Changes in Tumor Metabolism as Readout for Mammalian Target of 
Rapamycin Kinase Inhibition by Rapamycin in Glioblastoma

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Abstract  

Purpose: Inhibition of the protein kinase mammalian target of rapamycin (mTOR) is being evaluated for treatment of a variety of malignancies. However, the effects of mTOR inhibitors are cytostatic and standard size criteria do not reliably identify responding tumors. The aim of this study was to evaluate whether response to mTOR inhibition could be assessed by positron emission tomography (PET) imaging of tumor metabolism.

Experiment Design: Glucose, thymidine, and amino acid utilization of human glioma cell lines with varying degrees of sensitivity to mTOR inhibition were assessed by measuring in vitro uptake of [18F]fluorodeoxyglucose ([18F]FDG), [18F]fluorothymidine ([18F]FLT), and [3H]L-tyrosine before and after treatment with the mTOR inhibitor rapamycin. The tumor metabolic activity in vivo was monitored by small-animal PET of tumor-bearing mice. The mechanisms underlying changes in metabolic activity were analyzed by measuring expression and functional activity of enzymes and transporters involved in the uptake of the studied imaging probes.

Results: In sensitive cell lines, rapamycin decreased [18F]FDG and [18F]FLT uptake by up to 65% within 24 hours after the start of therapy. This was associated with inhibition of hexokinase and thymidine kinase 1. In contrast, [3H]L-tyrosine uptake was unaffected by rapamycin. The effects of rapamycin on glucose and thymidine metabolism could be imaged noninvasively by PET. In sensitive tumors, [18F]FDG and [18F]FLT uptake decreased within 48 hours by 56 ± 6% and 52 ± 8%, respectively, whereas there was no change in rapamycin-resistant tumors.

Conclusions: These encouraging preclinical data warrant clinical trials evaluating [18F]FDG and [18F]FLT-PET for monitoring treatment with mTOR inhibitors in patients.

Glioblastoma multiforme is the most common malignant primary brain tumor in adults. Despite advances in diagnosis and standard therapies such as surgery, radiation, and chemotherapy, the prognosis remains poor (1, 2).

Mammalian target of rapamycin (mTOR) is a key signaling molecule involved in the growth of glioblastoma and other tumor types (2). It is a serine-threonine kinase that functions in the phosphatidylinositol 3'-kinase/Akt signaling pathway (3) by integrating extracellular signals (growth factors and hormones) with amino acid availability, and intracellular energy status to control translation rates and metabolic processes (4). In response to mitogenic stimuli, mTOR regulates the phosphorylation of p70S6 kinase, promoting translation of proteins involved in cellular proliferation, activation of angiogenesis, and metastasis formation.

Rapamycin is an antibiotic that, after binding to FKBP-12 (FK506 binding protein), inhibits mTOR function with affinity and specificity (5). It has been used for several years as an immunosuppressive agent (6). More recently, rapamycin has been identified as a cytostatic agent with significant activity in various human cancer cell lines and tumor models (7). Based on these data, phase I, phase II, and phase III trials are being conducted for evaluation of rapamycin and its derivatives in a variety of cancers (3, 7), including glioblastomas (8, 9).

Although there is a strong rationale and preclinical evidence for the effectiveness of rapamycin as an antitumor agent, clinical trials evaluating rapamycin therapy have been limited by less than ideal techniques for assessing tumor responses to cytostatic drugs that might not, or only slowly, induce tumor shrinkage. Thus, conventional size criteria (WHO or Response Evaluation Criteria in Solid Tumors) are not readily applicable to rapamycin therapy and may underestimate the effectiveness of treatment (10). In this study, we evaluated whether positron emission tomography (PET) imaging of tumor metabolism may be used to assess tumor response to rapamycin early in the course of therapy. This approach is based on several studies that show that mTOR activity plays an important role in glucose and amino acid metabolism (11–14) and leads to cell cycle arrest in the G1 phase (15, 16). Thus, we hypothesized that blocking of mTOR signaling by rapamycin may modulate glucose and amino acid utilization as well as thymidine uptake of cancer cells. These effects of rapamycin could be imaged clinically by
PET. Glucose metabolism of cancer is assessed by PET with the glucose analogue [18F]fluorodeoxyglucose ([18F]FDG). In addition, several radiolabeled amino acids and amino acid analogues are available that permit imaging of tumor amino acid transport (17). Inhibition of cellular proliferation by rapamycin could potentially be imaged with the thymidine analogue [3H]fluorothymidine ([3H]FLT; ref. 18), because accumulation of [3H]FLT has been shown to be low during the G1 phase (19). Thus, PET imaging with readily available imaging probes could provide an early readout for rapamycin activity in clinical trials. The objectives of the present study were to dissect in cell culture the effects of rapamycin on tumor cell metabolism and to use animal models to determine which imaging probe is best suited for monitoring rapamycin effects in vivo.

Materials and Methods

Cell lines and reagents. Rapamycin was obtained from LC Laboratories and dissolved in ethanol. Human malignant glioma cell lines U87 (with PTEN loss), LN-229 and LN-18, both with wild-type PTEN (20), were purchased from American Type Culture Collection. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen), 4 mmol/L glutamine, and 100 units/ml penicillin/streptomycin at 37°C in 5% CO2.

Growth curve analysis. Cells were plated in six-well plates (Costar) at 1 x 105 per well. After 24 h, different concentrations of rapamycin (0.1-10 mmol/L) were added to each well. Viable cell numbers from triplicate wells were determined every 24 h for 3 d by trypsin blue exclusion using the Vi-Cell XR instrument (Beckman Coulter).

Western blot analysis. Immunoblotting was done on 20 μg of total protein from either whole-cell lysates or membrane extracts. After SDS-PAGE, the protein was electrophoretically transferred to a polyvinylidene difluoride membrane at 100 V for 1 h at room temperature and then blocked using 5% (w/v) evaporated milk in TBS containing 0.01% (v/v) Tween 20 (TBST) for 1 h at room temperature. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies. The next day, the blots were washed in TBST buffer and incubated with goat anti-mouse or goat anti-rabbit horseradish peroxidase–conjugated secondary antibody for 1 h at room temperature. After washing in TBST buffer (three times, 10 min each wash), the immunoreactive proteins were finally visualized using ECL Plus (Amersham Biosciences). The antibodies included anti-human phospho-p70s6, p70S6, phospho-S6kinase, S6kinase, PTEN, thymidine kinase 1 (TK1), and β-actin (Cell Signaling Technology). They also included anti-human GLUT-1 and GLUT-3, Na,K-ATPase (Abcam), and CD98 [human amino acid transporter heavy chain (4F2hc)].

In vitro tracer uptake studies. Synthesis and quality control of [18F]FDG and [18F]FLT were conducted by the University of California at Los Angeles Cyclotron Facility, as previously described (21). For both [18F]FDG and [18F]FLT, the radiotracer purity was >99% and the specific activities were >1,000 Ci/mmol. 3-0-[methyl-3H]glucose (3-OMeG; specific activity, 60 Ci/mmol) and [3H]-tyrosine (specific activity 60 Ci/mmol) were obtained (Moravek Biochemicals). For all uptake studies, cells were plated in six-well plates (Costar) at 1 x 105 cells per well 1 d before the experiment. All experiments were done in triplicate and repeated at least once.

To measure [18F]FDG and [18F]FLT uptake, fresh cell culture medium containing [18F]FDG or [18F]FLT at 1 μCi/ml was added to each well. Glucose-free medium was used for [18F]FDG uptake studies. Cells were then incubated with [18F]FDG or [18F]FLT at 37°C for 60 min. Cells were then rinsed twice in ice-cold PBS and then harvested with trypsin to determine cell-associated fluorescence-18 radioactivity, using a Packard 5600 gamma counter (Packard).

For measurement of glucose transport, we used [3H]3-OMeG. 3-OMeG is a glucose analogue that cannot be phosphorylated by hexokinase (22); hence, the initial transport rate of 3-OMeG is a measure of glucose transport activity. Cells were incubated with [3H]3-OMeG (1 μCi/mL) and cold 3-OmeG from 10 s to 3 min at a final concentration of 0.05 mmol/L 3-OMeG, as previously described (22). Cells were then washed with ice-cold PBS supplemented with 100 mmol/L phosphate and lysed with 0.1% SDS. The cell lysates were collected; scintillation fluid (ICN) was added; and [3H] radioactivity was measured in a Packard 2-counter. Time-activity curves for cellular uptake of 3-OMeG were fitted by using Graph Pad Prism 4.0 (Graph Pad Software).

For measurement of [3H]-tyrosine, uptake studies were done with Na+-free uptake medium [125 mmol/L choline-Cl, 4.8 mmol/L KCl, 1.3 mmol/L CaCl2, 1.2 mmol/L MgSO4, 25 mmol/L HEPES, 1.2 mmol/L KH2PO4, and 5.6 mmol/L glucose (pH 7.4)] and pre-incubated for 10 min at 37°C. The medium was then replaced by medium containing [3H]-tyrosine at 1 μCi/mL. Following a 5-min uptake, cells were washed and radioactivity uptake was measured as described for 3-OMeG. Uptake studies were done in the presence or absence of sodium and an inhibitor of L-type amino acid transporter, 2-aminobicyclo(2,1,1)-heptane-2-carboxylic acid (BCH). Uptake of all tracers was quantified as both disintegrations per minute per 106 viable (trypan blue negative) cells and as a ratio of the intracellular activity concentration and the activity concentration in the medium (cell to medium ratio) as previously described (19, 23). The cell to medium ratio describes to which extent a tracer has been concentrated within a cell and is comparable with a tumor to background ratio in a PET study. Both variables (uptake per viable cells and cell to medium ratio) showed similar changes in response to rapamycin therapy. Therefore, only the results based on the cell to medium ratio are shown in the following sections.

Cell cycle analysis. Cells were stained with propidium iodide (Calbiochem) and RNase A (Sigma) in a hypotonic buffer. The DNA content was then analyzed by a FACScan flow cytometer (Becton Dickinson) using the CellQuest 3.1 software (Becton Dickinson) for acquisition and the ModFit LT 2.0 software (Verity) for analysis. For assessment of cellular apoptosis, cells were stained with FITC-conjugated Annexin V from the Apoptosis Detection Kit II (BD Biosciences) and analyzed by a FACScan (Becton Dickinson), gating out doublets and clumps using pulse processing and collecting fluorescence above 620 nm.

Hexokinase assay. Cells were harvested, washed twice in cold PBS, and lysed in extraction buffer [45 mmol/L Tris-HCl (pH 8.2), 50 mmol/L KH2PO4, 10 mmol/mL glucose, 11.1 mmol/L monothioglycerol, 0.5 mmol/L EDTA, 0.2% Triton X-100]. Whole-cell lysates were evaluated by a standard glucose-6-phosphate dehydrogenase–coupled spectrophotometric assay (24). The reaction was started by addition of extract content and then analyzed by a FACScan flow cytometer (Becton Dickinson).

Xenograft models. Severe combined immunodeficient (Scid/Scid) mice were purchased from the Jackson Laboratory. All animal manipulations were conducted with sterile techniques following the guidelines of the University of California at Los Angeles Animal Research Committee. U87 and LN-229 cell lines were used to generate xenografts in mice; the LN-18 cell line failed to reproducibly form tumors in SCID mice. Cells (1 x 105-5 x 105 per mouse) growing exponentially in culture were resuspended in PBS and Matrigel (BD Biosciences) and injected s.c. at the right shoulder of the animal. Mice bearing xenografts of U87 or LN-229 cells underwent either [18F]FDG- or [18F]FLT-PET/computed tomography (CT) scans when the tumor size had reached ~100 mm2 (2-3 wk after tumor cell injection). Following the PET/CT study, groups of six mice were treated once with 3 mg/kg rapamycin i.p. or vehicle as previously described (16, 25). Two days later, a follow-up PET/CT study was done using the same image acquisition variables. Following the second PET scan, tumors were excised and half of the tumor tissue was homogenized. Protein was
extracted and S6 phosphorylation and TK1 levels were assessed by Western blot analysis as described above for the cell culture studies. The other half of the tumor tissue was fixed in 4% buffered formalin, processed, and embedded in paraffin. The tissue-embedded paraffin blocks were cut and slides were taken for immunohistochemical staining with an anti–Ki-67 antibody (1:100 dilution; DAKO Corp.). Tissue sections of each tumor were also stained with Harris’ H&E.

To assess the effect of repeated rapamycin treatment on tumor growth, groups of six mice bearing U87 or LN-229 xenografts were treated either with vehicle or with 3 mg/kg rapamycin i.p. every other day. Tumor size was measured every other day by calipers. Tumor volume was calculated using the following equation: tumor volume = 0.5 × (greatest diameter) × (shortest diameter)².

**PET and CT imaging.** MicroPET/CT scans were done using the microPET FOCUS 220 PET scanner (ref. 26; Siemens Preclinical Solutions) and MicroCAT II CT scanner (Siemens Preclinical Solutions). Mice were fasted for 12 h before [18F]FDG injection and placed on a heating pad (30°C) starting 30 min before [18F]FDG injection (27). For tracer injection and imaging, mice were anesthetized using 1.5% to 2% isoflurane. Mice were imaged in a chamber that minimizes positioning errors between PET and CT to less than 1 mm (28). Imaging was started 60 min after an i.p. injection of 200 μCi of [18F]FDG (27) or [18F]FLT via tail vein. Image acquisition time was 10 min. Images were reconstructed by filtered back-projection, using a ramp filter with a cutoff frequency of 0.5 Nyquist. Image counts per pixel per second were calibrated to activity concentrations (Bq/mL) by measuring a 3.5-cm cylinder phantom filled with a known concentration of [18F]FDG. Immediately after the PET scan, the mice underwent a 7-min microCT scan using routine image acquisition variables (70 kVp, 90 mA-s with 2-mm aluminum filters).

For display, activity concentrations were expressed as percent of the decay-corrected injected activity per gram of tissue by using the AMIDE software (29). Spherical regions of interest (2-mm diameter) were placed in the area of the tumor with the highest [18F]FDG or [18F]FLT uptake. To account for interindividual differences in [18F]FDG or [18F]FLT biodistribution, a spherical region of interest with a diameter of 2 mm was placed in the mediastinum for [18F]FDG and the liver for the [18F]FDG-PET studies as previously described (21, 30). Tumor [18F]FDG uptake was expressed as the ratio between the mean activity concentrations in the tumor region of interest divided by the mean activity concentration in the liver or mediastinum, respectively. Regions of interest were defined on fused PET/CT images generated by the AMIDE software to ensure reproducible positioning of the regions of interest.

**Statistical analysis.** Results are shown as mean ± 1 SE. Comparisons were made by two-tailed t-tests as well as by ANOVA as appropriate. Significant associations by ANOVA were further analyzed by Bonferroni post hoc tests. P < 0.05 was considered as statistically significant.
Rapamycin did not induce apoptosis in U87 cells even at the highest tested concentration.

**Influence of rapamycin on the L-type amino acid transport system.** In untreated cells, the cell to medium ratio was >10 after the 5-minute uptake period. As shown in Fig. 4A, treatment with rapamycin for 24 hours at 0.1, 1, and 10 nmol rapamycin did not affect [³H]-tyrosine uptake in U87 cells. BCH, a competitive inhibitor of the amino acid transport system L, completely blocked the [³H]-tyrosine uptake of U87 cells, whereas sodium depletion had no significant effect on [³H]-tyrosine uptake (data not shown). Because these findings indicated that the amino acid transport system L mediates uptake of tyrosine in U87 cells, we did FACS analysis to determine the cell surface expression of 4F2hc (CD98), the heavy chain of the L-type transporters LAT1 and LAT2 (Fig. 4B and C). These studies showed that U87 cells express 4F2hc but there was no inhibition of 4F2hc expression following rapamycin treatment (Fig. 4C).
Fig. 2. Influence of rapamycin on cellular glucose utilization. A to C, [18F]FDG uptake of the three studied cell lines treated with rapamycin at the indicated concentrations or vehicle. D, effect of rapamycin on the expression of Glut1 and Glut3. U87 cells were incubated with vehicle or 10 nmol/L of rapamycin for 24 h. Then, the expression of Glut1 and Glut3 was determined by Western blots of whole-cell lysates and cell membrane fractions. Fraction purity was confirmed by performing Western blots for Na⁺,K⁺-ATPase (cell membrane marker). E, effect of rapamycin on 3-OMeG uptake. U87 cells were incubated with vehicle or 0.1, 1, and 10 nmol/L of rapamycin for 24 h. Then, 3-OMeG uptake was measured for 3 min. F, effect of rapamycin on hexokinase activity. U87 cells were incubated with vehicle or with 0.1, 1, and 10 nmol/L of rapamycin for 24 h. Whole-cell extracts were prepared to assess the hexokinase activity. *, P < 0.05; **, P < 0.01 compared with vehicle control.
Fig. 3. Influence of rapamycin on [18F]FLT uptake, TK1 expression, cell cycle distribution, and apoptosis. A to C, [18F]FLT uptake of the three studied cell lines treated with rapamycin at the indicated concentrations or vehicle. D, effect of rapamycin on TK1 expression. U87, LN-18, and LN-229 cells were incubated with the indicated concentrations for 24 h. E, cell cycle distribution of rapamycin-treated cells and controls. U87 cells were incubated with the indicated concentrations of rapamycin for 24 h. Cells were then stained with propidium iodide and subjected to FACS. F, Annexin V staining of U87 cells 24 h after treatment with 10 nmol/L rapamycin or vehicle. *, P < 0.05; **, P < 0.01 compared with vehicle control.
Rapamycin inhibits \[^{18}\text{F}]\text{FDG} and \[^{18}\text{F}]\text{FLT} uptake in vivo.

In U87 tumors treated with rapamycin, \[^{18}\text{F}]\text{FDG} uptake was reduced to 44 ± 6% of the baseline value \((P < 0.01)\) as shown in Fig. 5A and C. Liver activity did not change with rapamycin therapy \((83.2 ± 6.4 \text{ counts/pixel/s at baseline and } 84 ± 5.4 \text{ counts/pixel/s at follow up}); thus, the decrease of the tumor to liver ratio was not due to an increase in liver activity. In contrast to U87 tumors, \[^{18}\text{F}]\text{FDG} uptake did not significantly change in LN-229 tumors \((P = 0.3);\) Fig. 5B and D). After treatment with rapamycin, fasting serum glucose levels significantly increased in both xenograft models \((80 ± 7\text{ to } 141 ± 11 \text{ mg/mL in animals bearing U87 tumors and from } 70 ± 9 \text{ to } 144 ± 16 \text{ mg/mL in animals bearing LN-229 tumors, } P < 0.05 \text{ for both cell lines}). U87 and LN-229 xenografts, which received vehicle treatment, showed no significant changes of \[^{18}\text{F}]\text{FDG} uptake from the baseline to the follow-up scan \((P > 0.05 \text{ for both cell lines}). When comparing FDG uptake of rapamycin-treated tumors with that of vehicle-treated tumors, there was a highly significant difference for U87 tumors \((2.26 ± 0.22 \text{ versus } 5.1 ± 0.4, P = 0.001). In contrast, there was no significant difference for vehicle- and rapamycin-treated LN-229 xenografts \((4.9 ± 0.6 \text{ versus } 4.1 ± 0.4, P = 0.27)).

Treatment with rapamycin significantly reduced \[^{18}\text{F}]\text{FLT} uptake of U87 tumor to 48 ± 7% of baseline level \((P < 0.01). Mediastinal blood pool activity did not change with rapamycin therapy \((70 ± 1.2 \text{ counts/pixel/s before therapy and } 71 ± 3.2 \text{ counts/pixel/s after therapy}). In LN-229 tumors \((Fig. 5F and H)), no significant changes in \[^{18}\text{F}]\text{FLT} uptake were observed after rapamycin therapy \((P = 0.2)). When comparing FLT uptake of the rapamycin-treated tumors with that of the vehicle-treated tumors, there was a significant difference for U87 tumors \((0.8 ± 0.1 \text{ versus } 2.1 ± 0.2, P = 0.01). In contrast, there was no significant difference for vehicle- and rapamycin-treated LN-229 xenografts \((4.9 ± 0.5 \text{ versus } 5.1 ± 0.1, P = 0.18). Tumor size also did not significantly change at the time of the follow-up PET scan \((P > 0.3 \text{ for both LN-229 and U87 tumors). However, continued treatment with rapamycin efficiently inhibited tumor growth in U87, but not in LN-229, xenografts \((Fig. 6). Reduction of tumor \[^{18}\text{F}]\text{FDG} and \[^{18}\text{F}]\text{FLT} uptake correlates with rapamycin-induced growth inhibition in vivo.} Western blot analysis of tumor tissue excised after the follow-up PET scans showed that rapamycin therapy had abolished S6 protein phosphorylation in both U87 and LN-229 xenografts \((Fig. 6A). However, TK1 levels were inhibited only in U87 tumors. Histologic analysis provided no evidence for treatment-induced necrosis in U87 and LN-229 tumors \((Fig. 6B and C). However, Ki-67 staining was markedly reduced in U87 tumors \((Fig. 6B) but not in LN-229 xenografts \((Fig. 6C). When mice were treated with 3 mg/kg rapamycin every other day for 2 weeks, the volume of U87 xenografts slowly declined. Vehicle-treated U87 tumors showed a rapid exponential growth \((Fig. 6D). In contrast, treatment with rapamycin did not inhibit growth of LN-229 xenografts \((Fig. 6E).}

Discussion

This study shows that mTOR inhibition by rapamycin therapy can cause a rapid reduction of glucose and thymidine use of glioblastoma cell lines. These metabolic changes correlated well with rapamycin-induced growth inhibition.

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Fig. 4. Effects of rapamycin on the L-type amino acid transport system. A, \(\text{uptake of }^{[\text{H}]}\text{tyrosine. U87 cells were incubated with vehicle or with 0.1, 1, and 10 nmol/L of rapamycin for 24 h and then incubated with }1 \mu\text{Ci/mL }^{[\text{H}]}\text{-tyrosine for 5 min. B and C, cell surface expression of CD98. U87 cells were incubated with vehicle }\)\(B) \text{or 10 nmol/L rapamycin }\)\(C) \text{for 24 h; cells were then washed and incubated with an anti-CD98 primary antibody and then with a FITC-conjugated second antibody. Y-axis is side scatter (SSC). FACS analysis was then done to determine CD98 protein levels on the cell surface.}

Imaging, Diagnosis, Prognosis
Fig. 5. [(18)F]FDG- and [(18)F]FLT-PET/CT scans of tumor xenografts before and after treatment with rapamycin. U87 and LN-229 xenografts received either [(18)F]FDG-PET/CT or [(18)F]FLT-PET/CT scans before and after one dose of rapamycin (3 mg/kg), 48 h after the baseline scan. Axial [(18)F]FDG-PET/CT scans of representative tumor are shown in A and B. Changes in tumor [(18)F]FDG uptake are summarized in C and D. E and F show [(18)F]FLT-PET scans of U87 and LN-229 xenografts before and after one dose of rapamycin. Changes in tumor [(18)F]FLT uptake are summarized in F and H. **: P < 0.01; *: P < 0.05 for comparison of pretreatment and posttreatment scans.
If confirmed in clinical trials, these findings would suggest that PET imaging with FDG or FLT may be used to monitor treatment with rapamycin and other mTOR inhibitors in patients. In contrast, L-type amino acid transport, which is considered to mediate the uptake of most clinically available amino acid probes (17, 32), does not seem to reflect mTOR inhibition in glioblastomas. Therefore, PET imaging with $[^{18}\text{F}]$FDG or $[^{18}\text{F}]$FLT may be preferable to radiolabeled amino acids for monitoring treatment responses to rapamycin.

The rationale for monitoring tumor glucose and amino acid metabolism for assessing the effects of mTOR inhibitors is based on the well-established role of mTOR in cellular metabolism and growth factor signaling. Signaling by mTOR is activated through Akt-induced inhibition of the tuberous sclerosis complex TSC1/TSC2, which inhibits mTOR activity (33). In various tumor models, activation of Akt or HIF-1α has been shown to lead to increased expression of glycolytic enzymes and transporters (34, 35). These effects of Akt and HIF-1α were blocked by rapamycin, indicating that mTOR plays a role in regulating glucose use of cancer cells.

Transport of glucose across the plasma membrane of mammalian cells is the first step for glucose metabolism and is mediated by facilitative glucose transporter (GLUT) proteins. Overexpression of the GLUT1 and GLUT3 subtypes has been described in many cancers (36). Hexokinase, which converts glucose to glucose-6-phosphate, helps maintain the glucose concentration gradient that results in the movement of glucose into cells (37). The glucose analogue $[^{18}\text{F}]$FDG traces these first
two steps of glucose metabolism but cannot be metabolized further. Our results indicate that $^{[18F]}$FDG uptake and hexokinase activity are rapidly decreased in cells that are sensitive to rapamycin, whereas there is little effect on $^{[18F]}$FLT uptake of rapamycin in resistant cells. These metabolic effects of rapamycin could be imaged noninvasively by microPET within 48 hours after a single dose of rapamycin, suggesting that $^{[18F]}$FDG-PET may potentially be used to monitor rapamycin therapy in the clinic.

We found, however, that rapamycin therapy causes hyperglycemia in mice. Because $^{[18F]}$FDG competes with glucose for transport and phosphorylation, hyperglycemia can reduce tumor $^{[18F]}$FDG uptake. Hyperglycemia has also been reported in clinical studies of rapamycin analogues (10, 38). However, hyperglycemia cannot explain the observed reduction in $^{[18F]}$FDG uptake in U87 tumors because $^{[18F]}$FDG uptake of rapamycin-resistant LN-229 tumors was unchanged following rapamycin therapy. Furthermore, our in vitro studies show that rapamycin modulates glucose metabolic activity of cancer cells.

Rapamycin has cytostatic effects in a variety of preclinical tumor models. This effect is caused by a decrease of cyclin D1 expression and an increase in p27, leading to an arrest of cells in G1 (7). The thymidine analogue FLT is a substrate of TK1, whose expression levels and functional activity are low during the G1 phase of the cell cycle (31). TK1 activity is the key determinant for $^{[18F]}$FLT uptake by cancer cells (19). The uptake of $^{[18F]}$FLT also correlates with immunohistochemical proliferation markers such as Ki67 in glioblastoma (39). Based on this evidence, we tested the ability of $^{[18F]}$FLT-PET to monitor the cytostatic effects of rapamycin in glioblastoma. Inhibition of cellular proliferation by rapamycin caused a rapid decrease in $^{[18F]}$FLT uptake, which was paralleled by a decrease in TK1 protein expression and accumulation of cells in the G1 phase. These changes in $^{[18F]}$FLT uptake were also evident in vivo by microPET imaging and were allowed to differentiate between rapamycin-sensitive and rapamycin-insensitive tumors within 48 hours after start of therapy (Fig. 6).

PET/CT imaging of mice is associated with a considerably higher radiation exposure than PET/CT in humans. Our CT protocol resulted in a tumor dose of ~45 mGy (40) and the injection of 200 μCi $^{[18F]}$FDG or $^{[18F]}$FLT caused a tumor dose of ~200 to 400 mGy (41). Although this radiation dose is unlikely to have a significant effect on tumor growth, one cannot rule out that radiation exposure combined with the effects of fasting and anesthesia could have some effect on tumor growth, metabolism, or tracer biodistribution. To ensure that the observed changes in $^{[18F]}$FDG or $^{[18F]}$FLT uptake were not confounded by these factors, we also imaged vehicle-treated animals. In mice bearing U87 tumors, one dose of rapamycin significantly reduced FDG and FLT uptake compared with the vehicle-treated animals. In contrast, there were no significant differences between rapamycin- and vehicle-treated LN-229 tumors. In addition, we generated growth curves in mice that were not imaged by PET/CT. These growth curves showed a marked therapeutic effect of rapamycin on U87 xenografts, but not on LN-229 xenografts (Fig. 6).

The observed decrease in $^{[18F]}$FDG or $^{[18F]}$FLT uptake was not explained by cell death because there was no decrease in tumor size in vivo and no increase in the number of trypan blue or Annexin V–positive cells in vitro. These results are consistent with previous studies that have found that a cytotoxic response to rapamycin is infrequent (7). In clinical trials, tumor shrinkage in response to mTOR inhibition has been rare, even when patient survival was significantly improved (38).

Glioblastoma exhibit markedly increased uptake of neutral amino acids, which is believed to result from an increased expression and activity of the LAT-1 transporter (42, 43). The LAT-1 transport is one of the transporters of the L transport system, which transports large neutral amino acids such as tyrosine. The LAT-1 transporter is composed of two covalently linked protein chains called 4F2 light chain and 4F2 heavy chain (4F2hc) or CD98 (44). Studies have indicated that in growth-factor–deprived leukemia cells, rapamycin can inhibit Akt-stimulated amino acid uptake (45). In this context, rapamycin caused a translocation of 4F2hc from the plasma membrane to the cytosol. In contrast, in the presence of growth factors, inhibition of mTOR with rapamycin did not cause translocation of 4F2hc (46). In this study, we found that U87 cells expressed a high level of LAT1/4F2hc and that tyrosine uptake is mediated by the L system. However, treatment with rapamycin did not affect t-tyrosine uptake by U87 cell line despite significant growth inhibition. Thus, our data suggest that t-tyrosine and its analogues are less suited than $^{[18F]}$FDG and $^{[18F]}$FLT for monitoring rapamycin therapy in the clinic.

As models for rapamycin-sensitive and rapamycin-insensitive tumors, we used cell lines with and without deletion of the tumor-suppressor gene PTEN, because previous studies had indicated that loss of PTEN is associated with increased sensitivity to rapamycin (15, 47). In accordance with these previous observations, we found that growth of the PTEN null U87 cell lines was inhibited at low concentrations of rapamycin (0.1 nmol/L), whereas 100-fold higher concentrations had no growth-inhibitory effect on PTEN wild-type LN-229 cells. However, LN-18 cells, which also express wild-type PTEN (20), showed a similar sensitivity to rapamycin as U87 cells in vitro, indicating that PTEN status does not completely reflect cell vulnerability to mTOR inhibition. Similar observations have been made by others in glioblastoma (48) and other tumor types (47). The lack of reliable molecular markers to predict tumor responsiveness to rapamycin emphasizes the need for techniques to monitor tumor response early in the course of therapy. Furthermore, rapamycin showed a concentration-dependent inhibition of the phosphorylation of the mTOR targets 70S6K and ribosomal protein S6 proteins in PTEN wild-type LN-229 cells, although no inhibition of cell growth was observed in this cell line. Similarly, one dose of rapamycin abolished S6 phosphorylation in vivo, but did not inhibit growth of LN-229 xenografts. This suggests that phosphorylation of p70S6K and S6 are not essential in regulating the growth of LN-229 cells. This confirms previous observations (48) that inhibition of phosphorylation of P70S6K and S6 are not sufficient for a growth inhibitory effect of rapamycin. Thus, phosphorylation of S6, which has been used to monitor rapamycin therapy in clinical trials, is a marker of target inhibition, but not necessarily of tumor response (49, 50).

In conclusion, the results of the present study suggest that imaging of tumor glucose and thymidine utilization may be useful for monitoring the growth-inhibitory effects of rapamycin. If validated in clinical trials, PET imaging with $^{[18F]}$FDG or $^{[18F]}$FLT-PET may provide useful information on the growth-inhibitory effects of rapamycin.
[18F]FLT could complement pretherapeutic analysis of the PTEN status and assessment of changes in S6 phosphorylation. In contrast, imaging of L-type amino acid transport with tyrosine analogues is unlikely to assess tumor response early in the course of therapy. This study also illustrates how in vitro studies and small-animal PET imaging can be used to select molecular imaging probes for further evaluation in clinical trials.

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

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References
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Changes in Tumor Metabolism as Readout for Mammalian Target of Rapamycin Kinase Inhibition by Rapamycin in Glioblastoma

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