Target validation of G-protein coupled receptors

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G-protein coupled receptors (GPCRs) represent possibly the most important target class of proteins for drug discovery. Over 30% of clinically marketed drugs are active at this receptor family. These drugs exhibit their activity at <10% of all known GPCRs. A major challenge for the pharmaceutical industry is to associate the many novel GPCRs with disease to identify the drugs of the future. This process consists of a collection of experimental paradigms that together can be loosely labelled 'target validation'.

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▼ G-protein coupled receptors (GPCRs) are widely expressed in the body and play a fundamental role in physiology and pathophysiology. As such, they are potential targets for therapeutic intervention in many, if not most, diseases. Of the ~500 currently marketed drugs, greater than 30% are modulators of GPCR function (Table 1) [1]. In 2000, 26 of the top 100 pharmaceutical products were compounds that target GPCRs accounting for sales of over US$23.5 billion. This represents ~9% of total global pharmaceutical sales [2], thus making the GPCR superfamily the most successful of any target class in terms of therapeutic benefit and commercial sales (Table 2).

GPCR agonist and antagonist drugs have therapeutic benefit across a broad spectrum of diseases, including pain (opioid receptor agonists such as morphine), asthma (β_2-adrenoceptor agonists such as salmeterol), peptic ulcers (histamine receptor antagonists such as ranitidine) and hypertension (angiotensin receptor antagonists such as losartan). To develop these compounds, efficient methods have been established to enable the cloning and expression of their cognate receptors. In parallel, rapid and efficient screening methods have been developed to enable the screening of compound libraries of many hundreds of thousands, or indeed millions, of chemical entities to readily identify small molecule 'drug-like' ligands that either activate or block receptor action. This infrastructure is now being used within the pharmaceutical industry to attempt to further exploit members of this target class to identify the blockbuster drugs of the future.

Following the publication of a rough draft of the human genome sequence [3,4] and the rapid progression of bioinformatics to help identify and classify novel genes, it has been estimated that of the 35,000 or so human genes, approximately 750 are GPCRs. Almost half of these sequences are likely to encode sensory receptors, leaving around 400 receptors that could be considered to be potential drug targets, of which ~30 are the targets of currently marketed drugs. The natural ligand has been identified for a further 210 receptors, which leaves around 160 so-called 'orphan' receptors with no known ligand or function (Fig. 1). Identifying the physiological role of such receptors, which in turn may provide some insight into their role in the pathophysiology of disease, is a daunting task. However, based on the track record of success for this target family, it is believed that therapeutic intervention at these novel receptors will have major benefit in a wide range of human diseases. The challenge for the pharmaceutical industry is to match the receptor with the disease using a collection of techniques that have been loosely labelled 'target validation'.

In silico target validation

GPCRs can be classified into three major families according to sequence homology. Family A represents the largest subgroup of receptors and includes catecholamine, neuropeptide, chemokine, glycoprotein, lipid and nucleotide receptors. Family B contains receptors for a large number of peptides such as calcitonin-gene-related peptide (CGRP) and calcitonin,

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Table 1. Molecular targets of known drugs [1]

Target	Number of drugs (n = 483)
GPCRs	217
Enzymes	135
Hormones and factors	53
Unknown	34
Ion channels	24
Nuclear receptors	10
DNA	10

and family C is the metabotropic family containing the metabotropic glutamate receptors (mGluRs), γ-amino butyric acid ($GABA_B$) receptors and the calcium-sensing receptor.

For many orphan receptors there is currently little information available beyond the gene sequence. As the first step in target validation, the DNA sequence of the orphan receptor is compared with the sequences of liganded receptors, and where they are closely related it is sometimes possible to predict the likely cognate ligand of the orphan receptor. Put simply, such comparisons give an indication as to the nature of the likely ligand, be it a nucleotide, biogenic amine or peptide. However, the generation of structures and accurate models of receptor binding sites is still in its infancy, and for the most part we are not yet able to accurately predict which ligand is likely to bind a novel receptor simply from an analysis of the sequence of that receptor. In fact, sequence homology can be misleading; for example, a receptor originally known as $P2Y_7$ (BLT_1) was thought to be a nucleotide receptor based on its similarity to P2Y receptors (Fig. 2), but was shown to be activated by an unrelated ligand, leukotriene B4 [5]. However, there have been successes using this approach. For example, the initial demonstration that OGR-1 could act as a high affinity receptor for the lipid sphingosylphosphorylcholine [6] probably facilitated the more recent identification of two related orphan GPCRs, TDAG-8 and G2A, as receptors for the lipids psychosine [7] and lysophosphatidylcholine (LPC) [8], respectively. The ligands for the fourth member of this receptor subfamily, GPR 4, have recently been identified. As would be predicted from sequence homology, the ligands for GPR4 are LPC and sphingosylphosphorylcholine [9].

Table 2. Best-selling therapeutics targeted at GPCRs in 2000 [2]

Product	Receptor(s)	Indication	Company
Zyprexa®	Serotonin 5-HT_2 and dopamine	Schizophrenia or antipsychotic	Eli Lilly, Indianapolis, IN, USA
Claritin®	Histamine H_1	Rhinitis or allergy	Schering-Plough, Kenilworth, NJ, USA
Risperdal™	Serotonin 5-HT_2	Schizophrenia	Johnson & Johnson, Titusville, NJ, USA
Imigran™	Serotonin 5-$HT_{1B/1D}$	Migraine	GlaxoSmithKline, Harlow, UK
Cozaar®	Angiotensin AT_2	Hypertension	Merck and Co., Whitehouse Station, NJ, USA
Serevent®	β2-adrenoceptor	Asthma	GlaxoSmithKline
Singulair®	BLT_1	Asthma	Merck and Co.
Gastridin™	Histamine H_2	Peptic ulcer	Merck and Co.
Zantac/Tagamet™	Histamine H_2	Peptic ulcer	GlaxoSmithKline
Zirtec™	Histamine H_1	Rhinitis or allergy	Pfizer, Sandwich, UK
BuSpar®	Serotonin 5-HT_{1a}	Anti-depressant	BMS, New York, NY, USA
Gaster®	Histamine H_2	Peptic ulcer	Yamanouchi, Tokyo, Japan

Abbreviation: BMS, Bristol-Myers Squibb.

Biological target validation

Target validation occurs throughout the process of drug discovery and there are many levels of experiments that can add weight to a target's ability to modify disease. A truly validated drug target is one that improves disease outcome when it is modulated in humans. However, this level of validation is expensive and time consuming and confidence can generally be gained in a target well before clinical experiments are performed. Evidence of a target's involvement in disease is gathered from a variety of sources, including an analysis of the pattern of expression of the target in normal and diseased tissue, *in vitro* and *in vivo* gene modulation using molecular biology techniques, studies using small molecules and other modulators *in vitro* and *in vivo*, and from genetic studies such as chromosomal location, disease association and transgenic models.

Inevitably, the majority of biological target validation experiments are performed in laboratory species. Hence,

one has to remain aware that differences can exist in terms of expression profile, receptor pharmacology and function between humans and the species being studied, and between different laboratory species. The expression pattern of a receptor is often different between species and in some cases might be absent altogether. For example, compared with humans, rodents possess an additional serotonin receptor, the 5-HT_{5B} receptor. For this reason expression analysis is often confirmed using human tissue samples. To ensure that the pharmacology of the orphan receptor is similar in humans and the species being studied, it is always necessary to clone and characterize both the human receptor and species orthologues. Should the pharmacology or expression pattern differ, for example between humans and rats, it might be necessary to perform target validation experiments in a species, such as the hamster, in which the differences might be less marked. Similarly, there is the possibility that the physiological role of the receptor might be different in the laboratory species and in humans. For this reason it is necessary, if possible, to study receptor function in both animal species and in primary or immortalized human cell lines. Such target validation data has to be interpreted with caution and, if possible, human data should be obtained to confirm the conclusions drawn from studies in other species.

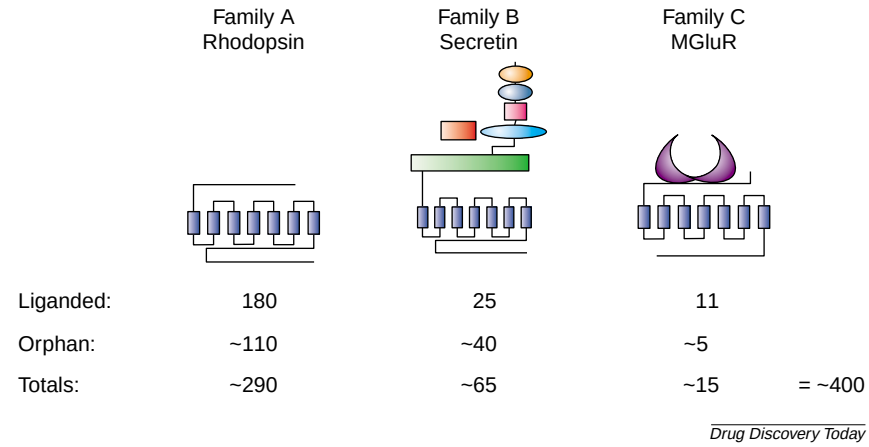

Figure 1. Schematic representation of the number of liganded and orphan G-protein coupled receptors (GPCRs) in the human genome. These figures have been estimated following the completion of the human genome sequence. The olfactory receptors are not included in this figure.

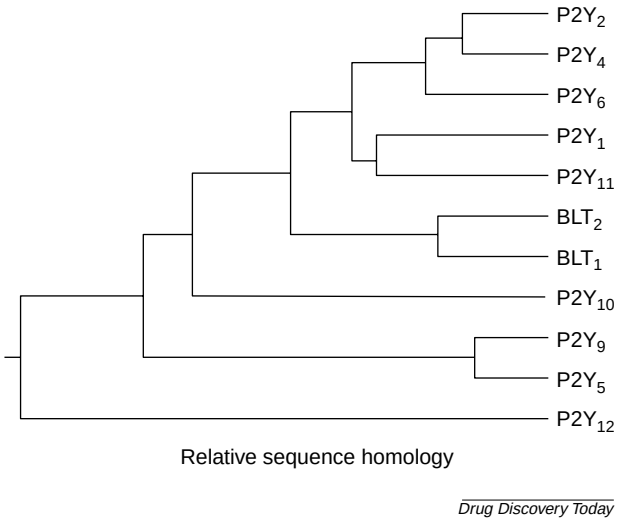

Figure 2. Dendrogram showing the nucleotide sequence homology of members of the family of purinergic–leukotriene receptors. Such dendrograms of related receptors are often used to predict the likely ligand of an orphan G-protein coupled receptor (GPCR). Currently, six human genes $P2Y_1$ (U42030), $P2Y_2$ (U07225), $P2Y_4$ (X91852), $P2Y_6$ (AF007892), $P2Y_{11}$ (AR084814) and $P2Y_{12}$ (AF321815) have been shown to be functionally active nucleotide receptors. BLT_1 (U33448) and BLT_2 (AB008193) are leukotriene B_4 receptors. $P2Y_5$ (AF000546), $P2Y_9$ (U90322) and $P2Y_{10}$ (AF000545) are orphan receptors but are suspected to have nucleotide or leukotriene ligands. Numbers in brackets in the figure legend are the Genbank accession number for each gene sequence.

Expression profiling

Expression profiling of GPCRs is one of the earliest steps in target validation. The goal of such studies is to identify the tissues and cell types in which the receptor is expressed and to determine if the pattern of expression is altered in disease. Put simply, if a receptor is only expressed in the brain it might be a target in CNS disorders but is unlikely to be a target in respiratory disease. The analysis of the pattern of receptor mRNA expression can be performed by northern blotting or quantitative polymerase chain reaction (qPCR) analysis [10]. The qPCR assay enables the detection and real-time quantitation of the level of receptor mRNA in an experimental sample. In this assay, short oligonucleotide probes complementary to the sequence of the receptor are used in a PCR reaction to selectively amplify a short region of the receptor mRNA. The amount of PCR product is quantified using a third fluorescently labelled oligonucleotide probe. In such experiments the site and level of expression of receptor RNA is determined in panels of normal and disease tissue. This type of analysis is simple and quick to perform, particularly in the case of

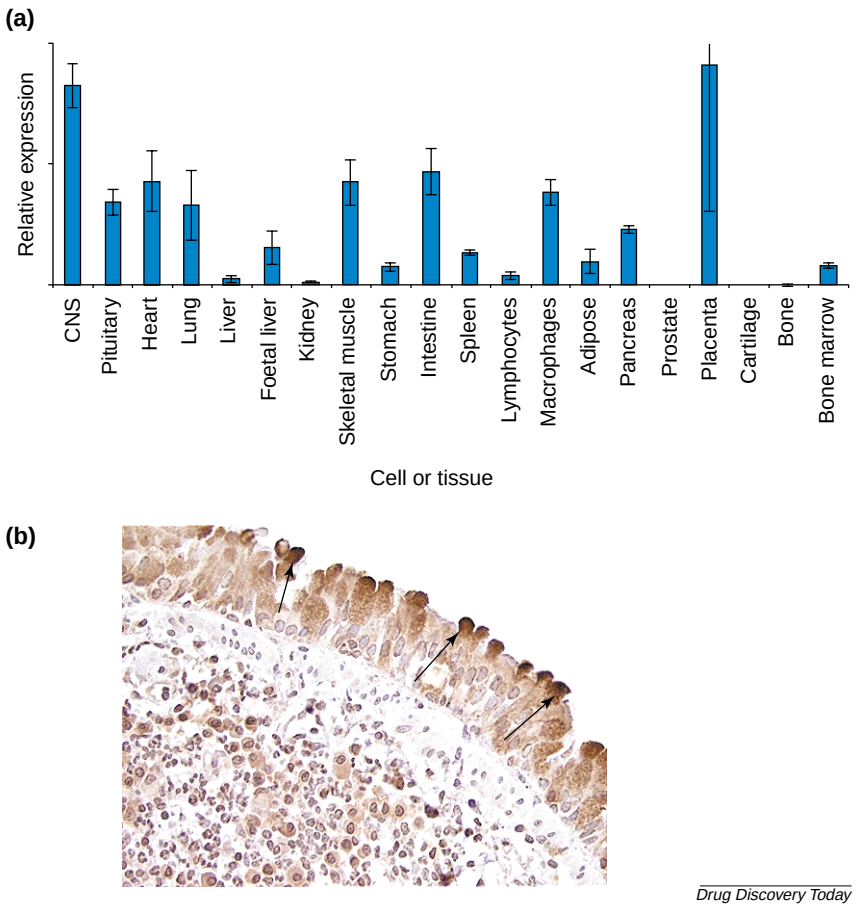

Figure 3. Determination of the site of expression of the purinergic $P2Y_1$ receptor using **(a)** quantitative Polymerase Chain Reaction (qPCR) analysis to examine the relative level of receptor mRNA (normalized to expression of a control gene) in a wide range of human tissues, indicating that the receptor is expressed in several tissues, including human lung (reproduced, with permission, from [83]), and **(b)** immunohistochemistry to determine the cellular localization of receptor protein expression in a single tissue, the mouse lung. In this section, strong immunostaining, indicative of receptor expression, is observed in Clara cells in the airway epithelium (indicated by the arrows). In such studies mRNA analysis is usually performed to identify, at a relatively crude level, the site of expression of the receptor. This is followed by immunohistochemistry to examine the precise site of receptor expression.

qPCR, in which many tissue samples can be analyzed simultaneously, but which rarely reveals highly restricted patterns of expression.

Because this analysis is most easily performed using gross tissue samples, small numbers of specialized cells that might have high levels of expression can easily be missed. To obtain a greater level of detail regarding the site of receptor expression, *in situ* hybridization experiments or immunohistochemistry must be carried out. *In situ* hybridization can be performed at an early stage because only the receptor gene sequence needs to be known for preparing synthetic oligonucleotide hybridization probes. Immunohistochemistry is a more powerful technique because it confirms expression of the protein, but for this antibodies to the target GPCR need to be generated and good quality tissue samples must be available. Obtaining antibodies directed against the extracellular domains of GPCRs has proved notoriously difficult because of the relatively short sequence and constrained nature of the extracellular loops and, for many receptors, the short nature of the N-terminal domain. However, antibodies have been successfully raised to receptors, such as members of the chemokine receptor family, which possess a relatively large (>30 aa), N-terminal domain. These antibodies have been used in target validation studies to implicate such receptors in disease (see later).

An example of the level of detail obtained using different expression profiling techniques for the $P2Y_1$ receptor is shown in Fig. 3. In this example, qPCR analysis on the level of expression of the $P2Y_1$ receptor indicated that the mRNA for this receptor was found in a wide range of tissues (Fig. 3). A more detailed analysis of the site of expression of the receptor in human lung tissue using immunohistochemistry with a $P2Y_1$-specific antibody showed strong immunostaining in Clara cells in the airway epithelium (Fig. 3), and also in mast cells and eosinophils [11]. Such a pattern of expression has led to speculation that this receptor has a role in airway disorders such as asthma or chronic obstructive pulmonary disease (COPD).

Reverse pharmacology

One direct means of revealing the physiological function of an orphan GPCR is to identify either its endogenous 'natural ligand' or a synthetic, surrogate ligand. This ligand–receptor pairing can then be used to investigate the G-protein coupling and effector-regulating specificities of the receptor in question, and mutagenesis studies can be employed to identify key amino acid residues within the receptor that are involved in ligand binding. Such functional information and tissue distribution data, coupled

with knowledge of the physiological role of the endogenous ligand, can provide invaluable information regarding the potential role of a novel GPCR in disease.

Historically, endogenous ligands were isolated and pharmacologically characterized by following their modulating effects at defined tissues in *ex vivo* whole-tissue experiments. The identification of the cognate receptor for such ligands was performed by complex and resource-intensive protein purification and expression cloning techniques. The advent of high-throughput DNA sequencing technologies and the wealth of genomic information this generated led to a shift from ligand-based to sequence-based receptor discovery. The sheer number of novel GPCR sequences being identified meant that there were insufficient pharmacological tools to assign their pharmacology and function. This has led to the generation of a novel strategy for characterizing orphan GPCRs, which is often referred to as 'reverse pharmacology' [12].

Put simply, reverse pharmacology uses an orphan GPCR as 'bait' to identify its ligand or 'prey' (Fig. 4). In this strategy, the orphan receptor is transfected into cells, which are then exposed to a variety of naturally occurring molecules that might mediate their biological effects via binding to and activation of the transfected receptor. Activation of the orphan receptor can then be measured by changes in second messenger responses. There are several key criteria that should be considered when adopting this approach to improve the likelihood of identifying novel ligand–receptor pairings. First, a suitable expression system that provides the necessary trafficking, G-protein and downstream signalling machinery to enable successful functional GPCR expression is crucial. Such systems include the widely used mammalian cell lines Chinese Hamster Ovary (CHO) and Human Embryonic Kidney (HEK) 293, *Xenopus* oocytes, melanophores [13] and the yeast *Saccharomyces cerevisiae* [14,15]. Second, receptor expression should be confirmed in order to improve confidence in this strategy. In some systems successful functional expression can be monitored because of the ability of the transfected GPCR to exhibit G-protein activation and signal transduction in the absence of a ligand ('constitutive activity'). mRNA analysis using northern blotting or qPCR can also be used to monitor expression in a recombinant system, although this does not provide information as to the level of protein expressed or its localization in the cell. Antibodies can also be generated and used to confirm receptor expression, but a simpler alternative is to add a short epitope tag such as haemagglutinin (HA) or Myc to the N-terminus of the receptor and then confirm cell surface expression by fluorescence-activated cell sorting (FACS) analysis using a commercially available antibody to the epitope tag.

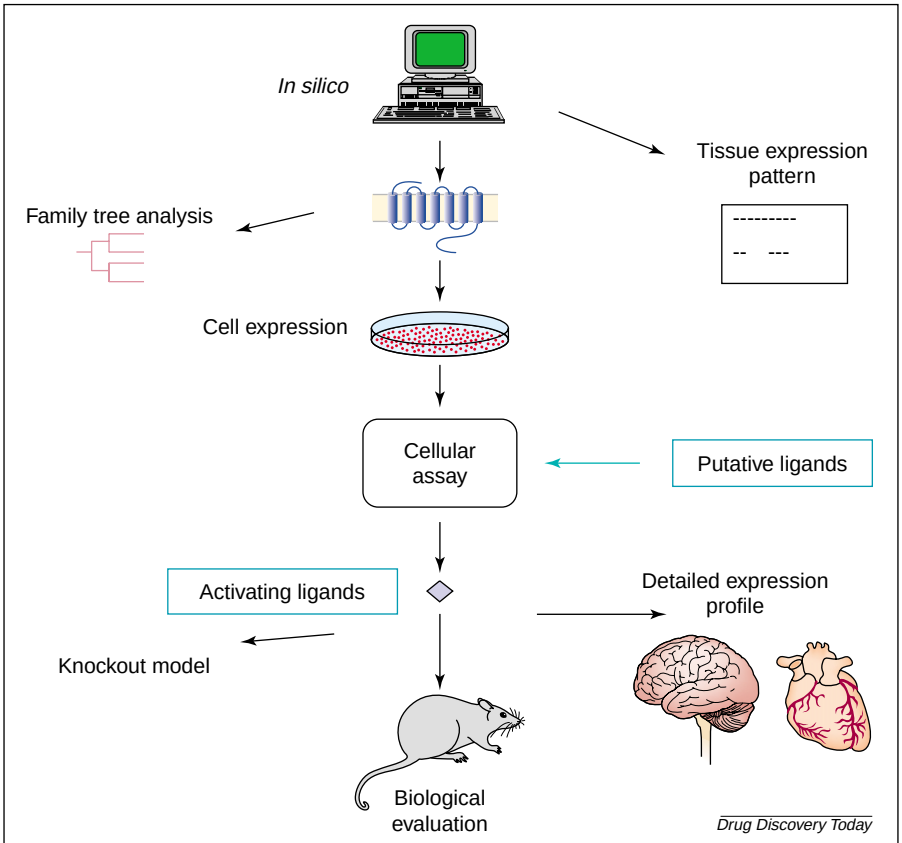

Figure 4. Schematic representation of the reverse pharmacology approach to characterizing orphan G-protein coupled receptors (GPCRs) (modified, with permission, from [84]). Following the identification of a novel receptor, the sequence of this receptor is compared to known receptors using bioinformatics tools to provide the first indication of the likely ligand for the receptor. At this time, analysis is also performed to identify the site of receptor expression according to the localization of mRNA for the receptor. If the receptor is of interest, it is then cloned and expressed to derive a suitable assay system for the identification of putative ligands. In the event of the identification of a ligand this can be used to probe receptor function in disease models. At this time a more detailed analysis of the site and time of expression of the receptor would be performed.

Commonly used methods for the identification of ligands at orphan GPCRs

The choice of assay is of paramount importance for the success of a ligand fishing strategy. For an orphan receptor

the G-protein signalling pathway is often unknown. Thus to maximize the chance of success the assay system must be as generic as possible to allow for the detection of a wide range of signalling mechanisms, but also be amenable to HTS such that the activity of a large number of ligands can be readily measured. Such assay systems in mammalian cells rely mainly on measuring changes in intracellular cAMP or calcium levels, either directly or via the use of reporter gene assays. Perhaps the most successful screen protocol used to identify ligands at orphan GPCRs has been through the detection of ligand-mediated increases in intracellular calcium concentration using the Fluorescent Imaging Plate Reader (FLIPR™; Molecular Devices, Sunnyvale, CA, USA) for detection. This instrument allows the simultaneous detection of changes in intracellular calcium concentration in every well of a 96- or 384-well assay plate. In such assays the orphan GPCR is usually transiently transfected into mammalian cells in the presence of one or more of a cocktail of promiscuous G-proteins such as $G\alpha_{16/15}$ [16], or with chimaeric G-proteins such as $G\alpha_{qi5}$ or $G\alpha_{qs5}$ [17] in which the N-terminal five amino acids of $G\alpha_q$ have been replaced with the corresponding amino acids of $G\alpha_i$ or $G\alpha_s$ to facilitate receptor coupling to calcium mobilization. This approach has been used with considerable success for ligand pairing (Table 3), although it does rely upon the orphan receptor being able to couple to one of the G-proteins provided in the experiment. By focusing on a single assay read-out the speed at which the activity of a wide range of ligands can be profiled is increased.

An interesting alternative ectopic system in which to express orphan GPCRs are melanophore cells derived from the neural crest of *Xenopus laevis* [13]. These cells contain the dark brown pigment melanin within intracellular organelles called melanosomes. These melanin-containing organelles can be dispersed evenly throughout the cell by activation of adenylate cyclase or phospholipase C, or aggregated near the nucleus following inhibition of adenylate cyclase [18,19]. Pigment aggregation leads to an overall lightening of the colour of the cells, whereas dispersion leads to cell darkening. Pigment translocation in melanophores can easily be detected colorimetrically within a few minutes following the activation of effector molecules, thereby providing a fast, sensitive and versatile reporter technology for measuring activation of GPCRs coupled to multiple G-protein families. One particular feature of this assay that makes it highly attractive for orphan GPCR screening is that receptors expressed in melanophores are usually efficiently coupled to *Xenopus* G-proteins to regulate pigment dispersion. Following transient expression of the receptor, constitutive activity can usually be observed, thus providing an indication of successful expression and the mechanism of receptor coupling before the start of the ligand screening experiment [20]. Although highly successful in terms of receptor coupling, melanophore technology requires the availability of expertise in the culture and transfection of melanophores, and is established in relatively few laboratories. This technology may have application for the study of orphan GPCRs for which functional expression in mammalian cells is difficult to achieve.

The presence of endogenous receptors, particularly in mammalian heterologous expression systems, which results in 'background' responses to ligands, has also to be considered when choosing an expression system for a ligand fishing experiment. Engineered yeast strains that have had the endogenous GPCR genetically deleted and which then provide a 'null' background for ligand fishing experiments are ideally suited to addressing this problem [14,15]. Several groups have successfully expressed mammalian GPCRs in yeast. The assay has been configured such that receptor signalling leads to an increase in the level of expression of a β-galactosidase reporter gene. To facilitate receptor coupling a variety of synthetic G-proteins have been developed and stably expressed in yeast. Reporter expression can be readily determined in the assay plate using one of a variety of commercially available colourimetric, fluorescent or chemiluminescent assay reagents. This assay is a simple and cheap alternative to mammalian expression, requiring the availability of little specialist equipment. However, although successful expression can be achieved for many GPCRs, there remains a significant proportion of GPCRs for which functional expression in yeast has not been demonstrated.

Successful identification of ligands at orphan GPCRs

Once receptor expression has been achieved in a suitable system, screening for ligands can commence. The ability to marry orphan receptors with their endogenous ligand partner arguably provides the most powerful means of validating the role of a receptor in the pathophysiology of disease. The number of ligand–orphan GPCR pairings have increased exponentially over the past two years. Indeed, there are often multiple publications describing such pairings [such as Histamine H4 and melanin-concentrating hormone (MCH) receptors] – a true testament to the importance that both the pharmaceutical industry and academia place on this approach to associate novel GPCRs with a biological and physiological role.

Receptors are usually screened against known GPCR ligands together with bioactive molecules of unknown mechanism of action. This approach has recently led to the pairings of peptidergic (motilin, MCH, urotensin II, neuropeptide FF and AF, neuromedin U) [10,21–28], lipid

Table 3. Ligand–orphan GPCR pairings identified using reverse pharmacology strategy (modified from Ref. [74]).

Orphan receptor	Assay used	Ligand	Ligand source	Ligand properties	Year found	Refs
ORL-1	cAMP	Nociceptin/Orphanin FQ	Brain extract	Novel	1995	[43]
Orexin-1 and 2	$[Ca^{2+}]_i$	Orexin-A and B	Brain extract	Novel	1998	[44,45]
GPR10	Arachidonic acid	Prolactin-releasing peptide	Brain extract	Novel	1998	[46]
APJ	Ext. pH	Apelin	Stomach extract	Novel	1998	[47]
GHS-R	$[Ca^{2+}]_i$	Ghrelin	Stomach extract	Novel	1999	[48]
GPR14	$[Ca^{2+}]_i$	Urotensin II	Brain extract, synthetic ligand	Known	1999	[24,27]
KIAA0001	Yeast	UDP–glucose	Synthetic ligand	Novel	2000	[41]
AXOR12 (GPR54)	$[Ca^{2+}]_i$	KiSS-1	Synthetic ligand, placental extract	Novel	2001	[49]
G2A	$[Ca^{2+}]_i$	Lysophosphatidylcholine	Synthetic ligand	Known	2001	[8]
CIRL	Radioligand binding	Latrotoxin	Synthetic ligand	Surrogate	1997	[75]
BRS-3	Oocytes	Bombesin	Synthetic ligand	Surrogate	1993	[76]
CRLR	Oocytes	CGRP	Synthetic ligand	Known	1998	[77]
AZ3B	$[Ca^{2+}]_i$, Oocytes	C3a	Synthetic ligand	Known	1996	[78]
EDG1,3,5,6 and 8	$[Ca^{2+}]_i$, cAMP	S1P	Synthetic ligand	Known	1998–2000	[29,32,33, 35]
EDG2, 4 and 7	$[Ca^{2+}]_i$, cAMP	LPA	Synthetic ligand	Known	1998–2000	[30,31,34]
HG57 (Cys-LT1R)	$[Ca^{2+}]_i$	LTD4	Synthetic ligand	Known	1999	[79]
GPR38	$[Ca^{2+}]_i$	Motilin	Synthetic ligand	Known	1999	[21]
SLC-1 (MCH1)	$[Ca^{2+}]_i$	Melanin-concentrating hormone	Synthetic ligand, brain extract	Known	1999	[22]
OGR-1	$[Ca^{2+}]_i$	Sphingosyl-phosphorylcholine	Synthetic ligand	Known	2000	[6]
PSECO146 (CysLT2R)	$[Ca^{2+}]_i$, Oocytes	LTC4 and D4	Synthetic ligand	Known	2000	[36,37]
GPR16 (BLT2)	cAMP, radioligand binding	LTB4	Synthetic ligand	Known	2000	[38]
FM-3/4	$[Ca^{2+}]_i$	Neuromedin U	Synthetic ligand	Known	2000	[25,26,28]
GPRv53 (H4-R)	cAMP, radioligand binding	Histamine	Synthetic ligand	Known	2000	[39]
HLWAR77	$[Ca^{2+}]_i$	Neuropeptides FF and AF	Synthetic ligand	Known	2000	[10,80]
P2Y12	Oocytes	ADP	Synthetic ligand	Known	2001	[81]
CRTH2	$[Ca^{2+}]_i$	Prostaglandin D2	Synthetic ligand	Known	2000	[82]
MCH2	$[Ca^{2+}]_i$	Melanin-concentrating hormone	Synthetic ligand	Known	2001	[23,42]
TDAG-8	cAMP	Psychosine	Synthetic ligand	Known	2001	[7]
TA_1, TA_2	Oocytes	Trace amines (tyramine)	Synthetic ligand	Known	2001	[40]

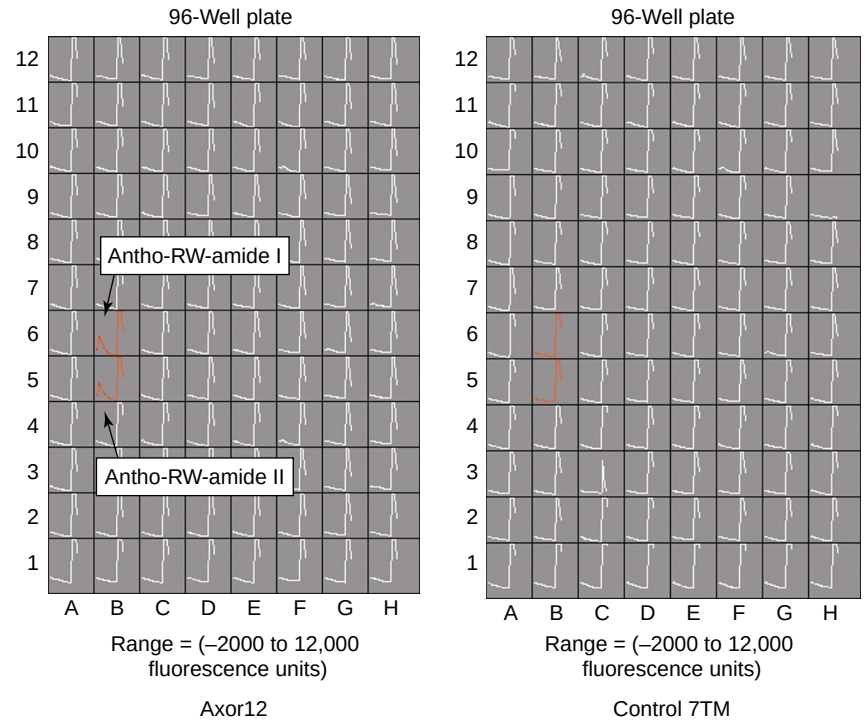

Figure 5. The identification of surrogate ligands for the orphan G-protein coupled receptor (GPCR) AXOR12. Human Embryonic Kidney (HEK) 293 cells, transiently transfected with the receptor together with a cocktail of chimeric G-proteins to facilitate receptor coupling to phospholipase C, were plated into a 96-well microtitre plate. Using the FLIPR™ instrument (Molecular Devices, Sunnyvale, CA, USA) changes in the level of intracellular calcium can be simultaneously detected in cells plated within every well of a 96-well microtitre plate. Following the addition of a different ligand to each well of the plate, AXOR12 is shown to mediate an increase in the level of intracellular calcium in response to antho-RW amides I and II, two surrogate agonist peptides isolated from sea anemone with a common C-terminal amidated motif. This response is not seen with a control GPCR. The large response seen in each assay well is to a control ligand known to produce an increase in intracellular calcium in HEK 293 cells and is used as an internal control in the experiment. AXOR 12 has now been shown to function as a receptor for KiSS-1 (metastin), a related carboxy-terminally amidated 54 amino acid peptide isolated from human placenta [49,50].

(sphingosine-1-phosphate, sphingosylphosphorylcholine, psychosine, lysophosphatidylcholine, lysophosphatidic acid) [6–8,29–35], leukotriene [5,36–38] and biogenic aminergic (histamine H4 and trace amine receptors) [39,40] ligands to different orphan GPCRs (Table 3). This approach has also led to the identification of a novel ligand, UDP–glucose, as a putative bioactive agent acting via a GPCR [41]. A typical ligand fishing experiment in mammalian cells is depicted in Fig. 5.

Biological extracts obtained from tissues, biological fluids and supernatants can also be used in ligand fishing experiments, but purification of the 'active entity' from such extracts can require a significant investment of time. This approach has been successfully employed to identify orphan GPCRs for several bioactive peptides such as urotensin II [27], neuromedin U [28] and MCH [42], and has also led to the identification of several novel peptides, such as nociceptin [43], orexins [44,45], prolactin-releasing peptide [46], apelin [47], ghrelin [48] and KiSS-1 (metastin) [49,50], and their cognate receptors (Table 3).

In addition, ligand–GPCR pairings have been made by matching the tissue distribution pattern of an orphan receptor with the site of action or expression pattern of a known ligand. For example, the expression pattern of an orphan GPCR, which was identified as a receptor for neuropeptide Y (NPY1R), perfectly matched that of neuropeptide Y [51,52].

Compound or peptide libraries are screened to identify 'surrogate agonists' that may then be used as tool compounds. The ability of many GPCRs to display agonist independent or 'constitutive activity' following expression in melanophores [20] has provided a system tailored to the identification of both agonists and inverse agonists in the same experiment. The identification of either natural or surrogate ligands can provide tools with which to investigate the biology of a receptor either *in vivo* or in tissues. Such tools can be employed to develop high-throughput screens to identify other potent novel small-molecule modulators that might have some therapeutic application.

Characterization of liganded GPCRs

Several approaches have been adopted to derive evidence that a particular receptor–ligand combination is involved in disease pathology. In the following paragraphs we discuss the application of five complementary approaches that have been used to validate GPCRs as drug targets in animal models of disease: (1) studies of the effects of the natural ligand; (2) studies of the effects of synthetic antagonists; (3) the use of antagonist antibodies; (4) the application of antisense DNA technology and overexpression strategies; and (5) transgenic animal studies. Once again, it is important to note that it is the combination of the application of these technologies that provides the various 'pieces of the jigsaw' to produce a package of data that implicates a receptor in disease.

The identification of a novel endogenous ligand–GPCR pairing undoubtedly provides a significant platform of information from which to further explore the physiological function of a particular receptor and to validate its role in disease. This is particularly so if there is existing literature on the physiological role of the ligand, which can be combined with knowledge of the expression pattern of the receptor. A good example of this was the discovery of the orexins (or hypocretins) and their associated GPCRs [44,45]. De Lecea and coworkers [45] identified two orexin peptides in the lateral hypothalamus, a region known as a major regulatory centre for autonomic and endocrine homeostasis. At the same time, Sakurai *et al.* [44] used a reverse pharmacology approach to identify the same two neuropeptides as ligands for two closely related orphan receptors. Taken together, these findings rapidly facilitated the identification of the role that these peptides played in feeding and energy metabolism in rodents [53,54], as well as in the regulation of sleep [55]. Furthermore, it was recently reported that the gene encoding the orexin precursor (prepro-orexin) was downregulated in obese mice [56]. Intracerebroventricular injection of orexin A stimulates food intake in rats. This effect is blocked by the selective orexin-1 receptor antagonist SB334867 [57], suggesting that antagonists at this receptor may have potential as treatments in obesity.

The somatostatin-like peptide urotensin II, originally isolated from fish spinal cord, has been demonstrated to be the most potent mammalian vasoconstrictor identified, to date. The finding that this peptide was a natural ligand for the orphan receptor GPR14 immediately indicated that antagonists at this receptor might have therapeutic benefit for a variety of cardiovascular disorders [24].

In further studies, MCH has been shown to be a natural ligand for the orphan GPCR, SLC-1 [22]. MCH has long been demonstrated to regulate a variety of functions in the mammalian brain, including feeding behaviour. Direct injection of MCH into rat brain has been shown to stimulate feeding, and transgenic mice lacking MCH eat less and are lean. Expression analysis of SLC-1 has strongly implicated this receptor as the MCH receptor involved in the regulation of food consumption, leading to the hypothesis that antagonists to this receptor might have therapeutic benefit in obesity.

In another example, the biological mechanism of action of neuromedin U (NMU) has remained unknown since the first isolation of this ligand in 1985. In recent years, NMU has been identified as the natural ligand of two receptors, FM-3 and FM-4 [25,26,28]. NMU has potent activity on smooth muscle, is widely distributed in the gut and CNS, and has been implicated in the control of feeding. Intracerebroventricular injection of NMU significantly decreases food intake in rats, leading to the hypothesis that agonists at the FM-3 or FM-4 receptors (now known as tNMUR1 and NMUR2) might have therapeutic benefit in obesity.

Following the pairing of an orphan receptor with a natural ligand it is possible to establish screening systems to identify receptor antagonists. Such activities can involve the screening of many hundreds of thousands of chemical entities using HTS to identify novel compounds with activity and selectivity at the receptor being studied that can then be used in target validation studies. Using this approach, the specific orexin-1 receptor antagonist, SB334867, was identified [57]. This ligand has been demonstrated to block the analgesic effects of orexin A in rat and mouse models of hypoalgesia and nociception and, as already mentioned, will block feeding behaviour in rats. This demonstrates that in addition to a role in the control of feeding, the orexin-1 receptor has a potential role in the modulation of nociceptive transmission and agonists at this receptor might have efficacy in pain therapy [57].

The finding that antibodies to cell-surface receptors often possess antagonist activity, presumably as a consequence of steric hindrance of receptor–ligand interaction, has been used to investigate the role of many cytokine, growth factor and integrin receptors in both normal physiology and disease. The use of antibodies to study the role of GPCRs in disease has been limited to a relatively small subset of receptors because of the technical difficulties associated with the generation of antibodies to GPCRs. However, if available, blocking antibodies are powerful target validation tools. There have been some successful studies, most notably for receptor families such as the chemokine receptors. Antibodies to the N-terminus of the CCR2 receptor block ligand activation of the receptor in recombinant cell lines and prevent monocyte chemotaxis, thus implicating this receptor in monocyte recruitment during inflammation [58]. Similarly, antibodies to the CCR3 chemokine receptor have been demonstrated to block ligand-induced eosinophil chemotaxis, again suggesting that antagonists to this receptor might possess efficacy in inflammatory disorders such as asthma [59]. Antibodies to the human CCR9 chemokine receptor block agonist-mediated migration of blood T-lymphocytes and thymocytes, implicating this receptor both in the regulation of T-cell development and the mucosal immune response [60]. Blocking antibodies have also been used to demonstrate that a variety of GPCRs, including the CCR5 [61] and CXCR4 [62] receptors, act as co-receptors for HIV entry, indicating that antagonists at these receptors might possess antiviral activity.

Antisense technology relies upon the synthesis of short DNA oligonucleotides complementary to the nucleotide sequence of the receptor of interest. Following delivery to a cell system or whole animal, antisense oligonucleotides hybridize to the messenger RNA of the receptor being studied and cause a selective degradation of the target RNA by several processes, including degradation by RNase H [63], thus resulting in a decrease in the level of receptor expression. Antisense RNA molecules are simple and quick to synthesize and can be used to rapidly generate disease association data for a target receptor, particularly in *in vitro* and *in vivo* experiments. However, there are relatively few examples of the successful use of antisense technology for the target validation of GPCRs or indeed other targets. The reasons for this remain unclear, but include concerns over the specificity and stability of the antisense molecule, issues with delivery (particularly *in vivo*), and the observation that it is difficult to completely eliminate mRNA for the receptor being studied; inhibition of RNA levels by 90% could have no effect on the level of receptor protein.

The application of antisense technology to the functional analysis of GPCRs has been extensively reviewed elsewhere [64]. One early use of this methodology to generate disease association of a recently de-orphanized GPCR was performed by Meunier and coworkers [43]. Intracerebroventricular injection into mice of a 27-nucleotide antisense oligonucleotide to the ORL-1 receptor implicated this receptor as a possible drug target for pain. Following injection of the antisense molecule, mice were studied in a hot-plate behavioural test. In this test, mice treated with the antisense molecule demonstrated an increased latency to rearing and escape-jumping, implying that the animals were less susceptible to pain. This led to the conclusion that antagonists at this receptor might be analgesic. Antisense DNA targeted to the vasoactive intestinal peptide (VIP-1) receptor has been used to implicate this receptor in T-cell development [65]. As a final example, antisense to the μ-opioid receptor has been shown to block the analgesic actions of morphine in mice, thus leading to the conclusion that this receptor is the molecular target of this drug [66].

In addition to information gained from the knockout of receptor expression in cells and tissues, it can also be informative to study the effects of overexpression of a receptor using readily available gene delivery techniques. For example, overexpression of the α2-adrenoceptor in insulin-secreting beta cells resulted in a reduction in insulin secretion and insulin content of these cells, thus implicating abnormalities in expression or function of $G\alpha_i$-coupled receptors as a potential contributory factor in type II diabetes [67].

The application of gene deletion experiments in transgenic animals has also been used to generate data supporting the role of GPCRs in disease. In such studies, the gene encoding the receptor being studied is deleted from the germline of mice to generate animals that no longer express the receptor. Although such studies have proved invaluable in the characterization of some receptors, these experiments are technically complex. They often take over a year from induction to the generation of data, and a negative result must be treated with caution as it is always possible that adaptive changes may have occurred within the animal to compensate for the loss of the disrupted gene.

There are at least 100 examples in published literature of GPCR-deleted transgenic mice. More than half of these exhibit phenotypes implicating particular GPCRs in disease, however, many only display such phenotypes upon physiological challenge. This is an important caveat: the consequences of the loss of a receptor might only be revealed in the appropriate behavioural test. Hence, the design of behavioural tests in which to study the physiological consequences of gene deletion is at least as important as the design of the gene deletion experiment and represents an ongoing challenge for behavioural pharmacology. As examples, mice lacking the neurokinin NK_1 receptor no longer exhibit the rewarding effects of opiate drugs, thus implicating this receptor as a possible therapeutic target for the control of drug abuse [68]. Transgenic mice lacking the M_3 muscarinic receptor are hypophagic and lean, thus invoking this receptor in the regulation of food intake [69]. Transgenic studies have implicated the ORL-1 receptor in auditory function. Mice deficient in this receptor possess greater learning ability and have better memory than control animals [70]. Transgenic mice deficient in the PAC_1 receptor (pituitary adenylate cyclase activating polypeptide type 1 receptor) possess strongly reduced anxiety-like behaviour, suggesting that antagonists at this receptor might have efficacy in behavioural disorders such as anxiety [71].

The functional $GABA_B$ receptor is a heterodimer of two GPCRs – the $GABA_{B1}$ and $GABA_{B2}$ sub-units. To identify whether the $GABA_{B1}$ subunit is required for all $GABA_B$ receptor pharmacology, transgenic mice were generated in which this receptor had been deleted. In these animals all pre- and post-synaptic $GABA_B$ receptor function was absent. This observation strongly suggested that the $GABA_{B1}$ subunit is essential for all $GABA_B$ receptor pharmacology, thus indicating that there are probably no further $GABA_B$ receptors awaiting molecular identification. In addition, the epileptic phenotype of heterozygote animals indicated that both $GABA_B$ receptor agonists and antagonists might have therapeutic benefit in the treatment of neurological and psychiatric disorders in which attentional processing is impaired [72].

Finally, Conklin and coworkers have recently pioneered an elegant approach to receptor target validation through the construction of opioid receptors that no longer respond to endogenous peptides, but which can still be activated by synthetic small-molecule drugs. These RASSLs (Receptors Activated Solely by Synthetic Ligands) can then be introduced into animals, using targeted expression systems, and, following administration of the synthetic compound, can be used to clarify the role of that particular receptor or signalling pathway in a relevant physiological system or in a diseased state [73].

Closing remarks

In recent times, genome mining has led to the identification of a large number of novel GPCRs. Such receptors are likely to respond to ligands from a broad spectrum of chemical classes, ranging from small molecules to large peptides. All of these orphan GPCRs are potential therapeutic targets. Based upon the historical success of GPCR drugs, the pharmaceutical industry is investing billions of dollars in the race to validate orphan GPCRs as drug targets. As outlined in this review, the successful association of a new receptor with a disease state relies on a combination of techniques from a wide range of disciplines, including bioinformatics, genetics, molecular biology, cell biology, histology, HTS and of course analytical, molecular and *in vivo* pharmacology. It is the combination of each of these pieces of the jigsaw that provides a sufficient body of information to lead to the hypothesis that modulation of a particular receptor will have therapeutic benefit in disease. How will we know when a GPCR has been successfully validated? The answer to this question remains many years away and will follow the demonstration in the clinic that a drug targeting what was once an orphan receptor has lifestyle-improving efficacy in a human disease.

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