Molecular Imaging of Inflammation in Osteoarthritis Using a Water-Soluble Fluorocoxib

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ABSTRACT: Clinical imaging approaches to detect inflammatory biomarkers, such as cyclooxygenase-2 (COX-2), may facilitate the diagnosis and therapy of inflammatory diseases. To this end, we report the discovery of *N*-[(rhodamin-X-yl)but-4-yl]-2-[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]-acetamide chloride salt (fluorocoxib D), a hydrophilic analog of fluorocoxib A. Fluorocoxib D inhibits COX-2 selectively in purified enzyme preparations and cells. It exhibits adequate photophysical properties to enable detection of COX-2 in intact cells, in a mouse model of carrageenan-induced acute footpad inflammation and inflammation in a mouse model of osteoarthritis. COX-2-selectivity was verified either by blocking the enzyme's active site with celecoxib or by molecular imaging with nontargeted 5-carboxy-X-rhodamine dye. These data indicate that fluorocoxib D is an ideal candidate for early detection of inflammatory or neoplastic lesions expressing elevated levels of COX-2.

KEYWORDS: Molecular imaging, cyclooxygenase-2, inflammation, osteoarthritis, fluorocoxib D

Osteoarthritis is an irreversible musculoskeletal degenerative disease that damages hips, knees, neck, lower back, and/or small joints of the hands, affecting nearly 50 million people in the United States, a number projected to increase to 67 million by the year 2030.^{1–3} It is evident that low-grade chronic inflammation is one of the leading causes of osteoarthritis development and progression.⁴ Studies have shown that mediators of inflammation, including IL-1 β , TNF- α , and chemokines, contribute to activation of NF- κ B signaling that induces cyclooxygenase-2 (COX-2) expression in the pathogenesis of osteoarthritis. COX-2 is one of two cyclooxygenase enzymes that catalyze the formation of prostaglandins from arachidonic acid.^{5–7} COX-2-derived prostaglandins, particularly prostaglandin E₂, not only affect normal cellular functions but also facilitate the inflammatory response.^{8–10} Thus, COX-2 is an ideal molecular target for early detection of osteoarthritis.

We previously reported the discovery of fluorocoxib A (Figure 1a), a fluorescent 5-carboxy-X-rhodamine (5-ROX)-labeled COX-2-selective inhibitor that has proven successful in the visualization and early detection of COX-2 in inflammation and cancer in animal models including intestinal polyps, skin adenocarcinomas, and human tumor xenografts in mice; footpad inflammation in rats and mice; and spontaneous bladder and oral tumors in dogs.^{11–16} Thus, clinical development of fluorocoxib A promises to facilitate the early detection

and accurate surgical resection of cancers in the skin, colon, esophagus, and bladder. However, translation of fluorocoxib A to the clinic has been hampered by the compound's lack of solubility in aqueous solutions appropriate for human administration.^{12,11,15} As fluorocoxib A is a zwitterion bearing no net charge, we hypothesized that a decarboxy analog of fluorocoxib A, bearing a net positive charge, would exhibit improved aqueous solubility, thereby facilitating its use as an *in vivo* molecular probe for selective visualization of COX-2 in inflammatory and malignant lesions. Here, we report the discovery of *N*-[(rhodamin-X-yl)but-4-yl]-2-[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetamide, named fluorocoxib D, a decarboxy analog of fluorocoxib A. We also provide results from our initial exploration of fluorocoxib D's potential to serve as a probe for *in vivo* visualization of COX-2 using a mouse model of osteoarthritis.

The synthesis of decarboxy-5-ROX dyes required a symmetric condensation of 2 equiv of 8-hydroxyjulolidine

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Figure 1. (a) Chemical structures of indomethacin and fluorocoxib A. (b) Chemical synthesis of fluorocoxib D: (i) 4-formylbenzoic acid, 60% aqueous H_2SO_4 , 150 °C, 24 h, (ii) N,N,N',N' -tetramethyl- O -(N -succinimidyl)uronium tetrafluoroborate, triethylamine, dichloromethane 25 °C, 2 h, (iii) N -(4-aminobutyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)acetamide hydrochloride, triethylamine, dichloromethane, 25 °C, 16 h.

with 1 equiv of 4-formylbenzoic acid, all of which were combined and stirred for 24 h in 60% sulfuric acid at 160 °C. The overall process required one Fries rearrangement and two sequential Friedel–Crafts-type electrophilic aromatic substitution reactions for the formation of the xanthene skeleton. The final conjugation required an N -succinimidyl activation of the carboxylic acid group of the ROX dye followed by coupling with indomethacin-butylamine to afford the product, fluorocoxib D (Figure 1b).

Using ultraviolet (UV) spectroscopy, we determined that fluorocoxib D is soluble in water containing 13% dimethyl sulfoxide at a concentration of 0.25 mg/mL (Figure 2a). In this solvent system, fluorocoxib A is not soluble. DMSO exhibits a certain degree of anti-inflammatory activity; however, it has only a narrow window of efficacy. Studies have shown that anti-inflammatory properties of DMSO are due to the inhibition of ERK1/2, p38, PI3K, and JNK pathways,¹⁷ but there is no evidence that supports its association in inhibition of COX-2 pathway.

We evaluated fluorocoxib D's net charge in a range of pHs. Within the error of the UV instrumentation, the peak of the absorption spectrum of fluorocoxib D was found to be unaffected by pH in the range from 2 to 12, a result in accord with the predicted unchanged charge state within this pH range including the physiological pH 7.4. Further, we confirmed the presence of a positive charge on fluorocoxib D by an agarose gel electrophoresis. In an octanol/water binary system, fluorocoxib D and fluorocoxib A exhibited cLogP values of 0.75 and 3.0, respectively (Figure 2b). These results

Figure 2. (a) Solubility of fluorocoxib D in water containing 13% dimethyl sulfoxide. (b) cLogP of fluorocoxib D and fluorocoxib A. (c,d) Fluorescence excitation and emission properties of fluorocoxib D in ethanol (RFU, relative fluorescence units). (e) Photophysical quantum yield (ϕ) and molar extinction coefficient (ϵ) of fluorocoxib A and D. (f) Inhibition of purified COX-1 and COX-2 proteins by fluorocoxib D.

confirm the higher hydrophilicity of fluorocoxib D as compared to that of fluorocoxib A.

Fluorocoxib D exhibited fluorescence excitation at $\lambda_{\text{max}} = 577$ nm and emission at $\lambda_{\text{max}} = 603$ nm (Figure 2c,d). We calculated the quantum yield ($\phi_{577/603\text{nm}} = 0.9$) of fluorocoxib D by comparing its fluorescence with the fluorescence of fluorocoxib A, and we determined its extinction coefficient ($\epsilon_{540\text{nm}} = 16 \text{ mM}^{-1} \text{ cm}^{-1}$) (Figure 2e).

The *in vitro* COX isozyme inhibitory activity of fluorocoxib D was evaluated using purified proteins.¹¹ We purified COX-1 from ovine seminal vesicular glands and human COX-2 from baculovirus-infected Sf9 cells using previously reported procedures.^{18,19} Varying concentrations of fluorocoxib D were preincubated with hematin-reconstituted COX-2 or its isozyme, COX-1, at 25 °C for 17 min and 37 °C for 3 min followed by the addition of 50 μM [$1\text{-}^{14}\text{C}$]arachidonic acid ($\sim 55 \text{ mCi/mmol}$). A mixed solvent system (ether/methanol/citric acid, 30:4:1) was used to terminate the reaction. The organic layer was spotted on a TLC plate to separate and detect radiolabeled prostaglandin products, which were quantified by a radioactivity counter (Bioscan, Inc.). The assay identified fluorocoxib D as a selective COX-2 inhibitor (COX-2 $\text{IC}_{50} = 0.23 \mu\text{M}$, COX-1 $\text{IC}_{50} > 4 \mu\text{M}$) (Figure 2f).

We evaluated fluorocoxib D's ability to inhibit COX-2 in human 1483 head and neck squamous cell carcinoma (1483 HNSCC) cells that express elevated levels of the COX-2 enzyme, as previously reported and confirmed by immunoblot using a commercially available antibody against human COX-2 (Figure 3a).¹¹ The 1483 HNSCC cells were cultured using a published method.¹¹ Cells were treated with fluorocoxib D for

Figure 3. (a) Immunoblot analysis of COX-2 in 1483 HNSCC cells. (b) Inhibition of COX-2 in 1483 HNSCC cells by fluorocoxib D. (c) Fluorescence microscopic imaging of 1483 HNSCC cells treated with fluorocoxib D. (d) Fluorescence microscopy of 1483 HNSCC cells treated with celecoxib followed by fluorocoxib D.

30 min at 37 °C followed by the addition of [1-¹⁴C]-arachidonic acid (5 μM, ~55 mCi/mmol) for 20 min at 37 °C. The reaction was quenched with a solvent mixture (ether/methanol/citric acid, 30:4:1). After layer separation, the organic layer was spotted on a TLC plate and developed in ethyl acetate/dichloromethane/glacial acetic acid (75:25:1). By use of a radioactivity counter (Bioscan, Inc.), the radiolabeled prostaglandin products were quantified. In this assay, fluorocoxib D inhibited COX-2 with an IC₅₀ of 0.4 μM (Figure 3b).

Fluorescence microscopy was used to evaluate fluorocoxib D's capacity for targeted visualization of COX-2 in 1483 HNSCC cells.¹¹ Cells at 60% confluency were incubated with 200 nM fluorocoxib D for 30 min at 37 °C. In some cases, we preincubated cells with 5 μM celecoxib for 20 min to block the COX-2 active site prior to treatment with fluorocoxib D (200 nM). We washed the cells three times and imaged them on a fluorescence microscope (Zeiss Axiovert 25) with a propidium iodide filter. Cells incubated with fluorocoxib D alone exhibited strong perinuclear fluorescence (Figure 3c) that was blocked by preincubation with celecoxib (Figure 3d). These data suggest that the uptake and retention of fluorocoxib D in the cells were due to selective binding to COX-2.

We determined the *in vivo* plasma half-life of fluorocoxib D in C57BL/6J mice. All animal procedures were performed according to a protocol approved by the Vanderbilt Institutional Animal Care and Use Committee (IACUC). Following intravenous administration of the probe (1.41 mg/kg), blood samples were collected by cardiac puncture at 0.08, 0.5, 1, 2, or 4 h. The blood samples were mixed with heparin and centrifuged to collect the plasma layer for analysis by LC–

MS/MS. The fluorocoxib D in plasma samples was quantified against a standard curve. The plasma half-life of fluorocoxib D was 40 min (Figure 4a). The short half-life of fluorocoxib D represents the quick clearance of the unbound probe from nontargeted organ sites to significantly reduce the background noise.

Figure 4. (a) Plasma levels of fluorocoxib D following intravenous administration to mice. (b) *In vivo* imaging of footpad inflammation using fluorocoxib D (*t* = 1 h). (c) Quantification of uptake of fluorocoxib D in inflamed vs control footpads.

With this information in hand, we evaluated the feasibility of using fluorocoxib D to target COX-2 in a mouse footpad inflammation model in which COX-2-derived prostaglandins play a major role in inducing maximum edema at 24 h after carrageenan injection.¹¹ This model offers COX-2-targeted imaging of inflammation in an edematous footpad as compared to the contralateral noninflamed control footpad lacking COX-2 expression. An aqueous solution of carrageenan (1%) was injected subcutaneously in the rear right footpad of female C57BL/6 mice (volume, 50 μL; dose, 20 mg/kg; route, sc), and vehicle alone was injected into the contralateral footpad. Following a 24 h period to allow induction of maximum footpad inflammation, we confirmed the expression of COX-2 in the inflamed footpad by microscopic examination of tissue sections obtained from the inflamed and contralateral feet using immunofluorescence (Figure S1). The inflamed footpad

expressed high levels of COX-2 compared to minimal COX-2 in the noninflamed footpad.

For *in vivo* imaging, animals bearing an inflamed footpad were dosed with fluorocoxib D (1 mg/kg, ip) dissolved at a concentration of 250 μ g/mL in water containing 13% dimethyl sulfoxide. *In vivo* imaging was conducted at various time points from 15 min to 4 h postinjection of fluorocoxib D on a Xenogen IVIS 200 system. A high uptake of fluorocoxib D was detected in the inflamed footpad compared to the noninflamed normal footpad based on fluorescent signals that were reproducibly detected at the inflamed sites throughout the time course of the experiment. Maximal signals were detected at 1 h postinjection (Figure 4b left mouse). We quantified the probe accumulation in the footpad at 1 h by image analysis using AMIDE software ($n = 6$, $p = 0.001$).²⁰ The analysis showed significantly higher uptake of fluorocoxib D in inflamed tissues compared to noninflamed tissues (Figure 4c). To test the hypothesis that the fluorescent signal was due to COX-2-targeted delivery of fluorocoxib D, we pretreated the animals bearing an inflamed footpad with a high dose of celecoxib (10 mg/kg in dimethyl sulfoxide, ip) 1 h prior to fluorocoxib D injection (1 mg/kg, ip). The animals were imaged *in vivo* at 1 h after fluorocoxib D injection. The imaging revealed minimal fluorescence in the celecoxib-pretreated inflamed footpad (Figure 4b,c right mouse), indicating that the uptake in this model of inflammation was COX-2-targeted.

For selective visualization of inflammation in osteoarthritis, we employed a mechanical post-traumatic osteoarthritis (PTOA) animal model in wild-type C57Bl/6J mice.²¹ The PTOA model of noninvasive repetitive joint loading was induced by subjecting the left knee joint of mice (anesthetized with 3% isoflurane) to 50 cycles of compressive mechanical loading at 9 N. This procedure was repeated three times per week over a period of 2 weeks. The nanoString nCounter Inflammation panel was used to quantify expression of 254 genes in the knee joint samples. Of these genes, expression of COX-2 (as indicated by mRNA levels) was dramatically increased in PTOA knee tissues (Figure 5a). We evaluated fluorocoxib D's ability to target COX-2 in inflammation at the PTOA site. We administered fluorocoxib D dissolved in water containing 13% dimethyl sulfoxide (1 mg/kg, iv) to mice bearing PTOA in the left knee. *In vivo* imaging was performed by a Xenogen IVIS 200 instrument. The uptake of fluorocoxib D allowed a clear delineation of PTOA starting at 0.5 h and extending to 4 h postadministration. Maximum uptake was observed at 1 h postinjection of fluorocoxib D with minimal uptake at the contralateral normal knee (Figure 5b and Figure S2). In contrast to the high and selective uptake of fluorocoxib D, minimal uptake of the nontargeted free decarboxy-5-ROX dye was observed at the PTOA site expressing high levels of COX-2 enzyme (Figure 5c,d).²⁰ These *in vivo* experiments support the utility of fluorocoxib D as a feasible molecular imaging agent for detection of inflammation in osteoarthritis.

In conclusion, we report the discovery of fluorocoxib D, a selective COX-2 inhibitor in both purified enzyme and cellular preparations that exhibits adequate hydrophilic, photophysical, and pharmacokinetic properties to enable optical imaging of COX-2 in intact cells and *in vivo*. The probe targets COX-2 in carrageenan-induced acute footpad inflammation and inflammation in osteoarthritis without off-target accumulation. Extensive control experiments validate the specificity of fluorocoxib D in both models. These experiments provide

Figure 5. (a) Elevated COX-2 mRNA levels in PTOA knee tissues compared to healthy knee tissues. Error bars represent standard error, and an unpaired parametric *t* test with Welch's correction (not assuming similar distribution) was performed to assess statistical difference ($p < 0.001$). (b) *In vivo* visualization of inflammation in osteoarthritis (left knee = PTOA, right knee = healthy control) using fluorocoxib D (dose = 1 mg/kg, iv, $t = 1$ h). (c) Imaging of PTOA using the free decarboxy-5-ROX dye as a control probe. (d) Quantitative measurement of fluorocoxib D or free dye uptake in PTOA knees vs healthy knees by AMIDE software.

proof-of-principle for the utility of fluorocoxib D in the diagnosis of inflammatory diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmmedchemlett.9b00512>.

Synthesis and characterization of all compounds and other experimental methods; supplementary Figures S1 and S2 (PDF)

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Author Contributions

Md.J.U. designed and synthesized decarboxy-5-carboxy-X-rhodamine and fluorocoxib D, designed *in vitro* and *in vivo* experiments, interpreted the data, and wrote the manuscript. A.V. performed scale-up synthesis of fluorocoxib D for biochemical and biological evaluation. H.N. performed characterization of COX-2 in inflamed tissues using immuno-

fluorescence assay. B.C.C. performed fluorescence microscopy, developed the mouse footpad inflammation model, and assisted with animal imaging experiments. C.G.O. conducted the purification of fluorocoxib D. P.J.K. conducted HPLC and LC–MS/MS analysis. T.E.K. developed the mouse model of osteoarthritis and performed *in vivo* imaging. S.K.B. carried out the characterization of COX-2 in osteoarthritic tissues. J.O.M. determined the photophysical properties and neat charge of fluorocoxib D. M.M. measured the partition coefficient of fluorocoxib D and fluorocoxib A from octanol/water binary systems. A.M.A. confirmed fluorocoxib D as a positive charged organic compound by agarose gel electrophoresis. R.J.C. provided support for immunofluorescence analysis. C.L.D. provided support for osteoarthritis imaging. L.J.M. provided laboratory space and financial support for the study, edited the manuscript, and supervised the overall conduct of the research program.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

COX-2, cyclooxygenase-2; IL-1 β , interleukin 1 β ; TNF α , tumor necrosis factor α ; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells; 1483 HNSCC, 1483 head and neck squamous cells carcinoma; ROX, carboxy-X-rhodamine; PTOA, post-traumatic osteoarthritis

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