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Biological Characterization of F-18-Labeled Rhodamine B, a Potential Positron Emission Tomography Perfusion Tracer

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Abstract

Introduction—Myocardial infarction is the leading cause of death in western countries, and positron emission tomography (PET) plays an increasing role in the diagnosis and treatment planning for this disease. However, the absence of an F-18-labeled PET myocardial perfusion tracer hampers the widespread use of PET in myocardial perfusion imaging (MPI). We recently reported a potential MPI agent based on F-18-labeled rhodamine B. The goal of this study was to more completely define the biological properties of F-18-labeled rhodamine B with respect to uptake and localization in an animal model of myocardial infarction and to evaluate the uptake F-18-labeled rhodamine B by cardiomyocytes.

Methods—A total of 12 female Sprague Dawley rats with a permanent ligation of the left anterior descending artery (LAD) were studied with small-animal PET. The animals were injected with 100–150 μ Ci of F-18-labeled rhodamine B diethylene glycol ester ($[^{18}\text{F}]\text{RhoBDEGF}$) and imaged two days before ligation. The animals were imaged again two to ten days post-ligation. After the post-surgery scans, the animals were euthanized and the hearts were sectioned into 1 mm slices and myocardial infarct size was determined by phosphorimaging and 2,3,5-triphenyltetrazolium chloride staining (TTC). In addition, the uptake of $[^{18}\text{F}]\text{RhoBDEGF}$ in isolated rat neonatal cardiomyocytes was determined by fluorescence microscopy.

Results—Small-animal PET showed intense and uniform uptake of $[^{18}\text{F}]\text{RhoBDEGF}$ throughout the myocardium in healthy rats. After LAD ligation, well defined perfusion defects were observed in the PET images. The defect size was highly correlated with the infarct size as determined ex vivo by phosphorimaging and TTC staining. In vitro, $[^{18}\text{F}]\text{RhoBDEGF}$ was rapidly internalized into rat cardiomyocytes with ~40 % of the initial activity internalized within the 60 min incubation time. Fluorescence microscopy clearly demonstrated localization of $[^{18}\text{F}]\text{RhoBDEGF}$ in the mitochondria of rat cardiomyocytes.

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Conclusion—Fluorine-18-labeled rhodamine B diethylene glycol ester ($[^{18}\text{F}]\text{RhoBDEGF}$) provides excellent image quality and clear delineation of myocardial infarcts in a rat infarct model. In vitro studies demonstrate localization of the tracer in the mitochondria of cardiac myocytes. In combination, these results support the continued evaluation of this tracer for the PET assessment of myocardial perfusion.

Keywords

rhodamine; PET; perfusion; infarct; fluorescence; imaging

1. Introduction

Myocardial infarction is the leading cause of death in western countries, and myocardial perfusion imaging (MPI) is an important tool in the evaluation of myocardial ischemia and infarction [1, 2]. Positron emission tomography (PET) imaging offers several significant advantages over single-photon emission computed tomography (SPECT) imaging in this application including more straightforward attenuation correction, which is important in the evaluation of obese patients and women with large or dense breasts; the ability to measure myocardial blood flow, which is important in the detection of balanced ischemia; and higher spatial resolution [3–5]. While PET tracers labeled with radionuclides other than ^{18}F have been used for MPI, these suffer from significant limitations. For example, $[^{13}\text{N}]\text{NH}_3$ can only be used at sites with cyclotrons because of the short half-life of ^{13}N (10 min), and the high cost of the $^{82}\text{Sr}/^{82}\text{Rb}$ generator limits its use to sites with high patient throughput. These limitations have led to considerable interest in the development of an ^{18}F -labeled MPI radiopharmaceutical [6, 7], particularly as the development of regional production facilities for $[^{18}\text{F}]\text{FDG}$ has demonstrated the feasibility of centralized production of ^{18}F -labeled radiopharmaceuticals.

Several ^{18}F -labeled MPI radiopharmaceuticals are currently in various stages of development. These include Flurpiridaz (Lantheus Medical Imaging, Inc.), which is currently in Phase 3 clinical trials [8–12], and BFPET (Fluoropharma) for which Phase 1 clinical trials were recently completed [13, 14]. In addition to these compounds, we recently reported the development of ^{18}F -labeled esters of rhodamine B as a potential MPI radiopharmaceutical [15, 16]. In a comparative study of different rhodamine B esters, we found that the ^{18}F -labeled diethylene glycol ester of rhodamine B ($[^{18}\text{F}]\text{RhoBDEGF}$) is optimal in terms of stability and pharmacokinetics [17]. In this report, we describe the more complete biological characterization $[^{18}\text{F}]\text{RhoBDEGF}$, including its ability to delineate perfusion defects in a rat model of myocardial infarction and its accumulation within the mitochondria of neonatal rat cardiomyocytes in vitro.

2. Materials and methods

2.1 General

Rhodamine B lactone (>97%) was purchased from MP Biomedical (Solon, OH). Diethylene glycol ditosylate was purchased from TCI (Waltham, MA). Extra dry reagent grade acetonitrile was obtained from Thermo Scientific. Kryptofix (K2.2.2) (98%) was obtained from Sigma-Aldrich. Potassium carbonate (99.97%) was purchased from Alfa Aesar (Ward Hill, MA). Fluorine-18 (as F^- in water) was purchased from Cardinal Healthcare (Woburn, MA) and the Brigham and Women's Hospital BICOR facility (Boston, MA).

2.2 Purification and quality control

For semi-preparative high-performance liquid chromatography (HPLC), an ISCO system comprised of an ISCO V 113 4 variable wavelength UV-visible detector (operated at $\lambda = 550$ nm), ISCO 2300 HPLC pumps, and a Grace Apollo C18 column (10 $\times$ 250 mm, 5 μ m) was used. The radiometric HPLC detector was comprised of Canberra nuclear instrumentation modules and optimized for 511-keV photons. The solvent system was: 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The following gradient was used for purification of the ^{18}F -labeled product: 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. Analytical HPLC was carried out using a HITACHI 7000 system including an L-7455 diode array detector, an L-7100 pump, a radiometric gamma detector similar to that described above, and a D-7000 interface using an LaChrom PuroSphere Star C18e column (4 $\times$ 30 mm, 3 μ m). The solvent system was: 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 gradient was 0–15 min (30–70% B), 15–25 min (70% B).

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 gamma counter or imaged using phosphorimaging plates (Fuji Medical Systems) which were subsequently visualized using a Fujifilm BAS-5000 system. The resulting images were assayed using ImageJ software 1.43u (National Institutes of Health).

2.3 Radiosynthesis of ^{18}F -labeled rhodamine B diethylene glycol ester [^{18}F]RhoBDEGF

The rhodamine B diethylene glycol ester was prepared by a one-pot two-step procedure as recently described [17]. Briefly, after addition of Kryptofix® and K_2CO_3 to an aqueous [^{18}F]fluoride solution, the mixture was azeotropically dried in a Pierce vial using acetonitrile, and diethylene glycol ditosylate dissolved in 0.5 mL anhydrous acetonitrile was added to the dried residue. The mixture was heated at 90 °C for 10 min in the sealed Pierce vial. After rapid cooling to room temperature, a solution of rhodamine B lactone in 0.8 mL anhydrous acetonitrile and DIPEA (3–5 drops) were added into the vial. The vial was then sealed, the Teflon cap was pierced with a 30-gauge ventilation needle, and the reaction mixture was heated at 160 °C for 30 min to give the crude ^{18}F -labeled rhodamine, which was purified by semi-preparative HPLC. Fractions containing the radioactive product were collected, combined, and dried under a stream of nitrogen at 65 °C. For animal experiments, the radioactive product was dissolved in 10% ethanol/saline and filtered using a pre-sterilized 0.2 μ m centrifugal filter (Eppendorf). The identity and purity of the radioactive product were confirmed by analytical HPLC and/or radio-TLC.

2.4 Small-animal PET imaging studies

Animal studies were carried out under a protocol approved by the Children's Hospital Boston Institutional Animal Care and Use Committee. For PET imaging studies animals were injected with 100 μ L of [^{18}F]RhoBDEGF (3.7–5.6 MBq, 100–150 μ Ci) via the tail vein 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 a single 60-min image. 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 mm³). Image analysis was performed using the ASIPRO software package (Siemens Medical Solutions). For perfusion defect analysis, the tracer distribution defects were quantified in the PET images for each short-axis slice using the ImageJ. The defect size was calculated as the percentage of the total left ventricle.

2.5 Myocardial infarct model

Rats were initially anesthetized with 4% isoflurane in an induction chamber and then anesthetized by intramuscular injection of a mixture of Ketamine (75 mg/kg body weight) and Xylazine (5 mg/kg body weight). A power light with flexible horns was used to illuminate the neck of the rat, the tongue was retracted, and endotracheal intubation was gently performed with a 16-gauge angiocatheter. Lidocaine (2 ml/kg, 0.1% solution) was then injected intraperitoneally to prevent ventricular arrhythmias. The animal was placed on a rodent ventilator (Harvard Apparatus, Model 683 or 680) and ventilated with 2% isoflurane in 95% oxygen at a flow rate of 1.5 L/min and a stroke volume of 2.5 ml at 50–60 stroke/min. A 1.5 cm vertical left parasternal skin incision exposed the underlying pectoralis muscles, which were then retracted. The fifth intercostal space was opened and the 5th and 6th ribs separated with a small retractor to expose the heart. The left coronary artery was carefully examined and a 6-0 silk suture was passed through the epicardial layer around the origin (approximately 5 mm below the edge of the left atrial appendage) of the main trunk of the left coronary artery. The coronary artery was then occluded by tying the ligature. Ischemia was verified visually by the regional appearance of paleness on the surface of the left ventricle distal to the ligation. The chest cavity was closed and animals received 0.1 mg/kg buprenorphine intraoperatively for analgesia. After removal from the ventilator, animals were placed in a recovery cage with a water-circulating heating pad and a supply of oxygen for about 30 min.

2.6 Phosphorimaging and TTC staining

Following the post-infarct PET scans, animals were euthanized by CO₂ asphyxia. The hearts were excised, rinsed with PBS, and dried carefully before storing at –20 °C for 1–2 h. The frozen hearts were then sectioned into ~1 mm slices in a cold room beginning at the apex and ending at the base. For phosphorimaging, heart slices were exposed to a phosphorimaging plate for 15–30 min. The plates were then removed in the dark and read out in the phosphorimager. The same slices were then incubated in 1% TTC (triphenyl tetrazolium chloride) solution at 37 °C for 20 minutes, fixed in formalin, and stored at 4°C for further analysis. For the evaluation of viable areas of the myocardium, both sides of the slices were photographed with a digital camera. The viable myocardium, stained as red, and the infarcted area, which was unstained (white), were manually traced for each slice using ImageJ. The infarct volume was calculated as a percentage of the total left ventricular volume. The phosphorimaging results were analyzed using the same method.

2.7 Cardiomyocyte isolation and culture

For each experiment, ventricular cardiomyocytes were isolated from 1 litter (6–9 pups) of 2-day-old Lewis rat pups using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corporation, LK003300). 24-well tissue culture plates with or without 12 mm No. 1 glass coverslips were coated with 1 µg/mL fibronectin (Sigma-Aldrich, F1141-1MG) and incubated at 37 °C for 24 h prior to cell plating. Cardiomyocytes were maintained in DMEM-F12 (1:1) (Gibco) medium containing 10% FBS (Atlanta Biologicals), 1% penicillin-streptomycin (Gibco), and 1% Fungizone (Gibco). The following day, cells were washed three times with PBS, and the medium was replaced. Six time points were used for uptake experiments: 0, 1, 5, 10, 30, and 60 min. After incubation with the ¹⁸F-labeled rhodamine compound, cells on coverslips were stained with either MitoTracker Green FM (Invitrogen, M-7514) or AlexaFluor 488-phalloidin (Life Technologies) in combination with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies) according to the manufacturer's directions and then mounted on slides and imaged using a FSX100 microscope (Olympus). For cell uptake experiments, the cardiomyocytes were grown directly on culture plates. The tracer [¹⁸F]RhoBDEGF was dissolved in serum-free medium to give a concentration of ~1 µCi/µL at the beginning of the experiments. Equal amounts were added to each vial. 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 1N 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]RhoBDEGF.

3. Results

3.1 Radiosynthesis of ^{18}F -labeled rhodamine B ester [^{18}F]RhoBDEGF

The ^{18}F -labeled rhodamine B ester [^{18}F]RhoBDEGF (Fig. 1) was successfully prepared according to the previously published method [17]. The product was isolated by semi-preparative HPLC in 97% radiochemical purity with a decay-corrected yield of $19\pm 1\%$. The total synthesis time including purification was 2 h.

3.2 Evaluation of myocardial infarction in rats

A total of 12 female Fisher rats were imaged with [^{18}F]RhoBDEGF on day 1 of the study. Pre-ligation PET images showed intense and homogeneous uptake of the tracer throughout the left ventricle as shown in representative short-axis images in Figure 2A. As a control, TTC staining was performed to confirm that all myocardial regions were 100% viable pre-ligation (Figure 2B). The homogeneous distribution of [^{18}F]RhoBDEGF was additionally confirmed by phosphorimaging (Figure 2C).

On day 3 of the study, each rat underwent permanent LAD ligation inducing a myocardial infarct (MI) that affected 15.6% to 32.6% of the heart as confirmed by TTC staining (vide infra). Animals were then allowed to recover from surgery for a minimum of two days.

Between days 5 and 20 of the study, the animals were again imaged under the same conditions as for the pre-LAD ligation scan. These PET images showed well-defined perfusion defects localized in the LAD-supplied area. Representative short-axis PET images of the pre- and post-LAD ligation PET scans are shown in Figure 3A and 3B, respectively. Pre- and post-LAD ligation PET scans, TTC staining, and phosphorimaging were obtained for three animals. Of the nine remaining animals of the study, several were used for the pre-ligation control studies (PET imaging, TTC staining, and phosphorimaging), several did not survive surgery or were euthanized post-surgery, and in the remaining animals phosphorimaging or TTC data were not acquired due to technical reasons. In the three animals for whom all of the data sets were available, the region of infarcted myocardium determined by TTC staining was positively correlated with the distribution of [^{18}F]RhoBDEGF determined by phosphorimaging (Pearson correlation coefficients 0.92–0.94, Fig. 3). The size of the defect measured ex vivo by TTC staining was compared to the defect size measured in the [^{18}F]RhoBDEGF microPET images and found to be highly and positively correlated ($r=0.999$; $P=0.011$). The size of the perfusion defect measured ex vivo by phosphorimaging was compared to the defect size measured by microPET imaging, and the correlation did not quite achieve significance ($r=0.995$; $P<0.06$). However, this is not surprising considering the physical difficulty of comparing ex vivo and in vivo images.

3.3 Uptake and localization of [^{18}F]RhoBDEGF in rat cardiomyocytes

In order to determine if [^{18}F]RhoBDEGF accumulates in mitochondria in the same manner as does non-radiolabeled [18] and ^{64}Cu -labeled rhodamine B [19], fluorescence microscopy experiments and cellular uptake studies were performed in rat cardiomyocytes. Due to the intrinsic fluorescent properties of rhodamine B [20], [^{18}F]RhoBDEGF could be directly visualized by fluorescence microscopy. Figure 4 shows a representative fluorescence

microscopy image of cardiomyocytes incubated with [^{18}F]RhoBDEGF. In figure 4A, the mitochondria are stained in green (MitoTracker Green), and figure 4B shows the mitochondria stained in red with [^{18}F]RhoBDEGF. Figure 4C shows the cell nuclei stained with DAPI (blue). The co-localization of [^{18}F]RhoBDEGF and MitoTracker in the mitochondria of the rat cardiomyocytes is clearly visualized in the overlaid image (Fig. 4D, Pearson correlation coefficient 0.98).

Additional experiments were performed using phalloidin to stain the cytoskeleton. In Figure 5A, the cytoskeleton is stained in green, the nuclei are stained in blue (DAPI) (Figure 5C), and [^{18}F]RhoBDEGF in red (Figure 5B). No localization of [^{18}F]RhoBDEGF was observed in the cytoskeleton as demonstrated in the overlaid image in Figure 5D.

The kinetics of the uptake of [^{18}F]RhoBDEGF in cardiomyocytes were also investigated. As shown in Figure 6, the tracer [^{18}F]RhoBDEGF is rapidly internalized into cardiomyocytes with $3.9 \pm 0.8\%$ of the initial activity internalized within 1 min. The uptake of [^{18}F]RhoBDEGF increased gradually over time with $43.1 \pm 7.7\%$ initial activity internalized at 1 h. Although an uptake plateau was not reached by the end of the study at 60 min, the study was not continued beyond this time because longer incubation times resulted in somewhat variable results due to the experimental stress on the isolated cardiomyocytes.

4. Discussion

Fluorine-18-labeled rhodamine B diethyleneglycol ester ([^{18}F]RhoBDEGF) was successfully prepared in reasonably good radiochemical yield (19%) and high radiochemical purity (97%) within 2 h using the previously described synthesis [17], which confirms the general suitability of the synthesis for future clinical studies.

The PET studies in rats with experimentally induced myocardial infarcts demonstrate the ability of [^{18}F]RhoBDEGF to delineate even small infarcts in vivo. These in vivo observations were confirmed ex vivo by comparison of the regions of tracer uptake as determined by phosphorimaging to those defined by TTC staining. Defect size determined by [^{18}F]RhoBDEGF PET images showed a very high and statistically significant correlation with MI size determined by TTC staining. The mean differences between the infarct size measured on the [^{18}F]RhoBDEGF microPET images the infarct size measured by TTC staining and phosphorimaging were 5.93% and 3.68%, respectively.

The results obtained in the present study are very similar to those obtained by Sherif, et al. using Flurpiradaz [21]. In that study, investigators also observed a high correlation between the size of the experimentally induced infarct measured ex vivo using TTC staining with the size of the infarct defined by uptake of the ^{18}F -labeled tracer ($r=0.89$; $P<0.01$; $n=31$). The mean difference between PET defect size and MI size by TTC staining was 2.01%. The overall survival of the animals post-surgery was also similar between the two studies (~75%).

Evaluation of the in vitro uptake of [^{18}F]RhoBDEGF in rat cardiac myocytes showed that, as expected from previous studies with non-radiolabeled rhodamines [18], the ^{18}F -labeled rhodamine ester of rhodamine B rapidly accumulates in these cells with more than 40% of the activity associated with the cells at 60 min.

A unique aspect of this tracer is that it is inherently a dual-modality imaging agent, incorporating both an ^{18}F label for PET imaging and the intrinsic fluorescence of the rhodamine dye [20]. 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 additional interventions. This allowed the direct

visualization of [^{18}F]RhoBDEGF accumulation in the mitochondria of the rat cardiac myocytes by fluorescence microscopy, which showed very high correlation with the accumulation of MitoTracker Green. This result demonstrates the potential utility of this radiopharmaceutical as a dual-modality tracer where the ^{18}F label might be exploited for the PET evaluation of myocardial perfusion and the fluorescence might be exploited for delineation of the infarct in vivo.

Similarly, since tracers that accumulate in mitochondria, such as $^{99\text{m}}\text{Tc}$ -MIBI, have also been used in tumor imaging [22–24], it may be possible to exploit the fluorescence of this tracer for the in vivo delineation of tumor extent during surgery.

5. Conclusion

PET imaging using [^{18}F]RhoBDEGF provides excellent image quality and allows the accurate evaluation of MI size in rat models. These results suggest that [^{18}F]RhoBDEGF may be a promising candidate for continued evaluation for possible use in PET assessment of myocardial perfusion. The dual modality properties may also allow other applications such as intraoperative visualization of regional myocardial perfusion or tumor imaging, where the fluorescence may be exploited to better define tumor margins.

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Figure 1.
The chemical structure of $[^{18}\text{F}]\text{RhoBDEGF}$.

Figure 2. PET imaging (short axis slices) shows uniform myocardial distribution of $[^{18}\text{F}]\text{RhoBDEGF}$ (3.7–5.6 MBq; 100–150 μCi) in healthy control rat heart (60 min summed image, anesthesia: 2–4% isoflurane) (A). Control heart was stained red by TTC showing 100% viability (B). Homogeneous uptake of $[^{18}\text{F}]\text{RhoBDEGF}$ in healthy control as determined by phosphorimaging (C).

Figure 3.

(A) Representative PET images (short axis slices) of $[^{18}\text{F}]\text{RhoBDEGF}$ prior to permanent ligation of the left anterior descending artery (LAD). (B) Post-Ligation PET scan showing diffusion defects with infarcted myocardium as determined by TTC staining (infarcted tissue unstained by TTC) (C), and distribution of $[^{18}\text{F}]\text{RhoBDEGF}$ in infarcted myocardium determined by phosphorimaging (D) with high correlation between MI and defect size. Corresponding Pearson-correlation coefficients range from 0.92–0.94. All data is taken from the same animal. PET scans were acquired for 60 min beginning immediately after injection of 3.7–5.6 MBq (100–150 μCi) $[^{18}\text{F}]\text{RhoBDEGF}$, anesthesia – 2–4% isoflurane. The animals were sacrificed 60 min post-injection, and tissue samples were collected for TTC staining and phosphorimaging as described in the Materials and Methods section.

Figure 4. Fluorescence microscopy images of rat cardiomyocytes showing mitochondria stained by MitoTracker Green dye (A), $[^{18}\text{F}]\text{RhoBDEGF}$ (B), nuclei stained by DAPI (C), and the overlay (D). The tracer $[^{18}\text{F}]\text{RhoBDEGF}$ clearly co-localizes in the mitochondria of rat cardiomyocytes (Pearson correlation coefficient 0.98).

Figure 5. Fluorescence microscopy images of rat cardiomyocytes showing the cytoskeleton stained by phalloidin dye (A), $[^{18}\text{F}]\text{RhoBDEGF}$ (B), nuclear DNA stained by DAPI (C), and the overlay (D). No co-localization of the tracer $[^{18}\text{F}]\text{RhoBDEGF}$ and the cytoskeleton is observed.

Figure 6.
Time-dependent uptake of [^{18}F]RhoBDEGF into rat cardiomyocytes.

Table 1

Comparison of defect size as the percentage of the myocardium measured by the various methods.

Animal	Defect Size (%)		
	TTC staining	Phosphorimaging	microPET
1	15.6	15.1	16.3
2	21.3	22.8	22.4
3	32.6	32.7	33.7