

Synchronized renal tubular cell death involves ferroptosis

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Receptor-interacting protein kinase 3 (RIPK3)-mediated necroptosis is thought to be the pathophysiologically predominant pathway that leads to regulated necrosis of parenchymal cells in ischemia–reperfusion injury (IRI), and loss of either Fas-associated protein with death domain (FADD) or caspase-8 is known to sensitize tissues to undergo spontaneous necroptosis. Here, we demonstrate that renal tubules do not undergo sensitization to necroptosis upon genetic ablation of either FADD or caspase-8 and that the RIPK1 inhibitor necrostatin-1 (Nec-1) does not protect freshly isolated tubules from hypoxic injury. In contrast, iron-dependent ferroptosis directly causes synchronized necrosis of renal tubules, as demonstrated by intravital microscopy in models of IRI and oxalate crystal-induced acute kidney injury. To suppress ferroptosis in vivo, we generated a novel third-generation ferrostatin (termed 16-86), which we demonstrate to be more stable, to metabolism and plasma, and more potent, compared with the first-in-class compound ferrostatin-1 (Fer-1). Even in conditions with extraordinarily severe IRI, 16-86 exerts strong protection to an extent which has not previously allowed survival in any murine setting. In addition, 16-86 further potentiates the strong protective effect on IRI mediated by combination therapy with necrostatins and compounds that inhibit mitochondrial permeability transition. Renal tubules thus represent a tissue that is not sensitized to necroptosis by loss of FADD or caspase-8. Finally, ferroptosis mediates posts ischemic and toxic renal necrosis, which may be therapeutically targeted by ferrostatins and by combination therapy.

regulated cell death | programmed cell death | ferroptosis | necroptosis | apoptosis

Regulated cell death may result from immunologically silent apoptosis or from immunogenic necrosis (1–3). Necroptosis, the best-characterized pathway of regulated necrosis, involves activation of receptor-interacting protein kinase 3 (RIPK3)-mediated phosphorylation of mixed lineage kinase domain-like protein (pMLKL) and subsequent plasma-membrane rupture, which was demonstrated in several disease states, including ischemia–reperfusion injury (IRI) in all organs analyzed (2–6); however, none of these previous studies clearly investigated the mode of cell death in the primary parenchymal cells. Therefore, it remained possible that the protective effects reported upon application of the necroptosis inhibitor necrostatin-1 (Nec-1) and for RIPK3-ko mice involve vascular, nonparenchymal effects. This possibility has been ruled out in non-IRI settings by conditional tissue targeting of proteins involved in the prevention of

spontaneously occurring necroptosis, such as RIPK1, and components that regulate its ubiquitinylation status [linear ubiquitinylation chain assembly complex (LUBAC), cellular inhibitors of apoptosis proteins (cIAPs), caspase-8, and Fas-associated protein with death domain (FADD)] in the gastrointestinal tract (7, 8), the skin (9, 10), the liver (11), and immune cells (12, 13), all of which result in spontaneous RIPK3-mediated tissue necroptosis and inflammation (7–9, 11, 12, 14–17).

Ferroptosis is an iron-dependent necrotic type of cell death that occurs due to lipid peroxide accumulation, which routinely is prevented by glutathione peroxidase 4 (GPX4), a glutathione-(GSH)-dependent enzyme, and therefore depends on the functionality of a glu/cys antiporter in the plasma membrane referred to as system X_c-minus (18–20). Ferroptosis has been reported to cause several diseases and may be interfered with in vitro by the small molecule ferrostatin-1 (Fer-1) (18); however, Fer-1 was suggested to have low in vivo functionality due to potential metabolic and plasma instability.

Significance

Cell death by regulated necrosis causes tremendous tissue damage in a wide variety of diseases, including myocardial infarction, stroke, sepsis, and ischemia–reperfusion injury upon solid organ transplantation. Here, we demonstrate that an iron-dependent form of regulated necrosis, referred to as ferroptosis, mediates regulated necrosis and synchronized death of functional units in diverse organs upon ischemia and other stimuli, thereby triggering a detrimental immune response. We developed a novel third-generation inhibitor of ferroptosis that is the first compound in this class that is stable in plasma and liver microsomes and that demonstrates high efficacy when supplied alone or in combination therapy.

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faint plasma membrane of untreated tubules did not change in 16-86-treated tubules [a novel third-generation ferrostatin we generated (Fig. 3)], vehicle-treated and especially erastin-treated tubules showed membrane protrusions and obvious signs of deformation and functional insufficiency (Fig. 2B). When erastin was applied into the tubule, a similar type of cell death occurred even without fatty-acid depletion (Movie S4). In another well-established lactate dehydrogenase (LDH) release-based assay of ex vivo tubulotoxicity (29), hydroxyquinoline-iron-induced cell death, we found Fer-1 and the Nox-inhibitor GKT to be protective whereas Nec-1 did not show any protection (Fig. 2C).

Ferroptosis Mediates IRI-Mediated Immune-Cell Infiltration of the Cremaster Muscle. The concept of immunogenic necrotic cell death proposes to trigger an autoamplification loop, which is triggered by a necroinflammatory microenvironment. To understand how ferroptosis attracts immune cells during hypoxia/reperfusion situations, we performed musculus cremaster IRI assays and read out the immune-cell infiltration by intravital microscopy (Fig. 2D–F) and quantified rolling, adherent, and transmigrated cells in the presence and absence of ferrostatins (Fig. 2F and G and Fig. S4A and B). Less immune-cell infiltration into the postischemic area suggested either that Fer-1 directly inhibits leukocyte transmigration or that the local proinflammatory microenvironment was less chemoattractive, presumably by less damage-associated molecular pattern (DAMP) release due to less ferroptotic cell death.

Ferroptosis Mediates Tubule Necrosis in Oxalate Nephropathy, but Not in an LPS-Induced Septic Shock Model. Having identified ferroptosis to be of relevance in acute postischemic injury, we sought to investigate another model of acute renal failure, oxalate nephropathy, which has recently been established (30). Intrarenal CaOx crystal deposition was identical in all groups (Fig. S5A and B). However, neutrophil infiltration and expression levels of proinflammatory cytokines (CXCL-2, IL-6), kidney injury molecule 1 (KIM-1), and the p65 subunit of NF- κ B were statistically significantly reduced upon a once daily i.p. application of Fer-1 (Fig. S5E). Importantly, Fer-1 also significantly reduced the tubular injury score (Fig. S5D) and the functional serum markers of kidney injury creatinine and urea (Fig. S5C). Because RIPK3-deficient mice have been demonstrated to be resistant to sepsis models, we also investigated Fer-1 in the model of LPS-induced shock (Fig. S5F), but no difference compared with vehicle-treated mice was evident. Therefore, ferroptosis seems to be a valuable target for both postischemic and toxic acute kidney injury. However, given the poor plasma stability of Fer-1, we sought to develop a more stable compound for the in vivo applications.

Generation of a Third-Generation Ferrostatin with Increased Plasma and Metabolic Stability for in Vivo Studies. Given the promising results of the cremaster-IRI and oxalate nephropathy studies and the previously published efficacy of second-generation ferrostatins in models of Huntington's disease, periventricular leukomalacia, and tubular necrosis (29), we wondered whether the reported poor plasma stability of Fer-1 could be further enhanced by structure-relation analysis. We therefore first evaluated the in vitro metabolic stability of one of the highly potent Fer-1 analogs, SRS15-72B (29), in liver microsomes of mice. SRS15-72B was cleared rapidly with a half-life of $t_{1/2} = 2$ min (Table S1). To achieve a more metabolically stable Fer-1 analog, we created an imine-type structure (SRS15-72A) that showed high stability in microsomes with a half-life of $t_{1/2} = 154$ min (Table S2). This metabolically stable analog turned out not to be sufficiently stable in the plasma due to its ethyl ester moiety (Table S3), a functional group well-known to be more susceptible to hydrolysis in plasma (31). Because a previous study showed that the ethyl ester is important to maintain the potency of

Fer-1, we aimed at improving the plasma stability, while maintaining high microsomal stability by creating additional imine analogs with isopropyl (SRS16-80) and *tert*-butyl (SRS16-86) esters (Fig. 3A). These analogs allowed us to comparatively study the plasma stability of the ethyl, isopropyl, and *tert*-butyl esters, respectively. Among these three ester analogs (SRS15-72A, SRS16-80, and SRS16-86), the *tert*-butyl ester analog (SRS16-86) showed the highest stability in plasma (Table S4). In addition, SRS16-86 maintains high stability in the microsomal compartment [intrinsic clearance (CL_{int}) (mL/min per g liver < 0.5)] (Table S5). For in vivo experiments, we therefore used 16-86 and used it in comparison with 16-79, an inactive derivate (Fig. 3B). We confirmed the ferroptosis-inhibiting ability of 16-86 and 16-79 in vitro by FACS analysis of erastin-treated HT-1080 cells and in NIH 3T3 cells (Fig. 3C). In both necroptosis and ferroptosis, we failed to detect cleaved caspase 3 (Fig. 3D).

Ferroptosis Mediates Necrotic Tubular Cell Death in Renal Ischemia-Reperfusion Injury. We applied active (16-86) and inactive (16-79) compounds to a model of severe ischemia-reperfusion injury (IRI), which we described before (4). Upon 40 min of ischemia before reperfusion, we know that all WT mice die between 48 h and 72 h, which can be anticipated from the 48-h serum creatinine values above 2.0 mg/dL. As demonstrated in Fig. 4A–D, creatinine levels of all vehicle-treated C57BL/6 mice exceeded 2.0 mg/dL and showed strong morphologic damage and high serum urea levels whereas Fer-1-treated and, to a greater extent,

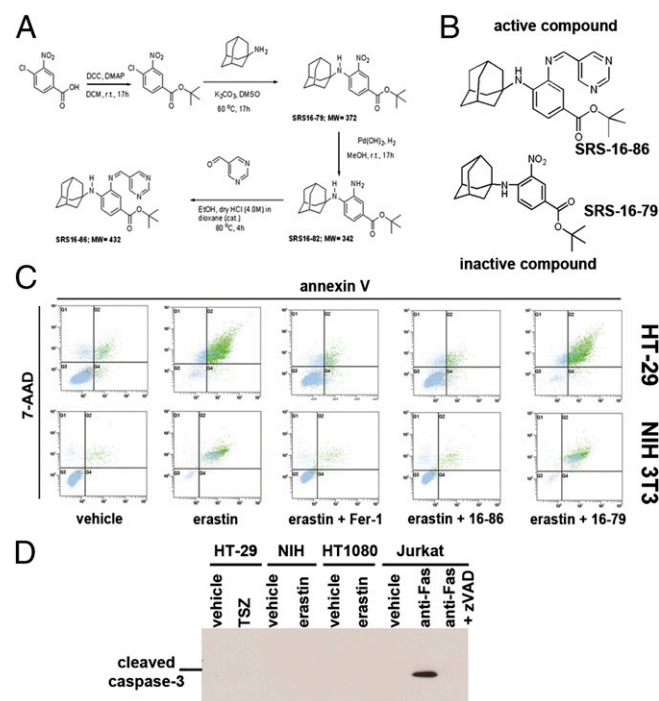


Fig. 3. Generation and in vitro testing of a ferrostatin derivative for in vivo applications. (A) Synthetic route of the most microsomal and plasma stable ferrostatin analog (SRS16-86). (B) Model structure of the two novel ferrostatin derivatives SRS16-79 (inactive compound) and SRS16-86 (active compound). (C) FACS analysis for the necrotic marker 7AAD and phosphatidylserine accessibility (annexin V) in HT1080 and NIH 3T3 cells treated with either vehicle or 50 μ M erastin in the presence or absence of 1 μ M Fer-1, 16-86, or 16-79. (D) Absence of cleaved caspase-3 in necroptosis and ferroptosis. Western blot of cleaved caspase-3 in lysates from 100 ng/mL TNF α plus 1 μ M Smac mimetics plus 25 μ M zVAD-treated HT-29 cells, 50 μ M erastin-treated NIH 3T3 cells, and 50 μ M erastin-treated HT1080 cells for 24 h. Monoclonal anti-Fas-treated Jurkat cells (100 ng/mL) served as positive control. TSZ, TNF α /SMAC-mimetic/zVAD.

16-86-treated mice were protected from functional acute renal failure (Fig. 4 *C* and *D*) and structural organ damage (Fig. 4 *A* and *B*) after IRI. Mice treated with 16-79 in a similar manner did not show any differences compared with the vehicle-treated controls. We did not observe any side effects from treatment with the same dose of 16-86 4 wk after application. The effect of 16-86 exceeded that of any single compound we previously experienced to be protective from renal IRI.

Ferrostatis Further Increase the Protective Effect of [Necrostatin-1/Sanglifehrin A] Combination Therapy in Renal IRI. Given that Nec-1 protects from renal IRI to a lesser extent than 16-86, and given that interference with mitochondrial permeability transition (MPT)-induced regulated necrosis (MPT-RN) by the compound sanglifehrin A (SfA) also mildly protects from IRI, with marked additive protective effects in combination therapy with Nec-1 (4, 5), we sought to further extend the combination therapy by investigating the influence of 16-86 and 16-79 in [Nec-1 + SfA]-treated mice. Because the effect of the [Nec-1 + SfA] treatment could be investigated only in a severe ischemia model and we aimed to investigate additive protective effects by 16-86, we further increased the ischemic duration to a model of ultrasevere IRI (see *Materials and Methods* for details). In such settings, even the combination therapy with [Nec-1 + SfA] did not fully rescue creatinine values and organ damage (Fig. 4 *E–H*). Addition of 16-86, but not 16-79, reduced plasma levels of serum urea and serum creatinine, suggesting

that a triple combination therapy with [Nec-1 + SfA] plus 16-86 is superior in the prevention of renal IRI compared with the double-combination therapy with [Nec-1 + SfA]. In addition, this result indicates either that at least three independent pathways of regulated necrosis may be involved in IR-mediated organ damage or that inhibition of overlapping elements with SfA and Nec-1 are incomplete.

Discussion

Parenchymal cell necrosis, but not apoptosis, which seems to be minimally involved in the pathogenesis of acute kidney injury (32), triggers the release of DAMPs, some of which can trigger regulated necrosis and therefore initiate a necroinflammatory feedback loop. In clinical routine, immunosuppression is a standard procedure, but an anti-cell death therapy does not exist apart from cyclosporine (5). Therefore, defining the precise mechanisms that trigger regulated necrosis is essential for the development of new drugs. Our data indicate that alternative interpretations apart from “pure” necroptosis exist and suggest that ferroptosis is of importance in renal tubules, which, other than the skin, the gastrointestinal tract, or immune cells, do not depend on a necroptosis-inhibiting complex, at least not of FADD and caspase-8. To date, it cannot be ruled out that previously described protection against ischemia–reperfusion injury in diverse organs is mediated by increased perfusion rather than direct protection from parenchymal necroptosis. Our results regarding the cerulein-induced pancreatitis are in line with this

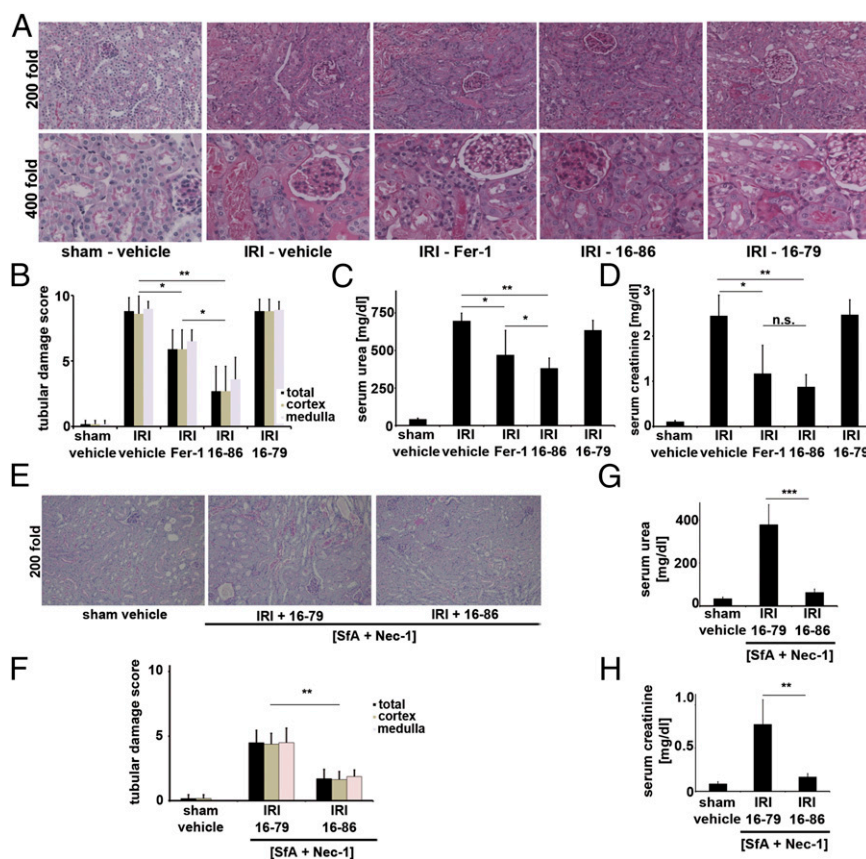


Fig. 4. Ferroptosis significantly contributes to renal ischemia–reperfusion injury. (*A* and *B*) Representative PAS stainings and quantification of renal damage from mice treated with severe ischemic durations before reperfusion. Mice were killed 48 h after reperfusion. (*C* and *D*) Functional markers of acute kidney injury after severe IRI (as in *A* and *B*). (*E* and *F*) Histologic PAS staining and quantification of sham operation or ultrasevere IRI (50 min of ischemia before reperfusion) in WT mice treated with [Nec-1 + SfA] combination therapy together with either 16-79 or 16-86. (*G* and *H*) C57BL/6 were treated as in *E*, and functional markers of acute kidney injury were measured 48 h after the onset of reperfusion. n.s., not significant; * $P = 0.05–0.02$, ** $P = 0.02–0.001$, *** $P \leq 0.001$; $n = 10$ per group in all experiments).

hypothesis and are in contrast to previously published data (33, 34). Several unanswered questions remain: Why are RIPK3-deficient mice protected in several models of IRI if the parenchymal cells do not undergo necroptosis? Why do necrostatins protect such tissues from organ failure if necroptosis is not the predominant mechanism of parenchymal cell death? Endothelial cell-specific deletion of RIPK3 will help to answer these open questions.

Materials and Methods

See *SI Materials and Methods* for detailed descriptions.

Mice. All WT mice reported in this study were on C57BL/6 background. Eight- to 12-wk-old male C57BL/6 mice (average weight ~23 g) were used for all WT experiments, unless otherwise specified. Caspase-8 fl/fl mice were kindly provided by Razuella Hakem (Department of Medical Biophysics, University of Toronto, Toronto and Ontario Cancer Institute, University Health Network, Toronto). FADD fl/fl mice were generated by and provided by M.P. Doxycyclin-inducible renal tubule-specific Pax8-rtTA; Tet-on-Cre mice have been published (28) and were kindly provided by Tobias B. Huber (Renal Division, University Medical Center Freiburg, Freiburg, Germany). RIPK3-deficient mice were kindly provided by Vishva M. Dixit (Genentech, San Francisco, CA). All in vivo experiments were performed according to the Protection of Animals Act, after approval of the German local authorities or the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan, and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (35), after approval from the University of Michigan IACUC or by the local authorities responsible for the approval at Ghent University. In all experiments, mice were carefully matched for age, sex, weight, and genetic background.

Histology, Immunohistochemistry, and Evaluation of Structural Organ Damage.

Organs were dissected as indicated in each experiment and infused with 4% (vol/vol) neutral-buffered formaldehyde, fixated for 48 h, dehydrated in a graded ethanol series and xylene, and finally embedded in paraffin. Stained sections were analyzed using an Axio Imager microscope (Zeiss). Kidney damage was quantified by two experienced pathol-

ogists in a double-blind manner on a scale ranging from 0 (unaffected tissue) to 10 (severe organ damage). The following parameters were chosen as indicative of morphological damage to the kidney after ischemia-reperfusion injury (IRI): brush border loss, red blood cell extravasation, tubule dilatation, tubule degeneration, tubule necrosis, and tubular cast formation. These parameters were evaluated on a scale of 0–10, which ranged from not present (0), mild (1–4), moderate (5 or 6), severe (7 or 8), to very severe (9 or 10). Each parameter was determined on at least four different animals.

Statistics. For all experiments, differences of datasets were considered statistically significant when *P* values were lower than 0.05, if not otherwise specified. Statistical comparisons were performed using the two-tailed Student *t* test. Asterisks are used in the figures to specify statistical significance (**P* < 0.05; ***P* < 0.02; ****P* < 0.001). *P* values in survival experiments (Kaplan–Meier plots) were calculated using GraphPad Prism, ver. 5.04 software. Statistics are indicated as SD unless otherwise specified.

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