The Antimalarial Drug Proguanil Is an Antagonist at 5-HT₃ Receptors

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ABSTRACT

Proguanil is an antimalarial prodrug that is metabolized to 4-chlorophenyl-1-biguanide (CPB) and the active metabolite cycloguanil (CG). These compounds are structurally related to meta-chlorophenyl biguanide (mCPBG), a 5-hydroxytryptamine 3 (5-HT₃) receptor agonist. Here we examine the effects of proguanil, and its metabolites on the electrophysiology and ligand-binding properties of human 5-HT₃A receptors expressed in Xenopus oocytes and human embryonic kidney 293 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 (Kₛ = 2.97 and 11.4 μM), and radioligand competition (Kᵢ = 4.89 and 0.41 μM). At higher concentrations, CPB was a partial agonist (EC₅₀ = 14.1 μM; Iₗ/Iₗmax = 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 gastrointestinal 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, cytochrome 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-hydroxytryptamine 3 (5-HT₃) receptor agonist meta-chlorophenyl 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 that are responsible for fast excitatory and inhibitory neurotransmission in the central and peripheral nervous systems. The family includes nicotinic acetylcholine (nACh), 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 (which is 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., 2010; 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 chemotheraphy, radiotherapy, and general anaesthesia (Thompson, 2013). 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; Pfizer, Surrey, UK), agonist activity is seen at 5-HT₃ receptors, and it is likely that this is responsible for the most commonly reported side effect, which is nausea. As gastrointestinal 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...
quarine, chloroquine, and mefloquine (Lariat; Roche, Basel, Switzerland), 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/noncompetitive antagonist actions (Sieb et al., 1996; Ballester et al., 2005; Thompson and Lummis, 2008; Lummis et al., 2011).

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

**Materials and Methods**

Quinapril was from Sigma-Aldrich (St. Louis, MO), mCPBG from Tocris (Bristol, UK), CP from Fluorochem (Hadfield, UK), and CG from Santa Cruz Biotechnology Inc. (Dallas, TX). Purity was ≥98% for all compounds. In particular, NMR analysis by both the manufacturer and our own facilities showed that CP was not contaminated with the isomer mCPBG. Human 5-HT3A (accession number P46098) subunit cDNA was kindly provided by J. Peters (Dundee University, Dundee, UK).

**Oocyte Maintenance.** *Xenopus laevis* oocyte–positive females were purchased from Nasco (Fort Atkinson, WI) 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, and 5 mM HEPES, pH 7.5); defolliculated in 1.5 mg ml−1 collagenase type 1A for 1.5–2 hours; washed again in four changes of ND96; and stored in ND96 containing 2.5 mM sodium pyruvate, 50 mM gentamycin, and 0.7 mM theophylline.

**Cell Culture.** Human embryonic kidney (HEK) 293T cells were grown on 90-mm round tissue culture plates as monolayers in Dulbecco’s modified Eagle’s medium/F12 (Gibco/Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (BioConcept, Alschwil, 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 linearized plasmid cDNA template using the mMessage mMACHINE Ultra T7 Transcription kit (Ambion, Austin, TX). 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 postinjection.

5-HT3A subunit cDNA was cloned into pcDNA3.1 for expression in HEK293T cells. Cells were transiently transfected with this cDNA using polyethylenimine (25 kDa, linear, powder; Polysciences Inc., Eppelheim, Germany). Thirty microliters of polyethylenimine (1 mg ml−1), 5 μl of cDNA, and 1 ml of Dulbecco’s modified Eagle’s medium were incubated for 10 minutes at room temperature, added dropwise to a 90-mm plate of media, and incubated for 2 days before harvesting.

**Electrophysiology.** Using a two-electrode voltage clamp, *Xenopus* oocytes were routinely clamped at −60 mV using an OC-725 amplifier (Warner Instruments, Hamden, CT), NI USB-6341 X Series DAQ Device (National Instruments, Newbury, UK), and the Strathclyde Electrophysiology Software package v4.7.3 (University of Strathclyde, Glasgow, UK). Microelectrodes were fabricated from borosilicate glass (GC120TF-10; Harvard Apparatus, Edenbridge, UK) using a two-stage horizontal pull (P-97; Sutter Instrument, Novato, CA) 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 × 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 of 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 of HEPES buffer (pH 7.4) containing 0.6 nM [3H]granisetron and differing concentrations of competing ligands. Nonspecific binding was determined with 1 mM quipazine or 10 μM tetrodotoxin, which gave similar results. Incubations were terminated by filtration onto Whatman GF/B filters (Sigma-Aldrich) and radioactivity measured using a Tri-Carb 2100TR (PerkinElmer, Waltham, MA) scintillation counter.

**Data Analysis.** All data analysis was performed with GraphPad Prism v5.00 (GraphPad Software, Inc., La Jolla, CA). The peak current was measured for a range of concentrations and normalized to the maximum peak current for the same oocyte. For inhibition curves, antagonists were routinely coapplied in the presence of 2 μM 5-HT or continuously applied for 1 minute before the coapplication of 2 μM 5-HT. A 2-minute 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:

\[
y = \frac{I_{\text{max}} - I_{\text{min}}}{1 + 10^{\frac{[A]}{K_a} - x}}
\]

where \( I_{\text{min}} \) is the baseline current, \( I_{\text{max}} \) is the peak current evoked by agonist, \( [A] \) is the concentration of agonist needed to evoke a half-maximal response, \( x \) is the ligand concentration, and \( K_a \) is the Hill slope. \( K_a \) was estimated from IC50 values using the Cheng-Prusoff equation with the modification by Leff and Dougall (1993):

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

where \( K_a \) 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, \( IC_{50} \) is the agonist EC50, and \( n_H \) is the Hill slope of the agonist.

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

\[
\log([EC_{50} / EC_{50}]) = \log(L) - \log K_a
\]

where \( EC_{50} \) and \( EC_{50} \) are values in the presence and absence of antagonist (dose ratio), \( L \) is the concentration of antagonist, and \( K_a \) 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):

\[
pEC_{50} = - \log(LI - 10^{-\log C}) - \log C
\]

where \( pEC_{50} \) is the negative logarithm of the agonist EC50, \( L \) 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 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 \( K_a \) 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:

\[
L + R \rightarrow LR
\]

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:
where onset and recovery of an antagonist response can be used to estimate \( k_{+1} \) and \( k_{-1} \):

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

and

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

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

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

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

where \( y \) is bound ligand, \( A_{\max} \) 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 analyzed by iterative curve fitting according to:

\[
y = A_{\min} + A_{\max} - A_{\min} \frac{1}{1 + 10^{(y - \text{pIC}50) / \text{Hill slope}}}
\]

where \( A_{\min} \) is the minimum signal, \( A_{\max} \) 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:

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

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 Modeling.** The protein sequence of the human 5-HT3a subunit (accession number P46098) was aligned with a tropisetron-bound acetylcholine-binding protein template (PDB ID 2WNC) using FUGUE (Shi et al., 2001). With Modeler 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 ab initio in Chem3D Ultra 7.0 (CambridgeSoft, Cambridge, UK) and energy-minimized using the included MM2 force field. The binding site was defined as being within 5 Å of the \( \alpha \)-carbon of W183, a residue that is centrally located in the binding site and is important for 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 (DeLano Scientific LLC, San Francisco, CA).

**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 desensitized over the time course of the applications. Plotting current amplitude against a series of agonist concentrations allowed the data to be fitted with eq. 1. For 5-HT, this gave a \( pEC_{50} \) of 5.77 ± 0.04 (EC\(_{50}\) = 1.70 μM; \( n = 9 \)) and Hill slope of 1.68 ± 0.23, and for mCPBG a \( pEC_{50} \) of 5.45 ± 0.07 (EC\(_{50}\) = 3.55 μM; \( n = 8 \)) and Hill slope of 1.53 ± 0.28 (Fig. 2A). The relative maximal current of mCPBG compared with 5-HT was similar (\( I/I_{\max} \) = 0.94 ± 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 ± 0.06 (IC\(_{50}\) = 1.81 μM; \( n = 8 \)) with a Hill slope of 1.03 ± 0.13. This IC\(_{50}\) gave a 5.0, 1.74 μM (eq. 2). Inhibition was fully reversible after 1 minute of washing and was unaltered by preapplication (Fig. 2C, upper panel). To test whether proguanil blocked the 5-HT3 receptor channel, experiments were performed at holding potentials of −40 and +40 mV. The biguanide side chain of proguanil is basic and thus positively charged under physiologic conditions (calculated pKa 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 and +40 mV revealed

![Fig. 1. Chemical structures of the compounds used in this study. 5-HT (A) and mCPBG (B) are established 5-HT3 receptor agonists. Proguanil (C) is an antimalarial prodrug that is converted to CPB (D) and the active metabolite CG (E) by a cytochrome P450–dependent process in the liver.](image-url)
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, 10, 20, and 40 μM) 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 ± 0.09; R² = 0.98) and a pA₂ value of 5.77 ± 0.47 (Kb = 1.70 μM). The Kb was similar (2.87 μM) if the data were fitted using a nonlinear regression method (eq. 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 a two-electrode voltage clamp, 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 monoexponential functions (Fig. 4; Table 2) that were not significantly affected by the much slower underlying desensitization of the 5-HT current response (τoff = 39 ± 5 seconds; n = 9). When the reciprocal of these rates was plotted against antagonist

<table>
<thead>
<tr>
<th>Proguanil Concentration (μM)</th>
<th>pEC₅₀</th>
<th>EC₅₀</th>
<th>nH</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (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>
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concentration, the rates of onset of inhibition ($t_{on}$) for proguanil increased linearly with the antagonist concentration, while recovery ($t_{off}$) was unaltered, as predicted by eq. 5 (Fig. 4B). The association rate ($k_{on}$) was determined from the slope of the $1/t_{on}$ curve and the dissociation rate constant from the $y$-axis intercept. The association rate for proguanil was $4.0 \times 10^4$ M$^{-1}$ s$^{-1}$, and the dissociation rate was $0.23$ s$^{-1}$, giving a $K_D$ of $4.35$ $\mu$M (eq. 6); direct measurements of dissociation gave the same value for $k_{off}$ (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 electrophysiologic methods described here and provide further support for a simple competitive mechanism of action.

**Competitive Binding with [3H]Granisetron.** To further test for a competitive behavior, we measured [3H]granisetron binding in the presence of increasing concentrations of proguanil. Proguanil displayed concentration-dependent competition with $0.6$ nM [3H]granisetron ($K_D$; Fig. 5). The average $pK_I$ (eq. 11) of proguanil calculated from a series of separate competition-binding curves was $5.58 \pm 0.10$ ($K_I = 2.61$ $\mu$M; $n = 7$). This value is similar to the affinities measured using electrophysiologic methods and provides further support for a competitive mode of action.

**Docking Studies.** Based on the pharmacologic evidence that proguanil binds at the orthosteric binding site, we used a bioinformatics approach to dock proguanil into a homology model of the 5-HT$\textsubscript{3}$ receptor. Docking of proguanil yielded ligand orientations that fell into four distinct poses that we designated I, II, III, and IV based on 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 toward 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, and the biguanide side chain is pointing toward 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 toward 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 evidence in the literature to support our docking (see 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 1 of the top 10 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 toward 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-HT₃ Receptor Currents.** Proguanil is metabolized to CG and CPB by cytochrome P450 enzymes in the liver. To determine whether these two metabolites also influence 5-HT₃ 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 pIC₅₀ of 5.83 ± 0.04 (IC₅₀ = 1.48 μM; n = 8) and a Hill slope of 1.11 ± 0.15. This yielded a Kᵣ of 1.42 μM, which was similar to that measured for the parent compound proguanil (eq. 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 EC₅₀ values by Schild plot gave a pA₂ of 5.53 (Kᵣ = 2.95 μM).

CPB reversibly inhibited 2 μM 5-HT responses with a pIC₅₀ of 5.36 ± 0.06 (IC₅₀ = 4.36 μM; n = 7) and a Hill slope of 1.10 ± 0.16, which yielded a Kᵣ of 4.19 μM (eq. 2). CPB caused similar parallel and surmountable shifts in 5-HT concentration-response curves that gave a pA₂ of 4.94 (Kᵣ = 11.4 μM) when analyzed by Schild plot (Fig. 7D). In addition to these inhibitory effects, at higher concentrations, CPB was also a low-efficacy partial agonist with a pEC₅₀ of 4.85 ± 0.12 (EC₅₀ = 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 preapplication or holding potential (Fig. 7, E and F). 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 [³H]Granisetron.** To confirm our electrophysiologic results, CG and CPB were also examined using radioligand competition with 0.6 nM [³H]granisetron. This gave a pKᵢ value of 5.31 ± 0.11 (Kᵢ = 4.89 μM; n = 6) for CG, which was similar to the affinity calculated by the Cheng-Prusoff equation (eq. 2) or Schild analysis (eq. 3). CPB had a higher affinity than CG, with a pKᵢ of 6.39 ± 0.07 (Kᵢ = 0.41 μM; n = 5) that was 10- to 27-fold higher than values calculated by the Cheng-Prusoff equation (eq. 2) or Schild analysis (eq. 3). For both ligands, these results are consistent with a competitive mode of action.

![Fig. 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 six additional curves. Data were normalized to [³H]granisetron binding in the absence of antagonist and fitted with eq. 10. Data from each of the separate curves were averaged to give the mean ± S.E.M. shown in the text. (Inset) A typical saturated binding curve for [³H]granisetron alone.](image-url)
Fig. 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 gray), complementary face (right, dark gray), 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.
Discussion

This study describes the effects of the antimalarial compound proguanil and its metabolites, CG and CPB, on human 5-HT₃ receptors. All were relatively potent inhibitors of 5-HT-mediated currents, with IC₅₀ 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 radiolabeled antagonist [³H]granisetron. For CPB, a low-efficacy partial agonism was also seen at higher concentrations.

Fig. 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 (eq. 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 (C). (E) The levels of inhibition caused by 3 µM CG or 3 µM CPB were not altered when the compounds were coapplied (Co) with 2 µM 5-HT or preapplied (Pre) for 1 minute followed by immediate coapplication (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 and +40 mV (P > 0.05, paired t test; CG, −40 mV = 0.45 ± 0.07, n = 5; +40 mV = 0.44 ± 0.07, n = 5; CPB, −40 mV = 0.69 ± 0.05, n = 5; +40 mV = 0.66 ± 0.03, n = 6). IC₅₀ values and Hill slopes derived from the curves can be found in the text.
5-HT3 receptors are highly expressed in the gut, where they are also a bimodal agonist, as are the related antidiabetics phenformin and buformin. Gastrointestinal 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 IC50 (pKi = 2.71 ± 0.05; n = 5) (unpublished data) 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 mCPBG. 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-hydroxy in the agonists 5-HT, dopamine, m-tyramine, and mCPBG (Meiboom et al., 2013; Thompson and Lummis, 2013). In our own study, the transfer of Cl from the 3- (mCPBG) 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 of >10 μM (Dukat et al., 1996). In contrast, mCPBG, which also contains a 3-Cl but has an unsubstituted terminal amine rather than N,N-dimethyl, has an affinity in the low- to mid-nanomolar 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; mCPBG 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 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 mCPBG binding have been examined in detail and may be similar for the structurally related compound proguanil. To explore this possibility, we docked proguanil and mCPBG into the 5-HT3 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), that form the "aromatic box" that is an established component of the 5-HT3 receptor-binding site and influence mCPBG binding when mutated. In a comprehensive Ala scan of loop E, only residues Y143 and Y153 affected mCPBG binding, suggesting specific interactions that are supported by the orientations of mCPBG (Fig. 6E) and proguanil poses I–III (Fig. 6, A–C) in our models (Venkataraman et al., 2002). Differences between the actions of mCPBG in rodent and human receptors have been entirely attributed to residues in loop C with important contributions made by F226 and Y234.
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 (Fig. 6, B–D). 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., 2008). 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 affect a wide range of agonists and antagonists without altering receptor expression (Spier and Lummis, 2000; Thompson et al., 2010; Nys et al., 2013).

Importantly, none of the poses extended toward 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 therefore favor 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, GABA_A, and nACh receptors (Ballester et al., 2005; Thompson et al., 2007; Thompson and Lummis, 2008). At 0.10 nACh and muscle-type acetylcholine receptors, quinine and chloroquine also have additional noncompetitive actions, while the antimalarial mefloquine (Lariam) is largely non-competitive at low concentrations, but displaces [3H]granisetron at higher concentrations (Sieb et al., 1996; Ballester et al., 2005; Thompson et al., 2007). Given that proguanil has been reported to affect acetylcholine responses in the cardiovascular 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 homeric 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 an 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: Thompson.
Conducted experiments: Thompson.
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Wrote or contributed to the writing of the manuscript: Thompson, Lochner.

References
Kesters et al., 2013; Hassaine et al., 2014).


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