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A biased ligand for OXE-R uncouples Gα and Gβγ signaling within a heterotrimer

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Differential targeting of heterotrimeric G protein versus β-arrestin signaling are emerging concepts in G protein–coupled receptor (GPCR) research and drug discovery, and biased engagement by GPCR ligands of either β-arrestin or G protein pathways has been disclosed. Herein we report on a new mechanism of ligand bias to titrate the signaling specificity of a cell-surface GPCR. Using a combination of biomolecular and virtual screening, we identified the small-molecule modulator Gue1654, which inhibits Gβγ but not Gα signaling triggered upon activation of $G\alpha_i$-βγ by the chemoattractant receptor OXE-R in both recombinant and human primary cells. Gue1654 does not interfere nonspecifically with signaling directly at or downstream of Gβγ. This hitherto unappreciated mechanism of ligand bias at a GPCR highlights both a new paradigm for functional selectivity and a potentially new strategy to develop pathway-specific therapeutics.

GPCRs are transmembrane proteins that share a common superstructure consisting of an extracellular N terminus and an intracellular C terminus connected by seven α-helical domains[1–5]. Their biological importance has inspired intensive investigations on many fundamental aspects concerning their function and regulation and is reflected by the fact that GPCRs belong to the most commonly targeted protein families for prescribed medicines[6]. GPCRs use extracellular and transmembrane regions to sense a variety of physiological stimuli such as neurotransmitters, hormones, chemokines, lipids and nucleotides, among many others. Despite the chemical diversity of their activating ligands, GPCRs do share a common mechanism of relaying information from outside the cell to its interior. Upon binding to extracellular stimuli, GPCRs signal either through heterotrimeric Gα-βγ proteins from different families ($G\alpha_i$ or $G\alpha_o$ ($G\alpha_{i/o}$), $G\alpha_{q/11}$, $G\alpha_s$, $G\alpha_{12/13}$) and/or—as more recently appreciated—through β-arrestin proteins[5,7–10]. For a large number of GPCRs, engagement of multiple G proteins has been described, separate from or in parallel with stimulation of β-arrestin pathways[5,8–14]. Meanwhile, several ligands have been characterized, the pharmacology of which depends on the individual receptor signaling endpoints that are being examined[5,8,10–17]. Ligands that differentially affect downstream pathways are now referred to as biased ligands or functionally selective ligands, and the terms functional selectivity, ligand bias, agonist trafficking of receptor responses and pluridimensional efficacy have been coined to describe effector-dependent pharmacology of GPCR modulators[5,13,14,18]. Some of the biased agonists have evolved as invaluable tools to deconvolute complex signaling patterns of GPCRs both *in vitro* and *in vivo*[5,9,12]. It could even be envisaged that the concept of ligand bias will translate into new and more specific approaches for therapeutic intervention and hence become meaningful *in vivo*[8,9]. Biased ligands are defined by their ability to selectively or preferentially activate one or more effector pathways over others; for example, discriminate between individual heterotrimeric G protein pathways or between G protein and β-arrestin signaling[5,13,14,17–19]. Biased ligands may also show opposing efficacies in a pathway-dependent manner[5,10,13,18,20,21]. Apparently, there is heterogeneity in both the G proteins and β-arrestins that are available for interaction with activated receptors. The numerous examples of biased ligands have resulted in the appreciation that individual ligands may stabilize distinct receptor conformations and that a single receptor may adopt multiple, ligand-specific conformational states[5,10,11,13,14,18,20].

In this study, we present a concept for ligand bias that is, to our knowledge, unprecedented, using the receptor for the lipid mediator 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-oxo-ETE), referred to as OXE-R[22–24], as a model system. OXE-R is a $G\alpha_{i/o}$-sensitive receptor that is expressed in eosinophils, neutrophils, basophils and monocytes. Its endogenous agonist 5-oxo-ETE acts as a potent chemoattractant for these cells[22–24]. A large number of downstream signaling events induced by 5-oxo-ETE–bound OXE-R are mediated via $G\alpha_{i/o}$-βγ heterotrimeric G proteins[22–24]. Yet, the pathophysiological role of this ligand–receptor complex is not well understood, in part because of the lack of pharmacological tools for studying this receptor.

Herein, we report on the identification of the first small-molecule inhibitor for the OXE-R, Gue1654, with a hitherto unappreciated mechanism of action: Gue1654 selectively disrupts Gβγ but not $G\alpha_i$ signaling triggered upon activation of the $G\alpha_i$-βγ heterotrimer by OXE-R. Such a Gβγ-biased mechanism represents an expansion in the chemical space for GPCR ligands and offers an entirely new

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concept of selective interference with the signaling repertoire of cell-surface receptors and, therefore, a potentially new strategy to develop signaling pathway–selective therapeutics.

RESULTS

Receptor-based identification of a new OXE-R modulator

To identify antagonists for OXE-R, we performed a biomolecular screening in Ca^{2+} mobilization assays on OXE-R–expressing cells using a subset of our in-house small-molecule repository of compounds from natural sources and synthetic ligands. To accomplish this, we pretreated human embryonic kidney (HEK293) cells that were stably transfected to express both OXE-R and the promiscuous $G\alpha_{16}$ protein with the potential receptor modulators, which were tested for their ability to inhibit intracellular Ca^{2+} flux upon stimulation with the agonist 5-oxo-ETE. The primary screen yielded two structurally closely related ligands (Gue1157 (**1**) and Gue1158 (**2**); **Fig. 1a**) that consistently showed about 30% inhibition of 5-oxo-ETE–mediated Ca^{2+} flux (**Supplementary Results**, **Supplementary Fig. 1a**) but did not trigger Ca^{2+} mobilization on their own (**Supplementary Fig. 1b**), and thus they did not mask inhibition of agonist responses in this assay. Synthetic routes to both inhibitors are shown in **Supplementary Figure 2**, and a detailed description of synthesis and compound characterization can be found in **Supplementary Methods**. To examine whether the antagonists retain the capacity to inhibit OXE-R in its natural environment, we chose to use chemotactic activation of human eosinophils as a functional assay. Preincubation of eosinophils with Gue1157 substantially reduced 5-oxo-ETE–mediated shape change (**Supplementary Fig. 3a**), whereas no inhibition of shape change by Gue1157 was observed when eosinophils were stimulated with prostaglandin D_2 (**Supplementary Fig. 3b**), indicating that the antagonistic effect is specific to OXE-R. However, Gue1157 caused unexpected, non-specific activation of neutrophils that also express OXE-R[23,24], and higher concentrations of this ligand (100 μM) activated both neutrophils and eosinophils (**Supplementary Fig. 3c**,**d**). To identify additional OXE-R antagonists and minimize biomolecular screening efforts, we chose to perform a virtual screen of the ZINC database (http://zinc.docking.org/)[25] using Gue1157 and Gue1158 as bait.

Prior to the virtual screen, the structural requirements for the interaction between the ligands and OXE-R were defined by computational modeling (**Supplementary Fig. 4a**,**b**) guided by the recently solved X-ray structure of the chemokine receptor CXCR4 (ref. 2).

On the basis of this computational model, a virtual screen of the ZINC database of commercially available compounds was performed. The best 100 compounds were sorted by the Autodock scoring function and docked into the OXE-R model using the AutoDock Vina virtual screening tools[26]. From the top 100 compounds, 10 were chosen by visual inspection, acquired from commercial vendors and tested for inhibition of OXE-R–mediated Ca^{2+} release, and a single active compound was identified (ZINC code 11852816, hereafter referred to as Gue1654 (**3**); **Fig. 1a**). Gue1654 lacks the protonated amine group as compared with Gue1157 and Gue1158, but otherwise it anchors the OXE-R in a manner similar to the other tested compounds (**Supplementary Fig. 4c**).

Apparently, the entire molecule, including all of its pharmacophoric elements, is required for biological activity, as fragmentation of Gue1654 into smaller pieces did not allow identification of fragments or functional groups capable of inhibiting OXE-R–mediated Ca^{2+} flux (**Supplementary Table 3**). The fragments were designed in an attempt to not only identify new directions for synthetic optimization but also generate compounds with improved physicochemical properties, as Gue1654 is rather lipophilic compared

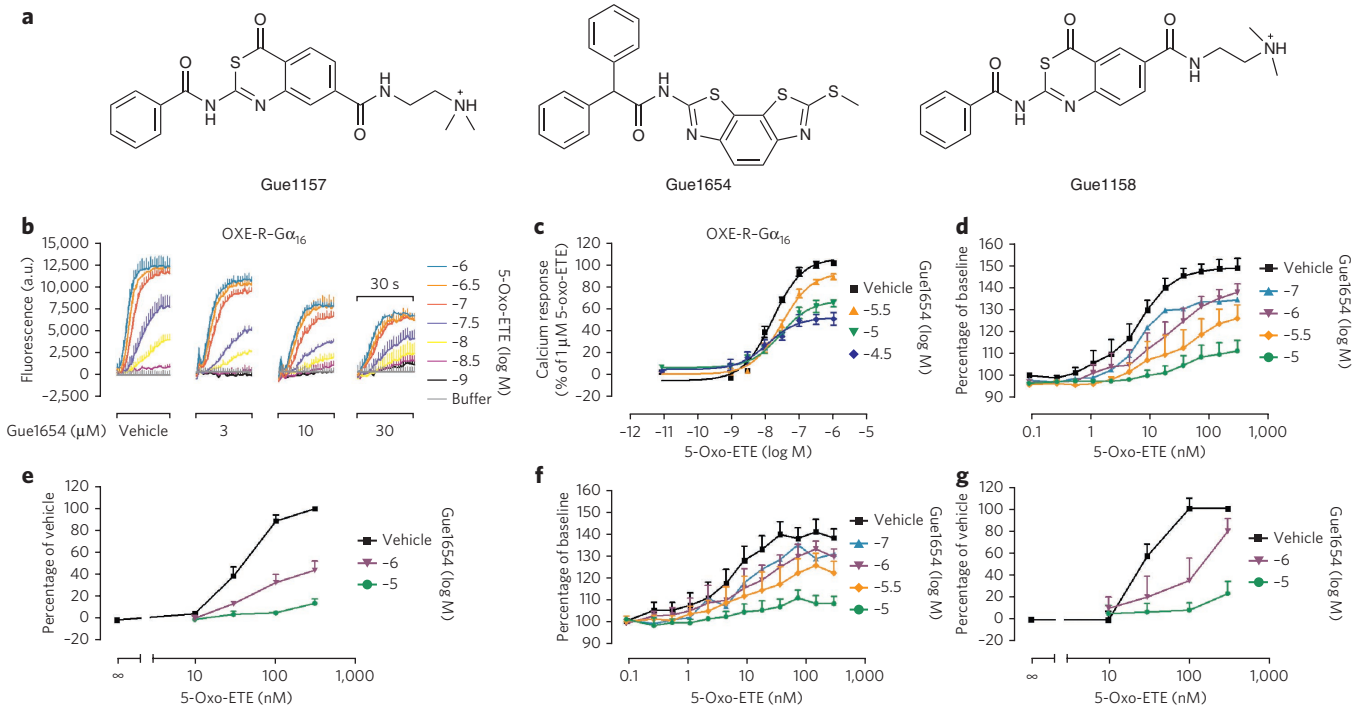

Figure 1 | Gue1654 is a functional antagonist of OXE-R in human recombinant and primary cells. (**a**) Chemical structures of Gue1157, Gue1158 and Gue1654. (**b**,**c**) Gue1654 antagonism of intracellular Ca^{2+} mobilization mediated by OXE-R. OXE-dependent Ca^{2+} mobilization in the absence (vehicle) or presence of the indicated Gue1654 concentrations (**b**). Traces are from one representative out of three independent experiments. Concentration-effect curves of 5-oxo-ETE in the absence (vehicle) and presence of Gue1654 (**c**). Data are mean values ± s.e.m. from at least three separate experiments. (**d**,**e**) Concentration-dependent inhibition by Gue1654 of neutrophil shape change (**d**) and neutrophil chemotaxis (**e**) triggered upon stimulation of cells with 5-oxo-ETE. (**f**,**g**) Concentration-dependent inhibition by Gue1654 of eosinophil shape change (**f**) and eosinophil chemotaxis (**g**) triggered upon stimulation of cells with 5-oxo-ETE. Data in **d**–**g** represent mean values + s.e.m. of three independent experiments. A.u., arbitrary units.

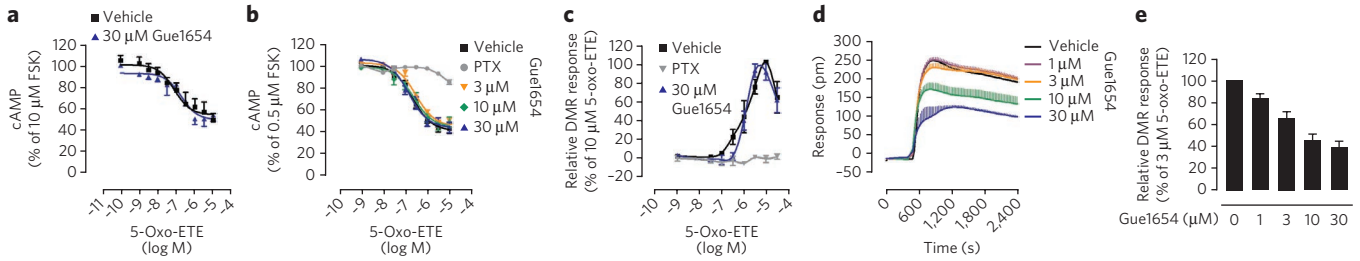

Figure 2 | Gue1654 inhibits $G\alpha_{16}$- but not $G\alpha_i$-dependent signaling of OXE-R in recombinant and primary cells. (**a**) Inhibition of forskolin (FSK)-stimulated cAMP production by 5-oxo-ETE in the absence (vehicle) and presence of Gue1654 in human neutrophils. Gue1654 does not interfere with the ability of 5-oxo-ETE to lower intracellular cAMP production. (**b**) Inhibition of forskolin-stimulated cAMP production by 5-oxo-ETE in the absence (vehicle) and presence of the indicated Gue1654 concentrations in HEK293 cells stably transfected to coexpress OXE-R and $G\alpha_{16}$. PTX pretreatment abolishes the functional 5-oxo-ETE response and confirms its $G\alpha_i$ origin. Gue1654 fails to inhibit OXE-R signaling through $G\alpha_i$ in the HEK293 cellular background. (**c**) Lack of inhibitory efficacy of Gue1654 on the OXE-R-$G\alpha_i$ signaling pathway, as determined in DMR assays. HEK293 cells transiently transfected to express OXE-R were stimulated with 5-oxo-ETE in the absence (vehicle) or presence of Gue1654. $G\alpha_i$ origin of the functional response was verified by abrogation of the agonist DMR signal with PTX. (**d**,**e**) Gue1654 attenuates OXE-R-$G\alpha_{16}$ signaling in a concentration-dependent manner. HEK293 cells stably expressing OXE-R and $G\alpha_{16}$ were pretreated with PTX to silence $G\alpha_i$ activity, and DMR was monitored after stimulation with 3 µM 5-oxo-ETE. Data in **a** are mean values + s.e.m., and data in **b** and **c** are mean values ± s.e.m. of at least three independent experiments. Depicted in **d** are original optical traces that are representative of at least three independent experiments, and data in **e** represent mean values + s.e.m. of three such experiments.

with Gue1157 and Gue1158 (logD values at pH 7.4 as a measure for lipophilicity are 5.28 (Gue1654), 1.02 (Gue1157) and 0.87 (Gue1158); **Supplementary Methods**, **Supplementary Fig. 5** and **Supplementary Tables 1** and **2**).

To further characterize Gue1654 in a number of different cellular assays, we also prepared it by chemical synthesis (**Supplementary Methods** and **Supplementary Fig. 6**).

Gue1654 interdicts various OXE-R signaling routes

Gue1654 was of particular interest as it had a number of favorable properties: (i) it inhibits OXE-R–mediated Ca^{2+} mobilization in a concentration-dependent, albeit noncompetitive, manner (**Fig. 1b**,**c**) but does not trigger a Ca^{2+} response when applied alone (**Supplementary Fig. 7a**); (ii) no inhibition of Ca^{2+} flux was observed when cells were treated with carbachol to stimulate endogenous $G\alpha_q$-linked muscarinic receptors (**Supplementary Fig. 7b**); (iii) efficacious and concentration-dependent inhibition of neutrophil shape change and chemotaxis was observed with no signs of nonspecific activation even at high concentrations (**Fig. 1d**,**e**); (iv) likewise, shape change and chemotaxis of human eosinophils that also express the OXE-R were effectively attenuated (**Fig. 1f**,**g**). Inhibition by Gue1654 of 5-oxo-ETE responses in human leukocytes was specific, as the antagonist was completely ineffective when chemotactic activation of neutrophils and eosinophils was achieved by stimulation with the chemoattractant C5a (**Supplementary Fig. 8a**) or the lipid mediator prostaglandin D_2 (**Supplementary Fig. 8b**), respectively.

Gue1654 does not impair all cellular signaling of OXE-R

In addition to increasing cytosolic calcium ion concentrations, OXE-R is also known to inhibit forskolin-stimulated cyclic AMP (cAMP) production via coupling to $G\alpha_{i/o}$ proteins, as observed with Chinese hamster ovary (CHO) cells transfected to stably express this receptor[22]. Inhibition of cAMP formation by 5-oxo-ETE in human primary cells has not been reported to date. We therefore established cAMP inhibition assays in primary human neutrophils and determined whether Gue1654 similarly inhibits 5-oxo-ETE–induced responses via this signaling route. To our surprise, Gue1654 was completely inactive at concentrations sufficient to attenuate neutrophil shape change or chemotaxis (**Fig. 2a**, compare with **Fig. 1d**,**e**). The lack of inhibitory activity of Gue1654 was also assessed using cAMP inhibition assays in HEK293 cells exogenously expressing OXE-R (**Fig. 2b**). Our data support the notion that this pattern of 'selective antagonism' is apparently independent of the cellular background or expression of OXE-R. In fact, the lack of antagonism of Gue1654 is also observed when OXE-R functionality is examined in label-free dynamic mass redistribution (DMR) assays that exclusively monitor $G\alpha_{i/o}$ downstream events triggered by OXE-R (**Fig. 2c**). In contrast, Gue1654 behaves as antagonist when label-free DMR assays are designed to exclusively capture OXE-R signaling downstream of $G\alpha_{16}$ (**Fig. 2d**,**e**), an observation that is congruent with the results from the initial Ca^{2+} mobilization assays, where $G\alpha_{16}$ coexpression was required to guide OXE-R to the Ca^{2+} mobilization pathway. Notably, Gue1654 preincubation had no impact on $G\alpha_{16}$ activation by the chemoattractant receptor CRTH2 (**Supplementary Fig. 9**), indicating that it operates via OXE-R but not via inhibition of signaling at or downstream of $G\alpha_{16}$. Taken together, these results show that Gue1654 apparently represents an antagonist with functional selectivity that is incompetent to impair $G\alpha_{i/o}$ downstream signaling of OXE-R.

A new mechanism of signaling bias at OXE-R

To further corroborate this biased antagonism profile, Gue1654 was examined for its effect on additional neutrophil responses, known to be mediated by engagement of $G\alpha_{i/o}$ proteins. 5-Oxo-ETE is known to induce rapid increases of intracellular Ca^{2+} in human neutrophils in a PTX-sensitive manner[24,27], implicating $G\alpha_i$-βγ proteins in the signaling process. We confirm PTX sensitivity of 5-oxo-ETE–mediated Ca^{2+} flux in human neutrophils (**Fig. 3a**); notably, however, Gue1654 pretreatment completely abolished this $G\alpha_{i/o}$-dependent response (**Fig. 3b**). As a control, Gue1654 had no effect on interleukin-8–dependent increases of Ca^{2+} in neutrophils, confirming that the antagonistic effect is specific to OXE-R (**Supplementary Fig. 10**). Essentially identical results were obtained when human eosinophils were exposed to 5-oxo-ETE and intracellular Ca^{2+} was recorded (**Fig. 3c**,**d**). These cells are also known to transmit their Ca^{2+} signals in a $G\alpha_{i/o}$-sensitive manner[24]. Many chemoattractant activators of leukocytes signal via $G\alpha_{i/o}$-linked GPCRs, and their Ca^{2+} signaling can be specifically obliterated in the presence of pertussis toxin (PTX), which inactivates heterotrimeric $G\alpha_{i/o}$-βγ proteins[28]. However, it has become apparent that PTX-sensitive chemoattractant responses such as Ca^{2+} mobilization or even chemotaxis in leukocytes are not transduced by $G\alpha_{i/o}$ subunits but instead are triggered by the Gβγ complexes released upon $G\alpha_i$-βγ heterotrimer activation[28–30]. Indeed, the $G\alpha_i$-βγ–phospholipase C β2/3 (PLCβ2/3) module was found to be responsible

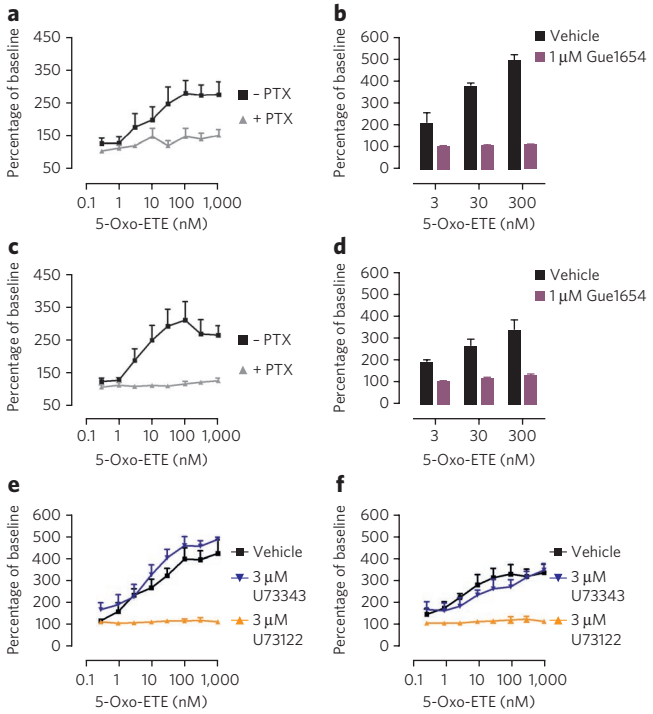

Figure 3 | Gue1654 is an efficacious antagonist of 5-oxo-ETE–dependent Ca^{2+} flux in human neutrophils and eosinophils. (**a**) 5-Oxo-ETE mobilizes Ca^{2+} from intracellular stores in human neutrophils in a PTX-sensitive manner. (**b**) 5-Oxo-ETE–mediated Ca^{2+} flux is completely blunted when neutrophils are preincubated with Gue1654. (**c**) 5-Oxo-ETE mobilizes Ca^{2+} from intracellular stores in human eosinophils in a PTX-sensitive manner. (**d**) 5-Oxo-ETE–mediated Ca^{2+} flux in eosinophils is completely abrogated upon preincubation of the cells with Gue1654. (**e**,**f**) 5-Oxo-ETE–mediated Ca^{2+} release in human neutrophils (**e**) and eosinophils (**f**) is sensitive to pretreatment with the phospholipase Cβ inhibitor U73122 but not with its inactive analog U73343. Data in **a**–**f** represent mean values + s.e.m. of three separate experiments.

for chemoattractant-induced PLC activation and Ca^{2+} mobilization[31] as mice lacking both PLCβ2 and PLCβ3 were unresponsive to chemoattractants in PLC and Ca^{2+} flux assays[32]. In line with this postulated mechanism, inhibition of Ca^{2+} flux in eosinophils and neutrophils was sensitive to pretreatment with the PLC inhibitor U73122 but not its inactive analog (**Fig. 3e**,**f**). Hence, the apparent paradox; that is, inhibition by Gue1654 of PTX-sensitive Ca^{2+} flux but lack of antagonistic efficacy in cAMP assays could only be rationalized if Gue1654 was competent to differentially modulate $G\alpha_{i/o}$- versus Gβγ-triggered signaling events, a mode of biased antagonism that is, to our knowledge, unprecedented.

Gue1654 inhibits Gβγ but not Gαi signaling of OXE-R

To further ascertain whether Gue1654 permits $G\alpha_{i/o}$ but not Gβγ signaling of OXE-R, we established two functional assays to exclusively monitor Gβγ-dependent downstream events. First, HEK293 cells were transiently transfected to coexpress OXE-R, $G\beta_1$ and $G\gamma_2$, and Gβγ-mediated inositol monophosphate (IP1) production was quantified as a measure of receptor activity. 5-Oxo-ETE–mediated IP1 production was increased significantly ($P < 0.0001$) and mediated by Gβγ, as pretreatment with the small-molecule Gβγ inhibitor gallein[33] completely abolished the response (**Fig. 4a**). In contrast, gallein hardly affected IP1 accumulation induced upon stimulation of endogenous $G\alpha_q$-linked muscarinic receptors, in agreement with the inability of small-molecule Gβγ inhibitors to dampen $G\alpha_q$-mediated cellular responses[33]. Importantly, Gue1654, which was ineffective when applied alone, completely abrogated OXE-R–mediated IP1 production but had no impact on IP1 production triggered by stimulation of endogenous $G\alpha_q$-linked muscarinic receptors (**Fig. 4a**), confirming that inhibition of IP1 accumulation is specific to OXE-R. In addition, Gue1654 did not affect Gβγ-mediated IP1 accumulation induced upon stimulation of the $G\alpha_i$-linked muscarinic M2 receptor, further corroborating that it acts through OXE-R but does not inhibit Gβγ downstream signaling in general (**Supplementary Fig. 11**).

Exclusive inhibition by Gue1654 of Gβγ signaling was also observed when OXE-R activity was captured with an assay that records Gβγ-mediated opening of G protein–regulated inwardly rectifying K^+ (GIRK) channels. It is well established that Gβγ subunits, 'released' upon activation of $G\alpha_{i/o}$-coupled receptors, directly interact with GIRK channels and thereby modulate channel opening[34,35]. The ability of GIRK channels to also conduct thallium ions (Tl^+) has recently been exploited in Tl^+ flux assays as a new platform to monitor Gβγ-mediated signaling triggered by $G\alpha_{i/o}$-coupled GPCRs[36]. As we expected, treatment of HEK cells expressing GIRK1/2 transfected to coexpress the OXE-R and preloaded with the fluorescent indicator dye FluoZin-2 AM responded with

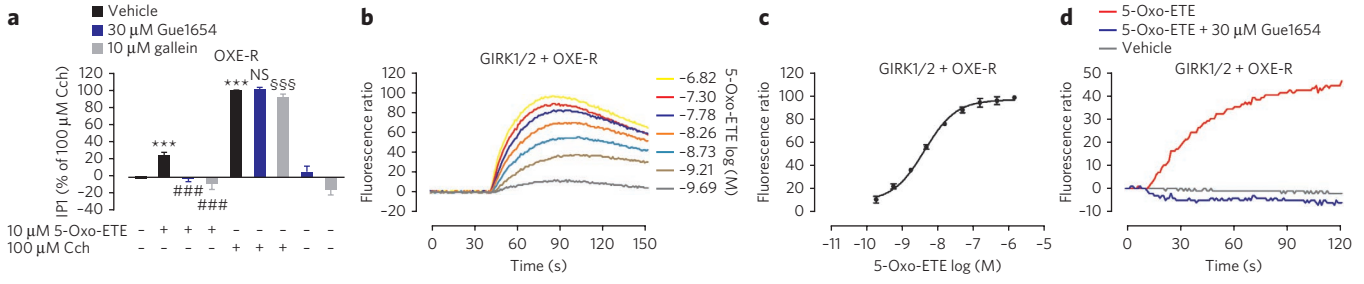

Figure 4 | Gue1654 is a specific inhibitor of OXE-R–Gβγ signaling but does not interfere nonspecifically with signaling at or downstream of Gβγ. (**a**) 5-Oxo-ETE increases IP1 production in HEK293 cells transfected to express OXE-R, $G\beta_1$ and $G\gamma_2$ in a Gβγ-dependent manner. 5-Oxo-ETE–mediated IP1 production is completely blunted by pretreatment with Gue1654 or gallein. IP1 production triggered upon stimulation with carbachol (Cch) via endogenous $G\alpha_q$-linked muscarinic receptors is unaffected by gallein and Gue1654. Data are mean values + s.e.m. (n = 3–8, quadruplicate determinations). ***, significant compared to basal IP1; ###, significant compared to 5-oxo-ETE; §§§, significant compared to carbachol ($P < 0.001$ according to one-way ANOVA with Bonferroni's multiple comparison test); NS, nonsignificant compared to carbachol. (**b**) HEK cells expressing GIRK1/2 cotransfected to express the OXE-R were loaded with the Tl^+ indicator dye FluoZin-2 AM, and Tl^+ flux was recorded after stimulation with the indicated concentrations of 5-oxo-ETE. All traces were vehicle-corrected (mean values of representative experiment, n = 3). (**c**) Concentration-response curve for 5-oxo-ETE calculated from the slopes of the individual traces in the time window of 10 s after stimulus addition and normalized to a maximally effective concentration of 5-oxo-ETE (mean values ± s.e.m., n = 3). (**d**) Pretreatment with 30 μM of Gue1654 of HEK cells expressing both GIRK1/2 and OXE-R dampens Tl^+ flux induced by 2 nM of 5-oxo-ETE. Mean values of traces, representative of three individual experiments are shown.

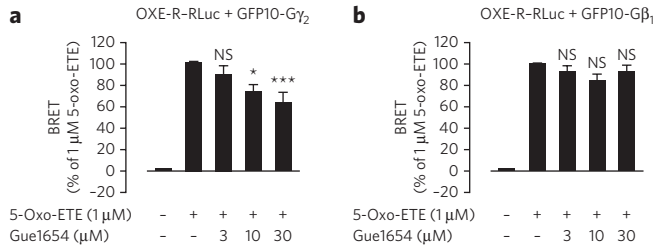

Figure 5 | Gue1654 spatially separates OXE-R from the Gβγ complex. (**a**,**b**) BRET assay between OXE-R fused to RLuc and GFP10-Gγ_2 (**a**) or GFP10-Gβ_1 (**b**) in HEK293 cells expressing the labeled constructs along with unlabeled Gα_{i2}, Gβ_1 or Gγ_2. Data in **a** and **b** are mean values + s.e.m. of 2–7 independent experiments, each performed in triplicate. In **a**, $^*P < 0.05$ and $^{***}P < 0.001$ compared to 5-oxo-ETE–mediated BRET; in **b**, NS is nonsignificant compared to 5-oxo-ETE–mediated BRET according to one-way ANOVA with Bonferroni's multiple comparison test.

robust and concentration-dependent increases in Tl^+ flux (**Fig. 4b**,**c**). No alterations of Tl^+ flux upon application of 5-oxo-ETE were observed in HEK cells expressing GIRK1/2 but lacking the OXE-R (**Supplementary Fig. 12**). In agreement with the postulated mechanism for GIRK channel activation, 5-oxo-ETE responses were completely blunted in cells pretreated overnight with PTX (**Supplementary Fig. 13**) or in cells pretreated for 30 min with the OXE-R antagonist Gue1654 (**Fig. 4d** and **Supplementary Fig. 14**). Notably, Gue1654 was ineffective when Gβγ-mediated opening of GIRK channels was triggered with serotonin via the G$\alpha_{i/o}$-sensitive 5-HT1a receptor (**Supplementary Fig. 15**), again corroborating the notion that Gue1654 inhibits Gβγ signaling of the OXE-R but does not blunt function of Gβγ-mediated effectors in general.

Gue1654 spatially separates OXE-R from the Gβγ complex

To the best of our knowledge, all functionally selective ligands reported so far for GPCRs can discriminate between subfamilies of distinct Gα-βγ heterotrimers or between G protein and β-arrestin signaling[5,8–14,17,19,21]. However, differentiation between Gα and Gβγ signaling of distinct heterotrimers in a receptor-dependent manner has not been achieved to date. To gain mechanistic insight into this new mode of biased antagonism, we assessed whether Gue1654 affects the proximity between or the relative orientation of the receptor and the Gβγ complex using a bioluminescence resonance energy transfer (BRET) assay based on protein-protein interactions. Because previous studies suggested that BRET between the receptor and Gβγ is an indicator of conformational changes propagated from an activated receptor to the G protein[37], and given that a computational OXE-R–Gα-βγ model based on the crystal structure of the β_2-adrenergic receptor in complex with the Gs heterotrimer (Protein Data Bank code 3SN6)[38] suggested that the OXE-R C terminus expands intracellularly toward the N termini of Gβ and Gγ (**Supplementary Fig. 16**), we reasoned that such an assay should prove useful to unravel whether Gue1654 may prevent the 5-oxo-ETE–bound receptor from achieving a conformation required to relay receptor activity to G protein activation. To this end, OXE-R was tagged at its C terminus with the energy donor *Renilla reniformis* luciferase (RLuc), and Gβ_1 and Gγ_2 subunits were N-terminally fused to the energy acceptor GFP10 (refs. 37,39). Addition of RLuc to the receptor's C terminus did not alter its functionality, as determined in label-free DMR assays (**Supplementary Fig. 17**), and functionality of the Gβ and Gγ BRET probes was described previously[39]. Upon stimulation with 5-oxo-ETE, we observed robust engagement of Gβγ, indicating either *de novo* association between OXE-R and Gβγ or a conformational change between a preformed OXE-R–Gβγ complex in which the energy donor and energy acceptor move closer together (**Fig. 5a**,**b**).

Remarkably, the 5-oxo-ETE–induced BRET response between receptor and Gγ was reduced by 40% in the presence of Gue1654 (**Fig. 5a**), whereas receptor-Gβ BRET was hardly affected (**Fig. 5b**). These data suggest that Gue1654 prevents, at least in part, communication between the ligand-activated OXE-R and the Gβγ complex by altering their proximity or relative orientation, respectively. Such molecular rearrangement is conceivable given the flexibility of the N-terminal α-helices of Gβ and Gγ and the spatial flexibility of the receptor's C tail (**Supplementary Fig. 18**). Gue1654, when applied alone, did not affect basal OXE-R–Gβγ BRET (**Supplementary Fig. 19a**,**b**), reflecting its inability to separate a preformed receptor–Gβγ complex.

Notably, when BRET was accomplished between the nicotinic acid receptor HM74A fused to RLuc and the respective Gβγ probe (GFP10-Gγ_2), no inhibition of protein-protein interaction was achieved by Gue1654 (**Supplementary Fig. 20**), suggesting that interaction between OXE-R and the Gγ component of the Gβγ complex is specifically inhibited by Gue1654. Finally, Gue1654 was completely ineffective when tested for its ability to inhibit opening of the Gα_i nucleotide-binding pocket (**Supplementary Fig. 21**), as determined in BRET assays with appropriate sensors for the Gα_i subunit and the Gβγ complex (Gα_{i1}–91-RLuc and GFP10-Gγ_2, respectively)[37,39]. The lack of antagonism in this assay is in good agreement with the absence of antagonist efficacy in G$\alpha_{i/o}$-based signaling assays. Together, these results provide further support for the hypothesis that Gue1654 interferes with agonist-induced receptor signaling in a manner that permits Gα_i but precludes Gβγ activity within an individual Gα-βγ heterotrimer.

DISCUSSION

GPCRs communicate with cells by disassembling or rearranging G protein Gα-βγ heterotrimers into Gα and Gβγ complexes, which in turn affect numerous intracellular effectors[5,37,40]. GPCRs may also signal via β-arrestin proteins, which have different effector pathways than G proteins[5,8,9,12]. Ligand bias is an emerging concept in GPCR basic research and drug discovery, and a number of functionally selective ligands for GPCRs have been described that either discriminate between distinct Gα-βγ heterotrimers[5,11,19] or differentially affect heterotrimeric G protein versus β-arrestin signaling[5,8–10,17,20]. We show here, for what is to our knowledge the first time, that functionally selective ligands may also discriminate between Gα- and Gβγ-mediated downstream events. A small-molecule antagonist Gue1654 was identified that shows no inhibition of Gα_i-dependent signaling of the chemoattractant receptor OXE-R in both recombinant and human primary cells but completely prevents signaling via the Gβγ route, as evidenced in Ca^{2+} assays on primary human leukocytes and also in IP1 and Tl^+ flux assays in human recombinant cells engineered to overexpress OXE-R. The latter two recombinant assays rely completely on transduction of signals from receptors by the G protein βγ subunits.

This previously unknown mode of ligand bias offers a new perspective to fine-tune GPCR signaling and suggests that modulation of receptor function by pharmacological tools and, ultimately, medical therapeutics may be much more flexible and specific than previously anticipated. This would be particularly so if the remaining Gα-βγ subfamilies (Gα_s, G$\alpha_{q/11}$ and G$\alpha_{12/13}$) were equally susceptible to this differential mode of inhibition.

It is widely accepted that different ligands stabilize distinct conformational states of receptors[5,8,11,12,14]. Apparently, Gue1654 selectively stabilizes a receptor conformation that preserves activation by 5-oxo-ETE of Gα_i-mediated downstream events such as inhibition of forskolin-activated adenylyl cyclase but completely precludes propagation of the Gβγ-mediated signal.

Models of receptor–G protein interaction presume that Gβγ subunits either have a passive role for nucleotide exchange on Gα (for example, contributing by scaffolding Gα at the plasma membrane via the prenylated C terminus of Gγ) or, alternatively, participate actively as suggested by the gear-shift and the lever-arm hypothesis[7,30,41]. Although the exact mechanism by which GPCRs trigger Gα nucleotide exchange remains to be fully elucidated, all of these models imply that decreasing the proximity between an activated receptor and the Gβγ dimer should lead to impaired G protein activation.

Indeed, BRET experiments monitoring proximity between an OXE-R–RLuc fusion protein and the Gβγ complex revealed that robust recruitment by OXE-R of Gγ was reduced by about 40% in the presence of Gue1654, whereas proximity between OXE-R and Gβ was hardly affected in the Gue1654-occupied receptor. Remarkably, this partial reduction of receptor-Gγ BRET was sufficient to completely silence Gβγ- but not Gα-dependent downstream signaling of OXE-R. We speculate that this partial BRET inhibition is the basis for Gβγ-biased antagonism, whereas complete inhibition of OXE-R–Gβγ BRET may be associated with silencing of the entire Gα-βγ heterotrimer signaling repertoire. Indeed, the inverse agonist ICI118551 for the β_2 adrenergic receptor that completely blocked isoproterenol-induced energy transfer between receptor and Gβγ also abrogated $G\alpha_s$ activity of the receptor[39]. Similarly, when protein-protein interaction between the α2A adrenoceptor and Gβγ was prevented by the α2A receptor antagonist RX821002, $G\alpha_i$ activation of the receptor was completely blunted[37]. Gue1654, in contrast, neither hindered $G\alpha_i$-GTP from activating its downstream effectors nor interfered with opening of the $G\alpha_i$ nucleotide-binding pocket (**Supplementary Fig. 21**)[37,39]. Inhibition of Gβγ but not Gα signaling by a given GPCR is unexpected because it is generally assumed that once the heterotrimeric G protein is activated by agonist binding to the GPCR, the subsequent actions of Gβγ and Gα proceed independently of the receptor. Inhibition of Gβγ but not Gα signaling is therefore difficult to reconcile with the model that G protein α and βγ subunits dissociate upon receptor activation. Our data rather support the notion that G protein subunits α and βγ are likely to undergo an intramolecular rearrangement during the receptor-mediated GDP-GTP on-off cycle[37,40]. In this complex of receptor, Gα and Gβγ, Gue1654 acts to keep Gβγ apart from the receptor, which is sufficient to preclude Gβγ-effector activation yet insufficient to impair nucleotide exchange on Gα.

Such a pathway-specific form of antagonism can only be explained by an allosteric phenomenon; Gue1654 causes the natural agonist 5-oxo-ETE to change its signaling pattern from activation of Gα and Gβγ responses to exclusive Gα activation, which can only be rationalized by simultaneous co-occupation of OXE-R by both ligands. Remarkably, OXE-R occupation by Gue1654 shares common structural features with the chemokine receptor CXCR4 bound to the small-molecule antagonist IT1t[2]: IT1t binds CXCR4 in a cavity between transmembrane domains 2, 3 and 7. In addition, 1-oleoyl-*R*-glycerol (OLC) inserts into the transmembrane domain bundle from the lipid bilayer via the interface between transmembrane domains 5 and 6 (ref. 2). The remarkable structural similarity between OLC and 5-oxo-ETE led us to propose, as a working hypothesis, similar simultaneous binding poses for IT1t and OLC to CXCR4 and Gue1654 and 5-oxo-ETE to OXE-R, respectively (**Supplementary Fig. 22**, compare to **Supplementary Fig. 4c**). The insurmountable nature of antagonism observed with Gue1654 in the various functional assays is in good agreement with such an allosteric mode of action.

A new class of small molecules specifically designed to inhibit signaling of Gβγ responses without affecting functionality of Gα proteins[30,33] is noteworthy. These Gβγ inhibitors are thought to improve therapeutic options for a number of diseases in which Gβγ subunits represent central participants[30]. Inhibition of Gβγ by small-molecule interaction with the 'hot spot' on this protein complex interferes with downstream signaling in a receptor-independent but partially effector-selective way[30,33]. In contrast, Gue1654 offers the perspective to suppress Gβγ signaling in a strictly receptor-dependent manner. Notably, many drug-screening assays are designed to detect the effects of signaling downstream of Gα proteins, such as the increase of intracellular Ca^{2+} or IP1s for $G\alpha_q$-linked receptors or the increase or decrease of cAMP for $G\alpha_s$- and $G\alpha_i$-coupled receptors, respectively, or are based on the detection of β-arrestin recruitment[5,8,9,12]. Such assays may not be equally suitable to detect signaling via the Gβγ pathway. Screening of a compound library for OXE-R antagonists using a classical endpoint assay on the natural $G\alpha_{i/o}$ signaling pathway of this receptor would not have permitted identification of Gue1654. The findings of this study are therefore highly relevant for design of appropriate assays in drug discovery and development.

In conclusion, we report identification and characterization of the first GPCR antagonist with notable disparity of efficacy for $G\alpha_i$-mediated versus Gβγ-mediated cellular events. Discrimination of receptor signaling on the level of an individual Gα-βγ heterotrimer has not been achieved to date and suggests an ever-increasing number of relevant conformational states that may be adopted by GPCRs and be linked to defined signaling routes. In general, our study opens new vistas for GPCR signaling with considerable implications for the design of new classes of therapeutics to achieve an unprecedented level of fine-tuning of receptor responses *in vivo* as well as increases the scope for therapeutically targeted selective drug effects.

METHODS

Cell culture and transfection. HEK293 cells were maintained in DMEM supplemented with 10% (v/v) fetal calf serum (FCS), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Flp-In Chinese hamster ovary cells stably expressing the M2 receptor (M2-CHO cells) were cultured in Ham's nutrient mixture F-12 (HAM-F12) supplemented with 10% (v/v) FCS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine. HEK293 cells were transiently transfected 24 h after seeding using the Ca^{2+} phosphate coprecipitation method[42], except for in the BRET studies, where electroporation was used for efficient DNA transfer. For IP1 assays, cells were transfected using a total amount of 20 μg cDNA (3 μg OXE-R, 6 μg β_1 and 3 μg γ_2, 8 μg pcDNA3.1).

Generation of stable HEK OXE-R–$G\alpha_{16}$ cells. HEK293 cells were transfected to coexpress the OXE receptor OXE-R[23] and the $G\alpha_{16}$ protein, which recognizes all GPCR classes. Stable clones were maintained in DMEM supplemented with 10% (v/v) FCS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and the selection agents hygromycin B (250 μg ml^{-1}) and geneticin (400 μg ml^{-1}).

Ca^{2+} mobilization assays in HEK293 OXE-R–$G\alpha_{16}$ cells. HEK293 OXE-R–$G\alpha_{16}$ cells were seeded at a density of 60,000 cells per well into poly-D-lysine–coated 96-well tissue culture plates, and intracellular Ca^{2+} was quantified 24 h later using the Calcium4 assay kit (Molecular Devices) as described previously[43].

cAMP accumulation assay in recombinant cells. The amount of intracellular cAMP was quantified as described previously in detail[44] with the HTRF-cAMP dynamic kit (Cisbio) on a Mithras LB 940 reader (Berthold Technologies) following the manufacturer's instructions.

cAMP accumulation assay in human primary cells. The amount of intracellular cAMP in human neutrophils was quantified with the time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay kit LANCE Ultra (PerkinElmer) as per manufacturer's instructions on the Mithras LB 940 reader.

IP1 accumulation assay. The amount of intracellular IP1 in HEK293 cells transfected to transiently express the OXE-R along with $G\beta_1$ and $G\gamma_2$ or in M2-CHO cells (100,000 cells per well) were quantified as described previously[45] using the HTRF-IP1 kit (Cisbio) as per manufacturer's instructions. For quantification of IP1 in the absence of $G\alpha_i$ activity, cells were pretreated for 18–24 h with 100 ng ml^{-1} PTX. Viability of PTX-treated cells was verified with 300 μM of the pan–G protein agonist AlF_4^-.

Tl^+ flux assays. Tl^+ flux in HEK GIRK1/2 cells transiently transfected to coexpress the OXE-R was recorded as described previously[36].

DMR assays (Corning Epic Biosensor measurements). DMR assays were performed on a beta version of the Corning Epic biosensor as described previously in detail[42,46]. Briefly, HEK293 OXE-R–Gα_{16} cells or HEK293 cells transfected to transiently express OXE-R or a OXE-R–RLuc fusion protein were grown to confluence for 20–24 h on fibronectin-coated Epic biosensor 384-well microplates. Cells were then washed twice with Hank's Balanced Salt Solution containing 20 mM HEPES and were kept for at least 1 h in the Epic reader at 28 °C. DMR was monitored before (baseline read) and after the addition of compound solutions for 2,400 s.

BRET assay. All BRET measurements were performed using HEK293 cells transiently cotransfected by electroporation with the indicated BRET partners.

Molecular modeling and virtual screening. Generation of the OXE-R model, ligand docking and virtual screening of the commercially available ZINC database (http://zinc.docking.org/) are described in **Supplementary Methods**.

Preparation of human leukocytes. Citrated whole blood was obtained from healthy non-atopic volunteers after written informed consent in agreement with the Institutional Review Board of the Medical University of Graz. Polymorphonuclear leukocytes (PMNLs; containing neutrophils and eosinophils) were prepared by dextran sedimentation of erythrocytes and Histopaque gradient centrifugation[47]. Purified eosinophil preparations were obtained by negative magnetic selection using antibody cocktails (CD2, CD14, CD16, CD19, CD56 and glycophorin A) and colloidal magnetic particles from StemCell Technologies.

Chemotaxis. Purified eosinophils were resuspended in assay buffer at 2×10^6 cells ml^{-1}, and 50 µl of the cell suspension were loaded into the top wells of 48-well microBoyden chemotaxis chamber. Thirty microliters of assay buffer or agonists were placed into the bottom wells of the chamber, separated from the bottom wells by a polyvinylpyrrolidone-free polycarbonate filter with 5-µm pores. The chamber was incubated at 37 °C for 1 h in a humidified incubator. Migrated cells were enumerated by flow cytometry[48].

Leukocyte shape change assay. Aliquots of PMNLs were mixed with agonists at a final volume of 100 µl and stimulated for 4 min at 37 °C. We added 250 µl of ice-cold fixative solution, and changes in the cell shape were estimated as the increase of forward scatter by flow cytometry. Eosinophils were distinguished from neutrophils by autofluorescence in the FL-1 and FL-2 channels of the flow cytometer.

Ca^{2+} flux in eosinophils and neutrophils. The amount of intracellular Ca^{2+} in eosinophils was analyzed by flow cytometry. PMNLs were treated with 2 µM of the acetoxymethyl ester of FLUO-3 (Invitrogen) in the presence of 0.02% (w/v) Pluronic F-127 for 60 min at 21–23° C. Changes in the amount of intracellular Ca^{2+} was detected by flow cytometry as the increase of the fluorescence of the Ca^{2+}–sensitive dye FLUO-3 in the FL-1 channel. Eosinophils were identified as CD16-negative cells, and neutrophils were identified as CD16-positive cells.

Calculations and data analysis. Quantification of DMR signals for concentration-effect curves was performed by calculation of the maximum value between 500 s and 1,800 s. All optical DMR recordings were buffer-corrected. For data normalization, indicated as relative response (%), top levels of concentration-effect curves were set at 100% and bottom levels at 0%. Data calculation and half-maximum effective concentration (EC_{50}) value determination by nonlinear regression was performed using Prism 4.02 (GraphPad).

Statistical analyses. Data are shown as mean ± or + s.e.m. for *n* observations. Comparisons of groups were performed using one-way analysis of variance with Bonferroni's multiple comparison test.

Additional methodology. Additional details are available in **Supplementary Methods**.

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Author contributions

S.B. designed and performed experiments and provided important ideas. L. Peters, P.A.O., A.B., V.K., C.D.W., R.S., P.L., J.G. and S.H. designed and performed experiments. A.G. and L. Pardo created the receptor model, performed the virtual screening and contributed to discussion. R.T. and T.U. established synthesis of 5-oxo-ETE, edited the manuscript and contributed to discussion. C.D.W., L. Pardo, K.M., M.G. and A.H. designed research, contributed to discussion and edited the manuscript. E.K. designed research and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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