Multi-pathway *in vitro* pharmacological characterisation of specialised pro-resolving G protein-coupled receptors (SPM-GPCRs)

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Running title

Pharmacological profiling of specialised pro-resolving GPCRs

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List of abbreviations

ACT-389949, N-(2-([4-(1,1-difluoroethyl)-1,3-oxazol-2-yl]methyl)-2H-1,2,3-triazol-4-yl)-2-methyl-5-(3-methylphenyl)-1,3-oxazole-4-carboxamide; ALX, lipoxin A4 receptor; AnxA1, annexin A1; BRET, bioluminescence resonance energy transfer; Compound 43, N-(4-chlorophenyl)-N'-[2,3-dihydro-1-methyl-5-(1-methylethyl)-3-oxo-2-phenyl-1H-pyrazol-4-yl]-urea; FACS, Fluorescence-activated cell sorting; FPR2, formyl peptide receptor 2; LTB₄, leukotriene B₄; LXA₄, lipoxin A₄; N-Arachidonoyl Glycine, NAGly; NPD1, neuroprotectin D1; PMN, polymorphonuclear leukocytes; RvD1, resolvin D1; RvD2, resolvin D2; RvE1, resolvin E1; SAA, serum amyloid A; SPM, specialised pro-resolving mediators

ABSTRACT

Specialised pro-resolving mediators (SPMs) and their cognate G protein-coupled receptors are implicated in autoimmune disorders including chronic inflammation, rheumatoid arthritis, systemic sclerosis, and lupus erythematosus. To date six GPCRs have been paired with numerous endogenous and synthetic ligands. However, the function and downstream signalling of these receptors remains unclear. To address this knowledge gap, we systematically expressed each receptor in a HEK293-Flp-In-CD8a-FLAG cell system. Each receptor was pharmacologically characterised with both synthetic and putative endogenous ligands across different signalling assays, covering both G protein-dependent (G_s , G_i and G_q), and independent mechanisms (β -arrestin2 recruitment). Three orphan GPCRs previously identified as SPM receptors (GPR18, GPR32 and GPR37) failed to express in HEK293 cells. While we were unsuccessful in identifying an endogenous ligand for FPR2/ALX, with only a modest response to fMLP, we did reveal clear signalling bias away from ERK1/2 phosphorylation for the clinically tested agonist ACT-389949, adding further evidence for its poor efficacy in two phase I studies. We also identified neuroprotectin D1 as a new BLT₁ agonist, implying an alternative target for the neuroprotective effects of the ligand. We confirmed activity for RvE1 at BLT₁ but failed to observe any response at the chemerin₁ receptor. This study provides some much-needed clarity around published receptor-ligand pairings but indicates that the expression and function of these SPM GPCRs remains very much context-dependent. In addition, the identification of signalling bias at FPR2/ALX may assist in guiding design of new FPR2/ALX agonists for the treatment of autoimmune disorders.

SIGNIFICANCE STATEMENT

To our knowledge, this is the first study to comprehensively show how several natural mediators and synthetic ligands signal through three specialised pro-resolving mediator GPCRs using multiple ligands from different classes across four to six endpoint signalling assays. This study investigates receptor poly-pharmacology and discovers new ligand pairings, refutes others, and identifies biased agonism in FPR2/ALX pharmacology. This study further highlights the potential of these receptors in treating specific autoimmune diseases including rheumatoid arthritis, systemic scleroderma, and systemic lupus erythematosus.

INTRODUCTION

Resolution of inflammation involves specialised pro-resolving mediators (SPMs) that tightly regulate inflammation and tissue repair. Following the pro-inflammatory phase, where polymorphonuclear leukocytes (PMNs) are recruited to the injury site, resolution describes the process of PMN apoptosis and efferocytosis by infiltrating macrophages. During this phase a range of SPMs are released from apoptotic PMNs and recruit macrophages that drive resolution (Park et al., 2020). Chronic inflammation results from inadequate or dysregulated resolution (Fullerton and Gilroy, 2016), however, the role that individual SPMs and their target G protein-coupled receptors (GPCRs) play in these processes remains to be fully elucidated.

SPMs are a diverse set of lipid mediators synthesised from polyunsaturated fatty acids and include E- and D-series resolvins, protectins, maresins, and lipoxins (Chiang and Serhan, 2017). SPMs, and the pro-resolving proteins chemerin, annexin A1 (AnxA1) and serum amyloid A (SAA), are reported to mediate their effects via six GPCRs: leukotriene B₄ receptor 1 (BLT₁), chemerin receptor 1 (chemerin₁), *N*-Formyl peptide receptor 2/lipoxin A₄ receptor (FPR2/ALX), and the orphan receptors GPR18, GPR32, and GPR37 (Park et al., 2020).

This system is highly complex, reportedly exhibiting both ligand poly-pharmacology (a ligand interacting with multiple target receptors) and receptor promiscuity (a receptor that binds multiple endogenous ligands; Arienti et al., 2019; Park et al., 2020). These SPM-GPCR pairings have been determined using a range of different techniques and assay formats.

BLT₁ is a well-established target of LTB₄ (Yokomizo et al., 1997), that when activated inhibits cAMP accumulation (Arita et al., 2007; Yokomizo et al., 1997), generates calcium influx (Ito et al., 2002; Yokomizo et al., 1997), and drives Akt/PI3K activation and NFκB inhibition (Arita et al., 2007). It has been suggested that RvE1 is a partial agonist with low nanomolar affinity for BLT₁, capable of antagonising LTB₄ (Arita et al. 2007). *In vivo*

observations of RvE1 activity at BLT₁ and chemerin₁ – which displays higher affinity for RvE1 than chemerin itself (Arita et al., 2007) – produced mixed results. RvE1-mediated increases in phagocytosis by macrophages (Ohira et al., 2010) was abolished in *Cmklr1* knockout mice (Laguna-Fernandez et al., 2018), while RvE1-mediated inhibition of PMN recruitment in a zymosan-A-induced peritonitis model was significantly reduced in *Ltb4r1* knockout mice (Arita et al., 2007).

FPR2/ALX, that was orphanised by its high sequence homology with FPR1 and activation by the low affinity formyl peptide *N*-formylmethionine-leucyl-phenylalanine (fMLP) (Ye et al., 1992), is reportedly a target of multiple SPMs, including lipoxin A4 (LXA4), SAA, LL37, AnxA1, and Resolvin D1 (RvD1; Krishnamoorthy et al., 2010).

The receptor is also amenable to the peptides WKYMVm and WKVMVM and the investigational small molecule, ACT-389949 (Maciuszek et al., 2021). In the context of this study, β -arrestin2-mediated FPR2/ALX internalisation by ACT-389949 is thought to underpin loss of therapeutic efficacy in phase I clinical trials (NCT02099071 and NCT0209920; Stalder et al., 2017), suggesting that clinical development of FPR2/ALX ligands with bias away from β -arrestin2 recruitment will improve therapeutic efficacy.

The breadth of SPM ligand and GPCR target pairings, coupled with the different *in vitro* and *in vivo* techniques used indicates a need for clearer, more consistent determination of these designations. In this study we performed a systematic pharmacological exploratory characterisation of 12-15 commercially available SPM ligands from multiple classes (including resolvins, protectins, lipoxins, peptides, lipids, and synthetic molecules) at six SPM GPCRs stably expressed in HEK293 cells across assays targeted at key signalling pathways: cAMP accumulation (stimulation and inhibition), ERK1/2 phosphorylation, GTP γ ³⁵S binding, calcium, and β -arrestin2 recruitment.

We both confirm existing ligand-receptor pairings, including RvE1 as a low potency agonist of BLT₁, and present evidence that questions current pairings. For example, we detected no

activity of RvE1 at chemerin₁. We confirm that fMLP is a weak agonist at FPR2/ALX but were unable to confirm activity of other proposed endogenous ligands at this receptor. More interestingly, we identify new pairings, including neuroprotectin D1 (NPD1) – recently identified as a GPR37 ligand (Bang et al., 2021; Bang et al., 2018) – as an agonist of BLT₁. Finally, using bias analysis, we determined that the FPR2/ALX agonist ACT-389949 is strongly biased away from ERK1/2 phosphorylation relative to the synthetic peptide WKYMVm, providing further context for its limited clinical efficacy.

MATERIALS AND METHODS

Cell line generation and culture

Stably expressing cell lines for each receptor (FPR2/ALX, BLT₁, chemerin₁, GPR18, GPR32 and GPR37) were generated by transfecting HEK293 cells with cDNA encoding for HEK293-Flp-In-CD8a-FLAG-‘GPCR’, at a concentration of 2.4 µg and a cDNA:transfection reagent ratio of 1:2.5, using lipofectamine (LTX; ThermoFisher Scientific, Waltham, MA, USA). Clonal populations were identified and maintained in normal growth medium (Dulbecco’s modified eagle’s medium; DMEM; Thermo Fisher Scientific, 10% foetal bovine serum; FBS; Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO₂. Cells were periodically selected using 200 µg/mL hygromycin B (Sigma-Aldrich, St Louis, MO, USA). All cells were routinely tested for mycoplasma monthly using an in-house developed PCR test and appropriate primers covering 98% of all mycoplasma species.

mRNA extraction, cDNA preparation and qPCR gene expression

Messenger RNA was extracted from the tissue using the Isolate II RNA kit (Bioline, Alexandria, NSW, Australia) as per the manufacturer’s instructions. Complementary DNA was prepared using the Tetro cDNA Synthesis Kit (Bioline, Alexandria, NSW, Australia), as per manufacturer’s instructions. The qPCR reaction mixture was prepared using the SybrGreen I Master Mix (Roche, North Ryde, NSW, Australia) as per manufacturer’s instructions. The qPCR was run using the Sybr Green protocol using a CFX384 Touch™ (Bio-Rad, Gladesville, NSW, Australia) using the following settings: 95°C for 2 min then 40 cycles of 95°C for 15 s, and 62°C for 1 min to measure primer melting temperature. Data were normalised the housekeeper genes *GAPDH* and *ACTB2* with a Ct value of >35 being deemed not detected. Primers (Gene Works, Melbourne, VIC, Australia) used for the study are described in **Table 1**.

Fluorescence-activated cell sorting (FACS) analysis of surface and total expression

Surface receptor expression was measured using FACS analysis, represented by FLAG tag expression. Briefly, 1,000,000 cells were transferred into 1.5 mL Eppendorf tubes and centrifuged at 400 x g for 5 min at 4°C and the supernatant removed. All subsequent steps were performed on ice. The resulting pellet was resuspended in 100 µL of blocking buffer (0.01% Na azide, PBS, 5% BSA, 2mM EDTA) and incubated on ice for 30 min. Following this incubation, 100 µL of 10 µg/mL anti-FLAG tag Alexa Fluor-488 (AF-488) conjugated monoclonal antibody (Invitrogen, Waltham, MA, USA) prepared in blocking buffer was added. The samples were incubated for a further 1 h on ice. To account for cell viability 0.5 uL of 1 mg/mL propidium iodide (PI) was then added to each sample and incubated for further 10 minutes. Following the incubation, the samples were centrifuged at 400 x g for 5 min at 4°C and the supernatant aspirated. The samples were washed once with 100 µL wash buffer (0.04% Na Azide, PBS, 0.5% BSA, 2mM EDTA) and resuspended. The plates were centrifuged at 400 x g for 5 min at 4°C once more and the supernatant aspirated. The final sample was resuspended in 200 µL wash buffer prior to analysis using a FACS Canto II flow cytometer (BD Biosciences, North Ryde, NSW, Australia). Settings used were as follows: AF-488 was detected by a blue laser (488nm) with 530/30 in voltage of 310. Front scatter, side scatter and PI voltages of 250, 450, 380 were applied without compensation. Total receptor expression was measured using the same method described above but with the addition of 0.5% TWEEN® 20 (Sigma-Aldrich, St Louis, MO, USA) in the blocking and wash buffers.

Cyclic AMP (cAMP) accumulation

The accumulation of cAMP was measured using a LANCE ULTRA cAMP Assay Kit (Perkin Elmer, Waltham, MA, USA). Briefly, cells were seeded into transparent 384-well plates at 5,000 cells per well in 25µL stimulation buffer (HBSS, 5mM HEPES, 0.1% BSA and 20 µM rolipram, pH 7.4) at 37°C with 5% CO₂ for 20 min. Compounds were serial-diluted in stimulation buffer directly from stock solutions to 7.4x the final assay concentration indicated

in results. Cells were stimulated with 5 μ L of each agonist for 10 min. cAMP accumulation was stimulated for 20 min by addition of either 5 μ L FSK (5 μ M final assay concentration; for G_i -mediated response), or stimulation buffer (for G_s -mediated response). cAMP levels were detected by the addition of 6.25 μ L Eu-CAMP and 6.25 μ L ULight, pre-diluted in detection buffer (50nM HEPES, 10nM CaCl_2 , 0.35% Triton X-100) at 1:50 and 1:150 respectively and incubated for 60 min at room temperature (RT), in the dark. Fluorescence was measured using an Envision plate reader (Perkin Elmer, Waltham, MA, USA). In all assays a standard curve was generated to allow quantification of cAMP.

ERK1/2 phosphorylation assay

Phosphorylated extracellular signal-related kinase 1/2 (pERK1/2) was measured using AlphaScreen SureFire assay kits (Perkin Elmer, Waltham, MA, USA). Cells were seeded into transparent poly-D-lysine-coated (PDL; 50 μ g/mL; Sigma-Aldrich, St Louis, MO, USA) 96-well plates at 40,000 cells per well and incubated overnight in 90 μ L serum-free DMEM (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C/5% CO_2 . Compounds were serial-diluted in serum-free DMEM from stock at 10x final concentration. All compounds were profiled in the pERK1/2 assay using time-to-peak as agonist exposure time (generally 5 min; *data not shown*). Responses were terminated by removal of drugs and addition of 100 μ L SureFire lysis buffer (TGR Biosciences, Thebarton, SA, Australia), followed by agitation on a plate shaker for 5 min. 5 μ L cell lysate was then added to a white ProxiPlate (Perkin Elmer, Waltham, MA, USA) followed by 8 μ L of 100:600:3:3 ($v^{-1}v^{-1}v^{-1}v^{-1}$) SureFire activation buffer/SureFire reaction buffer/AlphaScreen donor/acceptor beads. All plates were secured with a top seal and incubated for 60 min at 37°C in 5% CO_2 in the dark. Fluorescence was measured using an Envision plate reader (Perkin Elmer, Waltham, MA, USA). FBS (10% $v^{-1}v^{-1}$) was used as a positive control.

GTP γ ³⁵S binding

Cells were grown to 80–90% confluence in 500cm² cell-culture trays at 37°C in 5% CO₂. All subsequent steps were conducted at 4°C to avoid receptor degradation. The cell-culture medium was removed and replaced with ice-cold buffer (10 mL per tray; 10 mM HEPES, 0.9% w/v NaCl, 0.2% w/v EDTA, pH 7.4). Cells were scraped from the trays into a 50 mL Corning tube and centrifuged at 250 x g for 5 min. The supernatant was aspirated and 10 mL per 500 cm² tray of wash buffer (10 mM HEPES, 10 mM EDTA, pH 7.4) added. Pellets were prepared following homogenization (Werker, ultra-turrax, position 6, 4 x 5 s bursts) and centrifugation at 48,000 x g at 4°C (Beckman Avanti J-251 Ultracentrifuge) for 30 min. The supernatant was discarded and the pellet re-homogenised in wash buffer and recentrifuged. The final pellet was suspended in ice-cold assay buffer (10 mM HEPES, 0.1 mM EDTA, pH 7.4) at a concentration of 1–2 mg/mL. Protein concentration was determined by a bicinchoninic acid assay, using BSA as a standard and aliquots maintained at –80 °C until required. Compounds were prepared at 100x concentration in 100% DMSO, and 2.5 µL added to each well in a 96-well assay plate together with 200 µL of assay buffer (20mM HEPES, 10mM MgCl₂, 100mM NaCl, 1mM EDTA, pH 7.4), with 0.1% BSA and 30 µg per mL saponin (added fresh on the day of experimentation), containing 50 µg per mL membranes. 3.7 µM guanosine 5'-diphosphate sodium salt (GDP) was added and the plates incubated at room temperature with gentle agitation for 30min. 50µL of [³⁵S]-guanosine 5'-(γ-thio)triphosphate (GTPγ³⁵S) at a final concentration of 300 pM was then added to each well and incubated at RT with gentle agitation for 40 min. Bound and free GTPγ³⁵S were separated by rapid vacuum filtration using a FilterMateTM Cell Harvester (Perkin Elmer, Waltham, MA, USA) and 96-well GF/C filter plates and rapidly washed three times with ice cold wash buffer (50 mM Tris-HCL, 10 mM MgCl₂, 100 mM NaCl, pH 7.6). After drying (> 4 h), 40 µL of MicroscintTM 20 (Perkin Elmer, Waltham, MA, USA) was added to each well and the radioactivity quantified using single photon counting on a MicroBeta² microplate scintillation counter (Perkin Elmer, Waltham, MA, USA).

Calcium assays

Assays were performed in isotonic buffer (146 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1 mM MgSO₄, 1.5 mM NaHCO₃, 10 mM D-glucose, 10 mM HEPES, 2.5 mM probenecid and 0.5 % BSA, pH 7.4). Assay buffer stock was prepared without probenecid or BSA, sterile filtered and stored at 4 °C until required. For assay, 100 mL of buffer was prepared by adding 0.625 mL of 400 mM probenecid (dissolved in 1 M NaOH) to 90 mL stock buffer, and pH adjusted to 7.4, before being made to the final volume of 100 mL. BSA (0.5% w/v) was added, and buffer used within 24 h.

Cells were seeded into PDL-coated 96-well plates in growth medium at a density of 50,000 cells per well. The following day, plates containing a confluent monolayer of cells were washed once with 200 µL assay buffer, before being loaded with 1 µM Fluo-8-AM in 90 µL assay buffer (Abcam, Cambridge, United Kingdom) and incubated 1 h at 37°C. Compounds were serial-diluted from stock at 10x concentrations and added on a FlexStation 3 microplate reader (Molecular Devices; Sunnyvale, CA, USA). Fluorescence was detected using 485 nm excitation and 525 nm emission filters. Calcium influx responses were measured by the peak fluorescence post ligand addition minus the average basal fluorescence measured. All assays were performed in duplicate, and data normalised to the response of 100 µM ATP.

β-arrestin2 recruitment

β-arrestin2 recruitment assays were performed using Bioluminescence Resonance Energy Transfer (BRET) technology, with Rluc8-fused receptor donors and a Venus-fused β-arrestin2 acceptor (Savage et al., 2013). Bicistronic plasmids containing both receptor donor and β-arrestin2 acceptor for all 6 receptors of interest (CD8a-FLAG-'GPCR'-Rluc8-IRES-βArr2-Venus) were generated for transfection into HEK293 parental cells, a method that guarantees co-expression of both BRET acceptor and donor within the same cells. This bicistronic vector system generates 4-7-fold higher expression of the downstream cistron (Bochkov and Palmenberg, 2006), therefore producing β-arrestin2 acceptor in excess of receptor donor within each co-expressing cell. HEK293 cells were plated at 500,000 cells

per well in 6-well plates in normal growth medium and incubated overnight at 37°C in 5% CO₂. Transfection was performed using a polyethylenimine (PEI) transfection protocol. For each well, 2µg DNA of each receptor/β-arrestin2 bicistronic plasmid were diluted in 125mM NaCl to a final volume of 125µL and combined with 125 µL of 125mM NaCl containing 15µg PEI and incubated at RT for 5 min. PEI/plasmid mix was added to each well and incubated for 24 h at 37°C in 5% CO₂.

Transfected cells were collected by pipetting and medium removed by centrifugation before being resuspended in phenol red-free DMEM (Thermo Fisher Scientific, Waltham, MA, USA) with 5% FBS (3 mL media per well collected). Cells were plated into PDL-coated 96-well white CulturPlates (Perkin Elmer, Waltham, MA, USA) at 50µL per well. Cells were allowed to adhere overnight. Medium was removed and replaced with 80 µL serum-free phenol red-free DMEM for 1 h (37°C), with 10 µL Coelenterazine h (100µM; NanoLight Technology, Pinebox, AZ, USA) added after 55 min, prior to reading BRET1 (480 and 530 nm emission) signals on the PheraStar (BMG Labtech, Mornington, VIC, Australia). After 3 consistent 'basal' reads, 10 µL of compounds were administered in phenol red-free DMEM (Thermo Fisher Scientific, Waltham, MA, USA) and the plate read for a further 11 reads. Each plate contained 'control' wells, where DMEM with no compound was added.

Compounds and Reagents

All compounds were supplied from Cayman Chemical (Ann Arbor, MI, USA) with the exceptions of serum amyloid A and fMLP (Sigma Aldrich, St Louis, MO, USA), chemerin (Enzo Life Sciences, Redfern, NSW, Australia), and WYKMVm (Tocris Bioscience, Bristol, United Kingdom). Compound 43 was a generous gift from Dr Cheng Xue Qin (Monash University, Melbourne, VIC, Australia). ACT-389949 was synthesised by Servier, Suresnes, France. All SPM ligands were serially diluted directly from stock (or intermediate, where noted in **Table 2**) to concentrations indicated in results for each assay in either assay buffer (cAMP accumulation; HBSS, 5 mM HEPES, 0.1% BSA and 20 µM rolipram, pH 7.4; [Ca²⁺]_i

elevation; 146 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1 mM MgSO₄, 1.5mM NaHCO₃, 10 mM D-glucose, 10 mM HEPES, 2.5 mM probenecid and 0.5 % BSA, pH 7.4, and GTPγ³⁵S binding; 100% DMSO) or assay media (ERK1/2 phosphorylation assay; serum-free DMEM (cat #11995-065, Thermo Fisher Scientific, Waltham, MA, USA) and β-arrestin2 recruitment; serum-free, phenol red-free DMEM supplemented with 1mM sodium pyruvate. Radioligand was supplied by PerkinElmer (Waltham, MA, USA). Cell culture and molecular biology reagents were supplied by Life Technologies (Melbourne, Australia) or Thermo Fisher Scientific (Waltham, MA, USA). Radioligand was supplied by Perkin Elmer (Waltham, MA, USA). Cell culture and molecular biology reagents were supplied by Life Technologies (Melbourne, VIC, Australia).

Data analysis

All experiments were analysed by nonlinear regression using Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA). All values reported in the tables are mean + SEM for the indicated number of experiments. For the single concentration analysis, data are presented as the mean + SD normalised to each individual assay control for 3-5 different assay formats. The full concentration response curve data are presented as normalised to the chosen reference ligand for that receptor e.g., FPR2/ALX; WKYMVm, BLT₁; LTB₄, and chemerin₁; chemerin and are shown as mean + SEM.

qPCR data were obtained by normalising the raw expression of genes against two housekeeping genes *GAPDH* and *ACTB2* and calibrator samples, where $\Delta\Delta C_T$ values using the equation as below on Bio-Rad CFX Manager software (Bio-Rad Laboratories software, Hercules, CA). Grouped data are shown as + SD.

$$\text{Normalised Expression}_{\text{sample (GOI)}} = \frac{RQ_{\text{sample (GOI)}}}{(RQ_{\text{sample (Ref 1)}} \times RQ_{\text{sample (Ref 2)}})^{\frac{1}{n}}}$$

RQ refers to the relative quantity of a sample. Ref is the reference housekeeping gene in each sample, where Ref 1 is *GAPDH* and Ref 2 is *ACTB2*. GOI is the gene of interest and 'n' refers to the number of reference targets.

Flow cytometry data was gated against parental HEK293 cells, and the level of expression was presented as the percentage of cells positively stained for FLAG-Alexa Fluor 488 in relation to parental HEK293 or as the mean fluorescence intensity (MFI) of AF488. The data was analysed using FlowJo V10 (LLC, Ashland). Grouped data are shown as mean + SD.

Agonist concentration-response curves were fitted to sigmoidal (variable slope) using a four-parameter logistic equation:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(LogEC_{50} - X)^{nH}}}$$

where Y is the response measured, Top and Bottom denote the maximal and minimal asymptotes, respectively, and 'nH' represents the Hill slope (constrained to unity where appropriate).

β-arrestin2 recruitment data were analysed in MARS Data Analysis Software (BMG Labtech, Ortenburg, Germany) to determine BRET ratio (480nm/530nm), ΔBRET ratios were calculated by subtracting average 'basal' pre-addition signal (to account for minor plating/signal differences) and ΔΔBRET ratios were calculated by further subtracting average 'control' curves (to account for signal drift over time), before analysing the area under the curve of the subsequent time-resolved ΔΔBRET.

Ligand bias at FPR2/ALX was analysed using the method of relative transduction ratios as a measure of ligand bias (Kenakin and Christopoulos, 2013; Riddy et al., 2017). Log(τ/KA) values, which consider both relative potency and maximal response at each pathway, were

normalised to $\Delta\text{Log}(\tau/\text{KA})$ with respect to the $\text{Log}(\tau/\text{KA})$ value for WKYMVm in each pathway, and further normalised ($\Delta\Delta\text{Log}(\tau/\text{KA})$) to cAMP inhibition as the reference pathway (Riddy et al., 2017) using Microsoft Excel (2010); bias plots were generated using the radar plot feature. Where ACT-389949 displayed no response at ERK phosphorylation, it was given the arbitrary minimal value of $10^{\Delta\Delta\text{Log}(\tau/\text{KA})} = 0.001$ for graphing purposes. fMLP was excluded from this analysis due to the incomplete definition of the concentration-response curves.

RESULTS

The stable expression of FPR2/ALX, LTB4R (BLT₁) and CMKLR1 (chemerin₁) in HEK293 cells was confirmed by assessing mRNA and protein expression levels. *FPR2/ALX*, *LTB4R* and *CMKLR1* had similar relative mRNA expression as measured by $\Delta\Delta C_T$ (Fig. 1A). As each receptor was tagged with an N-terminal FLAG epitope, cell-surface protein expression could be assessed by flow cytometry. Percentage of cell surface expression, as shown in Fig. 1B and quantified in Fig. 1C, revealed that FPR2/ALX and LTB4R were both highly expressed, whilst CMKLR1 was more modestly expressed at the cell surface. Using mean fluorescence intensity as a readout of expression within a cell, LTB4R was more abundantly expressed than both FPR2/ALX and CMKLR1 (Fig. 1D). Total expression levels revealed a similar pattern (Supp Fig. 1A-B). After successfully demonstrating suitable levels of expression these cell lines were next assessed for their pharmacological responses.

Of the 15 compounds profiled across the different signalling assays for FPR2/ALX, only four ligands displayed an appreciable response (Fig. 2A-D; Table 3; Supp Fig. 2). As the peptide WKYMVm behaved as a full agonist in each assay, all data was normalised to this reference control. Robust responses were obtained to synthetic peptides (WKYMVm; Fig. 2A) and small molecules (Compound 43 and ACT-389949; Fig. 2B-C). WKYMVm produced a rank order of potencies of GTP γ ³⁵S binding = inhibition of cAMP = [Ca²⁺]_i elevation >> pERK1/2 > β -arrestin2 recruitment (Fig. 2A; Table 3; Supp Fig. 2A-E). Compound 43 also displayed full agonism at FPR2, but with a different rank order of potencies: inhibition of cAMP = pERK1/2 = [Ca²⁺]_i elevation >> GTP γ ³⁵S binding = β -arrestin2 recruitment (Fig. 2B; Table 3; Supp Fig. 2A-E).

ACT-389949, a clinically tested FPR2/ALX small molecule agonist, produced a response in each assay for FPR2/ALX with a rank order of potency of inhibition of cAMP >> [Ca²⁺]_i elevation > GTP γ ³⁵S binding > β -arrestin2 recruitment >> pERK1/2 (Fig. 2C; Table 3; Supp Fig. 2A-E). Interestingly, its response in the pERK1/2 assay was severely reduced compared

to the other pathways tested (Fig. 2C; Supp Fig. 2C). A time course was also performed but no response was observed over 1-hour (*data not shown*)

Of all the putative endogenous ligands profiled at FPR2/ALX, only the non-selective peptide, fMLP, produced measurable responses for cAMP inhibition, GTP γ ³⁵S binding and calcium elevation at the highest concentrations tested (Fig. 2D; Table 3; Supp Fig. 2). No compound produced an increase in cAMP accumulation, suggesting no apparent G_s coupling (Table 3; Supp Fig. 2B).

In the single concentration assay, RvE1, NPD1 and, to a lesser extent LXA4, produced [Ca²⁺]_i elevation responses. However, extension to full concentration response curves failed to produce any meaningful potency estimates (Table 3; Supp Fig. 2D).

Given the failure of ACT-389949 to stimulate pERK1/2, we quantified the extent of the signalling bias for the two small molecule FPR2 agonists. Biased agonism implies that different ligands may induce distinct signalling profiles at the same receptor leading to individual cellular outcomes (Kenakin and Christopolous, 2013; Wootten et al, 2018). Using WKYMVm as a reference agonist and the inhibition of cAMP assay as the reference pathway, $\Delta\Delta\text{Log}(\tau/K_A)$ values (Fig. 2E) show that Compound 43 has strong bias *towards* pERK1/2 and β -arrestin2 recruitment but is markedly biased *away* from GTP γ ³⁵S binding. In addition, ACT-389949 was clearly biased *away* from both GTP γ ³⁵S binding and [Ca²⁺]_i elevation, whereas it was unbiased relative to WKYMVm for the recruitment of β -arrestin2.

BLT₁ was similarly profiled across the same set of assays. The endogenous ligand LTB4 produced full agonist responses in each assay, with rank order potencies of cAMP > pERK1/2 > GTP γ ³⁵S binding >> [Ca²⁺]_i elevation = β -arrestin2 recruitment (Fig. 3A; Table 4; Supp Fig. 3A-D). RvE1, an SPM ligand reported to activate multiple SPM GPCRs including BLT₁, induced responses in three of the five assays with a rank order of potency of inhibition

of cAMP > GTP γ ³⁵S binding > pERK1/2 (Fig. 3B; Table 4,). It produced a minimal response in [Ca²⁺]_i elevation (Supp Fig. 3C), which could not be robustly quantified (Table 4) and failed to activate β -arrestin2 recruitment (Fig. 3B; Table 4; Supp Fig. 3D).

Interestingly, NPD1 was identified as a BLT₁ agonist, demonstrating modest activity in the inhibition of cAMP and GTP γ ³⁵S binding assays (Fig. 3C; Table 4; Supp Fig. 3A), though it failed to produce a response in the remaining assays (Fig. 3C; Table 4; Supp Fig. 3B-D). Similarly, chemerin, an endogenous ligand of the chemerin₁ receptor, displayed weak agonist activity in the inhibition of cAMP assay (Fig. 3C; Table 4; Supp Fig. 3A-D). Both RvD1 and RvD2 (1 μ M) robustly inhibited FSK-induced cAMP accumulation, though neither yielded robust potency estimates in a full concentration response format (Table 4; Supp Fig. 2A). No signalling bias was observed for any ligand at BLT₁ (*data not shown*).

At the chemerin₁ receptor its cognate ligand, chemerin, produced a response in only the inhibition of cAMP and pERK1/2 assays (Fig. 4A; Table 5; Supp Fig. 4A-D). No response was observed in the GTP γ ³⁵S binding, [Ca²⁺]_i elevation or β -arrestin2 recruitment assays. The reported chemerin₁ agonist, RvE1, was inactive in all assays (Fig. 4B; Table 5; Supp Fig. 4A-D). Unlike chemerin and RvE1, NAGly, LTB₄ and NPD1 all produced a clear response in the [Ca²⁺]_i elevation assay when profiled at a single high concentration, though none yielded robust potency estimates in a concentration response format (Table 5; Supp Fig. 2C).

Cell lines for the orphan GPCRs, GPR18, GPR32, and GPR37, were generated using the same HEK293-Flp-In-CD8a-FLAG system as described above. Stable transfection yielded modest mRNA expression for *GPR18* and *GPR37* (Supp Fig. 5A), though this expression translated into very limited surface and total receptor expression as measured by FACS (Supp Fig. 5B-C). Despite a similar stable transfection regimen, there was no detectable

mRNA expression of *GPR32*. Although the expression was extremely low, we profiled each receptor with the same set of ligands across most of the assays as described above.

Perhaps unsurprisingly none of the ligands yielded activity in any assay (Supp Figs. 6A-E), except for small responses (~2-fold over basal) for NPD1 at GPR32 (Supp Fig. 7B) and LXA4 at GPR37 (Supp Fig. 8B) in the cAMP accumulation assay (G_s). Further expression systems for GPR18 (tetracycline inducible HEK293-FlpIn-TREx and transient expression in HEK293 cells using a bicistronic vector containing both the receptor and β -arrestin2) failed to generate any functional responses (*data not shown*).

DISCUSSION

Although in recent years the field of resolution pharmacology has gathered interest and ligand pairings of SPM GPCRs have been extensively investigated, there have been many inconsistencies in these findings. The role of receptor poly-pharmacology and ligand promiscuity adds extra complexities to this field (Park et al., 2020). To complement published findings, we carried out a comprehensive and methodical pharmacological analysis of six SPM GPCRs, using putative endogenous and synthetic ligands across multiple signalling assays.

FPR2/ALX has been paired with multiple pro-inflammatory and pro-resolving ligands, including LXA4, RvD1, AnxA1 and SAA (Ernst et al., 2004; He et al., 2003; Krishnamoorthy et al., 2010; Maderna et al., 2010). We found, in contrast to previous findings, that none of these ligands showed any activity in FPR2 assays, despite a consistent method of ligand handling. Interaction of these ligands with FPR2/ALX has mostly been described in radioligand binding studies, which can reflect selectivity of ligand and competitor thus producing misleading results, and in knockout mouse models (Dufton et al., 2010; Fiore et al., 1992; Krishnamoorthy et al., 2012; Norling et al., 2012) with limited data from functional studies.

The pro-resolving effects of LXA4, including stimulation of efferocytosis, have been suggested to be mediated by FPR2/ALX activation (Maderna et al., 2010). However, other studies questioned these effects with LXA4 unable to produce functional responses in $[Ca^{2+}]_i$ elevation and β -arrestin2 recruitment assays in FPR2-expressing cells (Christophe et al., 2002; Forsman et al., 2011). RvD1 induced β -arrestin2 recruitment at sub-nanomolar potency in the DiscoverX PathhunterTM system when FPR2/ALX was expressed in HEK293 or CHO cell backgrounds (Krishnamoorthy et al., 2010). This result was somewhat surprising, as typically, β -arrestin recruitment potencies from this assay format are close to

their binding affinity (Riddy et al., 2012); this result would suggest other RvD1 functional potencies in the picomolar range.

However, using BRET rather than enzyme complementation, no effect of RvD1 (or any other SPM) was detected in the β -arrestin2 recruitment assay, highlighting clear inconsistencies within the field. AnxA1, a glucocorticoid-mediator protein, also showed no activity in any assay, again at odds with previous western blot analysis of AnxA1-activated ERK1/2 in FPR2/ALX transfected HEK293 cells (Hayhoe et al., 2006).

LXA4, RvD1 and AnxA1 exert pro-resolving physiological effects, including inhibition of neutrophil infiltration, enhancement of apoptosis and efferocytosis of apoptotic neutrophils, and downregulation of proinflammatory mediators (El Kebir et al., 2009; Lee and Surh, 2013; Norling et al., 2012). These actions are putatively linked to FPR2, so their lack of activity in our signalling assays is not clear. It may be possible that in a native context additional receptors or scaffolding proteins (absent in HEK293 cells) are required for these physiological effects, or due to limitations in compound availability and solubility, their effects may only be observed at concentrations higher than those tested within this study.

The SPM ligands, particularly the putative endogenous lipoxins and resolvins, are notoriously labile and prone to issues associated with handling, storage, and preparation. However, wherever possible all ligands were prepared and diluted as recommended by manufacturers and consistent with best practice in the field. For example, we observed robust signalling with RvE1 at BLT₁ indicating that ligand preparation is an unlikely explanation for its lack of activity at other receptors.

The synthetic FPR2/ALX ligands, WKYMVm and Compound 43, induced robust responses across all FPR2/ALX signalling assays, corroborating published data (Forsman et al., 2011; Qin et al., 2017; Zhou et al., 2007). However, in our hands the first-in-class investigational

agonist, ACT-389949, failed to induce ERK1/2 phosphorylation, whilst generating a robust response at other pathways. ACT-389949 progressed to phase I clinical trials but was discontinued due to lack of efficacy on neutrophil recruitment in an LPS challenge model in healthy human subjects (Stalder et al., 2017). Follow-up pharmacological studies revealed G_q-independent transient rises in intracellular Ca²⁺ and recruitment of β-arrestin (Lind et al., 2019), which are further confirmed by our findings.

Activation of the MEK/ERK pathway is crucial for the pro-inflammatory function of neutrophils, including phagocytosis, delayed apoptosis and activation of NADPH oxidase to form neutrophil extracellular trap (NET), that helps destroy microbes (Hakim et al., 2011). Conversely, inhibition of MEK-dependent ERK activation significantly impairs LPS-stimulated neutrophil mediated release of pro-inflammatory cytokines (CXCL8, CCL3, CCL4; Simard et al., 2015), that are essential for further recruitment of neutrophils and other phagocytic immune cells (Tecchio and Cassatella, 2016). ERK1/2 activation is also linked to host immune responses including macrophage activation (Willmann et al., 2020), uptake of mycobacteria (Li et al., 2015) and key effector functions of CD8+ T cells (Crawford et al., 2013). ERK1/2 signalling is implicated in multiple inflammatory diseases, including autoimmune disorders. Defective ERK1/2 signalling is hypothesised to contribute to lupus pathogenesis, causing gene dysregulation and autoreactivity in T cells (Gorelik and Richardson, 2010) and there is T cell-ERK1/2 phosphorylation impairment in patients with active lupus (Deng et al., 2001).

ACT-389949's failure to induce ERK1/2 phosphorylation in HEK293 cells may provide further evidence to explain its limited clinical efficacy, in addition to the observation that it stimulates robust receptor internalisation. Collectively, these effects may need to be considered when developing new synthetic FPR2/ALX small molecule agonists with greater clinical efficacy.

We confirmed previous reports that RvE1 is a BLT₁ agonist, with potencies in the mid-high nanomolar range across different assays. However, previous studies identified RvE1 in serum at low picomolar concentrations (Lotfi et al., 2020), perhaps questioning the physiological role of this ligand at BLT₁. We also verified robust chemerin activity at chemerin₁ in both cAMP inhibition and ERK1/2 phosphorylation assays. However, the ligand showed no activity in GTPγ³⁵S binding, [Ca²⁺]_i elevation or β-arrestin2 recruitment assays. These data contrast previous studies (Luangsay et al. 2009; Wittamer et al. 2003), that showed GTPγ³⁵S binding and [Ca²⁺]_i elevation to chemerin in chemerin₁-CHO-K1 cells and mouse bone marrow-derived dendritic cells and peritoneal macrophages. This further underpins the critical role of cell background in studies of chemerin₁ function. In addition, it should be noted that differences in assay kinetics, incubation time, and temperature may further influence these results (Herenbrink et al., 2016).

A significant finding of the study was the identification of new ligand-receptor pairings, with chemerin at concentrations >10 nM and NPD1 at nanomolar concentrations activating BLT₁ in cAMP and GTPγ³⁵S binding assays.

NPD1 is predominantly found in the brain and is known to have neuroprotective actions (Bazan, 2007). It was recently paired with the orphan receptor, GPR37 (Bang et al., 2021; Bang et al., 2018). As a potential additional receptor for NPD1, BLT₁ is also broadly expressed in the human brain, notably in neurons and glial cells of the hippocampus, entorhinal cortex, basal forebrain, cingulate gyrus, Brodmann area 46 and cerebellum (Emre et al., 2020). This wide-ranging expression, that includes immune cells, suggests that BLT₁ may play a role in the neuroprotective actions of NPD1.

Conversely, this study failed to replicate some previously reported ligand-receptor pairings. For example, RvE1 did not induce a response when profiled at chemerin₁. Other studies have also failed to show an effect of RvE1 on chemerin₁, including attenuation of PMN

migration in chemerin₁^{-/-} cells (Arita et al., 2005) and displacement of [³H]RvE1 by LTB₄, but not chemerin on human neutrophils (Arita et al., 2007). Activation of BLT₁ but not chemerin₁ within our study supports these original findings and may suggest that the pro-resolving effects of RvE1 on neutrophil function are due to BLT₁ activation.

Despite our broader aim to examine the pharmacological profile of six GPCRs, the orphans, GPR18, GPR37, and GPR32 proved extremely difficult to express, as measured at mRNA or protein level. It is difficult to ascertain why these orphan receptors failed to express. All six GPCRs were generated using the same HEK293-Flp-In-CD8a-FLAG cell system. It is unlikely, but possible, that the FLAG epitope impairs expression, though it is commonly used tag for measuring GPCR expression (Beerepoot et al., 2013). GPR18 has been expressed in other recombinant studies, which suggests that it has high constitutive activity (Finlay et al., 2016). If so, this could hinder prolonged cell surface location in a nominally stable expression system, though other attempts at transient expression in our hands yielded similar results. Relatively little is known about GPR32 or GPR37, but a similar mechanism may explain poor expression; alternatively, it could reflect the cell background.

In summary, a systematic, multi-pathway evaluation of a range of putative endogenous and synthetic SPM ligands at six GPCRs has revealed two new ligands for BLT₁ and confirmed some previous pairings. However, in several cases, notably for FPR2, we were not able to replicate previous studies. It may be that different cell backgrounds (e.g., CHO, THP-1 or HL-60s) or deeper signalling analyses (e.g., TruPath G protein-coupling; Olsen et al., 2020) are required to fully establish the detailed pharmacology of some of these putative endogenous ligands. However, we did reveal marked signalling bias downstream of FPR2 for synthetic agonists, which may further help guide the design of FPR2/ALX agonists for the treatment of multiple inflammatory diseases, including autoimmune disorders.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Merlin, Park, Allinne, Langmead, Riddy

Conducted experiments: Merlin, Park, Vandekolk, Riddy

Contributed new reagents or analytic tools: Fabb

Performed data analysis: Merlin, Park, Vandekolk, Riddy

Wrote or contributed to the writing of the manuscript: Merlin, Park, Summers, Langmead, Riddy

¹Merlin and Park contributed equally to this manuscript.

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FOOTNOTES

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Table 1. Sequences of qPCR primers used in this study

Gene	Forward	Reverse
<i>FPR2/ALX</i>	GCCTTTTGGCTGGTTCCTGTGT	CCAGACTGGATGCAGGACACAA
<i>LTB4R</i>	CCTGTGTCACTATGTCTGCGGA	ATCGCCTTGGTGCGTAGCTTCT
<i>CMKLR1</i>	CTCACTCCCAAATGGACCCTGT	CACACGATGGTGAGGTAGCAAG
<i>GPR18</i>	CTTCTGCCAGATTCTTGGAGCTC	GTTCTTTGGCGTACTTCGGCTG
<i>GPR32</i>	GTGATCGCTCTTGTTCCAGGAAG	TGCGTGCCATACGGAAGACAGT
<i>GPR37</i>	CTTCCGCTGGTCATCTTCC	TGCACAGAGCACATAAGGTG

Table 2. Details of compounds used in this study. Assay specific compound dilutions and solvents used are described in the Methods, and Compounds and Reagents section

Compound name	Supplier	Catalogue number	FW/kDa	Stock concentration (μM)	Storage (°C)	Storage solvent	Note
Resolvin D1 (RvD1)	Cayman Chemical	10012554	376.5	664	-80	Ethanol	
Resolvin D2 (RvD2)	Cayman Chemical	10007279	376.5	664	-80	Ethanol	
Resolvin E1 (RvE1)	Cayman Chemical	10007848	350.5	142.7	-80	Ethanol	
Chemerin	Enzo Life Sciences	ALX-522-142-C010	16	10	-80	Water	
Annexin A1 (AnxA1)	Cayman Chemical	19881	40.48	24.7	-80	Water	
Lipoxin A4 (LXA4)	Cayman Chemical	90410	352.5	283.7	-80	Ethanol	
Arachidonoyl Glycine (NAGly)	Cayman Chemical	90051	361.5	138312.6	-80	Ethanol	Stock was diluted to a 1383 μM intermediate in ethanol prior to serial dilution for assays.
LL-37 (trifluoroacetate salt)	Cayman Chemical	24461	4493.3	222.6	-80	Water	
Serum Amyloid A (SAA)	Sigma Aldrich	SRP4324	11.5	87	-20	0.1% Acetic acid	
N-Formylmethionyl-leucyl-phenylalanine (fMLP)	Sigma Aldrich	F3506	437.6	2285.5	-80	DMSO	Stock was diluted to 500 μM intermediate in DMSO prior to serial dilution for assays.
Leukotriene B4	Cayman	20110	336.5	297.2	-20	Ethanol	

(LTB4)	Chemical						
Neuroprotectin D1 (NPD1)	Cayman Chemical	10010390	360.5	138.7	-80	Ethanol	
WKYMVm (synthetic peptide)	Tocris Bioscience	1800/1	856.11	1000	-20	Water	
Compound 43 (synthetic small molecule)	Anthem Biosciences	Custom made	384.86	1000	-20	DMSO	
ACT-389949 (synthetic small molecule)	Servier	Custom made	428.39	10000	+4	DMSO	Stock was diluted to either 100 μ M or 10 μ M intermediate in DMSO and serial diluted in DMSO.

TABLE 3. Potency (pEC₅₀) and Emax (%) values for the agonist activity of the different ligands at FPR2/ALX in HEK293-Flp-In-CD8a-FLAG-‘GPCR’ stably expressing cell lines across six signalling assays. Data represent the means ± SEM. Where n=2 this represents ligands tested at a single concentration in duplicate experiments. *ne* = no effect, *nt* = not tested, *nd* = not determined, (*sc*) = single concentration

FPR2/ALX	cAMP (Gi) (n=3-4)		cAMP (Gs) (n=3-4)		pERK1/2 (n=3-6)		GTPγ ³⁵ S (n=3-5)		[Ca ²⁺] _i elevation (n=2-6)		β-arrestin2 (n=2-5)	
	pEC ₅₀	Emax	pEC ₅₀	Emax	pEC ₅₀	Emax	pEC ₅₀	Emax	pEC ₅₀	Emax	pEC ₅₀	Emax
RvD1	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
RvD2	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
RvE1	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<6 (<i>sc</i>)		<i>ne</i>	
Chemerin	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
AnxA1	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
LXA4	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<6 (<i>sc</i>)		<i>ne</i>	
NAGly	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
LL-37	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
SAA	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
fMLP	4.7±0.3	<i>nd</i>	<i>ne</i>		<i>ne</i>		4.2±0.1	<i>nd</i>	4.8±0.1	<i>nd</i>	<i>ne</i>	
LTB4	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
NPD1	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<6 (<i>sc</i>)		<i>ne</i>	
WKYMVm	9.5±0.2	105.9±6.9	<i>ne</i>		8.0±0.2	108.3±11.7	9.7±0.1	101.9±2.5	9.5±0.4	100.2±16.6	7.3±0.1	107.5±8.0
Compound 43	6.8±0.2	120.8±9.7	<i>ne</i>		6.9±0.2	107.3±12.3	5.3±0.1	88.7±5.7	6.9±0.2	91.5±6.9	5.2±0.1	85.3±11.9

ACT-389949	9.8±0.3	97.3±19.1	<i>ne</i>	<i>n/e</i>	11.0±1.7	8.0±0.1	96.3±1.4	8.4±0.2	111.0±11.2	7.4±0.2	102.8±11.5
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TABLE 4. Potency (pEC_{50}) and E_{max} (%) values for the agonist activity of the different ligands at BLT₁ in HEK293-Flp-In-CD8a-FLAG-‘GPCR’ stably expressing cell lines across six signalling assays. Data represent the means $\pm$ SEM. Where $n=2$ this represents ligands tested at a single concentration in duplicate experiments. *ne* = no effect, *nt* = not tested, *nd* = not determined, (*sc*) = single concentration

BLT ₁	cAMP (Gi) (n=3-6)		pERK1/2 (n=3-4)		GTPγ ³⁵ S (n=3-5)		[Ca ²⁺] _i elevation (n=2-3)		β-arrestin2 (n=2-4)	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}
RvD1	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
RvD2	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
RvE1	7.6 $\pm$ 0.2	110.2 $\pm$ 11.9	7.9 $\pm$ 0.5	~100	7.2 $\pm$ 0.1	94.4 $\pm$ 4.2	<6 (<i>sc</i>)		<i>ne</i>	
Chemerin	8.1 $\pm$ 1.1	<i>nd</i>	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
AnxA1	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
LXA4	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
NAGly	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
LL-37	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
SAA	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
fMLP	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
LTB4	10.5 $\pm$ 0.1	104.7 $\pm$ 2.9	9.4 $\pm$ 0.1	104.4 $\pm$ 3.7	8.9 $\pm$ 0.1	97.9 $\pm$ 3.7	7.4 $\pm$ 0.1	104.0 $\pm$ 7.0	7.2 $\pm$ 0.1	107.0 $\pm$ 3.6
NPD1	7.5 $\pm$ 0.3	104.4 $\pm$ 16.7	<i>ne</i>		6.5 $\pm$ 0.1	66.0 $\pm$ 5.8	<i>ne</i>		<i>ne</i>	

TABLE 5. Potency (pEC₅₀) and Emax (%) values for the agonist activity of the different ligands at chemerin₁ in HEK293-Flp-In-CD8a-FLAG-‘GPCR’ stably expressing cell lines across six signalling assays. Data represent the means ± SEM. Where n=2 this represents ligands tested at a single concentration in duplicate experiments. *ne* = no effect, *nt* = not tested, (*sc*) = single concentration

Chemerin ₁	cAMP (Gi) (n=3-4)		pERK1/2 (n=3-4)		GTPγ ³⁵ S (n=5)		[Ca ²⁺] _i elevation (n=2-3)		β-arrestin2 (n=2-4)	
	pEC ₅₀	Emax	pEC ₅₀	Emax	pEC ₅₀	Emax	pEC ₅₀	Emax	pEC ₅₀	Emax
RvD1	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
RvD2	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
RvE1	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
Chemerin	9.9±0.5	107±23.0	8.6±0.2	100±0.0	<i>ne</i>		<i>ne</i>		<i>ne</i>	
AnxA1	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
LXA4	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
NAGly	<i>ne</i>		<i>ne</i>		<i>ne</i>		<7 (<i>sc</i>)		<i>ne</i>	
LL-37	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
SAA	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
fMLP	<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>		<i>ne</i>	
LTB4	<i>ne</i>		<i>ne</i>		<i>ne</i>		<6 (<i>sc</i>)		<i>ne</i>	
NPD1	<i>ne</i>		<i>ne</i>		<i>ne</i>		<6 (<i>sc</i>)		<i>ne</i>	

FIGURE LEGENDS

Figure 1

Expression of *FPR2/ALX*, *LTB4R* and *CMKLR1* in recombinant receptor expressing HEK293-Flp-In-CD8a-FLAG cell lines. (A) Relative expression of the three receptors. Grouped data are shown as individual data points and mean + SD. (n=4). (B) Representative of flow cytometry gating strategy for measurement of surface protein expression. Surface protein expression presented as (C) % of the total cell population positively stained with FLAG-Alexa Fluor (AF) 488 relative to the parental HEK293 cells or (D) the mean fluorescence intensity (MFI) of AF488. Grouped data are shown as individual data points and mean + SD. (n=6).

Figure 2

Concentration-response curves of the FPR2/ALX agonists (A) WKYMVm, (B) Compound 43 (C), ACT-389949, and (D) fMLP, in HEK293-Flp-In-CD8a-FLAG-FPR2/ALX cells across five signalling pathways where all ligand responses are normalised to the maximal effect of WKYMVm. Grouped data are shown as mean + SEM. (n=3-9). (E) Data from (A-C) were used to construct a web of bias for responses of ACT-389949 and Compound 43 relative to the reference agonist WKYMVm and normalised to the reference pathway inhibition of cAMP accumulation [axis represents $10^{\Delta\Delta\text{Log}(\tau/\text{KA})}$].

Figure 3

Concentration-response curves of the BLT₁ agonists (A) LTB₄, (B) RvE1, (C) NPD1, and (D) chemerin, in HEK293-Flp-In-CD8a-FLAG-BLT₁ cells across five signalling pathways where all ligand responses are normalised to the maximal effect of LTB₄. Grouped data are shown as mean + SEM. (n=3-6).

Figure 4

Concentration-response curves of the chemerin₁ agonists (A) chemerin, and (B) RvE1, in HEK293-Flp-In-CD8a-FLAG-chemerin₁ cells across five signalling pathways where responses are normalised to the maximal chemerin effect. Grouped data are shown as mean + SEM. (n=3-8).

Figure 1

Figure 2

Figure 3

Figure 4