Cell-Proliferation Imaging for Monitoring Response to CDK4/6 Inhibition Combined with Endocrine-Therapy in Breast Cancer: Comparison of $^\text{[18F]}$FLT and $^\text{[18F]}$ISO-1 PET/CT

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**Abstract**

**Purpose:** Cyclin-dependent kinase 4/6 (CDK4/6) inhibitors in combination with endocrine-therapy have emerged as an important regimen of care for estrogen receptor (ER)-positive metastatic breast cancer, although identifying predictive biomarkers remains a challenge. We assessed the ability of two PET-proliferation tracers, $^\text{[18F]}$FLT and $^\text{[18F]}$ISO-1, for evaluating response to CDK4/6-inhibitor (palbociclib) and ER-antagonist (fulvestrant).

**Experimental Design:** To determine the effect of CDK4/6 inhibition combined with estrogen-blockade, we assessed cell proliferation in six breast cancer cell lines after 1, 3, and 6 days of treatment with palbociclib and/or fulvestrant. These data were correlated to in vitro radiotracer assays and results were verified by longitudinal $^\text{[18F]}$FLT and $^\text{[18F]}$ISO-1 micro-PET imaging performed in MCF7 tumor-bearing mice.

**Results:** All palbociclib-sensitive cell lines showed decreased $^\text{[18F]}$FLT accumulation and S-phase depletion after treatment, with both measures augmented by combination therapy. In contrast, these cells showed changes in $^\text{[18F]}$ISO-1 analogue-binding and G0 arrest only after prolonged treatment. MicroPET imaging of MCF7 xenografts showed a significant decrease in $^\text{[18F]}$FLT but no changes in $^\text{[18F]}$ISO-1 uptake in all treated mice on day 3. On day 14, however, mice treated with combination therapy showed a significant decrease in $^\text{[18F]}$ISO-1, corresponding to G0 arrest, while maintaining reduced $^\text{[18F]}$FLT uptake, which corresponded to S-phase depletion.

**Conclusions:** Our data suggest complementary roles of $^\text{[18F]}$FLT and $^\text{[18F]}$ISO-1 PET in evaluating tumor-proliferation after combined CDK4/6 inhibitor and endocrine therapy in breast cancer. $^\text{[18F]}$FLT is more sensitive to immediate changes related to cell-cycle arrest and transition to G0 quiescence (3, 4). One emerging strategy to overcome this resistance involves the cross-talk between the ER pathway and cell cycle by combining endocrine therapy with cell-cycle targeted agents such as CDK4/6 inhibitors. This approach utilizes CDK4/6 inhibition to arrest cancer cells at G1, so that, when combined with ER modulators, they will synergistically inhibit cancer growth resulting in tumor cell senescence (5, 6). Palbociclib is an FDA approved oral inhibitor of CDK4/6 and is increasingly used as the first-line or second-line treatment of ER-positive metastatic breast cancer together with endocrine-based therapy in postmenopausal women (7, 8). Despite the efficacy of this combined approach and its documented impact on progression-free survival, not all patients benefit from the combination therapy. The results of the PALOMA-3 trial showed that 30 of 72 (42%) premenopausal patients with breast cancer treated with palbociclib and fulvestrant demonstrated disease progression or death (9). Additionally, although well tolerated, CDK4/6 inhibitors do carry some toxicity and may not be needed in women who would respond well to endocrine therapy alone. Biomarkers that can monitor changes in tumor proliferation early in the course of therapy to identify patients most likely to benefit from this combinational therapy versus endocrine therapy alone might therefore improve...
treatment selection for patients with ER-positive breast cancer. The preclinical study described herein was designed to test the feasibility of this approach.

Currently, Ki-67 expression in tissue is the clinical standard technique for evaluating cell proliferation, however this technique is invasive, requiring serial biopsies with potential sampling errors and underestimation of tumor heterogeneity (10, 11). Correlation of Ki-67 to cell proliferation markers such as 3’-deoxy-3’-[18F]fluorothymidine ([18F]FLT) has been shown for several tumor types including breast cancer; however, the literature is controversial (12, 13).

Molecular imaging with PET utilizing radiotracers that are precursors for DNA synthesis such as [18F]FLT is an attractive alternative. [18F]FLT targets the activity of the thymidine salvage pathway of DNA synthesis and quantifies the S-phase fraction of cycling cells, reflecting tumor proliferation (14). [18F]FLT PET has been used for monitoring treatment response in various tumor types such as lung, head and neck, and breast cancer both in preclinical and clinical studies (15, 16). However, changes in [18F]FLT retention following treatment is variable depending on the tumors proliferation rate and treatment regimen (17). A multicenter phase II ACRIN trial demonstrated a positive correlation of Ki-67 to cell proliferation markers such as [18F]FLT and [18F]ISO-1 PET/CT imaging in different tumors for predicting and monitoring tumor-proliferation changes.

Materials and Methods

Cell lines

To study the correlation between changes in tumor proliferation in response to CDK4/6 inhibition and [18F]FLT and [18F]ISO-1 activity, six human breast cancer cell lines with different ER status and a range of reported sensitivities to palbociclib (22) were selected (Supplementary Table S1). All cell lines were obtained from ATCC in the beginning of the study period (August 2017) and cultured as recommended on the ATCC website. All experiments were performed within 6 months of cell-line purchase. Phenol red-free medium supplemented with 10% charcoal/dextran-treated FBS were used for endocrine-based therapies. Experiments were carried out with cells below 85% confluence for the experiments as
the primary focus of this study was evaluation of tumor cell proliferation.

**Treatment regimen**

For each experiment, cells were plated at the desired concentration (depending on plating efficiency) and were allowed to grow for 24 hours prior to being exposed to treatment regimens. Estrogen receptor negative cells were plated and treated with 500 nmol/L palbociclib (PD0332991; SelleckChem), whereas ER-positive cells were treated with 500 nmol/L palbociclib, 100 nmol/L fulvestrant (ICI-182-780; Tocris-Bioscience), or a combination of both. Fulvestrant was included in the treatment regimen in ER-positive cells to evaluate the role of combination therapy (7). The treatment doses were selected based on the results of previous reports (23–25). Cells were treated with the indicated agents or DMSO controls for 1, 3, and 6 days and medium were replaced every other day.

**Cellular proliferation assay and immunofluorescence of Ki-67**

For cell-proliferation studies, 10,000 to 15,000 cells were seeded in 12-well plates and treated as described. At each time-point, cells were collected and cell numbers counted using Automated Cell Counter (Countess; ThermoFisher Scientific). To confirm cell growth data and to relate findings to the standard proliferation marker in the clinical setting, we investigated Ki-67 expression using indirect immunofluorescence. Cells were initially cultured on slides, treated as above, fixed in 4% paraformaldehyde, and permeabilized with 0.5% triton X-100. They were incubated with mouse anti-human Ki-67 antibody (Invitrogen) followed by goat–anti-mouse Alexa Fluor-488 conjugated secondary antibody (Invitrogen).

Fluorescent sections were then imaged on a Zeiss Axio Observer (The Netherlands) and Zeiss Zen software was used for quantification of Ki-67 labeling index (percent of positively-labeled/negatively-labeled cells). An average of four regions was used for calculations.

**Cell-cycle analysis**

Cell-cycle analysis was used in cell-line studies to track the different impact of palbociclib versus fulvestrant on the cell cycle, which is incompletely captured by the Ki-67 assay. Cell-cycle distribution was assessed using FACScalibur flow cytometer (BD-Immunocyte Systems). A multiplexed staining technique was used to enable a targeted assessment of cell-cycle changes to reflect the contribution of each drug in growth inhibition. We used EdU for S-phase assessment and Ki-67 to estimate G0 versus G1 accumulation for assessing quiescence (26). Expression of Ki-67 has been reported during active phases of cell cycle (G1, S, G2, M phases), accordingly Ki-67 negative population in G1–G0 region can represent G0 phase (27). Briefly, 20,000 to 40,000 cells were plated in six-well plates and after the treatment period, cells were pulse labeled with EdU (ThermoFisher Scientific) for 1-hour before harvesting. Harvested cells were fixed in 70% ethanol (1 x 10^6 cells/mL) and stored at −20°C until analysis (28). Fixed cells were initially stained with Alexa Fluor 488 anti-human Ki-67 Antibody (Biolegend) according to the manufacturer’s protocol and subsequently stained with propidium iodide (Sigma-Aldrich). Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (ThermoFisher Scientific) was used for accurate measurement of changes in S-phase. Data were analyzed with FlowJo software (Tree Star).

**Western blot analysis**

TMEM97 is a conserved integral membrane protein that was recently identified as containing the σ2-receptor. It has been unofficially renamed as “TMEM97/σ2-receptor,” and has been shown to have important role in regulating cell survival, morphology, and differentiation (29). Accordingly, protein expression levels of TMEM97 was evaluated as a biochemical surrogate assay for quantifying σ2 receptors and correlated to [18F]ISO-1 ligand binding which is a measure of receptor density. For this purpose, cells were plated in 150-mm dishes and treated similar to prior experiments and were harvested and lysed in RIPA-lysis buffer. Protein content was separated by gel electrophoresis. TMEM97 expression was detected using rabbit anti-human TMEM97 antibody (AbiViva System-Biology). The intensity of band was normalized using ratio relative to β-actin as a housekeeping gene.

**[18F]FLT uptake**

[18F]FLT was prepared as a service by our institution’s cyclotron facility, as previously described (30). The radiochemical purity of [18F]FLT was >95% as analyzed with analytical HPLC. Specific activity of [18F]FLT ranged from (5,500 Ci/mmol) 203,500 GBq/mmoll to (8,000 Ci/mmol) 296,000 Ci/mmoll. To measure the impact of treatments on [18F]FLT trapping, we measured cell uptake as a function of tracer exposure time in culture. Cells were plated in 96-well plates at 10,000 to 25,000 cells per well and treated according to the described protocol (14). [18F]FLT (20 kBq/well) was added and incubated at 37°C for 5, 10, 15, 30, 45, and 60 minutes. Subsequently, radioactivity was assayed on Wizard automatic gamma-counter (PerkinElmer).

**[125I]RHM-4 σ2-ligand-binding assay**

[125I]RHM-4, chemical analogue of [18F]ISO-1, is a σ2-receptor ligand that is used for in vitro pharmacologic assays (31). Given the similar binding properties of [18F]ISO-1 and [125I]RHM-4 and their equivalence in prior in vitro studies (32) and longer half-life of 125I (59-days vs. 109-minutes), [125I]RHM-4 has been used as a practical analogue for in vitro binding studies. [125I]RHM-4 was prepared by an iododeannylation reaction of the corresponding tributyltin precursor (33). Cells were cultured in 150-mm dishes (500,000–1.5 x 10^6 cells per plate, depending on plating efficacy and growth rate of cell line) and treated according to the protocol. The cells were mechanically scraped, homogenized, followed by saturation binding assay. This approach is tailored to the properties of [125I]RHM-4 (and [18F]ISO-1) as tracers of receptor-mediated binding and has shown to be an optimal evaluation of σ2-receptor ligand uptake (34). Briefly, the cell membranes were incubated with [125I]RHM-4 (0.02–9 nmol/L) for 90 minutes and the nonspecific binding was defined. Membrane-associated receptor bound ligands were filtered, collected, and counted on a Perkin-Elmer Wizard gamma-counter. The equilibrium dissociation constant (K_D) and maximum number of binding sites (B_max) were determined by a nonlinear regression analysis using Prism GraphPad (version 6.0).

**Xenograft model of human breast cancer**

To evaluate the performance of [18F]FLT and [18F]ISO-1 PET in predicting response to cell-cycle targeted treatment in the preclinical setting, we selected the MCF7 xenograft model, which is an ER-positive cell line with high sensitivity to both fulvestrant and palbociclib, as confirmed by our in vitro studies. All animal
experiments were approved by the Institutional Animal Care and Use Committee. Female SCID mice (6-week-old) were purchased from Charles River Laboratories (Wilmington, MA) and housed in laminar flow cabinets under specific pathogen-free conditions. The body weight and behaviors of mice were monitored on regular basis. Mouse xenograft models were prepared by implanting an estradiol pellet (0.72 mg, 60-day release; Innovative Research) 72 hours prior to the subcutaneous injection of 8 to 10 × 10^6 MCF7 cells in 200 μL of DMEM:Matrilix (BD-Biosciences) in the left flank. All invasive procedures were performed under general anesthesia using isoflurane. Tumor growth was monitored with caliper measurement and volume was calculated as V = (length × width^2)/2. Once the tumors reached an average volume of 100 mm^3 (6 weeks), the tumor-bearing mice were randomized into four groups (n = 4) and treated with vehicle (normal saline), palbociclib, fulvestrant (Faslodex; AstraZeneca-Pharmaceuticals), or combination of both for 14 days (23, 24). Palbociclib was administered daily via oral gavage (75 mg/kg). Although this differs from the cyclical use of CDK4/6 inhibitors in patients, prior publication in mouse model support the efficacy of this approach (24, 35). Fulvestrant was injected intramuscularly (5 mg/mice) weekly (35, 36). For the combination-treatment group, mice received both agents. The design of the study was longitudinal and [18F]FLT and [18F]ISO-1 PET/CT scans were performed in the same animals at baseline, then repeated on days 3 and 14 of treatment.

\[ \text{SUV}_{\text{max}} \], which is commonly used in both clinical and preclinical studies (20, 38) and supported by mouse model support the efficacy of this approach (24, 35). Fulvestrant was injected intramuscularly (5 mg/mice) weekly (35, 36). For the combination-treatment group, mice received both agents. The design of the study was longitudinal and [18F]FLT and [18F]ISO-1 PET/CT scans were performed in the same animals at baseline, then repeated on days 3 and 14 of treatment.

\[ \text{SUV}_{\text{max}} \] of PET and CT images. Circular-3D region of interest (ROI) were drawn manually to assess the maximum tracer accumulation in the tumors and muscles were frozen, cryo-sectioned (10-μm thick), and exposed to a phosphor imaging plate to assess the distribution of radioactivity. Consecutive tissue slices were also prepared for immunofluorescent study as described below. Digital autoradiography of tissue sections was performed after overnight exposure (Typhoon FLA7000; GE-Healthcare Biosciences). The tumor-to-muscle ratio was calculated for each radiotracer (41). These analyses were performed on four to six tumor sections for each animal.

Xenograft tissue analysis

Immunofluorescent staining: For tissue analysis of markers of proliferation, we stained frozen tumor sections for Ki-67 and TUNEL. Frozen sections were fixed in 4% paraformaldehyde and after permeabilization, the cells were incubated with mouse anti-human Ki-67 antibody (Santa Cruz Biotechnology) and rabbit anti-human TUNEL antibody (Invitrogen). Alexa Fluor 568 conjugated secondary antibody (Invitrogen) were then applied. Ki-67 and TUNEL labeling index was calculated as above.

Flow-cytometric analyses: Portion of freshly excised tumors were minced and dissociated with an enzyme cocktail consisting of 0.04% collagenase (Gibco) and 0.05%DNAase I (Worthington Biochemical) followed by digestion with 0.25% trypsin/EDTA (Gibco). The suspension was then filtered through 40 μm cell strainer to obtain single cell suspension. Flow cytometric analysis of DNA content and Ki-67 was performed based on simultaneous analysis of Ki-67 and propidium iodide (27). The percentage of cells in Go/G1, S-phase were calculated and correlated to changes in [18F]ISO-1 and [18F]FLT PET/CT, respectively.

Statistical analysis

Data were expressed as mean ± SE. For in vitro phase, Mann–Whitney U test was used to determine statistical significance between experimental and control groups. Longitudinal [18F]FLT and [18F]ISO-1 uptake parameters were compared within and between cohorts using the nonparametric Wilcoxon rank-sum test, respectively. Correlations were calculated using linear regression, and expression as Pearson correlation coefficients. Statistical analysis was performed using SPSS statistic software package. For all experiments, P < 0.05 was considered significant.

Results

Concurrent inhibition of CDK4/6 and ER leads to growth delay and decreased tumor cell proliferation in ER-positive cell lines

Combination treatment with palbociclib and fulvestrant in MCF7 and MDA-MB134 resulted in significant inhibition of proliferation on day 1 (Fig. 2A). On day 3, treatment with palbociclib demonstrated a significant growth inhibition in MCF7 and MDA-MB134. Inhibition of ER enhanced the antiproliferative effect of palbociclib (P = 0.009 for MDA-MB134 and P = 0.012 for MCF7), indicating additive effects compared with individual agents. For example, only the combination of palbociclib and fulvestrant showed significant growth inhibition at day 1, and combined therapy showed a greater reduction in Ki-67 on day 6 compared with either agents alone. There were no significant differences in Ki-67 labeling index on day 1 of treatment. However, changes in Ki-67 correlated with cell-proliferation assay on day 3 and 6 (r = 0.78 and 0.89 for MCF7, respectively). There was significant reduction in Ki-67 expression on day 6 of combination therapy in MCF7 and MDA-MB134 when compared with...
palbociclib alone (Fig. 2B), confirming the enhanced growth inhibitory effect of combination-therapy ($P = 0.040$ for MDA-MB134 and $P = 0.020$ for MCF7). ER-negative cell lines revealed significant growth inhibition in HCC38 and MDA-MB231 after 1 day of treatment ($P = 0.006$ and 0.008, respectively; Supplementary Fig. S1A). In agreement with findings of Finn and colleagues (22), treatment with 500 nmol/L palbociclib did not have significant effects on proliferation or Ki-67 expression of HCC1806 and UACC732 (Fig. 2; Supplementary Fig. S1).

ER-modulator fulvestrant, in combination with CDK4/6 inhibitor palbociclib, induces cell-cycle arrest at G1, which progresses to G0 arrest over time. Early and persistent G1 arrest with associated S-phase depletion was observed in the ER-positive cell lines, MCF7 and MDA-MB134, after palbociclib and to lesser extent fulvestrant on day 1 (Fig. 3). There was a time-dependent increase in number of cells in G0 in all treated groups. After 3 days of treatment with fulvestrant, a notable increase in the fraction of cells in G0 was observed. Addition of palbociclib to fulvestrant led to further increase in percentage of cells accumulating in G0. Combination therapy resulted in significantly higher G0 accumulation in MCF7 cells when compared with palbociclib or fulvestrant alone on day 6 (34% vs. 11%, $P = 0.001$ for palbociclib and 34% vs. 22%, $P = 0.016$ for fulvestrant). Palbociclib resulted in early arrest at G1 to S transition resulting in S-phase depletion in HCC38 and MDA-MB231 on day 1 ($P < 0.0001$). There was subsequent increase in the percentage of cells in G0 after 3 days of treatment (HCC38, 10% vs. 3%; $P = 0.005$ and MDA-MB231, 11% vs. 3.4%; $P = 0.001$), which was further augmented on day 6 (Supplementary Fig. S2). The cell-cycle distribution of the palbociclib-resistant UACC732 was not affected by treatment with palbociclib; fulvestrant resulted in small but significant S-phase reduction ($P = 0.012$ on day-1).

In vitro cellular $[^{18}F]$FLT uptake measures early changes in cell cycle in breast cancer cell lines: correlation with S-phase depletion

Incubation with palbociclib resulted in marked decrease in $[^{18}F]$FLT uptake in the sensitive cell lines as early as day 1 ($P < 0.0001$; Fig. 4A). In ER-positive cell lines, fulvestrant induced a moderate uptake reduction ($-42\%$ for MCF7 and $-52\%$ for MDA-MB134, on day 1). The combination therapy groups showed enhanced reduction, irrespective of incubation time ($-95\%$ for MCF7 and $-93\%$ for MDA-MB134, on day 1; Supplementary Table S2). However, differences in uptake when comparing palbociclib with combination therapy group were not significant. The decline in $[^{18}F]$FLT uptake correlated significantly with flow cytometric S-phase depletion (e.g., $r = 0.98$, $P < 0.0001$ for MCF7 on day 3). The results of $[^{18}F]$FLT uptake also correlated with Ki-67 expression on day 3 and 6 of treatment (Supplementary Table S3; $r = 0.74$ and 0.69, $P = 0.009$ and $P = 0.01$ for MCF7 on day 3 and 6, respectively). Similarly, treatment with palbociclib lead to marked decrease in $[^{18}F]$FLT uptake in the sensitive ER-negative cell lines ($P < 0.0001$; Supplementary Fig. S3, panel I).

It is notable that the magnitude of changes in $[^{18}F]$FLT uptake and S-phase were different from changes in Ki-67 expression. For example, 3 days of combination therapy in MCF7 cells resulted in 37% reduction in Ki-67 expression whereas there was 94% and 97% decrease in $[^{18}F]$FLT uptake and S-phase, respectively. This was expected given that palbociclib induces G1 arrest, which would diminish S-phase, and thus $[^{18}F]$FLT uptake, but not Ki-67, which is positive in all phases of the cell-cycle besides G0 (27).

Figure 2.

In vitro antiproliferative effect of 500 nmol/L palbociclib (PD), 100 nmol/L fulvestrant (Fulv), or combination of both in ER-positive cell lines: (A) cell proliferation assay and (B) Ki-67 expression. *, $P < 0.05$; #, $P < 0.01$; and +, $P < 0.001$, compared with control group of the same treatment period.
In vitro $\sigma_2$-receptor binding reflects delayed treatment changes in cell cycle, corresponding to $G_0$ accumulation

We evaluated saturation binding of $[^{125}\text{I}]$RHM-4 on day 3 and 6 of treatment in sensitive cell lines. On day 3, we did not identify significant differences in $K_d$ and $B_{\text{max}}$ values between treated cells and controls. However, there was significant reduction in $B_{\text{max}}$ after 6 days of treatment (Fig. 4B; Supplementary Fig. S3, panel II, $P = 0.002$ and $P = 0.01$; combination therapy vs. controls of MDA-MB134 and MCF7, respectively), indicating a reduction in $\sigma_2$ expression. Accordingly, in MCF7 and MDA-MB134 cells, 6 days of treatment with combination therapy resulted in significant reduction in TMEM97 expression, in line with the results of $[^{125}\text{I}]$RHM-4 binding (Fig. 4B). The results of $[^{125}\text{I}]$RHM-4 binding also correlated with Ki-67 expression on day 6 ($r = 0.68$, $P = 0.01$ for MCF7). Furthermore, HCC38 and MDA-MB231 treated with palbociclib for 6 days resulted in a...
significant decrease in TMEM97 expression. Palbociclib-resistant cells, HCC1806 and UACC732, did not show a significant difference in TMEM97 expression compared with controls.

\[18\text{F}]\text{FLT PET assesses early treatment response in MCF7 xenografts after 3 days of treatment with CDK4/6 inhibitor and/or ER modulator.}

There was a significant increase in tumor size only in control mice on day 14 (P = 0.02; Supplementary Fig. S4). Mean baseline \([18\text{F}]\text{FLT/T/M ratio was 1.98 ± 0.37 with a significant reduction in treated tumors, on day 3 (42% reduction in FLT T/M for palbociclib, } P = 0.009, 25% reduction for fulvestrant, } P = 0.01, \) and 44% reduction for combination group, } P = 0.004; Fig. 5, panel A and C). Follow-up \([18\text{F}]\text{FLT/CT on day 14 revealed a persistent reduction in T/M ratio in all the treated mice, when compared with controls (40% decrease in uptake in combination therapy group).}

\[\text{Delayed changes in [18F]ISO-1 PET correlate with tumor quiescence status in MCF7 xenografts after 14 days of combination therapy.}

Baseline \([18\text{F}]\text{ISO-1 T/M ratio was 2.64 ± 0.69. Three days of treatment with palbociclib, fulvestrant, or combination therapy did not result in significant changes in [18\text{F}]\text{ISO-1 T/M (P = 0.99, 0.45, and 0.34, respectively; Fig. 5, panel II A and C). On day 14, combination therapy caused significant reduction in uptake to 1.87 ± 0.12 (27% mean reduction; } P = 0.021). On day 14, T/M ratio in palbociclib and fulvestrant monotherapy groups was 2.24 ± 0.89 and 2.1 ± 0.32, respectively, which was only slightly lower compared with baseline (7% and 13% reduction, respectively), and the difference was not significant (P = 0.086 and 0.051, respectively). We note that modest T/M ratio in for \([18\text{F}]\text{ISO-1 in the MCF-7 xenograft model limits contrast recovery from microPET images. This effect is better demonstrated in the tumor autoradiographic data described below.

\[\text{Inhibition of ER enhances the antiproliferative effect of palbociclib in vivo: correlation of microPET/CT findings with autoradiography and tissue biomarkers of proliferation.}

Two tumors from each group were assessed by \textit{ex vivo} autoradiography of \([18\text{F}]\text{FLT and [18\text{F}]ISO-1.}

Tumor-to-muscle ratios quantified from \textit{ex vivo} analysis supported PET/CT findings. \([18\text{F}]\text{ISO-1 uptake revealed a decreasing trend in all treated groups with significantly lower uptake in fulvestrant and combination therapy mice (Fig. 6A). A significantly lower \([18\text{F}]\text{FLT uptake was observed in all treated mice without any appreciable difference across the treatment groups (Fig. 6B). Treatment with palbociclib or fulvestrant led to a significant decrease in Ki-67 and TMEM97 on day 14 (Fig. 6D) with pronounced reduction in the combination therapy mice. To further evaluate whether the changes in \([18\text{F}]\text{FLT and [18\text{F}]ISO-1 corresponded to the markers of proliferation, we correlated the Ki-67 and TMEM97 scoring with autoradiographic T/M ratio of both tracers (Fig. 6C). We detected a strong correlation between Ki-67 and \([18\text{F}]\text{FLT uptake (r = 0.87), whereas TMEM97 and Ki-67 demonstrated a moderate correlation with \([18\text{F}]\text{ISO-1 uptake (r = 0.57 and 61, respectively; Supplementary Fig. S5B and S5C). Cell-cycle distribution of the tumors was affected by all three treatment regimens, demonstrating significant S-phase depletion in all treatment groups when compared with controls (Fig. 5, panel IB). Palbociclib-treated tumor cells experienced a mild but significant arrest in G0 (P = 0.022 vs. control). Treatment with fulvestrant resulted in significant arrest in G0 (P = 0.008 vs. control). The combination therapy led to enhancement of the G0 quiescence (34% in combination vs. 19% in palbociclib group; } P = 0.031 and 23% in fulvestrant group; } P = 0.014; Fig. 5, panel IB). The changes in \([18\text{F}]\text{FLT uptake correlated with S-phase depletion on flow cytometry (r = 0.78), in line with our \textit{in vitro} results. We also noticed a reverse correlation between G0 arrest and \([18\text{F}]\text{ISO-1 uptake (r = 0.57).}

\[\text{Discussion}

In this preclinical study, we explored a potential strategy for predicting response to combination therapy with palbociclib and fulvestrant in breast cancer. We assessed two proliferation radiotracers, \([18\text{F}]\text{FLT and [18\text{F}]ISO-1, with the hypothesis that the combination of imaging biomarkers might be able to discern distinct patterns of response for the individual agents and their combined use. Several studies have evaluated the performance of the individual tracers (15, 17, 19) but this study is the first to evaluate both tracers side-by-side. We analyzed the antiproliferative effect of combined CDK4/6 inhibition and ER modulation on a panel of breast cancer cell lines and found that both \([18\text{F}]\text{FLT and [18\text{F}]ISO-1 measure the changes in cell cycle, which correspond to cell-line drug sensitivity, but with different magnitude and time course. We noted that while \([18\text{F}]\text{FLT provides a larger indication of response to the individual agents, [18\text{F}]ISO-1 had superior performance in identifying the added effect of combining an ER-targeted agent with CDK4/6 inhibitor. Next, a MCF7 xenograft using cancer model was used to prove the potential of the individual radiotracers}]

\[\text{PET Imaging of Cell Proliferation in Breast Cancer

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Figure 5.

Panel I (FLT PET/CT)

A, Quantitative tumor uptake as T/M ratio in controls and treated mice, showing persistent reduction of T/M ratio in treated mice on days 3 and 14.

B, Changes in S-phase fraction relative to controls on day 14 of treatment, demonstrating comparable S-phase reduction across different treatment groups.

C, Representative PET/CT images of one mouse from the combination treatment group, demonstrating dramatic decline of [18F]FLT uptake on day 3, which is persistent on day 14.

Panel II (ISO-1 PET/CT)

A, Quantitative tumor uptake as T/M ratio in controls and treated mice, showing delayed reduction of T/M ratio in treated mice, seen only on day 14.

B, G0 arrest relative to controls on day 14 of treatment, more pronounced in the combination therapy group.

C, Representative PET/CT images of one mouse from the combination treatment group, demonstrating delayed decline in [18F]ISO-1 uptake on day 14.

Figure 5.
Panel I: [18F]FLT uptake after treatment with palbociclib (PD), fulvestrant (Fulv), or combination of both at baseline and days 3 and 14 of treatment. A, Quantitative tumor uptake as T/M ratio in controls and treated mice, showing persistent reduction of T/M ratio in treated mice on days 3 and 14. B, Changes in S-phase fraction relative to controls on day 14 of treatment, demonstrating comparable S-phase reduction across different treatment groups. C, Representative PET/CT images of one mouse from the combination treatment group, demonstrating dramatic decline of [18F]FLT uptake on day 3, which is persistent on day 14.

Panel II: [18F]ISO-1 uptake after treatment with palbociclib (PD), fulvestrant (Fulv), or combination of both at baseline and days 3 and 14 of treatment. A, Quantitative tumor uptake as T/M ratio in controls and treated mice, showing delayed reduction of T/M ratio in treated mice, seen only on day 14. B, G0 arrest relative to controls on day 14 of treatment, more pronounced in the combination therapy group. C, Representative PET/CT images of one mouse from the combination treatment group, demonstrating delayed decline in [18F]ISO-1 uptake on day 14.
The early effect of palbociclib in cells was arrest of G1 to S progression with drastic S-phase depletion and G1 accumulation on day 1 in all palbociclib-sensitive cell lines, regardless of ER status. In agreement with these findings, $[^{18}F]$FLT uptake in cells reduced after short-term exposure to palbociclib and/or fulvestrant in palbociclib-sensitive and/or ER-positive cell lines, respectively. Addition of fulvestrant in ER-positive cells led to gradual G0 accumulation that corresponded with time-dependent arrest at G0–G1 starting after 3 days of treatment, as indicated by cell-cycle assays in the cell-line studies. When treatment was continued for 6 days, substantial progression of cells to G0 was achieved in the ER-positive luminal-A cells, especially for combined therapy, whereas maintaining S-phase depletion. Although, the changes in $[^{18}F]$FLT uptake mirrored S-phase changes, it failed to predict the subsequent changes due to delayed G0 arrest. Accordingly, for combined therapy, $[^{18}F]$FLT predominantly reflected the contribution of palbociclib whereas changes due to fulvestrant remains under-valued. To address this shortcoming, we interrogated the associated cell-cycle changes and their influence on $\sigma_2$-receptor radiotracers to assess whether this technique can predict transition of cells from proliferating status to quiescence. In our study, changes in $[^{125}I]$RHM-4 binding was time dependent and a significant reduction was noted only on day 6 in ER-positive luminal cells treated with combinational therapy. These findings demonstrated a significant reversed correlation of $[^{125}I]$RHM-4 binding with accumulation of cells in G0 on day 6, indicating induction of quiescence mainly due to the contribution of fulvestrant and its synergy with palbociclib.

We selected MCF7 cell line, for in vivo testing in xenograft models as the best available cell-line representative of the most common form of breast cancer in the clinical practice, the luminal-A subtype (46). We performed PET/CT at baseline and after 3 and 14 days of treatment to reflect the immediate and delayed posttreatment changes that were noted in vitro. Significant reduction in $[^{18}F]$FLT T/M uptake was noticed in all the treated groups.
mice both on day 3 and 14 of treatment (approximately 18%–50% reduction), correlating to S-phase reduction, corresponding to in vitro findings, supporting the role of \([18F]\)FLT as an early indicator of therapeutic efficacy. Despite the robust posttreatment changes in \([18F]\)FLT, we did not detect any significant differences between our treatment groups and also different timelines, indicating a limitation of \([18F]\)FLT as a stand-alone biomarker to identify the contribution of each component of the combinational therapy. Our results revealed no significant change in \([18F]\)ISO-1 uptake after 3 days of treatment, despite the dramatic \([18F]\)FLT changes. The simplest explanation for this observation is that the short-term treatment leads to early changes in S-phase fraction and arrest at G1. Continued exposure leads to transition to an increase in the fraction of cells in the quiescent (G0) phase, not well reflected by \([18F]\)FLT. However, the increase in quiescence was well measured by \([18F]\)ISO-1 PET imaging, showing a significant decline in uptake after 14 days of combinational therapy correlated with G0 arrest on tissue analysis.

Histologic assessments of proliferation markers validated the strong correlation between \([18F]\)FLT uptake and Ki-67 expression after 14 days of treatment, in line with previous reports (47). Expression of TMEM97 also paralleled the uptake of \([18F]\)ISO-1, which confirmed the PET results by indicating that the decrease in tracer uptake was due to a real change in expression of proliferation marker, \(\alpha\)-receptor, and not a consequence of decreased delivery of radiotracer. However, the correlation between TMEM97 labeling index and \([18F]\)ISO-1 T/M ratio was only moderate that might be due to the fact that we used a tissue-based technique by immunofluorescence for quantification of TMEM97 and future studies using more direct quantification technique are required before drawing a conclusion.

Our study has several limitations. The animal model in this study was used as a proof of concept and only a single cell line was used. We note that MCF-7 cells are somewhat modestly proliferative and larger changes may be noted in more proliferative human tumors. We also note that the daily administration of palbociclib used in this study differs from cyclic use in patients (9) and may need to be considered in translation of the results to patient studies. We also noted modest T/M ratios for \([18F]\)ISO-1 in our MCF-7 xenograft, which might also be increased in human breast cancer (20). Additionally, optimizing the timing of sequential PET imaging was challenging to ensure timely imaging to reflect posttreatment changes at the similar interval for both radiotracers and at the same time avoid contamination of signals. In order to minimize the effect of first radiotracer on the subsequent set of imaging we allowed 18 to 20 hours interval to allow decay of the tracer. These issues will need to be considered if the approach is tested in future patient studies. It is notable that the proliferation tracers used in this study are not specific to BC or the mentioned combinational treatment and the xenograft model is a proof of concept for validating the in vitro behavior of each tracer. This concept can be applied in the setting of other cell-cycle targeted treatment regimens in oncology.

\([18F]\)FLT and \([18F]\)ISO-1 PET probes can evaluate the time-course of changes that occurs in response to cell-cycle targeted therapy targeting CDK4/6, endocrine agents, and their combination in a preclinical model of breast cancer. Comparison of the two radiotracers revealed that \([18F]\)FLT measures immediate changes in S-phase as a predominate effect of targeting CDK4/6, providing a very early prediction of tumor response, whereas \([18F]\)ISO-1 can assess relatively delayed changes reflecting cell-cycle arrest and transition to quiescence. Future studies are needed to determine the timing of \([18F]\)ISO-1 and \([18F]\)FLT PET/CT imaging for prediction and monitoring tumor-proliferation changes in the setting of cell-cycle targeted therapy; however, these early results indicate the potential of this approach to guide combined CDK4/6 inhibitors and anti-ER therapy in breast cancer and merit further preclinical studies and early-phase clinical studies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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