TARGETING tGLI1-POSITIVE BREAST CANCER WITH KETOCONAZOLE

BY

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Dedication

I would like to dedicate this text to all those who have suffered this dreadful disease.

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List of Abbreviations

AR: Androgen receptor

AREG: Amphiregulin

AS-ON: Antisense oligonucleotides

BBB: Blood-brain barrier

BCBM: Breast cancer brain metastases

BCC: Basal cell carcinoma

BTB: Blood-tumor barrier

BTC: Betacellulin

cDNA: Complementary DNA

CK: Cytokeratin

CSC: Cancer stem cells

CTC: Circulating tumor cells

DMEM: Dulbecco's Modification of Eagle's Medium

DMSO: Dimethyl sulfoxide

EGFR: Epidermal growth factor receptor

EPGN: Epigen

EREG: Epiregulin

ERα: Estrogen receptor α

FBS: Fetal bovine serum

GBM: Glioblastoma

GLI1: Glioma-associated oncogene homolog 1

HB-EGF: Heparin-binding EGF-like growth factor

HER2: Human epidermal growth factor receptor 2

Hh: Hedgehog

Hhip: Hedgehog-interacting protein

IACUC: Institutional Animal Care and Use Committee

I.P.: Intraperitoneal

LNA: Locked nucleic acid

miR: MicroRNA

miRNA: MicroRNA

nt: Nucleotides

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PEG-300: polyethylene glycol 300

PR: Progesterone receptor

P/S: Penicillin-Streptomycin solution

PUMA: p53-upregulated modulator of apoptosis

PTCH1: Patched-1

qPCR: Quantitative polymerase chain reaction

RFP: Red fluorescent protein

RT-PCR: Reverse transcription polymerase chain reaction

SHH: Sonic Hedgehog

SKBRM: Brain-metastatic variant of SKBR3

SKBRM-GLI1: Brain metastatic SKBR3 cells overexpressing GLI1

SKBRM-tGLI1: Brain metastatic SKBR3 cells overexpressing tGLI1

SMO: Smoothened

TAE: Tris-acetate-EDTA

TGF-α: Transforming growth factor-α

tGLI1: Truncated glioma-associated oncogene homolog 1

TNBC: Triple-negative breast cancer

WBRT: Whole brain radiotherapy

WFSM: Wake Forest School of Medicine

Abstract

Despite improvements in early detection and therapies, breast cancer remains the second leading cause of cancer-related death in women and the second most common cancer to metastasize to the brain. Current standard of care for breast cancer brain metastases (BCBM) involves surgical resection of individual lesions and whole brain radiotherapy (WBRT) for multiple lesions, however, effective FDA-approved drugs for patients with intracranial progression remain an area of unmet need. Our laboratory discovered an alternative splice variant of glioma-associated oncogene homolog 1 (GLI1) in glioblastoma (GBM), termed truncated GLI1 (tGLI1), that is a gain-of-function GLI1 transcription factor and demonstrates tumor-specific expression. Previous results in our laboratory have implicated tGLI1 in promoting breast cancer stem cells (CSCs) and BCBM. These potent oncogenic and metastasis-promoting effects and tumor-specific expression make tGLI1 an ideal therapeutic target. Using a synthetic lethality chemical screen approach, we found that ketoconazole, an FDA-approved azole antifungal, was able to kill tGLI1-expressing cancer cells with increased efficacy against the CSC subpopulation. Additionally, ketoconazole selectively inhibited the progression of tGLI1-positive BCBM *in vivo.* Mechanistic studies suggest that ketoconazole-dependent cell kill is, in part, mediated through downregulation of the stemness genes CD44 and OCT4. Collectively, our results demonstrate that ketoconazole is an effective inhibitor of CSCs and brain metastasis of tGLI1-positive breast cancer. These findings provide the rationale to further develop novel tGLI1 inhibitors, based on the structure of ketoconazole for the treatment of BCBM.

Introduction

The American Cancer Society estimates that approximately 266,120 new breast cancer cases will be diagnosed, and 40,920 deaths will occur due to breast cancer in the U.S. alone in 2018 [1]. It is estimated that breast cancer will remain the most common cancer diagnosis and the second most common cancer-related cause of death in women in 2018. In a group of 8 women, one is projected to develop the disease while 1 in 37 women are expected to succumb to their illness. Despite the 5-year survival for women diagnosed with the localized disease remaining high at approximately 99%, the survival rate plummets to 27% once distant metastases form [1].

Breast cancers are classified into five different subtypes based on expression levels of the hormone receptors, estrogen receptor α (ERα) and progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), cytokeratins (CKs) 5 and 6, and claudins 3, 4, and 7. The five subtypes are luminal A, luminal B, HER2-enriched, claudin-low (mesenchymal), and basal-like [2, 3]. Tumors classified as luminal A are positive for ERα and PR, and have low Ki-67 expression corresponding to histologically low-grade presentations. Luminal B tumors are also ER- and PR-positive but express high levels of HER2 and are often high-grade. HER2-enriched tumors, which show amplification and high expression of the *ERBB2* gene and several other genes of the *ERBB2* amplicon, are negative for ERα and PR, but positive for HER2 and Ki-67. Claudin-low (mesenchymal) and basal-like tumors, collectively known as triple-negative breast cancer (TNBCs) because they lack expression of for ERα, PR, and HER2, do not overexpress several genes that typify myoepithelial cells of normal breast tissue, such as luminal CKs, smooth-muscle–specific markers, and certain integrins. Additionally, claudin-low tumors are generally high-grade tumors with low expression of Ki-67, E-cadherin, and claudins 3, 4, and 7 [4]. Basal-like

tumors tend to be TNBC that are positive for CKs, epidermal growth factor receptor (EGFR), and Ki-67 [5, 6].

These distinct transcriptional and genomic aberrations that differentiate the five subtypes of breast cancer suggest these variants may arise from different transformed progenitor or stem cells, each with distinct biologic properties [7-9]. Furthermore, these subgroups differ in prognosis and responses to therapy. The low-grade luminal A tumors are indolent and sensitive to hormone therapy such as antiestrogens. Luminal B tumors and tumors that are HER2/ER-positive have incomplete sensitivity to endocrine therapy, while HER2-positive tumors, which have a more aggressive nature, are sensitive to trastuzumab, an anti-HER2 antibody. Basal-like tumors also tend to be more aggressive compared to the luminal subtypes, though they can be especially sensitive to chemotherapy [10].

The likelihood of metastasizing to the brain differs drastically between subtypes [11-13]. An extensive study of the metastatic patterns of early-stage breast cancer patients found luminal tumors to be significantly associated with increased bone metastases but less lung or brain metastases [12]. Patients with HER2-enriched and basal-like tumors had a 3.6- to 5.3-fold increased risk to develop brain metastases compared to those with luminal A tumors [12]. A similar study by Smid et al. independently confirmed that patients presenting with HER2-positive and basal-like breast cancers were more likely to suffer brain metastases whereas patients with luminal tumors were more likely to present with bone metastases. Gene expression profiling of these tumors revealed that both basal-like tumors as well as brain metastatic tissue, regardless of breast cancer subtype, showed upregulation of WNT signaling and upregulation of the genes involved in cell cycle control [14].

Cancer stem cells (CSCs), though not unanimously accepted, are believed to be the primary impetus behind metastasis. The CSC theory is based on experimental evidence demonstrating that tumors are a heterogenous mass of cells that includes rare undifferentiated CSCs responsible for maintaining the tumor cell population [15]. These cells possess a number of key characteristics that also define normal stem cells, such as self-renewal, asymmetric division, and resistance to radiation and chemotherapeutic intervention [16]. For a CSC to form a metastasis, it must first separate from the primary tumor, intravasate into the circulatory system, evade anoikis, extravasate at the target organ, and adapt to and grow within the new microenvironment while avoiding immune system surveillance. Therefore, a CSC that is to colonize the brain should present with upregulation of signaling pathways that confer these abilities.

The HER2 pathway is one of the most researched pathways in the management of breast cancer. The HER2 receptor is a 185 kDa tyrosine kinase receptor and a member of the EGFR family of receptor tyrosine kinases. HER2 is found to be overexpressed in about 30% of primary breast cancers [17, 18], and confers an aggressive phenotype [19]. Compared to other types of breast cancer, HER2-positive tumors have a higher incidence of brain metastases with up to 50% of HER2-positive breast cancer patients developing intracranial metastases [20]. Although there are no known HER2-specific ligands to date, Honarvrar et al. developed an A9 nonapeptide derived from the trastuzumab-Fab portion capable of specifically binding HER2-positive cells leading to internalization of the construct [21]. HER2 possesses a functional tyrosine kinase domain that can be activated upon interactions with ligand-activated EGFR or HER3 [22-24], or homodimerization [25]. Activation of HER2 proteins leads to phosphorylation of downstream signaling proteins and the subsequent activation of multiple pathways implicated in breast cancer progression such as the STAT3,

RAS-MAPK, and PI-3K pathways. Previous work published by our laboratory has demonstrated the ability of HER2 to directly interact with the p53-upregulated modulator of apoptosis (PUMA) leading to its phosphorylation and subsequent degradation [26]. This activity promotes tumor cell survival suggesting HER2 is important for breast cancer progression.

EGFR is a transmembrane receptor tyrosine kinase, similar to HER2, that homodimerizes, heterodimerizes, or trans-autophosphorylates following ligand binding. EGFR-expressing basal-like tumors have a high likelihood of metastasizing to the brain [5, 12]. Seven ligands are known to bind and activate mammalian EGFR including EGF, heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor-α (TGF-α), betacellulin (BTC), amphiregulin (AREG), epiregulin (EREG), and epigen (EPGN) [27]. Once activated by phosphorylation, EGFR initiates multiple signaling cascades that promote cell proliferation, cell survival, and tumor progression [28-31]. EGFR has been implicated in potentiating circulating tumor cells (CTCs) and may play a role in brain-tropism. CTCs isolated from breast cancer patients with brain metastases were shown to overexpress specific proteins for brain metastasis compared to lung metastasis including EGFR, HER2, Heparanase, and Notch1. Expression of these proteins in breast cancer CTCs contributed to an invasive phenotype and ability to colonize the brain [32].

The Hedgehog (Hh) signaling pathway has been shown to promote the proliferation of adult stem cells from various tissues, including primitive hematopoietic cells, mammary and neural stem cells [33-35]. The signaling activity of this pathway has also been deemed critical for normal embryonic development, organogenesis, and adult tissue maintenance and renewal [36-41]. In the absence of Hh ligand, the pathway is inactive whereby the tumor

suppressor, Patched-1 (PTCH1), constitutively represses Smoothened (SMO) activity, leading to cytoplasmic sequestration of the glioma-associated oncogene homolog 1 (GLI1) transcription factor. Upon binding of Hh to PTCH1, SMO activity is derepressed allowing for nuclear translocation of GLI1. First discovered to be amplified in glioblastoma (GBM), GLI1 is a zinc-finger transcription factor that translocates to the nucleus and activates the transcription of target genes by binding to their promoters [42]. The primary target genes of GLI1 include the *PTCH1*, *PTCH2*, and *GLI1* genes [43]. Other GLI1 target genes include Hedgehog-interacting protein (Hhip) [44], cell cycle regulators (*CCND2* and *CCNE1*) [45], *BCL2* [46], *MYCN* [47], *ABCG2* [48], *FGF4* [49], *VEGFA* [50], *FOXM1* [51], and members of the WNT signaling pathway [52]. Notably, several of these target genes, such as *FOXM1*, *MYCN*, *BCL2*, lead to tumorigenesis, angiogenesis, proliferation and stemness, all of which are key characteristics of malignant tumors [53]. Aberrant GLI1 expression in breast cancer has been implicated in the initiation and proliferation of tumors, progression of malignant phenotype, invasion and metastasis, and the expansion of breast cancer stem cells [54]. Furthermore, high expression levels of sonic Hh (SHH) and GLI1 are reported to correlate with a higher histological grade and poor prognosis, such as shorter relapse-free survival and overall survival [55-61].

Our laboratory discovered an alternative splice variant of GLI1 in GBM which we termed truncated GLI1 (tGLI1) [62]. This truncated form arises from an in-frame deletion of 41 amino acids corresponding to the entirety of exon III and part of exon IV. Despite this, tGLI1 retains the functional domains of GLI1 and is activated by the SHH-PTCH1-SMO signaling pathway [62]. tGLI1 demonstrates tumor-specific expression in that it is detected in GBM, breast cancer, and most recently invasive hepatocellular carcinoma, but not in normal brain or breast tissue or hepatocytes [62-66]. Additional work published by our lab has demonstrated that tGLI1 is

a gain-of-function GLI1 transcription factor that, in addition to being able to regulate GLI1 target genes, acquires the ability to upregulate seven genes not regulated by GLI1, specifically *VEGFA*, *VEGFC*, *VEGFR2*, *TEM7*, *HPSE*, *CD24*, and *CD44* [62-64, 67-69]. These genes are known to promote cell proliferation, angiogenesis, migration and invasion of cancer cells, all of which are characteristics of tumors that are likely to metastasize. Furthermore, other groups independently confirmed the roles of tGLI1 in breast cancer angiogenesis [70] and gliomas [71], and reported the roles of tGLI1 in invasive hepatoma [72], and pancreatic/breast cancer in immune evasion [73].

In this study, we sought to identify tGLI1 inhibitors that could be developed further to treat tGLI1-positive BCBM. Given tGLI1's tumor-specific expression and potent oncogenic effects, tGLI1 is an ideal therapeutic target. Using a synthetic lethality chemical screen approach, we found that ketoconazole, an FDA-approved azole antifungal, was able to kill tGLI1-expressing cells without a concomitant effect on GLI1-expressing cells. We observed that ketoconazole was particularly efficacious against the tGLI1-positive CSC population without affecting normal cells. Ketoconazole also proved effective in preventing the engraftment and progression of tGLI1-positive BCBM in our *in vivo* mouse models. Furthermore, mechanistic studies suggest that downregulation of the stemness genes *CD44* and *OCT4* may play an important role in ketoconazole-elicited cell kill in tGLI1-expressing breast cancer cells. Collectively, our results demonstrate that ketoconazole is an effective inhibitor of CSCs and brain metastasis of tGLI1-positive breast cancer.

Chapter 1: Materials and Methods

Cell culture and reagents

Human breast cancer cell lines MCF10A, BT-20, and SKBR3 were purchased from ATCC (Manassas, VA) and cultured as specified by ATCC. HMLE, a kind gift from the Weinberg laboratory, were cultured in MEBM™ Mammary Epithelial Basal Medium (Lonza CC-3151) supplemented with MEGM™ Mammary Epithelial SingleQuots™ (Lonza CC-436), 10% fetal bovine serum (FBS, Corning 35-10-CV) and 1% Penicillin-Streptomycin solution (P/S, Corning 30-002-CI). MDA-MB-231, brain-metastatic MDA-MB-231-BRM, and the lung-metastatic MDA-MB-231-LM cell lines are from the Massagué laboratory. Brain-metastatic variant of SKBR3 (SKBRM) was a kind gift from Dr. Watabe (Wake Forest Baptist Medical Center, Winston-Salem, NC). SKBRM are brain metastatic cells derived from parental SKBR3 cells through three rounds of *in vivo* selection [74]. The expression plasmids for GLI1 and tGLI1 were previously developed in our laboratory [62]. Drug libraries (L1200, L1300, L1400) were purchased from Selleck Chemicals (Houston, TX). CD44 and OCT4 expression plasmids were purchased from Sino Biological, Inc. (Beijing, China).

Isogenic Cell Lines

Lentiviruses containing vector, GLI1 or tGLI1 were generated as described previously [63, 75]. Breast cancer cell lines were infected with virus at an MOI of 30 for 72 hours. Expression was confirmed by red fluorescent protein (RFP) signal and cells were grown under puromycin selection. To ensure cell purity, each cell line was sorted by RFP signal using the FACS Aria instrument.

Synthetic Lethality Screen

Adherent MDA-MB-231 and MDA-MB-231-BRM cell lines stably expressing the control vector, GLI1-, or tGLI1-overexpression vectors were cultured in Dulbecco's Modification of Eagle's Medium (DMEM, Corning 10-013-CMR) supplemented with 10% FBS (Corning 35-10-CV) and 1% P/S (Corning 30-002-CI). Cells were harvested during the exponential growth phase, seeded at $4x10^3$ cells per well in 96-well white bottom plates (Greiner Bio-One 655083), and incubated at 37°C, 5% CO_2 for 24 hours. Cells were subsequently treated with various concentrations of test compound or vehicle control for 48 hours. The concentration of DMSO (vehicle) was 1% for all treatments. Viability was determined using the CellTiter-Blue® Cell Viability Assay (Promega G8080) according to the manufacturer's instructions.

Colony Formation Assay

Adherent cells in the exponential growth phase were harvested and seeded at 250 cells per well into 12-well plates (Corning 3513) and allowed to grow for 24 hours. Culture medium was subsequently replaced with fresh media containing various concentrations of ketoconazole or vehicle control. Treatment medium was replaced every 48 hours during the 10-day treatment window. Cells were washed once with phosphate-buffered saline (PBS) before being stained for 1 hour with 1.5% Crystal Violet (Millipore Sigma HT90132) diluted in 20% ethanol. Plates were washed with tap water and allowed to dry before colonies were counted.

Mammosphere Assay

Adherent cells were harvested and seeded at a density of $1x10^3$ to $4x10^3$ cells per well in 24-well ultra-low attachment plates (Corning 3473) with Dulbecco's modified Eagle's medium/F12 (Gibco 11320033) containing 2% B27 (Gibco 17504044), 20 ng/mL rEGF (Millipore Sigma PHG0311), 4 μg/mL insulin (Millipore Sigma 12585014), and 100 ng/mL

SHH (Millipore Sigma GF174). Beginning 24 hours after seeding, mammospheres were treated with varying concentrations of ketoconazole or its derivatives. Mammospheres were cultured for 7–14 days and supplemented with 100 μL of fresh treatment prepared in mammosphere medium every 48 hours. The number of spheres per well were counted under 5× objective.

Selective knockdown of tGLI1 using antisense oligonucleotides (AS-ONs)

Control or tGLI1 specific locked nucleic acid (LNA) AS-ONs were custom designed and purchased from Qiagen. Sequence for the negative control LNA AS-ON is /56-FAM/*A*A*C*A*C*G*T*C*T*A*T*A*C*G*C. BLAST analysis did not show binding of the control to any gene. Sequence for the tGLI1-targeting AS-ON is /56-FAM/$^{+}$C^{+}A^{+}A^{+}CT*T*G*A*C*T*T*C^{*+}T^{+}G^{+}TC. Phosphorothioated bases are indicated by * whereas LNA bases are labeled by +. Knockdown of tGLI1 was conducted as described previously [63]. Briefly, BT-20 cells were transfected for 48 hours with control or tGLI1 AS-ON using Lipofectamine 2000 (Invitrogen 11668027). Cells were subsequently harvested for RT-PCR or seeded for a mammosphere assay.

Animal Housing

Female athymic nude (*nu*/*nu*) mice 6-8 weeks of age purchased from Charles River Laboratories (Wilmington, MA) were used. All colonies were housed in a pathogen-free facility of the Animal Research Program at Wake Forest School of Medicine (WFSM) under a 12:12-hour light/dark cycle and fed irradiated rodent chow *ad libitum*. Animal handling and procedures were approved by the WFSM Institutional Animal Care and Use Committee (IACUC) (protocol #A15-122).

Pharmacokinetic Study

Mice were inoculated with $2x10^5$ actively growing SKBRM cells overexpressing tGLI1 (SKBRM-tGLI1) in 100 μL ice cold PBS as per the method originally described by Conley et al. [76]. Tumor engraftment was monitored biweekly via bioluminescent imaging. Once sufficiently sized tumors had formed at 10 days following inoculation, mice received a single intraperitoneal (I.P.) treatment of either vehicle or 50 mg/kg ketoconazole. Twenty minutes after treatment, mice were sacrificed, blood collected by intracardiac bleed, and recovered brains were washed three times in ice cold PBS prior to being flash frozen in the vapor phase of liquid nitrogen. Mass spectrometry analysis was performed by the Metabolomics and Proteomics Shared Resource.

***In Vivo* Tumor Prevention Study**

Twenty-four hours prior to intracardiac inoculation into the left ventricle, mice received a single 100 μL I.P. treatment of either vehicle or 50 mg/kg ketoconazole dissolved in 100% polyethylene glycol 300 (PEG-300). Mice were slowly inoculated with $2x10^5$ actively growing SKBRM-tGLI1 in 100 μL ice cold PBS. Each group contained 10 mice. For bioluminescent imaging, xenograft-bearing mice were injected with d-luciferin intraperitoneally at 100 mg/kg body weight and imaged twice weekly using the IVIS Lumina LT Series III imager from PerkinElmer (Waltham, MA) to monitor tumor progression. Treatments were administered three times per week until study termination according to our IACUC-approved humane endpoints encompassing ambulation, body weight, and grooming habits.

***In Vivo* Tumor Treatment Study**

Mice were inoculated with $2x10^5$ actively growing SKBRM cells overexpressing either GLI1 (SKBRM-GLI1) or tGLI1 (SKBRM-tGLI1) in 100 μL ice cold PBS. Tumors were allowed to

engraft for 13 days after which mice were randomized into either vehicle or 50 mg/kg ketoconazole treatment groups. Each of the 4 groups contained 12 mice. Tumor progression was monitored via biweekly bioluminescent imaging and mice received three I.P. treatments per week. The study was terminated according to our IACUC-approved humane endpoints encompassing ambulation, body weight, and grooming habits.

Extraction and Cryopreservation of Brains

At the conclusion of the studies, mice were sacrificed by overdosing with 200 µL of a mixture of 114 mg/kg ketamine and 17 mg/kg xylazine. Euthanasia was confirmed by cervical dislocation. Following decapitation, brains were rapidly excised and placed in PBS on ice. For *ex vivo* bioluminescent imaging, brains were incubated in a mixture of 100 µL d-luciferin and 6 mL PBS at room temperature for 5 minutes. Brains were carefully imaged before being embedded in NEG-50 Frozen Section Medium (Richard-Allen Scientific 6502) and stored at -80°C.

Quantitative RT-PCR of miRNAs from mouse serum

Following exsanguination, serum was collected by allowing the blood to clot at room temperature for 25 min. The clot was removed via centrifugation at 2000 x *g* for 15 minutes at 4°C. The supernatant was removed and stored at -80°C until further analysis. To further remove any cellular debris, the serum was centrifuged for an additional 15 minutes at 3000 x *g* and 4°C. Next, 200 µL of the biofluid was mixed with 53.6 µL of ExoQuick© Exosome Precipitation Solution (System Biosciences EXOQ5A-1). The mixture was incubated on ice for 30 minutes and precipitated exosomes were pelleted by centrifugation for 30 minutes at 1500 x *g* at 4°C; supernatant was aspirated from the tube and discarded. The pellet was resuspended in 250 µL of the provided Resuspension Buffer and the protein concentration was

determined to estimate the quantity of exosomes present in the sample. Next, 500 µg of protein equivalent of the exosome in 250 µL was added to the provided Microsphere Beads. The samples were mixed at room temperature on an inverting shaker for 15 min. After mixing, the samples were centrifuged for 5 minutes at 6000 x *g* at 4°C. The supernatant containing the purified exosomes was removed and stored at -80°C until further processing. Total miRNA was isolated from purified exosomes using the miRNeasy kit from Qiagen (217004), which is specifically designed for purification of RNA 18 nucleotides (nt) and larger, according to the manufacturer's instructions. MiRNA complementary DNA (cDNA) was then synthesized using the TaqMan™ Advanced miRNA cDNA Synthesis kit from Applied Biosciences (A28007). Quantitative PCR (qPCR) was carried out using the TaqMan™ Fast Advanced Master Mix (Applied Biosciences 4444557).

Western Blots

Western blot analysis was performed as previously described [62, 77]. Immunoblotting antibodies included androgen receptor (Cell Signaling, D6F11), GLI1 (Cell Signaling; 2643), Nanog (Cell Signaling; 4903), Sox2 (Cell Signaling; 4900), α-tubulin (Sigma), and β-actin (Sigma).

CD44/OCT4 Rescue

CD44 (HG12211-UT) and OCT4 (HG13137-UT) mammalian expression plasmids were received from Sino Biological and transformed into DH5α competent cells (ThermoFisher 18265017). Positive transformants were selected for using 50 µg/mL Ampicillin. Plasmid DNA was isolated from bacteria using the SpinSmart Plasmid DNA Miniprep kit (Thomas Scientific, Inc. 1158P42) according to the manufacturer's instructions. Plasmid purity was demonstrated using a 1% agarose gel. Expansion and purification of the correct plasmids was verified via

PCR. SKBRM-tGLI1 cells were transfected with either the empty control vector (CV011), CD44, or OCT4 expression plasmids using XtremeGene HP (Millipore Sigma 6366244001) in T25 flasks (Corning CLS430639). To one T25 flask containing $1x10^6$ cells in 5 mL growth medium, 200 μL of transfection mix comprised of 6 μg plasmid DNA and XtremeGene HP at a ratio of 3 μL:1 μg plasmid in OptiMEM reduced-serum medium (Gibco 31985070) was added to each flask. Cells were allowed to grow for 48 hours at 37°C and 5% CO_2 before being seeded for a mammosphere assay.

Visualization of Plasmid Purity and PCR product using an Agarose Gel

A 1% or 1.5% (w/v) agarose gel was prepared by dissolving 0.50 g or 0.75 g OmniPur agarose (Millipore Sigma 2120-OP) in 50 mL Tris-acetate-EDTA (TAE) buffer, respectively. Once the solution cooled to below 60°C, 1.5 μL of SYBR® Safe DNA dye (Invitrogen S33102) was added and the solution was poured into an electrophoretic gel case and allowed to solidify. The case was then filled with TAE running buffer. Lanes were loaded with either 5 μL of 1 kb DNA ladder (Millipore Sigma D0428) or 0.1 μg plasmid or PCR product diluted to 12 μL with 6X DNA Gel Loading Dye (ThermoFisher R0611) and ddH_2O. Samples were electrophoresed for 45 minutes to 1 hour at 70V. Gels were imaged on the Amersham Imager 600 (GE Healthcare Life Sciences 29083461).

PCR

GE Healthcare illustra™ RNAspin Mini Isolation Kit (45-001-162) was used to isolate total RNA and RT was performed using the Superscript III First-Strand cDNA synthesis system (Invitrogen 18080051). Relative gene expression levels were visualized using a 1% or 1.5% agarose gel.

Statistical analyses

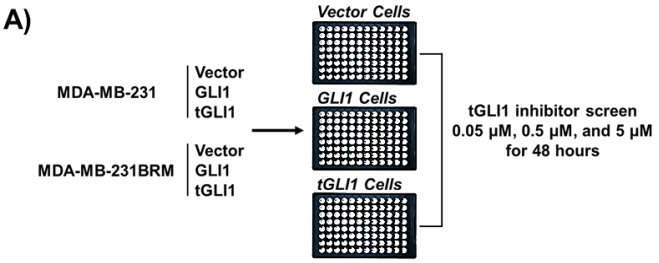
Results are represented as mean ± SEM unless otherwise noted. Fisher's exact test and Student's t-test were performed using GraphPad Prism 5.

Chapter 2: Results

Synthetic lethality screen identified compounds preferentially inhibiting the growth of tGLI1-expressing breast cancer cells.

To identify potential tGLI1 inhibitors, we took a synthetic lethality approach in designing our chemical screen [78, 79]. The screen was performed in MDA-MB-231 and MDA-MB-231BRM lines engineered to express an empty vector, GLI1-, or tGLI1-expression vector (Figure 1A). 1520 different compounds from three commercial libraries were screened. We selected inhibitors that resulted in a viability fold change of less than 0.50 at a dose of 5 μM

A)



B)

				Cytotoxic effect [Fold Change over Vehicle Control]								
				MDA-MB-231								
				Vector			GLI1			tGLI1		
Compound	Library	Pathway	Target	0.05 μM	0.5 μM	5 μM	0.05 μM	0.5 μM	5 μM	0.05 μM	0.5 μM	5 μM
AG-490 (Tyrphostin B42)	KI	Protein Tyrosine Kinase	JAK, EGFR	1.20	1.17	1.21	1.08	1.42	1.02	0.95	1.32	0.57
JNJ-7706621	KI	Cell Cycle	Aurora Kinase, CDK	1.82	1.04	0.17	1.23	1.11	0.14	1.06	0.40	0.14
BMS-265246	KI	Cell Cycle	CDK	1.74	1.30	0.28	1.24	1.00	0.33	0.85	0.87	0.22
Crenolanib (CP-868596)	KI	Protein Tyrosine Kinase	PDGFR	0.93	0.91	0.28	1.11	0.96	0.11	1.23	0.82	0.17
FRAX597	KI	Cytoskeletal Signaling	PAK	0.90	0.90	0.58	1.25	1.01	0.69	1.14	0.73	0.16
Ketoconazole	FDA		P450 (e.g. CYP17)	1.01	1.23	1.57	1.36	1.25	1.37	0.72	0.69	0.65
Valacyclovir HCl	FDA		Others	1.01	1.05	0.88	1.11	0.96	1.04	1.18	0.77	0.78
Chlorzoxazone	FDA		Others	1.31	0.92	0.00	1.41	1.02	0.00	0.82	0.72	0.00
Andrographolide	NP			0.96	0.99	0.39	1.01	1.15	0.43	1.09	0.66	0.36
Chlorogenic acid	NP			0.95	1.23	1.08	1.13	1.07	1.05	1.06	0.87	0.79
Fumalic acid (Ferulic acid)	NP			1.16	1.27	1.11	1.28	1.11	1.07	0.99	0.70	0.67
Gramine	NP			1.24	1.30	1.22	1.24	1.01	1.18	1.02	0.16	0.41
Kaempferol	NP			1.01	1.34	0.85	1.35	1.12	0.77	0.97	0.22	0.50
Morin hydrate (Aurantica)	NP			0.93	1.22	1.01	1.35	1.11	1.09	1.04	1.22	0.17
Chrysophanic acid (Chrysophanol)	NP		EGFR, mTOR	1.29	0.96	0.00	1.12	0.98	0.00	1.34	0.79	0.00
Apocynin (Acetovanillone)	NP			1.37	1.07	1.16	1.10	1.05	1.21	1.46	0.85	0.79

C)

				Cytotoxic effect [Fold Change over Vehicle Control]								
				MDA-MB-231-BRM								
				Vector			GLI1			tGLI1		
Compound	Library	Pathway	Target	0.05 μM	0.5 μM	5 μM	0.05 μM	0.5 μM	5 μM	0.05 μM	0.5 μM	5 μM
Roscovitine (Seliciclib,CYC202)	KI	Cell Cycle	CDK	0.97	0.93	0.94	1.06	1.02	0.94	0.96	1.03	0.61
JNJ-7706621	KI	Cell Cycle	Aurora Kinase, CDK	1.00	1.17	0.56	1.16	1.17	0.95	1.11	0.63	0.48
Ruxolitinib (INCB018424)	KI	JAK/STAT	JAK	1.12	1.03	0.96	1.16	1.10	0.95	1.05	0.74	0.66
BMS-265246	KI	Cell Cycle	CDK	1.16	1.21	1.17	0.82	1.24	0.97	1.18	1.27	0.54
Indirubin	KI	PI3K/Akt/mTOR	GSK-3	1.09	1.14	1.03	1.09	1.10	0.93	0.99	1.14	0.53
WYE-125132 (WYE-132)	KI	PI3K/Akt/mTOR	mTOR	0.96	0.97	0.75	0.90	0.91	0.76	0.93	0.86	0.56
KX2-391	KI	Angiogenesis	Src	1.20	1.23	1.00	1.22	1.21	1.09	1.19	1.16	0.65
SB415286	KI	PI3K/Akt/mTOR	GSK-3	1.10	1.02	0.89	1.02	1.02	0.87	1.03	1.05	0.56
Ketoconazole	FDA		P450 (e.g. CYP17)	1.09	0.98	1.01	1.03	1.01	0.98	0.65	0.67	0.64
Silibinin (Silybin)	NP		P450	1.10	1.10	0.98	1.01	1.06	0.99	1.06	1.06	0.58

Figure 1. Synthetic lethality screen identified compounds preferentially inhibiting the growth of tGLI1-expressing breast cancer cells. A) Overview of the setup of the tGLI1 inhibitor screen. MDA-MB-231 and MDA-MB-231BRM cell lines stably expressing the control vector, GLI1-, or tGLI1-overexpression vectors were seeded in 96-well plates and treated in triplicate with a test compound consisting of 1520 different compounds. After 48 hours of exposure to 0.05 μM, 0.5 μM, or 5 μM of test compound, cell viability was assessed using CellTiter-Blue® Cell Viability Assay. **B-C)** Compounds demonstrating a cytotoxic effect in MDA-MB-231 or MDA-MB-231BRM cell lines. Fold change values shown are relative to the vehicle treatment within each cell line. Selected candidate compounds (hits) produced a viability fold change of <0.50 at 5 μM in any cell line or a viability fold change of <0.80 at 5 μM in the tGLI1-expressing cell line. KI, Kinase Inhibitor; NP, Natural Product. Student's t-test was used to compute *p*-values.

regardless of selectivity and tGLI1 selective compounds that produced a viability fold change of less than 0.80 at 5 μM (Figure 1B-C). Of the 25 compounds that fulfilled either of the hit

criteria, only ketoconazole, an FDA-approved antifungal, was active in both cell lines and preferentially targeted tGLI1-expressing cells.

Ketoconazole selectively targets the tGLI1-positive breast cancer stem cell population.

Given tGLI1's role in promoting breast cancer stem cells (unpublished data; not shown), a small population of cells thought to be responsible for cancer progression, metastasis, and recurrence [80]; we wanted to determine if ketoconazole could target this cell population. Because MDA-MB-231 and MDA-MB-231BRM cells are unable to form mammospheres, a colony formation assay was performed. The colony formation assay is an *in vitro* assay that assesses the ability of a cell population to clonally expand, an indicator of undifferentiated cancer cells [81, 82]. Briefly, cells were seeded at a low density and treated with vehicle or ketoconazole for 10 days after which colonies were counted following Crystal Violet staining. Treatment with ketoconazole significantly reduced colony formation of tGLI1-expressing MDA-MB-231 and MDA-MB-231BRM cells beginning at the 100 pM dose relative to both the vector and GLI1-expressing cells (Figure 2A), suggesting ketoconazole targets the CSC subpopulation. There was no significant reduction in colony formation in either vector or GLI1-expressing cell line with ketoconazole treatment. To further investigate the ability of ketoconazole to inhibit the CSC subpopulation, we performed a mammosphere formation assay to examine the ability of ketoconazole to inhibit CSC [83]. tGLI1 expression has been shown to be induced when CSCs are enriched under mammosphere culture conditions (unpublished data; not shown). In agreement with the colony formation assay, treatment with ketoconazole significantly reduced the mammosphere forming ability of tGLI1-overexpressing SKBRM cells without affecting the vector control or GLI1-overexpressing cells (Figure 2B).

Upon treatment of immortalized human astrocytes (UC1), normal brain endothelial cells (HBMEC), and two mammary epithelial cell lines (HMLE and MCF10A), only 10 μM ketoconazole resulted in a significant reduction in viability in UC1, which is 5-fold higher than the dose required to elicit an effect on cancer stem cells (Figure 2C). Given the pronounced

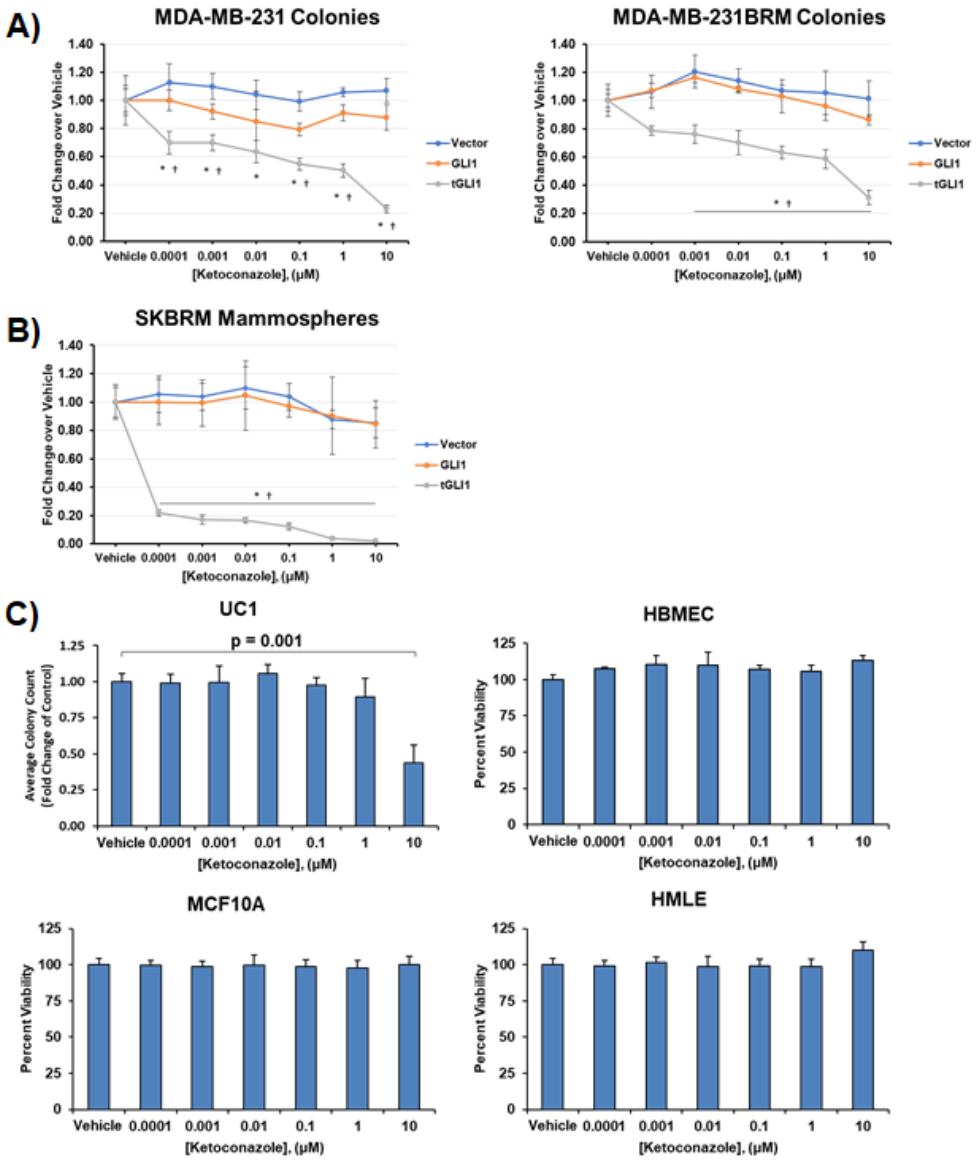


Figure 2. Ketoconazole selectively targets the tGLI1-positive breast cancer stem cell population. **A-B)** tGLI1 ectopic expression sensitizes the cancer stem cell population to ketoconazole treatment. Panel A, Colony formation assay showing the dose-response effect of ketoconazole in MDA-MB-231 and MDA-MB-231-BRM ectopically expressing vector, GLI1, or tGLI1. Panel B, Mammosphere formation assay showing the dose-response effect of ketoconazole in SKBRM ectopically expressing vector, GLI1, or tGLI1. Statistical significance denoted by symbols; **P*<0.05 relative to SKBRM-Vec, †*P*<0.05 relative to SKBRM-GLI1. **C)** Viability assays showing ketoconazole is not toxic to normal human astrocytes (UC1), brain endothelial (HBMEC), or breast epithelial (MCF10A and HMLE) cells. The results are expressed relative to the vehicle control (1% dimethyl sulfoxide (DMSO) treated). Student's t-test was used to compute *p*-values.

effect of ketoconazole on CSC at the low dose of 100 pM, we believe that ketoconazole directly targeted the CSC population. Additionally, ketoconazole only affects cells that overexpress tGLI1 but not the vector control or GLI1, suggesting tGLI1 expression may be required for ketoconazole to affect these cells.

tGLI1 expression is required for ketoconazole's ability to target tGLI1-expressing breast cancer cells.

We have previously shown tGLI1 expression to be induced under mammosphere-forming (CSC) conditions, but not when cells are grown as a monolayer (unpublished data; not shown). Given this information, we wanted to compare the ability of ketoconazole to inhibit the CSC subpopulation versus total cell population. Parental MCF7, BT-20, and MDA-MB-231-LM cells were concurrently grown as either monolayers or mammospheres, and then treated with increasing concentrations of ketoconazole. The CellTiter-Blue® Viability and mammosphere assays were used to assess ketoconazole-induced toxicity in monolayer versus mammosphere cultures, respectively. In all three cell lines shown to undergo tGLI1 enrichment when grown as mammospheres, we found significant reduction in mammosphere formation, but not monolayer viability upon treatment with ketoconazole (Figure 3A), suggesting that ketoconazole is more effective in targeting tGLI1-expressing CSCs.

Next, we wanted to determine if tGLI1 knockdown would abolish the sensitivity of cells to ketoconazole treatment. Briefly, parental BT-20 cells were transiently transfected with either a non-targeting locked nucleic acid (LNA) anti-sense oligonucleotide (AS-ON) or a tGLI1 targeting AS-ON before being seeded in mammosphere-forming conditions [63]. The phosphorothioated bases and LNA bases of the oligonucleotides preclude nuclease-mediated degradation. The AS-ON include a 5' 6-FAM fluorescent dye attachment to allow for

visualization of transfection efficiency. From a sample of 700 cells, the transfection efficiency for the non-targeting AS-ON oligo was estimated to be 49.6% while the transfection efficiency of the tGLI1 AS-ON was slightly lower at 32.7%. Untransfected parental BT-20 cells were included in the experiment to monitor the effects of AS-ON transfection on mammosphere

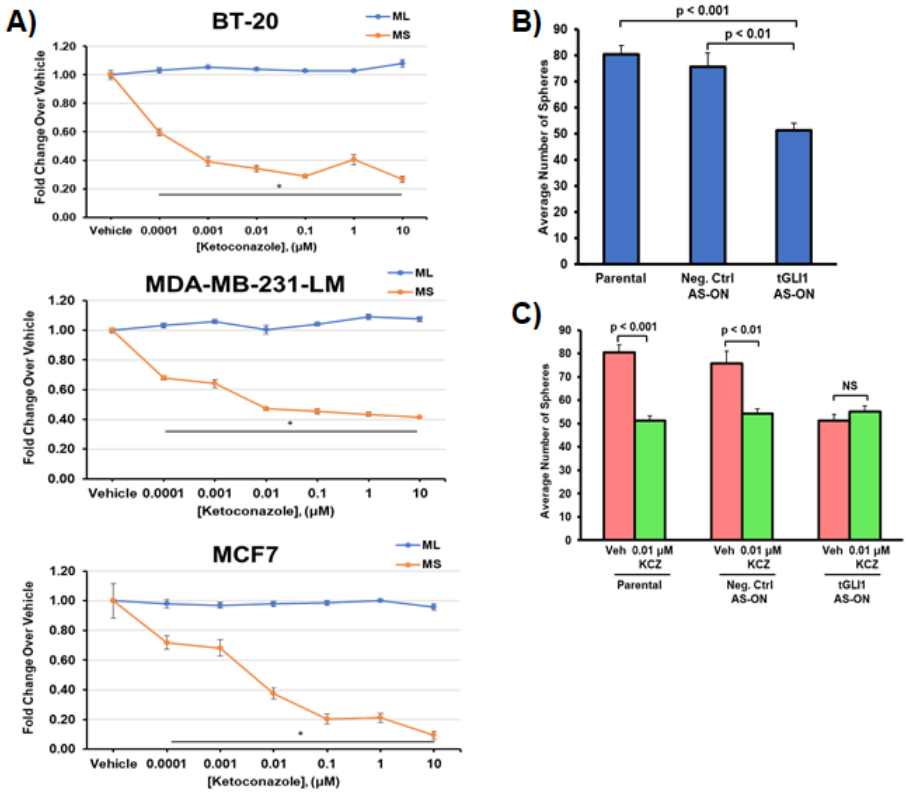


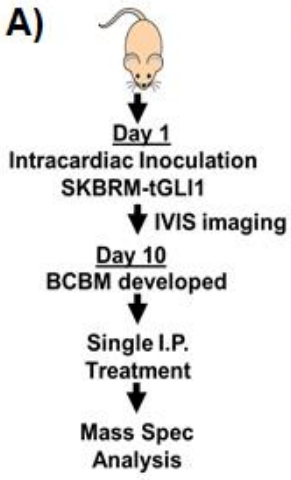
Figure 3. tGLI1 expression is required for ketoconazole's ability to target tGLI1-expressing breast cancer cells.
A) Comparison of the effect of treatment with increasing concentrations of ketoconazole on parental cell lines grown as monolayer (ML) or mammospheres (MS). Viability of ML was determined by CellTiter-Blue® Viability assay while the number of spheres was used to assess the stem cell population. The results are expressed relative to the vehicle control (1% dimethyl sulfoxide (DMSO) treated). Statistical significance denoted by asterisks; **P*<0.05 relative to ML. **B)** Transfection with a tGLI1 AS-ON significantly reduced the mammosphere-forming ability of BT-20 cells compared to the parental cell line and cells transfected with the non-targeting control AS-ON. **C)** Mammosphere assay showing the number of mammospheres formed by parental BT-20, BT-20s transfected with control AS-ON, and BT-20s transfected with tGLI1 AS-ON after 7 days of treatment with vehicle (1% DMSO) or 0.1 μM ketoconazole. Student's t-test was used to compute *p*-values.

formation and stability. Transient transfection with the non-targeting AS-ON did not significantly affect mammosphere formation by BT-20 cells (Figure 3B). However, transfection with the tGLI1 AS-ON significantly reduced the average number of

mammospheres compared to both the parental cells and those transfected with the control AS-ON (Figure 3B) confirming our previous results (unpublished data; not shown) [63]. Despite being transiently transfected, both AS-ON were present in mammospheres after 10 days in culture (data not shown). Upon treatment with 0.01 μM ketoconazole, we observed a significant reduction in mammosphere formation in the parental cells and cells transfected with the non-targeting AS-ON, but not in cells with tGLI1 knockdown (Figure 3C), indicating the requirement of tGLI1 in ketoconazole-mediated suppression of CSCs. Collectively, these results demonstrate that tGLI1 is required for the ability of ketoconazole to inhibit the breast CSC population.

Ketoconazole penetrates the blood-brain barrier of female athymic mice.

Since published literature provides conflicting evidence on ketoconazole's ability to cross the BBB [84-87], we performed an *in vivo* pharmacokinetic study to determine the brain tissue concentration of ketoconazole in treated mice by mass spectrometry (Figure 4A). Mice were intracardially inoculated with actively growing SKBRM-tGLI1 cells and tumor engraftment was monitored with biweekly IVIS imaging for 10 days. Mice with and without detectable



B)

Whole Brain Samples

Treatment	BCBM Status	Concentration (ng/mg)
Vehicle	No Met	Undetected
	With Met	Undetected
50 mg/kg KCZ	No Met	>>> 6.250
	With Met	>>> 6.250

Figure 4. Ketoconazole penetrates the blood-brain barrier of athymic mice.
A) Overview of the pharmacokinetic study to assess brain penetrance by ketoconazole. **B)** Detection of ketoconazole according to metastasis status and treatment. N=3 per group.

tumors were randomized into either vehicle or 50 mg/kg ketoconazole treatment groups (N=3/group). Each mouse received a single 100 μL treatment of either vehicle or 50 mg/kg ketoconazole by intraperitoneal injection 20 minutes before sacrifice. Brains were then rapidly excised, and flash frozen in the vapor phase of liquid nitrogen prior to storage at -80°C. Whole brain samples were homogenized, and ketoconazole content was determined on a Shimadzu LCMS-8050 triple-quadrupole mass spectrometer combined with Shimadzu Nexera UPLC system using caffeine as an internal standard. The brain tissue concentration of ketoconazole was found to greatly exceed 6.250 ng/mg tissue whereas ketoconazole was not detected in any of the control mice (Figure 4B). This finding was true for samples with and without brain metastases indicating that ketoconazole can permeate an intact BBB in our *in vivo* mouse model.

Systemic administration of ketoconazole inhibits development of breast cancer brain metastases *in vivo*.

Given our findings demonstrating ketoconazole can cross the BBB and its ability to selectively target tGLI1-positive breast cancer cells *in vitro*, we then wanted to determine if ketoconazole could be used to inhibit the establishment of BCBM *in vivo* (Figure 5A). Athymic female *nu/nu* mice were pretreated with 100 μL of either vehicle or 50 mg/kg ketoconazole by I.P. injection. Twenty-four hours later, mice were inoculated with actively growing SKBRM-tGLI1 into the left ventricle. Each group contained 10 mice. Successful inoculation was confirmed approximately an hour later by IVIS imaging. Treatments were administered three times per week and tumor progression was monitored twice weekly by IVIS imaging. Organs were harvested and imaged *ex vivo* to confirm tumors were present in the organs and not the surrounding tissue. Measuring the weights of the mice over time showed no acute toxicity due to drug treatment (Figure 5B). Two mice from the ketoconazole group were removed from

analysis due to unsuccessful intracardiac inoculations. The results showed that ketoconazole

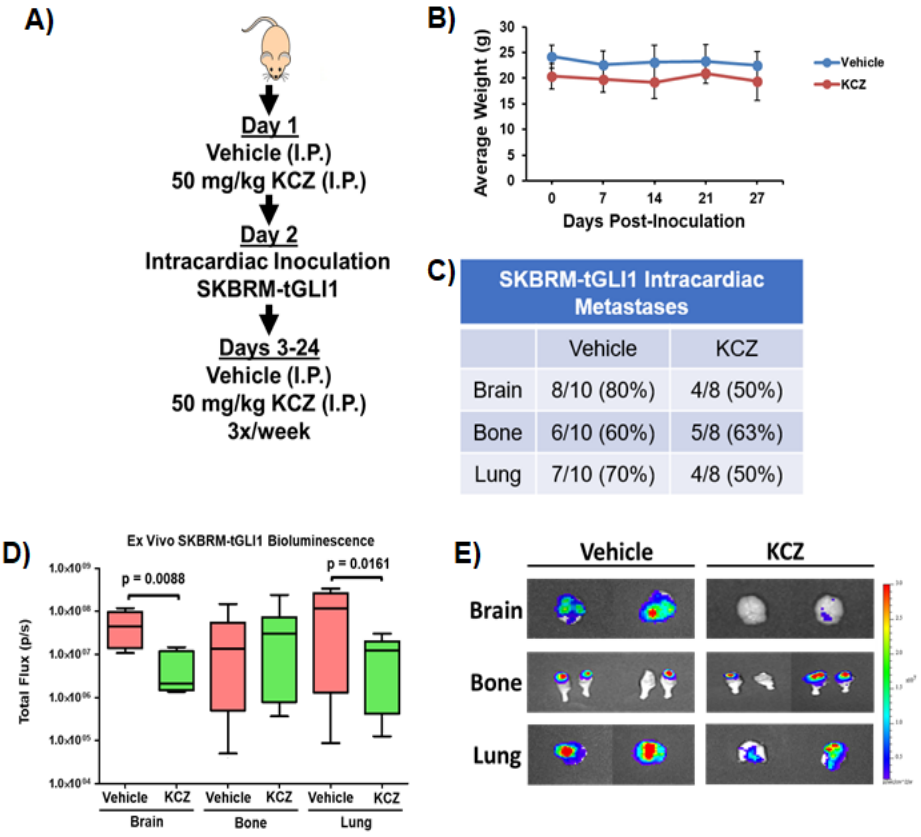


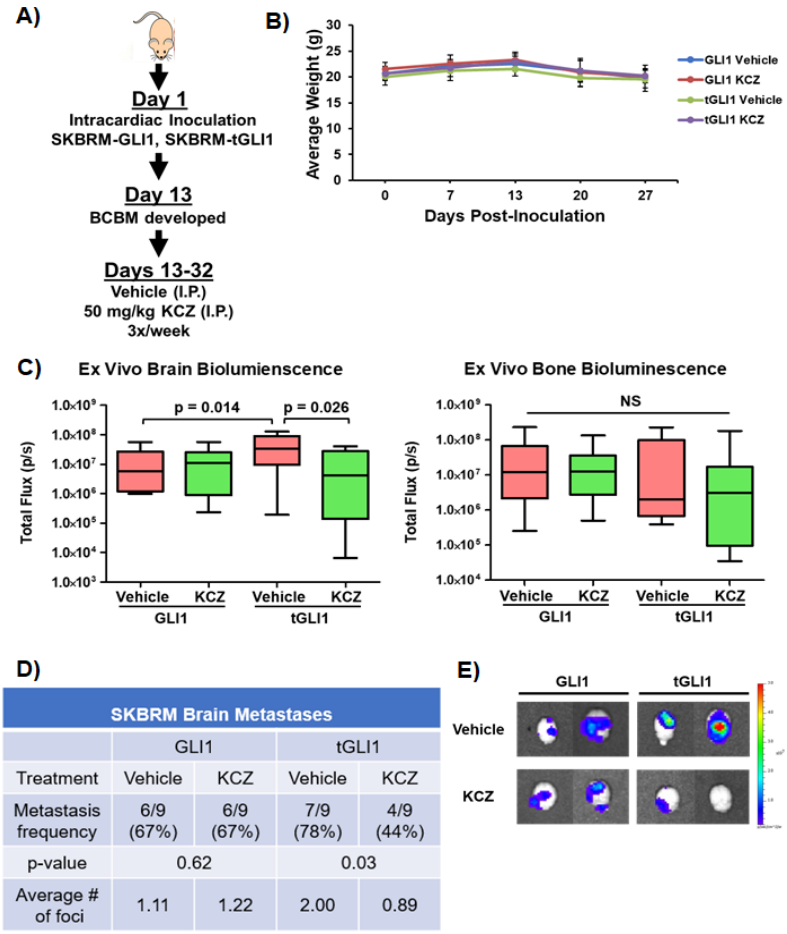
Figure 5. Systemic administration of ketoconazole inhibits the development of breast cancer brain metastasis *in vivo*.
A) Overview of the tumor prevention model. **B)** Average animal weight tracked over the course of the experiment. **C)** Incidence of intracardiac metastases. **D)** Brain, bone, and lung *ex vivo* bioluminescence was quantified. **E)** Representative *ex vivo* IVIS images. N=8-10 per group. Student's t-test was used to compute *p*-values.

reduced the incidence of BCBM compared to the vehicle treatment (Figure 5C). We observed that 8/10 (80%) of the vehicle-treated mice presented with brain metastases, 6/10 (60%) with bone metastases, and 7/10 (70%) with lung metastases compared to 4/8 (50%), 5/8 (63%), and 4/8 (50%) presenting with brain, bone, and lung metastases, respectively, in the ketoconazole-treated group (Figure 5C). Quantification of the bioluminescent signal, a surrogate measure of tumor volume, revealed that tGLI1-expressing brain and lung metastases treated with ketoconazole were significantly smaller than their vehicle-treated counterparts (Figure 5D). There was no significant reduction in the size of bone metastases with ketoconazole treatment.

Representative *ex vivo* images are shown in Figure 5E. These findings suggest ketoconazole can prevent the development and progression of breast cancer brain and lung metastases, but not bone metastases.

Ketoconazole selectively reduces the *in vivo* progression of established tGLI1-positive breast cancer brain metastases.

Our previous animal study could not be used as evidence that ketoconazole could be used to treat already established tGLI1-positive BCBM because ketoconazole could also target circulating cancer cells in addition to established brain metastases. Since our evidence suggests ketoconazole kills tGLI1-positive breast cancer cells, it can be reasonably argued that the ketoconazole pretreatment killed a portion of the breast cancer cells before they reached their target organs, and this is what lead to the difference in tumor size between the treatment groups. Furthermore, it could also be argued that even though ketoconazole can cross the BBB, it cannot cross the blood-tumor barrier (BTB) and accumulate to a sufficient concentration to inhibit tGLI1-postive tumor growth. Therefore, we conducted a second *in vivo* study to address these arguments by determining if ketoconazole can be used to treat existing tGLI1-expressing BCBM (Figure 6A). Anesthetized mice were inoculated with either SKBRM-GLI1 or SKBRM-tGLI1 cells into the left ventricle. Tumors were allowed to engraft for 13 days after which mice were randomized into either vehicle or 50 mg/kg ketoconazole treatment groups. Each of the 4 groups contained 12 mice. Tumor progression was monitored via biweekly bioluminescent imaging and mice received three I.P. treatments per week. Measuring the weights of the mice over time showed no acute toxicity due to drug treatment (Figure 6B). Organs were harvested and imaged *ex vivo* to confirm tumors were present in the organs and not the surrounding tissue. Three mice from each group, a total of 12 mice, were removed from analysis due to unsuccessful inoculations or early death. Brain *ex vivo* bioluminescent imaging



SKBRM Brain Metastases				
	GLI1		tGLI1	
Treatment	Vehicle	KCZ	Vehicle	KCZ
Metastasis frequency	6/9 (67%)	6/9 (67%)	7/9 (78%)	4/9 (44%)
p-value	0.62		0.03	
Average # of foci	1.11	1.22	2.00	0.89

Figure 6. Ketoconazole selectively reduces the *in vivo* progression of established tGLI1-positive breast cancer brain metastases.
A) Overview of the tumor treatment model. **B)** Average animal weight tracked over the course of the experiment. **C)** Brain and bone *ex vivo* bioluminescence was quantified. **D)** Incidence of SKBRM-GLI1 and SKBRM-tGLI1 brain metastases and number of foci. Tumor foci within mouse brains were determined by *ex vivo* IVIS imaging. *P*-values calculated by Fisher's exact test. **E)** Representative *ex vivo* IVIS images of brains. N=12 per group. Student's t-test was used to compute *p*-values.

showed that ketoconazole treatment significantly reduced the size of tGLI1-expressing brain metastases but not GLI1-expressing brain metastases (Figure 6C). Furthermore, untreated tGLI1-expressing brain metastases were significantly larger than the untreated GLI1-expressing metastases providing evidence to further support tGLI1's role in promoting the aggressiveness of BCBM (Figure 6C). Ketoconazole was also able to reduce the incidence of detectable BCBM in mice with tGLI1-expressing tumors, but not those with GLI1-expressing tumors (Figure 6D). We also observed evidence ketoconazole may be useful in suppressing multifocal brain cancer. In these patients, an increasing number of foci correlates with

worsening survival rates [88, 89]. After calculating the number of foci in each brain sample, we found that ketoconazole treatment substantially reduced the number of foci in mice inoculated with tGLI1-expressing cells but not in mice inoculated with GLI1-expressing cells (Figure 6D). Furthermore, untreated mice inoculated with tGLI1-expressing cells presented with more foci compared to untreated mice harboring GLI1-expressing cells further supporting tGLI1's role in promoting the aggressiveness of BCBM. Analysis of bone metastases in this study found no significant relationships. Representative *ex vivo* images of brains are shown in Figure 6E. These results provide further evidence that ketoconazole selectively targets tGLI1-positive breast cancer and that ketoconazole crosses the blood-brain and blood-tumor barriers.

To further confirm the ability of ketoconazole to cross the BTB, we excised a tumor sample from the brain of a mouse with tGLI1-positive BCBM and determined the ketoconazole concentration in the tumor sample by mass spectrometry. The excised sample was confirmed to be SKBRM-tGLI1-derived tumor tissue by visualization of RFP expression. The concentration of ketoconazole in this tumor sample was found to be 6.47 ± 0.837 ng/mg tissue confirming that ketoconazole crosses the BTB and is present in an appreciable quantity in the tumor.

Ketoconazole reduces the expression of exosomal miR-1246 and miR-1290 *in vivo*.

Previous data has demonstrated that tGLI1 upregulates two exosomal miRNAs (miR), miR-1246 and miR-1290, which are important in tumor initiation and progression (unpublished data; not shown). Exosomes are small extracellular vesicles that contain growth factors, DNA, and miRNAs which may be secreted by primary tumors to prime the tumor microenvironment for development of secondary metastases [90-92]. MiR-1246 has been shown to promote cell proliferation, invasion, and drug resistance in breast cancer [93] while miR-1290 expression

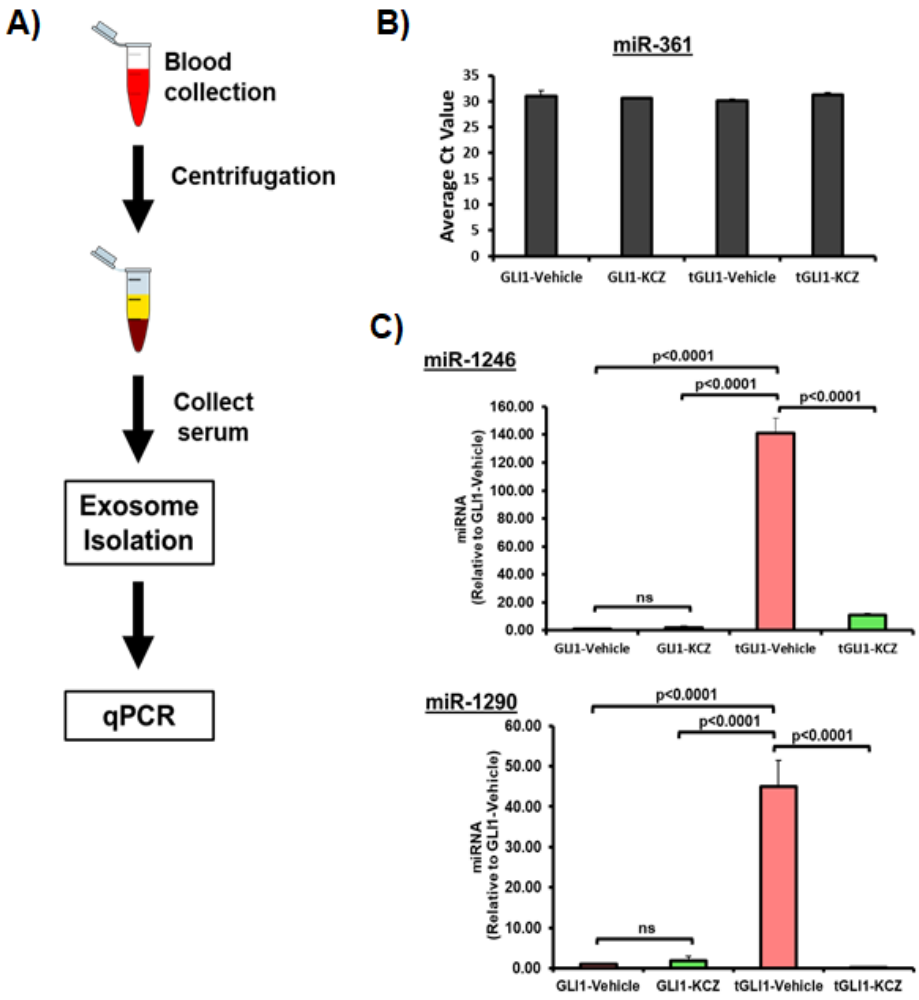


Figure 7. Ketoconazole reduces the expression of exosomal miR-1246 and miR-1290 *in vivo*.

A) Overview of miRNA analysis workflow. **B)** Ketoconazole treatment and GLI1/tGLI1 status does not affect miR-361 expression. **C)** Systemic ketoconazole treatment reduces expression of miR-1246 and miR-1290 by tGLI1-positive tumors. MiRNA expression levels were first normalized to miR-361 before expressing results relative to the GLI1-vehicle group. Ct, cycle threshold. Student's *t*-test was used to compute *p*-values.

has been found to be upregulated in the HER2-enriched and TNBC subtypes which are more likely to metastasize to the brain [94]. In our model, we believe these exosomal miRNAs, which are secreted by tGLI1 tumors, to prime the brain microenvironment for colonization by breast cancer stem cells through promoting angiogenesis and activation of astrocytes. Therefore, we wanted to determine if ketoconazole could reduce expression of these miRNAs as a potential mechanism by which BCBM are inhibited.

At the time of sacrifice, blood was collected and centrifuged to isolate exosomes from the purified serum. MicroRNAs were then isolated from the exosomes and their expression levels were analyzed by qPCR (Figure 7A). To control for decreased expression as a result of smaller tumor size due to ketoconazole treatment, miRNA expression levels were first normalized to miR-361, one of the control miRNAs recommended by the manufacturer. We confirmed miR-361 expression was unaffected by ketoconazole treatment or GLI1 and tGLI1 status (Figure 7B). Importantly, we found both miR-1246 and miR-1290 to be significantly upregulated in the untreated tGLI1 samples compared to the untreated GLI1 samples confirming our previous results that tGLI1 upregulates these miRNAs (Figure 7C). Furthermore, we observed that ketoconazole treatment significantly reduced miR-1246 and miR-1290 expression in the ketoconazole-treated tGLI1 group, but not in the treated GLI1 group compared to their respective controls (Figure 7C). These findings suggest that ketoconazole may inhibit BCBM via suppression of miRNAs-1246/1290-mediated breast cancer brain-tropism.

Ketoconazole inhibits tGLI1-positive breast cancer via an androgen receptor-independent mechanism.

Given our evidence demonstrating ketoconazole's ability to selectively target tGLI1-positive breast cancer, we wanted to uncover the mechanism behind this activity. One of the known targets of ketoconazole is the androgen receptor (AR) [95, 96]. Therefore, we hypothesized that ketoconazole could preferentially target tGLI1-positive cells through inhibiting AR. To investigate this potential mechanism, we began by analyzing the breast

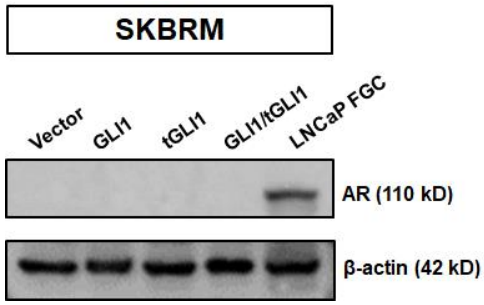


Figure 8. Ketoconazole inhibits tGLI1-positive breast cancer via an androgen receptor-independent mechanism. Expression of androgen receptor (AR) in breast cancer cell lines (SKBRM) and prostate carcinoma cell line (LNCaP FGC) by western blot. β-actin was used as a loading control.

cancer cell lines used in our models for expression of AR. SKBRM cells expressing either vector, GLI1, tGLI1, or GLI1/tGLI1 were analyzed, alongside the prostate carcinoma cell line LNCaP FGC, by western blot for AR expression. The results show that the SKBRM cell lines used in our *in vitro* and *in vivo* models do not express AR to any appreciable extent (Figure 8). This precludes ketoconazole from inhibiting tGLI1-positive breast cancer by targeting AR.

Ketoconazole targets tGLI1-positive breast cancer not through degrading the tGLI1 protein.

We then speculated that tGLI1-expressing cells may become dependent on expression of tGLI1 for their survival, and that ketoconazole may selectively degrade tGLI1, thereby leading to increased cell kill only in tGLI1-driven cancer cells. To test this hypothesis, isogenic MDA-MB-231-BRM cell lines were grown in colonies and treated with either vehicle or ketoconazole. Their expression of GLI1 and tGLI1 was determined by western blotting analysis. We observed that the expression levels of GLI1 and tGLI1 are not reduced with ketoconazole treatment, indicating that ketoconazole does not kill tGLI1-expressing cells via degradation of tGLI1 (Figure 9).

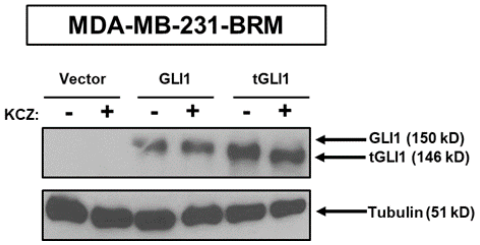


Figure 9. Ketoconazole targets tGLI1-positive breast cancer not through degrading the tGLI1 protein. Western blot analysis of isogenic MDA-MB-231-BRM cell lines treated with ketoconazole (KCZ). Tubulin was used as a loading control.

Ketoconazole inhibits tGLI1-positive breast cancer stem cells, in part, by downregulating the stemness genes CD44 and OCT4.

As a gain-of-function transcription factor, tGLI1 is known to upregulate several stemness genes not regulated by GLI1, such as CD44 [63] and OCT4 (unpublished data; not shown), which are important for pluripotency and stem cell self-renewal. tGLI1 upregulates known GLI1

target stemness genes, Nanog and Sox2 (unpublished data; not shown). To begin working to elucidate the mechanism behind ketoconazole's selective inhibition of tGLI1-positive breast cancer, we tested whether ketoconazole could inhibit expression of these genes. Isogenic MDA-MB-231-BRM and SKBRM cell lines were grown as colonies or mammospheres, respectively, and treated with ketoconazole for 10 days. Colonies and mammospheres were then harvested and subjected to western blot analysis. We observed that treatment with ketoconazole did not reduce expression of Nanog or Sox2 in either stem cell population, suggesting that ketoconazole does not kill tGLI1-expressing breast cancer via downregulation of these genes (Figure 10A). Given the evidence suggesting expression of CD44 and OCT4 may be involved in ketoconazole's selective inhibition of tGLI1-positive breast cancer stem cells (data not shown), we then reasoned that if involved, forced expression of CD44 or OCT4 may rescue SKBRM-tGLI1 mammospheres from ketoconazole intervention. To test this hypothesis, CD44 and OCT4 mammalian expression plasmids were transiently transfected into SKBRM-tGLI1 cells before the cells were seeded in mammosphere-forming conditions. We observed that under the treatment of 0.1 μM ketoconazole, cells transfected CD44 and OCT4 formed significantly more mammospheres compared to the vector control. Interestingly, both CD44 and OCT4 were also able to rescue at the 1 μM dose, but transfection with OCT4 resulted in significantly more mammospheres formed compared to transfection with CD44 (Figure

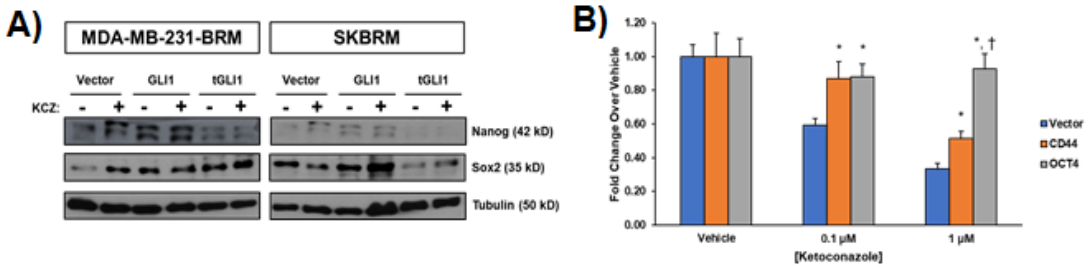


Figure 10. Ketoconazole inhibits tGLI1-positive breast cancer stem cells, in part, by downregulating the stemness genes CD44 and OCT4. A) Expression levels of Nanog and Sox2 in the MDA-MB-231-BRM and SKBRM stem cell population after treatment with ketoconazole. **B)** Mammosphere assay demonstrating stem cell rescue from ketoconazole treatment by overexpression of CD44 and OCT4. **P*<0.05 relative to vector, †*P*<0.05 relative to CD44. Student's t-test was used to calculate *p*-values.

10B). These findings suggest both CD44 and OCT4 play an essential role in ketoconazole's selective inhibition of tGLI1-positive breast cancer stem cells with OCT4 potentially playing a more significant role.

Chapter 3: Discussion

Despite improvements in early detection and therapies, breast cancer remains the second leading cause of cancer-related death in women [1] and second most common cancer to metastasize to the brain [97]. Brain metastases frequently occur in metastatic breast cancer patients, with approximately 10%–16% of patients developing symptomatic brain metastases and another 10% of patients noted to have asymptomatic brain involvement in post-mortem autopsies [97, 98]. Current standard of care for BCBM involves surgical resection of individual lesions and WBRT for multiple lesions resulting in a median survival of 23.5 months for good-performance patients [99]. However, a consensus has yet to be reached on subsequent therapy for those with intracranial progression and effective, FDA-approved, drugs for this indication remain an area of unmet need [100]. We have previously published data demonstrating the discovery of the novel gain-of-function GLI1 transcription factor, tGLI1, and its role in breast cancer progression and metastasis (unpublished data; not shown) [62-65, 67-69]. Our laboratory also has generated results indicating an important role that tGLI1 plays in promoting breast CSCs and breast cancer metastasis to the brain (unpublished data; not shown). Given tGLI1's tumor-specific expression and potent metastasis-promoting effects, we believe tGLI1 to be an ideal therapeutic target and sought to identify a tGLI1 inhibitor to treat tGLI1-positive breast cancer.

The SHH pathway is critical for normal tissue maintenance, renewal, and regeneration and has also been deemed a critical determinant of cancer initiation, progression, and metastasis in many cancer types [33-41]. Given the relevance of this signaling pathway in multiple cancers, several inhibitors have been synthesized to target this pathway. Vismodegib and Sonidegib are SMO-inhibitors approved by the FDA to treat basal cell carcinoma (BCC)

[101, 102]. Despite being used to treat basal cell carcinoma, these SMO-inhibitors have found limited success treating other solid tumors such as breast cancer. BCC is characterized by SMO hyperactivity most frequently caused by genetic inactivation of PTCH1 or by oncogenic SMO mutations [103-105]. This could explain, in part, why these SMO-inhibitors are effective against BCC, but not other solid tumor types in which SMO hyperactivity is not found. As one would expect when targeting an upstream molecule, upon testing these compounds in our synthetic lethality screen, neither compound demonstrated tGLI1 selectivity (data not shown). The Hh signaling pathway has also been targeted through direct inhibition of the terminal effector GLI1. GANT61 is a small molecule GLI1 inhibitor that binds to the DNA-binding pocket of GLI1 precluding GLI1 from binding to its target genes and upregulating their transcription [106]. Interestingly, GANT61 induced significant cytotoxicity in *in vitro* models of colon cancer, however, this compound has failed to progress beyond the preclinical phase [107]. Given the lack of success SMO-inhibitors have demonstrated in treating breast cancer and the possibility for GLI1 and tGLI1 to be activated through noncanonical pathways, we reasoned that direct inhibition of tGLI1 could have therapeutic potential.

Using a synthetic lethality approach to compound screening, we found that ketoconazole, an FDA-approved azole antifungal, specifically targeted tGLI1-expressing breast cancer cells (Figure 1B and 1C). Further experimentation demonstrated pronounced suppression of the tGLI1-positive CSC population while leaving normal cell types found in the brain tumor microenvironment unaffected (Figure 2), indicating the potential utility of ketoconazole in treating brain malignancies.

Using two animal models, we showed that ketoconazole treatment can prevent development of breast cancer brain metastasis, and selectively reduce the growth of established BCBM (Figures 5 and 6). In addition to promoting breast cancer brain metastasis via upregulation of stemness genes, another potential mechanism by which tGLI1 mediates breast cancer brain-tropism is through regulating exosomal miRNAs. Exosomes are small extracellular vesicles that contain growth factors, DNA, and miRNAs which may be secreted by primary tumors to prepare the microenvironment for development of secondary metastases [90-92]. It may be the case that tGLI1-positive CSCs secrete exosomal miRNAs to prime the brain for colonization; however, this will need to be verified. Inhibiting secretion of these miRNAs could potentially be a mechanism underlying the inhibition tGLI1-postive brain metastases by ketoconazole.

Previous evidence suggests that tGLI1-overexpression transforms normal mammary epithelial cells into CSC-like cells as indicated by increased mammosphere formation and anchorage-independent growth (unpublished data; not shown). This finding suggests tGLI1 may be involved in mammary tumorigenesis. First, additional studies determining the role of tGLI1 in malignant transformation of normal breast cancer cells are needed. To assess this, conditional transgenic mouse models may be established to knockout the *GLI1* gene and knock in the human *tGLI1* gene. These floxed mice would then be crossed with mammary-specific Cre mice, such as MMTV-Cre. After establishing this transgenic mouse model, ketoconazole could be introduced to ascertain its ability to inhibit tGLI1-dependent malignant transformation of mammary cells, and breast tumor growth and progression.

Our studies on ketoconazole prompt additional work to test its efficacy using additional mouse models of breast cancer, including the transgenic tGLI1-knockin mouse, patient-

derived xenograft (PDX) mouse models, and tumor implantation mouse models using additional breast cancer cell lines. Since ketoconazole is an FDA-approved drug, it can be evaluated in pilot or phase I clinical trials for the treatment of breast cancer patients with brain metastases. In summary, the results presented in this project are of significant clinical and biological importance in clinical management of breast cancer brain metastasis.

Conclusion

In this study, we made the following novel and substantial observations: 1) ketoconazole, an azole antifungal, selectively inhibits tGLI1-positive breast cancer stem cells; 2) tGLI1 expression is required for ketoconazole's ability to inhibit breast cancer stem cells; 3) ketoconazole can penetrate the BBB and BTB *in vivo*; 4) development of tGLI1-positive BCBM can be prevented by systemic treatment with ketoconazole *in vivo*; 5) ketoconazole selectively reduces the progression of established tGLI1-positive BCBM *in vivo*; and 6) selective inhibition of tGLI1-positive breast cancer stem cells is, in part, mediated by downregulation of CD44 and OCT4, but not by targeting AR or degrading tGLI1 protein. Collectively, these results demonstrate the therapeutic potential of ketoconazole in patients with tGLI1-positive BCBM.

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EDUCATION

2018 **M.S. in Biomedical Science**, Wake Forest University Graduate School of Arts and Sciences, Winston-Salem, NC

2014 **B.S. in Chemistry; Emphasis: Biochemistry**, The University of North Carolina at Chapel Hill, Chapel Hill, NC
B.A. in Philosophy, The University of North Carolina at Chapel Hill, Chapel Hill, NC
Minor in Spanish for the Professions

RESEARCH AND WORK EXPERIENCE

2017 – Present **Graduate Student Researcher,** Wake Forest University Graduate School of Arts and Sciences, Winston-Salem, NC (research advisor: Hui-Wen Lo, PhD)
Thesis topic: Targeting tGLI1-positive Breast Cancer with Ketoconazole
- Identify and characterize compounds capable of selectively inhibiting our actionable target tGLI1
- Confirm compound efficacy through phenotypic *in vitro* and *in vivo* models
- Determine the mechanism of action behind inhibition of tGLI1-positive tumors by ketoconazole

2016 – 2017 **Scientist II,** ScitoVation, LLC, Research Triangle Park, NC
(research advisor: Rebecca Clewell, PhD)
Research focus and duties:
- Primary researcher for the estrogen receptor case study to assess 133 prioritized chemicals for estrogenic activity
- Implemented barcode scanning system for chemical inventory to improve stocktaking, established SOP
- Prepared quarterly reports to update sponsors on project progress

2016 **Scientist I,** ScitoVation, LLC, Research Triangle Park, NC
(research advisor: Michelle Miller, PhD)
Research focus and duties:
- Developed a multiplex high-throughput, fit-for-purpose *in vitro* assay to measure uterine estrogenic activity
- Investigated glucocorticoid receptor-dependent adipogenesis
- Assisted in development of direct double strand break assay to study DNA damage
- Managed chemical inventory and chemical safety documentation

2015 - 2016 **Research Intern,** The Hamner Institutes for Health Sciences, Institute for Chemical Safety Sciences, Research Triangle Park, NC
(research advisors: Michelle Miller, PhD and Chad Deisenroth, PhD)
Research focus and duties:

- Explored compatibility of an impedance-based cell analysis platform with a uterine epithelial adenocarcinoma (Ishikawa) cell line to study uterine proliferative effects of endocrine disrupting chemicals
- Examined estrogen receptor expression using colorimetric assays in response to estrogen disrupting chemicals
- Validated assay to measure adipokine secretion in PPARγ-dependent adipogenesis

PUBLICATIONS

Hartman J, Beames T, Parks B, **Doheny D**, Song G, Efremenko A, Yoon M, Foley B, Deisenroth C, McMullen P, Clewell R. 2018. An *in vitro* approach for prioritization and evaluation of chemical effects on glucocorticoid receptor mediated adipogenesis. Toxicology and Applied Pharmacology **355**: 112-126.

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Selected Abstracts and Presentations (* presenting author)

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