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Serotonin Signals are Essential for the Survival of Breast Cancer Cells

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14. ABSTRACT

Our hypothesis addresses the idea that serotonin is important for the growth/survival of breast cancer cells. We tested 5 different types of breast cancer cell lines and have shown that the transcripts for both synthesizing and responding to serotonin signals are present in every type of cell, but each exhibits its own unique profile of expression. We removed serotonin from the cell culture medium and found that the cells transcriptionally responded to the presence or absence of exogenous serotonin through a feedback pathway.

We found that several 5-HT receptor antagonists caused a loss of viability in breast cancer cells. The most potent inhibition was obtained by the use of a selective 5-HT_{1B} antagonist. The observed inhibition was significantly better than Tamoxifen. Mechanistically, we have shown that blocking the 1B receptor resulted in a loss of the Akt activity, leading to apoptosis. In addition, we found that highly proliferative cancer cells are more sensitive to 5-HT_{1B} antagonist-induced cell death compared to resting normal cells. Normal MCF-10A cells grown in three dimensional basement membrane assays undergo minimal apoptosis following 5-HT_{1B} antagonist treatment. In contrast, highly proliferative MCF-10A cells overexpressing the mutant form of ErbB2 are extremely sensitive to 5-HT_{1B} antagonist-induced apoptosis.

Taken together we believe that these data suggest that the 5-HT_{1B} receptor plays a crucial role in the survival of breast cancer cells.

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INTRODUCTION

It was known since the late 1800's that the sera from blood contains a vasoconstrictive activity (*1*). By 1948, the activity was purified and named serotonin for its ability to activate smooth muscle cells to produce a tonic response (2). It was later shown that it was also a neurotransmitter (*3*). Serotonin's role as a neurotransmitter sparked a wide-spread interest among scientists. Two years later, Benditt (*4*) reported that chlorpromazine, the first widely used psychiatric drug, acted as a 5-HT receptor antagonist. The commercial success of chlorpromazine prompted the scientific community to exploit serotonin's role as a neurotransmitter, as can be seen by the vast majority of publications that followed this discovery. Consequently, there has been very little research concerning its role as an activator of cellular pathways. One generally considers serotonin to be a neurotransmitter; it is easily overlooked that only 5% of the body's serotonin resides within the central nervous system (CNS). The vast majority of the body's 5-HT is found within the gastrointestinal tract and major organs of the body. From an evolutionary perspective, serotonin (or serotonin-like molecules) is ancient; it is present in plants and fungi where it provides signals for wound healing and growth (*5*). In fact, serotonin's major role appears to be as a cellular activator and as an agent of differentiation in development within a wide variety of species, ranging from drosophila to humans. In this concept award, we have pursued the hypothesis that serotonin contributes to the growth and survival signals in breast cancer cells.

Serotonin Signals are Essential for the Survival of Breast Cancer Cells

Our statement of work involved two tasks. The first was to establish that serotonin is an autocrine growth factor in breast cancer tumors. Tryptophan Hydroxylase 1 (TPH1) is the rate-limiting enzyme responsible for synthesizing serotonin from tryptophan. This enzyme is expressed in breast cancer cells and gives rise to a constitutive background of serotonin. As a part of our proposed task 1, we intended to use shRNA constructs to knockdown TPH1. We purchased a variety of shRNAs targeting TPH1 from OPEN Biosystems. We diligently worked on the transfection of the MCF-7 cells (in consultation with OPEN Biosystems). We were having difficulty getting a knockdown with our (validated) positive control targeting GAPDH. After nine months, OPEN Biosystems informed us that the only way to get the shRNAs into the MCF-7 cells was to transduce them with a Lentiviral vector (which we are currently doing). Thus, we were unable to accomplish this part of the task. We were able to make good progress with the rest of this task. The other major task outlined in our statement of work was to determine whether the inhibition of serotonergic receptor signals in breast cancer cells cause apoptosis. We made substantial progress on this task. The results are outlined below. The data show that serotonin, or at least signals from its cognate receptors, are essential for the growth of breast cancer cells.

Breast Cancer Cell lines Used:
As a control, we used mRNA harvested from normal healthy breast cells (purchased from Applied Biosystems). The cells used for this final report are:

- **MCF-10A** –estrogen receptor-positive (ER-positive), non-tumorogenic, immortalized epithelial cells
- **NeuT** –MCF-10A cells that have been engineered to over express the ErbB-2 receptor and are aggressively tumorogenic.
- **MCF-7** –estrogen receptor positive , weakly tumorogenic, epithelial cells derived from the pleural effusion of an adenocarcinoma
- **BT-483**-estrogen receptor positive, tumorogenic, derived from a solid invasive tumor
- **BT-20**-estrogen receptor negative (ER-negative), tumorogenic, derived from a solid invasive tumor
- **MDA MB 231**-estrogen receptor negative, minimal Her2/neu expression, very tumorogenic, derived from an adenocarcinoma
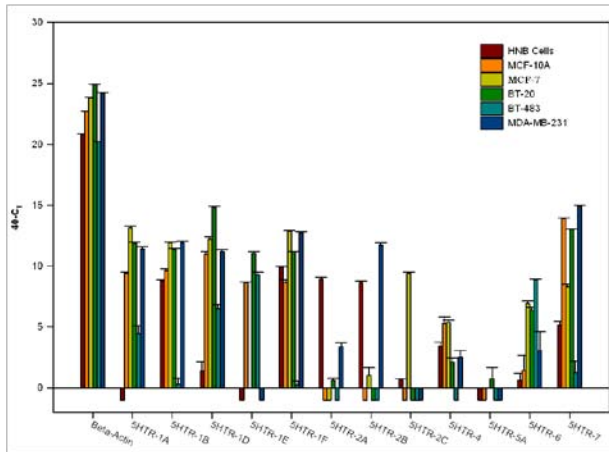

Figure1. *Normal and breast cancer cell lines express transcripts for the serotonin receptors.* Shown above are the relative expression levels for the transcripts amplified by the designated TaqMan probes. Samples were run in triplicate. A 40 cycle run for the qPCR study was used in acquiring these data. The data is expressed as the total number of cycles minus the cycle number at which the signal was observed ($40-C_T$), see methods for details. Probes that did not have a transcript were attributed an arbitrary value of 41, so they are plotted at -1 to avoid confusion.

The serotonergic pathway is present in breast cancer cells.

We choose to use clonal breast cancer cell lines that have very different origins and are well-characterized. The cell lines used in this study represent a variety of breast cancer phenotypes/genotypes; some are estrogen receptor positive and some are estrogen receptor negative and they range
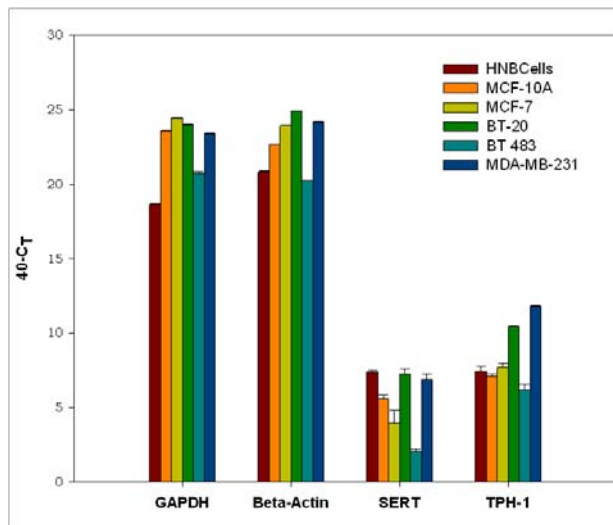
Figure 2. *Normal and breast cancer cell lines express transcripts to transport and synthesize serotonin*. Shown above are the relative expression levels for the transcripts amplified by the designated TaqMan probes. A 40 cycle run for the qPCR study was used in acquiring these data. The data is expressed as the cycle number at which the signal was observed minus the total number of cycles ($40-C_T$).

from non-tumorigenic to aggressively tumorigenic. The data obtained with the cell lines were compared with the commercially available mRNA harvested from healthy, normal breast cells [HNBC].

To characterize the presence or absence of the serotonergic 'machinery' in these various cells, we probed the cells for the expression of the relevant mRNAs (Figure 1). The real time PCR study showed that the pattern of expressed receptors was unique to each of the cells. All of the cells expressed the 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_6, and 5-HT_7 receptors transcripts. In reviewing the profile of the various transcripts, we were unable to find a distinctive expression pattern for the tumorigenic cells. We currently do not have enough information to adequately interpret the significance of the receptor expression patterns. It should also be noted that there is cross-talk among the receptors when they are coupled to signal transduction pathways (*6*), meaning that the overall pattern of expressed proteins may be more important than the focus on any individual receptor.
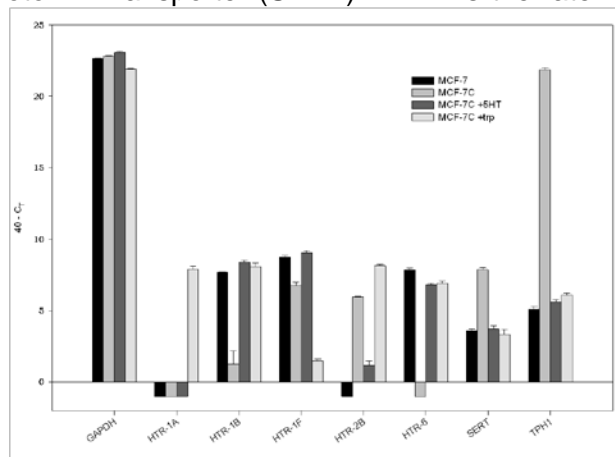
In addition to probing for the presence of the 5-HT receptors, we also screened the mRNA pool for transcripts corresponding to Tryptophan Hydroxylase 1 (TPH1) and the Serotonin Transporter (SERT). TPH1 is the rate-limiting enzyme responsible for synthesizing serotonin from tryptophan and SERT allows for the cell's uptake of 5-HT from the local environment. All of the cells probed had mRNAs encoding these two proteins (Figure 2); thus, all of these cells possess the basic elements required to produce and process serotonergic signals.

On average, the sera used to supplement the tissue culture media contains about 2μM serotonin (*7*); which is enough serotonin to saturate most of the 5-HT receptors. We cultured MCF-7 cells in the presence or absence of exogenous serotonin in order to ascertain whether this would affect the transcriptional levels of the pathway components. Cells grown in the absence of exogenous serotonin were cultured in media containing charcoal-dextran filtered sera for at least 4 passages; these cells have been referred to as MCF-7C ('C' for charcoal). Charcoal-dextran filtering of the sera effectively removes the 5-HT, but also filters out other small molecules. To ensure that any of the observed effects were exclusively due the absence of 5-HT, we added serotonin or its precursor,

Figure3. *Breast cancer cell line, MCF-7 exhibits a feedback mechanism in response to tryptophan and serotonin.*
MCF-7 were grown with (MCF-7) or without (MCF-7C) serotonin for 4 passages then supplemented with either serotonin (125µM) or tryptophan (125µM) for 4 hours before being processed to evaluate the mRNA levels of the targets. Each samples were run in triplicate, the graph represent the relative expression of the samples run for 40 cycles. Each signal is represented by 40 minus the threshold cycle. Probes that did not have a transcript were attributed an arbitrary value of 41.

tryptophan to the media for 4 hours prior to harvesting the RNA for analysis (Figure 3). Adding back serotonin, or its metabolic precursor, restored the transcription back to the original baseline levels showing that the cells have a 'feed-back' system triggered by serotonin.

When the charcoal-filtered serum was used to replace the normal media, all of the various breast cancer cell lines responded by increasing their transcription level of TPH1 and/or SERT; this may reflect the dependence of the cells on serotonin. The *notable* exception to this observation was the BT-20 cells; which uniformly died. If a source of serotonin was constantly provided as a supplement at the same time as the charcoal-filtered media, the cells could be rescued for several passages (we are currently following up this observation).

The role of 5-HT receptors in cell growth.

Table 1

	MCF-10A	MCF-7	BT-483	BT-20	MDA-MB-231
Er	-	+	+	-	-
ErbB2	-	-	-	-	-
SB-216641 (5HT$_{1B}$)	9.6	6.75	8.55	10.8	3.81
BRL 15572 (5HT$_{1D}$)	>40	>40	>40	>40	>40
SB258585 (5HT$_6$)	>40	>40	>40	>40	>40
SB269970 (5HT$_7$)	>40	>40	>40	>40	>40
tamoxifen (N/A)	13.51	35.22	N/A	18.14	13.8

Having established that breast cancer cells have serotonergic receptors, we studied the effects of selectively antagonizing individual receptors. For these studies, we obtained a panel of selective inhibitors for a number of 5-HT receptors .We have used this panel of antagonists in a viability assay to screen the various breast cancer cell lines (Table 1). We show representative data of the MTS assays of two different cell lines; the ER-positive MCF-7 cells and the ER-negative MDA-MB-231 cells using the results of the most significant drugs used (Figure 4). Of all the drugs tested, the best inhibition for all of the cell li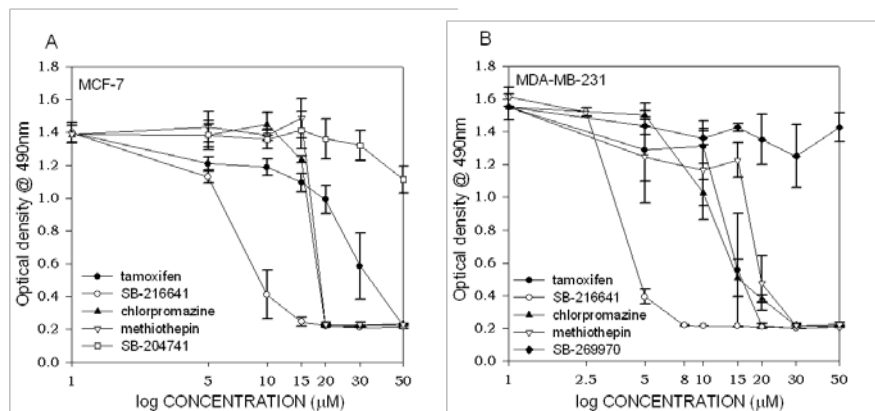nes was observed with the selective 1B antagonist, SB-216641. Methiothepin (a general type 1, type 6, and type 7 receptor inhibitor) was used to show that generalized inhibition of the 1B receptor also resulted in the loss of cellular viability, in accordance with its relative affinity for the 1B receptor. We have also used another chemically different, highly selective 1B inhibitor, SB-224289, with similar results; to show that it is not an unexpected result of a single compound (Figure 5). We have screened all the breast cancer cell lines listed for the effect of inhibiting the 5-HT$_{1B}$ receptor signals. All of the cells were inhibited
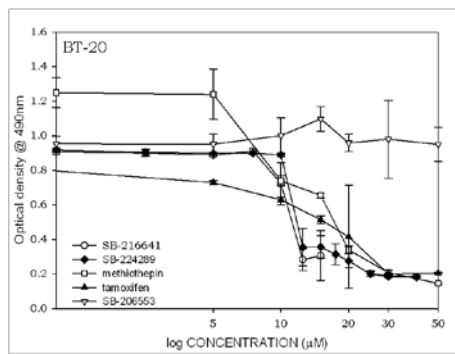

Figure4. *Inhibition of the serotonin receptors can affect breast cancer cell viability.*

Cells were incubated for 72h with respective drugs. Cell viability was assessed by an MTS assay where higher OD represents metabolically active cells. Cell lines (A) MCF-7, (B) MDA-MB-231. Drugs used: the solid circles refer to Tamoxifen; the open circles refer to SB-216641, the selective 5HT$_{1B}$ inhibitor; the solid triangles refer to chlorpromazine, an FDA approved serotonin/dopamine receptor inhibitor; the open inverted triangles refer to Methiothepin, a general 5HT$_{1,2,6,7}$ inhibitor; the open squares refer to SB-204741, a selective 5HT$_{2B}$ inhibitor; the solid diamonds refer to SB-269970, a selective 5HT$_7$ inhibitor.

and showed an IC$_{50}$ for the SB-216641 between 5-10 μM. The data for the SB-216641 is considerably better than Tamoxifen, under our assay conditions (Table 1).

The type 7 receptor is expressed by all of the different cell lines but is not known to affect viability (*6*); its inhibition with SB-269970 has no effect on the growth/viability of the cells (figure 4*B*). As an internal control, we tested, on MCF-7 cells, a 5-HT$_{2B}$ selective antagonist, SB-204741, which did not express a transcript encoding this receptor (figure 4*A*); we showed

Figure 5. *Inhibition of the serotonin receptors can affect breast cancer cell viability.*
BT-20 Cells were seeded 24h prior to treatment, and then incubated for 72h with drugs. Cell viability was assessed by measuring the reduction of MTS by metabolically active cells. Drug used : the open circles refer to SB-216641, a selective $5HT_{1B}$ inhibitor; the closed diamonds refer to SB-224289, a specific inhibitor of $5HT_{1B}$; the open squares refer to Methiothepin, a general $5HT_{1,2,6,7}$ inhibitor; the solid triangles refer to Tamoxifen; the open inverted triangles refer to SB-206553, a selective $5HT_{2B,2C}$ inhibitor.
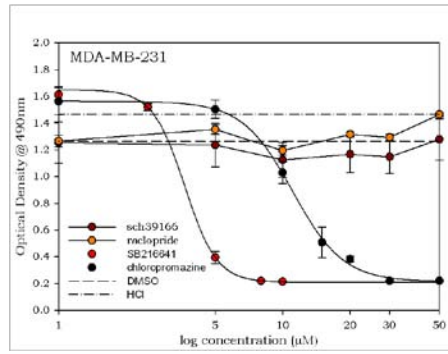

Figure6. *Chlorpromazine effect is exclusively due to inhibition through the serotonin receptors.*
MDA-MB-231 Cells were seeded 24h prior to treatment, and then incubated for 72h with drugs. Cell viability was assessed by measuring the reduction of the MTS compound into a colored formazan product by metabolically active cells. The sch39166 is a potent inhibitor of the dopaminergic receptors D1, D2, D4, and D5; while the raclopride is a potent and selective inhibitor of D2/D3 (thus all of the dopaminergic receptors were screened between these two inhibitors).

that it does not inhibit growth in those cells. We also used an FDA-approved drug, with a well-established safety record, Chlorpromazine [commonly known as Thorazine]. It belongs to a class of drugs known as phenothiazines and is classified as a serotonin-dopamine inhibitor. The chlorpromazine inhibited the cells and specificity was validated by using dopaminergic receptor inhibitors, which had no effect on the growth of the cells (Figure 6). We can then say that the inhibition is solely due to inhibition of the serotonin receptors. Thus, reducing signals from serotonin, specifically through the 5-HT_{1B} receptor significantly reduces viability of breast cancer cells.

We were unable to test the role of 5-HT_{1E}, 5-HT_{1F}, and 5-HT_5; since there are no selective inhibitors available for these receptors.

The 5-HT_{1B} receptor antagonist blocks phosphorylation of protein kinase B (Akt-1)

The data with respect to the 5-HT_{1B} receptor indicates that its inhibition results in a loss of cell viability. As a preliminary means of addressing the u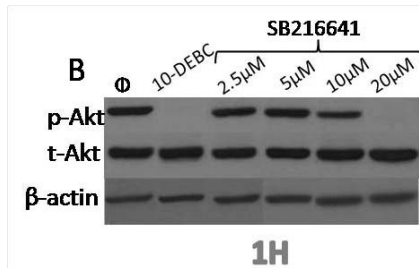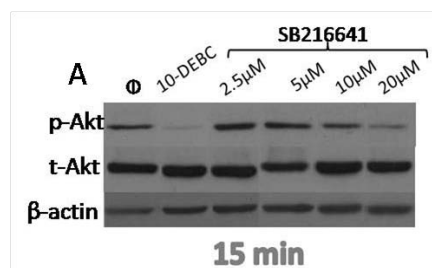nderlying mechanism, we explored the 1B receptor's signal transduction pathway. In addition to its inhibitory effects on adenyl cyclase, the 5-HT_{1B} receptor is also known to transduce signals through the Akt 1 (protein kinase B) pathway (*8, 9*). There is an increasing amount of evidence suggesting that deregulation of the Akt pathway is involved in the maintenance/survival of malignant cells

Figure7. *Inhibition of $5HT_{1B}$ leads to apoptosis through the Akt1 pathway.*
Cells were seeded 24h prior to treatment and incubated for 15min (A) or 1h (B) with vehicle (⊘), 20μM 10-DEBC (a selective inhibitor of Akt), 2.5, 5, 10 or 20μM SB-216641 a specific $5HT_{1B}$ inhibitor, whole cell extracts were probes on a nitrocellulose membrane for phospho-Akt1 (ser473), total Akt (pan-Akt) and β-actin was used as endogenous control.
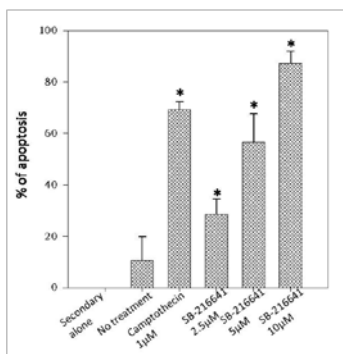
Figure 8. *Breast cancer cells undergo apoptosis when the 5HT$_{1B}$ is inhibited.* MDA-MB-231 Cells were seeded 24h prior to treatment; cells were then treated for another 24h before being processed. For each sample 3 pictures were taken, 50 dapi stained nucleus were counted randomly on each picture, then, counted the double positives (DAPI and cleaved-caspase3 positive). * p<0.001

including protecting them from apoptosis (*10*). It has been seen that breast cancer progression *in vivo*, often depends upon the constitutive activation of Akt1 (*11*). We used Western Blot analysis to study the effects of SB-216641 on Akt activity in MDA-MB-231 cells. Exponentially growing cells were plated in the presence of SB-216641. Results show that SB-216641 inhibits the phosphorylation within 15 min (figure 7A); after 1 hour of treatment, 20µM of SB-216641 was able to completely inhibit activation of Akt, similarly to our positive control, 10-DEBC, a specific p-Akt1 inhibitor (figure 7B). In order to confirm that the loss of the phospho-Akt correlates with apoptosis, we treated MDA-MB-231 cells with SB-216641 and stained the cells for the presence of activated caspase 3. Camptothecin, a topoisomerase I inhibitor, was used as a positive control for this study. This assay was quantitated by measuring (counting) DAPI stained nuclei versus activated Caspase 3 staining (Figure 8). .

Effect of 5-HT$_{1B}$ receptor inhibition on the 3 dimensional growth of breast cells

Our data indicate that the 1B receptor provides a survival signal for breast cancer cells grown in a traditional 2-dimensional (2D) cell culture system. The loss of growth corresponded with an immediate loss of the phospho-Akt1 signal followed by the activation of caspase-3, inferring that the loss of the 1B signal results in apoptosis. To test whether the 1B-induced apoptosis affects resting cells, we tested MCF-10A in 3D conditions where they are known to growth arrest after forming acini.
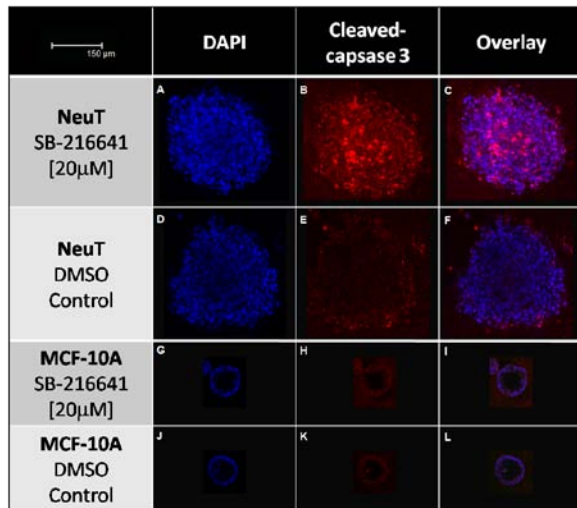

Figure 9. *The 5-HT$_{1B}$ receptor inhibitor (SB-216641) induces apoptosis in tumorigenic cells.* The confocal pictures of the 3D cell culture shown here have been stained with an anti-cleaved Caspase 3 antibody (red; B,E,H,K) or a nuclear DAPI stain (blue; A,D,G,J). Panel A-C are NeuT cells (MCF-10A overexpressing ErbB2) that have been treated with 20µM SB-216641. Panel D-F are NeuT cells that have been treated with DMSO as a vehicle control. Panels G-I are regular MCF-10A treated with SB-216641, panels J-L are MCF-10A vehicle control.

Mammary epithelial cells naturally form a 3D structure known as an acinus. It is a berry-shaped, hollow gland into which milk is secreted and is created by a single continuous layer of epithelial cells. *In vivo*, this structure is encased by a layer of myoepithelial cells and a basement membrane composed of extracellular matrix (ECM) proteins that provide structural support and functional information. This acinar architecture can be mimicked *in vitro* by culturing mammary epithelial cells completely surrounded by exogenous matrix (Matrigel). Epithelial cells grown in 3D cultures form these acinus-like spheroids with a hollow lumen and establish basal apical polarity (*12*). Using this system, we studied the induction of apoptosis in MCF-10A cells (which form normal acini) and NeuT cells (which have an uncontrolled, invasive growth pattern). The NeuT cells are MCF-10A cells that have been engineered to overexpress a mutant, constitutively active form of the ErbB2 receptor and emulate an aggressive breast cancer tumor (based on the constructs described by Hulit et al. (*13*)). When grown in Matrigel, the MCF-10A cells form acini via a hollowing process of the

lumen; known as anoikis. The transformed NeuT cells do not respond to the normal signals for apoptosis and, consequently, form a large, amorphous mass. Both cells were treated with SB-216641 and were screened for apoptotic death with an antibody recognizing activated Caspase 3. The MCF-10A cells were placed in 3D culture for 14 days, allowed to form growth arrested acini-like structures and then treated with 20 μM SB-216641 for 48 hours. MCF-10A acini showed no significant difference when compared with the vehicle controls, with almost no detection of apoptosis. In contrast, the same treatment with the NeuT cells resulted in significant induction of apoptosis as judged by the heavy, punctuate staining with the anti-cleaved Caspase 3 antibody (Figure 9). This experiment shows that in a 3D cell culture model, normal cells remain unaffected after treatment with a 1B receptor inhibitor while the same concentration of the drug induces apoptosis in oncogenic-expressing acini.

Materials & Methods

Cell lines
Cell culture media were obtained from Invitrogen (Carlsbad, Ca) MCF-7, BT-20, BT-483, MDA-MB-231 and MCF-10A (all from ATCC, Manassas, VA) were maintained following ATCC's recommendations. Except MDA-MB-231: DMEM + 10% FBS + 1% Pen/Strep; and MCF-10A: DMEM-F12 + 5% Horse serum + 0.02%EGF + 0.05% hydrocortisone + 0.01% cholera toxin + 0.1% insulin + 1% Pen/Strep.
All the serotonin antagonists as well as the p-Akt1 inhibitor 10-DEBC, and the dopamine inhibitors were obtained from Tocris (Ellisville, Mu). The serotonin, tryptophan, phenylalanine and tamoxifen were obtained from sigma-Aldrich (St. Louis, MO)

Charcoal filtration
50mL of FBS was incubated with 1g of activated charcoal over-night; the charcoal was then separated by 2 centrifugations of 20min at $2x10^3$g. The culture medium was prepared as usual using charcoal filtered sera (*14*). Because charcoal filtration also removes small charged molecules, MCF-7 had to be supplemented with estradiol.

Western blot
Whole cell extracts were prepared using RIPA buffer supplemented with phosphatase inhibitor. The electrophoresis was run using a 10% SDS-PAGE then transferred onto a nitrocellulose membrane. The membrane was blocked in TBST+5%milk. The membrane was incubated with primary antibody in TBST+5%BSA over night at 4°C (p-Akt 1:800, pan-Akt 1:1000, β-actin 1:5000 all obtained from Cell Signaling Danvers, MA). The secondary antibodies (Cell Signaling, Danvers, MA) (1:5000) were diluted in TBST+5%BSA. The detection was performed using ECL technique using HyGlo reagent (Denville scientific, South Plainfield NJ), HyBlot films (Denville scientific, South Plainfield NJ) and the Kodac developer M35A X-OMAT processor.

Real-Time PCR
Commercially available human normal breast cells mRNA (HNBcells) was used as the reference sample (Ambion, Austin, TX). The RNA from the cells was extracted using the *RNAqueous kit* (Ambion, Austin, TX). This step was followed by the synthesis of cDNA using *Verso cDNA* kit (Abgene, Epsom, Surrey, UK). The final profile was performed using validated TaqMan probes (Applied Biosystems, Foster City, CA) and Perfecta mix (Fisher scientific Pittsburgh, PA) and using the ABI7500 instrument (Applied biosystems, Foster City, CA) for 40 cycles. Relative quantification of real time PCR data is usually performed using the delta-delta C_T method described by Livak & Schmittgen (*15*). This analysis technique works well as long as a signal is obtained in the PCR data collection. If no signal is obtained within 40 cycles, we arbitrarily assigned that sample a C_T value of 41 (one cannot reasonably use zero). For relative comparisons, we are using the cycle number at which a signal is observed (C_T) and subtracting that from the total number of cycles (40-C_T). With this calculation, a higher number indicates a higher copy number. If no signal is observed the final value will be a -1.

Cell Viability Assay

The viability assay was performed in 96well plates, flat bottom, using 10^4 cells per well for MCF-10A and MDA-MB-231 and 1.5×10^4 cells per wells for MCF-7, BT-20, BT-483. 24 hours after plating cells were treated for 72 hours. The viability was then measured using Celltiter 96 [MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] (Promega, Madison, WI) at 490nm.

3D Morphogenesis Assay.

Assays were performed as previously described by Reginato et al. (*16*). Briefly, MCF-10A cells or MCF-10A-ErbB2 (NeuT) cells were resuspended in assay medium **(**DMEM/F12 supplemented with 2% donor horse serum, 10 μg/ml insulin, 1 ng/ml cholera toxin, 100 μg/ml hydrocortisone, 50 U/ml penicillin, 50 μg/ml streptomycin, as well as 5 ng/ml EGF). Eight-well chamber slide (BD Falcon) were coated with 45 µl Matrigel per well and 5,000 cells were plated per well in assay medium containing final concentration of 2% matrigel. Assay medium containing 2% Matrigel was replaced every four days. For studies with inhibitors, morphogenesis assay was performed as described until the medium was replaced with assay medium supplemented with vehicle control, and a serotonin antagonist (SB-216641) for time indicated.

Immunofluorescence

Cells were plated 24h prior to the experiment on glass coverslips. After treatment, cells were fixed with PBS +5% formaldehyde, washed with PBS+100mM glycine then permeabilized with PBS+0.1% triton X100 and blocked with PBS+1%BSA. Cells were then incubated with cleaved-caspase 3 antibody (Cell Signaling Danvers, MA) (1:500) in PBST over night. Cells were incubated with secondary antibody (Cell Signaling, Danvers, MA) (1:400) in PBS+1%BSA. Finally coverslips were washed with PBS, rinsed in ddH$_2$O and mounted with a drop of DAPI (0.5 ng/ml) and Prolong (an antifade reagent; Invitrogen, Carlsbad, CA), and dried overnight. Immunofluorescence of 3D structures was performed as previously described by Reginato et al *(16)*. Confocal analysis was performed by using the Leica DM6000 B Confocal Microscope. Images were generated using the Leica Confocal Imaging Software SP2 AOBS System.

Apoptosis Assay

An immunofluorescence analysis was performed using an antibody to the activated caspase 3 protein. All of the cells were DAPI stained. Pictures of the stained samples were taken without zoom, no overlay was performed. We divided each pictures into 4 panels and counted 50 Dapi positive nucleus total. A transparent sheet was used to mark the 50 nucleus. The same transparent sheet was then overlaid on the cleaved-caspase3 pictures and double positive were counted. Each sample was done in triplicate; a one-way ANOVA was used to analyze significance.

Key Research Accomplishments

❖ We have provided the first demonstration of multiple 5-HT receptors in Breast Cancer Cells.

❖ We have shown that Breast Cancer Cells transcriptionally respond to the presence or absence of exogenous serotonin.

❖ Inhibition of the 5-HT$_{1B}$ receptor in breast cancer cells results in Akt-mediated programmed cell death. In these studies, we have also used FDA-approved serotonin inhibitors (that also target the 1B receptor), such as Chlorpromazine, and shown that they also induce apoptosis.

❖ We found that highly proliferative cancer cells [mimicking Tamoxifen-resistant cells] are more sensitive to 5-HT$_{1B}$ antagonist-induced cell death compared to resting normal cells. Normal MCF-10A cells grown in three dimensional basement membrane assays undergo minimal apoptosis following 5-HT$_{1B}$ antagonist treatment [after treatment they appeared healthy and formed normal acini]. In contrast, highly proliferative MCF-10A cells overexpressing the mutant form of ErbB2 are extremely sensitive to 5-HT$_{1B}$ antagonist-induced apoptosis.

Reportable Outcomes

There were no reportable outcomes at the conclusion of this study. We are currently trying to finish adding some additional data in order to write and submit a manuscript on this study.

Conclusions

Normally, nature safe guards against tumor formation by monitoring genomic stability and inducing programmed cell death when the stability is in jeopardy. Cancer cells, by definition, have lost their ability to respond to the normal signals for apoptosis. To delay or cure the malignancy, systemic treatments are used. The current treatments focus on cytotoxic, hormonal, and immunotherapeutic agents; they are active at the beginning of therapy in 90% of primary breast cancers and 50% of metastases. Unfortunately, at one point, resistance to therapy is not only common but expected. One of the key strategies in developing new treatments in breast cancer patients is to provoke apoptosis in the tumor cells without damaging the surrounding quiescent cells [our FDA-approved drugs have a proven track-record of NOT causing peripheral damage in patients]. The other key goal in breast cancer research is to discover new ways to circumvent resistance. One of the current therapeutic targets is activated Akt1. Recent research indicates that constant signaling originating from Akt1 provides a crucial survival signal for many type of breast cancers and accounts for the progression of the disease.

Any potentially successful breast cancer drug must be able to target the tumor cells without invasively destroying the surrounding healthy tissue. We used a 3D cell culture system to show that chemical inhibition of the 1B receptor has no effect on the integrity of the normal acinar structure. However, oncogene-overexpressing acini were still highly sensitive to antagonist induced programmed cell death in 3D culture. These studies indicate that continuously dividing tumorigenic cells are more sensitive to inhibition of the $5-HT_{1B}$ receptor. This approach may also provide a viable strategy for treating resistant tumors therapy. The concern of inevitable resistance seems to be avoided because the serotonin receptors are located on the outside of the cell and their primordial role in the growth may prevent their loss.

In terms of practical implications, the vast array of FDA approved drugs with well documented safety profiles that block serotonergic receptors may act as a 'proof-of-principle' to show the importance of these pathways in cancer patients. The next step is to test the in vivo efficacy of this approach on a murine tumor model.

There is an emerging interest of the new clinical information that could be obtained by using 'old' drugs for new indications; Medicare recently changed its policies to expand coverage of drugs for off-label uses to treat cancer patients. We believe that our results point toward a new area of research that has strong clinical potential for the use of serotonin inhibitors as single agents or in combination with traditional chemotherapy to treat cancer. Thus, a broader and more in-depth examination of the role of serotonin in breast cancer may open a new pathway for treating patients.

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