**18F-FAZA PET Imaging Response Tracks the Reoxygenation of Tumors in Mice upon Treatment with the Mitochondrial Complex I Inhibitor BAY 87-2243**

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**Abstract**

**Purpose:** We describe a noninvasive PET imaging method that monitors early therapeutic efficacy of BAY 87-2243, a novel small-molecule inhibitor of mitochondrial complex I as a function of hypoxia-inducible factor-1α (HIF1α) activity.

**Experimental Design:** Four PET tracers [18F-FDG, 18F-Fpp (RGD)2, 18F-FLT, and 18F-FAZA] were assessed for uptake into tumor xenografts of drug-responsive (H460, PC3) or drug-resistant (786-0) carcinoma cells. Mice were treated with BAY 87-2243 or vehicle. At each point, RNA from treated and vehicle H460 tumor xenografts (n = 3 each) was isolated and analyzed for target genes.

**Results:** Significant changes in uptake of 18F-FAZA, 18F-FLT, and 18F-Fpp(RGD)2 (P < 0.01) occurred with BAY 87-2243 treatment with 18F-FAZA being the most prominent. 18F-FDG uptake was unaffected. 18F-FAZA tumor uptake declined by 55% to 70% (1.21% ± 0.10% ID/g to 0.35 ± 0.1% ID/g; n = 6, vehicle vs. treatment) in both H460 (P < 0.001) and PC3 (P < 0.05) xenografts 1 to 3 days after drug administration. 18F-FAZA uptake in 786-0 xenografts was unaffected. Decline occurred before significant differences in tumor volume, thus suggesting 18F-FAZA decrease reflected early changes in tumor metabolism. BAY 87-2243 reduced expression of hypoxia-regulated genes CA IX, ANGPTL4, and EGLN-3 by 99%, 93%, and 83%, respectively (P < 0.001 for all), which corresponds with reduced 18F-FAZA uptake upon drug treatment. Heterogeneous expression of genes associated with glucose metabolism, vessel density, and proliferation was observed.

**Conclusions:** Our studies suggest suitability of 18F-FAZA-PET as an early pharmacodynamic monitor on the efficacy of anticancer agents that target the mitochondrial complex I and intratumor oxygen levels (e.g., BAY 87-2243). Clin Cancer Res; 21(2): 1–12. ©2014 AACR.

**Introduction**

Studies into the gene and protein expression profile on cancers (1) have resulted in novel strategies to monitor anticancer therapies by radiotracers (2, 3). Such approaches are important as clinical investigations show that expanding tumors lead to heterogeneous patterns of tissue hypoxia (4–6). This has the initial consequence of impeding growth but over time, there will be selection for tumor subpopulations that grow even more aggressively and invasively under hypoxia (7). Clinical investigations carried out over the last two decades have demonstrated that the prevalence of hypoxic tissue areas [i.e., O2 tensions (pO2 values) ≤2.5 mmHg or %O2 of 0.3] is characteristic of locally advanced solid tumors and is a relevant factor in the pathophysiology of the tumor (7). In addition, hypoxic tumors are also known to be especially resistant to radiotherapy (8). Thus, it is evident that hypoxia influences multiple, critical steps in the process of tumorigenesis, often leading to a more malignant and metastatic stage. It is also evident that additional monitoring tools, such as molecular imaging, are required to adjust therapies such that they are more appropriate to the cancer profiles of the individual patient. To achieve this, it is vital to understand how to abrogate such a response. Ultimately, one also needs reagents that could potentially resolve tumor hypoxia.

Recently, the experimental drug BAY 87-2243 has been identified that acts by impacting the hypoxia-induced activity of the HIF1α pathway, principally by inhibiting the activity of mitochondrial complex I (9). A chemically related drug candidate, BAY 84-7296, sensitizes tumors to radiotherapy through the same mechanism, that is, by resolving cancer hypoxia via inhibition of mitochondrial complex I (10). HIF1α is a transcription factor that plays a key role in initiating and maintaining eukaryotic responses to hypoxic and free radical stress (7, 8, 11, 12). In cell
Cell culture of H460, PC3, and 786-0

in PBS. Ethanol in PBS. Vehicle solution was 20% solutol/10% ethanol prepared as a suspension in a solution of 20% solutol and 10% pyrazol-1-yl)methyl\[piperazine; chemical structure disclosed in ref. 9; shown in Supplementary Fig. S4) was prepared as a suspension in a solution of 20% solutol and 10% ethanol in PBS. Vehicle solution was 20% solutol/10% ethanol in PBS.

Cell culture of H460, PC3, and 786-0

The human cancer cell lines used in this study were: H460 lung carcinoma cells, PC3 prostate cancer cells, and 786-0 renal cell carcinomas. All lines were purchased from the ATCC and pre-tested for the presence of endotoxins and mycoplasma. No other authentication was done. H460 lung carcinoma cells were grown in RPMI1640 (high glucose), 2 mmol/L GlutaMax, and 1 mmol/L sodium pyruvate, 1.5 g/L sodium bicarbonate (Gibco BRL/Invitrogen), 10% FBS, and 1% penicillin (100 U/mL)/streptomycin (100 mg/mL). PC3 prostate cancer cells were propagated in F12/Kaighn medium with 2 mmol/L GlutaMax, 1.5 g/L sodium bicarbonate (Gibco BRL/Invitrogen), and 10% FBS with 1% penicillin (100 U/mL)/streptomycin (100 mg/mL). 786-0 renal cell carcinoma cultures were grown in RPMI1640 medium, 10% FBS, and 1% penicillin (100 U/mL)/streptomycin (100 mg/mL).

Mouse tumor xenograft model

Animal protocols were approved by the Stanford Administrative Panel on Laboratory Animal Care. Fourteen- to 16-week-old, female nude mice (Charles River Laboratories, Inc.) were inoculated subcutaneously on both scapular shoulder blades with 5 × 10^6 human cancer cells per side. The mice were anesthetized with 2% isoflurane in oxygen at 2 L/minute during the injections. The tumor xenografts were grown to at least 200 mm^3 (3). For H460 cells, a period of 7 to 10 days after injection was needed while 786-0 and PC3 cells required 4 to 6 weeks. The majority of xenografted mice were used for small-animal PET studies. Small-animal PET scans were done before vehicle or drug treatment and at defined times after treatment (see Results section). To associate tumor growth kinetics with changes in radiotracer uptake, tumor volume measurements (taken via calipers) were performed at various time points throughout the course of the experiments. At the end of studies, the mice were sacrificed and the tumors excised and frozen in optimum cutting temperature medium (OCT) for immunohistochemical analysis. In one set of experiments, the xenografts were used exclusively for real-time PCR studies and selected tumors were isolated at various times before and after drug or vehicle delivery.

Preclinical experimental protocols

Time-dependency experiment. Small-animal PET scans were performed 1 day before the oral administration of either vehicle (20% solutol/10% EtOH in PBS) or BAY 87-2243 (9 mg/kg in vehicle solution). Scans were then performed 1, 3, 5, or 7 days after the initiation of the drug or vehicle treatments. Treatments were given via oral gavage daily. Tumor size was also monitored daily starting at day 1 through the end of studies. Sample size was n = 6 tumors in 3 mice. At the end of each study, mice were pretreated with pimonidazole (9 mg/kg, tail vein injection 1.5 hours before autopsy), sacrificed, and tumors frozen in OCT. Samples were then analyzed via immunohistochemistry and real-time PCR (RT-PCR) to determine molecular response to treatment.

Specificity experiments. Time dependency of tumor growth and radiotracer uptake in the presence of escalating concentrations of BAY 87-2243 was compared among the treated groups and with the vehicle-control groups. Three separate cohorts of mice (n = 6 tumor in 3 mice per cohort) were given either vehicle-control (20% solutol/10% EtOH in PBS), 3 mg/kg BAY 87-2243 (in solutol vehicle), or 9 mg/kg BAY 87-2243. Follow-up specificity experiments at 1 mg/kg BAY 87-2243 and at 9
mg/kg BAY 87-2243 were also undertaken. As a negative control, equivalent preclinical experiments were done on 786-0 xenografts.

Radiotracer production and purification

Synthesis of \(^{18}\text{F}-\text{Fpp}(\text{RGD})_2\). All chemicals obtained commercially were of analytical grade (Sigma-Aldrich) and used without further purification. \(^{18}\text{F}-\text{Fpp}(\text{RGD})_2\) was synthesized as per a standardized protocol (14). Finished reaction products were purified and analyzed via reversed phase high-performance liquid chromatography as described in a previous publication (14). Radiochemical yield was 50% ± 14% (n = 10) with specific activity of 18.5 × 10^5 Bq \(^{18}\text{F}/\text{umole FPP (RGD)}_2\). Radiochemical purity was 95%.

Synthesis of \(^{18}\text{F}-\text{FDG}\). The synthesis of \(^{18}\text{F}-\text{FDG}\) was performed according to a previous publication (15). Tail vein injection of tracer was completed on 6-hour fasted athymic nude mice (female, 14–16 weeks). Radiochemical yield was 63% ± 11% (n = 10) with specific activity of 5 × 10^5 Bq \(^{18}\text{F}/\text{umole FDG}\). Radiochemical purity was 95%.

Synthesis of \(^{18}\text{F}-\text{FLT}\). The synthesis of \(^{18}\text{F}-\text{FLT}\) was performed according to a previous publication (3). Radiochemical yield was 13% ± 1% (n = 10) with specific activity of 37 × 10^5 Bq \(^{18}\text{F}/\text{umole FLT}\). Radiochemical purity was 95%.

Synthesis of \(^{18}\text{F}-\text{FAZA}\). \(^{18}\text{F}-\text{FAZA}\) (Supplementary Fig. S4) was synthesized as described previously (16–19). Radiochemical yields were approximately 21% at end of the bombardment with specific activity of 9.8 × 10^5 Bq \(^{18}\text{F}/\text{umole FAZA}\). Typically, 7 to 11 GBq of \(^{18}\text{F}-\text{FAZA}\) were isolated with radiochemical purity of generally more than 95%.

Small-animal PET scanning

An R4 microPET (Siemens Medical Solutions USA, Inc.) was used for imaging, which has an approximate resolution of 2 mm in each axial direction (20). After a tail vein injection of \((3.7 ± 0.3) \times 10^6\) Bq of \(^{18}\text{F}-\text{FP-PRGD}_2\), \(^{18}\text{F}-\text{FLT}\), \(^{18}\text{F}-\text{FDG}\), or \(^{18}\text{F}-\text{FAZA}\) in 100 µL of PBS, a 3-minute prone acquisition scan was performed approximately 60 minutes after injection (90 minutes for \(^{18}\text{F}-\text{FAZA}\)). A heating pad, heat lamp, or hot water was used to dilate the tail veins for injections.

Except for those injected with \(^{18}\text{F}-\text{FDG}\), the mice were maintained under isoflurane anesthesia during the injection and scanning periods only. In between, the mice were given ad libitum access to food and water. For mice injected with \(^{18}\text{F}-\text{FDG}\), the mice were fasted 6 hours before the injections and were kept under anesthesia between the injection and scanning sessions. For all experiments, the mice were kept warm using a heating pad or heat lamp while under anesthesia to maintain a body temperature of around 35°C. microPET images were reconstructed with the ordered subsets expectation maximization algorithm (21) using 16 subsets and 4 iterations. Attenuation correction was not performed.

microPET image analysis

Ellipsoidal 3-dimensional regions of interest (ROI) were manually drawn around the edge of the tumor xenograft activity by visual inspection using AMIDE software (22).

Immunohistochemical analysis

In nude mice bearing preestablished H460 tumor xenografts (80 mm³ in size; n = 3 per group), either a single or dual oral treatment was performed with vehicle or BAY 87-2243 (9 mg/kg). One day after, pimonidazole (9 mg/kg, Natural Pharmacia International) was injected via the tail vein. Mice were sacrificed 1.5 hours after the pimonidazole injection. Tumors wereexcised and processed for pimonidazole staining according to the protocol provided by Natural Pharmacia International (23).

Immunohistochemical assays for CD31 were done on H460 xenografts after one, two, and four treatments of vehicle or BAY 87-2243 (9 mg/kg). Primary antibody was a rat anti-mouse-CD 31 (MEC 13.3; Fa BD Pharmingen), diluted 1:250. Secondary antibody was a biotinylated goat anti-rat antibody (Fa BD Pharmingen), diluted 1:100. All tissues were counterstained with hematoxylin. Calculations of percent hypoxic fraction (PHF) and percent vascular fraction (PVF) was determined by using ImageJ to trace and then to integrate the areas of both positive staining and the total tumor. The hypoxic and vascular fractions were then determined by obtaining the ratio for area of positive staining over total tumor area.

Real-time PCR analysis

Nude mice bearing preestablished H460 tumor xenografts (grown for 10 days) were treated orally with vehicle or BAY 87-2243 (9 mg/kg every day, qd). RNA from xenograft tumors was isolated from satellite animal groups either before treatment or after 1 and 3 days of treatment with BAY 87-2243 according to published methods (9). Expression levels of the HIF1α target genes were quantified by RT-PCR pursuant to established protocols (9). Data were normalized to α-actin and were given in arbitrary units as the mean of n = 3 animals/treatment group ± SD.

Statistical analysis

Statistical analysis was performed either with Excel 2011 (Microsoft) or with GraphPad Prism version 6.00 (Graph Pad Software). Paired t-tests were used when paired data from the same mouse were compared. Unpaired t-tests were used when two groups of data were compared. When multivariate analyses for groups of more than 3 were needed, ANOVA techniques were employed. Correlation coefficients were calculated when two groups of nominal variables were compared. A significant value of P < 0.05 was used. Data are reported as mean ± SD.

Results

BAY 87-2243 impacts growth of H460 and PC3 but not 786-0

Tumor xenografts of H460 (Fig. 1A), PC3 (Fig. 1B), and 786-0 (Fig. 1C) were established as test models. For both H460 and PC3, the treatment with BAY 87-2243 significantly abrogated tumor growth \((P < 0.001 \text{ vehicle vs. treatment, } n = 6 \text{ tumors per condition})\) when compared with the vehicle controls (Fig. 1A and B). BAY 87-2243 did not affect the kinetics of tumor growth for 786-0 xenografts (Fig. 1C, n = 6 tumors per condition) when compared with vehicle controls. In no cases did oral administration of BAY 87-2243 affect the total weight of any animal model that was studied (Supplementary Fig. S1).
BAY 87-2243 reduces 18F-FAZA uptake and growth in H460 lung carcinoma xenografts in a dose-dependent manner

To examine the concordance between tracer uptake and drug efficacy, we examined uptake of [18F]-FDG, [18F]-FPP(RGD)₂, [18F]-FLT, 18F-FAZA at time points before, 1 day after, and 3 days after vehicle or drug treatment. In H460 xenografts, reduction of tracer uptake by BAY 87-2243 was most pronounced for 18F-FAZA (Fig. 2A and B). One day after drug treatment, 18F-FAZA uptake declined by 50% compared with prescan controls (0.1%ID/g to 0.55% ID/g, n = 6, P < 0.001 vehicle vs. treatment). By day 3, a significant difference (P < 0.01 vehicle vs. treatment, n = 6 tumors in 3 mice per treatment) in tumor volume between vehicle and treated groups became apparent and this occurred with significantly (P < 0.001) abrogated 18F-FAZA uptake in the tumors from the treated cohorts. Thus, changes in 18F-FAZA uptake occurred before significant changes in tumor size.

To determine whether BAY 87-2243 reduces both tumor growth and 18F-FAZA uptake in a dose-dependent manner, murine models of H460 xenografts were given escalating doses of the drug (3 and 15 mg/kg, respectively). At both drug concentrations, tumor sizes started to be reduced 24 to 48 hours after initial drug treatment. However, the degree of tumor shrinkage was positively related to dosage of BAY 87-2243, namely, the percent reduction of tumor size was greater for input of 15 mg/kg/d BAY 87-2243 than for a dosage of 3 mg/kg/d (n = 6 tumor per group, Fig. 2E); however, the differences were not significant. In a second set of experiments where the effects of dosage 1 and 9 mg/kg BAY 87-2243 were examined, the dose-dependent effect on tumor shrinkage with drug treatment was more pronounced and significant (P < 0.001, ANOVA vehicle vs. treatment group, n = 6 tumors per group, Supplementary Fig. S7B). Such findings were consistent with separate investigations by Ellinghaus and colleagues (9).

To determine whether the initial 18F-FAZA findings from H460 could be extended to other human cancer models, we performed...
similar studies in mice bearing human PC3 xenografts. When time-dependent $^{18}$F-FAZA uptake studies were performed in the presence of vehicle versus 9 mg/kg/d BAY 87-2243 oral gavage, $^{18}$F-FAZA uptake was significantly reduced by nearly 70% in the drug-treated cohorts (1.2 ± 0.1%ID$_{mean}$/g to 0.50 ± 0.1%ID$_{mean}$/g, n = 6 tumors per group, $P < 0.001$ vehicle vs. treatment; Fig. 3A and B). For PC3 xenografts, a significant reduction ($P < 0.001$) in $^{18}$F-FAZA uptake occurred as early as one day after initiation of drug treatment, before any significant differences in tumor volume appeared between vehicle and drug-treated animals (Fig. 3C and D). Differences in $^{18}$F-FAZA uptake persisted to day 3 after initiation of the drug treatment, at which

Figure 2. Pharmacodynamic effects of vehicle (•) and BAY 87-2243 (9 mg/kg, □) on reduction of $^{18}$F-FAZA uptake in the H460 tumor xenograft model in both single dose and dose-dependent manners. A, single voxel plane coronal microPET scans (tracer is $^{18}$F-FAZA) of nude mice containing H460 tumor xenografts. Scans performed on mice before treatment (left column) and 1 (middle column) and 3 days (right column) after treatment for either vehicle (upper panel) or 9 mg/kg BAY 87-2243 (lower panel). B, quantitative assessments of $^{18}$F-FAZA uptake of corresponding tumor xenografts. Uptake expressed in terms of %ID$_{mean}$/g. Vehicle cohorts represented by solid bars while hatched, open bars represent uptake in tumors from drug-treated animals. C, significant ($P < 0.001$ vehicle vs. treatment) percent loss in %ID$_{mean}$/g values, 1 day after start of drug treatment. D, relative loss in uptake occurred before significant percent decline in tumor volume ($P < 0.001$). N = 6 tumor over three mice per cohort. ± or +, $P < 0.001$, vehicle vs. treatment. Tumors indicated by white arrows. E, tumor growth kinetics of H460 xenografts for given oral gavages of vehicle or 3 and 15 mg/kg of BAY 87-2243. F, quantitative assessments of $^{18}$F-FAZA uptake of corresponding tumor xenografts. Uptake expressed in terms of %ID$_{mean}$/g. N = 6 tumor over three mice per cohort. ± or +, $P < 0.001$, vehicle vs. treatment. Tumors indicated by white arrows.
point there occurs a significant abrogation of tumor growth with BAY 87-2243 oral gavage. In a similar finding to that with the H460 cohorts, the BAY 87-2243 reagent reduced both 18F-FAZA and tumor growth for PC3 xenografts in a dose-dependent manner (Supplementary Fig. S2).

Drug treatment reduces neither 18F-FAZA uptake nor growth in BAY 87-2243–resistant human 786-0 renal cell carcinoma xenografts

BAY 87-2243 did not inhibit the growth of 786-0 human renal cell carcinoma xenografts (Fig. 1C). When 18F-FAZA small-animal PET was performed on these animals, the scans revealed no significant difference in 18F-FAZA uptake between vehicle treated mice and those treated with BAY 87-2243 (oral gavage, 9 mg/kg; Figs. 1C and 4).

Lack of effect by BAY 87-2243 on 18F-FDG uptake in murine models of H460 and PC3 xenografts

When BAY 87-2243 was administered to nude mice carrying H460 tumor xenografts, no change in 18F-FDG uptake was observed (Fig. 5A) between vehicle- and BAY 87-2243–treated mice at 3 days after treatment (scans for 1 day after treatment was not done). For mice carrying the human-derived PC3 xenografts, no significant difference in 18F-FDG was observed between vehicle- and BAY 87-2243–treated mice at 1 and 3 days after treatment (Fig. 5B).

BAY 87-2243 reduces 18F-FLT uptake in both H460 human lung cancer and human PC3 prostate cancer xenografts

When the effect of BAY 87-2243 on 18F-FLT uptake was examined, by day 1 after treatment, there was a 25% reduction in %IDmean/g in treated H460 xenografts compared with vehicles (4.3 ± 0.3 vs. 3.0 ± 0.3 vehicle vs. treatment, P < 0.01, n = 6 tumors per group; Fig. 5C) and there was a reduction by 26% of 18F-FLT uptake for PC3 xenografts (3.9 ± 0.2 vs. 2.9 ± 0.2 vehicle vs. treatment, P < 0.01, n = 6 tumors per group, Fig. 5D). The capacity of BAY 87-2243 to reduce 18F-FLT uptake became more apparent by day 5 when it was observed that drug-treated H460 xenografts had %IDmean/g values that were reduced by 28% compared with vehicle-controls (4.1 ± 0.3 vs. 3.0 ± 0.3 vehicle vs. treatment, P > 0.01, n = 6 tumors per group; Fig. 5A), whereas in PC3 xenografts, the equivalent study yielded a reduction of 43% for drug-treated animals compared with vehicle-treated animals (4.0 ± 0.2 vs. 2.4 ± 0.2 vehicle vs. treatment, P < 0.01, n = 6 tumors per group; Fig. 5B). Neither drug nor vehicle treatment significantly affected mouse weight during the time course of this study (data not shown).

Induction of 18F-FPP(RGD)2 uptake by BAY 87-2243 on H460 tumor xenograft models

When the effect of BAY 87-2243 on 18F-FPP(RGD)2 uptake was examined (Fig. 5E), the drug increased %IDmean/g over

Figure 3.
Pharmacodynamic (PD) effects of BAY 87-2243 on the reduction of 18F-[FAZA] uptake in the PC3 prostate carcinoma xenograft model: A, single voxel plane coronal uPET scans (tracer is 18F-FAZA) of nude mice containing H460 tumor xenografts. Scans performed on mice before treatment (left column) and 1 (middle column) and 3 days (right column) after treatment for either vehicle (top) or for 9 mg/kg BAY 87-2243 (bottom). B, quantitative assessments of 18F-FAZA uptake of corresponding tumor xenografts. Uptake expressed in terms of %IDmean/g. Vehicle cohorts represented by black bars while patterned bars represent uptake in tumors from drug-treated animals. C, significant (P < 0.001 vehicle vs. treatment) percent loss in %IDmean/g values, 1 day after start of drug treatment. D, relative loss in uptake occurred before significant percent decline in tumor volume (P < 0.001). N = 6 tumor over three mice per cohort. * or +, P < 0.001, vehicle vs. treatment. Tumors indicated by white arrows.
BAY 87-2243 suppresses HIF1α target gene expression in the H460 tumor xenograft model at both 1 day and 3 days after drug delivery

Analysis of HIF1α target gene expression by RT-PCR revealed that BAY 87-2243 reduced expression of the hypoxia-regulated genes CA IX, ANGPTL4 and EGLN-3 by 99%, 93% and 83% respectively (Fig. 6C–I, P < 0.001 for three), which corresponds to reduced 18F-FAZA uptake upon drug treatment. EGLN-2, a gene not controlled by HIF-1α, was unaffected (Fig. 6J). Reduced 18F-FLT uptake was not accompanied by reduced expression of Tki (Supplementary Fig. S5). Expression levels of genes associated with glucose metabolism did not fully match unchanged 18F-FDG uptake: GLUT-1 expression and GLUT-3 expression were reduced while HK-2 decreased by 75% (Supplementary Fig. S6, P < 0.01). We detected low integrin beta-3 (ITGB3) expression in untreated xenografts but were unable to detect transcripts after BAY 87-2243 treatment (Supplementary Fig. S5).

Discussion

BAY 87-2243 is effective in inhibiting the growth of human lung and prostate cancer xenografts (H460 and PC3) in nude mice, whereas the human renal cell carcinoma 786-0 was shown to be resistant to BAY 87-2243 treatment. Of the four tracers studied, BAY 87-2243 affected most dramatically the uptake of 18F-FAZA (before significant changes in tumor volume). Thus, from a pragmatic standpoint, 18F-FAZA appears to be the most promising of these tracers for clinical translation. The decrease in 18F-FAZA uptake with BAY 87-2243 challenge suggests that the drug exerts its action through a process of reoxygination. Indeed, tumor reoxygination by BAY 87-2243 via inhibition of mitochondrial complex I has been reported by previous investigators.18

The dramatic change in 18F-FAZA uptake does not preclude the potential success of other radiotracers that was studied to date. The known capability of BAY 87-2243 and related compounds to resolve tumor hypoxia (9, 10) would inevitably have downstream impact on the tumor microenvironment, on tumor proliferation and on glucose metabolism (27–30). However, while BAY 87-2243 consistently downregulated hypoxia-associated gene expression in parallel with a diminution of 18F-FAZA tracer uptake, we did not observe such consistent changes in the expression of gene targets associated with angiogenesis, glucose metabolism, or proliferation. In turn, the changes in uptake of the corresponding tracers were not as consistent as we had originally surmised. These findings illustrate the complex manner in which changes in the hypoxic status of a tumor affects downstream biologic processes. The mutational status of a tumor (Kras, GLUT1, etc.) most likely will affect the response of a cancer cell to hypoxia as well (30). To tease out these complex interactions (and to assign appropriate tracers to track such processes) will involve several careful studies in the future.

18F-FAZA belongs to a class of tracers known as nitroimidazoles. A number of nitroimidazole-based tracers [e.g., misonidazole (MISO) or fluoromisonidazole (FMISO)] have seen clinical PET utilization (18, 31, 32). 18F-labeled fluoroazomycin arabinoside (Supplementary Fig. S4) or 18F-FAZA may

Figure 4.
Pharmacodynamic (PD) effects of BAY 87-2243 on 18F-FAZA uptake in the 786-0 renal cell carcinoma xenograft model. A, single voxel plane coronal microPET scans (tracer is 18F-FAZA) of nude mice containing 786-0 tumor xenografts. Scans performed on mice before treatment (left column) and 1 (middle column) and 3 days (right column) after treatment for either vehicle (top) or for 9 mg/kg BAY 87-2243 (bottom). B, quantitative assessments of 18F-FAZA uptake of corresponding tumor xenografts. Uptake expressed in terms of %IDmean/g. Vehicle cohorts represented by solid, black bars while open, spotted bars represent uptake in tumors from drug-treated animals. Tumors indicated by white arrows.

BAY 87-2243 (qd × 1) reduces pimonidazole and CD31 immunostaining in the H460 tumor xenograft model but with different kinetics

In nude mice bearing preestablished H460 tumor xenografts (80 mm3 in size), a single oral treatment was performed with vehicle or BAY 87-2243 (9 mg/kg). Pimonidazole staining (Fig. 6A, arrows) occurred inhomogeneously throughout the tumor and tended to describe irregular, circular patterns. Such patterns were consistent with previous observations (25, 26) of solid tumors. Treatment with 9 mg/kg BAY 87-2243 resulted in significant reduction in pimonidazole staining (Fig. 6B), a result that is consistent with the reduction in 18F-FAZA uptake in BAY 87-2243–treated H460 xenografts.

The PHF of H460 xenografts at 80 mm3 declines from 28.1% ± 4.5% to 2.6% ± 1.2% (Fig. 6E, P < 0.001, vehicle vs. treatment, n = 3) with one treatment of BAY 87-2243 (9 mg/kg). Two to three administrations of BAY 87-2243 are required to induce a significant decrease in PVH (i.e., from 17.8% ± 3% in vehicle-treated mice to 10.2% ± 3.7% after two treatments to 4.5% ± 1.5% after four BAY 87-2243 treatments; Fig. 6C–F, P < 0.005, n = 3).
represent some advance upon preexisting nitroimidazole tracers because it is more readily diffusible across cell membrane yet more readily alkylating at neutral pH (31). Consequently, this may yield greater signal to noise (i.e., hypoxic tissue-to-normoxic tissue activity concentration) ratios than other nitroimidazoles such as FMISO, intravenous iodoacetate, or MISO (16, 18, 33). Nevertheless, to establish translatability of 18F-FAZA, further studies on additional types of human hypoxic tumors are needed.

With increasing hypoxia, superoxide anions accumulate. Nitroimidazoles readily accept electrons from these highly reactive species and through a multistep process driven by hypoxia...
Figure 6.
Pharmacodynamic effects on the effects of BAY 87-2243 (qd × 1) on the reduction of pimonidazole immunostaining in the H460 lung carcinoma xenograft model. In nude mice, bearing preestablished H460 tumor xenografts (80 mm² in size), a single oral treatment was performed with vehicle or BAY 87-2243 (9 mg/kg). One day after the treatment, hypoxyprobe (60 mg/kg, Natural Pharmacia International) was injected via the tail vein. Mice were sacrificed 1.5 hours after the hypoxyprobe injection. Tumors were excised and processed for pimonidazole staining according to the protocol provided by Natural Pharmacia International. Histologic samples were processed for both vehicle (A) and drug-treated H460 xenografts (B). BAY 87-2243 also promoted a decline in CD31 staining with respect to vehicle (C and D) but the kinetics is slower; to see a decrease, a dosage of qd × 4 was required. Kinetics of the loss of %PHF; E) and (%PVF; F) was also calculated. BAY 87-2243 suppresses HIF1α target gene expression in the H460 lung carcinoma xenograft model at qd × 1 and qd × 3 dosing. Nude mice bearing preestablished H460 tumor xenografts (grown for 10 days) were treated orally with vehicle or BAY 87-2243 (9 mg/kg every day). RNA from xenograft tumors was isolated from satellite animal groups either before treatment or after 1 and 3 days of treatment with BAY 87-2243. Expression levels of the HIF target genes carbonic anhydrase 9 (CA9; G), angiopoietin-like 4 (ANGPTL4; H), and HIF-prolyl hydroxylase-3 (EGLN-3; I) and the non-HIF target gene HIF-prolyl hydroxylase-2 (EGLN-2; serving as a negative control; J) was quantified by RT-PCR. Data were normalized to β-actin and are given in arbitrary units as the mean of n = 3 animals/treatment group ± SD.
activated nitroreductase (e.g., xanthine oxidase, lipooxgenases, and NADPH oxidases) the hydroxylamine moiety of nitroimidazoles becomes reduced to a relatively inactive amine derivative (34). However, intermediates along this pathway are known to be highly alkylating and thus, readily form covalent bonds with resident macromolecules (31, 35). The molecular construct is then trapped within the cell. Thus, the level of trapping is reflective of the amount of nitroreductase activity in the cell and therefore of the extent of hypoxia in tissue (31).

Changes in tumor oxygenation (i.e., pO2) are presumably related to changes in tumor vascularity and accessibility of blood-borne tracers to tumor (36). Thus, any abrogations in 18F-FAZA uptake as seen with BAY 87-2243 treatment may be a reflection of changes in accessibility by the nitroimidazole-like tracer to the vasculature of the cancer (and consequently, accessibility to the cancer tissue itself). If tracer accessibility would be truly compromised due to disruption of vascular normality by BAY 87-2243 treatment, then one would expect a general decline of all tracers employed in our studies. In fact, we observe tracer uptake being either increased ([18F]-FFP(RGD1)), decreased ([18F]-FLT), or unchanged([18F]-FDG) following administration of BAY 87-2243. Thus, the data in aggregate would suggest that changes in 18F-FAZA uptake tracks biochemical and genomic changes induced by hypoxic- (or hypoxia-like) stress occurring with BAY 87-2243 administration rather than a physiologic alteration of blood vessel patency that results in reduced accessibility of tracer to tumor.

In assessing for the presence of pimonidazole immunostaining (an established marker for hypoxia), histologic sections show a marked decline within one day after BAY 87-2243 treatment (Fig. 6A and B). The reliability of such histologic observations has been confirmed by follow-up molecular expression studies of HIF1α-associated genes (carbonic anhydrase 9 (CA9), angiopeitnin-like 4 (ANGPTL4), and HIF-prolyl hydroxylase-3 (EGLN-3); see Fig. 6), which demonstrated that BAY 87-2243 significantly inhibits the expression of the corresponding genes. The subsequent loss in CD31 staining in H460 xenografts would suggest that the process of reoxygenation eventually diminishes vessel density within treated H460 xenografts. However, such an interpretation should be taken with caution as no such result after hypoxia resolution occurred with squamous cell carcinoma xenografts (10).

The contribution of BAY 87-243 to maintenance of prolyl hydroxylase activity has been proposed previously (9) based upon the observations that: (i) BAY 87-2243 acts principally through inhibition of oxidative phosphorylation via inhibition of the mitochondrial complex I of the respiratory chain and (ii) HIF1α stabilization was abrogated by inhibition of the complex I component of the mitochondrial respiratory chain. Hellbig and colleagues (10) showed that a chemically similar drug, BAY-84-7296, resolved tumor hypoxia through the same mechanism, namely, by inhibition of the mitochondrial complex I. With drug challenge, hypoxia and hypoxic ROS activity are decreased and subsequently, there is less degradation of prolyl hydroxylase which leads to destabilization of HIF1α. One consequence of reoxygenation is the loss of nitroreductase activity (13), thereby leaving less nitroimadazolamine products to be metabolized. As the FAZA tracer is a nitroimidazolamine, this would result in less 18F-FAZA tracer being trapped in tumors. 18F-FAZA uptake is thus an effective monitor of BAY 87-2243 efficacy as it can be argued that tracer uptake is related to the ability of the drug to form mitochondrial ROS and thereby reflect the degree to which HIF1α activity (and hence response to hypoxia) has been compromised. This mechanism can also explain the lack of change in 18F-FAZA uptake with BAY 87-2243 treatment for 786-0 xenografts as 786-0 cells have high glycolytic activity and are not dependent upon oxidative phosphorylation (37, 38). Phenotypically, 786-0 cells present an oxygenated phenotype (as opposed to a hypoxic one) and thus baseline 18F-FAZA uptake would also be low. Functional VHL-1 complexes in 786-0 cells are lost (39), thus the lack of change in tracer uptake with BAY 87-2243 treatment would be consistent with a drug that targets the functionality of the VHL-1 complex in relation to O2 levels. In Ellingshaus and colleagues (9), BAY 87-2243 treatment of renal carcinoma RCC4 cells (that also possess a VHL loss-of-function mutation) did not show any effect on the constitutively activated HIF1α pathway. This further supports our view of BAY 87-2243’s mode of action, that is, no direct effect on HIF1α or components of the HIF1α pathway.

Taken together, we present here evidence that BAY 87-2243 abrogates hypoxia via complex I inhibition in responsive models such as H460 cells and PC3 cells. Most observations such as reduced FAZA tracer uptake and HIF1α pathway suppression can be explained by such reoxygeenation of the formerly hypoxic tumor areas. Responsive tumors are thus characterized by the occurrence of hypoxic areas, which is not fulfilled with the 786-0 model, where 18F-FAZA uptake is unchanged. Furthermore, H460 and PC3 cells harbor functional VHL, which means that HIF1α pathway is activated upon hypoxia thereby resulting in enhanced glycolysis that can contribute to ATP supply. Upon inhibition of complex I by BAY 87-2243, ATP generation via oxidative phosphorylation is diminished and at the same time reoxygenation abrogates HIF1α pathway activation along with glycolysis-driven ATP generation. The concurrent reduction of ATP supply by complex I inhibition and diminished glycolysis together with suppression of growth-promoting factors generated by an activated HIF1α pathway may explain, in part, the observed growth inhibition of the H460 and PC3 xenograft models. However, the lack of significant alterations of 18F-FDG uptake upon BAY 87-2243 treatment suggests that the impact on diminished glycolysis/ATP supply remains uncertain. One needs to compare ATP levels on drug treatment between responsive and nonresponsive models to resolve these conundrums.

One direction in future studies will involve delineating the impact of BAY 87-2243 subsequent to changes in hypoxic status. Promising avenues include effect of drug on entry of pentose into the tricarboxylic acid cycle, on glucose transport, on thymidine salvage, and on integrin induction. Furthermore, the inhibition of mitochondrial complex I (9, 10) by BAY 87-2243 might provide a new approach for sensitization toward localized radiotherapy of formerly hypoxic tumors that have now become reoxygenated (10). Such studies should provide crucial insights on targeting select patient populations and on improving choice and use of radiotracers for clinical monitoring of cancer therapies.

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References


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