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Heparanase mediates a novel mechanism in lapatinib-resistant brain metastatic breast cancer[1,2]

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Abstract

Heparanase (HPSE) is the dominant mammalian endoglycosidase and important tumorigenic, angiogenic, and pro-metastatic molecule. Highest levels of HPSE activity have been consistently detected in cells possessing highest propensities to colonize the brain, emphasizing the therapeutic potential for targeting HPSE in brain metastatic breast cancer (BMBC). Lapatinib (Tykerb) is a small-molecule and dual inhibitor of human epidermal growth factor receptor1 and 2 (EGFR and HER2, respectively) which are both high-risk predictors of BMBC. It was approved by the US Food and Drug Administration for treatment of patients with advanced or metastatic breast cancer. However, its role is limited in BMBC whose response rates to lapatinib are significantly lower than those for extracranial metastasis. Because HPSE can affect EGFR phosphorylation, we examined Roneparstat, a non-anticoagulant heparin with potent anti-HPSE activity, to inhibit EGFR signaling pathways and BMBC onset using lapatinib-resistant clones generated from HER2-transfected, EGFR-expressing MDA-MB-231BR cells. Cell growth, EGFR pathways, and HPSE targets were assessed among selected clones in the absence or presence of Roneparstat and/or lapatinib. Roneparstat overcame lapatinib resistance by inhibiting pathways associated with EGFR tyrosine residues that are not targeted by lapatinib. Roneparstat inhibited the growth and BMBC abilities of lapatinib-resistant clones. A molecular mechanism was identified by which HPSE mediates an alternative survival pathway in lapatinib-resistant clones and is modulated by Roneparstat. These results demonstrate that the inhibition of HPSE-mediated signaling plays important roles in lapatinib resistance, and provide mechanistic insights to validate the use of Roneparstat for novel BMBC therapeutic strategies.

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Introduction

The family of epidermal growth factor receptors (HER/ErbB) plays pivotal roles in the regulation of breast cancer progression and metastasis [1]. One of the four HER family members, epidermal growth factor receptor1 (EGFR, HER1, or ErbB1) is overexpressed in 25% to 80% of breast cancers [2], while another, epidermal growth factor receptor2 (HER2, *neu*, or ErbB2), is amplified and/or overexpressed in approximately 20% to 30% of primary breast

Abbreviations: ANOVA, analysis of variance; BR, HER2-transfected MDA-MB-231BR; BMBC, brain metastatic breast cancer; COX-2, cyclooxygenase-2; DME/F-12, Dulbecco's modified Eagle's/F-12 medium; ERK, extracellular signal-regulated kinase; EGFR, human epidermal growth factor receptor1; FACS, fluorescence activated cell sorting; FAK, focal adhesion kinase; FBS, fetal bovine serum; HER2, human epidermal growth factor receptor2; HPSE, heparanase; HS, heparan sulfate; Ls/Lr BR clones, lapatinib-sensitive/lapatinib-resistant BR clones; MAPK, mitogen-activated protein kinase; MMP-9, matrix metalloprotease-9; PBS, phosphate-buffered saline; PI3K, phosphoinositide 3-kinase; STR, short tandem repeat

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[2] Author contributions: L.Z. performed experiments, data analysis, and wrote the manuscript; M.W. carried out colony formation and ELISA assays, and J.N. completed ELISA and HPSE assays and cell culture. D.M. designed and supervised experiments, and edited the manuscript.

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cancers [3]. Lapatinib (Tykerb, GW572016) is a small-molecule and dual inhibitor of EGFR and HER2. Its use was approved by the US Federal and Drug Administration for therapeutic combinations with capecitabine for the treatment of patients overexpressing HER2 that have received prior therapy, including anthracycline and the humanized HER2 antibody trastuzumab (Herceptin) [4]. Brain metastatic breast cancer (BMBC) is frequently detected in patients overexpressing HER2 and EGFR [5]. However, either systemic or recent targeted therapies using lapatinib are only minimally effective with BMBC response rates far lower (approximately 5%) than those with extracranial metastases [6–8].

Molecular mechanisms countering lapatinib resistance are not fully understood and subject of intense investigation because of their therapeutic implications. Roles of lapatinib in cell survival and proliferation are dependent and selective upon EGFR and HER2 phosphorylation and tyrosine kinase catalytic activity [4,6–8]. For example, EGFR and HER2 signal through the phosphoinositide 3-kinase (PI3K) pathway and lapatinib was shown to block downstream signaling via PI-3K/Akt and mitogen-activated protein kinase (MAPK) pathways in breast cancer cells by interrupting baseline and ligand-stimulated activity [6,7,9]. However, not all EGFR/HER2-expressing breast cancer cells respond to lapatinib, particularly if cells overexpress EGFR such as the brain-seeking MDA-MB-231BR variant [5,7].

Heparanase (HPSE) is the dominant mammalian endoglycosidase (endo-β-D-glucuronidase), cleaving heparan sulfate (HS) to fragments which retain biological activity. By this action, HPSE releases important HS/heparin-binding angiogenic and growth factors, affecting their levels and biological potency [10–12]. HPSE functions are not limited to HS cleavage or the release of HS-sequestered growth factors but also affect clustering, shedding, and mitogenic activity of HS proteoglycans, e.g., cell surface syndecans, which are the main HPSE targets [10–13]. Heparanase activity correlates with the metastatic potential of cancer cells, a notion that is well-supported experimentally and clinically [10–19]. Of relevance, highest HPSE levels have been consistently detected in tumor cells selected to possess highest propensities to colonize the brain [14–16] with the recent evidence for its expression in patient-isolated breast cancer circulating tumor cells competent to generate brain metastasis in xenografts [20]. Apart from its well-characterized enzymatic activity, heparanase was also shown to exert enzymatic-independent functions, e.g., acting as a signal transducer and regulator of cell adhesion [21] and cytoskeletal dynamics [22]. A recent report also showed that HPSE augmented EGFR phosphorylation that correlated with head and neck tumor progression [18]. Heparanase can thus initiate broad effects that dramatically alter the microenvironment and stimulate tumor cell growth and metastasis. Altogether, these notions raised the following questions – Is HPSE implicated in lapatinib resistance of breast cancer cells expressing EGFR and HER2? If so, by which mechanism(s)?

We hypothesized that HPSE is implicated in mechanisms of lapatinib resistance of breast cancer cells expressing EGFR/HER2, and promotes alternative signaling pathways which are not inhibited by lapatinib, with HPSE inhibition suppressing tumor growth and BMBC. To examine this, we used Roneparstat, a chemically modified heparin lacking anticoagulant activity and a potent inhibitor of heparanase activity [19,23]. We selected and used lapatinib-resistant clones generated from the human brain-colonizing MDA-MB-231BR breast cancer cell line [24], then studied Roneparstat-mediated actions related to the lapatinib resistance in these clones. We show that the inhibition of HPSE activity by Roneparstat overcomes lapatinib resistance and suppresses cell growth in vitro and BMBC onset in vivo. These findings provide the molecular basis to potentially employ Roneparstat in therapeutic approaches of lapatinib-resistant breast cancers, particularly breast cancer brain metastasis.

Materials and Methods

Tissue Culture, Establishment of Lapatinib-Resistant Clones, and Clones Characterization

The human brain metastatic MDA-MB-231BR cell line was obtained from Dr. Toshiyuki Yoneda (The University of Texas Health Science Center-San Antonio, TX). It was derived from MDA-MB-231 parental cells by six sequential cycles of selection followed by cell injection into the internal carotid artery of nude mice, and resulting in augmented abilities to generate brain metastasis over the parental counterpart [24]. The MDA-MB-231BR clone *transfected with HER2* (named BR for brevity; original cells possess low HER2 levels) (Figure S3) [5] was provided by Dr. Patricia Steeg (National Cancer Institute, Bethesda, MD). MDA-MB-231 parental, MDA-MB-231BR, and BR variant cell lines were authenticated by short tandem repeat (STR) DNA fingerprinting analyses for 16 loci, and data compared to the database of the Characterized Cell Line Core at MD Anderson Cancer Center (Houston, TX). Lapatinib-resistant cell lines were obtained from surviving BR cells exposed to increasing lapatinib concentrations (0.1, 0.5, 1, 5, and 10 μmol/L) in DMEM/F12 culture medium for 4 to 5 weeks. Resistance at each dose was assessed by comparing the growth of each resistance derivative to parental BR cells. Next, surviving cells were treated chronically in vitro with lapatinib (1 μmol/L) [25]. Medium supplemented with this lapatinib concentration was changed every 2 to 3 days with cells being continuously exposed to lapatinib for a three-months period, and resulting in the generation of BR lapatinib-resistant (BR-Lr) clones. Lapatinib-sensitive (BR-Ls) clones were also obtained from BR cells using the limiting dilution method (single-cell colonies) in 96-well plates [25]. BR-Lr and BR-Ls clones were cultured in Dulbecco's Modified Eagle Medium plus F12 (DMEM/F12) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), and 1% penicillin and streptomycin (Life Technologies). All BR-Lr clones were cultured in a humidified, 5% CO_2 incubator at 37°C, passaged twice weekly along the same schedule, and used only at low passage and if *Mycoplasma* negative. Experimental metastasis assays were periodically performed to test cell in vivo tumorigenic abilities. These studies were accomplished per protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine, and involved all steps of animals sacrifice and amelioration of suffering. Cell cycle analyses based upon fluorescence activated cell sorting (FACS) were carried out using the FACSCalibur instrument at the flow cytometry core facility of Baylor College of Medicine, and analyzed using a CellQuest software (BD Bioscience).

Antibodies, Reagents, and Inhibitors

Primary antibodies include mouse anti-human heparanase monoclonal antibody which was obtained from Cedarlane Laboratories (Burlington, NC). Rabbit anti-human heparanase was kindly provided by Dr. Israel Vlodavsky (The Rappaport Institute-Technion, Haifa, Israel). The other primary antibodies were purchased from Cell Signaling (Danvers, MA). Secondary antibodies included: goat anti-rabbit IgG [H+L]-HRP and goat anti-mouse IgG [H+L]-HRP that were purchased from Santa Cruz