Measuring Dynamic Changes in the Labile Iron Pool in Vivo with a Reactivity-Based Probe for Positron Emission Tomography

Ryan K. Muir,†‡#, Ning Zhao,§# Junnian Wei,§ Yung-hua Wang,§ Anna Moroz,§ Yangjie Huang,§ Ying-Chu Chen,† Renuka Sriram,§ John Kurhanewicz,§ Davide Ruggero,∥⊥ Adam R. Renslo,*†∥ and Michael J. Evans*†§∥

†Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, California 94158, United States
‡Graduate Program in Chemistry & Chemical Biology, University of California, San Francisco, San Francisco, California 94158, United States
§Department of Radiology and Biomedical Imaging, University of California, San Francisco, San Francisco, California 94158, United States
∥Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, San Francisco, California 94158, United States
⊥Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, California 94158, United States

Supporting Information

ABSTRACT: Redox cycling of iron powers various enzyme functions crucial for life, making the study of iron acquisition, storage, and disposition in the whole organism a worthy topic of inquiry. However, despite its important role in biology and disease, imaging iron in animals with oxidation-state specificity remains an outstanding problem in biology and medicine. Here we report a first-generation reactivity-based probe of labile ferrous iron suitable for positron emission tomography studies in live animals. The responses of this reagent to systemic changes in labile iron disposition were revealed using iron supplementation and sequestration treatments in mice, while the potential of this approach for in vivo imaging of cancer was demonstrated using genetically and pathologically diverse mouse models, including spontaneous tumors arising in a genetically engineered model of prostate cancer driven by loss of PTEN.

INTRODUCTION

Iron is a transition metal nutrient whose redox cycling is utilized by iron-dependent enzymes to perform various biological functions essential to life.1,2 The storage, transport, and utilization of iron is highly regulated in normal cells and tissues due to the potential of free ferrous iron to promote Fenton chemistry and the formation of reactive oxygen species. Accordingly, iron in biology is stored and transported in an inert ferric state, bound with high affinity by proteins including ferritin (storage) and transferrin (transport). Unloading of iron from transport and storage proteins involves reduction to the soluble ferrous state and entry into the cytosolic labile iron pool (LIP),3,4 from which iron is utilized to produce essential enzyme cofactors including iron heme1 and iron–sulfur clusters.5,6 Dysregulation of iron homeostasis occurs in diverse disease states significantly impacting public health, including neurodegenerative disease,7 cardiovascular disease,8 inflammation,9 and cancer.10,11

Given its important role in biology and disease, the development of chemical reagents to detect iron with metal-ion and oxidation-state specificity is an important problem in chemical biology.12,13 One of the earliest and most widely used probes, calcein AM, is a chelation-based reagent that

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Unfortunately exhibits poor selectivity for Fe(II) over Fe(III) and other metal ions. Improved metal and oxidation-state selectivity was later achieved in the first generation of reactivity-based iron probes, such as Rho-Nox1 and IP-1. Subsequently, Rensto and co-workers introduced the reactivity-based probe TRX-PURO, an iron-caged form of puromycin that is activated by Fe(II)-promoted fragmentation of a 1,2,4-trioxolane (TRX) moiety. Immunofluorescence imaging of TRX-PURO treated cells affords improved sensitivity compared to Rho-Nox1 and IP-1, but its use in mice requires the laborious ex vivo analysis of mouse tissues. Caged fluorescent probes are also unsuitable for in vivo studies due to the limited tissue penetration of short wavelength probes. Caging of d-aminoluciferin with the TRX moiety used in TRX-PURO produced the iron probe ICL-1, which enables imaging of labile iron in live, luciferase-expressing mice.

Despite the advances described above, reactivity-based imaging of labile ferrous iron in nontransgenic animals and humans remains an outstanding problem in biology and medicine. We considered that this challenge might be met in Fe(II)-sensitive, TRX-based radiotracers for positron emission tomography (PET). Incorporation of positron-emitting radionuclides such as fluorine-18 or carbon-11 in biologically active small molecules generally has minimal pharmacological impact. Moreover, PET is used widely in both basic science and clinical settings, affording 3-D noninvasive views of an experimental animal model or human patient. 18F radioisotope is also widely available for routine preclinical and clinical use, and has a convenient 110 min half-life that allows sufficient time for radiotracer preparation and imaging, while ensuring a relatively low radiation dose in patients.

The degradation mechanism of 1,2,4-trioxolane antimalarial agents like artefenomel (Figure 1A) has been widely studied, with Fe(II)-promoted cleavage of the peroxide bond and a subsequent β-scission reaction producing carbon-centered radicals that can be intercepted with stable radical reagents like TEMPO, or by nearby biological macromolecules when formed in cells. This radical trapping chemistry thus provides a mechanism by which a putative PET reagent would become sequestered in cells and tissues in an Fe(II)-dependent fashion. Accordingly, we designed the trioxolane-based reagent 18F-TRX in which a short linker bearing 18F is introduced on the general approach described for the synthesis of artefenomel and related antimalarial trioxolanes. Full details regarding the synthesis of TRX-amine and 18F-TRX analytical standard are provided in the Supporting Information.

Herein, we describe the synthesis of 18F-TRX, a first-generation reactivity-based PET probe of labile ferrous iron. We show that this new scaffold retains Fe(II)-dependent reactivity in vitro and accumulates in cells in an Fe(II)-dependent fashion. We describe mouse pharmacokinetic and biodistribution studies in healthy mice with macro- and microdosing, where we observed relatively rapid elimination of 18F-TRX from the blood pool and significant radiotracer uptake in small intestine at 60 min postinjection. The iron-sensitivity of 18F-TRX biodistribution in vivo was confirmed using iron supplementation and withholding strategies. Finally, we show enhanced accumulation of 18F-TRX in human and mouse tumors (up to ~5% ID/g) compared to normal reference tissue compartments like blood and muscle. Overall, this study marks a first step toward a general method for sensitive and selective imaging of labile iron in living animals and suggests a new approach for functional imaging of cancer.

**RESULTS AND DISCUSSION**

**Radiosynthesis of 18F-TRX.** The synthesis of 18F-TRX required the novel 1,2,4-trioxolane precursor reagent TRX-amine in which an amine-bearing side chain has been introduced at a bridgehead position on the adamantane ring (Scheme 1). This reagent was synthesized in eight steps, based on the general approach described for the synthesis of artefenomel and related antimalarial trioxolanes. Full details regarding the synthesis of TRX-amine and 18F-TRX analytical standard are provided in the Supporting Information.

The radiosynthesis of 18F-TRX began with the automated preparation of 18F-N-succinimidyl 4-fluorobenzoate. Using an ELIXYS automated radiosynthesizer, 18F-SFB was prepared in 75 min to a decay-corrected radiochemical yield of approximately 70%. For the coupling reaction, 20 mCi of 18F-SFB was added to TRX-amine (5 mg of a formate salt) and 10% (v/v) DIPEA in anhydrous DMF (1 mL). The reaction was stirred at 40 °C for 30 min. The reaction produced only one major radioactive peak, which comigrated with the 18F-TRX standard (Figure 2A). The crude reaction was purified using semipreparative HPLC (1:10 CH3CN:H2O to 19:1 CH3CN:H2O over 20 min) to obtain the radiotracer 18F-TRX to a decay-corrected radiochemical yield of 67 ± 7.2%. The purity of the compound was verified by reinjection on semiprep HPLC (Figure 2B). 18F-TRX was concentrated, and immediately reconstituted for additional in vitro or animal studies (see the Experimental Section). The specific activity of

**Figure 1.** (A) Structure of artefenomel and 18F-TRX. (B) Reaction with Fe2+ yields 18F-TRX-linked biomolecule adducts that are sequestered in cells at site(s) of reaction.
In Vitro Studies Show That TRX Analogues React with Fe(II) and Cross-Link Cellular Proteins. To confirm that \(^{18}\text{F-TRX}\) retains reactivity with Fe(II), the purified compound was exposed to aqueous ammonium Fe(II) sulfate (FAS, 30 mg/mL). After 30 min, \(^{18}\text{F-TRX}\) was entirely consumed and cleanly converted to a single radioactive byproduct as observed on reverse phase rad-HPLC (Figure 2C). This result indicates that the reactivity of \(^{18}\text{F-TRX}\) with Fe(II) is highly regioselective, as was expected based on earlier mechanistic studies\(^26\) of the antimalarial compounds on which Fe(II) is highly regioselective, as was expected based on earlier mechanistic studies\(^26\) of the antimalarial compounds on which \(^{18}\text{F-TRX}\) is based. To confirm that the \(^{18}\text{F-bearing side chain in}\) \(^{18}\text{F-TRX}\) becomes sequestered in cells following reaction with Fe(II), we prepared the nonradioactive “clickable” probe HC2-TRX (Figure 3A) in which an alkyne function replaces the fluorine atom in \(^{18}\text{F-TRX}\). Fluorescence cell imaging of PC3 cells treated with HC2-TRX followed by fixing and copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC) reaction with Alexa488-azide were consistent with irreversible labeling of cytoplasmic targets (Figure 3B). An in-gel TAMRA fluorescence analysis confirmed the promiscuous labeling of proteins in PC3 cells treated with HC2-TRX (Figure 3C). Moreover, pretreating PC3 cells with ferric ammonium citrate (FAC) or the iron chelator desferrioxamine (DFO) prior to HC2-TRX revealed more and less labeling, respectively, consistent with iron-dependent activation. These findings with HC2-TRX indicate that the analogously positioned \(^{18}\text{F}\) atom in \(^{18}\text{F-TRX}\) would similarly become sequestered in cells in an Fe(II)-dependent fashion, as designed.

\(^{18}\text{F-TRX Biodistribution Is Fe(II)-Dependent in Normal Mouse Tissues. To determine the biodistribution of \(^{18}\text{F-TRX}\) in clinically relevant mouse models, the radiotracer was injected into immunocompetent C57Bl/6j mice and studied over time. Region of interest analysis of a dynamic PET/CT acquisition from 0 to 60 min postinjection revealed several enlightening trends. First, \(^{18}\text{F-TRX}\) rapidly cleared from the mediastinal blood pool at a rate \sim 60\times faster than what we observed from a classic PK assessment of \(^{18}\text{F-TRX}\) (Figure 4A and Figure S2A). Moreover, \(^{18}\text{F-TRX}\) rapidly accumulated in liver from 0 to 60 s, suggesting that the radiotracer may be metabolized and cleared through this organ (Figure 4B,C) as is the case for artefenomel in humans.\(^31\) Kidney accumulation of the radiotracer was also observed, but the uptake was significantly lower than liver. Moreover, kidney uptake plateaued within 30 s postinjection, suggesting that (as with artefenomel) renal clearance is not the dominant mechanism of clearance of radiotracer. Focal uptake in a region of the small intestine was also observed early after radiotracer injection, and the accumulation steadily increased from 400 to 3600 s (Figure S2B). This observation is also consistent with a model of hepatobiliary excretion. To study radiotracer distribution over a broader window of time, ex vivo biodistribution studies were conducted at 30, 60, and 90 min postinjection of \(^{18}\text{F-TRX}\) in a separate cohort of mice (Figure 4D). These studies generally corroborated the PET findings, showing that \(^{18}\text{F-TRX}\) uptake was dominant in the liver, components of the small intestine, and the kidneys. Moreover, radiotracer uptake generally increased from 30 to 90 min in these tissues. Radiotracer uptake was also observed in the spleen, pancreas, stomach, large intestine, and lungs. Uptake in the brain was very low, consistent with previous observations that other TRX conjugates do not cross the blood–brain barrier.\(^31\)

To better understand the localization of the radiotracer within regions of the small intestine, components of the GI tract were excised from a representative mouse 60 min postinjection, and radiotracer biodistribution was assessed with PET/CT (Figure 4E). This study suggested that radiotracer uptake was predominant in the duodenum and jejunum. Comparatively lower uptake was observed in the ileum, cecum, and a truncated segment of the large intestine (purged manually to remove fecal matter).

We next tested whether \(^{18}\text{F-TRX}\) biodistribution was impacted by exogenous treatments designed to alter tissue concentrations of ferrous iron. 20 minutes prior to i.v. injection of \(^{18}\text{F-TRX}\), immunocompetent mice were treated with an i.p. bolus of PBS, ferric ammonium citrate (FAC, 20 mg/kg), desferrioxamine (50 mg/kg, DFO), deferiprone (50 mg/kg, DFP), or a mixture of FAC (20 mg/kg) precomplexed with deferroxamine (50 mg/kg, FAC + DFO). The use of a ferric iron source ensures that bioconversion to ferrous iron by normal cellular processes is a necessary prelude to reaction with \(^{18}\text{F-TRX}\). The biodistribution of the radiotracer at 60 min postinjection was strikingly different between treatment arms on PET/CT (Figure 5A). For instance, while \(^{18}\text{F-TRX}\) most visibly accumulated in the liver of mice treated with PBS, FAC treatment elevated uptake in the liver, small intestine, and gall bladder. Iron depleting strategies (e.g., DFO, FAC + DFO, DFP) reduced radiotracer uptake in the liver, while clearly redistributing \(^{18}\text{F-TRX}\) to components of the small intestine. Quantitative biodistribution studies showed a statistically significant increase in \(^{18}\text{F-TRX}\) uptake in the liver, spleen, pancreas, and duodenum of mice treated with FAC versus those treated with PBS (Figure 5B and Figure S3). Moreover, treatment with iron chelating agents significantly reduced \(^{18}\text{F-TRX}\) uptake in virtually all tissues, with the notable exceptions of the duodenum, ileum, cecum, and large intestine in the FAC + DFO treatment arm. In summary, these data demonstrate that \(^{18}\text{F-TRX}\) biodistribution is substantially influenced by exogenous treatments designed to modulate tissue concentrations of ferrous iron.

\(^{18}\text{F-TRX Detects Tumor Xenografts Sensitive to Trioxolane Prodrugs. We next evaluated if \(^{18}\text{F-TRX}\) can\)
Figure 2. Synthesis and isolation of $^{18}$F-TRX. (A) Representative semipreparative rad-HPLC traces showing, from top, the UV spectrum at 254 nm for TRX-amine starting material, the UV spectrum at 254 nm for the pure $^{18}$F-TRX analytical standard, the UV spectrum at 254 nm for complex radiochemistry reaction mixture at 40 min, and the rad spectrum of the complex radiochemistry reaction mixture at 40 min. Peaks of interest are labeled with the retention time in minutes. (B) Semipreparative rad-HPLC traces of the reinjected fraction isolated after purification. The retention time in minutes of the peak of interest representing $^{18}$F-TRX is labeled. (C) A rad spectrum collected 30 min after incubation of $^{18}$F-TRX in aqueous ammonium Fe(II) sulfate (30 mg/mL) showing disappearance of the peak corresponding to $^{18}$F-TRX and appearance of a new peak with a different retention time.

Figure 3. Cellular probe HC$_2$-TRX promiscuously labels proteins in PC3 cells. (A) Structure of cellular "clickable" probe HC$_2$-TRX. (B) Representative fluorescence images of PC3 cells treated with HC$_2$-TRX for 6 h and visualized by CuACC reaction with Alexa488-azide (green, FITC channel); cell nuclei are visible in the DAPI channel (blue). Scale bars denote 50 μm. (C) In-gel TAMRA fluorescence analysis reveals promiscuous protein labeling following incubation of PC3 cells with HC$_2$-TRX. Enhanced labeling is observed when PC3 cells were pretreated (2 h) with the Fe(III) source FAC (500 μM) before treatment with HC$_2$-TRX. Conversely, PC3 cells pretreated with the iron chelator DFO (300 μM) showed reduced labeling intensity. Coomassie (Coo) staining is used as a loading control.
detect human tumors derived from cell lines previously shown to harbor sensitivity to TRX-based iron(II)-activatable prodrugs.32 Biodistribution studies were first conducted in intact male nu/nu mice bearing subcutaneous PC3 xenografts.

Figure 4. An in vivo assessment of the biodistribution of $^{18}$F-TRX in tumor naive immunocompetent mice. (A) Plasma concentration–time curve for $^{18}$F-TRX administered via tail vein injection in C57Bl/6J mice revealing a plasma half-life of ~30 min. (B) Time activity curves derived from region of interest analysis of a 1 h dynamic PET scan reveal radiotracer biodistribution in vivo. The radiotracer rapidly clears from the blood (red) with a calculated serum half-life of about 25 s. The radiotracer is also sequestered within 60 s by the liver (tan) and kidneys (blue), with no additional accumulation from 60 to 3600 s. Liver uptake is significantly higher than what is observed in kidneys. Also, $^{18}$F-TRX accumulation in the small intestine (gray) steadily increases from 0 to 3600 s, consistent with a model of hepatobiliary clearance for the radiotracer. Little uptake is observed in the muscle (green). Figure S2C shows the location of the manually drawn regions of interest. (C) Maximum intensity projections sampled serially over short time frames from the dynamic acquisition show the biodistribution of the radiotracer in normal tissues over time. A diffuse signal is observed at early time points, which gradually consolidates into the liver, kidney, and small intestine. (D) Biodistribution data acquired at 30, 60, and 90 min postinjection of $^{18}$F-TRX show continuous accumulation of the radiotracer in many abdominal organs. The highest uptake was observed in the liver, kidneys, and small intestine. Radiotracer accumulation was low in the blood pool and muscle, as expected from the MIPs. (E) A PET/CT shows the biodistribution of $^{18}$F-TRX in components of the gastrointestinal tract after dissection from a mouse. Prominent uptake was observed in the duodenum and jejunum, while comparatively lower radiotracer uptake was noted in the stomach, ileum, cecum, and large intestine. Liver, kidneys, and muscle are included for perspective on the upper and lower bound of radiotracer uptake in tissues.
tumors showed that 18F-TRX was well-distributed through the analysis of the spatial distribution of the radiotracer in U251 tumor, the model with the highest 18F-TRX uptake, was the Pb-Cre:Ptenfl/fl mouse with invasive adenocarcinoma was observed in nearly all organs, including the liver and components of the gastrointestinal tract. Pretreatment with various iron depleting agents, including desferrioxamine (DFO), FAC complexed with DFO, and deferiprone (DFP), generally reduced radiotracer uptake in organs and accelerated clearance. The MIPs, while inherently semiquantitative, were derived from decay-corrected PET data with scale bars adjusted to the range 0–50% ID/g to enable gross comparison. (B) Biodistribution data collected at 60 min postinjection show the percent changes in radiotracer uptake for selected organs in each treatment arm compared to mice receiving PBS. Relative increases in radiotracer uptake due to FAC treatment were observed in nearly all organs, while iron depleting treatments generally reduced organ uptake of the radiotracer. Treatment with FAC + DFO enhanced radiotracer uptake in components of the gastrointestinal tract, which may reflect accelerated clearance of the radiotracer.

at 30, 60, and 90 min postinjection of 18F-TRX. The uptake of the radiotracer steadily increased from 30 to 90 min postinjection. Radiotracer levels in the tumor significantly exceeded blood and muscle at 90 min postinjection with a tumor to blood ratio of 1.97 ± 0.4 and a tumor to muscle ratio of 1.90 ± 0.3 (Figure 6A and Figure S4A,B).

We further tested if 18F-TRX can detect PC3 tumors implanted in the renal capsule, which provides a better vascularized environment than subcutaneous implants. Biodistribution studies conducted 90 min postinjection of 18F-TRX showed equivalent radiotracer uptake in a PC3 tumor embedded in the renal capsule compared to the extent of uptake in subcutaneous PC3 tumors (Figure 6B and Figure S5). 18F-TRX uptake was also significantly higher than background (blood and muscle) in subcutaneous EKVX and U251 tumors, two models of human lung adenocarcinoma and glioblastoma, respectively (Figure 6B and Figure S6). The U251 tumor, the model with the highest 18F-TRX uptake, was visually obvious on small animal PET/CT (Figure 6C). Ex vivo analysis of the spatial distribution of the radiotracer in U251 tumors showed that 18F-TRX was well-distributed through the xenograft, with the regions of highest uptake appearing to have the densest cellularity on H&E (Figure 6D). Collectively, these data show that genetically and pathologically diverse models of human cancer harbor high avidity for 18F-TRX in vivo.

We next conducted a pilot imaging study in a genetically engineered mouse model of prostate cancer. A 10 month old Pb-Cre:Pten+/fl mouse with invasive adenocarcinoma was treated with 18F-TRX, and after 90 min, the whole prostate was resected post mortem and imaged with PET/CT. Radiotracer uptake was visually higher in the prostate tissue compared to seminal vesicles and muscle (Figure 6E). Moreover, 18F-TRX uptake was predominant in the prostate tissue, and not observed in the cysts that routinely develop in this disease model.

**CONCLUSION**

Here we reported the design, synthesis, and characterization of 18F-TRX, a reactivity-based PET radiotracer that enables quantitative imaging of labile Fe(II) in living animals, including its application to several clinically relevant human and mouse cancer models. Based on an antimalarial with Fe(II)-dependent pharmacology, 18F-TRX can react with Fe(II) and become sequestered in tissues at the site(s) of its Fe(II)-promoted activation in vivo. Biodistribution studies and ex vivo imaging revealed high levels of radiotracer uptake in liver and small intestine that could be altered by pretreatment with bioavailable iron sources (FAC) or iron chelators (DFO). Furthermore, 18F-TRX was capable of detecting elevated Fe(II) levels in tumor compared to blood pool and muscle in PC3, EKVX, and U251 xenograft models and in a genetic prostate cancer model. These results suggest that labile Fe(II) represents an actionable analyte for cancer imaging with PET in animals.

**EXPERIMENTAL SECTION**

**Radiosynthesis of 18F-TRX.** 18F-SFB was prepared in 75 min to a decay-corrected radiochemical yield of approximately 70%. 18F-SFB was transferred from the ELIXYS on a C18 SepPak cartridge, and the 18F-SFB was eluted from the cartridge using neat CH3CN. The acetonitrile was removed under vacuum and a gentle stream of N2(g), and to the 18F-SFB (20 mCi) was added TRX-amine (5 mg, as formate salt) and 10% (v/v) DIPEA in anhydrous DMF (1 mL). The reaction was...
stirred at 40 °C. Reaction progress was monitored by rad-HPLC, and the reaction was terminated at 30 min, as the coupling of 19F-SFB to TRX-amine under analogous conditions was complete at 30 min. The crude reaction was purified using semipreparative HPLC (1:10 CH₃CN:H₂O to 19:1 CH₃CN:H₂O over 20 min) to obtain the radiotracer ¹⁸F-TRX to a decay-corrected radiochemical yield of 67 ± 7.2%. The purity of the compound was verified by reinjection on semiprep HPLC.

Figure 6. ¹⁸F-TRX detects tumor tissue in vivo in genetically and pathologically diverse cancer models. (A) Biodistribution data acquired at 30, 60, and 90 min postinjection of ¹⁸F-TRX in male nu/nu mice with subcutaneous PC3 xenografts. The radiotracer uptake in the tumor continually increases from 30 to 90 min, consistent with a reactivity-based mechanism of action. Moreover, radiotracer uptake in the tumor exceeds the level observed in blood and muscle at 90 min postinjection, two standard reference compartments for background radiotracer accumulation. The human prostate cancer model PC3 was prioritized as it was previously shown to be highly sensitive to an Fe(II)-sensitive TRX prodrug bearing a chemotherapeutic payload. * P < 0.01 compared to blood and muscle. Figure S4 shows the biodistribution values for the entire repertoire of tissues from this animal cohort as well as the tumor to normal tissue ratios. (B) Biodistribution data acquired 90 min postinjection of ¹⁸F-TRX shows radiotracer uptake in tumor exceeding background for PC3 tumors implanted in the renal capsule (rcPC3), and subcutaneous EKVX and U251 tumors (scEKVX, scU251). Figure S5 shows a MR image highlighting the tumor burden in renal capsule. Figure S6 shows the complete biodistribution data sets, and the tumor to normal tissue ratios. (C) PET/CT imaging data showing uptake of ¹⁸F-TRX in tumor and normal tissues for mice bearing subcutaneous U251 tumors. The data were acquired at 90 min postinjection. (D) H&E (left) and digital autoradiography (right) showing ¹⁸F-TRX distribution within a representative section of U251 tumors. ¹⁸F-TRX appears to be present in all regions of the slice, with the highest relative uptake appearing to colocalize with the area of densest cellularity on H&E. (E) (left) A photograph of the surgically excised whole prostate (Pr.), a piece of muscle from the hindlimb (Mu.), and the seminal vesicles (SV) of a 10 month old Pb-Cre:Ptenfl/fl mouse with fully invasive adenocarcinoma. Cysts extending from the anterior prostate are evident by eye. (middle) A volume rendered CT image of the tissues acquired on a small animal PET/CT. (right) A volume rendered PET/CT image of ¹⁸F-TRX uptake in the tissues acquired 90 min postinjection of ¹⁸F-TRX. The image clearly shows relatively higher accumulation of radiotracer in the diseased prostate compared to muscle or seminal vesicles. ¹⁸F-TRX was excluded from the cysts, as expected.
**Cell Culture and Cell/Protein Labeling Experiments.** The PC3, EKVC, and U251 cell lines were obtained from ATCC (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium (VWR) supplemented with 10% fetal bovine serum (Gemini Bio) and 1× penicillin/streptomycin (Life Technologies). Cells were cultured at 37 °C supplemented with 5% CO₂.

For the fluorescence cell imaging experiment, PC3 cells were cultured in a 96-well Greiner μClear plate until reaching 75% confluence. Media was then removed and replaced with media containing trizolone HC₂-TRX (20 μM, 0.1% DMSO) or vehicle (0.1% DMSO). After 6 h, cells were washed with PBS twice and then fixed with 4% paraformaldehyde for 10 min at room temperature (rt). Cells were washed with PBS twice and incubated with PBS supplemented with 0.1% Triton X-100 for 5 min. Cells were then washed with PBS three times, then incubated for 1 h with click master mix (5 mM sodium ascorbate, 2 mM THPTA, 500 μM CuSO₄, 10 μM Alexa488-azide), and then washed with PBS three times. Cells were then treated with PBS containing Hoechst nuclear stain for 10 min, washed with PBS twice, and imaged with an IN Cell 6500 automated cell imager at 40× magnification.

For the protein labeling and in-gel fluorescence experiment, PC3 cells were cultured in 6-well plates until reaching 75% confluence. Media was then removed and replaced with fresh media or fresh media containing FAC (500 μM) or DFO (300 μM). After 2 h, media was removed and replaced with media containing HC₂-TRX (20 μM, 0.1% DMSO) or vehicle (0.1% DMSO). After 6 h, cells were washed with PBS twice then incubated in cold PBS for 5 min. Cells were collected using a plastic cell scraper and pelleted. The pellet was resuspended in 100 μL of cold 0.1% NP40 lysis buffer (100 mM Heps, 150 mM NaCl, 0.1% NP40, pH 7.5) with 5 mg/mL EDTA free protease inhibitor (Roche) for 30 min on ice. The following lysate was centrifuged at 13,000 rpm for 10 min. Protein concentration was determined using Pierce BCA protein assay kit (Thermo Fisher) and subsequently normalized to 1 mg/mL. To 50 μL of this lysate was added the click reaction cocktail (final concentrations: 25 μM TAMRA-Azide, 1 mM CuSO₄, 0.1 mM TBTA, 1 mM TCEP), and it was incubated at room temperature for 1 h in the dark.

Protein was precipitated by addition of 1 mL of cold methanol and cooled to ~80 °C. Protein was pelleted by centrifugation at 14,000 rpm for 5 min at 4 °C. Methanol was decanted, and the protein pellet was washed with 1 mL of cold methanol and pelleted again as described. Methanol was decanted, and the pellet was resuspended in 50 μL of 1× laemml buffer (Bio-Rad) supplemented with β-mercaptoethanol. Sample was boiled for 5 min, and 25 μg of protein was loaded onto Bolt 12% Bis-Tris Plus Gel (Thermo Fisher). Fluorescence was visualized by a ChemiDoc system and displayed in a grayscale. Following imaging, total protein was visualized by Coomassie stain.

**Animal Experiments.** To reconstitute ¹⁸F-TRX for animal studies, the probe was trapped on a C18 Sep-Pak cartridge, and eluted with a small volume of ethanol. Ethanol was removed at 50 °C under vacuum and a gentle stream of N₂(g) to afford neat ¹⁸F-TRX. Formulation of ¹⁸F-TRX for in vivo studies proved challenging due to the very poor aqueous solubility of this material. After some experimentation, we adopted a formulation comprising 10% DMSO in a 20 mM aqueous sodium phosphate solution at pH 3 used previously by Charman and co-workers for IV pharmacokinetic studies of artefenomel. A 5 mg/kg dose of ¹⁸F-TRX in 100 μL of this formulation was well-tolerated in mice when administered by tail vein injection.

All animal experiments were conducted under the approval from Institutional Animal Care and Use Committee (IACUC) at UCSF. Male nu/nu or C57BL6/J mice (4–6 weeks) were purchased from Charles River. All the mice were well-housed in the USCF with free access to food and water. Nu/nu mice were inoculated with 2–5 × 10⁶ PC3 cells in a mixture of media (RPMI) and Matrigel (Corning) (1:1 v/v) subcutaneously into one flank. Tumors in mice were palpable within 3–4 weeks after the implanting. Male Rag2 RAGN12 mice (Taconic) were used for the renal capsule tumor implants. The mice were anesthetized with isoflurane (2–3%) and performed with a dorsal midline incision (0.5 cm). Via pressuring on the muscle wall, one kidney was pulled gently and carefully through the small incision. PC3 tumor cells (5 × 10⁵ in 50 mL of PBS) were then injected into the pocket under the kidney capsule, which was lifted from the kidney parenchyma. The kidney was placed back to the body of mice, and then the skin incision was closed using 3 surgical sutures. Carprofen (5–10 mg/kg) was used to treat the mice for easy recovery. Mice were observed carefully over 24 h for the signs of postoperative bleeding, pain, and (or) other complications. After surgery, a 14 T Agilent MRI was used to monitor the tumor progression for the following 7–14 days.

**Small Animal PET/CT.** ¹⁸F-TRX (~300 μCi) was injected via tail vein in 100–150 μL of 10% DMSO in a 20 mM aqueous sodium phosphate solution. For treatment studies, vehicle (100 μL PBS), FAC (20 mg/kg in PBS, 100 μL), both FAC (20 mg/kg in PBS, 100 μL) and DFO (50 mg/kg in PBS, 100 μL), DFP (30 mg/kg in PBS, 100 μL), or DFO (30 mg/kg in PBS, 100 μL) were injected intraperitoneally 20 min prior to the intravenous injection of ¹⁸F-TRX. The mice were anesthetized with 2–3% isoflurane, and imaged with a Siemens Inveon microPET/CT. For dynamic acquisitions, the mice were anesthetized prior to injection, and injected while positioned on the scanner bed. All imaging data were decay-corrected, reconstructed, and analyzed with AMIDE software. Maximum intensity projections (MIPs) were generated by AMIDE software. Regions of interest (ROI) were manually placed to calculate SUV data from the dynamic acquisitions.

**Biodistribution Studies.** Mice were euthanized with CO₂(g) asphyxiation and dissected at dedicated time points postinjection. The blood and tissues were removed, washed, dried, and weighed. The activity of each tissue was measured with a γ counter. All data were decay-corrected. PRISM software was used to express a percentage (% ID/g) of the injected dose per gram of tissue.

**Digital Autoradiography.** Post mortem, tumors were flash frozen in OCT on liquid nitrogen. The tissue was sectioned with a microtome into 20 μm thickness slices and mounted on glass slides. The slides were exposed in a GE phosphor storage screen for 10 radionuclide half-lives. The phosphor screen was developed on an Amersham Typhoon 9400 phosphorimager. The images were processed using ImageJ software. H&E staining was performed by the Pathology core facility at UCSF.

**Statistics.** All statistical analysis was performed using PRISM v6.0 or ORIGIN software. An unpaired, two-tailed Student’s t test was used to determine statistically significant
differences in the data. Changes at the 95% confidence level (P < 0.05) were reported as statistically significant.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.9b00240.

Additional experimental details, figures, and schemes including time activity curves, coronal PET/CT images, biodistribution data, MR image, and synthesis schemes (PDF)

## AUTHOR INFORMATION

### Corresponding Authors

*Phone: 415-514-9698. Fax: 415-514-4507. E-mail: adam.renslo@ucsf.edu.*
*Phone: 415-353-3442. Fax: 415-353-9425. E-mail: michael.evans@ucsf.edu.*

### ORCID

Ning Zhao: 0000-0003-3699-3369
Renuka Siram: 0000-0003-3505-2479
Adam R. Renslo: 0000-0002-1240-2846
Michael J. Evans: 0000-0003-4947-1316

### Author Contributions

*R.K.M. and N.Z. contributed equally. R.K.M., N.Z., D.R., A.R.R., and M.J.E. designed compounds and conceived experiments. R.K.M. synthesized TRX-amine, 19F-TRX, and A.R.R., and M.J.E. designed compounds and conceived HC2-TRX. R.S. and J.K. performed the radiosynthesis. N.Z., J.W., Y.W., A.M., and Y.H. conducted all animal and imaging experiments. R.K.M. and Y.-C.C. performed the in vitro uptake and cellular cross-linking assay with HC2-TRX. J.W. and N.Z. were involved in the development of the 19F-tracer. A.R.R., and M.J.E. designed compounds and conceived experiments.*

### Notes

The authors declare the following competing financial interest(s): A.R.R. declares equity in Tatara Therapeutics, Inc. which seeks to develop Fe(II)-activated therapies. The REMEDY project is supported by a grant from the US National Institutes of Health (R01 Grant AI105106). R.K.M. acknowledges funding from the NIH Research Training Grant in Chemistry and Chemical Biology (T32 GM064337). M.J.E. acknowledges funding from the American Cancer Society (Research Scholar Grant 130635-RSG-17-005-01-CCE). M.J.E. and A.R.R. acknowledge funding from the Congressionally Directed Medical Research Program (W81XWH1810763 and W81XWH1810754).

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