**A reactivity-based 18F-labeled probe for PET imaging of oxidative stress in chemotherapy-induced cardiotoxicity**

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**Abstract:**

Oxidative stress underlies the pathology of many human diseases, including the Doxorubicin induced off-target cardiotoxicity in cancer chemotherapies. Since current diagnostic procedures are only capable of monitoring cardiac function, a non-invasive means of detecting biochemical changes in redox status prior to irreversible functional changes is highly desirable for both early diagnosis and prognosis. We designed a novel 18F-labeled molecular probe, 18F-FPBT, for the direct detection of superoxide *in vivo* using positron emission tomography (PET). 18F-FPBT was radiosynthesized in one-step by nucleophilic radiofluorination. *In vitro*, 18F-FPBT showed rapid and selective oxidation by superoxide (around 60% in 5 min) compared to other physiological ROS. In healthy mice and rats, 18F-FBPT is distributed to all major organs in the first few minutes post-injection and is rapidly cleared via both renal and hepatobiliary routes with minimal background retention in the heart. In a rat model of Doxorubicin-induced cardiotoxicity 18F-FBPT showed significantly higher (*P*<0.05) uptake in the hearts of treated animals compared to healthy controls. These results warrant further optimization of 18F-FBPT for clinical translation.

**Introduction**

Reactive oxygen species (ROS) are generated as normal by-products of metabolism in the electron transport chain, and play an integral role in the regulation of cell growth, neurotransmission, and the immune response.(*1*) However, uncontrolled ROS production leads to the oxidation of DNA, proteins, and lipids, underlying the pathogenesis of many cardiovascular and neurodegenerative diseases, as well as cancers and inflammatory conditions.(*2-7*) In the cardiovascular system, elevated ROS are responsible for tissue injury during ischemia/reperfusion, and have been linked to the progression from cardiac hypertrophy to heart failure, the evolution of atherosclerotic plaques, and the cardiac and microvascular dysfunction associated with diabetes. ROS production has also been linked to the cardiotoxicity of cancer chemotherapeutic agents, which severely limits their dosimetry and effectiveness.(*8-11*) Most notably, the cardiotoxicity induced by Doxorubicin, a widely used cancer chemotherapy agent, has been linked to ROS generation.(*12,13*)

A clinically translatable means of noninvasively identifying and quantifying elevated ROS production *in vivo* would be highly desirable for both diagnostic and prognostic purposes, and the development and evaluation of emerging cardioprotective approaches.(*14*)

Several radiolabeled small molecules have been reported to indirectly detect oxidative stress by positron emission tomography.(*15,16*) Radiotracers based on the fluorescent probe dihydroethidium have been tested in rodent models of superoxide associated cardiotoxicity and neuroinflammation.(*17,18*) While some of these designs are promising, none have yet progressed to clinical evaluation, and the search for optimal ROS-sensing PET radiotracer designs continues.

Here, we describe a novel chemical scaffold with potential utility as 18F-labeled PET tracers for the direct detection of ROS *in vivo*. Phenyl benzothiazoles were identified as promising candidates due to their low molecular weight, well established chemistry, susceptibility to oxidation by superoxide, and physicochemical properties that are favourable to cell membrane and blood-brain barrier permeability.(*19,20*) Our tracer, [18F]3-(3-fluoropropyl)-2-phenyl-2,3-dihydrobenzo[*d*]thiazole, 18F-FPBT was designed to be sufficiently lipophilic in its reduced form to facilitate cell membrane and blood brain barrier penetration, while sufficiently hydrophilic when oxidized to become transiently retained in cells under oxidative stress to provide ROS-sensitive PET contrast (**Figure** **1**). Herein, we report the synthesis, 18F-labeling, *in vitro*, and preclinical *in vivo* evaluation of 18F-FPBT in healthy mice and rats, as well as in a rat model of Doxorubicin-induced cardiotoxicity.



**Figure 1. Proposed mechanism of PET tracer, 18F-FPBT for the direct detection of intracellular reactive oxygen species.**

**Materials and Methods**

**General Information**

HPLC analysis was performed with an Agilent 1200 HPLC system equipped with a 1200 series diode array detector. Radio-HPLC analysis was performed with an Agilent 1200 HPLC system equipped with a series diode array detector and Raytest GABI Star radioactivity detector. 18F-Fluoride was purchased from either the PET Center at St. Thomas’ Hospital or Alliance Medical UK. All reagents were purchased from Sigma-Aldrich and were used without further purification. Radiochemical yield was calculated as percentage of purified tracer to starting activity. Radiochemical conversion was calculated based on the HPLC peak AUCs. Preclinical PET/CT images were acquired using a NanoScan PET/CT (Mediso, Budapest, Hungary) scanner. Data are represented as mean ± SD, unless stated otherwise. Chromatographs and graphs were plotted on GraphPad Prism 8.

**Chemical Synthesis**

The synthesis and characterization of the non-radioactive reference compound **1**, intermediates, **2**, **3**, **4**, and radiolabeling precursor **5** is described in the supplementary material.

**Radiosynthesis**

**Radiosynthesis of 18F-FPBT ([18F] 1)**

18F-Fluoride (~1.2 GBq) in water was trapped in a QMA cartridge (Waters Sep-Pak light, pre-treated with 10 mL of water), and released with 1.0 mL of Kryptofix 222 and potassium carbonate mixture (30:15 mM) in acetonitrile/water (85:15) into a 5 mL Wheaton vial. After removing the solvents by heating at 110 °C under a stream of nitrogen for 15 min, azeotropic distillation with anhydrous acetonitrile (400 µL) was repeated twice at 90 °C for another 15 min. A solution of **5** (6 mg, 16 µmol) in anhydrous acetonitrile (400 µL) was then added and heated at 80 °C for 15 min. The reaction was cooled to room temperature, quenched by addition of water (100 µL), and purified by semi-preparative HPLC. 3-(3-[18F]Fluoropropyl)-2-phenyl-2,3-dihydrobenzo[d]thiazole (18F-FPBT, **[18F] 1**) was purified with a ZORBAX column (300SB-C18, semi-preparative 9.4 X 250 mm, 5 µm) using acetonitrile and water as the mobile phase, at a flow rate of 3.0 mL/min. The following gradient was used: from 50 % to 90 % acetonitrile in 15 min; kept at 90 % acetonitrile for 10 min; from 90 to 50 % acetonitrile in 5 min. **[18F] 1** has a HPLC retention time of 12.5 min. It was collected from the HPLC column and diluted to 10 % acetonitrile in water. It was trapped onto a Sep-Pak C-18 light cartridge (pre-activated with 5 mL methanol followed by 5 mL water). The cartridge was washed with 2 mL of water and **[18F] 1** was then eluted with 1 mL of ethanol in to give **[18F] 1** in 60 ± 20 % radiochemical conversion, decay corrected isolated RCYs around 33 ± 8 %, and >98 % radiochemical purity (n = 10).

**Radiosynthesis of 18F-FPBT-Ox ([18F] 3)**

**[18F]** **3** was obtained by reacting **[18F] 1** (~50 MBq, 1.0 mL in PBS containing 10 % ethanol) with excess of potassium superoxide (10 mg) until >90 % oxidation was observed by radio-HPLC in about 15 min. **[18F] 3** has a HPLC retention time of 4.5 min under the same HPLC setting as **[18F] 1**. **[18F] 3** was used in the same formulation as the reduced tracer **[18F] 1** (n = 3).

***In vitro* characterization**

**Lipophilicity**

The lipophilicity of 18F-FPBT and 18F-FPBT-Ox was determined by a conventional partition method between 1-octanol and phosphate buffered saline (PBS), pH 7.4. 1-Octanol was pre-saturated with PBS before use. The radiotracer (~2 KBq) was added to a mixture of PBS (200 µL) and 1-octanol (200 µL) in a 1.5 mL Eppendorf vial (n = 6). The mixture was vigorously agitated at RT for 5 min and then centrifuged at 3000 g for 10 min. A 100 µL aliquot from each layer was drawn for measurement in a gamma counter. The LogDoct/PBS was calculated as follows: log [(cpm in the 1-octanol layer – cpm 1-octanol blank)/(cpm in the PBS layer – cpm PBS blank)].

**Vial stability**

18F-FPBT (1 – 4 MBq) in 100 % ethanol or 5 % ethanol in PBS containing sodium ascorbate (0.01 mg/mL) was kept at room temperature for 4 hrs and then analyzed by radio-HPLC.

**Serum stability**

18F-FPBT (~3.0 MBq) in 5 % ethanol in PBS (200 µL) containing sodium ascorbate (0.01 mg/mL) was incubated with rat serum (200 µL) at 37 °C for 1 min or 1 h. Subsequently, plasma proteins were precipitated from the supernatant with ice cold acetonitrile (1.5 mL) and samples were centrifuged (3 min, 13,000 rpm). The residual activity in the pellet was <3 %. The supernatant (~2.0 MBq, 1.0 mL) was analyzed by radio-HPLC. HPLC method: ZORBAX column (300SB-C18, semi-preparative 9.4 X 250 mm, 5 µm) using acetonitrile and water as the mobile phase, at a flow rate of 3.0 mL/min. The following gradient was used: from 50 % to 90 % acetonitrile in 15 min; kept at 90 % acetonitrile for 10 min; from 90 to 50 % acetonitrile in 5 min.

18F-FBPT-Ox (2-4 MBq, 50 µL in 10% EtOH/PBS) was added to serum (500 µL) and incubated at 37 °C for 5, 15, and 30 min, respectively. Plasma proteins were precipitated with 5-sulfosalicylic acid water solution (10% *w/v*, 1 mL) and centrifuged (3 min, 13,000 rpm). The supernatant was removed and diluted with 500 µL of D.I. H2O and analyzed via radio-HPLC. A control experiment was performed where 18F-FBPT-Ox (2 MBq, 50 µL in 10% EtOH/PBS)was added to 500 µL of PBS, incubated at 37 °C for 30 minutes. 5-Sulfosalicylic acid water solution (10% *w/v*, 1 mL) was added, thoroughly mixed then diluted with 500 µL of D.I. H2O and analyzed via radio-HPLC. HPLC method: ZORBAX column (300SB-C18, semi-preparative 9.4 X 250 mm, 5 µm) Solvent A: H2O, Solvent B: MeCN; Flow rate: 3.0 mL/min; 0 – 5 min, 0% of B; 5 – 20 min, 0 – 50% of B; 20 – 30 min, 50 – 0% of B.

**Reactivity of 18F-FPBT to various reactive oxygen species**

18F-FPBT (0.5 – 2.0 MBq) was reacted with potassium superoxide (source of superoxide, 1 mg), hydrogen peroxide (100 µM), Fenton-reaction generated hydroxyl radicals (100 µM), Iron (II) sulfate heptahydrate (100 µM), tert-butyl hydroperoxide (100 µM), tert-butoxy radical (100 µM), and 3-morpholinosydnomine (source of peroxynitrite (100 µM) for 5 min or diethylamine NONOate (source of nitric oxide, 100 µM) for 30 min at room temperature after which it was subjected to radio-HPLC analysis and % oxidation was calculated.

**Animal studies**

All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 under Project License numbers PPL P96678ED7 and PPL 70/8482. Male Wistar rats were obtained from Envigo Ltd. and C57BL/6 mice from Charles Rivers. All animals went through a 7-day acclimatisation period at the Biological Services Unit at St. Thomas’ Hospital prior to any experiments. 18F-FPBT was formulated in ~5 % ethanol in PBS, containing 0.01 mg/mL sodium ascorbate for injection. 18F-FPBT-Ox was formulated in ~5 % ethanol in PBS. All PET imaging studies were performed using a Nano-Scan PET/CT (Mediso, Budapest, Hungary) scanner. All PET/CT data were reconstructed with the Monte Carlo-based full-3D iterative algorithm Tera-Tomo (Mediso Medical Imaging Systems, Budapest, Hungary). All procedures were performed under 2 % isoflurane in oxygen anesthesia.

**PET/CT imaging of healthy mice**

C57BL/6 mice (male, 25 ± 2 g, n = 3) were placed on the PET scanner bed and injected with 18F-FPBT (1.7 ± 0.4 MBq) through the tail vein at the start time of PET acquisition. A dynamic PET scan was performed for 60 min, followed by a CT scan and *ex vivo* biodistribution. The PET data was reconstructed into 5 frames of 60 seconds, 3 frames of 300 seconds, and 4 frames of 600 seconds. Regions of interest (ROIs) were drawn and quantified using VivoQuant software (v3.0, inviCRO, LLC, Boston, USA). Organ uptake was calculated as standard uptake values (SUV) and data are reported as mean ± SD.

**Post-mortem biodistributions in healthy rats**

Wistar rats (male, 319 ± 20 g) were injected intravenously *via* the tail vein with either 18F-FPBT (1.0 ± 0.4 MBq) or 18F-FPBT-Ox (1.1 ± 0.5 MBq). The animals were sacrificed by cervical dislocation at 1, 5, or 30 min post-injection (n = 3/group). Organs and tissues of interest were harvested, weighed, and the radioactivity measured in a gamma counter. Organ uptake was calculated as percentage injected dose per gram of tissue mass (% ID/g). Data are reported as mean ± SD.

**Rat model of Doxorubicin-induced cardiotoxicity**

**Minipump implantation**

Male Wistar rats (280 – 300 g) were used in all experiments. Subcutaneous 7-day osmotic pumps (Alzet), containing either Doxorubicin (Cambridge Bioscience, 30 mg/kg cumulative dose) or vehicle (sterile 0.9% NaCl), were inserted into rats under 2 % isoflurane in 100 % oxygen.

**Echocardiography**

In order to assess cardiac function all rats were subjected to a cardiac ultrasound (Vevo 770TM, VisualSonics) one day prior to and on day 6 of osmotic pump implantation. Rats were anaesthetized with 2 % isoflurane in 100 % oxygen and maintained at 37 °C via a homoeothermic platform and rectal thermometer. High-resolution parasternal left ventricle (LV) long axis M-mode and B-mode images were obtained using a RMV710B transducer. Images were analysed offline using Vevo Software to determine LV function. Data are reported as mean ± SD. Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparisons test.

**PET/CT imaging in rat model of Doxorubicin-induced cardiotoxicity**

Wistar rats that had received either Doxorubicin treatment (male, 267 ± 19 g, n = 6) or saline (male, 303 ± 9 g, n = 4) *via* osmotic pumps for 7 days were injected with 18F-FPBT (2.6 ± 1.5 MBq) *via* the tail vein on the PET/CT scanner bed. The time of injection coincided with the beginning of PET acquisition in order to obtain dynamic tracer uptake information. PET scans with the chest area in the field of vision were acquired for 30 min, followed by a CT scan, after which the animals were sacrificed and organs harvested, weighed, and gamma-counted as for the biodistribution protocol. The hearts were frozen in liquid nitrogen for gamma counting and oxidative stress biomarker assays. The PET data was reconstructed into three frames: 0 – 3 min, 3 – 10 min, and 10 – 30 min. For the time activity curve, the PET data was reconstructed into 1 min per frame in the first 5 min and then 5 minutes per frame between 5 to 30 min. Using VivoQuant software (v3.0, inviCRO, LLC, Boston, USA), regions of interest (ROIs) were drawn in the left ventricle and blood inside the myocardium. Radioactivity uptake in the heart was calculated as standard uptake value ratio (SUVR) between left ventricle and blood. Data are reported as mean ± SD. Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparisons test.

***Ex vivo* heart biomarkers of oxidative stress**

Total glutathione concentration was determined by an enzyme recycling method.(*21*) Lipid peroxidation was evaluated using the lipid peroxidation (MDA) assay kit from Sigma-Aldrich, which measures the colorimetric product that results from the reaction of malondialdehyde (MDA) present in the tissue with thiobarbituric (TBA) acid. Iron content was measured in isolated mitochondria and cytosolic fractions using the iron colorimetric assay kit from BioVision following the manufacture’s protocol.

***In vivo* blood stability of 18F-FBPT-Ox**

Wistar rats (male, 320 ± 20 g) were intravenously injected with 18F-FPBT-Ox (~15 MBq). The animals were sacrificed by cervical dislocation at 30 min post-injection (n = 2). Blood samples were collected in heparin-coated tubes. After centrifugation (5 min, 13,000 rpm), the plasma (∼1 mL) was separated. Plasma proteins were subsequently precipitated with 5-sulfosalicylic acid water solution (10% *w/v*, 1.5 mL) and centrifuged (5 min, 13,000 rpm). The supernatant was removed and diluted with 500 µL of D.I. H2O and analyzed via radio-HPLC. HPLC method: ZORBAX column (300SB-C18, semi-preparative 9.4 X 250 mm, 5 µm) Solvent A: H2O, Solvent B: MeCN; Flow rate: 3.0 mL/min; 0 – 5 min, 0% of B; 5 – 20 min, 0 – 50% of B; 20 – 30 min, 50 – 0% of B. HPLC eluent was collected every minute in 30 vials separately and submitted to gamma counting. The counts per minute in each vial was plotted against the corresponding time point.

**Statistical Analyses**

Statistical analyses were performed using Graphpad prism 9 software. Statistical comparisons between two groups were determined by unpaired t-test with Welch’s correction, and comparisons between multiple groups were determined by one way ANOVA with Tukey’s correction.

**Results**

**Synthesis**

The non-radioactive reference compound of 18F-FPBT and its iodinated precursor for nucleophilic radiofluorination were prepared in three steps from commercially available starting materials (**Figure 2**). 2-Phenylbenzo[*d*]thiazole **2** was synthesized by reacting 2-aminobenzenethiol with benzoic acid in the presence of polyphosphoric acid.(*22*) *N*-alkylation of 2-phenylbenzo[*d*]thiazole **2** took place with 3-iodopropyl trifluoromethanesulfonate or 3-fluoropropyl trifluoromethanesulfonate to yield the triflate salts **3** and **4,** which were subsequently reduced with sodium borohydride to obtain the non-radioactive reference compound **1** and precursor **5** for 18F-labeling.



**Figure 2. Precursor and non-radioactive reference compounds synthesis and radiosynthesis of 18F-FPBT and 18F-FPBT-Ox.** Reagents and conditions: (i) benzoic acid, polyphosphoric acid, 150 °C, 24 hrs, 67 %; (ii) 3-iodopropyl trifluoromethanesulfonate or 3-fluoropropyl trifluoromethanesulfonate, NaHCO3, nitrobenzene, r.t., 24 hrs, 20 % for **3** and 32 % for **4**; (iii) NaBH4, THF, methanol, r.t., 20 min, 52 % for **1** and 56 % for **5**; (iv) K18F, K2CO3, Kryptofix 2.2.2., acetonitrile, 80 °C, 15 min, RCYs 33 ± 8 % (n = 10); (v) KO2, PBS, r.t., 15 min, RCYs 93 ± 2 % (n = 5).

**Radiosynthesis**

18F-FPBT was radiosynthesized by a one-step 18F-labeling of alkyl iodide precursor **5** with high radiochemical conversion (60 ± 20 %, n = 10) as indicated by radioHPLC chromatogram of the crude reaction mixture. (**Figure 3A**) The decay corrected isolated radiochemical yields (RCYs) were consistently around 33 ± 8 % (n = 10) with >98 % radiochemical purity. The manual radiosynthesis, purification, and formulation of 18F-FPBT took around 2.5 hrs from the end of bombardment till formulation. The oxidized analogue, 18F-FPBT-Ox, was obtained by reacting 18F-FPBT with excess of potassium superoxide in PBS in 15 min, and isolated with >90 % radiochemical purity. The identity of both 18F-labeled compounds was confirmed by HPLC co-elution with the corresponding nonradioactive reference compounds (**Figures 3B and Supplementary Figure 1**). The molar activity of 18F-FPBT was measured as 168 ± 39 GBq/mol (n = 3) when starting with around 1.2 GBq of 18F-fluoride.

**Lipophilicity measurements**

18F-FPBT has a LogD of 1.00 ± 0.08 (n = 6) and its oxidized form 18F-FPBT-Ox has a significantly lower LogD of -1.00 ± 0.04 (n = 6) determined using a variation of the conventional shake-flask method.

***In vitro* stability of 18F-FPBT and 18F-FPBT-Ox**

18F-FPBT is stable in both pure ethanol and 5 % ethanol in PBS in the presence of ascorbic acid (0.01 mg/mL) for 4 h (not tested for longer). When incubated in rat serum at 37 °C, about 90 % of 18F-FPBT was intact in 1 hour (**Figure 3C**). The 18F-FPBT-Ox also exhibits excellent serum stability (**Figure 3D**).



**Figure 3. Production and co-elution HPLC chromatogram, and serum stability**. (**A**) HPLC chromatogram of crude 18F-FPBT radiolabeling reaction mixture; (**B**) Co-elution of purified 18F-FPBT with its non-radioactive reference compound **1**; (**C**) and (**D**) Serum stability of 18F-FPBT and 18F-FPBT-Ox at 37 °C determined by radioHPLC with two different HPLC methods.

***In vitro* reactivity of 18F-FPBT towards various ROS**

18F-FPBT showed rapid (in 5 min) and selective oxidation 58 ± 8 % (n = 4) by superoxide, which can be partially inhibited to 31 ± 5 % (n = 3) in the presence of ascorbate (1 mg/mL) (**Figure** **4**). In contrast, 18F-FPBT had little or no reactivity to other biologically relevant ROS.



**Figure 4. Chemoselectivity of 18F-FPBT**. Reactivity of 18F-FPBT to different oxidants (O2-• - superoxide; H2O2 – hydrogen peroxide; Fe(II) – iron(II); HO• - hydroxyl radical; TBHP – tert-Butyl hydroperoxide; t-BuO• - tert-butoxy radical; ONOO• - peroxynitrite; •NO – nitric oxide; AA – ascorbic acid); data represent mean of 2-4 experiments +/- SEM.

**18F-FPBT** ***in vivo* PET/CT imaging of healthy mice**

18F-FPBT had fast blood clearance. It was rapidly taken up and cleared by the heart, lungs, spleen, and brain with low non-specific background retention in these organs. The radiotracer is excreted by both renal and hepatobiliary routes in less than 1 hour (**Supplementary Figure 3**).

**Postmortem biodistributions in healthy rats**

*Ex vivo* biodistribution studies in healthy Wistar rats also showed rapid uptake of 18F-FPBT by major organs including the brain, heart, lung, and spleen, and fast clearance from the blood pool at 1 min post-injection (**Figure 5A**). 18F-FPBT was excreted by both renal and hepatobiliary routes. Conversely, 18F-FPBT-Ox shows significantly reduced uptake in major organs and was quickly cleared by the kidneys and excreted through the urine (**Figure 5B**).



**Figure 5. Biodistribution of 18F-FPBT and 18F-FPBT-Ox in healthy rats.** *Ex vivo* tissue biodistribution of 18F-FPBT (**A**) and 18F-FPBT-Ox (**B**) in healthy Wistar rats at 1, 5, and 30 min after intravenous injection. Data represented as mean ± SEM (n = 3/group).

**Rat model of Doxorubicin-induced cardiotoxicity**

Either Doxorubicin or saline was delivered to two groups of rats via osmotic pumps to develop the animal model of Doxorubicin-induced cardiotoxicity and control group. From the day of minipump implantation to day of imaging (7 days), animals in the saline control group maintained their weight with small variations [1.3 ± 2.5 % (n = 4) increase] while Doxorubicin treated animals consistently lost weight [16.2 ± 8.9 % (n = 6) decrease]. Rats treated with Doxorubicin showed statistically significant reduced (15 %) left ventricular ejection fraction at day 6 compared to their baseline values at day 0. However, echocardiography cannot detect statistical difference of the left ventricular ejection fraction between Doxorubicin-treated animals and their time-matched vehicle controls at day 6 (**Figure 6B**).

**PET/CT imaging in rat model of Doxorubicin-induced cardiotoxicity**

Dynamic PET/CT scan (30 min) was performed to the rats that received either Doxorubicin or saline. The PET data of each animal was reconstructed into three frames: 0–3, 3–10, and 10–30 min. Standard uptake value ratio (SUVR) between left ventricle and blood was calculated to determine the radioactivity retention in the heart of each animal. The Doxorubicin-treated rats showed higher myocardial radioactivity retention than the vehicle control group in the PET imaging (**Figure 6C**). The SUVRLV/blood of the Doxorubicin-treated rats was statistically significantly (p<0.05) higher than the vehicle control group (**Figure 6D**). In the time activity curve extracted from PET data (**Supplementary Figure 4 a and b**), the median myocardial SUV values of the Dox treated animals are higher than their median blood SUV values in the 30 min PET scan. While the median myocardial and blood SUV values of the saline treated control group are very similar at all the time points. In addition, the median SUVRLV/blood of Dox treated animals are also higher than the saline treated control group in the 30 min PET scan (**Supplementary Figure 4 c**).



**Figure 6. PET/CT imaging of 18F-FPBT in a rat model of Doxorubicin-induced cardiotoxicity.** (**A**) Experimental design and timeline. (**B**) Ejection fraction for control (saline, n = 4) and treated (Doxorubicin, n = 6) Wistar rats before and after treatment. (**C**) Representative sagittal, coronal, and axial images (3-10 min) from co-registered PET/CT imaging of 18F-FPBT in Wistar rats, following 7-day exposure to saline (n = 4, top) or Doxorubicin (n = 6, bottom). (**D**) SUVR LV/blood between 0-3, 3-10, and 10-30 min post-injection of 18F-FPBT. Data represented at median ± interquartile range.

***In vivo* blood stability of 18F-FBPT-Ox**

Subsequently, we examined the *in vivo* blood stability of 18F-FPBT-Ox at 30 min post IV injection. Plasma proteins were precipitated and the supernatant was analyzed by radio-HPLC. The HPLC eluent was collected every minute in 30 vials separately and submitted to gamma counting. The counts per minute in each vial was plotted against the corresponding time point. Increased gamma counts (**Supplementary Figure 5**)were only observed very close to the HPLC retention time of the 18F-FPBT-Ox (**Figure 3 D**).

***Ex vivo* heart biomarkers of oxidative stress**

Cardiac oxidative stress biomarkers including glutathione, maldonaldehyde, mitochondrial iron, and cytosolic ironwere measured following the established literature methods. The levels of these biomarkers were marginally higher in the Doxorubicin treated group comparing to the saline control group. **(Supplementary Figure 6)**

**Discussion**

The radiosynthesis of 18F-FPBT was achieved in a late-stage one-step radiolabeling with sufficiently high yield to make it suitable for translation into a fully automated radiosynthesizer such as GE FASTlab. We systematically investigated the reactivity and chemoselectivity of 18F-FPBT with various bio-relevant ROS *in vitro* and observed rapid and selective oxidation by superoxide, which can be significantly inhibited by large excess of ascorbic acid. We suspect the redox potential, half-life, and the steric hindrance of the ROS played a combination role in the chemoselectivity of 18F-FPBT towards various ROS oxidation. The redox potential of hydroxyl radical (2.33 E°/V) is significantly higher than superoxide (0.94 E°/V). *(23)* However, hydroxyl radical has a much shorter half-life (10-10 second) than that of the superoxide (10-6 second). *(24)* Therefore, 18F-FPBT appears to be inert to hydroxyl radical but reactive towards superoxide. Such chemoselectivity of 2-benzothiazolines towards superoxide rather than hydroxyl radical has also been observed by others. For example, Zhang has reported that the 2-(2-pyridil)-benzothiazoline was oxidized by superoxide to form 2-(2-pyridil)-benzothiazole but not by the hydroxyl radical.(20) This might also be the reason why 18F-FPBT has shown some reactivity toward other longer half-life ROS such as nitric oxide (a few seconds), peroxynitrite (10-3 second) and hydrogen peroxide (stable) despite they have much lower redox potential than hydroxyl radical. *(24)* On the other hand, alkylperoxyl radical (~1.0 E°/V)such as tert-butoxy radical has similar redox potential as the superoxide (0.94 E°/V) but has significantly higher steric hindrance. *(23)* This could explain that no oxidation was observed when 18F-FPBT was treated with tert-butoxy radical. The X-ray structure of 2-phenylbenzothiazoline indicates that the phenyl ring is perpendicular to the benzothiazoline, which increases the steric hindrance of C2 proton from oxidant attack. *(19)*

Next, the LogD of 18F-FPBT and 18F-FPBT-Ox were determined to be around 1 and -1, respectively. This raises the possibility that the lipophilic 18F-FPBT can penetrate the cell membrane and react with intracellular superoxide. After oxidation, the hydrophilic 18F-FPBT-Ox would be trapped inside the cells and generate contrast for PET imaging of oxidative stress. The biodistribution of both compounds in healthy rats demonstrated that 18F-FPBT has rapid uptake by all major organs including the brain. Conversely, 18F-FPBT-Ox showed significantly reduced uptake in these organs and was quickly cleared by the kidneys. The PET/CT imaging of 18F-FPBT in healthy mice provided further evidence that 18F-FPBT has fast uptake and clearance by heart, lung, and brain, that generates low background for imaging oxidative stress in these organs. Additionally, there was little radioactivity accumulation in the bone by both rats and mice indicating that 18F-FPBT is inert toward defluorination *in vivo*.

To assess whether 18F-FPBT can detect oxidative stress *in vivo*, PET imaging was performed in a rat model of Doxorubicin-induced cardiotoxicity, which is characterized by mitochondrial dysfunction leading to excess intracellular superoxide generation.(*11-13*) Increased myocardial radioactivity uptake was observed in the PET images of Doxorubicin treated rats comparing to the saline control group. The Doxorubicin treated animals also had significantly higher LV to blood SUV ratio than the saline control group in the same period. Post-mortem biodistribution studies at 30 min post IV injection of 18F-FPBT did not show increased uptake in the heart of Doxorubicin-treated animals. However, this represents a single late time-point and does not account for radiotracer bioavailability. PET/CT imaging overcomes these limitations of sampling bias and these results give us the confident that 18F-FPBT can be used to detect oxidative stress in cardiac pathologies. Moreover, blood metabolite analysis has shown that 18F-FPBT-Ox is stable in blood at least 30 min post IV injection, which further supports the trapping mechanism of 18F-FPBT post ROS oxidation.

*Ex vivo* measurements of cardiac biomarkers of oxidative stress (glutathione, malondialdehyde and iron accumulation) were marginally elevated by Doxorubicin treatment, but did not reach statistical significance (**Supplementary Figure 6**), largely due to the inter-individual variability in response, which is a well characterized aspect of Doxorubicin cardiotoxicity, both experimentally and in patients; this phenomenon is in fact one of the prime motivators for developing imaging agents which would enable personalized medicine approaches for titrating chemotherapeutic regimes to maximize therapeutic effectiveness while minimizing cardiac risk.

18F-FPBT is a novel small molecule probe that can be readily prepared by nucleophilic 18F-labeling in one step with good radiochemical conversion. 18F-FPBT exhibits chemoselectivity towards superoxide oxidation. The radiotracer was taken up by major organs and excreted through both renal and hepatobiliary routes rapidly. These characteristics would in principle make 18F-FPBT a good candidate for clinical translation. Although further characterization in additional models of oxidative stress is required, we observed an increased uptake in the hearts of Doxorubicin-treated rats when compared to controls, highlighting the potential of 18F-FPBT as a PET tracer to noninvasively detect oxidative stress *in vivo*.

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**ASSOCIATED CONTENT**

**Supporting information:**

* The synthesis and characterization of the non-radioactive reference compound **1**, intermediates, **2**, **3**, **4**, and radiolabeling precursor **5**;
* HPLC chromatograms of 18F-labeled compounds co-elution with the corresponding non-radioactive reference compounds and quality control;
* 18F-FPBT *in vivo* PET/CT imaging of healthy mice;
* Time-activity curves of 18F-FPBT in rats;
* Blood stability of 18F-FPBT-Ox 15 min post intravenous injection;
* *Ex vivo* measurements of cardiac biomarkers of oxidative stress;
* 1H, 13C NMR, and 19F spectra for all compounds prepared.

**Disclosure**

A UK patent application has been filed on 29 July 2020 and that it has a United Kingdom Patent Application No.: 2011787.5. The authors declared no conflict of interest.

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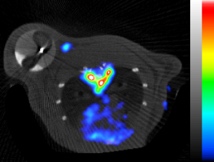
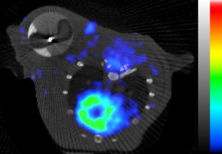
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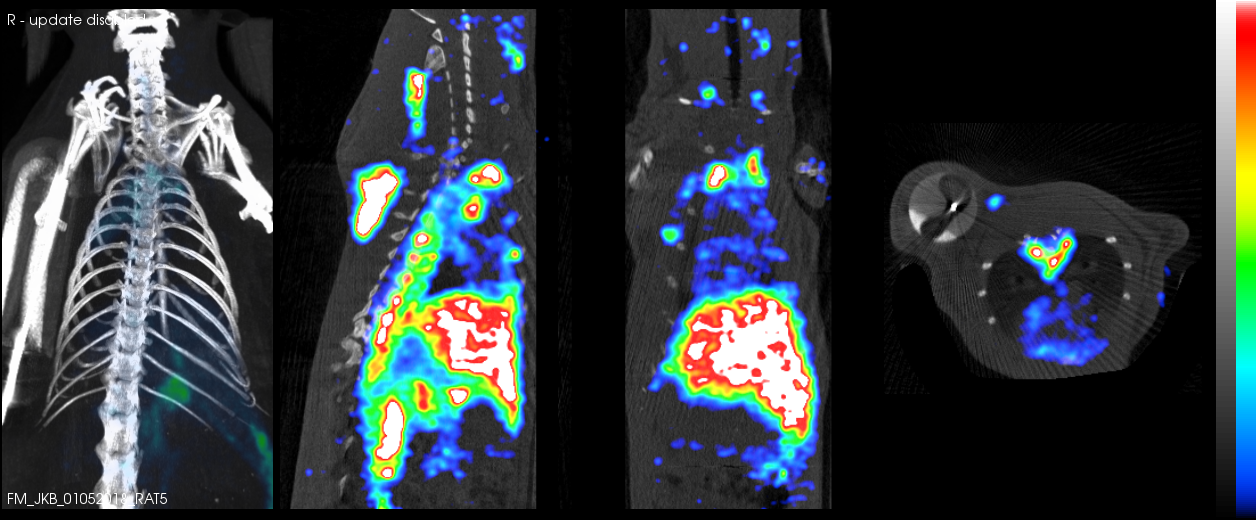
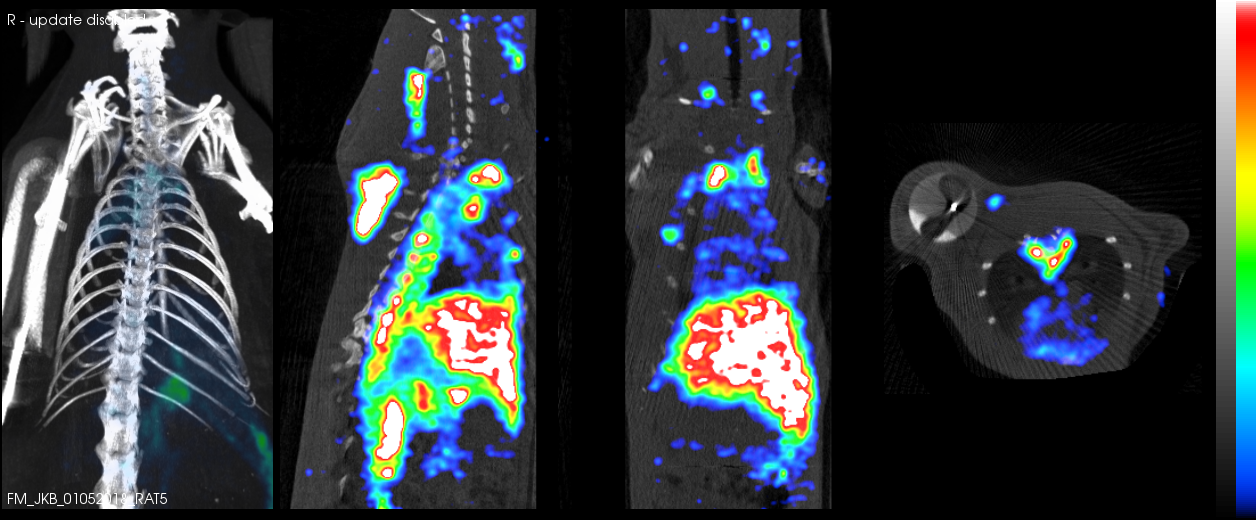


healthy

Oxidative stress

left ventricle

left ventricle



2

2

0.2

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