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Chemoenzymatic radiosynthesis of 2-deoxy-2-[18F]fluoro-D-trehalose ([18F]-2-FDTre): A PET radioprobe for in vivo tracing of trehalose metabolism

Santiago Peña-Zalbidea,b,1, Ashley Y.-T. Huangc,1, Herbert W. Kavunja1, Beatriz Salinasa,b,f, Manuel Descoa,b,f,g, Christopher Drake, Peter J. Woodruff, Juan J. Vaqueroa,b,*, Benjamin M. Swartsc,*

a Dept. Bioingeniería e Ingeniería Aeroespacial, Universidad Carlos III de Madrid, Madrid, Spain
b Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain
c Department of Chemistry and Biochemistry, Central Michigan University, Mount Pleasant, MI, United States
d SOFIE Co, Culver City, CA, United States
e Department of Chemistry, University of Southern Maine, Portland, ME, United States
f Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain
g Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Spain

ABSTRACT
Trehalose analogues bearing fluorescent and click chemistry tags have been developed as probes of bacterial trehalose metabolism, but these tools have limitations with respect to in vivo imaging applications. Here, we report the radiosynthesis of the [18F]-modified trehalose analogue 2-deoxy-2-[18F]fluoro-D-trehalose ([18F]-2-FDTre), which in principle can be used in conjunction with positron emission tomography (PET) imaging to allow in vivo imaging of trehalose metabolism in various contexts. A chemoenzymatic method employing the thermophilic TreT enzyme from Thermoproteus tenax was used to rapidly (15–20 min), efficiently (70% radiochemical yield; ≥ 95% radiochemical purity), and reproducibly convert the commercially available radiotracer 2-deoxy-2-[18F]fluoro-D-glucose ([18F]-2-FDG) into the target radioprobe [18F]-2-FDTre in a single step; both manual and automated syntheses were performed with similar results. Cellular uptake experiments showed that radiosynthetic [18F]-2-FDTre was metabolized by Mycobacterium smegmatis but not by various mammalian cell lines, pointing to the potential future use of this radioprobe for selective PET imaging of infections caused by trehalose-metabolizing bacterial pathogens such as M. tuberculosis.

1. Introduction
Trehalose, a non-reducing disaccharide composed of two glucose units linked via a 1,1-α,α-glycosidic bond (Fig. 1A), has diverse and important roles in biology and human health. It is abundant in bacteria, yeast, fungi, plants, and invertebrates, where it fulfills functions in stress protection, energy storage, and pathogenesis [1,2]. Although trehalose is not produced by mammals, it is of high significance to human health. Trehalose's ability to preserve biomaterials has led to its inclusion in thousands of pharmaceutical, food, and cosmetics products [3]. In addition, trehalose induces autophagy and mitigates disease burden in multiple murine models, which has led to studies on its mechanism(s) of action and therapeutic potential [4,5]. Trehalose is also critical to many human-resident microbes. Numerous bacterial pathogens require trehalose for viability and virulence [2]. For example, Mycobacterium tuberculosis (Mtb) has unique trehalose metabolic pathways that are essential for pathogenesis and represent valuable targets for drug and diagnostic development [6,7]. Many gut bacteria, some of which can become lethal pathogenic (e.g., Clostridium difficile), can also utilize trehalose [8–12].

The ability to image trehalose metabolism in vivo is expected to stimulate basic and applied research in various areas of biomedical interest. First, because many bacteria possess dedicated trehalose uptake and processing pathways that are absent from humans [2], trehalose-based probes may selectively accumulate in bacteria and thus enable targeted imaging of bacterial infections. Such tools are urgently needed in the clinic [13] given the massive health burdens of increasingly drug-resistant nosocomial pathogens such as C. difficile, S. aureus, and P. aeruginosa (722,000 cases, 75,000 deaths in the US in 2011 [14]) as well as global pathogens such as Mtb (10.0 million cases, 1.3 million deaths estimated in 2017 [15]). Infection imaging tools will enable pathophysiology and antibiotic development studies in animals, and they could revolutionize clinical infection management by enhancing diagnosis, staging, and therapy monitoring [13,16,17]. Targeting

in vivo approach for a broad range of PET probes, most notably 2-deoxy-[18F]fluoro-D-glucose (2-FDG), which is widely available and the standard probe for the development of infection imaging tools is an attractive approach, as recently exemplified by the development of maltose-, maltohexaose-, and sorbitol-based in vivo imaging probes [18–20]. In this context, trehalose-based probes are attractive candidates for bacterial infection imaging, a notion which was first put forth in the context of Mtb imaging by the Barry and Davis groups [21]. Second, trehalose has gained traction as a human therapeutic due to its ability to induce autophagy and alleviate disease burden in models of neurodegeneration and non-fatty alcoholic liver disease, but the current in vivo mechanistic understanding of these phenomena is incomplete [5,22–33]. The ability to directly trace trehalose metabolism in vivo will overcome a critical barrier to mechanistic inquiry in these fields and may inform optimal administration route and dosing as trehalose advances to clinical utility.

To date, several probes have been developed that permit tracking of trehalose metabolism in vitro, but pose problems for in vivo imaging [34]. 14C-labeled trehalose is commercially available and has been used to probe trehalose metabolism in diverse biological systems [35–42], but it cannot be used for imaging. Fluorescently-tagged [21,43–45] and clickable [46,47] trehalose analogues have been introduced over the past several years and used to image trehalose metabolism in cells. However, these probes are not suitable for non-invasive in vivo imaging due to disadvantages such as the size of the detectable moiety, the need for a secondary labeling step, and reliance on optical imaging. In addition, the multi-step chemical syntheses of these probes are inefficient and require synthetic expertise, limiting their accessibility. Positron emission tomography (PET) imaging employing deoxy-[18F]fluoro-α-trehalose (18F-FDTre) probes (structures shown in Fig. 1B) is an ideal approach for in vivo imaging of trehalose metabolism to support the applications discussed above. PET is a non-invasive, clinically established technique for rapid, quantitative, 3-D in vivo imaging of (often disease-associated) metabolic processes deep within the body. The most extensively used radionuclide for PET is fluorine-18, whose beneficial nuclear and physical properties have led to its incorporation into a broad range of PET probes, most notably 2-deoxy-[18F]fluoro-α-glucose (18F-2-FDG), which is widely available and the standard probe for a range of research and clinical PET imaging applications [48]. 18F-FDTre–PET is a logical merger of the trehalose-based probe concept with the established capabilities and broad/expanding accessibility of PET.

A major challenge to the development of 18F-FDTre probes is that the short half-life (109.7 min) of fluorine-18 requires rapid synthetic methods capable of generating the radioprobe from the radionuclide in ~2 h or less. Radiosynthesis using Sn2 reactions (e.g., between sugar triflates and nucleophilic 18F-fluoride) can be considered, but these routes have lengthy and inefficient precursor syntheses due to the challenges associated with trehalose’s 1,1-α,α-glycosidic bond and C2 symmetry [46]. Moreover, “cold” 18F-fluorinations of protected trehalose intermediates are slow and have moderate yields [21,49,50]. A significantly more streamlined and efficient approach would be to access 18F-FDTre from the corresponding 18F-FDG, which would be particularly attractive for the 2-position isomer, as 18F-2-FDG is widely commercially available (over a million 18F-2-FDG–PET scans are done annually in the US). Given that naturally occurring trehalose biosynthetic pathways utilize glucose or glucose phosphates as acceptor substrates to generate trehalose, there is an opportunity to adapt these pathways—if they are sufficiently substrate tolerant—to enable the biocatalytic conversion of 18F-FDG analogues to 18F-FDTre analogues [34]. This concept was originally explored in 2011, when a chemoenzymatic method inspired by the OtsAB trehalose biosynthetic pathway was used to convert non-radioactive 19F-2-FDG into 19F-2-FDTre (Scheme 1A) [21]. While this method can potentially be adapted to the radiosynthesis of 18F-2-FDTre, it used three enzymes and was limited to the production of only one regioisomer [21]. In 2014, we developed a one-step chemoenzymatic method for trehalose analogue synthesis enlisting the substrate-permissive trehalose synthase (TreT) from Thermoproteus tenax [51,52], and in 2016 we applied this method to the synthesis of non-radioactive 19F-2-, 19F-3-, and 19F-6-FDTre analogues from the corresponding 19F-FDG analogues (Scheme 1B) [53]. These reactions featured quantitative conversion of substrate to product in ≤60 min, suggesting that TreT may be suitable for radiosynthesis [53]. Despite these advances, to date the radiosynthesis of 18F-2-FDTre has not been reported in the literature. Here, we describe the successful application of TreT catalysis to the rapid one-step radiosynthesis of 18F-2-FDTre, as well as preliminary uptake evaluation of this radioprobe in mammalian and mycobacterial cells.

Fig. 1. Structures of (A) trehalose and (B) regioisomeric 18F-labeled FDTre analogues as PET probes proposed for in vivo imaging of trehalose metabolism. This study focuses on the radiosynthesis of 18F-2-FDTre.

2. Results and Discussion

2.1. Kinetic analysis of TreT-catalyzed synthesis of 18F-2-FDTre from 18F-2-FDG

Our prior work demonstrated that Thermoproteus tenax TreT, which is a thermostable enzyme that couples glucose and UDP-glucose to form trehalose [54], is substrate tolerant and can be employed to efficiently convert various glucose analogues into the corresponding trehalose analogues in high yield (with the main exception being 4-position-modified analogues) [51,52]. We subsequently showed that TreT is active on 18F-FDG analogues and can generate 18F-FDTre analogues [53]. However, we did not analyze the kinetic properties of these pathways—if they are sufficiently substrate tolerant—to enable the biocatalytic conversion of 18F-FDG analogues to 18F-FDTre analogues [34]. This concept was originally explored in 2011, when a chemoenzymatic method inspired by the OtsAB trehalose biosynthetic pathway was used to convert non-radioactive 19F-2-FDG into 19F-2-FDTre (Scheme 1A) [21]. While this method can potentially be adapted to the radiosynthesis of 18F-2-FDTre, it used three enzymes and was limited to the production of only one regioisomer [21]. In 2014, we developed a one-step chemoenzymatic method for trehalose analogue synthesis enlisting the substrate-permissive trehalose synthase (TreT) from Thermoproteus tenax [51,52], and in 2016 we applied this method to the synthesis of non-radioactive 19F-2-, 19F-3-, and 19F-6-FDTre analogues from the corresponding 19F-FDG analogues (Scheme 1B) [53]. These reactions featured quantitative conversion of substrate to product in ≤60 min, suggesting that TreT may be suitable for radiosynthesis [53]. Despite these advances, to date the radiosynthesis of 18F-2-FDTre has not been reported in the literature. Here, we describe the successful application of TreT catalysis to the rapid one-step radiosynthesis of 18F-2-FDTre, as well as preliminary uptake evaluation of this radioprobe in mammalian and mycobacterial cells.

Scheme 1. (A) OtsAB-inspired three-step synthesis of 2-FDTre using hexokinase, trehalose-6-phosphate synthase (TPS), and alkaline phosphatase. (B) TreT-catalyzed one-step synthesis of FDTre regiosomers. Both methods can potentially convert the widely commercially available PET probe 18F-2-FDG into the corresponding trehalose probe 18F-2-FDTre.
reactions, which would provide useful information for predicting/understanding reaction outcomes and optimizing reaction conditions if needed. Here, we obtained the kinetic parameters of TreT-catalyzed conversion of $^{19}$F-2-FDG to $^{19}$F-2-FDTre analogues by employing a commercially available luminescence-based glycosyltransferase assay (UDP-Glo, Promega). TreT reactions were run in 50 mM Tris-HCl buffer with the donor (UDP-glucose) held at a saturating concentration of 1 mM and the acceptor (glucose or $^{19}$F-FDG isomers) varied in concentration from 0 to 10 mM for durations of 0–10 min. UDP released in the reactions was quantified by the luminescence assay and Michaelis–Menten analyses were performed (Fig. 2). The kinetic parameters obtained for the natural acceptor glucose ($K_{m} = 0.31 \pm 0.02$ mM; $V_{max} = 0.80 \pm 0.22$ μM min$^{-1}$) were similar to those observed when $^{19}$F-2-FDG was employed as the acceptor substrate ($K_{m} = 1.45 \pm 0.20$ mM; $V_{max} = 0.35 \pm 0.01$ μM min$^{-1}$). Although $^{19}$F-2-FDG exhibited moderately higher $K_{m}$ and lower $V_{max}$ values than glucose, these data suggested that TreT utilizes 2-FDG with comparable efficiency to glucose, potentially allowing conversion of $^{19}$F-2-FDG to the corresponding $^{18}$F-labeled trehalose analogue.

2.2. TreT-catalyzed radiosynthesis of $^{18}$F-2-FDTre from $^{18}$F-2-FDG

Encouraged by our promising results using non-radioactive substrates reported previously [53] and in this study, we next sought to develop a protocol for the rapid radiosynthesis of $^{18}$F-2-FDTre from commercially available $^{18}$F-2-FDG. The molar concentration of $^{18}$F-2-FDG in a commercial batch is typically in the picomolar range—much lower than the 10 mM concentration we used for semi-preparative scale TreT-catalyzed synthesis of $^{19}$F-2FDTre. While unlabeled $^{18}$F-2-FDG can be added to radiolabeled $^{18}$F-2-FDG to increase the molar concentration of substrate, this would decrease the specific activity of the tracer, which is often undesirable for PET imaging applications. Thus, the most significant change in adapting the established TreT method to $^{18}$F-2-FDTre radiochemistry was quantified by the luminescence assay and Michaelis–Menten analyses if needed. Here, we obtained the kinetic parameters of TreT-catalyzed conversion of $^{19}$F-2-FDG to $^{19}$F-2-FDTre analogues by employing a commercially available luminescence-based glycosyltransferase assay (UDP-Glo, Promega). TreT reactions were run in 50 mM Tris-HCl buffer with the donor (UDP-glucose) held at a saturating concentration of 1 mM and the acceptor (glucose or $^{19}$F-FDG isomers) varied in concentration from 0 to 10 mM for durations of 0–10 min. UDP released in the reactions was quantified by the luminescence assay and Michaelis–Menten analyses were performed (Fig. 2). The kinetic parameters obtained for the natural acceptor glucose ($K_{m} = 0.31 \pm 0.02$ mM; $V_{max} = 0.80 \pm 0.22$ μM min$^{-1}$) were similar to those observed when $^{19}$F-2-FDG was employed as the acceptor substrate ($K_{m} = 1.45 \pm 0.20$ mM; $V_{max} = 0.35 \pm 0.01$ μM min$^{-1}$). Although $^{19}$F-2-FDG exhibited moderately higher $K_{m}$ and lower $V_{max}$ values than glucose, these data suggested that TreT utilizes 2-FDG with comparable efficiency to glucose, potentially allowing conversion of $^{19}$F-2-FDG to the corresponding $^{18}$F-labeled trehalose analogue.

Radio-HPLC analysis employing an aminopropyl column was used to monitor reactions, showing that (i) $^{18}$F-2-FDG was fully converted to $^{18}$F-2-FDTre in only 15 min; and (ii) the $^{18}$F-2-FDTre radiochemical purity of the product was high (Fig. 3A). Injection of $^{19}$F-2-FDG alone gave a single peak with a retention time ($t_{R}$) of 14 min. When the TreT reaction was analyzed following 10 min, some unreacted $^{18}$F-2-FDG ($t_{R} = 14$ min) was observed along with a major new peak for $^{18}$F-2-FDTre ($t_{R} = 27$ min). At 15 min reaction time, the $^{18}$F-2-FDG was completely consumed and a single peak corresponding to the $^{18}$F-2-FDTre product was present. The non-decay corrected radiochemical yield and purity for this reaction were 70% and > 99%, respectively. The $t_{R}$ trends for the starting material and product were in agreement with those previously observed for non-radioactive compounds using a similar HPLC method [51]. To confirm the identity of the radio-HPLC peak at $t_{R} = 27$ min as $^{18}$F-2-FDTre, we performed a modified radiochemical reaction with additional “cold” $^{19}$F-2-FDG doped in, then separated the mixture by HPLC and collected the peak of interest ($t_{R} = 27$ min). After the radioactivity dissipated, the sample was analyzed by ESI-MS and confirmed to be $^{19}$F-2-FDTre (Fig. 3C; base peaks observed: positive mode, [M + Na]$^+$ = 367.0; and negative mode, [M + Cl]$^-$ = 379.1). The chromatogram shown in Fig. 3A is representative of several reaction trials, which all had non-decay corrected $^{18}$F-2-FDTre radiochemical yields of approximately 70% and radiochemical purities of ≥ 95%. The radionuclidic purity of synthetic $^{18}$F-2-FDTre was established by confirming that the decay time and photopeak (511 keV) of the product matched fluorine-18. The chemical stability of $^{18}$F-2-FDTre was also confirmed by incubating newly-synthesized material in phosphate-buffered saline (PBS) at 37°C with shaking and re-analyzing the sample by radio-HPLC, which in the chromatograms showed no changes over 6 h (data not shown).

Automation of radiosynthetic protocols is a critical step for commercialization of radioprobes. With this in mind, we sought to demonstrate that TreT-catalyzed production of $^{18}$F-2-FDTre is amenable to automation on an ELIXYS FLEX/HEM automated radiosynthesizer (SOFIE Co., Culver City). The manual synthesis steps were translated into an ELIXYS sequence, reflecting the optimized conditions described above. The sequence was executed, using commercially available $^{18}$F-2-FDG (PetNet solutions, Culver City), and analytical HPLC demonstrated nearly quantitative conversion of $^{18}$F-2-FDG to $^{18}$F-2-FDTre in this format (Fig. 3B). While no further purification was attempted, ELIXYS is fully compatible with a wide range of cartridge-based purification protocols; thus automating the purification protocol established for manual $^{18}$F-FDTre radiosynthesis should not pose a significant challenge. Taken together, our data demonstrate that the TreT catalysis method is capable of rapidly and reproducibly generating radiochemically pure $^{18}$F-2-FDTre from commercially available $^{18}$F-2-FDG via manual or automated radiosynthesis.
2.3. Comparison of $^{18}$F-2-FDTre uptake by *M. smegmatis* and mammalian cell lines

Because various bacterial pathogens possess dedicated trehalose transporters but mammals are not known to, we hypothesized that $^{18}$F-FDTre PET radioprobes would be taken up more efficiently by bacteria than by mammalian cells, and thus may allow for selective imaging of bacterial infections in vivo. In prior work, we showed that *M. smegmatis*—a frequently used fast-growing non-pathogenic model organism for the global pathogen *Mtb*—can uptake three non-radioactive $^{19}$F-FDTre regioisomers, including $^{19}$F-2-FDTre, via the trehalose-specific transporter LpqY-SugABC [53]. However, these experiments were performed with micromolar concentrations of $^{19}$F-FDTre analogues, which far surpasses the trace concentration of $^{18}$F-radioprobe that would be available in vivo. Furthermore, no findings on FDTre uptake by mammalian cells have been reported to date.

To address these questions, we performed an uptake analysis of radiolabeled $^{18}$F-2-FDTre comparing *M. smegmatis* and several mammalian cell lines, including human mesenchymal stem cells (hMSC) and the tumor cell lines AR42J and HT29 (rat pancreatic and human colorectal adenocarcinoma, respectively). Mammalian cells were incubated in PBS containing ~5μCi of freshly synthesized $^{18}$F-2-FDTre for 60 min, then washed and assessed for radioactivity. *M. smegmatis* cells were subjected to essentially the same procedure, along with controls using bacteria that were heat-killed and bacteria that were co-tREATED with competing excess unlabeled trehalose. As shown in Fig. 4, while the hMSC, AR42J, and HT29 mammalian cells showed virtually no probe uptake (<1% of $^{18}$F-2-FDTre in the medium), *M. smegmatis* exhibited efficient, linear, and time-dependent uptake probe (~30% uptake after 3 h). Accumulation of $^{18}$F-2-FDTre in *M. smegmatis* was abolished if the bacteria were either heat-killed or co-incubated with excess unlabeled trehalose (<1% uptake), demonstrating that probe uptake is active and specific for trehalose metabolism. Together, these data support our hypothesis that trehalose-metabolizing bacteria such as *M. smegmatis* will indeed accumulate $^{18}$F-2-FDTre more efficiently than mammalian cells. Furthermore, at least in the *in vitro* experimental conditions employed, even very low (picomolar) concentrations of $^{18}$F-2-FDTre can be taken up by bacteria. Finally, evaluation of $^{18}$F-2-FDTre...
in a plasma protein binding assay showed that >99% of the radioprobe remained unbound to porcine serum proteins (data not shown), indicating that it will not be disadvantageously absorbed by plasma components during in vivo applications.

3. Conclusion

The development of 18F-FDTre–PET technology is motivated by the prospect of imaging trehalose metabolism in vivo, which is perhaps of most interest for the selective imaging of infections caused by trehalose-metabolizing bacterial pathogens (e.g., Mtb). Building on the earlier successes of fluorescent and clickable trehalose analogues, we recently applied our TreT catalysis method to produce three non-radioactive 19F-FDTre regioisomers in high yield. Herein, we translated these findings to the rapid one-step radiosynthesis of 18F-2-FDTre by exploiting TreT, which is attractive from numerous standpoints. Most importantly, even when using the extremely low (picomolar) concentrations of 18F-2-FDG starting material in commercial preparations, TreT allowed quantitative conversion of 18F-2-FDG to 18F-2-FDTre under mild conditions in only 15–20 min. This is beneficial because no unlabeled substrate needs to be added to the reaction to increase substrate concentration, thus avoiding reduction of the tracer’s specific activity. Another advantage of this process is that the starting material, 18F-2-FDG, is widely commercially available, meaning that the TreT method can tap into existing infrastructure to make 18F-2-FDTre readily accessible to virtually any radiopharmacy or PET imaging facility in the world. TreT is a thermostable enzyme, which has practical advantages such as extended shelf life and heating of reactions to increase rate and avoid microbial contamination. In addition to a manual synthesis format, we performed TreT-catalyzed preparation of 18F-2-FDTre on an automated radiosynthesis module, which should facilitate the adoption of this radiophobe and synthetic method by others. Furthermore, we recently reported a bead-immobilized version of TreT [55], which will allow enzyme reuse and will further simplify the radiosynthesis process by eliminating the enzyme removal step of the purification. Finally, we previously showed that TreT catalysis can generate non-radioactive 18F-3- and 18F-6-FDTre regioisomers, so the corresponding radioactive 18F-labeled analogues should be accessible through the procedures reported herein; indeed, the radiosyntheses of the precursors, 18F-3- and 18F-6-FDG, have been published [56,57] and can easily be coupled to TreT catalysis. Also in this work, we showed that an avirulent mycobacterial model organism, M. smegmatis, can uptake trace concentrations of radioisynthetic 18F-2-FDTre via trehalose-specific metabolism, likely via LpqY-SugABC-mediated transport across the plasma membrane. Given the lack of radioprobe uptake by various mammalian cell lines (Fig. 4), there is motivation to perform in vitro uptake studies in additional trehalose-utilizing bacteria and pursue in vivo imaging studies in relevant animal infection models. The chemoenzymatic synthetic methods reported in this study will facilitate these future directions.

4. Experimental

4.1. Materials and reagents

TreT was expressed and purified from E. coli as previously described [52]. The UDP-Glo assay kit, including ultra-pure UDP-glucose, were obtained from Promega. 18F-2-FDG was purchased from IBA Molecular (Madrid, Spain) or PetNet Solutions (Culver City, CA). Non-radioactive 18F-2-FDG was purchased from CarboSynth. UDP-Glucose was obtained from Sigma or Abcam. AG1-X8 mixed-bed ion exchange resin and PolyPrep columns were purchased from Bio-Rad Laboratories.

4.2. Enzyme kinetics

Kinetic properties of TreT-catalyzed synthesis of trehalose and 18F-2-FDTre were obtained by measuring the production of UDP using the UDP-Glo luminescence glycosyltransferase assay (Promega), essentially as previously described [53,55]. TreT (0.8 μg) was incubated in the presence of 1 mM UDP-glucose, 20 mM MgCl2, 300 mM NaCl, and 50 mM Tris-HCl buffer (pH 8.0) in the absence (negative control) or presence of acceptor substrate. The acceptors evaluated were glucose and 19F-2-FDG, each tested over a concentration range of 0–10 mM. For each concentration, UDP production was assessed at four time points (immediately, 2, 5, and 10 min) to ensure linearity of the enzymatic reaction. Reactions were set up in 96 well plates with UDP-glucose, glucose or 19F-2-FDG, MgCl2, and buffer added to the wells first, then the reactions were initiated by addition of TreT enzyme to achieve a final volume of 25 μL at room temperature. The reactions were incubated at room temperature for 0–10 min, then 25 μL UDP detection reagent were added, which quenched the reaction and coupled UDP production to a luciferase reaction. After incubation at room temperature for 60 min, the luminescence signal was recorded using a microplate reader (Tecan Infinite M200 Pro). The luminescence signal was fitted to a standard curve made from a dilution series of known UDP concentrations measured in the same 96-well microplate. Relative light units (RLUs) given by the luminescence reader were converted to UDP concentration values, which were subsequently processed in GraphPad Prism v. 6.02 to obtain Michaelis–Menten plots and calculate kinetic parameters Km and Vmax. Data shown are representative of two independent trials. Reported Km and Vmax values are the average from the two trials with standard deviation given.

4.3. Radiosynthesis and characterization of 18F-2-FDTre

To a 0.5 or 1.5 mL microcentrifuge tube containing a solution of commercially obtained 18F-2-FDG in 0.9% NaCl, reactants were added sequentially to achieve final concentrations of 40 mM UDP-glucose, 20 mM MgCl2, ~10 μM TreT enzyme, and enough Tris buffer (50 mM Tris, 300 mM NaCl, pH 7–8) to achieve the desired volume if needed. After gently pipetting up and down three times, the tube was capped and incubated for 15 min at 70 °C with shaking in a Grant Bio PMHT Thermoshaker inside a cell. The reaction mixture was transferred to an Amicon Ultra-15 centrifugal filter unit (nominal molecular weight limit (NMWL) 10 kDa) pre-rinsed 3× with deionized water. The filter unit was centrifuged at 14,000 rpm for 10 min, then the filtrate was collected and loaded onto a Bio-Rad Poly-Prep column (0.8 × 4 cm) pre-packed with pre-equilibrated Bio-Rad AG 501-X8(D) mixed-bed ion exchange resin (biotechnology grade, 20–50 mesh, H+ + OH− form, with blue-to-gold indicator dye to monitor resin exchange capacity). A
ratio of 75 mg resin per 100 μL solution was used. The initial eluate containing the void volume was discarded, then the probe-containing eluate was collected. The total synthesis time including purifications was 30 min.

To determine radiochemical yield and radiochemical purity, the product was analyzed by radio-HPLC using an Agilent 1200 Series HPLC system equipped with an Imtakt UK-Amino 250 × 46 mm column, a Raytest gamma detector, and Gina software for monitoring radioactivity. The HPLC method, which was adapted from our previous report [51], utilized isotopic elution with a pre-mixed mobile phase of 80% acetonitrile in water at a flow rate of 0.4 mL/min. Radiochemical yield was determined by dividing the product activity by the starting activity and multiplying it by the radiochemical purity. Radiochemical purity was determined by calculating the area under the curves in the radiochromatogram. The radionuclidic purity of the product was determined by using a portable spectrometer (Hamamatsu Photonics radiation detection module CI2137) and calculating decay time using a PTW Curiementor 4 activimeter.

To confirm product identity by mass spectrometry (MS), a radio-synthesis of 18F-2-FDG was performed as described above, except 18F-2-FDGA was added to a final concentration of 0.2 mCi to generate sufficient non-radioactive 18F-2-FDG product to allow MS analysis. Following the reaction, radio-HPLC was used to employ the method described above to iso-olate the presumed radioactive 18F-2-FDG and non-radioactive 18F-2-FDG species by obtaining eluate from tR = 27 min. After decay of 18F occurred, the non-radioactive sample was analyzed by electrospray ionization (ESI) MS in positive and negative mode using a HCT-Ultra ion trap mass spectrometer.

Automated conversion of 18F-2-FDG to 18F-2-FDG was performed on an ELIXYS FLEX/CHRM automated radiosynthesizer (SOFIE Co., Culver City, CA) using standard ELIXYS cassettes, reagent vials (W9862599N, Wheaton; Millville, NJ), magnetic stir bars (SMB-0605-GLC, stirbars.com; Huntersville, SC), and reagent vials (62413PF-2, Voigt; Lawrence, KN). An automated program ("sequence") was created, based on the optimized manual radiosynthesis, by assembling a short sequence of intuitive "unit operations" (macros) using a drag-and-drop interface, and specifying appropriate parameters for each. A "reaction mix" (0.55 mL total volume, 50 mM Tris buffer, pH 8.0) containing T. tenax TreT enzyme (450 μL of a 951 μg/mL solution), UDP-glucose (50 μL of a 0.4 M solution), and MgCl2 (5 μL of a 2 M solution) was loaded into the ELIXYS cassette prior to starting the sequence. The sequence commenced with the addition of 18F-2-FDG (433 μg) to 20 μL of the reaction to the reactor using the EXTERNAL ADD unit operation. The "reaction mix" was then added using the ADD unit operation (N.B., ~100 μL will not enter the reactor due to the dead volume of the ELIXYS cassette), to give a solution of 18F-2-FDG, TreT (840 μg/L/mL), UDP-glucose (40 mM), MgCl2 (20 mM) in ~50 mM Tris buffer, which was then heated at 70°C for 20 min. The solution was cooled, and a sample withdrawn for analytical HPLC (method was adapted from that described above for manual synthesis).

4.4. Cellular uptake experiments

For mammalian cells, frozen stocks of each cell type were thawed and diluted in PBS prior to initiating uptake experiments with 106 cells. For M. smegmatis, liquid cultures were grown in Löwenstein–Jensen medium at 37°C to mid-log phase, then uptake experiments were initiated with 108 colony-forming units (CFU)/mL of the bacteria in PBS. For both mammalian cells and M. smegmatis, ~5 μCi of newly synthesized 18F-2-FDG-Trt was added to the cell suspensions, which were then incubated at 37°C with gentle shaking for 60 min. Next, cells were pelleted by centrifugation at 13,000 rpm for 5 min and washed with ice-cold PBS three times. The cells were resuspended in PBS and radioactivity was measured using an automated gamma well counter (Genesys Gamma-1, Laboratory Technologies, Inc.). For the M. smegmatis heat-killing control, 105 CFU/mL of bacteria were heat-killed at 80°C for 60 min prior to performing uptake experiments as described above. For the M. smegmatis competition experiment, 106 CFU/mL of bacteria were subjected to uptake experiments as described above but in the presence of 1 μM unlabeled native trehalose. For the M. smegmatis uptake time course, 106 CFU/mL of bacteria were subjected to uptake experiments as described above but aliquots were taken and analyzed at 0, 10, 30, 60, 90, and 180 min time points (note: due to the sample washing steps prior to gamma well counting, there was a ~15–25 min delay time between the “end point” and activity measurement, during which some additional uptake could have occurred). Mammalian cell viability following uptake experiments was >90% as determined by trypan-blue staining.

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