Src Family Kinases as Novel Therapeutic Targets to Treat Breast Cancer Brain Metastases

Siyuan Zhang1,6, Wen-Chien Huang1, Lin Zhang1,4, Chenyu Zhang1, Frank J. Lowery1,4, Zhaoxi Ding1, Hua Guo1, Hai Wang1, Suyun Huang2, Aysegul A. Sahin3, Kenneth D. Aldape3, Patricia S. Steeg5, and Dihua Yu1,4

Abstract

Despite better control of early-stage disease and improved overall survival of patients with breast cancer, the incidence of life-threatening brain metastases continues to increase in some of these patients. Unfortunately, other than palliative treatments there is no effective therapy for this condition. In this study, we reveal a critical role for Src activation in promoting brain metastasis in a preclinical model of breast cancer and we show how Src-targeting combinatorial regimens can treat HER2+ brain metastases in this model. We found that Src was hyperactivated in brain-seeking breast cancer cells derived from human cell lines or from patients’ brain metastases. Mechanistically, Src activation promoted tumor cell extravasation into the brain parenchyma via permeabilization of the blood–brain barrier. When combined with the EGFR/HER2 dual-targeting drug lapatinib, an Src-targeting combinatorial regimen prevented outgrowth of disseminated breast cancer cells through the induction of cell-cycle arrest. More importantly, this combinatorial regimen inhibited the outgrowth of established experimental brain metastases, prolonging the survival of metastases-bearing mice. Our results provide a rationale for clinical evaluation of Src-targeting regimens to treat patients with breast cancer suffering from brain metastasis. Cancer Res; 73(18); 1–11. ©2013 AACR.

Introduction

Although modern multimodality therapies have improved the overall survival of patients with breast cancer, many of these survivors ultimately develop brain metastases, with a median survival of less than one year (1, 2). Neither chemotherapies nor targeted therapies are clinically effective in treating life-threatening established brain metastases. Current clinical options for patients with brain metastasis are very limited. Most of these patients undergo whole-brain radiotherapy (WBRT) or stereotactic radiosurgery. As severe cognitive complications may accompany these aggressive treatments, particularly WBRT, the quality of life associated therewith is poor (3). Despite the better control of primary disease and systemic metastases (4), the increasing incidence of brain metastasis further underpins an imperative need for developing novel therapies to treat overt brain metastases and/or prevent outgrowth of brain micrometastases.

Clinically, patients with either HER2-amplified (HER2+ or triple-negative breast cancer (TNBC) have a significantly higher incidence of brain metastasis relapse (2, 5). Interestingly, the Src family of nonreceptor tyrosine kinases (referred to as “Src”, hereafter) is critical for both types of breast cancer. In HER2+ breast cancer, Src activation maximizes the HER2/HER3 interaction and serves as a convergent point of multiple upstream signals, thus conferring resistance to anti-HER2 therapies (6–8). TNBC cells are preferentially sensitive to Src-targeting small-molecule inhibitors, indicating Src targeting is a promising strategy for TNBC (9, 10). In addition, blood–brain barrier (BBB)-permeable Src inhibitors have been considered for the treatment of glioblastoma and leukemia in the central nervous system (11, 12). This collective evidence suggests that targeting Src, coupled with anti-HER family agents, is a promising strategy for the treatment of breast cancer brain metastasis.

Here, we show a unique role of Src activation in disrupting the BBB and promoting breast cancer brain metastasis. A combinatorial regimen of Src and the EGFR/HER2 inhibitor lapatinib-inhibited brain metastasis incidence and significantly extended the overall survival of metastasis-bearing mice. These data exemplify the clinical potential of Src-targeting strategies for patients suffering from brain metastases.

Materials and Methods

Reagents and cell culture

BT474.m1 (BT474) and MDA-MB-231.brain-seeking (Br) cells were previously described (6, 13, 14). MD Anderson
Cancer Center (MDACC; Houston, TX) Cell Line Characterization Core Facility verified the identities of above cell lines. MDA-MB-231 HER2 cells were generated as described previously (15). Lentiviral-based expression vector pLOVE and packaging vectors were from Addgene. Src and fluorescence proteins vectors were generated via Gateway cloning. Lentiviral-based pLKO.1 Src short hairpin RNA (shRNA) was purchased from Sigma. All antibodies were purchased from Cell Signaling Technology.

**Reverse phase protein array**

Reverse phase protein array (RPPA) was conducted in MDACC Functional Proteomics core facility. Briefly, cellular proteins were denatured by 1% SDS, serial diluted, and spotted on nitrocellulose-coated slides. Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody. The signal obtained was amplified using a DakoCytomation-catalyzed system and visualized by 3,3'-diaminobenzidine (DAB) colorimetric reaction. The slides were analyzed using customized Microvige Software (VigeneTech Inc.). Each dilution curve was fitted with a logistic model (“super curve fitting” developed at MDACC) and normalized by median polish. Antibodies that did not show progressive and consistent changes from parental, Br.1 to Br.2 were excluded from further analysis. The distance (Dis = v1−v2) between the normalized log values of parental cell (control) and Br.2 were compared. Antibodies with distance more than 0.1 were selected for clustering. The data clustering was conducted using Cluster 3.0 (centered by gene; followed by hierarchically clustering by gene and array using complete linkage) and TreeView.

**Western blotting**

Western blotting was done as previously described (16). Briefly, at the end of designated experiments, cells were lysed in cell lysis buffer (20 mmol/L Tris at pH 7.0, 1% Triton-X 100, 0.5% NP-40, 250 mmol/L NaCl, 3 mmol/L EDTA, and protease inhibitor cocktail). Proteins were separated by SDS-PAGE and transferred onto a polyvinyldene difluoride membrane. After each membrane was blocked with 5% milk for 1 hour, it was probed with various primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 1 hour at room temperature before being visualized with enhanced chemiluminescence reagent.

**Trans-BBB invasion assay**

Human brain microvascular endothelial cells (HBMEC) were purchased from Cell Systems. Immortalized mouse astrocytes were a gift from Dr. Isaiah J. Fidler (MDACC). HBMEC (5,000 cells) were plated on fibronectin-coated Transwell inserts with 8 μm pores. 24 hours later, astrocytes (5,000 cells) were seeded on the bottom side of inserts. Four days later, tumor cells were seeded in FBS-free medium on top of the inserts and immersed into medium containing 10% FBS for 24 hours. Fluorescent protein-labeled cancer cells transmitting to bottom chambers were counted.

**Electric cell-substrate impedance sensing assay**

Briefly, 8W10E chamber slides were coated with type I collagen and then plated with 100,000 mouse endothelial cells. Eight hours later, tumor cells (500,000) were seeded onto the endothelial cell monolayer. The transendothelial cell impedance was monitored in real-time by ECIS detector model 1600R (Applied BioPhysics).

**BBB permeability assays**

BBB permeability was measured by fluorescent tracers as described before (17). Briefly, BBB tracer dyes (1% sodium fluorescein in PBS, molecular weight 376 or 2% Evans blue in PBS, molecular weight 960,82) were injected intravenously on the same day as intracarotid injection of tumor cells. Forty-eight hours later, whole mouse brains were dissected and imaged using a fluorescence stereomicroscope equipped with filter sets for GFP and DsRed. The mean fluorescence intensity of dissected brains was compared by ImageJ software (NIH).

**Cell proliferation, invasion, and adhesion assays**

These assays were conducted as we have previously described (8). Briefly, cells (5,000/well) were seeded in 96-well plates and treated for 72 hours. Cell proliferation was determined by MTT assay or Click-iT EdU Cell Proliferation Assays (Life Technology). Percentage of inhibition of cell proliferation was calculated as [1-(treated cells/untreated cells) × 100]. For invasion assays, a Transwell was coated with 15% Matrigel for 2 hours. Tumor cells (5,000/well) were plated in FBS-free medium. 10% PBS-containing medium was used as chemical attractant. For adhesion assay, a 96-well plate was coated with fibronectin for 1 hour at 37°C before blocking the plate with 0.5% bovine serum albumin (BSA) in medium. About 100,000 cells were seeded and incubated for 30 minutes. After 2,000 rpm shaking for 1 minute, the cells that had adhered onto the plate were stained with crystal violet and counted.

**EdU cell-cycle assays**

Cell-cycle analysis was conducted using Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit from Life Technology. Briefly, EdU was added into cell-culture medium to a final concentration of 10 μmol/L 2 hours before the endpoint of the experiments. Then, cells were washed once with 3 mL of 1% BSA in PBS, fixed using Click-iT fixative, and incubated for 15 minutes in saponin-based permeabilization solution. Cells were then treated with Click-iT reaction cocktail according to manufacturer’s instruction for 30 minutes before flow cytometry analysis.

**Immunofluorescence**

Cell proliferation and apoptosis were examined by immunofluorescence-based PathScan Apoptosis and Proliferation Multiplex IF Kit from Cell Signaling. Briefly, after washing with PBS, cells were fixed with 4% formaldehyde. Samples were blocked with PBS per 5% normal goat serum for 1 hour before incubation with a primary antibody cocktail overnight at 4°C. Samples were then incubated with secondary antibodies before examination using confocal microscope.
**In vivo experiments**

Animal experiments were conducted under approved MDACC Institutional Animal Care and Use Committee protocols. To develop the brain metastasis models, female Swiss nude mice were injected with tumor cells [250,000 cells in 0.1 mL Hank’s Balanced Salt Solution (HBSS) for MDA-MB-231 and 500,000 cells in 0.1 mL HBSS for BT474.m1] into the right common carotid artery. After development of overt metastases, whole brains were dissected and disaggregated by using Tenbroeck homogenizer briefly in Dulbecco’s Modified Eagle Medium/F-12 medium before plating on tissue culture dishes. Two weeks later, tumors cells recovered from brain tissue were collected and expanded as brain-seeking sublines (Br.1). For drug treatment assays, inhibitors were prepared in vehicle (0.5% hydroxypropyl-methylecelulose with 0.1% Tween 80) and administered via oral gavage (25 mg/kg/d saracatinib and 100 mg/kg/d lapatinib). At the end of experiments, brains were excised for imaging and histologic examination. Ten serial sagittal sections every 300 microns throughout the brain were analyzed as described (14).

**Patient samples and immunohistochemistry**

Tissues were collected under protocols approved by the Institutional Review Board. Archived paraffin-embedded tumor samples were coded with no patient identifiers and subjected to standard immunohistochemistry (IHC) analysis. IHC staining was conducted as previously described (18). Briefly, after deparaffinization and rehydration, 4 µm sections were subjected to heat-induced epitope retrieval (0.001 mol/L EDTA for pSrc-Y416 and 0.01 mol/L citrate for cyclin D1). After blocking with 1% goat serum and incubating with primary antibody at 4°C overnight, slides underwent color development with DAB and hematoxylin counterstaining. Ten visual fields from different areas of each tumor were evaluated by 2 independent pathologists. Cyclin D1 staining was calculated as percentage of nuclear-positive cells per field (%) and normalized by the total cancer cell number in each field.

**Statistical analysis**

For quantitative data with normal distribution, the Student t test was used for comparing 2 groups. Differences of IHC staining were compared using $\chi^2$ test. Survival analysis was conducted using Kaplan–Meier model with two-sided log-rank test. All P values are two-tailed. A difference with $P < 0.05$ (two-sided) was considered statistically significant.

**Results**

**Development of novel brain metastasis cell line model with endogenous HER2 amplification**

All currently available models for studying brain metastasis have severe limitations (19). Among other limitations, none of the current models are representative of breast cancers with endogenous HER2 amplification (HER2+). Therefore, to model HER2+ breast cancer brain metastasis, we used a subline of the HER2+ tumorigenic BT474 human breast cancer cell line, BT474.m1 (BT474), to select for brain-seeking (Br) cells in vivo. Tumor cells were injected into the right-side common carotid artery (Fig. 1A, left). Three months later, BT474 cells formed overt brain metastases predominantly in the right hemisphere of the brain. Rare micrometastasis in left hemisphere could be detected. No metastasis was observed in other organs. Overt brain metastasis lesions derived from BT474 cells have a moderately invasive phenotype (Fig. 1A, right, hematoxylin and eosin stain, H&E) with a high-level of HER2 expression (Fig. 1A, right, IHC HER2), although metastatic formation occurs with low efficiency. The metastatic lesions were isolated and used for additional cycles of in vivo selection of Br.2 and Br.3 sublines.

**Src signaling is upregulated in brain-seeking cell lines and human brain metastases**

To reveal the mechanisms contributing to brain metastasis, we conducted RPPA analyses comparing BT474.Br sublines with the parental line (Fig. 1B and Supplementary Table S1). In vivo selective pressure led to an increase of Src protein expression in the brain-seeking subline BT474.Br.2 (Fig. 1B and C, left). This upregulation was accompanied by a strong increase in Src activity, indicated by increased phosphorylation of Src on tyrosine 416 (pSrc–Y416; Fig. 1C, left), pSrc–Y416 and phospho-Paxillin (Y118; a Src downstream target) were also significantly elevated in the Br subline of MDA-MB-231 (Fig. 1C, right). A consistent change of Akt phosphorylation could not be detected. To validate the clinical relevance, we compared pSrc–Y416 levels between resected brain metastases and primary breast tumors (unmatched) by IHC analyses (Fig. 1D). Forty-five primary breast tumors and 46 unmatched brain metastasis tumors were collected. Of all the primary tumors, 31.1% were HER2+ and 51.1% were estrogen receptor-positive (ER+). Of brain metastases, 43.3% were HER2+ and 33.3% were ER+. Src activation, indicated by strong membrane staining of pSrc–Y416 staining, was significantly increased in brain metastases compared with primary breast tumors (65.7% vs. 34.3% IHC 3+, $P = 0.008$). Moreover, a significant increase of pSrc–Y416 ($\chi^2, P = 0.045$) was observed in HER2-amplified tumors (Supplementary Table S2).

**Src activation in tumor cells promotes the invasion of simulated BBB in vitro and brain metastasis in vivo**

To determine the functional role of Src activation in brain metastasis, we expressed either V5-tagged wild-type Src (Src-WT) or constitutively activated Src (Src–Y527F) in MDA-MB-231 and BT474 parental cells. Src activation was validated by Western blotting (Fig. 2A and Supplementary Fig. S1A). Next, we examined key brain metastasis-related properties in vitro in Src-activated cells. One distinct feature of the brain is the presence of the BBB, which represents the first line of defense in preventing extravasation of disseminated tumors cells into the brain (20, 21). To test role of Src activation in cell extravasation, we used an in vitro model of BBB by coculturing brain endothelial cells and astrocytes on each side of the membrane of a Transwell (Fig. 2B, left; ref. 22). Src–Y527F 231.Br cells were significantly (2.5-fold) more efficient in passing through the simulated BBB ($P = 0.002$; Fig. 2B, right). Similarly, transfection of either Src-WT or Src–Y527F into BT474 parental cells significantly promoted invasion (2.0-fold and 2.2-fold increase, respectively; Supplementary Fig. S1B and S1C). Next, we knocked down Src in brain-seeking sublines using 2 independent Src shRNA constructs (Src.sh648 and Src.sh1579). Stable
transfection of Src shRNAs led to downregulation of Src, dramatic inhibition of Src activation (Fig. 2C, left) and significant suppression of invasion of the simulated BBB in both MDA-MB-231.Br (3.14-fold) and BT474.Br (4.74-fold) models (Fig. 2C, right). In vivo brain metastasis assays further tested the role of Src activation in promoting brain metastasis. We injected an equal mixture (1:1 ratio) of Src-activated MDA-MB-231 cells labeled with tdTomato Red (Src-Y527F.TdRed) and control cells labeled with GFP (vec.GFP). Remarkably, 35 days after injection, mice developed overt brain metastases dominated by strong red fluorescent signals (Src-Y527F cells; Fig. 2D, left). As a byproduct of the model, intracarotid injection of tumor cells occasionally generated extracranial lesions. Interestingly, the extracranial tumors observed had a similar ratio of parental (GFP-labeled) and Src-activated (TdRed-labeled) cells (Fig. 2D), in sharp contrast with a dominant brain metastatic tumor formation by the Src-activated cells. We also carried out the same experiment by injecting tdTomato red-labeled MDA-MB-231 cells that stably overexpress wild-type Src and control cells labeled with GFP (vec.GFP). Consistently, overt brain metastases contained significantly more Src-overexpressing cells than vector control cells ($P = 0.0051$; Supplementary Fig. S2). These in vivo observations pointed to a unique role of Src activation in the development of brain metastasis.

Src activation-mediated disruption of BBB facilitates tumor-cell extravasation

We next examined whether Src-activated cells may disrupt the BBB integrity to facilitate brain metastasis. We first tested the impact of Src activation in cancer cells on the integrity of human primary endothelial cell tight junctions in real-time using an electric cell-substrate impedance sensing (ECIS) assay \textit{in vitro} (Fig. 3A). Compared with a sustained transendothelial cell impedance after the plating of control MDA-MB-231 cells (231.Vec), cells expressing Src-WT or Src-Y527F showed a decreased impedance across the endothelial cell monolayer, indicating more disruption of the endothelial cell tight junctions.

Figure 2. Src activation in tumor cells promotes brain metastasis \textit{in vitro} and \textit{in vivo}. A, Western blotting examining the exogenous expression of Src wild-type (WT) and Src-Y527F mutant in MDA-MB-231 parental cells. B, trans-BBB assay. Left, schematic of the experimental setup and representative pictures. TdRed, tdTomato red. Right, fold increase of trans-BBB invasion after Src activation. C, left, Western blotting examining efficiency of Src knockdown by two independent shRNA constructs targeting Src; right, quantification of fold decrease of trans-BBB invasion after knockdown of Src. D, \textit{in vivo} brain metastasis assay. Left, schematic of the experiment procedures and representative pictures of metastatic tumors and extracranial tumors; right, quantification of normalized fluorescence intensity of GFP or TdRed tumors. Quantitative data are presented as mean ± SEM.
junctions (Fig. 3A). In addition, compared with 231.Vec cells, injection of 231.Src-Y527F cells into mice resulted in a much greater compromise of BBB integrity in vivo, as measured by the accumulation of 2 BBB integrity tracers in the brain parenchyma (Fig. 3B and C). Compared with vector control cells, Src-activated cells induced a 2-fold increase of accumulation of both small molecular weight BBB tracer (sodium fluorescein) and high molecular weight tracer (Evans blue) in the brain parenchyma (Fig. 3C). Concordantly, 5 days after intracarotid injection, significantly ($P = 0.0132$) more Src-activated cells had extravasated successfully into the brain parenchyma than the 231.Vec control cells, likely due to a leaky BBB (Fig. 3D).

**Src-targeting combinatorial treatments inhibit experimental brain metastasis**

We next sought to preclinically test the efficacy of targeting Src for brain metastasis treatment. Although once considered a promising targeted therapy for HER2$^+$ breast cancer brain metastases, lapatinib, an EGFR and HER2 dual-targeting small-molecule inhibitor, showed only a modest efficacy (6%) as a monotherapy in treating brain metastases in limited clinical studies (23, 24). We reasoned that a combination of the small-molecule Src inhibitor saracatinib plus lapatinib might be a superior regimen for HER2$^+$ brain metastases therapy. To support this hypothesis, we first showed that saracatinib abolished cell migration in a wound-healing assay (Supplementary Fig. S3) and significantly enhanced the inhibitory effects of lapatinib on trans-BBB invasion and the adhesion of 231.Br cells transfected with HER2 to the extracellular matrix (Fig. 4A). Similarly, saracatinib and lapatinib treatment each inhibited the invasion of BT474.Br cells ($\sim$60% and $\sim$75% inhibition, respectively) and combinatorial treatment further enhanced the inhibitory effect to about 90% (Supplementary Fig. S4). Moreover, compared with either single-drug treatment, the combinatorial treatment more effectively inhibited proliferation of BT474.Br and 231.Br cells (Fig. 4B). Together, the data indicated that Src-targeting combinatorial treatments can more effectively inhibit both metastasis-related properties and proliferation of brain-seeking cancer cells.

Next, we investigated this combinatorial regimen for both prevention and treatment of breast cancer brain metastasis...
HER2 cells were treated with treatments. MDA-MB-231.Br. and adhesion capability by different metastasis. A, inhibition of invasion HER2 and saracatinib (5 μmol/L), or combination for designated time. Left, invasion assay; right, adhesion assay. B, MTT assay examining the inhibition of cell proliferation. Cells were treated as A for 72 hours. C, prevention of brain metastases by combinatorial regimen. Mice were treated with vehicle, saracatinib (25 mg/kg), lapatinib (100 mg/kg), or combination daily. Left, representative picture of brain collected at the end of experiments. Top right, schematic of the experimental procedure; bottom right, quantification of metastatic tumors after designated treatments. D, overall survival of tumor-bearing mice after treatments. For 231.Br.HER2 model, mice received vehicle, saracatinib (25 mg/kg), lapatinib (100 mg/kg), or combination treatment by daily gavage starting 10 days after injection. For BT474. Br model, mice received vehicle, saracatinib (25 mg/kg), lapatinib (50 mg/kg), or combination treatment by daily gavage starting 7 days after injection. Overall survival was compared with log-rank test between vehicle group and combinatorial treatment group. All quantitative data are presented as mean ± SEM.

**Figure 4.** Combinatorial treatment of lapatinib and Src inhibitor inhibits HER2+ experimental brain metastasis. A, inhibition of invasion and adhesion capability by different treatments. MDA-MB-231.Br. HER2 cells were treated with vehicle, saracatinib (1 μmol/L), lapatinib (5 μmol/L), or combination for designated time. Left, invasion assay; right, adhesion assay. B, MTT assay examining the inhibition of cell proliferation. Cells were treated as A for 72 hours. C, prevention of brain metastases by combinatorial regimen. Mice were treated with vehicle, saracatinib (25 mg/kg), lapatinib (100 mg/kg), or combination daily. Left, representative picture of brain collected at the end of experiments. Top right, schematic of the experimental procedure; bottom right, quantification of metastatic tumors after designated treatments. D, overall survival of tumor-bearing mice after treatments. For 231.Br.HER2 model, mice received vehicle, saracatinib (25 mg/kg), lapatinib (100 mg/kg), or combination treatment by daily gavage starting 10 days after injection. For BT474. Br model, mice received vehicle, saracatinib (25 mg/kg), lapatinib (50 mg/kg), or combination treatment by daily gavage starting 7 days after injection. Overall survival was compared with log-rank test between vehicle group and combinatorial treatment group. All quantitative data are presented as mean ± SEM.

**in vivo.** First, oral gavage of saracatinib led to a prominent suppression of pSrc-Y416 in brain metastasis lesions derived from 231.Br.HER2 cells in mice, indicating the BBB permeability of saracatinib (Supplementary Fig. S5). We then tested saracatinib for prevention of brain metastasis with treatment started at day 0 after intracarotid injection, before tumor cell extravasation (Fig. 4C). Saracatinib decreased the overall incidence of brain metastasis (4 of 10 mice had brain metastases) compared with vehicle-treated group (9 of 10 mice), which is likely due to reduced tumor cell extravasation due to Src inhibition. Histologic examination 30 days after tumor cell injection revealed that the combinatorial regimen of lapatinib and saracatinib exhibited significantly (P = 0.036, Student t test) better efficacy in reducing overt brain metastases, The combinatorial treatment effectively reduced the large brain metastases derived from 231.Br.HER2 cells (Fig. 4C, left). We next tested the efficacy of the combinatorial regimen in treatment of established metastases. Combinatorial treatment significantly (P = 0.004, log-rank test) extended overall survival of brain metastases-bearing mice (Fig. 4D, left, 231.Br.HER2 model). In the BT474 model, consistent with 231.Br model, lapatinib or saracatinib alone had only modest effects on prolonging the survival of brain metastases-bearing mice, whereas combinatorial treatment significantly extended the survival time of these mice (median survival of 43 days in vehicle control group vs. 103 days in combinatorial treatment group, P = 0.0177; Fig. 4D, right). Collectively, these data strongly support the potential clinical application of this combinatorial regimen of targeted therapies for the treatment of brain metastases.

**Combinatorial treatment more effectively suppresses Akt signaling and induces apoptosis**

To explore the mechanisms underlying the therapeutic efficacy of combinatorial treatment, we conducted PathScan Protein Array analysis for signaling changes after combinatorial treatment of brain-seeking BT474.Br cells. As expected, combinatorial treatment with lapatinib and saracatinib completely abolished Src phosphorylation (Fig. 5A, top) and suppressed the phosphorylation of multiple receptor tyrosine kinase.
kinases (RTK), including HER2, HER3, and Eph family receptors (Fig. 5A). In addition, phosphorylation of both Akt and Erk was also inhibited (Fig. 5A). Western blotting validated that cotreatment with lapatinib and saracatinib more significantly inhibited HER2 and Akt phosphorylation than either individual treatment (Fig. 5B). Saracatinib treatment of BT474.Br cells further inhibited cell proliferation and promoted lapatinib-induced PARP cleavage. The phosphorylation of histone H3 serine 10 (a proliferation marker) in BT474.Br cells was greatly diminished in combination treatment group compared with the control group (Fig. 5C). Concurrently, cleaved PARP, an apoptosis marker, was detected in combinatorial therapy-treated BT474.Br cells but not in control- or single drug-treated cells (Fig. 5A, and data not shown). Consistently, a sub-G₁ detection assay (Fig. 5D) showed that combinatorial treatment of BT474.Br cells resulted in more than 60% apoptotic cells compared with less than 40% apoptosis by lapatinib single treatment (Fig. 5D).

**Combinatorial treatment suppresses cell-cycle progression**

A recent study has suggested that inhibition of Src disrupts pyruvate kinase M2 (PKM2)-dependent β-catenin transactivation of cyclin D1 (25). To investigate whether this mechanism may also contribute to the inhibition of proliferation of BT474.Br and 231.Br cells, we further analyzed the cell-cycle profile after combinatorial treatment. Cell-cycle profiling coupled with EdU incorporation assay...
using 231.Br model revealed that inhibition of Src and HER2 signaling by combinatorial therapy led to a decrease of EdU incorporation (Fig. 6A, top), an indicator of reduced DNA synthesis. Correspondingly, significantly more cells were arrested at G1 phase (Fig. 6A, bottom). Furthermore, while neither saracatinib nor lapatinib single treatment could significantly reduce cyclin D1 expression in either BT474.BR or 231.Br.HER2 cells, combinatorial therapy led to a prominent decrease of cyclin D1 protein expression in both cell lines (Fig. 6B and C and Supplementary Fig. S6). In BT474.Br cells, combined treatment also led to a strong induction of p27 protein expression (Fig. 6B). Thus, Src-targeting combinatorial treatment further enhanced the therapeutic effects on cell-cycle regulatory proteins. In accordance with these data, IHC staining of brain metastasis tumors from mice injected with 231.Br.HER2 cells also showed that combinatorial therapy led to fewer tumor lesions with a significant decrease of cyclin D1-positive cells compared with single treatments (Fig. 6D).

**Discussion**

To develop novel therapies for brain metastases, it is imperative to understand the mechanism of the disease. Brain metastasis is a complex and inefficient process. As such, in vivo models that simulate the development of brain metastasis are limited (19, 26). None of the currently brain metastasis models adequately represent breast cancers with endogenous HER2 amplification. Intracranial injection of HER2+ tumor cells directly into brain parenchyma (27) bypassed the most critical obstacle of brain metastasis, the BBB, failing to faithfully model the brain metastasis. Previously, in lieu of endogenously HER2+ models, the 231.Br model was transfected with HER2 for use in brain metastasis preclinical study (14). However, this model’s problems include the facts that HER2 is exogenously overexpressed and HER3, which is crucial for HER2 signaling, is not expressed at all (14, 15). BT474 cells have endogenous HER2 amplification and here, we show that they readily form moderately invasive brain metastatic tumors. Histologically, BT474 brain metastasis tumors have a distinct

*Offprint requests: *S. M. Sullivan, Department of Neurology, University of North Carolina at Chapel Hill, 2107 CDs 221時代, Chapel Hill, NC 27599.

**Fig. 6.** Combinatorial treatment of lapatinib and saracatinib inhibits cell-cycle progression. A, EdU incorporation assay showing G1 arrest induced by drug treatment. 231.Br.HER2 cells were treated as B. Twenty-four hours after treatments, cells were pulse labeled for EdU for another 2 hours before fluorescence-activated cell sorting analysis. B, Western blotting showing the inhibition of cyclin D1 and induction of p27 expression by drug treatment in BT474.Br cells. Cells were treated with lapatinib, saracatinib alone, or combination for 24 hours. C, Western blotting showing the inhibition of cyclin D1 and induction of p27 expression by drug treatment in 231.Br.HER2 cells. Cells were treated with lapatinib, saracatinib alone, or combination for 24 hours. D, IHC for cyclin D1 expression in experimental metastasis tumors. Tumors from in vivo brain metastasis assay (Fig. 4c) were stained for cyclin D1 expression: representative picture of IHC staining (top); quantification of nuclear-positive staining of cyclin D1 (bottom). Quantitative data are presented as mean ± SEM.
metastasis pattern from that of the MDA-MB-231 model (Fig. 1A). Importantly, the BT474 model might more faithfully represents the therapeutic response of HER2† tumor to anti-HER2 therapies, which makes it an ideal model for testing HER2-targeting combinatorial therapies for HER2† tumor brain metastasis.

Our studies shed new light on the potential clinical application of Src inhibitor-containing regimens in the treatment of HER2† breast cancer brain metastasis. While Src inhibitor holds promise in treating metastatic disease, Src inhibitor monotherapy clinical trials were unsuccessful (28). Previous Src inhibitor trials were conducted in unselected patients, which may be one underlying reason for the overall low response rate observed. To improve the clinical benefit of Src-targeting therapies, it will be important to preselect patients whose tumors have Src activation before applying Src inhibitors. Indeed, a number of preclinical studies showed a strong correlation between Src pathway activation with the response to Src inhibitors and the importance of hyperactivated Src signaling for the ideal therapeutic efficacy of Src inhibitors (29, 30). In breast cancer cells, the expression of Yes-associated protein-1 (YAP1), Src pathway-associated protein, strongly correlated with a response to the Src inhibitor dasatinib (10). Consistently, our RPPA analysis of brain-seeking breast cancer sublines revealed Src activation along with a prominent increase of YAP phosphorylation at S127, an indicator of YAP activation (Fig. 1B). Collectively, these data indicate a super activation of Src pathway in brain metastases and provide the rationale to target Src activation for treating brain metastasis. Preselection of patient groups using Src activation biomarker may improve the clinical efficacy of Src-targeting therapies, especially for patients with brain metastasis.

Src is a key downstream transducer of many RTKs, especially EGFR and HER2 (8, 31). The crosstalk between Src signaling and RTKs facilitates RTK-addicted tumor growth (32). For HER2† tumors, we and others recently showed that targeting Src signaling significantly sensitized resistant tumors to anti-HER2 therapies (7, 6). In this study, we also showed that Src inhibitor plus lapatinib extended the overall survival of mice bearing HER2† brain metastasis tumors. As clinical studies have indicated that lapatinib has only moderate therapeutic efficacy for brain metastasis (14, 23), it is conceivable that combining Src inhibitors with lapatinib should enhance the efficacy in treating brain metastasis, especially for HER2† breast cancer brain metastasis. Notably, we also observed additive effects of this combinatorial regimen in inhibiting brain metastasis of the triple-negative MDA-MB-231.Br cells (Fig. 4B). This suggested that the combinatorial therapy might also be efficacious for treatment of brain metastasis derived from TNBC. In addition, our data on Src promoting the extravasation of disseminated tumor cells, an early step in the metastatic cascade, emphasized the importance of choosing the right therapeutic window. To obtain the optimum efficacy in treating and preventing brain metastasis, clinical testing of Src-targeting combinatorial therapies in the after adjuvant treatment setting is warranted (33). Applying rationally designed combinatorial therapies in adjuvant setting may not only reduce the risk of metastatic recurrence, but also prevent the outgrowth of secondary metastases.

In summary, our studies functionally and mechanistically define an important role of Src activation in promoting breast cancer brain metastasis. The high efficacy of the combinatorial regimen of lapatinib plus saracatinib for prevention and treatment of HER2† experimental brain metastases may complement the limited existing clinical options for patients with brain metastasis.

Disclosure of Potential Conflicts of Interest

P.S. Steeg has commercial research support from Geron. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: S. Zhang, W.-C. Huang, C. Zhang, Z. Ding, A.A. Sahin, P.S. Steeg, D. Yu

Development of methodology: S. Zhang, W.-C. Huang, L. Zhang, Z. Ding

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Zhang, W.-C. Huang, L. Zhang, C. Zhang, F.J. Lowery, Z. Ding, H. Guo, D. Yu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Zhang, W.-C. Huang, L. Zhang, H. Guo, H. Wang, A.A. Sahin, K.D. Alldape, D. Yu

Writing, review, and/or revision of the manuscript: S. Zhang, W.-C. Huang, F.J. Lowery, K.D. Alldape, P.S. Steeg, D. Yu

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Zhang, W.-C. Huang, S. Huang, K.D. Alldape, D. Yu

Study supervision: S. Zhang, D. Yu

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References


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