SUPPLEMENTAL MATERIAL

Neuronal Death Following Hemorrhagic Stroke *in vitro* Shares Features of Ferroptosis and Necroptosis

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Chemicals and Reagents

DPQ (cat. #14450) was obtained from Cayman Chemical. 3-Methyladenine (#BML-AP502-0025), Mdivi-1 (#BML-CM127-0010) Necrostatin-1 (#BML-AP309-0020), Trolox (#ALX-270-267-M100), and z-VAD-fmk (#ALX-260-138-R100) were purchased from Enzo Life Sciences. Bafilomycin A1 (#B-1080), Cyclosporine A (#C-6000), Olaparib (#O-9201), SB 203580 (#S-3400), SP600125 (#S-7979), U0126 (#U-6770) were obtained from LC Laboratories. Necrostatin-1 inactive (#480066), mouse Tumor Necrosis Factor-α (#GF027), and U0124 (#662006) were from Millipore, Hemoglobin (#0855914) from MP Biomedicals, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay, #G4100) from Promega, Erastin from Selleck Chemicals (#S7242). Actinomycin D (#A1410), Chloroquine (#C6628), Cycloheximide (#01810) Deferoxamine (#D9533), Ferrostatin-1 (#SML0583), Hemin (#H9039), L-homocysteate (#H9633), N-acetylcysteine (#A7250), Rapamycin (#R8781), collagenase (#C2399), protease inhibitor cocktail (#P8340), Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA, #E0396), sodium orthovanadate (#S6508), mouse anti-γ-tubulin (clone GTU-88, #T6557, 1:20,000), mouse anti-β-actin (clone AC-74, #A5316, 1:20,000), and Tween 20 (#P7949) were obtained from Sigma. Triton X-100 (#161-0407), Quick Start Bradford Reagent (#500-0205) and Protein Dual Color Standard (#161-0374) were purchased from Bio-Rad. Dulbecco's modified Eagle's medium (DMEM, #11965118), MEM GlutaMAX Supplement (#41090101), fetal bovine serum (#16140071), horse serum (#26050088), penicillin-streptomycin (#15140163), Live/dead assay (#L3224), NuPAGE 4-12% Bis-Tris protein gels (#NP0335 and #NP0336), MES SDS Running Buffer (#NP0002), Taqman RIP1 (#Mm00436354_m1), RIP3 (Mm00444947_m1) mouse primers, β-actin endogenous control VIC (#4352341E), Taqman RNA-to-CT 1-Step Kit (#4392656), and MicroAmp 96-well Reaction Plates (#4346906) were purchased from Thermo Fisher Scientific. Laemmli SDS Sample Buffer (#BP-110R), Transfer Buffer (#BP-190), and Tris-Buffered Saline (#BM-300) were obtained from Boston BioProducts. Methanol (#BDH1135) was purchased from VWR. Rabbit anti-ERK1/2 (#9102, 1:5000) and rabbit anti-phospho-ERK1/2 (#9101, 1:1000, recognizing phosphorylation of threonine 202 and tyrosine 204 of ERK1 or threonine 185 and tyrosine 187 of ERK2) antibodies were obtained from Cell Signaling. Rabbit anti-phospho-S166 RIP1 antibody (1:2000) was provided by P.J.G., J.B., and J.F. (GlaxoSmithKline). Nitrocellulose membrane 0.2µm (#10600001) was from GE Healthcare. Odyssey Blocking Buffer (#927-40010), goat anti-rabbit 680RD (#926-68071, 1:20,000), and goat anti-mouse 800CW (#926-32210, 1:20,000) were purchased from LI-COR Biosciences. NucleoSpin RNA isolation kit (#740955) was obtained from

Clontech. Epon-812 (#14120), glutaraldehyde (#16220), osmium tetroxide (#19100), and propylene oxide (#20401) were purchased from Electron Microscopy Sciences.

3-Methyladenine, Actinomycin D, Bafilomycin A1, Cycloheximide, Cyclosporine A, DPQ, Erastin, Ferrostatin-1, Mdivi-1, Necrostatin-1, Necrostatin-1 inactive, Olaparib, Rapamycin, SB 203580, SP600125, U0124, U0126, and z-VAD-fmk were dissolved in DMSO. Chloroquine, Deferoxamine, Hemoglobin, N-acetylcysteine were dissolved in water, Tumor Necrosis Factor-α in PBS, and Trolox in ethanol. Hemin was dissolved in NaOH and further diluted in water to 10mM stock solution. L-homocysteate was dissolved in MEM and further diluted in water to 250mM stock solution.

Detailed Statistical Analysis

1) Supporting Figure 1:

- 0.5 and 1μM Ferrostatin-1: Kolmogorov-Smirnov test, $Z=0.925$, $p=0.359$; Levené test, $F(4,20)=2.293$, $p=0.095$; one-way ANOVA, $F(4,20)=17.960$, $p<0.001$, partial-$\eta^2=0.782$; posthoc Bonferroni $p<0.001$
- 25, 50, and 100μM Deferoxamine: Kolmogorov-Smirnov test, $Z=0.929$, $p=0.354$; Levené test, $F(4,15)=1.343$, $p=0.300$; one-way ANOVA, $F(4,15)=24.168$, $p<0.001$, partial-$\eta^2=0.866$; posthoc Bonferroni $p<0.001$
- 1mM N-acetylcysteine: Kolmogorov-Smirnov test, $Z=1.000$, $p=0.270$; Levené test, $F(4,15)=1.939$, $p=0.156$; one-way ANOVA, $F(4,15)=9.255$, $p=0.001$, partial-$\eta^2=0.712$; posthoc Bonferroni $p=0.001$
- 10 and 100μM Trolox: Kolmogorov-Smirnov test, $Z=0.879$, $p=0.423$; Levené test, $F(4,25)=1.530$, $p=0.224$; one-way ANOVA, $F(4,25)=10.580$, $p<0.001$, partial-$\eta^2=0.629$; posthoc Bonferroni $p=0.015$ for 10μM, $p<0.001$ for 100μM Trolox
- U0126: Kolmogorov-Smirnov test, $Z=0.984$, $p=0.288$; Levené test, $F(8,73)=3.173$, $p=0.004$; Kruskal-Wallis test, $\chi^2(8,N=82)=55.242$, $p<0.001$, $\eta^2=0.682$; since variances were not homogenous across groups, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at $\alpha=0.05/k$, with $k=12$ (comparison of four different concentration of U0126 and U0124 vs. vehicle-treated cells and additional four comparisons of U0126 vs. U0124). Thus, differences were only significant when p was smaller than corrected $\alpha=0.0042$. posthoc Mann-Whitney U $p<0.001$ for 5, 10, and 20μM U0126 vs. vehicle as well as for 5 and 10μM of U0126 vs. the same concentrations of U0124
- 50 and 100μM Necrostatin-1 vs. vehicle: Kolmogorov-Smirnov test, $Z=0.657$, $p=0.781$; Levené test, $F(8,28)=2.227$, $p=0.056$; one-way ANOVA, $F(8,28)=3.853$, $p=0.004$, partial-$\eta^2=0.524$; posthoc Bonferroni $p=0.003$ for 50μM, $p=0.004$ for 100μM, but not vs. the same concentration of Necrostatin-1i ($p>0.05$)
- 2x2 contingency table: Fisher's exact test, two-tailed $p=1.00$

2) Supporting Figure 2:

- 0.1, 0.5, and 1μM Ferrostatin-1: Kolmogorov-Smirnov test, $Z=1.205$, $p=0.109$; Levené test, $F(4,25)=2.428$, $p=0.074$; one-way ANOVA, $F(4,25)=19.812$, $p<0.001$, partial-$\eta^2=0.760$; posthoc Bonferroni $p=0.004$ for 0.1μM, $p<0.001$ for 0.5 and 1μM

- 25 and 50μM Deferoxamine: Kolmogorov-Smirnov test, $Z=0.495$, $p=0.967$; Levené test, $F(4,15)=2.157$, $p=0.124$; one-way ANOVA, $F(4,15)=6.869$, $p=0.002$, partial-$\eta^2=0.647$; posthoc Bonferroni $p=0.001$ for 25μM, $p=0.028$ for 50μM DFO
- 10, 20, and 30μM N-acetylcysteine: Kolmogorov-Smirnov test, $Z=1.240$, $p=0.092$; Levené test, $F(5,30)=2.331$, $p=0.067$; one-way ANOVA, $F(5,30)=50.071$, $p<0.001$, partial-$\eta^2=0.893$; posthoc Bonferroni $p=0.010$ for 10μM, $p<0.001$ for 20 and 30μM NAC
- 100μM Trolox: Kolmogorov-Smirnov test, $Z=0.829$, $p=0.498$; Levené test, $F(4,15)=0.996$, $p=0.440$; one-way ANOVA, $F(4,15)=7.652$, $p=0.001$, partial-$\eta^2=0.671$; posthoc Bonferroni $p=0.002$
- U0126: Kolmogorov-Smirnov test, $Z=1.438$, $p=0.032$; Levené test, $F(8,45)=7.941$, $p<0.001$; Kruskal-Wallis test, $\chi^2(8,N=54)=47.158$, $p<0.001$, $\eta^2=0.890$; since variances were not homogenous across groups, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at $\alpha=0.0042$ considered significant (see above). posthoc Mann-Whitney U $p=0.004$ for 5, 10, and 20μM U0126 vs. vehicle and 1, 5, 10, and 20μM U0126 vs. the same concentrations of U0124
- 50 and 100μM Necrostatin-1 vs. vehicle and the same concentrations of Necrostatin-1i: Kolmogorov-Smirnov test, $Z=0.750$, $p=0.627$; Levené test, $F(8,36)=1.342$, $p=0.255$; one-way ANOVA, $F(8,36)=39.220$, $p<0.001$, partial-$\eta^2=0.897$; posthoc Bonferroni $p<0.001$)

3) Supporting Figure 3:

- A, phospho-ERK/ERK protein fold-change: median of 1.21 at 2 hours, 2.45 at 4 hours, 2.13 at 8 hours, and 2.61 at 16 hours of 100μM hemin, 2.00 at 8 hours of 5mM glutamate; Kolmogorov-Smirnov test, $Z=0.908$, $p=0.382$; Levené test, $F(5,24)=2.684$, $p=0.046$; Kruskal-Wallis test, $\chi^2(5,N=30)=14.790$, $p=0.011$, $\eta^2=0.510$; since variances were not homogenous across groups, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at $\alpha=0.05/k$, with $k=5$ (comparison of 2, 4, 8, and 16 hours 100μM hemin as well as 8 hours 5mM glutamate treatment to 0 hours). Thus, differences were only significant when p was smaller than corrected $\alpha=0.01$. posthoc Mann-Whitney U $p=0.005$ for 4, 8, 16 hours 100μM hemin and 8 hours 5mM glutamate treatment
- B, phospho-ERK/ERK protein fold-change at 8 hours: median of 1.638 for 100μM hemin, 0.377 for 10μM U0126, 0.640 for 10μM U0126 with hemin, 0.981 for 10μM U0124, 1.831 for 10μM U0124 with hemin; Kolmogorov-Smirnov test, $Z=1.095$, $p=0.182$; Levené test, $F(5,36)=4.112$, $p=0.005$; Kruskal-Wallis test, $\chi^2(5,N=42)=31.457$, $p<0.001$, $\eta^2=0.767$; since variances were not homogenous across groups, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at $\alpha=0.05/k$, with $k=9$ (comparison of vehicle vs. hemin for all three conditions, comparison of the three vehicle and three hemin conditions against each other). Thus, differences were only significant when p was smaller than corrected $\alpha=0.0056$. posthoc Mann-Whitney U $p=0.001$ for vehicle vs. 100μM hemin or vs. 10μM U0126,

p=0.003 for 10μM U0124 vs. 10μM U0124 with hemin as well as 10μM U0126 with hemin vs. hemin or vs. 10μM U0124 with hemin

- C, phospho-ERK/ERK protein fold-change: mean±SD of 1.313±0.332 at 3 hours, 1.773±0.380 at 6 hours, 1.355±0.309 at 12 hours, and 1.737±0.802 at 24 hours of ICH; Kolmogorov-Smirnov test, $Z=0.786$, $p=0.566$; Levené test, $F(4,28)=1.740$, $p=0.169$; one-way ANOVA, $F(4,28)=4.288$, $p=0.008$, partial-$\eta^2=0.380$; posthoc Bonferroni p=0.014 for ICH 6h and p=0.034 for ICH 24h vs. sham
- D, RIP1 mRNA fold-change in hemin: median of 0.725 at 2 hours, 0.870 at 4 hours, 4.939 at 8 hours, and 14.932 at 16 hours; Kolmogorov-Smirnov test, $Z=1.991$, $p=0.001$; Levené test, $F(4,40)=17.916$, $p<0.001$; Kruskal-Wallis test, $\chi^2(4,N=45)=31.323$, $p<0.001$, $\eta^2=0.712$; since variances were not homogenous across groups, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at $\alpha=0.05/k$, with k=4 (comparison of 2, 4, 8, and 16 hours 100μM hemin to 0 hours). Thus, differences were only significant when p was smaller than corrected $\alpha=0.0125$. posthoc Mann-Whitney U p=0.003 hours for 8 and $p<0.001$ for 16 hours compared to 0 hours treatment
- D, RIP3 mRNA fold-change in hemin: median of 0.752 at 2 hours, 0.959 at 4 hours, 2.769 at 8 hours, and 3.307 at 16 hours; Kolmogorov-Smirnov test, $Z=2.147$, $p<0.001$; Levené test, $F(4,40)=9.425$, $p<0.001$; Kruskal-Wallis test, $\chi^2(4,N=45)=14.449$, $p=0.006$, $\eta^2=0.328$; since data were not normally distributed and variances not homogenous, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at $\alpha=0.0125$ considered significant (see above). posthoc Mann-Whitney U p=0.003 for 16 hours compared to 0 hours treatment
- D, RIP1 mRNA fold-change in ICH: mean±SD of 1.000±0.171 for sham vs. 3.206±0.187 for ICH at 24 hours; Kolmogorov-Smirnov test, $Z=0.702$, $p=0.709$; Levené test, $F(1,4)=0.078$, $p=0.794$; Student's t-test, $t(4)=-15.084$, $p<0.001$, $r=0.987$
- D, RIP3 mRNA fold-change in ICH: mean±SD of 1.000±0.100 for sham vs. 15.742±2.616 for ICH at 24 hours; Kolmogorov-Smirnov test, $Z=0.765$, $p=0.602$; Levené test, $F(1,4)=7.261$, $p=0.054$; Student's t-test, $t(4)=-9.752$, $p=0.001$, $r=0.970$
- D, phospho-RIP1 S166 protein fold-change: median of 1.430 at 2 hours, 1.595 at 4 hours, 2.240 at 8 hours, 1.960 at 12 hours, 1.130 at 24 hours, 1.620 at 12 hours + Nec-1, 8.015 at 8 hours 100ng/ml TNFα + 5μM zVAD, and 1.430 at 8h TNFα/zVAD + Nec-1; Kolmogorov-Smirnov test, $Z=2.299$, $p<0.001$; Levené test, $F(8,63)=5.049$, $p<0.001$; Kruskal-Wallis test, $\chi^2(8,N=72)=40.666$, $p<0.001$, $\eta^2=0.573$; since data were not normally distributed and variances not homogenous, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at $\alpha=0.05/k$, with k=10 (comparison of 2, 4, 8, 12, 24 hours, 12 hours + Nec-1, 8 hours TNFα/zVAD ± Nec-1 to 0 hours, 12 hours + Nec-1 to 12 hours, and 8 hours TNFα/zVAD + Nec-1 to 8 hours TNFα/zVAD). Thus, differences were only significant when p was smaller than corrected $\alpha=0.005$. posthoc Mann-Whitney U $p<0.001$ for 4-12 hours hemin and 8 hours TNFα/zVAD compared to 0 hours

treatment and p=0.001 for 8 hours TNFα/zVAD + Nec-1 compared to 8 hours TNFα/zVAD.

4) Supporting Figure 4:

- B, necrotic cells: mean±SD of 6.941±7.467% for vehicle- vs. 39.538±14.503% for hemin-treated cells; Kolmogorov-Smirnov test, Z=0.419, p=0.995; Levené test, F(1,4)=1.306, p=0.317; Student's t-test, t(4)=-3.461, p=0.026, r=0.816
- B, apoptotic cells: mean±SD of 0.741±1.283% for vehicle- vs. 0.794±1.375% for hemin-treated cells; Kolmogorov-Smirnov test, Z=0.997, p=0.273; Levené test, F(1,4)=0.038, p=0.855; Student's t-test, t(4)=-0.049, p=0.963, r=0.020
- C, mitochondrial size: mean±SD of 4.967±0.277% area mitochondria/cytoplasm for vehicle- vs. 6.498±1.279% for hemin-treated cells; Kolmogorov-Smirnov test, Z=0.728, p=0.665; Levené test, F(1,4)=3.887, p=0.120; Student's t-test, t(4)=-2.027, p=0.113, r=0.637

5) Supporting Figure 5:

- A, 1µM Ferrostatin-1: Kolmogorov-Smirnov test, Z=1.452, p=0.029; Levené test, F(7,48)=3.422, p=0.005; Kruskal-Wallis test, $\chi^2(7,N=56)=34.678$, p<0.001, $\eta^2=0.631$; since data was not normally distributed and variances were not homogenous across groups, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at α=0.05/k, with k=7 (comparison of addition of Ferrostatin-1 at 0-12 hours compared with hemin treatment alone). Thus, differences were only significant when p was smaller than corrected α=0.00714. posthoc Mann-Whitney U p=0.002 for addition of Ferrostatin-1 at 0 to 8 hours compared with hemin treatment alone
- A, 100µM Deferoxamine: Kolmogorov-Smirnov test, Z=0.988, p=0.283; Levené test, F(7,34)=2.500, p=0.035; Kruskal-Wallis test, $\chi^2(7,N=42)=28.583$, p<0.001, $\eta^2=0.697$; since variances were not homogenous across groups, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at α=0.05/k, with k=7 (comparison of addition of Deferoxamine at 0-12 hours compared with hemin treatment alone). Thus, differences were only significant when p was smaller than corrected α=0.00714. posthoc Mann-Whitney U p=0.004 for addition of Deferoxamine at 0 to 4 hours compared with hemin treatment alone
- A, 1mM N-acetylcysteine: Kolmogorov-Smirnov test, Z=0.923, p=0.362; Levené test, F(7,42)=1.715, p=0.132; one-way ANOVA, F(7,42)=22.360, p<0.001, partial-$\eta^2=0.788$; posthoc Bonferroni p<0.001 for addition of N-acetylcysteine at 0 or 2 hours compared with hemin treatment alone
- A, 100µM Trolox: Kolmogorov-Smirnov test, Z=1.016, p=0.253; Levené test, F(7,33)=1.894, p=0.102; one-way ANOVA, F(7,33)=12.291, p<0.001, partial-$\eta^2=0.723$; posthoc Bonferroni p<0.001 for addition of Trolox at 0 to 4 hours compared with hemin treatment alone, p=0.005 for 6 hours, and p=0.035 for 8 hours
- A, 10µM U0126: Kolmogorov-Smirnov test, Z=1.354, p=0.051; Levené test, F(7,37)=0.459, p=0.857; one-way ANOVA, F(7,37)=40.239, p<0.001, partial-

η^2=0.884; posthoc Bonferroni p<0.001 for addition of U0126 at 0 to 8 hours compared with hemin treatment alone

- A, 100µM Necrostatin-1: Kolmogorov-Smirnov test, Z=0.493, p=0.968; Levené test, F(7,37)=0.699, p=0.673; one-way ANOVA, F(7,37)=10.982, p<0.001, partial-η^2=0.884; posthoc Bonferroni p<0.001 for addition of Necrostatin-1 at 0, 2, and 6 hours, p=0.001 for 4 hours, and p=0.019 for 8 hours compared with hemin treatment alone
- B, RIP1 mRNA fold-change: median of 1.863 for 100µM hemin, 0.677 for 10µM U0126, 1.387 for 10µM U0126 with 100µM hemin, 0.930 for 10µM U0124, and 1.954 for 10µM U0124 with 100µM hemin; Kolmogorov-Smirnov test, Z=1.270, p=0.080; Levené test, F(5,42)=5.902, p<0.001; Kruskal-Wallis test, χ^2(5,N=48)=19.411, p=0.002, η^2=0.413; since variances were not homogenous across groups, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at α=0.05/k, with k=9 (comparison of vehicle vs. hemin for all three conditions, comparison of the three vehicle and three hemin conditions against each other). Thus, differences were only significant when p was smaller than corrected α=0.0056. posthoc Mann-Whitney U p<0.001 for 100µM hemin vs. vehicle, p=0.027 for 100µM hemin with 10µM U0126 vs. 10µM U0126, p=0.046 for 100µM hemin with 10µM U0124 vs. 10µM U0124, and p=0.248 for 10µM U0126 with 100µM hemin vs. 100µM hemin
- C, RIP3 mRNA fold-change: median of 2.572 for 100µM hemin, 0.711 for 10µM U0126, 1.793 for 10µM U0126 with 100µM hemin, 0.870 for 10µM U0124, and 1.896 for 10µM U0124 with 100µM hemin; Kolmogorov-Smirnov test, Z=1.317, p=0.062; Levené test, F(5,42)=14.034, p<0.001; Kruskal-Wallis test, χ^2(5,N=48)=37.207, p<0.001, η^2=0.792; since variances were not homogenous across groups, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at α=0.05/k, with k=9 (comparison of vehicle vs. hemin for all three conditions, comparison of the three vehicle and three hemin conditions against each other). Thus, differences were only significant when p was smaller than corrected α=0.0056. posthoc Mann-Whitney U p<0.001 for 100µM hemin vs. vehicle, p=0.002 for 100µM hemin with 10µM U0126 vs. 10µM U0126, p=0.001 for 100µM hemin with 10µM U0124 vs. 10µM U0124, and p=0.074 for 10µM U0126 with 100µM hemin vs. 100µM hemin
- D, phospho-RIP1 S166 protein fold-change: median of 1.370 at 4 hours, 1.460 at 4 hours + 10µM U0126, 1.910 at 8 hours, 1.700 at 8 hours + 10µM U0126, 2.220 at 12 hours, 1.600 at 12 hours + 10µM U0126, 1.390 at 12 hours + 100µM Necrostatin-1, 2.120 at 8 hours 100ng/ml TNFα + 5µM zVAD, and 1.370 at 8h TNFα/zVAD + Nec-1; Kolmogorov-Smirnov test, Z=1.865, p=0.002; Levené test, F(9,59)=10.257, p<0.001; Kruskal-Wallis test, χ^2(9,N=69)=30.598, p<0.001, η^2=0.450; since data were not normally distributed and variances not homogenous, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at at α=0.05/k, with k=9 (comparison of 4, 8, and 12 hours hemin, and 8 hours TNFα/zVAD to 0 hours and respective time point + 10µM U0126 or 100µM Necrostatin-1). Thus,

differences were only significant when p was smaller than corrected $\alpha=0.0056$. posthoc Mann-Whitney U: $p=0.017$ for 4 hours, $p<0.001$ for 8 and 12 hours hemin as well as 8 hours TNFα/zVAD vs. 0 hours of hemin treatment; $p=0.721$ for 4 hours + 10µM U0126, $p=0.225$ for 8 hours + U0126, and $p=0.277$ for 12 hours + U0126 vs. respective time point without U0126; $p=0.064$ for 12 hours + Necrostatin-1 vs. 12 hours alone; $p=0.009$ for 8 hours TNFα/zVAD + Necrostatin-1 compared to 8 hours TNFα/zVAD.

6) Supporting Figure 6:

- A, cell survival Necrostatin-1 dose-response with 0.5µM U0126 (sub-threshold dose) in hemin toxicity: Kolmogorov-Smirnov test, $Z=1.069$, $p=0.203$; Levené test, $F(8,54)=2.211$, $p=0.041$; Kruskal-Wallis test, $\chi^2(8,N=63)=34.542$, $p<0.001$, $\eta^2=0.557$; since variances were not homogenous across groups, nonparametric omnibus test Kruskal-Wallis was performed followed by posthoc Mann-Whitney U with Bonferroni correction at $\alpha=0.05/k$, with $k=8$ (comparison of 0.5 and 10µM U0126 and 10, 50, and 100µM Necrostatin-1 to hemin treatment alone; cotreatment of 0.5µM U0126 and different dosage of Necrostatin-1 vs. Necrostatin-1 alone). Thus, differences were only significant when p was smaller than corrected $\alpha=0.00625$. posthoc Mann-Whitney U $p=0.004$ for 10µM U0126, $p=0.009$ for 100µM Necrostatin-1 vs. hemin alone, $p=0.565$ for 10µM Necrostatin-1 + 0.5µM U0126 vs. 10µM Necrostatin-1, $p=0.085$ for 50µM Necrostatin-1 + 0.5µM U0126 vs. 50µM Necrostatin-1, $p=0.225$ for 100µM Necrostatin-1 + 0.5µM U0126 vs. 100µM Necrostatin-1
- B, cell survival Necrostatin-1 dose-response with 0.5µM U0126 (sub-threshold dose) in hemoglobin toxicity: Kolmogorov-Smirnov test, $Z=1.158$, $p=0.137$; Levené test, $F(8,36)=1.232$, $p=0.309$; one-way ANOVA, $F(8,36)=41.880$, $p<0.001$, partial-$\eta^2=0.903$; posthoc Bonferroni $p<0.001$ for 50 and 100µM Necrostatin-1 ± 0.5µM U0126 and 10µM U0126, $p=1.000$ for 10, 50, and 100µM Necrostatin-1 + 0.5µM U0126

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Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
Experimental groups and study timeline	☑ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. ☑ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. ☐ An overall study timeline is provided. N/A
Inclusion and exclusion criteria	☐ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article. N/A
Randomization	☑ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. ☑ Type and methods of randomization have been described. ☑ Methods used for allocation concealment have been reported.
Blinding	☐ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. ☑ Blinding procedures have been described with regard to masking of group assignment during outcome assessment.
Sample size and power calculations	☑ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.
Data reporting and statistical methods	☑ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. ☐ Baseline data on assessed outcome(s) for all experimental groups have been reported. N/A ☐ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. N/A ☑ Statistical methods used have been reported. ☑ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.
Experimental details, ethics, and funding statements	☑ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. ☐ Different sex animals have been used. If not, the reason/justification is provided. N/A ☑ Statements on approval by ethics boards and ethical conduct of studies have been provided. ☑ Statements on funding and conflicts of interests have been provided.