Biased agonists of GPR84 and insights into biological control

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Abstract

GPR84 was first identified as an open reading frame encoding an orphan Class A G protein coupled receptor in 2001. Gpr84 mRNA is expressed in a limited number of cell types with the highest levels of expression being in innate immune cells, M1 polarised macrophages and neutrophils. The first reported ligands for this receptor were medium chain fatty acids with chain lengths between 9 and 12 carbons. Subsequently a series of synthetic agonists that signal via the GPR84 receptor were identified. Radioligand binding assays and molecular modelling with site-directed mutagenesis suggest the presence of three ligand binding sites on the receptor, but the physiological agonist(s) of the receptor remain unidentified. Here, we review the effects of GPR84 agonists on innate immune cells following a series of chemical discoveries since 2001. The development of highly biased agonists has helped to probe receptor function in vitro, and the challenge remaining is to follow the effects of biased signalling to the physiological functions of innate immune cell types.

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Abbreviations:

BMDM, Bone marrow-derived macrophage; C10, capric acid; DIM, diindolylmethane; MCFA, medium-chain fatty acid; SAR, structure-activity relationship; 3-OH-C10, 3-hydroxy capric acid.

Abstract

GPR84 was first identified as an open reading frame encoding an orphan Class A G protein coupled receptor in 2001. *Gpr84* mRNA is expressed in a limited number of cell types with the highest levels of expression being in innate immune cells, M1 polarised macrophages and neutrophils. The first reported ligands for this receptor were medium chain fatty acids with chain lengths between 9 and 12 carbons. Subsequently a series of synthetic agonists that signal via the GPR84 receptor were identified. Radioligand binding assays and molecular modelling with site-directed mutagenesis suggest the presence of three ligand binding sites on the receptor, but the physiological agonist(s) of the receptor remain unidentified. Here, we review the effects of GPR84 agonists on innate immune cells following a series of chemical discoveries since 2001. The development of highly biased agonists has helped to probe receptor function *in vitro* , and the challenge remaining is to follow the effects of biased signalling to the physiological functions of innate immune cell types.

Keywords

GPCR biased agonism, GPR84, orphan GPCRs, GPCRs and drug development, macrophage biology

GPCRs as key mediators of cell signalling and cell physiology

The human genome encodes around 800 transmembrane proteins with a conserved architecture containing seven hydrophobic transmembrane spanning regions. Most of these TM7 proteins can transmit intracellular signals via G proteins, hence the name G protein coupled receptors. GPCRs are the largest class of membrane receptors in human cells. Of the 800 known GPCRs, around 400 are involved in olfaction, 33 are taste receptors, 10 are involved in light perception and 5 are pheromone receptors. That leaves around 350 non-sensory GPCRs that mediate cell signalling including many aspects of endocrinology, cellular physiology and immunity (S. P. AlexanderChristopoulos *et al.* , 2021). The first classification scheme for GPCRs across all eukaryotes divided them into six different classes based on conserved structures (Kolakowski, 1994) and subsequent phylogenetic studies identified five broad families of human GPCRs; Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2 and Secretin comprising the GRAFS classification system (Schiöth & Fredriksson, 2005). The Glutamate (Class C) family includes the metabotropic glutamate receptors, the $GABA_B$ receptors and three taste receptors. The Rhodopsin (Class A) receptor family responds to a wide variety of small molecules, neurotransmitters, peptides and hormones, including but not limited to dopamine, cannabinoids, opioids, chemokines and adenosine. The adhesion receptors (aGPCRs) are the second largest family of GPCRs with 33 human orthologues (Folts, Giera *et al.* , 2019). The aGPCRs have large extracellular N-termini that can be proteolytically cleaved leading to signalling. Mutations in aGPCRs have been shown to contribute to or increase susceptibility to human diseases including Usher Syndrome, attention deficit hyperactivity disorder and Tourette disorder. Recently mutations in ADGRE2/EMR2 have implicated EMR2 as a mechanoreceptor in mast cells and macrophages (Boyden, Desai *et al.* , 2016; I, Tseng*et al.* , 2020). The Frizzled family consists of 11 GPCRs and the Secretin family 15 GPCRs in the human genome. Secretin family receptors signal in response to polypeptide hormones including glucagon-like peptide-1.

The single copy gene encoding GPR84 is uninterrupted by introns and codes for 396 amino acid orthologues in both humans and mice (Wittenberger, Schaller *et al.* , 2001; Yousefi, Cooper *et al.* , 2001). On its discovery in 2001, prior to the deorphanisation of the FFARs, GPR84 could not be assigned a subfamily based on similarity to other known GPCRs (Wittenberger, Schaller *et al.* , 2001). Later, a phylogenetic tree analysis highlighted that GPR84 and FFAR4 bear only a distant evolutionary relationship with FFARs 1, 2 and 3 (Ichimura, Hirasawa *et al.* , 2009). In 2013 the IUPHAR noted there was insufficient evidence to classify GPR84 as FFAR5 (Davenport, Alexander *et al.* , 2013).

GPR84 is conserved across vertebrates dating back at least 400-550 Myr (Schulze 2022). A short 20 years on from its identification, a majority of the research on GPR84 has indicated that it has major roles in inflammation, given the receptor is highly expressed by immune cells, induced by inflammatory stimuli, and augments pro-inflammatory effector functions (Luscombe, Lucy *et al.* , 2020). Deletion of *GPR84* in mice does not lead to embryonic death or any obvious growth or fertility defect, suggesting that this GPCR does not play a non-redundant role in host physiology. It has been suggested to involve the host detection of bacterial quorum sensing molecules or metabolites from dying bacteria (Peters, Rabe *et al.* , 2020; Schulze, Kleinau*et al.* , 2022).

A brief history of GPR84

In *2001* Yousefi, Cooper, *et al* . (2001) reported the cloning and expression analysis of a transcript identified in human neutrophils. Among other chemokine receptor like genes the authors identified the *EX33* gene encoding a 396 amino acid protein. Transcription profiling identified higher levels of *EX33* mRNA in neutrophils and eosinophils than B- and T-lymphocytes with high expression in bone marrow and lung. An independent study by Wittenberger, Schaller, *et al* . (2001) identified 14 expressed sequence tags with homology to GPCRs including GPR84 - identical to the open reading frame of *EX33* (Fig 1).

In *2005* the pharmaceutical company Tularik generated the first *GPR84*$^{-/-}$ knockout mouse and reported a modest phenotype for these animals (Venkataraman & Kuo, 2005). *GPR84*$^{-/-}$ T- and B- lymphocyte proliferation in response to a range of mitogens was normal but the authors reported that stimulation of T-cells with anti-CD3 antibodies led to increased Interleukin-4 and Interleukin-5 production. *GPR84*$^{-/-}$ mice showed no difference in antibody response to ovalbumin as an antigen.

In *2006* Wang, Wu, *et al* . (2006) published the first report of agonists that activated the GPR84 receptor expressed in transfected cells. The authors transfected CHO cells with human or murine GPR84 expression vectors, an aequorin reporter gene and a mixture of G proteins. mGPR84 transfected CHO cells showed Ca^{2+} responses to the medium chain fatty acids (MCFAs) capric acid (C10; Fig 2), undecanoic acid (C11), and lauric acid (C12). Similar results were obtained using stably transfected CHO cells using cAMP inhibition assays and [^{35}S]GTPγS assays that could be blocked by treatment with pertussis toxin. Importantly the same paper reported significant *Gpr84* expression in human and murine macrophages and that the identified MCFA ligands enhanced secretion of IL-12 p40 expression in the murine RAW macrophage cell line treated with LPS. Wang, Wu, *et al* . (2006) also described diindolylmethane (DIM; Fig 2) as a surrogate agonist for GPR84.

In *2013* Suzuki, Takaishi, *et al* . (2013) reported a synthetic GPR84 agonist 6-n-octylaminouracil (6-OAU; Fig 2). The authors showed that 6-OAU elicited human neutrophil and macrophage chemotaxis and amplified the production of the pro-inflammatory cytokine TNFα from LPS treated macrophages. Further evidence for the pro-inflammatory effects of GPR84 agonists came from recruitment of polymorphonuclear leukocyte and macrophages following injection of 6-OAU into a rat dorsal air pouch.

In *2015* it was reported that *GPR84*$^{-/-}$ mice did not develop mechanical or thermal hypersensitivity following sciatic nerve ligation, a phenotype the authors attributed to reduced pro-inflammatory effects in *GPR84*$^{-/-}$ macrophages (Nicol, Dawes *et al.* , 2015). In the same year Audoy-Remus, Bozoyan, *et al* . (2015) showed that *GPR84*$^{-/-}$ crossed with the APP/PS1 mouse model of Alzheimer's Disease showed greater dendritic degeneration and cognitive decline compared to wild-type mice. The authors saw no difference between *GPR84*$^{-/-}$ and wild-type mice in endotoxaemia or the experimental autoimmune encephalomyelitis model of multiple sclerosis. The GPR84 antagonist GLPG1205 failed to meet efficacy endpoints in a PhII clinical trial for ulcerative colitis (Vermeire, Reinisch *et al.* , 2017) (NCT02337608).

In *2016* Zhang, Yang, *et al* . (2016) used HEK cells transfected with hGPR84 and $G_{\alpha 16}$ to screen a library of 160,000 small molecules and identified the potent GPR84 agonist, ZQ-16, also known as 2-HTP (Fig 2).

Liu, Zhang, *et al* . (2016) identified the most potent agonist of the time, LY-237, also known as cpd 51 (Fig 2).

In *2017* PBI-4050 failed to meet efficacy endpoints in the treatment of idiopathic pulmonary fibrosis (Khalil, Manganas *et al.* , 2019) (NCT02538536).

In *2018* Gaidarov, Anthony *et al* . (2018) identified the natural product embelin (Fig 2) as a potent GPR84 agonist and synthesised and tested other dihydroxybenzoquinone analogues with varying alkyl tail chain lengths. The authors reported that GPR84 activation drives human neutrophil chemotaxis and primes amplification of oxidative burst while implicating macrophage GPR84 activation with reverse cholesterol transport in macrophage foam cells. In the same year Recio, Lucy, *et al* . (2018) reported that the GPR84 agonist 6-OAU augmented macrophage inflammatory responses and enhanced macrophage phagocytosis. Activation of GPR84 was also shown to trigger ROS release from TNFα or Latrunculin-A primed neutrophils (Sundqvist, Christenson*et al.* , 2018).

In *2019* Lucy, Purvis *et al* . (2019) reported a novel biased agonist DL-175 (Fig 2) that was active in the $G\alpha_i$pathway but inactive in the β-arrestin pathway. Dietary supplementation with lauric acid, perilla oil, and DIM together, which target both GPR84 and FFAR4 and were formulated using colonic release capsules, was found to reduce energy intake in obese adults (Peiris, Aktar *et al.* , 2022) (NCT04292236).

In *2020* GPR84 antagonist GLPG1205 failed to meet primary endpoint outcomes for the treatment of idiopathic pulmonary fibrosis (Strambu, Seemayer *et al.* , 2023) (NCT03725852). Köse, Pillaiyar,*et al* . (2020) reported an agonist radioligand, [^{3}H]PSB-1584, which was used to measure the binding affinities of other agonists, including DIM which is shown to allosterically increase the specific binding of [^{3}H]PSB-1584.

In *2022* Marsango, Ward, *et al* . (2022) identified key residues in the intracellular loop 3 of GPR84 which undergo GRK2/3-mediated phosphorylation in an agonist-dependent manner, revealing a step in the mechanism underlying the β-arrestin bias of DL-175.

Insights into GPR84 Biology

Immune Cell GPCRs

Given the importance of GPCRs and their agonists in cell-cell communication it is not surprising that GPCRs play central roles in regulation of the immune system. In drawing up Table I we have sought to include examples from all aspects of innate and adaptative immunity. Recognition and response to antigens by B lymphocytes is dominated by the B cell receptor for antigen in the context of MHC restriction, both of which are dominated by kinase signalling rather than GPCRs.

In a recent review Jason Cyster delineates the multiple roles of specific GPCRs in humoral immunity and B lymphocyte biology (Lu & Cyster, 2019). Efficient humoral antibody responses rely on optimising relatively rare interactions between antigen presenting cells and patrolling B lymphocytes within secondary lymphoid organs, with the CCR7 and the CCR6 chemokine receptors playing an important role in this process and the $S1P_1R$ receptor being important for lymphocyte recirculation. B lymphocyte differentiation, and their tropism to different host tissues continues to be directed by Class A GPCRs including CXCR4, CXCR3, CCR9 and CCR10 (Lu & Cyster, 2019).

Class A GPCRs play multiple roles in T cell biology through chemotaxis and immune cell homeostasis. One such receptor is CCR4 which came to attention following single cell RNA sequencing of human adult T cell leukemia / lymphoma (ATLL) cells (Nakagawa, Schmitz *et al.* , 2014). All the ATLL mutant CCR4 receptors responded more strongly in chemotaxis assays and p-Akt signalling assays to CCR4 chemokines and showed a loss of receptor internalisation following ligand binding. Enhanced PI3K / Akt activation conferred a survival advantage on cells expressing mutant CCR4 and hence identified CCR4 as a therapeutic molecular target in ATLL.

Another Class A GPCR chemokine receptor whose multiple roles in leukocyte homeostasis were revealed by rare germline mutations is the CXCR4 receptor the C-terminus of which is mutated in WHIM syndrome. WHIM stands for warts, hypogammaglobulinemia, infections and myelokathexis (retention of neutrophils in the bone marrow). Patients also have fewer B and T lymphocytes which leads to increased human papilloma virus infections, the cause of warts.

The majority of GPCRs listed in Table I are Class A GPCRs and their ligands are considered inflammatory mediators be they secreted proteins (e.g. chemokines), peptides (e.g. fMLF), or lipid mediators (e.g. prostaglandins and leukotrienes). The biological effects of the GPCRs listed in Table I are most often recognised in innate immune cells but signalling via these receptors is manifest in multiple cell types for instance in the stromal cells of lymphoid tissues or in adaptive immune cells. Consideration of the breadth of molecules that can act as Class A GPCR ligands listed in Table I and further consideration of GPCR deorphanisation leaves open the possibility that the physiological ligand of the GPR84 receptor remains to be discovered and that it plays a role in host immunity.

Effects of GPR84 agonists on innate immune cells

We know that GPR84 is expressed predominantly in immune cell types and is highly upregulated by inflammatory stimuli (Luscombe, Lucy *et al.* , 2020). Once expressed, activation of GPR84 using surrogate agonists results in an enhancement of inflammatory processes in a cell-type dependent manner. In innate immune cells, particularly monocytes/macrophages and neutrophils, research using surrogate GPR84 agonists has uncovered a role for this receptor in the mobilisation of inflammatory mediators, chemotaxis, and phagocytosis (Table II).

A review of the effects of GPR84 agonists on specific cells involved in innate immunity highlights the inflammatory effects of this receptor. The secretion of inflammatory cytokines, chemokines, and prostanoids, as well as ROS production, NETosis, and degranulation are regulated by GPR84 (Table II). In almost all cases, the effect of GPR84 activation is an increase in the mobilisation of pro-inflammatory mediators, or a decrease in anti-inflammatory mediators. In just two cases to date were opposite effects observed. Firstly, a decrease in CCL2 secretion with 6-OAU treatment was observed by Reyes, Kim, *et al* . (2021) in RAW264.7 macrophages infected with *Brucella abortus* . Secondly, a decrease in *Tnf* expression was observed by Ohue-Kitano, Nonaka,*et al* . (2023) with 6-OAU treatment of palmitate-stimulated RAW264.7 cells. Conversely, 6-OAU increased CCL2 secretion in primary murine M-CSF differentiated and LPS-stimulated bone marrow-derived macrophages (BMDMs) (Recio, Lucy *et al.* , 2018), and 6-OAU increased TNFα secretion in PMA-differentiated LPS-stimulated U937 macrophage cells (Suzuki, Takaishi *et al.* , 2013). This seeming contradiction only serves to highlight the variability of responses between cell types and stimulation paradigms, and indeed, potential context-dependent responses of GPR84.

GPR84 agonists have been demonstrated to act as chemoattractants, promoting chemotaxis, migration, and motility. For example, stimulation with both 6-OAU and embelin has been shown to induce chemotaxis in primary human and murine neutrophils and monocytes (Table II). However, results with macrophages are less clear. Evidence from immortalised cell lines suggest 6-OAU can act as a chemoattractant in PMA-differentiated U937 macrophages (Lucy, Purvis *et al.* , 2019; Suzuki, Takaishi*et al.* , 2013) but not primary murine M-CSF-differentiated LPS-stimulated BMDMs (Recio, Lucy *et al.* , 2018). There is a growing appreciation that the underlying mechanisms of macrophage migration differs from those of its monocytic precursors (Rumianek & Greaves, 2020). However, given the relative scarcity of reports on GPR84-mediated chemotaxis, cell-type differences in GPR84-mediated chemotaxis remain unclear.

The observation of enhanced bacterial adhesion and phagocytosis has been a more recent development in the field, and GPR84 agonists have been shown to promote these pathways (Table II). In contrast to observations in chemotaxis, reports of phagocytosis are almost exclusively made in macrophages. Here, 6-OAU has been shown to enhance adhesion to bacteria, enhance phagocytic activity, and enhance the number of phagocytic cells (Recio, Lucy *et al.* , 2018). GPR84 was also identified in a CRISPR screen for regulators of cancer

cell phagocytosis (Kamber, Nishiga*et al.* , 2021). Here, stimulation with 6-OAU enhanced the phagocytosis of PMA-differentiated LPS-stimulated U937 macrophages and LPS-stimulated J774 macrophages of Ramos lymphoma target cells (Kamber, Nishiga *et al.* , 2021). Only one report showed small decreases in adhesion and uptake of live bacteria when stimulating RAW264.7 macrophages with 6-OAU (Reyes, Kim *et al.* , 2021). As with the previously discussed chemokine readout, the live infection context with pathogenic and immune-evasive *Brucella* spp. and *Salmonella*spp. may relate to the observations of decreased CCL2 secretion and bacterial adhesion and uptake and certainly warrants further investigation.

The use of specific GPR84 agonists has undoubtedly proven highly useful in interrogating the physiological role of this receptor. While there is still ongoing debate over the endogenous agonist, particularly whether it is some form of MCFA, we believe it is still necessary to use potent surrogate agonists to support conclusions made with MCFAs which are low potency, are often used at concentrations above 100 μM, and consequently lack selectivity for GPR84. Furthermore, the discovery and characterisation of biased and allosteric agonists has opened new avenues to probe the biology of innate immune cells and should be widely adopted alongside the use of 'balanced' agonists such as 6-OAU. For example, Lucy, Purvis, *et al* . (2019) compared 6-OAU and DL-175 and found that only 6-OAU promotes chemotaxis in PMA-differentiated LPS-stimulated U937 macrophages, while both agonists promote chemotaxis in human monocytes. Similar comparisons were made by Mikkelsen, Arora,*et al* . (2022), noting that 3-hydroxy capric acid (3-OH-C10; Fig 2) promotes neutrophil but not monocyte migration, and that 2- or 3-hydroxylated MCFAs appear to promote migration while their non-hydroxy conterparts did not.

Delineating the effects of GPR84 signalling bias at the level of physiological readouts is a challenging task and requires systematic investigation. As can be seen, the variety of cell-types and stimulation paradigms completely obscures comparisons that could be made about agonist-specific results in the secretion of cytokines, chemotaxis, or phagocytosis. Studying the function of GPR84 in primary cells poses three challenges; first, the receptor requires transcriptional upregulation that subsides over time (e.g. LPS-stimulation upregulates*Gpr84* with a peak at 8 h in BMDMs), second, some responses require priming (e.g. TNFα-priming prior to GPR84-mediated ROS release in neutrophils), and thirdly, activation of GPR84 will sometimes only augment existing responses (e.g. 6-OAU only augments C5a-mediated migration in BMDMs, or fMLP- or C5a-mediated ROS production in neutrophils). We therefore encourage researchers to attempt multiple stimulation paradigms when testing for agonist-mediated effects.

GPR84 as a therapeutic target

GPCRs as targets

With 475 drugs targeting 108 unique GPCRs it is estimated that GPCRs are targets for ˜35% of approved drugs (Hauser, Attwood*et al.* , 2017; Insel, Sriram *et al.* , 2019). Around 15% of the ˜350 non-sensory GPCRs are targets for therapeutic drugs but why do 85% of GPCRs remain untargeted by molecular therapies? One obvious answer is that many non-sensory GPCRs remain orphans with no known physiological agonists (Morfa, Bassoni *et al.* , 2018). Although GPR84 lacks a known physiological agonist it has been targeted in a number of randomised clinical trials. Other deorphanised GPCRs such as members of the chemokine receptor family that play a central role in inflammatory cell recruitment in pre-clinical models have proven difficult to target using small molecules (Schall & Proudfoot, 2011). However, recent FDA approval of a small molecule complement C5a receptor antagonist Avacopan for ANCA-associated vasculitis shows that this problem can be overcome with good medicinal chemistry, good target validation and appropriate financial incentive (Jayne, Merkel *et al.* , 2021).

While orphan GPCRs have long been considered an untapped source of new drugs, in his recent review Paul Insel argues that application of unbiased, hypothesis generating methodologies to quantify GPCR expression in cells and tissues (GPCRomics) can lead to the discovery of disease-relevant GPCRs that contribute to functional responses and pathophysiology (Insel, Sriram *et al.* , 2019). Application of GPCRomics to

cancer cells and tumours may identify GPCRs following the example of CCR4 and may find application as potential biomarkers and maybe even therapeutic targets in cancer (Insel, Sriram *et al.* , 2018). Given the expression of GPR84 by macrophages it will be interesting to look at GPR84 expression by tumour associated macrophages in a range of solid tumours.

Orphan GPCRs and Class A GPCR deorphanisation

Approximately 30% of the ˜400 non-olfactory human GPCRs have not been definitively paired with endogenous ligands and are hence designated as "orphan" receptors (S. P. H. Alexander, Christopoulos*et al.* , 2019; Hauser, Gloriam *et al.* , 2020; Laschet, Dupuis *et al.* , 2018). During the 'Golden Age' of GPCR deorphanisation, which can be defined as the late 1990's and early 2000s, endogenous agonists for approximately 10 GPCRs were identified every year. Pharmacology companies invested significant resources into 'reverse pharmacology' approaches to characterise GPCRs identified in the completed human genome sequence. Although the number of GPCR deorphanisations has fallen over the last decade there have been notable successes which owe much to the application of new strategies e.g. bioinformatics (Foster, Hauser *et al.* , 2019).

P2RY8 was recently deorphanised by searching for bioactive molecules in bile and culture supernatants of cell lines, revealing S-geranylgeranyl-L-glutathione as the agonist which regulates B cell confinement to germinal centres (Lu, Wolfreys *et al.* , 2019). The chemotactic peptide agonist for the T-cell receptor GPR15, GPR15L and encoded by *C10ORF99* , was discovered following searches for open reading frames with similarity to chemokines, screening porcine colon tissue extracts for activity, and using comparative genomics and bioinformatics (Foster, Hauser *et al.* , 2019; Ocón, Pan *et al.* , 2017; Suply, Hannedouche *et al.* , 2017). On the other hand, numerous proposals have been made for the lipid agonist of GPR55, including its role as a putative third cannabinoid receptor or lysophosphatidic acid receptor, and has also been characterised as a chemotactic receptor for lysophosphatidylglucoside in monocytes and macrophages (X. Li, Hanafusa *et al.* , 2021).

Where to start looking for the true GPR84 ligand?

Consideration of recent successes in deorphanising Class A GPCRs might suggest new strategies to follow in the continuing hunt for endogenous agonists of the GPR84 receptor. So where should we start looking for the endogenous (or exogenous) GPR84 ligand(s)?

Expression profiling of murine *Gpr84* mRNA conducted by our laboratory suggested expression in the atherosclerotic lesions of$ApoE^{-/-}$ mice, a result which should be confirmed using *in situ* RNA hybridisation or ideally immunohistochemistry (Recio, Lucy *et al.* , 2018). Perhaps fractionation of the modified lipids found in atherosclerotic lesions could identify novel lipid agonists, a strategy similar to that used to identify the P2RY8 ligand. *Gpr84* mRNA expression by murine microglia suggest the CNS as a potential site of GPR84 ligands. Current medium chain fatty acid agonists do not exclude the possibility of protein or peptide agonists for the GPR84 receptor but without more spatial and disease related information it is hard to see how a similar strategy to that used to identify CARTp as the GPR160 ligand could be employed.

Finally, can we exclude an exogenous ligand as the true physiological agonist of GPR84? The evolutionary conservation of the GPR84 receptor in vertebrate but not avian species might support the idea of this Class A receptor in sensing pathogens or pathogen derived products (Schulze, Kleinau *et al.* , 2022). Schulze, Kleinau, *et al* . (2022) used cAMP inhibition assays of transiently transfected mammalian GPR84 orthologues to test the bacterial quorum sensing MCFAs *cis* -2-C10 and *trans* -2-C10. The authors' cAMP signalling data revealed low potency activity. More recently, Peters, Rabe, *et al* . (2020) proposed 3-OH-C10 as a GPR84 signalling component of LPS and data showed an increase in 3-OH-C10 in stationary cultures of *E. coli* . Using M1 polarised THP-1 cells they demonstrated that 3-OH-C10 signalling via GPR84 involved $G_{\alpha 15}$ and p-Akt signalling.

Signalling bias at GPR84

With the discovery of GPR84 agonist MCFAs, lipid mimetics, and other natural products, functional assays began to uncover signalling bias. This began with the exploration of structure-activity relationships (SAR) around DIM derivatives with regard to the canonical G-protein and β-arrestin pathways. Most of the compounds were biased towards the G-protein pathway, exemplified by PSB-16671 (Fig 2), while only a few compounds were found to have limited bias towards the β-arrestin pathway (Pillaiyar, Köse *et al.* , 2017). These derivatives are also known to act allosterically (Mahmud, Jenkins *et al.* , 2017), adding further texture to the downstream signalling of GPR84. Further SAR around 6-OAU lipid mimetics highlighted the variability of cAMP versus β-arrestin signalling, ranging from unbiased compounds such as PSB-1584, to compounds such as PSB-16434 (Fig 2) which had a 79-fold pathway selectivity towards cAMP (Pillaiyar, Köse *et al.* , 2018). Additionally, a cyclopropane-containing MCFA isolated from the marine bacterium *Labrenzia* sp. 011 has been shown to recruit β-arrestin without affecting cAMP production in GPR84 stable cell lines, demonstrating bias towards β-arrestin from natural product MCFAs (Amiri Moghaddam, Dávila-Céspedes *et al.* , 2018). Biased signalling resulting from natural agonists has been shown to underlie context-specific signalling in other inflammation associated GPCRs, which are exemplified by the 20 chemokine receptors and their distinct responses to the 50 described chemokines (Eiger, Boldizsar *et al.* , 2021).

A ligand-based virtual screen and subsequent hit optimisation led to the discovery of DL-175, which is a potent and selective G-protein biased agonist with no detectable recruitment of β-arrestin (Lucy, Purvis*et al.* , 2019). As an *in vitro* tool this compound has found use in dissecting G-protein versus β-arrestin pathway effects in macrophages and neutrophils (Fredriksson, Holdfeldt *et al.* , 2022; Lucy, Purvis *et al.* , 2019; Mårtensson, Sundqvist *et al.* , 2021). In its initial biological characterisation, it was found that DL-175 confers distinct functional responses in macrophages when compared to the balanced agonist 6-OAU (Lucy, Purvis *et al.* , 2019).

The currently described range of GPR84 agonists point to a marked system bias of this receptor in favour of G_i-mediated pathways over the β-arrestin pathway (Fig 3). We and others have observed trends that favour the G-protein pathway within the chemical series of the orthosteric agonists 6-OAU (Pillaiyar, Köse *et al.* , 2018), DL-175, and some MCFAs (Lucy, Purvis *et al.* , 2019; Mikkelsen, Arora *et al.* , 2022; Peters, Rabe *et al.* , 2020). Indeed, it has been suggested that this system bias is a physiological property of GPR84 in its proposed function in innate immunity (Peters, Rabe *et al.* , 2020).

In contrast, compounds with bias towards the β-arrestin pathway are more commonly seen within the DIM class of allosteric agonists, although these are also micromolar potency and low affinity compounds (Köse, Pillaiyar *et al.* , 2020; Pillaiyar, Köse *et al.* , 2017). The absence of a physiological agonist with which to set as a reference ligand when determining bias factors remains a challenge to medicinal chemistry projects, though the evidence to date indicates that GPR84 is inherently poorly coupled to the β-arrestin pathway. With few exceptions, GPR84 agonists with sub-micromolar cAMP potencies are also biased towards cAMP (Fig 3). Furthermore, activity in the β-arrestin pathway is also highly variable, as analogues within the major ligand classes of MCFAs, 6-OAU derivatives, and DIM derivatives have been shown to have greater correlation between binding and cAMP potency than between binding and β-arrestin potency (Köse, Pillaiyar *et al.* , 2020). Nonetheless, multiple distinct methods have shown that GPR84 can indeed couple to β-arrestins and it remains an important pathway to investigate. This system bias is another mechanism by which cellular contexts dictate the resulting signals and physiological responses and have been observed in other inflammatory GPCR pairings such as CXCR4 and the arrestin-coupled ACKR3, CCR2 and the arrestin-coupled D6R, and the C5a1 receptor and the arrestin-coupled C5a2 receptor (Pandey, Kumari*et al.* , 2021; Yen, Schafer *et al.* , 2022).

As β-arrestins are part of the canonical desensitisation and internalisation pathways of GPCRs, an inherent system bias away from this pathway has broad implications for receptor regulation and drug design. The lack of efficacy of DL-175 in the GPR84-β-arrestin pathway has been demonstrated using tagged receptor and arrestin systems by chemiluminescent enzyme fragment complementation (Lucy, Purvis *et al.* , 2019;

Mårtensson, Sundqvist *et al.* , 2021) and BRET (Marsango, Ward *et al.* , 2022), as well as by measuring arrestin translocation to the membrane in enhanced bystander BRET assays (Fredriksson, Holdfeldt *et al.* , 2022). It is now also known that two key threonine residues in intracellular loop 3 are phosphorylated by GRK2/3 following agonist stimulation with ZQ-16, but not DL-175 (Marsango, Ward *et al.* , 2022). Phosphorylation of these residues, Thr^{263} and Thr^{264}, allow for subsequent interactions with β-arrestin and β-arrestin-2 (Marsango, Ward*et al.* , 2022). This is consistent with the idea that bias is orchestrated by GRK proteins in response to certain agonist-induced receptor conformations, which then influence β-arrestin interactions and signalling, rather than directly favouring or disfavouring interactions with β-arrestin itself (Choi, Staus *et al.* , 2018; Zidar, Violin*et al.* , 2009).

Functional results in primary cells using the GRK2/3 inhibitor cmpd101 support the physiological involvement of GRK2/3 in GPR84 desensitisation. The label-free impedance response of BMDMs stimulated with DL-175 is more prolonged than the response to 6-OAU, but interestingly pre-treatment with cmpd101 results in a 6-OAU response that mirrors DL-175 alone (Lucy, Purvis *et al.* , 2019). In neutrophils, the ROS response induced by ZQ-16 was also prolonged by pre-treatment with cmpd101, whereas the response of DL-175 was not affected (Fredriksson, Holdfeldt *et al.* , 2022). Further work is needed to determine the involvement of GRK2/3 on the kinetics of these responses and direct readouts such as live-cell cAMP or β-arrestin recruitment would be especially valuable. However, both the impedance response of BMDMs and ROS production in neutrophils when stimulated with DL-175 were terminated in relatively short timeframes, suggesting an alternative mechanism of desensitisation. By comparison to FPR2, a direct coupling of GPR84 to the actin cytoskeleton has been hypothesised (Fredriksson, Holdfeldt *et al.* , 2022). The importance of desensitisation in drug design can be highlighted by the use of $S1P_1$-desensitising agonists for the treatment of multiple sclerosis. Agonists causing persistent $S1P_1$signalling are sought for their endothelial protective properties (Grailhe, Boutarfa-Madec *et al.* , 2020). Likewise, in infection research, agonist-induced internalisation of the HIV coreceptor CCR5 is an effective strategy for viral entry inhibition, and a further desirable effect is to achieve this with minimal receptor activation and the concomitant inflammatory response (Kazmierski, Bifulco *et al.* , 2003).

GPR84 has been shown to activate transducer proteins including $G_{i/o}$, $G_{12/13}$, G_{15}, GRK2/3, and β-arrestin1/2 (Gaidarov, Anthony *et al.* , 2018; Marsango, Ward *et al.* , 2022; Peters, Rabe *et al.* , 2022; J. Wang, Wu *et al.* , 2006) which couple to effector molecules including β-catenin, DOK3, NLRP3, and phospholipase C (Dietrich, Yang*et al.* , 2014; Gao, Qu *et al.* , 2020; Peters, Rabe *et al.* , 2022; Zhang, Chen *et al.* , 2022), small GTPases such as ras/rho and dynamin (Peters, Rabe *et al.* , 2022), kinases PI3K, Akt, ERK1/2, JNK, and p38 (Gao, Qu *et al.* , 2020; Meng, Zhang*et al.* , 2017; Park, Yoon *et al.* , 2018; Recio, Lucy*et al.* , 2018), and the transcription factors NF-ϰB and STAT3 (Recio, Lucy *et al.* , 2018; Yin, Cheng *et al.* , 2020). Further investigation into the effector proteins downstream of GPR84 may be important when assessing physiological efficacy.

For example, positive allosteric modulators at the G_q-coupled M_1 muscarinic acetylcholine receptor with similar selectivity profiles and similar effects on the binding and calcium responses of acetylcholine were still found to differentially potentiate, i.e. bias, receptor coupling to phospholipases C and D (Marlo, Niswender *et al.* , 2009). Further evaluation revealed that PLD activity is necessary for M_1-dependent long-term depression in the prefrontal cortex, an effect that is implicated in targeting M1 for the treatment of cognitive deficits in schizophrenia and Alzheimer's disease (Moran, Xiang *et al.* , 2019).

In the case of GPR84, the best evidence to the importance of bias is the suggestion that the G_i-based agonist DL-175 promotes equivalent levels of phagocytosis but not chemotaxis as 6-OAU (Lucy, Purvis *et al.* , 2019). However, given the differences in assay time points, cell types, and concentration-response profiles between these assays and those for cAMP production and β-arrestin recruitment, it remains a possibility that other effectors are involved in the distinct responses between phagocytosis and chemotaxis. For example, it has recently been shown that GPR84 couples to $G_{\alpha 15}$ which results in phospholipase C activity, ERK phosphorylation, and calcium signalling (Peters, Rabe *et al.* , 2022). Given that GPCRs can not only promiscuously couple to a number of G-proteins, but can also 'switch' in coupling preferences over a signalling

time course (Cawston, Redmond *et al.* , 2013), the G_{α} and $G_{\alpha 15}$ pathways may be important pharmacological descriptors to monitor in future experiments.

Conclusions and Future Vision

Since its discovery, GPR84 has undergone a large growth in research activity and clinical trials. While the physiological agonist remains elusive, experiments using potent surrogate agonists from multiple chemical classes demonstrate that GPR84 activation on innate immune cells is pro-inflammatory and results in the activation of cell effector functions. While the physiological context also remains unknown, experiments to date indicate that GPR84 agonists augment inflammation by promoting the mobilisation of inflammatory mediators, stimulating migration and chemotaxis, and enhancing phagocytosis. However, the influence of biased signalling on these responses remains unclear. Further utilisation of recently developed biased and allosteric agonists may help to shed light on this question. In addition, we have highlighted that GPR84 seems to be inherently poorly coupled to the β-arrestin pathway. Most surrogate agonists reported to date exhibit a G-protein bias, and there is a trend towards higher G-protein bias with higher potency agonists. Following receptor desensitisation, either directly using functional assays or potentially by following the phosphorylation of recently identified residues on GPR84, could therefore be an important readout when characterising agonist activity in primary immune cells.

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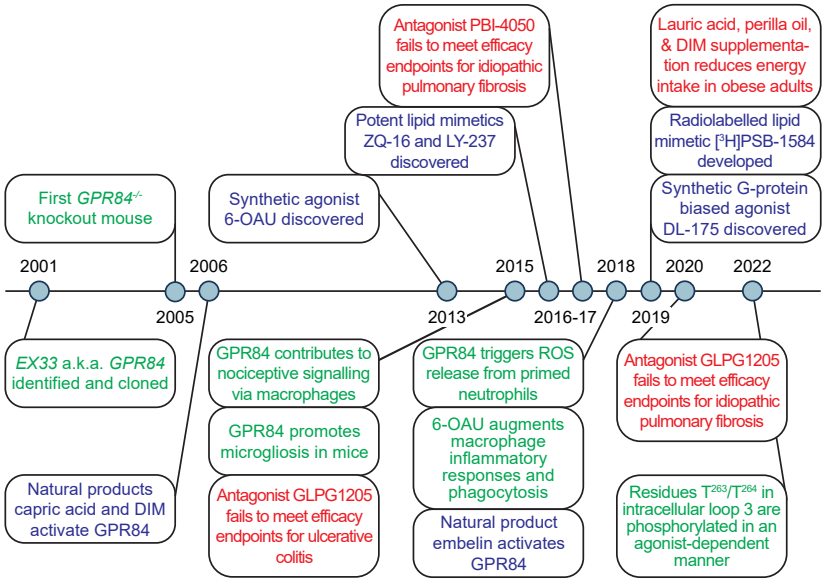

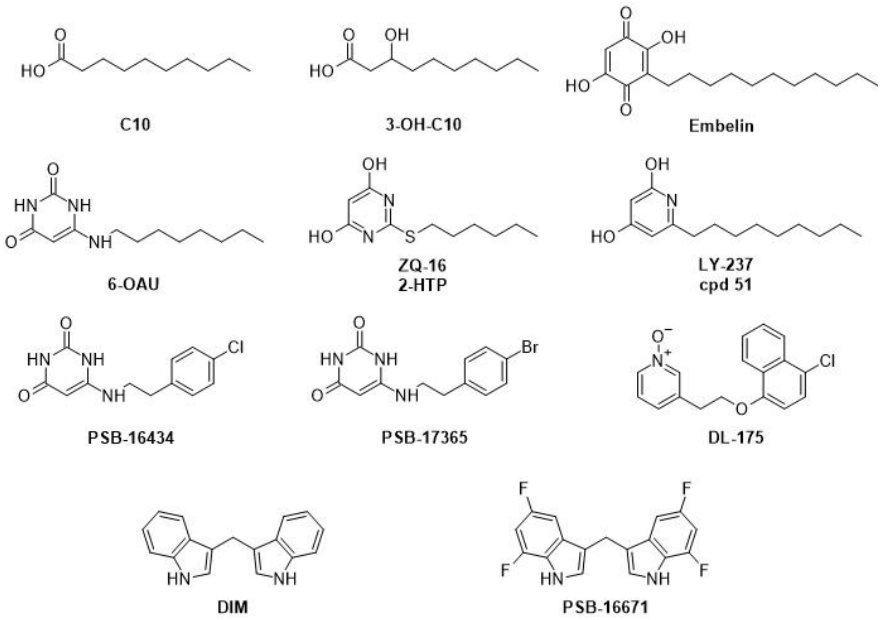

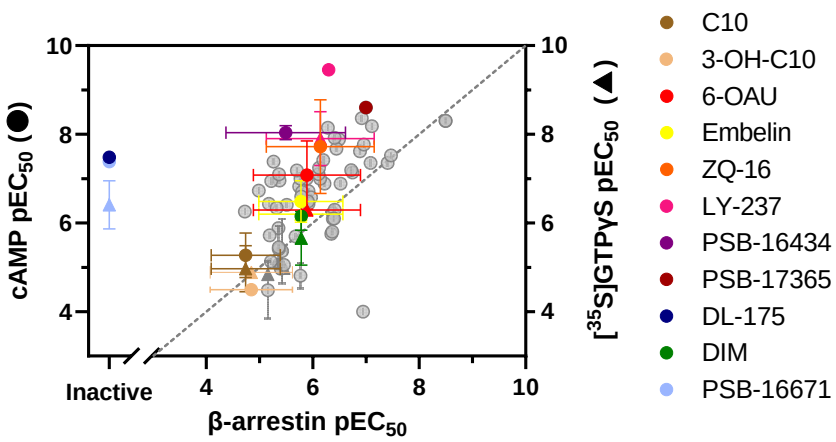

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