SEROTONIN RECEPTOR 5A SIGNALING PATHWAY IN BREAST CANCER

IDENTIFYING SIGNALING PATHWAYS DOWNSTREAM OF THE SEROTONIN RECEPTOR 5A IN BREAST CANCER

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LAY ABSTRACT

Accumulating data suggests that the progression of breast cancer is driven by a rare population of breast tumor-initiating cells (BTIC). BTIC lie dormant during conventional therapy and initiate recurrence after such therapies are withdrawn. Hence, there is an urgent need to develop drugs that target BTIC that can be combined with the current standard of care to improve the durability of remission. With the latter objective in mind, our lab previously determined that antagonists of serotonin signaling target BTIC. One of the agents that we identified in our screen inhibits the activity of serotonin receptor 5A (HTR5A). The exact signaling mechanism whereby inhibition of HTR5A leads to a loss in BTIC activity was enigmatic. Hence, this thesis aims to elucidate the signaling pathways downstream of HTR5A in breast cancer. Knowledge of the latter will help identify a plausible mechanism in addition to identifying biomarkers of therapy efficacy.

ABSTRACT

Breast cancer therapy resistance and disease recurrence are driven by an infrequent population of stem-like tumor cells, termed breast cancer stem cells or tumor-initiating cells (BTIC). Whereas drugs that target BTIC could be combined with conventional therapies to provide durable remissions, identifying such agents has been difficult. To achieve the latter, our lab screened more than 35,000 compounds for their capacity to reduce the activity of BTIC-enriched mouse mammary tumorspheres, wherein we identified numerous antagonists of multiple serotonin receptors (HTRs). The serotonergic antagonist that prevented sphere formation with the highest potency is a highly selective antagonist of HTR5A, SB-699551. We subsequently demonstrated that this agent affects BTIC activity in breast tumor cell lines representative of all clinical and molecular subtypes of breast cancer. Whereas the primary target of SB-699551 is known, the downstream signaling pathways responsible for its anti-BTIC effect remains enigmatic. The goal of this thesis work was to elucidate the signaling pathways downstream of HTR5A in human breast tumor cell lines. We used a phospho-proteomic approach to establish that treatment of human SB-699551 affects the phosphorylation of proteins involved in the $G\alpha_i$-coupled and the PI3K/AKT/mTOR signaling axes. Moreover, we demonstrated that selective antagonists of PI3K, AKT, and mTOR phenocopied the effect of SB-699551 in tumorsphere forming assays. Taken together, our data suggests that SB-699551 elicits its effect through the PI3K/AKT/mTOR signaling pathways downstream of HTR5A.

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LIST OF ABBREVIATIONS

4EBP1: 4E binding protein 1

5-HT: 5-hydroxytryptamine, serotonin

5-HTTP: 5-hydroxytryptophan

ABC: ATP-binding cassettes

ATF1: Activating transcription factor 1

BTIC: Breast tumor-initiating cells

cAMP: Cyclic adenosine monophosphate

CNS: Central nervous system

CREB: cAMP-response element binding protein

CRC: Colorectal cancer

EGFR: Epidermal growth factor receptor

EMT: Epithelial to mesenchymal transition

ER: Estrogen receptor

FOXO: Forkhead box proteins

GPCR: G-protein coupled receptor

GRK: G-protein coupled receptor kinases

HER2: Human epidermal growth factor receptor 2

HTR: 5-HT receptor/serotonin receptor

IC_{50}: Half-maximal inhibitory concentration

MAO: Monoamine oxidase

MAPK: Mitogen activated protein kinases

MESC: Mammary epithelial stem cells

mTOR: Mammalian target of rapamycin

p38 MAPK: p38 mitogen-activated protein kinase

PDK1: 3-phosphoinositide-dependent protein kinase 1

PHLPP1/2: PH domain and leucine-rich repeat protein phosphatase

PI3K: Phosphoinositide 3-kinase

PIP2: Phosphatidylinositol 4, 5-bisphosphate

PIP3: Phosphatidylinositol 3,4,5- triphosphate

PKA: Protein kinase A

PP2A; Protein phosphatase 2

PPA: Proteomic profiler array

PR: Progesterone receptor

PRAS40: Proline-rich AKT substrate

PTEN: Phosphatase and tensin homolog

PTHrP: Parathyroid hormone-related protein

ROS: Reactive oxygen species

RTK: Receptor tyrosine kinases

S6K1: Ribosomal protein S6 kinase

S6RP: S6 ribosomal protein

SERT: Serotonin reuptake transporter

SIN1: Stress-activated map kinase interacting protein 1

TNBC: Triple negative breast cancer

TNM: Tumor, lymph node, metastasis

TPH: Tryptophan 5-monooxygenase

TSC1/2: Tuberous sclerosis complex 1 and 2

WNK1: Lysine deficient protein kinase 1

INTRODUCTION

Cancer

Cancer is a disease that results from uncontrolled cell proliferation and comprises both hematological malignancies and solid tumors of epithelial origin. The latter typically originates as a localized, microscopic tumor *in situ*, but without therapeutic intervention the tumor cells can spread to surrounding tissues or metastasize to distant organs through the circulatory system[1]. Metastatic disease is invariably fatal as tumors disrupt essential organ functions due to their growth. Despite advancements in screening technologies and the development of novel, targeted therapies, cancer remains a leading cause of mortality and disability in the Canadian population. Indeed, cancer is the second leading cause of death in Canada. Nearly 1 in 2 Canadians will be diagnosed with cancer during their lifetime and 50% of those diagnosed succumb to the disease[2].

The development of cancer is attributed to genetic alterations acquired through different sources such as hormones, chemicals, radiation, infection and hypoxia[3,4]. For example, the accumulation of genetic mutations in proto-oncogenes like *RAS*[5] and *MYC*[6] or in tumor suppressor genes such as *BRCA1/2*[7] and *TP53*[8] impair cellular processes such as apoptosis, or proliferation, leading to uncontrolled tumor growth. The hyperplasia and dysplasia of tumors induce angiogenesis, providing the nutrients required to sustain tumorigenesis.

Whereas each cancer has its own idiosyncrasies, eight hallmarks of cancer have been proposed, which comprise common features that enable tumor development and

metastasis[9]. These hallmarks include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction[9]. These hallmarks are evident in carcinomas of several epithelial organs including skin, lung, and breast. For example, breast tumors that overexpress growth factor receptors have increased activation of Ras[10]. Moreover, germline mutations in *BRCA1* gene, which significantly increase the chances of developing breast and ovarian cancers, are associated with basal-like breast tumors[11].

Breast cancer

Breast cancer originates from epithelial cells that populate the mammary gland[12]. The human mammary gland is composed of lobules and ducts. Lobules are the milk-producing units of the gland, whereas ducts transport milk to the nipple during lactation. Breast carcinoma is classified as non-invasive (carcinoma *in situ*) or invasive depending on whether the tumor has penetrated the basal membrane. Whereas carcinoma *in situ* is manageable with surgery, invasive carcinomas can spread to surrounding tissues and/or metastasis to distant organs of the body and hence these tumors often require systemic therapy[13].

Breast cancer incidence

Breast cancer is the leading cause of cancer-related death among women globally. According to 2018 estimates, 2.1 million women were diagnosed with breast cancer and nearly 500,000 succumbed to the disease[14]. It is estimated that over 26, 300 Canadian women were diagnosed with breast cancer in 2017 and 5,000 women died from the disease[2]. Although breast cancer treatments are improving as new technologies, chemical agents, and treatment strategies are discovered, the incidence of breast cancer is steadily increasing[2,15]. Innovative strategies for classifying breast cancer have aided treatment strategies, outcome predictions and the development of novel, targeted therapies.

Breast cancer subtypes

Breast cancer is a genetically and phenotypically heterogeneous group of diseases that be classified according to their differences[12]. Clinically, the subtypes are minimally defined by the expression of the estrogen receptor (ER), the progesterone receptor (PR), the human epidermal growth factor receptor 2 (HER2), and Ki-67[16]. Extensive gene expression profiling of breast tumors has broadened our understanding of their molecular heterogeneity. The molecular subtype classification aids in prognosis and determining appropriate therapeutic strategy[16].

Whereas the main molecular subtypes are luminal A, luminal B, HER2-enriched, and triple negative/basal-like, other molecular subtypes have also been described. Luminal A tumors express ER and/or PR, but lack HER2 expression and have a lower proliferative capacity as established by reduced Ki-67 expression. Similarly to luminal A tumors,

luminal B tumors are ER-positive, PR-positive and HER2-negative, but their Ki67 staining profiles suggest that the cells are highly proliferative[17]. Hence, luminal B is a more aggressive breast cancer subtype than luminal A[17]. The HER2-enriched subtype overexpress HER2 and may or may not express ER and/or PR. Lastly, tumors of the basal-like/triple-negative breast cancer (TNBC) subtype do not express ER, PR, or HER2[18]. As a consequence, there are no targeted therapies available to treat TNBC.

Tumor, lymph node involvement, and metastasis (TNM) staging

Developed by the American Joint Committee on Cancer, TNM staging is used to determine the extent of cancer progression using anatomical properties[19]. It establishes whether the tumor is in early stages of development, which is associated with a good prognosis, or whether the cancer is in an advanced stage of development with or without metastases, which translates to a poor prognosis. TNM staging classification ascertains the stages using three factors, tumor size and location (T), lymph node invasion (N), and extent of metastasis (M)[20]. TNM staging influences prognosis, therapeutic approaches and evaluation of therapeutic results.

Tumor (T)	T1	Tumor size < 2cm
	T2	2cm < Tumor size < 5cm
	T3	5cm < Tumor size
	T4	Tumor has grown into chest wall or skin or has become inflammatory breast cancer
Lymph Node (N)	N0	No cancer found in lymph nodes
	N1	Metastasis in 1-3 auxiliary lymph nodes
	N2	Metastasis in 4-9 auxiliary lymph nodes
	N3	Metastasis in 10 or more auxiliary lymph nodes
Metastasis (M)	M0	Cancer has not metastasized
	M1	Cancer has metastasized to other body parts

Table 1: TNM staging criteria for scoring each stage

TNM Staging system			
Stage 0	T1	N0	M0
Stage IA	T1	N0	M0
Stage IB	T1	N1mi	M0
Stage IIA	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Table 2: TNM staging classification for breast cancer

Tumor grade

Tumor grade is a histological estimation of the extent of differentiation within a tumor. High-grade tumors comprise poorly differentiated, more stem-progenitor like cells, whereas low-grade tumors consist of well-differentiated cells that are characteristic of normal tissues[21]. Tumor grade serves as a prognostic indicator, outlining the aggressiveness of the tumor cells populating the tumor[22]. The Nottingham grading system is used when grading breast tumors. It determines tumor grade based on the extent of tubule formation, the size and shape of the nucleus in tumor cells, and the frequency of dividing tumor cells[23]. Each category is scored out of three, and the score is compiled and classified into three different grades, G1, G2 and G3[23] **(Table 2)**. Tumor grade along with other factors dictate patient prognosis as well as therapeutic options.

Grade		Tumor Differentiation Status
G1	Low grade	Well differentiated
G2	Intermediate grade	Moderately differentiated
G3	High grade	Poorly differentiated

Table 3: The Nottingham tumor grading system

Current therapies for breast cancer (Treatment)

Surgery, chemotherapy, radiation and hormonal therapy comprise the standard of care for patients that experience breast cancer. Surgery is generally the initial treatment option and can be either a lumpectomy, where the tumor and some surrounding tissue is excised or a mastectomy, where the entire breast is removed. Surgery is coupled with systemic radiation or chemotherapy to kill cancer cells that escape surgery and to reduce the risk of recurrence[24]. Chemotherapy and/or endocrine therapies are used in an adjuvant or neo-adjuvant setting to compliment the surgery depending on characteristics of the tumor such as its size or its subtype.

Tumors within the luminal and normal-like subtypes are treated with endocrine therapy. Endocrine therapies comprise inhibitors of estrogen biosynthesis or estrogen analogues that inhibit ER directly. Whereas HER-2-enriched tumors historically exhibited poor survival, the development of HER-2 directed monoclonal antibodies like trastuzumab (Herceptin), have significantly extended the survival of these patients. Triple negative breast tumors lack ER, PR, or HER2 expression and consequently there are no targeted therapies available to treat these tumors. Instead, patients are administered repeated rounds of cytotoxic chemotherapy and/or radiation. Chemotherapeutic agents consist of taxanes

(mitotic inhibitors) and anthracyclines (DNA intercalators). The genomic and molecular mechanism of chemoresistance in triple negative breast cancer patients remains largely unknown, partly due to the extensive intratumoral heterogeneity and the inability to detect genomic alterations in rare subpopulations[25].

Chemoresistance

Resistance to anticancer agents develops in 50% of the patients, leading to poor overall survival[26]. Indeed, clinical studies conducted on the efficacy of the chemotherapeutic doxorubicin reported that 40% of early-stage breast cancer patients experienced a relapse in the first 5 years after treatment[27,28]. Additionally, 30% of the patients that experienced relapse succumbed to the disease, and this was independent of the treatment regimen used[27,28]. Similar to chemotherapeutic agents, resistance to hormonal and HER2-targeted agents is also evident. Recurrences in patients treated with hormonal and HER2-targeted therapies occurs at a frequency of 30% and 20-25%, respectively, with recurring tumors evolving resistance to the respective therapeutic agents[29–32]. For example, ER+ breast tumor cells have been reported to evolve resistance to antiestrogen therapies by reducing expression of an ER gene cluster and inducing expression of receptor tyrosine kinases (RTK) including EGFR and HER2[33–35]. Indeed, long-term deprivation of ER+ breast tumor cells can result in independence of ER signaling, where breast tumor cells adapt to signal through alternative pathways to decrease their dependency from ER signaling[33–35]. Hence, breast tumor cells can develop resistance to current anticancer agents.

Resistance to anticancer agents is a major cause of therapy failure. Therapy resistance leads to disease recurrence and aids metastases, resulting in poor clinical outcomes for patients. Chemoresistance can take two forms, primary resistance and acquired resistance. Primary resistance describes a lack of therapeutic response to chemotherapeutic agents due to de novo characteristics, whereas acquired resistance arises after selection of drug-resistance tumor cells during treatment.

There are many molecular mechanisms associated with chemoresistance: increased expression of ATP-binding cassette (ABC) transporters that efflux small molecules, mutations in tumor suppressor genes such as *BRCA1* or *TP53*, alterations in mitochondria mediated apoptosis pathways, reduced activity of DNA repair enzymes, increased autophagy to fulfill high metabolic and energy demands, and induction of an epithelial-to-mesenchymal transition (EMT)[36]. EMT is a cellular program which drives epithelial cells to several phenotypic states associated with greater migratory capacity, drug resistance, and the ability to form metastases, thereby contributing to cancer relapse[37,38]. To try and overcome these clinical limitations, inhibitors of other signaling pathways have been developed such as receptor tyrosine kinases like the epidermal growth factor receptor (EGFR). However, activation of new signaling pathways can allow tumor cells to bypass this and reduce the effectiveness of these agents.

Several of the above resistance mechanisms have been attributed to the activity of an infrequent subpopulation of breast tumor cells, termed breast cancer stem cells or breast tumor-initiating cells (BTIC)[39–41]. BTIC have been reported to overcome the stress of radiation by upregulating Wnt-mediated DNA repair pathways[39]. Moreover, BTIC

overexpress ABC transporters providing a general resistance mechanism to efflux anticancer agents out of the cells[40,41]. Consequently, the frequency of BTIC increases in residual breast tumors after neo-adjuvant chemotherapy as revealed by the expression of BTIC markers such as CD44[42]. Moreover the residual tumors overexpress ABC transporters compared to the surrounding non-tumor cells[42]. BTIC share properties with mammary epithelial stem cells (MESC), like their ability to self-renew and to differentiate. Hence, whereas BTIC make up only a minor fraction (~0.01%) of the total tumor cell population, their existence has significant therapeutic implications[43]. Taken together these data suggest that there is an urgent need for novel drugs that can target the infrequent BTIC population.

Mammary epithelial stem cells

Adult organs are maintained by an infrequent population of stem cells that self-renew, creating new copies of themselves and differentiate, acquiring tissue-specific functions. Hence, tissues can be described according to this cellular hierarchy[44].

MESC are an infrequent population of stem cells that reside in the mammary gland and their activity populates the different mammary epithelial cellular lineages[44]. MESC differentiate into epithelial progenitor cells, which differentiate again to generate cells from three different lineages: ductal epithelial cells, alveolar cells and myoepithelial cells. These three different cell types combine to give rise to the three-dimensional lobulo-alveolar structure of the adult mammary gland. Proteins present in milk are synthesized by the alveolar cells, whereas ductal epithelial cells generate the lumen of the ducts. The

contractile myoepithelial cells populate the basal layer of the ducts and facilitate the secretion of milk into the lumen[44].

Cancer stem cell model

The cancer stem cell model postulates that human tumors, like normal tissues, can be described in terms of a cellular heirarchy[45]. The tumorigenic BTIC reside at the apex of the hierarchy and non-tumorigenic, aberrantly differentiated progeny at its base[45]. Whereas the cellular origin of BTIC is unknown, they may be produced by the accumulation of genetic/epigenetic modifications in tissue-specific cells[45]. BTIC are functionally defined through their capacity to seed tumors in immune-compromised mice that retain the phenotypical heterogeneity of the tumors from which they were isolated[45,46]. BTIC generate progeny that lack stem cell properties and are the source of the abundant population of non-tumorigenic tumor cells.

Accumulating data suggests that the non-tumorigenic population can dedifferentiate into their stem-like predecessors through an epithelial-to-mesenchymal transition (EMT).[47] Hence, differentiation and EMT may act in a dynamic an equilibrium between BTIC and non-BTIC, maintaining both tumor cell populations. This bidirectional equilibrium has therapeutic implications. BTIC are resistant to conventional therapies[48] including radiation[39] and chemotherapy[42]. Cytotoxic therapies principally eradicate non-BTIC. Consistent with the latter, tumors after neoadjuvant chemotherapy comprise a higher frequency of BTIC[49]. Hence, the development of BTIC-targeting agents should be

combined with existing standard of care therapies so that both BTIC and their non-tumorigenic progeny, which are a potential reservoir of BTIC, are eradicated.

Breast tumor initiating cells

Although first discovered in acute myeloid leukemia[46], TIC have been identified in tumors of the breast[43], brain[50], skin[51], gastro-intestinal tract[52,53], prostate[54], and head and neck[55]. In fact, breast cancer was the first malignancy of solid epithelial tumors reported to follow the cancer stem cell model.[43] Although tumors are clonally derived from a single cell of origin, tumors are comprised of heterogeneous population of cells with different morphological characteristics and immunophenotypes. This allows isolation of a distinct subpopulation of cells, such as BTIC, using antibodies to surface markers specific to that particular subpopulation. Consequently, BTIC are enriched using florescence activated cell sorting with antibodies to cell surface markers, $CD44^{+}CD24^{-}$, and this method yields BTIC frequency up to 1%[43]. With additional sorting for BTIC markers such as ALDH1+, this frequency can be increased up to 5%[56]. However, this frequency proves insufficient for drug screening purposes BTIC. Hence, researchers used *in vitro* and *in vivo* functional assays to model the activity of BTIC.

Many studies have reported that tumors arising in transgenic mouse mammary tumor models comprise a cellular hierarchy that follows the cancer stem cell model[57,58]. Moreover, our lab previously determined that the tumors arising in three independent transgenic mouse mammary tumor models comprise a high BTIC frequency[59]. This BTIC frequency could be maintained *in vitro* by culturing the tumor cells as three-dimensional,

non-adherent spheres, termed tumorspheres in a serum-free, chemically-defined media. We exploited the latter to conduct a high-throughput phenotypic screen with a library of approximately 35,000 compounds to identify agents that target BTIC[60]. Compounds that reduced the tumorsphere cell viability by more than 50% were considered hits and the half-maximal inhibitory concentrations (IC_{50}) were determined independently using freshly sourced compounds. Surprisingly, a large fraction of hits from the bioactive subset comprised drugs involved in neurotransmission, namely antagonists of the serotonergic system[60].

Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is a biogenic monoamine that has several biological functions in the central nervous system (CNS) as well as in select cells in the periphery. Derived from tryptophan, an essential amino acid obtained primarily through the diet, 5-HT is synthesized via two enzymatic reactions.

The first step is catalyzed by tryptophan 5-monooxygenase (TPH), which converts tryptophan into 5-hydroxytryptophan (5-HTP)[61]. This step is performed by TPH2 in the CNS and TPH1 in the periphery. Subsequently, 5-HTP is decarboxylated by aromatic amino acid decarboxylase to generate 5-HT[61]. Although both enzymes are essential for 5-HT production, TPH is the rate limiting enzyme[62–65]. Following synthesis, 5-HT is packaged into vesicles by vesicular monoamine transporter for release into the extracellular space.

Once 5-HT enters the extracellular space, it binds to one of several 5-HT receptors (HTRs) that transduce signals intracellularly. In the CNS, 5-HT activity is terminated when it either diffuses away from the receptors or is transported into the cells via the serotonin reuptake transporter (SERT). Once taken up by SERT, 5-HT is degraded by monoamine oxidase (MAO) through oxidative deamination. The deamination of 5-hydroxytryptamine by MAO produces 5-hydroxyindolacetyl aldehyde, which is converted into 5-hydroxyindolacetic acid by aldehyde dehydrogenase, the principle metabolite excreted in the urine. 5-HT is also converted into melatonin by tissue specific enzymes in the pineal gland and the retina.

The pharmacological classification of serotonin receptors (HTR) identifies 14 HTRs that comprise 7 receptor subfamilies[66]. Transcripts encoding the HTRs can be differentially spliced yielding a wider array of receptor subtypes. The diversity in receptor subtypes contributes to multiple and often opposing effects of 5-HT. The activity of 5-HT is determined by the characteristics of the receptor to which it binds and the intracellular signaling pathway coupled to the receptor.

The HTR3 subfamily of receptors are pentameric ligand-gated ion channels that transport ions including sodium, potassium and calcium across the plasma membrane[67]. Consequently, in the CNS activation of HTR3 results in plasma membrane depolarization. The remaining HTR subtypes are members of the G-protein coupled receptor (GPCR) superfamily of seven transmembrane-spanning receptors, the largest family of receptors in the human genome[68]. Stimulation of GPCR induces a conformation change that alters the proximity of the intracellular domain allowing it to interact with the heterotrimeric G-

proteins[68]. Heterotrimeric G-proteins consists of three subunits: Gα, Gβ, and Gγ, with the Gα family comprising four subfamilies: Gs, G_i/G_0, G_q/G_{11}, and G_{12}/G_{13}[68]. The HTR4, HTR6, and HTR7 subtypes are coupled to Gs and hence stimulation of these receptors leads to adenylate cyclase activation and increased cAMP levels. The HTR1A-F, HTR5A and HTR5B receptors are G_i/G_0 coupled and their activation inhibits adenylate cyclase, causing a decrease in cAMP levels. Lastly, the HTR2A-C receptors are Gq/G_{11} coupled. 5-HT binding these receptors activates phospholipase C, phospholipase C cleaves the plasma membrane phospholipid, phosphatidylinositol 4, 5 bisphosphate into diacylglycerol and inositol triphosphate, thus inducing intracellular calcium release.

Aside from the $G\alpha_{i/o}$ subunit, the Gβγ-subunit has been implicated with the PI3K pathway. Indeed, Gβγ-subunits have been found to directly interact with the PI3K catalytic subunit, p110γ[69]. Furthermore, Gβγ-subunits stimulated PI3K activity by 60-fold (EC_{50} ~20nM) as measured using phosphatidylinositol 3,4,5- triphosphate (PIP3) production[69]. Another study reports that Gβγ-subunits recruit the PI3K to cellular membrane through interaction with its regulatory subunit, p101[70]. Moreover, recruitment of PI3K to cellular membrane is insufficient to increase its activity. Interestingly, they report that Gβγ-subunits directly stimulate the P110γ subunit to increase PI3K activity as measured by PIP3 abundance[70]. In fact, Gβγ-subunits are able to activate PI3K even without recruitment of the p101 regulatory subunit. Collectively, this evidence suggests a link between G-protein coupled receptors and the recruitment and activation of the PI3K pathway.

Independent of the downstream signaling mechanisms that HTRs are coupled to, 5-HT binding to these receptors and release of g-proteins is mediated by G-protein coupled

receptor kinases (GRK). GRK are a family of serine/threonine kinases that phosphorylate the C-terminus and/or the third intracellular loop of the ligand bound receptor after receptor activation[71]. Subsequently, the C-terminus and/or the third intracellular loop phosphorylation recruits β-arrestin proteins to uncouple the receptor from its associated G-proteins and initiate internalization via clathrin coated vesicles, attenuating its signaling and yielding intracellular vesicles termed endosomes[71]. The endosomes are directed to lysosomes for degradation or recycled back to the plasma membrane.

Whereas these β-arrestin proteins were traditionally thought to be only responsible for receptor recycling, many studies have shown that they are also coupled to oncogenic signaling pathways[72]. Specifically, β-arrestins have been implicated in initiating their own signals to activate various kinases by exploiting their scaffolding function [73–75]. The capacity of GPCRs to signal through β-arrestins signaling is dictated by the number of serine-threonine motifs in the C-terminus[76]. The serine-threonine motifs on the receptor C-terminus and the pattern of agonist induced ubiquitination of β-arrestins determines the stability of the receptor-β-arrestins complex. High levels serine-threonine motifs promote tighter complexes and these complexes can function as 'signalosomes', scaffold complexes associated with endosomes[76]. Receptors with lower serine-threonine motifs induce transient ubiquitination of β-arrestins and form only transient signaling complexes[76]. Shenoy et al report that that the stimulation of β—adrenergic receptor results in increased ERK1/2 phosphorylation through β-arrestin signaling, independent of G-protein activation[77]. However, there is evidence suggesting that ERK1/2 activation in response to β—adrenergic receptor activation is not a result of β-arrestins signaling. Indeed, O'Hayre

et al report that β-arrestins are dispensable for β—adrenergic receptor based ERK1/2 activation[78]. Hence, the signaling role of β-arrestins remain unclear.

Serotonin's role in mammary epithelium homeostasis

5-HT has several regulatory functions in maintaining mammary epithelial homeostasis, especially during mammary gland development in pregnancy and lactation. In fact, all 5-HT receptors have been detected in mammary epithelial cells with the exception of HTR6[79] and 5-HT production is high during the period of breast feeding[80]. Nipple stimulation during suckling stimulates prolactin secretion which upregulates the expression of TPH1 and subsequent 5-HT synthesis[81]. Important for milk production, 5-HT activates HTR2B to induce the expression of parathyroid hormone-related protein (PTHrP). PTHrP acts through osteoblast to stimulate osteoclastic bone demineralization necessary for calcium release, which is essential for milk production[82].

Another role for 5-HT in mammary gland homeostasis occurs during involution. Weening leads to milk filling the alveoli, which stimulates 5-HT synthesis. 5-HT stimulates HTR7 which results in cyclic adenosine monophosphate (cAMP)-mediated activation of protein kinase A (PKA) and p38 mitogen-activated protein kinase (p38 MAPK) signaling. Activation of p38 MAPK signaling leads to disruption of tight junction integrity between mammary epithelial cells and initiates apoptosis leading to involution[80,83]. The involution process reverses the structural integrity of the mammary gland to its pre-pregnancy form.

Serotonin in breast cancer

Many studies have demonstrated that several HTRs are expressed in human breast tumor cell lines as established by microarray data, immunohistochemistry, or Western blotting, including HTR1B, HTR1D, HTR1E, HTR1F, HTR2A, HTR2B, HTR2C, HTR3, HTR4, HTR5A, HTR6 and HTR7[79,84–87]. Interestingly, 5-HT stimulates the proliferation of breast tumor cells and this effect can be prevented by treating the cells with HTR antagonists[85–88]. The dysregulation of 5-HT activity in mammary epithelial homeostasis can contribute to breast cancer. Hence, this provided a rational for us to investigate the serotonergic antagonists for their capacity to reduce BTIC frequency.

To establish a connection between serotonin and BTIC and to identify other receptors that may be involved in BTIC activity, we tested antagonists of HTRs that were not among the compounds in the small molecule library. Interestingly, one of the most potent inhibitors at reducing tumorsphere cell viability and tumorsphere formation was SB-699551, a selective antagonist of serotonin receptor 5A (HTR5A)[60]. Moreover, SB-699551 reduced tumorsphere formation by breast tumor cell lines representative of all molecular subtypes[89].

Additionally, a panel of six selective HTR5A antagonists were tested for their capacity to reduce tumorsphere formation[90]. Compounds with higher selectively for HTR5A showed greater potency at reducing tumorsphere formation. Two HTR5A antagonists tested were (S)-isomers of the guanidine-type antagonists. These (S)-isomer compounds were more selective for HTR5A than their (R)-isomer counterparts.

Consequently, the (S)-isomer HTR5A antagonists were more potent at inhibiting tumorsphere formation than the (R)-isomer compounds, thereby demonstrating enantiomeric selectivity.

Moreover, in an *ex vivo* assay, SB-699551 treated HCC1954 tumorsphere cells transplanted into immunocompromised mice displayed reduced tumor formation frequency as compared to vehicle treated tumorsphere cells (not published)[90]. Moreover, in an *in vivo*, SB-699551 treatment in combination with Docetaxel significantly reduced tumor volume and growth than either treatment alone or vehicle (not published). Collectively, this suggests that SB-699551 has BTIC targeting properties.

Additionally, we have previously shown that breast tumor cells of mouse and human origin synthesize 5-HT and express SERT[60]. Pharmacological inhibition of SERT with SSRI reduced the frequency of BTIC in these cells as established by *in vitro* sphere-forming assays and *ex vivo* cell transplantation assays. Moreover, these agents synergize with chemotherapy to inhibit tumor growth *in vivo.*

Based on the knowledge of its activity in synapses, SERT inhibition is expected to increase extracellular 5-HT levels and stimulate proliferation. There are several theories that may explain why this does not occur. First, SERT activity is coupled to oncogenic intracellular signaling pathways inducing AKT and MAPK[91]. In fact, many SERT-interacting GTPases, phosphatases, and kinases have been identified, suggesting a SERT signaling role[91]. Indeed, in pulmonary smooth muscle cells, SERT plays a vital in transactivation of PDGFRβ to achieve 5-HT mediated hypertrophy[92,93]. Moreover, the 5-

HT that is taken up by SERT is degraded by MAO-A to generate reactive oxygen species (ROS)[94]. These ROS can mediate nuclear translocation of phosphorylated ERK1/2 that may be downstream of HTR[95,96]. Taken together, these mechanisms highlight methods that SERT can enhance mitogenic signaling by HTR.

HTR5A

Cloned and characterized in 1994[97], HTR5A was discovered to be a member of the superfamily of GPCR.[98] HTRA couples to the $G\alpha_{i/0}$ subunit and consequently the binding of 5-HT to HTR5A inhibits adenylate cyclase activity, reducing the production of cAMP[71]. Rodents have been shown to express two functional HTR5 receptors, HTR5A and HTR5B[99]. However, while humans possess both *HTR5A* and *HTR5B* genes, the coding sequence of HTR5B is interrupted by stop codons, thereby making the gene non-functional[100]. Notably, homozygous *HTR5A* knockout mice are also phenotypically normal, in fact, they display an increased tendency to explore the novel environment, indicative of improved cognitive ability[101]. Moreover, SB-699551 phenotypically recapitulates the effects of *HTR5A* knockout and has also been shown to improve schizophrenia symptoms[102].

PI3K/AKT/mTOR signaling

The phosphoinositide 3-kinase (PI3K) pathway has emerged in recent years as a crucial regulator of cell metabolism, growth, proliferation and survival[103]. Dysregulation of the PI3K pathway is a characteristic of many human malignancies.

PI3Ks are divided into three classes based on structural and enzyme-kinetic differences: class I, class II, and class III. The class I PI3Ks are abnormally activated in breast cancer[104]. Mammalian class I PI3Ks are divided into class IA and IB based on different types of receptor that regulate their activation. Class IA and IB PI3Ks are activated by RTKs and GPCRs, respectively[103]. PI3Ks function as heterodimeric lipid kinases comprised of a regulatory subunit and a catalytic subunit. Class IA PI3K catalytic subunits p110α, p110β, p110δ are encoded in humans by *PIK3CA*, *PIK3CB*, and *PIK3CD*, respectively. Class IA regulatory subunits are encoded by *PIK3R1*, *PIK3R2*, and *PIK3R3*. By contrast, the class IB PI3K p110γ catalytic subunit is encoded by *PIK3CG*, which interacts with either the p101 or the p87 regulatory subunits, which are encoded by *PIK3CR5* or *PIK3R6*, respectively[105].

Due to the several regulatory functions of the PI3K pathway, activity is tightly controlled by a multistep process. The presence of activating signals recruit PI3Ks to the cell membrane, liberating the catalytic subunit from the regulatory subunit. The catalytic subunit phosphorylates phosphatidylinositol 4, 5-bisphosphate (PIP2) lipids to generate PIP3. The secondary messenger PIP3 coordinates AKT localization to the plasma membrane through specific lipid-binding domains, where AKT is phosphorylated at threonine 308 (T308) by 3-phosphoinositide-dependent protein kinase 1 (PDK1), leading to partial AKT activation[106].

AKT functions as the central mediator of the PI3K pathway and activates the downstream signaling pathways, including mammalian target of rapamycin (mTOR). Partially activated AKT phosphorylates stress-activated map kinase interacting protein 1

(SIN1) at T86, which is a regulatory subunit exclusive to the mammalian target of rapamycin complex 2 (mTORC2) [107]. Phosphorylation of SIN1 relieves the inhibition of mTORC2, thereby activating it. This creates a positive feedback loop whereby activated mTORC2 can in turn phosphorylate AKT on serine 473 (S473), fully activating it[107].

Fully active AKT phosphorylates and inactivates proline-rich AKT substrate (PRAS40) and tuberous sclerosis complex 1 and 2 (TSC1/2)[108]. Inactivation of PRAS40 and TSC1/2 terminate their inhibitory effects on mammalian target of rapamycin complex 1 (mTORC1). Activated mTORC1 phosphorylates 4E binding protein 1 (4EBP1) and ribosomal protein S6 kinase (S6K1) thus which results in activation of ribosomal protein S6 (S6RP), promoting protein synthesis and cellular proliferation[109].

Additionally, AKT also phosphorylates and inhibits the pro-apoptotic forkhead box proteins (FOXO)[110], promoting cell growth. Fully active AKT mediates cellular functions including angiogenesis, metabolism, growth, proliferation, survival, protein synthesis, transcription, and apoptosis. Termination of AKT signaling occurs through dephosphorylation at S473 and T308. Dephosphorylation of AKT at T308 is accomplished by protein phosphatase 2 (PP2A) and S473 by the PH domain and leucine-rich repeat protein phosphatase (PHLPP1/2). Furthermore, PIP3 is reverted to PIP2 by the phosphatase and tensin homolog (PTEN) to completely inhibit AKT signaling[111]. AKT signaling termination is crucial to prevent dysregulation of homeostatic cell functions. In fact, elevated PI3K signaling is associated with breast cancer tumorigenesis, drug resistance, and poor clinical outcome[112].

MATERIALS AND METHODS

Cell Culture

All breast cancer cells lines were acquired from the American Type Culture Collection (ATCC) and propagated following to their respective protocols provided. Adherent cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute media (RPMI). The media was supplemented 10% fetal bovine serum (Gibco), 1mg/ml fungizone (Gibco), and 1% penicillin/streptomycin (Gibco). Cells were incubated at 37°C and 5% CO_2 in 75cm^2 or 150 cm^2 flasks (Thermo Scientific). Cells were grown to confluency (70-90%) before being harvested with 0.25% trypsin-EDTA (Gibco) and seeded into new flasks.

Tumorsphere-formation Assays

Tumorspheres were mechanically triturated in fresh stem cell media, and dispersed cells were plated into 96-well plate at a density of 30,000 viable cells/mL or 6000 cells in 200µl of media per well. Compounds of interest were serially diluted with DMSO and added in triplicate to the wells. Cells were incubated for four days at 37°C and 5% CO_2 before they were manually counted. IC_{50} values were generated based on the percentage of spheres counted in the negative control wells (DMSO). The IC_{50} values and the graphs were generated with GraphPad Prism 7.

Proteomic Profiler Array

The Proteomic Profiler Array was conducted following the instructions provided by the manufacturer (R&D Systems, #ARY003B). The membranes were first blocked in Array Buffer 1 for one hour at room temperature on a rocking platform. Following blocking, membranes were exposed to total extracts (600µg) from MCF-7 cells treated with vehicle (DMSO) or SB-699551 suspended in Array Buffer 1 overnight at 4°C. Next day, the membranes were washed and incubated with Detection Antibody Cocktail A/B in Array Buffer 2/3 for two hours at room temperature on a rocking platform. The membranes were washed again and then incubated with Streptavidin-Horseradish Peroxidase secondary antibody for 30 minutes at room temperature on a rocking platform. After the final wash, the membranes were exposed to the Chemi Reagent for one minute and placed in an autoradiography film cassette. The membranes were exposed to X-Ray films (Thermo Scientific) and the films were scanned using a Hewlett-Packard scanner.

Western Immunoblotting

Total extracts from breast tumor cell were collected after treatment with SB-699551 or vehicle (DMSO) using RIPA (50mM Tris-HCl pH 8.0, 150mM NaCl, 0.5% Na deoxyCholate, 0.1% Sodium dodecyl sulfate, 1% NP40, protease and phosphatase inhibitors). Lysates were centrifuged at 5000 *g* for five minutes at 4°C and the supernatant was loaded as protein samples. Following 8-12% SDS-PAGE separation, proteins were

transferred to nitrocellulose membranes. Blocking was performed using 5% Bovine Serum Albumin (Thermo Scientific), and the antibodies for proteins of interest were diluted using the blocking buffer (1:5000) and incubated overnight at 4°C on a rocking platform. Membranes were washed thrice using 1 x Tris buffer solution plus Tween 20 (TBST) for five minutes per wash at room temperature. Following washes, the membranes were exposed to Goat anti-Rabbit antibody (A21076, life technologies) diluted in blocking buffer for one hour at room temperature. The membranes are washed again before being imaged using the LICOR Odyssey CLx. The membranes were then incubated with Rat alpha-tubulin antibody (MA1-80017, Invitrogen) diluted in blocking buffer for one hour at room temperature. The membranes are washed and then exposed to Donkey anti-Rat secondary antibody (SA5-10032, Invitrogen) diluted in blocking buffer for one hour at room temperature. Finally, the membranes are imaged again to detect alpha tubulin using the LICOR Odyssey CLx. All quantifications of western blots was performed using LICOR provided, Image Studio 2.0 software. Antibodies for P-AKT (S473) (#4060), P-AKT (T308) (#13038), AKT (#2938), P-PRAS40 (#2997), PRAS40 (#2691), P-P70S6K (#9205), P70S6K (#9202), P-S6RP (#2211), S6RP (#2217), P-4E-BP1 (#2855), 4EBP1 (#9644), P-CREB (#9198), CREB (#9197), P-ERK (#4370), ERK (#9102), P-FOXO1 (#9461), Fox01 (#2880) were purchased from Cell Signaling.

Cell Viability Assays

Cell viability assays were performed in 96-well dishes where the cells were seeded at 6K cells or 10K cells per well density in 200 µl media. After 24h, 1µl of compounds serially diluted in DMSO were added to the media in the cells. Next day, 20µl of Presto

Blue (Thermo Scientific) was added to each well (1:10 ratio), and Presto Blue reduction was read (530nM excitation / 590nM emission) after 1.5h of incubation using the Biomek DTX880 multimode detector (Beckman Coulter). Raw intensity values were converted to % residual activity using this formula:

$$\% \text{ residual activity} = \frac{\text{Sample reading} - \text{Negative control}}{\text{Positive control} - \text{Negative control}} \times 100\%$$

The IC_{50} values were calculated using GraphPad Prism 7. For calculation purposes, the vehicle was noted as 1nM concentration of the test compound.

Compounds Suppliers

SB-699551 was procured from Dalriada Therapeutics, API-2 (2151) was obtained from Tocris Chemicals, while Buparlisib (S2247), AZD8055 (S1555), and MK-2206 (S1087), were purchased from Selleckchem. Rapamycin (R5000) was obtained from LC Laboratories.

RESULTS

HTR5A signals through the PI3K/AKT/mTOR signaling axes.

To elucidate the signaling pathways downstream of HTR5A, we treated human breast tumor cells with SB-699551 and assessed the changes in phosphorylation of signaling proteins using the Proteomic Profiler Array (PPA). The PPA assesses the phosphorylation levels of 43 signaling proteins using phospho-specific antibodies in a sandwich ELISA manner.

To accomplish the latter we collected protein lysates from MCF-7 cells treated with SB-699551 or its vehicle (0.5% DMSO) for 30 minutes and then analyzed the lysates using the PPA (**Fig. 1A**). We quantified the mean pixel density of each spot on the X-ray film, which is indicative of the total abundance of the phosphorylated species of each signaling proteins at the time of isolation. The proteins that displayed the greatest changes in phosphorylation in response to SB-699551 are shown in **Fig. 1B** and the mean pixel densities are graphed in **Fig. 1C.**

We observed an increase in the phosphorylation of the cAMP-response element binding protein (CREB) in response to SB-699551. HTR5A is coupled to $G\alpha_i$ and hence inhibition of HTR5A prevents the $G\alpha_i$ subunit from inhibiting adenylate cyclase thus increasing cAMP. The accumulation of cAMP activates PKA, which in turn phosphorylates and activates CREB. Hence the increase in CREB phosphorylation observed with SB-699551 treatment is consistent with its inhibition of HTR5A.

We also found that AKT phosphorylation was reduced at residues S473 and T308. Moreover, the phosphorylation of two of its downstream substrates, lysine deficient protein kinase 1 (WNK1) and PRAS40 was also reduced in response to SB-699551 treatment. PRAS40 functions as an inhibitory regulator of mTOR complex 1. Phosphorylation of PRAS40 by AKT mitigates its inhibition of mTORC1 and promotes mTOR signaling. Hence, reduction in PRAS40 phosphorylation results in inhibition of mTORC1 and its downstream targets like P70S6K. This explains our observation that SB-699551 also reduces the phosphorylation of P70S6K. Taken together, the observations from the PPA indicate that inhibition of HTR5A with SB-699551 in the MCF-7 cell line reduces the activity of the $G\alpha_i$-coupled and AKT/mTOR signaling pathways.

We wished to validate these observations by Western immunoblotting using independently purchased antibodies to the targets of interest. Furthermore, we wanted to determine the temporal kinetics of the phosphorylation of target proteins after SB-699551 treatment. To this end we treated human breast tumor cells with SB-699551 for 5, 10, 30, and 60 minutes and isolated protein at each of these time intervals. We then probed for the total and phosphorylated species of each protein using Western blotting. The relative abundance of each phosphoprotein was determined by dividing the mean pixel densities of bands corresponding to each phosphoprotein with their respective total protein. The fold change of each phosphoprotein after SB-699551 treatment are presented in **Table 4**. To verify the PPA results, the experiment was performed in the MCF-7 cell line. To establish whether these observations were maintained in breast tumor cells with a different molecular

subtype, the experiment was performed with a triple negative breast tumor cell line, MDA-MB-157.

In agreement with our PPA results, we detected an increase in CREB phosphorylation after the treatment of both cell lines with SB-699551 **(Figure 2A)**. SB-699551 treatment caused an increase in pCREB, occurring as early as 5 minutes after treatment and peaking at 10 minutes. The increase in pCREB was maintained as late as 60 minutes **(Figure 2A)**. Interestingly, the antibodies that detect CREB phosphorylation and total CREB also binds to the activating transcription factor 1 (ATF1), a member of the cyclic AMP-dependant transcription family. ATF1 is a target of PKA, which also phosphorylates CREB[113]. We determined that the pATF1 abundance in both MCF-7 and MDA-MB-157 cells followed the same temporal pattern of pCREB **(Figure 2A)**. These findings suggest that SB-699551 acts through the $G\alpha_i$-coupled HTR5A.

We found that treatment of the breast tumor cell lines with SB-699551 also decreases the phosphorylation of AKT (S473), occurring as early as 5 minutes and peaking at 30 minutes in MCF-7 cells and at 60 minutes in the MDA-MB-157 cell line **(Figure 2B)**. Despite observing a minimal reduction in phosphorylation of AKT (T308) in the PPA, we observed a substantial decrease in pAKT (T308) by Western immunoblotting. This may be due to the differences in sensitivity of the antibodies and/or the techniques used in the PPA and Western immunoblotting experiments. Both pAKT (S473) and pAKT (T308) followed a similar temporal pattern in response to SB-699551 treatment in each cell line. Interestingly, we observed increases in pAKT (S473) and (T308) phosphorylation in the

vehicle-treated cells over time. This may be the result of the growth factors present in the serum added to the cells at the time of treatment[114].

We also wanted to verify the alterations in PRAS40 and P70S6K phosphorylation that we observed in the PPA. PRAS40 phosphorylation was reduced in a time-dependent manner after treatment with SB-699551 in both cell lines **(Figure 2C)**. The decrease in pPRAS40 peaked at 10 minutes and was sustained until 60 minutes. Furthermore, the reduction in P70S6K phosphorylation in the PPA was also reproduced **(Figure 2C)**. SB-699551 reduced P70S6K phosphorylation with the greatest reduction occurring after 60 minutes of treatment in both cell lines. Taken together, the results of the PPA and Western immunoblotting suggest that inhibition of HTR5A by SB-699551 affects signaling via $G\alpha_i$-coupled and AKT/mTOR signaling pathways downstream of HTR5A.

We also investigated the phosphorylation of S6RP, a substrate of P70S6K. Although we observed a slight reduction in S6RP phosphorylation in response to SB-699551 exposure in the MCF-7 cell line **(Figure 2C)**, the reduction was not mirrored in the MDA-MB-157 cell line. This may be the result of differences in the kinetics of phosphorylation or phosphatases in the two cell lines.

Inhibition of HTR5A by SB-699551 reduced the phosphorylation of the pro-apoptotic transcription factor FOXO1

We wished to investigate which downstream effectors of AKT signaling may be effected by SB-699551. We chose to look at forkhead box protein 1 (FOXO1), a transcription factor involved in apoptosis, because of its regulation by AKT. Activated

AKT promotes cell survival by phosphorylating and inactivating FOXO1. Phosphorylation of FOXO1 prevents its translocation to the nucleus where it induces expression of pro-apoptotic genes such as BCL2 family protein, Bim[115]. Indeed, a recent study reports that SB-699551 inhibition of Htr5a reduced phosphorylation of FOXO1 and upregulated the expression of FOXO1 in mouse embryonic hematopoietic stem and progenitor cells[116]. This resulted in upregulated expression of *bim* and *fasl*, pro-apoptotic genes regulated by FOXO1, and reduced survival of mouse embryonic hematopoietic stem and progenitor cells[116]. To this end, we decided to investigate phosphorylation status of FOXO1 in response to HTR5A inhibition with SB-699551.

To determine whether inhibition of HTR5A results in an alteration of FOXO1 phosphorylation in breast tumor cell lines, we conducted Western immunoblotting using antibodies specific to pFOXO1 and total FOXO1. Treatment of MCF-7 cells with SB-699551 decreased the phosphorylation of FOXO1, which peaked at 60 minutes, coupled with an increase in total FOXO1 **(Figure 2D).** In MDA-MB-157 cells, the reduction in FOXO1 phosphorylation was most apparent at 30min, also coupled with an increase in total FOXO1 protein. Overall, SB699551 treatment slightly reduced phosphorylation of FOXO1, suggesting increased FOXO1 activity in response to HTR5A inhibition.

Whereas we did detect very slight changes in FOXO1 and S6RP phosphorylation, they were not as significant as those observed for the hits mentioned previously. Indeed, we observed that the phosphorylation alterations occurred earlier in MCF-7 than MDA-MB-157 cells. For example, the reduction of AKT phosphorylation peaked at 10 and 30 minutes in MCF-7 cells, whereas it peaked at 60 minutes in MDA-MB-157 cells. Hence,

we wondered whether the changes in abundance of downstream phosphoproteins or transcription factors would require a longer incubation period with SB-699551 or an increased concentration of the compound.

To test whether the kinetics of protein phosphorylation differed with longer exposure by SB-699551, we treated MDA-MB-157 cells with the compound for 24h. The lysates collected from the SB-699551- and vehicle-treated cells were probed for pFOXO1 and total FOXO1. As expected, the IC_{90} of SB-699551 reduced phosphorylation of FOXO1 drastically after the lengthened incubation period **(Figure 3).** The reduction in FOXO1 phosphorylation was more than 9-fold. Overall, these observations suggest that longer term exposure to SB-699551 resulted in a greater reduction of FOXO1 phosphorylation, thereby increasing FOXO1 activity.

Seeing a time-dependent effect of SB-699551 on FOXO1 encouraged us to probe S6RP. To this end, we also probed for S6RP phosphorylation and total S6RP. The phosphorylation of S6RP was reduced drastically in MDA-MB-157 cells after a 24h treatment with SB-699551 **(Figure 3)**, more so than with SB-699551 treatment at 1h **(Figure 2C).** Following this, we wondered whether the reduced phosphorylation of AKT was also maintained after 24h. To investigate the latter, we probed for AKT (S473) phosphorylation and total AKT. Interestingly, AKT phosphorylation remained low after a 24h treatment with SB-699551. By contrast, there was no difference in the abundance of total AKT. Hence, these findings suggest that the kinetics of phosphorylation of different signaling proteins change with longer treatment intervals.

We also probed for the phosphorylation of 4EBP1 as this protein is a direct substrate of mTORC1. In accordance with our previous observations, the phosphorylation of 4EBP1 was reduced after a 24-hour exposure to SB-699551 **(Figure 3)**. However, unlike other proteins, the total 4EBP1 species was also reduced at the IC_{90} concentration of SB-699551. Through normalization of pixel densities of phosphorylated 4EBP1 to total 4EBP1, we determined that the reduction in phosphorylation was greater than the reduction of total 4EBP1 with SB-699551 exposure. This decrease in total 4EBP1 is explained by its instability when unphosphorylated as reported by Yanagiya et al[117,118]. Hypophosphorylated species of 4EBP1 are heavily ubiquitinated and degraded, and the degree of ubiquitination is associated with the extent of 4EBP1 phosphorylation[118]. Moreover, hypophosphorylated 4EBP1 inhibits eIF4E by competing with eIF4G for a common binding site[119]. eIF4E and eIF4G are subunits of the eIF4F cap-binding complex that interact directly with 5' cap of mRNA and mediates 5' cap dependant translation. Hence reduction in eIF4E would result in decreased protein translation, which may contribute to the anti-BTIC effects of SB-699551 that we observed.

Selective antagonists of kinases downstream of HTR5A phenocopy the effects of SB-699551.

We previously established that treatment of human breast tumor cell lines with SB-699551 targets BTIC as established by *ex vivo* and *in vitro* functional assays[90]. Hence, if the signaling pathways that we identified are indeed necessary for BTIC activity, then perturbation of these kinases may elicit a similar phenotypic effect as that of SB-699551.

To investigate the latter, we used small molecule selective antagonists of each kinase. We selected the PI3K inhibitor, Buparlisib; two AKT inhibitors, MK2206 and API2; the mTOR complex 1 inhibitor, Rapamycin; the mTOR complex 2 inhibitor, JRAB2011 and a rapalog that inhibits both complex 1 and 2 of mTOR, AZD8055. We assessed the capacity of these kinase inhibitors to reduced tumorsphere formation *in vitro* using quantitative sphere forming assays with MDA-MB-157 **(Figure 4A)** and MCF-7 **(Figure 4B)** breast tumor cell lines. Moreover, to determine whether the kinase inhibitors affected cell viability, we also measured their capacity to reduce PrestoBlue, which is an indirect measure of the latter.

The sphere-forming assays showed that all the kinase inhibitors reduced tumorsphere formation of MDA-MB-157 and MCF-7 tumor cells. The IC_{50} values of the kinase antagonists ranged from 50nM to 1µM. Buparlisib, the PI3K inhibitor reduced tumorsphere formation in both cell lines with similar IC_{50} values, however, it only reduced the cell viability of MCF-7 tumorsphere cells. Similarly, the two AKT inhibitors MK2206 and API2, also reduced tumorsphere formation in both cell lines. Whereas MK2206 reduced cell viability of both MCF-7 and MDA-MB-157 tumorspheres, the effect in MCF-7 cells was much more pronounced. API2 did not affect the cell viability of tumorsphere from either cell line.

The mTORC1 inhibitors, Rapamycin and AZD8055, reduced tumorsphere formation of MDA-MB-157 and MCF-7 cells and they also had a marginal effect at reducing the cell viability of the two cell lines. The mTORC2 inhibitor, JRAB2011, reduced the tumorsphere formation and cell viability in both MDA-MB-157 and MCF-7

cells. Taken together, these observations indicate that inhibition of the PI3K/AKT/mTOR pathways using selective antagonists phenocopies the effect of SB-699551 in sphere-forming assays.

DISCUSSION

The high recurrence rate of breast cancer following conventional therapy is attributed to an infrequent population of drug-resistant BTIC[39–41]. Previously, our lab identified serotonergic antagonists that inhibit BTIC activity including the HTR5A selective antagonist, SB-699551[90]. Hence, it is important to elucidate the signaling pathways affected by inhibitors of HTR5A activity. Furthermore, knowledge of the HTR5A regulated pathways will identify biomarkers of therapy efficacy. To identify HTR5A signaling pathways we used a phospho-proteomic approach, wherein we determined that treatment of human breast tumor cell lines with SB-699551 affected signaling via the $G\alpha_i$-coupled and the PI3K/Akt/mTOR axes.

We determined that the latter occurs in human breast tumor cell lines of both the luminal A and triple-negative breast cancer subtypes. This is consistent with our previous observations where we found that SB-699551 reduces tumorsphere-formation independently of the subtype modeled by the breast tumor cell lines. Using the PPA and Western immunoblotting we established that treatment of human breast tumor cell lines with SB-699551 alters the phosphorylation of numerous intracellular signaling proteins in the $G\alpha_i$-coupled and the PI3K/Akt/mTOR signaling pathways **(Figure 5)**. SB-699551 increased phosphorylation of CREB and ATF1, which are two transcriptional proteins downstream of $G\alpha_i$-coupled pathway. Furthermore, SB-699551 treatment reduced phosphorylation of AKT, which phosphorylates PRAS40. Hypophosphorylated PRAS40 inhibits mTORC1 activity. The reduced activity of mTORC1 renders it unable to phosphorylate P70S6K and 4EBP1. Hypophosphorylated P70S6K leads to inactive S6RP,

thereby reducing mRNA translation. Moreover, hypophosphorylated 4EBP1 is bound to eIF4E, which is required for 5' cap dependent protein translation. Additionally, reduced AKT activity results in decreased FOXO1 phosphorylation, which activates FOXO1 to upregulate expression of apoptotic genes. We also demonstrated that inhibition of AKT, PI3K, or mTOR with selective antagonists phenocopies the effect of SB-699551 *in vitro* by reducing the tumorsphere-forming capacity of MDA-MB-157 and MCF-7 cells. Taken together, our data suggests that SB-699551 affects intracellular signaling pathways that are consistent with on-target activity and identifies a biologically plausible mechanism of its action.

5-HT mediated HTR5A stimulation cause G_{γ} to exchange GDP for GTP, this exchange results in disassociation of the trimeric G-protein complex and allows the $G\alpha_i$ subunit to inhibit adenylate cyclase. Impairment of adenylate cyclase activity perturbs the conversion of ATP to cAMP, and because cAMP is required for PKA activity, reduced cAMP abundance suppresses PKA function. Downregulation of PKA activity prevents phosphorylation of CREB and ATF1 at S133 and S63 respectively. Hence, successful stimulation of HTR5A would result in dephosphorylated CREB and ATF1. Consequently, inhibition of HTR5A would result in the opposite effect, an increase in CREB and ATF1 phosphorylation. The latter is observed with our data when breast tumor cells are treated with SB-699551, a selective inhibitor of HTR5A. This further validates that SB-699551 is functioning through its intended target, HTR5A.

We observed reduced FOXO1 phosphorylation in response to HTR5A inhibition. Interesting, FOXO proteins have been associated with maintenance of cancer stem cell

properties[120,121]. One recent study reported that the inhibition of the PI3K or AKT pathways reduced the frequency of $CD44^{High}/CD24^{Low}$ BTIC in the MDA-MB-231 breast tumor cell line[121]. Moreover, the study demonstrated that the reduction of BTIC frequency by inhibition of PI3K/AKT signaling was facilitated by the inhibition of FOXO3 phosphorylation and consequent increased activity of Bim1. Elevated Bim1 activity was shown to be accompanied with reduced cell viability and loss of E-cadherin, resulting in reduced the capacity of the cells to form tumorspheres[121]. Hence, FOXO1 may play a similar role in breast tumor cell lines, which would explain the apoptotic and BTIC targeting properties of HTR5A inhibition with SB-699551. Overall, our results support the notion that AKT inhibition of the FOXO1 mediated pro-apoptotic pathway downstream of HTR5A may be essential to protect BTIC from apoptosis.

We also observed that selective inhibitors of the PI3K/AKT/mTOR pathways abrogated tumorsphere formation capacity of tumorsphere-derived cells from MCF-7 and MDA-MB-157 cell lines. Consistent with our data, a recent study shows the use of MK-2206 to target CD133(+) colorectal cancer (CRC) tumor-initiating cells to reduce their capacity to form colonospheres *in vitro* and initiate tumor formation *in vivo*[122]. Moreover, another study reports that hepatocellular carcinoma cells enriched for CD90(+) tumorsphere forming cells were more tumorigenic and resistant to the chemotherapeutic agent, doxorubicin[123] and this resistance could be overcome using a selective inhibitor of PI3K/AKT. Furthermore, the abundance of phosphorylated AKT1 was elevated in tumorsphere-forming cells treated with doxorubicin but reduced when treated with

PI3K/AKT inhibitor. Hence, this suggests that PI3K/AKT/mTOR signaling is highly active in tumor-initiating cells.

Although we observed alterations in the phosphorylation of PI3K/AKT/mTOR proteins in response to SB-699551 in both MCF-7 and MDA-MB-157 cell lines, the effect was more marked in MCF-7 cells. This may be explained by the *PIK3CA* copy number amplification in MCF-7 cells and a mutation that promotes constitutive activity of the PI3K pathway[124]. Consequently, this results in a greater intracellular abundance of active PI3K compared to the MDA-MB-157 cell line. Notably, although MCF-7 is the only cell line with the additional *PIK3CA* copy number and constitutive active PI3K mutation, the effect of HTR5A inhibition of tumorsphere-forming cells is evident in MDA-MB-157 as well. Hence, the mechanism downstream of HTR5A is essential for BTIC activity in breast tumor cell lines of different molecular subtypes, even those that do not have constitutive PI3K signaling.

Consistent with the latter, there were also obvious differences in the sensitivity of MCF-7 and MDA-MB-157 cells to some of the kinase inhibitors. For example, Buparlisib reduced the cell viability of MCF-7 tumorsphere-derived cells to a greater extent than MDA-MB-157 tumorsphere-derived cells. Interestingly, PI3K/AKT antagonists have been used either alone or in combination with chemotherapy to reduce the growth rate of breast tumor xenografts derived from cell lines that possess the *PIK3CA* mutation or abnormally active PI3K signaling[125–127]. For example, a combination of Buparlisib and Tamoxifen has been shown to attenuate the growth of MCF-7 xenografts in Balb/c nude mice[125]. Moreover, MK2206 synergized with chemotherapeutics such as Doxorubicin,

Camptothecin, 5-fluorouracil, Docetaxel, Paclitaxel and Carboplatin to inhibit the proliferation of human tumor cell lines that have elevated PI3K/AKT signaling including MCF-7 cells[126,127]. The capacity for MK2206 to augment the efficacy of existing anticancer agents is explained by its inhibition of AKT phosphorylation, which contributes to resistance against many of the conventional chemotherapeutic agents[126]. Moreover, using sulforhodamine B assays, MK2206 has been shown to have greater sensitivity against breast tumor cell lines that possess the *PIK3CA* mutations, such as MDA-MB-463, BT474, and MCF-7[127]. This is consistent with our results where MK2206 or Buparlisib had a more potent effect on reducing the viability of tumorsphere-cells derived from MCF-7 tumorspheres than those derived from MDA-MB-157 tumorspheres.

Several studies have documented the use of PI3K, AKT, or mTOR inhibitors to inhibit the growth of human breast tumor cells[128]. However, resistance to these inhibitors frequently develops due to compensatory mechanisms between signaling pathways. For example, whereas rapamycin has been studied extensively for its capacity to inhibit BTIC activity, resistance develops due to signaling feedback mechanisms between mTOR and AKT[128]. Rapamycin treatment inhibits the phosphorylation of P70S6K and 4EBP1, however, rapamycin also simultaneously increases the phosphorylation of AKT and eIF4E[128].

To overcome this hurdle, researchers have used AKT and mTOR inhibitors in combination[129,130]. For example, Rapamycin and MK2206 have been used together to completely inhibit the PI3K/AKT/mTOR pathways in breast tumor cell lines representing all molecular subtypes[129,130]. Evidently, Rapamycin and MK2206 combination treatment

suppresses 4EBP1 and P70S6K phosphorylation more effectively than either agent alone[129]. Moreover, Rapamycin and MK2206 combination treatment reduces the growth of tumor xenografts derived from ZR-75-1 and MDA-MB-468 human breast tumor cell lines *in vivo* more significantly than either agent alone[130]. Hence, a BTIC-targeting mechanism involving PI3K/AKT/mTOR is consistent with biological mechanisms proposed by others.

CONCLUSION

We have established that HTR5A signals through the $G\alpha_i$-coupled and PI3K/AKT/mTOR signaling pathways. The PPA identified changes in phosphorylation of several key proteins associated with the PI3K/AKT/mTOR pathways. Western immunoblotting validated these observations and helped identify additional proteins that are associated with apoptosis, protein synthesis and BTIC. Using PI3K, AKT, and mTOR kinase inhibitors in sphere-forming assays, we phenocopied the effect of SB-699551. Collectively, these observations suggest that HTR5A maintains BTIC activity through the PI3K/AKT/mTOR signaling axis.

REFERENCES

1. Fidler, I. J. Origin and biology of cancer metastasis. *Cytometry* (1989). doi:10.1002/cyto.990100602

2. Canadian Cancer Society. Canadian Cancer Statistics 2017. *Can. Cancer Stat.* (2017).

3. Weinberg, R. A. Finding the anti-oncogene. *Scientific American* (1988). doi:10.1038/scientificamerican0988-44

4. Nelson, D. A. *et al.* Hypoxia and defective apoptosis drive genomic instability and tumorigenesis. *Genes Dev.* (2004). doi:10.1101/gad.1204904

5. Downward, J. Targeting RAS signalling pathways in cancer therapy. *Nature Reviews Cancer* (2003). doi:10.1038/nrc969

6. Finver, S. N. *et al.* Sequence analysis of the MYC oncogene involved in the t(8;14)(q24;q11) chromosome translocation in a human leukemia T-cell line indicates that putative regulatory regions are not altered. *Proc. Natl. Acad. Sci.* (2006). doi:10.1073/pnas.85.9.3052

7. Friedenson, B. The BRCA1/2 pathway prevents hematologic cancers in addition to breast and ovarian cancers. *BMC Cancer* (2007). doi:10.1186/1471-2407-7-152

8. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. V. & Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science (80-.).* (1990). doi:10.1126/science.2144057

9. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* (2011). doi:10.1016/j.cell.2011.02.013

10. von Lintig, F. C. *et al.* Ras activation in human breast cancer. *Breast Cancer Res. Treat.* (2000). doi:10.1023/A:1006491619920

11. Sørlie, T. *et al.* Repeated observation of breast tumor subtypes in independent gene

expression data sets. *Proc. Natl. Acad. Sci.* (2003). doi:10.1073/pnas.0932692100

12. Stingl, J. & Caldas, C. Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. *Nature Reviews Cancer* (2007). doi:10.1038/nrc2212

13. Malhotra, G. K., Zhao, X., Band, H. & Band, V. Histological, molecular and functional subtypes of breast cancers. *Cancer Biology and Therapy* (2010). doi:10.4161/cbt.10.10.13879

14. Jemal, A., Bray, F. & Ferlay, J. Global Cancer Statistics. *CA Cancer J Clin* (2011).

15. Canada, C. C. S. S. *Canadian Cancer Statistics 2014*. *Canadian Cancer Statistics* (2013).

16. Dai, X. *et al.* Breast cancer intrinsic subtype classification, clinical use and future trends. *Am. J. Cancer Res.* (2015).

17. Cheang, M. C. U. *et al.* Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. *J. Natl. Cancer Inst.* (2009). doi:10.1093/jnci/djp082

18. Heitz, F. *et al.* Triple-negative and HER2-overexpressing breast cancers exhibit an elevated risk and an earlier occurrence of cerebral metastases. *Eur. J. Cancer* (2009). doi:10.1016/j.ejca.2009.06.027

19. Cancer, A. J. C. on. *American Joint Committee on Cancer Staging Manual*. *Springer* (2010).

20. Brierley, J. D., Gospodarowicz, M. K. & Wittekind, C. TNM classification of malignant tumours - 8th edition. *Union Int. Cancer Control* (2016). doi:10.1002/ejoc.201200111

21. Roylance, R. *et al.* Comparative genomic hybridization of breast tumors stratified by histological grade reveals new insights into the biological progression of breast cancer. *Cancer Res.* (1999).

22. Tian, Z. *et al.* Prognostic significance of tumor grading and staging in mammary

carcinomas with neuroendocrine differentiation. *Hum. Pathol.* (2011). doi:10.1016/j.humpath.2010.11.014

23. Elston, C. W. THE ASSESSMENT OF HISTOLOGICAL DIFFERENTIATION IN BREAST CANCER. *Aust. N. Z. J. Surg.* (1984). doi:10.1111/j.1445-2197.1984.tb06677.x

24. Dhankhar, R. *et al.* Advances in novel drug delivery strategies for breast cancer therapy. *Artificial Cells, Blood Substitutes, and Biotechnology* (2010). doi:10.3109/10731199.2010.494578

25. Dexter, D. L. & Leith, J. T. Tumor heterogeneity and drug resistance. *J. Clin. Oncol.* (1986). doi:10.1200/JCO.1986.4.2.244

26. Liedtke, C. *et al.* Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. *J. Clin. Oncol.* (2008). doi:10.1200/JCO.2007.14.4147

27. Jones, S. E. *et al.* Phase III trial comparing doxorubicin plus cyclophosphamide with docetaxel plus cyclophosphamide as adjuvant therapy for operable breast cancer. *J. Clin. Oncol.* (2006). doi:10.1200/JCO.2006.06.5391

28. Mansi, J. L. *et al.* Five-year outcome for women randomised in a phase III trial comparing doxorubicin and cyclophosphamide with doxorubicin and docetaxel as primary medical therapy in early breast cancer: An Anglo-Celtic Cooperative Oncology Group Study. *Breast Cancer Res. Treat.* (2010). doi:10.1007/s10549-010-0989-6

29. Ali, S. & Coombes, R. C. Endocrine-responsive breast cancer and strategies for combating resistance. *Nature Reviews Cancer* (2002). doi:10.1038/nrc721

30. Minuti, G. *et al.* Increased MET and HGF gene copy numbers are associated with trastuzumab failure in HER2-positive metastatic breast cancer. *Br. J. Cancer* (2012). doi:10.1038/bjc.2012.335

31. Voduc, K. D. *et al.* Breast cancer subtypes and the risk of local and regional relapse. *J. Clin. Oncol.* (2010). doi:10.1200/JCO.2009.24.9284

32. Vogel, C. L. *et al.* Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J. Clin. Oncol.* (2002). doi:10.1200/JCO.20.3.719

33. Miller, T. W. *et al.* Hyperactivation of phosphatidylinositol-3 kinase promotes escape from hormone dependence in estrogen receptor-positive human breast cancer. *J. Clin. Invest.* (2010). doi:10.1172/JCI41680

34. Guttilla, I. K. *et al.* Prolonged mammosphere culture of MCF-7 cells induces an EMT and repression of the estrogen receptor by microRNAs. *Breast Cancer Res. Treat.* (2012). doi:10.1007/s10549-011-1534-y

35. Creighton, C. J. *et al.* Activation of mitogen-activated protein kinase in estrogen receptor α-positive breast cancer cells in vitro induces an in vivo molecular phenotype of estrogen receptor α-negative human breast tumors. *Cancer Res.* (2006). doi:10.1158/0008-5472.CAN-05-4363

36. Housman, G. *et al.* Drug resistance in cancer: An overview. *Cancers* (2014). doi:10.3390/cancers6031769

37. Balaji, S. A., Udupa, N., Chamallamudi, M. R., Gupta, V. & Rangarajan, A. Role of the drug transporter ABCC3 in breast cancer chemoresistance. *PLoS One* (2016). doi:10.1371/journal.pone.0155013

38. Zhang, Y. & Weinberg, R. A. Epithelial-to-mesenchymal transition in cancer: complexity and opportunities. *Frontiers of Medicine* (2018). doi:10.1007/s11684-018-0656-6

39. Buchholz, T. A. *et al.* WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells. *Proc. Natl. Acad. Sci.* (2007). doi:10.1073/pnas.0606599104

40. Hansen, S. N. *et al.* Acquisition of docetaxel resistance in breast cancer cells reveals upregulation of ABCB1 expression as a key mediator of resistance accompanied by discrete upregulation of other specific genes and pathways. *Tumor Biol.* (2015). doi:10.1007/s13277-015-3072-4

41. Dean, M. ABC transporters, drug resistance, and cancer stem cells. *J. Mammary Gland Biol. Neoplasia* (2009). doi:10.1007/s10911-009-9109-9

42. Li, X. *et al.* Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J. Natl. Cancer Inst.* (2008). doi:10.1093/jnci/djn123

43. Clarke, M. F., Al-Hajj, M., Morrison, S. J., Wicha, M. S. & Benito-Hernandez, A. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci.* (2003). doi:10.1073/pnas.0530291100

44. Watson, C. J. & Khaled, W. T. Mammary development in the embryo and adult: a journey of morphogenesis and commitment. *Development* (2008). doi:10.1242/dev.005439

45. Dick, J. E. Stem cell concepts renew cancer research. *Blood* (2008). doi:10.1182/blood-2008-08-077941

46. Lapidot, T. *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* (1994). doi:10.1038/367645a0

47. Bierie, B. *et al.* Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. *Proc. Natl. Acad. Sci.* (2011). doi:10.1073/pnas.1102454108

48. Sachlos, E. *et al.* Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells. *Cell* (2012). doi:10.1016/j.cell.2012.03.049

49. Bao, S. *et al.* Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* (2006). doi:10.1038/nature05236

50. Singh, S. K. *et al.* Identification of a cancer stem cell in human brain tumors.

Cancer Res. (2003). doi:10.1038/nature03128

51. Fang, D. *et al.* A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res.* (2005). doi:10.1158/0008-5472.CAN-05-1343

52. Kato, K. *et al.* Characterization of side-population cells in human normal endometrium. *Hum. Reprod.* (2007). doi:10.1093/humrep/del514

53. O'Brien, C. A., Pollett, A., Gallinger, S. & Dick, J. E. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* (2007). doi:10.1038/nature05372

54. Collins, A. T., Berry, P. A., Hyde, C., Stower, M. J. & Maitland, N. J. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res.* (2005). doi:10.1158/0008-5472.CAN-05-2018

55. Wolf, G. T. *et al.* Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc. Natl. Acad. Sci.* (2007). doi:10.1073/pnas.0610117104

56. Ginestier, C. *et al.* ALDH1 Is a Marker of Normal and Malignant Human Mammary Stem Cells and a Predictor of Poor Clinical Outcome. *Cell Stem Cell* (2007). doi:10.1016/j.stem.2007.08.014

57. Vaillant, F. *et al.* The mammary progenitor marker CD61/β3 integrin identifies cancer stem cells in mouse models of mammary tumorigenesis. *Cancer Res.* (2008). doi:10.1158/0008-5472.CAN-08-1949

58. Cicalese, A. *et al.* The Tumor Suppressor p53 Regulates Polarity of Self-Renewing Divisions in Mammary Stem Cells. *Cell* (2009). doi:10.1016/j.cell.2009.06.048

59. Kurpios, N. A. *et al.* Single Unpurified Breast Tumor-Initiating Cells from Multiple Mouse Models Efficiently Elicit Tumors in Immune-Competent Hosts. *PLoS One* (2013). doi:10.1371/journal.pone.0058151

60. Hallett, R. M. *et al.* Serotonin transporter antagonists target tumor-initiating cells

in a transgenic mouse model of breast cancer. *Oncotarget* (2016). doi:10.18632/oncotarget.10614

61. Brenner, B. *et al.* Plasma serotonin levels and the platelet serotonin transporter. *J. Neurochem.* (2007). doi:10.1111/j.1471-4159.2007.04542.x

62. Noguchi, T., Nishino, M. & Kido, R. Tryptophan 5-hydroxylase in rat intestine. *Biochem. J.* (1973).

63. Tong, J. H. & Kaufman, S. Tryptophan hydroxylase. Purification and some properties of the enzyme from rabbit hindbrain. *J. Biol. Chem.* (1975).

64. Tyce, G. M. Origin and metabolism of serotonin. *J. Cardiovasc. Pharmacol.* (1990). doi:10.1097/00005344-199000163-00002

65. Champier, J. *et al.* Evidence for tryptophan hydroxylase and hydroxy-indol-o-methyl- transferase mRNAs in human blood platelets. *Life Sci.* (1997). doi:10.1016/S0024-3205(97)00234-8

66. Hoyer, D. *et al.* International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol. Rev.* (1994).

67. Peroutka, S. J. Molecular biology of serotonin (5-HT) receptors. *Synapse* (1994). doi:10.1002/syn.890180310

68. Baldwin, J. M. Structure and function of receptors coupled to G proteins. *Curr. Opin. Cell Biol.* (1994). doi:10.1016/0955-0674(94)90134-1

69. Leopoldt, D. *et al.* Gβγ stimulates phosphoinositide 3-kinase-γ by direct interaction with two domains of the catalytic p110 subunit. *J. Biol. Chem.* (1998). doi:10.1074/jbc.273.12.7024

70. Brock, C. *et al.* Roles of Gβγ in membrane recruitment and activation of p110γ/p101 phosphoinositide 3-kinase γ. *J. Cell Biol.* (2003). doi:10.1083/jcb.200210115

71. Benovic, J. L. *et al.* Phosphorylation of the mammalian β-adrenergic receptor by cyclic AMP-dependent protein kinase. Regulation of the rate of receptor phosphorylation and dephosphorylation by agonist occupancy and effects on coupling of the receptor to the stimulatory guanine. *J. Biol. Chem.* (1985).

72. Perry, S. J. *et al.* Targeting of cyclic AMP degradation to β2-adrenergic receptors by β-arrestins. *Science (80-.).* (2002). doi:10.1126/science.1074683

73. Lefkowitz, R. J. & Shenoy, S. K. Transduction of receptor signals by β-arrestins. *Science* (2005). doi:10.1126/science.1109237

74. Shenoy, S. K. & Lefkowitz, R. J. Seven-Transmembrane Receptor Signaling Through -Arrestin. *Sci. Signal.* (2005). doi:10.1126/stke.2005/308/cm10

75. Shenoy, S. K. & Lefkowitz, R. J. β-arrestin-mediated receptor trafficking and signal transduction. *Trends in Pharmacological Sciences* (2011). doi:10.1016/j.tips.2011.05.002

76. Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G. & Barak, L. S. Differential affinities of visual arrestin, βarrestin1, and βarrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J. Biol. Chem.* (2000). doi:10.1074/jbc.M910348199

77. Shenoy, S. K. *et al.* β-arrestin-dependent, G protein-independent ERK1/2 activation by the β2 adrenergic receptor. *J. Biol. Chem.* (2006). doi:10.1074/jbc.M506576200

78. O'Hayre, M. *et al.* Genetic evidence that b-arrestins are dispensable for the initiation of b2-adrenergic receptor signaling to ERK. *Sci. Signal.* (2017). doi:10.1126/scisignal.aal3395

79. Pai, V. P., Marshall, A. M., Hernandez, L. L., Buckley, A. R. & Horseman, N. D. Altered serotonin physiology in human breast cancers favors paradoxical growth and cell survival. *Breast Cancer Res.* (2009). doi:10.1186/bcr2448

80. Stull, M. A. *et al.* Mammary gland homeostasis employs serotonergic regulation of epithelial tight junctions. *Proc. Natl. Acad. Sci.* (2007). doi:10.1073/pnas.0708136104

81. Matsuda, M. *et al.* Serotonin regulates mammary gland development via an autocrine-paracrine loop. *Dev. Cell* (2004). doi:10.1016/S1534-5807(04)00022-X

82. Hernandez, L. L., Gregerson, K. A. & Horseman, N. D. Mammary gland serotonin regulates parathyroid hormone-related protein and other bone-related signals. *Am. J. Physiol. Metab.* (2012). doi:10.1152/ajpendo.00666.2011

83. Pai, V. P. & Horseman, N. D. Biphasic regulation of mammary epithelial resistance by serotonin through activation of multiple pathways. *J. Biol. Chem.* (2008). doi:10.1074/jbc.M802476200

84. Kopparapu, P. K., Tinzl, M., Anagnostaki, L., Persson, J. L. & Dizeyi, N. Expression and localization of serotonin receptors in human breast cancer. *Anticancer Res.* (2013).

85. Sonier, B., Arseneault, M., Lavigne, C., Ouellette, R. J. & Vaillancourt, C. The 5-HT2A serotoninergic receptor is expressed in the MCF-7 human breast cancer cell line and reveals a mitogenic effect of serotonin. *Biochem. Biophys. Res. Commun.* (2006). doi:10.1016/j.bbrc.2006.03.080

86. Ballou, Y. *et al.* 5-HT serotonin receptors modulate mitogenic signaling and impact tumor cell viability. *Mol. Clin. Oncol.* (2018). doi:10.3892/mco.2018.1681

87. Gautam, J. *et al.* Tryptophan hydroxylase 1 and 5-HT7 receptor preferentially expressed in triple-negative breast cancer promote cancer progression through autocrine serotonin signaling. *Mol. Cancer* (2016). doi:10.1186/s12943-016-0559-6

88. Zhang, W. *et al.* Antiproliferative activities of the second-generation antipsychotic drug sertindole against breast cancers with a potential application for treatment of

breast-to-brain metastases. *Sci. Rep.* (2018). doi:10.1038/s41598-018-33740-0

89. Gwynne, W. D. *et al.* Serotonergic system antagonists target breast tumor initiating cells and synergize with chemotherapy to shrink human breast tumor xenografts. *Oncotarget* (2017). doi:10.18632/oncotarget.16646

90. William D. Gwynne1#, Mirza S. Shakeel1#, Adele Girgis-Gabardo1, Kwang H. Kim1, Anna Dvorkin-Gheva1, Methvin Isaac2, Rima Al-awar2, and J. A. H. Selective Antagonists of Serotonin Receptor 5A Target Human Breast Tumor Initiating Cells. (2019). (Unpublished),

91. Zhong, H., Sánchez, C. & Caron, M. G. Consideration of allosterism and interacting proteins in the physiological functions of the serotonin transporter. *Biochemical Pharmacology* (2012). doi:10.1016/j.bcp.2011.09.020

92. Liu, Y., Li, M., Warburton, R. R., Hill, N. S. & Fanburg, B. L. The 5-HT transporter transactivates the PDGFβ receptor in pulmonary artery smooth muscle cells. *FASEB J.* (2007). doi:10.1096/fj.06-8058com

93. Ren, W., Watts, S. W. & Fanburg, B. L. Serotonin transporter interacts with the PDGFβ receptor in PDGF-BB-induced signaling and mitogenesis in pulmonary artery smooth muscle cells. *Am. J. Physiol. Cell. Mol. Physiol.* (2011). doi:10.1152/ajplung.00237.2010

94. Tipton, K. F., Boyce, S., O'Sullivan, J., Davey, G. P. & Healy, J. Monoamine Oxidases: Certainties and Uncertainties. *Curr. Med. Chem.* (2004). doi:10.2174/0929867043364810

95. Lawrie, A. *et al.* Interdependent serotonin transporter and receptor pathways regulate S100A4/Mts1, a gene associated with pulmonary vascular disease. *Circ. Res.* (2005). doi:10.1161/01.RES.0000176025.57706.1e

96. Bianchi, P., Pimentel, D. R., Murphy, M. P., Colucci, W. S. & Parini, A. A new hypertrophic mechanism of serotonin in cardiac myocytes: receptor-independent

ROS generation. *FASEB J.* (2005). doi:10.1096/fj.04-2518fje

97. Rees, S. *et al.* Cloning and characterisation of the human 5-HT5A serotonin receptor. *FEBS Lett.* (1994). doi:10.1016/0014-5793(94)01209-1

98. Hannon, J. & Hoyer, D. Molecular biology of 5-HT receptors. in *Serotonin and Sleep: Molecular, Functional and Clinical Aspects* (2008). doi:10.1007/978-3-7643-8561-3_6

99. Matthes, H. *et al.* Mouse 5-hydroxytryptamine5A and 5-hydroxytryptamine5B receptors define a new family of serotonin receptors: cloning, functional expression, and chromosomal localization. *Mol. Pharmacol.* (1993).

100. Nelson, D. 5-HT5 Receptors. *Curr. Drug Target -CNS Neurol. Disord.* (2005). doi:10.2174/1568007043482606

101. Grailhe, R. *et al.* Increased exploratory activity and altered response to LSD in mice lacking the 5-HT(5A) receptor. *Neuron* (1999). doi:10.1016/S0896-6273(00)80712-6

102. Nikiforuk, A., Hołuj, M., Kos, T. & Popik, P. The effects of a 5-HT5A receptor antagonist in a ketamine-based rat model of cognitive dysfunction and the negative symptoms of schizophrenia. *Neuropharmacology* (2016). doi:10.1016/j.neuropharm.2016.01.035

103. Hemmings, B. A. & Restuccia, D. F. PI3K-PKB / Akt Pathway. *Cold Spring Harb. Perspect. Biol.* (2012). doi:10.1101/cshperspect.a011189

104. Yang, S. X., Polley, E. & Lipkowitz, S. New insights on PI3K/AKT pathway alterations and clinical outcomes in breast cancer. *Cancer Treatment Reviews* (2016). doi:10.1016/j.ctrv.2016.03.004

105. Thorpe, L. M., Yuzugullu, H. & Zhao, J. J. PI3K in cancer: Divergent roles of isoforms, modes of activation and therapeutic targeting. *Nature Reviews Cancer* (2015). doi:10.1038/nrc3860

106. Alessi, D. R. *et al.* Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol* (1997). doi:S0960-9822(06)00122-9 [pii]

107. Yang, G., Murashige, D. S., Humphrey, S. J. & James, D. E. A Positive Feedback Loop between Akt and mTORC2 via SIN1 Phosphorylation. *Cell Rep.* (2015). doi:10.1016/j.celrep.2015.07.016

108. Haar, E. Vander, Lee, S. il, Bandhakavi, S., Griffin, T. J. & Kim, D. H. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat. Cell Biol.* (2007). doi:10.1038/ncb1547

109. Holz, M. K. The role of S6K1 in ER-positive breast cancer. *Cell Cycle* (2012). doi:10.4161/cc.21194

110. Guertin, D. A. *et al.* Ablation in Mice of the mTORC Components raptor, rictor, or mLST8 Reveals that mTORC2 Is Required for Signaling to Akt-FOXO and PKCα, but Not S6K1. *Dev. Cell* (2006). doi:10.1016/j.devcel.2006.10.007

111. Song, M. S., Salmena, L. & Pandolfi, P. P. The functions and regulation of the PTEN tumour suppressor. *Nature Reviews Molecular Cell Biology* (2012). doi:10.1038/nrm3330

112. Mayer, I. A. & Arteaga, C. L. The PI3K/AKT Pathway as a Target for Cancer Treatment. *Annu. Rev. Med.* (2015). doi:10.1146/annurev-med-062913-051343

113. Sun, P., Lou, L. & Maurer, R. A. Regulation of activating transcription factor-1 and the cAMP response element-binding protein by Ca2+/calmodulin-dependent protein kinases type I, II, and IV. *J. Biol. Chem.* (1996). doi:10.1074/jbc.271.6.3066

114. Gross, S. M. & Rotwein, P. Akt signaling dynamics in individual cells. *J. Cell Sci.* (2015). doi:10.1242/jcs.168773

115. Gilley, J., Coffer, P. J. & Ham, J. FOXO transcription factors directly activate bim

gene expression and promote apoptosis in sympathetic neurons. *J. Cell Biol.* (2003). doi:10.1083/jcb.200303026

116. Lv, J., Wang, L., Gao, Y., Ding, Y.-Q. & Liu, F. 5-hydroxytryptamine synthesized in the aorta-gonad-mesonephros regulates hematopoietic stem and progenitor cell survival. *J. Exp. Med.* (2017). doi:10.1084/jem.20150906

117. Gingras, A. C. *et al.* Regulation of 4E-BP1 phosphorylation: A novel two step mechanism. *Genes Dev.* (1999). doi:10.1101/gad.13.11.1422

118. Yanagiya, A. *et al.* Translational Homeostasis via the mRNA Cap-Binding Protein, eIF4E. *Mol. Cell* (2012). doi:10.1016/j.molcel.2012.04.004

119. Mader, S., Lee, H., Pause, A. & Sonenberg, N. The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. *Mol. Cell. Biol.* (2015). doi:10.1128/mcb.15.9.4990

120. Zhang, X. *et al.* FOXO1 is an essential regulator of pluripotency in human embryonic stem cells. *Nat. Cell Biol.* (2011). doi:10.1038/ncb2293

121. Gargini, R., Cerliani, J. P., Escoll, M., Antõn, I. M. & Wandosell, F. Cancer stem cell-like phenotype and survival are coordinately regulated by Akt/FoxO/bim pathway. *Stem Cells* (2015). doi:10.1002/stem.1904

122. Malkomes, P. *et al.* Selective AKT Inhibition by MK-2206 Represses Colorectal Cancer-Initiating Stem Cells. *Ann. Surg. Oncol.* (2016). doi:10.1245/s10434-016-5218-z

123. Zhang, X.-L., Jia, Q., Lv, L., Deng, T. & Gao, J. Tumorspheres Derived from HCC Cells are Enriched with Cancer Stem Cell-like Cells and Present High Chemoresistance Dependent on the Akt Pathway. *Anticancer. Agents Med. Chem.* (2015).

124. Wu, G. *et al.* Somatic mutation and gain of copy number of PIK3CA in human

breast cancer. *Breast Cancer Res.* (2005). doi:10.1186/bcr1262

125. Chen, I. C. *et al.* Phosphatidylinositol-3 Kinase Inhibitors, Buparlisib and Alpelisib, Sensitize Estrogen Receptor-positive Breast Cancer Cells to Tamoxifen. *Sci. Rep.* (2017). doi:10.1038/s41598-017-10555-z

126. Hirai, H. *et al.* MK-2206, an Allosteric Akt Inhibitor, Enhances Antitumor Efficacy by Standard Chemotherapeutic Agents or Molecular Targeted Drugs In vitro and In vivo. *Mol. Cancer Ther.* (2010). doi:10.1158/1535-7163.mct-09-1012

127. Sangai, T. *et al.* Biomarkers of rsponse to Akt inhibitor MK-2206 in breast cancer. *Clin. Cancer Res.* (2012). doi:10.1158/1078-0432.CCR-12-1141

128. Sun, S. Y. *et al.* Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res.* (2005). doi:10.1158/0008-5472.CAN-05-0917

129. Mi, W., Ye, Q., Liu, S. & She, Q.-B. AKT inhibition overcomes rapamycin resistance by enhancing the repressive function of PRAS40 on mTORC1/4E-BP1 axis. *Oncotarget* (2015). doi:10.18632/oncotarget.3920

130. Chen, J., Crawford, R. & Xiao, Y. Vertical inhibition of the PI3K/Akt/mTOR pathway for the treatment of osteoarthritis. *Journal of Cellular Biochemistry* (2013). doi:10.1002/jcb.24362

TABLES & FIGURES

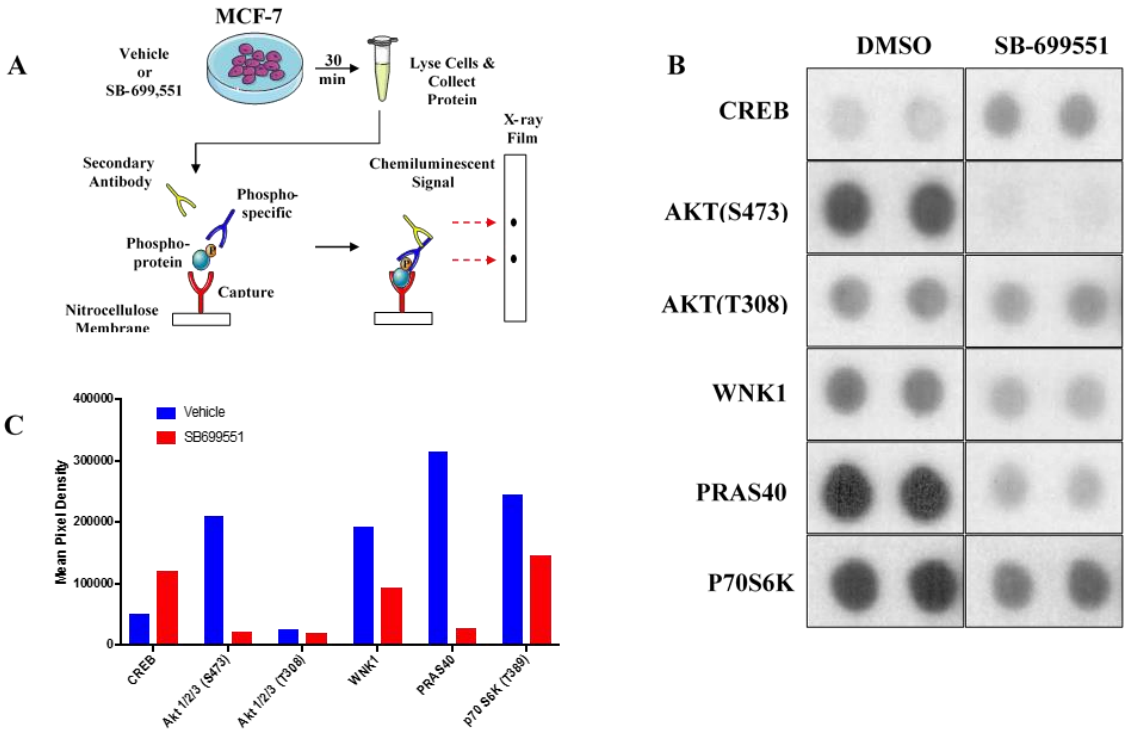

Figure 1. HTR5A signals via canonical G_i coupling and through the Akt/PRAS40/mTOR axes. (A) MCF7 cells were incubated with DMSO (vehicle; 0.1%) or SB-699551 (IC_{90}) for 30 minutes. Total extracts were collected and used in the Phospho-Proteomic Array. **(B)** Phospho-proteins with the greatest change in pixel density in response to SB-699551 treatment are shown. **(C)** Graph shows the pixel density values of phosphoproteins depicted in (B) for the vehicle and SB-699551 treatment. SB-699551 treatment results in an increase in CREB phosphorylation and a reduction in phosphorylation of proteins involved in the PI3K/mTOR pathways.

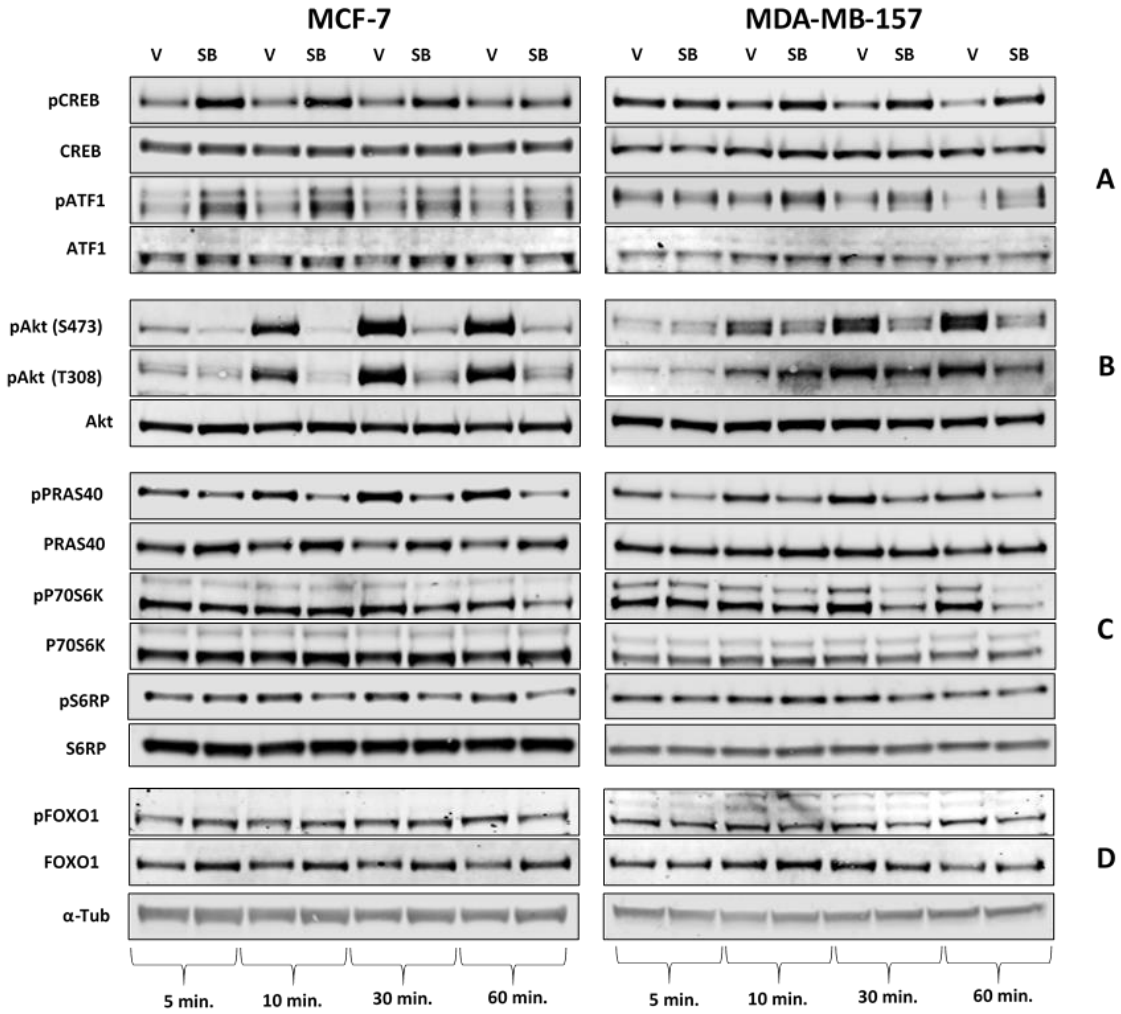

Figure 2. SB-699551 inhibits AKT/mTOR signaling pathways downstream of the serotonin receptor. MCF7 and MDA-MB-157 cells were incubated with the vehicle (DMSO) or SB-699551 (IC_{90}) for the indicated time intervals. Total extracts were subjected to SDS-PAGE followed by immunoblotting with antibodies against indicated proteins. **(A)** SB-699551 increased phosphorylation of CREB and ATF1 in both cell lines. **(B)** SB-699551 reduced phosphorylation of AKT, and downstream proteins **(C)** PRAS40, P70S6K, and **(D)** FOXO1.

Table 4. Quantifying differences in abundance of intracellular phosphoproteins after treatment with SB-699551.

Phospho-protein	MCF-7				MDA-MB-157			
	5	10	30	60	5	10	30	60
pCREB	1.6	2.1	1.8	1.4	1.5	1.3	1.8	2.0
pATF1	1.5	2.1	1.5	1.5	1.2	1.7	2.0	1.7
pAkt(S473)	6.7	24.1	8.9	6.2	1.5	2.2	2.9	1.6
pAkt(T308)	3.8	12.3	5.7	3.2	1.3	2.4	1.6	3.2
pPRAS40	1.5	3.8	3.8	2.8	1.4	2.4	2.3	2.0
pP70S6K	1.2	1.5	1.8	2.5	1.1	1.6	3.4	4.3
pS6RP	1.1	1.3	1.1	1.6	1.2	1.1	1.2	1.0
pFOXO1	1.7	1.1	1.5	2.0	1.0	2.0	1.1	2.2

The mean pixel density of each band was normalized to its respective loading control. Phosphoprotein abundance was determined by normalizing to the total protein. The fold increase (blue) or decrease (red) in phosphoprotein abundance after treatment with SB-699551 at different time intervals.

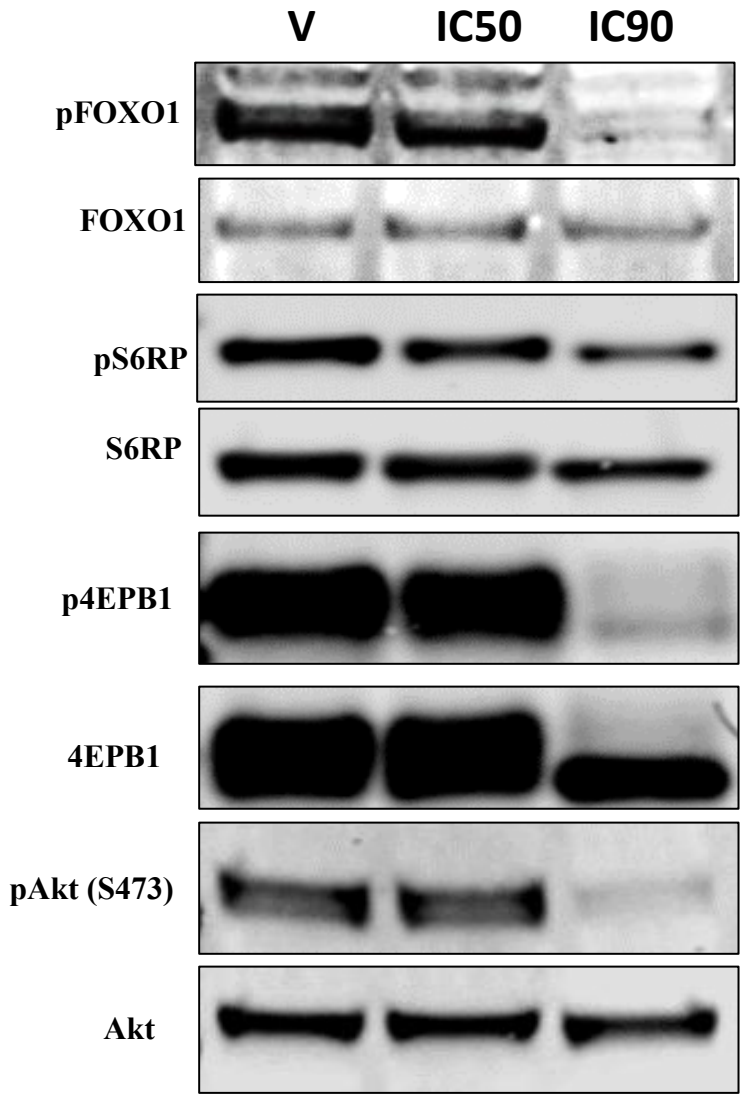

Figure 3. SB-699551 inhibits cell growth by reducing phosphorylation S6RP, FOXO, and 4EBP1. MDA-MB-157 cells were incubated with the vehicle (DMSO) or SB-699551 (IC_{50} & IC_{90}) for 24h. Total extracts were subjected to SDS-PAGE followed by immunoblotting with antibodies against indicated proteins. Reduction in phosphorylation of AKT, S6RP, 4EBP1 and FOXO1 was detected in response to SB-699551.

Table 5. Quantifying differences in abundance of intracellular phosphoproteins after treatment with SB-699551 for 24h.

Phospho-protein	MDA-MB-157	
	IC50	IC90
pS6RP	1.3	1.7
pFOXO1	1.3	9.3
p4EBP1	1.2	4.4
pAkt(S473)	1.3	7.6

The mean pixel density of phosphoprotein and total protein bands was calculated using image studio and normalized to their respective α-tubulin loading controls. Relative phosphoprotein abundance was determined by normalizing phosphoprotein pixel density to that of the total protein. The fold decrease (red) in phosphoprotein abundance after treatment with SB-699551 at various time intervals is shown.

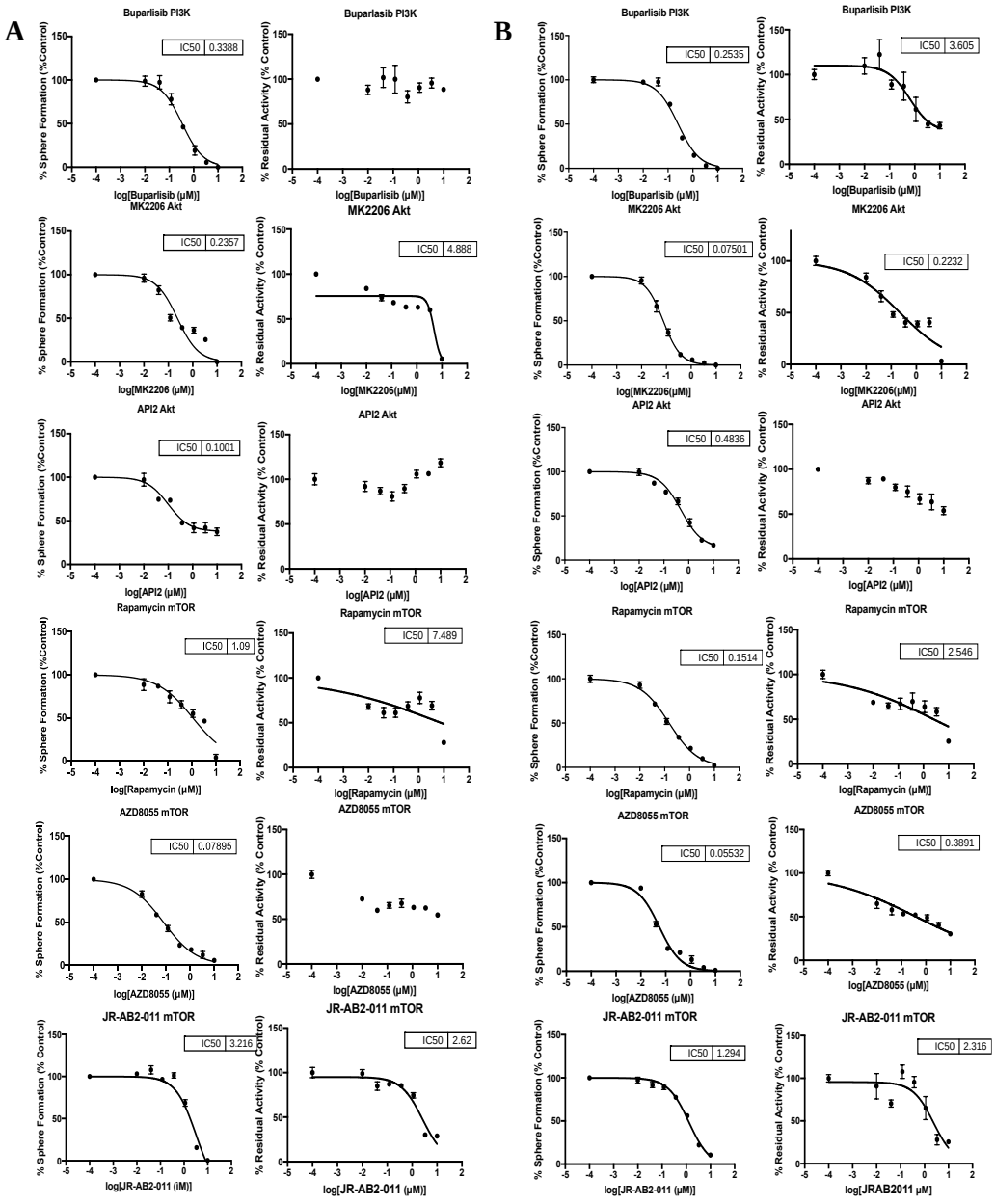

Figure 4. PI3K/Akt/mTOR pathway inhibitors phenocopy the effects of the SB-699551. Dispersed cells from MDA-MB-157 (A) and MCF-7 (B) tumorspheres were suspended in serum-free, chemically-defined media and the effect of serial dilutions of the various compounds assayed for their capacity to reduce sphere-formation. Inhibitors of AKT, API2 and MK2206, as well as PI3K inhibitor, Buparlisib, reduced sphere-formation in dose-dependent fashion. Similarly, mTOR inhibitors, rapamycin, AZD8055, and JRAB2011 also reduce sphere-formation.

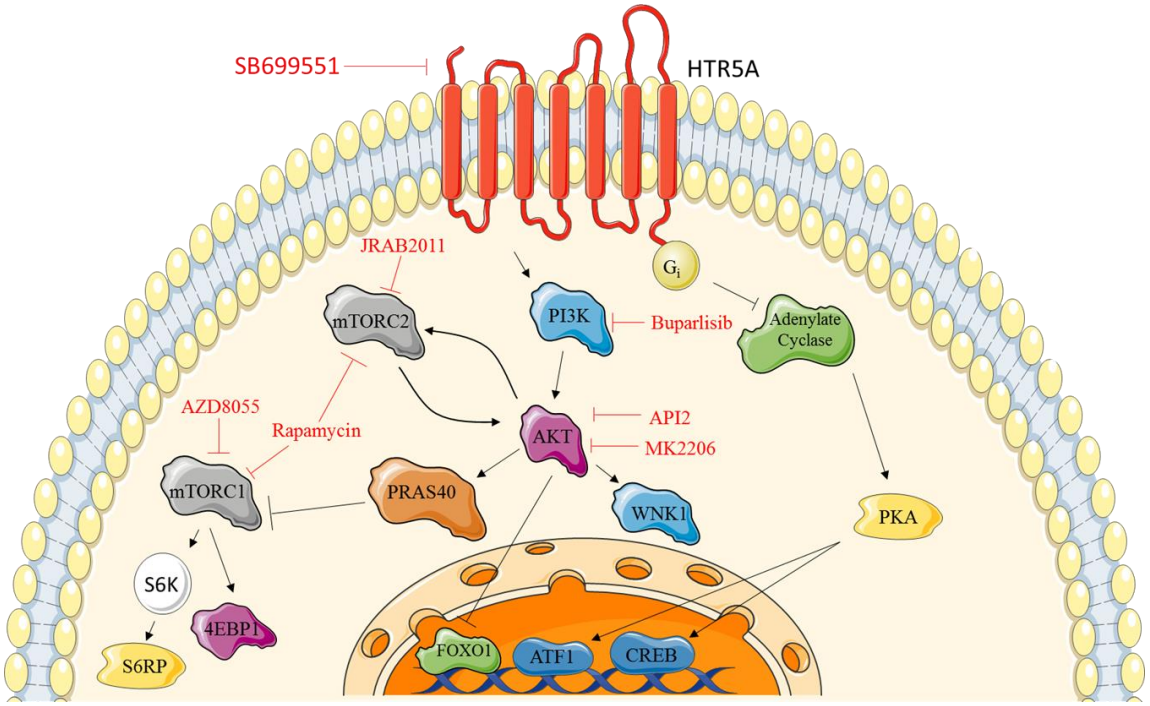

Figure 5. SB-699551 antagonist inhibits HTR5A and affects downstream canonical G-protein signaling and signaling via the PI3K/AKT/mTOR pathways. Inhibition of HTR5A G_i protein by SB-699551 antagonist expectedly increase adenylate cyclase, which generates cAMP and stimulates PKA inducing CREB and ATF1 phosphorylation. Moreover, SB-699551 reduces activity of PI3K/Akt/mTOR pathway proteins such as AKT, PRAS40, FOXO1, and S6RP. Inhibition of several proteins in the PI3K/Akt/mTOR pathways with selective antagonists phenocopy the effects of SB-699551 treatment. These findings suggest that HTR5A signals through the PI3K/Akt/mTOR pathways.

APPENDIX

Summary of accomplishments:

Presentations:

May 13, 2019	Poster presentation at the 2019 Faculty of Health Science Seminar
Dec 4, 2018	Participated in the biochemistry departmental seminar with Hassell lab
Nov 12-14, 2018	Poster presentation at the 2018 Till & McCulloch meeting

Awards:

May 2019	Poster presentation at the Faculty of Health Science Research Plenary
November 2018	Travel Award from Ontario Institute for Regenerative Medicine
	Poster presentation at the Stem Cell Network conference
2017-2018	Ontario Graduate Fellowship

Publications:

Co-first author

William D. Gwynne#, **Mirza S. Shakeel#**, Adele Girgis-Gabardo, Kwang H. Kim, Anna Dvorkin-Gheva, Methvin Isaac, Rima Al-awar, and J. A. H. *Selective Antagonists of Serotonin Receptor 5A Target Human Breast Tumor Initiating Cells*. (2019). (Unpublished)

Second author

William D. Gwynne, **Mirza S. Shakeel**, Jianhan Wu, Robin M. Hallett, Adele Girgis-Gabardo, Anna Dvorkin-Gheva, and J. A. H. Monoamine oxidase A expression and activity are associated with clonal tumorsphere-formation by human breast tumor cells. *Cell. Mol. Biol. Lett. (under Rev.* (2019).