Intratumoral Modeling of Gefitinib Pharmacokinetics and Pharmacodynamics in an Orthotopic Mouse Model of Glioblastoma

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Abstract

Like many solid tumors, glioblastomas are characterized by intratumoral biologic heterogeneity that may contribute to a variable distribution of drugs and their associated pharmacodynamic responses, such that the standard pharmacokinetic approaches based on analysis of whole-tumor homogenates may be inaccurate. To address this aspect of tumor pharmacology, we analyzed intratumoral pharmacokinetic/pharmacodynamic characteristics of the EGFR inhibitor gefitinib in mice with intracerebral tumors and developed corresponding mathematical models. Following a single oral dose of gefitinib (50 or 150 mg/kg), tumors were processed at selected times according to a novel brain tumor sectioning protocol that generated serial samples to measure gefitinib concentrations, phosphorylated extracellular signal-regulated kinase (pERK), and immunohistochemistry in 4 different regions of tumors. Notably, we observed up to 3-fold variations in intratumoral concentrations of gefitinib, but only up to half this variability in pERK levels. As we observed a similar degree of variation in the immunohistochemical index termed the microvessel pericyte index (MPI), a measure of permeability in the blood–brain barrier, we used MPI in a hybrid physiologically-based pharmacokinetic (PBPK) model to account for regional changes in drug distribution that were observed. Subsequently, the PBPK models were linked to a pharmacodynamic model that could account for the variability observed in pERK levels. Together, our tumor sectioning protocol enabled integration of the intratumoral pharmacokinetic/pharmacodynamic variability of gefitinib and immunohistochemical indices followed by the construction of a predictive PBPK/pharmacodynamic model. These types of models offer a mechanistic basis to understand tumor heterogeneity as it impacts the activity of anticancer drugs. Cancer Res; 73(16); 5242–52. ©2013 AACR.

Introduction

Glioblastoma multiforme, also called grade 4 astrocytoma, is the most aggressive and frequent of all primary brain tumors, accounting for about 54% of all gliomas (CBTRUS report 2011), with a median survival of less than 1 year from diagnosis (1, 2). Although new combination drug regimens have generated excitement and positive responses, they ultimately fail as tumors enact numerous drug-resistant mechanisms. These failures are often attributed to pharmacodynamics or cellular mechanisms that have their origins in either preexisting or adaptive genetic alterations that manifest as biochemical network unresponsive to the selected drugs. Regional analysis from glioblastoma multiforme samples has revealed extensive intratumoral heterogeneity in genetic composition and protein expression (3), including variable EGFRvIII expression (4) and EGFR and PDGFR gene amplification patterns across the same tumor sample (5). In such a scenario, reliance on singular bulk tumor samples to map individual genomic profiles and prioritize therapeutic strategies could be inaccurate as a means to advance personalized medicine.

In addition to pharmacodynamic-based treatment failures, it is also reasonable to expect that pharmacokinetic-based failures also contribute to poor drug responses. In this case, tumor concentrations would be insufficient to inhibit intended targets that could be accentuated by intratumoral biologic heterogeneity. Glioblastoma multiforme is a heterogeneous, highly invasive brain tumor characterized by a highly disorganized vasculature and a compromised blood–brain barrier (BBB; ref. 6). Intratumoral variability within glioblastoma multiformes has been observed for morphology and functioning of the vasculature (7, 8), vessel permeability (9), interstitial fluid pressure (10), necrosis, and cell proliferation and density (11–14) that may contribute to heterogeneous drug distribution. Much of the limited data in this regard were based on the use of quantitative autoradiography, a sensitive technique that is limited by the resources need to synthesize radiolabeled drugs and their lack of specificity (10, 15–18). Other attempts to
examine regional pharmacokinetic variability have used unlabeled drugs and mass spectrometry for drug concentration measurements; however, the measurements were sparse preventing a systematic analysis that would support the development of models (19). Of course, it is to be expected that clinical investigations of brain tumor concentrations are limited by sample availability, yet there is an indication that intratumoral drug concentrations of various chemotherapeutic drugs (20, 21), and some targeted anticancer agents like imatinib (22) and gefitinib (23) were quite variable. All these studies lend support to the hypothesis that ineffective chemotherapy could be due to pharmacokinetic-based failures.

Given the rise in the importance of tumor heterogeneity and the lack of systematic investigations to detail such heterogeneity with respect to both pharmacokinetics and pharmacodynamics, we applied our recently developed tumor sampling protocol, which assigns adjacent tumor sections to either pharmacokinetics, pharmacodynamics, or immunohistochemical (IHC) analyses, to characterize the intratumoral pharmacokinetic/pharmacodynamic variability of gefitinib.

Materials and Methods

Materials

U87/EGFRvIII and U87/PTEN cell lines were a generous gift from Dr. Webster Cavenee (University of California San Diego, San Diego, CA) and Dr. Paul Mischel (University of California at Los Angeles, Los Angeles, CA), respectively. U87-MG cell line was purchased from the American Type Culture Collection. U87, U87/PTEN, and U87/EGFRvIII cells were cultured in Dulbecco’s Modified Eagle Medium with 10% FBS and supplemented with penicillin/streptomycin. The cells were maintained in a humidified incubator at 37°C with 5% CO₂.

Cell culture

U87, U87/PTEN, and U87/EGFRvIII cells were cultured in Dulbecco’s Modified Eagle Medium with 10% FBS and supplemented with penicillin/streptomycin. The cells were maintained in a humidified incubator at 37°C with 5% CO₂.

Orthotopic tumor implantation

The procedure for brain tumor implantation was similar to that reported previously (24). Briefly, nude mice (Male NIH Swiss nude mice, nu/nu, 5–7 weeks old) were anesthetized and secured in a stereotactic apparatus. A suspension of glioma cells (U87/EGFRvIII) was prepared fresh from culture (10⁵ cells/µL in PBS) and injected (5 µL) into the caudate putamen. After one week, mice were monitored once a day for symptoms related to tumor growth that included an arched back, unsteady gait, and loss of body weight. Mice were entered into the pharmacokinetic/pharmacodynamic studies when they showed a total body weight loss of 10% or more on 2 consecutive days.

In vivo gefitinib treatment and sampling

Gefitinib was administered at a dose of 50 or 150 mg/kg by oral gavage. A serial sacrifice design was used with blood and tumor samples collected from 3 mice at each scheduled time point (i.e., 0, 0.5, 1, 2, 4, 8, 18, and 24 hours) following drug administration. The brain tumors were serially sectioned using a cryostat to obtain samples for pharmacokinetics, pharmacodynamics, and IHC analyses in 4 distinct regions separated by 1 mm intervals starting from the periphery (region R1) to the center (region R4) of each tumor as we recently reported (25).

Drug analysis (liquid chromatography/tandem mass spectrometry)

Previously validated liquid chromatography/tandem mass spectrometry methods were used to quantitate gefitinib in plasma (26) and brain tumor samples (25).

pERK analysis

A highly sensitive electrochemiluminescence method was used to analyze phosphorylated extracellular signal-regulated kinase (pERK) in brain tumor sections collected from various regions of brain tumor, as described previously (25). The assay was run according to the manufacturer’s protocol with the phospho and total extracellular signal-regulated kinase (ERK) ratios expressed as a fraction of baseline (untreated tumors). Phospho/total ERK, which was set to 1.

Immunohistochemistry

Cryosections (12 µm) from frozen tumors were allowed to dry in air for 30 minutes followed by fixation in 4% methyl-aldehyde paraformaldehyde at room temperature. The sections were then immersed in 100% methanol and treated with deoxyribonuclease digestion. The sections were then treated with 2 N HCl for 15 minutes, and then washed with PBS. The sections were then incubated with terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL; apoptosis) or double stained with anti-CD31 and anti-P-glycoprotein using diluted primary and secondary antibodies, followed by nuclear staining with 4’-6-diamidino-2-phenylindole. The detailed staining protocol can be found in the Supplementary Methods section.

The images of the cryosections were acquired (1.0 µm/pixel) as grayscale, using an automated fluorescent microscope (Zeiss Axioplan 2E, Carl Zeiss Microscopy) controlled by AxiVision software with subsequent image analyses processed by MetaMorph microscopy automation and image analysis software (Molecular Devices). The threshold values of each tumor section image were identified by positive staining and then semiquantitated by measuring the number (for TUNEL/apoptosis staining) or pixel area (for CD31 and SMA) of positive staining in the region of interest. The number of total TUNEL-positive cells per total area of each selected region was presented as the number of apoptotic cells per mm² of tissue area. Microvessel density (MVD) was calculated as the total CD31-positive pixel area × 100/total pixel area of the
selected region. The microvessel pericyte index (MPI) required dual positive staining analysis of CD31 in combination with SMA as follows:

\[
\text{MPI} = \frac{\text{Overlapping area of CD31- and SMA-positive staining}}{\text{Total area of CD31-positive stain}} \times 100
\]

The microvessel transporter index (MTI) that provided a measure of the efflux transporter P-glycoprotein (Pgp) expression on microvessels was similarly calculated as

\[
\text{MTI} = \frac{\text{Overlapping area of CD31- and Pgp-positive staining}}{\text{Total area of CD31-positive stain}} \times 100
\]

The images were pseudocolored for presentation using MetaMorph software.

Pharmacokinetic and pharmacodynamic modeling

Pharmacokinetic and pharmacodynamic data analyses were conducted with the SAAMII simulation and modeling program (version 2. University of Washington, Seattle, WA). The analyses were based on a sequential modeling approach of first defining the pharmacokinetic models and then the pharmacodynamic models. A sequential modeling approach was also applied to obtain the pharmacokinetic models specifically, hybrid physiologically based pharmacokinetic (PBPK) models were derived by first fitting the model to the combined gefitinib plasma concentrations from both dose levels to obtain fixed forcing functions (see Table 1) that were then used to fit the tumor models to the observed gefitinib tumor concentration data.

On the basis of the significant inverse correlation between gefitinib intratumoral concentrations and the MPI, 3 unique hybrid PBPK models of gefitinib were developed corresponding to three MPI groups, indicative of variable BBB permeability. To achieve this goal, the regional pharmacokinetic/pharmacodynamic data at each dose level were segregated into three groups according to their corresponding MPI values as shown in Fig. 1A. The MPI values in each tumor were ranked from 1 to 4. The tumor pharmacokinetic/pharmacodynamic measurements corresponding to regions with a MPI rank = 1 from both dose levels were grouped as the low MPI group, whereas those corresponding to regions with a MPI rank = 4 formed the high MPI data group. The data from the intermediate MPI ranks (2 and 3) were combined to obtain a medium MPI group. The PBPK models, consisting of a two-compartment systemic disposition model and a two-compartment permeability–limited brain tumor model, were individually fit for each MPI group to gefitinib intratumoral concentration data that produced three different sets of tumor pharmacokinetic parameters. The rate equations for the plasma forcing function and the tumor compartments are provided in the Supplementary Materials and Methods.

The following pharmacokinetic parameters were fixed on the basis of previously reported values: oral absorption rate constant (\(K_a\)), oral bioavailability (26), and for the tumor pharmacokinetic models, tumor blood flow rate (\(Q_t = 3\) mL/h; ref. 27). The mean volume of tumor (\(V_t\)) was fixed as 0.001 mL based on its measured value and then assigned as the volume of tumor vascular compartment (\(V_{ve}\)) and extravascular compartment (\(V_{ev}\)) based on a reported volume fraction (28, 29). The gefitinib tumor to plasma partition coefficient was determined as the average of the ratio of observed tumor per plasma AUC (\(R\)) obtained from each dose level. The Michaelis-Menten constant (\(K_m\)) for active efflux of gefitinib by Pgp at the BBB was estimated by using Bayesian constraints based on reported values (30). All other parameters were estimated by fitting the model to the observed gefitinib concentrations using maximum likelihood estimation. These parameters were: central compartment elimination rate constant (\(k_{el}\)), volume distribution for the central compartment (\(V_c\)), intercompartment transfer rate constants (\(k_{12}\) and \(k_{21}\)), maximum rate of active efflux from tumor extravascular to vascular compartment (\(V_{max}\)), tumor vascular to extravascular transcellular mass transfer coefficient (\(h\)), and paracellular transport rate constant (\(K_p\)). Once the hybrid PBPK model was finalized, the associated pharmacokinetic variables were set as constant and linked to the pharmacodynamic model.

The fractional inhibition of pERK in tumors, using untreated tumors to obtain baseline pERK values, after gefitinib administration was used as the pharmacodynamic response and represented as a three-compartment sequence of a link compartment connected to a target [i.e., phosphorylated EGFR (pEGFR) response (i.e., pERK)] model. The link compartment consisted of an inhibitory \(I_{max}\) model to link gefitinib concentrations in tumor (\(C_t\)) to the pharmacodynamic model. The rate equations are provided in the Supplementary Materials and Methods.

It was assumed that the variability in the pERK response was solely driven by gefitinib tumor concentrations based on their slightly higher degree of variability that was attributed to changes in BBB permeability. It was necessary to segregate the pharmacodynamic models into low- and high-dose groups to obtain the best-fit models.

The pharmacodynamic model parameters estimated were the \(IC_{50}\) (gefitinib concentrations for 50% inhibition of pEGFR), \(K_{in}\) (the zero-order formation rate for pEGFR), and \(K_{out}\) (the first-
Intratumoral Pharmacokinetic/Pharmacodynamic Modeling

A novel tumor sampling protocol was used to integrate pharmacokinetics, pharmacodynamics, and IHC measurements from each tumor following administration of single doses of 50 and 150 mg/kg of gefitinib to mice bearing intracerebral tumors. Tumor samples collected at the dose of 150 mg/kg gefitinib showed an intratumoral variability of gefitinib concentrations in the range of 1.2- to 2.4-fold over the entire 24-hour time course and about a 1.5-fold difference in the AUC(0-24 h) between the regions with highest and lowest gefitinib concentrations (Fig. 2A). Parallel to the regional tumor pharmacokinetics, corresponding pharmacodynamic (pERK) measurements varied 1.2- to 4.4-fold over 24 hours, with a similar area between the effect curves (ABEC), a measure of the cumulative degree of inhibition between the regions with highest and lowest gefitinib concentrations (Fig. 2C). Although intratumoral variability at 150 mg/kg of gefitinib was low, gefitinib concentrations tended to increase moving from the tumor peripheral toward the central regions, which produced an expected opposite trend in pERK values (Supplementary Fig. S1A and S1B).

Regional variability in intratumoral pharmacokinetics/pharmacodynamics in mice administered 50 mg/kg of gefitinib was slightly greater than at the higher dose level and over 24 hours, gefitinib intratumoral concentrations ranged from 1.6- to 3-fold, with a 2-fold difference in the AUC(0-24 h) in the regions with highest and lowest gefitinib concentrations (Fig. 2B). The corresponding intratumoral pharmacodynamic variability

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**Results**

**Intratumoral pharmacokinetic/pharmacodynamic variability in gefitinib**

A novel tumor sampling protocol was used to integrate pharmacokinetics, pharmacodynamics, and IHC measurements from each tumor following administration of single doses of 50 and 150 mg/kg of gefitinib to mice bearing intracerebral tumors. Tumor samples collected at the dose of 150 mg/kg gefitinib showed an intratumoral variability of gefitinib concentrations in the range of 1.2- to 2.4-fold over the entire 24-hour time course and about a 1.5-fold difference in the AUC(0-24 h) between the regions with highest and lowest gefitinib concentrations (Fig. 2A). Parallel to the regional tumor pharmacokinetics, corresponding pharmacodynamic (pERK) measurements varied 1.2- to 4.4-fold over 24 hours, with a similar area between the effect curves (ABEC), a measure of the cumulative degree of inhibition between the regions with highest and lowest gefitinib concentrations (Fig. 2C). Although intratumoral variability at 150 mg/kg of gefitinib was low, gefitinib concentrations tended to increase moving from the tumor peripheral toward the central regions, which produced an expected opposite trend in pERK values (Supplementary Fig. S1A and S1B).

Regional variability in intratumoral pharmacokinetics/pharmacodynamics in mice administered 50 mg/kg of gefitinib was slightly greater than at the higher dose level and over 24 hours, gefitinib intratumoral concentrations ranged from 1.6- to 3-fold, with a 2-fold difference in the AUC(0-24 h) in the regions with highest and lowest gefitinib concentrations (Fig. 2B). The corresponding intratumoral pharmacodynamic variability
was in the range of 1.2- to 1.9-fold with a 1.4-fold difference in ABEC in the regions with highest and lowest gefitinib concentrations (Fig. 2D). Of some interest and opposite to the high-dose trend, the gefitinib concentration gradient decreased in moving from the tumor periphery towards the central regions (Supplementary Fig. S2A and S2B).

The viability of the tumor sampling protocol was shown by use of another EGFvIII brain tumor model, specifically the LN229/EGFRvIII-PTEN. Mice bearing these tumors were administered 150 mg/kg gefitinib orally and processed similar to the U87/EGFRvIII study. This preliminary study focused on the assessment of intratumoral variability in gefitinib concentrations that showed a similar degree of variability as in the U87/EGFRvIII model; ranging from 1.6- to 3.2-fold intratumoral differences over the 24-hour sampling period (Supplementary Materials and Methods and Supplementary Table S2).

**Intratumoral variability in biologic characteristics**

To identify biologic characteristics of the tumor that could explain the variability in gefitinib intratumoral concentrations, MVD, MPI, and apoptotic cells (MVD, MPI, and apoptotic cells/mm²) in the tumor regions adjacent to the pharmacokinetic and pharmacodynamic measurements were analyzed, first in the high-dose 150 mg/kg group. There was a small but significant difference (P < 0.05) in the MVD between the tumor peripheral and central regions, being lower at the tumor center (Fig. 3A–C), consistent with a previous report (31). It seemed counterintuitive that the regions with high MVD values had lower gefitinib concentrations; however, the MPI, an index of microvessel pericyte coverage and a more accurate indicator of BBB integrity, showed significantly (P < 0.05) higher values in the tumor peripheral regions compared with the central regions (Fig. 3D–F), and inversely correlated with the gefitinib concentration gradient. There was no significant difference in the intratumoral apoptotic cells measurements as a function of tumor region and this was not further considered as a potential determinant of drug distribution at the lower 50 mg/kg dose. Therefore, the integrity of the BBB as measured by the MPI was found to be the most significant determinant of gefitinib brain tumor distribution in the high-dose group.

The MPI values in the low-dose gefitinib group also showed significant (P < 0.05) intratumoral variability. Although, unlike the high-dose group, the high MPI values were located more centrally (Supplementary Fig. S3A), but still showed an inverse correlation to the intratumoral gefitinib concentration gradient. Given the concurrence between MPI and intratumoral gefitinib concentrations at both dose levels (Fig. 4), we concluded that BBB integrity was the primary determinant of regional gefitinib tumor concentrations.

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**Figure 2.** Intratumoral variability in gefitinib pharmacokinetic/pharmacodynamic following single oral doses of 150 mg/kg and 50 mg/kg gefitinib to mice-bearing intracerebral U87/vIII tumors. A and B, gefitinib brain tumor concentrations at 150 mg/kg (A) and 50 mg/kg (B). C and D, pharmacodynamic responses on the basis of pERK (fraction of baseline pERK) at 150 mg/kg (C) and 50 mg/kg (D) in the regions with the lowest and highest variability. All observed points represent the mean ± SD.
As gefitinib is reported to be a substrate for Pgp (32), a small subset of tumors (n = 4) from the 50 mg/kg dose level were stained for Pgp and quantified as the MTI (microvessel transporter index). MTI values indicated a significantly greater Pgp expression on microvessels in the tumor center as compared with the peripheral region. Also the regional MTI measurement and the corresponding MPI in each of these tumor regions showed a significant positive correlation, reflecting that tumor microvessels with a more intact BBB are associated with a greater Pgp expression (Supplementary Fig. S3B–S3I) and could be an additional factor responsible for variability in gefitinib pharmacokinetics/pharmacodynamics.

Regional pharmacokinetic/pharmacodynamic models of gefitinib on the basis of differential BBB permeability

The best-fit PBPK models consisted of a two-compartment systemic disposition model and a two-compartment permeability–limited model for the tumor (Fig. 1B). The latter two-compartment vascular–extravascular model attributed to the importance of BBB integrity as a key determinant in gefitinib brain tumor distribution. Specifically, the tumor model incorporated passive transcellular diffusion, active efflux, and passive paracellular transport processes. The active efflux term represented Pgp-mediated BBB efflux and was based on the more than dose-proportional increase in gefitinib AUC in tumor (Supplementary Table S1). Transcellular diffusion is a

Figure 3. Intratumoral immunohistochemical analysis of MVD and MPI in U87vIII brain tumors following single oral doses of 150 mg/kg gefitinib; data points, mean ± SD values from each tumor region from all the animals in the study (n = 21). A, regional variability in MVD showing decreasing MVD values from tumor periphery to center (P < 0.05). B and C, representative images of MVD (CD31 staining) at tumor periphery and center, respectively. D, regional variability in MPI showing decreasing MPI values from tumor periphery to center (significant difference, P < 0.05). E and F, representative images of microvessel pericyte coverage (α-SMA staining) at tumor periphery and center, respectively. Each image was acquired as a grayscale image at a resolution of 1.0 μm per pixel and then pseudocolored for presentation.

Figure 4. Regional gefitinib brain tumor concentrations as a function of the corresponding regional MPI (as determined by IHC analysis) values following single doses of 50 mg/kg and 150 mg/kg gefitinib orally (all mice and regions included). A significant negative correlation was found, Pearson correlation coefficient, -0.19, P = 0.014.
bidirectional process driven by concentration gradients across the BBB. Paracellular transport of gefitinib is a unique mechanism that accounted for unidirectional entry of protein-bound gefitinib across the disrupted BBB. The best-fit model supported this was not a bidirectional process. The disrupted BBB caused by the growth of the tumor created large endothelial cell gaps that are highly permeable to water soluble compounds and large molecular weight proteins like albumin (7). The size of the endothelial gaps is much larger than the steric diameter of albumin and hence could be a significant source of drugs like gefitinib that are highly bound to plasma proteins. In this case, plasma protein-bound gefitinib passing into the tumor interstitium could dissociate and form a dynamic equilibrium with unbound drug and contribute to the pool of free drug able to traverse intracellularly and interact with its receptor or diffuse back into blood. The predicted plasma and tumor concentration–time profiles agreed well with the observed values in all 3 MPI (low, medium, and high) data groups (Fig. 5).

Several interesting findings supported our hypothesis that variable intratumoral BBB integrity was the primary mechanism for variability in the tumor pharmacokinetics of gefitinib (Table 2). First, the predicted area under the tumor concentration–time curve (AUC) for gefitinib adhered to the following order, low MPI AUC > medium MPI AUC > high MPI AUC, consistent with the relationship between MPI and BBB integrity, especially at the low dose. This trend was slightly offset at the high gefitinib dose with the medium MPI AUC being minimally less than the AUC for the high-MPI group. Second, the difference between the low and high-MPI tumor AUC in the low-dose group was more (2-fold higher AUC in low MPI group) than in the high-dose group, where the low and high-MPI tumor AUCs were more similar. One likely reason for this observation is that at the high dose of gefitinib, Pgp-mediated efflux at the BBB is saturated preventing any further increase in efflux at high concentrations. This phenomenon, by allowing more gefitinib to penetrate the BBB, may also have contributed to the lower intratumoral variability observed at the 150 mg/kg dose level. Third, $V_{\text{max}}$ values, indicative of the maximum Pgp-mediated transport rate, were in the rank order of high MPI $V_{\text{max}}$ > medium MPI $V_{\text{max}}$ > low MPI $V_{\text{max}}$ that directly corresponded to the observation of lower Pgp expression found in the regions with low MPI (Supplementary Fig. S3B–S3I), and consistent with a previous report that found Pgp expression negatively correlated with immature cerebral capillaries (33). Finally, the transcellular diffusion coefficient, $h$, which is a product of BBB permeability and its surface area, was found to be almost 5-fold more in the low-MPI group as compared with the medium MPI group and 8-fold more as compared with the high-MPI group (low MPI $h$ >

![Figure 5](image-url)

Figure 5. Intratumoral pharmacokinetic modeling of gefitinib in athymic mice-bearing intracerebral U87vIII tumors following either 50 mg/kg or 150 mg/kg single oral doses. The model predicted (—, 50 mg/kg; —, 150 mg/kg) and observed (mean + or – SD; n = 3; ●, 50 mg/kg; ▲, 150 mg/kg) gefitinib concentrations are presented for plasma (A), low MPI tumor group (B), medium MPI tumor group (C), and high MPI tumor group (D).
medium MPI "h" > high MPI "h"), consistent with the low MPI regions having a more permeable vasculature due to poor pericyte coverage. The paracellular transport rate term ($K_p$) was not significantly different between the three MPI groups, indicating that its contribution is relatively constant across all MPI groups and that the transcellular transport is the major contributor to the intratumoral pharmacokinetic variability.

Once the hybrid PBPK model for each MPI group was established, the models were linked to pharmacodynamic models that characterized the pERK profiles as shown in Fig. 1B. A three-compartment sequence of a link compartment connected to a target-response model (26) provided the best-fit pharmacodynamic model. Given our assumption that MPI-mediated differences in the PBPK models drove changes in the pERK response, a separate pharmacodynamic model for each MPI group was not required. Instead, a pharmacodynamic model was fitted to the combined pERK data from all the MPI groups, yet segregated by dose (Supplementary Fig. S4 for each MPI group) generating 2 sets of pharmacodynamic model parameters (Table 3). This approach attests to the similar pERK profiles for each MPI group. Attempts to fit pharmacodynamic models for each MPI group or for the combined pERK data for both dose levels resulted in less accurate fits than those based on dose level. The baseline pEGFR0 and $I_{max}$ values were set equal to 1 based on the assumption that the phosphorylation of EGFR was not inhibited in the absence of gefitinib and that it could be fully inhibited at high gefitinib tumor concentrations, respectively. To reduce parameter estimation difficulties, the value of $K_{in}$, a variable representing the formation of pEGFR, was fixed in the low-dose group to a value equal to that obtained from the high-dose pharmacodynamic model and indicated that the synthesis rate of pEGFR was not influenced by dose. The $IC_{50}$ values were found to be dose-dependent with a higher value (3-fold) obtained at the 150 mg/kg dose level. The transfer rate constant, $k_{tr}$, a variable that

### Table 2. Brain tumor pharmacokinetic parameters of gefitinib in regions corresponding to low (less intact BBB), medium, and high MPI (more intact BBB) regions

<table>
<thead>
<tr>
<th>Variables</th>
<th>Units</th>
<th>High MPI group</th>
<th>Medium MPI group</th>
<th>Low MPI group</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_p$</td>
<td>mL/h</td>
<td>0.0024 (23%)</td>
<td>0.0022 (18%)</td>
<td>0.0012 (68%)</td>
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<tr>
<td>$K_{in}$</td>
<td>ng/g</td>
<td>2,026.65 (10%)</td>
<td>2,048.51 (10%)</td>
<td>2,003.35 (10%)</td>
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<tr>
<td>$V_{max}$</td>
<td>ng/h</td>
<td>20.07 (29%)</td>
<td>18.27 (23%)</td>
<td>6.47 (55%)</td>
</tr>
<tr>
<td>$h$</td>
<td>mL/h</td>
<td>0.0006 (63%)</td>
<td>0.0009 (37%)</td>
<td>0.005 (34%)</td>
</tr>
<tr>
<td>$R^a$</td>
<td>mL/h</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>$Q_a^a$</td>
<td>mL/h</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>AUC high dose(p&lt;24)</td>
<td>h x ng/g</td>
<td>1.68E+05</td>
<td>1.67E+05</td>
<td>1.70E+05</td>
</tr>
<tr>
<td>AUC low dose(p&lt;24)</td>
<td>h x ng/g</td>
<td>2.27E+04</td>
<td>2.54E+04</td>
<td>4.91E+04</td>
</tr>
</tbody>
</table>

*aValues fixed either on the basis of literature or experimentally determined values.

### Table 3. Brain tumor pharmacodynamic model variables of gefitinib determined in mice-bearing intracerebral U87vIII tumors

<table>
<thead>
<tr>
<th>Variables</th>
<th>Units</th>
<th>High dose model</th>
<th>Low dose model</th>
</tr>
</thead>
<tbody>
<tr>
<td>$IC_{50}$</td>
<td>ng/g</td>
<td>41,969.54 (44%)</td>
<td>16,073.65 (42%)</td>
</tr>
<tr>
<td>$k^a$</td>
<td>1/h</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$K_{in}$</td>
<td>1/h</td>
<td>2.02 (11%)</td>
<td>2.02(^a)</td>
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<tr>
<td>$K_{out}$</td>
<td>1/h</td>
<td>2.58 (10%)</td>
<td>2.74 (5%)</td>
</tr>
<tr>
<td>$k_{tr}$</td>
<td>1/h</td>
<td>0.32 (18%)</td>
<td>1.34 (18%)</td>
</tr>
<tr>
<td>Predicted ABEC (high MPI group)</td>
<td>a</td>
<td>9.53</td>
<td>7.57</td>
</tr>
<tr>
<td>Predicted ABEC (medium MPI group)</td>
<td>a</td>
<td>9.48</td>
<td>7.64</td>
</tr>
<tr>
<td>Predicted ABEC (low MPI group)</td>
<td>a</td>
<td>9.72</td>
<td>8.45</td>
</tr>
</tbody>
</table>

*NOTE: Values represent mean (% coefficient of variation) of model fitted variables unless otherwise stated.

*aUnits of ABEC: [Fraction of baseline pERK × time (h)].

*The parameter “k” was used to link gefitinib tumor concentrations to the pEGFR target compartment and fixed at a value of 1.

*Values fixed either on the basis of literature or experimentally determined values.
reflects signal transduction efficiency from pEGFR to pERK showed an inverse relationship with dose indicative of reduced signaling efficiency at higher gefitinib concentrations. The model that predicted ABEC, which provides a measure of cumulative degree of inhibition (34), indicated there was a minimal difference in the ABEC values as a function of the MPI groups in both the high- and low-dose groups; yet, dose-dependent increases in the model-predicted ABEC values were obtained: being 1.26-fold, 1.24-fold, and 1.15-fold for the high, medium, and low MPI groups, respectively (Table 3).

Discussion

Most preclinical pharmacokinetic/pharmacodynamic studies in brain tumor-bearing mice are based on measurements from whole tumor homogenates that rely on the assumption of homogeneous drug distribution (i.e., single averaged drug concentration) for interpretation. However, it is well known that tumors are heterogeneous with regard to vascularity, blood flow, and interstitial fluid pressure and more recently genomic characteristics that could impact both pharmacokinetic and pharmacodynamic variability, and ultimately, personalized medicine (5). As individualized medicine moves forward, an accounting of intratumoral pharmacokinetic/pharmacodynamic heterogeneity is necessary to not only understand why drugs may be inactive, but further to offer computational approaches to mitigate the effects of tumor heterogeneity. Models of regional pharmacokinetics/pharmacodynamics provide a tool to design improved dosing schedules to attain therapeutic relevant pharmacokinetic concentrations in the entire tumor that will produce optimal pharmacodynamic effects. It can be appreciated that such pharmacokinetic/pharmacodynamic models will become increasingly important as multidrug combinations are designed that may be subject to drug–drug interactions, both in the pharmacokinetic sphere as well as in the pharmacodynamic sphere where drugs act on the same and intersecting cell signaling pathways. The current study characterized the intratumoral pharmacokinetic and pharmacodynamic characteristics of gefitinib, a model EGFR inhibitor, and as a first investigation in this area, began to unravel how such data may be modeled to enhance our understanding of intratumoral pharmacokinetics/pharmacodynamics.

A pivotal advance in our approach was to develop a tumor sampling scheme that was readily accessible and provided regional pharmacokinetic and pharmacodynamic measurements that could be related to biologic characteristics of the tumor measured by immunohistochemistry. Adjacent serial sections were assigned to pharmacokinetics, pharmacodynamics, or IHC analyses and in this particular investigation, four such regions were obtained at 1 mm intervals in the coronal direction. Both gefitinib concentration and pERK measurements required about five adjacent 12 μm tissue sections or about 1 mg of brain tumor, whereas, IHC analyses could be completed on each 12 μm section that were interdigitated between the pharmacokinetic and pharmacodynamic sections that served as a means to bin the pharmacokinetic and pharmacodynamic data. This procedure, by combining adjacent tissue sections, does lump or average the measurements, yet much less so than whole-tumor homogenates and with potential improvements in assay sensitivities improved resolution can be expected. Overall, the tumor sectioning protocol permitted each animal to contribute regional pharmacokinetic/pharmacodynamic/IHC measurements that facilitated our understanding of tumor heterogeneity.

Before the pharmacokinetic/pharmacodynamic investigations in brain tumor-bearing mice, in vitro pharmacodynamic biomarker studies were conducted in U87vIII cells to confirm that pERK could be used as a pharmacodynamic marker (Supplementary Fig. S5). The in vivo studies were then completed at two dose levels to assess dose-dependent changes in intratumoral pharmacokinetics/pharmacodynamics; the high gefitinib dose of 150 mg/kg was based on previous preclinical studies that showed this dose to be the highest nontoxic oral dose and a dose of 50 mg/kg was chosen based on previous studies related to gefitinib’s interactions with Pgp at the BBB (32). As the brain tumor vasculature has been reported to show regional variability in Pgp expression (higher at the tumor periphery; ref. 35), it was important to include Pgp in our analysis.

The orthotopic brain tumor model possessed mutant EGFR (EGFRvIII) that is more sensitive to gefitinib than the widely used parental line, U87MG. The EGFRvIII variant has been reported to form a discrete well circumscribed intracerebral tumor mass, with a more aggressive phenotype than the U87MG tumors (36) and hence, more likely to recapitulate the phenotype of brain tumors in patients. The intratumoral variability of gefitinib pharmacokinetics [AUC_{0–24h}] was about 2-fold and comparable with a 3-fold intratumoral variability reported for gefitinib brain tumor concentrations in a single patient receiving a multiple-dose regimen of 500 mg/day (23). The slightly lower intratumoral variability in gefitinib brain tumor concentrations observed at the 150 mg/kg gefitinib dose could be attributed to saturation of efflux transporters at the BBB, and thus, limit Michaelis–Menten transport that is sensitive to concentration changes and likely to exhibit greater variability. The variability in the intratumoral concentrations of gefitinib in U87MG tumors (36) and hence, more likely to recapitulate the phenotype of brain tumors in patients. The intratumoral variability of gefitinib pharmacokinetics [AUC_{0–24h}] was about 2-fold and comparable with a 3-fold intratumoral variability reported for gefitinib brain tumor concentrations in a single patient receiving a multiple-dose regimen of 500 mg/day (23). The slightly lower intratumoral variability in gefitinib brain tumor concentrations observed at the 150 mg/kg gefitinib dose could be attributed to saturation of efflux transporters at the BBB, and thus, limit Michaelis–Menten transport that is sensitive to concentration changes and likely to exhibit greater variability. The variability in the intratumoral concentrations of gefitinib did not directly translate into the same degree of pharmacodynamic variability based on pERK measurements that were fairly uniform especially after high-dose administration (Fig. 2C) and could indicate gefitinib brain tumor concentrations were sufficiently high to produce near maximal inhibition of pERK. Although pERK regional variability was, modest it should be appreciated that drugs that exhibit steep concentration-response curves will be prone to greater variability; apparently, the gefitinib concentration-pERK relationship in U87vIII tumors is not.

The tumor vasculature exhibited significant intratumoral variability and was depicted according to MPI values. Many studies have reported the role of pericytes in regulating vascular integrity and maturity via regulation of endothelial cell proliferation, formation of tight junctions, and the composition of the extracellular matrix around vessels (37–39). The negative correlation obtained between MPI and gefitinib concentrations indicated that a more mature or functionally intact BBB (higher MPI) was a factor that impaired the delivery of
gefitinib to brain tumors. Antiangiogenic treatment can influence tumor distribution of coadministered drugs both increased and decreased drug delivery has been reported, which is related to a so-called vascular normalization process associated with increased pericyte coverage, decreased permeability, and IFP (24, 40–42). Although our study design prevents drawing directly from these analyses we do show that a more functionally intact BBB limits gefitinib distribution into tumors, which is in agreement with previous reports on gefitinib brain distribution (43, 44).

The pharmacokinetic characteristics of gefitinib were most different in the low- and high-MPI groups that were explained by different BBB transport, and in terms of model parameters, were attributed to transcellular diffusion (i.e., $h$) and active efflux by Pgp ($K_{\text{m}}$). Both parameters, $h$ and $V_{\text{max}}$, were found to be MPI-dependent, which is consistent with previous reports that pericytes regulate vascular integrity and maturity (37–39). One of these studies showed that the lack of pericyte coverage was associated with destabilized and permeable tumor vessels, resulting in increased metastasis (37–39). Another study showed greater biotin leakage into mouse brain that was attributed to lower pericyte coverage of brain microvessels. These reports support our contention that higher transcellular diffusion would be expected in vessels with less pericyte coverage or low MPI. The $V_{\text{max}}$ dependence on MPI was consistent with the observed lower Pgp expression in the regions with low MPI and a previous report that found Pgp expression negatively correlated with immature cerebral capillaries (33). Paracellular transport ($K_{p}$) of gefitinib was independent of MPI status, which seems reasonable because the basis for paracellular transport of gefitinib is large endothelial cell gaps that are characteristic of the physically disrupted BBB found in brain tumors (45).

Tumor pERK profiles predicted by the model agreed with the observed results in the low- and high-dose groups in all MPI groups. The pharmacodynamic model showed a dose-dependent increase in IC$_{50}$, a similar phenomenon that has been observed previously (46, 26, 47), yet not always attached to a definitive mechanism. In one case, a dose-dependent change in EC$_{50}$ was reported when a single drug acted simultaneously on two different receptors or targets (48). In a similar context, although gefitinib is known for its highly selective and potent EGFR inhibition, it targets at least 20 other kinases, albeit with higher IC$_{50}$ values than for EGFR. One such target is high concentrations, as it inhibits another intracellular kinase called cyclin G-associated kinase (49), that negatively regulates EGFR signaling, (50). By removing this negative feedback mechanism, more gefitinib is required to inhibit the intended target (pEGFR) and thus, the higher IC$_{50}$ value at the high-dose level. We do not have direct evidence to support this mechanism but the plausibility of this dose dependency was supported by the model.

In summary, the study successfully illustrates how intratumoral heterogeneity in the biologic characteristics of the tumor can result in variable drug exposure and pharmacodynamic response, albeit to a limited extent in this case. Nonetheless, the ability to characterize regional pharmacokinetic/pharmacodynamic information from tumors, made accessible through a tumor sectioning protocol, is advantageous to understand potential reasons for drug inactivity that traditional pharmacokinetic/pharmacodynamic studies based on tumor homogenates cannot offer. By combining pharmacokinetic/pharmacodynamic/IHC measurements regional PBPK/pharmacodynamic models, highlighted by variable BBB integrity, were developed that had the drugs’ efficacy been under evaluation could be used to examine alternate dosing strategies to attain therapeutically relevant concentration and pharmacodynamic effects throughout the tumor. Intratumoral pharmacokinetic/pharmacodynamic models provide an additional means to improve chemotherapy for glioblastoma multiformes.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Sharma, J.M. Gallo
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Sharma, H. Lv, J.M. Gallo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Sharma, J.M. Gallo
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Study supervision: J.M. Gallo

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