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Therapy response monitoring of the early effects of a new BRAF inhibitor on melanoma xenograft in mice: evaluation of 18F-FDG-PET and 18F-FLT-PET

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Inhibition of the V600E mutated BRAF kinase gene (BRAFV600E) is an important and effective approach to treating melanomas. A new specific small molecule inhibitor of BRAFV600E, PLX3603, showed potent melanoma growth-inhibiting characteristics in preclinical studies and is currently under clinical investigation. In this study we investigated the feasibility of 18F-FDG and 18F-FLT-PET to monitor the early effects of the BRAFV600E inhibitor in mice with melanoma xenografts. SCID/beige mice with subcutaneous (s.c.) A375 melanoma xenografts, expressing BRAFV600E, received the BRAFV600E inhibitor twice daily orally (0, 25, 50 and 75 mg/kg). At 1, 3 and 7 days after start of therapy, the uptake of 18F-FDG in the A375 tumors was observed by ex vivo biodistribution analysis. Administration of 75 mg/kg BRAF inhibitor for 1, 3 and 7 days resulted in a significantly decreased 18F-FDG uptake in A375 tumors (41, 35 and 51%, respectively). 18F-FLT uptake in the A375 tumors was low at baseline and no significant changes in 18F-FLT uptake were observed at any of the doses administered. These effects were corroborated by serial in vivo 18F-FDG and 18F-FLT-PET imaging. These data demonstrate that 18F-FDG-PET can be used as an imaging biomarker to noninvasively evaluate the early effects of PLX3603. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: BRAF inhibitor; FDG-PET; FLT-PET; therapy response monitoring; biomarker

1. INTRODUCTION

Metastatic melanoma is an aggressive disease and patients have a poor prognosis. The median survival time for patients with metastasized melanoma is a mere 8–9 months and 3-year survival is <15% (1). Only a few effective therapies for metastatic melanoma are available, such as chemotherapy with dacarbazine and immunotherapy with interferon-α and interleukin-2, although their advantages in terms of survival and response rate are moderate.

In the search for new therapy targets, a genomic screen for mutations in signaling molecules in melanoma revealed numerous mutations, particularly in members of the mitogen-activated protein kinase (MAPK) pathway (2). Mutations in the NRAS gene have been observed in ~20% of all melanomas, whereas mutations in the BRAF gene have been observed in ~50% of all melanomas. These mutations lead to constitutive kinase activation, independent of upstream signaling. This continuous activation of the NRAS/BRAF/MAPK/ERK signal transduction pathway stimulates melanocyte proliferation and is therefore a key driving force for the development of melanoma. Nearly 90% of all of the mutations observed in the BRAF gene are a single-point mutation, which results in a valine-to-glutamic acid substitution at residue 600 (BRAFV600E). Owing to its high prevalence, the selective inhibition of the oncogenic BRAFV600E is a prime therapeutic approach for treatment of metastatic melanoma (3).

Vemurafenib (PLX4032) is the first selective small molecule inhibitor of BRAFV600E that has been approved for clinical use to treat patients with BRAFV600E-positive metastatic melanoma. In clinical trials, treatment with vemurafenib resulted in a response rate of 53–81%, a median overall survival (OS) of 13.2–15.9 months, a median progression-free survival (PFS) of 6.9 months and a 63% decrease in risk of death compared with treatment with dacarbazine (median OS of 9.6 months and a median PFS of 1.6 months) (4–7). Dabrafenib, another specific BRAFV600E inhibitor, showed similar clinical response rates (50%) and median PFS (5.1 months) to vemurafenib (8). Although not life-threatening,
treatment with vemurafenib and dabrafenib may result in serious adverse events, including the increased incidence of cutaneous squamous cell carcinoma (cuSCC, in approximately 20% of the patients) and keratoacanthoma and the possible subsequent development of additional malignant lesions (9).

Recently, a new inhibitor of BRAFV600E (PLX3603) was developed, which in pre-clinical studies showed promising melanoma growth-inhibiting characteristics. PLX3603 inhibited cell proliferation and MAPK signaling in several BRAFV600E-expressing cancer cell lines more potent than vemurafenib and in mice with melanoma xenografts it showed increased anti-tumor activity in vivo compared with vemurafenib (10). More importantly, PLX3603 had no effect on the tumor growth of A431 SCC xenografts, whereas vemurafenib treatment stimulated its growth (10). A phase I clinical dose escalation study showed an improved safety profile for PLX3603 (11). No dose-limiting toxicities were observed, adverse events were mild to moderate and only 7% of the patients developed cuSCC (11). Efficacy of PLX3603 treatment was encouraging: 18 out of 44 patients showed stable disease, while 14 patients showed a partial response (11).

Continued activation of the MAPK-pathway, owing to mutated BRAF, has been directly linked to activation of glucose metabolism and proliferation. For instance, ERK stimulates glucose metabolism via the LKB1-AMP pathway and the expression of the glucose transporter, GLUT1 (12). Phase II clinical trials with vemurafenib and dabrafenib may result in serious adverse events were mild to moderate and only 7% of the patients developed cuSCC (11). Efficacy of PLX3603 treatment was encouraging: 18 out of 44 patients showed stable disease, while 14 patients showed a partial response (11).

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percentage injected dose per gram for the voxels within the VOI$_{50\%}$ assuming a density of 1 g/ml.

2.6. Immunohistochemistry

Directly following dissection, the A375 tumors were fixed in 4% paraformaldehyde. Tumors were dehydrated in a graded series of ethanol, embedded in paraplast and 6 μm sections were cut. Sections were stained for phosphorylated ERK (pERK), glucose transporter 1 (GLUT1) and Ki67, a marker for cell proliferation. In short, antigen was retrieved in citrate buffer (10 mM, pH 6.0, 95–100 °C), after which sections were incubated with H$_2$O$_2$ (3%) to inactivate endogenous peroxidase activity. Sections were pre-incubated with normal goat serum (20%, pERK and GLUT1) or normal swine serum (20%, Ki67) to block nonspecific binding sites.

Sections were incubated with rabbit-anti-GLUT1 antibody (1:200, R13-9052-P, Neomarkers, Fremont, CA, USA), rabbit-anti-pERK antibody (1:100, 4376, Cell Signaling Technology, Danvers, MA, USA) or rabbit-anti-Ki67 antibody (1:100, RM9106, Thermo Scientific, Waltham, MA, USA) for 2 h. Sections incubated with the GLUT1 and pERK antibodies were incubated with biotinylated goat-anti-rabbit secondary antibody (1:200, VectaStain, Vector Laboratories, Burlingame, CA, USA) for 1 h and followed by incubation for 30 min with VectaStain ABC reagent. The sections incubated with the Ki67 antibody were incubated with swine-anti-rabbit-peroxidase antibody (1:500, Dako, Glostrup, Denmark). Peroxidase activity was stained with 3,3′-diaminobenzidine (Powervision DAB, Immunologic, Duiven, The Netherlands) in the presence of H$_2$O$_2$ and all sections were counterstained with hematoxylin.

2.7. Statistical Analysis

All data are presented as mean values ± standard deviation. The size of the experimental groups (n) is given in parentheses. Differences between groups were assessed by one-way ANOVA and Bonferroni post hoc test or by Student’s t-test. Statistical significance was set at p < 0.05 (two-tailed) and probabilities are indicated by asterisks (* p < 0.05; ** p < 0.01; *** p < 0.001).

3. RESULTS

3.1. Effect PLX3603 on Tumor Growth

Administration of PLX3603 inhibited A375 tumor growth. Compared with vehicle-treated animals, a dose of 25 mg/kg resulted in a 34% decrease in tumor size after 7 days of treatment (p = 0.004; Fig. 1). Higher doses (50 and 75 mg/kg) resulted in 39 and 29% tumor size reduction after 3 days of treatment (p = 0.003 and p = 0.02, respectively) and 56 and 49% after 7 days of treatment (p < 0.0001; Fig. 1).

3.2. 18F-FDG and 18F-FLT Biodistribution Studies

The biodistribution data of 18F-FDG and 18F-FLT in the mice with s.c. A375 tumors during treatment with PLX3603 are summarized in Figs 2 and 3, respectively. Increased uptake of 18F-FDG compared with blood levels was observed in all tissues investigated, while increased 18F-FLT uptake was only observed in spleen, kidney, intestines and bone marrow. A dose-dependent decrease in 18F-FDG uptake in the A375 melanoma xenografts was observed after treatment with PLX3603. One day after administration of 75 mg/kg PLX3603 (three doses) the 18F-FDG uptake in the tumor (2.9 ± 0.5 %ID/g) was significantly reduced by 40% (p = 0.006) compared with vehicle treatment (4.8 ± 1.0 %ID/g; Fig. 2a). After 3 days, the administration of both 50 mg/kg (3.4 ± 0.8 %ID/g) and 75 mg/kg (3.3 ± 0.6 %ID/g) PLX3603 resulted in significantly decreased uptake of 18F-FDG in the tumor (33 and 35% respectively, p = 0.033) compared with vehicle treatment (5.1 ± 0.6 %ID/g; Fig. 2b). Seven days after administration of the three doses, 25 mg/kg (2.9 ± 0.4 %ID/g), 50 mg/kg (2.3 ± 0.6 %ID/g) and 75 mg/kg (2.2 ± 0.6 %ID/g), there was a significant reduction of the uptake of 18F-FDG in the tumor (36, 49 and 51% respectively, p < 0.0001) compared with vehicle treatment (4.5 ± 0.6 %ID/g; Fig. 2c).

The administration of PLX3603 had no consistent effect on 18F-FLT uptake in the tumor (Fig. 3). 18F-FLT uptake in the A375 tumor decreased after treatment with 75 mg/kg PLX3603 for 1 and 7 days (36%, p = 0.041 and 61%, p = 0.039, respectively). However, this decrease was accompanied by decreased blood levels of 18F-FLT (35%, p = 0.031 and 32%, p = 0.014, respectively; Fig. 3). It appears that the reduction of uptake of 18F-FLT in the tumor was the result of enhanced clearance of 18F-FLT from the blood (Fig. 3). Indeed, 18F-FLT tumor-to-blood ratios did not significantly change compared with tumor-to-blood ratios of vehicle treated animals after 1 (p = 0.933), 3 (p = 0.798) or 7 days (p = 0.133) of treatment with PLX3603 (data not shown).

3.3. 18F-FDG and 18F-FLT-PET/CT

18F-FDG uptake in A375 melanoma xenografts was clearly visualized in the 3D PET/CT images (Fig. 4a). Three days after the start of PLX3603 treatment, 18F-FDG uptake in the tumor was lower. This decrease in 18F-FDG uptake was sustained up to 7 days of treatment. 18F-FDG uptake was further observed in muscle, brown adipose tissue, kidney, intestines, heart and brain, 18F-FLT uptake in tail is likely to be injection related. Quantification of the 18F-FDG-PET signal in the A375 melanoma xenograft VOI showed a significantly decreased 18F-FDG uptake after 3 (40%, P = 0.012) and 7 days (38%, P = 0.006) after the start of treatment, compared with basal 18F-FDG uptake (Fig. 4b). 18F-FLT PET/CT images revealed a relative low uptake of 18F-FLT in A375 melanoma xenografts, which was not further reduced after treatment with PLX3603 (Fig. 5a). High uptake of 18F-FLT was observed in the kidney, spleen, intestines and bone marrow. Quantification of the 18F-FLT-PET signal in the A375...
tumor VOI confirmed that the $^{18}$F-FLT uptake in the tumor did not change during the treatment with PLX3603 (Fig. 5b).

3.4. Immunohistochemistry

Staining for pERK in A375 melanoma xenografts was reduced after 3 days of treatment with 75 mg/kg PLX3603, indicating inhibition of the MAPK pathway (Fig. 6). The decreased uptake of $^{18}$F-FDG in A375 tumors was not accompanied by reduced expression of GLUT1 (data not shown), which may be explained by the relatively small changes in $^{18}$F-FDG uptake and the involvement of other proteins, such as hexokinases, in cellular uptake of $^{18}$F-FDG. Similarly, no change in Ki67 expression was observed in tumors of animals treated with PLX3603, which may be due

Figure 2. $^{18}$F-FDG uptake (percentage of injected dose per gram tissue, %ID/g) in tissues of SCID/beige mice ($n = 5$) with s.c. A375 melanoma xenografts after 1 (a), 3 (b) and 7 days (c) of treatment with PLX3603 (25, 50 and 75 mg/kg, administered orally, twice daily.)
to low baseline expression of Ki67 in the A375 tumors (data not shown).

4. DISCUSSION

The dose- and time-dependent effects of PLX3603 treatment on 18F-FDG-uptake in A375 xenografts observed in this study, show that 18F-FDG-PET is a potential imaging biomarker to noninvasively evaluate the pharmacodynamic effects of the BRAFV600E inhibitor PLX3603 on melanoma in an early phase, whereas these effects could not be assessed with 18F-FLT-PET. Ex vivo biodistribution analysis revealed a significant 40% decrease in 18F-FDG uptake as early as 1 day after administration of PLX3603 at 75 mg/kg, before any change in tumor size was apparent. This decrease persisted during the 7 day dosing regimen.

Figure 3. 18F-FLT uptake (%ID/g) in tissues of SCID/beige mice (n = 5) with s.c. A375 melanoma xenografts after 1 (a), 3 (b) and 7 days (c) of treatment with PLX3603 (25, 50 and 75 mg/kg, administered orally, twice daily).
(35 and 51% at 3 and 7 days, respectively). Lower doses of PLX3603 also resulted in a reduced \(^{18}\)F-FDG uptake in the tumor after longer exposure: a dose of 50 mg/kg resulted in a decreased \(^{18}\)F-FDG uptake after 3 days, whereas at a dose of 25 mg/kg \(^{18}\)F-FDG uptake only decreased significantly after 7 days. Immuno-histochemical analysis of the tumor showed a decreased expression of pERK after 3 days of treatment with PLX3603, confirming a pharmacological effect of PLX3603 on the inhibition of the MAPK pathway.

The observed effects of PLX3603 on \(^{18}\)F-FDG uptake in A375 melanoma xenografts could also be monitored by \textit{in vivo} \(^{18}\)F-FDG-PET imaging. After 3 and 7 days of treatment with 75 mg/kg PLX3603, a 40 and 38% reduction in \(^{18}\)F-FDG uptake in the A375 xenografts was detected with \(^{18}\)F-FDG-PET, which is comparable to the \textit{ex vivo} biodistribution analysis. Other small-molecule BRAF\(^{V600E}\) inhibitors, RAF256 (100 mg/kg) and vemurafenib (50 mg/kg), also induced reduced \(^{18}\)F-FDG uptake in A375 melanoma xenografts (17,18). In patients with metastatic melanoma, treatment with vemurafenib for 15 days resulted in an 87% reduction of \(^{18}\)F-FLT uptake, although the reduced uptake did not predict responses as measured by RECIST (19). The results of the present study show that \(^{18}\)F-FDG-PET can be used as a pharmacodynamic marker to monitor the dose response and time course of the response of PLX3603, and therefore \(^{18}\)F-FDG-PET can be a useful tool in the clinical development of PLX3603, although caution is needed when extrapolating data from nude mouse xenograft tumor models to melanoma in men.

A few studies have shed light on the mechanism how BRAF\(^{V600E}\) inhibition could lead to the reduction of uptake of \(^{18}\)F-FDG in the tumor. Inhibition of the MAPK-pathway, including inhibition via BRAF, is directly linked to pathways that regulate glucose metabolism. For instance, inhibition of BRAF\(^{V600E}\) results in the activation of the LKB1-AMP pathway, via ERK, which negatively influences glucose metabolism (13).

Although \textit{in vitro} studies have shown reductions in \(^{18}\)F-FLT uptake in some melanoma cells lines after treatment with the BRAF\(^{V600E}\) inhibitors RAF265 and vemurafenib (17,20), in this study we show that PLX3603 did not induce decreased \(^{18}\)F-FLT uptake in the A375 melanoma xenografts, as measured by \textit{ex vivo} biodistribution analysis and \(^{18}\)F-FLT-PET. It is not uncommon that \(^{18}\)F-FLT uptake does not reflect changes in tumor proliferation. Proliferating tumor cells are not only dependent on uptake of thymidine via the nucleotide transporter (salvage pathway), but thymidine can also be synthesized \textit{de novo} from deoxyuridine monophosphate via thymidine synthetase. Furthermore, in mice thymidine plasma levels tend to be high and endogenous thymidine may compete with \(^{18}\)F-FLT (21), which may explain the low \(^{18}\)F-FLT uptake at baseline and the lack of reduced \(^{18}\)F-FLT uptake in the A375 xenografts upon BRAF\(^{V600E}\) inhibitor treatment. These results do not exclude \(^{18}\)F-FLT-PET to
monitor the effects of PLX3603 per se, as baseline 18F-FLT in the present melanoma model was relatively low, possibly precluding measurement of further reduction of FLT uptake.

This study shows that treatment with the specific BRAF<sup>V600E</sup> inhibitor PLX3603 results in inhibition of the MAPK-pathway in A375 melanoma xenografts, as demonstrated by a decreased pERK expression. The reduced MAPK-pathway activity probably results in a reduced glucose metabolism in A375 tumors, which is reflected by the decreased uptake of 18F-FDG as assessed by 18F-FDG-PET. Furthermore, 18F-FDG-PET allowed the monitoring

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**Figure 5.** (a) 18F-FLT-PET/CT images of a representative mouse at day -1, 1, 3 and 7 of treatment with PLX3603 (75 mg/kg, administered orally, twice daily). White arrows indicate 18F-FLT uptake in the A375 melanoma xenograft. (b) 18F-FLT uptake (%ID/g) in A375 melanoma xenografts as determined from PET signal at -1, 1, 3 and 8 days of treatment with PLX3603 (75 mg/kg, administered orally, twice daily), in three mice.

**Figure 6.** pERK immunohistochemistry on sections of A375 melanoma xenografts of SCID/beige mice treated with 75 mg/kg PLX3603 (a–c) or vehicle only (d–f) for 1 (a, d), 3 (b, e) and 7 days (c, f).
of dose responses and time course effects of PLX3603 activity. These data demonstrate the potential of 18F-FDG-PET as a pharmacodynamic marker to noninvasively evaluate the early effects of the BRAF<sup>V600E</sup> inhibition by PLX3603 and to further define optimal dosing and timing of PLX3603.

**Acknowledgements**

The authors gratefully acknowledge B. Lemmers, K. Lemmens, I. Lamers and H. Arts for expert assistance in the animal experiments.

**Conflict of interest**

Edwin Geven received a Roche Postdoctoral Fellowship grant. Stefan Evers, Tapan Nayak, Mats Bergström and Fei Su are employees of F. Hoffmann-La Roche, the sponsor of the study.

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