Synthesis and Evaluation of a ^{18}F -Labeled 4-Ipomeanol as an Imaging Agent for CYP4B1 Gene Prodrug Activation Therapy

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Abstract

We report the development of a ^{18}F -labeled 4-ipomeanol (4-IM), which is metabolized by the CYP4B1 enzyme, to image tumors and monitor enzyme-activating anticancer prodrugs. The fluorine-substituted derivative, 1-(3-furyl)-4-hydroxy-5-fluoro-1-pentanone (F-4-IM, **1**), was synthesized from 3-furaldehyde. [^{18}F]F-4-IM ([^{18}F]**1**) was prepared in 20%–35% radiochemical yield by a fluorine-18 displacement reaction, followed by reduction and deprotection of the ketal group, and was shown to be stable (>96% at 2 hours) in human serum at 37°C. The biodistribution of [^{18}F]F-4-IM in normal rats was high in the lung, where CYP4B1 gene is preferentially expressed. We transduced C6-glioma cells with a retrovirus-expressing CYP4B1 (C6-CYP4B1). Evaluation of CYP4B1 expression was confirmed by reverse transcription polymerase chain reaction and MTT assay. Cell assays were carried out using C6 and C6-CYP4B, and the uptake of [^{18}F]F-4-IM in these cells was compared with that in parental controls. The uptake ratio of [^{18}F]F-4-IM was 2.8-fold higher in C6-CYP4B1 compared with that in parental cells at 1 hour, whereas [^3H]4-IM was taken up at similar rates in both cell lines after 6 hours. These results suggest that [^{18}F]F-4-IM could be a promising PET imaging agent with potential to be used for imaging of CYP4B1-transfected tumor cells, as well as for monitoring CYP4B1 enzyme/prodrug interactions.

Keywords: 4-ipomeanol, CYP4B1, fluorine-18, gene prodrug activation therapy

Introduction

Gene prodrug activation therapy (GPAT), also known as suicide gene therapy for cancer, is an approach in which the activation of a prodrug by an exogenously expressed enzyme in tumor cells leads to tumor cell death.¹ Among the many enzyme-prodrug systems, cytochrome P450 (CYP) enzymes play a role in the oxidative metabolism of numerous drugs, chemical carcinogens, and environmental contami-

nants.² The CYP4B1 isozyme efficiently converts the prodrugs, 4-ipomeanol (4-IM), **2** and 4-aminoanthracene (4-AA) to anticancer alkylating metabolites, which are highly reactive and bind covalently to cells at the site of conversion. CYP4B1 is found in many rodents and ruminants, but not in humans. 4-IM is a naturally occurring pulmonary cytotoxin that was first isolated from sweet potatoes roots infected with a common fungus,^{3–5} and its bioactivity in cell culture and animal models suggested it could be a potential cancer

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chemotherapeutic agent. However, human clinical trials have shown that 4-IM had unexpected hepatotoxicity and lacked efficacy.^{6–10} In contrast, Smith *et al.* showed that overexpression of CYP4B1 increased the sensitivity of normal murine cells to 4-IM.¹¹ Rabbit CYP4B1 efficiently converts the prodrug 4-IM to alkylating metabolites, which are especially cytotoxic to tumor cells, whereas the equivalent human CYP4B1 isozyme have low activity (<1%) against this compound.^{2,12} Therefore, transfer of rabbit CYP4B1 to tumor cells might lead to their death by conversion of the 4-IM or 2-AA to highly toxic alkylating metabolites. The major pathway responsible for the metabolism of 4-IM by CYP enzymes involves furan ring opening, leading to the formation of unsaturated α,β -aldehyde/ketones of dialdehyde reactive metabolites that bind immediately to nucleophilic tissue macromolecules near the site of formation.^{6,7,13–17} Recently, 4-IM has been studied as a potential prodrug for P450-directed gene therapy in liver and brain cancer.^{12,18,19}

Among the radionuclides, positron-emitting fluorine-18 has adequate physical properties for applications that require imaging gene expression. Here we designed fluorine-18 labeled 4-IM (Fig. 1) to monitor the mechanism of rabbit CYP4B1-mediated prodrug conversion in rat C6-glioma cells (C6). To label the proximal end of carbon (1-(3-furyl)-4-hydroxy-5-[¹⁸F]fluoro-1-pentanone, [¹⁸F]4-IM), we prepared the mesylated precursor that could be readily labeled with the fluoride-18 ion. Subsequently, we reduced it at the carbonyl group and hydrolyzed at the ketal group. In this report, we describe the details of the radiofluorination of ligand-targeting CYP4B1 expression, as well as its metabolic stability, *in vivo* tissue biodistribution in normal rats, and the uptake in CYP4B1 gene transfected C6-glioma cells (C6-CYP4B1).

Materials and Methods

Reagents and equipment

Chemicals and reagents were purchased from Sigma-Aldrich and High-performance liquid chromatography (HPLC) solvents from Fisher Scientific. ¹H and ¹³C NMR spectra were recorded on a Varian Gemini-200 or Varian Gemini-400 and chemical shifts were reported in parts per million (ppm, δ units). Electron impact (EI) and chemical ionization (CI) mass spectra were obtained on a GC/MS QP5050A spectrometer (Shimadzu). Fast atom bombardment (FAB) mass spectra were obtained on a JMS 700 (Jeol Ltd.). HPLC was carried out on a Waters Co. system with a semi-preparative column (Alltech, Econosil silica gel, 10 μ , 10 $\times$ 250 mm), and simultaneously monitored by a Waters 2487 instrument (254 nm) and Raytest GABI γ -detector. Radio-TLC was performed on Merck F₂₅₄ silica plates and

analyzed on a Bioscan AC-3000 scanner. An automated gamma counter (1480 Wizard 3[™]; Perkin Elmer Life Sciences) was used to measure radioactivity in the cell binding uptake and biodistribution studies. Total RNA Extraction Kit (Intron Biotechnology) was used for the preparation of RNA in cells. MTT cell growth and colorimetric cell growth assays were performed using an MTT assay kit (Sigma-Aldrich). The 2-aminoanthracene (2-AA) used in prodrug treatments was purchased from Sigma-Aldrich.

Synthesis

The preparation of target compound, 1-(3-furyl)-4-hydroxy-5-fluoro-1-pentanone (**1**), and mesylate **12** as a precursor for fluorine-18 labeling were synthesized in 12 and 9 steps, respectively, from 3-furaldehyde as shown in Scheme 1.

Structural parameters

2-(3-Furyl)-1,3-dithiane (4). To a solution of 1,3-propanedithiol (2.08 mL, 20.73 mmol) in dried tetrahydrofuran (30 mL), was carefully treated with borontrifluoride-diethyletherate (2.63 mL, 20.73 mmol) under a nitrogen atmosphere at 0°C. After 3-furaldehyde (1.79 mL, 18.84 mmol) was added, the reaction mixture was refluxed for 1 hour. The reaction was quenched by crashed-ice, and then saturated sodium bicarbonate was poured. The crude product was extracted by methylene chloride (20 mL $\times$ 3), dried over anhydrous sodium sulfate. The product **4** (3.4 g, 92%) was crystallized with ethylacetate and hexane as a white crystal: mp 64.4°C–66.0°C; ¹H NMR (200 MHz, CDCl₃) δ 7.52 (t, *J* = 0.8 Hz, 1H), 7.37 (t, *J* = 1.8 Hz, 1H), 6.50 (t, *J* = 0.8 Hz, 1H), 5.11 (s, 1H), 3.04–2.80 (m, 4H), 2.19–1.82 (m, 2H); ¹³C NMR (50 MHz, CDCl₃) δ 141.5, 138.7, 122.5, 108.3, 39.7, 29.2, 23.6; MS (EI) *m/z* 186 (M⁺). Anal. calcd. for C₈H₁₀OS₂: C, 51.58; H, 5.41. Found: C, 51.59; H, 5.40.

2-(3-Furyl)-2-(3-butene)-1,3-dithiane (5). A solution of 2-(3-furyl)-1,3-dithiane (**4**, 1.58 g, 8.52 mmol) in anhydrous THF (50 mL) was treated with 1.6 M *n*-buthyllithium (5.9 mL, 9.37 mmol) carefully at –78°C under a nitrogen atmosphere. After the reaction mixture was stirred for 30 minutes at –78°C. 4-Bromobutene (10.1 mL, 9.96 mmol) was added, and then maintained for 2 hours with stirring. The reaction was quenched by crashed-ice, and then extracted with methylene chloride (20 mL $\times$ 3). The combined organic layers were dried over anhydrous sodium sulfate, and then evaporated under reduced pressure. The product was obtained by flash column chromatography (5% EtOAc/hexane) provided **5** (1.86 g, 91%) as colorless oil: ¹H NMR (200 MHz, CDCl₃) δ 7.51 (t, *J* = 0.8 Hz, 1H), 7.41 (t, *J* = 1.8 Hz, 1H), 6.46 (t, *J* = 0.8 Hz, 1H), 5.80–5.64 (m, 1H), 5.01–4.90 (m, 2H), 2.93–2.63 (m, 4H), 2.17–1.84 (m, 6H); ¹³C NMR (50 MHz, CDCl₃) δ 143.4, 142.3, 137.4, 128.7, 114.8, 110.7, 59.7, 42.8, 28.1, 27.4, 25.8; MS (EI) *m/z* 240 (M⁺). HRMS (EI) *m/z* C₁₂H₁₆OS₂ calcd: 240.0643; found: 240.0635.

1-(3-Furyl)-4-penten-1-one (6). To a stirred solution of 2-(3-furyl)-2-(3-butene)-1,3-dithiane (**5**, 232 mg, 0.97 mmol) in 6 mL of acetone was added copper (II) oxide (92 mg, 1.16 mmol), anhydrous copper chloride (313 mg, 2.33 mmol), and dropwise 180 μ L of *N,N*-dimethylformamide. The

FIG. 1. Structure of F-4-IM and 4-IM.

reaction mixture was heated for 2 hours at 60°C. After ethylacetate was poured, the mixture was filtered. The combined organic solution was washed with saturated sodium bicarbonate and brine. The solution was dried over anhydrous sodium sulfate, and then evaporated under reduced pressure. The product was obtained by flash column chromatography (10% EtOAc/hexane) provide **6** (126 mg, 73%) as colorless oil: ^1H NMR (200 MHz, CDCl_3) δ 8.01 (d, $J=0.8$ Hz, 1H), 7.41 (t, $J=1.6$ Hz, 1H), 6.74 (t, $J=0.8$ Hz, 1H), 6.00–5.80 (m, 1H), 5.09–4.94 (m, 2H), 2.85–2.78 (m, 2H), 2.48–2.37 (m, 2H); ^{13}C NMR (50 MHz, CDCl_3) δ 194.1, 147.0, 144.1, 137.0, 127.6, 115.3, 108.5, 39.4, 28.0; MS (EI) m/z 150 (M^+). HRMS (EI) m/z $\text{C}_9\text{H}_{10}\text{O}_2$ calcd: 150.0681; found: 150.0686.

1-(3-Furyl)-4-penten-1-one, ethylene ketal (7). To a stirred solution of **6** (2.72 g, 16.99 mmol) and *p*-toluenesulfonic acid (160 mg, 0.85 mmol) in 20 mL of toluene was added ethylene glycol (1.42 mL, 25.48 mmol). The mixture was placed with Dean stack and refluxed for 12 hours. The solvent was removed, and then dissolved with dichloromethane. The mixture was penetrated on anhydrous sodium sulfate, and evaporated under reduced pressure. Flash column chromatography (5% EtOAc/hexane) provided **7** (3.36 g, 98%) as colorless oil: ^1H NMR (200 MHz, CDCl_3) δ 7.40–7.35 (m, 2H), 6.33 (t, $J=0.9$ Hz, 1H), 5.89–5.72 (m, 1H), 5.05–4.89 (m, 2H), 4.03–3.82 (m, 4H), 2.21–1.94 (m, 4H); ^{13}C NMR (50 MHz, CDCl_3) δ 141.5, 138.3, 136.5, 126.1, 112.5, 107.0, 105.7, 63.0 ($\times 2$), 37.1, 26.2; MS (EI) m/z 194 (M^+). HRMS (CI) m/z $\text{C}_{11}\text{H}_{15}\text{O}_3$ calcd: 195.1021; found: 195.1025.

1-(3-Furyl)-4,5-dihydroxy-1-pentanone, ethylene ketal (8). The solution of potassium ferricyanide (1.5 g, 4.57 mmol), potassium carbonate (632 μL , 4.57 mmol), and $(\text{DHQ})_2\text{PHAL}$ in 10 mL of *t*-BuOH: H_2O (v/v 1:1) was stirred for 10 minutes, and then added potassium osmate dihydrate (5.6 mg, 1 mol%), methanesulfonamide (244 mg, 1.52 mmol), and **7** (295 mg, 1.52 mmol). The reaction mixture was stirred at 0°C. After 1 hour, the reaction was added sodium sulfite (274.5 mg, 2.29 mmol). After stirring for 40 minutes, the mixture was washed by 2N KOH for removing methanesulfonamide. The resulting mixture was extracted with ethyl acetate (70 mL $\times 3$). The combined extracts were dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The product **8** (267 mg, 77%) was obtained by flash column chromatography (70% EtOAc/hexane) as a white oil: ^1H NMR (200 MHz, CDCl_3) δ 7.32–7.27 (m, 2H), 6.92 (brs, 1H), 6.25 (t, $J=1.3$ Hz, 1H), 3.98–3.76 (m, 4H), 3.64–3.45 (m, 3H), 3.34–3.25 (m, 1H), 2.10–1.81 (m, 2H), 1.55–1.43 (m, 2H); ^{13}C NMR (50 MHz, CDCl_3) δ 141.6, 138.3, 125.8, 107.0, 105.9, 70.3, 64.9, 63.02, 62.97, 33.8, 25.4; MS (FAB) m/z 228 (M^+). HRMS (FAB) m/z $\text{C}_{11}\text{H}_{16}\text{O}_5$ calcd: 228.0998; found: 228.0992.

1-(3-Furyl)-4-hydroxy-5-(tert-Butyl-diphenyl-silanyloxy)-1-pentanone, ethylene ketal (9). To a stirred solution of **8** (2.95 g, 12.39 mmol) and imidazole (1.01 g, 14.87 mmol) in 30 mL of dichloromethane was added TBDPSCI (3.7 mL, 13.63 mmol) at 0°C dropwise. After stirring for 60 minutes, the reaction was quenched by ice-crashed water, and then poured saturated sodium bicarbonate. The resulting mixture was extracted with dichloromethane (70 mL $\times 3$). The combined extracts were dried over anhydrous sodium sulfate,

and evaporated under reduced pressure. The product **9** (5.43 g, 94%) was obtained by flash column chromatography (20% EtOAc/hexane) as colorless oil: ^1H NMR (200 MHz, CDCl_3) δ 7.68–7.63 (m, 4H), 7.48–7.33 (m, 8H), 6.31 (t, $J=1.3$ Hz, 1H), 4.01–3.82 (m, 4H), 3.78–3.45 (m, 3H), 2.65 (d, $J=3.6$ Hz, 1H), 2.18–1.84 (m, 2H), 1.55 (t, $J=7.0$ Hz, 2H), 1.06 (s, 9H); ^{13}C NMR (50 MHz, CDCl_3) δ 143.2, 140.0, 135.52, 135.53, 133.3, 129.7, 127.7, 108.7, 107.6, 71.8, 68.0, 64.75, 64.69, 60.3, 35.5, 26.9, 19.2; MS (FAB) m/z 467 ($\text{M}+\text{H}^+$). HRMS (FAB) m/z $\text{C}_{27}\text{H}_{35}\text{O}_5\text{Si}$ calcd: 467.2254; found: 467.2263.

1-(3-Furyl)-5-(tert-Butyl-diphenyl-silanyloxy)-pentan-1,4-dione, ethylene ketal (10). To a solution of **9** (606 mg, 1.30 mmol) in dichloromethane, Dess-Martin reagent (664.3 mg, 1.56 mmol) was added at 0°C for 2 hours. After that, $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ was portionwise at room temperature for 1 hour. The mixture was extracted with dichloromethane and dried over anhydrous sodium sulfate. The organic layer was evaporated under reduced pressure, the product **10** (543 mg, 90%) was obtained by flash column chromatography (10% EtOAc/hexane) as a colorless oil: ^1H NMR (200 MHz, CDCl_3) δ 7.68–7.63 (m, 4H), 7.46–7.36 (m, 8H), 6.33 (t, $J=1.3$ Hz, 1H), 4.19 (s, 2H), 4.00–3.83 (m, 4H), 2.65 (d, $J=7.3$ Hz, 2H), 2.22 (t, $J=7.6$ Hz, 2H), 1.10 (s, 9H); ^{13}C NMR (50 MHz, CDCl_3) δ 209.3, 143.3, 140.0, 135.5, 132.8, 129.9, 127.8, 127.5, 108.6, 107.1, 69.6, 64.8 ($\times 2$), 60.3, 33.2, 26.7, 19.2; MS (CI) m/z 465 ($\text{M}+\text{H}^+$). HRMS (CI) m/z $\text{C}_{27}\text{H}_{33}\text{O}_5\text{Si}$ calcd: 465.2097; found: 465.2094.

1-(3-Furyl)-5-hydroxy-pentan-1,4-dione, ethylene ketal (11). To a stirred solution of **10** (464 mg, 1.00 mmol), in 6 mL of acetonitrile, was added tetra-*n*-butylammonium fluoride hydrate (316 mg, 1.00 mmol) at 0°C. After stirring for 30 minutes, acetonitrile was evaporated under reduced pressure. The product **11** (222 mg, 98%) was obtained by flash column chromatography (30% EtOAc/hexane) as colorless oil: ^1H NMR (200 MHz, CDCl_3) δ 7.39–7.35 (m, 2H), 6.30 (t, $J=1.3$ Hz, 1H), 4.22 (s, 2H), 4.00–3.82 (m, 4H), 2.71 (brs, 1H), 2.49 (t, $J=7.0$ Hz, 2H), 2.27 (t, $J=6.6$ Hz, 2H); ^{13}C NMR (50 MHz, CDCl_3) δ 208.9, 143.5, 140.1, 127.2, 108.5, 106.7, 68.0, 64.8 ($\times 2$), 33.3, 32.8; MS (CI) m/z 227 ($\text{M}+\text{H}^+$). HRMS (CI) m/z $\text{C}_{11}\text{H}_{15}\text{O}_5$ calcd: 227.0919; found: 227.0923.

1-(3-Furyl)-5-(methanesulfonyloxy)-pentan-1,4-dione, ethylene ketal (12). To a stirred solution of **11** (563 mg, 2.49 mmol) and triethylamine (521 μL , 3.74 mmol) in 25 mL of dichloromethane was added methanesulfonyl chloride (193 μL , 2.49 mmol) at 0°C dropwise. After stirring for 30 minutes, the reaction was quenched by ice-crashed water, and then poured saturated sodium bicarbonate. The resulting mixture was extracted with dichloromethane (40 mL $\times 3$). The combined extracts were dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The product **12** (696 mg, 92%) was obtained by flash column chromatography (40% EtOAc/hexane) as a colorless oil: ^1H NMR (200 MHz, CDCl_3) δ 7.39–7.36 (m, 2H), 6.31 (t, $J=1.0$ Hz, 1H), 4.81 (s, 2H), 4.03–3.83 (m, 4H), 3.18 (s, 3H), 2.53 (t, $J=7.3$ Hz, 2H), 2.28 (t, $J=6.8$ Hz, 2H); ^{13}C NMR (50 MHz, CDCl_3) δ 202.0, 143.4, 139.9, 127.1, 108.4, 106.5, 71.5, 64.7 ($\times 2$), 38.5, 33.0, 32.8; MS (EI) m/z 304 (M^+). HRMS (EI) m/z $\text{C}_{12}\text{H}_{16}\text{O}_7\text{S}$ calcd: 304.0617; found: 304.0612.

1-(3-Furyl)-5-fluoro-pentan-1,4-dione, ethylene ketal (13). To a stirred solution of **12** (304 mg, 1.00 mmol), in 6 mL of acetonitrile, was added tetra-*n*-butylammonium fluoride hydrate (316 mg, 1.00 mmol), and then heated at 100°C. After stirring for 30 minutes, acetonitrile was evaporated under reduced pressure. The product **13** (132 mg, 58%) was obtained by flash column chromatography (10% EtOAc/hexane) as a colorless oil: ¹H NMR (400 MHz, CDCl₃) δ 7.40–7.36 (m, 2H), 6.33 (t, *J* = 0.7 Hz, 1H), 4.80 (d, *J* = 47.6 Hz, 2H), 4.00–3.86 (m, 4H), 2.64 (dt, *J* = 7.0, 2.2 Hz, 2H), 2.27 (t, *J* = 7.0 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 206.4 (d, *J* = 19.3 Hz), 143.5, 140.1, 127.1, 108.6, 106.7, 84.9 (d, *J* = 183.6 Hz), 64.8 (×2), 32.69, 32.63; MS (CI) *m/z* 229 (M+H⁺). HRMS (CI) *m/z* C₁₁H₁₄O₄F calcd: 229.0876; found: 229.0875.

1-(3-Furyl)-4-hydroxy-5-fluoro-1-pentanone, ethylene ketal (14). To a stirred solution of **13** (285 mg, 1.25 mmol) in 6 mL of ethanol was added NaBH₄ (95 mg, 2.50 mmol) at room temperature. After stirring for 1 hour, ethanol was evaporated under reduced pressure. The product **14** (282 mg, 98%) was obtained by flash chromatography (50% EtOAc/hexane) as a colorless oil: ¹H NMR (200 MHz, CDCl₃) δ 7.39–7.36 (m, 2H), 6.32 (t, *J* = 0.8 Hz, 1H), 4.51–4.11 (m, 2H), 4.05–3.85 (m, 5H), 2.57 (d, *J* = 4.0 Hz, 1H), 2.13–2.02 (m, 2H), 1.59 (t, *J* = 6.8 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 143.4, 140.0, 127.3, 108.6, 107.4, 86.8 (d, *J* = 167.6 Hz), 70.1 (d, *J* = 18.9 Hz), 64.8, 64.7, 35.2, 25.9 (d, *J* = 6.8 Hz); MS (CI) *m/z* 229 (M+H⁺). HRMS calcd for C₁₁H₁₄O₄F 229.0876, found 229.0877.

1-(3-Furyl)-4-hydroxy-5-fluoro-1-pentanone (**1**). To a stirred solution of **14** (290 mg, 1.26 mmol) in a mixture of acetone-water (*v/v* = 2:1, 6 mL) was added pyridinium *p*-toluenesulfonate (32 mg, 0.126 mmol) at 100°C. After stirring for 1 hour, acetone was evaporated under reduced pressure. The product **1** (195 mg, 83%) was obtained by flash column chromatography (40% EtOAc/hexane) as a colorless oil: ¹H NMR (200 MHz, CDCl₃) δ 8.07–8.06 (m, 1H), 7.44 (t, *J* = 0.8 Hz, 1H), 6.77–6.76 (m, 1H), 4.50–4.24 (m, 2H), 3.96–3.89 (m, 1H), 3.00–2.96 (m, 2H), 2.65 (d, *J* = 1.6 Hz, 1H), 1.99–1.76 (m, 2H); ¹³C NMR (50 MHz, CDCl₃) δ 195.0, 147.4, 144.3, 127.4, 108.5, 86.8 (d, *J* = 168.3 Hz), 69.6 (d, *J* = 18.9 Hz), 36.0, 25.8 (d, *J* = 6.8 Hz); MS (CI) *m/z* 187 (M+H⁺). HRMS calcd for C₉H₁₂O₃F 187.0770, found 187.0770.

Fluorine-18 labeling procedure for [¹⁸F]**1**

[¹⁸F]Fluoride was produced in a MC-50 cyclotron by the ¹⁸O(*p,n*)¹⁸F reaction. A volume of 100–200 μL [¹⁸F]fluoride (8.0–17.0 GBq) in water was added to a vacutainer containing *n*-Bu₄NHCO₃ (40% aq., 2.70 μL, 3.65 μmol). The azeotropic distillations were carried out each time with 300 μL aliquots of CH₃CN at 85°C under a stream of nitrogen. A fluorine-18 ion displacement reaction of **12** (2 mg, 6.5 μmol) with *n*-Bu₄N[¹⁸F]F in acetonitrile (300 μL) was carried out in a reaction vial at 100°C for 10 minutes. After the reaction, the vial was cooled in an ice bath. The solvent was removed under a gentle stream of nitrogen at room temperature, and then NaBH₄ (2 mg, 52.8 μmol) and EtOH (200 μL) were added to the vial and stirred for 10 minutes at room temperature. After removing the ethanol, the mixture was diluted with CH₂Cl₂ (200 μL × 2) and filtered through silica Sep-pak. To remove ketal groups, the residue was treated with pyr-

ridinium *p*-toluenesulfonate (1 mg, 4.0 μmol) in acetone/water (*v/v* = 2:1, 200 μL) at 100°C for 10 minutes. Acetone was removed with a gentle stream of nitrogen, and then added saturated NaHCO₃ (300 μL) and CH₂Cl₂ (300 μL × 2). The extracted organic layer was evaporated and injected onto normal phase HPLC with the help of 35% ethyl acetate in hexane and purified. The desired compound was collected from HPLC (*t_R* = 15 minutes; Econosil silica gel; 10 μ; 10 × 250 mm; EtOAc/hexane = 35:65 (*v/v*); 254 nm; flow rate: 4 mL/minutes). After the solvent was evaporated, [¹⁸F]**1** was used for subsequent biological studies.

In vitro stability assessment

The stability of [¹⁸F]**1** was assayed by monitoring the Radio-TLC profile and determining its radiochemical purity. To determine the *in vitro* serum stability, 100 μL of [¹⁸F]**1** in 10% EtOH/saline was incubated with 0.5 mL of human serum at 37°C for 2 hours, and the solution was analyzed by radio-TLC scanner.

Cells

Rat C6-glioma cells (C6) were purchased from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose (WelGENE), supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO) and antibiotics (100 units/mL penicillin G and 10 μg/mL streptomycin; GIBCO) at 37°C in a humidified 5% CO₂ atmosphere.

Construction of CYP4B1 retroviral expression vector

cDNA for CYP4B1 was obtained from rabbit lung tissue and cloned into the pcDNA3.1/Hygro vector (Invitrogen) generating pcDNA-CYP4B1.²⁰ The nucleotide sequence for rabbit CYP4B1 was referred to GenBank No. NM_001082103.1. To construct the retroviral expression vector, the expression of CYP4B1 gene was introduced downstream of the long terminal repeats in the MFG vector backbone (kindly provided by Dr. Kwon, KIRAMS, Republic of Korea) by using NotI/BamHI and named as pMFG-CYP4B1.

Establishment of C6-CYP4B1 using the retroviral vector

The pMFG-CYP4B1 was introduced into H29D retrovirus packaging cell culture. The retroviral titers were measured between 1 × 10⁵ and 5 × 10⁵ infection units/mL. C6 were seeded in a six-well plate at a concentration of 3 × 10⁵ cells/well 1 day before retrovirus infection and infected by exposing the cell monolayer to 700 μL of retroviral vector in 300 μL of serum free media for 4 hours in the presence of 8 μg/mL of polybrene (Sigma-Aldrich). The transfected C6 cells were plated at a density of 1 cell/well and kept for 10 days. These stably transfected cells were selected with CYP4B1 expression by reverse transcription polymerase chain reaction (RT-PCR).

Evaluation of CYP4B1 expression in C6-CYP4B1

Reverse transcription polymerase chain reaction. Total RNA extraction was performed from 5 × 10⁵ cells of C6-CYP4B1 by using Easy-spin™ (DNA free) Total RNA Extraction Kit (iNtRON Biotechnology), according to the

manufacturer's protocol. The total RNA was used as a template to produce cDNA using the SuperScript III First-Strand Synthesis kit (Invitrogen), according to the manufacturer's protocol. The resulting cDNAs were amplified using 2.5 units of Taq polymerase and 10 pmol of each primer (Table 1) in a GeneAmp PCR System (Applied Biosystems). The PCR conditions were as follows: for 35 cycles of denaturation (95°C for 30 seconds), annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds. The final extension was performed at 72°C for 7 minutes. Amplification products were confirmed by ethidium bromide staining after products were subjected to electrophoresis on a 2% agarose gel.

Prodrug activation cytotoxicity

MTT assay. C6 and C6-CYP4B1 were seeded in 96-well plates in triplicate at a density of 2×10^3 cells/well. After 24 hours, cell culture media containing various concentrations of 2-aminoanthracene (2-AA; Sigma-Aldrich) ranging from 0 to 0.1 mM were added and incubated for 96 hours. The number of surviving cells was determined using the MTT assay (*In vitro* Toxicology Assay Kit MTT based; Sigma-Aldrich). Next, 10 μ L of MTT reagent was added to each well and incubated for 2 hours at 37°C. When a purple precipitate was clearly visible under the microscope, 100 μ L of Detergent Reagent was added to all wells, and the plate was placed in the dark at room temperature for 30 minutes. MTT color development was measured and analyzed using an ELISA reader (Bio-Rad) with a single filter (595 nm).

In vivo biodistribution in normal rats

Fisher 344 rats (180–200 g body weight, $n=5$) were obtained from Charles River laboratory and handled in accordance with the guidelines of the Korea Institute of Radiological and Medical Sciences. The animals were anesthetized with ethyl ether before injection of radiotracer and remained anesthetized through the study. Rats were injected with 7.4 MBq of [18 F]1 in 0.5 mL of 10% EtOH/saline through the tail vein. Biodistribution was performed at 10, 30, 60, and 120 minutes post-injection. Blood was obtained through cardiac puncture, and the tissue sample of selected liver, lung, heart, spleen, kidneys, stomach, small intestine, large intestine, muscle, and femur were obtained from normal rats. All selected samples were weighed and the radioactivity was measured using a NaI crystal well-type gamma counter, applying a decay correction. Counts were compared with those of standards, and the data are expressed as percentage

of injected radioactivity per gram of tissue (%ID/g) for some selected organs as the mean value.

Comparative cellular uptake of [18 F]F-4-IM and [3 H]4-IM

C6 and C6-CYP4B1 were seeded in six-well plates in triplicate at a density of 1×10^6 or 5×10^5 cells/well 1 day before cellular uptake of [18 F]F-4-IM or [3 H]4-IM. After 24 hours, cells were incubated with [18 F]F-4-IM (0.037 MBq) or [3 H]4-IM (0.037 MBq; Moravek Biochemicals and Radiochemicals) with 10 μ M of 4-IM, at a concentration of 1–4 μ Ci/well for an additional 0, 10, 30, 60, 180, and 360 minutes. After incubation, the supernatants were removed; cells were immediately rinsed with cold phosphate-buffered saline (PBS), and stripped with 1% trypsin-EDTA or cells were lysis with 0.2% SDS. [18 F]F-4-IM-trapped cells were harvested in plastic test tubes by centrifugation at 1,000 rpm for 3 minutes. The supernatant was removed, and cell pellets were washed twice with cold PBS. [3 H]4-IM-trapped lysates were harvested in plastic counter tubes and 10 mL of liquid scintillation cocktail (ULTIMA GOLD; PerkinElmer, Inc.) was added, followed by vortexing at 4°C. The CYP4B1 expression level was determined by gamma counting or beta counting of [18 F]F-4-IM or [3 H]4-IM uptake in cells for 1 minute, respectively. The level of binding of [18 F]F-4-IM and [3 H]4-IM was expressed as the percentage of binding to C6-CYP4B1 relative to that of C6.

Statistical analysis

Comparisons between groups were performed using IBM SPSS Statistics 19 software. Data were analyzed using the two-way factorial analysis of variance (ANOVA) followed by Bonferroni *post-hoc* tests with a p -value less than 0.05 were considered as significant.

Results

Chemistry

Using 1,3-propandithiol as a protecting group of aldehyde in 3-furaldehyde (3), compound 4 was prepared in the presence of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in 92% yield. The olefin 5 was synthesized with *n*-butyllithium and 4-bromo-1-butene with excellent yields, followed by deprotection of 1,3-dithiane with copper(II) oxide and copper chloride in DMF/acetone under

TABLE 1. OLIGONUCLEOTIDES USED FOR POLYMERASE CHAIN REACTION

Oligonucleotide	Sequence (5' → 3')
CYP4B1	
Sense	ATA GGA TCC ATG CTC GGC TTC CTC TCC
Antisense	ATA TCT AGA CTA CTT CTC AGC CTT GGG G
Human and Murine Beta-actin	
Sense	GTG GGG CGC CCC AGG CAC CAG GGC
Antisense	CTC CTT AAT GTC ACG CAC GAT TTC

FIG. 2. High-performance liquid chromatography (HPLC) profile of the reaction mixture.

FIG. 3. HPLC profile of the collected [¹⁸F]1 with the authentic compounds (1 and 14).

mild condition to produce compound **6** in 73% yield. The olefin **6** was quantitatively reprotected with ketal to avoid enolic conversion under basic condition. The ketal-protected compound **7** was dihydroxylated under K₃Fe(CN)₆, K₂CO₃, K₂OsO₄·2H₂O, and (DHQ)₂PHAL condition to yield compound **8** in 77% yield, and then selectively protected using *tert*-butoxydiphenylsilyl chloride at the terminal hydroxyl moiety to obtain compound **9**. Compound **9** was oxidized using Dess-Martin reagent to yield compound **10** in 90% yield. The cleavage of the *tert*-butoxydiphenylsilyl group occurred under tetra-*n*-butylammonium fluoride in tetrahydrofuran to give compound **11**, quantitatively. Mesylation of the alcohols was carried out with methanesulfonyl chloride and triethylamine to give mesylate **12** in 92% yield and

subsequent fluorination using tetra-*n*-butylammonium fluoride (TBAF) gave compound **13** in 58% yield. This mesylate products **12** also was used as the precursor for the synthesis of [¹⁸F]1. Compound **14** was prepared by reduction with NaBH₄, and then followed deketalization in the presence of pyridinium *p*-toluenesulfonate (PPTS) to give authentic compound **1** in 83% yield.

Radiolabeling

The process for preparation of 1-(3-furyl)-4-hydroxy-5-[¹⁸F]fluoro-1-pentanone ([¹⁸F]F-4-IM, [¹⁸F]1) also includes 3 steps—the displacement of [¹⁸F]fluoride, reduction, and the deprotection of ketal groups—as shown in Scheme 1. Each progress was monitored by radio-TLC and HPLC. Formation of [¹⁸F]13 from the mesylated compound **12** and fluorine-18 was checked by radio-TLC. After the reduction of ketone in the presence of NaBH₄ in ethanol, the obtained [¹⁸F]14 was purified using a silica Sep-Pak cartridge. The reaction was also monitored by radio-TLC until the compound [¹⁸F]13 peak disappeared. The target compound, [¹⁸F]1, was prepared from deprotection of the ketal group by PPTS and purified by HPLC (Fig. 2). The final radiochemical yield was 20%–35% through 3 steps, leading to >95% of radiochemical purity. The specific activity was 92–133 GBq/μmol and total preparation time required about 120 minutes. For the identification of [¹⁸F]1, the collected HPLC fraction was matched with the authentic compound **1** (Fig. 3).

Stability of [¹⁸F]F-4-IM ([¹⁸F]1) in human serum

The results of [¹⁸F]F-4-IM analysis in human serum showed that the intact has over 96% stability for up to 120

SCHEME 1. (a) 1,3-propanedithiol, BF₃·Et₂O, THF, N₂, 0 to 70°C, 1.5 hours; (b) *n*-BuLi, THF, 4-bromo-1-butane, −78°C, 2.5 hours; (c) Cu(II)O, CuCl₂, DMF, acetone, 60°C, 2 hours; (d) ethylene glycol, toluene, 120°C, 12 hours; (e) K₃Fe(CN)₆, K₂CO₃, K₂OsO₄·2H₂O, (DHQ)₂PHAL, *t*-BuOH:H₂O (v/v = 1:1), sodium sulfite, 0 to r.t., 2 hours; (f) TBDPSCl, imidazole, CH₂Cl₂, 0°C to r.t., 1 hour; (g) Dess-Martin oxidation, 0°C, 2 hours; (h) *n*Bu₄NF, THF, 0°C, 30 minutes; (i) MsCl, CH₂Cl₂, 0°C, 0.5 hours; (j) 1 M *n*Bu₄NF in THF, 100°C, 0.5 hours for **13** and *n*Bu₄N[¹⁸F]F, CH₃CN, 100°C, 10 minutes for [¹⁸F]13; (k) NaBH₄, EtOH, r.t., 1 hour for **14** and NaBH₄, EtOH, r.t., 10 minutes for [¹⁸F]14; (l) PPTS, acetone/H₂O, 100°C, 1 hour for **1** and PPTS, acetone/H₂O, 100°C, 10 minutes for [¹⁸F]1.

FIG. 4. Radio-TLC chromatogram of stability in human serum (left: 0 min, right: 120 min).

minutes (Fig. 4). This indicated that [^{18}F]1 was sufficiently stable to be used in further biological studies.

In vivo studies

The biodistribution data of radioactivity of [^{18}F]F-4-IM bound to tissues in normal rats are summarized in Table 2. The data are presented as percentage of injected dose per gram of tissues (%ID/g). After 1 hour postinjection, tissue distribution studies showed the uptake of [^{18}F]F-4-IM to be highest in the lung (%ID/g = $25.83\% \pm 2.90\%$), followed by the liver (%ID/g = $6.43\% \pm 0.87\%$). The initial level of tissues in gastrointestinal and urinary system was high and decreased in a time-dependent manner. An exception was in the lung, in which the tracer was retained for the duration of the experimental. In addition, bone uptake (%ID/g = $1.73\% \pm 0.17\%$ at 1 hour) was low and similar to those of other tissues, such as muscle, heart, stomach, and blood. PET images in normal rat also showed that [^{18}F]F-4-IM uptake was high in liver, lung, and kidney but radioactivity of other tissues was relatively low (Fig. 5).

Confirmation of CYP4B1 gene expression

To investigate the gene expression of the CYP4B1, we performed RT-PCR. CYP4B1-specific primers were designed to produce PCR products of 650-bp (540-bp of human and murine beta-actin). A specific band was detected in C6-CYP4B1 but not in parental cells (Fig. 6). This indicated that the CYP4B1 gene is expressed following retroviral transduction.

TABLE 2. BIODISTRIBUTION OF [^{18}F]F-4-IM IN NORMAL RATS

Tissue	Time point			
	10 min	30 min	60 min	120 min
Blood	6.19 ± 0.47	4.23 ± 0.52	2.93 ± 0.38	1.33 ± 0.49
Liver	9.78 ± 0.97	8.33 ± 0.65	6.43 ± 0.87	5.21 ± 0.69
Lung	16.98 ± 0.99	25.32 ± 7.06	25.83 ± 2.90	22.26 ± 2.77
Heart	6.61 ± 0.60	3.67 ± 0.40	2.07 ± 0.31	0.96 ± 0.15
Spleen	6.62 ± 0.54	3.62 ± 1.77	3.34 ± 0.52	2.39 ± 0.21
Kidneys	12.97 ± 1.04	17.68 ± 2.34	12.39 ± 2.07	7.91 ± 1.02
Stomach	4.15 ± 0.84	2.22 ± 0.71	1.30 ± 0.29	2.44 ± 2.38
S. intestine	8.81 ± 1.64	12.40 ± 0.88	14.57 ± 1.70	9.17 ± 1.47
L. intestine	4.96 ± 0.55	2.75 ± 0.33	1.49 ± 0.23	14.14 ± 4.33
Muscle	5.48 ± 0.74	2.94 ± 0.37	1.30 ± 0.32	0.52 ± 0.21
Femur	4.06 ± 0.49	2.63 ± 0.43	1.73 ± 0.17	1.23 ± 0.14

Comparison of prodrug activation therapy between C6 and C6-CYP4B1

To evaluate the therapeutic efficacy of prodrug targeting CYP4B1, the survival rates of C6 and C6-CYP4B1 treated with 2-AA were determined using an MTT assay. As shown in Figure 7, the survival rate of C6-CYP4B1 was lower compared with C6 in a dose-dependent manner. Specifically, the 50% lethal dose (LD_{50}) of C6-CYP4B1 was 0.03 mM following 96 hours treatment with 2-AA. In two-way factorial ANOVA test, a significant difference showed in all group-specific and concentration differences (with F -value 2028.162, 477.024, and $p < 0.005$). Interaction effect of group and concentration was significant difference (with F -value 92.403 and $p < 0.005$).

Measurement of CYP4B1 activity by [^{18}F]F-4-IM or [^3H]4-IM

To evaluate the sensitivity of radiolabeled 4-IM analogs for GPAT, a cellular uptake assay was carried out using C6 and C6-CYP4B1 in a paired format to compare [^{18}F]F-4-IM and [^3H]4-IM. In the *in vitro* cell uptake study, [^{18}F]F-4-IM and [^3H]4-IM showed comparable results as shown in Figure 8. In C6-CYP4B1, the increase in uptake of both compounds was proportional to the incubation time. However, the specific uptake of [^{18}F]F-4-IM in C6-CYP4B1 was about 2.8-times higher than that in C6 at 1 hour ($p < 0.01$), whereas it required up to 3 hours for [^3H]4-IM to achieve a similar effect ($p < 0.001$). In two-way factorial ANOVA test, a significant difference showed in all group-specific and time differences (with F -value 128.394, 49.769, and $p < 0.005$ for [^{18}F]F-4-IM; F -value 441.110, 149.292, and $p < 0.005$ for [^3H]4-IM). Interaction effect of group and time was significant difference (with F -value 50.502 and $p < 0.005$ for [^{18}F]F-4-IM; F -value 151.166 and $p < 0.005$ for [^3H]4-IM).

Discussion

In this study, a 4-IM derivative was designed for imaging of CYP4B1-transfected tumor cells and investigated as a potential ligand for fluorine-18 labeling. The successful GPAT study depends on effective transfer and expression of the desired genes into the target cell and tissues. Among viral and nonviral techniques, the simplest way of therapeutic transgene delivery is injecting naked DNA but this method is relatively less efficient than those of viral methods.²¹ Viral techniques use various classes of viruses as a tool for gene delivery by genetic modification of retroviruses, adenoviruses, herpesviruses, and others. Because the best established vector system

FIG. 5. PET images of $[^{18}\text{F}]$ F-4-IM in normal rat.

for achieving stable transduction due to integration into the target cells is retrovirus vector, we selected retroviral system as CYP4B1 gene delivery. As examples of the concept for GPAT, stably expressing clones of 9L and U87 glioma cells with the CYP4B1 plasmid DNA were sensitized about 20-fold to 4-IM with an efficient bystander effect.¹⁹

The radiolabeling method we have developed here lay the groundwork for the synthesis of the fluorine substituted 4-IM and fluorine radiolabeled form. As shown in Scheme 1, we prepared the mesylated precursor (12), which was synthesized in 9 steps from 3-fluraldehyde. We considered a number of approaches that would be suitable for the preparation of the fluorine-substituted 4-IM derivative. In our studies, we were unable to effect fluoride ion substitution on the corresponding the mesylated precursor without the use of the ethylene ketal protection group, because in the final fluorination step of the mesylated precursor, the carbon may intramolecularly displace the mesyl group leading to the corresponding C- or O-alkylated undesirable products. To label the proximal end of carbon in 4-IM, the process for preparation of 1-(3-furyl)-4-hydroxy-5-fluoro-1-pentanone contains 3 steps: the displacement of fluoride ion using tetra-*n*-butylammonium fluoride hydrate, the reduction of keton using NaBH_4 and the deprotection of the ketal group using PPTS.

$[^{18}\text{F}]$ 4-IM was synthesized via the same route as the preparation of F-4-IM. Various conditions were explored for the preparation of $[^{18}\text{F}]$ 4-IM from the mesylated precursor. The results from reaction conditions optimized to give $[^{18}\text{F}]$ 4-IM in 20%–35% radiochemical yield (decay corrected for 120 minutes): first step- $n\text{Bu}_4\text{N}[^{18}\text{F}]\text{F}$ in acetonitrile at 100°C for 10 minutes, second step- NaBH_4 in ethanol at room temperature for 10 minutes, third step-PPTS in a mixture of acetone-water at 100°C for 10 minutes. The reaction mixture

was then purified by HPLC (Econosil silica gel; 10μ ; $10\times 250\text{ mm}$), and the retention time of $[^{18}\text{F}]$ 4-IM was 15 minutes using a 36:65 mixture of ethyl acetate:hexane as the eluants (Fig. 2). As shown Figure 3, the collected HPLC fraction was analyzed by HPLC. This was identified as the fluorine-18 labeled 4-IM by comparison with authentic samples that we had prepared (cf., Scheme 1).

Percentage of the remaining $[^{18}\text{F}]$ F-4-IM was about 98% after 1 hour and over 96% even after 2 hours when the radiotracer was incubated with human serum at 37°C and analyzed by radio-TLC, indicating high *in vitro* stability.

In biodistribution studies, the radioactivity of $[^{18}\text{F}]$ F-4-IM was greater in the lung, in which CYP4B1 is preferentially expressed, than in all other organs at all time points. The uptake of $[^{18}\text{F}]$ F-4-IM shows a similar biodistribution pattern as that of $[^3\text{H}]$ 4-IM.²² In addition, Low levels of fluoride incorporation into bone implied that the injected compounds remained largely intact throughout the course of the experiment and is consistent with the results of the *in vitro* serum stability study. The gene expression of CYP4B1 in C6-CYP4B1 was confirmed with RT-PCR (Fig. 6). Using this C6-CYP4B1, we measured the cell survival in the presence of 2-AA with MTT assay. As shown in Figure 7, the survival rates of C6-CYP4B1 was more rapidly decreased with increasing 2-AA dose compared with C6. This result indicated that the therapeutic efficacy of 2-AA is dependent on CYP4B1 expression, and that the metabolic conversion of this prodrug under these conditions resulted in enhanced tumor cell death.

FIG. 6. Confirmation of CYP4B1 gene expression in C6-CYP4B1.

FIG. 7. Prodrug activation therapy as measured by the MTT assay. $p < 0.001$ ($> 0.01\text{ mM}$).

FIG. 8. *In vitro* cellular uptake of [¹⁸F]-4-IM (left) or [³H]-4-IM (right) in C6 and C6-CYP4B1 cells. **p* < 0.01, ***p* < 0.001.

To evaluate the sensitivity of radiolabeled 4-IM analogs to the CYP4B1 expression, a cellular uptake assay was performed using [¹⁸F]-4-IM and [³H]-4-IM. Levels of [¹⁸F]-4-IM in C6-CYP4B1 was rapidly increased about 2.8-fold, compared to that in C6 at 1 hour. [³H]-4-IM, on the other hand, had low levels of accumulation in C6-CYP4B1 at 1 hour, and then reached the similar level at 3 hours. Despite the same electron configuration of the fluorine and hydrogen atom, cell uptake of two radiolabeled 4-IM analogs may not be the same in our comparison studies because of their different electronegativity. Therefore, [¹⁸F]-4-IM selectively bound to the CYP4B1 protein and could be used to identify transfected cells within a short period of time (~1 hour) postinjection.

Conclusion

In conclusion, F-4-IM (1), a fluorinated 4-IM analogue targeting CYP4B1, was synthesized and labeled with fluorine-18. [¹⁸F]-4-IM, a radiolabeled small-molecule ligand that can be used to detect CYP4B1 expression, showed high stability and selective accumulation in the lung, an organ in which CYP4B1 is constitutively expressed in the rats. In addition, our studies using C6-glioma cells overexpressing CYP4B1 indicated that [¹⁸F]-4-IM is a promising imaging candidate for use in GPAT. These results suggest that [¹⁸F]-4-IM should be further studied to evaluate its utility for PET imaging of CYP4B1 transfected cancer cells.

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Disclosure Statement

No competing financial interests exist.

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