Are Capecitabine and the Active Metabolite 5-FU CNS Penetrable to Treat Breast Cancer Brain Metastasis? 

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ABSTRACT

Brain metastasis (BM) is increasingly diagnosed in Her2 positive breast cancer (BC) patients. Lack of effective treatment to breast cancer brain metastases (BCBMs) is probably due to inability of the current therapeutic agents to cross the blood-brain barrier. The central nervous system (CNS) response rate in BCBM patients was reported to improve from 2.6%–6% (lapatinib) to 20%–65% (lapatinib in combination with capecitabine). Lapatinib is a poor brain penetrant. In this study, we evaluated the CNS penetration of capecitabine and hoped to interpret the mechanism of the improved CNS response from the pharmacokinetic (PK) perspective. Capecitabine does not have antiproliferative activity and 5-fluorouracil (5-FU) is the active metabolite. Capecitabine was orally administered to mouse returning an unbound brain-to-blood ratio (Kp,uu,brain) at 0.13 and cerebrospinal fluid (CSF)-to-unbound blood ratio (Kp,uu,CSF) at 0.29 for 5-FU. Neither free brain nor CSF concentration of 5-FU can achieve antiproliferative concentration for 50% of maximal inhibition of cell proliferation of 4.57 μM. BCBM mice were treated with capecitabine monotherapy or in combination with lapatinib. The Kp,uu,brain value of 5-FU increased to 0.17 in the brain tumor in the presence of lapatinib, which is still far below unity. The calculated free concentration of 5-FU and lapatinib in the brain tumor did not reach the antiproliferative potency and neither treatment showed antitumor activity in the BCBM mice. The CNS penetration of 5-FU in human was predicted based on the penetration in preclinical brain tumor, CSF, and human PK and the predicted free CNS concentration was below the antiproliferative potency. These results suggest that CNS penetration of 5-FU and lapatinib are not desirable and development of a true CNS penetrable therapeutic agent will further improve the response rate for BCBM.

Introduction

Central nervous system (CNS) metastases are more often diagnosed in breast cancer (BC) patients than before probably because of longer survival with current therapeutic options and improvements in diagnostic techniques (Stemmler and Heinemann, 2008). Patients with BC overexpressing Her2 proto-oncogene have higher risk to develop CNS metastasis than other BC phenotypes (Pestalozzi et al., 2006). In a recent published data, a total of 37% (377/1012) enrolled patients diagnosed as Her2 positive metastatic BC had developed CNS metastasis (Bruinšky et al., 2011). Median survival was significantly shorter for patients with brain metastasis (BM) (26.3 months) than those without BM (44.6 months).

Lapatinib, an epidermal growth factor receptor and Her2 dual inhibitor, was approved in combination with capecitabine to treat advanced or metastatic BCs overexpressing Her2 proto-oncogene. Lapatinib assessed in Her2 positive BCBM patients returned a low CNS response rate at 2.6%–6% (lapatinib) to 20%–65% when the patients were treated with lapatinib in combination with capecitabine (Bartolotti et al., 2013). Chefrour et al. (2012) elucidated the mechanism for the synergistic benefit in a subcutaneous BC mouse model. Their study showed that lapatinib and capecitabine modulate each other’s molecular determinants of response and that concomitant dosing seems to be the optimal way for the combination treatment in the subcutaneous BC mouse model. However, the mechanism of improved clinical objective CNS response rate after the combination of lapatinib and capecitabine has not been studied from the pharmacokinetic (PK) perspective. Lapatinib is a substrate of P-gp and BCRP, the two major efflux transporters localized on the luminal side of the blood-brain barrier (BBB) (Polli et al., 2008). The passive permeability of lapatinib studied in the MDCK cell line was 0.27–1.1 × 10⁻⁶ cm/s, which was far below the desired permeability (>15 × 10⁻⁶ cm/s) for CNS drugs (Mahr Doan et al., 2002; Wager et al., 2010). The total brain-to-blood ratio (Kp,brain) of lapatinib was only 0.04 in normal mice, which was improved to 1.7 in mdr1a(b¹/b¹)/bcrp(¹/¹) knockout mice, suggesting that efflux transporters limited penetration of lapatinib into the CNS (Polli et al., 2009).

The CNS penetration of capecitabine and its active metabolite(s) are not well understood after oral administration of capecitabine, in the presence and absence of lapatinib. Capecitabine is a prodrug and sequentially metabolized to 5'-DFCR, 5'-DFUR, and finally to ABBREVIATIONS: AUC, area under curve; BBB, blood-brain barrier; BC, breast cancer; BM, brain metastasis; CNS, central nervous system; CSF, cerebrospinal fluid; DMSO, dimethylsulfoxide; ER, efflux ratio; 5-FU, 5-fluorouracil; Glfs, concentration for 50% of maximal inhibition of cell proliferation; Kp,brain, brain-to-blood ratio; Kp,CSF, cerebrospinal fluid-to-blood ratio; Kp,uu,brain, unbound brain-to-blood ratio; Kp,uu,CSF, cerebrospinal fluid-to-unbound blood ratio; LM, leptomeningeal metastasis; MTD, maximal tolerable dose; PK, pharmacokinetic.
5-fluorouracil (5-FU) by carboxylesterase, cytidine deaminase, and thymidine phosphorylase in liver and tumor (Onodera et al., 2000; Tsukamoto et al., 2001a,b; Desmoulin et al., 2002). The in vitro antiproliferative potency [concentration for 50% of maximal inhibition of cell proliferation (GI50)] of capcetabine and the metabolites was evaluated in Her2 BT474C1 cells. Capcetabine and its active metabolite 5-FU were assessed in MDCKII-MDR1 and MDCKII-BCRP transport assays because efflux liability and passive permeability are two critical parameters in CNS drug delivery.

We initially evaluated the CNS penetration of capcetabine and 5-FU in wild-type mice. Subsequently, we investigated the CNS penetration of capcetabine and 5-FU in brain tumor and adjacent normal brain tissue in BCBM xenograft model mice when capcetabine was orally dosed alone at the maximal tolerable dose (MTD), 400 mg/kg or in combination with laptatinib at a MTD dose of 75 mg/kg. CNS penetration of Kp,brain and cerebral spinal fluid (CSF)-to-blood ratio (Kp,CSF) were calculated by the area under curve (AUC) ratio. Combining the in vivo unbound fraction in blood and brain homogenates, the unbound brain-to-blood ratio (Kp,unbound,brain), and CSF-to-unbound blood ratio (Kp,unbound,CSF) were estimated as critical parameters in assessing CNS penetration. Efficacy of capcetabine as a single agent or in combination with laptatinib at their corresponding MTD doses was evaluated in BCBM nu/nu mice carrying brain metastatic tumors of Her2-overexpressing BT474C1 cells. The PK-antitumor activity in human was also explored. The concentration of 5-FU in human CNS was predicted based on the CNS penetration in preclinical brain tumor, preclinical CSF, and human plasma PK at the approved clinical dose. Our results indicated that CNS penetration of capcetabine and 5-FU and current Her2 target-specific therapeutic agents are not optimal and development of a true CNS penetrable chemo-agent and Her2 inhibitor are needed to treat BCBM.

Materials and Methods

BT474C1 cells were provided by R&D center Móndal, AstraZeneca. Cell culture medium and reagents were supplied by Invitrogen (Grand Island, NY). Capcetabine, 5'-DFCR, 5'-DFUR, and 5-FU were purchased from Sigma-Aldrich (St. Louis, MO). Laptatinib was synthesized by AstraZeneca. Artificial CSF was purchased from Harvard Apparatus (Holliston, MA). The equilibrium dialysis device and cellulose membranes with molecular weight cutoffs of 12,000 to 14,000 were purchased from HTDialysis, LLC (Gales Ferry, CT).

In Vitro Antiproliferative Potency. The antiproliferative activity was evaluated by using Hoechst and the propidium iodide staining assay (Yu et al., 2012). Growth inhibition, by Prism (GraphPad Software, San Deigo, CA).

Determination of Unbound Fraction in Mouse Blood, Brain Homogamate, Cell Culture Medium, and Human Plasma. The in vitro unbound fraction of capcetabine, 5-FU, and laptatinib in mouse blood, brain homogenate, cell culture medium (10% fetal bovine serum in the medium) and human plasma was determined by using an equilibrium dialysis device. Blood and brain were collected from nu/nu mice. Blood was diluted with 1× Dulbecco’s phosphate-buffered saline (DPBS). Brain was homogenized with 3× DPBS. Diluted blood or brain homogenate was spiked with capcetabine or 5-FU to a final concentration of 5 μM. Cell culture medium or human plasma was spiked with 5-FU to a final concentration of 5 μM. The dialysis apparatus was maintained on a rotator (set at 300 rpm) at 37°C for 4 hours. The unbound fractions (fu) were corrected using eq. 3 to yield an estimate of fu in the blood, brain, cell culture medium, or plasma.

Materials and Methods

BT474C1 cells were provided by R&D center Móndal, AstraZeneca. Cell culture medium and reagents were supplied by Invitrogen (Grand Island, NY). Capcetabine, 5'-DFCR, 5'-DFUR, and 5-FU were purchased from Sigma-Aldrich (St. Louis, MO). Laptatinib was synthesized by AstraZeneca. Artificial CSF was purchased from Harvard Apparatus (Holliston, MA). The equilibrium dialysis device and cellulose membranes with molecular weight cutoffs of 12,000 to 14,000 were purchased from HTDialysis, LLC (Gales Ferry, CT).

In Vitro Antiproliferative Potency. The antiproliferative activity was evaluated by using Hoechst and the propidium iodide staining assay (Yu et al., 2014). In brief, cells were seeded in a 96-well plate at 6 × 10³ cells/well for 24 hours prior to treatment. Capcetabine, 5’-DFCR, 5’-DFUR, and 5-FU were dissolved in dimethylsulfoxide (DMSO) to obtain a 10 mM stock solution followed by serial dilution to prepare dosing solutions. The final concentration of DMSO used in the corresponding wells did not exceed 0.3% (v/v). Control cells were treated with the same volume of DMSO. All experiments were performed in triplicate. After 72 hours of treatment, cells were incubated with 10 M of Hoechst 34580 (Invitrogen) and 1.5 M of propidium iodide for 30 minutes, and then detected by Acumen × 3 (TTP). Viability was expressed as a percentage relative to the vehicle controls. The concentration-response curve was conducted to obtain the GI50 value, the concentration at 50% of growth inhibition, by Prism (GraphPad Software, San Deigo, CA).

Determination of Efflux Transport in MDCKII-MDR1 and MDCKII-BCRP Cell Lines. In vitro human P-gp or BCRP transport assays were performed as described previously (Wang et al., 2005). In brief, cells were seeded onto polycarbonate Transwell filter membranes (Millipore Corporation, Billerica, MA) at a density ≈450,000 cells/cm² and monolayers were ready for studies 3 days later. Capcetabine and 5-FU were dissolved at 10 mM in DMSO and diluted to 1 μM for test. Each determination was performed in duplicate. Membrane transfer of Lucifer yellow was also measured for each monolayer to assess the integrity of the cell monolayers. All samples were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). The passive permeability (Pexact) was assessed in the presence of GF120918 according to eq. 1 (Tran et al., 2004) and percent recovery was calculated according to eq. 2.

\[
P_{\text{exact}} = \frac{-(V_d \times V_c)}{(V_d + V_c)}/A/t \times \ln \left[1 - \frac{(V_d + V_c) \times C_i}{(V_d \times C_d + V_c \times C_i)}\right]
\]

Recovery % = 100 × \left[\frac{(V_c \times C_i) + (V_d \times C_d)}{(V_d \times C_d)}\right]

where Vd is the volume in the donor chambers (0.1 ml in A to B, 0.3 ml in B to A); Vc is the volume in the receiver chambers (0.3 ml in A to B, 0.1 ml in B to A); Cc and Cd are the final concentrations of transport compound in donor and receiver chambers, respectively; Cc is the initial concentration in the donor chamber (μM), and A is the surface area for the transport, i.e., 0.11 cm²

Determination of Unbound Fraction in Mouse Blood, Brain Homogmate, Cell Culture Medium, and Human Plasma. The in vitro unbound fraction of capcetabine, 5-FU, and laptatinib in mouse blood, brain homogenate, cell culture medium (10% fetal bovine serum in the medium) and human plasma was determined by using an equilibrium dialysis device. Blood and brain were collected from nu/nu mice. Blood was diluted with 1× Dulbecco’s phosphate-buffered saline (DPBS). Brain was homogenized with 3× DPBS. Diluted blood or brain homogenate was spiked with capcetabine or 5-FU to a final concentration of 5 μM. Cell culture medium or human plasma was spiked with 5-FU to a final concentration of 5 μM. The dialysis apparatus was maintained on a rotator (set at 300 rpm) at 37°C for 4 hours. The unbound fractions (fu) were corrected using eq. 3 to yield an estimate of fu in the blood, brain, cell culture medium, or plasma.

\[
f_a = 1/\left[D \times (1/f_u - 1)\right]
\]

where D and fu represent the dilution factor and unbound fraction determined in diluted blood or brain homogenate, respectively.

Animals. Female BALB/c mice and female nu/nu mice were obtained from the Vital River Company (Beijing). The animals were allowed acclimation at 3 days under standard environmental conditions with 12:12 hour light/dark cycles. All experimental protocols were approved by the Institute Animal Care and Use Committee at Innovation China Center, AstraZeneca.

BCBM nu/nu Mouse Model. Six-to-eight week old, female nu/nu mice (n = 8 per group; Vital River, Beijing) were intracranially injected with 0.5 × 10⁵ of luciferase transfected BT474C1 cells into the right brain at stereotactic coordinates at 2.5 mm to the right of the bregma and 1 mm anterior to the coronal suture, and 3 mm deep. One week after tumor cell injection, the viable hypoxic tumor was identified by noninvasive bioluminescent imaging (Xenogen, Caliper Life Sciences, Hopkinton, MA). Bioluminescent images were obtained weekly by using a Xenogen small animal imager (IVIS Imaging System) (Xenogen, Caliper Life Science, Hopkinton, MA) equipped with a living image. Mouse body weight was monitored twice a week as a surrogate marker of general toxicity. The mouse was euthanized whenever a 20% loss of initial body weight was reached.

Determination of CNS Penetration in BALB/c and BCBM nu/nu Mice. Capcetabine or laptatinib was formulated in 0.5% hydroxypropyl methylcellulose (aq. (w/v). Capcetabine was orally administered to female BALB/c mice at the MTD dose of 400 mg/kg. Dosing volume was 10 ml/kg. The mouse was sacrificed after isoflurane anesthesia followed by collection of CSF, blood, and brain at 0.83, 0.16, 0.25, 0.5, 1, 2, 4, 7, 16, and 24 hours postdose (n = 3 each time point). After anesthetization, the skin of the neck was shaved and the surgical site was swabbed with iodine followed by 70% ethanol. A sagittal incision of the skin was made inferior to the occiput. The subcutaneous tissue and muscles were separated by blunt dissection with forceps. A pair of microtretors was used to hold the muscle apart. The mouse was laid down so that the head formed a nearly 135° angle with the body. Dura mater at the cisterna magna was exposed and the CSF space was visible. The dura mater was blotted with a sterile cotton swab. Then, the dura mater at cisterna magna was penetrated through by using the indwelling single wing needle (26 G) at a nearly 45° angle with the body. The CSF then flowed into the indwelling single wing needle. The indwelling single wing needle was carefully removed and connected to a 1 ml syringe. The collected CSF samples (8–10 μl from each mouse) were injected into a premarked 0.5 ml Eppendorf tube and frozen immediately in liquid
nitrogen prior to sample analysis. Five microliters of CSF was mixed with same volume of artificial CSF with 5% mouse plasma (Seehusen et al., 2003). Whole blood was mixed with 3× deionized water. Brain was weighted and homogenized with 3× DPBS (w/v). Samples of diluted blood, CSF, and brain homogenate were immediately frozen in liquid nitrogen and subsequently stored at approximately −70°C until analysis.

The area under the concentration-time curve truncated at the last reported concentration (AUC0–t) was calculated by Phoenix 6.2 (Pharsight Corporation, Mountain View, CA). Brain penetration of total Kp,brain and Kp,CSF were calculated by the AUC ratio using eqs. 4 and 5,

\[
K_{p,\text{brain}} = \frac{AUC_{\text{brain}}}{AUC_{\text{blood}}} \quad (4)
\]

\[
K_{p,\text{CSF}} = \frac{AUC_{\text{CSF}}}{AUC_{\text{blood}}} \quad (5)
\]

Combining the in vitro unbound fraction in blood and brain, the Kp,uu,brain and Kp,uu,CSF were estimated by using eqs. 6 and 7,

\[
K_{p,\text{uu,brain}} = K_{p,\text{brain}} \times f_u,\text{brain} \quad / f_u,\text{blood} \quad (6)
\]

\[
K_{p,\text{uu,CSF}} = K_{p,\text{CSF}} \times f_u,\text{blood} \quad (7)
\]

To assess the drug penetration difference between normal brain tissue and brain tumor, BCBM mice were orally dosed with lapatinib (75 mg/kg), and 2 hours after the same animal was orally administered capcitabine at 400 mg/kg. Blood and brain were collected at 1 hour postdose with capecitabine. Brain was harvested and divided into two parts, left brain (normal brain tissue) and right brain (brain tumor), because tumor cells were intracranially injected into the right brain.

**Bioanalytical**. All samples from the in vitro protein binding study; MDCKII-MDR1, MDCKII-BCRP studies; and samples of blood, CSF, and brain homogenates from in vivo studies were processed by deproteination by using acetonitrile containing appropriate internal standards. Analytes were quantified by the API4000 instrument (Applied Biosystems, Foster City, CA) equipped by Agilent HP 1200 series liquid chromatography (Agilent Technologies, Santa Clara, CA) or Waters ACQUITY UPLC integrated system (Waters, Milford, MA) in a multiple reaction monitoring mode. The analytical column (Gemini C18, 4.6 × 150 mm, 5 μm, Torrance, CA); Waters BEH C18, 2.1 × 50 mm, 1.7 μm) was maintained at ambient temperature. The mass transitions used for quantification were m/z 358.1–153.9 for capcitabine, 128.9–422.5 for 5-FU, and 579.1–470.2 for lapatinib in negative mode. Refer to the Supplemental Material for more details.

**In vivo Efficacy Study by Xenogene Imaging**. The antitumor activity was investigated in the BCBM mice carrying xenograft tumors of luciferase transfected Her2 amplified cells. On the day of imaging study, mice were intraperitoneally injected with luciferin (150 mg/kg of luciferin, Xenogen). Ten minutes after luciferin injection, the image was acquired by autoexposure. Mice were orally administered with capcitabine monotherapy or in combination with lapatinib. Animals received an oral dose of lapatinib at 75 mg/kg twice a day, and 2 hours after the first dose of lapatinib the animals received an oral dose of capcitabine at 400 mg/kg every day. The dose used for both drugs was the MTD in mice determined from a pilot study. Lapatinib at 75 mg/kg twice daily is in the range of clinical equivalent dose estimated by equivalent plasma exposure. Capcitabine at 400 mg/kg every day was below the clinical relevant dose due to dose limiting toxicity in mice. The clinical relevant dose of capcitabine at ~1200 mg/kg in mice was estimated by equivalent free plasma exposure of 5-FU. Mice in the control group were administered 0.5% hydroxypropyl methylcellulose at 10 ml/kg.

**Prediction of Human CNS PK for 5-FU**. Kp,uu,CSF is often preserved across species for nontransporter substrates (Di et al., 2013). 5-FU is neither a P-gp nor a BCRP substrate. The free CNS concentration of 5-FU in humans was estimated by using the Kp,uu,brain and Kp,uu,CSF in mice multiplied by the human unbound plasma concentration (Chu et al., 2007).

**Statistical Analysis**. Data are presented as the mean ± S.D. except for the clinical data in Table 4, which are presented as the mean ± 90% confidence interval (CI), and the efficacy data in Fig. 2, which are presented as geometric mean ± S.E. A Student’s two-tailed unpaired t test was used to determine statistical significance. The significance level was P < 0.05.

**Results**

**In Vitro Antiproliferative Potency**. The antiproliferative potency of capcitabine, 5'-DFCR, 5'-DFUR, and 5-FU was evaluated in BT474C1 cells. Capcitabine, 5'-DFCR, and 5'-DFUR did not have antiproliferative activity up to 30 μM. 5-FU was determined as the active metabolite of capcitabine with antiproliferative GI50 at 5.24 ± 2.45 μM (681 ± 319 ng/ml). Both prodrug Capcitabine and its active metabolite 5-FU were selected for further characterization.

**Passive Permeability and Efflux Transport**. The results of passive permeability and efflux transport of capcitabine and 5-FU are given in Table 1. Good recovery (>70%) was observed in all incubations. Capcitabine was found to be a BCRP substrate with an efflux ratio (ER) at 7.44 in the absence of GI20198, which returned to 0.70 in the presence of GI20198. In the MDCKII-MDR1 cell assay, the ER of capcitabine was 1.59 in the absence of GI20198 and 0.46 in the presence of GI20198. The ER difference was 3.46-fold (1.59/0.46), which was greater than 2, suggesting that capcitabine may be a P-gp substrate. 5-FU was determined to be neither a P-gp nor a BCRP substrate because the ER was close to 1 in the absence and presence of GI20198. However, the passive permeability of 5-FU was < 2 × 10⁻⁶ cm/s, which was much lower than the desired permeability (>15 × 10⁻⁶ cm/s) for CNS drugs (Mahar Doan et al., 2002). Low passive permeability indicated that 5-FU would take longer to reach distribution equilibrium between peripheral blood and CNS compartments. In a separate PK study, 5-FU was intravenously administered to mice and the observed total blood clearance was 139 ml/min/kg (~90% mouse hepatic liver blood flow) and Vss of 0.9 l/kg (data not shown). The rapid elimination of 5-FU from the body and low-to-moderate volume of distribution may therefore take away the opportunity of 5-FU from entering the CNS.

**TABLE 1**

Permeability and efflux transport of capcitabine and 5-FU in MDCKII-MDR1 or MDCKII-BCRP cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Assay</th>
<th>GI20198</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P&lt;sub&gt;app&lt;/sub&gt; (× 10⁻⁶ cm/s)</td>
<td>Efflux Ratio</td>
</tr>
<tr>
<td></td>
<td>A &gt; B</td>
<td>B &gt; A</td>
</tr>
<tr>
<td>Capcitabine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDCKII-MDR1</td>
<td>No</td>
<td>3.74 (2.52, 4.96)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3.09 (3.23, 2.86)</td>
</tr>
<tr>
<td>MDCKII-BCRP</td>
<td>No</td>
<td>3.53 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>4.21 (4.02, 4.41)</td>
</tr>
<tr>
<td>5-FU</td>
<td></td>
<td>0.99 ± 0.09</td>
</tr>
<tr>
<td>MDCKII-BCRP</td>
<td>Yes</td>
<td>1.08 ± 0.08</td>
</tr>
<tr>
<td>5-FU</td>
<td>Yes</td>
<td>2.16 (1.20, 3.11)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1.92 (1.75, 2.08)</td>
</tr>
</tbody>
</table>

Data are presented as mean (individual value) or mean ± S.D.
Unbound Fraction in Mouse Blood, Brain Homogenate, Cell Culture Medium, and Human Plasma. The unbound fraction of capecitabine, lapatinib, and 5-FU in mouse blood, brain homogenate, cell culture medium, and human plasma was determined by using equilibrium dialysis. Binding of lapatinib was very high (>99.9%) in all matrices. Given the low molecular weight (mol. wt. = 359 for capecitabine and 130 for 5-FU) and hydrophilic properties (c log P = 0.8 for capecitabine and −0.6 for 5-FU), both compounds showed low binding to blood protein and brain tissue. The fu,blood and fu,brain were 0.571 and 0.446 for capecitabine and 0.479 and 0.378 for 5-FU, respectively. The fu,plasma was 0.951 for 5-FU in human plasma. The unbound fraction of 5-FU in cell culture medium was 0.872. The free in vitro antiproliferative potency of 5-FU was corrected by the unbound fraction in the cell culture medium (0.872) to be 4.57 ± 2.14 μM (594 ± 278 ng/ml).

CNS Penetration in BALB/c and BCBM nu/nu Mice. CNS penetration of capecitabine and 5-FU was assessed after oral administration of capecitabine at the MTD dose of 400 mg/kg to female BALB/c mice. Blood concentration after 2 hours, brain concentration at 0.083 hour and after 2 hours, and CSF concentration at 0.083 hour and after 1 hour for 5-FU were all below the limit of quantification. The AUC values in blood, brain, and CSF were used to calculate Kp,brain and Kp,CSF. Kp,brain was 0.04 and 0.15 and Kp,CSF was 0.13 and 0.13 for capecitabine and 5-FU, respectively. Combining the in vitro unbound fraction in blood and brain homogenate, both Kp,brain and Kp,CSF were calculated to be 0.03 and 0.25 for capecitabine and 0.13 and 0.29 for 5-FU, respectively (Table 2). The brain penetration of 5-FU in our study was comparable to the studies conducted by Bourke et al. (1973) (Kp,brain = 0.09 and Kp,CSF = 0.43 in primates after IV injection of 5-FU-2,14C), Kerr et al. (1984) (Kp,CSF = 0.11–0.48 in monkey after IV injection of 5-FU), and Heier et al. (1986) (Kp,CSF = 0.015–0.06 in human after IV injection of 5'-DFUR). These data suggested that capecitabine and 5-FU had very poor brain penetration and low CSF penetration. After oral administration of capecitabine at 400 mg/kg to mice, the free drug concentrations of 5-FU in relation to its in vitro potency are shown in Fig. 1; neither free brain nor CSF concentration of 5-FU can achieve free in vitro antiproliferative GI50 at 594 ng/ml in the Her2-amplified BT474C1 cell line.

The brain penetration of capecitabine, 5-FU, and lapatinib in brain tumor and adjacent normal brain tissue is shown in Table 3 and the Supplemental Material for checking raw data and calculation. The time point at 1 hour was selected based on the PK study in BALB/c mice. The average Kp,brain of 5-FU at 1 hour was 0.15, which was similar to the Kp,brain at 0.17 calculated by AUC. Luciferase-transfected BT474C1 cells were intracranially injected to the right brain and the immuno-histochemistry imaging results confirmed that the tumor signal almost occupied the whole right brain (data not shown). The brain penetration of capecitabine and lapatinib was generally similar between brain tumor and normal brain tissue. The Kp,brain/free blood concentration ratio of 5-FU in brain tumor was 0.13 (p < 0.05), which is 2.17× higher than that in adjacent normal brain tissue (Kp,brain/free blood = 0.06), when lapatinib was orally administered as a single agent to mice. The improved brain penetration for 5-FU in brain tumor was still low because the Kp,brain/free blood was far below 1. The brain penetration of 5-FU was higher after combination of capecitabine and lapatinib. The Kp,brain of 5-FU increased to 0.17 but did not reach statistical significance and the Kp,brain of lapatinib stayed unchanged after combination of capecitabine and lapatinib. Kp,brain/free blood could not be calculated because the unbound fractions in blood and brain were too small to quantify. The brain concentrations after the combination did not reach the GI50 value and the concentration causing 90% inhibition of the desired activity for 5-FU and lapatinib, respectively (data not shown). A concentration causing 90% inhibition of the desired activity cover is required for the anti-Her2 agent to elicit pharmacological efficacy (Nakagawa et al., 2009; Murakami et al., 2012), and the concentration causing 90% inhibition of the desired activity for lapatinib was 927 nM (539 ng/ml).

In Vivo Efficacy Study. To further address whether the BBB disruption in brain tumor would be sufficient to drive desired efficacy, female nu/nu mice bearing Her2 BCBM tumors were treated with vehicle (0.5% hydroxypropyl methylcellulose), capecitabine monotherapy (400 mg/kg every day), or in combination with lapatinib (75 mg/kg twice a day). No significant loss of body weight was observed for all groups.
during the experimental period. As shown in Fig. 2, A and B, the tumor growth curves between the vehicle and drug treatment groups almost overlapped, suggesting that there was no antitumor efficacy after oral administration of capecitabine monotherapy or in combination with lapatinib in BCBM mice carrying xenograft tumors of Her2-overexpressing BT474C1 cells. No antitumor efficacy was probably ascribed to both limited brain penetration and suboptimal dose (lower than clinical relevant dose due to toxicity).

Projected CNS Penetration in Human for 5-FU. The CNS concentration of 5-FU in human was predicted based on the CNS penetration in preclinical species (\(K_{\text{p,uu,brain}}\) at 0.17 in the brain tumor in BCBM mice and \(K_{\text{p,uu,CSF}}\) at 0.29 in wild-type BALB/c mice) and human unbound PK at the approved clinical dose (Table 4). The predicted free \(C_{\text{max}}\) concentrations of 5-FU in human brain and CSF were 5–10× lower than the in vitro antiproliferation activity.

Discussion

Our study investigated the CNS penetration of capecitabine and its active metabolite 5-FU in an attempt to understand the mechanism of the clinically observed combination effect of lapatinib and capecitabine for the treatment of BCBM from the PK perspective. 5-FU showed antiproliferative activity, GI50 at 4.57 \(\mu\text{M}\), in the in vitro BT474C1 assay. Despite not being a substrate of P-gp and BCRP in vitro, 5-FU has low passive permeability and high clearance, which makes it difficult to penetrate the CNS. Both capecitabine and 5-FU had

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time Point (hour)</th>
<th>Capecitabine Monotherapy</th>
<th>Lapatinib</th>
<th>Capecitabine + Lapatinib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal Brain Tissue</td>
<td>Normal Brain Tissue</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brain Tumor</td>
<td>Brain Tumor</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(K_{\text{p,brain}})</td>
<td>(K_{\text{p,uu,brain}})</td>
<td>(K_{\text{p,brain}})</td>
</tr>
<tr>
<td>Capecitabine</td>
<td>1</td>
<td>0.06 ± 0.03</td>
<td>0.05 ± 0.02</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>5-FU</td>
<td>1</td>
<td>0.08 ± 0.02</td>
<td>0.06 ± 0.02</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not applicable (due to protein binding of lapatinib > 99.9% in brain tissue and blood); NC, not conducted; refer to the Supplemental Material to check the raw data and calculations. \(p < 0.05\).

Fig. 2. Monitoring of tumor growth by bioluminescence imaging in the Her2 BCBM mouse model. Data are presented as geometric mean ± S.E., n = 6. (A) Capecitabine (Δ) at 400 mg/kg every day or vehicle of 0.5% hydroxypropyl methylcellulose (♦) was orally administrated to BCBM mice. The study was stopped on day 22. For each day, there were no significant differences between the vehicle and capecitabine treatment groups. (B) Lapatinib at 75 mg/kg twice a day + capecitabine at 400 mg/kg every day (□) or vehicle of 0.5% hydroxypropyl methylcellulose (♦) was administered orally to BCBM mice. The study was stopped on day 17. For each day, there were no significant differences between the vehicle and capecitabine + lapatinib treatment groups.
brain penetration impairment as indicated by distribution disequilibrium between the blood and CNS compartments (K_{p,uu,brain} = 0.13 and K_{p,uu,CSF} = 0.29). The K_{p,uu,brain} of 5-FU in the brain tumor was increased to 0.17 but did not reach statistical significance after combination of capecitabine and lapatinib in the BCBM mouse. Neither free brain nor CSF concentration of 5-FU in the mouse model or in human can achieve the in vitro antiproliferative potency. Coadministration of capecitabine and lapatinib did not show antitumor activity in the BCBM mouse. Because the clinical relevant dose of capecitabine estimated by free plasma exposure of 5-FU should be at ~1200 mg/kg in mouse, no antitumor efficacy is probably ascribed to both limited brain penetration and suboptimal dose (lower than clinical relevant dose due to toxicity). Our study suggests the penetration of the drugs into brain tumor was improved but still not well enough. Another barrier between CNS and periphery is the blood-CSF barrier, which is more fenestrated and permeable than the BBB (Abbott et al., 2010). In the present study, 5-FU did have higher penetration into CSF than in brain. However, the projected human CNS concentration of 5-FU at the approved clinical dose of capecitabine was still lower than the in vitro antiproliferative activity, suggesting that systemic dose of capecitabine should have little benefit in treating leptomeningeal metastasis (LM) from BC. This may explain the current clinical practice for the treatment of BC LM; 79% of the LM patients were treated by intrathecal chemotherapy, whereas only 13% of the LM patients were selected for systemic administration of capecitabine (Niwinska et al., 2013).

The BBB became structurally and functionally compromised in macroscopic brain metastasis with brain tumor beyond 1–2 mm in diameter (Eichler et al., 2011). In addition to changes in blood vessel permeability, the reduction in P-gp expression level in brain metastasis to 5% and 40% of normal brain was also observed. These changes could make it possible for large hydrophilic chemotherapies and P-gp substrates to get into the brain tumor in the treatment of BCBM disease. Taskar et al. (2012) investigated the distribution of 14C-lapatinib in Her2 overexpressing experimental BCBM mouse by using quantitative autoradiography and chromatography. We made our calculations on the basis of the Taskar et al. (2012) data and found that the brain penetration (K_{p,brain}) of lapatinib in the BCBM mice model improved from 0.013 in normal brain tissue and 0.090 in brain tumors at 2 hours postdose and 0.028 in normal brain tissue and 0.262 in brain tumors at 12 hours postdose, respectively. Brain penetration of 14C-lapatinib was heterogeneous among the brain tumors and within individual tumors, K_{p,brain} was 0.025, 0.071, and 0.228 for 12.5%, 70%, and 17.5% of brain tumors. The average K_{p,brain} for a tumor was 0.081, and different regions within the same tumor had varied K_{p,brain} ranging from 0.004 to 0.576. In our study, the estimated free brain and CSF concentration (C_{u,max,brain} and C_{CSF}) of 5-FU in human were 5–10$ times lower than the in vitro antiproliferative activity. However, the concentration of 5-FU and lapatinib in certain regions of brain tumors may be sufficient to elicit antitumor activity. This could partially explain the observed clinically improved CNS response after combination of lapatinib and capecitabine from the PK perspective. In light of the heterogeneous brain penetration within and among tumors, true CNS penetrable therapeutic agents should further improve the response rate for BCBM.

Trastuzumab (trade name Herceptin), a humanized monoclonal antibody directly targeting the Her2 (erbB2/neu) oncoprotein, is associated with longer time to disease progression and longer survival in peripheral metastatic BC patients who received trastuzumab plus chemotherapy (Slamon et al., 2001; Hudis, 2007). Trastuzumab has no effect, or a limited effect, in controlling Her2 positive brain metastasis; it has been observed that the cause of death in Her2 positive BC patients due to developed brain metastases was 45.7% in a pre-trastuzumab treatment group and 59.5% in a post-trastuzumab treatment group (Park et al., 2009). This is consistent with the fact that trastuzumab hardly penetrates into CNS (CSF-to-serum ratio at 0.003) (Pestalozzi and Brignoli, 2000) because of its large molecular size. Pertuzumab (trade name Perjeta) was recently approved for use in combination with trastuzumab and docetaxel in patients with Her2 positive metastatic BC. Pertuzumab is also a monoclonal antibody, and should be similar to trastuzumab in brain penetration.

Neratinib (Puma Biotechnology, Inc.) is a dual inhibitor of Her2 and EGFR kinases under investigation for the treatment of early and late stage Her2 positive BC. Neratinib was investigated in patients with HER2 positive metastatic BC that has metastasized to the brain. The efficacy result was released at the American Society of Clinical Oncology 2014 annual meeting (Freedman, 2014). Under treatment of neratinib monotherapy, the median progression free survival of the 40 evaluable patients with BM was 1.9 months and the median overall survival was 8.7 months, as released by Puma Biotechnology, Inc. (http://www.pumbabiotechnology.com/pr/20140601.html) Neratinib had little effect on BCBM in this trial with an overall CNS response rate of 7.5% (Freedman, 2014). In contrast, the median progression free survival for neratinib was 4.5 months and the median overall survival was 19.7 months in treating patients with Her2 positive locally advanced or metastatic BC (Martin et al., 2013).

In conclusion, CNS penetration of current chemotherapies and targeted anti-Her2 therapies are not desirable. The PK-efficacy relationship could not be well established for the observed improved clinical response in BCBM patients when lapatinib was coadministered with capecitabine. Development of a true CNS penetrable therapeutic agent is necessary, which will further improve the response rates and overall survival of the BCBM patients.

**Authorship Contributions**

*Participated in research design: J. Zhang, Cheng.*

*Conducted experiments: Yan, Xie, Zhong, Lv, X. Zhang.*

*Performed data analysis: L. Zhang, Li, Bai.*

*Wrote or contributed to the writing of the manuscript: J. Zhang, Cheng.*

**References**

