Selective Visualization of Cyclooxygenase-2 in Inflammation and Cancer by Targeted Fluorescent Imaging Agents

Md. Jashim Uddin1, Brenda C. Crews1, Anna L. Blobaum1, Philip J. Kingsley1, D. Lee Gorden2, J. Oliver McIntyre2, Lynn M. Matrisian2, Kotha Subbaramaiah4, Andrew J. Dannenberg4, David W. Piston3, and Lawrence J. Marnett1

Abstract

Effective diagnosis of inflammation and cancer by molecular imaging is challenging because of interference from nonselective accumulation of the contrast agents in normal tissues. Here, we report a series of novel fluorescence imaging agents that efficiently target cyclooxygenase-2 (COX-2), which is normally absent from cells, but is found at high levels in inflammatory lesions and in many premalignant and malignant tumors. After either i.p. or i.v. injection, these reagents become highly enriched in inflamed or tumor tissue compared with normal tissue and this accumulation provides sufficient signal for in vivo fluorescence imaging. Further, we show that only the intact parent compound is found in the region of interest. COX-2–specific delivery was unambiguously confirmed using animals bearing targeted deletions of COX-2 and by blocking the COX-2 active site with high-affinity inhibitors in both in vitro and in vivo models. Because of their high specificity, contrast, and detectability, these fluoroceoxibs are ideal candidates for detection of inflammatory lesions or early-stage COX-2–expressing human cancers, such as those in the esophagus, oropharynx, and colon.

Cancer Res; 70(9); 3618-27. ©2010 AACR.

Introduction

Molecular imaging presents exciting opportunities for the selective detection of specific cell populations, such as those bearing markers of disease (1, 2). Cyclooxygenase-2 (COX-2) is an attractive target for molecular imaging because it is expressed in only a few normal tissues and is greatly upregulated in inflamed tissues as well as many premalignant and malignant tumors (3, 4). COX-2 is an important contributor to the etiology of inflammation and cancer as illustrated by the efficacy of COX-2–selective inhibitors as anti-inflammatory agents, cancer preventive agents, and adjuvant cancer therapeutic agents (5). The importance of COX-2 in tumor progression has been thoroughly documented in the esophagus and colon where COX-2 is detected in premalignant lesions and its levels seem to increase during tumor progression (6–8). The importance of COX-2 in survival and response to therapy has been elegantly shown by Edelman and colleagues (9) who reported that non–small cell lung cancer patients expressing high levels of COX-2 in their tumors have reduced survival compared with patients expressing low levels of COX-2. Patients with high tumor expression of COX-2 benefit from the combination of carboplatin and gemcitabine plus the COX-2 inhibitor, celecoxib, whereas patients with low expression exhibit a poorer response to carboplatin/gemcitabine/celecoxib than to carboplatin/gemcitabine alone (9).

Positron emission tomography or single-photon emission computed tomography imaging agents (18F-, 11C-, or 123I-labeled COX-2 inhibitors) have been described for nuclear imaging (10–17). These have all been based on the diarylhetrocycle structural class analogous to celecoxib and rofecoxib. Although selective uptake into macrophages or tumor cells expressing COX-2 has been shown in vitro for some compounds, such selectivity has not been rigorously shown in vivo and significant nonspecific binding has been observed (18). Thus, despite recognition of the potential of COX-2–targeted imaging agents, in vivo proof-of-concept for this strategy is lacking.

Fluorescent COX-2 inhibitors are attractive candidates as targeted imaging agents. Such compounds have the advantage that each molecule bears the fluorescent tag and the compounds are nonradioactive and stable. Thus, they can be used conveniently for cellular imaging, animal imaging, and clinical imaging of tissues in which topical or endoluminal illumination is possible (e.g., esophagus, colon, and upper airway through endoscopy, colonoscopy, and bronchoscopy, respectively). Prior work from our laboratory showed that fluorescent COX-2 inhibitors can be useful biochemical probes of protein binding but these earlier compounds were...
and [1-14C]arachidonic acid (50 μmol/L, approximately 55–57 mCi/mmol, Perkin-Elmer). For the time-dependent inhibition assay, hematin-reconstituted COX-1 (44 nmol/L) or COX-2 (66 nmol/L) was preincubated at 25°C for 17 minutes and 37°C for 3 minutes with varying inhibitor concentrations in DMSO followed by the addition of [1-14C]arachidonic acid (50 μmol/L) for 30 seconds at 37°C. Reactions were terminated by solvent extraction in Et2O/CH3OH/1 mol/L citrate (pH 4.0; 30:4:1). The phases were separated by centrifugation at 2,000 g for 2 minutes and the organic phase was spotted on a TLC plate (EMD Kieselgel 60, VWR). The plate was developed in EtOAc/CH2Cl2/glacial AcOH (75:25:1) and radiolabeled products were quantified with a radioactivity scanner (Bioscan, Inc.). The percentage of total products observed at different inhibitor concentrations was divided by the percentage of products observed for cells preincubated with DMSO.

**Materials and Methods**

Synthesis and characterization of all compounds is described in Supplementary Data.

**Inhibition assay using purified COX-1 and COX-2.** Cyclooxygenase activity of ovine COX-1 or human COX-2 was assayed by a method that quantifies the conversion of [1-14C]arachidonic acid to [1-14C]prostaglandin products. Reaction mixtures of 200 μL consisted of hematin-reconstituted protein in 100 μmol/L Tris-HCl (pH 8.0), 500 μmol/L phenol, and [1-14C]arachidonic acid (50 μmol/L, approximately 55–57 mCi/mmol, Perkin-Elmer). For the time-dependent inhibition assay, hematin-reconstituted COX-1 (44 nmol/L) or COX-2 (66 nmol/L) was preincubated at 25°C for 17 minutes and 37°C for 3 minutes with varying inhibitor concentrations in DMSO followed by the addition of [1-14C]arachidonic acid (50 μmol/L) for 30 seconds at 37°C. Reactions were terminated by solvent extraction in Et2O/CH3OH/1 mol/L citrate (pH 4.0; 30:4:1). The phases were separated by centrifugation at 2,000 g for 2 minutes and the organic phase was spotted on a TLC plate (EMD Kieselgel 60, VWR). The plate was developed in EtOAc/CH2Cl2/glacial AcOH (75:25:1) and radiolabeled products were quantified with a radioactivity scanner (Bioscan, Inc.). The percentage of total products observed at different inhibitor concentrations was divided by the percentage of products observed for cells preincubated with DMSO.

**Fluorescence microscopy of RAW264.7 cells or 1483 HNSCC cells.** RAW264.7 cells were plated on 35-mm MatTek dishes (MatTek Corp.) such that the cells were 40% confluent and human 1483 HNSCC cells were 60% confluent on the day of the experiment. The RAW264.7 cells were activated for 6 hours in serum-free DMEM with 200 ng/mL LPS and 10 μg/mL IFN-γ. Both cell lines were incubated in 2.0 mL HBSS/Tyrode’s buffer with 200 nmol/L compound 1, 2, or 3 for 30 minutes at 37°C. To block the COX-2 active site, the cells were preincubated with 10 μmol/L indomethacin or 5 μmol/L celecoxib for 20 minutes before the addition of compound 1 or 2. The cells were then washed briefly thrice and incubated in HBSS/Tyrode’s buffer for 30 minutes at 37°C. Following the required washout period, the cells were imaged in 2.0 mL fresh HBSS/Tyrode’s buffer on a Zeiss Axiosvert 25 Microscope with the propidium iodide filter (0.5–1.0 second exposure, gain of 2). All treatments were performed in duplicate dishes in at least three separate experiments.

**Confocal microscopy of 1483 cells treated with compound 2/mitotrackerGR.** 1483 HNSCC were plated in MatTek dishes (MatTek Corp.) and grown to 60% to 70% confluence for 48 hours. DMSO or compound 2 (100 nmol/L) was added to each dish containing 2.0 mL HBSS/Tyrode’s buffer for 30 minutes at 37°C. After four quick HBSS washes, cells were incubated for 30 minutes in 2.0 mL HBSS/Tyrode’s buffer and imaged with a Zeiss LSM510 confocal microscope using a 63 × 1.4 NA plan-Apochromat objective lens. To visualize cellular mitochondria, 100 nmol/L Mitotracker GR was added for 15 minutes at 37°C followed by three quick washes before imaging. Four hundred eighty-eight nanometer excitation was used to image Mitotracker GR through a 500- to 530-nm bandpass filter and compound 2 was imaged using 532 nm excitation and collection through a 565- to 615-nm bandpass filter. The pinhole was set to 1 Airy unit and images were collected throughout the focus of the cells. To assure a full sampling of the perinuclear region, analysis was performed on the optical sections through the middle of the nucleus.

**In vivo imaging of COX-2 in inflammation.** Carrageenan (50 μL 1% in sterile saline) was injected in the rear left footpad of female C57BL/6 mice, followed by compound 1 or 2 (1 mg/kg, i.p.) at 24 hours postcarrageenan. Animals were grown at passage 8 to 18, Mycoplasma negative by a PCR detection method, in DMEM/F12 + 10% FBS + Antibiotic/Antimycotic in six-well plates to 60% confluence. Serum-free medium (2 mL) was added and the cells were treated with inhibitor dissolved in DMSO (0–5 μmol/L, final concentration) for 30 minutes at 37°C followed by the addition of [1-14C]-arachidonic acid (10 μmol/L, approximately 55 mCi/mmol) for 20 minutes at 37°C. Reactions were terminated by solvent extraction in EL2O/CH2OH/1 mol/L citrate (pH 4.0; 30:4:1) and the organic phase was spotted on a 20 × 20 cm TLC plate (EMD Kieselgel 60, VWR). The plate was developed in EtOAc/CH2Cl2/glacial AcOH (75:25:1) and radiolabeled products were quantified with a radioactivity scanner (Bioscan, Inc.). The percentage of total products observed at different inhibitor concentrations was divided by the percentage of products observed for cells preincubated with DMSO.

**Cell culture and in vitro intact cell metabolism assay.** HCT116, ATCC CCL-247 human colorectal carcinoma cells, passage 8 to 18, Mycoplasma negative by a PCR detection method (Sigma VenorGena) were grown in DMEM (Invitrogen/Life Technologies) + 10% fetal bovine serum (FBS; Atlas) to 70% confluence. RAW264.7, ATCC TIB-71 murine macrophage-like cells, passage number 8 to 15, Mycoplasma negative by a PCR detection method were grown in DMEM + 10% heat-inactivated FBS to 40% confluence (six-well plates, Sarstedt) and activated for 7 hours in 2 mL serum-free DMEM with 200 ng/mL lipopolysaccharide (LPS; Calbiochem) and 10 μg/mL IFN-γ (Calbiochem). Human 1483 head and neck squamous cell carcinoma (HNSCC) cells (20), derived, characterized, and provided by Dr. Peter Sacks (New York University School of Dentistry, New York, NY), were negative by a PCR detection method, in DMEM/F12 + 10% FBS + Antibiotic/ Antimycotic in six-well plates to 60% confluence. Serum-free medium (2 mL) was added and the cells were treated with inhibitor dissolved in DMSO (0–5 μmol/L, final concentration) for 30 minutes at 37°C followed by the addition of [1-14C]-arachidonic acid (10 μmol/L, approximately 55 mCi/mmol) for 20 minutes at 37°C. Reactions were terminated by solvent extraction in EL2O/CH2OH/1 mol/L citrate (pH 4.0; 30:4:1) and the organic phase was spotted on a 20 × 20 cm TLC plate (EMD Kieselgel 60, VWR). The plate was developed in EtOAc/CH2Cl2/glacial AcOH (75:25:1) and radiolabeled products were quantified with an absorbance scanner (Bioscan, Inc.). The percentage of total products observed at different inhibitor concentrations was divided by the percentage of products observed for cells preincubated with DMSO.

www.aacrjournals.org Cancer Res; 70(9) May 1, 2010 3619

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2010 American Association for Cancer Research.
were imaged 3 hours later in a Xenogen IVIS 200 (DsRed filter, 1.5 cm depth, 1 s). For comparison, animals also were
dosed with compound 3, which does not inhibit COX-2. To test
further the molecular target for compound 2, parallel experi-
ments were performed using COX-2 (−/−) mice. Experiments
also were performed in which compound 1 was administered
to the same animals by repetitive i.p. injection on days 1, 3, 5,
and 7 to monitor the time course of compound uptake follow-
ing carrageenan induction of inflammation.

Establishment of xenografts in nude mice. Female nude
mice, NU-Fox1nu, were purchased at 6 to 7 weeks of age
from Charles River Laboratories. Human 1483 HNSCC cells
and HCT116 colorectal carcinoma cells were trypsinized
and resuspended in cold PBS containing 30% Matrigel such
that 1 × 10⁶ cells in 100 μL were injected s.c. on the left flank.
The HCT116 or 1483 xenografts required only 2 to 3 weeks of
growth.

In vivo imaging of nude mice with xenografts. Female nude
mice bearing medium-sized HCT116 or 1483 xenograft
tumors on the left flank were dosed by i.p. injection with
2 mg/kg compound 2 or by retro-orbital injection with
1 mg/kg compound 2. The animals were lightly anesthetized
with 2% isoflurane for fluorescence imaging in the Xenogen
IVIS 200 with the DsRed filter at 1.5-cm depth and 1-second
exposure (2). For the COX-2 active site–blocking experi-
ments, nude mice bearing 1483 xenografts were predosed by
i.p. injection with 2 mg/kg indomethacin at 24 hours
and 1 hour before dosing with compound 2 (2 mg/kg, i.p.).

Pharmacokinetics of candidate compounds. Female nude
mice with medium-sized 1483 HNSCC xenograft
tumors on the left flank were injected i.p. with 2 mg/kg com-
ound 2. At 0, 0.5, 3, 12, and 24 hours, the mice (n = 4 for each
time point, duplicate experiments) were anesthetized with
isoflurane. Blood samples were taken by cardiac puncture
into a heparinized syringe into a 1.5-mL heparinized tube
on ice, followed by removal of the liver, kidney, contralateral
leg muscle, and xenograft tumor. All organs/tissues were
rinsed briefly in ice-cold PBS, blotted dry, weighed, and
snap frozen in liquid nitrogen. The blood samples were
centrifuged at 4°C at 6,000 rpm for 5 minutes and the
plasma was transferred to clean tubes and frozen at
80°C.

Compound 2 was extracted by homogenizing the tissue
into a heparinized syringe into a 1.5-mL heparinized tube
on ice, followed by removal of the liver, kidney, contralateral
leg muscle, and xenograft tumor. All organs/tissues were
rinsed briefly in ice-cold PBS, blotted dry, weighed, and
snap frozen in liquid nitrogen. The blood samples were
centrifuged at 4°C at 6,000 rpm for 5 minutes and the
plasma was transferred to clean tubes and frozen at
–80°C.

Compound 2 was extracted by homogenizing the tissue
in 100 mmol/L Tris (pH 7.0) buffer and mixing an ali-
quots of the homogenate with 1.2× volume of acetonitrile.
The acetonitrile was removed and the samples were dried,
reconstituted, and analyzed through reversed-phase high-
performance liquid chromatography (HPLC)-UV using a
Phenomenex 10 × 0.2 cm C18 or a Phenomenex 7.5 × 0.2 cm
Synergi Hydro-RP column held at 40°C. The samples were
quantified against a standard curve prepared by adding
compound 2 to tissue homogenates of undosed animals
followed by the workup described. Cocrhomatography was
performed with multiple columns and elution conditions as
described in the Supplemental Data.

In vivo imaging of Min mice. C57BL/6 APC-Min mice
maintained on a high-fat (11%) diet for 18 weeks developed
20 to 30 intestinal polyps per mouse. Before imaging, Min
mice were anesthetized (2% inhaled isoflurane) for retro-
orbital injection of compound 2 at 1 mg/kg. At 2 hours post-
jection, the mice were euthanized and the intestines were
resected, washed with PBS, and fixed in 10% formalin before
ex vivo imaging by fluorescence dissecting microscopy (Zeiss
M2Bio; n = 5).

Results

COX-2 is a potentially ideal target for molecular imaging
because its active site (and the active site of COX-1) is buried
deep inside each subunit of the homodimeric protein (21–23).
Access to the active site is controlled by a constriction
that separates it from a large opening in the membrane-
binding domain that we have termed the lobby (Supple-
mentary Fig. S1). All substrates or inhibitors bind in the lobby
and then diffuse through the constriction into the active site
(24). The constriction is composed of Tyr-355, Glu-524, and
Arg-120 and serves as the binding site for the carboxylic acid
group of substrates and certain inhibitors (25). We have
reported that neutral derivatives (esters and amides) of certain
carboxylic acid inhibitors (e.g., indomethacin) bind to COX-2
but not to COX-1 (26). A three-dimensional structure of
COX-2 complexed to such a conjugate has not been solved
but structures of related complexes suggest the indomethacin
unit binds in the active site with the tethered amide, breech-
ing the constriction and projecting into the lobby (22, 27).
These structural and functional analyses provide the design
principles for the construction of COX-2–targeted imaging
agents.

Synthesis of candidate compounds and cellular imaging.
Three carboxylic acid cores—i.e., indomethacin, a celecoxib
carboxylic acid derivative, and an indolyl carboxamide ana-
logue of indomethacin—were tethered through a series of
alkylenediamines, pipерazines, polyethylene glycol, or pheny-
lediamines to a diverse range of fluorophores. The fluo-
rophores attached included dansyl, dabsyl, coumarin,
fluorescein, rhodamine, alexa-fluor, nile blue, cy5, cy7, near
IR, and IR dyes as well as lanthanide chelators. Nearly
200 compounds were synthesized and each conjugate was
tested for its ability to selectively inhibit COX-2 in assays
using purified proteins in vitro. Promising molecules were
tested for their ability to inhibit COX-2 in LPS-treated
RAW264.7 macrophages. Preliminary experiments indicated
that indomethacin conjugates bound most tightly and selec-
tively to COX-2; therefore, most of the compounds synthe-
sized were derived from this core.

Indomethacin conjugates to dansyl, dabsyl, coumarin,
fluorescein, and rhodamine-derived fluorophores exhibited
promising COX-2 inhibition and selectivity both in vitro
and in intact cells. The carboxy-X-rhodamine (6-ROX- and
5-ROX)–based conjugates, 1 and 2, displayed the best bal-
ance of cellular activity and optical properties (λmax = 581
nm, Φmax = 603 nm) and were used for all subsequent experi-
ments (Table 1). A detailed kinetic analysis indicated that
1 and 2 require lengthy preincubations with COX-2 to
achieve maximal inhibition but once bound, they dissociate
very slowly (Supplementary Fig. S2). Thus, they are slow,
tight-binding inhibitors with very low rates of association

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>Structure</th>
<th>Purified enzyme IC₅₀ (μmol/L)</th>
<th>Cell IC₅₀ (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>COX-1</td>
<td>COX-2</td>
</tr>
<tr>
<td>1</td>
<td><img src="image1" alt="Structure" /></td>
<td>&gt;25</td>
<td>0.83</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2" alt="Structure" /></td>
<td>&gt;25</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3" alt="Structure" /></td>
<td>&gt;25</td>
<td>&gt;4</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4" alt="Structure" /></td>
<td>&gt;25</td>
<td>&gt;4</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5" alt="Structure" /></td>
<td>&gt;25</td>
<td>&gt;4</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Structure" /></td>
<td>0.92</td>
<td>0.21</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="Structure" /></td>
<td>&gt;25</td>
<td>0.14</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8" alt="Structure" /></td>
<td>0.05</td>
<td>0.75</td>
</tr>
</tbody>
</table>

NOTE: Assays were conducted as described in Materials and Methods. IC₅₀ for inhibition of ovine COX-1 or human COX-2. Compounds also were tested in intact RAW264.7 macrophages (Cell IC₅₀).

Abbreviations: ND, no inhibition detected up to 5 μmol/L; NT, not tested.
and dissociation. Compounds 1 and 2 were less potent than celecoxib or rofecoxib as inhibitors of COX-2 (Table 1). A negative control molecule (3) was synthesized that contained 6-ROX bound to indomethacin through a shorter ethylenediamine tether, which eliminated COX-2 inhibition (Table 1).

The human head and neck cancer cell line, 1483, which expresses high levels of COX-2 (28), exhibited strong labeling with compounds 1 or 2 (Fig. 1A). Preincubation of the cells with the COX-2–selective inhibitor, celecoxib, prevented the labeling of 1483 cells by either compound (Fig. 1B). In all of these in vitro experiments, the labeling seemed to be intracellular, so confocal microscopy was performed to verify the localization. Incubation of compound 2 with 1483 cells led to the perinuclear labeling of membraneous structures that appeared to be endoplasmic reticulum or Golgi (Fig. 1C). The perinuclear labeling correlated well to multiple previous reports of the intracellular localization of COX-2 (29–32). Incubation of the same cells with Mitotracker showed that the mitochondria did not colocalize with compound 2 (Fig. 1C).

The mouse macrophage–like cell line, RAW 264.7, does not express COX-2 and exhibited very weak labeling with 1 or 2 (e.g., Supplementary Fig. S3A), whereas LPS-pretreated cells labeled more strongly (Supplementary Fig. S3B). The labeling of the COX-2–expressing RAW cells by 1 or 2 was prevented by pretreatment of the cells with indomethacin (Supplementary Fig. S3C) or celecoxib, which block the COX-2 active site. Importantly, no labeling was observed when either control or LPS-pretreated RAW cells were incubated with compound 3, which does not inhibit COX-2 (Supplementary Fig. S3D). The extent of compound 2 uptake increased at 4 hours with the appearance of COX-2 protein. A further increase in uptake was not observed at 7 hours although there was higher COX-2 protein as detected by Western blotting (Supplementary Fig. S4). Comparison of the amount of compound 2 taken up at 7 hours to the amount of COX-2 estimated by Western blotting in the LPS-treated cells suggested a stoichiometry of binding of 0.90 (Supplementary Data).

**Imaging carrageenan–induced inflammation.** Compounds 1 and 2 seemed promising based on these in vitro imaging experiments, so their potential for in vivo imaging was evaluated using carrageenan-induced inflammation in the mouse footpad, human tumor xenografts in nude mice, and spontaneous tumors arising in mouse models. The mouse footpad model is well documented for the role of COX-2–derived prostaglandins as a major driving force for the acute edema that results 24 hours after carrageenan injection into the paw (33). One of the significant advantages of this animal model of inflammation is the ability to image the inflamed footpad compared with the noninflamed contralateral footpad, which does not express COX-2. We injected female C57BL/6 mice with 50 μL 1% carrageenan in the rear left footpad, followed by compound 1 or 2 (1 mg/kg, i.p.) at 24 hours postcarrageenan. Animals were imaged 3 hours later in a Xenogen IVIS 200 (DsRed filter, 1.5 cm depth, 1 s). Both compounds 1 and 2 targeted the swollen footpad with an average 4.5-fold increase in fluorescence over that of the contralateral, uninjected footpad (Fig. 2). For comparison,
animals also were dosed with compound 3, which does not inhibit COX-2. In Fig. 2A, the left mouse was dosed with compound 3 and the right mouse with compound 1. Compound 3 yielded minimal fluorescence in the inflamed paw compared with the contralateral paw, whereas compound 1 yielded a strong signal in the inflamed paw. To test further the molecular target of compounds 1 or 2, parallel experiments were performed using mice bearing targeted deletions in COX-2. Figure 2B depicts the fold difference in the compound 2–derived fluorescence signal in the 24-hour carrageenan-injected footpad over the control footpad for wild-type versus COX-2 (−/−) mice. The COX-2 null mice consistently showed approximately a 40% increase in signal in the swollen footpad apparently due to nonspecific binding. This contrasts with a 400% to 600% increase in the swollen footpad in wild-type mice. Finally, experiments were conducted to evaluate the uptake of compound 1 during the resolution of inflammation. Following carrageenan injection, compound 1 was administered i.p. 1, 3, 5, and 7 days later and the animals were imaged. Uptake of compound 1 was maximal at 24 hours but declined thereafter (Fig. 2C). Attempts to estimate active COX-2 protein by the quantification of prostaglandins in paw extracts were unsuccessful because of poor recovery.

Imaging COX-2–expressing tumors. The results in the footpad inflammation model show that COX-2–targeted fluorescent conjugates are taken up in inflamed paws of COX-2–expressing mice but not in COX-2 null animals. We next evaluated the ability of these compounds to target COX-2 in human tumor xenografts. Female nude mice were injected in the left flank with HCT-116 or 1483 cells and the xenografts were allowed to grow to approximately 750 to 1,000 mm³. Animals were dosed by retro-orbital injection with compound 2 (1 mg/kg) then lightly anesthetized with 2% isoflurane in preparation for imaging. No fluorescence was observed during the first 60 minutes postinjection, but signal was reproducibly detected in the COX-2–expressing 1483 tumors starting at 3 to 5 hours and persisted as long as 26 hours postinjection. At 3.5 hours postinjection, the HCT116 tumor, which does not express COX-2 (34), showed minimal fluorescence (Fig. 3A) whereas the 1483 tumor exhibited bright fluorescence (Fig. 3B). In another control experiment, nude mice bearing 1483 xenografts were treated with the fluorophore alone, 5-ROX (2 mg/kg, i.p.), which is neither an inhibitor of COX-2 nor COX-1. No signal from 5-ROX alone accumulated in the tumors at any time point. This result showed that the fluorophore moiety was not responsible for the tumor uptake of compound 2, supporting the conclusion that the difference in labeling of 1483 and HCT116 xenografts is due their differential in COX-2 expression.

Nude mice with 1483 xenografts were pretreated with either DMSO or indomethacin in DMSO (2 mg/kg, i.p.) before compound 2 dosing (2 mg/kg, i.p.). At 3 hours postinjection, the DMSO-pretreated mice showed strong fluorescence in their tumors (Fig. 3C) compared with weak signals in the tumors of the indomethacin-pretreated mice (Fig. 3D). In the mouse xenograft model, indomethacin was able to block

Figure 2. In vivo labeling of COX-2–expression in inflammation by compound 1, 2, or 3. A, C57BL/6 mouse with carrageenan-induced inflammation in the left foot pad. The left mouse was dosed with the negative control molecule 3 (1 mg/kg, i.p.) and the right mouse was dosed with compound 1 (1 mg/kg, i.p.). Both mice were imaged at 3 h postinjection. B, fold increase of fluorescence in inflamed versus contralateral paw of wild-type (WT) and COX-2 (−/−) mice at 3 h postinjection of compound 2 (1 mg/kg, i.p.; n = 6). RFU, relative fluorescence unit. C, carrageenan was injected in the rear left footpads of female C57BL/6 mice, followed by dosing compound 1 (1 mg/kg, i.p.) 24 h later. Animals were reinfected with compound 1 at 3, 5, and 7 d postcarrageenan (n = 9). Mice were imaged at 3 h after compound injection. The plot shows the fold increase of fluorescence in swollen versus contralateral foot (n = 6).
92 ± 6% (n = 8) of the COX-2–expressing tumor uptake of compound 2.

We next investigated whether the COX-2 inhibitory activity of our imaging probes correlated with their in vivo efficacy in targeting COX-2–expressing tumors. Nude mice bearing 1483 xenografts were dosed (2 mg/kg, i.p.) with compound 4 (no COX inhibition at 3 μmol/L), compound 5 (30% COX-2 inhibition at 3 μmol/L), and compound 2 (90% COX-2 inhibition at 3 μmol/L; Table 1). At 3.5 hours postinjection, fluorescence from the tumor region was directly proportional to the compound potency as a COX-2 inhibitor (Supplementary Fig. S5).

Experiments were conducted to determine the identity of the fluorescent material(s) detected in vivo and to monitor the time course of its distribution and tissue uptake following injection of compound 2 into nude mice bearing 1483 human tumor xenografts. Extracts of plasma, liver, kidney, tumor, and adjacent muscle were quantitatively analyzed by HPLC at different times after i.p. administration of the compound. A single fluorescent compound was detected in all the extracts, which coeluted with a standard of compound 2 in multiple HPLC systems. This compound displayed an identical mass spectrum to the unmetabolized parent molecule, compound 2 (Fig. 4A). The time courses of uptake and distribution of compound 2 in plasma and various tissues are displayed in Fig. 4B. Compound 2 was rapidly distributed following i.p. administration and reached nearly maximal levels in plasma, liver, and kidney 30 minutes after injection. Compound 2 levels declined substantially over the course of 12 to 24 hours to a small fraction of its initial levels in all three of these compartments. In contrast, the time course for uptake of compound 2 into the 1483 tumors lagged substantially and required ∼3 hours to reach near maximal levels. The levels of compound 2 remained relatively high in the tumor so by 24 hours, the tumor levels were as high as the levels in liver or kidney. This indicates both slow uptake and release of compound 2 into and out of the tumor.

APCmin mice bear the same mutation (Apc−) that is causative for familial adenomatous polyposis in human beings and these mice primarily develop small intestinal tumors that express COX-2 (35, 36). Crossing APCmin mice with COX-2 (−/−) mice reduces intestinal tumor development by 85% and treatment of APCmin mice with COX-2 inhibitors also reduces tumorigenesis (37, 38). APCmin mice (ages 18–20 wk, fed a high-fat diet) were injected retro-orbitally with compound 2 (1 mg/kg), and after 2 hours, animals were sacrificed and their intestines were removed. The tissue was washed thoroughly with PBS, opened longitudinally, and imaged. Figure 5A shows the low background fluorescence of a section of small intestine without polyps. A single polyp (Fig. 5B) and a five-polyp cluster (Fig. 5C) displayed high fluorescence, with greatly increased detection compared with bright-field visualization. The signal enrichment of compound 2 in the polyps was estimated to be >50:1. COX-2 expression in the polyps seems to be required for this selective uptake although other factors beside the level of COX-2 protein may contribute to the relative enrichment over surrounding normal tissue.

Discussion

These studies show the feasibility of specific in vivo targeting of COX-2 in inflammatory lesions and tumors using organic fluorophores tethered to indomethacin through an amide linkage. Compounds 1 and 2 display a very high degree of selectivity of uptake by inflammatory tissue and tumors in live animals relative to surrounding normal tissue or muscle as determined by either imaging or mass spectrometry. This selectivity seems greater than that reported in previous literature reports of fluorescent tumor imaging in which the ratio of tumor fluorescence was compared with muscle fluorescence (39). Uptake of our compounds requires the expression
of COX-2 at the target site and declines as the level of COX-2 decreases. Although uptake into inflamed or tumor tissue seems to be slower than expected from simple distribution in the body, the kinetics of compound release seem to be extremely slow, thus leading to a detectable buildup of the label. Similar results are observed by both imaging compounds 1 or 2 (Figs. 2 and 3) or by direct quantitative analysis of compound 2 (Supplementary Fig. S4; Fig. 4).

To achieve this success, ~200 compounds were evaluated as candidate COX-2–targeted imaging agents. Although a significant percentage showed COX-2 inhibitory activity against purified protein, only a fraction of these compounds inhibited COX-2 activity in intact cells, and of those, most did not possess fluorescence properties suitable for in vivo imaging. Among the compounds that emerged from our development pathway, only compounds 1 and 2 exhibited sufficient metabolic stability to survive long enough to distribute to inflammatory lesions or xenograft tumors. The low overall success rate (~1%) likely underscores why COX-2–targeted imaging agents have proven difficult to develop.
The specificity for COX-2 binding of these compounds was illustrated by multiple observations: (a) only cultured cells that express COX-2 took up fluorocoxibs and uptake was inhibited by the COX inhibitors indomethacin and celecoxib. The intracellular localization of the probes matches that of COX-2 protein and the stoichiometry of uptake was ∼0.9 molecule of beacon per subunit; (b) uptake into inflamed over noninflamed tissue was blocked by indomethacin pretreatment of the animals and was not observed in COX-2 (−/−) animals. No uptake was observed with a close structural analogue of compound 1 that does not inhibit COX-2; (c) uptake into COX-2–expressing tumors was blocked by indomethacin pretreatment of the animals and a correlation was found between the amount of light emission from the tumor and the COX-2 inhibitory potency of the beacon. The nontargeted fluorophores 5-ROX and 6-ROX did not accumulate in COX-2–expressing xenografts; and (d) >95% of the fluorescent material present in the tumors is the unmetabolized parent compound. Thus, in vitro and in vivo studies provide strong support for the conclusion that binding to COX-2 is the major determinant of uptake into inflamed, premalignant, or malignant tissue. Although the stoichiometry of compound 2 binding to COX-2 protein was estimated to be 0.9 in activated RAW cells, such high stoichiometry cannot be assumed in all situations. The extent of uptake in cells, inflamed tissue, or tumors will depend upon several factors such as the permeability of COX-2–expressing cells to the probe, kinetics of binding and release from the COX-2 active site, vascularization of the tissue, and possible expulsion of the probes by transporters. Further studies will be needed to explore in greater detail the quantitative aspects of the use of these compounds in vivo.

Compounds 1 and 2 represent the first feasible reagents for clinical detection of tissues containing high levels of COX-2 in settings amenable to fluorescent excitation and analysis by surface measurement or endoscopy (e.g., skin, esophagus, intestine, and bladder). Although such fluorocoxibs will not be useful for applications in internal organs that are not accessible for optical imaging, their development provides rigorous proof of concept for the feasibility of molecular targeting of COX-2 in inflammatory lesions, premalignant lesions, and tumors.

** Disclosure of Potential Conflicts of Interest**

L.J. Marnett: sponsored research and consulting, XL Tech Group. The other authors disclosed no potential conflicts of interest.

**Acknowledgments**

We thank S.K. Dey for the COX-2 null animals and Melissa Turman for the assistance with molecular graphics.

**Grant Support**

Research and Center grants from the NIH (CA86283, CA89450, CA105296, CA68485, CA60867, CA126588, CA111469, and GM72048), the Medical Free-Electron Laser Program of the US Department of Defense, XL TechGroup, and New York Crohn’s Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 07/16/2009; revised 02/20/2010; accepted 02/23/2010; published online 04/29/2010.

**Figure 5.*** In vivo labeling of COX-2–expression in intestinal polyps by compound 2. C57BL/6J-Min/+ mice bearing small intestinal polyps were euthanized at 2 h after retro-orbital injection of compound 2 (1 mg/kg) and small intestines were washed, opened, and examined by dissecting fluorescence microscopy. A, section of small intestine with no polyp, 90-millisecond exposure. B, single polyp, 90-millisecond exposure. C, polyp cluster, 90-millisecond exposure.


Selective Visualization of Cyclooxygenase-2 in Inflammation and Cancer by Targeted Fluorescent Imaging Agents


Cancer Res 2010;70:3618-3627.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/70/9/3618

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/05/10/70.9.3618.DC1

Cited articles
This article cites 39 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/9/3618.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/70/9/3618.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.