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

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Running title: CNS penetration of 5-FU to treat breast cancer brain metastasis

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Abbreviations

BC, breast cancer; bm, brain metastasis; BCbm, breast cancer brain metastasis; Her2, human epidermal growth factor receptor-2; EGFR, epidermal growth factor receptor; BBB, blood brain barrier; CNS, central nervous system; CSF, Cerebrospinal fluid; MTD, maximal tolerable dose; MDR, multidrug resistance; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; ER, efflux ratio; $K_{p,brain}$, brain-to-blood ratio; $K_{p,CSF}$, CSF-to-blood ratio; $K_{p,uu,brain}$, unbound brain-to-blood ratio; $K_{p,uu,CSF}$, CSF-to-unbound blood ratio; GI50, the concentration for 50% of maximal inhibition of cell proliferation; IC90, the concentration causing 90% inhibition of the desired activity
Abstract

Brain metastasis is increasingly diagnosed in Her2 positive breast cancer patients. Lack of effective treatment to the breast cancer brain metastases (BCbm) is probably due to inability of the current therapeutic agents to cross BBB. 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 hope to interpret mechanism of the improved CNS response from pharmacokinetic perspective. Capecitabine doesn’t have antiproliferative activity and 5-FU is the active metabolite. Capecitabine was orally administered to mouse returning $K_{p,uu,\text{brain}}$ at 0.13 and $K_{p,uu,\text{CSF}}$ at 0.29 for 5-FU. Neither free brain nor CSF concentration of 5-FU can achieve antiproliferative GI$_{50}$ of 4.57 µM. BCbm mice were treated with capecitabine monotherapy or in combination with lapatinib. $K_{p,uu,\text{brain}}$ of 5-FU increased to 0.17 in the brain tumor in the presence of lapatinib, which is still far below the unity. The calculated free concentration of 5-FU and lapatinib in the brain tumor didn’t 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 suggested 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 the improvement in diagnostic techniques (Stemmler and Heinemann, 2008). Patients with breast cancer over-expressing human epidermal growth factor receptor-2 (Her2) proto-oncogene have higher risk to develop CNS metastasis than other breast cancer phenotypes (Pestalozzi et al., 2006). In a recent published data, total of 37% (377/1012) enrolled patients diagnosed as Her2 positive metastatic breast cancer has developed CNS metastasis (Brufsky et al., 2011). Median survival was significantly shorter for patients with brain metastasis (26.3 months) than those without brain metastasis (bm) (44.6 months).

Lapatinib, an EGFR (epidermal growth factor receptor) and Her2 dual inhibitor, was approved in combination with capecitabine to treat advanced or metastatic breast cancers over-expressing Her2 proto-oncogene. Lapatinib has been assessed in Her2 positive BCbm patients returned a low CNS response rate at 2.6–6% (Lin et al., 2008; Lin et al. 2009). The CNS response rate was improved to 20–65% when the patients were treated with lapatinib in combination with capecitabine (Bartolotti et al., 2013). Chefrour and his co-workers in 2012 (Chefrour et al., 2012) have elucidated the mechanism for the synergistic benefit in 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 subcutaneous BC mice model. However, the mechanism of improved clinical objective CNS response rate after combination of lapatinib and capecitabine has not been studied.
from pharmacokinetic (PK) perspective. Lapatinib was a substrate of P-gp and BCRP, the two major efflux transporters localized on the luminal side of blood-brain barrier (BBB) (Polli et al., 2008). Passive permeability of lapatinib studied in MDCK cell line was 0.27~1.1×10^{-6} \text{ cm/s} which was far below the desired permeability (>15 \times 10^{-6} \text{ cm/s}) for CNS drugs (Kelly et al., 2002; Wager et al., 2010). The total brain-to-blood ratio of lapatinib was only 0.04 in normal mice, which was improved to 1.7 in \textit{mdr1a/b(-/-)/bcrp(-/-)} 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 pro-drug and sequentially metabolized to 5′-DFCR, 5′-DFUR and finally to 5-fluorouracil (5-FU) by carboxylesterase, cytidine deaminase and thymidine phosphorylase in liver and tumor (Tsukamoto et al., 2001a; Tsukamoto et al., 2001b; Desmoulin et al., 2002; Onodera et al., 2000). The \textit{in-vitro} antiproliferative potency (GI50) of capecitabine and the metabolites was evaluated in Her2 BT474C1 cells. Capecitabine and its active metabolite 5-FU were assessed in MDCKII-MDR1 and MDCKII-BCRP transport assays as efflux liability and passive permeability are two critical parameters for CNS drug delivery.

We have initially evaluated the CNS penetration of capecitabine and 5-FU in wild type mice. Subsequently, we investigated the CNS penetration of capecitabine and 5-FU in brain tumor and adjacent normal brain tissue in BCbm xenograft model mice when capecitabine was orally dosed alone at the maximal tolerable dose (MTD) 400 mg/kg or in combination with lapatinib at MTD dose of 75 mg/kg. CNS penetration of total brain-
to-blood ratio ($K_{p,\text{brain}}$) and cerebral spinal fluid (CSF)-to-blood ratio ($K_{p,\text{CSF}}$) were calculated by the Area Under Curve (AUC) ratio. Combining \textit{in-vitro} unbound fraction in blood and brain homogenates, $K_{p,\text{uu,brain}}$ (unbound brain-to-blood ratio) and $K_{p,\text{uu,CSF}}$ (CSF-to-unbound blood ratio) were estimated as critical parameters in assessing CNS penetration. Efficacy of capecitabine as a single agent or in combination with lapatinib 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 capecitabine 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 breast cancer brain metastasis.
Materials and Methods

Materials

BT474C1 cells were provided by R&D center Möndal, AstraZeneca. Cell culture medium and reagents were supplied by Invitrogen. Capecitabine, 5′-DFCR, 5′-DFUR and 5-FU were purchased from Sigma. Lapatinib was synthesized by AstraZeneca. Artificial CSF was purchased from Harvard. The equilibrium dialysis device and cellulose membranes with molecular weight cutoff 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 propidium iodide staining assay (Yu et al., 2014). In brief, cells were seeded in a 96-well plate at 6×10³ cells/well at 24 hr prior to treatment. Capecitabine, 5′-DFCR, 5′-DFUR and 5-FU were dissolved in 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 hr of treatment, cells were incubated with 10 M of Hoechst 34580 (Invitrogen) and 1.5 M of PI for 30 min, and then detected by Acumen X3 (TTP). Viability was expressed as a percentage relative to the vehicle controls. The concentration-response curve was conducted to obtain the GI50 value, concentration at 50% of growth inhibition, by Prism (GraphPad Software, San Deigo, CA, USA).
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, USA) at a density ~450,000 cells/cm² and monolayers were ready for studies 3 days later. Capecitabine 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 LC-MS/MS. The passive permeability (Pexact) was assessed in the presence of GF120918 according to equation 1 (Tran et al., 2004) and percent recovery was calculated according to equation 2,

\[ P_{\text{exact}} = \frac{V_d \times V_r}{V_d + V_r} \times \frac{A}{t} \times \text{Ln} \left[ 1 - \frac{(V_d + V_r) \times C_r}{(V_d \times C_d + V_r \times C_r)} \right] \]  

(1)

\[ \text{Recovery\%} = 100 \times \frac{(V_r \times C_r) + (V_d \times C_d)}{(V_d \times C_0)} \]  

(2)

Where \( V_d \) is the volume in the donor chambers (0.1 mL in A to B, 0.3 mL in B to A), \( V_r \) is the volume in the receiver chambers (0.3 mL in A to B, 0.1 mL in B to A); \( C_d \) and \( C_r \) are the final concentrations of transport compound in donor and receiver chambers, respectively. \( C_0 \) is the initial concentration in the donor chamber (µM). \( A \) is the surface area for the transport, i.e. 0.11 cm².

Determination of unbound fraction in mouse blood, brain homogenate, cell culture medium and human plasma.
The *in-vitro* unbound fraction of capecitabine, 5-FU and lapatinib in mouse blood, brain homogenate, cell culture medium (10% FBS in the medium) and human plasma was determined by using equilibrium dialysis device. Blood and brain were collected from nu/nu mice. Blood was diluted with 1× DPBS. Brain was homogenized with 3× PBS. Diluted blood or brain homogenate was spiked with capecitabine 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 hr. The unbound fractions (fu) were corrected using equation 3 to yield an estimate of fu in the blood, brain, cell culture medium or plasma

\[
f_u=1/(D×(1/f'_u-1)+1)\tag{3}
\]

where D and f_u' 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 Beijing Vital River CO.LTD. The animals were allowed for acclimation for at least 3 days under standard environmental conditions with 12:12 hr light/dark cycle. 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 0.5×10⁶ 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 (BLI) (Xenogen, Caliper Life Sciences, USA). BLIs were obtained weekly by using a Xenogene small animal imager (IVIS Imaging System) equipped with living image. Mouse body weight was monitored twice a week as a surrogate marker of general toxicity. Mouse was euthanized whenever a 20% loss of initial body weight was reached.

**Determination of CNS penetration in BALB/c mouse and BCbm nu/nu mouse**

Capecitabine or lapatinib was formulated in 0.5% hydroxypropyl methylcellulose aq. (HPMC, w/v). Capecitabine was orally administered to female BALB/c mice at the MTD dose of 400 mg/kg. Dosing volume was 10 mL/kg. Mouse was sacrificed under isoflurane anesthesia followed by collection of CSF, blood and brain at 0.083, 0.16, 0.25, 0.5, 1, 2, 4, 7, 16 and 24 hr post dose (n=3 each time point). After anesthetization, 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 microretrators was used to hold the muscle apart. The mouse was laid down so that the head formed a nearly 135° angle with body. Dura mater at the cisterna magna was exposed and the CSF space was visible. The dura mater was blotted with 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 pre-marked 0.5 mL eppendorf tube and frozen immediately in liquid nitrogen prior to sample analysis. Five microliter 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 (AUC_{0-t}) was calculated by Phoenix 6.2 (Pharsight Corporation, Mountain View, CA). Brain penetration of total $K_{p,\text{brain}}$ and $K_{p,\text{CSF}}$ were calculated by AUC ratio using equation 4 and 5,

$$K_{p,\text{brain}} = \frac{\text{AUC}_{\text{brain}}}{\text{AUC}_{\text{blood}}}$$  \hspace{1cm} (4)

$$K_{p,\text{CSF}} = \frac{\text{AUC}_{\text{CSF}}}{\text{AUC}_{\text{blood}}}$$  \hspace{1cm} (5)

Combining in-vitro unbound fraction in blood and brain, unbound brain-to-blood ratio ($K_{p,\text{uu,brain}}$) and CSF-to-unbound blood ratio ($K_{p,\text{uu,CSF}}$) were estimated by using equation 6 and 7,

$$K_{p,\text{uu,brain}} = K_{p,\text{brain}} \times f_{u,\text{brain}} / f_{u,\text{blood}}$$  \hspace{1cm} (6)

$$K_{p,\text{uu,CSF}} = K_{p,\text{CSF}} / f_{u,\text{blood}}$$  \hspace{1cm} (7)

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

**Bioanalysis**

All samples from *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 API4000 instrument (Applied Biosystems, Foster City, CA, USA) equipped with an Agilent HP 1200 series liquid chromatography (Angilent Technologies, Santa Clara, CA, USA) or an Waters ACQUITYTM UPLC integrated system (Waters, Milford, MA, USA) in a multiple reaction monitoring mode. The analytical column (Gemini C18 from Phenomenex Co., 4.6 × 150 mm, 5 µm; 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 to 153.9 for capecitabine, 128.9 to 42.2 for 5-FU and 579.1 to 470.2 for lapatinib in negative mode. Refer to supplemental data 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, image was acquired by auto exposure. Mice were orally administered with capecitabine monotherapy or in combination with lapatinib. Animals
received twice daily (BID) oral dose of lapatinib at 75 mg/kg and 2 hr after the first dose of lapatinib, the animals received once daily (QD) oral dose of capecitabine at 400 mg/kg. Dose used for both drugs were the maximal tolerable dose 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. Capecitabine at 400 mg/kg QD was below the clinical relevant dose due to dose limiting toxicity in mice. The clinical relevant dose of capecitabine at ~1200 mg/kg in mice was estimated by equivalent free plasma exposure of 5-FU. Mice in control group were administered with 0.5% HPMC at 10 mL/kg.

**Prediction of Human CNS PK for 5-FU**

$K_{p,uu}$ is often preserved across species for non-transporter substrates (Di et al., 2013). 5-FU was neither a P-gp nor BCRP substrate. Free CNS concentration of 5-FU in human was estimated by using the $K_{p,uu,brain}$ and $K_{p,uu,CSF}$ in mice multiplied by human unbound plasma concentration (Chu et al., 2007).

**Statistical analysis**

Data are presented as the mean ± S.D. except that clinical data in Table 4 that are presented as the mean ± 90% CI and efficacy data in Figure 2 are presented as geometric mean ± S.E. A Student’s two-tail 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 capecitabine, 5'-DFCR, 5'-DFUR and 5-FU was evaluated in BT474C1 cells. Capecitabine, 5'-DFCR and 5'-DFUR did not have antiproliferative activity up to 30 µM. 5-FU was determined as the active metabolite of capecitabine with antiproliferative GI₅₀ at 5.24 ± 2.45 µM (681 ± 319 ng/mL). Both pro-drug Capecitabine 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 capecitabine and 5-FU were shown in Table 1. Good recovery (>70%) was observed in all incubations. Capecitabine was found to be a BCRP substrate with efflux ratio (ER) at 7.44 in the absence of GF120918 which returned to 0.70 in the presence of GF120918. In the MDCKII-MDR1 cell assay, ER of capecitabine was 1.59 in the absence of GF120918 and was 0.46 in the presence of GF120918. The ER difference was 3.46 fold (1.59/0.46) which was greater than 2, suggesting that capecitabine may be a P-gp substrate. 5-FU was determined as neither a P-gp nor a BCRP substrate as the ER was close to 1 in the absence and presence of GF120918. 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 (Kelly 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 mouse and the observed total blood
clearance was 139 mL/min/kg (~90% mouse hepatic liver blood flow) and V_{ss} of 0.9 L/kg (data not shown). Rapid elimination of 5-FU from body and low to moderate volume of distribution may therefore take away the opportunity of 5-FU from entering CNS.

**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 matrixes. Given the low molecular weight (MW = 359 for capecitabine and 130 for 5-FU) and hydrophilic properties (cLogP = 0.8 for capecitabine and -0.6 for 5-FU), both compounds showed low binding to blood protein and brain tissue. The f_{u,blood} and f_{u,brain} were 0.571 and 0.446 for capecitabine, 0.479 and 0.378 for 5-FU, respectively. The f_{u,plasma} was 0.951 for 5-FU in human plasma. The unbound fraction of 5-FU in cell culture medium was 0.872. Free in-vitro antiproliferative potency of 5-FU was corrected by unbound fraction in cell culture medium (0.872) to be 4.57 ± 2.14 µM (594± 278 ng/mL).

**CNS penetration in BALB/c mouse and BCbm nu/nu mouse**

CNS penetration of capecitabine and 5-FU was assessed after oral administration of capecitabine at the MTD dose 400 mg/kg to female BALB/c mice. Blood concentration after 2 hr, brain concentration at 0.083 hr and after 2 hr, and CSF concentration at 0.083 hr and after 1 hr for 5-FU were all below limit of quantification. The AUC values in blood, brain and CSF were used to calculate K_{p,brain} and K_{p,CSF}. K_{p,brain} was 0.04 and 0.15 and K_{p,CSF} was 0.13 and 0.13 for capecitabine and 5-FU, respectively. Combining in-vitro
unbound fraction in blood and brain homogenate, both \( K_{p,\text{uu,brain}} \) and \( K_{p,\text{uu,CSF}} \) 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 (\( K_{p,\text{brain}} = 0.09 \) and \( K_{p,\text{CSF}} = 0.43 \) in primates after IV injection of 5-FU\(-2^{14}\text{C}\)) (Bourke et al., 1973), Kerr et al (\( K_{p,\text{CSF}} = 0.11-0.48 \) in monkey after IV injection of 5-FU) (Kerr et al., 1984) and Heier et al (\( K_{p,\text{CSF}} = 0.015-0.06 \) in human after IV injection of 5'-DFUR) (Heier et al., 1986). 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 mouse, the free drug concentrations of 5-FU in relationship with its \textit{in-vitro} potency are shown in Figure 1. Neither free brain nor CSF concentration of 5-FU can achieve free \textit{in-vitro} antiproliferative GI\textsubscript{50} at 594 ng/mL in the Her2 amplified BT474C1 cell line.

Brain penetration of capecitabine, 5-FU and lapatinib in brain tumor and adjacent normal brain tissue is shown in Table 3 and supplemental data for checking raw data and calculation. Time point at 1 hr was selected based on PK study in BALB/c mice. The average \( K_{p,\text{brain}} \) of 5-FU at 1 hr was 0.15, which was similar to the \( K_{p,\text{brain}} \) at 0.17 calculated by AUC. Luciferase transfected BT474C1 cells were intracranially injected to the right brain and the immune-histochemistry imaging results confirmed that tumor signal almost occupied the whole right brain (data not shown). Brain penetration of capecitabine and lapatinib was generally similar between brain tumor and normal brain tissue. The \( K_{p,\text{uu,brain}} \) (free brain tumor to free blood concentration ratio) of 5-FU in brain tumor was 0.13 (\( p < 0.05 \)) which is 2.17\times higher than that in adjacent normal brain tissue (\( K_{p,\text{uu,brain}} = 0.06 \)), when capecitabine was orally administered as a single agent to mice.
The improved brain penetration for 5-FU in brain tumor was still low as the $K_{p,uu,\text{brain}}$ was far below 1. Brain penetration of 5-FU was higher after combination of capecitabine and lapatinib. The $K_{p,uu,\text{brain}}$ of 5-FU increased to 0.17 but not reaching statistically significance and the $K_{p,\text{brain}}$ of lapatinib stayed unchanged after combination of capecitabine and lapatinib. $K_{p,uu,\text{brain}}$ for lapatinib cannot be calculated as the unbound fractions in blood and brain were too small to quantify. The brain concentrations after the combination didn’t reach the $GI_{50}$ and $IC_{90}$ for 5-FU and lapatinib, respectively (data not shown). $IC_{90}$ cover is required for anti-Her2 agent to elicit pharmacological efficacy (Nakagawa et al., 2009; Murakami et al., 2012) and the $IC_{90}$ 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% HPMC), capecitabine monotherapy (400 mg/kg QD) or in combination with lapatinib (75 mg/kg BID). No significant loss of body weight was observed for all groups during the experimental period. As shown in Figure 2A and 2B, the tumor growth curves between vehicle and drug treatment groups almost overlapped suggesting that there was no anti-tumor efficacy after oral administration of capecitabine monotherapy or in combination with lapatinib in BCbm mouse carrying xenograft tumors of Her2-overexpressing BT474C1 cells. No anti-tumor efficacy was probably ascribed to both limited brain penetration and sub-optimal 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_{p,uu,brain} at 0.17 in the brain tumor in BCbm mice and K_{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_{max} concentrations of 5-FU at in human brain and CSF were 5~10\times lower than the in-vitro anti-proliferation activity.
Discussions

Our study investigated the CNS penetration of capecitabine and its active metabolite 5-FU in an attempt to understand the mechanism of clinical observed combination effect of lapatinib and capecitabine for the treatment of breast cancer brain metastasis from pharmacokinetic perspective. 5-FU showed antiproliferative activity GI50 at 4.57 µM 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 brain penetration impairment as indicated by distribution disequilibrium between the blood and CNS compartments (Kp,uu,brain = 0.13 and Kp,uu,CSF = 0.29). The Kp,uu,brain of 5-FU in the brain tumor was increased to 0.17 but not reaching statistically significance after combination of capecitabine and lapatinib in 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. Co-administration of capecitabine and lapatinib did not show anti-tumor activity in the BCbm mouse. As the clinical relevant dose of capecitabine estimated by free plasma exposure of 5-FU should be at ~1200 mg/kg in mouse, no anti-tumor efficacy is probably ascribed to both limited brain penetration and sub-optimal dose (lower than clinical relevant dose due to toxicity). Our study suggests 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 BBB (Abbott et al., 2010). In the present study, 5-FU did have higher penetration into CSF than that into brain. However, the projected human CSF 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 to treat leptomeningeal metastasis (LM) from breast cancer. This may explain the current clinical practice for the treatment of breast cancer leptomeningeal metastasis. 79% of the LM patients were treated by intrathecal chemotherapy, whereas only 13% of the LM patients were selected for systemic administration of capecitabine (Niwińska et al., 2013).

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, it was also observed the reduction in P-gp expression level in brain metastasis to 5% and 40% of normal brain. Those changes could bring opportunity to large hydrophilic chemotherapies and P-gp substrates to get into the brain tumor for the treatment of BCbm disease. Taskar and co-workers (Taskar et al., 2012) investigated the distribution of $^{14}$C-lapatinib in Her2 overexpressing experimental BCbm mouse by using quantitative autoradiography and chromatography. We made calculation on the basis of Taskar’s data and found that brain penetration ($K_{p,\text{brain}}$) of lapatinib in BCbm mice model improved from 0.013 in normal brain tissue and 0.090 in brain tumors at 2 hr post dose; 0.028 in normal brain tissue and 0.262 in brain tumors at 12 hr post dose, respectively. Brain penetration of $^{14}$C-lapatinib was heterogeneous among the brain tumors and within individual tumor, $K_{p,\text{brain}}$ was 0.025, 0.071 and 0.228 for 12.5%, 70% and 17.5% of brain tumors. The average $K_{p,\text{brain}}$ for a tumor was at 0.081, and different regions within the same tumor have varied $K_{p,\text{brain}}$ ranging from 0.004 to 0.576. In our study, the estimated free brain and CSF concentration ($C_{u,\text{max,brain}}$ and $C_{\text{CSF}}$) of 5-FU in human were 5~10× lower than its in-vitro antiproliferative activity. However, the concentration of 5-FU and lapatinib in certain region of brain tumors may be sufficient to
elicit antitumor activity. This could partially explain the observed clinical improved CNS response after combination of lapatinib and capecitabine from pharmacokinetic 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 targeted the Her2 \((erbB2/neu)\) oncoprotein, was associated with a longer time to disease progression and a longer survival in peripheral metastatic breast cancer patients who received trastuzumab plus chemotherapy (Slamon et al., 2001; Hudis, 2007). Trastuzumab has no or limited effect in controlling Her2 positive brain metastasis as observed that cause of death in Her2 positive BC patients due to developed brain metastases was 45.7% in pre-trastuzumab treatment group and 59.5% in post trastuzumab treatment (Park et al., 2009). This is consistent with the fact that trastuzumab hardly penetrated into CNS (CSF-to-serum ratio at 0.003 (Pestalozzi and Brignoli, 2000) because of large molecular size. Pertuzumab (trade name Perjeta) was recently approved for use in combination with trastuzumab and docetaxel in patients with Her2 positive metastatic breast cancer. 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 breast cancer. Neratinib was investigated in patients with HER2 positive metastatic breast cancer that has metastasized to the brain. The efficacy result was released at the ASCO 2014 annual meeting. Under treatment of neratinib monotherapy, the median progression free survival
(PFS) of the 40 evaluable patients with brain metastasis was 1.9 months and the median overall survival (OS) was 8.7 months released by Puma Biotechnology Inc. Neratinib had little effect on breast cancer brain metastases in this trial with an overall CNS response rate of 7.5% (Freedmann, 2014). In contrast, median PFS for neratinib was 4.5 months and median OS was 19.7 months in treating patients with Her2 positive locally advanced or metastatic breast cancer (Martin et al., 2013).

In conclusion, CNS penetration of current chemotherapies and targeted anti-Her2 therapies are not desirable. PK-efficacy relationship cannot be well-established for the observed improved clinical response in BCbm patients when lapatinib was co-administered 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: Jinqiang Zhang, Cheng

Conducted experiments: Yan, Xie, Zhong, Lv, Xiuhua Zhang

Performed data analysis: Lingli Zhang, Li, Bai

Wrote or contributed to the writing of the manuscript: Jinqiang Zhang, Cheng
References


Freedman RA (2014) TBCRC 022, Phase II trial of neratinib for patients (Pts) with human epidermal growth factor receptor 2 (HER2+) breast cancer and brain metastases (BCBM), *Proceding of the 50th annual meeting of the American Society of Clinical Oncology for breast cancer*; 2014 May 30-Jun 3, Chicago, IL. Abstract 528, ASCO.


Taskar KS, Rudraraju V, Mittapalli RK, Samala R, Thorsheim HR, Lockman J, Gril B, Hua E, Palmieri D, Polli JW, Castellino S, Rubin SD, Lockman PR, Steeg PS, and


FIGURE LEGENDS

Fig. 1. Free concentration of 5-FU in blood (♦), brain (▲) and CSF (■) after oral administration of capecitabine at 400 mg/kg to wild type female BALB/c mice and relationship with the free antiproliferative GI_{50} (--) of 5-FU in BT474C1 cells. Data are presented as mean ± S.D.

Fig. 2. Monitoring of tumor growth by bioluminescence imaging in Her2 BCbm mice model. Data are presented as geometric mean ± S.E., n=6. (A) Capecitabine (Δ) at 400 mg/kg QD or vehicle of 0.5%HPMC (♦) was orally administrated to BCbm mice. Study was stopped on day-22. For each day, there were no significant differences between vehicle and capecitabine treatment groups. (B) Lapatinib at 75 mg/kg BID + capecitabine at 400 mg/kg QD (□) or vehicle of 0.5%HPMC (♦) was administered orally administrated to BCbm mice. Study was stopped on day-17. For each day, there were no significant differences between vehicle and capecitabine + lapatinib treatment groups.
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TABLES

Table 1. Permeability and efflux transport of capecitabine and 5-FU in MDCKII-MDR1 or MDCKII-BCRP cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Assay</th>
<th>( P_{\text{exact}} \times 10^{-6} \text{ cm/s} )</th>
<th>Efflux ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GF120918</td>
<td>A&gt;B</td>
<td>B&gt;A</td>
</tr>
<tr>
<td>Capecitabine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDCKII-MDR1</td>
<td>No</td>
<td>3.74 [2.52, 4.96]</td>
<td>5.93 [6.51, 5.36]</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3.09 [3.32, 2.86]</td>
<td>1.41 [1.10, 1.72]</td>
</tr>
<tr>
<td>MDCKII-BCRP</td>
<td>No</td>
<td>3.53 ± 0.55</td>
<td>26.3 [24.2, 28.4]</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>4.21 [4.02, 4.41]</td>
<td>2.96 [2.61, 3.30]</td>
</tr>
<tr>
<td>5-FU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDCKII-MDR1</td>
<td>No</td>
<td>0.99 ± 0.09</td>
<td>1.36 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1.08 ± 0.08</td>
<td>1.15 ± 0.03</td>
</tr>
<tr>
<td>MDCKII-BCRP</td>
<td>No</td>
<td>2.16 [1.20, 3.11]</td>
<td>3.18 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1.92 [1.75, 2.08]</td>
<td>2.17 [2.90, 1.44]</td>
</tr>
</tbody>
</table>

Data are presented as mean [individual value] or mean ± SD.
Table 2. PK and CNS penetration of capecitabine and 5-FU after oral administration of capecitabine at 400 mg/kg to BALB/c mouse

<table>
<thead>
<tr>
<th>Compound</th>
<th>PK parameters</th>
<th>Matrix</th>
<th>CNS penetration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blood</td>
<td>Brain</td>
</tr>
<tr>
<td>Capecitabine</td>
<td>Tmax (hr)</td>
<td>0.25</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Cmax (ng/mL)</td>
<td>64000</td>
<td>2406</td>
</tr>
<tr>
<td></td>
<td>AUC_{0-t} (ng·h/mL)</td>
<td>33615</td>
<td>1482</td>
</tr>
<tr>
<td></td>
<td>AUC_{0-inf} (ng·h/mL)</td>
<td>33643</td>
<td>1484</td>
</tr>
<tr>
<td>5-FU</td>
<td>Tmax (hr)</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Cmax (ng/mL)</td>
<td>315</td>
<td>25.9</td>
</tr>
<tr>
<td></td>
<td>AUC_{0-t} (ng·h/mL)</td>
<td>335</td>
<td>50.7</td>
</tr>
<tr>
<td></td>
<td>AUC_{0-inf} (ng·h/mL)</td>
<td>338</td>
<td>57.5</td>
</tr>
</tbody>
</table>

\[ K_{p,brain} = \frac{AUC_{brain}}{AUC_{blood}}; \quad K_{p,CSF} = \frac{AUC_{CSF}}{AUC_{blood}} \]

\[ K_{p,uu,brain} = K_{p,brain} \times f_{u,brain} / f_{u,blood}; \quad K_{p,uu,CSF} = K_{p,CSF} / f_{u,blood} \]
Table 3. Brain penetration of capecitabine and 5-FU in brain tumor and adjacent normal brain tissue

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time point</th>
<th>Normal brain tissue</th>
<th>Brain tumor</th>
<th>Normal brain tissue</th>
<th>Brain tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(hr)</td>
<td>K_p,brain</td>
<td>K_p,uu,brain</td>
<td>K_p,brain</td>
<td>K_p,uu,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>
<td>0.05±0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05±0.02</td>
<td>0.5±0.02</td>
<td>0.05±0.03</td>
<td>0.05±0.02</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>
<td>0.15±0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.17±0.03</td>
<td>0.13±0.02</td>
<td>0.12±0.05</td>
<td>0.22±0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.15±0.07</td>
<td>0.12±0.05</td>
<td>0.17±0.08</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>1</td>
<td>NC^a</td>
<td>NC^a</td>
<td>NC^a</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NC^a</td>
<td></td>
<td>NA^b</td>
</tr>
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<td></td>
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<td>NC^a</td>
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<td>0.12±0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA^b</td>
</tr>
</tbody>
</table>

^aNC: not conducted

^bNA: not applicable due to protein binding of lapatinib >99.9% in brain tissue and blood.

*p < 0.05

Refer to supplemental data for checking raw data and calculation.
Table 4. Projected human CNS penetration of 5-FU after oral administration of capecitabine

<table>
<thead>
<tr>
<th>Compound (analyte)</th>
<th>Clinical dose</th>
<th>C_{\text{plasma, max}} (ng/mL)</th>
<th>C_{\text{u,plasma, max}} (ng/mL)</th>
<th>C_{\text{u,brain, max}} (ng/g)</th>
<th>C_{\text{CSF, max}} (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capecitabine 2000 mg/m^2/day</td>
<td>472 [343-650]</td>
<td>449 [326-618]</td>
<td>76.3 [55.4-105]</td>
<td>130 [94.5-179]</td>
<td></td>
</tr>
<tr>
<td>5-FU</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capecitabine 2000 mg/m^2/day + lapatinib 1250 mg/day</td>
<td>375 [274-512]</td>
<td>357 [261-487]</td>
<td>60.7 [44.4-82.8]</td>
<td>104 [75.7-141]</td>
<td></td>
</tr>
</tbody>
</table>

K_{p,u,brain} at 0.17 from preclinical brain tumor in BCbm mouse was used to estimate the free brain concentration of 5-FU; K_{p,u,CSF} at 0.29 from BALB/c mouse was used to estimate CSF concentration of 5-FU.

Data are presented as mean [90% CI].