The binding characteristics and orientation of a novel radioligand with distinct properties at 5-HT$_3$A and 5-HT$_3$AB receptors

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**ABSTRACT**

VUF10166 (2-chloro-3-(4-methyl piperazin-1-yl)quinoxaline) is a ligand that binds with high affinity to 5-HT$_3$ receptors. Here we synthesise $[^{3}H]$VUF10166 and characterise its binding properties at 5-HT$_3$A and 5-HT$_3$B receptors. At 5-HT$_3$A receptors $[^{3}H]$VUF10166 displayed saturable binding with a $K_d$ of 0.18 nM. Kinetic measurements gave monophasic association ($6.25 \times 10^{-7}$ M$^{-1}$ min$^{-1}$) and dissociation (0.01 min$^{-1}$) rates that yielded a similar $K_d$ value (0.16 nM). At 5-HT$_3$AB receptors two association $(6.15 \times 10^{-7}, 7.23$ M$^{-1}$ min$^{-1}$) and dissociation (0.024, 0.162 min$^{-1}$) rates were seen, yielding $K_d$ values (0.38 nM and 22 nM) that were consistent with values obtained in saturation ($K_d = 0.74$ nM) and competition ($K_i = 37$ nM) binding experiments respectively. At both receptor types, specific binding was inhibited by classical 5-HT$_3$ receptor-selective orthosteric ligands (5-HT, allosterton, $\delta$-tubocurarine, granisetron, mCPBG, MDL72222, quipazine), but not by non-competitive antagonists (bilibalide, ginkgolide B, picrotoxin) or competitive ligands of other Cys-loop receptors (ACh, bicuculline, glycine, gabazine). To explore VUF10166 ligand–receptor interactions we used in silico modelling and docking, and tested the predictions using site directed mutagenesis. The data suggest that VUF10166 adopts a similar orientation to 5-HT$_3$ receptor agonists bound in AChBP (varenicline) and 5HTBP (5-HT) crystal structures.

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1. Introduction

5-HT$_3$ receptors are transmembrane ligand-gated ion-channels that are responsible for fast synaptic neurotransmission in the central and peripheral nervous systems. They are composed of five subunits, each of which contains an extracellular, a transmembrane and an intracellular domain (Thompson et al., 2008a; Miller and Smart, 2012). In vivo 5-HT$_3$ receptor activation can result in nausea and vomiting, and for over three decades competitive antagonists of these receptors have been used to alleviate these symptoms arising from cancer therapy and general anaesthetics. There is also a limited use of antagonists for treating irritable bowel syndrome and pre-clinical interest in the use of partial agonists for the same disorder (Thompson and Lummis, 2007; Walstab et al., 2010; Thompson, 2013).

There are currently five 5-HT$_3$ receptor subunits (5-HT$_3$A–5-HT$_3$E), with further complexity arising from splice variants and species differences (Walstab et al., 2010). 5-HT$_3$A subunits can form homomorphic receptors, but the subunits 5-HT$_3$B–5-HT$_3$E must combine with 5-HT$_3$A subunits to function. The functional properties of these receptor subtypes have been reported by several groups, but to date only the pharmacologies of 5-HT$_3$A and 5-HT$_3$AB receptors have been studied in detail (Holbrook et al., 2009; Walstab et al., 2010; Thompson et al., 2013; Thompson and Lummis, 2013). Until recently only pore-blocking antagonists were known to have different properties at 5-HT$_3$A and 5-HT$_3$B receptors, and these differences could be attributed to the varying pore-lining amino acids of the 5-HT$_3$A and 5-HT$_3$B subunits (Thompson and Lummis, 2013). However, the utility of these compounds is limited as they tend to be of low affinity ($\mu$M range)

**Abbreviations:** 5-HT, 5-hydroxytryptamine; nACh, nicotinic acetylcholine; GABA, gamma-aminobutyric acid; HEK, human embryonic kidney; AChBP, acetylcholine binding protein; 5HTBP, an AChBP mutant modified to resemble the 5-HT$_3$R binding site; VUF101066, 2-chloro-3-(4-methyl piperazin-1-yl)quinoxaline.

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and also target other receptor types. More recently there have been descriptions of two compounds with other sites of action that discriminate between 5-HT₃A and 5-HT₃AB receptor subtypes. One of these, topotecan, primarily an anticancer drug, was found to inhibit 5-HT₃A and potentiate 5-HT₃AB receptors, although this compound also has a relatively low (μM) potency (Nakamura et al., 2013). The second compound is VUF10166 (2-chloro-3-(4-methylpiperazin-1-yl)quinoxaline), which is highly potent, with an affinity at 5-HT₃A receptors (pKᵝᵢ ~ 10) that is ~100-fold greater than at 5-HT₃AB receptors (Thompson et al., 2012). We previously showed that VUF10166 binds to the orthosteric binding site of both 5-HT₃A and 5-HT₃AB receptors (formed at the interface of two 5-HT3A subunits, A+A−) and that a second, allosteric, binding site (A+B−) in the 5-HT₃AB receptor was responsible for causing ligands at the A+A− binding site to dissociate more rapidly.

Here we perform a detailed characterisation of VUF10166 binding to 5-HT₃A and 5-HT₃AB receptors with a radiolabelled version of this compound and use mutagenesis to explore the residues that interact with VUF10166 at the A+A− binding site.

2. Experimental procedures

2.1. Synthesis of [³H]VUF10166

60 μl [³H]methyl nosylate (0.7 GBq/ml, 19 mCl/ml) in hexane/ethyl acetate (10/2 v/v) was injected into a closed reaction screwcap reaction vessel and the solvent evaporated under argon at 60 °C. 2-chloro-3-(4-methylpiperazin-1-yl)quinoxaline hydrochloride (7.2 mg, 0.025 mmol) in dry DMF (150 μl) and DIPEA (30.7 μl, 0.176 mmol)
were added for 1 h at room temperature. The reaction was quenched with 500 μL semi-prep HPLC eluent and subjected to semi-preparative HPLC purification, using a Reprosphere C18-DE 5 μm, 50’x8 mm column as stationary phase (Dr. Maisch, Ammerbuch-Entringen, Germany) and acetonitrile/water 75/25 (v/v) with 0.1% diisopropylethylamine as eluent at a flow of 3 ml min⁻¹, with UV monitoring at 254 nm (Jasco UV-1575, Jasco, de Meern, Netherlands). 30 µL of fractions were collected, 5 µL of each added to 5 ml scintillation fluid, and counted for 1 min in a beta well counter (Rac Beta 1219 LCS, LKB-Wallac, Netherlands). Fractions containing 2-chloro-3-(4-[1H]methylyperazin-1-yl)quinoxinol were diluted with 45 ml sterile water and passed over a preconditioned Waters tC18 plus Sep-Pak, washed with 20 ml of water, and the product obtained by elution with 1.5 ml ethanol; 35 MBq water and passed over a preconditioned Waters tC18 column, washed with 7.4), homogenised and frozen. After thawing, they were washed with HEPES buffer, diluted for 2 min incubation at 0°C, labelled for 2 min with [3H]VUF10166 at relevant concentration of radioligand at 0°C, using 2 mM quipazine. Equilibrium reactions were incubated for at least 3 h for [3H]VUF10166 binding at 5-HT3A receptors.

2.4.2. Competition binding

All data were fitted with GraphPad Prism 4.03. Individual saturation binding experiments were fitted to Eqn (1), and the values averaged to obtain mean ± sem.

Table 1

| Receptor     | k_on (M⁻¹ min⁻¹) | k_off (min⁻¹) | K_d (nM) | K_s (nM) | Saturation
|--------------|------------------|--------------|----------|----------|-------------|
| VUF10166     |                  |              |          |          |             |
| 5-HT3A       | 6.25 × 10⁷       | 0.010        | 0.16     | 0.18 ± 0.04 (11) | 0.24 ± 0.11 (12) |
| 5-HT3AB      | 6.15 × 10⁷       | 0.024        | 0.38     |          |             |
| VBR43694     | 7.23 × 10⁷       | 0.162        | 22.4     |          |             |
| 5-HT3A       | 5.90 × 10⁷       | 0.064        | 1.08     | 0.68 ± 0.05 (12) | 0.12 |
| 5-HT3AB      | 1.20 × 10⁷       | 0.074        | 0.62     | 0.74 ± 0.10 (4) |             |

1 Competition binding was performed with [3H]BRL43694 and unlabelled VUF10166. k_on and k_off were calculated from plots of K_d versus ligand concentration (Figs. 1 and 5). – not determined.

2 Not significantly different to 5-HT3A (p > 0.05, Student’s t-test).

3. Results

3.1. [3H]VUF10166 binding at 5-HT3A receptors

[3H]VUF10166 showed high affinity saturable binding at 5-HT3A receptors with low levels (<5%) of non-specific binding. The K_d value was similar to the K_i value from competition of unlabelled
3.2. VUF10166 kinetic parameters at 5-HT3A receptors

Association curves for [3H]VUF10166 were best fit with a single exponential function (Fig. 1b), and the resultant rates (k\textsubscript{obs}) plotted against ligand concentration to yield k\textsubscript{on} and k\textsubscript{off} (Fig. 1c, Table 1). The value for k\textsubscript{on} was similar to values determined directly from k\textsubscript{obs} values using Equ (5) (8.24 x 10\textsuperscript{7} M min\textsuperscript{-1}). Dissociation of [3H] VUF10166 in the presence of excess cold quipazine was also monophasic (Fig. 1d), with k\textsubscript{off} values that were similar to those determined from plots of k\textsubscript{obs} against ligand concentration (Table 1). K\textsubscript{d} values calculated from these kinetic measurements (Equ (4)) were similar to those derived from the saturation and competition binding (Table 1). These results indicate [3H] VUF10166 binding can be best described by a simple bi-molecular binding scheme.

3.3. Specificity of binding

A range of competitive and non-competitive ligands of 5-HT3 and related Cys-loop receptors were tested for their ability to compete with [3H]VUF10166 binding (Table 2). All tested 5-HT3 receptor competitive ligands (agonists and antagonists) displaced [3H]VUF10166 binding. Binding was unaffected by the non-competitive ligands bicalutamide, ginkgolide and picrotxin, or the majority of competitive ligands of other Cys-loop receptors. Exceptions were strychnine (glycine receptor antagonist) and nicotine (nACh receptor agonist); these were later shown to also compete with [3H]granisetron.

Previously we showed that unlabelled VUF10166 does not compete with [3H]epibatidine at nACh receptors (the closest pharmacologically related receptor) (Thompson et al., 2012). Here we performed saturation binding experiments on nACh receptors using [3H]VUF10166 which revealed no specific saturable binding (data not shown).

These results show that classical 5-HT3 receptor competitive antagonists continue to interact with [3H]VUF10166, showing it binds to the orthosteric site.

### Table 2

| Compound       | pIC\textsubscript{50} 5-HT\textsubscript{3A} | pIC\textsubscript{50} 5-HT\textsubscript{3AB} |
|---------------|--------------------------|--------------------------|
| Allosetron    | 11.14 ± 0.01 (4)         | 11.15 ± 0.10 (4)         |
| Quipazine     | 8.84 ± 0.03 (4)          | 8.60 ± 0.75 (5)          |
| MDL27222      | 12.90 ± 0.01 (3)         | 13.23 ± 0.11 (1)         |
| mCPBG         | 7.49 ± 0.06 (4)          | 6.07 ± 0.20 (3)          |
| Granisetron   | 10.48 ± 0.08 (4)         | 10.35 ± 0.10 (3)         |
| d-Tubocurarine| 5.41 ± 0.06 (4)          | 5.44 ± 0.30 (4)          |
| 5-HT          | 4.54 ± 0.07 (3)          | 4.49 ± 0.09 (3)          |
| ACh           | NB (4)                   | NB (3)                   |
| GABA          | NB (4)                   | NB (3)                   |
| Glycine       | NB (4)                   | NB (3)                   |
| Gabazine      | NB (4)                   | NB (3)                   |
| Bicuculline   | NB (5)                   | NB (3)                   |
| Strychnine    | 5.83 ± 0.09 (4)          | 6.26 ± 0.01 (2)          |
| Picrotoxin    | NB (3)                   | NB (3)                   |
| Bilobalide    | NB (3)                   | NB (2)                   |
| Ginkgolide    | NB (3)                   | NB (3)                   |
| Nicotine      | 6.81 ± 0.23 (4)          | 6.76 ± 0.09 (2)          |

3.4. Granisetron binding at 5-HT\textsubscript{3A} receptors

To compare [3H]VUF10166 with a well-established 5-HT\textsubscript{3} receptor competitive ligand, experiments were also conducted using [3H]granisetron. As expected, [3H]granisetron showed high affinity binding at 5-HT\textsubscript{3A} receptors (Table 1). Competition binding with a range of known 5-HT\textsubscript{3} receptor agonists and antagonists gave K\textsubscript{d} values similar to those determined using competition with [3H] VUF10166 (Table 3) and to those published elsewhere (Brady et al., 2001). Similar to [3H]VUF10166, nicotine and strychnine competed with [3H]granisetron.

[3H]granisetron association rates were best fit with a monophasic curve. k\textsubscript{obs} increased with free ligand concentration and a straight line was fitted (Fig. 1e) to yield the k\textsubscript{on} and k\textsubscript{off} values in Table 1. K\textsubscript{d} values calculated from these kinetic measurements (Equ (4)) were in agreement with affinities calculated from our saturation binding studies (Table 1). Dissociation was also monophasic and the rate agreed well with that from our k\textsubscript{obs} versus concentration plots described above (Fig. 1f).

These observations show that using a well-established radio-labelled 5-HT\textsubscript{3} receptor antagonist ([3H]granisetron) we are able to accurately reproduce the binding characteristics reported elsewhere and, similar to [3H]VUF10166, they are consistent with a simple bi-molecular binding scheme.

3.5. Homology modelling & docking

To gain insights into the residues that potentially interact with VUF10166 at the orthosteric site (A–A interface), five 5-HT\textsubscript{3}A receptor homology models were generated and in silico docking of VUF10166 performed on each one (Fig. 2). A total of 50 docked poses were generated and for each of these the amino acids within 5 Å of VUF10166 were identified (Table 4). 26% of residues were common to all models, comparable to a previous docking study with granisetron, where 31% of residues were common to all of the predicted binding orientations (Thompson et al., 2005). A selection of these residues were chosen for mutagenesis based upon the following criteria, 1) side chains accessible to the ligand, 2) residues known to interact with other 5-HT\textsubscript{3} ligands or, 3) residues present in a limited number of docked poses to provide support for specific orientations. Of the 39 amino acids identified, 23 were mutated to cysteine (Fig. 3); cysteine substitution of these residues was shown to be able to interact with all of the Cys mutants have been previously shown to express on the cell-surface, and the residue positions have been similarly used for the study of our radioligand standard, [3H]granisetron (Thompson et al., 2005, 2011).

3.6. Effects of mutations

The binding affinity of [3H]VUF10166 at each of the mutant receptors is shown in Table 5, and their locations in Fig. 4. Changing 3 of the 23 residues resulted in no significant change in affinity,
suggesting these residues do not play a role in ligand binding (I71, K112, S114). For the remaining 20 mutants there were differences in the binding affinities when compared to wild type receptors, indicating that these residues may have a role in VUF10166 binding. For 9 of these residues $[^3H]$VUF10166 had reduced affinities (R92, L126, N128, I139, R145, Q151, Y153, H185, F226) and for 11 no saturable binding ($K_d > 10 \text{ nM}$) was detected (R58, W90, E129, Y141, Y143, T179, T181, W183, H185, D189, Y234, E236). All these mutant receptors have been previously shown to express in oocytes (Thompson et al., 2012).

These data show that $[^3H]$VUF10166 binds to the orthosteric site and are consistent with our findings that $[^3H]$VUF10166 competes with other 5-HT$_3$ receptor competitive ligands.

### 3.7. VUF10166 binding at 5-HT$_3$AB receptors

VUF10166 was previously shown to discriminate between 5-HT$_3$ receptors subtypes (Thompson et al., 2012) and so binding properties of the new radioligand were also tested at 5-HT$_3$AB receptors. $[^3H]$VUF10166 showed high affinity binding at 5-
HT₃AB receptors, but unlike 5-HT₃A receptors, it was complex and could not be fit with a single site model (Fig. 5a). Dissociation of [³H]VUF10166 at these receptors was best fit with a double exponential curve, which contained both a fast and a slow component; the latter was not significantly different (p < 0.05) to the single rate measured at 5-HT₃A receptors (Fig. 5b, Table 1). Association curves were monophasic (Fig. 5c), but when kobs was plotted against radioligand concentration, the data were also best approximated by a two site fit (Fig. 5d, Table 1). At concentrations of [³H]VUF10166 < 3 nM the koff and kon values were similar to 5-HT₃A receptors; below 3 nM, average kon values determined from kobs (Equ. (5)) were also similar to 5-HT₃A receptors (8.77 × 10⁷ M⁻¹ min⁻¹). At concentrations >3 nM, koff and kon had slower rates that yielded a Kd (22.4 nM; Equ. (4)) close to the value from competition binding (36.7 nM; Table 1). Competition binding with a range of ligands was performed using 0.6 nM [³H]VUF10166 and Ki values were similar to values at 5-HT₃A receptors (Table 2).

Table 4
Residues within 5 Å of docked VUF10166 in 5 different homology models of the 5-HT₃A receptor binding site.

| Residue | Model 1 | Model 2 | Model 3 | Model 4 | Model 5 |
|---------|---------|---------|---------|---------|---------|
| Ser | 1 | 1 | 1 | 1 | 1 |
| Asp | 2 | 2 | 2 | 2 | 2 |
| Glu | 3 | 3 | 3 | 3 | 3 |
| Thr | 4 | 4 | 4 | 4 | 4 |
| Ser | 5 | 5 | 5 | 5 | 5 |
| Asp | 6 | 6 | 6 | 6 | 6 |
| Glu | 7 | 7 | 7 | 7 | 7 |
| Thr | 8 | 8 | 8 | 8 | 8 |
| Ser | 9 | 9 | 9 | 9 | 9 |
| Asp | 10 | 10 | 10 | 10 | 10 |
| Glu | 11 | 11 | 11 | 11 | 11 |
| Thr | 12 | 12 | 12 | 12 | 12 |
| Ser | 13 | 13 | 13 | 13 | 13 |
| Asp | 14 | 14 | 14 | 14 | 14 |
| Glu | 15 | 15 | 15 | 15 | 15 |
| Thr | 16 | 16 | 16 | 16 | 16 |
| Ser | 17 | 17 | 17 | 17 | 17 |
| Asp | 18 | 18 | 18 | 18 | 18 |
| Glu | 19 | 19 | 19 | 19 | 19 |
| Thr | 20 | 20 | 20 | 20 | 20 |
| Ser | 21 | 21 | 21 | 21 | 21 |
| Asp | 22 | 22 | 22 | 22 | 22 |
| Glu | 23 | 23 | 23 | 23 | 23 |
| Thr | 24 | 24 | 24 | 24 | 24 |
| Ser | 25 | 25 | 25 | 25 | 25 |
| Asp | 26 | 26 | 26 | 26 | 26 |
| Glu | 27 | 27 | 27 | 27 | 27 |
| Thr | 28 | 28 | 28 | 28 | 28 |
| Ser | 29 | 29 | 29 | 29 | 29 |
| Asp | 30 | 30 | 30 | 30 | 30 |
| Glu | 31 | 31 | 31 | 31 | 31 |
| Thr | 32 | 32 | 32 | 32 | 32 |
| Ser | 33 | 33 | 33 | 33 | 33 |
| Asp | 34 | 34 | 34 | 34 | 34 |
| Glu | 35 | 35 | 35 | 35 | 35 |
| Thr | 36 | 36 | 36 | 36 | 36 |
| Ser | 37 | 37 | 37 | 37 | 37 |
| Asp | 38 | 38 | 38 | 38 | 38 |
| Glu | 39 | 39 | 39 | 39 | 39 |
| Thr | 40 | 40 | 40 | 40 | 40 |
| Ser | 41 | 41 | 41 | 41 | 41 |
| Asp | 42 | 42 | 42 | 42 | 42 |

Fig. 3. An amino acid sequence alignment showing the positions of residues mutated in this study (white text, grey boxes). The six recognised binding loops are indicated by black lines above the text. Positions of β-sheets are shown by grey lines beneath the text. Numbering of residues and structural features are taken from the AChBP protein crystal structure (Cole et al., 2004). The proteins are the human 5-HT₃A subunit (P46098) and Lymnaea stagnalis AChBP (58154).
These results show that [3H]VUF10166 has different binding properties at 5-HT3A and 5-HT3AB receptors. In the latter effects are complex and some only become apparent at higher concentrations of [3H]VUF10166.

3.8. Granisetron binding at 5-HT3AB receptors

Unlike [3H]VUF10166, [3H]granisetron saturation binding at 5-HT3AB receptors yielded \( K_d \) values that were the same as those at 5-HT3A receptors, as reported elsewhere (Table 1) (Brady et al., 2001). Association (Fig. 5e), dissociation (Fig. 5f) and \( K_i \) values from competition binding (Table 3) were also the same as those at 5-HT3A receptors.

These results show that the binding properties of [3H]granisetron are the same at 5-HT3A and 5-HT3AB receptors unlike those of [3H]VUF10166.

4. Discussion

[3H]VUF10166 binds specifically and with high affinity to 5-HT3A and 5-HT3AB receptors, with evidence of a second, lower affinity, binding site in 5-HT3AB receptors. The effects of this second site are apparent at concentrations of [3H]VUF10166 > 3 nM, and are consistent with previous work that identified an additional allosteric binding site for unlabelled VUF10166 at the A\( + \)B\( + \)/C0 interface (Thompson et al., 2012). Docking of this competitive ligand into the orthosteric (A\( + \)A\( + \)/C0) binding site, combined with data from mutagenesis, suggest that VUF10166 is oriented with its quinuclidine rings close to W183 and its basic nitrogen extended towards loop E. Individual residues, many of which have been previously shown to be important in studies of other 5-HT3 receptor ligands (including \( d \)-tubocurarine, granisetron, lerisetron, \( meta \)-chloro-phenylbiguanide and tropisetron) are also important for VUF10166 binding (Hope et al., 1999; Mochizuki et al., 1999;
4.1. The role of loop A residues

VUF10166 binding was abolished by Cys substitution of E129, slightly modified by L126C (4-fold change in $K_d$) and not altered by N128C. E129 was previously identified as an important 5-HT$_3$ receptor binding residue and may form a hydrogen bond with bound ligand, which is consistent with our data (Price et al., 2008). However, data from 5HTBP (a modified AChBP with high affinity binding for 5-HT$_3$ receptor ligands) suggest that E129 may hydrogen bond with the side chain of T179 (Kesters et al., 2013), and therefore might have primarily a structural role. L126 may also have a structural role but is less important as the effects of altering this residue were small, while N128 has been shown to play a role in gating but not binding (Price et al., 2008; Kesters et al., 2013).

4.2. The role of loop B residues

Loop B has been previously identified as both a critical structural component of the binding pocket, and it contributes to ligand binding. W183 is especially important as a constituent of the ‘aromatic box’ that exists in all Cys-loop receptor binding sites (Beene et al., 2002; Thompson et al., 2008b; Duffy et al., 2012). Other residues (T179, H185, D189) are known to stabilise the binding site structure via hydrogen bonds (Thompson et al., 2008b; Kesters et al., 2013). It is therefore not surprising that all our loop B mutations altered or abolished $[^3]H$VUF10166 binding and we suggest that T181 and W183 interact with VUF10166 while T179, H185 and D189 have a structural role.

4.3. The role of loop C residues

F226 and Y234 are also constituents of the aromatic box and mutations here alter or eliminate VUF10166 binding. F226A has no effect on granisetron binding affinity, indicating this residue is more important for VUF10166 binding (Thompson et al., 2005). In 5HTBP Y234 (Y193) interacts with 5-HT and also contributes to a conserved water network that stabilises the granisetron-bound structure (Kesters et al., 2013); a conserved water network is also seen at this location in many AChBP crystal structures and may be important in many Cys-loop receptors. E236C also abolished VUF10166 binding, consistent with studies where substitutions affect binding of both CR65630 and granisetron, as well as altering the maximal current and EC$_{50}$ of 5-HT responses (Schreiter et al., 2003; Nyce et al., 2010). However E236 mutations may adversely affect the correct assembly of the binding site rather than interfering with specific ligand interactions as Nyce et al. (2010) and Schreiter et al. (2003) showed that some E236 mutant receptors are trapped within the cell. As this hypothesis is supported by the lack of interactions in the 5HTBP structure, we consider it unlikely that E236 contributes to VUF10166 binding (Kesters et al., 2013).

4.4. The role of loop D residues

W90 is another aromatic box residue that contributes to binding. In 5HTBP the equivalent residue (W53) is involved in van der Waals interactions with granisetron and W90 may have a similar role in binding VUF10166 (Spier and Lummis, 2000; Price and Lummis, 2004; Thompson et al., 2005; Yan and White, 2005). Substitutions at W90 decrease the affinity of other potent 5-HT$_3$ receptor-specific ligands such as curare, lerisetron and 5-HT (Yan et al., 1999; Venkataraman et al., 2002a) R92 interacts with grani-setron in 5HTBP (R55), and the effects of its substitution on the affinity of VUF10166, ondansetron, granisetron and MDL72222, suggest an interaction with all of these ligands (Thompson et al., 2005; Yan and White, 2005).

4.5. The role of loop E residues

All of the mutations in loop E (Y141, Y143, R145, Q151, Y153) caused significant changes to $[^3]H$VUF10166 binding. In the 5HTBP crystal structure granisetron does not extend towards loop E, but instead lies horizontally between loops B and D, similar to the orientations of the closely related ligands tropisetron (2WNC) and cocaine (2PGZ) in AChBP. In contrast, in 5HTBP 5-HT hydrogen bonds with the backbone carbonyls of I104 (Y141 in 5-HT$_3$) and I116 (Y153), and has hydrophobic interactions with M114 (Q151), explaining why 5-HT activation is strongly affected by mutations at these locations, but effects on granisetron are less apparent (Venkataraman et al., 2002b; Price and Lummis, 2004; Thompson et al., 2011; Kesters et al., 2013). Here the affinity of VUF10166 was decreased 10-fold by Y153C and abolished by Y143C, indicating

Venkataraman et al., 2002a; Price and Lummis, 2004). The residues are discussed in more detail below.
that bound VUF10166 extends towards, and may interact with, loop E residues. As VUF10166 is also a low efficacy partial agonist at μM concentrations, and must therefore induce the same structural changes as 5-HT, it is likely it adopts an orientation that at least partially mimics that of 5-HT.

4.6. The orientation of VUF10166 in the ligand binding pocket

Our results show that VUF10166 binding is affected by many of the residues previously identified as important for binding 5-HT3 receptor antagonists, while mutation of R58, I71, K112 and S114, which are close to VUF10166 in models 1, 2 and 5, did not alter its affinity, suggesting that these models are less probable. Also in model 1 the predicted ligand orientations do not extend towards Loop E and yet residues here were important for VUF10166 binding. Similarly R145 is within 5 Å of VUF10166 in model 2, but our mutagenesis data show that altering this residue has little effect on binding affinity. Model 3 seems unlikely as these poses are positioned closer to the complimentary face of the binding site, and do not significantly interact with key principal face residues such as

Fig. 5. Radioligand binding at 5-HT₃AB receptors. (a) Binding at 5-HT₃AB receptors could not be well fit with a standard one site model; deviation occurs at a radioligand concentration of ~3 nM (arrow). Inset competition binding of unlabelled VUF10166 with [³H]granisetron. (b) Dissociation was best fit with a double exponential at 5-HT₃AB receptors (0.010 ± 0.003 min⁻¹ and 0.227 ± 0.056 min⁻¹, n = 8). (c) Association was mono-exponential, but a plot of kₐ against radioligand concentration. (f) revealed two components, showing that it was rate-limited at higher concentrations. (e) The association of [³H]granisetron was best fit with a mono-exponential function, but unlike [³H]VUF10166, the fit of kₐ against the radioligand concentration was linear at across all concentrations, yielding the values for kₐ and kₐ in Table 1. (f) Consistent with this plot, dissociation of [³H] granisetron was also best described by a single exponential function (kₐ = (0.012 ± 0.002 min⁻¹, n = 5)) that was not significantly different to 5-HT₃A receptors (p > 0.05, Student’s t-test).
T181, W183 and Y234. Models 4 and 5 have quite similar docked poses with only F226 distinguishing them; F226C mutant receptors had a 7-fold lower affinity than wild type receptors suggesting that this residue is close enough to interfere with VUF10166 binding, which would best fit with model 4.

In previous work we presented a structure-activity study (SAR) of VUF10166 analogues (Verheij et al., 2012; Thompson et al., 2013) and the active analogues from these studies would fit well into model 4 in two distinct orientations (Fig. 6). These data showed substitutions of the chlorine atom in VUF10166 (Fig. 6a, region 2) are poorly tolerated, suggesting an important interaction at this location; in both poses in Fig. 6 the chlorine atom is closely located to R92 and W90. In contrast, substitutions in regions 1 and 3 are fairly well tolerated, providing that they are not too large; neither of the poses in Fig. 6 are sterically restricted around these regions of VUF10166. The poses also explain the importance of the charged N-methylpiperazine nitrogen atom, as there are possible cation–π interactions with W183 and Y234 in one pose, with these residues contributing to π–π stacking of the quinoxaline ring in the other.

We therefore suggest that the docked poses in model 4 are most consistent with the mutagenesis data described here and our previously published SAR. It is difficult to predict whether the N-
methylpiperazine ring or the quinolazine ring is positioned toward loop E, but the orientation in Fig. 6c is most reminiscent of vare-nicline co-crystallised into AChBP (PDBID = 4AFG & 4AFT) and 5-HT in 5HTBP (2YMD), both of which are agonists at 5-HT3 receptors (Billem et al., 2012; Rucktooa et al., 2012). This similarity in orien-
tation may explain why VUF10166 also displays partial agonist activity (Thompson et al., 2012). However, it should be stressed that we must exercise caution when making these predictions as the 
physiological relevance of these structures have not yet been fully 
ascertained, for example three ligand molecules have been 
observed in a single AChBP binding site, something we would not have predicted (Brams et al., 2011; Stornaiuolo et al., 2013).

5. Conclusion

Our results show that VUF10166 interacts with several of the core binding site residues found at the A+1A− interface and, com-
bined with homology modelling and ligand docking, we propose it 
adopts an orientation similar to that of other 5-HT3 receptor ago-

References

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