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

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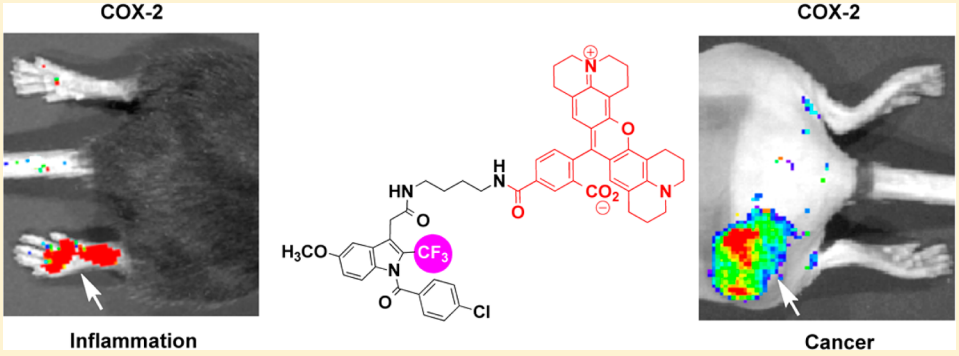
Supporting Information



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_3-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_3-fluorocoxib A is a slow, tight binding inhibitor of COX-2 that exhibits low nanomolar inhibitory potency. While CF_3-fluorocoxib A and fluorocoxib A are similar in structure, CF_3-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_3-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_3-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_3-indomethacin, CF_3-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.[1] 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.[2] 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.[3] COX-2 overexpression is an early event in tumorigenesis, and it plays a role in tumor progression.[4] Selective COX-2 inhibitors are useful in the treatment of various cancers.[5,6] 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.[7] 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_3-fluorocoxib A to inflammatory tissues and human tumor xenografts (Figure 1).

CF_3-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.[8]

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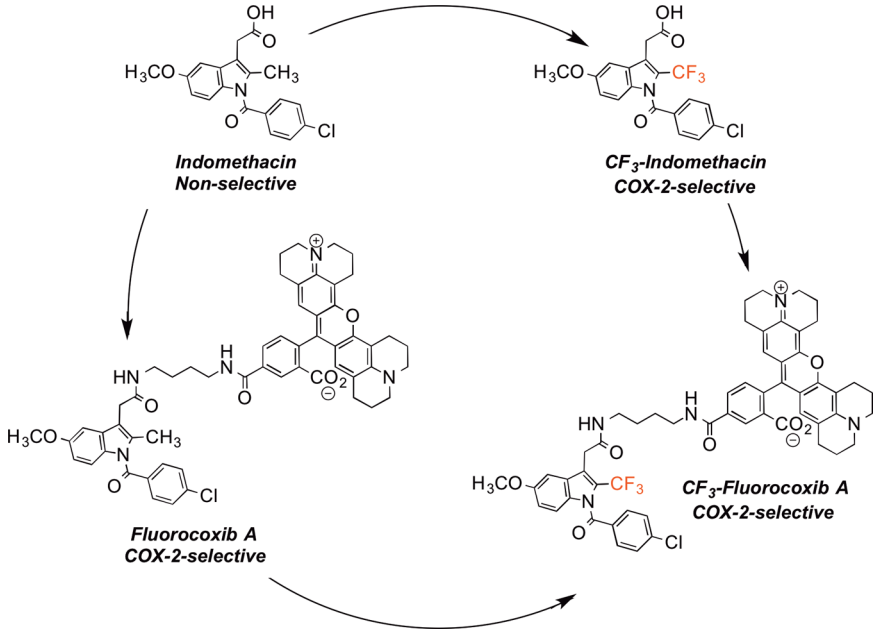
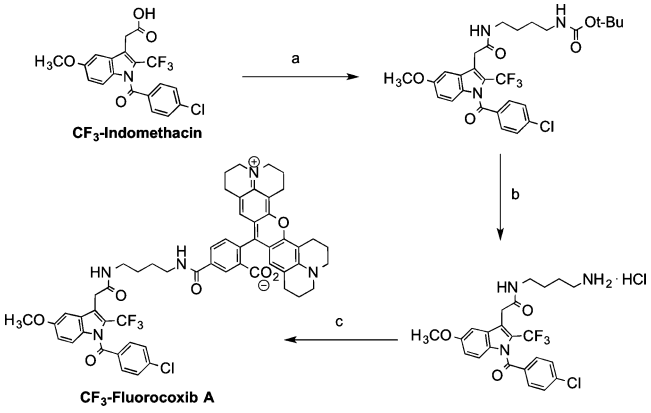


Figure 1. Chemical evolution of COX-2-selective inhibitors from nonsteroidal anti-inflammatory drug indomethacin.

The CF_3-indomethacin was then coupled with mono *N*-*tert*-butoxycarbonyl-butylenediamine 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). Treatment 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*-succinnimidyl ester (5-ROX NSE)[9] 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-chlorobenzoyl)-5-methoxy-2′-trifluoromethyl-1*H*-indol-3-yl}-acetamide (CF_3-fluorocoxib A) (Scheme 1). Synthetic procedures and the characterization of all the new compounds are described in the Supporting Information.

Scheme 1[a]



[a]Reagents and conditions (a) H_2N-$(CH_2)_4$-NH-BOC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 1-hydroxybenzotriazole hydrate, *N*,*N*-diisopropylethylamine, dimethyl formamide, 25 °C, 16 h; (b) HCl (gas), CH_2Cl_2, 0—25 °C 1 h; (c) 5-carboxy-X-rhodamine *N*-succinimidyl ester, triethylamine, dimethylsulfoxide, 25 °C, 16 h.

CF_3-fluorocoxib A was assayed against purified COX-2 or COX-1 by a previously reported thin layer chromatography assay.[10] We found that CF_3-fluorocoxib A is a selective and potent inhibitor of COX-2. The calculated LogP values of CF_3-fluorocoxib A and fluorocoxib A are 6.96 and 6.34, respectively. The IC_{50} value for inhibition of COX-2 is 0.56 μM with no inhibition of COX-1 up to 25 μM. CF_3-fluorocoxib A was assayed in RAW264.7 murine macrophage-like cells to check for membrane permeability and subsequent COX-2 inhibition.[11] The IC_{50} value for inhibition of COX-2 by CF_3-fluorocoxib A was 0.08 μM. Further, the ability of CF_3-fluorocoxib A to inhibit COX-2 in 1483 head and neck squamous cell carcinoma (HNSCC) cells was assayed.[7] CF_3-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-^{14}C]-arachidonic acid (~55 mCi/mmol). CF_3-Fluorocoxib A inhibited COX-2 with an IC_{50} 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_3-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_3-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_{obs}) as a function of CF_3-fluorocoxib A

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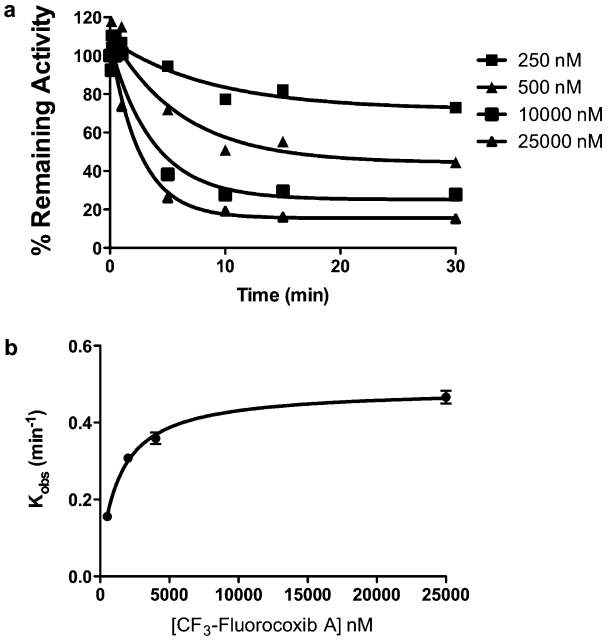

Figure 2. Kinetics of the time-dependent inhibition of COX-2 by CF_3-fluorocoxib A. The purified COX-2 enzyme was reconstituted with heme and preincubated with the inhibitor at 37 °C for various times (0, 0.5, 1, 3, 5, 10, 15, and 30 min) prior to the addition of the substrate (50 μM). (a) Time-dependent inhibition of COX-2 by CF_3-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_3-fluorocoxib A and fluorocoxib A demonstrate similar affinities for initial complex formation, as indicated by their K_I values (1.5 ± 0.35 and 1.7 ± 2.3 μM, respectively). The forward rate constants (k_2) are also similar for these compounds (CF_3-fluorocoxib A, k_2 = 0.004 s^{-1}; fluorocoxib A, k_2 = 0.005 s^{-1}), and the reverse rate constants (k_{-2}) are essentially the same (CF_3-fluorocoxib A, k_{-2} = 0.001 s^{-1}; fluorocoxib A, k_{-2} = 0.001 s^{-1}). The low rate constant for dissociation of the tightly bound complex is a strong contributing factor toward the potency of the compounds.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [EI] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^* \quad (1)$$

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.[12] 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 μL 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_3-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_3-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_3-fluorocoxib A (1 mg/kg, i.p.). At 3 h postinjection of CF_3-fluorocoxib A, we lightly anesthetized the animals with 2% isoflurane and imaged them using the Xenogen IVIS200 camera. There was no enrichment of CF_3-fluorocoxib A in the inflamed paw compared to the control paw (Figure 3b). Figure 3c displays the relative uptake of CF_3-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_3-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^3. Animals were injected (1 mg/kg, i.p.) with CF_3-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_3-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_3-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_3-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_3-fluorocoxib A in liver, kidney with clearance in both urine and feces. CF_3−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_3-fluorocoxib A and fluorocoxib A are similar in structure, dissimilarities or improved properties were observed for CF_3-fluorocoxib A in inhibition assays with a series of site-directed COX-2 mutants (Table 1).[13] The compounds described earlier by our laboratory were conjugates of the nonselective NSAID, indomethacin, with fluorophores.[7] The present compound is a conjugate of a COX-2-selective inhibitor, CF_3-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_3 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_3-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

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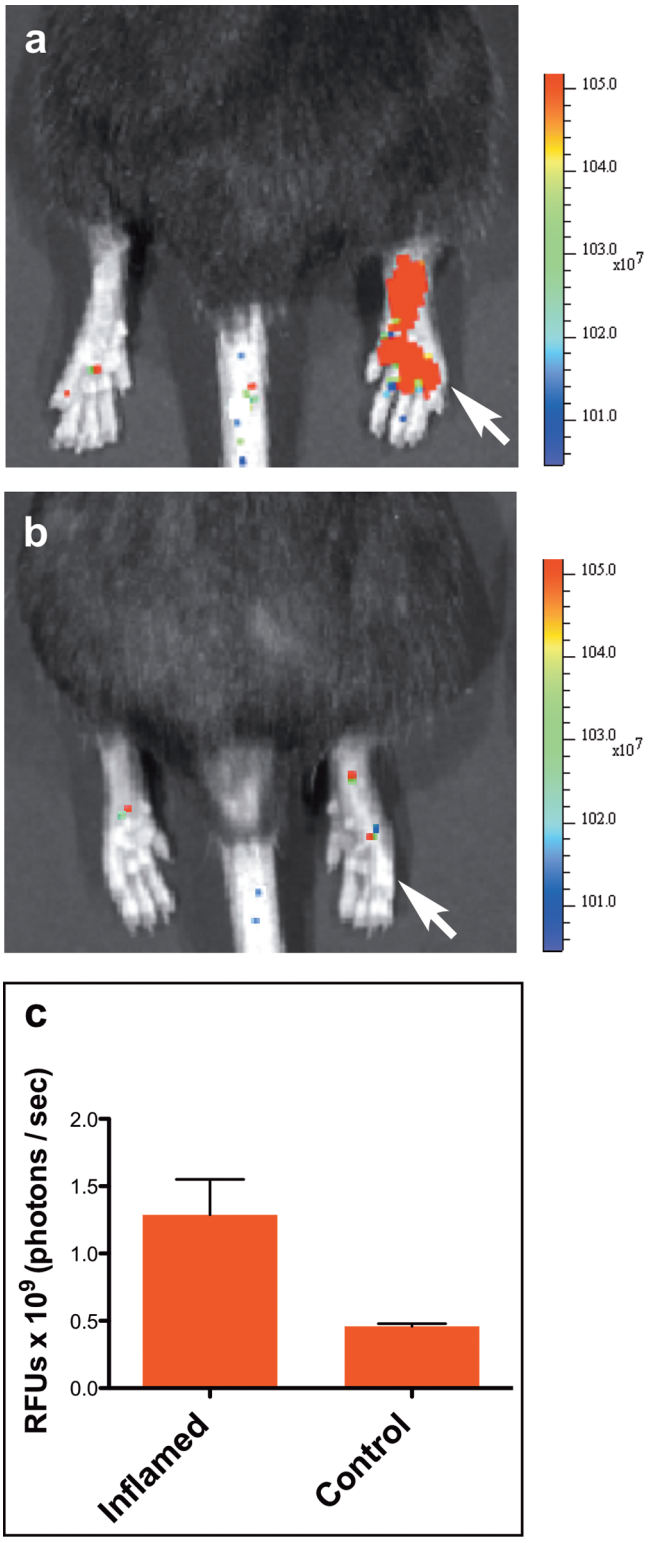

Figure 3. In vivo labeling of COX-2 expression in C57BL/6 mouse footpad inflammation by CF_3-fluorocoxib A. The inflammation was developed by injecting 50 μL 1% carrageenan into the right hind footpad. (a) At 24 h postcarrageenan, the mouse was injected with CF_3-fluorocoxib A (1 mg/kg, i.p.). At 3 h postinjection of CF_3-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_3-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_3-fluorocoxib A (1 mg/kg, i.p.). At 3 h postinjection of CF_3-fluorocoxib A, the mouse was lightly anesthetized with 2% isoflurane and imaged in the Xenogen IVIS 200 optical imaging system. There was minimal uptake of CF_3-fluorocoxib A in the inflamed footpad that was comparable to the noninflamed foot. (c) Quantitation of CF_3-fluorocoxib A uptake in inflamed vs control footpad at 3 h postinjection of the agent (from data in panel a).

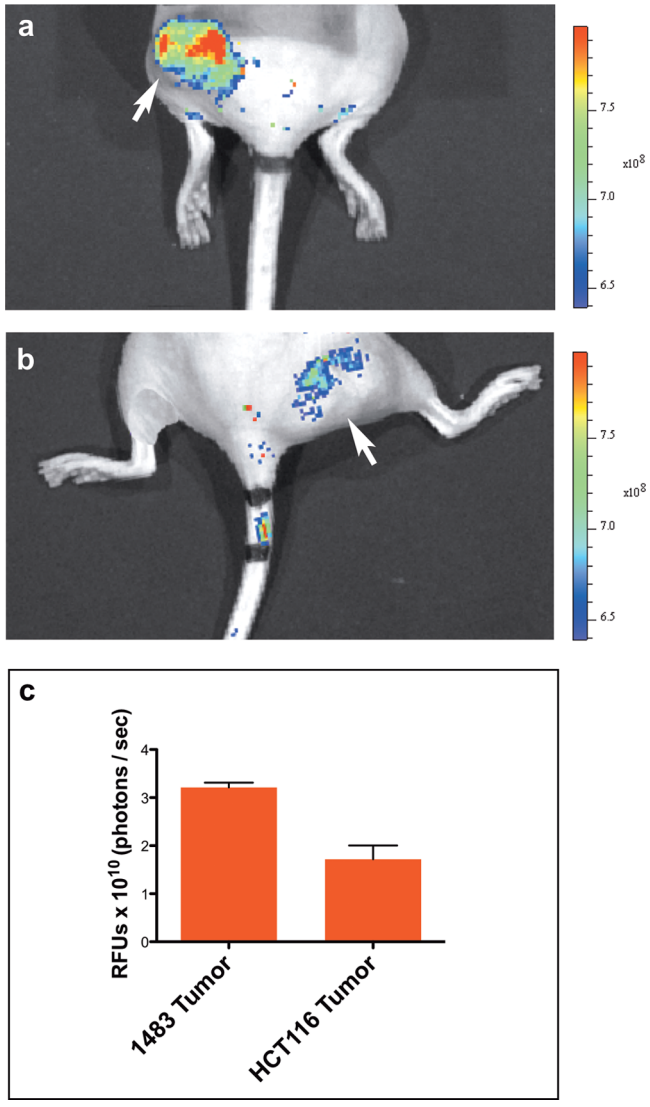

Figure 4. In vivo labeling of COX-2-expressing cancer by CF_3-fluorocoxib A. Female nude mice bearing COX-2-expressing 1483 HNSCC and COX-2-null HCT116 tumors were dosed with CF_3-fluorocoxib A (1 mg/kg, i.p.). The animals were imaged at 4 h postinjection of CF_3-fluorocoxib A in a Xenogen IVIS200 optical imaging instrument. (a) A significant uptake of CF_3-fluorocoxib A was documented in the COX-2-expressing 1483 tumors (arrow). (b) Minimal uptake of CF_3-fluorocoxib A was documented in the COX-2-negative HCT116 tumors. (c) Quantitation of CF_3-fluorocoxib A uptake in COX-2-expressing 1483 tumors and COX-2-negative HCT116 tumors at 4 h postinjection of the agent.

CF_3-fluorocoxib A binds with these residues more tightly then that of the 2′-methyl group of fluorocoxib A. Improved properties of CF_3-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′-

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Table 1. Inhibition of Wild-Type Ovine COX-1, Wild-Type Mouse COX-2, and Mouse COX-2 Mutants by Fluorocoxib A and CF_3-Fluorocoxib A

wild-type or mutant enzymes	fluorocoxib A (IC_{50} μM)[a]	CF_3-fluorocoxib A (IC_{50} μM)[a]
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

[a]IC_{50} 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_3-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_3-fluorocoxib A has been synthesized and evaluated as a potent fluorescent COX-2-specific inhibitor for optical imaging. The fluorescent CF_3-fluorocoxib A inhibited COX-2 selectively in purified protein as well as in intact inflammatory and cancer cells. CF_3-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_3-fluorocoxib A and fluorocoxib A share similar structural features, CF_3-fluorocoxib A is a more potent inhibitor of wtCOX-2 and of a series of COX-2 mutants. CF_3-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_3-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_3-fluorocoxib A in inflamed tissues and tumors. Thus, CF_3-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.

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Notes

The authors declare no competing financial interest.

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