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**Function of the Blood-Brain Barrier and Restriction of Drug Delivery to Invasive Glioma Cells: Findings in a Orthotopic Rat Xenograft Model of Glioma**

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**Running Title:** BBB Restricts Drug Delivery to Glioma in Rat Xenograft Model

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**List of Abbreviations**

ABC - ATP-binding cassette

GBM – Glioblastoma Multiforme

TKI - tyrosine kinase inhibitor

CNS - central nervous system

BBB - blood-brain barrier

P-gp - P-glycoprotein

Bcrp - breast cancer resistance protein

Abcb1/Mdr1 - gene encoding the murine P-glycoprotein

Abcg2/Bcrp1 - gene encoding the murine breast cancer resistance protein

MS - mass spectrometry

LC - liquid chromatography

GF120918 (elacridar) - N-[4-[2-(6,7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)ethyl]-5-methoxy-9-oxo-10H-acridine-4-carboxamide]

FVB - Friend leukemia virus strain B

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## **Abstract**

Despite aggressive treatment with radiation and chemotherapy, recurrence in glioblastoma (GBM) is inevitable. The objective of this study was to show that the blood-brain barrier (BBB), though a combination of tight junctions and active efflux transporters in the brain microvasculature, can significantly restrict delivery of molecularly-targeted agents to invasive glioma cells. Transgenic mice lacking P-glycoprotein (P-gp) and breast cancer resistance protein (Bcrp) were used to study efflux of erlotinib at the BBB. A U87 rat xenograft model of GBM was used to investigate the regional distribution of erlotinib to the tumor and brain regions surrounding the tumor. The effect of concurrent administration of elacridar on regional tumor distribution of erlotinib was evaluated. We show that erlotinib transport across an intact BBB is significantly restricted due to P-gp and Bcrp-mediated efflux transport. We then show that the BBB is sufficiently intact in areas of brain adjacent to the tumor core to significantly restrict erlotinib delivery. Inhibition of P-gp and Bcrp by the dual inhibitor elacridar dramatically increased erlotinib delivery to the tumor core, rim and normal brain. These results provide conclusive evidence of the impact of active efflux at the BBB on delivery of molecularly-targeted therapy to different tumor regions in glioma. These data also support the possibility that the repeated failure of clinical trials of new drugs for glioma may be in part due to a failure to achieve effective concentrations in invasive tumor cells that reside behind an intact BBB.

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## **Introduction**

Glioblastoma (GBM) is an invasive tumor and should be regarded as a disease of the entire brain (Berens and Giese, 1999, Agarwal, 2011b). Despite recent advances in surgery, radiotherapy, and chemotherapy, there is no effective treatment for GBM, and progression of the disease is almost always the cause of death. The median survival of patients with GBM is only 16-19 months (Grossman et al., 2010). A fatal characteristic of GBM is the invasive growth of tumor cells into normal brain tissue surrounding the main tumor mass, making complete surgical resection not feasible. Tumor invasion in GBM has been reported as early as in 1938, when Hans-Joachim Scherer described the diffuse invasion of GBM by defining secondary patterns that reflected growth of tumor in neighboring brain tissue (Scherer, 1938). In 1961, Matsukado showed that more than 50% of untreated brain tumors spread into the contralateral hemisphere (Matsukado et al., 1961). Even radical surgical approaches, such as complete removal of the tumor-bearing hemisphere (hemispherectomy), do not prevent recurrence, and have been discontinued (Dandy, 1928; Bell and Karnosh, 1949). Therefore, further progress in treating this disease requires that the invasive glioma cells, that can reside centimeters away from the main tumor mass, are effectively treated (Agarwal et al., 2011b).

Advances in our understanding of the molecular pathogenesis of GBM have led to the development of many promising small molecule tyrosine kinase inhibitors (TKIs) that inhibit critical signaling pathways essential for growth and development of the tumor. However, clinical trials evaluating these molecularly-targeted TKIs in GBM have mostly resulted in disappointing failures (Huang et al., 2009). Amongst the various hypotheses developed to explain the failure of promising molecularly targeted agents in GBM, one that has often been overlooked is impaired delivery of drugs to their intracellular targets. The central nervous system (CNS) is protected by

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the blood-brain barrier, which restricts the passage of most small and large molecules into the brain (Pardridge, 2005). ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp, Abcb1) and the breast cancer resistance protein (Bcrp, Abcg2), constitute a vital component of this barrier that restricts CNS drug penetration by active efflux out of the brain (Schinkel and Jonker, 2003). It has been shown that these two “gatekeepers” restrict the entry of several molecularly-targeted and other anti-cancer agents into the brain (Chen et al., 2009; Agarwal et al., 2010; Agarwal et al., 2011a; Agarwal et al., 2011c). Certainly, restricted drug delivery to the target can help explain the inefficacy of otherwise potent molecularly-targeted agents in GBM (Agarwal et al., 2011b).

Several recent studies have suggested that drug delivery to the tumor is not restricted in GBM. These studies concluded that the BBB is overcome by residual damage from radiotherapy, and/or by the pathological infiltrative characteristics of GBM that compromises the functional integrity of the BBB (Hofer and Frei, 2007). These conclusions were based on the finding that drug concentrations in tumor (resected tissue) were several-fold greater than plasma concentrations. However, these tissues are frequently contaminated with residual blood, which increases measured drug concentrations in the “tumor”. Therefore, drug concentration measurements in resected brain tissues can be misleading and should be cautiously interpreted. Moreover, a significant finding in all of the above studies is that drug concentrations in areas distant from the tumor core (non-contrast enhancing regions) were several-fold lower than that in the tumor core (contrast-enhancing areas) (Fine et al., 2006; Rosso et al., 2009; Pitz et al., 2011). This suggests that while the BBB may be disrupted at or near the tumor core, it most certainly is intact near the growing edge of the tumor, regions where invasive tumor cells may reside. An intact BBB along with its functional efflux transport systems in such regions of the brain can significantly impede

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drug delivery to the invasive tumor, which, in almost all cases, is more important to treat after resection of the primary tumor.

The objective of this study was to show that: 1) the blood-brain barrier can be heterogeneously disrupted in GBMs and can be intact in areas away from the primary tumor, and 2) an intact BBB in such areas, along with its functional efflux transport systems, can significantly restrict delivery of molecularly-targeted agents to invasive glioma. With use of a xenograft model of GBM, we show that the BBB restricts delivery of the molecularly-targeted agent erlotinib to the brain and brain tumor. Furthermore, we show that pharmacological inhibition of P-gp- and Bcrp-mediated active efflux at the BBB results in a significant increase in drug concentration in the brain, even in the tumor core where the BBB is thought to be disrupted.

## **Materials and Methods**

### **Chemicals**

Erlotinib hydrochloride and AG1478 were procured from LC Labs (Woburn MA). Elacridar (GF120918) was obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). Ammonium formate and acetonitrile were HPLC grade and were obtained from Sigma-Aldrich (St. Louis, MO).

### **Steady-State Brain Distribution of Erlotinib in FVB Mice**

Steady-state brain distribution of erlotinib was examined in male FVB wild-type, *Mdr1a/b<sup>-/-</sup>*, *Bcrp1<sup>-/-</sup>* and *Mdr1a/b<sup>-/-</sup>Bcrp1<sup>-/-</sup>* mice (*n = 4 per genotype*) of a FVB genetic background (Taconic Farms, Inc.). All animals were 8 to 10 weeks old at the time of experiment. Animals were

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maintained under temperature-controlled conditions with a 12-h light/dark cycle and unlimited access to food and water. All studies were carried out in accordance with the guidelines set by the Principles of Laboratory Animal Care (National Institutes of Health) and were approved by The Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

Erlotinib was infused to steady-state using Alzet osmotic mini pumps (Durect Corporation, Cupertino, CA). A 15 mg/ml solution of erlotinib in DMSO was filled in the minipumps (model 1003D) and the pumps were equilibrated by soaking them overnight (12 hours) in sterile saline solution at 37°C. The pump operated at a flow rate of 1  $\mu$ L/hr yielding a constant rate infusion of 15  $\mu$ g/hr (0.6 mg/hr/kg). Mice were anesthetized using 5 % isoflurane (Boynton Health Service Pharmacy, Minneapolis, MN) and were maintained under anesthesia using 2 % isoflurane in oxygen. The abdominal cavity was shaved and cleaned and a small midline incision was made in the lower abdomen under the rib cage. An incision was made in the peritoneal wall directly beneath the cutaneous incision and the primed pump was inserted into the peritoneal cavity. The musculoperitoneal layer was closed with sterile absorbable sutures and the skin incision was closed using sterile wound clips. The animals were allowed to recover on a heated pad. Erlotinib half-life in mice has been reported to be approximately 1 hour (Marchetti et al., 2008), so an infusion lasting 48 hours was considered to be sufficient to attain steady-state in both brain and plasma. The animals were euthanized 48 hours post surgery by using a CO<sub>2</sub> chamber. Blood was collected by cardiac puncture and whole brain was harvested. Plasma was obtained by centrifuging the blood sample at 3500 rpm for 10 minutes. Plasma and brain specimens were stored at - 80 °C until analysis by LC-MS/MS.

Brain concentrations were corrected for drug present in residual blood in brain by using the correction factor described by Dai et al.(Dai et al., 2003). Briefly, Dai and coworkers showed

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that brain space for plasma in brain vasculature is 1.4% of a FVB mouse brain. Using the measured plasma concentration, amount of drug in the brain vasculature was determined and the amount of drug in brain was corrected for drug present in vasculature using the following equation –

*Corrected Brain Amount =*

*Measured Brain Concentration – (1.4% × Brain Weight) × Measured Plasma Concentration*

### Glioblastoma cells

Human glioblastoma U87 cells transfected with wild-type EGFR were grown in short-term cell culture (7–14 days) at 37°C with 5% CO<sub>2</sub> in DMEM supplemented with 10% fetal bovine serum, and 1% antibiotic/antimycotic (Invitrogen, Carlsbad, CA, USA). Immediately before inoculation, cells were harvested and suspended in PBS to a concentration of 5 × 10<sup>4</sup> cells/μl.

### *Orthotopic Xenograft Model*

All experiments in the xenograft model were performed on a protocol approved by the Cleveland Clinic Institutional Animal Care and Use Committee. Animals were fed a standard rodent diet, and maintained in a pathogen-free environment. Athymic male nude rats (RNu, Charles River), 6-7 wks of age were obtained from Charles River Laboratories (Wilmington, MA) and housed in the Biological Resources Unit of the Cleveland Clinic. Rats were anesthetized by an intraperitoneal injection of ketamine and xylazine and inoculated with U87 cells (2.5 × 10<sup>5</sup> cells in a total volume of 5 μl) by injection into the right frontal region using a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Tumor was allowed to grow for three weeks before being used for further studies.

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### **Regional Tumor Distribution of Erlotinib**

Tumor-bearing rats were randomly assigned to one of the three groups ( $n = 6$  per group); control (no erlotinib treatment), erlotinib treatment with perfusion, and erlotinib treatment without perfusion. The rats in the treatment groups received 20 mg/kg/day erlotinib (in 0.5% methylcellulose) by oral gavage for 5 days. All animals were sacrificed 30 minutes after the last erlotinib dose followed by collection of blood and brain tissue. Rats in the perfusion group underwent cardiac perfusion with saline prior to collection of brain. Brain tissue was visually dissected into 3 parts: the tumor, the brain tissue adjacent to the tumor, and the contralateral hemisphere. Plasma was separated by centrifuging the blood at 3500 rpm for 10 minutes. Tissue specimens were flash frozen in liquid nitrogen and stored at -80°C until further analysis. Tissue specimens from each group were analyzed for erlotinib concentrations.

### **Effect of Elacridar on Distribution of Erlotinib to the Tumor**

In a separate study, tumor-bearing rats were divided into three groups ( $n = 4$  per group): control (no erlotinib treatment), erlotinib treatment, and treatment with erlotinib plus elacridar. The rats in the treatment groups received 20 mg/kg/day erlotinib (in 0.5% methylcellulose) by daily gavage for 3 days. Rats in the erlotinib plus elacridar group received an additional dose of 10 mg/kg elacridar by intravenous administration into the jugular vein 30 minutes before each erlotinib dose. Animals were sacrificed 30 minutes after the last erlotinib dose followed by collection of blood. All rats were then perfused with saline and brain tissue was harvested. Plasma and brain tissue were processed as above. All samples were stored at -80°C until further analysis.

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### **Quantification of Erlotinib in Brain and Plasma by LCMS-MS**

The concentration of erlotinib in mouse plasma and brain homogenate was determined by high pressure liquid chromatography (HPLC) coupled with mass spectrometry. Prior to analysis, frozen samples were thawed at room temperature. Brain samples were homogenized using 3 volumes of ice-cold 5% bovine serum albumin in phosphate-buffered saline using a tissue homogenizer (Fisher Scientific, Pittsburgh, PA). 100 µL of specimen plasma and brain homogenate were spiked with 50 ng of internal standard, tyrphostin (AG1478), and alkalinized by addition of 100 µL of a pH 11 buffer (1 mM sodium hydroxide, 0.5 mM sodium bicarbonate). Samples were extracted by vigorous vortexing with 1 mL of ice cold ethyl acetate followed by centrifugation at 7500 rpm for 15 minutes at 4°C. A volume of 750 µL of the organic layer was transferred to fresh polypropylene tubes and dried under nitrogen. Samples were reconstituted in 100 µL mobile phase and transferred to glass auto sampler vials. A volume of 10 µL was injected in the HPLC system using a temperature controlled autosampling device maintained at 10°C. Chromatographic analysis was performed using an Agilent Model 1200 separation system (Santa Clara, CA, USA). Separation of analytes was achieved using an Agilent Eclipse XDB-C18 RRHT threaded column (4.6 mm ID x 50mm, 1.8 µ) fitted with an Agilent C18 guard column (4.6 mm ID x 12.5mm, 5 µ) (Santa Clara, CA, USA). The mobile phase was composed of acetonitrile: 20mM ammonium formate (containing 0.1% formic acid) (45:55 v/v), and was delivered at a flow rate of 0.25 mL/min. The column effluent was monitored using a Thermo Finnigan™ TSQ® Quantum 1.5 detector (San Jose, CA, USA). The instrument was equipped with an electrospray interface, and controlled by the Xcalibur version 2.0.7 data system. The samples were analyzed using an electrospray probe in the positive ionization mode operating at a spray voltage of 4500V for both erlotinib and the internal standard. The spectrometer was

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programmed to allow the [MH]<sup>+</sup> ion of erlotinib at m/z 395.14 and that of internal standard at m/z 316.68 to pass through the first quadrupole (Q1) and into the collision cell (Q2). The collision energy was set at 14V for erlotinib and 9V for tyrphostin. The product ions for erlotinib (m/z 278.91) and the internal standard (m/z 300.9) were monitored through the third quadrupole (Q3). The scan width and scan time for monitoring the two product ions were 1.5 m/z and 0.5 s, respectively. The assay was sensitive over a range of 2.5 ng/ml to 1 µg/ml with the coefficient of variation being less than 15% over the entire range.

### Statistical Analysis

Comparison between groups were made using SigmaPlot, version 11 (Systat Software, Inc., Point Richmond, CA). Statistical difference between two groups was tested by using the two-sample t-test and significance was declared at  $p < 0.05$ . Multiple groups were compared by one way analysis of variance with the Holm-Sidak post-hoc test for multiple comparisons at a significance level of  $p < 0.05$ .

## Results

### P-gp and Bcrp Mediated Efflux Restricts CNS Delivery of Erlotinib

The influence of P-gp and Bcrp on the transport of erlotinib across the BBB was examined by studying its steady-state brain distribution in FVB mice. After a continuous intraperitoneal infusion lasting for 48 hours, the steady-state plasma concentrations of erlotinib ranged from  $72 \pm 42$  ng/ml in the wild-type mice to  $99 \pm 22$  ng/ml in the *Mdr1a/b<sup>-/-</sup>Bcrp1<sup>-/-</sup>* mice. Steady-state plasma concentrations were not significantly different from each other between the four mouse

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groups (**Table 1**). Steady-state brain concentrations were  $4.3 \pm 5.5$  ng/gm in the wild-type,  $12 \pm 2.8$  ng/gm in the *Mdr1a/b*<sup>-/-</sup>,  $4.9 \pm 2.9$  ng/gm in the *Bcrp1*<sup>-/-</sup>, and  $34 \pm 17$  ng/gm in the *Mdr1a/b*<sup>-/-</sup> *Bcrp1*<sup>-/-</sup> mice. The corresponding steady-state brain-to-plasma ratios were  $0.02 \pm 0.02$  in the wild-type mice and increased to  $0.09 \pm 0.06$  (4.5-fold) in the *Mdr1a/b*<sup>-/-</sup>,  $0.07 \pm 0.02$  in the *Bcrp1*<sup>-/-</sup> (3.5-fold), and to  $0.62 \pm 0.27$  (31-fold) in the *Mdr1a/b*<sup>-/-</sup> *Bcrp1*<sup>-/-</sup> mice (**Figure 1**). This indicates that these two ABC transporters efflux erlotinib at the BBB and their absence in the combined P-gp/Bcrp knockout mice results in a dramatic enhancement in brain distribution of erlotinib.

### **Regional Brain Delivery of Erlotinib in GBM Model**

The impact of the BBB in restricting delivery of erlotinib to the tumor was investigated using the U87 rat xenograft model of GBM. Tumor-bearing rats were administered a daily dose of erlotinib and concentrations in three different brain regions were determined. We first studied the effect of perfusion, prior to tissue collection, on the concentrations of erlotinib in the brain. Residual blood remaining in the brain vasculature can contribute to drug concentration measurements in brain, resulting in significant error, especially for drugs that do not penetrate the BBB well. Erlotinib concentrations in all three brain areas decreased significantly when brain was perfused with saline prior to harvesting (**Figure 2A, 2B**). Drug concentrations were approximately 34% lower in the tumor core at  $1.83 \pm 0.54$   $\mu\text{g/gm}$ , while that in the tumor rim and normal brain decreased by more than 50% to  $0.39 \pm 0.19$   $\mu\text{g/gm}$  and  $0.37 \pm 0.22$   $\mu\text{g/gm}$ , respectively ( $p < 0.05$ ). These findings suggest that drug remaining in residual blood within the brain vasculature, within gross tumor or brain, can contribute significantly to total brain concentrations, especially where there are low concentrations in the brain parenchyma, thereby introducing significant error to overall results. All subsequent experiments were therefore

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conducted after perfusing the brain using saline to avoid overestimation of drug concentrations and misinterpretation of results.

Comparison of erlotinib in the three brain regions showed significant heterogeneity in brain distribution of erlotinib. Erlotinib concentrations in the tumor core were  $1.83 \pm 0.54 \mu\text{g/gm}$  and decreased significantly to  $0.39 \pm 0.19 \mu\text{g/gm}$  (4.7-fold) in the brain around the tumor and to  $0.37 \pm 0.22 \mu\text{g/gm}$  (5-fold) in the contralateral hemisphere ( $p < 0.05$ , **Figure 3**). The tissue-to-plasma concentration ratio was  $0.51 \pm 0.06$  in the tumor core and decreased significantly to  $0.10 \pm 0.03$  in the tumor rim and  $0.09 \pm 0.03$  in the normal contralateral hemisphere ( $p < 0.05$ ). This indicates that while drug delivery is not completely restricted by the BBB in the core, the intact BBB definitely limits transport of erlotinib to sites both immediately adjacent (rim) and significantly distant from the tumor core (normal brain).

### Effect of Pharmacological Inhibition of P-gp and Bcrp on Regional Erlotinib Delivery

Inhibition of P-gp and Bcrp using small molecule inhibitors is a possible strategy to enhance brain penetration of substrate drugs. We investigated this by using the dual P-gp/Bcrp inhibitor elacridar (GF120918) in combination with erlotinib. Erlotinib concentrations in all three brain regions increased significantly in rats that were administered elacridar concurrently with erlotinib, compared to those which were treated with erlotinib alone (**Figure 4A**). Concentrations in the tumor core increased by 4-fold while that in the brain around tumor and normal brain increased by more than 12-fold compared to the vehicle treated group ( $p < 0.05$ ). In the group that received both elacridar and erlotinib, concentrations in three brain regions were not statistically different from each other, with the tissue-to-plasma ratio reaching a value of ~ 1 (**Figure 4B**). This indicates that pharmacological inhibition of P-gp and Bcrp by elacridar dramatically enhances erlotinib delivery, especially to the tumor periphery and normal tissue,

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where the BBB is intact. Comparison of erlotinib levels in Figure 4A and Figure 2 and 3 reveals that tissue concentrations in this study were lower than the previous studies. It is possible that the lower tissue concentrations in this study can be related to difference in experiments as a result of differences in effect of tumor on the BBB. Importantly, the relative magnitude of the difference between the three regions remained the same (compare Figure 4B with Figure 2B).

## **Discussion**

GBM is an invasive disease that affects the whole brain. The infiltrative nature of the disease makes treatment particularly challenging, because surgery does not remove tumor cells that have invaded into normal brain areas, and protective mechanisms such as the BBB shield and protect these invasive cells from chemotherapeutic agents (Agarwal et al., 2011b). Recent studies have questioned the role of the BBB in limiting delivery and thus efficacy of chemotherapy, based on findings that drug concentrations in tumor (resected tumor tissue) were several-fold higher than that in plasma (Hofer and Frei, 2007; Blakeley et al., 2009; Pitz et al., 2011). However, these concentrations do not represent drug levels in other areas of the brain, especially regions where the BBB may be intact and capable of restricting drug delivery. Given that the target in question includes invasive tumor cells away from the site of surgical resection, drug concentrations in these invasive sites are of paramount importance. We have demonstrated that BBB is intact in a rat xenograft model of GBM, especially in areas that are distant from the central tumor mass. This intact BBB restricts the delivery of the molecularly-targeted agent erlotinib, a substrate for P-gp and Bcrp, to the brain and brain tumor. We show that drug concentrations in sites away from the main tumor, such as the tumor rim and the contralateral normal hemisphere, are several-fold lower than that in the tumor core. Furthermore, we demonstrate that pharmacological

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inhibition of the two efflux transporters results in a dramatic increase in drug distribution to the entire brain. Finally, we have also demonstrated that measurement of drug levels obtained from clinical samples may be unreliable due to contamination by drug in the tumor vasculature. Studies that use resected clinical tissue to demonstrate that a drug can cross the BBB should be viewed with caution. Similarly, studies that claim that a drug crosses the BBB following evaluation of drug levels in the tumor mass only in a xenograft model, especially when saline perfusion has not been performed, should also be viewed with caution.

The brain distribution study in FVB mice shows that erlotinib is a substrate for both P-gp and Bcrp and that together the two transporters significantly restrict its brain penetration (**Figure 1**). The impact of P-gp and Bcrp on brain penetration of erlotinib has been previously shown by several other studies (de Vries et al., 2010; Kodaira et al., 2010; Elmeliogy et al., 2011). Our results are consistent with these reports and show that erlotinib distribution to the brain increases dramatically when both P-gp and Bcrp are absent at the BBB (**Figure 1**). This finding is similar to our previous studies with other TKIs where we reported that P-gp and BCRP cooperate at the BBB and compensate functionally for each other's loss (Chen et al., 2009; Agarwal et al., 2010; Agarwal et al., 2011c). In a recent study, using a highly sensitive liquid chromatography-tandem mass spectrometry method to determine the quantitative expression of membrane transporters at the BBB of the three transgenic mouse genotype, we showed that P-gp or Bcrp is completely absent at the BBB in the single P-gp-knockout and Bcrp-knockout mice and the combined P-gp/Bcrp knockout mice, and that the loss of one transporter did not influence the expression of the other (Agarwal et al., 2012). This validation of the transgenic mouse model further supports the hypothesis that the greater than additive increase in brain penetration in absence of P-gp and

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Bcrp is due to a simple functional compensation between the two transporters at the BBB, rather than an upregulation of a transporter.

We used the U87 rat xenograft model of GBM to show that the BBB is intact and restricts delivery of erlotinib to the brain, especially to areas away from the tumor core. Erlotinib concentrations in the brain around the tumor and the normal brain were up to 7-fold lower than that in plasma (**Figure 3**). The tissue-to-plasma ratio of 0.14 in the normal brain and 0.17 in the rim were similar to the steady-state brain-to-plasma ratios in the FVB wild-type mice and indicate that the BBB may be fully intact in these areas. The tissue-to-plasma ratio of less than unity in the tumor core (0.52) suggests that the BBB is not completely disrupted even in the tumor core and restricts drug penetration to a small extent. Recently, there have been several studies that have investigated the impact of the blood-brain barrier in restricting drug delivery to the tumor (Fine et al., 2006; Hofer and Frei, 2007; Blakeley et al., 2009; Rosso et al., 2009; Pitz et al., 2011). Pitz and coworkers showed that concentrations of many anti-cancer drugs in contrast-enhancing areas of the tumor were generally higher than the corresponding plasma concentrations (Pitz et al., 2011). Likewise, tumor concentrations of paclitaxel and temozolomide have also been reported to be higher than that in plasma (Fine et al., 2006; Rosso et al., 2009). All these studies determined drug concentrations in resected tumor tissue and reported significantly high concentrations of chemotherapeutic agents therein, concluding that drug delivery to the tumor is not restricted in GBM. Many of the above studies also report that drug concentrations in non-contrast enhancing regions of the brain (areas distant from the tumor core) were several-fold lower than that in the contrast-enhancing tumor (Fine et al., 2006; Blakeley et al., 2009; Rosso et al., 2009; Pitz et al., 2011). These findings imply that while the pathological characteristics of GBM compromises the integrity of the BBB in or near the tumor

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core, leading to higher drug concentrations, the BBB most certainly is intact near the growing edge of the tumor. Invasive cells residing in such areas may remain shielded from chemotherapy and eventually give rise to the recurrent tumor. Ultimately, it is the areas of invasive tumor that are the critical issue in controlling this disease; the areas of enhancing tumor (tumor mass) often can be removed surgically. Thus, conclusions drawn on the basis of drug concentrations in a resected tumor can be highly misleading and do not represent the status of the BBB throughout the entire brain in GBM.

Many small molecule anti-cancer TKIs are substrates for P-gp and Bcrp, and therefore they do not cross the BBB to a significant extent (Dai et al., 2003; Chen et al., 2009; Lagas et al., 2009; Polli et al., 2009; Agarwal et al., 2010; Agarwal et al., 2011c). Consequently, inhibition of these two transporters has been proposed as a possible strategy to enhance brain penetration of substrate drugs. Elacridar (GF120918) is a dual inhibitor of P-gp and Bcrp that was developed to reverse multi-drug resistance seen in cancer (Hyafil et al., 1993). Several preclinical studies have shown that brain distribution of dual P-gp/BCRP substrates increase dramatically when elacridar is administered concomitantly (Breedveld et al., 2005; Chen et al., 2009; Lagas et al., 2009; Agarwal et al., 2010; Agarwal et al., 2011c). We therefore used elacridar to investigate its influence on delivery of erlotinib to the brain and brain tumor.

When elacridar was administered concurrently with erlotinib, concentrations in all three brain regions increased significantly compared to the vehicle-treated group (**Figure 4**). In the elacridar-treated group, the tissue-to-plasma ratio in all three brain regions was approximately 1, indicating that when the two transporters are inhibited, there is no restriction to the transport of erlotinib across the BBB. The fact that elacridar increased erlotinib delivery to the tumor core as well, suggests that the BBB is not completely disrupted even in the tumor core and has

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functional efflux transporters, capable of restricting drug delivery. Thus, inhibition of P-gp and Bcrp can be an attractive strategy to enhance delivery of substrate drugs across the BBB and thereby improve their efficacy.

In conclusion, this study shows the impact of an intact BBB on the delivery of a molecularly-targeted agent to the brain and brain tumor. We show that drug concentrations in the tumor core are not representative of those in the entire brain and should not be used as a guide for adequacy of drug delivery to the brain. Finally, we show that concurrent administration of a modulator of drug transporters, such as elacridar, can be used as a strategy to enhance delivery of substrate chemotherapeutic agents to the brain.

Several clinical trials evaluating the EGFR inhibitor erlotinib have reported disappointing results (Peereboom et al., 2009; Prados et al., 2009; Reardon et al., 2009; Raizer et al., 2010). The fact that recurrence occurs in areas away from the margins of surgical resection is indicative that invasive glioma cells in these areas are not being effectively treated. Confusion over the status of the BBB in glioma has stemmed from reports that use the findings of high drug concentrations in resected tumor tissue to represent drug delivery to the entire brain. This study shows that studies of that type can be highly misleading since the BBB may not be disrupted in areas of brain away from the tumor and drug concentrations in these areas can be significantly lower. Therefore, successful targeting of the invasive glioma cells will require enhanced delivery of chemotherapeutic agents across an intact BBB.

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**Acknowledgments**

The authors would like to dedicate this manuscript to the memory of Juan Palomo, M.D.

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**Authorship Contributions**

***Participated in research design:*** Agarwal, Manchanda, Vogelbaum, Ohlfest and Elmquist

***Conducted experiments:*** Agarwal and Manchanda

***Contributed new reagents or analytic tools:*** Vogelbaum and Elmquist

***Performed data analysis:*** Agarwal, Vogelbaum and Elmquist

***Wrote or contributed to the writing of the manuscript:*** Agarwal, Vogelbaum , Ohlfest and Elmquist

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**Footnotes**

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## **Figure Legends**

**Figure 1. Steady-state brain distribution of erlotinib in FVB wild-type, *Mdr1a/b*<sup>-/-</sup>, *Bcrp1*<sup>-/-</sup> and *Mdr1a/b*<sup>-/-</sup>*Bcrp1*<sup>-/-</sup> mice.** Brain-to-plasma ratios after a 0.6 mg/hr/kg intraperitoneal infusion for 48 hours. The values are presented as mean  $\pm$  S.D. (\* p<0.05, compared to wild-type; n = 4 per mouse genotype).

**Figure 2. Effect of Perfusion on Regional Distribution of Erlotinib.** Erlotinib concentrations (A) and brain-to-plasma ratios (B) in tumor core, rim and normal brain were significantly lower in rats that were perfused with saline (gray bars) prior to collection of brain tissue compared to the non-perfused rats (black bars). This indicates that residual drug in brain vasculature can contribute significantly to total brain levels. Therefore, drug concentration measurements in brain tissues that are not perfused or corrected can be misleading and should be cautiously interpreted. The values are presented as mean  $\pm$  S.D. (\* p<0.05, compared to tumor core; † p<0.05, compared to non-perfused group; n = 6 per group).

**Figure 3. Regional Distribution of Erlotinib in the U87 Rat Xenograft Model.** Erlotinib concentrations in tumor core were significantly greater than those in the brain around the tumor and normal brain (C/L hemisphere). The corresponding brain-to-plasma ratios (*inset*) suggests that the BBB may be intact in areas away from the tumor, indicated by the restricted delivery of erlotinib to the brain around tumor and the normal contralateral hemisphere. The values are presented as mean  $\pm$  S.D. (\* p<0.05, compared to tumor core; n = 6).

**Figure 4. Influence of Elacridar on Regional Distribution of Erlotinib.** Erlotinib concentrations (A) in tumor core, rim and normal brain increased significantly in the elacridar treated group (gray bars) compared to control (black bars). Erlotinib brain-to-plasma ratio (B) increased significantly and was approximately 1 in all three brain regions, indicating that when

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these two transporters are inhibited there is no restriction to the delivery of erlotinib to the brain.

This indicates that concurrent administration of a modulator of drug transporters, such as

elacridar, can be used as a strategy to enhance delivery of substrate chemotherapeutic agents to the brain. The values are presented as mean  $\pm$  S.D. (\* p<0.05, compared to tumor core; † p<0.05, compared to vehicle control; n = 4 per group).

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## Tables

**Table 1. Steady-state plasma and brain concentrations of erlotinib in wild-type, *Mdr1a/b*<sup>-/-</sup>, *Bcrp1*<sup>-/-</sup> and *Mdr1a/b*<sup>-/-</sup>*Bcrp1*<sup>-/-</sup> mice after a constant intraperitoneal infusion at a rate of 0.6 mg/hr/kg**

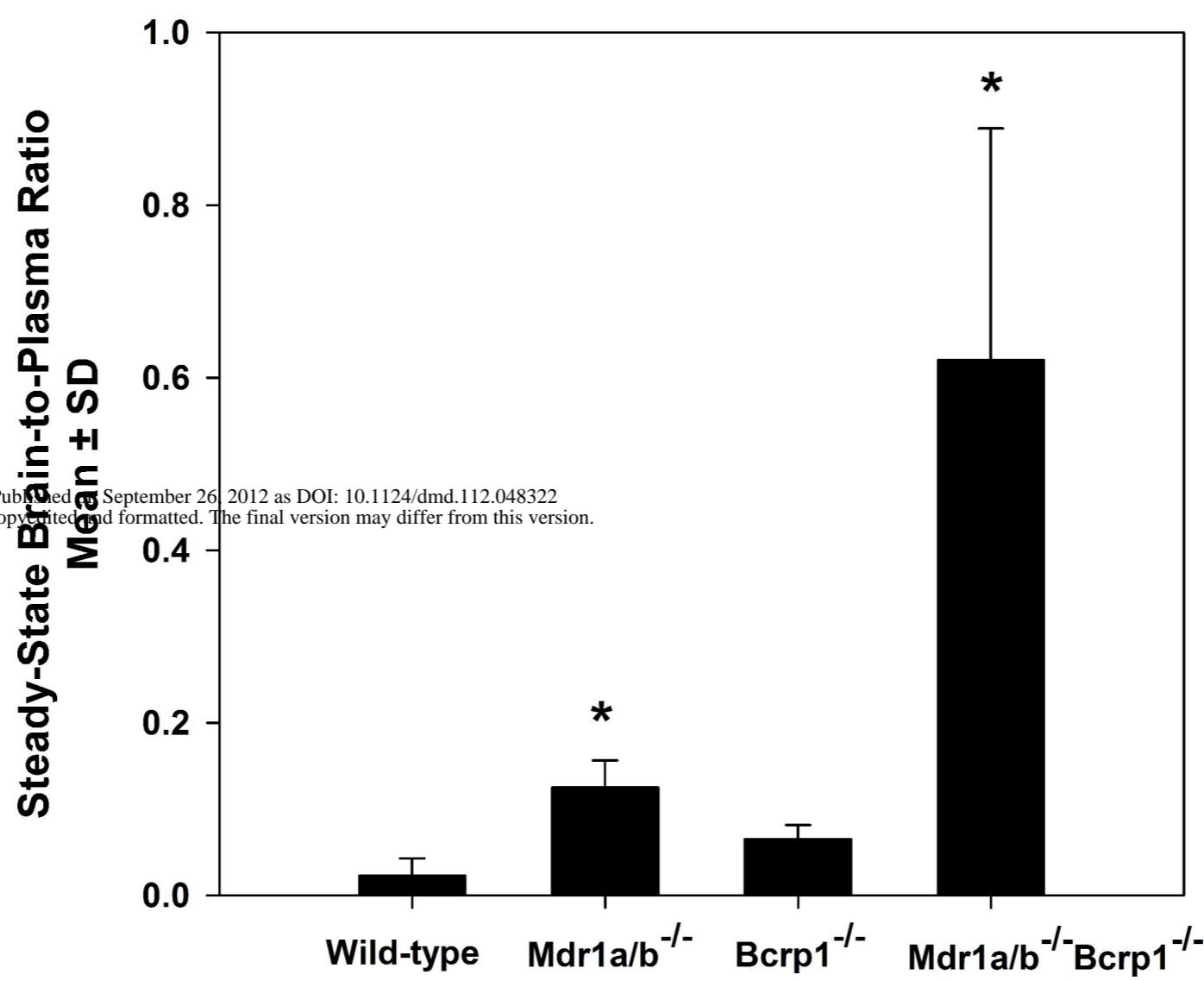
Genotype	n	Plasma Css	Brain Css	Brain-to-Plasma Ratio
		(ng/ml)	(ng/gm)	
FVB (wild-type)	4	72 ± 41	4.3 ± 5.5*	0.02 ± 0.02
<i>Bcrp1</i> <sup>-/-</sup>	4	71 ± 3	4.9 ± 2.9*	0.07 ± 0.02
<i>Mdr1a/b</i> <sup>-/-</sup>	4	81 ± 44	12 ± 2.8*,†	0.09 ± 0.06†
<i>Mdr1a/b</i> <sup>-/-</sup> <i>Bcrp1</i> <sup>-/-</sup>	4	99 ± 21	34 ± 17†	0.62 ± 0.27†

Data presented as mean ± S.D.

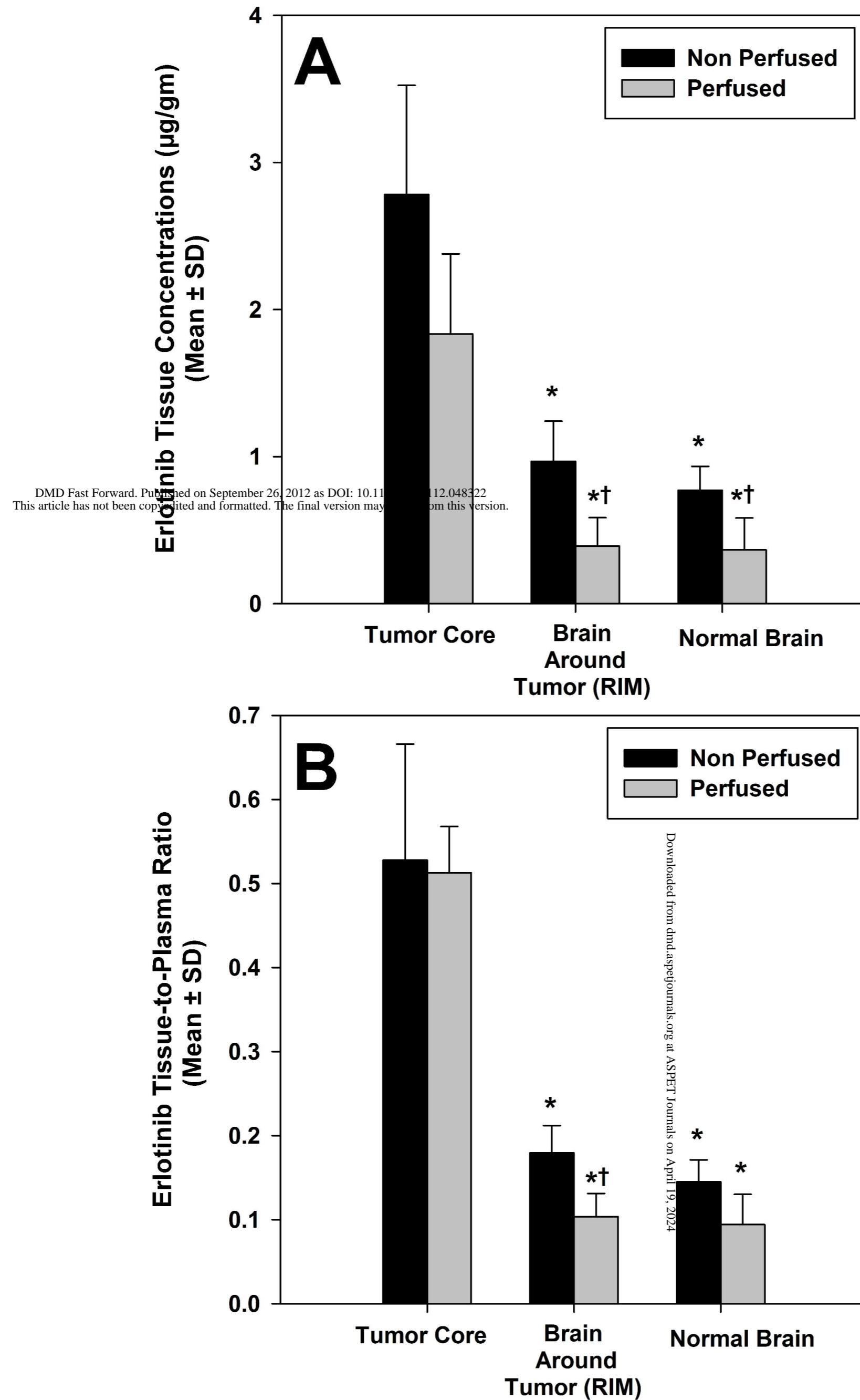
\* p < 0.05 compared to corresponding plasma concentration

† p < 0.05 compared to wild-type group

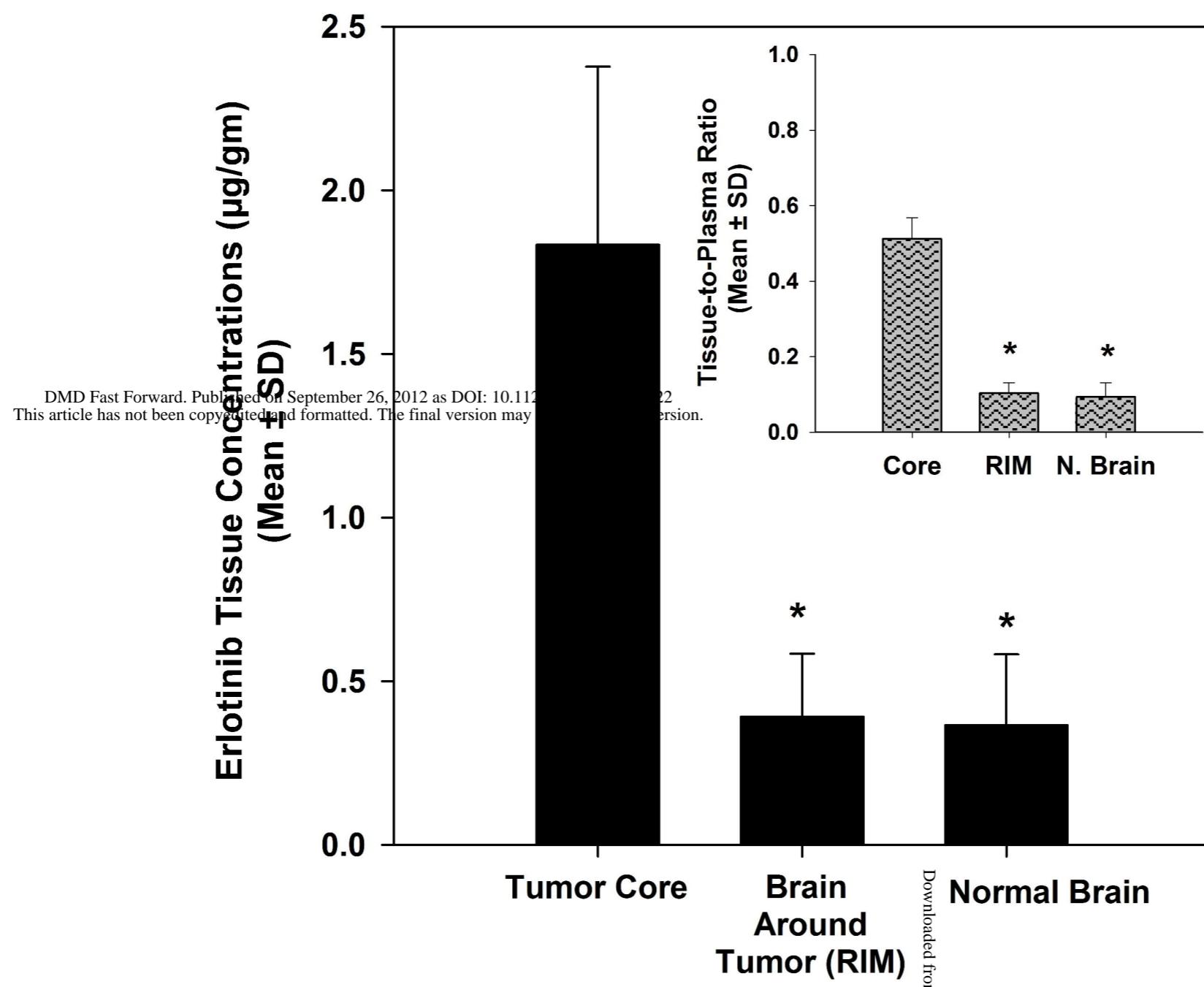
# Figure 1



## Figure 2



## Figure 3



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## Figure 4

