-yl[2-1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]acetamide} \) (ref. 25). Fluorocoxib A is a weak inhibitor of purified ovine COX-1 and a strong inhibitor of purified human COX-2 enzyme (\( \text{IC}_{50} = 25 \mu\text{mol/L} \), \( \text{IC}_{50} = 0.7 \mu\text{mol/L} \), respectively). Fluorocoxib A inhibits lipopolysaccharide- and IFN-\( \gamma \)-induced Cox-2 activity in murine macrophage-like cells RAW 264.7 (\( \text{IC}_{50} = 0.31 \mu\text{mol/L} \); refs. 25, 26). Fluorocoxib A was evaluated using carrageenan-induced acute inflammation in the mouse footpad, human tumor xenografts in nude mice, and in mice with spontaneous tumors (25) in vivo. Human colorectal cancer HCT-116 xenografts that do not express COX-2, exhibited minimal fluorescence, whereas the human SCC 1483 xenografts with high levels of COX-2 expression exhibited bright fluorescence after fluorocoxib A administration (25). Nude mice with 1483 xenografts pretreated with indomethacin [2 mg/kg, intraperitoneally (i.p.)] 2 hours before fluorocoxib A administration (2 mg/kg, i.p.) show 92% ± 6% (n = 8) reduction of the fluorocoxib A uptake in these tumors (25). In addition, the COX-2 nontargeted fluorophores, such as 5-ROX and 6-ROX alone, did not accumulate in COX-2–expressing tumor xenografts, supporting specificity of fluorocoxib A uptake in these tumors (25). In addition, the COX-2 nontargeted fluorophores, such as 5-ROX and 6-ROX alone, did not accumulate in COX-2–expressing tumor xenografts, supporting specificity of fluorocoxib A uptake in COX-2–expressing tumors in vivo. We also confirmed specific fluorocoxib A uptake in COX-2–expressing colorectal adenocarcinomas in dogs during endoscopy in vivo (27).

Often, usage of rodent models for testing of new imaging tracers or therapeutic drugs fails in bench-to-bedside translation. To fill this gap, we evaluated fluorocoxib A for the detection of TCCs, using not only a rodent model with K9TCC xenografts but also dogs with naturally occurring urinary bladder carcinomas during cystoscopy in vivo. In this study, we confirmed selective fluorocoxib A uptake by COX-2–expressing primary K9TCC cells, COX-2–expressing K9TCC xenografts tumors in nude mice, and K9TCC during cystoscopy in dogs.

Material and Methods
Fluorocoxib A was synthesized according to the previously described method (25, 26). The chemical structure of fluorocoxib A is shown in Fig. 1.

Antibodies and other reagents
Antibody for COX-2 were obtained from Cayman Chemical Corporation; E-cadherin and actin were purchased from Santa Cruz Biotechnology; F4/80 was obtained from Abcam; phosphorylated p-65 (p–p65) was obtained from BD Biosciences; and lysozyme and cytokeratin were purchased from Dako.

Human cell lines
Human transitional cell carcinomas T-24 and UMUC-3 were purchased from American Type Culture Collection in 2010. Human T-24 cells were maintained in McCoy’s media, and human UMUC-3 cells were maintained in EMEM media, respectively, supplemented with 10% FBS, 100 IU penicillin, and 100 \( \mu\text{g/mL} \) streptomycin and grown in an atmosphere of 5% \( \text{CO}_2 \) at 37°C. Cell lines were authenticated via short tandem repeat (STR) DNA profiling by Genetica DNA Laboratories just prior manuscript submission.

Isolation of primary K9TCC cells
K9TCC primary cells were isolated from tumor biopsies from client-owned dogs with TCCs during cystoscopy procedures according to approved the University of Tennessee (Knoxville, TN) Institutional Animal Care and Use Committee (IUT IACUC) protocol. The biopsies confirmed by veterinary pathologists were trypsinized and the isolated cells were cultured in RPMI-1640 media supplemented with 10% FBS and antibiotics for 24 hours. Colonies of K9TCC cells were further expanded and after reaching 80% confluence, the K9TCC cells were subcultured and early passages of cells were used for experiments. Primary K9TCC#1 Lillie cells were obtained in 2009; K9TCC#2 Dakota cells were obtained in 2011, and K9TCC#3 Buffy cells were obtained in 2012. Cell lines were tested to prove the epithelial cell origin by E-cadherin and cytokeratin expressions in vitro and tested for tumorigenic behavior of cells using xenograft mouse model in vivo.

COX-2 immunofluorescence in K9TCC
Primary K9TCC cells were plated on 4-chamber slides (Nalge Nunc) and grown for 24 hours. Then the cells were treated with 1 \( \mu\text{mol/L} \) fluorocoxib A for 1 hour, washed, and fixed in 4% paraformaldehyde. The nonspecific binding of
the antibodies was blocked for 30 minutes at room temperature. The cells were incubated with COX-2 primary antibodies overnight at 4°C and incubated with secondary anti-rabbit AlexaFluor 488nm antibodies for 1 hour at room temperature in darkness. The nuclei of cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue color). Slides were evaluated by fluorescence confocal microscopy.

Animals

All animal studies were conducted in accordance with the UT IACUC–approved protocols and in accordance with the NIH guidelines. Four- to 5-week-old female athymic nude Foxn1nu mice were purchased from Harlan Laboratories and Taconic Laboratories. Enrolled client-owned female dogs with bladder TCC with weights of 10 to 20 kg were intravenously injected with fluorocoxib A (1 mg/kg) over 20 minutes and then after 18 to 24 hours imaged during cystoscopy using Karl Storz imaging system.

Fluorocoxib A uptake by K9TCC and UMUC-3 xenograft tumors detected by IVIS Lumina system in vivo

Two primary K9TCC cancer cell lines with confirmed COX-2 expression and human UMUC-3 lacking expression of COX-2 were subcutaneously implanted in female athymic mice. All tested mice developed subcutaneous tumors with a size of approximately 0.8 to 1.5 cm³ within 3 weeks after implantation with K9TCC (1.5 × 10⁶ mixed 1:1 with Matrigel) and UMUC-3 (3 × 10⁶ mixed 1:1 with Matrigel).

To evaluate the ability of fluorocoxib A to target COX-2 in K9TCC#1Lillie xenografts tumors, mice were injected with fluorocoxib A (1 mg/kg, i.v.) and then up to 24 hours imaged using Xenogen IVIS Lumina with DsRed filters with excitation 500 to 550 nm and emission 575 to 650 nm and background 460 to 490 nm. Obtained total flux (p/s) and averaged radiance (p/s/cm²/sr) of labeled regions of interest (ROI) of selected tissues were analyzed.

To prove the specificity of fluorocoxib A binding to COX-2–expressing TCCs in vivo, we carried out 2 additional experiments using COX-2–expressing K9TCC#2Dakota in xenograft mice pretreated with selective COX-2 inhibitor—celecoxib (10 mg/kg) 4 hours before injecting with fluorocoxib A (1 mg/kg). In addition, we used UMUC-3 human TCCs with no COX-2 expression. The xenograft tumors were imaged up to 24 hours postinjection with fluorocoxib A using IVIS system. The normalized averaged radiance of each tissue to blood was evaluated and compared between groups. After imaging, tissues were formalin-fixed and paraffin-embedded for histologic analysis. Histology and expression of COX-2 and E-cadherin in K9TCC#2Dakota and UMUC-3 xenograft tumors were assessed by immunohistochemical (IHC) analysis.

Fluorocoxib A uptake by heterogeneous K9TCC during cystoscopy

All cystoscopy and imaging procedures in dogs were conducted by a board-certified veterinary internal medicine specialist (J.W. Bartges) in accordance with standard

Figure 2. COX-2 expression in K9TCC in vitro. A, representative images of COX-2 in K9TCC by IHC (brown color). Inset image show no detection of COX-2 in normal canine urothelium. B, expression of E-cadherin confirms the epithelial cell origin of K9TCC expressing COX-2. (C and D) COX-2, (E) F4/80, (F) p-p65, and (G) lysozyme expressions in macrophages of the submucosa layer of canine bladder inflammation.
veterinary care and the UT IACUC-approved protocol as described previously (27). Five client-owned female dogs with bladder cancer lesions were enrolled in our study. The owners signed a consent form to agree enrolling their pets to this study to evaluate fluorocoxib A uptake in TCCs during cystoscopy. Fluorocoxib A was administrated i.v. 1 mg/kg over 20 minutes using a catheter, followed by up to 24-hour uptake by COX-2–expressing bladder tumor cells. The concentration of fluorocoxib A (1 mg/kg) was determined on the basis of results from our pharmacokinetic and safety studies (27). Cystoscopy was used to evaluate the bladder cancer lesion, specificity of fluorocoxib A uptake to obtain biopsy samples for diagnosis. The client-owned dogs were returned to owners after complete recovery from the cystoscopic examination and anesthesia.

**Immunohistochemistry**

Xenograft tumors from athymic mice and primary tumor biopsies from dogs with TCCs were formalin-fixed paraffin-embedded and sectioned at 7 μm. After deparaffinization and rehydration, the sections were stained for COX-2 (1:500 overnight at 4°C), E-cadherin (1:500 overnight at 4°C), cytokeratin (1:800 overnight at 4°C), p-p65 (1:1,000 for 1 hour at room temperature), F4/80 (1:500 for 30 minutes at room temperature), or lysozyme (1:400 for 30 minutes at room temperature) using IHC protocol as described previously (27). The images were captured by DP71 camera (Hunt Optics and Imaging) attached to Olympus microscope BX41 or Leica Leitz DMRB with microscopic objectives 4, 20, and 40 using the Cell-Sens Standard imaging software (Hunt Optics and Imaging) and Adobe Photoshop CS5 (Adobe). The stained slides were reviewed by M. Cekanova, and the percentage of COX-2–positive normal and neoplastic epithelial cells was scored as either absent (−), low (+, <20% of positive tumor cells/×40 objective), moderate (++, 20%–50% positive tumor cells/×40 objective), or high (+++, >50% positive tumor cells/×40 objective).

**Western bloting**

Human and canine TCC cells were cultured in media with or without serum for 24 hours. The cells were lysed, proteins were extracted and used for detection of COX-2 expression following the Western blotting protocol as described previously (27).

**Statistical analysis**

Statistical analyses of obtained data were conducted using the Student t test. Results were considered statistically significant at *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

**Results**

**COX-2 expression in canine bladder lesions**

The expression of COX-2 in bladder lesions was assessed by IHC analysis. We evaluated 16 carcinomas and 8 acute and chronic inflammatory lesions of canine urinary bladder specimens obtained from tissue repository bank at University of Tennessee College of Veterinary Medicine (UT CVM). IHC analysis of COX-2 expression revealed absent (1/16), low (1/16), moderate (4/16), or high (10/16) levels of COX-2–positive K9TCC cells. The representative image of high levels of COX-2–positive cells in K9TCC (brown color) is shown in Fig. 2A. Canine bladder carcinomas showed strong expression of COX-2 in the cytoplasm with perinuclear localization in the tumor cells. The COX-2–positive cells of bladder tumors were predominantly localized at the periphery of K9TCC cancer, whereas the centers of K9TCC.

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**Figure 3.** Fluorocoxib A uptake by COX-2–expressing K9TCC/iLilie cells in vitro. A, DIC image of primary K9TCC/iLilie cells. B, specific uptake of fluorocoxib A (1 μmol/L; 1 hour) by K9TCC/iLilie (red color; C) with COX-2 expression detected by Alexa Fluor488 nm (green color; D) and counterstained with DAPI for nuclei detection (blue color). E, merged (B and C) images to confirm overlap of fluorocoxib A uptake with COX-2 expression. F, merged (B–D) images.
tumors contained mostly COX-2–negative cells. The normal urothelium did not show any expression of COX-2 (inset in Fig. 2A). The expression of E-cadherin (brown color) is shown in Fig. 2B to confirm the epithelial cell origin of tested K9TCC. K9TCC showed reduced E-cadherin expression as compared with normal urothelium (inset in Fig. 2B) confirming one of the characteristic of invasive adenocarcinomas.

We evaluated the expression of COX-2 in the biopsies from dogs with acute and chronic inflammation (n = 8).
The histology of these tissues revealed mild edema and moderate multifocal inflammatory cell infiltrates that were predominantly composed of perivascular plasma cells, eosinophils, lymphocytes, neutrophils, and macrophages. No COX-2 expression was detected in submucosa and urothelium with neutrophilic and lymphocytic cells inflammation (Supplementary Fig. S1). Only the infiltrating perivascular macrophages in the submucosa showed positive COX-2 expression (brown color) as shown in Fig. 2C and D. We conducted additional IHC for detection of F4/80 (macrophage marker), p-p65 (active p-p65, inflammatory marker), and lysozyme (macrophage marker) expressions to confirm the presence of inflammatory macrophages in the submucosa as shown in Fig. 2E–G.

**Fluorocoxib A uptake by K9TCC cells in vitro**

To confirm the reactivity of fluorocoxib A to canine COX-2, we isolated K9TCC primary cancer cells from biopsies obtained during cystoscopy procedures. The primary K9TCC cells (K9TCC#1Lillie Fig. 3A, bright field using differential interference contrast microscopy, DIC) were incubated with 1 μmol/L fluorocoxib A for 1 hour and stained with COX-2–specific antibodies labeled with Alexa Fluor 488 dye. As shown in Fig. 3B, fluorocoxib A (red color) penetrated into the cytoplasm of K9TCC and specifically bound to COX-2 (green color) located at perinuclear areas as shown in Fig. 3C. The nuclei of K9TCC were counterstained with DAPI staining (blue color) as shown in Fig. 3D. Colocalization of COX-2 and fluorocoxib A uptake was confirmed by overlaying the images as shown in Fig. 3E (yellow color). The overlaid image of fluorocoxib A, COX-2, and DAPI is shown in Fig. 3F.

**Fluorocoxib A uptake by K9TCC xenograft tumors in vivo**

Canine TCC#1Lillie cells were isolated from tumor with confirmed COX-2, E-cadherin, and cytokeratin expressions as shown in Fig. 4A by immunohistochemistry. The isolated K9TCC#1Lillie cells were subcutaneously implanted in female nude mice. The body weights of mice bearing the tumors were shown in Fig. 4B, a. Tested nude mice (n = 5) developed subcutaneous tumors with 100% incidence with a size of approximately 1 to 1.8 cm³ within 3 weeks after injection with K9TCC (1.5 × 10⁶ with Matrigel/mouse) as shown in Fig. 4B, b. To evaluate the ability of fluorocoxib A to target COX-2 in K9TCC#1Lillie xenograft tumors, 4 mice were injected with fluorocoxib A (1 mg/kg, i.v.) and after up to 24 hours imaged using IVIS Lumina System. K9TCC#1Lillie xenograft tumors exhibited bright fluorescence signal (intensive red yellow color in ROI green circle) as shown in Fig. 4C, a. Specific and selective uptake of fluorocoxib A by K9TCC#1Lillie xenograft tumors were confirmed by comparing the total flux of dissected tissues (in blue circles ROI) such as lung, liver, kidney, spleen, brown fat, heart, muscle, pancreas, blood, and background (ROI in yellow circle) ex vivo as shown in Fig. 4C, b. The averaged radiance (p/s/cm²/sr) of each tissue was normalized to blood as shown in Fig. 4D. K9TCC#1Lillie xenograft tumors showed 3.5-fold increased fluorocoxib A uptake as compared with blood. Specific uptake of fluorocoxib A in K9TCC#1Lillie tumor was also confirmed by Karl Storz PDD imaging system ex vivo. Fluorocoxib A was detected in xenograft tumors as shown in top image of Fig. 4E, a, with pink color and no detection of fluorocoxib A uptake was detected in liver as shown in the bottom image of Fig. 4E, b. To confirm specific uptake of fluorocoxib A by COX-2–expressing K9TCC#1Lillie, we conducted histologic evaluation of dissected K9TCC#1Lillie xenograft tumors for COX-2 expression. Histologic analysis of dissected K9TCC#1Lillie xenograft tumors stained with hematoxylin and eosin revealed that tumors were encapsulated with partial central necrosis and with moderate number of mitotic figs. K9TCC#1Lillie cells showed positive perinuclear COX-2 expression (brown color) as shown in Fig. 4F, a. In addition, infiltrated macrophages in stroma expressed COX-2 enzyme (brown color). Membrane E-cadherin expression (brown color) in K9TCC#1Lillie xenograft tumors confirmed the epithelial cell origin of K9TCC#1Lillie as shown in Fig. 4F, b.

**Specificity of fluorocoxib A uptake by COX-2–expressing TCC xenograft tumors in vivo**

To prove specific uptake of fluorocoxib A by COX-2–expressing tumors in vivo, we used K9TCC#2Dakota xenograft tumor mice that were pretreated with the selective COX-2 inhibitor, celecoxib (10 mg/kg, i.v.) 4 hours before intravenous administration of fluorocoxib A (1 mg/kg). In addition, we used UMUC-3 human TCC cells that have no expression of COX-2 as shown in Fig. 5.
K9TCC#2Dakota xenograft tumors expressing COX-2 had strong specific uptake of fluorocoxib A as compared with other organs as shown in Fig. 5A, a. Fluorocoxib A uptake by K9TCC#2Dakota xenograft tumors was significantly reduced by approximately 70% (P < 0.005) when mice (n = 3/group) were pretreated with celecoxib (4 hours before injecting with fluorocoxib A as shown in Fig. 5A, b and 5B). Furthermore, UMIC-3 xenograft tumors showed no uptake of fluorocoxib A due to lack of COX-2 expression as shown in Fig. 5A, c and 5B. K9TCC#2Dakota xenograft tumors had positive perinuclear COX-2 expression in cells by IHC (brown color) as shown in Fig. 5C, a. Positive E-cadherin expression confirmed epithelial cell origin of TCC as shown in Fig. 5C, b. Primary K9TCC#2Dakota tumor with confirmed COX-2 and E-cadherin expressions by IHC is shown in Supplementary Fig. S2A (a and b). The UMIC-3 xenograft tumors had no expression of COX-2 but had a positive E-cadherin expression as shown in Fig. 5C (c and d). The size of developed xenograft tumors using K9TCC#2Dakota and UMIC-3 in athymic mice over a 3-week period are shown in Supplementary Fig. S2B (a and b). In addition, we observed that UMIC3 tumors of approximately the same size were more vascularized than K9TCC xenograft tumors as shown in enlarged images of xenograft tumors in Supplementary Fig. S2B. The expression of COX-2 was confirmed by Western blot analysis in primary K9TCC#2Dakota but not in UMIC-3 cells as shown in Fig. 5D. Taken together, these data showed that fluorocoxib A uptake was specific for COX-2–expressing tumors and fluorocoxib A was not accumulated in tumors due to increased vascular permeability in tumors.

Detection of COX-2–expressed K9TCC by fluorocoxib A during cystoscopy using Karl Storz imaging system

We evaluated this novel optical agent, fluorocoxib A, in dogs with naturally occurring heterogeneous bladder tumors as a more realistic and suitable translational animal model than rodents for human cancer. Five client-owned female dogs with bladder lesions were enrolled in our study for cystoscopy. Representative images of normal urothelium of canine bladder wall under bright light and fluorescence light during cystoscopy procedure are shown in Fig. 6A (a and b), respectively. No uptake of fluorocoxib A was detected in normal urothelium 24 hours after 1 mg/kg i.v. administration during cystoscopy as shown in Fig. 6A, b. Specific uptake of fluorocoxib A by COX-2–expressing K9TCC#3Buffy was visualized by bright pink color as shown in representative images of heterogeneous K9TCC#3Buffy using bright light (left images Fig. 6B a, c, e, and g) and fluorescence light (right images Fig. 6B b, d, f, h) during cystoscopy. The similar heterogeneous expression profile of COX-2 detected by fluorocoxib A was observed in additional K9TCC cases as shown in the representative images of Fig. 6B.

Confirmed COX-2 expression in K9TCC biopsy samples after fluorocoxib A uptake

Small tissue K9TCC samples were obtained during cystoscopy of dogs with K9TCC imaged using fluorocoxib A. The biopsy samples were divided for histology analysis and for isolation of primary cancer K9TCC cells. COX-2 expression (brown color) was confirmed in fluorocoxib A uptake K9TCC#3Buffy using IHC as shown in Fig. 6C. We successfully isolated primary K9TCCs from obtained biopsy samples from dogs after fluorocoxib A imaging, and we confirmed COX-2 expression in established primary K9TCC#3Buffy in the presence or absence of serum in the culture medium for 24 hours using Western blot analysis as shown in Fig. 6D. Both K9TCC primary cells showed higher levels of COX-2 expression than human T-24 or COX-2–negative UMIC-3 TCC cell lines (Figs. 5D and 6D). Increased expression of COX-2 was detected in K9TCC#2Dakota when cultured in medium containing serum.

Discussion

Because immunodeficient rodent models cannot accurately simulate the in vivo behavior patterns of heterogeneous human tumors, many new imaging agents fail in bench-to-bedside translation. Dogs with naturally occurring tumors provide a large animal model that is more translatable, can better assess the efficacy of the molecular imaging agents to visualize heterogeneous tumors, are more applicable because the dog’s larger body size allows endoscopy/cystoscopy procedures, and are more similar to human cancers with regard to histologic appearance and biologic behavior rather than chemically or genetically induced rodent cancer models (28–30). Usage of primary canine cancer cells isolated from spontaneously occurring tumors is a more suitable model than stable cell lines. In our study, we isolated primary K9TCC to verify the specificity of fluorocoxib A binding to canine COX-2 in vitro and in vivo.

COX-2–positive bladder tumors in dogs show strong expression of COX-2 in cytoplasm with perinuclear localization in the tumor and surrounding stromal cells that mimics the pattern seen in human tumors (2, 29, 31–33). COX-2 expression shows a significantly proportional increase (P < 0.05) with advanced-stage bladder carcinomas (12, 13, 34). The COX-2–positive cells in K9TCC were predominantly localized at the tumor edges supporting the evidence that COX-2 is involved in tumor invasiveness and metastasis (34–36). The epidemiologic studies indicate that the development of the bladder carcinomas is closely associated with chronic inflammation of the urinary tract, but the underlying mechanisms are still unknown (14, 37). COX-2 is markedly expressed in K9TCC, suggesting that chronic inflammation stimulates the production of COX-2 leading to the development of bladder carcinomas. We evaluated the expression of COX-2 in the biopsy samples obtained during cystoscopy in dogs (n = 8) diagnosed with acute and chronic inflammation. We included bladder inflammation to check the COX-2 expression, which might
create false-positive uptake of fluorocoxib A during examination of neoplastic lesions in the bladder. As shown in Supplementary Fig. S1, no COX-2 expression was detected in neutrophilic and lymphocytic inflammation regions. Only the infiltrated perivascular macrophages in the submucosa showed positive COX-2 expression (Fig. 2C and D), suggesting that only the presence of macrophages might give a false-positive signal from fluorocoxib A uptake. However; the limited number of macrophages and the location in the submucosa in contrast to COX-2–expressing tumor cells at the periphery of the tumor might not be sufficient for the fluorescence signal from fluorocoxib A in macrophages to be detected. Additional studies using fluorocoxib A uptake in dogs diagnosed with bladder inflammation are planned for further investigation.

To prove fluorocoxib A selective binding to COX-2–expressing tumors in vivo, we pretreated mice with the COX-2–selective inhibitor, celecoxib (10 mg/kg, i.v.) before fluorocoxib A injection (n = 3). Dissected tissues imaged ex vivo (from the top on left side: tumor, lung, pancreas; from top in middle row: heart, liver, kidney; from the top on right side: fat and blood; background ROI in yellow circle) using IVIS System, B, averaged radiance (p/s/cm²/sr) of each tissue was normalized to blood. The fluorocoxib A uptake by K9TCC#2Dakota xenograft tumors were significantly blocked by 70% by pretreatment with celecoxib. Values represent mean ± SE (n = 3), paired Student t test: P < 0.005. The UMUC-3 xenograft tumors with no COX-2 expression showed no uptake of fluorocoxib A. C, COX-2 (a) and E-cadherin expressions (b, brown color) in K9TCC#2Dakota xenograft tumors and (c and d) UMUC-3 xenograft tumors counterstained with hematoxylin (nuclei, blue color). D, confirmed COX-2 expression in established primary K9TCC#2Dakota and COX-2–negative UMUC-3 TCC cells in presence or absence of serum in culture media for 24 hours using Western blot analysis. Actin was used as loading control.
UMUC3 xenograft tumors. We also observed that UMUC3 tumors were more vascularized than K9TCC xenograft tumors as shown in enlarged images of xenograft tumors in Supplementary Fig. S2B. Taken together, our data confirmed that fluorocoxib A uptake is specific for COX-2–expressing tumors and fluorocoxib A is not accumulated in tumors due to increased vascular permeability in tumors. Our findings are in agreement with a previously published study using human colorectal cancer HCT-116 xenografts with no expression of COX-2, which exhibited minimal uptake of fluorocoxib A, whereas the human head-and-neck SCC 1483 xenografts with high levels of COX-2 expression, exhibited high uptake of fluorocoxib A (25). The tumor-to-normal tissues ratio of fluorocoxib A was up to 5:1 that make this imaging agent highly sensitive and selective for detection of COX-2–expressing tumors.

Tumor detection and characterization of COX-2 expression might be useful for COX-2–targeted treatments to increase the survival of canine (38) and human patients. Numerous studies have provided evidence that inhibition of COX-2 pathways may have significant benefits for cancer treatment and prevention (19, 28, 31, 39–42), particularly in bladder cancer (41, 43–45). Interestingly, patients with COX-2–negative, non–small cell lung cancer (NSCLC) fared worse if treated with celecoxib as adjuvant treatment to carboplatin and gemcitabine than patients given chemotherapy alone (46). None of the currently used imaging agents in clinic have the ability to detect COX-2–expressing tumors.

Although we only evaluated fluorocoxib A in bladder cancer, data obtained from this study can be useful for testing of fluorocoxib A in other types of COX-2–expressing cancers, such as breast, lung, pancreas, head and neck, and prostate carcinomas in dogs and people. In our previously published study, we have shown a specific uptake of fluorocoxib A in canine colorectal adenocarcinomas during colonoscopy in vivo (27). Exploration of this approach in the dog model provides an opportunity to assist with more rapid translation of fluorocoxib A to clinical use in people for detection of COX-2–expressing tumors. Fluorocoxib A may help improve the visualization of COX-2–expressed cancers and help identify appropriate patients for NSAID treatment.
Disclosure of Potential Conflicts of Interest
L.I. Marnett has ownership interest (including patents) in licensed patents. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: M. Cekanova, M.J. Uddin, J.W. Bartges, A. Legendre, L.I. Marnett
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