

$[^{64/67}\text{Cu}]$ -FAP theranostics in combination with immunotherapy in a preclinical model of TNBC

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Abstract

Rationale:

Fibroblast activation protein (FAP) is a prominent transmembrane protein in the tumor microenvironment (TME) that promotes tumorigenesis. FAP has been a recent target for radionuclide-conjugated peptides for PET imaging and targeted radionuclide therapy (TRT). The objective of this study is to develop a theranostic approach using [$^{64/67}\text{Cu}$]-FAP2287 in combination with immunotherapy (IMT) to treat triple-negative breast cancer (TNBC).

Methods

4T1 tumor-bearing BALB/c mice (N = 20) were injected and imaged with 100 μCi [^{64}Cu]-FAP2287 PET at 1, 4, and 24h to quantify baseline FAP expression followed by autoradiography. 4T1-tumor bearing BALB/c mice (N = 42) were divided into Control, IMT, 800 μCi [^{67}Cu]-FAP2287 or 4)[^{67}Cu]-FAP2287 + IMT and 1600 μCi [^{67}Cu]-FAP2287, or [^{67}Cu]-FAP2287 + IMT (N = 5–10/group) and imaged with [^{64}Cu]-FAP2287 PET followed by treatment. All [^{67}Cu]-FAP2287 groups were imaged with SPECT at 4, 24, and 48h. Tumor growth and survival rates were monitored. Standard uptake values (SUV) quantified, statistical analysis and probability of survival were done with significance at $p < 0.05$.

Results

At baseline, there was a strong positive correlation between [^{64}Cu]-FAP2287 PET SUV_{Mean} and autoradiography ($R^2 = 0.7806, p < 0.001$). No changes in [^{67}Cu]-FAP2287 retention over time was observed following the IMT. [^{64}Cu]-FAP2287 SUV_{Mean} proved to be a reliable predictor of survival with 800 μCi ($R^2 = 0.84, p = 0.03$) and 1600 μCi ($R^2 = 0.62, p = 0.01$). FAP-TRT increased survival compared to controls and IMT. 1600 μCi [^{67}Cu]-FAP2287 + IMT significantly decreased tumor volume ($p = 0.0003$) and increased survival ($p = 0.0005$) compared to [^{67}Cu]-FAP2287.

Conclusion

We can noninvasively target and predict response to FAP radiolabeled with elementally paired theranostic probe, [$^{64/67}\text{Cu}$]-FAP2287 in TNBC. IMT synergistically increased response to FAP-TRT and can guide personalized approaches to improve treatment efficacy.

INTRODUCTION

Triple-negative breast cancer (TNBC) represents about 15–20% of breast cancer and does not overexpress the hormone receptors estrogen (ER), progesterone (PR) and human epidermal growth factor (HER2) receptors and is one of the leading causes of cancer related deaths in females (1). 2.3 million breast cancer cases are reported every year and 15–20% of those reported cases are categorized as TNBC (2–4). With no overexpression of targetable cell membrane proteins, the standard-of-care for TNBC includes chemotherapy, radiation, and more recently, immunotherapy (IMT) is being incorporated at both early and late-stage disease (5–9). Despite inducing increases in T cell activation and infiltration (10–13), IMT only improves the prognosis in a portion of TNBC patients due to the ever-evolving tumor microenvironment (TME) (14). In particular, the TNBC TME is composed of several pro- and anti-tumorigenic factors which contribute to therapeutic response including stromal cells, blood vessels, tumor-associated macrophages, extracellular matrix (ECM), and cancer associated fibroblasts (CAFs) (15–18). CAFs play a pivotal role in cancer metastasis, ECM remodeling, therapeutic resistance, angiogenesis, decreased immunogenicity, and increased mesenchymal transition (19, 20). CAFs and tumor cells with high mesenchymal transition express fibroblast activation protein (FAP), a type II membrane-bound glycoprotein belonging to the dipeptidyl peptidase family (21). Studies have previously demonstrated that high FAP expression is correlated with increased local tumor invasion, increased risk of lymph node metastases, and decreased survival of patients with several types of cancer (22). As there is a clinical need to create new combination strategies for treatment, clinical and pre-clinical studies have explored targeting FAP for targeted radionuclide therapy (TRT) to treat several types of solid tumors that are hard to treat with standard-of-care (23).

TRT induces double-stranded DNA breaks causing immunogenic cell death (ICD) but can also lead to immunomodulation and increased infiltration of immune cells. Others have demonstrated that radiation, whether through external beam or TRT, can increase response to IMT across various tumor types (24–27). Theranostic pairs for FAP TRT have been primarily developed using radionuclides such as [^{68}Ga , ^{18}F] for diagnosis and [^{177}Lu] for therapy. However, these radionuclides have inherently different half-lives, chemistry, and chelators which can significantly influence the pharmacological behavior of the radiotracer, resulting in different distribution profiles (28, 29). For personalized TRT strategies, pre-treatment dosimetry studies are performed with the diagnostic radionuclide of the theranostic pair to optimize injected activity and predict the therapeutic absorbed doses, distribution, accumulation, metabolism, elimination and decay rates in tissues of interest. It has been shown that both the chelator and the isotope alter the distribution when using the same targeting probe (30–32). Elementally paired isotopes, such as [^{64}Cu] and [^{67}Cu], have similar chemistry and pharmacokinetic profiles, which could address the limitations of the current gold standard [$^{68}\text{Ga}/^{177}\text{Lu}$] in theranostic applications. [^{64}Cu], with a half-life of 12.7 h, can be used for diagnostic imaging via positron emission tomography (PET), while [^{67}Cu], with a half-life of 2.58 days, decays by β^- and γ -rays which can be used for therapy and imaged using single photon emission computed tomography (SPECT) imaging (33).

The objective of this study is to develop a theranostic approach to establish FAP as a targeting molecule for treating TNBC in syngeneic mouse models of disease that have intact immune systems, validate

[^{64/67}Cu] radionuclide pair targeting FAP in TNBC, and evaluate its synergistic effects with IMT. Evaluating the relationship of chemically-paired theranostic approaches targeting FAP and optimizing radiopharmaceutical development has an opportunity to improve patient care, increasing both therapeutic effectiveness and predictive capabilities on a personalized basis [30]. FAP2287 is a murine surrogate to FAP2286, a clinically relevant FAP peptide used in theranostic clinical trials (e.g. NCT04939610, NCT06880757, NCT04621435). FAP2287 is a cyclic peptide proven to have sensitivity to FAP with improved properties such as binding affinity and plasma stability to mouse (34, 35). We hypothesize that [⁶⁴Cu] radiolabeled with FAP2287 will be predictive of treatment outcomes to [⁶⁷Cu]-FAP2287 treatment and that the addition of IMT after FAP-TRT will increase therapeutic response. Our study represents some of the first work looking at Cu radiolabeling of FAP2287, chemically paired [^{64/67}Cu] for FAP theranostics, and combining IMT with FAP-TRT for the treatment of TNBC. Overall, our study aims to investigate novel elementally matched theranostic agents, modulate the tumor microenvironment to optimize combination targeted treatments, and improve overall survival in TNBC preclinical models.

METHODOLOGY

Cell Culture and Tumor Model Development

4T1 (mouse TNBC cell line syngeneic to BLAB/c mice) cells were acquired from ATCC (catalog number: CRL-2539-LUC2) and cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Fisher, 11-875-119) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. E0771 (mouse TNBC cell line syngeneic to C57BL/6 mice), TS/A (mouse ER+ cell line syngeneic to BALB/c mice) and TUBO (rat HER2+/neu cell line syngeneic to BALB/c mice) purchased from ATCC (Manassas, VA), syngeneic breast cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% FBS, 1% Sodium Pyruvate and 1% L-Glutamine. All cells were maintained at 37°C and 5% CO₂. Cells were maintained at passage numbers below 25 at a confluency of 75–80%. Cells were evaluated for FAP expression via western blot. Female 6- to 12-week-old BALB/c mice (Charles River Laboratories) were injected with 2x10⁵ 4T1 cells suspended in phosphate buffer saline in the right 3rd mammary fat pad for development of an orthotopic model of TNBC. Tumor volumes of 45-140mm³ (9 days post inoculation) were enrolled in the study. Mice (N = 42) were randomly divided into 6 treatment groups: namely, 1) Control (N = 6), 2) IMT (N = 6), 3) 800μCi [⁶⁷Cu]-FAP2287 (N = 5), 4) 800μCi [⁶⁷Cu]-FAP2287 + IMT (N = 6), 5) 1600μCi [⁶⁷Cu]-FAP2287 (N = 9), or 6) 1600μCi [⁶⁷Cu]-FAP2287 + IMT (N = 10). Animals were treated with one dose of 50μl [⁶⁷Cu]-FAP2287 via intravenous (IV) retro-orbital injection, either 100μl IMT (100μg Anti-CTLA4 (InVivoMab BE0164) +200μg Anti-PD1(InVivoMab, BE0146)), or 100μl saline via intraperitoneal (IP) injections on day 0, 3, 6 and 9. Tumors were measured every other day with calipers and tumor volume was calculated using the formula $\left(\frac{4}{3} * 3.14 * \left(\frac{l}{2}\right) * \left(\frac{b}{2}\right) * \left(\frac{l+b}{4}\right)\right)$, to monitor longitudinal changes. Mice were categorized into responders and non-responders based on tumor volume changes using the immune response evaluation criteria in solid tumors (iRECIST) with a 20%

decrease in tumor volume from baseline were considered responders. Endpoints for tumor-bearing animals include sustained tumor volume of 1500 mm³ or 20% loss of body weight.

Radiochemistry of Copper-64 and Copper-67 labeling of FAP2287

Copper-64 [⁶⁴Cu] was produced via the ⁶⁴Ni(p,n)⁶⁴Cu reaction. Nickel-64 (Isoflex, San Francisco, CA, USA) was electroplated into a 1mm thick gold backing and placed behind a 1mm aluminum degrader. The target was bombarded at 18MeV on the degrader (13MeV on target) and 40uA for 2–4 h. After the bombardment, the target was dissolved in 9M HCl, loaded on a 2.5g AG1x8 resin column, and washed with 15mL of 9M HCl. Co-produced cobalt-61 was eluted with 4mL of 4M HCl (36). ⁶⁴Cu was eluted with 5mL of 0.1M HCl, dried at 100°C under vacuum, and reconstituted in 0.1M HCl. Copper-67 [⁶⁷Cu] was purchased from The Idaho Accelerator Center, Idaho State University, Pocatello, ID.

4μg of FAP2287 (clovis Oncology) (in aqueous solution, 1mg/1mL) mixed with 50mM ammonium acetate (2:1, v/v) and 0.1M gentisic acid (1:1, v/v) and either 0.1mCi (37MBq) ⁶⁴Cu or 0.8mCi (296MBq) of [⁶⁷Cu] for 15min at 400 rpm at 95°C. The radiolabeling yield was assessed using thin layer chromatography (TLC) with 50mM DTPA solution and iTLC (glass microfiber chromatography paper impregnated with a silica gel, Agilent).

Biological validation of FAP expression in 4T1 TNBC model

In Vitro Western Blot Assessment

A range of syngeneic breast cancer cells subtypes (TNBC, ER+, HER2+) were cultured (4T1, TUBO, E0771 and TS/A) and lysed for 10 min with radioimmunoprecipitation assay (RIPA) buffer (Roche Applied Science, Indianapolis, IN, USA) on ice to isolate the proteins. Protein concentrations from all samples were determined using the Bicinchoninic Acid (BCA) Protein assay. Samples were run and probed using Abcam rabbit FAP primary antibody (EPR20021) 1:1000 overnight and with anti-rabbit secondary IgG antibody with the dilution of 1:1000 for 3 h (37). The film was developed for 30 min, and the bands were visualized using Fiji ImageJ software (public domain JAVA image processing program, National Institute of Health, Bethesda, MA). FAP expression of each cell line was normalized to β-actin expression and analyzed.

In Vivo [⁶⁴Cu]-FAP2287 PET for Imaging

4T1 FAP+ tumor bearing BALB/c mice (N = 20) were intravenously injected with 4μg/100μCi of [⁶⁴Cu]-FAP2287 and imaged at three different time points (1, 4, and 24 h) post-injection with pre-clinical small animal PET/CT (Sofie Biosciences GENXT, Dulles, VA) to optimize FAP2287 nuclear imaging. Anesthesia was maintained with 2% isoflurane in air, and body temperature was maintained at 36°C. 20-min static PET was acquired followed by a 5-min CT for anatomical reference. DICOMs with Bq/ml were converted

to standardized uptake values (SUV) using the formula

$$SUV = \frac{\text{concentration of the radiotracer} \times \text{body weight}}{\text{decay corrected dose}}$$
 (VivoQuant Version 5.2, Invivo, Needham). Regions of interest (ROIs) including the tumor and contralateral quadricep muscle were manually identified with CT and [⁶⁴Cu]-FAP2287 uptake were quantified for those regions using SUV_{mean}, SUV_{max}, SUV_{median}, SUV_{peak}, Tumor-to-muscle ratio (TMR) and histogram analysis.

Ex Vivo Autoradiography

Following [⁶⁴Cu]-FAP2287 PET imaging, (N = 20) mice were euthanized, and tumors were excised and sliced into 1mm sections. All the slices were placed on a cassette (GE Healthcare) along with standards (1nCi, 2nCi and 10nCi). A film was placed on a cassette and exposed for 3 hours in the dark. The film was then placed in an Amersham Typhoon Scanner, and a Phosphor image was obtained (sensitivity, 4000; pixel size, 50μm). Autoradiography scans were then calibrated and quantified (VQ Autoradiography Calibration Tool). ROIs of the tumor sections were semi-automatically generated using global thresholding and regional smoothing. Mean, max, and peak autoradiography concentrations were calculated for the combination of individual tumor sections (38).

Theranostic paired [Cu]-FAP2287 Nuclear Imaging

4T1 tumor bearing BALB/c mice were imaged with 100 μCi [⁶⁴Cu]-FAP2287 4 h post-injection as previously described. The following day, mice were administered with 800μCi or 1600μCi [⁶⁷Cu]-FAP2287 therapeutics and underwent SPECT images for 30 min followed by CT using (MILabs U-SPECT⁶, Netherlands) at 4, 24 and 48 h post-injection. A phantom scan with a known concentration at 0.8mm voxel with nine iterations and no post filtering (MILabs) was used to identify a calibration factor and SPECT images were reconstructed. The reconstructed images were then attenuation corrected with the CT. SPECT images were filtered through a gaussian filter of 0.6mm, decay corrected and converted from μCi/cc to SUV (VivoQuant). ROIs of tumor and muscle (background) were segmented with CT and [⁶⁷Cu]-FAP2287 uptakes per region were quantified. Mice weights were regularly taken and monitored for any physical changes.

Pilot investigation of metastasis following treatment with bioluminescence Imaging (BLI)

Mice with longstanding survival (> 75 days) following treatment protocols were injected with 100μl D-luciferin (115144-35-9, GoldBio, Olivette, MO) via intraperitoneal injection and a 5-min bioluminescence was acquired (IVIS Lumina III (Perkin Elmer, Waltham, MA). Quantification of total flux (p/s) was performed in primary tumor injection sites and visible areas with metastasis (Living Image, Waltham, MA). Mice were rechallenged with 2x10⁵ 4T1 tumors left 3rd mammary fat pad and the tumors were measured to assess tumor growth [38].

Statistical Analysis

Two-tailed unpaired t-test and one-way ANOVA were used to determine the differences in [^{64}Cu]-FAP2287 uptake between the two imaging groups to determine retention of radiotracer over time. Multiple t-tests with adjusted α values were used to evaluate the differences in tumor volume of different treatment groups over time. Kaplan Meier's estimator was used to analyze the probability of survival. Pearson's correlation was used to determine the correlation between SUV values and autoradiography values and correlation between the probability of survival and [^{64}Cu]-FAP2287 SUV. P values < 0.05 were considered significant. The Bliss test of synergy was used to identify the synergistic effects of the combination therapy (39, 40). Mice were categorized into responders and non-responders based on tumor volume changes using the immune response evaluation criteria in solid tumors (iRECIST) (41).

RESULTS

In vitro, in vivo and ex vivo biological validation of FAP expression in 4T1 TNBC model

Baseline protein expression of FAP in different syngeneic mouse breast cancer cell lines, including TS/A, 4T1, E0771 and TUBO was quantified (Supplemental Fig. 1A), showing that 4T1 (TNBC) tumor cells have increased FAP expression compared to the other syngeneic breast cancer cell lines, (Supplemental Fig. 1B).

The uptake and retention of [^{64}Cu]-FAP2287 in the tumor was evaluated using PET at 1, 4, and 24 h post-injection. Transverse images from the static scan (Fig. 1A), reveal that there is an uptake of radiotracer in the tumor, which is retained through 24 h. Qualitatively, there is heterogeneity in the spatial distribution of the radiotracer in the tumor both *in vivo* imaging and *ex vivo* autoradiography. *Ex vivo* autoradiography revealed increased retention in the peripheral regions compared to the core. Autoradiography showed a positive correlation to PET imaging (Fig. 1B), with SUV_{mean} values being positively correlated with the mean values from autoradiography ($R^2 = 0.7806$, $p < 0.001$).

Tumor uptake of chemically paired radioisotopes [Cu] conjugated with FAP2287

To understand the correlation of pharmacokinetics between ^{64}Cu and ^{67}Cu , 4T1 tumor bearing BALB/c mice were imaged with [^{64}Cu]-FAP2287 followed by [^{67}Cu]-FAP2287 SPECT within 24 hrs, as shown in the coronal images of [^{64}Cu]-FAP2287 PET and [^{67}Cu]-FAP2287 SPECT (Fig. 2A). These images demonstrate the heterogeneity of both radiotracer uptake in the tumor, with specific regions having high [^{64}Cu]-FAP2287 and [^{67}Cu]-FAP2287 uptake and [^{67}Cu]-FAP2287 retention over time. Using the average SUV_{mean} of the tumor across the cohort as a threshold, [^{64}Cu]-FAP2287 uptake per tumor was stratified into two groups demonstrating high and low uptake (Fig. 2B). When comparing the high and low [^{64}Cu]-

FAP2287 groups to its respective [^{67}Cu]-FAP2287 uptake, there was a significantly higher amount of [^{67}Cu]-FAP2287 in the high PET signal cohort compared to the low cohort ($p = 0.026$) (Fig. 2C).

[^{67}Cu]-FAP2287 in combination with IMT demonstrates synergistic response

[^{67}Cu]-FAP2287 SPECT images (Fig. 3A) across time points show an average of $51.33 \pm 16.53\%$ washout of the radiotracer from the tumor region by 48h. There was no significant differences [^{67}Cu]-FAP2287 retention measured by SUV changes, in 48h following the administration of IMT compared to tumors that did not receive IMT (Fig. 3B-C). Mice were imaged and treated following the timeline displayed in (Fig. 4A). IMT alone demonstrated no differences in tumor curves compared to saline control, confirming the tumor is not responsive to checkpoint inhibitors alone ($p > 0.05$). A tumor growth curve shows therapeutic response from both the doses (800/1600 μCi) of [^{67}Cu]-FAP2287 with and without the addition of IMT (Fig. 4B and D). Mice treated with 800 μCi [^{67}Cu] + IMT had significantly reduced tumor burden compared to the controls ($p = 0.002$) by day 17 post treatment (Fig. 4B). Mice treated with 1600 μCi [^{67}Cu]-FAP2287 compared to controls had significantly reduced tumor volumes ($p = 0.0004$) and combination group had significantly reduced tumor volumes compared to the controls ($p = 0.00025$), IMT ($p = 0.00066$), and [^{67}Cu]-FAP2287 ($p = 0.0014$) (Fig. 4D) by day 17 from the beginning of treatment. When comparing longitudinal survival on day 52, the 800 μCi [^{67}Cu]-FAP2287 group had a significantly increased probability of survival compared to the controls ($p < 0.01$). The combination group of 800 μCi [^{67}Cu]-FAP2287 plus IMT had significantly increased probability of survival compared to controls ($p < 0.001$) and IMT alone ($p = 0.04$) (Fig. 4C). The 1600 μCi [^{67}Cu]-FAP2287 group showed significantly higher probability of survival compared to control ($p = 0.0053$). The combination treatment cohort (1600 μCi [^{67}Cu]-FAP2287 plus IMT) had a significantly higher probability of survival compared to controls ($p < 0.0001$), IMT ($p < 0.01$) and [^{67}Cu]-FAP2287 ($p < 0.01$) (Fig. 4E). The Bliss test of synergy done at 5 different time points (on days 1, 6, 9, 11 and 16 from treatment) revealed synergy in the combination treatment with both the higher and lower dose of [^{67}Cu]-FAP2287 with the higher combination group tumor volume having significantly reduced tumor volumes compared to the Bliss test line ($p = 0.006$ Fig. 4F and G). Using the iRECIST criteria we observed that the response rate was 33.3% from 800 μCi [^{67}Cu]-FAP2287, 66.7% from 800 μCi [^{67}Cu]-FAP2287 + IMT, none from 1600 μCi [^{67}Cu]-FAP2287 and 70% from 1600 μCi [^{67}Cu]-FAP2287 + IMT treated groups. The response rate for controls and IMT treatments was 0%. We observed any changes in body weight or physiological changes in these treated mice, we observed no physical changes or decrease in body weight over time (supplemental Fig. 2).

[^{64}Cu]-FAP2287 PET imaging as a reliable predictor for response to [^{67}Cu]-FAP2287 TRT

To analyze if [^{64}Cu]-FAP2287 PET can be a reliable predictor of overall survival to [^{67}Cu]-FAP2287, [^{64}Cu]-FAP2287 SUV_{mean} were plotted against probability of survival of the mice that received 800/1600 μCi [^{67}Cu]-FAP2287 alone (not in combination with IMT) to observe if there is a correlation between the two entities. The SUV values were positively correlated with days of survival in both the lower and higher dose of [^{67}Cu]-FAP2287 dose with 800 μCi (Fig. 5A, $R^2 = 0.84$, $p = 0.03$) and 1600 μCi (Fig. 5B, $R^2 = 0.62$, $p = 0.01$), suggesting [^{64}Cu]-FAP2287 PET is a reliable predictor for long-term response to [^{67}Cu]-FAP2287 TRT but there was a decrease in correlation between probability of survival and [^{64}Cu]-FAP2287 SUV_{mean} .

Exploratory preliminary assessment of long-term anti-tumor response

To assess metastatic tumor burden (as 4T1 tumors metastasize to organs such as lungs, liver, brain, etc.) (42), we performed BLI imaging on long-standing survivors on day 75 from tumor implantation and observed that for our combination treatment, there were $N = 1$ mouse/group (out of remaining 7 from 1600 and 4 from 800 μCi) with no primary tumors. On day 88 from tumor implantation, $N = 1$ per lower and higher doses had achieved a complete response with no recurrence or metastasis of the primary tumor, which was confirmed using BLI (supplemental Fig. 3A). These mice were rechallenged with tumor cells (exploratory), and tumor growth was monitored closely every two days. We observed that the mouse that had received that 1600 μCi showed increased resistance to secondary tumor growth and significantly decreased rates of tumor growth compared to the mouse that received 800 μCi ($p < 0.0001$) which had relapse in primary tumor growth (supplemental Fig. 3B).

DISCUSSION

FAP has been defined as a good target for solid tumors across many cancer types, which propelled translational theranostic trials and success of those trials across many cancers including TNBC. FAP2286 is a cyclic peptide which is a human equivalent of FAP2287 and is already in several clinical trials, and cyclic peptides have demonstrated higher binding affinity, specificity, and retention in the tumor region compared to the linear peptides [40]. However, there have been limited pre-clinical studies demonstrating combination of TRT approaches with IMT. This work builds upon what others, such as Zboralski, D et. al. previously showed where [^{177}Lu]-FAP2287 studied preclinically with transduced MCA205-mFAP cells and paired [^{68}Ga] PET imaging revealed increased enhanced response to anti PD-1 following FAP TRT [41]. Our study builds upon this work with different dosing of TRT in a syngeneic model with naturally available overexpression of FAP and using novel chemically-similar theranostic pairs. Further, we demonstrate long-standing response using combination approaches of FAP-TRT and checkpoint inhibitors in tumors that are resistant to immunotherapy alone (35, 43).

Our studies build upon the growing work of others that have previously demonstrated robust responses of breast cancer to TRT in preclinical studies. TRT causes DNA damage that leads to immunomodulation of TME and induces ICD, which leads to increased infiltration of CD4 + and CD8 + T cells in the TME [44–

46]. Brenda Gibbens-Bandala B, et. al, used [^{177}Lu]-Bombesin-PLGA (paclitaxel) for treatment of TNBC human-cell line (MDA-MB-231) tumors and revealed synergy through increased higher cell death in combination with chemotherapy, paclitaxel. Kasten BB, et. al, showed the use of [^{212}Pb]-225.28 for reduced tumor burden in the TNBC human cell line xenografts. Breast cancer is known to have an immunogenically cold TME with low infiltrating T cells. Due to its low immunogenicity, TNBC has varied response to IMT which have been shown both clinically and preclinically (44–46). With the addition of immunotherapy, there is an increase in activation and infiltration of T cells and other effector cell populations, which mediate cell death and enhance therapeutic effects. However, very few studies have evaluated the use of combination therapies with TRT and IMT. Few such studies reviewed the current standard [^{177}Lu] TRT with IMT in several pre-clinical studies including Kleinendorst, S. C., et. al, and Kerr, C. P., et. al, compared several preclinical and clinical studies with β [^{177}Lu], α [^{212}Pb] TRT and external beam radiation in combination with IMT and observed an increase in anti-immune response with TRT with further ideas to optimize TRT dosing in combination with IMT (26, 47). Similar were observed in a preclinical study using immunotherapy response syngeneic tumors, E0771 in C57/B16 mouse model, treated with IMT in combination with ^{177}Lu TRT showing increased survival and decreased tumor volumes in the combination group compared to the other treatment groups (48). Our work compliments these and expands upon their work, demonstrating [^{67}Cu]-FAP2287 TRT in combination with IMT in a model that is not responsive to IMT and revealed reduced tumor volume, increased the probability of survival, and almost doubled the survival rate of mice with aggressive 4T1 TNBC tumors.

Eisazadeh, R., et. al, showed that high tumor-to-background ratio of [^{68}Ga]Ga-PSMA-11 PET can be a significant prognostic for [^{177}Lu]Lu-PSMA therapy and associated with improved survival in a clinical study. Ozkan, E., et. al, observed that [^{68}Ga]Ga-PSMA PET is a good way to predict patients who will benefit from [^{177}Lu]Lu-PSMA treatment based on pretreatment SUV metrics that were all statistically significant factor in prediction of PSA-based response and RECIP disease control in clinical settings (49, 50). Other clinical studies Sharma, R., et. al, and Lee, H., et. al, showed similar trends for predicting response to peptide receptor radionuclide therapy, [^{177}Lu]-DOTATATE in neuroendocrine tumors using [^{68}Ga]-DOTATATE PET (51, 52). Our study bridges the gap between the current treatment options, IMT, with new elementally-paired FAP TRT using [$^{64/67}\text{Cu}$]-FAP2287 TRT to enhance the efficacy of IMT and to identify predictive approaches to personalize treatment in a preclinical syngeneic TNBC model. We successfully were able to demonstrate that [^{64}Cu]-FAP2287 PET can predict response to [^{67}Cu]-FAP2287 TRT alone and can be used as a diagnostic tool to stratify groups based on baseline FAP expression for increased response to TRT in preclinical model of TNBC. Cingoranelli, S, et. al, previous showed that with the use of elementally paired isotopes in pre-clinical models of prostate cancer [^{43}Sc]Sc-PSMA-617 was able to predict probability of survival for both low and high doses of [^{47}Sc]Sc-PSMA-617 treatment. We successfully were also able to demonstrate that [^{64}Cu]-FAP2287 PET can predict response to [^{67}Cu]-FAP2287 TRT alone and can be used as a diagnostic tool to stratify groups based on baseline FAP expression for increased response to TRT in pre-clinical model of TNBC (53). However, one drawback of this study design was that the survival data for the combination treatment mice was censored following

the death of mice from other treatment groups. Therefore, we could not correlate the [^{64}Cu]-FAP2287 PET values to the probability of survival for the combination group to assess if [^{64}Cu]-FAP2287 PET can be a reliable predictor of response to [^{67}Cu]-FAP2287 + IMT (supplemental Fig. 4A and B). Theranostic approaches using [^{67}Cu]-FAP2287 in combination with IMT provide an opportunity to non-invasively target a critical component of the TME to enhance clinical decision making for treating TNBC. Lower and higher doses of [^{67}Cu]-FAP2287 successfully reduced tumor burden and enhanced the efficacy of IMT in 4T1 tumor-bearing mice, which led to an increase in the probability of survival. With the higher dose of [^{67}Cu]-FAP2287, there is a higher rate of ICD which enhances the therapeutic effects of IMT and successfully reduces the tumor burden compared to the lower dose of [^{67}Cu]-FAP2287, with no visible side effects. We observed a correlation between [^{64}Cu]-FAP2287 SUV_{mean} to days of survival when treated with [^{67}Cu]-FAP2287 TRT. We also observed that our combination therapy with both 800 and 1600 μCi doses of [^{67}Cu]-FAP2287 and IMT had synergistic effects resulting in enhanced treatment response and reduced tumor volumes with the higher dose of [^{67}Cu]-FAP2287. These results emphasize the importance of combination therapy approaches to modulate the TME and overcome treatment resistance in TNBC. Importantly, there is very little work done in establishing a relation between the diagnostic and therapeutic agent for predicting response. We identified [^{64}Cu]-FAP2287 as a good diagnostic tool to differentiate between responders and non-responders of [^{67}Cu]-FAP2287 TRT to streamline the process of treating TNBC cases with TRT and IMT, allowing to stratify TRT based on baseline FAP expression for enhanced treatment efficacy.

Despite exciting preclinical results in preclinical response and improve therapeutic efficacy, future directions are still needed to optimize [^{67}Cu]-FAP2287 dosing, possibly identifying the lowest amount of [$^{64/67}\text{Cu}$]-FAP2287 that can be used and to identify how multiple doses of delivered TRT affect combination therapy. Identifying the best dosing strategy could further optimize patient care and identify opportunities for best tumor burden eradication (and reduction of future metastasis). This study only used one dose of TRT and could have potentially further increased effectiveness or reduced metastatic burden by optimizing the dosing or personalizing dose amount based on PET imaging. Additionally, this study shows an exploratory investigation into long-term survival and re-challenging. There is a need to characterize the mechanism behind the potential long-term immune surveillance and the effect of memory T cells in stunting primary tumor growth following [$^{64/67}\text{Cu}$] FAP2287 TRT. We observed that animals with complete disappearance of the primary lesion following combination treatment had decreased tumor growth when rechallenged. Studies exploring synergy in combination therapy and long-term immune surveillance are needed to better characterize long-term benefits. While this study focused on chemically paired isotopes, future studies could evaluate the ability of [^{64}Cu]-FAP to predict response to TRT, such as ^{177}Lu , that are currently in clinical trials, as it provides longer half-life imaging compared to current [^{68}Ga] and [^{18}F] tracers used.

In conclusion, our study highlights the significance of TRT to modulate and sensitize the TME to increase treatment efficacy of IMT response and improve overall treatment outcomes. Using chemically paired

radionuclides could help personalize treatment strategies and give a non-invasive approach to provide clinically relevant metrics for treatment selection in patients with TNBC patients, as Cu-64 imaging provide predictive capabilities of long-term survival from Cu-67 treatment. [^{64/67}Cu]-FAP2287 TRT has potential to expand to several other cancer types, including pancreatic ductal adenocarcinoma, head and neck cancer, and colorectal cancer, which have high FAP expression in their TME.

Abbreviations

TNBC

triple-negative breast cancer

FAP

fibroblast activation protein

TRT

Targeted Radionuclide Therapy

PET

Positron Emission Tomography

SPECT

Single Photon Emission Computed Tomography

TME

Tumor Microenvironment

IMT

Immunotherapy

CTLA-4

cytotoxic T-lymphocyte associated antigen 4

PD-1

programmed cell death receptor 1

ICD

Immunogenic cell death

CAF

Cancer associated fibroblast

SUV

Standardize uptake value VQ:Vivo Quant

TMR

tumor to muscle ratio

ROI

Regions of interest

CT

Computed tomography

BLI

Bioluminescence Imaging

Declarations

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY

Data generated in this study will be made available upon request.

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Figures

Fig 1

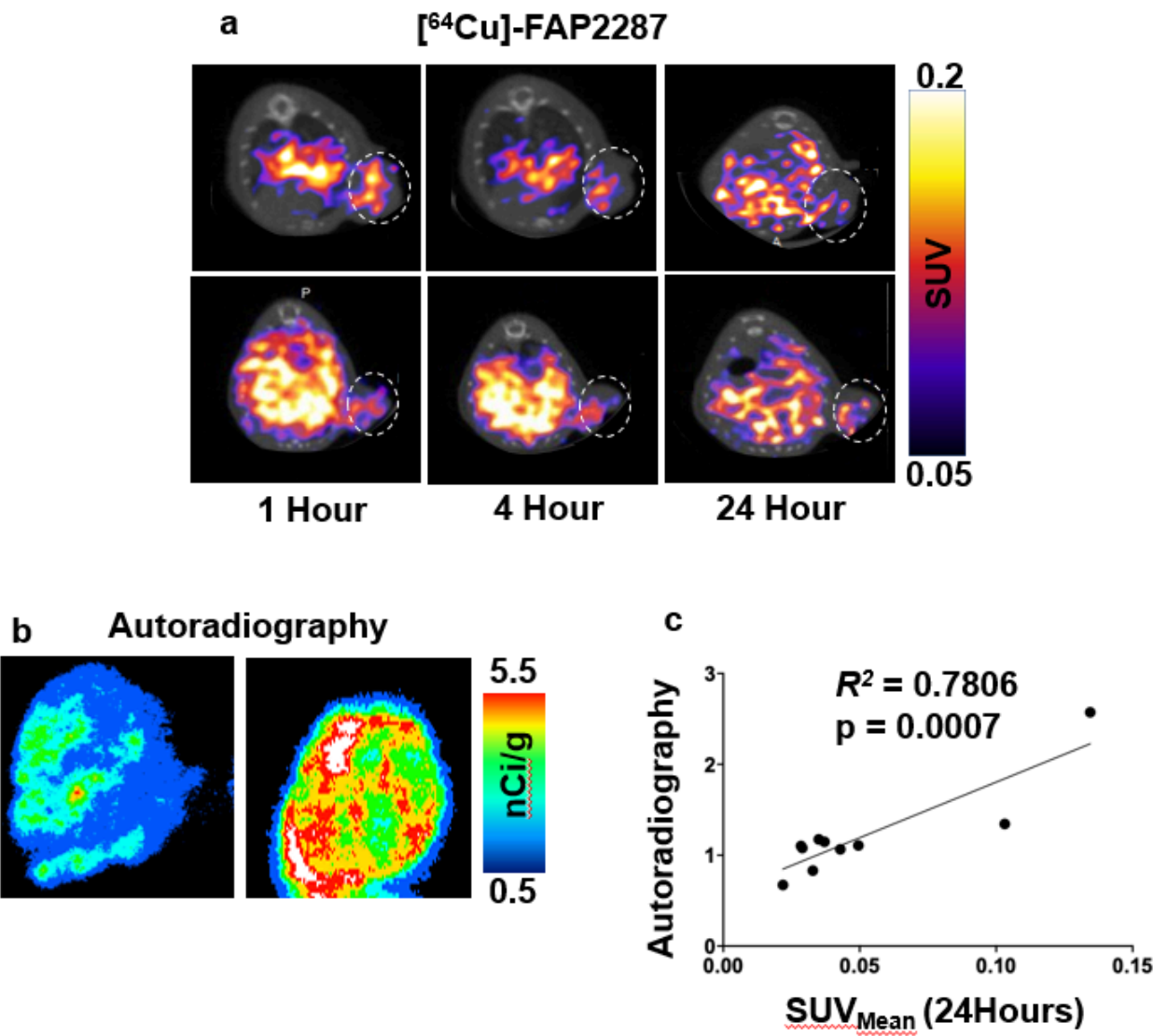


Figure 1

Ex Vivo biological validation of FAP expression in 4T1 TNBC model. (a) Representative transverse in vivo [⁶⁴Cu]-FAP2287 PET imaging in 4T1 tumor bearing Balb/c mice at 4, 24 and 48h p.i. with the white dotted circle highlighting the tumor region. (b) Autoradiography showing radiotracer concentration the tumor region obtained following 24h imaging time point. (c) Strong Positive correlation between [⁶⁴Cu]-FAP2287 SUV_{Mean} (24h) with radiotracer concentration recorded in autoradiography ($R^2=0.7806$, $p=0.0007$)

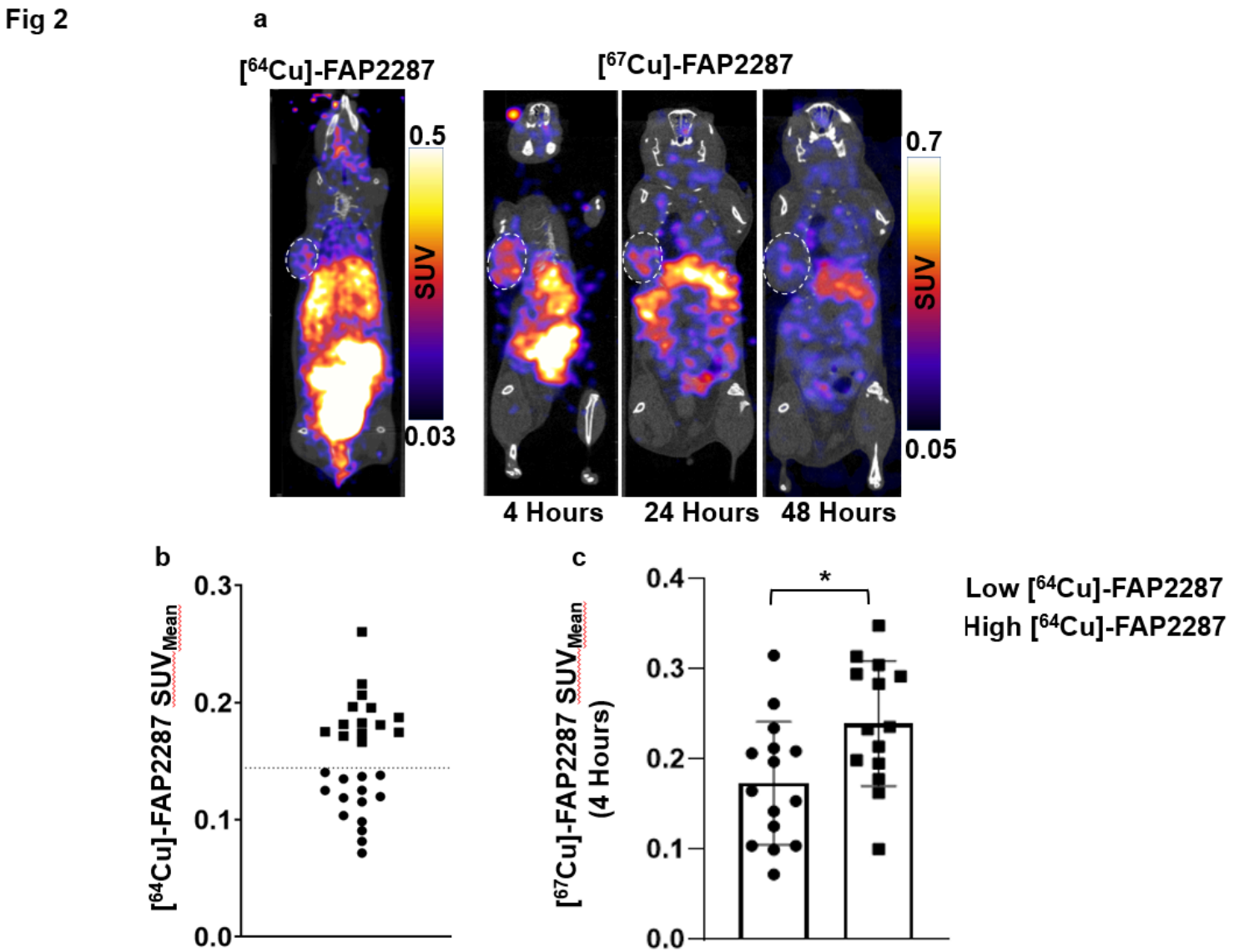


Figure 2

Positive correlation between chemically-paired radioisotopes [^{64/67}Cu]-FAP2287. (a) Representative coronal images of [⁶⁴Cu]-FAP2287 PET 4h p.i. and [⁶⁷Cu]-FAP2287 SPECT 4, 24 and 48h p.i. showing quantifiable radiotracer uptake and increased retention in the tumor region highlighted by the white dotted circle. (b) Graphical representation of thresholding of mice into higher and lower [⁶⁴Cu]-FAP2287

SUV_{Mean} (c) Graphical representation showing significant differences in [⁶⁷Cu]-FAP2287 SUV_{Mean} (4h p.i.) between groups of low and high [⁶⁴Cu]-FAP2287 SUV_{Mean}

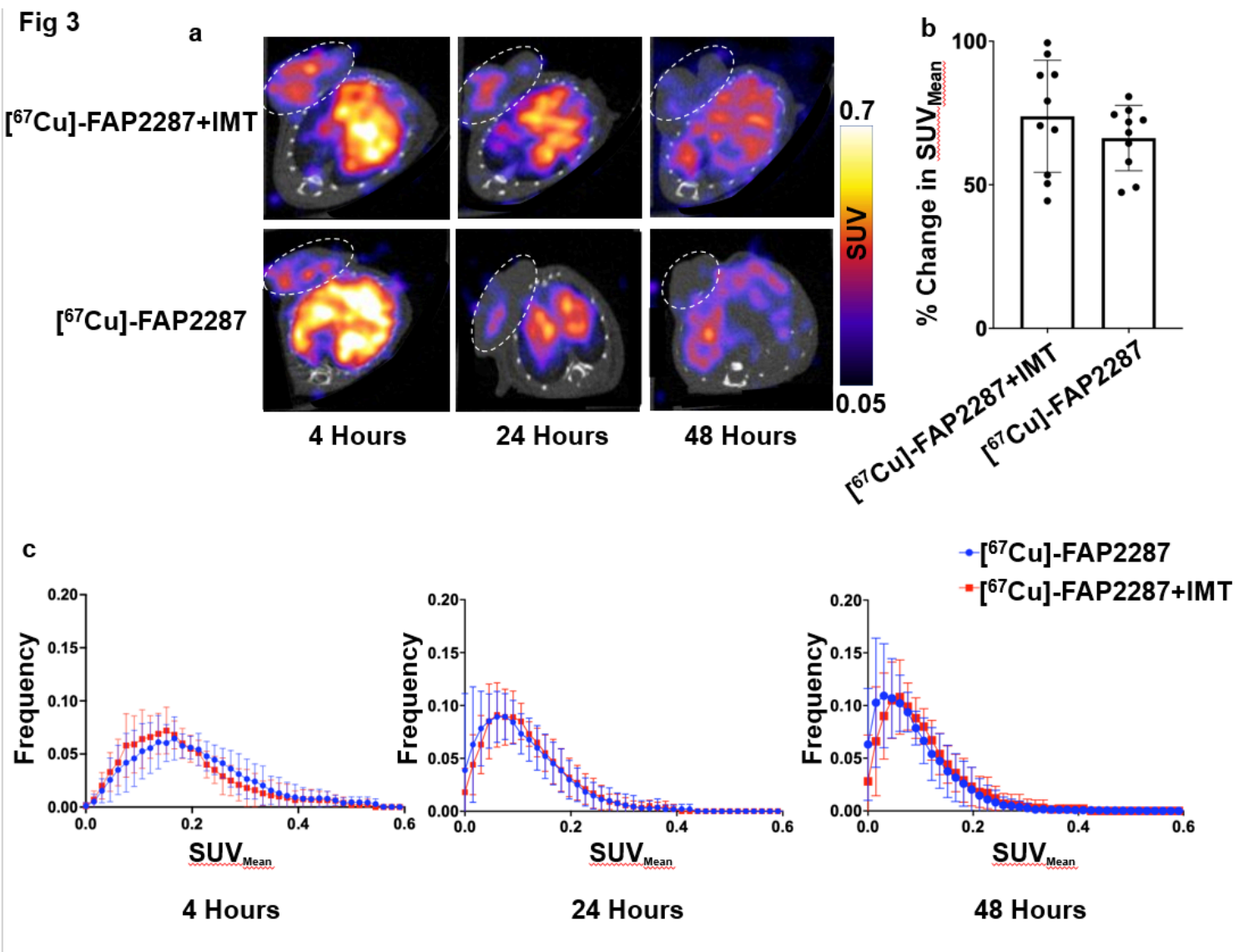


Figure 3

Effects of IMT in [⁶⁷Cu]-FAP2287 retention. (a) Transverse [⁶⁷Cu]-FAP2287 SPECT images at 4, 24 and 48h p.i., there was no difference in [⁶⁷Cu]-FAP2287 retention between the groups in the tumor region highlighted by the white dotted circle. (b) Graphical representation of % retention of radiotracer 48h p.i. showing no statistical differences with the addition of IMT. (c) Histogram analysis of SUV_{Mean} indicating similar frequency distribution in both the groups

Fig 4

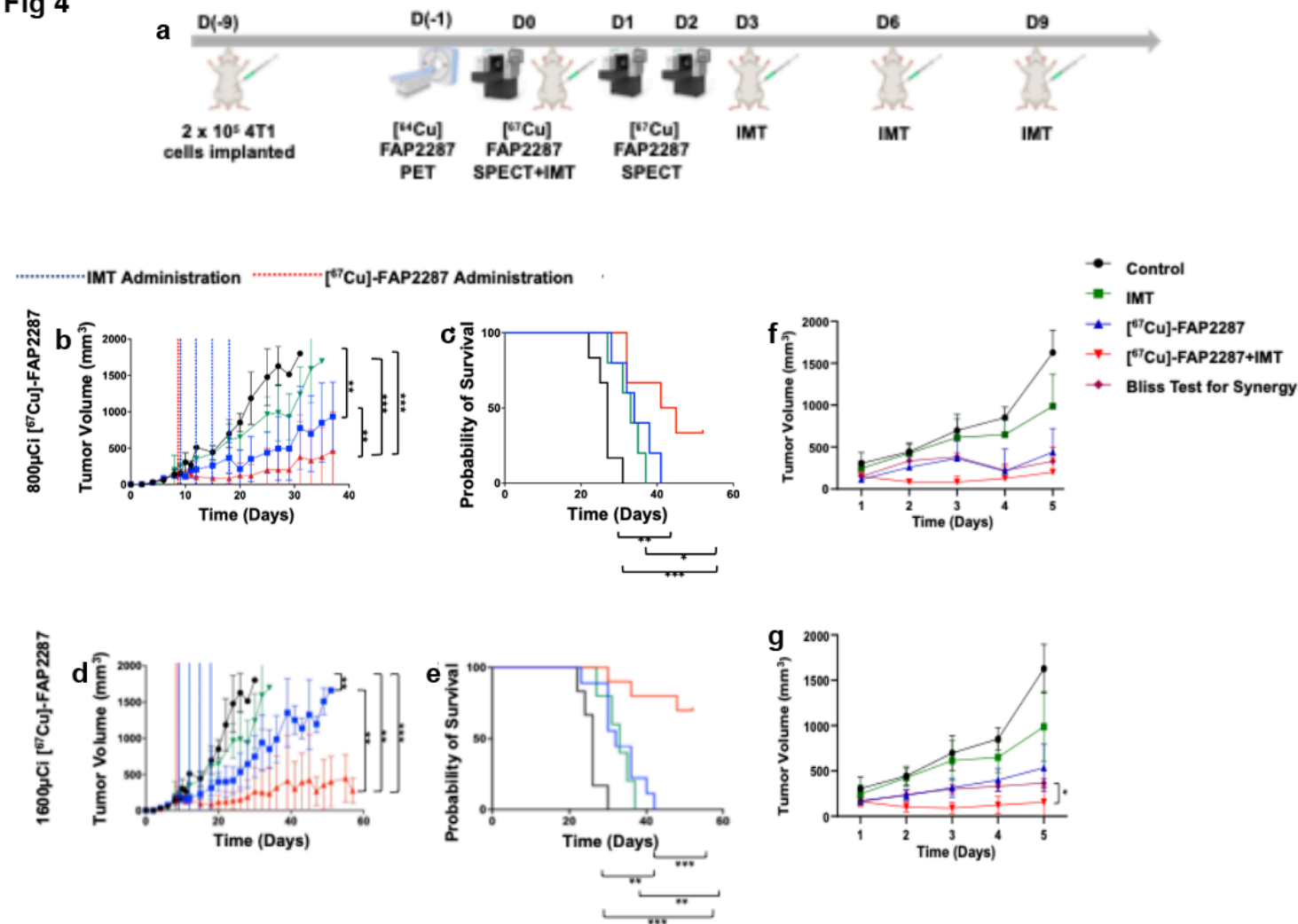


Figure 4

Response of 4T1 tumors to $[^{67}\text{Cu}]\text{-FAP2287}$ in combination with IMT. (a) Experimental timeline for treatment. Tumor growth curve in 4T1 model administered with (b) 800 μCi (d) 1600 μCi $[^{67}\text{Cu}]\text{-FAP2287}$ suggesting that higher dose of $[^{67}\text{Cu}]\text{-FAP2287}$ significant decreases tumor burden in the combination group compared to single treatment groups. Probability of survival with (c) 800 μCi (e) 1600 μCi $[^{67}\text{Cu}]\text{-FAP2287}$ indicating significant increase in probability of survival with 1600 μCi combination group compared to all the other groups. (f) 800 μCi (g) 1600 μCi observed tumor volume in all treatment groups at five different time points and expected tumor volumes of the combination groups from the bliss test of synergy showing higher tumor volume compared to the observed combination tumor volumes suggesting synergy in the combination treatment

Fig 5

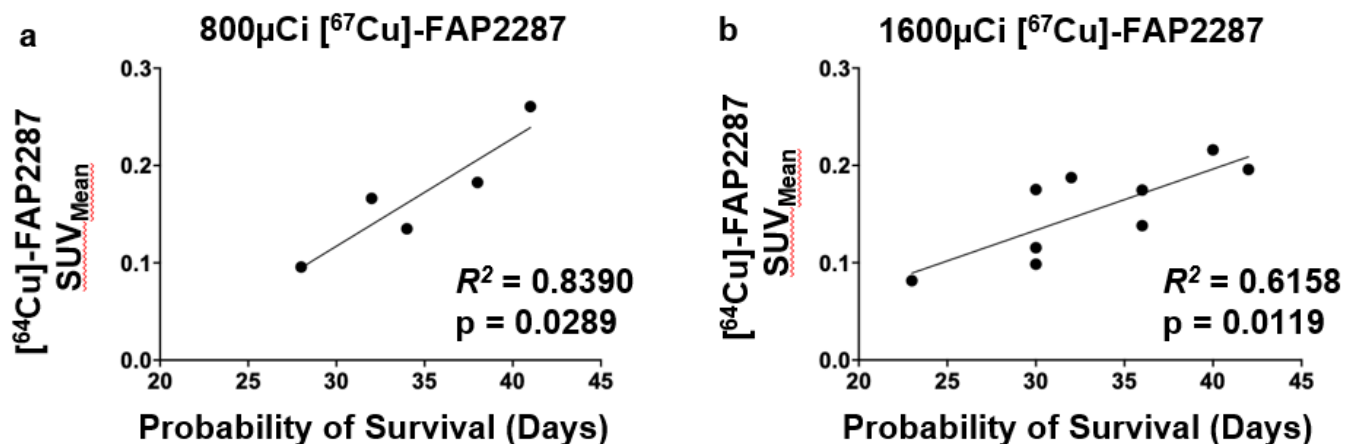


Figure 5

[⁶⁴Cu]-FAP2287 PET imaging as a reliable predictor for response to [⁶⁷Cu]-FAP2287 TRT. Positive correlation trend between probability of survival and [⁶⁴Cu]-FAP2287 SUV_{Mean} in mice treated with (a) 800uCi of [⁶⁷Cu]-FAP2287 ($R^2=0.839$, $p=0.0289$) and (b) 1600uCi of [⁶⁷Cu]-FAP2287 ($R^2=0.6158$, $p=0.0119$) showing significant strong positive correlation between SUV_{Mean} and median survival in both cohorts

Supplementary Files

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