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REVIEW ARTICLE

Identifying ligands at orphan GPCRs: current status using structure-based approaches

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GPCRs are the most successful pharmaceutical targets in history. Nevertheless, the pharmacology of many GPCRs remains inaccessible as their endogenous or exogenous modulators have not been discovered. Tools that explore the physiological functions and pharmacological potential of these 'orphan' GPCRs, whether they are endogenous and/or surrogate ligands, are therefore of paramount importance. Rates of receptor deorphanization determined by traditional reverse pharmacology methods have slowed, indicating a need for the development of more sophisticated and efficient ligand screening approaches. Here, we discuss the use of structure-based ligand discovery approaches to identify small molecule modulators for exploring the function of orphan GPCRs. These studies have been buoyed by the growing number of GPCR crystal structures solved in the past decade, providing a broad range of template structures for homology modelling of orphans. This review discusses the methods used to establish the appropriate signalling assays to test orphan receptor activity and provides current examples of structure-based methods used to identify ligands of orphan GPCRs.

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Abbreviations

CRE, cAMP response element; ECL, extracellular loop; HTS, high-throughput screening; ICL, intracellular loops; NFAT, nuclear factor of activated T cells; RE, response element; RMSD, root mean squared deviation; SAR, structure activity relationship; SBDD, structure-based drug design; SRE, serum response element; SRF, serum response factor; TM, transmembrane; VLS, virtual ligand screening

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Tables of Links

TARGETS					
GPCRs[a]					**Enzymes**[b]
5-HT_{1B} receptor	GPR4	GPR32	GPR78	MRGPRD	AC
5-HT_{2B} receptor	GPR6	GPR34	GPR82	MRGPRE	ERK1
α_{1A}-adrenoceptor	GPR10	GPR35	GPR83	MRGPRF	ERK2
β_1-adrenoceptor	GPR11	GPR37	GPR84	MRGPRG	MAPK
β_2-adrenoceptor	GPR12	GPR37L1	GPR85	MRGPRX1	MEK
δ receptor	GPR13	GPR39	GPR87	MRGPRX2	MEKK
κ receptor	GPR14	GPR40	GPR88	MRGPRX4	PKC
A_{2A} receptor	GPR15	GPR43	GPR92	MT_1 receptor	PLC
AT_1 receptor	GPR16	GPR45	GPR97	NOP receptor	Raf
BB_3 receptor	GPR17	GPR50	GPR99	OPN3	Ste7
CXCR4	GPR18	GPR51	GPR139	OPN4	Ste11
CXCR7 (ACKR3)	GPR19	GPR52	H_1 receptor	OPN5	
D_2 receptor	GPR20	GPR55	H_4 receptor	$P2Y_1$ receptor	
D_3 receptor	GPR21	GPR61	LGR4	P2RY8	
FFA1 receptor	GPR22	GPR62	LGR5	TAAR2	
FFA2 receptor	GPR25	GPR63	LGR6	TAAR5	
FFA3 receptor	GPR26	GPR65	M_3 receptor	TAAR6	
GPR1	GPR27	GPR68	MAS1	TAAR8	
GPR3	GPR31	GPR75	MAS1L	TAAR9	

LIGANDS
Acetate
ATP
cAMP
GW9508
IP_3
Linoleic acid
Nateglinide
TAK-875

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b]Alexander *et al.*, 2015a,b).

GPCRs are membrane proteins that can be activated by a diverse range of extracellular stimuli, triggering intracellular signalling events that mediate numerous physiological responses. Consequently, GPCRs, especially those of the rhodopsin-like family, are regularly exploited as drug targets (Garland, 2013; Kinch *et al.*, 2015). However, the pairing of GPCRs with their respective endogenous agonists remains a challenge for pharmacologists. The sequencing and annotation of the human genome has revealed the full extent of the GPCR superfamily, identifying over 800 GPCR genes, of which 342 are non-olfactory (Fredriksson *et al.*, 2003). Related GPCRs were quickly recognized and sorted by phylogeny (Fredriksson *et al.*, 2003; Bjarnadottir *et al.*, 2006); however, more than 150 GPCRs remain orphans (Davenport *et al.*, 2013), including 86 rhodopsin-like receptors (Table 1). Furthermore, only a small subset of GPCRs (~10%) is actually targeted by existing drugs (Garland, 2013). Therapeutic candidates are currently under development for many more receptors whose pharmacology is well understood. However, orphan GPCRs may also prove to be valuable therapeutic targets and experiments with orphan receptor knockout mice may provide insights into the physiological roles and therapeutic potential of these receptors (Davenport *et al.*, 2013). If the orphan GPCR in question is activated by a yet unknown endogenous ligand, the discovery of this ligand is of paramount importance; however, it is not clear that every orphan GPCR necessarily requires an endogenous ligand, as some of them instead rely upon constitutive activity or heteromerize to achieve activation (Civelli *et al.*, 2013). Regardless of their endogenous means of stimulation, identification of surrogate (non-natural) ligands that can modulate the activity of orphan GPCRs will be invaluable for unlocking both their pharmacology and function.

Traditionally, reverse pharmacology has been used to identify endogenous and/or surrogate ligands for orphan GPCRs. This involves the expression of the orphan GPCR in a cellular system and testing large libraries of ligands, in a high-throughput manner, for activity in a functional assay. Reverse pharmacology has proved successful for matching many orphan GPCRs with their cognate ligands (reviewed by Wise *et al.*, 2004; Oh *et al.*, 2006; Civelli *et al.*, 2013), but newer strategies are needed as the success rate of this deorphanization approach has waned in the last few years. This is evident by the deorphanization of only 15 rhodopsin-like GPCRs since the initial publication of IUPHAR's receptor list (Foord *et al.*, 2005; Davenport *et al.*, 2013; Southern *et al.*, 2013) and the lack of independent validation of ligand-receptor pairings (http://www.guidetopharmacology.org/latestPairings.jsp).

The past decade has seen the elucidation of an increasing number of GPCR X-ray crystal structures, largely due to improved stabilization methods through protein engineering, the identification of tightly bound ligands and to the development

Table 1

Rhodopsin-like orphan GPCRs and the reach of crystal structure templates for homology modelling

Rhodopsin-like GPCRs[a]	Solved crystal structures (PDB[b])	Orphan GPCR	Best % TM identity within subclass (template)[c]	Best % TM identity overall (template)
α-subclass 107 GPCRs	Bovine rhodopsin (1F88)	GPR3	28% (D_3)	—
(26 orphans; 24%)	Human β_2-adrenoceptor (2RH1)	GPR6	26% ($S1P_1$)	—
	Turkey β_1-adrenoceptor (2VT4)	GPR12	28% ($S1P_1$)	—
	Human adenosine A_{2A} (3EML)	GPR21	28% (M_3)	—
	Human dopamine D_3 (3PBL)	GPR22	23% (H_1)	—
	Human histamine H_1 (3RZE)	GPR45	30% ($5\text{-}HT_{1B}$)	-
	Human $S1P_1$ (3V2Y)	GPR50	28% (D_3)	—
	Human M_2 muscarinic (3UON)	GPR52	28% (M_3)	29% (OX_2)
	Rat M_3 muscarinic (4DAJ)	GPR61	25% (D_3)	—
	Human $5\text{-}HT_{1B}$ (4IAQ)	GPR62	20% ($5\text{-}HT_{1B}$)	20% (NTS_1)
	Human $5\text{-}HT_{2B}$ (4IB4)	GPR63	30% (β_1-adrenoceptor)	—
	Human LPA_1 (4Z34)	GPR88	27% (D_3)	—
		GPR119	29% ($S1P_1$)	—
		GPR135	29% (D_3)	—
		GPR152	21% (D_3)	23% (NTS_1)
		GPR153	15% (H_1)	19% (δ)
		GPR160	15% ($5\text{-}HT_{2B}$)	16% ($P2Y_{12}$)
		GPR162	17% (D_3)	—
		OPN3	35% (Rhodopsin)	—
		OPN4	30% (β_1-adrenoceptor)	—
		OPN5	28% (Rhodopsin)	—
		TAAR2	33% (β_2-adrenoceptor)	—
		TAAR5	39% (β_1-adrenoceptor)	—
		TAAR6	33% ($5\text{-}HT_{1B}$)	—
		TAAR8	32% ($5\text{-}HT_{1B}$)	—
		TAAR9	35% (β_2-adrenoceptor)	—
β-subclass 41 GPCRs	Rat NTS_1 (4GRV)	BB_3 receptor	32% (OX_2)	—
(7 orphans; 17%)	Human OX_2 (4RNB)	GPR37	21% (OX_2)	24% (CCR5)
		GPR37L1	23% (NTS_1)	—
		GPR39	38% (NTS_1)	—
		GPR75	20% (NTS_1)	21% (CXCR4)
		GPR83	35% (OX_2)	—
		GPR150	18% (NTS_1)	19% (δ)
γ-subclass 64 GPCRs	Human CXCR4 (3ODU)	GPR1	34% (AT_1)	—
(7 orphans; 11%)	Human μ opioid receptor (4DKL)	GPR15	35% (AT_1)	—
	Human κ opioid receptor (4DJH)	GPR20	32% (κ)	—
	Human NOP receptor (4EA3)	GPR25	35% (AT_1)	—
	Mouse δ opioid receptor (4EJ4)	GPR32	31% (δ)	—
	Human CCR5 (4MBS)	GPR151	22% (NOP)	—
	Human AT_1 (4YAY)	GPR182	28% (NOP)	—
δ-subclass 74 GPCRs	Human PAR1 (3VW7)	GPR4	31% ($P2Y_1$)	—
(39 orphans; 53%)	Human FFA1 (4PHU)	GPR17	33% (PAR1)	—
	Human $P2Y_{12}$ (4NTJ)	GPR18	28% ($P2Y_1$)	28% (AT_1)
	Human $P2Y_1$ (4XNV)	GPR19	20% ($P2Y_1$)	29% (D_3)

(Continues)

Table 1

(Continued)

Rhodopsin-like GPCRs[a]	Solved crystal structures (PDB[b])	Orphan GPCR	Best % TM identity within subclass (template)[c]	Best % TM identity overall (template)
		GPR26	19% (PAR1)	24% (D_3)
		GPR27	18% (FFA1)	28% (5-HT_{2B})
		GPR31	30% ($P2Y_1$)	—
		GPR34	32% ($P2Y_{12}$)	—
		GPR35	27% ($P2Y_1$)	30% (κ)
		GPR55	24% (PAR1)	29% (CCR5)
		GPR65	32% ($P2Y_1$)	—
		GPR68	27% (PAR1)	28% (δ)
		GPR78	23% (FFA1)	—
		GPR82	22% ($P2Y_1$)	23% (δ)
		GPR84	24% ($P2Y_{12}$)	25% (D_3)
		GPR85	18% (FFA1)	25% (5-HT_{2B})
		GPR87	47% ($P2Y_1$)	—
		GPR101	14% (PAR1)	20% (β_2-adrenoceptor)
		GPR132	31% ($P2Y_1$)	—
		GPR161	22% ($P2Y_1$)	27% (5-HT_{1B})
		GPR171	37% ($P2Y_{12}$)	—
		GPR173	16% (PAR1)	24% (5-HT_{2B})
		GPR174	33% ($P2Y_1$)	—
		GPR183	29% (PAR1)	—
		LGR4	19% ($P2Y_{12}$)	22% (AT_1)
		LGR5	16% ($P2Y_{12}$)	19% ($S1P_1$)
		LGR6	17% ($P2Y_{12}$)	20% (CCR5)
		MAS1	20% (PAR1)	—
		MAS1L	17% (FFA1)	19% (M_2)
		MRGPRD	19% (PAR1)	20% (κ)
		MRGPRE	19% (FFA1)	—
		MRGPRF	17% (FFA1)	18% (M_2)
		MRGPRG	20% (FFA1)	—
		MRGPRX1	19% ($P2Y_1$)	23% (κ)
		MRGPRX2	19% ($P2Y_1$)	24% (AT_1)
		MRGPRX3	18% ($P2Y_{12}$)	22% (μ)
		MRGPRX4	20% ($P2Y_1$)	24% (μ)
		P2RY8	38% (PAR1)	—
		P2RY10	32% ($P2Y_1$)	—
Unclassified orphans		GPR139	—	21% (δ)
		GPR141	—	19% ($P2Y_{12}$)
		GPR142	—	22% (NTS_1)
		GPR146	—	19% (CXCR4)
		GPR148	—	16% (κ)
		GPR149	—	14% (FFA1)
		GPR176	—	23% (NOP)

[a]Receptor numbers are according to the IUPHAR/BPS Guide to PHARMACOLOGY (Alexander *et al.,* 2015a); probable pseudogenes were excluded.
[b]The PDB ID of the first instance of the unique GPCR structure is given.
[c]Sequence identity was determined based on the Needleman and Wunch algorithm (Needleman and Wunsch, 1970), following alignment of the orphan receptor sequence to the TM regions of all crystallized GPCRs.

of improved crystallization protocols [e.g. the development of lipidic cubic phase crystallization (Caffrey and Cherezov, 2009)] and crystallographic procedures [e.g. the development of femtosecond crystallography (Liu *et al.,* 2014)]. Structures have typically been solved as fusion proteins with the T4-lysozyme or thermostabilized apocytochrome bRIL substituted for the highly flexible third intracellular loop or N-terminus; more recently, stabilizing mutations along with nanobodies have been used [discussed in Chun *et al.* (2012); Mujic-Delic *et al.* (2014); Ghosh *et al.* (2015); Jazayeri *et al.* (2015)]. This increasing availability of GPCR structural information has allowed a shift from traditional high-throughput screening (HTS) methods to cheaper and efficient virtual ligand screening (VLS) approaches for the identification of novel ligands at GPCRs.

This review will consider two aspects of these newer screening approaches: (i) establishing suitable screening assays for measuring orphan GPCR activation and (ii) the current status of structure-based drug design (SBDD) in the context of orphan GPCRs. We have have also hypothesized what we expect could become a universal pipeline for orphan GPCR drug discovery, outlined in Figure 1.

Experimental strategies for detection of orphan GPCR activation

Before attempting to deorphanize a GPCR, a number of issues need to be considered. Broadly speaking, it is important to establish whether there is sufficient preliminary evidence to suggest a significant physiological function of the orphan GPCR of interest, such as knockout mouse models, that would justify it being a potentially useful clinical target. Tissue expression profiles will also indicate the function of a particular orphan GPCR (Petryszak *et al.,* 2015). Once an orphan target is identified, it is imperative that a robust, facile and relatively high-throughput assay is established, either for screening of chemical libraries or for the validation of potential *in silico* 'hits'. GPCRs mediate various physiological responses; however, as their name indicates, almost all of them signal via coupling to a heterotrimeric G protein (made up of α, β and γ subunits), particularly by interacting with the Gα subunit. Moreover, upon activation, most GPCRs recruit the internalization and scaffolding proteins β-arrestins 1 and 2 (Luttrell and Lefkowitz, 2002). Several screening modalities based on both of these interactions have been developed and have their advantages and disadvantages. Other approaches could include, for example, the use of pathway-specific inhibitors or siRNA screens.

If G protein-coupling assays are used for detection of receptor activation, elucidation of the Gα subtype involved in receptor coupling is critical. A frequently employed strategy for identifying the coupling capacity of a GPCR is the use of receptor constitutive activity, a phenomenon whereby a GPCR (or indeed any receptor) signals in the absence of its activation by a ligand. Constitutive activity is now widely accepted as an intrinsic feature of GPCRs, particularly when they are overexpressed in heterologous *in vitro* expression systems, and it is also observed in *in vivo* studies (Damian *et al.,* 2012; Corder *et al.,* 2013). Constitutive activity upon overexpression of a GPCR can be used to identify the G protein involved in

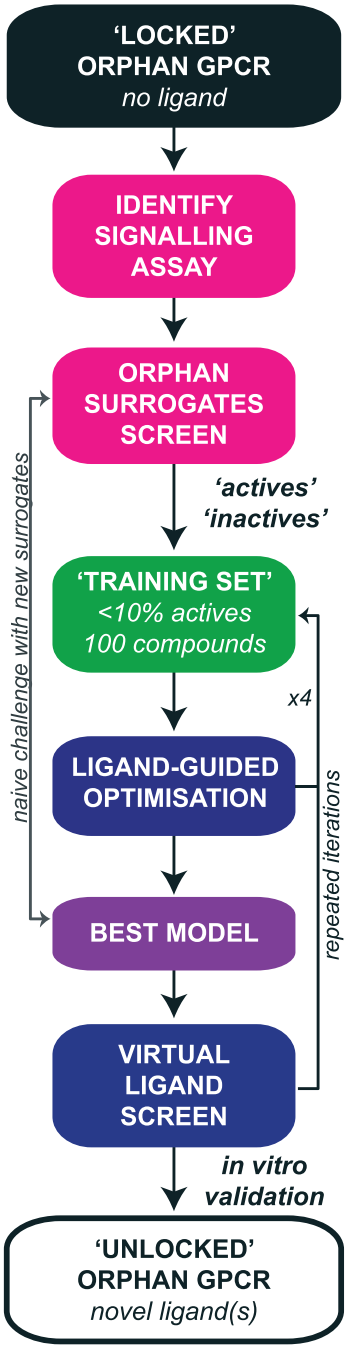


Figure 1

A putative pipeline for unlocking the pharmacology of orphan GPCRs by combining targeted HTS and SBDD. Combining both *in vitro* screening and hit validation with VLS at ligand-optimized homology models is one rational approach to unlocking orphan GPCRs. First, an appropriate signalling assay must be identified, and then, a small library is screened for low affinity hits. This surrogate screen informs the composition of a ligand training set that can be used for the iterative ligand-guided optimization of a homology model. Ideally, a subset of active and inactive ligands is set aside for naïve challenge of the best model to validate performance. VLS is then performed and hits validated *in vitro* by at least one assay readout. Additional site-directed mutagenesis data and structure–activity relationships can further feed into repeated iterations of homology model optimization and VLS.

signalling by a particular orphan GPCR. However, given the lack of pharmacological controls when studying orphan GPCRs, it is imperative that any findings from such studies are validated using multiple second messenger assays (described in depth by Zhang and Xie, 2012a), particularly in mammalian cells. This is particularly important in overexpression studies as promiscuous coupling may result from high receptor abundance.

One of the simplest and least expensive assays for measuring multiple G protein pathways is that developed by Simon Dowell and Andrew Brown at GlaxoSmithKline, in which the yeast pheromone response pathway has been manipulated for the study of mammalian GPCRs (Brown *et al.,* 2000; Dowell and Brown, 2009; Ngo *et al.,* 2015). Different yeast strains have been engineered to express yeast Gpa1p/human Gα subunit chimeras, allowing the study of GPCR activation by the four Gα subunit families in a single assay. GPCR constitutive coupling is revealed by both growth and β-galactosidase activity (Figure 2A and B). The first time this assay was used to identify orphan GPCR coupling through constitutive activity was with GPR43 (now known as FFA2), where it was found to be $G\alpha_i$ and $G\alpha_q$ coupled (Brown *et al.,* 2003). The yeast strain expressing FFA2 was then screened with a small ligand library, and acetate was found to increase β-galactosidase activity above constitutive levels. Importantly, acetate activation of FFA2 via $G\alpha_i$ or $G\alpha_q$ was then confirmed by [^{35}S]-GTPγS binding and increases in [Ca^{2+}] levels, respectively (Brown *et al.,* 2003). Despite the versatility of the yeast system, which allows different G protein coupling pairs to be tested, it has not necessarily been the primary platform for initial G protein coupling screens of orphan GPCRs. In fact, the yeast assay was used as a secondary validation of $G\alpha_s$ coupling of GPR26 and $G\alpha_{13}$ coupling of GPR35 (Jones *et al.,* 2007; Jenkins *et al.,* 2010).

Another approach for initially screening constitutive activity is to use a transcription factor response element (RE) reporter gene assay, a highly sensitive but not necessarily specific platform for ligand screening. In brief, a reporter gene construct, commonly luciferase, is cloned downstream of REs that are targeted by distinct second messengers (cAMP, ERK1/2, Ca^{2+}, RhoA; Figure 2C) (Cheng *et al.,* 2010). For example, luciferase gene transcription is induced by cAMP RE (CRE) following cAMP activation ($G\alpha_s$), or serum RE (SRE) activation downstream of $G\alpha_{i/o}$ mediated ERK1/2 phosphorylation (Cheng *et al.,* 2010). This approach has been used to reveal the coupling mechanism of GPR133, a member of the adhesion GPCR family. Consistent with GPR133 being a $G\alpha_s$-coupled receptor, the assay revealed that of the three transcription factors, CRE, SRE and nuclear factor of activated T-cells (NFAT), GPR133 signalled only via CRE to increase luciferase activity (Bohnekamp and Schoneberg, 2011). This observation was validated using chimeric $G\alpha_{q(s)}$, where the last five amino acids of $G\alpha_q$ were replaced by the corresponding $G\alpha_s$ amino acids, thus leading to increased inositol phosphate accumulation as a result of GPR133's constitutive signalling (Bohnekamp and Schoneberg, 2011).

Although the coupling mechanisms of a few orphan GPCRs have been proposed using similar approaches (Ge *et al.,* 2008; Muller *et al.,* 2013), they have not been validated with a second assay. The ambiguity and difficulty with working with orphan GPCRs is best highlighted by the case of GPR139. Here, multiple independent groups have used HTS to identify surrogate ligands for GPR139, but the signalling assays used to identify the cognate G protein involved produced contradictory results. GPR139 was proposed to be coupled to $G\alpha_s$ (Hu *et al.,* 2009), $G\alpha_{i/o}$ (Susens *et al.,* 2006) and/or $G\alpha_q$ (Susens *et al.,* 2006; Shi *et al.,* 2011; Isberg *et al.,* 2014). Overall, the evidence favours GPR139 being a $G\alpha_q$-coupled receptor; however retesting the identified ligands in a second assay would provide better validation. This further highlights the necessity for careful validation of the proposed G protein interaction involved in orphan GPCR signalling, before moving forward.

The screening assays described above detect classical G protein coupled signalling, but it is clear that the recruitment of β-arrestin by GPCRs results in signalling via several G protein-independent pathways (Lefkowitz and Shenoy, 2005). Indeed, β-arrestin screens should not be contingent on the G protein coupling repertoire of the orphan GPCR. However, ligand screening using various β-arrestin recruitment approaches has met with variable success. For example, HTS of a panel of orphan GPCRs using a diverse library made up of natural and synthetic compounds (10 500 compounds) revealed novel surrogate ligands for the orphans MRGPRX2, GPR88 and GPR97, although the majority of orphans failed to register hits through the PathHunter β-arrestin recruitment assay (Southern *et al.,* 2013). Moreover, a screen of GPR35 with the Prestwick Chemical Library using BRET-based β-arrestin 2 recruitment both confirmed existing hits and identified a number of novel ligands at both the human and rat orthologues of the receptor (Jenkins *et al.,* 2010). β-arrestin recruitment assays have also been used to confirm proposed endogenous ligand pairings of orphan GPCRs, including CXCR7, GPR84, GPR92 and GPR99 (Yin *et al.,* 2009; Southern *et al.,* 2013). Finally, a very recent study by Roth and colleagues used a modified β-arrestin Tango assay to identify and confirm that the K_{ATP}-channel blocker, nateglinide, is a surrogate ligand for MRGPRX4 (Kroeze *et al.,* 2015). Importantly, their open-source platform proposed many other surrogate ligands at 91 orphan and poorly characterized GPCRs, providing an invaluable starting point for the scientific community in their quest to study orphan GPCRs (Kroeze *et al.,* 2015). It should be noted however that β-arrestin screens, in principle, should only capture agonists because antagonists or inverse agonists do not efficiently activate this pathway. This is a crucial point given that there are known endogenous inverse agonists of GPCRs (Milligan, 2003; Breit *et al.,* 2006). It is also important to note that, particularly in the age of biased agonism where ligands can display distinct second messenger signals (Reiter *et al.,* 2012; Kenakin, 2013), no single assay can be applied to all orphan GPCRs without the possibility of missing crucial hits. Indeed, it is possible that future screening campaigns will need to incorporate multiple endpoints and modalities [including allosterism (Wootten *et al.,* 2013)].

Using GPCR crystal structures for VLS

Once the signalling pathway of the orphan GPCR of interest has been confirmed, the search for endogenous or surrogate ligands begins. Ideally, this would be performed directly via HTS; however, this is often not financially feasible, and there is no commercial or academic library of biological metabolites (in contrast to synthetic chemical compounds) including peptides and small proteins. Therefore, we need to look for alternative approaches such as structure-based VLS for

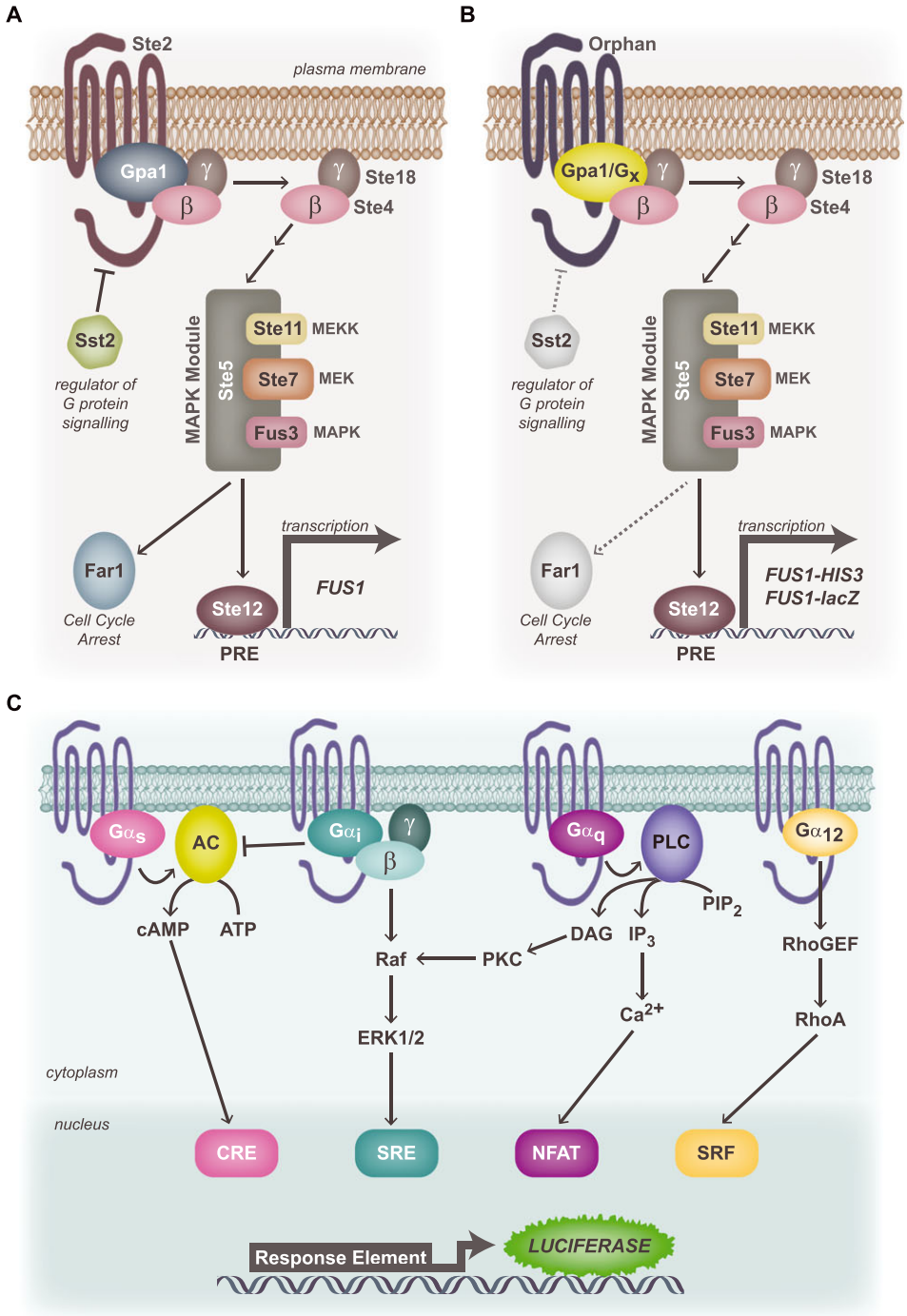


Figure 2

Assays for the identification of G protein signalling pathways using the constitutive activity of an orphan GPCR. (A) Yeast GPCR signalling pathway. The yeast pheromone receptor, Ste2, couples to the endogenous yeast heterotrimeric G proteins (Gα = Gpa1) to drive Gβγ-mediated (MAPK) pathway activation and Ste12-mediated transcription via the pheromone-response element (PRE). MEK: MAPK kinase; MEKK: MEK kinase. (B) Modified yeast G protein signalling assay: chimeric versions of Gpa1 bearing the final five amino acids of each of the human Gα proteins have been introduced into yeast strains lacking each of the yeast GPCR, Ste2, the regulator of G protein signalling, Sst2, and the cell cycle arrest protein, Far1. Upon transformation with the orphan GPCR of interest, constitutive coupling to a specific G protein chimera drives the MAPK module to stimulate transcription of HIS3 (facilitates selection and expansion in HIS3-deficient media) and the lacZ reporter via FUS1. (C) Mammalian reporter assays for transcription factors downstream of each of the major G protein families. Constitutive coupling via $G\alpha_s$ leads to stimulation of AC and generation of cAMP, which stimulates transcription of luciferase that lies downstream of a cAMP response element (CRE). Likewise, $G\alpha_i$ activity is reported via the serum response element (SRE) upon Gβγ signalling to ERK1/2 MAPK; $G\alpha_q$ via nuclear factor of activated T-cells (NFAT) and $G\alpha_{12}$ via serum response factor (SRF) response element.

identifying the ligand in a direct screen of metabolites or indirectly for identifying chemical modulators as a first step towards the deorphanization.

Structure-based VLS, or docking-based VLS, has long been the mainstay of SBDD for the identification of novel ligands at various protein targets. It involves docking large and chemically diverse libraries of small molecules into a protein crystal structure or homology model of interest. The selection of small molecules for biological testing is generally based on docking score, chemical diversity, predicted interactions with key residues and other criteria. Small molecules that cause a biological response are termed hits and act as novel chemical scaffolds for hit-to-lead development. The first demonstration of the theoretical applicability of VLS of potential metabolites against a GPCR for deorphanization was shown in 2003 (Cavasotto *et al.,* 2003). Thus, in the absence of crystal structures at that time, the challenge moved from the screen itself to the construction of a credible three-dimensional model of the binding pocket.

As of November 2015, structures have been solved for 30 unique GPCRs, including two secretin-like (Hollenstein *et al.,* 2013; Siu *et al.,* 2013), two glutamate-like (Dore *et al.,* 2014; Wu *et al.,* 2014) and one frizzled-like (Wang *et al.,* 2013) families of GPCRs. Structure-based VLS using the GPCR crystal structures themselves has been very successful in identifying new ligands, with particularly high hit rates (Table 2) [for more comprehensive examples, see de Graaf and Rognan (2009); Kooistra *et al.* (2013); Andrews *et al.* (2014)]. This shows that crystal structures are an accurate representation of a native GPCR and that structural accuracy of the docking target is important for VLS. However, one must consider the crystallized receptor conformation and the binding pocket space when docking and conducting VLS, as both depend on the nature of the co-crystallized ligand, that is, whether it is an agonist or antagonist, a peptide or small molecule (Qin *et al.,* 2015), and whether the structure reflects an inactive, active-like or fully active receptor state (Lebon *et al.,* 2011; Rasmussen *et al.,* 2011a, 2011b). Studies conducted using an antagonist-bound crystal structure identified only antagonists, with the exception of the κ-opioid receptor and muscarinic M_3 receptor screens, where one agonist and one partial agonist were identified respectively (Kruse *et al.,* 2013; Negri *et al.,* 2013). This contrasts with studies using crystal structures of GPCRs in an active-like or fully activated state. Using the fully activated β_2-adrenoceptor crystal structure, Weiss and colleagues only retrieved novel agonists (27% hit rate; 6/22) (Weiss *et al.,* 2013). However, in a separate study with the active crystal structure of the 5-HT_{1B} receptor, only three agonists were revealed out of five ligands tested (from 11 hits) (Rodriguez *et al.,* 2014), while screening at a dopamine D_2 receptor homology model based on the fully activated β_2-adrenoceptor was unsuccessful (Weiss *et al.,* 2013). Intriguingly, VLS using the active-like adenosine A_{2A} receptor crystal structure only yielded novel antagonists (Rodriguez *et al.,* 2015). The authors not only provide evidence of chemical bias towards A_{2A} antagonists within the screening database, but also towards β_2-adrenoceptor and 5-HT_{1B} receptor agonists, thus explaining the discrepancies (Rodriguez *et al.,* 2015). Despite this, GPCR agonists have been identified using homology models built on inactive structures (de Graaf and Rognan, 2009). Aside from chemical bias in screening databases, this highlights the importance of an accurate conformation of the binding pocket that can favourably accommodate agonist ligands, that is, optimization of the binding pocket is critical (discussed further below). It also reveals the need to further understand the intricate mechanism of receptor activation at the atomic level.

GPCR homology models

Given that no *bona fide* orphan receptor has been crystallized to date, application of SBDD for orphan GPCRs must rely on the use of homology models. A homology model is an atomic-level approximation of the structure of a protein, generated by comparative modelling of the target based upon the closest experimentally-determined crystal structure (called a template).

Table 2

Examples of the types of hits identified from structure-based virtual screening using published crystal structures

Crystal structure (PDB ID)	Receptor conformation	Hit rate (actives/ligands tested)	Reference[a]
Adenosine A_{2A} receptor (3EML)	Inactive	41% (23/56)	(Katritch *et al.*, 2010a)
β_2-adrenoceptor (2RH1)	Inactive	24% (6/25)	(Kolb *et al.,* 2009)
CXCR4 chemokine receptor (3ODU)	Inactive	17% (4/23)	(Mysinger *et al.,* 2012b)
Dopamine D_3 receptor (3PBL)	Inactive	20% (5/20)	(Carlsson *et al.,* 2011)
Histamine H_1 receptor (3RZE)	Inactive	73% (19/26)[b]	(de Graaf *et al.,* 2011)
κ-opioid receptor (4DJH)	Inactive	18% (4/22) (one agonist identified)	(Negri *et al.,* 2013)
M_3 muscarinic ACh receptor (4DAJ)	Inactive	50% (8/16) (one partial agonist identified)	(Kruse *et al.,* 2013)
Adenosine A_{2A} receptor (2YDO and 2YDV)	Active-like	45% (9/20) (No agonists identified)	(Rodriguez *et al.,* 2015)
5-HT_{1B} receptor (4IAQ)	Active-like	50% (11/22) [three agonists identified (five tested)]	(Rodriguez *et al.,* 2014)
β_2-adrenoceptor (2RH1)	Full active	27% (6/22) (only agonists identified)	(Weiss *et al.,* 2013)

[a]For a more comprehensive table of SBDD using published crystal structures, see Andrews *et al.* (2014).
[b]Fragments (<22 heavy atoms) were screened.

Template selection remains a critical step in building reliable homology models, particularly for GPCRs, given their sequence and/or ligand diversity. Considerations other than overall or local homology between a GPCR of interest and the template sequence, as discussed above, also include the receptor conformational state and the shape of the binding pocket of the crystal structure template. These issues are continually being addressed with the increasing number of crystal structures solved, leading to wider template selection for homology modelling of GPCRs.

It has been estimated that a receptor must have at least 30–35% sequence similarity to its template for accurate homology modelling in the absence of any additional refinement (which would usually involve ligand-guided optimization or knowledge gained from site-directed mutagenesis) (Kufareva *et al.,* 2011; Beuming and Sherman, 2012; Katritch *et al.,* 2012). Presently, all subclasses of the rhodopsin-like family of GPCRs are represented by at least one crystal structure. Table 1 and Figure 3 highlight the transmembrane (TM) sequence homology of the remaining rhodopsin-like orphan GPCRs in each of the subclasses, demonstrating not only the range of current crystal structures for orphan GPCR homology modelling but also the need for more crystal structures to continually expand this range (only 10 of 86 orphans have ≥35% identity to a GPCR with a solved structure; Figure 3). It is important to note, however, that some orphan GPCRs do not have ≥35% identity to any GPCR, crystallized or not. In these cases, further optimization of homology models will be necessary. Meanwhile, the ideal homology model template for a given orphan may not always reside in the same GPCR subclass, as different subclasses of GPCRs may share similar binding modes. For example, both β-subclasses and γ-subclasses bind to endogenous peptides and GPCR homology models built using distantly related templates can be successful (Kufareva *et al.,* 2011; Rataj *et al.,* 2014). Indeed, 31 of the 86 rhodopsin-like orphan GPCRs have higher TM sequence homology with a template from another receptor subclass (Table 1).

GPCR Dock is a community-wide evaluation of the progress of molecular modelling and ligand docking methods for GPCRs (Michino *et al.,* 2009; Kufareva *et al.,* 2011; Kufareva *et al.,* 2014). For a given competition round, a recently solved GPCR crystal structure(s) is withheld from publication, and research groups are called upon to submit their blinded prediction of the solved ligand–receptor complex, with particular focus on the ligand interactions within the receptor-binding pocket. At the time of the CXCR4 GPCR Dock in 2010, no crystal structures of γ-subgroup GPCRs had been solved. Thus, homology models could only rely on structures from the α-subgroup. Despite distant homology (and ~25% sequence identity), the best performing CXCR4 model, built using the β_2-adrenoceptor crystal structure, achieved a TM backbone root mean squared deviation (RMSD) of 2.21 Å and captured 36% of ligand–receptor atomic contacts correctly when compared with the reference CXCR4 crystal structure. Interestingly, this was the only model that performed 'well' with respect to identifying ligand–receptor contacts.

Another approach to building homology models that has been gaining popularity is the use of multiple structural templates, which enable modelling of regions of the orphan GPCR based on higher local sequence identity, thereby creating a 'chimeric' template. Such an approach was applied to

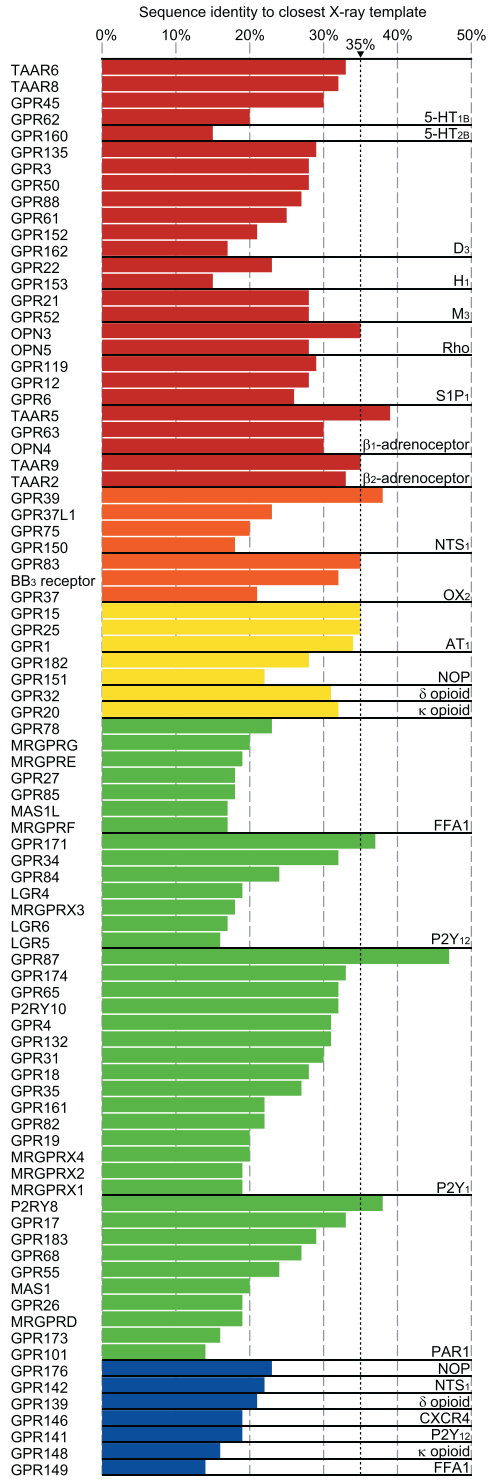


Figure 3

Orphan GPCRs ranked according to their closest crystal structure template. Each orphan GPCR (left) was ranked according to the crystal structure with which it shares the highest TM sequence identity, independent to GPCR subgroup (expressed as a percentage; listed in Table 1). The minimum identity required for VLS at a homology model in the absence of further refinement is 35% (dotted line). The homologous crystal structure is shown in the bottom right-hand corner of each box. Red: α subgroup of rhodopsin-like GPCRs; orange: β-subgroup; yellow: γ-subgroup; green: δ-subgroup; blue: unclassified.

the orphan GPR17, where separate templates for the TM domains, and extracellular (ECL) and intracellular loops (ICL) were chosen with respect to sequence identity: the final model comprised TM regions based on bovine rhodopsin, ECL1 and ICL2 based on the human β_2-adrenoceptor, ECL2 on the turkey β_1-adrenoceptor, and ECL3 based on the A_{2A} receptor (Eberini *et al.,* 2011). Critically, five novel ligands were identified by screening against this mixed-template homology model of GPR17 (100% hit rate in validation assays) (Eberini *et al.,* 2011). The use of chimeric templates is not unprecedented; it has been shown previously that matching TMs to individual templates improved the RMSD of the binding site area of models to their reference crystal structure (Worth *et al.,* 2011; Latek *et al.,* 2013). However, other evaluation metrics were not considered, such as ligand–receptor contact-based methods (Kufareva and Abagyan, 2012). Furthermore, homology models developed with multiple templates remain to be thoroughly tested in VLS studies.

Outside of community-wide assessments like GPCR Dock, we do not have the luxury of a direct comparison between our homology model(s) and a reference crystal structure to provide feedback on how well we have portrayed the best representation of the binding pocket. Thus, other evaluations of the accuracy of a homology model are needed. In the context of this review, the most appropriate method to assess homology model(s) is by their performance in VLS, or more specifically, the ability of VLS to identify novel ligands that can be experimentally validated using the signalling assays discussed earlier (Figure 2). Furthermore, while building homology models with low sequence identity to the template structure can be daunting, the orientation of the binding pocket side chains is important (as mentioned above) – a process that can be optimized.

Optimization of orphan GPCR homology models and their use in VLS

Traditionally, homology models have been refined around a single ligand, with selection of the appropriate docking pose between ligand and receptor guided by existing site-directed mutagenesis data. Explicit flexibility of the known interacting side chains is generally used to allow a degree of ligand-induced fit of the binding pocket. However, invariably, this introduces some bias towards recognition of the original docked ligand. Similarly, a crystal structure is a static image of an otherwise dynamic entity that is capable of adopting multiple conformations. This is best exemplified by a study where a ligand-guided optimized dopamine D_3 receptor structure identified novel D_3 ligands with a higher hit rate (56%) than the non-refined crystal structure (20%) (Carlsson *et al.,* 2011; Lane *et al.,* 2013). For these reasons, it is important for VLS to optimize the modelled binding pocket residues to accommodate a diverse range of ligands.

Recently, multi-step ligand-guided optimization approaches have been developed to overcome this problem (Figure 1) (Carlsson *et al.,* 2011; Mysinger *et al.,* 2012b; Rueda *et al.,* 2012; Kolaczkowski *et al.,* 2013). Firstly, multiple conformations of the homology model are generated with specific importance given to the binding pocket, generally by building multiple models based on different templates or by elastic network models. The different conformations are then subjected to docking of a ligand training set made up of chemically diverse known 'active' ligands and 'inactive' decoy ligands. The screening performance of the models is then assessed by receiver operating characteristic curves, plotting the rate of true positives against false positives in the hit list ordered by decreasing predicted binding score, where the AUC can be calculated. Other measures have been introduced to improve the assessment of the early development of active ligands in VLS (Truchon and Bayly, 2007; Katritch *et al.,* 2010b). Model conformations that can best distinguish between active and inactive ligands are selected, and this process is repeated iteratively until there is no further improvement in the model's performance. At this point, the fact that the model or ensemble of models can reliably retrieve known active ligands is taken as a sign that the same model will be able to identify novel hits from a large chemical database. The significance of considering VLS performance when optimizing models is evident in a comparison of the two best submitted models of the A_{2A} receptor from GPCR Dock 2008 (Katritch *et al.,* 2010b). Even though the traditionally optimized model by Costanzi was ranked first by the competition's criteria, the model's VLS performance was characterized by poor early enrichment of active ligands when compared with a ligand-guided optimized model by Katritch/Abagyan (Katritch *et al.,* 2010b). This highlights the importance of considering VLS performance when assessing homology models, particularly in cases where the motivation for constructing the homology model is to discover novel ligands.

Optimized homology models have themselves still produced mixed hit rates (Table 3) [extensive examples are given in de Graaf and Rognan (2009); Kooistra *et al.* (2013)], relative to the crystal structures (Table 2), but the fact that hits have been found for orphan GPCRs is promising. Undoubtedly, the major limitation of orphan GPCR homology models for structure-based VLS is the lack of ligands for model optimization and validation. Smaller screening campaigns [10–50k samples (Valler and Green, 2000)] can play a complementary role in SBDD in this instance, as any surrogate ligands identified can then be used to populate a VLS training set. This strategy was employed for the immune-related orphan GPR34 (Liebscher *et al.,* 2011), where a screening campaign of 17 565 compounds identified six novel ligands that were validated using two measures of cAMP (Diaz *et al.,* 2013). Next, a crude GPR34 homology model was refined with iterative changes to rotamers of key side chains found in the binding cavity, until the model was optimized for the six identified ligands from the initial screen. Biological testing of 2954 compounds (from an in-house database of 1.25 million small molecules), led to the identification of five hits (of which two hits were the same ligands identified in the initial screen; 0.17% hit rate, 5/2954) (Diaz *et al.,* 2013). These results should be encouraging to orphan GPCR researchers, despite the dismal success rate, as they demonstrate that it is possible to optimize homology models and achieve novel hits from VLS by using ligands from a smaller biological screening campaign (Figure 1). Furthermore, there are a number of simple and modifiable methodological explanations for why the hit rate was low; for example, the composition of the training set was suboptimal with a ratio of active to inactive ligands at 0.03%, whereas the popular inactive or decoy generating database, DUD-e (recommends 2% and up to 10% of active ligands) has been used in training sets (Carlsson *et al.,* 2011; Katritch *et al.,* 2012; Mysinger *et al.,* 2012a, 2012b; Rueda *et al.,* 2012).

Table 3

Examples of structure-based VLS using GPCR homology models

Homology model	Model development strategy	Hit rate	Reference
Adenosine A_{2A} receptor	Energy minimization; mapping of site-directed mutagenesis data onto model	4% (10/230)	(Langmead *et al.*, 2012)
CXCR4 chemokine receptor	Ligand-guided optimization	4% (1/24)	(Mysinger *et al.*, 2012b)
Dopamine D_3 receptor	Ligand-guided optimization	23% (6/26)	(Carlsson *et al.*, 2011)
Histamine H_4 receptor	Manual adjustment of key residues; validated with known ligands and decoys	6% (16/255)	(Kiss *et al.*, 2008)
Orphan GPCRs			
GPR17	Multiple templates; energy minimization	100% (5/5)	(Eberini *et al.*, 2011)
GPR34	Similar to ligand-guided optimization; iterative changes to side chain rotamers until enriched for active ligands	0.17% (5/2954)	(Diaz *et al.*, 2013)
GPR40 (FFA1)	Monte Carlo torsional sampling of binding pocket residues	10% (1/10)	(Tikhonova *et al.*, 2008)

A reasonable question concerning orphan GPCRs is whether a crude homology model may actually be sufficient to retrieve hits, without ligand optimization and validation of the model. Certainly, this was successful for the GPR17 example cited earlier, where the multi-template-based GPR17 homology model was only subjected to energy minimization (Eberini *et al.*, 2011); in the absence of a training set, top ranking docked compounds were subjected to a further round of molecular dynamics-based energy minimization steps before selection (Eberini *et al.*, 2011). While a crude model cannot be validated prior to performing VLS, we can at least hypothesize which residues are necessary for ligand interactions by a binding pocket analysis: are charged residues compensated for? What types of residues are positioned at conserved interaction sites for GPCRs? Building upon these observations, the residue side chains can then be checked and made accessible for small molecule docking in the binding pocket, whether by manual adjustment or by fumigation methods (Nilmeier and Jacobson, 2008; Abagyan and Kufareva, 2009). One might suggest allowing some explicit flexibility within the hypothesized key binding pocket residues while conducting VLS; however, this remains computationally challenging when docking millions of compounds, leaving full conformational searches impractical. Some inherent flexibility in the binding pocket may be accounted for by screening using multiple binding pocket conformations (Totrov and Abagyan, 2008; Vilar and Costanzi, 2013). It must be stressed, however, that a robust signalling assay for the orphan GPCR should be established before conducting a blind VLS, as the hits are unlikely to have particularly high affinity.

Although there are few examples of successful VLS using orphan GPCR homology models, these models have been used to provide insights into the binding mode of proposed ligands. For example, postulated interactions based upon docking to orphan homology models were confirmed by site-directed mutagenesis for FFA1/GPR40, FFA2/GPR43, FFA3/GPR41, GPR55, GPR183 and the bombesin BB_3 receptor (Gonzalez *et al.*, 2008; Kotsikorou *et al.*, 2011; Schmidt *et al.*, 2011; Zhang *et al.*, 2012b), amongst many other examples. Such studies defining and validating the binding pocket of these orphans will be valuable for future VLS as they can inform docking selection criteria. Although these models were each optimized around a single ligand and thus have the potential to generate a biased homology model, this may not be limiting. Furthermore, the key interacting residues can also facilitate structure-based pharmacophore development.

Application of pharmacophores for orphan GPCR ligand discovery

Pharmacophores are an alternative way to view the contacts made between ligand and receptor, where a pharmacophore defines the spatial arrangement of chemical features that characterize the interactions necessary for eliciting a desired biological effect at a protein target of interest. Structure-based pharmacophore features can be selected from key residues that have been identified by site-directed mutagenesis or those based on hypothesized ligand–protein interactions derived from grid-based interaction energies of small chemical probes (Barillari *et al.*, 2008; Sanders *et al.*, 2012; Drwal and Griffith, 2013). These three-dimensional features are then used to filter out and identify small molecules that map well onto the pharmacophore, taking into account the shape of the binding pocket. This is why optimizing the binding pocket of orphan GPCR homology models is important. Currently, the use of structure-based pharmacophores to identify novel ligands for GPCRs has only been described for the β_2-adrenoceptor and GPR40 (now known as FFA1) (Tikhonova *et al.*, 2007; Barillari *et al.*, 2008). The application of structure-based pharmacophores to orphan GPCRs is probably limited in cases where the binding pocket has been modelled without additional refinement, although mapping ligands of lower stringency can be considered.

Intuitively, ligand-based pharmacophores must complement the structure-based pharmacophores. Thus, instead of using known or hypothesized binding site residues as features, a ligand

set is used to build the pharmacophore. Based upon the assumption that the ligands share a similar binding mode, compounds with a range of activity profiles for the target are compiled and common features likely to favour ligand binding inform pharmacophore composition. Ligand-based pharmacophores offer an alternative way to identify novel ligands at GPCRs with little structural information but where a range of known ligands have been identified, for example, at the α_{1A}-adrenoceptor (Ngo *et al.,* 2013). However, transferring this approach to orphan GPCRs is again hindered by a lack of ligands, although ligand-based pharmacophores were used to identify aromatic L-α-amino acids as the putative endogenous agonists for GPR139 (Isberg *et al.,* 2014). Here, a ligand scaffold was initially identified by HTS and then subjected to structure–activity relationship (SAR) studies, with a series of derivatives then used to build the ligand-based pharmacophore (Shi *et al.,* 2011; Isberg *et al.,* 2014). The authors noted that the central linker of the identified ligands resembled peptide backbones and screened a dipeptide database against the pharmacophore. This resulted in the identification of aromatic L-α-amino acids as the putative endogenous agonists for GPR139 (Isberg *et al.,* 2014), setting a precedent for inferring endogenous agonists of orphan GPCRs through identification of surrogate ligands (GPR139 is still considered an orphan GPCR according to the IUPHAR/BPS Concise Guide to PHARMACOLOGY; www.guidetopharmacology.org). It also further highlights the major complementary role that HTS can play in *in silico* identification of orphan GPCR ligands.

A case study of FFA1: from orphan to drug target

Drug discovery at the free fatty acid 1 receptor (FFA1; previously known as GPR40) is the prime example of an orphan receptor to drug target pipeline, combining both HTS and SBDD. FFA1 is a target for metabolic disorders, including type 2 diabetes, as stimulation of pancreatic beta cell expressing FFA1 receptors leads to an increase in glucose-stimulated insulin secretion (reviewed by Mancini and Poitout, 2013). The GPR40 gene was discovered as part of a family of four genes (FFA1-3 and GPR42, a presumed pseudogene) (Sawzdargo *et al.,* 1997; Stoddart *et al.,* 2008). The FFA1 receptor was deorphanized 6 years later via two independent screens of approximately 1000–1500 ligands, which demonstrated that long-chain free fatty acids (>C6) activated FFA1 receptors in [Ca^{2+}] flux assays (Briscoe *et al.,* 2003; Itoh *et al.,* 2003). Although the FFA1 receptor was thus deorphanized, fatty acids are poor agonists and are not amenable to SAR studies [highlighted by a failure of one study to improve upon the ligand efficiency of short-chain fatty acids at FFA2 and FFA3 receptors (Schmidt *et al.,* 2011)], thereby preventing hit-to-lead development. Further efforts were needed to identify small molecules activating FFA1 receptors; a novel small molecule was discovered via HTS and, following SAR, led to the highly potent FFA1 agonist GW9508 (Figure 4) (Briscoe *et al.,* 2006; Garrido *et al.,* 2006).

Tikhonova and colleagues have used a computational approach to ligand discovery at FFA1 receptors, as detailed in a series of elegant papers combining molecular modelling with site-directed mutagenesis (Sum *et al.,* 2007; Tikhonova *et al.,* 2007, 2008). Using bovine rhodopsin as the structural template, a FFA1 homology model was built, and Monte Carlo torsional sampling was applied to binding pocket residues. Both GW9508 and the endogenous agonist, linoleic acid, were docked into the model, and predicted interacting residues were validated experimentally by site-directed mutagenesis. By building upon this knowledge (Sum *et al.,* 2007; Tikhonova *et al.,* 2007), the authors were able to subject their validated homology model to VLS studies that combined both traditional docking and structure-based pharmacophore approaches, ultimately identifying six novel

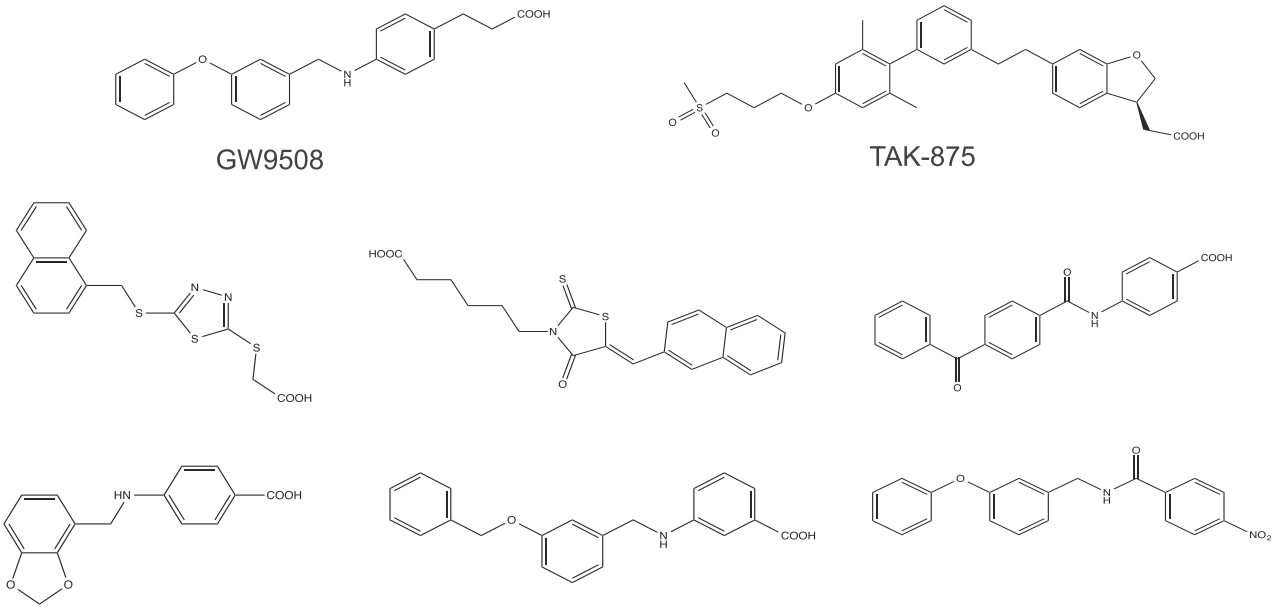
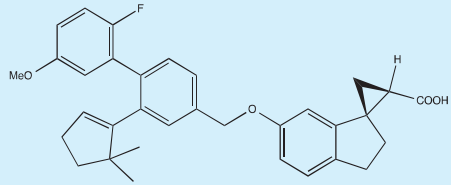
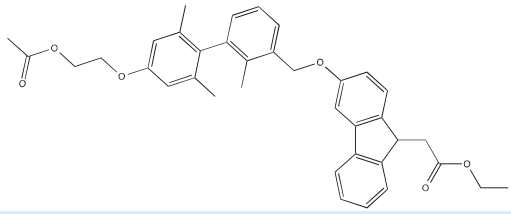
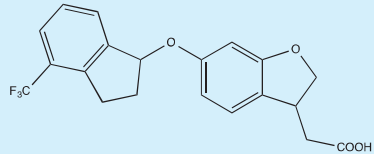
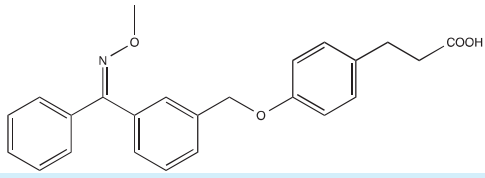
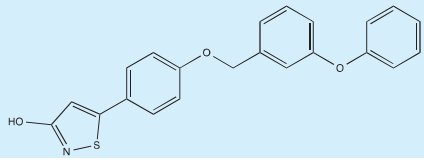
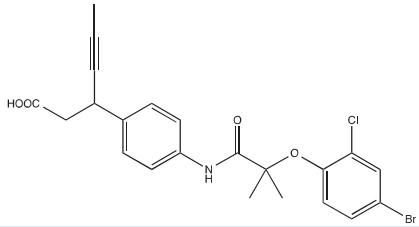
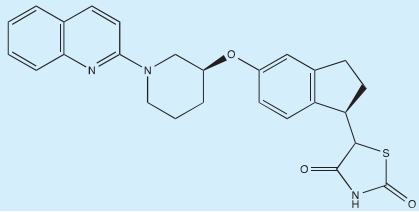
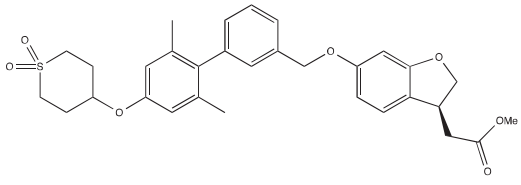


Figure 4
Structures of GW9508, TAK-975 and novel FFA1 agonists identified by virtual screening.

Table 4

Patented chemical scaffolds as FFA1 agonists

Pharmaceutical company	Patent application number	Patented scaffold (and derivatives thereof)
Amgen	US2011190330	
Astellas Pharma	US2012035196	
Boehringer Ingelheim	WO2012072691	
Connexios Life Sciences	WO2012011125	
Mochida Pharmaceutical	US2012157459	
Sanofi	WO2012004269	
Schering Corporation	US2011312995	
Takeda	US8088821	

Adapted from (Harrison, 2012).

chemical scaffolds for the FFA1 receptor (Figure 4) (6/52; 11.5% hit rate) (Tikhonova *et al.,* 2008). This provides further evidence of the strength of VLS, driving the discovery of multiple surrogate ligands from an initial HTS hit. Interestingly, comparison of hits with patented scaffolds of FFA1 agonists reveals that VLS was able to retrieve unique chemical motifs (Figure 4, Table 4), indicating both the utility of VLS and that opportunities remain for the development of second-generation FFA1 agonists (Tikhonova *et al.,* 2008).

Pharmaceutical companies have been actively chasing FFA1 as a therapeutic target, demonstrated by the number of patented compounds in Table 4. Although identified by traditional HTS (Sasaki *et al.,* 2011), Takeda's TAK-875 was the first FFA1 allosteric agonist to complete clinical development and significantly improved glycaemic control of type 2 diabetes in two independent phase-II and one phase-III trials (Burant *et al.,* 2012; Kaku *et al.,* 2013, 2015). The therapeutic development of TAK-875 appears to have been abandoned due to liver toxicity (Takeda, 2015); however, it proved useful in receptor crystallization (Srivastava *et al.,* 2014). The crystal structure provided visual validation of the FFA1 binding pocket, with the carboxylic acid moiety of TAK-875 interacting with R183 (5.39) and R258(7.35) as predicted with GW9508 and linoleic acid from homology modelling (Sum *et al.,* 2007; Tikhonova *et al.,* 2007; Srivastava *et al.,* 2014) [Ballesteros and Weinstein numbers in parentheses (Ballesteros and Weinstein, 1995)]. The FFA1 crystal structure also confirmed the allosteric nature of TAK-875 and suggested other potential binding pockets that can be targeted, which, in turn, may prove useful in future SBDD efforts aimed at identifying potent agonists with better safety profiles.

Concluding remarks

Orphan GPCRs represent an experimental black box – we rarely have information about their activation, signal transduction or (patho)physiological functions. However, we have reasons to believe that they will become easy to use once we start to fill in the gaps, because GPCRs have historically been good therapeutic targets. Clearly, the process of GPCR deorphanization and *in vivo* phenotyping would be greatly hastened by the identification of surrogate ligands, either through traditional HTS or via VLS. Currently, the use of SBDD for the identification of surrogate ligands for orphan GPCRs is somewhat limited and will probably still require some kind of small screening campaign for scaffold identification (Figure 1). Importantly, our experimental toolkit is constantly expanding, with a steady supply of new GPCR crystal structures and methods for homology model optimization, although approaches for assessing the performance of homology models is an area needing further development. Furthermore, it is still unclear just how 'accurate' a homology model needs to be to reliably predict hits for orphan GPCRs, although visual inspection of the positioning of key conserved residues such as that in transmembrane 3 (3.32) (Venkatakrishnan *et al.,* 2013), and generation of multiple homology models based upon multiple templates, will increase confidence in their use. Finally, it is important to consider ligand-independent roles of orphan GPCRs, including modulation of signalling through heteromerization (Smith and Milligan, 2010), for example, GPR50 and the MT_1 melatonin receptor (Levoye *et al.,* 2006), GPR88 and the D_1 dopamine receptor (Marley *et al.,* 2013) and even GPR3 complexes with β-arrestin and amyloid precursor protein (Thathiah *et al.,* 2009; Nelson and Sheng, 2013). Although it is likely that more than the abovementioned GPCRs signal through such non-canonical mechanisms (i.e. without an endogenous ligand), this does not preclude the development of surrogate ligands. Much remains to be discovered about orphan GPCR pharmacology; surrogate ligands from VLS can only augment the rate of translation of these interesting receptors to genuine drug targets.

Note added during revision

During the revision of our manuscript, a seminal paper was published by Bryan Roth and Brian Shoichet in which they used a very similar combined experimental and computational approach to that discussed herein to identify surrogate ligands for the proton receptors, GPR68 and GPR65 (Huang *et al.,* 2015).

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Conflict of interest

R. A. is a founder of Molsoft, LLC. Other authors declare no conflicts of interest.

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