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Supporting Information

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Supporting Information

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Content

Experimental Section

Materials and Instruments

All solvents (H_20, DMSO) were of the highest grade commercially available (≥99.9% HPLC grade, Sigma-Aldrich) and used as received. γ-Terpinene (γ-T; >97%, Sigma-Aldrich), was percolated on alumina and silica before each experiment to remove impurities and traces of stabilizer. Chemicals were purchased from Sigma-Aldrich and TCI, and were used without any additional purification: 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH; 97%, Sigma-Aldrich), Triton X-100 (*t*-octylphenoxypolyethoxyethanol, for molecular biology, Sigma-Aldrich), methyl linoleate (MeLH; 98%, TCI), L-α-phosphatidylcholine (from egg yolk, Type XI-E, 100 mg/mL in chloroform, ≥99%, solution, Sigma-Aldrich), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; 97%, Sigma-Aldrich), α-tocopherol (95.5%, Sigma-Aldrich), 2,2,5,7,8-pentamethyl-6-chromanol (PMC; 97%, Sigma-Aldrich).

0.1 M buffer solution at pH 7.4 was prepared in a 50-mL flask using 101 mg Na_2HPO_4, 17 mg NaH_2PO_4 and 438 mg NaCl, bringing to volume with MilliQ water.

0.1 M buffer solution at pH 4.5 was prepared in a 200 mL volumetric flask by weighing 3.692 g of CH_3COONa and adding 0.628 ml of neat CH_3COOH, bringing to volume with MilliQ water.

The STY-BODIPY was synthesized according to literature procedure.[1]

UV–visible measurements were performed using a Cary 1E (Varian) spectrometer coupled to Cary Temperature Control (Varian). Dynamic light scattering (DLS) measurements were conducted using a Zetasizer Nano ZS90 (Malvern Instruments). The concentration of O_2 was measured using an optical oxygen meter FireStingO_2 (PyroScience GmbH).

Autoxidation kinetics in micelles.

Micellar suspension of MeLH preparation. In a scintillation vial, 22 µL of methyl linoleate (MeLH) and 10 mL of 17.6 mM Triton X-100, were mixed by a vortex stirrer for 3 min. Next, 10 mL of phosphate buffer solution 0.1 M (pH 4.5 or pH 7.4) was added, and the mixture was stirred again for 3 min. The final concentration of MeLH and Triton X-100 in the micellar system was 3.3 mM for lipid and 8.8 mM for surfactant.[2]

Inhibited Co-autoxidation of MeLH in micellar system. A 3 mL quartz cuvette was loaded with 1.8 mL of micellar suspension of MeLH, 10 μL of 2 mM STY-BODIPY (λ=564 nm, ε=94000 $M^{-1}cm^{-1}$) in DMSO and 200 μL of 100 mM AAPH in phosphate buffer solution. The solution was thoroughly mixed. The cuvette was placed into the thermostatted sample holder of a UV−vis spectrophotometer and allowed to equilibrate to 37 °C. The absorbance at 564 nm was monitored for 10−20 min to ensure that the reaction was proceeding at a constant rate, after which a small aliquot (2 μL, 4 μL or 10 μL) of a 1 mM in DMSO solution of the test antioxidant was added.

Autoxidation of Egg-Yolk phosphatidylcholine liposomes

Egg-Yolk PC Liposomes Preparation. 150 μL of a solution 100 mg/mL in chloroform of egg yolk phosphatidylcholine was added in a round-bottom flask and the solvent was then evaporated under argon and the lipids were vacuum-dried 1h to yield a thin film. The lipid film was hydrated with 1 mL of a 10 mM phosphate buffered-saline (PBS) solution containing 150 mM NaCl (pH 7.4), yielding a 20 mM lipid suspension. The lipid suspension was then extruded 21 times using a mini extruder equipped with a polycarbonate membrane with 100 nm pores.[2] The diameter was measured by Dynamic Light Scattering (DLS) at 37°C.

Inhibited Autoxidation of Egg-Yolk PC Liposomes. To a 1 mL graduated flask were added 500 μL of liposome suspension 20 mM in PBS, 30 μL of 100 mM AAPH in PBS solution and made up to the mark with PBS solution and mixed. Next, 600-1000 μL of the mixture was transferred to a vial and used to measure O_2 consumption during autoxidation using a Fire Sting O_2 optical oxygen meter (PyroScience GmbH) at 37 °C. The rate of O_2 consumption was monitored for 10-15 minutes until it was ensured that the reaction was proceeding at a constant rate, after which 10 μL of a 600 μM solution in DMSO of the test antioxidant was added.

Inhibited Autoxidation of Egg-Yolk PC Liposomes studied by the confocal microscope

The samples consisted in 1 mM PCL in PBS pH 7.4, with AAPH 3 mM and STY-BODIPY 1 μM. A control without AAPH was also prepared. The antioxidants were added at 5 μM concentration. The liposome suspensions were incubated at 37 °C for 1 h, then put into a IDIBI chamber and analysed with a confocal microscope. The experiments were performed in duplicate. The peroxidation extent of PCL was assessed by measuring the ratio between the red and green emission intensity of the reduced and the oxidized forms of STY-BODIPY, respectively.

Autoxidation of 1,4-cyclohexadiene in acetonitrile

Autoxidation experiments were performed by measuring the oxygen consumption in a two-channel gas uptake apparatus, immersed in a thermostatted bath, based on Validyne DP15 pressure transducer developed in our laboratory.[3]

All the autoxidation experiments were initiated by the thermal decomposition of AIBN at 30 °C, in acetonitrile (MeCN). In a typical experiment, an air-saturated solution of the oxidizable substrate 1,4-cyclohexadiene (0.26 M) containing AIBN (0.025 M) in MeCN is equilibrated at 30 ◦C with an identical reference solution containing an excess of 2,2,5,7,8-pentamethyl-6-hydroxychromane (125 mM) so to block any radical chain in the reference during the experiment. After reaching a constant O_2 consumption, a stock solution of base in MeCN is injected in the sample flask. Final concentration 0.1 mM.

Cell line and culture conditions.

Human neuroblastoma cells SH-SY5Y (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM (EuroClone, Pero, Italy) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (MilliporeSigma, Burlington, MA, USA). Cells were grown in a humidified incubator with 95% air and 5% CO_2 at 37°C.

Cell viability assay

Cell viability was assessed by resazurin-based assay following as previously reported.[4] Briefly, cells were seeded in 96-well plates at 2×10^4 cells/well density (Optiplate, Perkin Elmer). After 24 hours to allow adhesion, cells were treated with different concentrations of γ-terpinene (γ-T), 1,4-cyclohexadiene (CHD), 50 μM deferoxamine (DFO) or 0.4 μM ferrostatin-1 (Fer-1) for 1 hour dissolved in complete medium. After this time, cells were washed with Hanks balanced buffer solution (HBSS) and exposed to 400 nM of RSL3 dissolved in complete medium for 18 hours. Then, cells were washed again with HBSS and incubated with resazurin (4 mg/ml, 100 μL per well) dissolved in complete medium for 3 hours. The fluorescence emission was recorded using a plate reader (EnSpire, Perkin Elmer) at λ_{exc} 560 nm, λ_{em} 590nm.[4]

Lipid peroxidation assay

The determination of lipid peroxidation induced by RSL3 treatment in SH-SY5Y cells was performed using the lipid peroxidation sensor dye BODIPY® 581/591 C11 (ThermoFisher Scientific) according to the method described by Pap et al.[5]

Cells were seeded in black 96-well plates (Optiplate, PerkinElmer) at a density of 1 x 10^4 cells/well and incubated overnight to allow adhesion. The cells were then pre-treated with 400 nM γ-T, 400 nM 1,4-CHD or 400 nM Ferrostatin-1. The cells were then washed with HBSS, stained with 1 µM BODIPY® 581/591 C11 and exposed to 400 nM RSL3 dissolved in complete medium for 3 or 24 hours. BODIPY® 581/591 C11 fluorescence was measured using a multiplate reader (EnSpire, PerkinElmer). Alternatively, cells were seeded onto an 8-well chambered coverslip (µ-Slide 8 Well, (Ibidi, Germany) according to the manufacturer's instructions. After 24 hours to allow adhesion, cells were pre-treated with 50 nM γ-T for 1 hour. The cells were then washed with HBSS, stained with 1 µM BODIPY® 581/591 C11 and exposed to 400 nM RSL3 dissolved in complete medium for 6 hours. After this time, the cells were washed with HBSS and images were captured using a Nikon C1si confocal microscope (Nikon, Tokyo, Japan). Green and red fluorescence intensities were quantified using standard ImageJ software tools.

Expression analysis

Quantitative real-time RT–qPCR

Total RNA was extracted from SH-SY5Y cells using Monarch Total RNA Miniprep Kit (NEB) following the manufacturer's instructions. For gene expression of human Gpx4, GSR and Nrf2, (500 ng) was used for retrotranscription using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Thermo Fisher) following the manufacturer's instructions. RT–qPCR was performed with PowerTrack SYBR Green Master Mix (Thermo Fisher) and 0.5 µmol/L primers in an ABI 7500 real-time PCR system (Thermo Fisher). Primers for gene expression were designed with Primer3 (https://primer3.ut.ee/). The primers used are:

hGPX4qpcr_Fw 5’-ACCGAAGTAAACTACACTCAGC-3’,

hGPX4qpcr_Rv 5’-CGGCGAACTCTTTGATCTCTTC-3’,

hGSR Fw 5’-GACCTATTCAACGAGCTTTAC-3’,

hGSR Rv 5’-CAACCACCTTTTCTTCCTTG-3’,

hNrf2 Fw 5’-CGTTTGTAGATGACAATGAGG-3’,

hNrf2 Rv 5’-AGAAGTTTCAGGTGACTGAG-3’,

hACTB Fw 5’-CCTGGCACCCAGCACAAT-3’,

hACTB Rv 5’-GGGCCGGACTCGTCATACT-3’,

All target genes were normalized to the corresponding endogenous control (human beta-actin, ACTB) using the ΔΔCt comparative method.

Analysis of autoxidation rates by the co-oxidation model

The co-oxidation model is a system in which two different oxidizable substrates RH and EH, simultaneously undergo autoxidation following the equations 1-10, where **In** is the initiator producing R$^{\bullet}$ radicals at a rate R_i. In our case, EH = γ-terpinene and the radical EOO$^{\bullet}$ is represented by HOO$^{\bullet}$ or $O_2^{\bullet-}$, depending on the pH. A thorough explanation of co-oxidation kinetics is available in literature.[8]

$$\mathrm{In} \rightarrow \mathrm{R}^{\bullet} \quad (1)$$

$$\mathrm{R}^{\bullet} + \mathrm{O_2} \rightarrow \mathrm{ROO}^{\bullet} \quad (2)$$

$$\mathrm{E}^{\bullet} + \mathrm{O_2} \rightarrow \mathrm{EOO}^{\bullet} \quad (3)$$

$$\mathrm{ROO}^{\bullet} + \mathrm{RH} \rightarrow \mathrm{ROOH} + \mathrm{R}^{\bullet} \quad (4)$$

$$\mathrm{ROO}^{\bullet} + \mathrm{EH} \rightarrow \mathrm{ROOH} + \mathrm{E}^{\bullet} \quad (5)$$

$$\mathrm{EOO}^{\bullet} + \mathrm{EH} \rightarrow \mathrm{EOOH} + \mathrm{E}^{\bullet} \quad (6)$$

$$\mathrm{EOO}^{\bullet} + \mathrm{RH} \rightarrow \mathrm{EOOH} + \mathrm{R}^{\bullet} \quad (7)$$

$$2\ \mathrm{ROO}^{\bullet} \rightarrow \text{non radical products} \quad (8)$$

$$\mathrm{ROO}^{\bullet} + \mathrm{EOO}^{\bullet} \rightarrow \text{non radical products} \quad (9)$$

$$2\ \mathrm{EOO}^{\bullet} \rightarrow \text{non radical products} \quad (10)$$

At the steady state, the O_2 consumption rate (R_{ox}) is given by equation 11.

$$R_{ox} = \frac{\left\{k_4 k_7 [RH]^2 + 2k_5 k_7 [RH][EH] + k_5 k_6 [EH]^2\right\} R_i^{1/2}}{\left\{k_8 k_7^2 [BH]^2 + k_9 k_5 k_7 [RH][EH] + k_{10} k_5^2 [EH]^2\right\}^{1/2}} \quad (11)$$

In the assumption that the consumption of the STY-BODIPY probe is proportional to O_2 consumption, equation 11 can be used to model the trend of STY-BODIPY consumption rates in the system MeLH / γ-T in TritonX-100 micelles initiated by AAPH.

Equation 11 predicts that EH behaves as antioxidant at low concentrations if $k_5 > k_4$, $k_9 > k_8$ and $k_{10} > k_8$. The reactivity of EOO$^{\bullet}$ with RH and EH determines the shape of the R_{ox}/[EH] plot.

Keeping in mind that the trend lines reported in Figures 1B and 1C in the main article are intended mainly for qualitative purposes, they were obtained by relating the consumption of STY-BODIPY to

R_{ox} by equation 12, by using the rate constants collected in Table S1 and with R_i = 4.4 x 10^{-9} Ms^{-1} (experimentally measured).

$$R_{ox} = 220\, d[\text{STY-BODIPY}]/dt \quad (12)$$

The propagation constant for the autoxidation of MeLH in Triton X-100 micelles (k_4) is 62 $M^{-1}s^{-1}$.[7,8] The oxidizability of MeLH ($k_p/\sqrt{2k_t}$) in Triton X-100 micelles can be calculated from the rate of O_2 consumption experiments performed under the same conditions as 2.0 $M^{-1/2}s^{-1/2}$ at pH 7 and 3.0 $M^{-1/2}s^{-1/2}$ at pH 4.[5] From these values, the termination rate constant ($2k_t$) for MeLH autoxidation in Triton X-100 micelles can be obtained as 1.2×10^3 $M^{-1}s^{-1}$ at pH 7.4. The very low value of $2k_t$ found in Triton X-100 micelles depends on the difficulty of radical-radical encounter in this heterogeneous system.

Table S1. Kinetic rate constants for the co-oxidation model of MeLH autoxidation in Triton X-100 micelles in the presence of γ-terpinene. The numbers in bold were taken from the literature or calculated from the oxidizability of MeLH in micelles and were kept constant.

Rate constant	pH 4.5 ($M^{-1}s^{-1}$)	pH 7.4 ($M^{-1}s^{-1}$)
k_4	**62**	**62**
k_5	600	500
k_6	50	50
k_7	15	15
k_8	**1200**	**1200**
k_9	$5x10^3$	$5x10^3$
k_{10}	$3X10^4$	$3X10^4$

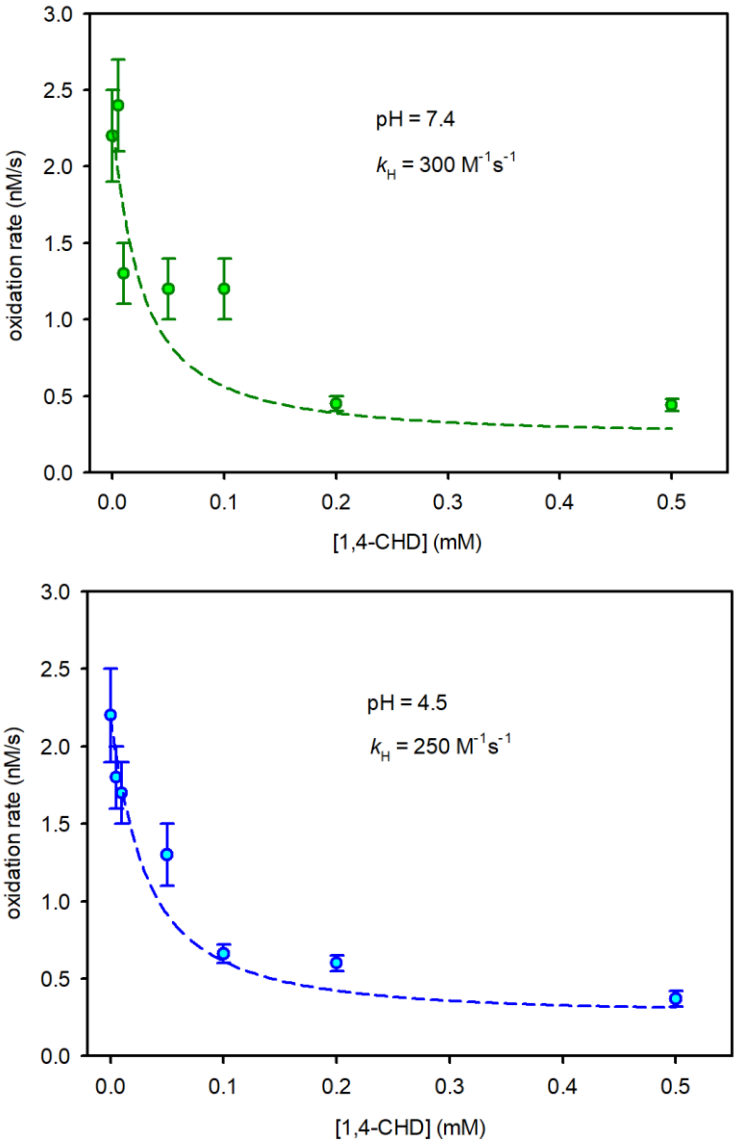

Figure S1. Rate of STY-BODIPY (10 μM) consumption as function of the concentration of 1,4-cyclohexadiene during the autoxidation of MeLH (2.7 mM) in Triton X-100 micelles (8.0 mM) initiated by AAPH (10 mM) at 37 °C, in buffered water at pH 7.4 and 4.5.

Table S2. Different organic bases, pK_a of their conjugated acids in MeCN and water.

Base	pK_a MeCN	Reference	pK_a water	Reference
pyridine	12.53	11	5.25	11
tert-octylamine	≈ 18	12	10.7	13
piperidine	19.35	12	11.3	14
2,2,6,6-tetramethylpiperidine	19.7	Estimated from ref 15	11.8	14
tetrabutylammonium hydroxide	>23	Estimated from ref 17	>13	16

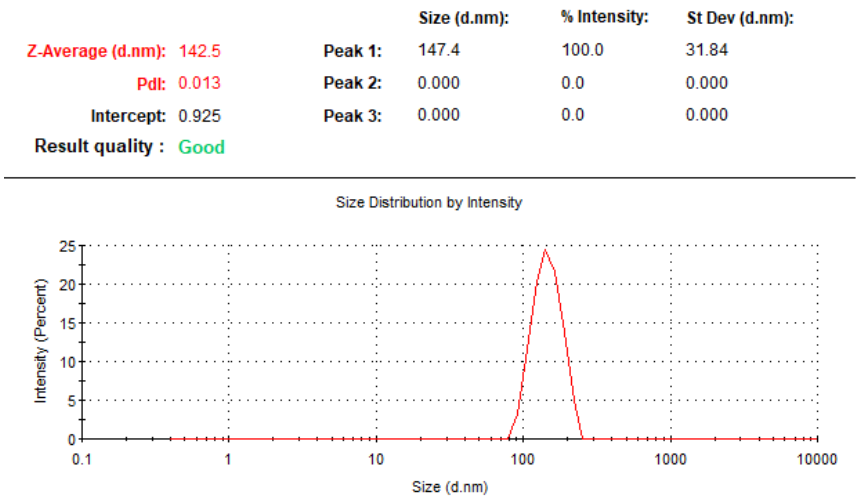

Figure S2. DLS analysis of liposomes.

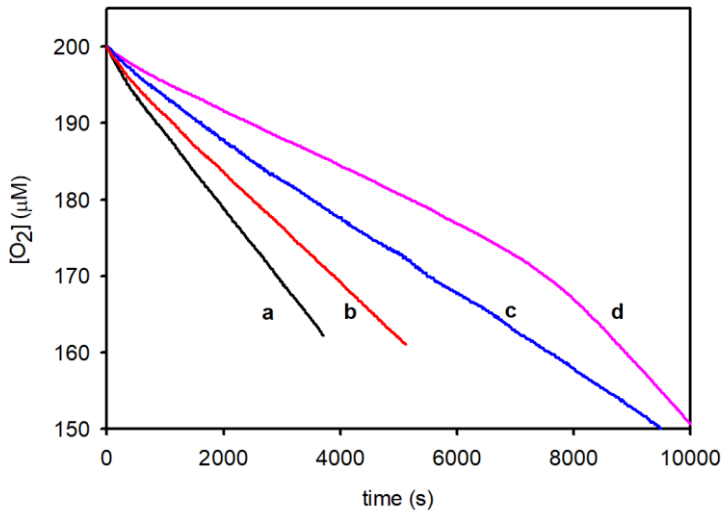

Figure S3. Consumption of dissolved O_2 measured during the autoxidation of PCL (10 mM) initiated by AAPH (3 mM) at 37 °C at pH 7.4 without antioxidants (a) and in the presence of 5 μM of: γ-terpinene (b), α-tocopherol (c), 2,2,5,7,8-pentamethyl-6-chromanol (d).

Table S3. Kinetic parameters obtained from peroxidation studies of PCL: slope of the O_2 consumption in the absence and in the presence of the antioxidants, duration of the inhibition period, rate constant for the reaction with peroxyl radicals.

	$\Delta[O_2]/dt$ (nMs^{-1})	Inhibition period (t) s	k_{inh} ($M^{-1}s^{-1}$)
Without inhibitor	12±2		
PMHC (5 μM)	3.2±0.3	7700±100	(1.6±0.3)×104
α-TOH (5 μM)	4.5±0.4	ND	$(4.5\pm0.6)\times10^3$
γ-terp (5 μM)	6.8±0.6	ND	$(2.4\pm0.4)\times10^3$

The inhibition rate constant was calculated by the following equation, by using the known propagation constant of linoleic acid (62 $M^{-1}s^{-1}$) and the concentration of the polyunsaturated lipids in PCL (about 15%, corresponding to about 2 mM). The rate of initiation by AAPH was 1.8 nMs^{-1}, measured from preliminary experiments by using Trolox (hydrosoluble analogue of α-TOH) as antioxidant.

$$-\frac{d[O_2]}{dt} = \frac{k_p[PCL]R_i}{2k_{inh}[AH]} + R_i$$

This gave results in good agreement with literature in the case of α-TOH, as the reported k_{inh} is 4.3×10^3 $M^{-1}s^{-1}$.[8]

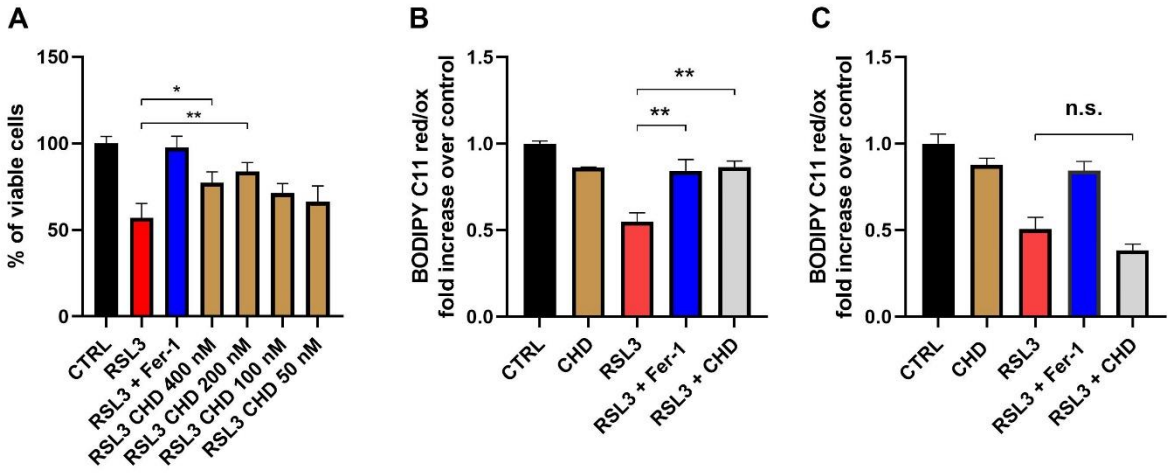

Figure S4. A) Cell viability determined by resazurin assay. SH-SY5Y cells were pretreated with different concentrations of 1,4-cyclohexadiene (CHD) for 1 hour and then incubated for 18 hours with 400 nM RSL3. Pretreatment with 50 µM deferoxamine (DFO) or 400 nM Ferrostatin-1 (Fer-1) was used as a positive control. Data are reported as mean ± s.e.m (n = 4, one-way ANOVA * $p \leq 0.05$; ** ≤ 0.01 vs. RSL3). B) Membrane peroxidation determination using BODIPY 581/591 C11. Cells were pre-treated with 400 nM CHD or Fer-1 and then incubated for 3 hours or 24 hours (C) with 400 nM of the ferroptosis inducer RSL3. Oxidation of the probe results in a shift of fluorescence emission from red to green. Data are presented as fluorescence intensity ratio of reduced/oxidized BODIPY-C11 emission. Data are reported as mean ± s.e.m. (n=3, one-way ANOVA, ** $p \leq 0.01$).

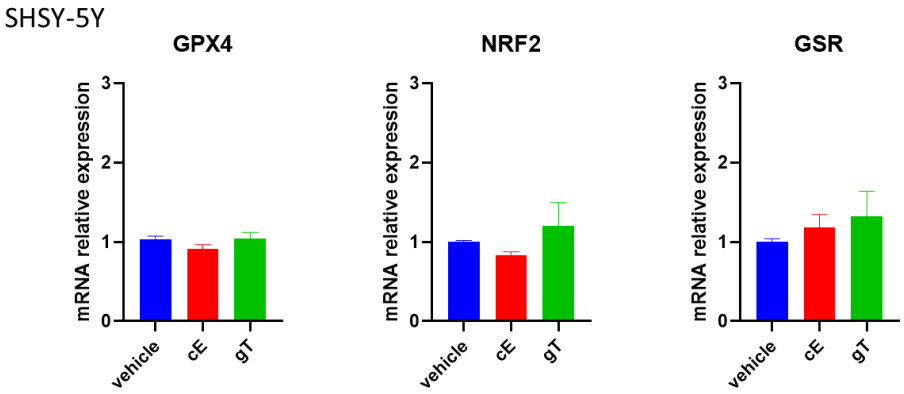

Figure S5. Analysis of GPX4, NRF2, and GSR expression in cells treated for 24 hours with 400 nM of 1,4 cyclohexadiene (CHD) or γ -Terpinene (γ-T). Data are reported as fold change over vehicle treated cells (CTRL).

References

(1) Haidasz, E. A.; Van Kessel, A. T. M.; Pratt, D. A. A *J. Org. Chem.* **2016**, *81*, 737–744

(2) Konopko, A.; Litwinienko, G. *J. Org. Chem.* **2022**, *87*, 1698−1709

(3) Amorati, R.; Baschieri, A.; Valgimigli, L. *J. Chem.* **2017**, 1-12

(4) Daniel F. Gilbert and Oliver Friedrich (eds.), Cell Viability Assays: Methods and Protocols, Methods in Molecular Biology, vol. 1601, DOI 10.1007/978-1-4939-6960-9_1, © Springer Science+Business Media LLC 2017.

(5) Pap, E.H.W.; Drummen, G.P.C.; Winter, V.J.; Kooij, T.W.A.; Rijken, P.; Wirtz, K.W.A.; Op Den Kamp, J.A.F.; Hage, W.J.; Post, J.A *FEBS Lett.* **1999**, 453, 278–282.

(6) C. Diquigiovanni, N. Rizzardi, A. Kampmeier, I. Liparulo, F. Bianco, B. De Nicolo, E. Cataldi-Stagetti, E. Cuna, G. Severi, M. Seri, M. Bertrand, T. B. Haack, A. Della Marina, F. Braun, R. Fato, A. Kuechler, C. Bergamini, E. Bonora, *Open Biol*. **2024**, *13*, 230040.

(7) A. J. Valente, L. A. Maddalena, E. L. Robb, F. Moradi, J. A. Stuart, *Acta Histochem.* **2017,** *119*, 315–326.

(8) Howard, J. A.; Yamada, T. *J. Am. Chem. Soc.* **1981**, 103, 7102–7106

(9) Barclay, L.R.C., Baskin, K.A., Locke, S.J., and Vinqvist, M.R. *Can. J. Chem*. **1989**, 67, 1366–1369.

(10) R. Shah, L. A. Farmer, O. Zilka, A. T. M. Van Kessel, D. A. Pratt, *Cell Chem. Biol.* **2019**, *26*, 1–14

(11) I. Kaljurand, A. Kütt, L. Sooväli, T. Rodima, V. Mäemets, I. Leito, I. A. Koppel. Extension of the Self-Consistent Spectrophotometric Basicity Scale in Acetonitrile to a Full Span of 28 pKa Units: Unification of Different Basicity Scales. *J. Org. Chem.* **2005**, 1019–1028

(12) S. Tshepelevitsh, A. Kütt, M. Lõkov, I. Kaljurand, J. Saame, A. Heering, P. G. Plieger, R. Vianello, I. Leito. On the Basicity of Organic Bases in Different Media. *Eur.J. Org. Chem.* **2019**, 31, 6735-6748.

(13) S. T. J. Droge, J. L. M. Hermens, J. Rabone, S. Gutsell, G. Hodges. Phospholipophilicity of CxHyN+ amines: chromatographic descriptors and molecular simulations for understanding partitioning into membranes. Environ. Sci.: *Processes Impacts*, **2016** 18, 1011-1023

(14) D. Yang, G. Zuccarello, B. R. Mattes. Physical Stabilization or Chemical Degradation of Concentrated Solutions of Polyaniline Emeraldine Base Containing Secondary Amine Additives. *Macromolecules* **2002**, 35, 5304-5313.

(15) E. Rossini, D. Bochevarov, E. W. Knapp. Empirical Conversion of pKa Values between Different Solvents and Interpretation of the Parameters: Application to Water, Acetonitrile, Dimethyl Sulfoxide, and Methanol. *ACS Omega* **2018**, 3, 2, 1653–1662

(16) S. Jin, R. Sanii, B.-Q. Song, M. J. Zaworotko. Crystal Engineering of Ionic Cocrystals Sustained by the Phenol–Phenolate Supramolecular Heterosynthon. *Cryst. Growth Des.* **2022**, 22, 7, 4582–4591.

(17) K. Kaupmees, A. Trummal, I. Leito *Croat. Chem. Acta* **2014**, 87, 385–395