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RESEARCH PAPER

Inhibition of allergen-induced dermal eosinophilia by an oxoeicosanoid receptor antagonist in non-human primates

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Background and Purpose: 5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), acting via the OXE receptor, is unique among 5-lipoxygenase products in its ability to directly induce human eosinophil migration, suggesting its involvement in eosinophilic diseases. To address this hypothesis, we synthesized selective indole-based OXE receptor antagonists. Because rodents lack an OXE receptor orthologue, we sought to determine whether these antagonists could attenuate allergen-induced skin eosinophilia in sensitized monkeys.

Experimental Approach: In a pilot study, cynomolgus monkeys with environmentally acquired sensitivity to *Ascaris suum* were treated orally with the "first-generation" OXE antagonist **230** prior to intradermal injection of 5-oxo-ETE or *Ascaris* extract. Eosinophils were evaluated in punch biopsy samples taken 6 or 24 hr later. We subsequently treated captive-bred rhesus monkeys sensitized to house dust mite (HDM) allergen with a more recently developed OXE antagonist, ***S*-Y048**, and evaluated its effects on dermal eosinophilia induced by either 5-oxo-ETE or HDM.

Key Results: In a pilot experiment, both 5-oxo-ETE and *Ascaris* extract induced dermal eosinophilia in cynomolgus monkeys, which appeared to be reduced by **230**. Subsequently, we found that the related OXE antagonist ***S*-Y048** is a highly potent inhibitor of 5-oxo-ETE-induced activation of rhesus monkey eosinophils in vitro and has a half-life in plasma of about 6 hr after oral administration. ***S*-Y048** significantly inhibited eosinophil infiltration into the skin in response to both intradermally administered 5-oxo-ETE and HDM.

Conclusions and Implications: 5-Oxo-ETE may play an important role in allergen-induced eosinophilia. Blocking its effects with ***S*-Y048** may provide a novel therapeutic approach for eosinophilic diseases.

1 | INTRODUCTION

5-Oxo-6,8,11,14-eicosatetraenoic acid (**5-oxo-ETE**) is a potent eosinophil chemoattractant that is formed by the action of 5-hydroxyeicosanoid dehydrogenase (Powell, Gravelle, & Gravel, 1992) on the **5-lipoxygenase** product 5*S*-hydroxy-6,8,11,14-eicosatetraenoic acid (**5S-HETE**; Figure 1). 5-Oxo-ETE induces a variety of responses in eosinophils, including calcium mobilization, actin polymerization, L-selectin shedding, surface expression of CD11b (Powell, Gravel, & Halwani, 1999) and CD69 (Urasaki, Takasaki,

Abbreviations: 230, 5-(5-chloro-2-hexyl-1-methyl-1H-indol-3-yl)-3-methyl-5-oxopentanoic acid; **264**, 5-(6-chloro-2-hexyl-1H-indol-1-yl)-3-methyl-5-oxopentanoic acid; 5-LO, 5-lipoxygenase; 5-oxo-ETE, 5-oxo-6,8,11,14-eicosatetraenoic acid; HDM, house dust mite; ***S*-Y048**, (*S*)-5-(5-chloro-2-(6-(3-chlorophenyl)hexyl)-1-methyl-1*H*-indol-3-yl)-3-methyl-5-oxopentanoic acid; ***S*-Y048M**, (*S*)-5-(5-chloro-2-((*S*)-6-(3-chlorophenyl)-1-hydroxyhexyl)-1-methyl-1*H*-indol-3-yl)-3-methyl-5-oxopentanoic acid; TBS, Tris-buffered saline

Nagasawa, & Ninomiya, 2001), activation of the respiratory burst (Czech et al., 1997) and degranulation (O'Flaherty et al., 1996). Among lipid meditators, it is the most powerful chemoattractant for human eosinophils. It also induces the migration of these cells through the basement membrane (Guilbert et al., 1999) and endothelial cell monolayers (Dallaire et al., 2003) by a combination of its chemoattractant effects and stimulation of the release of **MMP-9**. In addition to its direct effects on eosinophils, it can indirectly increase their survival by inducing **GM-CSF** release from monocytes (Stamatiou et al., 2004). The effects of 5-oxo-ETE on eosinophils are enhanced by cytokines and chemokines, including GM-CSF (O'Flaherty et al., 1996), **IL-5**, (Guilbert et al., 1999), **eotaxin**, and **RANTES** (Powell, Ahmed, Gravel, & Rokach, 2001).

Cellular responses to 5-oxo-ETE are mediated by the G protein-coupled oxoeicosanoid (**OXE**) receptor, which is encoded by the *OXER1* gene (Bäck et al., 2014; Hosoi et al., 2002; Jones et al., 2003; Takeda, Yamamoto, & Haga, 2002) and is coupled to $G\alpha_{i/o}$. It is most highly expressed on eosinophils (Jones et al., 2003) and basophils (Iikura et al., 2005; Sturm et al., 2005), and to a lesser extent on neutrophils (Jones et al., 2003), monocytes (Sturm et al., 2005), and macrophages (Jones et al., 2003). Downstream signalling is effected by activation of several pathways, including phospholipase Cβ, PKC, ERK, and p38 MAPK (Blättermann et al., 2012; Langlois et al., 2009). Most of its effects are mediated by the βγ G protein subunits, with only its inhibitory effect on AC being mediated by the α_i subunit (Blättermann et al., 2012; Konya et al., 2014).

Orthologues of the OXE receptor are found in many species, with the notable exception of rodents, which has limited progress in understanding the pathophysiological role of 5-oxo-ETE. Activation of the OXE receptor by 5-oxo-ETE has been demonstrated to be important for host defence in zebrafish, being required for the infiltration of leukocytes to sites of tissue damage (Enyedi, Kala, Nikolich-Zugich, & Niethammer, 2013). In humans, we have shown that intradermal injection of 5-oxo-ETE induces the infiltration of eosinophils into the skin, with asthmatic subjects displaying a much stronger response than healthy control subjects (Muro et al., 2003). Furthermore, it has recently been shown that antigen challenge of human asthmatic subjects who are sensitive to house dust mite (HDM) allergen results in elevation of 5-oxo-ETE levels in exhaled breath condensate (Kowal, Gielicz, & Sanak, 2017). 5-Oxo-ETE may also play a role in the development of nasal polyps. It has been reported to be formed by epithelial cells from nasal polyps and was shown to increase the levels of eosinophil cationic protein in organ cultures derived from this tissue (Lin et al., 2018). The above studies raise the possibility that 5-oxo-ETE may be an important mediator in eosinophilic diseases such as atopic dermatitis, allergic rhinitis, and asthma, making the OXE receptor an attractive target for therapeutic intervention.

We recently initiated a program to identify synthetic OXE receptor antagonists by creating conformationally restricted compounds using an indole scaffold to which were attached regions of the 5-oxo-ETE molecule that are essential for activation of its receptor. We identified

What is already known

- The 5-lipoxygenase product 5-oxo-ETE is a potent eosinophil chemoattractant that acts through the OXE receptor.
- We have prepared selective OXE receptor antagonists that block the in vitro effects of 5-oxo-ETE.

What does this study add

- This is the first study to examine the in vivo efficacy of an OXE antagonist.
- ***S*-Y048** inhibits both 5-oxo-ETE- and allergen-induced eosinophil infiltration into the skin of monkeys.

What is the clinical significance

- This is the first evidence for a pathophysiological role for 5-oxo-ETE in mammals.
- The OXE receptor antagonist ***S*-Y048** may serve as a novel therapeutic agent in eosinophilic diseases.

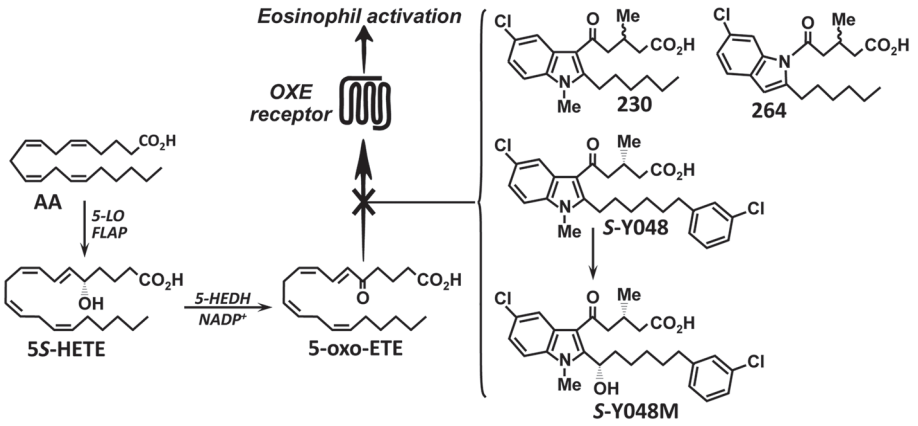

FIGURE 1 Formation of 5-oxo-ETE and inhibition of its actions by OXE receptor antagonists

two compounds, **230** and **264** (Figure 1), with in vitro potencies of about 30 nM (Gore et al., 2014) and oral bioavailability in monkeys (Cossette et al., 2016; Reddy et al., 2018). Both compounds contain a chiral carbon due to the presence of a methyl group in the 3-position of the carboxyl side chain, with the *S*-enantiomers accounting for nearly all of the antagonist activity (Gore et al., 2014; Patel et al., 2014). Because the alkyl side chain of these antagonists is a target for cytochrome P450-catalysed ω-oxidation, we introduced a phenyl group in an attempt to minimize this (Chourey et al., 2018). Further modification led to our most potent antagonist, the *S*-enantiomer of **Y048** (i.e. ***S*-Y048**), which has an in vitro IC_{50} of about 20 pM in inhibiting 5-oxo-ETE-induced calcium mobilization in human neutrophils (Ye et al., 2019). ***S*-Y048** is selective for the OXE receptor and does not affect the responses of other relevant receptors to their ligands, including **CCR3** (eotaxin), the **BLT1** receptor (**leukotriene B4**) and the **DP2** receptor (**prostaglandin D2**). It also has a much longer plasma half-life after oral administration to cynomolgus monkeys compared to **230** and **264**. As shown in Figure 1, ***S*-Y048** is converted to a major long-lasting plasma metabolite (***S*-Y048M**) that we identified as α*S*-hydroxy-***S*-Y048**. This compound is formed by the stereospecific hydroxylation of the methylene group of ***S*-Y048** α to the indole. The synthetic compound was found to have equivalent potency to ***S*-Y048** in inhibiting 5-oxo-ETE-induced calcium mobilization (Ye et al., 2019).

Although 5-oxo-ETE is a well-established in vitro activator of human eosinophils (Powell & Rokach, 2015), with the exception of a single study in zebrafish (Enyedi et al., 2013), there is no information on its pathophysiological role, due in large part of the lack of mouse models. However, the present availability of potent and selective OXE receptor antagonists now provides the opportunity to address this question in mammalian species that possess this receptor. The objective of the current study was to determine whether an OXE receptor antagonist could inhibit allergen-induced infiltration of eosinophils into the skin of non-human primates. We initially conducted a pilot study in cynomolgus monkeys with natural sensitivity to *Ascaris suum*, in which **230** and **264** were administered by oral gavage prior to allergen challenge. This was followed by a second study in which ***S*-Y048** was administered to HDM-sensitized rhesus monkeys.

2 | METHODS

2.1 | Animals

Studies were performed on both cynomolgus and rhesus monkeys. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*. Male wild-caught cynomolgus monkeys (2–2.5 kg), with environmentally acquired sensitivity to *A. suum*, housed at INRS-Institut Armand-Frappier, Laval, Quebec, were used for the pilot studies with **230** and **264**. These experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the local institutional animal care committee. Animals were housed in pairs in cages with a 12-hr light/12-hr dark schedule. They received a diet consisting of Teklad Global chow for nonhuman primates supplemented with fresh fruit, vegetables and breakfast cereal. They had unlimited access to water.

Studies with ***S*-Y048** were conducted at the California National Primate Research Center, University of California, Davis, using captive-bred adult male rhesus monkeys (10.1–16.7 kg) and were approved by the UC Davis Institutional Animal Care and Use Committee. Care and housing of animals before, during, and after treatment complied with the provisions of the Institute of Laboratory Animal Resources and conformed to practices established by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). Animals were socially paired whenever possible and housed indoors in cages with a 12-hr light/12-hr dark schedule. They received a diet consisting of Purina monkey chow supplemented with produce and a continuous access to water.

2.2 | Effects of 230 and 264 on dermal eosinophilia in cynomolgus monkeys

Animals were first acclimated to oral gavage by repeating this procedure over three successive days. At the end of three days the monkeys were weighed to permit calculation of the correct dose. Three days later, all food was withdrawn at the end of the afternoon. The following morning, the appropriate amounts of racemic **230** (Gore et al., 2014) or racemic **264** (Reddy et al., 2018), synthesized as described previously, were dissolved in ethanol and diluted with 10 volumes of 20-mM $NaHCO_3$, pH 8.0. After vortexing, either vehicle alone, or the resulting antagonist suspensions (4.4 $ml \cdot kg^{-1}$) were administered by oral gavage at doses of either 30 $mg \cdot kg^{-1}$ (**230**) or 75 $mg \cdot kg^{-1}$ (**264**). After 30 min, 5-oxo-ETE (5 μg in 100 μl of 2.5% ethanol), *A. suum* extract (40 μl), or vehicle were injected intradermally using a 28-gauge needle at previously shaved sites on the backs of the monkeys. *A. suum* was obtained as a defatted lyophilized solid from Greer Labs Inc., Lenoir, NC., and prepared in PBS at a concentration of 0.78 $mg \cdot ml^{-1}$. After 6 hr (5-oxo-ETE) or 24 hr (*Ascaris*) the monkeys were anaesthetized by intramuscular injection of ketamine (5 $mg \cdot kg^{-1}$) and acepromazine (0.5 $mg \cdot kg^{-1}$). Skin samples were then obtained by punch biopsy (3-mm diameter), fixed in 10% formalin and embedded in paraffin. For the experiments with *Ascaris*, two additional identical doses of antagonist were administered after 8 and 16 hr. The plasma levels of **230** (Cossette et al., 2016) and **264** (Reddy et al., 2018) in blood samples (1 ml), collected 24 hr after injection of *Ascaris*, were measured by reversed-phase HPLC as described previously.

To visualize eosinophils, sections were deparaffinized in xylene, dehydrated in ethanol and washed in Tris-buffered saline (TBS). Sections were immersed in 0.2% Triton for 30 min, washed three times for 5 min in TBS and incubated with Dako Universal Blocking solution

for 15 min. After draining, sections were incubated overnight at 4 °C with a mouse monoclonal antibody against full-length human eosinophil cationic protein (Diagnostics Development, Uppsala, Sweden; clone EG2; batch number 593-101), diluted 1:50 (16 μg·ml^{-1} final concentration). After three 5-min washes in TBS, sections were incubated with biotinylated polyclonal rabbit anti-mouse IgG (Dako E0354, lot 00078773, diluted 1:100; 12 μg·ml^{-1} final concentration) for 1 hr, washed in TBS, and incubated with Streptavidin-AP for 30 min. Sites of immunoreactivity were visualized by incubation with Fast Red. Sections were counterstained with haematoxylin and mounted with aqueous medium. Immunoreactive cells were counted under blinded conditions at 200× magnification and expressed as numbers of positive cells per square millimetre of tissue. One section was evaluated in its entirety (between 20 and 40 fields per section, depending on its size) for each injection site. An IgG1 isotype antibody (R&D systems, Minneapolis, MN; Clone 11711; Catalogue number MAB002; RRID:AB_357344; 16 μg·ml^{-1} final concentration) was used as a negative control, whereas sections from human nasal polyps were stained as a positive control. The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology*.

2.3 | Preparation of rhesus monkey leukocytes

Rhesus monkey blood in heparinized tubes obtained from CiToxLAB, Montreal, Canada was treated with Dextran 500 for 45 min at room temperature and subjected to hypotonic lysis of red blood cells. After centrifugation, the unfractionated leukocytes (36 ± 2% neutrophils and 1.5 ± 0.3% eosinophils) were suspended in PBS (Cossette et al., 2016).

2.4 | Evaluation of actin polymerization in eosinophils

Unfractionated leukocytes were incubated for 30 min on ice with allophycocyanin-labelled anti-CD49d (2 μl per 10^6 cells; BioLegend, San Diego, CA; Mouse IgG, clone 9F10; lot number B17246; RRID: AB_2130041), washed with PBS, and suspended in PBS containing Ca^{2+} (1.8 mM) and Mg^{2+} (1 mM) at a concentration of 5.5 × 10^6 cells·ml^{-1} (cf. Cossette et al., 2015). Aliquots (100 μl) were then pre-incubated for 5 min at 37 °C with vehicle (1-μl DMSO) or ***S*-Y048**, followed by addition of either vehicle (10 μl of PBS containing Ca^{2+}, Mg^{2+}, and 0.1% BSA) or 5-oxo-ETE (10 nM). The incubations were terminated 20 s later by the addition of formaldehyde (37%; final concentration 8.5%) and kept on ice for 30 min. After addition of a mixture of lysophosphatidylcholine (30 μg in 23.8-μl PBS) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin (49 pmol in 6.2-μl methanol; final concentration, 0.3 μM), the samples were incubated overnight in the dark at 4 °C. Immediately prior to analysis by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA), PBS (200 μl, containing 1% formaldehyde) was added to each sample. Eosinophils were identified by high side scatter and high expression of CD49d.

2.5 | Evaluation of leukocyte migration

Vehicle (PBS containing Ca^{2+}, Mg^{2+}, and 0.3% BSA) or 5-oxo-ETE (100 nM) was added to the bottom wells of 48-well microchemotaxis chambers with Sartorius cellulose nitrate filters (8-mm pore size; 140-mm thickness), whereas leukocytes (150,000 cells in PBS containing Ca^{2+}, Mg^{2+}, and 0.4% OVA) were added to the top wells as previously described (Cossette et al., 2015). Different concentrations of ***S*-Y048** were added to both top and bottom wells. After incubation of the chambers for 2 hr at 37 °C in 5% CO_2 and humidified air, the cells were stained with haematoxylin followed by chromotrope 2R, and the numbers on the bottom surfaces counted in five different fields at a magnification of 400×. All incubations were performed in triplicate.

2.6 | Assessment of calcium mobilization

Leukocytes (10^7 cells·ml^{-1}) were incubated with the acetoxymethyl ester of fluo-3 (2 mM) in the presence of Pluronic F-127 (0.02%) for 60 min at 23 °C as previously described (Cossette et al., 2015). After centrifugation (200× *g*, 10 min), the cells were suspended in PBS to give a concentration of 50 × 10^6 cells·ml^{-1} and treated with allophycocyanin-labelled mouse anti-human CD49d (2 μl per 10^6 cells; RRID: AB_2130041) for 30 min at 0 °C. After addition of PBS (1 ml) and centrifugation, the pellet was resuspended in PBS to give a concentration of 2 × 10^6 leukocytes·ml^{-1}. PBS (50 μl) containing Ca^{2+} (36 mM) and Mg^{2+} (20 mM) was added to 1-ml aliquots of the suspended leukocytes. Either vehicle or ***S*-Y048** was added 5 min later, followed after 45 s by the addition of 5-oxo-ETE (10 nM) and A23187 (10 μM) after a further 45 s. During this time, fluorescence was measured by flow cytometry using a FACSCalibur instrument (Becton-Dickinson, San Jose, CA). Neutrophils were gated out on the basis of moderate forward and side scatter and low staining with anti-CD49d. Although eosinophils were also identified based on high staining with anti-CD49d and high side scatter, their numbers were too low to obtain meaningful results.

2.7 | Pharmacokinetics of *S*-Y048 in rhesus monkeys

***S*-Y048** was dissolved in ethanol (25 mg·kg^{-1}) and stored at −80 °C before use. On the morning of the experiment, the ethanolic solution was thawed,vortexed and the required amount was added to 10 volumes of 20-mM $NaHCO_3$ (pH 8.0). The resulting suspension (2.2 ml·kg^{-1}; 9.1% EtOH) was immediately vortexed and administered by nasogastric intubation. A second identical dose of ***S*-Y048** was administered 8 hr later. ***S*-Y048** and ***S*-Y048M** were measured in blood samples (2 ml) taken after various times by reversed-phase HPLC as described previously (Ye et al., 2019).

2.8 | Effects of *S*-Y048 on dermal eosinophilia in HDM-sensitized rhesus monkeys

Animals (n = 6) were first sensitized by a series of subcutaneous injections of HDM (*Dermatophagoides pteronyssinus*; 60-μg protein with 1-mg alum; 1 ml total volume per injection) at weeks 0, 1, 2, and 3 (Figure 5). Four weeks after the initial injection, the monkeys were skin-tested to confirm that they exhibited wheal and flare reactions to HDM as previously described (Schelegle et al., 2001). On week 7, monkeys were sedated with **ketamine** (5-30 mg·kg^{-1}) and the vehicle for ***S*-Y048** (2.2 ml·kg^{-1}) was administered by nasogastric intubation, followed 30 min later by intradermal injection of 5-oxo-ETE (5 μg in sterile saline containing 2.5% ethanol), HDM diluted in sterile saline (1:1,000) and vehicle (100 μl), each at two separate sites on the shaved dorsal thorax of each animal (i.e. a total of 6 injection sites per animal). A second dose of vehicle was administered by nasogastric intubation 8 hr after the intradermal injections. After a further 16 hr, monkeys were sedated with ketamine (5–30 mg·kg^{-1}) and dexmedetomidine (0.0075–0.015 mg·kg^{-1}) prior to the acquisition of 4-mm skin punch biopsies at each injection site. Sedation was reversed with atipamezole (administered at the same dose as dexmedetomidine) at the conclusion of the procedure. Monkeys received ketoprofen (2 mg·kg^{-1}) for analgesia after the skin biopsy procedure. Skin biopsy specimens were fixed in 4% paraformaldehyde for 72 hr and embedded in paraffin. This was repeated 5 and 10 weeks later, when ***S*-Y048** was administered by nasogastric intubation at doses of 5 or 10 mg·kg^{-1}, respectively. Blood samples for HPLC analysis were obtained just prior to treatment with ***S*-Y048**, 1 hr later, just prior to the second dose, and after 24 hr.

Eosinophils were evaluated in one section for each biopsy site from each paraffin block after staining with haematoxylin and eosin and scanning the slides at 60× under oil with an Olympus VS110 whole slide scanning system. Visiopharm image analysis and stereology software (Hørsholm, Denmark) were used to sample individual slide scans. Depending on the size, 30–100 fields were imaged and counted for each slide. Volume fraction counts for eosinophils relative to tissue were determined by Stereology Toolbox software, as previously described. (Chou et al., 2005). Prior to evaluation, images were deidentified with regard to individual animal and treatment group.

2.9 | Statistical analysis

The data and statistical analysis comply with the recommendations of British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). The statistical significance of differences among multiple groups was evaluated by two-way repeated measures ANOVA using SigmaStat software. The Bonferroni test was used as a multiple comparison method when F in ANOVA achieved $P < .05$ and there was no significant variance inhomogeneity. Differences between two groups were evaluated using Student's paired t-tests. Data are presented either as individual values or as means ± SEM. The criterion for statistical significance was a probability of less than 5%. There were 6 animals in each group.

2.10 | Materials

The following were prepared by total chemical synthesis:5-oxo-ETE (Khanapure et al., 1998), racemic **230** (Gore et al., 2014), racemic **264** (Reddy et al., 2018), and ***S*-Y048** (Ye et al.,2019). The sources of the following reagents were: Streptavidin-AP, acetoxymethyl ester of fluo-3, Pluronic F-127, and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin (NBD-phallacidin), alum (Imject Alum Adjuvant) (Thermo Fisher Scientific Inc, Waltham, MA, USA); Fast Red (SIGMAFAST Fast RedTR/Naphthol AS-MX tablets, chromotrope 2R, and Dextran 500 (Sigma Aldrich Canada, Oakville, ON, Canada), hematoxylin (Canadawide Scientific, Ottawa, ON, Canada); Dako Universal Blocking solution (Agilent, Santa Clara, CA, USA). Ascaris suum (defatted lyophilized solid) and Dermatophagoides pteronyssinus were obtained from Greer Labs Inc.,Lenoir, NC, USA. Microchemotaxis chambers were obtained from Neuro Probe Inc., Cabin John, MD, USA and Sartorius cellulose nitrate filters (8 μm pore size; 140 mm thickness) were purchased from Neuro Probe Inc, Gaithersburg,MD, USA.

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

3 | RESULTS

3.1 | Pilot study with cynomolgus monkeys

We initially conducted a study in cynomolgus monkeys to determine whether 5-oxo-ETE could induce eosinophil infiltration into the skin in this species, as it does in humans (Muro et al., 2003). Although there was a considerable variability among individual animals, as shown in Figure 2a, intradermal injection of 5-oxo-ETE consistently significantly increased eosinophil numbers in skin biopsy samples, taken 6 hr later, from 1 ± 0.3 cells·mm^{-2} at sites injected with vehicle to 20 ± 14 cells·mm^{-2} at sites injected with 5-oxo-ETE (5 μg). We then did some preliminary experiments to determine whether an OXE antagonist could inhibit this response. Some of our early data were from unpaired experiments and the results were uninformative because of the large degree of variability among animals. However, in the two paired experiments that we conducted, in which animals initially received vehicle by gavage and 5 weeks later received **230** (30 mg·kg^{-1}), 5-oxo-ETE-induced eosinophilia was inhibited by the OXE receptor antagonist (Figure 2b).

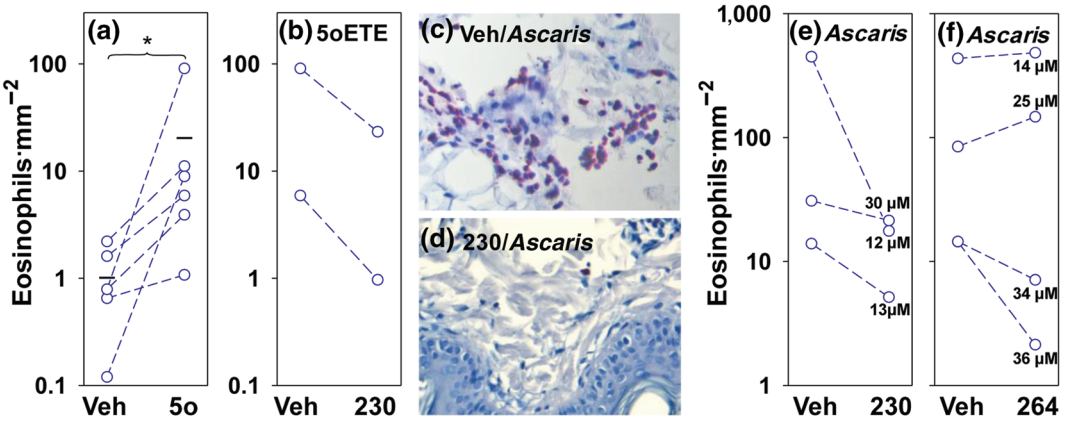

FIGURE 2 Effects of **230** on eosinophil infiltration into the skin of cynomolgus monkeys. (a) Eosinophil numbers in skin punch biopsy sections obtained 6 hr after intradermal injection of 5-oxo-ETE (5 μg). $^{*}P < .05$. (b) Effects of **230** (30 mg·kg^{-1}) compared to vehicle, both administered by oral gavage, on 5-oxo-ETE-induced dermal eosinophilia. (c) Skin section stained with an antibody to eosinophil cationic protein 24 hr after intradermal injection of an *Ascaris* extract (three in 100 dilution) and oral administration of vehicle (0.5 hr before and 8 and 16 hr after injection of *Ascaris* extract). (d) As in panel (c), except that **230** (3 × 30 mg·kg^{-1}) was administered instead of vehicle. (e) Effects of **230** on *Ascaris*-induced dermal eosinophilia. (f) Effects of **264** (3 × 75 mg·kg^{-1}) on *Ascaris*-induced dermal eosinophilia. The numbers on the right in panels (e) and (f) denote the plasma concentrations of **230** and **264** after 24 hr

The cynomolgus monkeys in this study had environmentally acquired sensitivity to *A. suum*, to which they exhibited an eosinophilic response. Three animals treated with vehicle by gavage (three times; 8-hr apart) received an intradermal injection of *Ascaris* 30 min after the first gavage. Evaluation of punch biopsy sections, taken 24 hr later, revealed a robust infiltration of eosinophils in response to *Ascaris* (Figure 2c,e). After 5 weeks, **230** (30 mg·kg^{-1} every 8 hr) was administered to each of these animals. In each case, the numbers of eosinophils in biopsy samples were considerably lower following treatment with **230** compared to vehicle (Figure 2d,e). High plasma levels of **230** (18 ± 6 μM) were measured at the end of the experiment (Figure 2e). Similar experiments were performed with **264** (3 × 75 mg·kg^{-1}), but this compound did not have a consistent effect, with decreases in eosinophil numbers in two animals and increases in two others (Figure 2f). Despite the 2.5-fold higher dose of **264**, its plasma concentration after 24 hr (28 ± 8 μM) was not significantly higher than that of **230**. The two animals that displayed increased eosinophil numbers after **264** had lower plasma levels compared to those with lower eosinophil numbers, but it seems unlikely that this could explain the lack of response to **264**.

3.2 | Effects of *S*-Y048 on 5-oxo-ETE-induced activation of rhesus monkey leukocytes

Because of the inter-animal variability in the data from the above environmentally sensitized cynomolgus monkeys, we abandoned this model in favour of the HDM-sensitized rhesus monkey model established at UC Davis. By the time that it took to organize these studies we had identified a more potent and metabolically-resistant selective OXE receptor antagonist, ***S*-Y048** (Ye et al., 2019).

Before embarking on a very costly study on its in vivo efficacy, we wanted to ensure that the in vitro inhibitory effects of ***S*-Y048** were similar in rhesus monkeys and humans. Because of the relatively small amounts of rhesus monkey blood available to us, we could only conduct a limited number of experiments, using a preparation of mixed leukocytes (36 ± 5% neutrophils; 1.5 ± 0.3% eosinophils). As shown in Figure 3a, ***S*-Y048** strongly inhibited 5-oxo-ETE (10 nM)-induced actin polymerization in rhesus monkey eosinophils (which were gated out using anti-CD49d) with an IC_{50} of 0.34 ± 0.12 nM (n = 4), similar to the IC_{50} of 0.59 ± 0.09 nM (n = 6) that we observed for human eosinophils. It also inhibited leukocyte migration in response to 5-oxo-ETE (100 nM) with an IC_{50} of 30 ± 25 nM (n = 2; Figure 3b). Although these mixed leukocyte samples did not contain sufficient numbers of eosinophils for accurate measurement of calcium transients, we were able to examine the effects of ***S*-Y048** on 5-oxo-ETE-induced calcium mobilization in neutrophils using flow cytometry. Compared to vehicle-treated cells (Figure 3c), ***S*-Y048** (0.1 nM) nearly completely blocked the response to 5-oxo-ETE (10 nM; Figure 3d).

3.3 | Pharmacokinetics of *S*-Y048 in rhesus monkeys

We examined the plasma levels of ***S*-Y048**, following administration by nasogastric intubation of two doses of 5 mg·kg^{-1}, given 8-hr apart (Figure 4). High levels of ***S*-Y048** rapidly appeared in the blood, with its plasma concentration rising to a maximum of nearly 20 μM by 2 hr and then declining ($t_{½}$ ~6 hr). After the second dose, the plasma levels of ***S*-Y048** rose sharply to about 30 μM within 1 hr and declined to about 12 μM at 24 hr. The level of the active metabolite of ***S*-Y048** (i.e., ***S*-Y048M)** at 24 hr was about 5 μM.

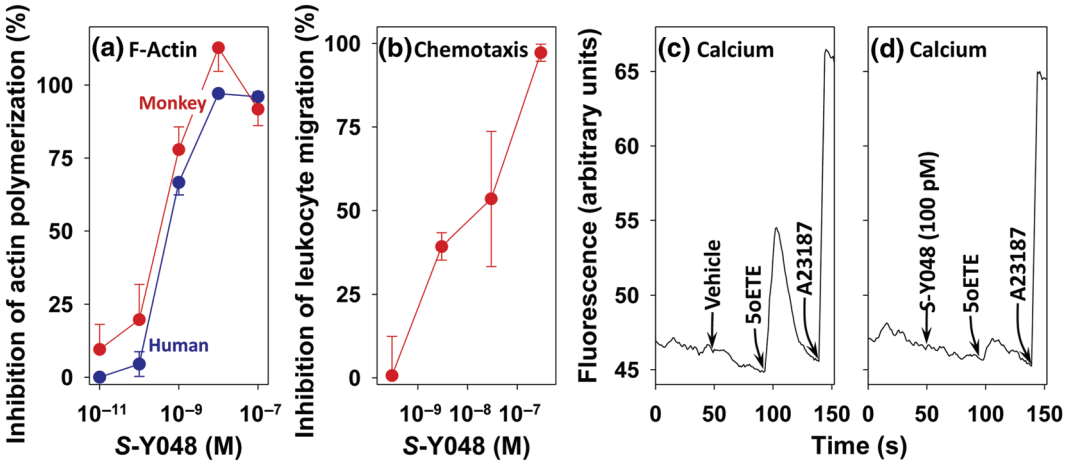

FIGURE 3 Effects of ***S*-Y048** on 5-oxo-ETE-induced activation of rhesus monkey leukocytes in vitro. (a) Effects of ***S*-Y048** on actin polymerization induced by 5-oxo-ETE (10 nM; n = 4). (b) Effects of ***S*-Y048** on migration of leukocytes through nitrocellulose filters in response to 5-oxo-ETE (100 nM; n = 2). (c) Calcium mobilization in neutrophils in response to 5-oxo-ETE (10 nM; n = 1). Vehicle was added 45 s before 5-oxo-ETE. (d) Effect of ***S*-Y048** (100 pM) on 5-oxo-ETE-induced calcium mobilization

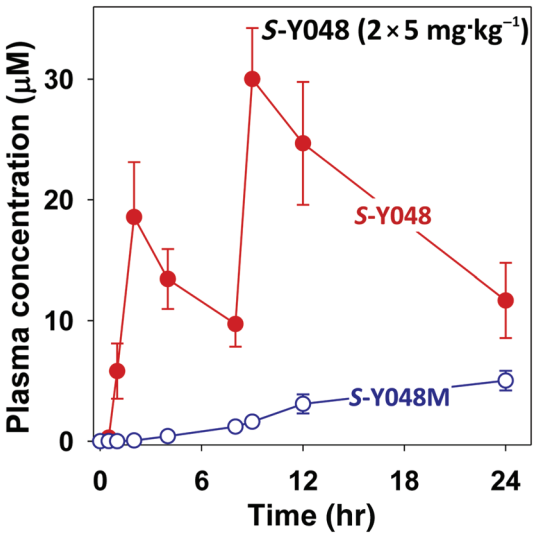

FIGURE 4 Pharmacokinetics of ***S*-Y048** in rhesus monkeys. A suspension of ***S*-Y048** in 20-mM $NaHCO_3$ was administered by nasogastric intubation to rhesus monkeys (n = 3) at the beginning of the experiment and again 8 hr later at a dose of 5 mg·kg^{-1}. Blood samples were taken at various time points (0.5, 1, 2, 4, 8, 9, 12, and 24 hr) and plasma was extracted and analysed for ***S*-Y048** and ***S*-Y048M** as described in Section 2

3.4 | *S*-Y048 inhibits 5-oxo-ETE-induced eosinophil infiltration into rhesus monkey skin

Rhesus monkeys (n = 6) were sensitized by four weekly subcutaneous injections of HDM. To ensure that the sensitization procedure was successful, a skin test was performed with a positive reaction being defined as a wheal and flare response with a diameter of 2 mm or more beyond the diameter of a negative control (diluent) injection. All six animals exhibited a positive skin test of between 2.4 and 8.95 mm relative to control (average, 5.2 mm with a SD of ± 2.25). There was no correlation between the magnitude of HDM skin test reactivity prior to initiation of ***S*-Y048** treatment and eosinophil volume in skin biopsy samples obtained in subsequent experiments.

The sensitized animals were first treated with vehicle, administered by nasogastric intubation, followed 30 min later by intradermal injection of either vehicle (2.5% ethanol in saline) or 5-oxo-ETE (5 μg) (Figure 5). Administration of vehicle was repeated after 8 hr and a punch biopsy sample obtained after 24 hr. This process was repeated twice, after successive intervals of 5 weeks, except that instead of vehicle, suspensions of ***S*-Y048** (two times either 5 or 10 mg·kg^{-1}, 8-hr apart) were administered by nasogastric intubation. As shown in Figure 6a, five out of the six animals responded to 5-oxo-ETE with an increased volume ratio of eosinophils in skin biopsy samples, whereas no eosinophils were detected in the remaining animal, either in the presence or absence of 5-oxo-ETE. Overall, eosinophil volume fractions relative to total tissue volume within individual biopsies significantly increased from 5 ± 2 × 10^{-5} to 29 ± 9 × 10^{-5}. The lower dose of ***S*-Y048** completely inhibited the response to 5-oxo-ETE (Figure 6b,c;). The higher dose of ***S*-Y048** also significantly inhibited 5-oxo-ETE-induced eosinophilia, reducing eosinophil volume fractions in five out of the six monkeys. However, in this case, eosinophil volume was higher in the single animal in which eosinophils were previously undetectable (open circles, Figure 6b).

3.5 | *S*-Y048 inhibits HDM-induced eosinophil infiltration

In conjunction with the experiment described above with 5-oxo-ETE, HDM was also administered intradermally at separate sites. The eosinophilic response to HDM was much stronger than that to 5-oxo-ETE alone (Figure 7a,c,d). Both the 5 and 10 mg·kg^{-1} doses of ***S*-Y048** reduced the fractional volume of skin eosinophils in five out of the six monkeys, with the remaining animal showing no effect compared

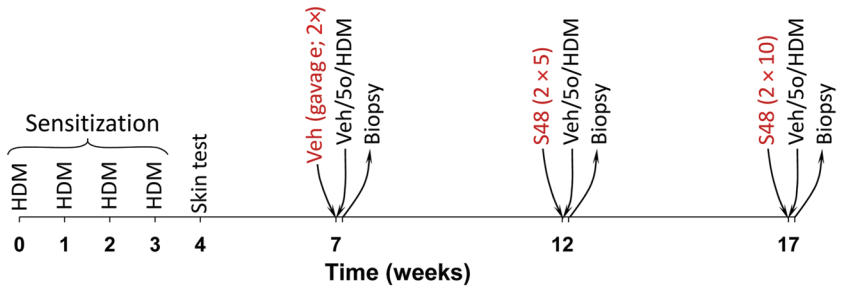

FIGURE 5 Protocol for evaluation of the effects of ***S*-Y048** on dermal eosinophilia induced by 5-oxo-ETE or HDM

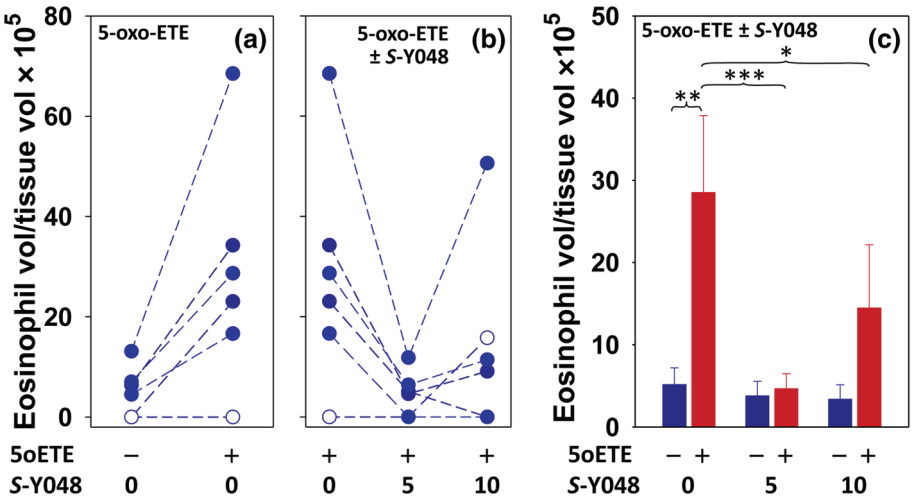

FIGURE 6 Inhibition of 5-oxo-ETE-induced dermal eosinophil infiltration by ***S*-Y048**. (a) Vehicle was administered by nasogastric intubation to rhesus monkeys 1 hr before and 7 hr after intradermal injection of 5-oxo-ETE (5 μg). After 24 hr, skin punch biopsy samples were obtained. Point counts of eosinophils relative to point counts of tissue were obtained from 30 to 100 image fields randomly sampled from each slide. (b) As in panel (a), except that instead of vehicle, ***S*-Y048** was administered by nasogastric intubation at doses of either 5 or 10 mg·kg^{-1}. The open circles in panels (a) and (b) show data from the single animal that did not respond to the antagonist. (c) Representation of means of data in panels (a) and (b). $^{*}P < .05$

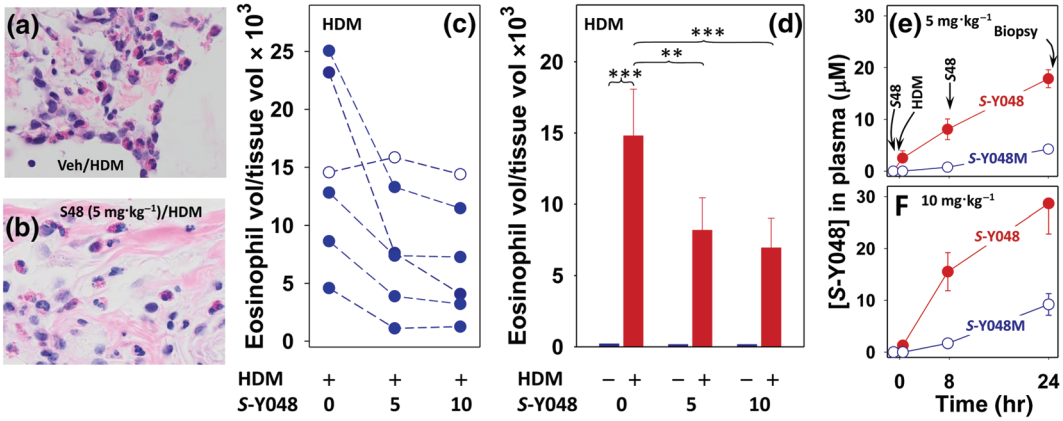

FIGURE 7 Inhibition of HDM-induced dermal eosinophil infiltration by ***S*-Y048**. (a) Skin section stained by haematoxylin and eosin, obtained 24 hr after intradermal injection of HDM accompanied by nasogastric administration of vehicle 30 min before and 8 hr after HDM. (b) As in panel (a), except that instead of vehicle, ***S*-Y048** (2 × 5 mg·kg^{-1}) was administered by nasogastric intubation. (c) Effect of ***S*-Y048** (2 × 5 mg·kg^{-1} or 2 × 10 mg·kg^{-1}) on HDM-induced dermal eosinophilia. (d) Representation of mean data from panel (c). $^{**}P < .05$;. (e) Plasma levels of ***S*-Y048** and ***S*-Y048M** after administration by nasogastric intubation of a dose of 5 mg·kg^{-1} of ***S*-Y048** in the experiments depicted in panels (c) and (d). Blood samples were obtained 1 hr after intradermal injection of HDM, just before administration of the second dose of ***S*-Y048**, and 24 hr after HDM. (f) As in panel (e), except that the dose of ***S*-Y048** was 2 × 10 mg·kg^{-1}

to the vehicle-treated control (Figure 7b,c). The effects of both doses were highly significant (Figure 7d). The plasma levels of ***S*-Y048** were measured 1 hr after initial administration of the antagonist, just before the second dose, and at the end of the experiment (Figure 7e,f). High levels were observed 7 and 25 hr after treatment with HDM with both doses. There did not appear to be any correlation between the magnitude of the inhibitory effect of ***S*-Y048** and its plasma concentration in individual monkeys (data not shown).

4 | DISCUSSION

Because of the high expression of the OXE receptor on human eosinophils and the potent stimulatory effects of 5-oxo-ETE on human, monkey and feline eosinophils, we postulated that this receptor may play an important role in eosinophilic diseases. Given the lack of rodent gene knockout models, we sought to identify selective OXE receptor antagonists that could be used to address this question and potentially serve as novel therapeutic agents in eosinophilic diseases. Our initial lead compounds were indoles containing a chiral methyl group on one of their side chains, with nearly all of the antagonist activity residing in the *S*-enantiomer (Figure 1). We used racemic mixtures containing equal amounts of the *S* and *R* enantiomers of **230** and **264** in our earlier in vivo studies (Figure 2) because of the difficulty in synthesizing sufficient quantities of the pure active *S*-enantiomers. However, we subsequently developed a procedure to synthesize substantial amounts of the chiral intermediate that is required for the synthesis of the active enantiomers of these antagonists (Reddy et al., 2015), which we were able to use to prepare the *S*-enantiomer of **Y048** that was used in the in vivo experiments shown in Figures 6 and 7.

We had initially hoped to use the cat as an experimental model because this species is highly susceptible to asthma (Dye et al., 1996) and feline granulocytes respond very strongly to 5-oxo-ETE (Cossette et al., 2015). However, our OXE receptor antagonists have only weak effects in this species, presumably due to differences between the feline and human OXE receptors, which are only about 75% identical. We next focused on the cynomolgus monkey, which was available to us locally. Our initial feasibility studies showed that both **230** (Cossette et al., 2016) and **264** (Reddy et al., 2018) are potent in vitro OXE receptor antagonists in this species and appear rapidly in the blood following oral administration. This led us to investigate whether they could inhibit eosinophil infiltration following in vivo administration to these animals. We first confirmed that 5-oxo-ETE elicits eosinophil infiltration into the skin after intradermal injection, as we have shown in humans (Muro et al., 2003). We then sought to determine whether this response could be blocked by OXE receptor antagonists. We initially had hoped to be able to use data from unpaired experiments, but the responses were too variable, and we could only use data from experiments in which each animal served as its own control. The limited paired data that we obtained in these experiments with only two animals suggests that **230** might inhibit 5-oxo-ETE-induced eosinophilia, but clearly, additional experiments would have been required to prove this. The eosinophilic response to *A. suum* was considerably stronger than that to 5-oxo-ETE and was also highly variable among animals. This response was reduced by **230** in all three animals from which paired data were obtained. In contrast, eosinophil numbers were reduced in only two of the four animals that received **264**. For this reason, and because of its superior pharmacokinetic profile, we focused on a modified compound related to **230** (i.e. ***S*-Y048**) in our further studies.

Although we were encouraged by the results of our initial experiments with **230** in cynomolgus monkeys, we concluded that the variability in the responses of these animals to antigen challenge would make it difficult to draw firm conclusions. Others have used this approach using wild-caught cynomolgus monkeys that had been environmentally exposed to *A. suum* (Tomkinson, Morton, Stevens, Bowden, & Tepper, 2009), but their animals were selected from a larger group based on screening for antigen sensitivity and thus were more homogeneous than the ones in our study. To reduce the variability among animals, we decided to take advantage of the well-established model developed at UC Davis, in which rhesus monkeys are sensitized by weekly subcutaneous injections of HDM (Schelegle et al., 2001).

We had previously shown that **230** and **264** have very similar potencies in blocking in vitro activation of cynomolgus monkey and human eosinophils (Cossette et al., 2016; Reddy et al., 2018). However, we had thus far only examined the ability of ***S*-Y048** to inhibit 5-oxo-ETE-induced calcium mobilization in human neutrophils. Before embarking on our in vivo studies with this compound in rhesus monkeys, we first confirmed that it is a potent OXE receptor antagonist in this species, blocking the effects of 5-oxo-ETE on actin polymerization in eosinophils, leukocyte migration and calcium mobilization.

We also confirmed that high levels of ***S*-Y048** appear in the blood shortly after oral administration to rhesus monkeys. At a dose of 2×5 mg·kg^{-1}, the plasma concentrations of ***S*-Y048** were maintained at between approximately 10 and 30 μM throughout the experiment, about twice the levels that we previously observed with cynomolgus monkeys (Ye et al., 2019). The concentration of ***S*-Y048M**, which is formed by the stereospecific addition of an *S*-hydroxyl group to the methylene group in the arylalkyl side chain adjacent to the indole (Ye et al., 2019), rose to about 5 μM by 24 hr. Since its potency is similar to that of ***S*-Y048** in blocking the action of 5-oxo-ETE, this metabolite could contribute to its anti-inflammatory effect at longer time points.

Because we were uncertain of its in vivo potency in rhesus monkeys, we wanted to be sure to select a dose of ***S*-Y048** that would block the actions of 5-oxo-ETE for the duration of the experiment. We initially selected a dose of 2×5 mg·kg^{-1} because this resulted in relatively high plasma concentrations as discussed above. Although ***S*-Y048** is extremely potent in inhibiting calcium mobilization in human neutrophils (IC_{50}, 0.02 nM; Ye et al., 2019), it is less potent in inhibiting actin polymerization in monkey eosinophils (IC_{50}, 0.34 nM) and still less potent in an in vitro chemotaxis assay (IC_{50} 30 nM), so we wanted to be sure to use a sufficiently high concentration to block the OXE receptor in vivo. The initial dose we used nearly completely

blocked dermal eosinophil infiltration in response to 5-oxo-ETE and inhibited the response to HDM by about 50%.

We also tested a higher dose of ***S*-Y048** (2×10 mg·kg^{-1}), which had a similar effect on HDM-induced eosinophilia, but appeared to have a lesser, although still statistically significant, effect on the response to 5-oxo-ETE compared to the 5 mg·kg^{-1} dose. The higher dose of ***S*-Y048** may have been well above the dose required for maximal efficacy and not necessarily expected to have a greater effect than the 5-mg dose. Because of the high cost of these experiments we were unable to test lower doses of ***S*-Y048**. This apparent inconsistency may have been due to a greater per cent variability in the response to 5-oxo-ETE because of the smaller numbers of eosinophils present in sections from sites at which 5-oxo-ETE was injected compared to HDM, which induced a stronger response. The weaker response to exogenous 5-oxo-ETE might be due to its fairly rapid dissipation after injection of a single dose due to rapid conversion to its much less active ω-hydroxy (Powell, MacLeod, Gravel, Gravelle, & Bhakar, 1996) and 6,7-dihydro (Berhane, Ray, Khanapure, Rokach, & Powell, 1998) metabolites and incorporation into cellular lipids (O'Flaherty, Taylor, & Thomas, 1998). In contrast, 5-oxo-ETE released endogenously in response to antigen may have been produced over a longer period of time, resulting in a more sustained proinflammatory effect.

Although the inter-animal variability in the data that we obtained from HDM-sensitized rhesus monkeys was much less than with the wild-caught cynomolgus monkeys used in the study with **230**, it was still considerable, as noted above. This is not unexpected, as the rhesus monkeys are outbred and are therefore expected to exhibit diversity in their responses. This could be due to differences among individual animals in the expression of other eosinophil chemoattractants such as **eotaxin** (Menzies-Gow et al., 2002) and **PGD_2** (Satoh et al., 2006), which may also contribute directly to the eosinophilic response and have been shown to have synergistic effects with 5-oxo-ETE (Powell et al., 2001; Schratl et al., 2006). There could also be variable expression of different cytokines such as GM-CSF, which has synergistic interactions with 5-oxo-ETE. (O'Flaherty et al., 1996; Stamatiou et al., 2004). Nevertheless, we were able to demonstrate significant in vivo eosinophilic responses to 5-oxo-ETE and HDM in this species that were inhibited by ***S*-Y048**.

In conclusion, this is the first study to demonstrate in vivo efficacy of an OXE receptor antagonist and the first to provide evidence for a pathophysiological role in mammals for the 5-lipoxygenase product 5-oxo-ETE. The only other study demonstrating an in vivo role for 5-oxo-ETE showed that in zebrafish, morpholino-induced knockdown of *oxer1* reduced leukocyte infiltration in response to wounding (Enyedi et al., 2013), which would be consistent with the inhibitory effects on eosinophil infiltration of our OXE receptor antagonist in the present study. Our data provide the first evidence that 5-oxo-ETE may be an important mediator in allergic reactions associated with eosinophil infiltration, which is supported by the increased levels of 5-oxo-ETE found in exhaled breath condensate following exposure of human subjects to HDM (Kowal et al., 2017) and after exposure of human bronchial segments to anti-IgE (Kolmert et al., 2018). Because of its high potency and long lifetime in vivo ***S*-Y048** could be a novel therapeutic agent for the treatment of allergic eosinophilic diseases.

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CONFLICT OF INTEREST

W.S.P. and J.R. have been granted a patent covering **230** and **264** and have applied for a patent covering ***S*-Y048**. There are no conflicts of interest for the other authors.

AUTHOR CONTRIBUTIONS

L.A.M. and C.C. coordinated the in vivo experiments and evaluated the skin eosinophils. C.C. also conducted in vitro experiments on leukocytes and performed the HPLC analyses. Q.Y., S.C., and C.N.R. performed the chemical syntheses. W.S.P., L.A.M., and J.R. designed the study. W.S.P, L.A.M., and J.R. wrote or contributed to the writing of the manuscript.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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