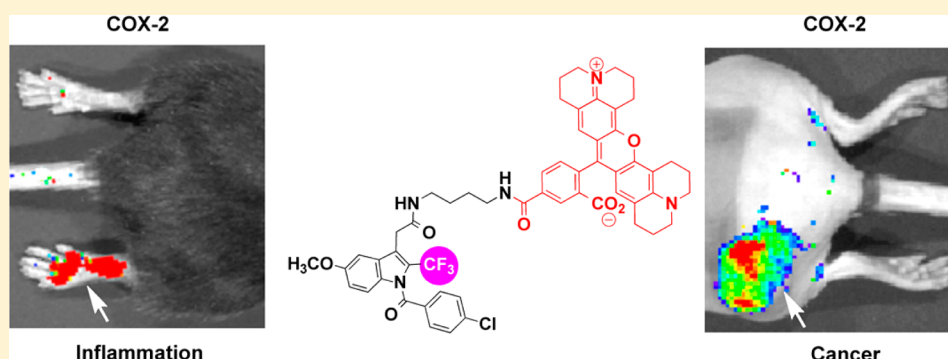


# Trifluoromethyl Fluorocoxib A Detects Cyclooxygenase-2 Expression in Inflammatory Tissues and Human Tumor Xenografts

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## Supporting Information



**ABSTRACT:** Fluorocoxib A is an effective COX-2-targeted optical imaging agent, used for in vivo detection of inflammatory tissues and premalignant and malignant tumors that express elevated levels of COX-2 (Uddin et al. *Cancer Res.* **2010**, 70, 3618–3627). In an effort to discover novel optical probes for COX-2, a trifluoromethyl analogue of fluorocoxib A (CF<sub>3</sub>-fluorocoxib A) was synthesized and evaluated for its ability to inhibit COX-2 in vitro purified enzyme and human cancer cell lines. Kinetic analysis revealed that CF<sub>3</sub>-fluorocoxib A is a slow, tight binding inhibitor of COX-2 that exhibits low nanomolar inhibitory potency. While CF<sub>3</sub>-fluorocoxib A and fluorocoxib A are similar in structure, CF<sub>3</sub>-fluorocoxib A shows improved potency in inhibition of wtCOX-2 and with a series of site-directed COX-2 mutants. After intraperitoneal injection, selective uptake of CF<sub>3</sub>-fluorocoxib A is detected in inflamed mouse paws compared to noninflamed contralateral paws by optical imaging, and uptake is blocked by pretreatment with the COX-2 inhibitor, celecoxib. Selective uptake is also detected in the COX-2-positive human tumor xenografts (1483 HNSCC) as compared with the COX-2-negative tumor xenografts (HCT116) in an in vivo nude mouse tumor model. These in vitro and in vivo studies suggest that binding to COX-2 is the major determinant of uptake of CF<sub>3</sub>-fluorocoxib A into the inflamed tissues and tumor xenografts. Thus, this new COX-2-targeted imaging probe should find utility in the detection and evaluation of COX-2 status in naturally occurring malignancies.

**KEYWORDS:** Cyclooxygenase-2 (COX-2), CF<sub>3</sub>-indomethacin, CF<sub>3</sub>-fluorocoxib A, optical imaging, inflammation, cancer

Cyclooxygenases (COX) catalyze the biotransformation of arachidonic acid into a wide variety of prostaglandins, which are important biological mediators of inflammation.<sup>1</sup> COX-1 is constitutively expressed in most normal tissues, where it performs housekeeping functions, such as maintenance of vascular tone and cytoprotection of the gastric mucosa.<sup>2</sup> COX-2 is an inducible enzyme that is expressed in inflammation, where it modulates edema and pain, and in proliferative diseases, where it promotes growth and enhances metastasis.<sup>3</sup> COX-2 overexpression is an early event in tumorigenesis, and it plays a role in tumor progression.<sup>4</sup> Selective COX-2 inhibitors are useful in the treatment of various cancers.<sup>5,6</sup> Therefore, COX-2 can be used as a target for imaging of inflammation and cancer with fluorescently conjugated COX-2 inhibitors. We recently reported the synthesis and evaluation of fluorocoxib A for the selective

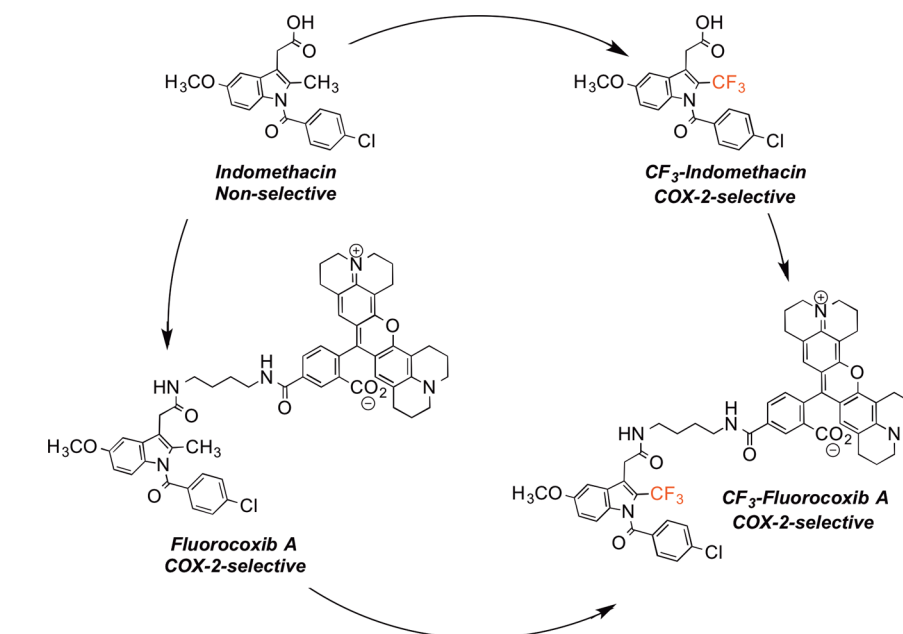
visualization of COX-2 in inflammatory and malignant lesions.<sup>7</sup> Herein, we report the synthesis and evaluation of a trifluoromethyl analogue of fluorocoxib A as a selective COX-2 inhibitor in purified protein and cells. We also describe the enzyme–inhibitor binding kinetics and in vivo delivery of CF<sub>3</sub>-fluorocoxib A to inflammatory tissues and human tumor xenografts (Figure 1).

CF<sub>3</sub>-indomethacin was synthesized using a Fisher indole cyclization of 5,5,5-trifluorolevulinic acid lactone with 1-(4-methoxyphenyl)-1-(4-chlorobenzoyl)hydrazine hydrochloride under acidic conditions, as described in a previous report.<sup>8</sup>

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**Figure 1.** Chemical evolution of COX-2-selective inhibitors from nonsteroidal anti-inflammatory drug indomethacin.

The CF<sub>3</sub>-indomethacin was then coupled with mono *N*-*tert*-butoxycarbonyl-butylendiamine using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 1-hydroxybenzotriazole hydrate, and *N,N*-diisopropylethylamine to afford *t*-butyl 4-[2-{1-(4-chlorobenzoyl)-5-methoxy-2'-trifluoromethyl-1*H*-indol-3-yl}acetamido]butylcarbamate (Scheme 1). Treat-

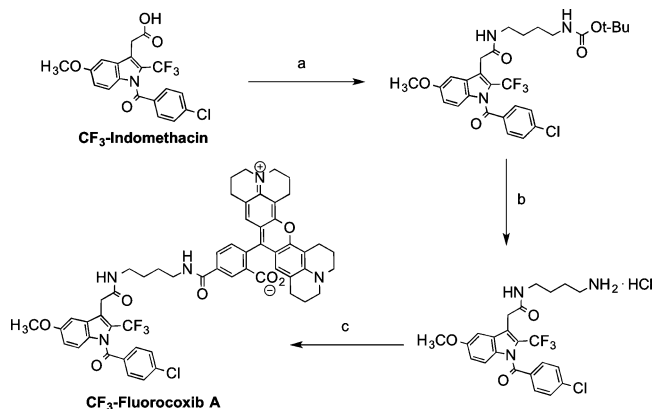
ment of 4-[2-{1-(4-chlorobenzoyl)-5-methoxy-2'-trifluoromethyl-1*H*-indol-3-yl}acetamido]butylcarbamate with HCl (gas) gave *N*-(4-aminobutyl)-2-{1-(4-chlorobenzoyl)-5-methoxy-2'-trifluoromethyl-1*H*-indol-3-yl}acetamide hydrochloride. 5-Carboxy-X-rhodamine *N*-succinimidyl ester (5-ROX NSE)<sup>9</sup> was reacted with the free amine of *N*-(4-aminobutyl)-2-{1-(4-chlorobenzoyl)-5-methoxy-2'-trifluoromethyl-1*H*-indol-3-yl}acetamide hydrochloride in the presence of triethylamine to give *N*-{(5-carboxy-X-rhodaminyl)but-4-yl}-2-{1-(4-chloro-

benzoyl)-5-methoxy-2'-trifluoromethyl-1*H*-indol-3-yl}-acetamide (CF<sub>3</sub>-fluorocoxib A) (Scheme 1). Synthetic procedures and the characterization of all the new compounds are described in the Supporting Information.

CF<sub>3</sub>-fluorocoxib A was assayed against purified COX-2 or COX-1 by a previously reported thin layer chromatography assay.<sup>10</sup> We found that CF<sub>3</sub>-fluorocoxib A is a selective and potent inhibitor of COX-2. The calculated LogP values of CF<sub>3</sub>-fluorocoxib A and fluorocoxib A are 6.96 and 6.34, respectively. The IC<sub>50</sub> value for inhibition of COX-2 is 0.56 μM with no inhibition of COX-1 up to 25 μM. CF<sub>3</sub>-fluorocoxib A was assayed in RAW264.7 murine macrophage-like cells to check for membrane permeability and subsequent COX-2 inhibition.<sup>11</sup> The IC<sub>50</sub> value for inhibition of COX-2 by CF<sub>3</sub>-fluorocoxib A was 0.08 μM. Further, the ability of CF<sub>3</sub>-fluorocoxib A to inhibit COX-2 in 1483 head and neck squamous cell carcinoma (HNSCC) cells was assayed.<sup>7</sup> CF<sub>3</sub>-fluorocoxib A was incubated with 1483 HNSCC cells at several concentrations (0–5 μM) for 30 min followed by the addition of 10 μM [1-<sup>14</sup>C]-arachidonic acid (~55 mCi/mmol). CF<sub>3</sub>-Fluorocoxib A inhibited COX-2 with an IC<sub>50</sub> value of 0.59 μM.

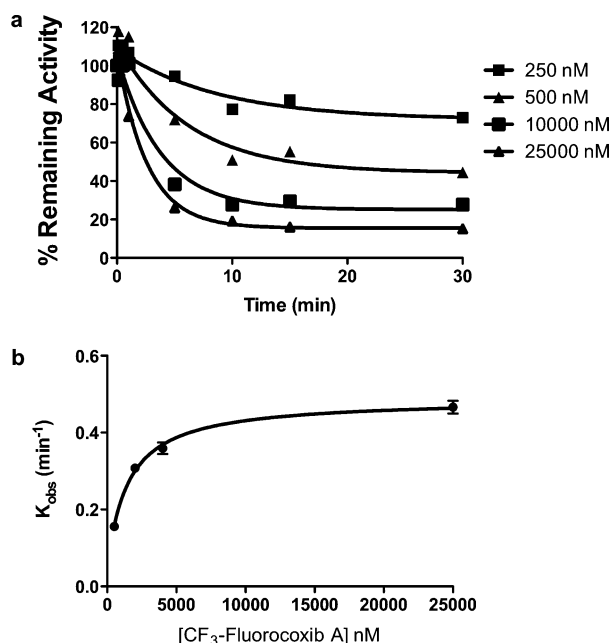
Nearly all COX inhibitors, whether nonselective, like indomethacin, or COX-2-selective, like celecoxib, bind with the enzyme in a noncovalent manner. The one notable exception is aspirin, which irreversibly inactivates COX-1 and COX-2 through covalent modification of an active site serine residue. Indomethacin and celecoxib are examples of slow, tight-binding COX inhibitors. They rapidly establish an equilibrium with a loosely bound enzyme–inhibitor complex, which slowly converts to a much more tightly bound complex (eq 1). Figure 2a shows the time- and concentration-dependent inhibition of mCOX-2 by CF<sub>3</sub>-fluorocoxib A. The time-dependency of COX-2 inhibition is clearly evident, as it requires approximately 10 min to achieve maximal inhibition. Note that the plateau of 15% activity remaining at high CF<sub>3</sub>-fluorocoxib A concentrations suggests some reversibility of the tightly bound enzyme–inhibitor complex. Figure 2b displays a plot of the observed single exponential rate constants for inhibition (*k*<sub>obs</sub>) as a function of CF<sub>3</sub>-fluorocoxib A

#### Scheme 1<sup>a</sup>



<sup>a</sup>Reagents and conditions (a) H<sub>2</sub>N-(CH<sub>2</sub>)<sub>4</sub>-NH-BOC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 1-hydroxybenzotriazole hydrate, *N,N*-diisopropylethylamine, dimethyl formamide, 25 °C, 16 h; (b) HCl (gas), CH<sub>2</sub>Cl<sub>2</sub>, 0–25 °C 1 h; (c) 5-carboxy-X-rhodamine *N*-succinimidyl ester, triethylamine, dimethylsulfoxide, 25 °C, 16 h.

ment of 4-[2-{1-(4-chlorobenzoyl)-5-methoxy-2'-trifluoromethyl-1*H*-indol-3-yl}acetamido]butylcarbamate with HCl (gas) gave *N*-(4-aminobutyl)-2-{1-(4-chlorobenzoyl)-5-methoxy-2'-trifluoromethyl-1*H*-indol-3-yl}acetamide hydrochloride. 5-Carboxy-X-rhodamine *N*-succinimidyl ester (5-ROX NSE)<sup>9</sup> was reacted with the free amine of *N*-(4-aminobutyl)-2-{1-(4-chlorobenzoyl)-5-methoxy-2'-trifluoromethyl-1*H*-indol-3-yl}acetamide hydrochloride in the presence of triethylamine to give *N*-{(5-carboxy-X-rhodaminyl)but-4-yl}-2-{1-(4-chloro-



**Figure 2.** Kinetics of the time-dependent inhibition of COX-2 by CF<sub>3</sub>-fluorocoxib A. The purified COX-2 enzyme was reconstituted with heme and preincubated with the inhibitor at 37 °C for various times (0, 0.5, 1, 3, 5, 10, 15, and 30 min) prior to the addition of the substrate (50  $\mu$ M). (a) Time-dependent inhibition of COX-2 by CF<sub>3</sub>-fluorocoxib A at the indicated concentrations. (b) Secondary plot of  $k_{obs}$  versus inhibitor concentration used to generate values for  $K_i$ ,  $k_2$ , and  $k_{-2}$ .

concentration, which allows the determination of the equilibrium constant for initial association ( $K_i = k_{-1}/k_1$ ) and the forward and reverse rate constants for the conversion to the tightly bound enzyme–inhibitor complex ( $k_2$  and  $k_{-2}$ , respectively, eq 2). CF<sub>3</sub>-fluorocoxib A and fluorocoxib A demonstrate similar affinities for initial complex formation, as indicated by their  $K_i$  values ( $1.5 \pm 0.35$  and  $1.7 \pm 2.3$   $\mu$ M, respectively). The forward rate constants ( $k_2$ ) are also similar for these compounds (CF<sub>3</sub>-fluorocoxib A,  $k_2 = 0.004$  s<sup>-1</sup>; fluorocoxib A,  $k_2 = 0.005$  s<sup>-1</sup>), and the reverse rate constants ( $k_{-2}$ ) are essentially the same (CF<sub>3</sub>-fluorocoxib A,  $k_{-2} = 0.001$  s<sup>-1</sup>; fluorocoxib A,  $k_{-2} = 0.001$  s<sup>-1</sup>). The low rate constant for dissociation of the tightly bound complex is a strong contributing factor toward the potency of the compounds.



where  $K_i = k_{-1}/k_1$  and

$$k_{obs} = \frac{k_2^*[I]}{K_i + [I]} + k_{-2} \quad (2)$$

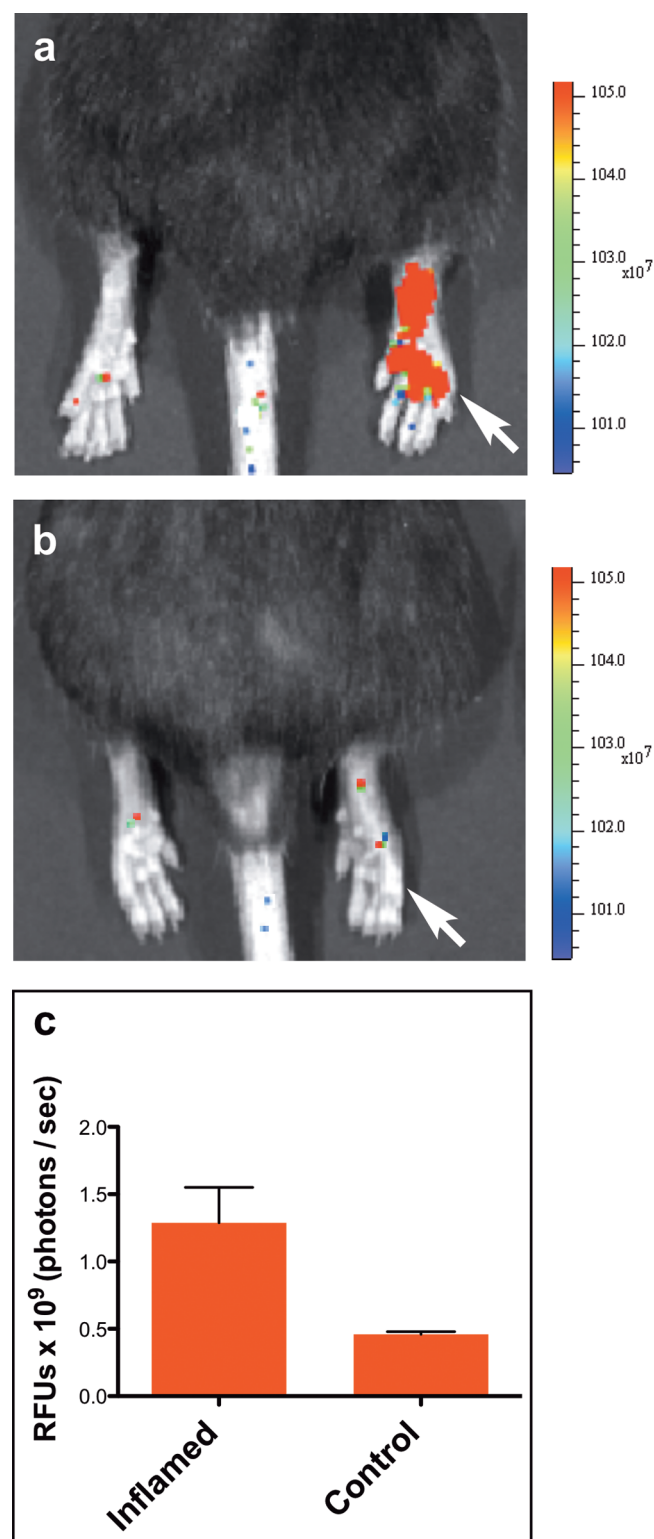
The C57BL/6 mouse footpad model is a well-established system for the study of COX-2-dependent inflammation. COX-2-derived prostaglandins have a significant role in the acute edema induced by carrageenan injection into the paw.<sup>12</sup> A major advantage of this inflammation model is the ability to image the inflamed mouse footpad in comparison to the noninflamed contralateral footpad without COX-2 expression. We injected 50  $\mu$ L of 1% carrageenan in the rear right footpad of each mouse (body weight 20–25 g) and waited 24 h for inflammation to develop. Then, we injected the fluorescent CF<sub>3</sub>-fluorocoxib A (1 mg/kg) intraperitoneally (i.p.) dissolved

in dimethyl sulfoxide. After 3 h, the animals were lightly anesthetized with 2% isoflurane and placed in a Xenogen IVIS200 optical imaging system. CF<sub>3</sub>-fluorocoxib A targeted the inflamed footpad selectively over the contralateral control footpad (Figure 3a). The dependence of compound uptake on COX-2 in the inflammatory tissues was confirmed by blocking the COX-2 active site with celecoxib. We administered celecoxib dissolved in dimethyl sulfoxide (50 mg/kg, i.p.) at 24 h postcarrageenan and waited 1 h for absorption and blockage of the COX-2 active site prior to dosing with CF<sub>3</sub>-fluorocoxib A (1 mg/kg, i.p.). At 3 h postinjection of CF<sub>3</sub>-fluorocoxib A, we lightly anesthetized the animals with 2% isoflurane and imaged them using the Xenogen IVIS200 camera. There was no enrichment of CF<sub>3</sub>-fluorocoxib A in the inflamed paw compared to the control paw (Figure 3b). Figure 3c displays the relative uptake of CF<sub>3</sub>-fluorocoxib A in the inflamed footpad versus the control footpad by image analysis of the data in Figure 3a ( $n = 3$ ,  $p = 0.02$ ). Also, imaging was performed at 5 and 30 min points, where significant probe distribution was observed in both paws.

We next evaluated the ability of CF<sub>3</sub>-fluorocoxib A to target COX-2 in human tumor xenografts. Female nude mice were injected in the left hip with COX-2-expressing human 1483 HNSCC cells or in the right hip with COX-2-null human colorectal carcinoma (HCT116) cells. The tumor xenografts were allowed to grow to approximately 800–1000 mm<sup>3</sup>. Animals were injected (1 mg/kg, i.p.) with CF<sub>3</sub>-fluorocoxib A. At 4 h postinjection, the animals were lightly anesthetized with 2% isoflurane and placed in the Xenogen IVIS 200 optical imaging system. A significant uptake of CF<sub>3</sub>-fluorocoxib A was documented in the COX-2-expressing 1483 tumors (Figure 4a), where as only a minimal uptake was observed in the COX-2-null HCT116 tumors (Figure 4b). Figure 4c displays quantification of the uptake of CF<sub>3</sub>-fluorocoxib A in the 1483 and HCT116 tumors obtained from image analysis ( $n = 4$ ,  $p = 0.01$ ). This suggests that the difference in uptake of CF<sub>3</sub>-fluorocoxib A in 1483 and HCT116 xenografts is due to their differential in COX-2 expression. A significant peritoneal accumulation was detected at the earlier time points, which is due the distribution of the CF<sub>3</sub>-fluorocoxib A in liver, kidney with clearance in both urine and feces. CF<sub>3</sub>-Fluorocoxib A exhibits promise for in vivo detection of COX-2-expressing tumors that are deep-seated, such as tumors located in bladder, colon or intestine using noninvasive endoscopic techniques.

Although, CF<sub>3</sub>-fluorocoxib A and fluorocoxib A are similar in structure, dissimilarities or improved properties were observed for CF<sub>3</sub>-fluorocoxib A in inhibition assays with a series of site-directed COX-2 mutants (Table 1).<sup>13</sup> The compounds described earlier by our laboratory were conjugates of the nonselective NSAID, indomethacin, with fluorophores.<sup>7</sup> The present compound is a conjugate of a COX-2-selective inhibitor, CF<sub>3</sub>-indomethacin, with a fluorophore. It possesses superior selectivity and binding characteristics for COX-2. The inhibition of COX-2 by fluorocoxib A is due to the key interactions of the 2'-CH<sub>3</sub> group with the residues Ala-527, Val-349, Ser-530, and Leu-531 that form a small hydrophobic pocket. Mutation of Val-349 to Ala increases the potency of fluorocoxib A by ~2-fold due to enlargement of the pocket, whereas mutation to Leu reduces the pocket size and decreases the potency of fluorocoxib A by ~6-fold. A more intense trend was observed for CF<sub>3</sub>-fluorocoxib A, where it showed an improved potency against Val-349 to Ala mutant and Val-349 to Leu mutant, suggesting that the 2'-trifluoromethyl group of

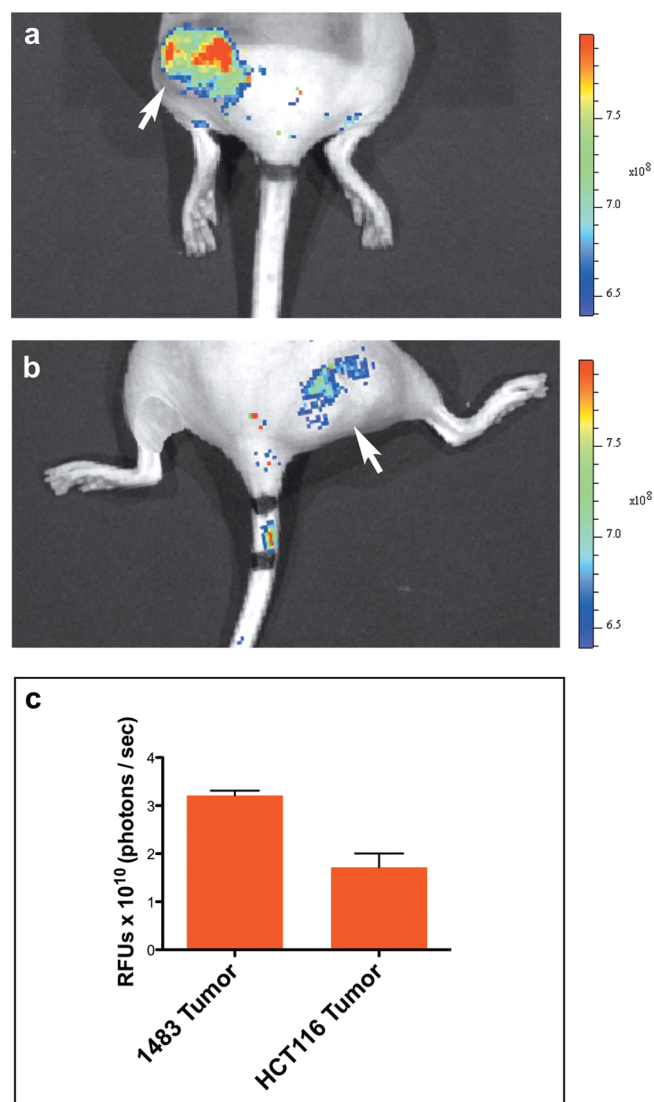




**Figure 3.** In vivo labeling of COX-2 expression in C57BL/6 mouse footpad inflammation by CF<sub>3</sub>-fluorocoxib A. The inflammation was developed by injecting 50  $\mu$ L 1% carrageenan into the right hind footpad. (a) At 24 h postcarrageenan, the mouse was injected with CF<sub>3</sub>-fluorocoxib A (1 mg/kg, i.p.). At 3 h postinjection of CF<sub>3</sub>-fluorocoxib A, a mouse was lightly anesthetized with 2% isoflurane and imaged in the Xenogen IVIS 200 optical imaging system. A significant uptake of CF<sub>3</sub>-fluorocoxib A was documented in the inflamed footpad. (b) At 24 h postcarrageenan, the mouse was predosed with celecoxib (50 mg/kg, i.p.) 1 h before the administration of CF<sub>3</sub>-fluorocoxib A (1 mg/kg, i.p.). At 3 h postinjection of CF<sub>3</sub>-fluorocoxib A, the mouse was

**Figure 3.** continued

lightly anesthetized with 2% isoflurane and imaged in the Xenogen IVIS 200 optical imaging system. There was minimal uptake of CF<sub>3</sub>-fluorocoxib A in the inflamed footpad that was comparable to the noninflamed foot. (c) Quantitation of CF<sub>3</sub>-fluorocoxib A uptake in inflamed vs control footpad at 3 h postinjection of the agent (from data in panel a).



**Figure 4.** In vivo labeling of COX-2-expressing cancer by CF<sub>3</sub>-fluorocoxib A. Female nude mice bearing COX-2-expressing 1483 HNSCC and COX-2-null HCT116 tumors were dosed with CF<sub>3</sub>-fluorocoxib A (1 mg/kg, i.p.). The animals were imaged at 4 h postinjection of CF<sub>3</sub>-fluorocoxib A in a Xenogen IVIS200 optical imaging instrument. (a) A significant uptake of CF<sub>3</sub>-fluorocoxib A was documented in the COX-2-expressing 1483 tumors (arrow). (b) Minimal uptake of CF<sub>3</sub>-fluorocoxib A was documented in the COX-2-negative HCT116 tumors. (c) Quantitation of CF<sub>3</sub>-fluorocoxib A uptake in COX-2-expressing 1483 tumors and COX-2-negative HCT116 tumors at 4 h postinjection of the agent.

CF<sub>3</sub>-fluorocoxib A binds with these residues more tightly than that of the 2'-methyl group of fluorocoxib A. Improved properties of CF<sub>3</sub>-fluorocoxib A were also observed with Ser-530 to Ala mutant, Arg-120 to Ala mutant, or Val-89 to Trp/Ser-119 to Trp double mutant, suggesting that the 2'-

**Table 1.** Inhibition of Wild-Type Ovine COX-1, Wild-Type Mouse COX-2, and Mouse COX-2 Mutants by Fluorocoxib A and CF<sub>3</sub>-Fluorocoxib A

wild-type or mutant enzymes	fluorocoxib A (IC <sub>50</sub> μM) <sup>a</sup>	CF <sub>3</sub> -fluorocoxib A (IC <sub>50</sub> μM) <sup>a</sup>
wt hCOX-2	0.70	0.56
wt oCOX-1	>25	>25
V349A	0.38	0.18
V349L	>4	0.72
S530A	0.32	0.16
R120A	2.00	0.36
V89W/S119W	1.30	0.51

<sup>a</sup>IC<sub>50</sub> values are μM and represent time-dependent inhibition and average determinations from three experiments.

trifluoromethyl group, the *n*-butyl diamide tether, or the 5-ROX fluorophore of CF<sub>3</sub>-fluorocoxib A interacts with the respective residues more tightly than the 2'-methyl group, the *n*-butyl diamide linker group, or the 5-ROX fluorophore group of fluorocoxib A.

In summary, CF<sub>3</sub>-fluorocoxib A has been synthesized and evaluated as a potent fluorescent COX-2-specific inhibitor for optical imaging. The fluorescent CF<sub>3</sub>-fluorocoxib A inhibited COX-2 selectively in purified protein as well as in intact inflammatory and cancer cells. CF<sub>3</sub>-fluorocoxib A is a slow and tight binding inhibitor of COX-2 with similar binding kinetics for COX-2 as the parent fluorocoxib A. Although CF<sub>3</sub>-fluorocoxib A and fluorocoxib A share similar structural features, CF<sub>3</sub>-fluorocoxib A is a more potent inhibitor of wtCOX-2 and of a series of COX-2 mutants. CF<sub>3</sub>-fluorocoxib A displays good selectivity of uptake in inflammatory tissues and COX-2-expressing tumors compared to control tissues or COX-2-negative tumors. Uptake of CF<sub>3</sub>-fluorocoxib A requires the expression of COX-2 at the target site. Uptake is reduced when the COX-2 active site is preblocked or in the absence of COX-2 expression in the target site. These in vitro and in vivo studies provide support for the conclusion that high specificity and tight binding to the COX-2 enzyme is the major determinant of uptake and retention of CF<sub>3</sub>-fluorocoxib A in inflamed tissues and tumors. Thus, CF<sub>3</sub>-fluorocoxib A represents a new optical imaging reagent for the detection and evaluation of COX-2 status in naturally occurring malignancies.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Full synthetic procedures and analytical and spectral characterization data of the synthesized compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

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### Notes

The authors declare no competing financial interest.

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