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[¹⁸F](2S,4R)4-Fluoroglutamine PET Detects Glutamine Pool Size Changes in Triple Negative Breast Cancer in Response to Glutaminase Inhibition

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Abstract

Glutaminolysis is a metabolic pathway adapted by many aggressive cancers, including triple-negative breast cancers (TNBC), to utilize glutamine for survival and growth. In this study, we examined the utility of [¹⁸F](2S,4R)4-fluoroglutamine ([¹⁸F]4F-Gln) PET to measure tumor cellular glutamine pool size, whose change might reveal the pharmacodynamic (PD) effect of drugs targeting this cancer-specific metabolic pathway. High glutaminase (GLS) activity in TNBC tumors resulted in low cellular glutamine pool size assayed via high-resolution ¹H magnetic resonance spectroscopy (MRS). GLS inhibition significantly increased glutamine pool size in TNBC tumors. MCF-7 tumors, with inherently low GLS activity compared to TNBC, displayed a larger baseline glutamine pool size that did not change as much in response to GLS inhibition. The tumor-to-blood-activity-ratios (T/B) obtained from [¹⁸F]4F-Gln PET images matched the distinct glutamine pool sizes of both tumor models at baseline. After a short course of GLS inhibitor treatment, the T/B values increased significantly in TNBC, but did not change in MCF-7 tumors. Across both tumor types and after GLS inhibitor or vehicle treatment, we observed a strong positive correlation between T/B values and tumor glutamine pool size measured using MRS ($R^2=0.71$). In conclusion, [¹⁸F]4F-Gln PET tracked cellular glutamine pool size in breast cancers with differential GLS activity and detected increases in cellular glutamine pool size induced by GLS inhibitors. This study accomplished the first necessary step towards validating [¹⁸F]4F-Gln PET as a PD marker for glutaminase-targeting drugs.

Keywords

Glutamine; Glutaminase; Triple Negative Breast Cancer; PET; MRS; Glutaminolysis; Precision Medicine

Introduction

Glutamine (Gln) is the most abundant amino acid in the blood. In addition to participating in cellular protein synthesis, Gln is a substrate in the **glutaminolysis** pathway (diagram in Fig 1), which is highly active in many aggressive forms of human cancers, including triple

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Disclosure

The authors disclose no potential conflicts of interest.

negative breast cancer (TNBC), glioma, lymphoma, pancreatic and lung cancers (1–7). The first and rate-limiting step of glutaminolysis is catalyzed by mitochondrial glutaminase (*GLS*), which converts Gln to glutamate. Glutamate can then be used to fuel the tricarboxylic acid (TCA) cycle, from which intermediates (e.g., citrate) can be exported and used in biosynthesis (e.g., of lipid for cell membrane). By activating glutaminolysis, cancers are able to utilize both glycolysis (the Warburg effect) and the TCA cycle to fulfill their demands for energy and biosynthesis. Consequently, cancers with active glutaminolysis may become “addicted” to Gln for growth and proliferation (8–11). To exploit this tumor-specific metabolic alteration, small molecule inhibitors targeting Gln metabolism have been developed (12–16). Candidates that inhibit the rate-limiting enzyme, *GLS*, have shown promising results in preclinical studies of solid tumors including TNBC (1,2). Some have advanced to clinical trials (e.g., NCT02071862)(17). However, clinical laboratory tools have not been developed to identify patients whose tumors might benefit from *GLS*-targeted therapy or measure the pharmacodynamic (PD) effect of *GLS*-inhibiting drugs. This would not only require tissue biopsy, but also the ability to maintain the enzyme-inhibitor binding status during assay process. A non-invasive *in vivo* imaging marker capable of assessing glutaminolysis and the PD effect of *GLS* inhibitors could overcome these limitations and guide early drug trials.

The fluorine-18 labeled glutamine analog, [¹⁸F](2*S*,4*R*)4-Fluoroglutamine ([¹⁸F]4F-Gln), detectable by positron emission tomography (PET), has recently been developed to study glutamine transport and kinetics (18,19). Based on an earlier report that this glutamine analog is taken up by cells via the same transporters as Gln (20), but is minimally metabolized in tumor cells (19,21), we performed studies to test the feasibility of using [¹⁸F]4F-Gln PET to measure changes in cellular glutamine pool size in response to *GLS* inhibition in breast cancers. We chose to use two breast cancer models with distinct *GLS* activities: TNBC exhibiting high *GLS* activity and estrogen receptor positive (ER+) breast cancer with inherently low *GLS* activity (1). This feasibility study is the first necessary step towards establishing [¹⁸F]4F-Gln PET as a pharmacodynamic (PD) marker of drugs targeting glutaminolysis.

Materials and Methods

Human TNBC (HCC1806 and HCC38) and ER+ (MCF-7) breast cancer cell lines purchased from ATCC (Manassas, VA) over the span of 2012–2016 were authenticated by the cell bank using the Short Tandem Repeat DNA profiling method and were passaged less than six months after resuscitation. Cells were maintained in complete culture media consisting of RPMI 1640 supplemented with 10% FBS (no antibiotics were used). These lines were chosen based on the distinct *GLS* activities reported previously (1) and confirmed in our study: 35–55 nmol/min/mg protein in the TNBC lines vs. 5 nmol/min/mg protein in MCF-7. Cells within 50 passages were used for the study. Radiosynthesis of [¹⁸F]4F-Gln followed previously established methods (18,22). The minimum specific activity of [¹⁸F]4F-Gln at the end of synthesis (EOS) was 40mCi/μmol and the maximal molar dose was 8.75 nanomoles based on the injected dose specified below. Two *GLS* inhibitors were examined in this study: BPTES (bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide) (13) was purchased

(Sigma) and CB-839 (1) was supplied by Calithera, Inc. (Palo Alto, CA) via a material transfer agreement between institutes.

In vitro cell uptake of [^{18}F]4F-Gln

HCC1806 (25,000 cells/well) and MCF-7 (30,000 cells/well) were attached to a 96-well plate overnight. CB839 stock solution (10 mM in DMSO) was serially diluted in complete media to 1 μM (final DMSO concentration was 0.05%) and incubated with cells for 4 h. Control cells were incubated in media containing 0.05% DMSO. To study tracer uptake, the culture media was replaced by PBS (with Ca^{++} and Mg^{++} to facilitate normal cell functions such as uptake and attachment) containing 5 mM glucose, 100 μM *L*-Gln (Sigma), 1 μM CB839 (or 0.05% DMSO), plus [^{18}F]4F-Gln ligand (~300,000 cpm/well in nano- to picomolar concentration). At specified time points (5, 15, 30, 60 and 120 min), supernatant was aspirated and the wells were washed twice with cold PBS (without Ca^{++} and Mg^{++}). Samples are counted on the gamma counter (2470 WIZARD2, Perkin Elmer, Waltham, MA) and the amount of protein per well was estimated by the Lowry method. Of note, owing to reversible nature of *GLS* inhibition mediated by CB839, the drug was present during tracer uptake period. The concentration of cold *L*-Gln was optimized to 100 μM so that it did not block [^{18}F]4F-Gln uptake while preventing the tracer from being depleted during the 2-hour uptake study.

Tumor implantation and treatment

All animal studies were approved by the animal care and usage committee (IACUC) of the University of Pennsylvania.

Xenografts from HCC1806, HCC38, and MCF-7 were established in NCR athymic nu/nu mice (female, 7 weeks old, Charles River) as we described previously (23). Only one type of cell was implanted in a single location in each mouse (the flank). Briefly, 1 million HCC1806 cells in PBS, 10 million HCC38 cells in Matrigel (1:1 mixed with PBS), or 5 million MCF-7 cells in Matrigel were implanted subcutaneously in the flank of the mouse. One week before MCF-7 cells implantation, a 60-day-release tablet containing 17 β -estradiol (Innovative Research America, Sarasota, FL, USA) was implanted under the skin of the neck region. Compared to HCC38, HCC1806 xenografts grew more robustly. While both were used in this study, the majority of the data presented in Results section were from HCC1806.

We examined two highly selective, potent, and reversible inhibitors of *GLS* in this study. BPTES was used to treat the TNBC (HCC1806) model in a pilot study, while CB839 was used to treat both the TNBC and MCF-7 models in subsequent studies. BPTES (dissolved in DMSO and diluted in saline to the final concentration of 0.8 mg/mL containing 4% DMSO) was administered via intraperitoneal injection at 12.5 mg/kg/day for 6 days. CB839, dissolved in the vehicle solution (VEH) of 25% (w/v) hydroxypropyl- β -cyclodextrin in 10 mmol/L citrate (pH 2), was administered via oral gavage (200 mg/kg twice daily); control mice received the same volume (0.25 mL) of VEH. A total of 4–6 doses (2–3 days) of CB839 or VEH were administered. Since CB839 has fast clearance and its inhibitory effect on *GLS* is slowly reversible (1), this short treatment course ensured tumor exposure to the drug, without substantial changes in tumor volume.

HPLC-based analysis of [^{18}F]4F-Gln metabolites

After 4 doses of CB839 or VEH treatment, mice were injected with [^{18}F]4F-Gln (same activity as for imaging –see below). One hour after injection, the tumor was harvested and the blood was collected via cardiac puncture. All samples were kept on ice during processing. The plasma was obtained by centrifuging the blood (3000 *g* for 10 minutes at 4°C) and was mixed with 1 mL acetonitrile (ACN). The mixture was vortexed thoroughly and then followed by another round of centrifugation (3000 *g* for 10 min at 4°C). The supernatant and the pellet were separated and counted with a gamma counter (2480 series, Perkin Elmer, Waltham, MA) to determine the extraction efficiency. Part of the supernatant (> 200 μL) was then filtered with a 0.45 μm filter and 200 μL of the filtered supernatant was injected into an analytical metabolite HPLC (1200 series, Agilent Technologies, Santa Clara, CA) equipped with an Astec[®] CHIROBIOTIC[®] T Chiral HPLC column (5 μm , 25 cm $\times$ 4.6 mm, Sigma-Aldrich, St. Louis, MO) and a fraction collector (Waters, Milford, MA). The mobile phase consisted of 80:20 methanol:water (v/v) and the flow rate was 1 mL/min. A fraction was collected every 30 seconds and counted with the Perkin Elmer gamma counter. The activity in each fraction was then plotted against the fraction number. The areas under the curve from each peak were summed to determine the percentage of activity from the parent ligand ([^{18}F]4F-Gln) vs. metabolite(s). Fractions #9 to #14 represented the parent ligand ([^{18}F]4F-Gln) as validated by HPLC analysis of the cold compound; all other peaks were considered metabolites. Our HPLC method can separate [^{18}F]4F-Gln versus [^{18}F]4F-glutamate (Supplemental Information or SI, Fig 1). The latter was part of the "metabolites" fraction reported in our study.

For HPLC analysis of tumor tissue, the tumor tissue was first homogenized in 1 mL ice-cold PBS. Subsequently, 1 mL ice-cold denaturing solution (3:1 ACN: water v:v) was added to denature the proteins and the mixture was vortexed. The supernatant and pellet were separated by centrifugation (3000 *g* for 10 min at 4°C) and were counted. The filtration of supernatant and HPLC analyses followed the same protocol for plasma as detailed above.

In vivo PET studies and image analysis

Each mouse underwent pre- and post-treatment PET scans. PET studies were performed on a dedicated small animal scanner (A-PET). Technical details of the A-PET are similar to the commercial Mosaic HP scanner from Philips Healthcare (Cleveland, OH) as described before (24). Briefly, the scanner uses 2 mm $\times$ 2 mm $\times$ 10 mm lutetium (yttrium) orthosilicate (LYSO) crystals with 2.3-mm pitch, resulting in a transverse spatial resolution of 2.3 mm and axial spatial resolution of 2.4 mm. The detector diameter is 19.7 cm with a length of 11.6 cm. The latter allows the field-of-view to encompass the whole mouse, including the tail. The system is fully 3D, without interplane septa. Images were reconstructed with the 3D row-action maximum likelihood algorithm (3D RAMLA) (25) into 1 mm³ isotropic resolution. Random subtraction and decay correction were applied; scatter and attenuation correction were not used. During imaging, the mouse was sedated using 1L/min oxygen containing 1.5% isoflurane via a nose cone and prone on a heating pad.

Dynamic PET scans were used for a pilot study - to determine the optimal imaging time for uptake measures for subsequent studies. Each scan was started immediately after tracer

(300–350 μCi) injection with a temporal resolution of 5 min/frame $\times$ 12 frames for one hour of imaging. PET images were analyzed using AMIDE software (<http://amide.sourceforge.net/>). To measure blood activity, an ROI of fixed size ($2 \times 2 \times 2 \text{ mm}^3$ box) was placed on the anatomical location of the heart, noting that tracer accumulation in myocardium did not exceed blood pool background. For tumors, a single spherical ROI with a volume of $\frac{1}{8}$ (12.5%) the size of the tumor volume as measured by calipers prior to the PET scan was centered on the tumor area with maximal uptake. This approach is akin to the “peak” ROI used in clinical PET analysis (26). Other single ROI methods ($\frac{1}{4}$ and $\frac{1}{2}$ of caliper-based tumor volume) were also examined as detailed in the SI. In such cases, the ROI often covered portions of tumor with low tracer uptake (non-viable or cystic tumor). Time-activity curves (TACs) of the blood and tumor were constructed as T/B or %ID/g, calculated from the Eq. (1), where WM_ROI was the ROI enclosing the whole mouse.

$$\% \text{ID/g} = \frac{\text{tumor_ROI_mean_counts} \times \text{tumor_ROI_volume}}{[\text{WM_ROI_mean_counts} \times \text{WM_ROI_volume}]} \times \frac{1}{\text{tumor_ROI_volume} \times 1 \text{ g/cm}^3} \times \frac{1000 \text{ mm}^3}{\text{cm}^3} \times 100\%$$

(1)

TACs of T/B revealed a time window of 30–45 min after injection when the T/B reached equilibrium. In subsequent studies using CB839, a static 15-minute PET scan protocol, starting at 30 min after [^{18}F]4F-Gln injection, was applied and T/B values estimated from static PET images were used to estimate tumor Gln pool size. The post-treatment scan was timed at 4 h after the last gavage of CB839 to ensure the absorption of the drug.

Since the whole tumor homogenate was used for MRS-based Gln pool size measurement (see section below), we also estimated T/B value from the entire tumor using a multiROI approach in which planar ROIs were manually defined on each image slice to cover the whole tumor (detailed procedure described in SI).

^1H MR spectroscopy quantification of tumor glutamine pool size from tumor extracts

Gln concentration in cell, tumor, and blood samples was estimated by ^1H magnetic resonance spectroscopy (MRS) of aqueous metabolite extraction. After HCC1806 or MCF-7 cells were incubated with 1 μM CB839 or 0.05% DMSO in culture media for 24 h, cell pellet (from $\sim 10^7$ cells) was collected by centrifuge (1000 g for 10 min). Methanol:water (1:1 v:v total 500 μL per pellet) was added and metabolites were extracted in the aqueous phase (27). The protein content of the cell pellet was estimated by the Lowry method. The extracted sample was then lyophilized and prepared for MRS analysis in the same manner as for tumors described below.

After post-treatment PET imaging, the whole tumor was harvested and freeze-clamped between a pair of aluminum tongs which were pre-cooled in liquid nitrogen and stored at -80°C until extraction. The blood was collected via cardiac puncture under deep anesthesia. Plasma was separated from blood and was extracted in a similar fashion as cells. To extract soluble metabolites from the tumor, the sample was first weighed (the weight ranges from

0.14–0.74 g) and then pulverized in liquid nitrogen using a suitable size mortar/pestle. Perchloric acid (PCA, 6.6%) was added at 3 mL per gram of tissue to the pulverized sample, which was thawed and homogenized followed by centrifugation (11,000 g for 20 min at 4°C). The pH of the supernatant was adjusted to 7.0 ± 0.2 , using KOH and PCA in graded concentrations. The precipitated potassium perchlorate was removed by centrifugation (1000 g for 10 min at 4°C). The sample was then lyophilized and stored at -80°C freezer.

For high resolution ^1H MRS, the lyophilized powder was dissolved in 0.5 mL D_2O and filtered into a 5 mm (diameter) NMR tube. A glass capillary containing trimethylsilyl propanoic acid (TMSP) reference solution was calibrated inside the NMR tube to be 0.408 mM. ^1H MRS was performed on a 400 MHz vertical bore spectrometer (Avance DMX 400, Bruker Instruments Inc., Germany) using a 5-mm radio-frequency coil. ^1H MR spectra were analyzed by NUTS® software (Acorn NMR, Livermore, CA) with the reference peak set at 0 ppm. The integration of a metabolite peak(s) in an MR spectrum is proportional to its concentration. To determine the Gln concentration in the NMR tube, the integrated Gln-H4 resonance peaks (at 2.48–2.42 ppm) relative to the reference peak was multiplied by 0.408 mM. Multiplying the Gln concentration by sample volume (0.5 mL) gave the amount of Gln in the sample. The amount of Gln was normalized to the tissue wet weight, total protein in the cell pellet, and plasma volume, yielding Gln concentration in the tumor tissue ($\mu\text{mole}/\text{gram}$), tumor cells ($\mu\text{mole}/\text{gram}$ of protein) and plasma ($\mu\text{mole}/\text{mL}$), respectively. In spectra obtained from the 400 MHz MR spectrometer, there was no overlap of Gln-H4 peaks with glutamate-H4 peaks or with other metabolites at the detection sensitivity of MRS, assuring no contamination in the Gln pool size estimation.

Data are presented as mean $\pm$ SEM unless otherwise noted. Statistical tests were performed using GraphPad Prism 6. Two-sided student t-tests were used to evaluate significance at the $\alpha = 0.05$ level. To examine correlation, the squared Pearson correlation coefficient (R^2) was calculated in GraphPad Prism 6 or Excel.

Results

Change of Gln concentration (pool size) in cells, tumors and plasma in response to GLS blockade

^1H MRS quantification revealed that after a short course (2–3 days) of CB839 treatment, Gln concentration in TNBC (HCC1806) tumor extracts increased from 0.31 ± 0.10 $\mu\text{mole}/\text{gram}$ without GLS inhibition to 1.35 ± 0.20 $\mu\text{mole}/\text{gram}$ after CB839 treatment, a 4.3-fold increase ($P = 0.007$, Fig 1A). The invasive nature of the MRS assay did not allow pre- versus post-treatment comparison; instead, samples from CB839-treated mice were compared with VEH-treated ones as controls (without *GLS* inhibition). This increase of cellular Gln pool is the most immediate metabolic consequence of *GLS* inhibition (Fig 1E). In HCC1806 cells taken from culture, the increase of cellular Gln pool size induced by *GLS* inhibitor was even more dramatic (>20 -fold, Fig 1B). In contrast, the MCF-7 tumors exhibited a higher Gln concentration without *GLS* inhibition (3.08 ± 0.390 $\mu\text{mole}/\text{gram}$) and a moderate increase afterwards (3.90 ± 0.23 $\mu\text{mole}/\text{gram}$, $P > 0.05$, Fig 1C). After CB839 treatment, the plasma Gln concentration were similar in TNBC or MCF-7 bearing mice (both were the same strain), therefore, the data were pooled into CB839 and VEH groups without discriminating

tumor models: the plasma Gln concentration was 0.62 ± 0.04 mM after CB839 compared to 0.25 ± 0.02 mM after VEH treatment (~ 2.5 -fold increase) (Fig 1D, $P < 0.001$).

Cellular uptake of [^{18}F]4F-Gln in response to GLS blockade

The initial uptake (up to 30 min) of [^{18}F]4F-Gln into TNBC and MCF-7 cells was similar in the presence of CB839 versus DMSO, suggesting that the Gln transporter activities were not blocked by *GLS* inhibitor. For the HCC1806 cells, tracer uptake plateaued at a higher level after *GLS* inhibition compared to DMSO-treated cells ($P < 0.001$): the tracer uptake in CB839-treated cells was > 3 -fold of VEH-treated cells at 60 min, and > 2 -fold at 120 min (both $P < 0.0001$) (Fig 2A). The reduced [^{18}F]4F-Gln activity in DMSO-treated HCC1806 cells at later time points (60–120 min) was consistent with tracer efflux corresponding to a small Gln pool size due to *GLS* activity (Fig 1B). Compared to HCC1806 cells, [^{18}F]4F-Gln uptake in MCF-7 cells were higher without *GLS* inhibition, and CB839 treatment induced a much smaller increase in tracer accumulation (Fig 2B).

Lack of significant metabolic conversion of [^{18}F]4F-Gln in tumors and blood

Centrifugation of tumor homogenates led to 90 ± 1 % ($n=12$) of the activity in the supernatant while 10 ± 1 % ($n=12$) in the pellet, suggesting minimal tracer incorporation into macromolecules. We compared different methods in our pilot studies to denature and precipitate proteins: ACN/water (used here) or ACN combined with trichloroacetic acid (10%), and the results (% activity in pellet) were almost identical (data not shown), suggesting that ACN/water can efficiently denature proteins to release small molecules while it does not require adjusting pH of the supernatant. HPLC analysis of supernatant revealed the parent ligand was the dominant species accounting for 85% of the total activity, similar in both tumor type and treatment groups, while 9–14% of activity was summed from other peaks in the HPLC profile (Fig 3A).

Total blood activity was divided between RBC (37 ± 4 %, $n=6$) and plasma (63 ± 4 %, $n=6$). In plasma samples, 25 ± 4 % ($n=6$) of the activity was found in the pellet while 75 ± 4 % ($n=6$) was found in the supernatant. HPLC analysis of the supernatant suggested that the majority (82–87%) of activity was from the parent ligand and a small fraction (16%) was from metabolites (Fig 3B). Findings in Fig 3 confirmed that [^{18}F]4F-Gln was the primary species in the tumor and blood. The modest amount of labeled metabolites seen in the blood, and an even smaller average metabolite fraction in the tumor, suggests that the contribution of labeled metabolites to the tumor PET signal was small and unlikely to have a significant influence on image-derived measures such as the T/B ratio. This should be further addressed in future studies examining kinetics of [^{18}F]4F-Gln uptake in tumors.

In vivo [^{18}F]4F-Gln PET detects tumors with distinct Gln pool size

In our pilot study (described in Fig 4), we treated TNBC model with a commercially available *GLS* inhibitor, BPTES, or VEH ($n=3$ for each group) and performed 1-hour dynamic scans. In a BPTES treated mouse, a clear difference in TAC of the tumor relative to the blood at baseline (Fig 4A) versus post treatment was observed (Fig 4B). T/B ratios increased post-treatment with BPTES (Fig 4C), but were decreased in a VEH-treated mouse compared to pre-treatment (Fig 4D). Complete pre and post-treatment [^{18}F]4F-Gln PET data

sets were obtained from two (out of 3) BPTES-treated and one (out of 3) VEH-treated mice. From these data sets, we were able to identify a time window (30–45 min post tracer injection) during which tumor uptake and blood clearance of the tracer reached approximate steady state. T/B values obtained from this window (between the red lines in Fig 4B) revealed a 51% increase over baseline in one and 8% increase in the other BPTES-treated mouse, whereas an 18% *decrease* was observed in the VEH-treated mouse.

Visual assessment of the images agreed with numerical values: HCC1806 tumors (arrows) were less visible in baseline PET images, but showed more prominent uptake after BPTES treatment (Fig 4E, both images were windowed at 10% ID/g- detailed in Supplemental Methods). Robust FDG uptake was observed in both pre- and post-BPTES PET images (Fig 4F), suggesting FDG is not a PD marker of *GLS* inhibition, as expected. This pilot study was used to devise a short static PET scan protocol (15 min) for the subsequent studies described below.

To further test the utility of [¹⁸F]4F-Gln PET for detecting changes of Gln pool size, we treated the mice with CB839, a potent *GLS* inhibitor currently being evaluated in clinical trials. After a short treatment course (2–3 days), mice underwent a 15-min PET scan starting at 30 min after tracer injection. In TNBC-bearing mice, while T/B values exhibited some variation at baseline, T/B values increased in *all* CB839-treated mice ($P < 0.05$ using two-tailed paired t-test) and decreased in 3 out of 4 VEH-treated mice (Fig 5A **left panel**). In receptor-positive MCF-7 tumors, pre-treatment T/B values were higher compared to TNBC tumors ($P < 0.05$, t-test of the mean without assumption of equal variance), and changes in T/B values after CB839 vs. VEH treatment were not significant (Fig 5A **right**). Similar to BPTES treatment, HCC1806 tumors exhibited enhanced [¹⁸F]4F-Gln PET signal on the post-CB839 images compared to pre-treatment (Fig 5B **upper**). In contrast, MCF-7 tumors showed strong [¹⁸F]4F-Gln PET signals on both pre- and post-CB839 treatment images (Fig 5B **bottom**). In TNBC tumors, T/B value increased $34 \pm 7\%$ after CB839 treatment in comparison to an $11 (\pm 12)\%$ decrease after VEH treatment ($P < 0.05$, t-test of the mean percent change, without assumption of equal variance, Fig 5C **left**). Notably, the increase of T/B induced by CB839 treatment was accompanied by a $6 (\pm 7)\%$ decrease in tumor size, whereas the T/B decrease after VEH-treatment was accompanied by a $35 (\pm 20)\%$ increase in tumor size (Fig 5C **right**), suggesting that the changes in T/B after CB839 or VEH treatment were not due to partial volume effect resulting from small tumor size or change in tumor size after treatment.

We next examined the correlation between T/B values obtained from post-treatment PET images vs. Gln pool size measured from the same tumors (which were harvested after imaging). We identified a strong correlation (Fig 5D, $R^2 = 0.71$, $P = 0.0002$), where T/B values obtained using the multiROI method (in SI). When T/B values from single ROI ($1/8$ of caliper size) method were used, R^2 was 0.44. This suggests that the multiROI method might better account for the heterogeneity of the tumor, leading to a stronger correlation, noting that MRS-based Gln pool size estimates used extracts from homogenates of the entire tumor.

To evaluate whether the image analysis methods were robust to different readers or the usage of different sized tumor ROIs, we compared T/B values obtained by two readers and from

various ROI methods ($\frac{1}{8}$, $\frac{1}{4}$ and $\frac{1}{2}$ of caliper size or multiROI). In the single ROI methods, the ROI size was proportional to caliper-based tumor volume. This approach reduced the impact of tumor size variations on T/B measurements and appeared to be robust to different readers or ROI size; the T/B values obtained from the two readers were highly correlated ($R^2 = 0.99$, SI Fig 2). Strong correlations were also observed between single ROI of different sizes ($\frac{1}{8}$ versus $\frac{1}{4}$ or $\frac{1}{2}$ of caliper size) ($R^2 = 0.94$, SI Fig 3). The correlation between the single ROI ($\frac{1}{8}$ caliper) versus multiROI method was still strong, but slightly lower ($R^2 = 0.80$, SI Fig 4). As the latter covered the entire tumor, it likely averaged across the heterogeneous PET signals from portions of the tumor that were viable, cystic, or necrotic; such heterogeneity was most notable in TNBC model and visualized in the PET images (e.g., Fig 5B upper panel).

Since T/B index represents an approximation of tracer's cellular distribution volume fraction (DV), we also estimated DV by analyzing the dynamic PET images (SI Fig 5A) with the Logan graphical method and 2-compartment modeling (SI Fig 5B–C). The late linearity revealed from Logan plot (SI Fig 5B) and the small rate constant k_3 (0.008/min derived from modeling fit in SI Fig 5D) indicated minimal tracer trapping and nearly completely reversible tracer exchange between blood and tissue. This was consistent with the small fraction of activity (10%) trapped in macromolecules in the pellet of ACN-extracted tissues, and supported our hypothesis that [^{18}F]4F-Gln DV traces the cellular Gln pool. Preliminary analysis using Logan analysis showed expected changes in DV after glutaminase inhibition (28). This approach will provide a theoretical frame for using T/B as an index of the cellular Gln pool size, since the static imaging using the T/B ratio is a clinically feasible procedure.

Discussion

TNBC represents an aggressive subtype of breast cancer. However, unlike other subtypes of breast cancer that express specific receptors such as the ER or HER2, TNBC lacks a specific target for subtype-specific therapy (29). Preclinical studies of GLS inhibitors support that inhibition of glutamine metabolism might provide a therapeutic strategy for TNBC since some TNBC exhibit high levels of *GLS* activity combined with low expression/activity of glutamine synthetase (*GLUL*) (30,31). We sought to develop a non-invasive imaging marker to identify glutaminolytic breast cancers and to measure the impact of *GLS*-targeted therapy. The overarching goal is to develop markers to help guide clinical trials and inform clinical use of glutamine-targeted drugs. In this early study, our strategy was to measure cellular glutamine pool size as an inverse indicator of glutaminolysis. We first demonstrated that a short course of CB839 treatment led to a significant increase of cellular Gln pool size in TNBC tumors and cultured cells (Fig 1A–B), suggesting that an *in vivo* measure of cellular Gln pool size could be used to assess the PD effect of *GLS* inhibitors. The increase of Gln pool size in TNBC tumors after *GLS* inhibition cannot be explained by the elevated plasma Gln level following administration of the *GLS* inhibitors since (1) the increase of tumor Gln concentration (> 4-fold) exceeded the increase in plasma concentration, (2) despite similar plasma Gln level increases, Gln concentration did not increase significantly in the MCF-7 tumors, and (3) in TNBC cells cultured in a standard Gln concentration, a significant increase in cellular Gln concentration was observed after CB839 treatment (Fig 1B).

We then tested a glutamine analog, [^{18}F]4F-Gln, as a PET probe to measure cellular Gln pool size *in vivo*. For this agent to be able to trace the cellular Gln pool size, it must meet two requirements: (1) It enters and leaves the cell via Gln transporters. (2) Once in the cell, it remains as the parent ligand (i.e., it is not metabolized). Competitive inhibition studies performed in previous studies indicated that [^{18}F]4F-Gln is transported by cellular Gln transporters including ASCT2 (SLC1A5) (20). Minimal metabolism had been shown earlier (19,21) and was confirmed in this study. Additional data from our study showed that the majority (85%) of the radioactivity in both tumor types was from [^{18}F]4F-Gln, and this fraction was not affected by *GLS* inhibitor treatment. Furthermore, the small fraction (10%) of radioactivity trapped in the macromolecules (pellet) and the late washout of radioactive label from cultured TNBC cells suggest nearly completely reversible transport. These properties of [^{18}F]4F-Gln make it a suitable probe for tracing cellular Gln pool size.

We chose two breast cancer models that exhibit distinct levels of glutaminolysis, specifically, in the activity of the rate-limiting enzyme *GLS*: TNBC (HCC1806) was shown to have high *GLS* activity whereas ER+ (MCF-7) has low *GLS* activity. Results from these two models (Fig 1, 2 and 5) consistently diverged along this metabolic signature, both at baseline and after treatment with *GLS* inhibitors. *In vivo* [^{18}F]4F-Gln PET results indicate that the tumor-to-blood-activity ratio, T/B, can serve as an index of cellular Gln pool size. This index provides a marker for *in vivo* *GLS* activity and for the PD effect of *GLS* inhibitors: The high *GLS* activity in untreated TNBC tumors is associated with a small cellular Gln pool size. Thus, [^{18}F]4F-Gln competed with a small pool of *native* Gln for efflux, resulting in low tracer retention and low pre-treatment T/B value. With the increase in cellular Gln pool after *GLS* inhibition, [^{18}F]4F-Gln competed with a larger pool of native Gln molecules for efflux, leading to greater retention of the radioactive tracer (and greater T/B values). Conversely, tumors with low *GLS* activity have larger Gln cellular pools at baseline – and thus higher [^{18}F]4F-Gln T/B – with minimal impact of *GLS* inhibition on probe retention.

Quantitative comparison of *in vivo* PET imaging results to MRS assay of tumor extracts provided further support for our proposed method. Comparison of post-therapy T/B values and the Gln pool sizes in both tumor models revealed a good correlation ($R^2 = 0.71$, Fig 5D). Interestingly, *GLS* inhibition in TNBC induced a 1.3-fold increase of Gln pool size measured by T/B, whereas the actual Gln pool size measured by MRS increased by 4.3-fold. The underestimation of Gln pool size by T/B can be explained by competition of plasma Gln with [^{18}F]4F-Gln for cell uptake, resulting in lower T/B values when plasma Gln level is elevated. With the 2.5-fold increase in plasma Gln, the increase in T/B implies an overall $1.3 \times 2.5 = 3.3$ -fold increase in Gln pool size as estimated by imaging. This compares well to the 4.3-fold actual increase in Gln pool size.

Some limitations of this study include examination of a limited number of breast cancer subtypes and reliance on primarily static imaging uptake measures. Future studies should examine a wider range of breast cancer subtypes as well as other glutaminolytic cancers, and validate the T/B marker with kinetic analysis of [^{18}F]4F-Gln uptake. Of note, in considering clinical translation of this method, tumors with high *GLS* activities exhibited low [^{18}F]4F-Gln PET signals (Fig4E and 5B) and as such, might be mistaken for an absence of tumor.

However, a robust FDG uptake was observed in the TNBC model (Fig 4F) as well as in TNBC patients (32). Therefore, a paired FDG PET scan could guide interpretation of [^{18}F]4F-Gln PET.

Taken together, the current study provides a foundation for future studies to explore the utility of [^{18}F]4F-Gln PET in human breast cancers and other tumor types. These early results support the need for additional studies that examine the kinetics of [^{18}F]4F-Gln and the T/B index in response to a range of doses and exposure periods of *GLS* inhibitors. Such studies could firmly establish [^{18}F]4F-Gln PET derived tracer uptake measures as predictive and PD markers for *GLS*-targeted therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Tumor tissue and cellular glutamine concentration after CB839 or Vehicle treatment (A) Gln concentration (µmole/gram of tissue wet weight) in TNBC tumors after 4–6 doses of CB839 (n=4) or VEH (n=4) treatment. (B) Gln concentration (µmole/gram of protein) in HCC1806 cells after 24 h incubation with culture media containing 1µM CB839 or VEH (n=2 for each group). (C) Gln concentration in MCF-7 tumors after 4–6 doses of CB839 (n=4) or VEH (n=3) treatment (P=0.07). (D) Plasma Gln concentration in CB839 (n=4) or VEH (n=4) treated mice (pooled from mice bearing HCC1806 or MCF-7 tumors). NS= not significant. (E) Diagram of cancer-specific glutaminolysis pathway (grey arrow), in which the first and rate-limiting step catalyzed by *GLS* is inhibited by small molecule drugs including BPTES and CB839.

Figure 2. Cell uptake of [^{18}F]4F-Gln tracer

(A) Tracer uptake in HCC1806 cells which were incubated for 4 h with CB839 (n=4 for each time point) or VEH (n=4 for each time point). (B) Tracer uptake in MCF-7 cells (the same sample size as in A). CB839 (black) or VEH (white). * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$

Figure 3. Lack of significant metabolic conversion of [¹⁸F]4F-Gln in the tumor and blood
 (A) The fraction of radioactivity in parent ligand vs. metabolites in the supernatant of protein-denatured homogenates of tumor tissue (A, n=3 for each group), and in the supernatant of protein-denatured plasma samples (B, n=3 for each group).

Figure 4. Tumor-to-blood-ratio (T/B) time course in mice bearing TNBC tumors treated with GLS inhibitor BPTES

(A) Activity-time-course (TAC) of the blood and tumor from a single mouse at baseline and (B) after 6 daily treatments of BPTES. (C–D) T/B time course obtained from pre- and post-treatment PET images were plotted for the same BPTES-treated mouse as in (A–B) and a VEH-treated mouse. Note the relative position of pre- and post-treatment T/B time courses were reversed in BPTES compared to VEH treated mouse. (E) PET images summed from 30–45 min of dynamic scan and registered to the similar anatomical plane revealed the markedly enhanced conspicuity of the tumor after BPTES treatment. (F) Robust FDG uptake at baseline and post-BPTES treatment. Arrows in E and F point to tumors. Red lines in B defined a time period (30–45 min) when the tracer uptake and clearance became steady state. In this pilot study, each group was started with $n=3$ mice; complete data sets (baseline and post-treatment) were obtained from 2 mice in BPTES group and 1 mouse in VEH group.

Figure 5. T/B estimated from [^{18}F]4F-Gln PET tracks tumor Gln pool and its change induced by CB839

(A) Pre and post-treatment T/B values from TNBC and MCF-7 tumors reveal their differential responses to *GLS* inhibition ($n=4$ for each group). (B) PET images are shown for a mouse bearing HCC1806 and one bearing MCF-7 tumor scanned pre- and post-CB839 treatment. Note a maximal color scale of 12%ID/g used for MCF-7 tumors while 10%ID/g for TNBC tumor (detailed in Supplemental Methods). (C) Percent change of T/B and tumor size from pre- to post-treatment (Group size is the same as in A). (D) Correlation between post-treatment T/B values (y-axis) versus tumor Gln concentrations (x-axis) ($R^2=0.71$, $P=0.0002$). Fourteen out of 16 tumors imaged in (A) were harvested after PET imaging for MRS measurement. T/B values were obtained from multiROI method described in SI.