Investigating the pharmacology of novel 5-HT_3 receptor ligands; with the potential to treat neuropsychiatric and gastrointestinal disorders

by

Alexander Roberts

A thesis submitted to the University of Birmingham for the Degree of Doctor of Philosophy

Institute of Clinical Sciences

College of Medical and Dental Sciences

University of Birmingham

February 2020

Abstract

The 5-hydroxytryptamine (5-HT; serotonin) 5-HT_3 receptor is an excitatory ligand-gated ion channel expressed in for example the brain and the gastrointestinal tract. Two major subtypes of the receptor have been studied in the most detail; the homomeric 5-HT_3A receptor and the heteromeric 5-HT_3AB receptor. 5-HT_3 receptor antagonists are used clinically to treat chemotherapy induced and post-operative nausea and vomiting, and demonstrate symptomatic relief in diarrhoea-predominant irritable bowel syndrome (IBS-d); but unfortunately, these medications cause adverse effects such as constipation or rarely ischemic colitis in the latter condition. This study has characterised the pharmacology of two structurally distinct 5-HT_3 receptor partial agonists (vortioxetine and CSTI-300); and identified the unique binding properties of the cryptic orthosteric modulator 5-chloroindole (Cl-indole) for the human (h) 5-HT_3 receptor. Vortioxetine is a multi-modal antidepressant, which displays affinity for the 5-HT transporter as well as a multitude of 5-HT receptors, including the 5-HT_3 receptor. The first part of this study has identified vortioxetine as a relatively high affinity, competitive 5-HT_3 receptor partial agonist with an intrinsic efficacy of approximately 40-50% that of 5-HT. Given the safety of vortioxetine in patients, it has the potential to be trialled in other conditions for which 5-HT_3 receptor antagonists demonstrate efficacy but cause adverse effects, such as IBS-d or even schizophrenia. The second section of this study has identified CSTI-300 as a selective, high affinity 5-HT_3 receptor partial agonist (intrinsic activity 30-40% that of 5-HT). Moreover, in a rodent model of IBS-d, CSTI-300 demonstrated comparable efficacy to alosetron, a 5-HT_3 receptor antagonist with established efficacy in treating IBS-d. CSTI-300 is predicted to be able to relieve symptoms of IBS-d in patients without eliciting the side effect profile associated with 5-HT_3 receptor antagonism. The final part of this study has demonstrated that the binding mechanism of Cl-indole for the 5-HT_3A receptor is modulated by 5-HT_3 receptor agonists, but not 5-HT_3 receptor antagonists. This could have implications in designing 5-HT_3 receptor allosteric ligands for potentially treating conditions such as IBS-d.

Acknowledgements

I would firstly like to thank Professor Nicholas Barnes for the opportunity to work for Celentyx and in the academic lab. I am grateful for his knowledge, experience and advice during my time here. I would also like to thank Dr Andy Powel for his advice and help.

Furthermore, I am also grateful to Dr Gillian Grafton for all her help and expertise. I wish to give many thanks to Dr Fay Stewart, Lindsay Bentley, Tina Tang and Gillian Mackie, for all the fun nights out and adventures.

To my wife Jess, for all your support and patience, and for organising our wedding. To my son Evan, I hope I can make you proud.

“You don’t expect to be at the top of the mountain the day you start climbing”

Ron Dennis

Table of Contents

Figures and Tables

Appendix Tables

List of abbreviations

(h) 5-HT_3A or 5-HT_3AB receptor: Human 5-HT_3A or 5-HT_3AB receptor

5-CT: 5-Carboxamidotryptamine

5-HI: 5-hydroxyindole

5-HIAA: 5-hydroxyindoleacetic acid

5-HT: 5-hydroxytryptamine

5-HTBP: 5-HT binding protein

5-HTP: 5-hydroxytryptophan

5-OHIP: 5-hydroxyindalpine

AChBP: Acetylcholine binding protein

AUC: Area under curve

BDI: Beck depression inventory

BDNF: Brain derived neurotrophic factor

B_{max}: Maximum specific binding of a radioligand at receptor

BSA: Bovine serum albumin

cAMP: Cyclic AMP

CB: Cannabinoid receptor

CCK: Cholecystokinin

CINV: Chemotherapy nausea and vomiting

Cl: Clearance of drug from the body

Cl-indole: Chloroindole

C_{max}: Maximum concentration of a drug in plasma

CNS: Central nervous system

COM: Cryptic orthosteric modulator

CTZ: Chemoreceptor trigger zone

CYP: Cytochrome P_{450} enzyme family

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulphoxide

EC_{50}: Concentration of an agonist to a response half of the maximum

ED_{50}: 50% effective dose

ED_{90}: 90% effective dose

EDTA: Ethylenediaminetetraacetic acid

ELIC: Erwinia ligand-gated ion channel

E_{max}: Maximum response (efficacy) of an agonist at a receptor

ENS: Enteric nervous system

FBS: Foetal bovine serum

FDA: Food and drugs administration

Fluo-4 AM: Fluo-4 acetoxymethyl ester

GABA: gamma(γ)-aminobutyric acid

GIT: Gastrointestinal tract

GPCR: G-protein coupled receptor

HAM-A: Hamilton anxiety rating scale

HAM-D: Hamilton depression rating scale

HBSS: Hank's balanced salt solution

HEK293: Human embryonic kidney 293

hERG: Human ether-a-go-go-related gene

HPLC: High performance liquid chromatography

I: Ionomycin

i.p.: Intraperitoneal

IBS: Irritable bowel syndrome

IBS-c: Constipation-predominant IBS

IBS-d: Diarrhoea-predominant IBS

IBS-m: Mixed IBS

IBS-QOL: IBS quality of life questionnaire

IC_{50}: Half-maximal inhibitory concentration

IPANS: Intrinsic primary afferent nerves

K_d: Equilibrium dissociation constant of a radioligand

K_i: Equilibrium dissociation constant of a ligand

LGIC: Ligand-gated ion channel

LSD: Lysergic acid diethylamide

LTP: Long term potentiation

MADRS: Montgomery–Åsberg Depression Rating Scale

MAO: Monoamine oxidase

mCPBG: Meta-chlorophenylbiguanide

MDD: Major depressive disorder

nAChR: Nicotinic acetylcholine receptor

NAM: Negative allosteric modulator

NDA: New drug application

NOAEL: No observed adverse effect level

p.o.: Per os (oral administration)

PAM: Positive allosteric modulator

PANSS: Positive and negative syndrome scale

PBG: Phenyl biguanide

PBS: Phosphate buffered saline

PCPA: Para-chlorophenylalanine

PEI: Polyethyleneimine

PLC: Phospholipase C

PMA: Phorbol 12-myristate 13-acetate

PNS: Peripheral nervous system

PONV: Post-operative nausea and vomiting

PSS: Panic associated symptom scale

PVT: Polyvinyltoluene

RIC3: Resistance to inhibitors of cholinesterase 3

s.c.: Subcutaneous

SDS: Sheehan Disability Scale

SERT: Serotonin transporter

SNRI: Serotonin–norepinephrine reuptake inhibitors

SPA: Scintillation proximity assay

$T_{1/2}$: Half-life of a drug *in vivo*

T_{max}: Time to achieve maximum drug concentration in plasma

TPH: Tryptophan Hydroxylase

Tris: T*ris*(hydroxymethyl)aminomethane

V_d: Volume of distribution

1 Introduction

1.1 Discovery of 5-HT

The discovery of 5-hydroxytryptamine (5-HT, serotonin, Figure 1) began with Erspamer and colleagues who in 1940 identified a molecule in the gastrointestinal tract (GIT), which mediated contraction of smooth muscle (Erspamer et al., 1952). This molecule was found to be secreted by enterochromaffin cells which line the GIT. Based on the localisation and chemical structure, this compound was named enteramine (entero-from the GIT, amine-due to its monoamine structure). In 1948, Rapport et al identified serotonin (sero-serum, tonin-impacted vascular tone), as a plasma-borne mediator of vasoconstriction (Rapport et al., 1948). Erspamer then demonstrated that these two molecules were identical, and the term 5-hydroxytryptamine was born. 5-HT was found in various brain regions (hippocampus, hypothalamus and cortex) (Amin et al., 1954). Moreover, Twarog and colleagues extracted 5-HT from the brains of a variety of animals (rat, rabbit and dog), and demonstrated it was active in a functional assay using the isolate heart from *venus mercenaria* (Twarog et al., 1953).

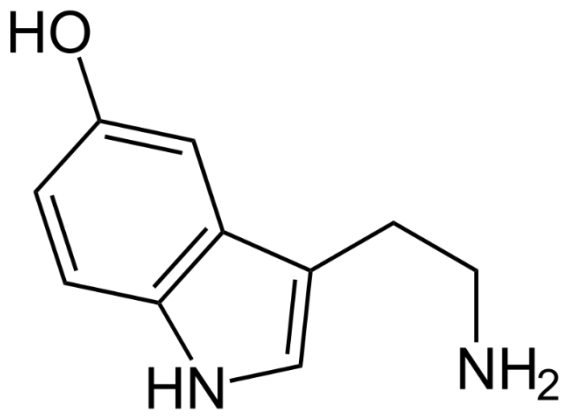

Figure 1. The structure of 5-HT.

1.2 Localisation of 5-HT

The majority of 5-HT within the human body can be found in enterochromaffin cells, which reside in the GIT. Tryptophan is converted into the 5-HT precursor 5-hyroxytryptohan (5-HTP) by tryptophan hydroxylase (TPH). 5-HT is generated from 5-HTP by 5-hydroxytryptohan decarboxylase. 5-HT is catabolised into inactive metabolite 5-hydroxyindole acetic acid (5-HIAA) by monoamine oxidase (MAO). TPH was initially cloned from rabbit pineal gland (Grenett et al., 1987). It was discovered that knock-out mice for TPH lack peripheral 5-HT, and after screening the human genome, a second TPH isoform (TPH2) was discovered on chromosome 12 (Walther et al., 2003). mRNA for *Tph1* was predominantly detected in mouse duodenum, whilst *Tph2* mRNA was found in the mouse brain (Walther et al., 2003). Moreover, Sakowski and colleagues used antibodies selective for TPH1 and TPH2 to elucidate the localisation of these enzymes (Sakowski et al., 2006). To further complement the mRNA studies, an antibody recognising TPH1 labelled cells in the basolateral side of mouse gastrointestinal mucosa, consistent with enterochromaffin cells in the gut (Sakowski et al., 2006). TPH2 protein was labelled in cell bodies and axons in mouse mesencephalon, a pattern consistent with serotonergic neurons (Sakowski et al., 2006). Although this suggests TPH1 and TPH2 have restricted expression, both isoforms have been detected (and shown to be functional) in first trimester placental tissue, implying expression of these isoforms maybe more complex than first thought (Laurent et al., 2017).

There is also evidence of polymorphisms in TPH correlating with diseases (Jun et al., 2011; Israelyan et al., 2019). Indeed, there are associations with TPH1 polymorphisms and diarrhoeal symptoms in irritable bowel syndrome (Jun et al., 2011). The R441H polymorphism in human TPH2 has been identified in a selection of patients with treatment resistant depression (Israelyan et al., 2019). Mice expressing R439H (the murine homolog of R441H) had reduced brain 5-HT levels and demonstrated depressive behaviours (Israelyan et al., 2019). Interestingly there was a reduction in 5-HT immunoreactive cell bodies in the Enteric Nervous System (ENS), and these mice had slowed gastrointestinal motility (Israelyan et al., 2019). This implies a potential link between synthesis of central 5-HT with development of

peripheral serotonergic neurons and disruption to gastrointestinal function plus mood disorders.

In the GIT, 5-HT functions as a local hormone, mediating contraction of intestinal smooth muscle, as well as modulating gut activity through neurotransmitter activity within the ENS. In addition, platelets take up 5-HT from the blood through the serotonin transporter (SERT). 5-HT is then released by platelets to induce vasoconstriction by contracting vascular local smooth muscle during thrombus formation. 5-HT also functions as a neurotransmitter in the nervous system. Within the Central Nervous System (CNS), 5-HT-ergic neurons arise from the raphe nuclei found within the brainstem (Gothert, 2013). These serotonergic fibres ascend to innervate areas such as the cerebral cortex, hippocampus, limbic areas and cerebellum, with 5-HT implicated in mood, emotion, cognition and homeostatic functions. 5-HT neurons also descend to the spinal cord and are involved in modulating sensory and motor responses (Gothert, 2013). Similar to other monoamines (dopamine and noradrenaline) and acetylcholine, serotonin influences the activity of excitatory or inhibitory interneurons, through a plethora of receptors.

1.3 5-HT receptors

The physiological actions of 5-HT are mediated though interactions with its many different receptor subtypes; which are distributed throughout the human body. The first initial description of 5-HT receptors was in the guinea pig intestine by Gaddum and Picarelli, who designated receptors depending on their sensitivity to morphine (M) or dibenzyline (D) (Gaddum et al., 1957). Bennett and Aghajanian demonstrated that rat brain homogenate contained [^{3}H]-Lysergic acid diethylamide ([^{3}H]-LSD) binding sites, and the level of [^{3}H]-LSD binding could be reduced by 5-HT (which is structurally similar to LSD). Specific [^{3}H]-LSD binding sites were found in rat cortex, striatum, hippocampus, diencephalon, raphe nuclei and the cerebellum (Bennett et al., 1974). In 1979, Peroutka and Snyder used different radioligands ([^{3}H]-5-HT, [^{3}H]-LSD or [^{3}H]-spiroperidol) to distinguish two different 5-HT receptors (named 5-HT$_1$ and 5-HT$_2$ receptors) based on their differential binding sensitivity to those radioligands ([^{3}H]-5-

HT bound 5-HT_1 receptors, [^{3}H]-spiroperidol bound 5-HT_2 receptors and [^{3}H]-LSD bound to both receptors with similar affinity) (Peroutka et al., 1979). More selective pharmacological tools and molecular biology has enabled the discovery of 14 distinct 5-HT receptor subtypes (Table 1). The majority of 5-HT receptors are G-protein coupled receptors (GPCRs). 5-HT_1 and 5-HT_{5A} receptors are inhibitory 5-HT receptors, coupling to G_i protein to inhibit cyclic adenosine monophosphate (cAMP) production and activate K^+ channels. 5-HT_2 receptors mobilise Ca^{2+} through phospholipase C (PLC) dependent G_q protein stimulation (Hoyer et al., 1989). The 5-HT_4, 5-HT_6 and 5-HT_7 receptors all signal through the G_s protein. The 5-HT_3 receptor is unusual among monoamine receptors because it is the only ligand-gated ion channel (LGIC), permeable to Na^+, K^+ and Ca^{2+} ions.

Table 1: 5-HT receptors and their function

Receptor	Transduction mechanism	Biological activity	Example drug/ligand	Side effects
5-HT_{1A}	G_i protein.	Somatodendritic autoreceptor (decrease 5-HT release).	Buspirone: partial agonist used to treat anxiety.	Light-headiness, nausea and vomiting.
5-HT_{1B}	G_i protein.	Presynaptic autoreceptor (decrease 5-HT release). Also, vasoconstriction of coronary and cerebral vasculature.	Sumatriptan: agonist used to treat migraines.	Dizziness, vertigo, sedation.
5-HT_{1D}	G_i protein.	Autoreceptor (decrease 5-HT release).	BRL15572: antagonist.	N/a.
5-ht_{1e}	G_i protein.	Yet to be observed in native tissue.	BRL54443: agonist.	N/a.
5-HT_{1F}	G_i protein.	Inhibits neurogenic dural inflammation.	Lasmiditan: agonist used to treat migraines.	Dizziness, fatigue, vertigo.
5-HT_{1P}	Unknown	Elicits slow depolarisation of myenteric neurones.	5-OHIP: agonist	N/a.

Table 1 cont.

5-HT$_{2A}$	G_q protein.	Increase firing of cortical glutamatergic and GABAergic neurones.	Pimavanserin: inverse agonist/antagonist used to treat Parkinson's disease psychosis.	Somnolence, headache, peripheral oedema.
5-HT$_{2B}$	G_q protein.	Proliferation and differentiation of cardiac tissue. Anorexigenic.	Fenfluramine: agonist used to treat obesity.	Cardiac hypertrophy (now withdrawn from the market).
5-HT$_{2C}$	G_q protein.	Stimulate pro-opiomelanocortin release from hypothalamus.	Lorcaserin: agonist used to treat obesity.	Headache, dizziness, nausea.
5-HT$_3$	Na^+, Ca^{2+} and K^+ LGIC.	Elicit emetic response. Stimulate intestinal motility and visceral sensation.	Alosetron: antagonist used to treat diarrhoea-predominant irritable bowel syndrome.	Constipation, headache, ischemic colitis (rare).
5-HT$_4$	G_s protein.	Promote gastric motility. Stimulate cortical acetylcholine release.	Cisapride: agonist used to treat constipation-predominant irritable bowel syndrome.	Tachycardia (now withdrawn).
5-HT$_{5A}$	G_i protein.	Activates K^+ channels to inhibit neuronal firing in the prefrontal cortex.	ASP5736: antagonist	N/a.
5-HT$_6$	G_s protein.	Modulate release of neurotransmitters (acetylcholine, dopamine, noradrenaline, glutamate).	Clozapine: antagonist/inverse agonist used to treat schizophrenia.	Sedation, hypotension, agranulocytosis.
5-HT$_7$	G_s protein.	Elicits a hypothermic response.	JNJ18038683: antagonist	N/a.

1.3.1 5-HT$_{1A}$ receptors

The 5-HT$_{1A}$ receptor functions primarily as a somatodendritic autoreceptor on raphe neurons, mediating feedback control of 5-HT signalling through inhibition of neuronal

activity (Barnes et al., 1999). This receptor is also found postsynaptically in the for example, hippocampus, septum and cingulate gyrus. Buspirone, a 5-HT_{1A} receptor partial agonist, is used clinically in the treatment of anxiety disorders (Pytliak et al., 2011). Side effects are generally mild (light headiness, nausea and vomiting) (Strawn et al., 2018).

1.3.2 5-HT_{1B} receptors

The 5-HT_{1B} receptor is located on presynaptic raphe terminals as an autoreceptor. The 5-HT_{1B} receptor is also found as a heteroreceptor, expressed by cholinergic neurons (Hoyer et al., 2002). In addition, activation of this receptor mediates vasoconstriction of cerebral and coronary arteries. Therapeutically, the -triptan class of drugs (e.g. sumatriptan) offer relief as anti-migraine agents with few adverse effects (dizziness, sedation, vertigo) (Maghbooli et al., 2014). This is achieved through stimulation of 5-HT_{1B} (and 5-HT_{1D}) receptors on cerebral vasculature, which initiates vasoconstriction after circulating 5-HT elicits vasodilation (Pytliak et al., 2011).

1.3.3 5-HT_{1D} receptors

The 5-HT_{1D} receptor has similar pharmacology to the 5-HT_{1B} receptor (both receptors display high affinity for sumatriptan) (Nichols et al., 2008). However, in the human brain, 5-HT_{1D} receptor expression is lower in comparison to the 5-HT_{1B} receptor. The 5-HT_{1D} receptor can be found in the cortex, hippocampus and substantia nigra (Barnes et al., 1999). The selective antagonist BRL15572 has been used to investigate the function of the 5-HT_{1D} receptor *in vivo* (De Vries et al., 1998).

1.3.4 5-ht_{1e} receptors

Due to a lack of *in vivo* functional data, the 5-ht_{1e} receptor is denoted in lower case. Expression of the receptor clone in heterologous expression systems has demonstrated that the 5-ht_{1e} receptor couples negatively to G_i protein, and so is grouped with the other 5-HT_1 receptors. BRL54443, a 5-ht_{1e} antagonist was used to

investigate the potential function of the 5-ht$_{1e}$ receptor in rat (Granados-Soto et al., 2010).

1.3.5 5-HT$_{1F}$ receptors

Similar to the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors, the 5-HT$_{1F}$ receptor displays affinity for sumatriptan, suggesting a role for this receptor in the antimigraine activity of sumatriptan (Pytliak et al., 2011). 5-HT$_{1F}$ binding sites have been discovered in the hippocampus and dorsal raphe nucleus, which strongly correlates with its mRNA distribution (Barnes et al., 1999). The 5-HT$_{1F}$ agonist lasmiditan has been approved to treat migraines, with dizziness, fatigue and vertigo common side effects (Neeb et al., 2010).

1.3.6 5-HT$_{1P}$ receptors

The 5-HT$_{1P}$ receptor is a novel receptor which has been characterised *in vivo* but has yet to be cloned and studied in recombinant expression systems (Galligan, 2007). [^{3}H]-5-hydroxyindalpine (5-OHIP) has been shown to label the 5-HT$_{1P}$ in mouse, rabbit and guinea pig gut (Branchek et al., 1988). The 5-HT$_{1P}$ receptor has been shown to elicit a slow excitatory post synaptic potential in the myenteric plexus of guinea pig and increase the frequency of peristalsis in murine colon (Galligan, 2007; Mitchell et al., 2009).

1.3.7 5-HT$_{2A}$ receptors

The 5-HT$_{2A}$ receptor is the ‘D’ receptor which Gaddum originally described (Richard Green, 2006). 5-HT$_{2A}$ receptor stimulation elicits vascular smooth muscle contraction as well as functioning in platelet aggregation (Hoyer et al., 2002). Within the CNS, this receptor is distributed within the cortex, nucleus accumbens and hippocampus. Hallucinogenic drugs (e.g. LSD) and atypical antipsychotics such as clozapine demonstrate high affinity for this receptor (Bockaert et al., 2006). Recently, pimavanserin has been developed as a 5-HT$_{2A}$ inverse agonist/antagonist used to

treat Parkinson's disease psychosis (Zhang et al., 2019). Side effects including headache, somnolence and peripheral oedema have been experienced by patents taking this drug (Zhang et al., 2019).

1.3.8 5-HT$_{2B}$ receptors

Knock-out of the 5-HT$_{2B}$ receptor is embryonic lethal in mouse, implying this is the only essential serotonin receptor. During neural development, the 5-HT$_{2B}$ receptor is involved in neuronal mitosis, although there is low expression of this receptor in the adult CNS (Barnes et al., 1999; Berger et al., 2009). Stimulation of the 5-HT$_{2B}$ receptor also appears to trigger mitosis of myofibroblasts in the heart valves. Fenfluramine, a potent 5-HT$_{2B}$ receptor agonist, was used in the treatment of obesity, but was withdrawn due to the valvopathies associated with potential 5-HT$_{2B}$ receptor activation (Berger et al., 2009; Pytliak et al., 2011). The 5-HT$_{2B}$ receptor may also be implicated in colonic motility in rodent (Barnes et al., 2019.

1.3.9 5-HT$_{2C}$ receptors

The 5-HT$_{2C}$ receptor is unique amongst serotonergic receptors in the respect it undergoes RNA editing to generate a multitude of different splice variants (Pauwels, 2000). 5-HT$_{2C}$ receptor agonists have been shown to be anxiogenic *in vivo* (with the converse true of antagonists), which correlates with receptor expression in cortical and limbic areas of the CNS (Barnes et al., 1999). Furthermore, lorcaserin, a 5-HT$_{2C}$ receptor agonist, has received marketing authorisation (new drug application) by the FDA (Food and drugs administration) to reduce food intake of obese patients. Side effects are mild and have been listed as headache, dizziness and nausea (Elhag et al., 2019).

1.3.10 5-HT$_4$ receptors

The 5-HT$_4$ receptor was the first 5-HT receptor to be discovered using functional studies (cAMP production) rather than radioligand binding experiments (Barnes et al.,

1999). In human, the 5-HT_4 gene has 9 splice variants (Bockaert et al., 2006). In the CNS, the 5-HT_4 receptor modulates acetylcholine release and facilitates the generation of long-term potentiation (LTP), implicating this receptor in cognition, learning and memory (Hoyer et al., 2002). In the colon, 5-HT_4 receptors stimulate acetylcholine release to contract colonic smooth muscle and elicit peristalsis (Hoyer et al., 2002). 5-HT_4 partial agonists (e.g. cisapride) were effective in treating constipation-predominant irritable bowel syndrome (IBS-c), but induced cardiac side effects (tachycardia) due to expression of 5-HT_4 receptors by heart muscle (Berger et al., 2009).

1.3.11 5-HT_{5A} receptor

The 5-HT_{5A} receptor has now been recognised as a functional 5-HT receptor, with evidence suggesting it couples to the G_i protein to reduce adenylyl cyclase activity (Barnes et al, 2019). Early 5-HT_{5A} receptor knock-out work demonstrated evidence of *in vivo* 5-HT_{5A} receptor activity, and recent *ex vivo* experiments have suggested 5-HT_{5A} receptors mediate K^+ currents in rodent prefrontal cortex brain slices (Grailhe et al., 2001; Goodfellow et al., 2012). 5-HT_{5A} receptors show widespread CNS expression and display high affinity for the non-selective 5-HT agonists; LSD and 5-CT (5-Carboxamidotryptamine) (Tanaka et al., 2012). The selective 5-HT_{5A} antagonist ASP5736 appears to ablate schizophrenic symptoms in an animal model (Yamazaki et al., 2014).

1.3.12 5-ht_{5b} receptor

Expression of the mouse 5-ht_{5b} receptor in a heterologous expression system will yield a functional receptor, but with the transduction mechanism not fully understood. In humans, a stop codon in the first exon means the protein is unlikely to be functional (Grailhe at al., 2001; Barnes et al, 2019).

1.3.13 5-HT_6 receptors

The 5-HT_6 receptor is predominantly expressed in CNS regions including the nucleus accumbens, striatum and hippocampus. There is limited expression outside the human brain, although mRNA has been found in the stomach and adrenal glands (Barnes et al., 1999). A non-functional splice variant of the human 5-HT_6 receptor has been discovered (Olsen et al., 1999). This receptor couples positively to adenylyl cyclase, and has been demonstrated to increase acetylcholine release in the CNS, implicating the 5-HT_6 receptor in cognition (Hoyer et al., 2002). Clozapine demonstrates high affinity for the 5-HT_6 receptor, suggesting a role for this receptor in schizophrenia (Pytliak et al., 2011). Patients receiving clozapine report sedation, hypotension and agranulocytosis as adverse effects (Zhang et al., 2019).

1.3.14 5-HT_7 receptors

The 5-HT_7 receptor is expressed in the hippocampus, thalamus and hypothalamus, where for the latter brain region it is believed to be involved in thermoregulation and the circadian rhythm (Bockaert et al., 2006). Three splice variants of the 5-HT_7 receptor exist in humans, with each differing in the length of the C-terminus and demonstrate differences in G-protein coupling efficacy and desensitisation (Barnes et al., 1999). Similar to the 5-HT_6 receptor, clozapine displays high-affinity inverse-agonist activity at the 5-HT_7 receptor (Nichols et al., 2008). In addition, the selective 5-HT_7 receptor antagonist JNJ18038683 demonstrated some anti-depressant activity in mouse, but this was not translated in human studies (Bonaventure et al., 2012).

1.3.15 5-HT_3 receptor

The 5-HT_3 receptor is the only serotonergic (and monoaminergic) receptor which is a LGIC, eliciting an excitatory response in cells through passage of Na^+ and Ca^{2+} ions. The 5-HT_3 receptor was originally, designated the ‘M’ receptor by Gaddum, due to the fact morphine blocked 5-HT induced muscle contraction in guinea pig ileum (Gaddum et al., 1957). Radioligand binding studies in 1987 first identified 5-HT_3 receptor binding sites in rat and human brain (Kilpatrick et al., 1987; Barnes et al., 1989). In 1991,

using isolated cDNA from NCB-20 cells, the function of the 5-HT_3 receptor was explored, and demonstrated it to be an excitatory LGIC with structural similarities to the nicotinic acetylcholine receptor (nAChR). Moreover, it was confirmed to be a member of the cys-loop family of ionotropic receptors, which also includes the nAChR, $GABA_A$ (gamma[γ]-aminobutyric acid) and glycine receptor (Maricq et al., 1991).

1.4 Cys-loop receptors

Cys-loop receptors are a large family of LGICs conserved across both eukaryotic and prokaryotic cells. In humans, this family includes the nAChR, $GABA_A$ receptor, glycine receptor and the 5-HT_3 receptor. Briefly, the structure of these proteins can be divided into three domains; a large extracellular N-terminus for ligand binding (which encompasses the cysteine loop), a transmembrane domain for ion conduction, an intracellular loop for receptor trafficking and function, and a small extracellular C-terminus. Upon receptor activation by agonist binding, a conformational shift permits ion flux across the cell membrane. To promote excitatory synaptic transfer, 5-HT_3 receptors and nAChR conduct Na^+ ions (and possibly Ca^{2+} ions depending on nAChR receptor subtype). $GABA_A$ and glycine receptors on the other hand permit Cl^- influx to induce fast inhibitory synaptic transfer.

1.5 5-HT_3 receptor structure

5-HT_3 receptor binding sites were first identified in rat cortex using the selective radioligand [^{3}H]-GR65630. The affinity of antagonists to compete off [^{3}H]-GR65630 correlated well with their ability to block depolarisation of rat vagus nerve induced by 5-HT (Kilpatrick et al., 1987). The first structural information regarding the 5-HT_3 receptor came in 1991 when Maricq and colleagues isolated 5-HT_3 receptor cDNA from NCB-20 cells. It was demonstrated to have 487 amino acids, with a molecular weight of 56 kDa. The 5-HT_3 receptor showed sequence similarity to the α7 nAChR, with four transmembrane domains and a large N-terminal extracellular loop which contained cysteine loops (Maricq et al., 1991).

The 5-HT_3 receptor is composed of five protein subunits (identical or non-identical) surrounding a channel spanning the cell surface membrane (Figure 2). Homology modelling using the closely-related nAChR, along with the acetylcholine binding protein (AChBP, secreted by *Lymnaea stagnalis* at synapses to terminate cholinergic transmission) and prokaryotic LGICs have aided in elucidating the structure of these subunits. The large extracellular N-terminus forms the ligand binding domain (where the classic cysteine-loops are located) between two juxtaposed subunits. One protein (termed the principle subunit) contributes 3 loops (noted A, B and C) and these loops form the binding site for orthosteric ligands with 3 loops (D, E and F) from the adjacent complementary subunit. Research has shown that the majority of the amino acids in this structure aid in maintaining the binding pocket as well as playing a part in channel gating (Thompson et al., 2006; Barnes et al., 2009). The structure of the mouse 5-HT_3A receptor has been dissected using crystal structure techniques. It was demonstrated that each receptor subunit was formed by a twisted β-sandwich generating the extracellular domain and four helical transmembrane domains (Figure 2) (Hassaine et al., 2014; Basak et al., 2018).

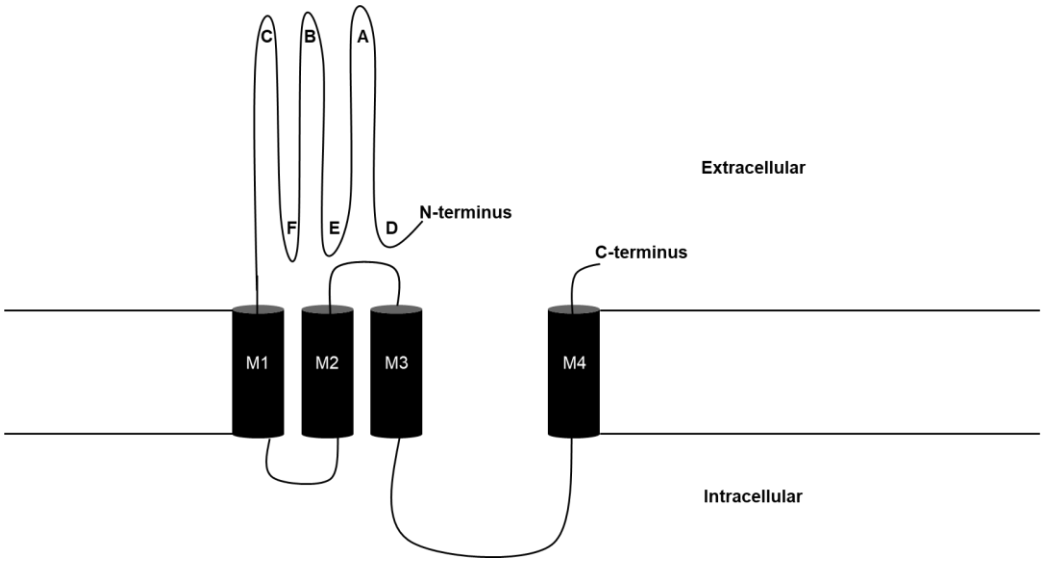

Figure 2. The basic structure of the 5-HT_3 receptor.

This figure highlights the general location of the principle (A, B, C) and complementary (D, E, F) loops within the N-terminus of the 5-HT_3 receptor. The transmembrane domains can also be seen, with M2 forming the channel pore. The large intracellular loop between M3 and M4 serves as a modulatory domain in receptor function; and the small C-terminus is important for receptor trafficking (Thompson et al., 2007).

Studies have found selective roles for various amino acid residues within the N-terminal loops. In the mouse 5-HT_3 receptor, Glutamate 106 has a critical role in 5-HT_3 receptor pharmacology. Mutating this residue to aspartate caused the affinity of the radioligand [^{3}H]-GR65630 to be decreased compared to wild-type receptor (Boess et al., 1997). Furthermore, in competition binding assays, the affinity for 5-HT and ondansetron, granisetron and renzapride were also decreased at the mutant receptor. Although the potency of 5-HT was similar in the mutant receptor compared to wild-type receptor, the Hill coefficient was reduced to unity in the mutant receptor compared to approximately 2 in the wild type receptor, implying a lack of cooperative binding of 5-HT for the mutant receptor (Boess et al., 1997). It was suggested that this residue may form a hydrogen bond with 5-HT (Boess et al., 1997). Furthermore, mutating asparagine 128 to glycine or lysine caused the potency of 5-HT to be reduced, whilst the converse was true when this residue was changed to valine (Price et al., 2008). Any mutations to glutamate 129 generated a receptor that failed to respond to 5-HT or demonstrate any [^{3}H]-granisetron binding. It was deduced therefore that asparagine 128 is involved in channel gating, meanwhile glutamate 129 is implicated in ligand binding, similar to glutamate 106 (Price et al., 2008). Within loop B, tryptophan 183 is thought to be critical in forming a cation-π interaction with 5-HT. This was determined by incorporating unnatural amino acids into the 5-HT_3 receptor and evaluating its function using electrophysiology. Introducing a fluorine atom into tryptophan prevents the formation of cation-π interactions (Beene et al., 2002). The potency of 5-HT is reduced compared to wild type receptor when more fluorine atoms are incorporated into tryptophan 183. Interestingly, the EC_{50} of 5-HT is not impacted when fluorine atoms are added to tryptophan 90 (in loop D), suggesting this residue does not form cation-π interactions with 5-HT (Beene et al., 2002).

The most inter-species variability is noticed within loop C, and hence the species-specific differences in drug action. Tyrosine residues are thought to be important for 5-HT_3 receptor function (Price et al., 2004). Mutating tyrosine 50 or tyrosine 91 generated receptors which did not reach the cell surface and were also not functional in electrophysiology experiments. Moreover, tyrosine 234 in loop C was shown to be important in either ligand binding or receptor gating, because mutating this amino acid generated a non-functional receptor (Price et al., 2004). Using a photo-crosslinking

granisetron analogue, it was discovered this ligand covalently bound to methionine 228 in loop C, suggesting an important role for this residue in antagonist binding to the 5-HT_3A receptor (Jack et al., 2018). The conformation of loop C correlates with occupancy of ligand, and functional channel gate (i.e. antagonists leave the loop in an open shape, whilst agonists induce a closed shape) (Hassaine et al., 2014; Basak et al., 2018).

Within loop D of the complementary subunit, tryptophan 90 is important for ligand binding. Mutating this residue to tyrosine generated a receptor for which 5-HT displayed reduced potency and [^{3}H]-granisetron demonstrated reduced affinity. This suggests this residue is important for ligand binding (Spier et al., 2000). This is also true for tryptophan 183, which also displayed slower desensitisation kinetics. Tryptophan 95, when mutated to tyrosine, produced a receptor which did not reach the cell surface, implicating this amino acid in receptor formation or assembly (Spier et al., 2000). Within loop E, tyrosine residues have been shown to be important for ligand binding and 5-HT_3 receptor function. By mutating tyrosine 141 to alanine or serine, the 5-HT induced agonist response was similar to wildtype receptor, but the affinity for [^{3}H]-granisetron was reduced, implicating this residue in antagonist binding and not receptor gating (Beene et al., 2004). Tyrosine 143 is thought to be involved in 5-HT_3 receptor function, because 5-HT displayed decreased potency at alanine or serine mutants; although [^{3}H]-granisetron binding affinity remains unaffected. 5-HT_3 receptors in which tyrosine 153 is mutated to alanine or serine displayed reduced affinity for [^{3}H]-granisetron and an increased EC_{50} for 5-HT; which implicated this residue in both channel gating and ligand binding (Beene et al., 2004).

Like many transmembrane proteins, the 5-HT_3 receptor undergoes post-translational modifications before cell surface expression. One example is N-linked glycosylation. It has been shown that preventing N-linked glycosylation, either pharmacologically (using tunicamycin) or genetically (mutating various asparagine residues within the N-terminus), causes a reduction in binding of [^{3}H]-granisetron (Monk et al., 2004). Immunoreactivity studies showed that cell surface receptor expression is prevented by tunicamycin. It was concluded therefore that N-glycosylation is necessary for

stabilising the receptor at the cell surface after transit from the endoplasmic reticulum (Monk et al., 2004).

A conserved proline residue (proline 256) in the M1 domain have been shown to be essential in 5-HT_3 receptor function. 5-HT fails to elicit a response at the 5-HT_3 receptor when this proline residue is mutated to alanine, glycine or leucine probing (Dang et al., 2000). Interestingly, these mutations do not affect the level of [^{3}H]-granisetron binding. The M2 domain contains an α-helix which is known to line the channel pore and play an essential role in determining ion conductance (both size and flux) (Peters et al., 1992; Lummis, 2012). By mutating various residues within the transmembrane domain, the charge selectivity can be altered so the 5-HT_3 receptor now conducts anions (Lummis, 2012). The M2 domain forms straight helices which lie parallel to the pore axis, with aspartate 105 at the extracellular end. This is then followed by hydrophobic rings (isoleucine, valine, leucine), which act as a gate to stop ion conduction, and finally threonine, serine and glutamate form the intracellular end (Hassaine et al., 2014; Basak et al., 2018). There is a large intracellular loop between the M3 and M4 transmembrane domains. This loop contains potential phosphorylation sites, and serves a modulatory role in receptor trafficking, modulation and ion conductance (Walstab et al., 2010; Lummis, 2012). The M4 transmembrane domain lies close to the cys loops and has a role in channel gating.

It was established that the final residues of the C-terminus of the 5-HT_3 receptor is critical in receptor formation (Butler et al., 2009). If the last two (glutamine 453 and tyrosine 454) residues from the C-terminus are deleted, there is a significant reduction in the formation of [^{3}H]-granisetron binding sites (Butler et al., 2009). Moreover, this is also replicated when the final alanine amino acid is deleted. The importance of alanine residues in the C-terminus is further strengthened when additional alanine residues are added to the C-terminus; or when the before-mentioned glutamine and tyrosine are mutated to alanine because these modifications lead to an increase in the formation of 5-HT_3 receptor binding sites greater than wild type receptor (Butler et al., 2009). Cell surface expression of 5-HT_3 receptor (visualised by

immunocytochemistry) is also diminished when the final residues of the C-terminus are deleted (Butler et al., 2009).

1.6 Subunits of the 5-HT_3 receptor

Like the other Cys-loop receptors, the 5-HT_3 receptor is composed of 5 protein subunits. In 1991 the first subunit (termed 5-HT3A) was found to be able to generate a functional homomeric receptor in expression systems, unlike nAChR which require the assistance of chaperone proteins such as RIC-3 (resistance to inhibitors of cholinesterase 3) to transit to the cell surface (Lummis, 2012). It was however noticed that the single channel conductance of the 5-HT_3A receptor in expression systems (e.g. human embryonic kidney [HEK] 293 cells) does not correspond to what is seen in native tissue (e.g. rabbit nodose ganglion neurons). This inconsistency could not be explained until 1999 when a second 5-HT_3 subunit (termed 5-HT3B) was identified by screening the human genome (Dubin et al., 1999). The 5-HT3B subunit is approximately 44% identical to the 5-HT3A subunit. Unlike the 5-HT3A subunit, the 5-HT3B protein does not form a functional receptor in cell cultures, instead it requires the 5-HT3A subunit to form a non-identical heteromeric receptor complex (the 5-HT_3AB receptor) (Davies et al., 1999). Surface labelling of the 5-HT3B subunit was only present when the 5-HT_3A protein was co-transfected in HEK293 cells (Monk et al., 2001; Reeves et al., 2006). Co-expression of these two subunits generates a receptor with high single channel conductance, and therefore it has similar biophysical properties to native tissue. Since the discovery of the 5-HT3B subunit, the 5-HT3C, 5-HT3D and 5-HT3E subunits have all been identified (Niesler et al., 2003; Niesler et al., 2007; Jensen et al., 2008; Barnes et al., 2009).

The 5-HT_3A and 5-HT_3AB receptors are the two most commonly studied 5-HT_3 receptor isoforms; and these two subtypes have subtle differences in pharmacology and biophysical properties. One of the biggest differences between the 5-HT_3A and 5-HT_3AB receptor is single channel conductance, which is greater in the 5-HT_3AB receptor compared to the 5-HT_3A receptor. The reason behind this was determined using chimeric constructs of 5-HT_3A and 5-HT_3AB receptors (Kelley et al., 2003).

Three arginine residues within the cytoplasmic loop between the M3 and M4 (Figure 1) transmembrane domains in the 5-HT_3A receptor are responsible for the low single channel conductance (Kelley et al., 2003). When these residues are mutated to QDA (glutamine, aspartate, alanine) as seen in the 5-HT_3AB receptor, the single channel conductance of the 5-HT_3A receptor matches that of the 5-HT_3AB receptor (Kelley et al., 2003). The 5-HT_3A receptor also has an inwardly rectifying current-voltage relationship whereas this correlation is linear for the 5-HT_3AB receptor (Davies et al., 1999; Dubin et al., 1999). In addition, the 5-HT_3A receptor has increased Ca^{2+} permeability and slower desensitisation kinetics when contrasted against the 5-HT_3AB receptor (Davies et al., 1999; Dubin et al., 1999; Stewart et al., 2003). An aspartate residue in the 5-HT_3A receptor may be responsible for this difference in Ca^{2+} conductance. Mutating aspartate 293 in the 5-HT_3A receptor has been shown to reduce calcium permeability, to a conductance similar to the 5-HT_3AB receptor (Livesey et al., 2008).

It is important to note that 5-HT and other orthosteric ligands bind at an A-A interface in both the homomeric and heteromeric receptor subtypes (Lochner et al., 2010). Therefore, both receptor isoforms show similar pharmacology, although 5-HT has slightly higher affinity for the 5-HT_3A receptor compared to the 5-HT_3AB receptor (Brady et al., 2001). With that, ligands such as picrotoxin show marginally different sensitivities to each receptor subtype (Brady et al., 2001; Thompson et al., 2013). The compound VUF10166 displayed different pharmacology at the 5-HT_3A and 5-HT_3AB receptors. This ligand demonstrated lower affinity at the 5-HT_3AB receptor compared to the 5-HT_3A receptor; and whilst it behaved as a competitive ligand at the 5-HT_3A receptor, VUF10166 appeared to show a non-competitive interaction at the 5-HT_3AB receptor (Thompson et al., 2012). Functionally, the 5-HT_3A receptor recovered from VUF10166 inhibition; yet at the 5-HT_3AB receptor the recovery from VUF10166 inhibition was faster (Thompson et al., 2012). The pharmacology of VUF10166 was further explored using [^{3}H]-VUF10166. At the 5-HT_3A receptor, [^{3}H]-VUF10166 labelled a population of saturable binding sites with high affinity and labelled a similar density of receptors compared to [^{3}H]-granisetron (Thompson et al., 2014). At the 5-HT_3AB receptor, saturation radioligand binding studies demonstrated that the binding of [^{3}H]-VUF10166 did not fit a one site model hypothesis. Below 3.0 nM, the binding

of [^{3}H]-VUF10166 appeared similar at both receptor subtypes. It was suggested that above 3.0 nM, [^{3}H]-VUF10166 interacts with an A-B binding site at the 5-HT$_3$AB receptor (Thompson et al., 2014).

Positive staining for the 5-HT3B subunit has been detected within the rat hippocampus, with a diffuse population of cells showing similar morphology and distribution to interneurons, which is comparable to the 5-HT3A protein (Monk et al., 2001; Reeves et al., 2006). mRNA for the 5-HT3B subunit has been detected in the human brain (caudate, hippocampus, amygdala, thalamus and cerebral cortex) (Davies et al., 1999; Dubin et al., 1999). Within the Immunoreactivity for the 5-HT3B protein has also been identified in the human hippocampus (Brady et al., 2007).

Recently, novel 5-HT$_3$ receptor subunits (5-HT3C, 5-HT$_3$D and 5-HT$_3$E) have been identified and characterised (Niesler et al., 2007). The *HTR3C* gene was found on chromosome 3 following a search of the human genome using the 5-HT3A and 5-HT3B protein sequences (Karnovsky et al., 2003). The two most recent genes, *HTR3D* and *HTR3E* were cloned from human kidney tissue (Niesler et al., 2003). The *HTR3C*, *HTR3D* and *HTR3E* genes are between 82.8-90.1% identical, with each protein subunit showing between 64.8-74.3% similarities. However, when compared to the 5-HT3A and 5-HT3B subunits, the similarity is between 26.8-39.8% (Niesler et al., 2003). The 5-HT3C, 5-HT3D and 5-HT3E subunits are conserved across other mammalian species (e.g. dog and chimpanzee) but are not present in rodent (Holbrook et al., 2009). Interestingly, the 5-HT3D subunit has a truncated N-terminus which is missing the cysteine loops, suggesting this subunit cannot contribute to a functional agonist binding site. The 5-HT3C subunit showed a similar expression pattern to 5-HT3A and 5-HT3B mRNA, being found in brain, colon, intestine, lung and stomach (Niesler et al., 2003). mRNA for 5-HT3D and 5-HT3E subunits show more of a restrictive expression pattern; with the 5-HT3D mRNA being localised to kidney, colon and liver, whilst the 5-HT3E subunit was found in colon, intestine and brain (Niesler et al., 2003; Holbrook et al., 2009). Immunofluorescence studies demonstrated expression of the 5-HT3C, 5-HT3D and 5-HT3E subunits within neurons of the submucosal and myenteric plexus, and co-localised with the 5-HT3A protein

(Kapeller et al., 2011). This suggests 5-HT_3 receptor function in the gastrointestinal tract may be mediated by a plethora of different 5-HT_3 receptor subunit combinations.

Using immunocytochemistry, it was discovered that the 5-HT3C, 5-HT3D and 5-HT3E proteins do not reach the cell surface when expressed without the 5-HT3A subunit in transfected HEK293 cells (Niesler et al., 2007). In addition, there is no specific [^{3}H]-GR65630 binding when the subunits are expressed in HEK293 cells in the absence of the 5-HT3A protein. These subunits cannot elicit a functional response without the 5-HT3A protein present (Niesler et al., 2007; Holbrook et al., 2009). Niesler and colleagues used immunoprecipitation experiments to demonstrate that when these novel subunits are expressed in HEK293 cells along with the 5-HT3A subunit, heteromeric receptor complexes are generated at the cell surface. These heteromers (5-HT_3AC, 5-HT_3AD, and 5-HT_3AE) all displayed similar affinity for the radioligand [^{3}H]-GR65630, although there were more binding sites detected in HEK293 cells expressing the 5-HT_3AD and 5-HT_3AE receptors, compared to 5-HT_3A and 5-HT_3AC receptors. In functional calcium assays, 5-HT and meta-chlorophenylbiguanide (mCPBG) had similar potency across the different receptor combinations. However, the maximal response generated by 5-HT was greater at the 5-HT_3AD and 5-HT_3AE receptors, and reduced at the 5-HT_3AC receptor (compared to the 5-HT_3A receptor) (Niesler et al., 2007). Conversely, Holbrook and colleagues demonstrated that 5-HT responses were reduced at the 5-HT_3AC and 5-HT_3AE receptors compared to the 5-HT_3A receptor, whilst this response was similar at the 5-HT_3D receptor (Holbrook et al., 2009). Moreover, mCPBG had higher intrinsic efficacy at the 5-HT_3A receptor compared to the 5-HT_3AC, 5-HT_3AD and 5-HT_3AE receptors (Price et al., 2017).

1.7 5-HT_3 receptor localisation

Although initially discovered in the GIT, subsequent research has since pinpointed the 5-HT_3 receptor to being located within the human CNS and immune system. 5-HT_3 receptor binding sites have been identified in the human brain using the high-affinity radioligand [^{3}H]-(S)-zacopride (Parker et al., 1996). The highest level of specific [^{3}H]-(S)-zacopride binding was found in the nucleus tractus solitarius and area postrema (Barnes et al., 1989; Parker et al., 1996). Here the 5-HT_3 receptor functions within the

chemoreceptor trigger zone (CTZ) to elicit the emetic response. This brainstem area serves an important chemosensory role (not being protected by the blood-brain barrier), and stimulation of 5-HT_3 receptors causes to the vomiting reflex (Thompson et al., 2007). This knowledge contributed to the development of potent and selective 5-HT_3 receptor antagonists (known collectively as the –setrons e.g. ondansetron and granisetron) which are utilised therapeutically for post-operative nausea and vomiting (PONV) as well as cancer chemotherapy-induced nausea and vomiting (CINV).

In addition, regions of the human forebrain also show discrete expression of the 5-HT_3 receptor. Specific [^{3}H]-(S)-zacopride binding was identified in the hippocampus (specifically the granule layer of the dentate gyrus and CA2 region of pyramidal cells) (Parker et al., 1996). Lower levels of [^{3}H]-(S)-zacopride binding were detected in the nucleus accumbens, putamen and caudate nucleus (Parker et al., 1996). These higher brain centres imply a role for the 5-HT_3 receptor in cognition, along with neuropsychiatric disorders such as schizophrenia, depression, anxiety and addiction. Research and clinical trials have been conducted to investigate the potential beneficial effects of 5-HT_3 receptor antagonists for these before-mentioned neurological disorders (Thompson et al., 2007; Walstab et al., 2010).

Within the GIT, the 5-HT_3 receptor is localised to vagal afferents, enteric motor neurons, interstitial cells of Cajal and intrinsic primary afferent nerves (IPANS) (Gershon, 2004; Sanger, 2008; Fakhfouri et al., 2012; Fidalgo et al., 2013). Immunohistochemistry and RT-PCR have showed that both the 5-HT_3A and 5-HT_3B subunits are expressed in the submucosal plexus. Functionally, 5-HT and selective 5-HT_3 receptor agonists induced an excitatory response in gut tissue which can be blocked by prior application of a 5-HT_3 receptor antagonist (Michel et al., 2005). Enterochromaffin cells, which lie in the base of crypts in the GIT, release 5-HT in response to various stimuli; including intraluminal distension or vagal nerve stimulation. 5-HT can then induce responses through interactions with enterocytes or the ENS (Spiller, 2007; Manocha et al., 2012). The 5-HT_3 receptor located on these nerve terminals serves a significant role for visceral sensation, plus information transfer between the gut and brain via the ENS (Nam et al., 2018).

5-HT_3 receptors are thought to be implicated in the pathophysiology of diarrhoea-predominant irritable bowel syndrome (IBS-d); where they appear to be overactive. There is also evidence suggesting a genetic component to IBS-d (Niesler, 2011). The c._76G>A variant of the *HTR3E* gene has been associated with IBS-d in female patients (Kapeller et al., 2008). This polymorphism is found in the untranslated region of the *HTR3E* gene, where after being studied *in vitro*, it was discovered that the microRNA miR510 binds to this region of the gene (Kapeller et al., 2008). Interestingly, miR510 plays an inhibitory role in *HTR3E* expression, and the c._76G>A polymorphism reduces miR510 binding, leading to increased expression (determined by a reporter gene assay) (Kapeller et al., 2008). *In situ* hybridisation studies demonstrated co-localisation of *HTR3E* and miR510 in human enterocytes(Kapeller et al., 2008). In addition, the c.-42C>T variant in the *HTR3A* gene has been shown to correlate with worse anxiety symptoms associated with IBS-d and increased amygdala activity (Kapeller et al., 2008; Kilpatrick et al., 2011; Niesler, 2011; Celli et al., 2017). Similar to the c._76G>A variant of the *HTR3E* gene, this polymorphism leads to increased expression of the 5-HT_3A receptor *in vitro*, suggesting a mechanism behind the phenotype associated with this gene variant (Niesler, 2011; Celli et al., 2017). A variant of the *HTR3C* gene (p.N163K rs6766410) was found to be associated with IBS-d patients for whom ondansetron improves stool consistency, implying a potential genotype correlating with response to treatment (Gunn et al., 2019). 5-HT_3 receptor antagonists such as alosetron did provide therapeutic relief but were withdrawn and distribution was restricted due to severe side-effects (this will be developed further, see section 1.11) (Hoyer et al., 2002; Gershon, 2004; Sanger, 2008).

There is emerging evidence that 5-HT via the actions of multiple receptors (including the 5-HT_3 receptor) modulates the activity of various cells of the immune system. Monocytes and T cells have been shown to express 5-HT_3A receptor mRNA (Fiebich et al., 2004). The exact role of the 5-HT_3A receptor in immunomodulation remains to be elucidated; however, given 5-HT is thought to be pro-inflammatory, this receptor may play a key role in inflammation. Moreover, the intrinsic Ca^{2+} permeability of the 5-HT_3 receptor could be important for intracellular signalling and potentially cytokine expression or synthesis in immune cells. Indeed, it has been demonstrated that tropisetron will decrease IL-2 synthesis in human T-cells stimulated with either phorbol

12-myristate 13-acetate (PMA) or *Staphylococcal enterotoxin* B (Vega et al., 2005). Furthermore, tropisetron decreased the production of proinflammatory cytokines (IL-1β and IL-6) in mouse brain following Lipopolysaccharide-induced neuroinflammation (Yu et al., 2018).

In fibromyalgia, a condition with widespread chronic body pain and inflammation, it has been evaluated that tropisetron improves pain (measured by visual analogue score), and associated depression, anxiety and sleep disturbances (symptoms highly associated with fibromyalgia), whilst being well tolerated (Färber et al., 2000; Haus et al., 2000; Marcus, 2009). Furthermore, tropisetron has demonstrated efficacy in treating rheumatoid arthritis (Hrycaj, 2004). In addition, tropisetron reduced hyperalgesia and mechanical allodynia in rats with neuropathic pain elicited by spinal compression (Nasirinezhad et al., 2016). Taken together, this suggests a peripheral and central role for 5-HT_3 receptors in pain and inflammation.

The inflammatory nature of 5-HT in the gastrointestinal tract has been studied using a *Tph1* knock-out mouse model (Ghia et al., 2009). Indeed, experimental colitis (elicited by application of dextran sulphate) was more severe in wild-type mouse compared to *Tph1* knock-out mouse (Ghia et al., 2009). The knock-out mice had reduced macrophage infiltration and decreased levels of proinflammatory cytokines (IL-1β, IL-6, myeloperoxidase and tumour necrosis factor alpha) compared to their wild-type counterparts (Ghia et al., 2009). This anti-inflammatory phenotype was also mirrored with pharmacological inhibition of TPH1 using Para-chlorophenylalanine (PCPA) (Ghia et al., 2009). This suggests 5-HT plays a major role in the development of gastrointestinal inflammation. Moreover, the levels of IL-1β, IL-6 and tumour necrosis factor alpha were decreased by alosetron following experimentally-induced colitis in mouse (Motavallian et al., 2019). Taken together, this suggests the 5-HT_3 receptor has an important role in both central and systemic inflammation and could be used as a drug target for inflammatory conditions.

1.8 5-HT_3 receptor pharmacology

As previously mentioned, antagonists at the 5-HT_3 receptor (including but not limited to: ondansetron, granisetron, palonosetron) are prescribed for CINV and PONV (Billio et al., 2010). As these drugs are relatively safe and expression of the 5-HT_3 receptor is readily detectable in higher brain centres, studies have been conducted to elucidate if 5-HT_3 receptor antagonists have potential in various neuropsychiatric conditions. These include schizophrenia, anxiety and substance abuse.

Excess levels of 5-HT are thought to be involved in IBS-d (Bearcroft et al., 1998). Alosetron (a selective, high affinity 5-HT_3 receptor antagonist) was prescribed as a treatment and was beneficial in IBS-d patients by improving visceral sensation, gut motility and secretions (Manocha et al., 2012). The mild side effect of constipation was noted; however, post-marketing surveillance identified more severe adverse effects such as life-threatening ischemic colitis. The FDA has sternly regulated the prescription of alosetron after its withdrawal in 2000, with only a select group of patients being allowed access to the drug. This 'black box' approach to prescription of alosetron has now been withdrawn by the FDA. Moreover, cilansetron again demonstrated efficacy in treating IBS-d, but similar to alosetron, there were incidences of ischemic colitis and so this drug was never marketed (Chey et al., 2005; Grover et al., 2014). A structurally different 5-HT_3 receptor antagonist ramosetron, which shows efficacy in treating IBS-d, has been approved in Japan (Grover et al., 2014; Min et al., 2015; Fukudo et al., 2016; Qi et al., 2018).

In a clinical trial, the 5-HT_3 receptor antagonist tropisetron displayed anxiolytic effects (Lecrubier et al., 1993). In the rodent elevated plus maze model of anxiety, 5-HT_3 receptor antagonists appeared anxiolytic. This is consistent with data suggesting 5-HT_3A receptor knock-out mice show reduced anxious behaviour in stressful situations (Barnes et al., 1999; Thompson et al., 2006).

The 5-HT_3 receptor could be an attractive target for schizophrenia as it modulates midbrain dopamine signalling. Blocking this receptor will inhibit over-active dopaminergic neurons in rats (Hoyer et al., 2002; Nichols et al., 2008). The atypical antipsychotic clozapine potently inhibits 5-HT induced currents at the 5-HT_3 receptor (Hermann et al., 1996). Inhibition occurs at concentrations of clozapine which are detected in patient plasma, suggesting blockade of 5-HT_3 receptors may contribute to the clinical efficacy of clozapine (Hermann et al., 1996). In patients with schizophrenia, ondansetron improved p50 auditory gating, without having any benefit on the positive or negative symptoms of schizophrenia (E Adler et al., 2005). Furthermore, schizophrenic patients administered with either tropisetron or granisetron in combination with the atypical antipsychotic risperidone demonstrated a significant improvement in general psychopathy score (specifically an improvement in negative symptom score) (Khodaie-Ardakani et al., 2013; Noroozian et al., 2013). There is also evidence of ondansetron improving the cognitive impairment associated with schizophrenia (Zheng et al., 2019).

The concept that 5-HT_3 receptor antagonists can modulate the dopaminergic system has also led to research in substance abuse/addiction. In animal studies, 5-HT_3 receptor antagonists diminished the increase in midbrain dopamine levels measured after administration of morphine or cocaine; which also corresponded behaviourally to a decrease in locomotor activity and reduced self-consumption of potentially abusive drugs (Thompson et al., 2007; Walstab et al., 2010). In 5-HT_3A receptor knock-out mice, the 5-HT_3 receptor antagonist LY-278, 584 did not decrease alcohol in-take, this was only seen for wild-type mouse, suggesting the 5-HT_3A subunit is important for the impact of 5-HT_3 receptor antagonists on ethanol intake (Hodge et al., 2004). Ondansetron also diminished alcohol cravings for early onset alcoholics but not late onset alcoholics (Johnson et al., 2002).

1.9 Introduction to allosteric modulation

Many receptors and proteins (including GPCRs, ion channels, and kinases) have multiple ligand-binding sites. These sites include the orthosteric (competitive) binding

site, whereby the endogenous ligand interacts (e.g. 5-HT at the 5-HT_3 receptor), and any synthetic molecules binding to this site are orthosteric or competitive ligands (e.g. ondansetron at the 5-HT_3 receptor). Alternatively, some compounds appear to interact with proteins through a non-competitive manner, binding at a site that is distinct from the orthosteric site (so-called allosteric ligands/modulators). The 5-HT_3 receptor continually transitions between an open and closed state (even in the absence of agonist); and agonists shift the equilibrium of the 5-HT_3 receptor more towards the open state. Allosteric modulators cannot influence this equilibrium, but instead moderate the function of agonist. Ligands which increase the function of a receptor in the presence of orthosteric agonist are positive allosteric modulators (PAMs). Molecules which decrease protein activity whilst agonist is bound are negative allosteric modulators (NAMs) (Wenthur et al., 2014). PAMs can also be classified by how the affect channel kinetics; type I PAMs do not affect receptor desensitisation, whilst type II PAMs slow receptor desensitisation (Thomsen et al., 2012). Many allosteric agents also increase the affinity of orthosteric ligand. By only subtly influencing protein (in this case receptor) function, allosteric modulators offer a new and potentially more beneficial area of pharmacology (Thompson, 2013; Wenthur et al., 2014).

However, some allosteric ligands function through a more complex mechanism. For example, the $GABA_B$ receptor ligand CGP7930 increased the potency and efficacy of the endogenous agonist GABA at the $GABA_B$ receptor, akin to a PAM (Binet et al., 2004). In addition, CGP7930 stimulated functional responses (measured by accumulation of Inositol triphosphate 3) at the $GABA_B$ receptor in the absence of GABA. Furthermore, whilst the actions of GABA at the $GABA_B$ receptor can be blocked by the competitive antagonist CGP54626; this is not true for CGP7930 (Binet et al., 2004). It is thought that GABA and CGP7930 bind to different subunits of the dimeric $GABA_B$ receptor, with GABA interacting with the extracellular domain and CGP7930 binding to the heptahelical domain of the $GABA_B$ receptor. These ligands were then termed ‘orthosteric protomer’ and ‘allosteric protomer’ respectively (Binet et al., 2004; Schwartz et al., 2007).

Clinical examples of allosteric modulation at Cys-loop receptors are benzodiazepines (e.g. diazepam) which demonstrate activity as a PAM at the $GABA_A$ receptor. These drugs only increase the activity of the $GABA_A$ receptor when GABA is bound (and interact with a distinct modulatory site). Clinically, these drugs are safer than barbiturates because of a reduced overdose risk.

1.10 5-HT_3 receptor allosteric modulation

The 5-HT_3 receptor has multiple modulatory sites separate from the orthosteric binding site (Figure 3). A diverse range of molecules (endogenous and exogenous, natural and synthetic) modulate agonist-induced responses at the 5-HT_3 receptor.

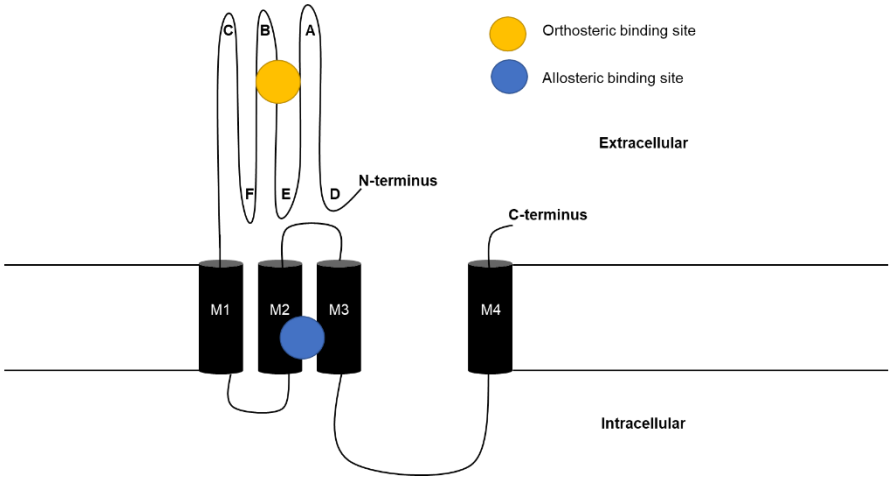

Figure 3. Potential ligand binding sites at the 5-HT_3 receptor.

'Classic' orthosteric ligands (e.g. 5-HT, ondansetron) interact within the N-terminus of the 5-HT_3 receptor, whilst allosteric ligands interact with a different site on the 5-HT_3 receptor, including the transmembrane domain (Walstab et al., 2010).

Ligands acting at the endocannabinoid receptors (CB_1 and CB_2 receptors) modify the activity of the 5-HT_3 receptor via allosteric mechanisms. Synthetic CB_1 receptor agonists such as WIN55, 212-2 and JWH-015 all inhibited 5-HT-induced inward currents at the 5-HT_3A receptor in a concentration-dependent manner (Barann et al., 2002). This activity is also true for the endocannabinoid anandamide and the plant-derived Δ^9-THC; as well as LY320135 and cannabidiol, both of which are CB_1 receptor

antagonists (Barann et al., 2002; Yang et al., 2010). This suggests activity at the endocannabinoid receptor does not correlate with allosteric actions at the 5-HT_3A receptor. The cannabinoid mechanism of action is not thought to be via channel block (Barann et al., 2002; Yang et al., 2010). Cannabinoid ligands interacted with an allosteric site at the 5-HT_3 receptor (demonstrated by the ligands not displacing [^{3}H]-GR65630 in radioligand binding studies) (Barann et al., 2002; Yang et al., 2010). It is known cannabinoids are anti-emetic, and this beneficial therapeutic factor may be mediated via interactions at the 5-HT_3A receptor (Barann et al., 2002; Walstab et al., 2010; Yang et al., 2010). It must also be noted however that CB_1 agonists are sedative, and this may contribute towards their anti-emetic actions.

Various terpenes and pungent substances display allosteric activity at the 5-HT_3A receptor. Both carvacrol and thymol increased the efficacy and potency of 5-HT at the 5-HT_3A receptor, with carvacrol slightly more potent that thymol. In addition, both of these molecules displayed weak activity as partial agonists at the 5-HT_3 receptor, with intrinsic efficacies approximately 15% of 5-HT (Ziemba et al., 2015). The responses elicited by carvacrol and thymol could be blocked using the selective 5-HT_3 receptor antagonist tropisetron. Furthermore, these ligands have no efficacy at the mouse 5-HT_3A receptor. Both carvacrol and thymol demonstrated activity at chimeric mouse/human 5-HT_3A receptors (N-terminus of mouse with transmembrane and C-terminus of human receptor); but had no effect at a chimeric human/mouse 5-HT_3A receptor (N-terminus of human with transmembrane and C-terminus of mouse receptor) (Lansdell et al., 2015). This suggests that carvacrol and thymol interact with the transmembrane or C-terminal domain of the 5-HT_3A receptor to elicit a response, unlike 5-HT which binds to the N-terminus (Lansdell et al., 2015). Furthermore, other related terpene molecules demonstrated allosteric inhibitory effects, including citronellol and linalool, which inhibited 5-HT induced responses by 98% and 93% respectively. Although efficacious, both of these ligands displayed low affinity, with functional IC_{50} concentrations in the high micromolar range (Ziemba et al., 2015).

Using the NCB-20 mouse neuroblastoma cell line, it was shown that ethanol (at concentrations considered to be intoxicating) potentiated currents induced by 5-HT

and 2-methyl-5-HT at the 5-HT_3A receptor. Ethanol had no functional effect at the 5-HT_3A receptor in absence of agonist. The impact of ethanol at the 5-HT_3A receptor is more apparent at lower 5-HT concentrations (below 1.0 μM) compared to higher concentrations of 5-HT (10 μM or above). Ethanol also increased the rate of decay for the 5-HT current. This suggests that pharmacologically relevant concentrations of ethanol influenced the activity of the 5-HT_3 receptor (Lovinger, 1991). To further investigate the mechanism of action of ethanol at the 5-HT_3 receptor, single channel electrophysiology was undertaken. Single channel conductance of the 5-HT_3A receptor can be measured using a 5-HT_3A-QDA mutant, which incorporates the glutamine, aspartate and alanine residues from the 5-HT_3AB receptor. Ethanol increased the single channel activity and the number of open 5-HT_3A-QDA receptors (Feinberg-Zadek et al., 2010). Moreover, at the mouse 5-HT_3AB receptor, ethanol appeared to have no impact on 5-HT induced currents (Hayrapetyan et al., 2005).

Menthol decreased currents elicited by 5-HT at the h5-HT_3A receptor in a pharmacologically relevant concentration-dependent manner (Barann et al., 2008; Walstab et al., 2014). The activity of menthol appeared to be allosteric, as menthol did not impact 5-HT binding at the 5-HT_3A receptor, nor did it compete with [^{3}H]-GR56630 for 5-HT_3A binding sites. The efficacy of menthol was similar at both the 5-HT_3A and 5-HT_3AB receptors (Ashoor et al., 2013). This may be of interest as menthol has been used in the treatment of functional GIT disorders (Walstab et al., 2014).

Although structurally similar to 5-HT, 5-hydroxyindole (5-HI) did not display agonist activity at the 5-HT_3 receptor expressed endogenously in mouse NIE-115 neuroblastoma cell line. Instead, 5-HI increased the efficacy and potency of 5-HT through an allosteric mechanism (van Hooft et al., 1997). 5-HI also slowed desensitisation of the 5-HT_3 receptor. The Hill slope of 5-HT concentration curves remains approximately 2 in the presence/absence of 5-HI; implying 5-HI does not affect the cooperativity of 5-HT at the 5-HT_3A receptor (van Hooft et al., 1997).

The intravenous anaesthetic propofol reversibly inhibited 5-HT induced currents in a concentration-dependent manner at the 5-HT_3A receptor, without having any activity in the absence of 5-HT. The rate at which the 5-HT_3A receptor desensitised (due to activation by 5-HT) is increased by propfol (Barann et al., 2008).

Trattnig and colleagues identified PU02 as a non-competitive inhibitor of 5-HT_3 receptors. PU02 displayed similar potency at inhibiting 5-HT induced responses at the 5-HT_3A, 5-HT_3AB, 5-HT_3AC, 5-HT_3AD and 5-HT_3AE receptors (Trattnig et al., 2012). In addition, PU02 also reduced calcium currents elicited at the α7 nAChR, albeit with lower potency compared to 5-HT_3 receptors. PU02 did not displace [^{3}H]-GR65630 from 5-HT_3A receptor binding sites, suggesting an allosteric mechanism of action (Trattnig et al., 2012). Mutagenesis studies identified that PU02 interacted with the transmembrane domains of the 5-HT_3 receptor (Trattnig et al., 2012).

mCPBG has similar potency as an agonist at the 5-HT_3A and 5-HT_3AB receptor. However, whilst it appeared to be a partial agonist at the 5-HT_3A receptor; it demonstrated activity as a super-agonist at the 5-HT_3AB receptor, with an intrinsic efficacy 2.75x greater than that of 5-HT (Miles et al., 2015). Mutating glutamate 124 within loop A of the 5-HT_3A subunit caused mCPBG to lose all functional activity. However, when this mutant subunit is introduced into to the 5-HT_3AB receptor, mCPBG still retains activity as a partial agonist. This suggests that mCPBG can act at a B-A binding site at the 5-HT_3AB receptor, unlike most agonists which appear to act at an A-A interface allosteric (Miles et al., 2015).

Current research suggests that 5-chloroindole (Cl-indole) is a potent PAM at the 5-HT_3 receptor (Newman et al., 2013; Powell et al., 2016). Using functional calcium assays and electrophysiology, Cl-indole had no activity at the 5-HT_3A receptor in the absence of agonist (Powell et al., 2016). At the 5-HT_3A receptor, Cl-indole increased the efficacy of 5-HT and 5-HT_3 receptor partial agonists. Moreover, Cl-indole also demonstrated similar activity at the 5-HT_3AB receptor. Further, in radioligand binding assays, the affinity of 5-HT (but not the 5-HT_3 receptor antagonist tropisetron) for 5-

HT_3A receptor binding sites was increased by Cl-indole (Newman et al., 2013). Cl-indole did not impact 5-HT_3 receptor desensitisation or resensitisation. Interestingly, after 5-HT is removed following co-application of 5-HT and Cl-indole, Cl-indole induces a tail current after the initial 5-HT response. This tail current can be blocked by ondansetron (Powell et al., 2016). Moreover, Cl-indole does not bind to the 5-HT_3A receptor, but in the presence of 5-HT or other 5-HT_3 receptor agonists, Cl-indole will compete for [^{3}H]-granisetron occupied 5-HT_3A receptor binding site. This suggests that 5-HT can induce Cl-indole to interact with the 5-HT_3A receptor through an orthosteric mechanism, whilst remaining potentially allosteric in the absence of 5-HT (Powell et al., 2016). Cl-indole was termed a cryptic orthosteric modulator (COM), because of its ability to interact with the 5-HT_3A receptor in through an orthosteric mechanism once 5-HT has activated the receptor.

Furthermore, clinically relevant drugs (including: fluoxetine, flupentixol, fluphenazine, haloperidol and levomepromazine) are known to interact with the 5-HT_3 receptor through non-competitive mechanisms (Choi et al., 2003; Rammes et al., 2004). Although fluoxetine has no efficacy at 5-HT_3 receptors, it inhibited 5-HT induced currents in NCB-20 cells through a concentration-dependent manner (Choi et al., 2003). Moreover, fluoxetine also increased the rate of 5-HT_3 receptor desensitisation, acting through a voltage independent mechanism. In rat GIT, fluoxetine treatment decreased 5-HT_3 receptor expression, suggesting a potential impact on receptor downregulation (Freeman et al., 2006). In addition, the metabolite norfluoxetine also blocked 5-HT_3 receptors with higher potency compared to fluoxetine (Choi et al., 2003). This suggests some of the therapeutic efficacy of fluoxetine may be due to 5-HT_3 receptor blockade.

It has been shown that various antipsychotics inhibit the 5-HT_3 receptor through an allosteric mechanism of action. Typical antipsychotics (including flupentixol, fluphenazine, haloperidol and levomepromazine) have been shown to block 5-HT induced responses at the 5-HT_3 receptor (Rammes et al., 2004). The inhibition was voltage independent, which implies the antagonism is not through channel block. As 5-HT_3 receptors modulate dopaminergic activity in the mesolimbic and mesocortical

pathways; it is suggested that blockade of these receptors by antipsychotics may contribute to their therapy (Rammes et al., 2004).

Ginger and its pungent components have been shown to allosterically modulate the 5-HT_3 receptor. These compounds elicited a concentration-dependent decrease in 5-HT-evoked calcium responses in HEK293 cells expressing the 5-HT_3A or 5-HT_3AB receptor; and was replicated in electrophysiology experiments with transfected oocytes (Walstab et al., 2013; Ziemba et al., 2015). This was replicated in *ex vivo* experiments using human myenteric plexus. Moreover, these compounds did not displace [^{3}H]-GR65630 from 5-HT_3 receptor binding sites, and failed to increase the potency of 5-HT; suggesting a non-competitive interaction with the 5-HT_3 receptor (Walstab et al., 2013). Interestingly, ginger has been shown to be effective in treating CINV and IBS, both of which are thought to involve 5-HT_3 receptor activity (Chang et al., 2018; Fifi et al., 2018).

1.11 Irritable bowel syndrome

Irritable bowel syndrome (IBS) is the most prevalent functional gastrointestinal disorder, with approximately 11% of the population affected (Canavan et al., 2014; Enck et al., 2016; Lacy et al., 2016). IBS is more common in women and younger adults (25-54-year olds) (Canavan et al., 2014; Lacy et al., 2016). As this condition has a multifactorial pathophysiology, there is no definite treatment, rather a symptom-based management treatment is utilised. Although no known cause, the symptoms are clearly identifiable: pain and altered gut activity as defined by Rome IV criteria (Schmulson et al., 2017). This defines irritable bowel syndrome as recurrent abdominal pain for at least one day a week for the last six months; and at least two of the following: increased or painful defecation, change in stool frequency and change in stool form or appearance (Drossman et al., 2016; Schmulson et al., 2017). The altered gut activity means IBS can be grouped as diarrhoea or constipation predominant IBS (IBS-d or IBS-c respectively), which can be defined by the Bristol stool scale, or mixed IBS, which is a combination of both (Chey et al., 2015; Lacy et al., 2016). IBS-d is defined by having more than 25% of bowel movement on the

Bristol stool scale subtypes 6-7 (i.e. liquid stool) or less than 25% on the Bristol stool scale subtypes 1-2 (i.e. hard stool) (Drossman et al., 2016). Treatment for IBS-d is often symptom based, e.g. loperamide as an antidiarrheal agent. Often antidepressants are prescribed to potentially alleviate the psychological side of IBS-d (e.g. depression and anxiety) as well as pain (Enck et al., 2016).

The dysmotility, hypersensitivity and secretomotor abnormalities seen in IBS-d are thought to be due to altered enterochromaffin cell activity and the elevated levels of 5-HT in the gut (Bearcroft et al., 1998; Park et al., 2009). 5-HT plays a major role in the communication between the CNS and GIT through the ENS to influence visceral sensation and motor function. The ENS releases 5-HT to initiate motility and visceral perception. 5-HT is also secreted following mucosal stimulation, where it activates IPANs. IPANs synapse with excitatory ascending interneurons and descending inhibitory interneurons, which activate or inhibit motor neurons respectively. 5-HT_3 receptors are expressed by post synaptic afferent neurons of the PNS. This pathway is thought to be involved in visceral perception and nociception. 5-HT_3 receptor antagonists such as granisetron or alosetron have been shown to block this pathway to reduce sensation (Crowell, 2004). In IBS-d, there is an increased intestinal motor response following meal ingestion or in response to cholecystokinin (CCK). Visceral hypersensitivity is also a factor in IBS-d, with patients showing increased sensation in response to stimuli (Chey et al., 2015).

In IBS-d, there is evidence to suggest alteration in gastrointestinal 5-HT signalling. In a study of 8-12-year old children with IBS-d, it was demonstrated that there were increased levels of 5-HT in rectal mucosa, which correlated strongly with a reduction in the expression of SERT; suggesting the increased 5-HT is due to reduced uptake rather than increased synthesis (because levels of TPH1 were consistent between the control group and IBS-d cohort (Faure et al., 2010). Consistent with this, platelets in patients with IBS-d show reduced expression and binding affinity of SERT, and the ration of 5-HIAA:5-HT is reduced, implying a decrease in the metabolism of 5-HT contributes to the elevated levels (Camilleri, 2009). Furthermore, SERT in IBS-d patients also demonstrated reduced [^{3}H]-5-HT uptake (Foley et al., 2011). Bearcroft

and colleagues used HPLC (high performance liquid chromatography) to evaluate plasma 5-HT levels in healthy volunteers and IBS-d patients following ingestion of a carbohydrate-rich meal. It was found that patients with IBS-d had significantly higher concentrations of plasma 5-HT compared to healthy controls, and there was a longer duration of detectable 5-HT in the plasma of IBS-d patients compared to healthy volunteers (Bearcroft et al., 1998; Park et al., 2009). Indeed, compared to healthy control subjects, IBS patients have increased plasma 5-HIAA and reduced urinary 5-HIAA, suggesting a dysfunction of 5-HT metabolism in IBS patients (Thijssen et al., 2016; Yu et al., 2016). Given the knowledge ondansetron is relatively safe in patients experiencing nausea and vomiting, and that 5-HT_3 receptors are known to influence the gastrointestinal tract, it was suggested ondansetron could show promise in treating IBS-d. This idea was then strengthened with the findings that raised plasma 5-HT levels are recorded in IBS-d patients (Manning et al., 2011; Moore et al., 2013; Stasi et al., 2014). The use of 5-HT_3 receptor antagonists such as alosetron in IBS-d appeared to initially be a success, with increased fluid absorption, reduced gut movement and improved visceral sensation all reported in IBS-d patients (Thompson et al., 2007; Walstab et al., 2010).

Several systemic reviews and meta-analyses have evaluated the efficacy and adverse effects of 5-HT_3 receptor antagonists in clinical trials for IBS-d. A meta-analysis assessing the impact of ramosetron on IBS-d in 4 randomised clinical trials (821 patients taking ramosetron and 811 patients receiving placebo) found that compared to placebo, ramosetron improved stool consistency, abdominal pain/discomfort, abnormal bowel habits to relieve overall IBS-d symptoms (Qi et al., 2018). 65% (78/120 patients) who took ondansetron reported relief of IBS-d symptoms (improved abdominal pain, reduction in the frequency of loose stool or diarrhoea) (Garsed et al., 2014). Similarly, 54% patients (930/1725) who took alosetron or cilansetron reported a global improvement in abdominal pain and IBS-d symptoms (Andresen et al., 2008). In addition, 81 out of 132 patients (61.8%) receiving ramosetron reported an overall improvement in IBS-d symptoms (Fukudo et al., 2016). A separate study demonstrated that IBS-d symptoms persisted in 49% of patients taking alosetron, whilst for placebo this number was 64% (Ford et al., 2009). Zheng and colleagues evaluated 12 studies looking at the impact of alosetron, cilansetron and ramosetron

on IBS-d symptoms. All three drugs had similar efficacy, with 51% (1990 patients) describing a global improvement in IBS-d symptoms, compared to 33% (939) patients receiving placebo (Zheng et al., 2017).

However, in all of these cases, adverse effects (which in some cases were serious) for 5-HT_3 receptor antagonists have been reported. There was an increased incidence of hard stool and constipation when IBS-d patients received ramosetron compared to placebo (Qi et al., 2018). 24 clinical trials assessing alosetron had been finished by November 2000 (around the same time GSK withdrew alosetron from market) (Chang et al., 2006). Some 11,874 patients had received alosetron (3,500 for placebo), and there was an incidence of ischemic colitis for 0.15% who took alosetron (none for placebo) (Chang et al., 2006; Camilleri, 2017). In addition, serious constipation was seen in 0.1% of patients who were prescribed alosetron, compared to 0.06% who received placebo (Chang et al., 2006). Moreover, it was reported that 24% (604/2526 patients) taking either alosetron or cilansetron experienced constipation (Andresen et al., 2008). This is a similar proportion to patients receiving ramosetron (19.7%) (Fukudo et al., 2016). Furthermore, Zheng and colleagues reported that whilst 3% of those receiving placebo reported constipation, the incidence of constipation was 9%, 16% and 23% for those taking ramosetron, cilansetron and alosetron respectively; with there being 9 cases of ischemic colitis in just the alosetron cohort (Zheng et al., 2017). Clearly, 5-HT_3 receptor antagonists have efficacy in treating IBS-d, but cause serious adverse effects.

Of interest, these cases of ischemic colitis appear to be unique for IBS-d patients receiving 5-HT_3 receptor antagonists. 5-HT_3 receptor antagonists (e.g. ondansetron) have been prescribed to treat CINV and PONV, with side effects being mild (Egerton-Warburton et al., 2014). A large study examining a variety of clinical trials found that none out of 58,412 patients, none reported harm when receiving granisetron, ondansetron, palonosetron or tropisetron, despite showing clear efficacy in treating CINV (Tricco et al., 2016). This is also the case for ramosetron and ondansetron, with only headache reported as a side effect when being treated for PONV (Yokoi et al.,

2017). Constipation has been reported for patients receiving granisetron or ondansetron for the treatment of CINV (Billio et al., 2010).

Pumosetrag (DDP, MKC-733), is a high-affinity selective 5-HT_3 receptor partial agonist, which was also trialled for IBS (both subtypes) (Evangelista, 2007). Pumosetrag accelerated gastric emptying in rat, and also enhanced GIT transit in guinea pig; displaying an intrinsic efficacy of 40% and 20% (relative to full agonist) respectively (Evangelista, 2007). Contractile responses elicited by pumosetrag in rat or guinea pig colon were blocked with ondansetron, demonstrating the selectivity of pumosetrag for the 5-HT_3 receptor (Chetty et al., 2008). In early phase I trials, pumosetrag delayed gastric emptying and small intestinal transit in healthy volunteers (Coleman et al., 2003; Evangelista, 2007). Moreover, in phase II trials, pumosetrag increased percentage elimination and stool consistency in IBS-c patients (Fujita et al., 2005; Evangelista, 2007). However, side effects (flushing, headache and nausea), which are likely to be 5-HT_3 receptor mediated, were all reported as adverse effects, and lead to the withdrawal of pumosetrag (Coleman et al., 2003; Evangelista, 2007; Choung et al., 2014).

Manning and colleagues have recently developed a series of structurally-related ligands which display activity as high-affinity 5-HT_3 receptor partial agonists (Manning et al., 2011; Moore et al., 2013; Manning et al., 2014). One of these ligands, CSTI-300 (Figure 4), has been investigated further in this thesis (Section 3.2), as a potential lead candidate drug to treat IBS-d.

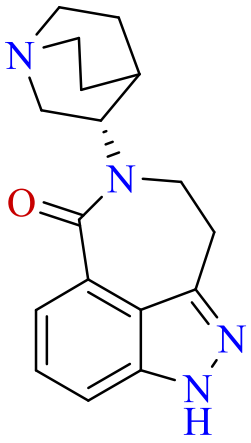

Figure 4. The structure of CSTI-300

Eluxadoline, a μ receptor agonist and δ receptor antagonist, was approved for IBS-d in the USA in May 2015 and Europe in September 2016 (Breslin et al., 2012; Fragkos, 2017). Opioid receptors are expressed in the ENS and by muscle cells, and μ receptor agonists have been known to delay gastric emptying and intestinal transit (Fragkos, 2017). Eluxadoline has been shown to decrease contraction of Guinea pig ileum (Fragkos, 2017). Eluxadoline demonstrated efficacy (decreased abdominal pain and improved stool constancy) in 2 randomised, double-blind placebo-controlled trials, with constipation and nausea listed as common adverse effects. However due to potential additive effects it is not prescribed alongside 5-HT_3 receptor antagonists. Eluxadoline demonstrated efficacy after week 4 of being prescribed, relieving symptoms such as abdominal pain and bloating, as well as improving stool consistency (Özdener et al., 2017). Although common (43.8-60.2% of patients), adverse effects (nausea and vomiting, constipation) were well tolerated, with only 0.6-1.7% discontinuing the drug (Lacy et al., 2016; Fragkos, 2017). After-marketing surveillance has reported rare cases of pancreatitis, but the prevalence is not yet clear as this drug has only recently been introduced to the clinic (Camilleri, 2017).

Another novel drug of interest for treating IBS-d is the melatonin receptor agonist agomelatine. Enterochromaffin cells produce melatonin, which is thought to modulate pain and GIT motility; and IBS-d patients show low salivary levels of melatonin (Ng et al., 2018). Agomelatine, which has higher affinity for melatonin receptors (MT_1 and

MT_2) compared to the endogenous ligand, as well as a longer half-life (~2 hours compared to 30-50 minutes for melatonin) is currently used to treat depression. 5 trials have shown positive results for agomelatine in IBS-d, measured by improved abdominal pain (Ng et al., 2018). This drug could be a novel adjunct for treating IBS-d.

1.12 Vortioxetine

Vortioxetine (Figure 5) is a multi-model drug developed by Lundbeck, which was approved in 2013 for the treatment of major depressive disorder (MDD) in the USA and Europe. Microdialysis assays showed that vortioxetine raises monoamine (5-HT, noradrenaline and dopamine) levels in the medial prefrontal cortex and hippocampus, consistent with the actions of some other antidepressant drugs in the clinic. However, it is reported that vortioxetine also antagonises 5-HT_3 receptors expressed by GABAergic interneurons in the hippocampus, which leads to an increase in glutamatergic transmission in pyramidal neurons to potentiate LTP; a relevant mechanism that may underlie the improved cognition function evident in elderly patients receiving the drug (Dale et al., 2013; Alvarez et al., 2014; Sanchez et al., 2015). Vortioxetine, through blockade of SERT and interaction with a multitude of monoaminergic receptors, increases the levels of 5-HT, noradrenaline, dopamine, acetylcholine and histamine in rat brain (specifically prefrontal cortex and ventral hippocampus (Mork et al., 2012). Chronic treatment with vortioxetine significantly increased 5-HT levels (a 2-fold increase compared to vehicle) in the ventral hippocampus, and increased social interaction in rats (Mork et al., 2012). Vortioxetine also enhances memory function in rat, which clinically translates to improved cognitive performance in elderly patients with MDD (Schatzberg et al., 2014).

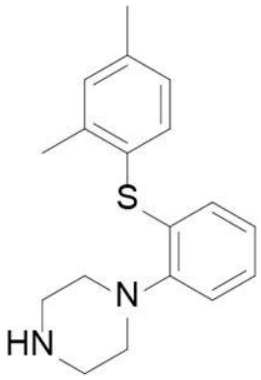

Figure 5. The structure of vortioxetine.

According to a short-term (6-8 week) clinical trial, vortioxetine appeared to have efficacy in treating MDD, with 8 out of 12 trials being positive for vortioxetine against placebo (3 trials were non-significant, and 1 trial failed). Furthermore, it was discovered that patients were responding to vortioxetine after two weeks, with a maximum response noted during the fourth week of treatment (Alvarez et al., 2014; Sanchez et al., 2015). Longer term trials (12 weeks) demonstrated that the rate of MDD relapse was significantly different between vortioxetine and placebo, highlighting that the drug maintains its therapeutic activity (Alvarez et al., 2014; Sanchez et al., 2015).

Several meta-analyses have evaluated the clinical efficacy and tolerability of vortioxetine compared against placebo and to other antidepressants. One such review examined 57 studies (18,326 patients in total), and found that all antidepressants (including vortioxetine) were beneficial in improving either the Hamilton depression rating scale (HAM-D) or the Montgomery–Åsberg Depression Rating Scale (MADRS) score (Llorca et al., 2014). Briefly, both of these scales assess various factors to evaluate the level of depression the person is experiencing. Factors include (but are not limited to): depressed mood, suicidal thoughts, insomnia, decreased sleep and appetite (Hamilton, 1960; Montgomery et al., 1979). Moreover, vortioxetine had

comparable efficacy when compared against other antidepressants, and was better tolerated then desvenlafaxine, sertraline and venlafaxine (Llorca et al., 2014). In separate studies, a statistically significant reduction in MADRS score was seen for patients taking vortioxetine compared to placebo (Berhan et al., 2014; Pae et al., 2015). Again, there was no difference in efficacy between vortioxetine and other antidepressants (serotonin–norepinephrine reuptake inhibitors [SNRI] or agomelatine) (Pae et al., 2015).

In a large systematic review, looking at 522 double-blind, randomised, placebo-controlled trials, vortioxetine was identified as one of the more efficacious antidepressants (defined by reduction of over 50% of total score on a standardised observer scale for depression e.g. the HAM-D), when compared against placebo and other antidepressants (Cipriani et al., 2018). Moreover, Thase and colleagues assessed 11 studies (3,304 patients taking vortioxetine, 1,824 taking placebo). The response rates (measured by an improvement in MADRS score) were significantly higher in the 5.0, 10 and 20 mg vortioxetine compared to placebo (Thase et al., 2016). 655/1783 (36.7%) of patients taking placebo demonstrated an improvement in MADRS score. For vortioxetine; 5.0 mg, n=496/989 (50.2%), 10 mg, n=501/1026 (48.8%), 15 mg, n=202/436 (46.3%), 20 mg, n=412/799 (51.6%) displayed higher rates of response (Thase et al., 2016). In terms of remission (defined by a MADRS score of $\leq$ 10), only 10 and 20 mg vortioxetine were statistically significant compared to placebo (placebo, n=425/1783 (23.8%); vortioxetine 5.0 mg, n=304/989 (30.7%), 10 mg, n=310/1026 (30.2%), 15 mg, n=125/436 (28.7%), 20mg, n=258/799 (32.3%) (Thase et al., 2016). This pattern of 15 mg vortioxetine not causing an improvement in MARDS score was also noted when Baldwin and colleagues assessed the impact of vortioxetine on the Hamilton Anxiety Rating Scale (HAM-A) (Baldwin et al., 2016). Similar to the HAM-D and MARDS, the HAM-A scale examines a variety of factors (e.g. anxious mood, tension, fears, somatic pains) to evaluate the level of anxiety a patient is suffering from (Hamilton, 1959). Furthermore, compared to placebo, vortioxetine significantly improved patient functioning (defined by an improvement in the Sheehan Disability Scale [SDS]), i.e. symptoms were less likely to disrupt work, social life or family life (Sheehan et al., 1996; Florea et al., 2017).

However, it was reported that a significantly larger number of patients reported adverse effects when taking vortioxetine compared to placebo, with the odds of experiencing nausea was 3 x higher for vortioxetine compared to placebo (Berhan et al., 2014). Approximately 25% of patients who took vortioxetine experienced nausea (compared to 9% who received placebo), 4% of patients experienced vomiting (3% for placebo) and 4% of patients suffered from constipation (1% for placebo). These adverse effects were tolerated, with only 8% of patients who received vortioxetine withdrawing from treatment (Schatzberg et al., 2014). In addition, it was reported that the most common adverse effect experienced by those taking vortioxetine (20 mg) was nausea (20% compared to 5% for placebo) (Meeker et al., 2015). In a separate analysis, nausea was the biggest reason for discontinuation, with 0.3% of patients taking placebo withdrawing, compared to: 1.5%, 1.6%, 2.4% and 4.3% of patients taking 5.0, 10, 15 and 20 mg of vortioxetine discontinuing, suggesting the probability of experiencing nausea is dose dependent (Baldwin et al., 2016). In addition, vortioxetine had no significant effect on weight gain or sexual dysfunction; both of which are issues seen in other antidepressants (Alvarez et al., 2014; Sanchez et al., 2015).

There appears to be however conflicting data on the tolerability of vortioxetine. One study found the number of people discontinuing due to adverse effects was higher in the other antidepressant treatment group (SNRIs and agomelatine) compared to vortioxetine (Pae et al., 2015). Similarly, a large systemic review comparing various antidepressants found vortioxetine was also one of the best tolerated drugs, with a low dropout rate, when contrasted against placebo and other antidepressants (Cipriani et al., 2018).

Vortioxetine has a multi-modal pharmacological profile. Receptor binding assays demonstrated the drug has relatively high affinity (K_i) for the 5-HT_3 (3.7 nM) and 5-HT_7 (19 nM) receptors and also the 5-HT transporter, SERT (1.6 nM) (Bang-Andersen et al., 2011). Vortioxetine also interacted with the 5-HT_{1A} (15 nM) and 5-HT_{1B} (33 nM) receptors and displayed agonist and partial agonist activity respectively (measured by a cAMP assay) (Bang-Andersen et al., 2011; Mork et al., 2012; Dale et al., 2013). In

contrast the interaction of vortioxetine with the 5-HT_3 receptor was described as being an antagonist, although electrophysiological recordings using oocytes expressing the 5-HT_3A receptor demonstrated agonist action on first administration but not for subsequent application (Bang-Andersen et al., 2011; Ladefoged et al., 2018). Vortioxetine appeared to show a similar binding orientation to 5-HT_3 receptor antagonists in computational ligand docking assays. Furthermore, in functional membrane potential assays, mutations to valine 202 (located in loop F) appeared to impact the potency of vortioxetine as an antagonist, a residue previously not thought to play a role in ligand binding (Ladefoged et al., 2018). Consistent with 5-HT_3 receptor antagonist activity, vortioxetine (and ondansetron) both inhibited transient bradycardia elicited by intravenous administration of 5-HT to rats (Mork et al., 2012). Given the interaction of vortioxetine with the 5-HT_3 receptor, the nausea and vomiting experienced by patients can be attributed to this pharmacological mechanism of action. Hepatic cytochrome P_{450} enzymes metabolise vortioxetine (with linear kinetics) to an inactive metabolite. Vortioxetine also does not induce nor inhibit those enzymes (Alvarez et al., 2014; Sanchez et al., 2015).

Interestingly, a study has demonstrated anti-inflammatory activity of vortioxetine. Nanomolar concentrations of vortioxetine (relevant concentrations to what has been found in the plasma of patients taking this drug) inhibit superoxide anion production from PMA stimulated human macrophages (Talmon et al., 2018). Moreover, treating monocytes with vortioxetine before differentiating them into macrophages will reduce PMA induced superoxide anion production; as well as increasing the expression of an anti-inflammatory marker (peroxisome proliferator-activated receptor gamma) and decreasing the expression of the proinflammatory cytokine tumour necrosis factor alpha (Talmon et al., 2018).

1.13 Aims

The aim of this research is to characterise the pharmacology of two 5-HT_3 receptor partial agonists (vortioxetine and CSTI-300), and to investigate the binding mechanism of Cl-indole at the 5-HT_3 receptor.

The first section of this study is an investigation into the pharmacology of vortioxetine, a novel antidepressant, at the human (h) 5-HT_3A and h5-HT_3AB receptors. This is the first detailed examination of vortioxetine in both functional (intracellular calcium assays) and receptor binding techniques at the 5-HT_3 receptor. It was also evaluated whether vortioxetine could show promise in IBS-d by reducing 5-HT activity in an *in vitro* model of IBS-d.

The next section of this study is a comprehensive assessment of CSTI-300, a selective 5-HT_3 receptor partial agonist with the therapeutic potential to treat IBS-d. Similar to the vortioxetine studies, the pharmacology of CSTI-300 is explored at the h5-HT_3A and h5-HT_3AB receptors using functional and radioligand binding assays. In addition, CSTI-300 is assessed as a potential treatment for IBS-d in a rodent *in vivo* model, and its efficacy compared to a current marketed therapeutic for IBS-d, the 5-HT_3 receptor antagonist alosetron. The pharmacokinetics of CSTI-300 in a variety of species (mouse, dog, rat and mini-pig) are also explored.

The last section of this work is analysing how 5-HT_3 receptor ligands (agonists and antagonist) impact how Cl-indole binds to the h5-HT_3A and h5-HT_3AB receptors.

1.14 Hypotheses

The first hypothesis is that vortioxetine will demonstrate activity as a high-affinity, competitive 5-HT_3 receptor partial agonist in *in vitro* assays using recombinant expression systems. This will allow for the speculation that vortioxetine could be a viable therapeutic option for treating IBS-d, where there is known 5-HT_3 receptor dysfunction.

The second hypothesis is that CSTI-300, much like vortioxetine, will display activity in *in vitro* assays as a high-affinity 5-HT_3 receptor partial agonist. Furthermore, *in vivo* studies will demonstrate that CSTI-300 is effective in reducing colonic sensitivity in rat and is active in the rat Bezold-Jarisch model. This will enable conclusions to be drawn about whether CSTI-300 is a viable candidate to potentially treat IBS-d.

The third hypothesis is that Cl-indole will demonstrate an unusual binding mechanism, in that the presence of 5-HT (or indeed any 5-HT_3 receptor ligand) may allow Cl-indole to interact with the 5-HT_3 receptor differently compared to Cl-indole alone.

2 Methods

2.1 Cell culture

All tissue culture work was carried out under a type II microbiology safety hood to ensure sterility. All reagents used were either purchased sterile or filter sterilised through 0.22 µm filters. HEK293 cells stably expressing the following constructs: h5-HT_3A receptor (HEK3A cells) or the h5-HT_3AB receptor (HEK3A3B cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, cat. D5796) complemented with (v/v) 10% Foetal Bovine Serum (FBS, Sigma-Aldrich, cat. F7524) and 1% Penicillin-Streptomycin (100 units penicillin, 0.1 mg/ml streptomycin, Sigma-Aldrich, cat. P4333) (Brady et al., 2001). Cells were passaged when 90% confluent. This was achieved by initially aspirating media from the cells and washing with phosphate buffered saline (PBS, without calcium and magnesium, Thermo Fisher, cat. 14200067). 1 ml of trypsin/EDTA (0.5 g/l trypsin and 0.2 g/l EDTA [Ethylenediaminetetraacetic acid], Sigma-Aldrich, cat. T3924) was added to the cells to detach them from the T75 tissue culture flask (Corning, cat. BC301). After five minutes at 37°C, cells were then resuspended in 9 ml of media (to inactivate the trypsin/EDTA). Generally, the cells were split 1:3 (3 ml of out 9 ml the cells were in were added to a new tissue culture flask) and volume made up to 15 ml with DMEM. Table 2 lists the selection antibiotics (and concentration) used to maintain protein expression in the different cell lines. These antibiotics were added every 2 weeks. After no more than 40 passages, cells were discarded, and new aliquots of cells taken from liquid nitrogen.

Table 2: The selection antibiotics which were added to transfected HEK cells to ensure target protein expression.

Cell line	Antibiotic (concentration)
HEK3A	Geneticin (250 µg/ml, Thermo Fisher, cat 10131019)
HEK3A3B	Geneticin (250 µg/ml) and zeocin (80 µg/ml, Thermo Fisher, cat. R25001)

Cells were stored long term in liquid nitrogen. Briefly, cells were detached from the tissue culture flask using trypsin/EDTA before being resuspended in DMEM. Cells were pelleted at 400 x g for 5 minutes with supernatant discarded. The pellet was resuspended in freezing media (90% FBS, 10% dimethyl sulphoxide [DMSO], Sigma-Aldrich, cat. D2650) and transferred immediately to – 80°C in Mr Frosty (Thermo Fisher, cat. 5100-0050). This enabled a freezing rate of -1°C/minute. After 24 hours cells were stored in the vapour phase of liquid nitrogen.

To recover cell lines from liquid nitrogen, cells were rapidly thawed at 37°C, and 1 ml of media was gradually added to the cell suspension, before adding a further 10 ml of DMEM. Cells were pelleted by centrifugation (400 x g, 5 minutes). Supernatant was aspirated, and the pellet was resuspended in fresh media, before being transferred to a T-75 tissue culture flask. After 24 hours, media was replaced (to ensure removal of any residual DMSO) and cells were maintained as above. Antibiotics (for selection) were not added until cells had been in culture for at least one week.

2.2 Preparing whole cell lysates

To generate a whole cell lysate for radioligand binding experiments, media was aspirated off confluent cells (HEK3A or HEK3A3B cells) in a T75 tissue culture flask and the cells were washed in 5 ml PBS. Adherent cells were scraped off using a cell scraper (Sarstedt, cat. 83.1830) in 5 ml PBS. Cells were then resuspended in a further 5 ml of PBS and centrifuged at 400 x g for 5 minutes. The supernatant was discarded, and the pellet was stored long-term at -80°C or resuspended in Tris(hydroxymethyl)aminomethane buffer pH 7.4 (Tris, Sigma-Aldrich, cat. T1503) and lysed by sonication for immediate use.

2.3 Bradford assay

The Bradford protein assay uses the negatively charged Coomassie Brilliant Blue G-250 dye, which binds to basic amino acids (Bradford, 1976). In this situation, the Bradford reagent absorbs at 595 nm. A range of Bovine serum albumin (BSA, Sigma-Aldrich, cat. A9418) standard concentrations (0.1 to 1.4 mg/ml) was used to calibrate the Eppendorf BioPhotometer Plus spectrophotometer, enabling the concentration of protein in a whole cell lysate to be determined. 20 µl of BSA standard (diluted in 25 mM tris buffer) was combined with 1 ml of Bradford reagent (Sigma-Aldrich, B6916) in a polystyrene cuvette and left to stand for 5 minutes at room temperature. A blank, which was 20 µl of tris buffer, was also included. Absorbance (at 595 nm) was then measured on the spectrophotometer. For whole cell lysates, the same procedure was applied.

2.4 Intracellular calcium assays

2.4.1 Agonist experiments

To assess the functional activity of the h5-HT_3A or h5-HT_3AB receptor *in vitro*, intracellular calcium assays were undertaken using a fluorescent calcium binding dye. When confluent, HEK3A or HEK3A3B cells were trypsinised and resuspended in 10 ml DMEM. Cells were counted using a haemocytometer and diluted to $1x10^6$ cells/ml in DMEM. Finally, $1x10^5$ cells were seeded per well onto poly d-lysine (100 µg/ml, Sigma-Aldrich, cat. P7280) coated black-sided, clear-bottomed 96 well plates (Sigma-Aldrich, cat. CLS3603). Poly d-lysine was needed to assist in cell adhesion. Cells were left overnight in a humidified incubator at 37°C to reach confluency.

After 24 hours, 300 ml of HBSS (Hank's Balanced Salt Solution, Thermo Fisher, cat. 1416506) was prepared from a 10x stock and the pH adjusted to 7.4. After discarding the media, cells were washed twice in HBSS. Fluo-4 acetoxymethyl (AM) ester (Thermo Fisher, cat. F14201) was initially dissolved in DMSO before being diluted in

HBSS to a final concentration of 5.0 μM (Newman et al., 2013). Cells were loaded with Fluo-4AM for 1 hour at room temperature in the dark on a rotating table. Fluo-4AM contains an acetoxymethyl ester, which renders the molecule cell permeant due to increased lipophilicity. Once the Fluo-4AM is inside the cell, non-specific esterases cleave the ester group to render the Fluo-4 active (able to bind calcium) and cell impermeant. After the 1 hour incubation, excess extracellular Fluo-4AM was discarded and cells washed twice in HBSS, before being incubated for 30 minutes in 100 μl HBSS to allow complete de-esterification of the Fluo-4AM.

Fluorescence was read at 495 nm/515 nm (excitation/emission spectra) using the Flex Station (Molecular Devices); which is an automated pipetting system which also reads fluorescence. The Flex Station applied drugs at three predetermined time points, and also reads fluorescence every 3 seconds. Generally, Cl-indole or vehicle was added at 20 seconds, agonist was applied at 80 seconds and ionomycin (750 ng/ml) at 320 seconds. Ionomycin punctures holes in the endoplasmic reticulum and thereby releases intracellular calcium to bind to cytosolic fluo-4. Ionomycin showed that the cells could still respond at the end of the experiment, as well as demonstrating the maximum calcium response the cells could achieve (Figure 6).

For data analysis, maximal response elicited by drug was subtracted from baseline (before addition of agonist). This was then taken as a percentage of the maximal agonist response and plotted against drug concentration.

2.4.2 Antagonist studies

To verify the calcium responses elicited by agonists at the 5-HT_3 receptor were 5-HT_3 receptor dependent, cells were preincubated with granisetron (500 nM) 30 minutes before they were analysed on the Flex station was above.

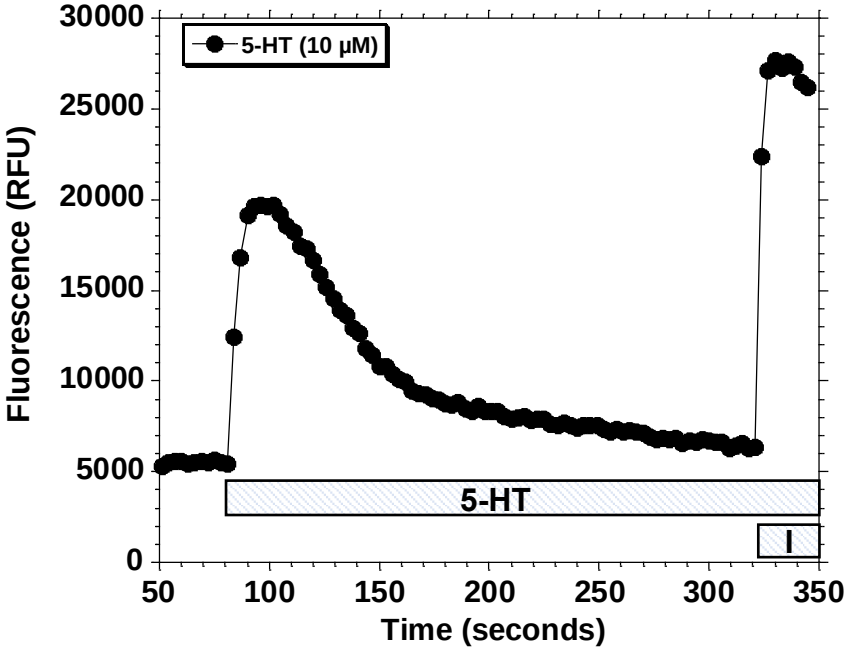

Figure 6: Ionomycin elicits the maximal calcium response in HEK3A cells

The ionomycin (I) response demonstrated that cells at the end of the intracellular calcium assay could still respond to stimulation and provided the maximum calcium response the cells could achieve.

2.5 Radioligand binding

2.5.1 Competition radioligand binding

The binding characteristics of various 5-HT_3 receptor ligands for the 5-HT_3 receptor can be studied using receptor binding techniques. HEK3A or HEK3A3B cell lysates were prepared as previously described and kept on ice in a glass beaker.

Radioligand binding studies were carried out similar to before (Monk et al., 2004). Each condition was performed in triplicate. Each binding tube (Sarstedt, cat. 55.526.005) contained a final volume of 300 µl after drug(s), radioligand and cell lysate were added in that order. Drugs and radioligand were prepared in tris buffer (25 mM, pH=7.4). The radioligand used, [^{3}H]-granisetron (Perkin Elmer, cat. Net1030250uc), is a high-affinity selective radioligand for the 5-HT$_3$ receptor, which was used at a final concentration of ~ 0.5 nM. The selective 5-HT$_3$ receptor antagonist ondansetron (10 µM) was used to define non-specific binding.

After addition of drug(s), radioligand and cell lysate, samples were left for one hour at room temperature to allow [^{3}H]-granisetron or [^{3}H]-vortioxetine binding to reach equilibrium before the reaction was terminated rapidly using a Brandel cell Harvester onto pre-soaked GF/B filter papers (Brandel, cat. FP105). The filter papers were soaked in 0.3% Tris/polyethyleneimine (PEI, Sigma-Aldrich, cat. 03880) to assist in the protein binding to the filter paper. For [^{3}H]-granisetron, the filter paper was washed 3 x 3 seconds with ice-cold tris buffer. 4 ml of scintillation fluid (Optiphase Hisafe-3, Perkin Elmer, cat. 1200-437) was added to the filter paper and the samples were left for at least three hours before being counted on a Liquid Scintillation Counter (Packard).

2.5.2 Saturation radioligand binding

To demonstrate vortioxetine or CSTI-300 is a competitive antagonist, saturation radioligand binding was used to assess whether vortioxetine or CSTI-300 decreases the affinity of [^{3}H]-granisetron for the 5-HT$_3$ receptor. Cell lysates were prepared the same as for competition radioligand binding. A range of [^{3}H]-granisetron concentrations (0.1 – 30 nM) were used in the absence (vehicle) or presence of vortioxetine or CSTI-300, with ondansetron (10 µM) used to define non-specific binding. Samples were then incubated and harvested as above. Each condition was performed in duplicate. Specific binding (total binding – non-specific binding) was plotted against [^{3}H]-granisetron concentration using Kaleidagraph. Kaleidagraph was also used to draw Scatchard plots (Bound vs Bound/Free).

2.5.3 Scintillation Proximity Assay

Scintillation proximity assays (SPA) depend upon copper PVT (Polyvinyltoluene) beads which will bind His-tagged proteins which in turn are bound to a radioligand. The copper PVT beads contain scintillant which converts the β energy emitted by ^{3}H into light. The light is measured by a scintillation counter and converted back to units of radioactivity. This technique is useful when the protein target is not located within the cell surface membrane and so may not be retained by the filter paper used in traditional filtration receptor binding techniques.

To investigate the binding characteristics of Cl-indole (±5-HT) or alosetron at the $A1B2D1_w$ 5-HTBP (5-HT binding protein), SPA experiments were undertaken as has been described previously (Kesters et al., 2013). 25 µl of competing drug(s), 25 µl of [^{3}H]-granisetron (~8.0 nM), 25 µl of copper His-tagged PVT beads (2 mg/ml, Perkin Elmer cat. RPNQ0095) and 25 µl of 5-HTBP (a gift from Chris Ulens, University of Leuven, 1.0 µg/ml) were prepared in buffer (10 mM HEPES [Sigma-Aldrich, cat. H3375]/0.05 %Tween-20 [Sigma-Aldrich, cat. P1379) and added to white 96 well optiPlates (Perkin Elmer cat. 6005290). The reaction was left for 90 minutes at room temperature on a rotating table. The plate was centrifuged at 400 x g for 1 minute to allow the SPA beads to settle. A TopCount (Perkin Elmer) was used to measure radioactivity remaining on the SPA beads.

2.5.4 Single cell electrophysiology

Approximately 18 hours prior to electrophysiology assays, HEK3A cells were seeded directly onto 13 mm diameter glass coverslips coated with poly-L-lysine (100 µg/ml; Sigma-Aldrich, cat. P7280) and fibronectin (40 µg/ml; Sigma-Aldrich, cat. F1056), at a density of 2 x 10^{4} HEK3A cells per coverslip. Macroscopic currents were recorded in the whole-cell recording mode of the patch-clamp technique. Cells were superfused at 2.0 ml/min with an extracellular solution composed of (in mM) NaCl 140, KCl 2.8, $CaCl_2$ 1.0, glucose 10, HEPES 10, pH 7.4 adjusted with NaOH. Patch electrodes were

pulled from borosilicate glass (O.D. 1.2 mm, I.D. 0.69 mm; Harvard Apparatus, Edenbridge, UK) using a P-97 puller (Sutter, Novato, CA, USA) and filled with intracellular solution consisting of (in mM) 140 CsCl, 2.0 $MgCl_2$, 10 HEPES, 1.0 EGTA, 1.0 Mg-ATP and 0.3 Na-GTP; pH adjusted to 7.3 with CsOH (osmolarity ~285 mOsm). Patch electrodes typically had open tip resistances of 4-7 MΩ. Membrane currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Wokingham, UK), low-pass Bessel filtered at 1.0 kHz and digitized at 10 kHz by a Digidata 1550B (Molecular Devices). Experiments were performed at room temperature with the cells voltage-clamped at -60 mV.

Agonist-evoked currents were elicited by pressure ejection (1.3 bar; Picospritzer III; Parker Hannifin, Pine Brook, NJ, USA) of agonist from patch pipettes placed ~30 µm from the recorded cell.

2.5.5 Rat Bezold-Jarisch model *in vivo*

Male Sprague-Dawley rats (233-260 g) were fasted overnight prior to use. Dose volume for all solutions was 10 ml/kg. For studies investigating a dose-response to CSTI-300 (0.1, 0.3, 1.0, 3.0 mg/kg), alosetron (0.1 mg/kg) or vehicle (2.0% Tween-80), test substance was administered orally (by oral gavage) to each group of six animals. One hour later, 5-HT (0.3 mg/kg, i.v.)-induced bradycardia was recorded in anesthetised (1,500 mg/kg urethane, i.p.) animals. For studies analysing the time effect of CSTI-300 (1.0 mg/kg), the drug was administered orally to each group of six animals. One, two, four and eight hour(s) later, 5-HT (0.3 mg/kg, i.v.)-induced bradycardia was recorded in anesthetised (1,500 mg/kg urethane, i.p.) animals. Lead II ECG was obtained with subdermal needle electrodes in the upper right shoulder and the lower left inner thigh. The ECG was recorded using a bio amplifier (AD Instruments, Australia) and analysed by data acquisition and analytic system (Power Lab/8 SP, AD Instruments, Australia) with a frequency of 60 Hz. Each bradycardia event was recorded for 30 seconds after injection of 5-HT. Following completion of the study, terminal blood was collected for subsequent analysis of plasma CSTI-300 levels. Olan Ricerca (Concord, Ohio, USA) performed the experiments. All aspects

of this work including housing, experimentation and disposal of animals were performed in general accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D. C., 1996). I was given the raw data in the form of a report. I analysed the data by calculating the percentage net decrease in bpm (mean±SEM, n=6 rats) and plotting this against either CSTI-300 (or alosetron) concentration, or against time. I also performed statistical analysis (a Shapiro-Wilk procedure to test for normality, followed Kruskal-Wallis test with Dunn's multiple comparisons). The study was not powered.

2.5.6 Rat colon distension model of IBS-d

A previously described IBS-d model was used to evaluate the activity of CSTI-300 versus alosetron (Banner et al., 1995; Banner et al., 1995). This *in vivo* model applies a noxious colo-rectal distension to conscious rats by acute balloon inflation and the effects observed as abdominal muscle contraction with the threshold pressure noted as the readout. Thus, under ether anaesthesia (ether), a 6-7 cm long latex balloon was carefully inserted intra-anally to a position ~1 cm beyond the ano-rectal verge. The cannula from the balloon, which was taped to the tail to prevent expulsion of the balloon, was connected to a coloured-water filled manometer and via a 3-way tap to a syringe pump. Throughout the procedure the singly housed animals (male Wistar rats; 180-250 g) were allowed unrestricted movement within a 20x20x14 cm clear acrylic box.

After recovery from the balloon insertion procedure, a ramp inflation of the colo-rectal balloon at a rate of 20 mm water per minute was performed until the visceromotor response (abdominal muscle contraction) was observed. At this point the pressure was noted and released immediately by opening the 3-way tap. The inflation procedure was repeated at 5 minute intervals until 3 stable responses were recorded.

Rats (n=10) were then dosed with 5-HTP (10 mg/kg s.c.), and 5 minutes later a ramp inflation of the colo-rectal balloon was performed until the visceromotor response was

observed. The inflation procedure was repeated at 5 minute intervals until 3 stable responses were recorded before subsequent treatment; either alosetron (0.01-1.0 mg/kg; 1.0 ml/kg s.c.), CSTI-300 (0.01-1.0 mg/kg; 1.0 ml/kg s.c.) or vehicle (0.9% NaCl; 1.0 ml/kg s.c.). The colonic distensions were performed at 5 minute intervals for a further 45 minutes with effective pressures monitored. As soon as the last time point was recorded, blood was collected from the rats to enable the concentration of CSTI-300 (or alosetron) to be assessed. Rats were euthanised (CO_2) following completion of the study. The experiments were carried out by Medicilon (Shanghai, China). The protocol complied with and was approved by the Institutional Animal Care and Use Committee (Shanghai Medicilon Inc., Shanghai, China) and Shanghai Medicilon Inc is accredited by the NIH Office of laboratory Animal Welfare (OLAW; https://olaw.nih.gov/home.htm) and AAALAC (www.aaalac.org). I was given the raw data in Microsoft Excel, and from this I calculated the percentage response (mean±SEM, n=10), and plotted this against time. I also performed statistical analysis (a Shapiro-Wilk procedure to test for normality, followed by a 2-way ANOVA with subsequent Tukey's multiple comparisons test, with Bonferroni correction for multiple comparisons). The study was not powered.

2.5.7 Dog behavioural and emesis model

A total of 6 naïve male Beagle dogs (10.15-11.45 kg) were administered with CSTI-300 (1.0 mg/kg; 5.0 ml/kg p.o.) or vehicle (sterile water, 5.0 ml/kg p.o.). Each animal was monitored continuously (by an observer and a video recording) for least 4 hours post-treatment (which would have been extended up to 6 hours if any remarkable observations had been evident during the first 4 hours post-dose). During the observation period, each animal was observed primarily for signs of behavioural disturbances or emesis. Emesis was defined by retching and vomiting; "retch" was defined as the action, and "vomit" was defined as the delivery of gastric content through the retching action. If either or both of these emetic characteristics had occurred, they would have been counted independently and recorded on a raw data sheet. Observations for other adverse events were also performed (e.g. abdominal contractions, excessive salivation, state of faecal matter [e.g. normal, loose, diarrhoea], general distress, vocalising [e.g. excessive barking or whimpering],

atypical behaviour, atypical feeding behaviour, atypical drinking behaviour, indication of pain, or mortality). With no emesis or adverse observations, the observation at the time point was noted as 'normal'. Animals were also observed for 1 hour at 23-24 hrs post-dose and the above observations assessed. If any emesis had been evident or any adverse observation, the animals would have been monitored periodically until normal behaviour was resumed (with the above observations monitored and recorded).

Six hours after treatment, a peripheral venous blood sample was taken (~1 ml/ sample into sodium heparin collection tubes) from each animal for bioanalysis of the plasma concentrations of CSTI-300 by HPLC-MS. Briefly; plasma samples (30 µl) were mixed with 150 µl 0.1% formic acid in water and transferred to a 96-well plate for injection. The liquid chromatography system was comprised of a Waters Ultra Performance Liquid Chromatography (UPLC, Waters Corporation, USA) equipped with an ACQUITY UPLC binary solvent manager, ACQUITY UPLC Autosampler Module, ACQUTIY UPLC sample organiser and ACQUITY UPLC column heater HT. Samples (3.0 µl) were injected on to an ACQUITY UPLC BEH C18 1.7 µm (50 mm×2.10 mm) column and the mobile phase ran at 600 µl/minute. Post-column mass spectrometric analysis was performed using an API 4000 (triple-quadrupole) instrument from Applied Biosystems/MDS Sciex with an ESI Ionsource. The data acquisition and control system were created using Analyst 1.5.1 Software from Applied Biosystems/MDS Sciex. A calibration curve using CSTI-300 was created to enable quantification of CSTI-300 levels in the plasma. At study termination, all animals were transferred to the stock animal collection. Experiments were performed by Medicilon (Shanghai, China). The protocol for the dog behavioural and emesis model complied with and was approved by the Institutional Animal Care and Use Committee (Shanghai Medicilon Inc., Shanghai, China). I was given the data in the form presented in this thesis (Table 11).

2.5.8 Pharmacokinetic studies

The pharmacokinetics of CSTI-300 was investigated in male CD-1 mice (20-25 g, n=3), male Sprague-Dawley rats (288-322 g, n=3), male Beagle dogs (8.0-12 kg, n=3-4) and Gottingen mini-pigs (8.9-10.0 kg, n=3). CSTI-300 was administered either intravenously (i.v.) or orally (p.o.) in sterile water as vehicle, with blood samples taken at pre-determined times into K_2 EDTA anti-coagulant tubes. For oral studies (drug administered by oral gavage), the dose volume was: 10 ml/kg, 5.0 ml/kg, 2.0 ml/kg, and 5.0 ml/kg for mouse, rat, dog and mini-pig, respectively. For intravenous studies, the dose volume was: 5.0 ml/kg, 1.0 ml/kg, 2.0 ml/kg and 1.0 ml/kg for mouse, rat, dog and mini-pig, respectively. Plasma samples underwent protein precipitation with acetonitrile containing 0.1% formic acid. After sonication for 1 minute followed by vortex mixing for 2 minutes and refrigeration for 30 minutes, supernatants were transferred to a 96-well filter plate and filtered in a centrifuge. Filtered extracts (200 µl) were transferred into separate 96-well plates and blow dried under nitrogen at 40°C. The residue was reconstituted in 100 µl of mobile phase and submitted for LC/MS/MS analysis. CSTI-300 was quantified with Analyst 1.4.2 software using a calibration curve. Plasma concentration data were analysed with WinNonlin 5.2 software using a non-compartmental model to calculate pharmacokinetic parameters. Mouse and rat studies were carried out by NoAb Biodiscoveries (Mississauga, Ontario, Canada) after approval of procedures by the NoAb BioDiscoveries animal care committee and performed in accordance with the principles of the Canadian Council on Animal Care. Following collection of blood from the last time point, mice were terminated by cervical dislocation and rats euthanised by CO_2. Dog experiments were performed by Covance Laboratories Inc. (Madison, Wisconsin, USA) in compliance with Animal Welfare Act Regulations (9 CFR 3). Following completion of the study, the dogs were returned to the stock colony. Mini-pig studies were undertaken by Calvert laboratories (Scott Township, Pennsylvania, USA), and animals were treated in accordance with the USDA Animal Welfare Act (9 CFR Parts 1, 2 and 3) and the conditions specified in the Guide for the Care and Use of Laboratory Animals (ILAR, 1996, National Academy Press). For all pharmacokinetic studies (mice, rat, dog and mini-pig), I received the raw data in Microsoft Excel. I calculated the mean±SEM and plotted CSTI-300 concentration against time.

2.6 Data analysis and statistics

The fluorescence responses measured by the Flex station were recorded by Softmax Pro software (Molecular Devices, Berkshire, UK). For radioligand binding, inhibition curves and iterative curve fitting were drawn using Kaleidagraph (Synergy, Pennsylvania, USA) defined by the four-parameter logistic equation:

$$b = \frac{B_{max}[L]^n}{[L]^n + K^n}$$

Where b=bound radioligand, B_{max}=maximum binding, K=the molar concentration of competing drug to decrease specific binding by half, L=molar concentration of competing compound and n=Hill coefficient.

The Cheng-Prussoff equation was used to calculate the K_i of competing ligand:

$$\mathrm{K_i} = \frac{\mathrm{IC_{50}}}{1 + \frac{[\mathrm{RL}]}{\mathrm{K_d}}}$$

Where the IC_{50} is from the competing ligand, [RL] is the concentration of free radioligand and K_d is the dissociation constant of the radioligand.

2-way ANOVA with subsequent Tukey's multiple comparisons test (with Bonferroni correction for multiple comparisons) were used to evaluate potential treatment differences in the rat IBS-d colon model; a Shapiro-Wilk procedure tested normality. A Kruskal-Wallis test with Dunn's multiple comparisons was used to test any differences in the impact of 5-HT_3 receptor agonist (5-HT, vortioxetine or CSTI-300) upon Cl-indole affinity for the h5-HT_3A receptor; and to investigate any differences between CSTI-300 or alosetron compared to vehicle in the rat Bezold-Jarisch reflex. A Mann-Whitney U test was utilised to determine if the impact of CSTI-300 or vortioxetine upon [^{3}H]-granisetron affinity for the h5-HT_3 receptor was significant; and used to analyse the impact of 5-HT upon rat heart rate in the Bezold-Jarisch reflex. A Mann-Whitney U test was also undertaken to see if the impact of Cl-indole upon vortioxetine affinity for the h5-HT_3 receptor was significant. It was also used to determine if there was a difference in the kinetics of a 5-HT plus vortioxetine induced

response compared to a 5-HT-induced response at the h5-HT_3A receptor. In addition, a Wilcoxon signed-rank sum test was utilised to examine any difference caused by Cl-indole upon the Hill number for vortioxetine at the h5-HT_3 receptor in competition radioligand binding assays. Post-hoc tests were only conducted if p was <0.05 and the variance of the data was homogenous. Significance criterion was $p<0.05$. Prism (GraphPad, California, USA) was used for statistical analysis.

2.7 Drugs

5-HT (Sigma-Aldrich cat. H7752), alosetron (Albany Molecular Research Inc.), Cl-indole (Sigma-Aldrich cat. C47604), CSTI-300 (Consynance Therapeutics Inc), DDP (Albany Molecular Research Inc.), granisetron (SmithKline Beecham), ionomycin (Sigma-Aldrich, cat. I0634), metoclopramide (Research Biomedicals Incorporated, cat. M-117), ondansetron (Tocris, cat. 2891), PBG (phenyl biguanide, Sigma-Aldrich, cat. 164216), quipazine (Tocris, cat. 0629), s-zacopride (Dalalonde), vortioxetine (Richard Myerson, University of Birmingham).

3 Results

3.1 The anti-depressant vortioxetine is an agonist at the h5-HT_3 receptor

3.1.1 The affinity of vortioxetine at the h5-HT_3 receptor

In competition radioligand binding studies, vortioxetine competed for [^{3}H]-granisetron occupied h5-HT_3A and h5-HT_3AB receptor binding sites with relatively high affinity (Figure 7; Table 3). The affinity of vortioxetine for the h5-HT_3A and h5-HT_3AB receptors was comparable. At the h5-HT_3A receptor, vortioxetine displayed a relatively high Hill number ($p<0.05$ when compared against unity), indicating this ligand demonstrated positive cooperative binding at this receptor. This was not seen for the h5-HT_3AB receptor, where the Hill number was approximately unity, which is not uncommon for agonists at the 5-HT_3AB receptor (Dubin et al., 1999; Brady et al., 2001). The affinity of vortioxetine was increased at both the h5-HT_3A and h5-HT_3AB receptors by the COM Cl-indole (30 µM, Figure 8; Table 3, $p<0.05$).

Table 3: Summary data from Figure 7. The affinity (K_i) of vortioxetine at h5-HT_3A or h5-HT_3AB receptors. The affinity of vortioxetine for each receptor isoform was increased by Cl-indole (30 µM) (*p<0.05 when compared to vortioxetine + vehicle, using Mann-Whitney U test). **p<0.05 when compared to unity (i.e. 1.0) using Wilcoxon Signed Rank Test. Data represents mean±SEM, n=7-8 (h5-HT_3A receptor) and n=5 (h5-HT_3AB receptor).

5-HT_3A receptor	**K_i (nM)**	**Hill number**
Vortioxetine + vehicle (0.1% DMSO)	8.2±1.1	1.43±0.07**
Vortioxetine + Cl-indole (10 µM)	6.2±0.9	1.46±0.07**
Vortioxetine + Cl-indole (30 µM)	5.0±0.8*	1.39±0.11**
5-HT_3AB receptor	**K_i (nM)**	**Hill number**
Vortioxetine + vehicle (0.1% DMSO)	11.4±1.7	0.86±0.06
Vortioxetine + Cl-indole (10 µM)	10.6±3.0	1.01±0.07
Vortioxetine + Cl-indole (30 µM)	5.8±0.9*	0.93±0.04

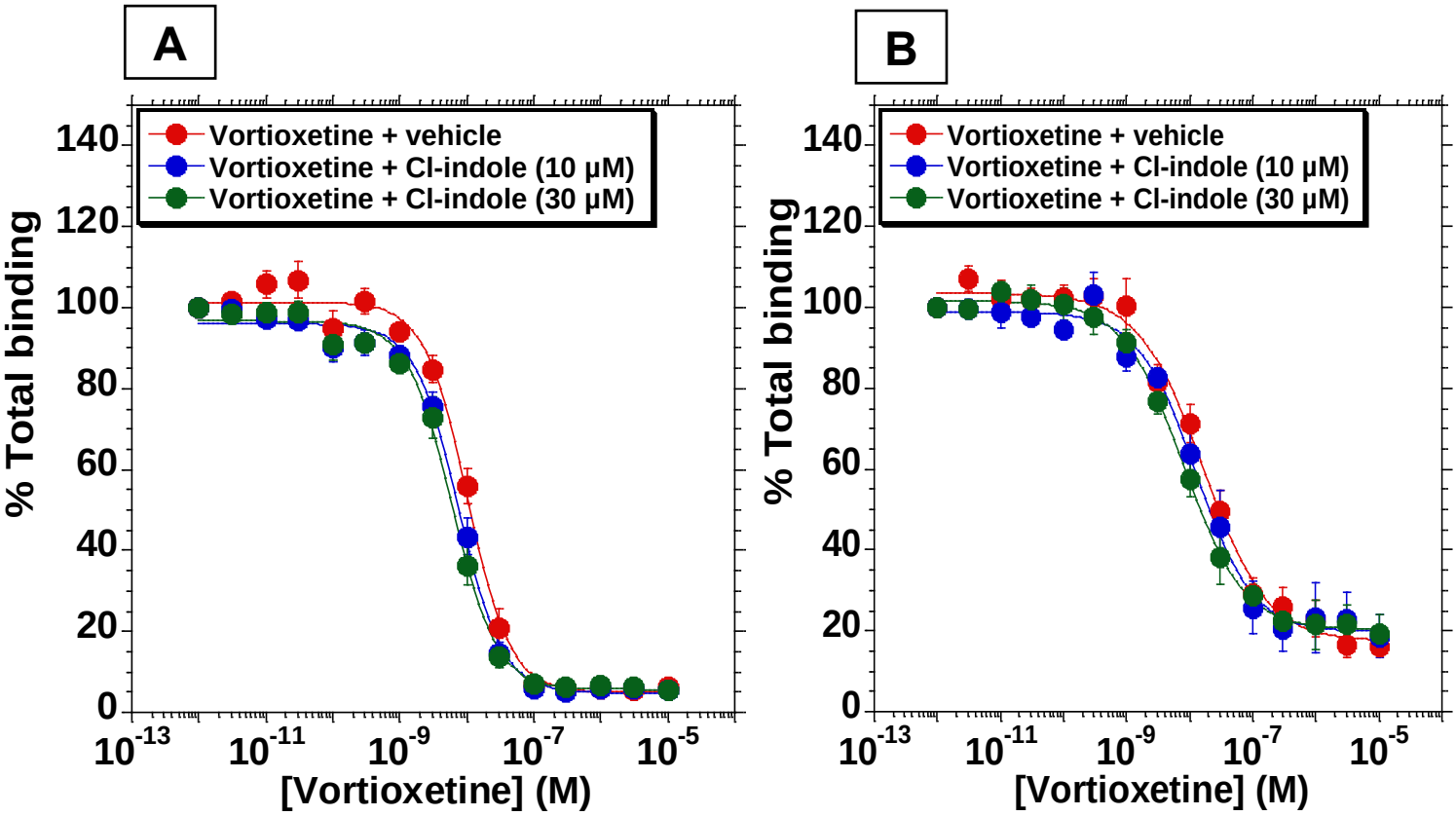

Figure 7. Vortioxetine demonstrated high affinity for 5-HT$_3$ receptors.

The ability of vortioxetine to compete with [^{3}H]-granisetron for the binding of h5-HT$_3$A (A) or h5-HT$_3$AB (B) receptor sites. Cl-indole, a 5-HT$_3$ receptor selective COM, increased the affinity of vortioxetine at both receptor subtypes. Data represents mean±SEM, n=7-8 (h5-HT$_3$A receptor) and n=5 (h5-HT$_3$AB receptor).

It has been demonstrated previously that Cl-indole (up to 100 µM) does not bind to the orthosteric site of h5-HT$_3$A receptor. However, the presence of approximate EC$_{50}$ concentrations of 5-HT induce Cl-indole to bind to the h5-HT$_3$A receptor through a competitive mechanism (Powell et al., 2016). It was investigated whether this phenomenon could also be replicated with vortioxetine. Indeed, in the absence of agonist, Cl-indole demonstrated non-competitive binding to the h5-HT$_3$A receptor. Yet, sub-maximal concentrations of vortioxetine (30 nM and lower), and 5-HT (200 nM) allow Cl-indole to compete with [^{3}H]-granisetron for the h5-HT$_3$A receptor (Figure 5; Table 4). The increase in Cl-indole affinity caused by vortioxetine (30 nM) or 5-HT (200 nM) was also statistically significant ($p<0.01$, Kruskal-Wallis with Dunn's multiple comparisons test).

Table 4: Summary data from Figure 8. Both vortioxetine and 5-HT (200 nM) increased the affinity of Cl-indole for the h5-HT_3A receptor.

Data represents mean±SEM of five experiments. * indicates $p<0.01$ when compared to Cl-indole + vehicle (Kruskal-Wallis with Dunn's multiple comparisons test).

Ligand	**Cl-indole IC_{50} (μM)**
Cl-indole + vehicle	457±70
Cl-indole + 5-HT (200 nM)	30±5*
Cl-indole + vortioxetine (1.0 nM)	256±71
Cl-indole + vortioxetine (3.0 nM)	160±26
Cl-indole + vortioxetine (10 nM)	85±24
Cl-indole + vortioxetine (30 nM)	20±4*

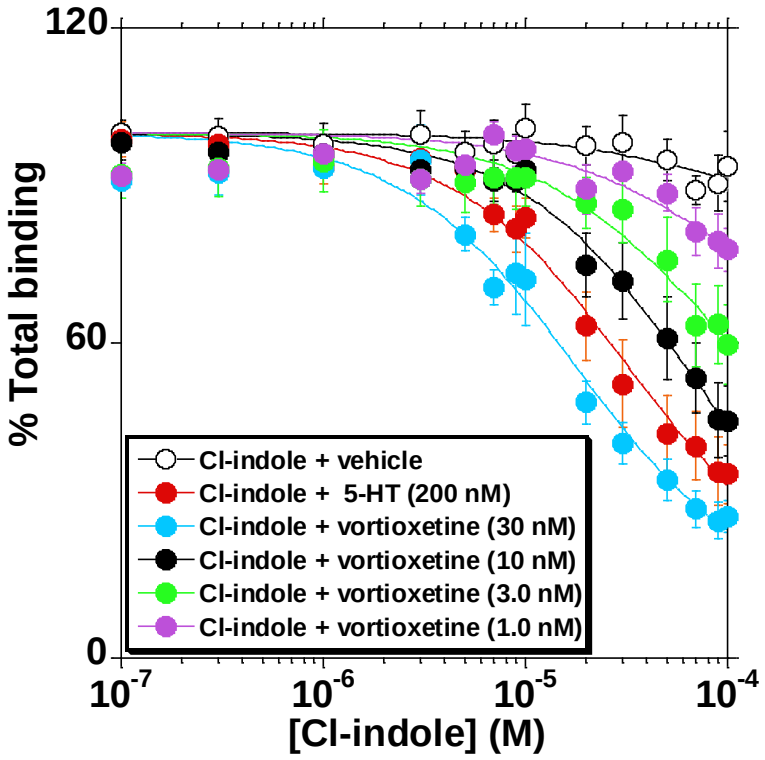

Figure 8. Vortioxetine modulated the binding mechanism of Cl-indole.

Vortioxetine (or 5-HT) converted Cl-indole from a non-competitive ligand to a competitive ligand. Data represents mean±SEM of 5 independent experiments.

3.1.2 Vortioxetine is a competitive drug at the h5-HT$_3$A receptor

Saturation radioligand binding was undertaken in order to demonstrate that vortioxetine interacts with the h5-HT$_3$A receptor through a competitive mechanism. From Figure 9 and Table 5 it can be noted that the presence of vortioxetine (10 nM) decreased the affinity (K_d) of [^{3}H]-granisetron for the h5-HT$_3$A receptor ($p<0.05$, Mann-Whitney U test). However, the density of receptors labelled by a saturating concentration of [^{3}H]-granisetron (B_{max}) remained unaffected by the presence of vortioxetine (10 nM) when compared to vehicle (Figure 9; Table 5).

Table 5: Summary data from Figure 9. Vortioxetine displayed a competitive binding interaction with the h5-HT_3A receptor.

The affinity (K_d) of [^{3}H]-granisetron is approximately trebled by vortioxetine, but the number of labelled binding sites (B_{max}) is not affected by vortioxetine (mean±SEM, n=5), indicating a competitive interaction at the h5-HT_3A receptor. The impact of vortioxetine upon the K_d of [^{3}H]-granisetron was statistically significant ($p<0.05$, Mann-Whitney U test) but was not statistically significant for the B_{max}.

Treatment	K_d (nM)	B_{max} (fmol/mg)	Hill number
Vehicle	0.52±0.08	3558±404	0.92±0.05
With vortioxetine	1.61±0.62	3414±537	0.91±0.09

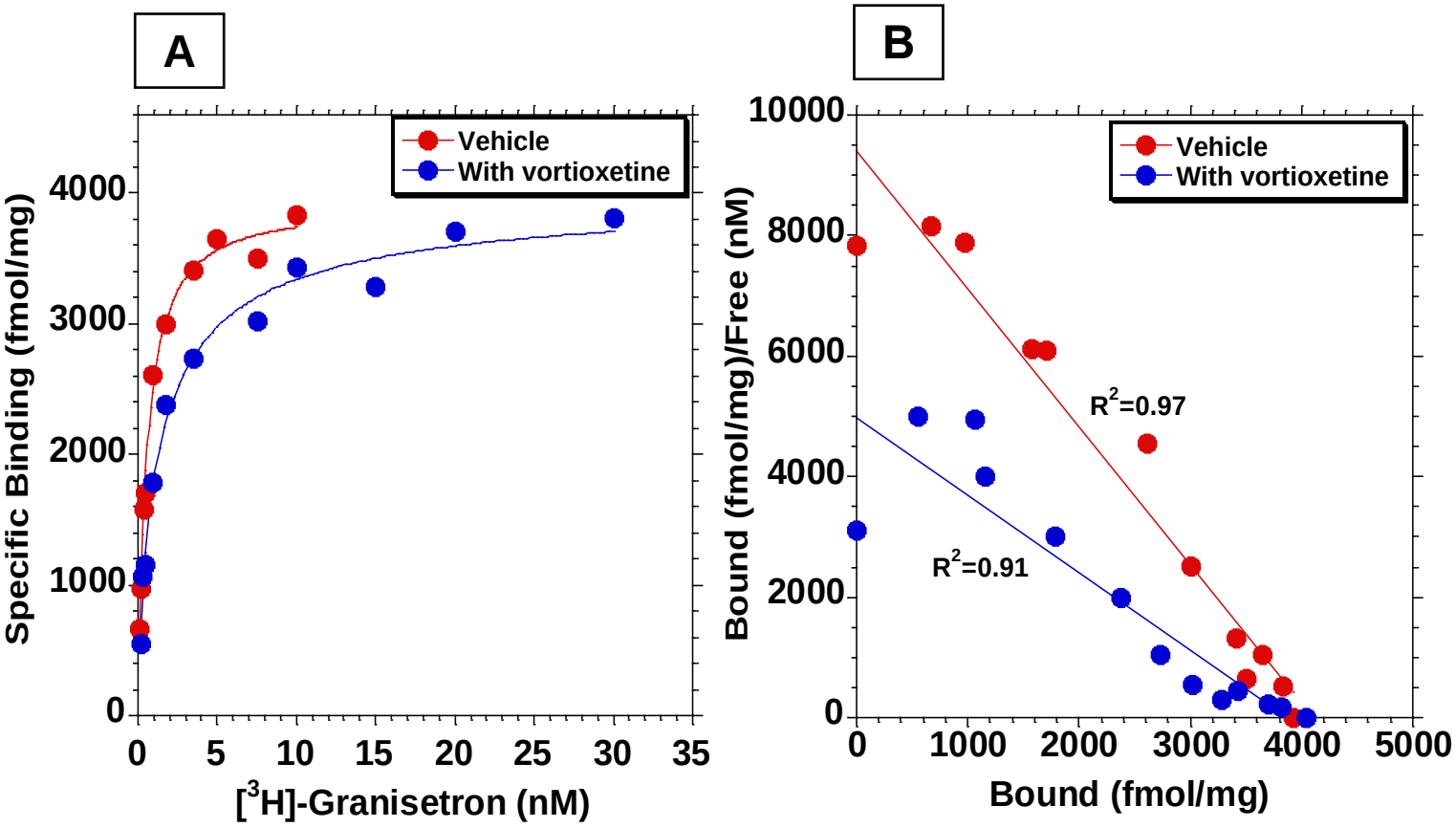

Figure 9. Vortioxetine is a competitive ligand at the h5-HT_3A receptor.

The affinity of [^{3}H]-granisetron for the h5-HT_3A receptor in the absence (vehicle) or presence of vortioxetine (10 nM) A: Saturation curve. B: Scatchard transformation of the data. Representative data of five repeats.

3.1.3 Functional studies of vortioxetine at the h5-HT_3 receptor

Intracellular calcium assays were utilised to investigate the functional activity of vortioxetine at the 5-HT_3 receptor. Both 5-HT and vortioxetine activated the h5-HT_3A and h5-HT_3AB receptors as agonists with differing degrees of efficacy and potency (Figures 10 and 11; Table 6). Maximal responses elicited by vortioxetine were approximately 50% and 40% relative to 5-HT at the h5-HT_3A and h5-HT_3AB receptor respectively. The potency of vortioxetine (and 5-HT) was comparable at both receptor subtypes, and vortioxetine was ~ 3x more potent than 5-HT (Table 6) at both receptor subtypes. The maximum response elicited by both 5-HT (10 µM) and vortioxetine (10 µM) was reduced at the h5-HT_3AB receptor compared to the h5-HT_3A receptor. Moreover, the response induced by vortioxetine at the h5-HT_3A receptor appeared to decay to baseline faster than 5-HT, although at the h5-HT_3AB receptor both 5-HT and vortioxetine responses returned to baseline at a similar rate (Figures 10 and 11). 30 minute pre-treatment with the selective 5-HT_3 receptor antagonist granisetron (500 nM), blocked maximal responses elicited by 5-HT and vortioxetine at both of the receptor subtypes, demonstrating the selectivity of vortioxetine and 5-HT in this assay.

Table 6: Summary data from Figures 10 and 11. Vortioxetine is a 5-HT_3 receptor agonist.

Potency (EC_{50}) and efficacy (E_{max}) of 5-HT and vortioxetine h5-HT_3A and h5-HT_3AB receptors. E_{max} presented as percentage of 5-HT response. Data presented as mean±SEM, n=5.

5-HT_3A receptor	**EC_{50} (nM)**	**E_{max}**	**Hill Number**
5-HT	571±134	103±2	5.19±1.32
Vortioxetine	195±25	50±7	3.45±0.77
5-HT_3AB receptor	**EC_{50} (nM)**	**E_{max}**	**Hill Number**
5-HT	571±109	102±2	2.34±0.45
Vortioxetine	195±68	38±2	1.82±0.26

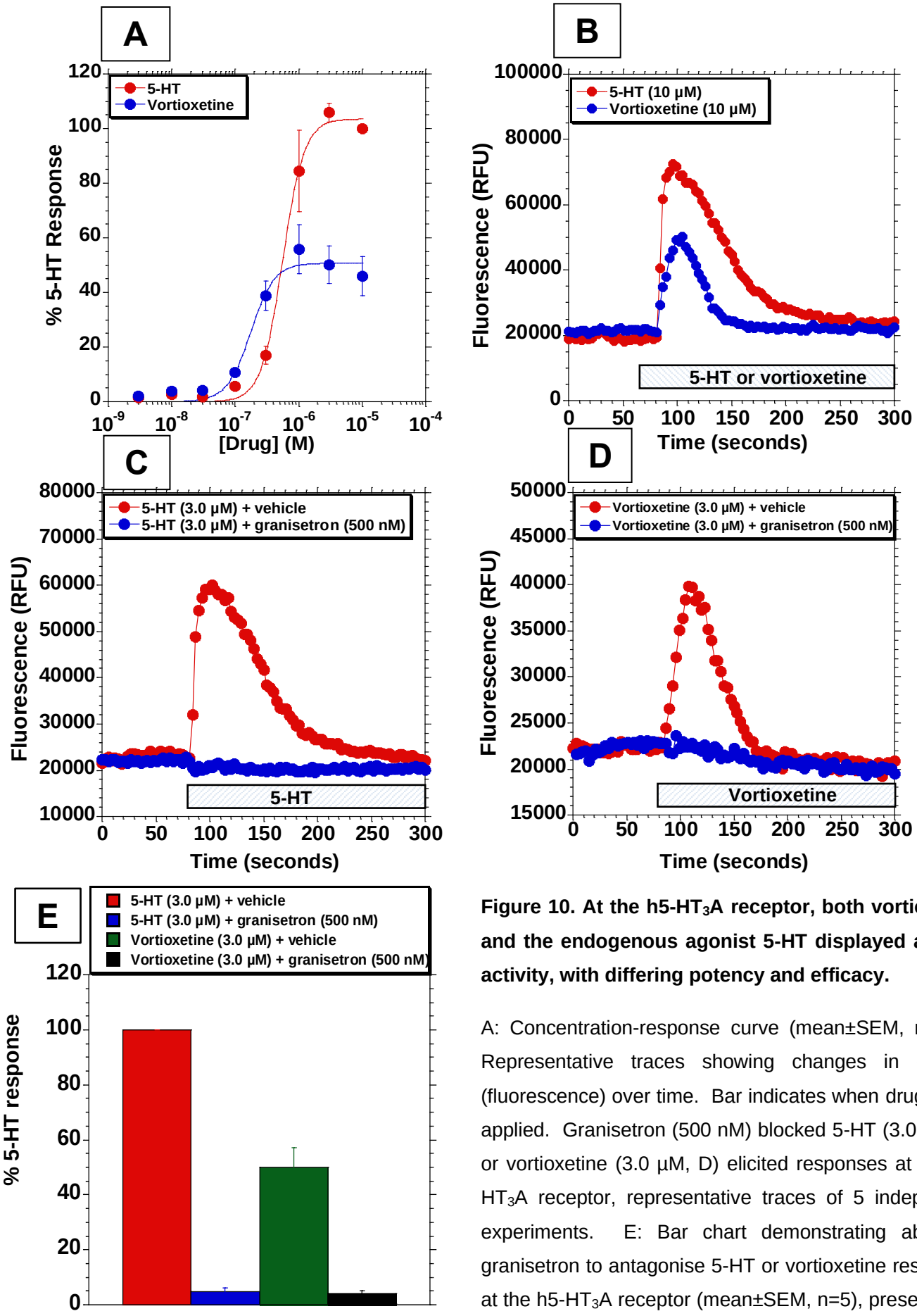

Figure 10. At the h5-HT$_3$A receptor, both vortioxetine and the endogenous agonist 5-HT displayed agonist activity, with differing potency and efficacy.

A: Concentration-response curve (mean±SEM, n=5) B: Representative traces showing changes in calcium (fluorescence) over time. Bar indicates when drugs were applied. Granisetron (500 nM) blocked 5-HT (3.0 µM, C) or vortioxetine (3.0 µM, D) elicited responses at the h5-HT$_3$A receptor, representative traces of 5 independent experiments. E: Bar chart demonstrating ability of granisetron to antagonise 5-HT or vortioxetine responses at the h5-HT$_3$A receptor (mean±SEM, n=5), presented as % 5-HT response.

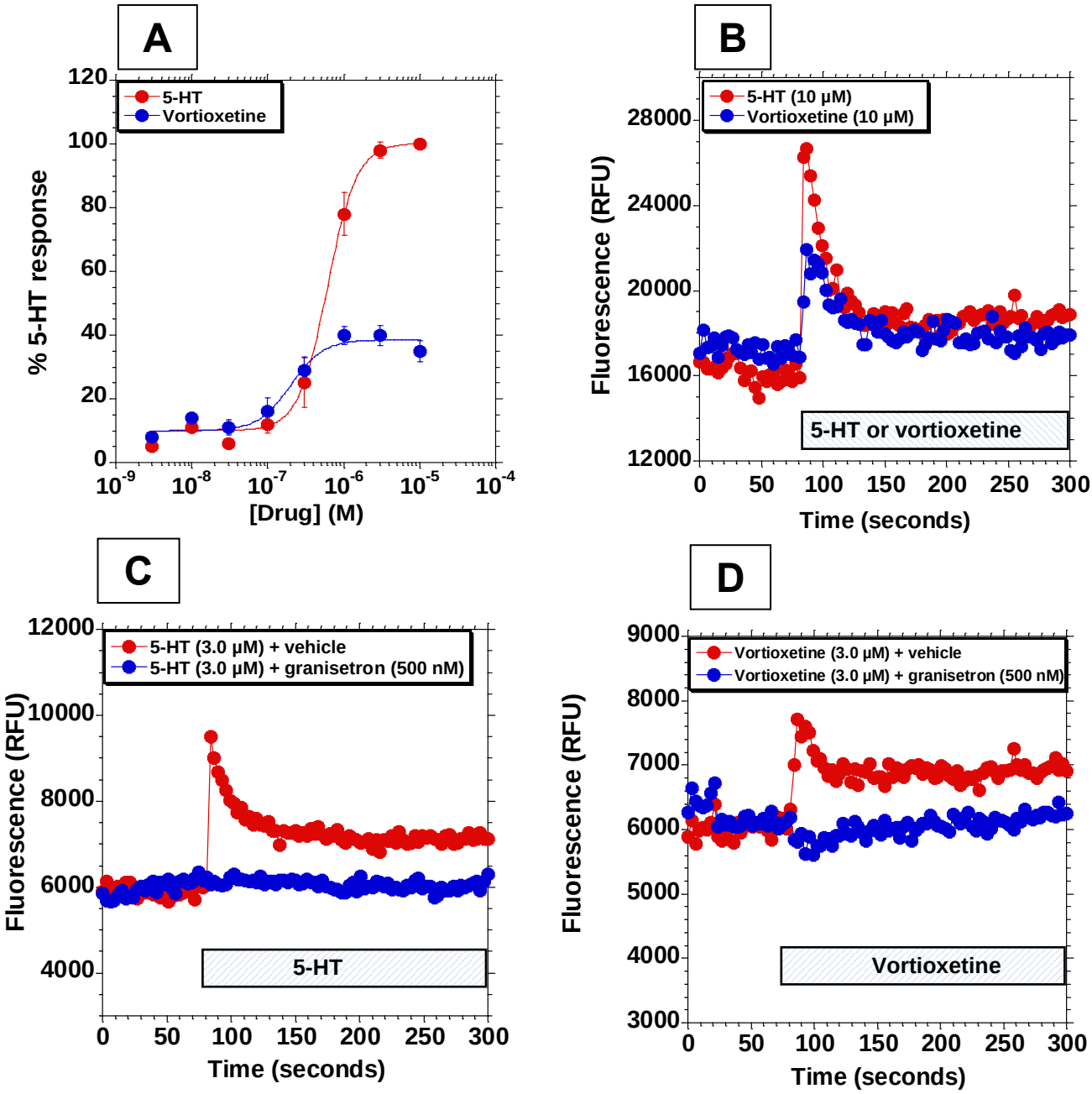

Figure 11. At the h5-HT$_3$AB receptor, both vortioxetine and the endogenous agonist 5-HT displayed agonist activity, with differing potency and efficacy.

A: Concentration-response curve (mean±SEM, n=5) B: Representative traces showing changes in calcium (fluorescence) over time. Bar indicates when drugs were applied. Granisetron (500 nM) to blocked 5-HT (3.0 µM, C) or vortioxetine (3.0 µM, D) elicited responses at the h5-HT$_3$AB receptor. Representative traces of 5 independent experiments. E: Bar chart demonstrating ability of granisetron to antagonise 5-HT or vortioxetine responses at the h5-HT$_3$AB receptor (mean±SEM, n=5), presented as % 5-HT response.

3.1.4 Vortioxetine inhibited 5-HT-induced responses at the h5-HT$_3$A receptor.

In functional calcium studies, alosetron (10 µM) completely blocked the 5-HT (1.0 µM) elicited response at the h5-HT$_3$A receptor (Figure 12). Yet, in the presence of vortioxetine (10 µM) some h5-HT$_3$A receptor activity is still retained. However, the 5-HT response in the presence of vortioxetine decayed faster compared to 5-HT in the absence of vortioxetine. The $t_{1/2}$ time for the decay was 139±3 s for 5-HT, and 122±2 s for vortioxetine, which was statistically significant ($p<0.01$, Mann-Whitney U test). This suggests that vortioxetine can reduce h5-HT$_3$A receptor function but still retain some receptor activity.

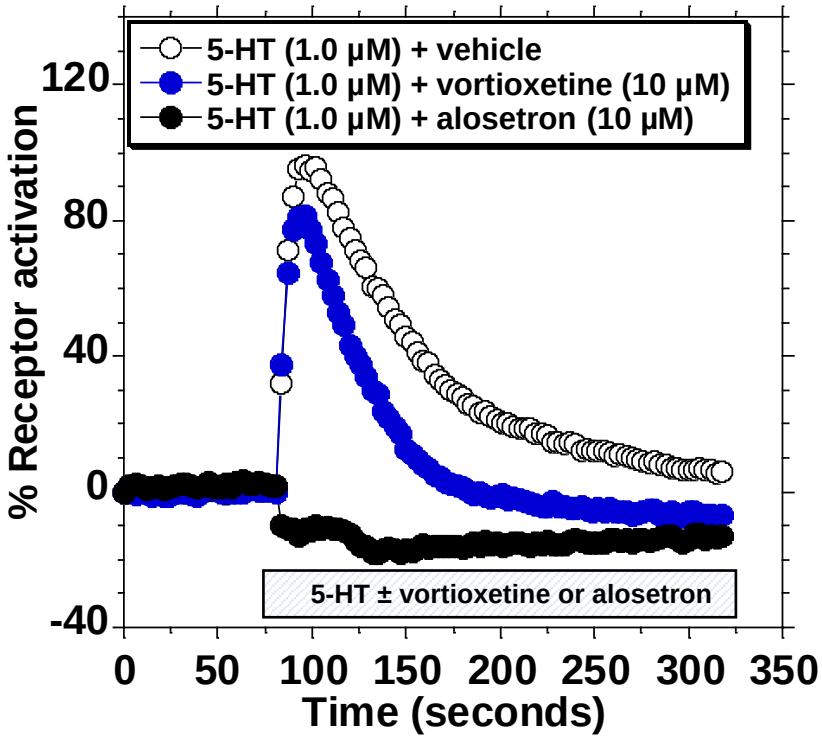

Figure 12. The impact of vortioxetine upon a 5-HT-mediated response at the h5-HT$_3$A receptor.

Ability of vortioxetine (10 µM) or the selective 5-HT$_3$ receptor antagonist alosetron (10 µM) to inhibit a 5-HT (1.0 µM) mediated increase in intracellular calcium at the h5-HT$_3$A receptor, when applied together. Mean traces of n=7-8 independent experiments presented.

3.2 CSTI-300; a novel 5-HT_3 receptor partial agonist with potential to treat patients with irritable bowel syndrome

3.2.1 CSTI-300 in competition radioligand binding studies

CSTI-300 and 5-HT competed for h5-HT_3A or h5-HT_3AB receptor binding sites with [^{3}H]-granisetron. CSTI-300 demonstrated around 100-fold and 350-fold greater affinity compared to 5-HT (Figure 13; Table 7) at the h5-HT_3A and h5-HT_3AB receptor respectively. Both CSTI-300 and 5-HT displayed comparable affinity at both the h5-HT_3A and h5-HT_3AB receptors. The Hill number for CSTI-300, like 5-HT, was above unity when competing for the h5-HT_3A receptor (Table 7). This however was not the case for the h5-HT_3AB receptor.

Table 7: Summary data from Figure 13. **CSTI-300 is a high-affinity 5-HT_3 receptor ligand.**

The affinity (K_i) of CSTI-300 or 5-HT for the h5-HT_3A and h5-HT_3AB receptors. Data represents mean±SEM, n=5.

5-HT_3A receptor	**K_i (nM)**	**Hill Number**
CSTI-300	2.26±0.48	1.19±0.16
5-HT	327±62	1.49±0.19
5-HT_3AB receptor	**K_i (nM)**	**Hill Number**
CSTI-300	1.59±0.09	0.70±0.13
5-HT	563±89	0.91±0.07

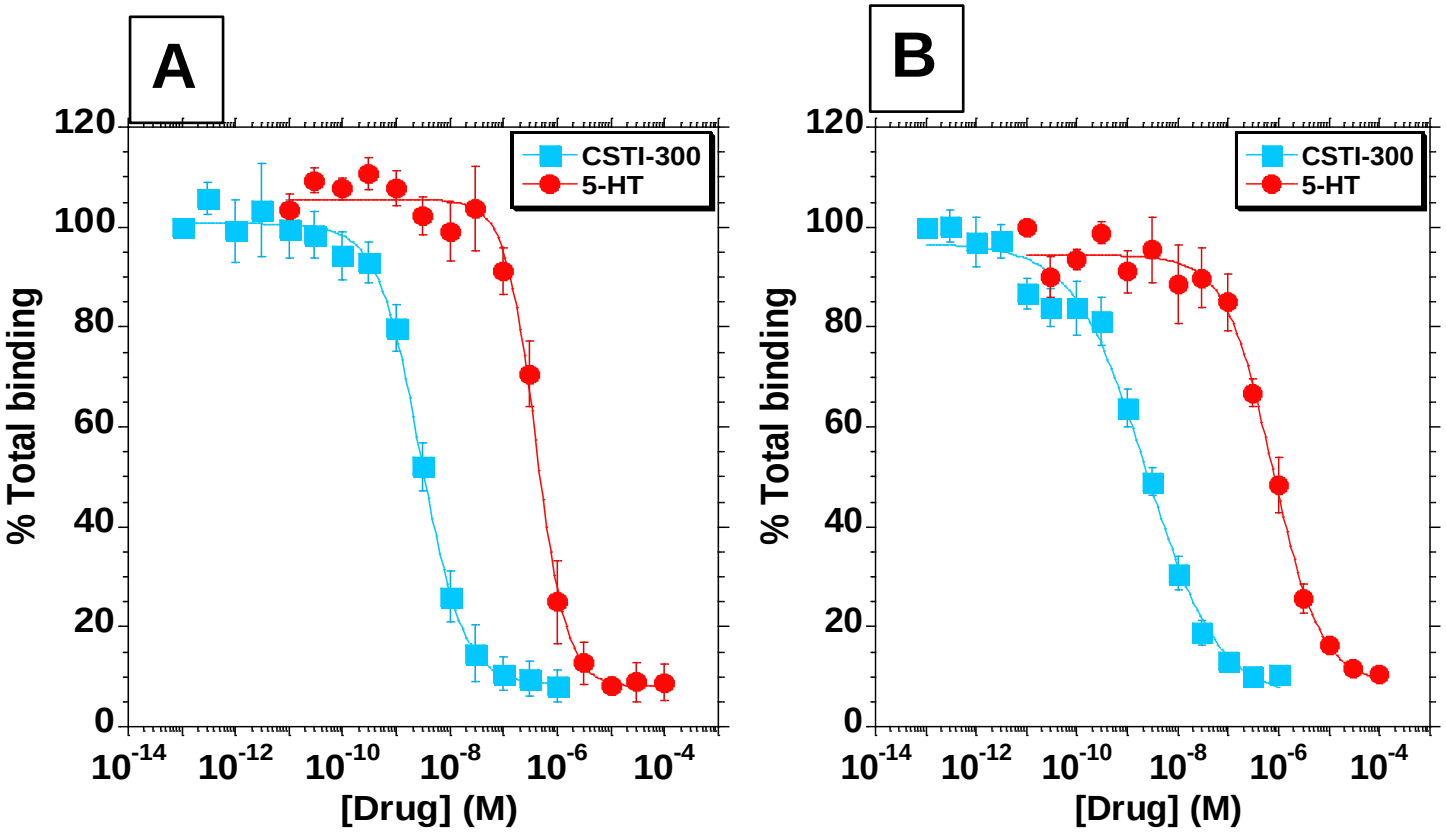

Figure 13. CSTI-300 displayed high-affinity binding for the h5-HT$_3$ receptor.

Ability of CSTI-300 or 5-HT to compete for [^{3}H]-granisetron binding to h5-HT$_3$A (A) or h5-HT$_3$AB (B) receptors stably expressed in HEK293 cells. Data represents mean±SEM, n=5.

It has been demonstrated previously that Cl-indole does not compete with [^{3}H]-granisetron for the h5-HT$_3$A receptor. However, 5-HT will induce Cl-indole to competitively bind to the h5-HT$_3$A receptor (Powell et al., 2016). This phenomenon of 5-HT is also shared with CSTI-300, and so is able reveal the ability of Cl-indole to compete for [^{3}H]-granisetron binding to the h5-HT$_3$A receptor (Figure 14; Table 8). The degree to which the affinity of Cl-indole for the h5-HT$_3$A receptor is increased by CSTI-300 is concentration-dependent. The impact of CSTI-300 (3.0 or 5.0 nM) or 5-HT (200 nM) upon Cl-indole affinity for the h5-HT$_3$A receptor was statistically significant ($p<0.05$-0.01, Kruskal-Wallis test with Dunn's multiple comparisons test).

Table 8: Summary data from Figure 14. CSTI-300 and 5-HT (200 nM) increased the affinity of Cl-indole for the h5-HT$_3$A receptor.

Data represents mean±SEM, n=5. *p<0.05, **p<0.01, Kruskal-Wallis test with Dunn's multiple comparisons test.

Ligand	Cl-indole IC_{50} (μM)
Cl-indole alone	500±182
Cl-indole + 5-HT (200 nM)	25±6*
Cl-indole + CSTI-300 (1.0 nM)	63±14
Cl-indole + CSTI-300 (3.0 nM)	30±4*
Cl-indole + CSTI-300 (5.0 nM)	24±5**

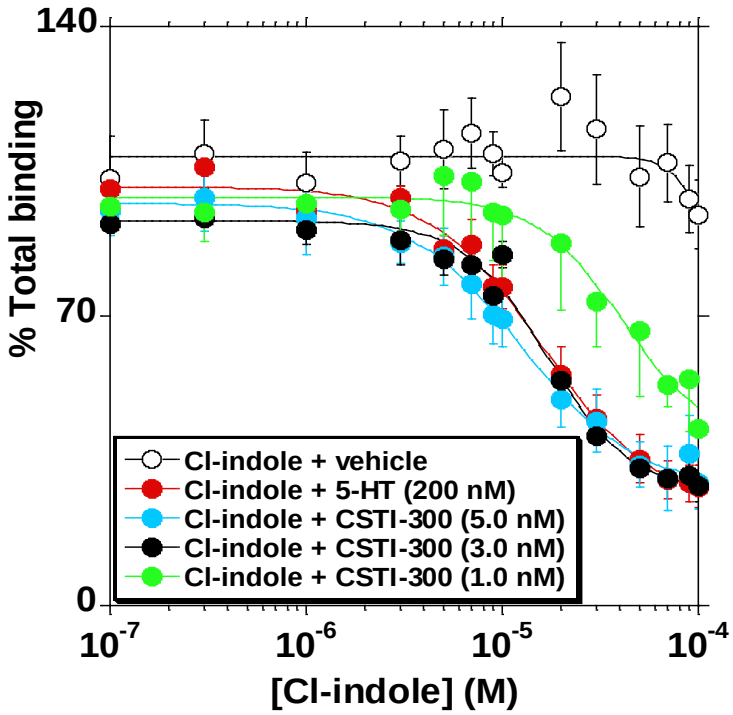

Figure 14. CSTI-300 and 5-HT alter the binding mechanism of Cl-indole at the h5-HT$_3$A receptor.

Both CSTI-300 and 5-HT (200 nM) allowed Cl-indole to compete with [^{3}H]-granisetron for the h5-HT$_3$A receptor. Data presents mean±SEM, n=5.

3.2.2 CSTI-300 in saturation radioligand binding studies

In order to demonstrate that at the h5-HT_3A and h5-HT_3AB receptors, CSTI-300 is a competitive ligand, saturation binding using [^{3}H]-granisetron was undertaken (Figure 15; Table 9). At the h5-HT_3A and h5-HT_3AB receptors, presence of CSTI-300 (3.0 nM and 2.0 nM respectively) reduced the affinity of [^{3}H]-granisetron ($p<0.05$ using Mann-Whitney U test for both receptor isoforms), without impacting the density of labelled receptors, indicating a competitive interaction (Figure 15; Table 9).

Table 9: Summary data from Figure 15. CSTI-300 displayed a competitive interaction with the h5-HT_3 receptor.

The presence of CSTI-300 decreased the affinity (K_d) of [^{3}H]-granisetron for h5-HT_3A and h5-HT_3AB receptors ($p<0.05$, Mann-Whitney U test), but did not impact upon the density of labelled receptors (B_{max}). Data represents mean±SEM, n=5.

5-HT_3A receptor	**K_d (nM)**	**B_{max} (fmol/mg)**	**Hill Number**
Vehicle	0.38±0.05	3414±582	1.16±0.10
With CSTI-300	1.42±0.19	3264±552	1.52±0.48
5-HT_3AB receptor	**K_d (nM)**	**B_{max} (fmol/mg)**	**Hill Number**
Vehicle	0.95±0.21	769±83	0.66±0.06
With CSTI-300	3.40±0.84	845±159	0.83±0.06

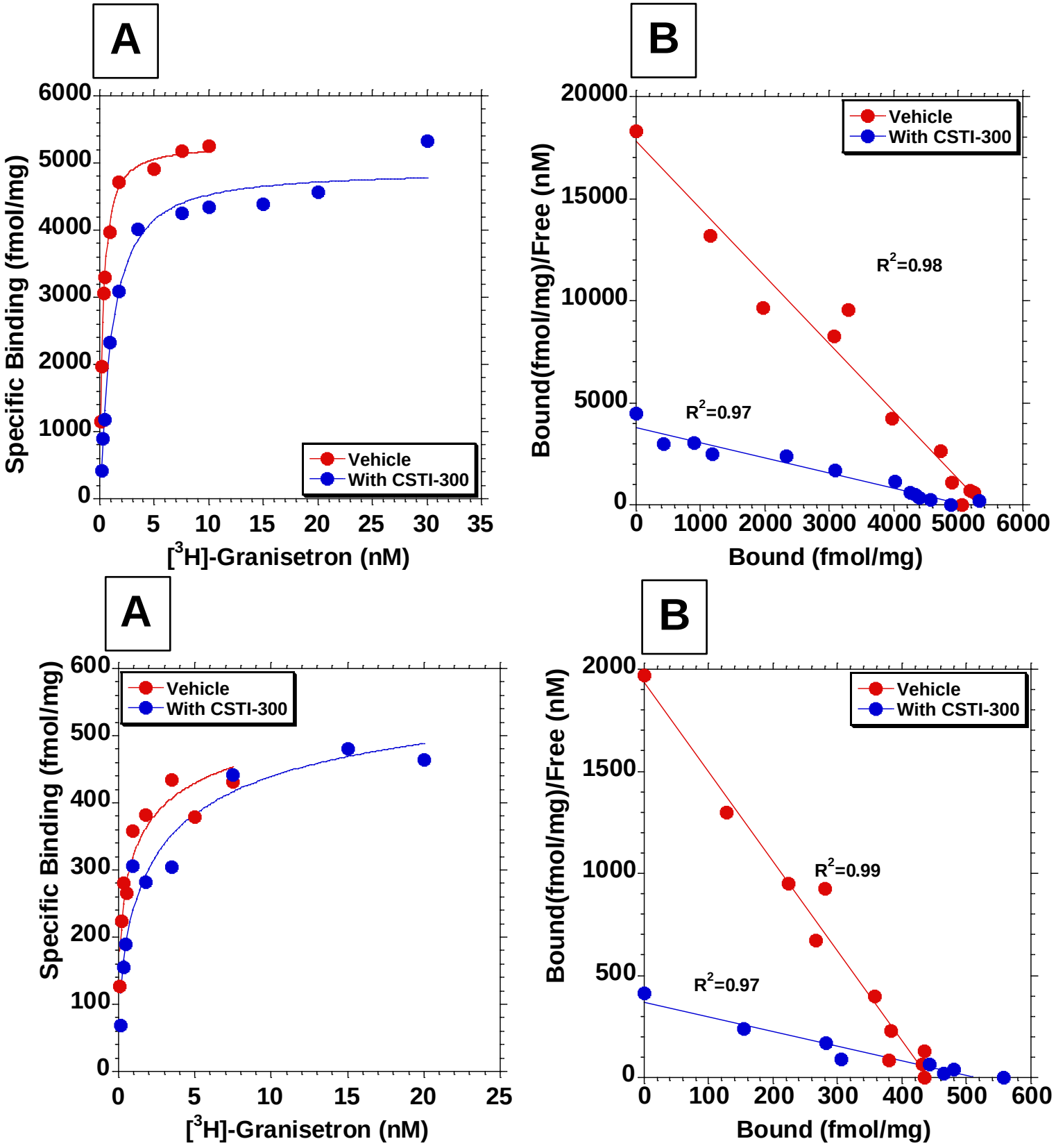

Figure 15. CSTI-300 is a competitive h5-HT$_3$ receptor partial agonist.

The impact of CSTI-300 upon the affinity of [^{3}H]-granisetron for h5-HT$_3$A (3.0 nM, upper panels) and h5-HT$_3$AB (2.0 nM, lower panels) receptors. A: Saturation curve. B: Scatchard transformation of the data. Representative of five repeats.

3.2.3 CSTI-300 in functional *in vitro* assays

Using functional intracellular calcium assays, both 5-HT and CSTI-300 displayed activity as agonists at the h5-HT_3A and h5-HT_3AB receptors (Figures 16 and 17; Table 10). CSTI-300 was approximately 16x more potent at activating the h5-HT_3A receptor compared to 5-HT, and had an intrinsic efficacy of around 40% relative to 5-HT. At the h5-HT_3AB receptor, CSTI-300 displayed intrinsic activity of approximately 30%, and was around 40x more potent than 5-HT. Although the potency of 5-HT was similar at both receptor isoforms, the potency of CSTI-300 was marginally higher at the h5-HT_3A receptor compared to the h5-HT_3AB receptor. The Hill slope for CSTI-300 was lower than unity for both the h5-HT_3A and h5-HT_3AB receptors, whereas for 5-HT the Hill slope was greater than unity at both receptor isoforms. The calcium response induced by CSTI-300 at the h5-HT_3A and h5-HT_3AB receptors was also slower to reach a peak and returned to baseline slower compared to 5-HT. Maximal responses elicited by 5-HT and CSTI-300 at the h5-HT_3A and h5-HT_3AB receptors were blocked by preincubation with the selective 5-HT_3 receptor antagonist granisetron (500 nM).

Table 10: Summary data from Figures 16 and 17. CSTI-300 is a potent h5-HT_3 receptor agonist.

Efficacy (E_{max}) and potency (EC_{50}) of CSTI-300 or 5-HT at the h5-HT_3A and h5-HT_3AB receptors, where efficacy is presented as percentage 5-HT response. Data represents mean±SEM, n=5.

5-HT_3A receptor	**E_{max}**	**EC_{50} (nM)**	**Hill number**
CSTI-300	41±7	25±5	0.85±0.14
5-HT	104±1	400±17	3.28±0.46
5-HT_3AB receptor	**E_{max}**	**EC_{50} (nM)**	**Hill number**
CSTI-300	30±4	12±2	0.83±0.17
5-HT	101±1	525±71	2.30±0.17

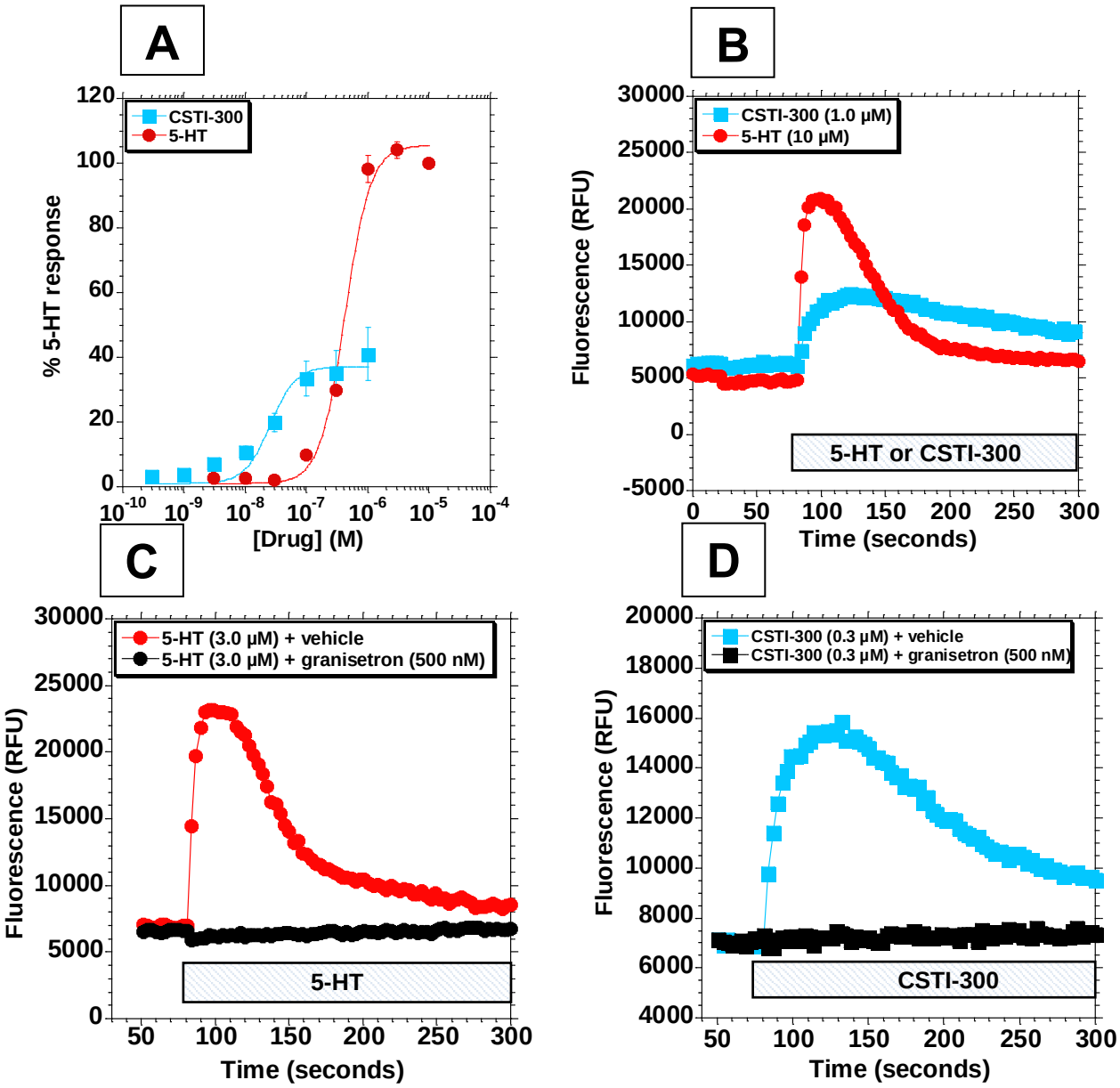

Figure 16. Both CSTI-300 or 5-HT displayed agonist activity at the h5-HT$_3$A receptor, assessed by an increase in [Ca^{2+}]$_i$.

A: Concentration-dependent activity of 5-HT and CSTI-300 (mean±SEM, n=5) B: Representative traces displaying change in calcium (fluorescence) over time of 5-HT (10 µM) or CSTI-300 (1.0 µM). The responses elicited by 5-HT (3.0 µM, C) or CSTI-300 (0.3 µM, D) at the h5-HT$_3$A receptor were blocked with granisetron (500 nM, 30 minutes preincubation).

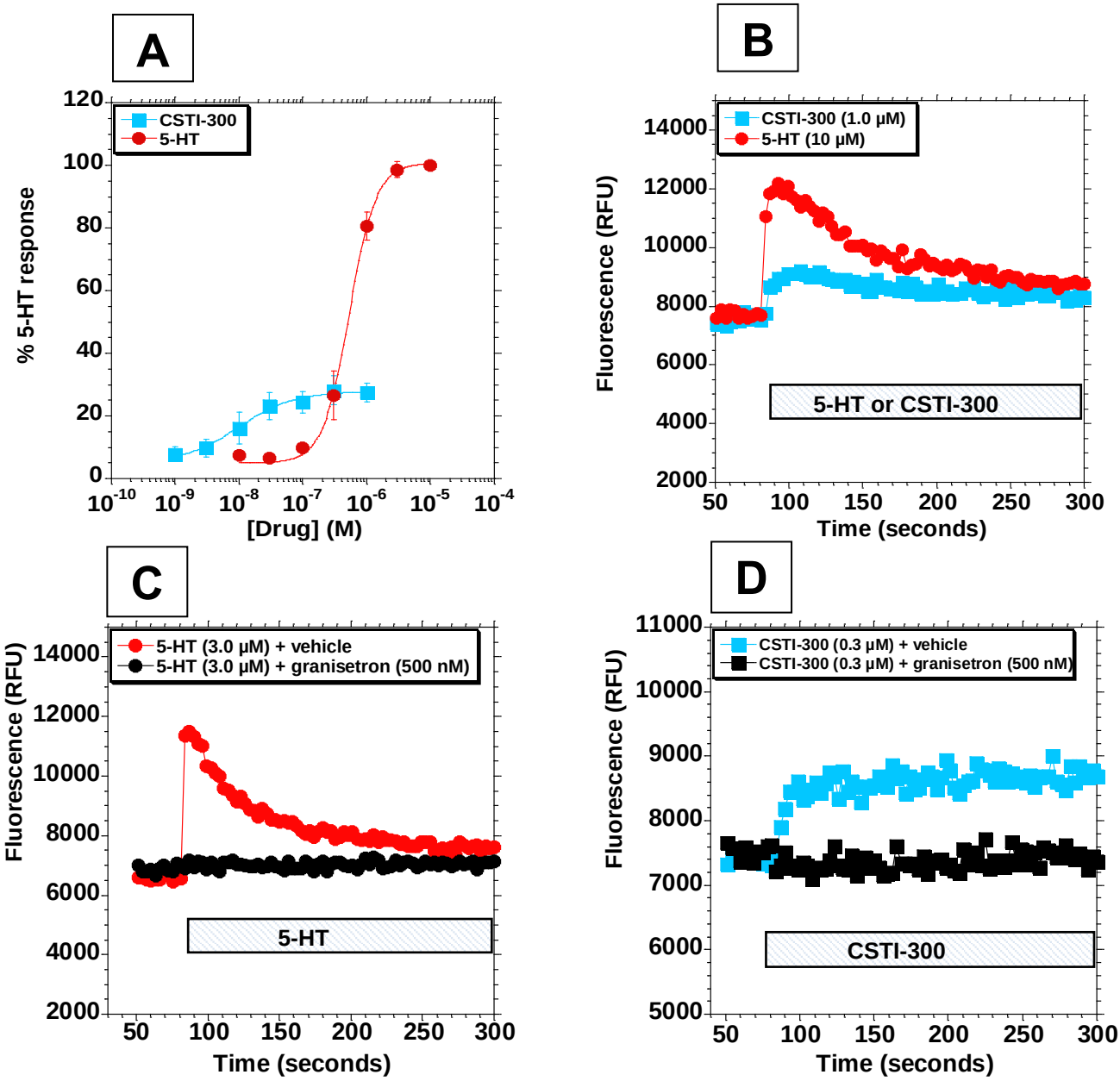

Figure 17. Both CSTI-300 or 5-HT displayed agonist activity at the h5-HT$_3$AB receptor, assessed by an increase in [Ca^{2+}]$_i$.

A: Concentration-dependent activity of 5-HT and CSTI-300 (mean±SEM, n=5) B: Representative traces displaying change in calcium (fluorescence) over time of 5-HT (10 µM) or CSTI-300 (1.0 µM). The responses elicited by 5-HT (3.0 µM, C) or CSTI-300 (0.3 µM, D) at the h5-HT$_3$AB receptor were blocked with granisetron (500 nM, 30 minutes preincubation).

3.2.4 CSTI-300 as a h5-HT$_3$A receptor functional antagonist

In the presence of a maximal 5-HT concentration (1.0 µM), a saturating concentration of CSTI-300 (10 µM) decreased the level of h5-HT$_3$A receptor activity down to 40% (compared to 100% for 5-HT alone). This suggests CSTI-300 could reduce the activity of the h5-HT$_3$A receptor in the presence of endogenous agonist 5-HT, but still retain some receptor activity (Figure 18). This is not seen when 5-HT is co-applied with alosetron (10 µM), which blocked the 5-HT response completely.

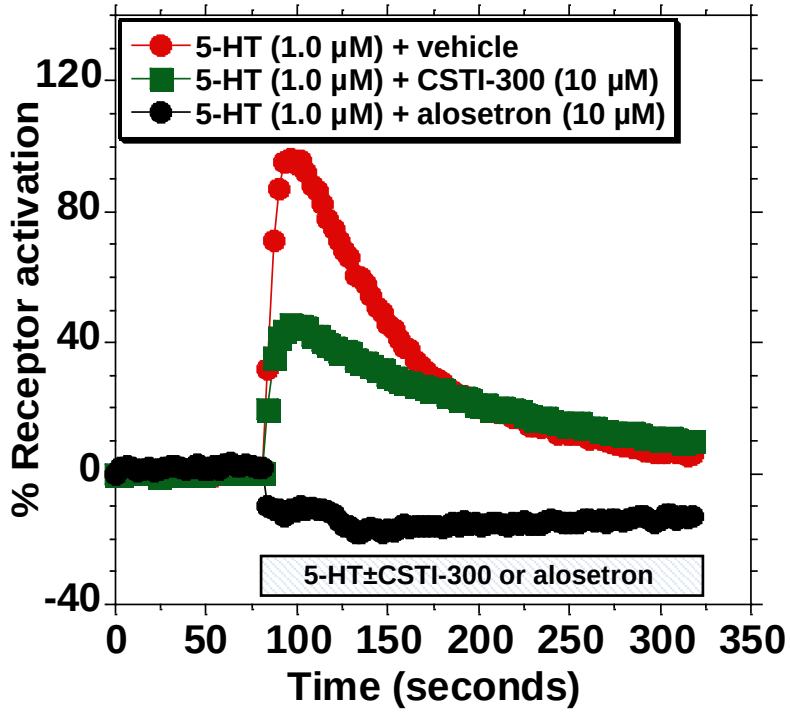

Figure 18. CSTI-300 inhibited 5-HT mediated responses through the h5-HT$_3$A receptor.

The selective 5-HT$_3$ receptor antagonist alosetron (10 µM) prevented a 5-HT (1.0 µM) induced response at the h5-HT$_3$A receptor when applied simultaneously. When 5-HT and CSTI-300 (10 µM) are co-applied there was still h5-HT$_3$A receptor activity. Mean traces of n=8 independent experiments presented.

3.2.5 The kinetics of CSTI-300 at the h5-HT$_3$A receptor

Single cell electrophysiology was used to further examine the kinetics of CSTI-300 (and 5-HT) activation of the h5-HT$_3$A receptor. At a holding potential of -60 mV, a maximal 5-HT concentration (10 µM; 60 second application) elicited an inward current of 1,256±231 pA (n=5; Figure 19). The time constant of the rising phase was 0.28±0.07 seconds (n=5), which decayed slowly back to baseline. A two-exponential curve fit was best used to model the decay, with time constants of 57.6±26.5 ms and 245.8±50.3 ms (n=5). In contrast, a maximal concentration of CSTI-300 (100 nM; 60 second application) mediated a smaller inward current (248±74 pA; n=9; Figure 17), approximately 20% that of 5-HT at the h5-HT$_3$A receptor. In addition, the rise time was slower (3.64±0.29 s; n=9) as was the decay time (one-exponential fit; 260.9±31.9 ms; n = 9) when compared to 5-HT.

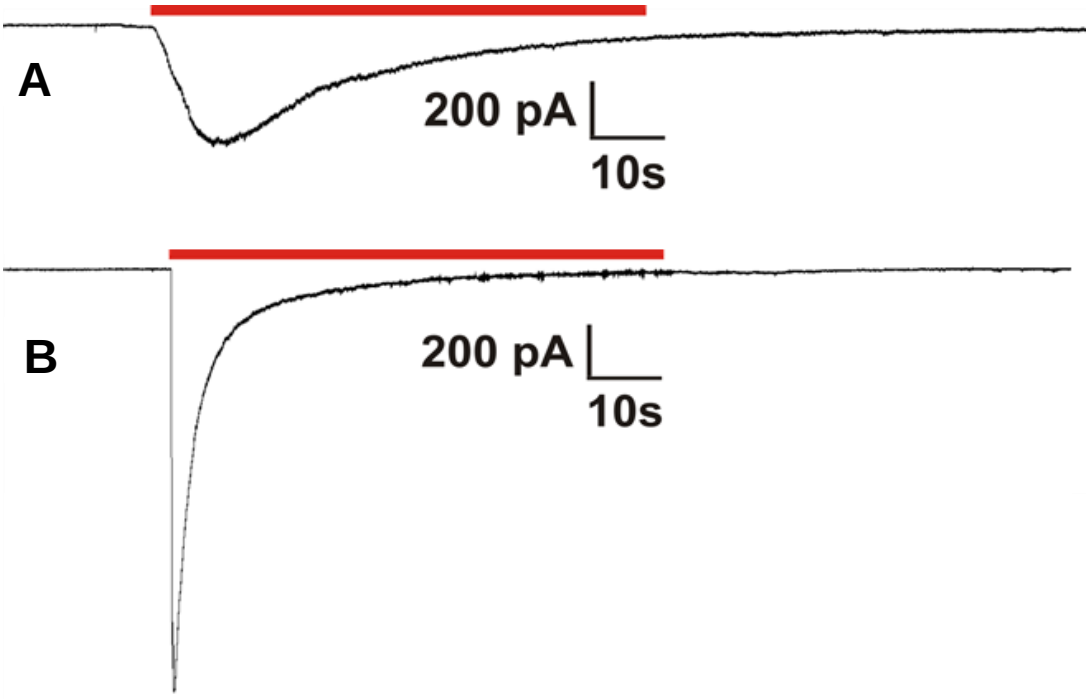

Figure 19. The kinetics of a CSTI-300 or 5-HT induced current at the h5-HT$_3$A receptor.

An inward current in h5-HT$_3$A receptor expressing HEK293 cells was elicited by a maximal concentration of CSTI-300 (100 nM, A) or 5-HT (10 µM, B). Red bar indicates 60 second application of drug using a Picospritzer. Data are representative of 5 (5-HT) or 9 (CSTI-300) separate experiments. Work performed in collaboration with Dr Gillian Grafton.

3.2.6 The activity of CSTI-300 activity in rat Bezold-Jarisch reflex

The efficacy of 5-HT_3 receptor antagonists has historically been evaluated using the Bezold-Jarisch reflex in rat. Briefly, intravenous administration of 5-HT will induce transient bradycardia through stimulation of vagal afferent 5-HT_3 receptors (Cote et al., 2004). Prior oral administration of a 5-HT_3 receptor antagonist will prevent this reflex (Fozard, 1984; Cohen et al., 1989; Eglen et al., 1995). Transient bradycardia was evoked by intravenous administration of 5-HT (0.3 mg/kg) in rats (Bezold-Jarisch reflex; basal heart rate 422±15 bpm; maximum decrease of 66±3%; mean±SEM, n=6; $p<0.01$). Prior oral treatment with alosetron (0.1 mg/kg) or CSTI-300 (0.1-3.0 mg/kg) prevented this reflex in a dose-dependent manner ($p<0.01$; Figure 18). The effect of CSTI-300 (1.0 mg/kg; representing an approximate ED_{90} dose) to prevent the Bezold-Jarisch reflex induced by 5-HT was evident for at least 4 hrs following a single oral administration ($p<0.05$-0.01; Figure 18). At various time points during the study, plasma concentration of CSTI-300 were determined to be 16.6±4.5 nM (1 h), 9.1±0.9 nM (2 h), 9.1±0.8 nM (4 h), and 3.3±0.2 nM (8 h) (mean±SEM, n=6), supporting a correlation of the level of inhibition of the Bezold-Jarisch reflex achieved with the CSTI-300 plasma concentrations when considering the 5-HT_3 receptor K_i (approximately 2.0 nM) and the rat plasma protein binding (17±5%; mean±SEM, n=3) for CSTI-300. For example, the estimated free concentration of CSTI-300 in the plasma at 4 h and 8 h was 7.6 nM and 2.7 nM, respectively; concentrations that are on the concentration-responsive portion of the competition curve generating the K_i value (Figure 13).

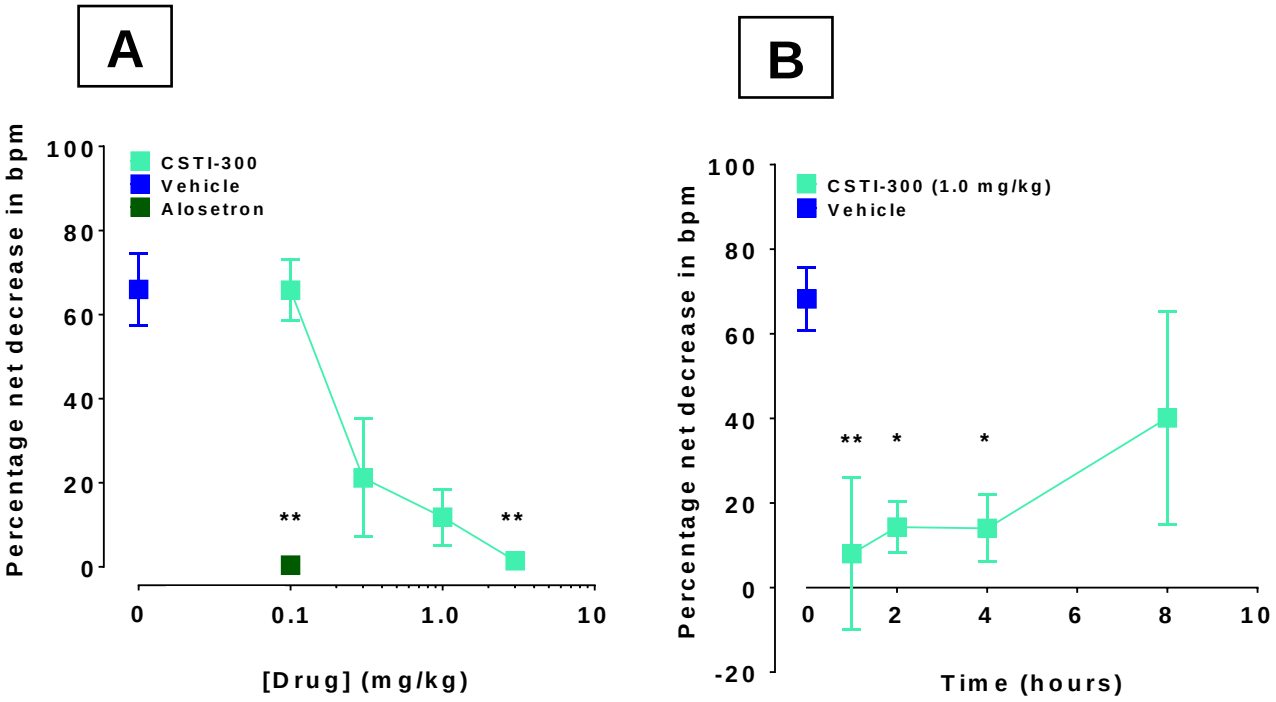

Figure 20. CSTI-300 and alosetron both block the 5-HT-induced Bezold–Jarisch reflex in rat *in vivo*.

A: There was a dose-dependent inhibition by CSTI-300 (p.o.) upon the 5-HT-induced Bezold-Jarisch reflex. The efficacy of CSTI-300 was similar to alosetron (0.1 mg/kg p.o.). The ability of alosetron (0.1 mg/kg) and CSTI-300 (3.0 mg/kg) to prevent the decrease in 5-HT-induced heart rate compared to vehicle was statistically significant (**p<0.01; Kruskal-Wallis test with Dunn's multiple comparisons). B: Even 4 hours after CSTI-300 administration (1.0 mg/kg p.o., estimated ED_{90} dose), the 5-HT-induced Bezold-Jarisch reflex was still being prevented. 5-HT (0.3 mg/kg i.v.) was used to evoke the bradycardia associated with the Bezold-Jarisch reflex. Data represent mean±SEM, n=6 rats. The effect of CSTI-300 (1.0 mg/kg) upon heart rate at 1, 2 and 4 hours after dosing was also statistically significant (**p<0.01, *p<0.05; Kruskal-Wallis test with Dunn's multiple comparisons) compared to vehicle. Work outsourced to Olan Ricerca Biosciences (http://www.ricerca.com/), data analysed and plotted by myself.

3.2.7 The activity of CSTI-300 in an *in vivo* rodent IBS-d model

A major symptom of IBS-d is increased visceral sensation and perception (Lacy et al., 2016). To assess the potential of CSTI-300 for treating IBS-d, it was first evaluated in a rodent *in vivo* IBS-d model based on previous studies (Banner et al., 1995; Banner et al., 1995). CSTI-300 was examined alongside the selective $5\text{-}HT_3$ receptor

antagonist alosetron (which is also currently marketed as a therapeutic for IBS-d). Sub-cutaneous application of 5-hydroxytryptophan (5-HTP; 10 mg/kg) markedly increased the rat's sensitivity to abdominal contractions (Figure 21). Both alosetron and CSTI-300 reversed this increase in colonic sensitivity, with CSTI-300 showing similar efficacy to alosetron (Figure 19). The reduction is sensitivity elicited by alosetron or CSTI-300 was statistically significant ($p<0.01$ to $p<0.0001$, using 2-way ANOVA with Bonferroni adjustment; Supplementary Tables 1, 2 and 3 in Appendix).

5-HT_3 receptor stimulation induces emesis, and this occurred previously when a 5-HT_3 receptor partial agonist (DDP, MKC-733, pumosetrag) was given to IBS-c patients or healthy adults (Coleman et al., 2003; Fujita et al., 2005). A maximal oral dose of CSTI-300 (1.0 mg/kg, p.o.) did not induce emesis in dogs (Table 11). The concentration of plasma CSTI-300 six hours after dosing would be saturating the 5-HT_3 receptor, implying if CSTI-300 did causes emesis it would have done. Moreover, CSTI-300 did not alter the faeces or the behaviour of the dogs.

Table 11: An oral dose of CSTI-300 (1.0 mg/kg) did not alter the faeces of dogs, nor did it induce emesis or affect the behaviour of the dogs.

The free plasma concentration taken six hours after oral dosing was between 10 – 70x the K_i for the h5-HT_3A receptor (dog plasma protein binding 30±3%). Data from six dogs. Work outsourced to Medicilon (http://www.medicilon.com/).

Treatment	**Emetic episodes (either retching or vomiting)**	**State of faeces**	**General observations**	**Plasma [CSTI-300] 6 hrs post dose Mean±SD (range)**
CSTI-300 (1.0 mg/kg p.o.)	No emetic episodes for any of the six dogs.	Normal for all six dogs [see methods for definition].	No abnormal observations [see methods for definition].	105±87 nM (31-227 nM)

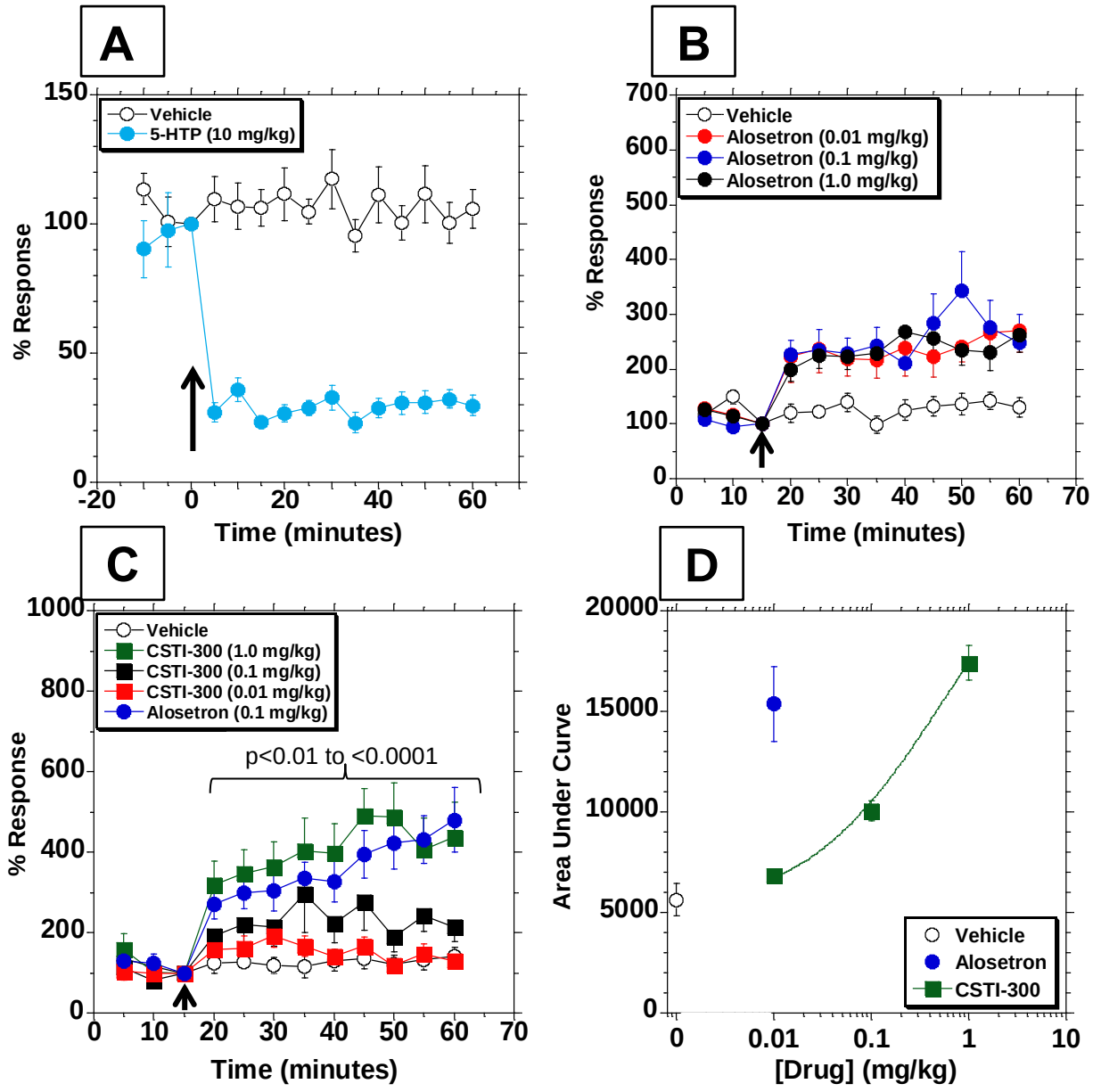

Figure 21. CSTI-300 and alosetron displayed similar efficacy in a rodent *in vivo* model of IBS-d, measured by a reduction in colonic sensitivity.

A: The precursor to 5-HT, 5-HTP (10 mg/kg, s.c. arrow indicates administration), increased the sensitivity of rats to colon distension. Alosetron reserved this increase in colonic sensitivity (B & C, arrow indicates administration of vehicle or alosetron where appropriate) and so did CSTI-300 (C, arrow indicates administration of vehicle, CSTI-300 or alosetron where appropriate). D: Dose-response curve for CSTI-300, generated by calculating area under the curve from graph C, yielded an ED_{50} of 0.55 mg/kg. Data plotted as mean±SEM, n=10 rats. The impact of alosetron or CSTI-300 was statistically significant ($p<0.01$ to <0.0001). Work outsourced to Medicilon (http://www.medicilon.com/), data analysed and plotted by myself.

3.2.8 The pharmacokinetics of CSTI-300

The pharmacokinetics of CSTI-300 has been evaluated in dog, rat, mouse and mini-pig (Figures 22, 23, 24 and 25; Table 12). The $t_{1/2}$ for CSTI-300 was comparable between rat, mouse and dog, with the half-life being faster when CSTI-300 was given as an intravenous injection (at 1.0 mg/kg) compared to oral administration. The half-life of CSTI-300 was longer in minipig when administered intravenously compared to the other species (rat, mouse and dog). Following intravenous administration of CSTI-300 (1.0 mg/kg), the $t_{1/2}$ was comparable between rat (1.6±0.2 hrs; n=3), dog (1.6±0.2 hrs; n=4) and mini-pig (2.4±1.3 hrs, n=3), with the mouse displaying faster pharmacokinetics (0.4±0.06 hrs; n=3). The $t_{1/2}$ for CSTI-300 was slower following oral administration; mouse (10 mg/kg), rat (1.0 and 5.0 mg/kg), dog (10 and 30 mg/kg) and mini-pig (10 and 30 mg/kg), with values: 2.4± 0.4, 2.3±0.7 and 4.4±2.7, 3.7±0.8 and 3.4±0.6, and 3.0±1.2 and 3.0±0.4 hrs, respectively. The volume of distribution values was moderate in the species evaluated (3.0-14.6 L/kg). The clearance values for all species were also moderate, with an inverse relationship between clearance value and species size. Thus, lower clearance values were apparent in the larger species, dog and mini-pig (1.3 and 2.2 L/h/kg, respectively), compared to rodent (mouse and rat; 8.2 and 6.2 L/h/kg, respectively). The oral bioavailability was good in mice and rat (24-41 %F) and excellent in dog and mini-pig (60-100 %F). The metabolites of CSTI-300 are not available in the public domain, but no issues have been reported. A supra-proportional exposure (AUC) of CSTI-300 was apparent with increasing doses of drug within the species examined. After oral administration, the C_{max} and AUC are increased for matched or similar doses in the larger animals (dog and mini-pig) versus the rodent species (mouse and rat).

Table 12: Summary data from Figures 22, 23, 24 and 25. The pharmacokinetics of CSTI-300 in a variety of animals.

Data represented as Mean±SD. [a]Area under the plasma concentration versus time curve from 0 to the last time point CSTI-300 was quantifiable in plasma. [b]Time of maximum observed concentration of CSTI-300 in plasma. [c]Maximum observed concentration of compound in plasma. [d]Apparent half-life of the terminal phase of elimination of CSTI-100 from plasma. [e]Total body clearance of CSTI-300. [f]Volume of distribution. [g]Oral bioavailability; F = (AUC_{last} po x $Dose_{iv}$)/ (AUC_{last} iv x $dose_{po}$).

Species (n)	**Dose (mg/kg)**	**AUC_{last} [a] (h*ng/ml)**	**T_{max}[b] (hours)**	**C_{max}[c] (ng/ml)**	**$T_{1/2}$[d] (hours)**	**CL[e] (L/h/kg)**	**Vd[f] (L/kg)**	**%F[g]**
Mouse (3)	1.0, iv	105±5	0.083±0	237±16	0.4±0.1	8.2	12.8	
Mouse (3)	10, po	435±4	0.5±0	172±36	2.4±0.4			41
Rat (3)	1.0, iv	163±27	0.083±0	235±28	1.6±0.2	6.2±1.0	14.6±4.3	
Rat (3)	1.0, po	39.8±3.0	0.8±0.1	15±10	2.3±0.7			24
Rat (3)	5.0, po	331±102	0.8±1.1	138±68	4.4±2.7			41
Dog (4)	1.0, iv	764±135	0.083±0	497±48	1.6±0.2	1.3±0.2	3.0±0.3	
Dog (3)	0.3, po	205±105	0.5±0	86.1±34.4	1.6±0.18			89
Dog (3)	1.0, po	1010±712	0.5±0	364±157	2.2±1.1			100
Dog (4)	10, po	12098±1076	0.4±0.1	3498±725	3.7±0.8			100
Dog (4)	30, po	39115±13049	0.25±0	9223±1464	3.4±0.6			100
Mini-pig (3)	1.0, iv	444±39	0.083±0	510±109	2.4±1.3	2.2±0.2	7.7±4.9	
Mini-pig (3)	10, po	2669±328	2.67±1.2	568±203	3.0±1.2			60
Mini-pig (3)	30, po	9180±948	2.0±0	2424±516	3.0±0.4			69

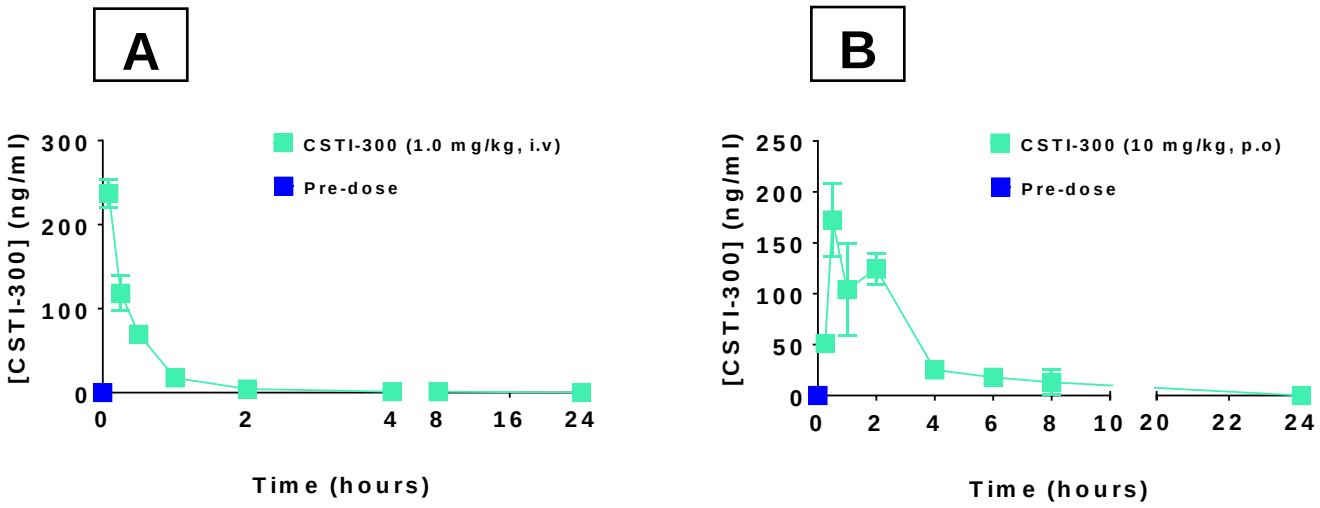

Figure 22. The pharmacokinetics of CSTI-300 in mouse.

After administration of CSTI-300 either intravenously (A) or orally (B), CSTI-300 was metabolised with a half-life of approximately 2.4 hours for oral administration or 0.4 hours for intravenous administration. Data represents mean±SD, from 3 mice. Work outsourced to NoAb BioDiscoveries, data analysed and plotted by myself.

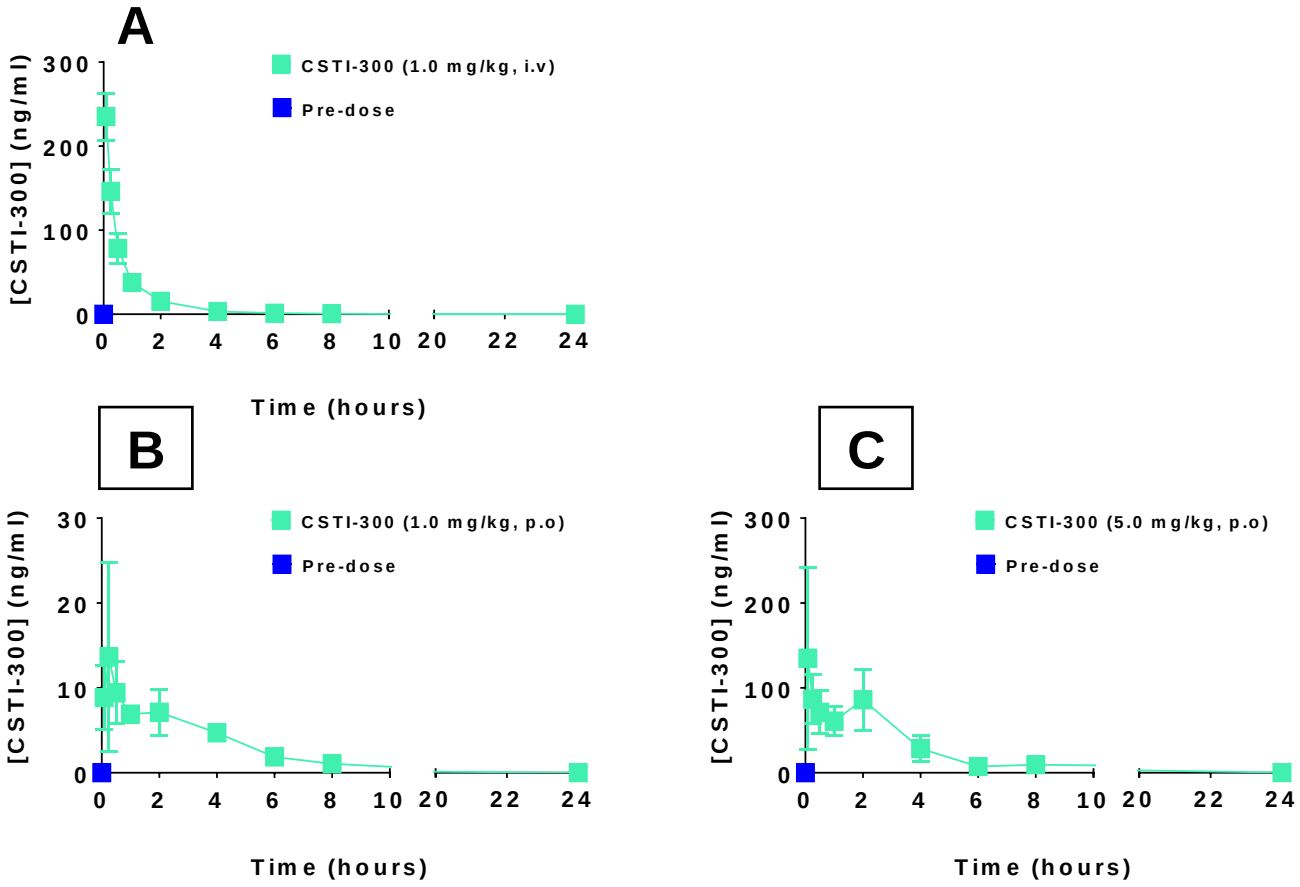

Figure 23. The pharmacokinetics of CSTI-300 in rat.

After administration of CSTI-300 either intravenously (A) or orally (B; C), CSTI-300 was metabolised with a half-life of approximately 2 hours for 1.0 mg/kg or 4 hours for 5.0 mg/kg. A half-life of 1.6 hours was recorded for intravenous administration. Data represents mean±SD, from 3 rats. Work outsourced to NoAb BioDiscoveries, data analysed and plotted by myself.

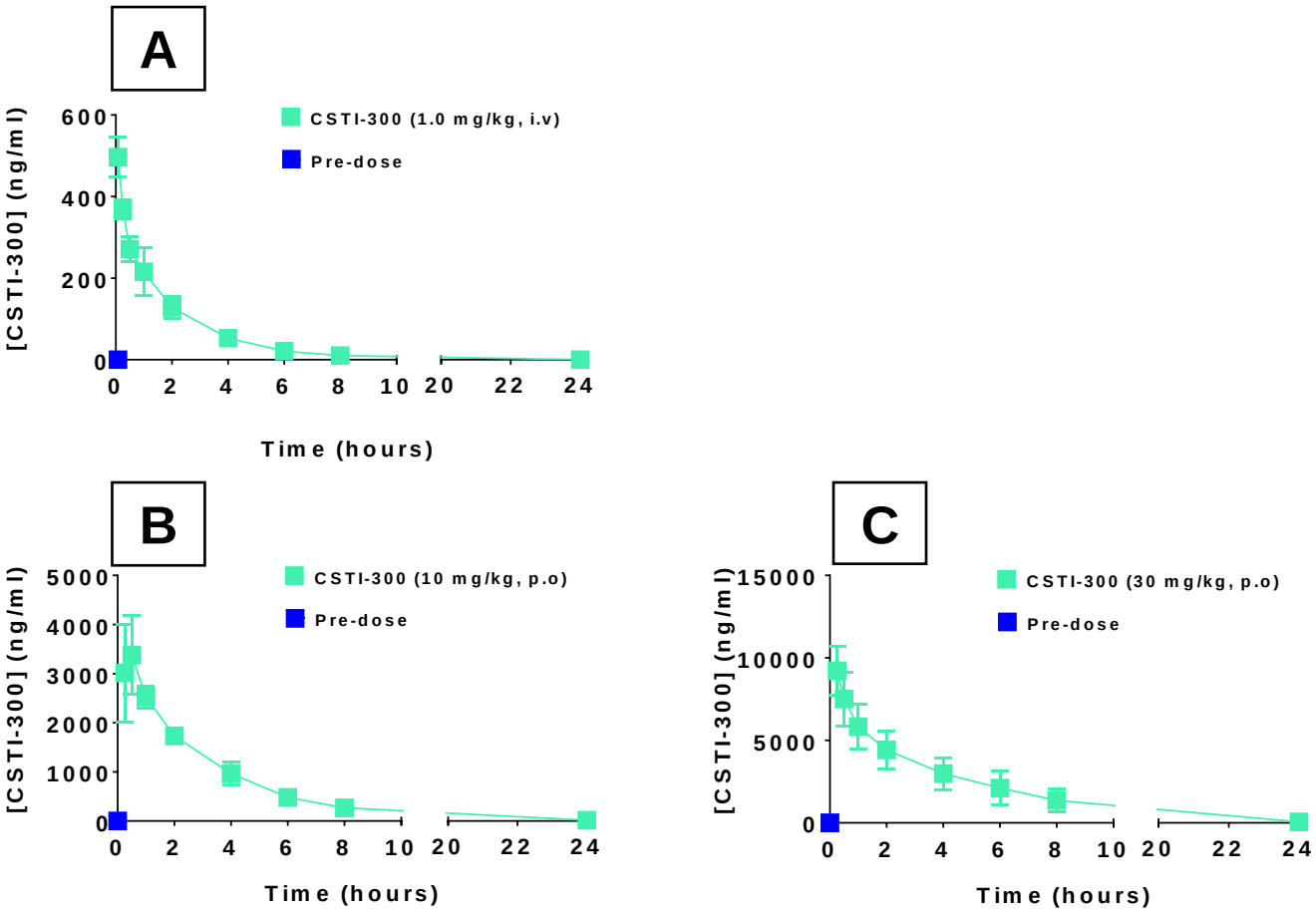

Figure 24. The pharmacokinetics of CSTI-300 in dog.

After administration of CSTI-300 either intravenously (A) or orally (B; C), CSTI-300 was metabolised with a half-life of approximately 4 hours for oral administration, and 1.6 hours for intravenous administration. Data represents mean±SD, from 4 dogs. Work outsourced to Covance (https://www.covance.com/), data analysed and plotted by myself.

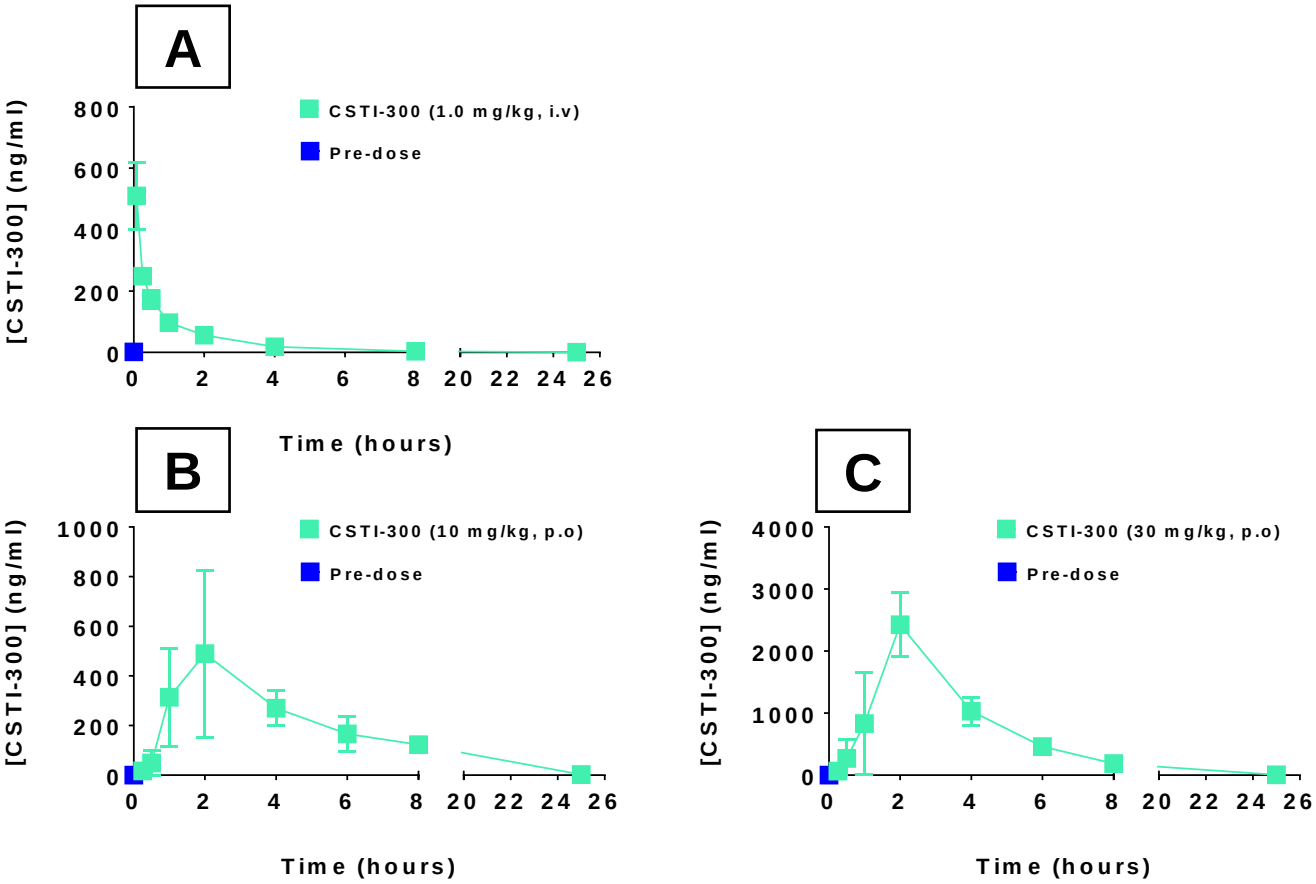

Figure 25. The pharmacokinetics of CSTI-300 in mini-pig.

After administration of CSTI-300 either intravenously (A) or orally (B; C), CSTI-300 was metabolised with a half-life of approximately 3 hours for oral administration or 2.4 hours for intravenous administration. Data represents mean±SD, from 3 mini-pigs. Worked outsourced to by Calvert labs (http://calvertlabs.com/), data analysed and plotted by myself.

CSTI-300 also has desirable therapeutic characteristics to make it an ideal drug for market (Table 13). CSTI-300 demonstrated no significant efficacy at a range of other receptors, transporters or ion channels and does not interact with members of the cytochrome P_{450} enzyme (CYPs) family, suggesting a low chance of drug-drug interactions. CSTI-300 was also well tolerated in a variety of species (dog, rat and monkey) (Table 13).

Table 13: Summary of additional pharmacological and pharmaceutical characteristics of CSTI-300.

High affinity and selectivity for 5-HT_3 receptor	No significant (>50%) off-target activity (screened @ 1.0 µM) (See supplementary Table 4 in Appendix).
Low probability of drug/drug interactions	CYPs (1A2, 2B6, 2C9, 2C19, 2D6, 3A4) IC_{50} > 100 µM
Good oral PK in rodent, dog and monkey	F_{mice}= 42%, $t_{1/2}$ = 4.2 h F_{dog} = 100%, $t_{1/2}$ = 3.7 h F_{monkey} = 26%, $t_{1/2}$ = 4.9 h
Large margin of safety	NOAEL (no-observed-adverse-effect-level) = 300 mg/kg/day (7 day dosing in rat); ~300 x ED_{90} conc. Good tolerability in dog and monkey study; no emesis or loose stool at 1.0 mg/kg (~10 x ED_{90}) Non-mutagenic in micro-Ames Test
Excellent pharmaceutical/biophysical properties	Molecular Weight < 350 Daltons Log P = 1.95 Water soluble (>400 mg/mL)

3.3 The binding mechanism of Cl-indole at the 5-HT_3 receptor

3.3.1 5-HT alters the binding mechanism of Cl-indole but not PBG

Cl-indole is a relatively selective and potent modulator at the 5-HT_3 receptor. It has been shown previously to increase the efficacy and potency of 5-HT (plus other 5-HT_3 receptor agonists) at the h5-HT_3A receptor (Newman et al., 2013). Cl-indole also displayed non-competitive binding at the h5-HT_3A receptor (i.e. it did not displace [^{3}H]-granisetron from h5-HT_3A receptor binding sites). This has also been replicated (Figure 26), and it has been demonstrated that sub-maximal concentrations of 5-HT (up to 200 nM) induce Cl-indole to compete with [^{3}H]-granisetron for the h5-HT_3A receptor and increase the affinity of Cl-indole for the h5-HT_3A receptor (Figure 26; Table 14). The impact of 5-HT upon the affinity of Cl-indole for the h5-HT_3A receptor was statistically significant ($p<0.05$ to $p<0.001$, using Kruskal-Wallis test with Dunn's multiple comparisons). Binding of the weak 5-HT_3 receptor partial agonist phenyl biguanide (PBG) for the h5-HT_3A receptor is not impacted by the same concentrations of 5-HT.

Table 14: Summary data from Figure 26. **The affinity of Cl-indole is increased by 5-HT (compared to Cl-indole in the absence of 5-HT) at the h5-HT_3A receptor.**

However, the affinity of PBG for the h5-HT_3A receptor was not affected by 5-HT. *p<0.05, ***p<0.001 when compared to Cl-indole + vehicle, using Kruskal-Wallis with Dunn's multiple comparisons test. The data represents mean±SEM, n=3-8.

[5-HT] (nM)	**IC_{50} (μM)**	
	Cl-indole	**PBG**
0 (Vehicle)	179±20	15±2
10	77±12	14±1
30	49±12	14±1
100	27±6*	15±3
200	10±1***	10±2

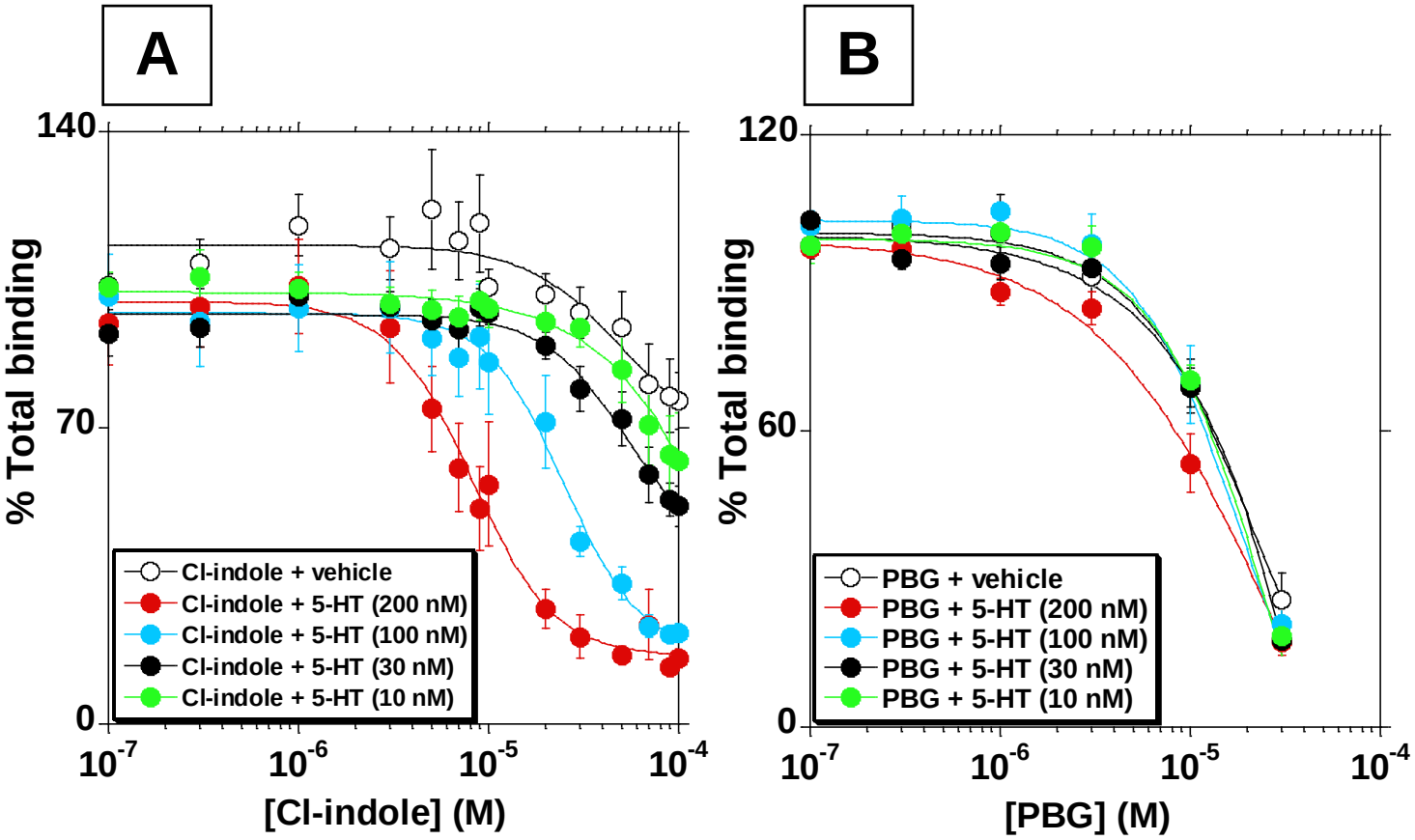

Figure 26. 5-HT impacted the binding of Cl-indole, not PBG, for the h5-HT$_3$A receptor.

The affinity of Cl-indole (A), but not the 5-HT$_3$ receptor partial agonist PBG (B) was increased by sub-maximal concentrations of 5-HT at the h5-HT$_3$A receptor. Data represents mean±SEM, n=3-8.

3.3.2 The affinity of Cl-indole for the h5-HT$_3$A receptor is modulated by a variety of 5-HT$_3$ receptor partial agonists.

In the presence of Cl-indole, the maximal response elicited by a 5-HT$_3$ receptor partial agonist is greater compared to the partial agonist alone (Newman et al., 2013). It was therefore evaluated whether 5-HT$_3$ receptor partial agonists (quipazine, DDP and s-zacopride) could have the same effect as 5-HT and increase the affinity of Cl-indole for the h5-HT$_3$A receptor (Figure 27; Table 15). Despite having lower intrinsic efficacies than 5-HT, all three partial agonists caused Cl-indole to compete out [^{3}H]-granisetron from h5-HT$_3$A receptor binding sites. To ensure a valid comparison, the concentrations of partial agonists used are approximately their K_i.

Table 15: Summary data from Figure 27. The affinity of Cl-indole was increased by 5-HT (200 nM) and a variety of 5-HT_3 receptor partial agonists at the h5-HT_3A receptor. Note: Cl-indole + vehicle and Cl-indole + 5-HT (200 nM) were included with each experiment for the different partial agonist, so have been included for each different ligand. The data represents mean±SEM, n=3-5.

Ligand	**Cl-indole IC_{50} (μM)**
Cl-indole + vehicle	187±45
Cl-indole + 5-HT (200 nM)	30±4
Cl-indole + quipazine (0.3 nM)	144±30
Cl-indole + quipazine (1.0 nM)	92±21
Cl-indole + quipazine (3.0 nM)	32±5
Ligand	**Cl-indole IC_{50} (μM)**
Cl-indole + vehicle	136±16
Cl-indole + 5-HT (200 nM)	29±3
Cl-indole + DDP (0.1 nM)	223±82
Cl-indole + DDP (0.3 nM)	150±30
Cl-indole + DDP (1.0 nM)	50±6
Ligand	**Cl-indole IC_{50} (μM)**
Cl-indole + vehicle	160±28
Cl-indole + 5-HT (200 nM)	52±13
Cl-indole + s-zacopride (1.0 nM)	99±14
Cl-indole + s-zacopride (2.0 nM)	47±6

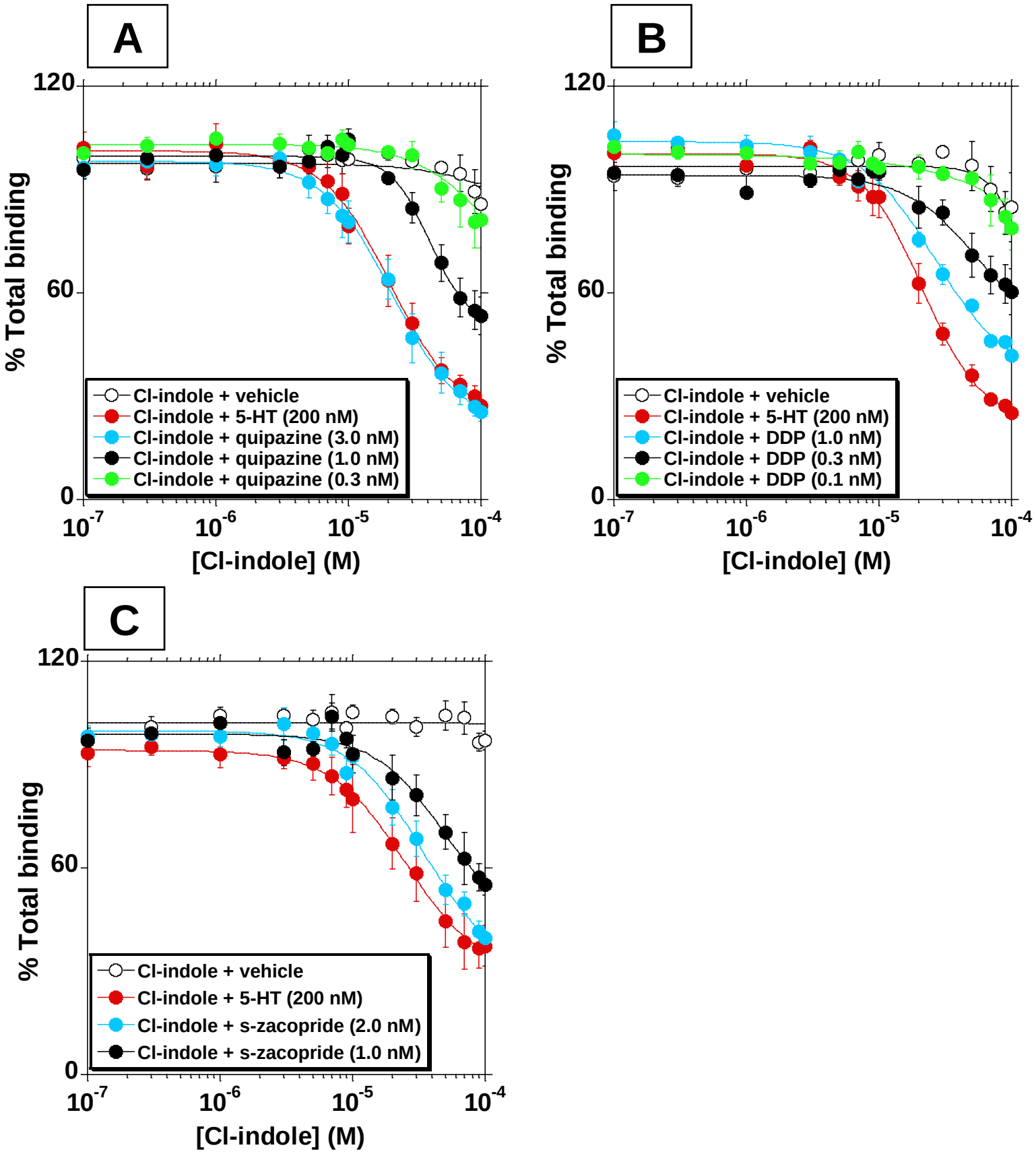

Figure 27. The impact of 5-HT upon Cl-indole binding for the h5-HT$_3$A receptor was replicated by other 5-HT$_3$ receptor agonists.

The affinity of Cl-indole was increased by 5-HT (200 nM), and by other 5-HT$_3$ receptor partial agonists, quipazine (A), DDP (B) and s-zacopride (C) at the h5-HT$_3$A receptor. Data represents mean±SEM, n=3-5.

The IC_{50} for Cl-indole at the h5-HT_3A receptor in the presence of quipazine (3.0 nM), DDP (1.0 nM) or s-zacopride (2.0 nM) is comparable to what was determined for Cl-indole + 5-HT (200 nM), demonstrating that the affinity of Cl-indole can be increased by a variety of 5-HT_3 receptor partial agonists with different intrinsic activities.

3.3.3 5-HT_3 receptor antagonists do not affect the binding of Cl-indole at the h5-HT_3A receptor

To determine whether any 5-HT_3 receptor ligand (i.e. intrinsic efficacy wasn't necessary) could increase the affinity for Cl-indole at the h5-HT_3A receptor, the impact of 5-HT_3 receptor antagonists (metoclopramide or ondansetron) upon Cl-indole binding for the h5-HT_3A receptor was investigated (Figure 28; Table 16). To allow a comparison with the 5-HT_3 receptor partial agonists (Figure 27; Table 15), concentrations approximate to the K_i of antagonists were used. Whilst the affinity of Cl-indole for the h5-HT_3A receptor was increased by 5-HT (200 nM), neither metoclopramide nor ondansetron altered the affinity of Cl-indole (Figure 28; Table 16). In the presence of 5-HT_3 receptor antagonist, Cl-indole did not compete with [^{3}H]-granisetron for the h5-HT_3A receptor binding sites.

Table 16: Summary data from Figure 28. The affinity of Cl-indole was increased by 5-HT (200 nM), but not by 5-HT_3 receptor antagonists at the h5-HT_3A receptor. Note: Cl-indole + vehicle and Cl-indole + 5-HT (200 nM) were included with each experiment for the different antagonist, so have been included for each different ligand. The data represents mean±SEM, n=3-4.

Ligand	**Cl-indole IC_{50} (μM)**
Cl-indole + vehicle	302±101
Cl-indole + 5-HT (200 nM)	30±7
Cl-indole + metoclopramide (30 nM)	311±38
Cl-indole + metoclopramide (100 nM)	364±75
Cl-indole + metoclopramide (300 nM)	361±75
Cl-indole + metoclopramide (1000 nM)	167±7
Ligand	**Cl-indole IC_{50} (μM)**
Cl-indole + vehicle	187±45
Cl-indole + 5-HT (200 nM)	30±4
Cl-indole + ondansetron (0.3 nM)	269±95
Cl-indole + ondansetron (1.0 nM)	251±67
Cl-indole + ondansetron (3.0 nM)	239±30

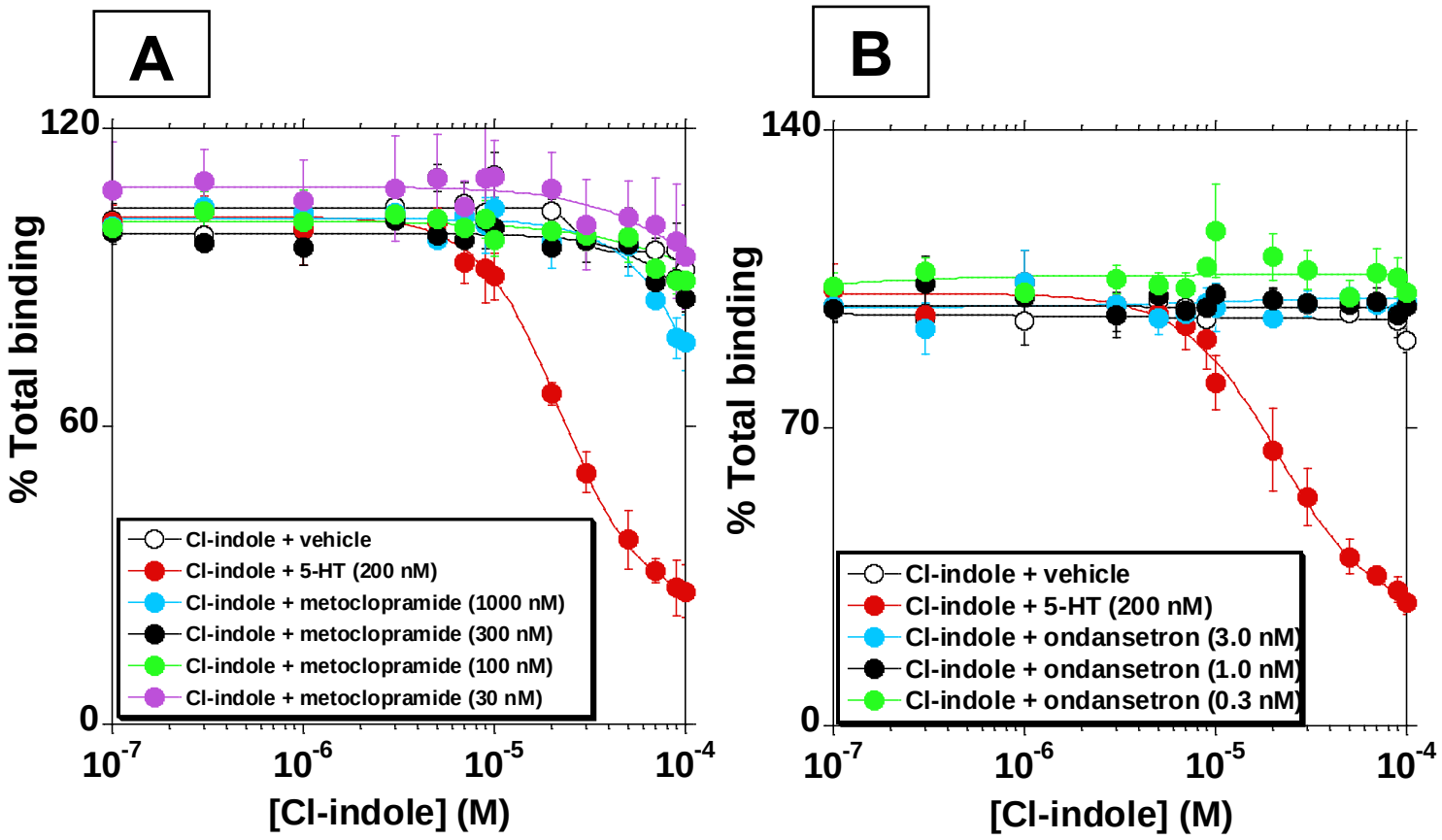

Figure 28. 5-HT$_3$ receptor antagonists do not have the same effect as 5-HT upon Cl-indole binding for the h5-HT$_3$A receptor.

The affinity of Cl-indole was increased by 5-HT (200 nM), but not by 5-HT$_3$ receptor antagonists, metoclopramide (A) and ondansetron (B) at the h5-HT$_3$A receptor. Data represents mean±SEM, n=3-4.

3.3.4 Cl-indole binding to the 5-HTBP

The 5-HTBP was generated by aligning the AChBP (which does bind 5-HT, albeit with lower affinity compared to acetylcholine) with the sequence of the h5-HT$_3$A receptor, and identifying homologous ligand binding residues in the extracellular domain (Kesters et al., 2013). The following mutations were made in the AChBP: S92E, V140L, K141T and Y53W, homologous to E129 in loop A, L178 and T179 in loop B, and W90 in loop D of the h5-HT$_3$A receptor (Kesters et al., 2013). It was determined whether the binding mechanism of Cl-indole to the h5-HT$_3$A receptor could be replicated using the 5-HTBP. Due to the lower affinity of the 5-HTBP for [^{3}H]-granisetron, SPA was used to measure the binding of [^{3}H]-granisetron to the 5HTBP rather than traditional filtration methods. Unlike what was seen for the h5-HT$_3$A receptor, Cl-indole displaced [^{3}H]-granisetron from the 5-HTBP, and the affinity of Cl-indole was not increased by 5-HT (30 µM) (Figure 29; Table 17). Moreover, 5-HT and

alosetron both competed for [^{3}H]-granisetron occupied 5-HTBP binding sites, albeit with lower affinities than what would be seen for the h5-HT$_3$A receptor (Figure 29; Table 17) (Hirata et al., 2007).

Table 17: Summary data from Figure 29. The affinity of Cl-indole was decreased by the presence of 5-HT (30 µM) at the 5-HTBP.

Alosetron and 5-HT both compete for the 5-HTBP with [^{3}H]-granisetron. The data represents mean±SEM, n=3.

IC$_{50}$ (µM)			
Cl-indole + vehicle	**Cl-indole + 5-HT (30 µM)**	**Alosetron**	**5-HT**
55±11	227±43	2.0±0.6	8.5±1.3

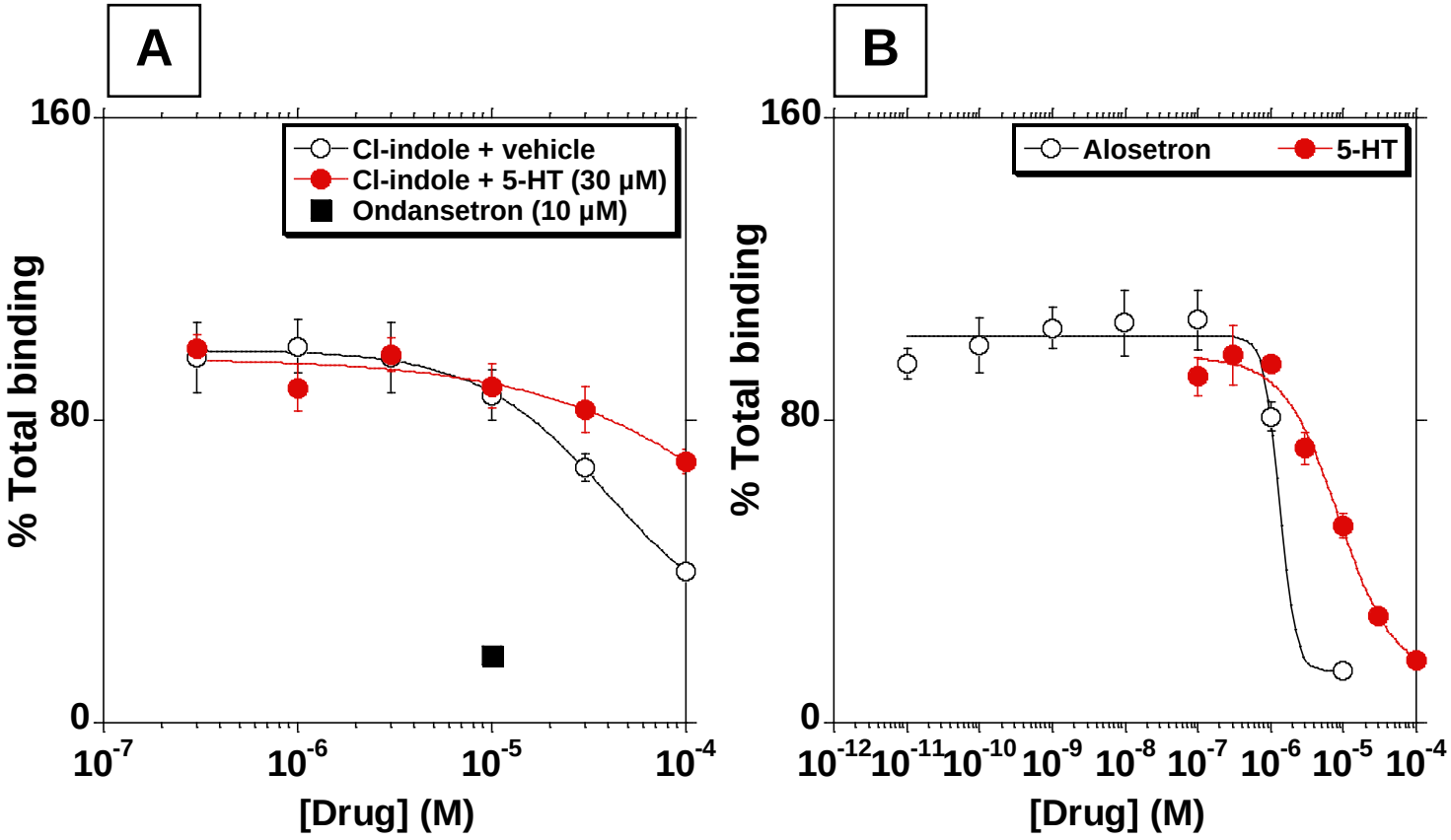

Figure 29. The 5-HTBP does not replicate Cl-indole binding to the h5-HT$_3$A receptor.

The affinity of Cl-indole was not affected by 5-HT (30 µM) at the 5-HTBP (A). Alosetron and 5-HT compete with [^{3}H]-granisetron for 5-HTBP binding sites with lower affinity compared to what would be expected for the 5-HT$_3$A receptor (B). Data represents mean±SEM, n=3.

3.3.5 5-HT does not influence Cl-indole binding to the h5-HT_3AB receptor

Cl-indole has similar functional activity at the h5-HT_3AB receptor as it does at the h5-HT_3A receptor, increasing the maximal response and potency of 5-HT (Figure 30; Table 18). The potency of 5-HT was approximately doubled at both receptor subtypes in the presence of Cl-indole (10 µM). Moreover, the maximal response elicited by 5-HT increased 2-fold at the h5-HT_3A receptor in the presence of Cl-indole. At the h5-HT_3AB receptor, the increase in maximal 5-HT response was also apparent in the presence of Cl-indole, albeit not quite as dramatic (a 50% increase). It was investigated whether Cl-indole demonstrated the same binding strategy at the h5-HT_3AB receptor as it does at the h5-HT_3A receptor. Cl-indole did not displace [^{3}H]-granisetron from h5-HT_3AB receptor binding sites (Figure 31). However, the presence of sub-maximal 5-HT concentrations did not induce Cl-indole to compete for h5-HT_3AB receptor binding sites with [^{3}H]-granisetron nor did 5-HT increase the affinity of Cl-indole for the h5-HT_3AB receptor (Figure 31; Table 19).

Table 18: Summary data from Figure 30. **The impact of Cl-indole upon 5-HT potency and efficacy at the 5-HT_3A and 5-HT_3AB receptors.**

At both the h5-HT_3A and the h5-HT_3AB receptors, Cl-indole (10 µM) increases the potency and efficacy of 5-HT in intracellular calcium assays. E_{max} presented as percentage 5-HT + vehicle, data represents mean±SEM, n=3.

5-HT_3A receptor	**E_{max}**	**EC_{50} (nM)**	**Hill number**
5-HT + vehicle	119±1.9	329±76	2.65±0.62
5-HT + Cl-indole (10 µM)	241±33	195±39	2.91±0.20
5-HT_3AB receptor	**E_{max}**	**EC_{50} (nM)**	**Hill number**
5-HT + vehicle	106±1	367±22	1.86±0.25
5-HT + Cl-indole (10 µM)	156±16	181±33	1.98±0.23

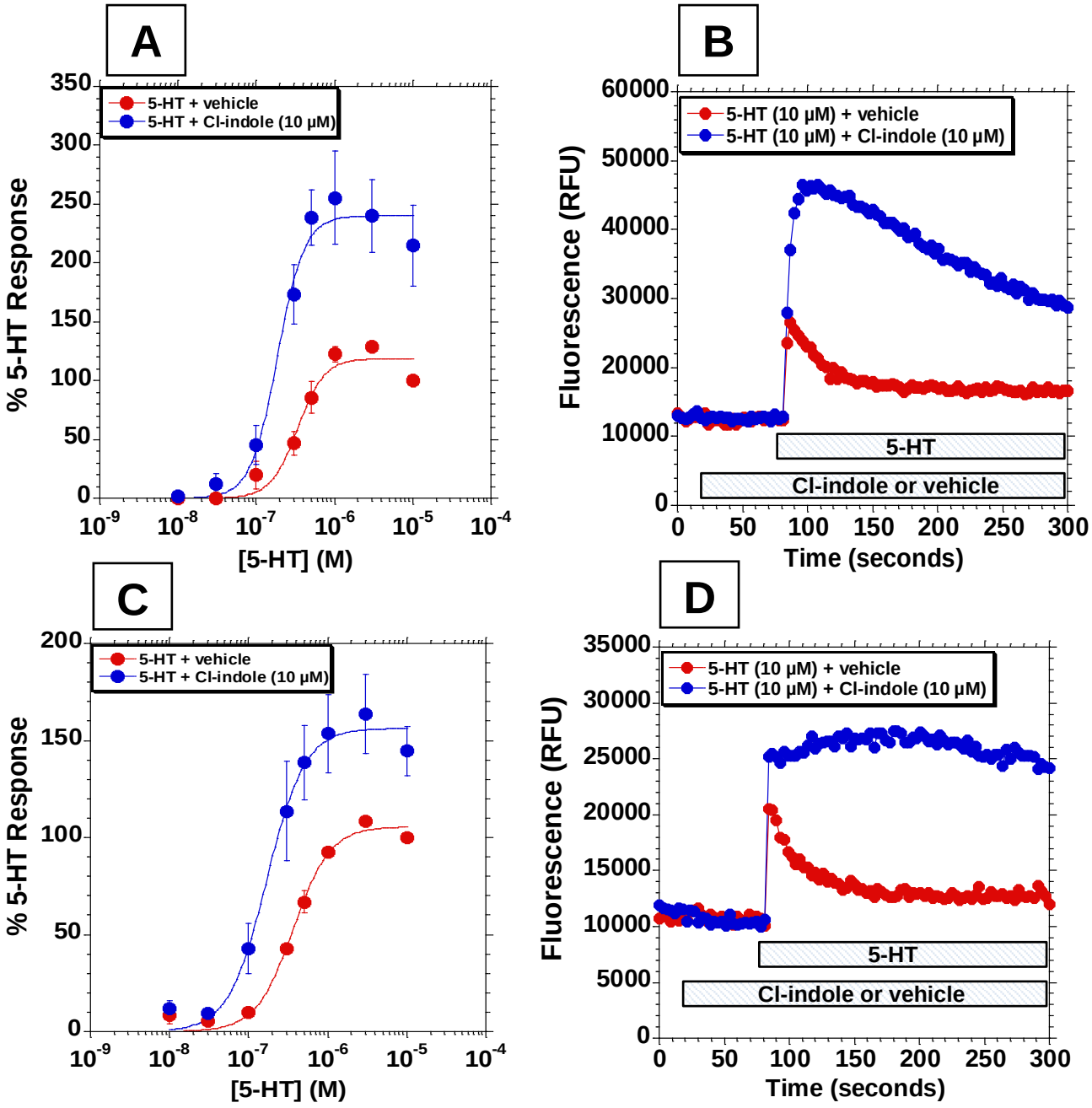

Figure 30. Cl-indole increases the efficacy and potency of 5-HT at the h5-HT$_3$A and h5-HT$_3$AB receptors.

Concentration-response curve (A) and Representative traces showing changes in calcium (fluorescence) over time (B) for the h5-HT$_3$A receptor. Bar indicates when drugs were applied. Concentration-response curve (C) and Representative traces showing changes in calcium (fluorescence) over time (D) for the h5-HT$_3$AB receptor. Bar indicates when drugs were applied. Data represents mean±SEM, n=3, presented as % 5-HT response for concentration-response curves.

Table 19: Summary data from Figure 31. The affinity of Cl-indole was unchanged by 5-HT at the h5-HT_3AB receptor.

Unlike at the h5-HT_3A receptor, 5-HT did not impact the affinity of Cl-indole at the h5-HT_3AB receptor. The data represents mean±SEM, n=5.

[5-HT] (nM)	**Cl-indole IC_{50} (µM)**
0 (Vehicle)	237±35
10	345±20
30	262±62
100	224±40
200	240±52

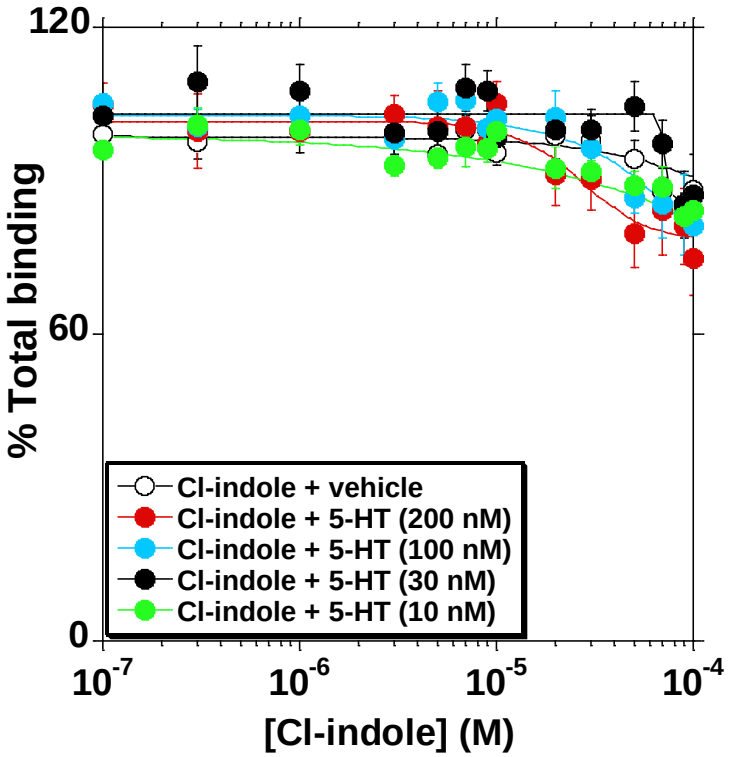

Figure 31. Cl-indole binding at the h5-HT$_3$AB receptor.

The affinity of Cl-indole was unaffected by 5-HT at the h5-HT$_3$AB receptor, unlike what was seen for the h5-HT$_3$A receptor. Data represents mean±SEM, n=5.

4 Discussion

4.1 Vortioxetine is a 5-HT_3 receptor partial agonist

4.1.1 The pharmacology of vortioxetine at the h5-HT_3 receptor

From this *in vitro* study of vortioxetine at the h5-HT_3 receptor, it has been discovered that vortioxetine is a high affinity, competitive 5-HT_3 receptor partial agonist, with similar pharmacology (in terms of potency and efficacy) at the h5-HT_3A and h5-HT_3AB receptor isoforms. Furthermore, the ability of co-applied vortioxetine to reduce (but not fully block) a maximal 5-HT-induced response at the h5-HT_3A receptor suggests this drug may have potential in treating conditions where the 5-HT_3 receptor is over-active, such is the case in IBS-d.

At the time of investigation, the present studied provided the first detailed pharmacological investigation into the activity of vortioxetine at h5-HT_3 receptors. Recent work by Ladefoged and colleagues was published during the end of these studies and after the data in this thesis was presented at conferences. Vortioxetine demonstrated high affinity for the h5-HT_3A and h5-HT_3AB receptors, and similar to the endogenous agonist 5-HT, the affinity of vortioxetine for h5-HT_3 receptors was increased by the COM Cl-indole (Newman et al., 2013). Moreover, the affinity of Cl-indole for the h5-HT_3A receptor was increased by vortioxetine in a concentration-dependent manner, similar to what has been shown previously for 5-HT in this thesis. These binding characteristics of vortioxetine (which are shared also by 5-HT) support the idea that vortioxetine has functional agonist activity at the h5-HT_3 receptor.

In functional studies, in which the ability of a 5-HT_3 receptor agonist to activate the 5-HT_3 receptor (measured by an increase in $[Ca^{2+}]_i$), vortioxetine displayed partial agonist activity, with an intrinsic efficacy (relative to the endogenous full agonist 5-HT)

of approximately 50% at the h5-HT_3A receptor and 40% at the h5-HT_3AB receptor. As may have been expected, the maximal responses are lower for both vortioxetine and 5-HT at the h5-HT_3AB receptor. This is due to the reduced calcium permeability of the h5-HT_3AB compared to the h5-HT_3A receptor (Davies et al., 1999). However, because these cell lines are not clonal (i.e. each cell does not generate the same number of cell surface receptors), the difference in calcium response could apparently be due to differences in 5-HT_3 receptor expression. Furthermore, the responses elicited by 5-HT and vortioxetine were selective for the 5-HT_3 receptor, because preincubation with the selective 5-HT_3 receptor antagonist granisetron blocked 5-HT_3 receptor activity. Granisetron was used in this assay as a selective 5-HT_3 receptor antagonist to verify the selectively of the 5-HT and vortioxetine induced responses at the h5-HT_3 receptor.

The first initial investigation of vortioxetine used electrophysiology to assess the functional activity of vortioxetine at the 5-HT_3A receptor (both rat and human isoforms) (Bang-Andersen et al., 2011). It was established that vortioxetine is a 5-HT_3 receptor partial agonist for the human receptor (it displayed no functional activity at the rat 5-HT_3 receptor). However, after vortioxetine elicited an inward current at the h5-HT_3A receptor, subsequent applications of vortioxetine (or 5-HT) fail to evoke any response (Bang-Andersen et al., 2011). This is unlike what occurs when 5-HT activates the h5-HT_3A receptor, where under appropriate conditions, further applications of 5-HT still elicit currents of a similar magnitude. This unusual mechanism of vortioxetine lead to the authors describing it as a ‘functional antagonist’, i.e. vortioxetine doesn’t block the 5-HT_3 receptor through a competitive manner (like a classic antagonist, e.g. ondansetron), but rather impacts the receptor such that it can no longer respond to agonist, possibly by desensitisation or receptor internalisation.

Different studies have shown that the 5-HT_3 receptor undergoes internalisation/recycling after treatment with selective agonists or antagonists. Using radioligand binding studies, it was demonstrated that treatment of HEK293 cells expressing the h5-HT_3A receptor with palonosetron (1.0 nM) for 24 hours caused a reduction (approximately 50%) in [^{3}H]-palonosetron surface binding sites, suggesting internalisation of the receptor (Rojas et al., 2010). The effect of palonosetron on cell

surface h5-HT_3A receptor expression was shown to occur after 15 minutes of palonosetron treatment (receptor expression was decreased by 30%). 2 hours of palonosetron treatment had a similar effect on h5-HT_3A receptor surface expression as did 24 hour palonosetron treatment (Rojas et al., 2010). Furthermore, this was translated in functional studies, whereby palonosetron treatment caused a decrease in the calcium response elicited by 5-HT at HEK293 cells expressing the h5-HT_3A receptor (Rojas et al., 2010). Moreover, the potent 5-HT_3 receptor agonist mCPBG also caused 5-HT_3 receptor internalisation (Ilegems et al., 2004). Immunocytochemistry studies demonstrated that after 2 hours treatment with mCPBG (500 nM), the majority of mouse 5-HT_3A receptors were internalised. It could be possible therefore that the inhibitory actions of vortioxetine are due to 5-HT_3 receptor internalisation, rather than purely a competitive block.

Interestingly, in functional experiments, the calcium response evoked by vortioxetine decayed to baseline faster than the response induced by 5-HT at both the h5-HT_3A and h5-HT_3AB receptors. This was not seen for other 5-HT_3 receptor partial agonists (e.g. DDP, CSTI-300, quipazine), whereby the response appeared to decay slower than that of 5-HT in intracellular calcium assays (Newman et al., 2013). Moreover, electrophysiology assays have demonstrated that 5-HT_3 receptor partial agonists (e.g. mCPBG or SR 57227A) often have similar desensitisation kinetics compared to 5-HT (van Hooft et al., 1996; Nakamura et al., 2019). This could further imply that vortioxetine causes a more rapid desensitisation of the h5-HT_3 receptor compared to other 5-HT_3 receptor agonists.

Functional studies were undertaken where a saturating concentration of 5-HT (1.0 µM) and vortioxetine (10 µM) were co-applied to the h5-HT_3A receptor. In this assay, vortioxetine was compared alongside alosetron as a 5-HT_3 receptor antagonist. Alosetron was used so vortioxetine can be compared with a marketed therapeutic for IBS-d. Compared to 5-HT alone, the calcium response caused by 5-HT and vortioxetine returned to baseline significantly faster than 5-HT alone (as defined by $t_{1/2}$ time). It is interesting to speculate the mechanism behind this. 5-HT could (having lower affinity, and so likely to interact with the receptor first) be eliciting the initial

response, and then vortioxetine begins to compete out 5-HT and cause the receptor to desensitise more rapidly. However, this must be approached with some caution. Firstly, the functional calcium assays are a population measurement, i.e. it is analysing average fluorescent responses of a number of cells in a 96 well plate, rather than at the single cell level (unlike electrophysiology). Therefore, actual comments about the receptor state (i.e. whether it is desensitised) cannot be said without examining the impact of vortioxetine at the single cell or single channel level. However, this could be one of the mechanisms for which vortioxetine causes 5-HT_3 receptor antagonism.

5-HT_3 receptor agonists will cause receptor desensitisation. The distinct desensitisation kinetics of 5-HT and vortioxetine are consistent with previous findings that desensitisation in the presence of partial agonists exhibit a different profile to full agonists (Corradi et al., 2014). The rate of recovery from desensitisation is dependent on 5-HT_3 receptor agonist (and not dependent on agonist concentration or agonist reassociation with the receptor), and it could be speculated that vortioxetine is an agonist which simply shifts the 5-HT_3 receptor into such a state that it cannot recover from desensitisation (van Hooft et al., 1997). It may be of interest to determine if this desensitisation also occurs when vortioxetine is applied to the h5-HT_3A receptor in the presence of 5-HI, an allosteric modulator which is known to reduce desensitisation kinetics (van Hooft et al., 1997).

In addition, the natural agonist 5-HT (at high concentrations) is known to cause channel block (Corradi et al., 2014). It is interesting to think that the small decrease in response elicited by vortioxetine at 3.0 and 10 µM compared to 1.0 µM could be due to channel block; although this must be resolved using single channel activity to assess the open time kinetics (Corradi et al., 2014).

4.1.2 The efficacy and tolerability of vortioxetine in depression

The efficacy and tolerability of vortioxetine has been studied in depth. One such review assessing 18,326 patients across 57 studies found that vortioxetine had similar

efficacy to other antidepressants (e.g. duloxetine, sertraline, escitalopram) using an improvement in the HAM-D or MADRS scale as an endpoint in evaluating depression (Llorca et al., 2014). In addition, vortioxetine was demonstrated to be more efficacious than placebo in reducing the MADRS score (Berhan et al., 2014). Moreover, vortioxetine is also effective at helping MDD patients achieve remission (which rarely occurs for other antidepressants) (Thase et al., 2016). Vortioxetine is also effective in treating anxiety and improving overall patient functioning (Baldwin et al., 2016; Florea et al., 2017). Indeed, a large review (encompassing 194 studies, 34,196 patients) identified vortioxetine (alongside escitalopram) as one of the more efficacious drugs in treating depression (with fluoxetine and reboxetine amongst the worst performers) (Cipriani et al., 2018).

The highest density of 5-HT_3 receptor expression in the brain is within the nucleus tractus solitarius of the brainstem (Parker et al., 1996). Activation of 5-HT_3 receptors (specifically in the chemoreceptor trigger zone) will induce the emetic response (Miner et al., 1987; Bermudez et al., 1988). Therefore 5-HT_3 receptor agonists have rarely been tested clinically. Indeed, only low doses of a 5-HT_3 receptor agonist (SR 57227A) could be used to investigate the impact of 5-HT_3 receptor activation on sleep (Staner et al., 2001). This was because higher doses of the drug could elicit an emetic response. Furthermore, in phase II trials of pumosetrag (DDP733, MCK-733), for IBS-c, nausea and vomiting were regularly described as common adverse effects, with over 25% of patients reporting these side effects (Evangelista, 2007; Choung et al., 2014).

As may have been anticipated, nausea and vomiting are regularly recorded as an adverse effect when prescribed vortioxetine. One meta-analysis calculated the odds of experiencing nausea were 3x higher for vortioxetine compared to placebo, which has led to a higher drop-out rate for vortioxetine compared to placebo (Berhan et al., 2014; Pae et al., 2015). 20% of patients receiving vortioxetine (20 mg) reported nausea, whilst this was the case for only 5% of patients taking placebo. Of note, 1.0 mg vortioxetine caused a similar rate of nausea as placebo, suggesting a dose-

dependent effect of vortioxetine on nausea (indicative of a receptor driven event for this adverse effect, rather than a non-specific effect) (Meeker et al., 2015).

However, despite this clear issue with vortioxetine, many studies have concluded that vortioxetine is one of the better tolerated antidepressants. Indeed, vortioxetine has been described as being better tolerated than desvenlafaxine and sertraline (Llorca et al., 2014). Similar to their efficacy, both vortioxetine and escitalopram are the better tolerated antidepressants, (as was fluoxetine interestingly, despite its limited effectiveness), whilst amitriptyline and reboxetine were poorly tolerated (Cipriani et al., 2018).

There are various ideas that support targeting the 5-HT_3 receptor for treating depression. Presynaptic 5-HT_3 receptors in the brainstem trigger release of GABA (Kohler et al., 2016). This will in turn lead to an inhibition of the release of neurotransmitters (e.g. dopamine, noradrenaline and 5-HT), which are thought to be implicated in the neurobiology of depression. Therefore blocking 5-HT_3 receptors could lead to an increase in these neurotransmitters through a disinhibition mechanism (Kohler et al., 2016). Stahl devised a network theory to try and explain why 5-HT_3 receptor antagonism might culminate into antidepressant efficacy. It relies on the idea that 5-HT can regulate its own release through a simple 3 neuron system (Stahl, 2015). Serotonergic neurons input onto GABAergic neurons within the hippocampus or prefrontal cortex. 5-HT release will stimulate 5-HT_3 receptors on these inhibitory interneurons within these higher brain centres (Stahl, 2015). These GABAergic neurones will inhibit pyramidal neurons which project back into the raphe nucleus to stimulate 5-HT release. Therefore 5-HT release into the hippocampus or prefrontal cortex can lead to a downstream inhibition of 5-HT release through 5-HT_3 receptors on these GABAergic neurons. Vortioxetine can have a dual impact on neurotransmitter release. Vortioxetine, through blockade of SERT, will increase 5-HT release; and also through antagonism of 5-HT_3 receptors will also lead to an increase in 5-HT, as well as noradrenaline and acetylcholine (Stahl, 2015). Moreover, vortioxetine can further elevate brain 5-HT levels through desensitisation of raphe 5-HT_{1A} receptors (Kohler et al., 2016). It is interesting of note that increasing CNS levels

of acetylcholine is thought to be important in cognition, and vortioxetine has been demonstrated to improve cognition in elderly patients suffering with MDD (Sanchez et al., 2015).

There is also evidence for pharmacological targeting of the 5-HT_3 receptor as a treatment for depression. The novel 5-HT_3 receptor antagonist QCM-4 (which has similar potency as ondansetron in blocking 2-methyl 5-HT induced contractions of guinea pig ileum) has been evaluated for depression or anxiety using *in vivo* mice studies (Mahesh et al., 2011; Kurhe et al., 2014; Kurhe et al., 2017). QCM-4 (and reference drug escitalopram) was shown, in a variety of behavioural studies (sucrose consumption, forced swim test, tail-suspension test, elevated plus maze and open field test) to reverse the anxiogenic effects of sequential application of a variety of mild stresses (such as forced swim test, elevated plus maze and tail suspension test) (Kurhe et al., 2014). Moreover, biochemical markers of oxidative stress (e.g. brain levels of thiobarbituric acid reactive substance or nitrate levels) were shown to be reduced by QCM-4 or escitalopram, having been increased by the stressful environment imposed on the mice (Kurhe et al., 2014). In addition, QCM-4 and escitalopram also reduced the depressive-like phenotype caused by feeding mice a high-fat diet (Kurhe et al., 2017). Again, biochemical markers of depression caused by a high-fat diet (e.g. decreased hippocampal cAMP, brain derived neurotrophic factor [BDNF] and 5-HT) were all reversed by QCM-4 and escitalopram (Kurhe et al., 2017). In addition, the number of pyknotic neurons in the hippocampus was increased by the high-fat diet, and reversed by QCM-4 and escitalopram, demonstrating that this ligand has similar efficacy to a marketed antidepressant on behavioural, biochemical and histological markers of anxiety or depression (Kurhe et al., 2014; Kurhe et al., 2017).

There is recent evidence that the tricyclic antidepressant amitriptyline displays affinity for the 5-HT_3 receptor. Using endogenous 5-HT_3 receptor expression in the mouse NCB-20 neuroblastoma cell line, amitriptyline inhibited 5-HT-induced currents through a competitive interaction (Park et al., 2018). This inhibition occurred at concentrations of amitriptyline which have been detected in the plasma of patients receiving this drug

(Montgomery et al., 1979). The mechanism of inhibition was thought to be due to increased rate of 5-HT_3 receptor desensitisation. This implies that 5-HT_3 receptor inhibition may be an important factor in anti-depressant activity, given also fluoxetine will reduce 5-HT_3 receptor activity at concentrations relevant to its clinical impact (Pato et al., 1991; Choi et al., 2003).

Clinically used 5-HT_3 receptor antagonists have also been studied for their potential to treat depression. Rats administered ondansetron had reduced immobility in the forced swim test compared to rats treated with placebo (Betry et al., 2015). Moreover, ondansetron and paroxetine combined caused a greater reduction still in time spent immobile in the same assay. In biochemical studies, ondansetron and paroxetine caused a greater increase in hippocampal 5-HT release compared to paroxetine (or ondansetron) alone; an effect which was also noted when ondansetron was combined with citalopram (Mork et al., 2012; Betry et al., 2015).

Some studies have assessed 5-HT_3 receptor antagonists in treating depression or anxiety disorders in humans. Dépôt and colleagues looked at the impact of ondansetron on CCK induced behavioural changes in healthy subjects. Administration of CCK is known to produce anxiety and panic attacks in people. Acute administration of ondansetron (2.0 mg) caused a decrease in PSS (Panic associated symptom scale) score (which like HAM-A measures behaviours including dizziness, trembling/shaking, abdominal distress etc.) (Argyle et al., 1991; Depot et al., 1999). Moreover, plasma levels of adrenocorticotropic hormone (a biochemical marker for stress) were decreased in the ondansetron treatment group compared to the placebo cohort. Furthermore, ondansetron abolished the emotion potentiated startle effect in healthy volunteers (Harmer et al., 2006). In all studies, ondansetron was well tolerated and did not affect mood. It is apparent that 5-HT_3 receptor antagonists are efficacious in treating depression and anxiety. Whereas these drugs simply provide a competitive block at the 5-HT_3 receptor, vortioxetine appears to inhibit a 5-HT_3 receptor mediated response through a slightly different mechanism of action, by increasing the rate at which the response decays.

4.1.3 5-HT_3 receptor antagonists in treating schizophrenia

5-HT_3 receptor antagonists may have use in other neuropsychiatric conditions, including schizophrenia. A variety of animal and clinical trial data supports this theory. In a small trial of 22 schizophrenic patients (currently receiving antipsychotics such as olanzapine or risperidone), tropisetron (a high-affinity 5-HT_3 receptor antagonist with α7 nAChR partial agonist activity) was found to decrease the p50 auditory gating, 1 hour after administration (Macor et al., 2001; Koike et al., 2005). Furthermore, patients receiving tropisetron as an adjunct to risperidone (an atypical antipsychotic with no 5-HT_3 receptor activity) demonstrated a reduction in negative symptom score; which corresponded into a decrease in general psychopathy score (Noroozian et al., 2013). Tropisetron interestingly did not appear to have any impact on the positive symptoms of schizophrenia. Side effects were mild, and consistent with what might be expected with 5-HT_3 receptor antagonism; constipation was experienced in 30% of patients receiving tropisetron (Noroozian et al., 2013).

This impact of 5-HT_3 receptor antagonists on psychosis is mirrored with other 5-HT_3 receptor antagonists too. Granisetron (which unlike tropisetron does not display relevant α7 nAChR pharmacology), when prescribed along with risperidone to schizophrenic patients resulted in a significant improvement in negative symptoms when compared to placebo; yet did not have a significant effect on the positive symptoms of schizophrenia (Khodaie-Ardakani et al., 2013). Like tropisetron and granisetron, ondansetron combined with risperidone significantly improved the negative symptoms of schizophrenia which culminated in a significant improvement in psychopathology compared to placebo (Akhondzadeh et al., 2009). Moreover, as before, ondansetron (and risperidone) did not impact on the positive symptoms compared to risperidone alone (Akhondzadeh et al., 2009). Given that vortioxetine has a complicated pharmacological profile (akin to clozapine), it may be speculated that vortioxetine could also target the positive symptoms of schizophrenia (Warnez et al., 2014).

Ondansetron has also demonstrated efficacy in treating psychosis when administered alongside a typical antipsychotic (haloperidol). A 12-week trial with 58 patients receiving haloperidol and ondansetron, and 63 patients receiving haloperidol and placebo was conducted (Zhang et al., 2006). Those who were administered ondansetron with haloperidol showed an improvement in negative symptoms and an improved PANSS (positive and negative syndrome scale) score compared to placebo. The PANSS examines a variety of positive symptoms (including delusions, conceptional disorganisation and hallucinatory behaviour), as well as negative symptoms (e.g. blunted affect, emotional withdrawal and stereotyped behaviour) and general psychopathology (such as disorientation, poor attention and a lack of judgement and insight) (Kay et al., 1987). Moreover, the clinical response rate (defined as a greater than 30% reduction in PANSS score) was 62.5% for patients receiving ondansetron alongside haloperidol, compared to 40.3% for placebo and haloperidol (Zhang et al., 2006). Similar to tropisetron and risperidone, more patients receiving ondansetron experienced constipation compared to placebo; however, the frequency of nausea and vomiting was reduced for those receiving ondansetron compared to placebo (Zhang et al., 2006). Of course, given vortioxetine is a 5-HT_3 receptor partial agonist, it may elicit nausea and vomiting as an adverse effect (as has been recorded in clinical trials) (Berhan et al., 2014; Pae et al., 2015).

A review conducted by Kishi and colleagues investigating 6 randomised control trials (311 patients) found that there was a significant effect for 5-HT_3 receptor antagonists for treating the negative symptoms of schizophrenia when co-administered with antipsychotics (Kishi et al., 2014). This resulted in a significant decrease in PANSS. Although constipation was more common with 5-HT_3 receptor antagonists, the dropout rate was similar to placebo, suggesting these drugs are well tolerated by patients with schizophrenia (Kishi et al., 2014).

What is of most interest regarding 5-HT_3 receptor antagonism and schizophrenia is the ability of these ligands to treat the negative symptoms of schizophrenia. These symptoms have similarities with symptoms of depression (e.g. diminished emotional responsiveness, emotional and social withdrawal). These clinical manifestations are

not thought to be due to hyperdopaminergic activity (which antipsychotics target to treat the classic positive symptoms); and often are not treatable by typical and atypical antipsychotics (Remington et al., 2016). Vortioxetine could offer therapy for these affective symptoms experienced in schizophrenia through 5-HT_3 receptor antagonism and its intrinsic ability to treat depression; whilst being generally very well tolerated. As it appears vortioxetine can reduce a 5-HT_3 receptor mediated response, it could be trialled as an adjunct to treat schizophrenia, just as other 5-HT_3 receptor antagonists have been tried with some success (Kishi et al., 2014).

4.1.4 5-HT_3 receptor antagonists as a therapy for IBS-d

As was previously mentioned in the introduction, current treatment for IBS-d is limited and does not meet the patient need for symptom relief. There is evidence to suggest 5-HT_3 receptor antagonist could prove useful in treating this condition. However, although these ligands demonstrated efficacy in treating the symptoms of IBS-d, a number of patients experienced constipation; and more rarely ischemic colitis (approximately 1:750 patients taking alosetron) (Chang et al., 2006; Camilleri, 2017). Patient advocacy lead to the reinstatement of alosetron, albeit with a 'black-box' warning label, which has now been lifted (FDA.gov, 2016). Given the ability of vortioxetine to reduce a 5-HT_3 mediated response, it could have potential to treat IBS-d, without the manifestation of severe adverse effects.

However, there is a clear hypothesis to treat IBS-d using a 5-HT_3 receptor partial agonist. A 5-HT_3 receptor partial agonist, in the presence of excess endogenous 5-HT, would demonstrate activity as an antagonist, whilst preventing full blockade of 5-HT_3 receptor function, which therefore should prevent the associated side effects experienced by patients with IBS-d when taking 5-HT_3 receptor antagonists(Nam et al., 2018). Vortioxetine, which displays activity as a 5-HT_3 receptor partial agonist, could be a viable therapeutic for IBS-d. It is interesting to note of a case study whereby a female patient expressing depression with IBS-d was prescribed vortioxetine (Aydin et al., 2018). She suffered from IBS-d and was diagnosed with MDD. After being prescribed paroxetine and sertraline (both drugs were withdrawn due to adverse side

effects), she was offered vortioxetine. Vortioxetine treated her depression (her HAM-D score was decreased by almost half), and also influenced her IBS-d (Aydin et al., 2018). After 4 weeks of treatment, her IBS-d was in remission with abdominal pain, cramping and frequency to defecate all being reduced. After 7 months of treatment, there was no sign of IBS-d or depression. Indeed, vortioxetine was shown to improve quality of life in IBS patients, although impact on bowel function was not investigated (Seddighnia et al., 2020). Furthermore, Manning and colleagues have developed a number of high-affinity 5-HT_3 partial agonists which show potential in treating IBS-d (see also CSTI-300 discussion section) (Manning et al., 2011; Moore et al., 2013).

There is a strong link between depression and IBS-d. A review used an IBS quality of life questionnaire (IBS-QOL) and the Beck Depression Inventory (BDI) to assess the link between depression in IBS-d patients. The IBS-QOL evaluates how IBS-d patients feel, examining food avoidance, social reaction etc, and the BDI, similar to HAM-D and MARDS, scores on feelings such as feeling sad, suicidal thoughts, sexual interest (Beck et al., 1961; Patrick et al., 1998). Depression was diagnosed more frequently in IBS-d (46%, 40 patients) compared to healthy controls (9%, 5 patients) (Kopczynska et al., 2018). In addition, the IBS-QOL was worse in IBS-d patients compared to control subjects (Kopczynska et al., 2018). There is therefore precedence for using vortioxetine to treat IBS-d, if not for treating any depressive symptoms alone. Moreover, as vortioxetine is well tolerated by patients, and so safety is unlikely to be an issue (Cipriani et al., 2018).

Further studies to identify this could involve treating 5-HT_3 receptor transfected HEK293 cells with vortioxetine, and assessing its functional activity using electrophysiology, before then visualising cell surface h5-HT_3A receptor expression by immunocytochemistry or flow cytometry. This would allow a correlation between actual functional vortioxetine response and any potential impact it has on 5-HT_3 receptor internalisation. In addition, simply examining whether treatment with vortioxetine causes receptor downregulation (measured by immunocytochemistry or flow cytometry) could help to elucidate its mechanism of action. Moreover, given the complexity of the 5-HT_3 receptor architecture, it may be of importance to study the

pharmacology of vortioxetine at other 5-HT_3 receptor subtypes (e.g. 5-HT_3AC, 5-HT_3AD, 5-HT_3AE). Given there is speculation about whether vortioxetine could be efficacious in treating schizophrenia or IBS-d, it may be relevant to study the *in vivo* effect of vortioxetine (and compare to clinically-used drugs) in animal models for these conditions (Banner et al., 1995; Banner et al., 1995; Marcotte et al., 2001).

A limitation of this study is only two 5-HT_3 receptor isoforms have been studied (5-HT_3A and 5-HT_3AB), and given there is at least 5-HT_3AC, 5-HT_3AD, 5-HT_3AE (plus potentially other subunit combinations), these need to be considered, especially given expression of these receptors in human GIT and possibly brain (Holbrook et al., 2009). Similarly, conclusions about the effectiveness of vortioxetine in schizophrenia or IBS-d cannot be substantiated without testing in *in vivo* models.

In summary, vortioxetine has been characterised as relatively high affinity, competitive h5-HT_3 receptor partial agonist. The antagonist nature of vortioxetine (as reported in the literature) could be due to vortioxetine desensitising the 5-HT_3 receptor into a state whereby agonist can no longer activate the receptor; or possibly by internalising the receptor. The activity of vortioxetine (and the fact it is a well-tolerated therapeutic used clinically) means it could be used to treat other disorders where 5-HT_3 receptor blockade is useful. These disorders include the negative symptoms of schizophrenia, depression/anxiety and IBS-d.

4.2 Evaluating the pharmacology of a novel 5-HT_3 receptor partial agonist CSTI-300

4.2.1 The *in vitro* and *in vivo* pharmacology of CSTI-300

This study presents the results of an in-depth pharmacological investigation of CSTI-300, at the 5-HT_3 receptor. *In vitro* assays were utilised to establish the affinity, potency and efficacy of CSTI-300 at the h5-HT_3 receptor; with CSTI-300 demonstrating a near-identical pharmacological profile at the two most studied 5-HT_3 receptor subtypes, the 5-HT_3A and 5-HT_3AB receptors. Moreover, *in vivo* (rat Bezold-Jarisch reflex and rat colon distension model) assays highlight the ability of CSTI-300 to attenuate a 5-HT_3 receptor mediated response (transient bradycardia and colonic sensitivity respectively) with similar efficacy to alosetron, suggesting the potential of CSTI-300 to treat IBS-d and carcinoid syndrome, conditions whereby there the pathology is thought to be due to excessive 5-HT_3 receptor activity (Bearcroft et al., 1998).

In radioligand binding assays, CSTI-300 displayed relatively high affinity (approximately 100-fold higher than the endogenous ligand 5-HT) for the h5-HT_3A and h5-HT_3AB receptor. Moreover, whilst in the absence of agonist Cl-indole fails to interact with the orthosteric binding site of the h5-HT_3A receptor; both CSTI-300 (and 5-HT) were able to allow Cl-indole to compete for [^{3}H]-granisetron occupied h5-HT_3A receptor binding sites (Powell et al., 2016). CSTI-300 also displayed a competitive interaction with the h5-HT_3A and h5-HT_3AB receptors, demonstrated by its ability to reduce the affinity of [^{3}H]-granisetron for these 5-HT_3 receptors without influencing the density of labelled 5-HT_3 receptor binding sites. The affinity of CSTI-300 for the h5-HT_3 receptor is similar to the ondansetron, a first-generation 5-HT_3 receptor antagonist (used to treat CINV and PONV, and used 'off-label' to treat IBS-d) as well as being ~10x lower affinity compared to the very high affinity 5-HT_3 receptor antagonist, alosetron (Hirata et al., 2007). This may be an advantageous property of CSTI-300, as it is speculated that the serious constipation that some IBS-d patients experience when taking alosetron is due to the high 5-HT_3 receptor affinity. In functional *in vitro*

assays, CSTI-300 demonstrates potent partial agonist efficacy (approximately 30-40% of 5-HT), with responses elicited being selective for the h5-HT_3 receptor. Granisetron was used in this assay as a selective 5-HT_3 receptor antagonist to verify the selectively of the 5-HT and CSTI-300 induced responses at the h5-HT_3 receptor.

In electrophysiology assays examining the kinetics of h5-HT_3A receptor activation by CSTI-300 or 5-HT, it was determined that the decay rate of the h5-HT_3A receptor current was approximately 4x slower for CSTI-300 compared to 5-HT. This is of interest because should CSTI-300 cause rapid 5-HT_3 receptor desensitisation (akin to 5-HT), then this may replicate the impact of 5-HT_3 receptor antagonists upon 5-HT_3 receptor activity in IBS-d patients, which could potentially cause constipation, which is associated with 5-HT_3 receptor antagonists.

CSTI-300 demonstrated activity in the rat Bezold-Jarisch reflex. In this assay, rapid transient bradycardia is elicited by intravenous administration of 5-HT stimulating 5-HT_3 receptors on cardiac vagal terminals (Cote et al., 2004). This bradycardia can be prevented with prior oral administration of 5-HT_3 receptor antagonists, and so has been used historically to characterise many selective 5-HT_3 receptor antagonists (Eglen et al., 1995). Both alosetron and CSTI-300 were able to reverse the 5-HT-induced bradycardia event, demonstrating that these ligands both engage 5-HT_3 receptors *in vivo*. Furthermore, this also reinforces the concept that in the presence of excess 5-HT, CSTI-300 displays activity as a 5-HT_3 receptor antagonist. The ability of CSTI-300 to behave as an antagonist and block the 5-HT-induced reflex in this model is consistent with structurally different 5-HT_3 receptor partial agonists (Campiani et al., 1997; Lopez-Tudanca et al., 2003; Butini et al., 2009). In previous studies using a mouse model of the Bezold-Jarisch reflex, CSTI-300 displayed similar efficacy in its ability to block the 5-HT dependent bradycardia (Moore et al., 2013; Manning et al., 2014). Furthermore, when administered intravenously, CSTI-300 was able to evoke the Bezold-Jarisch reflex, with efficacy approximately 40% of 5-HT (Moore et al., 2013; Manning et al., 2014). Furthermore, oral administration of alosetron was able to block the CSTI-300 evoked bradycardia (unpublished work). This reinforces the selectivity of CSTI-300 for the 5-HT_3 receptor. Given CSTI-300 also does not interact with

several other receptors or ion channels, this suggests CSTI-300 is less likely to elicit off-target side effects, important in any marketed therapeutic.

After demonstrating that CSTI-300 engages 5-HT_3 receptors *in vivo*, an established rat model designed to replicate the visceral sensitivity experienced by IBS-d patients was used to investigate the efficacy of CSTI-300 (Houghton et al., 2002). This assay was selected to investigate the efficacy of CSTI-300 (and to compare against the clinically effective drug, alosetron) because the most debilitating factor in IBS-d is visceral pain. A comparable rodent model was used previously to establish the efficacy of 5-HT_3 receptor antagonists (e.g. granisetron, bemesetron) in reducing visceral sensitivity (Banner et al., 1995; Banner et al., 1995). In this assay, slow inflation of an intra-colonic balloon delivers a noxious stimulus to rats, which respond with a visceromotor reflex. The pressure required to deliver this visceromotor response is the quantitative readout (Banner et al., 1995; Banner et al., 1995). Subcutaneous administration of the precursor of 5-HT (5-HTP) increased the sensitivity of the rats to colonic distension, i.e. lower balloon pressures evoked the visceromotor response. 5-HTP will be converted to 5-HT in the colon thus increasing gastrointestinal 5-HT, which is evident in patients with IBS-d (Bearcroft et al., 1998; Dunlop et al., 2005; Enck et al., 2016). Again, similar to what was shown before, a 5-HT_3 receptor antagonist (the clinically validated alosetron in this case) reversed the increase in colonic sensitivity induced by 5-HTP. This also supports the concept that increased 5-HT_3 receptor activity is primarily responsible for the visceral pain and discomfort experienced by IBS-d patients. CSTI-300 displayed comparable efficacy to alosetron to reduce the colonic distension elicited by 5-HTP. To elicit a comparable response, the dose of CSTI-300 was approximately 10-fold that of alosetron, which mirrors the difference in affinity these two drugs have for the 5-HT_3 receptor (Hirata et al., 2007).

Functional intracellular calcium assays were used as an *in vitro* model to try and replicate the gut microenvironment with excess endogenous 5-HT. Thus, a maximal concentration of CSTI-300 (or alosetron for comparison) was applied to HEK293 cells expressing the h5-HT_3A receptor in the presence of a saturating 5-HT concentration.

Alosetron was used so CSTI-300 can be compared with a marketed therapeutic for IBS-d. Whilst alosetron ablated the 5-HT activity, the 5-HT response in the presence of CSTI-300 was approximately 40% that of 5-HT alone. This suggests that CSTI-300 can, in the presence of 5-HT, reduce 5-HT_3 receptor activity whilst still retaining some tone upon the 5-HT_3 receptor. This may be of importance for CSTI-300 to not cause constipation or ischemic colitis in IBS-d patients, which is thought to arise from complete 5-HT_3 receptor blockade caused by 5-HT_3 receptor antagonists. Theoretically, by the presence of CSTI-300 still allowing 5-HT_3 receptor activity, gut activity can be restored to 'normal' (Figure 32).

4.2.2 The 5-HT_3 receptor is a viable target for treating IBS-d

There is well established evidence that pharmacologically targeting the 5-HT_3 receptor is beneficial in IBS-d (Enck et al., 2016; Camilleri et al., 2017). A diverse variety of selective 5-HT_3 receptor antagonists demonstrate efficacy in treating IBS-d. In a randomised trial investigating the potential for ondansetron to treat IBS-d, it was discovered 65% of patients reported relief from IBS-d symptoms (including average urgency scores and average frequency of defecation) (Garsed et al., 2014). Furthermore, over 50% of patients reported a global improvement in abdominal pain and discomfort as well as stool consistency when talking either alosetron, cilansetron or ramosetron (Andresen et al., 2008; Ford et al., 2009; Fukudo et al., 2016; Zheng et al., 2017; Qi et al., 2018). However, a common adverse effect consistently associated with these drugs in IBS-d patients is constipation, and in the case of alosetron and cilansetron, rare ischemic colitis. The statistics regarding occurrences of constipation has been studied most in alosetron, following its initial withdrawal from the market by manufactures GSK in 2000. Out of 11,874 patients across 26 trials, the rate of serious constipation was 0.1% in patients receiving alosetron compared to 0.06% for those receiving placebo (Chang et al., 2006). This is also true for the other 5-HT_3 receptor antagonists previously discussed (Andresen et al., 2008; Ford et al., 2009; Fukudo et al., 2016; Zheng et al., 2017). Given CSTI-300 is a partial 5-HT_3 receptor agonist, it is predicted constipation or ischemic colitis is unlikely to occur.

What must be taken into consideration is the complication of ischemic colitis associated with 5-HT_3 receptor antagonists appears to be unique with IBS-d patients. 5-HT_3 receptor antagonists are routinely used to treat emesis associated with chemotherapy or after surgery. No serious adverse effects have been recorded, although constipation was noted from a variety of large clinical trials investigating the efficacy and safety of 5-HT_3 receptor antagonists (i.e. ondansetron, granisetron or ramosetron) in CINV or PONV (Atkinson et al., 2006; Miura et al., 2013; Candiotti et al., 2014).

The mechanism of action of CSTI-300 is shown in Figure 32. Increasing 5-HT_3 receptor activity is correlated with increasing gut activity, thus IBS-d is associated with high levels of 5-HT_3 receptor functioning. Whereas alosetron (or indeed any 5-HT_3 receptor antagonist) potentially completely blocks 5-HT_3 receptor activity and causes constipation, CSTI-300 is anticipated to reduce 5-HT_3 receptor activity to such an extent it potentially restores normal gut functioning, and thus does not cause constipation. In addition, the more modest affinity for the 5-HT_3 receptor that CSTI-300 has compared to alosetron will potentially allow local 5-HT to have more of an influence upon receptor activity. This is a concept shared with ondansetron, which has similar affinity to CSTI-300, and for which ischemic colitis is not seen in IBS-d patients, possibly due to 5-HT being able to out-compete ondansetron and allow some 5-HT_3 receptor activity (Garsed et al., 2014). However, ondansetron is known to activate the hERG ion channel (human ether-a-go-go-related gene), which in long-term administration could lead to cardiac side effects (Kuryshev et al., 2000; Chandrakala et al., 2008). This should not be an issue for CSTI-300, given it does not interact with hERG, meaning CSTI-300 should be a safe, effective, long-term treatment for IBS-d.

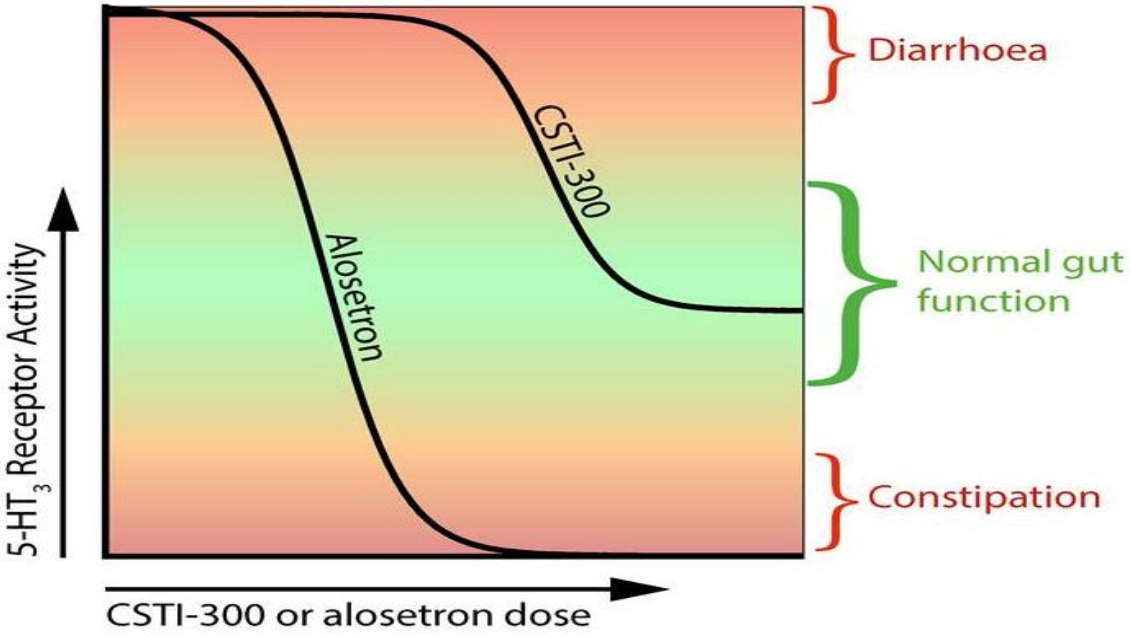

Figure 32. Theoretical mode of CSTI-300 function.

Elevated or reduced 5-HT_3 receptor activity is thought to be responsible for diarrhoea or constipation, respectively. Alosetron, a 5-HT_3 receptor antagonist, may cause constipation through 5-HT_3 receptor blockade. CSTI-300 is anticipated to reduce the activity of the 5-HT_3 receptor, leading to normal gut function, without complete blockade and hence will less likely cause constipation.

Previous studies have assessed 5-HT_3 receptor partial agonists for treating IBS. Pumosetrag (DDP, MKC-733) was evaluated for constipation dominant IBS rather than diarrhoea predominant. Interestingly, the intrinsic efficacy of pumosetrag varied depending on the species studied, with activity ranging from 20% to 100% of full agonist in rat or mouse colon (Evangelista, 2007; Chetty et al., 2008). Similar to CSTI-300, in the Bezold-Jarisch reflex in mouse, pumosetrag elicited transient bradycardia approximately 65% that of 5-HT. In rats treated with dopamine to delay gastric emptying, pumosetrag increased the rate of gastric motility (Evangelista, 2007). In healthy subjects, pumosetrag showed prokinetic activity, measured by an increase in migrating motor complexes in the duodenum (Coleman et al., 2003). In IBS-c patients, pumosetrag improved symptoms associated with bowel motility by increasing percent elimination and frequency of defecation (Fujita et al., 2005; Evangelista, 2007). As may have been anticipated with a 5-HT_3 receptor partial agonist, a common side effect

experienced by those administered with pumosetrag was nausea and vomiting, which ultimately lead to its withdrawal from further research (Coleman et al., 2003; Fujita et al., 2005; Evangelista, 2007). It may be of concern that this issue could arise when CSTI-300 is assessed in humans. However, oral administration of CSTI-300 to dog failed to elicit emesis, despite plasma concentrations of the drug reaching levels that would saturate the 5-HT_3 receptor. Moreover, there was no suggestion of an increase in gastric motility, and the behaviour of the dogs was unaffected by the drug. Furthermore, in pharmacokinetic studies in a variety of species (mouse, dog and mini-pig), CSTI-300 failed to evoke any emetic episodes, and these studies included supramaximal doses of CSTI-300 (up to 30 mg/kg). This data supports the theory that CSTI-300 could treat IBS-d, without evoking emesis or increasing gastrointestinal motility associated with 5-HT_3 receptor activation. In addition, CSTI-300 failed to interact with a large number (100) of receptors/channels, suggesting this drug will have limited off-target adverse effects.

As was mentioned previously (section 4.4.1, page 111), there is a case study demonstrating the efficacy of the 5-HT_3 receptor partial agonist vortioxetine in treating a patient with IBS-d and concurrent depression (Aydin et al., 2018). After 4 weeks of treatment, there was a global improvement in gastrointestinal symptoms (abdominal pain and frequency to defecate). As CSTI-300 has similar intrinsic activity to vortioxetine (see section 3.1), this further implies that CSTI-300 will be efficacious in treating the pain and discomfort patients with IBS-d experience.

Given the intrinsic activity of CSTI-300 at the 5-HT_3 receptor, it is interesting to speculate whether this drug could show promise in treating IBS-c as well as IBS-d. In contrast to IBS-d, 5-HT levels are lower in IBS-c patients. Following meal ingestion, plasma levels of 5-HT will rise in healthy volunteers as well as IBS-d patients, but this is not recorded in IBS-c patients (Dunlop et al., 2005; Atkinson et al., 2006). Furthermore, plasma concentrations of the 5-HT breakdown product, 5-HIAA, were lower in IBS-c patients compared to control subjects; both under fasting conditions and following meal ingestion (Dunlop et al., 2005; Atkinson et al., 2006). CSTI-300 might therefore be beneficial in increasing 5-HT_3 receptor activity to a level which

results in ‘normal gut functioning’ rather than constipation (Figure 32). Currently, prucalopride, a selective 5-HT_4 receptor agonist, is used to treat IBS-c when laxatives have failed (Thayalasekeran et al., 2013; Jiang et al., 2015). However, it has been shown that less than 50% of IBS-c patients report relief from symptoms when taking prucalopride (Jadav et al., 2013). Although speculative, CSTI-300 could show promise in treating IBS-c. Furthermore, the ability of CSTI-300 to modulate 5-HT_3 receptor such that it should result in normal gut functioning could lead to this drug demonstrating efficacy in IBS-m (mixed IBS). In this condition, symptoms fluctuate between constipation and diarrhoea (Lacy et al., 2009). By keeping 5-HT_3 receptor activity relatively consistent, CSTI-300 be a viable therapeutic for IBS-m, where currently no effective treatments are indicated.

4.2.3 Targeting the 5-HT_3 receptor to treat carcinoid syndrome

There is also potential for CSTI-300 to offer symptomatic relief to patients with carcinoid syndrome, whereby the associated diarrhoea is often debilitating. Gastrointestinal carcinoid tumours have an incidence rate of 1.5-2 per 100,000, with 64% of tumours arising from the small intestine and rectum (ileal tumours being the most common cause of carcinoid syndrome) (Ha et al., 2012). The frequency of carcinoid syndrome in patients with carcinoid tumours is increasing; with 11% of patients being diagnosed with carcinoid syndrome in 2000, compared to 19% of patients in 2011, perhaps due to improved diagnosis (Halperin et al., 2017). Most symptoms of carcinoid syndrome are caused by a hypersecretion of amines (most commonly 5-HT), resulting in raised plasma 5-HT levels (Spiller, 2007). The most common symptom experienced by patients with carcinoid syndrome is diarrhoea, which is reported in 83% of patients (49% also experience flushing) (Ha et al., 2012; Oronsky et al., 2017). Diagnosis of carcinoid syndrome is based on clinical presentation and urinary 5-HIAA levels, which will be raised compared to a healthy control (Lauffer et al., 1999; Ha et al., 2012; Oronsky et al., 2017; Rastogi et al., 2018).

A variety of treatments have been established for treating carcinoid syndrome. Traditionally, the first-line treatment for carcinoid syndrome was long acting forms of

somatostatin analogues (octreotide and lanreotide), both of which demonstrated a response rate of 50% (Riechelmann et al., 2017). Somatostatin is known to reduce gut hormone secretion via interactions with somatostatin receptors (SST_{1-5}) (Hoyer et al., 1995; Patel, 1999). Indeed, it was established octreotide decreased 5-HT release from isolated human carcinoid tumour cells (Wängberg et al., 1991). However, many patients receiving either of these drugs experience refractory carcinoid syndrome, whereby possibly due to tachyphylaxis, hormone related symptoms are no longer controlled by this treatment, and a new therapy is required (Riechelmann et al., 2017).

PCPA, a TPH inhibitor, the rate-limiting enzyme in 5-HT synthesis, has been trialled for treatment with some efficacy (flushing and diarrhoea were relived in 50-60% of patients, and urinary 5-HIAA was decreased by 80%) (Kvols, 1986; Ha et al., 2012). However, associated neuropsychiatric side effects (including confusion and depression) made this treatment option unusable (Kvols, 1986).

An oral peripherally acting TPH inhibitor, telotristat ethyl, was developed by Lexicon pharmaceuticals and approved to treat carcinoid syndrome in the USA and Europe in February and September 2017, respectively. This pro-drug is metabolised to the active telotristat, which cannot cross the blood-brain barrier and so should limit any CNS based adverse effects (Chan et al., 2018). Pre-clinical studies demonstrated that telotristat decreased plasma 5-HT in mice with experimental colitis, and also decreased levels of proinflammatory cytokines including IL-1β and IL-6 (Chan et al., 2018). In phase II clinical trials, telotristat decreased bowel movement in 23% of patients, and reduced urinary 5-HIAA by at least 50% (Masab et al., 2017; Chan et al., 2018). In these small studies, telotristat was well tolerated, with most side effects being gastrointestinal, and any serious adverse effects were not attributed to the drug (Masab et al., 2017).

Two phase III trials have been conducted to investigate telotristat, TELESTAR and TELECAST. Both examined the effects of 250 mg, 500 mg telotristat and placebo on 135 patients with carcinoid syndrome (Kulke et al., 2017; Pavel et al., 2018). In the

TELESTAR trials, at both doses of drug, bowel movement was reduced by approximately 40% (20% of patients receiving placebo responded) (Kulke et al., 2017). Urinary 5-HIAA was reduced by 78% and 87% respectively by 250 mg and 500 mg telotristat respectively; with placebo reducing urinary 5-HIAA in 10% of patients (Kulke et al., 2017). These primary outcomes were also replicated in the TELECAST trials (Pavel et al., 2018). Furthermore, 40% of patients receiving telotristat were defined as durable responders (they had over 30% decrease in bowel movement for more than 50% of the duration of the trial) in the TELECAST trials (Pavel et al., 2018). Patients receiving telotristat reported an improvement in quality of life compared to placebo, and although nausea was a reported side effect, no subject withdrew from the trial because of it, reinforcing the tolerability of this drug (Kulke et al., 2017; Masab et al., 2017). Although telotristat appears safe in these trials, the effects of telotristat need to be monitored by long-term post marketing surveillance, just in case any issues arise, especially given it will be decreasing total gastrointestinal 5-HT. As CSTI-300 only targets the 5-HT_3 receptor and has no off-target effects, it may be a safer long-term option in carcinoid syndrome (although again that needs to be evaluated).

A number of small studies have also been conducted to examine the impact of 5-HT_3 receptor antagonists to treat carcinoid syndrome. Administration of tropisetron improved diarrhoea (smaller stool size and less frequent defecation) in three cases of carcinoid syndrome, whilst also being well tolerated (Anderson et al., 1987). Furthermore, ondansetron improved the diarrhoea and vomiting associated with carcinoid syndrome in a single patient (Platt et al., 1992). This was also replicated in a separate study, whereby ondansetron improved diarrhoea (as well as nausea and vomiting) in 6 patients presenting with carcinoid syndrome (Wymenga et al., 1998). In addition, ondansetron offered relief to patients with carcinoid syndrome for whom octreotide and lanreotide failed to work or had refractory carcinoid syndrome (Kiesewetter et al., 2013; Kiesewetter et al., 2018). Clearly there is evidence that 5-HT_3 receptor antagonism has efficacy in treating carcinoid syndrome. In the knowledge 5-HT levels in patients with carcinoid syndrome are highly elevated, it is even more likely that a partial agonist would be useful, by displaying activity as a full antagonist. In addition, given the ability of CSTI-300 to reduce 5-HT_3 receptor activity

in the presence of excess 5-HT, this drug could be a viable therapeutic for treating the diarrhoea associated with carcinoid syndrome.

It may be of importance to further study the *in vitro* pharmacology of CSTI-300 at other 5-HT_3 receptor isoforms (5-HT_3AC, 5-HT_3AD, 5-HT_3AE), especially given their expression in the gastrointestinal tract (Kapeller et al., 2011). Moreover, CSTI-300 (and for comparison, alosetron), may need to be validated in an animal model of diarrhoea (5-HTP induced diarrhoea) to further demonstrate its effectiveness in IBS-d (Pascual et al., 2002). Furthermore, it may be of interest to see what impact CSTI-300 has upon experimentally-induced colitis, given alosetron appears to reduce inflammation (Motavallian et al., 2019). Finally, the safety of CSTI-300 should be investigated in long-term animal toxicology studies.

One limitation of this study is the use of only male rats in the colon distension model, which needs to be acknowledged given IBS-d impacts women more than men, and any possibly any sex differences in the gut of male and female rats (Afonso-Pereira et al., 2018). The pharmacology of CSTI-300 at the other heteromeric 5-HT_3 receptor complexes has not been considered, which may be crucial in the *in vivo* activity of CSTI-300, especially given expression of 5-HT3C, 5-HT3D and 5-HT3E subunits in human colon (Kapeller et al., 2011). The cell lines used to study the *in vitro* pharmacology of CSTI-300 are not clonal, i.e. each cell does not generate the same receptor density. Similarly, CSTI-300 may influence the 5-HT_{1P} receptor, and any impact this has on gastrointestinal function should be appreciated.

In summary, CSTI-300 is a potent, relatively high affinity, selective 5-HT_3 receptor partial agonist. In the mouse Bezold-Jarisch reflex, CSTI-300 was able to both elicit transient bradycardia (a response approximately 40% of 5-HT) and block 5-HT-induced bradycardia, indicative of 5-HT_3 receptor antagonistic properties in the presence of excess 5-HT. Furthermore, CSTI-300 demonstrated comparable efficacy to alosetron, an approved therapeutic for IBS-d, in a rodent model of the visceral sensation experienced by many IBS-d patients. Yet despite its intrinsic activity at the

5-HT_3 receptor, CSTI-300 did not evoke emesis in a variety of species (mouse, dog and mini-pig) studied. CSTI-300 could undergo further *in vivo* assays to determine if it slows or delays gastric motility (assessed by bead expulsion in rodent), with the animal having been first treated with a compound to increase colonic motility (e.g. dopamine). The safety and tolerability of CSTI-300 will be further studied by long-term toxicology studies in mini-pig. By maintaining basal 5-HT_3 receptor activity, CSTI-300 is predicted to increase the risk/benefit ratio. By not fully inhibiting the 5-HT_3 receptor, and therefore allowing some 5-HT signalling, CSTI-300 is predicted not to cause the constipation associated with 5-HT_3 receptor antagonists when used to treat IBS-d. CSTI-300 is also anticipated to have success in treating the diarrhoea associated with carcinoid syndrome, which is thought to be 5-HT_3 receptor mediated, and for which limited therapies are available.

4.3 The binding mechanism of Cl-indole at the h5-HT_3 receptor

4.3.1 Cl-indole has a unique binding model at the h5-HT_3 receptor

This study presents the findings from investigating the binding mechanism of the COM Cl-indole at the h5-HT_3 receptor; and how understanding this mechanism may be of importance for identifying potential novel 5-HT_3 receptor ligands for therapy. Although Cl-indole appears to interact with the h5-HT_3A receptor through a non-orthosteric binding site, it is apparent that in the presence of 5-HT_3 receptor agonist, Cl-indole will compete for the orthosteric h5-HT_3A receptor binding site. This is true for a variety of structurally diverse 5-HT_3 receptor agonists (with differing intrinsic efficacies), but this is not the case when a 5-HT_3 receptor antagonist is present. Interestingly, Cl-indole does not interact with the agonist binding site at the h5-HT_3AB receptor, even when 5-HT_3 receptor agonist is present, suggesting Cl-indole has a different mechanism of action at this heteromeric receptor compared to the homomeric h5-HT_3A receptor.

In the absence of 5-HT_3 receptor agonist, Cl-indole does not displace bound [^{3}H]-granisetron from h5-HT_3A orthosteric binding sites. However, the presence of 5-HT_3 receptor agonist (at sub-maximal concentrations) appears to impact the h5-HT_3A receptor so that Cl-indole (at concentrations above 3.0 µM, which demonstrate functional activity *in vitro*) now competes out [^{3}H]-granisetron in a concentration-dependent manner, akin to an orthosteric agonist. It is also interesting that the intrinsic activity of agonist, which range from 100% (5-HT) to 5% (s-zacopride) does not appear to correlate with ability to increase the affinity of Cl-indole for the h5-HT_3A receptor (i.e. all agonists allow Cl-indole to interact with the orthosteric site of the h5-HT_3A receptor). Interestingly, the affinity of a weak 5-HT_3 receptor partial agonist PBG is not impacted by 5-HT, suggesting this is a unique binding strategy for allosteric and not orthosteric ligands. It is hypothesised that Cl-indole (in the presence of 5-HT or a 5-HT_3 receptor partial agonist) will bind to the h5-HT_3A receptor and behave as a full agonist. This is responsible for the increased functional response elicited by agonist in the presence of Cl-indole (Newman et al., 2013).

The ability of 5-HT_3 receptor ligands to allow Cl-indole to interact with the orthosteric binding site of the h5-HT_3A receptor appears to be unique to agonists, as two 5-HT_3 receptor antagonists (metoclopramide and ondansetron) fail to influence the binding of Cl-indole for the h5-HT_3A receptor. This may occur because when bound, 5-HT_3 receptor antagonists do not induce a conformational change in the 5-HT_3 receptor; whilst it is predicted that this will occur when a 5-HT_3 receptor agonist binds. Moreover, it is thought that this conformational change is also involved in the positive cooperativity which has been well established for 5-HT_3 receptor agonists. Given the structural similarities between Cl-indole and other 5-HT_3 receptor agonists, it could transpire therefore that once agonist is bound, through positive cooperative mechanisms, Cl-indole is able to interact with other binding sites on the 5-HT_3 receptor which would otherwise engage agonist.

This mechanism of orthosteric binding may in part explain the functional activity of Cl-indole. It has been established that in *in vitro* and *ex vivo* functional assays Cl-indole behaves as a selective, relatively potent type I PAM at the 5-HT_3 receptor, i.e. it does not impact upon receptor desensitisation (Newman et al., 2013; Powell et al., 2016). Cl-indole has no efficacy at (and does bind to) the h5-HT_3A receptor in the absence of agonist, yet Cl-indole will increase the potency and efficacy of agonist (Newman et al., 2013). Further studies have elucidated the possible mechanism of Cl-indole action. In single cell electrophysiology, after removal of 5-HT following co-application of 5-HT and Cl-indole at the h5-HT_3A receptor, a tail current is recorded (Powell et al., 2016). Cl-indole appears to be responsible for this tail current, as the amplitude of the current is independent of initial 5-HT concentration. Furthermore, given the tail current can be blocked with prior application of ondansetron, it suggests Cl-indole has an orthosteric mechanism of action, which is only revealed when agonist is present (Powell et al., 2016). This complements the findings that the presence of agonist allows Cl-indole to compete for orthosteric binding sites at the h5-HT_3A receptor. This led to reidentifying Cl-indole as a cryptic orthosteric modulator rather than a positive allosteric modulator.

Interestingly, the binding mode of Cl-indole is not replicated at the h5-HT_3AB receptor. Even in the presence of 5-HT (300 nM), Cl-indole failed to interact with the orthosteric binding site of the h5-HT_3AB receptor, and the affinity of Cl-indole remained unaltered by 5-HT. This further strengthens the idea that the h5-HT_3AB receptor only has one A-A binding site (which orthosteric agonists can interact with) (Lochner et al., 2010). Moreover, Cl-indole does demonstrate efficacy at the h5-HT_3AB receptor, suggesting it may have activity as both an orthosteric ligand (at the h5-HT_3A receptor in the presence of agonist) and an allosteric ligand at the h5-HT_3AB receptor. Cl-indole may, like mCPBG, interact with A-B binding sites at the h5-HT_3AB receptor (Miles et al., 2015). Unlike at the 5-HT_3A receptor, in competition binding assays, agonists do not display positive cooperativity (see competition binding assays in vortioxetine or CSTI-300 results section) when interacting with the h5-HT_3AB receptor. Furthermore, despite Cl-indole demonstrating activity at the h5-HT_3AB receptor (to increase agonist efficacy) Cl-indole failed to elicit tail currents (Powell et al., 2016). This reiterates the principle that 5-HT allows Cl-indole to interact with an A-A orthosteric binding site at the h5-HT_3A receptor allowing a functional effect of Cl-indole. The unusual mechanism of action of Cl-indole is similar to the ago-allosteric modulator CGP7930 at the $GABA_B$ receptor (Schwartz et al., 2007). The main difference with Cl-indole however is the orthosteric activity is only revealed when agonist is present (Powell et al., 2016).

At the 5-HTBP, which is generated by mutating the AChBP so it is able to bind [^{3}H]-granisetron, 5-HT failed to demonstrate the same impact upon Cl-indole binding as was shown at the h5-HT_3A receptor. This suggests that the 5-HTBP is not necessarily a good model to use for trying to elucidate 5-HT_3 receptor binding interactions.

Lummis and colleagues have also tried to determine the mechanism of action for Cl-indole, using a chimera composed of the mouse 5-HT_3A receptor extracellular domain and the transmembrane domain of the ELIC (Erwinia ligand-gated ion channel), which is a prokaryotic homologue of Cys-loop receptors (Thompson et al., 2012; Price et al., 2018). After demonstrating this chimera has similar pharmacology to wild-type mouse 5-HT_3A receptor, it was discovered that 5-HT elicited responses are inhibited by Cl-

indole at this chimeric receptor, in contrast to what has been shown at the 5-HT_3 receptor. This may suggest that although Cl-indole interacts with the N-terminus of the 5-HT_3 receptor, the transmembrane is required for its functional activity. However, this work must be replicated using a h5-HT_3 receptor-ELIC chimera. Perhaps the lack of a transmembrane domain in the 5-HTBP is also partially the reason Cl-indole fails to replicate its binding mechanism with it.

4.3.2 Allosteric ligands interacting with other Cys-loop receptors

Allosteric ligands with unusual mechanisms of action of been studied in depth at other Cys-loop receptors. One of the best studied examples of ago-allosteric modulation is the compound 4BP-TQS, which demonstrates selective activity at the α7 nAChR. Initial characterisation of 4BP-TQS identified that this molecule, which unlike the compound it was based on (TQS, a type II PAM), demonstrates agonist activity at the α7 nAChR (TQS does not activate the α7 nAChR in the absence of agonist) (Gill et al., 2011). Interestingly, whereas a current elicited by acetylcholine at the α7 nAChR has a rapid onset and fast desensitisation, the current evoked by 4BP-TQS has a slower onset, and doesn't desensitise (Gill et al., 2011). Single channel studies identified that whilst acetylcholine elicits infrequent, short openings; 4BP-TQS induced responses have a longer mean open time and a shorter close time (Palczynska et al., 2012). This suggests that 4BP-TQS fundamentally activates the α7 nAChR through a different mechanism compared to classic nicotinic agonists such as acetylcholine. This complements the studies involving Cl-indole by highlighting how distinct the mechanism of action for allosteric ligands is compared to receptor agonists.

Mutational studies have attempted to pinpoint the binding site for 4BP-TQS (and it's active isomer, GAT107) (Thakur et al., 2013). Mutating methionine 253 to leucine (found in the transmembrane domain 2 region of the α7 nAChR) causes the effect of 4BP-TQS (and TQS) to be lost, whilst acetylcholine still retains activity (Gill et al., 2011). Moreover, mutations to tryptophan 148, which is close to the acetylcholine binding site, appear to have no impact of 4BP-TQS activity whilst reducing the affinity of acetylcholine (Gill et al., 2011). Mutations to other sites in the ligand binding domain

(e.g. tryptophan 55, tyrosine 93, cysteine 116) all render acetylcholine inactive, yet GAT107 activity is unaffected (Papke et al., 2014; Horenstein et al., 2016). Furthermore, the α7 nAChR selective antagonist methyllycaconitine blocks any response induced by 4BP-TQS, suggesting that the orthosteric binding site is still necessary for receptor activation by 4BP-TQS (Gill et al., 2011; Gill et al., 2013). It appears that 4BP-TQS activity is dependent somewhat on an interaction between the allosteric transmembrane domain and the orthosteric binding site. Indeed, modelling studies demonstrated that when GAT107 binds to the α7 nAChR, it triggers closure of loop C in the ligand binding domain, which is also seen when α7 nAChR agonists such as epibatidine bind (Grazioso et al., 2015).

4.3.3 Allosteric ligands in *in vivo* models of disease

Allosteric modulators (and ago-allosteric modulators or COMs) have many advantages as a clinical target compared to classic orthosteric ligands. Whilst an orthosteric binding site at a transmembrane receptor may have similarity across a family (e.g. 5-HT receptors), allosteric binding sites are often unique to each receptor, allowing receptor sub-type specific selectivity (Wang et al., 2009; Rocheville et al., 2010). This may lead to allosteric ligands having less adverse effects due to fewer interactions with 'off target' receptors, channels or transporters, leading to a safer biological profile (Rocheville et al., 2010; Grover, 2013). Ago-allosteric modulators will also have a dual effect, by having intrinsic efficacy and also being able to increase the efficacy and potency of endogenous ligand (Schwartz et al., 2006). Furthermore, an additional level of receptor chemistry increases the diversity of drug-like chemical spaces available to exploit (Rocheville et al., 2010).

There are several examples of allosteric (or ago-allosteric) modulators which demonstrate efficacy in *in vitro* assays and also display activity in *in vivo* models of various diseases. The previously mentioned selective α7 nAChR ago-allosteric modulator GAT107 was investigated for its impact on inflammatory or neuropathic pain in mice. In control conditions, GAT107 did not impact motor activity or coordination in mice, demonstrating a promising safety profile of GAT107 (Bagdas et al., 2016).

GAT107 reduced formalin induced nociceptive behaviour in mice; this response was blocked when mice were pre-treated with methyllycaconitine and was not apparent in α7 nAChR knock-out mice (Bagdas et al., 2016). Furthermore, GAT107 reversed lipopolysaccharide induced inflammatory pain and also reduced neuropathic pain (caused by sciatic nerve injury) (Bagdas et al., 2016). GAT107 does therefore appear to have potential to treat a plethora of different types of allodynia, with limited off-target effects. Indeed, it may be of interest to investigate the effect of Cl-indole in an *in vivo* assay, for example an animal model of constipation, whereby increasing 5-HT_3 receptor activity should increase gastrointestinal function (Evangelista, 2007; Taschler et al., 2015).

Allosteric ligands which display affinity for GPCRs have also been studied, including BQCA at the M_1 muscarinic receptor. In functional calcium assays, BQCA increased the potency of acetylcholine at the M_1 muscarinic receptor without demonstrating activity at any other muscarinic receptors (Grover, 2013). This translated in mouse models of schizophrenia, where for example BQCA reversed amphetamine induced hyperlocomotion (Grover, 2013). Allosteric ligands do therefore appear to have potential to treat a complication conditions where orthosteric small molecules may have limited efficacy or adverse effects.

Understanding the mechanism of action of Cl-indole at the h5-HT_3 receptor may aid in developing more potent ligands (which may have therapeutic benefit in IBS-c). Moreover, it may lead to the synthesis of NAMs. NAMs may have therapeutic potential in treating IBS-d, by subtly overactive reducing 5-HT_3 receptors without complete blockade (which is thought to be responsible for the constipation experienced by patients receiving 5-HT_3 receptor antagonists, see vortioxetine and CSTI-300 discussion sections).

Future experiments examining this unusual activity of Cl-indole include ligand docking computational studies to see if Cl-indole interacts with additional binding sites at the h5-HT_3A receptor as well as the orthosteric binding site. It may also be of interest to

see if mutations to the h5-HT_3A receptor which ablate 5-HT activity or binding also have the same effect for Cl-indole binding, or indeed if there are any residues which not impact 5-HT function but do impact Cl-indole binding. A photo-reactive Cl-indole (which covalently labels the 5-HT_3 receptor under UV conditions) could be used to identify the exact amino acid residues Cl-indole interacts with in the presence of 5-HT.

One limitation of this study is the binding mechanism of Cl-indole has been studied indirectly. To further cement how Cl-indole interacts with the h5-HT_3 receptor, the binding of Cl-indole may need to be monitored directly, possibly using [^{3}H]-labelled Cl-indole or a photo-reactive version.

Cl-indole demonstrates a unique pharmacological profile at the h5-HT_3 receptor. The presence of orthosteric agonist, not antagonist, allows Cl-indole to compete with [^{3}H]-granisetron for h5-HT_3A receptor binding sites; a mechanism which may explain its functional activity. To further elucidate the mechanism of Cl-indoles action, mutational studies to identify if specific residues within the orthosteric binding site of the h5-HT_3A receptor impact not only agonist activity but the functioning and binding of Cl-indole. Understanding the mechanism of Cl-indole action may aid in developing other allosteric 5-HT_3 receptor ligands, which could show therapeutic promise in IBS-d.

To conclude, this present thesis provides a detailed pharmacological investigation into three structurally diverse 5-HT_3 receptor ligands (vortioxetine, CSTI-300 and Cl-indole), and how each through different mechanisms of action could provide therapeutical promise in treating a variety of conditions (schizophrenia, IBS, carcinoid syndrome) where malfunction of the 5-HT_3 receptor is reported.

Appendix

Supplementary Table 1: Statistical analysis for Figure 21A.

Statistics were performed using 2-way ANOVA ($p \leq 0.05$) with Tukey post-hoc test.
ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

A= Vehicle

B=5-HTP (10 mg/kg)

-10 mins		**5 mins**		**20 mins**		**35 mins**		**50 mins**	
A vs. B	ns	A vs. B	****	A vs. B	****	A vs. B	****	A vs. B	****
- 5 mins		**10 mins**		**25 mins**		**40 mins**		**55 mins**	
A vs. B	ns	A vs. B	****	A vs. B	****	A vs. B	****	A vs. B	****
0 mins		**15 mins**		**30 mins**		**45 mins**		**60 mins**	
A vs. B	ns	A vs. B	****	A vs. B	****	A vs. B	****	A vs. B	****

Supplementary Table 2: Statistical analysis for Figure 21B.

Statistics were performed using 2-way ANOVA ($p \leq 0.05$) with Tukey post-hoc test. ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

B=Vehicle

C=Alosetron (0.01 mg/kg)

D=Alosetron (0.1 mg/kg)

E=Alosetron (1.0 mg/kg)

5 mins		**20 mins**		**35 mins**		**50 mins**	
B vs. C	ns	B vs. C	ns	B vs. C	*	B vs. C	*
B vs. D	ns	B vs. D	*	B vs. D	**	B vs. D	***
B vs. E	ns	B vs. E	ns	B vs. E	**	B vs. E	*
C vs. D	ns	C vs. D	ns	C vs. D	ns	C vs. D	ns
C vs. E	ns	C vs. E	ns	C vs. E	ns	C vs. E	ns
D vs. E	ns	D vs. E	ns	D vs. E	ns	D vs. E	ns
10 mins		**25 mins**		**40 mins**		**55 mins**	
B vs. C	ns	B vs. C	ns	B vs. C	ns	B vs. C	*
B vs. D	ns	B vs. D	ns	B vs. D	ns	B vs. D	**
B vs. E	ns	B vs. E	ns	B vs. E	***	B vs. E	ns
C vs. D	ns	C vs. D	ns	C vs. D	ns	C vs. D	ns
C vs. E	ns	C vs. E	ns	C vs. E	ns	C vs. E	ns
D vs. E	ns	D vs. E	ns	D vs. E	ns	D vs. E	ns
15 mins		**30 mins**		**45 mins**		**60 mins**	
B vs. C	ns	B vs. C	ns	B vs. C	ns	B vs. C	**
B vs. D	ns	B vs. D	ns	B vs. D	*	B vs. D	ns
B vs. E	ns	B vs. E	ns	B vs. E	*	B vs. E	**

C vs. D	ns	C vs. D	ns	C vs. D	ns	C vs. D	ns
C vs. E	ns	C vs. E	ns	C vs. E	ns	C vs. E	ns
D vs. E	ns	D vs. E	ns	D vs. E	ns	D vs. E	ns

Supplementary Table 3: Statistical analysis for Figure 21C.

Statistical analysis was performed using a 2-way ANOVA with Tukey post-hoc test. ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

A= Vehicle

B= CSTI-300 (1.0 mg/kg)

C= CSTI-300 (0.1 mg/kg)

D= CSTI-300 (0.01 mg/kg)

E= Alosetron (0.1 mg/kg)

5 mins		**20 mins**		**35 mins**		**50 mins**	
A vs. B	ns	A vs. B	*	A vs. B	****	A vs. B	****
A vs. C	ns	A vs. C	ns	A vs. C	****	A vs. C	ns
A vs. D	ns	A vs. D	ns	A vs. D	ns	A vs. D	ns
A vs. E	ns	A vs. E	*	A vs. E	****	A vs. E	****
B vs. C	ns	B vs. C	ns	B vs. C	ns	B vs. C	****
B vs. D	ns	B vs. D	ns	B vs. D	*	B vs. D	****
B vs. E	ns	B vs. E	ns	B vs. E	ns	B vs. E	ns
C vs. D	ns	C vs. D	ns	C vs. D	*	C vs. D	ns
C vs. E	ns	C vs. E	ns	C vs. E	ns	C vs. E	**
D vs. E	ns	D vs. E	ns	D vs. E	*	D vs. E	****
10 mins		**25 mins**		**40 mins**		**55 mins**	
A vs. B	ns	A vs. B	*	A vs. B	****	A vs. B	****
A vs. C	ns	A vs. C	ns	A vs. C	ns	A vs. C	**
A vs. D	ns	A vs. D	ns	A vs. D	ns	A vs. D	ns
A vs. E	ns	A vs. E	*	A vs. E	**	A vs. E	****
B vs. C	ns	B vs. C	ns	B vs. C	ns	B vs. C	ns

B vs. D	ns	B vs. D	ns	B vs. D	***	B vs. D	***
B vs. E	ns	B vs. E	ns	B vs. E	ns	B vs. E	ns
C vs. D	ns	C vs. D	ns	C vs. D	ns	C vs. D	ns
C vs. E	ns	C vs. E	ns	C vs. E	ns	C vs. E	ns
D vs. E	ns	D vs. E	ns	D vs. E	*	D vs. E	****
15 mins		**30 mins**		**45 mins**		**60 mins**	
A vs. B	ns	A vs. B	***	A vs. B	****	A vs. B	****
A vs. C	ns	A vs. C	ns	A vs. C	**	A vs. C	ns
A vs. D	ns	A vs. D	ns	A vs. D	ns	A vs. D	ns
A vs. E	ns	A vs. E	*	A vs. E	****	A vs. E	****
B vs. C	ns	B vs. C	ns	B vs. C	**	B vs. C	*
B vs. D	ns	B vs. D	ns	B vs. D	****	B vs. D	****
B vs. E	ns	B vs. E	ns	B vs. E	ns	B vs. E	ns
C vs. D	ns	C vs. D	ns	C vs. D	*	C vs. D	ns
C vs. E	ns	C vs. E	ns	C vs. E	ns	C vs. E	***
D vs. E	ns	D vs. E	ns	D vs. E	**	D vs. E	****

Supplementary Table 4: Pharmacological selectivity of CSTI-300.

CSTI-300 (1.0 μM) tested for the ability to interact with a wide variety of named targets assessed by the screening assays performed by either Cerep (www.cerep.fr), ChanTest (www.criver.com) or MDS Pharma Services (www.mdsps.com).

Target	Company	Species
5-HT_{1A} receptor	Cerep and MDS Pharma Services	Human
5-HT_{1B} receptor	Cerep	Rat
5-HT_{1D} receptor	Cerep	Rat
5-HT_{2A} receptor	Cerep	Human
5-HT_{2B} receptor	Cerep	Human
5-HT_{2C} receptor	Cerep	Human
5-HT_{4e} receptor	Cerep	Human
5-ht_{5a} receptor	Cerep	Human
5-HT_{6} receptor	Cerep	Human
5-HT_{7} receptor	Cerep	Human
Adenosine A_1 receptor	MDS Pharma Services	Human
Adenosine A_{2A} receptor	MDS Pharma Services	Human
Adenosine A_3 receptor	MDS Pharma Services	Human
Adrenergic α_{1A} receptor	MDS Pharma Services	Rat
Adrenergic α_{1B} receptor	MDS Pharma Services	Rat
Adrenergic α_{1D} receptor	MDS Pharma Services	Human
Adrenergic α_{2A} receptor	MDS Pharma Services	Human
Adrenergic α_1 receptor	MDS Pharma Services	Human

Adrenergic α_2 receptor	MDS Pharma Services	Human
Androgen (Testosterone) receptor	MDS Pharma Services	Rat
BK ($K_{Ca}1.1$)	ChanTest	Human
Bradykinin B_1 receptor	MDS Pharma Services	Human
Bradykinin B_2 receptor	MDS Pharma Services	Human
Calcium Channel L-Type, Benzothiazepine	MDS Pharma Services	Rat
Calcium Channel L-Type, Dihydropyridine	MDS Pharma Services	Rat
Calcium Channel L-Type (Cav1.2)	ChanTest	Human
Calcium Channel N-Type	MDS Pharma Services	Rat
Calcium Channel T-type (Cav3.2)	ChanTest	Human
Chloride Channel-1	ChanTest	Human
Cysteinyl Leukotriene $CysLT_1$ receptor	MDS Pharma Services	Human
Cystic Fibrosis Transmembrane Conductance Regulator	ChanTest	Human
Dopamine D_1 receptor	MDS Pharma Services	Human
Dopamine D_{2S} receptor	MDS Pharma Services	Human
Dopamine D_3 receptor	MDS Pharma Services	Human
Dopamine $D_{4.2}$ receptor	MDS Pharma Services	Human
Dopamine Transporter	MDS Pharma Services	Human
Endothelin ET_A receptor	MDS Pharma Services	Human
Endothelin ET_B receptor	MDS Pharma Services	Human
Epidermal Growth Factor	MDS Pharma Services	Human
GABA Transporter	MDS Pharma Services	Rat

$GABA_A$, Flunitrazepam receptor	MDS Pharma Services	Rat
$GABA_A$, Muscimol receptor	MDS Pharma Services	Rat
$GABA_{B1A}$ receptor	MDS Pharma Services	Human
Glucocorticoid receptor	MDS Pharma Services	Human
GPR103 receptor	MDS Pharma Services	Human
HCN2	ChanTest	Human
HCN4	ChanTest	Human
hERG	ChanTest and MDS Pharma Services	Human
Histamine H_1 receptor	MDS Pharma Services	Human
Histamine H_2 receptor	MDS Pharma Services	Human
Histamine H_3 receptor	MDS Pharma Services	Human
IK ($K_{Ca}3.1$)	ChanTest	Human
Imidazoline I_2 receptor	MDS Pharma Services	Rat
Interleukin IL-1 receptor	MDS Pharma Services	Mouse
Kainate receptor	MDS Pharma Services	Rat
K_{ATP}	MDS Pharma Services	Hamster
$K_{ir}2.1$	ChanTest	Human
$K_{ir}3.1/3.4$	ChanTest	Human
$K_{ir}6.2/SUR2A$	ChanTest	Human
$K_v1.3$	ChanTest	Human
$K_v1.5$	ChanTest	Human
$K_v4.3$	ChanTest	Human
KvLQT/mink	ChanTest	Human

Melatonin MT_1 receptor	MDS Pharma Services	Human
Muscarinic M_1 receptor	MDS Pharma Services	Human
Muscarinic M_2 receptor	MDS Pharma Services	Human
Muscarinic M_3 receptor	MDS Pharma Services	Human
Nicotinic α-BGTX-insensitive ($\alpha_4\beta_2$) receptor	Cerep	Rat
Nicotinic α-BGTX-sensitive (α_7) receptor	Cerep	Rat
$Na_v1.5$ Phasic	ChanTest	Human
$Na_v1.5$ Tonic	ChanTest	Human
Neuropeptide Y Y_1 receptor	MDS Pharma Services	Human
Neuropeptide Y Y_2 receptor	MDS Pharma Services	Human
Nicotinic Acetylcholine receptor	MDS Pharma Services	Human
Nicotinic Acetylcholine α_1 Bungarotoxin receptor	MDS Pharma Services	Human
NMDA, Agonism receptor	MDS Pharma Services	Rat
NMDA, Glycine receptor	MDS Pharma Services	Rat
NMDA, Phencyclidine receptor	MDS Pharma Services	Rat
Noradrenaline Transporter	MDS Pharma Services	Human
Oestrogen ERα receptor	MDS Pharma Services	Human
Opiate δ receptor	MDS Pharma Services	Human
Opiate κ receptor	MDS Pharma Services	Human
Opiate μ receptor	MDS Pharma Services	Human
$P2X_1$ receptor	ChanTest	Human
$P2X_3$ receptor	ChanTest	Human
Phorbol Ester	MDS Pharma Services	Mouse

Platelet Activating Factor	MDS Pharma Services	Human
Prostanoid EP_4 receptor	MDS Pharma Services	Human
Purinergic P2X receptor	MDS Pharma Services	Rabbit
Purinergic P2Y receptor	MDS Pharma Services	Rat
Rolipram	MDS Pharma Services	Rat
Serotonin Transporter	MDS Pharma Services	Human
Sigma σ_1 receptor	MDS Pharma Services	Human
Sigma σ_2 receptor	MDS Pharma Services	Rat
Sodium Channel Site 2	MDS Pharma Services	Rat
Tachykinin NK_1 receptor	MDS Pharma Services	Human
Thyroid Hormone	MDS Pharma Services	Rat
TRPV1	ChanTest	Human

References

Afonso-Pereira F, Dou L, Trenfield SJ, Madla CM, Murdan S, Sousa J*, et al.* (2018). Sex differences in the gastrointestinal tract of rats and the implications for oral drug delivery. European Journal of Pharmaceutical Sciences 115**:** 339-344.

Akhondzadeh S, Mohammadi N, Noroozian M, Karamghadiri N, Ghoreishi A, Jamshidi AH*, et al.* (2009). Added ondansetron for stable schizophrenia: a double blind, placebo controlled trial. Schizophr Res 107**:** 206-212.

Alvarez E, Perez V, & Artigas F (2014). Pharmacology and clinical potential of vortioxetine in the treatment of major depressive disorder. Neuropsychiatr Dis Treat 10**:** 1297-1307.

Amin AH, Crawford TBB, & Gaddum JH (1954). The distribution of substance P and 5-hydroxytryptamine in the central nervous system of the dog. The Journal of Physiology 126**:** 596-618.

Anderson JV, Coupe MO, Morris JA, Hodgson HJ, & Bloom SR (1987). Remission of symptoms in carcinoid syndrome with a new 5-hydroxytryptamine M receptor antagonist. British Medical Journal (Clinical research ed) 294**:** 1129-1129.

Andresen V, Montori VM, Keller J, West CP, Layer P, & Camilleri M (2008). Effects of 5-hydroxytryptamine (serotonin) type 3 antagonists on symptom relief and constipation in nonconstipated irritable bowel syndrome: a systematic review and meta-analysis of randomized controlled trials. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association 6**:** 545-555.

Argyle N, Deltito J, Allerup P, Maier W, Albus M, Nutzinger D*, et al.* (1991). The Panic-Associated Symptom Scale: measuring the severity of panic disorder. Acta psychiatrica Scandinavica 83**:** 20-26.

Ashoor A, Nordman JC, Veltri D, Yang KH, Shuba Y, Al Kury L*, et al.* (2013). Menthol inhibits 5-HT3 receptor-mediated currents. The Journal of pharmacology and experimental therapeutics 347**:** 398-409.

Atkinson W, Lockhart S, Whorwell PJ, Keevil B, & Houghton LA (2006). Altered 5-hydroxytryptamine signaling in patients with constipation- and diarrhea-predominant irritable bowel syndrome. Gastroenterology 130**:** 34-43.

Aydin O, & Ünal-Aydin P (2018). Vortioxetine treatment for major depressive disorder with the co-morbidity of irritable bowel syndrome with diarrhoea: a case report. Psychiatry and Clinical Psychopharmacology 28**:** 107-109.

Bagdas D, Wilkerson JL, Kulkarni A, Toma W, AlSharari S, Gul Z*, et al.* (2016). The alpha7 nicotinic receptor dual allosteric agonist and positive allosteric modulator GAT107 reverses nociception in mouse models of inflammatory and neuropathic pain. British journal of pharmacology 173**:** 2506-2520.

Baldwin DS, Florea I, Jacobsen PL, Zhong W, & Nomikos GG (2016). A meta-analysis of the efficacy of vortioxetine in patients with major depressive disorder (MDD) and high levels of anxiety symptoms. Journal of Affective Disorders 206**:** 140-150.

Bang-Andersen B, Ruhland T, Jørgensen M, Smith G, Frederiksen K, Jensen KG*, et al.* (2011). Discovery of 1-[2-(2,4-Dimethylphenylsulfanyl)phenyl]piperazine (Lu AA21004): A Novel Multimodal Compound for the Treatment of Major Depressive Disorder. Journal of Medicinal Chemistry 54**:** 3206-3221.

Banner SE, Carter M, & Sanger GJ (1995). 5-Hydroxytryptamine3 receptor antagonism modulates a noxious visceral pseudoaffective reflex. Neuropharmacology 34**:** 263-267.

Banner SE, & Sanger GJ (1995). Differences between 5-HT3 receptor antagonists in modulation of visceral hypersensitivity. British Journal of Pharmacology 114**:** 558-562.

Barann M, Linden I, Witten S, & Urban BW (2008). Molecular actions of propofol on human 5-HT3A receptors: enhancement as well as inhibition by closely related phenol derivatives. Anesth Analg 106**:** 846-857, table of contents.

Barann M, Molderings G, Bruss M, Bonisch H, Urban BW, & Gothert M (2002). Direct inhibition by cannabinoids of human 5-HT3A receptors: probable involvement of an allosteric modulatory site. Br J Pharmacol 137**:** 589-596.

Barnes JM, Barnes NM, Costall B, Ironside JW, & Naylor RJ (1989). Identification and characterisation of 5-hydroxytryptamine 3 recognition sites in human brain tissue. J Neurochem 53**:** 1787-1793.

Barnes NM, Ahern GP, Becamel C, Bockaert J, Camilleri M, Chaumont-Dubel S, *et al*. (2019). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine; pharmacology and function. Pharmacological reviews: ahead of e-print.

Barnes NM, Hales TG, Lummis SC, & Peters JA (2009). The 5-HT3 receptor--the relationship between structure and function. Neuropharmacology 56**:** 273-284.

Barnes NM, & Sharp T (1999). A review of central 5-HT receptors and their function. Neuropharmacology 38**:** 1083-1152.

Basak S, Gicheru Y, Samanta A, Molugu SK, Huang W, Fuente M*, et al.* (2018). Cryo-EM structure of 5-HT3A receptor in its resting conformation. Nat Commun 9**:** 514.

Bearcroft CP, Perrett D, & Farthing MJ (1998). Postprandial plasma 5-hydroxytryptamine in diarrhoea predominant irritable bowel syndrome: a pilot study. Gut 42**:** 42-46.

Beck AT, Ward CH, Mendelson M, Mock J, & Erbaugh J (1961). An inventory for measuring depression. Arch Gen Psychiatry 4**:** 561-571.

Beene DL, Brandt GS, Zhong W, Zacharias NM, Lester HA, & Dougherty DA (2002). Cation-pi interactions in ligand recognition by serotonergic (5-HT3A) and nicotinic acetylcholine receptors: the anomalous binding properties of nicotine. Biochemistry 41**:** 10262-10269.

Beene DL, Price KL, Lester HA, Dougherty DA, & Lummis SCR (2004). Tyrosine Residues That Control Binding and Gating in the 5-Hydroxytryptamine₃ Receptor Revealed by Unnatural Amino Acid Mutagenesis. The Journal of Neuroscience 24**:** 9097-9104.

Bennett JL, & Aghajanian GK (1974). d-LSD binding to brain homogenates: possible relationship to serotonin receptors. Life sciences 15**:** 1935-1944.

Berger M, Gray JA, & Roth BL (2009). The expanded biology of serotonin. Annual review of medicine 60**:** 355-366.

Berhan A, & Barker A (2014). Vortioxetine in the treatment of adult patients with major depressive disorder: a meta-analysis of randomized double-blind controlled trials. BMC psychiatry 14**:** 276.

Bermudez J, Boyle EA, Miner WD, & Sanger GJ (1988). The anti-emetic potential of the 5-hydroxytryptamine3 receptor antagonist BRL 43694. British journal of cancer 58**:** 644-650.

Betry C, Overstreet D, Haddjeri N, Pehrson AL, Bundgaard C, Sanchez C*, et al.* (2015). A 5-HT3 receptor antagonist potentiates the behavioral, neurochemical and electrophysiological actions of an SSRI antidepressant. Pharmacology, biochemistry, and behavior 131**:** 136-142.

Billio A, Morello E, & Clarke MJ (2010). Serotonin receptor antagonists for highly emetogenic chemotherapy in adults. The Cochrane database of systematic reviews**:** Cd006272.

Binet V, Brajon C, Le Corre L, Acher F, Pin JP, & Prezeau L (2004). The heptahelical domain of GABA(B2) is activated directly by CGP7930, a positive allosteric modulator of the GABA(B) receptor. J Biol Chem 279**:** 29085-29091.

Bockaert J, Claeysen S, Becamel C, Dumuis A, & Marin P (2006). Neuronal 5-HT metabotropic receptors: fine-tuning of their structure, signaling, and roles in synaptic modulation. Cell Tissue Res 326**:** 553-572.

Boess FG, Steward LJ, Steele JA, Liu D, Reid J, Glencorse TA*, et al.* (1997). Analysis of the ligand binding site of the 5-HT3 receptor using site directed mutagenesis: importance of glutamate 106. Neuropharmacology 36**:** 637-647.

Bonaventure P, Dugovic C, Kramer M, De Boer P, Singh J, Wilson S*, et al.* (2012). Translational evaluation of JNJ-18038683, a 5-hydroxytryptamine type 7 receptor antagonist, on rapid eye movement sleep and in major depressive disorder. The Journal of pharmacology and experimental therapeutics 342**:** 429-440.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72**:** 248-254.

Brady CA, Dover TJ, Massoura AN, Princivalle AP, Hope AG, & Barnes NM (2007). Identification of 5-HT3A and 5-HT3B receptor subunits in human hippocampus. Neuropharmacology 52**:** 1284-1290.

Brady CA, Stanford IM, Ali I, Lin L, Williams JM, E. Dubin A*, et al.* (2001). Pharmacological comparison of human homomeric 5-HT3A receptors versus heteromeric 5-HT3A/3B receptors. Neuropharmacology 41**:** 282-284.

Branchek TA, Mawe GM, & Gershon MD (1988). Characterization and localization of a peripheral neural 5-hydroxytryptamine receptor subtype (5-HT1P) with a selective agonist, 3H-5-hydroxyindalpine. J Neurosci 8**:** 2582-2595.

Breslin HJ, Diamond CJ, Kavash RW, Cai C, Dyatkin AB, Miskowski TA*, et al.* (2012). Identification of a dual δ OR antagonist/μ OR agonist as a potential therapeutic for diarrhea-predominant Irritable Bowel Syndrome (IBS-d). Bioorganic & Medicinal Chemistry Letters 22**:** 4869-4872.

Butini S, Budriesi R, Hamon M, Morelli E, Gemma S, Brindisi M*, et al.* (2009). Novel, potent, and selective quinoxaline-based 5-HT(3) receptor ligands. 1. Further structure-activity relationships and pharmacological characterization. J Med Chem 52**:** 6946-6950.

Butler AS, Lindesay SA, Dover TJ, Kennedy MD, Patchell VB, Levine BA*, et al.* (2009). Importance of the C-terminus of the human 5-HT3A receptor subunit. Neuropharmacology 56**:** 292-302.

Camilleri M (2009). Serotonin in the Gastrointestinal Tract. Current opinion in endocrinology, diabetes, and obesity 16**:** 53-59.

Camilleri M (2017). Medical Therapies in the Pipeline for Irritable Bowel Syndrome. Gastroenterology & hepatology 13**:** 550-552.

Camilleri M, & Boeckxstaens G (2017). Dietary and pharmacological treatment of abdominal pain in IBS. Gut 66**:** 966-974.

Campiani G, Cappelli A, Nacci V, Anzini M, Vomero S, Hamon M*, et al.* (1997). Novel and highly potent 5-HT3 receptor agonists based on a pyrroloquinoxaline structure. J Med Chem 40**:** 3670-3678.

Canavan C, West J, & Card T (2014). The epidemiology of irritable bowel syndrome. Clin Epidemiol 6**:** 71-80.

Candiotti KA, Ahmed SR, Cox D, & Gan TJ (2014). Palonosetron versus ondansetron as rescue medication for postoperative nausea and vomiting: a randomized, multicenter, open-label study. BMC pharmacology & toxicology 15**:** 45.

Celli J, Rappold G, & Niesler B (2017). The Human Serotonin Type 3 Receptor Gene (HTR3A-E) Allelic Variant Database. Human Mutation 38**:** 137-147.

Chan DL, & Singh S (2018). Developments in the treatment of carcinoid syndrome – impact of telotristat. Therapeutics and Clinical Risk Management 14**:** 323-329.

Chandrakala R, Vijayashankara CN, Kumar KK, & Sarala N (2008). Ondansetron induced fatal ventricular tachycardia. Indian journal of pharmacology 40**:** 186-187.

Chang L, Chey WD, Harris L, Olden K, Surawicz C, & Schoenfeld P (2006). Incidence of ischemic colitis and serious complications of constipation among patients using alosetron: systematic review of clinical trials and post-marketing surveillance data. Am J Gastroenterol 101**:** 1069-1079.

Chang WP, & Peng YX (2018). Does the Oral Administration of Ginger Reduce Chemotherapy-Induced Nausea and Vomiting?: A Meta-analysis of 10 Randomized Controlled Trials. Cancer Nurs.

Chetty N, Irving HR, & Coupar IM (2008). Effects of the novel 5-HT3 agonist MKC-733 on the rat, mouse and guinea pig digestive tract. Pharmacology 81**:** 104-109.

Chey WD, & Cash BD (2005). Cilansetron: a new serotonergic agent for the irritable bowel syndrome with diarrhoea. Expert Opin Investig Drugs 14**:** 185-193.

Chey WD, Kurlander J, & Eswaran S (2015). Irritable bowel syndrome: a clinical review. Jama 313**:** 949-958.

Choi JS, Choi BH, Ahn HS, Kim MJ, Rhie DJ, Yoon SH*, et al.* (2003). Mechanism of block by fluoxetine of 5-hydroxytryptamine3 (5-HT3)-mediated currents in NCB-20 neuroblastoma cells. Biochem Pharmacol 66**:** 2125-2132.

Choung RS, Locke GR, 3rd, Francis DD, Katzka D, Winkle PJ, Orr WC*, et al.* (2014). Novel partial 5HT3 agonist pumosetrag reduces acid reflux events in uninvestigated GERD patients after a standard refluxogenic meal: a randomized, double-blind, placebo-controlled pharmacodynamic study. Neurogastroenterol Motil 26**:** 13-20.

Cipriani A, Furukawa TA, Salanti G, Chaimani A, Atkinson LZ, Ogawa Y*, et al.* (2018). Comparative efficacy and acceptability of 21 antidepressant drugs for the acute treatment of adults with major depressive disorder: a systematic review and network meta-analysis. The Lancet.

Cohen ML, Bloomquist W, Gidda JS, & Lacefield W (1989). Comparison of the 5-HT3 receptor antagonist properties of ICS 205-930, GR38032F and zacopride. Journal of Pharmacology and Experimental Therapeutics 248**:** 197.

Coleman NS, Marciani L, Blackshaw E, Wright J, Parker M, Yano T*, et al.* (2003). Effect of a novel 5-HT3 receptor agonist MKC-733 on upper gastrointestinal motility in humans. Aliment Pharmacol Ther 18**:** 1039-1048.

Corradi J, & Bouzat C (2014). Unraveling mechanisms underlying partial agonism in 5-HT3A receptors. J Neurosci 34**:** 16865-16876.

Cote F, Fligny C, Fromes Y, Mallet J, & Vodjdani G (2004). Recent advances in understanding serotonin regulation of cardiovascular function. Trends in molecular medicine 10**:** 232-238.

Crowell MD (2004). Role of serotonin in the pathophysiology of the irritable bowel syndrome. Br J Pharmacol 141**:** 1285-1293.

Dale E, Zhang H, Leiser SC, Chao Y, Yang C, Plath N*, et al.* (2013). P.2.e.004 Vortioxetine (Lu AA21004) disinhibits pyramidal cell output and enhances theta rhythms and long-term plasticity in the hippocampus. European Neuropsychopharmacology 23, Supplement 2**:** S394.

Dang H, England PM, Farivar SS, Dougherty DA, & Lester HA (2000). Probing the Role of a Conserved M1 Proline Residue in 5-Hydroxytryptamine₃ Receptor Gating. Molecular pharmacology 57**:** 1114-1122.

Davies P, Pistis M, Hanna M, Peters J, Lambert J, Hales TG*, et al.* (1999) *The 5-HT3B subunit is a major determinant of serotonin-receptor function*. vol. 397.

De Vries P, Sánchez-López A, Centurión D, Heiligers JPC, Saxena PR, & Villalón CM (1998). The canine external carotid vasoconstrictor 5-HT1 receptor: blockade by 5-HT1B (SB224289), but not by 5-HT1D (BRL15572) receptor antagonists. European Journal of Pharmacology 362**:** 69-72.

Depot M, Caille G, Mukherjee J, Katzman MA, Cadieux A, & Bradwejn J (1999). Acute and chronic role of 5-HT3 neuronal system on behavioral and neuroendocrine changes induced by intravenous cholecystokinin tetrapeptide administration in humans. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 20**:** 177-187.

Drossman DA, & Hasler WL (2016). Rome IV-Functional GI Disorders: Disorders of Gut-Brain Interaction. Gastroenterology 150**:** 1257-1261.

Dubin AE, Huvar R, D'Andrea MR, Pyati J, Zhu JY, Joy KC*, et al.* (1999). The Pharmacological and Functional Characteristics of the Serotonin 5-HT3A Receptor Are Specifically Modified by a 5-HT3B Receptor Subunit. Journal of Biological Chemistry 274**:** 30799-30810.

Dunlop SP, Coleman NS, Blackshaw E, Perkins AC, Singh G, Marsden CA*, et al.* (2005). Abnormalities of 5-hydroxytryptamine metabolism in irritable bowel syndrome. Clinical Gastroenterology and Hepatology 3**:** 349-357.

E Adler L, M Cawthra E, A Donovan K, G Harris J, Nagamoto H, Olincy A*, et al.* (2005) *Improved P50 Auditory Gating With Ondansetron in Medicated Schizophrenia Patients*. vol. 162.

Egerton-Warburton D, Meek R, Mee MJ, & Braitberg G (2014). Antiemetic use for nausea and vomiting in adult emergency department patients: randomized controlled trial comparing ondansetron, metoclopramide, and placebo. Annals of emergency medicine 64**:** 526-532.e521.

Eglen RM, Lee CH, Smith WL, Johnson LG, Clark R, Whiting RL*, et al.* (1995). Pharmacological characterization of RS 25259-197, a novel and selective 5-HT3 receptor antagonist, in vivo. Br J Pharmacol 114**:** 860-866.

Elhag W, El Ansari W, Razaq S, Elsherif M, & Mustafa I (2019). Lorcaserin vs. Phentermine among non-surgical and surgical obese patients: Anthropometric, glycemic, lipid, safety and cost outcomes. Ann Med Surg (Lond) 45**:** 75-81.

Enck P, Aziz Q, Barbara G, Farmer AD, Fukudo S, Mayer EA*, et al.* (2016). Irritable bowel syndrome. Nat Rev Dis Primers 2**:** 16014.

Erspamer V, & Asero B (1952). Identification of Enteramine, the Specific Hormone of the Enterochromaffin Cell System, as 5-Hydroxytryptamine. Nature 169**:** 800.

Evangelista S (2007). Drug evaluation: Pumosetrag for the treatment of irritable bowel syndrome and gastroesophageal reflux disease. Current opinion in investigational drugs (London, England : 2000) 8**:** 416-422.

Fakhfouri G, Rahimian R, Ghia J-E, Khan WI, & Dehpour AR (2012). Impact of 5-HT3 receptor antagonists on peripheral and central diseases. Drug Discovery Today 17**:** 741-747.

Färber L, Stratz T, Brückle W, Späth M, Pongratz D, Lautenschläger J*, et al.* (2000). Efficacy and tolerability of tropisetron in primary fibromyalgia--a highly selective and competitive 5-HT3 receptor antagonist. German Fibromyalgia Study Group. Scandinavian journal of rheumatology Supplement 113**:** 49-54.

Faure C, Patey N, Gauthier C, Brooks EM, & Mawe GM (2010). Serotonin Signaling Is Altered in Irritable Bowel Syndrome With Diarrhea but Not in Functional Dyspepsia in Pediatric Age Patients. Gastroenterology 139**:** 249-258.

Fda.gov. (2016). *Lotronex (alosetron hydrochloride) Information*. [online] Available at: https://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatients andProviders/ucm110450.htm [Accessed 27 Jan. 2019].

Feinberg-Zadek PL, & Davies PA (2010). Ethanol stabilizes the open state of single 5-hydroxytryptamine(3A)(QDA) receptors. The Journal of pharmacology and experimental therapeutics 333**:** 896-902.

Fidalgo S, Ivanov DK, & Wood SH (2013). Serotonin: from top to bottom. Biogerontology 14**:** 21-45.

Fiebich BL, Akundi RS, Seidel M, Geyer V, Haus U, Muller W*, et al.* (2004). Expression of 5-HT3A receptors in cells of the immune system. Scandinavian journal of rheumatology Supplement 119**:** 9-11.

Fifi AC, Axelrod CH, Chakraborty P, & Saps M (2018). Herbs and Spices in the Treatment of Functional Gastrointestinal Disorders: A Review of Clinical Trials. Nutrients 10.

Florea I, Loft H, Danchenko N, Rive B, Brignone M, Merikle E*, et al.* (2017). The effect of vortioxetine on overall patient functioning in patients with major depressive disorder. Brain and Behavior 7**:** e00622.

Foley S, Garsed K, Singh G, Duroudier NP, Swan C, Hall IP*, et al.* (2011). Impaired uptake of serotonin by platelets from patients with irritable bowel syndrome correlates with duodenal immune activation. Gastroenterology 140**:** 1434-1443.e1431.

Ford AC, Brandt LJ, Young C, Chey WD, Foxx-Orenstein AE, & Moayyedi P (2009). Efficacy of 5-HT3 antagonists and 5-HT4 agonists in irritable bowel syndrome: systematic review and meta-analysis. Am J Gastroenterol 104**:** 1831-1843; quiz 1844.

Fozard JR (1984). MDL 72222: a potent and highly selective antagonist at neuronal 5-hydroxytryptamine receptors. Naunyn-Schmiedeberg's archives of pharmacology 326**:** 36-44.

Fragkos KC (2017). Spotlight on eluxadoline for the treatment of patients with irritable bowel syndrome with diarrhea. Clin Exp Gastroenterol 10**:** 229-240.

Freeman SL, Glatzle J, Robin CS, Valdellon M, Sternini C, Sharp JW*, et al.* (2006). Ligand-induced 5-HT3 receptor internalization in enteric neurons in rat ileum. Gastroenterology 131**:** 97-107.

Fujita T, Yokota S, Sawada M, Majima M, Ohtani Y, & Kumagai Y (2005). Effect of MKC-733, a 5-HT receptor partial agonist, on bowel motility and symptoms in subjects with constipation: an exploratory study. J Clin Pharm Ther 30**:** 611-622.

Fukudo S, Kinoshita Y, Okumura T, Ida M, Hayashi K, Akiho H*, et al.* (2016). Effect of ramosetron in female patients with irritable bowel syndrome with diarrhea: a phase III long-term study. J Gastroenterol 51**:** 874-882.

Gaddum JH, & Picarelli ZP (1957). Two kinds of tryptamine receptor. British Journal of Pharmacology and Chemotherapy 12**:** 323-328.

Galligan JJ (2007). Actions of sumatriptan on myenteric neurones: relief from an old headache in the enteric nervous system? Neurogastroenterol Motil 19**:** 1-3.

Garsed K, Chernova J, Hastings M, Lam C, Marciani L, Singh G*, et al.* (2014). A randomised trial of ondansetron for the treatment of irritable bowel syndrome with diarrhoea. Gut 63**:** 1617-1625.

Gershon MD (2004). Review article: serotonin receptors and transporters -- roles in normal and abnormal gastrointestinal motility. Alimentary pharmacology & therapeutics 20 Suppl 7**:** 3-14.

Ghia JE, Li N, Wang H, Collins M, Deng Y, El–Sharkawy RT*, et al.* (2009). Serotonin Has a Key Role in Pathogenesis of Experimental Colitis. Gastroenterology 137**:** 1649-1660.

Gill JK, Chatzidaki A, Ursu D, Sher E, & Millar NS (2013). Contrasting properties of alpha7-selective orthosteric and allosteric agonists examined on native nicotinic acetylcholine receptors. PLoS One 8**:** e55047.

Gill JK, Savolainen M, Young GT, Zwart R, Sher E, & Millar NS (2011). Agonist activation of alpha7 nicotinic acetylcholine receptors via an allosteric transmembrane site. Proc Natl Acad Sci U S A 108**:** 5867-5872.

Goodfellow NM, Bailey CDC, & Lambe EK (2012). The native serotonin 5-HT(5A) receptor: electrophysiological characterization in rodent cortex and 5-HT(1A)-mediated compensatory plasticity in the knock-out mouse. The Journal of neuroscience : the official journal of the Society for Neuroscience 32**:** 5804-5809.

Gothert M (2013). Serotonin discovery and stepwise disclosure of 5-HT receptor complexity over four decades. Part I. General background and discovery of serotonin as a basis for 5-HT receptor identification. Pharmacological reports : PR 65**:** 771-786.

Grailhe R, Grabtree GW, & Hen R (2001). Human 5-HT(5) receptors: the 5-HT(5A) receptor is functional but the 5-HT(5B) receptor was lost during mammalian evolution. Eur J Pharmacol 418**:** 157-167.

Granados-Soto V, Argüelles CF, Rocha-González HI, Godínez-Chaparro B, Flores-Murrieta FJ, & Villalón CM (2010). The role of peripheral 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E and 5-HT1F serotonergic receptors in the reduction of nociception in rats. Neuroscience 165**:** 561-568.

Grazioso G, Sgrignani J, Capelli R, Matera C, Dallanoce C, De Amici M*, et al.* (2015). Allosteric Modulation of Alpha7 Nicotinic Receptors: Mechanistic Insight through Metadynamics and Essential Dynamics. J Chem Inf Model 55**:** 2528-2539.

Grenett HE, Ledley FD, Reed LL, & Woo SL (1987). Full-length cDNA for rabbit tryptophan hydroxylase: functional domains and evolution of aromatic amino acid hydroxylases. Proceedings of the National Academy of Sciences of the United States of America 84**:** 5530-5534.

Grover AK (2013). Use of allosteric targets in the discovery of safer drugs. Med Princ Pract 22**:** 418-426.

Grover M, & Camilleri M (2014). Ramosetron in irritable bowel syndrome with diarrhea: new hope or the same old story? Clin Gastroenterol Hepatol 12**:** 960-962.

Gunn D, Garsed K, Lam C, Singh G, Lingaya M, Wahl V*, et al.* (2019). Abnormalities of mucosal serotonin metabolism and 5-HT3 receptor subunit 3C polymorphism in irritable bowel syndrome with diarrhoea predict responsiveness to ondansetron. Alimentary Pharmacology & Therapeutics 50**:** 538-546.

Ha J, & A T (2012) *Gastrointestinal Carcinoid Tumours – A Review*. vol. 2.

Halperin DM, Shen C, Dasari A, Xu Y, Chu Y, Zhou S*, et al.* (2017). Frequency of carcinoid syndrome at neuroendocrine tumour diagnosis: a population-based study. Lancet Oncol 18**:** 525-534.

Hamilton M (1959). The assessment of anxiety states by rating. Br J Med Psychol 32**:** 50-55.

Hamilton M (1960). A rating scale for depression. J Neurol Neurosurg Psychiatry 23**:** 56-62.

Harmer CJ, Reid CB, Ray MK, Goodwin GM, & Cowen PJ (2006). 5HT(3) antagonism abolishes the emotion potentiated startle effect in humans. Psychopharmacology (Berl) 186**:** 18-24.

Hassaine G, Deluz C, Grasso L, Wyss R, Tol MB, Hovius R*, et al.* (2014). X-ray structure of the mouse serotonin 5-HT3 receptor. Nature 512**:** 276-281.

Haus U, Varga B, Stratz T, Spath M, & Muller W (2000). Oral treatment of fibromyalgia with tropisetron given over 28 days: influence on functional and vegetative symptoms, psychometric parameters and pain. Scand J Rheumatol Suppl 113**:** 55-58.

Hayrapetyan V, Jenschke M, Dillon GH, & Machu TK (2005). Co-expression of the 5-HT(3B) subunit with the 5-HT(3A) receptor reduces alcohol sensitivity. Brain Res Mol Brain Res 142**:** 146-150.

Hermann B, Wetzel CH, Pestel E, Zieglgansberger W, Holsboer F, & Rupprecht R (1996). Functional antagonistic properties of clozapine at the 5-HT3 receptor. Biochemical and biophysical research communications 225**:** 957-960.

Hirata T, Keto Y, Funatsu T, Akuzawa S, & Sasamata M (2007). Evaluation of the Pharmacological Profile of Ramosetron, a Novel Therapeutic Agent for Irritable Bowel Syndrome. Journal of Pharmacological Sciences 104**:** 263-273.

Hodge C, Kelley S, Bratt A, Iller K, P Schroeder J, & Besheer J (2004) *5-HT3A Receptor Subunit is Required for 5-HT3 Antagonist-Induced Reductions in Alcohol Drinking*. vol. 29.

Holbrook JD, Gill CH, Zebda N, Spencer JP, Leyland R, Rance KH*, et al.* (2009). Characterisation of 5-HT3C, 5-HT3D and 5-HT3E receptor subunits: evolution, distribution and function. J Neurochem 108**:** 384-396.

Horenstein NA, Papke RL, Kulkarni AR, Chaturbhuj GU, Stokes C, Manther K*, et al.* (2016). Critical Molecular Determinants of α7 Nicotinic Acetylcholine Receptor Allosteric Activation: SEPARATION OF DIRECT ALLOSTERIC ACTIVATION AND POSITIVE ALLOSTERIC MODULATION. Journal of Biological Chemistry 291**:** 5049-5067.

Houghton LA, Lea R, Jackson N, & Whorwell PJ (2002). The menstrual cycle affects rectal sensitivity in patients with irritable bowel syndrome but not healthy volunteers. Gut 50**:** 471-474.

Hoyer D, Bell GI, Berelowitz M, Epelbaum J, Feniuk W, Humphrey PP*, et al.* (1995). Classification and nomenclature of somatostatin receptors. Trends in pharmacological sciences 16**:** 86-88.

Hoyer D, Hannon JP, & Martin GR (2002). Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacology Biochemistry and Behavior 71**:** 533-554.

Hoyer D, Waeber C, Schoeffter P, Palacios JM, & Dravid A (1989). 5-HT1C receptor-mediated stimulation of inositol phosphate production in pig choroid plexus. A pharmacological characterization. Naunyn Schmiedebergs Arch Pharmacol 339**:** 252-258.

Hrycaj P (2004). Serotonin type 3 receptor antagonist tropisetron in the treatment of chronic inflammatory rheumatic conditions – preliminary clinical experience. Scandinavian Journal of Rheumatology 33**:** 55-58.

Ilegems E, Pick HM, Deluz C, Kellenberger S, & Vogel H (2004). Noninvasive imaging of 5-HT3 receptor trafficking in live cells: from biosynthesis to endocytosis. J Biol Chem 279**:** 53346-53352.

Israelyan N, Del Colle A, Li Z, Park Y, Xing A, Jacobsen JPR*, et al.* (2019). Effects of Serotonin and Slow-Release 5-Hydroxytryptophan on Gastrointestinal Motility in a Mouse Model of Depression. Gastroenterology 157**:** 507-521.e504.

Jack T, Leuenberger M, Ruepp MD, Vernekar SKV, Thompson AJ, Braga-Lagache S*, et al.* (2018). Mapping the Orthosteric Binding Site of the Human 5-HT3 Receptor Using Photo-crosslinking Antagonists. ACS chemical neuroscience.

Jadav AM, McMullin CM, Smith J, Chapple K, & Brown SR (2013). The association between prucalopride efficacy and constipation type. Tech Coloproctol 17**:** 555-559.

Jensen AA, Davies PA, Brauner-Osborne H, & Krzywkowski K (2008). 3B but which 3B and that's just one of the questions: the heterogeneity of human 5-HT3 receptors. Trends in pharmacological sciences 29**:** 437-444.

Jiang C, Xu Q, Wen X, & Sun H (2015). Current developments in pharmacological therapeutics for chronic constipation. Acta Pharm Sin B 5**:** 300-309.

Johnson BA, Roache JD, Ait-Daoud N, Zanca NA, & Velazquez M (2002). Ondansetron reduces the craving of biologically predisposed alcoholics. Psychopharmacology 160**:** 408-413.

Jun S, Kohen R, Cain KC, Jarrett ME, & Heitkemper MM (2011). Associations of tryptophan hydroxylase gene polymorphisms with irritable bowel syndrome. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 23**:** 233-e116.

Kapeller J, Houghton LA, Monnikes H, Walstab J, Moller D, Bonisch H*, et al.* (2008). First evidence for an association of a functional variant in the microRNA-510 target site of the serotonin receptor-type 3E gene with diarrhea predominant irritable bowel syndrome. Hum Mol Genet 17**:** 2967-2977.

Kapeller J, Moller D, Lasitschka F, Autschbach F, Hovius R, Rappold G*, et al.* (2011). Serotonin receptor diversity in the human colon: Expression of serotonin type 3 receptor subunits 5-HT3C, 5-HT3D, and 5-HT3E. J Comp Neurol 519**:** 420-432.

Karnovsky AM, Gotow LF, McKinley DD, Piechan JL, Ruble CL, Mills CJ*, et al.* (2003). A cluster of novel serotonin receptor 3-like genes on human chromosome 3. Gene 319**:** 137-148.

Kay SR, Fiszbein A, & Opler LA (1987). The positive and negative syndrome scale (PANSS) for schizophrenia. Schizophr Bull 13**:** 261-276.

Kelley SP, Dunlop JI, Kirkness EF, Lambert JJ, & Peters JA (2003). A cytoplasmic region determines single-channel conductance in 5-HT3 receptors. Nature 424**:** 321.

Kesters D, Thompson AJ, Brams M, van Elk R, Spurny R, Geitmann M*, et al.* (2013). Structural basis of ligand recognition in 5-HT3 receptors. EMBO Rep 14**:** 49-56.

Khodaie-Ardakani MR, Seddighi S, Modabbernia A, Rezaei F, Salehi B, Ashrafi M*, et al.* (2013). Granisetron as an add-on to risperidone for treatment of negative symptoms in patients with stable schizophrenia: randomized double-blind placebo-controlled study. J Psychiatr Res 47**:** 472-478.

Kiesewetter B, Duan H, Lamm W, Haug A, Riss P, Selberherr A*, et al.* (2018). Oral Ondansetron Offers Effective Antidiarrheal Activity for Carcinoid Syndrome Refractory to Somatostatin Analogs. Oncologist.

Kiesewetter B, & Raderer M (2013). Ondansetron for diarrhea associated with neuroendocrine tumors. N Engl J Med 368**:** 1947-1948.

Kilpatrick GJ, Jones BJ, & Tyers MB (1987). Identification and distribution of 5-HT3 receptors in rat brain using radioligand binding. Nature 330**:** 746-748.

Kilpatrick LA, Labus JS, Coveleskie K, Hammer C, Rappold G, Tillisch K*, et al.* (2011). The HTR3A polymorphism c. -42C>T is associated with amygdala responsiveness in patients with irritable bowel syndrome. Gastroenterology 140**:** 1943-1951.

Kishi T, Mukai T, Matsuda Y, & Iwata N (2014). Selective serotonin 3 receptor antagonist treatment for schizophrenia: meta-analysis and systematic review. Neuromolecular Med 16**:** 61-69.

Kohler S, Cierpinsky K, Kronenberg G, & Adli M (2016). The serotonergic system in the neurobiology of depression: Relevance for novel antidepressants. Journal of psychopharmacology (Oxford, England) 30**:** 13-22.

Koike K, Hashimoto K, Takai N, Shimizu E, Komatsu N, Watanabe H*, et al.* (2005). Tropisetron improves deficits in auditory P50 suppression in schizophrenia. Schizophr Res 76**:** 67-72.

Kopczynska M, Mokros L, Pietras T, & Malecka-Panas E (2018). Quality of life and depression in patients with irritable bowel syndrome. Prz Gastroenterol 13**:** 102-108.

Kulke MH, Horsch D, Caplin ME, Anthony LB, Bergsland E, Oberg K*, et al.* (2017). Telotristat Ethyl, a Tryptophan Hydroxylase Inhibitor for the Treatment of Carcinoid Syndrome. J Clin Oncol 35**:** 14-23.

Kurhe Y, Mahesh R, & Devadoss T (2017). Novel 5-HT3 receptor antagonist QCM-4 attenuates depressive-like phenotype associated with obesity in high-fat-diet-fed mice. Psychopharmacology (Berl) 234**:** 1165-1179.

Kurhe Y, Radhakrishnan M, Gupta D, & Devadoss T (2014). QCM-4 a novel 5-HT3 antagonist attenuates the behavioral and biochemical alterations on chronic unpredictable mild stress model of depression in Swiss albino mice. The Journal of pharmacy and pharmacology 66**:** 122-132.

Kuryshev YA, Brown AM, Wang L, Benedict CR, & Rampe D (2000). Interactions of the 5-hydroxytryptamine 3 antagonist class of antiemetic drugs with human cardiac ion channels. The Journal of pharmacology and experimental therapeutics 295**:** 614-620.

Kvols LK (1986). Metastatic carcinoid tumors and the carcinoid syndrome. A selective review of chemotherapy and hormonal therapy. Am J Med 81**:** 49-55.

Lacy BE, & Moreau JC (2016). Diarrhea-predominant irritable bowel syndrome: Diagnosis, etiology, and new treatment considerations. J Am Assoc Nurse Pract 28**:** 393-404.

Lacy BE, Weiser K, & De Lee R (2009). The Treatment of Irritable Bowel Syndrome. Therap Adv Gastroenterol 2**:** 221-238.

Ladefoged LK, Munro L, Pedersen AJ, Lummis SCR, Bang-Andersen B, Balle T*, et al.* (2018). Modeling and Mutational Analysis of the Binding Mode for the Multimodal Antidepressant Drug Vortioxetine to the Human 5-HT3A Receptor. Molecular pharmacology 94**:** 1421-1434.

Lansdell SJ, Sathyaprakash C, Doward A, & Millar NS (2015). Activation of human 5-hydroxytryptamine type 3 receptors via an allosteric transmembrane site. Molecular pharmacology 87**:** 87-95.

Lauffer JM, Zhang T, & Modlin IM (1999). Review article: current status of gastrointestinal carcinoids. Aliment Pharmacol Ther 13**:** 271-287.

Laurent L, Deroy K, St-Pierre J, Côté F, Sanderson JT, & Vaillancourt C (2017). Human placenta expresses both peripheral and neuronal isoform of tryptophan hydroxylase. Biochimie 140**:** 159-165.

Lecrubier Y, Puech AJ, Azcona A, Bailey PE, & Lataste X (1993). A randomized double-blind placebo-controlled study of tropisetron in the treatment of outpatients with generalized anxiety disorder. Psychopharmacology 112**:** 129-133.

Livesey MR, Cooper MA, Deeb TZ, Carland JE, Kozuska J, Hales TG*, et al.* (2008). Structural determinants of Ca2+ permeability and conduction in the human 5-hydroxytryptamine type 3A receptor. J Biol Chem 283**:** 19301-19313.

Llorca PM, Lancon C, Brignone M, Rive B, Salah S, Ereshefsky L*, et al.* (2014). Relative efficacy and tolerability of vortioxetine versus selected antidepressants by indirect comparisons of similar clinical studies. Current medical research and opinion 30**:** 2589-2606.

Lochner M, & Lummis SC (2010). Agonists and antagonists bind to an A-A interface in the heteromeric 5-HT3AB receptor. Biophys J 98**:** 1494-1502.

Lopez-Tudanca PL, Labeaga L, Innerarity A, Alonso-Cires L, Tapia I, Mosquera R*, et al.* (2003). Synthesis and pharmacological characterization of a new benzoxazole derivative as a potent 5-HT3 receptor agonist. Bioorg Med Chem 11**:** 2709-2714.

Lovinger DM (1991). Ethanol potentiation of 5-HT3 receptor-mediated ion current in NCB-20 neuroblastoma cells. Neurosci Lett 122**:** 57-60.

Lummis SCR (2012). 5-HT3 Receptors. Journal of Biological Chemistry 287**:** 40239-40245.

Macor JE, Gurley D, Lanthorn T, Loch J, Mack RA, Mullen G*, et al.* (2001). The 5-HT3 antagonist tropisetron (ICS 205-930) is a potent and selective alpha7 nicotinic receptor partial agonist. Bioorg Med Chem Lett 11**:** 319-321.

Maghbooli M, Golipour F, Moghimi Esfandabadi A, & Yousefi M (2014). Comparison between the efficacy of ginger and sumatriptan in the ablative treatment of the common migraine. Phytother Res 28**:** 412-415.

Mahesh R, Devadoss T, Pandey DK, & Yadav SK (2011). Quinoxalin-2-carboxamides: synthesis and pharmacological evaluation as serotonin type-3 (5-HT3) receptor antagonists. J Enzyme Inhib Med Chem 26**:** 610-615.

Manning DD, Cioffi CL, Usyatinsky A, Fitzpatrick K, Masih L, Guo C*, et al.* (2011). Novel serotonin type 3 receptor partial agonists for the potential treatment of irritable bowel syndrome. Bioorg Med Chem Lett 21**:** 58-61.

Manning DD, Guo C, Zhang Z, Ryan KN, Naginskaya J, Choo SH*, et al.* (2014). The discovery of diazepinone-based 5-HT3 receptor partial agonists. Bioorg Med Chem Lett 24**:** 2578-2581.

Manocha M, & Khan W (2012) *Serotonin and GI Disorders: An Update on Clinical and Experimental Studies*. vol. 3.

Marcotte ER, Pearson DM, & Srivastava LK (2001). Animal models of schizophrenia: a critical review. J Psychiatry Neurosci 26**:** 395-410.

Marcus DA (2009). Fibromyalgia: Diagnosis and treatment options. Gender Medicine 6**:** 139-151.

Maricq A, Peterson A, Brake A, Myers R, & Julius D (1991). Primary structure and functional expression of the 5HT3 receptor, a serotonin-gated ion channel. Science 254**:** 432-437.

Masab M, & Saif MW (2017). Telotristat ethyl: proof of principle and the first oral agent in the management of well-differentiated metastatic neuroendocrine tumor and carcinoid syndrome diarrhea. Cancer Chemother Pharmacol 80**:** 1055-1062.

Meeker AS, Herink MC, Haxby DG, & Hartung DM (2015). The safety and efficacy of vortioxetine for acute treatment of major depressive disorder: a systematic review and meta-analysis. Systematic Reviews 4**:** 21.

Michel K, Zeller F, Langer R, Nekarda H, Kruger D, Dover TJ*, et al.* (2005). Serotonin Excites Neurons in the Human Submucous Plexus via 5-HT3 Receptors. Gastroenterology 128**:** 1317-1326.

Miles TF, Lester HA, & Dougherty DA (2015). Allosteric activation of the 5-HT3AB receptor by mCPBG. Neuropharmacology 91**:** 103-108.

Min YW, & Rhee P-L (2015). The clinical potential of ramosetron in the treatment of irritable bowel syndrome with diarrhea (IBS-D). Therap Adv Gastroenterol 8**:** 136-142.

Miner WD, Sanger GJ, & Turner DH (1987). Evidence that 5-hydroxytryptamine3 receptors mediate cytotoxic drug and radiation-evoked emesis. British journal of cancer 56**:** 159-162.

Mitchell NA, Pepperell E, Ociepka S, Brown JD, Witherington J, Tuladhar B*, et al.* (2009). 5-hydroxyindalpine, an agonist at the putative 5-HT receptor, has no activity on human recombinant monoamine receptors but accelerates distension-induced peristalsis in mouse isolated colon. Neurogastroenterol Motil 21**:** 760-e748.

Miura S, Watanabe S, Sato K, Makino M, Kobayashi O, Miyao H*, et al.* (2013). The efficacy of triplet antiemetic therapy with 0.75 mg of palonosetron for chemotherapy-induced nausea and vomiting in lung cancer patients receiving highly emetogenic chemotherapy. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer 21**:** 2575-2581.

Monk SA, Desai K, Brady CA, Williams JM, Lin L, Princivalle A*, et al.* (2001). Generation of a selective 5-HT3B subunit-recognising polyclonal antibody; identification of immunoreactive cells in rat hippocampus. Neuropharmacology 41**:** 1013-1016.

Monk SA, Williams JM, Hope AG, & Barnes NM (2004). Identification and importance of N-glycosylation of the human 5-hydroxytryptamine3A receptor subunit. Biochem Pharmacol 68**:** 1787-1796.

Montgomery SA, & Asberg M (1979). A new depression scale designed to be sensitive to change. Br J Psychiatry 134**:** 382-389.

Montgomery SA, McAuley R, Rani SJ, Montgomery DB, Braithwaite R, & Dawling S (1979). Amitriptyline plasma concentration and clinical response. British medical journal 1**:** 230-231.

Moore NA, Sargent BJ, Manning DD, & Guzzo PR (2013). Partial agonism of 5-HT3 receptors: a novel approach to the symptomatic treatment of IBS-D. ACS chemical neuroscience 4**:** 43-47.

Mork A, Pehrson A, Brennum LT, Nielsen SM, Zhong H, Lassen AB*, et al.* (2012). Pharmacological effects of Lu AA21004: a novel multimodal compound for the treatment of major depressive disorder. J Pharmacol Exp Ther 340**:** 666-675.

Motavallian A, Minaiyan M, Rabbani M, Mahzouni P, & Andalib S (2019). Anti-inflammatory effects of alosetron mediated through 5-HT(3) receptors on experimental colitis. Res Pharm Sci 14**:** 228-236.

Nakamura Y, Kondo M, Koyama Y, & Shimada S (2019). SR 57227A is a partial agonist/partial antagonist of 5-HT3 receptor and inhibits subsequent 5-HT- or SR 57227A-induced 5-HT3 receptor current. Biochemical and biophysical research communications 508**:** 590-596.

Nam Y, Min YS, & Sohn UD (2018). Recent advances in pharmacological research on the management of irritable bowel syndrome. Arch Pharm Res.

Nasirinezhad F, Hosseini M, Karami Z, Yousefifard M, & Janzadeh A (2016). Spinal 5-HT3 receptor mediates nociceptive effect on central neuropathic pain; possible therapeutic role for tropisetron. J Spinal Cord Med 39**:** 212-219.

Neeb L, Meents J, & Reuter U (2010). 5-HT(1F) Receptor agonists: a new treatment option for migraine attacks? Neurotherapeutics 7**:** 176-182.

Newman AS, Batis N, Grafton G, Caputo F, Brady CA, Lambert JJ*, et al.* (2013). 5-Chloroindole: a potent allosteric modulator of the 5-HT3 receptor. British journal of pharmacology 169**:** 1228-1238.

Ng QX, Soh AYS, Lim DY, & Yeo WS (2018). Agomelatine, a novel therapeutic option for the management of irritable bowel syndrome. J Clin Pharm Ther.

Nichols DE, & Nichols CD (2008). Serotonin Receptors. Chemical Reviews 108**:** 1614-1641.

Niesler B (2011). 5-HT3 receptors: potential of individual isoforms for personalised therapy. Current Opinion in Pharmacology 11**:** 81-86.

Niesler B, Frank B, Kapeller J, & Rappold GA (2003). Cloning, physical mapping and expression analysis of the human 5-HT3 serotonin receptor-like genes HTR3C, HTR3D and HTR3E. Gene 310**:** 101-111.

Niesler B, Walstab J, Combrink S, Moller D, Kapeller J, Rietdorf J*, et al.* (2007). Characterization of the novel human serotonin receptor subunits 5-HT3C,5-HT3D, and 5-HT3E. Molecular pharmacology 72**:** 8-17.

Noroozian M, Ghasemi S, Hosseini SM, Modabbernia A, Khodaie-Ardakani MR, Mirshafiee O*, et al.* (2013). A placebo-controlled study of tropisetron added to risperidone for the treatment of negative symptoms in chronic and stable schizophrenia. Psychopharmacology (Berl) 228**:** 595-602.

Olsen MA, Nawoschik SP, Schurman BR, Schmitt HL, Burno M, Smith DL*, et al.* (1999). Identification of a human 5-HT6 receptor variant produced by alternative splicing. Molecular Brain Research 64**:** 255-263.

Oronsky B, Ma PC, Morgensztern D, & Carter CA (2017). Nothing But NET: A Review of Neuroendocrine Tumors and Carcinomas. Neoplasia 19**:** 991-1002.

Özdener AE, & Rivkin A (2017). Eluxadoline in the treatment of diarrhea-predominant irritable bowel syndrome. Drug Des Devel Ther 11**:** 2827-2840.

Pae CU, Wang SM, Han C, Lee SJ, Patkar AA, Masand PS*, et al.* (2015). Vortioxetine: a meta-analysis of 12 short-term, randomized, placebo-controlled clinical trials for the treatment of major depressive disorder. Journal of psychiatry & neuroscience : JPN 40**:** 174-186.

Palczynska MM, Jindrichova M, Gibb AJ, & Millar NS (2012). Activation of alpha7 nicotinic receptors by orthosteric and allosteric agonists: influence on single-channel kinetics and conductance. Molecular pharmacology 82**:** 910-917.

Papke RL, Horenstein NA, Kulkarni AR, Stokes C, Corrie LW, Maeng CY*, et al.* (2014). The activity of GAT107, an allosteric activator and positive modulator of alpha7

nicotinic acetylcholine receptors (nAChR), is regulated by aromatic amino acids that span the subunit interface. J Biol Chem 289**:** 4515-4531.

Park S-Y, Park M-H, Yoon K-W, Cho S-B, Lee W-S, Park C-H*, et al.* (2009). Plasma 5-hydroxytryptamine concentration and its correlation with psychopathology in patients with irritable bowel syndrome. Gut Liver 3**:** 26-30.

Park YS, Myeong SH, Kim IB, & Sung KW (2018). Tricyclic antidepressant amitriptyline inhibits 5-hydroxytryptamine 3 receptor currents in NCB-20 cells. Korean J Physiol Pharmacol 22**:** 585-595.

Parker RMC, Barnes JM, Ge J, Barber PC, & Barnes NM (1996). Autoradiographic distribution of [3H]-(S)-zacopride-labelled 5-HT3 receptors in human brain. Journal of the Neurological Sciences 144**:** 119-127.

Pascual D, Alsasua A, Goicoechea C, & Martín MI (2002). The involvement of 5-HT3 and 5-HT4 receptors in two models of gastrointestinal transit in mice. Neuroscience Letters 326**:** 163-166.

Patel YC (1999). Somatostatin and Its Receptor Family. Frontiers in Neuroendocrinology 20**:** 157-198.

Pato MT, Murphy DL, & DeVane CL (1991). Sustained plasma concentrations of fluoxetine and/or norfluoxetine four and eight weeks after fluoxetine discontinuation. Journal of clinical psychopharmacology 11**:** 224-225.

Patrick DL, Drossman DA, Frederick IO, DiCesare J, & Puder KL (1998). Quality of life in persons with irritable bowel syndrome: development and validation of a new measure. Dig Dis Sci 43**:** 400-411.

Pauwels PJ (2000). Diverse signalling by 5-hydroxytryptamine (5-HT) receptors. Biochem Pharmacol 60**:** 1743-1750.

Pavel M, Gross DJ, Benavent M, Perros P, Srirajaskanthan R, Warner RRP*, et al.* (2018). Telotristat ethyl in carcinoid syndrome: safety and efficacy in the TELECAST phase 3 trial. Endocr Relat Cancer 25**:** 309-322.

Peroutka SJ, & Snyder SH (1979). Multiple serotonin receptors: differential binding of [3H]5-hydroxytryptamine, [3H]lysergic acid diethylamide and [3H]spiroperidol. Mol Pharmacol 16**:** 687-699.

Peters JA, Malone HM, & Lambert JJ (1992). Recent advances in the electrophysiological characterization of 5-HT3 receptors. Trends in Pharmacological Sciences 13**:** 391-397.

Platt AJ, Heddle RM, Rake MO, & Smedley H (1992). Ondansetron in carcinoid syndrome. The Lancet 339**:** 1416.

Powell AD, Grafton G, Roberts A, Larkin S, O'Neill N, Palandri J*, et al.* (2016). Novel mechanism of modulation at a ligand-gated ion channel; action of 5-Cl-indole at the 5-HT3 A receptor. British journal of pharmacology 173**:** 3467-3479.

Price KL, Bower KS, Thompson AJ, Lester HA, Dougherty DA, & Lummis SC (2008). A hydrogen bond in loop A is critical for the binding and function of the 5-HT3 receptor. Biochemistry 47**:** 6370-6377.

Price KL, Hirayama Y, & Lummis SC (2017). Subtle Differences among 5-HT3AC, 5-HT3AD, and 5-HT3AE Receptors Are Revealed by Partial Agonists. ACS chemical neuroscience.

Price KL, & Lummis SC (2004). The role of tyrosine residues in the extracellular domain of the 5-hydroxytryptamine3 receptor. J Biol Chem 279**:** 23294-23301.

Price KL, & Lummis SCR (2018). Characterization of a 5-HT3-ELIC Chimera Revealing the Sites of Action of Modulators. ACS chemical neuroscience 9**:** 1409-1415.

Pytliak M, Vargova V, Mechirova V, & Felsoci M (2011). Serotonin receptors - from molecular biology to clinical applications. Physiological research 60**:** 15-25.

Qi Q, Zhang Y, Chen F, Zuo X, & Li Y (2018). Ramosetron for the treatment of irritable bowel syndrome with diarrhea: a systematic review and meta-analysis of randomized controlled trials. BMC Gastroenterol 18**:** 5.

Qi Q, Zhang Y, Chen F, Zuo X, & Li YJBG (2018). Ramosetron for the treatment of irritable bowel syndrome with diarrhea: a systematic review and meta-analysis of randomized controlled trials. 18**:** 5.

Rammes G, Eisensamer B, Ferrari U, Shapa M, Gimpl G, Gilling K*, et al.* (2004). Antipsychotic drugs antagonize human serotonin type 3 receptor currents in a noncompetitive manner. Molecular psychiatry 9**:** 846-858, 818.

Rapport MM, Green AA, & Page IH (1948). Serum vasoconstrictor, serotonin; isolation and characterization. J Biol Chem 176**:** 1243-1251.

Rastogi V, Singh D, Mazza JJ, Yang D, Parajuli D, & Yale SH (2018). Flushing Disorders Associated with Gastrointestinal Symptoms: Part 1, Neuroendocrine Tumors, Mast Cell Disorders and Hyperbasophila. Clin Med Res.

Reeves DC, & Lummis SC (2006). Detection of human and rodent 5-HT3B receptor subunits by anti-peptide polyclonal antibodies. BMC Neurosci 7**:** 27.

Remington G, Foussias G, Fervaha G, Agid O, Takeuchi H, Lee J*, et al.* (2016). Treating Negative Symptoms in Schizophrenia: an Update. Current Treatment Options in Psychiatry 3**:** 133-150.

Richard Green A (2006). Neuropharmacology of 5-hydroxytryptamine. British Journal of Pharmacology 147**:** S145-S152.

Riechelmann RP, Pereira AA, Rego JF, & Costa FP (2017). Refractory carcinoid syndrome: a review of treatment options. Ther Adv Med Oncol 9**:** 127-137.

Rocheville M, & Garland SL (2010). An industrial perspective on positive allosteric modulation as a means to discover safe and selective drugs. Drug Discovery Today: Technologies 7**:** e87-e94.

Rojas C, Thomas AG, Alt J, Stathis M, Zhang J, Rubenstein EB*, et al.* (2010). Palonosetron triggers 5-HT(3) receptor internalization and causes prolonged inhibition of receptor function. Eur J Pharmacol 626**:** 193-199.

Sakowski SA, Geddes TJ, Thomas DM, Levi E, Hatfield JS, & Kuhn DM (2006). Differential tissue distribution of tryptophan hydroxylase isoforms 1 and 2 as revealed with monospecific antibodies. Brain Research 1085**:** 11-18.

Sanchez C, Asin KE, & Artigas F (2015). Vortioxetine, a novel antidepressant with multimodal activity: review of preclinical and clinical data. Pharmacol Ther 145**:** 43-57.

Sanger GJ (2008). 5-hydroxytryptamine and the gastrointestinal tract: where next? Trends Pharmacol Sci 29**:** 465-471.

Schatzberg AF, Blier P, Culpepper L, Jain R, Papakostas GI, & Thase ME (2014). An overview of vortioxetine. The Journal of clinical psychiatry 75**:** 1411-1418.

Schmulson MJ, & Drossman DA (2017). What Is New in Rome IV. J Neurogastroenterol Motil 23**:** 151-163.

Schwartz TW, & Holst B (2006). Ago-allosteric modulation and other types of allostery in dimeric 7TM receptors. J Recept Signal Transduct Res 26**:** 107-128.

Schwartz TW, & Holst B (2007). Allosteric enhancers, allosteric agonists and ago-allosteric modulators: where do they bind and how do they act? Trends Pharmacol Sci 28**:** 366-373.

Seddighnia A, Tadayon Najafabadi B, Ghamari K, Noorbala AA, Ebrahimi Daryani N, Kashani L*, et al.* (2020). Vortioxetine effects on quality of life of irritable bowel syndrome patients: A randomized, double-blind, placebo-controlled trial. Journal of Clinical Pharmacy and Therapeutics 45**:** 97-104.

Sheehan DV, Harnett-Sheehan K, & Raj BA (1996). The measurement of disability. Int Clin Psychopharmacol 11 Suppl 3**:** 89-95.

Spier AD, & Lummis SCR (2000). The Role of Tryptophan Residues in the 5-Hydroxytryptamine3 Receptor Ligand Binding Domain. Journal of Biological Chemistry 275**:** 5620-5625.

Spiller R (2007). Recent advances in understanding the role of serotonin in gastrointestinal motility in functional bowel disorders: alterations in 5-HT signalling and metabolism in human disease. Neurogastroenterol Motil 19 Suppl 2**:** 25-31.

Stahl SM (2015). Modes and nodes explain the mechanism of action of vortioxetine, a multimodal agent (MMA): blocking 5HT3 receptors enhances release of serotonin, norepinephrine, and acetylcholine. CNS Spectr 20**:** 455-459.

Staner L, Linker T, Toussaint M, Danjou P, Roegel JC, Luthringer R*, et al.* (2001). Effects of the selective activation of 5-HT3 receptors on sleep: a polysomnographic study in healthy volunteers. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology 11**:** 301-305.

Stasi C, Bellini M, Bassotti G, Blandizzi C, & Milani S (2014). Serotonin receptors and their role in the pathophysiology and therapy of irritable bowel syndrome. Tech Coloproctol 18**:** 613-621.

Stewart A, Davies PA, Kirkness EF, Safa P, & Hales TG (2003). Introduction of the 5-HT3B subunit alters the functional properties of 5-HT3 receptors native to neuroblastoma cells. Neuropharmacology 44**:** 214-223.

Strawn JR, Mills JA, Cornwall GJ, Mossman SA, Varney ST, Keeshin BR*, et al.* (2018). Buspirone in Children and Adolescents with Anxiety: A Review and Bayesian Analysis of Abandoned Randomized Controlled Trials. J Child Adolesc Psychopharmacol 28**:** 2-9.

Talmon M, Rossi S, Pastore A, Cattaneo CI, Brunelleschi S, & Fresu LG (2018). Vortioxetine exerts anti-inflammatory and immunomodulatory effects on human monocytes/macrophages. British journal of pharmacology 175**:** 113-124.

Tanaka KF, Samuels BA, & Hen R (2012). Serotonin receptor expression along the dorsal-ventral axis of mouse hippocampus. Philos Trans R Soc Lond B Biol Sci 367**:** 2395-2401.

Taschler U, Eichmann TO, Radner FPW, Grabner GF, Wolinski H, Storr M*, et al.* (2015). Monoglyceride lipase deficiency causes desensitization of intestinal cannabinoid receptor type 1 and increased colonic μ-opioid receptor sensitivity. British journal of pharmacology 172**:** 4419-4429.

Thakur GA, Kulkarni AR, Deschamps JR, & Papke RL (2013). Expeditious synthesis, enantiomeric resolution, and enantiomer functional characterization of (4-(4-bromophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-sulfonamide (4BP-TQS): an allosteric agonist-positive allosteric modulator of alpha7 nicotinic acetylcholine receptors. J Med Chem 56**:** 8943-8947.

Thase ME, Mahableshwarkar AR, Dragheim M, Loft H, & Vieta E (2016). A meta-analysis of randomized, placebo-controlled trials of vortioxetine for the treatment of major depressive disorder in adults. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology 26**:** 979-993.

Thayalasekeran S, Ali H, & Tsai HH (2013). Novel therapies for constipation. World J Gastroenterol 19**:** 8247-8251.

Thijssen AY, Mujagic Z, Jonkers DMAE, Ludidi S, Keszthelyi D, Hesselink MA*, et al.* (2016). Alterations in serotonin metabolism in the irritable bowel syndrome. Alimentary Pharmacology & Therapeutics 43**:** 272-282.

Thompson AJ (2013). Recent developments in 5-HT3 receptor pharmacology. Trends in Pharmacological Sciences 34**:** 100-109.

Thompson AJ, Alqazzaz M, Ulens C, & Lummis SCR (2012). The pharmacological profile of ELIC, a prokaryotic GABA-gated receptor. Neuropharmacology 63**:** 761-767.

Thompson AJ, & Lummis SC (2006). 5-HT3 receptors. Current pharmaceutical design 12**:** 3615-3630.

Thompson AJ, & Lummis SCR (2007). The 5-HT(3) receptor as a therapeutic target. Expert opinion on therapeutic targets 11**:** 527-540.

Thompson AJ, & Lummis SCR (2013). Discriminating between 5-HT3A and 5-HT3AB receptors. British Journal of Pharmacology 169**:** 736-747.

Thompson AJ, Verheij MH, de Esch IJ, & Lummis SC (2012). VUF10166, a novel compound with differing activities at 5-HT(3)A and 5-HT(3)AB receptors. The Journal of pharmacology and experimental therapeutics 341**:** 350-359.

Thompson AJ, Verheij MH, Verbeek J, Windhorst AD, de Esch IJ, & Lummis SC (2014). The binding characteristics and orientation of a novel radioligand with distinct properties at 5-HT3A and 5-HT3AB receptors. Neuropharmacology 86**:** 378-388.

Thomsen MS, & Mikkelsen JD (2012). Type I and II positive allosteric modulators differentially modulate agonist-induced up-regulation of alpha7 nicotinic acetylcholine receptors. J Neurochem 123**:** 73-83.

Trattnig SM, Harpsoe K, Thygesen SB, Rahr LM, Ahring PK, Balle T*, et al.* (2012). Discovery of a novel allosteric modulator of 5-HT3 receptors: inhibition and potentiation of Cys-loop receptor signaling through a conserved transmembrane intersubunit site. J Biol Chem 287**:** 25241-25254.

Tricco AC, Blondal E, Veroniki AA, Soobiah C, Vafaei A, Ivory J*, et al.* (2016). Comparative safety and effectiveness of serotonin receptor antagonists in patients undergoing chemotherapy: a systematic review and network meta-analysis. BMC medicine 14**:** 216.

Twarog BM, & Page IH (1953). Serotonin content of some mammalian tissues and urine and a method for its determination. The American journal of physiology 175**:** 157-161.

van Hooft JA, van der Haar E, & Vijverberg HP (1997). Allosteric potentiation of the 5-HT3 receptor-mediated ion current in N1E-115 neuroblastoma cells by 5-hydroxyindole and analogues. Neuropharmacology 36**:** 649-653.

van Hooft JA, & Vijverberg HP (1996). Selection of distinct conformational states of the 5-HT3 receptor by full and partial agonists. British journal of pharmacology 117**:** 839-846.

van Hooft JA, & Vijverberg HP (1997). Full and partial agonists induce distinct desensitized states of the 5-HT3 receptor. J Recept Signal Transduct Res 17**:** 267-277.

Vega Ldl, Muñoz E, Calzado MA, Lieb K, Candelario-Jalil E, Gschaidmeir H*, et al.* (2005). The 5-HT3 receptor antagonist tropisetron inhibits T cell activation by targeting the calcineurin pathway. Biochem Pharmacol 70**:** 369-380.

Walstab J, Kruger D, Stark T, Hofmann T, Demir IE, Ceyhan GO*, et al.* (2013). Ginger and its pungent constituents non-competitively inhibit activation of human recombinant and native 5-HT3 receptors of enteric neurons. Neurogastroenterol Motil 25**:** 439-447, e302.

Walstab J, Rappold G, & Niesler B (2010). 5-HT(3) receptors: role in disease and target of drugs. Pharmacol Ther 128**:** 146-169.

Walstab J, Wohlfarth C, Hovius R, Schmitteckert S, Röth R, Lasitschka F*, et al.* (2014). Natural compounds boldine and menthol are antagonists of human 5-HT3 receptors: implications for treating gastrointestinal disorders. Neurogastroenterology & Motility 26**:** 810-820.

Walther DJ, Peter J-U, Bashammakh S, Hörtnagl H, Voits M, Fink H*, et al.* (2003). Synthesis of Serotonin by a Second Tryptophan Hydroxylase Isoform. Science 299**:** 76.

Wang L, Martin B, Brenneman R, Luttrell LM, & Maudsley S (2009). Allosteric modulators of g protein-coupled receptors: future therapeutics for complex physiological disorders. The Journal of pharmacology and experimental therapeutics 331**:** 340-348.

Wängberg B, Nilsson O, Theodorsson E, Dahlström A, & Ahlman H (1991). The effect of a somatostatin analogue on the release of hormones from human midgut carcinoid tumour cells. British journal of cancer 64**:** 23-28.

Warnez S, & Alessi-Severini S (2014). Clozapine: a review of clinical practice guidelines and prescribing trends. BMC Psychiatry 14**:** 102-102.

Wenthur CJ, Gentry PR, Mathews TP, & Lindsley CW (2014). Drugs for Allosteric Sites on Receptors. Annual Review of Pharmacology and Toxicology 54**:** 165-184.

Wymenga AN, de Vries EG, Leijsma MK, Kema IP, & Kleibeuker JH (1998). Effects of ondansetron on gastrointestinal symptoms in carcinoid syndrome. Eur J Cancer 34**:** 1293-1294.

Yamazaki M, Harada K, Yamamoto N, Yarimizu J, Okabe M, Shimada T*, et al.* (2014). ASP5736, a novel 5-HT5A receptor antagonist, ameliorates positive symptoms and cognitive impairment in animal models of schizophrenia. European Neuropsychopharmacology 24**:** 1698-1708.

Yang KH, Galadari S, Isaev D, Petroianu G, Shippenberg TS, & Oz M (2010). The nonpsychoactive cannabinoid cannabidiol inhibits 5-hydroxytryptamine3A receptor-mediated currents in Xenopus laevis oocytes. The Journal of pharmacology and experimental therapeutics 333**:** 547-554.

Yokoi A, Mihara T, Ka K, & Goto T (2017). Comparative efficacy of ramosetron and ondansetron in preventing postoperative nausea and vomiting: An updated systematic review and meta-analysis with trial sequential analysis. PLoS One 12**:** e0186006.

Yu F-Y, Huang S-G, Zhang H-Y, Ye H, Chi H-G, Zou Y*, et al.* (2016). Comparison of 5-hydroxytryptophan signaling pathway characteristics in diarrhea-predominant irritable bowel syndrome and ulcerative colitis. World J Gastroenterol 22**:** 3451-3459.

Yu Y, Zhu W, Liang Q, Liu J, Yang X, & Sun G (2018). Tropisetron attenuates lipopolysaccharide induced neuroinflammation by inhibiting NF-κB and SP/NK1R signaling pathway. J Neuroimmunol 320**:** 80-86.

Zhang H, Wang L, Fan Y, Yang L, Wen X, Liu Y*, et al.* (2019). Atypical antipsychotics for Parkinson's disease psychosis: a systematic review and meta-analysis. Neuropsychiatr Dis Treat 15**:** 2137-2149.

Zhang ZJ, Kang WH, Li Q, Wang XY, Yao SM, & Ma AQ (2006). Beneficial effects of ondansetron as an adjunct to haloperidol for chronic, treatment-resistant schizophrenia: a double-blind, randomized, placebo-controlled study. Schizophr Res 88**:** 102-110.

Zheng W, Cai D-B, Zhang Q-E, He J, Zhong L-Y, Sim K*, et al.* (2019). Adjunctive ondansetron for schizophrenia: A systematic review and meta-analysis of randomized controlled trials. J Psychiatr Res 113**:** 27-33.

Zheng Y, Yu T, Tang Y, Xiong W, Shen X, Jiang L*, et al.* (2017). Efficacy and safety of 5-hydroxytryptamine 3 receptor antagonists in irritable bowel syndrome: A systematic review and meta-analysis of randomized controlled trials. PLOS ONE 12**:** e0172846.

Ziemba PM, Schreiner BS, Flegel C, Herbrechter R, Stark TD, Hofmann T*, et al.* (2015). Activation and modulation of recombinantly expressed serotonin receptor type 3A by terpenes and pungent substances. Biochemical and biophysical research communications 467**:** 1090-1096.