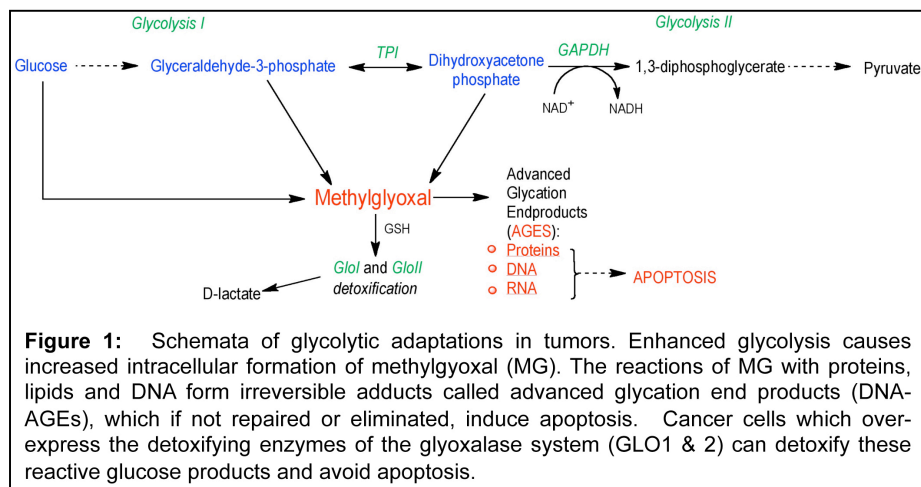## Introduction and Hypothesis

Breast to brain metastases (BBM) in HER2$^+$ and triple negative (TN) patients is major clinically unmet need and increasingly relevant for improving survival and quality of life. The brain remains impenetrable to most new molecular therapeutics, despite their effectiveness outside the central nervous system. This is leading to increased incidence of brain metastases in patients with breast cancer [1-3]. Presently, the options are limited to surgery and radiation (XRT) which are antiquated with respect to the current generation of molecular therapeutics. BBMs occur after a latency period in which women often have good control of systemic disease. This late occurrence of brain metastases in women with breast cancer is particularly devastating because it occurs in the setting of effective systemic control. However, this latency also offers scientists and clinicians an opportunity to develop new therapeutics that could prevent brain metastases in high-risk women, as well as treatment options for brain metastases once they are diagnosed.

Most breast carcinomas display the Warburg Effect. This metabolic shift from normal mitochondrial respiration toward upregulated glycolysis is initiated as an adaptive response to the hypoxic tumor microenvironment. Constant detoxification of toxic metabolites, specifically methylglyoxal (MG), by the enzyme glyoxalase (Glo1) is required to avert apoptosis (Figure 1). **We hypothesize** that disruption of this adaptive metabolic response in tumors will result in the accumulation of toxic glycolytic metabolites and induce cancer cell specific apoptosis. We have identified bromobenzyl-glutathione-dicyclopentyl-ester (GloX1) as a candidate drug for targeting the metabolic detoxification strategy employed by breast cancer cells in the brain. We will investigate GloX1 alone and in combination with radiation (XRT), which is the current standard of clinical treatment.

Figure 1: Schemata of glycolytic adaptations in tumors. Enhanced glycolysis causes increased intracellular formation of methylgyoxal (MG). The reactions of MG with proteins, lipids and DNA form irreversible adducts called advanced glycation end products (DNA-AGEs), which if not repaired or eliminated, induce apoptosis. Cancer cells which over-express the detoxifying enzymes of the glyoxalase system (GLO1 & 2) can detoxify these reactive glucose products and avoid apoptosis.

Specific Aims

AIM 1: To determine the cytotoxicity of GLO1 inhibition in BBM fresh cultures from surgical specimens *ex vivo*.
    1.1. To derive low passage *ex vivo* BBM cell lines from fresh surgical specimens from patients (IRB #05901).
    1.2. To develop pharmacodynamic assays to quantify GLO1 inhibition in GloX1 treated cells to determine the PD and PK efficacy of GloX1 to establish a working range for the *in vivo* studies.
    1.3. To determine the sensitivity of low passage BBM cell lines to GLO1 inhibition alone or in combination with radiation (XRT). We expect to observe tumor inhibition by GloX1 and therapeutic synergy between the two treatments *ex vivo*.

AIM 2: To determine the efficacy of GloX1 for inhibiting the proliferation of orthotopic, metastatic breast-brain cells *in vivo*.
2.1. To create patient derived, orthotopic xenograft models by implanting dissociated cells from patient BBM lines established in Aim 1 into the brains of NOG (NOD/Shi-*scid*/IL-2Rγ^{null}) mice (active Animal Protocol #10044).
    2.2. To use histological and molecular markers to demonstrate that BBM cells directly implanted into the mice brains are phenotypically characteristic of the patient BBM from which they were derived.
    2.3. To determine the efficacy of GloX1, XRT, and GloX1 + XRT treatment by evaluating tumor progression in animals with verified brain metastases subsequent to xenografting. This aim will serve as the pre-clinical foundation for considering new experimental permutations as well as the potential for clinical trials.

Progress Report

Clinical evidence for Glo1-mediated survival can be found by examining lactate levels in tumors, since Glo1 converts MG into lactate. Elevated lactate levels are often found in tumors, and correlate with poor patient prognosis, decreased disease-free or metastasis-free survival and shorter overall survival in a variety of cancers [4]. In fact, the production of lactate can be non-invasively quantified by *in situ* observation using Proton Magnetic Resonance Spectroscopy (MR SPECT) [Figure 2A]. Further proof of upregulated glycolytic metabolism can be provided by Positron Emission Tomography (PET) showing
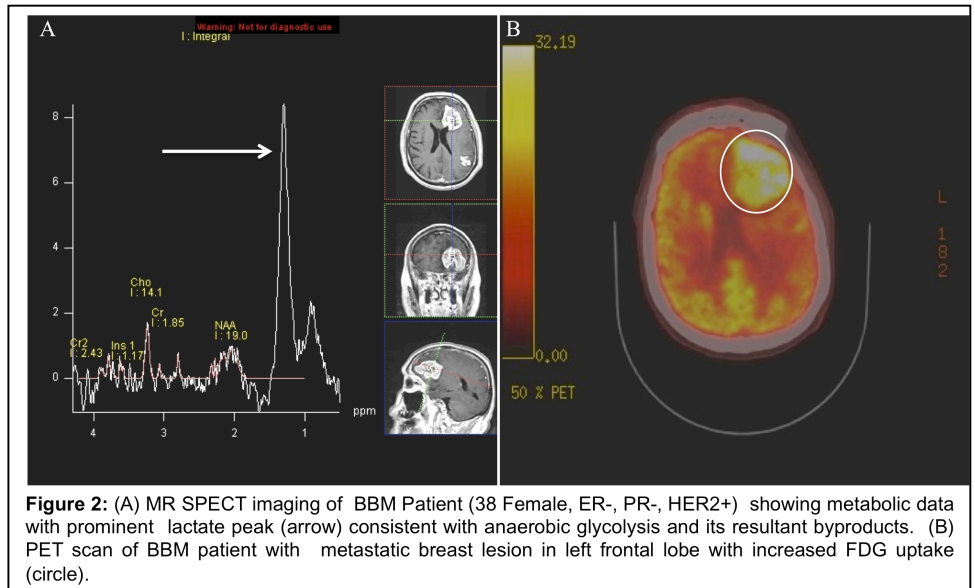

Figure 2: (A) MR SPECT imaging of BBM Patient (38 Female, ER-, PR-, HER2+) showing metabolic data with prominent lactate peak (arrow) consistent with anaerobic glycolysis and its resultant byproducts. (B) PET scan of BBM patient with metastatic breast lesion in left frontal lobe with increased FDG uptake (circle).

increased fludeoxyglucose (FDG) uptake in brain metastases relative to normal brain [Figure 2B]. This body of empirical evidence suggests that Glo1 mediated detoxification is active in brain metastases and that inhibition of Glo1 is a therapeutic strategy. Although the therapeutic targeting of enzymes in the glycolysis pathway has been previously explored, treatment efficacy has been limited because of the unavoidable inhibition of shared glycolysis pathways in normal brain cells [5]. However, we show that Glo1 is a unique and ideal target because it is selectively over-expressed in BBM tumors and its expression is limited in surrounding normal brain tissue indicating that a Glo1 inhibitor should selectively target the tumor tissue without affecting the normal brain cells (Figure 3). Similarly, comparison of GLO1 mRNA expression levels in the breast-to-brain metastatic cells and brain trophic cell line MDA-MB-231Br (231Br), human neural progenitor cells (NPC), neurons, and astrocytes reveals that GLO1 is overexpressed only in the BBM cells (Figure 4A). Since XRT increases GLO1 expression in tumors and enhances their radioresistance, a combinatorial approach including GloX1 should improve the radiation response by neutralizing the anti-apoptotic action of GLO1 overexpression. This experimental design is further rationalized by the fact that XRT is the current standard of care for brain metastases and included in treatment arms within clinical trials. Accordingly effects of XRT and GloX1 will be investigated.
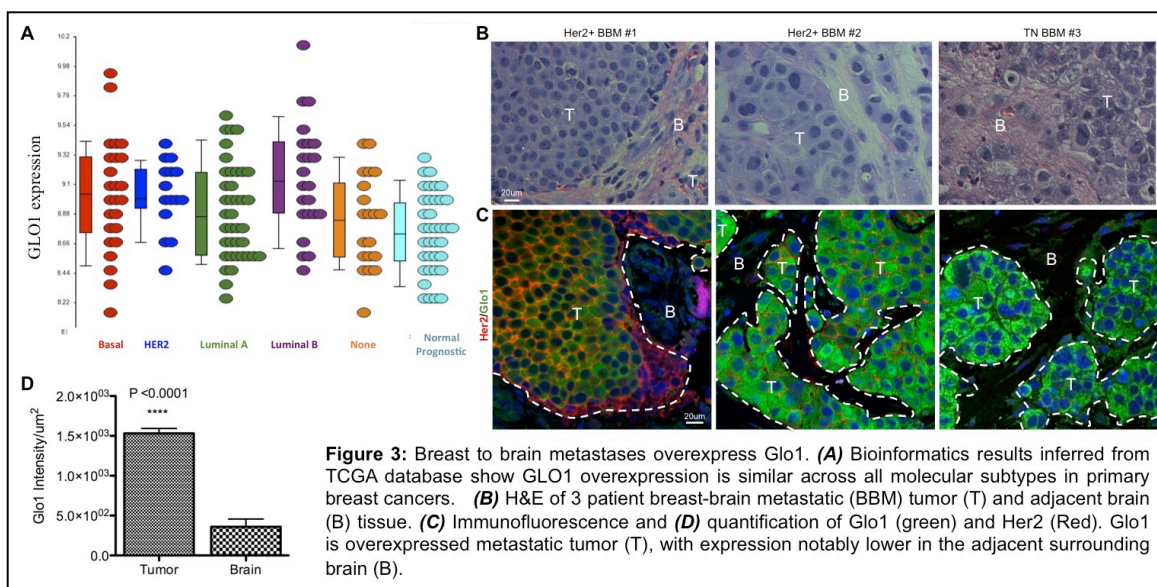

Figure 3: Breast to brain metastases overexpress Glo1. *(A)* Bioinformatics results inferred from TCGA database show GLO1 overexpression is similar across all molecular subtypes in primary breast cancers. *(B)* H&E of 3 patient breast-brain metastatic (BBM) tumor (T) and adjacent brain (B) tissue. *(C)* Immunofluorescence and *(D)* quantification of Glo1 (green) and Her2 (Red). Glo1 is overexpressed metastatic tumor (T), with expression notably lower in the adjacent surrounding brain (B).

The shortcomings of cell line derived models is highlighted by discouraging rates of translational efficacy failure for many *in vitro* and pre-clinical models in early human clinical trials [14]. Accordingly our experiments rely solely on new minimally passaged cells from surgical specimens. Our data reveals the treatment of BBM cells with the Glo1 inhibitor GloX1 leads to potent cell growth inhibition *ex vivo,* and increases in DNA-AGEs even at low micromolar (µM) concentrations (Figure 4C,D). GloX1 however, did not significantly affect the growth of brain NPC, neurons, or astrocyte cell lines indicating that GloX1 specifically targeted pathway unique to the BBM cells. *Other compounds have been reported as Glo1 inhibitors, but they fail to cross the BBB making them unsuitable for the treatment of brain metastases.* In contrast, the BBB permeability of our selective GloX1 inhibitor, GloX1, has been demonstrated using microdialysis studies [6].

We have adopted a tumor grafting approach using NOD/Shi-*scid*/IL-2Rγ^{null} mice to test if GloX1 is effective at inhibiting tumor progression *in vivo*. BBM cells were xenografted and then treated with GloX1. Our findings confirm that GloX1 crosses the blood brain barrier and decreases growth of breast to brain metastases (Figure 5). Results from the *in vivo* studies using animal models will serve as the pre-clinical foundation for considering new experimental permutations and the potential for clinical trials. We are currently working to determine the sensitivity of low passage patient BBM cells (active IRB # 05901) to Glo1 inhibition in combination with XRT. Because XRT increases Glo1 expression in brain tumors and enhances their radioresistance [7], the combinatorial approach that includes GloX1 is expected to improve the radiation response[7]. Furthermore, because XRT is the current standard of care for brain metastases novel therapeutics often need to be investigated in an adjuvant setting in clinical trials. Even when women undergo surgical resection, they receive post-operative radiation to decrease the incidence of local recurrence. As such, XRT is offered to any patient interested in additional treatment for their brain metastases
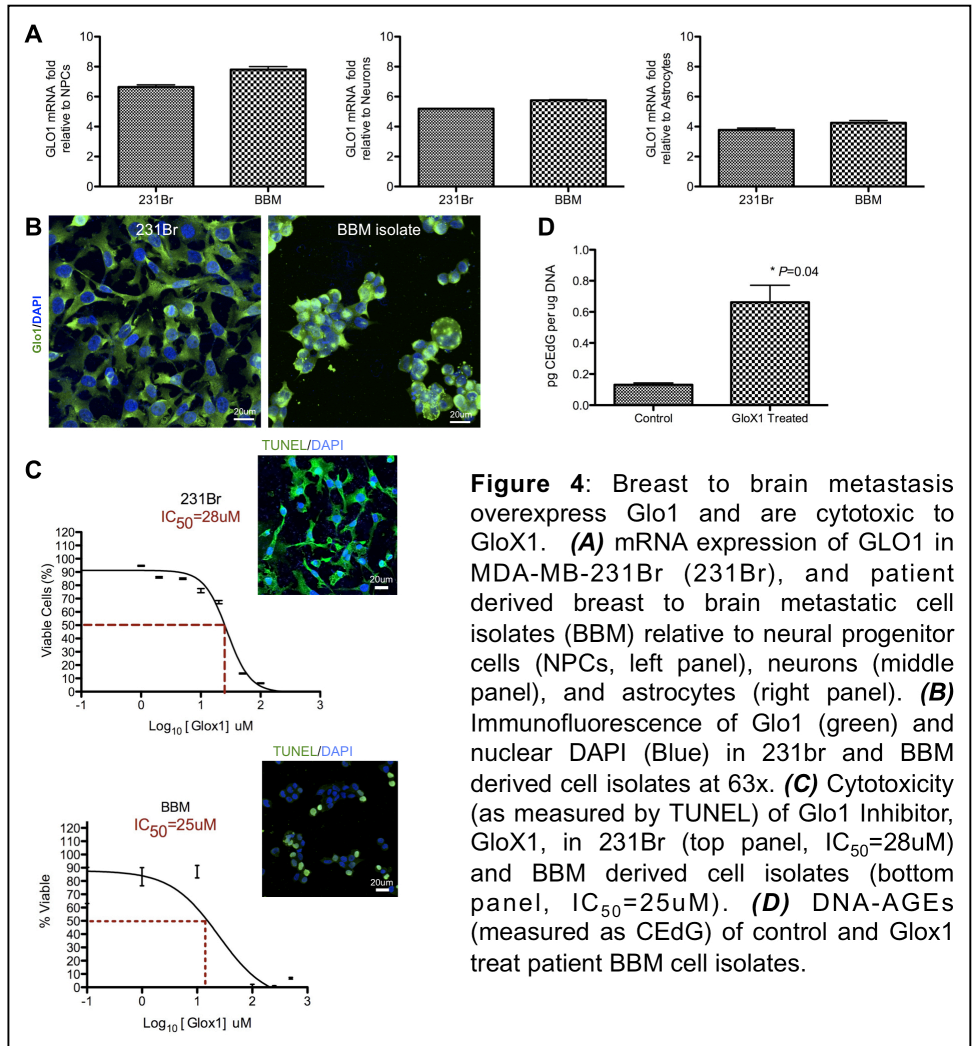

Figure 4: Breast to brain metastasis overexpress Glo1 and are cytotoxic to GloX1. **(A)** mRNA expression of GLO1 in MDA-MB-231Br (231Br), and patient derived breast to brain metastatic cell isolates (BBM) relative to neural progenitor cells (NPCs, left panel), neurons (middle panel), and astrocytes (right panel). **(B)** Immunofluorescence of Glo1 (green) and nuclear DAPI (Blue) in 231br and BBM derived cell isolates at 63x. **(C)** Cytotoxicity (as measured by TUNEL) of Glo1 Inhibitor, GloX1, in 231Br (top panel, IC$_{50}$=28uM) and BBM derived cell isolates (bottom panel, IC$_{50}$=25uM). **(D)** DNA-AGEs (measured as CEdG) of control and Glox1 treat patient BBM cell isolates.
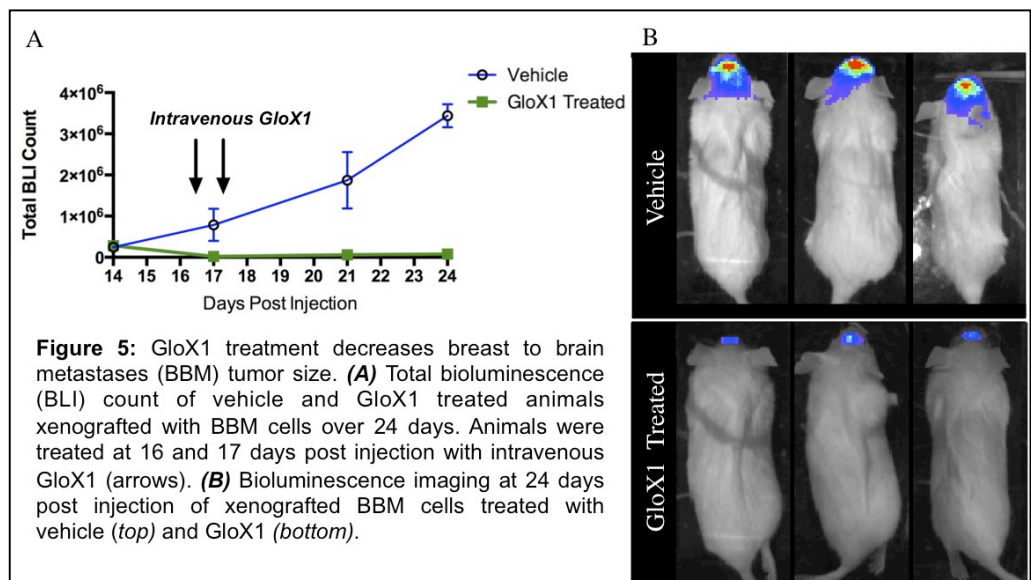

Figure 5: GloX1 treatment decreases breast to brain metastases (BBM) tumor size. **(A)** Total bioluminescence (BLI) count of vehicle and GloX1 treated animals xenografted with BBM cells over 24 days. Animals were treated at 16 and 17 days post injection with intravenous GloX1 (arrows). **(B)** Bioluminescence imaging at 24 days post injection of xenografted BBM cells treated with vehicle (*top*) and GloX1 (*bottom*).

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