Supplemental data

Nano-targeted induction of dual ferroptotic mechanisms eradicates high-risk neuroblastoma

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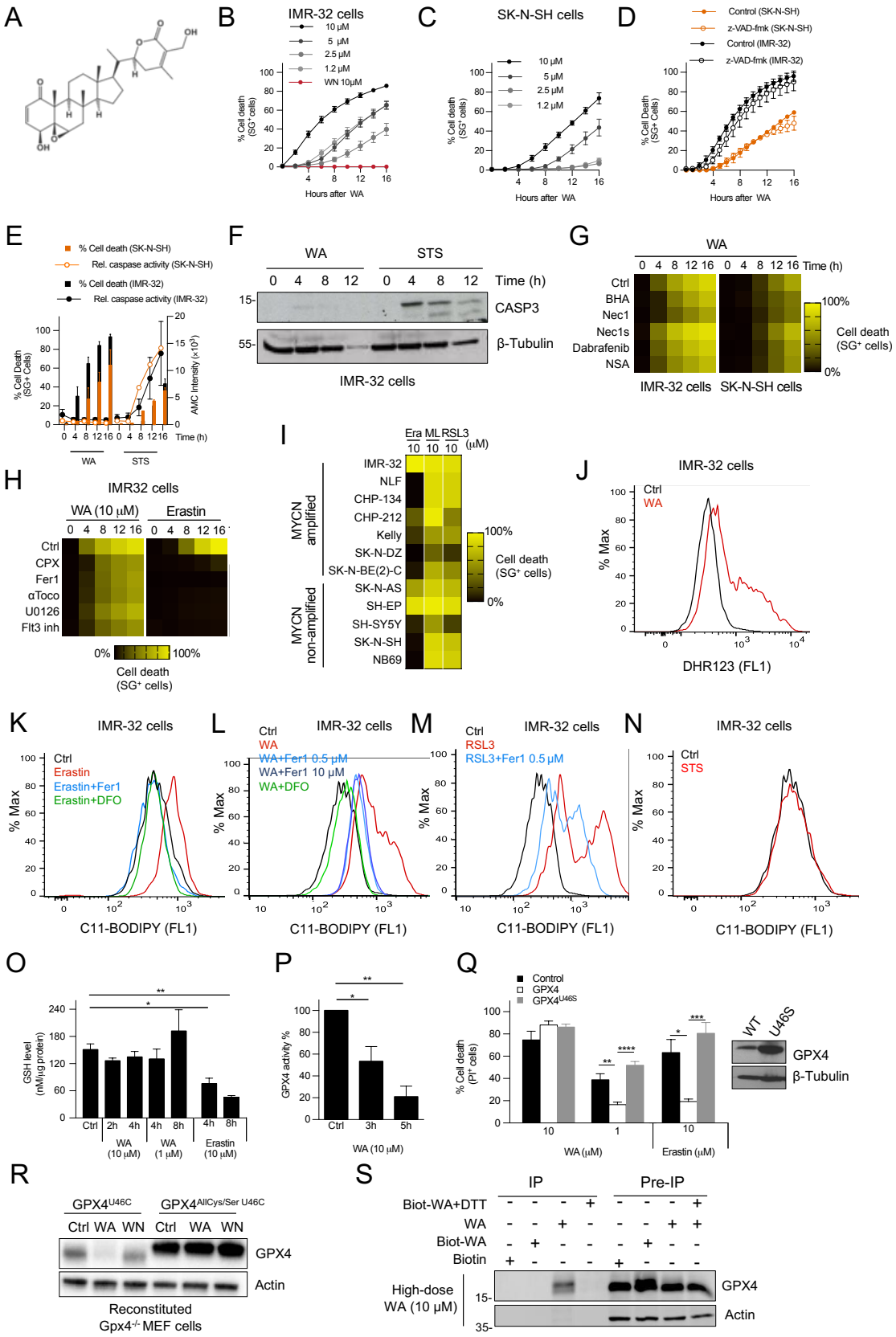
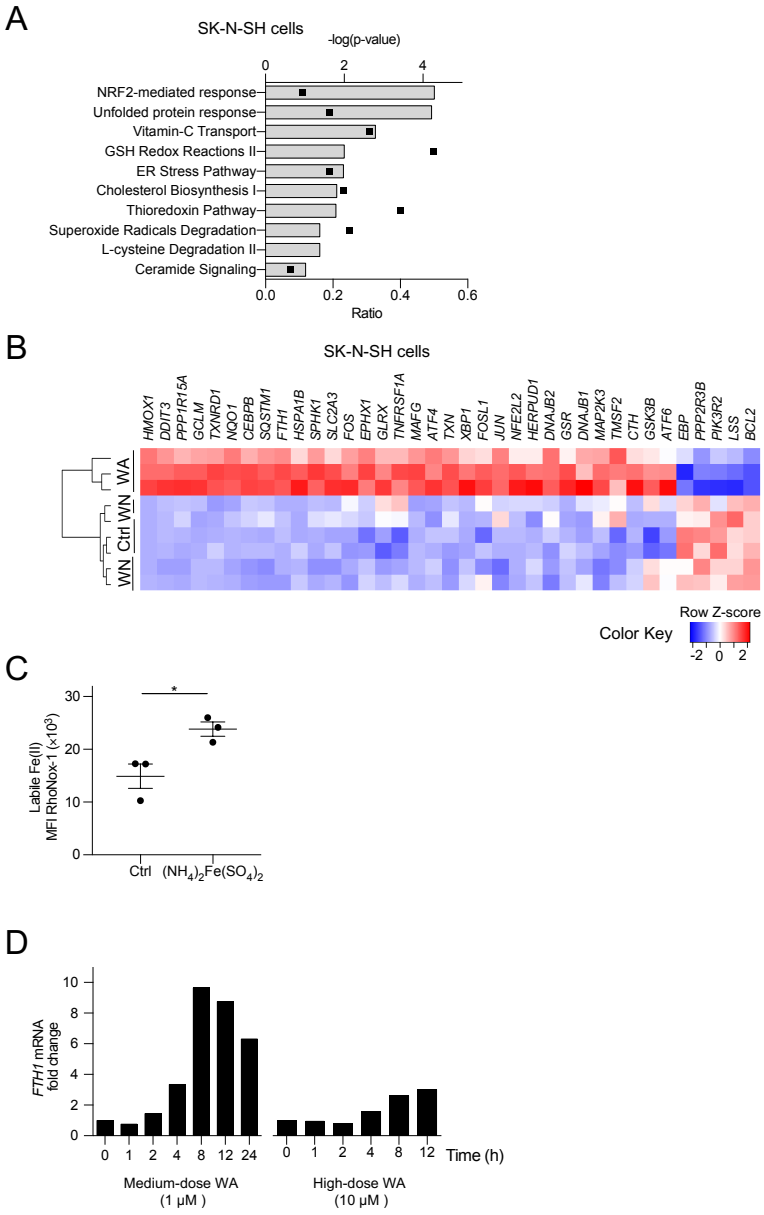


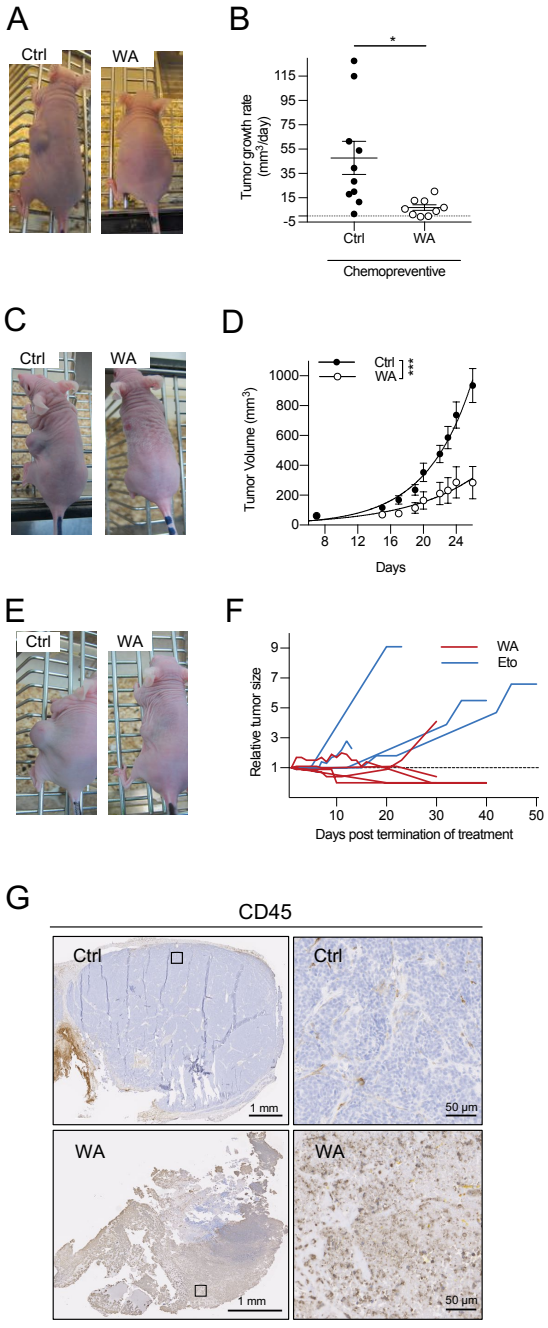
Supplemental Figure 1

Supplemental Figure 1. (A) Structure of Withaferin A. (B) Percentage of cell death triggered by different concentrations of WA compared to the structurally related inactive withanolide, viz. withanone (WN), in IMR-32 cells. The combined results of at least four independent experiments are shown. Error bars represent SEM. (C) Percentage of cell death induced by different concentrations of WA in SK-N-SH cells. The combined results of three independent experiments are shown. Error bars represent SEM. (D) Percentage of cell death triggered by WA in IMR-32 and SK-N-SH cells in presence of the pan caspase inhibitor z-VAD-fmk. The combined results of three independent experiments are shown. Error bars represent SEM. (E) Caspase substrate cleavage (DEVD-AMC fluorescence) after exposure of IMR-32 and SK-N-SH cells to WA and staurosporine (STS). The combined results of five independent experiments are shown. Error bars represent SEM. (F) Western blot analysis revealing caspase 3 activation of cells treated with WA or STS. (G) Heatmap representing cell death sensitivity of IMR-32 and SK-N-SH cells after exposure to WA in presence of necroptosis inhibitors including the multi-target inhibitor butylated hydroxyanisole (BHA), the RIPK1 inhibitors necrostatin-1 (Nec1) and necrostatin-1s (Nec1s), the RIPK3 inhibitors dabrafenib and the hMLKL inhibitor necrosulfonamide (NSA). See also Supplemental Table 2. (H) Heatmap representing the sensitivity of IMR-32 cells after exposure to WA (10 μM) and erastin (10 μM) in presence of different ferroptosis inhibitors including the iron chelator ciclopiroxolamine (CPX), the lipid peroxidation inhibitor ferrostatin-1 (Fer1), the lipophilic antioxidant α-tocopherol, the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) and the kinase inhibitors U0126 and Flt3-inhibitor. See also Supplemental Table 3. (I) Heatmap representing cell death sensitivity of various high-risk neuroblastoma cell lines after exposure to erastin (Era), ML162 (ML) and RSL3 (RSL). See also Supplemental Table 1. (J) Flow cytometric analysis of general ROS production by WA in IMR-32 cells using ROS sensor (DHR123). (K-N) Flow cytometric analysis of the lipid peroxidation sensor (C11-BODIPY-581/591 dye) on live-gated cells (Sytox® Blue-negative cells) after treating IMR-32 cells with (K) erastin, (L) a medium-dose of WA (1 μM), (M) RSL3 (10 μM), or (N) staurosporine (STS). (O) GSH level after treatment with different (medium, high-) or erastin (10 μM). Time points were selected shortly before the onset of cell death. The combined results of at least two independent experiments are shown. Error bars represent SEM. (P) GPX4 activity in IMR-32 cells after treatment with WA (10 μM). The combined results of three independent experiments are shown. Error bars represents SEM. (Q) Percentage of cell death in control (parental), overexpressing GPX4 and $GPX4^{U46S}$ (catalytic inactive mutant) IMR-32 cell treated with WA and erastin. Western blot analysis reveals GPX4 overexpression and β-tubulin expression. (R) Western blot analysis revealing GPX4 expression in GPX4-depleted MEFs reconstituted with $GPX4^{U46C}$ or $GPX4^{\text{AllCys/Ser U46C}}$ 5h after treatment with withaferin A (WA) and withanone (WN) (10 μM). (S) Western blot analysis revealing GPX4 and actin after immunoprecipitation of biotin-WA in lysates of IMR-32 cells treated with a high-dose (10 μM) of biotin-WA in presence/absence of 1,4-Dithiothreitol (DTT) (200 μM). The combined results of three or four independent experiments are shown for respectively erastin and WA. Error bars represent SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$, one-way ANOVA (O), two-way/One-way ANOVA (Q).



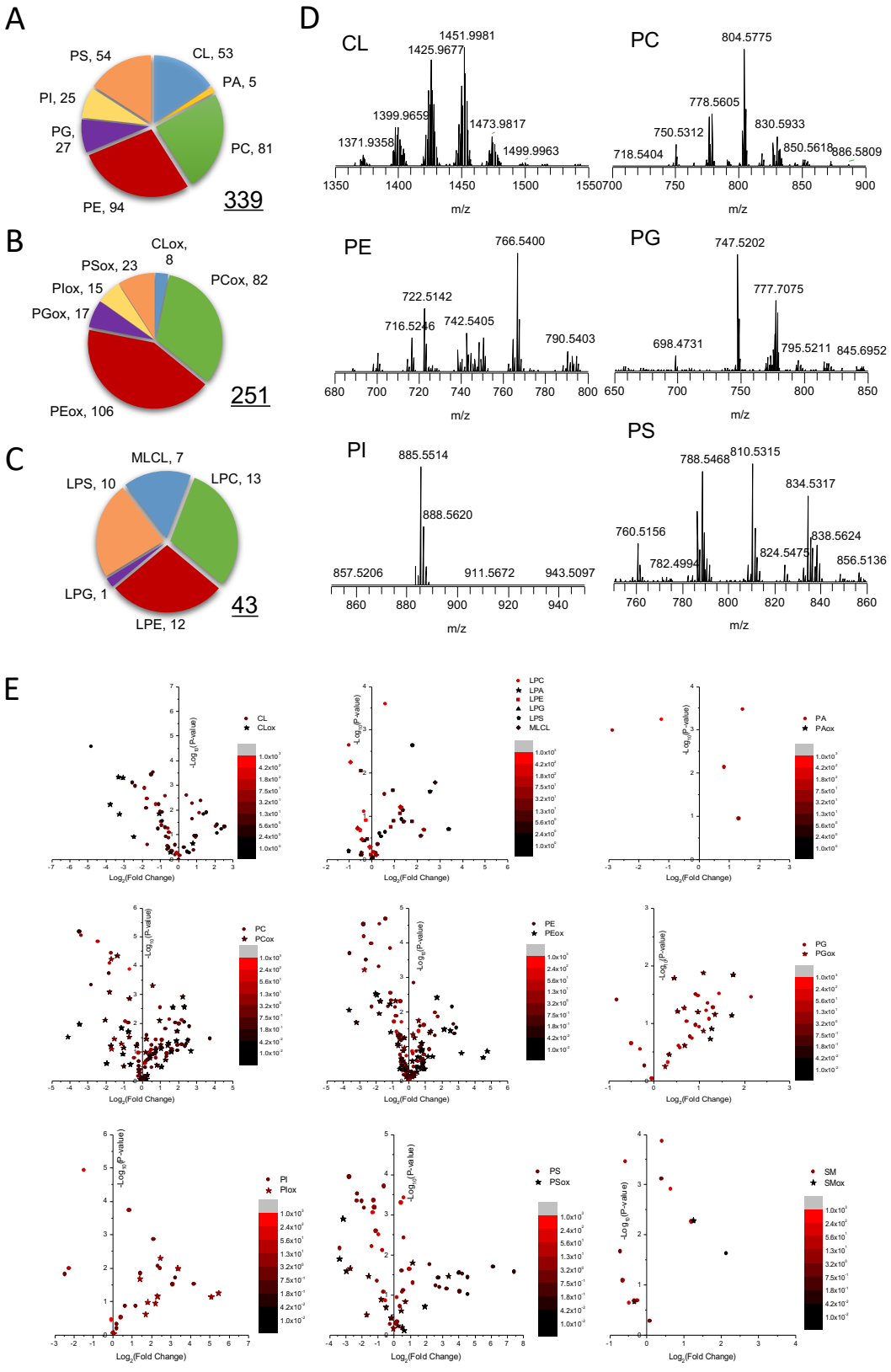
Supplemental Figure 2

Supplemental Figure 2. (A) Ingenuity pathway analysis of genes differentially expressed after WA-treatment in SK-N-SH cells. Top enriched oxidative stress related canonical pathways are ranked by -log (p value) and displayed along x axis in a decreased order based on significance (p value<0.05). (B) Heatmap representing gene expressions of control (Ctrl), WA and WN treated SK-N-SH cells that belong to the top 10 ranked (IPA) oxidative stress related canonical pathways. Color key legend represents Z-scores values ranging from green (low expression) to red (high expression). (C) Cellular levels of labile Fe(II), using RhoNox-1, in function of time in response to $(NH_4)_2Fe(SO_4)_2$ (300 µM). (D) FTH1 mRNA fold change (quantified by RT-qPCR) in IMR-32 cells after treatment with a medium-dose (1 µM) and high-dose WA (10 µM). *p<0.05, two-tailed t-test (C).



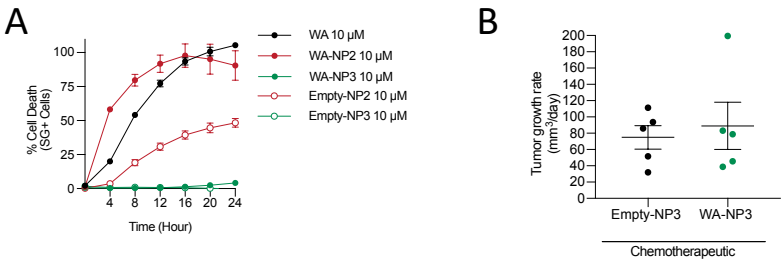
Supplemental Figure 3

Supplemental Figure 3. (A) Pictures of neuroblastoma tumor xenografts in nude mice and (B) quantification of tumor growth rates after chemopreventive treatment regime with Ctrl (vehicle) or withaferin A (WA). The combined results of 2 independent experiments are shown. Each point indicates an individual mouse. Error bars represents SEM, n=10 (Ctrl), n=9 (WA). (C) Pictures of neuroblastoma tumor xenografts in nude mice and (D) quantification of tumor growth rates after therapeutic treatment regime (60 mm^3 at the start of injection) with Ctrl (vehicle) or WA. Error bars represent SEM, n=5. (E) Pictures of neuroblastoma tumor xenografts in nude mice after therapeutic treatment regime (100-200 mm^3 at the start of injection). (F) Quantification of the relative tumor size in mice upon termination of treatment with WA or Etoposide (Eto). (G) Immunohistochemical staining revealing immune cell infiltration using CD45 antibody in tumor sections after therapeutic treatment regime with Ctrl (vehicle) or WA. ***p<0.001; *p<0.05, two-tailed t-test (B), F-test (D).



Supplemental Figure 4

Supplemental Figure 4. (A) The distribution and number of non-oxygenated phospholipid species in six major classes were determined by MS/MS and quantified by high resolution mass spectrometry with mass accuracy less than 5 ppm. (B) Based on the detected polyunsaturated species of phospholipids, potential substrates for oxidation, 251 possible oxygenated species of phospholipids were screened out and quantified by high resolution mass spectrometry with mass accuracy less than 5 ppm. (C) Additionally, 43 species of phospholipid hydrolysis products, lyso-phospholipids, have been detected in five major classes of phospholipids. (D) Representative MS spectra of each phospholipid class. (E) Volcano plots of WA induced changes in the levels of phospholipids (log2 (fold-change), X-axis) vs significance (-log10(p-Value), Y-axis) of neuroblastoma and neuroblastoma treated with WA (Two-tailed t-test).



Supplemental Figure 5

Supplemental Figure 5. (A) Percentage of cell death in function of time triggered by WA-NP2, WA-NP3, Empty-NP2 and Empty NP3 and WA in IMR-32 cells. (B) Quantification of tumor growth rates after therapeutic treatment regime (100-200 mm^3 at the start of injection) with empty (Empty-NP3) or WA encapsulated nanoparticle (WA-NP3). Each point indicates an individual mouse. Error bars represents SD, n=5/group.

Supplemental Video 1. Time-lapse of SK-N-SH cells treated with WA (10 µM). Staining: SytoxGreen & Lysotracker red staining.

Supplemental Video 2. Time-lapse of IMR-32 cells treated with WA (10 µM). Staining: SytoxGreen & Lysotracker red staining.

Supplemental Video 3. Visualization of docking of WA with GPX4 showing predicted covalent binding with cysteine107.

Supplemental Table 1. **Cell death percentage in various neuroblastoma cell lines after exposure to WA, etoposide and cisplatin**

	WA 5 µM		WA 10 µM		WA 20 µM		Eto 5 µM		Eto 10 µM		Eto 20 µM		
Cell line	% CD	SE	% CD	SE	% CD	SE	% CD	SE	% CD	SE	% CD	SE	N
IMR-32	82.2	1.2	91.5	0.9	86.9	9.0	41.2	9.2	35.2	11.2	25.7	9.5	2
NLF	65.0	2.0	77.2	2.5	85.2	2.1	3.4	0.7	4.7	0.7	5.5	0.7	1
CHP-134	47.2	1.0	60.8	1.3	93.6	6.4	32.6	3.4	29.1	2.2	20.9	2.2	2
CHP-212	0.0	0.0	36.0	8.0	100.0	3.0	0.0	1.0	0.0	1.0	1.0	0.0	1
Kelly	18.5	0.8	61.3	7.6	83.6	8.0	0.7	0.7	0.7	0.7	3.4	3.2	2
SK-N-DZ	46.9	4.8	66.8	3.8	91.1	2.2	0.0	0.7	0.3	2.1	44.1	0.9	1
SK-N-BE(2)-C	30.9	0.2	38.0	6.4	48.3	0.4	0.0	0.0	0.0	1.0	1.0	0.0	2
SK-N-AS	1.9	0.2	43.4	9.0	85.3	3.4	0.5	0.5	1.0	0.0	1.0	0.0	2
SH-EP	3.0	0.0	52.0	11.6	93.8	1.1	0.0	0.0	0.0	0.0	0.0	0.0	2
SH-SY5Y	63.2	17.6	88.0	2.7	88.8	0.2	16.0	3.0	24.6	4.4	29.3	5.7	2
SK-N-SH	15.3	0.3	45.6	3.6	75.3	10.3	16.0	1.0	18.0	1.0	20.0	1.0	2
NB69	1.4	0.9	45.5	0.6	68.6	1.7	7.3	1.6	8.7	0.3	9.7	0.7	2

N represents number of independently performed experiments. CD, Cell Death; WA, withaferin A; Eto, etoposide; cis, cisplatin. SE represents. SE represents SEM if N>1.

Supplemental Table 1 cont.

	Eto 200 µM		Cis 5 µM		Cis 10 µM		Cis 20 µM		Cis 200 µM		
Cell line	% CD	SE	% CD	SE	% CD	SE	% CD	SE	% CD	SE	N
IMR-32	37.3	3.0	1.2	1.2	2.6	0.8	3.5	1.2	39.1	0.9	2
NLF	27.1	0.5	1.9	1.4	3.3	1.9	5.4	1.9	36.9	7.5	3
CHP-134	22.3	1.8	1.2	1.2	1.8	0.5	6.4	1.3	43.1	9.8	4
CHP-212	28.1	2.5	1.1	0.1	1.7	0.1	2.9	0.0	30.5	8.5	2
Kelly	6.2	0.2	0.0	0.0	0.3	0.0	0.1	0.7	4.3	1.8	2
SK-N-DZ	40.0	2.4	4.6	0.5	9.2	4.1	11.6	5.3	10.9	0.7	2
SK-N-BE(2)-C	0.9	0.9	0.3	0.3	0.0	0.0	0.0	0.0	5.3	1.0	2
SK-N-AS	2.6	1.4	43.4	9.0	85.3	3.4	0.5	0.5	29.8	4.9	2
SH-EP	2.8	0.5	0.5	0.0	0.2	0.1	2.3	1.9	13.5	3.3	4
SH-SY5Y	15.6	2.0	1.9	1.6	3.0	0.8	9.8	7.0	41.9	5.3	3
SK-N-SH	4.4	1.9	2.1	1.3	1.2	0.7	1.9	1.0	14.0	5.2	2
NB69	43.9	2.4	0.1	0.1	0.0	0.0	4.4	1.7	42.3	11.3	2

Supplemental Table 1 cont. **Cell death percentage in various neuroblastoma cell lines after exposure to Erastin, ML162 and RSL3**

	Erastin 10 µM		ML162 10 µM		RSL3 10 µM		
Cell line	% CD	SE	% CD	SE	% CD	SE	N
IMR-32	100.7	1.9	95.8	0.8	91.6	5.4	2
NLF	4.3	1.6	89.1	3.1	85.1	5.5	3
CHP-134	19.5	9.4	81.6	10.9	86.5	11.0	2
CHP-212	33.0	13	98.2	2.1	48.7	4.6	2
Kelly	26.0	10	77.5	20.0	59.4	20.1	2
SK-N-DZ	3.4	1.7	35.9	3.8	18.7	2.2	2
SK-N-BE(2)-C	9.0	3.5	68.8	21.6	52.0	12.3	2
SK-N-AS	62.3	12.8	84.3	6.1	73.3	6.5	2
SH-EP	93.4	10.9	98.3	2.6	91.3	1.6	2
SH-SY5Y	13.2	2.6	58.8	7.1	23.3	2.7	2
SK-N-SH	6.3	4.3	88.1	4.3	77.9	3.9	3
NB69	4.5	1.2	92.2	7.7	83.5	5.2	3

Supplemental Table 2. **Cell death percentage in IMR-32 and SK-N-SH cells after exposure to WA in presence of necroptosis inhibitors**

		0h		4h		8h		12h		16h			
Cell line	Inhibitor	% CD	SEM	% CD	SEM	% CD	SEM	% CD	SEM	% CD	SEM	N	p value
IMR-32	Ctrl	1.1	0.3	32.2	5.1	62.6	3.4	78.0	2.9	87.5	2.3	13	
	BHA	1.8	0.5	9.1	2.6	41.7	8.0	61.7	9.5	75.4	8.3	5	***
	Nec1	0.5	0.2	9.3	5.3	31.2	12.2	47.2	11.1	61.2	7.8	3	***
	Nec1s	2.3	1.0	41.5	3.8	73.4	3.4	86.4	2.6	91.9	2.9	3	ns
	Dabrafenib	0.2	0.2	43.7	3.0	64.1	1.8	77.9	1.0	82.8	0.3	2	ns
	NSA	1.1	0.4	26.9	9.6	55.7	7.1	69.0	5.7	78.2	4.4	5	ns
SK-N-SH	Ctrl	0.5	0.1	1.4	0.5	15.6	2.1	40.5	2.7	62.7	2.7	12	
	BHA	0.6	0.2	1.3	0.3	12.4	1.1	27.4	1.9	40.0	2.9	8	*
	Nec1	0.0	0.0	0.6	0.5	8.3	1.5	26.2	0.8	42.9	3.1	3	ns
	Nec1s	0.7	0.5	0.6	0.3	13.5	2.2	42.8	5.1	67.4	5.6	2	ns
	Dabrafenib	0.0	0.0	0.3	0.1	9.0	0.9	27.6	4.1	50.4	7.2	2	ns
	NSA	0.2	0.2	1.0	0.6	12.0	2.5	33.0	4.3	53.6	5.7	4	ns

N represents number of independently performed experiments. ***$p<0.001$, *$p<0.05$. CD, Cell Death; WA, withaferin A.

Supplemental Table 3. Cell death percentage in IMR-32 and SK-N-SH cells after exposure to WA and erastin in presence of ferroptosis inhibitors

			0h		4h		8h		12h		16h			
Cell line	Trigger	Inhibitor	% CD	SEM	% CD	SEM	% CD	SEM	% CD	SEM	% CD	SEM	N	P value
IMR-32	Erastin 10µM	Ctrl	0.9	0.3	4.6	1.6	38.8	8.8	83.0	3.6	98.9	2.1	9	
		CPX	1.3	1.2	1.8	1.4	2.4	1.9	4.8	2.6	10.9	3.9	2	***
		Fer1	0.4	0.1	0.5	0.1	0.5	0.1	0.4	0.2	1.4	0.6	4	***
		α-Toco	0.8	0.3	1.5	0.1	2.7	1.1	3.2	1.2	3.5	0.6	3	***
		NDGA	1.6	0.7	1.3	0.2	1.6	0.2	3.3	0.7	10.1	0.9	3	***
		U0126	0.6	0.3	1.7	0.3	3.0	1.5	3.5	1.7	4.0	0.6	3	***
		Flt3 inh	2.3	0.8	1.5	0.4	2.3	0.4	2.8	0.5	3.1	0.6	3	***
IMR-32	WA 10µM	Ctrl	0.8	0.2	39.9	3.7	68.5	2.9	82.4	2.8	90.8	2.3	12	
		CPX	0.8	0.5	6.6	3.2	36.7	2.5	49.4	0.4	54.8	2.3	2	***
		Fer1	0.2	0.1	10.9	4.2	35.8	6.0	49.7	5.1	59.0	4.6	5	***
		α-Toco	0.8	0.3	11.6	3.9	34.5	10.7	47.5	11.0	57.1	10.4	3	**
		NDGA	1.5	0.5	16.1	3.0	37.0	4.6	47.6	4.4	55.8	4.1	4	***
		U0126	0.8	0.3	16.3	1.7	43.7	0.8	57.7	1.2	67.9	1.7	4	*
		Flt3 inh	1.5	0.5	11.8	5.7	32.5	9.7	44.1	10.2	53.0	9.9	4	***
IMR-32	WA 1µM	Ctrl	1.2	0.4	6.7	1.9	32.8	5.0	58.3	4.3	75.2	2.8	10	***
		CPX	0.2	0.1	0.1	0.1	0.9	0.4	2.0	1.0	8.0	1.2	4	***
		Fer1	0.9	0.4	0.2	0.1	0.7	0.3	1.5	0.8	7.4	1.9	6	***
		α-Toco	0.5	0.2	0.4	0.2	1.4	0.8	2.8	1.6	10.5	0.9	4	***
		NDGA	0.6	0.6	2.7	1.1	3.8	1.4	5.8	3.0	12.0	5.3	2	***
		U0126	0.9	0.3	0.7	0.5	1.8	1.1	4.8	1.3	16.8	1.5	4	***
		Flt3 inh	1.1	0.5	0.0	0.0	0.7	0.4	2.3	1.1	10.4	0.9	4	***
SK-N-SH	WA 10µM	Ctrl	0.8	0.2	2.0	0.6	18.0	2.0	44.0	2.4	66.6	3.0	12	***
		CPX	0.0	0.0	0.3	0.1	8.4	2.9	16.1	5.6	21.0	5.7	2	***
		Fer1	0.7	0.2	1.5	0.4	8.6	1.9	20.3	3.1	33.6	4.7	9	***
		α-Toco	0.9	0.4	1.5	0.3	9.8	0.7	19.6	0.8	29.9	1.1	4	***
		NDGA	1.04	0.58	3.2	0.75	19.8	3.07	32	2.6	42	0.79		
		U0126	1.0	0.4	1.0	0.2	9.2	0.5	21.1	1.1	33.7	2.1	3	***
		Flt3 inh	0.3	0.1	1.2	0.7	9.1	2.5	18.4	3.0	29.1	3.3	4	***

N represents number of independently performed experiments .***p<0.001, *p<0.05. CD, Cell Death; WA, withaferin A.

Supplemental Table 4. Binding energy and distance covalent bond of WA, altretamine and ML162 as predicted by covalent method on PDB crystal structure of human GPX4 (2OBI)

GPX4 binding site	WA		Altretamine		ML162	
	Estimated G (K.cal/mol)	Bond length	Estimated G (K.cal/mol)	Bond length	Estimated G (K.cal/mol)	Bond length
Cys 10	Non favorable	-	-2.8	-	-2.9	-
Cys 37	Not surface exposed					
Cys 66	-4.8	-	-2.8	-	-3.3	-
Cys 75	Not surface exposed					
Cys 107	-5.7	3.6	-3.6	3.6	-4.3	5
Cys 148	-	-	-2.4	-	-2.8	-

Supplemental Table 5. Cell death percentage in IMR-32 cells after exposure to different concentrations of hemin in presence of ferroptosis inhibitors

	50 µM		25 µM		12 µM		6 µM		0 µM			
Inhibitor	% CD	SEM	% CD	SEM	% CD	SEM	% CD	SEM	% CD	SEM	N	$P^{\#}$ value
Ctrl	94.5	5.4	67	7.8	30.3	9.5	15.5	8.3	4.4	2.2	5	
ZnPP	20.8	8.1	9.6	4.6	7.6	4.3	3.3	2.7	20.8	8.1	3	***
Fer1	4.7	1.2	2.9	1.1	2.9	0.7	4.0	1.2	4.7	1.2	5	****
DFO	2.5	1.0	1.4	0.8	2.8	1.3	4.0	1.8	18.3	3.9	5	****
NDGA	22.6	0.5	15.2	3.5	17.1	5.3	15.9	7.5	18.3	3.8	2	****
Zileuton	27.8	7.5	30.9	19.8	20.8	8.8	16.8	6.7	14.4	6.7	3	****

N represents number of independently performed experiments. # P values calculated for hemin 50 µM. ****p<0.0001,***p<0.001, CD, Cell Death.

Supplemental Table 6. Cell death percentage in IMR-32 cells after exposure to different concentrations of $(NH_4)_2Fe(SO_4)_2$ in presence of ferroptosis inhibitors

	600 µM		300 µM		150 µM		75 µM		0 µM			
Inhibitor	% CD	SEM	% CD	SEM	% CD	SEM	% CD	SEM	% CD	SEM	N	$P^{\#}$ value
Ctrl	48.9	8.3	39.6	9.4	23.8	5.8	11.8	3.7	1.3	0.3	4	
Fer1	0.8	0.4	0.4	0.1	3.8	2.5	1.4	0.8	0.8	0.5	3	****
NDGA	2.9	1.3	3.2	0.7	4.7	0.6	4.6	0.1	6.6	0.9	3	****
Zileuton	5.3	1.8	5.5	3.3	4.5	2.4	6.0	2.6	5.0	2.4	3	****

N represents number of independently performed experiments. # P values calculated for $(NH_4)_2Fe(SO_4)_2$ ****p<0.0001,***p<0.001, CD, Cell Death.