PREDICTIVE MODELING OF FERROPTOSIS SUSCEPTIBILITY IN RENAL CELL CARCINOMA

By

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ABSTRACT

Ferroptosis is a recently discovered type of programmed cell death that is implicated in many cancers and neurodegenerative diseases. It is dependent on the accumulation of iron and lipid reactive oxygen species. If targeted, this mechanism could be used as an alternative mediator of cell death in cancers which have become resistant to apoptosis. It has been shown that large b cell lymphomas and renal cell carcinomas are most susceptible to this regulated cell death. Characterizing and understanding this process is imperative for developing effective treatments using this mechanism. Predicting a cell type's susceptibility to ferroptosis could aid in this treatment process and further develop the key factors in ferroptosis. A mathematical model was developed to predict this outcome in the renal cell carcinoma context. Multi-level analyses of predictor variables were performed; a single feature linear regression model proved to be the most effective. After extensive validation, ferroptosis susceptibility was accurately predicted using labile iron pool measurements. With further extension into various types of cancers and contexts, this can be a useful tool in determining a cell's ability to be induced into ferroptosis, furthering the research in this breakthrough mechanism.

KEYWORDS: Renal cell carcinoma, ferroptosis, cancer biology, GPX4, linear regression

INTRODUCTION

Regulated (RCD) pathways are an essential target for cancer therapies. One of those pathways is ferroptosis. The characterization of this cell death is in high demand. Furthermore, predicting this mechanism in cell lines and other models has the ability to aid the research process significantly. Understanding the behavior prior to expensive and time sensitive assays is crucial to progressive research. Modeling the behavior of cells in this setting gives an insight to researchers as to what to expect and what research direction is most effective.

Creating a space for ferroptosis as the field of cancer research quickly delves into computational techniques and open-source data is crucial to this effort. Establishing databases early on provide a way for researchers to input their data and collaborate. Adding functionality with numerical analyses and predictive tools can only accelerate the progress in this field, as does increasing the ways in which researchers can collaborate.

The multitude of biological assays has made predictive modeling very data intensive. While consistency and data quality is essential, choosing the most impactful features is also of utmost importance. Steps taken when choosing the most impactful features include a thorough data cleaning and data quality check, followed by an exploratory data analysis. This analysis will show any possible relationship within the data, while possibly exposing confounding effects. Other considerations include feasibility checks, understanding any challenges that may come with gathering such features in the future. Ensuring that the input data is easily collected, reducing time commitment and increasing cost effectiveness, will greatly improve the accessibility of the model.

By using multiple analytical tools, predictive modeling in the biological space has become much more accurate. Adding descriptive statistics and numerical trends to biological experiments can aid in the applicability and reproducibility. By taking into account accessibility and ease of use when considering feature selection, the developer can ensure the model has the biggest impact possible.

After proving the predictive capability (the ability to numerically and accurately calculate our outcome variable) of modeling in cell lines, moving onto more complex contexts such as mouse models is critical to proving relevancy in translational science. It is expected that this setting will be more difficult, however adapting the model to a more heterogenous environment is essential and could be just as effective at predicting response.

In summary, modeling this newly discovered type of cell death, ferroptosis, can add insight to a rapidly growing biological research field. It can use computational tools to bring researchers together whilst adding functionality to already built platforms. Focusing on biologically relevant and accessible features for this model can increase accessibility and understanding of this process. This also has the ability to open up more opportunities for study in heterogeneous contexts with the hope of finding relevancy in translational science.

SPECIFIC AIMS

AIM ONE:

Determine significant predictors of ferroptosis susceptibility by utilizing published iron-dependent pathways. Examine which markers have the most predictive power and specify the level of

confidence through literature review. Recent curation of these markers have been published in a ferroptosis specific database, this will also be surveyed.[1] By understanding and visualizing the iron-dependent portion of this mechanism, more possibilities for treatment targets may become available.

AIM TWO:

A) Develop a model using ferroptosis markers to determine susceptibility to ferroptosis in renal cell carcinoma cell lines. This model will take in multiple input variables that have proven to be indicative of ferroptosis sensitivity. Binary classification methods will be optimized to accurately predict the outcome of ferroptosis sensitivity. Susceptibility will be measured by the levels of treatment required to induce ferroptosis in each cell line. By creating computational procedures to predict ferroptotic susceptibility, research in this field will become less dependent on labor and time expensive biological assays.

B) Prove transferability by assessing the prediction power of this model on a cell line in a different cancer context, such as a prostate cancer cell line, which has also shown to be implicated in this mechanism.[2] This will aid in understanding how the iron-dependent pathways may be applicable across tumor types and subtypes.

By further exploration of this type of cell death, this model will provide new mechanisms for which resistant tumor types may be treated. This includes (a) the ability to test and validate the ferroptotic sensitivities of different cell line and treatment combinations more efficiently, (b) a framework for which to apply this model to different cancer settings, or even to other research settings such as neurodegenerative diseases, and (c) a contribution to the known iron-dependent pathways that effect many of the 'hallmarks of cancer'.[3]

BACKGROUND

Ferroptosis, a newly characterized type of regulated cell death, has the potential to be a promising target for cancer treatments. This is similar to how other cell death mechanisms have proven effective at treating cancer in the past. Utilizing ferroptosis as an alternate treatment path for multi-resistant tumor types could be used to add another level of defense against these cancers. This cell death mechanism is dependent on an increase in lipid peroxidation and iron accumulation. The former has been heavily characterized, but there is still a lack of exploration of the iron dependent processes that induce this cell death. By targeting different arms in this pathway, these cancerous cells can be induced into regulated cell death.

3.1 Context

In the cancer context, inducing ferroptosis selectively within these malfunctioning cells is the ultimate goal. However, there are other contexts within the biomedical field that have other opportunities for ferroptosis as a treatment. For example, in neurological disorders such as Alzheimer's disease and ischemic strokes, patients can benefit from protecting those cells within the brain from ferroptosis using ferroptotic inhibiting agents. The mechanisms include iron chelation, lipid peroxidation, and toxic lipid neutralization.[4] Characterizing ferroptosis, no matter the motivation, would add to the possibilities for treatments in many different types of patients.

3.2 History

Ferroptosis was discovered and named in 2012 as an 'iron-dependent form of non-apoptotic cell death'.[5] Two years later glutathione peroxidase (GPX4) was shown to be a regulator of the process.[6] This discovery shaped further research in the field. Characterization continued and lipid

metabolism was published as having necessary involvement.[7] Recent publications continue to produce new identifiers. The acceleration of discoveries in this field, as seen in figure 1, illustrate the need for further research, with the hope of creating effective cancer treatments, in the future.

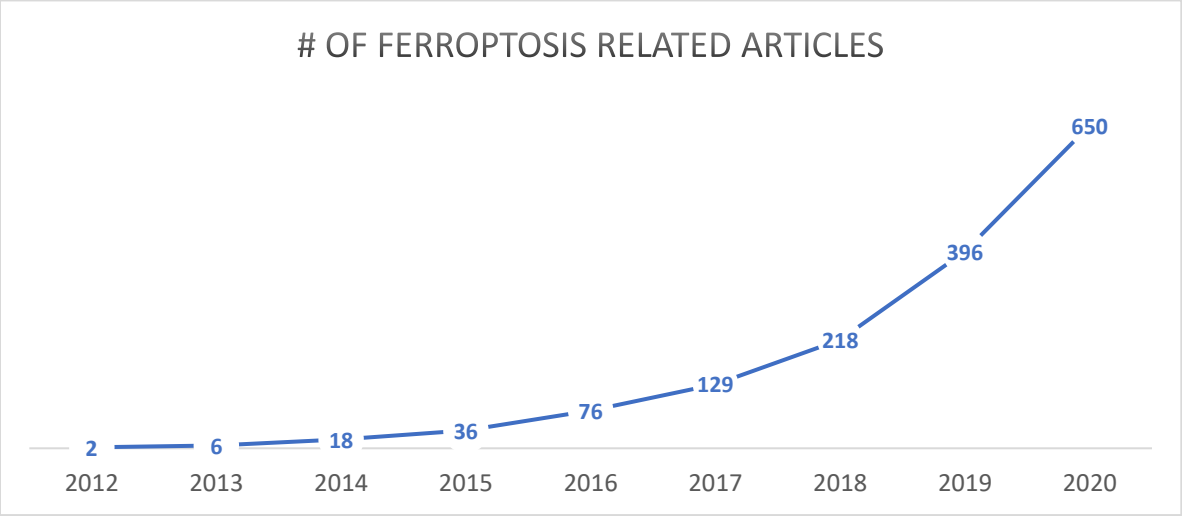


Figure 1: A continuous increase of the keyword "ferroptosis" in the PubMed database over the last ten years.[8]

3.3 Biology

Currently, the underlying biology of ferroptosis is under investigation as the field has seen an exponential increase in interest. The areas of focus include lipid peroxidation, iron accumulation, cystine/cystathionine transportation, and an increase in oxidative stress.[9] There are a multitude of regulators within the ferroptosis mechanism, with the most significant being glutathione peroxidase 4 (GPX4). This regulator ensures that reactive oxygen species are being removed thus decreasing toxicity within the cell.[6] Other key points within the mechanism include the cystine/glutamate antiporter, or system xCT. This functions by importing cystine, which provides glutathione for the regulatory GPX4 system. The system has been shown to protect cells again

ferroptosis.[10] As more characterizations are being discovered, the more potential there is to manipulate and influence this mechanism.

3.4 Inducing agents

Previous publications have found the most effective small molecules in inducing ferroptosis. (1S-3R)-RSL3, stereochemistry is essential here, and erastin being the main treatments to force a cell into this programmed cell death. RSL3 affects the function of GPX4, limiting its ability to process lipid ROS leading to an accumulation of this resulting in cell death.[6] Erastin inhibits the cystine/glutamate antiporter leading to an imbalance of antioxidants and depleting the glutathione levels, thus resulting in cell death.[5] These two treatments have been some of the most studied treatments in this field of research, however there are other ferroptosis inducing agents. These include sulfasalazine and sorafenib, which also attack the system xCT.[11] Attacking multiple distinct points in this pathway aids in the understanding of the underlying mechanism.

3.5 Labile iron pool

Iron contained in the labile iron pool (LIP) of a cell is a key measurement in understanding the iron-dependent pathways. It is considered to be free iron and is able to undergo reactions that increase the cell's toxicity leading to ferroptosis.[12] While the LIP is homeostatic, it does remain in healthy amounts in a control cell. These levels are tightly regulated using uptake, storage, and release mechanisms and are dependent on iron regulatory proteins. At consistent levels, the LIP is used for supplying iron to metabolic and proliferative processes.[13,14] The LIP can be found in many different areas of the cell, including the cytoplasm, mitochondria, nucleus, and lysosome. The majority of iron found in these areas are Fe^{2+}.[15] While characterizing these intracellular iron

pools has been proven difficult, the advances in assays has been significant, with each assay having a separate advantage.[16] Being able to build models off this assay data could aid in validating the efficacy and influence of these assays, while progressing the already complex field of metallomics.

3.6 Drug sensitivity

There are multiple metrics of drug sensitivity, each measuring the impact of treatment over time on an individual cell line. The metrics include IC_{50}, E_{max}, GR_{50}, GI_{50}, and GR_{max}. There have been recent publications suggesting the legitimacy of GR_{50} over others, especially the traditional measurement, IC_{50}. GR_{50} is the dose at which the treatment reduces the growth rate of the cell line to 50%. In contrast to IC_{50} which measures the dose at which the control cell count is reduced by 50%. Hafner et al. showed that the GR_{50} metric was more resistant to experiment inconsistencies such as cell seeding number than other metrics of drug sensitivity through correlation analyses.[17] Considering all possible metrics of a feature and choosing the most robust is crucial to developing the optimal predictive tool.

3.7 Database

FerrDb is an excellent tool in understanding the interactions with drugs and cell types. It currently contains hundreds of curated markers, inducing agents, and associated disease that are involved in the ferroptosis mechanism.[1] It also has the capability to act as a collaborative space where researchers can add their newly discovered characterizations. Implementing a predictive tool to this database would add to the functionality and capability for other ferroptosis researchers within the community.

3.8 Renal Cell Carcinoma

Renal cell carcinoma (RCC) is in the top ten most malignant cancers in the United States.[18] It also has been shown to be one of the most sensitive cancer types to ferroptosis.[6] This is true across multiple subtypes within RCC due to the increase in lipid activity and iron metabolism.[19,20] With this in mind, exploring the mechanism in this context could yield results that support this as a further treatment option for this lethal cancer.

After deciphering which cancers are most sensitive to ferroptosis, it is important to understand why. It has been shown that the hypoxia-inducible factor (HIF) pathway is responsible for ferroptosis susceptibility within clear cell renal cell carcinomas (CCRCC), the prominent subtype of renal cancers. This is due to HIF-1/2α's ability to promote lipid storage and accumulation. After depletion of HIF-2α, the CCRCC became much more resistant to ferroptosis.[19] Further exploration into other cancer contexts could elucidate why others are more resistant to this pathway.

3.9 Statistical Models

Numerous statistical models have been developed in the past that identify and characterize the pathways that lead to the more traditional mechanisms of cell death. These include apoptosis, necroptosis, and necrosis. Specific to pathways implicated in those mechanisms, these models have seen success using logic gates and probabilistic methods to determine a cell type's fate.[21] These methods are useful to help understand the most likely sequence of events that occur in the pathway and are dependent on computationally simulated workings of a cell. These methods have been extended to ferroptotic cell death in recent years. The biochemical focus has remained on the effects of the more understood aspect of ferroptosis, lipid peroxidation; while the modeling aspect has focused on decision-based approaches.[22] Building upon these models as the biochemistry

processes are being discovered can provide essential information for treatment development. Focusing on creating a numerical predictive model can aid the research process within the lab, as it is based on assays over simulation.

MATERIALS & METHODS

Cell culture

All cell lines used can be found in Supplementary Table 1. Cells were maintained at 37°C in a humidified incubator at 5% CO_2. Cells were grown in appropriate media as specified and supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco).

Cell proliferation and viability assay

6,000 cells were seeded on day 1, treatment was added on day 2, and cell viability was determined using CellTiterBlue (Promega) on day 5. Both treatments, RSL3 and erastin were purchased from Selleck Chem.

FeRhoNox-1

Labile iron (Fe^{2+}) was detected using FeRhoNox-1 (GORYO Chemical) fluorescent imaging probe. The cells were seeded on a poly-l-lysine coated coverslip in a 6-well plate for 24hrs. FeRhoNox-1 was dissolved in dimethyl sulfoxide to produce a 1 mM solution, which was further diluted to a final concentration of 5 μM in HBSS. This diluted solution was prepared fresh. The cells were washed with HBSS three times. The cells were incubated in the diluted solution at 37°C for 1 hour. The cells were again washed with HBSS three times. The coverslip was removed from the well and placed upside down with a drop of buffer on a slide. The slide was scanned using the appropriate filter, ensuring that the cells did not dry out.

FerroFarRed

To validate the above assay, labile iron was detected via SiRhoNox (GORYO Chemical). Cells were seeded and cultured overnight on a poly-l-lysine coated coverslips. Culture medium was removed the following day then rinsed twice gently with HBSS. A final concentration of 5 μM of SiRhoNox in a HBSS was added to the plate and incubated for 1 hour at 37°C. The cells were washed with HBSS three times. The coverslip was removed from the well and placed upside down with a drop of buffer on a slide. The slide was scanned using the appropriate filter, ensuring that the cells did not dry out.

MDA

All cell lines were seeded on poly-l-lysine coated coverslips (Sigma Aldrich) in a 6-well plate for 24 hours. Media was removed and the cells were gently washed with PBS twice. The cells were fixed and permeabilized with 4% paraformaldehyde (PFA) in PBS. Then incubated at room temperature for 15 minutes. The cells were washed with PBS three times, then blocked with 5% goat serum (ThermoFisher) in PBS for 1 hour at room temperature. The cells were incubated with mouse anti-malondialdehyde (1F83) at a 1:100 dilution in PBS with 1% BSA overnight at 4°C. The cells were washed with TBST three times. The cells were incubated with goat anti-mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 at a 1:1000 dilution at room temperature for 1 hour. The cells were then washed with TBST three times. DAPI mountant (ThermoFisher) was used to adhere coverslips upside down on a slide. The slide was then scanned using the appropriate filter.

Western blot

Cells were seeded at $3x10^5$ cells per well in a 6-well plate. Cells were washed with ice cold PBS and lysed with RIPA buffer (Sigma) after 24 hours. Phosphatase inhibitor cocktail set II and protease inhibitor cocktail set III (EMD Millipore) were added at the time of lysis. Lysates were centrifuged at 15,000g x 10 min at 4°C. Protein concentrations were calculated using a BCA assay (Thermo Scientific). Proteins were resolved using the NuPAGE Novex Mini Gel system on 4% to 12% Bis-Tris Gels (Invitrogen). Equal amounts of cell lysates (15- 20 μg of protein) were resolved with SDS-PAGE then transferred to membranes. The membrane was probed with iron dependent primary antibodies, DMT, FTH1 etc. (Cell Signaling Technology) overnight at 4°C. The membrane was then washed three times and incubated with fluorescent secondary antibodies for 1 hour at room temperature. The secondary was removed and the membrane was washed three times. The membrane was cut and scanned using LI-COR (Lincoln, NE) Odyssey Imaging System.

Bodipy

All cell lines were seeded at $2x10^5$ cells per well on poly-lysine-coated coverslips (Sigma Aldrich) in a 6-well plate for 24 hours. The media was aspirated and each coverslip was washed with PBS three times. 2 μM BODIPY staining solution was prepared fresh in PBS. The cells were incubated in this solution for 15 minutes at 37 °C. The coverslips were then washed with PBS three times and placed onto a slide face down. The slides were then imaged.

Quantification method

Fluorescent images, FeRhoNox-1, FerroFarRed, and Bodipy, were quantified using ImageJ (v1.53). Western blot protein abundance signal was quantified using ImageStudio (v5.2.5).

Data analysis

All data analysis was done using r (v4.0.2). Viability curves were generated using the package GR-metrics (v1.16.0). Statistical analysis was done using the package jtools (v2.1.1)

RESULTS

Data quality

After analyzing multiple assays including bodipy, cellular iron, gene expression through western blot, anti-malondialdehyde expression through immunofluorescence, it was determined that iron assay measurements are the most consistent data type gathered. After quantifying protein abundance from western blot scans, the signal gathered was not consistent. These inconsistencies have been published about frequently within recent years.[23] No relationships were discovered likely due to the lack of quality in this data type. The bodipy signal, which measures lipid amount, remained consistent with excellent quality, however, there was no indication of a relationship between strength of signal and ferroptosis sensitivity. While the anti-MDA immunofluorescence signal showed a direct correlation and good consistency and quality of the data, the biology behind understanding oxidative stress in basal levels has yet be solidified. It was determined that creating a predictive model using more biologically relevant features at the basal level would be of greater significance.

Genetically modified cells were used to confirm the treatment induced ferroptosis trend. The GPX4 gene was knocked out in the ACHN cell line. This induces the cell into ferroptosis by removing that key regulator from the pathway. The GPX4 gene is no longer able to protect the cell from ferroptosis, which means that the GPX4 knockout (KO) cell does not need any treatment to induce ferroptosis, having a GR_{50} value of zero. This will be an important control for further analyses.

Iron signal

In figure 2, the spectrum of FeRhoNox-1 signal is shown. ACHN has a much higher signal compared to TK10. TK10 is resistant to both inducing agents and has a low iron signal. In contrast, ACHN is significantly more sensitive to both ferroptosis inducing agents and has a high iron level, showing the direct correlation. Finally, the GPX4 KO cell line is shown as an extreme control. Again, the KO cell line requires no chemical treatment to achieve cell death through ferroptosis, thus the iron signal is expected to be greatest. This data point was not included in the regression analysis as it uses an entirely different mechanism to achieve cell death. However, showing that it lies at the maximum point in ferroptosis proves our relationship between iron signal and ferroptosis inducibility.

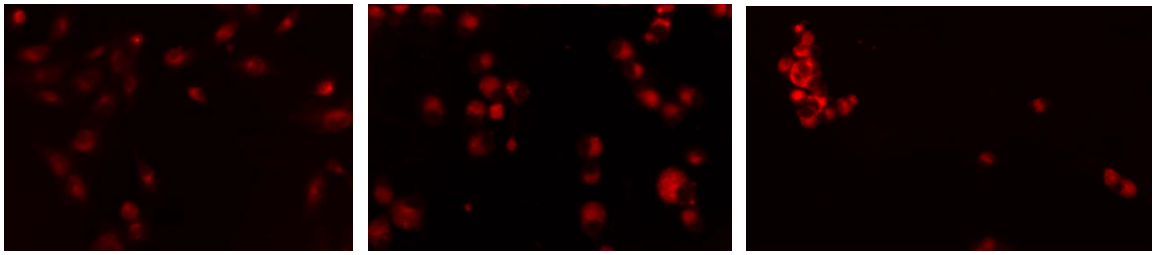
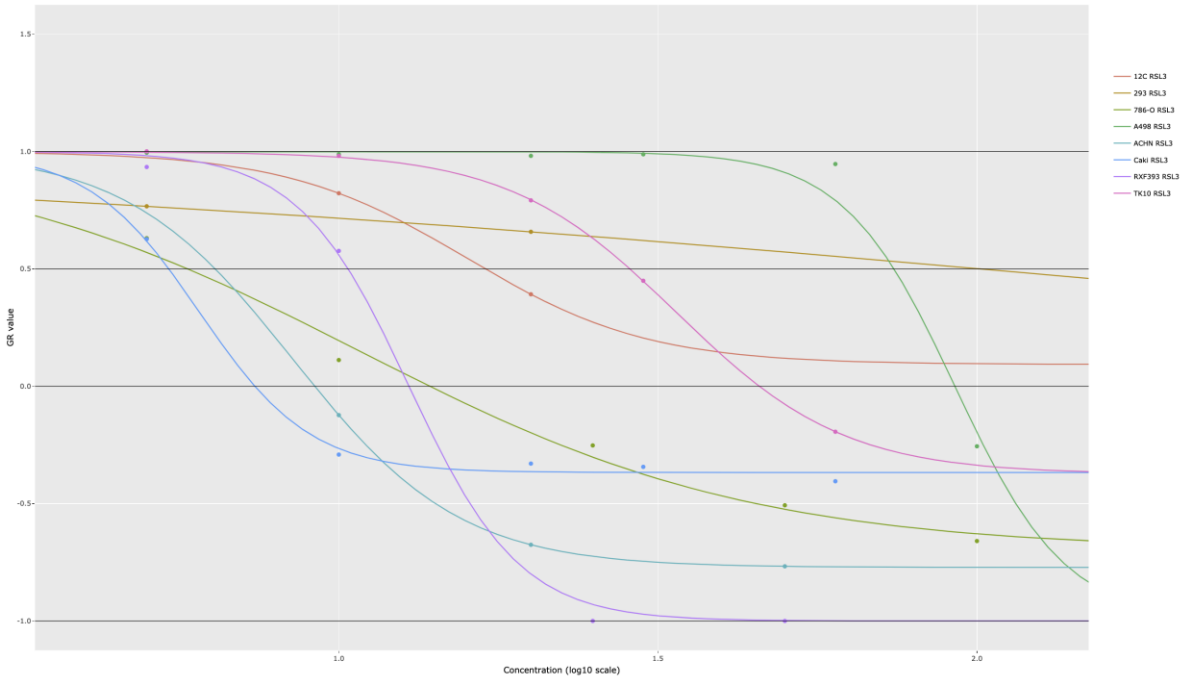


Figure 2: FeRhoNox-1 images of cell lines, TK10, ACHN, and GPX4 KO respectively.

Viability graphs

The difference in cell line sensitivity can be seen in the GR curves in figure 3. If it is shallower, the cell is more resistant to that certain treatment. If the curve is deeper or occurs shortly after initiation, the cell line is more sensitive to that inducing agent. The cell lines react similarly to different inducing agents, however not identically. This is illustrative of the fact that these inducing

agents affect different parts of the ferroptosis pathway, proving the ability to probe this mechanism from separate regulators.



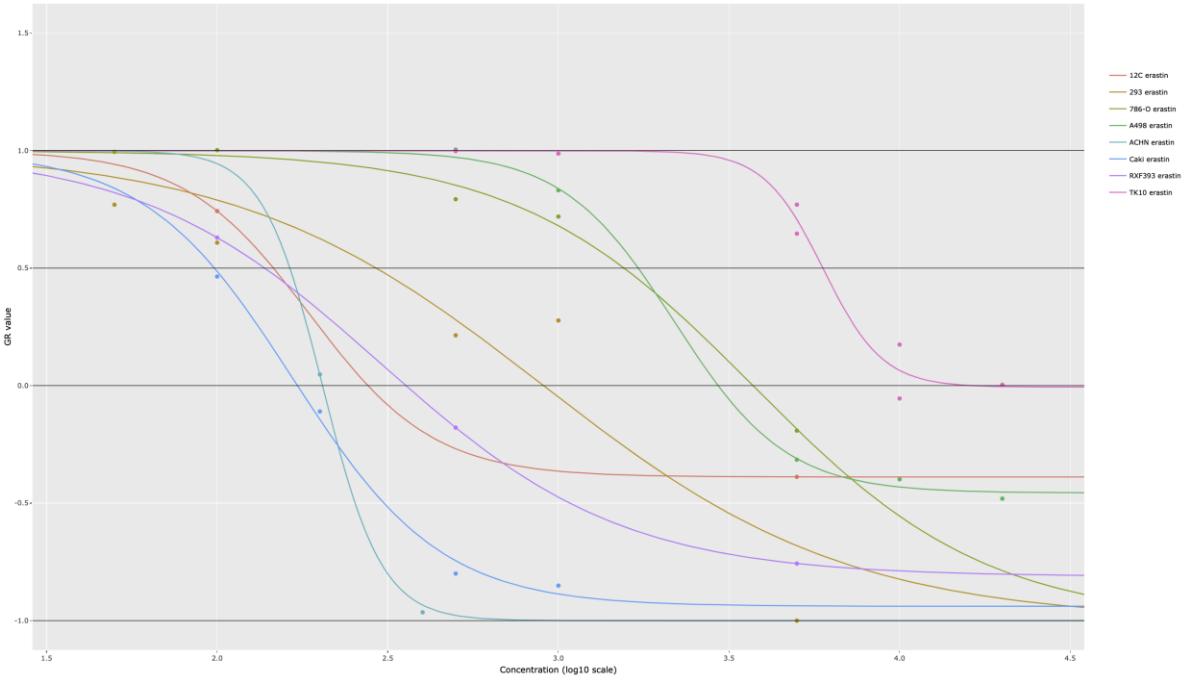


Figure 3: GR curves for eight renal cell carcinoma cell lines, using varying does of RSL3 and erastin respectively.

Correlation graphs:

In figure 4, a direct relationship between iron signal and drug sensitivity is shown. If more treatment is needed to induce a cell into ferroptosis, the cell has a lower iron signal. Therefore, if a cell is resistant to ferroptosis, it will have a low FeRhoNox-1 signal. If the cell is sensitive to the ferroptosis inducing agent, it will have a low GR_{50} value and a high iron signal.

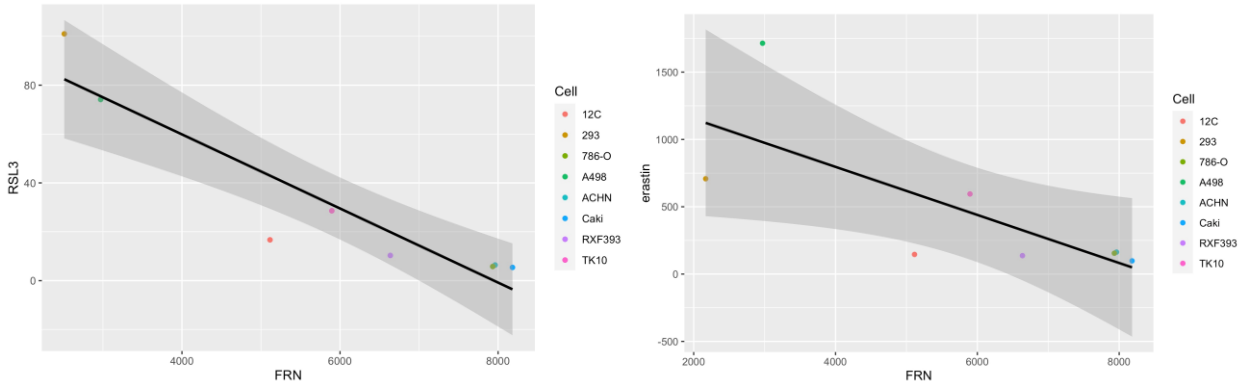


Figure 4: Scatter plot and best fit line for FeRhoNox-1 signal count and GR_{50} values for both inducing agents.

RSL3 is overall a more potent drug, meaning the necessary dosage to have an effect is significantly lower than erastin. This should be considered when looking at the difference in variability between inducing agents. If a drug has a larger dosing range, there will be higher variability when plotting in this context.

If the GPX4 KO cell signal were to be shown in figure 4, it would lie far to the right with a GR_{50} value of zero. Proving that the genetically modified cell is an extreme control for inducing ferroptosis.

Confirmation assay

To ensure that the results seen in the iron signal determined by FeRhoNox-1 were true, iron signal was also measured using a separate assay, FerroFarRed. The trend can be seen again in figure 5, validating the feature selection of LIP and therefore, the model.

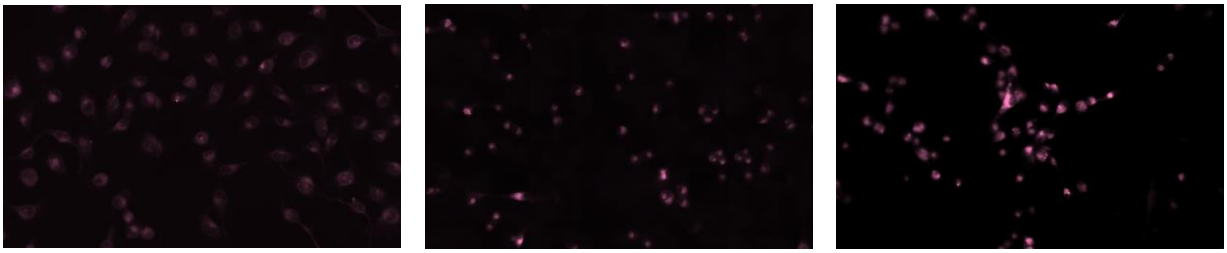


Figure 5: FerroFarRed images of cell lines, TK10, ACHN, and GPX4 KO respectively.

In figure 6, the trend also holds. The relationship matches that to FerRhoNox-1, supporting that this can be a reliable method of predicting ferroptosis.

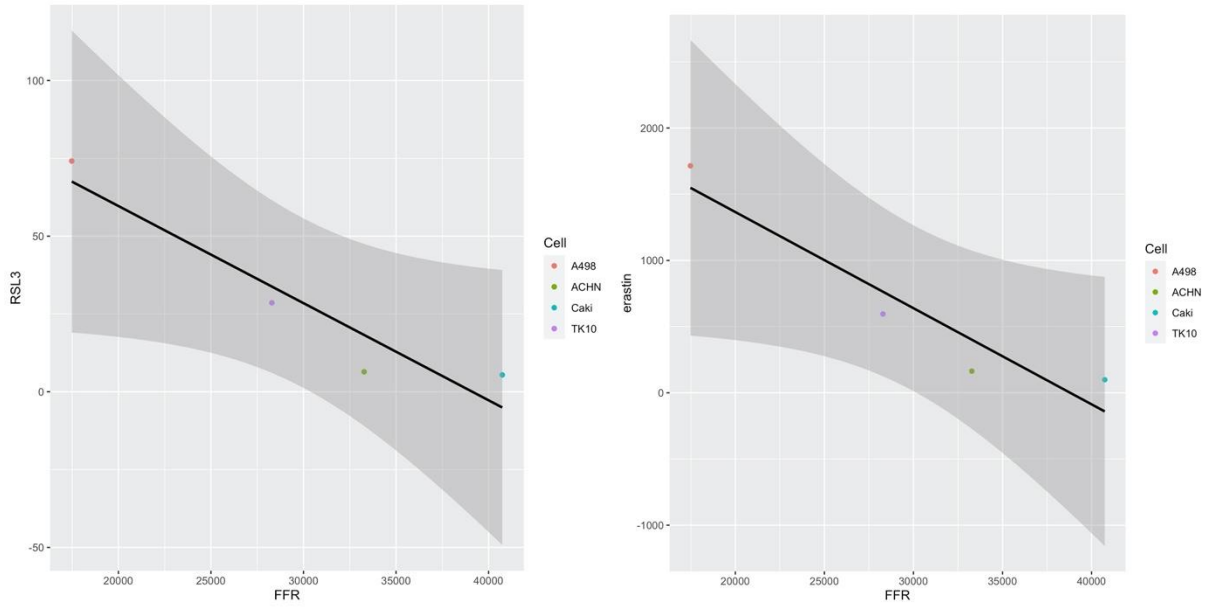


Figure 6: Scatter plot and best fit line for FerroFarRed signal count and GR50 values for both inducing agents. Not all cell lines measured, used for validation.

Model specification

The measurements of the linear regression model in figure 6 show that it is statistically significant with p-values lower than 0.05. It also shows the variability accounted for with the R^2. An R^2 value of 0.84 signifies that 84% of the variability within the data is explained using this model. That number decreases for erastin due to the dosing range, as mentioned earlier. This is also apparent in the confidence intervals shown in figure 7. The range is significantly larger for the erastin model.

A linear regression model was selected as optimal after a thorough feature selection process was completed. After assessing all possible features, the iron signal data showed to be the most consistent and reliable. Since that input variable showed a direct correlation with the output variable, only one feature was used. This ensured that the most accessible model was created. With a single feature and a direct correlation, linear regression is the best possible model for these data.

Observations	8
Dependent variable	RSL3
Type	OLS linear regression

F(1,6)	37.24
R²	0.86
Adj. R²	0.84

	Est.	S.E.	t val.	p
(Intercept)	120.52	15.55	7.75	0.00
FRN	-0.02	0.00	-6.10	0.00

Standard errors: OLS

	Est.	2.5%	97.5%	t val.	p
(Intercept)	120.52	82.46	158.57	7.75	0.00
FRN	-0.02	-0.02	-0.01	-6.10	0.00

Standard errors: OLS

Observations	8
Dependent variable	erastin
Type	OLS linear regression

F(1,6)	7.98
R²	0.57
Adj. R²	0.50

	Est.	S.E.	t val.	p
(Intercept)	1577.12	417.79	3.77	0.01
FRN	-0.19	0.07	-2.82	0.03

Standard errors: OLS

	Est.	2.5%	97.5%	t val.	p
(Intercept)	1577.12	554.83	2599.40	3.77	0.01
FRN	-0.19	-0.35	-0.03	-2.82	0.03

Standard errors: OLS

Figure 7: Model information, including variability accounted for, statistical significance, and confidence intervals, using FeRhoNox-1 as independent variable and GR_{50} for inducing agents as dependent variable.

Prostate results

Measurements of iron signal in the prostate cancer cell line validated the trend of low iron signal/low ferroptosis susceptibility. V16D has almost no reaction to the ferroptosis inducing agents, the cells also have incredibly low iron signal. Seen in figure 8 is the viability curve for V16D, this calculates to an infinite GR_{50}.

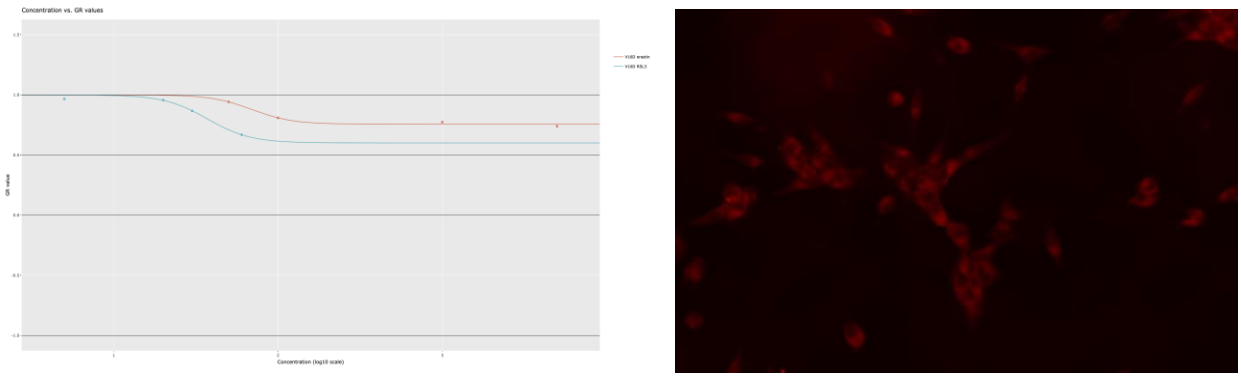


Figure 8: The GR curves and FeRhoNox-1 signal for a prostate cell line.

The assumption that a cancer type is more sensitive to ferroptosis than other cancer types is of utmost importance. Building a predictive model off of the most sensitive cancer does not lend to a generalizable model. This illustrates the boundaries of the current model. Building a model based on the most sensitive context ensures ability to induce the cell lines into ferroptosis. Extending that model to the one of the most resistant contexts, prostate cancer, proves more challenging. Closing this gap is a necessary step in further extension of this model.

DISCUSSION

It has been shown that using solely iron-dependent probes to predict ferroptosis within a cell line is possible. In the process of proving this, the LIP measurement was deemed the most effective and consistent assay signal out of a multitude of assays. Deciphering the most relevant and predictive features for a given model is one of these most important aspects in prediction tools, and the data quality analysis was the foundation for building this relevant and predictive model. This step in the process weeded out many possible features for this model, leaving a single feature linear regression as the most accessible and accurate option of predicting ferroptosis.

During the model building process, the original intent was to build a binary classification model to predict high or low susceptibility categories. However, with the selection of one sole, high quality feature, this was no longer possible or useful. Thus, a quantitative model was then selected. The complexity of the model was expected to more significant, however when creating accessible predictive tools for the research community, simplicity is of more importance.

Proving that this methodology is possible is just as important as outlining the boundaries. As seen when generalizing this model, some amount of sensitivity to ferroptosis is necessary, therein lies the boundary. However, this does open biological questions that could inform the research community about ferroptosis resistance and how this may be seen in patient populations.

Implementing this model as a collaborative tool within the ferroptosis research community could significantly aid advancement in the field. Databases with predictive functionality continue to speed up discovery, with the goal of being the able to provide increasingly individualized treatment options to the patient population. Further investigation into other functionalities, similar to what is shown here, is ever more impactful to cancer research.

Limitations

The scope of this project remains in the cell line model and was not extended into more complex contexts. It was also limited to two cancer types and two treatments, increasing these numbers could greatly enhance the model.

Future Work

This is a rapidly developing field of research and opportunities for future work can be found everywhere. Due to the anti-MDA immunofluorescence signal that showed promising results with quality and correlation, further research into the effect that basal levels of oxidative stress have on a cell type's sensitivity to ferroptosis could elucidate more key players in this mechanism. As mentioned throughout, expanding this model to other cell lines and inducing agents could increase the interpretability, functionality, and relevancy.

By measuring and averaging the LIP in organoids or mouse models, the error can be calculated and relevancy towards these contexts can be assessed. In an organoid, providing a way to predict ferroptosis susceptibility can create a method to model this in a heterogenous context. In a tumor environment, understanding the susceptibility within each cell type could predict the ability to induce that given tumor into ferroptosis, providing an effective treatment to lower the tumor burden within a patient. By extending this prediction tool to more heterogenous contexts give necessary information to prove efficacy in translational medicine.

CONCLUSION

Proving a direct relationship to ferroptosis susceptibility is key to researching this biological mechanism in many contexts. In addition, making this a numerical model that can calculate sensitivity adds functionality to the prediction. This research provides a base foundation of knowledge and understanding that can later be expanded to other cancer types with a clearer view of relevant mechanisms. This research also provides an example of co-design of computational models with biochemical processes, when used with more frequency could greatly impact the field of cancer research and provide necessary insight into patient treatments.

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APPENDIX

Table 1

CELL LINE	SOURCE	MEDIA
ACHN	ATCC	DMEM
CAKI-1	ATCC	McCoy's
TK10	ATCC	RPMI 1640
A498	ATCC	RPMI 1640
RXF-393	ATCC	DMEM
12C	ATCC	DMEM
293	ATCC	DMEM
786-O	ATCC	RPMI 1640
V16D	ATCC	RPMI 1640