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Characterizing new fluorescent tools for studying 5-HT$_3$ receptor pharmacology

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ABSTRACT

The pharmacological characterization of ligands depends upon the ability to accurately measure their binding properties. Fluorescence provides an alternative to more traditional approaches such as radioligand binding. Here we describe the binding and spectroscopic properties of eight fluorescent 5-HT$_3$ receptor ligands. These were tested on purified receptors, expressed receptors on live cells, or *in vivo*. All compounds had nanomolar affinities with fluorescent properties extending from blue to near infra-red emission. A fluorescein-derivative had the highest affinity as measured by fluorescence polarization (FP; 1.14 nM), flow cytometry (FC; 3.23 nM) and radioligand binding (RB; 1.90 nM). Competition binding with unlabeled 5-HT$_3$ receptor agonists (5-HT, *m*CPBG, quipazine) and antagonists (granisetron, palonosetron, tropisetron) yielded similar affinities in all three assays. When cysteine substitutions were introduced into the 5-HT$_3$ receptor binding site the same changes in binding affinity were seen for both granisetron and the fluorescein-derivative, suggesting that they both adopt orientations that are consistent with co-crystal structures of granisetron with a homologous protein (5HTBP). As expected, *in vivo* live imaging in anaesthetized mice revealed staining in the abdominal cavity in intestines, but also in salivary glands. The unexpected presence of 5-HT$_3$ receptors in mouse salivary glands was confirmed by Western blots. Overall, these results demonstrate the wide utility of our new high-affinity fluorescently-labeled 5-HT$_3$ receptor probes, ranging from *in vitro* receptor pharmacology, including FC and FP ligand competition, to live imaging of 5-HT$_3$ expressing tissues.

1. Introduction

High-affinity, radiolabeled 5-HT$_3$ receptor antagonists are commercially available and have been frequently used to characterize 5-HT$_3$ receptors in live cells and in *in vitro* binding assays (Barnes et al., 2009; Brady et al., 2001). However, fluorescent ligands can provide alternative opportunities for quantifying binding interactions using fast, economical and information rich methods that do not generate radioactive waste and can be more easily adapted to high-throughput technologies.

Fluorometric methods have been used in high-resolution microscopy to observe phenomena, such as the movement and internalization of cell-surface receptors, distances between fluorophores and the physical environments within proteins, among other uses (Lavis and Raines, 2008; Wysocki and Lavis, 2011). For quantitative cellular and molecular pharmacological measurements flow cytometry (FC) and fluorescence polarization (FP) can

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); ACh, acetylcholine; FACS, fluorescence activated cell sorting; FC, flow cytometry; FLAG, peptide tag DYKDDDDK; FP, fluorescence polarization; G-AD, granisetron-acridone; G-CN, granisetron-coumarin; G-FL, granisetron-fluorescein; G-TMR, granisetron-5-tetramethylrhodamine; G-TO, granisetron-thiazole orange; G-RhB, granisetron-rhodamine B; G-R101, granisetron-rhodamine 101; G-SiR, granisetron-Si-rhodamine; *m*CPBG, *meta*-chlorophenylbiguanide; PTX, picrotoxin; RB, radioligand binding.

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be used. However, the challenge with these techniques is the creation of fluorescent tracers that have a sufficiently high affinity for the protein target and give suitable fluorescent signals that are sufficiently higher than background. Here, we use the 5-HT_3 receptor as a model system to test the utility of fluorophores conjugated to a competitive antagonist of this receptor.

The 5-HT_3 receptor is a ligand-gated ion channel related to nicotinic acetylcholine (nAChRs), γ-amino butyric acid ($GABA_A$) and glycine receptors (Thompson et al., 2010a). These receptors are referred to as the Cys-loop family and are located in the cell membranes of central and peripheral synapses where they are responsible for fast neurotransmission. All of these receptors consist of an extracellular domain, a transmembrane domain and an intracellular domain. When agonists bind to extracellular binding sites a conformational change opens a transmembrane pore, allowing ions to enter the cell. Compounds that compete at these sites and prevent opening of the pore are known as competitive antagonists, and several are marketed as antiemetic drugs that alleviate symptoms resulting from chemotherapy, radiotherapy and general anesthesia (Thompson and Lummis, 2007; Walstab et al., 2010). 5-HT_3 receptor competitive antagonists are also less frequently used to treat irritable bowel syndrome (IBS), and there is interest in partial agonists for treating the same disorder (Moore et al., 2013). There have also been suggestions that 5-HT_3 receptor antagonists could be used to control neurological disorders such as depression, drug abuse, schizophrenia, fibromyalgia, pruritus and pain (Thompson and Lummis, 2007; Walstab et al., 2010). Recent literature shows that new 5-HT_3 receptor ligands continue to be identified, with particular interest in allosteric modulators and sub-type selective ligands (Thompson and Lummis, 2013). These will inevitably require further pharmacological characterization and new tools to allow the efficient analysis of receptor binding will be useful.

Here we describe the spectroscopic and ligand binding properties of a wider range of nanomolar affinity fluorophores that use granisetron as a ligand scaffold. We provide mutagenesis data that shows (i) the fluorescent probes bind to the same site as the granisetron scaffold, (ii) describe two pharmacological assays (flow cytometry and fluorescence polarization) which give robust and reproducible results that are the same as those determined using radioligand methods, and (iii) show the utility of the fluorophores in determining the localization of receptors in *in vivo* imaging. As such, these results represent the first description of a molecular toolkit that encompasses the whole fluorescent spectrum, making it appealing for a broad range of 5-HT_3 receptor studies.

2. Experimental procedures

2.1. Chemicals & drugs

Granisetron, palonosetron and tropisetron were synthesized according to published procedures (Bermudez et al., 1990; Clark et al., 1993; Langlois et al., 1993; Vernekar et al., 2010). 5-HT (serotonin) creatinine sulfate, acetylcholine (ACh) chloride, quipazine maleate, γ-aminobutyric acid (GABA), glycine and picrotoxin (PTX; an equimolar mixture of picrotoxinin and picrotin) were obtained from Sigma Aldrich (St. Louis, MO, USA). *m*CPBG was from Tocris. Human 5-HT3A (Accession: 46098) subunit cDNA was kindly provided by J. Peters (Dundee University, UK). The synthesis of fluorescent granisetron derivatives G-CN, G-FL and G-RhB (Fig. 1A) was described previously (Simonin et al., 2012; Vernekar et al., 2010). Fluorescent granisetron conjugates G-SiR, G-TO, G-R101, G-TMR and G-AD (Fig. 1A) were synthesized accordingly (for detailed description of synthetic procedures, for full spectroscopic characterization and purity assessment see Supplementary data). Briefly, fluorophores were either purchased (rhodamine 101 and acridone; Sigma Aldrich) or synthesized according to published procedures (Egawa et al., 2011; Holzhauser et al., 2010; Kvach et al., 2009), and coupled to the granisetron core via linkers.

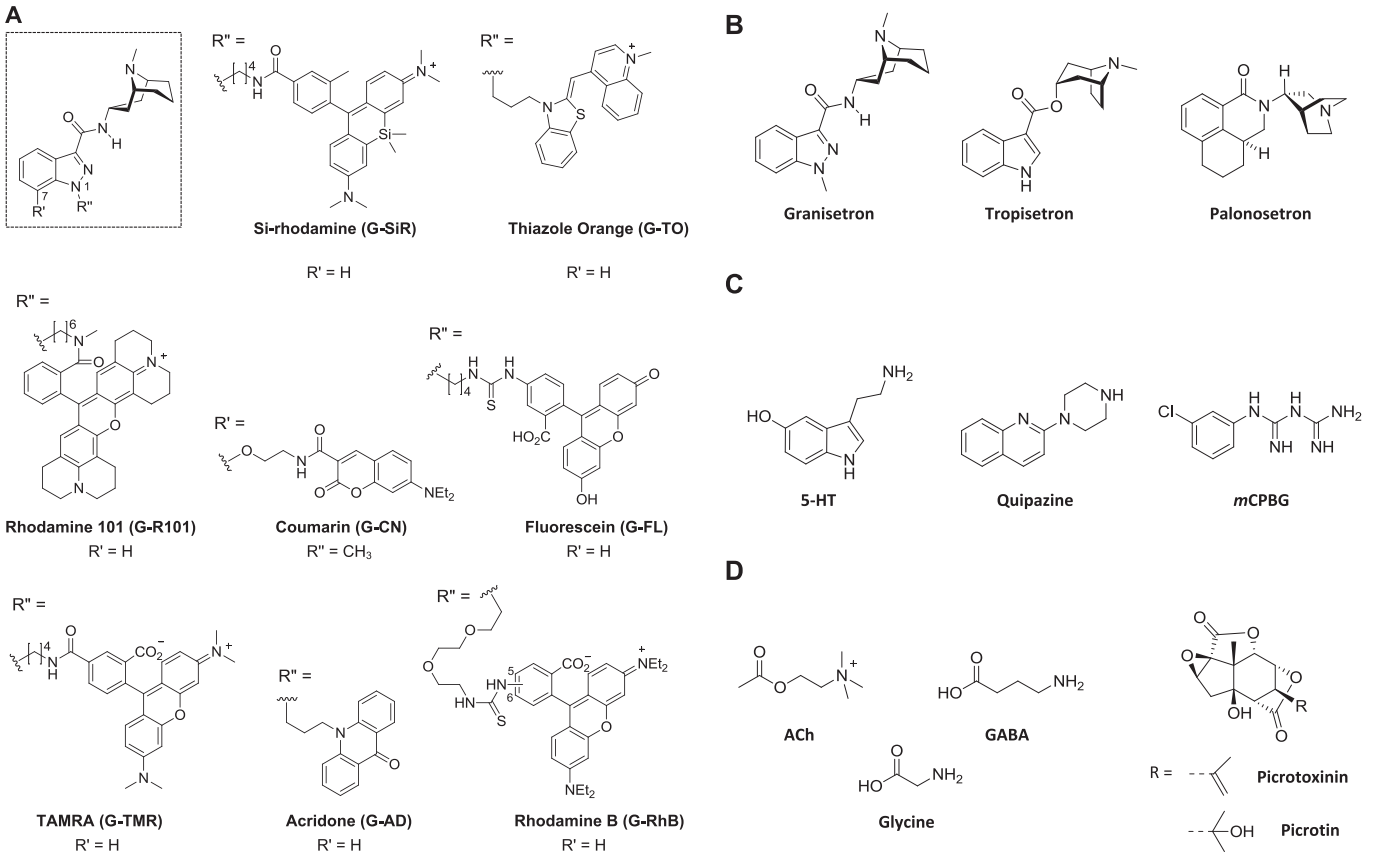

Fig. 1. A). Chemical structures of the fluorescent granisetron conjugates described here. Chemical structures of antagonists (B), agonists (C) and negative controls (D) used in competition assays.