

Evaluation of [^{18}F]gefitinib as a molecular imaging probe for the assessment of the epidermal growth factor receptor status in malignant tumors

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Abstract

Purpose Gefitinib, an inhibitor of the epidermal growth factor receptor–tyrosine kinase (EGFR-TK), has shown potent effects in a subset of patients carrying specific EGFR-TK mutations in advanced non-small-cell lung cancer. In this study, we asked whether PET with [^{18}F]gefitinib may be used to study non-invasively the pharmacokinetics of gefitinib in vivo and to image the EGFR status of cancer cells.

Materials and methods Synthesis of [^{18}F]gefitinib has been previously described. The biodistribution and metabolic stability of [^{18}F]gefitinib was assessed in mice and vervet monkeys for up to 2 h post injection by both micropositron emission tomography (PET)/computed tomography (CT)

scans and postmortem ex vivo tissue harvesting. Uptake levels of radiolabeled gefitinib in EGFR-expressing human cancer cell lines with various levels of EGFR expression or mutation status were evaluated both in vivo and in vitro.

Results MicroPET/CT scans in two species demonstrated a rapid and predominantly hepatobiliary clearance of [^{18}F]gefitinib in vivo. However, uptake levels of radiolabeled gefitinib, both in vivo and in vitro, did not correlate with EGFR expression levels or functional status. This unexpected observation was due to high nonspecific, nonsaturable cellular uptake of gefitinib.

Conclusion The biodistribution of the drug analogue [^{18}F]gefitinib suggests that it may be used to assess noninvasively the pharmacokinetics of gefitinib in patients by PET imaging. This is of clinical relevance, as insufficient intratumoral drug concentrations are considered to be a factor for resistance to gefitinib therapy. However, the highly nonspecific cellular binding of [^{18}F]gefitinib may preclude the use of this imaging probe for noninvasive assessment of EGFR receptor status in patients.

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Introduction

The epidermal growth factor receptor (EGFR) signaling pathway contributes to a number of processes important to cancer development and progression. Overexpression or mutation of EGFR has been observed in several common tumors including non-small-cell lung cancer (NSCLC) [1], head-and-neck cancer [2], and glioblastoma [3]. The dereg-

ulation of EGFR–tyrosine kinase (EGFR-TK) initiates diverse signal transduction pathways in tumor cells and has a profound effect on their biology. Activation of the EGFR-TK provides signals that drive proliferation, metastasis, angiogenesis, and enhanced cell survival. Therefore, EGFR has been viewed as an attractive target for novel antitumor therapies [4].

In recent years, several small-molecule EGFR inhibitors have been developed for treatment of a variety of malignant tumors [1, 2, 4]. One of those is gefitinib (Iressa, ZD1839), a fluorine-containing anilinoquinazoline, which has recently been approved for the treatment of patients with advanced NSCLC [5, 6]. Gefitinib binds to the intracellular tyrosine kinase domain of EGFR and inhibits EGFR function with nanomolar affinity [7]. Gefitinib and other small-molecule EGFR kinase inhibitors, such as erlotinib (Tarceva), can induce dramatic clinical responses but only in 10–15% of NSCLC patients [8]. At present, the mechanisms for gefitinib sensitivity and resistance are only partially understood [9]. Thus, there is a great need for new approaches to better characterize EGFR expression and function in patients.

A large number of molecular probes for neurological imaging have been developed on the basis of drugs binding to cellular receptors or inhibiting specific enzymes involved in neuronal function [10]. In the present study, we applied a similar approach for imaging EGFR levels in cancer cells and asked whether EGFR receptor status might be imaged with ^{18}F -labeled gefitinib. Murali et al. [11] and our group [12] have independently developed strategies for labeling gefitinib at the 4' position with fluorine-18. Gefitinib contains fluorine-19 at the 4' position; therefore, radiolabeling with fluorine-18 is expected to produce only a minor isotopic effect on the biochemical properties of gefitinib. Two other groups have reported the synthesis of carbon-11-labeled gefitinib [13, 14] for positron emission tomography (PET) imaging of EGFR expression. Since ^{11}C - and ^{18}F -radio-labeled analogs are chemically identical to gefitinib (Fig. 1), they could potentially be used to image EGFR expression

and to study noninvasively the tissue pharmacokinetics of gefitinib in patients. In humans, pharmacokinetic studies of gefitinib by oral administration have shown that the drug is extensively distributed out of the blood into body tissues and is rapidly cleared from the plasma, with the peak plasma concentration of gefitinib reached at 1 h [15]. Hence, with the radioactivity half-life of 2 h, fluorine-18-labeled gefitinib can be used to image the early time points of the in vivo biodistribution of gefitinib. This is also of high clinical relevance, as insufficient intratumoral drug concentrations are being discussed as a potential factor for the low response rates to gefitinib in clinical trials [16, 17].

Traditional drug studies do not provide the means to examine the tissue pharmacokinetics of the drug throughout the body. Therefore, it is difficult to determine (1) whether the drug reaches the target and also (2) whether the dose levels in patients that produce the desired pharmacological effect was achieved. To evaluate whether [^{18}F]gefitinib may be used to assess gefitinib pharmacokinetics and to image the EGFR receptor status of malignant tumors in patients, we first studied the biodistribution and metabolic stability of gefitinib in mice and vervet monkeys. We then measured uptake of gefitinib, both in vivo and in vitro, in tumor cell lines with various degrees of EGFR expression and different sensitivity to EGFR kinase inhibitor therapy because of specific mutations of the EGFR kinase domain.

Materials and methods

Radiolabeled gefitinib

Synthesis and quality control of [^{18}F]gefitinib was performed as previously described [12], with the radiochemical purity greater than 99% and the specific activity greater than 222 GBq/ μmol (>6,000 Ci/mmol). [^3H]gefitinib (specific activity of 236 GBq [6.4 Ci]/mmol) was obtained from Moravsek Biochemicals (Brea, CA) and used for in vitro studies.

Biodistribution of gefitinib in mice

All animal experiments were approved by the UCLA Animal Research Committee. Five non-tumor-bearing mice were studied by microPET/computed tomography (CT) imaging for up to 2 h. Imaging was performed with a microPET FOCUS 220 PET scanner [18] (Siemens Pre-clinical Solutions, Knoxville, TN) and MicroCAT II CT scanner (Siemens Preclinical Solutions). Mice were placed on a heating pad (30°C) and anesthetized using 1.5–2% isoflurane, starting 15 min before injection. [^{18}F]gefitinib (7.4–18.5 MBq [200–500 μCi]) was injected via the tail vein, while the animals were positioned on the scanner bed

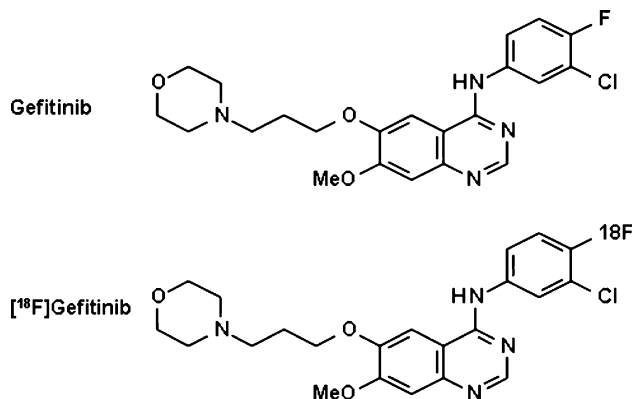


Fig. 1 Chemical structure of gefitinib and [^{18}F]gefitinib

before a dynamic PET study was acquired for 2 h. Mice were then placed in an imaging chamber that minimizes positioning errors between PET and CT to less than 1 mm [18]. PET images were reconstructed by filtered back projection, using a ramp filter to yield an image resolution of 1.7 mm. Matrix size of the reconstructed images was $128 \times 128 \times 95$, and the voxel size was 0.40 mm^3 . 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 fluorine-18. Immediately after the PET scan, mice underwent a 7-min microCT scan, using routine image acquisition parameters [18]. The microCT scan was used for anatomical localization of the tissue concentrations of [^{18}F]gefitinib over time in the microPET study.

To determine temporal changes of tracer concentration in various organs, spherical regions of interests (ROIs, 2 mm diameter) were placed in the area of the organ that exhibited the highest [^{18}F]gefitinib uptake as determined by visual inspection. To ensure accurate anatomical positioning of the ROIs in the various organs, ROIs were placed on fused PET/CT fusion images generated by the AMIDE software [19]. Activity concentrations was quantified as standardized uptake values (SUVs) using the formula: $\text{SUV} = \text{tissue activity concentration (Bq/ml)} / \text{injected dose (Bq)} \times \text{body weight (g)}$.

To validate noninvasive tracer uptake measurements by PET, three mice were injected intravenously with 1.85 MBq (50 μCi) of [^{18}F]gefitinib. The mice were killed 1 h later, and various organs were harvested and weighed. The radioactivity concentration in each tissue was measured by a Packard 5600 gamma counter (Packard, Meriden, CT), and expressed as SUV.

Imaging of vervet monkeys with [^{18}F]gefitinib

To study the biodistribution of [^{18}F]gefitinib in nonhuman primates, two vervet monkeys were fasted for 6 h, anesthetized using 25 mg/kg ketamine and 1–2% isoflurane, and imaged with an ECAT EXACT HR/HR+ scanner (Siemens/CTI, Knoxville, TN) [20]. After an initial transmission scan for attenuation correction, animals were injected intravenously with 370 MBq (10 mCi) of [^{18}F]gefitinib. Five whole-body studies were acquired between 0 and 90 min post injection. Each whole-body study consisted of five bed positions. The acquisition time per bed position was 1 min in the first scan and increased by 1 min for each of the following scans. To determine the time course of [^{18}F]gefitinib uptake in various organs, the AMIDE software was used to draw ROIs (5 mm diameter) in the area of the organ with the highest tracer uptake, as described above. This procedure was repeated for each scan, and time–activity curves were created for the decay-corrected radioactivity concentrations.

Metabolic stability of gefitinib in mice

A dose of 7.4–18.5 MBq (200–500 μCi) of [^{18}F]gefitinib was injected into groups of at least three mice. The animals were killed at different time points (5, 25, 60, 75, 120 min) post injection. Blood was collected with heparin-coated syringes, and the plasma fraction was separated by centrifugation at 3,500 rpm for 5 min. Ether was immediately added to the plasma samples [21]. The ether phase was then removed, and the postextraction phase was then counted in the gamma counter. The ether phase was then evaporated, dissolved in methanol, and loaded onto silica gel-precoated thin-layer chromatography (TLC) plates ($5 \times 20 \text{ cm}$, 250 μm layer thickness; Sigma, St. Louis, MO). [^{18}F]gefitinib was also spotted onto a TLC plate and run as a standard alongside the samples. Elution was performed with MeOH/0.1 N NaCl (4:1) as the mobile phase, and radioactivity on the TLC plates was located by electronic autoradiography with a Automatic TLC-Linear Analyzer (Berthold, Oak Ridge, TN). Pre-extraction blood samples and extracted blood residues were counted in a gamma counter for determination of the extraction efficiency.

Cell cultures

To determine whether [^{18}F]gefitinib can specifically accumulate in EGFR-expressing tumors, we selected a variety of tumor cell lines harboring different EGFR expression levels or mutational status. To determine the effect of EGFR expression levels on gefitinib uptake, we selected the U87 and U87-EGFR glioblastoma cell lines. U87 cells express only low levels of EGFR. The U87-EGFR cells were generated by the stable transfection of U87 with the EGFR gene [22]. These cells were kindly provided by Dr. Paul Mischel (UCLA, Los Angeles, CA). Both cell lines were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, CA), 2 mg/ml glucose, 100 U/ml of penicillin, and 100 U/ml of streptomycin (Invitrogen).

To study the effect of EGFR kinase mutations on the uptake of gefitinib, we measured the two human NSCLC cell lines, H3255 and H1975. H3255 cells carry a L858R point mutation, which causes them to be highly sensitive to gefitinib treatment [23]. In contrast, H1975 harbors both L858R and the secondary mutation T790M. The T790M mutation interferes with the binding of gefitinib to the kinase domain and confers resistance to gefitinib [1, 24]. H1975 cells were obtained from the American Tissue Culture Collection (Rockville, MD); H3255 cells were kindly provided by Dr. Bruce Johnson (Dana Farber Cancer Institute, Boston, MA). Both cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Omega Scientific), 2 mg/ml glucose, 100 U/ml of

penicillin, and 100 U/ml of streptomycin (Invitrogen). All cells were grown at 37°C in an atmosphere of 5% CO₂.

Xenograft model

Severe combined immunodeficient (*Scid/Scid*) mice were purchased from Jackson Laboratories (Bar Harbor, MN). All animal manipulations were performed with the sterile technique following the guidelines of the UCLA Animal Research Committee. U87, U87-EGFR, H3255, and H1975 cells growing exponentially in culture were removed from the plate with trypsin, resuspended in phosphate-buffered saline (PBS) and Matrigel (BD Biosciences, Bedford, MA), and injected subcutaneously into the right hind leg or flank of *SCID* mice at $1\text{--}2 \times 10^6$ cells per mouse. Animals underwent a dynamic microPET/CT scan of 90 min duration after tumors had grown to an approximate size of 6–7 mm.

Cellular uptake of radiolabeled gefitinib

U87, U87-EGFR, H3255, and H1975 cells were plated in six-well plates (Costar, Cambridge, MA) at 1×10^5 cells per well 1 day before the uptake experiment. On the day of the experiment, cells were first incubated at 37° with 20 μM cold gefitinib or vehicle in a serum-free cell culture medium. One hour later, [³H]gefitinib at a final concentration of 1 nM (222 Bq [0.006 μCi]/ml) was added to each well. The cells were then incubated for 1 h at 4°C. Preliminary studies indicated that under this condition, there is no significant depletion of the radiolabeled gefitinib from the culture media. After the incubation period, wells were rinsed twice in cold PBS, lysed with 1% sodium dodecyl sulfate, and measured for cell-associated radioactivity in scintillation cocktail (Scintisafe, Fisher, New Jersey, NJ) using a Packard Beta counter (Packard). Radioactivity uptake values were normalized to the number of viable (trypan blue-negative) cells (ViCell XR, Beckman-Coulter, Fullerton, CA). To estimate the intracellular concentration of [³H]gefitinib, the diameter of 500 detached cells was measured using the ViCell XR system. The cellular volume was then approximated by calculating the volume of a sphere with the same diameter, as previously described [25].

Western blot analysis

EGFR and phospho-EGFR expression levels were assessed by Western blotting of cell lysates, prepared as previously described [26]. For detection of phospho-EGFR, cells were treated with 20 μM of gefitinib or vehicle, then stimulated with 100 ng/ml EGF for 15 min before preparation of the lysates. We used rabbit anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-phospho-EGFR

(Y1173) mAb (Santa Cruz Biotechnology), and rabbit anti-actin antibody (Sigma) for the primary antibody. Peroxidase-conjugated goat anti-rabbit IgG and peroxidase-conjugated goat anti-mouse IgG (both from Jackson ImmunoResearch, West Grove, PA) were used as the secondary antibodies for the Western blots. All blots were performed according to the manufacturer's instructions.

Tracer kinetic analysis

Tumor time–activity curves were fitted using to a standard two-tissue compartment model [27]. In this model, one compartment (q_1) represents the free and nonspecifically bound gefitinib, and the other compartment (q_2) represents specifically bound gefitinib. The flux between these two compartments and gefitinib in the blood is described by the following two differential equations:

$$\frac{dq_1(t)}{dt} = (k_2 + k_3)q_1(t) + k_4q_2(t) + K_1u(t) \quad (1)$$

$$\frac{dq_2(t)}{dt} = k_3q_1(t) - k_4q_2(t) \quad (2)$$

where t is the time after injection and $u(t)$ represents the concentration of gefitinib in the plasma. K_1 describes the transfer rate from plasma to tissue interstitial space, k_2 accounts for efflux from tissue back into plasma plus non-specific binding effects, while k_3 and k_4 describe specific binding. The total concentration of gefitinib in the tissue, $y(t)$, is written as the sum of tracer in both tissue compartments plus the amount of tracer in the tissue blood fraction (f_b), as follows:

$$y(t) = q_1(t) + q_2(t) + f_b \cdot u(t) \quad (3)$$

Because gefitinib showed no uptake by the myocardium, a 1.5-mm spherical ROI was placed in the left ventricle to measure the input function. Using this model, time–activity curves derived from the tumor tissue were fitted using the UCLA Kinetic Imaging System (KIS) [28]; that is, five unknown parameters were estimated (K_1 , k_2 , k_3 , k_4 , f_b). To assess the amount of specifically bound gefitinib, the binding potential BP_P was calculated as recently defined by an international consensus paper [27]:

$$BP_P = \frac{K_1k_3}{k_2k_4} \quad (4)$$

BP_P refers to the ratio at equilibrium of specifically bound radioligand to total radioligand in the plasma [27].

In addition, the total distribution volume of V_T of gefitinib at equilibrium [27] was estimated by Logan analysis. For

this analysis, $\int_0 c_{\text{tumor}}(t)dt/c_{\text{tumor}}(t)$ was plotted against $\int_0 c_{\text{plasma}}(t)dt/c_{\text{tumor}}(t)$. The slope of the linear portion of this plot was used to determine V_T . All tracer kinetic analyses were performed using the KIS software [28].

Statistical analysis

Quantitative data are expressed as mean $\pm$ 1 standard deviation (SD). Differences between experimental groups were tested by analysis of variance and Bonferroni post-hoc tests. Differences were considered significant when $P < 0.05$.

Results

Biodistribution of [^{18}F]gefitinib in mice

Figure 2a shows the time course of the biodistribution of [^{18}F]gefitinib in a non-tumor-bearing mouse. Immediately

after injection, [^{18}F]gefitinib accumulated in the lungs, and within 5 min, started to accumulate in the liver and gallbladder. By 1 h, both the hepatobiliary and the renal elimination pathways of [^{18}F]gefitinib dominated the whole-body distribution, with the highest activity concentrations in the gallbladder, urinary bladder, and parts of the intestines. Moderate activity concentrations were evident in the kidneys and liver. The quantitative analysis of the radioactivity concentration in various tissues over time is shown in Fig. 2b. Within 5 min, radioactivity measured in the liver was at SUV 5.3 ± 0.6 . This corresponds to approximately 30% of the total injected activity. After this phase of initial rapid excretion, blood clearance of gefitinib was slow, with the activity concentrations in the blood remaining almost stable between 15 and 120 min post injection. The postmortem biodistribution study confirmed the results of the measurements derived from the dynamic microPET study (Table 1). At 1 h post injection, the activity concentration of [^{18}F]gefitinib in the intravenously injected

Fig. 2 Biodistribution of [^{18}F]gefitinib in mice. **a** Normal (nontumor-bearing) mice were subjected to a microPET/CT scan after intravenous injection of 7.4 MBq (200 μCi) of [^{18}F]gefitinib. **b** Quantitative analysis of [^{18}F]gefitinib uptake in various tissues over time. Error bars indicate 1 SD

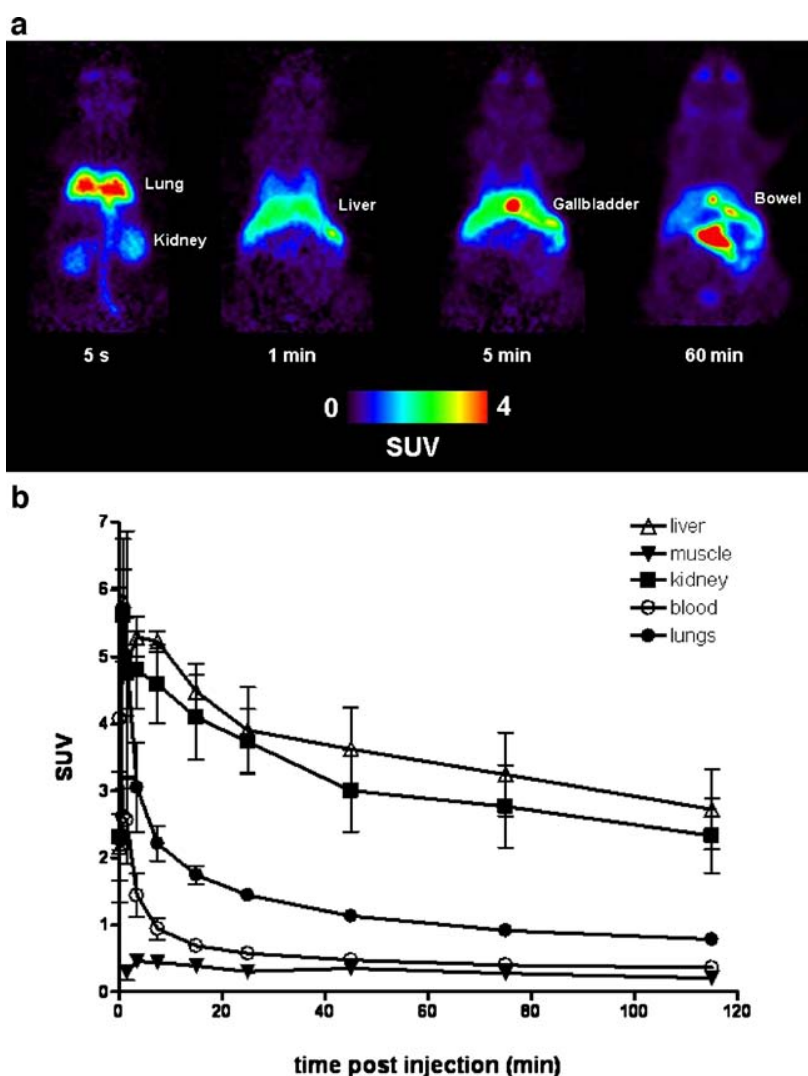
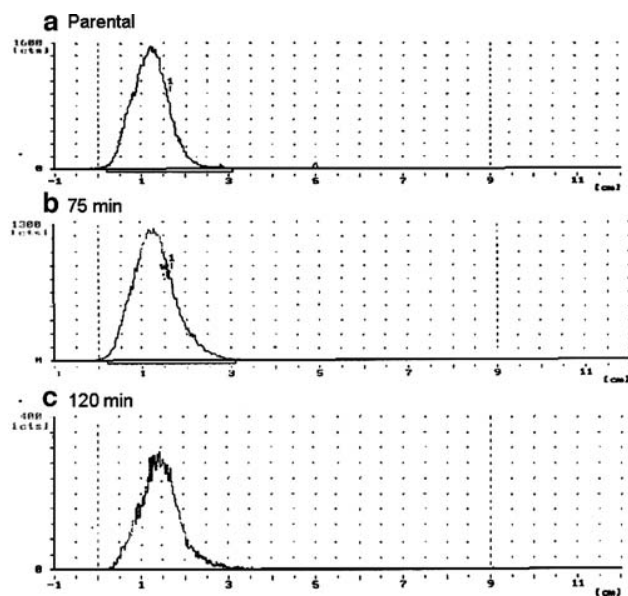


Table 1 Ex vivo biodistribution of [^{18}F]gefitinib in mice at 2 h postinjection ($n=3$)

	SUV
Organs	
Blood	0.36 ± 0.09
Muscle	0.22 ± 0.01
Skin	0.33 ± 0.19
Brown fat	0.92 ± 0.28
Submaxillary glands	2.69 ± 0.73
Liver	3.28 ± 0.62
Stomach	1.51 ± 0.68
Colon	2.27 ± 1.01
Kidney	3.21 ± 1.01
Lung	1.14 ± 0.17
Heart	0.46 ± 0.03
Brain	0.15 ± 0.04
Bladder	1.24 ± 0.69
Tumor xenografts	
H3255	0.22 ± 0.09
H1975	0.15 ± 0.10
U87	0.14 ± 0.05
U87-EGFR	0.14 ± 0.06

animals was similar to the results from the microPET images. Thin-layer radiochromatograms (Fig. 3) indicated that more than 97% of the radioactivity in the plasma represented the nonmetabolized gefitinib at 120 min post injection.

**Fig. 3** TLC radiochemical profiles of plasma extracts obtained from mice injected with 7.4 MBq (200 μCi) of [^{18}F]gefitinib. **a** [^{18}F]gefitinib; **b**, **c** plasma extracts obtained at 75 and 120 min post injection. X-axis, length of TLC plate. Y-axis, intensity (arbitrary units)

Biodistribution of [^{18}F]gefitinib in vervet monkeys

Analysis of the biodistribution of [^{18}F]gefitinib in vervet monkeys over time in various tissues is shown in Fig. 4. Overall, a similar pattern of radioactivity concentrations was observed. However, the hepatobiliary and renal excretion was significantly slower than in mice, as would be expected.

Tumor uptake of [^{18}F]gefitinib in vivo

Figure 5a shows the in vivo time course of gefitinib uptake for the studied tumor types in mice. Representative PET/CT images at 60 min post injection are shown in Fig. 5b. In the PET images, the tumors show no focal uptake when compared to surrounding skeletal muscle. The time–activity curves confirm that tumor uptake of [^{18}F]gefitinib remained low. For all tumors, the tumor-to-blood ratio was less than 1 during the duration of the PET scan (Fig. 5a). Further analysis of the data by nonlinear regression analysis revealed that in all studied tumors, BP_P [^{18}F]gefitinib was low (Table 2). Furthermore, BP_P was virtually identical for U87 xenografts (no EGFR expression by Western blot analysis, Fig. 6a) and U87-EGFR xenografts (overexpression of EGFR, Fig. 6a). Logan analysis also demonstrated only modest differences between V_T among the studied cell lines despite marked differences in EGFR expression levels (Table 2, Fig. 6a). The fractional blood volume (f_b) was found to be low (<5%) for all studied tumors.

[^3H]gefitinib uptake in EGFR in vitro

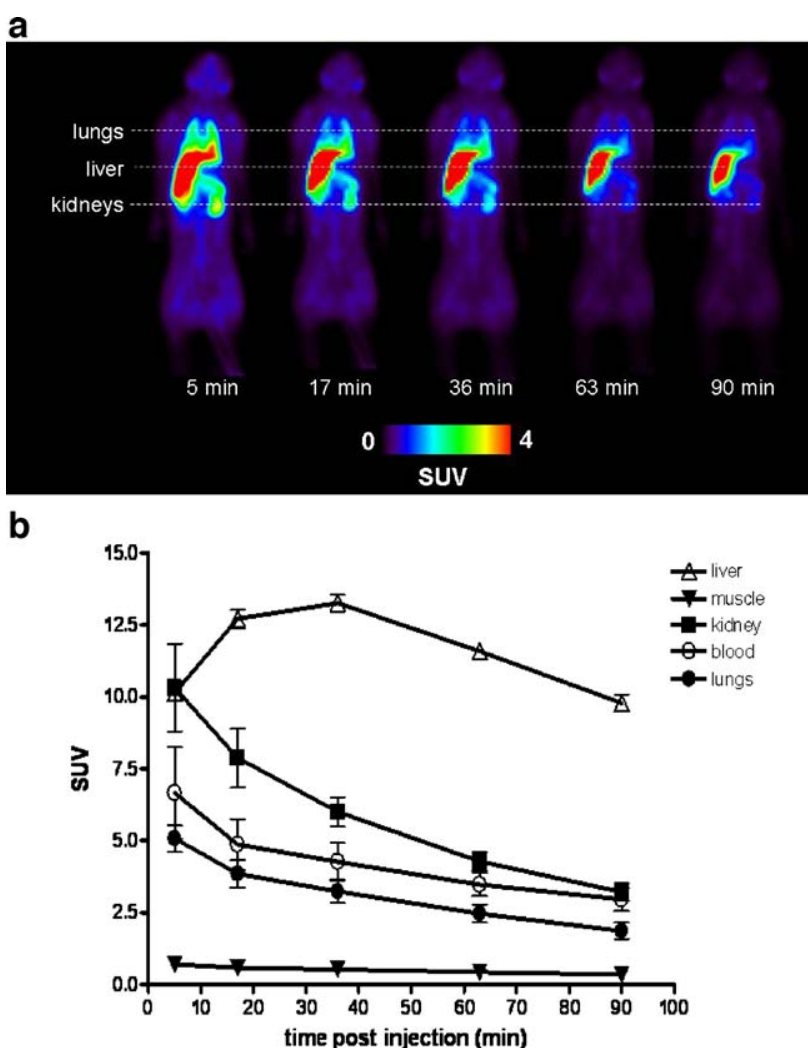
To explain this unexpected result of similar uptake levels in tumors with different EGFR mutational and/or expression status, we performed uptake studies of gefitinib in cell cultures. There was no significant difference between U87 and U87-EGFR cell uptake ($P=0.91$; Fig. 6b) despite marked differences in EGFR expression (Fig. 6a). The addition of 20 μM of unlabeled gefitinib did not significantly reduce uptake of radiolabeled gefitinib in U87 ($P=0.22$), U87EGFR ($P=0.31$), and H1975 cells ($P=0.66$). There was a significant 62% decrease in uptake by H3255 cells ($P=0.005$). In the presence of 20 μM gefitinib, in which we observed inhibition of EGFR phosphorylation in U87, U87-EGFR, and H3255 (Fig. 6c), the estimated activity concentration in the cells was at least 100 times higher than in the culture medium, suggesting a high degree of nonspecific binding.

Discussion

This study indicates that the radiolabeled EGFR kinase inhibitor, [^{18}F]gefitinib, does not show EGFR-specific

Fig. 4 Biodistribution of [^{18}F] gefitinib in vervet monkeys.

a Vervet monkeys were subjected to a PET/CT scan after intravenous injection of 185 MBq (5 mCi) of [^{18}F] gefitinib. **b** Quantitative analysis of [^{18}F] gefitinib uptake in various tissues over time. Error bars indicate 1 SD



accumulation in human tumor xenografts. This makes it unlikely that PET with [^{18}F] gefitinib may be used to images EGFR expression levels in patients. Nevertheless, the lack of EGFR-specific tracer uptake does not necessarily mean that [^{18}F] gefitinib can have no impact for patient studies. Because the imaging probe [^{18}F] gefitinib is chemically identical to the drug gefitinib, [^{18}F] gefitinib can be used as a tracer to study the tissue pharmacokinetics of gefitinib and to determine intratumoral drug concentrations non-invasively. This may provide important insights into the mechanism underlying gefitinib resistance.

Gefitinib is a nanomolar inhibitor of EGFR-TK activity, with an IC_{50} at 20 nM for inhibition of EGFR phosphorylation in vitro [7]. This IC_{50} is higher than that of receptor ligands used in PET imaging of malignant tumors. For example, Ga-DOTA-TOC inhibits binding of somatostatin to the somatostatin 2 receptor with an IC_{50} of 2.5 nM [29]. However, it has been reported that EGFR-overexpressing

tumor xenografts express about 1,000 fmol EGFR per mg protein [30]. This corresponds to an EGFR concentration of about 100 nM [31]. For such high receptor concentrations, one would consider the affinity of gefitinib as sufficient for PET imaging, as the expected bound-to-free ratio at steady state ($B_{\text{max}}/K_d \approx B_{\text{max}}/\text{IC}_{50}$) is about 5 [31]. Based on these promising in vitro characteristics, we explored the possibility of using [^{18}F] gefitinib for the non-invasive imaging of EGFR expression in tumors. However, our data indicate that the in vitro and in vivo [^{18}F] gefitinib uptake in mouse models did not correlate with EGFR expression in the various tumor types that were tested. Lack of correlation with EGFR expression was most likely not because of metabolic instability of [^{18}F] gefitinib, as we did not detect radiolabeled metabolites by TLC during the duration of the PET scans. TLC analysis has been reported previously for the use of detection of metabolites of radiolabeled quinazoline derivatives in rodent blood and plasma

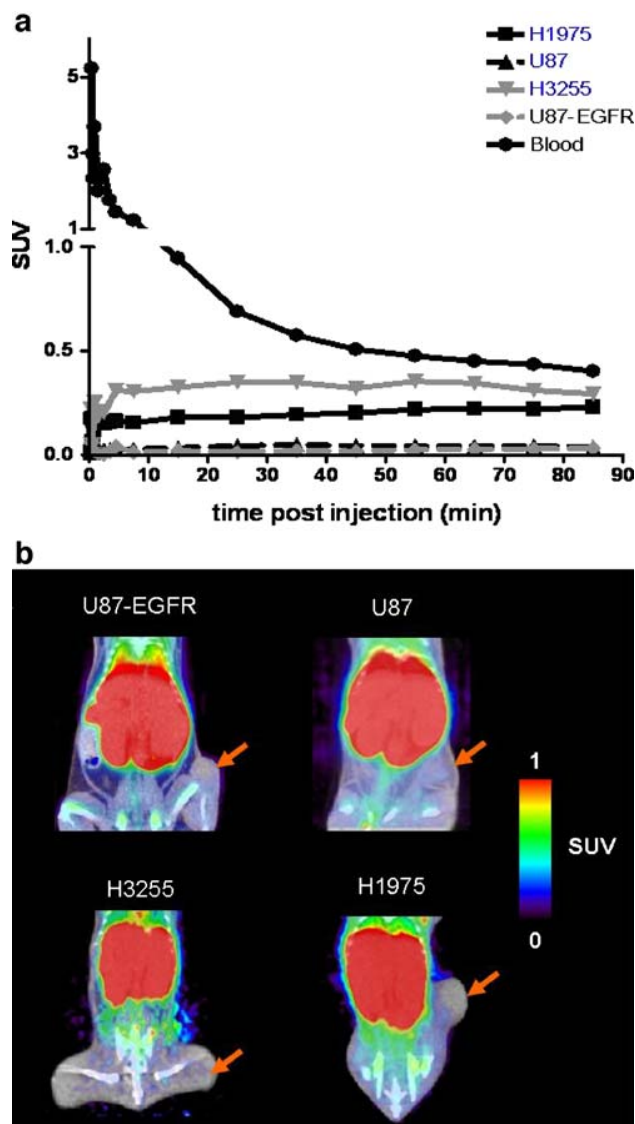


Fig. 5 Tumor uptake of [^{18}F]gefitinib in mice. **a** Quantitative analysis of [^{18}F]gefitinib uptake in various tumors over time in mice. Error bars indicate 1 SD. **b** microPET/CT images of tumor-bearing mice at 1 h post intravenous injection of 7.4 MBq (200 μCi) of [^{18}F]gefitinib

[21, 32]. The metabolic stability that we observed is also consistent with other reports, where more than 90% of gefitinib in the plasma stays intact for up to 8 h after receiving oral doses of gefitinib [33]. This represents a significant advantage to previously described radiolabeled EGFR kinase inhibitors, which were rapidly degraded in vivo [21, 32].

The lack of EGFR-specific tumor uptake in vivo is likely explained by both the rapid hepatobiliary excretion and also the nonspecific uptake of tissues with low EGFR expression. In the cell culture studies, we noticed that in the presence of high concentrations of unlabeled gefitinib, cell uptake of the radiolabeled analogue was not blocked,

Table 2 Results of the tracer kinetic analysis of [^{18}F]gefitinib uptake in tumor-bearing mice

Tumor xenografts	BP _p (two-compartment model)	V_T (Logan analysis)
H3255 ^a	1.14 $\pm$ 0.03	1.21 $\pm$ 0.09
H1975 ^a	0.29 $\pm$ 0.02	0.58 $\pm$ 0.13
U87 ^b	0.73	0.82
U87-EGFR ^b	0.69	0.98

^a $n=2$

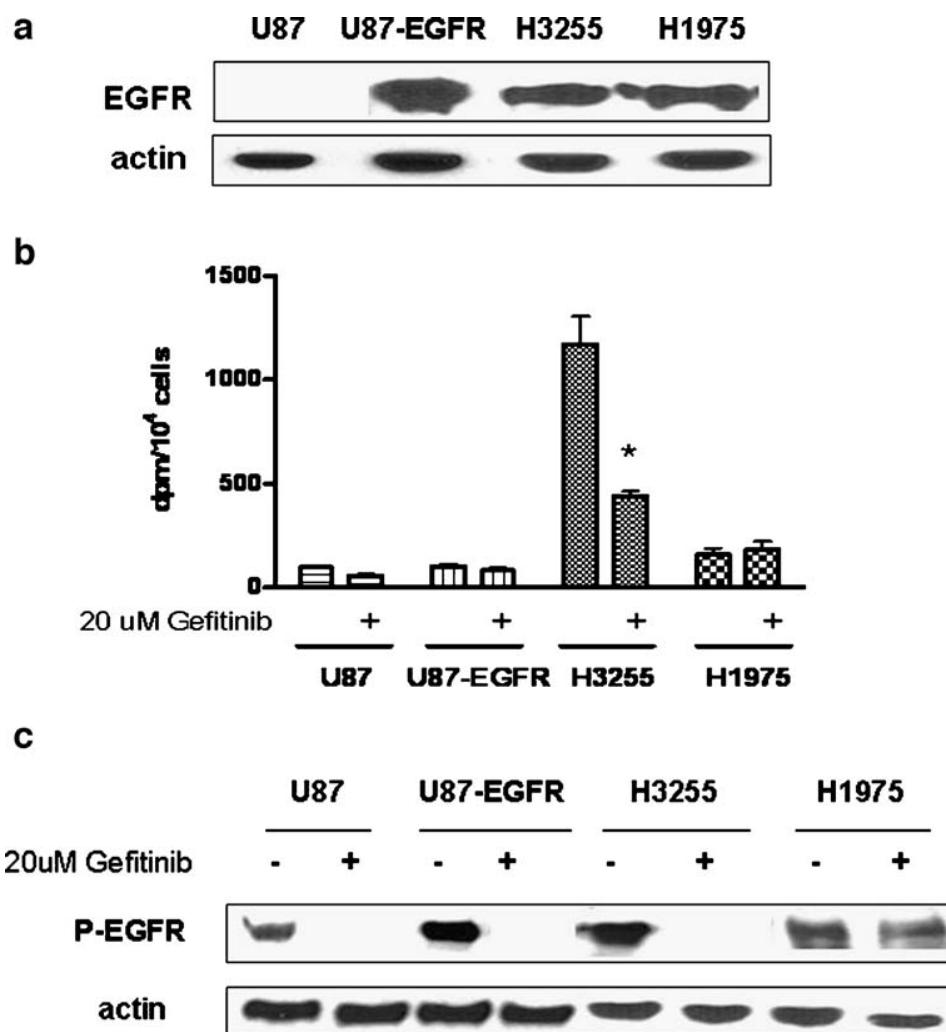
^b $n=1$

and the cellular concentration of [^3H]gefitinib was approximately 100-fold higher than in the cell culture medium. This is most likely due to the lipophilicity of Gefitinib ($\log P=3.46$) [7], which may result in nonspecific binding to the cell membrane. Furthermore, gefitinib, in common with other small molecule kinase inhibitors, binds not only to EGFR kinase but also to several other protein kinases with similar affinity [34]. Tracer kinetic analysis of the PET data confirmed these in vitro findings and demonstrated no significant differences in the binding potential (BP_p) of gefitinib for xenografts with and without EGFR overexpression.

It would be very challenging to directly measure the amount of gefitinib bound to the EGFR or other receptors, as gefitinib does not form a covalent bond with the receptor. Consequently, the EGFR/[^3H]gefitinib complex would not be stable during routine conditions for separating EGFR from other cellular proteins. Therefore, we used an indirect approach to demonstrate that the amount of [^3H]gefitinib bound to EGFR is small compared to total cellular uptake of [^3H]gefitinib. We studied a syngeneic model of two glioblastoma cell lines (U87 and U87-EGFR). These cell lines are genetically identical, except for the overexpression of EGFR by U87-EGFR cells (see Fig. 6a). Nevertheless, [^3H]gefitinib uptake of U87-EGFR cells was not significantly different from U87 cells. Thus, we can exclude the possibility that binding to EGFR significantly contributed to the total [^3H]gefitinib uptake of U87-EGFR cells. Consequently, the amount of specifically bound [^3H]gefitinib must be low compared to free or nonspecifically bound [^3H]gefitinib.

Moreover, when we added cold gefitinib at a high concentration of 20 μM , we saw no significant decrease in [^3H]gefitinib uptake by U87-EGFR cells (Fig. 6b). To prove that this concentration of cold gefitinib completely blocks EGFR, we measured EGF-induced EGFR autophosphorylation, a standard test for assessing EGFR inhibition by gefitinib. As expected from literature data [23, 35], we found that 20 μM of gefitinib completely blocked EGF-induced EGFR phosphorylation (Fig. 6c). In contrast,

Fig. 6 In vitro studies of the various cancer cell lines. **a** Western blot of EGFR from cell lysates. **b** Cellular uptake of [^{3} H] gefitinib. Cells were treated with or without 20 μ M of gefitinib for 1 h, then cellular accumulation levels of [^{3} H] gefitinib were determined after 1 h incubation with [^{3} H] gefitinib. Uptake values are shown as dpm per 10^4 cells. Error bars indicate 1 SD. Asterisk indicates $P < 0.05$ in comparison with H3255 cells without pre-treatment with 20 μ M gefitinib (t test). **c** Western blot of phospho-EGFR from cell lysates



20 μ M of cold gefitinib did not significantly decrease the uptake of [^{3} H] gefitinib by U87-EGFR cells, again confirming that the amount of [^{3} H] gefitinib bound to the EGFR is small compared to free or nonspecifically bound gefitinib.

Finally, uptake of [^{3} H] gefitinib in U87-EGFR cells was comparable to H1975 cells. H1975 cells harbor a well-characterized mutation of the EGFR receptor that interferes with the binding of gefitinib [24]. We could confirm this by showing that 20 μ M gefitinib does not inhibit EGFR autophosphorylation in this cell line (Fig. 6c). Thus, if specific binding of gefitinib to the EGFR would determine the total cellular uptake of [^{3} H] gefitinib, [^{3} H] gefitinib uptake would have to be significantly lower in H1975 than in U87-EGFR cells, as U87-EGFR and H1975 cells express similar amounts of EGFR (Fig. 6a).

H3255 cells demonstrated a higher [^{3} H] gefitinib uptake than U87-EGFR cells. Furthermore, [^{3} H] gefitinib uptake was significantly reduced by 20 μ M of cold gefitinib. This observation may be related to the fact that H3255 cells

harbor a mutation of the EGFR kinase domain, which increases its affinity to gefitinib [23]. However, even for this cell line, the degree of specific binding was not high enough for in vivo imaging, as tumor xenografts of H3255 cells were not visualized with positive contrast in mice. Tracer kinetic analysis confirmed a low binding potential of [^{18} F] gefitinib in H3255 tumors as well as the other studied xenografts (Table 2).

A rapid and predominant hepatobiliary clearance of [^{18} F] gefitinib was observed in both mice and monkeys. This clearance pattern translates into high liver uptake and intestinal excretion in images, which can render the evaluation of areas around the abdomen and pelvis very difficult. A similar biodistribution has been observed for other radio-labeled EGFR kinase inhibitors tested in mice [21, 32, 36]. Nonetheless, assessment of tumor uptake of [^{18} F] gefitinib in other body regions appears feasible, especially in the lungs and the brain. These two areas are of special interest, as gefitinib has been used clinically for treatment of lung cancer and glioblastomas [1, 3].

Using radiolabeled gefitinib to noninvasively measure drug levels within the tumor tissue may provide important insights into the mechanism behind the inadequate response of gefitinib in patients as revealed in several clinical trials (reviewed in [9]). The dose of conventional cytotoxic agents is generally limited by side effects, whereas for cytostatic drugs (including EGFR kinase inhibitors) that inhibit specific signal transduction pathways, target inhibition and antitumor activity can occur at significantly lower concentrations than the maximum tolerated dose [5]. Accordingly, there has been much debate on how to best select the dose of targeted drugs in phase II and III studies [16]. For EGFR kinase inhibitors, this problem is further complicated by the fact that EGFR amplification and specific mutations of the EGFR kinase domain have been shown to markedly increase the sensitivity of tumors to this class of drugs. Thus, if a certain dose of an EGFR kinase inhibitor is selected on the basis of a response rate in a phase II study, this dose may not be appropriate for a different patient population that has different frequencies of EGFR kinase mutations or EGFR amplifications. In fact, it has been hypothesized that the recent failure of gefitinib in a large phase III study may have been due to the fact that the tissue concentration gefitinib was not sufficient to inhibit EGFR kinase activity in patients without EGFR kinase mutations [16]. This hypothesis could be tested in patients by measuring the tissue pharmacokinetics of gefitinib by PET with [^{18}F]gefitinib. Furthermore, gefitinib is a substrate of the ABCG2 multidrug transporter, a member of the adenosine triphosphate binding cassette (ABC) transporter superfamily, and expression of this transporter has been shown to be associated with resistance to gefitinib therapy [17]. This resistance mechanism could also be studied by measuring uptake and clearance of [^{18}F]gefitinib by PET.

McKillop et al. [33] have previously reported that [^{14}C]gefitinib is significantly accumulated in tumors expressing wild-type EGFR, with a tumor/plasma ratio of 2.4 at 2 h and 6.5 after 8 h. This difference in observation between their study and ours may be related to the considerably higher dose of gefitinib that was used in their study. McKillop et al. administered 50 mg/kg of [^{14}C]gefitinib orally, which is more than 1,000 times higher than the tracer amount of [^{18}F]gefitinib that was used in our study. The plasma concentration of [^{14}C]gefitinib in their report was $\sim 10\ \mu\text{M}$ at 2 h after drug administration. This means that the plasma concentration was 50 times higher than the IC_{50} value of gefitinib, suggesting that the measured tumor uptake may have been related to the mass effect of the high dose and unlikely to be receptor specific. Further investigation in determining uptake and clearance of [^{18}F]gefitinib as a function of the injected dose would aid in reconciling the reported differences between the findings of McKillop et al. and our results. The difference in (1) route of gefitinib

administration and (2) the tumor cell lines used also might have contributed to the differing results.

Recently, Pal et al. [36] reported the development of a [^{124}I]radiolabeled irreversible EGFR kinase inhibitor. In mice bearing tumors overexpressing wild-type EGFR, the tumor/muscle ratio was more than 5 at 1 h postinjection. However, the tumor/blood ratio was less than 1 at the same time point. As with [^{18}F]gefitinib and other radiolabeled EGFR kinase inhibitors [21, 32], there was also rapid hepatobiliary clearance. Therefore, the authors suggest that improved water solubility may be required to optimize the pharmacokinetics of this class of molecular imaging agents. However, EGFR kinase and other protein kinase inhibitors bind to an intracellular target and reach this target by passive diffusion through the cell membrane. Consequently, all these drugs need to be sufficiently lipophilic to reach their targets. Thus, high lipophilicity is likely one of the fundamental problems of using radiolabeled protein kinase inhibitors as imaging agents.

Conclusion

[^{18}F]gefitinib did not accumulate specifically in EGFR-expressing tumor xenografts in this study, regardless of whether wild-type or gefitinib-responsive mutants were concerned. This unexpected result illustrates that the development of targeted imaging probes involves more than radiolabeling targeted drugs. Targeted drugs can provide a starting point for the development of imaging probes, but reduction in unspecific binding is necessary to achieve tumor-specific uptake with a good target-to-background ratio. This optimization is likely to be challenging for imaging probes based on protein kinase inhibitors, because these probes generally reach their target by passive diffusion through the cell membrane, therefore generally needing to be lipophilic. Nevertheless, [^{18}F]gefitinib may be clinically useful for studying noninvasively the tissue pharmacokinetics of gefitinib. Measurement of intratumoral drug concentration by PET with [^{18}F]gefitinib is expected to provide important insights into the mechanisms of gefitinib resistance in clinical studies.

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