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Agonists and antagonists induce different palonosetron dissociation rates in 5-HT_3A and 5-HT_3AB receptors

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ABSTRACT

Palonosetron is a potent 5-HT_3 receptor antagonist with a unique structure and some unusual properties. Here we explore the properties of palonosetron at heterologously expressed 5-HT_3A and 5-HT_3AB receptors. We used receptors expressed in HEK293 cells, and functionally analysed them using a membrane potential sensitive dye in a Flexstation, which revealed IC_{50}s of 0.24 nM and 0.18 nM for 5-HT_3A and 5-HT_3AB receptors respectively. Radioligand binding studies with [^{3}H]palonosetron revealed similar K_ds: 0.34 nM for 5-HT_3A and 0.15 nM for 5-HT_3AB receptors. Kinetic studies showed palonosetron association and dissociation rates were slightly faster in 5-HT_3AB than 5-HT_3A receptors, and for both subtypes dissociation rates were ligand-dependent, with antagonists causing more rapid dissociation than agonists. Similar ligand effects were not observed for [^{3}H]granisetron dissociation studies. These data support previous studies which show palonosetron has actions distinct to other 5-HT_3 receptor antagonists, and the slow rates observed for agonist induced dissociation ($t_{1/2}$ > 10 h) could at least partly explain the long duration of palonosetron effects *in vivo*.

1. Introduction

5-HT_3 receptors are members of the Cys-loop family of ligand-gated ion channels, membrane proteins that are responsible for fast excitatory and inhibitory synaptic neurotransmission in central and peripheral nervous systems, and the targets for a number of important therapeutics. 5-HT_3 receptor antagonists are routinely used in the management of post-operative, radiotherapy-induced and chemotherapy-induced nausea and vomiting and for treating irritable bowel syndrome in patients that do not respond adequately to conventional therapies. A range of other therapeutic applications has also been suggested (reviewed in (Thompson and Lummis, 2007; Walstab et al., 2010)). Antagonists are often referred to as the "setrons", and include ondansetron, granisetron and palonosetron (Fig. 1). These drugs are potent (K_d = nM – pM), long lived *in vivo*, and most are highly selective for the 5-HT_3 receptor. Furthermore, they are usually well tolerated and display only mild, transient side-effects, making them the preferred choice of drug in most instances (Aapro, 2004; Blower, 1995; Eglen et al., 1995; Hirata et al., 2007).

Palonosetron has a different structure from the other 5-HT_3 antagonists (Fig. 1), and some distinctive properties. The first published accounts of these properties were in 1995, when radioligand binding experiments demonstrated that it bound to 5-HT_3 receptors with high potency and selectivity, and *in vivo* data showed an anti-emetic efficacy greater than or equal to that of ondansetron or granisetron (Bonhaus et al., 1995; Eglen et al., 1995; Wong et al., 1995). At that time, however, it was not clear that there are multiple 5-HT_3 receptor subunits, (A-E), in addition to alternative splice variants, thus providing the potential for a wide range of different 5-HT_3 receptor subtypes. Heteromeric assemblies of 5-HT3A plus 5-HT3C, 5-HT3D or 5-HT3E subunits have not yet been extensively studied, but their biophysical properties appear similar to homomeric 5-HT_3A receptors (see (Niesler, 2011) and (Walstab et al., 2010) for reviews). 5-HT_3AB receptors, however, have been extensively investigated in heterologous systems, and have differing concentration–response curves (increased EC_{50} values and shallower Hill slopes), increased single channel conductance (5-HT_3A = sub-pS; 5-HT_3AB = 16–30 pS), an increased rate of desensitisation, reduced Ca^{2+} permeability and a non-linear current–voltage relationship (Davies et al., 1999; Kelley et al., 2003;

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); HEK, human embryonic kidney; IC_{50}, concentration of antagonist required for half-maximal inhibition; K_d, affinity constant.

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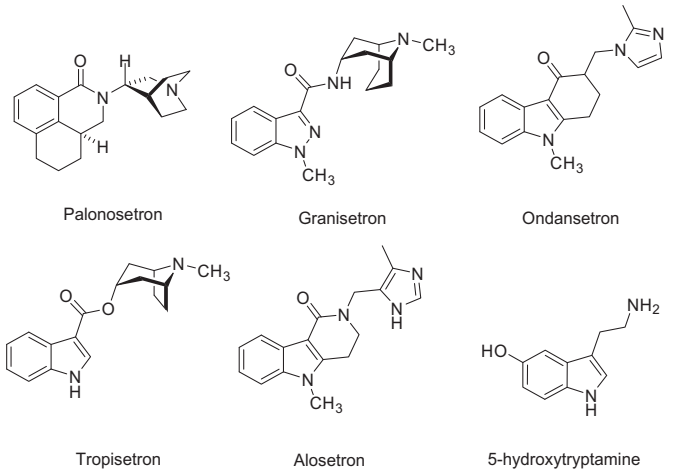

Fig. 1. Chemical structures of 5-HT and clinically used 5-HT_3 receptor competitive antagonists.

Livesey et al., 2008). To determine if there are differences in the affinity and association and dissociation rates of palonosetron in 5-HT_3A and 5-HT_3AB receptors, we here explore the effects of palonosetron on 5-HT_3 receptor function and binding in these receptor subtypes.

2. Materials and methods

2.1. Materials

All cell culture reagents were obtained from Gibco BRL (Paisley, U.K.), except foetal calf serum which was from Labtech International (Ringmer, U.K.). [^{3}H]granisetron (84 Ci mmol^{-1}) was from PerkinElmer (Boston, Massachusetts, USA). [^{3}H]-palonosetron (37.2 Ci/mmol) was custom synthesised for Helsinn Healthcare (Lugano, Switzerland), and both this and the unlabelled form of palonosetron were kindly gifted by Helsinn Healthcare (Lugano, Switzerland). All other reagents were of the highest obtainable grade.

2.2. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were maintained on 90 mm tissue culture plates at 37 °C and 7% CO_2 in a humidified atmosphere. They were cultured in DMEM:F12 (Dulbecco's Modified Eagle Medium/Nutrient Mix F12 (1:1)) with GlutaMAX™ I media containing 10% foetal calf serum and passaged when confluent. For radioligand binding studies cells in 90 mm dishes were transfected using PEI and incubated for 3–4 days before use. For functional studies cells were plated on 96 well plates, transfected using the Neon transfection system (Invitrogen) and incubated 1–2 days before assay. Mutagenesis reactions were performed using QuikChange (Agilent Technologies Inc., CA, USA) using human 5-HT_{3A} or 5-HT_{3B} receptor subunit cDNA (accession numbers: P46098 or O95264) in pcDNA3.1 (Invitrogen, Paisley, UK). Subunit numberings have been altered to the aligning residues in the mouse 5-HT_3A receptor.

2.3. Radioligand binding

Methods were as previously described (Lummis et al., 1993), with minor modifications. Briefly, transfected HEK293 cells were washed twice with phosphate buffered saline (PBS) at room temperature and scraped into 1 ml of ice-cold HEPES buffer (10 mM, pH 7.4) containing the following proteinase inhibitors (PI): 1 mM EDTA, 50 μg ml^{-1} soybean trypsin inhibitor, 50 μg/ml bacitracin and 0.1 mM phenylmethylsulphonyl fluoride. Cells were homogenised, freeze-thawed, washed with HEPES buffer, and 50 μg of the crude cell membrane preparation incubated in 0.5 ml HEPES buffer containing [^{3}H]granisetron or [^{3}H]palonosetron at a range of concentrations for saturation binding, or at 0.3 nM and 0.1 nM respectively for competition binding and association/dissociation studies. Non-specific binding was determined using 10 μM quipazine. Equilibrium reactions were incubated for at least 1 h or 24 h for [^{3}H]granisetron or [^{3}H]palonosetron respectively at 4 °C. Dissociation was initiated with unlabelled ligands to give a final concentrations of 100 μM (5-HT), 10 μM (quipazine), 1 μM (MDL72222) or 100 nM (palonosetron). All samples were terminated by vacuum filtration using a Brandel cell harvester onto GF/B filters pre-soaked in 0.3% polyethyleneimine. Radioactivity was determined by scintillation counting using a Beckman LS6000SC (Fullerton, California, USA).

2.4. Fluorescent studies

These were performed as previously described (Price and Lummis, 2005). Briefly, cells were gently rinsed twice with buffer (10 mM HEPES, 115 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, pH 7.4) and 100 μl fluorescent membrane-potential sensitive dye (Molecular Devices) added. Cells were then incubated at room temperature for 45 min before assay. For inhibition studies, palonesetron was added either with the dye, ensuring a 45 min pre-incubation, or simultaneously with 5-HT (co-application). Fluorescence was measured in a FLEXstation™ (Molecular Devices Ltd., Wokingham, UK) every 2 s for 200 s using the acquisition software SOFTmax® PRO v4.3. Control (buffer alone) or 5-HT (0.001 μM–30 μM) was added to each well at 20 s. Typical responses are shown in Fig. 2.

2.5. Data analysis

Data were analysed by iterative curve fitting using Prism software (GraphPad, San Diego, California, USA). Determination of K_i values was performed using the Cheng–Prusoff equation. Values are presented as mean ± SEM, $n = 3–6$.

3. Results

3.1. Functional studies

Examination of palonosetron inhibition of 5-HT_3 receptors expressed in oocytes revealed very slow recovery after washout, with <10% of the original response being recovered after a 10 min wash (data not shown). We therefore determined the inhibitory effects of palonosetron on 5-HT-induced responses using 5-HT_3 receptors expressed in HEK293 cells loaded with membrane sensitive fluorescent dye where washout is not required. Preliminary experiments revealed palonosetron required at least 5 min incubation before application of 5-HT to reveal its full inhibition, and thereafter it was preincubated for 45 min. Data revealed different apparent potencies of palonosetron at 5-HT_3A receptors, depending upon whether it was preincubated (pIC_{50} = 9.73 ± 0 0.13;

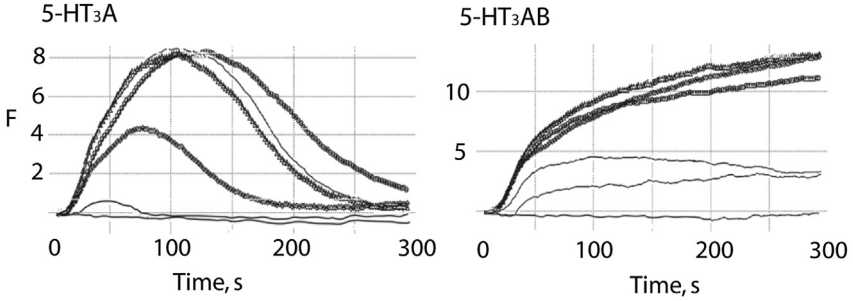

Fig. 2. Typical Flexstation responses of HEK293 cells expressing 5-HT_3A and 5-HT_3AB receptors. 5-HT at various concentrations (0–30 μM) was added at 20 s. Note the shapes of the responses, which are different in homomeric and heteromeric receptors. F = arbitrary fluorescent units.