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# Inhibitory and mechanistic investigations of oxo-lipids with human lipoxygenase isozymes ☆

Michelle M. Armstrong, Giovanni Diaz, Victor Kenyon, Theodore R. Holman *

*Chemistry and Biochemistry Department, University of California, Santa Cruz, CA 95064, United States*

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ABSTRACT

Oxo-lipids, a large family of oxidized human lipoxygenase (hLOX) products, are of increasing interest to researchers due to their involvement in different inflammatory responses in the cell. Oxo-lipids are unique because they contain electrophilic sites that can potentially form covalent bonds through a Michael addition mechanism with nucleophilic residues in protein active sites and thus increase inhibitor potency. Due to the resemblance of oxo-lipids to LOX substrates, the inhibitor potency of 4 different oxo-lipids; 5-oxo-6,8,11,14-(*E*,*Z*,*Z*,*Z*)-eicosatetraenoic acid (5-oxo-ETE), 15-oxo-5,8,11,13-(*Z*,*Z*,*Z*,*E*)-eicosatetraenoic acid (15-oxo-ETE), 12-oxo-5,8,10,14-(*Z*,*Z*,*E*,*Z*)-eicosatetraenoic acid (12-oxo-ETE), and 13-oxo-9,11-(*Z*,*E*)-octadecadienoic acid (13-oxo-ODE) were determined against a library of LOX isozymes; leukocyte 5-lipoxygenase (h5-LOX), human reticulocyte 15-lipoxygenase-1 (h15-LOX-1), human platelet 12-lipoxygenase (h12-LOX), human epithelial 15-lipoxygenase-2 (h15-LOX-2), soybean 15-lipoxygenase-1 (s15-LOX-1), and rabbit reticulocyte 15-LOX (r15-LOX). 15-Oxo-ETE exhibited the highest potency against h12-LOX, with an $IC_{50} = 1 \pm 0.1$ μM and was highly selective. Steady state inhibition kinetic experiments determined 15-oxo-ETE to be a mixed inhibitor against h12-LOX, with a $K_{ic}$ value of $0.087 \pm 0.008$ μM and a $K_{iu}$ value of $2.10 \pm 0.8$ μM. Time-dependent studies demonstrated irreversible inhibition with 12-oxo-ETE and h15-LOX-1, however, the concentration of 12-oxo-ETE required ($K_i = 36.8 \pm 13.2$ μM) and the time frame ($k_2 = 0.0019 \pm 0.00032\ s^{-1}$) were not biologically relevant. These data are the first observations that oxo-lipids can inhibit LOX isozymes and may be another mechanism in which LOX products regulate LOX activity.



## 1. Introduction

Human lipoxygenases (hLOX) generate hydroperoxyeicosatetraenoic acid (HpETE) and hydroperoxyoctadecadienoic acid (HpODE) as their primary products from polyunsaturated fatty acids, such as arachidonic acid (AA)[1] and linoleic acid (LA), respectively.[2] These hydroperoxide products are in turn reduced by cellular glutathione peroxidase to the secondary alcohol product, hydroxyeicosatetraenoic acid (HETE) and hydroxyoctadecadienoic acid (HODE), respectively.[3,4] The electrophilic oxo-lipids, such as 5-oxo-ETE, 15-oxo-ETE, 12-oxo-ETE, and 13-oxo-ODE, are derived either from HpETE and HpODE, such as in the macrophage[5] or from the corresponding HETE and HODE, by a dehydrogenase-mediated oxidation.[6,7] These oxo-lipids are of interest because they are important biological molecules, whose interaction with LOX isozymes has not been fully explored.

The role of the various oxo-lipids in biology is significant and expanding. 5-oxo-6,8,11,14-(*E*,*Z*,*Z*,*Z*)-eicosatetraenoic acid (5-oxo-ETE) is a multi-functional oxo-lipid that has been found to

*Abbreviations:* LOX, lipoxygenase; hLOX, human lipoxygenase; h15-LOX-2, human epithelial 15-lipoxygenase-2; h15-LOX-1, human reticulocyte 15-lipoxygenase-1; h12-LOX, human platelet 12-lipoxygenase; s15-LOX-1, soybean 15-lipoxygenase-1; h5-LOX, human leukocyte 5-lipoxygenase; r15-LOX, rabbit reticulocyte 15-LOX; AA, arachidonic acid; LA, linoleic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 12-HpETE, 12-hydroperoxyeicosatetraenoic acid; 12-oxo-ETE, 12-oxo-5,8,10,14-(*Z*,*Z*,*E*,*Z*)-eicosatetraenoic acid; 12-oxo-ETEre, 10,11-dihydro-12-oxo-ETE; 12-HETrE, 10,11-dihydro-12-HETE; 15-HpETE, 15-hydroperoxyeicosatetraenoic acid; 15-oxo-ETE, 15-oxo-5,8,11,13-(*Z*,*Z*,*Z*,*E*)-eicosatetraenoic acid; 5-HpETE, 5-hydroperoxyeicosatetraenoic acid; 5-oxo-ETE, 5-oxo-6,8,11,14-(*E*,*Z*,*Z*,*Z*)-eicosatetraenoic acid; LA, linoleic acid; 13-HpODE, 13-(*S*)-hydroperoxyoctadecadienoic acid; 13-HODE, 13-hydroxy-9*Z*,11*E*-octadecadienoic acid; 13-oxo-ODE, 13-oxo-9,11-(*Z*,*E*)-octadecadienoic acid; 5-HEDH, 5-hydroxyeicosanoid dehydrogenase; 15-PGDH, 15-hydroxyprotaglandin dehydrogenase; 12-HEDH, 12-hydroxyeicosanoid dehydrogenase; CYP2S1, human cytochrome P450; GSH, glutathione; fmk, fluoromethylketone-substituted ligand; RSK1/2, p90 ribosomal protein S6 kinase; PPARγ, peroxisome proliferator activated receptor γ; CF, cystic fibrosis; IEC, intestinal epithelial cells.

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* Corresponding author. Tel.: +1 831 459 5884; fax: +1 831 459 2935.
*E-mail address:* holman@ucsc.edu (T.R. Holman).

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stimulate proliferation in cancer cell lines through a specific $G_{\alpha i}$-coupled receptor.[8–10] 5-oxo-ETE also plays an important role in the asthmatic inflammatory response,[11] gastrointestinal diseases[12] and activation of peroxisome proliferator-activated receptor γ (PPARγ) transcriptional activity.[13] Another oxo-lipid which plays a role in the cell is 12-oxo-5,8,10,14-(*Z*,*Z*,*E*,*Z*)-eicosatetraenoic acid (12-oxo-ETE). Powell et al.[14,15] observed that 12-oxo-ETE had effects on cytosolic calcium levels at concentrations of 10 μM, but Naccache et al.[16] reported calcium effects as low as 10 nM. The third oxo-lipid generated from AA is 15-oxo-5,8,11,13-(*Z*,*Z*,*Z*,*E*)-eicosatetraenoic acid (15-oxo-ETE). For this oxo-lipid, an esterified, 15-oxo-ETE phospholipid has been detected in patients with cystic fibrosis (CF)[13] and shown to activate transcriptional activity in PPARγ.[12] Activation of PPARγ expression in CF mice ameliorates disease severity, suggesting that 15-oxo-ETE might potentially act to lower inflammation in CF.[13] Finally, there is an oxo-lipid generated from LA, 13-oxo-9,11-(*Z*,*E*)-octadecadienoic acid (13-oxo-ODE), which was found to be an endogenous ligand to PPARγ in intestinal epithelial cells (IEC). 13-Oxo-ODE mediated the activation of PPARγ to reduce mucosal damage and down-regulate inflammation in several mouse models of intestinal colitis,[17] implicating it as a possible therapeutic target for the treatment of inflammatory bowel disease.[17]

Chemically, the oxo-lipids, like 5-oxo-ETE, 15-oxo-ETE, 12-oxo-ETE and 13-oxo-ODE, are unique in that they contain an α, β unsaturated carbonyl that can readily react with nucleophiles, such as proteins and glutathione (GSH), via Michael addition reaction, resulting in covalent modifications. The reversible conjugation of 13-oxo-ODE by GSH[18,19] has been shown to occur by both enzymatic and non-enzymatic pathways, with the conjugate being exported from the cell via an energy-dependent process.[20] Similar synthetic molecules that form covalent linkages to their targets have been considered as therapeutics, but have traditionally been disfavored due to concerns for their off-target reactivity, either through direct tissue damage or through haptenization of proteins, which could elicit an immune response.[21] However, as selectivity and drug resistance remain a serious issue for reversible inhibitors, a resurgence of interest in this class of therapeutics has emerged. For example, Taunton and co-workers,[22] developed a fluoromethylketone-substituted ligand (fmk), which irreversibly inactivates p90 ribosomal protein S6 kinase (RSK1/2) in human cells at nanomolar concentrations by modifying an active site cysteine, without inhibiting over 130 other kinases.[22] A similar covalent inhibitor, JNK-IN-8, was discovered as a specific, irreversible intracellular inhibitor against the mitogen-activated kinase JNK.[23] JNK-IN-8 inhibits phosphorylation of c-Jun, a direct substrate of JNK, by covalent modification of a conserved cysteine residue in the ATP-binding motif.[23] Both of these studies argue against the widely held view that electrophilic inhibitors are inherently nonselective[22] and therefore it is possible that the oxo-lipids target non-conserved, non-catalytic cysteines in many proteins in the cell, such as lipoxygenase.

Due to the fact that oxo-lipids have interesting biological properties, that they are potential covalent modifiers and that they have similar structures to LOX substrates, we hypothesized that oxo-lipids could potentially inhibit LOX isozymes at concentrations that are biologically relevant. This hypothesis is reinforced by the fact that certain LOX isozymes have non-catalytic cysteines in their active sites,[24] which could serve as nucleophiles to oxo-lipids. In the current work, we present inhibitory data of a variety of oxo-lipids (5-oxo-ETE, 15-oxo-ETE, 12-oxo-ETE, and 13-oxo-ODE) against LOX isozymes (h5-LOX, h15-LOX-1, human platelet 12-lipoxygenase (h12-LOX), human epithelial 15-lipoxygenase-2 (h15-LOX-2), soybean 15-lipoxygenase-1 (s15-LOX-1), and rabbit reticulocyte 15-LOX (r15-LOX)) and demonstrate that certain oxo-lipids are LOX inhibitors.

## 2. Materials and methods

### 2.1. Materials

All commercial fatty acids (Sigma–Aldrich Chemical Company) were re-purified using a Higgins HAIsil Semi-Preparative (5 mM, 250 × 10 mm) C-18 column. Solution A was 99.9% MeOH and 0.1% acetic acid; solution B was 99.9% $H_2O$ and 0.1% acetic acid. An isocratic elution of 85% A: 15% B was used to purify all fatty acids, which were stored at −80 °C for a maximum of 6 months. HPLC grade solvents were used for both semi-preparative HPLC purification and analytical HPLC analysis of LOX products. Large scale product purification was achieved by using a C18HAIsil 250 × 10 mm semi-preparative column, whereas a C18HAIsil 250 × 4.6 mm analytical column was used for product separation in tandem with MS/MS analysis. Both columns were purchased from Higgins Analytical (Mountain View, CA). All other chemicals were reagent grade or better and were used without further purification.

### 2.2. Protein expression

All the LOX isozymes used in this publication were expressed and purified as previously published (h5-LOX,[28] h12-LOX,[25] h15-LOX-1[25] and s15-LOX-1,[26] h15-LOX-2[27] and r15-LOX[29]).

### 2.3. General procedure for the synthesis of oxo-lipids

The synthesis of all oxo-lipids consists of two steps, the first step is enzymatic while the second step is synthetic. In the synthesis of 13-oxo-ODE, s15-LOX-1 is reacted with linoleic acid (LA) in 100 mL of 100 mM Borate (pH 9.2) generating 13-HpODE. 15-Oxo-ETE is generated by reaction between h15-LOX-2 and 40 μM arachidonic acid (AA) in 100 mL of 25 mM HEPES (pH 7.5), generating 15-HpETE. 12-Oxo-ETE is generated by reaction between h12-LOX and 40 μM AA in 100 mL 25 mM HEPES (pH 8.0), generating 12-HpETE. 5-Oxo-ETE is generated by reaction between 5-LOX and 40 μM AA in 100 mL 25 mM HEPES (pH 7.3), 0.3 mM $CaCl_2$, 0.1 mM EDTA, 0.2 mM ATP, generating 5-HpETE. The reactions are quenched with 1–2% acetic acid and extracted using dichloromethane (DCM). The formation of 13-HpODE, 15-HpETE, 12-HpETE and 5-HpETE are monitored at 234 nm with a Perkin Elmer Lambda 40 UV/vis spectrophotometer. The second step is an overnight synthetic reaction in which the hydroperoxy products are reacted with acetic anhydride and pyridine at 4 °C in a 1:1 ratio to generate 13-oxo-ODE, 15-oxo-ETE, 12-oxo-ETE and 5-oxo-ETE, respectively. The reactions are quenched with cold Milli-Q water for 2 h. The oxo-lipids are purified via high performance liquid chromatography (HPLC) using a Higgins HAIsiL Semi-Preparative C-18 column. Solution A was 99.9% ACN and 0.1% acetic acid; solution B was 99.9% $H_2O$ and 0.1% acetic acid. An isocratic elution of 55% A: 45% B was used to purify each oxo-compound. The retention times for each oxo-lipid at 280 nm are as follows: 13-oxo-ODE (30 min), 15-oxo-ETE (33 min), 12-oxo-ETE (90 min) and 5-oxo-ETE (70 min). Analytical analysis was performed by liquid chromatography–mass spectrometry (LC–MS/MS). Solution A was 99.9% $H_2O$ and 0.1% formic acid; solution B was 99.9% ACN and 0.1% formic acid. Oxo-lipids were injected onto a Phenomenex Synergi (4 μM, 150 mm × 4.6 mm) C-18 column attached to a Thermo LTQ LC–MS/MS. The elution protocol consisted of 200 μL/min, with an isocratic mobile phase of 45% solution A and 55% solution B. Negative ion MS/MS was utilized (collision energy of 35 eV) to determine the fragmentation patterns of all the oxo-lipids. 13-Oxo-ODE, parent $m/z = 293$, fragments $m/z = 113$, 249, 293; 15-oxo-ETE, parent $m/z = 317$, fragments $m/z = 113$, 273, 299; 12-oxo-ETE, parent $m/z = 317$, fragments $m/$