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Pharmacological Properties of *Chromobacterium violaceum* Violacein at the Human Serotonin 2C Receptor

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Abstract

The monoamine neurotransmitter serotonin (5-HT) plays a role in many physiological responses by interacting with various receptor subtypes. The 5-HT_{2C} receptor subtype is a 7-transmembrane, G protein-coupled receptor (GPCR) that is involved in neuronal excitability, spatial learning, mood, and appetite. The microorganism *Chromobacterium violaceum* produces a purple pigment, violacein, which can be extracted and purified. Violacein has antibiotic, antileishmanial, antifungal and antitumoral properties in various cancer cell lines. Violacein is derived from the amino acid tryptophan as is 5-HT and therefore, the two have similar chemical structures. However, no one has reported the activity of violacein at 5-HT receptors. Therefore the Fentress lab decided to investigate whether or not violacein had an effect on 5-HT_{2C} receptor trafficking. Human Embryonic Kidney (HEK) 293 cells expressing fluorescently-tagged 5-HT_{2C} receptor were treated with 5-HT, violacein, water or vehicle and then cells were fixed and visualized with fluorescent microscopy. Violacein treatment did not cause receptor internalization. Recent studies suggest that the 5-HT_{2C} receptor can activate the JAK/STAT pathway. To see if violacein can modulate this pathway, HEK 293 cells expressing 5-HT_{2C} receptor were treated with either 5-HT, violacein, or pretreated with violacein followed by incubation with 5-HT. Phosphorylation states of JAK2 and STAT3 were examined by immunoblotting. Results determined that 5-HT_{2C} receptor activation had no effect on JAK2 phosphorylation and that violacein blocked STAT3 phosphorylation. Primary radioligand binding determined that violacein has a low affinity for 5-HT_{2C} receptor but has a higher affinity for adrenergic receptors. Future studies will examine G protein-coupling by measuring phosphoinositide hydrolysis and cAMP assay to investigate adrenergic pathways.

Keywords

Violacein; Serotonin 2C Receptor; Serotonin; Chromobacterium violaceum; Cell Signaling; JAK/STAT Pathway

Introduction

Serotonin (5-HT) is a neurotransmitter synthesized from the amino acid tryptophan. It plays a role in many physiological responses such as appetite, mood, cognition, sleep, arousal, and

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Conflict of Interest

There are no conflicts of interest.

aggression by binding to 14 serotonin receptor subtypes. The 5-HT_{2C} receptr subtype is a 7-transmembrane, G protein-coupled receptor (GPCR] that is specifically involved in neuronal excitability, spatial learning, mood, and appetite [1], This receptor has been shown to activate signaling pathways downstream of the $G_{\alpha q/11}$ protein as well as other intracellular proteins that activate G protein-independent pathways.

The microbe *Chromobacterium violaceum* produces a purple pigment, violacein, which can be extracted from cells and purified. Violacein can kill other bacterial cells and induce cell death in various cancer cells [2], Violacein is also a derivative of tryptophan and therefore is similar in structure to 5-HT, however, no one has ever reported the activity of violacein at 5-HT receptors.

Many studies have shown constitutive activation of signal transducers and activators of transcription 3 (STAT3) in a wide variety of human tumors [2–4], The Fentress lab recently discovered that the 5-HT_{2C} receptor can activate STAT3 (unpublished observation). Given that violacein induces cell death in cancer cells which could involve STAT3 and it has similar structure to 5-HT, it was hypothesized that violacein will bind 5-HT_{2C} receptors to regulate ta activity. The antipsychotic olanzapine is known to desensitize both the 5-HT_{2A} and 5-HT_{2C} receptors. The Muma group recently published that chronic olanzapine treatment in mice can increase phosphorylation of STAT3 and caused a dose-dependent desensitization of the PLC pathway [5]. The serotonin 2C receptor is only found in the central nervous system and is a target for drug therapy in a variety of disorders, making it an ideal subtype to investigate the effects of violacein in the brain.

This study may lead to the development of violacein as a treatment for disorders the 5-HT_{2C} receptor is involved in such as obesity, depression, and schizophrenia. Though cancer cells are not being tested in this study, it may shed light on violacein's effect on the JAK/STAT signaling pathway that would lead to those investigations. This information would prove helpful to drug developers and clinicians.

Materials and Methods

Bacterial Strains

Chromobacterium violaceum (CV) strains were collected from the Tennessee Mining Company site in Ducktown, TN. Soil and water samples were taken by Dr. Terrance Johnson and yielded several different CV strains. In this study violacein was extracted from *Chromobacterium violaceum* 14N23.

Violacein extraction

Violacein was extracted as described by Mehta., *et al.* from pure culture of *Chromobacterium violaceum* strain 14N23. The bacteria was grown in 50 mL Lysogeny broth [LB) media for 48 hours in a rotary shaker set at 175 rpm, 30°C. This culture was mixed with 50 mL of ethanol and centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was added to a separatory funnel with an equal volume of chloroform. The funnel was shaken to mix and allowed to sit for lhr allowing the phases to separate. The

precipitate purple phase was collected into a pre-weighed beaker, then allowed to air-dry. The dry extracts were suspended into DMSO at a concentration of 1.0 mg/1.0 mL [6,7],

Cell culture

293 Human Embryonic Kidney cells (ATCC CRL-1573) were obtained from American Tissue Culture Collection [Manassus, VA). Cells were transfected via electroporation or by using X-fect Transfection Reagent kit (Takara Bio USA, Inc.) to express the INI isoform. Cells were also obtained from Dr. Ron Emeson's laboratory at Vanderbilt University. These cells stably expressed the 5-$HT_{2C\text{-}IDI}$ isoform of the receptor.

Crystal Violet Cytotoxicity Assay

Untransfected HEK293 cells were plated into a 24-well plate and grown until ~50% confluency. Cells were serum starved for 4 hours prior to treatments. Cells were treated with 100 μMol, 10 μMol, 1 μMol, 100 nMol, 10 nMol or 1 nMol of violacein or 100 μMol or 100 nMol of DMSO and incubated at 37°C with 5% CO_2 for 30 minutes. Cells were fixed with 100% methanol then stained with 5% crystal violet for 10 minutes. After washing, cells were solubilized in 0.1M Na citrate and 200 uL was transferred to microwell plate for measurements at 595 nm using BioRad iMark Microplate reader.

Immunocytochemistry

HEK293 cells were transiently transfected with yellow fluorescent protein-tagged 5-HT2C receptor in an 8-well chamber slide. Cells were incubated with 10 μMol of drug and incubated at 37°C with 5% C02 for either 30 minutes or 1 hr. After treatment, media was removed and the cells washed with IX PBS. Cells were fixed with 4% PFA for 15 minutes. Fixative was removed and the cells washed with IX PBS. The wells were removed and the slide coverslipped before viewing.

Treatment and cell lysate preparation

Cells were split into separate 100 mm plates, and serum starved for 16 hours prior to treatments. Cells were treated with 10 μMol 5-HT (Sigma-Aldrich, St. Louis, MO), 10 μMol olanzapine (Sigma-Aldrich, St. Louis, MO), 10 μMol of violacein or vehicle and incubated at 37°C with 5% CO_2 for either 30 minutes or pretreated with drug for 15 minutes then treated with serotonin for 30 minutes, then washed with cold phosphate buffered saline (PBS) for 5 minutes. Cells were lysed using radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with PhosSTOP (Roche Life Science, Branford, CT), PMSF (Sigma-Aldrich, St. Louis, MO) and leupeptin (Sigma-Aldrich, St. Louis, MO) on ice. Cells were then scraped from plates and sonicated at 10% amplitude then transferred to 1.5 mL tubes, and stored at −80°C.

Gel electrophoresis and immunoblotting

Protein concentrations were determined via the bicinchoninic acid (BCA) protein assay kit (Pierce Life Technologies, Carlsbad, CA] and 45 μl of each sample were diluted to equal protein concentrations and loaded into NuSep 10% SDS-PAGE precast gels (NuSep Holdings Ltd., Homebush NSW 2140, Australia), and run for 45 minutes at 200V. Proteins

were transferred to a nitrocellulose membrane overnight at 4°C. Nitrocellulose membranes were blocked in 3% BSA in transfer buffer for 2 hr. Nitrocellulose membranes were washed 3 times in transfer buffer, cut in half and incubated in primary rabbit anti-phospho-STAT3 (GeneTex, Irvine, CA, STAT3 phosphorylated Tyr705), primary rabbit anti-phospho-JAK2 (Millipore, Temecula, CA) or primary goat anti-β-actin antibody (Abeam Inc., Cambridge, MA) overnight at 4°C on a shaker. Antibodies were diluted to 1:500,1:2500 and 1:1000, respectfully. Anti-phospho-JAK2 and anti-phospho-STAT3 membranes were washed 3 times, and incubated in alkaline phosphatase conjugated secondary goat-anti-rabbit IgG (Millipore, Temecula, CA) at a 1:2500 dilution for 1 hour at room temperature. Anti-β-actin membranes were probed with alkaline phosphatase conjugated secondary rabbit anti-goat IgG antibody (Abeam Inc., Cambridge, MA) at a dilution of 1:5000 for 1 hour at room temperature. Nitrocellulose membranes were washed 3 times in transfer buffer, and were then incubated in BCIP/NBT color developing solution (Millipore, Temecula, CA) until protein bands were visualized (10 – 20 minutes).

Competition binding

Violacein was extracted and sent to Dr. Bryan Roth's Psychoactive Drug Screening Program at University of North Carolina at Chapel Hill where competition binding experiments were performed [8]. This program tests compounds against a wide range of neurotransmitter receptors and transporters. It is important to note that the 5-$HT_{2C\text{-}INI}$ isoform is what was tested. Flp-IN HEK was used to create stable cells and membrane fractions for one 96-well plate have at least 500 cpm/well when assayed at .5–1.0× K_d value of ^{3}[H]-mesulergine.

Data analysis

Densitometry of immunoblots was performed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD). GraphPad Prism 7 was used to preform two-way ANOVA for optical density of immunoblots and one-way ANOVA for luciferase data. Level of significance set at $p < 0.05$.

Results and Discussion

Violacein is not toxic to HEK293 cells

To establish the cytotoxicity of violacein on normal cells, untransfected HEK293 cells were tested and cell viability was assessed by a crystal violet assay. Cells were treated with 100 μMol, 10 μMol, 1 μMol, 100 nMol, 10 nMol or 1 nMol of violacein or 100 μMol or 100 nMol of DMSO. There were no significant differences in cell viability between any of the concentrations of violacein or DMSO (Figure 1). Previous studies have shown violacein to be toxic to HEK293 cells at 0.998 ± 0.058 μg/mL [9].

Violacein treatment appears to increase 5-$HT_{2C\text{-}INI}$ receptor on the cell's surface

Agonist binding and constitutive activation typically lead to internalization of receptors. In order to determine if violacein effects 5-HT_{2C} receptor trafficking, cells transiently expressing the fluorescently labelled receptor were treated with 5-HT, violacein, H_2O or DMSO then washed, fixed and coverslipped. Viewing these cells with fluorescent microscopy (Figure 2) revealed that the H_2O and DMSO treated cells displayed some

receptor internalization, which suggests that the receptor was constitutively active. The 5 - HT treated cells appeared to have more receptor trafficking, shown by more fluorescence intracellularly.

This is to be expected with agonist binding. The cells treated with violacein seemed to maintain the receptor at the membrane.

JAK2 phosphorylation is not altered by 5-$HT_{2C\text{-}IDI}$ receptor activation

STAT phosphorylation is usually preceded by JAK phosphorylation, therefore this study examined JAK2 phosphorylation as it relates to STAT3 activation since JAK2 phosphorylates STAT3 via the 5-HT_{2A} receptor. We examined untransfected cells and HEK293 cells stably expressing 5-$HT_{2C\text{-}IDI}$ receptor treated with 5-HT, olanzapine, violacein and DMSO. As shown in figure 3, band densities did not vary between untransfected cells and transfected cells for all treatment groups.

Violacein blocks basal STAT3 phosphorylation at 5-$HT_{2C\text{-}IDI}$ receptors

Previous data has shown that phosphorylation of STAT3 is stimulated by 5-$HT_{2C\text{-}IDI}$ receptor treatment with 5-HT and with vehicle treatment due to constitutive activity [2,10]. Conversely, olanzapine treatment blocked constitutive phosphorylation of STAT3. To determine the effect of violacein on this activation, HEK293 cells stably expressing the 5-$HT_{2C\text{-}IDI}$ receptor were stimulated with violacein for 30 minutes or for 15 minutes, followed by a 30 minute 5-HT treatment. As shown in figure 4a, violacein treatment decreased phosphorylation of STAT3 similar to olanzapine, though the ANOVA did not find significant differences due to outliers [Figure 4b]. Pre-treatment with violacein followed by treatment with serotonin confirmed that violacein did not damage the receptor as serotonin was able to bind and induce STAT3 phosphorylation.

Violacein has a low affinity for 5-HT2C-INI receptor but higher affinity for adrenergic receptors

In viewing this data [Figure 5), note that total binding would be indicated by 0% inhibition and nonspecific binding at 100%. Significant binding is greater than 50%, meaning more than half of the violacein was bound to receptors. Violacein showed 17.3% for 5-$HT_{2C\text{-}INI}$ receptor, which is not significant, suggesting low affinity for the receptor. Alpha ID is at 49.2 and Alpha 2C at 43.6, suggesting nearly significant inhibition and binding for these adrenergic receptors.

There have been no previous reports of violacein's effects on serotonin receptors so this was an exploratory study. The results of the receptor trafficking experiments suggest that violacein treatment maintains the receptor at the membrane as compared to serotonin treatment. This suggests that violacein is preventing activation. This could occur as a result of violacein binding the receptor, blocking agonist-independent activation. Without being able to quantify these images, it is not clearly determine a quantifiable difference between the treatment groups. Future studies could include subcellular fractionation or surface biotinylation to verify and quantify receptor presence in the cell membrane.

Earlier studies [5,11] showed that the 5-HT_{2A} receptor can aerate the JAK/STAT pathway and that activation is decreased by treatment with olanzapine. JAK2 phosphorylation typically leads to STAT3 phosphorylation. This study showed no difference between untransfected cells and transfected cells in JAK2 phosphorylation, suggesting JAK2-independent activation of STAT3. Data in the Fentress ab determined that 5-HT_{2C} receptor can also activate this pathway [10]. Olanzapine is characterized as an antagonist but the current study as well as the Curtis studies suggests inverse agonist activity at the 5-HT_{2C} receptor by blocking constitutive phosphorylation of STAT3. Violacein treatment reduced constitutive phosphorylation as well, which suggests it may serve as an inverse agonist or antagonist at the receptor.

The 5-HT_{2C} receptor is not the only effector of STAT3. Though 5-HT_{2C} is the only serotonin receptor in the HEK293 cells used in these experiments, muscarinic receptors that can stimulate the phospholipase C pathway are endogenous to the cells [12]. MAP kinases and small G proteins also activate STAT3 [13]. The ability of violacein to block STAT3 phosphorylation may be a reflection of interaction with these other proteins. This can be tested by modifying the competition binding studies previously mentioned with the named receptors and proteins.

The 5-HT_{2C} receptor is also able to undergo ligand biased signaling. Backstrom, Chang [14] determined that partial agonist lysergic acid diethylamide (LSD] induces a different signaling cascade from endogenous serotonin. Investigation into the phospholipase D and ERK1/2 pathway activation could be helpful and verifying which pathway violacein is acting on to block STAT3 phosphorylation. In addition, 5-HT_{2C} receptor can form dimers and oligomers that influence activation and functionality; receptor interact with antagonist show a tendency to the monomeric state [14,15], Fluorescence resonance energy transfer experiments can be done to determine if violacein has an effect on receptor dimerization.

Competition binding helps determine the relative affinity a ligand has for a receptor. Primary radioligand binding assays performed by the Psychoactive Drug Screening Program determined violacein had a much higher affinity for the Alpha ID and 2C adrenergic receptors than the 5-HT_{2C} receptor. Alpha ID is the predominant adrenergic receptor in epicardial coronary arteries [16] and Alpha 2C is required for normal presynaptic release of neurotransmitter in the heart [17]. Risperidone is an antagonist for Alpha 2C and 5-HT_{2C} receptor used to treat schizophrenia, irritability due to autism and bipolar 1 disorder. It does have a higher affinity for the 5-HT_{2A}receptor than 5-HT_{2C} receptor but risperidone has a K_i of 50 nMol at the 5-HT_{2C} receptor. Olanzapine has a K_i of 11 nMol, while 5-HThas a kD of 5.1 nMol in the 5-$HT_{2C\text{-}INI}$ receptor isoform. Violacein may have a similar pharmacological profile to risperidone or an inverse agonist. It is also noted that violacein exhibited some stimulation of binding with the 5-HT_{1A}, 5-HT_{1B} and 5-HT_{2A} receptors. The 5-HT_{2C} receptor was originally in the 5-HT_1 receptor family and has 78% homology to 5-HT_{2A} receptor [18].

Conclusion

This is the first study investigating the effects of violacein on 5-HT2C receptors. Preliminary results in HEK 293 transiently expressing the INI isoform suggested that violacein does not cause internalization of the receptor. HEK cells expressing the IDI isoform were used to investigate the effect of violacein on STAT3 phosphorylation. Receptor trafficking and constitutive activity of 5-HT_{2C} receptor are effected by RNA editing. Experiments in this study used both the 5-$HT_{2C\text{-}INI}$ and 5-$HT_{2C\text{-}IDI}$ receptor isoforms. The IDI isoform is only edited at the E site and it is expected to maintain constitutive activity as the immunoblotting results suggest. It is important to note, however, that the most prevalent isoform in the brain is 5-$HT_{2C\text{-}VSV}$ at 32% while INI consists of 7% and IDI less than 1% [19]. It is likely that violacein will have a different effect if tested on various isoforms and variants of the receptor.

Previous studies showed that 5-HT_{2C} receptor stimulates JAK2-independent activation of STAT3 and violacein treatment blocked basal phosphorylation. Competition binding assay revealed that violacein has a low affinity for 5-HT_{2C} receptor but a high affinity for Alpha ID and 2C adrenergic receptor. Violacein may be a low affinity inverse agonist for the 5-$HT_{2C\text{-}IDI}$ receptor by suppressing constitutive activity. Typically, drugs that are used to treat mood disorders are given to patients chronically, they will receive multiple doses following a certain schedule. These drugs often take time and require several doses to show any change in a patient. For example, a standard antidepressant can take between 7 to 20 days to exert some initial effects [20], These experiments only treated cells briefly with violacein. Chronic treatment with an *in vivo* model displaying schizophrenic or anxiety phenotype would more clearly indicate violacein's therapeutic effect. Future studies should also include PI hydrolysis to verily violacein's effect on phospholipase C signaling. To investigate violacein's effect on adrenergic receptors, similar *in vitro* studies can be performed and the detection of cAMP measured.

Acknowledgements

The Fentress lab would like to thank Dr. Ron Emeson and his laboratory for the HEK cells stably expressing the 5-$HT_{2C\text{-}IDI}$ receptor.

Abbreviations

5-HT	Serotonin
JAK	Janus Kinase
STAT	Signal Transducer and Activator of Transcription

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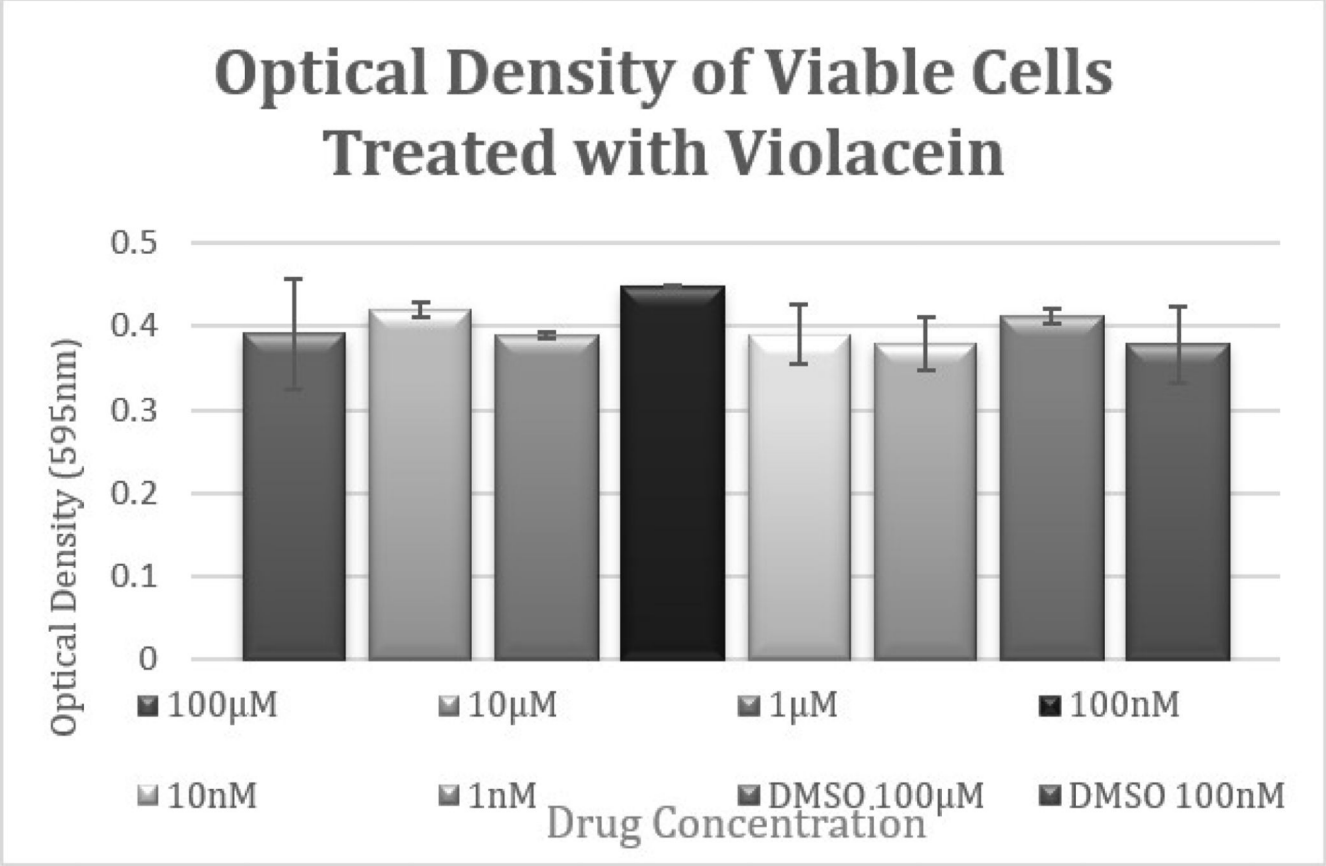

Figure 1:
Crystal Violet Stain of Nuclei in Viable Cells. HEK293 cells stably expressing 5-HT2CR were treated with various concentrations of violacein or DMSO for 30 minutes. Fixed dye that is solubilized correlates to cell number.

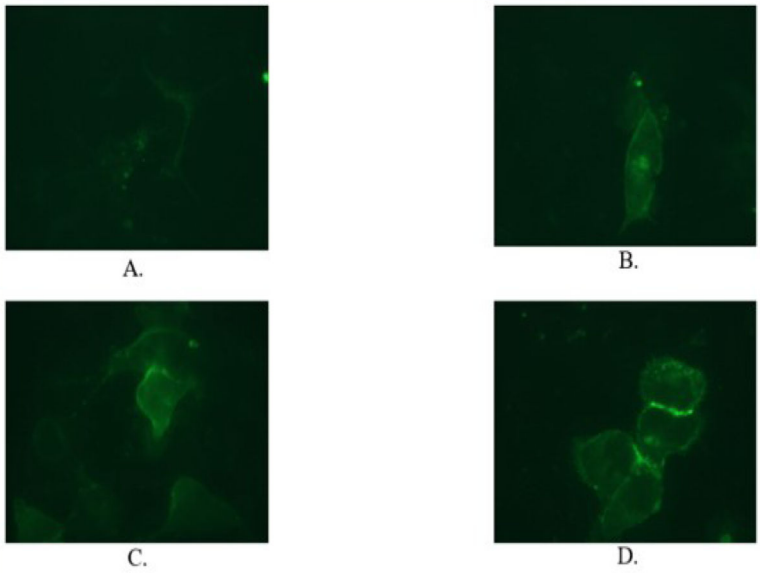

Figure 2:

Immunocytochemistry of 5-HT_{2C} receptor. HEK293 cells were transfected with fluorescently tagged 5-$HTR_{2C\text{-}INI}$ receptor and visualized by fluorescent imaging after 30 minutes of drug stimulation. A Water. B. DMSO. C. 5-HT. D. violacein.

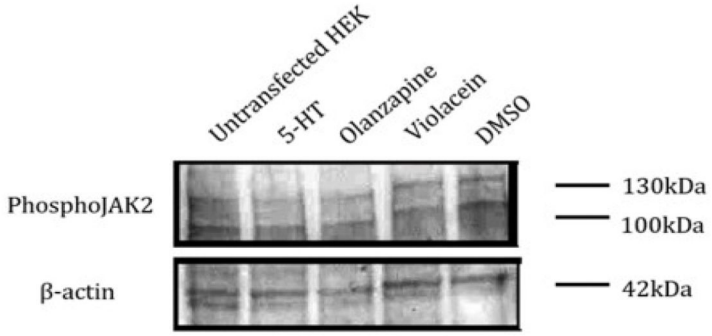

Figure 3:
Phosphorylation of JAK2 by 5-HT2C Receptor. Western blot ofphosphorylatedJAK2 in HEK293 cells.

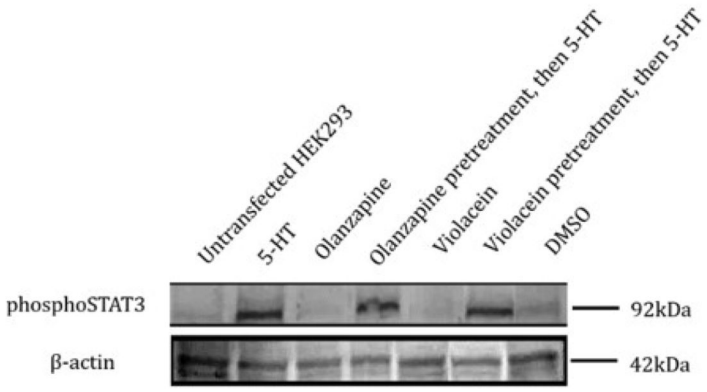

Figure 4a:

Phosphorylation ofSTAT3 by the 5-HT_{2C} receptor. Western blot of phosphorylated STAT3 in HEK293 cells with or without the 55-HT_{2C}R after 15 minutes pretreatment with olanzapine or violacein.

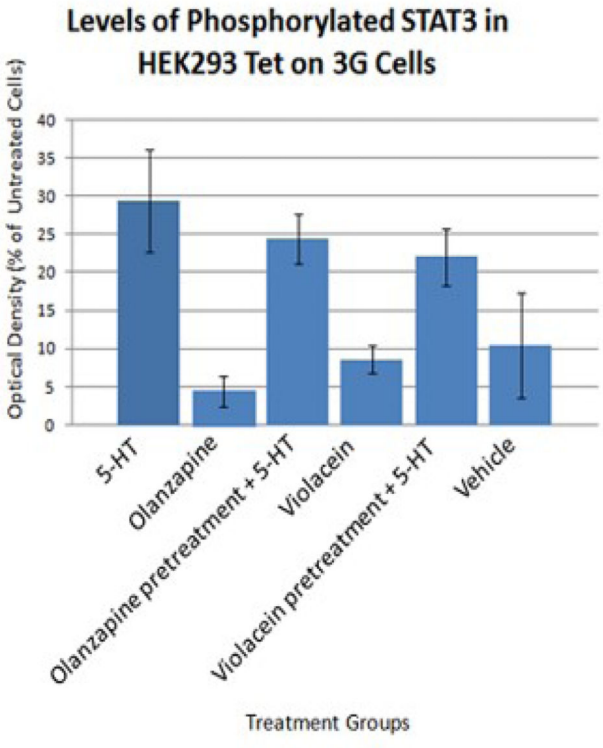

Figure 4b:

Optical Density ofPhospho-STAT3. Optical density of phosphorylated STAT3 in HEK293 cells with 5-$HT_{2C\text{-}IDI}$ receptor after drug treatment. (ANOVA indicates significant difference between all treatments $p > 0.04$, n=3. No significant comparisons indicated by Tukey's).

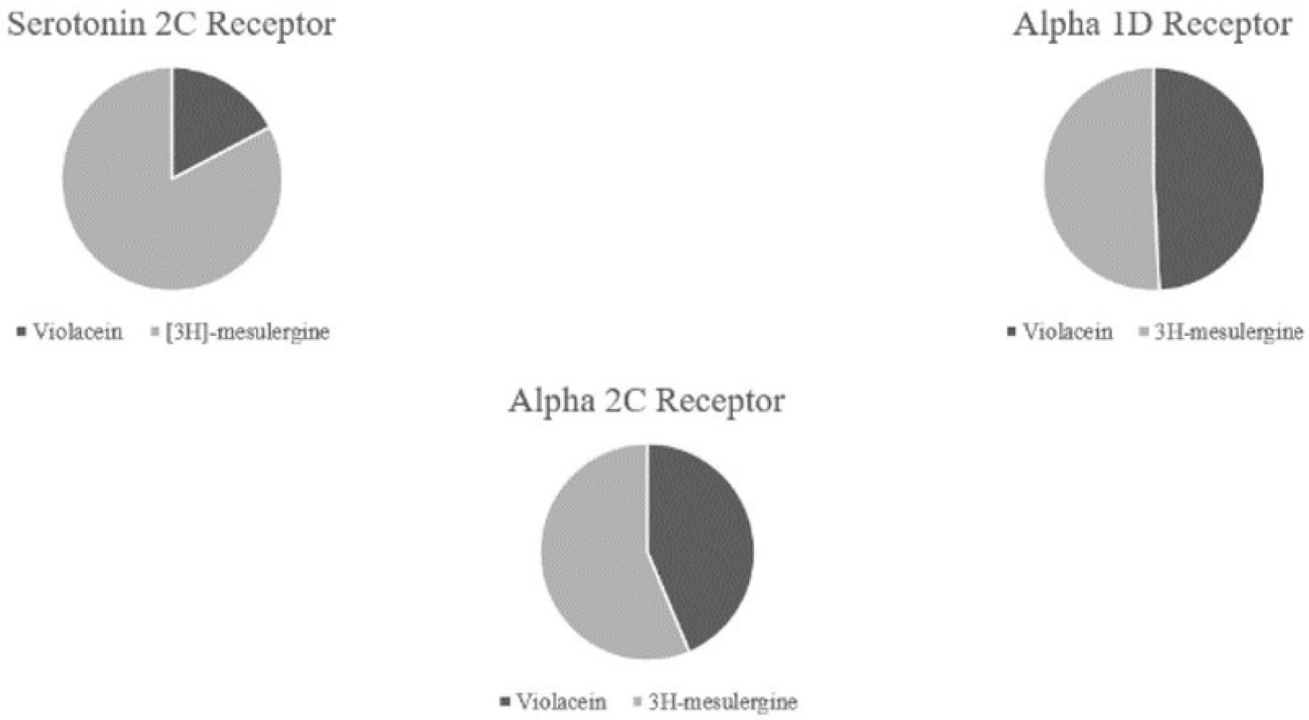

Figure 5:
Illustrated results from primary radioligand binding assay.

Table 1:

Tabular results from primary radioligand binding assay. **Significant inhibition is** considered > 50%. negative inhibition represents a stimulation of binding.

Target	Mean % Inhibition
5-HT1A	−15.5
5-HT1B	−13.4
5-HT1D	3.9
5-HT1E	7.6
5-HT2A	−14.4
5-HT2B	−7.4
5-HT2C	17.3
5-HT3	−13.5
5-HT5A	−8.1
5-HT6	3.4
5-HT7	10.6
AlphalA	−26.6
AlphalB	−1.6
AlphalD	49.2
Alpha2A	−15.9
Alpha2B	1.5
Alpha2C	43.6
Betal	−12
Beta2	−2.9
P Rat Brain s	−7.2
D1	6.4
D2	2.5
D3	−18.3
D4	−6.7
D5	−7.8
DAT	7.4
DOR	−8.8
GABAA	13.1
H2	−2.6
H3	3.9
H4	−13.5
KOR	13.9
Ml	1.7
M2	−16.2
M3	−18.7
M4	5.3

Target	Mean % Inhibition
M5	−23.2
MOR	16.8
NET	11.1
PBR	12.5
SERT	7.6
Sigma 1	−13.9
Sigma 2	−16.3