Integrated mapping of pharmacokinetics and pharmacodynamics in a patient-derived xenograft model of glioblastoma

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Therapeutic options for the treatment of glioblastoma remain inadequate despite concerted research efforts in drug development. Therapeutic failure can result from poor permeability of the blood-brain barrier, heterogeneous drug distribution, and development of resistance. Elucidation of relationships among such parameters could enable the development of predictive models of drug response in patients and inform drug development. Complementary analyses were applied to a glioblastoma patient-derived xenograft model in order to quantitatively map distribution and resulting cellular response to the EGFR inhibitor erlotinib. Mass spectrometry images of erlotinib were registered to histology and magnetic resonance images in order to correlate drug distribution with tumor characteristics. Phosphoproteomics and immunohistochemistry were used to assess protein signaling in response to drug, and integrated with transcriptional response using mRNA sequencing. This comprehensive dataset provides simultaneous insight into pharmacokinetics and pharmacodynamics and indicates that erlotinib delivery to intracranial tumors is insufficient to inhibit EGFR tyrosine kinase signaling.

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Glialblastoma (GBM) is the most common and aggressive form of primary parenchymal brain tumor. Treatment typically involves surgery with concurrent radiotherapy and chemotherapy; however, prognosis remains poor with a median survival of just 14–16 months. While numerous potential factors contribute to the poor efficacy of otherwise promising GBM therapies, one major but controversial limitation is heterogeneous drug delivery across the blood–brain barrier and the blood–tumor barrier (hereafter jointly referred to as the BBB). The BBB provides both physical and biochemical barriers to drug delivery into normal brain and excludes the majority of oncologic drugs. The BBB is generally considered to be disrupted in GBM, evidenced by accumulation of normally brain-impenetrant gadolinium (Gd) contrast agent in tumor regions on magnetic resonance (MR) images. However, the relationship between imaging-detectable contrast levels and concentrations of small molecules is not well characterized. Moreover, numerous image-guided biopsy studies and patterns of failure following gross total resection of all contrast-enhancing tumor demonstrate, categorically, that a significant portion of all GBM invade tissues beyond the contrast-enhanced regions. Thus, non-uniform disruption of the BBB may influence drug delivery throughout GBM tumors, and the resulting heterogeneous pharmacodynamic (PD) impact on tumor cell survival may be a critical factor limiting the efficacy of many therapies tested in GBM.

A common genetic feature of GBM is overexpression of epidermal growth factor receptor (EGFR). EGFR gene amplification occurs in ~40% of GBM, and almost half of these tumors have additional truncation or point mutations that result in ligand-independent, high-level constitutive signaling. Disregulated EGFR signaling promotes cell proliferation, migration, invasiveness, and impaired apoptosis. Erlotinib is a first-generation EGFR tyrosine kinase inhibitor, FDA-approved for the treatment of non-small cell lung cancer, that has significant activity in adenocarcinoma with activating mutations in the EGFR kinase domain. Based on the frequency of activation and importance of EGFR signaling, there has been sustained interest in evaluating various EGFR inhibitors in GBM. In pharmacokinetic (PK) studies, the measured concentration of erlotinib in patient cerebrospinal fluid was similar to efficacious concentrations in pre-clinical studies; however, little is known about how CSF concentrations of erlotinib relate to the concentrations and heterogeneity of erlotinib distribution in human brain and tumor tissues. Pre-clinical models, erlotinib has limited distribution into the normal mouse brain, and—consistent with the concept that heterogeneous distribution across the BBB in tumors might limit efficacy—only marginal activity was observed in clinical trials testing erlotinib in patients with newly diagnosed or recurrent GBM.

Erlotinib is known to be a substrate for the major efflux proteins in the BBB, P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). The active efflux mechanism limiting erlotinib permeability across an intact BBB, as found in invasive regions of glioma, may be in part responsible for the lack of adequate delivery and hence efficacy in GBM. While these clinical studies demonstrated that erlotinib is not a viable therapy for GBM, there are numerous other small molecule EGFR inhibitors in development with variable distribution across the BBB. Thus, the study reported herein provides a ‘failure analysis’ to understand the limitations of erlotinib tumor distribution and to describe a multi-modal platform that can be used to evaluate and nominate the most promising drugs for evaluation in clinical testing for GBM.

The successful evaluation of new treatments for GBM requires better understanding of the influence of a number of physiologic and pharmacologic parameters. Here we present a novel, multimodal approach to probe these parameters in a GBM patient-derived xenograft (PDX) model in response to erlotinib. Briefly, MR imaging T2-weighted (T2W) hyperintense and T1-weighted Gd contrast-enhanced (T1Gd) image regions allowed approximate delineation of tumor. Ex vivo tissue sections were analyzed by matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) to analyze drug and drug metabolite distribution, and ions identified as metabolic biomarkers of the tumor allowed accurate delineation of tumor regions. Imaging of serial sections by stimulated Raman scattering (SRS) microscopy produced high resolution images of the distribution of proteins, lipids and heme, enabling differences in brain and tumor tissue micro-architecture to be assessed. MS images were coregistered with the MR images to provide a comprehensive understanding of drug distribution across various imaging parameters. The MALDI MSI evaluation of drug levels was paired with laser capture microdissection (LCM) and subsequent analysis of transcriptional response in regions of heterogeneous drug delivery. A comparative study between intracranial (IC) and flank PDX was included in the experimental design to evaluate differences in drug delivery and the effect of the BBB on treatment response at the transcriptional and protein signaling levels. Collectively, this multimodality analysis established a spatially resolved PK-PD model for erlotinib and demonstrates that this platform is a robust and comprehensive assessment tool, that could be applied to essentially any novel therapeutic strategy targeting GBM or other tumor where differential drug distribution may be an important factor governing therapeutic response.

**Results**

**Multimodality analysis for integrated PK and PD.** This study was designed to investigate the distribution of and cellular response to erlotinib, an EGFR inhibitor, in both flank and IC GBM PDX models and is outlined in Fig. 1. Mice in both flank and IC groups were dosed with placebo, low (33 mg kg$^{-1}$) or high (100 mg kg$^{-1}$) dose erlotinib, and an additional flank group was dosed at ultra-low dose (5 mg kg$^{-1}$). Drug levels in plasma, brain, and flank tumors were determined by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Tissue samples were then serially sectioned and processed for multimodality imaging. Detailed analyses (MRI, 3D MALDI MSI, histology, SRS microscopy, and mRNA sequencing) were performed on tissue from one high dosed animal with IC PDX to provide comparative data for integrated PK and PD. Further analyses were performed on tissue from animals with flank and IC PDX under different doses of erlotinib to investigate dose-dependent response and differences in drug delivery and distribution.

**Heterogeneous drug distribution observed by 3D MALDI MSI.** Brain tissue from an animal bearing an IC tumor and dosed with 100 mg kg$^{-1}$ erlotinib was harvested and sectioned for multimodal imaging. 3D MALDI MSI acquired on a 9.4 T Fourier transform ion cyclotron resonance (FT-ICR) MS was used to measure the distribution of drug and metabolite in relation to endogenous biomolecules. Coronal sections of the tumor region were taken every 160 μm, as outlined in Fig. 2a, b. Serial sections were stained with H&E for comparison. Images of ions with m/z 394.1760 (Δppm = 0.25) (Fig. 2b and Supplementary Figure 1) indicate that the parent drug (erlotinib) is distributed with higher intensity in the highly cellular tumor regions compared with normal brain parenchyma. The drug is not distributed homogeneously through tumor, but instead appears with varied intensity in the x, y, and z dimensions. Images of ions with m/z...
metabolites (M13/M14) were detected with higher intensity between the blood vessels and the drug over the full tissue section demonstrating the ability of SRS imaging to provide detailed spatial and chemical information that could not be provided by traditional histology.

Overlaid, co-registered images of erlotinib (MALDI-MSI) and SRS images provided a means to investigate drug distribution and its correlation with the brain tissue structure, morphology, and chemical components (Fig. 3d–h). Images demonstrated that the protein rich tumor core contained higher concentrations of the drug in relation to histology and MR imaging (T1Gd and T2W MR images), a non-rigid registration was applied to a combined dataset, and erlotinib was mapped to the tumor region. Drug signal intensity was highest within the regions of high contrast in MR images, as measured using a mask image of three different regions of interest (ROIs) segmented from T1Gd and T2W MR images (Fig. 2d., e). The low intensity of erlotinib at the invasive margins of the tumor (beyond contrast enhanced region of MR images) suggests that significant fractions of the tumor cells are underexposed (Fig. 2d). Taken together, these data indicate that erlotinib drug levels were elevated but highly variable within the tumor.

High-resolution imaging with SRS microscopy. SRS microscopy was used to provide high spatial resolution images of chemical constituents of the brain and tumor microenvironments. SRS images of protein, lipid and heme (absorption) in serial sections of brain used for 3D MALDI were acquired (see Fig. 3). Multicolor SRS images revealed cellular morphologies, and demonstrated relative levels of different chemical classes in cells and tissue (Fig. 3b): protein (blue), lipid (green), and heme (red). Higher levels of protein were detected in the tumor core due to the highly-packed cancer cell bodies (Fig. 3b–e). At the brain/tumor boundary, compression or reorganization of the myelinated axon fibers at the tumor margin and cancer cells invading into the normal brain tissue were observed (Fig. 3f–g). In the normal brain structure, white matter (high lipid content, in green) and gray matter (high protein content, in blue) were distinguished due to the lipid/protein concentration difference (Fig. 3h–i). Corresponding H&E images (Fig. 3c–i) of serial sections demonstrated the ability of SRS imaging to provide detailed spatial and chemical information that could not be provided by standard histology.

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Drug delivery is limited at the invasive tumor edge. Quantitative MALDI MSI was performed to measure absolute concentration per pixel in brain and flank tumors under the low and high doses of erlotinib. A tissue mimetic model containing known quantities of erlotinib was analyzed by MALDI MSI and a calibration curve was produced (Supplementary Figure 4). The tumor biomarker, indicating similar specificity for the target region.

Comparative analysis of MRI and MALDI MSI. MR images and 3D MALDI images from the same animal were co-registered to establish the relationships among BBB disruption, drug distribution, and tumor microenvironment. To assess the distribution of drug in relation to histology and MR imaging (T1Gd and T2W MR images), a non-rigid registration was applied to a reduced MALDI MSI dataset, and erlotinib was mapped to the aligned coordinates. Overlaid images between drug and T2W MR images can be seen in Fig. 2d. Registration between the drug ion image and H&E histology reveals the extent of heterogeneity in drug distribution within the tumor region (see Supplementary Figure 3). Regions of high contrast were similar in T1Gd and T2W MR images, Fig. 2d. Drug signal intensity was highest within the regions of high contrast in MR images, as measured using a mask image of three different regions of interest (ROIs) segmented from T1Gd MR and erlotinib images (Fig. 2d, e). The low intensity of erlotinib at the invasive margins of the tumor (beyond contrast enhanced region of MR images) suggests that significant fractions of the tumor cells are underexposed (Fig. 2d). Taken together, these data indicate that erlotinib drug levels were elevated but highly variable within the tumor.

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calculated drug concentration in the normal brain, ROI (tumor regions defined by bisecting k-means clustering of MALDI MSI data) and flank tumors measured by MALDI MSI were similar to those measured using an analytical LC–MS/MS assay in normal and tumor-bearing (ipsilateral to tumor site) tissue, thereby validating the use of MALDI MSI for erlotinib quantification (Table 1). Quantitative MALDI MSI, was only performed on one animal in each treatment group, but agreement with LC–MS/MS measurements (n = 3 for flank, n = 2 for IC) suggests the animals analyzed by MALDI MSI were representative of the group and also provides a measure of variation between animals. The brain-to-plasma ratios of erlotinib obtained in this study were similar to those previously measured in a rat glioma model.26 Average concentration of erlotinib (measured by MALDI MSI) in IC PDX dosed at 33 and 100 mg kg\(^{-1}\) were found to be similar (2354.2 and 2182.3 ng g\(^{-1}\), respectively), with slightly higher concentration detected in the tumor dosed at 33 mg kg\(^{-1}\) (Table 1). This could be due to a relatively well vascularized tumor situated closer to the brain surface in the 33 mg kg\(^{-1}\) dosed animal (Supplementary Figure 5). Further segmentation of the IC tumor into the core and invading edge (Supplementary Figure 6) reveals ~two-fold decrease in drug concentration at the edge relative to the tumor core (at 100 mg kg\(^{-1}\) dose). No difference in drug concentration was observed in flank tumors between the core and edge. The concentration of erlotinib was similar in IC tumors dosed at 33 and 100 mg kg\(^{-1}\), the average concentrations of erlotinib in flank tumor was dose-dependent at 2426.8 and 8211.6 ng g\(^{-1}\), for 33 and 100 mg kg\(^{-1}\), respectively. These concentration values were next used to correlate dose-related response detected in further analysis of IC and flank tumors.

Correlation coefficients between drug and heme were calculated between different image regions (Supplementary Table 2), providing an indication of the availability of the drug (to tumor cells), alongside absolute concentration of drug in the region. Broadly, higher correlation between drug and heme was observed in IC tumors compared to flank, suggesting that the drug is less...
available in the IC tumor, regardless of the relative concentration. Higher correlation between drug and heme was also observed in the IC tumor edge compared with corresponding tumor core.

**Erlotinib activity is reduced in IC tumors relative to flank.** Tissue immunofluorescence (IF) was used to visualize and relatively quantify EGFR and phosphorylated-EGFR (p-EGFR) in a further set of IC and flank tumors under placebo, low and high dose of erlotinib. Hoechst images were used to define tumor ROIs based on regions of high cellularity; the tumor was segmented from normal brain (Supplementary Figure 7), and flank tumors were segmented from background. Average intensity for total EGFR and p-EGFR was calculated (see Fig. 4). Total EGFR was detected with higher relative intensity in IC tumor vs. normal brain tissue (Supplementary Figure 7). There was no significant difference in total EGFR in IC tumor regions between placebo and treatment with either low or high dose of erlotinib (t = 1.183, degrees of freedom (df) = 2, p = 0.358). In flank tumors however, there was a decrease in the amount of total EGFR after treatment with high dose erlotinib, relative to placebo (t = 4.213, df = 4, p = 0.0136). A similar relationship between flank and IC tumors was observed in relative levels of p-EGFR; on average there was no change in p-EGFR in IC tumors treated with 100 mg kg$^{-1}$ erlotinib, relative to placebo (t = 0.162, df = 2, p = 0.8859), however, heterogeneity in signal intensity was observed in all IC tumors, which could mask localized regions of response, see Supplementary Figure 8. A decrease was observed in p-EGFR in flank tumors after treatment with low (t = 8.562, df = 4, p = 0.001) and high (t = 13.069, df = 4, p = 0.0002) dose erlotinib, relative to placebo. This result is consistent with a dose–response relationship where a lower availability of erlotinib in IC tumors vs. flank results in insufficient suppression of EGFR activity.

**A low dose of erlotinib inhibits EGFR in flank.** Phosphoproteomic analysis was performed on tissue samples from flank tumors to assess phosphorylation changes in signaling pathways in response to different doses of erlotinib. Since phosphoproteomic analyses require considerable tissue quantities and are sensitive to post-resection ischemia, our studies were limited to larger and more quickly resectable flank tumors$^{30}$. Phosphoproteomic analysis identified 210 unique phosphotyrosine-containing peptides across the four treatment groups (vehicle, 5, 33, 100 mg kg$^{-1}$). Quantifying these phosphorylation sites across the tumor treatment conditions revealed inter-tumor heterogeneity in the pre-treatment and ultra-low dose erlotinib (5 mg kg$^{-1}$) tumors (see Fig. 5). Upon treatment with erlotinib
(33 and 100 mg kg$^{-1}$), tumor heterogeneity decreased as a consequence of similar responses to signaling inhibition. For these higher dose conditions, we observed a pronounced inhibition of the target (EGFR), proximal adaptor proteins (Gab1, Shc1), and downstream signaling (MAPK1, MAPK3) suggesting that 33 mg kg$^{-1}$ is an adequate dose to inhibit EGFR signaling in flank tumors. Despite the decrease in heterogeneity on treatment, individual tumors treated with 33 or 100 mg kg$^{-1}$ erlotinib demonstrated increased phosphorylation of selected nodes, potentially indicative of resistance pathways beginning to emerge in these tumors (Supplementary Dataset 1). Although no consensus resistance pathways were detected in all of the treated tumors, p130Cas (BCAR1) and PI3K (p110/p85) tyrosine phosphorylation were increased in several tumors in response to 5 and 100 mg kg$^{-1}$ erlotinib.

Table 1 Comparison of tissue-to-plasma ratio and concentrations of erlotinib measured by MALDI MSI and LC-MS/MS in IC and flank tumors and normal brain, whole brain and tumor bearing brain in animals treated with a low dose of erlotinib (33 mg kg$^{-1}$) and a high dose (100 mg kg$^{-1}$)

| Tumor location/region | Low dose (MALDI-MSI) (ng g$^{-1}$) | High dose (MALDI-MSI) (ng g$^{-1}$) | Low dose (LC-MS/MS) (ng g$^{-1}$) | High dose (LC-MS/MS) (ng g$^{-1}$) |
|-----------------------|-----------------------------------|-----------------------------------|----------------------------------|-----------------------------------|
| **Flank tumor**       | 2426.8                            | 8211.6                            | 2476.6 ± 1339.9                  | 6267.2 ± 2544.7                  |
| **Brain tissue**      |                                   |                                   |                                  |                                   |
| Normal brain*         | 619.3                             | 1082.9                            | 608.4 ± 145.2                    | 3218.8                            |
| Tumor only            | 2354.2                            | 2182.3                            | NA                               | NA                                |
| Tumor core            | 2411.5                            | 2787.1                            | NA                               | NA                                |
| Tumor edge            | 2011.2                            | 1446.5                            | NA                               | NA                                |
| Whole brain*          | NA                                | NA                                | 471.2 ± 282.6                    | 1337.6 ± 400.4                    |
| Tumor-bearing brain*  | NA                                | NA                                | 719.7 ± 355.0                    | 3677.2                            |
| **Tissue-to-plasma ratio** |                                  |                                   |                                  |                                   |
| Flank tumor           | NA                                | NA                                | 0.54 ± 0.11                      | 0.63 ± 0.18                       |
| Normal brain*         | NA                                | NA                                | 0.11 ± 0.04                      | 0.18 ± 0.08                       |
| Whole brain*          | NA                                | NA                                | 0.10 ± 0.02                      | 0.14 ± 0.02                       |
| Tumor bearing brain*  | NA                                | NA                                | 0.14 ± 0.08                      | 0.21 ± 0.09                       |

*Data are presented as mean (over ROI in MALDI MSI data, n = 1) or mean ± standard deviation (intra-animal LC-MS/MS measurement, n = 2 for brain, n = 3 for flank)

*Non-tumor bearing brain (left hemisphere; contralateral to the injection site) obtained from GBM12 intracranial (orthotopic) xenografts

*Brain tissue obtained from mouse bearing flank GBM12 xenografts

*Right hemisphere (ipsilateral to the injection site) obtained from GBM12 intracranial xenografts

*NA—not analyzed
33 mg kg⁻¹. To assess the functional relevance of these increased phosphorylation sites, additional time points or co-treatment, for instance with PI3K inhibition, would be required.

**Dose-dependent transcriptional response in flank and IC.**

Transcriptional response to variable erlotinib concentration in flank tumors was evaluated by mRNA sequencing. We selected differentially expressed genes (q < .05) from a group comparison of the different treatment groups (5 mg kg⁻¹ vs. placebo, 33 mg kg⁻¹ vs. placebo group, and 100 mg kg⁻¹ vs. placebo group) and utilized Ingenuity comparison analysis to identify canonical pathways activated and repressed (Z > 1.5 and q < .05). We observed unique and shared pathway signatures among treatment groups (Fig. 6a). All erlotinib-treated groups had activated eukaryotic initiation factor 2 (EIF2), peroxisome proliferator-activated receptor (PPAR), AMP-activated protein kinase (AMPK) signaling and decreased actin cytoskeleton and Ephrin B signaling compared to placebo treated tumors. Additionally, Rho GTPase activity signature was decreased and RhoGDI signaling was increased. Specifically, CDC42, RhoA, and Rac signaling were decreased in erlotinib-treated tumors compared to placebo. We observed that treatment with 5 mg kg⁻¹ erlotinib was insufficient to inactivate EGF, hepatocyte growth factor (HGF), 14-3-3, protein kinase A, integrin, and phospholipase C signaling pathways. Compared to flank tumors with low dose erlotinib, higher erlotinib doses activated the G1/S cell cycle checkpoint and interleukin 9 (IL9) pathway, and repressed EGF, cyclin, and death receptor signaling pathways.

To correlate drug exposure/response findings from the flank tumors to IC tumor response, LCM was used to dissect IC tumor tissue (dosed with 100 mg kg⁻¹ erlotinib) into four separate quadrants (Fig. 6b). MALDI MS ion images of the drug in a serial tissue section was used to determine the relative concentration of drug in each quadrant dissected (Fig. 6b, c). Relative drug concentration per quadrant varied from 100% in quadrant R1 to 75% in quadrant L1 and 64.5% and 61.09% in quadrants R2 and L2, respectively. Cluster analysis of transcripts from each quadrant show dose-related response highlighting heterogeneity in IC drug distribution and associated response. For this analysis, all genes were taken into account, limiting the differential gene expression. The overall transcripts from IC tumor tissue (dosed with 100 mg kg⁻¹ erlotinib) clustered between transcripts from flank tumors treated with ultra-low and low dose erlotinib. The centre portions of IC tumors dosed with 100 mg kg⁻¹ received similar concentration of drug as flank tumors treated with 33 mg kg⁻¹ and the edge portions of IC tumors received ~two-fold less, as determined by MALDI MSI quantification. Transcripts from IC tumors are therefore consistent with the dose–response relationship observed in flank tumors, Fig. 6d.

**Integration of phosphoproteomic and mRNA sequencing data.**

We have previously shown that correlating phosphoproteomic data across different tumors enabled the identification of activated signaling networks; here we applied this same approach to identify co-regulated networks of phosphorylation sites across tumors and treatment conditions. Hierarchical clustering analysis

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**Fig. 5** In vivo phosphorylation changes in erlotinib-treated flank tumors reveal inhibition of EGFR signaling. **a** Hierarchical clustering (Euclidian distance) of phosphopeptide changes for GBM12 flank tumors treated with 5, 33, and 100 mg kg⁻¹ erlotinib (n = 4) and displayed relative to a vehicle-treated flank tumor measured in both of the analyzed TMT-10-plex MS runs (E1 and E2). **b** Phosphoprotein interaction network based on STRING for members of cluster 1 in **a** and represented by their gene name.
of the correlation matrix of the 210 tyrosine phosphorylation sites across tumors yielded a few clusters (Fig. 7a, Supplementary Dataset 1), including a large, highly correlated cluster for most of the phosphorylation sites in the middle of the clustergram. This large cluster demonstrates that, despite the inter-tumor heterogeneity, most of the sites were co-regulated across tumors and treatment conditions. Smaller sub-clusters were also evident within the data, including a cluster of highly co-regulated sites comprised of four EGFR phosphopeptides, phosphorylation sites on the proximal adaptor proteins and direct EGFR substrates SHC and GAB1, the activation loop phosphorylation sites on the proximal adaptor proteins and direct EGFR substrates SHC and GAB1, the activation loop phosphorylation sites, ARHGAP35, and Annexin.”}

mRNAseq data led to the identification of two large, anti-correlated clusters (Fig. 7b) comprised of many smaller sub-clusters. These large clusters were predominantly defined by sites that increase or decrease upon EGFR inhibition, and are anti-correlated due to their differential response to erlotinib.

Quantitative phosphoproteomic and mRNASeq results were correlated across tumors to attempt to identify co-regulated networks of signaling and transcripts (Fig. 7c, Supplementary Dataset 2–3). This analysis was dominated by the direction of the fold-change on treatment with inhibitor, with most of the phosphorylation sites and approximately half of the transcripts de-correlated clusters (Fig. 7b) comprised of many smaller sub-clusters. These large clusters were predominantly defined by sites that increase or decrease upon EGFR inhibition, and are anti-correlated due to their differential response to erlotinib.

For instance, a cluster of phosphorylation sites with some of the strongest correlation/anti-correlation with mRNA includes six ERK1 and ERK2 MAPKs are known to be directly downstream of EGFR/SHC/GAB1 activation, and p190RhoA is involved in the EGFR pathway. ERK5 signaling is comprised of four EGFR phosphopeptides, phosphorylation sites within the data, including a cluster of highly co-regulated sites across tumors and treatment conditions. Smaller sub-clusters were also evident within the data, including a cluster of highly co-regulated sites comprised of four EGFR phosphopeptides, phosphorylation sites on the proximal adaptor proteins and direct EGFR substrates SHC and GAB1, the activation loop phosphorylation sites on the proximal adaptor proteins and direct EGFR substrates SHC and GAB1, the activation loop phosphorylation sites, ARHGAP35, and Annexin.
A1, all well characterized members of the EGFR signaling network. As shown in Fig. 7d, phosphorylation sites on given proteins were not necessarily correlated with their respective transcripts; for instance the phosphorylation of EGFR, the proximal adaptor protein GAB1, and the downstream MAPK1 proteins all decrease significantly with increasing erlotinib concentration, while their respective transcript levels either increase (EGFR, GAB1) or decrease slightly (MAPK1). As this example demonstrates, acquisition of mRNA and protein phosphorylation data on the same tumors enables the determination of whether the change in phosphorylation was likely due to altered signaling, rather than altered protein expression due to transcription and translation.

**Discussion**

Differences in the overall average drug concentration in IC compared to flank tumors do not sufficiently account for the lack of efficacy of erlotinib in GBM. Overall average drug concentrations of erlotinib in IC tumors at low and high doses were similar to flank tumors at the low dose. Tissue IF reveals that decreased levels of p-EGFR were associated with both low and high dose of erlotinib relative to placebo in flank tumors, but not in IC tumors with similar erlotinib concentration. Considering these results together it would appear that the same concentration of drug in IC and flank tumors did not have the same effect on EGFR. Closer inspection of MALDI MSI data, however, revealed that different concentrations of erlotinib were detected in different regions of the IC tumor; with a higher concentration (~two-fold higher) measured in the core of the tumor vs. the edge. Furthermore, co-localization between heme and erlotinib images provided an indication of the availability of the drug, i.e., whether the drug had left the blood vessels and entered brain/tumor tissue. Higher correlation between drug and heme was observed in IC tumors compared to flank, which suggested that the drug was less available in IC tumors. The highest correlation between drug and heme was observed in the tumor edge, further suggesting that the drug was least available to the infiltrating portion of the tumor. So, while the tumor core received quantities of drug that had the desired therapeutic effect in flank, the tumor edge did not. The combination of lower concentration of drug and low availability at the tumor edge could be a mechanism of failure of erlotinib.

Registration of SRS microscopy images and MALDI ion images of erlotinib enabled the distribution of drug to be related to chemical composition of tissue. Erlotinib showed an almost inverse relationship with regions of high lipid concentration. The region of highest intensity in the drug image corresponded with...
the protein-rich tumor core. Lower relative concentrations of drug were detected in and at the interface with the lipid-rich regions of white matter. The combination of these two imaging modalities demonstrates how drug distribution relates to global chemical content of various tissues and further demonstrates the limited and heterogeneous nature of drug distribution in the tumor. Differences in non-specific binding in different tumor regions can lead to varying concentrations of free (active) drug due to region-specific changes in the fraction unbound. Differences in the chemical composition of the tissue, such as the tumor core, peripheral tumor rim, normal brain, and even a tumor in the flank, may lead to differences in free drug concentrations, and hence be a factor in determining efficacy.\(^{34}\)

Registration of T1Gd and T2W MR images with MALDI ion images of erlotinib demonstrated that drug distribution overlapped with the contrast-enhanced portion of the tumor and the area of high drug intensity was larger than the contrast-enhanced portion. This suggested that Gd contrast agent and erlotinib have different distributions, as would be expected of molecules with different physico-chemical properties and PK, but which is mostly disregarded when using contrast enhancement MR to indirectly evaluate drug distribution. Further, although there were generally increased levels of drug within the MR image abnormality, the drug levels were highly variable. This heterogeneity in drug delivery to both the highly cellular tumor regions and the diffusely invaded regions of the brain could provide an opportunity for tumor cells to escape exposure supporting the continued tumor progression seen experimentally.\(^{14}\)

Activation or inhibition of protein tyrosine phosphorylation signaling networks should lead to consequent changes in transcriptional networks, which may themselves feed back to alter phospho-signaling; together, these regulatory networks control many of the cellular responses to perturbations, including treatment with kinase inhibitors. Integrated phosphoproteomics and mRNA sequencing data revealed that inhibition of protein signaling pathways did not necessarily correlate with respective transcription levels. Changes in the phosphorylation of EGFR, GAB1, and MAPK1 with erlotinib treatment were not correlated with their respective transcripts, which may suggest that the change in phosphorylation was due to altered signaling and not due to transcription and translation. Tissue IF data provided the bridge between response in flank and IC tumors and showed that EGFR and p-EGFR were not inhibited at the limited IC drug exposure.

Analysis of mRNA isolated from four quadrants of the high-dose IC tumor, suggested that the IC-treated tumors were more similar to these two lower dose flank cohorts at the transcript level. While the differential gene expression and ingenuity pathway for the IC quadrants was limited from having taken all genes into account (filtering out low abundance genes and batch sequence effect), the overall result provided evidence that similar dose-related response was observed in IC and flank tumors exposed to relatively similar drug levels. It should be noted that IC tumors were analyzed following a single dose of erlotinib (2 h post dose), whereas flank tumors were dosed three times over 3 days and sacrificed 6 h post final dose. After two hours, the concentration of drug in IC tumors is similar to those measured in low dose flank (by MALDI MSI) and transcriptomic analysis of mRNA signatures validates the drug concentration. In addition, mRNA transcripts change quickly (within 1–2 h) which is consistent with the IC tumor transcript signature being similar to that from flank tumor at low dose.

Considering the wider implications of this study it is important to account for model specific limitations. The integrity of the BBB in the mouse is clearly a critical factor for assessment of PK; a recent comparison of BBB permeability in the athymic nude mouse and an immunocompetent mouse found no difference in drug (ponatinib, another tyrosine kinase inhibitor) concentration in plasma and normal brain tissue.\(^{34}\) Compromise of the BBB in brain tumors has been shown to be largely ascribed to tumor cell secretion of cytokines which leads to localized effects on tight junctions, as well as other structural features of the BBB.\(^{35}\) This cytokine effect on the BBB has shown to be present in athymic mice\(^{35}\) suggesting that the PDX model described herein is a suitable model for PK/PD investigations in GBM. The use of PDX models requires a deficient immune system and therefore the inflammatory response will be compromised; in future work we will consider the use of genetically engineered mouse models of GBM in order to retain and study the effect of an intact immune system. In addition to the possible issues surrounding the use of PDX models; in this work we considered a single GBM xenograft model, it is likely that due to heterogeneity in genotype and phenotype between different GBM tumors, differences in PK and PD would be observed in other models.

Overall we propose that significant portions of IC PDX tumors received insufficient drug levels to successfully inhibit EGFR tyrosine kinase signaling and that this might be due to both limitations imposed by the BBB and tissue characteristics. This conclusion was drawn based on imaging the distribution of erlotinib in relation to other chemical and functional markers, and demonstrates the power of the multimodal platform in establishing relationships between drug PK and PD.

**Methods**

**Animals.** All studies involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at Mayo Clinic. Xenografts were established in female athymic nude mice (Hsd:athymic Nude-Foxn1Neco, ages 6–7 weeks; Envigo, Indianapolis, IN) as previously described.\(^{36,37}\) The GBM12 PDX model has been previously described\(^{38,39}\), full genotypic and phenotypic characterization is available from the Mayo Clinic PDX National Resource (http://www.mayo.edu/research/labs/translational-neuro-oncology/mayo-clinic-brain-tumor-patient-derived-xenograft-national-resource). A study into GBM12 IC PDX model survival with erlotinib treatment demonstrated a modest improvement in overall survival in mice treated with either 100 or 150 mg kg\(^{-1}\) erlotinib daily for two weeks or until moribund, compared with a control group, demonstrating the suitability of this model/drug combination for the study described herein. The provenance of the PDX models used have been confirmed by short tandem repeat analysis with a comparison to the original patient tumor or germline DNA sample. All of the PDX models are maintained by serial heterotopic tumor passage at Mayo Clinic. Screening and testing of these PDX models for Mycoplasma contamination has been negative. Mice were stratified by tumor size and randomized to treatment groups using the StudyLog database program (http://www.studylog.com/). Mice with established IC or flank tumors were dosed with a single dose of placebo, 33 or 100 mg kg\(^{-1}\) erlotinib. Two hours post dose mice were euthanized by CO\(_2\) inhalation and brain and plasma were harvested and flash frozen on dry ice for multi-omics analysis.

Adaptation phosphoproteomics and mRNA sequencing experiments were performed on a further set of mice with established flank tumors and were treated when tumor volume reached 250–350 mm\(^3\). Treatments were placebo, 5, 33, or 100 mg kg\(^{-1}\) erlotinib daily for 3 days (total three doses) and 6 h after last dose mice were euthanized with CO\(_2\) inhalation and tumor tissue and plasma were harvested and flash frozen on dry ice for subsequent analysis. This study included five mice per treatment group, the selection of animal number was based on the feasibility of the downstream analyses (proteomics, RNAseq, MALDI) across multiple treatment groups. The number of mice used for each analysis is stated for each method separately. All mice were observed daily by a veterinarian blinded to the treatment received. The technician performing the tumor measurements was also blinded to the treatment received.

**Sample preparation for MALDI MSI.** Brain or flank tumors were dissected and snap frozen by immersion in liquid nitrogen. Tissues were placed at −25 °C 1 h before use, and tissue sections were prepared using a Microm HM550 cryostat (Thermo Scientific, Waltham, MA) with the microtome, and then briefly chilled at 20 °C and the specimen holder at −19 °C. Cryosections of 12-μm thickness were thaw mounted onto ITO-coated microscopic slides (Bruker Daltonics, Billerica, MA) for MALDI MSI and onto optical slides for hematoxylin, eosin (H/E) staining, and Tissue IF. For MALDI MSI, the mounted sections were dried for 15 min in a dehydrator prior to matrix application. 2,5-dihydroxybenzoic acid matrix (DHB, 160 mg ml\(^{-1}\) solution in methanol/0.2% TFA 70:30 vol/vol) was deposited using a TM-sprayer (HTX imaging, Carboro, NC) with the following conditions: flow
rate, 90 μL min⁻¹; spray nozzle velocity, 1200 mm min⁻¹; spray nozzle tempera-
ture, 73 °C; nitrogen gas pressure, 10 psi; track spacing, 2 mm; number of passes, 4.

Tissue mimetic sample preparation for quantitative MALDI. 100 μL aliquots of control mouse brain tissue homogenate were transferred into 7 pre-weighted 1.5 mL Eppendorf tubes and then each tube was weighed to provide an accurate mass for total quantity of homogenate transferred. Based on the weight of the tissue homogenate in each tube the appropriate concentration of drug standard was added for a final concentration of 100, 50, 10, 5, 1, 0.5, 0.1, 0.05 μg μL⁻¹ (drug/homogenate). Based on the work of Groseclose et al.29, we developed a 3D model of their negative mode and printed it using a 3D printer. The negative mode was filled with 40% gelatin and placed at –80 °C for 2 h. Once frozen, the assembly was placed into the chamber of a cryostat held at –40 °C, the negative mode was removed, and the frozen cylinder of 40% gelatin was separated from the outer tube by removing the cap and sliding out the 40% gelatin. The 100 μL aliquots of homogenate were then transferred into separate cores in the frozen cylinder of 40% gelatin inside the cryostat chamber. The tissue model was placed at –80 °C for 24 h prior to sectioning. The tissue mimetic model was then sectioned at 12-μm thickness using a Microm HM535 cryostat and sections were mounted onto ITO-coated microscopic slides next to the tissue sections for MSI.

**Tissue IF staining and microscopy.** Localization of total and phosphorylated (Tyr1173) EGFR in tumor sections from flank and brain tumors was examined via IF staining. Briefly, 8 μm tumor sections were fixed on glass slides in 4% paraformaldehyde for 10 min at room temperature (RT) and rinsed with PBS. Then, the slides were permeabilized in 0.1% Triton X-100 for 3 min. After washing with PBS, blocking buffer (5% goat serum/TBS) was applied to block unspecified staining for 1 h at RT. The sections were incubated with primary antibody (anti-EGFR 1:200 (ab32077) or anti-phospho-EGFR 1:50 (ab52578)) in 0.5% goat serum/TBS-0.05% Tween at 4 °C overnight, and subsequently the secondary antibodies (AlexaFluor® 488, A11036 and AlexaFluor® 594, A11032) conjugated with the primary antibodies were added and the sections were stained with Hoechst. A negative control leaving out the primary antibody was included. Fluorescence images were acquired using a Zeiss Observer Z.1 microscope equipped with ≥20 Plan-APOCHROMAT lens and AxioCam MR3 camera. Fluorescence images of Hoechst and EGFR/p-EGFR were acquired using a fluorescence microscope (Axio Observer Z.1) operated in an epifluorescence setup (488 nm excitation/495 nm emission) and with a FLA 3000 (Hitachi) scanning confocal laser scanning microscope (fluorescence cube was non-linearly aligned to both H&E histology and MRI using the automatic alignment quality was visually assessed.

**Automatic multi-modal non-rigid image registration.** The MALDI MSI datacube was not linearly aligned to both H&E histology and MRI using the automatic t-distributed stochastic neighbor embedding (t-SNE) based registration method42. Briefly, the dimensionality reduction method of the t-SNE25 was first used to reduce the dimensionality of the MSI dataset into two-dimensions where each coordinate represents t-SNE features. These 3D t-SNE features were spatially organized as such each t-SNE feature represents an image that was used to feed a color channel in a L*a*b* color system, and the resultant combined image is called t-SNE image. Of note, the spectra were projected while preserving their local characteristics, in a single map representation space. In this way pairs of similarities such as similar spectra were projected close to each other whereas dissimilar ones were projected farther apart. This translates to forming molecularly distinct structures in the t-SNE image and thus establish spatial correspondences that would guide the registration with other structure/anatomy-rich images, such as MRI and H&E.

The t-SNE image was then non-linearly aligned to its corresponding 2D MR image. A 2D MR image was manually selected from the MRI volume at similar location to that of a MALDI section. In this alignment process, the t-SNE image was first linearly warped using Aline transformation (T_A) to capture global distortion, registration, rotation, translation, and shearing. Then the entire image was further warped using the BSpline transformation (T_B) to correct for local differences. And finally, each m/z image was transformed using T_A and T_B, respectively, to be spatially aligned with the MR image. This alignment process was repeated but after replacing the MR image with the corresponding histological image. The alignment quality was visually assessed.

**Phosphoproteomic analysis.** Tumor material (whole flank tumor) was collected from mice (n = 4 per treatment group: vehicle, 5 mg kg⁻¹ erlotinib, 33 mg kg⁻¹ erlotinib, and 100 mg kg⁻¹ erlotinib) and snap frozen in liquid nitrogen. Tumors were homogenized by sonication in 8 M urea supplemented with 1 mM sodium hydroxide, and then were tightly focused onto the sample using a water-immersion objective (X1 PLAN N 25 ×, NA 1.05; Olympus). Imaging was realized by raster scanning the laser beams across the sample. Frequency difference between the two beams determines the Raman shifts to image. By tuning the pump wavelength, multicolor imaging could be achieved. The laser source was remote controlled through the RS-232 interface. A single FOV was 350 × 350 μm. Each FOV was acquired with 512 × 512 pixels in about one second. Tiling imaging was conducted using a pre-determined stage (MS2006, ASI) with partial overlap of the two beams. A Si photodiode detector was used to convert the pump light to electrical signals for detection. Labview programming (National Instruments Inc.) integrated the whole system for automatic multi-color and large-tissue image acquisition. Lipids were imaged (green) at 2854 cm⁻¹ attributed to CH2 stretching vibration from the fatty acid long chains; proteins were imaged (blue) at 2940 cm⁻¹ mainly attributed to CH3 vibration of the chemical bonds in the amino acid backbone (red) was imaged at 2800 cm⁻¹ due to two-color two-photon absorption. Imaging resolution is ~500 nm. Scale bar, 350 μm.
orthovandate (Na3VO4), 0.1% Nonidet P-40, protease inhibitor cocktail (Roche) (1 tablet/10 mL), and PhosStop (Roche) (1 tablet/10 mL). Protein concentration was measured by a bicinchoninic acid (BCA) assay (Pierce) and proteins were reduced with 10 mM dithiothreitol (DTT) for 1 h at 56 °C, alkylated with 50 mM iodoacetamide for 1 h at RT, and diluted fourfold with 100 mM ammonium acetate at pH 8.9. Proteins were digested using trypsin (sequencing-grade, Promega; 1 μg trypsin/50–50 mg total protein) overnight at RT. Enzyme activity was quenched by acidification of the sample with 1% acetic acid. Peptides were fractionated and concentrated on a C18 Sep-Pak Plus cartridge (Waters) and eluted with 40% acetonitrile, 0.1% acetic acid. Organic solvent was evaporated in a SpeedVac vacuum centrifuge. 400 μg aliquots of each sample were aliquoted and frozen in liquid nitrogen for 5 min, lyophilized and stored at −80 °C. Tumors were fixed and labeled with tandem mass tag (TMT)–10-plex Mass Tag Labeling Kits (Thermo)44. One vehicle-treated and one erlotinib-treated (5 mg kg⁻¹) tumor was included in each of a total of two TMT–10-plex analyses. Thus, allowing them to serve as normalization channel, as well as technical replicates. For TMT labeling, tumor peptide aliquots (400 μg peptide for each channel) were resuspended in 100 μL of 70% (vol/vol) ethanol, 30% (vol/vol) 0.5 M triethylammoniumbicarbonate at pH 8.5, and incubated with TMT reagent resuspended in 40 μL anhydroly acetonitrile at RT for 1 h. The samples were concentrated, combined, and concentrated to dryness using a SpeedVac centrifuge. Phosphopeptides were enriched using phosphophoryl immunoprecipitation and immobilized metal ion chromatography (IMAC)44. In brief, due to samples were resuspended in IP buffer (100 mM Tris–HCl 1% Nonidet P-40 at pH 7.4) and incubated with protein G agarose beads conjugated with 12 μg of anti-phospho tyrosine antibodies: 4G10 (Millipore), PY-100 (Cell Signaling Technologies), and PT-66 (Sigma) overnight at 4 °C. Due to differences in sequence in preferences of phosphopeptide capture, we combined three different antibodies to maximize the sequence coverage of different phosphorylated tyrosine-containing peptides by the immunoprecipitation. Beads were washed with IP buffer and rinse buffer (100 mM Tris–HCl at pH 7.4) and subsequently peptides were eluted with 100 mM glycine at pH 2.5 for 30 min at RT and acidified with TFA. Next, phosphopeptides were additionally enriched by IMAC using Fe-NTA Agarose beads. Bound peptides were eluted with 250 mM NaH2PO4 and loaded onto a precolumn [100 μm ID × 10 cm packed with 10 μm C18 beads (YMC gel, ODS-A, 12 nm, S-10 μm, AA12511)], which was rinsed with 0.2 M acetic acid for 10 min before LC–MS analysis44. Raw mass spectral data files were loaded into Proteome Discoverer version 1.4.1.14 (Thermo) and searched against the human SwissProt database (including 20,215 sequences) using Mascot version 2.4 (Matrix Science). TMT reporter quantification was extracted and isoleted corrected in Discoverer. Tandem mass spectra were matched with an initial mass tolerance of 10 ppm on precursor masses and 15 nuno for fragment ions. Cysteine carbami- domethylmation, TMT-labeled lysine, and protein N-terminal were searched as fixed modifications. Oxidized methionine, and phosphorylation of serine, threonine, and tyrosine were searched as variable modifications. Minimal peptide length was seven amino acids. Only peptides with an ion score > 20 were included in the final peptide list. Peptide quantification was normalized based on median. Protein quantification obtained from a crude peptide analysis to correct for minor variation in sample amount among TMT channels. For each peptide, relative quantitation was represented as a ratio between TMT ion intensities for each analyzed tumor and the included normalization channel. The protein network was obtained by using the STRING database (version 10.0)28. All active interaction sources except text mining were included and to ensure high confidence, a con- fidence score over 0.9 was required. Further network analysis and visualization were performed by using the Cytoscape platform (version 2.8)47.

RNA isolation and sequencing. ArcturusXT™ LCM System was used to capture cells from four regions of the IC tumor section. Total RNA from each captured region was isolated using the ARCTURUS™ PicooPure™ RNA Isolation Kit according to manufacturer’s protocol. For flank tumors, total RNA was isolated using RNAeasy Mini Kit (Qiagen) according to manufacturer’s protocol.

Total RNA (50 ng) for each region was used to generate whole transcriptome libraries for RNA sequencing using Illumina TruSeq RNA Sample Prep Kit (catalog # MS-122-1001) following the manufacturer’s recommendation. Poly-A mRNA selection was performed using oligo(dT) magnetic beads, and libraries were enriched using the TruSeq PCR Master Mix and primer cocktail. Amplified products were cleaned and quantified using the Agilent Bioanalyzer and Invtrogen Qubit. The clustered flowcell was sequenced on the Illumina HiSeq 2500 for paired 100-bp reads using Illumina’s TruSeq SBS Kit V3 (catalog #FC-401-3001).

Transcriptome alignment and differential expression analysis. Lane level fastq files were aligned together if they were sequenced across multiple lanes. These fastq files were then aligned with STAR 2.3.1 to GRCh37.62 using ensembl63. genes.gtf as GTF file. Changes in transcript expression were calculated with Cuffdiff 2.1.1 in FPKM format using upper-quietile normalization. Clustering was per- formed using R heatmap.2 package with Euclidean Distance and McQuitty clus- tering method. Files submitted to the European Nucleotide Archive (ENA).
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