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Chemogenomic Discovery of Allosteric Antagonists at the GPRC6A Receptor

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SUMMARY

GPRC6A is a Family C G protein-coupled receptor recently discovered and deorphanized by our group. This study integrates chemogenomic ligand inference, homology modeling, compound synthesis, and pharmacological mechanism-of-action studies to disclose two noticeable results of methodological and pharmacological character: (1) chemogenomic lead identification through the first, to our knowledge, ligand inference between two different GPCR families, Families A and C; and (2) the discovery of the most selective GPRC6A allosteric antagonists discovered to date. The unprecedented inference of pharmacological activity across GPCR families provides proof-of-concept for in silico approaches against Family C targets based on Family A templates, greatly expanding the prospects of successful drug design and discovery. The antagonists were tested against a panel of seven Family A and C G protein-coupled receptors containing the chemogenomic binding sequence motif where some of the identified GPRC6A antagonists showed some activity. However, three compounds with at least ~3-fold selectivity for GPRC6A were discovered, which present a significant step forward compared with the previously published GPRC6A antagonists, calindol and NPS 2143, which both display ~30-fold selectivity for the calcium-sensing receptor compared to GPRC6A. The antagonists constitute novel research tools toward investigating the signaling mechanism of the GPRC6A receptor at the cellular level and serve as initial ligands for further optimization of potency and selectivity enabling future ex vivo/in vivo pharmacological studies.

INTRODUCTION

The Nobel laureate Sir James Black has stated that "the best way to find a new drug is to start from an old" (Raju, 2000). In lead identification practice, old ligands may be new to other targets. Such inference of ligands between targets (target hopping) is typically based on the principle that "similar targets have similar ligands." This is the strategy of chemogenomics, an evolving combination of ligand chemoinformatics and protein target biology that has demonstrated repeated successes in lead discovery, primarily at G protein-coupled receptors (GPCRs) (Garland and Gloriam, 2011a; Harris and Stevens, 2006). The novelty and strength of chemogenomics is that target similarity is defined based on the binding site characteristics able to form ligand interactions rather than evolutionary patterns or natural ligand families, which have long been the choice of bioinformaticians or pharmacologists, respectively. By adopting this "ligands' view to target similarity," patterns of target similarity can be detected, and ligand-binding correlated, where the classical measures cannot be used even across diverse receptors recognizing very heterogeneous physiological ligand types, including peptides, lipids, and small molecules (Gloriam et al., 2009; Harris and Stevens, 2006).

GPCRs make up one of the largest protein families in humans (Venter et al., 2001) and are cell-surface receptors that can be activated by a broad range of ligands (Bockaert and Pin, 1999). A large proportion of current drugs (27%–45%) exert their effect via GPCRs, including well-known examples such as morphine and propranolol, but the majority of the receptors in this protein superfamily are as yet untapped for potential therapies (Drews, 2000; Hopkins and Groom, 2002; Overington et al., 2006). Chemogenomic techniques have unique capabilities and advantages in the analysis of GPCRs. First, they are not dependent on target structural data. The significance of this relates to the fact that there are more than 400 human nonolfactory GPCRs distributed over five families (Fredriksson et al., 2003), while crystal structures are limited to six receptors (Cherezov et al., 2007; Chien et al., 2010; Jaakola et al., 2008; Palczewski et al., 2000; Warne et al., 2008; Wu et al., 2010) and one family (Family A/*Rhodopsin* family). Second, chemogenomic approaches can identify similarities between targets that are very distantly or not related using classical classifications based on evolutionary relationships. Whereas GPCRs are believed to be structurally conserved, all consisting of a bundle of seven transmembrane (TM) protein helices (TMHs), the sequence identities between members of different families is extremely low as a result of a huge evolutionary gap; the GPCR families separated already before the split of the nematodes from the chordate lineages (Fredriksson and Schiöth, 2005). Finally, chemogenomic methods can provide receptor activity relationships also for

Table 1. The 2-Phenyl-Indole Moieties Paired with Binding Sequence Motifs

Moiety	Indole	NH	Phenyl	Whole Structure
Interaction	Aromatic-Aromatic	Hydrogen bond to backbone	Hydrophobic	Charge neutrality
Receptor residue positions	5.47^{T760}, 6.48^{W797}, 6.51^{F800}, and 6.52^{I801}	5.46^{I759}	3.33^{G667}, 3.37^{T671}, 4.56^{V724}, 4.57^{L725}, and 5.46^{I759}	3.36^{F670}, 3.37^{T671}, 5.47^{T760}, 6.51^{F800}, and 6.52^{I801}
Allowed amino acids	Aromatic: F,W,Y, and H	NOT very large (W) or basic (KR)	Aromatic or hydrophobic: F,W,Y,H,A,I,L,M, and V	Not charged residues: D,E,K, and R
No. required matches	2/4	1/1	2/5	5/5

Ligand moieties and interacting receptor residue positions with allowed amino acids are color-coded. The binding sequence motif has been adapted from previous analyses (Bondensgaard et al., 2004). Residue positions are denoted using Ballesteros-Weinstein indices (Ballesteros and Weinstein, 1995; Garland and Gloriam, 2011b) and with the mouse GPRC6A amino acid in superscript.

ligands that are structurally unrelated to and/or bind in a different (allosteric) site than the endogenous agonist, and therefore have a strong applicability in the design of synthetic drugs.

The GPRC6A receptor belongs to a small family of dimeric GPCRs, Family C/*Glutamate* family, which includes eight metabotropic glutamate receptors (mGluRs), the calcium sensing receptor (CaR), the γ-aminobutyric acid type B receptor ($GABA_B$) as well as several taste and orphan receptors (Bräuner-Osborne et al., 2007). The Family C receptors have a characteristic large, extracellular, ligand-binding "Venus-flytrap" domain, a cysteine-rich region, a 7TM bundle and an intracellular C terminus (Bräuner-Osborne et al., 2007). The GPRC6A receptor is physiologically activated by L-α-amino acids, with a preference for the naturally occurring basic amino acids L-arginine, L-lysine and L-ornithine (Kuang et al., 2005; Wellendorph et al., 2005). Depending on which signaling pathway is studied, the receptor is also positively modulated (Christiansen et al., 2007; Kuang et al., 2005) or directly activated (Pi et al., 2005) by divalent cations. GPRC6A has a broad expression profile in humans, mice, and rats (Kuang et al., 2005; Pi et al., 2005; Wellendorph and Bräuner-Osborne, 2004; Wellendorph et al., 2007). The broad ligand recognition and tissue expression have obscured the elucidation of the physiological function of the GPRC6A receptor, however, with the generation of GPRC6A knockout mice, potential physiological roles have emerged, among these severe metabolic and endocrinological disturbances (Oury et al., 2011; Pi et al., 2008; Wellendorph et al., 2009b). However, large discrepancies have been observed between different strains of knockout mice (Pi et al., 2008; Wellendorph et al., 2009b) that underline the strong need for selective pharmacological tool compounds, which can be employed to study the physiological function of the GPRC6A receptor in a broader context.

Until now, only two antagonists have been identified for GPRC6A, the calcimimetic calindol and the calcilytic NPS 2143 (Faure et al., 2009). These suffer from a lack of selectivity as they were developed to modulate CaR rather than GPRC6A function and possess ~30-fold higher potency on CaR compared to GPRC6A (Faure et al., 2009). In addition, both compounds suffer from low solubility and only partial inhibition of GPRC6A responses are thus obtained at the highest possible ligand concentrations (Faure et al., 2009), further limiting their value as pharmacological tools to investigate the GPRC6A receptor. Strikingly, whereas both ligands inhibit GPRC6A and bind in overlapping (allosteric) binding sites in the CaR TM bundle (Miedlich et al., 2004; Petrel et al., 2004), they display opposite actions at CaR: negative and positive modulation by NPS 2143 and calindol, respectively. Inherently, it is of essence to pursue new approaches to ligand identification that are directed at GPRC6A as the primary target and, preferably, can adequately define the binding mode and rationale for activity at this receptor. In the present study, we therefore undertook a chemogenomic approach to identify a novel GPRC6A antagonist scaffold.

This study reports two findings of significant novelty. First, it comprises a pioneering inter-GPCR-family ligand inference, from Family A to C. The successful extrapolation of ligand scaffold activity is remarkable because of the low sequence homology between the families and the lack of structural data for the TM region of Family C GPCRs. Second, we disclose the identification of the most selective GPRC6A antagonists reported to date, along with an allosteric binding site in the 7TM region as supported by receptor mutation and ligand substitution. Selective antagonists constitute important research tools toward the elucidation of the physiological and therapeutic relevance of GPRC6A.

RESULTS

Chemogenomic Ligand Inference to the GPRC6A Target

The binding modes of three known Family A GPCR privileged structures; 2-phenyl-indole, 1,1′-biaryl-2-acid and 4-aryl-piperidine; have been determined previously, by sequence analysis and ligand docking, and translated into binding sequence motifs (Bondensgaard et al., 2004; Garland and Gloriam, 2011b). The privileged structures are substructures of a large number of known ligands for Family A GPCRs (Garland and Gloriam, 2011b), but have never been reported to have affinity in other GPCR families. Here, we matched their binding sequence motifs to the GPRC6A protein sequence pointing out 2-phenyl-indole as a candidate ligand scaffold.

Table 1 pairs the 2-phenyl-indole moieties with proposed interacting GPCR residues. These residues and their physicochemical character (aromatic, hydrophobic, charged etc.) constitute the binding sequence motif, which is matched toward GPRC6A and related receptor protein sequences in Figure 1. The indole moiety can have aromatic interactions with a hydrophobic core site located deep inside the GPCR TM bundle. Two aromatic residues in positions 5.47, 6.48, 6.51, or 6.52 are

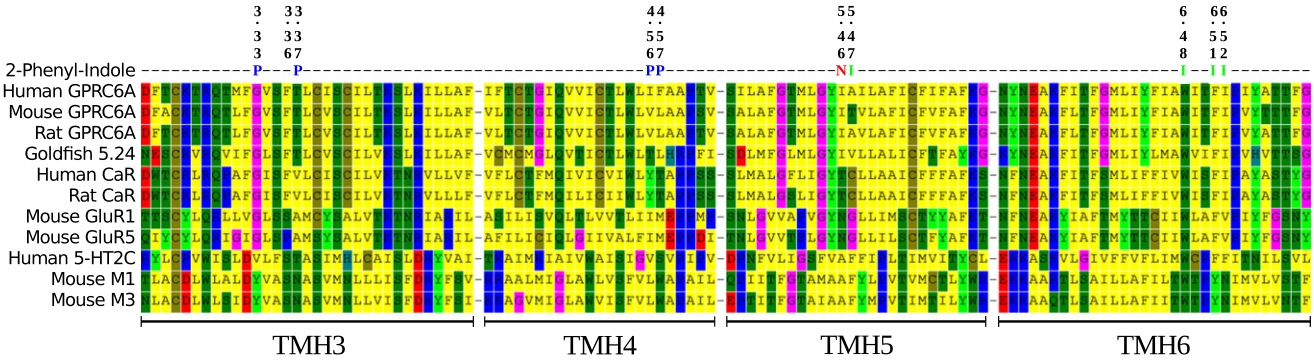

Figure 1. The 2-Phenyl-Indole Binding Motif Matched against the Protein Sequences of Assayed Receptors

Protein sequence alignments of TMH3-6 in GPRC6A (human, mouse, and rat), the 5.24 receptor (goldfish), CaR (human and rat), and 5-HT_{2C} (human). The 2-phenyl-indole binding sequence motif is shown in the top row detailing the residue positions and the moieties they interact with denoted as: indole (I), indole NH (N), and phenyl (P). Position 3.36 has no specific interaction, but should be uncharged.

considered sufficient to stabilize the indole. GPRC6A has aromatic residues in positions 6.48 and 6.51 (W and F, respectively) and the residues in 5.47 and 6.52 are hydrophobic (A and I, respectively). The indole nitrogen functionality (NH) is hypothesized to form a hydrogen bond to the helical backbone carbonyl of residue position 5.46. Too large (W) or basic (K or R) side-chains would sterically hinder this interaction, which is critical for 2-phenyl-indole binding. GPRC6A has an isoleucine in this position and thus matches also this part of the binding sequence motif. Furthermore, the 2-phenyl moiety resides in an area enclosed by five residues from TMHs 3, 4, and 5, of which at least two should be aromatic or hydrophobic. GPRC6A has two hydrophobic residues (4.56 and 5.46) and one aromatic residue (4.57). Finally, for the whole structure to be accommodated, a close strong charge does not seem to be permitted as it would interfere with its aromatic, hydrophobic character. No charge has been observed at any of the positions 3.36, 3.37, 5.47, 6.51, or 6.52 within known targets or GPRC6A.

Discovery of 3-Substituted 2-Phenyl-Indoles as Novel Antagonists at Mouse GPRC6A

We purchased and assayed a selection of 25 compounds incorporating the 2-phenyl-indole scaffold and a variety of structurally diverse substituents at the indole 3-position (R group in Table 1). The compounds were initially tested for their ability to modulate the L-ornithine-induced response at mouse GPRC6A (mGPRC6A) at EC_{25} (enhancement) or EC_{80} (antagonism) of L-ornithine, using a previously reported inositol phosphate (IP) turnover assay in tsA201 cells cotransfected with mGPRC6A and $G\alpha_{qG66D}$ (Christiansen et al., 2007). From this screening, no allosteric enhancers, and hence no agonists, were identified. In contrast, nine compounds (36%) displayed antagonistic effects with IC_{50} values in the 18–67 μM range (see Table S1 available online).

Selectivity Assessment and Prioritization of Active Compounds

Active compounds were tested against the rat CaR (closest mammalian homolog) and the human 5-HT_{2C} receptor (chemogenomically related Family A receptor; see Figure 5). GPRC6A antagonists **1**–**3** (Figure 2) were found to be inactive at these two receptors (Figure 3A; Table S1). In addition, **3** was tested inactive at the goldfish 5.24 receptor (closest overall homolog in all species, Figure S1). In order to further evaluate the selectivity of compounds **1**–**3** they were tested on two additional Family A (muscarinic acethylcholine receptors M_1 and M_3) and Family C (metabotropic glutamate receptors $mGlu_1$ and $mGlu_5$) GPCRs, which all contain the 2-phenyl-indole binding sequence motif. **1** and **3** displayed some activity at these receptors, whereas **2** did not (Table 2). However, all three compounds displayed at least ~3-fold higher potency at GPRC6A than any of the other tested receptors. All seven receptors screened for off-target activities contain the 2-phenyl-indole binding sequence motif (Figure 1). Thus, the selectivity has to be explained by additional interactions formed by the 3-indole substituents. The IC_{50} values of compounds **1**–**3** are very similar (18–27 μM) (Figure 3A and Table 2). Compound **2** is the most selective compound but like **1** suffer from low solubility and a labile carboxylic ester in the side-chain. Compound **3** is less selective, but is devoid of the labile carboxylic ester linkage and has significantly improved aqueous solubility over compounds **1** and **2** (Table 3). Ligand **1** has better aqueous solubility than compound **2**. Thus, further molecular pharmacological studies focused on antagonists **1** and **3**, representing both identified ligand classes. To confirm their chemical structures these were synthesized by us. Both compounds were spectroscopically identical and displayed IC_{50} values similar to the purchased compounds.

Validation of Activity in a Secondary Assay

To confirm the inhibitory activity of the 2-phenyl-indole compounds at mGPRC6A, **3** was tested in a *Xenopus laevis* expression system that is independent on coexpression of chimeric G proteins and does not risk effects from L-amino acids in the media/buffers (Christiansen et al., 2007; Kuang et al., 2005; Wellendorph et al., 2005). In accordance with the IP assay, 100 μM of compound **3** significantly depressed the L-ornithine-induced response at a submaximal agonist concentration, but was devoid of agonist activity (Figure S1).

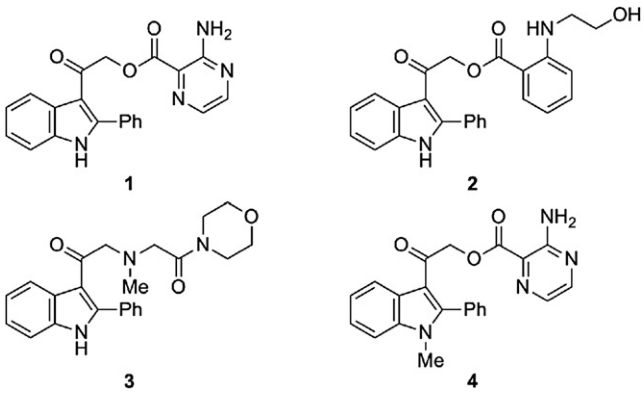

Figure 2. Chemical Structures of Molecules 1–4
Chemical structures of mGPRC6A antagonists **1**–**3** and an inactive *N*-methyl analog **4**.

Compounds 3 and 1 Are Allosteric Antagonists at mGPRC6A

L-Ornithine concentration-response curves were generated in the presence of increasing concentrations of **3** (Figure 3B). This Schild analysis revealed that **3** causes a marked attenuation of L-ornithine-mediated signaling with no significant effect on agonist potency, thus, demonstrating that this compound acts with a noncompetitive mode of action. Similar results were also obtained for **1** (Figure S2).

Validation of the Binding Mode by Receptor Mutations

The most specific ligand interaction in the binding sequence motif (Table 1) is the hydrogen bond between the NH of the 2-phenyl-indole and a carbonyl in the helical backbone of receptor position 5.46^{I759}. We hypothesized that mutation of this residue; an isoleucine in mGPRC6A, to a large or basic amino acid would interfere with bond formation and consequently constructed tryptophan and arginine mutants. Testing of these mutants in the IP turnover assay revealed an unchanged responsiveness to the orthosteric ligand L-ornithine compared to wild-type mGPRC6A (Table S2). However, in stark contrast the mutated receptors were not antagonized by **1** or **3** in the presence of 500 μM L-ornithine (Figure 3C; Table S2). These data support that **1** and **3** critically interact with 5.46^{I759} in the 7TM domain of mGPRC6A. This was further corroborated by ligand docking into a structure model of the mGPRC6A 7TM domain (Figure 4).

Additional Validation of the Binding Mode through Ligand Substitution

We synthesized an *N*-methylated analog of compound **1**, **4** (Figure 2), which is unable to hydrogen bond to the receptor backbone due to replacement of the indole NH hydrogen with a methyl group. As anticipated, analog **4** had no agonistic or antagonistic activity when tested in the IP turnover assay (Figure 3D), further substantiating the binding mode.

DISCUSSION

Lead generation by chemogenomic ligand inference has earlier been demonstrated for the melanin-concentrating hormone (MCH_1), prostanoid DP_2 (a.k.a. GPR44/CRTh2), somatostatin 5 (sst_5), and anaphylatoxin (C3a) receptors (see Table 1 in Garland and Gloriam, 2011b). Furthermore, the three privileged structures studied here, although just a subset of the available, constitute the core scaffolds of more than 100 ligands for 84 GPCR targets (S. Garland and D.E.G., unpublished data). Remarkably, hitherto all chemogenomic ligand inferences and privileged structures apply only to Family A receptors and this report for GPRC6A, which belongs to Family C, is the first of its kind, to our knowledge. This represents a breakthrough for the development of drugs and pharmacological tool compounds for receptor families in which structure-based ligand identification/design and the number of known ligands are still very limited.

The pioneering proof-of-concept for inter-GPCR-family ligand inference provided in this study is fundamental as it clearly has achieved what the traditional classifications or similarity measures, i.e., evolutionary or pharmacological receptor relationships, cannot (Davies et al., 2011; Garland and Gloriam, 2011a). Specifically, the chemogenomic ligand inference covered allosteric ligands and crossed GPCR families. The difference between evolutionary and chemogenomic receptor relationships is illustrated in Figure 5. The left tree, based on the whole TM region to represent a conventional evolutionary phylogenetic analysis, shows a large separation of the Family C (GPRC6A and CaR, red) and A GPCRs (black). In contrast, in the right, chemogenomic tree, which is focused on the 2-phenyl-indole binding site, the difference (distance) between the two families is not bigger than it is between several Family A members.

Of the 25 compounds, 9 (36%) displayed activity in the 10 μM range. These results are very satisfactory for an initial ligand discovery effort, especially when considering that no 2-phenyl-indole ligands have been reported for any Family C receptors previously. Additional testing of the active 2-phenyl-indole compounds revealed that 3 of the 25 compounds are selective antagonists at the GPRC6A receptor with no activity at CaR or $5\text{-}HT_{2C}$. Further testing of these three compounds at two muscarinic acetylcholine receptors and two metabotropic glutamate receptors revealed that all three compound also displayed selectivity for GPRC6A over these receptor subtypes albeit compound **1** and **3** had some inhibitory activity on some of the subtypes (Table 2). However, compared to the two only previously published GPRC6A antagonists, the CaR selective compounds calindol and NPS 2143, all three compounds show a superior selectivity profile for GPRC6A.

The GPCR binding sequence motif for the 2-phenyl-indole suggested that the hydrogen bond interaction between the indole NH and the 5.46^{I759} residue in mGPRC6A is essential for binding. This was supported by the fact that mutations of this residue to large amino acids, predicted to interfere with the formation of the hydrogen bond, resulted in lack of effect for compounds **1** and **3**. This was not due to alterations in the responsiveness of the mGPRC6A receptor, as the mutants responded similar to the wild-type receptor to the natural ligand L-ornithine. The confirmed hydrogen bond interaction with the indole NH together with the observed affinities renders it reasonable to assume that also the interactions of the indole and phenyl groups correspond to those in Family A GPCRs (Bondensgaard et al., 2004). To further substantiate this hypothesis we

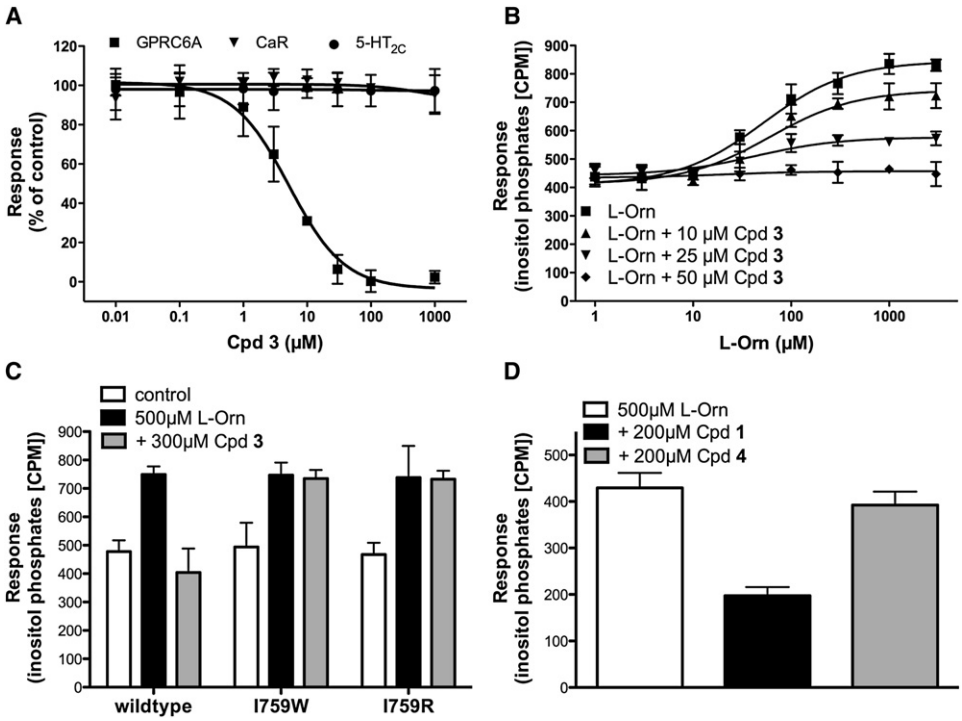

Figure 3. Pharmacological Characterization of Compounds 1, 3, and 4 on Mouse GPRC6A, Rat CaR and Human 5-HT$_{2A}$ Transiently Expressed in tsA201 Cells

(A) Concentration-response curves of **3** on the mouse GPRC6A, rat CaR and human 5-HT$_{2C}$ receptors in the presence of 500 μM L-ornithine (L-Orn), 4 mM Ca^{2+} and 50 nM serotonin, respectively.

(B) Schild analysis of **3**: concentration-response curves of L-Orn in the presence of various concentrations of **3** at the mouse GPRC6A receptor.

(C) Effect of L-Orn and **3** on wild-type and mutated mouse GPRC6A receptors.

(D) Pharmacological characterization of **1** and its *N*-methylated analog **4** on mouse GPRC6A showing that **1** is an antagonist whereas **4** is inactive. The formation of inositol phosphates was determined as described in Experimental Procedures. Results are expressed as counts per minute and are means ± SD (n = 3).

synthesized the *N*-methylated analog of **1** (providing **4**), which would prevent the hydrogen bond interaction between the hydrogen of the indole NH and the backbone carbonyl at 5.46^{I759}. As predicted **4** was inactive likely due to lack of this hydrogen bond. Collectively, the mutations in the receptor and the *N*-methyl "mutation" of the compound provide compelling evidence that the binding site for the 2-phenyl-indole compounds is located in a conserved pocket within the 7TM, which is further supported by the Schild analysis of the effect of **1** and **3** on mGPRC6A indicating that the compounds are

Table 2. Selectivity of Compounds 1–3

Receptor	GPRC6A	5-HT$_{2C}$	CaR	5.24	M_1	M_3	$mGlu_1$	$mGlu_5$
Compound	IC_{50}/pIC_{50} ± SEM/independent experiments (n)							
1	27	>100	>100	n.d.	62	>100	>100	>100
	4.58 ± 0.09				4.22 ± 0.06			
	9				4			
2	18	>100	>100	n.d.	>100	>100	>100	>100
	4.74 ± 0.09							
	4							
3	18	>100	>100	>100	75	91	>100	56
	4.75 ± 0.26				4.14 ± 0.05	4.06 ± 0.08		4.26 ± 0.05
	5				4	4		3

Antagonist activity of compounds **1–3** at GPRC6A and seven other G protein-coupled receptors containing the 2-phenyl indole binding sequence motif. All receptors are coupled to the G_q pathway and were tested using an inositol phosphate turnover assay except for responses at 5.24 that were obtained using *Xenopus* oocyte electrophysiology. The following submaximal (~EC_{80}) concentrations of endogenous agonist were used: 500 μM L-ornithine (GPRC6A), 50 nM serotonin (5-HT$_{2C}$), 4 mM Ca^{2+} (CaR), 30 μM L-ornithine (5.24), 1.6 μM acetylcholine (m1), 86 μM acetylcholine (m3), 60 μM L-glutamate ($mGlu_1$), and 17 μM L-glutamate ($mGlu_5$). n.d., not determined.

Table 3. Potency, Solubility, and Calculated logP of Compounds 1–3

Compound	IC_{50} (μM)	Observed Solubility in Assay Buffer (mM)	Calculated LogP in Octanol/Water (Schrödinger QikProp)
1	27	0.2	3.9
2	18	0.1	5.0
3	18	1.0	2.2

noncompetitive antagonists binding to an allosteric site. Furthermore, the inactivity of **1** and **3** on the 5.46^{I759} mutants and the lack of activity of **1–3** on some of the other receptor subtypes tested (Table 2), demonstrate that the antagonists do indeed act via the GPRC6A receptor and not another receptor expressed endogenously in the transfected cells.

GPRC6A is one of the most recently deorphanized GPCRs. This receptor is activated by a range of L-α-amino acids such as L-arginine, L-lysine and L-ornithine and is positively modulated/activated by the divalent cations Ca^{2+} and Mg^{2+} (Christiansen et al., 2007; Kuang et al., 2005; Pi et al., 2005; Wellendorph et al., 2005). The omnipresent nature of these ligands in living beings and their multiple targets severely limit their use as pharmacological tools for ex vivo and in vivo studies of physiological functions. We and the groups of Quarles and Karsenty have therefore generated GPRC6A knockout mice, which, however, have provided ambiguous results (Pi et al., 2008; Wellendorph et al., 2009b), presumably due to differences in genetic background or gene targeting strategy (Conigrave and Hampson, 2010; Wellendorph et al., 2009a). To firmly establish the physiological role of the GPRC6A receptor novel selective pharmacological tools are thus required. Here, we have presented, to our knowledge, the first such tools that will be valuable for future cellular studies. For example, recent studies have reported putative GPRC6A mediated signaling in cell lines endogenously expressing the GPRC6A receptor, but used either no controls or siRNA as control (Oury et al., 2011; Pi et al., 2010; Pi and Quarles, 2011). In our view, the three antagonists reported here would be of value as additional controls in such studies although potential inhibition of other GPCRs would have to be considered (Table 2). The compounds also represent ligands that may be optimized for higher potency and selectivity to enable ex vivo and in vivo studies or unlock potential therapeutic effects.

It is well established that the 7TM domain of Family C receptors contains an allosteric binding site, which has previously been targeted by drug-like molecules for the metabotropic glutamate, CaR, and $GABA_B$ receptors (Bräuner-Osborne et al., 2007). Indeed, the positive allosteric modulator cinacalcet acting on CaR was the first allosteric GPCR modulator to be marketed as drug (Jensen and Bräuner-Osborne, 2007) and allosteric modulators for several Family C receptors are actively being pursued by a number of pharmaceutical companies (Froestl, 2010; Jensen and Bräuner-Osborne, 2007; Nicoletti et al., 2010). The GPRC6A antagonists identified in this study have overcome the problem that the previously identified GPRC6A antagonists, calindol and NPS 2143, have with a lack of selectivity. Both compounds were originally designed to modulate CaR rather than GPRC6A function and are ~30 times more potent at CaR than GPRC6A (Faure et al., 2009). The new

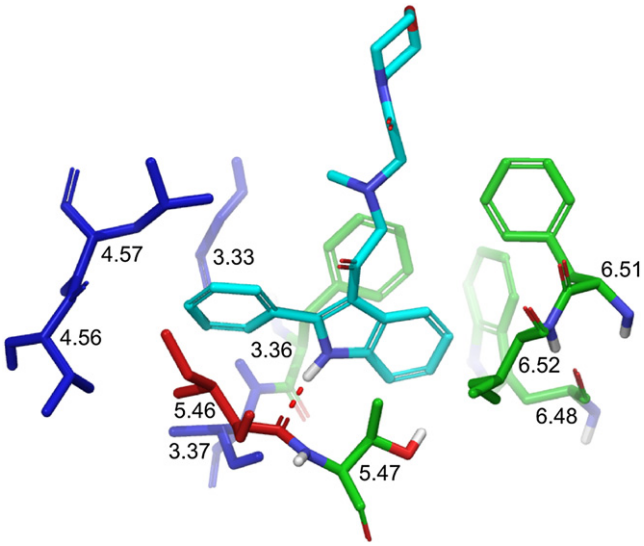

Figure 4. Compound 3 Docked in the Mouse GPRC6A Model
Residues are color-coded according to their interacting moiety in the 2-phenyl-indole binding sequence motif; Green: Indole, Red: Indole NH and Blue: Phenyl. Residues are indexed using Ballesteros-Weinstein nomenclature (Ballesteros and Weinstein, 1995).

GPRC6A antagonists and calindol both contain an indole; however, this fragment constitutes a building block in medicinal chemistry at numerous target families. It is only when the 2-phenyl is added that the structure adopts a preference for the GPCR superfamily, and furthermore substitutions at the 3-position are required to gain affinity and selectivity (S. Garland and D.E.G., unpublished data). The current antagonists have a distinct site of action from calindol, which by mutagenesis and modeling has been proposed to bind at the top of TM helix 6 and 7 at the extracellular interface (Faure et al., 2009).

Homology models are commonly used tools for structure-based drug design and lead identification through virtual screening. However, models of sufficient quality are extremely difficult to generate for most GPCR families, as templates are limited to only Family A (six receptors; Cherezov et al., 2007; Chien et al., 2010; Jaakola et al., 2008; Palczewski et al., 2000; Scheerer et al., 2008; Warne et al., 2008; Wu et al., 2010) and interfamily sequence conservation is very low making the production of reliable alignments challenging. However, in a few analyses, alignments between GPCR families have been generated by anchoring TM helices on evolutionarily conserved residues and validation through mutagenesis of binding residues (Binet et al., 2007; Surgand et al., 2006; deGraaf et al., 2011). There are also several successful examples of how Family C receptor homology models have been used to predict ligand binding modes confirmed by mutagenesis (Bräuner-Osborne et al., 2007; Faure et al., 2009). Our Family A–C alignment is validated by the 2-phenyl-indole activity, which would not be possible without conservation of the binding site, as well as the receptor mutation that pinpoints the most crucial ligand interaction. It would be highly warranted to gather the collected support for inter-GPCR-family alignments that can be used by the scientific community for the building of homology models. To that end,

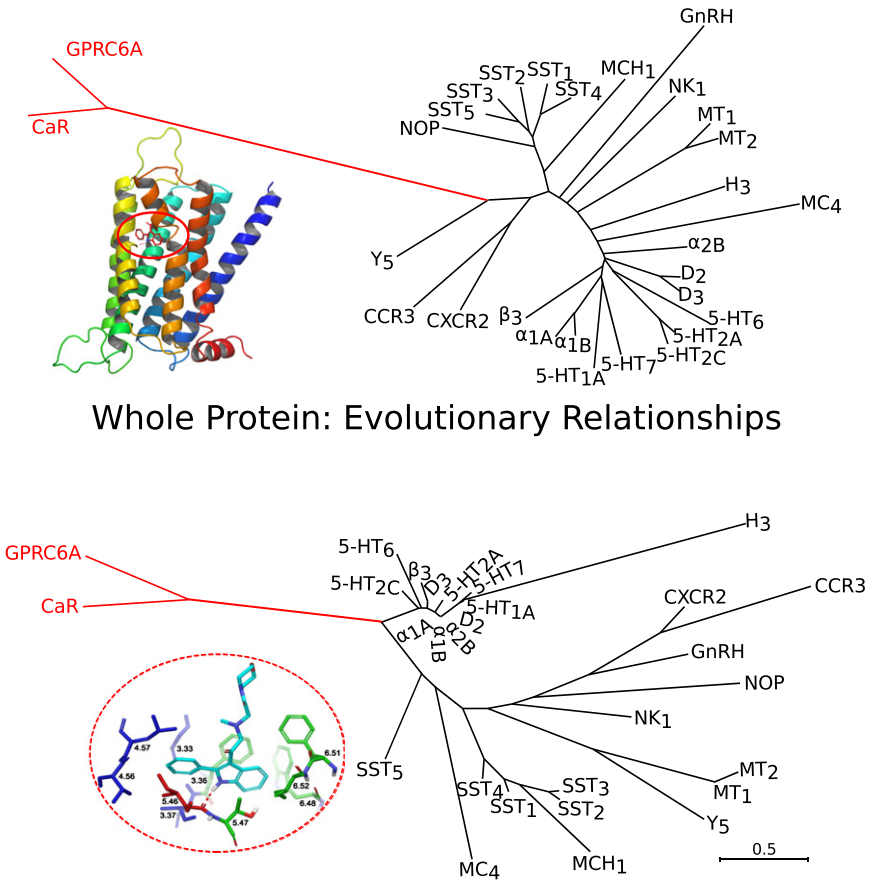

Figure 5. Comparison of Evolutionary and Chemogenomic Receptor Relationships

A comparison of evolutionary and chemogenomic receptor relationships based on the full TM region (left) or the 2-aryl-indole binding site (right). The trees include 27 Family A GPCRs (black) with known 2-aryl-indole containing ligands and the Family C receptors GPRC6A and CaR (red). Branch lengths indicate receptor (dis)similarities. The trees were produced with Neighbor of the Phylip package using the JTT substitution matrix.

we are currently performing a comprehensive mutagenesis study on the 7TM domain of $mGlu_5$ to test the validity of our current alignment, which we plan to further substantiate by additional mutagenesis on GPRC6A. The latter would potentially further improve our model of the docked antagonists (Figure 4), and thus provide an important platform for rational design of novel GPRC6A antagonist analogs with improved potency and selectivity.

Based on chemogenomic analysis we have identified allosteric antagonists for Family C by inference of privileged structures binding to Family A. Hitherto, this approach had only been applied within Family A, but our chemogenomic analysis indicated that the binding sequence motif for 2-phenyl-indoles was preserved in remotely related Family C receptors including GPRC6A and this was subsequently confirmed experimentally. This demonstrates that the use of privileged structures and receptor binding sequence motifs can be applied in a much broader context than previously realized. In addition to in silico ligand inference, chemogenomics may also be used to predict the identity of unwanted targets; consequently it represents a new, more rational means for the selection of off-target screening panels. Moreover, it could also be used to rationalize polypharmacology and possibly aid the design of drugs displaying activity at a profile of (chemogenomically) similar targets. Most chemogenomic analyses have been performed on GPCR targets, but there are also substantial data for protein kinases (Harris and Stevens, 2006; Vieth et al., 2004) and even interprotein family comparisons based on crystal structures (Weill and Rognan, 2010). It can therefore be anticipated that chemogenomic techniques will gain a wide implementation as more structural ligand binding mode data are deposited.

SIGNIFICANCE

GPCRs form one of the largest protein families in human (Venter et al., 2001) and are the targets of 27%–45% of marketed drugs (Drews, 2000; Hopkins and Groom, 2002; Overington et al., 2006). This study presents, to our knowledge, the first ever ligand inference across GPCR families. This is remarkable because of the low sequence homology between the families and the lack of structural data for other than the Family A class. This proof-of-concept for in silico approaches against Family C targets based on Family A templates greatly expands the prospects of drug design and discovery. Specifically, it facilitates alignment and receptor 3D homology model building as well as potential inference of further ligands binding in the TM domain. This domain encompasses most of the many Family A ligands, but is an allosteric binding site for Family C receptors that instead are physiologically activated by binding in a large amino-terminal "Venus-flytrap" domain. Allosteric ligands have a range of advantages including the possibility to gain increased selectivity, fine-tune an endogenous response or generating functionally selective responses. GPRC6A was recently discovered and deorphanized by our group (Christiansen et al., 2007; Kuang et al., 2005; Pi et al., 2005; Wellendorph and Bräuner-Osborne, 2004; Wellendorph et al., 2005). The antagonists 1–3 constitute novel research tools for investigating the signaling mechanism of the GPRC6A receptor at the cellular level. They also constitute lead structures for further optimization of potency and selectivity, which would enable elucidation of the wider physiological function and therapeutic relevance of GPRC6A, a receptor already linked to severe metabolic and endocrinological disturbances (Oury et al., 2011; Pi et al., 2008; Wellendorph et al., 2009b).

EXPERIMENTAL PROCEDURES

Alignment of the Transmembrane Helices of Family A and C GPCRs

MEGA version 4.0 was used to produce ungapped protein sequence alignments of the seven TM helices of the majority of the human Family A and all Family C receptors, respectively. Subsequently, a Family A to C alignment was made anchoring the TM helices on residues conserved in a large number of receptor homologs and orthologs (Bjarnadóttir et al., 2006; Gloriam et al., 2007; Haitina et al., 2009; Lagerström et al., 2006). TMH3 was anchored on C3.26 that forms a conserved disulphide bond to ECL2. THM4 was aligned based on P4.60, which is conserved in the glutamate receptors, TAS1R, GPRC6A, and CaR. TMH5 was fit to give the best overall alignment using the alignment program MUSCLE inside MEGA being placed in between the disulphide bonding cysteine residue in ECL2 and TMH6. These structural elements are close and restrict the alignment of TMH5 in Family C receptors. In GPRC6A, they are separated by only 3 residues on each side. THM6 was anchored on W6.48.

Chemogenomic Binding Motif-Based Ligand Inference

We utilized a chemogenomic approach to assess whether the privileged structures 2-acid-1,1′-biphenyl, 2-phenyl-indole or basic 4-aryl-piperidine, which are substructures of a large number of Family A GPCRs ligands (Bondensgaard et al., 2004), were also likely to bind in GPRC6A. The binding modes of the three privileged structures had previously been determined by docking and mutagenesis and are conserved among targets (Bondensgaard et al., 2004; Garland and Gloriam, 2011b). For each privileged structure, a binding sequence motif defined: (1) which positions (sites) in the sequence alignment that interact with the ligand and (2) which of the natural amino acids that can mediate the given ligand interaction at each positions. The positions were indexed using Ballesteros-Weinstein indexing (Ballesteros and Weinstein, 1995) and amino acid flavors as simple sets, for example F, W, Y, or H for aromatic interactions. The binding sequence motifs were matched against the aligned GPRC6A protein sequence. Twenty-five compounds were purchased that contain the 2-phenyl-indole scaffold (only matching binding motif) with structurally diverse substituents on the indole 3-position.

GPRC6A modeling and ligand docking

A homology model of mouse GPRC6A was constructed with MODELER version 9v6. The high-resolution human β_2-adrenergic crystal structure (Cherezov et al., 2007) was retrieved from PDB (PDB ID: 2RH1) and used as template. Five hundred models were constructed using the default settings and the model with the best DOPE score was chosen. Compound **3** was manually docked in the mGPRC6A model using Maestro (Schrödinger LCC).

Culturing and Transfection of tsA201 and CHO cells

tsA201 cells (Chahine et al., 1994) were cultured in GlutaMAX-I DMEM medium, supplemented with 10% dialyzed fetal bovine serum and penicillin (100 U/ml)/streptomycin (100 mg/ml) at 37°C in a humidified atmosphere of 5% ambient CO_2. Cells were transfected using PolyFect (QIAGEN, West Sussex, UK) with mouse GPRC6A, human 5-HT_{2C}, rat CaR, goldfish 5.24, and human M_1 or human M_3 plasmid DNA as previously described (Bonner et al., 1987; Christiansen et al., 2006, 2007). To enable efficient coupling of mGPRC6A and mutant receptors to phospholipase C, the receptors were coexpressed with $G\alpha_{qG66D}$ (1:1 transfection ratio) (Christiansen et al., 2007; Heydorn et al., 2004). For the IP turnover assay, transfected cells were split into poly-D-lysine coated 96-well plates the day before assaying and grown to confluence in inositol-free DMEM medium supplemented with antibiotics, serum and 0.15 MBq/ml *myo*-[2-^{3}H]inositol (GE Healthcare, Buckinghamshire, UK).

CHO cells stably expressing the rat $mGlu_1$ or $mGlu_5$ receptors were cultured in GlutaMAX-I DMEM medium, supplemented with 10% dialyzed fetal bovine serum, 1% L-proline and penicillin (100 U/ml)/streptomycin (100 mg/ml) at 37°C in a humidified atmosphere of 5% ambient CO_2 as previously described (Hermit et al., 2004).

IP Turnover Assay

The assay on transfected tsA was carried out as previously described (Christiansen et al., 2007; Wellendorph et al., 2005). In brief, the cells were prewashed for 2 × 2 hr at 37°C with buffer containing Hank's balanced salt Solution (HBSS) containing 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mg/ml BSA [pH 7.4]) (Kuang et al., 2005). The cells were washed and preincubated with buffer or allosteric modulator in 50 μl assay buffer (HBSS containing 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM LiCl [pH 7.4]) for 30 min at 37°C. Following this preincubation, the cells were stimulated with 50 μl of agonist with or without modulator in assay buffer for 30 min at 37°C. The reactions were stopped by exchanging the buffer with 50 μl 10 mM ice-cold formic acid and incubating the cells at 4°C for at least 30 min. Yttrium silicate scintillation proximity assay beads (PerkinElmer, Waltham, CA, USA) were used for measuring radioactivity from generated [^{3}H]IP, as previously described (Brandish et al., 2003; Christiansen et al., 2007). Radioactivity was quantified in a Packard TopCount microplate scintillation counter and responses read as counts per minute. All data points were performed in triplicate and experiments performed in at least three independent repetitions. The IP turnover assay on CHO cells was performed as previously described (Hermit et al., 2004), which is the same protocol as detailed above, except from omission of the 2 × 2 hr prewashing step.

Electrophysiology

Preparation of oocytes from *Xenopus laevis* frogs and injection with in vitro-transcribed cRNA encoding mGPRC6A were carried out as described previously (Wellendorph et al., 2005). In brief, mGPRC6A in pGEMHE-3Z was linearized with SapI, transcribed to cRNAs with mMessage mMachine kits (Ambion, Austin, TX, USA) and cRNA injected into harvested healthy-looking stage V–VI oocytes. Whole cell currents were recorded from oocytes 4–5 days after injection using two-electrode voltage clamp at −60 mV in ND96 solution (in mM): NaCl (96), KCl (2), MgCl2 (1), CaCl2 (1.8), HEPES (hemi-Na salt; 5) supplemented with sodium pyruvate (2.5), theophylline (0.5) and 50 μg/ml gentamycin (pH 7.4). Recordings were performed at room temperature by means of a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA, USA), a MacLab 2e recorder (AD Instruments, Sydney, NSW) and LabChart (AD Instruments). Oocytes were voltage-clamped at −60 mV using glass microelectrodes filled with 3 mM KCl (0.5–1.5 MΩ). The preparation was continually perfused with ND96 solution. The ligands were dissolved in ND96 and applied to the oocytes by gravity-driven perfusion. Currents were digitized at 100 Hz.

Construction of mGPRC6A Mutants

Single amino acid replacement was carried out by the QuikChange method (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The identity of each mutant was confirmed by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Compound Synthesis

To supply sufficient amounts of ligands **1** and **3** and to confirm their chemical structures they were synthesized in-house. Both compounds, as well as the *N*-methylated analog **4**, were synthesized from commercially available 2-phenyl-indole using a Friedel-Craft acylation approach. All spectral data for ligands **1** and **3** were identical to that for the commercially available compounds, confirming their identities. (full details are available in the Supplemental Experimental Procedures).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables, two figures, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.09.012.

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