

# Resolving the Role of Lipoxygenases in the Initiation and Execution of Ferroptosis

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## Supporting Information

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**Materials.** C<sub>15</sub>-THN<sup>1</sup>, Fer-1<sup>2</sup>, Lip1<sup>3</sup>, RSL3<sup>2,3</sup>, erastin<sup>4,5</sup>, and STY-BODIPY<sup>6</sup>, Oleic 10-hydroperoxide<sup>7</sup>, Cholesterol 7-hydroperoxide<sup>8</sup> were synthesized according to literature procedures. Egg phosphatidylcholine, AIBN, MeOAMVN, BODIPY-C11<sup>581/591</sup>, NDGA, Zileuton, PD146176, CAY10649, CJ-13610,  $\alpha$ -TOH, ML210, baicalein, cinnamyl-3,4-dihydroxy- $\alpha$ -cyanocinnamate, MEM media with/without phenol red, Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), penicillin-streptomycin, Antibodies, AquaBluer and reagents for synthesis were purchased from commercial sources and used as received.

**Inhibited Autoxidation of Cumene.** Cumene was washed thrice with 1 M aqueous NaOH, dried over MgSO<sub>4</sub>, filtered, distilled under vacuum and purified by percolating through silica, then basic alumina. To a cuvette containing 1.25 mL cumene was added 1.18 mL chlorobenzene and the solution equilibrated for 5 minutes at 37°C. The cuvette was blanked and 12.5  $\mu$ L of 2 mM PBD-BODIPY in 2,3,5-trichlorobenzene was added followed by 50  $\mu$ L of 0.3 M AIBN in chlorobenzene and the solution was thoroughly mixed. After 20 minutes, an aliquot of LOX inhibitors or RTA stock solution (1 mM) in chlorobenzene was added and the loss of absorbance at 591 nm followed. The inhibition rate constant ( $k_{inh}$ ) and stoichiometry ( $n$ ) was determined for each experiment according to Eq. (1) and (2), respectively. Kinetic data are given as averages of three independent measurements.

**Inhibited Autoxidation of Egg-PC Liposomes.** To a cuvette of 2.34 mL of 10 mM PBS at pH 7.4 was added liposomes (125  $\mu$ L of 20 mM stock in PBS at pH 7.4) and the solution equilibrated for 5 minutes at 37°C (liposome formation from egg-PC was described elsewhere).<sup>1</sup> The cuvette was blanked and 10  $\mu$ L of 2 mM STY-BODIPY in DMSO was added followed by 10  $\mu$ L of 0.05 M MeOAMVN in acetonitrile and the solution was thoroughly mixed. After 5 minutes, an aliquot of LOX inhibitors or RTA stock solution (1 mM) in DMSO was added and the loss of absorbance at 565 nm followed. The inhibition rate constant ( $k_{inh}$ ) and stoichiometry ( $n$ ) was determined for each experiment according to Eq. (1) and (2), respectively. Kinetic data are given as averages of three independent measurements. Indistinguishable results were obtained in select control experiments where the antioxidant was added prior to liposome extrusion.

**Cell Culture.** All cell lines were cultured at 37 °C in a 5% CO<sub>2</sub> atmosphere unless otherwise indicated. HEK-293 cells were cultured in MEM with 10% FBS, 1% 100 $\times$  non-essential amino acid solution, 1 mM sodium pyruvate and 1% penicillin-streptomycin. Cells were passaged by dissociation with 0.05% trypsin and 0.2% EDTA every two to three days. LOX overexpressing stable cell lines were cultured in MEM with 10% FBS, 1% 100 $\times$  non-essential amino acid solution, 10 mM glutamine and 1 g/L geneticin. Pfa1 cells were cultured in DMEM with 10% FBS, 1% 100 $\times$  non-essential amino acid solution, 1 mM sodium pyruvate and 1% penicillin-streptomycin. All cell experiments were carried out as a minimum of three biological replicates.

**Overexpression of 5, 12- and 15-LOX in HEK 293 cells.** Cells were plated to 30% confluency and were transfected with a recombinant pcDNA3/*alox5*, *alox12* and *alox15* constructs using LipoJet reagent according to the manufacturer's recommendations. The cells were passaged to 35 mm plates after 1 day and colonies were screened using selection medium containing 1 g/L geneticin. Stable expression was verified every few passages.

**Immunoblotting.** Protein extracts were prepared by lysing either HEK293 cells, LOX overexpressing cells or Pfa1 cells in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein loading buffer (62.5 mM Tris [pH 6.8], 25% glycerol, 2% SDS, 0.1% bromophenol blue, 5% 2-mercaptoethanol). After separation by 10% PAGE, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immuno-Blot PVDF; Bio-Rad Laboratories), and blocked in 5% dry milk, Tris-buffered saline. The membrane was probed with an antibody specific for 5, 12- and 15-LOX, GPX4 and  $\beta$ -actin (Novusbio). Binding of the primary antibody was detected using a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Novusbio) and visualized by ECL chemiluminescence reaction (Pierce ECL, Thermo Scientific).

**Determination of H(P)ETEs by UPLC/MS/MS.** LOX overexpressing cells were harvested and lysed in Tris-HCl [pH 7.4] in the presence of 1% protease inhibitor cocktail (Sigma Aldrich). The residue was vortexed at 10,000 rpm for 10 minutes and the supernatants were incubated with 70  $\mu$ M arachidonic acid (AA, Nu-Chek Prep) at 37 °C for 10 min. The reactions were terminated with 1 volume of methanol and 1.5 mg/mL solution of TCEP (Sigma Aldrich). The samples were stored at room temperature for 90 min to ensure complete reduction of the HPETEs to HETE takes place. For inhibition studies, incubations with inhibitors were carried out for 10 min on ice prior to the addition of AA. Briefly, 10  $\mu$ M prostaglandin B<sub>2</sub> (Cayman Chemical) was added as internal standard along with 15  $\mu$ l of 1M HCl for acidification followed by extraction using C-18 solid phase extraction columns (Thermo Scientific) as per manufacturer's protocol. The eluent in methanol was evaporated to dryness and resuspended in 100  $\mu$ l methanol. Aliquots (5  $\mu$ l) were injected on a Waters Acquity C<sub>18</sub> column (2.1 mm  $\times$  50 mm, 1.7- $\mu$ m particle size) in a Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system. HETEs were eluted isocratically with a mixture of acetonitrile, methanol, water and acetic acid (42:25:33:0.007) and detected using MS/MS analysis. Eicosanoids were detected in the negative ion mode by multiple reaction monitoring of  $m/z$  333  $\rightarrow$  235 for PGB<sub>2</sub>,  $m/z$  319  $\rightarrow$  115 for 5-HETE,  $m/z$  319  $\rightarrow$  179 for 12-HETE, and  $m/z$  319  $\rightarrow$  219 for 15-HETE. Each experiment was performed in technical triplicate and reproduced independently three times, error bars represent SD from the mean.

**Determination of H(P)ETEs by UPLC/MS/MS.** LOX overexpressing cells were plated in a 10-cm plate and allowed to adhere overnight. The next day, media was removed, the cells were washed with PBS and suspended in media containing 5  $\mu$ M RSL3 and 40  $\mu$ M AA. The cells were harvested using a cell scraper at appropriate time points and flash frozen in liquid nitrogen. After thawing, the cells were treated with 5 mL of MeOH containing the internal standard, 1 mg/mL BHT and 1.5 mg/mL TCEP, followed by sonication to lyse the cells. BHT was added in order to suppress any air oxidation of the PUFAs present in the sample. The mixture was left on a shaker for 30 minutes at room temperature to ensure complete reduction of HPETEs. 10 mL of chloroform and 1mL of 0.9% NaCl solution was added followed by shaking for 1 hour to ensure maximal extraction of lipids. The tubes were centrifuged to separate the organic and aqueous

layers. The lipids were recovered by removing the bottom chloroform layer. The mixture was re-extracted using 5 mL chloroform and the two organic layers were combined. The combined chloroform layers were dried in a vacuum dryer at 20 °C. The lipids were re-suspended in 500 µL MeOH containing BHT, followed by the addition of 500 µL KOH (1.5%) solution and incubated at 60°C for 30 minute to saponify all the phospholipids. After incubation, the solution was acidified using 500 µL HCl (2 M) solution. The mixture was purified using the above protocol and analyzed on Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) system.

**Induction of Ferroptosis Induced by Gpx4 Inhibition with (1S,3R)-RSL3.** HEK293 cells, the LOX-overexpressing cells and Pfa1 cells (3,000 in 100 µL) were seeded in 96-well plates and incubated overnight. The next day the media was removed, the cells were washed twice with PBS and the cells were suspended in new media for 30 minutes before addition of (1S, 3R)-RSL3 in a final volume of 100 µl. Cell viability was assessed 4 hours later using the AquaBluer assay (MultiTarget Pharmaceuticals, LLC) according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Experiments are carried out with six-technical replicates ( $n = 6$  wells of a 96-well plate) and performed independently with a minimum of three biological replicates.

**Induction of Ferroptosis Induced by GSH Depletion with Erastin.** HEK293 cells, the LOX-overexpressing cells and Pfa1 cells (3,000 in 100 µL) were seeded in 96-well plates and incubated overnight. The next day the media was removed, the cells were washed twice with PBS and the cells were suspended in new media for 30 minutes before addition of erastin in a final volume of 100 µl. Cell viability was assessed 24 hours later using the AquaBluer assay (MultiTarget Pharmaceuticals, LLC) according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Experiments are carried out with six-technical replicates ( $n = 6$  wells of a 96-well plate) and performed independently with a minimum of three biological replicates.

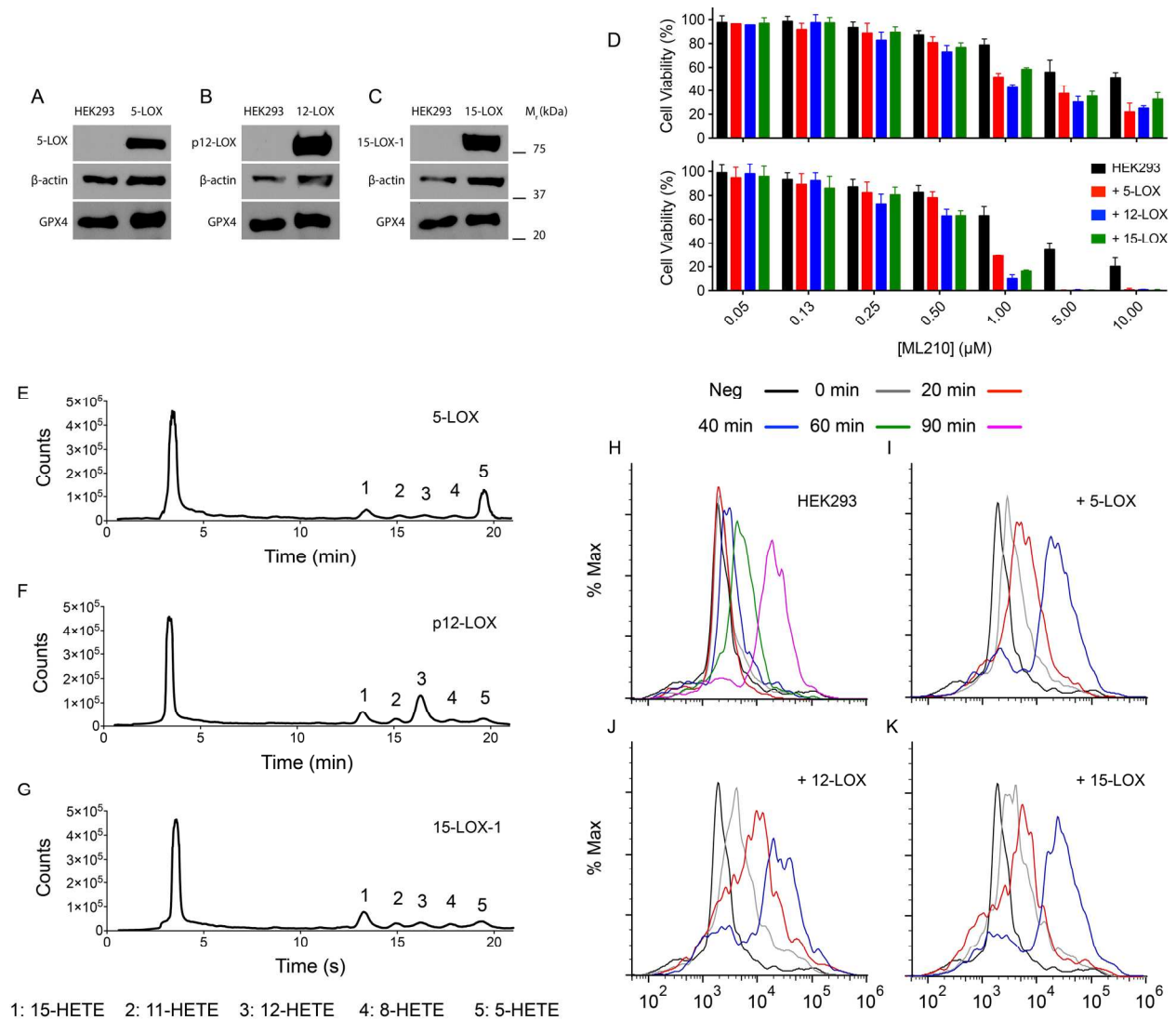
**Induction of Ferroptosis Induced by ML210.** HEK293 cells and the LOX-overexpressing cells (3,000 in 100 µL) were seeded in 96-well plates and incubated overnight in the presence or absence of 40 µL LA. The next day the media was removed, the cells were washed with PBS and the cells were suspended in new media containing 10 µM ML210 in a total volume of 100 µl. Cell viability was assessed at either 24 hours (LA-treated cells) or 48 hours (LA-untreated cells) using the AquaBluer assay (MultiTarget Pharmaceuticals, LLC) according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Experiments are carried out with six-technical replicates ( $n = 6$  wells of a 96-well plate) and performed independently with a minimum of three biological replicates.

**Cellular Lipid Peroxidation.** HEK293 and LOX overexpressing cells were cultured in 12-well plates ( $1 \times 10^5$  cells/mL) overnight. The media was removed and the cells were washed with PBS three times and then treated with 1 µM BODIPY-C11<sup>581/591</sup> in media and incubated at 37 °C in dark for 30 minutes after which the cells were trypsinized and AA (70 µM) in DMSO was added. Cells were then analyzed by flow cytometry ( $\lambda_{\text{ex}} = 488$  nm;  $\lambda_{\text{em}} = 525 \pm 25$  nm). Cells treated with DMSO as vehicle were used as negative control. Experiments are carried out with three-technical replicates and performed independently with a minimum of three biological replicates.

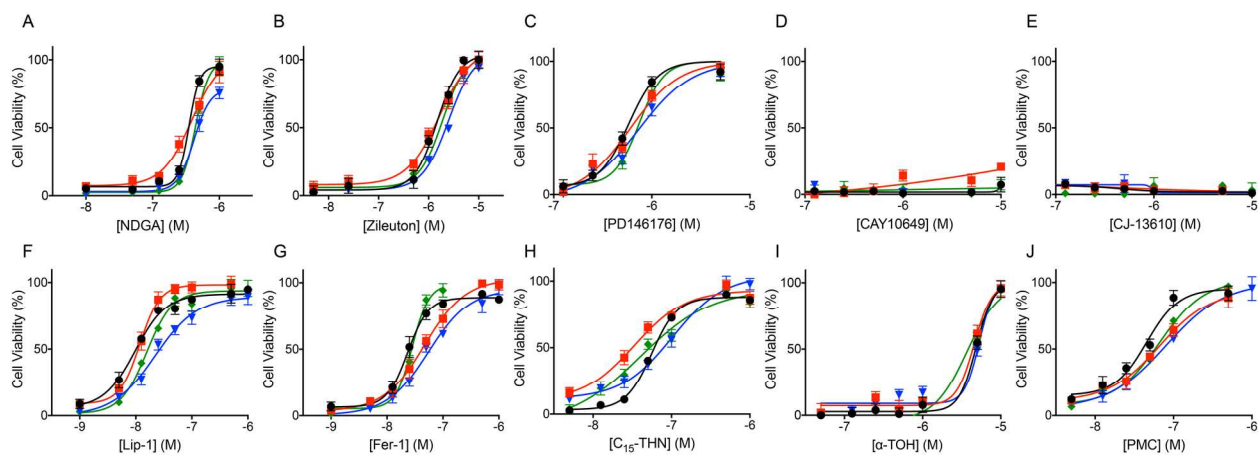
**Inhibition of Ferroptosis Induced by Gpx4 Inhibition with (1*S*,3*R*)-RSL3.** HEK293, LOX overexpressing cells or Pfa1 cells (3,000 in 100  $\mu$ L) were seeded in 96-well plates and cultured overnight. The next day the media was removed, the cells were washed twice with PBS and the cells were suspended in new media for 30 minutes before addition of RTAs and LOX inhibitors. Ferroptosis was induced using (1*S*, 3*R*)-RSL3 (10  $\mu$ M) 30 minutes after incubation of compounds. Cell viability was assessed 6 hours later using the AquaBluer assay (MultiTarget Pharmaceuticals, LLC) according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Experiments are carried out with six-technical replicates ( $n = 6$  wells of a 96-well plate) and performed independently with a minimum of three biological replicates.

**Inhibition of Ferroptosis Induced by ML210.** HEK293 and LOX overexpressing cells (3,000 in 100  $\mu$ L) were seeded in 96-well plates and cultured overnight in the presence of 40  $\mu$ M LA. The next day the media was removed, the cells were washed with PBS and the cells were suspended in new media for 30 minutes before addition of RTAs and LOX inhibitors. Ferroptosis was induced using ML210 (10  $\mu$ M) concomitant with the administration of compounds. Cell viability was assessed 24 hours later using the AquaBluer assay (MultiTarget Pharmaceuticals, LLC) according to the manufacturer's instructions. Cell viability was calculated by normalizing the data to untreated controls. Experiments are carried out with six-technical replicates ( $n = 6$  wells of a 96-well plate) and performed independently with a minimum of three biological replicates.

**Inhibition of LOX Overexpressing Cells Using Deuterated AAs.** Cells were plated in a 96-well plate and allowed to adhere overnight. The next day, media was removed and the cells were washed with PBS. Clear media containing the coumarin-triarylphosphine probe (10  $\mu$ M) was added and allowed to incubate for 15 minutes. After incubation, a solution of AA was added and the fluorescence of the probe was measured over time in a plate reader at 37°C (excitation wavelength = 340 nm; emission wavelength = 425 nm). The initial rates were used to determine the rate of product formation. Experiments are carried out with three-technical replicates ( $n = 3$  wells of a 96-well plate) and performed independently with a minimum of three biological replicates.



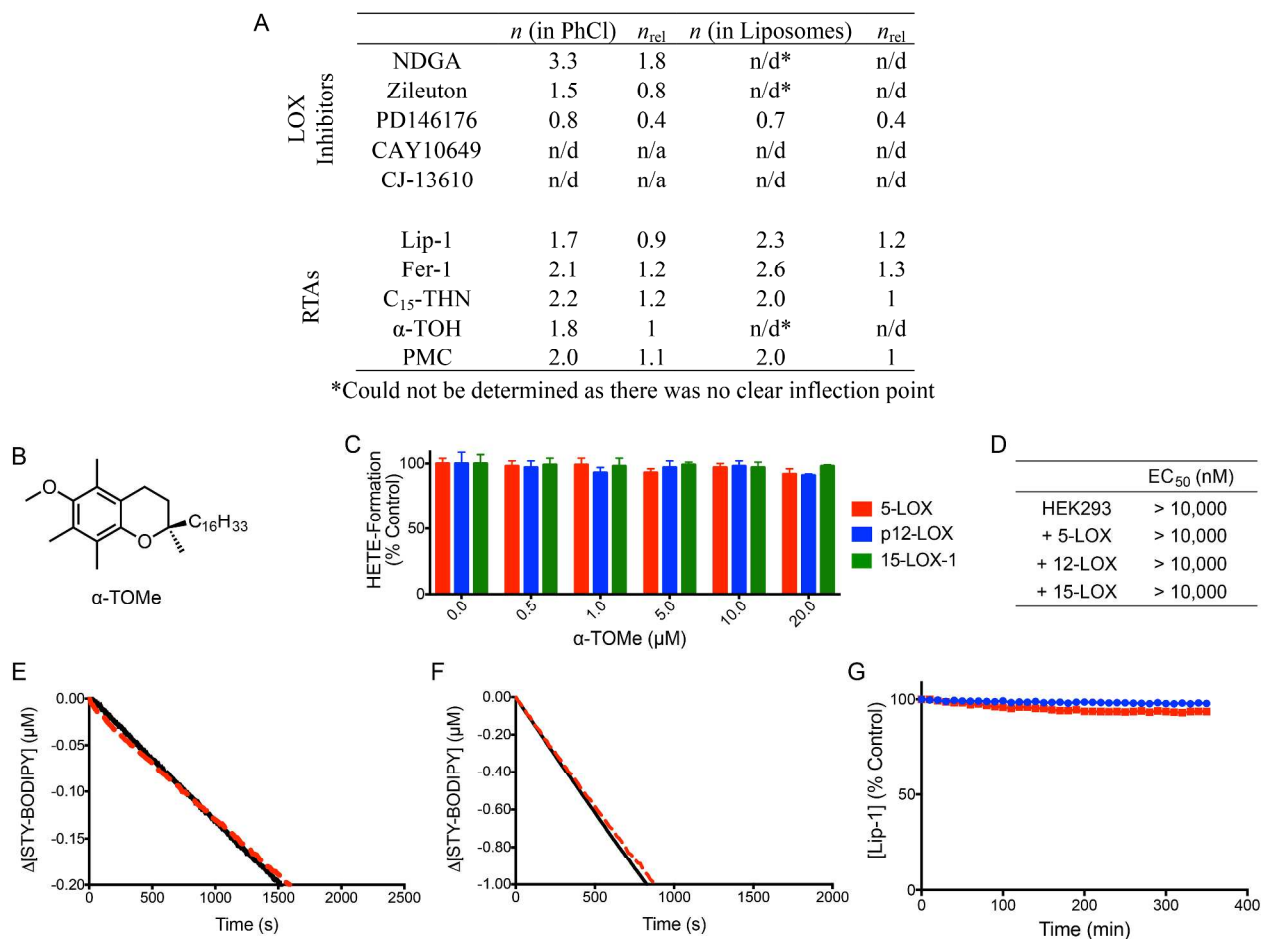
**Figure S1.** Western blots showing GPX4 expression in HEK293, 5-LOX (A), 12-LOX (B) and 15-LOX (C) cells. (D) ML210 induces ferroptosis in each of the cell lines following 48-hour incubation (top) and 24-hour incubation (bottom) in the presence of 40  $\mu\text{M}$  linoleic acid. LC/MS chromatograms of lipid extracts of lysates from 5-LOX (E), p12-LOX (F) and 15-LOX-1 (G) following incubation with 70  $\mu\text{M}$  arachidonic acid for 10 minutes. Flow cytograms of  $\text{C}_{11}$ -BODIPY treated cells supplemented with 5  $\mu\text{M}$  RSL3 in HEK293 (H), 5-LOX (I), 12-LOX (J) and 15-LOX (K) cell lines.



**Figure S2.** Dose-response curves for inhibition of RSL3-induced ferroptosis by various LOX inhibitors: (A) NDGA, (B) Zileuton, (C) PD146176, (D) CAY10649, (E) CJ-13610 and RTAs: (F) Lip-1, (G) Fer-1, (H) C<sub>15</sub>-THN, (I)  $\alpha$ -TOH, (J) PMC in HEK293 (●), + 5-LOX (■), + 12-LOX (▼) and + 15-LOX (◆) cells.

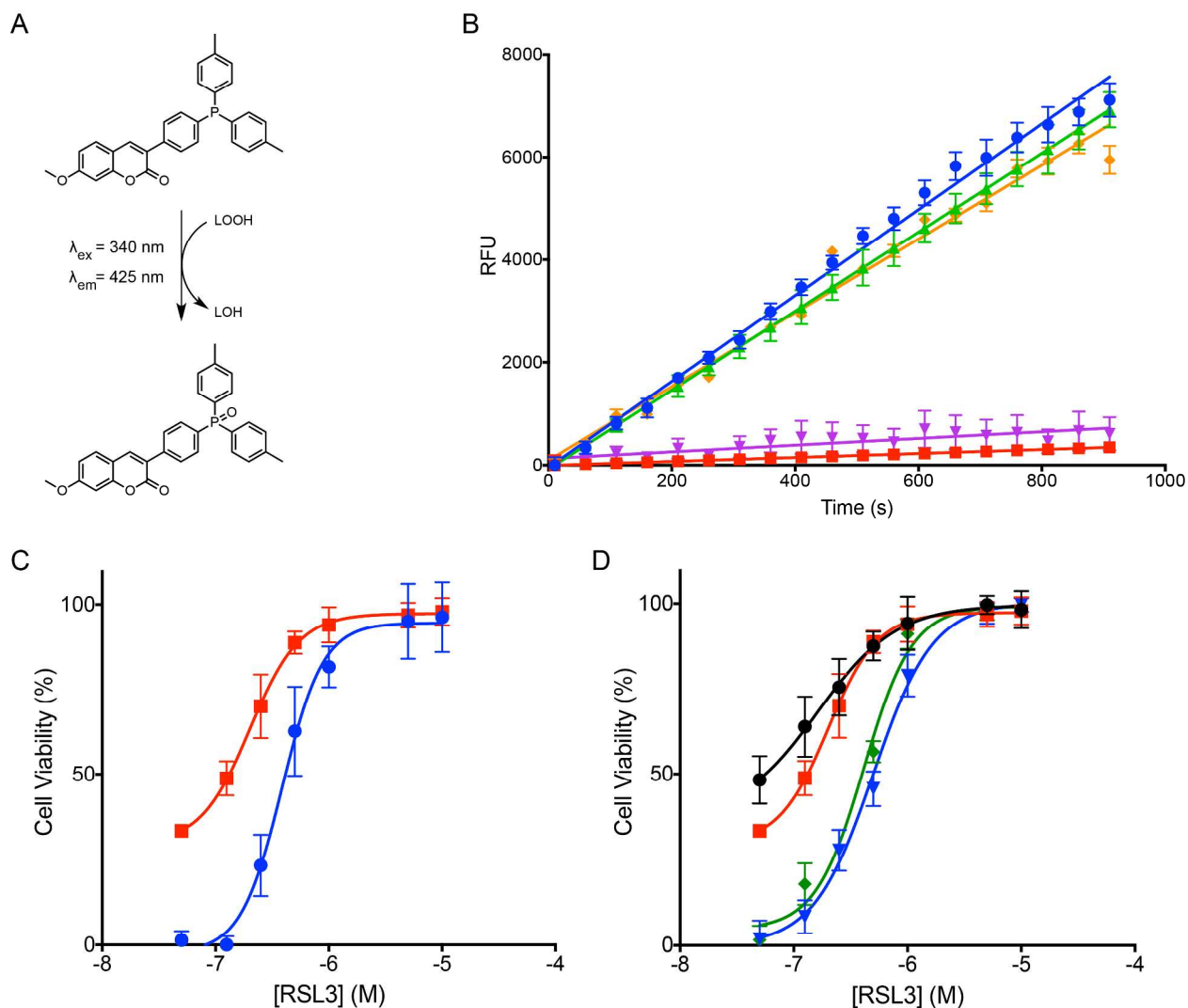
	EC <sub>50</sub> (nM)			
	HEK293	+ 5-LOX	+ 12-LOX	+ 15-LOX
NDGA	1041	1181	922	1057
Zileuton	887	1454	1402	1089
PD146176	158	253	188	210
CJ-13610	> 10,000	> 10,000	> 10,000	> 10,000
Lip-1	11	14	24	14
Fer-1	20	48	35	32

**Table S1.** Potency of representative RTAs and LOX inhibitors as inhibitors of ML210-induced ferroptosis in HEK293, + 5-LOX, + 12-LOX and + 15-LOX cells.



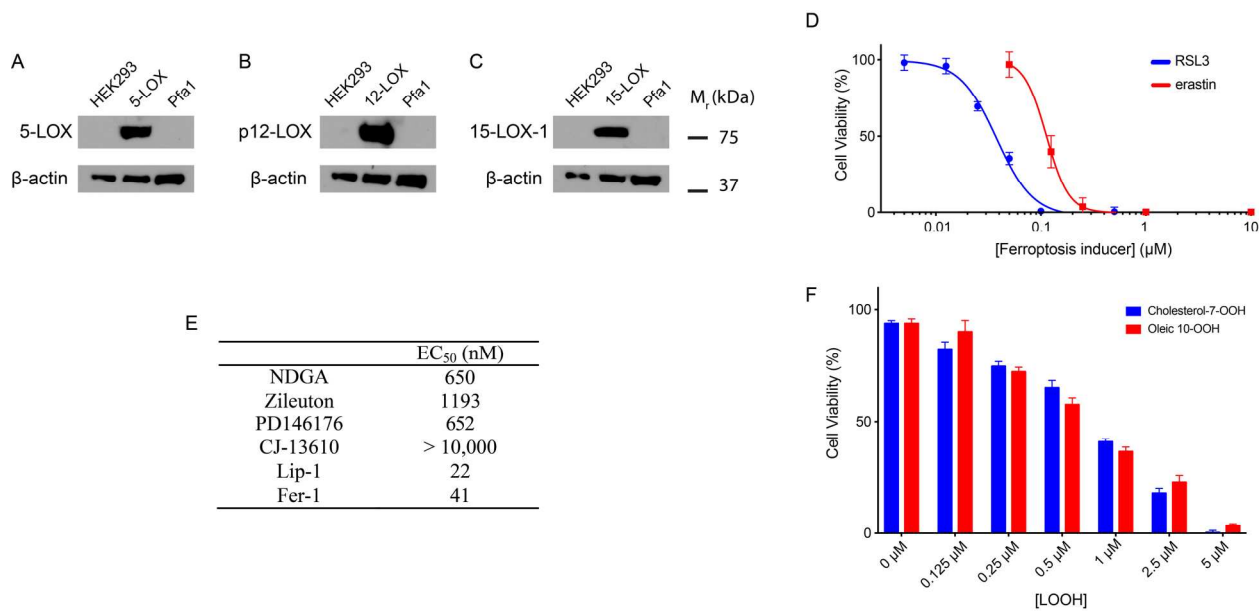
**Figure S3.** Reaction stoichiometry ( $n$ ) for LOX inhibitors and RTAs in cumene and liposome autoxidations determined using Eq. 2 from Figure 3 (A); relative values of  $n$  are given for easy comparison to  $\alpha$ -TOH. Structure of  $\alpha$ -TOMe (B), its inhibitory activity against 5-LOX, p12-LOX and 15-LOX-1 (C), and potency as inhibitor of RSL3-induced ferroptosis in the four different cell lines (D). Co-autoxidations of cumene (3.6 M) and STY-BODIPY (10  $\mu$ M) initiated by AIBN (6 mM) in chlorobenzene at 37°C (black) and inhibited by 2  $\mu$ M of  $\alpha$ -TOMe (red, - - -) (E). Co-autoxidations of egg phosphatidylcholine liposomes (1.0 mM) and STY-BODIPY (10  $\mu$ M) suspended in phosphate-buffered saline (10 mM) at pH 7.4 initiated by MeOAMVN (0.2 mM) at 37°C (black) and inhibited by 2  $\mu$ M of  $\alpha$ -TOMe (red, - - -) (F). Stability of Lip-1 (10  $\mu$ M) in the absence (●) or presence (■) of oleic 10-hydroperoxide (50  $\mu$ M) in PBS buffer at 37°C monitored by the loss in absorbance of Lip-1 by UV-Vis spectroscopy (G).



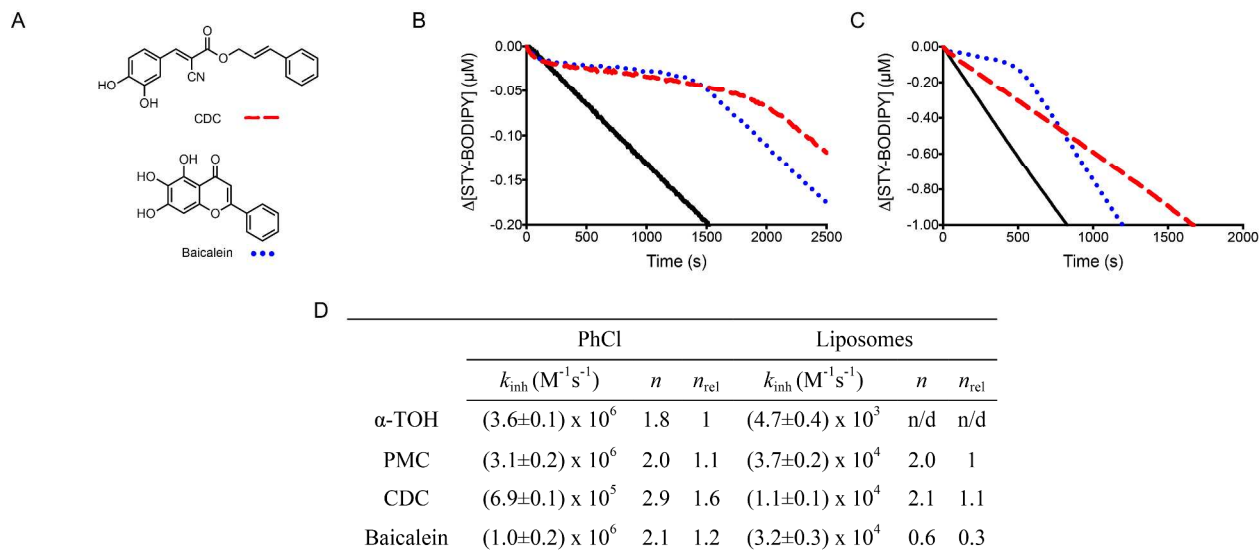


**Figure S4.** (A) Reaction of fluorogenic coumarin-triarylphosphine conjugate with lipid hydroperoxides. (B) Representative data for LOX-activity obtained from 12-LOX cells determined using the coumarin-triarylphosphine probe (10  $\mu\text{M}$ ) in the presence of 70  $\mu\text{M}$  of either AA (●),  $d_6$ -AA (■),  $7,7-d_2$ -AA (▲),  $10,10-d_2$ -AA (▼) and  $13,13-d_2$ -AA (◆). (C) Cell viability determined for 5-LOX cells with varying concentrations of RSL3, cultured in the absence (●) or presence of 10  $\mu\text{M}$  CJ-13610 (■) using AquaBluer 4-hours post induction. (D) Cell viability determined for HEK293 (●), 5-LOX (■), 12-LOX (▼) and 15-LOX (◆) cells with varying concentrations of RSL3 cultured in the presence of 10  $\mu\text{M}$  CJ-13610 using AquaBluer 4-hours post induction.

The production of LOOH from LOX cells in the presence of different PUFAs (Figure S4B, C and D) was determined using our coumarin-triarylphosphine probe. The probe undergoes fluorescence enhancement upon reaction with hydroperoxides which can be monitored using a microplate reader.<sup>9-11</sup>

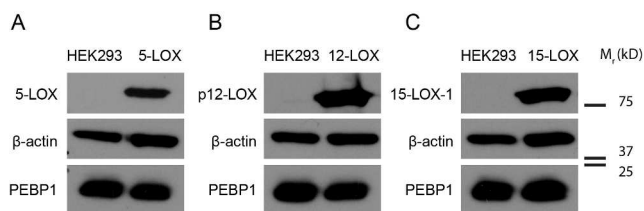


**Figure S5.** Western blots showing expression levels of 5-LOX (A), p12-LOX (B) and 15-LOX-1 (C) in Pfa1 cells compared alongside HEK293 and LOX-overexpressing cells. (D) Cell viability LD<sub>50</sub> curves for ferroptosis initiated with RSL3 (—) and erastin (—) in Pfa1 cells. (E) EC<sub>50</sub> values obtained from RSL3 induced ferroptosis in Pfa1 cells, inhibited by RTAs and LOX inhibitors. (F) Sensitization of Pfa1 cells to ferroptosis with 0.05 μM RSL3 in the presence of various concentrations of exogenously prepared cholesterol-7-OOH (blue) or oleic 10-OOH (red).

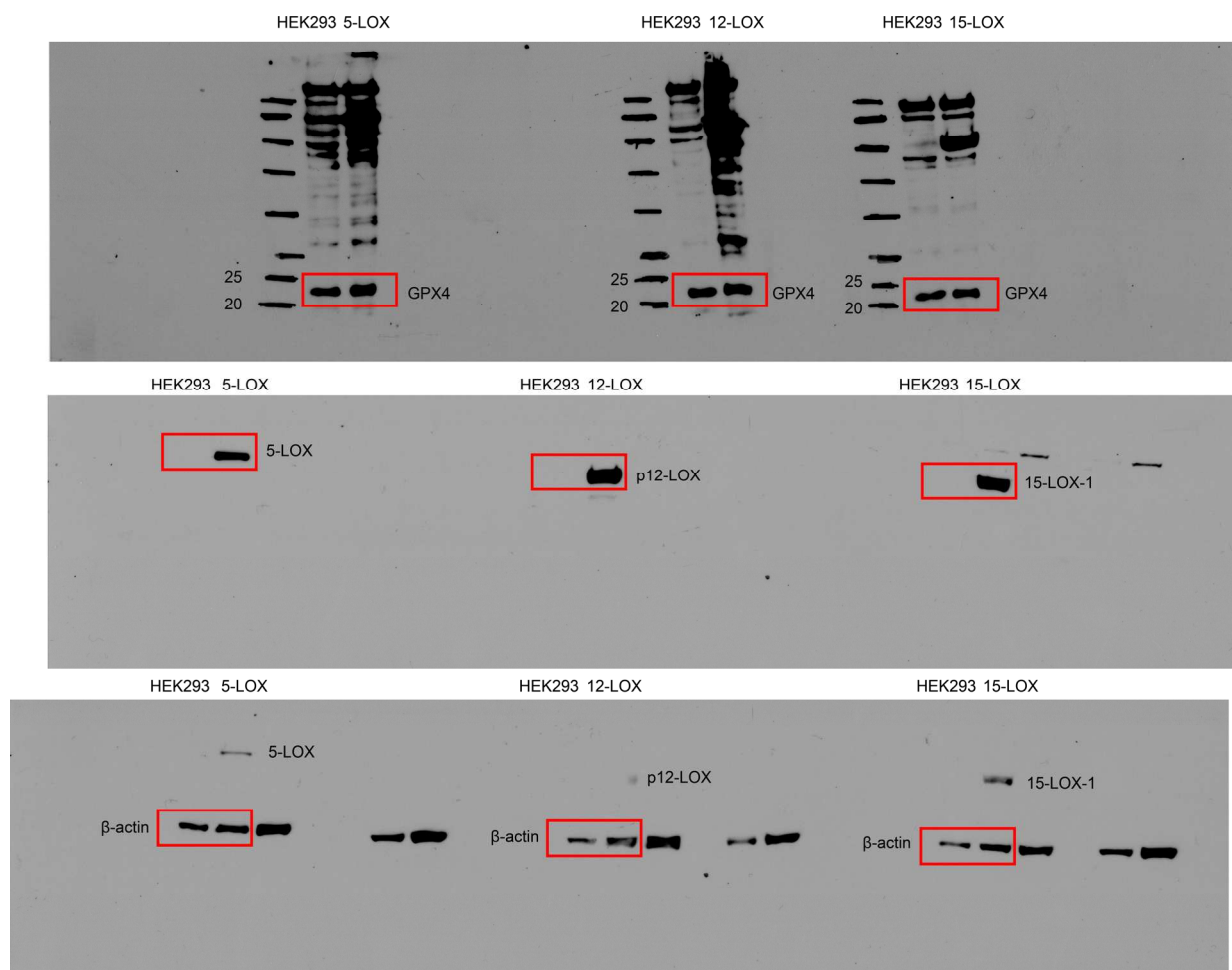


**Figure S6.** (A) Structures of commonly used LOX inhibitors, CDC and Baicalein. (B) Co-oxidations of cumene (3.6 M) and STY-BODIPY (10 μM) initiated by AIBN (6 mM) in chlorobenzene at 37°C (black) and inhibited by 2 μM of CDC (red, ---) and Baicalein (blue, ●●) (C) Co-oxidations of egg phosphatidylcholine liposomes (1.0 mM) and STY-BODIPY (10 μM) suspended in phosphate-buffered saline (10 mM) at pH 7.4 initiated by MeOAMVN (0.2 mM) at 37°C (black) and inhibited by 2 μM of CDC (red, □□□) and Baicalein (blue, ●●

•). (D) Rate constants ( $k_{inh}$ ) and reaction stoichiometry ( $n$ ) determined for CDC and Baicalein in cumene and liposome autoxidations compared along with  $\alpha$ -TOH and PMC.



**Figure S7.** Western blots showing expression levels of PEBP1 in 5-LOX (A), 12-LOX (B) and 15-LOX (C) overexpressing cells compared alongside HEK293 cells.



**Figure S8.** Uncropped blots.

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