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PET Radiotracers for Imaging the Proliferative Status of Solid Tumors

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Synopsis

Two different strategies have been developed for imaging the proliferative status of solid tumors with the functional imaging technique, Positron Emission Tomography (PET). The first strategy uses carbon-11 labeled thymidine and/or, more recently, fluorine-18 labeled thymidine analogs. These agents are a substrate for the enzyme thymidine kinase-1 (TK-1) and provide a pulse label of the number of cells in S phase. The second method for imaging the proliferative status of a tumor uses radiolabeled ligands that bind to the sigma-2 receptor which has a 10-fold higher density in proliferating (P) tumor cells versus quiescent (Q) tumor cells. This article compares and contrasts the two different strategies for imaging the proliferative status of solid tumors, and describes the strengths and weaknesses of each approach.

Keywords

Cell proliferation; thymidine analogs; sigma-2 receptors; PET imaging

Introduction

The initiation and progression of tumors involve deregulation of the cell cycle leading to uncontrolled cell proliferation. Consequently, measurement of the proliferative status can provide useful information regarding the prognosis and aggressiveness of tumors, and this

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information can be used to guide treatment protocols in clinical practice. Frequently, the chemotherapeutic agents and radiotherapy regimens used to treat cancer have serious side effects due to normal tissue toxicity that leads to poor patient tolerability. In addition, many of the newer “targeted therapies” are also limited by resistance, normal tissue toxicity, and other adverse events that reduce drug efficacy¹. A measurement of the proliferative status of tumors, which is defined as the fraction of cells progressing through the cell cycle, may identify patients who are at a higher risk of recurrence and can benefit from individual tailoring of radiotherapy, chemotherapy, targeted therapy, or a combination of these modalities. A change in the proliferative status of a tumor during or after treatment also has the potential to serve as a predictor of response and allow further tailoring of therapy.

Positron Emission Tomography (PET) is an imaging technique that provides a means of measuring disease-based changes in cell function at the molecular level. The use of PET in the field of oncology has focused largely on the use of [¹⁸F]2-fluorodeoxyglucose ([¹⁸F]FDG), a metabolic tracer which measures differences in glucose utilization between tumors and normal tissue. Although [¹⁸F]FDG has proven to be an important PET radiotracer in the diagnosis and staging/restaging of tumors, differences in glucose utilization do not provide sufficient information to identify an appropriate strategy for treating solid tumors². The uptake of [¹⁸F]FDG by solid tumors also does not correlate strongly with their proliferative status because it often simply reflects tumor cell viability³. In addition, [¹⁸F]FDG uptake is dependent upon the number of infiltrating inflammatory cells, or the oxygenation status of the tumor cells. Therefore, while [¹⁸F]FDG is the primary radiotracer typically used for diagnosing and staging/restaging tumors, it is not capable of providing information regarding which chemotherapy regimen or radiation therapy schedule to use based on the proliferative status of the tumor.

Another metabolism-based imaging strategy for assessing a tumor’s proliferative status uses the radiolabeled amino acid [¹¹C]methionine, which measures differences in the rates of protein synthesis between normal and tumor cells. Initial studies have shown a good correlation between [¹¹C]methionine uptake and the S-phase fraction of both non-small-cell lung carcinoma⁴ and breast cancer^{5, 6}. However, the low tumor uptake and low tumor: background ratios indicate that [¹¹C]methionine is not an ideal agent for imaging the proliferative status of these tumors⁷. Recent studies in brain tumor patients have yielded mixed results. One study in patients with newly diagnosed brain tumors (n = 41) showed no correlation between the [¹¹C]methionine uptake measured by the standardized uptake ratio (SUV) method and the Ki-67 labeling index of the tumor specimen⁸. Another study demonstrated a modest correlation between the [¹¹C]methionine SUV and the Mib-1 labeling index in patients diagnosed with diffuse astrocytoma (n = 21; r = 0.63), but no such correlation for patients diagnosed with oligodendroglioma or oligogastrocytoma⁹. These data indicate that tumor type may be an important factor when seeking a correlation between radiotracer uptake and the proliferative status of brain tumors in PET imaging studies.

Two strategies have emerged for imaging the proliferative status of solid tumors with PET. The first strategy involves the use of radiolabeled thymidine analogs that utilize the salvage pathway of DNA synthesis for their uptake¹⁰. This imaging strategy provides a measure of the number of tumor cells in S-phase during the tracer uptake and image acquisition period of a PET scan. The second strategy involves the use of radiotracers that image the sigma-2 receptor status of solid tumors. Previous studies have shown that sigma-2 receptors are expressed in a 10-fold higher density in cycling, proliferating tumor cells versus tumor cells that are driven into quiescence by nutrient deprivation^{11–13}. Although not immediately obvious, these two imaging strategies look at two completely different aspects of cell proliferation; radiolabeled thymidine analogs provide a pulse label of the S-phase fraction of a solid tumor, whereas the sigma-2 receptor imaging agents provide a measure of the ratio of cycling, proliferating (P) to quiescent (Q) tumor cells (i.e., the P: Q ratio) in a solid tumor.

S-Phase Fraction versus P: Q Ratio

The loss of the ability of a cell to control its progression through the cell cycle, resulting in uncontrolled cell growth, is a hallmark of cancer¹⁴. As a result, most tumors have a higher S-phase fraction, the phase of the cell cycle where DNA synthesis occurs, than the normal tissue from which they were derived. Historically, measurement of the S-phase fraction of a tumor or cells growing under culture conditions has been a reliable method for measuring cell proliferation. The reagents used to measure the S-phase fraction are usually [³H]thymidine or the thymidine analog, 5-bromodeoxyuridine (BrdU). Since the de novo pathway of DNA synthesis involves the conversion of uridine to thymidine via the catalytic action of thymidylate synthetase, the incorporation of [³H]thymidine or BrdU into DNA occurs exclusively through the salvage DNA synthesis pathway. The key step in the salvage pathway involves the phosphorylation of the 5'-hydroxy position of thymidine by thymidine kinase-1 (TK-1).

Normally, TK-1 is synthesized at the G1/S boundary and consists of 234 amino acids with a monomeric molecular weight of 25.5 kDa. The enzyme exists as a high catalytic-activity tetrameric complex in S-phase, and as a lower catalytic activity dimer in the G2/M phases of the cell cycle^{15, 16}. Phosphorylation of Ser-13 by cyclin-dependent kinases is thought to be a key step in the degradation of TK-1 which declines to near zero in early G₁. There is no expression of the enzyme in quiescent (G₀) cells. Since TK-1 is usually catalytically active only in S phase, tumor cells growing under cell culture conditions or as tumor allografts or xenografts implanted in a laboratory animal must be exposed to [³H]thymidine or BrdU every 8 hours (i.e., the average length of S phase) *for at least 1.5 to 2 cell cycle times if all* the cycling cells are to be labeled with a thymidine analog (Figures 1 and 2). Consequently, the measurement of tritium- or BrdU-labeled DNA receiving only *a single pulse* of [³H]thymidine or BrdU will underestimate the total number of cycling, proliferating cells because those cells in the G₁ and G₂/M phases of the cell cycle will not be labeled. In addition, some tumor cells are TK-1 deficient (TK-1⁻) and rely exclusively on the de novo DNA synthesis pathway. In these tumors, measurements of radiolabeled thymidine, radiolabeled thymidine analogs, or BrdU incorporation will not correlate with their proliferative status.¹⁰

A number of studies have provided evidence suggesting that there is an altered regulation of TK-1 in tumor cells¹⁰. For example, TK-1 has been shown to be catalytically-active in the S, G₂, and M phases of the cell cycle in a number of leukemia cell lines^{17, 18}. HeLa cervical carcinoma cells have been shown to have TK-1 activity in all phases of the cell cycle, including G₁. Mouse fibroblasts transformed with DNA tumor viruses display a TK-1 activity profile similar to HeLa cells¹⁷. Truncation of the human TK-1 C-terminus by 30–40 amino acids, or changing Ser-13 to an alanine residue that prevents phosphorylation by cyclin-dependent kinases results in a constitutively-active TK-1^{19, 20}. These data suggest that some tumors will be comprised of cells with TK-1 activity in multiple phases of the cell cycle, others will have normal regulation of TK-1, and yet others will be TK-1 deficient. Therefore, pulse-labeling studies using either BrdU, radiolabeled thymidine, or a radiolabeled thymidine analog may not represent a true measure of even the S phase fraction because incorporation is dependent upon the regulation of TK-1.

Another property of solid tumors is that as they increase in size they can quickly outgrow their blood supply and become hypoxic and/or deprived of nutrients necessary to sustain the high energy requirements for cell proliferation. When this occurs, cycling, P cells can exit the cell cycle and enter a prolonged quiescence (Q) state (Figures 1 and 2)^{21–25}. A *quiescent tumor cell* is distinct from *most quiescent normal cells* because they remain undifferentiated and can be recruited back into a cycling, P cell state once the conditions of hypoxia and/or nutrient deprivation are eliminated^{12, 13, 26, 27}. All solid tumors, even those as small as 2–5 mm in size, contain both P and Q cells. The ratio of the P cells to Q cells (the P:Q ratio) is defined as

the proliferative status of solid tumors^{11–13}. A number of molecular markers have been used to distinguish between the P and Q cell populations in a solid tumor, including the presence of Ki-67^{24, 28, 29}, proliferating cell nuclear antigen (PCNA)²⁹, ribonucleotide reductase M1 subunit (RRM1)³⁰, and chromatin assembly factor-1 (CAF-1)³¹. Of these molecular markers, Ki-67 is generally considered the “gold standard” for measuring the proliferative status of a broad spectrum of solid tumors²⁴. However, determining the percentage of cells in a tumor that are labeled with [³H]thymidine or BrdU is also a valid method for measuring the proliferative status of solid tumor provided that the animal is injected with [³H]thymidine or BrdU every 8 hours over 1.5 – 2 cell cycle times to insure that all of the cycling cells are labeled (Figure 2)¹³.

Imaging the Proliferative Status of Tumors with Thymidine and Its Analogs

The first imaging studies of tumor proliferation used [¹¹C]thymidine labeled in both the 5-methyl group and the carbonyl position designated by the asterisks in Figure 3^{32–35}. The radiosynthesis of [¹¹C]thymidine is rather complicated and difficult to automate for routine production. In addition, the rapid in vivo metabolism and complex kinetic model needed to quantify a tumor’s proliferative status has limited the utility of [¹¹C]thymidine as a PET radiotracer. The short half-life of carbon-11 ($t_{1/2} = 20.4$ min) also places significant time constraints on both image acquisition and metabolite analysis that further complicates imaging studies with [¹¹C]thymidine.

Because of its relatively long half-life ($t_{1/2} = 109.8$ min) fluorine-18 is the preferred radionuclide for clinical PET imaging studies. Therefore, a second generation of thymidine analogs for PET imaging were developed by substitution of the hydroxyl groups of the sugar moiety of thymidine with fluorine-18^{36–39}. The first ¹⁸F-labeled thymidine analog reported was [¹⁸F]FLT³⁸ which involved the replacement of the 3'-hydroxy group of thymidine with the ¹⁸F radiolabel (Figure 3). Since [¹⁸F]FLT lacks the 3'-hydroxy group, it is not incorporated into DNA and is trapped in tumor cells following phosphorylation of the 5'-hydroxy group by TK-1 in a manner analogous to the trapping of [¹⁸F]FDG in cells via phosphorylation by hexokinase^{38, 40}. Other analogs have been developed by attaching the ¹⁸F-radiolabel to the 2'-beta position of thymidine. Examples of these analogs include the radiotracers [¹⁸F]FMAU and [¹⁸F]FBAU (Figure 3)^{41–43}. Because these analogs contain the 3'-hydroxy group, they can be readily incorporated into DNA and provide a direct measure of DNA synthesis. Of the two, [¹⁸F]FMAU has been studied the most in animal tumor models. Although [¹⁸F]FMAU is incorporated into DNA, it is a poorer substrate for TK-1 than [¹⁸F]FLT. In addition, unlike [¹⁸F]FLT, [¹⁸F]FMAU is a substrate for the mitochondrial enzyme thymidine kinase-2 (TK-2), which is involved in mitochondrial DNA synthesis. Given its low specificity for TK-1 and the higher background due to the uptake in normal tissue mitochondria, [¹⁸F]FMAU is slightly less desirable than [¹⁸F]FLT for imaging the proliferative status of solid tumors.³

One of the fundamental limitations of imaging a tumor’s proliferative status with thymidine analogs such as [¹⁸F]FLT and [¹⁸F]FMAU is that they are administered as a pulse label. The test subject, either a tumor bearing animal or cancer patient, is given a bolus intravenous injection of the labeled thymidine analog, and then the imaging data is acquired over a period ranging from 60 – 120 min. Consequently, PET imaging studies with radiolabeled DNA precursors provide only a “snapshot” of the small number of cycling, proliferating tumor cells that happen to be in the S-phase during radiotracer uptake and data acquisition.

Because the proliferating cells within a tumor grow asynchronously, some of them will always be in each of the four phases of the cell cycle. Therefore, the proliferating cells that are in the G₁-, G₂-, and M-phase of the cell cycle will not be labeled by [¹⁸F]FLT or [¹⁸F]FMAU, assuming TK-1 is regulated normally. Further complicating the situation, the potential doubling

time (i.e., the cell cycle time) of human tumors is the highly variable, typically ranging on the order of one to several days. Because the length of the S phase in mammalian cells is relatively constant at 8–10 hours, only a small percentage of proliferating tumor cells with long cell cycle times will be in the S phase during the pulse labeling with a thymidine analog. Thus, the thymidine analogs such as [^{18}F]FLT and [^{18}F]FMAU will theoretically underestimate the actual number of cycling, proliferating cells and the P:Q ratio in a solid tumor, if TK-1 is catalytically active only during S phase.

In spite of these potential limitations, a number of clinical imaging studies have shown both a good correlation and a relatively steep slope in plots of the uptake of [^{18}F]FLT versus the Ki-67 labeling index in breast ⁴⁰, lung ^{44–46}, brain ^{8, 9, 47–50}, and colon tumors ^{51, 52}. A key requirement for obtaining a high correlation between [^{18}F]FLT uptake and the Ki-67 labeling index involves the method used for analyzing the PET data. Although most imaging studies reported to date have used SUV for measuring [^{18}F]FLT uptake in tumors, a number of more recent studies have shown that a graphical (i.e., Patlak) analysis is needed to give a high correlation between the tracer uptake and the Ki-67 labeling index. This Patlak analysis requires the measurement of metabolite-corrected arterial blood samples (i.e., the input function) in addition to a 45–95 min dynamic acquisition scan in order to quantify the [^{18}F]FLT uptake ^{40, 46, 48, 49}. This imaging protocol is considerably more complicated than the typical intravenous injection of [^{18}F]FLT followed by a 15 min emission scan acquired at 60 min after injection of the radiotracer. A summary of the clinical studies reported to date using [^{18}F]FLT to image a tumor's proliferative status is provided in Table 1.

Finally, the uptake of [^{18}F]FLT in normal tissues can also place limitations on the type of clinical imaging studies conducted with this radiotracer. [^{18}F]FLT is metabolized by forming glucuronide that is excreted via the hepatobiliary system. The high uptake of [^{18}F]FLT and its glucuronide metabolite by the liver usually limits its ability to image tumors in the abdominal cavity. There is also a high uptake of [^{18}F]FLT in bone that reflects the high proliferative activity of hematopoietic cells in bone marrow (Figure 4)³. Consequently, the high uptake of [^{18}F]FLT in bone can limit the ability of this radiotracer to define the extent of tumors near or invading bony structures.

Recent studies have also demonstrated that [^{18}F]FLT PET imaging can be used to assess the response to chemotherapy ³⁶. For example, Kenny et al ⁵³ reported that serial imaging studies in breast cancer patients demonstrated a reduction in [^{18}F]FLT uptake at 1 week after combination chemotherapy with 5-fluorouracil, epirubicin, and cyclophosphamide (FEC). This reduction in uptake exceeded test-retest variability, and preceded any physical change in tumor diameter. This reduction in [^{18}F]FLT uptake was observed using both the Patlak compartmental modeling and SUV methods. An earlier study by Pio et al. ⁵⁴ demonstrated that a change in [^{18}F]FLT SUV values after a single dose of chemotherapy in breast cancer patients had a higher correlation with post-treatment changes in the glycoprotein cancer antigen 27.29 (CA27.29), than with the change in [^{18}F]FDG SUV values. It has also been shown that the relative change in [^{18}F]FLT SUV values at 1 week after treatment with Gefitinib clearly delineates between the responders and nonresponders who have lung tumors ⁵⁵. Similar results have been reported for, i) glioma patients treated with bevacizumab and irinotecan ⁵⁶, and ii) lymphoma patients treated with CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) ⁵⁷. These data clearly demonstrate the potential utility of [^{18}F]FLT in determining a positive response to chemotherapy.

The Sigma-2 Receptor as a Biomarker for Imaging the Proliferative Status of Tumors

Another potential method for measuring a tumor's proliferative status with PET is to devise a way to image Ki-67 expression (Figure 1). Unfortunately, there are no small molecules having a high affinity for Ki-67 which can serve as lead compounds for PET radiotracer development. In lieu of imaging Ki-67, another option would be to identify a protein that behaves in a similar manner and has small molecules that bind to this protein. These molecules could then serve as lead compounds for PET radiotracer development. Over the past decade our group has shown that the sigma-2 receptor is regulated in a manner similar to Ki-67 and is a receptor-based biomarker of cell proliferation^{11–13}. Therefore, PET radiotracers that can image the sigma-2 receptor may provide an alternative strategy to the radiolabel DNA analogs for imaging the proliferative status of tumors.

Sigma receptors are a class of proteins that were originally thought to be a subtype of the opiate receptors⁵⁸. Subsequent studies revealed that sigma binding sites represent a distinct class of receptors. There are two well characterized subtypes of sigma receptors, sigma-1 and sigma-2. Sigma-1 receptors have a molecular weight of ~25 kDa, whereas the sigma-2 receptor has a molecular weight of ~21.5 kDa. The sigma-1 receptor gene has been cloned from guinea pig liver, human placental choriocarcinoma, rat brain, and mouse kidney⁶⁰. The sigma-2 receptor has not been sequenced or cloned. Historically, the differentiation of the sigma-2 receptor from the sigma-1 receptor has been based on the in vitro binding properties of two different radioligands, [³H](+)-pentazocine and [³H]1,3-di-*o*-tolylguanidine ([³H]DTG). [³H](+)-pentazocine has a high (~3 nM) affinity for the sigma-1 receptor and a low (>1,000 nM) affinity for the sigma-2 receptor, whereas [³H]DTG has a modest affinity (~25 nM) for both the sigma-1 and sigma-2 receptors^{58, 59}.

The role of the sigma receptors as potential biomarkers for breast cancer became apparent when it was shown that the radiotracer [¹²⁵I]N-(N-benzylpiperidin-4-yl)-4-iodobenzamide, which possesses a high affinity for both sigma-1 and sigma-2 receptors, labeled MCF-7 human breast tumor cells in vitro^{63, 64}. A subsequent study revealed that MCF-7 cells possess a high density of sigma-2 receptors, as measured by [³H]DTG in the presence of dextralorphan to mask sigma-1 sites⁶⁵. There was no detectable binding of the sigma-1 radiotracer, [³H](+)-pentazocine, to MCF-7 cells⁶⁵. Further studies indicated that many murine and human tumor cells possess a high density of sigma-2 receptors when grown under cell culture conditions. With the exception of LNCaP human prostate tumor cells which have an equal density of sigma-1 and sigma-2 receptors, and ThP-1 leukemia cells which have a higher density of sigma-1 versus sigma-2 receptors, the sigma-2 receptors density is much higher than the sigma-1 receptor density in all of the tumor cells evaluated to date⁶⁶.

Although in vitro binding studies suggest that the sigma-2 receptor is a potential target for imaging solid tumors,⁶⁶ these studies do not address whether or not there is a difference in the sigma-2 receptor density between proliferating and quiescent tumor cells. This issue was investigated in a series of in vitro and in vivo studies reported by Wheeler et al.^{11–13}. These studies used the well-characterized in vitro and in vivo mouse mammary adenocarcinoma model, 66, in which pure (>97%) populations of proliferative (66 P) and quiescent (66 Q) cells can be obtained under tissue culture conditions^{26, 27}. The 66 P cells have a cell cycle distribution and a tritiated thymidine labeling index characteristic of a mammalian cell line with a 13.8-h doubling time^{26, 27}. The 66 Q cells have predominantly a G₁ DNA content (>90%), a reduced cell volume (~50%), a mitotic and tritiated thymidine labeling index of virtually zero (<1%), and a reduced RNA content (~50%). 66 Q cells are viable (>98% trypan blue excluding) and can be recruited back into the P cell compartment^{26, 27}. Therefore, these

66 mouse breast tumor cells have all of the properties needed to determine if there is a difference in the sigma-2 receptor density between proliferating and quiescent tumor cells.

In the initial study ¹¹, the sigma-2 receptor density was measured in 3-day 66 P cells and in 7-, 10-, and 12-day 66 Q cells. Since the transition from 66 P cells to a pure population of 66 Q cells is not complete until day 7 in this in vitro model, the Q-cell populations in this study corresponded to about 1, 3, and 5 days into quiescence. The results of Scatchard studies revealed a receptor density of ~180,000 sigma-2 receptors/cell in 7-day 66 Q cells and ~510,000 sigma-2 receptors/cell in the corresponding 3-day 66 P cells to give a 7-day P:Q ratio of ~2.8:1. The sigma-2 receptor density of 10-day 66 Q cells was ~88,000 receptors/cell. The sigma-2 receptor density in the corresponding 3-day 66 P cells was ~840,000 receptors/cell, resulting in a P:Q ratio of ~9.5:1. The 12-day results were not statistically different from the 10-day results. These data suggest that at least a 3 day period in quiescence is required to maximize the loss of sigma-2 receptors from 66 cells; kinetics identical to those for the loss of Ki-67 during the 66 cell P to Q transition. Although the number of sigma-2 receptors in the 3-day 66 P cells appears to be exceptionally high (510,000 – 840,000 receptors/cell), this number is consistent with the number of sigma-2 receptors/cell reported in other tumor cell lines ⁶⁶.

In a follow-up study, these investigators measured the density of sigma-2 receptors during the P to Q and Q to P transitions ¹². When 10-day 66 Q cells were re-cultured, the Q cells rapidly entered the P-cell compartment with little or no delay (Figure 5A). The kinetics of the expression of sigma-2 receptors in 66 cells followed the population growth curve as expected. There was a rapid increase in the sigma-2 receptor density during the exponential growth phase that leveled off during early plateau phase. This represents the initial P to Q transition. The sigma-2 receptor density remained stable for 3 days, and then decreased during late plateau phase. The decrease in the sigma-2 receptor density in late plateau phase did not start until 5 days following reentry into quiescence. Therefore, the sigma-2 receptor density like many membrane-bound receptors is upregulation and downregulation over a 2–3 day period instead of the few hours for TK-1 and the cell cycle checkpoint proteins.

Finally, the sigma-2 receptor density of tumor xenografts derived from 66 cells correlated with the P: Q ratio determined by flow cytometry after labeling the proliferative cells with BrdU for ~2 cell cycles ¹³. In this study, tumor-bearing mice were injected with a dose of BrdU (100 mg/kg, i.p.) every 8 hours over a 48 hour period, and Scatchard studies were conducted on membrane preparations from one half of the tumor while flow cytometry studies were conducted on the dissociated tumor cells obtained from the other half (Figure 5B). A mathematical equation was derived that determined the P:Q ratio from a plot of the dpm/mg of tumor obtained at saturation from the Scatchard studies divided by the fraction of Q cells obtained from the flow cytometry studies against the P: Q ratio obtained from the flow cytometry studies. The sigma-2 receptor density P: Q ratio is estimated by calculating the ratio of the slope to the intercept determined from a linear regression analysis of the data (Figure 5B) ¹³. The results indicated that there is excellent agreement between the in vivo sigma-2 receptor density P:Q ratio (10.6) and the in vitro sigma-2 receptor density P:Q ratio (9.5).

The above studies demonstrate that the sigma-2 receptor is a good biomarker for measuring the P:Q ratio of solid tumors in vivo. That is, it is a protein that possesses kinetic properties similar to Ki-67 and other protein-based biomarkers of proliferation ²⁴⁻²⁸. The 10-fold higher density of sigma-2 receptors in P versus Q cells indicates that it should be possible to differentiate between tumors having a high proliferative status (i.e., high P:Q ratio) versus a low proliferative status (i.e., low P:Q ratio). Furthermore, the relatively high density of sigma-2 receptors in Q cells (~88,000 receptor/cell) also suggest that the sigma-2 receptor may be a useful biomarker for differentiating between quiescent tumor cells and normal cells since the density of sigma-2 receptors in normal tissues has been reported to be even lower than that of

quiescent tumor cells⁶⁶. In this regard, the sigma-2 receptor may be a more reliable marker than Ki-67 for determining the proliferative status of solid tumors since both *quiescent tumors* and *quiescent normal cells* are Ki-67 negative. However, further studies are needed to validate this hypothesis.

Development of Radiotracers for Imaging the Sigma-2 Receptor with PET

A class of compounds that has proven useful for developing PET radiotracers for imaging sigma-2 receptors are the conformationally-flexible benzamide analogs. The identification of this class of compounds as PET radiotracers for imaging sigma-2 receptors was a serendipitous discovery resulting from a structure-activity relationship study aimed at developing probes for imaging the dopamine D₃ receptor^{67, 68}. The lead compound for the D₃ studies was the benzamide analog, **1**, which has high affinity and modest selectivity for the D₃ versus D₂ receptors, but a log P that suggests it is not capable of readily crossing the blood-brain barrier (Figure 6). In order to prepare D₃ selective compounds having a lipophilicity that would more readily cross the blood-brain barrier, the 2,3-dichloropiperazine moiety of **1** was replaced with a 6,7-dimethoxytetrahydroisoquinoline ring to give compound **2** (Figure 6). Although this substitution increased the overall lipophilicity of the compound, it also resulted in an undesired reduction in affinity for the dopamine D₃ receptor. However, an unexpected observation was the dramatic increase in affinity for the sigma-2 receptors, and the unusually high selectivity for sigma-2 versus sigma-1 receptors. Consequently, compound **2** served as the lead compound for the development of carbon-11⁶⁹, fluorine-18⁷⁰, and bromine-76⁷¹ labeled probes for imaging the sigma-2 receptor with PET.

The first PET radiotracers based on the conformationally-flexible benzamide analogs were [¹¹C]**2–5**, whose synthesis involves labeling the corresponding ortho-hydroxy group with [¹¹C]methyl iodide. Although the affinities of [¹¹C]**2**, [¹¹C]**3**, [¹¹C]**4**, and [¹¹C]**5** for the sigma-2 receptor are similar, in vivo studies indicated that [¹¹C]**4** has the highest tumor uptake at all time points⁶². One explanation for this observation is that [¹¹C]**4** may have the optimal lipophilicity for tumor uptake since a parabolic relationship was observed between the % I.D./g of tumor and the calculated log P of the radiotracer (Figure 7). These data suggest that both the receptor affinity and lipophilicity are important properties that one must factor into the design of receptor-based tumor imaging agents. MicroPET and MicroCT imaging studies of [¹¹C]**4** in the human melanoma tumor MD-MBA-435 are shown in Figure 8A and clearly demonstrate its potential as a radiotracer for imaging the sigma-2 receptor status of solid tumors with PET. The presence of the bromine atom in the benzamide ring of compound **2** led to the preparation of [⁷⁶Br]**2**⁶⁴. Although [⁷⁶Br]**2** displayed high tumor uptake and excellent tumor to background ratios, the limited availability and high positron energy of Br-76, which degrades image resolution, limits the utility of [⁷⁶Br]**2** as a potential radiotracer in clinical PET studies.

Although the preclinical microPET imaging and tumor uptake studies of [¹¹C]**4** have shown promising results, the short half life of carbon 11 ($t_{1/2}$ = 20.4 min) is not ideal for the development of radiotracers that would have a widespread use in clinical PET imaging studies. The longer half-life of ¹⁸F ($t_{1/2}$ = 109.8 min) compared to ¹¹C places fewer time constraints on tracer synthesis, enables distribution to PET scanners within 2 hours of the cyclotron, and permits longer scan sessions that usually give higher tumor: normal tissue ratios. Therefore, the development of an ¹⁸F-labeled radiotracer was important for the clinical translation of imaging the σ_2 receptor status of human solid tumors with PET.

A limited number of ¹⁸F-labeled radiotracers based on the conformationally-flexible benzamide analogs have been evaluated in murine tumor models⁷⁰. The strategy involved replacement of the 2-methoxy group in the benzamide ring with a 2-fluoroethoxy group (Figure 6). The 2-fluoroethoxy- for -methyl substitution is a common method for incorporating a

fluorine-18 into a receptor-based imaging agent⁷⁰. In vivo tumor uptake and microPET imaging studies indicate that [¹⁸F]6 and [¹⁸F]7 are potential probes for imaging the sigma-2 receptor status of solid tumors with PET (Figure 6).⁷⁰ Although most of the initial preclinical evaluation of these probes involves imaging murine models of melanoma (Figure 8A), an important clinical use of these radiotracers may be determining the tumor margins and proliferative status of brain tumors in order to select appropriate radiotherapy regimens. For example, microPET studies of an intracranial 9L brain tumor demonstrated a higher tumor uptake and more favorable differentiation of tumor versus normal brain tissue with [¹⁸F]7 than that observed with [¹⁸F]FDG, the metabolic tracer used routinely in the diagnosis and staging of brain tumors (Figure 8B). The low tumor: background ratio of [¹⁸F]FDG is attributed to the high uptake of the radiotracer in normal brain tissue because glucose is the major energy source for the brain.

A limited number of studies have been conducted comparing the sigma-2 receptor imaging approach to the [¹⁸F]FLT approach. Studies with the ¹¹C-labeled radiotracer, [¹¹C]4, displayed higher tumor: blood and tumor: muscle ratios for the sigma-2 receptor-based imaging agent compared to that for [¹⁸F]FLT, whereas the tumor: fat ratios for the two radiotracers were similar⁶⁹. A microPET imaging study comparing [⁷⁶Br]2 and [¹⁸F]FLT also showed a much greater visualization of the tumor and higher tumor: background ratios for the sigma-2 receptor imaging agent (Figure 9)⁷¹. Clinical trials of [¹⁸F]6 are ongoing at Washington University in St. Louis. These trials will compare the images obtained with [¹⁸F]6 to those obtained with [¹⁸F]FLT, and determine if the relationship between each radiotracer and the Ki-67 labeling index is similar to that obtained with animal models.

Summary

The development of radiotracers for imaging the proliferative status of solid tumors has been an active area of research over the past decade. A noninvasive imaging measurement of a tumor's proliferative status may provide information that can be used for the identification of an appropriate treatment strategy for individual patients. For example, highly proliferative tumors are generally aggressive with a high metastatic potential that requires an aggressive initial treatment. Thus, they usually respond better to cell cycle specific agents (e.g., Ara-C and 5-fluorouracil) or hyperfractionated radiotherapy. In contrast, slowly proliferative tumors respond better to cell cycle nonspecific agents (e.g., cyclophosphamide and BCNU) or conventional radiation therapy. Furthermore, when imaging is conducted both pre- and post-treatment, a reduction in the proliferative status of a tumor has the potential to be used as an early indicator of the tumor's response to therapy.

Two strategies, radiolabeled thymidine analogs and sigma-2 receptor radiotracers, have been developed for imaging the proliferative status of solid tumors with PET. Radiolabeled thymidine analogs such as [¹⁸F]FLT provide an image that depends on the level of TK-1 activity in tumor cells. The uptake of [¹⁸F]FLT and its structural analogs reflects a "pulse label" of the number of cycling, proliferating cells that are in S-phase during the uptake and data acquisition period. However, since the regulation of TK-1 can be altered in many tumors, thymidine analogs only provide, i) an estimate of the number of cycling tumor cells in the S phase fraction if the cells regulate TK-1 similar to normal cells, ii) an estimate of the number of cycling tumor cells in the S/G₂/M phases if TK-1 is active through M phase, and iii) no estimate of the number of cycling tumor cells if the tumor cells are TK-1 deficient. Because TK-1 is low or nonexistent in the G₁ phase, thymidine analogs cannot differentiate among cycling, proliferative tumor cells in G₁, quiescent tumor cells in G₀, and normal cells which are predominantly in G₀. Consequently, the sigma 2 imaging approach may be a better strategy for, i) differentiating normal tissue from tumor tissue, and ii) estimating the P:Q ratio of a tumor than the radiolabeled analogs of thymidine. Although the sigma-2 receptor imaging strategy has shown great promise in animal models, clinical imaging studies must be conducted in

cancer patients in order to validate it as a viable alternative to radiolabeled thymidine analogs for determining the proliferative status of human solid tumors.

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- P : Q ratio has a profound effect on the outcome of chemotherapy and radiotherapy
- Tumors with a high P:Q ratio respond better to cell cycle specific agents (e.g., 5-FU and Ara-C) and/or hyperfractionated radiation therapy
- A change in the tumor's proliferative status can be used as an indicator of its response to radiation and chemotherapy

Figure 1.

Cartoon showing the characteristics of the P and Q cells in solid tumors. DNA is synthesized in the S phase of the cell cycle. TK-1 is normally synthesized at the G₁/S boundary and is active until early G₂. Proliferating cells are Ki-67-positive, whereas quiescent cells, including both quiescent tumor cells and quiescent normal cells, are Ki-67 negative.

Figure 2.

Flow cytometry of a mouse 66 mammary adenocarcinoma labeled with BrdU (100 mg/kg) every 8 h over a 48 h period, i.e. ~2 tumor cell cycle times. BrdU⁺ cells are proliferating (P) cells. Because the BrdU labeling occurred over 2 cell cycle times, the cycling P cells in all phases of the cell cycle are labeled. Cells that are BrdU⁻ are quiescent noncycling cells. The fluorescent probe, 7-AA distinguishes 66 cells that have a 2C DNA content (G₁/G₀ cells) from cells that have a 4C DNA content (G₂/M). Cells in S phase have an intermediate DNA content and usually are a small fraction of the total cells in a solid tumor.

* Denotes the position of the ^{11}C label.

Figure 3.
Structures of thymidine, BrdU, and the ^{18}F -labeled thymidine analogs that have been developed to determine the proliferative status of solid tumors with PET.

Figure 4. $[^{18}\text{F}]\text{FLT}$ -PET in a patient with locally advanced rectal cancer. Anterior and posterior projection images demonstrate intense $[^{18}\text{F}]\text{FLT}$ uptake in bone that reflects the proliferation of cells in bone marrow. There is increased accumulation of $[^{18}\text{F}]\text{FLT}$ in the rectal cancer (arrowhead in right panel). In addition, there is increased accumulation of $[^{18}\text{F}]\text{FLT}$ in the urinary bladder (arrow in the left panel), which is a normal finding.

Figure 5.

Validation of the sigma-2 receptor as a biomarker for imaging the proliferative status of solid tumors. **A:** Upregulation and downregulation of the sigma-2 receptors in 66 cells during the P to Q and the Q to P transitions. **B:** Determination of the sigma-2 receptor density P: Q ratio in solid 66 tumor xenografts grown in nude mice.

Figure 6. Structures of the conformationally-flexible benzamide analogs for imaging sigma-2 receptors with PET.

Figure 7. Relationship between the lipophilicity (log P) and tumor uptake of the ¹¹C-labeled conformationally-flexible benzamide analogs shown in Figure 6.

Figure 8.

PET and CT images of solid tumors with sigma-2 radiotracers and $[^{18}\text{F}]\text{FDG}$. **A:** MicroPET, microCT, and co-registered images of MDA-MB-435 melanoma tumors obtained 30 min after i.v. injection of $[^{11}\text{C}]\mathbf{4}$ and 60 min after i.v. injection of $[^{18}\text{F}]\mathbf{6}$. **B:** Co-registered microPET and microCT images of a rat intracranial 9L brain tumor obtained 60 min after i.v. injection of $[^{18}\text{F}]\mathbf{7}$ (top) or 60 min after i.v. injection of $[^{18}\text{F}]\text{FDG}$ (bottom).

Figure 9.

Comparison of the sigma-2 receptor imaging strategy (right) with the DNA precursor, [^{18}F]FLT (left) in EMT-6 tumors. Note the high contrast and high signal: noise ratio of the sigma-2 imaging agent, [^{76}Br]2, relative to [^{18}F]FLT. The tumor: background ratios of both tracers from biodistribution studies is shown in the graph.

Table 1[¹⁸F]FLT clinical studies: correlation with Ki-67

Reference	Cancer	Results
Vesselle et al. ⁴⁴	NSCLC (n = 11)	SUV: r = 0.84; K _i : r = 0.92
Buck et al. ⁴⁵	Pulmonary nodules (n = 15) ¹	SUV: r = 0.92
Muzi et al. ⁴⁶	NSCLC (n = 17)	K _i : r = 0.92
Choi et al. ⁴⁷	Glioma (n = 9)	SUV: r = 0.817
Kenny et al. ⁴⁰	Breast cancer (n = 12)	SUV: r = 0.79; K _i : r = 0.92
Ullrich et al. ⁴⁸	Glioma (n = 11)	SUV: NC ² ; K _i : r = 0.88 ³
Hatakeyama et al. ⁸	Glioma (n = 18)	SUV: r = 0.89
Francis et al. ⁵¹	Colorectal cancer (n = 10)	SUV: r = 0.8
[¹⁸ F]FLT: clinical studies monitoring response to therapy		
Pio et al. ⁵⁴	Breast cancer	Early changes in [¹⁸ F]FLT correlated with later changes of the tumor marker, CA27.29
Herrmann et al. ⁵⁷	Non-Hodgkin's Lymphoma	Early reductions in [¹⁸ F]FLT SUV detected positive response to R-CHOP/CHOP
Chen et al. ⁵⁶	Glioma	Can identify responders versus nonresponders after 1 – 2 weeks in patients treated with bevacizumab and irinotecan
Kenny et al. ^{40, 53}	Breast cancer	Patients responding to FEC ⁴ chemotherapy exhibited a 53% reduction in [¹⁸ F]FLT K _i value
Sohn et al. ⁵⁵	Adenocarcinoma of the lung	Patients responding to Gefitinib treatment displayed a 36% reduction in [¹⁸ F]FLT SUV

¹ Excluded 4 benign lesions from analysis;² no correlation;³ compartmental modeling showed a correlation of r = 0.88 for k₃ versus Ki-67 expression;⁴ 5-fluorouracil/epirubicin/cyclophosphamide