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Metabolism and pharmacokinetics of a potent *N*-acylindole antagonist of the OXE receptor for the eosinophil chemoattractant 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) in rats and monkeys

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ABSTRACT

We previously identified the indole **264** as a potent in vitro antagonist of the human OXE receptor that mediates the actions of the powerful eosinophil chemoattractant 5-oxo-ETE. No antagonists of this receptor are currently commercially available or are being tested in clinical studies. The lack of a rodent ortholog of the OXE receptor has hampered progress in this area because of the unavailability of commonly used mouse or rat animal models. In the present study, we examined the feasibility of using the cynomolgus monkey as an animal model to investigate the efficacy of orally administered **264** in future in vivo studies. We first confirmed that **264** is active in monkeys by showing that it is a potent inhibitor of 5-oxo-ETE-induced actin polymerization and chemotaxis in granulocytes. The major microsomal metabolites of **264** were identified by cochromatography with authentic chemically synthesized standards and LC-MS/MS as its ω2-hydroxy and ω2-oxo derivatives, formed by ω2-oxidation of its hexyl side chain. Small amounts of ω1-oxidation products were also identified. None of these metabolites have substantial antagonist potency. High levels of **264** appeared rapidly in the blood following oral administration to both rats and monkeys, and declined to low levels by 24 h. As with microsomes, its major plasma metabolites in monkeys were ω2-oxidation products. We conclude that the monkey is a suitable animal model to investigate potential therapeutic effects of **264**. This, or a related compound with diminished susceptibility to ω2-oxidation, could be a useful therapeutic agent in eosinophilic disorders such as asthma.

1. Introduction

Arachidonic acid is converted to a variety of pro- and anti- inflammatory mediators that have potent effects on both inflammatory and structural cells. Oxidation of arachidonic acid by 5-lipoxygenase (5-LO) results in the formation of proinflammatory leukotrienes (LTs) and 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) as well as anti-inflammatory lipoxins. 5-Oxo-ETE is formed by oxidation of 5*S*-hydroxy-6,8,11,14-eicosatetraenoic acid (5*S*-HETE) by 5-hydroxyeicosanoid dehydrogenase, which selectively oxidizes the 5*S*-hydroxyl group in the presence of $NADP^+$ (Powell et al., 1992). The synthesis of 5-oxo-ETE is regulated by the intracellular concentration of $NADP^+$, which normally is very low, but is rapidly increased under conditions of oxidative stress, activation of the respiratory burst in phagocytic cells, and cell death (Powell and Rokach, 2013).

The 5-LO pathway is important in host defence (Bailie et al., 1996) and contributes to the pathophysiology of inflammatory diseases, in particular asthma, in which case the 5-LO inhibitor zileuton and antagonists of the $cysLT_1$ receptor for LTD_4 (montelukast, zafirlukast, and pranlukast) are used clinically. Asthma is an inflammatory disease usually characterized by high levels of airway eosinophils, although other phenotypes also exist (Chung, 2016; Robinson et al., 2017). Eosinophils contribute to the pathology of asthma by releasing toxic mediators and proinflammatory cytokines (McBrien and Menzies-Gow,

Abbreviations: 5-HETE, 5*S*-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; 5-oxo-ETE, 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; DMP, Dess-Martin periodinane; PTSA, *p*-toluenesulfonic acid; DHP, 3,4-dihydro-2*H*-pyran; LT, leukotriene; TBDPS-Cl, *tert*-butyldiphenylsilyl chloride; TMS, tetramethylsilane; HRMS, High Resolution Mass Spectrometry

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2017). Interleukin-5 is important for the differentiation and survival of eosinophils, and antibodies against this cytokine or its receptor have been used therapeutically. Chemoattractants also play a critical role in the infiltration of eosinophils into the lungs. Among 5-LO products, 5-oxo-ETE and its metabolite 5-oxo-15-HETE are the only ones that have appreciable chemotactic effects on human eosinophils (Powell et al., 1995; Powell and Rokach, 2013). Furthermore, 5-oxo-ETE promotes the transendothelial migration of eosinophils (Dallaire et al., 2003) due to both its chemoattractant effects and its ability to induce the release of MMP-9 from these cells (Langlois et al., 2009). These effects are mediated by the G protein-coupled OXE receptor, which is highly selective for 5-oxo-ETE (Bäck et al., 2014) and 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), as well as various tumor cell lines (Sarveswaran and Ghosh, 2013).

Because of the potential involvement of 5-oxo-ETE in asthma and other eosinophilic diseases, the OXE receptor is an attractive therapeutic target. As no selective OXE receptor antagonists were available, we initiated a program to synthesize compounds that could selectively block this receptor. We initially identified a series of *N*-acylindoles that selectively blocked the activation of the OXE receptor in human neutrophils by 5-oxo-ETE. These compounds contained 5-oxo-valerate and hexyl groups in the 1- and 2-positions of the indole, respectively, mimicking the first 5 carbons of 5-oxo-ETE and the hydrophobic ω-end of the molecule (Gore et al., 2013). Addition of a chloro substituent in the 6-position of the indole increased potency from about 1.6 μM to 0.4 μM (Gore et al., 2013), and addition of a methyl group in the 3-position of the 5-oxovalerate side chain (compound **264**, Fig. 1) further increased potency to about 30 nM (Gore et al., 2014).

Unfortunately, we do not have the option of testing OXE receptor antagonists in mouse or rat models of asthma, because rodents do not possess an ortholog of the OXE receptor. Cats do have an OXE receptor with an amino acid sequence that is about 75% identical to the human OXE receptor. However, although 5-oxo-ETE is a very potent activator of feline eosinophils, **264** is only a weak antagonist of the feline OXE receptor (Cossette et al., 2015). We are therefore investigating the feasibility of using the monkey as an animal model to test the in vivo effects of **264**. The objectives of the current study were first to ensure that **264** is active in blocking 5-oxo-ETE-induced OXE receptor activation in monkeys and then to identify its major vivo metabolic pathways and determine whether significant levels appear in the blood following oral administration. We found that **264** is a potent OXE receptor antagonist in monkeys that appears rapidly in the blood following oral administration. Its major route of metabolism is ω2-oxidation of the hexyl side chain.

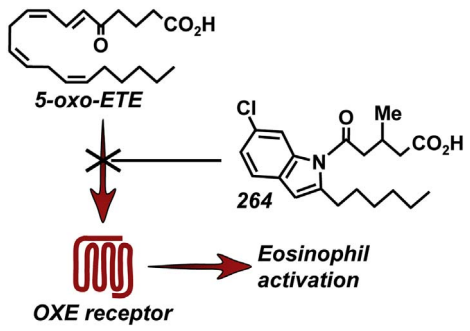

Fig. 1. Inhibition of the actions of 5-oxo-ETE by the OXE receptor antagonist **264**.

2. Materials and methods

2.1. Animals

Male and female cynomolgus monkeys weighing between 2.7 and 3.5 kg, housed at INRS-Institut Armand-Frappier, Laval, Quebec, and male Sprague-Dawley rats (200 to 250 g; 6 to 8 weeks old), housed at the Meakins-Christie Laboratories, were used for these studies. All experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the appropriate local institutional animal care committee.

2.2. Preparation of monkey leukocytes

Whole blood, collected in heparinized tubes from monkeys, was treated with Dextran 500 (Sigma-Aldrich) for 45 min at room temperature. After hypotonic lysis of red blood cells and centrifugation, the leukocytes were suspended in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4).

2.3. Evaluation of actin polymerization in monkey eosinophils and neutrophils

Leukocytes prelabeled with allophycocyanin (APC)-labeled mouse antihuman CD49d (BioLegend) as described previously (Cossette et al., 2015) were suspended in PBS containing Ca^{2+} (1.8 mM) and Mg^{2+} (1 mM). Aliquots of the leukocyte suspension (100 μl, 5×10^6 cells/ml) were preincubated for 5 min at 25 °C with either vehicle (1 μl DMSO) or **264** followed by the addition of 5-oxo-ETE (10 nM). The incubations were terminated after 20 s by the addition of formaldehyde (final concentration, 8.5%), and the samples were kept on ice for 30 min. Cytosolic F-actin was stained by treatment with a mixture of lysophosphatidylcholine (30 μg in 23.8 μl of PBS) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phallacidin (40.7 pmol in 6.18 μl methanol; final concentration, 1.36 μM) overnight in the dark at 4 °C. F-actin levels were measured by flow cytometry in eosinophils and neutrophils, which were distinguished from one another and from other leukocytes on the basis of side scatter and CD49d expression.

2.4. Measurement of leukocyte migration

Leukocyte migration was assessed as previously described (Monneret et al., 2001) using 48-well microchemotaxis chambers (Neuro Probe, Cabin John, MD) and Sartorius cellulose nitrate filters (8 μm pore size; 140-μm thickness; Neuro Probe). 5-Oxo ETE along with either vehicle (PBS containing 0.5% DMSO) or **264** were added to the bottom well in 30 μl PBS containing 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% BSA and 0.5% DMSO. Leukocytes (150,000 cells in PBS containing 0.05% BSA) and either vehicle or **264** were added in a total volume of 55 μl to each of the top wells. After incubation for 2 h at 37 °C, the filters were fixed with $HgCl_2$ and stained with hematoxylin and chromotrope 2R (Kay, 1970). The numbers of cells on the bottoms of the filters were counted in 5 different fields at a magnification of 400× for each incubation, each performed in triplicate.

2.5. Measurement of calcium mobilization in human neutrophils

The effects of **264** metabolites on 5-oxo-ETE-induced Ca^{++} mobilization in human neutrophils were examined as described previously (Gore et al., 2014). Neutrophils were prepared by dextran sedimentation and centrifugation over Ficoll-Paque and prelabeled with indo-1. Aliquots of the suspension were placed in a cuvette at 37 °C and the baseline fluorescence allowed to stabilize. **264** or one of its synthetic metabolites was then added, followed 2 min later by 5-oxo-ETE (10 nM) and, 1 min later, by digitonin (final concentration 0.1%). Fluorescence (λ_{ex}, 331 nm; λ_{em}, 410 nm) was measured with a Cary Eclipse