New Advances in Targeting the Resolution of Inflammation: Implications for Specialized Pro-Resolving Mediator GPCR Drug Discovery

Julia Park, Christopher J. Langmead,* and Darren M. Riddy*

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ABSTRACT: Chronic inflammation is a component of numerous diseases including autoimmune, metabolic, neurodegenerative, and cancer. The discovery and characterization of specialized pro-resolving mediators (SPMs) critical to the resolution of inflammation, and their cognate G protein-coupled receptors (GPCRs) has led to a significant increase in the understanding of this physiological process. Approximately 20 ligands, including lipoxins, resolvins, maresins, and protectins, and 6 receptors (FPR2/ALX, GPR32, GPR18, chemerin₁, BLT₁, and GPR37) have been identified highlighting the complex and multilayered nature of resolution. Therapeutic efforts in targeting these receptors have proved challenging, with very few ligands apparently progressing through to preclinical or clinical development. To date, some knowledge gaps remain in the understanding of how the activation of these receptors, and their downstream signaling, results in efficient resolution via apoptosis, phagocytosis, and efferocytosis of polymorphonuclear leukocytes (mainly neutrophils) and macrophages. SPMs bind and activate multiple receptors (ligand poly-pharmacology), while most receptors are activated by multiple ligands (receptor pleiotropy). In addition, allosteric binding sites have been identified signifying the capacity of more than one ligand to bind simultaneously. These fundamental characteristics of SPM receptors enable alternative targeting strategies to be considered, including biased signaling and allosteric modulation. This review describes those ligands and receptors involved in the resolution of inflammation, and highlights the most recent clinical trial results. Furthermore, we describe alternative mechanisms by which these SPM receptors could be targeted, paving the way for the identification of new therapeutics, perhaps with greater efficacy and fidelity.

KEYWORDS: specialized pro-resolving mediators, G protein-coupled receptor, biased signaling, allostery, resolution of inflammation

Excessive inflammation has been widely recognized as a ubiquitous component of various chronic diseases including asthma, cardiovascular, neurodegenerative and many autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus (SLE), and chronic granulomatous disease (CGD). More recently, a link between inflammation and metabolic diseases, including obesity, nonalcoholic steatohepatitis, and fibrosis has emerged.^{1,2} Defined by the four cardinal characteristics of *rubor* (redness), *calor* (warmth), *tumor* (swelling), and *dolor* (pain), acute inflammation is the body's natural defense response intended to provide protection from injury and external pathogens.^{3–5} However, evidence strongly suggests that uncontrolled chronic inflammation leads to the progression of significant pathophysiology.⁶

Under healthy conditions, acute inflammatory responses are self-limited and resolve independently by the complex and collaborative actions of immune cells and diverse cellular mediators. Chronic inflammation is ultimately the result of the

imbalance between the inflammatory response and the pro-resolving activity. The severity of the outcome of acute inflammation is heavily dependent on the efficacy of resolution.⁴ Indeed, it is suggested that chronic inflammation may be a result of “frustrated resolution” where the initial acute inflammation is not adequately resolved, leading to a defective immune response.^{3,7}

Traditionally, conventional anti-inflammatory therapies have targeted a reduction, or nullification, of the inflammatory response, but they are typically associated with many undesired side effects.⁸ For example, nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors cause gastrointestinal complications and renal toxicity.⁹ Furthermore,

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Figure 1. Resolution of inflammation. Following insult, injury or infection acute inflammation develops. Edema, followed by polymorphonuclear neutrophils (PMN) infiltration, occurs within minutes to hours, closely followed by the resolution of inflammation by monocytes and macrophages over hours to days. Specialized pro-resolving mediators (SPMs), including lipoxins (LXA₄), resolvins (D- and E-series), maresins, and protectins, are biosynthesized to facilitate resolution. Figure generated from our interpretation of multiple reviews and research articles.^{3,7,35,37,106,109}

some anti-inflammatory drugs require close and extensive monitoring due to their severe immunosuppressive effects, increasing the patient's risk to opportunistic infections.^{7,10} Therefore, current anti-inflammatory therapies leave an unmet medical need for the treatment of chronic inflammatory diseases.

With the advanced understanding of inflammation and its processes, pro-resolving strategies, including selectively targeting the G protein-coupled receptor (GPCR) upon which the specialized pro-resolving mediators (SPMs) exert their effects, have been proposed as a new way in targeting chronic inflammation. Here we will discuss alternative mechanisms in targeting these SPM receptors, including the potential of biased agonism and allosteric modulation, which may provide improved efficacy, resulting in greater patient outcomes.

RESOLUTION OF INFLAMMATION

Resolution marks the period between clearance of the injurious agent and dead polymorphonuclear leukocytes (PMNs), culminating in the return to homeostasis.³ Traditionally, the period of resolution was postulated to be passive, but now it is appreciated as a complex and active process that is tightly regulated by a wide range of cellular mediators (Figure 1).^{6,11–13} Once stimulus is removed, various regulatory mechanisms drive the innate immune system to dampen the production of pro-inflammatory mediators, including cytokines, chemokines, eicosanoids, and cell adhesion molecules, preventing interactions with their target receptors. For example, the CXC family of chemokines, which direct neutrophil migration toward the site of inflammation, is cleaved by matrix metalloproteinases (MMP) to end further influx of neutrophils, and thus prevents further unwanted tissue damage.^{14,15}

Efficient neutrophil apoptosis and their clearance by surrounding phagocytes are regarded as the most crucial step

in resolution.¹⁶ Apoptosis is defined as programmed cell death, intended to prevent the neutrophil from secreting its cytotoxic contents such as reactive oxygen species (ROS) and proteases into the extracellular environment.^{17,18} Apoptotic neutrophils undergo significant morphological changes including membrane blebbing and cellular shrinkage, resulting in cell detachment and organelle fragmentation.¹⁹ Apoptotic neutrophils also secrete various chemoattractants that act as “find-me” signals, including CX3C-chemokine ligand-1 (CX3CL1) and intracellular adhesion molecule 3 (ICAM3).^{19,20} Furthermore, downregulation of cell-surface molecules such as cluster of differentiation (CD)31, CD46, and CD47 act as “do-not-eat-me” signals, while expressing various “eat-me” signals, including phosphatidylserine (PtdSer) and calreticulin (CRT), that facilitate engulfment by phagocytes and robust efferocytosis.^{17,21}

PtdSer is recognized by membrane receptors, including brain-specific angiogenesis inhibitor (BAI1), stabilin 2, and T-cell immunoglobulin mucin domain protein family members (TIM1, TIM3, and TIM4).^{22–25} Recognition of PtdSer by BAI1 triggers the receptor to signal through ELMO1-DOCK180-RAC complex causing cytoskeletal rearrangement.²⁶ Furthermore, activated stabilin 2 interacts with phosphotyrosine-binding (PTB)-domain-containing engulfment adaptor protein 1 (GULP) and thymosin-β4.^{27,28} Additionally, various molecules including serum-component milk fat globule-epidermal growth factor 8 (MFG-E8) and protein S, act as “bridging molecules” between PtdSer and efferocytosis receptors including αvβ3 integrin, Mer tyrosine kinase (MerTK), and Tryp3-Axl-Mer (TAM).^{29,30}

The expression of CRT is detected by low-density lipoprotein (LDL)-receptor-related protein (CD91) further aiding efferocytosis.³¹ Phenotype switching of phagocytosing macrophages to “pro-resolution” is initiated by the secretion of anti-inflammatory cytokines interleukin-10 (IL-10) and trans-

Figure 2. Specialized pro-resolving mediators (SPMs). Overview of lipoxins, resolvins, maresins, and protectins biosynthesized from omega-3 polyunsaturated fatty acids (PUFA) including docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA).^{12,13,45,46,49,51–53,55,58,59,63}

forming growth factor-beta (TGF- β) and the downregulation of pro-inflammatory cytokine and chemokine release. These changes culminate in the promotion of tissue repair and wound healing.^{32,33} Macrophages involved in these processes are removed from the site of inflammation by lymphatic drainage or apoptosis.³⁴ Ultimately, it is this synergistic cooperativity between the different mechanisms that results in efficient efferocytosis.¹⁶

In addition to the process described above, the resolution of inflammation is further coordinated by specialized pro-resolving mediators (SPMs). These SPMs promote diverse pro-resolving and anti-inflammatory mechanisms such as assisting in the reduction of PMN migration and the secretion of tissue repair and wound healing molecules.^{7,35}

SPECIALIZED PRO-RESOLVING MEDIATORS

SPMs are lipid mediators that are actively biosynthesized from essential polyunsaturated fatty acids (PUFA) during inflammation (Figure 2).^{4,36} For comprehensive reviews, including structural elucidation, see refs 37 and 38.

Lipoxins. Lipoxin A4 (LXA₄; 5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid) and lipoxin B4 (LXB₄; 5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid) were the first lipid SPMs identified.³⁷ They are synthesized from omega-6 (ω -6) arachidonic acid (AA) by lipoxygenase (LOX) conversion and further converted by oxidation of 15-hydroxyperoxyeicosatetraenoic acid (15-HpETE).³⁷ Two routes of LXA₄ formation exist, one involving 12-LOX during peripheral blood platelet–leukocyte interactions,^{38,39} while the second requires a series of LOX that are dependent on the cell type. The conversion is mediated by 5-LOX in neutrophils and

15-LOX in erythrocytes and reticulocytes.^{37,40,41} Additionally, exogenous aspirin, often used prophylactically in the treatment of inflammation, leads to formation of “aspirin-triggered” (AT) lipoxins, named 15-epi-LX, where acetylation of cyclooxygenase-2 (COX-2) by aspirin leads to the enzyme converting AA into 15R-HETE, serving as a substrate for 5-LOX.⁴² It is perhaps through this process which highlights aspirin’s significant pro-resolution effects compared to other NSAIDs.^{4,7} It is interesting to note that statins which are widely used as potent cholesterol-lowering drugs, potentially contribute to the formation of 15-epi-LX by promoting the arachidonate conversion to 15-epi-lipoxin, justifying the clinical benefits and anti-inflammatory effects of statins and aspirin in the treatment of cardiovascular disorders.⁴³ *In vivo* biosynthesis of LXA₄ is triggered during the acute inflammation process where the interaction of PMNs with prostaglandin E2 (PGE₂) and PGD₂ activates 15-lipoxygenase to biosynthesize LXA₄.⁴⁴ In a murine model of peritonitis, the maximal level of LXA₄ is typically achieved by 2 h and declines within the first 24 h, corresponding with the increase in PMN infiltration.¹¹

E- and D-Series Resolvins. Resolvins are lipid mediators that are biosynthesized from ω -3 fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), derived from PUFA via enzymatic transformation, resulting in the production of E- and D-series resolvins, respectively.⁸

E-Series Resolvins. In hypoxic endothelial cells, aspirin-acetylated COX-2 oxygenates 18R-hydro(peroxy)-eicosapentaenoic acid (18R-HEPE). Activated PMNs convert this using 5-LOX into 5S(6)-epoxy-18R-HEPE, which is further hydro-

Table 1. Summary of Published GPCRs and Paired SPMs/Associated Ligands Involved in the Resolution of Inflammation^a

receptor	expression profile	species ortholog	G-protein coupling and/or β -arrestin	pro-resolving mediators	pK _D /pEC ₅₀	refs
FPR2/ALX (ALX, LXA ₄ R, FPRL1, FMLPX, formyl peptide receptor 2)	neutrophils, monocytes, macrophages, immature dendritic cells, T- and B-cells, epithelial cells, microglial cells, astrocytes, hepatocytes, endothelial cells, spleen, lung, kidney, leukocytes	human, mouse and rat	G _i /G _o , G _q /G ₁₁ β -arrestin	LXA ₄ AT-LXA ₄ RvD1 AT-RvD1 ANXA1 Ac2-26 SAA LL-37 LXA ₄ RvD1 RvD3 AT-RvD3 RvD5 NAGly RvD2 Abn-CBD O-1918 Chemerin RvE1 RvE2 LTB ₄ 12R-HETE RvE1 RvE2 NPD1 (PD1)	8.8–9.3/12 11.9 11.1 6.5/5.8–6.1 <6 6.6 6.0 9.7 11.1 ~11 ~11 ~11 8 8 antagonist 8.3 7.9 10 9.4 7.5 7.35 10 7.6	79, 95 93 95 95 157 107 158 159 95 95, 100 53 53 111 114, 117, 119, 120 64 64, 140 50 142 142 140 50, 47 65
GPR32 (DRV1, RVDRI, resolving D1 receptor)	myeloid cells, human umbilical vein cells, arterial tissue, venous tissue	human	not determined β -arrestin			
GPR18 (NAGly receptor)	PMNs, monocytes, macrophages, microglia, BV2 microglial cells	human and mouse	G _i /G _o , G _q /G ₁₁ G _s β -arrestin			
chemerin, (ChemR23, ERV1, RVER1, CMKLR1)	monocytes, macrophages, NK cells, myeloid cells, plasmacytoid dendritic cells, primary adipocytes, endothelial cells, circulating dendritic cells	human and mouse	G _i /G _o			
BLT ₁ (LTB ₄ R)	leukocytes, spleen, thymus, mast cells, monocytes, T- and B-lymphocytes, dendritic cells, endothelial cells	human, mouse, and rat	G _i /G _o , G _q /G ₁₁ β -arrestin			
GPR37 (EDNRBL, EDNRLB)	macrophages	human and mouse	G _i /G _o			

^aNomenclature and values as described in "The Concise Guide to Pharmacology 2017/18".¹²¹

lyzed to produce RvE1 (5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid).^{12,13} RvE2 (5S,18R-dihydroxy-6E,8Z,11Z,14Z,16E-eicosapentaenoic acid) is produced by the reduction of 5-LOX and 18R HEPE product, 5S-hydroperoxy,18-hydroxy-EPE in whole blood, while the production by PMNs is enhanced during hypoxia.⁴⁵ In contrast to RvE1 and RvE2, RvE3 (17R,18S-dihydroxy-5Z,8Z,11Z,13E,15E-eicosapentaenoic acid) is biosynthesised from 18-HEPE via the 12/15-LOX pathway in eosinophils.⁴⁶ Notably, EPA-derived 18-HEPE and 15-HEPE biosynthesised in hypoxic endothelial cells produce resolution effects by reducing the migration of PMNs, although the activity is much weaker in comparison to RvE1.¹³ As unacetylated COX-2 are not able to convert and produce 18-HEPE, aspirin, paracetamol, and indomethacin all trigger 18-HEPE production,¹³ whereas selective COX-2 inhibitors prevent this production. In inflammatory exudates, the precursor 18-HEPE EPA was shown to reach its peak level by 2 h and subsided to base levels within 12 h; however, endogenous RvE1 appeared at a delayed time point, accumulating between 48 and 72 h.⁴⁷ It is postulated that this result may be dependent on the cellular composition of the exudates utilized; as RvE1 appeared at an earlier time point in exudates of murine skin air pouch model,¹³ perhaps due to the difference in permeability between peritoneum and skin. RvE2 also appeared in murine peritoneal exudates, stimulated by zymosan A, with a corresponding time point to initial PMN infiltration and declining within 24 h.⁴⁸

D-Series Resolvins. D-Series resolvins (RvD1–RvD6) are biosynthetically produced from DHA by the action of LOX in PMNs and macrophages.¹² DHA is converted to a peroxide intermediate following two LOX steps. Hydrolysis of this product generates RvD1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid) and RvD2 (7S,16R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-docosahexaenoic acid), while reduction leads to the production of RvD5 (7S,17S-dihydroxy-4,8,10,13,15,19-docosahexaenoic acid).^{12,49,50} In the presence of aspirin, COX-2 in hypoxic endothelial cells transforms DHA from PUFA to 13-hydroxy-DHA or 17R-HDHA.¹² Activated PMNs further metabolizes these products to AT-RvD1, AT-RvD2 and other AT-D-series resolvins. RvD3 (4S,11R,17S-trihydroxy-5Z,7E,9E,13Z,15E,19Z-docosahexaenoic acid)^{12,51,52} and RvD4 (4S,5R,17S-trihydroxy-6E,8E,10Z,13Z,15E,19Z-docosahexaenoic acid)⁵³ originate from 4S,5S-epoxy-17S-hydroxy-docosahexaenoic acid, following hydrolysis of 4S-hydroperoxy-17S-hydroxy-docosahexaenoic acid, while RvD6 originates from peroxidase of the same precursor. Interestingly, the *in vivo* appearance of RvD3 following zymosan challenge in a peritonitis model is significantly increased to peak 48 h postinitiation, while RvD1, RvD2, and RvD5 peak earlier in the resolution phase (~6–24 h).⁵² RvD3 is most likely, therefore, produced by a subpopulation of macrophages, high in 15-LOX.^{52,54} *In vivo* production of RvD4, by an *S. aureus* dorsal pouch infectious model, appears even later with a sustained release over 72 h, indicating constitutive production and differential regulation.⁵³ RvD6 kinetics remain to be elucidated.

Protectins. Similar to maresins, the biosynthesis of protectins also arises from the formation of epoxide intermediates of DHA. Oxygenation of DHA into 17S-HpDHA via 15-LOX is converted to produce 16S,17S-epoxy-protectin where it is further hydrolyzed into protectin D1 (PD1, 10R,17S-dihydroxy-4Z,7Z,11E,13E,15Z,19Z-docosahex-

aenoic acid).⁵⁵ PD1 was found to accumulate in the ipsilateral hemisphere of the brain after focal ischemia resulting in enhanced wound healing and neuroprotection. Due to the location of expression, brain-specific PD1 is known as neuroprotectin D1 (NPD1).^{56,57} 15-LOX action on 17S-HpDHA can also produce 10S, 17S-diHDHA (PDx), an isomer of PD1, which also produces pro-resolving activity. In zymosan A-induced murine peritonitis model, the *in vivo* production of PD1 was shown to peak at 12 h.¹¹

Maresins. Maresins, or macrophage mediators in resolving inflammation, are a fourth family of SPMs.⁵⁸ These SPMs are biosynthesised from DHA by macrophages via the action of 12-LOX,⁵⁹ which catalyzes the oxygenation of DHA into 14-hydroperoxydocosahexaenoic acid (14-HpDHA), followed by the reduction to 13S,14S-epoxy-maresin.⁴⁹ The 13S-14S-epoxy-maresin is further converted to produce MaR1 (7R,14S-dihydroxy-4Z,8E,10E,12Z,16Z,19Z-docosahexaenoic acid) within human macrophages.⁶⁰ The complete stereochemistry of MaR1 was confirmed as 7R,14S-dihydroxy-docosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid.⁶¹ Conversion of 13S-14S-epoxy-maresin by a soluble epoxide hydrolase results in the formation of MaR2 (13R,14S-dihydroxy-4Z,7Z,9E,11E,16Z,19Z-docosahexaenoic acid).⁵⁸ The critical role of 12-LOX in the biosynthesis of maresins was demonstrated where 12/15-LOX-deficient mice showed a significant reduction in 14S-HDHA generation in a mouse zymosan A induced model of peritonitis.⁵⁸ *In vivo* production of MaR1 was discovered utilizing a murine model of acute respiratory distress syndrome, where its early production was detected during platelet–neutrophil interaction, where its level was significantly enhanced within 2 h of reaching a peak at 24 h.⁶²

■ SPMS MEDIATE THEIR EFFECTS THROUGH G-PROTEIN-COUPLED RECEPTORS (GPCRS)

Extensive studies have demonstrated the pro-resolving activities of SPMs occur as a result of activation of one or more of a family of GPCRs. To date, five key receptors have been identified: FPR2/ALX, chemerin₁, and BLT₁, and two still classified as orphan receptors, GPR32 and GPR18. Recently another orphan receptor, GPR37, has been suggested as the receptor for PD1.⁶⁴ Interestingly, more than 140 GPCRs still remain to be paired with their endogenous ligand, indicating the potential for the identification of yet further SPM receptors.⁶⁵

FPR2/ALX. N-Formyl peptide receptor 2/lipoxin A4 receptor (FPR2/ALX) is predominantly expressed on leukocytes, including neutrophils, monocytes, macrophages, dendritic cells (DC), naïve CD4 T-cells, and Th1, -2, -17 cells.^{66–69} FPR2/ALX is also expressed on nonimmune cells including endothelial, keratinocytes, and mesenchymal stem cells.⁶⁶ Furthermore, FPR2/ALX has been identified within the central nervous system, including the hippocampus and prefrontal cortex, indicating a possible role in learning, memory, and balance.⁷⁰ The surface expression of FPR2/ALX is significantly increased when pro-inflammatory stimuli are present, including tumor necrosis factor- α (TNF- α), platelet-activating factor (PAF), and IL-8.⁷¹ Orthologs of FPR2/ALX are present in both human and rodents.^{72,73} Endogenous and exogenous lipids, peptides, and proteins have been discovered to bind to and activate FPR2/ALX, producing both pro- and anti-inflammatory effects (Table 1).^{74,75} SPMs from both the lipoxin and resolvins families activate the

Figure 3. GPCRs activated by multiple subpopulation pro-resolving mediators. Pairing of SPMs and associate ligands to their cognate receptors have been much explored utilizing various methods including radioligand binding. Early research into SPM pharmacology focused primarily on the ligand activity; however, recent attention has shifted toward further elucidation of the signaling mechanism involved. To date, knowledge gaps remain in the signal transduction pathways leading to increasingly well-described physiological responses. Due to ligand poly-pharmacology and receptor pleiotropy, distinct opportunities to target these receptors via novel mechanisms exist, including biased signaling and allostery. Both mechanisms have been partially described for FPR2/ALX, but due to the vast number of SPMs and GPCRs identified, the combinations could be limitless, clearly highlighting significant opportunities in augmenting the resolution of inflammation. Figure generated from our interpretation of multiple reviews.^{63,89,95,113,130,140,160}

receptor with high potency, including LXA₄, AT-LXA₄ (15-epi-LXA₄), RvD1, AT-RvD1 (17-epi-RvD1), and annexin A1 (ANXA1). Furthermore, endogenous antagonists for the receptor have also been identified, including serum amyloid A (SAA) and cathelicidin (LL-37; Table 1 and Figure 3).

Originally, FPR2 was classified as an FPR receptor due to its activation by the low-affinity endogenous agonist *N*-formyl-methionyl peptide (fMLP).^{76,77} Screening of various receptors using radiolabeled [³H]-LXA₄ and subsequent GTPase activity, cells transfected with FPR2/ALX cDNA (pINF154) displayed specific and high-affinity LXA₄ binding.⁷⁸ Therefore, the receptor was reclassified as FPR2/ALX as LXA₄ displayed the highest affinity of all FPR2/ALX endogenous agonists.⁷⁹ However, LXA₄ binds to multiple receptors including GPR32, FPR3, and the nuclear estrogen receptor.⁸⁰ Furthermore, LXA₄ binds to the transcription factor aryl hydrocarbon receptor (AHR) to induce gene expression of many cytochrome P450 enzymes.⁸¹

Binding of LXA₄ induces stimulation of monocyte chemotaxis, macrophage differentiation, and efferocytosis.^{75,82} LXA₄ also reduces the adaptive immune response through decreasing memory B-cell antibody production and proliferation.⁸³ LXA₄ acts as positive feedback for FPR2/ALX expression by activation of the receptor promotor, thus upregulating FPR2/ALX expression level.⁸⁴ Various knockout mouse models have been used to demonstrate the interaction of LXA₄ with FPR2/ALX. Treatment of *Fpr2/Alx*^{-/-} mice with LXA₄ resulted in the absence of cell recruitment into dorsal air pouches inflamed with IL-1β, while LXA₄-induced resolution of ischemia-reperfusion (IR) injury was reduced in these knockout mice.^{85,86} Furthermore, isolated *Fpr2/Alx*^{-/-} macrophages failed to effectively clear apoptotic PMNs in response to LXA₄.⁷⁵ Finally, chemical inhibition of LXA₄-mediated resolution in a mouse model of pneumosepsis was shown with treatment of an FPR2/ALX antagonist BOC-2.⁸⁷ However, alternative FPR2/ALX agonists were not investigated.

Interestingly, LXA₄ binds to the seventh transmembrane (TM) domain and third extracellular loop (EL) of FPR2/ALX.⁸⁸ Due to this unusual binding location of LXA₄ compared to that of agonist-bound crystal structures of many GPCRs where the orthosteric pocket typically resides deep within TM 3 and 5, we might infer that FPR2/ALX is capable of binding multiple ligands simultaneously. In fact, during inflammation, the pro-inflammatory peptide ligand SAA is released and binds to FPR2/ALX to activate ERK1/2 phosphorylation and phosphoinositide 3-kinase (PI3K), while increasing NF-κB expression, resulting in enhanced PMN activation, differentiation, motility, and survival.⁸⁹ SAA binds in a distinct location to LXA₄ and is dependent on interactions with EL1 and 2.⁸⁹ When cobound LXA₄ acts as an allosteric inhibitor of SAA signaling, it thus reduces SAA-mediated PMN activation and survival.^{90,91} The cooperativity between allosteric interactions is always reciprocal; therefore, binding of SAA inhibits LXA₄ affinity and signaling.⁸⁸

In addition to LXA₄, the aspirin-triggered version, AT-LXA₄, enhances resolution by downregulating the expression of β2 integrin Mac-1 (CD11b/CD18), attenuating the signaling of myeloperoxidase (MPO) and suppressing human neutrophil apoptosis.⁹² AT-LXA₄ treatment increased phagocytosis of *E. coli* in a PI3K- and scavenger-receptor-dependent manner, while the FPR2/ALX gene was 6-fold upregulated in colonic biopsies of Crohn's disease patients.⁹³

RvD1 binds and activates FPR2/ALX with similar affinity and potency as LXA₄ (Table 1).⁹⁴ Binding of RvD1 results in zymosan-mediated macrophage phagocytosis and cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling and increases dihydronicotinamide-adenine dinucleotide phosphate (NADPH) oxidase activation while decreasing antiapoptotic protein expression.⁹⁵ Activation of FPR2/ALX by RvD1 also results in the regulation of specific microRNAs (miRNAs).^{4,96,97} For example, RvD1 selectively upregulates miR-208a in human macrophages overexpressing FPR2/ALX, which culminates in the downregulation of the suppressor of IL-10, programmed cell death protein 4 (PDCD4), therefore increasing IL-10 production.⁹⁶ In *Fpr2/Alx* overexpressing mice with peritonitis, RvD1 resulted in potent and anti-inflammatory effects that were attenuated in *Fpr2/Alx*^{-/-} mice. Furthermore, PMN number and levels of pro-inflammatory mediators were significantly reduced in the knockout animals.^{71,96} AT-RvD1 treatment also reduced leukocyte recruitment, neutrophil–platelet interaction, and downregulation of pro-inflammatory mediators, including IL-1β, IL-6, IL-8, and TNF-α.⁹⁸ Interestingly, AT-RvD1 (and the AT versions of RvD3 and LXA₄) inhibit tumor growth in an aggressive murine Lewis lung carcinoma (LLC) model.⁹⁹

Annexin A1 (ANXA1) is a glucocorticoid-regulated protein with high abundance in myeloid cells and is secreted by apoptotic neutrophils to limit their own recruitment.^{15,100} ANXA1 activates FPR2/ALX with low nanomolar potency, activating downstream signaling of mitogen-activated protein kinase (MAPK) and extracellular receptor kinase (ERK) phosphorylation¹⁰¹ (Table 1). Binding of ANXA1 leads to enhanced neutrophil apoptosis and efferocytosis of these apoptotic neutrophils by macrophages.^{100,102} Furthermore, ANXA1 modulates neutrophil adhesion and migration thereby selectively inhibiting PMN adhesion to the endothelium and recruitment.^{103,104}

There is some evidence to suggest that FPR2/ALX can form constitutive homodimers in a ligand-dependent manner.^{74,105} For example, binding of LXA₄ and ANXA1-derived peptide Ac2-26 leads to activation of p38MAPK pathway, enhancing IL-10 production.^{74,106} Conversely, the binding of SAA reduces the formation of these homodimers.⁷ Evidence suggests that Ac2-26 stimulates receptor heterodimerization of FPR2/ALX with the closely related receptor, FPR1, resulting in the activation of the Jun N-terminal kinase (JNK)–caspase-3 pathway to promote PMN apoptosis.^{74,91,106}

GPR32. GPR32 is predominantly expressed on human PMNs, monocytes, adipose tissue, and vascular endothelial cells.^{94,107} GPR32 is currently classified as an orphan receptor. It shares 29% sequence identity with FPR2/ALX and is the closest phylogenetically (Table 2 and Figure 4). RvD1 was identified as a potential agonist as its activation of GPR32,

Table 2. Percent Sequence Identity between the Six Human SPM GPCRs

	FPR2/ ALX	GPR32	GPR18	chemerin ₁	BLT ₁	GPR37
FPR2/ALX	100	29	18	26	23	10
GPR32		100	15	26	18	7
GPR18			100	17	15	8
chemerin ₁				100	21	9
BLT ₁					100	9
GPR37						100

Figure 4. Phylogenetic tree of the six human SPM GPCRs. The tree was generated using iTOL.¹⁶¹ cml1 = chemerin₁ and lt4r1 = BLT₁. GPR32 was identified as a receptor for RvD1,⁹⁴ RvD3, AT-RvD3, and RvD5.^{52,110} Sharing a close phylogenetic identity with GPR32, FPR2/ALX was initially discovered as an fMLP receptor;^{76,77} However, the receptor was later reclassified as a receptor for LXA₄ due to high affinity binding of this ligand.⁶⁸ Chemerin₁ has recently been reclassified following the identification of its cognate ligand, chemerin.^{134–136} BLT₁ binds numerous ligands including LTB₄, RvE1, and RvE2.^{50,140,142} GPR18 still remains as an orphan receptor with several endogenous and SPM ligands having been identified, including NAGly¹¹⁷ and RvD2,¹¹³ but yet remaining to be confirmed. GPR37 has recently been identified as a receptor for neuroprotection D1 (NPD1).⁶⁴

where [³H]-RvD1 bound to human leukocytes, and via screening of series of receptors involved in inflammation, RvD1 significantly lowered the TNF- α stimulated NF- κ B signaling in GPR32-overexpressing cells.⁹⁴ Despite the fact that RvD1 shows a higher affinity for GPR32 than FPR2/ALX, the interaction with GPR32 has not been extensively studied⁷¹ (Table 1 and Figure 3). Crucially, GPR32 exists as a pseudogene within rodents; therefore, any effect of RvD1 exhibited in animal studies can be surmised to be solely via FPR2/ALX.^{107,108} FPR2/ALX expression is downregulated in tissue-resident human macrophages indicating that RvD1 may act via GPR32 on these cells.¹⁰⁹ Furthermore, treatment of GPR32 expressing activated pro-inflammatory macrophages with RvD1 resulted in a change of phenotype to pro-resolving with increased phagocytosis and reduced secretion of pro-inflammatory cytokines.¹⁰⁷ Overexpression of GPR32 on human macrophages enhanced the phagocytosis of zymosan in response to RvD1, whereas short hairpin RNA (shRNA) knockdown of GPR32 opposed these effects.⁹⁴ Moreover, reduced PMN migration was ablated by treatment with an anti-GPR32 antibody.⁷¹ Interestingly, however, this effect was not observed with an anti-FPR2/ALX antibody. Increased RvD1 concentration did, however, reverse this effect, consistent with the higher potency of RvD1 on GPR32 versus FPR2/ALX.⁷¹ Clearly these data indicate that inhibition of GPR32 does not reduce the effect of RvD1, suggesting a level of redundancy within the system; an effect often seen in chemokine signaling. Of note, RvD3, AT-RvD3, and RvD5 have all been shown to activate GPR32 in a recombinant system of β -arrestin recruitment,^{52,110} further highlighting the potential redundancy. Using a high-throughput screen approach a selection of RvD1 mimetics were identified that shared the same binding pocket as RvD1 and activated both β -arrestin recruitment and cAMP.¹¹¹ However, very recently, no activity was shown for the pairing of GPR32 and RvD1, indicating further investigation into this receptor/ligand pairing is warranted.¹¹²

GPR18. GPR18 is abundantly expressed on PMNs, monocytes and macrophages.^{113–115} GPR18 still remains an orphan receptor, although several endogenous ligands have been proposed, including N-arachidonylglycine (NAGly)¹¹⁶ and RvD2¹¹³ (Table 1 and Figure 3). NAGly was identified via screening of a lipid library using CHO cells overexpressing GPR18. Both increases in intracellular Ca²⁺ and inhibition of forskolin-induced cAMP responses were observed, suggesting both G_{ai} and G_{aq} coupling.¹¹⁶ However, signaling by NAGly appears to be context-dependent, i.e., dependent on which cell

type or assay system is being studied. For example, in glioblastoma multiforme cell lines endogenously expressing GPR18, NAGly failed to induce ERK1/2 phosphorylation (pERK1/2).¹¹⁷ Moreover, confirmation of GPR18-NAGly pairing was observed in a β -arrestin assay using CHO cells overexpressing GPR18, but it was absent when expressed in human embryonic kidney 293 (HEK293) cells. As there is no supporting evidence from animal studies, interpretation of these findings is difficult.^{113,116,118} In addition to its role in resolution of inflammation, GPR18 also responds to endogenous and synthetic cannabinoid ligands, including N-arachidonoyl ethanolamine (AEA), 2-arachidonoyl glycerol (2-AG), Δ^9 -tetrahydrocannabinol (Δ^9 -THC), and arachidonoyl cyclopropylamide (ACPA),¹¹⁹ although, interestingly, GPR18 shows little structural similarity to the cannabinoid receptors CB₁, CB₂, and GPR55.¹²⁰

RvD2 was identified as a low nanomolar agonist for GPR18 using a β -arrestin recruitment assay, which was later confirmed using [³H]-RvD2 binding assays.^{113,121} Perhaps surprisingly, GPR18 has been shown to couple to G_{as}, as incubation of human macrophages, with RvD2, resulted in an increase in cAMP.¹¹³ Use of mass cytometry (CyTOF) RvD2-GPR18 interaction was found to involve STAT3 and PKA inhibition in RvD2-stimulated phagocytosis.¹²² In most cases, the GPCRs described in this review and those involved in PMN migration generally couple to G_{ai/o} perhaps indicting GPR18 as an atypical SPM receptor (Table 1 and Figure 3). RvD2 treated overexpressing GPR18 macrophages had increased phagocytosis of FITC-zymosan and *E. coli*, while GPR18^{-/-} macrophages showed reduced function.¹¹³ RvD2 treatment increased the expression of classical markers of anti-inflammatory macrophages, CD163 and CD206, while upregulating GPR18 expression.^{113,123} Furthermore, RvD2 activation of mouse Gpr18 resulted in reduced LPS- and ATP-stimulated inflammasomes in macrophages, which was abolished by O-1918, a GPR18/GPR55 antagonist.¹²⁴

In mouse models of zymosan A induced peritonitis and sepsis; RvD2 reduced IL-1 β , IL-17, and IL-10 secretion, caspase-1 activity, increased the anti-inflammatory macrophage population, and increased the survival rates of mice by restoring neutrophil movement preventing further tissue injury from neutrophils recruitment.^{124,125} Many of these effects were abolished in Gpr18^{-/-} mice.¹¹³ In patients with sepsis, GPR18 was found to be significantly reduced in PMNs, indicating a role in postinflammation resolution.¹²⁶ Clarification of GPR18's role in endocannabinoid signaling and resolution is required. However, with the discovery of CB₂ as a regulator of

inflammation,¹²⁷ GPR18 may appear a worthwhile target, especially as an alternative to developing selective cannabinoid agonists.

Chemerin Receptor 1. Chemerin receptor 1 (chemerin₁, ChemR23, or ERV1) is expressed on a wide range of immune cells including monocytes, macrophages, NK cells, myeloid cells, and DCs. Furthermore, these receptors have been identified on adipocytes and endothelial cells.^{128–130} The expression of chemerin₁ is more abundant on antigen-presenting cells (APCs) such as macrophages and DCs rather than neutrophils and T-lymphocytes,^{63,131} but its expression was shown to be upregulated in neutrophils in diseases including type 2 diabetes (T2D).¹³² Receptor expression is also upregulated by cytokines and toll-like receptor ligands during monocyte differentiation, while it has been suggested that the expression and function of chemerin₁ are restricted to pro-inflammatory macrophages.¹¹² Chemerin₁ was initially classified as an orphan GPCR with homology with the formyl peptide receptors¹³³ and anaphylatoxin C3a and C5a receptors,¹³¹ until it was recently paired with the chemotactic protein chemerin (Table 1 and Figure 3).^{134–136} Activation of chemerin₁ by chemerin results in G_{ai/o} coupling, leading to the release of intracellular calcium, inhibition of cAMP-, MAPK-, and ERK1/2-mediated signaling.¹³⁰ Receptor activation also results in the upregulation of phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), protein kinase B (Akt), and down-regulation of NF-κB signaling.¹³⁰

In addition to chemerin, RvE1 was identified as a second endogenous agonist via a screening campaign against a panel of GPCRs (Table 1 and Figure 3).⁶³ [³H]-RvE1 was shown to bind to chemerin₁ as competition binding with chemerin revealed complete displacement in membranes prepared from human PMNs. The anti-inflammatory effects of RvE1 were identified by chemerin₁-mediated attenuation of TNF-α-induced NF-κB. Treatment of macrophages with RvE1 reduced IL-12 levels and DC migration.⁶³ Furthermore, use of the MAPK inhibitor, PD98059, resulted in decreased RvE1-induced phosphorylation signaling and macrophage phagocytosis of apoptotic PMN.¹³⁷ Chemerin₁-overexpressing mice displayed decreased neutrophil inhibition and a significant increase in phagocytosis while reducing neutrophil infiltration.^{138,139}

However, some controversy exists with the pairing of RvE1 and chemerin₁. It has been suggested that due to the low expression of chemerin₁ on neutrophils the infiltration and apoptosis of these cells must be driven by an alternative mechanism.¹²⁸ However, this is questionable as other findings revealed the expression level of chemerin₁ was found to be markedly enhanced on human neutrophils during inflammation, including poorly controlled T2D.¹³² Like many resolvins, RvE1 binds to multiple receptors, including the leukotriene receptor, BLT₁, albeit with 100-fold lower affinity (Table 1). In *Blt₁^{-/-}* mice RvE1-mediated inhibition of neutrophil recruitment was significantly reduced in zymosan-induced peritonitis.^{128,140} However, these findings were further complicated as recent data revealed RvE1-enhanced phagocytosis by macrophages was abolished in *chemerin₁^{-/-}* mice.¹⁴¹ Furthermore, RvE2 was shown to be a partial agonist compared to RvE1 in a CHO-chemerin₁ β-arrestin recruitment assay.⁴⁸ However, no further information is available for this ligand, thus warranting further investigation into this potential ligand–receptor pairing.

Leukotriene BLT₁. The discovery of BLT₁ embarked when its cDNA was cloned as a high-affinity leukotriene B₄ (LTB₄) receptor using subtraction strategy (Table 1 and Figure 3),¹⁴² where LTB₄ was shown to induce an increase in intracellular Ca²⁺. LTB₄ is a potent lipid pro-inflammatory chemoattractant and, via BLT₁, induces T helper cell chemotaxis and early effector T-cell recruitment.¹⁴³ BLT₁ shares 21% sequence identity with chemerin₁ (Table 2 and Figure 4), and although this value is relatively low, BLT₁ was also revealed to be a receptor for RvE1, where binding of [³H]-RvE1 to human PMN membranes was displaced by the selective BLT₁ antagonist, U-75302. Furthermore, RvE1 inhibited adenylate cyclase activity and induced intracellular calcium mobilization in HEK293 cells overexpressing BLT₁, albeit with 100-fold less potency and reduced efficacy than LTB₄. Nevertheless, these data suggest that RvE1 acts as a partial agonist that competes with the LTB₄-mediated NF-κB activation and calcium mobilization, indicating a role of RvE1 in reducing BLT₁ induced inflammation.¹⁴⁰

Intravenous administration of RvE1 significantly reduced PMN infiltration in a zymosan A induced peritonitis model in wild-type mice, while this effect was abolished in *Blt₁^{-/-}* mice. However, increased concentrations of RvE1 opposed this effect indicating a BLT₁-independent role,¹⁴⁰ possibly via an alternative receptor, e.g., chemerin₁. However, as the expression level of chemerin₁ on PMNs is low, RvE1-mediated PMN infiltration reduction is most likely via BLT₁.^{128,144} Alternatively, an undiscovered receptor may also be responsible for these effects. Activation of BLT₁ by RvE1 also acts as a feedback mechanism for other SPMs, including increased production of LXA₄ in FPR2/ALX-mediated resolution of allergic lung inflammation.¹⁴⁵

RvE2 has been identified as an additional BLT₁ agonist (Table 1 and Figure 3);⁴⁵ a β-arrestin assay revealed RvE2 blocked LTB₄-mediated β-arrestin signaling with a similar potency as RvE1 indicating direct competition with LTB₄.⁴⁸ Various pro-resolving roles of RvE2 have been suggested including regulation of PMN infiltration and IL-10 production.⁴⁸ Furthermore, RvE1 promotes NADPH oxidase-mediated ROS generation via BLT₁ receptors, while RvE2 is unable to replicate these data, suggesting alternative roles in regulating ROS generation by neutrophils.¹⁴⁶

GPR37. GPR37, or parkin-associated endothelin-like receptor (Pael-R), was originally discovered through genomic library screening, in an attempt to find a novel neuropeptide receptor.¹⁴⁷ The receptor is predominantly expressed in the brain and has been linked to neurological disorders including Parkinson's disease and autism.^{148,149} Mutations within GPR37 lead to various autism spectrum disorders, modulation of dopamine reuptake, and oligodendrocyte cell differentiation.^{150–152} Recently the expression of GPR37 has been confirmed in mouse macrophages but not on microglia.⁶⁴ Neuroprotection D1 (NPD1, PD1) has been identified as a ligand for GPR37 as it induced a significant increase in intracellular calcium in both HEK293 cells overexpressing GPR37 and mouse peritoneal macrophages (Table 1 and Figure 3).⁶⁴ NPD1 also induced an increase in peritoneal macrophage phagocytosis of zymosan particles, an effect diminished in *Gpr37^{-/-}* mouse peritoneal macrophages. *Gpr37^{-/-}* mice also showed enhanced zymosan-induced upregulation of pro-inflammatory cytokines IL-1β and reduced levels of anti-inflammatory cytokines in inflamed skin.⁶⁴ More recently, GPR37 has also been suggested to be involved in the

protection against cell damage and inflammation after ischemic stroke, as *Gpr37*^{-/-} mice exhibited enhanced cell death and infarct size.¹⁵³ However, due to the clear role within the central nervous system (CNS), GPR37 may prove a less than desirable therapeutic target.

■ ACTIVATION OF OTHER RECEPTORS BY SPMs

Apart from the GPCRs described above, many SPMs activate other receptors including non-GPCRs, for example nuclear receptors. NPD1 activates peroxisome proliferator-activated receptor gamma (PPAR γ) where human neuronal-glial (HNG) cells cotransfected with hPPAR γ -GAL4 and MH100-tk-luc enhanced the PPAR γ transactivation reporter activity in a dose-dependent manner.¹⁵⁴ Transcriptional activity of PPAR γ was also significantly increased following treatment with 100 nM NPD1.¹⁵⁵ RvD1 has been postulated as a ligand for PPAR γ where its suppression of I κ B α degradation and NF- κ B p65 nuclear translocation were partially reversed by the PPAR γ inhibitor GW9662 in an LPS-induced acute lung injury model.¹⁵⁶ However, this pairing requires further study as RvD1 and RvE1 failed to activate the nuclear receptors including PPAR γ and retinoid X receptor- α when coexpressed in HEK293 cells linked to Gal4 DNA-binding domain.⁹⁴

■ DEVELOPMENT AND CLINICAL EVALUATION OF SPMs AND SYNTHETIC AGONISTS

SPMs are highly potent and efficacious endogenous ligands that have shown great potential in preclinical animal models. However, due to their delicate physicochemical nature being prone to metabolic inactivation and the complex chemical routes required to synthesize them,^{49,162,163} developing stable synthetic analogs to replicate the pro-resolving effects has been of interest. While some ligands have proved useful via topical application very few have progressed to clinical trials. RX-10045 (Resolvix and Celtic Therapeutics), a synthetic analog of RvE1, recently produced dose-dependent and statistically significant improvements in the treatment of dry-eye disease.¹⁶⁴ Furthermore, a 0.1% nanomicellar solution of the compound recently completed a phase 2 study for ocular inflammation and pain in cataract surgery, although no results have yet been disclosed (clinicaltrials.gov; NCT02329743). However, RX-10045 is a strong inhibitor of organic cation transporter-1 (OCT-1), suggesting novel formulation strategies may be required to achieve improved clinical efficacy.¹⁶⁵ A study using Lipinova, a combination of three DHA metabolites (17-HDHA, 18-HEPE, and 14-HDHA), to investigate the effects of inflammation on patients following orthopedic surgery is currently on hold following recruitment (clinicaltrials.gov; NCT03434236). Finally, a synthetic benzo-RvD1 analog, benzo-diacetylenic-17R-RvD1 methyl ester (BDA-RvD1), showed significantly high potency in reducing neutrophil infiltration in ischemia-reperfusion-initiated second organ injury and stimulating the phagocytosis of zymosan A particles.¹⁶⁶

Various stable analogs of LXA₄ have also shown promise in trials. Topical 15(R/S)-methyl-LXA₄ significantly reduced the severity of eczema with an efficacy similar to topical corticosteroid, Mometasone furoate (Eloson).¹⁶⁷ Inhaled 5(S),6(R)-LXA₄ methyl ester and BML-111, an LXA₄ agonist, displayed efficacy and safety in a pilot study of asthmatic children with acute moderate episodes, although efficacy was reduced compared to that of the standard of care (salbutamol).

Nevertheless, these data indicate an inhaled LXA₄ analog in combination with an LXA₄ receptor agonist may exhibit a novel therapeutic strategy for asthma.¹⁶⁸ Benzo-LXA₄ analogs have also portrayed diverse pro-resolving properties, where it was shown to reduce PMN infiltration and pro-inflammatory cytokine generation,¹⁶³ suppress renal fibrosis,¹⁶⁹ and attenuate obesity-induced adipose inflammation while restoring the ratio of pro-/anti-inflammatory macrophages.¹⁷⁰ Finally, a benzo-fused ring-modified LXA₄ analog (BLXA4-ME) is currently being assessed as a topical oral rinse treatment of gingivitis (clinicaltrials.gov; NCT02342691).

In addition to these LXA₄ analogs, whose effects are mostly via FPR2/ALX, extensive drug discovery efforts have been ongoing to develop selective and potent FPR2/ALX agonists. One of the first reported FPR2/ALX synthetic agonists was AR234245 from Arena Pharmaceuticals in 2004. Since then multiple pharmaceutical and academic groups have developed FPR2/ALX agonists, including Acadia Pharmaceuticals, Allergan, Amgen, and Daiichi Sankyo, many of which share a common aniline moiety, substituted at the para position; for a comprehensive view on these medicinal chemistry efforts, see ref 106. Since these initial efforts, Actelion Pharmaceuticals have become the leaders in this field with the development of the first-in-class anti-inflammatory agonist ACT-389949. In two phase 1 randomized double-blind studies, ACT-389949 resulted in a dose-dependent, long-lasting internalization of FPR2/ALX in leukocytes. Although promising, the inhibitory effect on leukocyte number appeared to be only transient, ~2 h postdose, with numbers reverting to baseline shortly thereafter. In addition, both pro- and anti-inflammatory cytokines were upregulated, while no effect was observed in an LPS inhalation model.¹⁷¹ Although safe and well-tolerated, receptor internalization clearly demonstrates the added challenges developing a potent and efficacious agonist and may explain the rapid rise in leukocytes following the initial decrease. No preclinical characterization of ACT-389949 has been reported; however, a recent study using CHO cells expressing FPR2/ALX has shown that ACT-389949 efficiently recruits β -arrestin with low nanomolar potency.¹⁷² As β -arrestin recruitment is a hallmark of receptor internalization these findings support those from the phase 1 trials.¹⁷³

■ CURRENT STATE OF SPM DRUG DISCOVERY AND NOVEL MODES OF TARGETING SPM GPCRS

It is clearly evident that the pharmacology of these SPM GPCRs is complex, multifaceted and in most cases incomplete. Great efforts have been made in the development of synthetic SPM analogs or small molecule agonists. However, this development has been markedly hampered by a lack of understanding of the mechanism(s) of SPM/ligand–receptor internalization, and signal transduction pathways—the potent tachyphylaxis of ACT-389949 attest to this. At present two key knowledge gaps exist. First, the greatest complexity is the identification of multiple pro-resolving mediators that act on multiple GPCRs. These ligands include not only the endogenous SPMs but also their aspirin-triggered forms. Moreover, the endogenous production of these SPMs is typically transient, which for the D-series resolvins (RvD1–RvD6) appear to be time-dependent during the resolution phase; thus, the kinetic variation in the release can lead to distinct ligand-induced signaling events.¹⁷⁴

Second, as described above, multiple binding sites have been identified at FPR2/ALX allowing both lipids (LXA₄) and

peptides (SAA, LL-37, ANXA1) to bind simultaneously. This allosteric binding can lead to both positive and negative outcomes. For example, binding of SAA results in the release of IL-8 by airway epithelial cells and can be detected in the bronchoalveolar lavage fluid of chronic obstructive pulmonary disease (COPD) patients, while coaddition of LXA₄ results in the inhibition of IL-8 release and decreased neutrophil recruitment.¹⁷⁵ However, during acute exacerbations, peripheral blood SAA levels are significantly increased, therefore dampening the inhibitory effects of LXA₄.¹⁷⁵ As many SPMs bind to multiple receptors, allosteric binding sites may be present on all SPM GPCRs. In fact, many of these ligands have been demonstrated to act allosterically on a number of non-SPM GPCRs. For example, treatment of LXA₄ in mouse brain led to cannabinoid-like effects, which were efficiently blocked by CB₁R antagonists.¹⁷⁶ Furthermore, the affinity of a range of cannabinoids, including anandamide, CP55940, and WIN55212-2 were increased in the presence of LXA₄, demonstrating clear allosteric cooperativity.¹⁷⁶ Similarly, the cathelicidin-related antimicrobial peptide, LL-37, acts as an allosteric modulator of P2X purinoreceptor 7 (P2 × 7) and C-X-C chemokine receptor 2 (CXCR2) receptors.^{177,178}

To date, six SPM GPCRs have been identified, three of which still remain orphan receptors, i.e., receptors for which identification of the endogenous ligand(s) has not been yet officially confirmed. Over 140 orphan GPCRs remain to be deorphanized,⁶⁶ indicating that perhaps even more SPM receptors could exist, which would be consistent with the vast number of SPMs identified. As such, ligand polypharmacology and receptor pleiotropy suggest distinct opportunities to target these receptors via novel mechanisms.

Biased Signaling. Previous dogma was that agonist binding would stabilize a single active conformation of the receptor and initiate the downstream signaling events.^{179,180} Although each ligand could have different efficacies, each agonist would activate a shared set of signaling transduction pathways.¹⁸⁰ However, it is now widely accepted that each ligand is able to stabilize a unique receptor conformation and selectively produce a signal that is “biased” toward a specific transduction pathway.¹⁸¹ The attraction of biased agonists in drug development is 4-fold.¹⁸² First, an agonist can be biased toward a favorable pathway, e.g., β -arrestin activation of parathyroid agonists for the treatment of osteoporosis.¹⁸³ Second, an agonist can be biased away from a detrimental or an undesired pathway that could result in off-target side effects, e.g., β -arrestin activation of CXCR3 reduces T-cell migration and induces inflammation.¹⁸⁴ Third, bias can be away from the desired pathway and inhibition of the endogenous agonist, e.g., angiotensin-mediated β -arrestin signaling and inhibition of angiotensin-mediated vasoconstriction in heart failure.¹⁸⁵ Finally, where different isoforms of the same receptor exist in discrete locations and display differential G-protein coupling (e.g., CXCR3 and histamine H₃R).^{186,187} Generally, bias is classified if differences are observed in signaling profiles.¹⁸² This phenomenon is extremely useful in GPCR drug discovery as the profile of a compound can be optimized to the most opportune signaling pathway. Finally, biased signaling can be affected by the binding kinetics of ligand in question.¹⁸⁸ In fact, ligand binding kinetics has now become a more appreciable aspect of drug discovery and should be given due attention.^{189–191}

Biased agonism has been identified for GPCRs where multiple endogenous agonists exist. For example, α -neo-

endorphin, Met-enkephalin-Arg-Phe, and endomorphin-1, endogenous agonists of the μ -opioid receptor (MOP), display bias away from receptor internalization, β -arrestin recruitment, and ERK1/2 phosphorylation in comparison to the synthetic peptide, DAMGO.¹⁹² Meanwhile, angiotensin 1 receptor (AT₁R), an agonist AT-(1–7) has been identified as a β -arrestin biased agonist involved in cardioprotection.¹⁹³ Therefore, given the large number of SPMs that pleiotropically activate multiple GPCRs, it is perhaps surprising that there is little information on biased signaling of the SPM GPCRs. However, some evidence does exist for FPR2/ALX, albeit using synthetic molecules. FPR2-activating pepducin F2 Pal10 appears biased away from β -arrestin in comparison to the FPR2-specific peptide agonist, WKYMVM, which resulted in reduced receptor internalization and impaired neutrophil chemotaxis.¹⁹⁴ More recently, Lau-[(I)-Aoc]-[Lys- β Nrpe]₆-NH₂, an analog of the FPR2-selective lipidated α -peptide/ β -peptoid agonist Lau-[(S)-Aoc]-[Lys- β NPhe]₆-NH₂, was developed with a similar bias away from β -arrestin recruitment.¹⁹⁵ Moreover, compound 17b, an FPR1/2 biased agonist for the treatment of myocardial infarction, revealed bias away from the detrimental FPR1/2-mediated calcium mobilization but retained ERK1/2 and Akt phosphorylation fundamental for pro-survival.¹⁹⁶

Although not strictly biased signaling, ANXA1, Ac2-26, and LXA₄ have been shown to induce biased homo and heterodimerization of FPR2/ALX and FPR1, both in recombinant HEK293 assay systems and neutrophils, leading to alterations in the signaling profile.¹⁰⁷ Concomitant binding of ANXA1 and Ac2-26 leads to homodimerization of FPR2/ALX resulting in the activation of MAPK and the release of the anti-inflammatory cytokine IL-10, while binding Ac2-26 and LXA₄ results in FPR2/ALX-FPR1 heterodimerization, activation of JNK/caspase-3, and neutrophil apoptosis. Formation of this heterodimer resulted in a loss of the inhibitory effects of the endogenous antagonists SAA and LL-37.^{75,107} However, the existence of physiologically relevant family A GPCR dimers still remains a subject of intense investigation. Evidence suggests only a small proportion of a single receptor population exist as dimers (<20%), and when observed, these events appear to be ligand-specific.¹⁹⁷ Although of great interest, no follow up study has been published in the intervening six years since this original discovery, but further investigation into this receptor dimerization is clearly warranted.

Of all the receptors described GPR32 could be the best placed for the observation of biased signaling. Although very little is known about the receptors signaling cascade four of the six D-series resolvins bind to GPR32.⁵³ Furthermore, there is evidence that each aspirin-triggered version binds, indicating a vast number of endogenous ligands for one receptor.⁵³ Interestingly, many of these ligands were identified using a β -arrestin recruitment assay where GPR32 was overexpressed,⁹⁵ indicating that a broader array of pathways would need to be assessed in an attempt to identify bias. However, differences in the receptor internalization have been identified in the absence or presence of the pro-inflammatory cytokine TNF- α , indicating possible inflammation-driven pathway bias.

Allostery. Along with bias, allosteric modulation of GPCRs has become an important addition to the drug discovery landscape. In addition to multiple orthosteric binding sites, where the natural ligand(s) for the receptor binds, additional binding sites have been discovered on many GPCRs. These allosteric binding sites are spatially distinct from the orthosteric

ligand-binding pocket, which enables the binding of two ligands simultaneously.¹⁹⁸ Binding of these ligands can occur independent of each other, producing their own intrinsic effect. However, when bound concomitantly the ligands exert reciprocal effects, a phenomenon termed cooperativity. Four predominant classes of allosteric modulator have been identified from drug discovery efforts (although potentially many more may exist): (i) positive allosteric modulators (PAM), which enhance the endogenous ligands activity; (ii) ago-PAMs, where the PAM also displays its own intrinsic efficacy; (iii) negative allosteric modulators (NAM), which oppose the endogenous ligand's activity; and (iv) neutral allosteric ligand (NAL), which binds but has no net effect on the endogenous ligand.¹⁹⁹

The benefit of an allosteric modulator compared to an orthosteric ligand is potentially 2-fold. First, generally allosteric binding sites are less conserved than their orthosteric binding site counterparts, therefore enabling the possibility of greater subtype selectivity, if necessary. Second, and perhaps more importantly, the effect of the modulator is limited by the degree of cooperativity and is therefore independent of ligand concentration, enabling greater levels of compound safety.¹⁹⁹ In essence, allosteric modulators are able to fine-tune the pharmacological response as desired. This effect enables the spatial and temporal nature of endogenous agonist signaling to be maintained, notably when changing the kinetics of release of certain SPMs during resolution are known.

As of 2018, 17 modulators have been approved or are currently under active clinical development. These modulators have been developed for a wide range of diseases from HIV (Maraviroc; NAM, CCR5) to hypercalcemia (Cinacalcet; PAM, calcium-sensing receptor) and schizophrenia (ASP4345; PAM, dopamine D1).²⁰⁰ This steady growth in the mechanistic understanding of allosteric modulators and the subsequent development of potential therapeutics clearly highlights a distinct opportunity in targeting SPM GPCRs. Although the exact binding sites of most SPMs are still to be elucidated, based on the knowledge gained from FPR2/ALX the probability of similar allosteric interactions occurring would appear high. As described above, GPR18 was originally designated as a cannabinoid receptor due to its binding of cannabinoid ligands, including NAGly and various synthetic molecules.¹²⁰ Therefore, as LXA₄ binds allosterically to CB₁R, allosteric modulation of GPR18 may also be a distinct possibility. Moreover, due to the lipid composition of many SPMs access to their binding site may be via the membrane lipid bilayer, as demonstrated by sphingosine-1-phosphate binding to its receptor (S1P₁R), AM841 and 2-AG binding to CB₂R, and vorapaxar binding to protease-activated receptor-1 (PAR1),^{201–204} thus potentially allowing simultaneous binding of ligands from the extracellular space. For a recent comprehensive review, see ref 205. Intriguingly, a cryo-EM structure of FPR2/ALX in complex with G_{ai} has been registered in the Research Collaboratory for Structural Bioinformatics-Protein Data Bank (RCSB-PDB; structure ID 60MM). To date, no description of the ligand(s) used is reported; however, this may reveal the structural confirmation of multiple binding sites.

Biased Allostery. As a composite of the preceding sections, allosteric modulators can in themselves generate biased signaling. For example, the ago component of an ago-PAM can display bias alone, while the PAM component can bias an otherwise unbiased agonist.^{206,207} Furthermore,

cooperativity can alter the bias depending on which endogenous ligand is present, a term known as probe dependency.²⁰⁷ Examples of biased modulators now exist including PAMs of muscarinic acetylcholine receptor 1 (M₁R) and metabotropic glutamate receptor 5 (mGlu₅R) and NAMs of the calcium-sensing receptor (CaSR) and prostaglandin F_{2α}, (PGF_{2α}).^{208–212} For comprehensive reviews, see refs 207 and 213.

■ APPLICATION TO SPM GPCR DRUG DISCOVERY

These novel mechanisms in targeting SPM GPCRs may be even more relevant following the disappointing phase 1 trial of the FPR2/ALX agonist, ACT-389949.¹⁶⁷ Due to the compound's efficient recruitment of β -arrestin,¹⁶⁸ internalization of the receptor was persistent, ultimately causing the ligand to act as a functional "antagonist". Therefore, a biased ligand away from β -arrestin may prove a more suitable candidate. Conversely, there is a growing body of work demonstrating intracellular signaling of receptors where either the initial signaling is prolonged (persistent signaling) or the alternative signaling pathways are activated,^{214,215} indicating that in some cases receptor internalization could be a valued characteristic or necessity.⁷⁶ Alternatively, development of an FPR2/ALX PAM to RvD1 could be worth considering, enabling greater control of receptor internalization when only the endogenous agonist is present. In fact, a biased PAM (away from β -arrestin) may be the ideal candidate. However, as the compound produced both pro- and anti-inflammatory cytokine release, other pathways (and/or receptors if dimers are involved) may have been implicit in its reduced clinical efficacy. Finally, as described above, COPD patients suffering from acute exacerbations may benefit from a selective NAM of SAA, typically elevated in peripheral blood, or a PAM of LXA₄ (or potentially a combination of both via probe dependency), thus restoring LXA₄'s inhibitory effect. Ultimately, due to the vast number of SPMs and GPCRs identified the combinations could be limitless, clearly highlighting significant opportunities in augmenting the resolution of inflammation. Nevertheless, complete pharmacological characterization of the signaling pathways is required, enabling target-specific decisions to be made.

■ CONCLUSION

Discovery of specialized pro-resolving mediators and their cognate receptors has led to a greater understanding of the resolution of inflammation. However, over 20 years have passed since the first identification of FPR2/ALX and LXA₄, and to date no compound has successfully reached the clinic for any SPM receptor. Although significant strides have been made in identifying the numerous ligand/receptor pairings, the downstream signaling observed is more complex, leading to increasingly well-described physiological responses. The information gained from multiple individual studies has allowed, in part, development of a representation of how resolution may occur;¹⁶⁰ nevertheless, many unanswered questions requiring investigation.

With the substantial increase in ligand/receptor function, including biased signaling and allosteric modulation, these new mechanisms add further complexity to an area already rich in challenges. However, these mechanisms should be seen as great opportunities, especially in light of the recently failed FPR2/ALX phase 1 trial. Perhaps the greatest hurdle to

overcome is in the complete understanding of receptor signaling, particularly in native immune cells, thus highlighting which ligand and/or pathway may require greater consideration. Nevertheless, with both pharmacological and analytical tools readily available, therapeutics for inflammatory diseases may soon be commonplace.

AUTHOR INFORMATION

Corresponding Authors

Christopher J. Langmead – Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville 3052, Australia; orcid.org/0000-0003-3483-1120; Email: chris.langmead@monash.edu

Darren M. Riddy – Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville 3052, Australia; orcid.org/0000-0003-0524-1498; Email: darren.riddy@monash.edu

Author

Julia Park – Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville 3052, Australia

Complete contact information is available at:
<https://pubs.acs.org/10.1021/acspsci.9b00075>

Notes

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