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5-HT2B Receptor-mediated Cardiac Valvulopathy

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5-HT_{2B} RECEPTOR-MEDIATED CARDIAC VALVULOPATHY

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Medicinal Chemistry at Virginia Commonwealth University

by

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List of abbreviations

5-HT	5-Hydroxytryptamine
BLAST	Basic local alignment search tool
BNP	Brain natriuretic peptide
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CGMP	Cyclic guanosine monophosphate
CNOS	Constitutive nitric oxide synthase
CNS	Central nervous System
COX	Cyclooxygenase
DAG	Diacyl glycerol
DMT	*N,N*-Dimethyltryptamine
DOET	1-(2,5-dimethoxy-4-ethylphenyl)propan-2-amine
DOB	1-(4-bromo-2,5-dimethoxyphenyl) propan-2-amine
DOI	1-(4-iodo-2,5-dimethoxyphenyl) propan-2-amine
DOX	1-(4-X-2,5-dimethoxyphenyl) propan-2-amine
ECM	Extracellular Matrix
EL2	Extracellular loop 2

ER	Endoplasmic reticulum
ERG	Ergotamine
ERK	Extracellular signal–regulated kinases
FDA	Food and drug administration
GABA	Gamma amino butyric acid
GAG	Glycosaminoglycans
GI	Gastrointestinal
GPCR	G-protein coupled receptors
HEK	Human embryonic kidney
HINT	Hydropathic Interactions
iNOS	Inducible nitric oxide synthase
IP3	Inositol triphosphate
IS	Interventricular septum
KO	Knockout
LA	Left atrium
LGICR	Ligand gated ion channel receptors
LOO	Leave-one-out
LOX	Lipooxygenase
LSD	Lysergic acid diethylamide
LV	Left ventricle
MAPK	Mitogen activated protein kinase
mCPP	Meta-chlorophenylpiperazine
MDA	Methylenedioxyamphetamine

MDMA	Methylenedioxymethamphetamine
mGlu2	Metabotropic glutamate receptor 2
mPFC	Medial prefrontal cortex
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa light chain-enhancer of activated B-cells
PDGFR	Platelet derived growth factor
PDZ	Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidyl Inositol 4,5-bisphosphate
PKC	Phosphokinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PLS	Partial least squares
PM	Papillary muscles
PTSD	Post-traumatic stress disorder
QSAR	Quantitative structure activity relationship
RA	Right atrium
RFEN	(*R)*-Fenfluramine
RNF	(*R)*-Norfenfluramine
ROS	Reactive oxygen species
SD	Sprague Dawley

SERT	Serotonin transporter
SFEN	(*S)*-Fenfluramine
SNF	(*S)*-Norfenfluramine
Src	Sarcoma proto-oncogene tyrosine-protein kinase
SSRI	Selective serotonin reuptake inhibitor
TACE	Tumor necrosis factor α converting enzyme
TFMPP	Trifluoromethylphenylpiperazine
TNF	Tumor necrosis factor
VHD	Valvular heart disease
VTA	Ventral tegmental area

Abstract

5-HT$_{2B}$ RECEPTOR-MEDIATED CARDIAC VALVULOPATHY

By Pallavi Nistala, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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5-HT$_{2B}$ receptor agonism causes cardiac valvulopathy, a condition characterized by thickening of the heart valves and as a result, regurgitation of blood within the heart. The anti-obesity drug fenfluramine, which was originally prescribed as an anorectic, was withdrawn from the market due to causing cardiac valvulopathy. Fenfluramine, after

metabolism by N-dealkylation, produces the metabolite norfenfluramine, which acts as a more potent valvulopathogen. The same was seen with MDMA (ecstasy), a popular drug of abuse, which is metabolized by N-dealkylation to produce MDA, a more potent valvulopathogen. Glennon and co-workers. studied a series of 2,5-dimethoxy-4-substituted phenylisopropylamines (DOX type) hallucinogens and determined their affinities at the three types of 5-HT_2 receptors. A high correlation was found between the affinities of these molecules at 5-HT_{2A} and 5-HT_{2B} receptors. Therefore, these hallucinogens have a high possibility of causing valvulopathy, which gives rise to a new class of valvulopathogens.

Since certain hallucinogens have the common phenylisopropylamine structural scaffold as that of MDA and norfenfluramine, we conducted 3D-QSAR studies to identify the common structural features of these molecules that are responsible for their high affinities. We were unable to obtain a suitable CoMFA and CoMSIA model for 5-HT_{2B} receptors, but we were able to obtain an internally and externally validated model for 5-HT_{2A} receptor affinities which indicated the hydrophobicity of the substituent at the 4-position was essential for high affinity. Following up with this evidence, we conducted a correlation analysis for the hydrophobicity (π-value) of the 4-position substituent and found a positive correlation between the π-value and the affinity of the molecules. The same results were not observed for the volume of the substituents.

We docked the molecules into the 5-HT_{2B} receptor and successfully generated models of the putative interactions made by the DOX molecules and the receptor. In order to compare their binding modes with respect to known valvulopathogens, we also generated

models for norfenfluramine and MDA. Our docking results revealed that DOX molecules bind in a more or less similar manner to valvulopathogens MDA and norfenfluramine. Ours is the first in silico model developed for the potent valvulopathogen MDA and the hallucinogenic DOX series of molecules.

I. Introduction

Cardiac valvulopathy is a condition wherein prolonged exposure of certain drugs (valvulopathogens) causes thickening of the bicuspid and tricuspid valves.[1] It is histologically characteristic and leads to regurgitation of blood within the ventricles, resulting in cardiac failure.[1] There are two classes of valvulopathogens - ergolines and phenylisopropylamines.[2] The anti-obesity drug fenfluramine was recalled in 1997 for causing valvulopathy.[3] Due to the structural similarity of fenfluramine with MDMA (ecstasy), Glennon and co-workers proposed that it has a potential to cause valvulopathy.[4] Follow up studies confirmed valvular damage seen in regular users of this empathogen.[4] Just like fenfluramine gets metabolized by N-dealkylation to produce a more potent valvulopathogen, MDMA also produces the potent N-demethlyated metabolite, MDA.[5]

Valvulopathy is caused due to agonism of 5-HT_{2B} receptors.[6] However, no evidence has yet confirmed if all agonists of 5-HT_{2B} receptors cause this condition. For instance, LSD is a 5-HT_{2B} agonist, yet there has been no evidence of it causing valvulopathy.[7] Therefore, it is unclear if it is due to a biased agonism of the 5-HT_{2B} receptor, or the receptor localization that may increase the likelihood of causing this condition. A majority of the ergolines involved in cardiac valvulopathy are prescribed to treat Parkinsonism (cabergoline, pergolide, bromocriptine and dihydroergocriptine).[7] The remainder are used to treat migraine (methysergide, ergotamine).[5] Low doses of

cabergoline are used to treat hyperprolactinemia (excess of prolactin secretion by the pituitary gland).[7] The lower dose of cabergoline in hyperprolactinemic patients puts them at a lower risk than Parkinson's patients.[7] In the case of cabergoline users of < 3 mg daily, they were five times more likely to develop valvulopathy, whereas those on > 3 mg daily were fifty times more likely.[7] Therefore, a high dose for prolonged periods of time vastly increases the likelihood of developing cardiac valve disease.[7] The SAR of these ergolines at 5-HT_{2B} receptors has been vaguely determined in that 8α-aminoergolines act as antagonists.[2]

Phenylisopropylamines are a class of hallucinogens and CNS-acting agents that are abused recreationally and used to treat CNS-related disorders.[8] Thus far, there have been no structure-affinity relationship studies conducted on phenylisopropylamines exclusively to determine the features that make norfenfluramine and MDA such potent valvulopathogens.

A series of hallucinogenic 2,5-dimethoxy-4-substituted phenylisopropylamine compounds (DOX) were studied by Glennon et al. to distinguish their selectivity at 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors.[9] The findings of this study showed a strong correlation between the affinity of these compounds at 5-HT_{2A} and 5-HT_{2B} receptors, indicating them to be directly proportional to each other.[9] It is therefore likely that this class of agents that bind with high affinity at 5-HT_{2A} receptors may bind with a proportionally high affinity to 5-HT_{2B} receptors as well, implicating them in cardiac valvulopathy. The functional activity of a majority of these compounds is unknown at 5-HT_{2B} receptors, but the 4-bromo and 4-iodo substituted compounds (DOB and DOI, respectively) are agonists.[10] Our goal is to determine the structure-activity relationships of these compounds by conducting 3D-

QSAR and docking studies, following up with HINT analysis to validate our docking studies.

The X-ray crystal structure of the 5-HT_{2B} receptor was determined with ergotamine and LSD, both structurally constrained analogs of the phenylisopropylamine scaffold.[11,12] In contrast, the isopropylamine system is freely rotatable in norfenfluramine, MDA and DOX molecules, which necessitated conformational studies. We used the template of the crystal structures of 4-ethyl and 4-methoxy substituted DOX molecules (DOEt and TMA) to sketch the remainder of the molecules.[13] The crystal structures for norfenfluramine and MDMA were available and were used to sketch them in Sybyl X2.1.1.[14,15]

The receptor-ligand interactions seen for LSD and ergotamine show the involvement of the transmembrane helices 3, 5, 6, and 7.[11,12] Findings from the study also determined the same interactions with norfenfluramine.[11] We have validated the quality of our models with respect to this prior model. There are no prior models for DOX molecules or MDA with the 5-HT_{2B} receptor, but multiple models have been generated with 5-HT_{2A} receptors for DOB and DOI, which are extensively studied for their high affinity at 5-HT_{2A} receptors.[16-18] Our docking studies will be compared against those of the prior models for reference. Nevertheless, the models were based on homology modeling and were published prior to the 5-HT_{2B} crystal structure, our model might be a more accurate and the latest representation of the ligand-receptor interactions.

In our studies, we will explore the similarities of valvulopathogens and DOX molecules, both with 3D-QSAR studies and docking. Our modeling will help provide insights of the potential ligand-receptor interactions for a new class of valvulopathogens.

II. Background

1. Valvulopathy

In September of 1997, the FDA and the pharmaceutical company Wyeth-Ayerst withdrew the popular anti-obesity drugs fenfluramine (Pondimin®) and dexfenfluramine (Redux®)

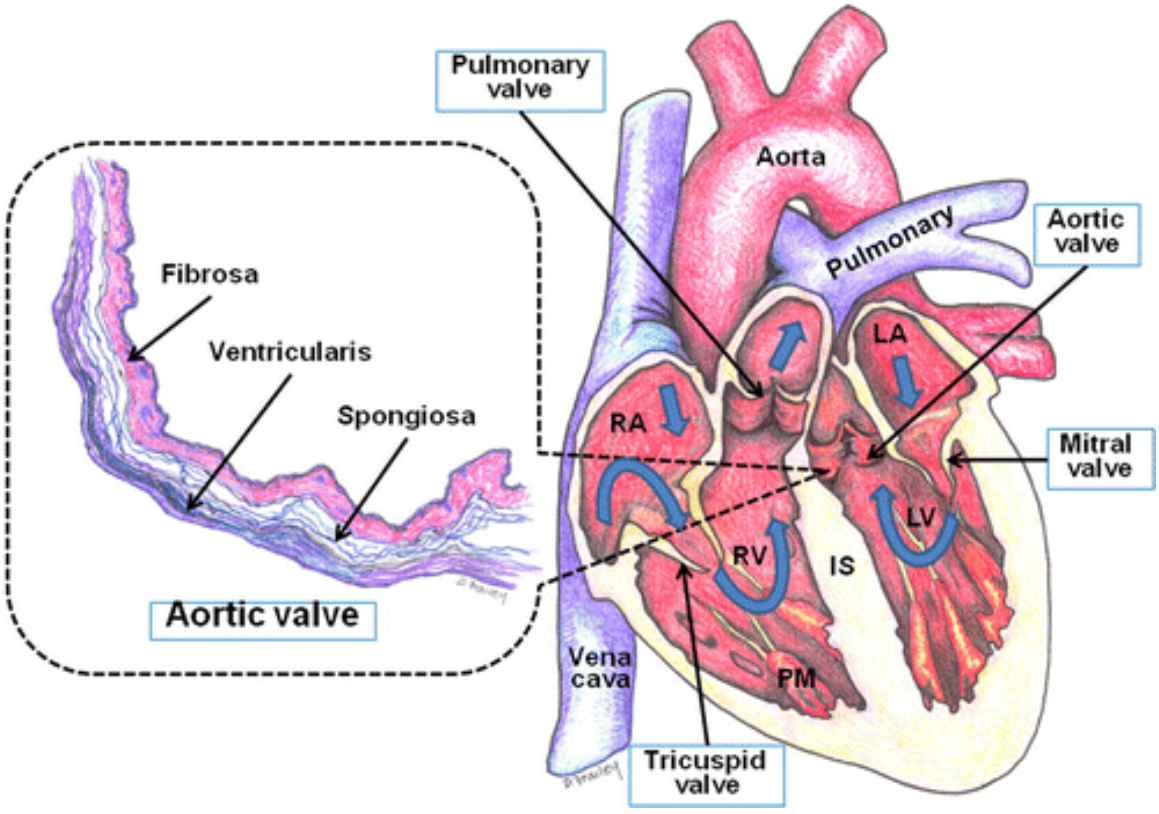


Figure 1. Diagram of the heart and the various types of atrial and ventricular valves affected in drug induced cardiac valvulopathy or valvular heart disease (VHD).[1]

(Left ventricle = LV; LA = Left atrium; RV = Right ventricle; RA = Right atrium; PM = Papillary muscles; IS = Interventricular septum)

from the market.[3] This Class 1 recall by the FDA was due to increased findings of abnormal echocardiograms in patients taking fenfluramine and the combination therapy of fenfluramine and phentermine (aka fen-phen) in a seminal paper published by Connolly et al.[19] Phentermine, however, showed no evidence of causing such anomalies. The findings were not unprecedented because a similar phenomenon was observed in ergot alkaloid (pergolide, cabergoline, dihydroergotamine) users.[1] Some patients undertaking anti-obesity treatment with fenfluramine and norfenfluramine underwent valve replacement surgery due to developing valvular disease.[6,20] The abnormal echocardiograms showed thickening of the cardiac valves (aortic, tricuspid and mitral) (Figure 1) and subsequent regurgitation of blood back to the ventricles. The heart valves are composed of three layers – the fibrosa – facing the outflow surface and composed of dense collagen, the spongiosa – composed of loose collagen and proteoglycans, and the ventricularis – facing the inflow surface composed of collagen and the inflow surface.[1] The extracellular matrix (ECM) of valvular tissue is responsible for the regular maintenance and repair of the valves. It is predominantly composed of proteoglycans, collagen, and elastin.[1] Changes in this ECM are responsible for producing heart diseases, including valvulopathy. The terms 'drug-induced valvulopathy' and 'valvular heart disease' (VHD) were thus used to identify this phenomenon.[1] VHD results in cardiac insufficiency and eventually death, due to the regurgitation of blood and the inability of the ventricles to maintain cardiac output.[1,20] The cause for this occurrence was unknown until its target was identified to be the 5-HT_{2B} receptor.[6] Further studies revealed that agonism of cardiac 5-HT_{2B} receptors caused valvular thickening.[6] The frequency of occurrence was varied in different case studies, i.e., 6%-30%. A meta-analysis of these

studies reported that one in eight patients receiving fenfluramine for more than ninety days developed valvulopathy.[20] Hence, the incidence of valvulopathy was governed by several factors such as dosage, length of exposure, signaling pathways associated with 5-HT_{2B} receptor agonism, and drug class.[1] This effect was not solely attributed to fenfluramine and related analogs. Other classes of agents such as ergolines, were also implicated.[7] Since thickening of heart valves was attributed to increased activation/agonism of the 5-HT_{2B} receptors in the heart, the endogenous ligand 5-HT was also on the gamut of suspects that caused valvulopathy.[1] An SD (Sprague Dawley) rat model of subcutaneous injection of 5-HT for seven days showed thickening of the valves characterized by increased glycosaminoglycans and a lower proportion of collagen.[21] Anorexigen-exposed valves have distinct histological features in comparison to other valvular disorders, such as floppy rheumatic valves or carcinoid heart disease, whereas carcinoid syndrome shows thickening of the valves, accompanied by increased vasculature and leukocytes.[6] Carcinoid syndrome is caused by elevated levels of serotonin produced by neuroendocrine tumors.[22] McDonald et al.[21] demonstrated that drug-induced valvulopathy is characterized by increased amounts of glycosaminoglycans (GAGs) in valvular tissue. The recall of norfenfluramine was followed by that of ergolines (e.g. pergolide, used to treat Parkinsonism).[23] Studies on 5-HT_{2B} receptor agonism are lacking in a specific preclinical animal model for the detection of valvulopathy.[1] However, they were demonstrated in SD rats and cynomolgus monkeys to show higher levels of 5-HT_{2B} receptor and serotonin transporter (SERT).[1] The valvulopathy due to agonism at 5-HT_{2B} receptors is caused by a specific downstream signaling pathway.[1] The 5-HT_{2B} receptor has multiple downstream effectors in producing its effects and while some are

unique to it, others are characteristic to the 5-HT_2 subfamily of receptors, such as G_q coupled signaling.[6]

2. Serotonin receptors

5-HT receptors are a group of centrally and peripherally distributed receptors involved in neurotransmission.[24,25] They are both excitatory and inhibitory in nature by their endogenous ligand 5-hydroxytryptamine (5-HT), aka serotonin.[24] 5-HT receptors are involved in the regulation of several other neurotransmitters such as dopamine, norepinephrine, gamma amino butyric acid (GABA), glutamate, and acetylcholine.[25] Due to their ubiquitous nature in the central nervous system, they are vital for several developmental processes such as memory, learning, and behavior in several vertebrate and invertebrate beings.[26] In humans, they are highly expressed in the medial prefrontal cortex (mPFC), which is responsible for executive function, decision making, and cognitive skills.[26,27] These receptors are the oldest neurotransmitters/hormones in evolution (700 – 800 million years) and have been expressed in a number of different organisms, including single cell eukaryotes such as paramecia, planaria, *Caenorhabditis elegans* (nematodes), drosophila, and man. [27] More recently, a novel serotonin receptor was found in the larvae of *Pieris rapae*, a small white butterfly.[28] The cDNA, named *Pr5-HT8,* has a relatively low similarity to any of the known serotonin receptor classes.[28] They were expressed and tested in HEK293 cells to show activation (due to an increase in intracellular calcium levels) with low (<10 nM) concentrations of 5-HT and did not affect intracellular cAMP levels even at high (>10 μM) concentrations of 5-HT.[28] These were

reported via a BLAST sequence search to be found exclusively in the insect genome, but not in the mammalian.[28]

Represented below (Figure 2) is a classification of 5-HT receptors. 5-HT receptors are primarily G-protein coupled receptors (GPCRs), except for 5-HT_3 receptors, which belong to the super family of Ligand Gated Ion Channel Receptors (LGICRs) (Figure 2 and 3).[25]

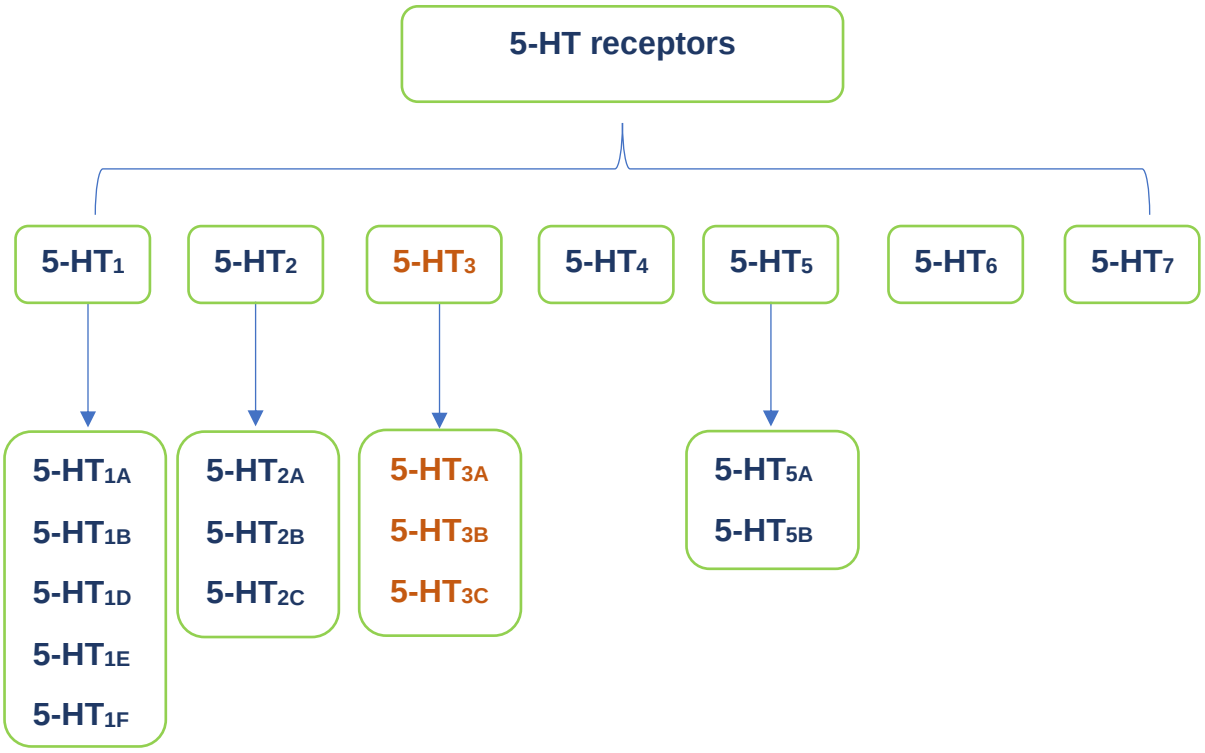


Figure 2. Representation of the 5-HT class and their subclasses of receptors.

Bennett and Snyder in 1976 were the first investigators that employed radioligand binding techniques in pursuit of discovering 5-HT receptors using rat cortical membrane preparations.[29] In 1979, Peroutka and Snyder helped distinguish 5-HT_1 and 5-HT_2 receptors using three radioligands, ^{3}[H]5-HT, ^{3}[H]spiperone, and ^{3}[H]LSD.[30] The initial

classification of 5-HT receptors was based on the ability of the receptors to be blocked by morphine (labeled as M), or dibenzyline (labeled as D).[27] Subsequently, the newer receptors were discovered between 1987 and 1992.[27] 5-HT_3 receptors were discovered when it was found they were unable to be blocked by either morphine or dibenzyline.[27]

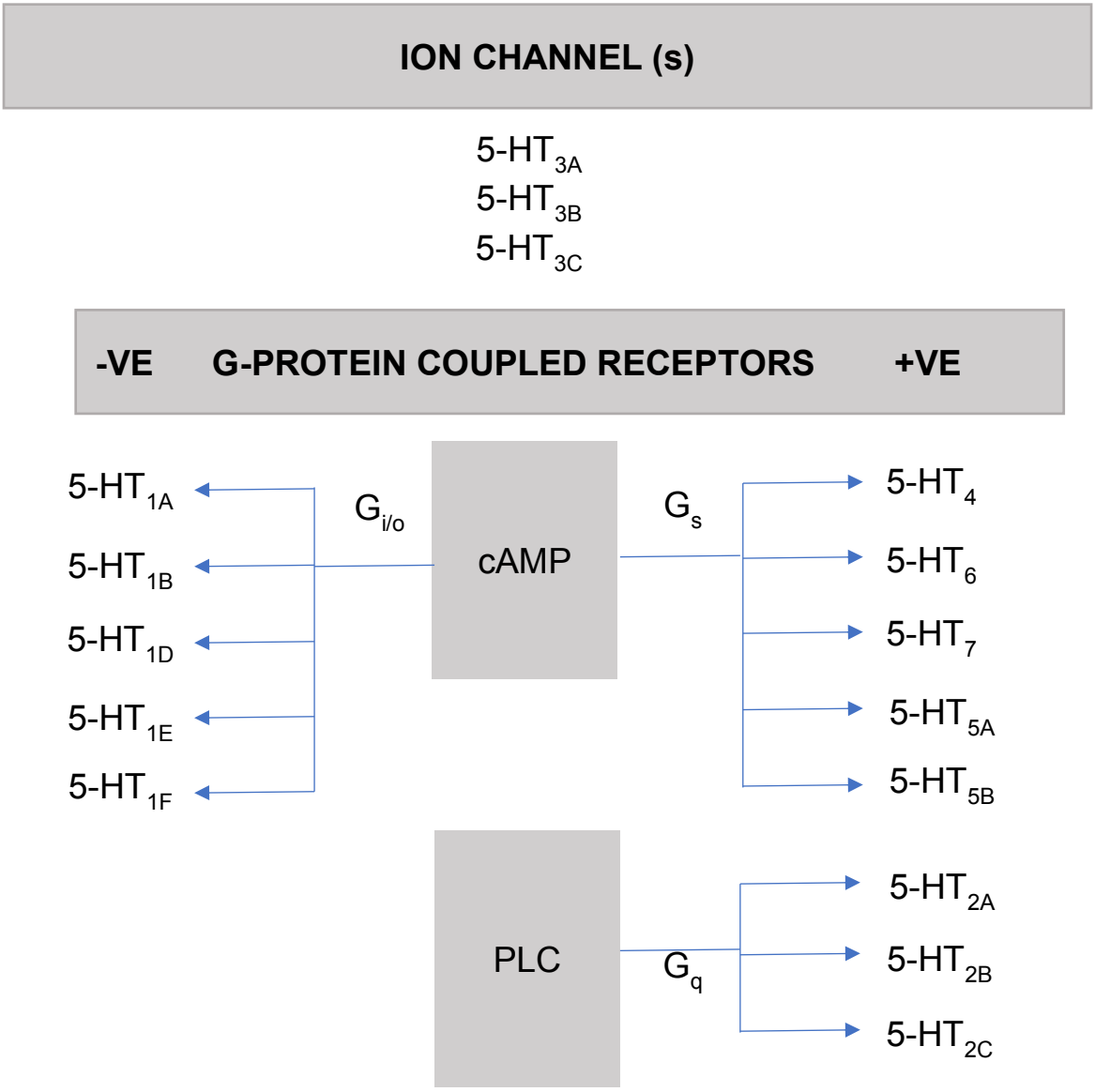


Figure 3. 5-HT receptors and representation of their signal transduction. (-VE = negative; +VE = positive). (cAMP = cyclic adenosine monophosphate; PLC = phospholipace C).

Streamlining the classification of 5-HT receptors began in 1993 when the Serotonin Club Receptor Nomenclature Committee laid down guidelines based on operational, structural, and transductional information (Figure 3).[27] The aforementioned three prerequisites, were equally weighted to facilitate proper classification.[27] The 5-HT_{1E} receptor is highly conserved and plays an important physiological role in mammalian brain.[27] Since its role was determined, it is no longer nominated in lower case, which indicates the physiological role has yet to be determined (example, 5- ht_{1E} in lieu of 5-HT_{1E}). Other subclasses (5-HT_{1F}, 5-HT_{5A}, 5-HT_{5B}, and 5-HT_6) have also recently received the uppercase nomenclature after their physiological functions were delineated.[27] 5-HT_{1F} receptors have been targeted to treat migraine[27] with indole derivatives and the proposed 5-$HT_{1B/1D}$ agonist naratriptan.[27] 5-HT_2 receptors are single protein molecules with a high degree of homology and are 458-471 amino acids in length. 5-HT_2 receptors are distributed centrally and peripherally. 5-HT_{2A} receptors are located in vascular smooth muscle, platelets, lung, the CNS and the GI tract. Initially 5-HT_{2B} receptors were found to be located peripherally, and 5-HT_{2C} receptors were located centrally.[25] The 5-HT_2 receptor was renamed 5-HT_{2A}, and the 5-HT_{1C} receptors were renamed 5-HT_{2C}.[25] Recent advancements on the structural information of 5-HT receptors including i.e., X-ray crystal structures of 5-HT_{2B} and 5-HT_3 receptors, enabled efforts in studying the mechanism of action/binding modes of known ligands at 5-HT_2 [PDB ID: 4IB4, 5TVN] and 5-HT_3 [PDB ID: 4PIR] receptors, respectively.[11,12,31]

"Higher end" 5-HT receptors such as 5-HT_5, 5-HT_6, and 5-HT_7, were more recently discovered, and their functions elucidated.[32] Thus, there was an initial lack of selective agents for the various types of 5-HT receptors.[33] This vacuum made characterizing the

variety of receptors a challenging task. However, through the years, selective agents have been developed for several serotonergic receptors. Additionally, the serotonin hypothesis of depression and related illnesses makes the serotonin receptor family a primary target for antidepressants on the market.[33]

The three types of 5-HT_2 receptors, 5-HT_{2A}, 5-$HT_{2B,}$ and 5-HT_{2C} receptors, have been extensively studied and have 46-50% homology.[34] They are grouped based on close sequence homology, the presence of similar exon and intron patterns, and the same transduction systems. They are coupled to $G_q/_{11}$ and increase the hydrolysis of PIP_2 to form inositol phosphate and diacyl glycerol (DAG) which results in an increase of intracellular calcium levels. Early on (i.e., late 1950s), 5-HT_2 receptors were the first to be discovered and were blocked by dibenzyline (D-type) as discovered by Gaddum and Picarelli,[27] but much later renamed as 5-HT_2 receptors by Peroutka and Snyder.[30] Gaddum and Picarelli also identified a second group of receptors (M-type) now recognized as 5-HT_3 receptors.[27] The three receptors were named based on agreement at the Serotonin Club Receptor Nomenclature Committee meeting in Houston.

a) 5-HT_{2A} receptors

5-HT_{2A} receptors are the quintessential 5-HT_2 receptors and have been studied for the longest time for their involvement in disease states such as mood related disorders.[24] Activation of 5-HT_{2A} receptors results in the activation of PLCβ and inositol phosphates, followed by Ca^{2+} release.[34] Depending on the nature of the tissue, different activation signals lead to different downstream effectors. For example, activation of 5-HT_{2A} receptors may also occur in conjunction with intracellular calcium release and Ca^{2+} influx from voltage-dependent calcium channels in vascular or tracheal smooth muscles.[34]

CNS-penetrating agonists of 5-HT_{2A} receptors were reported to possess hallucinogenic activity, and were widely studied, such as 1-(2,5-dimethoxy-4-iodophenyl)propan-2-amine (DOI), dimethyltryptamine (DMT), psilocybin, and mescaline to name a few, to develop radioligands and serve in conducting biological activity tests in 5-HT_2 type receptors.[8] Psilocybin (found in the *Psilocybe* genera of mushrooms), mescaline (peyote cactus), and DMT (in ingredients of ayahuasca brew) are naturally occurring hallucinogens. The abovementioned ligands , although do not bind exclusively to 5-HT_{2A} receptors, their selectivity can be harnessed to serve as radioligands for the other 5-HT_2 type receptors (5-HT_{2B} and 5-HT_{2C}).[25] 5-HT_{2A} receptors have served as an indispensable target for the study and development of antipsychotics. In the realm of antipsychotics, 5-HT_{2A} antagonists were amongst the more popular classes – for example, ketanserin, risperidone, trazodone, and clozapine, to name a few.[25] It was also noted that the *HTR2A* gene I197V allele polymorphism on chromosome 13 is responsible for the different responses upon treatment with clozapine.[35,36]

5-HT_{2A} receptors in recent years were found to dimerize with the metabotropic glutamate (mGlu2) receptor and facilitate increasing dopaminergic levels in the brain, which plays an important role in attention and learning.[27] Among their other functions, 5-HT_{2A} receptors are also responsible for mediating smooth muscle growth and potentiating growth factors.[27]

b) 5-HT_{2B} receptors

i. Distribution of 5-HT_{2B} receptors

5-HT_{2B} receptors belong to the G-protein coupled class of receptors and are distributed centrally and peripherally.[25] Their signaling is mediated primarily through the turnover of

phosphatidyl inositol. They were initially and extensively characterized in the rat stomach fundus where they triggered muscle contractions (hence, formerly known as 5-HT_{2F} receptors).[27] They were also initially cloned from rat cDNA.[34] These receptors are encoded by the HTR2B gene.[34] They are distributed peripherally in the stomach, intestine, pulmonary smooth muscles, myocardium, and osteoblasts.[34] In the rodent central nervous system, they are distributed in the dorsal raphae nucleus, cerebellum, lateral septum, dorsal hypothalamus, and amygdala.[34] Functionally, 5-HT_{2B} receptors are involved in presynaptic regulation of serotonergic neurons, vasoconstriction, normal cardiac development, and regulation of release of 5-HT from the serotonin transporter.[34]

Distribution of the receptors in humans is highly similar to that found in rodents.[34] They are distributed in cardiac musculature and valves, lungs, peripheral tissues, liver, kidneys, and prostate. Lower levels of expression are found in the cerebral cortex, pancreas, and spleen.[34] In humans, 5-HT_{2B} receptors undertake functions such as CNS regulation, digestion, normal cardiovascular development and repair. They are highly expressed early in murine embryos with a peak in expression at 8.5 days, as it plays a crucial role in neural crest and heart development.[34] 5-HT_{2B} KO (knockout) mice exhibit defects in the development of the heart and do not survive past mid-gestation.[34] Mice that do survive, reach adulthood with cardiomyopathy-like symptoms. Overall, the human receptor is 80% homologous to the rat receptor.[34]

ii. Signal transduction of 5-HT_{2B} receptors

Similar to 5-HT_{2A} and 5-HT_{2C} receptors, 5-HT_{2B} receptors are coupled to $G\alpha_q$ protein and increase intracellular levels of inositol phosphate (IP_3) and diacyl glycerol (DAG) (Figure 4).[34] Furthermore, their stimulation activates the arachidonic acid pathway via

phospohlipase A2 (PLA2), and a nitric oxide (NO) synthesis pathway via the C-terminal PDZ type 1 motif.[34] Stimulation of 5-HT_{2B} receptors triggers intracellular cyclic guanosine monophosphate (cGMP) production by activation of constitutive nitric oxide synthase (cNOS) and inducible NOS (iNOS).[34] NOS is also activate directly by calcium. Contraction of rat fundus muscle is mediated through Ca^{2+} influx via calcium dependent channels and activation of a phospholipase C (PLC)-stimulated increase in PIP_2 hydrolysis.[34]

The receptor is also coupled to β-arrestin, which is activated in the presence of agonists such as lysergic acid diethylamide (LSD) and ergotamine.[11,12] This is induced by changes in "trigger motifs" in GPCRs near the binding pocket to facilitate large scale helical movements to cause minute changes in the spatial arrangement of residues of each helix with respect to the residues in other helices. These residues are highly conserved, known as "microswitches".[12]

In a neuroendocrine serotonergic-differentiated teratocarcinoma cell line, stimulation of the 5-HT_{2B} receptor leads to activation of PLA2, followed by arachidonic acid release, which stimulates reactive oxygen species (ROS) synthesis via NADPH oxidase.[34] Downstream signaling leads to high levels of TNF-α, resulting in oxidative stress and neurodegeneration.[34]

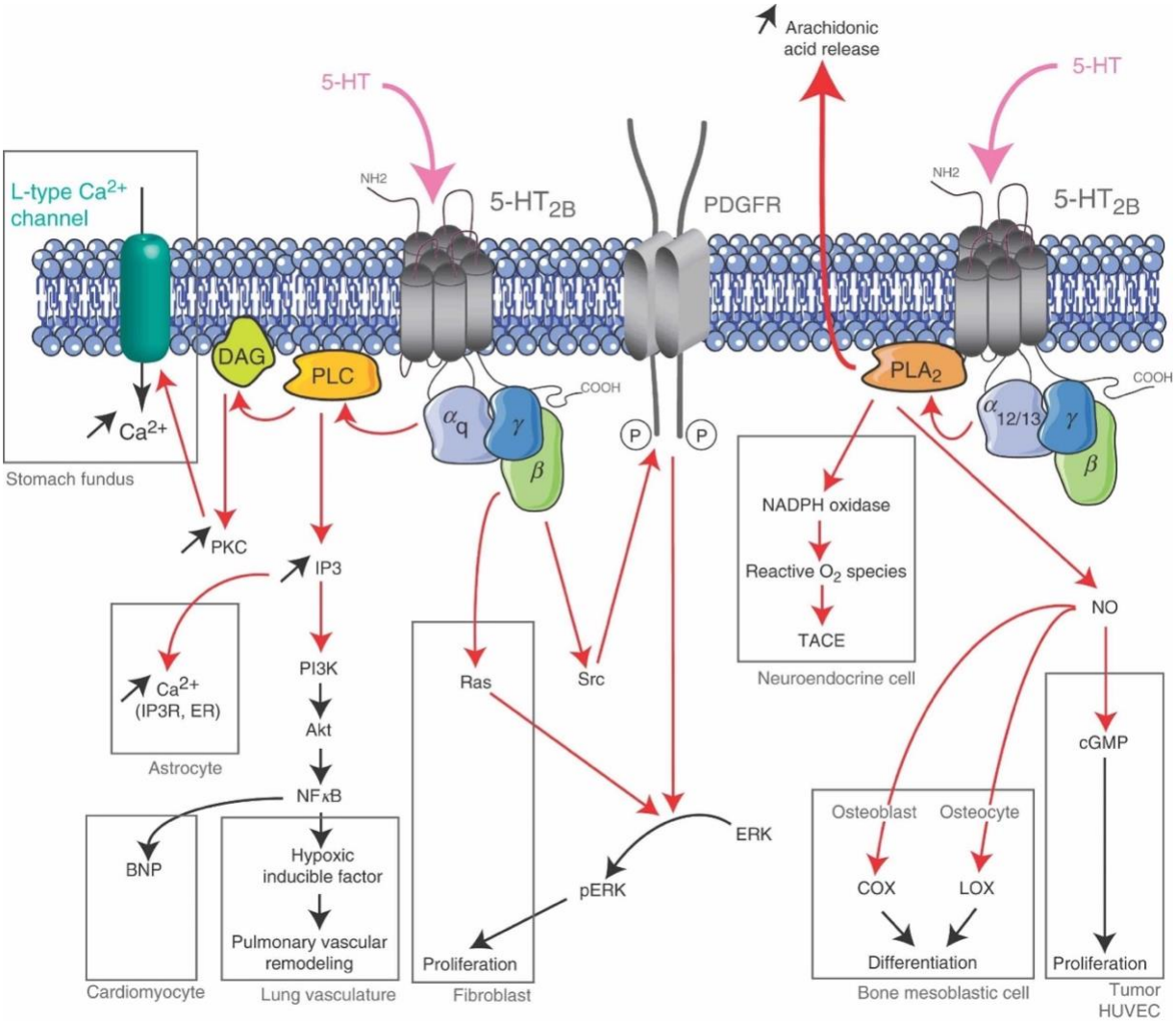


Figure 4. Downstream signaling effectors of 5-HT_{2B} receptors. (DAG = diacylglycerol; PDGFR = platelet derived growth factor receptor; PLA_2 = phospholipase A_2; PLC = phospholipase C; NADPH = nicotinamide adeninedinucleotide phosphate; IP_3 = Inositol triphosphate; ERK = extracellular signal-regulated kinase; ER = endoplasmic reticulum; PI3K = phosphoinositide 3-kinase; NFκB = nuclear factor κ-light-chain- enhancer of activated B cells; TACE = tumor necrosis factor α converting enzyme; BNP = brain natriuretic peptide; COX = cyclooxygenase; LOX = Lipooxygenase; Src = sarcoma proto-oncogene tyrosine-protein kinase.[34]

iii. Characteristics of 5-HT_{2B} receptors

The h5-HT_{2B} receptor consists of 402 amino acid residues. The recently published crystal structure of a 5-HT_{2B} receptor with LSD (PDB ID: 5TVN) has provided unique insight from the previously published crystal structure with ergotamine (PDB ID: 4IB4).[11,12] Due to the smaller size of LSD in comparison to ergotamine, LSD exhibits a longer residence time within the receptor and is seated deeper in the binding pocket compared to ergotamine.[12] LSD acts as a partial agonist and ergotamine is a full agonist at the 5-HT_{2B} receptor.[12] However, both exhibit biased agonism in that they stimulate the β-arrestin pathway.[12] The crystal structure also revealed the ergoline ring systems adopt distinct binding poses with respect to each other in the orthosteric binding site and that LSD never visited the conformation that was obtained by the small molecule crystal structure published by Baker et al. in 1972.[37] Additionally, the EL2 portion of the receptor forms a "lid" over this binding pocket, and the residue $L209^{EL2}$ forms a hinge by forming hydrophobic interactions with ring C of LSD.[12] The engagement of this residue, as suggested by mutagenesis studies, propagates β-arrestin signaling and has little to no effect on G_q signaling.[12]

The abovementioned knowledge of 5-HT_{2B} receptors will be vital in studying the consequence of agonists and antagonists in disease states such as valvulopathy, migraine, and irritable bowel syndrome.[34]

c) 5-HT_{2C} receptors

5-HT_{2C} receptors are widely distributed in the dopaminergic cell body regions of the substantia nigra and ventral tegmental area and terminal projection areas of the nucleus accumbens, striatum, and prefrontal cortex.[38] This class of 5-HT_2 receptors is

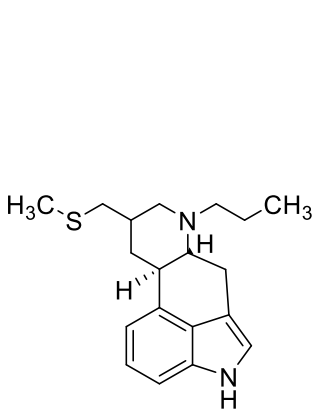
positively coupled to G_q and activation results in phosphoinositidyl inositol turnover. Their initial discovery was by finding receptors with high affinity to LSD and mesulergine, and low affinity for ketanserin.[39] However, due to their binding properties resembling 5-HT_{1A} and 5-HT_{1B} receptors, more than 5-HT_2 sites, they were initially named 5-HT_{1C} receptors.[39] They were later discovered by molecular cloning of the cDNA to have a high degree of homology to the 5-HT_2 receptor and were later named 5-HT_{2C} receptors.[39] Activation of these receptors results in the suppression of appetite. This property of 5-HT_{2C} receptors was used to create the anti-obesity drug lorcaserin[40] - a 5-HT_{2C} receptor agonist. Genetic evidence suggests that 5-HT_{2C} receptors stimulate reward-related behavior.[39] 5-HT_{2C} receptor KO mice have shown evidence of producing obesity and a proneness to seizure with more responsiveness to electrical stimulation.[39] The dopaminergic system is modulated by the signaling of 5-HT_{2C} receptors in the mesocorticolimbic system in the ventral tegmental area (VTA).[40] The system is also modulated by the constitutive activity of 5-HT_{2C} receptors.[41] The activity of 5-HT_{2C} receptors in the absence of ligand can be detected based on the PLC, and in most cases, 5-HT_{2C} receptors can be ten times as active as 5-HT_{2A} receptors.[41] This phenomenon is explained by the isomerization of receptors at different conformations and isoforms.[41] The 5-HT_{2C} receptors are unique in the aspect of undergoing RNA editing that converts five adenosine residues to inosine.[41] The various isoforms of 5-HT_{2C} receptors are attributed to this RNA editing feature that produces a difference in three amino acids in the second intracellular loop of the receptor.[41] The changes can affect the activity of the receptor due to less efficient G-protein coupling. Certain isoforms of 5-HT_{2C} receptors have been linked to depression and schizophrenia.[40]

Furthermore, 5-HT_{2C} receptors enhanced cocaine-induced elevations of dopamine in the nucleus accumbens.[40] The anorexigenic drug fenfluramine is also said to act via the activation of 5-HT_{2C} receptors by producing hypophagia, as evidenced by mice lacking functional 5-HT_{2C} receptors and the inability of fenfluramine to attenuate feeding.[42] In a behavioral satiety sequence (BSS) test, fenfluramine was able to enhance satiety in mice.[42] In mice lacking these receptors, it was unable to produce the same effect.[43] Other studies have been conducted to show strong evidence of the involvement of 5-HT_{2C} receptors in controlling feeding behavior, amongst which was *m*-chlorophenylpiperazine (mCPP) and trifluoromethylphenylpiperazine (TFMPP) - agonists at 5-$HT_{2B/2C}$ receptors and antagonists at 5-HT_{2A} receptors.[42] Due to the distribution of 5-HT_{2C} receptors in the VTA, hypothalamus, and amygdala, the main areas associated with emotional regulation, they are considered a potential target for the treatment of schizophrenia and depression.[42]

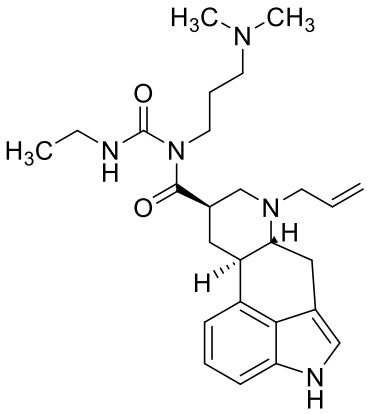
5-HT_{2C} receptors, as evidenced by the recently published crystal structure, exist as a homodimer.[44] The INI isoform of the 5-HT_{2C} receptor crystal structure was crystallized along with the agonist ergotamine and inverse agonist ritanserin.[44] Prior evidence was published by Herrick-Davis and co-workers that 5-HT_{2C} receptors are found in the choroid plexus epithelial cells.[45] The signaling properties of homodimers were investigated using ergotamine, a high affinity 5-$HT_{2A/2B/2C}$ receptor ligand.[46] Although it is debatable if the binding of ligands to one of both protomers of a dimeric complex is necessary or sufficient for G-protein signaling, it is known that the second protomer does not remain silent and plays a role in the signaling process.[45] The genetic polymorphism of 5-HT_{2C} receptors is associated with weight gain in patients on antipsychotic treatment. Obesity and diabetes are thus a comorbidity in patients expressing this polymorphism.[42]

3. Therapeutic agents and valvulopathy

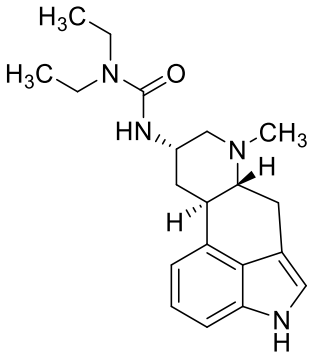
a) Ergot derivatives – pergolide (**1**) and cabergoline (**2**) are potent agonists at 5-HT_{2B} receptors.[47] It is, however, not a class effect seen in all ergolines (ergot derivatives) (Figure 5). Bromocriptine and 8α-aminoergolines such as lisuride (**3**) and terguride (**4**) lack agonist activity but are potent, silent antagonists at 5-HT_{2B} receptors.[48] Due to this reason, pergolide (**1**) – a dopamine receptor agonist was used in the treatment of Parkinson's disease, but withdrawn from the market in 2007 for causing serious damage to patients' heart valves (mitral and tricuspid).[5] Lisuride (**3**), interestingly, is an agonist at 5-HT_{2A} and 5-HT_{2C} receptors, but an antagonist at 5-HT_{2B} receptors.[48] This finding further helped reinforce the idea that 5-HT_{2B} receptor activation is necessary for causing valvulopathy. A study conducted by Schade et al.[49] investigated the involvement of other dopaminergic agonists used to treat Parkinsonism (bromocriptine (**5**), cabergoline (**2**), pergolide (**1**), pramipexole (**6**), and ropinirole (**7**)) also caused agonism of 5-HT_{2B} receptors. Pergolide (**1**) and cabergoline (**2**) were the only two molecules that caused valvulopathy.[49] Ergot derivatives such as ergotamine (**8**), dihydroergotamine (**9**), and methysergide (**10**), used to treat migraine, also cause cardiac valvulopathy.[49]



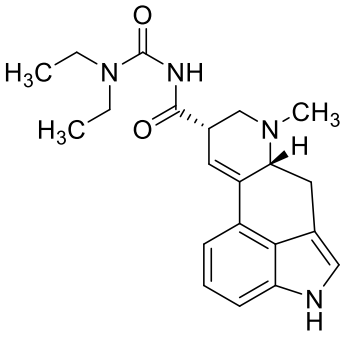
Pergolide (**1**)



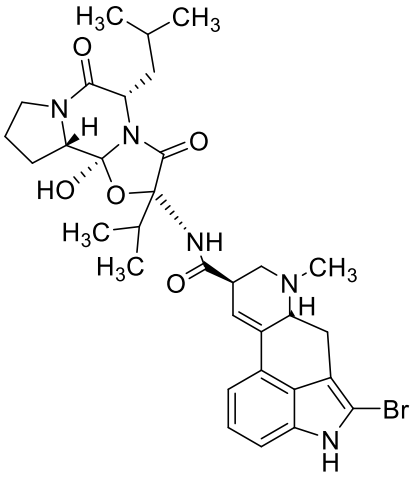
Cabergoline (**2**)



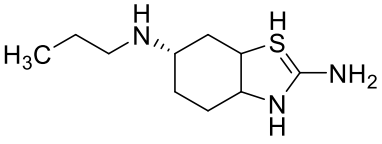
Lisuride (**3**)



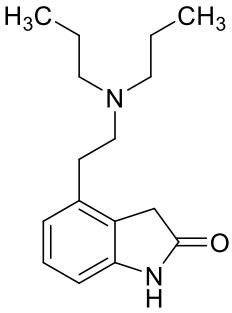
Terguride (**4**)



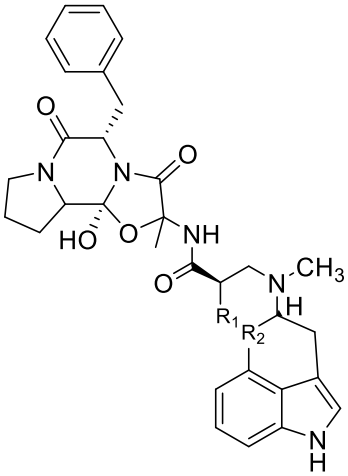
Bromocriptine (**5**)

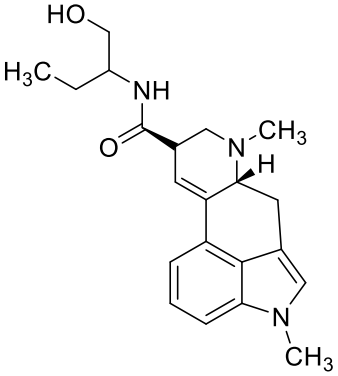


Pramipexole (**6**)



Ropinirole (**7**)





Methysergide (**10**)

R_1-R_2 = -HC=C-Ergotamine (**8**)
R_1-R_2 = -H_2C-CH- Dihydroergotamine (**9**)

Figure 5. Ergot and related derivatives examined for causing valvulopathy.

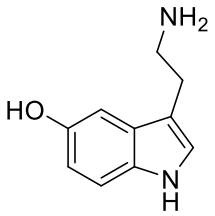
b) Fenfluramine – The compounds associated with fenfluramine consist of both optical isomers of fenfluramine (**11**), and the de-ethylated analog/metabolite, norfenfluramine (**12**). They were initially prescribed for the treatment of obesity as a serotonin transporter substrate/5-HT agonist due to their appetite suppressant pharmacological profile.[19] Fenfluramine (**11**) was initially introduced in the 1960's in France and in the 1970's in the US. It was marketed under tradenames Pondimin® (racemic mixture of fenfluramine), Redux® (the *S*- isomer of fenfluramine aka dexfenfluramine), and in combination therapy with phentermine as Fen-Phen®.[6] Phentermine being a dopamine transporter releaser, was shown to not be implicated in the valvular disease.[6] Norfenfluramine (**12**) isomers have higher affinity and potency at 5-HT_{2B} receptors than fenfluramine (**11**), making the metabolite (Figure 6) with higher side effects than the drug.[6] Approximately twenty years later, it was withdrawn from the market for producing cardiac valvulopathy.[3] Methylergonovine was found to be a less effective agonist than fenfluramine, yet it produces a more severe form of valvulopathy.[6]

Fenfluramine (**11**) Norfenfluramine (**12**)

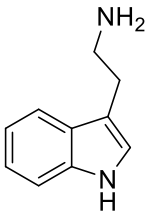
Figure 6. Metabolism of fenfluramine (**11**) to produce the more potent metabolite norfenfluramine (**12**).

Valvulopathy from fenfluramine is caused in doses ≥ 60 mg/kg.[50] Fenfluramine (**11**) is under clinical trials for the treatment of epilepsy and Dravet syndrome (severe myoclonic epilepsy of infancy).[50] In lower doses (10 - 20 mg/kg), fenfluramine (**11**) reduces epilepsy in infants and there has been no evidence of valvulopathy in the patients although it is premature to conclude if fenfluramine causes valvulopathy at such low doses.[50]

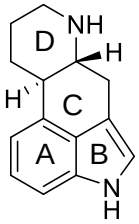
c) 5-HT – Serotonin (5-HT (**13**)) is the endogenous ligand of the 5-HT receptor class. Serotonin syndrome caused by neuroendocrine tumors secreting high levels of serotonin causes valvulopathy. In a study conducted by Elangbam et al.,[51] subcutaneous administration of 5-HT causes valvular thickening in SD rats. The study also highlights the importance of the serotonin transporter and its inhibition, further exacerbating valvulopathogenesis. However, the histological manifestation seen is different in anorexigen valves and serotonin-selective reuptake inhibitors.[51] 5-HT (**13**) is a tryptamine/indolealkylamine (**14**), however this effect is not seen with all tryptamines since there is no evidence of other tryptamines causing valvulopathy. Hence, the question arises, do all 5-HT_{2B} receptor agonists cause valvulopathy? Certain ergolines, which possess the basic indolealkylamine scaffold (Figure 7) cause valvulopathy but tryptamines themselves are not known to be involved in causing it, possibly due to lack of sufficient investigation. Most tryptamines are not used therapeutically and some are primarily hallucinogenic in nature.



5-HT (**13**)



Tryptamine/ indolealkylamine (**14**)



Ergoline (**15**)

Figure 7. Ergoline (**15**) contains the indolealkylamine scaffold (**14**), yet indolealkylamines (except for 5-HT (**13**)) have not been implicated in causing valvulopathy.

d) Lorcaserin – Lorcaserin (**16**) (Figure 8) is a potent agonist at 5-HT_{2B} receptors and is used to treat obesity by acting as an agonist at 5-HT_{2C} receptors.[52] Due to its potency (EC_{50} = 943 nM) and high affinity (K_i = 174 nM) for 5-HT_{2B} receptors,[52] it was studied for potential to cause cardiac valvulopathy in patients.[53] A study conducted by Thomsen et al.,[52] revealed lorcaserin (**16**) possessed approximately 104-fold functional selectivity for 5-HT_{2C} over 5-HT_{2B} receptors.

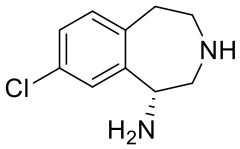


Figure 8. Lorcaserin (**16**).

The Psychoactive Drug Screening program at the National Institute of Mental Health has screened common therapeutic agents for agonist activity at 5-HT_{2B} receptors in the quest to identify agents causing valvulopathy.[54] The study found 27 agents among 2200 drugs and drug-like compounds.[54] Including the abovementioned compounds, there were five compounds not previously identified as valvulopathogens that behaved as

agonists at 5-HT_{2B} receptors: guanfacine, oxymetazoline, quinidine, xylometazoline, and fenoldopam.[54] Guanfacine and quinidine were used to treat hypertension and arrhythmia, respectively.[54] Being prescribed for prolonged periods, guanfacine and quinidine were scrutinized for their ability to induce valvulopathy.[54] However, due to possessing a lower potency at 5-HT_{2B} receptors than serotonin and norfenfluramine, they were not pursued.[54] Oxymetazoline, xylometazoline, and fenoldopam were of less concern since they were prescribed for shorter time periods. Among the drugs tested was ropinirole (**7**), which was a weak agonist (EC_{50} = 2570 nM) at 5-HT_{2B} receptors. Four cases of patients undergoing therapy with ropinirole (**7**) were reported with valvulopathy.[55] However, since the number of cases was not high enough to be statistically significant, it was not further pursued.[54]

4. Drugs of abuse and valvulopathy

a) 3,4-Methylenedioxymethamphetamine (MDMA) (**17**) - aka ecstasy, is a recreational Schedule I drug. In crystalline, powder form, it is referred to by its street name "Molly".[56] The use of MDMA (**17**) was most popular during the late 1990s and early 2000s. It acts as an empathogen and produces its effects primarily by acting as a releaser at SERT.[57] In addition, MDMA (**17**) also has affinity for the 5-HT_1 and 5-HT_2 class of receptors.[58] In a study conducted by Lyon et al.,[69] MDMA was investigated for its affinity for 5-HT_1 and 5-HT_2 receptors, along with its stereoselectivity. The (*R*)-MDMA isomer was found to have ~ 4-fold higher affinity than the (*S*)-isomer.[69] It was first synthesized by Merck in 1912 and was later rediscovered to study its effects as a psychopharmacological agent by Shulgin

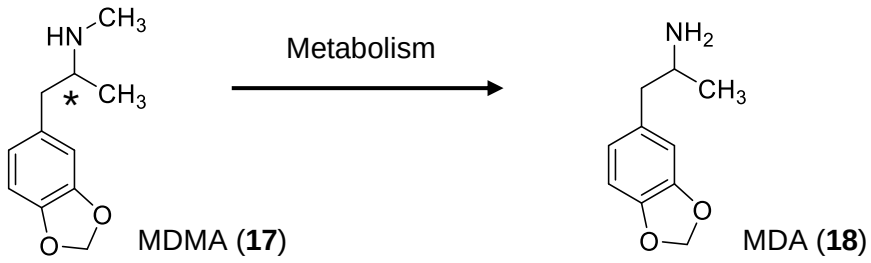


Figure 9. *3,4*-Methylenedioxymethamphetamine (MDMA) (**17**) and its metabolite *3,4*-methylenedioxyamphetamine (MDA) (**18**).

and Nichols, in 1978.[61] Years later, It was implicated in causing valvulopathy amongst chronic users.[4,60] It undergoes metabolism (Figure 9) *in vivo* to produce methylenedioxyamphetamine (MDA)(**18**), a more potent valvulopathogen than MDMA.[60] *In vitro* studies were conducted on cardiac valvular interstitial cells to study the activation of 5-HT_{2B} receptors.[4] However, MDMA is gaining popularity yet again in studies concerning the treatment of PTSD in war veterans, firefighters, and police officers who do not respond to conventional methods of treatments. [62,63]

A study conducted on 29 young adults using MDMA (**17**), blindly evaluated cardiac function with echocardiography.[60] Eight subjects taking MDMA (**17**) had abnormal

R = CH_3 = (*S*)-MDMA (**17a**)

R = H = (*S*)-MDA (**18a**)

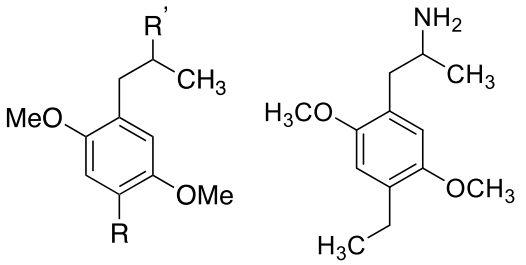
R = CH_3 = (*R*)-MDMA (**17b**)

R = H = (*R*)-MDA (**18b**)

Figure 10. Enantiomers of MDMA (**17**) - (*S*)-MDMA (**17a**) and (*R*)-MDMA (**17b**) and their metabolites – (*S*)-MDA (**18a**) and (*R*)-MDA (**18b**), respectively.

echocardiographic results.[60] Due to the prevalence of ecstasy use recreationally in young adults, it is a health risk to chronic users.[60] A typical dose of ecstasy (pill form) is 50-150 mg, and the second of 100 mg is usually taken after 3 hours, increasing the dosage to nearly 150-250 mg per usage.[57] In certain cases, MDMA is combined with other psychoactive drugs (most commonly, LSD) – a term referred to as "candy flipping".[64] Therefore, in order to reduce the amount of exposure in patients receiving experimental therapy, it is administered as a micro-dose.[65] The patients experienced significantly lower severity in PTSD symptoms following a twelve-month period of therapy.[65]

MDMA possesses a chiral center (Figure 9), the (*S)*-isomer (**17a**) (Figure 10) is a more potent serotonin releaser, whereas (*R*)-MDMA (**17b**) is more potent at 5-HT_{2B} receptors (EC_{50} = 900 nM) than (*S*)-MDMA (**17a**) (EC_{50} = 6000 nM), possessing approximately six-fold higher potency.[4] The eutomer of MDA is not known, i.e., (*R*)-MDA (**18b**) has an EC_{50} of 150 nM, its (*S*)-isomer (**18a**) has an EC_{50} of 100 nM, making them roughly equipotent.[60] In 1998, the X-ray crystal structure of (S)-MDMA (**17a**) was determined by Morimoto et al.[15] The phenylisopropylamine scaffold of MDMA (**17**) is common to hallucinogenic amphetamine analogs. According to the study, the alpha-methyl group and the amine group are transposed with respect to each other in comparison to other hallucinogenic phenylisopropylamines (**19**) (shown in Figure 11) 1-(2,4,5-trimethoxyphenyl)propan-2-amine (TMA; **19a**) and 1-(2,5-dimethoxy-4-ethylphenyl)propan-2-amine (DOET; **20**).[13]



Phenylisopropylamine scaffold **19**; TMA **(19a)** (R = OMe; R' = NH_2) DOB - **19b** (R= Br; R' = NH_2)

DOET (**20**)

Figure 11. Structures of TMA (**19a**), DOB (**19b**) and DOET (**20**).

The comparative differences may help determine the features necessary for high affinity 5-HT_{2B} binding (as seen in DOB; **19b**)) in order to study its pharmacological effects in producing valvulopathy.

b) LSD – LSD (**21**), shown in Figure 12, is a hallucinogenic recreational drug. It belongs to the ergoline class of compounds and is a synthetically derived drug, first synthesized by Albert Hoffman in 1938 at the Sandoz company in Basel, Switzerland.[59] It became popular in the 1970s in several experimental studies for the treatment of alcoholism, anxiety, and depression.[66] Its hallucinogenic effects are mediated due to its activity at the 5-HT_{2A} receptor as shown by Glennon et al.[67] LSD (**21**) possesses two chiral centers at the 5- and 8-positions, and therefore exists as four optical isomers: (*R, R*), (*R, S*) aka *iso*-LSD, (*S, R*) and (*S, S*). The (*R, R*) isomer is the most stable and possesses the highest affinity for the 5-HT_2 receptor class, while the other isomers are unstable and the 8-position epimerizes back from the (*S*)-configuration to (*R*)-configuration under basic conditions.[68] Due to its high affinity at 5-HT_2 receptor class, tritiated LSD is used as a radioligand for affinity studies. It acts as a potent agonist at 5-HT_{2B} receptors.[10] There have been no reports of cardiac valvulopathy caused by LSD (**21**). Perhaps, due to being a potent agonist and recruiting beta-arrestin pathway faster than a low efficacy agonist.

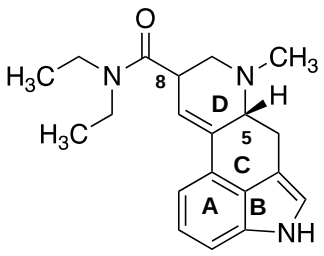
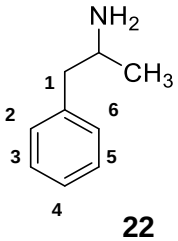


Figure 12. (5*R,8R)*-Lysergic acid diethylamide (LSD) (**21**).

The X-ray crystal structure of LSD (**21**) was first published by Baker et al. in 1972.[37] The constrained ring systems of LSD (**21**) confers near planarity to the molecule with the exception of the diethylamide side chain. The activity of LSD (**21**) was highly attributed to its conformation. Thus, it was important to determine if the natural crystallographic conformation is the bioactive conformation when binding to the 5-HT_{2A} receptor. The small molecule X-ray crystal structure was moderately predictive of the active state of LSD. However, in 2017 LSD was co-crystallized with the 5-HT_{2B} receptor that serves as a suitable model for the 5-HT_{2A} receptor.[12] The co-crystal structure and molecular dynamics simulations demonstrate LSD does not visit the conformation observed in the small molecule crystal structure.[12] This may be attributed to structural strain imposed by the crystal formation that influences the conformation of the molecule.

5. Phenylisopropylamines: structure-activity relationships

In 1984, Glennon et al. proposed that classical hallucinogens produce their effects by activating (agonists) 5-HT_{2A} receptors.[67] It has been established that hallucinogenic phenylisopropylamines primarily act as agonists at 5-HT_2 receptors, thus they qualify as classical hallucinogens by mediating their effects through the agonism of 5-HT_{2A} receptors.[67,70] They have varying degrees of selectivity at the three types of 5-HT receptors. When substituted at 2- and 5-position of a phenylisopropylamine system (**22**), the compounds are referred to as DOX compounds (Figure 13). Amongst hallucinogens, the DOX series possess the highest affinity for 5-HT_2 receptors.[70] DOX compounds are arylalkylamines, a class of widely studied hallucinogens.[70] DOI (**19c**) has been used to characterize and study 5-HT receptors for radioligand binding studies and functional tests.



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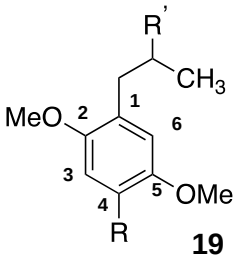


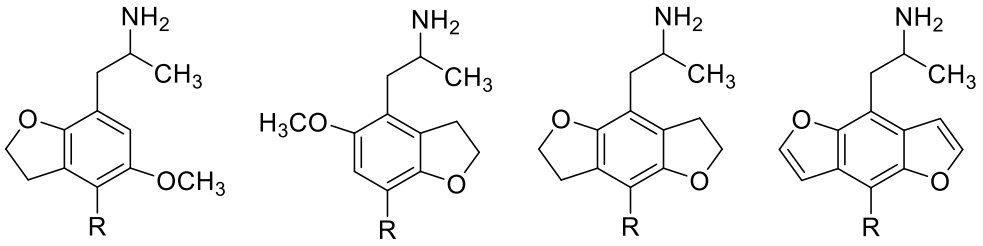
19

Figure 13. Phenylisopropylamine (amphetamine) (**22**), and general scaffold of 4-substituted 2,5-dimethoxyamphetamines (DOX compounds) (**19**) where R = I; R' = NH_2 for DOI (**19c**) R' may be primary/secondary/tertiary amine.

With one chiral center in the isopropylamine chain, they are di- or tri-substituted, typically with methoxy groups at 2- and 5-positions, and a variable substituent at the 4-position on the phenyl ring. The affinity increases with mono-, di- and tri- substitution (methoxylation).[70] The 2,4,5-substitution pattern is deemed ideal for high hallucinogenic activity.[71]

Structure-activity relationships have been previously established in the tests conducted on rat fundus muscle, and other isolated tissue preparations.[70] Subsequent studies utilized rat brain homogenates, and later, cloned human 5-HT_{2A} receptors. The general SAR derived from these systems is fairly consistent. The presence of an alpha-methyl group contributes to the affinity of phenylisopropylamines, in comparison to phenethylamines which have lower affinity. N,N-Dimethylation halves the affinity at 5-HT_2 receptors.[70] Addition of methyl group at the 4-position increases affinity, and no appreciable increase in affinity is seen with a 4-ethyl substitution. In addition, the activity of 4-substituted 2,5-dimethoxyphenylisopropylamines (**19**) exists as a continuum from agonist to antagonist based on the nature of the 4-position group.[70] The phenylpropyl substituent at the 4-position was the first compound of the DOX class of compounds that behaved as an antagonist, and these antagonists have a distinct structure affinity

relationship, as the methoxy substituents at 2- and 5- positions are less favorable than 2,3-, 3,5-, and 2,6-positions.[71] The 4-position modulates the affinity of phenylisopropylamines for the 5-HT_{2A} receptor, and a Hansch analysis of the substituents of the series conducted in a previous study suggests an increase in affinity at 5-HT_2 receptors with an increase in lipophilicity of the substituent at this position.[71] The high affinity of the DOX series may be attributed to the multiple substitutions at the phenyl ring.[70] Substituents with less bulk behave as agonists and adopt a distinct binding pose in contrast to antagonists which are bulkier and interact with more residues, thus accessing the secondary binding pocket.[72] Compounds with a bulky 4-position substituent behave as antagonists and no longer require the features necessary for 5-HT_{2A} binding, for example, removal of the methoxy substituents is tolerated in phenethylamines with a 4-phenylpropylamine substituent.[72]



24 **25** **26** **27**

Figure 14. Structures of methoxy-constrained phenylisopropylamines.

The orientation of the methoxy groups is preferred to be planar, which serves as evidence for the decreased affinity of the 2,4,5-trimethoxyamphetamine (TMA) (**19a**) in comparison to the more constrained compounds (e.g. **25**).[73] In order to further study the orientation of the two methoxy groups, constrained analogs of 2,5-dimethoxy-4-substituted compounds

were prepared systematically (Figure 14), by first individually constraining the 2-position and the 5-position methoxy group as seen in **24** and **25** respectively, and finally, both, to provide benzodihydrofuran ring, seen in **26**.[73]

The benzodihydrofuran ring constraining 5-position methoxy ring of DOB (**19b**) - **25**, showed an improvement in affinity to K_i = 3.1 nM in a rat prefrontal cortex preparation, and the benzodihydrofuran ring constraining both methoxy groups yielded the 'dragonfly' analog of DOB (**19b**) (i.e., **26** where R = Br), which drastically improved the affinity to 0.48 nM in a cloned 5-HT_{2A} receptor population.[73]

Aromatization of the dihydrofuran to furanyl rings resulted in **27**, leading to potent compounds (affinity and potency data not provided). Increasing the ring size of **25** to a dihydropyran resulted only in a slight/insignificant lowering of affinity (K_i = 3.7 nM).[73] However, the same structural changes did not produce the same effects in 3,4,5-methoxy substituted compounds, indicating they may bind in a different manner.[73] There was no apparent difference in affinity found in racemates of DOX related compounds upon alpha methylation, however, in compounds such as DOB (**19b**), DOM and MDA (**18**), the *R*(-)-enantiomer was the eutomer.[67] Phenylisopropylamines and aminotetralins were considered to be structurally similar to the ring C and D of ergolines.[73] The R-stereochemistry of the alpha methyl carbon in phenylisopropylamines and the *5*-position of LSD (**21**) was shown to be optimal for affinity at 5-HT_{2A} receptors.[73] These structural similarities have prompted studies which compare the binding poses of LSD to DOX series compounds.[73] To explore the favorable conformations of the alkylamine chain, cyclobutene analogs were prepared to identify if the extended chain is in the same plane as found in LSD, or away from the plane of the ring.[73] The phenylisopropylamines were

found to adopt a conformation with the alkylamine chain away/perpendicular to the plane of the benzene ring.

Binding modes: There has been statistically significant correlation of the binding of phenylisopropylamines at 5-HT_{2A} receptors, and 5-HT_{2B} and 5-HT_{2C} receptors.[9] The binding modes of DOB (**19b**) and DOI (**19c**) were initially studied in models of 5-HT_{2A} receptors by Parrish et al.,[16] and Runyon et al.[17] with a homology model constructed from the bovine rhodopsin sequence. Subsequently, a β_2 adrenergic receptor-based homology model was published by Kanagarajadurai, et al.[18] Since the 5-HT_{2A} receptor serves as a model for the 5-HT_{2B} receptor and vice versa, previous models serve as a suitable template to validate the docking results obtained with the 5-HT_{2B} receptor. The model proposed by Runyon, et al.,[17] was based on the premise of two distinct binding sites - 1 and 2 for agonist and antagonists, respectively. Site 1 engages residues in helices TM3, TM4, TM5, and TM6; and Site 2 – TM1, TM2, and TM7.[17] Transmembrane helices TM3 (Asp155$^{3.32}$, Ser159$^{3.36}$) and TM6 (Trp336$^{6.48}$, Phe339$^{6.51}$) are in the subset of shared between the two sites.[17] Included in this model of agonist binding mode of the 5-HT_{2A} receptor with common agonists such as 5-HT, LSD and dopamine, (dopamine also being an agonist at 5-HT_{2A} receptor).[17] The model of the 5-HT_{2A} receptor with bovine rhodopsin receptor as a template, proposed interactions that were seen with DOI (**19c**).[17] As predicted, compounds with small substituents as found in DOB (**19b**), adopt an agonist-preferred position engaging residues in Site 1.[17] The 4-position substituent binds in the interfacial region between TM5 and TM6. While addition of methyl and ethyl groups are tolerated at the 4-position, the ethyl group is thought to displace the aromatic ring from its initial site by 2.3 Å upon minimization.[17] Bulky 4-position substituted compounds are

oriented in a way as to access Site 2, adopting a more antagonist-like pose, as validated by functional activity studies.[17]

Stereoisomers of DOB (**19b**), *R*(-)-DOB and *S*(+)-DOB, both have high affinity for the 5-HT_{2A} receptor, with the *R*(-) isomer showing ~7-fold higher affinity (K_i = 0.29 nM) than the *S*(+)-isomer (K_i = 1.9 nM).[17] Modeling studies conducted on the isomers of DOB (**19b**) in the 5-HT_{2A} receptor by Parrish et al.,[16] asserts the differential orientation of the residues lining the agonist binding pocket. The models published by Parrish et al.[16] and Runyon et al.[17] differ in the π-stacked interaction between Phe340$^{6.52}$ and Phe243$^{5.47}$, which may be crucial for maintaining the orientation of the transmembrane helices. Mutation of Phe340$^{6.52}$ in 5-HT_{2A} receptors abolished affinity for DOX compounds, indicating the importance of aromatic interactions in their binding.[16] The aspargine (Asn344$^{6.55}$) on helix 6 is crucial for the binding of DOB (**19b**) as evidenced by its increased affinity for 5-HT_2 receptors, as it is highly conserved in 5-HT_2 receptors as opposed to 5-HT_1 receptors.[17]

6. DOB/DOI and their interactions observed in 5-HT_{2A} receptor models

a. Parrish model

The model proposed by Parrish et al. compares the binding of the (*R*)- and (*S*)-isomers of DOB (**19b**) within the 5-HT_{2A} receptor binding pocket.[16] In conjunction with functional activity studies indicating the (*R*)-isomer to have higher affinity and potency, the model examines the interactions with key residues found in the agonist binding pocket: Phe240$^{5.44}$, Phe243$^{5.47}$, Phe244$^{5.48}$, and Phe340$^{6.52}$, and Ser239$^{5.43}$.[16] The orientation of Ser239$^{5.43}$ is significantly altered for both enantiomers.[16] Phe243$^{5.47}$ is displaced in the (*S*)-isomer-docked receptor, while the 4-substituent of the (*R*)-isomer

forms a hydrophobic interaction with the residue.[16] The alpha methyl group undergoes van der Waals interactions and the aromatic ring, an edge to face interaction with Phe340$^{6.52}$ which forms a tight association between the ligand and the residue.[6] The residues Ser239$^{5.43}$, Phe243$^{5.47}$ and Phe340$^{6.52}$ show different orientations when the (*S*)-enantiomer binds but not with the (*R*)-enantiomer.[16] Phe339$^{6.51}$ on the other hand, shows no perturbation in the docked ligand models and is supported by mutagenesis data as not being strictly interacting with small molecule agonists.[16]

b. Runyon model

The 4-bromo substituent in DOB (**19b**) is oriented between TM5 and TM6.[17] The 2-methoxy group accepts a hydrogen bond from N343$^{6.55}$.[17] The 5-methoxy group is near Ser159$^{3.36}$, Thr160$^{3.37}$, and Ser242$^{5.46}$, and the methyl group of 5-methoxy and W336$^{6.48}$.[17] Other aromatic interactions are seen with Phe243$^{5.47}$, Phe339$^{6.51}$, and Phe340$^{6.52}$ (Figure 15). There is no H-bonding residue for the small 4-position substituents in the binding pose adopted by DOX compounds.[17] Larger 4-position substituents were found to be folded onto the ligands aromatic ring and the aromatic ring is displaced towards TM4.[17]

Antagonists of the DOX compounds place the phenyl ring between Asp155$^{3.32}$ and Val366$^{7.39}$. This pose is stabilized in the binding site through H-bonding of DOX amine moiety with Asp155$^{3.32}$ and Ser159$^{3.36}$, and the 5-methoxy group is stabilized with Trp151$^{3.28}$ and Ser226$^{x12.49}$, although they are not considered ideal.[17] The large substituents are oriented towards Site 2.[17]

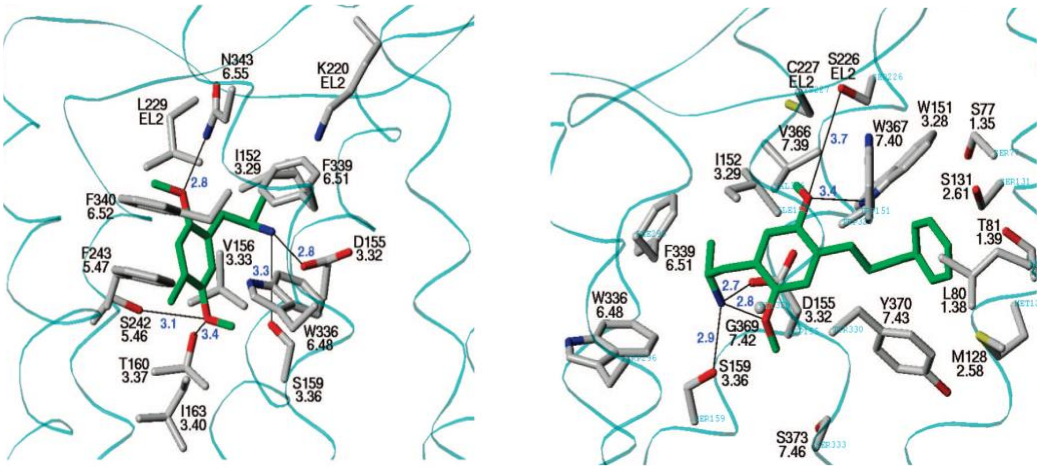


Figure 15. Model of (*R*)-DOI and 4-phenylpropyl substituted DOX compound docked in 5-HT$_{2A}$ receptor published by Runyon et al.[17]

The model constructed by Runyon et al.[17] presents a π-stacked interaction between F340$^{6.52}$ and F243$^{5.47}$. The validity of the model is based on the rotameric angle (χ_1) of the conserved residues Trp336$^{6.48}$ and Phe340$^{6.52}$ in the aromatic box, which are a part of the rotameric "toggle switch".[17] This switch is also known as an "ionic lock" found in rhodopsin-family GPCRs that has been proposed to link the cytoplasmic ends of TM3 and TM6.[74] Disruption of the lock is necessary but not sufficient for activation of the GPCRs, since partial agonists were as effective as full agonists in disrupting the lock.[75] The torsion angle of the residues with respect to the "proline kink" found in TM6, is responsible for the distance between the intracellular ends of TM3 and TM6 which forms the toggle switch.[17] The proline kink is a bend or kink found in helices of proteins and often plays a functional role.[75] At these kinks, the helices change direction and the hydrogen bonding pattern is broken.[75] They are identified by specifying an upper limit for differences in the angle of the helical axis of two sets of a specific number of residues (depending upon the platform), and annotating the residues which exceed the upper limit.[75] The rotameric angle (χ_1) for validation of the model is trans for the two residues, which was previously reported.[17]

c. Kanagarajadurai model

Kanagarajadurai et al.[18] developed a model of the 5-HT_{2A} receptor emplyoing the β_2 adrenergic receptor as a template. This model revealed a set of residues that were specific to 5-HT_{2A} receptors that aids in distinguishing it from other 5-HT_2 receptors. The docking study was conducted on endogenous ligands – 5-HT and dopamine and agonists – DOI (**19c**) and LSD, along with inverse agonists – clozapine and risperidone, and antagonists – ketanserin and haloperidol.[18] The study, was validated with mutagenesis experiments where the key residue Ser159 was predicted to be vital in anchoring the protonated amine of serotonin and other ligands.[18] Mutation of the residue reduced the affinity by 17.6-fold for serotonin and *N,N*-dimethyl-5-HT.[18] Mutation of Y370 diminishes affinity for serotonin and DOM but not for α-methyl-5-HT, further indicating a different binding mode for serotonin and α-methyl-5-HT, suggesting that the α-methyl group causes a difference in binding mode of tryptamines. Human 5-HT_{2A} share 43% homology with 5-HT_{2B} receptors and 54% with 5-HT_{2C} receptors.[18] The docked DOI (**19c**) was able to form a single cluster.[18] The model indicates interactions with TM3, TM5, TM6, TM7, ECL2 and ECL3.[18] The residues common to DOI (**19c**) and LSD are Ser239, Ser159, Asp231, Phe339, Phe340, Val235 and Gly238.[18] S239 is necessary for the binding of DOI (**19c**) as evidenced in mutagenesis but it is unclear if it may be due to secondary intramolecular interactions and if it may be necessary to create an activated receptor.[18] Summarized below (Table 1) is a comparison of the three models. Due to several confounding factors such as a difference in templates and the small size of the molecules in comparison to the binding pocket, there are differences found in the models proposed by the three groups.

Table 1. Comparison of prior models of DOX compounds docked in the 5-HT_{2A} receptor in chronological order

Substituents of DOX molecule	Residues (Parrish model, 2005)	Type of interaction	Residues (Runyon model, 2008)	Type of interaction	Residues (Kanagaraja-durai model, 2009)	Type of interaction
Amine	-	-	$D155^{3.32}$ $S159^{3.36}$	$F340^{6.52}$	D231	H-bond
Isopropyl chain	-	-	$V156^{3.33}$	van der Waals	V235 G238 S239	Hydrophobic Hydrophobic Not mentioned
α-Methyl group	$F340^{6.52}$	van der Waals	$I152^{3.29}$	$I152^{3.29}$	-	-
2-Methoxy group	-	-	$N343^{6.55}$	Hydrophobic	$N343^{6.55}$	Not mentioned/ van der Waals
5-Methoxy group	$S239^{5.43}$ $F243^{5.47}$	Not mentioned	$W151^{3.28}$ $S226^{X12.49}$ $S242^{5.46}$ $W366^{6.48}$	Hydrophobic Hydrophobic H-bond Hydrophobic	$S159^{3.36}$	H-bond
Phenyl ring	$F340^{6.52}$	Edge-to-face	$F243^{5.47}$ $W336^{6.48}$ $F340^{6.52}$	Hydrophobic Edge-to-face Hydrophobic	L229 $F340^{6.52}$	Hydrophobic Edge-to-face
4-Position substituent	-	-	$F243^{5.47}$	Hydrophobic	F339	Hydrophobic

III. Specific aims and rationale

Seventeen phenylisopropylamines of the DOX series were synthesized in the Glennon laboratory,[9] and tested at the three subtypes of 5-HT_2 receptors (5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}). These compounds were also compared for their binding affinity at 5-HT_{2C} vs 5-HT_{2A}, 5-HT_{2B} vs 5-$HT_{2A,}$ and 5-HT_{2B} vs 5-HT_{2C} receptors.[9] Cloned human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, and rat 5-HT_{2B} receptors were used as expression systems.[9] The compounds varied, primarily, in the 4-position substituent, which is a vital modulator of affinity.[9] Comparison of the receptor affinities showed a strong positive correlation between 5-HT_{2A} and 5-HT_{2C} receptor affinities (r = 0.93), and 5-HT_{2A} and 5-HT_{2B} receptor affinities (r = 0.92).[9] The slope for 5-HT_{2C} vs 5-HT_{2A} receptor (0.93) affinity was higher than that observed for 5-HT_{2B} vs 5-HT_{2A} affinity (0.53), indicating a less direct relationship of affinity for 5-HT_{2B} vs 5-HT_{2A} receptors.[9] This suggests a difference in the binding pockets of the two receptors.[9]

The availability of the X-ray crystal structure of the 5-HT_{2B} receptor[12] will serve as a tool for studying the interactions of the abovementioned phenylisopropylamines and serve as a point of comparison for the previously published 5-HT_{2A} receptor models with DOB (**19b**)/DOI (**19c**).[16-18] Although the functional activity of the phenylisopropylamines at 5-HT_{2B} receptors is not entirely known, extrapolations with the data available at 5-HT_{2A} receptors may serve in predicting the functional activities. The binding mode of

phenylisopropylamine 5-HT_{2A} agonists is distinct from that observed for antagonists in that the bulkier substituents access a secondary binding pocket and thus orients the phenyl ring and the 2,5-dimethoxy substituents differently.[17] If similarities are found in the binding modes at both receptor models, an SAR can be developed for 5-HT_{2B} agonists for this class of compounds.

Since the SAR of phenylisopropylamine 5-HT_{2A} receptor agonists has been well established, the strong positive correlation in the affinities at 5-HT_{2B} receptors vs 5-HT_{2A} receptors serves as a stepping stone to develop the SAR of 5-HT_{2B} receptor ligands of the phenylisopropylamine class.[9] Included in this class of 5-HT_{2B} agents, are also fenfluramine (**11**), norfenfluramine (**12**), MDMA (**17**) and MDA (**18**)–known valvulopathogenic agents at the commonly used dosage. Therefore, quantitative structure-activity relationship (QSAR) studies may serve as a useful tool to recognize the nature of ligands and their substitution pattern for high affinity at these receptors.

The rank order of selectivity of phenylisopropylamines is 5-HT_{2A} > 5-HT_{2B} > 5-HT_{2C}.[10] In contrast, (*R*)-fenfluramine, which possesses a phenylisopropylamine scaffold without the 2,4,5-substitution pattern, has about 2-fold higher selectivity for 5-HT_{2B} receptors in comparison with the 5-HT_{2A} receptors.[76] The rank order of affinity of fenfluramine and norfenfluramine isomers, as shown in Table 2, depicts the selectivity, where (*S*)-fenfluramine differs from (*R*)-fenfluramine in its rank order of affinity, and the norfenfluramine isomers are of the same order, with their highest affinity for 5-HT_{2C} receptors.[76] In general, (*R*)-norfenfluramine binds at all three receptor populations with twice the affinity of (*S*)-norfenfluramine.

Table 2. Affinities of fenfluramines and norfenfluramines at the three subtypes of 5-HT_2 receptors.[76]

Receptor	(S)-Fenfluramine K_i (nM) ± S.E.M	(*R*)-Fenfluramine K_i (nM) ± S.E.M	(*R*)-Norfenfluramine K_i (nM) ± S.E.M	(*S*)-Norfenfluramine K_i (nM) ± S.E.M
5-HT_{2A}	2470 ± 240	1430 ± 330	187 ± 10	267 ± 16
5-HT_{2B}	3920 ± 830	680 ± 16	56 ± 19	99 ± 12
5-HT_{2C}	2080 ± 480	1620 ± 340	27 ± 7	65 ± 23
Order (5-HT)	2C~2A> 2B	2B > 2C/2A	2C > 2B > 2A	2C > 2B > 2A

The specific aims of the current project are:

1. **To conduct 3D-QSAR studies on phenylisopropylamines to determine the nature of substituents that confer high affinity.**

   Approach:

   - Perform a comparative molecular field analysis (CoMFA) and/or comparative molecular similarity indices analysis (CoMSIA).

2. **To study the interactions of the phenylisopropylamines with the 5-HT$_{2B}$ receptor.**

   Approach:

   - Dock the phenylisopropylamines in the crystal structure of the receptor and study their binding poses.
   - Perform HINT (Hydropathic INTeraction) analysis to determine the quality of the model generated based on the HINT score obtained.

The models generated may provide insight on the functional activity of the compounds, based on difference in binding poses observed in agonistic vs. antagonistic phenylisopropylamines at the 5-HT$_{2A}$ receptor.

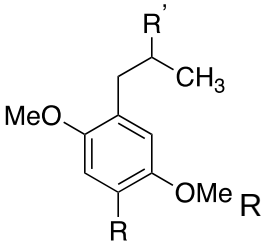
An indirect goal of these studies is to formulate SAR for the binding of phenylisopropylamines at 5-HT$_{2B}$ receptors. To date, only a few phenylisopropylamines have been shown to bind at 5-HT$_{2B}$ receptors (e.g., those shown later in Figure 3). And none of them has been examined for their valvulopathogenic action. Some phenylisopropylamines (e.g., MDMA and norfenfluramine) are recognized valvulopathogens, but to date, no 5-HT$_{2B}$ serotonin receptor SAR is available.

III. Results and discussion

GOAL 1. To conduct 3D-QSAR studies on phenylisopropylamines to determine the nature of substituents that confer high affinity.

The key step in conducting a 3D-QSAR study using CoMFA is a sufficiently large data set of approximately 20 molecules. Variation in substituents are made usually in preferably one, or two positions on a common scaffold. A CoMFA may be conducted on affinity data (K_i) or on potency (IC_{50}/EC_{50}) values (Table 3).

Table 3. Binding affinities of phenylisopropylamines (n = 17) at human 5-HT_{2B} receptors with two known agonists (blue).[9, 10]



	R	R'	Name	5-HT_{2B} K_i, nM (±SEM)	**5-HT_{2B} pK_i**	Functional activity
19a	-OCH_3	-NH_2	TMA	307.0 (21)	**6.51**	NA[a]
19b	-Br	-NH_2	DOB	26.9 (4)	**7.57**	Agonist
19c	-I	-NH_2	DOI	20.0 (3)	**7.70**	Agonist

19d	-H	$-NH_2$	DMA	1039.0 (143)	**5.98**	NA
19e	-F	$-NH_2$	DOF	227.0 (15)	**6.64**	NA
19f	-Cl	$-NH_2$	DOC	31.8 (2)	**7.50**	NA
19g	$-OC_2H_5$	$-NH_2$	MEM	763.0 (54)	**6.12**	NA
19h	$-COCH_3$	$-NH_2$	DOAC	313.0 (27)	**6.50**	NA
19i	$-NO_2$	$-NH_2$	DON	166.0 (28)	**6.78**	NA
19j	-CN	$-NH_2$	DOCN	774.0 (46)	**6.11**	NA
19k	$-nC_3H_7$	$-NH_2$	DOPR	54.4 (10)	**7.26**	NA
19l	$-nC_6H_{13}$	$-NH_2$	DOHx	30.3 (12)	**7.52**	NA
19m	$-tC_4H_9$	$-NH_2$	DOTB	24.6 (6)	**7.61**	NA
19.	$-CH_2\phi$	$-NH_2$	DOBZ	35.0 (3)	**7.46**	NA
19o.	-Br	$-N(CH_3)$	M-154	341.0 (49)	**6.47**	NA
19p.	-Br	$-NH-C_3H_7$	D-367	521.0 (6)	**6.28**	NA
19q.	-Br	$-N^+(CH_3)_3$	QDOB	>10,000	**NA**	-

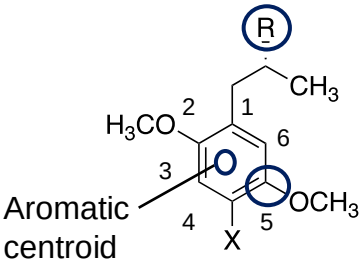
[a]NA*:* Not applicable since no studies were conducted.

1. CoMFA and CoMSIA studies on 5-HT_{2B} receptor affinities

By using the pK_i values of these ligands at 5-HT_{2B} receptors (Table 3), a 3D-QSAR study (CoMFA and CoMSIA) was conducted using Sybyl X2.1.1. CoMFA assesses the interactions of aset of molecules with an electrostatic grid to determine the steric and electrostatic substituents necessary for the affinity of a compound, whereas CoMSIA assesses similarities based on five criteria: steric, electrostatic, hydrophobic, hydrogen-bond donor and hydrogen-bond acceptor fields amongst the compounds that may confer affinity. The key step in conducting CoMFA was to obtain a proper alignment of three non-colinear points common to all the structures within the series. Upon attempting multiple points of alignments, the aromatic centroid, C5 of the benzene ring, and the nitrogen atom of the amine were found to be most suitable for common points of substitutions on the phenyl ring amongst the series of compounds (Figure 16A and 16B).

The initial CoMFA study including only DOX compounds (n = 16; QDOB (**19q**) has very low affinity K_i > 10,000 nM, hence not included) with 5-HT_{2B} receptor affinities yielded a q^2 value of -0.118 with a standard error of prediction of 0.893 with the optimal number of components being 1. For CoMSIA, a q^2 value of 0.612 was obtained with the standard error of prediction being 0.453. Further PLS analyses were conducted by the removal of the field having the lowest contribution, the optimal value was q^2 = 0.669 upon removal of electrostatic, donor and acceptor fields, leaving only steric and hydrophobic fields. Certain fields may be excluded if their contribution to the q^2 value is under 15%.

A



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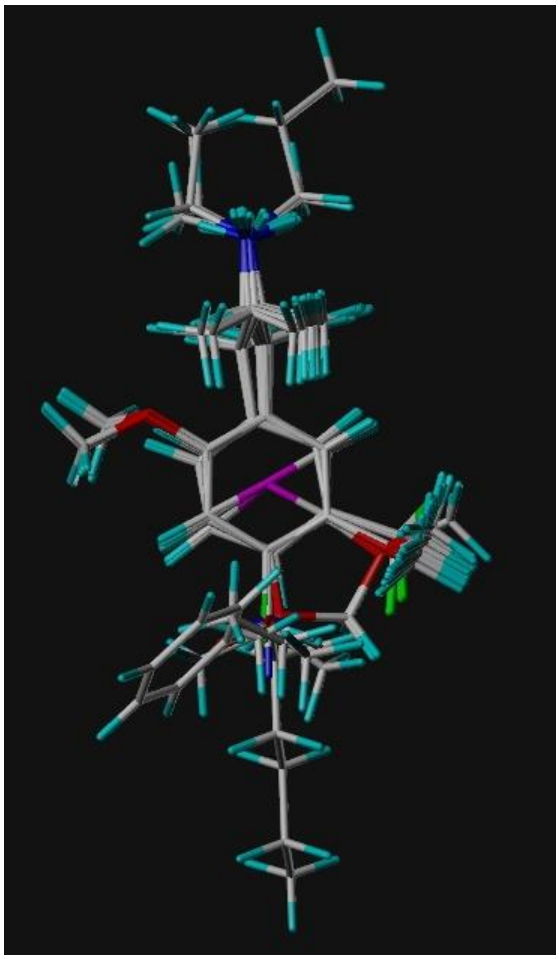


Figure 16. (A) General structure of DOX compounds (**19**), including points of alignment (blue). (B) Aligned compounds using the “Fit atom” command in Sybyl X2.1.1 (n = 22), includes the sixteen DOX compounds from the initial study, *R*- and *S*- isomers of MDA, and *R*- and *S*-isomers of norfenfluramine and fenfluramine.

A negative q^2 value is a sign of no model and is mainly due to a narrow range of affinity values, i.e., when the standard error of prediction (PRESS) is larger than the standard deviation.[77] The K_i values at 5-HT_{2B} receptors represent a 50-fold range.[9] Figure 17 provides a representation of the distribution of pK_i values at the 5-HT_{2B} receptor.

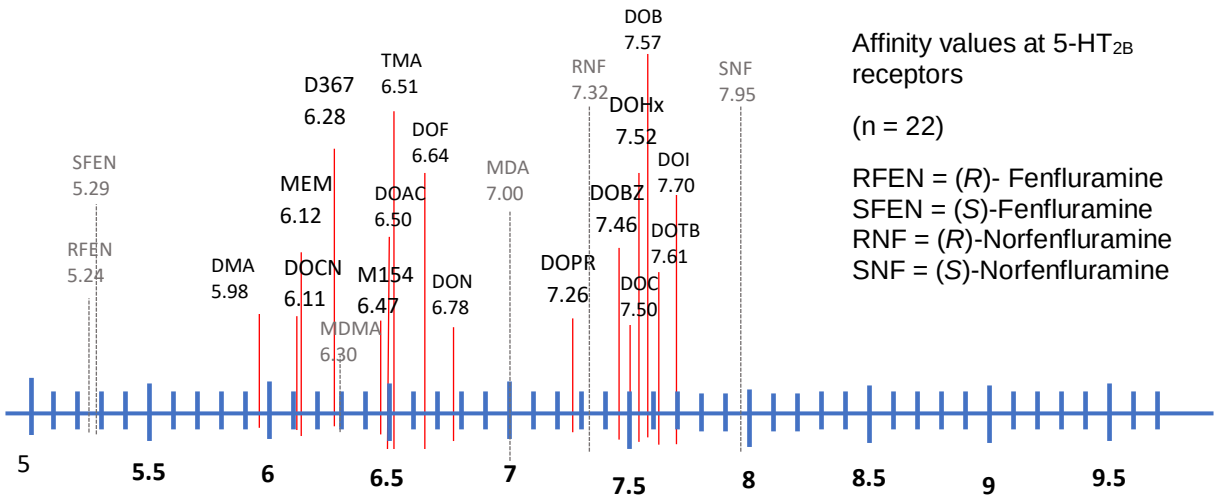


Figure 17. Distribution of pK_i values of phenylisopropylamines at 5-HT_{2B} receptors. Data in red represent the initial study (i.e., n = 16).

The value for q^2 did not improve beyond negative values for CoMFA after reducing column filtering or modifying the region file to include smaller step sizes. Thus, in order to increase the diversity of the dataset, the R and S isomers of the well-known 5-HT_{2B} ligands fenfluramine (**11**) and norfenfluramine (**12**) were added (n = 20).[6] The CoMFA on 5-HT_{2B} with fenfluramine (**11**) and norfenfluramine (**12**) isomers, improved the q^2 value to 0.240, which was not the case with CoMSIA (q^2 = 0.190).

Operating on the same principle of increasing diversity, known ligands of the 5-HT_{2B} receptor MDA (**18**) and MDMA (**17**) were added to the data set (n = 22). A major caveat to this addition is the lack of availability of individual enantiomeric affinities, therefore R

isomers were used in the CoMFA study, with the affinities of the racemic mixtures. The q^2 value for CoMFA was 0.325, while the CoMSIA model was not as robust, yielding q^2 = 0.181. This CoMSIA model was however fine-tuned to obtain a q^2 of 0.54 by excluding the fields that had the lowest contribution. By doing so, steric and hydrophobic fields were the highest contributors, operating at a 0.68:0.32 ratio. The CoMSIA representation is displayed in Figure 18 with the yellow and white areas representing favorable and unfavorable contour maps (80:20 ratio of favorable: unfavorable visualization) for hydrophobic substituents, and the blue and red contour map displaying favorable and unfavorable areas for steric bulk, respectively (Figure 18) This figure suggests hydrophobicity to be favorable at the 4- position, whereas it is not well tolerated at the amine, which can be determined from a cursory examination of the trend of the pK_i values in the data set. However, the q^2 value provides validation of this trend, suggesting better predictive power.

According to Cramer,[78] on the basis of CoMFA investigation of a steroid data set, the presence of constrained analogs is said to improve the predictivity of the model due to a known favorable conformation of the compound. Hence, (+) (5*R,8R*) LSD (**21**), the 5-HT_{2B} receptor co-crystal structure of which was recently reported, was added to this data set to determine if it might improve the model (pK_i = 8.43).[12] This brought about little improvement in the result (CoMFA q^2 = -0.334; CoMSIA q^2 = 0.279) (n = 23). In order to align the structures to more closely resemble LSD (**21**), the energy minimized structures of the data set were aligned with the crystal structure as the template using both atom fit as well as multifit approaches.

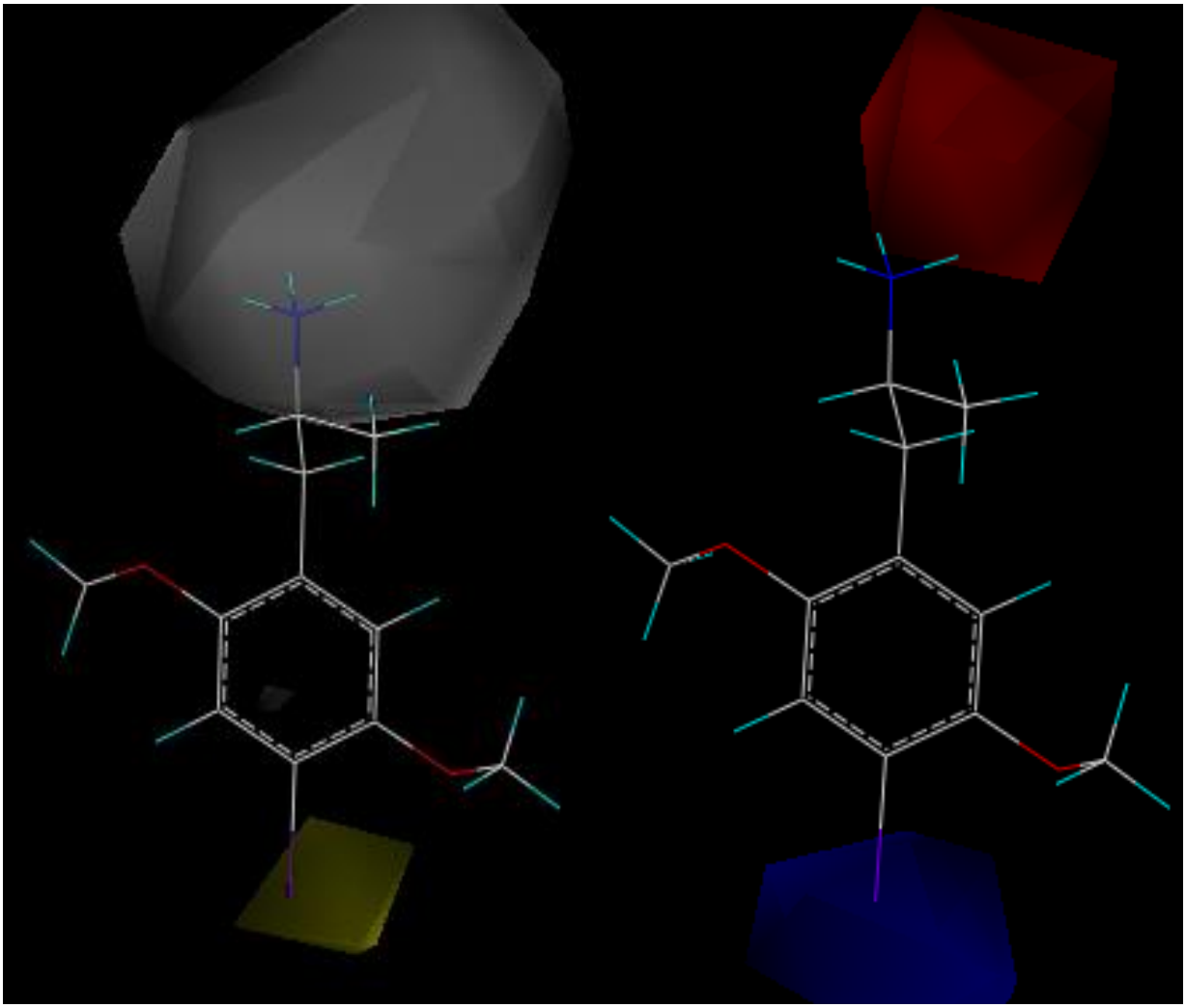


Figure 18. 5-HT_{2B} receptor (n = 16) hydrophobic (left) and steric (right) CoMSIA fields of DOI (**19c**). For the hydrophobic field, yellow and white areas indicate favorable and unfavorable substitutions; for the steric field, blue and red areas indicate favorable and unfavorable areas, respectively.

The above data are entirely based on atom fit, however multifit served little purpose, as the difference in energies between the energy-minimized structures and multifit modified structures was roughly 5 kcal/mol for the entire data set and the CoMFA q^2 was -0.350. Since the purpose of this modification was to mimic the structure of LSD (**21**) with phenylisopropylamines, the spring constant was modified to its reasonable limit (20 kcal/mol$Å^2$), and the desired conformer could not be constructed. The conformer of

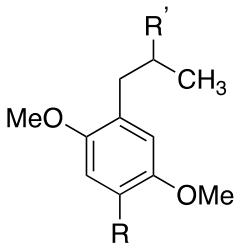
DOB (**19b**) that mimics LSD exists at 69.45 kcal/mol according to the top ranking (ChemPLP score) docking solution in GOLD v5.4, as opposed to its lowest energy conformer at 35.90 kcal/mol. A CoMFA study on the energy minimized structure of LSD was also conducted (data not included), but led to little improvement of the model.

There are certain trends observed in the affinity values of the compounds at the 5-HT_{2B} receptor, which on comparison with 5-HT_{2A} receptor affinities, provide clues about the differences in the binding pockets of both receptors. For example, compounds with increased 4-position chain length, (DOHx (**19l**), DOBZ (**19n**), and DOPR (**19k**)) display a >50-fold increase in 5-HT_{2A} receptor affinity relative to DMA (**19d**), whereas this is not the case with 5-HT_{2B} receptor affinity.[9] This is strongly evident in the case of DOHx (**19l**), where the affinity is 303 times greater at 5-HT_{2A} receptors than at 5-HT_{2B} receptors.

1. CoMFA study on 5-HT_{2A} receptor affinities

Since a potential reason for low predictivity was the narrow range of affinity values at 5-HT_{2B} receptors, CoMFA studies on the same compounds with affinity values at 5-HT_{2A} receptors (Table 4) were conducted as a comparative study, and yielded a q^2 of 0.398 (quaternary DOB or QDOB (**19q**) not included, n = 16) with a standard error of 1.027, and the CoMSIA study, $q^2 = 0.606$.

Table 4. Binding affinities of phenylisopropylamines (n = 17) at human 5-HT_{2A} receptors; 8 are known to be agonists, 3 partial agonists and one antagonist (blue, light blue and red respectively).[9,]



	R	R'	Name	5-HT_{2A} K_i, nM (±SEM)	**5-HT_{2A} pK_i**	Functional activity
19a	-OCH_3	-NH_2	TMA	57.9 (3.3)	**7.24**	Agonist[88]
19b	-Br	-NH_2	DOB	0.6 (0.1)	**9.22**	Agonist[87]
19c	-I	-NH_2	DOI	0.7 (0.006)	**9.15**	Agonist[5.5,87]
19d	-H	-NH_2	DMA	211.0 (15)	**6.68**	Partial agonist[87]
19e	-F	-NH_2	DOF	41.7 (1.8)	**7.38**	Agonist
19f	-Cl	-NH_2	DOC	1.4 (0.04)	**8.85**	Agonist[89]
19g	-OC_2H_5	-NH_2	MEM	73.0 (10.0)	**7.14**	Agonist[93]
19	-$COCH_3$	-NH_2	DOAC	80.5 (12.1)	**7.09**	NA[a]
19i	-NO_2	-NH_2	DON	5.5 (0.6)	**8.26**	Partial agonist[87]

19j	-CN	$-NH_2$	DOCN	45.7 (0.9)	**7.34**	NA
19k	$-nC_3H_7$	$-NH_2$	DOPR	0.9 (0.01)	**9.05**	Agonist[88-90]
19l	$-nC_6H_{13}$	$-NH_2$	DOHx	0.1 (0.03)	**10**	Antagonist[92]
19m	$-tC_4H_9$	$-NH_2$	DOTB	3.7 (0.2)	**8.43**	Partial agonist[91]
19n	$-CH_2\phi$	$-NH_2$	DOBZ	0.4 (0.08)	**9.40**	Antagonist[92]
19o	-Br	$-N(CH_3)$	M-154	94.2 (6.8)	**7.03**	NA
19p	-Br	$-NH-C_3H_7$	D-367	88.5 (5.8)	**7.05**	NA
19q	-Br	$-N^+(CH_3)_3$	QDOB	2155 (56)	**5.67**	NA

[a]NA: Not applicable since no studies were conducted.

The positive and robust q^2 value might be due to not only a higher range (2,110-fold) of affinity values, but also a good dispersion of pK_i values over 3.32 log units (QDOB not included, n = 17). Figure 19 displays the contour map obtained for 5-HT_{2A} receptor CoMFA, the red areas indicating unfavorable substitution at 3-position may be explained by the addition of fenfluramine and norfenfluramine isomers to the data set. The best CoMSIA model (Figure 20) indicates that hydrophobic substituents at 3-position detracts from affinity, and similar to 5-HT_{2B} receptors, a large (steric) hydrophobic substituent at 4-position is favorable. Any substitutions on the primary amine are not well tolerated at both 5-HT_{2A} and 5-HT_{2B} receptors.

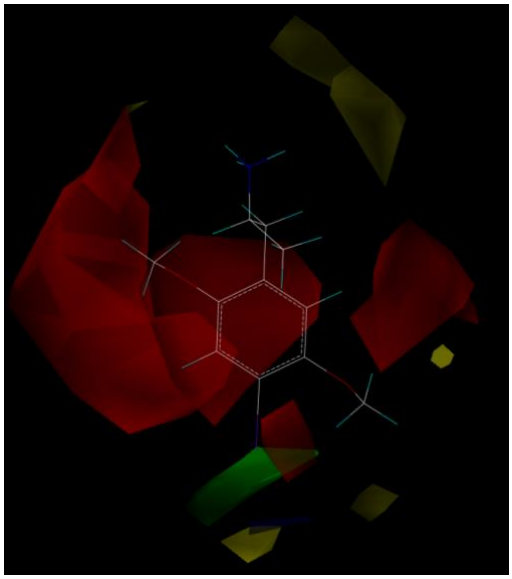


Figure 19. 5-HT_{2A} receptor CoMFA (n = 20) contour map. Blue and red areas represent favorable and unfavorable areas for electron withdrawing substituents, respectively. Green and yellow areas represent favorable and unfavorable areas for steric bulk, respectively.

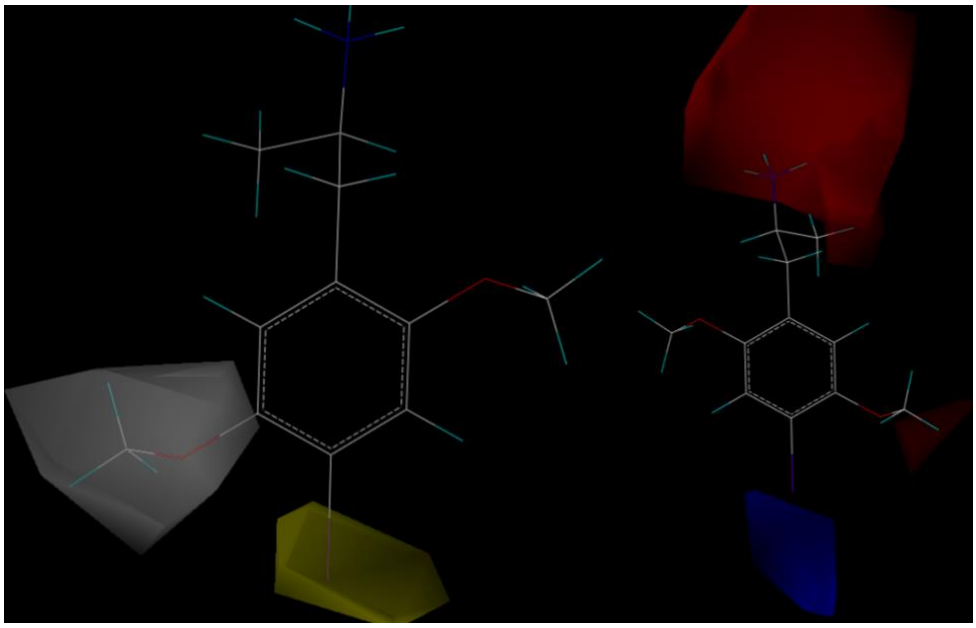


Figure 20. 5-HT_{2A} receptor (n = 20) hydrophobic (left) and steric (right) CoMSIA fields of DOI (**19c**). For the hydrophobic field, yellow and white areas indicate favorable and unfavorable substitutions; for the steric field, blue and red areas indicate favorable and unfavorable areas, respectively.

Figure 21 provides a visual representation of the distribution of pK_i values at 5-HT_{2A} receptors. To emphasize the importance of the range of pK_i values, adding QDOB to the 5-HT_{2A} CoMFA study barely showed appreciable improvement of the model (q^2 = 0.402 for CoMFA and q^2 = 0.302 for CoMSIA), however, addition of other well-known 5-HT_{2B} receptor ligands that also possess affinity for 5-HT_{2A} receptors showed a vast improvement of the 5-HT_{2A} model.

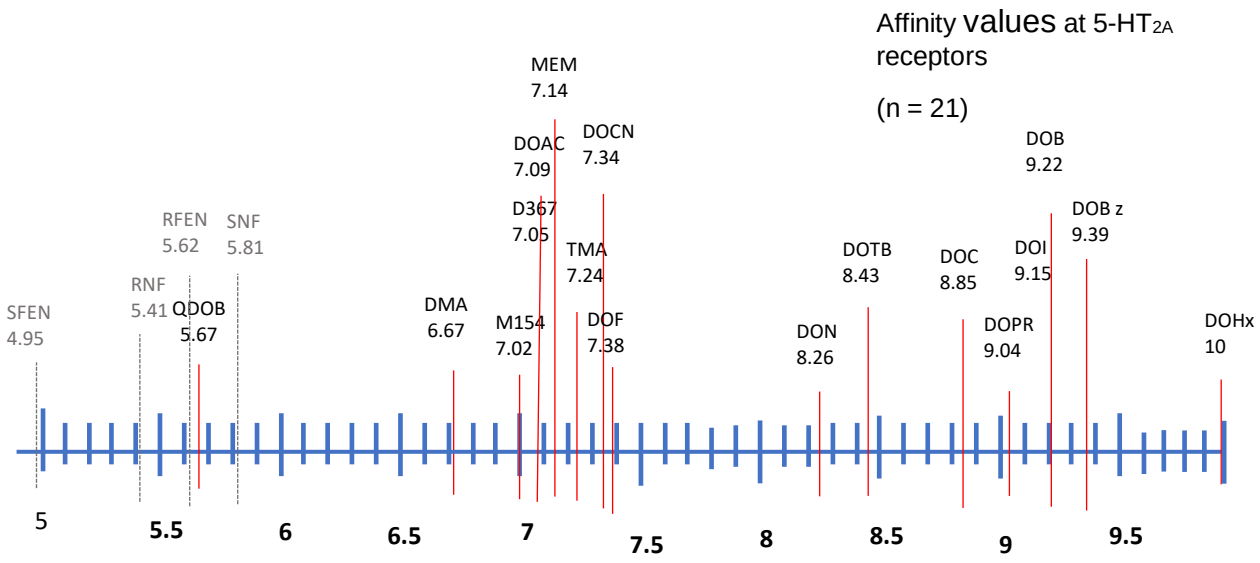


Figure 21. Distribution of pK_i values of phenylisopropylamines at 5-HT_{2A} receptors. DOX compounds are represented by red lines, fenfluramine and norfenfluramine isomers, grey lines.

The ligands used were the R and S isomers of fenfluramine (**11**), and norfenfluramine (**12**).[6] By adding these four compounds (n=21), the q^2 value for CoMFA increased to 0.685 with a standard error of prediction of 0.941 and the optimal number of components was 4. The CoMSIA, results also improved significantly, yielding a q^2 of 0.799.

Thus, with an improved distribution along with a fairly broad range of pK_i values, a good model was obtained, as evidenced by a comparative study of 5-HT_{2B} versus 5-HT_{2A}

affinity values. This is consistent with the literature on CoMFA studies that specifies that a good range and spread of values is recommended (typically 2.5 - 3 log units).[79-81] Thus, due likely to the low range of affinity values at the 5-HT_{2B} receptor, the CoMFA studies were unsuccessful. However, the CoMSIA study, although yielding 0.61 in q^2 value, did not withstand validation when divided into the training set and test set. Validation was performed by dividing the 16 compounds (Table 3 minus **19q**) into a training set of 12 compounds (**19d** – **19j**, **19l** - **19p**) and a test set of 4 compounds (**19a** – **19c, 19k**) after trial-and-error of modifying the training set and consequently, the test set, a q^2 value of 0.48 was obtained and the standard error of prediction was 0.216. Thus, the 5-HT_{2B} CoMSIA study was internally validated, but could not be externally validated. This leads to the conclusion that having a good distribution and range of affinity values is necessary for obtaining a good CoMFA/CoMSIA model.

Provided below are the results of the CoMFA and CoMSIA studies conducted on 5-HT_{2A} and 5-HT_{2B} receptor affinities (Table 5).

Table 5. CoMFA and CoMSIA summarized.

Model No.	No. of compounds	Compounds added	CoMFA q^2 (5-HT_{2B})	CoMSIA q^2 (5-HT_{2B})	CoMFA q^2 (5-HT_{2A})	CoMSIA q^2 (5-HT_{2A})
1.	n = 16	None	−0.209	0.612	0.398	0.606
2.	n = 20	RFEN, RNF, SFEN, SNF[a]	0.240	0.190	0.769	0.841
3.	n = 17	QDOB	−	−	0.402	0.302
4.	n = 21	QDOB, RFEN, RNF, SFEN, SNF	−	−	0.685	0.799
5.	n = 22	RFEN, RNF, SFEN, SNF, MDA, MDMA[b]	0.325	0.181	−	−
6.	n = 23	LSD, RFEN, RNF, SFEN, SNF, MDA, MDMA	-0.334	0.279		-

[a] RFEN = (R)-Fenfluramine; SFEN = (S)-Fenfluramine; RNF = (R)-Norfenfluramine; SNF = (S)-Norfenfluramine

[b] MDA = Methylenedioxyamphetamine; MDMA = Methylenedioxymethamphetamine

The 5-HT_{2A} receptor CoMSIA model yielding q^2 = 0.841 (Row 2 in Table 5) was validated by dividing the data set of 20 compounds into a training set and test set of 15 compounds (**19a**, **19c** – **19e**, **19g**-**19j**, SFEN (**11a**), and RFEN (**11b**)**)** (75%) and 5 compounds (**19b**, **19f**, and **19k**, SNF (**12a**) and RNF (**12b**)) (25%), respectively. The q^2 value of this model was 0.682 when the acceptor field was dropped. In order to test the influence of choice of training set on the q^2 value, DOB (**19d**) was replaced with DOF (**19e**) in the training set to increase the diversity of the steric factor, i.e., since the fluorine atom (in DOF) is similar in size to the hydrogen atom (DMA (**19d**)), which was already included in the training set, bromine was used as a suitable replacement as it is also a halogen, except with a larger radius. This replacement increased the q^2 value conservatively by 0.004 (= 0.680), and was accepted as the final model. The test set of 5 compounds for this model included **19e**, **19f**, **19k**, **12a** and **12b**. The choice of training set (remainder of the compounds, n = 15) was aimed at including the most diversity and redundancies to provide a good template of lipophilic, electrophilic, and steric properties. The abovementioned compounds of the test set are all replicated by similar compounds in the training set, therefore eliminating novel compounds. The standard error of estimate was 0.43, and the correlation coefficient (R^2 value) was 0.931. The average of the residuals was found to be 0.417. The training set was modified to be reduced to 14 compounds (**19a**- **19c**, **19d**, **19h-19j**, **19l-19p**, **11a**, and **11b**) and the test set increased to 6 compounds (**19e-19g**, **19k 12a, 12b**). As may have been expected, this reduced the q^2 value to 0.569, indicating the requirement for a more diverse and larger training set. The higher predictive power of CoMSIA over CoMFA is expected as there are similar (propyl, *tert*-butyl, hexyl, benzyl substituted) compounds in the data set and no

compounds with electron donating, or hydrophilic substitutions (for example, -OH, -NH_2), which may be required to gain clarity over the changing patterns of affinity when exploring chemical space. On the other hand, a CoMFA study on hallucinogenic phenylsopropylamines has been conducted in the past using Mescaline Units for biological activity at 5-HT_{2A} receptors.[82] The results of the study are consistent with the current findings that bulky substituents at the 4-position are favorable for activity, as was with the finding by Dommelsmith, et al.[83] This can be extrapolated to its affinity since high affinity is proportional to efficacy in 5-HT_{2A} ligands.[84] This is attributed to the presence of G-protein coupled receptors in a high affinity state and a low affinity state, and agonists are capable of selectively binding to, or stabilizing the active conformation of the receptor.[84] However, since DOTB (**19m**) and DOHx (**19l**) have high affinity, this assertion is contradicted as they act as partial agonists/antagonists and antagonists typically bind with high affinity to both states.[91,92] Additionally, the study by Seggel et al.[71] and a later published 3D-QSAR study by Zhang et al.[82] indicate the presence of an electron withdrawing substituent at the 4-position shows strong hallucinogenic activity (i.e., DOB (**19b**), DOC (**19f**), DOCN (**19j**). The meta substituted compounds, such as fenfluramine and norfenfluramine, however were found to be less active than the 3,4-disubstituted compounds such as MDMA (**17**).[60] Overall, the CoMSIA model for phenylisopropylamines was internally and externally validated for 5-HT_{2A} receptor affinities, and found to be conservatively predictive.

2. Conformational study

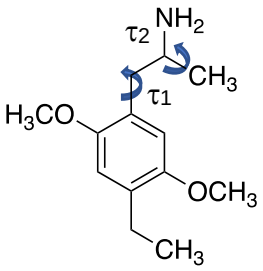
This study investigated 23 phenylisopropylamines, comprising 16 molecules of the DOX series, fenfluramine, norfenfluramine, MDA, and MDMA. LSD was included in

subsequent studies due to the presence of the phenylisopropylamine substructure. As discussed previously, the (*R*)-enantiomer is the eutomer and was hence used for conformational study. Due to the presence of six rotatable bonds on the DOX molecule, the conformation which was most likely to be bioactive, was to be determined as it may exist in several low energy conformations. The crystal structures of the compounds in this series were studied in order to compare their relative conformations in space. The alkylamine chain in the 2,4,5-substituted DOX series might be oriented in a different plane compared to the 3-substituted fenfluramines or the 3,4-disubstituted methylenedioxy compounds, due to steric hindrance of the amine with the 2-methoxy substituent. Therefore, the torsion angles of these compounds were measured before and after minimization of the crystal structure. A major caveat of performing a conformational study is that the molecules may not be at their global minimum (lowest energy of all the conformers), but might exist at a local minimum energy conformation within the receptor to fit the shape of the binding pocket. Additionally, crystal packing forces might influence the final conformation of the molecule by restricting freely rotatable bonds.[14] Therefore, the final conformation was based on the one in which there was minimal steric interaction between the methoxy and the amine substituent, i.e., the alkyl chain was oriented towards the 6-position on the phenyl ring.

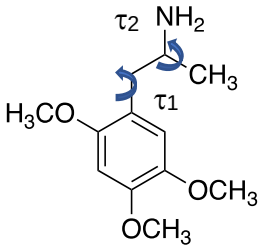
The crystal structures of 2,4,5-TMA,[36] MDMA,[15] norfenfluramine[15] and LSD[36] are known, although only the structure of MDMA was available in the Cambridge Crystallographic Database (CCD). Furthermore, the crystal structure of DOET, the 4-ethyl substituted DOX molecule, was also published by Horn, et al.[13] and was used as an

additional point of comparison for the DOX compounds. The torsion angles (τ) of the molecules about the alkylamine chain are as follows:

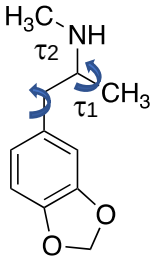
Table 6. Comparative study of torsion angles seen in various phenylisopropylamines.



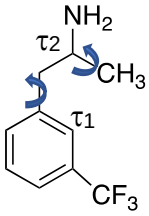
DOEt (**20**)



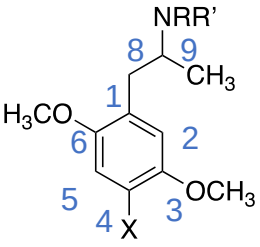
TMA (**19a**)



MDMA (**17**)



Norfenfluramine (**12**)



Rotatable bond	DOEt (**20**)	TMA (**19a**)	MDMA (**17**)	Norfenflur-amine (**12**)
C_6-C_1-C_7-C_8 (τ_1)	105.0	-114	108.24	110.50
C_1-C_7-C_8-C_9 (τ_2)	178.0	170	-66.47	50.57
C_1-C_7-C_8-N (τ_3)	-61.6	50	172.5	173.99
C_2-C_1-C_7-C_8 (τ_4)	-75.5	68	-70.14	-69.18

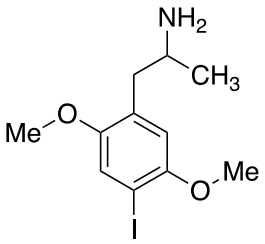
The torsion angles were obtained from the crystal structures of the abovementioned molecules.[13-15,36] The overall conformation of DOEt is similar to that of TMA (**19b**), wherein the torsion angles at the α-methyl groups (τ_2) are 178° and 170° respectively. In contrast, the corresponding torsion angle in MDMA is -66.47°, suggesting the substitution pattern influences the final conformation of the molecule. The energies of all the molecules were calculated prior to, and after minimization. The torsion angles varied after minimization, indicating a local minimum for the particular conformation.

Table 7. Torsion angles (τ_2) and energies of small molecule crystal structures before and after minimization.

Compound	Torsion angle before minimization	Energy before minimization (kcal/mol)	Torsion angle after minimization	Energy after minimization (kcal/mol)
DOEt (**20**)	178	11.18	170	6.25
TMA (**19a**)	170	12.20	172	5.09
Norfenfluramine (**12**)	50.57	21.79	60.11	4.13
MDMA (**17**)	-66.47	75.16	-51.11	13.87

The difference seen between the torsion angles of the DOX compounds (DOEt and TMA), norfenfluramine and MDMA suggests that there may be fewer similarities in their binding poses as well, owing to their substitution pattern. DOI was studied in detail to determine the various local minima that might exist. The two methoxy groups may orient towards the same plane (the terms *cis* and *trans* will be loosely used to describe the relative orientation of the methoxy groups) with respect to the plane of the ring.

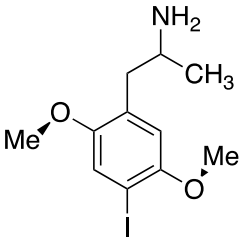
As per the crystal structure, the methoxy groups of DOEt are oriented along the plane of the phenyl ring, however, other low energy conformations might also exist. Since DOI is a part of the data set, it was sketched out in various conformations and the energies were recorded (Figure 22).



In plane

Before minimization: 9.97 kcal/mol

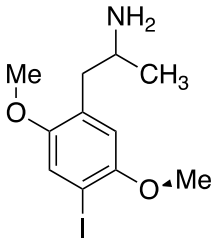
After minimization: 6.08 kcal/mol



Cis above plane

Before minimization: 7.10 kcal/mol

After minimization: 4.72 kcal/mol

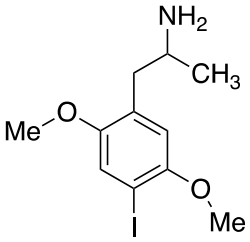


Rotamer with both methoxy groups oriented towards alkylamine chain

(2-methoxy flips forward by default)

Before minimization: 8.74 kcal/mol

After minimization: 5.59 kcal/mol

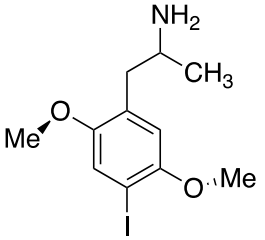


Rotamer with both methoxy groups oriented away from alkylamine chain

(5-methoxy flips forward by default)

Before minimization: 8.74 kcal/mol

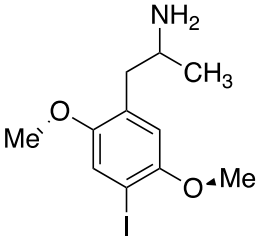
After minimization: 5.59 kcal/mol



Trans methoxy groups with 2-methoxy group above plane

Before minimization: 7.24 kcal/mol

After minimization: 4.87 kcal/mol



Trans methoxy groups with 5-OMe above the plane

(conformation unstable due to steric hindrance)

Before minimization: 7.26 kcal/mol

Figure 22. Conformational study of the methoxy groups of DOI.

All conformations did not minimize to the same energy due to the presence of various local minima for the molecule. The number of rotatable bonds is an overriding factor for the number of energy minima obtained. This is not expected in conformationally constrained molecules as they are confined to a rigid conformation and the molecule exists in fewer energy minima, as seen in LSD. Since DOX molecules have 6 rotatable bonds (τ_1-τ_4) as shown in Table 6, and one rotatable bond at each of the two methoxy groups. The conformational study details the various low energy conformations seen in 2,4,5-substituted DOX molecules. Due to the presence of multiple rotatable bonds, the exact bioactive conformation remains unknown. However, the conformation with the two methoxy groups trans to each other with the 2-OMe group below the plane, was chosen due to minimal likelihood of van der Waals' repulsions. The energy is highly influenced by the orientation of the methoxy group as it is in close quarters with the alkylamine system. This is supported by the higher affinity (K_i = 0.48 nM) seen in compound **26** (R = Br), which is conformationally constrained,[73] whereas DOB (**19b**) has an affinity of 26.9 nM, attributed partially to its freely rotatable bonds.[9]

A comparative study was conducted using CoMFA and CoMSIA with the affinity values at rat 5-HT_{2B} receptors which were reported in the same study, to determine if the rat 5-HT_{2B} receptor was more sensitive to the changes at the 4-position substituent.[9] Due to the unavailability of affinity values of other molecules such as fenfluramine (**11**), MDMA (**17**) and their metabolites (**12**, **18**) at rat 5-HT_{2B} receptors, the QSAR studies were limited to the DOX series. The best CoMFA model yielded a q^2 value of 0.163, suggesting that the rat 5-HT_{2B} receptor does not deviate far from the human 5-HT_{2B} receptor in terms of

sensitivity to the 4-position substitution. The CoMSIA study yielded a high q^2 of 0.49 by the exclusion of steric and electrostatic fields. This suggests a weak, if not a poor model.

Goal 2: To study the interactions of the phenylisopropylamines with the 5-HT_{2B} receptor.

The X-ray crystal structure of the 5-HT_{2B} receptor was determined by Wacker et al.[12] in 2017 with the partial agonist LSD bound to the orthosteric binding site. An earlier published crystal structure of the 5-HT_{2B} receptor co-crystallized with ergotamine,[11] revealed a difference in interactions between that of ergotamine (**8**) and LSD (**21**). While ergotamine protrudes from the binding pocket due to its large amide moiety connected to the ergoline system, LSD is seated more deeply in the binding pocket due to its smaller diethylamide moiety. This additionally explains the longer residence time of LSD in the binding pocket. Westkaemper and Glennon [85] earlier postulated a similarity of the binding of DOX compounds with that of LSD, and that they might bind similar to LSD in the binding pocket of the 5-HT_{2A} receptor. On this basis, the phenylisopropylamines were docked in the receptor and studied. The highly conserved residue $Asp135^{3.32}$ is vital for forming a salt bridge with the molecule for binding.[12] Docking studies were conducted with MDMA (**17**), MDA (**18**), fenfluramine, norfenfluramine and 16 phenylisopropylamines.

An important factor affecting the binding poses of the compounds that is taken into consideration is that while MDMA (**17**) and MDA (**18**) are substituted at 3- and 4-positions, fenfluramine (**11**) and norfenfluramine (**12**) are substituted only at 3-position and DOX compounds are substituted at 2-, 4-, and 5-positions. Therefore, the three classes of

compounds might adopt different binding poses, and the putative antagonists in the DOX series may adopt a distinct pose from that of agonists.

1. DOX molecules

The docking poses of the DOX series were varied and did not entirely dock in an overlapping manner owing to the mixed functional activities that were extrapolated from their 5-HT_{2A} receptor activities. Low affinity of certain molecules might also explain the differences in binding poses, thereby forming fewer interactions. In conjunction with the affinity values and the nature of the substituent, the size of the molecules in comparison to the ergolines were significantly smaller. Therefore, the docking radius was reduced from 10 Å to 8 Å, resulting in tighter clustering of the molecules around the conserved Asp135$^{3.32}$ residue.

Agonists of the DOX series are hallucinogens.[8] Therefore, they are assumed to bind in a similar manner to other hallucinogens such as LSD, wherein the phenyl ring and 5-methoxy group of DOI mimic the indole nucleus of LSD by forming an open ring analog and the 5-methoxy oxygen atom acting as the hydrogen-bond acceptor instead of nitrogen found in LSD. However, the crystal structure of the receptor reveals the indole nitrogen, H acts as a hydrogen bond donor to the backbone carbonyl of Gly221$^{5.42}$ residue.[12] Therefore, although the oxygen of the 5-methoxy group overlaps the nitrogen of the indole nucleus, it is unable to form the same interactions as LSD, but the similarities include the distance from the phenyl ring centroid to the amine (5.21 Å for LSD and 5.16 Å for DOI) as depicted in Figure 23, such that the aromatic ring of the ligand is able to form interactions with the hydrophobic residues lining the binding pocket and the amine forms an ionic salt-bridge with the highly conserved Asp135$^{3.32}$.

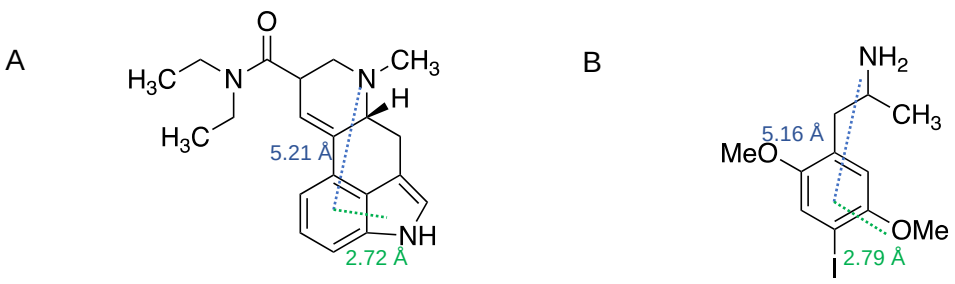


Figure 23. (A) Distances from the aromatic centroid to the basic amine and indolic nitrogen in LSD (B) Distances from the aromatic centroid to the basic amine and oxygen of the 5-methoxy group of DOI.

The DOX series displayed different binding poses based on the affinity and putative functional activities. For example, the analogs with smaller substituents than the propyl and hexyl chains – TMA, DOF, DOB, DOC, DOI, DON, DOAC and DOCN bind in the same manner such that they form close contact interactions with the hydrophobic residues.

Due to the large volume and the abundance of hydrophobic residues lining the orthosteric binding pocket, a majority of the protein-ligand interactions are hydrophobic in nature, as can be seen from the varying affinities of the molecules with their relative hydrophobicity. Hydropathic INTeractions (HINT) scores were measured and recorded for all of the molecules with the receptor to validate the model and determine the probability of the predicted binding pose. A HINT score is the sum of hydrophobic and electrostatic interactions between any two molecules.[94]

Table 8. HINT scores of DOX molecules (n = 10) with 5-HT_{2B} receptor.

Compound	HINT score	Hydrophobic	Polar
DMA (**19d**)	1380.77	864.38	1085.61
DOF (1**9e**)	2044.12	886.80	1702.91
DOC (**19f**)	1712.60	957.00	1534.11
DOB (**19b**)	1728.47	981.16	1549.37
DOI (**19c**)	1641.44	1016.72	1565.81
DON (**19i**)	1465.53	1024.15	1921.49
DOCN (**19j**)	1289.99	912.55	1610.46
DOAC (**19h**)	1370.77	1173.09	1515.22
TMA (**19a**)	1491.25	1090.09	1462.88
MEM (**19g**)	1250.06	1118.71	1067.86

In the 5-HT_{2A} receptor, Ser239$^{5.43}$ forms a hydrogen bond with the 5-methoxy substituent of the DOX molecule, whereas the corresponding residue in the 5-HT_{2B} receptor (i.e., Ser222$^{5.43}$ projects towards TM6, and thus, is unable to form the stabilizing hydrogen bond as demonstrated in previous studies.[96] In addition, Ser242$^{5.46}$, a polar residue, is present in the binding pocket of the 5-HT_{2A} receptor which facilitates a hydrogen bond with the 5-methoxy group. The 5-HT_{2B} receptor possesses an alanine residue (Ala225$^{5.46}$) in the same position, which might explain the lower affinity of DOX molecules at 5-HT_{2B} receptors compared to 5-HT_{2A} receptors. The DOX (putative agonist)

molecule forms interactions with Asp135$^{3.32}$ and Ser139$^{3.36}$ (Figure 24) as was seen in the Runyon model[17], and the 2-methoxy group, with Leu209^{EL2}, and Leu132$^{3.29}$, and the 5-methoxy group with Val136$^{3.33}$ and Gly221$^{5.42}$. The alpha-methyl group with the Trp337$^{6.48}$, and the hydrophobic substituent at the 4-position forms a hydrophobic interaction with the backbone of Met218$^{5.39}$, Phe217$^{5.38}$, and a weak electrostatic interaction with Ser222$^{5.43}$. Thus, the hydrophobic substituent is oriented towards TM5, forming primarily hydrophobic interactions, yet not capable of forming the halogen bond, but a weak electrostatic interaction at 3.3 Å with Ser222$^{5.43}$. The isopropylamine chain is oriented towards TM3 and TM6, forming a stable bidentate interaction with TM3. The abovementioned residues form a subset of the residues that interact with LSD in the receptor crystal structure.[28] The unsubstituted DMA (**19d**) and MEM (**19g**) adopt different binding poses. The poses for DMA (**19d**), D367 (**19p**) and M-154 (**19o**) are unprecedented and are distinct from that of the DOX series. Although the salt bridge is seen in all three models, the remainder of the molecule is free to form hydrophobic interactions with other residues in the binding pocket.

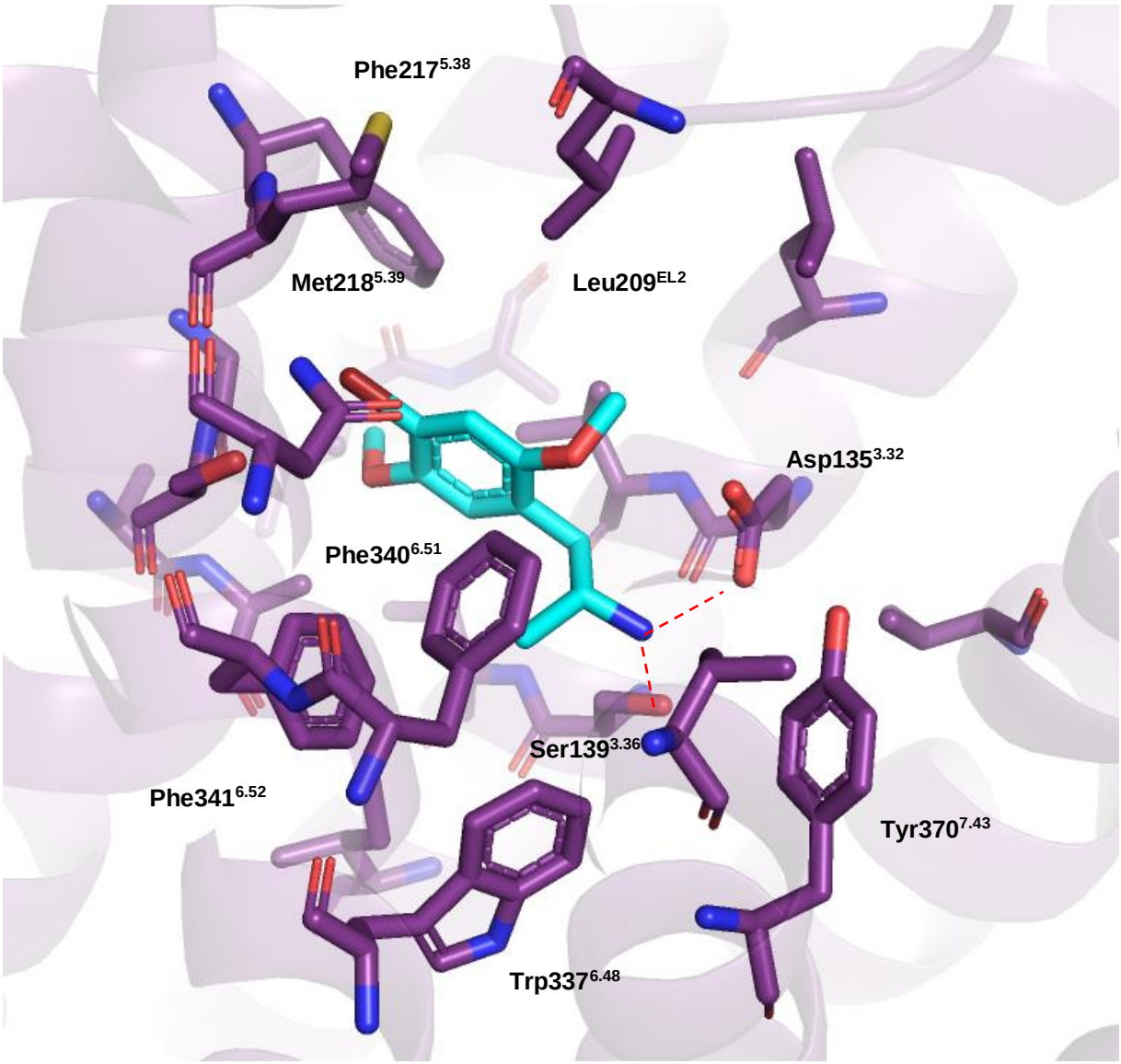


Figure 24. Structure of ligand DOB (**19b**) docked in the 5-HT$_{2B}$ receptor (PDB ID: 5TVN) represented in cyan and purple sticks, respectively. The dashed line indicates the interactions of the protonated amine with the amino acids Asp135$^{3.32}$ and Ser139$^{3.36}$ in transmembrane helix 3.

DOCN (**19j**) (Figure 25) form hydrogen bonds with the backbone of Ser222$^{5.43}$, and DON (**19i**) forms a hydrogen bond with Asn344$^{6.55}$. Despite these stabilizing interactions, they may be unfavorable for the overall affinity of the molecule. DOCN (**19j**) has an affinity of 774 nM at the 5-HT$_{2B}$ receptor, while DON (**19i**) has an affinity of 166 nM. A Craig plot of the nature of substituents supports these findings, since the nitrile group of DOCN has a negative π value (+σ, -π), while the nitro group has a positive π value (+σ, +π).

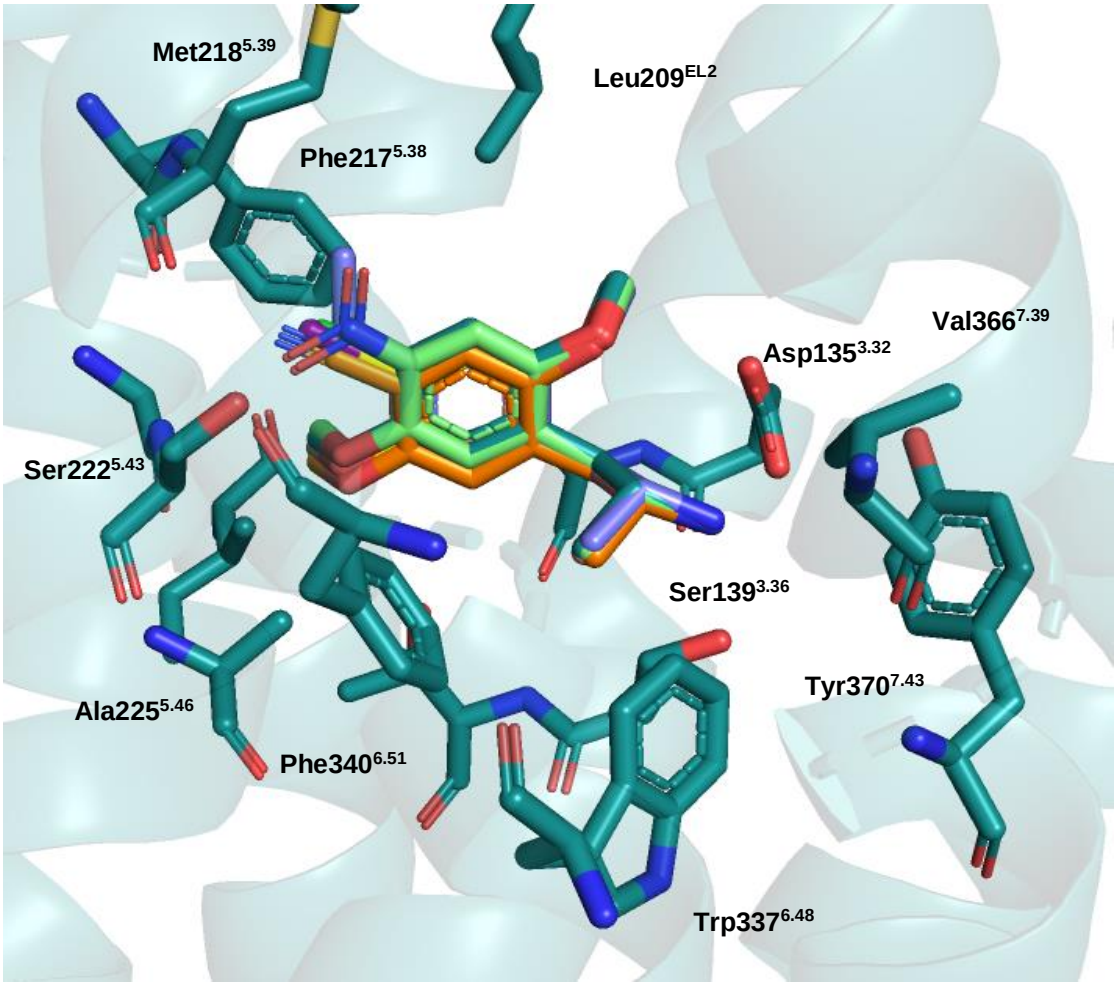


Figure 25. Common docking pose of TMA (**19a**) (purple blue), DOB (**19b**) (blue), DOI (**19c**) (yellow), DOF (**19e**) (dark green), DOC (**19f**) (bluegreen), DOCN (**19j**) (orange), DON (**19i**) (lime green) and DOAC (**19h**)(purple).

DOX molecules with larger 4-position substituents adopt a different binding pose in the receptor, such that the binding pocket might accommodate the large 4-position substituent, i.e., DOPR (**19k**) (*n*-propyl), DOHx (**19l**) (*n*-hexyl) and DOBZ (**19h**) (benzyl) (Figure 30). The distance from the Asp135$^{3.32}$ residue to the lower surface of the binding pocket is not large enough to accommodate 4-position substituents larger than a certain size. Therefore, the ring is oriented upside down such that the carbon chain accesses secondary binding pocket residues seen in the crystal structure of ergotamine (PDB ID: 4IB4).[11] Although the functional activity of these molecules is unknown at 5-HT$_{2B}$ receptors, they are known to act as antagonists at 5-HT$_{2A}$ receptors.[90] Therefore, agonists and antagonists might adopt different binding poses in the 5-HT$_{2B}$ receptor for DOX molecules. However, an exception was seen with DOPR, wherein it is an agonist at 5-HT$_{2A}$ receptor, but adopts a putative antagonist binding mode in the 5-HT$_{2B}$ receptor. Consequently, multiple binding modes were identified for phenylisopropylamines at the 5-HT$_{2B}$ receptor. Although these are distinct with respect to each other, they all engage the same residues that are seen with ergolines, yet form fewer interactions.

2. Norfenfluramine isomers

The phenylisopropylamine moiety of fenfluramine is mono-substitued, whereas most DOX molecules are tri-substituted, thereby increasing the possibilities of interactions with the receptor binding pocket. The difference in substitution pattern between DOX molecules and norfenfuramine or MDA influences the interaction of the molecule and their affinity for the receptor. The protonated amine forming a salt bridge with Asp135$^{3.32}$ is the common feature of all the molecules. A model previously proposed by Setola et al.[97] suggests the involvement of Val106$^{2.53}$ in TM2 in determining the stereoselective nature

of the receptor to the *R*- isomer, due to the its interaction with the α-methyl carbon as supported by mutagenesis data. Such involvement of TM2 in ligand binding was not seen in our model. A newer model of norfenfluramine was published in the same study as the crystal structure of the 5-HT_{2B} receptor with ergotamine, wherein the trifluoromethyl group forms a hydrophobic interaction with $Phe217^{5.38}$, was validated by mutagenesis to be essential for the efficacy of the molecule.[11] An F217A mutation reduced efficacy by ~10 fold.[11] Our findings are consistent with this model and show the same interactions. A HINT analysis of this model also shows it to be highly favorable. Mode 1 is similar to the newer model and shows interactions with TM5 residues (Figure 26, 27). Mode 2 shows interactions with TM2 (Figure 26), which is likely unfavorable, considering the residues of TM5 are highly involved in most ligand-receptor interactions as seen from the crystal structures.[11,36,96] 5-HT_{2B} receptor functional activity of these agents remains to be determined and will be the focus of a future study.

Table 9. HINT scores of norfenfluramine (**12**) isomers for both binding modes at the 5-HT_{2B} receptor.

Compound (mode)	HINT score	Hydrophobic	Polar
RNF (mode1)	1920.43	565.36	1236.90
SNF (mode1)	2531.42	544.97	2141.04
RNF (mode2)	1434.00	557.89	1101.29

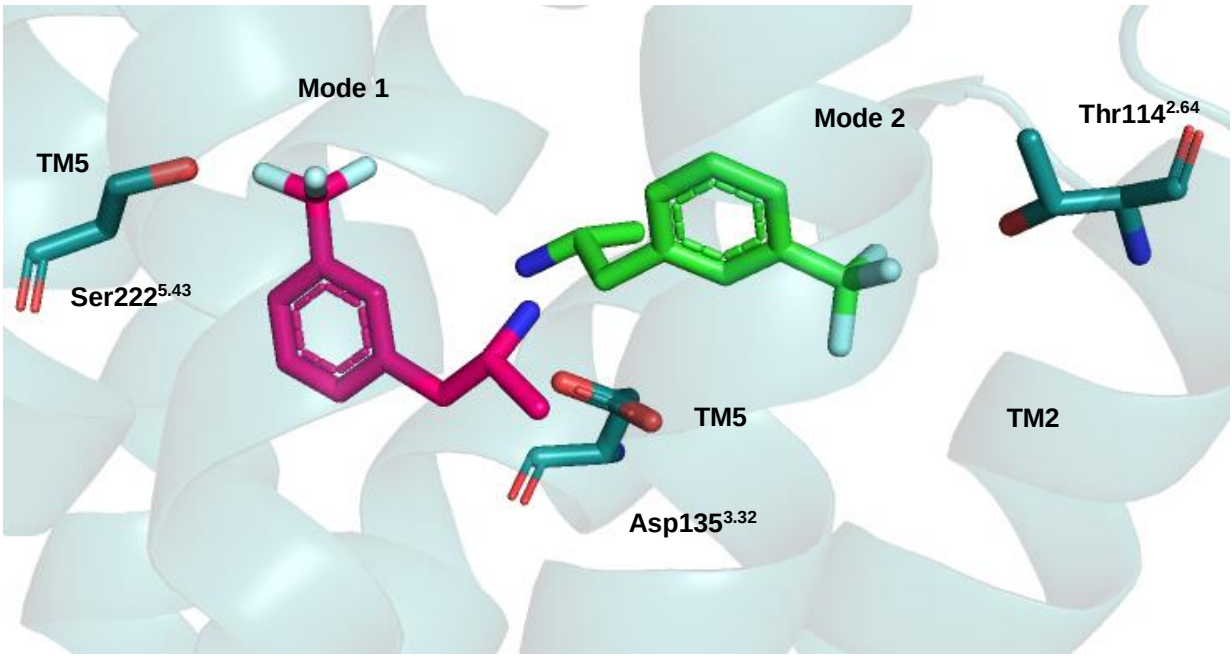


Figure 26. Binding poses of (*R*)-norfenfluramine (**12b**) - mode 1 and mode 2. Mode 1 (pink capped sticks) shows the molecule oriented towards TM5 and mode 2 (lime green capped sticks) towards TM2. Mode 1 is more likely to be the accurate binding mode.

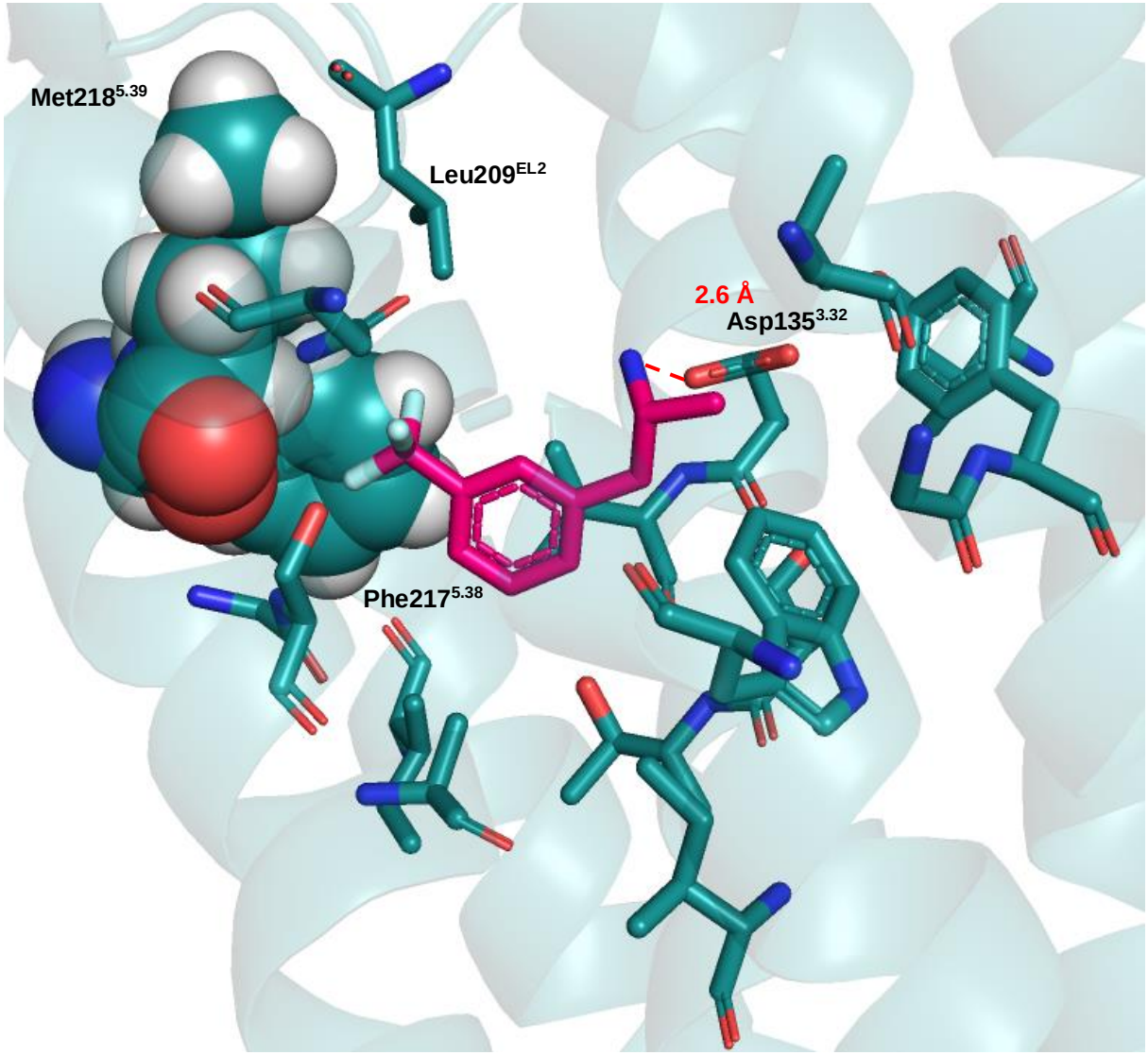


Figure 27. RNF (**12b**) docked in the 5-HT_{2B} receptor represented in hot pink and deep teal sticks, respectively. A salt bridge is formed with Asp135$^{3.32}$ and close contact interactions are formed with Phe217$^{5.38}$ and Met218$^{5.39}$ represented in spheres.

3. Binding modes of MDA and MDMA

The crystal structure of (*S*)-MDMA (**17a**) was used as a template to sketch and dock the (*R*)-MDMA (**17b**) and (*R*)-MDA (**18b**) molecules in the receptor. (*R*)-MDA forms

bidentate interactions with Asp135$^{3.32}$ (Figure 28), while (*R*)-MDMA does not. Two binding poses were generated for the MDA molecule, mode 1 mimicking the pose of DOX molecules, and mode 2 similar to the second pose of norfenfluramine (facing Thr114$^{2.64}$ of TM2) (Figure 29). A HINT analysis of the two poses helped establish mode 1 as the more favorable (Table 10). Since MDMA (**17**) is a ring-constrained analog of 3,4-dimethoxyphenylisopropylamine, and may even be compared to 2,4,5-TMA (**19a**). It possesses a higher affinity than TMA (**19a**), indicating that the constrained methoxy groups may be more favorable for the binding of the molecule. The lower affinity of the molecule (100 nM) in comparison to norfenfluramine (**12b**) (~15 nM) and DOB (**19b**)(26.9 nM), however cannot be attributed to the same property. Ours is the first model of both isomers of the valvulopathogen MDA ((*S*)-MDA (**18a**) and (*R*)-MDA (**18b**)) docked in the 5-HT$_{2B}$ receptor (Figure 28). It is representative of a probable binding pose of the molecule within the receptor, and provides useful insight into the binding of a different chemical class of valvulopathogens. The binding pose of MDMA (**17**) is similar to that of MDA (**18**), however the N-methyl group forms unfavorable interactions with, as seen in the final HINT score (Table 10).

The oxygen atom at the 4-position of the phenyl ring is within 3.0 - 3.5 Å of the backbone nitrogen and -OH group of Ser222$^{5.43}$, highly capable of forming a hydrogen bond. Majority of the interactions of MDA (**18**) and MDMA (**17**) are seen in TM3 (Asp135$^{3.32}$), TM5 (Phe217$^{5.38}$, Met218$^{5.39}$, Ala225$^{5.46}$), TM6 (Asn344$^{6.55}$) and TM7 (V366$^{7.49}$) (Figure 28). A weak hydrophobic interaction is seen with Leu209^{EL2} in the second extracellular loop. This residue is known to be involved in recruiting β-arrestin as highlighted by Wacker et al.[12] The (*S*)-isomer of MDMA (**17a**) was found to have a similar

binding mode as that of the (*R)*-isomer (Figure 28). Final HINT scores are provided in Table 10.

Table 10. HINT scores of MDA (**18**) and MDMA (**17**) based on their binding modes.

Compound	HINT score	Hydrophobic	Polar
(*R*)-MDA (mode 1)	2141.39	547.93	1659.54
(*R*)-MDMA (mode 1)	1620.15	703.77	1488.08
(*R*)-MDA (mode 2)	1227.65	533.16	1066.14
(*R*)-MDMA (mode2)	1435.43	675.21	1080.93
(*S*)-MDA	1731.04	565.85	992.45
(*S*)-MDMA	790.60	588.46	899.44

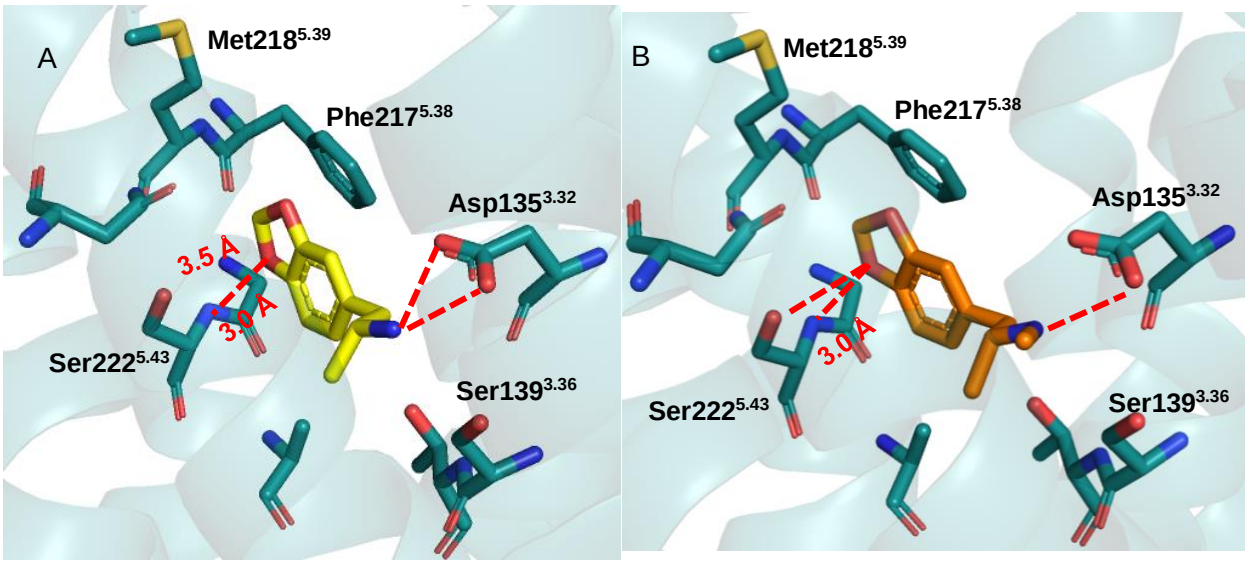


Figure 28. (A) (*R*)-MDA (**18b**) in yellow and (B) *(R)*-MDMA (**17b**) in orange docked in the 5-HT$_{2B}$ receptor. (*R*)-MDA (**18b**) forms bidentate interactions with Asp135$^{3.32}$. The methylenedioxy rings of (*R*)-MDA (**18b**) and (*R*)-MDMA (**17**) are within hydrogen-bonding distance of Ser222$^{5.43}$. MDMA (**17**) forms a hydrogen bond with Asp135$^{3.32}$. (Mode 1 is shown in both figures.)

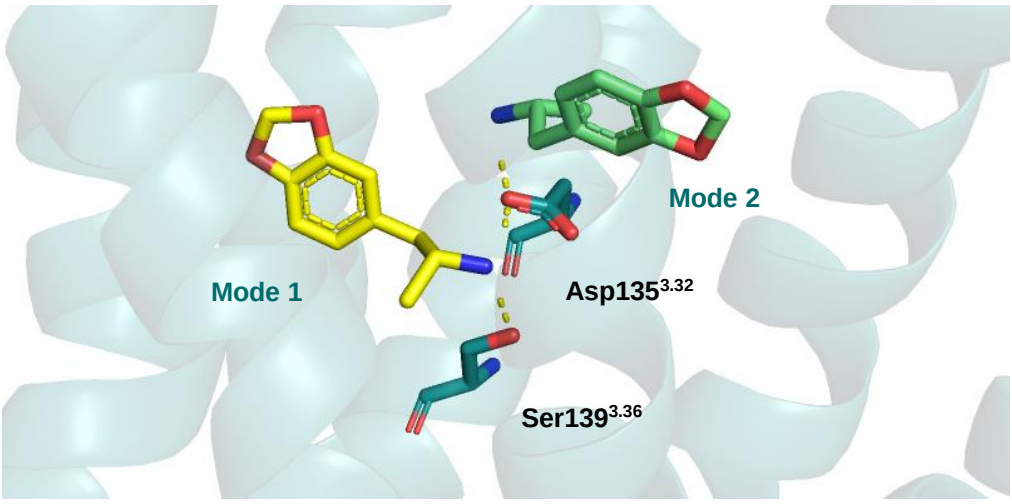


Figure 29. Binding poses of (*R*-MDA (**18**) (also seen with MDMA (**17**)) at the 5-HT$_{2B}$ receptor. Mode 1 is represented in yellow sticks and mode 2 is shown in green sticks. Asp135$^{3.32}$ and Ser139$^{3.36}$ are shown in deep teal sticks, dashed lines indicate potential hydrogen bonds.

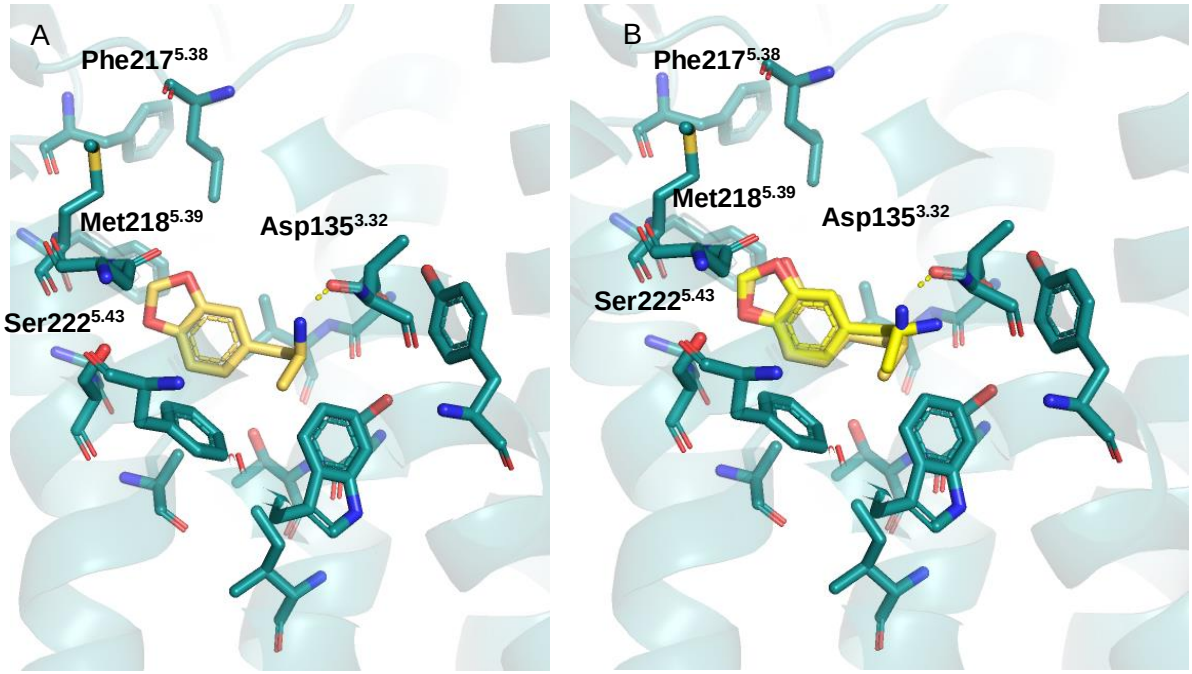


Figure 30. (A) Binding pose of (*S*)-MDA (**18a**) at the 5-HT$_{2B}$ receptor shown in pale yellow sticks. (B) Comparison of (*R*)-MDA (**18b**) and (*S*)-MDA (**18a**) binding poses shown in bright and pale yellow sticks respectively. (Mode 1)

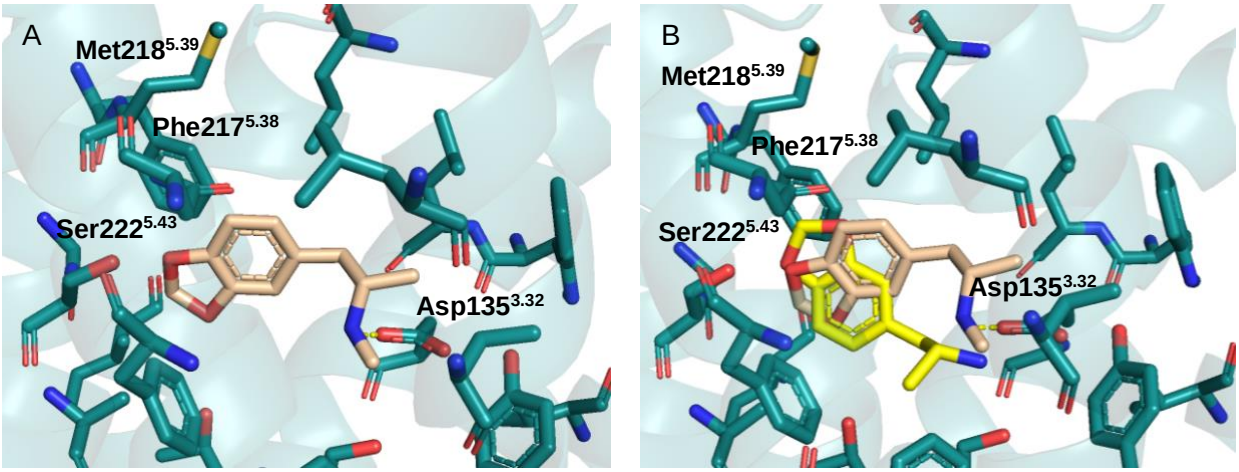


Figure 31. (A) Binding pose of (*S*)-MDMA (**17a**) at the 5-HT$_{2B}$ receptor shown in pale yellow sticks. (B) Comparison of (*R*)-MDMA (**17b**) and (*R*)-MDA (**18b**) binding poses shown in bright and pale yellow sticks respectively.

4. Comparison of recently published crystal structures

The crystal structure of the 5-HT$_{2B}$ receptor was recently published with four ligands lisuride (**3**), methylergonovine, methysergide (**10**) and LY266097 (PDB IDs: 6RDX, 6DRY, 6DRZ, 6DS0 respectively).[95] This was compared against that of the co-crystal structure with LSD (PDB ID: 5TVN)[12] (Table 11). The RMS deviation was

Table 11. Comparison of newly published crystal structures of 5-HT$_{2B}$ receptor with the previously published crystal structure 5TVN.[12]

PDB ID	Ligand	Functional activity	RMSD (5TVN template)
6DRX	Lisuride (**3**)	Antagonist	1.05 Å
6DRY	Methylergonovine	Agonist	0.426 Å
6DRZ	Methysergide (**10**)	Antagonist	1.362 Å
6DS0	LY266097	Antagonist	0.725 Å

measured in comparison to the Cα carbon of the backbone the template 5TVN. Since the RMS value was found to be within the recommended limit of significance (<4 Å), 5TVN, the crystal structure of 5-HT_{2B} receptor with LSD was pursued.

5. Overview of all docking poses

DOB (**19b**) (taken as template for binding of DOX series molecules) and RNF appear to bind in a somewhat similar manner (Figure 32A). This may provide clues into the potential of hallucinogenic phenylisopropylamines to cause cardiac valvulopathy. Figure 32B compares the binding poses of the hallucinogens DOB (**19b**) and LSD (**21**) with a binding pose of of valvulopathogen norfenfluramine (**12**), in Figure 32C the binding mode of DOB is shown with respect to valvulopathogens RNF and MDA (**18**). Two binding modes are seen for the phenylisopropylamines at the 5-HT_{2B} receptor, for probable agonists, antagonists. Secondary and tertiary amines may bind differently in comparison, and the 4-position unsubstituted DMA (**19d**) may bind differently in comparison to the other DOX molecules as there is no substituent to be anchored by the receptor.

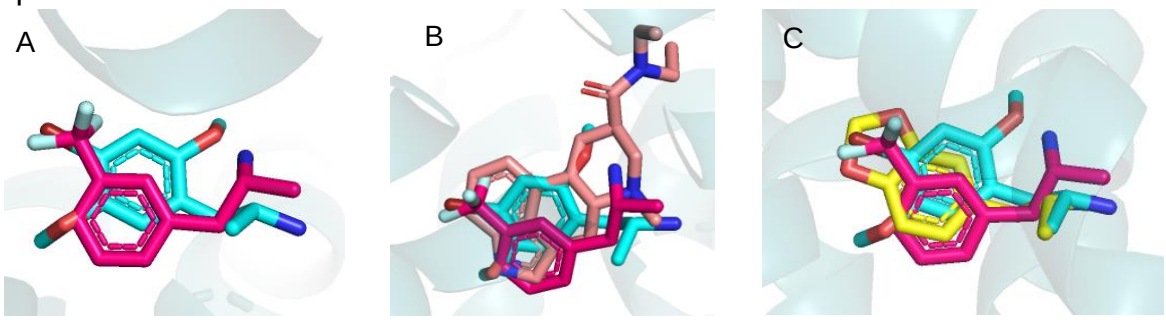


Figure 32. (A) Docking poses of RNF (**12b**) (hot pink) vs DOB (**19b**) (blue) at 5-HT_{2B} receptors (B) Comparison of binding poses of RNF (**12b**) and DOB (**19b**) relative to LSD (**21**)(salmon). (C) Comaprison of MDA (**18**) (yellow), RNF (**12b**) and DOB (**19b**).

6. Binding mode of bulky 4-position DOX molecules

The bulky 4-position substituent cannot be accommodated between TM3 and TM5, therefore the molecule is oriented towards TM7 wherein the volume of the cavity can accommodate larger substituents. For example, the volume of the bromo-substituent is 43.53 Å and that of the *n*-Pr substituent is 137.93 Å (as calculated in Sybyl X2.1.1). The largest substituent tolerated at the 4-position to adopt the common binding pose of DOX molecules is 84 Å, as seen in DOAC (**19h**) (Sybyl X2.1.1). The Asp135$^{3.32}$ – amine salt bridge is conserved in all the receptor-ligand interactions, however while smaller substituents are able to bind in the same manner, larger substituents are forced to orient towards the TM7 (Figure 33).

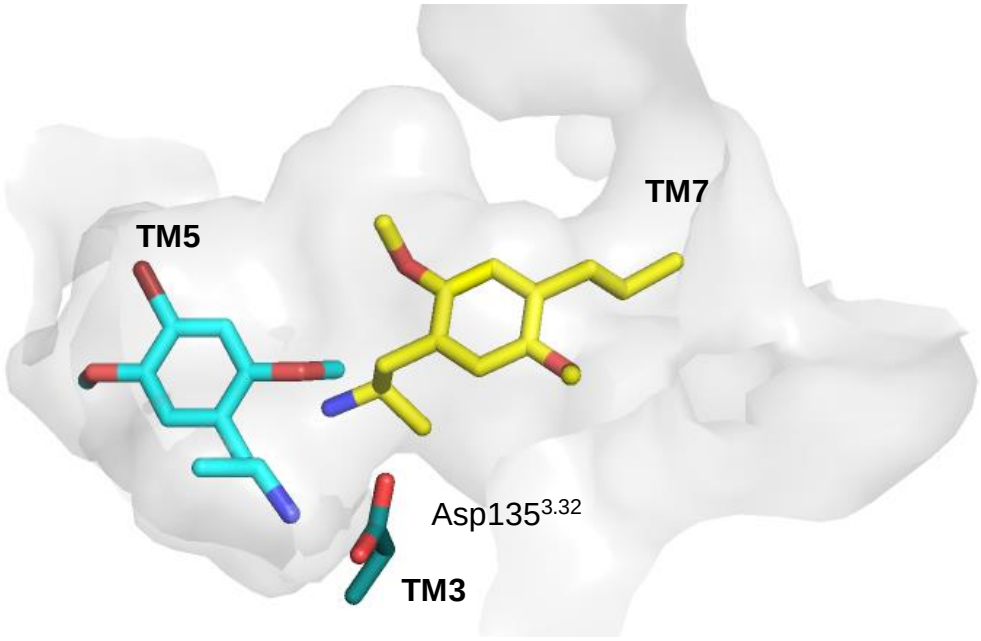


Figure 33. Comparison of DOB (**19b**)and DOPR (**19k**) depicted as blue and yellow capped sticks respectively, in the orthosteric binding pocket. DOPR (**19k**) is oriented in the opposite direction than DOB (**19b**). The propyl chain is oriented towards TM7 so as to accommodate the bulky group.

DOPR (**19k**), DOBZ (**19h**) and DOHx (**19l**) are able to bind in a nearly similar manner, yet the hexyl chain in DOHx (**19l**) and the benzyl group in DOBZ (**19h**) being longer than the propyl chain forces the molecule to orient itself further down in the binding pocket where there is space.

Table 12. HINT scores of DOX molecules with bulky 4-position substituents.

Compound	HINT score	Hydrophobic	Polar
DOPR	1156.22	999.22	1127.81
DOHx	1011.00	1310.01	1137.94
DOBZ	800.31	1066.11	764.08
DOTB	1389.66	1159.99	1106.95

This pose is the only one generated wherein the aspartate interaction is conserved, in DOHx (**19l**) (Figure 34) and DOBZ (**19h**) (Figure 35) however clashes were seen in this binding pose of the 2-methoxy group with Asp135$^{3.32}$.

Figure 34. (A) DOHx (**19l**) (blue) and DOB (**19b**) (warm pink) in the 5-HT$_{2B}$ receptor. (B) DOHx (**19l**), DOPR (**19k**)(yellow) and DOB (**19b**) (right) in the binding pocket. The propyl and hexyl groups fit into the cavity facing the *N*-terminus, away from binding pocket.

The same binding mode is not seen with DOTB however, due to its higher volume than length. As seen in Figure 36, it is oriented lower in the binding pocket, with the *t*-butyl group facing TM7. It adopts an unique binding mode, possibly due to being a partial agonist as seen in 5-HT$_{2A}$ receptors.[91]

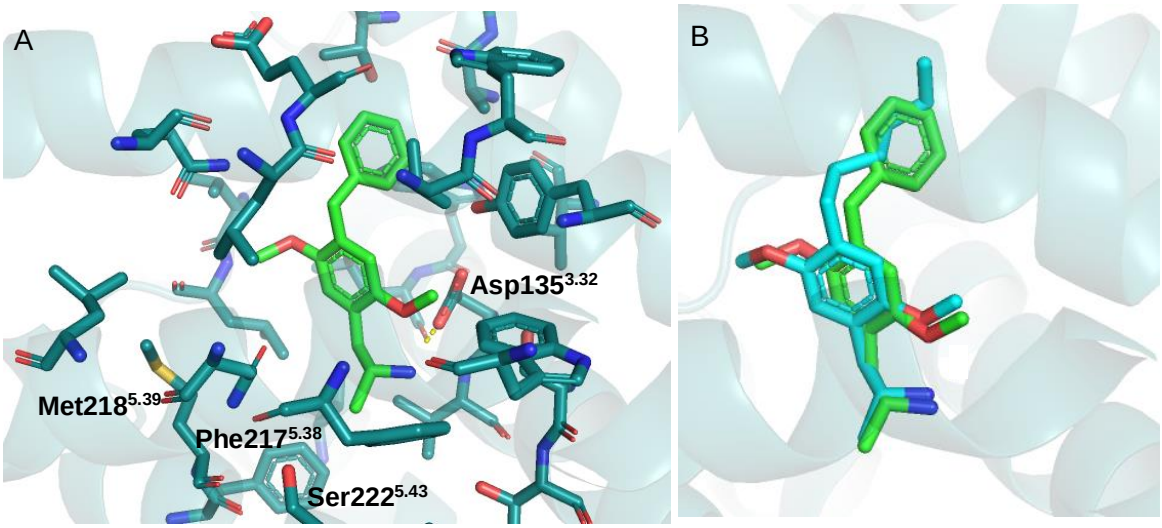


Figure 35. (A) DOBZ (**19h**) (lime green capped sticks) docked in the 5-HT$_{2B}$ receptor. (B) DOHx (**19l**) and DOBZ (**19h**) (blue and lime green capped sticks respectively) in the binding pocket. The hexyl and benzyl groups assume a similar conformation in the secondary binding pocket region.

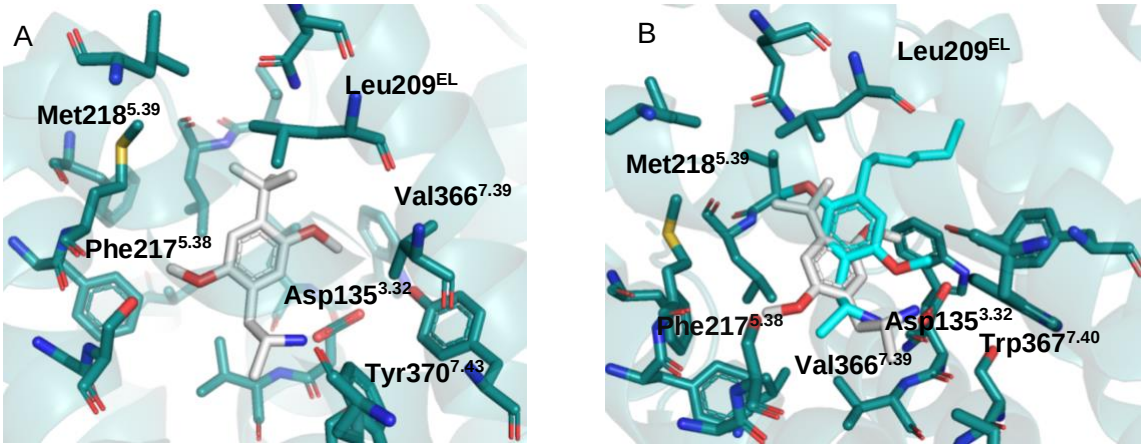


Figure 36. (A) DOTB (**19m**) (white sticks) in the 5-HT$_{2B}$ receptor. (B) DOTB (**19m**) (white sticks) and DOHx (**19l**) (blue sticks) in comparison. DOTB is oriented away from the hexyl group and towards TM7.

7. Binding of secondary and tertiary amines

D367 (**19p**) is a secondary amine analog of DOB (**19b**) with a *N*-propyl group and a significantly lower (20-fold) affinity than DOB (**19b**). The docking simulation suggests the molecule is situated higher than DOB (**19b**) in the binding pocket and forms interactions with the Asp135$^{3.32}$ and Tyr370$^{7.43}$ (Figure 37). The bromine atom of D367 (**19p**) faces the TM5 as does DOB (**19b**), it does not bind in the same way as the tertiary amine (M154 (**19o**)). D367 (**19p**) forms a single cluster upon docking and M154 (**19o**) forms several clusters. The best model was chosen based on the least number of clashes observed in the HINT scores.

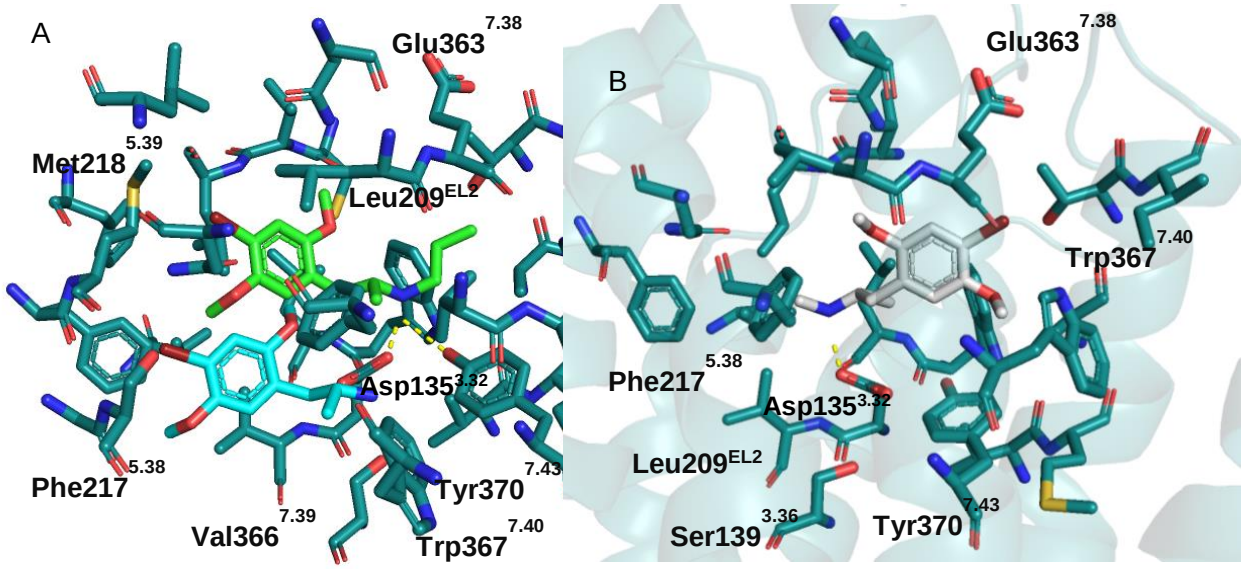


Figure 37. (A) D367 (**19p**) (secondary amine) and (B) M154 (**19o**) (tertiary amine) shown in lime green and grey sticks, respectively. DOB is shown in cyan sticks in figure A for reference.

8. Quantitative structure-activity relationships

The DOX molecules seemed to show a pattern of increasing 5-HT_{2B} receptor affinity with lipophilicity of the substituent at the 4-position (Table 13), which was also supported by our CoMSIA studies (Figure 18). In order to examine this, we conducted a correlation of the pK_i vs π-value for the varied substituents (n = 14).[9,98] A positive correlation (r = 0.753) was obtained for the π-values, with two clusters of points (Figure 38); however there was a poor distribution of affinity values of the compounds. The p value was below 0.05 and was therefore considered to be statistically significant. The correlation coefficient was improved (r =0.78) if DOHx was deleted from the set, therefore an outlier test was conducted to determine if DOHx was an outlier in the data set. The analysis was performed using the Statistical Package for the Social Sciences (IBM SPSS, New York, NY).

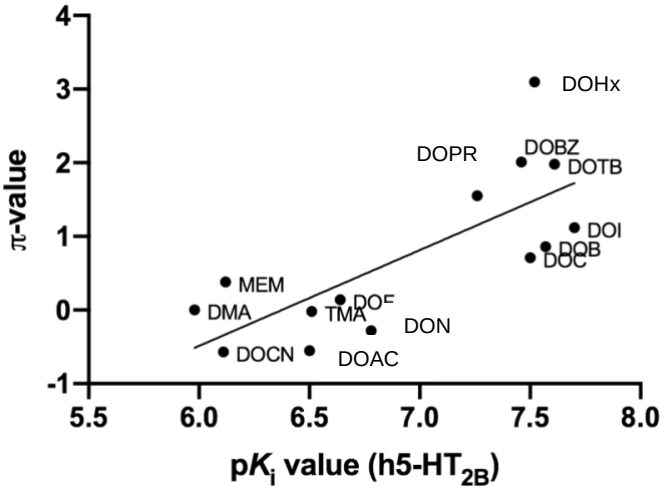


Figure 38. Plot of pK_i values of the DOX molecules versus the π values of the 4-position substituent (r = 0.753, p = 0.0018, n = 14).

The Z-score provides a standard deviation of 1 and a mean of 0, therefore a Z-score of >3 would indicate an outlier that is two standard deviations away from the original 1. The

range of Z-scores obtained (i.e., multiple agents were examined, including DOHx) was -1.38 to 1.45, suggesting there were no outliers in this model.

Table 13. QSAR parameter values for DOX series (*n* = 14)

Name	pKi (5-HT_{2B})	π-value[98]	Volume[a]
DMA	5.98	0	690.29
DOCN	6.11	-0.57	736.02
MEM	6.12	0.38	798.02
DOAC	6.5	-0.55	774.66
TMA	6.51	-0.02	752.38
DOF	6.64	0.14	700.52
DON	6.78	-0.28	748.64
DOPR	7.26	1.55	828.22
DOBZ	7.46	2.01	950.22
DOC	7.5	0.71	721.46
DOHx	7.52	3.1	987.63
DOB	7.57	0.86	733.82
DOTB	7.61	1.98	850.36
DOI	7.7	1.12	748.20

[a]Calculated for the entire molecule using SYBYL X2.1.1.

The volume of the substituents (calculated in Sybyl X2.1.1) was also investigated for their influence on the affinity of the molecules (Table 13). A poor correlation was affinity. Figure 39 shows a plot of the volume plotted against the affinity of the molecule.

However, when the π-value of the 4-position substituent was plotted against the volume

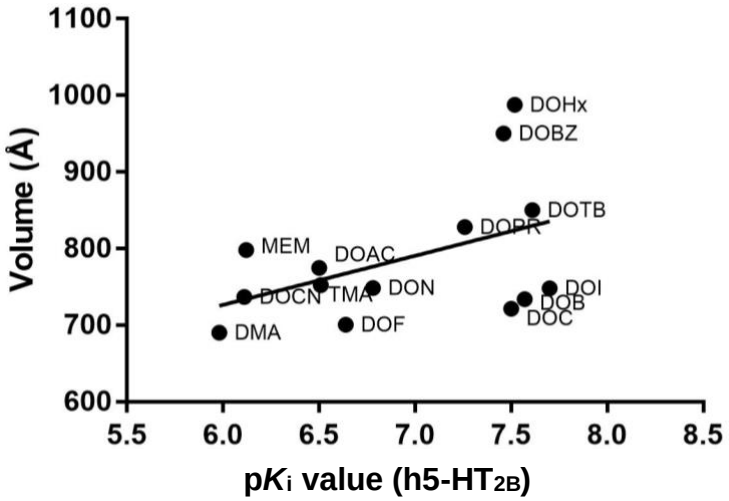


Figure 39. Plot of pK_i values of the DOX molecules versus the volume of the 4-position substituent (r = 0.455, p = 0.101, n = 14)

of the substituent, a strong positive correlation was seen (r = 0.819) (Figure 40).

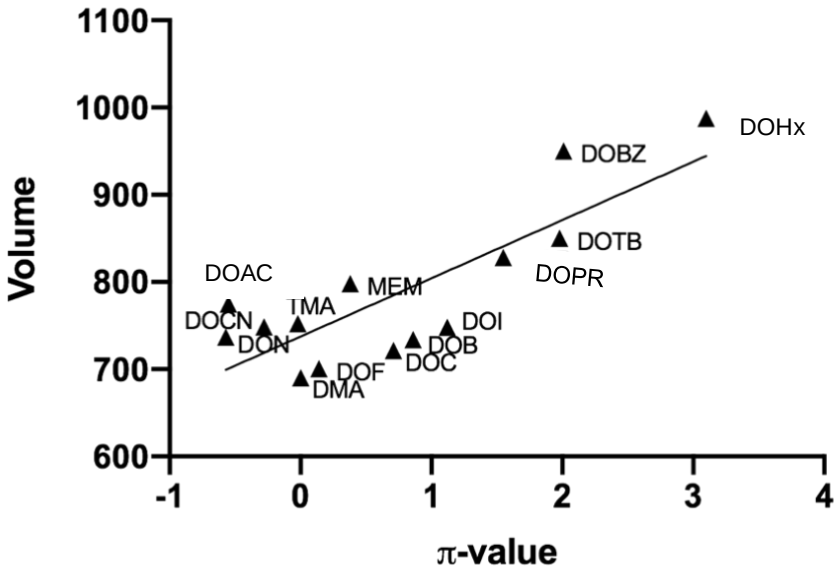


Figure 40. Positive correlation between the π-value and the volume of 4-position substituents (r = 0.819, p = 0.0003, n = 14).

The anomaly of a high correlation between hydrophobicity (π) and volume, but a low correlation between pK_i and volume was further investigated with a multiple regression analysis (Eq. 1).

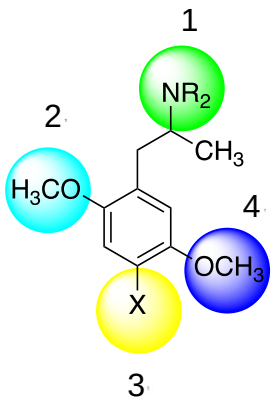
y = 0.66 (π) - 0.003 (volume) + 10.30 (Eq.1)

(n = 14; F = 10.11; r = 0.80; Standard error = 0.40)

The r value obtained was 0.80, and the ANOVA test resulted in the coefficients for π and volume to be 0.66 and -0.003, respectively. The overall p value was 0.001, indicating the results to be statistically significant. The overarching conclusion of this study is that the high affinity DOX molecules is due to a highly hydrophobic 4-position substituent, as also evidenced by docking studies wherein the orthosteric binding pocket is abundantly lined with hydrophobic residues.

V. Conclusions

The overall purpose of this study was to establish the potential of hallucinogens in causing cardiac valvulopathy mediated by agonism of 5-HT_{2B} receptors, by examining their structure-activity relationships and determining their interactions with the receptor. In the culmination of these studies, we found DOX molecules to adopt different binding poses owing to the varying size and nature of the 4-position substituents and the presence of primary, secondary, tertiary and quaternary amines.



The first structure-activity relationships of phenylisopropylamines as hallucinogens at 5-HT_{2B} receptors obtained from CoMSIA and statistical analyses suggest:

1. Primary amines are optimal for 5-HT_{2B} receptor binding.
2. The 2-OMe group appears unessential for 5-HT_{2B} affinity.
3. A substituent (electron-rich) seems to contribute.
4. Lipophilicity of the 4-position substituent as suggested by its π value, seems to contribute to 5-HT_{2B} receptor affinity. This might explain the (reduced) affinity of MDMA.

Having said this, it remains to be determined which of these agents in Table 2 act as agonists or antagonists. Agonists and antagonists might bind in a different manner. The DOX molecules investigated in the abovementioned studies were docked in the 5-HT_{2B}

receptor to study their interactions with the receptor. With the co-crystal structure of the receptor (PDB ID: 5TVN), we were able to compare two classes of hallucinogens ergolines and phenylisopropylamines (LSD and DOX). DOI has the highest binding affinity of all DOX molecules for the 5-HT_{2B} receptor. It occupies the same area as the LSD molecule, and the 5-methoxy substituent overlaps the indolic nitrogen of LSD. Similarities are seen with LSD and DOI such as the $Asp135^{3.32}$ – amine salt bridge, the aromatic interactions seen with residues in transmembrane helices 3, 5, 6, and 7. Common hydrophobic residues seen for DOI and LSD in the orthosteric binding pocket are $Phe340^{6.51}$, $Phe341^{6.52}$, $Val136^{3.33}$, and $Ala225^{5.46}$. Interaction with $Leu209^{EL2}$ in the extracellular loop-2 causes engagement of ß-arrestin as evidenced in the co-crystal structure with LSD (PDB ID: 5TVN). The iodo-substituent of DOI forms hydrophobic interactions with Leu209, suggesting that DOI may also be involved in ß-arrestin recruitment, yet there is not sufficient evidence to pinpoint if it has potential to cause cardiac valvulopathy. The DOX molecules with bulky 4-position substituent adopt an 'inverted' binding pose. The substituents engage residues in TM3 and TM7, as seen in ergotamine, which possesses a large amide substituent. These molecules are known to act as antagonists at 5-HT_{2A} receptors, providing us with evidence to predict their functional activities as well. An exception is seen with DOPR, as it is known to be a hallucinogen, (5-HT_{2A} agonist) but has the binding mode of a putative antagonist at the 5-HT_{2B} receptor. Hence, there is a structure-activity relationship observed, wherein the affinity of DOX molecules increases with hydrophobicity. This is not, however, seen with the volume of the substituents.

Overall, putative agonists of the phenylisopropylamine class all appear to adopt a similar binding pose such that the phenyl rings are more or less overlapping, and the substituents interact with the same residues. Norfenfluramine, MDA, and DOX molecules show similar binding modes in the docking studies. The differential binding modes, however, present as a caveat when conducting 3D-QSAR studies, since the alignment of molecules are based on the assumption that they all adopt a similar binding mode

The bulky 4-position-substituted (greater than 84 Å) DOX molecules adopt a different binding pose in comparison to accommodate the bulk of the molecule, as seen with DOPR, DOHx and DOBZ. The three substituents have high affinity as they access the secondary binding pocket residues in TM7. Therefore, the series might consist of agonists, partial agonists and antagonists. Functional activity studies are underway to determine the nature of the DOX molecules. The present studies suggest that the functional activity of DOX analogs at 5-HT_{2A} receptors might not be consistent with their functional activity at 5-HT_{2B} receptors. This remains to be determined.

VI. Experimental

A. CoMFA and CoMSIA

1. Software and Hardware

Conformational analyses and CoMFA studies descried here were carried out on Sybyl X2.1.1, a molecular modeling package available from Tripos Inc. Calculations were performed on a UNIX workstation at Virginia Commonwealth University (Department of Medicinal Chemistry).

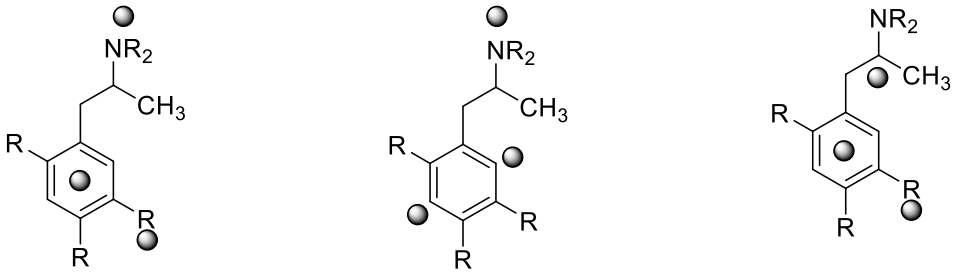
2. Sketching of molecules

All structures were sketched and minimized in Sybyl X2.1.1. The structures were minimized under Tripos force fields using Gasteiger Hückel charges and subject to 100,000 iterations. The dielectric constant was set at 4 to mimic the environment of the protein. Since the (*R*)-enantiomer was the eutomer for phenylisopropylamines at 5-HT_{2A} receptors, all structures were sketched with the (*R*) configuration. All the amines were protonated, as found in the protein. Phenylisopropylamines exist in several energy minima, and were hence sketched and minimized with various templates using the crystal structures of 5-HT_{2B} receptor ligands keeping in mind the multiple rationale that may dictate how the molecule may bind at the receptor. Initial studies were conducted by obtaining the co-crystal structure of LSD with the 5-HT_{2B} receptor, deleting the atoms

except those in the basic scaffold of phenylisopropylamines, and sketching the remainder. Acknowledging that the bioactive conformation of LSD co-crystal structure[12] does not visit the conformation seen in its small molecule crystal structure,[36] we chose the former. The biological data (affinity/K_i) of the molecules was obtained from the study conducted by Nelson, et al.[9] The K_i values of the compounds were expressed in molar units and converted to their negative logarithmic value (-log K_i = pK_i). The molecules were sketched, superimposed and saved in a molecular database (.mdb file), and the corresponding pK_i values were imported into a spreadsheet in Sybyl X2.1.1.

3. Alignment of molecules

The alignment is a crucial step in determining the quality of the model. With a view to ensure maximum overlap of the compounds present in the data set, three non-coplanar points were chosen to get a reliable model for 3D-QSAR analyses. Various alignments were used to obtain suitable overlap, with the lowest root mean square (RMS) deviation value being favorable. Following are the RMS values for each alignment. (Figure 41) Since alignment 1 brings about overlap of the vital components of the molecule, it was pursued for future CoMFA studies. A low RMS value may not necessarily include atom-to-atom overlap of the important components of the molecule.



Alignment 1	Alignment 2	Alignment 3
RMS value range: 0.001-0.089	RMS value range: 0.002-0.05	RMS value range: 0.002-0.6

Figure 41. Alignment of compounds attempted based on three non-coplanar points.

4. Calculation of CoMFA and CoMSIA descriptors

The studies were conducted on Sybyl X2.1.1 molecular modeling software suite. Steric and electrostatic interactions were calculated using an sp^3 hybridized carbon atom, and a (+1) charge as steric and electrostatic probes, respectively, in a grid with 2-Å spacing. Default values were of 30 kcal/mol was set as the maximum steric and electrostatic energy cutoff. During calculations of field energies at the lattice points, some values may exceed the selected cutoffs for electrostatic and steric energies. For the PLS analysis, the minimum column filtering was set at 2.0 kcal/mol to improve the signal to noise ratio by omitting those lattice points where energy variation was below the threshold. The column filtering value is the minimum level of energy variation allowed for a lattice point to be included in the PLS analysis. It can be reduced to improve the sensitivity of the PLS analysis. The following regression analysis was performed using the cross-validation by leave-one-out (LOO) method. The non-cross validated analysis was performed with the optimal number of components that yielded the highest R^2_{CV} (q^2) and the correlation coefficient r^2. The standard error of prediction values were also obtained in this study.

5. Partial least squares (PLS) analysis for CoMFA

The CoMFA study is performed by calculating and comparing the molecular steric (Lennard Jones) and electrostatic (Coulombic) fields of both active and inactive analogs. The energy values obtained are entered into columns in a CoMFA QSAR table. The table consists of thousands of descriptors for each molecule. A PLS analysis correlates these descriptors with the molecular structure and in turn, to the biological activity. The leave-one-out method and PLS analysis provide a statistically significant final CoMFA equation.

The result of this analysis is a 'cross validated r^{2}' (also known as q^2), an indicator of the quality of the model. A q^2 value of >1.0 suggests the model equation is predictive and when its value is negative, the model is not predictive. At 0.5, a q^2 value is considered significant. Optimum number of components is chosen for a non-validated analysis. Ideally, the number of components is 1/3rd of the total number of compounds. PLS analysis explains the differences in biological activity based upon the descriptors used in the calculations and the interactions of the molecules within the grid.

6. Selection of training set and test set

The training set was chosen to represent all electronic and steric characters of the 4-position substituents. The test set comprised of the remainder molecules, as described in the results and discussion section. The training set and test set were divided into 75% and 25% respectively.

7. Leave-one-out (LOO) method of cross validation for CoMFA

The leave-one-out method excludes one compound from the data set and a CoMFA model is generated to predict the biological activity of the deleted compound. This method validates the model externally by predicting the activity of a molecule absent from the dataset. The residual sum of squares or predicted residual error sum of squares (PRESS) describes the margin between the predicted versus actual biological activity, and the cross validated standard error of estimate (s_{CV}) provides the margin of error for the overall model. This method is run several times by deleting one molecule at a time each time generating a CoMFA model, typically based on the number of molecules present in the

data set. The final q^2 value determines the quality of the model and its predictivity, provided the model is not overfitted.

8. PLS analysis for CoMSIA

The steps for CoMFA and CoMSIA are the same, up to the alignment step. The PLS analysis for CoMSIA is different from that of CoMFA since there are five types of dependent variables calculated for the molecules of the data set: hydrogen bond donor, hydrogen bond acceptor, hydrophobic properties, steric, and electrostatic properties. CoMSIA differs from CoMFA in that it accounts for the similarity of the molecules in the study and provides a statistical correlation for the trends seen in that data set.

In a CoMSIA study (Table 14), the result consists of contribution of each field (donor/ acceptor/ hydrophobic/ steric/ electrostatic) and the q^2 value with the standard error of estimate. A dependent variable column can be excluded if it has less than 15% contribution to the model. Provided in Table 15 is the output obtained for the CoMFA models generated. The column filtering was reduced to 'fine tune' the q^2 value of the model. However, the pursuit of obtaining a high q^2 value bears certain risk of overfitting the model, and was hence not attempted any further.

Table 14. h5-HT_{2B} receptor affinities based CoMSIA study output.

Experimental settings	Donor (%)	Acceptor (%)	Hydrophobic (%)	Steric (%)	Electrostatic (%)	Optimum number of components	Column filtering	q^2
n = 20	13	15	45	12	15	4	0.01	0.36
n = 20 (column filtering default)	15	14	50.5	11	8	4	2	0.33
2° and 3° amines dropped (n = 16)	0.1	18.4	56.4	14.9	0.01	6	0.01	0.49
Excluded donor (low contribution)	-	20	60.8	11.8	0.07	5	0.01	0.49
Electrostatic dropped (low contribution)	-	15.1	68.7	16.1	-	5	0.01	0.49

Table 15. CoMFA study conducted on rat 5-HT_{2B} receptor affinity values.

Experimental settings	Std. error of prediction	Column filtering (kcal/mol)	Optimal number of components	Grid spacing	CoMFA q^2
Default	0.731	2	2	2Å	-0.110
Reduced grid spacing	0.732	2	2	1Å	-0.113
Reduced column filtering	0.744	1	2	2Å	-0.115
H-bond parameter[a]	0.635	2	2	2Å	0.163
H-bond parameter (reduced column filtering)	0.635	1	2	2Å	0.163
Reduced grid spacing	0.635	2	2	1Å	0.163

[a]Steric and electrostatic cutoff 50 kcal/mol

In order to further understand the models with low q^2 values for the human 5-HT_{2B} CoMFA, a comparative study was conducted with rat 5-HT_{2B} receptor affinities (Table 15 for CoMFA and Table 16 for CoMSIA), and subsequently with the human 5-HT_{2A} receptor

affinities. The data for 5-HT_{2A} receptor was found to be highly sensitive to changes in the 4-position substituents, a good range of affinity values were available. Table 17 and 18 provide the output obtained for CoMFA and CoMSIA models for 5-HT_{2A} receptors respectively.

9. CoMSIA on rat 5-HT_{2B} values

The CoMSIA studies using 5-HT_{2B} receptor binding affinity data were conducted to investigate if the quality of the model might improve by modulating various factors influencing the quality of the model (for example, column filtering, grid spacing, and number of dependent variable columns used for PLS). As Table 16 suggests, these factors play but a minor role in comparison with the biological activity data range. The q^2 value remains within a narrow range of 0.44-0.49, indicating the experimental settings, default or otherwise were suitable for obtaining a good CoMSIA model.

According to study conducted by Nelson et al.,[9] the affinity values of human 5-HT_{2B} and rat 5-HT_{2B} receptors were similar with a ratio of h5-HT_{2B}/r5-HT_{2B} ranging between 0.3 and 1.6. Therefore, these results were not entirely unexpected, owing to the narrow affinity range at r5-HT_{2B} receptors as well.

Table 16. CoMSIA studies conducted on rat 5-HT_{2B} receptor data with variations in experimental settings.

Experimental settings	Donor %	Acceptor %	Hydrophobic %	Steric %	Electro-static %	Column filtering	Optimal number of components	q^2
Default	15.3	10.2	52.9	12.8	8.88	2	2	0.44
Column filtering reduced	15.3	10.2	5.29	12.9	8.88	0.1	3	0.44
Electrostatic dropped (low contribution)	21.3	17.2	49.4	12.1	-	2	4	0.47
Steric dropped (low contribution)	21.2	17.4	61.5	-	-	0.1	3	0.49
Grid spacing reduced to 1Å	21.2	17.4	61.5	-	-	2	3	0.45

Table 17. CoMFA studies on human 5-HT_{2A} receptor affinities.

Experimental settings	Donor %	Acceptor %	Hydrophobic %	Steric %	Electro -static %	Column filtering	Optimal number of components	q^2
n = 16	13.8	12.7	47	22.5	4	2	5	0.606
n = 20								
Default (n = 21)	9.8	10.4	55.4	15.6	8.8	2	5	0.84 (pursued for investigation)
Electrostatic dropped (low contribution)								
Acceptor dropped (low contribution)	16.5	9	50.4	14.5	8.6	2	5	0.803
	20.3	8.4	50.9	20.4	-	2	5	0.809
	18.2	-	56.2	25.5	-	-	4	0.80

Experimental settings	Number of compounds	Column filtering	Optimal number of components	Grid spacing	q^2
Default	n = 16	2	4	2Å	0.398
Default	n = 17	2	3	2Å	0.402
Increasing number of compounds	n= 21	2	4	2Å	0.68

B. Docking and HINT analysis

1. Docking software

The molecules were docked using GOLD 5.4 (Cambridge Crystallographic Data Centre, Cambridge, UK) into the crystal structure of the 5-HT_{2B} receptor (PDB ID: 5TVN). GOLD is a program which uses genetic algorithm to find the best possible binding pose of the ligand in the receptor. The γ-carbon of conserved orthsteric binding pocket residue $Asp135^{3.32}$ was used to define the binding site and the radius was set at 10 Å around the residue. The model was first validated by re-docking the co-crystallized ligand (LSD) into the receptor and observing the solutions. LSD was docked thirty times, forming a single cluster with all poses matching that of the crystal structure and a robust ChemPLP score (80.48). The other ligands docked were molecules of the DOX series, both isomers of

MDMA, MDA, fenfluramine and norfenfluramine. Although their ChemPLP scores were lower than that of LSD (ranging from 40-50), it is not anamolous to obtain lower scores for a different class of molecules that were docked into the receptor.

2. Visualising and minimization of protein-ligand complexes

The docked ligands were merged with the receptor and minimized in Sybyl X2.1.1. The staged minimization function minimizes the protein side chains as well as the carbon backbone. Parameters for minimization were set in the same manner as that for sketching the molecules. The dielectric function was set at 4.0, using Gasteiger-Hückel charges in a Tripos force-field. The molecules were than extracted from the merged file and visually inspected for hydrogen bonding and other interactions with the receptor residues. Images were generated using PyMOL (Schrödinger, LLC, New York, NY)

3. HINT analysis

The HINT program is invoked from Sybyl 8.0 software. A HINT analysis provides a detailed report of the interacions of the ligand seen with the protein.[94] It helps quantify the level of interaction of the molecule with the receptor. The protein was partitioned using the dictionary option with the default settings.

4. Statistical analyses

All statistical analyses were performed on GraphPad Prism 8.0.0 (Graphpad Software Inc, La Jolla, CA), the Z score test was generated on the Statistical Program for the Social Sciences (SPSS, IBM, New York, NY).

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