Online Supplementary Data

Induction of ferroptosis-like cell death of eosinophils exerts synergistic effects with glucocorticoids in allergic airway inflammation

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Supplementary materials and methods

Reagents

Erastin, Ras-selective lethal small molecule 3 (RSL3), staurosporine, Z-VAD-FMK, spautin-1 and necrostatin-1 were purchased from Selleckchem (Houston, TX, USA). Artesunate (ART) was obtained from J&K (Beijing, China). Dexamethasone (DXMS) was obtained from the Second Affiliated Hospital of Zhejiang University (Hangzhou, China). Hydrogen peroxide (H_2O_2) solution was obtained from Aladdin (Shanghai, China). Deferoxamine (DFO), ferrostatin-1 (fer-1), liproxstatin-1 (lip-1), ferrous sulfate and MitoTEMPO were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ciclopirox olamine (CPX) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). N-acetylcysteine (NAC) and glutathione (GSH) were obtained from Beyotime (Shanghai, China). Recombinant human and mouse interleukin (IL)5 were obtained from PeproTech (Rocky Hill, NJ, USA). Antibody specific to glutathione peroxidase 4 (GPX4) was purchased from Abcam (Cambridge, MA, USA). Antibody specific to actin beta (ACTB) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit and anti-mouse secondary antibodies were from EarthOx (Millbrae, CA, USA). Primers for *Il4*, *Il13*, *Il25* and *Actb* were synthesized by Sangon Biotech (Shanghai, China).

Cell line and media

Mouse embryonic fibroblasts (MEFs) were obtained from ATCC (Manassas, VA, USA) and grown in DMEM with 10% foetal bovine serum (FBS). MEFs were cultured in culture incubators at 37°C with 5% CO_2, supplemented with penicillin and streptomycin.

Peripheral blood collection from patients

We recruited nine hypereosinophilic patients (three non-asthmatic and six asthmatic patients) from the clinical population at the Department of Respiratory and Critical Care Medicine of the Second Affiliated Hospital of Zhejiang University School of Medicine. Asthmatic patients were included if they met the criteria for asthma according to the Global Initiative for Asthma guidelines. All human subjects were from the Chinese Han population. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine, and all patients provided written informed consent and understood that their samples would be used for research.

Leukocytes were obtained by lysis of erythrocytes from the peripheral blood of all the enrolled patients. Peripheral eosinophils were purified from four of the six asthmatic patients using negative immunomagnetic bead selection as previously described.[1] The purity of peripheral eosinophils was regularly greater than 98%. Purified eosinophils and leukocytes were then seeded in 24-well plates with RPMI 1640, 15% FBS and 50 ng/mL human IL5.

Experimental animals

Male C57BL/6 mice (wild-type, 6 to 8 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Mice from each litter were randomized to different groups and housed for at least one week before initiation of the experiments. *Cd3δ* promoter *Il5* transgenic (Tg) mice were a generous gift from Professor James J Lee

(Department of Biochemistry and Molecular Biology, Mayo Clinic, USA). All mice were fed standard mouse chow and provided water. The room temperature was controlled at 20-22°C. Protocols were approved by the Ethics Committee for Animal Studies at Zhejiang University, China.

Allergic animal models and treatments

Mice for allergic model were sensitized intraperitoneally with 20 μg of chicken ovalbumin (OVA) (Sigma-Aldrich) emulsified in Imject alum (2.25 mg of $Al[OH]_3$/2 mg of $Mg[OH]_2$; Thermo Fisher Scientific, Waltham, MA, USA) in a total volume of 0.2 mL on days 0 and 14. On days 24, 25 and 26, mice were challenged with an aerosol of 1.5% OVA in sterile normal saline (NS) for approximately 40 minutes by means of ultrasonic nebulization (DeVilbiss, Somerset, PA, USA). The control mice were sensitized and challenged with the equal volume of NS.

Erastin (25 mg/kg) and RSL3 (10 mg/kg) were dissolved in dimethyl sulfoxide (DMSO) and administered intraperitoneally 2 hours after each OVA challenge. The controls were received equal dosage of DMSO for erastin or RSL3 group. ART (10 or 20 mg/kg) was dissolved in NS and delivered intraperitoneally once a day for 3 days before the first challenge, and 2 hours after each challenge. DXMS (0.25 or 0.5 mg/kg) was diluted in NS and administered intraperitoneally 2 hours after each challenge. In ART/DXMS groups, mice were injected with ART (10 mg/kg) alone once a day for 3 days before the first challenge, and then co-treated with ART (10 mg/kg) and DXMS (0.25 mg/kg) 2 hours after each challenge. Control mice were received the same volume of NS for ART, DXMS or

ART/DXMS group. Twenty-four hours after the last administration, all mice were sacrificed for analysis.

Bronchoalveolar lavage and lung histology

Mice were anaesthetized with 2% pentobarbital sodium, and the left lungs were lavaged three times with 0.4 mL of ice-cold phosphate buffer saline (PBS). The total bronchoalveolar lavage (BAL) cells were counted, and the rest of the bronchoalveolar lavage fluid (BALF) was centrifuged at 400 ×g for 15 minutes at 4°C. The cell pellet was suspended in 200 μL of PBS and stained with Wright-Giemsa stain (Baso, Guangdong, China). An analysis of differentiated cell counts in the BALF was performed by counting 200 total cells. Total protein concentration in BAL samples was quantified with a Pierce BCA-200 Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

After lavage, the left lungs were collected, fixed in 4% paraformaldehyde for more than 24 hours, and embedded in paraffin. Serial sections (5 mm) were sliced for haematoxylin and eosin (H&E) and periodic acid-schiff (PAS) staining. The inflammation score was assessed on a subjective scale of 0-3 based on published guidelines.[2] PAS-stained goblet cells in airway epithelium were scored using a numerical scoring system as described previously.[3]

Primary mouse BAL eosinophil culture

To harvest BAL eosinophils, allergic mice were anaesthetized, and BAL cells were

obtained with 0.8 mL of sterile PBS three times and cultured in 24-well plates with RPMI 1640 (10% FBS) at a density of 0.25×10^6 cell/well for 24 hours.

Primary mouse eosinophil isolation

Blood was collected from *Il5* Tg mice and separated by density centrifugation using Percoll (GE Healthcare, Little Chalfont, Buckinghamshire, UK) per the manufacturer's protocol, after which eosinophils were purified via negative selection with anti-CD4, anti-B220, anti-TER-199, and anti-CD8a-linked magnetic beads (BioLegend, San Diego, CA, USA). Purity (>99%) and viability (>99%) were confirmed by flow cytometric analysis. Eosinophils were grown in RPMI 1640 medium with 10% FBS and 10 ng/mL mouse IL5.

Flow cytometric analysis

The collected mouse BAL cells or blood leukocytes from patients were centrifuged at 400 ×g for 5 minutes at 4°C and resuspended in 50 μL of PBS. BAL cells were stained with anti-CD11c (APC; BD PharMingen, San Diego, CA, USA) and anti-Siglec-F (PE; eBioscience, San Diego, CA, USA). Blood leukocytes were stained with the following fluorescent dye-conjugated human antibodies: anti-CD45 (PE/Cy7; BioLegend), anti-Siglec-8 (APC; BioLegend) and anti-CCR3 (PE; eBioscience). All the samples were protected from light for 30 minutes at 4℃. Mouse BAL eosinophils were defined as Siglec-F^+/$CD11c^-$ cells. Human peripheral eosinophils were confirmed as Siglec-8^+/$CCR3^+$ cells. Isotype controls were used to define the gates.

Annexin V (FITC; MultiSciences, Hangzhou, China) and propidium iodide (PI) (PE;

MultiSciences) or 4',6-diamidino-2-phenylindole (DAPI) (MultiSciences) were used to assess the viability of cells. Double negative cells, annexin V^-/PI^- or annexin $V^-/DAPI^-$, were distinguished as viable cells. In the results of *in vitro* studies, cell viability was reported as a percentage relative to the control group which was considered as 100% of viability. In the results of *in vivo* studies, cell viability was shown as a percentage of annexin $V^-/DAPI^-$ eosinophils to the total number of BAL eosinophils. Data were acquired with a FACSCalibur flow cytometer (Cytoflex; Beckman Coulter, Miami, FL, USA) and analysed with FlowJo software (version 10; TreeStar, Ashland, OR, USA).

Analysis of reactive oxygen species (ROS)

Eosinophils were cultured in 12-well plates at 0.5×10^6 cells/well and treated with reagents. According to the manufacturer's instructions, after washing and resuspension, H_2DCFDA (5 μM), C11-BODIPY (581/591) (10 μM) or MitoSOX (5 μM) (all from Molecular Probes, Invitrogen, Carlsbad, CA, USA) was added to analyse the level of ROS. Cells were incubated for 30 minutes at 37°C and then washed in serum-free medium three times. Data were acquired with a FACSCalibur flow cytometer.

Transmission electron microscopy

Eosinophils from *Il5* Tg mice or BAL cells from allergic mice were fixed with 2.5% glutaraldehyde for 1 hour at room temperature and then at 4°C overnight. The samples were washed with PBS three times, treated with 1% osmium tetroxide in PBS for 1.5 hours and then stained with 2% uranyl acetate. After dehydration in an ascending ethanol series,

cells were embedded in embedding medium. Sections were stained with 1% uranyl acetate and 0.4% lead citrate, and photographed using transmission electron microscopy (TEM, Tecnai G2 Spirit 120 kV) at the Centre of Cyro-Electron microscopy, Zhejiang University.

Iron assay

An iron assay (Sigma-Aldrich) was used to detect the concentration of ferrous iron. The principle was as follows: A colorimetric (593 nm) product was produced by the reaction between iron and a chromagen, proportional to the iron present. Briefly, 2×10^7 cells were rapidly homogenized in 100 μL of iron assay buffer and centrifuged at 16,000 ×g for 10 minutes at 4°C. The supernatant was added to 96-well plates, brought to a volume of 100 μL, and incubated for 30 minutes at 25°C. Then, each well was mixed with 100 μL of an iron probe and incubated for 60 minutes in the dark. The absorbance at 593 nm was measured. The results were normalized to the cell total protein content according to the manufacturer's instructions.

GSH assay

Total GSH was determined by colorimetric microplate assay kits (Beyotime). In short, the collected cells were deproteinated and disrupted by repeated freeze-thawing. After centrifugation for 10 minutes (10,000 ×g at 4°C), supernatants were added to a 96-well plate, and the protocol strictly complied with the manufacturer's instructions. The absorbance at 412 nm was measured and used to calculate the content of GSH, since 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and GSH react to generate yellow

2-nitro-5-thiobenzoic acid. The data were normalized against the total protein level of the cells.

Western blot assay

Eosinophils were treated with different compounds and lysed in SDS-PAGE sample loading buffer supplemented with a protease inhibitor cocktail. Sonicated and denatured proteins were separated on SDS-PAGE gels and then transferred onto polyvinylidene difluoride membranes (Merck Millipore, Billerica, MA, USA). The membranes were immunoblotted with the relevant antibodies and probed with secondary antibodies. The blots were then visualized using a western blot detection system (Odyssey; Li-COR Bioscience, Lincoln, NE, USA). ACTB served as an internal control.

RNA isolation and reverse transcription quantitative PCR (qPCR)

Total RNA was extracted from tissues using TRIzol (Invitrogen). Reverse transcription was performed with reverse transcription reagents (Takara, Tokyo, Japan). The expression of mouse *Il4*, *Il13* and *Il25* was measured by Real-time qPCR on a StepOnePlus PCR system (Applied Biosystems, Foster City, CA, USA). All protocols were performed strictly according to the manufacturer's instructions. Data were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to *Actb* expression. The primers shown in supplementary table 1 were used to quantify mRNA levels.

Statistics

All related data are presented as the mean ± standard error of the mean (SEM). Comparisons between two groups were calculated by two-tailed Student's t-test, and significant differences between multiple groups were evaluated by one-way ANOVA with Tukey's HSD post hoc testing using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). The test statistics have been transformed into adjusted P values following Tukey multiple comparison testing. Differences were considered statistically significant when the P value was less than 0.05.

Supplementary references

1. Cherry WB, Yoon J, Bartemes KR, et al. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. *The Journal of allergy and clinical immunology* 2008;121(6):1484-90.
2. Lee KS, Lee HK, Hayflick JS, et al. Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *FASEB J* 2006;20(3):455-65.
3. McMillan SJ, Bishop B, Townsend MJ, et al. The absence of interleukin 9 does not affect the development of allergen-induced pulmonary inflammation nor airway hyperreactivity. *The Journal of experimental medicine* 2002;195(1):51-57.

Supplementary figure legends

Supplementary figure 1 FINs triggered the cell death of human eosinophils. (A) Gating strategy used to define the human eosinophil population (Siglec-8^+/$CCR3^+$) in the peripheral blood. Siglec-8^+/$CCR3^+$ cells were then sorted for Wright-Giemsa staining. (B) Representative flow cytometry plots of cell viability (x axis: annexin V; y axis: DAPI) at 24 hours for control and FIN-treated (erastin 40 μM, RSL3 4 μM, ART 200 μM) human eosinophils in leukocytes. (C) Peripheral leukocytes from non-asthmatic patients (*n*=3) with increased eosinophils were cultured with various concentrations of FINs for 24 hours prior to viability determination. Siglec-8^+/$CCR3^+$/annexin V^-/$DAPI^-$ cells were distinguished as viable peripheral eosinophils. Data are shown as mean ± SEM, analysed by one-way ANOVA. FINs, ferroptosis-inducing agents; DAPI, 4',6-diamidino-2-phenylindole; RSL3, Ras-selective lethal small molecule 3; ART, artesunate.

Supplementary figure 2 Representative flow cytometry plots for control and FIN-treated mouse eosinophils. (A) Representative flow cytometry plots of cell viability (x axis: annexin V; y axis: PI) at 24 hours for control and FIN-treated (erastin 30 μM, RSL3 2 μM, ART 100 μM) mouse eosinophils isolated from the peripheral blood of *Il5* transgenic mice.

(B) Gating strategy used to define the mouse eosinophil population (Siglec-F^+/$CD11c^-$) in BAL cells treated with FINs for 24 hours. (C) Representative flow cytometry plots of cell viability (x axis: annexin V; y axis: DAPI) at 24 hours for control and FIN-treated (erastin 30 μM, RSL3 2 μM, ART 100 μM) mouse BAL eosinophils from allergic mice. FIN, ferroptosis-inducing agent; PI, propidium iodide; RSL3, Ras-selective lethal small molecule 3; ART, artesunate; Il, interleukin; BAL, bronchoalveolar lavage; DAPI, 4',6-diamidino-2-phenylindole.

Supplementary figure 3 Apoptosis and necroptosis pathways in eosinophils, and the effects of lipid ROS inhibitors in FIN-treated eosinophils and MEFs. Eosinophils were isolated from the peripheral blood of *Il5* transgenic mice. Annexin V^-/PI^- cells were defined as viable eosinophils. (A) Eosinophils were cultured with staurosporine (20 nM) with or without Z-VAD-FMK (100 μM) for 24 hours. (B) Eosinophils were cultured with H_2O_2 (2 mM) with or without necrostatin-1 (100 μM) for 0.5 hours. (C) Transmission electron microscopy of eosinophils treated with DMSO (12 hours), staurosporine (20 nM, 12 hours) and H_2O_2 (2 mM, 0.25 hours). (D) Lipid ROS production at indicated times (6, 12, and 24 hours) was assessed by flow cytometry using C11-BODIPY in eosinophils. (E) Effect of fer-1 (2 μM) on lipid ROS production in eosinophils treated with FINs (erastin 30 μM, RSL3 2 μM, ART 100 μM) for 24 hours. (F) Effects of fer-1 (2 μM) and lip-1 (2 μM) on the cell viability of MEFs treated with FINs (erastin 2 μM, RSL3 1 μM, ART 100 μM) for 24 hours. (G) Effect of fer-1 on lipid ROS production in MEFs treated with FINs for 12 hours. All data are shown as mean ± SEM, analysed by one-way ANOVA. FIN, ferroptosis-inducing agent; MEFs, mouse embryonic

fibroblasts; Il, interleukin; PI, propidium iodide; H_2O_2, hydrogen peroxide; DMSO, dimethylsulfoxide; ROS, reactive oxygen species; fer-1, ferrostatin-1; RSL3, Ras-selective lethal small molecule 3; ART, artesunate; lip-1, liproxstatin-1.

Supplementary figure 4 Ferroptosis induction by FINs in eosinophils did not proceed through mitochondrial ROS. (A and B) Eosinophils isolated from the peripheral blood of *Il5* transgenic mice were cultured with FINs (erastin 30 μM, RSL3 2 μM, ART 100 μM). Mitochondrial ROS production at indicated times (6, 12, and 24 hours) was assessed by flow cytometry using MitoSOX. Representative histograms are shown in (A), and cumulative data expressed relative to the control are represented in (B). (C) Effect of MitoTEMPO (20 μM) on the lethality of ART (100 μM) treatment for 24 hours in eosinophils. Annexin V^-/PI^- cells were defined as viable cells. All data are shown as mean ± SEM, analysed by one-way ANOVA. FINs, ferroptosis-inducing agents; ROS, reactive oxygen species; Il, interleukin; RSL3, Ras-selective lethal small molecule 3; ART, artesunate; PI, propidium iodide.

Supplementary figure 5 FINs attenuated mucus hyperproduction induced by OVA, but had no effect on mouse weight and BAL protein. C57BL/6 mouse administrated with FINs (erastin 25 mg/kg, RSL3 10 mg/kg, ART 20 mg/kg) or vehicle control. (A) Experimental scheme for the FIN treatment *in vivo*. (B and C) Representative images (B) and the semi-quantified scorings (C) of PAS staining in mouse lung sections by FIN treatment. (D and E) Mouse weight (D) and total BALF protein (E) were determined. (F) Gating strategy used to define the mouse eosinophil population (Siglec-F^+/$CD11c^-$) in BAL cells from control

and allergic mice. Data are shown as the mean ± SEM of six to eight mice per group, analysed by one-way ANOVA (C) or Student's t-test (D, E). FINs, ferroptosis-inducing agents; OVA, ovalbumin; BAL, bronchoalveolar lavage; PAS, periodic acid-schiff; BALF, bronchoalveolar lavage fluid.

Supplementary table 1. Primer Sets for Real-time PCR Analysis

Genes (mouse)	Forward (5′–3′)	Reverse (5′–3′)
Il4	CCCCAGCTAGTTGTCATCCTG	CAAGTGATTTTTGTCGCATCCG
Il13	CAGCCTCCCCGATACCAAAAT	GCGAAACAGTTGCTTTGTGTAG
Il25	TATGAGTTGGACAGGGACTTGA	TGGTAAAGTGGGACGGAGTTG
Actb	AGAGGGAAATCGTGCGRGAC	CAATAGTGACCTGGCCGT

Supplementary table 2. Mean differences, 95% confidence intervals (CI) of difference and adjusted P values for compared groups

Figures	**Compared groups**	**Mean difference**	**95% CI of difference**	**Adjusted P Value**
Figure 1A	Erastin (0 vs 60 μM)	24.13	3.085 to 45.18	0.0181
	Erastin (0 vs 80 μM)	25.54	4.493 to 46.58	0.0112
	Erastin (0 vs 100 μM)	37.74	16.70 to 58.79	0.0001
	RSL3 (0 vs 4 μM)	49.46	14.81 to 84.11	0.004
	RSL3 (0 vs 6 μM)	70.6	35.94 to 105.3	0.0001
	RSL3 (0 vs 8 μM)	81.44	46.78 to 116.1	<0.0001
	ART (0 vs 100 μM)	20.48	0.3519 to 40.60	0.0447
	ART (0 vs 200 μM)	55.62	35.50 to 75.74	<0.0001

	ART (0 vs 400 μM)	79.7	59.57 to 99.82	<0.0001
Figure 1B	Erastin (0 vs 100 μM)	31.59	18.73 to 44.45	0.001
	RSL3 (0 vs 6 μM)	76.8	69.60 to 83.99	<0.0001
	ART (0 vs 200 μM)	40.12	17.31 to 62.93	0.0051
Figure 1C	Erastin (0 vs 20 μM)	40.12	35.71 to 44.53	<0.0001
	Erastin (0 vs 30 μM)	72.4	67.99 to 76.81	<0.0001
	Erastin (0 vs 40 μM)	92.41	88.00 to 96.82	<0.0001
	RSL3 (0 vs 2 μM)	62.39	47.09 to 77.69	<0.0001
	RSL3 (0 vs 4 μM)	80.52	65.23 to 95.82	<0.0001
	RSL3 (0 vs 6 μM)	98.3	83.00 to 113.6	<0.0001
	ART (0 vs 50 μM)	40.1	35.48 to 44.72	<0.0001
	ART (0 vs 100 μM)	63.95	59.33 to 68.56	<0.0001
	ART (0 vs 200 μM)	92.84	88.22 to 97.45	<0.0001
Figure 1D	Erastin (0 vs 12 hr)	14.92	13.43 to 16.42	<0.0001
	Erastin (0 vs 24 hr)	59.7	58.20 to 61.19	<0.0001
	RSL3 (0 vs 12 hr)	25.84	3.060 to 48.61	0.0274
	RSL3 (0 vs 24 hr)	74.85	52.07 to 97.63	<0.0001
	ART (0 vs 12 hr)	5.637	1.113 to 10.16	0.0169
	ART (0 vs 24 hr)	69.82	65.30 to 74.35	<0.0001
Figure 1E	Erastin (0 vs 20 μM)	30.73	23.82 to 37.64	<0.0001
	Erastin (0 vs 30 μM)	30.3	23.39 to 37.21	<0.0001
	Erastin (0 vs 40 μM)	39.03	32.13 to 45.94	<0.0001
	RSL3 (0 vs 2 μM)	50.08	42.54 to 57.63	<0.0001
	RSL3 (0 vs 4 μM)	83.27	75.73 to 90.81	<0.0001
	RSL3 (0 vs 6 μM)	88.48	80.94 to 96.02	<0.0001
	ART (0 vs 50 μM)	33.72	26.86 to 40.57	<0.0001
	ART (0 vs 100 μM)	48.16	41.31 to 55.01	<0.0001
	ART (0 vs 200 μM)	63.5	56.65 to 70.35	<0.0001
Figure 2C	ART (DMSO vs Spautin-1)	-21.05	-29.75 to -12.35	0.0003
Figure 2G	Erastin (CTL vs DFO)	-18.55	-19.91 to -17.18	<0.0001
	RSL3 (CTL vs DFO)	-40.72	-47.54 to -33.90	<0.0001
	ART (CTL vs DFO)	-55.36	-56.79 to -53.94	<0.0001
Figure 2H	Erastin (CTL vs CPX)	-17.93	-34.61 to -1.250	0.0357
	RSL3 (CTL vs CPX)	-39.75	-51.89 to -27.62	<0.0001
	ART (CTL vs CPX)	-48.82	-66.89 to -30.75	0.0001
Figure 2I	Erastin (CTL vs Fe(II))	27.08	20.04 to 34.13	<0.0001
	RSL3 (CTL vs Fe(II))	19.84	0.8208 to 38.85	0.0412

	ART (CTL vs Fe(II))	28.39	18.51 to 38.27	<0.0001
Figure 3B	Erastin (0 vs 24 hr)	-6.242	-7.242 to -5.243	<0.0001
	RSL3 (0 vs 24 hr)	-0.8781	-1.071 to -0.6849	<0.0001
	ART (0 vs 24 hr)	-2.992	-3.910 to -2.074	<0.0001
Figure 3C	Erastin (CTL vs NAC)	-23.77	-40.46 to -7.081	0.008
	RSL3 (CTL vs NAC)	-66.26	-74.52 to -58.01	<0.0001
	ART (CTL vs NAC)	-35.2	-42.46 to -27.94	<0.0001
Figure 3D	Erastin (CTL vs GSH)	-32.73	-45.41 to -20.06	0.0002
	RSL3 (CTL vs GSH)	-59.58	-67.01 to -52.16	<0.0001
	ART (CTL vs GSH)	-27.97	-45.20 to -10.75	0.0036
Figure 3E	Erastin (CTL vs NAC)	3.944	3.079 to 4.809	<0.0001
	Erastin (CTL vs GSH)	3.357	2.491 to 4.224	<0.0001
	RSL3 (CTL vs NAC)	1.249	0.9535 to 1.544	<0.0001
	RSL3 (CTL vs GSH)	1.066	0.7124 to 1.420	<0.0001
	ART (CTL vs NAC)	1.841	0.7965 to 2.886	0.0022
	ART (CTL vs GSH)	1.622	0.5518 to 2.692	0.0055
Figure 4A	Erastin (0 vs 12 hr)	1.068	0.8100 to 1.325	<0.0001
Figure 4C	ART (0 vs 6 hr)	0.1557	0.02634 to 0.2851	0.0179
	ART (0 vs 12 hr)	0.2648	0.1355 to 0.3942	0.0002
Figure 4D	Erastin vs Erastin+RSL3	42.86	29.72 to 56.01	<0.0001
	Erastin vs Erastin+ART	43.44	30.29 to 56.58	<0.0001
	RSL3 vs Erastin+RSL3	27.35	14.21 to 40.50	<0.0001
	RSL3 vs RSL3+ART	25.21	12.06 to 38.35	0.0002
	ART vs Erastin+ART	44.03	30.88 to 57.17	<0.0001
	ART vs RSL3+ART	41.31	28.16 to 54.45	<0.0001
Figure 5A	DMSO (CTL vs OVA)	-26.63	-35.71 to -17.54	<0.0001
	OVA (DMSO vs Erastin)	11	2.589 to 19.41	0.0047
	OVA (DMSO vs RSL3)	16.13	7.040 to 25.21	<0.0001
	NS (CTL vs OVA)	-24.33	-33.18 to -15.48	<0.0001
	OVA (NS vs ART)	14.83	5.983 to 23.68	0.0007
Figure 5B	DMSO (CTL vs OVA)	-49.88	-68.09 to -31.67	<0.0001
	OVA (DMSO vs Erastin)	23.87	7.013 to 40.73	0.0019
	OVA (DMSO vs RSL3)	32.61	14.40 to 50.82	<0.0001
	NS (CTL vs OVA)	-38.36	-52.96 to -23.77	<0.0001
	OVA (NS vs ART)	22.85	8.257 to 37.44	0.0015
Figure 5C	DMSO (CTL vs OVA)	-16.85	-25.22 to -8.478	<0.0001
	OVA (DMSO vs Erastin)	10.92	3.168 to 18.67	0.002

	OVA (DMSO vs RSL3)	14.01	5.643 to 22.38	0.0002
	NS (CTL vs OVA)	-11.77	-16.69 to -6.862	<0.0001
	OVA (NS vs ART)	8.542	3.630 to 13.45	0.0005
Figure 5E	DMSO (CTL vs OVA)	-2.625	-3.378 to -1.872	<0.0001
	OVA (DMSO vs Erastin)	1.25	0.5531 to 1.947	<0.0001
	OVA (DMSO vs RSL3)	1.125	0.3723 to 1.878	0.001
	NS (CTL vs OVA)	-2.833	-3.571 to -2.096	<0.0001
	OVA (NS vs ART)	1.167	0.4291 to 1.904	0.0014
Figure 5F (*Il13*)	DMSO (CTL vs OVA)	-2.691	-4.011 to -1.371	<0.0001
	OVA (DMSO vs Erastin)	2.114	0.8921 to 3.336	0.0001
	OVA (DMSO vs RSL3)	2.398	1.078 to 3.718	<0.0001
	NS (CTL vs OVA)	-1.387	-2.316 to -0.4572	0.0024
	OVA (NS vs ART)	1.423	0.4931 to 2.352	0.0019
Figure 5F (*Il25*)	DMSO (CTL vs OVA)	-2.406	-4.033 to -0.7781	0.0011
	OVA (DMSO vs Erastin)	2.277	0.7702 to 3.784	0.0008
	OVA (DMSO vs RSL3)	2.632	1.005 to 4.260	0.0003
	NS (CTL vs OVA)	-3.514	-6.014 to -1.013	0.0042
	OVA (NS vs ART)	3.614	1.113 to 6.115	0.0033
Figure 5G	OVA (DMSO vs Erastin)	19.11	11.67 to 26.55	<0.0001
	OVA (DMSO vs RSL3)	22.59	11.51 to 33.67	0.0008
	OVA (NS vs ART)	10.36	5.833 to 14.88	0.0005
Figure 5H	OVA (DMSO vs Erastin)	11.13	3.742 to 18.51	0.006
	OVA (DMSO vs RSL3)	13.27	4.951 to 21.6	0.0046
	OVA (NS vs ART)	7.22	2.996 to 11.45	0.0034
Figure 6A	DXMS (0 vs 1.2 mM)	92.1	87.42 to 96.78	<0.0001
	DXMS (0 vs 1.6 mM)	98.86	94.19 to 103.5	<0.0001
Figure 6B	DXMS (CTL vs Z-VAD-FMK)	-27.43	-35.47 to -19.38	<0.0001
Figure 6D	DXMS vs Erastin+DXMS	81.24	76.69 to 85.79	<0.0001
	Erastin vs Erastin+DXMS	80.27	75.72 to 84.82	<0.0001
Figure 6E	DXMS vs RSL3+DXMS	94.41	82.21 to 106.6	<0.0001
	RSL3 vs RSL3+DXMS	71.48	59.28 to 83.67	<0.0001
Figure 6F	DXMS vs ART+DXMS	78.86	73.60 to 84.12	<0.0001
	ART vs ART+DXMS	50.92	45.66 to 56.18	<0.0001
Figure 6G	NS (CTL vs OVA)	-74.45	-87.45 to -61.45	<0.0001
	OVA (NS vs ART 10+DXMS 0.25)	54.36	41.12 to 67.59	<0.0001
	OVA (NS vs DXMS 0.5)	57.79	40.7 to 74.87	<0.0001
	OVA (ART 10 vs ART 10+DXMS	22.4	8.631 to 36.18	0.0003

	0.25)			
	OVA (DXMS 0.25 vs ART 10+DXMS 0.25)	16.64	3.41 to 29.88	0.0073
Figure 6H	NS (CTL vs OVA)	-70.66	-88.03 to -53.30	<0.0001
	OVA (NS vs ART 10+DXMS 0.25)	53.72	36.04 to 71.4	<0.0001
	OVA (NS vs DXMS 0.5)	42.44	19.62 to 65.27	<0.0001
	OVA (ART 10 vs ART 10+DXMS 0.25)	33.65	15.25 to 52.05	<0.0001
	OVA (DXMS 0.25 vs ART 10+DXMS 0.25)	17.6	-0.08248 to 35.27	0.0516
Figure 6I	NS (CTL vs OVA)	-60.17	-72.23 to -48.10	<0.0001
	OVA (NS vs ART 10+DXMS 0.25)	54.67	42.39 to 66.96	<0.0001
	OVA (NS vs DXMS 0.5)	52.53	36.67 to 68.38	<0.0001
	OVA (ART 10 vs ART 10+DXMS 0.25)	19.95	7.165 to 32.73	0.0005
	OVA (DXMS 0.25 vs ART 10+DXMS 0.25)	10.38	-1.898 to 22.66	0.1338
Figure 6J (*Il4*)	NS (CTL vs OVA)	-1.814	-2.837 to -0.7914	0.0001
	OVA (NS vs ART 10+DXMS 0.25)	1.876	0.8346 to 2.917	<0.0001
	OVA (NS vs DXMS 0.5)	2.203	0.8589 to 3.547	0.0003
	OVA (ART 10 vs ART 10+DXMS 0.25)	0.9433	-0.1402 to 2.027	0.1149
	OVA (DXMS 0.25 vs ART 10+DXMS 0.25)	1.191	0.1501 to 2.232	0.0177
Figure 6J (*Il13*)	NS (CTL vs OVA)	-3.084	-4.865 to -1.303	0.0001
	OVA (NS vs ART 10+DXMS 0.25)	2.853	1.04 to 4.667	0.0005
	OVA (NS vs DXMS 0.5)	3.096	0.7547 to 5.437	0.0043
	OVA (ART 10 vs ART 10+DXMS 0.25)	1.78	-0.1072 to 3.667	0.073
	OVA (DXMS 0.25 vs ART 10+DXMS 0.25)	1.767	-0.04583 to 3.581	0.0593
Supplementary figure 1C	RSL3 (0 vs 6 μM)	90.98	43.66 to 138.3	0.0006
	RSL3 (0 vs 8 μM)	93.79	46.47 to 141.1	0.0005
	ART (0 vs 200 μM)	60.85	2.641 to 119.1	0.0404
	ART (0 vs 400 μM)	63.64	5.434 to 121.8	0.0322
Supplementary figure 3A	Staurosporine (CTL vs Z-VAD-FMK)	-34.17	-42.09 to -26.25	<0.0001
Supplementary	H_2O_2 (CTL vs Necrostatin-1)	-42.45	-49.63 to -35.27	<0.0001

figure 3B				
Supplementary figure 3E	Erastin (CTL vs Fer-1)	1.851	0.4915 to 3.211	0.0104
	RSL3 (CTL vs Fer-1)	2.489	0.3137 to 4.665	0.0263
	ART (CTL vs Fer-1)	3.63	1.618 to 5.641	0.0019
Supplementary figure 3F	Erastin (CTL vs Fer-1)	-64.56	-78.85 to -50.28	<0.0001
	Erastin (CTL vs Lip-1)	-66.11	-80.39 to -51.83	<0.0001
	RSL3 (CTL vs Fer-1)	-72.59	-91.76 to -53.41	<0.0001
	RSL3 (CTL vs Lip-1)	-74.65	-93.83 to -55.48	<0.0001
	ART (CTL vs Fer-1)	-54.11	-64.31 to -43.90	<0.0001
	ART (CTL vs Lip-1)	-48.44	-58.64 to -38.24	<0.0001
Supplementary figure 3G	Erastin (CTL vs Fer-1)	2.076	1.417 to 2.736	<0.0001
	RSL3 (CTL vs Fer-1)	1.884	1.584 to 2.184	<0.0001
	ART (CTL vs Fer-1)	2.656	2.198 to 3.115	<0.0001
Supplementary figure 4B	ART (0 vs 24 hr)	-1.761	-1.942 to -1.581	<0.0001
Supplementary figure 5C	DMSO (CTL vs OVA)	-2.75	-3.447 to -2.053	<0.0001
	OVA (DMSO vs Erastin)	1.375	0.7301 to 2.020	<0.0001
	OVA (DMSO vs RSL3)	1.917	1.220 to 2.613	<0.0001
	NS (CTL vs OVA)	-2.333	-3.523 to -1.144	0.0001
	OVA (NS vs ART)	1.833	0.6440 to 3.023	0.0018