Longitudinal Noninvasive Imaging of Progesterone Receptor as a Predictive Biomarker of Tumor Responsiveness to Estrogen Deprivation Therapy

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Abstract

Purpose: To investigate whether longitudinal functional PET imaging of mammary tumors using the radiopharmaceuticals [¹⁸F]FDG (to measure glucose uptake), [¹⁸F]FES [to measure estrogen receptor (ER) levels], or [¹⁸F]FFNP [to measure progesterone receptor (PgR) levels] is predictive of response to estrogen-deprivation therapy.

Experimental Design: [¹⁸F]FDG, [¹⁸F]FES, and [¹⁸F]FFNP uptake in endocrine-sensitive and -resistant mammary tumors was quantified serially by PET before ovariectomy or estrogen withdrawal in mice, and on days 3 and 4 after estrogen-deprivation therapy. Specificity of [¹⁸F]FFNP uptake in ERα mammalian tumors was determined by competition assay using unlabeled ligands for PgR or glucocorticoid receptor (GR). PgR expression was also assayed by immunohistochemistry (IHC).

Results: The levels of [¹⁸F]FES and [¹⁸F]FDG tumor uptake remained unchanged in endocrine-sensitive tumors after estrogen-deprivation therapy compared with those at pretreatment. In contrast, estrogen-deprivation therapy led to a reduction in PgR expression and [¹⁸F]FFNP uptake in endocrine-sensitive tumors, but not in endocrine-resistant tumors, as early as 3 days after treatment; the changes in PgR levels were confirmed by IHC. Unlabeled PgR ligand R5020 but not GR ligand dexamethasone blocked [¹⁸F]FFNP tumor uptake, indicating that [¹⁸F]FFNP bound specifically to PgR. Therefore, a reduction in FFNP tumor to muscle ratio in mammary tumors predicts sensitivity to estrogen-deprivation therapy.


Introduction

Breast cancer is the second most deadly cancer for women in the United States. About 80% of all newly diagnosed breast cancers are classified as estrogen receptor-α+ (ERα⁺; ref. 1). ERα, together with progesterone receptor (PgR) and HER2, is part of the standardized clinicopathologic evaluation of breast cancer. Because they provide important information to guide treatment decisions, accurate and reproducible assessment of the levels of these biomarkers is critical. ERα, PgR, and HER2 are routinely assayed using immunohistochemistry (IHC). HER2 amplification is also frequently detected by fluorescence in situ hybridization. However, discordance rates ranging from 9% to 39% were observed for PgR, depending on the techniques used to obtain biopsy specimens (2). This discrepancy can be partly explained by tumor heterogeneity leading to sampling error during biopsy (3). Therefore, noninvasive functional imaging of the whole lesion using positron emission tomography (PET) may provide a more complete molecular characterization of the tumor in its native setting. In addition, PET imaging is also an effective approach to monitor advanced metastatic disease during antitumor therapy when repeated biopsies may not be possible.

[¹⁸F]Fluoroestradiol ([¹⁸F]FES) and [¹⁸F]fluoro furanyl nonprogesterone ([¹⁸F]FFNP) are well-validated noninvasive molecular imaging radiopharmaceuticals for ERα and PgR, respectively (4, 5). [¹⁸F]FES is an estradiol analog that binds to ERα with high affinity and selectivity (6). Differences in FES uptake in multiple tumor sites within the same patient have demonstrated heterogeneity of metastases and highlight the value of using functional PET imaging to monitor changes in tumor characteristics with disease progression (7). In addition, tumor FES uptake before endocrine treatment is correlated with subsequent clinical response to therapy (8–10). Finally, blockade of tumor FES uptake after the initiation of tamoxifen or fulvestrant treatment is indicative of the pharmacodynamic effectiveness of the dosing

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Translational Relevance

Although endocrine therapies are the standard-of-care for patients with estrogen receptor-alpha-positive (ER\(\alpha\)) breast cancer, about half of the patients do not benefit from these treatments due to intrinsic or acquired resistance. Therefore, biomarkers that predict tumor responsiveness to endocrine therapies are highly valuable for identifying patients who will require alternative treatments. Noninvasive functional PET imaging offers a unique opportunity to serially follow the molecular changes in tumors in response to therapies. Using preclinical models of ER\(\alpha\) breast cancer, we demonstrated that a significant reduction in binding of [\(^{18}\)F]FFNP to progesterone receptor predicts a positive tumor response to estrogen-deprivation therapy. In contrast, [\(^{18}\)F]FES or [\(^{18}\)F]FDG uptake by tumors remained unchanged, suggesting that ER\(\alpha\) expression and glucose metabolism were not acutely affected by estrogen deprivation. Thus, measurement of ER\(\alpha\) function by longitudinal FFNP-PET imaging provides a more robust biomarker for response to endocrine deprivation therapies than measurement of ER\(\alpha\) level by FES-PET.

Materials and Methods

Cell cultures

The maintenance of the ER\(\alpha\)-PgR-STAT1\(+/−\) mammary tumor cell lines SSM2 and SSM3 has been described (15). MCF7 breast cancer cells from Benita Katzenellenbogen (University of Illinois) were maintained in Improved MEM Zinc\(++\) Option with phenol red supplemented with 5% heat-inactivated bovine calf serum and 5 nmol/L estradiol.

Mice

Six to 8-week-old wild-type (WT) 129S6/SvEv mice and athymic nude-Foxn1\(^{nu}\) mice were purchased from Taconic Farms and the National Cancer Institute, respectively, and maintained on an estrogen-free diet (Harlan Teklad). A total of 1.2 \(×\) 10\(^6\) SSM2 or SSM3 tumor cells were transplanted into the right thoracic mammary fat pads of WT mice. Nude mice were ovariectomized, fed drinking water supplemented with 10 \(μ\)g/mL 17\(β\)-estradiol for 7 days, and subsequently transplanted with 5 \(×\) 10\(^6\) MCF7 cells. Tumors were measured with calipers at two perpendicular diameters every 3 to 4 days and the average diameters were used for analyses. Tumor-bearing mice were ovariectomized when the SSM2 or SSM3 tumors measured about 5 mm in diameter. All animal experiments were carried out according to the guidelines of the American Association for Laboratory Animal Science under an approved protocol by the Animal Studies Committees and performed in AAALAC-accredited specific pathogen-free facilities at Washington University School of Medicine in St. Louis.

Radiopharmaceuticals, biodistribution assay, and small-animal \(μ\)PET/CT

[\(^{18}\)F]FDG was provided by the Cyclotron Facility at Washington University School of Medicine in St. Louis. [\(^{18}\)F]FES and [\(^{18}\)F]FFNP were synthesized using optimized methods as described previously (5, 14, 16, 17). The specific activities for both [\(^{18}\)F]FES and [\(^{18}\)F]FFNP averaged 7,700 \(±\) 3,000 Ci/mmol (average \(±\) SEM). Mice were injected intravenously with 11.1 MBq (300 \(μ\)Ci) of [\(^{18}\)F]FDG, 5.55 MBq (150 \(μ\)Ci) of [\(^{18}\)F]FES, or 1.11 MBq (30 \(μ\)Ci) of [\(^{18}\)F]FFNP. These injected doses correspond to approximately 20 or 4 pmol for the [\(^{18}\)F]FES and [\(^{18}\)F]FFNP agents, respectively, and at a tumor uptake of 5% ID/g, the tumor concentrations would be 1 or 0.2 pmol/g, which is far below the receptor levels in the tumors (approximately 30 pmol/g; ref. 14).

To examine whether [\(^{18}\)F]FFNP selectively binds to PgR, unlabeled ligand competitors were used to block [\(^{18}\)F]FFNP uptake in the SSM3 tumors. Progesterone (R5020; PerkinElmer) has high affinity for PgR (relative binding affinity (RBA) = 100), but does not bind the glucocorticoid receptor (GR) at significant levels (RBA = 2.6; ref. 18). Dexamethasone (MP Biomedicals) has high affinity for GR (RBA = 100), but does not have any appreciable affinity for PgR (RBA = 0.21; ref. 19). The IC\(_{50}\) of dexamethasone for GR in rat lung is 0.1 \(μ\)mol/L, whereas the IC\(_{50}\) of R5020 for PgR in sheep uterus is 0.05 \(μ\)mol/L (19). Blocking doses were determined on the basis of the IC\(_{50}\) and published results (20, 21). Dexamethasone

To assess tumor responsiveness before surgery and to guide treatment strategies after surgery, our findings that FFNP-PET is more effective than FES-PET and FDG-PET in predicting endocrine responsiveness provide strong support for noninvasive functional imaging as an informative imaging modality.
of [18F]FES (C) and [18F]FFNP (D) was determined in the harvested tissues 1 hour after FES or FFNP injection, respectively. % ID/g of tissue was analyzed.

Figure 1.
Response of the ERa STAT1−/− mammary tumors to estrogen-deprivation therapy, and in vivo tissue biodistribution of [18F]FES and [18F]FFNP, following therapy. A and B, mice bearing transplanted SSM3 (A) or SSM2 (B) mammary tumors were sham-operated (solid circles, n = 5) or ovariectomized (OVX; open circles, n = 5) when tumors were measured around 4 mm in diameter. Tumor growth was followed for 35 days after the surgery. C and D, SSM3-bearing mice were sham-operated (Sham; n = 5) or OVX for 2, 3, or 4 days (D2, D3, or D4, respectively; n = 5 per time point). The indicated organs were harvested. The biodistribution of [18F]FES (C) and [18F]FFNP (D) was determined in the harvested tissues 1 hour after FES or FFNP injection, respectively. % ID/g of tissue was analyzed. *, P < 0.05; ***, P < 0.001. ns, not significant.

(0.5 μmol) or R5020 (0.055 μmol) was injected into SSM3-bearing mice immediately before the injection of [18F]FFNP. For the in vivo tissue biodistribution assays, organs were harvested at 1 hour after injection and the radioactivity was measured. The percentage of injected dose per gram (% ID/g) of tissue was analyzed. For small-animal PET/CT studies, tumor-bearing mice were imaged before surgery (either sham-operation or ovariectomy; day 0), and 3 and 4 days after surgery (days 3 and 4). Because glucose levels are known to affect [18F]FDG uptake, tumor-bearing mice were fasted overnight the day before FDG imaging. For the MCF7 tumor study, estradiol was withdrawn from drinking water for 6 days starting on day 0. PET and CT images were acquired and analyzed as described previously (14). Brieﬂy, mice anesthetized with 1.5% to 2.0% isoflurane were scanned in the supine position in a small-animal PET/CT scanner (Inveon and Focus-220; Siemens Preclinical Solutions) 1 hour following the injection of radiotracer. Images were analyzed using Inveon Research Workplace 3.0. μPET and CT images are coregistered by visual alignment in all three planes (transverse, sagittal, and coronal). Regions of interest were manually drawn around the nonnecrotic areas of the tumor on the coregistered μPET/CT images. Activity in triceps muscle was used as nontarget tissue uptake. Activity measurements (Bq/cm³) were divided by the decay-corrected injected dose (Bq) and multiplied by 100 to obtain a tissue uptake index expressed as % ID/g of tissue. Tumor to muscle (T:M) ratio was calculated as the ratio of % ID/g of tumor to that of muscle.

Immunohistochemistry
Four-micrometer sections of formalin-fixed paraffin-embedded mammary tumors were deparaffinized, rehydrated, heated in citrate buffer (pH 6), and stained using antibodies against PgR (Dako; A0089; 1:100). Positive signal was developed according to instructions from the EnVision HRP System (Dako) or MACH 4 (Biocare) followed by DAB chromogen.

Statistical analyses
All numerical results are presented as mean ± standard error of mean (SEM). The unpaired t test was used to determine the statistical significance between control and experimental groups whereas the paired t test was implemented to compare across different time points within the same group of animals. All tests are two-sided and a P value ≤ 0.05 was considered significant.

Results
Kinetics of [18F]FES and [18F]FFNP uptake in endocrine-sensitive mammary tumors following ovarian ablation
A preclinical model of ERα breast cancer that faithfully recapitulates the pathophysiologic and developmental characteristics of human ERα breast cancer was used in this study (15, 22). One of the advantages of this preclinical model is the availability of paired ERα+ PgR+ endocrine-sensitive SSM3 and endocrine-resistant SSM2 mammary tumor cell lines, as potential imaging biomarkers can be examined and compared in the two tumor systems (refs. 14, 15; Fig. 1A and 1B). Ovariectomy (OVX) was...
carried out as the treatment strategy to eliminate estradiol production by the ovaries (Fig. 1A and 1B). Because the contribution of extragonadal aromatase activity in the mouse is negligible due to the lack of extragonadal-specific promoters in the mouse aromatase gene loci (23), OVX mirrors ovarian suppression plus aromatase inhibition (AI) for premenopausal women or AI alone for postmenopausal women. Using this treatment protocol, we observed that the growth of the SSM3 mammary tumors was blunted by estrogen-deprivation therapy (Fig. 1A). In contrast, progression of the SSM2 mammary tumors remained unaffected (Fig. 1B).

We first sought to determine whether an acute reduction in $[^{18}F]FES$ uptake could be observed in hormone-dependent SSM3 mammary tumors after estrogen-deprivation therapy. A tissue biodistribution study was carried out on sham-operated SSM3-bearing mice (Sham) and on tumor-bearing mice that were ovariectomized for 2, 3, or 4 days (D2, D3 or D4, respectively; Fig. 1C). Tumor, uterus, muscle, and blood were harvested from these mice, and radioactivity was measured in the harvested organs (Fig. 1C). Interestingly, $[^{18}F]FES$ uptake in the uterus was transiently reduced on day 2 after OVX and returned to basal levels on days 3 and 4 after OVX, mimicking a short-term transient regulation of uterine ER activity (24) previously noted by others (24).

In contrast with FES-PET, $[^{18}F]FFNP$ uptake in the SSM3 tumors was reduced gradually over the course of 4 days following OVX (Fig. 1D). By day 4 after OVX, tumor FFNP uptake was decreased by 40% compared with that in the tumors of sham-operated mice ($P = 0.01$). Taken together, these results indicate that $[^{18}F]FFNP$ uptake, but not $[^{18}F]FES$ uptake, in tumors is decreased acutely by depletion of ovarian hormones.

$[^{18}F]FFNP$ uptake in SSM3 tumors is specific to PgR

Because $[^{18}F]FFNP$ binds the glucocorticoid receptor (GR) with modest affinity (18), we examined whether $[^{18}F]FFNP$ uptake in the SSM3 tumors was specific to PgR. The unlabeled forms of the PgR-selective ligand R5020 or GR-selective ligand dexamethasone were injected into SSM3 tumor-bearing mice immediately before the injection of $[^{18}F]FFNP$ (5 mice per group). The indicated organs were harvested. The in vivo tissue biodistribution of $[^{18}F]FFNP$ was determined in the harvested tissues 1 hour after FFNP injection. Representative results from three independent experiments are shown. $P < 0.05$; $**P < 0.001$ ns, not significant.

Longitudinal FFNP-µPET/CT imaging of endocrine-resistant mammary tumors

Because the ER+$\alpha$ PR+$\alpha$ mammary tumor cell line SSM2 is nonresponsive to ovarian hormone-depletion (Fig. 1B), we hypothesized that PgR expression and thus $[^{18}F]FFNP$ uptake would not be affected acutely by OVX. Indeed, FFNP T:M ratios of sham and OVX mice were significantly lower than those in the sham mice on days 3 and 4 after the surgery ($P = 0.04$ and $P = 0.0005$, respectively; Fig. 3A and 3B). Consistent with the biodistribution data presented in Fig. 1D, the T:M ratio in the OVX group was steadily decreased by 15% from the basal level on day 0 to day 3 after OVX ($P = 0.01$), and by another 23% from day 3 to day 4 after OVX ($P = 0.009$). The overall reduction in T:M ratio was 35% on day 4 after OVX, compared with the baseline measurement on day 0 ($P = 0.0002$). Results obtained from IHC also indicated that the number of PgR+$\alpha$ tumor cells in OVX SSM3 tumors was significantly diminished compared with the SSM3 tumors in sham-operated mice (Fig. 3C). Importantly, tumor size in sham and OVX mice, as measured by caliper, did not differ on either days 3 or 4 post-surgery, indicating that the acute reduction in PgR expression in the OVX tumors occurs before a decrease in tumor size can be detected (Fig. 3D). Taken together, these data clearly demonstrate that a reduction in PgR expression in endocrine-responsive mammary tumor cells following downregulation of ER$\alpha$ signaling can be measured serially and noninvasively using $[^{18}F]FFNP$. 
expression is associated with downregulation of ERα signaling in endocrine-sensitive tumor (SSM3), whereas the lack of reduction in FFNP uptake and PgR expression is associated with endocrine-resistant tumor (SSM2).

Longitudinal FDG-µPET/CT imaging of endocrine-sensitive and -resistant mammary tumors

[18F]FDG is the most frequently used radiopharmaceutical to evaluate clinical response to therapies. We next examined whether FDG-PET would be suitable to monitor tumor response in the preclinical neoadjuvant setting of estrogen-deprivation therapy. As shown in Fig. 5A, we did not observe any changes in [18F]FDG uptake in the OVX-resistant SSM2 tumors following OVX. The T:M ratio of [18F]FDG uptake remained unchanged on days 3 and 4 after OVX compared with the basal level before OVX. There was also no statistically significant difference in the FDG T:M ratio between sham and OVX tumors at all three time points.

In contrast with the SSM2 tumors, as SSM3 tumor growth was blunted by OVX, we expected to observe a decrease in [18F]FDG uptake in the SSM3 tumors following OVX. To our surprise, the T:M ratio of [18F]FDG uptake was indistinguishable between the sham and OVX groups at all the time-points examined (Fig. 5B). There was also no difference in the T:M ratio between days 0, 3, and 4 in the OVX cohort. Within this time frame examined, the SSM3 tumors did not show significant tumor progression, as measured by caliper (Fig. 3D). Therefore, levels of [18F]FDG uptake remained unchanged in both endocrine-sensitive and -resistant mammary tumors.

Longitudinal FFNP-µPET/CT imaging of endocrine-sensitive MCF7 mammary tumors

To extend and validate our findings, we tested whether the endocrine-sensitive ERα+ MCF7 human breast tumors displayed a similar FFNP binding profile following estrogen depletion. Because the MCF7 xenograft tumors grew at a slower pace than the SSM3 tumors, we monitored [18F]FFNP uptake after 6 days of estrogen withdrawal (Fig. 6; /C0E2 group). Consistent with the SSM3 results, [18F]FFNP uptake in the MCF7 tumors was significantly reduced in the absence of estrogen on day 6 compared with the baseline levels on day 0 (Fig. 6; P = 0.0003). [18F]FFNP uptake was also lower in the estrogen-depleted group (–E2) compared with the estrogen-supplemented group (+E2) on day 6 (P = 0.0008). Taken together, rapid changes in [18F]FFNP uptake following estrogen deprivation were also observed in the prototypical ERα+ MCF7 breast cancer xenograft model.

Discussion

In this study, we demonstrated that serial FFNP-PET imaging of ERα+ mammary tumors before estrogen-deprivation therapy and shortly after treatment was an effective approach to inform treatment response. A significant drop in [18F]FFNP uptake levels in tumors posttreatment compared to pretreatment level predicted a positive response to estrogen-deprivation therapy.

![Figure 3. Longitudinal [18F]FFNP μPET/CT imaging of SSM3 tumor-bearing mice.](https://www.aacrjournals.org)

A, mice bearing SSM3 tumors were scanned 1 hour after [18F]FFNP injection. % ID/g of tumor and muscle were calculated. Ratio of % ID/g in tumor to % ID/g in muscle [i.e., tumor to muscle ratio (T:M)] from Sham (n = 6) and OVX mice before surgery (day 0; n = 6) and on days 3 and 4 after surgery (n = 6) were graphed: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant. B, 3D maximum intensity projection (MIP) from coregistered μPET and CT images of representative mice bearing SSM3 mammary tumors. Serial images from the same mouse in each group are shown. Arrows, tumors. C, representative PgR IHC images of SSM3 tumors on day 4 following surgery. D, mean tumor diameter of SSM3 tumors before surgery (day 0) and after surgery (day 3). No significant difference in tumor size was observed between the two cohorts of mice. Representative results of two independent studies are shown.
Although it is possible that the levels of [18F]FES uptake in endocrine-sensitive SSM3 tumors was detected 1 week after fulvestrant therapy (14). Together with the current findings, these results suggest that a decrease in [18F]FFNP tumor uptake precedes that in [18F]FDG tumor uptake. Therefore, monitoring acute changes downstream of ER signaling provides a more rapid assessment than measuring glucose metabolism in the tumors following estrogen deprivation.

In contrast with our current study, FDG-PET was demonstrated to be effective in predicting therapeutic response of the partial agonist tamoxifen or of low-dose estradiol therapy (9, 10, 29). We have previously demonstrated that tamoxifen or low-dose estradiol causes metabolic flare in tumors such that an increase in tumor glucose metabolism and [18F]FDG uptake 24 hours after estradiol administration is associated with a positive therapeutic response (9, 10, 29). It will be interesting to test whether FFNP-PET can also be used as an imaging biomarker in these settings where metabolic flare is observed, and whether it provides a signal that is more rapid and robust than that of FDG-PET, as it is shown in this current study with estrogen deprivation.

Quantification of tumor cell proliferation by IHC analysis of Ki67 antigen has also been proven to be an effective biomarker in day 4 following surgery. D. mean tumor diameter of SSM2 tumors before surgery (day 0) and after surgery (day 3). No significant difference in tumor size was observed between the two cohorts of mice. Representative results of two independent studies are shown.

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predicting outcome of endocrine therapies (26). Inhibition of tumor cell proliferation by antiestrogens or estrogen deprivation is closely correlated with better prognosis (28, 30). Because there is a strong concordance between Ki67 proliferation index and \[^{18}F\]FLT uptake in breast cancers (31), future studies assessing the effectiveness of FLT-PET as an imaging biomarker for endocrine treatments will be needed.

In conclusion, we demonstrated that serial FFNP-PET imaging was more effective than FDG-PET and FES-PET in predicting response to estrogen-deprivation therapy in preclinical models of ER\(^{+}\) breast cancer. In the context of our present study with estrogen-deprivation therapy and previous results with fulvestrant, one may extrapolate this biomarker approach to additional endocrine interventions, such as toremifene, which is also antagonistic in the breast. Measurement of acute changes in PgR expression by noninvasive FFNP-PET may be beneficial in a neoadjuvant setting so that a more aggressive treatment course can be implemented after surgery if the primary tumor is predicted to be endocrine-nonresponsive.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: S.R. Chan, A.M. Fowler, C.S. Dence, J.A. Katzenellenbogen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.R. Chan, A.M. Fowler, J.A. Allen, D. Zhou, C.S. Dence, T.L. Sharp, N.M. Fettig
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.R. Chan, A.M. Fowler, T.L. Sharp, N.M. Fettig, F. Dehdashti, J.A. Katzenellenbogen
Writing, review, and/or revision of the manuscript: S.R. Chan, A.M. Fowler, C.S. Dence, T.L. Sharp, N.M. Fettig, F. Dehdashti, J.A. Katzenellenbogen

Figure 5.
Longitudinal \[^{18}F\]FDG \(\mu\)PET/CT imaging of SSM2 (A) and SSM3 (B) mammary tumors. Top, mice bearing SSM2 (A) or SSM3 (B) tumors were scanned 1 hour after \[^{18}F\]FDG injection. % ID/g of tumor and muscle were calculated. Tumor to muscle ratio (T:M) of FDG uptake in SSM2 (n = 5 per group; A) and SSM3 (n = 6 per group; B) tumors from Sham and OVX mice before surgery (day 0) and on days 3 and 4 after surgery, ns, not significant. Bottom, 3D maximum intensity projection (MIP) from coregistered \(\mu\)PET and CT images of representative mice bearing SSM2 (A) and SSM3 (B) mammary tumors. Serial images from the same mouse in each group are shown. Arrows, tumors. Representative results of two independent studies are shown.

Figure 6.
Longitudinal \[^{18}F\]FFNP \(\mu\)PET/CT imaging of MCF7-bearing mice. Mice bearing MCF7 tumors were scanned 1 hour after \[^{18}F\]FFNP injection. Tumor to muscle ratio (T:M) of FFNP uptake in MCF7 human breast tumors before estrogen (E2) deprivation (day 0) and 6 days after E2 deprivation (day 6, \(-E\) group, n = 6). Estrogen was supplemented in the drinking water of control mice (day 0, \(+E\) group, n = 6). Representative of two independent experiments is shown. ***, \(P \leq 0.001\), ns, not significant.
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