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Characterizing Ferroptosis in the Model Invertebrate Hydra Vulgaris

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Characterizing Ferroptosis in the Model Invertebrate *Hydra vulgaris*

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Abstract

Ferroptosis is a form of regulated cell death distinct from apoptosis. It occurs in diverse animals and in plants, but has not been documented in *Hydra vulgaris*, a cnidarian species used for studies of stem cell biology and regeneration. Treatment with the ferroptosis-inducing chemicals diethyl maleate and Erastin caused cell death, suggesting that ferroptosis can be induced in *H. vulgaris*. The phenotypes are unlike those of apoptosis in *H. vulgaris*, and each inducer resulted in a different phenotypic response. Combining the ferroptosis inducers with ferroptosis inhibitors (Liproxstatin-1, Ferrostatin-1, and B-mercaptoethanol) was expected to decrease cell death, however this was not observed. This research begins to characterize ferroptosis in *Hydra vulgaris* using ferroptosis-inducing and ferroptosis-blocking compounds.

Introduction

Ferroptosis is a form of iron-dependent regulated cell death caused by accumulation of lipid peroxides (Dixon et al. 2012). This regulated cell death pathway is unlike that of apoptosis, necrosis, and other regulated cell death pathways in terms of the morphology, genetics, and molecular aspects of the pathway (Zhao et al., 2020). Ferroptosis contributes to normal development, and may also contribute to antiviral immune response targeting and diabetes (Zhao et al., 2020). Ferroptosis is also implicated in cell death associated with ischemic injury and with neurodegenerative diseases, including Alzheimer's and Parkinson's Diseases. It may also function to limit tumor cell survival (Stockwell et al. 2017). Ferroptosis has been described in vertebrates and *C. elegans*, and a ferroptosis-like form of cell death occurs in plants (Conrad et al. 2018; Jenkins et al. 2020). Better understanding ferroptosis in an organismal system is beneficial, as inducing this process may work to augment cancer therapies. Because some oncogenic pathways related to ferroptosis are proposed, cancer cells are thought to be extremely susceptible to induction of ferroptosis, making this process act as a potentially beneficial therapeutic treatment (Zhao et al., 2020).

The enzyme glutathione peroxidase 4 (GPX4) plays a key role in regulating the lipid peroxidation which characterizes ferroptosis.. Ferroptosis can be triggered by reduced activity of this phospholipid peroxidase; without its activity an increase of lipid peroxides occurs (Cao and Dixon 2016). The molecules contributing to GPX4 function (Figure 1) have the downstream effect of lowering lipid peroxides, reducing the chance of membrane disruption and bursting of the cell (Xie et al., 2016). Part of this pathway is the antiporter System Xc^- which brings cystine into the cell and glutamate out (Xie et al., 2016). The cystine is converted to cysteine and then used in the formation of glutathione (GSH) (Xie et al., 2016). Glutathione is a cofactor for GPX4, which reduces lipid peroxides to their non-reactive hydroxyl counterparts (Miotto et al., 2020). The oxidized lipids can originate from normal lipid metabolism, including mitochondrial fatty-acid metabolism, or from an increase in reactive oxygen species (Zhao et al., 2020). These alternative species can come from other metabolic processes which oxidize polyunsaturated fatty acids (PUFAs) (Xie et al., 2016; Zhao et al., 2020). If any of these molecules or enzymes in the

ferroptosis pathway become inactivated or are absent, a buildup of lipid peroxides occurs, which leads to membrane disruption by increasing membrane curvature as the lipid peroxides make their way into the layer (Li et al., 2020). This causes pores and micellization of the cell membrane as the shape change of the typical cylindrical phospholipids within the membrane are joined by the oxidized lipids which take a conical shape (due to enlargement of the hydrocarbon tails) (Li et al., 2020). The conical conformation is unable to pack into the bilayer, causing this disruption and ruptures the cell, leading to cellular death.

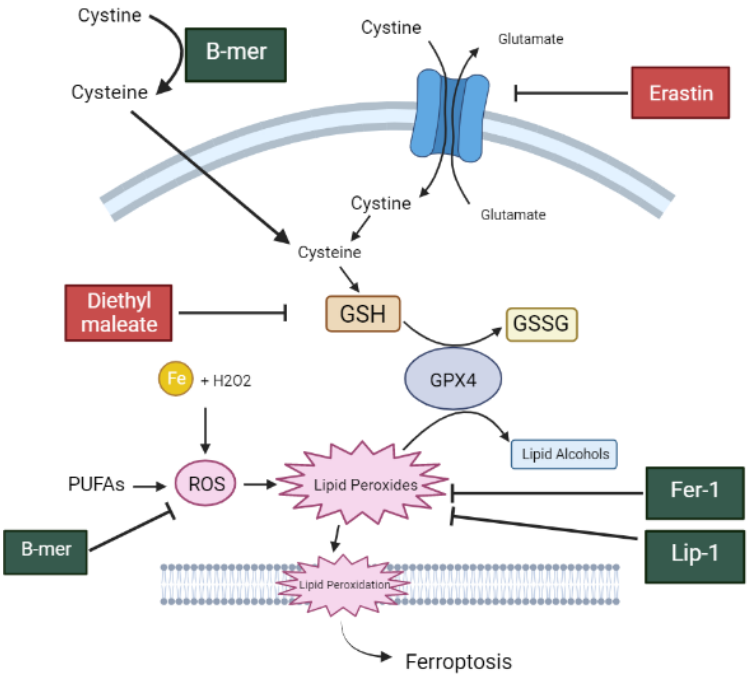


Figure 1: Targets of ferroptosis inducers and inhibitors. GPX4 plays a crucial role in controlling the level of lipid peroxides. GPX4 can be indirectly inhibited by Erastin or DEM. Inhibitors of ferroptosis include the statin-like lipid peroxide reducers Liproxstatin-1 and Ferrostatin-1, along with the general reducing agent β-mercaptoethanol.

Multiple reagents which can induce ferroptosis have been identified. One of these inducers is diethyl maleate (DEM). DEM is an electrophilic compound that depletes intracellular glutathione levels (Bannai, 1984). By interacting with the thiol (sulfhydryl) functional group of glutathione, DEM blocks disulfide bonds from forming via cofactor interaction with GPX4 (Casini et al., 1985). Without available glutathione, GPX4 will no longer reduce lipid peroxides. At small doses (less than 1μM DEM), an increase in cystine uptake was recorded in cell culture studies, indicating that activity of the glutamate-cystine antiporter system (System Xc^-) increased with decreased intracellular glutathione levels (Bannai, 1984). This result provides evidence that the System Xc^- is crucial in glutathione production within the cell.

Another inducer of ferroptosis is Erastin. Commonly used in cell culture for cancer therapy, Erastin is a known regulated cell death inducer (Zhao et al., 2020). This small molecule

causes accumulation of lipid peroxides by upstream inhibition of System Xc^-, among other molecular regulators (Zhao et al., 2020). By limiting intake of cystine, Erastin treatment depletes glutathione by reducing the levels of cysteine available in the cell to be converted to the glutathione required for GPX4 activity. Without GPX4's ability to degrade hydroperoxides, lipid peroxides and other reactive oxygen species accumulate (Zhao et al., 2020).

A number of reagents able to prevent or reverse ferroptosis have also been characterized. Statin-like ferroptosis inhibitors include Liproxstatin-1 (Lip-1) and Ferrostatin-1 (Fer-1). Lip-1 and Fer-1 are both lipid peroxide reducers, as they convert the peroxide into the hydroxyl counterpart through a metal-catalyzed reaction (Dong et al., 2015). The mechanism by which Lip-1 works to decrease these lipid peroxides is still being investigated, but Fer-1 appears to directly decrease levels of lipid peroxides that accumulate at the end of this ferroptosis pathway (Dong et al., 2015). The aromatic amine on Fer-1 works to reduce the oxidation (Xie et al., 2016). β-mercaptoethanol (β-mer) is another inhibitor. Acting as a general reducing agent, β-mer inhibit ferroptosis in multiple ways. β-mer converts cystine to cysteine outside of the cell. The cysteine produced can be transported into the cell without the involvements of system Xc^- (Xu et al., 2019). Because of this, β-mer should work well at decreasing the ferroptosis response to Erastin treatment, as Erastin blocks the System Xc^- antiporter. Because β-mer is also a general reducing agent, any β-mer that enters the cell may reduce ROS or lipid peroxides present, decreasing the accumulation of these oxidative species (Xu et al., 2019). When combining these inhibitors with the inducers, we expect a decrease in ferroptosis response.

The invertebrate *Hydra* is an attractive system for studying stem cell biology, regeneration, and nervous system architecture (Glauber, Dana, and Steele 2010; Bosch et al. 2017). It is a member of the phylum Cnidaria, a sister group to bilaterally symmetrical animals, and so provides insight into early-evolving features of animals (Bosch et al. 2017). This model organism is convenient to utilize as a large body of research exists on its cell and tissue dynamics. A convenient feature of *Hydra* is that their epithelial layers are directly exposed to their environment, making treatment with ferroptosis inducers and inhibitors easy. Both apoptosis and responses to oxidative stress in *Hydra* have also the subject of substantial research (Motamedi et al., 2019; Quinn et al., 2012). Ferroptosis in *Hydra* remains uncharacterized Because its cell and tissue dynamics are well understood and because of its phylogenetic position, *Hydra* has the potential to provide valuable insight into the process of ferroptosis.

The goal of this research is to characterize ferroptosis in *Hydra vulgaris*. Specific goals were to address the following questions. Does ferroptosis occur in the model organism *Hydra vulgaris*, and are some cell types more prone to this process? If exposed to the ferroptosis inducing chemicals Erastin and DEM, will *Hydra vulgaris* undergo cell death? If ferroptosis is occurring and *Hydra vulgaris* are exposed to ferroptosis inhibitors such as Liproxstatin-1, Ferrostatin-1, and β-mercaptoethanol, then will the effects of the inducers be reduced?

Methods

Animal culture

Experiments were conducted with the AEP strain of *Hydra vulgaris*. *Hydra* used were from cultures of genetically identical animals, fed three times per week on 48-hour-old brine shrimp nauplii (Brine Shrimp Direct). Cultures were maintained in *Hydra* medium (30 µM KNO_3, 80 µM $MgSO_4$, 0.5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1 mM $CaCl_2$). Animals treated with reagents that induced or inhibited ferroptosis were incubated in *Hydra* medium with the reagents added.

Induction of ferroptosis

To confirm that ferroptosis can be induced in *Hydra vulgaris*, treatment with diethyl maleate (DEM) and Erastin was conducted. I completed a dose curve for each inducer. For DEM, I tested the concentrations of 1µM, 10µM, 50µM, 100µM, 150µM, 200µM, 250µM, and 300µM, ranges that are commonly used in cell culture (Bannai, 1984). For Erastin, I tested the concentrations of 1µM, 10µM, 20µM, 40µM, 50µM, 100µM, and 300µM; the typical concentrations used in cell culture studies ranged from 10µM to 100µM (Sato et al., 2018). For 24 hours after the start of treatment, I monitored the stress response for each concentration. I also recorded sperm motility; looking at the animals under 40x magnification, I determined if sperm were moving within the testes along the body column of the male animals. The purpose of this initial test was to identify the lowest concentration at which the treated *Hydra* showed a dramatic difference from the DMSO control and death occurred within 24 hours. This was true for DEM at a concentration of 250µM and Erastin at a concentration of 20µM.

After treatment concentrations for the inducers were chosen, I repeated the experiment to determine the timing of the animals' responses. I monitored the effects of DEM and Erastin on the laboratory strain of *Hydra vulgaris*, AEP, over 24 hours, checking every hour for the first 12 hours, then again at 24 hours-post-treatment. Using 250µM DEM and 20µM Erastin, I placed AEP that had not been fed for 48 hours five to a well in a 12-well plate. Because Erastin is dissolved in DMSO, the negative control treatment was incubation in *Hydra* medium with DMSO at the same concentration as in the Erastin treatments. Once the solutions were made, I took off the *Hydra* media the animals were in, and replaced 1mL of the treatment solution (250µM DEM, 20µM Erastin, and DMSO).

To ensure that degradation of the inducers did not impact the results, I investigated the effects of the time of solution preparation on responses observed. I prepared the solutions using the same concentrations and let sit for 21 hours. Each hour post-treatment over the subsequent 12 hours and again at 24 hours, I recorded the stress response for that time. At the different time points, I determined the number of *Hydra* showing two or fewer tentacles, presence of clubbed tentacles, ectodermal thickening, body contraction, cells dissociating, and organismal death.

Combining Ferroptosis Inducers and Inhibitors

Once the experimental concentration for the inducers was chosen and the timing of the stress responses identified, I combined three different inhibitors with the two different inducers in order to determine whether the inhibitors could block or reduce the effects of the inducers. Looking at Liproxstatin-1 (Lip-1), β-mercaptoethanol (β-mer), and Ferrostatin-1 (Fer-1), I tested the individual inhibitors against both inducers. Based on concentrations of these inhibitors used in cell culture, I used 10μM Lip-1, 10μM Fer-1, and 50μM B-mercaptoethanol.

The treatments of the inhibitor series were as follows: a control of the inhibitor alone [either Fer-1(10μM), Lip-1(10μM), or β-mer (50μM)], DMSO alone (a standard concentration added across all solutions), Erastin (20μM), DEM (250μM), inhibitor + DEM, inhibitor + Erastin. A total of three experiments were conducted with this design, one for each inhibitor used. For each experiment, there were 5 animals in 2 wells of treatment, for a total of 10 animals per treatment. All were fed 48 hours prior to treatment. I placed 1mL of solution in the well, and checked over a period of 24 hours, recording the reactions of tentacle detachment, tentacle clubbing, body column contraction, ectodermal thickening, cell dissociation, and organismal death, quantifying the frequencies of each per well.

I conducted additional experiments with the inhibitor β-mer, having two replicates of a larger sample size experiment. I used animals that had been fed 48 hours prior, and placed 10 per well. Only using the treatments β-mer (50μM), Erastin (20μM), and β-mer + Erastin, I had four wells per treatment within a 12-well plate. This allowed a total of 40 animals per treatment, recording the response phenotypes separately for each well. I then ran t-tests using the frequencies of the phenotypes between each treatment.

Treatment with β-mer prior to inducer treatment

I incubated 5 animals in 2 wells for each treatment containing β-mer (treatment groups include β-mer (50μM), DMSO control (a standard concentration added across all solutions), Erastin (20μM), DEM (250μM), β-mer + DEM, β-mer + Erastin) for 1.5 hours, then replaced all media with fresh media containing the proper solution concentrations (with fresh inhibitor). I recorded over a period of 24 hours the responses of tentacle detachment, tentacle clubbing, body column contraction, ectodermal thickening, cell dissociation, and organismal death. I quantified the frequencies of each phenotype per well. I qualitatively compared the incubated treatment with non-incubated treatment.

Results

Induction of ferroptosis

Figure 2: Evidence that ferroptosis can be induced in *Hydra vulgaris*. Results 10 hours after treatment for the control, DEM (300µM), and Erastin (20µM) treatment at a magnification at 35x.

The experiments to establish a dose curve for the two inhibitors, DEM and Erastin, identified the concentrations of 250µM DEM and 20µM Erastin as the lowest concentrations that allowed for death at the 24 hour-mark while providing a distinct enough phenotypic response to characterize. Using these concentrations, I looked at the timing of the cell death response (Figure 3). The notable characteristics of DEM-induced stress and cell death response were tentacle clubbing, thickening of ectoderm of the body column, and rounded, dissociated cells (Figure 2). The thickening of the ectoderm and dissociating cells are unlike that of typical apoptotic responses for *Hydra vulgaris*. Sperm motility was maintained throughout this process, until organismal cell death when I could not decipher the testes amidst the thickened body column. For Erastin treatments, notable characteristics included tentacle detachment, tentacle clubbing, and heightened body column contraction (Figure 2). Tentacle detachment was unexpected, as this phenotypic response is not typical in *Hydra vulgaris* stress responses. The tentacles detachment consists of a complete dissociation of the tentacles from the head, leaving no remaining tentacle tissue attached. Around 90% of the animals could be classified as dead at the 24 hour-mark. Death classification is indicated by lack of body column extension and inability to repair cellular damage.

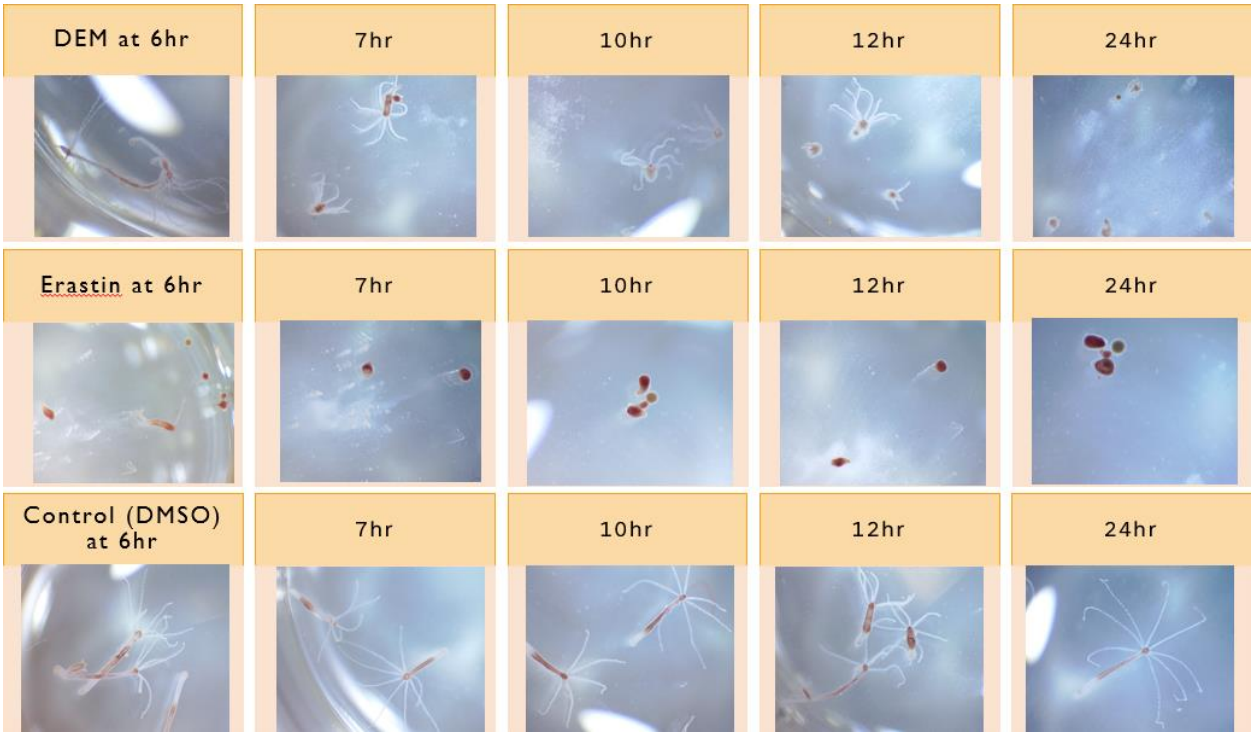


Figure 3: Timing of phenotypic response when treated with the ferroptosis inducers Erastin (20μM) and DEM (250μM) over the course of 24 hours at a magnification of 25x. DMSO control used where the concentration is equal to that of the highest inhibitor concentration (in this treatment, matching that of Erastin). Note the phenotypic response of tentacle clubbing, ectoderm thickening, and cell dissociation for DEM, and tentacle detachment, tentacle clubbing, and body column contraction for Erastin.

When looking at the stability of the inducers, I treated the animals with fresh solutions and 21 hour old solutions and compared between the two over the span of 24 hours. For DEM, similar phenotypic responses were observed at the same time points; around 6-8 hours post-exposure the animals showed tentacle clubbing, initial ectoderm thickening, and cell dissociation. All of the *Hydra vulgaris* were dead by 24 hours post treatment. With Erastin treatment, the freshness of the solution mattered; the fresh solution caused tentacle detachment at 1-3 hours post treatment, whereas the old solution caused detachment of tentacles between 6-7 hours post treatment. The animals in old solution also retained most of their tentacles – each one left clubbed. After 24 hours, the animals had contracted body columns, yet the old solution allowed for remaining tentacles and slightly less compacted bodies, while the fresh solution allowed for complete detachment of tentacles and spherical contraction. For each subsequent treatment with Erastin, the solution was made fresh.

Combining Ferroptosis Inducers and Inhibitors

After characterizing the cell death phenotype and dose curves for ferroptosis inducers, the animals were given treatments of inducers with inhibitors in order to determine if the inhibitor would decrease the responses to inducers. With Lip-1, there was no qualitative difference in the *Hydra vulgaris* response between the DEM and DEM+Lip-1 treatments at each recorded timepoint (Table 1, Figure 4). The frequencies of tentacle detachment, body column contraction,

and organismal cell death were similar between the two treatments. This is also true when comparing Erastin and Erastin+Lip-1 treatments (Table 1). The frequencies of tentacle clubbing, ectodermal thickening, and organismal death remained similar between the treatments, and no adequate statistical testing could be completed as there were not enough wells to compare.

Table 1: Frequencies for observed phenotypes, comprised of 2 wells with 5 AEP in each. Top: Frequencies of phenotypic responses of DEM, DEM+Lip-1, and Lip-1. Bottom: Frequencies of phenotypic responses of Erastin, Erastin+Lip-1, and Lip-1. Dashes indicate that no data could be provided for tentacle clubbing, as all of the tentacles were detached in those wells.

DEM Treated	5 hours post treatment			10 hours post treatment			21 hours post treatment		
	DEM	DEM+Lip	Lip	DEM	DEM+Lip	Lip	DEM	DEM+Lip	Lip
Detached Tentacles	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Clubbed Tentacles	0.00	0.00	0.00	1.00	1.00	0.00	1.00	1.00	0.30
Body Contraction	0.00	0.00	0.00	0.30	0.36	0.00	1.00	1.00	0.00
Ectodermal Thickening	0.00	0.00	0.00	0.90	0.82	0.00	1.00	1.00	0.00
Dead	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00
Dissociated Cells	No	No	No	Yes	Yes	No	Yes	Yes	No

Erastin Treated	5 hours post treatment			10 hours post treatment			21 hours post treatment		
	Erastin	Erastin+Lip	Lip	Erastin	Erastin+Lip	Lip	Erastin	Erastin+Lip	Lip
Detached Tentacles	0.80	0.70	0.00	1.00	0.90	0.00	1.00	1.00	0.00
Clubbed Tentacles	0.50	1.00	0.00	-	1.00	0.00	-	-	0.30
Body Contraction	0.00	0.00	0.00	0.90	0.90	0.00	1.00	1.00	0.00
Ectodermal Thickening	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00
Dead	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00
Dissociated Cells	No	No	No	Yes	Yes	No	No	No	No

Repeating this same setup and allowing for the same treatment groups, I tested the effectiveness of pre-treatment with the inhibitor Lip-1 to determine if prior exposure would decrease cell death. Upon incubation with Lip-1 for 1.5 hours before introduction to inducer exposure, similar results ensued. The tentacle detachment, tentacle clubbing, body column contraction, ectodermal thickening, and organismal cell death at 24 hours were similar between the inducer treatment and the inhibitor-incubated plus inducer treatments. The controls of only Lip-1 and only DMSO (DMSO controlled for the highest amount from chemicals, all wells brought up to that same amount) resulted in healthy *Hydra vulgaris* with no apparent tentacle detachment, body column contraction, or organismal death.

Figure 4: Qualitative phenotypic responses and comparison of inducers paired with Lip-1 (inhibitor) at a magnification of 25x. Concentrations include 250µM DEM, 20µM Erastin, and 10µM Lip-1. Left: DEM+Lip-1 at 11 and 24 hours post treatment, compared vertically with DEM. Mid: Control wells, only Lip-1 at 24 hours and only DMSO at 24 hours. Right: Erastin+Lip-1 at 11 and 24 hours post treatment, compared vertically with Erastin.

Using Fer-1 as the inhibitor, the treatments of DEM (250µM) and DEM+Fer(10µM) resulted in similar phenotypic responses and timing (Table 2, Figure 5). In the DEM treated group, the tentacle clubbing, ectoderm thickening, and organismal death occurred simultaneously in the inhibitor treatment as the no-inhibitor treatment. In contrast, comparing the Erastin (20µM) treated animals with the Erastin+Fer(10µM) animals, the Fer-1 group showed decreased frequency of body column contraction. At 24 hours post exposure, the Erastin+Fer-1 group had a few individuals that were not contracted or dead. The control group treated with Fer-1 alone displayed slight organismal stress as a few tentacles detached, but within 24 hours the tentacles started to grow back. This response was not shared by the Erastin or Erastin+Fer-1 treated groups. Replicates need to be completed for statistical analysis to determine significance of the relationship of Fer-1 and ferroptosis prevention or reversal.

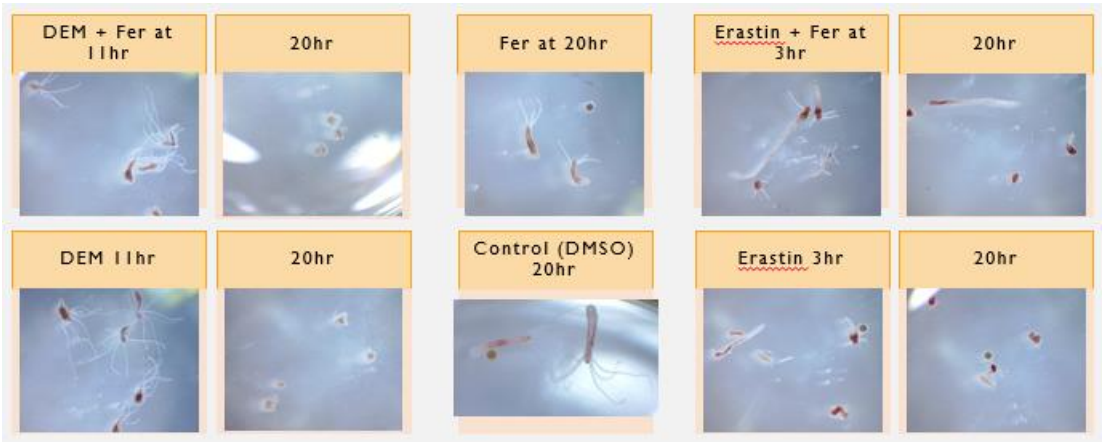


Figure 5: Qualitative phenotypic responses and comparison of inducers paired with Fer-1 (inhibitor) at a magnification of 25x. Concentrations include 250µM DEM, 20µM Erastin, and 10µM Fer-1. Left:

DEM+Fer-1 at 11 and 20 hours post treatment, compared vertically with DEM. Mid: Control wells, only Fer-1 at 20 hours and only DMSO at 20 hours. Right: Erastin+Fer-1 at 11 and 20 hours post treatment, compared vertically with Erastin.

Table 2: Frequencies for observed phenotypes, comprised of 2 wells with 5 AEP in each. Top: Frequencies of phenotypic responses of DEM, DEM+Fer-1, and Fer-1. Bottom: Frequencies of phenotypic responses of Erastin, Erastin+Fer-1, and Fer-1. Dashes indicate that no data could be provided for tentacle clubbing, as all of the tentacles were detached in those wells.

DEM Treated with Fer-1	3 hours post treatment			9 hours post treatment			20 hours post treatment		
	DEM	DEM + Fer	Fer	DEM	DEM+Fer	Fer	DEM	DEM+Fer	Fer
Detached Tentacles	0.10	0.10	0.20	0.10	0.00	0.30	0.10	0.00	0.40
Clubbed Tentacles	0.30	0.20	0.30	1.00	0.30	0.60	1.00	1.00	0.00
Ectodermal Thickening	0.00	0.00	0.00	0.90	1.00	0.00	1.00	1.00	0.00
Body Contraction	0.00	0.00	0.10	0.20	0.80	0.10	1.00	1.00	0.10
Dead	0.00	0.00	0.00	0.10	0.10	0.00	0.90	1.00	0.00
Dissociated Cells	No	No	No	Yes	Yes	No	No	No	No

Erastin Treated with Fer-1	3 hours post treatment			9 hours post treatment			20 hours post treatment		
	Erastin	Erastin+Fer	Fer	Erastin	Erastin+Fer	Fer	Erastin	Erastin+Fer	Fer
Detached Tentacles	0.50	0.30	0.20	0.90	0.70	0.30	1.00	0.80	0.40
Clubbed Tentacles	0.80	0.80	0.30	1.00	0.75	0.60	-	0.50	0.00
Ectodermal Thickening	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00
Body Contraction	0.10	0.00	0.10	0.30	0.30	0.10	0.80	0.30	0.10
Dead	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.10	0.00
Dissociated Cells	No	No	No	Yes	Yes	No	No	No	No

This same setup was repeated, using B-mer (50μM) as the inhibitor, where there were 5 *Hydra vulgaris* (AEP) to a well over 2 wells. The treatments of DEM (250μM) and DEM+B-mer resulted in similar phenotypic responses and timing (Figure 6). Again, DEM resulted in thickening of the ectoderm, tentacle clubbing, and death at around 24 hours post exposure. The DEM+B-mer had similar responses and timing, still resulting in organismal death at around 24 hours post exposure. Using Erastin (20μM) as the inducer of ferroptosis, the animals had tentacle detachment, body contraction, and organismal death around the 24 hour mark, and when combined with B-mer, most of these phenotypes ensued along the same timing. The only difference lies in the body column contraction timing with B-mer, as the *Hydra* had some continued body elongation past the 24 hour mark. Because their bodies were not contracted or they maintained movement control, the animals in this condition were not considered dead.

Figure 6: Qualitative phenotypic responses and comparison of inducers paired with β-mer (inhibitor) at a magnification of 25x. Concentrations include 250μM DEM, 20μM Erastin, and 50μM β-mer. Left: DEM+ β-mer at 11 and 23 hours post treatment, compared vertically with DEM. Mid: Control wells, only B-mer at 23 hours and only DMSO at 23 hours. Right: Erastin+ β-mer at 11 and 23 hours post treatment, compared vertically with Erastin.

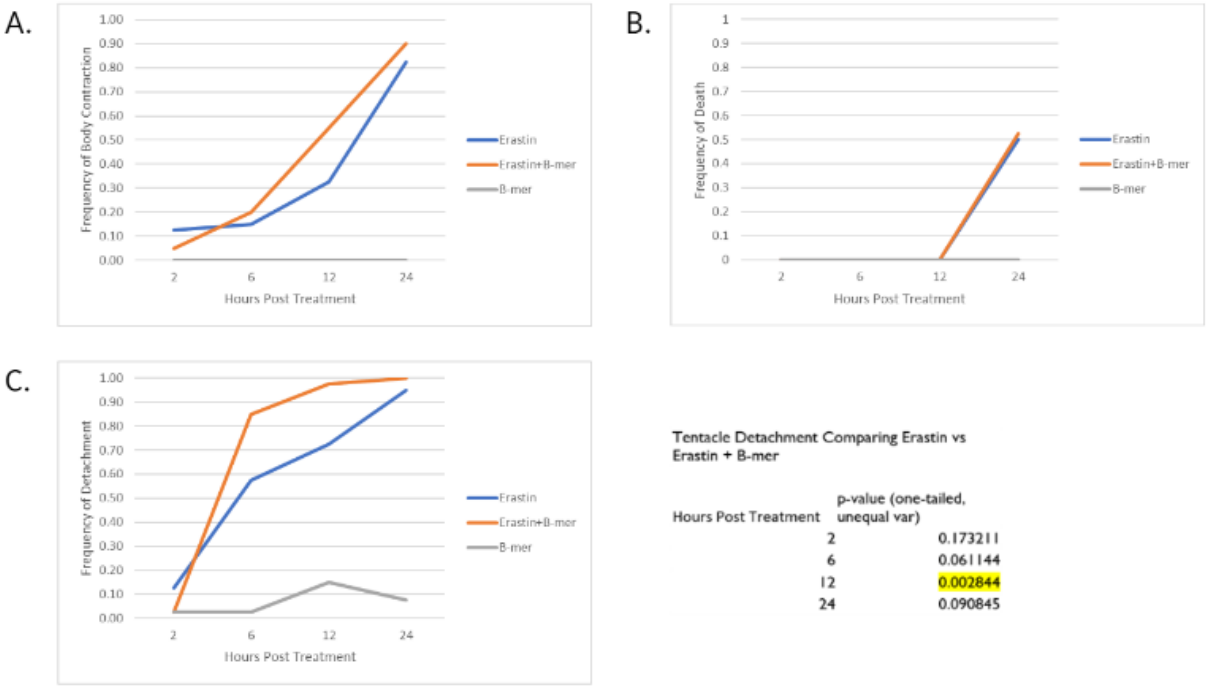
This same setup was repeated, but with an incubation of animals in β-mer for 1.5 hours prior to treatment with Erastin and DEM. These results (Table 3) indicate a potential trend of β-mer making a slight improvement to the ferroptosis response. Combined with DEM, β-mer made no difference in timing or organismal death at 24 hours. When combined with Erastin, there was less body column contraction and organismal death at the later time point, remaining consistent with the preliminary experiment combining Erastin and β-mer. The occurrence of dissociated cells within the wells also dissipated in the later hours, as these cells were associated with the tentacles and once the detached ones full disintegrated then there were little to no free-floated cells. The incubation in β-mer compared to no incubation resulted in similar phenotypic responses and timing, indicating that it is not necessary to incubate the animals in the inhibitor.

Table 3: Frequencies for observed phenotypes, comprised of 2 wells with 5 AEP in each. Top: Frequencies of phenotypic responses of DEM, DEM+ β-mer, and β-mer. Bottom: Frequencies of phenotypic responses of Erastin, Erastin+ β-mer, and β-mer. Dashes indicate that no data could be provided for tentacle clubbing, as all of the tentacles were detached in those wells.

Erastin Treated with B-mer	After 1.5hr Incubation			1 hour post treatment			11 hours post treatment			23 hours post treatment		
	Erastin	Erastin+B-mer	B-mer	Erastin	Erastin+B-mer	B-mer	Erastin	Erastin+B-mer	B-mer	Erastin	Erastin+B-mer	B-mer
Detached Tentacles	0.00	0.10	0.00	0.09	0.00	0.00	0.63	0.70	0.00	0.91	0.90	0.00
Clubbed Tentacles	0.00	0.00	0.18	0.45	0.20	0.81	1.00	1.00	0.80	1.00	0.00	0.20
Ectodermal Thickening	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.09	0.20	0.00
Body Contraction	0.00	0.10	0.36	0.00	0.10	0.00	0.63	0.10	0.10	0.63	0.30	0.00
Dead	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.63	0.20	0.00
Dissociated Cells	No	No	No	Yes	Yes	No	No	No	No	No	No	No

DEM Treated with B-mer	After 1.5hr Incubation			1 hour post treatment			11 hours post treatment			23 hours post treatment		
	DEM	DEM+B-mer	B-mer	DEM	DEM+B-mer	B-mer	DEM	DEM+B-mer	B-mer	DEM	DEM+B-mer	B-mer
Detached Tentacles	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Clubbed Tentacles	0.00	0.10	0.18	0.00	0.10	0.81	1.00	1.00	0.80	1.00	1.00	0.20
Ectodermal Thickening	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	1.00	1.00	0.00
Body Contraction	0.00	0.00	0.36	0.00	0.20	0.00	0.33	0.60	0.10	1.00	1.00	0.00
Dead	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00
Dissociated Cells	No	No	No	Yes	Yes	No	No	No	No	Yes	Yes	No

With this difference in response between the Erastin and Erastin+ β-mer treatments, these treatments were repeated in greater sample size in order to investigate this observation and determine statistical significance. Over 4 well, 10 AEP *Hydra vulgaris* were used with treatments of Erastin, Erastin+ β-mer, and β-mer. The amount of DMSO within the solution was standardized across the treatments. Over the course of 24 hours, the phenotypic responses seemed to continue to be similar between the inducer plus inhibitor and the inducer alone. The responses most notable in Erastin-treated animals are tentacle detachment, body column contraction, and organismal death, and when these phenotypes are compared between the two treatments there are no qualitative differences between them (Figure 7)



Tentacle Detachment Comparing Erastin vs Erastin + B-mer

Hours Post Treatment	p-value (one-tailed, unequal var)
2	0.173211
6	0.061144
12	0.002844
24	0.090845

Figure 7: Comparing Erastin, Erastin+ β-mer, and β-mer treatments on *Hydra vulgaris* with concentrations of treatments including 20μM Erastin and 50μM β-mer. (A) Frequency of body column contraction along a 24 hour period post exposure. (B) Frequency of organismal death over 24 hour period.

(C) Frequency of tentacle detachment over 24 hour period. To the right are the p-values of a t-test comparing Erastin and Erastin+ β-mer treatments, statistical significance highlighted.

In the preliminary experiment combining the inducers with β-mer, the Erastin+ β-mer showed a trend of prolonged body column elongation, yet with greater sample size this trend disappears. The Erastin+ β-mer have a greater frequency of causing negative responses from the animals. The largest qualitative difference between treatments with and without the inhibitor are indicated in tentacle detachment, where a statistical difference occurs with a t-test significance value of 0.0028 at the 12 hour mark. The Erastin+ β-mer treated animals had a higher occurrence of detached tentacles at this point. The β-mer only treated group did see loss in tentacles, yet with time the tentacles began to grow back.

Discussion

Ferroptosis is a regulated cell death pathway caused by an accumulation of lipid peroxides and free iron (Dixon et al. 2012). This pathway can be induced using specific chemicals that alter or block molecules necessary in the pathway. The lipid peroxide reducing enzyme GPX4 is crucial in preventing lipid peroxide accumulation which may cause rupturing of the cell membrane and ferroptosis (Miotto et al., 2020). The ferroptosis inducer DEM reduces GPX4 activity by binding to the GPX4 cofactor glutathione (Bannai, 1984; Jenkins et al. 2020). With GPX4 inactivated, lipid accumulation occurs and ferroptosis results. Erastin is also a known ferroptosis inducer, as it blocks the system Xc^- antiporter from allowing cystine into the cell (Xie et al., 2016). This causes a decrease in glutathione levels and thus a decrease in GPX4 activity (Xu et al., 2019).

The effects of these reagents have been studied in cell culture, but never before in the model organism *Hydra vulgaris*. When treated with the ferroptosis inducers Erastin and DEM, *Hydra vulgaris* undergo cell death. Ferroptosis has never been characterized in *Hydra*. The fact that Erastin and DEM are specific ferroptosis inducers provides evidence that this regulated cell death pathway is indeed able to occur in this model organism. The main cell death characteristics seen in these animals in this experiment is in their tentacles and body column (Figure 2). *Hydra* often show cellular stress first within the tentacles, and often shortening and clubbing occurs (Quinn et al., 2012). This can be seen in both ferroptosis inducer exposure, but also in typical apoptosis induction in *Hydra* (Motamedi et al., 2019). There are additional phenotypic responses that set responses to Erastin and DEM apart from apoptosis: tentacle detachment and ectodermal thickening (Figure 2). Tentacle detachment is the complete dissociation of the tentacles from the head, leaving no tissue behind. Because this dissociation is unique to just the base of the tentacles, this tissue may be prone to this cell death response, targeting changes in these specific cells. When exposed to DEM, *Hydra vulgaris* exhibits tentacle clubbing around 6-8 hours post exposure and thickening of the ectodermal layer at around 10 hours (Figure 3). The animals also exhibited cell dissociation, as rounded cells appear in the medium. By 24 hours, the animals are piles of cells, each one disintegrating. Erastin-exposed *Hydra vulgaris* underwent tentacle detachment, a response not seen when apoptosis is induced (Figure 2). This occurrence began

after the first hour, and by hour 7 most of the tentacles were detached and body column contracted (Figure 3). After 24 hours, the animals' body columns were continuously contracted to the point where they were spherical, at which point I considered the animals dead. There were dissociated cells in the Erastin treated wells at around 6 hours post treatment. There was an increased number of dissociated cells between the time points of 7-12 hours, then the amount dissipated with more time. These rounded, loose cells may be associated with the disintegration of the detached tentacles, as when the tentacle was completely gone from the well, so too were the dissociated cells.

It is interesting to note the difference between the ferroptosis responses of the Erastin treated and the DEM treated animals. First, the tentacle detachment caused by Erastin and the ectodermal thickening caused by DEM are not seen when apoptosis is induced in *Hydra* (Motamedi et al., 2019). The timing of these responses also differs between inducers (Figure 3). Erastin appears to influence *Hydra vulgaris* more quickly than DEM, causing detectable effects just 1-3 hours post exposure compared to DEM's 6-8 hours post exposure. Erastin works to inactivate the antiporter early in the ferroptosis pathway, causing a lack of cystine that decreases GPX4 activity, while DEM works to block the cofactor binding of glutathione with GPX4 (Xie et al., 2016). DEM may potentially not stop all of the glutathione from becoming a cofactor for GPX4, allowing a low level of GPX4 activity to remain within the cell. Alternatively, the time required for DEM to enter *Hydra* cells may explain the fact that it took longer to cause responses.

Ferroptosis can also be inhibited by certain chemicals such as lipid peroxide reducers, general reducers, and iron sequestering compounds (Dong et al., 2015). The statin-like inhibitors used here seemed to not reverse the effects of the ferroptosis inducers used (Figures 4 and 5). When DEM was combined with either Lip-1 or Fer, the *Hydra* did not show signs of improved health or prevention of organismal death. Each treatment resulted in the same timing of response and organismal death when compared to the inducer alone treatment. Because Lip-1 and Fer-1 target already formed lipid peroxides, the already accumulated lipid peroxide amounts could potentially have been too great for the inhibitors to undo the oxidative damage already underway (Dong et al., 2015).

Using the inhibitor β-mer combined with the inducer DEM, there were no qualitative differences between stress and cell death responses between the two treatments. In contrast, β-mer combined with Erastin was associated with reduced tentacle detachment as well as reduced organismal death at 24 hours after treatment (Figure 6). However, in the later experiment with three wells per treatment and twice as many animals per well, β-mer did not reduce tentacle detachment and organism death caused by Erastin (Figure 7). This could be due to the fact that ferroptotic cells tend to spread induction of this cell death to surrounding cells, acting as an amplification of further ferroptosis (Nishizawa et al., 2021). Because there were a greater number of animals per well (10 rather than 5), the increase in abundance of ferroptotic cells could potentially have caused cells that would have otherwise been fine to go into regulated cell death. β-mer might be expected to block ferroptosis because it is a strong reducing agent, able to decrease levels of lipid peroxides. One way that it could counter the effect of Erastin specifically

is by converting cystine to cysteine, making intracellular cysteine available without involvement of System Xc^- (Xu et al., 2019). This could explain why β-mer did not reduce the effect of DEM, as DEM sequesters glutathione after cysteine has entered the cell, thus still blocking GPX4 activation.

This project has only begun to explore the properties and potential roles of ferroptosis in *Hydra*. Additional questions which would be interesting to address include the following. Can ferroptosis and apoptosis be distinguished in *Hydra vulgaris* by using DAPI staining to detect the fragmentation of chromatin characteristic of apoptosis? Does crowding increase the response of *Hydra* to ferroptosis inducers? Does ferroptosis occur in response to heat shock in *Hydra vulgaris*, as in plants (Distéfano et al. 2017)? Can a polyunsaturated fatty acid induce ferroptosis in the developing gametes of *Hydra vulgaris*, as in the germ cells of *C. elegans*? Limitations of this study include the lack of concrete evidence that this cell death is actually ferroptosis and not another form or necrosis. The failure of ferroptosis inhibitors to block the effects of ferroptosis is not straightforward to explain. Altering the concentration of the inhibitor or using different inhibitors should provide additional insight. Looking at the molecular differences between apoptosis and ferroptosis in the nuclei will help to establish that this cell death mechanism is in fact not apoptosis, specifically looking for punctate nuclei known to occur in apoptotic *Hydra* (Motamedi et al., 2019).

Hydra provides a convenient whole animal model for the study of ferroptosis. Given the relevance of this process to cancer and neurodegenerative diseases, additional work to characterize ferroptosis in *Hydra* could be a source of medically valuable insights.

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