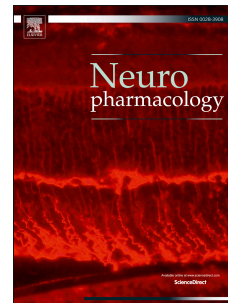# Accepted Manuscript

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5-HT

5-HT$_3$R

Scopolamine

Atropine

The Muscarinic Antagonists Scopolamine and Atropine are Competitive Antagonists at 5-HT₃ Receptors.

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ABSTRACT

Scopolamine is a high affinity muscarinic antagonist that is used for the prevention of post-operative nausea and vomiting. 5-HT$_3$ receptor antagonists are used for the same purpose and are structurally related to scopolamine. To examine whether 5-HT$_3$ receptors are affected by scopolamine we examined the effects of this drug on the electrophysiological and ligand binding properties of 5-HT$_3$A receptors expressed in *Xenopus* oocytes and HEK293 cells, respectively. 5-HT$_3$ receptor-responses were reversibly inhibited by scopolamine with an IC_{50} of 2.09 μM. Competitive antagonism was shown by Schild plot (pA_2 = 5.02) and by competition with the 5-HT$_3$ receptor antagonists [^{3}H]granisetron (K_i = 6.76 μM) and G-FL (K_i = 4.90 μM). The related molecule, atropine, similarly inhibited 5-HT evoked responses in oocytes with an IC_{50} of 1.74 μM, and competed with G-FL with a K_i of 7.94 μM. The reverse experiment revealed that granisetron also competitively bound to muscarinic receptors (K_i = 6.5 μM). In behavioural studies scopolamine is used to block muscarinic receptors and induce a cognitive deficit, and centrally administered concentrations can exceed the IC_{50} values found here. It is therefore possible that 5-HT$_3$ receptors are also inhibited. Studies that utilise higher concentrations of scopolamine should be mindful of these potential off-target effects.

1   **INTRODUCTION**

2   Scopolamine is a high-affinity (nM) muscarinic antagonist that is used to treat

3   post-operative nausea and vomiting, and motion sickness. As a research tool it is often

4   administered to induce cognitive dysfunction. At higher doses it can also produce

5   amnesia and compliance (Klinkenberg and Blokland, 2010). Atropine is a related

6   muscarinic antagonist from the same biosynthetic pathway as scopolamine and is used

7   as a cycloplegic and mydriatic in ophthalmology, and for the treatment of

8   bradychardia.

9   Scopolamine readily passes the blood brain barrier and it is believed that

10   inhibition of muscarinic receptors in the central nervous system causes a cholinergic

11   deficit that impairs memory (Klinkenberg and Blokland, 2010). As an age-related

12   deterioration in cognitive function is thought to be predominantly related to a decline

13   in cholinergic neurotransmission, scopolamine administration has often been used to

14   model dementia (Bartus, 2000). Scopolamine has therefore been extensively used for

15   preclinical and clinical testing of treatments for cognitive impairment (Bartolomeo et

16   al., 2000; Blin et al., 2009; Liem-Moolenaar et al., 2011).

17   In the clinic, $5-HT_3$ antagonists are mainly used for the treatment of nausea

18   and vomiting following cancer therapy and general anaesthesia (Thompson 2013;

19   Walstab *et al.*, 2010). Experimentally, they can also be administered to reverse

20   scopolamine-evoked learning and memory deficits (Barnes et al., 1990; Chugh et al.,

21   1991; Carli et al., 1997). In the brain $5-HT_3$ receptors are widely distributed in the

22   amygdala and hippocampus, regions of critical importance in memory and spatial

23   navigation, and involved in the control of emotional responses and their associated

24   disorders such as anxiety and depression (Gulyas et al., 1999; Thompson and

25   Lummis, 2007; Walstab et al., 2010). It is thought that the reversal of scopolamine-

26 induced cognitive dysfunction by 5-HT$_3$ receptor antagonists occurs by inhibiting pre-

27 synaptic 5-HT$_3$ receptors that modulate the functions of other neurotransmitters such

28 as acetylcholine, dopamine, γ-aminobutyric acid and glutamate in this region

29 (Seyedabadi et al., 2014). A similar mechanism is thought to underlie the anti-

30 anxiolytic and anti-depressive actions of 5-HT$_3$ antagonists.

31   5-HT$_3$ receptors are members of the Cys-loop family of ligand-gated ion

32 channels (LGIC). These are responsible for fast excitatory and inhibitory

33 neurotransmission in the central and peripheral nervous systems. The family includes

34 nicotininc acetylcholine (nACh), γ-aminobutyric acid (GABA$_A$) and glycine

35 receptors, which are all cell-surface, transmembrane ion channels. They consist of

36 five subunits that surround a central ion-conducting pore, and each subunit contains

37 three distinct functional regions that are referred to as the extracellular,

38 transmembrane and intracellular domains. The orthosteric binding site (that occupied

39 by the endogenous agonist) is located between the extracellular domains of adjacent

40 subunits, and is formed by the convergence of three amino acid loops from the

41 principal subunit (loops A - C) and three β-sheets (loops D - F) from the

42 complementary subunit (Thompson et al., 2008). Agonist binding results in the

43 opening of a central ion-conducting pore that is located within the transmembrane

44 domain (Peters et al., 2010; Hassaine et al., 2014). Ligands bind to both domains, but

45 the orthosteric binding site is the main drug target. These 5-HT$_3$ receptor competitive

46 antagonists have high affinities (nM) and conform to a pharmacophore that consists of

47 an aromatic group coupled to an azabicyclic ring via a carbonyl linker (fig 1). Both

48 atropine and scopolamine also have these structural features, suggesting that these

49 muscarinic antagonists could also bind at 5-HT$_3$ receptors (Thompson, 2013).

50   Here we use a combination of electrophysiology, radioligand binding, flow

51   cytometry and *in silico* ligand docking to provide evidence that, in addition to its

52   block of muscarinic receptors, scopolamine is also a competitive antagonist of 5-HT$_3$

53   receptors.

54

5-HT   Granisetron   SDZ-ICT 322   Tropisetron

Scopolamine   Atropine   [³H]*N*-Methylscopolamine

= [³H]

55
56
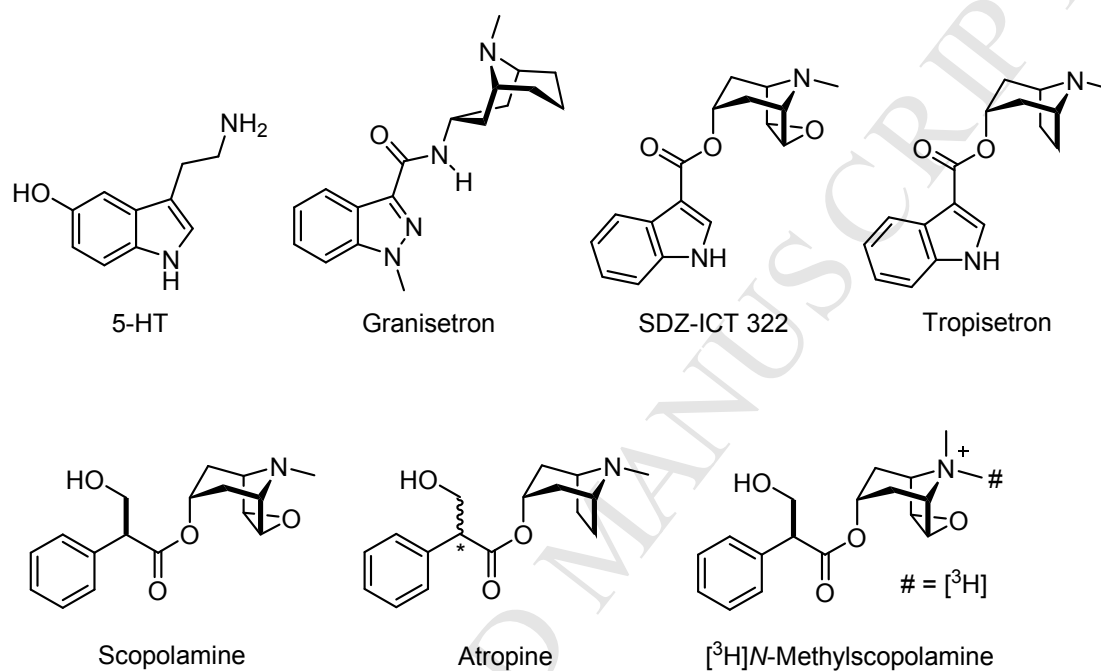
57   **Figure 1**

58   Chemical structures of endogenous agonist 5-HT, 5-HT$_3$ receptor antagonists

59   granisetron, tropisetron and SDZ-ICT 322, scopolamine, atropine and the radioligand

60   [³H]*N*-methylscopolamine. Note that scopolamine is a single enantiomer whereas

61   atropine is a mixture of epimers at the indicated (asterisk) carbon atom.

62

63   **MATERIALS AND METHODS**

64   **Materials:** Atropine and scopolamine were from Sigma-Aldrich (St. Louis,

65   MO, USA). [³H]*N*-methylscopolamine (84 Ci/mmol) was from Perkin Elmer (Boston,

3

66 MA, USA). Human 5-HT3A (Accession: 46098) subunit cDNA was kindly provided

67 by J. Peters (Dundee University, UK).

68 **Oocyte Maintenance:** *Xenopus laevis* oocytes were purchased from EcoCyte

69 Bioscience (Castrop-Rauxel, Germany) and maintained according to standard

70 methods (Goldin, 1992) in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM

71 HEPES, pH 7.4).

72 **Cell culture:** Human embryonic kidney (HEK) 293 cells were grown on 90

73 mm round tissue culture plates as monolayers in DMEM / F12 (Gibco, Life

74 Technologies, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma

75 Aldrich) at 37°C in a moist atmosphere containing 5% CO_2.

76 **5-HT$_3$ Receptor Expression**: 5-HT3A subunit cDNA was cloned into

77 pGEMHE for oocyte expression. cRNA was *in vitro* transcribed from linearised

78 plasmid cDNA template using the mMessage mMachine Ultra T7 Transcription kit

79 (Ambion, Austin, Texas, USA). Stage V and VI oocytes were injected with 50 nl of

80 100-600 ng / µl cRNA (5 - 25 ng injected), and currents were recorded 1 - 3 days

81 post-injection.

82 5-HT3A subunit cDNA was cloned into pcDNA3.1 for expression in HEK 293

83 cells. Cells were transiently transfected with this cDNA using polyethyleneimine

84 (PEI: 25 kDa, linear, powder, Polysciences Inc., Eppelheim, Germany). 30 µl of PEI

85 (1 mg ml^{-1}), 5 µl cDNA and 1 ml DMEM were incubated for 10 min at room

86 temperature, added drop wise to a 90mm plate, at 80 - 90% confluency, and incubated

87 for 2–3 days before harvesting.

88 **Muscarinic Receptor Preparation:** Muscarinic receptors were isolated from

89 the cerebral cortices of adult male Guinea pigs (200-300 g). Brains were dissected

90 into 10 mM Tris-HCl + 1 mM EDTA (pH 7.6) on ice and homogenised using a

4

91 Teflon-glass homogeniser with a motor-driven pestle (30 s, 300 rpm). The tissue was

92 pelleted 17,000 g for 30 min and the membranes resuspended, and then centrifuged

93 again using the same procedure. The final pellet was homogenised in 10 mM HEPES

94 buffer (pH 7.4) and used directly for radioligand binding. Experiments involving

95 animals were approved by the University of Cambridge Animal Welfare and Ethical

96 Review Body (PHARM 004/15).

97   **Radioligand Binding:** Saturation binding (8 point) curves were measured by

98 incubating either crude extracts of HEK 293 cells stably expressing 5-HT$_3$ receptors,

99 or Guinea pig membrane preparations, in 0.5 ml incubations containing 10 mM

100 HEPES buffer (pH 7.4) and 0.1 – 1 nM [^{3}H]granisetron or 1 – 10 nM [^{3}H]N-

101 methylscopolamine. Competition binding (10 point) was determined by incubating the

102 same receptors preparations in 0.5 ml HEPES buffer containing either 0.6 nM

103 [^{3}H]granisetron or 0.6 nM [^{3}H]N-methylscopolamine, and differing concentrations of

104 competing ligands. Non-specific binding was determined with 1 mM quipazine or 10

105 μM scopolamine respectively. Incubations were terminated by filtration onto

106 Whatman GF / B filters (Sigma Aldrich) wetted with HEPES buffer + 0.3 %

107 polyethyleneimine, followed by two rapid washes with ice-cold HEPES buffer.

108 Protein concentration was calculated using a Lowry protein assay with bovine serum

109 albumin standards (Lowry et al., 1951). Radioactivity was measured using a Tri-Carb

110 2100TR (Perkin Elmer, Waltham, MA, USA) scintillation counter.

111   **Flow Cytometry:** HEK 293 cells expressing the 5-HT$_3$ receptor were grown in

112 monolayers and harvested from 90 mm culture dishes using 10 ml Trypsin-EDTA

113 (Sigma Aldrich) for 10 min at 37°C. Digestion was terminated by the addition of 25

114 ml DMEM + 10% FBS and cells pelleted at low speed for 2 min. The pellet was

115 resuspended in 3 ml phosphate buffered saline (PBS: 137 mM NaCl, 8.0 mM

116   Na_2HPO_4, 2.7 mM KCl, 1.47 mM KH_2PO_4, pH 7.4) and cells filtered through a cell

117   strainer (BD Falcon, Franklin Lakes, NJ, USA). Competition binding was measured

118   by incubating HEK 293 cells with different concentrations of non-labeled ligands and

119   10 nM G-FL (Jack et al., 2015; Lochner and Thompson, 2015). After 10 min

120   incubation, cells were pelleted and rapidly washed in PBS before being resuspended

121   in the same buffer and analysed on a BD Accuri C6 flow cytometer (Becton,

122   Dickinson and Company, NJ, USA) at 488 nm excitation / 530 nm emission.

123   **Electrophysiology:** Using two electrode voltage clamp, *Xenopus* oocytes were

124   routinely clamped at -60 mV using an OC-725 amplifier (Warner Instruments,

125   Connecticut, USA), NI USB-6341 X Series DAQ Device (National Instruments,

126   Berkshire, UK) and the Strathclyde Electrophysiology Software Package (University

127   of Strathclyde, UK). Micro-electrodes were fabricated from borosilicate glass

128   (GC120TF-10, Harvard Apparatus, Edenbridge, Kent, UK) using a two stage

129   horizontal pull (P-1000, Sutter Instrument Company, California, USA) and filled with

130   3 M KCl. Pipette resistances ranged from 0.7 - 1.5 MΩ. Oocytes were routinely

131   perfused with ND96 at a rate of 15 ml min^{-1}. Drug application was via a simple

132   gravity fed system calibrated to run at the same rate. Antagonists were routinely co-

133   applied in the presence of 2 μM 5-HT or continuously applied for 1 min before the co-

134   application of 2 μM 5-HT. A 2 min wash was used between applications.

135   **Data Analysis:** All data analysis was performed with GraphPad Prism v5.00

136   (GraphPad Software, San Diego, CA, USA). For concentration-response curves, peak

137   currents were measured for each concentration of agonist and normalised to the peak

138   current in the same oocyte. For inhibition curves, the peak current response to 2 μM

139   5-HT was measured at in the absence or presence of antagonist and normalised to the

140   response to 2 μM 5-HT alone. The mean and S.E.M. for a series of oocytes was

141 plotted against agonist or antagonist concentration and iteratively fitted to the

142 following equation:

143

$$y = I_{min} + \frac{I_{max} - I_{min}}{1 + 10^{\log(EC_{50} - x)n_H}}$$

144 (Equ. 1)

145 where I_{min} is the baseline current, I_{max} is the peak current evoked by agonist, EC_{50} is

146 the concentration of agonist needed to evoke a half-maximal response, x is the ligand

147 concentration and n_H is the Hill slope. K_b was estimated from IC_{50} values using the

148 Cheng-Prusoff equation with the modification by Leff and Dougall (1993):((Leff and

149 Dougall, 1993)

$$K_b = \frac{IC_{50}}{((2 + ([A]/[A_{50}])^{nH})^{1/nH}) - 1}$$

150 (Equ. 2)

151 where K_b is the dissociation constant of the competing drug, IC_{50} is the concentration

152 of antagonist required to half the maximal response, [A] is the agonist concentration,

153 [A_{50}] is the agonist EC_{50}, and n_H is the Hill slope of the agonist.

154 Analysis of competitive inhibition was performed by Schild Plot according to

155 the following equation:

156 $$\log[(EC_{50}'/EC_{50}) - 1] = \log[L] - \log K_b$$

157 (Equ. 3)

158 where EC_{50}' and EC_{50} are values in the presence and absence of antagonist (Dose

159 Ratio), [L] is the concentration of antagonist, and K_b is the equilibrium dissociation

160 constant for the antagonist receptor interaction. Further analysis was performed using

161 the Gaddum-Schild equation (slope = 1) as recommended by Neubig *et al*

162 (2003):(Neubig et al., 2003)

163 $$pEC_{50} = -\log([L] + 10^{-pA_2}) - \log C$$

164 (Equ. 4)

165   where pEC_{50} is the negative logarithm of the agonist EC_{50}, $[L]$ is the antagonist

166   concentration, $\log C$ is a constant and pA_2 is the negative logarithm of the antagonist

167   concentration needed to double the concentration of agonist required in order to elicit

168   a response that is comparable to the original response in the absence of antagonist.

169   pA_2 is equal to the negative logarithm of K_b when the slope of the Schild plot is

170   exactly 1.

171   Kinetic parameters were determined according to the following model of a

172   simple bimolecular binding scheme:

173

$$L + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} LR$$

174   (Equ. 5)

175   where L is the free ligand concentration, R is receptor concentration, LR is the ligand-

176   receptor complex and k_{on} and k_{off} are the microscopic association and dissociation rate

177   constants. In a simple scheme such as this, the equilibrium dissociation constant (K_d)

178   is equal to the ratio of dissociation to association rate constants, such that:

179

$$K_d = \frac{k_{off}}{k_{on}}$$

180   (Equ. 6)

181   According to a one site binding model of the type shown, the time constants for the

182   onset and recovery of an antagonist response can be used to estimate k_{+1} and k_{-1}:

183

184                            $1/\tau_{off} = k_{-1}$ (Equ. 7)

185   and

186                      $1/\tau_{on} = k_{+1}[L] + k_{-1}$ (Equ. 8)

187   where τ_{on} refers to the time constant for the onset of inhibition, τ_{off} refers to recovery

188   from inhibition and $[L]$ is antagonist concentration.

189   Competition binding data were analysed by iterative curve fitting according to:

8

190
$$y = A_{\min} \frac{A_{\max} - A_{\min}}{1 + 10^{[L] - \log IC_{50}}}$$
(Equ 9)

191   K_i values were determined from the IC_{50} values using the Cheng-Prusoff

192   equation:

193
$$K_i = \frac{IC_{50}}{1 + [L]/K_d}$$
(Equ. 10)

194   where K_i is the equilibrium dissociation constant for binding of the unlabeled ligand,

195     $[L]$ is the concentration of labeled ligand and K_d is the equilibrium dissociation

196   constant of the labeled ligand.

197   **Docking:** We used a template of granisetron bound to 5HTBP (PDB ID

198   2YME); an AChBP chimaera with substitutions in the binding site that mimic the 5-

199   HT_3 receptor (Kesters et al., 2013). The three-dimensional structure of the

200   hydrochloride salt of scopolamine was extracted from the Cambridge Structural

201   Database (CSD, access code KEYSOW) and Chem3D Pro v14.0 (CambridgeSoft,

202   Cambridge, UK) was used to construct scopolamine based on the crystal structure.

203   The generated ligand was subsequently energy-minimised using the implemented

204   MM2 force field. Similarly, construction of the three-dimensional structure of the

205   protonated form of tropisetron was based on the crystal structure of *N*-methyl

206   tropisetron (CSD access code BEGLEG), and the three-dimensional structure of SDZ-

207   ICT 322 was based on the crystal structures of *N*-methyl tropisetron (for the indole

208   carboxylic moiety; CSD access code BEGLEG) and scopolamine (for the tricyclic

209   scopine moiety; CSD access code KEYSOW), followed by energy-minimisation. The

210   binding site was defined as being within 10 Å of the centroid of the aromatic side-

211   chain of W183, a residue that is centrally located in the binding site and is important

212   for the binding of other 5-HT_3 competitive ligands. The ligands were docked into this

213   site using GOLD Suite v5.3 (The Cambridge Crystallographic Data Centre,

214   Cambridge, UK) with the GoldScore function and default settings. For docking,

9

215   scopolamine was defined as flexible, while the C-C bond between the ester group and

216   the aromatic indole ring of SDZ-ICT322 and tropisetron was defined as rigid due to

217   conjugation. Ten docked poses were generated for each ligand and the poses

218   visualized with PyMol v1.7.5.0.

219

220   **RESULTS**

221   **Effects of scopolamine on 5-HT$_3$ receptor currents:** Application of 5-HT to

222   *Xenopus* oocytes expressing the 5-HT$_3$ receptor produced concentration-dependent,

223   rapidly activating, inward currents that slowly desensitised ($\tau = 42 \pm 5$ seconds; $n = 8$)

224   over the time-course of the applications. Plotting current amplitude against a series of

225   5-HT concentrations allowed the data to be fitted with Equ 1 to give a pEC_{50} of $5.65 \pm$

226   0.02 ($EC_{50} = 2.24$ µM, $n = 6$) and Hill slope of 2.06 ± 0.14 (fig 2A). Agonist responses

227   were completely inhibited by the established 5-HT$_3$ receptor-specific antagonist

228   granisetron (100 nM, *data not shown*). Uninjected oocytes did not respond to 5-HT.

229   Application of scopolamine to oocytes expressing 5-HT$_3$ receptors did not

230   elicit a response when applied alone, but caused a concentration-dependent inhibition

231   of the response during a co-application of 2 µM 5-HT (fig 2). The pIC_{50} value for

232   scopolamine was 5.68 ± 0.05 ($IC_{50} = 2.09$ µM, $n = 6$) with a Hill Slope of 1.06 ± 0.05.

233   This gave a K_b of 3.23 µM (Equ 2). The same concentration-dependent effect was also

234   seen when scopolamine was applied during the 5-HT application (fig 2C). Using this

235   protocol the onset of inhibition could be fitted with a mono-exponential function and

236   the reciprocal plotted against antagonist concentration to yield association (slope; k_{on}

237   $= 2.6 \times 10^4$ M^{-1} s^{-1}) and dissociation (*y*-axis intercept; 0.32 s^{-1}) rates that gave a K_d of

238   12.3 µM (fig 2D, Equ .6). Inhibition was fully reversible after 1 minute of washing

239   and was unaltered by a 1 min scopolamine pre-application (*data not shown*).

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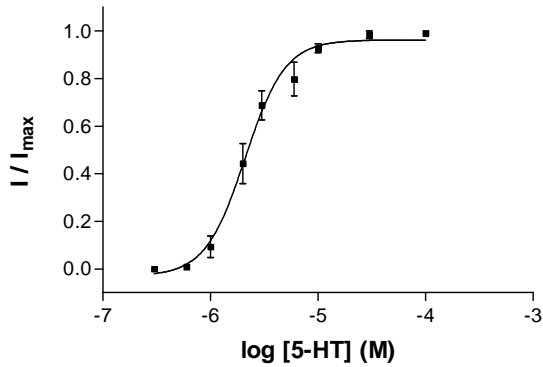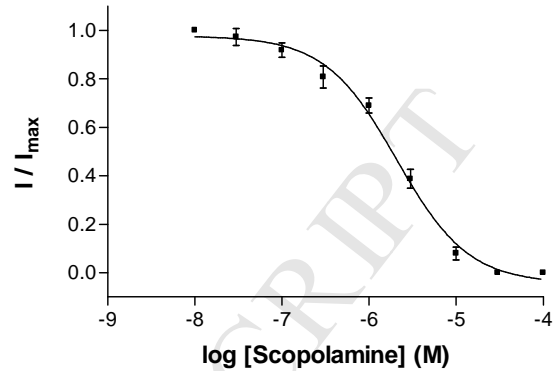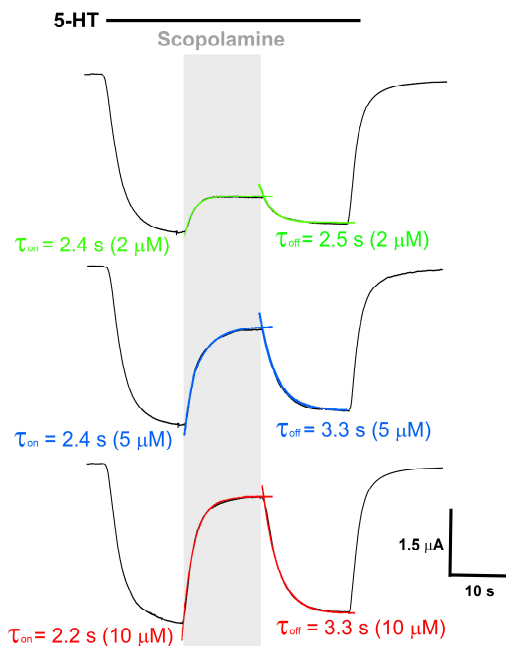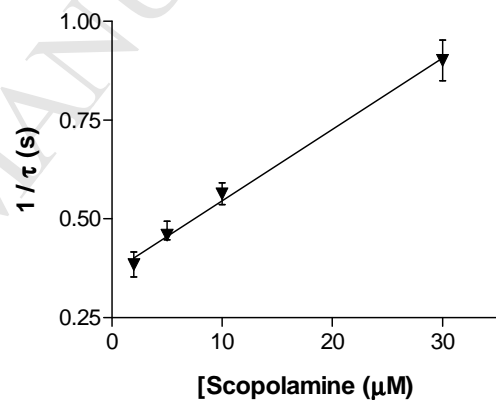
Figure 2

The effect of scopolamine on 5-HT$_3$ receptor currents. (**A**) Concentration-response curve for 5-HT. (**B**) Concentration-inhibition of the 2 μM 5-HT response by co-applied scopolamine. The data in 2A are normalised to the peak current response for each oocyte and represented as the mean ± S.E.M. for a series of oocytes. In fig 2B, inhibition by scopolamine is shown relative to the peak current response to 2 μM 5-

265 HT alone. For 5-HT curve fitting yielded a pEC_{50} of 5.65 ± 0.02 (EC_{50} = 2.24 µM, n =

266 6) and Hill slope of 2.06 ± 0.14. The pIC_{50} value for scopolamine was 5.68 ± 0.05

267 (IC_{50} = 2.09 µM, n = 6) with a Hill Slope of 1.06 ± 0.05. **(C)** Sample traces showing

268 the onset (τ_{on}) and recovery (τ_{off}) of scopolamine inhibition (*grey bar*) during a 2 µM

269 5-HT application (*filled bar*). **(D)** Onset of inhibition was well fitted by mono-

270 exponential functions to give k_{obs} (n = 17). A plot of the reciprocal of these time

271 constants versus the scopolamine concentration showed a linear relationship where

272 the slope = k_{on} (1.61 x 10^4 M^{-1} s^{-1}) and the *y*-axis intercept = k_{off} (0.37 s^{-1}).

273

274 **Mechanism of scopolamine block:** Increasing concentrations of scopolamine

275 (10 µM, 30 µM, 60 µM, 100 µM, 300 µM) caused a parallel rightward shift in the 5-

276 HT concentration-response curve, with no change in the maximal response (fig 3A,

277 table 1). A Schild plot of these results (fig 3B), yielded a gradient close to 1 (1.06 ±

278 0.10, R^2 = 0.97) and a pA_2 value of 5.03 ± 0.43 (K_b = 9.33 µM). The K_b estimate was

279 similar (2.88 µM) if the data were fitted using a nonlinear regression method (Equ. 4)

280 as recommended by Neubig *et al* (2003) and Lew and Angus (1995). These data

281 support a competitive mechanism of action, indicating that scopolamine binds to the

282 orthosteric binding site. (Lew and Angus, 1995)

283

284 **Table 1**

285 Parameters derived from concentration-response curves in the presence of increasing

286 concentrations of scopolamine.

[*Scopolamine*] *(µM)*	*pEC_{50}*	*EC_{50} (µM)*	*nH*	*n*
Control	5.65 ± 0.02	2.24	2.1	6
10	5.49 ± 0.04	3.23	2.2	4

30	5.15 ± 0.01	7.08	3.3	4
60	4.87 ± 0.03	13.5	3.4	4
100	4.84 ± 0.04	14.4	3.9	3
300	4.36 ± 0.03	43.6	2.5	5

287

288   **Binding at 5-HT$_3$ and muscarinic receptors:** To further test for a

289 competitive binding at the 5-HT$_3$ receptor, we measured competition of unlabelled

290 scopolamine with [^{3}H]granisetron, an established high-affinity competitive antagonist

291 at these receptors. Scopolamine displayed concentration-dependent competition with

292 0.6 nM [^{3}H]granisetron ($\sim K_d$, fig. 4), yielding an average pK_i (Equ. 10) of 5.17 ± 0.24

293 (fig 4; K_i = 6.76 µM, n = 3).

294

295   A   B
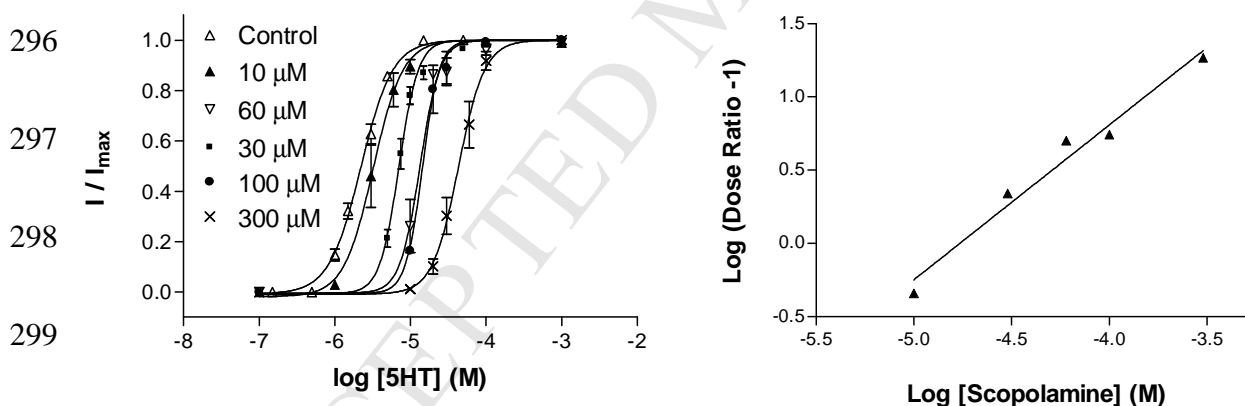
301 **Figure 3**

302 The mechanism of 5-HT$_3$ receptor inhibition by scopolamine. (**A**) Concentration-

303 response curves were performed in the absence or presence of the indicated

304 concentrations of scopolamine. The curves showed parallel dextral shifts with

305 maximal currents restored by increasing concentrations of 5-HT. Parameters derived

306 from these curves can be seen in table 1. (**B**) A Schild plot was created from the dose

307   ratios of the curves shown in 3A and fitted with Equ. 3. to yield a slope of 1.06 ± 0.10

308   ($R^2 = 0.97$) and a pA_2 of 5.03 ± 0.43 (K_b, 2.88 µM).

309

310

311   Saturation binding using radiolabelled scopolamine was also undertaken at 5-

312   HT$_3$ receptors. Although the K_i of scopolamine was too low to accurately measure

313   binding, the compound [^{3}H]N-methylscopolamine that we used contains a permanent

314   quaternary amine that increases its affinity at nicotinic receptors (fig. 1, Schmeller et

315   al., 1995). However, at concentrations of up to 10 nM, no saturable binding was

316   observed for this radioligand at 5-HT$_3$ receptors.

317   Competition of scopolamine was also measured at 5-HT$_3$ receptor by flow

318   cytometry with a fluorescently labelled form of granisetron (G-FL, (Jack et al.,

319   2015)). Concentration-dependent competition of G-FL with scopolamine gave an

320   average pK_i (Equ. 11) of 5.31 ± 0.09 (fig 4; $K_i = 4.90$ µM, $n = 8$). This is similar to the

321   affinities measured using electrophysiology and radioligand binding and provides

322   further support for a competitive mode of action.

323   In the reverse experiment, competition binding of granisetron with [^{3}H]N-

324   methylscopolamine was examined at muscarinic receptors. The IC_{50} for granisetron at

325   muscarinic receptors was 14.1 ± 3.1 µM ($n = 7$), yielding a K_i of 6.5 µM (Equ. 10).

326   **Properties of atropine:** Atropine is a structurally related muscarinic

327   antagonist (fig. 1). To test its pharmacological properties we performed measurements

328   using electrophysiology and flow cytometry. In oocytes expressing 5-HT$_3$ receptors,

329   atropine did not elicit a response when applied alone, but it caused concentration-

330   dependent inhibition of the 2 µM 5-HT-evoked response with a pIC_{50} of 5.76 ± 0.14

331   ($IC_{50} = 1.74$ µM, $n = 5$) and Hill Slope of 1.06 ± 0.05 (fig 5A). This yielded a K_b of

14

332 1.89 µM (Equ 2). Inhibition was fully reversible after 1 minute of washing and was

333 unaltered by pre-application (*data not shown*).

334 Competition of G-FL and atropine was also shown by flow cytometry (fig

335 5B). Concentration-dependent measurements were fitted to give a pK_i (Equ 10) of
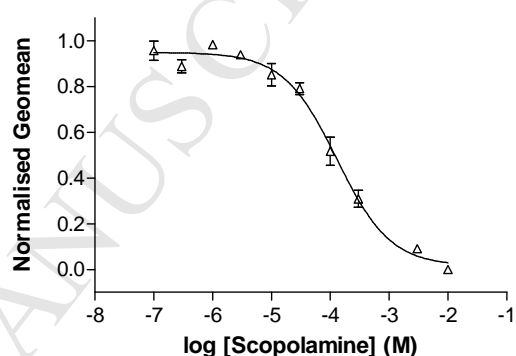
336 5.10 ± 0.16 ($K_i = 7.94$ µM, $n = 5$).

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338 **A**   **B**

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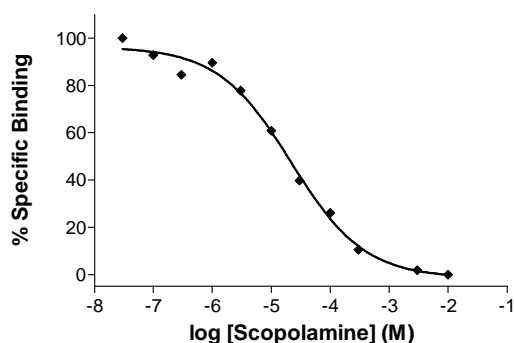
345 **Figure 4**

346 Competition of scopolamine with an established 5-HT$_3$ receptor antagonist. (**A**)

347 Radioligand binding curves for the competition of 0.6 nM [³H]granisetron and

348 varying concentrations of scopolamine at crude membrane extracts of 5-HT$_3$ receptors

349 from stably expressing HEK 293 cells. Data was normalised to [³H]granisetron

350 binding in the absence of antagonist and fitted with Equ. 10. The curve is

351 representative of 3 similar experiments, which gave an average pK_i of 5.17 ± 0.24 (K_i

352 $= 6.76$ µM, $n = 3$). (**B**) Flow cytometry, showing the competition of 10 nM G-FL (a

353 fluorescent derivative of granisetron; Jack et al., 2015) and varying concentrations of

354 scopolamine at 5-HT$_3$ receptors expressed on the surface of live HEK 293 cells. The

355 average pK_i of these experiments was similar to values from radioligand competition

356 $(5.31 \pm 0.09, K_i = 4.90$ µM, $n = 8$).

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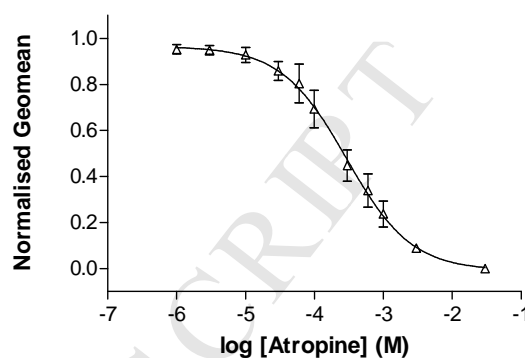359 A   B

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365 **Figure 5**
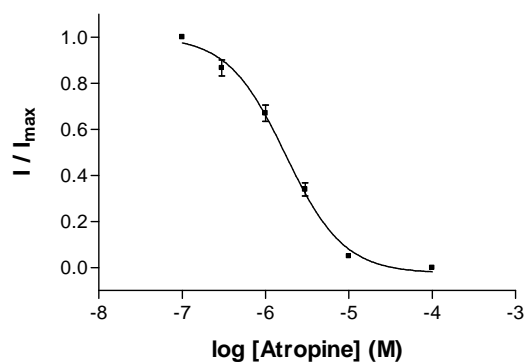
366 Effects of atropine on the electrophysiological responses to 5-HT and binding of G-

367 FL. (**A**) Concentration-inhibition of the 2 µM 5-HT response by co-applied atropine.

368 For each oocyte the responses in the presence of antagonist are normalised to the peak

369 current response to 5-HT alone and data represented as the mean ± S.E.M. for a series

370 of oocytes. Curve fitting yielded a pIC_{50} of 5.76 ± 0.14 (IC_{50} = 1.74 µM, n = 5) and

371 Hill Slope of 1.06 ± 0.05. (**B**) Flow cytometry, showing the competition of 10 nM G-

372 FL (a fluorescent derivative of granisetron; Jack et al., 2015) and varying

373 concentrations of atropine at 5-HT$_3$ receptors expressed on the surface of live HEK

374 293 cells. The affinity (pK_i = 5.10 ± 0.16, K_i = 7.94 µM, n = 5) of atropine calculated

375 from these experiments was similar to that measured using electrophysiology.

376

377   **Docking studies:** Based upon the evidence that scopolamine binds at the

378 orthosteric binding site we used a bio-informatics approach to probe possible ligand

379 orientations and try to understand why the affinity of scopolamine was lower than

380 other established 5-HT$_3$ receptor antagonists. To this end we chose a crystal structure

16

381   of a 5-HT$_3$ receptor-AChBP chimera (termed 5HTBP) complexed with granisetron

382   (PDB ID: 2YME) as a binding site model (fig 6A, Kesters et al., 2013). For the

383   purpose of validation we first removed granisetron from the template and re-docked

384   both this ligand and the closely related 5-HT$_3$ receptor antagonist, tropisetron, into the

385   binding site template. The proposed ligand orientations of these two antagonists were

386   almost identical to the binding pose from the crystal structure 2YME. This is

387   illustrated in fig. 6B where tropisetron is shown with its bicyclic moiety located

388   between the aromatic side chains of W90, W183 and Y234 and the flat indole ring is

389   sandwiched between loop C and R92 from loop D.

390   Following from our docking with established 5-HT$_3$ antagonists, we

391   performed docking with scopolamine. This yielded a docked pose cluster (fig. 6C)

392   that placed the scopine head of scopolamine at the same location as the azabicyclic

393   rings of granisetron and tropisetron, but owing to the flexibility of scopolamine and

394   the steric restraints imposed by the tight binding cavity, the hydroxyl of the carbonyl

395   linker was extended into a pocket at the rear of the binding site, displacing the

396   aromatic ring by ~ 3 Å towards the principal binding interface (fig 6D).

397   SDZ-ICT 322 (fig. 1), is a competitive, highly potent 5-HT$_3$ receptor

398   antagonist that contains key structural elements of both scopolamine and high affinity

399   5-HT$_3$ receptor antagonists such as granisetron and tropisetron (Blum et al., 1992); it

400   has the same tricyclic scopine moiety as scopolamine, which is rigidly linked to the

401   flat heteroaromatic group (indole) found in granisetron and tropisetron. Docking of

402   SDZ-ICT 322 into the 5-HT$_3$ receptor binding site predicted an orientation similar to

403   granisetron and tropisetron, with its aromatic indole group close to the side chain of

404   R92 from loop D and the scopine tricycle pointing towards the β-sheets of the

405   principal face, surrounded by the aromatic rings of W90, W183 and Y234 (fig. 6E).
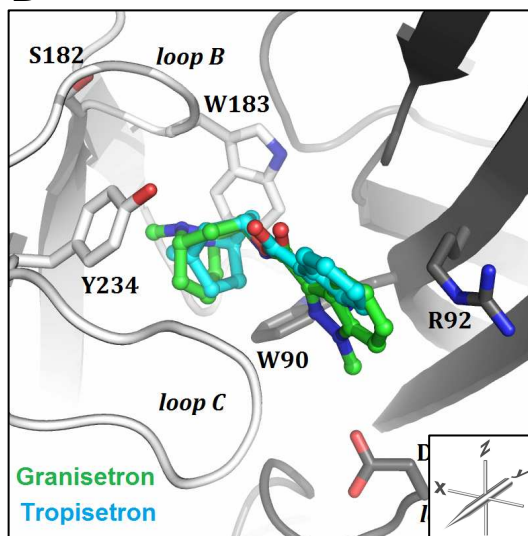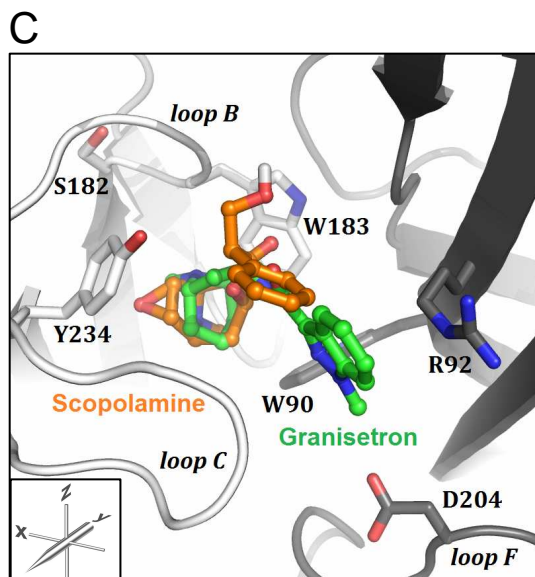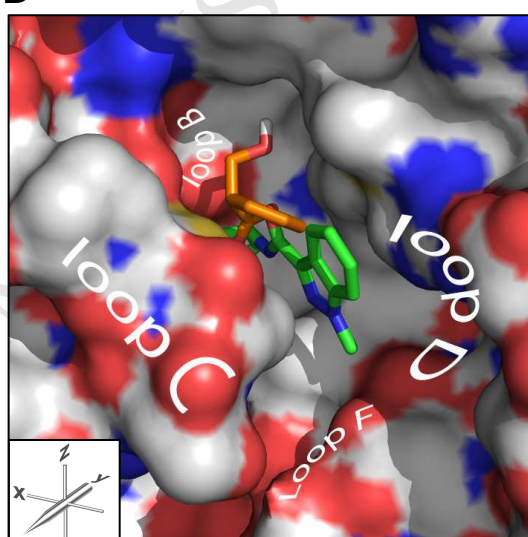
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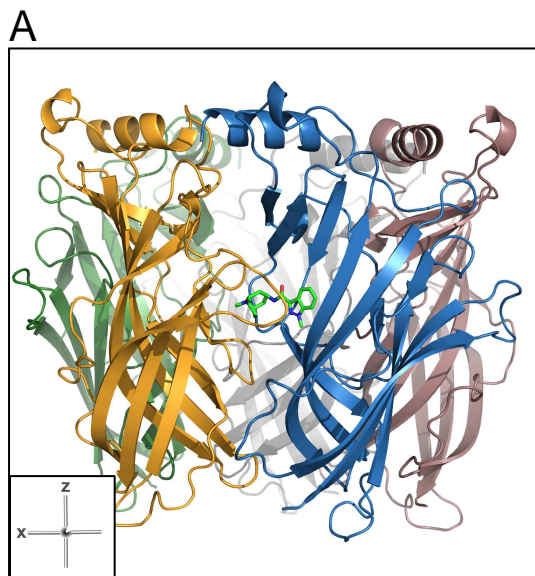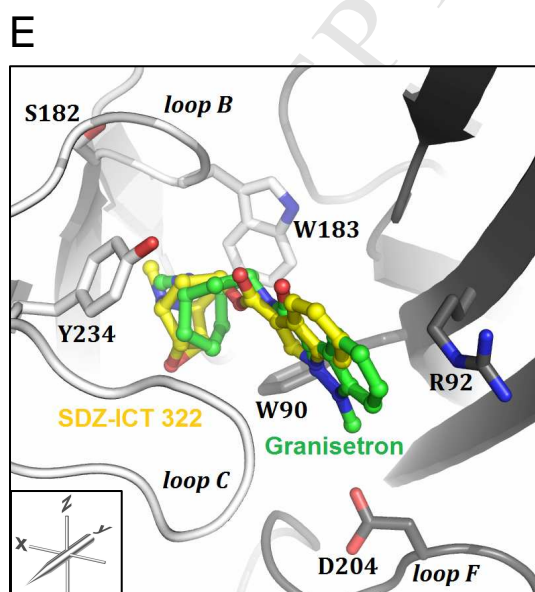
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431   **Figure 6**

432   epresentative examples of 5-HT$_3$ receptor antagonists (ball-and-stick representation)

433   docked into a 5-HT$_3$ receptor orthosteric binding site model (PDB ID: 2YME; a co-

434   crystal of granisetron bound to a mutant AChBP that contains residues from the 5-HT$_3$

435   receptor binding site (termed 5HTBP; Kesters et al., 2013) and important binding site

436   residues (stick representation). Principle face (left-hand side, light grey),

437   complementary face (right-hand side, dark grey). **(A)** 2YME from the side (*y*-axis)

438   showing the location of granisetron (green) in the orthosteric binding site at the

439   interface of two adjacent subunits. **(B)** Proposed binding pose for tropisetron (blue)

440   overlaying granisetron (green) from the co-crystal structure 2YME. **(C)** The proposed

441   binding pose for scopolamine (orange) showing its orientation in the 5-HT$_3$ binding

442   site. (**D**) A surface representation of 5HTBP bound with granisetron and an overlay of

443   docked scopolamine showing the hydroxyl of the carbonyl linker that, owing to steric

444   constraints, is located within a cavity at the rear of the binding site. It can be seen that

445   while the scopine head of scopolamine (orange) is at the same location as the

446   azabicyclic rings of granisetron (green), the steric bulk, flexibility and presence of a

447   hydroxyl in the linker region results in the aromatic ring being orientated away from

448   loops D and F. **(D)** In contrast, the proposed binding pose for SDZ-ICT 322 (yellow)

449   is more similar to that of granisetron. For chemical structures of the described ligands

450   see fig. 1.

451

452   **DISCUSSON**

453   This study describes the effects of scopolamine and atropine on human 5-HT$_3$

454   receptors. Both compounds were antagonists with µM potencies. For scopolamine,

455   binding at the orthosteric site was demonstrated by Schild analysis and competition

456   with the 5-HT$_3$ receptor antagonists [^{3}H]granisetron and G-FL. *In silico* docking

457   predicted that molecular features of the carbonyl linker of scopolamine may alter its

458   orientation within the binding site and could account for the lower potency when

459   compared to established 5-HT$_3$ receptor antagonists. Evidence for this is discussed in

460   more detail below.

461   The observation that scopolamine competitively inhibits 5-HT$_3$ receptor

462   responses was anticipated as it has structural similarities with other 5-HT$_3$ receptor

463   antagonists (fig 1) and ligand promiscuity at 5-HT$_3$ receptors has been reported

464   elsewhere. For example, epibatidine and tropisetron are high affinity agonists of α7

465   nACh and high affinity antagonists of 5-HT$_3$ receptors. Similarly, 5-HT$_3$ receptors

466   also have lower affinity competitive interactions with dopamine, acetylcholine,

467   nicotine, *d*-tubocurarine, chloroquine, varenecline and strychnine, as well as allosteric

468   modulators such as anaesthetics, alcohols, steroids and terpenoids and the non-

469   competitive antagonists picrotoxin, ginkgolides and mefloquine (Thompson and

470   Lummis, 2008; Thompson and Lummis, 2013; Thompson et al., 2014). It is perhaps

471   more surprising that the affinities of scopolamine and atropine were not higher given

472   their structural similarities to 5-HT$_3$ receptor antagonists that bind with nM affinities.

473   However, the lower affinities are likely to result from both scopolamine and atropine

474   having an aromatic ring that is not directly attached to the ester moiety that forms the

475   link with the bicyclic amine, a bond that is common to all 5-HT$_3$ receptor antagonists

476   (Thompson, 2013). The direct conjugation of the carbonyl (ester or amide) group with

477   the aromatic ring provides 5-HT$_3$ receptor antagonists with planarity and rigidity that

478   is crucial for potent inhibition and high-affinity binding (Hibert, 1994). Instead,

479   scopolamine and atropine have linkers that contain a tetrahedral carbon that carries a

480   polar hydroxymethyl substituent (fig 1). The importance of this region is highlighted

20

481   by SDZ-ICT 322, a ligand that is also a high affinity 5-HT$_3$ receptor antagonist (pA_2 =

482   10.6 in isolated rabbit vagus nerve, pK_d = 9.2 in N1E cells) but has the same scopine

483   tricyclic moiety as scopolamine directly linked to the aromatic indole ring (Blum et

484   al., 1992). This hypothesis is further supported by the low affinity of atropine which

485   contains the same tetrahedral carbon, while the close analogue tropane benzoate, with

486   a carbonyl linker, has high affinity at 5-HT$_3$ receptors (63 nM; Fozard 1989). We also

487   found that the potent 5-HT$_3$ receptor antagonist, granisetron, binds with a micromolar

488   affinity at muscarinic receptors, suggesting that while general conformations of these

489   ligands enable them to share common binding sites at both receptors, the linkers are

490   likely to confer the key structural elements that drive receptor selectivity.

491   To find further evidence for the importance of this linker region, we performed

492   docking into a homologue of the 5-HT$_3$ receptor that has been co-crystallised with the

493   antagonist granisetron in its binding site (Kesters et al., 2013). The predicted binding

494   pose for the high affinity antagonist SDZ-ICT 322 was similar to the orientations of

495   granisetron and tropisetron ligands in 5HTBP and AChBP co-crystal structures (fig

496   6E), which was anticipated given the similarity in their structures (fig. 1) and affinities

497   (Hibbs et al., 2009; Kesters et al., 2013). However, in scopolamine the tri-substituted

498   tetrahedral carbon between the scopine tricyclic moiety and the aromatic phenyl ring

499   leads to a kink in the molecular structure, unlike the high-affinity 5-HT$_3$ receptor

500   which are planar. In scopolamine this linker also contains a hydroxyl group. The

501   docking results lead us to speculate that the substituted tetrahedral carbon in

502   scopolamine creates increased bulk and ligand flexibility, while the polar hydroxyl

503   group is sterically restricted and occupies a cavity in the rear of the binding site. If

504   these predictions are correct, the differences in the linker region orientate scopolamine

505   away from residues in binding loops D and F (fig 6D), and the ligand no longer

506 engages with residues that are essential for high affinity binding (Thompson et al.,

507 2005; Thompson et al., 2006).

508 Scopolamine is generally regarded as a non-selective muscarinic receptor

509 antagonist with an affinity ≤ 1 nM. At higher concentrations it also blocks nicotinic

510 acetylcholine receptors (IC_{50} = 928 µM) and increases the expression of α7 nACh

511 receptors (Schmeller et al., 1995; Falsafi et al., 2012). When using scopolamine for

512 the prevention of motion sickness in humans, blood concentrations following

513 transdermal and combined oral administration have been reported to peak at ~0.37 ng

514 ml^{-1} within an hour (Nachum et al., 2001). Elsewhere, higher plasma concentrations

515 of 2.9 ng ml^{-1} are reported following intravenous administration (0.4 mg) to healthy

516 volunteers (Putcha et al., 1989). Both of these values are significantly lower than the

517 concentrations that affect 5-HT$_3$ receptors and it is unlikely that these receptors would

518 be inhibited. However, when scopolamine is used to induce cognitive dysfunction in

519 rodents, intraperitoneal or sub-cutaneous injections of up to 2 mg kg^{-1} are used

520 (Klinkenberg and Blokland, 2010). As a weight per volume this is the equivalent of

521 ~1 µM which is close to the IC_{50} at 5-HT$_3$ receptors. For centrally administered

522 scopolamine the focal concentrations at the site of administration can be as high as

523 140 µg µl^{-1} (460 µM), a concentration that is far in excess of its IC_{50} at 5-HT$_3$

524 receptors and would cause complete inhibition (Klinkenberg and Blokland, 2010).

525 The amygdala and hippocampus are of critical importance in implicit and

526 explicit memory, and this function is mediated via actions of both cholinergic and

527 serotonergic pathways. As scopolamine blocks muscarinic receptors with high affinity

528 it is used to induce cognitive dysfunction, but it is also known that 5-HT$_3$ receptor

529 antagonists alleviate these symptoms. Long-term potentiation (LTP, the neural

530 mechanism through which memory is formed) in the amygdala and hippocampus is

22

531 inhibited by 5-HT$_3$ receptor agonists and promoted by antagonists (Staubli and Xu,

532 1995). These effects are probably mediated via actions on the GABA-ergic synaptic

533 activity of interneurons, but may also result from activities at 5-HT$_3$ receptors that are

534 present outside of the hippocampus and would also be blocked by systemically

535 administered 5-HT$_3$ antagonists. If sufficiently high concentrations of scopolamine

536 were centrally administered we might expect a similar block of 5-HT$_3$ receptors which

537 could complicate the interpretation of its physiological effects. Pre-administering 5-

538 HT$_3$ antagonists to alleviate cognitive dysfunction might further complicate these

539 studies as their higher affinities and slower elimination from the body would prevent

540 scopolamine binding at 5-HT$_3$ receptors (Putcha et al., 1989). As mood disorders such

541 as anxiety and depression are also mediated by both cholinergic and serotonergic

542 pathways, the interpretation of scopolamine effects on these might be similarly

543 affected (Bétry et al., 2011).

544   In summary, we provide the first reported evidence that the drug scopolamine

545 inhibits the function of homomeric 5-HT$_3$ receptors via a competitive mode of action,

546 and suggest that the bond that links the kinked and more flexible structure of

547 scopolamine is responsible for the lower affinity when compared with other typically

548 flat and rigid 5-HT$_3$ receptor antagonists. Because the concentration of centrally

549 administered scopolamine can exceed the concentration that inhibits 5-HT$_3$ receptors,

550 it is likely that these receptors would be inhibited under this experimental paradigm,

551 and could influence LTP. Given this finding we believe that the potential effects at 5-

552 HT$_3$ receptors should be considered before centrally administering high

553 concentrations of this compound.

554

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23

556 **ACKNOWLEDGMENTS**

557

562

563 There are no conflicts of interest arising from this work.

564

565

566 **AUTHORSHIP CONTRIBUTIONS**

567

568 *Participated in research design:* AJT

569 *Conducted experiments:* AJT

570 *Contributed reagents or analytical tools:* -

571 *Performed data analysis:* AJT, ML

572 *Wrote or contributed to the writing of the manuscript:* AJT, ML

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- Muscarinic ligands scopolamine and atropine also have micromolar affinity at 5-HT$_3$ receptors.
- The 5-HT$_3$ receptor ligand granisetron also has micromolar affinity at muscarinic receptors
- Scopolamine has a tetrahedral carbon linker that is responsible for its lower affinity at 5-HT$_3$ receptors.
- Scopolamine is used as a preclinical model for inducing cognitive dysfunction.
- Use of high concentrations may inhibit 5-HT$_3$ receptors and complicate analysis.