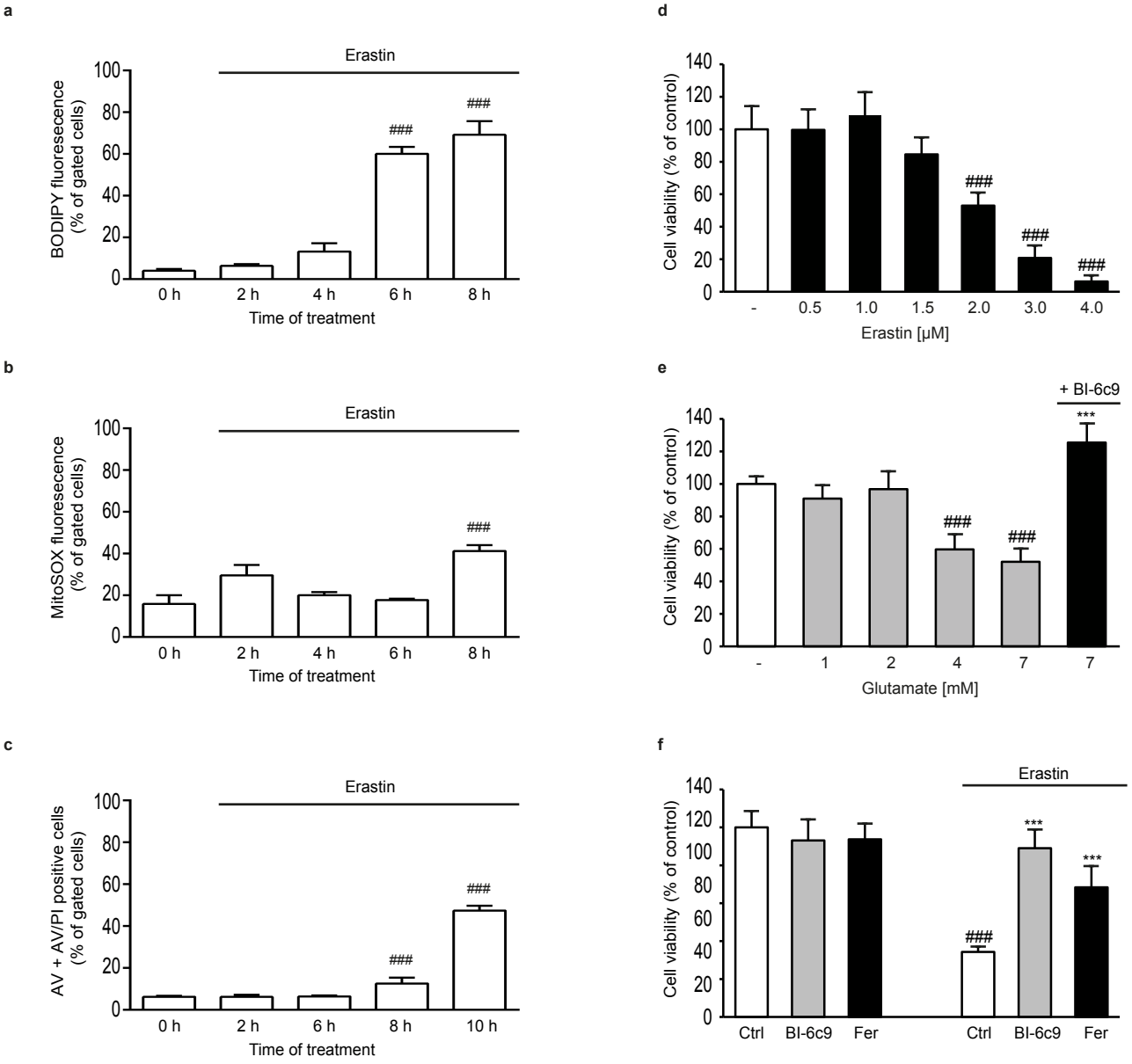
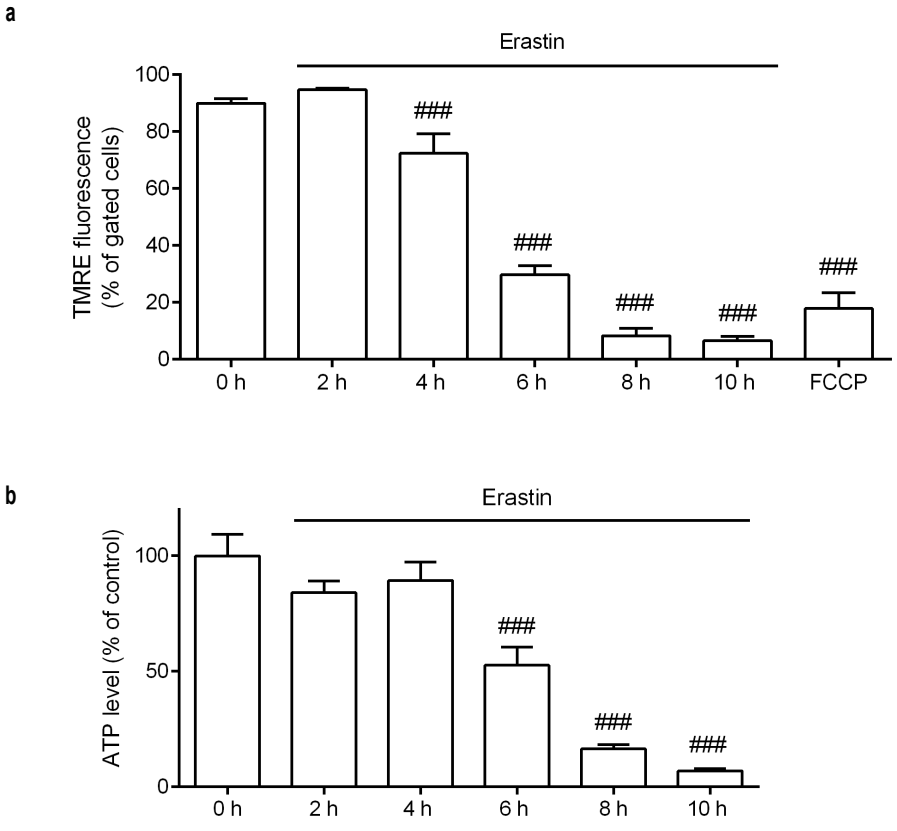
Supplemental figure 1: Ferrostatin-1 and BI-6c9 prevent oxytosis and ferroptosis in MEFs



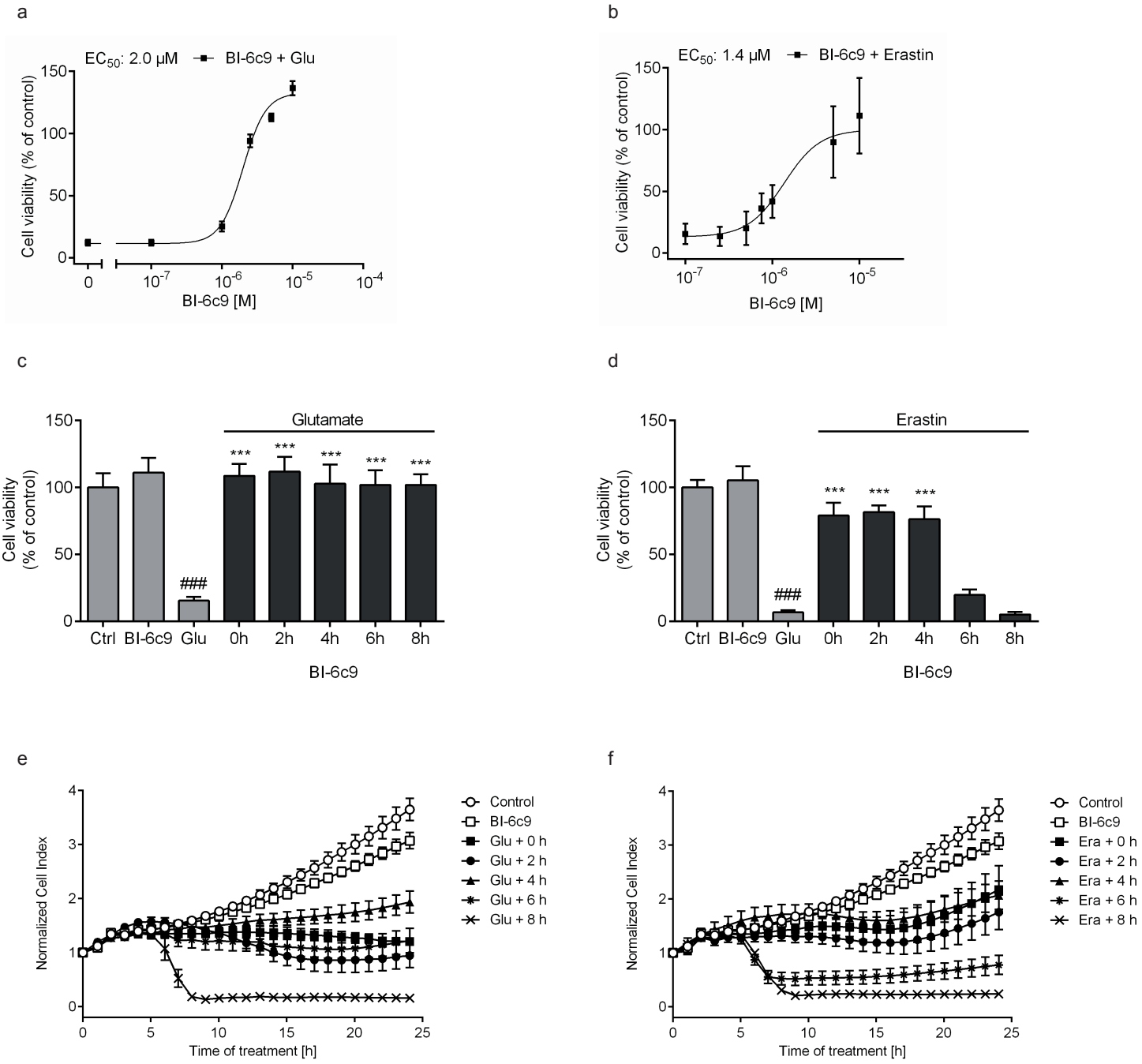
Supplemental figure 1: Ferrostatin-1 and BI-6c9 prevent oxytosis and ferroptosis in MEFs.
a: BODIPY staining and subsequent FACS analysis shows time-dependent increase in lipid peroxidation after erastin (1 µM) treatment in MEF cells. **b**: MitoSOX staining and subsequent FACS analysis revealed time-dependent mitochondrial ROS formation after erastin (1 µM) exposure in MEF cells. **c**: Annexin V/PI staining and subsequent FACS analysis showed time-dependent increase in Annexin V (Green fluorescence) and PI (Red fluorescence) positive cells after erastin (1 µM) exposure in MEF cells. **d**: MTT assay depicts dose-dependent toxicity of erastin in MEFs (17 h). n=8. **e**: MTT assay shows dose-dependent toxicity of glutamate in MEFs (17 h), which is prevented by BI-6c9. n=8. **f**: MTT assay revealed protection of BI-6c9 (10 µM) and ferrostatin-1 (2 µM) against erastin (1 µM, 16 h) toxicity (n=8). Data are given as mean + S.D. ###p<0.001 compared to untreated control; ***p<0.001 compared to erastin-/ glutamate-treated control (ANOVA, Scheffé's test).



Supplemental figure 2: TMRE and ATP time courses

a: TMRE staining and subsequent FACS analysis shows time-dependent loss of mitochondrial membrane potential after erastin (1 µM) treatment in HT-22 cells. FCCP (50 µM, 5 min) treatment is shown as a positive control. **b:** Measurement of ATP levels reveals a time-dependent loss of ATP production after erastin (1 µM) treatment in HT-22 cells. Data are given as mean + S.D. ###p<0.001compared to untreated control, ANOVA, Scheffé's test.

Supplemental figure 3: BI-6c9 prevents glutamate and erastin induced cell death

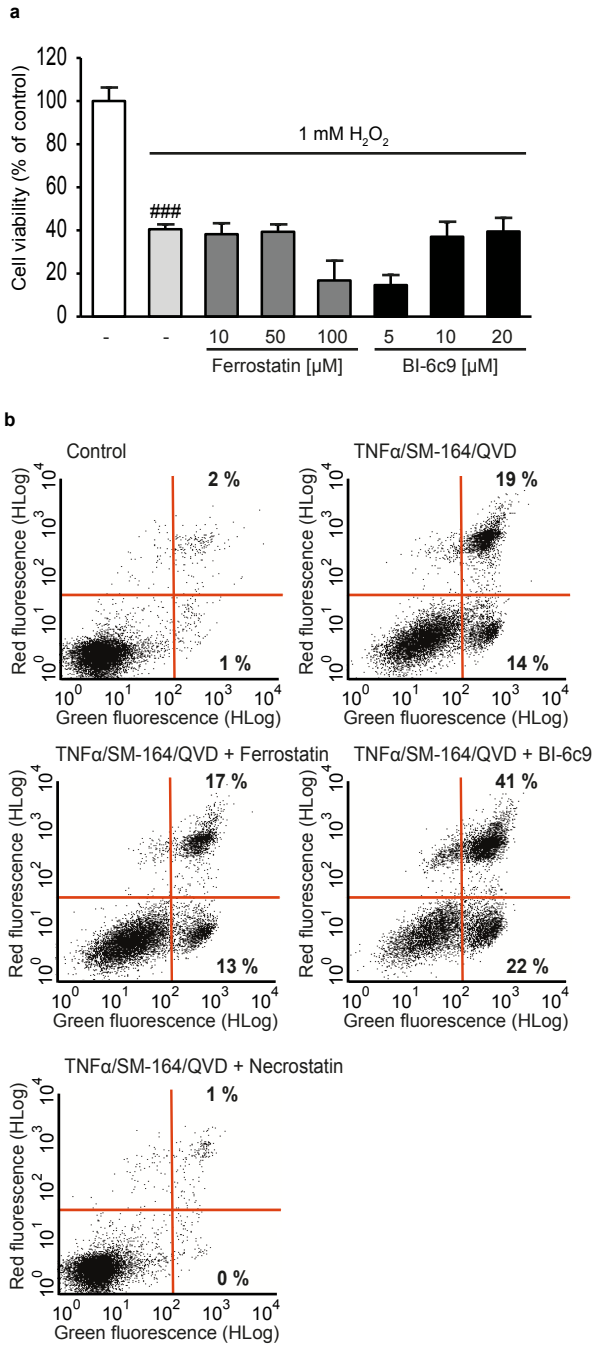


Supplemental figure 3: BI-6c9 prevents glutamate and erastin induced cell death

a: Concentration-response curve of BI-6c9 upon glutamate (3 mM, 14 h) treatment (EC_{50}= 2.0 µM, pEC_{50}= 5.70 ± 0.02, n=8). **b:** Concentration-response curve of BI-6c9 upon erastin (1.5 µM, 14 h) treatment. (EC_{50}= 1.4 µM, pEC_{50}= 5.87 ± 0.04, n=28). **c**: MTT assay revealed a protective effect of BI-6c9 (10 µM) until 8 hours after glutamate treatment (6 mM, 24 h) (n=8/treatment condition). **d**: MTT assay revealed a protective effect of BI-6c9 (10 µM) until 4 hours after erastin treatment (1 µM, 24 h) (n=8/treatment condition). **e**: Real-time impedance measurement with the Xcelligence system demonstrated a protective effect of BI-6c9 (10 µM) until 6 hours of glutamate treatment (10 mM). **f**: Real-time impedance measurement with the Xcelligence system demonstrated a protective effect of BI-6c9 (10 µM) until 4 hours of erastin treatment (1 µM).

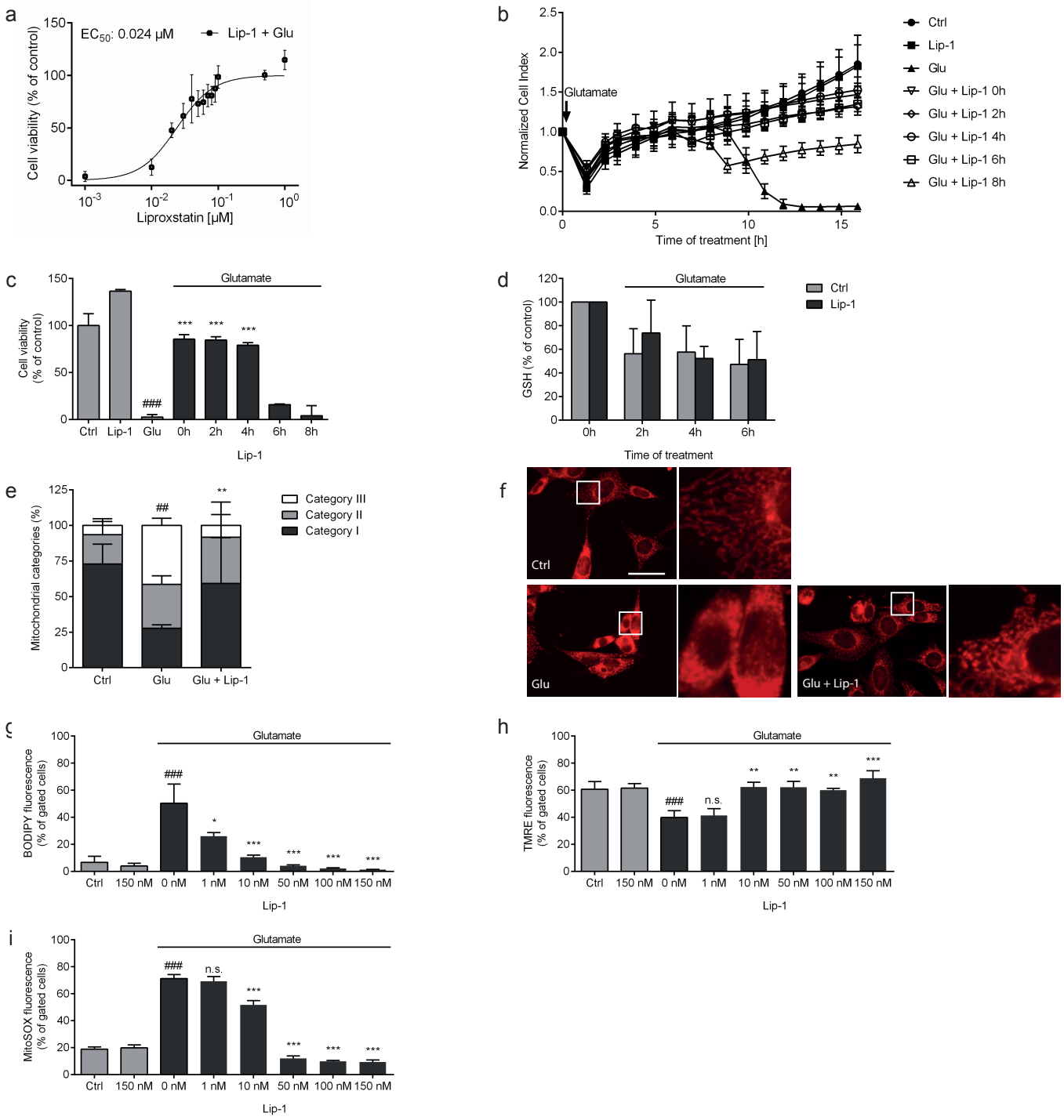
Data are given as mean + S.D. or ± S.D. ###p<0.001 compared to untreated control. ***p<0.001 compared to erastin-/ glutamate-treated control (ANOVA, Scheffé's test).

Supplemental figure 4: Ferrostatin-1 and BI-6c9 fail to prevent H_2O_2-induced cell death and necroptosis



Supplemental figure 4: Ferrostatin-1 and BI-6c9 fail to prevent H_2O_2-induced cell death and necroptosis.
a: MTT assay shows toxicity of H_2O_2 (1 mM, 16 h) in HT-22 cells which is not abolished by ferrostatin-1 or BI-6c9. n=8. b: Necroptosis induced by TNFα (100 ng/ml), SM-164 (50 nM) and QVD (10 µM) in HT-22 cells was analyzed by Annexin V/PI staining and subsequent FACS-analysis after 17 h of treatment. BI-6c9 (10 µM) and ferrostatin-1 (2 µM) fail to prevent necroptosis, while necrostatin fully abolishes cell death (representative FACS plots).

Supplemental figure 5: Liproxstatin-1 prevents glutamate-induced oxytosis

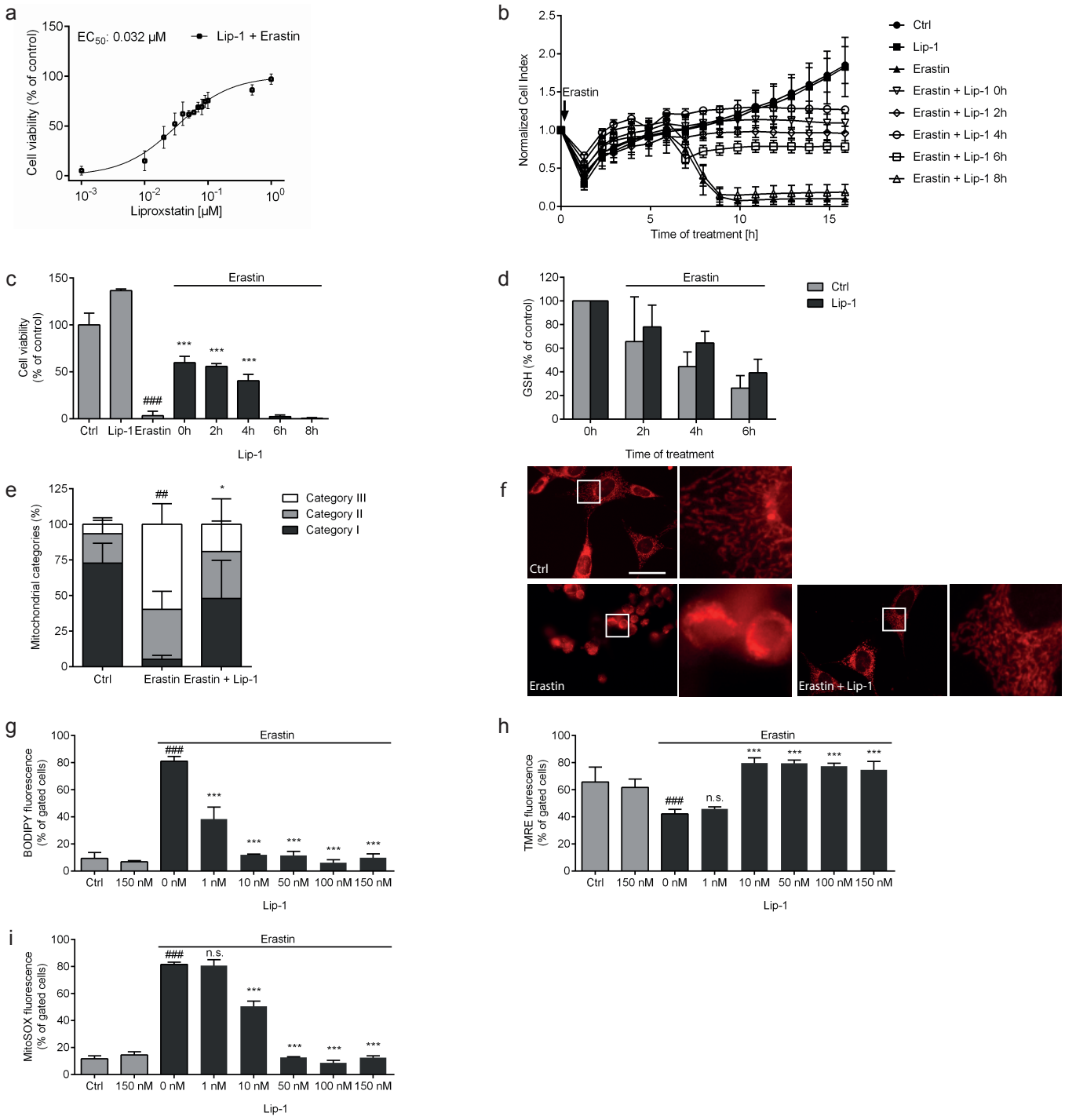


Supplemental figure 5: Liproxstatin-1 prevents glutamate-induced oxytosis

a: Concentration-response curve of Liproxstatin-1 (Lip-1) upon glutamate (5 mM, 16-17 h) treatment. Cell viability data were fitted from 0 for glutamate-treated control to 100 for highest protection at 1 µM Lip-1 (EC_{50}= 0.024 µM, pEC_{50}= 7.62 ± 0.04, n=3). **b:** Real-time impedance measurement revealed protection of Lip-1 (150 nM) against glutamate (6 mM) toxicity until 6-8 h post-treatment; (n=8/treatment condition). **c:** MTT confirmed protection of Lip-1 (150 nM) against glutamate (6 mM, 16-17 h) toxicity until 4-8 h post-treatment; (representative MTT assay; n=6/treatment condition). **d:** Measurement of glutathione (GSH) depicted rapid decrease of GSH after glutamate (6 mM) which could not be restored upon Lip-1 (150 nM) co-treatment; (n=4/treatment condition). **e:** Quantification of 500 cells counted blind to treatment (n=3) revealed Lip-1 dependent reduction of glutamate-induced (4 mM, 16 h) mitochondrial fission. **f:** Representative images (63x objective) showed mitochondrial morphology in the presence and absence of glutamate (4 mM, 16 h) ± Lip-1 (150 nM). Scale bar 50 µM. **g:** Glutamate treatment (8 mM, 8 h) increased lipid peroxide production (stained with BODIPY 581/591), which was blocked by co-treatment with concentrations of >10-50 nM Lip-1 (representative FACS measurement; n=3/treatment condition). **h:** Quantification of TMRE fluorescence showed that MMP was fully restored by >10 nM Lip-1 after glutamate exposure (8 mM, 16 h) (representative FACS measurement; n=3/treatment condition). **i:** Glutamate treatment (7 mM, 16 h) increased mitochondrial ROS production, which was blocked by co-treatment with concentrations of >10-50 nM Lip-1 (representative FACS measurement; n=3/treatment condition).

Data are given as mean ± S.D. ###p<0.001 compared to untreated control; ***p<0.001 compared to glutamate-treated control (ANOVA, Scheffe's test).

Supplemental figure 6: Liproxstatin-1 prevents erastin-induced ferroptosis

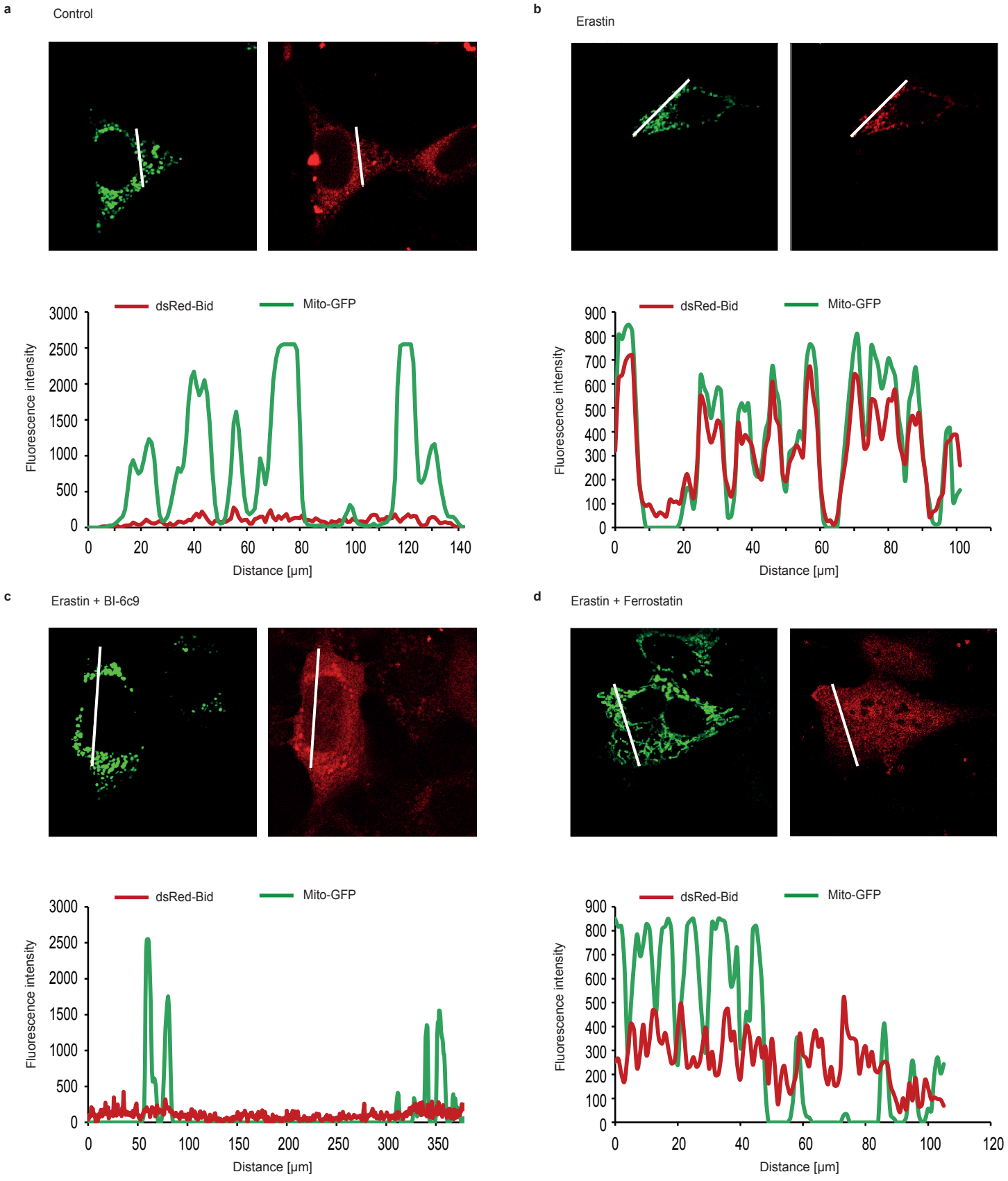


Supplemental figure 6: Liproxstatin-1 prevents erastin-induced ferroptosis

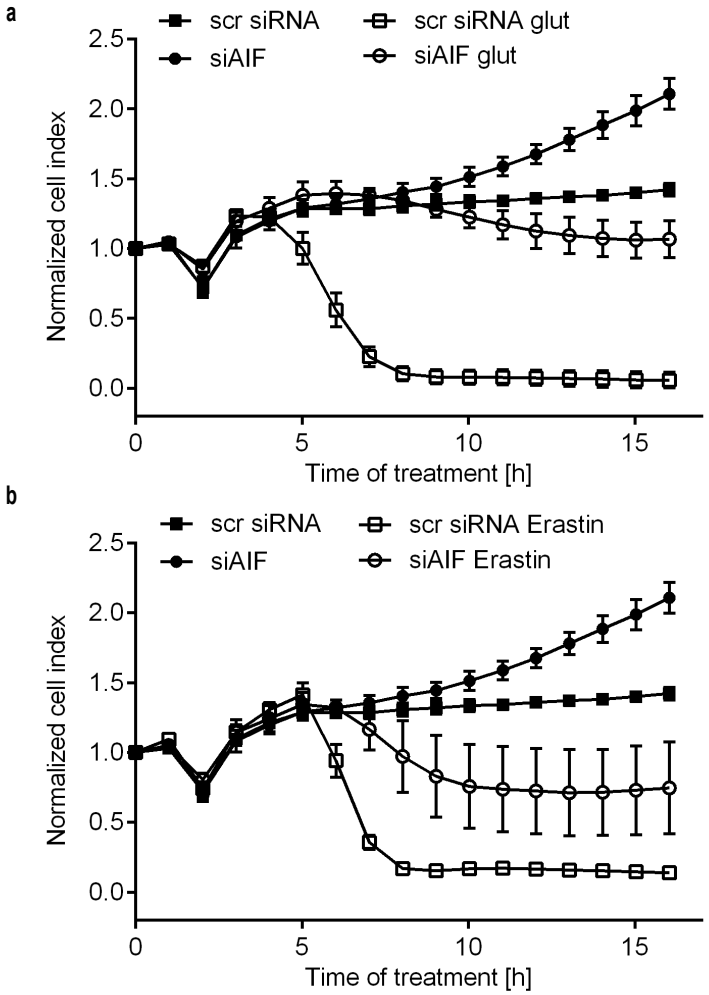
a: Concentration-response curve of Liproxstatin-1 (Lip-1) upon erastin (1 µM, 16-17 h) treatment. Cell viability data were fitted from 0 for erastin-treated control to 100 for highest protection at 1 µM Lip-1 (EC_{50}= 0.032 µM, pEC_{50}= 7.49 ± 0.03, n=3). **b:** Real-time impedance measurement revealed protection of Lip-1 (150 nM) against erastin (1 µM) toxicity until 4-6 h post-treatment; (n=8/treatment condition). **c:** MTT confirmed protection of Lip-1 (150 nM) against erastin (1 µM, 16-17 h) toxicity until 4 h post-treatment; (representative MTT assay; n=6/treatment condition). **d:** Measurement of glutathione (GSH) depicted rapid decrease of GSH after erastin (1 µM) which could not be restored upon Lip-1 (150 nM) co-treatment; (n=4/treatment condition). **e:** Quantification of 500 cells counted blind to treatment (n=3) revealed Lip-1 dependent reduction of erastin-induced (0.5 mM, 16 h) mitochondrial fission. **f:** Representative images (63x objective) showed mitochondrial morphology in the presence and absence of erastin (1 µM, 16 h) ± Lip-1 (150 nM). Scale bar 50 µM. **g:** Erastin treatment (1 µM, 8 h) increased lipid peroxide production (stained with BODIPY 581/591), which was blocked by co-treatment with concentrations of >1-10 nM Lip-1 (representative FACS measurement; n=3/treatment condition). **h:** Quantification of TMRE fluorescence showed that MMP was fully restored by >10 nM Lip-1 after erastin exposure (1 µM, 16 h) (representative FACS measurement; n=3/treatment condition). **i:** Erastin treatment (1 µM, 16 h) increased mitochondrial ROS production, which was blocked by co-treatment with concentrations of >10-50 nM Lip-1 (representative FACS measurement; n=3/treatment condition).

Data are given as mean ± S.D. ###p<0.001 compared to untreated control; ***p<0.001 compared to glutamate-treated control (ANOVA, Scheffe's test).

Supplemental figure 7: Quantification of Bid translocation upon glutamate and erastin exposure



Supplemental figure 7: Quantification of Bid translocation upon glutamate and erastin exposure.
HT-22 cells were co-transfected with pDSRed2-Bid (red) and mitochondrial targeted GFP. After 16 h of erastin (1 µM) treatment, cells were fixed and localization of Bid was analyzed by confocal microscopy. Analysis of fluorescence intensities (n=3/treatment condition) by ImageJ software revealed the co-localization of Bid and mitochondria by overlapping of green and red fluorescence after erastin **(b)** treatment with r=0.78±0.18, which was abolished by co-treatment with BI-6c9 with r=0.3±0.17 **(c)** and ferrostatin-1 with r=0.14±0.19 **(d)**, respectively. Under control conditions Bid did not co-localize with mitochondria **(a)**, r=0.31±0.05. Data are given as mean ± S.D.



Supplemental figure 8: AIF knockdown prevents oxytosis and ferroptosis
a, b: Real-time impedance measurement revealed protection of AIF knockdown (20 nM siAIF, 48 h) against glutamate (5.5 µM, a) and erastin (0.5 µM, b) toxicity compared to control siRNA (20 nM scr siRNA, 48 h).
Data are given as mean ± S.D.