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¹⁸F-labeled Rhodamines as Potential Myocardial Perfusion Agents: Comparison of Pharmacokinetic Properties of Several Rhodamines

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Abstract

Introduction—We recently reported the development of the [¹⁸F]fluorodiethylene glycol ester of rhodamine B as a potential positron emission tomography (PET) tracer for myocardial perfusion imaging (MPI). This compound was developed by optimizing the ester moiety on the rhodamine B core, and its pharmacokinetic properties were found to be superior to those of the prototype ethyl ester. The goal of the present study was to optimize the rhodamine core while retaining the fluorodiethyleneglycol ester prosthetic group.

Methods—A series of different rhodamine cores (rhodamine 6G, rhodamine 101, and tetramethylrhodamine) were labeled with ¹⁸F using the corresponding rhodamine lactones as the precursors and [¹⁸F]fluorodiethylene glycol ester as the prosthetic group. The compounds were purified by semipreparative HPLC, and their biodistribution was measured in rats. Additionally, the uptake of the compounds was evaluated in isolated rat cardiomyocytes.

Results—As was the case with the different prosthetic groups, we found that the rhodamine core has a significant effect on the *in vitro* and *in vivo* properties of this series of compounds. Of the rhodamines evaluated to date, the pharmacologic properties of the ¹⁸F-labeled diethylene glycol ester of rhodamine 6G are superior to those of the ¹⁸F-labeled diethylene glycol esters of

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rhodamine B, rhodamine 101, and tetramethylrhodamine. As with ^{18}F -labeled rhodamine B, [^{18}F]rhodamine 6G was observed to localize in the mitochondria of isolated rat cardiomyocytes.

Conclusions—Based on these results, the ^{18}F -labeled diethylene glycol ester of rhodamine 6G is the most promising potential PET MPI radiopharmaceutical of those that have been evaluated to date, and we are now preparing to carry out first-in-human clinical studies with this compound.

Keywords

Fluorine-18; rhodamine; myocardial perfusion imaging; PET

1. Introduction

Cardiovascular disease is a major health problem worldwide. Myocardial perfusion imaging (MPI) using non-invasive modalities such as SPECT (single-photon emission computer tomography) and PET (positron emission tomography) are the most widely used techniques for diagnosis and treatment planning in this disease. Currently, the primary method for MPI is SPECT using the single-photon emitters $^{99\text{m}}\text{Tc}$ -sestamibi, $^{99\text{m}}\text{Tc}$ -tetrofosmin, or ^{201}Tl . However, the limitations of SPECT, including the absence of a standardized attenuation correction method, the inability to perform quantitative measurements, the lower spatial resolution and sensitivity compared to PET as well as recurring shortages of $^{99\text{m}}\text{Tc}$ have increased interest in PET MPI [1, 2].

Despite the advantages of PET for MPI, its widespread clinical use is hampered by the practical limitations of the currently available PET perfusion agents: [^{13}N]NH₃, [^{15}O]H₂O, and [^{82}Rb]RbCl. The tracers [^{13}N]NH₃ and [^{15}O]H₂O are readily produced by accelerator methods, but the short half-lives of ^{13}N (10 min) and ^{15}O (2 min) restrict their use to clinical centers with an on-site cyclotron. Generator-produced ^{82}Rb ($t_{1/2} = 76$ s) can be used at clinics without access to cyclotrons, but the high cost of the $^{82}\text{Sr}/^{82}\text{Rb}$ generator requires high patient throughput for the generator to be cost effective. Other limiting factors of ^{82}Rb include its less than optimal myocardial extraction and its high positron energy, which results in decreased spatial resolution [1].

The limitations of the existing PET MPI agents and the near ideal physical properties of ^{18}F (β^+ , 0.635 MeV [97%]; $t_{1/2} = 110$ min) have increased interest in the development of ^{18}F -labeled myocardial perfusion tracers. An additional advantage of ^{18}F is that the 110 min half-life is sufficiently long enough to allow centralized production of ^{18}F -labeled radiopharmaceuticals and distribution to clinical centers without an on-site cyclotron, while still being short enough to allow repeated MPI studies of a patient on the same day.

In recent years, several ^{18}F -labeled compounds have been evaluated as possible myocardial perfusion agents including quaternary ammonium salts [3]; tetraphenylphosphonium compounds [4–7]; rotenone derivatives [8, 9]; and pyridazinone analogs, such as BMS-747158-02 (Flurpiridaz F18; Lantheus Medical Imaging, Inc.) [10–17]. Flurpiridaz is currently in Phase 3 clinical trials [18–22], and BFPET (Fluoropharma) has recently completed phase 1 clinical trials [7, 23].

Neither of these ^{18}F -labeled agents has, however, yet been approved by the FDA, and it is not certain that either, or both, will ultimately be approved. Given the clinical importance of MPI, the added clinical value of PET MPI, and the limitations of the existing PET MPI agents, the successful development of an effective ^{18}F -labeled MPI radiopharmaceutical is essential to the care of patients with cardiovascular disease.

We recently reported the development of ^{18}F -labeled esters of rhodamine B as potential MPI radiopharmaceuticals [24, 25]. The *in vivo* stability and pharmacokinetics of the ^{18}F -labeled rhodamine B ethyl ester were, however, less than optimal resulting in unfavorable liver uptake. In a study of different rhodamine B esters where we sought to reduce the liver uptake and increase the myocardial uptake of the tracer, we found that the ^{18}F -labeled diethylene glycol ester of rhodamine B (^{18}F 2) was superior to several other rhodamine B esters in terms of *in vivo* stability and pharmacokinetics [26]. In that study we also demonstrated the ability of ^{18}F 2 to delineate myocardial infarction in a rat [26]. The objective of the present study was to compare the pharmacokinetic properties of several different ^{18}F -labeled rhodamine dyes (i.e., rhodamine 6G, tetramethylrhodamine, and rhodamine 101, Fig. 1) and determine if they provided improved properties compared to ^{18}F 2.

2. Methods

2.1. General

Rhodamine 6G chloride was obtained from Acros (Fair Lawn, NJ). Tetramethylrhodamine lactone, rhodamine 101 lactone, and tetrabutylammonium fluoride (1 M in THF) were purchased from Sigma-Aldrich (St. Louis, MO). Diethyleneglycol bistosylate was purchased from TCI America (Philadelphia, PA). For the radiosynthesis, extra dry reagent grade acetonitrile (Thermo Scientific, Bellefonte, PA) and Kryptofix (K 2.2.2, 98%, Sigma-Aldrich) were used. Potassium carbonate (99.97%) was purchased from Alfa Aesar (Ward Hill, MA). Other solvents and reagents were of the highest grade commercially available and used without further purification. Thin-layer chromatography (TLC) was performed using silica gel IB-F coated plastic sheets from J. T. Baker (Phillipsburg, NJ). Nuclear magnetic resonance spectra were obtained using a 400 MHz Varian 400-MR system (Palo Alto, CA). Chemical shifts are given as parts per million (ppm) and are reported relative to tetramethylsilane. Coupling constants are reported in hertz (Hz). The multiplicity of the NMR signals is described as follows: s = singlet, d = duplet, t = triplet, q = quartet, m = multiplet. High-resolution mass spectra (ESI-MS mode) were obtained at the University of Illinois Mass Spectrometry Facility using a Micromass 70-VSE spectrometer. Fluorine-18 (as F^- in water) was purchased from Cardinal Healthcare (Woburn, MA) and the Brigham and Women's Hospital BICOR (Boston, MA). The purity of the nonradioactive (^{19}F) reference compounds was 95% as determined by analytical HPLC and NMR.

2.2. Purification and quality control

Analytical HPLC was carried out using a Hitachi 7000 system including an L-7455 diode array detector, an L-7100 pump, and a D-7000 interface. The radiometric HPLC detector was comprised of Canberra nuclear instrumentation modules and was optimized for 511 keV

photons. An LaChrom PuroSphere Star C18e column (4 mm × 30 mm, 3 μm) was used for analytical measurements. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) at a flow rate of 1 mL/min at room temperature. The solvent gradient was 0–15 min (30–70% B), 15–25 min (70% B). For semi-preparative HPLC, an ISCO system comprised of an ISCO V4 variable wavelength UV-visible detector (operated at 550 nm), ISCO 2300 HPLC pumps, a radiometric detector similar to that described above, and a Grace Apollo C18 column (10 mm × 250 mm, 5 μm) was used. Semi-preparative HPLC method A (isocratic): 40% solvent A (0.1% TFA in water), 60% solvent B (0.1% TFA in acetonitrile); flow rate, 5 mL/min; room temperature. Semi-preparative HPLC method B (gradient): 0–10 min (40% B); 10–30 min (40–50% B); 30–35 min (50–100% B); 35–40 min (100% B); flow rate, 5 mL/min; room temperature. Radiofluorination yields were determined by thin-layer chromatography using silica gel plates and chloroform/methanol (8:1 v/v) as the solvent. After development, the TLC strips were cut into 1 cm pieces and counted with a Packard Cobra γ counter.

2.3. Synthesis of non-radiolabeled reference compounds

2.3.1. 2-(2-Fluoroethoxy)ethyl tosylate (1)—Compound **1** was prepared using the previously described procedure [26]. Yield: 262 mg (83%). ¹H NMR (CDCl₃): δ 7.80 (2H, d, *J* = 8.26), 7.35 (2H, d, *J* = 8.20), 4.55 (m, 1H), 4.43 (m, 1H), 4.18 (t, 2H, *J* = 4.80), 3.71 (m, 3H), 3.63 (m, 1H), 2.45 (s, 3H).

2.3.2. Lactone precursors—The rhodamine 6G lactone precursor was prepared as previously described [27] and characterized by ¹H NMR and mass spectrometry.

2.3.3. Tetramethylrhodamine 2-(2-fluoroethoxy)ethyl ester, TFA salt (3)

Tetramethylrhodamine lactone (20 mg, 0.05 mmol) was dissolved in 10 mL of acetonitrile, and the solution was heated to 80 °C before adding 40 mg (0.15 mmol) of **1** dissolved in 2 mL of acetonitrile containing 0.1 mL (0.6 mmol) of DIPEA. The mixture was refluxed for 3 h and allowed to cool to room temperature. The condenser was replaced with a serum stopper equipped with a venting needle and the mixture was heated to 90 °C, allowing the reaction mixture to evaporate slowly to complete dryness (approx. 7 h). HPLC analysis of the reaction mixture revealed incomplete conversion (crude yield 40%). The crude product was dissolved in 3 mL acetonitrile and filtered through a C-18 Sep-Pak cartridge to remove the unreacted lactone, which is retained on the cartridge. In order to obtain high purity reference material, a small fraction of the crude product was further purified by semi-preparative HPLC using the same separation conditions as for the radioactive compound (HPLC method B). Yield (crude): 6 mg (20%). ¹H NMR (CDCl₃): δ 8.33 (dd, 1H, *J* = 1.21, 7.84), 7.82–7.73 (m, 2H), 7.33 (dd, 1H, *J*₁ = 7.34, *J*₂ = 0.96), 7.10 (d, 2H, *J* = 9.39), 6.94–6.88 (m, 4H), 4.52 (dt, 2H, *J*₁ = 47.77, *J*₂ = 4.03), 3.62–3.45 (m, 4H), 3.33 (s, 12H). ¹⁹F NMR (CDCl₃): −74.7 ppm, (s, 3F) −222.9 ppm, (m, 1F). HRMS *m/z* (%): calcd for C₃₂H₃₈FN₂O₄⁺ [*M*⁺] 477.2190, found 477.2183 (100%).

2.3.4. Rhodamine 6G 2-(2-fluoroethoxy)ethyl ester, chloride salt (4)—This compound was prepared using the same method as described for **3** starting with 138 mg (0.33 mmol) of rhodamine 6G lactone. The crude yield was 70% (by HPLC). The crude

product was dissolved in 3 mL acetonitrile and filtered through a C-18 Sep-Pak cartridge to remove the unreacted lactone. A high-purity sample was obtained by purifying a small amount of the crude product semi-preparative HPLC using the Method B. Yield: 89 mg (50%). ^1H NMR (CDCl_3 , 400 MHz): δ 8.35 (dd, 1H, $J = 1.34, 7.93$), 7.82–7.72 (m, 4H), 7.29 (dd, 1H, $J = 0.95, 7.42$), 6.72 (s, 2H), 6.63 (s, 2H), 4.50 (dt, 2H, $J_1 = 47.71, J_2 = 4.08$), 4.15 (t, 2H, $J = 4.71$), 3.60–3.47 (m, 8H), 2.30 (s, 6H), 1.40 (t, 6H, $J = 7.19$). ^{19}F NMR (CDCl_3): –222.8 ppm (m, 1F). HRMS m/z (%): calcd for $\text{C}_{32}\text{H}_{38}\text{FN}_2\text{O}_4^+$ [M^+] 505.2497, found 505.2503 (100%).

2.3.5. Rhodamine 101 2-(2-fluoroethoxy)ethyl ester, tosyl salt (5)—This compound was prepared from the rhodamine 101 lactone (215 mg, 0.44 mmol) and initially purified using a C-18 Sep-Pak cartridge to remove unreacted lactone as described above for **3**. A high-purity sample was obtained by C-18 column chromatography (22 mm $\times$ 350 mm) using 50% acetonitrile/water with 0.5% acetic acid as mobile phase. Yield (crude): 90 mg (30%). ^1H NMR (CDCl_3 , 400 MHz): δ 8.33 (dd, 1H, $J = 1.18, 7.84$), 7.85–7.70 (m, 4H), 7.27 (dd, 1H, $J = 0.98, 7.28$), 7.08 (d, 2H, $J = 8.02$), 6.52 (s, 2H), 4.55 (dt, 2H, $J_1 = 47.76, J_2 = 4.10$), 4.20 (t, 2H, $J = 4.63$), 3.75–3.46 (m, 12H), 3.04 (m, 4H), 2.66 (t, 4H, $J = 6.06$), 2.28 (s, 3H), 2.09 (m, 6H), 1.96 (m, 3H). ^{19}F NMR (CDCl_3): –222.9 ppm, (m, 1F). HRMS m/z (%): calcd for $\text{C}_{32}\text{H}_{38}\text{FN}_2\text{O}_4^+$ [M^+] 581.2816, found 581.2815 (100%).

2.4. Synthesis of ^{18}F -labeled rhodamines

The diethylene glycol esters [^{18}F]**3**, [^{18}F]**4**, and [^{18}F]**5** were prepared using the one-pot two-step procedure as previously described for [^{18}F]**2** [24–26]. Briefly, an aqueous [^{18}F]fluoride solution was dried azeotropically in a 3 mL Pierce Reacti-Vial in the presence of K 2.2.2, K_2CO_3 , and acetonitrile. Diethylene glycol ditosylate in 0.5 mL acetonitrile was added to the dried residue and the mixture was heated at 90° C for 10 min. After rapid cooling to room temperature, a solution of the rhodamine lactone in 0.8 mL acetonitrile and DIPEA (3–5 drops) were added to the vial. Further heating at 160° C for 30 min using a 30G ventilation needle gave the crude ^{18}F -labeled rhodamine, which was further purified by semi-preparative HPLC. For each compound, HPLC fractions containing the radioactive product were combined and dried under a stream of nitrogen at 65 °C. The radioactive product was then redissolved in either 10% EtOH/water for the partition coefficient measurements or 10% EtOH/saline for animal experiments. The integrity and purity of the final radioactive product was confirmed by analytical HPLC.

2.5. Partition coefficient (logD) measurements

The logD values for the compounds were determined as described previously [26]. Briefly, 3.7–5.6 MBq (100–150 μCi) of [^{18}F]**3**, [^{18}F]**4**, or [^{18}F]**5** in 20 μL 10% EtOH/water was added to a mixture of 480 μL phosphate-buffered saline (pH 7.4, PBS) and 500 μL octanol in 1.5 mL Eppendorf tubes. Samples were vortexed for 1 min and then centrifuged at 13,200 rpm for 5 min. From each sample, three 100- μL samples of both the PBS and the organic layer were transferred into plastic tubes, and the samples were counted with a Packard Cobra gamma counter. The experiments were performed in triplicate.

2.6. Small-animal PET imaging studies

All *in vivo* experiments were carried out in rats because of the previously observed instability of [^{18}F]2 in mouse serum [24]. All animal studies were carried out under a protocol approved by the Boston Children's Hospital Institutional Animal Care and Use Committee. Before injection, the solution was filtered through a sterilized centrifugal filter (0.2 μm). For PET imaging studies, animals were injected with 100 μL of [^{18}F]3, [^{18}F]4, or [^{18}F]5 (3.7–5.6 MBq, 100–150 μCi) and anesthetized with isoflurane (2–4% in air). Data acquisition was initiated as quickly as possible after injection of the tracer. Imaging was performed using a Siemens Focus 120 MicroPET scanner. Data were acquired for 60 min in list mode and reconstructed into either a single 60 min image or six 10 min frames. Reconstruction was performed using unweighted OSEM2D generating an image with a volume of $128 \times 128 \times 95$ voxels ($0.866 \times 0.866 \times 0.796 \text{ mm}^3$). Image analysis was performed using the ASIPro software package (Siemens Medical Solutions). Time-activity curves (TACs) were constructed by manually drawing regions of interest (ROI) within the left ventricular myocardium, the chest, and medially in the liver. All ROIs were then copied on each of the frames, and time-activity curves of the ROI mean values were generated.

2.7. Biodistribution studies

For each time point (15, 30, 60, 120 min), 3–5 animals were injected with 1.1–2.2 MBq (30–60 μCi) of the ^{18}F -labeled rhodamine in 100 μL 10% EtOH/saline via the tail vein, sacrificed by CO_2 asphyxia at the appropriate time, and weighed. Selected tissues were then excised, weighed and assayed for ^{18}F . The percent injected dose per gram (% ID/g) for each tissue was calculated by comparison of the tissue counts to a standard sample prepared from the injectate.

As part of the biodistribution study, a urine sample was obtained at 60 min post-injection and analyzed by HPLC to determine the identity of the excreted material.

2.8. Data Analysis

Statistical analysis was performed with Prism software (GraphPad Software Inc., La Jolla, CA) using a 2-tailed t test.

2.9. Cardiomyocyte uptake and fluorescent microscopy studies

Cardiomyocyte uptake and fluorescent microscopy studies were performed as previously described [26]. Briefly, ventricular cardiomyocytes were isolated from 2-day-old Lewis rat pups using the Neonatal Cardiomyocyte Isolation System (LK003300, Worthington Biochemical Corporation, Lakewood, NJ). For the cellular uptake experiments, [^{18}F]4 was dissolved in serum-free medium to give a concentration of $\sim 37 \text{ kBq}$ (1 μCi)/ μL at the beginning of the experiment. Six time points were used for uptake experiments: 0, 1, 5, 10, 30, and 60 minutes. After the appropriate incubation time, the supernatant was transferred into a separate tube for subsequent assay; the cardiomyocytes were washed with PBS, which was added to the supernatant; and the cells were harvested by addition of 1 N NaOH. The vials containing the combined supernatant and PBS washing solution and the harvested cells

were assayed in a gamma counter to determine percent tracer uptake over time. The cell uptake experiments were performed in triplicate with three different batches of [^{18}F]4.

For fluorescent microscopy imaging, cells were stained with either MitoTracker Green FM (M-7514, Life Technologies, Grand Island, NY) in combination with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies) according to the manufacturer's directions after incubation with the ^{18}F -labeled rhodamine compound, and then mounted on slides and imaged using an FSX100 microscope (Olympus, Waltham, MA).

3. Results and Discussion

We synthesized three new ^{18}F -labeled rhodamine dyes (rhodamine 6G, tetramethylrhodamine, and rhodamine 101) and compared their pharmacokinetic properties to those of rhodamine B ([^{18}F]2) to determine if, by changing the rhodamine core, we could further reduce the liver concentration and/or increase the rate of clearance from the liver while simultaneously maintaining or increasing tracer accumulation in the heart.

3.1. Synthesis of the non-radioactive (^{19}F) compounds

We prepared the non-radioactive compounds for use as reference materials for the HPLC characterization of the no-carrier-added ^{18}F compounds. These reference compounds were characterized by HRMS and proton and fluorine NMR. The crude ^{19}F compounds were purified by semi-preparative HPLC using the same elution conditions used for the purification of the ^{18}F compounds. The tosyldiethyleneglycol fluoride prosthetic group was prepared in good yield (80%) from the bistosylate and tetrabutylammonium fluoride as described previously.[26] Reaction of diethyleneglycol fluorotosylate with the rhodamine lactones in acetonitrile solution in the presence of DIPEA gave the corresponding esters in approximately 25% isolated yield.

3.2. Synthesis of ^{18}F -labeled rhodamine esters

The ^{18}F -labeled diethyleneglycol esters of ^{18}F -tetramethylrhodamine ([^{18}F]3), rhodamine 6G ([^{18}F]4), rhodamine 101 ([^{18}F]5) were prepared using a procedure similar to that previously described for the ^{18}F -labeled diethyleneglycol ester of rhodamine B ([^{18}F]2, Fig. 2) [26]. The identities of the ^{18}F -labeled rhodamines were confirmed by analytical HPLC using the non-radioactive compounds as reference materials. The total synthesis time was 120 min and the products were obtained in >97% radiochemical purity with decay-corrected yields of $18\pm1\%$, $19\pm1\%$, and $22\pm3\%$ for [^{18}F]3, [^{18}F]4, and [^{18}F]5, respectively. The specific activities of [^{18}F]3, [^{18}F]4, and [^{18}F]5 are all on the order of 310–320 MBq/ μmol (8.4–8.6 mCi/ μmol) (Table 1). The partition coefficients for the compounds were similar (1.90 to 1.97). These results are summarized in Table 1.

3.3. Small-animal PET imaging

Representative 60-min (summed) small-animal PET images obtained with [^{18}F]3, [^{18}F]4, and [^{18}F]5 are shown in Figure 3. These images clearly show high and persistent retention in the myocardium and high contrast between the myocardium and blood, liver, bones and lungs for [^{18}F]4 while [^{18}F]3 and [^{18}F]5 show lower uptake in the myocardium and higher

uptake in the liver. The heart is much more clearly defined with [^{18}F]4 than with [^{18}F]3 and [^{18}F]5, and the liver uptake is much lower.

The microPET images also reveal high accumulation of ^{18}F in the neck, as was observed for [^{18}F]2, possibly representing the parathyroid or salivary gland accumulation, and in the muscles of the forelimbs. The bone uptake in all cases is minimal, with the skeleton being barely visible, suggesting minimal *in vivo* defluorination.

Time-activity curves for [^{18}F]4 are shown in Figure 4. These curves show that, for [^{18}F]4, the initial difference (at 5 min p.i.) between the heart and the liver is much greater than was previously seen for [^{18}F]2 and the liver clearance is faster. Similarly to [^{18}F]2, the heart uptake of [^{18}F]4 remains constant through the study period with no washout. Thus, for [^{18}F]4, the heart concentration ($69 \pm 5 \text{ kBq/cm}^3$) at 5 min p.i. is somewhat higher than that in the liver ($59 \pm 2 \text{ kBq/cm}^3$) resulting in a heart-to-liver ratio of 1.2. Since the tracer rapidly washes out of the liver but not the heart, at 35 min p.i., the heart-to-liver ratio for [^{18}F]4 increases to 4.2. At 55 min p.i., the liver concentration of [^{18}F]4 decreases to 25% of the 5 min value and the heart-to-liver ratio increases to approximately 4.5.

3.4. Biodistribution Studies

Due to the lower myocardial uptake and high liver uptake of [^{18}F]3 and [^{18}F]5 in the microPET imaging study, a biodistribution study was only carried out for [^{18}F]4. The results of this study are summarized in Table 2.

As was seen in the small-animal PET images, the change from rhodamine B to rhodamine 6G leads to a significantly improved pharmacokinetic profile. The heart concentration of [^{18}F]4 reaches a plateau of 1.3% ID/g after 15 min and remains essentially constant over the entire 120 min study period with no washout of the tracer over that time. The absolute uptake by the heart is, however, somewhat lower than that seen for [^{18}F]2 (2.5% ID/g). On the other hand, the concentration of [^{18}F]4 in non-target tissues is significantly lower than that of [^{18}F]2. For example, at 60 min p.i. the liver uptake of [^{18}F]4 is 0.15% ID/g compared to 0.49% ID/g for [^{18}F]2. Similarly, at 60 min p.i. the ^{18}F concentration in the blood for [^{18}F]4 is 0.07% ID/g (vs. 0.25% ID/g for [^{18}F]2) and the ^{18}F concentration in the lungs is 0.58% ID/g (vs. 2.06% ID/g for [^{18}F]2). Results for other tissues follow a similar pattern. These results translate into much improved heart-to-non-target-tissue ratios. For example, at 60 min p.i. the heart-to-liver ratio for [^{18}F]4 is 12.0 compared to 5.1 for [^{18}F]2, and this value steadily increased throughout the 2-h study period, from 1.3 at 15 min p.i. to 16.1 at 2 h p.i. The heart-to-blood ratio also increased throughout the study period, from 10.1 at 15 min p.i. to 29.5 at 2 h p.i.

The concentration of the tracer in the kidneys remained relatively high and constant throughout the study, ranging from 10.2% at 15 min p.i. to 8.2% ID/g at 2 h while the concentration in the gut varied from 3.8% ID/g at 15 min to 1.0% ID/g at 2 h. Together these results suggest primarily renal excretion, in agreement with the small-animal PET images that show significant tracer accumulation in the bladder.

Analysis of urine samples (n=3) obtained at 60 min post-injection showed $79\pm 7\%$ of the material to be intact [^{18}F]**4**, which suggests that the compound is sufficiently stable for use in myocardial perfusion imaging.

There is significant accumulation of [^{18}F]**4** in both the thyroid/parathyroid/trachea and the mandibular salivary gland. The former is presumably due to accumulation of the compound in the parathyroid, as is seen with other lipophilic cationic compounds such as $^{99\text{m}}\text{Tc}$ -MIBI and $^{99\text{m}}\text{Tc}$ -tetrofosmin [28]. These tissues were collected because of the high uptake observed in the neck on the small-animal PET scans. As is seen with the cardiac uptake, the concentration of the tracer in these tissues remains fixed throughout the 2-h measurement period. If the uptake in the parathyroid is, in fact, [^{18}F]**4**, this suggests that it might also be useful for parathyroid imaging, similarly to $^{99\text{m}}\text{Tc}$ -MIBI [28]. Further studies are necessary to confirm exactly which these tissue(s) in the region actually accumulate ^{18}F and whether or not the accumulation reflects intact [^{18}F]**4**.

There is also accumulation of tracer in the spleen, which decreases slowly throughout the study. The concentration is, however, about 40% less than was seen for [^{18}F]**2** ($2.01\pm 0.61\%$ ID/g vs. $3.53\pm 0.57\%$ ID/g at 60 min p.i.). This result and the lung uptake, which is also lower than was observed for [^{18}F]**2** ($0.58\pm 0.19\%$ ID/g vs. $2.06\pm 0.16\%$ ID/g), may both be due to aggregation of the compound because of the relatively low specific activity (Table 1): Rhodamines are known to aggregate even at submicromolar concentrations [29]. The low specific activity (310–320 MBq/ μmol) is primarily the result of the small amount of ^{18}F used in these syntheses (typically 10 mCi), and pilot studies with larger amounts of ^{18}F show a proportionally higher value for the specific activity. However, as can be seen from the small-animal PET images, the low specific activity does not interfere with visualization of the heart. Finally, it is worth noting that there is minimal tracer uptake in the bone ($\sim 0.3\%$ ID/g) which does not increase during the study suggesting that there is no appreciable loss of ^{18}F from the compound.

The biodistribution of [^{18}F]**4** may be compared to that of other ^{18}F -labeled myocardial perfusion agents currently under development. Compared to BMS-747158-02 (Flurpiridaz F18; Lantheus Medical Imaging, Inc.), which is currently in late-stage clinical trials, the heart concentration of [^{18}F]**4** at 60 min p.i. ($1.77\pm 0.15\%$ ID/g) is similar to the $2.06\pm 0.83\%$ ID/g observed in a study in Wistar rats ($p = 0.51$) and somewhat lower than the $3.3\pm 0.3\%$ ID/g ($p < 0.001$) observed in Sprague-Dawley rats [11, 12, 30]. On the other hand, the liver concentration of BMS-747158-02 is somewhat higher and the clearance from the liver is somewhat slower than that of [^{18}F]**4**. The liver concentration of BMS-747158-02 decreases from $1.27\pm 0.10\%$ ID/g to $0.63\pm 0.16\%$ ID/g from 30 min to 120 min p.i. whereas the liver concentration of [^{18}F]**4** decreases from $0.14\pm 0.09\%$ ID/g to $0.05\pm 0.02\%$ ID/g in the same time frame. This is also reflected by a comparison of the heart-to-liver ratios. From 30 min to 120 min p.i., the heart-to-liver ratio of [^{18}F]**4** increases from 4.1 to 13.4 in contrast to BMS-747158-02 where the corresponding ratio only increases from 2.7 to 5.4 [12]. However, the heart-to-liver ratio for [^{18}F]**4** at 60 min p.i. (6.0) is similar to that of BMS-747158-02 (5.4 in Wistar rats and 3.7 in Sprague-Dawley rats) [11, 30]. In contrast, the heart-to-blood ratio of 19.3 for [^{18}F]**4** at 30 mins p.i., while quite high, is somewhat lower than that of BMS-747158-02 (30.0) [12].

Another ^{18}F -labeled myocardial perfusion tracer currently in clinical trials is BFPET (Fluoropharma), an ^{18}F -labeled analog of the tetraphenylphosphonium cation [7, 23]. This compound exhibits heart uptake similar to that of [^{18}F]4. For example, the heart uptake of BFPET in Sprague-Dawley rats is $1.51 \pm 0.04\%$ ID/g at 30 min, slightly higher than for [^{18}F]4 ($1.32 \pm 0.06\%$ ID/g, $p = 0.0007$), and $1.57 \pm 0.18\%$ ID/g at 60 min p.i., equivalent to that result for [^{18}F]4 ($1.77 \pm 0.15\%$ ID/g, $p = 0.1189$) [7]. The concentration of the two compounds in the liver is also very similar. At 30 min p.i. the liver concentration of BFPET is $0.18 \pm 0.05\%$ ID/g versus $0.15 \pm 0.11\%$ ID/g for [^{18}F]4, and at 60 min p.i. the liver concentration of BFPET is $0.17 \pm 0.03\%$ ID/g compared to $0.15 \pm 0.02\%$ ID/g for [^{18}F]4.

3.5. Cardiomyocyte Studies

A unique aspect of the rhodamine-based tracers is that rhodamines are inherently fluorescent making ^{18}F -labeled rhodamines intrinsic dual-modality imaging agents [31]. This allows the measurement of the uptake of the compound by cells by assaying the ^{18}F content while at the same time the intracellular localization of the tracer can be directly observed without the need for any additional interventions.

In order to determine if [^{18}F]4 accumulates in the mitochondria in the same manner as does non-radiolabeled rhodamine 6G [32] and ^{18}F -labeled rhodamine B diethylene glycol ester [^{18}F]2 [26], fluorescence microscopy experiments and cellular uptake studies were performed in rat cardiomyocytes. Figure 5 shows a representative fluorescence microscopy image of cardiomyocytes incubated with [^{18}F]4. Figure 5A shows the mitochondria stained in green (MitoTracker Green) while figure 5B shows the same cells stained in red with [^{18}F]4. Figure 5C shows the nuclei stained in blue (DAPI). In agreement with the results obtained for [^{18}F]2, [^{18}F]4 localizes in the mitochondria of rat cardiomyocytes as shown in the overlaid image in Figure 5D (Pearson correlation coefficient 0.85).

The uptake of [^{18}F]4 in rat cardiomyocytes were measured as previously described for [^{18}F]2 [26] and found to be similar (Figure 6). The ^{18}F -labeled rhodamine 6G diethylene glycol ester, [^{18}F]4, internalizes rapidly into rat cardiomyocytes with $4.6 \pm 0.7\%$ of the initial activity internalized within 1 min, similar to the results for the rhodamine B derivative, [^{18}F]2 ($3.9 \pm 0.8\%$ at 1 min). At 60 min, $55.0 \pm 6.8\%$ of the initial activity of [^{18}F]4 is internalized, not significantly higher than that of the rhodamine B derivative [^{18}F]2 ($43.1 \pm 7.7\%$, $p = 0.1153$). Incubation times exceeding 60 min resulted in variable results due to the experimental stress on the isolated cardiomyocytes, so the study could not be continued until an uptake plateau was reached.

One additional consideration in the in vivo evaluation of radiolabeled rhodamine dyes is that rhodamine 6G is a substrate of Pgp, the protein implicated in MDR1 drug resistance in cancer [33]. While this is not a factor in their evaluation as myocardial perfusion agents, it is an important consideration in their future evaluation as tumor imaging agents. Preliminary studies suggest that [^{18}F]4 is, in fact a substrate for Pgp [34], and studies are currently being carried out to more completely evaluate this possible application.

4. Conclusions

This comparison of several different ^{18}F -labeled rhodamines as potential myocardial perfusion agents demonstrated that ^{18}F -labeled rhodamine 6G (^{18}F 4) is superior to the first generation ^{18}F -labeled rhodamine tracer, rhodamine B (^{18}F 4), primarily in terms of its superior contrast between the heart and the liver. As was the case with all of the ^{18}F -labeled rhodamine dyes that have been evaluated to date, the uptake by the heart is rapid and does not decrease over time. In contrast to other ^{18}F -labeled rhodamines, the liver uptake of ^{18}F -labeled rhodamine 6G is both initially lower and decreases more rapidly. As was the case with ^{18}F -labeled rhodamine B, ^{18}F -labeled rhodamine 6G localizes in the mitochondria of isolated rat cardiac myocytes. In combination, these results strongly suggest that ^{18}F -labeled rhodamine 6G is a promising candidate for more extensive evaluation as a PET MPI tracer.

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Figure 1.
Rhodamine dyes discussed in this study.

Figure 2.
Radiosyntheses of the rhodamine esters: a) $\text{K}_2\text{CO}_3/\text{K}$ 2.2.2; MeCN, 90°C , 10 min. b) DIPEA, MeCN, 165°C , 30 min.

Figure 3. Coronal (top), sagittal (middle), and transverse (bottom) microPET images of [^{18}F]4 (A), [^{18}F]3 (B), and [^{18}F]5 (C). Animals were injected with (3.7–5.6 MBq, 100–150 μCi) of [^{18}F]3, [^{18}F]4, or [^{18}F]5, anesthetized with isoflurane (2–4% in air), and data were acquired for 60 min.

Figure 4. Time-activity curves for [^{18}F]rhodamine 6G ([^{18}F]4) derived from small-animal PET images.

Figure 5. Fluorescence microscopy images of rat cardiomyocytes showing mitochondria stained by MitoTracker green dye (A), $[^{18}\text{F}]\mathbf{4}$ (B), nuclei stained by DAPI (C), and the overlay (D). The tracer $[^{18}\text{F}]\mathbf{4}$ clearly co-localizes in the mitochondria of rat cardiomyocytes (Pearson correlation coefficient 0.85).

Figure 6.
Time-dependent uptake of [^{18}F]4 into rat cardiomyocytes. Error bars indicate estimated standard deviation.

Table 1

Specific activities and $\log D$ values for the three new rhodamine derivatives in comparison to the previously described rhodamine B ester [^{18}F]2.

Compound	Rhodamine	$\log D$	Specific Activity MBq/ μmol (mCi/ μmol)
[^{18}F]2	B	1.90 ± 0.01	310 (8.4)
[^{18}F]3	Me4	1.90 ± 0.01	310 (8.4)
[^{18}F]4	6G	1.96 ± 0.05	320 (8.6)
[^{18}F]5	101	1.97 ± 0.01	320 (8.6)

Table 2

Biodistribution of radioactivity after injection of [^{18}F]4 into rats^a

Tissue	Compound				
	[^{18}F]4				[^{18}F]2 ^b
	15 min	30 min	60 min	120 min	60 min
Blood	0.13 ± 0.03	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.25 ± 0.02
Heart	1.31 ± 0.46	1.32 ± 0.06	1.32 ± 0.29	1.77 ± 0.15	2.51 ± 0.16
Lung	1.08 ± 0.34	0.58 ± 0.14	0.58 ± 0.19	0.82 ± 0.19	2.06 ± 0.16
Liver	1.01 ± 0.14	0.15 ± 0.11	0.15 ± 0.02	0.11 ± 0.01	0.49 ± 0.04
Spleen	3.16 ± 1.30	2.01 ± 0.17	2.01 ± 0.61	1.86 ± 0.41	3.53 ± 0.57
Kidney	10.2 ± 3.7	7.90 ± 1.05	7.90 ± 0.99	8.21 ± 1.22	9.28 ± 0.66
Gut	3.83 ± 3.50	1.39 ± 0.78	1.39 ± 0.58	1.07 ± 0.28	1.69 ± 0.42
Skin	0.13 ± 0.04	0.15 ± 0.02	0.15 ± 0.04	0.16 ± 0.04	0.36 ± 0.04
Muscle	0.10 ± 0.02	0.12 ± 0.01	0.12 ± 0.03	0.14 ± 0.02	0.54 ± 0.07
Bone	0.30 ± 0.08	0.29 ± 0.03	0.29 ± 0.05	0.37 ± 0.05	0.77 ± 0.04
Thyroid/Trachea	1.23 ± 0.47	1.05 ± 0.34	1.05 ± 0.24	1.08 ± 0.12	-
Mandibular gland	1.74 ± 0.50	2.01 ± 0.26	2.01 ± 0.31	2.31 ± 0.54	-
Heart/blood ^c	10.1	18.9	18.9	29.5	10.0
Heart/lung ^c	1.2	2.3	2.3	2.2	1.2
Heart/liver ^c	1.3	8.8	12.0	16.1	5.1
Heart/bone ^c	4.4	4.6	4.6	4.8	3.3

^aResults are expressed as mean % ID/g ± ESD for n = 4.^bFrom ref. [26]^cTissue ratios