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AWARD NUMBER: W81XWH-11-1-0315

TITLE: Heparanase Mechanisms in Brain - metastatic Breast Cancer

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REPORT DATE: April 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE April 2013		2. REPORT TYPE Annual		3. DATES COVERED 1 April 2012 – 31 March 2013	
4. TITLE AND SUBTITLE Heparanase Mechanisms in Brain-Metastatic Breast Cancer				5a. CONTRACT NUMBER W81XWH-11-1-0315	
				5b. GRANT NUMBER W81XWH-11-1-0315	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dario Marchetti, PhD E-Mail: marchett@bcm.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine Houston, Texas 77030				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Brain-metastatic breast cancer (BMBC) is common in patients expressing epidermal growth factor receptor1 or 2 (EGFR or HER2). Lapatinib is a small-molecule inhibitor of EGFR and HER2 and EGFR/HER2-associated downstream signaling which result in the suppression of brain colonization propensities of in vivo-selected MDA-MB-231BR variant (BR for brevity). Conversely, heparanase (HPSE) is a potent tumorigenic, angiogenic, and pro-metastatic enzyme known to initiate effects which drastically alter the metastatic outcome. We selected lapatinib-sensitive and lapatinib-resistant clones (BR-Ls and BR-Lr) from BR parental cells and demonstrated that HPSE over-expression in BR-Lr but not BR-Lr clones. Addition of HPSE to BR-Lr cells resulted in EGFR phosphorylation and signaling, and to an augmented HPSE secretion and activity. Second, we used SST0001, a non-anticoagulant heparinoid with a potent anti-HPSE activity, and examined its action on BR-Lr/Ls clones. SST0001 effectively synergized with lapatinib to inhibit cell proliferation of BR-Lr cells. Similarly, HPSE inhibition was associated with reduced EGFR phosphorylation levels in those tyrosine residues not targeted by lapatinib (Y992); and reflecting reduced pSRC, pAKT, and pERK levels. Lastly, SST0001 in combination with lapatinib blocked tumor growth <i>in vivo</i> and BMBC by BR-Lr cells. These results provide novel insights into mechanisms responsible for lapatinib resistance in BMBC.					
15. SUBJECT TERMS Brain-metastatic breast cancer (BMBC), Heparanase (HPSE), EGFR, HER2, Lapatinib, BR-Lr, BR-Ls.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU		19b. TELEPHONE NUMBER (include area code)

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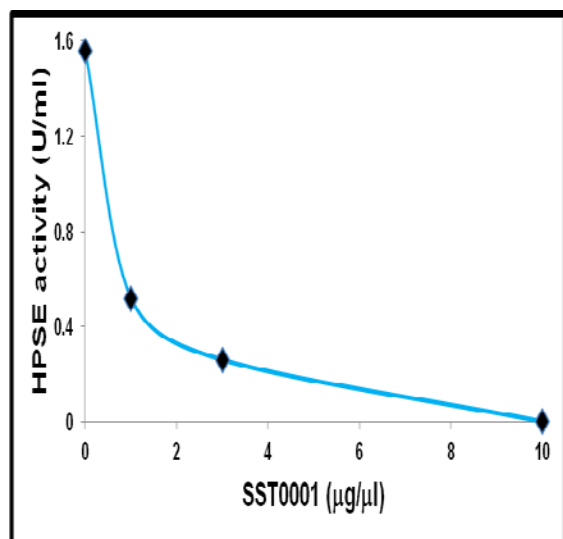
Introduction

Heparanase (HPSE) is the dominant endoglycosidase (endo- β -D-glucuronidase) in mammals, 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. Heparanase activity thus correlates with the metastatic potential of cancer cells, a notion that is well-supported experimentally and clinically. Of relevance, highest HPSE levels have been consistently detected in tumor cells selected to possess highest propensities to colonize the brain. Lapatinib (Tykerb, GW572016) is a selective small-molecule inhibitor of human epidermal growth factor receptor1 and 2 (EGFR and HER2, respectively) that was approved by the US Food and Drug Administration (FDA). Its role is however limited, particularly in brain metastatic breast cancer (BMBC) due to their resistance to lapatinib-based therapies. The current study was designed to test the hypothesis that HPSE mediates an alternative survival mechanism in lapatinib-resistant, BMBC-competent cells.

Body

This represents the second-year report for the DoD-CDMRP IDEA Award, and summarizes the progress made between April 1, 2012 and March 31, 2013. Of note, this progress report relates to completion of specific aim 2 and its sub-tasks since progress made for specific aim 1 and related sub-tasks was described in the Annual Report 1. Achievement of this aim has also resulted in a publication in *Cancer Research-Priority Report* (Zhang *et al.*, 71: 645-654, 2011). We have addressed specific aim 2 of the proposal and implemented sub-tasks per statement of work (SOW) for the second year of this Award. Findings pertaining each of these SOW subtasks have resulted in a manuscript submitted to *Clinical Cancer Research*, and are summarized below:

- 2a. Implement SST0001 toxicity and dose-dependent and dose optimization studies to determine its effects on: a) HPSE and HER2/EGFR phosphorylation; b) inhibition of *in vitro* cell adhesion, growth and invasion following SST0001 exposure to BMBC cells (months 13-14).



We have performed SST0001's dose optimization studies to determine its effects on the inhibition of HPSE (**Figure 1**). Secondly, we have derived lapatinib resistant-clones (BR-Lr) from MDA-MB231BR parental cells, and studied cell cycle profiles and quantitative analyses (**Figure 2**).

Figure 1 (left). Dose-dependent inhibition of HPSE activity by SST0001. Aliquots of 50 μ l of eluted active HPSE were treated with 0, 1, 3, and 10 μ g/ μ l SST0001 for 2 hours at 37°C. HPSE activity (U/ml) was then determined using the Heparan Sulfate Degrading Enzyme Assay kit™ (Takara, Inc.) per manufacturer's protocol.

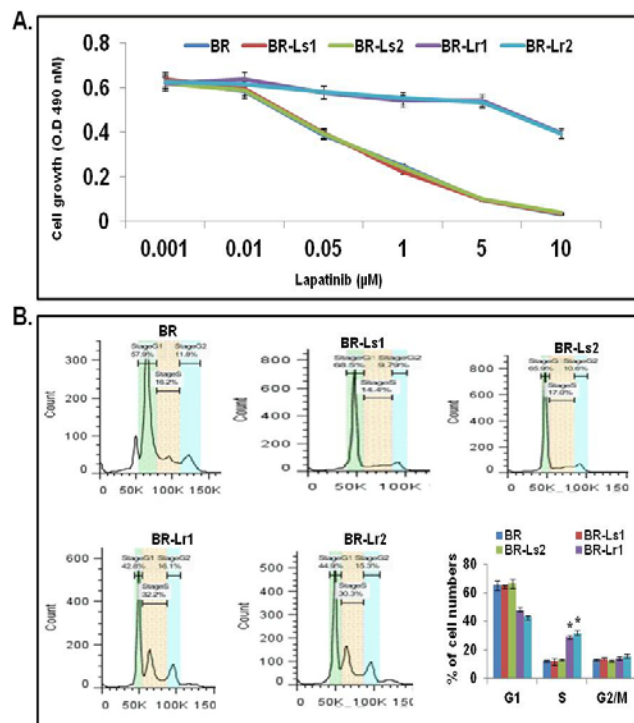


Figure 2 (left). Selection and growth of lapatinib resistant BR cells. **A.** Lapatinib-resistant cell lines were selected from surviving MDA-MB23BR cells (BR for brevity) exposed to different concentrations of lapatinib (0.1, 0.5, 1, 5 and 10 μmol/L) in DMEM/F12 culture medium) for 3 weeks, with the medium changed every 2-3 days. Surviving cells were followed by a chronic treatment of lapatinib (2 μmol/L) *in vitro*. Cells that developed resistance to lapatinib were then selected for amplification and characterization. **B.** Representative cell cycle profiles and quantitative analysis of MDA-MB231-BR parental and lapatinib-resistant and lapatinib-sensitive (BR-Lr and BR-Ls, respectively) cell profiles were determined by fluorescence-activated cell sorting. Quantitative analysis of respective cell cycle profiles are shown (* $p < 0.01$; $n = 5$).

Secondly, we have investigated the expression, activity, and secretion of HPSE in the lapatinib – resistant and lapatinib-sensitive (BR-Lr and BR-Ls, respectively) MDA-MB231BR clones we have derived. Highest HPSE concentration and activity were detected in BR-Lr clones (**Figure 3, below**).

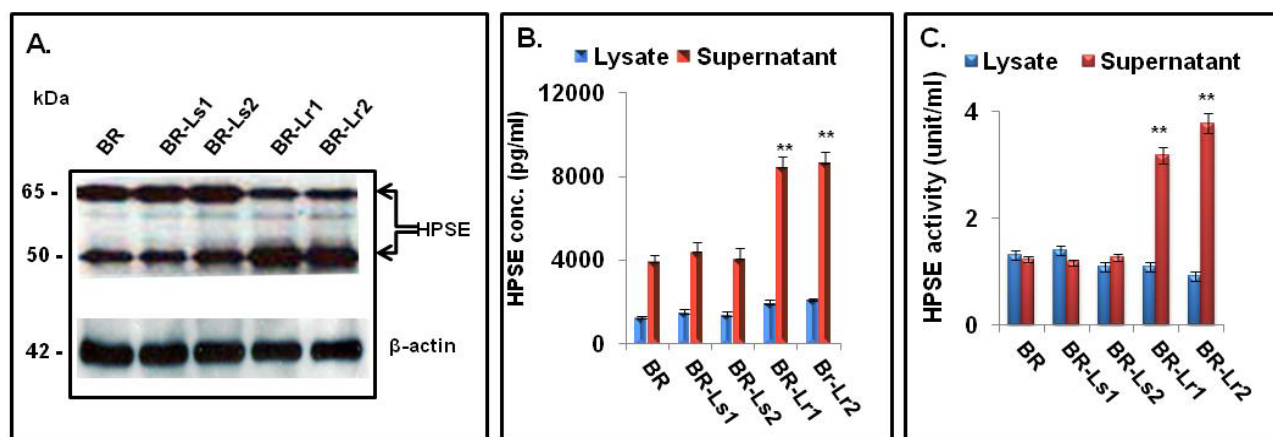


Figure 3. Heparanase (HPSE) expression and activity in selected MDA-231MB-BR lapatinib-resistant and sensitive cells (BR-Lr and BR-Ls, respectively). **A.** Western blotting analysis of heparanase protein expression in MDA-MB231BR (BR for brevity) cells and BR-Lr/Ls clones. **B.** Heparanase expression in cell supernatants and lysates of BR parental and BR-Lr/Ls clones by ELISA methodology. **C.** Heparanase activity of cell supernatants and lysates of BR parental cells and BR-Lr/Ls clones. HPSE activity assays were performed as previous described (9).

Thirdly, we studied the effects of SST0001 and lapatinib to synergistically inhibit BR-Lr cell growth using the 3D mammosphere models, and signaling of EGFR, a high-risk predictor for BMBC onset and HPSE target (**Figure 4**).

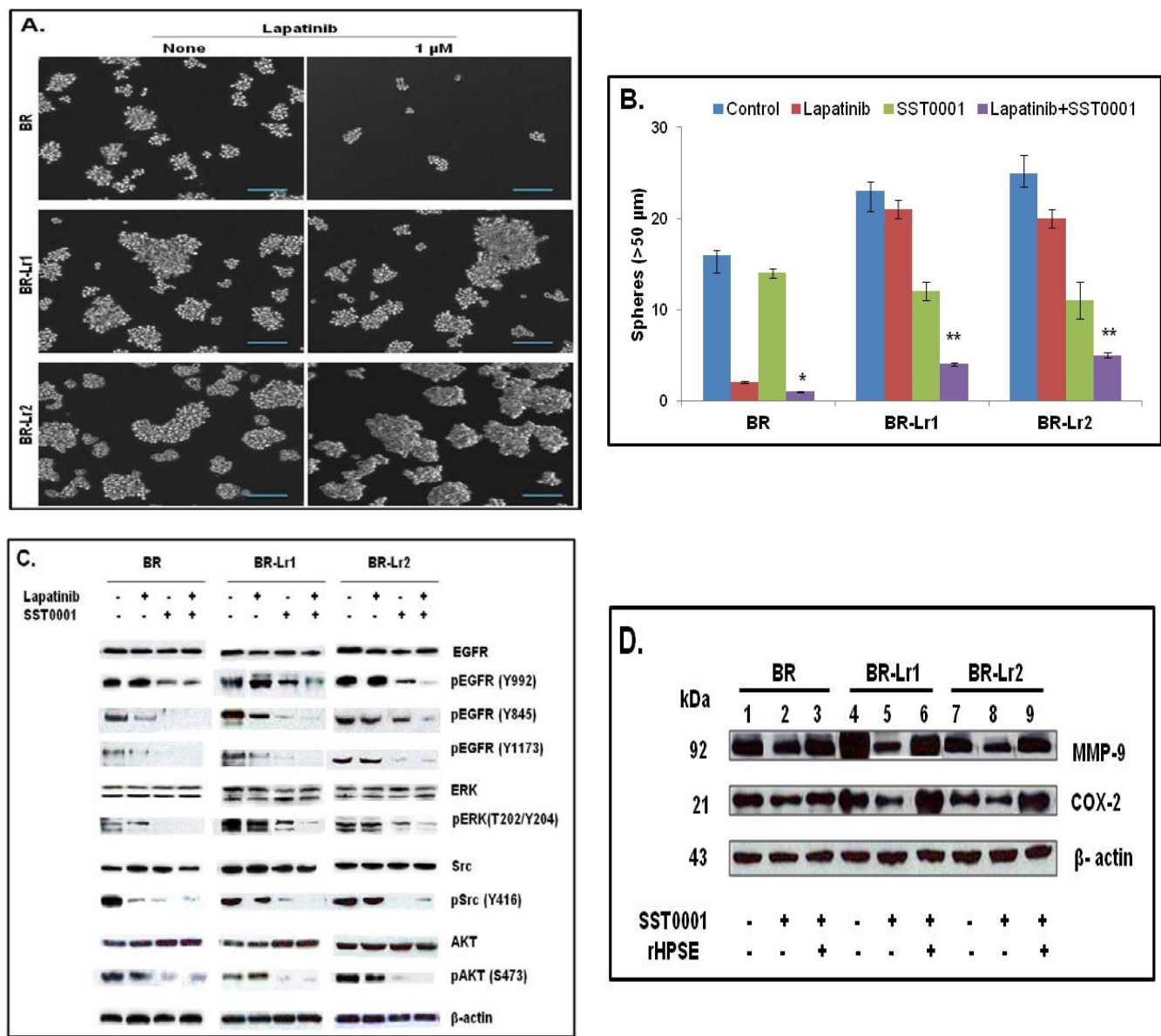


Figure 4. SST0001 and lapatinib synergistically inhibit BR-Lr cell growth. **A.** Mammosphere assays performed for MDA-231BR parental (BR for brevity) and BR lapatinib-resistant clones (BR-Lr1 and BR-Lr2, respectively) in the absence or presence of lapatinib (1 μ M). **B.** Quantitative analysis of mammospheres. Cells were seeded in six-well plates, and lapatinib (1 μ M) and SST0001 (5 μ M)(10, 11) were added to the plates, replenished every 2-3 days, and monitored for 14 days in cell culture at 37°C - 5% CO₂. Afterwards, colonies ($> 50 \mu$ m in size)

were counted, photographed, and their number quantitatively analyzed. **C.** EGFR signaling pathway of BR-Lr1 and BR-Lr2 clones. Cells were treated with lapatinib and SST0001 (same concentrations as per figure 4B) overnight (16 hrs), then treated with EGF (20 ng/ml) for 10 min, and phosphorylation of indicated signaling proteins was examined by Western blotting analysis with highly reproducible results (n = 5). **D.** Rescue assays of effects of SST0001 on HPSE-regulated proteins. Cells were treated with SST0001 (5 μ M) or SST0001 plus recombinant heparanase (rHPSE, 100 ng/ml) for 24 hrs, then MMP-9 and COX2 expression levels were examined by Western blotting analyses (MMP-9 and COX2 are known target of HPSE; 9). β -actin was the loading control.

- 2b. Perform *in vivo* experimental brain metastasis assays in animals (nu/nu mice) to examine whether SST0001 will prevent BMBC. Injection of human BMBC cells (231BR3) in nude mice via intracarotid artery. Five days after cell injection, deliver SST0001 by inserting Alzet pumps into animals, and monitor BMBC onset. Use of 40 animals, randomly assigned to receive SST0001 or vehicle: 20 in SST0001 treatment group, 20 in vehicle control group (months 15-17).

Because of high HPSE activity presence detected in selected BR-Lr clones, we hypothesized that SST0001 could overcome lapatinib resistance by inhibiting HPSE activity in these cells. Accordingly, we examined effects of SST0001 on primary tumor growth and metastasis by injecting these clones into nude mice, followed by various drug treatments (PBS, lapatinib with or without SST0001, and SST0001 alone). These reagents were administered to animals by Alzet osmotic pumps after 7 days of tumor cell injection. They were delivered for 28 days with daily monitoring for tumor development. Once observed, tumors were removed and their extent, size, and wet-weight were assessed among treatment modalities. Tumor size was approximately 40% smaller in lapatinib/SST0001 than lapatinib alone-treated animals (**Figure 5A**). The tumor weight was also reduced by 62% (p<0.0001) in lapatinib/SST0001 group compared to controls, and 22% lower (p<0.01) when compared to lapatinib only-treated animals. Further, to analyze whether combinatorial SST0001 and lapatinib inhibited BR-Lr cell - induced BMBC, treatments continued for additional two weeks within the animal sub-groups. Afterwards, mice brains were surgically removed and examined for BMBC presence. A distinct reduction (>60%) of metastatic breast cancer cell number in brains of SST0001-treated mice was observed when compared to lapatinib alone or control groups (**Figures 5B and 5C**). Data suggest that SST0001 is able to significantly inhibit BR-Lr cell-induced tumor growth and BMBC.

Figure 5A

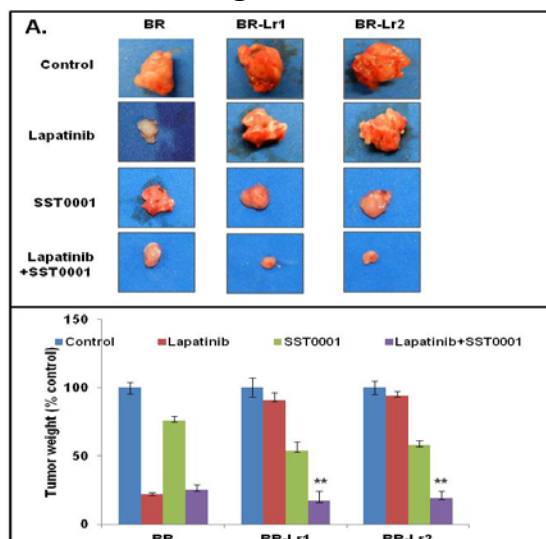


Figure 5B

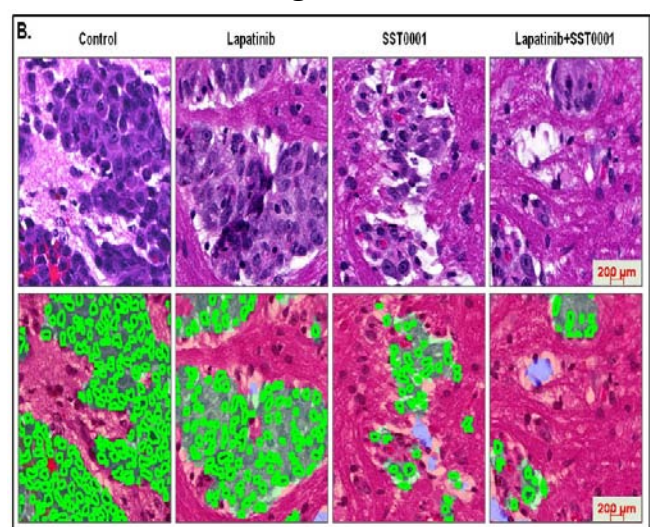


Figure 5C

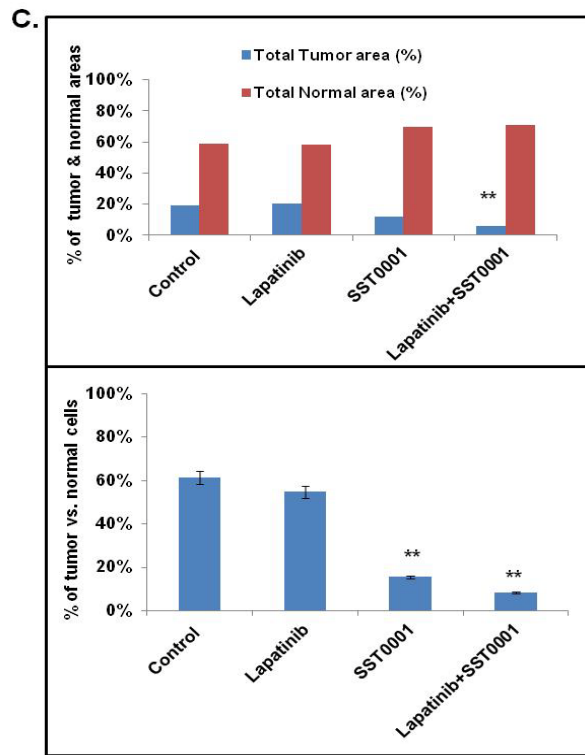


Figure 5. SST0001 and lapatinib synergistically inhibit tumor growth and BMBC (xenografts). **A.** BR parental and lapatinib-resistant BR cells (BR-Lr1 and BR-Lr2) were injected subcutaneously (1×10^6 cells/mouse; $n = 20$ mice per treatment group). Tumor growth was monitored daily by measuring the tumors' size (length and width) using a digital caliper. Tumors were then harvested and photographed (top). Tumor weight was calculated and compared among the various treatment groups (bottom). **B.** Representative images of Cri Vectra-Intelligent analysis of BMBC induced by BR-Lr cells. The Cri-Vectra-Intelligent imaging analysis system (Cambridge Research & Instrumentation, Inc.) was employed for the quantification of tumor cells at the single-cell level (green color) from H&E sections. The Vectra Intelligent™ software is based on machine-learning program to outline the tumor, recognize and distinguish significant histological features. **C.** H&E sections were randomly selected from animals with BR-Lr cell-induced BMBC. The graphic shows the quantification of BMBC tumor burden represented by total tumor areas and cells for the various treatment groups.

2c. Assessment of HPSE expression and activity in two additional BMBC cells (CN34-BrM2, and 231-BrM2) to demonstrate BMBC HPSE functions cross-species functions (months 15-17).

Because we derived and established lapatinib-resistant BR clones from highly brain metastatic MDA-MB231BR parental cells, we considered investigating these clones far more significant than studying CN34/231-BrM2 cells as more advanced and better substitutes. Therefore, we did not pursue this subtask because redundant to the achievements we have made.

- 2d. Perform *in vivo* experimental brain metastasis assays to examine whether SST0001 will prevent BMBC. Injection of human BMBC cells in nude mice via intracarotid artery. Five days after cell injection (with or without miR-1258 lentivirus), deliver SST0001 by inserting Alzet pump in animals, and monitor BMBC onset. Use of 80 animals, randomly assigned, to receive SST0001, miR-1258 lentivirus, or vehicle: 40 in SST0001 in treatment groups (two cell lines), 40 in vehicle control groups (months 16-18).

We have successfully carried out *in vivo* experimental brain metastasis assays to examine whether SST0001 will prevent BMBC, and results have been shown above. The portion of this subtask employing the delivery of mir1258 lentivirus was already achieved in year 1 of Award funding. Further, we considered adding the two compound synergistically may not be necessary because of the successful outcomes obtained by investigations within this two-year period.

- 2e. Perform *in vivo* experimental brain metastasis assays to examine whether SST0001 will prevent BMBC. Injection of human BMBC cells in nude mice (*nu/nu*) via intracarotid artery. Five days after cell injection, deliver SST0001 alone, lapatinib alone, or SST0001/lapatinib combinations by inserting Alzet pump in animals, and monitor BMBC onset. Use of 120 animals, randomly assigned, to receive treatment groups. 20 animals per treatment groups will be employed (months 17-22).

Since our research efforts for sub-task 2b have resulted in successful outcomes with data of statistical significance, we did not pursue this sub-task.

- 2f. Accrual of data, data tabulation and statistical analyses (months 17-23).

We have performed this task by employing this time period to organize and tabulate data, and assess the statistical significance of data accrued.

- 2g. Preparation and submittal of a manuscript for dissemination of results in peer-reviewed oncology journal (month 24).

We have performed this task by preparing a manuscript highlighting these findings. We have submitted this manuscript to the highly-regarded *Clinical Cancer Research* journal, and titled: "Heparanase mediates a novel mechanism in lapatinib-resistant brain metastatic breast cancer cells".

Key Research Accomplishments

Our laboratory has been able to obtain lapatinib-resistant and sensitive clones from MDA-MB-231BR cells and demonstrated that HPSE expression and activity play important role in lapatinib-resistant mechanisms towards BMBC onset. Furthermore, the research accomplished in our laboratory demonstrated that:

- SST0001 synergistically acts with lapatinib to significantly inhibit the growth and brain metastasis of lapatinib-resistant cells *in vitro* and *in vivo*.
- SST0001-mediated inhibition of tumor growth and metastasis is consistent with the compound's anti-heparanase activity, including the downregulation of matrix metalloproteinase-9 (MMP-9) and Cox-2 expression, two key target genes known to be regulated by HPSE.
- SST0001 treatment resulted in alterations of protein phosphorylation in EGFR, AKT and MAPK signaling. Thus, SST0001 and lapatinib synergistically inhibit tumor growth and brain metastasis.

Reportable outcomes

A manuscript that has resulted from this research has been submitted to the highly-regarded *Clinical Cancer Research* journal.

Conclusions

We have demonstrated that the dual inhibition of EGFR phosphorylation/signaling and HPSE activity by SST0001 suppresses BMBC *in vitro* cell growth and *in vivo* brain metastases. Our findings provide first-time evidence that HPSE plays important roles in lapatinib resistance of breast cancers homing to brain, and that the inhibition of HPSE is synergistic with lapatinib functionalities in BMBC. We consider these findings of high significance and the molecular basis for the development of novel therapeutic approaches treating lapatinib-resistant breast cancers, particularly BMBC. Further investigations of SST0001 and the outcome of ongoing clinical trials employing SST0001 (Phase I - multiple myeloma) will foster its potential as a novel therapeutic agent for more effective treatment of BMBC.

References

1. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* 2000; 19:3159-67.
2. Lu J, Steeg PS, Price JE, Krishnamurthy S, Mani SA, Reuben J, Cristofanilli M, Dontu G, Bidaut L, Valero V, Hortobagyi GN, Yu D. Breast cancer metastasis: challenges and opportunities. *Cancer Res* 2009; 69(12): 4951-62; Gril B., Palmieri D, Bronder JL, Herring JM, Vega-Valle E, Feigenbaum, Leiwehr D, Steinberg SM, Merino M J, Gilmer TM, Rubin, SD, Steeg PS. Effect of lapatinib on the outgrowth of metastatic breast cancer cells to the brain. *J Natl Cancer Inst.* 2008, 100(15):1092-103.
3. Cerra M, Cecco L, Montella M, Tuccillo F, Bonelli P, Botti G. Epidermal growth factor receptor in human breast cancer: comparison with steroid receptors and other prognostic factors. *Int J Bio Markers* 1995; 10: 136-42.
4. Slamon DJ, Godolphin W, Jones LA. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989; 244:707-12.

5. Vreys V and David G. Mammalian heparanase: what is the message? *J. Cell. Mol Med* 2007; 11(3): 427-52.
6. Marchetti D and Nicolson GL. Human heparanase: A molecular determinant of brain metastasis. *Adv Enz Reg* 2001; 41: 343-59.
7. Ilan N, Elkin M, Vlodavsky I. Regulation, function, and clinical significance of heparanase in cancer metastasis and angiogenesis. *Int J Biochem Cell Biol* 2006; 38 (12): 2018-39.
8. Zcharia E, Jia J, Zhang S, Baraz L, Lindahl U, Peretz T, Vlodavsky I, Li JP. Newly generated heparanase knockout mice unravel co-regulation of heparanase and matrix metalloproteinase *PLoS ONE* 2008; 4(4): e5181 (1-13).
9. Zhang L, Sullivan P, Suyama J, Marchetti D. EGF - induced heparanase nucleolar localization augments DNA Topoisomerase I activity in brain metastatic breast cancer. *Molecular Cancer Res.* 2010; 8(2): 278-90; Zhang L, Sullivan P, Goodman J, Gunaratne PH, Marchetti D. MicroRNA-1258 inhibits breast cancer invasion and metastasis by directly targeting heparanase. *Cancer Res. - Priority Report* 2011; 71: 645-54.
10. Naggi A, Casu B, Perez M, et al. Modulation of the heparanase-inhibiting activity of heparin through selective desulfation, graded N-acetylation, and glycol splitting. *J Biol Chem* 2005; 280:12103-13.
11. Ritchie JP, Ramani VC, Ren Y, Naggi A, et al. SST0001, a chemically modified heparin, inhibits myeloma growth and angiogenesis via disruption of the heparanase/syndecan-1 axis. *Clin Cancer Res* 2011; 17(6): 1382-93.