Alternative drug discovery approaches for orphan GPCRs

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G protein-coupled receptors (GPCRs) are well-known drug targets. However, a question mark remains for the more than 100 orphan GPCRs as current deorphanisation strategies failed to identify specific ligands for these receptors. Recent advances have shown that orphan GPCRs may have important functions that are ligand-independent. Orphan GPCRs can modulate the function of well-defined drug targets such as GPCRs with identified ligands and neurotransmitter transporters though physical association with those molecules. Thus, compounds that bind to orphan GPCRs and allosterically regulate the function of the interacting partner or even disrupt the interaction with the latter could become new drugs.

Current deorphanisation strategies

GPCRs constitute the largest family of membrane receptors. They are involved in virtually every biological process. They are typically expressed at the cell surface and possess well-defined orthosteric and allosteric ligand-binding pockets that can be targeted by small molecular weight compounds. Not surprising, many top-selling drugs target GPCRs. Based on the completed sequence of the human genome, approximately 400 non-odorant GPCRs can be predicted. During the last 20 years most of them were matched with their respective ligands using several different strategies. However, a considerable number of GPCRs, more than 100, are still orphans without identified ligand. In this review we will update recent progress on new ligand-independent functions of orphan GPCRs and discuss the potential of these orphan GPCRs as drug targets.

From the early days of GPCR cDNA cloning in the late 1980s until now, a strategy termed 'reverse pharmacology' (see Glossary) was used for the deorphanisation (see Glossary) of GPCRs [1]. This approach is based on the exogenous expression of the orphan GPCR in a suitable cell system. Activation of the receptor by exposure to a variety of potential ligands is monitored by changes in intracellular second messenger (see Glossary) levels involving heterotrimeric G proteins (Figure 1). As the number of identified natural ligands was rapidly outnumbered by the GPCRs discovered by DNA cloning, a modified version called 'orphan receptor strategy' emerged in the mid-1990s [2]. This strategy is also referred to as the 'tissue-extract based approach' where orphan GPCRs are exposed to tissue extracts instead of purified ligands. Activation of orphan receptors is again measured by changes in second messenger responses. Positive extracts are fractionated until the active component is isolated and characterised. Despite difficulties of assay specificity because of the complex composition of tissue extracts, the orphan receptor strategy proved to be useful for the identification of several natural ligands for orphan GPCRs [3–6].

More recently, approaches based on the cellular translocation of proteins that participate in the GPCR desensitisation/internalisation cycle, that is β-arrestin, have been added to the toolbox. This system commercialised as 'Transfluor technology' monitors the change in distribution of a β-arrestin-GFP construct from homogenous and diffuse intracellular localisation to aggregated distribution as a consequence of ligand-dependent internalisation [7,8]. Other strategies exploit the acidic nature of the endosomal recycling compartments and the pH-sensitive cyanine dye CypHer 5, which is non-fluorescent at pH 7.4 and fluoresces brightly in an

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GLOSSARY

Allosteric binding site : a binding site on the receptor that is topographically distinct from (does not exhibit any overlap with) the orthosteric site and which influences the functional effects of ligand binding to the orthosteric site.
Constitutive activity significant activity of GPCR in absence of ligand.
Deorphanisation : a process, which consists of the identification of a natural ligand acting on an orphan receptor.
Inverse agonist : molecule, which binds to the same receptor binding site as the endogenous agonist but exerts the opposite pharmacological effect.
Orphan 7TM protein : a seven-transmembrane protein without an assigned ligand, defined by a novel sequence with a certain degree of homology to other proteins with similar topology.
Orthosteric binding site : the primary binding site on the receptor that is recognized by the endogenous agonist.
Reverse pharmacology : strategy aiming to identify the endogenous ligand of a novel receptor.
Second messenger : cytosolic molecules such as cAMP or calcium, which are modulated upon activation of GPCRs.

acidic environment [9]. Surface exposed receptors are labelled with CypHer 5-labeled antibody and ligand-dependent internalisation is monitored by the occurrence of intense intracellular fluorescence. The expression pattern of receptors may provide another clue for ligand-receptor pairing. The nicotinic acid receptor GPR109B was identified based on its restricted expression pattern in adipose tissue and the spleen, the pharmacological sites of nicotinic acid action [10,11]. Generation of knock out animals has also been used to increase our understanding of the biological function of orphan GPCRs in the absence of knowledge about their endogenous ligand [12,13].

Limitation of current deorphanisation strategies

A recent update of the number of deorphanised GPCRs clearly shows that the deorphanisation rate slowed down considerably since 2004, with 4–6 deorphanisations per year (Figure 2). What might be the potential limitations of current strategies? Functional screening assays for orphan GPCRs traditionally focus on the monitoring of intracellular second messengers of G protein-dependent pathways. However, some receptors may not rely on the activation of heterotrimeric G proteins but rather on the activation of G protein-independent pathways [14]. This could be the case for GPCR C5L2 (also known as GPR77), which binds to C5a anaphylotoxin in binding assays, but which is completely inactive in functional G protein-dependent assays [15,16]. Whether this receptor is only a 'silent' (non-signalling) C5a anaphylotoxin scavenger or whether G protein-independent pathways are activated remains to be clarified [17]. Similarly, some chemokine receptors such as D6 receptors are known to bind to chemokines without inducing cell migration or G protein-dependent signalling [18].

The absence of specific GPCR-associated proteins in a given cell system may also represent a major obstacle for successful expression of functional receptors. Many GPCRs have indeed been reported to interact with several scaffolding (β-arrestin, homer, spinophilin) and cytoskeletal proteins (filamin A, cofilin) or so-called receptor activity modulating proteins (RAMPs). For some GPCRs, coexpression of these accessory proteins is obligatory to promote efficient surface expression, ligand binding and/or signal transduction. A well-known example is the calcitonin receptor-like receptor (CRLR) that requires RAMP for efficient transport to the cell surface and ligand binding [19]. Other GPCRs require coexpression with another GPCR. Accumulating structural, functional and pharmacological evidences suggest that GPCRs form dimers or higher order oligomers [20–22]. The best characterised example is the metabotropic γ-aminobutyric acid B ($GABA_B$) receptor heterodimer, where the coexpression of the $GABA_{B2}$ subunit is obligatory for the $GABA_{B1}$ subunit to reach the cell surface [23]. Similarly, coexpression of the β2-adrenergic receptor appears to promote α1D-adrenergic receptor surface expression and functional activity [24]. Moreover, coexpression of the different taste receptor subunits is necessary for the formation of functional receptors. Whereas $T1R_2/T1R_3$ heterodimers respond rather to sweet stimuli such as aspartame, the $T1R_1/T1R_3$ heterodimer responds to the umami taste of L-glutamate [25,26].

Taken together, these examples clearly indicate that new assays have to be designed to deorphanise the remaining orphan receptors. This includes the enlargement of functional readouts towards G protein-independent (i.e. β-arrestin-dependent) signalling pathways and the coexpression of appropriate interacting partners, which promote either cell surface expression or ligand binding. In addition, recent evidence suggests that orphan GPCRs could also have ligand-independent functions. Such a concept would offer additional options for future drug screening strategies as discussed below.

Ligand-independent functions of orphan 7TM proteins

Recent advances suggest that at least some of the remaining orphan receptors may have ligand-independent functions [27]. Considering that the term 'orphan GPCR' may be to restrictive, as some of them may be 'real' orphans and others are orphans with known ligand-independent functions for which ligand binding cannot be completely excluded, we will use the term orphan seven-transmembrane (7TM) protein in this review. Two types of ligand-independent functions have emerged so far, constitutive activity (see Glossary) of orphan 7TM proteins (see Glossary) and the modulation of the function of other proteins (i.e. non-orphan GPCRs or transporters) by hetero-oligomerisation with orphan 7TM proteins.

Constitutively active orphan 7TM proteins

The importance of agonist-independent constitutive activity of GPCRs in native tissues has been questioned for a long time, however, there is now significant evidence that this is physiologically relevant [28,29]. Some of the most convincing evidence comes from orphan 7TM proteins displaying a substantial degree of constitutive activity. Several virus-related (virus-encoded or virus-induced) orphan receptors are constitutively active. The human cytomegalovirus (HCMV) encodes three constitutively active GPCRs that exploit the cytokine system of the host cell to survive. Among these three receptors, UL33 is an orphan 7TM protein [30,31]. The Epstein Barr virus-induced receptor 2 (EBI2)

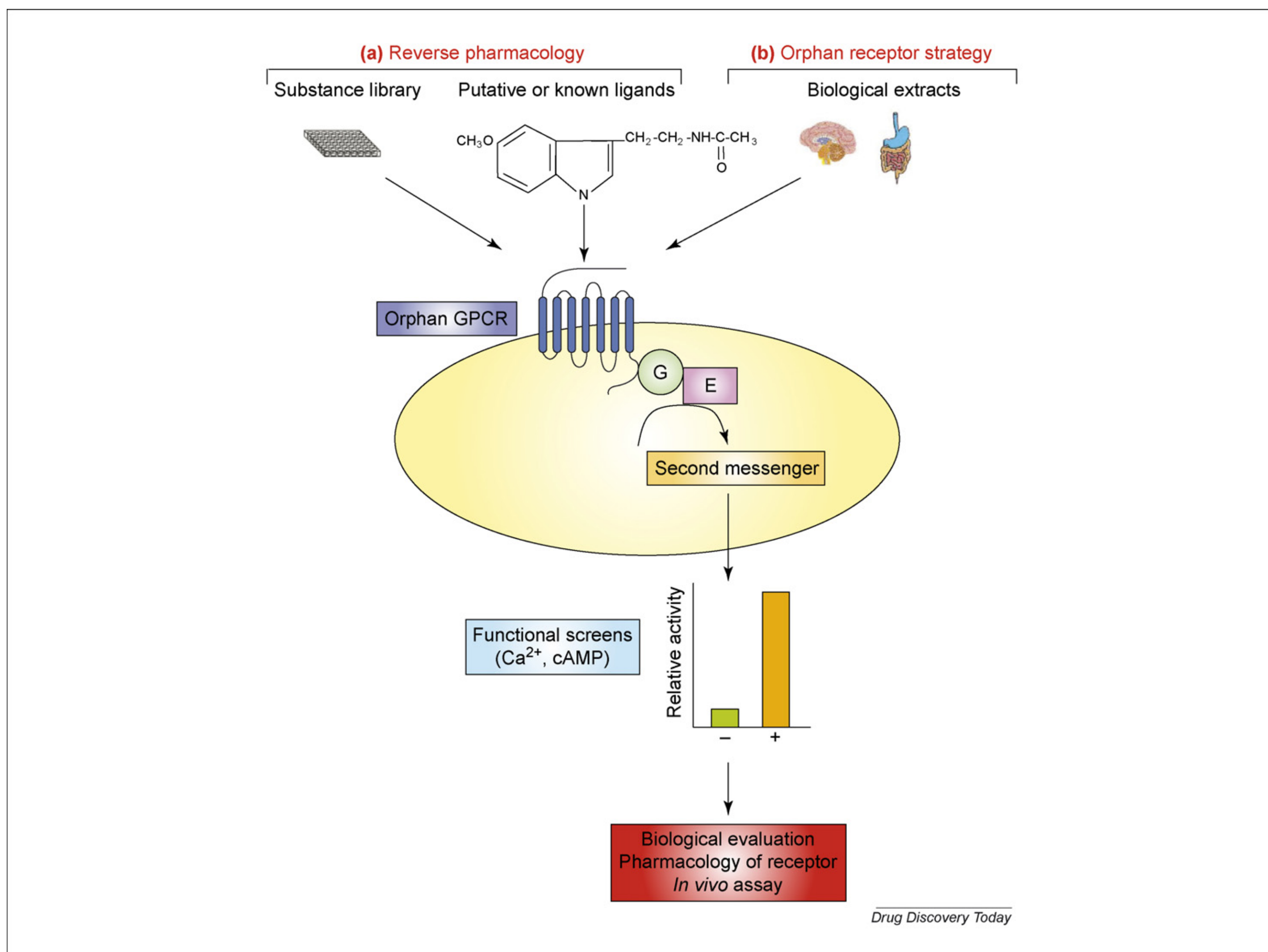

FIGURE 1

Schematic representation of current deorphanisation approaches. Orphan receptors are expressed in cells and G protein-dependent second messenger responses are monitored. **(a)** Reverse pharmacology approach. Orphan receptor-expressing cells are incubated in the presence of synthetic or natural (purified) compounds. **(b)** Orphan receptor strategy. Orphan receptor-expressing cells are incubated in the presence of tissue extracts, which are fractionated until an active component is isolated.

[32], originally identified as the most upregulated gene in EBV-infected cells, is an orphan 7TM protein, which constitutively signals through the inhibitory (G_i) subunit of G proteins [33]. Constitutive activity is also observed for endogenous, non-virus-related orphan GPCRs. GPR3, GPR6 and GPR12 constitute a subfamily with more than 60% homology that promote constitutive activation of the adenylyl cyclase pathway [34–36]. Similar observations have been made for the two homologous receptors GPR26 and GPR78 [37]. However, it should be mentioned that the possibility always remains that the apparent constitutive activity might actually reflect the presence of an endogenous ligand that is difficult to remove. This is illustrated by a recent study on GPR40, whose apparent constitutive activity can be explained by the occupation of receptor bindings sites with endogenous fatty acid ligands [38]. Screening programs for inverse agonists (see Glossary) should take into account such potential complications. Conversely, binding of endogenous inverse agonists may also exist and mask the ligand-binding site.

Association of orphan 7TM proteins with non-orphan GPCR

In line with the general notion that GPCRs can form dimers or higher order oligomers, some of these reported oligomeric complexes are composed of GPCRs with known ligands and orphan 7TM proteins [39]. This has been most convincingly demonstrated for several class C GPCRs. The $GABA_B$ receptor is composed of two functionally distinct subunits, $GABA_{B1}$ and $GABA_{B2}$. Whereas the $GABA_{B1}$ subunit binds the natural ligand, GABA, the $GABA_{B2}$ subunit couples to the heterotrimeric G protein. Importantly, the $GABA_{B2}$ subunit can be considered as an orphan 7TM protein without functional GABA binding site, a conclusion that is supported by an evolutionary trace analysis of the putative binding pocket [40]. An additional function of $GABA_{B2}$ is to promote cell surface expression of $GABA_{B1}$. A similar scenario has been reported for the Drosophila odorant receptor 83b (DOR83b), a 7TM protein with no apparent affinity for odorants that heterodimerise with classical odorant receptors such as DOR22a and DOR43a. Expression of DOR83b is required for

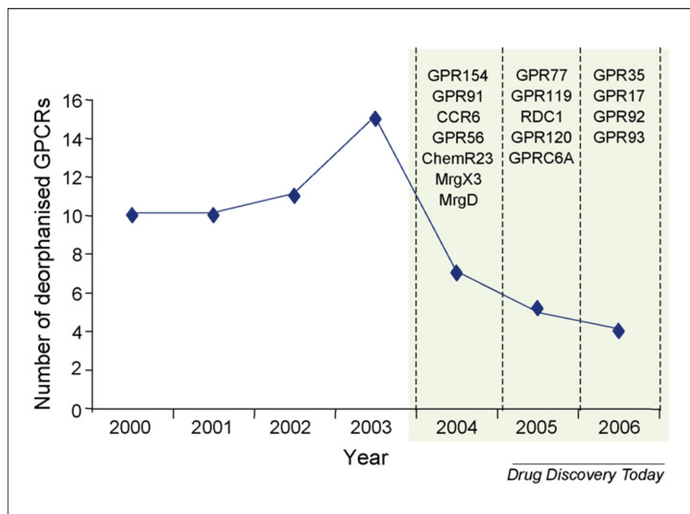

FIGURE 2

Success rate of deorphanisation between 2000 and 2006. Adapted from [67]. MrgD and MrgX3, Mas related gene D and X3; GPR, G Protein Coupled Receptor; CCR6, Chemokine CC motif Receptor 6; RDC1 or chemokine orphan receptor 1 (CMKOR1); GPRC6A, G-Protein-coupled Receptor family C 6 A; ChemR23, Chemerin Receptor 23.

functional membrane expression of DOR22a and DOR43a in olfactory sensory neurons [41,42]. The receptors for sweet and umami tastes are also composed of two receptor subunits, firstly the ligand-binding subunits $T1R_1$ or $T1R_2$, and secondly their orphan obligatory heterodimerisation partner $T1R_3$, which has no apparent ligand-binding capacity [43].

Two recent examples of the association of orphan 7TM proteins with non-orphan GPCRs show that orphans can also modify the signalling of their interacting partners. The Mas-related gene (Mrg) family members MrgD and MrgE have been shown to form heterodimers. ß-alanine binding to MrgD, when expressed alone, activates the ERK pathway and receptor internalisation. Coexpression of MrgD with the orphan MrgE potentiates ß-alanine-induced MrgD signalling and inhibits MrgD internalisation in the MrgD/MrgE heterodimer [44]. In the case of the heterodimer between the MT_1 melatonin receptor and the orphan GPR50 7TM protein, the orphan drastically inhibits ligand-promoted signalling of MT_1. Indeed, engagement of MT_1 with GPR50 inhibits high-affinity agonist binding, heterotrimeric G protein coupling and ß-arrestin binding in cells expressing both proteins heterologously and endogenously [39]. Consequently, GPR50 can be considered as a natural negative modulator of MT_1. Taken together, these examples show that orphan 7TM proteins may be important regulators of the function of GPCRs with known ligands by modulating their trafficking, signalling and pharmacological properties.

Association of orphan 7TM proteins with other membrane proteins

Orphan 7TM proteins can form heteromeric complexes containing proteins other than non-orphan GPCRs. The orphan GPR37, which is associated with Parkinson's disease has recently been reported to associate with the dopamine transporter DAT and to modulate its activity. GPR37-null mice show enhanced dopamine uptake in striatal membranes associated with increased DAT cell surface expression [45]. A further example concerns Smoothened (Smo), a 7TM protein that mediates the effects of Hedgehog (Hh) in embryonic development. Interestingly, Hh does not bind directly to Smo but rather to the 12TM receptor Patched (Ptch), which inhibits the constitutive activity of Smo. Binding of Hh to Ptch relieves this repression of Smo. Consequently, the latter can be considered as orphan 7TM protein whose activity is regulated by its binding partner Ptch [46].

Fast progress in the field of functional proteomics is likely to reveal further, unexpected, protein complexes that contain orphan 7TM proteins. One possibility is the complex formation between orphan 7TM proteins and ion channels as already shown for non-orphan GPCRs. Complexes containing the ß2-adrenergic receptor and the calcium activated potassium channel [47] or the L type calcium channel $Ca_v1.2$ [48], complexes between the dopamine D5 receptor and the $GABA_A$-ligand-gated channel and complexes between the nociceptin (ORL1) receptor and the voltage-gated calcium channel $Ca_v2.2$ [49] have been documented. It is important to note that the majority of these complexes are formed constitutively and do not require ligand activation of the receptor. For example, coexpression of ORL1 and $Ca_v2.2$ results in a constitutive voltage-dependent inhibition of the channel, a phenomenon that can be observed in dorsal root ganglion neurons.

Implications for drug design

Inverse agonists of orphan GPCRs with constitutive activity

Numerous GPCRs exhibit constitutive activity and the potential for identifying novel inverse agonists at such GPCRs has been recognised for drug discovery (Figure 3A) [28,50]. The only identified naturally occurring potent inverse agonist for GPCRs is the agouti-related peptide, which acts on the MC4 receptor to participate in body weight regulation [51,52]. Several potent synthetic inverse agonists have been developed for constitutively active GPCRs [50]. US28, a GPCR with constitutive activity that is encoded by the HCMV has also attracted attention. Recently, the first small nonpeptidergic inverse agonist for US28 has been described [53]. So far, no inverse agonist has been described for orphan 7TM proteins. As an initial step towards drug discovery, the identification of the biological function and signalling pathways used by these orphans will be of vital importance. The recent development of transcription-based reporter gene high throughput screening assays intended to identify the signalling pathways of orphan 7TM proteins such as GPR3, GPR12 and GPR26 should speed up this process [54].

Heterodimer-selective ligands

Development of selective ligands for dimeric (oligomeric) complexes between orphan 7TM proteins and non-orphan GPCRs constitutes a potential drug design strategy, which, however, still needs to prove its efficiency. Even the development of ligands that selectively target heterodimers of non-orphan GPCRs are still in its infancy. Two strategies are under current investigation for GPCR heterodimers: first, the design of small compounds that target receptor heterodimers, as proposed for 6′-GNTI, a compound that shows some selectivity for κ/δ opioid receptor heterodimers [55] and second bivalent ligands such as KDN21 that contains a δ and a κ opioid antagonist pharmacophore linked through a 21-atom

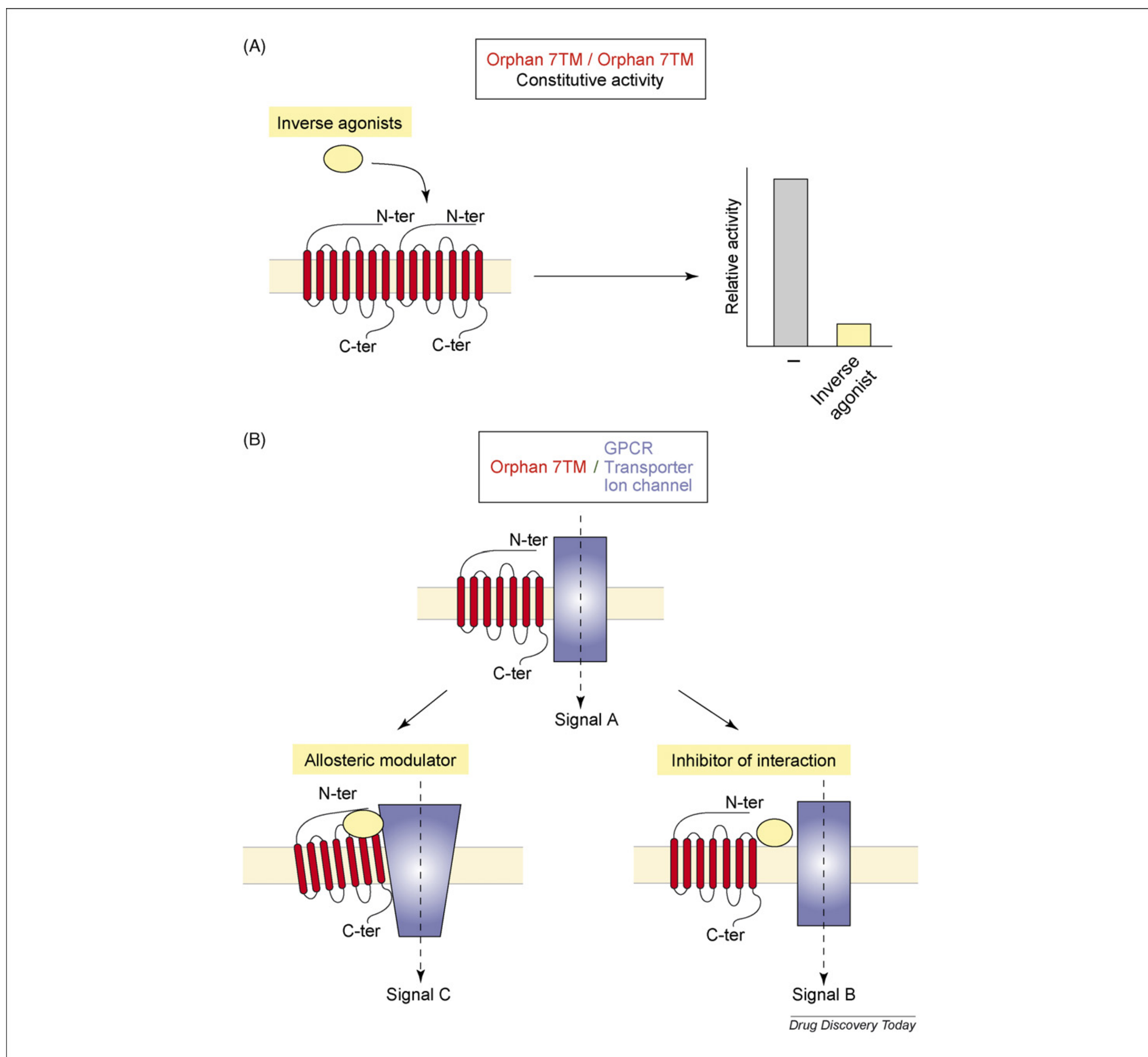

FIGURE 3

Design of 'drugs' for orphan 7TM proteins. **(A)** Inverse agonistes may inhibit the activity of constitutively active orphan 7TM proteins. **(B)** The interaction between orphan 7TM proteins and non-orphan GPCRs, neurotransmitter transporters or ion channels may be regulated by allosteric modulators that bind to the orphan 7TM protein to allosterically regulate the function of the interacting partner or may be inhibited by compounds that disrupt this interaction.

spacer. The first strategy could be applied to complexes between orphan 7TM proteins and non-orphan GPCRs.

Allosteric modulators

The therapeutical potential of allosteric modulators of GPCRs is gaining increasing acceptance. Two comprehensive state-of-the-art reviews have been published recently [56,57]. Allosteric modulators act per definition outside of the orthosteric binding site (see Glossary) of endogenous ligands (Figure 3B). Several examples illustrate the great potential of such modulators for orphan 7TM proteins. As described earlier, the $GABA_B$ receptor is composed of a ligand-binding competent $GABA_{B1}$ and the G protein coupling-competent $GABA_{B2}$ subunit. Although $GABA_{B2}$ has no functional orthosteric binding site, this subunit can be targeted by the CGP7930 compound, which allosterically enhances GABA binding to the heterodimer [58]. It is important to note that the $GABA_B$ receptor is a member of the class C GPCR subfamily that is characterised by a large bilobate extracellular orthosteric ligand-binding domain that binds GABA. Interestingly, CGP7930 and allosteric modulators of the structurally similar metabotropic glutamate receptors, preferentially target the 7TM domain in a manner similar to orthosteric ligands of class A GPCRs [59]. Similar observations have been made for taste receptors belonging to the class C subfamily. Lactisole and cyclamate have been proposed to

bind to the 7TM domain of the orphan $T1R_3$ subunit and thus allosterically regulate ligand binding to the bilobate extracellular orthosteric sites of $T1R_1$ and $T1R_2$ in their respective heterodimers. Collectively, this indicates that ligand binding competent GPCRs can be subject to allosteric regulation by orphan 7TM proteins in oligomeric complexes.

Targeting of protein–protein interfaces

An emerging class of compounds target the interacting interface between proteins. This interesting new class of molecules may become relevant for orphan 7TM proteins that modulate the function of other proteins by direct interaction (Figure 3B). The design and optimisation of such protein–protein interaction inhibitors requires detailed structural knowledge to identify relevant regions of the interface. Unfortunately, structural information, in particular of the 7TM domain, is still very poor and limited to the structure of the rhodopsin GPCR. Despite this general lack of knowledge, two specific examples illustrate the great potential of such compounds in the GPCR field.

The well-known antithrombotic compound clopidogrel and its active metabolite Act-Met have been shown to disrupt P2Y12 receptor oligomer complexes in platelets and to redistribute the receptors out of lipid rafts [60]. As raft-associated P2Y12 oligomers represent the functional form of the receptor, clopidogrel acts as an inhibitor of P2Y12 function. Although the precise mechanism of action of clopidogrel is currently unknown, modification of cysteine 97 within the first extracellular loop of P2Y12 by the free thiol of the Act-Met metabolite appears to be involved.

The pharmacological phenotype of the class B CRLR can be modulated through interaction with three distinct RAMPs [61]. Development of selective, high-affinity CGRP1 receptor (the heterodimer of CRLR and RAMP1) antagonists has been intensified in recent years because of mounting evidence that CGRP receptors are involved in the pathophysiology of migraine headache. Although RAMPs are not orphan 7TM proteins but rather single TM accessory proteins, the nature of CGRP receptor antagonists sheds light on possible mechanisms of action of compounds targeting interaction interfaces involving orphan 7TM proteins. Antagonists such as BIBN4096BS act in fact at the interface between CRLR and RAMP. Key sites for this interaction are tryptophane 74 of RAMP1 and the amino terminus of CRLR. These data suggest that the interface between a GPCR and other proteins can also constitute allosteric binding pockets [62,63]. Clearly, more precise structural information is needed to exploit the full therapeutic potential of this new class of compounds as shown for inhibitors of the p53 and Mdm2 interaction [64], iNOS dimerisation [65] and TNF-alpha trimerisation [66].

Concluding remarks and perspectives

To overcome the increasing resistance of the more than 100 remaining orphan 7TM proteins to current deorphanisation strategies, alternative approaches and concepts are needed. Whereas some of these orphans must be matched with their endogenous ligands, others may be true orphans for which no natural ligand does exist. However, even true orphans could become interesting drug targets as detailed in the present review. Elucidating the function of these orphans continues to be a priority for the understanding of their biological role and for drug discovery. Whereas the complete sequence of the human genome provided us with a fairly precise idea about the total number of orphan 7TM proteins, current progress in systems biology and proteomics will provide us with valuable insights in the orphan 7TM protein-containing complexes, to ultimately elucidate the *in vivo* function of these proteins. Clearly, more than 100 orphan 7TM proteins will keep us busy for several more years.

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