The Antimalarial Drug Proguanil is an Antagonist at 5-HT$_3$ Receptors.

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Abbreviations: nACh, nicotinic acetylcholine; GABA, γ-aminobutyric acid; CG, cycloguanil; CPB, 4-chlorophenyl-1-biguanide; mCPBG, meta-chlorophenyl biguanide; 5-HT, 5-hydroxytryptamine
ABSTRACT

Proguanil is an antimalarial prodrug that is metabolised to 4-chlorophenyl-1-biguanide (CPB) and the active metabolite cycloguanil (CG). These compounds are structurally related to 3-chlorophenyl-1-bigunaide (mCPBG), a 5-HT3 receptor agonist. Here we examine the effects of proguanil and its metabolites on the electrophysiological and ligand binding properties of human 5-HT3A receptors expressed in Xenopus oocytes and HEK293 cells respectively. 5-HT3 receptor-responses were reversibly inhibited by proguanil with an IC50 of 1.81 μM. Competitive antagonism was shown by a lack of voltage-dependence, Schild plot (Kb = 1.70 μM), and radioligand competition (Ki = 2.61 μM) with the 5-HT3 receptor antagonist [3H]granisetron. Kinetic measurements (K_on = 4.0 x 10^4 M^-1 s^-1; K_off = 0.23 s^-1) were consistent with a simple bimolecular reaction scheme with a Kb of 4.35 μM. The metabolites CG and CPB similarly inhibited 5-HT3 receptors as assessed by IC50 (1.48 μM and 4.36 μM respectively), Schild plot (Kb = 2.97 μM and 11.4 μM), and radioligand competition (Ki = 4.89 μM and 0.41 μM). At higher concentrations CPB was a partial agonist (EC50 = 14.1 μM; I/I_max, 0.013). These results demonstrate that proguanil competitively inhibits 5-HT3 receptors with an IC50 that exceeds whole-blood concentrations following its oral administration. They may therefore be responsible for the occasional gastrointestinal side-effects, nausea and vomiting reported following its use. Clinical development of related compounds should therefore consider effects at 5-HT3 receptors as an early indication of possible unwanted gastro-intestinal side-effects.
INTRODUCTION

Proguanil is a prophylactic antimalarial drug that is often taken in combination with other antimalarials such as atovaquone or chloroquine. In the liver, P450 enzymes convert it into 4-chlorophenyl-1-biguanide (CPB) and the active metabolite cycloguanil (CG). Proguanil belongs to the class of phenyl biguanides and has obvious structural similarities to the well-known 5-HT₃ receptor agonist 3-chlorophenyl-1-biguanide (mCPBG). It is therefore possible that proguanil interacts with 5-HT₃ receptors.

5-HT₃ receptors are members of the Cys-loop family of ligand-gated ion channels (LGIC) that are responsible for fast excitatory and inhibitory neurotransmission in the central and peripheral nervous systems. The family includes nicotinic acetylcholine (nACh), γ-amino butyric acid (GABA) and glycine receptors, all of which are cell-surface, transmembrane proteins. They are composed of five subunits that surround a central ion-conducting pore and each subunit contains three distinct functional regions that are referred to as the extracellular, transmembrane and intracellular domains. The orthosteric binding site (that occupied by the endogenous agonist) is found at the interface of two adjacent extracellular domains and is formed by the convergence of three amino acid loops from one subunit (loops A - C) and three β-sheets (loops D - F) from the other (Thompson et al., 2008a; Hassaine et al., 2014). The transmembrane domain contains twenty α-helices, four from each subunit (M1-M4), with M2 from each surrounding the central ion-conducting pore. The intracellular domain is largely unstructured and is responsible for trafficking, intracellular modulation and ion channel conductance (Peters et al., 2010, Hassaine et al., 2014). The extracellular and transmembrane domains are the main drug targets with competitive antagonists being of clinical relevance for alleviating the symptoms of nausea and vomiting associated with chemotherapy, radiotherapy and general anaesthesia.
There has also been limited use of competitive antagonists in the prevention of irritable bowel syndrome, and the use of partial agonists has been proposed for the same disorder (Moore et al., 2013). Off-target effects at 5-HT₃ receptors have also been described. For the smoking cessation drug varenicline (Champix®) agonist activity is seen at 5-HT₃ receptors and is likely that this is responsible for the most commonly reported side effect which is nausea. As gastro-intestinal side effects such as abdominal pain, constipation and vomiting are also sometimes experienced following the oral administration of proguanil, it is possible that 5-HT₃ receptors are similarly responsible (Wattanagoon et al., 1987). Other antimalarial drugs such as quinine, chloroquine and mefloquine (Lariam®) also have off-target effects at 5-HT₃ receptors, as well as at other members of the family. Quinine acts as a competitive antagonist at 5-HT₃ receptors, while chloroquine and mefloquine show mixed competitive / non-competitive antagonist actions (Thompson and Lummis, 2008; Lummis et al., 2011; Sieb et al., 1996; Ballestero et al., 2005).

In this study we use a combination of electrophysiology, radioligand binding and in silico ligand docking to provide evidence for the actions of the antimalarial drug proguanil and its metabolites at 5-HT₃ receptors.

MATERIALS AND METHODS

Materials: Proguanil was from Sigma-Aldrich (St. Louis, MO, USA), 3-chlorophenyl-1-biguanide (mCPBG) from Tocris (Bristol, Avon, UK), 4-chlorophenyl-1-biguanide (CPB) from Fluorochem (Hadfield, Derbyshire, UK) and cycloguanil (CG) from Santa Cruz Biotechnology Inc. (Dallas, Texas, USA). Purity was ≤ 98% for all compounds. In particular, NMR analysis by both the manufacturer and our own facilities showed that
CPB was not contaminated with the isomer mCPBG. Human 5-HT3A (Accession: 46098) subunit cDNA was kindly provided by J. Peters (Dundee University, UK).

**Oocyte Maintenance:** *Xenopus laevis* oocyte positive females were purchased from Nasco (Fort Atkinson, WI, USA) and maintained according to standard methods. Harvested stage V-VI *Xenopus* oocytes were washed in four changes of ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.5), de-folliculated in 1.5 mg ml⁻¹ collagenase Type 1A for 1.5 - 2 h, washed again in four changes of ND96 and stored in ND96 containing 2.5 mM sodium pyruvate, 50 mM gentamycin, 0.7 mM theophylline.

**Cell culture:** Human embryonic kidney (HEK) 293T cells were grown on 90 mm round tissue culture plates as monolayers in DMEM / F12 (Gibco, Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (FBS; BioConcept, Allschwil, Switzerland) at 37°C in a moist atmosphere containing 5% CO₂.

**Receptor Expression:** 5-HT3A subunit cDNA was cloned into pGEMHE for oocyte expression. cRNA was *in vitro* transcribed from linearised plasmid cDNA template using the mMessage mMachine Ultra T7 Transcription kit (Ambion, Austin, Texas, USA). Stage V and VI oocytes were injected with 50 nl of 100-500 ng / µl cRNA (5 - 25 ng injected), and currents were recorded 1 - 4 days post-injection.

5-HT3A subunit cDNA was cloned into pcDNA3.1 for expression in HEK293T cells. Cells were transiently transfected with this cDNA using polyethyleneimine (PEI: 25 kDa, linear, powder, Polysciences Inc., Eppelheim, Germany). 30 µl of PEI (1 mg ml⁻¹), 5 µl cDNA and 1 ml DMEM were incubated for 10 min at room temperature, added drop wise to a 90mm plate of 80 – 90% confluent HEK293T cells, and incubated for 2–3 days before harvesting.

**Electrophysiology:** Using two electrode voltage clamp (TEVC), *Xenopus* oocytes were routinely clamped at -60 mV using an OC-725 amplifier (Warner Instruments,
Connecticut, USA), NI USB-6341 X Series DAQ Device (National Instruments, Berkshire, UK) and the Strathclyde Electrophysiology Software Package v4.7.3 (University of Strathclyde, UK). Micro-electrodes were fabricated from borosilicate glass (GC120TF-10, Harvard Apparatus, Edenbridge, Kent, UK) using a two stage horizontal pull (P-97, Sutter Instrument Company, California, USA) and filled with 3 M KCl. Pipette resistances ranged from 0.8 - 1.5 MΩ. Oocytes were placed in a perfusion chamber made from 2 mm wide x 30 mm long silicon tubing that was cut in half lengthways (total volume ~ 0.1 ml), and were perfused with ND96 at a rate of 15 ml min⁻¹. Drug application was via a simple gravity fed system calibrated to run at the same rate.

**Radioligand Binding:** Saturation binding (8 point) curves were measured by incubating crude extracts of HEK293T cells stably expressing 5-HT₃ receptors in 0.5 ml HEPES buffer (pH 7.4) containing 0.1 – 20 nM [³H]granisetron. Competition binding (10 point) was determined by incubating the same cell extracts in 0.5 ml HEPES buffer (pH 7.4) containing 0.6 nM [³H]granisetron and differing concentrations of competing ligands. Non-specific binding was determined with 1 mM quipazine or 10 μM tropisetron which gave similar results. Incubations were terminated by filtration onto Whatman GF / B filters (Sigma Aldrich) and radioactivity measured using a Tri-Carb 2100TR (Perkin Elmer, Waltham, MA, USA) scintillation counter.

**Data Analysis:** All data analysis was performed with GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA). The peak current was measured for a range of concentrations and normalised to the maximum peak current for the same oocyte. For inhibition curves, antagonists were routinely co-applied in the presence of 2 μM 5-HT or continuously applied for 1 min before the co-application of 2 μM 5-HT. A 2 min wash was used between drug applications. The mean and S.E.M. for a series of oocytes was plotted against agonist or antagonist concentration and iteratively fitted to the following equation:
where \( I_{\text{min}} \) is the baseline current, \( I_{\text{max}} \) is the peak current evoked by agonist, \( EC_{50} \) is the concentration of agonist needed to evoke a half-maximal response, \( x \) is the ligand concentration and \( n_H \) is the Hill slope. \( K_b \) was estimated from \( IC_{50} \) values using the Cheng-Prusoff equation with the modification by Leff and Dougall (1993):

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

(Equ. 2)

where \( K_b \) is the dissociation constant of the competing drug, \( IC_{50} \) is the concentration of antagonist required to half the maximal response, \([A]\) is the agonist concentration, \([A_{50}]\) is the agonist \( EC_{50} \), and \( n_H \) is the Hill slope of the agonist.

Analysis of competitive inhibition was performed by Schild Plot according to the following equation:

\[
\log\left(\frac{EC_{50}'}{EC_{50}}\right) - 1 = \log[L] - \log K_b
\]

(Equ. 3)

where \( EC_{50}' \) and \( EC_{50} \) are values in the presence and absence of antagonist (Dose Ratio), \([L]\) is the concentration of antagonist, and \( K_b \) is the equilibrium dissociation constant for the antagonist receptor interaction. Further analysis was performed using the Gaddum-Schild equation (slope = 1) as recommended by Neubig et al (2003) and Lew and Angus (1995):

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

(Equ. 4)
where \( pEC_{50} \) is the negative logarithm of the agonist \( EC_{50} \), \([L]\) is the antagonist concentration, \( \log C \) is a constant and \( pA_2 \) is the negative logarithm of the antagonist concentration needed to double the concentration of agonist required in order to elicit a response that is comparable to the original response in the absence of antagonist. \( pA_2 \) is equal to the negative logarithm of \( K_b \) when the slope of the Schild plot is exactly 1.

Kinetic parameters were determined according to the following model of a simple bimolecular binding scheme:

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

(Equ. 5)

where \( L \) is the free ligand concentration, \( R \) is receptor concentration, \( LR \) is the ligand-receptor complex and \( k_{on} \) and \( k_{off} \) are the microscopic association and dissociation rate constants. In a simple scheme such as this, the equilibrium dissociation constant (\( K_d \)) is equal to the ratio of dissociation to association rate constants, such that:

\[
K_b = \frac{k_{-1}}{k_{+1}}
\]

(Equ. 6)

According to a one site binding model of the type shown, the rates of onset and recovery of an antagonist response can be used to estimate \( k_{+1} \) and \( k_{-1} \):

\[
1/\tau_{off} = k_{-1}
\]

(Equ. 7)

and

\[
1/\tau_{on} = k_{+1}[L] + k_{-1}
\]

(Equ. 8)
where $\tau_{on}$ refers to the rate of onset of inhibition, $\tau_{off}$ refers to recovery from inhibition and $[L]$ is antagonist concentration.

Radioligand saturation binding experiments were analysed by iterative curve-fitting according to:

$$y = \frac{A_{\text{max}} \cdot [L]}{K_d + [L]}$$

(Equ. 9)

where $y$ is bound ligand, $A_{\text{max}}$ is the maximum signal at equilibrium, $K_d$ is the equilibrium dissociation constant, and $[L]$ is the free concentration of labeled ligand.

Radioligand competition binding data were analysed by iterative curve fitting according to:

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

(Equ. 10)

where $A_{\text{min}}$ is the minimum signal, $A_{\text{max}}$ is the maximum signal, $[L]$ is the concentration of competing ligand and $IC_{50}$ the concentration of competing ligand that blocks half the signal.

$K_i$ values were determined from the $IC_{50}$ values using the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + [L]/K_d}$$

(Equ. 11)

where $K_i$ is the equilibrium dissociation constant for binding of the unlabeled ligand, $[L]$ is the concentration of labeled ligand and $K_d$ is the equilibrium dissociation constant of the labeled ligand.

**Homology Modelling:** The protein sequence of the human 5-HT3A subunit (accession number; P46098) was aligned with a tropisetron bound AChBP template (PDB ID;
2WNC) using FUGUE. With Modeller 9.9, five homology models were generated using default parameters and the best model selected using Ramachandran plot analysis. For the ligand, the protonated forms of proguanil and mCPBG were constructed \textit{ab initio} in Chem3D Ultra 7.0 (CambridgeSoft, Cambridge, UK) and energy-minimised using the MM2 force field. The binding site was defined as being within 5 Å of the α-carbon of W183, a residue that is centrally located in the binding site and is important for in the binding of other 5-HT₃ competitive ligands. The ligands were docked into this site using the GOLD docking program (version 3.0, The Cambridge Crystallographic Data Centre, Cambridge, UK) with the GOLDScore function and default settings. Ten docking poses were generated for each ligand and the poses visualized with PyMol v1.3.

**RESULTS**

**Effects of proguanil on 5-HT₃ receptor currents:** Application of 5-HT or mCPBG (Fig 1) to Xenopus oocytes expressing the 5-HT₃ receptor produced concentration-dependent, rapidly activating, inward currents that slowly desensitised over the time-course of the applications. Plotting current amplitude against a series of agonist concentrations allowed the data to be fitted with Equ 1. For 5-HT this gave a p$EC_{50}$ of $5.77 \pm 0.04$ ($EC_{50} = 1.70 \, \mu M$, $n = 9$) and Hill slope of $1.68 \pm 0.23$, and for mCPBG a p$EC_{50}$ of $5.45 \pm 0.07$ ($EC_{50} = 3.55 \, \mu M$, $n = 8$) and Hill slope of $1.53 \pm 0.28$ (Fig 2A). The relative maximal current of mCPBG compared to 5-HT was similar ($I/I_{\text{max}} = 0.94 \pm 0.05$), consistent with reports elsewhere (Michaelson et al., 2013; Thompson and Lummis, 2013). Agonist responses were completely inhibited by the established 5-HT₃ receptor-specific antagonists granisetron and palonosetron (100 nM, data not shown). Uninjected oocytes did not respond to 5-HT or mCPBG.
Application of proguanil alone did not elicit a response, but it caused concentration-dependent inhibition of the 2 µM 5-HT-evoked response (Fig 2b, Table 1). The pIC$_{50}$ value for proguanil was $5.74 \pm 0.06$ ($IC_{50} = 1.81$ µM, $n = 8$) with a Hill Slope of $1.03 \pm 0.13$. This $IC_{50}$ gave a $K_b$ of 1.74 µM (Equ 2). Inhibition was fully reversible after 1 minute of washing and was unaltered by pre-application (Fig 2C, upper panel). To test whether proguanil blocked the 5-HT$_3$ receptor channel, experiments were performed at holding potentials of -40 mV and +40 mV. The biguanide side-chain of proguanil is basic and thus positively charged under physiological conditions (calculated pK$_a$ values for the imine type NH groups are 8.2 and 10.4, respectively), so binding in the channel should be influenced by changes in the membrane holding potential. Comparison of proguanil inhibition at -40 mV and +40 mV revealed that the levels of inhibition were indistinguishable at these two potentials, showing that proguanil is unlikely to bind in the channel (Fig 2C, lower panel).

**Mechanism of proguanil block:** Increasing the concentration of proguanil (5 µM, 10 µM, 20 µM, 40µM) caused a parallel rightward shift in the 5-HT concentration-response curve, with no change in the maximal response (Fig 3A, Table 1). A Schild plot of these results (Fig 3B) yielded a gradient close to 1 ($1.08 \pm 0.09$, $R^2 = 0.98$) and a pA$_2$ value of 5.77 ± 0.47 ($K_b = 1.70$ µM). The $K_b$ was similar (2.87 µM) if the data were fitted using a nonlinear regression method (Equ. 4). These data support a competitive mechanism of action, indicating that proguanil binds to the same orthosteric binding site as 5-HT.

**A simple kinetic scheme for proguanil binding:** There are limitations to measuring microscopic rate constants using TEVC, but the experiments described in this study were performed under the same conditions which makes a comparison of relative rates valuable (Papke and Thinschmidt, 1998; Thompson et al., 2007). Inhibition of the 2 µM 5-HT response allowed the microscopic rates of association and dissociation for proguanil to be well fitted by mono-exponential functions (Fig. 4, Table 2) that were not significantly
affected by the much slower underlying desensitisation of the 5-HT current response ($\tau_{\text{off}} = 39 \pm 5 \text{ s, } n = 9$). When the reciprocal of these rates was plotted against antagonist concentration, the rates of onset of inhibition ($\tau_{\text{on}}$) for proguanil increased linearly with the antagonist concentration, while recovery ($\tau_{\text{off}}$) was unaltered, as predicted by Equ 5. (Fig. 4B). The association rate ($k_{\text{on}}$) was determined from the slope of the $1/\tau_{\text{on}}$ curve and the dissociation rate constant from the y-axis intercept. The association rate for proguanil was $4.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, and the dissociation rate was $0.23 \text{ s}^{-1}$, giving a $K_b$ of 4.35 µM (Equ. 6); direct measurements of dissociation gave the same value for $k_{\text{off}}$ (Table 2). This analysis is in agreement with a simple kinetic scheme that predicts a single population of binding sites with equal affinities. The affinities were similar to those determined using the other electrophysiological methods described here and provide further support for a simple competitive mechanism of action.

**Competitive binding with $[^3\text{H}]$granisetron:** To further test for a competitive behaviour we measured $[^3\text{H}]$granisetron binding in the presence of increasing concentrations of proguanil. Proguanil displayed concentration-dependent competition with 0.6 nM $[^3\text{H}]$granisetron ($\sim K_d$, Fig. 5). The average $pK_i$ (Equ 11) of proguanil calculated from a series of separate competition-binding curves was $5.58 \pm 0.10$ ($K_i = 2.61 \mu\text{M, } n = 7$). This value is similar to the affinities measured using electrophysiological methods and provides further support for a competitive mode of action.

**Docking studies:** Based upon the pharmacological evidence that proguanil binds at the orthosteric binding site we used a bio-informatics approach to dock proguanil into a homology model of the 5-HT$_3$ receptor. Docking of proguanil yielded ligand orientations that fell into four distinct poses that we designated I, II, III and IV based upon the orientation of the ligand and GOLD scores, (Olsen et al., 2004). In pose I (Fig. 6A) the phenyl ring of proguanil is located between the aromatic rings of W90, Y153, W183 and Y234, and the
biguanide side chain is pointing towards the membrane. There is a potential hydrogen bond between an imine NH group and the backbone carbonyl of W183. In pose II (Fig. 6B) the phenyl ring of proguanil is also located between the aromatic rings of W90, Y153, W183 and Y234, with the isopropyl group of the biguanide making a hydrophobic contact with the aromatic ring of Y143. In addition, the biguanide NH groups can potentially form hydrogen bonds with the hydroxyl group of Y234 on the principal face and the amide group of Q151 on the complementary face. In pose III (Fig. 6C) the biguanide moiety of proguanil is located in the aromatic box formed by the rings of W90, Y153, W183 and Y234 and potential hydrogen bonds are formed between the NH groups of proguanil and the side chains of loop C residues S230 and Y234, and side chains of loop E residues Q151 and Y153. In addition, the phenyl ring of proguanil engages in a hydrophobic interaction with the aromatic ring of Y143. Finally, pose IV (Fig. 6D) places the biguanide moiety in the aromatic box, but the phenyl ring of proguanil is pointing towards loop C. Potential hydrogen bonds are formed between biguanide NH groups and the hydroxyl groups of S230 and Y234, and the backbone carbonyls of S182 and W183.

Proguanil shares many structural similarities with mCPBG (Fig 1), for which there is literature evidence to support our docking (see the discussion). We therefore also docked mCPBG into the 5-HT3 receptor homology model. Contrary to the diversity of predicted binding models for proguanil, all but one of the top ten docking solutions predicted only one binding pose for mCPBG (Fig. 6E). In this pose, the phenyl ring of mCPBG is located in the center of the aromatic box formed by the rings of W90, Y153, W183 and Y234, and the biguanide side chain is pointing towards the aromatic ring of Y143, potentially forming a cation-π interaction. The average distance between the terminal nitrogen of the biguanide and the aromatic ring carbons of Y143 is approximately 5 Å, which is ideal for this attractive interaction. In addition, potential hydrogen bonds are formed between NH groups of the
biguanide moiety and side chains of Q151, S230 and Y234, and the backbone carbonyl of L184. This pose is most similar to pose II for proguanil.

**Effects of proguanil metabolites on 5-HT$_3$ receptor currents:** Proguanil is metabolised to CG and CPB by P450 enzymes in the liver. To determine whether these two metabolites also influence 5-HT$_3$ receptor currents, their effects on 2 µM 5-HT responses was measured at varying concentrations of each. CG reversibly inhibited these 5-HT responses with a pIC$_{50}$ of 5.83 ± 0.04 (IC$_{50}$ = 1.48 µM, n = 8) and a Hill slope of 1.11 ± 0.15. This yielded a $K_b$ of 1.42 µM that was similar to that measured for the parent compound proguanil (Equ. 2, Fig 7A). Similar to proguanil, the presence of increasing concentrations of CG also caused parallel dextral shifts in 5-HT concentration-response curves and inhibition was surmountable with increased concentrations of 5-HT (Fig 7C). An analysis of the changes in the resultant EC$_{50}$ values by Schild plot gave a pA$_2$ of 5.53 ($K_b = 2.95$ µM).

CPB reversibly inhibited 2 µM 5-HT responses with a pIC$_{50}$ of 5.36 ± 0.06 (IC$_{50}$ = 4.36 µM, n = 7) and a Hill slope of 1.10 ± 0.16, that yielded a $K_b$ of 4.19 µM (Equ 2). CPB caused similar parallel and surmountable shifts in 5-HT concentration-response curves that gave a pA$_2$ of 4.94 ($K_b = 11.4$ µM) when analysed by Schild plot (Fig 7D). In addition to these inhibitory effects, at higher concentrations, CPB was also a low efficacy partial agonist with a pEC$_{50}$ of 4.85 ± 0.12 (EC$_{50}$ = 14.1 µM, n = 6) and a maximal current response relative to 100 µM 5-HT of 0.013 ± 0.001 (Fig 8).

The effects of both metabolites were fully reversible after 1 minute of washing and were unaltered by pre-application or holding potential (Figs 7E & 7F). The surmountable inhibition, partial agonism and absence of voltage-dependence were consistent with a competitive mechanism of action for both ligands.

**Competition of proguanil metabolites with $[^3]$H]granisetron:** To confirm our electrophysiological results CG and CPB were also examined using radioligand competition
with 0.6 nM [\textsuperscript{3}H]granisetron. This gave a $pK_i$ value of $5.31 \pm 0.11$ ($K_i = 4.89 \mu M$, $n = 6$) for CG, that was similar to the affinity calculated by the Cheng Prusoff equation (Equ. 2) or Schild analysis (Equ. 3). CPB had a higher affinity than CG, with a $pK_i$ of $6.39 \pm 0.07$ ($K_i = 0.41 \mu M$, $n = 5$) that was 10 - 27 fold higher than values calculated by the Cheng Prusoff equation (Equ. 2) or Schild analysis (Equ. 3). For both ligands these results are consistent with a competitive mode of action.

**DISCUSSION**

This study describes the effects of the antimalarial compound proguanil and its metabolites, CG and CPB, on human 5-HT$_3$ receptors. All were relatively potent inhibitors of 5-HT-mediated currents with $IC_{50}$ values in the low micromolar range. Competition was shown by surmountable rightward shifts of 5-HT concentration-response curves in the presence of increasing concentrations of the compounds and competition with the radiolabelled antagonist [\textsuperscript{3}H]granisetron. For CPB a low efficacy partial agonism was also seen at higher concentrations.

Proguanil is used as an antimalarial drug in both monotherapy (e.g. Paludrine\textsuperscript{\textregistered}) or in combination with other antimalarial compounds (e.g. Malarone\textsuperscript{\textregistered}). Against \textit{P. falciparum} it is considered to be a safe and reliable drug and can be useful in special circumstances such as in children, pregnant women and regions of chloroquine-resistance (Bradley, 1993; Wangboonskul et al., 1993). Following oral administration of Paludrine (2 x 100 mg tablets) the whole-blood concentrations of proguanil peak at 4.1 \mu M after 2 - 4 hrs. For the metabolites CPB and CG, whole- blood concentrations peak at 0.3 \mu M within the same timeframe (Wattanagoon et al., 1987; Wangboonskul et al., 1993). 5-HT$_3$ receptors are highly expressed in the gut where they regulate motility, and are responsible for the vomiting reflex.
that occurs following the stimulation of gastro-intestinal vagal afferent nerves. Effects on these receptors may therefore be at least partially responsible for side effects such as abdominal pain, constipation and vomiting that are sometime experienced after administration of proguanil (Wattanagoon et al., 1987). Gastro-intestinal side-effects are also reported for the antimalarials quinine, chloroquine and mefloquine. These similarly inhibit 5-HT₃ receptors with \( IC_{50} \) values close to plasma concentrations and antagonise 5-HT₃ receptor-mediated gut contractions (Thompson and Lummis, 2008; Kelley et al., 2014). Strategies to find analogous drugs (e.g. phenoxypropoxybiguanides) may therefore wish to consider their effects on 5-HT₃ receptors as an indication of gut intolerance earlier in their development (Shearer et al., 2005; Zucca et al., 2013). It is also noteworthy that the first-line antidiabetic drug metformin (Bolamyn®, Glucophag®) is also a biguanide, as are the related antidiabetics phenformin and buformin. Gastro-intestinal disturbances are reported for all of these drugs and are the main reason (~ 30 %) for compliance issues in patients taking metformin (Pernicova and Korbonits, 2014). Metformin-evoked release of 5-HT is partially responsible, but gut concentrations of metformin can be close to the 2 mM \( IC_{50} \) (unpublished data; \( pK_i = 2.71 \pm 0.05, n = 5 \)) for this compound and may also have effects (Wilcock and Bailey, 1994).

The observation that proguanil competitively inhibits 5-HT₃ receptor responses was anticipated as there are structural similarities with the 5-HT₃ receptor agonist \( m \)CPBG. Indeed, previous structure-activity studies on arylbiguanides have reported the same affinities for CPB as our own, but did not explore agonist activities (Dukat et al., 1996; Glennon et al., 2003). In these studies, an important role for the 3-position substituent was reported, consistent with the importance of the 3-hydroxyl in the agonists 5-HT, dopamine, \( m \)-tryamine and \( m \)CPBG (Meiboom et al., 2013; Thompson and Lummis, 2013). In our own study the transfer of Cl from the 3- (\( m \)CPBG) to the 4-position (CPB) caused a dramatic change in
agonist efficacy, consistent with these earlier reports. A molecule similar to proguanil, with a terminal \(N,N\)-dimethyl group, and a 3-Cl rather than the 4-Cl found in proguanil, is reported to have an affinity > 10 \(\mu\)M (Dukat et al., 1996). In contrast, \(m\)CPBG which also contains a 3-Cl, but has an unsubstituted terminal amine rather than \(N,N\)-dimethyl, has an affinity in the low-mid nM range (Dukat et al., 1996; Hope et al., 1996). This suggests that the unsubstituted terminal amine is more optimal for high affinity binding than \(N,N\)-dimethyl, possibly due to reduced steric hindrance and its ability to act as a hydrogen bond donor; \(m\)CPBG may also have a slightly higher apparent affinity than proguanil because of the influence of gating efficacy on the apparent affinity of agonists (Colquhoun, 1998; Purohit and Grosman, 2006). Truncation of the biguanide chain to the shorter guanidine group, such as in \textit{meta}-chlorophenylguanidine does not affect affinity or agonist activity (Dukat et al., 2007), but further removal or replacement of one of the two nitrogen atoms in the truncated guanidine moiety abolishes agonist properties, showing that both the aromatic substituent and the nitrogen containing basic groups are needed for activation (Glennon et al., 2003).

The ligand-receptor interactions that are responsible for \(m\)CPBG binding have been examined in detail and may be similar for the structurally-related compound proguanil. To explore this possibility we docked proguanil and \(m\)CPBG into the 5-HT\(_3\) receptor ligand binding site. The docked pose clusters of both ligands were all located within 5 Å of residues Y143, Y153 (loop-E), W183 (loop-B), F226 and Y234 (loop-C), residues that form the ‘aromatic box’ that is an established component of the 5-HT\(_3\) receptor binding site and influence \(m\)CPBG binding when mutated. In a comprehensive Ala scan of loop-E, only residues Y143 and Y153 affected \(m\)CPBG binding, suggesting specific interactions that are supported by the orientations of \(m\)CPBG (fig 6E) and proguanil poses I – III (figs 6A - 6C) in our models (Venkataraman et al., 2002). Differences between the actions of \(m\)CPBG in rodent and human receptors have been entirely attributed to residues in loop-C with important
contributions made by F226 and Y234 (Mochizuki et al., 1999; Suryanarayanan et al., 2005). In our docked poses the formation of a hydrogen bond between NH groups of the biguanide moiety and the hydroxyl group and these residues was predicted for both mCPBG (fig 6E) and proguanil poses II – IV (figs 6B – 6D). In all of our docked poses, interactions with the loop-B residue W183 were also predicted. Loop-B forms a major structural component of the orthosteric binding site and mutations are poorly tolerated throughout the loop (Thompson et al., 2008b). In particular, Michaelson et al (2013) and Spier and Lummis (2000) reported that W183 was important for mCPBG binding and activation, and a direct ligand-receptor interaction is likely as the residue is centrally located in the binding site and mutations effect a wide range of agonists and antagonist without altering receptor expression (Spier and Lummis, 2000; Thompson et al., 2008a; Nys et al., 2013). Importantly, none of the poses extended towards loops-A or -F, both of which are unlikely to interact with proguanil or mCPBG; loop-F mutations have no effect on mCPBG and evidence from experiments on E129 supports a structural rather than a binding role as, 1) H-bonding is seen between loops-A and -B in 5-HTBP and 5-HT₃ receptor crystal structures and only H-bonding substitutions produce functional receptors, 2) E129 substitutions cause reduced cell-surface expression and, 3) effects of E129 mutations are the same regardless of the ligands studied (Jensen et al., 2006; Price et al., 2008; Kesters et al., 2013; Hassaine et al., 2014).

Of the four predicted poses for proguanil we favour pose II (fig 6B) which is the most similar to the main docked pose for mCPBG. In this pose the biguanide NH groups form hydrogen bonds with the side chains of Y234 and Q151, the phenyl ring of proguanil can establish hydrophobic interactions with the aromatic rings of W90, Y153 and W183, and the isopropyl end group of the biguanide is in hydrophobic contact with the aromatic ring of Y143. However, in contrast to mCPBG, proguanil acts as an antagonist and yet it is predicted to occupy a similar position. We therefore speculate that proguanil is similarly located, but
does not activate the receptor because it lacks the essential 3-Cl and terminal free amine moieties. This may be because proguanil does not have the capability to H-bond that the terminal unsubstituted NH₂ group has, nor the 3-substituent that is known to be necessary for receptor activation (Dukat et al., 2007). In previous studies direct ligand-receptor interactions and those mediated via a water molecule have been seen between 5-HTBP and 5-HT, and both the size and electronegativity of ligand substituents have been proposed to subtly limit the efficiency of conformational transitions that mediate channel opening (Kesters et al., 2013). With our static models such interactions are difficult to predict, but given the structural similarities between proguanil and mCPBG, and the effects of amino-acid substitutions on the binding of the latter, we believe that pose II is broadly representative (fig 6B).

Proguanil, CG and CPB behaved competitively, similar to the actions of the antimalarials quinine and chloroquine at 5-HT₃, GABA₂₆ and nACh receptors (Ballestero et al., 2005; Thompson and Lummis, 2007; Thompson and Lummis, 2008). At α9α10 nACh and muscle-type ACh receptors, quinine and chloroquine also have additional non-competitive actions while the antimalarial mefloquine (Lariam®) is largely non-competitive at low concentrations, but displaces [³H]granisetron at higher concentrations (Sieb et al., 1996; Ballestero et al., 2005; Thompson and Lummis, 2007). Given that proguanil has been reported to affect acetylcholine responses in the cardio-vascular system of dogs, it is possible that it also affects nACh receptors and that these antimalarials share conserved modes and sites of action throughout the Cys-loop family (Jindal, 1956).

In summary, we provide the first reported evidence that the antimalarial drug proguanil and its metabolites inhibit the function of homomeric 5-HT₃ receptors via competition. This work extends the number of antimalarials that are known to affect different members of the Cys-loop family, and because the concentration of proguanil in the blood
exceeds that which inhibits 5-HT3 receptors, may provide a reason for the occasional gastrointestinal side-effects experienced by patients consuming these biguanide drugs. Given that the development of related compounds has been previously discontinued as a result of unwanted nausea and vomiting we suggest an early assessment of promising leads on 5-HT3 receptors.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: AJT

Conducted experiments: AJT

Contributed reagents or analytical tools: -

Performed data analysis: AJT, ML

Wrote or contributed to the writing of the manuscript: AJT, ML
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Figure Legends

Figure 1
Chemical structures of the compounds used in this study. (A) 5-hydroxytryptamine (5-HT) and (B) 3-chlorophenyl-1-biguanide (mCPBG) are established 5-HT3 receptor agonists. (C) Proguanil is an antimalarial prodrug that is converted to (D) 4-chlorophenyl-1-biguanide (CPB) and the active metabolite (E) cycloguanil (CG) by a cytochrome P450-dependent process in the liver.

Figure 2
Agonist properties of 5-HT3 receptors and their inhibition by proguanil. (A) Concentration-response curves for 5-HT and mCPBG. (B) Concentration-inhibition of the 2 µM 5-HT response by proguanil. (C) Inhibition was unaltered by pre-application of proguanil for 1 min ($p > 0.05$, paired $t$-test; $Co = 0.61 \pm 0.05$, $n = 7$; $Pre = 0.62 \pm 0.05$, $n = 7$) and was not voltage-dependent ($p > 0.05$, paired $t$-test; $-40\text{mV} = 0.67 \pm 0.04$, $n = 6$; $+40\text{mV} = 0.62 \pm 0.05$, $n = 6$). (D) The upper panel shows a typical response to 2 µM 5-HT alone and inhibition of the same 5-HT concentration by varying concentrations of proguanil. The lower panel shows typical 2µM 5-HT responses and their inhibition by 3 µM proguanil at +40 mV and -40 mV. The grey bar above the traces indicates the application of 5-HT or 5-HT + proguanil. The data in 2A and 2B are normalised to the maximum peak response in each oocyte and
represented as the mean ± S.E.M. for a series of oocytes. Parameters derived from these curves can be found in the text and Table 1.

**Figure 3**

The mechanism of 5-HT3 receptor inhibition by proguanil. (A) Concentration-response curves were performed in the absence or presence of the indicated concentrations of proguanil. The curves showed parallel dextral shifts and maximal currents were restored by increasing concentrations of 5-HT. Parameters derived from these curves can be seen in Table 2. (B) A Schild plot was created from the EC50 values of the curves shown in 3A and were fitted with Equ. 3. to yield a pA2 of 5.77 ± 0.47 (Kb, 1.70 µM).

**Figure 4**

Kinetics of proguanil interactions at the 5-HT3 receptor. (A) Sample traces showing the onset (τon) and recovery (τoff) of proguanil inhibition (grey bar) during a 2 µM 5-HT application (black bar). (B) Association and dissociation constants for inhibition were well fitted by mono-exponential functions to give the values shown in Table 2. A plot of the reciprocal of these rates versus the proguanil concentration shows that the association rate (k_on) changed linearly with proguanil concentration, while the dissociation rate (k_off) did not alter significantly. This is consistent with a simple bi-molecular reaction scheme (Equ. 5 - 8).

**Figure 5**

An example binding curve for the competition of 0.6 nM [3H]granisetron and varying concentrations of proguanil at 5-HT3 receptors from transiently transfected HEK293T cells. The curve is a single experiment that is representative of 6 additional curves. Data was normalised to [3H]granisetron binding in the absence of antagonist and fitted with Equ. 10.
Data from each of the separate curves were averaged to give the mean ± S.E.M. shown in the text. *Inset* shows a typical saturated binding curve for [³H]granisetron alone.

**Figure 6**

Representative examples of docked ligands in the 5-HT₃ receptor orthosteric binding site, showing the orientation of the main residues that define these models. Proposed binding poses for proguanil (yellow, A – D) and mCPBG (blue, E) are shown. The principal face (left, light grey), complementary face (right, dark grey), peptide backbone (line representation) and side-chains (ball-and-stick representation) of residues discussed in the text are highlighted. Dotted lines indicate potential hydrogen bonds and are described in the text.

**Figure 7**

Properties of CG and CPB on 5-HT₃ receptors. (A) Both CG and CPB inhibited the 2 µM 5-HT response in a concentration-dependent manner. (B) Representative experiments of competition binding between 0.6 nM [³H]granisetron and increasing concentrations of CG and CPB; pIC₅₀ values calculated (Equ. 10) from these single curves were averaged with similar values from additional experiments and are shown as mean ± S.E.M. in the text. (C) 5-HT concentration-response curves were performed in the absence or presence of increasing concentrations of CG and showed parallel dextral shifts that were surmountable at higher concentrations of 5-HT. (D) Increasing concentrations of CPB had similar effects as those shown in panel 7C. (E) The levels of inhibition caused by 3µM CG or 3µM CPB were not altered when the compounds were co-applied with 2 µM 5-HT, or pre-applied for 1 min followed by immediate co-application (p > 0.05, paired t-test; CG, Co = 0.28 ± 0.02, n = 5; Pre = 0.26 ± 0.01, n = 5; CPB, Co = 0.49 ± 0.07, n = 6; Pre = 0.51 ± 0.08, n = 4). (F)
Inhibition by CG and CPB did not differ at -40 mV and +40 mV ($p > 0.05$, paired $t$-test; CG, 
-40mV = 0.45 ± 0.07, $n = 5$; +40mV = 0.44 ± 0.07, $n = 5$; CPB, -40mV = 0.69 ± 0.05 $n = 5$; 
+40mV = 0.66 ± 0.03, $n = 6$). $I_C50$ values and Hill slopes derived from the curves can be 
found in the text.

**Figure 8**

Agonist properties of CPB. **(A)** A concentration-response curve for CPB compared to the 
maximal (100 µM) 5-HT response. CPB was a partial agonist with a lower potency than 5-HT ($EC50 = 14.1$ µM, $I/I_{max} = 0.013 ± 0.001$, $n = 6$). A similar dual action agonist-antagonist 
action underpins the therapeutic effects of varenicline (Champix®) that relies upon the 
response of the partial agonist suppressing the response of the native ligand to enforce a 
reduced, but controlled activation. **(B)** Example traces for the indicated concentrations of 
CPB are shown. At > 300 µM CPB, the current returns to baseline more rapidly than at lower 
concentrations. For $m$CPBG this effect has been studied in detail and is believed to represent 
auto-inhibition of the receptor by agonist channel block at high concentrations (Hapfelmeier 
et al., 2003). NMR analysis showed that there was no contamination of CPB with the isomer 
$m$CPBG and the agonist response was therefore an effect of CPB alone.
Table 1

Parameters derived from concentration-response curves in the presence of increasing concentrations of proguanil.

<table>
<thead>
<tr>
<th>[Proguanil] (µM)</th>
<th>pEC$_{50}$</th>
<th>EC$_{50}$ (µM)</th>
<th>n$_H$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.77 ± 0.04</td>
<td>1.68</td>
<td>1.68</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>5.43 ± 0.03</td>
<td>3.71</td>
<td>1.52</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>5.30 ± 0.05</td>
<td>5.01</td>
<td>1.22</td>
<td>5</td>
</tr>
<tr>
<td>20</td>
<td>4.95 ± 0.05</td>
<td>11.2</td>
<td>1.30</td>
<td>6</td>
</tr>
<tr>
<td>40</td>
<td>4.72 ± 0.03</td>
<td>19.0</td>
<td>1.31</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 2

Rates of inhibition and recovery of 2 µM 5-HT responses in the presence of increasing concentrations of proguanil.

<table>
<thead>
<tr>
<th>[Proguanil]</th>
<th>$\tau_{on}$ (s)</th>
<th>$n$</th>
<th>$\tau_{off}$ (s)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0.21 ± 0.02</td>
<td>9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>0.25 ± 0.02</td>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0.35 ± 0.01</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0.70 ± 0.03</td>
<td>14</td>
<td>0.23 ± 0.01</td>
<td>15</td>
</tr>
<tr>
<td>20</td>
<td>1.04 ± 0.06</td>
<td>15</td>
<td>0.24 ± 0.02</td>
<td>12</td>
</tr>
<tr>
<td>30</td>
<td>1.42 ± 0.09</td>
<td>12</td>
<td>0.21 ± 0.02</td>
<td>12</td>
</tr>
</tbody>
</table>

A dashed line means that no value was measured at this concentration.
Figure 1

A

5-HT
(Full Agonist)

B

mCPBG
(Full Agonist)

C

Proguanil
(Antagonist)

D

CPB
(Partial Agonist)

E

Cycloguanil
(Antagonist)
Figure 2

A

![Graph showing I/I_max versus log[Agonist] (M) with symbols for 5-HT and mCPBG](image)

B

![Graph showing I/I_max versus log[Progwanil] (M)](image)

C

![Bar graph showing fractional inhibition with conditions Co and Pre](image)

D

![Graph showing current traces with conditions +40 mV, -40 mV, 30 μM, 10 μM, 5 μM, 1 μM, and control](image)
Figure 3

A

![Graph A](image)

B

![Graph B](image)
Figure 4

A

B

$1/\tau (s)$ vs. [Proguanil] (µM)

$\tau_{off} = 3.1$ s (10 µM)

$\tau_{on} = 2.5$ s (10 µM)

$\tau_{on} = 1.0$ s (20 µM)

$\tau_{on} = 0.6$ s (30 µM)

$\tau_{off} = 3.5$ s (20 µM)

$\tau_{off} = 3.5$ s (30 µM)

$\tau_{on}$

$\tau_{off}$

4 µA

10 s
Figure 6

A       B

C       D

E

Proguanil (Pose I) Proguanil (Pose II)

Proguanil (Pose III) Proguanil (Pose IV)

mCPBG
Figure 7
Figure 8

A

B