Tyrosine Kinase Inhibitor Gefitinib Enhances Topotecan Penetration of Gliomas

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Abstract

Gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, increases brain parenchymal extracellular fluid (ECF) accumulation of topotecan, a substrate of the ATP-binding cassette (ABC) transporters P-glycoprotein (Pgp/MDR-1) and breast cancer resistance protein (BCRP/ABCG2). The effect of modulating these transporters on topotecan penetration in gliomas has not been thoroughly studied. Thus, we performed intracerebral microdialysis on mice bearing orthotopic human gliomas (U87 and MT330) and assessed topotecan tumor ECF (tECF) penetration and the effect of gefitinib on topotecan tECF penetration and intratumor topotecan distribution. We found that topotecan penetration ($P_{tumor}$) of U87 was $0.96 \pm 0.25$ ($n = 7$) compared with that of contralateral brain ($P_{contralateral}$, $0.42 \pm 0.11$, $n = 5$; $P = 0.001$). In MT330 tumors, $P_{tumor}$ ($0.78 \pm 0.26$, $n = 6$) and $P_{contralateral}$ ($0.42 \pm 0.11$, $n = 5$) also differed significantly ($P = 0.013$). Because both tumor models had disrupted blood-brain barriers and similar $P_{tumor}$ values, we used U87 and a steady-state drug administration approach to characterize the effect of gefitinib on topotecan $P_{tumor}$. At equivalent plasma topotecan exposures, we found that $P_{tumor}$ after gefitinib administration was lower. In a separate cohort of animals, we determined the volume of distribution of unbound topotecan in tumor ($V_{tumor}$) and found that it was significantly higher in groups receiving gefitinib, implying that gefitinib administration leads to a greater proportion of intracellular topotecan. Our results provide crucial insights into the role that transporters play in central nervous system drug penetration and provide a better understanding of the effect of coadministration of transporter modulators on anticancer drug distribution within a tumor. Cancer Res; 70(11); 4499–508. ©2010 AACR.

Introduction

Most central nervous system (CNS) tumors, particularly gliomas, are resistant to chemotherapy (1). Drug resistance in brain tumors is not well understood but may involve poor tumor cell exposure (2), which can be caused by several mechanisms. One important mechanism involves active drug efflux from the endothelial cells forming the blood-brain barrier (BBB) by specific ATP-binding cassette (ABC) transporters (3). The irregular distribution of tumor vessels, the absence of lymphatics, and high oncotic pressure can also impede drug penetration to tumor cells distant from vessels (2). Further, some tumor cells can actively export xenobiotics via ABC transporters at the cell membrane (4). Intracellular drug exposure is crucial to the effectiveness of agents such as methotrexate (antimetabolite), paclitaxel (tubulin inhibitor), and the camptothecins (topoisomerase I inhibitors), to which cells overexpressing specific ABC transporters acquire resistance (5–7).

The semisynthetic camptothecin analogue topotecan is active against xenografted human CNS tumors (8, 9) but shows little efficacy against high-grade gliomas in clinical trials (10). Poor drug penetration may contribute to this outcome. We and others observed that topotecan penetration of brain parenchyma is restricted by at least two ABC transporters: breast cancer resistance protein (BCRP/ABCG2) and P-glycoprotein (Pgp/MDR1/ABCB1; refs. 11, 12). Further, we found that the tyrosine kinase inhibitor (TKI) gefitinib increased topotecan penetration into the brain extracellular fluid (ECF; refs. 11, 13). Gefitinib, an epidermal growth factor receptor inhibitor, also increased the bioavailability of oral irinotecan in mice (14) and humans (15) and reversed tumor cell resistance to topotecan and irinotecan (7, 16), likely via inhibition of BCRP and P-glycoprotein (Pgp/MDR1/ABCB1; refs. 11, 12). Several other TKIs are modulators of ABC transporters and have been studied, in combination with transporter-substrate chemotherapy agents, to overcome tumor drug resistance or to increase drug bioavailability (17–20).

The effect of ABC transporter inhibition on drug penetration of orthotopic brain tumor models is unknown. The BBB remains functionally intact at the margins of invasive brain tumors, where malignant cells migrate through vessel co-option to adjacent tissues (21, 22); therefore, inhibitors of active drug
efflux at the BBB may increase drug concentration in the tumor ECF (tECF; ref. 11). Efflux transporters have also been found in the neovasculature of the glioma tumor bulk (23, 24). However, because tumor-secreted angiogenic molecules (e.g., vascular endothelial growth factor) increase the permeability of vessels within the tumor bulk (25), it is unclear whether the activity of efflux transporters (and their inhibitors) is significant in these hyperpermeable tumor regions. Finally, if the tumor itself expresses ABC transporters, an inhibitor could act at the tumor cell level to increase topotecan intracellular penetration. Here, we used a combined microdialysis-tumor homogenate technique to compare the distribution of topotecan in the tECF and cells of orthotopic glioma xenografts in the presence versus absence of the pump inhibitor gefitinib. We hypothesized that gefitinib would increase intracellular drug accumulation in ABC transporter-expressing tumors with a permeable BBB by modulating cellular drug efflux.

Materials and Methods

Tumor lines

Two human glioma cell lines were used: U-87 MG (U87) and MT330. The U87 model has been extensively described (26). MT330 was created from a WHO grade 4 glioma sample obtained from a patient at University Methodist Hospital (Memphis, TN) as part of an Institutional Review Board–approved protocol. Both tumor lines, modified to stably express firefly luciferase (27), were donated by Dr. Andrew Davidoff (St. Jude Children’s Research Hospital; Memphis, TN).

Orthotopic tumor xenografts

Animal procedures were approved by the St. Jude Animal Care and Use Committee. CD1 athymic nude mice (8–12 wk old; Charles River Laboratories) were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine and injected i.p. with 120 mg/kg D-luciferin firefly (potassium salt; Caliper Lifesciences) and imaged under isoflurane anesthesia (IVIS-100 imaging system, Xenogen; ref. 29). Briefly, mice were injected i.p. with 120 mg/kg D-luciferin firefly (potassium salt; Caliper Lifesciences) and imaged under isoflurane anesthesia after 5 minutes. Tumor vascular permeability was assessed by contrast-enhanced magnetic resonance imaging (CE-MRI; see Supplementary Data for details).

Topotecan penetration of tECF

The concentration-time profiles of unconjugated drugs in tissue ECF can be characterized by microdialysis sampling coupled with an analytic method (30). We used this technique to measure the penetration of unconjugated topotecan lactone into tumor or brain ECF after a single i.v. injection of topotecan, as previously described (31). Briefly, a microdialysis guide cannula (MD-2255, Bioanalytical Systems) was inserted through the skull surface with dental cement and two screw anchors (MF-5182, Bioanalytical Systems). Microdialysis experiments were performed 14 to 21 days (U87) or 45 to 65 days (MT330) after tumor implantation in animals whose intracranial bioluminescence signal was at least 10⁸ photons per second or (in control animals) at least 3 days after implantation of the cannuila. On the day of the experiment, a microdialysis probe (MMB-1-5, Bioanalytical Systems) was inserted through the cannula, artificial cerebrospinal fluid (aCSF; ref. 31) was perfused at 0.5 μL/min, and the probe membrane was allowed to equilibrate for 1 hour. Topotecan (4 mg/kg) was then administered, and dialysate samples were collected every 18 minutes. Samples were analyzed for topotecan lactone and carboxylate with an automated microbore high-performance liquid chromatography (HPLC) fluorescence detection system until concentrations were unmeasurable (31). Probe recovery was calculated by retrodialysis as previously described (31). Using a previously described limited sampling strategy (13), we bled each mouse from the retro-orbital plexus 15, 60, and 180 minutes after the i.v. injection to assay plasma topotecan lactone. The unbound plasma topotecan concentration was calculated on the basis of a previous study (13), showing a 27% unbound fraction in CD1 nude mice. Unbound plasma topotecan levels were modeled by maximum a priori probability Bayesian estimation with ADAPT 5 software (Biomedical Simulations Resource; ref. 32). The prior parameter distribution was calculated by nonlinear mixed-effects modeling (NONMEM version VI; ref 33) in an independent group of mice receiving the same topotecan dose, as previously described (11).

The simulated concentration-time data from the ADAPT 5 model estimates were integrated to obtain the area under the concentration-time curve defining plasma exposure to unbound topotecan lactone (AUC_u,plasma). Similarly, unbound drug exposure in the dialyzed tissue (brain or tumor) ECF (AUC_u,ECF) was determined in each mouse (after correcting for probe recovery as previously described; ref. 31) by three-compartment analysis of combined plasma and tissue topotecan lactone pharmacokinetic data (11). Finally, the extent of penetration of the targeted tissue by unbound topotecan lactone (P_tissue) was calculated as the tissue (brain or tumor) ECF-to-plasma AUC ratio (34):

\[
P_{\text{tissue}} = \frac{\text{AUC}_{u,\text{tissueECF}}}{\text{AUC}_{u,\text{plasma}}}\]

The probe location was confirmed histologically at the end of all microdialysis experiments. In studies targeting tumor tissue, animals that had the probe outside the tumor were excluded from the analysis.

Western blots and immunohistochemistry for ABC transporters

Western blots of U87, MT330, and control cell lysates (Saos-2-BCRP, LLC-PK1-MDR1, LS180-MRP1, and Saos-2-MRP4, stably transfected with BCRP, Pgp, MRP1, and MRP4, respectively) were performed following previously published methods (35). Membranes were incubated with 1:400 anti-BCRP (Bxp-21; Kamiya Biomedical), 1:400 anti-Pgp (C219), 1:1,000 anti-MRP1

Kamiya Biomedical, 1:400 anti-Pgp (C219), 1:1,000 anti-MRP1...
A sample was obtained to characterize unbound next morning (12–penetration of tECF (was then reduced to 0.5 the back, and the incision was sutured. The aCSF flow rate saline (3 h, 37°C) was surgically inserted under the skin of ane, a topotecan-loaded pump previously primed in 0.9% aCSF at 2 6 and after (mean concentration of dialysate samples collected fraction in the tECF, we applied the tumor tissue components, in relation to the unbound drug the intracellular compartment or nonspecifically bound to

**Intratumor distribution of topotecan**

To determine the proportion of drug either entrapped in the intracellular compartment or nonspecifically bound to tumor tissue components, in relation to the unbound drug fraction in the tECF, we applied the "unbound drug volume of distribution in tumor" (V_u,tumor) parameter, adapted from Wang and Welty (36). V_u,tumor describes the relationship, at the steady state, between the total drug concentration in the tumor (assayed in homogenized tissue) and the unbound drug concentration in tECF (calculated by microdialysis). V_u,tumor is measured in mL/g tumor:

\[
V_{u,tumor} = \frac{A_{tot,tumor} - V_{tot,blood} \times C_{tot,blood}}{C_{ss,tECF}}
\]

where A_{tot,tumor} is the total quantity of drug per gram of tumor homogenate (including blood present in the tumor), V_{blood} is the volume of blood per gram of tumor, C_{tot,blood} is the total concentration of drug in blood, and C_{ss,tECF} is the measured concentration of unbound drug in the tECF.

We obtained experimental A_{tot,tumor} and C_{tot,blood} data by performing studies in additional groups of mice bearing U87 tumors using timing similar to that described above. One group, TPT25 (n = 8), received pump-infused topotecan at a constant rate of 25 μg/h. These animals were sacrificed 8 to 12 hours after insertion of the pump (at steady state), and blood and tumor tissues were harvested for HPLC drug assays, performed as already published (37). The effect of gefitinib on the V_u,tumor parameter was assessed in two additional groups of U87 tumor-bearing mice. The TPT25-GEF group (n = 7) received topotecan at 25 μg/h and received oral gefitinib (200 mg/kg) 8 hours before sacrifice. The TPT12-GEF group (n = 6) received an adjusted dose of topotecan (12.5 μg/h) to achieve systemic exposure (C_{ss,plasma}) comparable with that in the TPT25 group and received oral gefitinib (200 mg/kg) 8 hours before sacrifice. Tumors were immediately frozen and stored at ~80°C. For drug analysis, tumors were weighed and homogenized ultrasonically (CP-50, Cole-Parmer) at 4°C in water (10 μL/mg of tumor). The tumor suspension (200 μL) was mixed vigorously with 800 μL of cold methanol and centrifuged for 10 minutes at 14,000 rpm at 4°C. The supernatant was filtered in Spin-X Centrifuge filter tubes (Costar) at 14,000 rpm at 4°C for 10 minutes. Filters were stored at ~80°C for HPLC assay.

To estimate the V_{u,tumor} parameter in the TPT25 and TPT25-GEF groups, we used the mean C_{ss,tECF} values obtained in the previous steady-state microdialysis experiments. The value V_{blood} for intracranial U87 tumors in mice was obtained from the literature (4,1%; ref. 38). In the TPT2-GEF group, we performed a new set of microdialysis experiments (n = 3) to obtain the C_{ss,tECF} value (as described above) in animals bearing U87 tumors and colocalized microdialysis cannulae; in this group, gefitinib (200 mg/kg, oral) was administered 30 minutes before insertion of a pump dispensing 12.5 μg/h of topotecan. Dialysate samples were collected 8 to 10 hours after insertion, and a blood sample was obtained 8 hours after gefitinib administration.

**Intracellular accumulation of topotecan in vitro**

Cells (5 × 10^5 per well in 2 mL medium) were incubated overnight in six-well tissue culture plates (Becton Dickenson, and 2 mL of medium containing topotecan (final concentration, 1 μmol/L) with or without gefitinib (final concentration, 10 μmol/L) were then added. After incubation at 37°C for 1, 5, or 10 minutes, the medium was rapidly aspirated to terminate drug accumulation, and the wells were washed with ice-cold PBS (2 × 5 mL). Ice-cold water was added (500 μL/well), and cell suspensions were transferred to microcentrifuge tubes on ice. Samples were suspended ultrasonically, and 100 μL of suspension were added to 400 μL of cold methanol. The mixture was mixed vigorously and centrifuged for 10 minutes at 14,000 rpm at 4°C. The supernatant was filtered in a Costar Spin-X
Centrifuge filter tube at 14,000 rpm at 4°C for 3 minutes. Filtrates were stored at −80°C for HPLC assay. Samples were analyzed by HPLC as described previously (31).

**Drug sensitivity**

The antitumor activity of topotecan, gefitinib, and their combinations was determined in vitro in U87 and MT330 tumor models as previously described (6). After 4-hour incubation, the drug-containing medium was replaced with fresh medium and the proportion of viable cells was determined 4 days later.

**Statistics**

Aggregate data are presented as mean ± SD. Paired t test was used to compare variables in animals before and after a treatment (i.e., gefitinib). Student’s t test and the Mann-Whitney
test (for nonnormally distributed data) were used for nonpaired comparisons of two groups. For the comparison of more than two groups, a one-way ANOVA was performed followed by a post hoc t test with Bonferroni correction.

**Results**

**Glioma morphology and growth in vivo**

We studied drug penetration of gliomas by orthotopically implanting the most frequently used glioma cell line (U87) and a new low-passage primary tumor line (MT330). The tumor cells engrafted in 100% (58 of 58, U87) and 85% (17 of 19, MT330) of inoculated animals. U87 tumors, as previously described (39), were round and noninfiltrative (Fig. 1A); had profuse, uniform vascularization; and were easily dissected from surrounding brain under a dissecting microscope. In contrast, MT330 tumors infiltrated contiguous brain parenchyma, had irregular edges, had inhomogeneous core vasculature, and could be dissected only by using a bioluminescence-guided technique.

Tumor growth, as measured by increased bioluminescence signal (29), was also dissimilar (Fig. 1B): U87 tumors were >50 mm³ (assessed by terminal dissection) 2 to 3 weeks after implantation, and most animals showed neurologic signs (altered balance and lethargy) within 3 weeks. MT330 tumors grew slowly for several weeks before reaching the luminescence intensity and tumor volume (47 ± 14 mm³ as assessed by MRI, as specified in the Supplementary Data, at week 15; n = 5 mice) of U87 tumors.

**Permeability of the tumor vasculature**

We chose to characterize the BBB of our tumor models using a CE-MRI approach. If the BBB were disrupted, CNS drug penetration would be altered and it would be possible to assess the effect of ABC transporter inhibitors at the level of tumor cells (not tumor vessel). Using CE-MRI, we characterized the vascular integrity of both U87 and MT330, and images showed significant contrast enhancement, which confirmed an impaired BBB (Supplementary Fig. S1A). Homogeneous enhancement was observed in U87 tumors, significantly greater than enhancement in the contralateral brain tissue (Supplementary Fig. S1B). Contrast in MT330 tumors was inhomogeneous, with areas of high signal intensity that may have indicated heavy vascularization and/or necrosis.

**Topotecan penetration of tECF**

We anticipated greater drug concentrations within U87 and MT330 tECF than in normal brain tissue ECF because of the greater vascular permeability of the tumors. Our previous microdialysis studies showed the exposure of brain ECF to unconjugated topotecan lactone to be 21% to 36% that of plasma after a 4 mg/kg i.v. bolus injection in FVB mice (11, 13). Here, using an identical experimental setup, we first evaluated brain ECF penetration in nontumor-bearing CD1 nude mice; results were within the range previously reported (Pbrain = 0.30 ± 0.11, n = 7). We then compared tECF versus contralateral brain ECF penetration in animals bearing U87 and MT330 tumors. Unbound drug penetration of U87 tECF (Ptumor = 0.96 ± 0.25, n = 7) was 2.3 times that of contralateral brain ECF Pcontralateral = 0.42 ± 0.11, n = 5; P = 0.001, t test; Fig. 2A). In MT330 tumors, P tumor (0.78 ± 0.26, n = 6) and Pcontralateral (0.42 ± 0.11, n = 5) also differed significantly (2.2-fold; P = 0.013, t test). Pcontralateral values did not differ statistically from P brain values in the nontumor-bearing mice. Tumor targeting (% experiments with probe in tumor) was more...
accurate in the U87 model (88%) than in the MT330 model (55%). Figure 2B shows representative experiments.

**Expression of ABC transporters in tumor lines and xenografts**

Because one of our objectives was to evaluate the effect of inhibition of tumor cell ABC transporters on drug penetration, we examined the presence of these transporters by Western blot (in the cell lines) and immunohistochemistry (in the xenografts). As shown in Fig. 3A, BCRP, MRP1, and MRP4 were present in both tumor lines, whereas Pgp was not detected. In the U87 and MT330 xenografts, BCRP and MRP1 were strongly expressed, and Pgp and MRP4 were expressed moderately or weakly (Fig. 3B).

**Gefitinib shifts intratumor topotecan distribution toward the intracellular compartment**

To describe drug distribution in the tECF, vascular, and intracellular tumor compartments, we maintained a constant plasma drug concentration, determined the $C_{\text{ss,tecf}}/C_{\text{ss,plasma}}$ ratio ($P_{\text{tumor}}$), and estimated intratumor distribution ($V_{\text{u,tumor}}$) by using combined data sets obtained from microdialysis and homogenate samples. This strategy allowed us to (a) use fewer animals than studies using i.v. injections, which require serial tissue samples; (b) characterize the real-time effects of gefitinib on topotecan pharmacokinetics; and (c) achieve equivalent systemic exposures in animal groups that did and did not receive gefitinib.

We previously showed that gefitinib (likely through its interaction with ABC transporters) halves topotecan lactone plasma clearance (11, 13). In our steady-state studies, $C_{\text{ss,plasma}}$ (unbound lactone) was $34 \pm 8 \text{ ng/mL (}n = 15\text{)}$ after a 25 $\mu$g/h topotecan infusion but increased to $109 \pm 42 \text{ ng/mL after administration of gefitinib (}n = 15; \ P < 0.001\text{)}$. Figure 4A displays plasma unbound topotecan lactone concentration in all experiments in which gefitinib was administered 14 hours after topotecan pump insertion. In the TPT12-GEF group ($n = 6$), which received 200 mg/kg gefitinib before pump insertion, we corrected the topotecan dosage to 12.5 $\mu$g/h and achieved plasma exposure ($C_{\text{ss,plasma}}$) $33 \pm 2 \text{ ng/mL}$ similar to that in animals receiving 25 $\mu$g/mL without gefitinib.

We used the U87 model to characterize intratumor drug distribution with the steady-state approach because these tumors are easily identified and dissected, grow faster and more consistently, and have an ABC transporter expression profile similar to that of MT330 tumors. We first performed microdialysis experiments to define topotecan $C_{\text{ss,tecf}}$. After a 25 $\mu$g/h topotecan infusion, the mean $C_{\text{ss,tecf}}$ was $22 \pm 10 \text{ ng/mL (}n = 10\text{)}$ after administration of gefitinib (200 mg/kg), the mean $C_{\text{ss,tecf}}$ increased to $30 \pm 12 \text{ ng/mL (}n = 10; \ P = 0.019, \text{paired }t\text{ test)}$. Figure 4B shows a representative experiment in a single animal.

Microdialysis experiments were also performed in the TPT12-GEF group, which received 12.5 $\mu$g/h topotecan after 200 mg/kg gefitinib. $C_{\text{ss,tecf}}$ ($6.4 \pm 0.8 \text{ ng/mL, }n = 3$) was significantly lower than that in animals that had not received gefitinib ($P = 0.014, \text{Mann-Whitney test)}$.

When $P_{\text{tumor}}$ (i.e., $C_{\text{ss,tecf}}/C_{\text{ss,plasma}}$ ratio) was calculated, we observed lower ratios in both groups of animals receiving gefitinib than in the TPT25 group (without gefitinib; Fig. 4C). We hypothesized that gefitinib might have depleted
topotecan lactone from the tECF and induced drug accumulation in the intracellular compartment, likely by inhibiting ABC transporters in the tumor cells.

To test our hypothesis, we calculated the volume of distribution of unbound drug in tumor (\(V_{\text{u,tumor}}\)) to describe intratumor drug distribution \textit{in vivo}. New studies in U87 tumor-bearing mice provided the total (bound + unbound) topotecan lactone concentration at the steady-state in tumor homogenates (\(C_{\text{tot,tumor}}\), 47 ± 21, 142 ± 67, and 43 ± 17 ng/mg tumor) and blood (\(C_{\text{tot,blood}}\), 105 ± 33, 219 ± 81, and 109 ± 8 ng/mL) of the TPT25 (\(n = 8\)), TPT25-GEF (\(n = 7\)), and TPT12-GEF (\(n = 6\)) groups, respectively. The mean unbound topotecan \(C_{\text{u,tumor}}\) from the microdialysis experiments were then used to calculate \(V_{\text{u,tumor}}\). As shown in Fig. 5A, \(V_{\text{u,tumor}}\) was significantly higher in groups receiving gefitinib. Thus, after administration of gefitinib, a greater proportion of drug was intracellular or nonspecifically bound to tumor tissue components rather than as unbound drug in the tECF.

To further characterize intracellular topotecan accumulation in the presence of gefitinib, we incubated U87 and MT330 cells with topotecan (0.1 \(\mu\)mol/L) and gefitinib (1 or 10 \(\mu\)mol/L). Topotecan accumulation was enhanced as much as 36% by gefitinib in both U87 and MT330 cells in a dose-dependent manner (Fig. 5B). This finding supports the shift of topotecan distribution toward the intracellular compartment, likely by inhibiting ABC transporters in the tumor cells.

Effect of gefitinib on tumor cell sensitivity to topotecan

To assess whether gefitinib sensitizes tumor cells to topotecan, we measured the growth of U87 and MT330 cells with topotecan (0.1 \(\mu\)mol/L) and gefitinib (1 or 10 \(\mu\)mol/L). Topotecan accumulation was enhanced as much as 36% by gefitinib in both U87 and MT330 cells in a dose-dependent manner (Fig. 5B). This finding supports the shift of topotecan distribution toward the intracellular compartment in the presence of gefitinib.

Discussion

The ability of selected TKIs to enhance drug penetration of tumors by inhibiting ABC transporters \textit{in vivo} is not completely understood. Here, we used two glioma models—a cell line (U87) and a primary culture (MT330)—to assess the effect of the TKI gefitinib on the tumor penetration and intratumor distribution of topotecan both \textit{in vitro} and in orthotopic xenografts in CD1 athymic nude mice. Our results show that the pharmacologic interaction of the drugs results in increased intracellular tumor exposure to topotecan. The experimental model we used provides a novel method of assessing the effect of a modulator of drug efflux on the intratumor distribution of a chemotherapy agent in orthotopically engrafted brain tumors.

Our previous studies of topotecan-gefitinib and irinotecan-gefitinib combinations in transporter-overexpressing cells (6, 14, 35) and in brain penetration analyses (11, 13) showed that gefitinib inhibits at least two topotecan transporters: BCRP and Pgp. The inhibition of these two transporters in brain tumors could affect drug efflux at two levels: BBB vessels and tumor cells. We previously showed in an intact BBB...
model that gefitinib enhances drug penetration of the BBB (11, 13); in the present study, we focused on its effect at the tumor cell level. Therefore, we selected tumor models (U87 and MT330) that show increased vascular permeability (altered BBB) and ABC transporter expression. The U87 tumor vasculature does express multidrug resistance proteins like Pgp that may act as drug efflux pumps in intracerebral tumor models (23, 40). However, our contrast MRI (Supplementary Fig. S1) and microdialysis (Fig. 2) results, which confirm the increased permeability of the U87 tumor vessels, call into question the activity of this or other putative transporters at the tumor vessel level. At the cellular level, we found abundant BCRP expression in U87 and MT330 lines, consistent with clinical findings in gliomas (41). Topotecan is also a substrate of MRP4 (35) and likely of MRP1 (42), both of which we found in U87 and MT330 tumor lines (Fig. 3). To our knowledge, gefitinib inhibition of MRP4 and MRP1 has not been studied in detail, although a similar compound, erlotinib, did not alter MRP1-related drug resistance (17). Therefore, the activity of MRP1 and MRP4 in our tumor lines may at least partially explain their relatively high resistance to topotecan even in the presence of gefitinib.

The steady-state experimental approach allowed us to observe the real-time effects of gefitinib on the topotecan tECF-to-plasma concentration ratio in individual animals. The decrease in this ratio after gefitinib administration was confirmed in a group of gefitinib-exposed animals (TPT12-gef) whose topotecan dose was reduced to achieve systemic exposure equivalent to that achieved without gefitinib at a higher dosage (25 µg/h; equivalent plasma exposure helped to minimize the effect in which higher plasma levels can increase drug penetration of the intracellular compartment). These observations prompted us to investigate whether inhibition of transporters at the tumor cell level would drive drug distribution toward the tumor intracellular compartment. Data from our in vivo studies of homogenate-microdialysis concentration and from our in vitro studies of intracellular accumulation supported this shift in topotecan intratumor distribution. We did not evaluate intratumor gefitinib concentration, although good penetration was reported in subcutaneous glioma xenografts (gefitinib concentration >20 µmol/L for 8 h after a single oral dose of 55 mg/kg; ref. 43). In patients with gliomas who received gefitinib (500 mg, orally for 5 d) before tumor resection, gefitinib C_{ss} was 7 to 25 µmol/L in the tumor homogenate (44).

We attempted the steady-state experimental approach to study the effect of gefitinib on topotecan CNS penetration in nontumor-bearing mice. However, we did not obtain interpretable data from this experiment because the intact BBB limited CNS drug penetration in a way that, with our bioanalytic system, we could not detect topotecan in the ECF dialysates collected before the administration of gefitinib.

![Figure 5. Effect of gefitinib on topotecan accumulation in tumor xenografts in vivo and in tumor cells in vitro. A, V_{tumors} values at steady state in U87 tumors from mice receiving 25 µg/h topotecan (TPT25), 25 µg/h topotecan and 200 mg/kg gefitinib (TPT25-gef), or 12.5 µg/h topotecan and 200 mg/kg gefitinib (TPT12-gef; plasma exposure equivalent to that in the TPT25 group). Points, mean; bars, SD. *, P = 0.044; **, P = 0.002, one-way ANOVA, post hoc t test with Bonferroni correction. B, topotecan (TPT) accumulation in tumor cells in vitro in the presence and absence of gefitinib. Values are the percentage of the maximum accumulation in control cells (0.1 µmol/L topotecan, no gefitinib) at each time point. Points, mean (n = 3); bars, SD. *, P < 0.05; **, P < 0.01, compared with accumulation in control cells at the same time point, one-way ANOVA, post hoc t test with Bonferroni correction.](image-url)
A plausible reason for this limitation of the infusion approach is that slow drug infusions penetrate the intact CNS less efficiently compared with rapid bolus injections likely because the plasma concentration driving drug diffusion across the BBB is much lower in the case of the infusions (45). Increasing the infusion rate to achieve elevated plasma $C_{ss}$ in the nontumor-bearing animals might have rendered detectable drug levels in the ECF, but the toxicity of topotecan at such increased plasma $C_{ss}$ precluded performing this study.

We suggest that microdialysis alone is only partially informative in brain tumor drug penetration studies because (a) high drug concentrations in tECF would be expected, given the usually altered BBB, and (b) only drugs in the tECF are analyzed, whereas most chemotherapy agents have intracellular targets. Further, microdialysis measurement of drug concentration in intracranial tumors is complex (40, 46, 47). Microdialysis was recently used elegantly to identify very high methotrexate levels in the tECF of patients’ highly contrasting gliomas, but as mentioned above, these levels do not reveal the tumor cell penetration of the drug (46). New techniques like the one proposed by Langer and colleagues (48), combining positron emission tomography and microdialysis, may overcome the limitations of microdialysis alone and allow assessment of intracellular drug penetration in unresected human tumors.

To conclude, ours is the first report characterizing the role of drug efflux inhibitors in shifting the intratumor distribution of substrate drugs toward the cellular compartment in an orthotopic glioma model. Specific sampling and analysis methods are necessary to assess the intratumor distribution of anticancer drugs. Future studies to bring these concepts into the preclinical and clinical fields are warranted. One example is the use of irinotecan in combination with the antiangiogenic agent bevacizumab for recurrent glioma (49). As prolonged antiangiogenic therapy is reportedly associated with restoration of the BBB (50), future studies should address how the possible recovery of the BBB and inhibitors of drug efflux affect the penetration of brain tumors by irinotecan.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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