Neuronal Death After Hemorrhagic Stroke In Vitro and In Vivo Shares Features of Ferroptosis and Necroptosis

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Background and Purpose—Intracerebral hemorrhage leads to disability or death with few established treatments. Adverse outcomes after intracerebral hemorrhage result from irreversible damage to neurons resulting from primary and secondary injury. Secondary injury has been attributed to hemoglobin and its oxidized product hemin from lysed red blood cells. The aim of this study was to identify the underlying cell death mechanisms attributable to secondary injury by hemoglobin and hemin to broaden treatment options.

Methods—We investigated cell death mechanisms in cultured neurons exposed to hemoglobin or hemin. Chemical inhibitors implicated in all known cell death pathways were used. Identified cell death mechanisms were confirmed using molecular markers and electron microscopy.

Results—Chemical inhibitors of ferroptosis and necroptosis protected against hemoglobin- and hemin-induced toxicity. By contrast, inhibitors of caspase-dependent apoptosis, protein or mRNA synthesis, autophagy, mitophagy, or parthanatos had no effect. Accordingly, molecular markers of ferroptosis and necroptosis were increased after intracerebral hemorrhage in vitro and in vivo. Electron microscopy showed that hemin induced a necrotic phenotype. Necroptosis and ferroptosis inhibitors each abrogated death by >80% and had similar therapeutic windows in vitro.

Conclusions—Experimental intracerebral hemorrhage shares features of ferroptotic and necroptotic cell death, but not caspase-dependent apoptosis or autophagy. We propose that ferroptosis or necroptotic signaling induced by lysed blood is sufficient to reach a threshold of death that leads to neuronal necrosis and that inhibition of either of these pathways can bring cells below that threshold to survival. (Stroke. 2017;48:1033-1043. DOI: 10.1161/STROKEAHA.116.015609.)

Key Words: apoptosis ■ cell death ■ ferroptosis ■ intracerebral hemorrhage ■ necrosis

Although intracerebral hemorrhage (ICH) accounts for 15% of all strokes, it has the highest mortality rates among stroke subtypes, with ≤50% within 30 days after the insult.1,2 Treatment options are lacking, and translation of findings from the laboratory bench to the human bedside has been limited.

In ICH, a hydrostatic jet of blood emerging from the ruptured vessel causes direct tissue destruction immediately. In contrast, secondary injury, including perihematoma edema formation, inflammation, and cell death, occurs hours to days after the hemorrhagic event.3 Increasing evidence suggests that hemoglobin from the hematoma contributes to cell autonomous and non–cell autonomous neuronal injury.4 Specifically, infusion of lysed red blood cells into the striatum of rats leads to an increase in brain water content accompanied by neurological deficits in the forelimb placing task.5 Similarly, infusion of hemoglobin, as well as its oxidized product hemin, increases brain water content, inflammatory responses, and neuronal cell death.6,7 Furthermore, hemoglobin induces cell death both in vivo and in vitro.8,9

In the blood, haptoglobin and hemopexin act as hemoglobin and heme scavengers during hemolysis.9 After experimental ICH, expression of haptoglobin increases. Consequently, deletion of haptoglobin worsens neurological outcome, while mice overexpressing human haptoglobin 2 show less severe deficits after ICH.10 In vitro, haptoglobin prevents hemoglobin-induced neuronal toxicity.11 Similarly, knockout of hemopexin leads to an increase in lesion volumes and degenerating neurons and aggravates behavioral deficits after ICH in vivo.12

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Despite an increasing understanding of the role of hemoglobin in ICH, it remains unclear how cells die in response to bleeding in the brain, which is crucial to develop effective therapeutic strategies. Classical, the research community distinguished between 2 modes of cell death, that is, apoptosis and necrosis. Both apoptotic and necrotic cells have been identified in animal models of ICH, as well as in the perihematoma region after surgical evacuation in humans. However, caution must be taken when attributing apoptosis or necrosis to single biochemical markers because these markers are not specific for a single type of cell death. Moreover, recent studies discovered other forms of regulated nonapoptotic cell death, such as necroptosis and ferroptosis. To date, it is unclear to what extent the different forms of cell death contribute to ICH-induced toxicity.

In this study, we performed an unbiased screen of known pharmacological cell death inhibitors in hemoglobin- and hemin-induced toxicity in primary cortical neurons. We validated the identified cell death mechanisms using molecular markers in vitro and in vivo and performed electron microscopy to identify the morphological characteristics of ICH-induced cell death in vitro.

Materials and Methods

Chemicals and reagents are listed in the online-only Data Supplement.

Animals

Experimental procedures on mice were approved by the Weill Cornell Medicine Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Mice were purchased from Charles River Laboratories and housed at 68°F to 72°F; 30% to 70% humidity, under 12-hour light/dark cycle, with food (PicoLab Rodent diet 5053; LabDiet) and water freely accessible.

Mouse Model of ICH

ICH was induced in mice as previously described. Male C57BL/6 mice (8–10 weeks old) were randomly assigned to groups.

Cell Culture

Primary cortical neurons were obtained from CD-1(ICR) mice at embryonic day 15 as previously described and cultured at 37°C in modified Eagle’s medium containing 1% fetal bovine serum and 1% penicillin/streptomycin and treated when 70% confluent. Hippocampal neuroblasts (HT22 cells) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% penicillin/streptomycin and treated when 70% confluent.

Cell Viability

We determined cell viability as previously described at 14 to 18 hours after hemin or at 24 to 28 hours after hemoglobin exposure. Plates were measured at SpectraMax Plus Microplate Reader using SoftMax Pro v4.7.1 (both Molecular Devices, Sunnyvale, CA). We confirmed MTt assays (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Promega, Madison, WI) by Live/Dead assay (Thermo Fisher Scientific, Waltham, MA) and fluorescence microscopy at Nikon Eclipse TS100 microscope using Nikon DS-L3 (Nikon Instruments, Melville, NY).

Immunoblot Analysis

We prepared protein extracts using 1% Triton buffer (25 mMol/L, Tris pH 7.4, 100 mMol/L NaCl, 1 mMol/L EGTA, 1% Triton X-100, protease inhibitors, 2.5 mMol/L sodium orthovanadate). Samples were electrophoresed under reducing conditions on NuPAGE gels and transferred to nitrocellulose membrane. Antibodies against phospho extracellular signaling kinase 1/2 (ERK1/2), total ERK1/2, γ-tubulin, phospho-S166 receptor-interacting kinase 1 (RIP1), and β-actin were incubated overnight at 4°C. Secondary antibodies were incubated for 1 hour at room temperature. Proteins were detected using Odyssey infrared imaging system (LI-COR Biosciences).

RNA Extraction and Real-time Polymerase Chain Reaction

Total RNA was prepared using NucleoSpin RNA isolation kit according to established protocols. We performed real-time polymerase chain reaction using Taqman RNA-to-CT 1-Step Kit for mouse RIP1 (No. Mm00436354_m1) and RIP3 (Mm00444947_m1) at a 7500 Real-Time PCR System (Applied Biosystems). Expression levels were normalized to mouse β-actin endogenous control.

Electron Microscopy

Primary neurons were grown on poly-L-lysine-coated acar plastic coverslips. We fixed the cells overnight at 4°C in 0.05 mol/L phosphate buffer (pH 7.4) containing 2% glutaraldehyde and 0.1 mol/L sucrose. The coverslips were processed for electron microscopy as previously described, with modifications. We incubated the coverslips in 2% osmium tetroxide in phosphate buffer for 1 hour followed by embedding in Epon-812. Ultrathin sections (70 nm) using a Leica UC6 ultratome were collected on 400-mesh thin-bar copper grids (Electron Microscopy Sciences, Fort Washington, PA) and counterstained with 5% uranyl acetate and Reynolds’s lead citrate. Micrographs were taken on a Tecnai Biotwin transmission electron microscope (FEI, Hillsboro, OR). We quantified the percentage of cells displaying necrotic or apoptotic morphology (n=33–43/condition and replicate). Using ImageJ v1.49 (http://imagej.nih.gov/ij/), we measured mitochondrial size as percentage area of total area of the cytoplasm comprising mitochondria in total. Analysis was performed by an investigator blinded to treatment group assignment.

Statistical Analysis

We evaluated normality by Kolmogorov–Smirnov and variance homogeneity using Levene test. For normally distributed data, Mann–Whitney U test with α-correction according to Bonferroni to adjust for the inflation of type I error because of multiple testing. Data are represented as mean±standard deviation (SD) except for nonparametric data, in which case medians are given. For electron microscopy data, mean±standard error of the mean (SEM) is given because this is the convention in this field. A value of P<0.05 was considered statistically significant. For Kruskal–Wallis test followed by Mann–Whitney U test, P<0.05/k was used, with k as number of single hypotheses. To analyze contingency tables, Fisher’s exact test was used. For detailed statistical analyses, see online-only Data Supplement. We performed all statistical analyses with IBM SPSS version 21.

Results

Hemorrhagic Stroke In Vitro Shares Features of Ferroptotic and Necrotic Cell Death

Lysis of red blood cells after ICH leads to release of hemoglobin, which is further broken down into heme or its oxidized form hemin. Hemoglobin or heme have been used in vitro to study cell death in primary neurons and neuronal cell lines. Incubation of primary neurons with 100 μmol/L hemin led to a 50% reduction in cell survival (Figure 1A). This concentration is below the concentration estimated in vivo after ICH in humans.
Figure 1. Systematic pharmacological characterization of hemin-induced cell death in primary cortical neurons. Cell death is inhibited by chemical inhibitors of ferroptosis or necroptosis. A, Primary neurons treated with 100 μmol/L hemin (LD50) and chemical inhibitors effective in distinct cell death models (e.g., apoptosis, autophagy, ferroptosis) were examined. Values show mean±SD at representative concentration in brackets. Grayscale coding indicates the continuum from no protection in the presence of hemin (black) to maximal cell viability (white). B, Concentration–responses of inhibitors that inhibit hemin-induced death. Values represent mean±SD, except for U0126, where medians are given. *P<0.05 vs hemin, #P<0.05 vs U0124. C, Representative Live/Dead staining are shown. Scale bar =50 μm. D, Statistical analysis of profile of chemical inhibitors between operationally defined ferroptosis and hemin-induced toxicity revealed that hemin toxicity in primary neurons can be considered ferroptotic.
In a systematic pharmacological characterization, we found that several chemical inhibitors that collectively define a ferroptotic form of cell death inhibited hemin-induced death (Figure 1A). As with classically defined ferroptosis, the canonical ferroptosis inhibitor Ferrostatin-1 protected against hemin toxicity. Moreover, other inhibitors of ferroptosis, including the iron chelator deferoxamine, the glutathione prodrug N-acetylcysteine, the water-soluble lipid peroxidation inhibitor Trolox, and the ERK1/2 inhibitor U0126 (but not its inactive control), abrogated hemin toxicity (Figure 1B).

Consistent with ferroptosis being a dominant mode of death, caspase inhibitors (that by definition inhibit apoptotic death), inhibitors of protein or mRNA synthesis, inhibitors or activators of autophagy did not increase cell viability in the presence of hemin (Figure 1A). Live/Dead assays, which represent a visible measure of cell death in single cells, revealed results that were similar to quantitative measurements of cell death performed with MTT assays (Figure 1C).

To statistically address whether hemin-induced neuronal death is ferroptotic, we compared the profile of chemical inhibitors of hemin toxicity with operationally defined ferroptosis. We found a large overlap; statistical analysis revealed that cell death induced by hemin in primary neurons can be considered ferroptotic (Fisher’s exact test, two-tailed P=1.00).

Unexpectedly, an inhibitor of necroptosis (Necrostatin-1), a regulated form of necrosis and previously implicated in ischemic stroke, also blocked hemin-induced death (Figure 1A through 1C). The specificity of this inhibitor against RIP1 was suggested by the observation that a structural analog (Necrostatin-1i), with no activity toward RIP1, did not block hemin-induced death.

Together, these studies suggested that hemin-induced death in cortical neurons shares features of ferroptosis and necroptosis. Convergence of these distinct modes of cell death in a single cell death paradigm has not been described.

To confirm that the present profile of protective inhibitors can protect generally from other potential toxins of lysed blood in addition to hemin, we examined whether inhibitors of ferroptosis and necroptosis could abrogate hemoglobin-induced death. As expected from our hemin studies, inhibitors of ferroptosis and necroptosis abrogated hemoglobin-induced cell death (Figure 2).

Together, these findings suggest that neurons exposed to hemoglobin or hemin die via a similar ferroptotic and necrotic pathway.

**Hemorrhagic Stroke In Vitro and In Vivo Shows Molecular Features of Ferroptosis and Necroptosis**

We, thus, hypothesized that cell death after ICH in vitro is a mixture of ferroptosis and necroptosis. To establish the specificity of our chemical inhibitors, we assessed whether signaling molecules required for ferroptotic or necrototic death were activated using biochemical measurements.

Activation of mitogen-activated protein (MAP) kinase signaling involving phospho-ERK1/2 is important in ferroptosis induced by erastin in tumor cells bearing oncogenic Ras. We assessed whether ERK1/2 are activated by hemin in primary neurons evaluating total and phospho-ERK1/2 protein levels. We found an increase in phospho-ERK1/2 starting at 4 hours of hemin treatment (Figure 3A). As expected, this phosphorylation was inhibited by U0126, but not its inactive control U0124 (Figure 3B). Next, we wanted to confirm whether our in vitro findings translate to ICH in vivo. We found that phospho-ERK1/2 was significantly elevated at 6 and 24 hours after ICH in mice (Figure 3C).

RIP1 and RIP3 mediate necroptotic cell death, and prior studies demonstrated an increase in RIP1 and RIP3 message in association with this type of death. Both RIP1 and RIP3 mRNA levels increased after hemin treatment and after ICH (Figure 3D). However, increases in RIP1 and RIP3 mRNA do not necessarily indicate enhanced necroptosis because both proteins, especially RIP1, have other functions that do not lead to necroptosis. It is established that RIP1 kinase activity is required to execute necroptosis. Using an antibody against a known RIP1 autophosphorylation site at serine 166, we found a Necrostatin-1-sensitive increase in phospho-RIP1 after hemin treatment (Figure 3D).

**Hemorrhagic Stroke In Vitro Presents a Necrotic Morphology**

Historically, distinct mechanisms of cell death were discriminated by their morphology at an ultrastructural level. Cells undergoing necrosis/necroptosis display swelling of organelles, structural loss of mitochondria, disruption of the plasma membrane, and subsequent release of intracellular content. By contrast, cells that die by apoptosis show cell shrinkage, nuclear fragmentation, and formation of apoptotic bodies. In ferroptosis, shrunken mitochondria have been observed, while the plasma membrane remained intact, and formation of apoptotic bodies was absent.

To establish whether hemin toxicity involves ultrastructural features of necr(opt)osis and/or ferroptosis, we performed electron microscopy of primary neurons at 16 hours after hemin treatment. We chose this time because it is 8 hours before cell death has reached a steady state (24 hours), thus, increasing the likelihood to observe morphologies reflecting a continuum from early to late stages of death. Compared with vehicle treatment (Figure 4A, upper row), some of the hemin-treated neurons displayed a necrotic morphology, as reflected by a loss of plasma membrane integrity and disintegration of organelles (Figure 4A, middle row, left and middle). Other hemin-treated neurons presented an intact plasma membrane, but larger mitochondria (Figure 4A, lower row, left and middle). Neuronal processes of hemin-treated cells were no longer intact (Figure 4A, middle and lower row, right pictures). When we quantified the number of cells with either necrotic or apoptotic morphology, we found a significant increase in necrotic cells after hemin treatment, while we did not detect any apoptotic bodies (Figure 4B).

Because the sole positive morphological criterion for ferroptosis is shrunken mitochondria, we quantified mitochondrial size after hemin treatment. We did not find any shrunken mitochondria in any of the cells examined. In contrast, mean
mitochondrial size was larger in hemin-treated cells, although not statistically significant (Figure 4C); that is, the proportion of larger mitochondria increased, while that of smaller mitochondria decreased (Figure 4D).

**Hemin Induces Regulated Necrosis in Cortical Neurons by Recruiting Both Ferroptotic and Necroptotic Cell Death Mechanisms**

Because hemin-induced cell death shares pharmacological and molecular features of both ferroptosis and necroptosis, in the presence of ultrastructural features of necr(opt)osis, but not ferroptosis at 16 hours, we hypothesized that ferroptosis may induce an effector phase of death, leading to a necroptotic effector phase. If this were true, we predicted that the necroptosis inhibitor should have a longer therapeutic window than the ferroptosis inhibitors and that inhibiting ferroptosis should abrogate the increase in RIP1 and RIP3 gene expression and phospho-RIP1.

To assess the therapeutic window, primary neurons were treated with chemical inhibitors starting every 2 hours between 0 and 12 hours after hemin treatment. Necrostatin-1 was protective when added ≤ 8 hours. Similarly, Ferrostatin-1, Trolox, and U0126 abrogated hemin toxicity ≤ 8 hours. In contrast, the ferroptosis inhibitors N-acetylcysteine and deferoxamine...
had shorter protective windows of 2 and 4 hours, respectively (Figure 5A).

In line with the finding that the therapeutic window of Necrostatin-1 is not wider than that of the ferroptosis inhibitors, U0126 also failed to abrogate the increases in RIP1 (Figure 5B) and RIP3 (Figure 5C) gene expression and phospho-RIP1 (Figure 5D) after hemin treatment. Together, these data suggest that ferroptosis is not followed by downstream necroptosis in hemorrhagic stroke in vitro.

Alternatively, hemoglobin or hemin might elicit necroptotic (RIP1/3) or ferroptotic (phospho-ERK1/2) cell death mechanisms in parallel. If this is true, we expected that inhibition of both simultaneously would have an additive effect. Therefore, we investigated whether a subthreshold dose of U0126 would have an additive or synergistic effect on the dose–response of Necrostatin-1 protection. We found that addition of 0.5 μmol/L U0126 did not change the profile of protection by Necrostatin-1 (Figure 6A and 6B). This suggests that there might be a common yet to be identified denominator onto which both ferroptosis and necroptosis converge, which then leads to neuronal necroptosis (Figure 6C).

**Discussion**

The objective of this study was to use the leverage of an in vitro model of secondary injury in hemorrhagic stroke in cultured neurons to enhance understanding of cell death mechanisms after ICH that will allow more targeted and effective therapeutic strategies for this often fatal and highly morbid disease. We conclude that established toxic mediators from lysed blood, hemin, and hemoglobin, induced death in primary cortical neurons via features of ferroptotic or necroptotic cell death based on several criteria.

First, with the exception of cycloheximide, a panel of inhibitors of ferroptosis abrogated hemin- and hemoglobin-induced death (Figures 1 and 2). The overlap between hemorrhagic stroke in vitro and ferroptosis in terms of pharmacological inhibitors was large enough that cell death induced by hemin in primary neurons can be considered ferroptotic. Second, biochemical features consistent with ferroptosis (enhanced phospho-ERK1/2) were observed in HT22 cells exposed to hemin or 100 ng/mL tumor necrosis factor (TNF)α+5 μmol/L z-VAD-fmk for 8 hours (positive control). Necrostatin-1 served as confirmation for specificity of RIP1 kinase activity. Values represent median. *P<0.05 vs 0 hours hemin, ‡P<0.05 vs 8 h TNFα/zVAD.
Figure 4. Hemin-induced death in cortical neurons shows ultrastructural features of necrosis/necroptosis. A, Electron microscopic images of vehicle- (upper row) or hemin-treated cells (for 16 hours, middle and lower row). Some of the hemin-treated cells presented a necrotic phenotype with loss of plasma membrane integrity and disintegration of organelles (middle row, left and middle picture). Other hemin-treated cells displayed intact plasma membranes, but larger mitochondria (lower row, left and middle picture). Neuronal processes were no longer intact (middle and lower row, right pictures). Scale bars =500 nm. B, Hemin treatment increased the number of necrotic cells in culture. Note: we did not detect any apoptotic bodies in hemin-treated cells. Values represent mean±SEM. (as this is the convention in the electron microscopy field). *P<0.05 vs vehicle. C, Hemin treatment increased the mean percentage area of cytoplasm covered by mitochondria. D, Similarly, the frequency of appearance of larger mitochondria increased while the frequency of smaller mitochondria decreased. Values represent mean±SEM.
Figure 5. Ferroptosis is not upstream of necroptotic death signaling after hemin toxicity. A, Therapeutic window of protective ferroptosis and necroptosis inhibitors are shown. Primary neurons were treated with chemical inhibitors starting every 2 hours between 0 and 12 hours after hemin treatment and cell viability assessed at 16 hours. Values represent mean±SD, except for deferoxamine where medians are given. *P<0.05 vs hemin. B, RIP1 and (C) RIP3 mRNA expression was measured in primary neurons exposed to hemin and U0126 or its inactive analog U0124 for 8 hours. Values represent medians. *P<0.05 vs vehicle without hemin. D, Levels of phospho-RIP1 (normalized to β-actin) were measured in HT22 cells exposed to hemin and U0126 or 100 ng/mL tumor necrosis factor (TNF)α+5 μmol/L z-VAD-fmk for 8 hours (positive control). Necrostatin-1 served as confirmation for specificity of RIP1 kinase activity. Values represent median. *P<0.05 vs 0 hours.
necroptosis (Figure 4). In line with these morphological changes, the canonical necroptosis inhibitor, Necrostatin-1, but not its inactive analog prevented hemin- or hemoglobin-induced death in cortical neurons (Figures 1 and 2), and biochemical features of necroptosis (enhanced RIP1 and RIP3 mRNA expression and phospho-RIP1) were detected (Figure 3). Altogether, these data support the conclusion that experimental ICH induces cell death with features of ferroptosis and necroptosis.

Our studies raised the possibility that hemin or hemoglobin induce a ferroptotic affector pathway of death, leading to a necroptotic effector phase of cell death (Figure 6C). Contrary to this model, however, the therapeutic window for the necroptosis inhibitor Necrostatin-1 was not wider than the ferroptotic inhibitors Ferrostatin-1 and U0126. Moreover, U0126 did not abrogate the increase in RIP1 and RIP3 gene expression or phospho-RIP1 after hemin treatment (Figure 5). Although we cannot definitively exclude the possibility that the therapeutic window reflects differences in the time required for ferroptotic and necroptotic inhibitors to reach steady-state intracellular concentrations in vitro, the data suggest that necroptotic or ferroptotic signaling induced by hemin or hemoglobin is sufficient to reach a threshold of death that leads to neuronal necrosis. Inhibition of either one of these pathways can bring neurons below that threshold to survival (Figure 6). To our knowledge, hemin and hemoglobin are the first death stimuli described to involve a mixture of necroptotic and ferroptotic features.

Ferroptosis has not previously been shown to mediate ICH-induced cell death. However, 4 genes induced by a ferroptotic stimulus in cancer cells (ie, iron response element binding protein 2 [Ireb2], ATP synthase F0 complex subunit C3 [ATP5G3], citrate synthase [C3], and ATP ribosomal protein L8 [Rpl8]) are also induced after ICH in mice. Furthermore, Regan et al demonstrated that hemin activates ERK1/2 in astrocytes. This is in line with our findings demonstrating an
increase in phospo-ERK1/2 in neurons in vitro after hemin exposure and in vivo after ICH (Figure 3).

Other studies support the idea that necroptosis is at least partly involved in ICH. In vitro, pretreatment with Necrostatin-1 protects astrocytes subjected to hemin treatment for 5 hours.37 In a mouse model of ICH, intracerebroventricular administration of necrostatin-1 reduces cell death, brain edema, hematoma volumes, and neurological score deficits.38 Moreover, expression of RIP3 increases after ICH, while pretreatment with Necrostatin-1 diminishes the interaction of RIP3 with RIP1 and ameliorates cell death after ICH.39 Mice deficient in RIP3 show decreased cell death after ICH.40 This is consistent with our findings that RIP1 and RIP3 gene expression as well as phospo-RIP1 levels are increased after ICH in vitro and in vivo (Figure 3).

The apoptotic contribution to ICH-induced cell death has to date been unclear. Regan et al36 showed that the pan-caspase inhibitor z-VAD-fmk reduced cell death of cortical astrocytes at 4 hours but not at 8 hours after hemin treatment. Conversely, Laird et al37 demonstrated that pretreatment with z-VAD-fmk failed to protect after 5 hours of hemin toxicity in cortical astrocytes. Co-treatment with z-VAD-fmk increased cellular viability of microvascular cells after 18 hours of hemin treatment.41 In contrast, in human neuron-like cells exposed to hemin, z-VAD-fmk had no effect on cell viability.42 While hemoglobin treatment of rat cortical neurons induced upregulation of cleaved caspase-3 and increased caspase-3-like enzyme activity, pretreatment with z-VAD-fmk and other caspase inhibitors did not prevent cell death.43 After ICH in vivo, immunohistochemical expression of cleaved caspase-3 increased in rats44 and mice45 as did expression of apoptosis-related genes in rats.45

Similarly, the role of autophagy after ICH is not clear. Yang et al46 found that treatment of microglia with lysed blood in vitro resulted in toll-like receptor 4-mediated autophagy. Activated microglia caused neuronal death that was attenuated by 3-methyladenine. When administered intracerebroventricularly immediately after ICH onset, 3-methyladenine reduced microglia activation and brain damage.46 Furthermore, ICH induced immunohistochemical expression of the autophago-some marker light chain 3-II (LC3-II) in mice.38 Similarly, He et al47 reported an increase in LC3-II, the lysosomal marker cathepsin D, and vacuole formation after ICH in rats.

Surprisingly, when we investigated the morphological phenotype of cell death after hemin-induced neuronal toxicity, we found a necrotic phenotype with swelling of organelles and cell bodies and disruption of the plasma membrane. We did not observe any shrunken mitochondria, characteristic for ferroptosis, or apoptotic bodies (Figure 4). However, in animal models of ICH and in the perihematoma region after surgical evacuation in humans, both apoptotic and necrotic cells have been described.39,40 Because our morphological analysis was confined to hemin toxicity in neurons, the contribution of other cell death mechanisms in other cell types in vivo cannot be excluded.

The lack of protection by canonical, broad caspase inhibitors in neurons exposed to hemin, along with the necrotic morphology, seems to exclude apoptosis as a mechanism of death in vitro. Future studies will provide a head-to-head comparison of necroptotic, apoptotic, and ferroptotic inhibitors in vivo. It is likely that these studies will require intraventricular injections of drugs as the blood–brain barrier penetrability of many inhibitors is unclear. Our prediction is that ferroptotic and necroptotic inhibitors will be most effective. Consistent with this notion, the ferroptosis inhibitors deferoxamine48 and N-acetylcysteine (unpublished results) provide significant functional recovery in some models of ICH.

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Disclosures

The authors declare the following competing financial interest: P.J. Gough, Dr Bertin, and J. Finger are all employees and shareholders of GlaxoSmithKline. The other authors report no conflicts.

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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 (#C6000), 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 (#16140163), 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, A, DPQ, Erastin, propylene oxide (#20401) were purchased from Electron Microscopy Sciences.

**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-η²=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-η²=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-η²=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-η²=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, χ²(8,N=82)=55.242, p<0.001, η²=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 α=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 α=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-η²=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-η²=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; Levene test, F(4,15)=2.157, p=0.124; one-way ANOVA, F(4,15)=6.869, p=0.002, partial-η²=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; Levene test, F(5,30)=2.331, p=0.067; one-way ANOVA, F(5,30)=50.071, p<0.001, partial-η²=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; Levene test, F(4,15)=0.996, p=0.440; one-way ANOVA, F(4,15)=7.652, p=0.001, partial-η²=0.671; posthoc Bonferroni p=0.002

• U0126: Kolmogorov-Smirnov test, Z=1.438, p=0.032; Levene test, F(8,45)=7.941, p<0.001; Kruskal-Wallis test, χ²(8,N=54)=47.158, p<0.001, η²=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 α=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; Levene test, F(8,36)=1.342, p=0.255; one-way ANOVA, F(8,36)=39.220, p<0.001, partial-η²=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; Levene test, F(5,24)=2.684, p=0.046; Kruskal-Wallis test, χ²(5,N=30)=14.790, p=0.011, η²=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 α=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 α=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; Levene test, F(5,36)=4.112, p=0.005; Kruskal-Wallis test, χ²(5,N=42)=31.457, p<0.001, η²=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 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 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; Levene test, F(4,28)=1.740, p=0.169; one-way ANOVA, F(4,28)=4.288, p=0.008, partial-η²=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; Levene test, F(4,40)=17.916, p<0.001; Kruskal-Wallis test, χ²(4,N=45)=31.323, p<0.001, η²=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 α=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 α=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; Levene test, F(4,40)=9.425, p<0.001; Kruskal-Wallis test, χ²(4,N=45)=14.449, p=0.006, η²=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 α=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; Levene 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; Levene 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; Levene test, F(8,63)=5.049, p<0.001; Kruskal-Wallis test, χ²(8,N=72)=40.666, p<0.001, η²=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 α=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 α=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, χ²(7,N=56)=34.678, p<0.001, η²=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, χ²(7,N=42)=28.583, p<0.001, η²=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-η²=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-η²=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-
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; Levene test, F(7,37)=0.699, p=0.673; one-way ANOVA, F(7,37)=10.982, p<0.001, partial-η²=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; Levene test, F(5,42)=5.902, p<0.001; Kruskal-Wallis test, χ²(5,N=48)=19.411, p=0.002, η²=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; Levene test, F(5,42)=14.034, p<0.001; Kruskal-Wallis test, χ²(5,N=48)=37.207, p<0.001, η²=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 TNFa + 5μM zVAD, and 1.370 at 8h TNFa/zVAD + Nec-1; Kolmogorov-Smirnov test, Z=1.865, p=0.002; Levene test, F(9,59)=10.257, p<0.001; Kruskal-Wallis test, χ²(9,N=69)=30.598, p<0.001, η²=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 α=0.05/k, with k=9 (comparison of 4, 8, and 12 hours hemin, and 8 hours TNFa/zVAD to 0 hours and respective time point + 10μM U0126 or 100μM Necrostatin-1).
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 + 10\( \mu \)M 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 0.5\( \mu \)M 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 10\( \mu \)M U0126 and 10, 50, and 100\( \mu \)M Necrostatin-1 to hemin treatment alone; cotreatment of 0.5\( \mu \)M 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 10\( \mu \)M U0126, \( p = 0.009 \) for 100\( \mu \)M Necrostatin-1 vs. hemin alone, \( p = 0.565 \) for 10\( \mu \)M Necrostatin-1 + 0.5\( \mu \)M U0126 vs. 10\( \mu \)M Necrostatin-1, \( p = 0.085 \) for 50\( \mu \)M Necrostatin-1 + 0.5\( \mu \)M U0126 vs. 50\( \mu \)M Necrostatin-1, \( p = 0.225 \) for 100\( \mu \)M Necrostatin-1 + 0.5\( \mu \)M U0126 vs. 100\( \mu \)M Necrostatin-1.

- B, cell survival Necrostatin-1 dose-response with 0.5\( \mu \)M 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 100\( \mu \)M Necrostatin-1 ± 0.5\( \mu \)M U0126 and 10\( \mu \)M U0126, \( p = 1.000 \) for 10, 50, and 100\( \mu \)M Necrostatin-1 ± 0.5\( \mu \)M U0126.
# Stroke Online Supplement

## Table 1. Checklist of Methodological and Reporting Aspects for Articles Submitted to Stroke Involving Preclinical Experimentation

<table>
<thead>
<tr>
<th>Methodological and Reporting Aspects</th>
<th>Description of Procedures</th>
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| 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 prior 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.  
  - Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms.  
  - 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.  
  - Statements on approval by ethics boards and ethical conduct of studies have been provided.  
  - Statements on funding and conflicts of interests have been provided.  
  - N/A                                                                                                                                                                                                                   |