Early Detection of Erlotinib Treatment Response in NSCLC by 3′-Deoxy-3′-[\(^{18}F\)]-Fluoro-L-Thymidine (\([^{18}F\)]FLT) Positron Emission Tomography (PET)

The Harvard community has made this article openly available. **Please share** how this access benefits you. Your story matters.

**Citation**
Ullrich, Roland T., Thomas Zander, Bernd Neumaier, Mirjam Koker, Takeshi Shimamura, Yannic Waerzeggers, Christa L. Borgman, et al. 2008. Early detection of erlotinib treatment response in NSCLC by 3′-Deoxy-3′-[\(^{18}F\)]-Fluoro-L-Thymidine (\([^{18}F\)]FLT) positron emission tomography (PET). PLoS ONE 3(12): e3908.

**Published Version**
doi://10.1371/journal.pone.0003908

**Accessed**
August 12, 2017 6:55:57 PM EDT

**Citable Link**
http://nrs.harvard.edu/urn-3:HUL.InstRepos:10198625

**Terms of Use**
This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA

*(Article begins on next page)*
Early Detection of Erlotinib Treatment Response in NSCLC by 3’-Deoxy-3’-[18F]-Fluoro-L-Thymidine ([18F]FLT) Positron Emission Tomography (PET)

Roland T. Ullrich1,2, Thomas Zander6, Bernd Neumaier1, Mirjam Koker1, Takeshi Shimamura4,5, Yannic Waerzeggers1, Christa L. Borgman4,5, Samir Tawadros6, Hongfeng Li1, Martin L. Sos1, Heiko Backes1, Geoffrey I. Shapiro4,5, Jürgen Wolf6, Andreas H. Jacobs1,2,3, Roman K. Thomas1,6,7,9, Alexandra Winkeler1,2,9

1 Max Planck Institute for Neurological Research with Klaus-Joachim-Zülch-Laboratories of the Max Planck Society, Medical Faculty of the University of Cologne, Cologne, Germany, 2 Center for Molecular Medicine Cologne (CMMC), Cologne, Germany, 3 Klinikum Fulda, Fulda, Germany, 4 Department of Medical Oncology, Dana-Farber Cancer Institute, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, 5 Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, United States of America, 6 Department I of Internal Medicine and Center of Integrated Oncology Köln – Bonn, University of Cologne, Cologne, Germany, 7 Chemical Genomics Center of the Max Planck Society, Dortmund, Germany

Abstract

Background: Inhibition of the epidermal growth factor receptor (EGFR) has shown clinical success in patients with advanced non-small cell lung cancer (NSCLC). Somatic mutations of EGFR were found in lung adenocarcinoma that lead to exquisite dependency on EGFR signaling; thus patients with EGFR-mutant tumors are at high chance of response to EGFR inhibitors. However, imaging approaches affording early identification of tumor response in EGFR-dependent carcinomas have so far been lacking.

Methodology/Principal Findings: We performed a systematic comparison of 3’-Deoxy-3’-[18F]-fluoro-L-thymidine ([18F]FLT) and 2-[18F]-fluoro-2-deoxy-D-glucose ([18F]FDG) positron emission tomography (PET) for their potential to identify response to EGFR inhibitors in a model of EGFR-dependent lung cancer early after treatment initiation. While erlotinib-sensitive tumors exhibited a striking and reproducible decrease in [18F]FLT uptake after only two days of treatment, [18F]FDG PET based imaging revealed no consistent reduction in tumor glucose uptake. In sensitive tumors, a decrease in [18F]FLT PET but not [18F]FDG PET uptake correlated with cell cycle arrest and induction of apoptosis. The reduction in [18F]FLT PET signal at day 2 translated into dramatic tumor shrinkage four days later. Furthermore, the specificity of our results is confirmed by the complete lack of [18F]FLT PET response of tumors expressing the T790M erlotinib resistance mutation of EGFR.

Conclusions: [18F]FLT PET enables robust identification of erlotinib response in EGFR-dependent tumors at a very early stage. [18F]FLT PET imaging may represent an appropriate method for early prediction of response to EGFR TKI treatment in patients with NSCLC.

Introduction

Inhibition of the epidermal growth factor receptor (EGFR) tyrosine kinase by small molecule kinase inhibitors has evolved as a critical therapeutic strategy in non-small cell lung cancer (NSCLC). However, only a subset of patients responds to the treatment; most of these were found to carry activating mutations in EGFR [1,2,3]. Sensitive methods for mutation detection in clinical specimens have been developed that enable patient selection for genetically informed cancer therapy [4,5]. However, additional patients whose tumors lack EGFR mutations might also benefit from EGFR inhibitors.

Positron emission tomography using [18F]FDG PET is an effective means to staging of NSCLC patients and is now part of routine staging protocols [6,7]. Furthermore, [18F]FDG PET has been found to enable identification of NSCLC patients responding to chemotherapy [8] and in mice bearing EGFR-mutant tumors responding to gefitinib [9]. Given that EGFR inhibitor-induced
apoptosis in EGFR-mutant tumors is preceded by a pronounced cell cycle arrest [10], we hypothesized that imaging modalities reflecting tumor cell proliferation rather than glucose metabolism might afford even earlier measurements of tumor growth inhibition.

\[^{18}F\]-fluoro-L-thymidine (\[^{18}F\]FLT) PET has been developed as a specific marker to measure cellular proliferation at \[^{18}F\]FDG PET has been developed as a specific marker to measure cellular proliferation at \[^{18}F\]FDG [1]. As an analog substrate of thymidine, \[^{18}F\]FLT is phosphorylated by thymidine kinase 1 (TK1). TK1 is a cytosolic enzyme that is synthesized when proliferating cells enter the S-phase for DNA synthesis [12]. Moreover, \[^{[18}F\]FLT uptake values have been shown to correlate to tumor cell proliferation as assessed by Ki-67 immunostaining [13,14]. Thus, \[^{[18}F\]FLT PET might serve as an effective means to measure drug-induced cell cycle inhibition in vivo. Supporting this notion is the recent observation of an advantage of \[^{18}F\]FLT over \[^{18}F\]FDG in measuring response of BRAF V600E-mutant melanomas to treatment using \[^{18}F\]FLT PET rather indirectly reflects tumor cell proliferation and is therefore not a suitable marker for EGFR inhibition at that early stage of treatment. Thus, a therapy-induced reduction in \[^{18}F\]FDG PET signal is likely to be a later event, occurring during actual tumor shrinkage.

In summary, \[^{[18}F\]FLT PET enables detecting tumor cells arrested in G1 before morphological changes thereby providing a surrogate marker for erlotinib-induced apoptosis and tumor shrinkage at a very early time point. Thus, \[^{[18}F\]FLT PET might
Figure 1. Erlotinib treatment induces down-regulation of EGFR/EGFR-coupled signaling pathways and cell cycle arrest with subsequent induction of apoptosis in EGFR sensitive tumor cells. The erlotinib sensitive cell lines HCC827 and PC9 and the erlotinib-resistant cell line H1975 were treated with the indicated doses of erlotinib for 24 hours. Whole-cell lysates were subjected to immunoblotting with the indicated antibodies (A). PC9, HCC827 and H1975 cells were treated with erlotinib (0.5 μM) for 24h, 48h and 72h; nuclei were prepared, stained with propidium iodide and analyzed by flow cytometry. Results are shown for the G1 and S phases of the cell cycle (A). Apoptotic effects of erlotinib on EGFR-sensitive cell lines in comparison to the T790M mutant H1975 (B). Annexin V FACs was performed 12h, 24h, 36h, 48h, 72h and 96h after 0.5 μM erlotinib treatment. Images show Annexin V-positive cells after 48h in the different cell lines.
doi:10.1371/journal.pone.0003908.g001
be an appropriate method for the early identification of patients benefiting from EGFR TKI treatment.

Materials and Methods

Cell cultures
We used the EGFR-tyrosine kinase inhibitor (TKI) sensitive adenocarcinoma cell lines HCC827, PC9 and the resistant cell line H1975. All cell lines were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS, Roche Diagnostics, Mannheim, Germany), 1% penicillin and 1% streptomycin (P/S, Life Technologies) at 37°C in a 5% CO₂/95% air atmosphere.

Western blot analysis
Cells were serum-starved for 24h in the presence or absence of erlotinib. After preparation of cell lysates phosphorylation level of the proteins were determined using antibodies for total EGFR, phospho-EGFR (pEGFR) (both purchased from Biosource), total Akt and phospo-Akt (pAKT) (both obtained from Cell Signaling Technology).

Apoptosis assay
Cells were plated in 6-well plates, after 24h of incubation treated with erlotinib for 12h, 24h, 36h, 48h, 72h, and 96h and finally harvested after trypsinization. Then cells were washed with PBS, resuspended in Annexin-V binding buffer and finally stained with Annexin-V-FITC and PI. FACS analysis was performed on a FACS Canto Flow Cytometer (BD Biosciences, Germany) and results were calculated using FACS Diva Software.

Cell cycle analysis
Cells were fixed and then treated with RNase A (500 µg/ml). Following resuspension of the cells in propidium iodide and in sodium citrate cells were analysed for DNA content by flow cytometry.

Xenograft model
All animal procedures were in accordance with the German Laws for Animal Protection and were approved by the local animal committee and the Bezirksregierung Köln. Tumors were generated by s. c. injecting 5 × 10⁶ tumor cells into nu/nu athymic male mice. When tumors had reached a size of 100 mm³, animals were randomized into two groups, control (vehicle) and erlotinib-treated mice. Erlotinib (Tarceva) was dosed at 6% Captisol (CyDex, Inc., Lenexa, KS) in water for solution over night. All controls were dosed with the same volume of vehicle. After PET measurement mice were treated daily by oral gavage of 50mg/kg Tarceva. Tumor size was monitored every two days by measuring perpendicular diameters. Tumor volumes were calculated from the determination of the largest diameter and its perpendicular according to the equation [tumor volume = a × (b²/2)].

PET imaging
Tumor bearing mice were investigated using a R4 microPET scanner (Concord Microsystems, Inc., Knoxville, TN). [¹⁸F]FLT and [¹⁸F]FDG synthesis were performed as described previously [17,18]. No-carrier-added [¹⁸F]FLT was administered i.v. (tail vein) into experimental animals with a dose of 200 µCi/kg/mouse. No-carrier-added [¹⁸F]FDG was injected intraperitoneally (i.p.) with a dose of 300 µCi. Since the biodistribution of [¹⁸F]FDG is...
comparable for i.v. and i.p. injections after 60 min and i.p. injections allow for a more accurate dosage of tracer injection, we decided to use intraperitoneal injections for [18F]FDG as recently described [19,20]. All PET images were performed 60 min after injection. Data evaluation was based on a volume of interest (VOI) analysis of the entire tumor. For data analysis we used the maximal voxel radioactivity within the tumors. To determine the uptake ratio we chose the mediastinum as reference since we observed constant uptake for [18F]FLT and [18F]FDG in this region. Data were decay corrected and divided by the total injected dose to represent percentage injected dose per gram (%ID/g).

**Immunohistochemistry and TUNEL detection**

After the last PET measurements animals were sacrificed and s.c. tumors were extracted. After fixation (4% paraformaldehyde, 4°C, 24h; 30% sucrose, 4°C, 24h), tumors were embedded in tissue freezing medium (Jung, Nussloch, Germany) and cut in 10-μm frozen sections. H&E staining on the tissue was done according to standard protocols. Tumor proliferation was assessed using an anti-Ki-67 monoclonal antibody (1:200 dilution, KI6811C06, DCS, Hamburg, Germany), and the percentage of specifically stained cancer cells for Ki-67 was calculated. The number of Ki-67 positive nuclei was determined as percentage of Ki-67 stained nuclei per total number of nuclei in three representative tumor areas (F1+F2+F3/3 (%)) that contained the highest average fraction of labelled cells as described recently [14]. To quantify the number of apoptotic positive cells TUNEL was performed on cryostat tumor slices with the DeadEndTM TUNEL system (Promega) following the manufacturer’s directions. The average numbers of TUNEL positive were counted in three randomly selected fields in two tumor samples from each cell line.

**Statistical analysis**

Wilcoxon test was performed using SPSS software (release 11.0.1 SPSS, Inc., Chicago, IL, USA), statistical significance was set at p<0.05.

**Acknowledgments**

We thank Dr. Hasmann for assistance for the application of Tarceva and Annika Lindemann for great assistance of PET data acquiring.
Author Contributions

Conceived and designed the experiments: RTU TZ BN TS YW HB JW AHJ RKT AW. Performed the experiments: RTU MK TS YW CB ST HL MLS HB. Analyzed the data: RTU MLS HB. Contributed reagents/materials/analysis tools: RTU TZ BN MK ST GIS JW AHJ RKT AW. Wrote the paper: RTU TZ BN YW HL MLS HB JW AHJ RKT AW.

References

1. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, et al. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350: 2129–2139.

2. Pao W, Miller V, Zakowski M, Doherty J, Politi K, et al. (2004) EGFR receptor gene mutations are common in lung cancers from “never smokers” and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 101: 13306–13311.

3. Thomas RK, Nickerson E, Simons JF, Janne PA, Tengs T, et al. (2006) Sensitive mutation detection in heterogeneous cancer specimens by massively parallel picoliter reactor sequencing. Nat Med 12: 852–855.

4. Weber WA, Dietlein M, Hellwig D, Kirsch CM, Schicha H, et al. (2003) [PET with (18)F-fluorodeoxyglucose for staging of non-small cell lung cancer]. Nuklearmedizin 42: 135–144.

5. Buck AK, Schirrmacher V, Hetzel M, Von Der Heide M, Halter G, et al. (2002) 3-deoxy-3-[18F]fluorothymidine positron emission tomography for noninvasive assessment of proliferation in pulmonary nodules. Cancer Res 62: 3331–3334.

6. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, et al. (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92: 205–216.

7. Hamacher K, Czernin HH, Stocklin G (1986) Efficient stereospecific synthesis of no-carrier-added 2-[18F]-fluoro-2-deoxy-D-glucose using aminopolyether supported nucleophilic substitution. J Nucl Med 27: 235–238.

8. Machulla H, Blochet A, Kunzisch M, Pierr M, Wei R, et al. (2000) Simplified labeling approach for synthesizing 3-deoxy-3-[18F]fluorothymidine ([18F]-FLT). J Radiochem Nucl Chem 243: 843–846.

9. Fueger BJ, Czernin J, Hildebrandt I, Tran C, Halpern BS, et al. (2006) Impact of animal handling on the results of 18F-FDG PET studies in mice. J Nucl Med 47: 999–1006.

10. Schiffer WK, Mirrioune MM, Dewey SI. (2007) Optimizing experimental protocols for quantitative behavioral imaging with 18F-FDG in rodents. J Nucl Med 48: 277–287.

11. Shields AF, Grierin JR, Dohmen BM, Machulla HJ, StayanoEJC, et al. (1998) Imaging proliferation in vivo with [F-18]FLT and positron emission tomography. Nat Med 4: 1334–1336.