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Journal:	*ACS Chemical Biology*
Manuscript ID	cb-2019-00533t.R2
Manuscript Type:	Article
Date Submitted by the Author:	29-Aug-2019

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A biased agonist at immunometabolic receptor GPR84 causes distinct functional effects in macrophages

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ABSTRACT

GPR84 is an orphan G protein-coupled receptor which is expressed on immune cells and implicated in several inflammatory diseases. The validation of GPR84 as a therapeutic target is hindered by the narrow range of available chemical tools and consequent poor understanding of GPR84 pathophysiology. Here we describe the discovery and characterisation of DL-175, a potent, selective, and structurally novel GPR84 agonist and the first to display significantly biased signalling across GPR84-overexpressing cells, primary murine macrophages, and human U937 cells. By comparing DL-175 with reported GPR84 ligands, we show for the first time that biased GPR84 agonists have markedly different abilities to induce chemotaxis in human myeloid cells, while causing similar levels of phagocytosis enhancement. This work demonstrates that biased agonism at GPR84 enables the selective activation of functional responses in immune cells and delivers a high-quality chemical probe for further investigation.

INTRODUCTION

G-protein coupled receptors (GPCRs) expressed on the surface of immune cells control cellular functions that are critical for the maintenance of immune homeostasis and the mounting of inflammatory responses. While some immune GPCRs, such as the chemokine receptors, have been the subject of extensive research and actively pursued as drug targets, other GPCRs expressed throughout the immune system remain poorly characterised and represent a relatively unexploited area for treatments of inflammatory diseases.[1] The free fatty acid receptors (FFARs) are a subset of four immune-expressed GPCRs with metabolic intermediary fatty acids as their endogenous ligands, suggesting a role for dietary fatty acids in the regulation of immunity and inflammation.[2,3] The clinical potential of these receptors has been demonstrated by the small molecule agonist of the free fatty acid 1 receptor, fasiglifam, which showed efficacy in a Phase III trial for diabetes.[4]

GPR84 is the putative fifth fatty acid receptor and is highly expressed in the cells of myeloid lineage that constitute the innate immune system, including monocytes, macrophages and neutrophils in the periphery, and microglia in the brain.[5,6] Saturated fatty acids with chain lengths between C9 and C14 are activators of the receptor, although the weak potency of this interaction means that GPR84 officially remains an orphan receptor.[7] GPR84 expression in leukocytes is markedly upregulated by acute inflammatory stimuli such as lipopolysaccharide (LPS), and tissue from mice exposed to hyperglycaemic or dyslipidaemic conditions associated with chronic low-grade inflammation also shows elevated GPR84 mRNA.[8,9] Activation of the receptor in macrophages has been shown to result in increased cytokine secretion, immune cell migration, and enhanced phagocytosis.[7,9,10] These studies of GPR84 biology almost exclusively use a narrow range of fatty acids or fatty acid mimetics to activate the receptor which may not reflect the physiological activation of GPR84 *in vivo*, given the low potency of the purported natural fatty acid agonists. The possibility of other, as yet undiscovered, endogenous GPR84 ligands increases concern that additional aspects of GPR84 function may be hidden without the use of more diverse chemical tools.

A number of surrogate agonists for GPR84 have been identified in addition to fatty acids (Fig. 1), including natural product embelin **2**,[11] synthetic compound 6-octylaminouracil (6-OAU) **3**,[10] and structurally related compounds with improved potency **4** and **5**.[12,13] All reported orthosteric GPR84 agonists conform to a fatty acid mimetic structure with polar head groups appended to long alkyl or lipophilic chains, although 3,3-diindolylmethane and derivatives have been reported as ago-allosteric GPR84 modulators,[14] and dihydropyrimidinoisoquinolinones are described in the patent literature as GPR84 antagonists.[15] Recently, a series of uracil derivatives with potent activity at GPR84 were reported, including some compounds, such as **6**, with significant G-protein signalling bias.[16] Biased agonism is an emerging field in the study of GPCRs in which agonists for the same receptor can bring about different patterns of downstream signalling. In particular, examples of agonists that selectively activate canonical G-protein pathways or non-canonical β-arrestin dependent pathways have been reported, enabling the separation and selective induction of functional effects where they correspond to a specific pathway.[17] The limited range of GPR84 agonists and paucity of examples with significant signalling bias has meant that the implications of biased agonism at GPR84 on immune cell behaviour or function have not yet been explored.

In this study we set out to expand the range of tools available for investigating GPR84 biology and used a virtual screening strategy to discover a structurally novel agonist, DL-175, that exhibits biased signalling in GPR84-CHO cells. We show that this signalling bias translates into primary murine macrophages and human U937 macrophages, where DL-175 causes unique responses in impedance

signalling assays. Finally, we show that DL-175 and literature GPR84 agonist 6-OAU both cause similar phagocytosis enhancement in U937 macrophages but have drastically different abilities to induce directed migration in U937 macrophages and primary human monocytes. The functional consequences of GPR84 activation are therefore dependent on the ligand used, with significant implications for attempts at using small molecule tools to probe the pathophysiological role of GPR84.

Figure 1: Chemical structures of selected GPR84 agonists. Compounds **1-4** are commonly used GPR84 tool agonists. Compound **5** has been reported to have sub-nanomolar activity at the receptor, and **6** is reported to have G-protein signalling bias.

RESULTS AND DISCUSSION

Discovery of a novel GPR84 agonist

In the absence of a published crystal structure of GPR84, we used a ligand-based virtual screening methodology to identify novel GPR84 agonists (Fig. 2a). A literature dataset comprising 32 compounds structurally similar to 6-OAU was used to construct a predictive quantitative structure-activity relationship (QSAR) model (Forge, Cresset).[13] 10,000 structurally diverse compounds from an in-house library were then screened against the model and 45 compounds selected on the basis of predicted potency and fit to the model were tested in a GPR84-CHO cAMP inhibition assay (Supplementary Table 1). Several hit compounds were identified, including fatty acid mimetics with nanomolar potency. GPR84 active compounds were counter-screened against CHO cells expressing the G_i coupled CB_1 or G_s coupled β_2-adrenergic GPCRs, in addition to untransfected CHO cells (Supplementary Table 2). Among the hit compounds with genuine activity at GPR84, chloro-naphthol imidazole **7** was notable as a novel scaffold of GPR84 agonist (Supplementary Figure 1a-c) and was therefore chosen for further investigation.

We next set out to optimise the small molecule hit **7** into a compound with suitable properties for use as a chemical probe for GPR84.[18] Initially, a preliminary medicinal chemistry optimisation study (Fig. 2b) was performed to enhance the potency of the compound series. Replacing the imidazole heterocycle to pyrazole **8** or pyridyl regioisomers **9** and **11** resulted in a complete loss of activity. 3-pyridyl **10** however, showed enhanced activity and suggested the importance of a hydrogen bond acceptor in the 3-position of the heterocyclic moiety. Oxidation of 4-pyridyl **9** to the equivalent N-oxide **12** improved potency, while oxidation of 3-pyridyl **10** led to the most potent compound in the series, DL-175 (**13**), which has activity akin to that of 6-OAU. The GPR84 specificity of the observed inhibition of cAMP accumulation by DL-175 in GPR84-CHO cells was confirmed by the absence of response in untransfected CHO cells (Supplementary Figure 1d). Regioisomer 2-pyridine N-oxide **14** fails to activate GPR84 and was also confirmed not to inhibit the receptor (data not shown). Compound **14** can therefore serve as a useful, structurally related, inactive control to DL-175.

To establish the GPR84 selectivity of DL-175 against a broad range of receptors, we screened the compound in GPCR profiling panels. In a panel of 14 human GPCRs covering multiple receptor families, DL-175 (3 μM) showed no significant activation of any receptors in cAMP or Ca^{2+} flux functional assays (Supplementary Figure 2a). Notably, no activity was observed at the free fatty acid receptors (FFAR 1-4) which might be expected to have similar ligand binding sites to GPR84 given their similar lipid agonists. In a different GPCR panel using β-arrestin recruitment assays, DL-175 (3 μM) was inactive at all 168 GPCRs tested (Supplementary Figure 2b). Moreover, in antagonist mode, pre-treatment with 3 μM DL-175 did not significantly inhibit the activation of any of the 168 GPCRs with their respective reference agonists (Supplementary Figure 2c). DL-175 therefore has a clean selectivity profile across

a wide range of GPCR targets and in both second messenger functional assays and β-arrestin recruitment assays.

We further characterised DL-175 and its inactive counterpart **14** using chemical and metabolic stability studies. Incubating DL-175 and **14** at 37°C in neutral (pH 7.4), acidic (pH 2.5), or basic (pH 10) buffers resulted in negligible degradation after 3 hours (Supplementary Figure 3a-b). In the case of DL-175, more than 80% of compound remained even after 48 hours, and regardless of pH. In metabolic clearance assays (Supplementary Figure 3c-d), DL-175 showed good stability in murine S9 liver microsomes ($t_{1/2}$ = 73 min) but was rapidly cleared when exposed to whole mouse hepatocytes ($t_{1/2}$ < 10 min), likely precluding its use as an *in vivo* tool compound. The high potency, selectivity, and chemical stability of DL-175, coupled with its inactive negative control **14**, make it an ideal chemical probe for studying GPR84 biology *in vitro*.

Figure 2: Discovery and optimisation of a novel GPR84 agonist. (a) Overlay of 32 compounds used to generate 3D-QSAR model with common areas of positive charge (red), negative charge (blue), and hydrophobic regions (gold) highlighted. The structure of the initial screening hit **7** is shown with the indicated heterocyclic area of focus for the preliminary structure-activity relationship study. **(b)** Activity of compounds with imidazole heterocycle replacements in GPR84-CHO cAMP assay. Efficacy is relative to the maximum effect of capric acid (100 μM). Data shown as means ± SEM of n = 3–4 independent experiments.

DL-175 fails to recruit β-arrestin in GPR84-CHO cells

To understand if the structural novelty of DL-175 confers unique pharmacology at GPR84, we compared it to the most commonly used chemical tool agonist, 6-OAU, in GPR84-CHO cell signalling assays. In inhibition of cAMP accumulation assays, the two ligands show comparable potency and efficacy (Fig. 3a). To investigate the capacity of the ligands to activate alternative downstream signalling pathways, we monitored the recruitment of β-arrestin 2 following receptor activation using β-galactosidase enzyme fragment complementation technology (PathHunter, DiscoverX).[19] The reduced signal amplification of this assay format required relatively high concentrations of agonist to achieve saturating responses, so a non-toxic detergent was used to prevent aggregation of DL-175 and **14** causing spurious positive results (Supplementary Figure 4).[20] 6-OAU stimulation induced a luminescent signal corresponding to GPR84 and β-arrestin association in a dose-dependent fashion (Fig. 3b). Similarly, other GPR84 agonists embelin, ZQ-16, and **5** gave responses in the expected rank order of potency (Supplementary Figure 5).[16] In contrast DL-175 produces no response even at the highest concentration tested, suggesting a bias for G-protein signalling pathways (Fig. 3b). We therefore compared the activity of DL-175 to a previously reported G_i biased GPR84 agonist, PSB-16434 (**6**). DL-175 showed much less activation of arrestin signalling than PSB-16434 despite their relatively small difference in cAMP potency, consistent with DL-175 possessing significant signalling bias (Supplementary Figure 6a-b).

To further validate the G-protein bias of DL-175, we examined if the compound would block the action of a full β-arrestin agonist in GPR84-CHO cells. Pre-incubation with DL-175 reduced β-arrestin recruitment to GPR84 in response to 6-OAU (Supplementary Figure 6c), consistent with effective antagonism of arrestin signalling by DL-175. Finally, as efficacious β-arrestin recruitment can require the expression of G protein-coupled receptor kinase 2 (GRK2), we investigated if hGRK2 transfection would reveal DL-175 agonism of β-arrestin pathways. In fact, DL-175 remained entirely inactive for β-arrestin translocation in GPR84-CHO cells that overexpressed hGRK2 (Supplementary Figure 7). 6-OAU retained activity, but surprisingly showed reduced efficacy in cells with enhanced GRK2 expression, suggesting that other GRK isoforms such as GRK3, GRK5, and GRK6 are primarily involved in phosphorylation of GPR84 and subsequent arrestin recruitment.

The absence of detectable β-arrestin recruitment induced by DL-175 prevents the calculation of formal bias factors, but there is a clear preference for G-protein signalling pathways in comparison to 6-OAU. Given the significant difference in signalling pathway activation between the two ligands, a direct comparison will enable investigation of the consequences of functional selectivity at GPR84 and help in understanding the role of β-arrestin recruitment in creating functional responses following receptor stimulation.

Figure 3: DL-175 is a G-protein biased agonist in GPR84 transfected CHO cells. (a) Dose-response of 6-OAU and DL-175 in an assay measuring the inhibition of forskolin induced cAMP accumulation in GPR84-CHO cells. 6-OAU EC_{50} = 19 nM; DL-175 EC_{50} = 33 nM. Data pooled from n = 3 independent experiments and plotted as means ± SEM of the percentage of the response to forskolin in the absence of agonists. **(b)** Dose-response of agonists in a GPR84-CHO β-arrestin recruitment assay in the presence of 0.025% Tween-80. 6-OAU EC_{50} = 11 μM; DL-175 EC_{50} > 60 μM. Data plotted as the n-fold increase in luminescence over the response to DMSO and shown as means ± SEM from n = 3 independent experiments.

DL-175 induces novel signalling in macrophages

The downstream signalling induced by GPR84 agonists in transfected cell systems has been shown to poorly reflect the signalling observed in primary immune cells.[11] For this reason, we chose to further investigate our biased GPR84 agonist in primary murine bone marrow-derived macrophages (BMDMs) that express the receptor and associated signalling machinery at physiological levels. A label-free electrical cell impedance assay that enables rapid morphological changes to be monitored in real-time was employed to give an integrated readout of cellular signalling following GPR84 stimulation.[21]

As the expression of GPR84 mRNA in macrophages is heavily regulated by their activation state,[8,9] initially GPR84 agonists were tested in differentially polarised BMDMs and cellular impedance recorded (Fig. 4a-b). Both 6-OAU and DL-175 induced a rapid response in the BMDMs at 1 μM, with the Cell Index (CI) measure of cellular impedance peaking within 10 minutes before decaying to baseline at different rates. For both compounds, the greatest response was observed in LPS stimulated, pro-inflammatory M1 macrophages, with smaller responses observed in alternatively activated IL-4 stimulated M2 cells, and unstimulated M0 cells. These results correlate well with previously reported GPR84 mRNA expression data and subsequent experiments were performed with LPS stimulated macrophages to achieve maximal responses.[9]

Figure 4: DL-175 induces different responses in primary macrophages to other GPR84 agonists. (a) Impedance traces of differentially polarised BMDMs (M1 = 16 h LPS pre-treatment; M2 = 16 h IL-4 pre-treatment; M0 = vehicle pre-treatment) following stimulation with 1 μM GPR84 agonists **(a)** 6-OAU and **(b)** DL-175. **(c)** Normalised dose-response curves generated by quantifying impedance traces of M1 polarised BMDMs according to the magnitude of the initial peak. 6-OAU EC_{50} = 129 nM; DL-175 EC_{50} = 50.3 nM. **(d)** Wild-type M1 polarised BMDMs respond to C5a (10 nM) and GPR84 agonists (1 μM), and **(e)** the response to GPR84 agonists is ablated in GPR84-KO macrophages. **(f)** BMDM impedance traces for several GPR84 agonists (1 μM) and inactive control **14**. **(g)** Quantification of **(f)** showing area under the curve for 60 min following agonist addition. **(h)** Pre-treatment with PTX (200 ng/ml) for 16 h blocks impedance response of M1 polarised BMDMs to 6-OAU and DL-175 (1 μM) as illustrated in the quantification in **(i)** showing area under the curve up to 60 min. Impedance traces are shown as the pooled means from n = 3–4 biological replicates and dose-responses and quantifications are shown as pooled means ± SEM from 3-4 biological replicates. Statistical analysis was conducted using one-way ANOVA with Sidak's multiple comparison test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 for indicated comparisons.

Quantifying the M1 polarised BMDM impedance traces by the magnitude of the initial response demonstrated that 6-OAU (EC_{50} = 129 nM) and DL-175 (EC_{50} = 50 nM) have comparable potencies in primary macrophages (Fig. 4c), as expected from the similar EC_{50} values observed in transfected cell cAMP accumulation assays. The GPR84 specificity of these responses at 1 μM was demonstrated by

their absence in GPR84-knockout macrophages, which respond normally to complement component C5a (Fig. 4d-e). We next compared the impedance trace of DL-175 to a range of published GPR84 ligands. The structurally similar agonists 6-OAU, ZQ-16, and **5** (Fig. 1) each induce a rapid increase in cell index which returns to the baseline within 10 minutes (Fig. 4f). In contrast, DL-175 causes a sustained response in which the cell index remains elevated for over 30 minutes. Plotting the area under the curve of the impedance traces illustrates the differences between agonists and suggests novel signalling is occurring with DL-175 (Fig. 4g). The inactive DL-175 analogue **14** fails to induce any response in the BMDMs as expected. As GPCR mediated impedance responses are linked to changes in the actin cytoskeleton,[22] it is likely that DL-175 causes alternative cytoskeletal rearrangement when compared to other GPR84 agonists.

We next set out to establish if the difference in responses observed for the G-protein biased agonist DL-175 were a consequence of the absence of β-arrestin signalling. First, we used the $G_{\alpha i}$ inhibitor pertussis toxin (PTX) to block the expected G protein signalling pathway. The responses to both 6-OAU and DL-175 were ablated by PTX pre-treatment (Fig. 4h-i), suggesting that $G_{\alpha i}$ protein engagement is essential for creating the cytoskeletal response. This is consistent with reports suggesting that β-arrestin signalling alone is not detectable with label-free biosensor assays.[23] To investigate if β-arrestin interactions could modify the shape of the impedance response, we employed an inhibitor of G protein-coupled receptor kinases (GRK) 2 and 3, CMPD101, that would be expected to limit phosphorylation of GPR84 and thus indirectly prevent the recruitment of β-arrestin to the receptor.[24] We treated BMDMs with CMPD101 at various concentrations and then measured the impedance response to 6-OAU or DL-175 stimulation (Supplementary Figure 8a-b). Quantification by measuring the AUC demonstrates that increased GRK inhibition is associated with more sustained responses to 6-OAU, such that at high concentrations of CMPD101, the 6-OAU response resembles that of DL-175. GRK2/3 inhibition also results in a more sustained response to DL-175, although the effect is much less pronounced than for 6-OAU (Supplementary Figure 8c-d). These data suggest that differences in GRK regulation of GPR84 signalling underlie the singular macrophage response to DL-175. Both β-arrestin 2 and GRK2 are expressed in pro-inflammatory macrophages,[25] however arrestin knockdown experiments will be required to confirm if GPR84-arrestin interactions induced by GRKs are directly responsible for our cell impedance observations.

To determine if human macrophage populations would also exhibit differing responses to the GPR84 agonists, we used PMA differentiated human U937 macrophage-like cells that express GPR84 to a similar level as primary macrophages.[26] As expected, DL-175 stimulation of M1 polarised U937 cells resulted in a significantly more sustained impedance response than treatment with 6-OAU (Supplementary Figure 9). Inactive analogue **14** elicited no response, while pre-incubation with a reported GPR84 antagonist[9,15] blocked all responses except that to C5a, confirming the specificity of the induced signal. The differential signalling observed with DL-175 therefore extends to a human myeloid cell-line.

DL-175 and 6-OAU cause different functional responses in immune cells

As cytoskeletal rearrangement is fundamental to the initiation of macrophage functional responses such as phagocytosis and chemotaxis,[27,28] we next investigated if biased GPR84 agonists might show differences in their regulation of these processes. We have previously shown that 6-OAU activation of GPR84 in murine macrophages is associated with enhanced phagocytic capacity.[9] To see if DL-175 would have similar effects on phagocytosis, we used flow cytometry to measure the uptake of opsonised FITC labelled 2 μm beads in M1 polarised U937 macrophages (Fig. 5a-d). Stimulation with both 6-OAU and DL-175 (1 μM) prior to addition of beads led to a small, but statistically significant augmentation of bead phagocytosis (Fig. 5e-f), which GPR84 inactive DL-175 analogue **14** failed to replicate. To confirm that we were measuring phagocytosis of the beads and not simply adhesion, we

used a confocal fluorescence microscopy Z-stack to show that the beads were fully encapsulated within the cell (Supplementary Figure 10). Both GPR84 agonists therefore show similar effects on human U937 macrophage phagocytosis of opsonised beads, despite their different signalling bias.

We next determined the ability of the GPR84 agonists to act as chemoattractants, given the literature describing GPR84 as a chemotactic receptor in various immune cells.[10,15] For this we used a modified Boyden chamber assay where cells seeded into an upper chamber migrate along a chemoattractant gradient through a porous membrane, before adhering to an electrode in the lower chamber.[29] The migration of cells can therefore be observed in real time by increases in the cell index measure of cellular impedance.

Figure 5: 6-OAU and DL-175 both enhance phagocytosis in U937 macrophages. (a-d) Representative flow cytometry plots of U937 cells incubated with fluorescently labelled beads and different compound pre-treatments. Cells with beads are gated according to FITC fluorescence. **(e)** Representative histogram showing the frequency of cells with different levels of fluorescence. Peaks corresponding to different numbers of phagocytosed beads are visible. **(f)** Pooled data showing the percentage of cells with beads as n-fold change over DMSO. Data are means ± SEM for n = 6 independent experiments. Statistical analysis was conducted by one-way ANOVA with Sidak's multiple comparisons correction. ns = not significant, **P < 0.01 compared to DMSO treatment.

6-OAU provoked chemotaxis of M1 polarised U937 macrophages with a classical bell-shaped concentration dependence that is characteristic of chemotaxis (Fig. 6a-b).[30] In contrast, G-protein biased agonist DL-175 failed to induce significant migration of U937 cells at the same concentrations despite their comparable potencies in GPR84-CHO cAMP assays. The GPR84 specificity of the 6-OAU induced migration was demonstrated by pre-treatment with a GPR84 antagonist, which blocked migration of U937 cells to 6-OAU, while not affecting migration to complement component C5a (Fig. 6c-d). Pre-treatment with $G_{i/o}$ inhibitor PTX also blocked U937 cell chemotaxis towards 6-OAU, indicating that G-protein signalling is required for GPR84 mediated migration. The failure of G protein biased DL-175 to promote macrophage chemotaxis suggests that additional signalling machinery beyond G proteins must also be engaged following GPR84 stimulation to enable a migratory response. Biased signalling at the receptor therefore enables the selective activation of functional responses in human U937 macrophages.

Finally, we investigated the responses of primary human monocytes to the two GPR84 agonists. Monocytes express GPR84 in their basal state, but the role of the receptor in promoting monocyte chemotaxis has not yet been described despite the importance of the process in chronic disease states such as atherosclerosis.[31] We used magnetic-activated cell sorting to isolate primary human monocytes from samples taken from healthy volunteers and then confirmed that the resulting cell population comprised more than 80% $CD14^+$ monocytes using flow cytometry (Supplementary Figure 11). In real-time chemotaxis assays, both 6-OAU and DL-175 induced migration of human monocytes across a broad range of concentrations, demonstrating for the first time the role of GPR84 in regulating monocyte recruitment (Fig. 6e-f). However, the concentration dependence of monocyte migration for 6-OAU and DL-175 is quite different, despite their similar potency in GPR84-CHO cAMP assays. At 1 µM and above, for example, DL-175 is a significantly less effective chemoattractant than 6-OAU (Fig. 6e), but at lower concentrations DL-175 induces comparable migration (Supplementary Figure 11d). The two structurally distinct GPR84 agonists with relative signalling bias therefore exhibit distinct functional behaviour across multiple innate immune cell types, including primary human myeloid cells.

Figure 6: DL-175 is a less effective chemoattractant than 6-OAU for human U937 macrophages and human monocytes. (a) U937 cells migrate towards GPR84 agonists (1 μM) in a real time chemotaxis assa y, baseline corrected for migration towards vehicle. **(b)** Dose-response curves for U937 chemotaxis to GPR84 agonists quantified by slope analysis. **(c)** Real time chemotaxis of U937 cells towards C5a (10 nM) or 6-OAU (333 nM), pre-treated with either DMSO for 1 h, GPR84 antagonist (10 uM) for 1 h, or PTX (200 ng/ml) for 2 h. Data shown with each pre-treatment group baseline corrected for migration to vehicle. **(d)** Quantification by slope analysis shows how pre-treatments affect migration of U937 cells to C5a (10 nM) or 6-OAU (333 nM). **(e)** Primary human monocytes migrate towards GPR84 agonists (1 μM), baseline corrected for migration towards vehicle. **(f)** Dose-response curves for primary human monocyte chemotaxis to GPR84 agonists quantified by slope analysis. For **(a)**, **(c),** and **(e)** data are each representative figures of n = 3 independent experiments with 2–3 technical replicates per condition. For **(b)**, **(d),** and **(f)** data are pooled means ± SEM from n = 3 independent experiments and n = 3 donors for primary monocytes. Statistical analysis was conducted by two-way ANOVA with Sidak's multiple comparisons comparison. ns = not significant, * P < 0.05, ** P <0.01, *** P < 0.001, **** P < 0.0001 for 6-OAU vs DL-175 in **(b)** and **(f)** and indicated comparisons in **(d)**.

Discussion

In this study we report the discovery and characterisation of a structurally novel, potent, and selective GPR84 agonist chemical probe. Unlike previously reported ligands, DL-175 shows significant biased signalling at GPR84 in overexpressing cell-lines and induces markedly different signalling in primary murine macrophages and human U937 macrophages in an impedance assay. Through a direct comparison between our new probe and fatty acid mimetic 6-OAU, we show for the first time that activation of GPR84 with different agonists can produce distinct effects in key macrophage functional assays. GPR84 has previously been described as a pro-inflammatory receptor and a potential therapeutic target for chronic inflammatory diseases through the use of a narrow range of potent synthetic surrogate agonists.[9–11,32] Our work demonstrates that more diverse small molecule probes can reveal a greater range of GPR84 functional behaviour, and we provide a new chemical tool for further elucidation of the receptor's pathophysiological role.

It is important that chemical probes for biological targets are well characterised to avoid drawing erroneous conclusions with low-quality probes that may be unstable, poorly selective, or acting through non-specific mechanisms.[18,33] DL-175 is chemically stable across a wide pH range and therefore suitable for *in vitro* studies, even if metabolic liabilities may preclude its use *in vivo*. The off-target effects of GPR84 tool compounds such as embelin[34] and diindolylmethane[19,35] (DIM) can confound attempts to study GPR84 biology. Even reported GPR84 probes optimised by medicinal chemistry have shown poor selectivity, as was highlighted recently for DIM derivative PSB-16671 which retained the ability to activate G_i proteins in GPR84-KO neutrophils.[36] We used GPCR screening panels to demonstrate the selectivity of DL-175, and further used genetic knock-outs and GPR84 antagonists to demonstrate the GPR84 specificity of the responses elicited by DL-175 in GPR84-CHO cells, U937 cells, and primary murine macrophages. We also report inactive analogue **14** for use as a structurally matched negative control with DL-175. Further characterisation of DL-175 as a chemical probe will focus on determining its binding mode to GPR84. As DL-175 was developed through ligand comparison with compounds that occupy the orthosteric binding site, we predict that it also occupies the same pocket with the polar N-oxide motif acting as charge partner for the Arg172 residue involved in MCFA-GPR84 binding.[37] Future structural studies to confirm the binding mode of DL-175 may provide insight into the origin of signalling bias at GPR84.

We first observed the signalling bias of DL-175 in GPR84 over-expressing cell lines. The enhanced levels of receptor expression and artificial coupling in such systems do not always reflect physiologically relevant cells, exemplified by the observation that GPR84 agonists do not inhibit cAMP production in primary macrophages as seen in recombinant cells, but instead potentiates cAMP production through a $G_{i\beta\gamma}$ dependent mechanism.[11] We therefore sought to compare the agonists in more biologically representative systems. Impedance sensing technology allows the dynamic measurement of intracellular signalling using a label-free system appropriate for difficult-to-transfect primary cells. The

responses generated by cells in impedance sensing are not always easily attributable to specific pathways, although GPCR mediated impedance signals have been shown to be associated with actin cytoskeletal dynamics through Cytochalasin D inhibition of actin polymerisation.[22] We observed a marked difference in primary murine macrophage and human U937 macrophage responses to the biased ligands that was sensitive to GRK 2/3 inhibition. Notably, in GPR84-CHO cells, the difference in cellular impedance response between GPR84 agonists was not observed (data not shown), suggesting that this difference in response may only manifest in cells with physiological levels of receptor coupling. This further serves to highlight the limitations of comparing agonists with subtle downstream signalling differences in artificial transfected cell systems.

Consistent with their differing impacts on macrophage impedance signalling assays, 6-OAU and G-protein biased DL-175 have strikingly different abilities to provoke chemotaxis in human U937 macrophages. This finding is in line with a body of evidence implicating β-arrestin recruitment as a prerequisite for the initiation of chemotaxis. The β-arrestin dependence of chemotaxis was first shown by the impaired chemotaxis of β-arrestin 2 deficient lymphocytes towards CXCL12,[38] and has subsequently also been demonstrated in non-chemokine GPCRs such as protease-activated receptor 2 (PAR-2)[39] and angiotensin-II type 1A receptor (AT1AR).[40] Furthermore, the ability of a G-protein biased ligand to induce less chemotaxis by avoiding β-arrestin recruitment was recently demonstrated by comparing biased ligands at the CXCR3 receptor in human T-cells.[41] The role of β-arrestin in macrophage chemotaxis is relatively unexplored, although it has been shown that CCL5 induced chemotaxis of primary human macrophages requires cooperative signalling between G-protein and β-arrestin pathways.[42] Interestingly, G-protein biased DL-175 showed a greater capacity to induce chemotaxis in monocytes than macrophages, suggesting the initiation of monocyte migration is less dependent on β-arrestin signalling. Functionally selective ligands represent an opportunity to investigate how activation of chemotactic receptors without inducing chemotaxis may affect immune cell behaviour. DL-175, for example, could be envisaged to enhance macrophage phagocytosis and clearance of sites of inflammation by efferocytosis without also causing further recruitment of immune cells by chemotaxis.

Over a decade since medium-chain fatty acids (MCFAs) were first established as low-potency agonists for GPR84, it remains unproven as to whether circulating levels of MCFAs can be routinely high enough to activate the receptor *in vivo*. Dietary MCFAs are believed to be rapidly metabolised in the liver, such that the human plasma concentration of capric acid is generally below 0.5 μM: a level significantly below that required for appreciable GPR84 activation.[43] Alternative endogenous GPR84 ligands may activate different signalling pathways, or bind allosterically at one of the multiple GPR84 small molecule binding sites.[37] Precedent for this can be found in the chemokine system, where numerous endogenous ligands behave as biased agonists at the same receptor, creating distinct rather than redundant responses.[44,45] Moreover, both natural and synthetic chemokine receptor ligands with signalling bias have been demonstrated to induce different cell migratory responses,[41,46,47] with clear parallels to our study with synthetic GPR84 ligands.

The prospect of alternative, or additional, endogenous GPR84 ligands with signalling bias has far-reaching implications for the therapeutic targeting of the receptor. GPR84 is generally considered a pro-inflammatory receptor and the development of small molecule antagonists for the treatment of chronic inflammatory diseases is ongoing. However, the only clinical test of GPR84 antagonists to-date resulted in failure due to a lack of efficacy in phase II trials for ulcerative colitis, suggesting that the pathophysiological role of GPR84 remains poorly understood.[48] Other studies instead suggest that GPR84 activation may be beneficial in diseases such as atherosclerosis and Alzheimer's disease.[11,49] Exploiting functional selectivity at GPR84 may allow agonists to specifically activate beneficial pathways mediated by GPR84, for example phagocytosis, while avoiding adverse effects. High quality,

biased, chemical probes for GPR84, such as DL-175, will be invaluable in identifying and validating these alternative therapeutic opportunities.

ASSOCIATED CONTENT

Supporting Information

Supporting Information Available: This material is available free of charge via the Internet.

Supplementary Figures and Tables; biological and statistical methods; chemical synthesis; compound characterisation and spectra.

ACKNOWLEDGEMENTS

This work was supported by the BHF Centre of Research Excellence (RE/13/1/30181), and grants from the Novo Nordisk Foundation (NNF15CC0018346) and the British Heart Foundation (RG/15/10/23915).

AUTHOR CONTRIBUTIONS

DL designed the research, synthesised compounds, performed the experiments, analysed the data and wrote the paper. LZ, GP and CR prepared primary cells and performed flow cytometry experiments. CJRB synthesised compounds and performed waterLOGSY experiments. MC and GW supervised the chemistry. AJR and DRG conceived and supervised the project, designed the research, and wrote the paper. All authors edited the manuscript.

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569

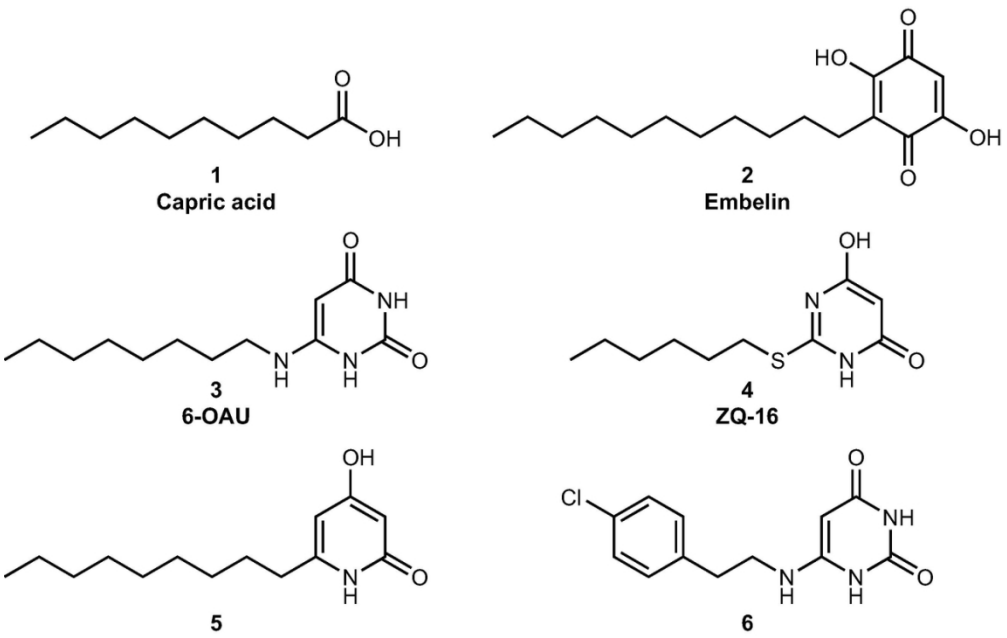
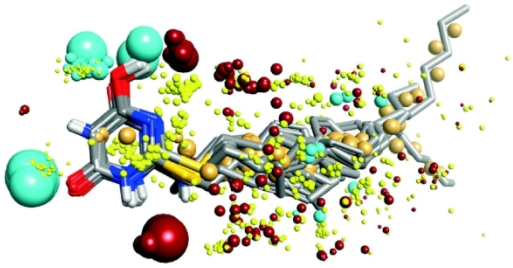


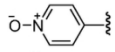
Figure 1

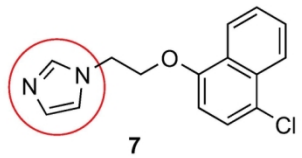
111x70mm (300 x 300 DPI)

a

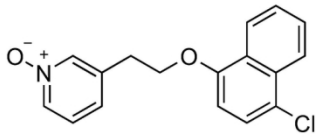


b

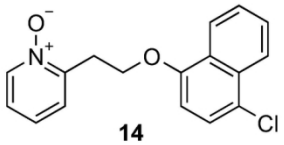
Compound	Heterocycle	EC_{50} (μM)	pEC_{50}	Efficacy (%)
Capric acid	—	1.23	5.91 ± 0.07	100
6-OAU	—	0.019	7.72 ± 0.06	97
7		1.23	5.91 ± 0.09	95
8		> 10	< 5	12
9		> 10	< 5	52
10		0.558	6.25 ± 0.02	96
11		> 10	< 5	6
12		0.285	6.55 ± 0.15	97
DL-175 (13)		0.033	7.48 ± 0.05	102
14		> 10	< 5	21



7
Screening hit
EC_{50} = 1.23 μM



DL-175 (13)
Optimised probe
EC_{50} = 0.033 μM



14
Inactive control
EC_{50} > 10 μM

Figure 2

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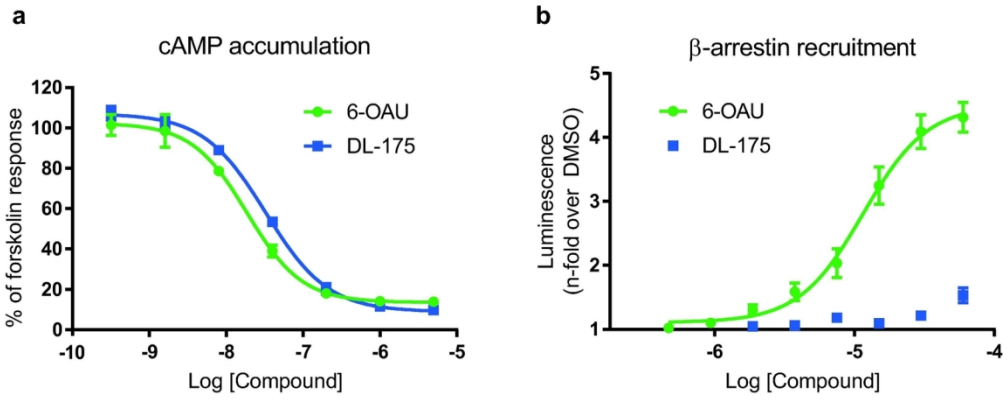

Figure 3

139x54mm (300 x 300 DPI)

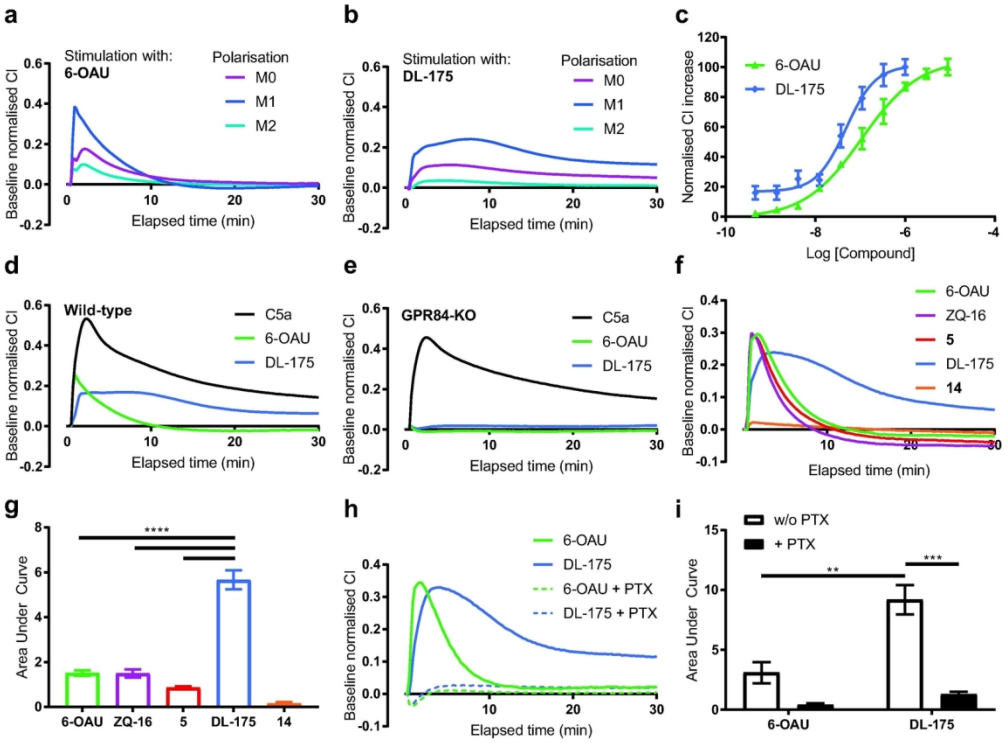

Figure 4

139x103mm (300 x 300 DPI)

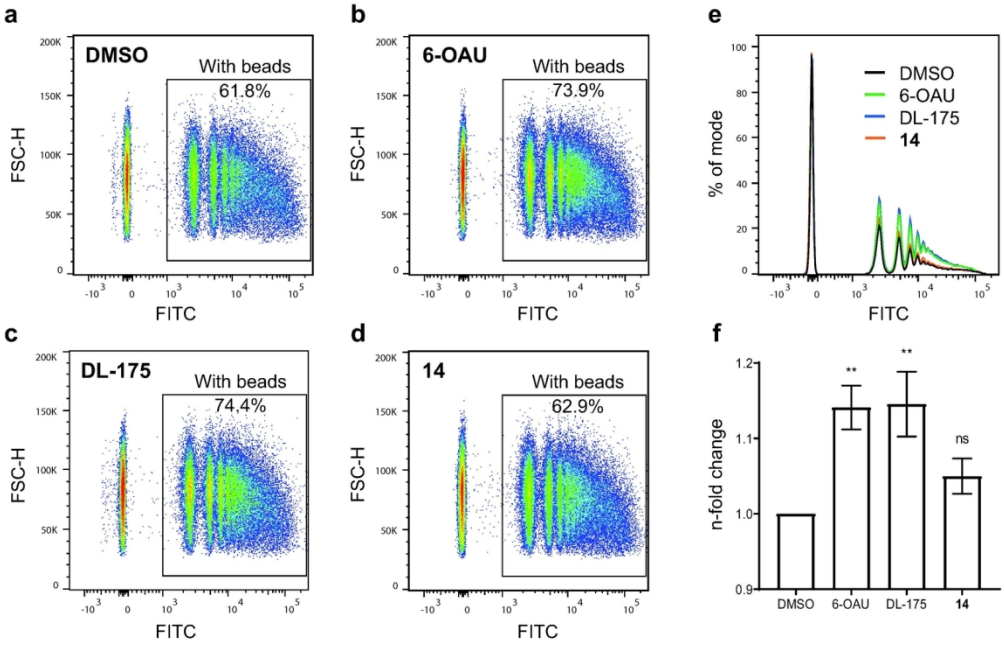

Figure 5

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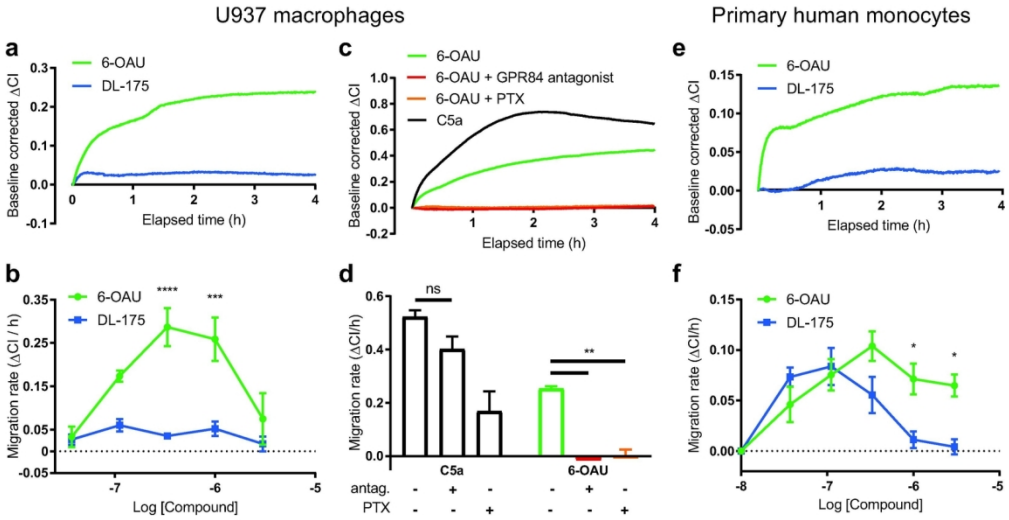

Figure 6

140x72mm (300 x 300 DPI)

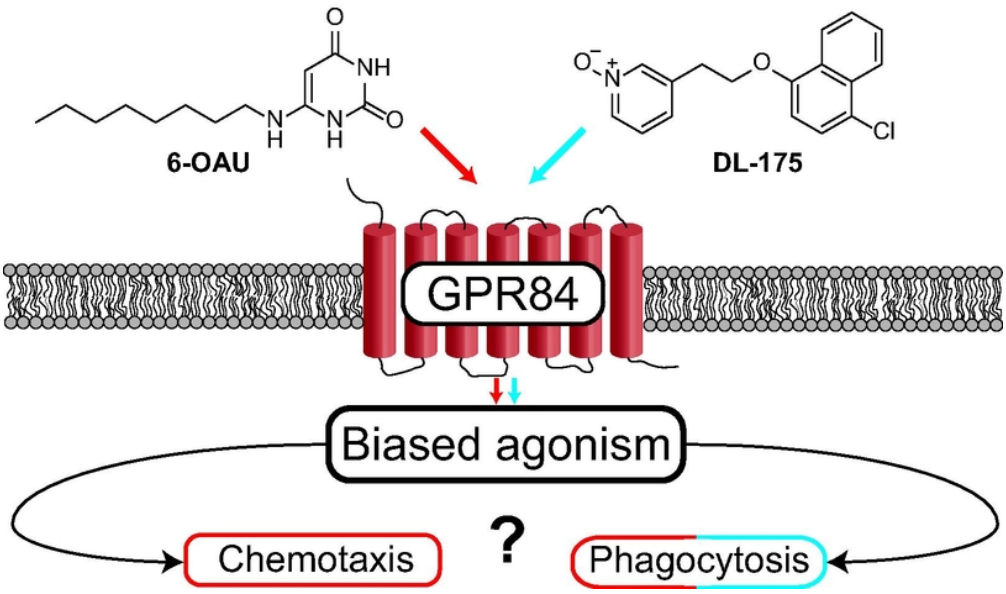

Table of Contents Graphic

68x40mm (300 x 300 DPI)