Review

Recent developments in 5-HT_3 receptor pharmacology

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Three decades ago the development of 5-hydroxytryptamine$_3$ (5-HT_3) receptor antagonists had a major impact on the treatment of nausea and vomiting, and the cloning of the receptor a decade later enabled researchers to better understand its physiology. In the last decade we have seen the publication of detailed molecular structures of closely related proteins, allowing us to reconcile the locations of binding-site residues with earlier pharmacological and biochemical studies. There are more than 500 5-HT_3 receptor ligands. The majority are competitive antagonists resulting from screens of structurally related analogues, but several non-competitive antagonists have also been described. Some ligands are noteworthy because they distinguish between receptor subtypes or have allosteric mechanisms. They will help us to further probe the physiological role of these receptors and could ultimately find applications in clinical and veterinary research. In this review, I consider recently identified ligands with an emphasis on their pharmacology and ligand–receptor interactions.

The 5-HT_3R

There are seven classes of 5-HT receptors (5-HT_1–5-HT_7). All are G-protein-coupled receptors except the 5-HT_3 receptor (5-HT_3R), which is a cation-selective ligand-gated ion channel. 5-HT_3R belongs to the superfamily of Cys-loop receptors responsible for fast synaptic neurotransmission in the central (CNS) and peripheral nervous systems (PNS). The family also includes nicotinic acetylcholine (nACh), $GABA_A$, and glycine receptors (GlyRs). The 5-HT3A subunit was first cloned in 1991 and although it forms functional homopentamers in recombinant systems, it does not entirely recapitulate the electrical responses seen in some native tissues [1]. Later, in 1999, the 5-HT3B subunit was identified [2]. 5-HT3B subunits do not form functional receptors when expressed alone, but in combination with 5-HT3A subunits they assemble as functional heteromers with properties more closely mimicking most native 5-HT_3 receptors. Since then, 5-HT3C, 5-HT3D, and 5-HT3E subunits have also been identified. None of these form functional homopentamers and they do not have a distinct biophysical or pharmacological fingerprint when coexpressed with 5-HT3A subunits [3,4].

5-HT_3R can be readily isolated from other 5-HT-activated receptors by several potent and selective ligands. Since the earliest descriptions of competitive ligands, such as bemesetron (MDL-72222) and tropisetron (ICS 205-930), there has been significant progress in developing compounds that target both native and recombinant 5-HT_3R, and many of the early compounds are now available as generics [5]. Many 5-HT_3R antagonists are licensed for the treatment of chemotherapy-induced (CINV), radiotherapy-induced, and postoperative nausea and vomiting, in which they strongly suppress the acute phase of emesis with a generally low incidence of side effects, of which constipation is the most frequent and troublesome [4]. More recently the very high-affinity antagonist palonosetron (RS 25259-197) entered the clinic and, because of its longer plasma persistence, is licensed for both acute and delayed CINV [6]. Novel routes of administration for some of the earlier first-generation drugs have also been recently developed, such as the first granisetron transdermal patch (Sancuso) for treatment of CINV [7].

Evidence suggests that 5-HT_3R ligands could also be of benefit in several other disorders, including depression, substance abuse, gastro-oesophageal reflux, fibromyalgia, pruritis, cognitive and psychotic disorders, and pain, but monotherapy trials have been unsuccessful so far [4,8]. Dual ligands with inherent activity at more than one target and bivalent compounds that contain conjugated ligands for targeting multiple receptors could be more successful because many of the disorders have complex aetiologies [9,10]. Targeting of the PNS and CNS, or the PNS alone, is also possible. Most 5-HT_3R antagonists freely cross the blood–brain barrier (BBB) and because of their actions on pre-synaptic and post-synaptic 5-HT_3R, could potentially have wide-ranging effects on numerous other transmitter systems. Targeting of the PNS can be achieved by modifying the physicochemical properties of antagonists to prevent them crossing the BBB [11,12]. There are clearly many avenues that are still to be exploited and the growing number of 5-HT_3R subunits and splice variants provides further potential for developing new and selective drugs [4].

Where do 5-HT_3R ligands bind?

In 1995, the 5-HT_3R was visualized by electron microscopy as a pentamer [13]. Higher-resolution studies of related proteins have since shown that the five subunits are arranged around a central ion-conducting pore, with each subunit comprising an extracellular domain (ECD), a transmembrane domain (TMD), and an intracellular domain (ICD) [14]. 5-HT_3R ligands bind in the ECD or TMD. The orthosteric binding sites (those occupied by endogenous agonist) are located in the ECD and are formed by six amino acid loops that converge at the interfaces of adjacent

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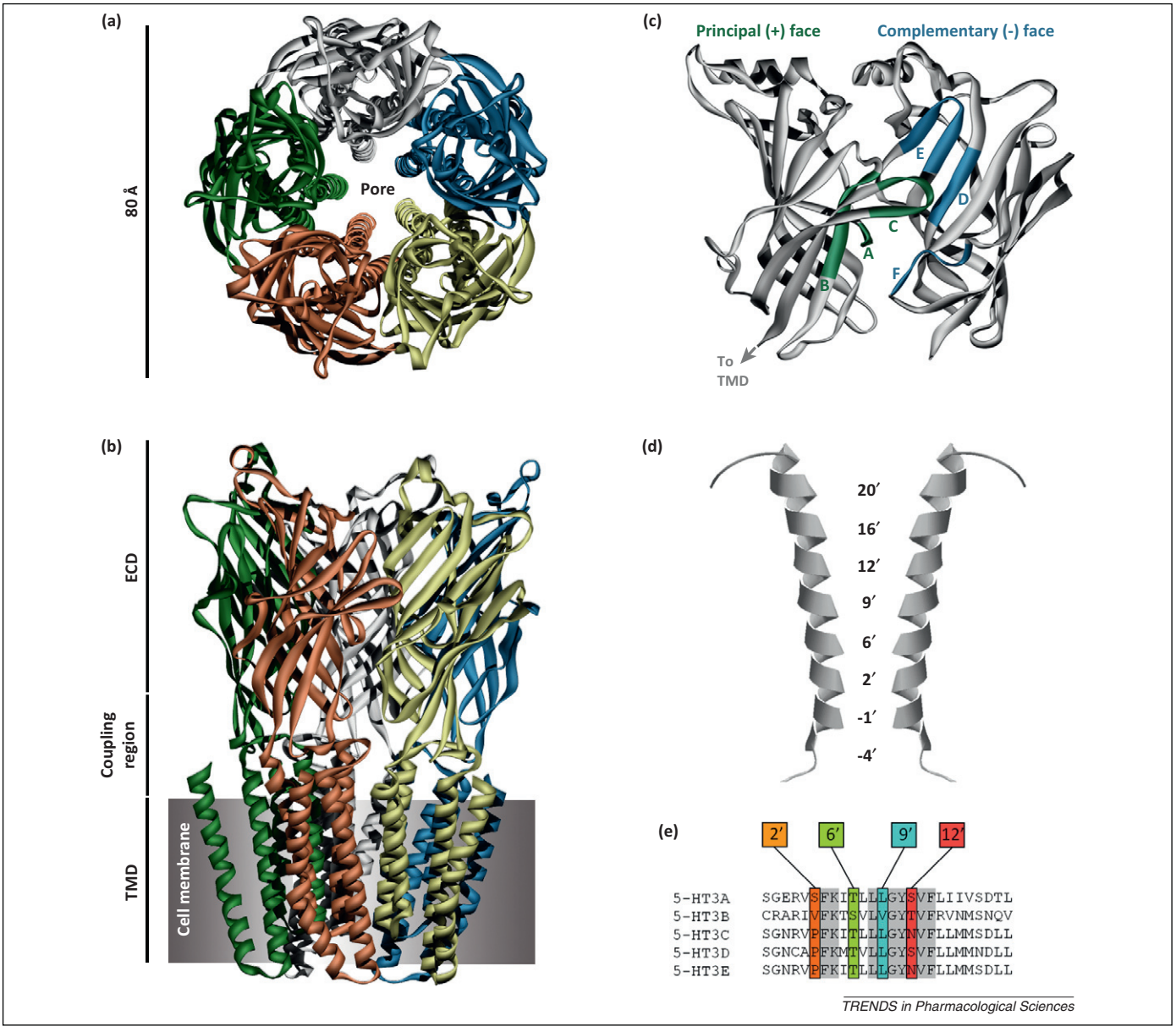

Figure 1. **Ligand binding sites in the 5-hydroxytryptamine$_3$ receptor (5-HT$_3$R)**. Topology of the 5-HT$_3$R with the extracellular domain (ECD) and transmembrane domain (TMD) seen from **(a)** above and **(b)** the side. Competitive antagonists (CAs) bind to the orthosteric binding sites in the ECD that are located at the interface of adjacent subunits, and one of these is shown in detail in **(c)**. In this cartoon image only two subunit ECDs are shown for clarity. Loops A–C are found on the principal face and loops D–F (which are in fact β-strands) on the complimentary face of the binding site, and are labelled accordingly. The importance of amino acids within these loops is reviewed in [14]. **(d)** The majority of noncompetitive antagonists (NCAs) bind in the channel formed by the convergence of TM2 α-helices from each of the five subunits. For clarity only two of the TM2 α-helices are shown here. The residues of the TM2 α-helices are referred to using a prime symbol (′) with residues numbered relative to a conserved charge at the intracellular end of the channel. **(e)** Alignment of TM2 regions from different 5-HT$_3$R subunits shows the positions of residues that affect the potency of NCAs at 5-HT$_3$A and 5-HT$_3$AB receptors.

subunits (Figure 1). These have been termed loops A–C from the principal (or +) face and loops D–E from the complementary (or –) face. Ligands that compete with the endogenous agonist are termed competitive antagonists (CAs). Noncompetitive antagonists (NCAs) bind to distinct and non-overlapping sites that are typically in the TMD. This is composed of four transmembrane α-helices (TM1–TM4) contributed by each subunit. The TM2 α-helices from each converge to form the central ion-conducting pore, and to aid comparisons of pores from different 5-HT$_3$R subunits and different Cys-loop receptors, a prime symbol (′) is used to describe the positions of channel-lining residues (Figure 1).

The orthosteric binding site in 5-HT$_3$AB receptors

Homomeric receptors contain only 5-HT3A subunits, and competitive ligands must bind to an A+A– interface. The affinities of competitive ligands at 5-HT$_3$AB receptors are similar, suggesting that 5-HT$_3$AB receptors may also contain an A+A– binding interface [15]. However, atomic force microscopy (AFM) predicts that the stoichiometry of the heteromeric 5-HT$_3$AB receptor is B-A-B-B-A, an arrangement that is comparable to the α and non-α subunits of nACh and GABA$_A$ receptors, but is inconsistent with the pharmacological evidence [16].

In an alternative approach, Lochner and Lummis exchanged aligned 5-HT3A and 5-HT3B subunit residues to

determine their impact on agonist (5-HT) and antagonist (granisetron) properties [17]. All 5-HT3A subunit substitutions affected the potency of 5-HT and the affinity of granisetron, but 5-HT3B subunit substitutions did not, suggesting a contribution by only A+ and A– interfaces. The presence of an A+A– interface in both homomeric and heteromeric receptors is also supported by disulfide trapping between cysteines on either side of the binding site [18]. 5-HT_3A and 5-HT_3AB receptors containing such mutations are unresponsive to 5-HT until the bonds are reduced by dithiothreitol (DTT), and following its washout, a gradual reduction in 5-HT peak current occurs as disulfide bonds reform. Spatial restraints mean that disulfide bonds can only exist between adjacent 5-HT3A subunits. Single cysteine substitutions also support an A+A– interface in both receptor types because only those in the 5-HT3A subunit affect function and [^{3}H]granisetron binding [18]; those in the 5-HT3B subunit have no effect. Covalent modification of the 5-HT3A subunit mutations by the thiol-reactive compound (2-aminoethyl) methanethiosulfonate (MTSEA) causes further functional changes that can be prevented by co-application of MTSEA with 5-HT or D-tubocurarine, proving that the residues are located in the binding site. Therefore, both functional and radioligand

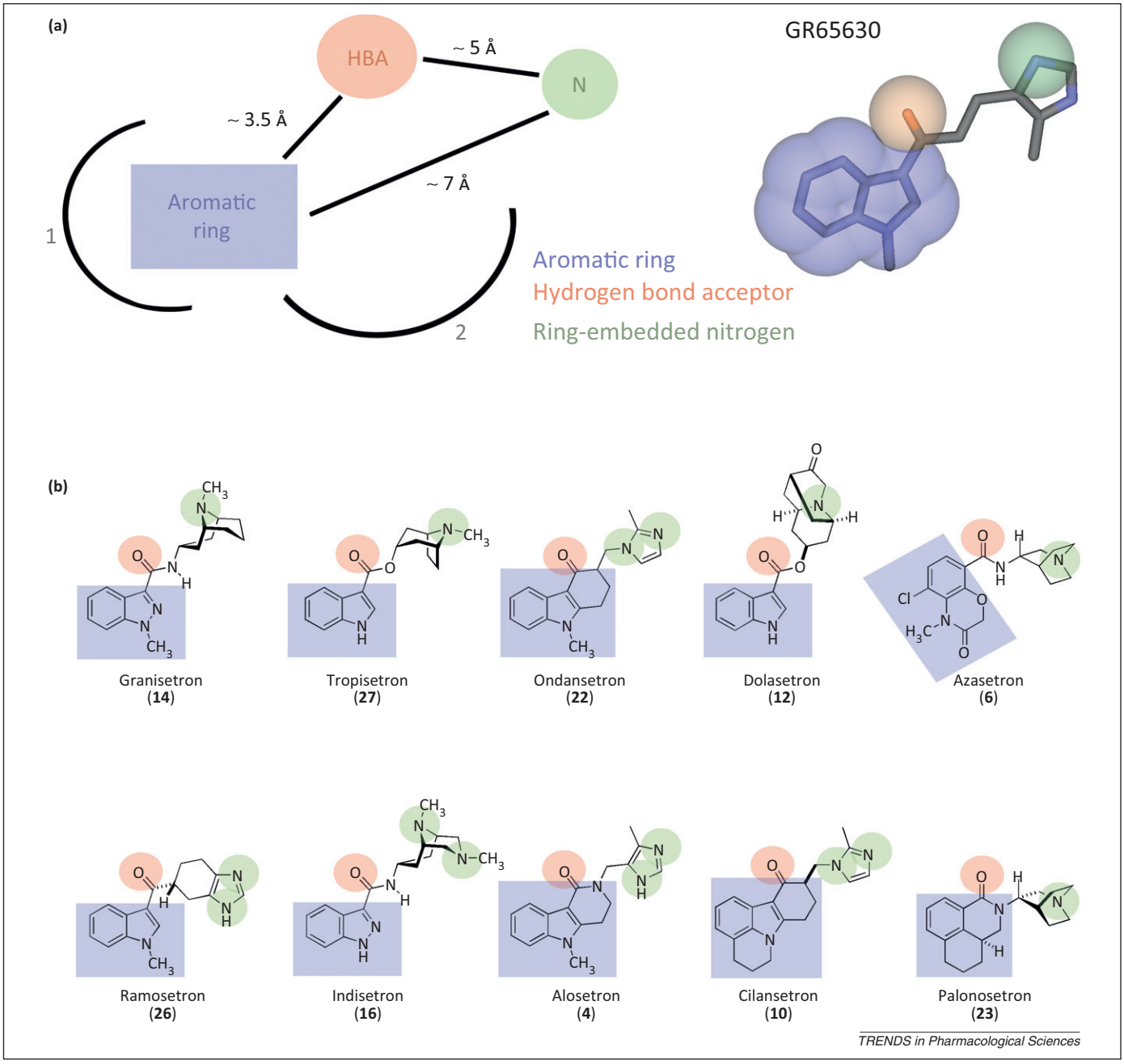

Figure 2. The 5-hydroxytryptamine$_3$ (5-HT_3) pharmacophore and clinically relevant ligands. **(a)** The 5-HT_3R pharmacophore is defined by three main points, an aromatic ring (purple), a hydrogen bond acceptor (HBA, orange) coplanar with the aromatic ring, and a ring-embedded basic nitrogen (green). In addition, there are several distance constraints, substituents around the aromatic ring are restricted (1), and hydrophobic interactions can be made opposite to the HBA (2). These features are highlighted in the crystal structure of GR65630 shown to the right of the panel. GR65630 is a potent and selective competitive antagonist (CA) that is no longer in clinical development, but is often used as a standard pharmacological tool. **(b)** Current 5-HT_3R therapeutic ligands with pharmacophore features highlighted in the same colours as in (a). These drugs are typically used for alleviation of postoperative, radiotherapy-induced, and chemotherapy-induced nausea and vomiting. Alosetron has been approved by the FDA for the treatment of irritable bowel syndrome under special conditions. Cilansetron was denied approval for a similar use, pending further clinical trials.

binding experiments consistently demonstrate the existence of an A+A– interface in both receptor types.

Competitive ligands

Although some 5-HT_3R agonists have recently been described [12,19,20], the majority of new competitive ligands are antagonists, including compounds with more complex properties such as fluorescent derivatives, allosteric ligands, dual ligands, and bivalent compounds [12,21–23]. These typically conform to a pharmacophore described by three interaction points and three distance constraints (Figure 2). Although a comprehensive description of these is beyond the scope of this review, it is briefly noted because all current therapeutic ligands conform to these general principles.

The major therapeutic group of 5-HT_3R CAs are known as setrons, but many of these ligands have been characterised for over two decades so they are not discussed here (Figure 2, Table 1). Recent attention has been paid to palonosetron (RS 25259-197), a second-generation setron. Palonosetron is highly selective for 5-HT_3R and has higher affinity, a longer plasma half-life and improved efficacy in preventing CINV than first-generation CAs [6]. However, such properties cannot adequately explain the improved clinical efficiency of palonosetron because other drugs would be expected to mimic these effects at higher dosage, or if administered at reduced intervals. Other properties may explain its profile. For example, an increased affinity with increasing concentration (positive cooperativity) and accelerated rate of [^{3}H]ligand dissociation provide

Table 1. Potency of selected 5-HT_3R ligands[a]

Compound[b]		5-HT_3A IC_{50} (μM)	5-HT_3AB IC_{50} (μM)[c]	5-HT_3A K_d/K_i (nM)[d]	Species	Action	Refs
1	2-Me-5-HT	4.1	12.1	224	Human	Agonist	[2,61]
2	5-HT	1.74	29.5	123	Human	Agonist	[45,61]
2	5-HT	1.80	2.0	294	Mouse	Agonist	[62,63]
3	Alisol extracts	~85	–	–	Human	NCA	[52]
4	Alosetron	–	–	0.40	Human	CA	[64]
5	Anandamide	0.13	–	NB	Human	NCA	[65]
6	Azasetron	0.036	0.005	3.16	Human	CA	[66]
7	Bilobalide	468	3100	NB	Human	NCA	[37]
8	Cannabidiol	0.6	–	NB	Human	NCA	[67]
9	Chloroquine	24.3	23.6	–	Human	CA	[45]
9	Chloroquine	11.8	–	24 200	Mouse	CA	[46]
10	Cilansetron	–	–	0.19	Rat brain	CA	[68]
11	Clozapine	0.08	–	–	Human	CA	[34]
12	Dolasetron	0.004	–	20.0	NG108-15	CA	[69]
13	Diltiazem	21.4	302	171 000	Human	CA and NCA	[37]
14	Granisetron	–	–	1.44	Human	CA	[61]
15	Ginkgolide B	727	3900	NB	Human	NCA	[37]
16	Indisetron	–	–	1.70	Rat	CA	[70]
17	Irinitecan	5.37	14.0	–	Human	CA	[71]
18	Methadone	14.1	41.1	–	Human	CA and NCA	[49]
19	Mefloquine	0.66	2.70	–	Human	CA and NCA	[45]
19	Mefloquine	9.36	–	35 700	Mouse	CA and NCA	[46]
20	mCPBG	2.6	–	67.6	Human	Agonist	[61]
21	Morphine	0.33	1.15	178 000	Human	CA and NCA	[15,47]
22	Ondansetron	–	–	4.90	Human	CA	[61]
23	Palonosetron	–	–	0.03	NG108-15	CA and NCA	[72]
–	Picrotoxin[e]	41.2	1135	NB	Mouse	NCA	[38]
24	Picrotoxinin	10.7	63.1	–	Human	NCA	[37]
25	Quinine	1.06	15.8	–	Human	CA and NCA	[45]
25	Quinine	13.4	–	15 000	Mouse	CA	[46]
26	Ramosetron	0.002	–	0.15	Human	CA	[73,74]
27	Tropisetron	–	–	3.85	NG108-15	CA	[48]
28	Tubocurarine	3.4	14.2	1585	Human	CA	[2,61]
29	Δ^9-THC	0.04	–	NB	Human	NCA	[65]
30	Varenicline	5.89	–	96.7	Human	Agonist	[33]
31	VUF10166	ND	0.04	0.04	Human	Agonist and allosteric	[28]
32	*Poria cocus* extracts	~3.0	–	–	Human	NCA	[53]

[a]Abbreviations: CA, competitive antagonist; NCA, noncompetitive antagonist; mCPBG, *meta*-chlorophenylbiguanide; NB, no binding detected in radioligand studies; –, experimental data not available; ND, value not determined because dissociation of this ligand was too slow to make equilibrium measurements.

[b]The molecular structures of the ligands are shown in Figures 2 and 3 and are numbered accordingly.

[c]IC_{50} values are from electrophysiological measurements.

[d]K_d/K_i values are derived from radioligand saturation or radioligand competition data.

[e]PTX is composed of two equimolar components, picrotoxinin and picrotin; picrotin is ineffective at 5-HT_3R. Additional values for 5-HT_3R ligands can be found in reviews [4] and [8].

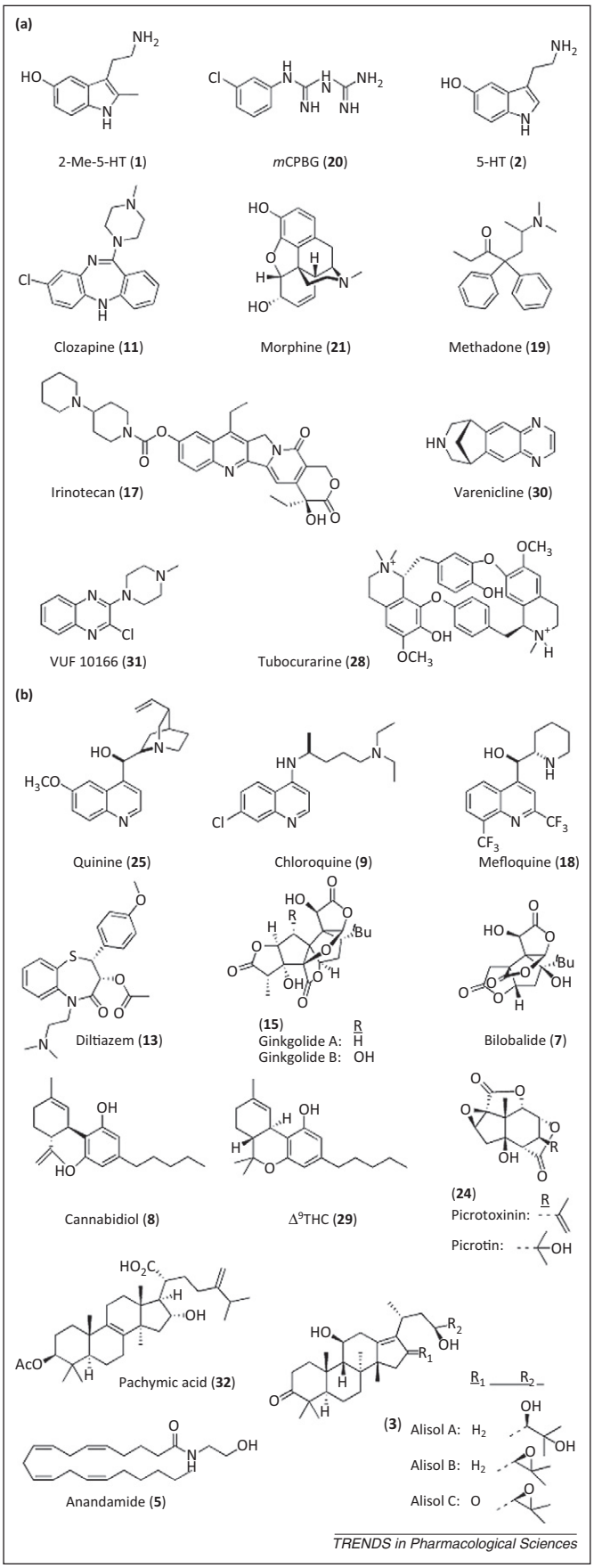

Figure 3. Compounds with activities at the 5-hydroxytryptamine$_3$ receptor (5-HT$_3$R). **(a)** Compounds that compete at the orthosteric binding site. **(b)** Noncompetitive antagonists that bind to locations other than the orthosteric binding site. None of the compounds shown in this figure are clinically relevant 5-HT$_3$R ligands (Figure 2), but some, such as diltiazem and quinine, have other clinical uses. Note that some of these compounds have mixed actions (both competitive and noncompetitive antagonist components), details of which can be found in the text and in Table 1.

evidence of allosteric modulation. Computational methods have predicted binding interactions at a possible allosteric site, but mutational evidence to confirm this is lacking [24,25]. Another possible explanation for its distinct profile is receptor internalisation, because 53% of radiolabelled palonosetron is still bound to receptors 2.5 h after removal, whereas granisetron and ondansetron are no longer detectable [6]. 5-HT$_3$A receptors leave the cell surface within minutes of incubation with palonosetron, and activation by 5-HT is concomitantly reduced. This effect could be due to slow dissociation kinetics, but the experiments were conducted over a time that exceeded that reported for full dissociation. Similar effects on internalisation have been reported for other 5-HT$_3$R ligands and at other Cys-loop receptors [26,27].

VUF10166 [2-chloro-(4-methylpiperazine-1-yl)quinoxaline] was the first CA found to have substantially differing potencies at 5-HT$_3$A and 5-HT$_3$AB receptors, suggesting a binding mode different from other 5-HT$_3$R antagonists (Figure 3A) [28]. VUF10166 competes with [^{3}H]granisetron with high affinity at A+A– binding sites in both receptor types, but is influenced by an additional allosteric site at the A+B– interface of heteromers, resulting in ~500-fold lower potency (Figure 4). This is supported by 5-HT3B subunit mutagenesis. Substitution of residues in the B+ interface have no effect on the functional properties or binding of VUF10166 at heteromeric receptors, but in the B– interface they produce 5-HT$_3$AB receptors with VUF10166 actions that closely resemble those of the homomer [28]. Ligands with differing potencies at 5-HT$_3$A and 5-HT$_3$AB receptors normally bind within the channel or elsewhere within the TMD (see below), but VUF10166 seems unlikely to bind here because its actions are unaltered by mutations within TM2 of 5-HT3A or 5-HT3B subunits, and no voltage dependence is seen even though VUF10166 is positively charged at physiological pH. At 5-HT$_3$A receptors, VUF10166 also has partial agonist activity (R_{max} = 0.24) at micromolar concentrations, but there is long-lived inhibition of subsequent 5-HT responses. At lower concentrations, agonist activity is not observable, but high-potency inhibition of 5-HT responses following preapplication most likely indicates that 5-HT$_3$A receptors accumulate in a desensitised state, similar to effects of low concentrations of other 5-HT$_3$R agonists (Box 1) [29]. Promiscuity of drug action across different Cys-loop receptors is common, but VUF10166 seems to have at least some selectivity for 5-HT$_3$R because, unlike several other 5-HT$_3$R ligands, no competition is seen at the closely related α7 nACh receptor [28,30,31].

Varenicline is a smoking cessation drug that competes with ACh as a partial agonist at α4β2 nACh receptors and enforces reduced but controlled activation of the receptor (Box 1) [32]. Varenicline is also a partial agonist at the 5-HT$_3$R and could be responsible for nausea, the most common adverse event reported in smoking cessation trials (40% of patients) and the reason for limiting its therapeutic

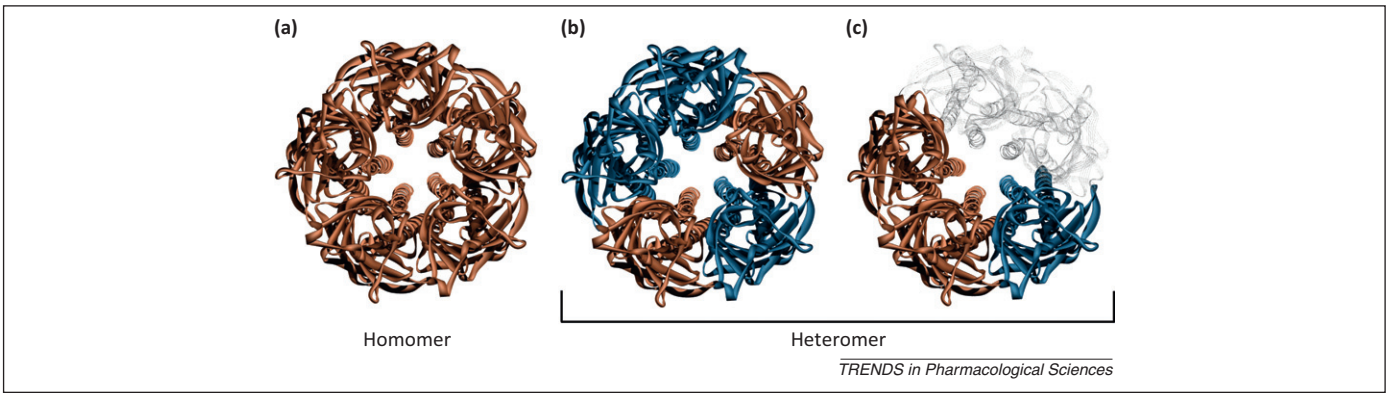

Figure 4. Stoichiometry of the 5-hydroxytryptamine$_3$ receptor (5-HT$_3$R). **(a)** The homomeric 5-HT$_3$R consists of five 5-HT3A subunits (orange) and the binding sites (A+A–) are located at the interfaces between them. **(b)** In a stoichiometry determined by atomic force microscopy, the arrangement of 5-HT3A and 5-HT3B subunits (blue) results in A+B–, B+A– and B+B– binding sites [16]. However, the majority of 5-HT$_3$R competitive antagonists (CAs) have similar affinities at both receptor types, suggesting that homomeric and heteromeric 5-HT$_3$R share a common binding site that does not exist in this stoichiometry. **(c)** Work has since shown that an A+A– interface exists in heteromeric receptors [17,18]. VUF10166 binds to this A+A– binding site and is influenced by VUF10166 binding at an A+B– interface [28]. In this figure the A+B– allosteric binding site has been placed adjacent to the A+A– interface because it is likely that a physical link is needed between the two binding sites to mediate the allosteric effect. The nature of the two remaining subunits cannot be determined from these studies and are not coloured.

Box 1. Inhibition by agonists

As well as activating 5-HT$_3$Rs, agonists also inhibit current responses, for which there are three potential mechanisms. First, inhibition may occur when low concentrations of agonists (insufficient to produce a detectable whole-cell current) are preapplied, inhibiting subsequent responses because of the accumulation of receptors in the desensitised state (occult desensitisation). This has been described for the actions of several 5-HT$_3$R agonists including VUF10166, 2-methyl-5-HT, and *meta*-chlorophenylbiguanide (mCPBG) [28,29]. Second, autoinhibition by channel blocking can manifest as depression of the agonist concentration–response curve at high concentrations, such as that seen with mCPBG [75]. This blocking is sometimes apparent as a tail current following agonist removal, and is most apparent when fast-switching drug perfusion is used. Third, a partial agonist can compete with a full agonist that causes reduced but controlled activation of the receptor. Therefore, the antagonism is merely suppression of the full agonist response when both partial and full agonists are present. This mechanism underlies the actions of varenicline at α4β2 nACh receptors and could also be a potential therapeutic application of 5-HT$_3$R partial agonists in the alleviation of irritable bowel syndrome [32].

dose. When mouse and human 5-HT$_3$R are compared, competition radioligand binding reveals similar affinities, but electrophysiology indicates greater sensitivity (IC_{50}) and efficacy (maximal current) at human receptors [33]. Different interacting residues in the binding site or differences in the activation pathway may underlie these differences, but these suggestions require confirmation. One clue may be provided by clozapine, which, like varenicline, is also a potent competitive ligand that antagonises mouse 5-HT$_3$R with higher potency (~tenfold) compared to human receptors [34]. Chimeras of human and mouse 5-HT3A subunits suggest that clozapine binds to a region close to the boundary of TM1 and the ECD (similar to some allosteric modulators; see below).

Noncompetitive antagonists

Over the past decade, the number of newly identified 5-HT$_3$R NCAs has grown (Figure 3B, Table 1). NCAs typically bind in the TMD and often have different potencies at 5-HT$_3$A and 5-HT$_3$AB receptors. These differences are typically due to differing pore-lining residues in TM2 of 5-HT3A and 5-HT3AB subunits, and for many NCAs the binding residues are known. The TM2 regions of the newly identified 5-HT3C–E subunits also differ, but NCA activity at these has yet to be reported (Figure 1E). Picrotoxin (PTX) was long considered a selective blocker of GABA$_A$ receptors, but became one of the first 5-HT$_3$R NCAs to be studied in detail; it also inhibits glycine receptors that lack a β-subunit [35]. PTX consists of equimolar quantities of picrotin (PTN) and picrotoxinin (PXN), but despite their structural similarity (Figure 3B), PTN is inactive at the 5-HT$_3$R, whereas PXN inhibits at micromolar concentrations [36]. Different potencies are observed at 5-HT$_3$A and 5-HT$_3$AB receptors, and replacement of 5-HT3A subunit residues with those aligning in the 5-HT3B subunit indicate that the 6′, 9′ and 12′ positions of TM2 are responsible for this difference [37,38]. The principal binding site for PTX is probably at the 6′ position, with the 9′ and 12′ positions influencing PTX as it descends through the narrowest region (9′–13′) of the pore. In GABA$_A$ and glycine receptors, blocking by PTX is also influenced by the 2′ and 6′ residues and is viewed at a similar position in co-crystal structures of the homologous invertebrate glutamate-gated chloride (GluCl) receptor, indicating that the PTX binding site is conserved across the family [39–41].

The terpene trilactones bilobalide (BB) and ginkgolide B (GB) have binding sites that overlap with PTX and are NCAs at several Cys-loop receptors, including 5-HT$_3$, GABA$_A$, and glycine receptors. They share the same binding site, so it is not surprising that their actions at 5-HT$_3$R are subtype-selective, and substitutions at 6′ and 12′ also abolish their inhibitory effects [37]. In addition, substitution of the 2′ residue with Ala causes GB to be trapped in the pore following channel closure, which is only fully relieved after several agonist applications; trapping was also found for BB, GA, and PTX at GABA$_A$ and glycine receptors, highlighting the similarities between family members, despite their differing ion selectivities [42,43]. Like the components of PTX, interactions of ginkgolides are specific; GA exerts only minimal inhibition at 1 mM despite only modest structural differences from GB (Figure 3B) [36]. BB and GB are also structurally similar

to PTX and are potent insecticides, but for some time it was unclear why they are not toxic to humans. A reason for this appears to be the presence of a 2′ Val residue in the channel of the related $GABA_A$ α1 subunit that renders them insensitive [44]. Mutation of this 2′ residue to Ala greatly increases the potency of these compounds at $GABA_A$ receptors (up to 10 000-fold) and accords with the 2′ Ala that is found in ginkgolide-sensitive $GABA_A$ receptors (RDL) from *Drosophila*. RDL receptors can be rendered insensitive by introducing the human 2′ Val, and the occurrence of mutations at this position correlates well with ginkgolide toxicity in insect bioassays [44].

The blocking potency of diltiazem (DTZ) also differs between 5-HT_3A and 5-HT_3AB receptors. At homomeric receptors, inhibition is voltage-dependent, and replacement of 5-HT3A subunit 7′ and 12′ residues with conserved counterparts abolishes the NCA component of inhibition [37]. However, residual inhibition remains because of a lower-affinity competitive antagonism that is equal at both receptor types. Therefore, the potency of DTZ differs at 5-HT_3A and 5-HT_3AB receptors because of the NCA component rather than the CA component, consistent with other CAs that bind to the A+A– interface of both receptors. The presence of both CA and NCA properties has been termed dual-action, mixed-action, or multi-modal antagonism, and, as noted below, is not confined to DTZ.

The antimalarial compounds quinine and mefloquine also show dual action at 5-HT_3AB receptors [45]. Mefloquine has the same potency and dual action at 5-HT_3A receptors, but quinine is 16-fold more potent at the homomer and solely competitive (Table 1). For mefloquine (quinine has not been tested), a small voltage dependence of the block is suggestive of binding within the channel [46]. The structurally related compound chloroquine is solely competitive at 5-HT_3A and 5-HT_3AB receptor and, like other CAs, has the same potency at both.

Morphine (which as an aside was instrumental in defining the 5-HT_3R concept 55 years ago) also has a dual action that consists of a low-affinity (μM) competitive component and a higher-affinity noncompetitive component of uncertain origin [47]. Competitive antagonism is evident in radioligand competition assays and the surmountable inhibition of 5-HT-evoked current responses when morphine is co-applied. A noncompetitive component becomes apparent when morphine is pre-applied, and the potency of this compound is reduced fourfold in the presence of the 5-HT3B subunit. Changes in potency following preapplication can result from failure of an inhibitor to reach equilibrium when co-applied with the agonist, or from accumulation of activated receptors in the desensitised state (Box 1). Because measurable agonist responses to morphine have not been observed, this suggests that the second of these possibilities is unlikely, but some caution may be needed because a similar apparent lack of agonist activity is seen for quipazine unless its efficacy is increased by co-application of the allosteric modulator trichloroethanol [48]. The NCA component of morphine appears not to be dependent on binding within the channel, because inhibition is not voltage-dependent and substitution of the whole 5-HT3A subunit TM2 region with the aligning 5-HT3B sequence does not affect morphine potency [47]. It is therefore conceivable that morphine binds to a site other than the orthosteric binding site formed by A+A– interfaces, although this remains to be proven. The analogue methadone also has greater potency when preapplied, and a similar fourfold reduction in potency is seen at the 5-HT_3AB receptor [49]. However, methadone inhibition is solely competitive at 5-HT_3A receptors but is insurmountable and voltage-dependent at 5-HT_3AB receptors, suggesting an additional channel component. This noncompetitive component is more sensitive to (*R*)-methadone than its stereoisomer (*S*)-methadone, unlike the competitive component, which is not stereoselective.

Δ^9-Tetrahydrocannabinol (Δ^9THC), the psychoactive component of cannabis, inhibits 5-HT currents with micromolar potency, but with slow kinetics of onset and offset [50]. Binding of [^{3}H]GR65630 is unaltered by Δ^9THC and the effects on 5-HT currents are insurmountable, suggesting it is an NCA. The related compounds cannabidiol and anandamide behave similarly, and inhibition for all three compounds depends on 5-HT_3R expression levels, a property that is difficult to explain [50,51].

There are many plant-derived NCAs with actions at 5-HT_3A receptors. PTX, quinine, α-thujone, resveratrol, ginkgolides, and Δ^9THC are discussed in detail elsewhere in this review, and others such as ginger extracts, ginseng and liquorice should also be noted [4]. Inhibition by alisol derivatives and extracts from the fungus *Wolfiporia extensa* has also been recently described, and their similar molecular structures, insurmountable effects, and voltage dependence suggest that they are all NCAs (Figure 3B, Table 1) [52,53]. Many of these NCAs can distinguish 5-HT_3A from 5-HT_3AB receptors, and such differences render them of some use in discriminating 5-HT_3R subtypes in primary tissues or confirming the expression of subunits in recombinant systems. However, potential therapeutic applications are severely hindered by their generally low potency and promiscuity.

Allosteric modulators

Allosteric modulators affect 5-HT_3R function by changing agonist sensitivity (EC_{50}), agonist efficacy (maximum response), and channel kinetics. Positive allosteric modulators achieve this by facilitating channel opening. Conversely, negative allosteric modulators increase the energy barrier for gating and reduce the probability that the channel will open. They bind in the ECD and TMD at sites distinct from the orthosteric binding site (Figure 1). There are several groups of relatively low-potency, promiscuous allosteric modulators, including *n*-alcohols, general anaesthetics, anti-depressants, cannabinoids, opioids, steroids, and natural compounds, but details of these have been reported elsewhere [4,54,55].

More recently, it was found that PU02 (6-[(1-naphthylmethyl)thio]-*9H*-purine) inhibits 5-HT_3A receptors by binding to a cavity in the upper region of the TMD [56]. Mutations at this site affect the actions of PU02 without affecting 5-HT activation or inhibition by ondansetron. Despite the different amino acids that are incorporated when the 5-HT3B subunit is coexpressed, the properties of PU02 are unaffected, and it is therefore possible that the allosteric site exists only at the A+A– interface. This contrasts with VUF10166 and several *n*-alcohols and inhaled

anaesthetics that display subtype selectivity [4,28]. The convulsant α-thujone also depends on the 5-HT3B subunit and purportedly increases the likelihood of 5-HT autoinhibition by channel blockade, a feature that particularly affects the slower 5-HT_3A receptor agonist response [57]. In support, α-thujone does not compete with [^{3}H]GR65630 at millimolar concentrations, and tail currents (brief reappearance of higher-amplitude current) that appear after removing 5-HT are not inhibited by the continued presence of α-thujone. Although its 5-HT_3R binding site is unknown, α-thujone shares some of its properties with 5-hydroxyindole (5-OHi), a compound that is affected by channel substitutions at the 15′ position [58]. Mutation at this position abolishes the potentiating effect of 5-OHi, enhances the efficacy of partial agonists, and enables 5-OHi to evoke currents in its own right [58]. However, caution may be needed when interpreting these effects because they could result from changes in ligand efficacy rather than direct effects on an allosteric binding site.

The microtubule-depolymerising agent colchicine was also recently identified as an allosteric modulator of 5-HT_3R, with differing actions at mouse and human receptors [59]. Colchicine does not compete with [^{3}H]granisetron binding at either mouse or human receptors, and this is supported by an insurmountable effect on 5-HT concentration–response curves at mouse receptors. By contrast, responses to low concentrations of 5-HT at the human 5-HT_3R are potentiated in a concentration-dependent manner (EC_{50} = 227 μM), whereas responses to 5-HT concentrations in excess of the EC_{50} (1.4 μM) are inhibited. The potentiation at human receptors is eliminated when a region corresponding to loops C and F and the pre-TM1 region are replaced with the aligned mouse sequence, and *vice versa*. Colchicine therefore binds to the interface between the ECD and TMD, where it affects the coupling of agonist binding to channel gating. The binding sites of several other allosteric compounds, such as *n*-alcohols, anaesthetics, and resveratrol, are also located in this general region (also an important determinant in other Cys-loop receptors), and this could be a rich area for the development of novel allosteric ligands [56,60].

Concluding remarks

5-HT_3R antagonists were described long before the 5-HT_3R was formally classified or cloned, and since their licensing in the 1980s the antagonists have revolutionised the treatment of adverse effects resulting from chemotherapy, radiotherapy, and general anaesthesia. The more recent development of palonosetron has further improved these treatments and can be used as a combination therapy (e.g., with dexamethasone) to confer additional benefits. Compounds with dual activities have attracted more attention recently, and this has produced ligands with actions at two or more targets. For the nACh agonist varenicline, this was not intended because it resulted in unwanted adverse effects, but its agonist actions at 5-HT_3R are now known and could provide the stimulus for reducing side effects while maintaining potency at nAChRs. Ligands that target different 5-HT_3R subtypes are lacking and although NCAs allow some discrimination, they are unlikely to be of major therapeutic value in the long term because they often have low affinity and their effects are too indiscriminate. The discovery of VUF10166 has shown that high-affinity subtype-specific ligands are possible, and with the recent identification of new subunits, this additional complexity provides potential opportunities for a larger variety of allosteric, CA, and NCA binding sites to exploit. This may allow selective targeting of specific cell types, especially if combined with recently developed methods for targeting the CNS or PNS. The association of 5-HT_3R single-nucleotide polymorphisms (SNPs) with several disorders and the clinical response to drugs also hold some promise, and may allow informed decisions on the type of therapeutic regime needed to improve care [4]. Although licensing of antagonists for the alleviation of 5-HT_3R-associated disorders has not been forthcoming, there is now a considerable variety of 5-HT_3R ligands with potential. CAs are a good option because they are selective and usually have higher affinities than NCAs. The remaining challenge is to use them to determine the physiological roles of the different 5-HT_3R subtypes, unequivocally prove their therapeutic benefits, and extend the range of clinical treatments that this class of receptors presents.

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