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A Boronate-Caged [^{18}F]FLT Probe for Hydrogen Peroxide Detection Using Positron Emission Tomography

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Supporting Information

ABSTRACT: Reactive oxygen species (ROS) play important roles in the development and progression of cancer and other diseases, motivating the development of translatable technologies for biological ROS imaging. Here we report Peroxy-Caged-[^{18}F]Fluorodeoxy thymidine-1 (PC-FLT-1), an oxidatively immolative positron emission tomography (PET) probe for H_2O_2 detection. PC-FLT-1 reacts with H_2O_2 to generate [^{18}F]FLT, allowing its peroxide-dependent uptake and retention in proliferating cells. The relative uptake of PC-FLT-1 was evaluated using H_2O_2-treated UOK262 renal carcinoma cells and a paraquat-induced oxidative stress cell model, demonstrating ROS-dependent tracer accumulation. The data suggest that PC-FLT-1 possesses promising characteristics for translatable ROS detection and provide a general approach to PET imaging that can be expanded to the *in vivo* study of other biologically relevant analytes.

Reactive oxygen species (ROS) are generated as a normal product of oxidative metabolism and act as essential signaling molecules in a diverse array of biological processes.[1] However, an imbalance in ROS regulation has been implicated in aging and several disease states, including chronic inflammation,[2,3] diabetes,[4−6] Alzheimer's,[7−10] and cancer.[11−14] In this context, observations of elevated concentrations of ROS in cancer cells compared to normal cells have been reported,[15] but methods with the potential to monitor ROS *in vivo* remain limited. To meet this need, we have initiated a program in molecular imaging for redox biology applications and have exploited the reaction-based cleavage of aryl boronates by H_2O_2 as a way to study the stress/signaling dichotomy of this major ROS.[16] The vast majority of these H_2O_2 indicators are restricted to cell-based imaging,[17] with limited reports of near-IR optical,[18] bioluminescence,[19,20] ^{13}C MRI,[21] and chemiluminescence[22] probes with *in vivo* potential. Additionally, the oxidation of aryl boronates has found elegant applications in drug-delivery,[23−25] pro-chelators,[26,27] mass spec probes,[28] and in activatable cell-penetrating peptides.[29]

Owing to high sensitivity, good spatial resolution, and low toxicity, positron emission tomography (PET) approaches to ROS detection have strong potential for clinical translation.[30,31] Recently, an ROS-responsive ^{18}F derivative of the fluorescent dye dihydroethidine was reported by Mach and coworkers.[32] Several of the most common PET tracers, including [^{18}F]-fluorodeoxyglucose ([^{18}F]FDG) and 3′-deoxy-3′-[^{18}F]-fluorothymidine ([^{18}F]FLT), mimic endogenous substrates that are transported into rapidly proliferating cells and subsequently phosphorylated, resulting in intracellular trapping of the radiotracer. Based on these considerations, we introduce a new reaction-based approach employing PET radiotracers that accumulate in cells following cleavage of a H_2O_2-sensitive moiety.

The clinical PET agent [^{18}F]FLT is a thymidine analogue that is transported into the cell during DNA replication via the equilibrative nucleoside transporter (ENT1) and then phosphorylated by thymidine kinase (TK1). However, unlike thymidine, [^{18}F]FLT is not subsequently phosphorylated by TK2/TK3 for incorporation into DNA but is instead trapped in the cell as its monophosphate, allowing for accumulation of the probe.[33−36] Owing to its uptake in a wide range of proliferating cells, we envisioned a prodrug-like strategy, where blocking of the 5′-OH of FLT with a H_2O_2-sensitive self-immolative linker would allow for an increase in signal from trapped FLT only in the presence of elevated levels of H_2O_2 and TK1. Therefore, we prepared Peroxy-Caged-[^{18}F]FLT-1 (PC-[^{18}F]FLT-1, Figure 1) based on this design. Accumulation of intracellular [^{18}F]FLT could potentially result from either extracellular oxidation-immolation of PC-[^{18}F]FLT-1 followed by transport into the cell by ENT1 or via passive diffusion of PC-[^{18}F]FLT-1 into the cell and subsequent intracellular oxidation-immolation. In both cases, [^{18}F]FLT would undergo phosphorylation by TK, resulting in trapped radiotracer and an accumulation in signal within proliferating cells with elevated levels of extra- or intracellular H_2O_2. Because this approach requires colocalization due to both ROS and TK1, it has the potential to be highly selective for tissues that are both highly proliferating and under oxidative stress. However, careful designs based on this concept are necessary, as the two independent steps may be unrelated biologically.

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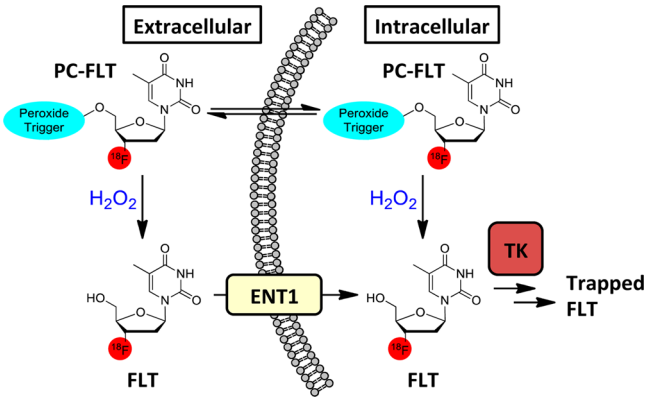
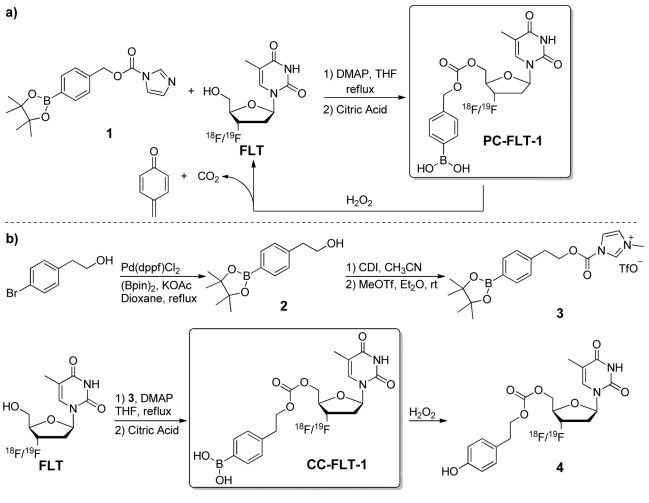


Figure 1. PC-[^{18}F]FLT-1, a PET radiotracer designed to exhibit a H_2O_2-dependent cellular accumulation of [^{18}F]FLT.

In designing a chemoselective H_2O_2-caged FLT tracer, we sought to utilize the oxidation-immolation of an aryl boronate *para* to a benzylic leaving group. A carbonate linkage was added to increase the kinetics of free FLT elimination upon oxidation, as decarboxylation would accompany quinone methide formation. PC-FLT-1 was prepared via coupling of [^{18}F/^{19}F]FLT with the imidazole carbamate **1** and subsequent conversion to the boronic acid (Scheme 1a). Oxidation of the

Scheme 1. Synthesis of PC-FLT-1 and CC-FLT-1



boronate by H_2O_2 provides the phenol, which decomposes to *para*-quinone methide, CO_2, and [^{18}F/^{19}F]FLT. We also designed and synthesized the control probe, Control-Caged-FLT-1 (CC-FLT-1, Scheme 1b), which exhibits similar properties but owing to its ethyl linker will not undergo conversion to FLT following oxidation by H_2O_2. Indeed, oxidation of CC-FLT-1 with H_2O_2 provides the phenol **4**, which does not go to FLT. The synthesis of the radioactive ^{18}F isotopomers follows a slightly modified coupling procedure (SI methods). Briefly, [^{18}F]FLT was prepared according to previously reported techniques,[37] and PC-[^{18}F]FLT-1 and CC-[^{18}F]FLT-1 were obtained by treating [^{18}F]FLT with the imidazole ester precursor **1** or **3** in acetonitrile with triethylamine and dimethylaminopyridine. Pinacol ester deprotection with 10% citric acid proceeded smoothly, and PC-[^{18}F]FLT-1 and CC-[^{18}F]FLT-1 were obtained in a 41 ± 14% (n = 5) and 44% (n = 1) radiochemical yield, respectively, from thymidine.

The reactivity of nonradioactive PC-[^{19}F]FLT-1 with H_2O_2 was characterized by monitoring its conversion to [^{19}F]FLT using HPLC (Figure 2). In the presence of H_2O_2 under

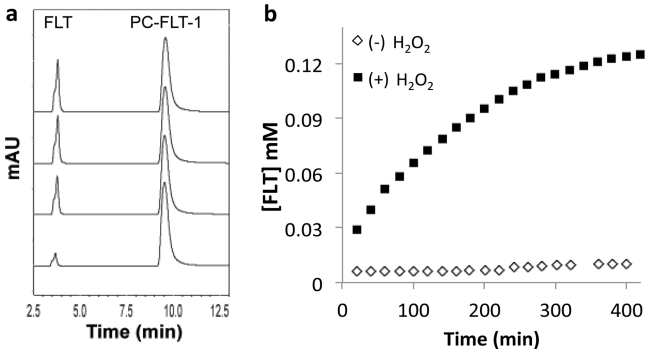


Figure 2. (a) HPLC traces of FLT (t_r = 3.8 min) generation from PC-FLT-1 (t_r = 9.5 min) in the presence of 100 μM H_2O_2 at 20 min (bottom trace), 2, 4, and 6 h (top trace). (b) Generation of FLT ±100 μM H_2O_2 over time.

simulated physiological conditions (20 mM, pH 7 phosphate buffer), consumption of PC-FLT-1 was observed along with concomitant formation of FLT, which provides a calculated pseudo-first-order rate constant of 6.9 ± 0.4 × 10^{-7} s^{-1} (Figure S1). Notably, no significant conversion from PC-FLT-1 to FLT could be detected in the absence of H_2O_2 (Figure 2b). Additional ROS reactivity assays show peroxynitrite at high, but not low, concentrations can also react (Figure S2), suggesting that this probe can be purposed toward reactive oxygen and/or nitrogen detection depending on the biological context.

Next the *in vitro* properties of PC-[^{18}F]FLT-1 were evaluated in UOK 262 renal carcinoma cells. Baseline uptake of PC-[^{18}F]FLT-1 was monitored in the absence of added peroxide, along with [^{18}F]FLT as a positive control (Figure 3a). Over the

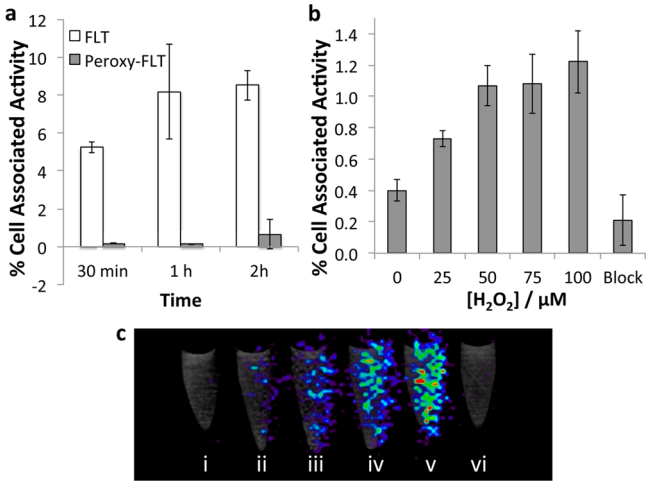


Figure 3. (a) Cellular uptake of [^{18}F] in UOK262 renal carcinoma cells under basal conditions, (−) H_2O_2, with PC-[^{18}F]FLT-1 and [^{18}F]FLT. b) Peroxide-dependent, (+) H_2O_2, [^{18}F] cellular uptake, and thymidine (1 mM) blocking of PC-[^{18}F]FLT-1. (c) MicroPET image of [^{18}F] cell uptake with PC-[^{18}F]FLT-1 in the presence of (i) 0, (ii) 25, (iii) 50, (iv) 75, (v) 100 μM H_2O_2 and (vi) 100 μM H_2O_2 + 1 mM thymidine (block).

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course of 2 h, cellular uptake of PC-[^{18}F]FLT-1 remains low (0.65 ± 0.78% of cell-associated activity at 2 h), whereas the positive control [^{18}F]FLT displays a continued increase over the course of the experiment. The observed low uptake for PC-[^{18}F]FLT-1 in the absence of exogenous peroxide addition is encouraging, as these data infer a low nonspecific background uptake for subsequent PET-based detection of ROS. PC-[^{18}F]FLT-1 responses to H_2O_2 concentrations ranging 0−100 μM over 1 h in UOK262 cells showed a H_2O_2-dependent accumulation of [^{18}F]FLT (Figure 3b), with a 3-fold increase in signal from 0.4 ± 0.07% cell associated activity at 0 μM H_2O_2 to 1.22 ± 0.2% cell associated activity at 100 μM H_2O_2 at 1 h (p = 0.0003). Moreover, the control radiotracer CC-[^{18}F]FLT-1 did not exhibit any significant change in cell uptake at 1 h ±100 μM H_2O_2 (p = 0.9) (Figure S3). Also, at 1 h with 100 μM H_2O_2, accumulation is effectively blocked by addition of 1 mM nonradioactive thymidine. This nearly quantitative amount of blocked activity suggests a high level of specific uptake of the PC-[^{18}F]FLT-1 probe. After counting, the cells were washed with pH 3 glycine followed by 1 M NaOH to release any surface bound or internalized activity. The separate fractions were counted (Figure S2), recombined, and then a MicroPET image was obtained, which illustrates the increase in PET signal for PC-[^{18}F]FLT-1 to H_2O_2 in a dose-dependent manner (Figure 3c).

Finally, we evaluated the ability of PC-[^{18}F]FLT-1 to sense endogenous ROS generation by stimulation of UOK262 cells with paraquat (Figure 4), a small-molecule inducer of ROS and

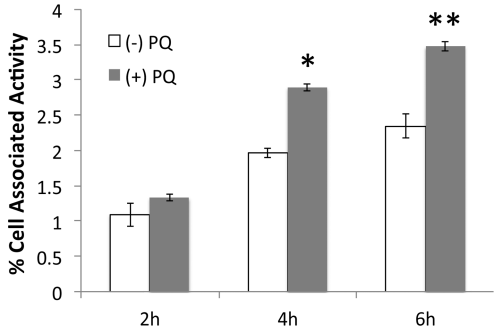

Figure 4. UOK262 uptake of [^{18}F]FLT upon PQ treatment with PC-[^{18}F]FLT-1. % Cell associated activity ± administration of 1 mM PQ (*p = 0.009, **p = 0.005 with respect to (−)PQ).

oxidative stress.[38] After 4 h of paraquat treatment, a significant increase in cell-associated activity was observed over control cells (p < 0.009 with respect to (−) paraquat (PQ)), establishing that PC-[^{18}F]FLT-1 is sensitive enough to detect changes in endogenous H_2O_2 levels.

To close, we have described a new type of reaction-based PET probe for molecular imaging of H_2O_2. PC-[^{18}F]FLT-1 utilizes a boronate oxidation to uncage the clinically used PET tracer [^{18}F]FLT in a H_2O_2-dependent manner, allowing for detection of changes in ROS levels in living cells. While we are encouraged by these proof-of-principle results, we recognize potential limitations of short ^{18}F lifetime vs ROS uncaging and linker cleavage, and as such, future improvements will seek to improve ROS detection kinetics by tuning of self-immolative linkers and signal amplification strategies. Current efforts are focused on preclinical imaging of oxidative stress in cancer with PC-[^{18}F]FLT-1 and related congeners as well as expanding the toolbox of reaction-based probes for PET imaging to study ROS in other potential disease states, with the long-term goal of translating these tracers to clinical settings.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic and experimental detail, including procedures for synthesis and radiosynthesis of compounds, ROS sensitivity assays, cell culture protocols and imaging. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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