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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$_3$AB 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 (6.25×10^7 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 (6.15×10^{-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, allosetron, *d*-tubocurarine, granisetron, *m*CPBG, MDL72222, quipazine), but not by non-competitive antagonists (bilobalide, 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.

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-HT3A–5-HT3E), with further complexity arising from splice variants and species differences (Walstab et al., 2010). 5-HT3A subunits can form homomeric receptors, but the subunits 5-HT3B–5-HT3E must combine with 5-HT3A 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$_3$AB receptors, and these differences could be attributed to the varying pore-lining amino acids of the 5-HT3A and 5-HT3B subunits (Thompson and Lummis, 2013). However, the utility of these compounds is limited as they tend to be of low affinity (μM 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-methylpiperazin-1-yl)quinoxaline.

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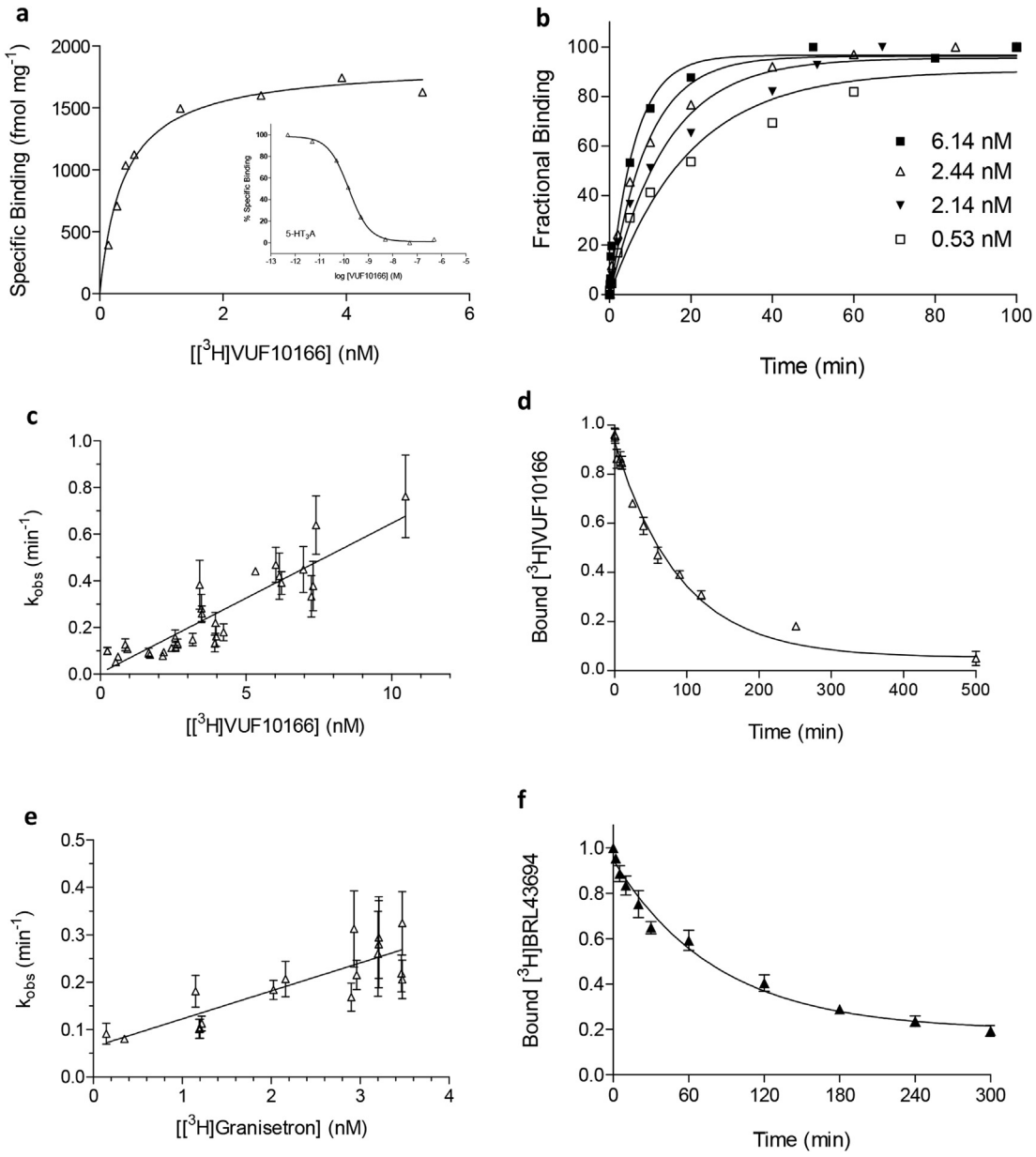

Fig. 1. Radioligand binding at 5-HT_3A receptors. (**a**) Representative binding curves for 5-HT_3A receptors. *Inset* competition binding of unlabelled VUF10166 with [^{3}H]granisetron. (**b**) Association of [^{3}H]VUF10166 was fit with a mono-exponential function to yield k_{obs}. (**c**) Linear regression was used to fit k_{obs} against the radioligand concentration, yielding the k_{on} (slope) and k_{off} (intercept at $y = 0$) values in Table 1. (**d**) Dissociation of [^{3}H]VUF10166 was best fit with a single exponential ($k_{off} = 0.011 \pm 0.001$ min^{-1}, $n = 4$). (**e**) For [^{3}H] granisetron, association was also best fit with mono-exponential functions that were used to plot k_{obs} against the concentration to yield the k_{on} and k_{off} values in Table 1. (**f**) Dissociation of [^{3}H]granisetron ($k_{off} = 0.011 \pm 0.001$ min^{-1}, $n = 5$).

and also target other receptor types. More recently there have been descriptions of two compounds with other sites of action that discriminate between 5-HT_3A and 5-HT_3AB receptor subtypes. One of these, topotecan, primarily an anticancer drug, was found to inhibit 5-HT_3A and potentiate 5-HT_3AB 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-HT_3A receptors (pK_i ~ 10) that is ~100-fold greater than at 5-HT_3AB receptors (Thompson et al., 2012). We previously showed that VUF10166 binds to the orthosteric binding site of both 5-HT_3A and 5-HT_3AB receptors (formed at the interface of two 5-HT3A subunits, A+A−) and that a second, allosteric, binding site (A+B−) in the 5-HT_3AB 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-HT_3A and 5-HT_3AB 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 [^{3}H]VUF10166

60 μl [^{3}H]methyl nosylate (0.7 GBq/ml, 19 mCi/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-(piperazin-1-yl)quinoxaline hydrochloride (7.2 mg, 0.025 mmol) in dry DMF (150 μl) and DIPEA (30.7 μL, 0.176 mmol)