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# Heparanase Mechanisms in Brain-Metastatic Breast Cancer

The identification of brain metastatic breast cancer (BMBC) mechanisms responsible for brain metastasis is imperative to develop new therapies. The relevance of heparanase (HPSE) in cancer invasion and metastasis has been established. Heparanase is the only known functional endoglycosidase in mammals and degrades heparan sulfate (HS), the main polysaccharide component of growth factor-binding proteoglycans. The therapeutic disruption of heparanase/HS function provides the opportunity to block multiple signaling pathways which are crucial for tumor cell adhesion, survival, and growth within the target organ microenvironment. Our hypothesis is that heparanase represents a novel target for the development of therapies for BMBC treatment. We propose aims to determine HPSE regulation by MiR-1258 in BMBC and to identify novel functions of SST0001 as synergies with lapatinib and/or miR-1258 in BMBC cell and animal models. We will address HPSE regulation at three levels: the first is to identify how heparanase is modulated translationally by miR-1258; the second is to study the efficacy of SST0001, a small-molecule glycol-split heparinoid, regulating post-translational HPSE activity; the third is to investigate synergies of the heparanase inhibitor SST0001 and lapatinib, and assess whether heparanase promotes the pathogenesis of BMBC by selectively foster resistance to lapatinib.
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Introduction

Heparanase (HPSE) is the dominant endoglycosidase (endo-β-D-glucuronidase) that cleaves heparan sulfate (HS) to fragments retaining biological activity in mammals. We demonstrated that levels of a specific microRNA, microRNA1258 or miR-1258, inversely correlate with the expression of an important glycosidase, named heparanase, its enzymatic activity, and cancer cell metastatic propensities with lowest expression in highly aggressive brain metastatic breast cancer (BMBC) cells. Second, we demonstrated that experimental brain metastasis are suppressed when miR-1258 is ectopically introduced into BMBC cells and these are applied to xenografts models. Third, because Lapatinib (Tykerb, GW572016) is a selective small-molecule inhibitor of EGFR and HER2 with limited roles in BMBC due to onset of drug resistance mechanisms, we demonstrated that HPSE mediates an alternative survival mechanism in lapatinib-resistant brain metastatic breast cancer cells. This, by employing a specific HPSE inhibitor, named SST001 (Roneparstat, received from Sigma-tau Research, Mendrisio, Switzerland) and prototypic glycol-split heparinoid.

Body

This represents the final report for the IDEA Award from breast cancer DoD-CDMRP. We have completed specific aims of this Award, and have implemented sub-tasks per statement of work provided. These are summarized below, per aims proposed:

Specific aim 1:

1a. Generate lentiviral vectors expressing miR-1258 and transduce parental (231P) and brain- metastatic BMBC cell lines (231BR1, -BR2, and -BR3) with miR-1258 lentivirus and corresponding control

1b. Determine the modulation of heparanase expression, activity in BMBC cells and respective non-brain metastatic counterparts

1c. Perform real-time PCR, Western blot analyses, and HPSE activity assays for transcripts and protein regulated by miR-1258

1d. Perform adhesion, migration, invasion assays in BMBC cells transduced with miR-1258 lentivirus or antisense control

1e. Carry-out experimental brain metastatic assays in animals (nu/nu mice) to demonstrate the in vivo miR-1258 physiological relevance by lentiviral deliver

1f. Detect miR-1258 by LNA-ISH in 50 formalin-fixed, paraffin-embedded paired BMBC clinical samples

1g. Complement LNA-ISH with IHC analyses to examine heparanase expression in same paired clinical samples. Compare digital determinations of LNA-ISH and IHC reactivities and analyze them for statistical significance
1h. Preparation and submittal of a manuscript for dissemination of results in a peer-reviewed oncology journal.

Specific aim 2:

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.

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 (MDA-MB231BR) 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.

2c. Perform in vivo experimental brain metastasis assays to examine whether SST0001 will prevent BMBC. Injection of human BMBC cells in nude mice. Five days after cell injection, deliver SST0001 by inserting Alzet pump in animals, and monitor BMBC onset. Use of animals, randomly assigned to receive SST0001 or vehicle.

2d. Perform in vivo experimental brain metastasis assays to examine whether SST0001 will prevent BMBC. Injection of human BMBC cells in nude mice. Five days after cell injection, deliver SST0001 alone, lapatinib alone, SST0001/lapatinib combinations by inserting Alzet pump in animals, and monitor BMBC onset.

2e. Accrual of data, data tabulation and statistical analyses.

2f. Preparation and submittal of a manuscript for dissemination of results in a peer-reviewed oncology journal.

Specific aim 1: Determine roles of miR-1258 in heparanase regulation and BMBC suppression.

The regulation of the HPSE gene and BMBC mechanisms are poorly understood. We hypothesized that heparanase represents a potential target for the development of novel therapies for BMBC, whose gene expression and modalities can be regulated by microRNA. Using miRanda and RNAhybrid, we identified microRNA-1258 (miR-1258) to directly target HPSE and suppress BMBC.

We have demonstrated that miR-1258 exerts its roles in BMBC cells by inhibiting heparanase expression and activity, and does so by directly targeting HPSE 3’-UTR (Figs. 1 and 2). To determine whether miR-1258 plays a role in breast cancer metastasis, notably to brain, six human breast cell lines were selected and examined for miR-1258 and heparanase expression. Their inverse correlation was demonstrated: the decrease of miR-1258 associated with an increase of HPSE content and correlated with metastatic abilities of these cell lines (Fig. 3A). Moreover, miR-1258 downregulated heparanase expression and activity (Figs. 2 and 4),
inhibited cell invasion (Fig. 4A), and suppressed the formation of brain metastasis in xenografts by 74% (Figs. 4B-D).

These findings introduce new concepts that links microRNA mechanisms with brain metastatic breast cancer by downregulating HPSE, providing the groundwork for heparanase-based therapeutics in patients with brain metastases, BMBC in particular.

Figure 1. MiR-1258 directly targets heparanase. A., Alignment of miR-1258 with HPSE 3'-UTRs. Complementary sequences of miR-1258 to mammalian HPSE 3'-UTRs are shaded red. The underlined sequence (5'-CCU-3') in the human HPSE mRNA, and common to other mammalian heparanases, denotes a deletion in the construct carrying Luc-HPSE 3'-UTR-del. Seed sequences of miR-1258 are underlined. Hsa = human; Ptr = pan troglodytes; Mmu = mus musculus; Rno = rat. B. and C., Effect of miR-1258 (wild type and mutant) on HPSE 3'-UTR luciferase reporters. Constructs carrying Luc-HPSE 3'-UTR (B.) or Luc-HPSE 3'-UTR-del (C.) were transfected in 293T cells with indicated miRNAs. MiR-100 expressed in a lentiviral construct was used as an additional control for miR-1258 specificity. After transfections (24 hr), cells were harvested and luciferase activity assays were performed. Bars represent the mean and standard deviation of three independent experiments.
Figure 2. MiR-1258 inhibits heparanase expression and activity in BMBC cells. BMBC cells (231BR3 and 231BR1 for brevity) were transiently transfected with anti-miR-1258 and negative control (NC), or stably transduced with lentiviral constructs expressing wild type miR-1258 or scrambled miR (scrambled). After 48 hr, cell lysates were prepared and examined simultaneously using Western blotting and the TakaRa heparan sulfate degrading enzyme assay kit (4). HPSE protein and enzyme activity in 231BR1 and 231BR3 cells (A.) and (B.). β-actin was used as a loading control. Bars represent the mean and standard deviation of three independent experiments. C. Effect of miR-1258 - mediated inhibition of HPSE and heparanase - regulated proteins. Same procedures as (A.) and (B.), expression clones carrying HPSE or scrambled controls were transduced into 231BR3 cells. Cell lysates were then examined for protein expression using antibodies indicated. Three independent experiments were performed, and analyses are shown.
Figure 3. MIR-1258 inversely correlates with BMBC ability and HPSE expression

3A. MIR-1258 inversely correlates with metastatic ability and HPSE expression in human breast cells

3B. MIR-1258 and HPSE expression in paired human mammary gland/carcinoma

3C. MIR-1258 and HPSE expression in paired human breast cancer/BMBC
Figure 3. MiR-1258 levels inversely correlate with metastatic ability of human breast cell lines and patient tissues. A., miR-1258 analyses in breast cell lines with differing metastatic propensities. (Top) miR-1258 levels were examined in six breast cell lines by TaqMan RT-PCR and data shown by fold change compared to human mammary epithelial cells (HMEC). RNU44 was used as endogenous control. (Bottom) Differential HPSE expression in same cell lines was examined by Western blotting. Based on their tumorigenic and metastatic potential, cell lines were classified into three groups: 1) non-tumorigenic HMEC and MCF-10A; 2) tumorigenic but non-metastatic SUM-149 and SUM-225; 3) BMBC 231BR1 and 231BR3 cells. β-actin was used as control for equal loading. B. and C., miR-1258 analyses of paired (mammary gland versus invasive ductal carcinoma, or primary versus BMBC) patient tissues using locked nucleic acid in situ hybridization (LNA-ISH). Hybridized, complementary probes were detected by a catalyzed reporter deposition using biotinylated tyramide followed by colorimetric detection of biotin with an avidin-alkaline phosphatase conjugate (n = 13 pairs). (Left) Representative LNA-ISH images of miR-1258 expression of normal mammary gland, invasive ductal carcinoma, and brain metastatic breast cancer, and quantification of miR-1258 signal intensity. Graph depicts relative miR-1258 expression in indicated tissues. While high miR-1258 levels were detected in the epithelial component of normal mammary gland, HPSE IHC negativity was consistently detected. The small insert in the HPSE IHC panel of normal breast seen at the periphery of patient’s tumor indicates HPSE IHC staining of tumor demonstrating strong heparanase expression. Each group represents the average of total signals in examined fields. (Right) Heparanase expression (IHC) in paired cases of normal mammary gland and invasive ductal carcinoma, and primary and brain metastatic carcinoma was examined by IHC.
Figure 4. MiR-1258 downregulates heparanase, inhibits BMBC cell invasion, and suppresses brain metastasis. A., miR-1258 downregulates heparanase. (Left) Cells (SUM-149, 231BR3) were transfected with miR-1258, anti-miR-1258 (inhibitor), and negative controls (NC) followed by Western blotting to detect HPSE expression. (Right) miR-1258 inhibits BMBC cell invasion. Chemo invasion analyses using Matrigel™ chambers were performed in parallel with Western blotting analyses for HPSE expression. Representative images of chamber inserts are shown. Cell invasive values were quantified (O.D. 560 nM) (n = 10). B., miR-1258 suppresses brain metastasis. 231BR3 cells stably expressing miR-1258 or scrambled-miR (scrambled) were injected intracardiacally into female nude mice (0.5 x 10^6 cells per mouse, 30 mice per group), respectively. After 6 weeks, mice were sacrificed, lungs and brains harvested, and analyzed for metastatic tumors. (Top) Incidence, and mean number of brain metastases in each group. (Middle) hematoxylin and eosin (H&E) visualization of BMBC and its suppression by miR-1258 compared to scrambled. (Bottom) The combined data (n = 3 assays) are shown graphically with dots representing the number of brain metastases from each mouse; the box represents the 10th and 90th percentile; and the black line in each box is the mean for each group. C., Heparanase expression was examined by IHC in experimental BMBC, and representatives IHC and H&E sections are shown (n = 4). D. Rescue experiments showing that a modulation of HPSE expression blocked miR-1258 – mediated effects on BMBC in vitro cell invasion and in vivo brain metastasis formation. Shown are representative images visualizing brain metastases in animals (H & E staining; n = 10 mice per treatment group). Bars represent the mean and standard deviation of two independent experiments.

Specific Aim 2: Identify novel functions of SST0001 as synergies with lapatinib and/or miR-1258 in BMBC cell and animal models.

We hypothesized that heparanase is implicated in initiating lapatinib resistance to EGFR-overexpressing breast cancer via activation of an alternative signaling pathway. In the present study, we first established lapatinib-resistant clones from MDA-MB-231BR (BR) human breast cancer cell line overexpressing EGFR, and then identified an HPSE-mediated resistant mechanism, including increased HPSE activity and secretion, and HPSE-mediated signaling pathway. Furthermore, we proved that dual inhibition of EGFR phosphorylation and HPSE activity by SST0001 suppresses BMBC cell growth and metastases in vitro and vivo. Our results provided first-time evidence that HPSE plays a role in lapatinib resistance, and inhibition of HPSE enhances lapatinib function in brain metastatic breast cancer. We consider this a significant information and molecular basis for the development of novel therapeutic approaches in treating lapatinib-resistant breast cancers, particularly brain metastatic breast cancer.

We selected lapatinib-resistant and sensitive clones from MDA-MB-231BR cells (Fig. 5) and demonstrated that HPSE expression and activity (Fig. 6) play important role in lapatinib-resistant mechanisms by demonstrating that lapatinib acts synergistically with SST0001 in these cells, resulting in alterations of protein phosphorylation in EGFR, AKT and MAPK signaling (Fig. 7). Further, we provided evidence that the SST0001/ lapatinib axis significantly inhibits the growth and metastasis of lapatinib-resistant cells in vitro and in vivo (Fig. 7 and 8).
Figure 5. Selection and growth of lapatinib resistant cells. A. Lapatinib-resistant cell lines were selected from the survival of MDA-MB23BR cells exposed to different concentrations (0.1, 0.5, 1, 5 and 10 µmol/L lapatinib in DMEM/F12 complete culture medium) for 3 weeks, with the medium changed every 2-3 days. The surviving cells were followed with chronic treatment of lapatinib (1 to 2 µmol/L) *in vitro*, and the cells that developed resistance to lapatinib were selected for amplification and characterization. B. Cell cycle profile and quantitative analysis. Representative cell cycles of MDA-231-BR parental and lapatinib-resistant cells were determined by fluorescence-activated cell sorting analysis and quantitative analysis of respective cell cycle profiles (*p < 0.01; n = 3*).
Figure 6. Heparanase expression and activity in the selected MDA-231MB-BR lap-resistant and sensitive cells (BR-Lr and BR-Ls, respectively). A. Western blot analysis of heparanase protein expression (9). B. Heparanase expression in indicated cell supernatant and lysates by ELISA. C. Heparanase activity assays were completed as previous described (9).

Figure 7. SST0001 and lapatinib synergistically inhibit lap-resistant cell growth. A. Mammosphere assay for MDA-231BR parental (BR) and lap-resistant cells (BR-Lr1 and – Lr2). Cells were seeded in six-well plates with reagents as indicated, and lapatinib (1µM) and SST0001 were added to the plates, replenished every 2-3 days, and monitored for 14 days in cell culture at 37°C - 5% CO2. B.
Quantitative analysis of mammospheres. After 14 days, colonies (> 50 µm²) were counted, photographed, and quantitatively analyzed. C. Signaling pathway in lapatinib-resistant cells. Cells were treated with lapatinib and SST0001 (same concentrations as A) overnight (16 hrs), then treated with EGF (20 ng/ml) for 10 min, and phosphorylation of indicated signaling proteins was examined by Western blot analysis. The results were reproducible (n = 3). D. Rescue assay of effects of SST0001 on HPSE-regulated proteins. Cells were treated with SST0001 (5 µM) or SST0001 plus recombinant heparanase (rHPSE, 100 ng/ml) for 24 hrs, and then MMP-9 and COX2 expression levels were examined by Western blot analysis. β-actin was the loading control.
Figure 8. SST0001 and lapatinib synergistically inhibit tumor growth and brain metastasis in mouse model. A. BR parental and lapatinib-resistant cells (BR-Lr1 and BR-Lr2) were injected subcutaneously (1 x 10^6 cells/mouse; n = 5 mice). Tumor growth was monitored daily by measuring the tumors’ length and width with a digital caliper. Tumors were harvested and photographed (top). Tumor weight was calculated and compared among the treatment groups (bottom). B. Representative images of Cri Vectra-Intelligent analysis of brain metastasis induced by lap-resistant cells. Cambridge Research & Instrumentation, Inc. Cri-Vectra-Intelligent imaging analysis system was selected for quantification of tumor cells from H&E sections. The Vectra Intelligent™ software is based on machine-learning program to outline the tumor, recognize and distinguish significant histological features, such cancerous or no normal tissue. C. H&E sections were randomly selected from 2-3 mice with metastatic brain tumors mediated by the lap-resistant cells. The graphic shows quantification of metastatic brain tumor burden represented by total tumor areas and cells.

Key Research Accomplishments

The purpose of our studies was to investigate microRNA - mediated mechanisms that alter invasive and metastatic modalities of BMBC through the downregulation of heparanase. We provide evidence that:

- A specific microRNA - miR-1258 - targets the heparanase gene and has profound effects on heparanase gene expression and function of this molecule in BMBC cell invasion and metastatic profiles;

- The ectopic expression and activity of miR-1258 negatively regulates heparanase expression and its unique endoglycosidase, and results in the inhibition of BMBC cell invasion and onset of brain metastasis.

- The development of miRNA-based approaches regulating heparanase is critical and of potential therapeutic value.

- Exposure of selected lapatinib-resistant MDA-MB231BR clones to SST0001 resulted in alterations of protein phosphorylation of EGFR, AKT and MAPK signaling, and synergistic with treating cells with lapatinib.

- SST0001, significantly inhibits the growth and metastasis of lapatinib-resistant cells in vitro and in vivo, and synergistic with lapatinib treatment.

Reportable outcomes

Specific aim 1:

Specific aim 2:

A manuscript has resulted from performing research of this aim, to be re-submitted. Copies of both manuscripts have been attached to this final report (Appendix)

Conclusions

As overall summary of this IDEA Award, we have identified a link between miR-1258 and heparanase, which represents a novel mechanism of endogenous regulation of this molecule. Our findings indicate that miR-1258 is a suppressor of breast cancer cell invasion and brain metastasis by targeting heparanase and heparanase - mediated pathways. Second, we provide first-time evidence that the combination of lapatinib and SST0001 is able to inhibit tumor growth and brain metastasis caused by lapatinib-resistant brain metastatic breast cancer cells. Coupled with the discovery of miR-1258/HPSE mechanisms, these advances may be important inroads to understand heparanase action, and miR-1258/SST0001 abilities to inhibit brain metastasis can have profound implications for the development and application of heparanase-based therapeutics in brain metastasis in general, brain metastatic breast cancer in particular.

References


Appendix:
