Light it Up: A Pre-Clinical Fluorescent Model to Illustrate Immunogenic Cell Death

by

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Submitted to the graduate degree program in Pharmaceutical Chemistry and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

Immunogenic cell death is a recently identified subset of apoptotic cell death that, when activated, may improve outcomes and remission rates in cancer patients. Calreticulin is an essential early-stage damage-associated molecular pattern that is triggered by certain drugs. It is thought that cancer chemotherapeutics and other agents proven to exhibit immunogenic cell death do so by creating endoplasmic reticulum stress that causes calreticulin to translocate to the cell membrane and activate more downstream signals to recruit the immune system. The current gold standard for the detection of immunogenic cell death is a lengthy and costly procedure involving immunocompetent mice vaccinated with cancer cells already exposed to the potentially immunogenic agent. This is not a realistic method to screen all agents for their potential to induce immunogenic cell death (ICD), but the only other pre-clinical models have been done in only one cancer cell line. This study aimed to generate a stable murine head and neck cancer cell line to be used for screening chemotherapeutics using fluorescence, assess the ability of these transfected cells to accurately predict ICD-inducing potential, and test newly modified chemotherapeutics for their potential to cause ICD. To do so, cells were transfected with a plasmid construct for the calreticulin protein and a red-fluorescent protein. The fluorescent model is not a replacement for the gold-standard detection of ICD, but rather enables a more high-throughput approach to the widespread testing of all clinically relevant cancer chemotherapeutics as well as specifically modified chemotherapeutics to induce ICD.

Acknowledgements

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Chapter 1: Introduction

1.1 Summary of Cancer Chemotherapy

The treatment of cancer has long fascinated scientists, politicians, and humanitarians. Due to the complex nature of the disease, curative chemotherapy has only been considered a realistic goal for a few decades. In fact, chemotherapy was only added to the standard of care for most cancers in the 1960s.[1] There were agents available prior to this time period, such as the nitrogen mustards discovered after their use in World War I, but the agents' intolerable side effects and lack of specificity for cancer cells made them nearly unusable. The rapid resistance cancer cells developed to these early agents was a problem as well. Through the 1940s, surgery alone was the standard of care, and in the 1950s, radiation was added.[2] As the pathophysiology and progression of the disease was unraveled, a problem with the surgery-only approach arose. With the understanding of metastasis-- when cancer cells from the tumor detach from the main cell and establish themselves at distant sites in the body--it became clear that surgery alone would not be enough to provide a cure as so many had thought before.

By the 1960s, these previously toxic agents had been chemically modified and new agents were discovered. Some toxicity remained, but the therapeutic benefits finally outweighed the risks. It was clinically beneficial for patients to receive these medications. The idea of combination chemotherapy, using multiple drugs with multiple mechanisms of action at the same time, was tested and proved to work. Chemotherapy officially became part of the guidelines and standards of care for most forms of cancer. These agents provided a direct effect onto cancer cells themselves, and had been strongly improved since the literal poisons of the early century.

As more and more researchers began to study cancer, it became clear that the disease differs depending on the originating site of the tumor.[2] However, discovery of these disease markers was slow until the targeted therapy revolution of the 1980s.[1] With specificity in mind and an ability to study cancer on a molecular level, it was possible to identify markers and molecular targets specifically for different kinds of cancer. Support for the idea of a single "magic bullet" to destroy all forms of the disease rapidly waned as it was shown over and over again to be an unrealistic goal. Different regimens including varied mechanisms of chemotherapy combined with surgery and radiation could be close to curative, but for patients with advanced metastatic disease, the treatment remained palliative.

One example of a breakthrough in targeted therapy includes the checkpoint inhibitors, useful in acute or chronic myeloid leukemia. In patients with a Philadelphia chromosome mutation, this type of leukemia upregulates a specific tyrosine kinase to rapidly grow and differentiate. Imatinib, or Gleevec, is used to inhibit this kinase which induces apoptosis in these cancerous cells. This has showed an improvement in progression-free survival time for a significant number of patients as compared to placebo.[3]

Another example of modern targeted therapy is immunotherapy using monoclonal antibodies.[4]This concept is popular in many chronic disease states outside of cancer including autoimmune disorders, and has dramatically changed the prognosis of patients with HER2-positive breast cancer. Before the advent of trastuzumab[5,6], or Herceptin, HER2-positive cancer was considered to be one of the deadliest forms of breast cancer. Due to the rapid proliferation and metastasis of this particular breast cancer, it was often resistant to treatment or detected too late. By identifying human epidermal growth factor receptor 2 as an overexpressed target

on these cancer cells, the development of an antibody to bind to its extracellular domain resulted in nearly curative outcomes in a previously aggressive disease. When used as an adjuvant to other chemotherapy, trastuzumab mediates antibody-dependent cellular cytotoxicity and improves overall outcomes including increased number of quality of life years and remission rates.

It wasn't until the late 1980s and early 1990s that the concept of a cancer vaccine arose.[1,7]Patients who had been successfully treated with chemotherapy years prior were living long enough that years after achieving remission—clinically undetectable cancer—the disease was coming back and it was much more difficult to treat. Combination chemotherapy to combat resistance worked for the initial case, but wasn't always a permanent solution for metastatic cancer. The idea of permanently sensitizing the immune system to a specific type of cell, as with infectious diseases, was there but remained undeveloped. Vaccination invokes the necessary aspect of immune system involvement[8]. However, the very nature of cancer requires evasion of the immune system to continue uncontrolled proliferation. In addition to this, many cancer chemotherapeutics cause bone marrow suppression and as a result, a weakened immune system. The concept of true vaccination in cancer is challenging, as vaccination would require activation of the already debilitated immune system by a stimulus that had previously been overlooked.

Ideally, it would be possible to stimulate even a weakened immune system and teach it to recognize a specific type of cancer via cancer-specific antigens. As the idea for a cancer vaccine developed and later shown to be effective and clinically relevant[9], there was more work being done on the role of the immune system in patients with cancer. It had been discovered

that anthracyclines were causing an unanticipated and previously unnoticed immune response.[10] This discovery demanded the observation that cell death was not as simple as previously presumed.

According to the old school of thought, there were only two kinds of cell death: apoptosis and necrosis.[11,12]Apoptosis happens thousands, even millions of times per day in the human body as the natural end to cells. These cells have divided so many times the risk of mutation is too high for the cell to safely continue dividing. This mechanism is one of the body's simplest and endogenous defenses against formation of cancerous cells. Because it happens so frequently and does not typically involve the immune system in patients, it was considered "tolerogenic" or allowable by the immune system, excluding patients with autoimmune disease. [13,14]On the other hand, necrosis is cell death as the direct result of a pathogen or some other stimulus killing cells prior to their planned end. It was thought that these two categories of cell death were entirely

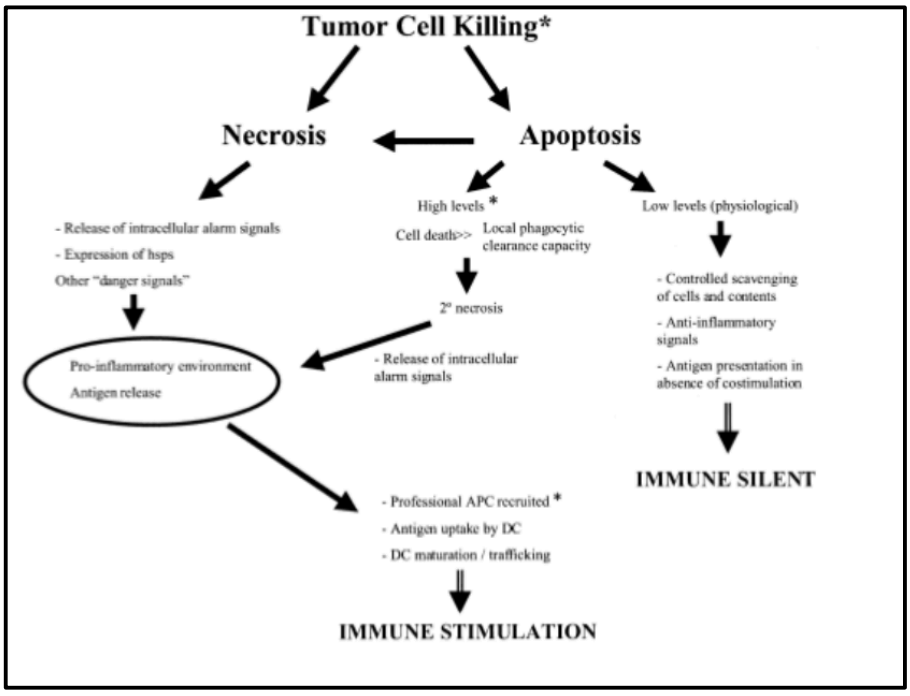


Figure 1. One of the earliest illustrations suggesting that both necrosis and apoptosis could be immunogenic. **Melcher et. al** *Apoptosis or necrosis for tumor immunotherapy: what's in a name?* J Mol Med 1999 77:824-83

discrete, until Casares et. al[10] showed that specifically anthracyclines stimulate the immune system to later recognize the types of tumors that have been proliferating.

1.2 Immunogenic Cell Death

The idea of immunogenic cell death as a specific form of apoptosis has since been studied

Bona fide* ICD-inducing drug	Confirmed** DAMPs activated
Bleomycin	CALR, ATP, Type I IFN, HMGB1
Bortezomib	CALR, Type I IFN, HMGB1
Cyclophosphamide	CALR, ATP, Type I IFN, HMGB1
Digoxin	CALR, ATP
Doxorubicin	CALR, ATP, Type I IFN, HMGB1
Epirubicin	CALR, ATP, HMGB1
Idarubicin	CALR, HMGB1
Mafosfamide	CALR, HMGB1
Mitoxantrone	CALR, ATP, Type I IFN, HMGB1
Oxaliplatin	CALR, ATP, Type I IFN
Patupilone	CALR, Type I IFN, HMGB1
*bona fide as tested by gold-standard ICD murine vaccination assay ** confirmed as tested—missing DAMPs from each box have yet to be determined. CALR = calreticulin, ATP = adenosine triphosphate, IFN = interferon, HMGB1 = high mobility group box 1, DAMP = damage-associated molecular patterns	

Table 1. A list of confirmed agents that induce ICD as challenged in mice and their confirmed DAMPs. Adapted from **Bezu et. al** *Combinatorial strategies for the induction of immunogenic cell death.* Front. Immunol 6:187 2015

extensively, but ultimately there is still largely conjecture as to why it may happen. It is entirely possible and highly likely that many pathways that result in this form of apoptosis have not yet been discovered. Not every anticancer agent is able to stimulate this response. Interestingly enough, agents even within the same class of chemotherapeutic drugs differ in their abilities to induce this type of response both in vitro and in vivo.

Immunogenic cell death is completely dependent on the establishment of a stress response.[15,16] That stress response must specifically activate certain signals that alert the adaptive immune system via dendritic cells. There is an increasing amount of clinical data that shows engaging the immune system remains clinically relevant even when the patient has been in stable remission.[17–19]The next step to maintaining curative chemotherapy and an alternative to the traditional idea of a cancer vaccine could be controlled activation of this type of cell death.

The key to inducing immunogenic cell death lies with a protein that is present in nearly every mammalian cell and does not under normal circumstances influence the immune system.[20]This protein, calreticulin, is usually found in the endoplasmic reticulum and is involved in the regulation of intracellular calcium release. However, in times of severe stress on the cell, it is able to act as an alarm and is translocated to the cell membrane where it affects the downstream release and secretion of other damage-associated molecular patterns (DAMPs) such as adenosine tri-phosphate (ATP) and high-mobility group box 1 (HMGB1) to amplify the stress response.

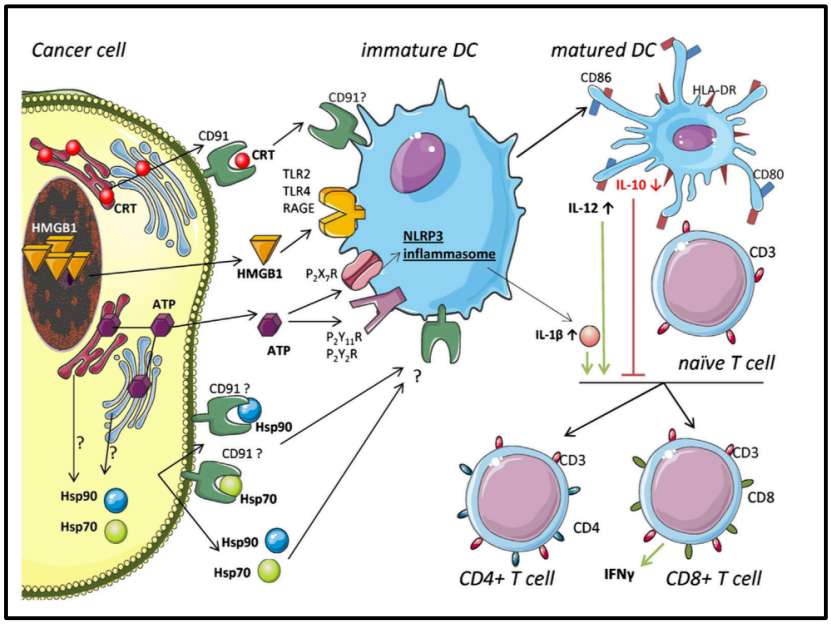


Figure 2. Schematic of immunogenic cell death. Calreticulin is translocated to the cell membrane, docks and activates other damage-molecular patterns. **A.M. Dudek et. al** Cytokine & Growth Factor Reviews 24 (2013) 319-333

HMGB1 in particular works on toll-like receptor 4 (TLR-4)[21]. TLR-4 is essential for an amplified immune response to these cells as it assists in the processing and cross-presentation of cancer cells to dendritic cells. Dendritic cells are essential components of the adaptive immune system response.[22] Without the amplification from TLR-4 activation, relapse of cancer has been shown in patients to be not only more frequent, but rapidly occurring. This immune response is what mediates suppression of tumor growth as well as determines long-term survival and maintenance of remission in animal models as well as patients.

As shown by anthracyclines, there are four currently identified DAMPs[14] required for the activation of the immune response. It is likely that there are more proteins that influence this pathway, but these four are essential to the anthracycline response. It is also possible that other drug classes may require more or different proteins to exhibit an immune response. However, it seems these four are a minimum requirement for the induction of immunogenic cell death, and stimulation of these four in particular to create an immune response has been seen in other drug classes as well.

The first required step is the extracellular exposure of calreticulin. This leads to secretion of ATP[23] to stimulate the recruitment of antigen-presenting cells, production of type I interferon, and the release of HMGB1 to activate TLR-4. By recruiting antigen-presenting cells, these DAMPs stimulate uptake processing and presentation of the antigens from the apoptotic cells to prime the adaptive immune response. If one of these four are not activated, it completely compromises the immunogenicity and does not result in ICD.

Since its discovery, many investigations into the mechanism of immunogenic cell death have occurred.[24–44]A large percentage of these studies focus specifically on calreticulin as the earliest

and one of the most important molecular targets for inducing immunogenic cell death. [45–53]These studies have shown that across all drug classes tested thus far, inability to stimulate translocation of calreticulin to the cell membrane equates to an inability to induce immunogenic cell death. It is suggested that endoplasmic reticulum stress is the cause of calreticulin translocation[54]. Cancer chemotherapeutic agents are not the only methods of inducing ICD, but they are some of the most convenient. Other methods include radiation and photodynamic therapy, and ICD is also shown in cells treated with cardiac glycosides.[55,56,57]

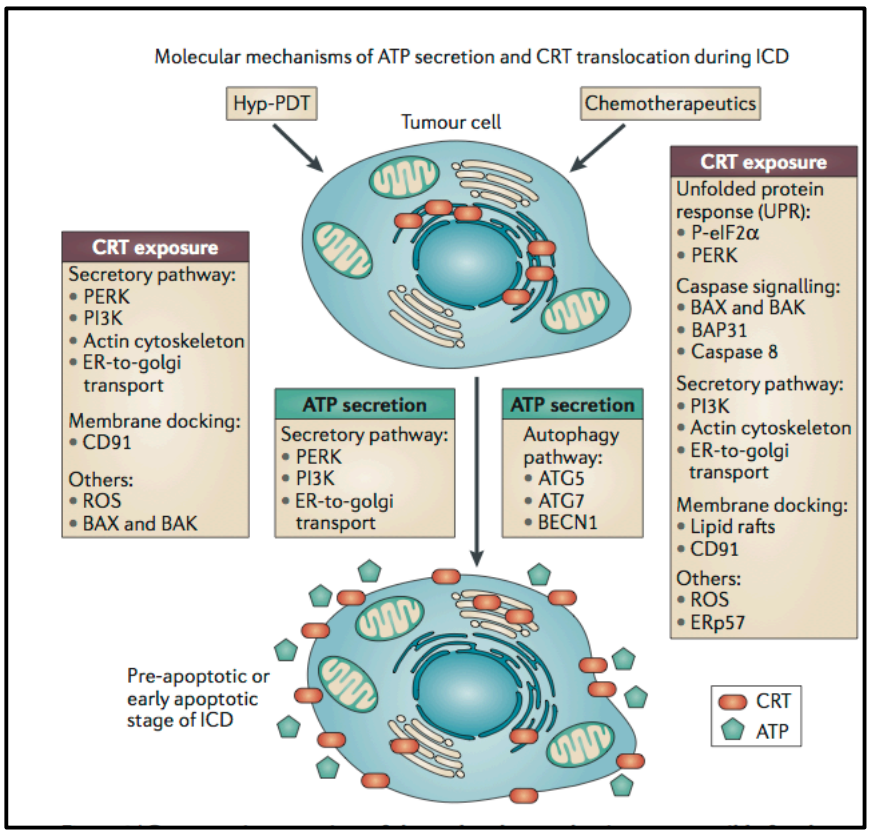


Figure 3. Illustration of molecular mechanisms of DAMPs in ICD. **Krysko et. al** *Immunogenic cell death and DAMPs in cancer therapy***.** Nat Rev Cancer 12:860-875 2012

While it is now possible to provide an explanation of how it happens, there is still no rapid method to indicate the possibility of a drug inducing immunogenic cell death, and there have been no specific drugs synthesized for the purpose of inducing immunogenic cell death. These investigations have led to a strong foundation that illustrates the physiology of immunogenic cell death at a cellular level, but the possibility remains largely untapped.[58–62] In addition, the high cost of exploring

the immune response in this manner has prevented the investigation of ICD in all chemotherapeutic products used in regimens today. Creating ICD is a new standard that chemotherapy regimens must be held to in order to maintain remission in patients. To make this feasible, modification of current agents available on the market is essential.[48,49] It is also necessary to be able to detect ICD potential in all cancer chemotherapeutic agents in widespread clinical use.

The current gold standard for the detection of immunogenic cell death[63] is not easily amenable to testing multiple drugs or concentrations. It is an expensive, slow process requiring immunocompetent murine models for vaccination assays. In these assays, tumor cells are exposed in-vitro to a chemotherapeutic agent or another stimulus that is purported to cause an immune response. The cells are washed and re-suspended in PBS, then injected into the flank of a mouse. Since the result is dependent on a functioning immune

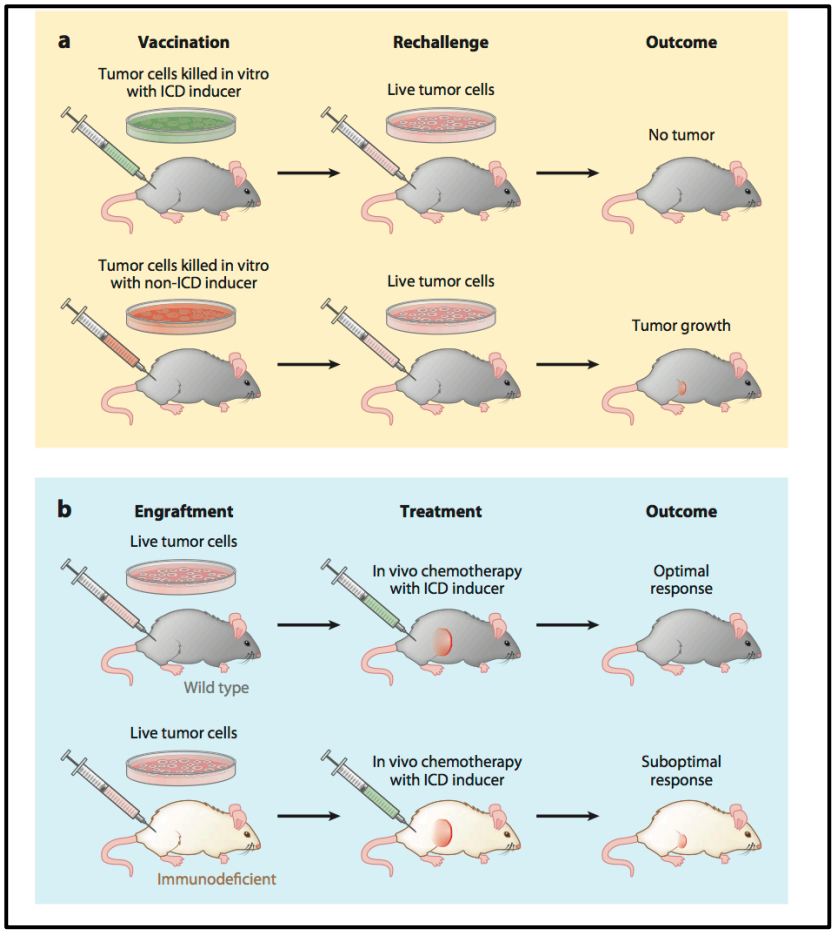


Figure 4. Illustration of the gold standard procedure for detection of ICD. **Kroemer et. al** *Immunogenic cell death in cancer therapy***.** Ann Rev Immunol 2013 31:51-72

system, the in-vitro exposure must occur in a murine cell line, as human cells will not grow tumors in immunocompetent mice. One week after injection, the same tumor cells are injected into the opposite flank and the mice are monitored for tumor growth. When the cells are exposed to doxorubicin, a typical inducer of immunogenic cell death, prior to injection, 80% of mice do not develop tumors. This "gold standard" is not easily affordable to the discovery of other immunogenic chemotherapeutics due to the cost and time commitment. Since the cells are murine cells in a murine model, there is still no guarantee that the response, if any, will relate to a similar response in a human model.

1.3 Platinum chemotherapy

Platinum compounds are used in over half of all patients receiving chemotherapy treatment.[64] These drugs work by chelating single-stranded DNA during cell division, causing a kink that stimulates programmed cell death.[65] Compared to more modern agents, these drugs are not particularly cancer specific. Their only specificity originates from the idea that in many cancers, the rate of cell division is increased. Platinums work only in actively dividing cells to stop those cells from further dividing. The overall mechanism of action remains relatively the same across every drug in the class, but there is only one agent that causes immunogenic cell death.

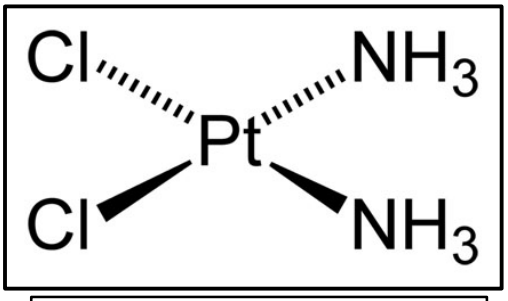


Figure 5: Structure of Cisplatin

Cisplatin is the oldest of these compounds[66] and is still used in some regimens today as it has been studied extensively and is an effective agent for direct killing.[67–69]It is a relatively

simple molecular structure with two chlorides to act as the leaving groups, leaving the central platinum open to chelation with nucleic acids. However, it has never been a curative treatment for chemotherapy due to the problems with resistance and side effects. The cisplatin resistance[70,71] has been studied extensively. Resistance caused by failure to achieve a drug concentration high enough to effectively kill cancer cells can be attributed to be decreased uptake of the drug rather than increased efflux of the drug by cancer cells. The dose limiting toxicity of cisplatin is nephrotoxicity, though there is a significant amount of bone marrow suppression that occurs as well as ototoxicity.

As an effort to reduce resistance and reduce side effects, other platinums were developed.[65,72]The first of these was carboplatin, modified by adding a dicarboxylate in place of the chloride leaving groups. By changing the central conformation, it showed an improved toxicity profile but did not confer an improvement to resistance. In fact, the level of cross-resistance was so high that eventually carboplatin fell out of favor, as other drugs with differing mechanisms of action allowed for a reduction in dose of cisplatin in combination therapy to improve the incidence of adverse events.

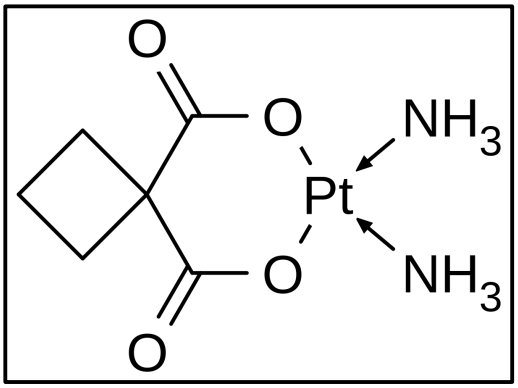


Figure 6: Structure of Carboplatin

A later version, oxaliplatin, was modified[52] to include two larger donating groups, changing the central platinum conformation to square planar. Due to its chemical structure, oxaliplatin has a slightly different mechanism of action and a different dose-limiting toxicity—neurotoxicity.[73,74]Though they are in the same class and have generally the same mechanism of

action, oxaliplatin does not exhibit cross-resistance to cisplatin, does not have the same dose-limiting toxicity, and is used in different cancer chemotherapy regimens. Additionally, oxaliplatin tends to exhibit fewer DNA adducts to achieve the same amount of growth

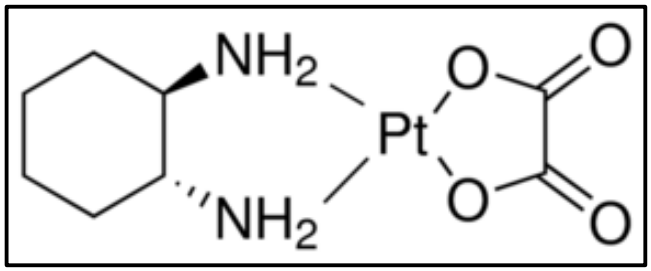


Figure 7: Structure of Oxaliplatin

inhibition, which is typically associated with crosslinked protein in the cytosol. Cisplatin is not effective in colon cancers, but oxaliplatin is first line for colon cancer in varying concentrations, and has also been approved for pancreatic cancer.[75,76]

However, their use in current regimens is not the only difference between the two most common platinums. After the discovery of doxorubicin to induce endogenous immunogenic responses, many other drugs were studied for this same response.[14] It was discovered that oxaliplatin alone of the platinums also had the ability to induce immunogenic cell death. This is often attributed to the fact that oxaliplatin causes endoplasmic reticulum stress. Cisplatin does not display immunogenic cell death unless this type of stress is exogenously added to the treatment regimen[77], in which case the immune response is restored. Thus, it is likely that cisplatin does not cause endoplasmic reticulum stress. These studies further proved that calreticulin motivated by endoplasmic reticulum stress is one of the earliest pathways required to induce immunogenic cell death.[34,50,78–84]

1.4 Niclosamide

Niclosamide is primarily classified as an anti-helminthic drug used to treat infectious agents such as tapeworms. It is given orally and has no major adverse events such as immunosuppression, neuropathy, or major organ toxicities[85]. By inhibiting glucose uptake, it stops oxidative phosphorylation and anaerobic metabolism. Recent investigations into the drug show beneficial properties as an adjuvant agent in regimens for cancer chemotherapies as an inhibitor of the STAT3 pathway[86]. Though it is not yet considered for routine clinical use and is

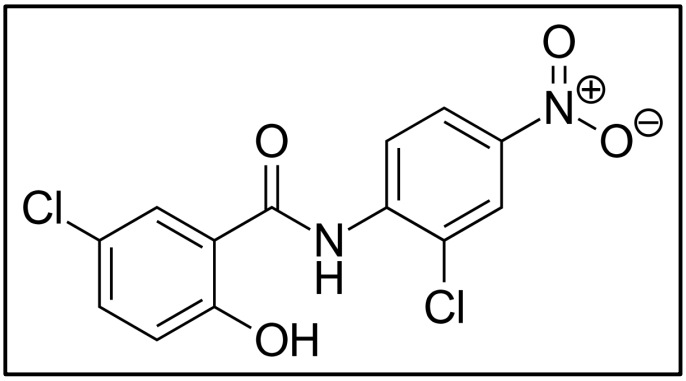


Figure 8: Structure of Niclosamide

not available in the U.S. due to lack of use, many early or pre-clinical studies show its efficacy in a wide variety of cancers including but not limited to prostate, renal, ovarian, oral, colon, lung, breast, osteosarcomas and leukemias. There is also some evidence that it may have activity against methicillin-resistant staphylococcus aureus[87], and may also inhibit early stages of Zika replication. Niclosamide has the potential to positively impact cancer chemotherapy when added to older chemotherapeutic agents.[88] Additionally, it is an old drug discovered in 1958 that is affordable and adds no risk of major adverse events to already hazardous chemotherapy regimens.

1.5 Purpose

In this project, we aimed to **(1)** adapt a pre-clinical model of immunogenic cell death to a new cell line using fluorescent tags, **(2)** characterize expression of immunogenic cell death in these lines using known ICD inducers, **(3)** assess the capacity of modified cytotoxic chemotherapeutics to induce ICD using this cell line as a pre-clinical model. The gold-standard model of immunogenic cell death was first illustrated in one of the earliest papers on the ICD-inducing properties of doxorubicin, an anthracycline. Providing an updated model in a new cell line has many benefits. It is cheaper, as there is no initial work using costly animals or employees trained in animal care. It is rapid, as potential ICD can be detected in under three days. With an updated pre-clinical model, screening agents for ICD becomes more high-throughput and more realistic to do on a larger scale.

Chapter 2: Materials and Methods

2.1 Summary

This project aimed to establish a rapid ICD detection method adapted from Golden et. al[56] in a new stable murine cell line expressing calreticulin and a fluorescent marker as well as test novel compounds to assess ICD potential. To do so, a plasmid construct was ordered from DNA 2.0 (Now ATUM) that encoded for calreticulin and resistance genes. After purifying the plasmid DNA, it could be used with lipofectamine transfection in several cell lines. After transfection, the cells containing plasmid expressing the calreticulin protein would emit fluorescence in the red fluorescent protein range, with an excitation of 554 nm and emission of 590 nm. The fluorescence would be displayed intracellularly, particularly near the nucleus as the protein is

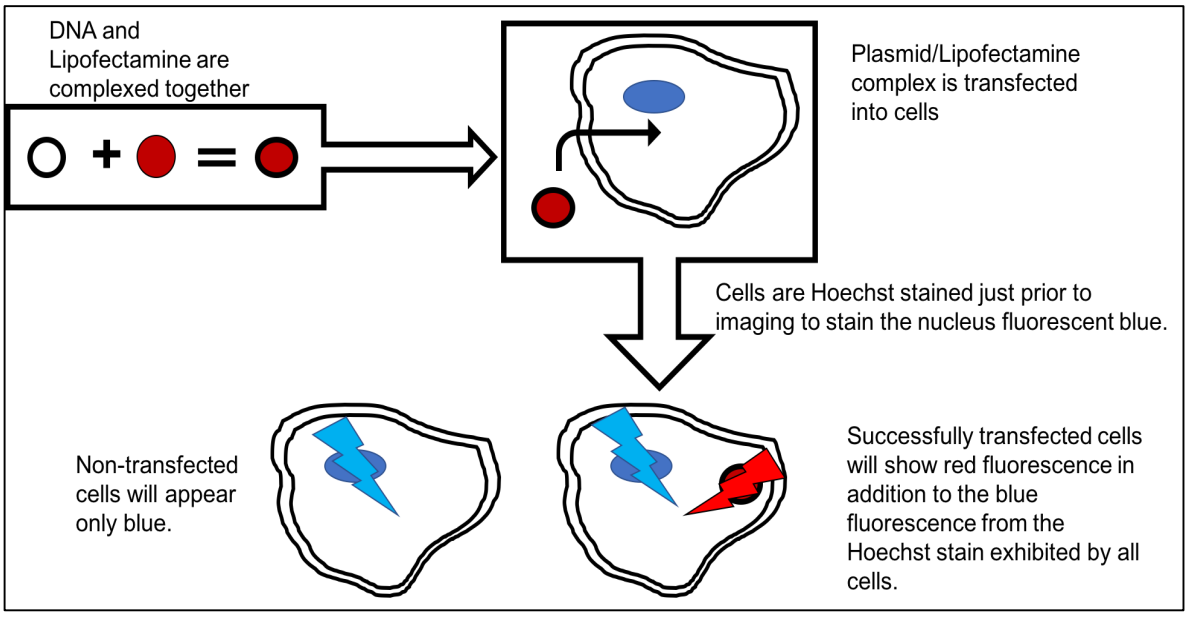


Figure 9: Phase 1 of the experimental process

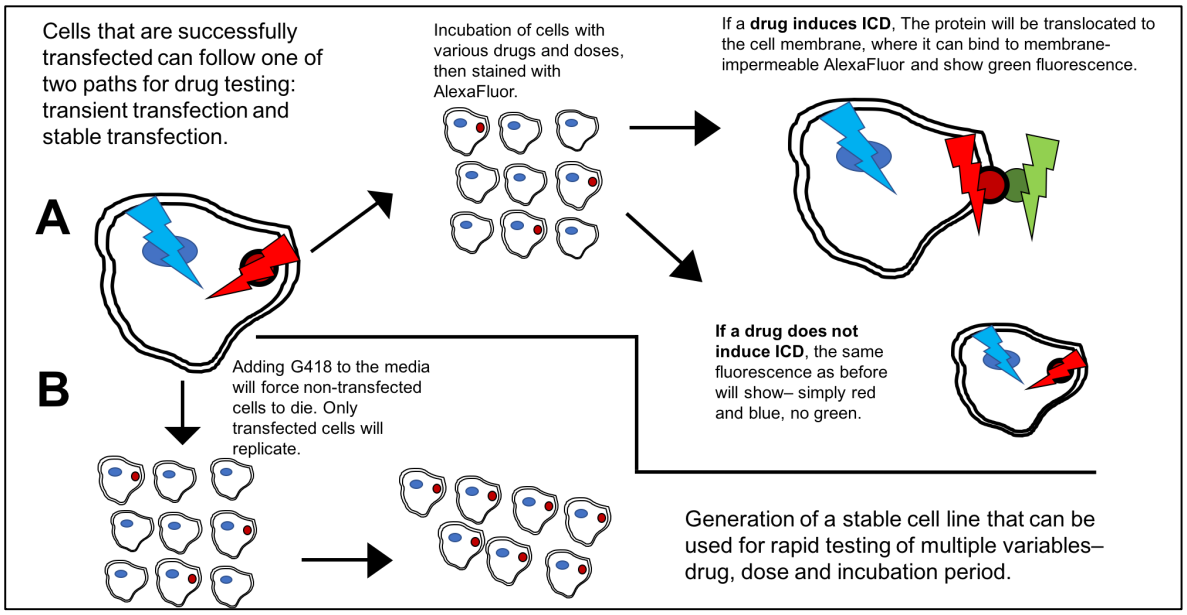


Figure 10: Phase 2 of the experimental process.
A. Transient transfection process. B. stable cell line process

normally displayed on the endoplasmic reticulum. For ease of imaging, cell nuclei would be stained with Hoechst dye[89,90], excitation of 361 nm and emission of 497 nm. After selection with antibiotics, all cells should contain plasmid and would fluoresce. The test drugs could then be applied. After short incubation with the drugs, it would be possible to tell if that drug caused

sufficient endoplasmic reticulum stress to induce immunogenic cell death. If the drug did cause immunogenic cell death, the calreticulin construct should move to the cell membrane where it would be displayed extracellularly. Rather than relying on visual red fluorescence alone, a secondary antibody of cell-impermeable HaloTag was used. This HaloTag would be able to bind and fluoresce to only external calreticulin, displaying at an excitation of 494 nm and an emission of 517 nm.

2.2 Isolation and purification of plasmid DNA

The plasmid obtained from DNA 2.0 contained a calreticulin construct, a red fluorescent protein, a G418 resistance gene for selection in cell culture, and an ampicillin resistance gene

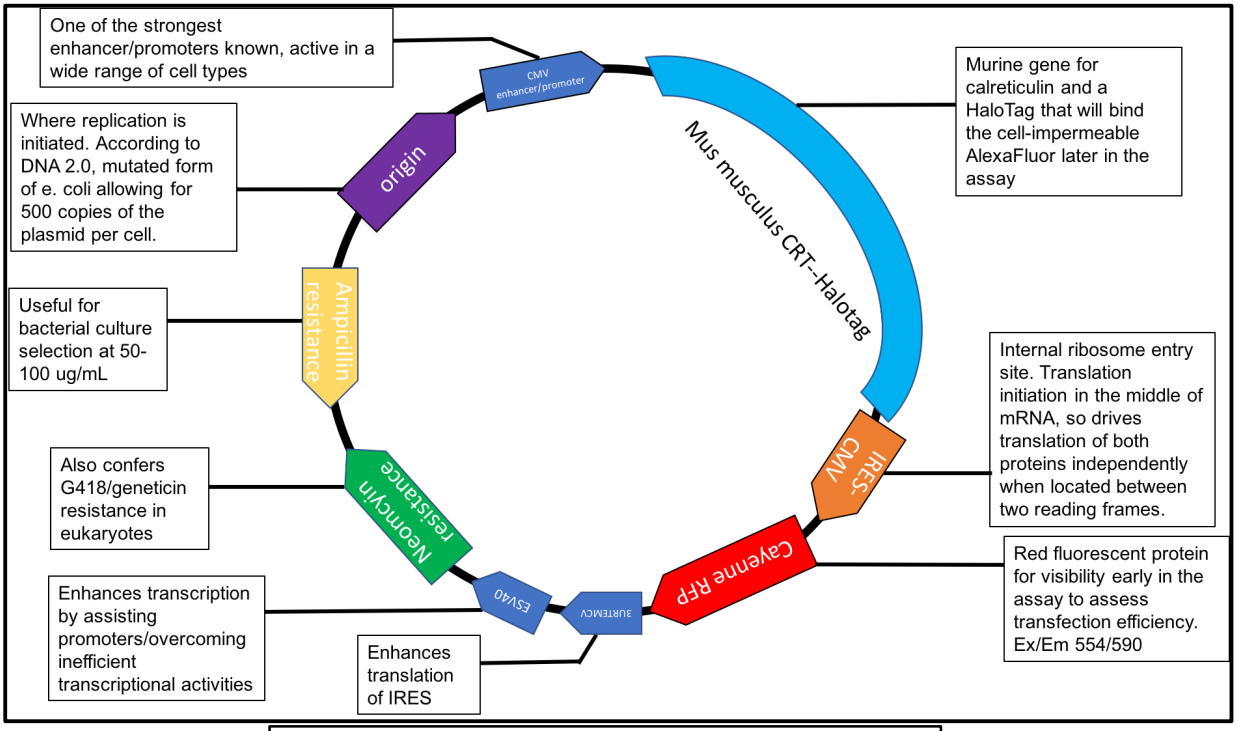


Figure 11: Plasmid map (Adapted from DNA 2.0)

for selection in bacterial culture. Initially, small quantities of the plasmid were generated using the bacterial stab given by DNA 2.0 and the PerfectPrep mini prep kit by 5 Prime. Bacteria

containing the plasmid construct were grown up overnight in LB media containing ampicillin and purification proceeded as directed by the manual. As transfection protocols changed, larger amounts of DNA were required to fit demand. A maxi prep kit--PureLink by Invitrogen—became essential. However, the first few attempts at generating a suitable concentration of DNA from the maxi prep kit yielded surprisingly low amounts of DNA. Due to this particular plasmid being a low copy plasmid, despite the mutated origin sequence, overnight incubation periods were extended to 12-14 hours at minimum, a range exceeding the upper limit recommended by the manufacturer. To further improve the yield, the pellet was spun down at higher speeds and in a centrifuge equipped specifically for large volumes of bacterial media as opposed losing purified DNA and bacteria at each step when using the same volume split among many tubes for a small centrifuge. The final step to improve the yield was using double the amount of bacterial culture media recommended by the manufacturer for low-copy plasmids.

2.3 Transfection

a. General procedure

Lipofectamine is a lipophilic agent used for plasmid transfection into cell culture that works by encapsulating and then transporting cationic nucleic acids across the lipophilic cell membrane. The lipofectamine used in this experiment was Lipofectamine 2000 (Invitrogen). The general procedure takes at least one day to prepare, one day to transfect, and another to select. Cells must be grown up overnight on a 6 well plate. The formation of the transfection complex is fairly straightforward. The procedure involves incubating at room temperature a

calculated amount of lipofectamine and a calculated amount of DNA separately for 5 minutes, each in 150 microliters of serum-free media. After the incubation period is up, the media containing lipofectamine and DNA is mixed together and incubated at room temperature for 20 minutes. After 20 minutes, the mixture is added to the 50-70% confluent cell culture and incubated overnight. In the morning, cells must be split. 24 hours later, the cells can begin selection with G418 at a concentration specific to each cell line.

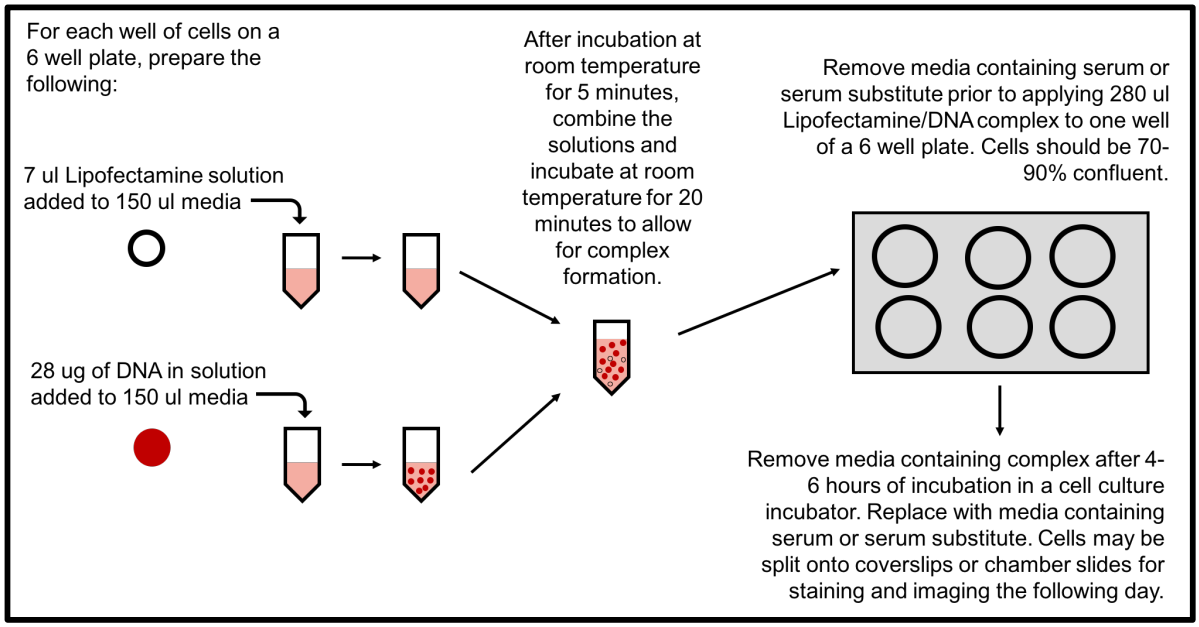


Figure 12: Sample transfection protocol (doses optimized for AT84 cells)

To optimize transfection, several variables were taken into account. The variables changed were the ratio of lipofectamine to DNA, with or without fetal bovine serum, confluency of the cells, and the length of time the transfection media was applied to the cells. To test if the transfection was successful, fluorescent imaging was performed.

b. MDA 1986 cell line

MDA 1986 is a human head and neck cancer cell line. It was cultured in DMEM with 10% fetal bovine serum or 10% synthetic serum, Fetalgro (RMBI). The cell line was not cultured in antibiotics as typical antibiotics, such as penicillin/streptomycin, used in cell culture may interact with the uptake of G418 and prevent effective selection. The initial experiments used complete culture media, incubated cells overnight in transfection media, and using a ratio of Lipofectamine: DNA of 1:1 as taken from the manufacturer's instructions. This procedure was modified throughout the optimization process and included removing fetal bovine serum during transfection, reducing the amount of incubation with transfection media to 4-6 hours, and testing various ratios of Lipofectamine: DNA including 1:1, 1:1.22, 1:2, 1:2.33, 1:3, 1:3.33, 1:4, 1.4:1, 2:1.

c. AT84 cell line

AT84 cells were acquired from the Paolini group in Italy and are a spontaneously occurring murine head and neck cancer. The cell line is cultured in RPMI 1640 with 10% fetal bovine serum or synthetic serum Fetalgro. The cell line was not cultured in antibiotics as typical antibiotics, such as penicillin/streptomycin, used in cell culture may interact with the uptake of G418 and prevent effective selection. Transfection procedures were modified from the manufacturer by omitting fetal bovine serum, incubating in transfection media for only 4-6 hours, and using a ratio of Lipofectamine: DNA of 1:4.

2.4 Selection of stable cell line

Cells were selected using G418 (Geneticin) obtained from Gibco. To identify the correct dose to use for each cell line, a cell toxicity curve was generated. Cells were plated in a 12 well plate and concentrations ranging from 0 μg/ml to 1000 μg /ml were tested in each well.

2.5 Imaging

Fluorescent imaging was performed on the Olympus IX83 microscope and images were obtained using the corresponding CellSens software. To assist with identifying location of the plasmid and finding the correct plane of cells, cell nuclei were dyed 10 minutes prior to imaging with Hoechst stain. The Hoechst stain at a concentration of 10 μg/ml was prepared in PBS immediately prior to use. Prior to application of the dye, cell media was removed and the cells were washed with PBS. Enough Hoechst 10 μg/ml solution was applied to cover the bottom of the well. Upon application, cells were incubated in the dark at room temperature for 5 minutes. The solution was removed and the cells were rinsed twice over with PBS to remove excess unbound stain. Clear cell media was used to further prevent interference with fluorescence.

Hoechst stain appears blue. The presence of red fluorescence was considered an accurate representation of the location of the calreticulin-containing plasmid when reasonably located near the blue-appearing nucleus bound to Hoechst stain. For the initial confirmation studies that transfection was successful prior to application of drug, cells were split onto poly-L-Lysine coverslips (Corning) and incubated overnight.

To induce translocation of the calreticulin protein to the cell membrane, cells were exposed to cisplatin as negative control, oxaliplatin, and modified niclosamide. Cell toxicity

assays were run to determine doses. Cells were added to a 96 well plate at a concentration of 3000 cells per well and exposed to varying concentrations of drug. Cell viability was assessed after 72 hours of treatment with the three drugs using resazurin blue.

Before applying the drugs to transfected cells, cells were split onto collagen-coated chamber slides. Cells were plated at 80% confluency when drug was applied. Oxaliplatin was first tested at concentrations of 500 μM, 100 μM, and 10 μM incubated with cells for 4 hours and then either 50 μl or 100 μl of 500 μM or 100 μl 100 μM for 12 and 24 hours. Cisplatin was tested at 300 μM incubated with cells for 12 and 24 hours. Either 20 μg or 40 μg of Niclosamide was applied to cells for 12 and 24 hours.

To confirm translocation of calreticulin to the cell membrane after exposure to these drugs, the cell-impermeable HaloTag AlexaFluor 488 was used. AlexaFluor was used according to the manufacturer's instructions.

Chapter 3: Results and Discussion

3.1 Transfection

a. MDA 1986

These cells were chosen for their similarity to cancers involved in treatment regimens with known ICD inducers as well as for their accessibility and use in previous projects in our lab. Optimization of transfection with these cells proved to be difficult and ultimately resulted in no establishment of a stable cell line.

The manufacturer states that lipofectamine can successfully be used in media containing up to 10% FBS. However, this resulted in an extremely low—close to undetectable—transfection

efficiency for this particular cell line, even with high amounts of lipofectamine and DNA. In the first few experiments, the only changed variable was the amount of DNA and lipofectamine used. For example, 3, 5 or 7 microliters of lipofectamine would be incubated according to the manufacturer's directions in serum-free DMEM, and varying concentrations of DNA—1 to 3 times the concentration of lipofectamine—would also be incubated in serum-free DMEM, but after mixing and incubating, the complexed solution was added to media containing serum and incubated overnight. After several tries and increasing the possibility of lipofectamine conditions to 3, 5, 7, 10 and 12 microliters each with 3, 5, 7, 10, and 12 micrograms of DNA respectively and still seeing an extremely low transfection efficiency, the FBS was removed from the process.

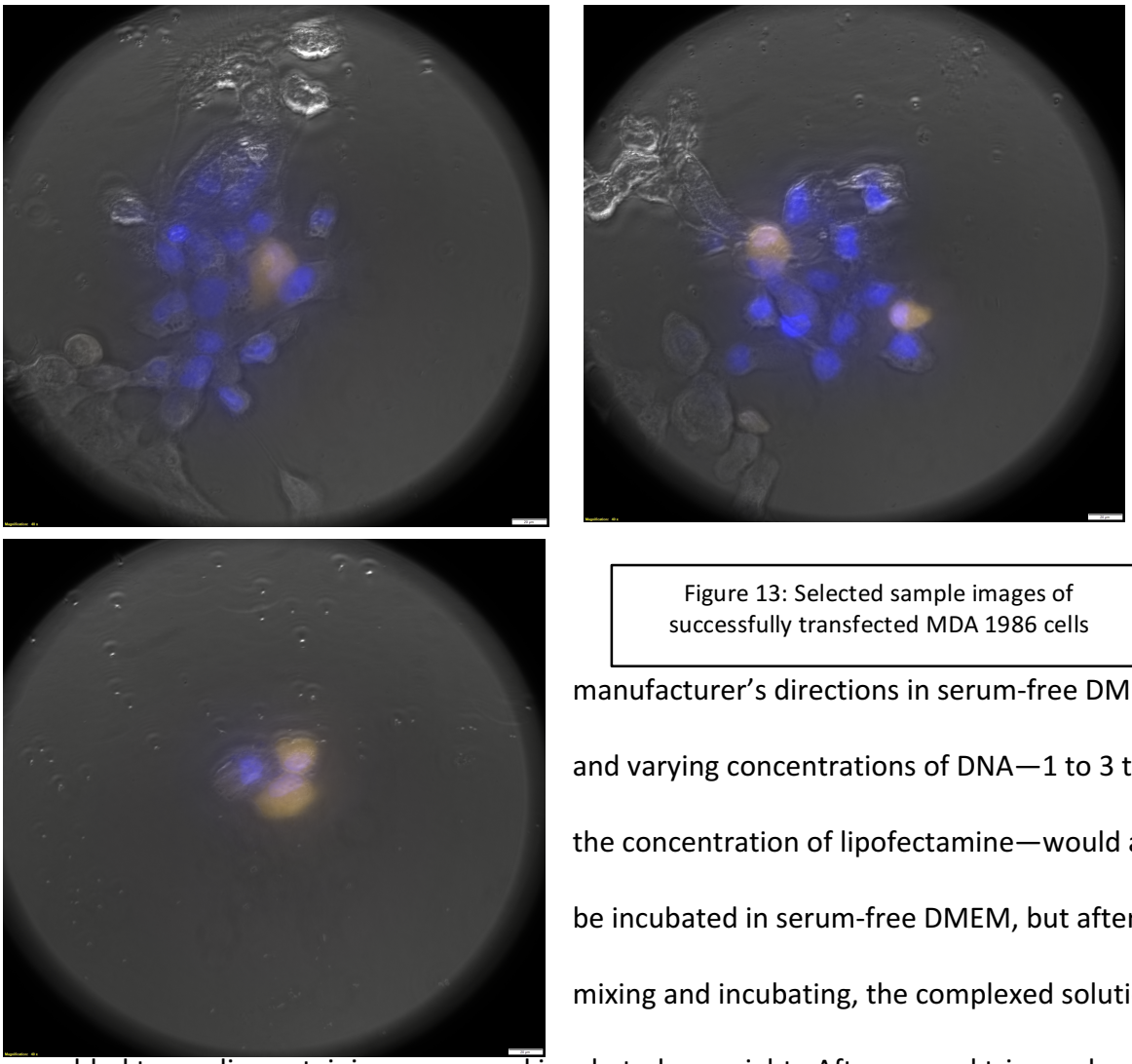


Figure 13: Selected sample images of successfully transfected MDA 1986 cells

After trying the same conditions again, it became clear that FBS was the problem, as transfection efficiency increased. While the manufacturer says that it is possible to use FBS, it is especially essential at cell lines with low transfection efficiency to leave it out. Mechanistically, this makes sense. Lipofectamine and other transfection agents used in this manner are often cationic to bind to anionic DNA. The polyplex is then neutral and can cross the cell membrane. However, serum albumin is also negatively charged, and it may competitively bind or at least interfere with the binding of DNA to lipofectamine.

After removing FBS, the transfection efficiency increased to 5% of cells in the well, which is extremely low. The optimization focus changed to the ratio of lipofectamine to DNA. The manufacturer states that in their cell lines, a 1:1 ratio is sufficient to see transfection. In these cells, the 1:1 or lower cells showed little to no transfection, whereas the 1:2 and larger ratios showed increasing amounts of successful transfection.

Transfection efficiency may increase or decrease based on the type of cells and their confluency. Some cell lines prefer less than 50% confluency to improve transfection, but the MDA 1986s preferred greater than 50% confluency and showed improved transfection efficiency when done under those conditions. After removing serum from the transfection media and improving the ratio, another tested condition showed that 60-70% confluency shows a slightly increased number of transfected cells.

The final step taken to improve transfection was reducing the time the complexed transfection solution was applied to cells. As indicated by the manufacturer, overnight or 8-12 hours of transfection time is appropriate. However, it is suggested that some cell lines are particularly sensitive to lipofectamine and DNA, and it may be cytotoxic. The final step to

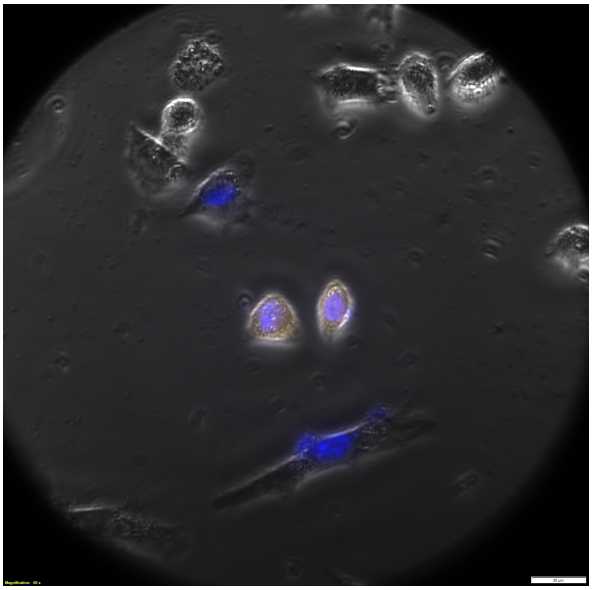
optimization with these cells was to reduce the time the complex was applied to anywhere from 4-6 hours. The cells looked healthier and were able to be visualized the next day, but it seemed the cells were a dead end as far as further optimizing the transfection protocol as less than 10% of all cells in the well were transfected after modifications to the procedure.

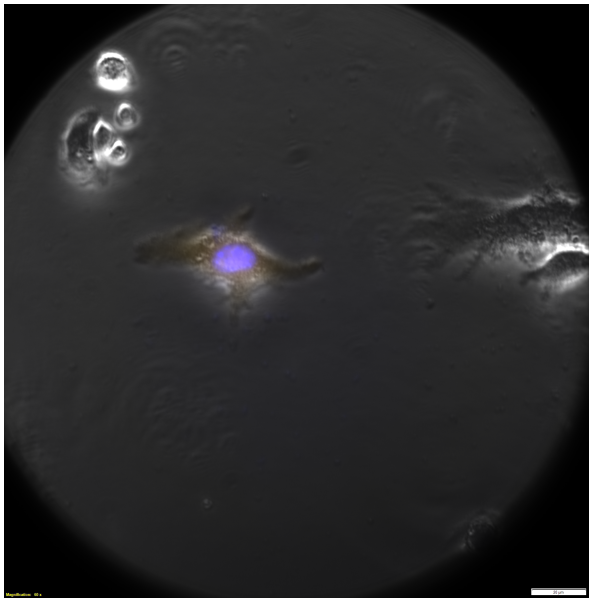
b. AT84

AT84 cells were the natural next step after the MDA 1986 cell line. There is strong evidence for these cells as a pre-clinical model as shown in work done by Paolini[91] et al and they are a murine cell line that would combine well with the next step of processing a clinical model as they can be injected into immunocompetent mice. Though the MDA 1986 cell line had its failures, the lessons learned remained. The cell line was transfected at minimum 70% confluency with ratios 1:2, 1:3, and 1:4 of Lipofectamine: DNA in serum-free media. Un-transfected cells were challenged side-by-side with serum free and plus serum media. The serum free cells did not look as healthy after an overnight incubation without serum. Rather than plating these cells in serum-free media overnight, as was done with the MDA 1986 cells, the wells containing these cells were replaced with serum free media just prior to incubation.

It was discovered that the AT84 cells preferred a ratio of 1:4 Lipofectamine: DNA and a shorter incubation time, closer to 4 hours than 6 hours. These cells were more amenable to transfection, and while efficiency was still not high, red fluorescence close to the blue fluorescence of the Hoechst stain indicated the protein was being expressed at 10%. This is still not ideal based on results stated by the manufacturer as well as by the protocol recommended by DNA 2.0, but enough cells had the plasmid to continue through with work to test drugs.

The cells expressing the plasmid and calreticulin protein did not image as well as non-transfected cells. The transfected cells were usually isolated and were not striated after overnight plating, unlike their non-transfected counterparts. The transfected cells generally did not look as healthy and this may have been contributing to the problem of stable cell line generation.





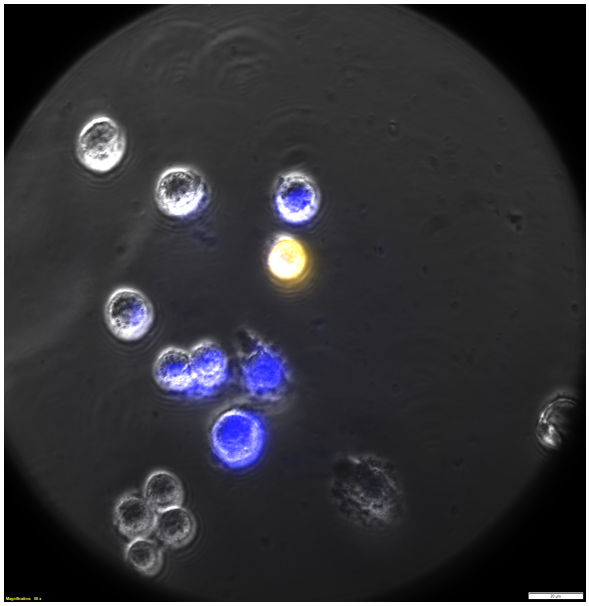


Figure 14: Selected initial images of successfully transfected AT84 cells

3.2 Selection

a. MDA 1986

Low transfection efficiency does not mean the cells are unusable; however, it does increase the amount of time it takes for the stable cell line to be formed. By selecting cells using the

G418 resistance conferred by the plasmid and growing up those cells in media with G418 for 4-7 days, 100% of cells in the flask should express the plasmid and therefore the protein. A selection curve was run on un-transfected MDA 1986 cells. After 5 days, it seemed that 100% of cells incubated with 300 μg/ml of G418 were dead and it was chosen as the optimal selection concentration. After applying 300 μg/ml G418 to transfected cells, there was significant cell death, and then improvement as the cells selected began to proliferate.

However, when imaged after 4 days of selection with 300 μg/ml G418, less than 1% of MDA1986 cells remained transfected as confirmed by detection of the red fluorescent protein. The selected cell line should have been stable, but did not succeed in keeping the plasmid. When replicating the selection curve results, cells at lower concentrations began dying faster than at higher concentrations and there was no direct curve. It is likely that the MDA 1986 cells have some level of intrinsic resistance to G418. This cell line has significantly less data and use in published papers as compared to many other cell lines, and it does not seem that others have attempted to use G418 on these cells in published work. The decision was made to discontinue work with this cell line, as it would not have been a good model to continue using in immunocompetent mice as it was a human cell line.

b. AT84

AT84 cells had been used previously in transfection and selected by Paolini et al in Italy at a concentration of 400 μg/ml. Cells were selected with G418 at this concentration for 4 days then split onto coverslips for imaging. Again, only 1-3% of cells in on the coverslip maintained the plasmid and protein expression as measured by the red fluorescent protein. It is possible that

the cells are not easily amenable to this type of transfection. Plasmid DNA is easily removed from the cell, whereas retroviral transfection methods make that more difficult by editing the host genome. A stable cell line was unable to be developed through this method, though the transient transfections were still able to accurately predict if a drug has the potential to induce immunogenic cell death. The pre-clinical model is viable, but the generation of a stable cell line for continuous, rapid testing of immunogenic cell death is not possible with this plasmid construct, cell line, and transfection agent.

3.3 Drug testing

a. Oxaliplatin

Oxaliplatin was first tested at 10, 100, and 500 μM concentrations incubated with the transiently transfected cell line for 4 hours. According to Obied et al, calreticulin translocation and exposure could occur as soon as one hour following endoplasmic reticulum stress. This was not true for this cell line at these concentrations, as there was no fluorescence indicating the presence of bound HaloTag AlexaFluor to surface-exposed calreticulin. These experiments using drug and AlexaFluor were done in chamber slides, which are wells small enough to contain only 7% of the cells split from a 6 well plate. After incubation with oxaliplatin for only four hours, expression of protein was significantly higher than seen 5 hours previously in the same transiently transfected cell line on a coverslip. The transfection efficiency was near 15%, an almost 5% increase.

It is extremely unlikely that by random chance, each of the 7 wells of transfected cells on the chamber slide randomly received more transfected cells than cells split from the same line onto coverslips. The difference must be exposure to oxaliplatin. It seems that oxaliplatin or

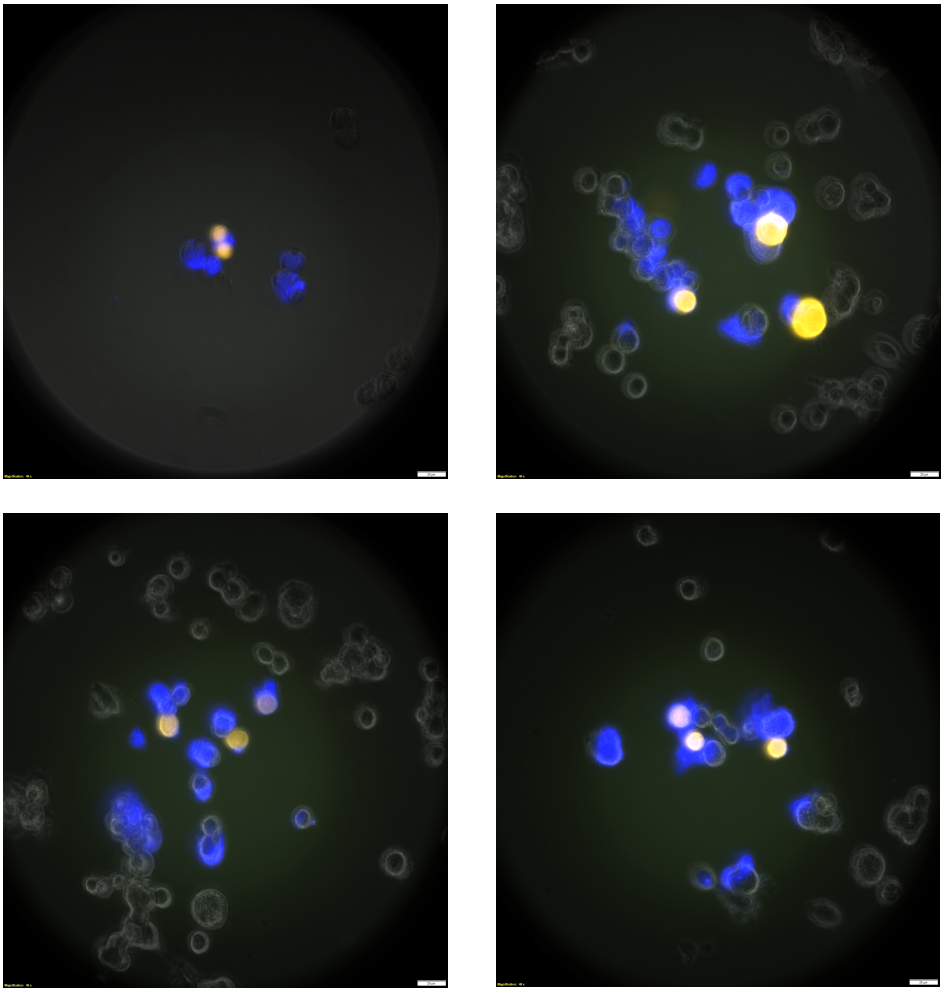


Figure 14: Selected sample images of successfully transfected AT84 cells after exposure to oxaliplatin after 4 hours at varying concentrations—500iNC mM, 100 mM and 10 mM

some other form of endoplasmic reticulum is necessary for selection and generation of a stable cell line, as the protein functions in these conditions and is less likely to be lost from the cell. If there is no purpose to holding onto a relatively large plasmid construct taking up valuable space in the cell, it would make sense for the cell to expel it during replication. However, in these conditions, soon after transfection, the protein is utilized, and expression is increased. These conditions also showed the first double transfection and expression by a single cell, showing more support for this condition as a requirement for selection.

The next conditions tested were 100 μM or 500 μM for 12 and 24 hours. Either 50 μl of solution or 100 μl of solution was applied to the chamber slides. While the 4-hour incubation period was not long enough to allow extracellular protein expression, the 12 and 24-hour incubations seemed to be too long. The amount of visible and fluorescent calreticulin was

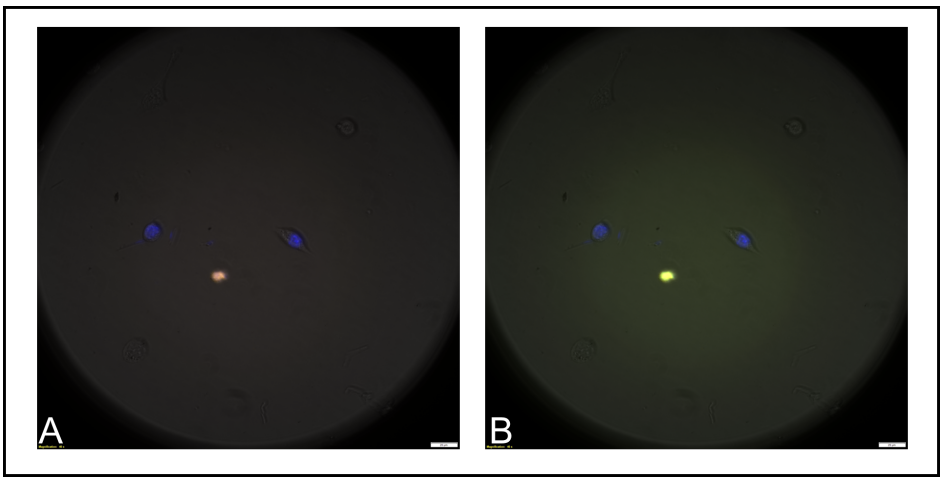


Figure 16: Calreticulin with halotag construct binds AlexaFluor.
- **A.** Microscopy with TRITC and DAPI filters showing location of cells and calreticulin protein.
- **B.** Microscopy with FITC, TRITC and DAPI filters

dramatically lower across every concentration, but there were red cells. A few cells both in the 12 and 24-hour incubations showed the classic display of ICD with the blue Hoechst-stained nucleus, red-orange calreticulin, and green AlexaFluor bound brightly to the cell membrane. While this is a low number of cells expressing the construct, the transient transfection does appropriately display immunogenic cell death in this cell line with oxaliplatin as the positive control. The decreased number of cells in each slide as compared to the 4-hour incubation could be attributed to a poor transfection, but also may be due to the rapid response of calreticulin. It is possible that dosing for 12 or 24 hours causes so much stress and cell death that the calreticulin has already responded and been flushed out of the cell, which is then lost

in changing cell media with AlexaFluor staining. More supporting evidence for this is the quality of cells at higher doses of oxaliplatin rapidly declines and the number of calreticulin-fluorescing cells in those conditions is also lower.

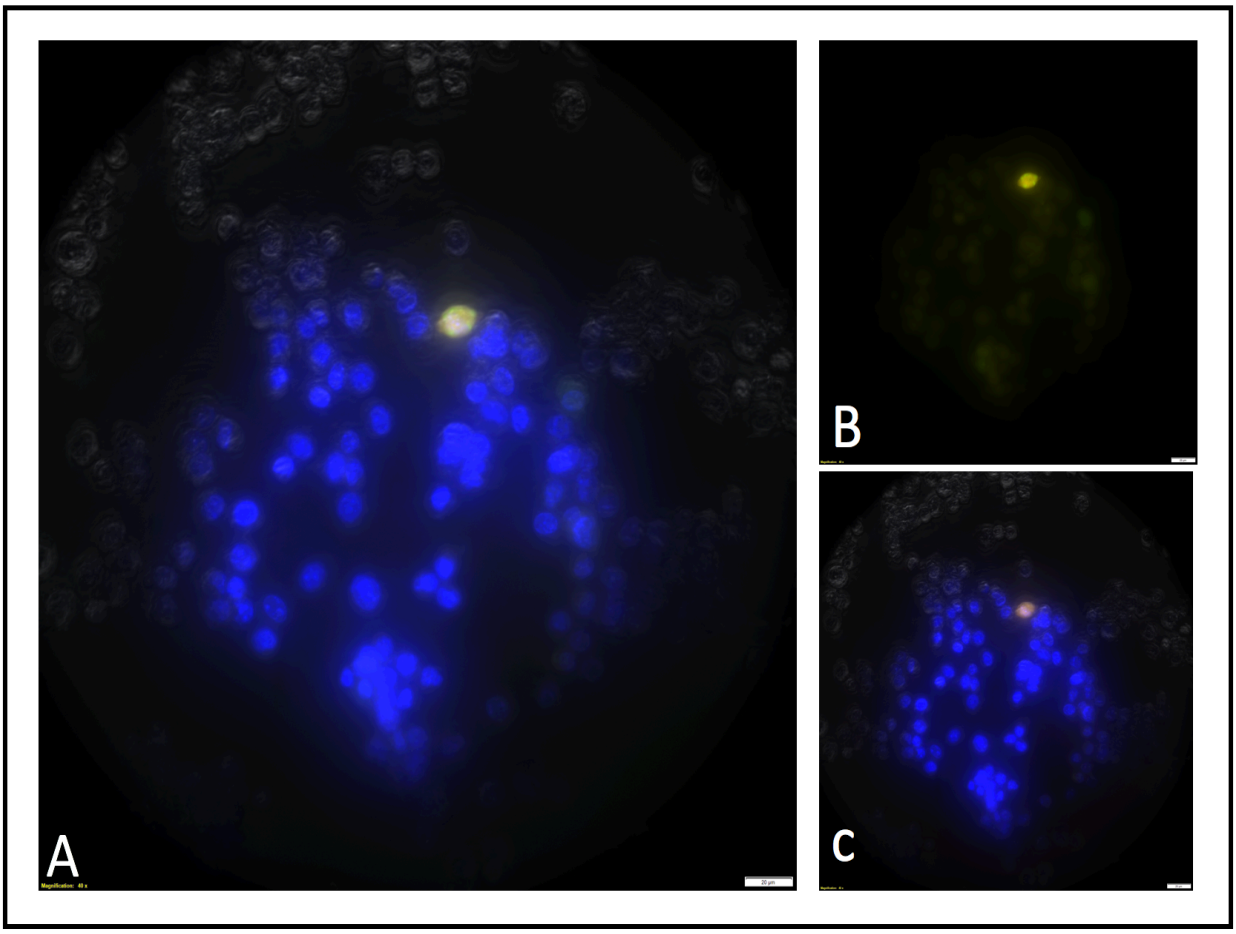


Figure 17: Calreticulin exposed on the surface of cells after treatment with oxaliplatin binds AlexaFluor.

- **A.** Fluorescence of Hoechst stain, RFP from Calreticulin construct, and Alexa Fluor
- **B.** Alexa Fluor florescence alone
- **C.** RFP and Hoechst stain fluorescence

b. Niclosamide

Though an old drug, niclosamide has been investigated recently for its properties in cancer chemotherapy. Though there is nothing in the literature to prove that it induces ICD, we hypothesize that it may. Niclosamide should undergo a full ICD investigation, including the bona-fide murine vaccination study. This is further supported from a mechanistic perspective, as it disrupts electron gradients in the mitochondria. A modified version of this agent was administered to cells and showed a significant effect on cell toxicity, after both 12 and 24-hour incubation with 40 μg niclosamide. In addition to the increased cell debris, it is essential to point out that in conditions where cells underwent incubation with niclosamide, the cells maintained expression (albeit low expression) of the calreticulin construct as illustrated by fluorescence. When cells were not exposed to agents causing endoplasmic reticulum stress, cells lost the ability to express the construct. In the conditions where cells were exposed to niclosamide for 24 hours, a few cells also bound the AlexaFluor, indicating that calreticulin had been translocated to the cell surface.

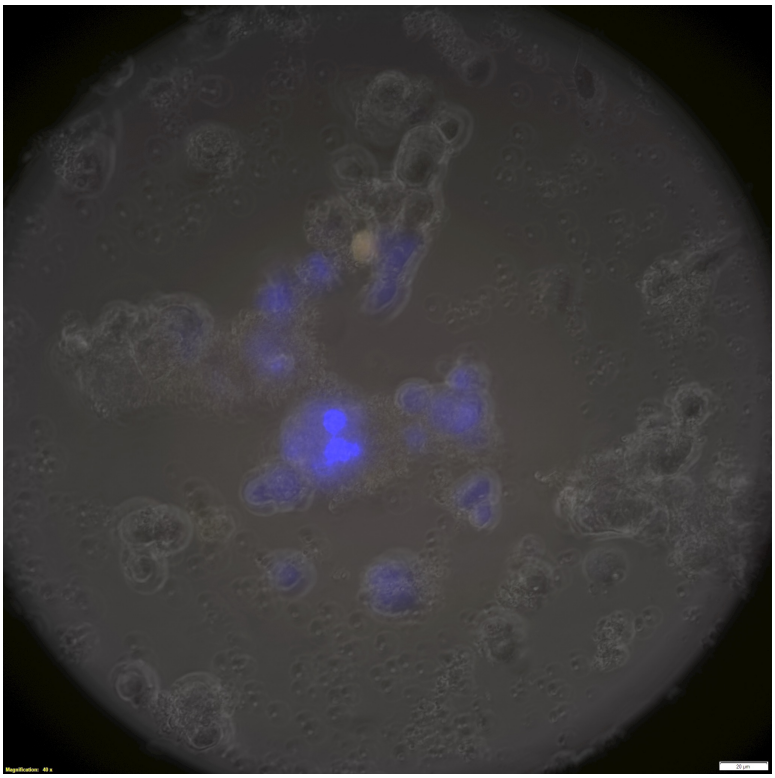


Figure 18: AT84 cells express calreticulin and increased amounts of cell debris after 12 hours of incubation with 20 μg modified niclosamide.

While these results alone are not enough to definitivelysay that niclosamide induces immunogenic cell death, it is a possibility that cannot be ignored. The solid foundation of pre-clinical evidence necessitates further study with this agent and ICD.

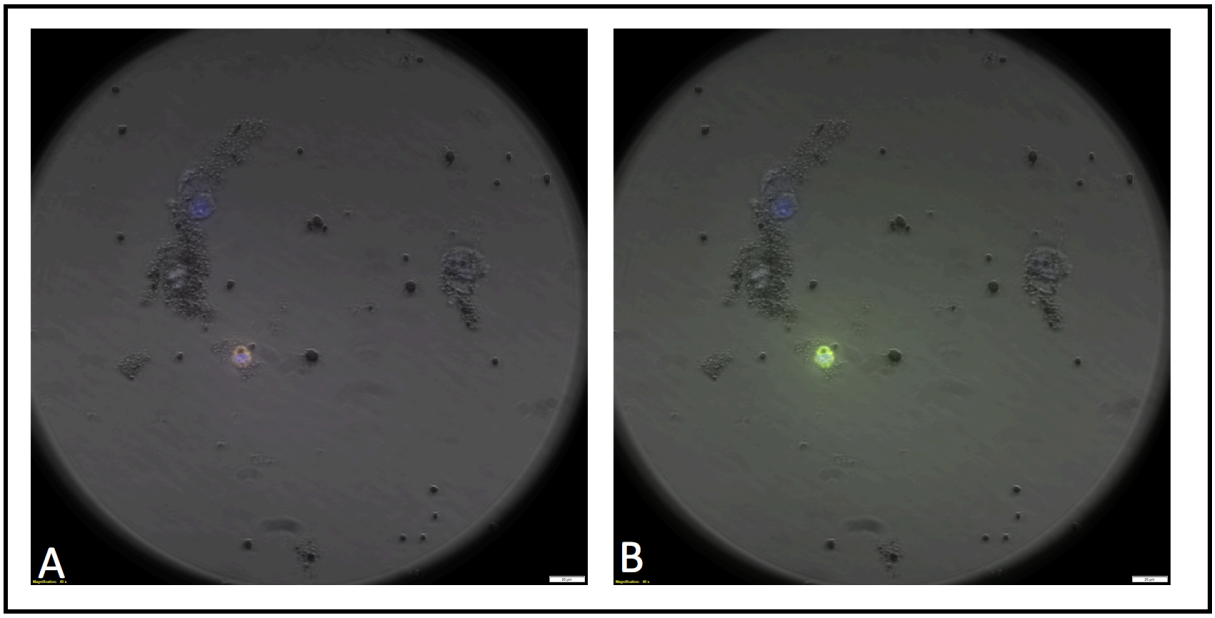


Figure 19: Transfected AT84 cells translocate calreticulin to the cell surface when exposed to niclosamide.
A. Hoechst and Calreticulin Fluorescence
B. Hoechst, Calreticulin, and AlexaFluor Fluorescence

c. Cisplatin

As expected, cells incubated with cisplatin did not show fluorescence with the AlexaFluor indicating that surface exposure of calreticulin (and thereby immunogenic cell death) was occurring. Cisplatin served as the negative control for this study. Interestingly enough, there were no cells in the four wells treated with cisplatin after either 12 or 24 hours of incubation expressing the expected calreticulin fluorescence. Combined with earlier clinical data from the 4-hour oxaliplatin incubation, it seems that the inability to select a stable cell line could require not just culturing with G418, but also low levels of a ER-stress-inducing agent as

well. An important note is that the cisplatin is clearly working on these cells due to the large amounts of cell debris and stained nucleic acids, it simply does not induce immunogenic cell death, confer stable transfection and maintenance of the calreticulin plasmid, or bind the AlexaFluor construct.

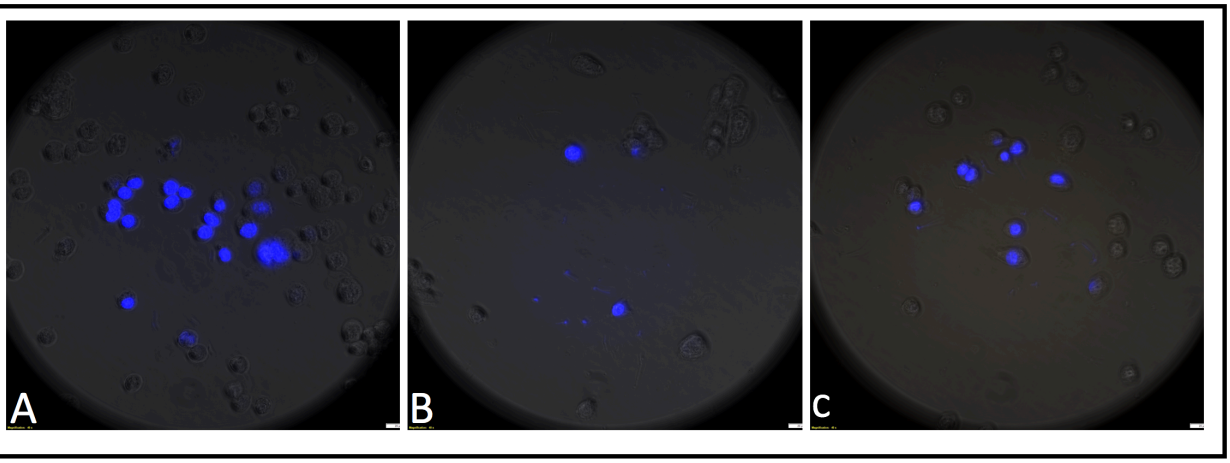


Figure 20: Representative images for transfected AT84 cells treated with cisplatin.

A. Incubation for 12 hours with 50 µl of 300 µM cisplatin
B. Visible loose nucleic acids after incubation of 100 µl 300 µM cisplatin for 12 hours.
C. 50 µl of 300 µM cisplatin incubated for 24 hours.

Chapter 4: Conclusion

4.1 The Impact of Immunogenic Cell Death

Harnessing the potential of immunogenic cell death could radically change the way cancer is treated in the modern world. By fully involving the immune system in a patient's anticancer regimen, it could greatly enhance cytotoxic therapy and improve the efficacy of newer agents such as checkpoint inhibitors. Remission rates would dramatically increase and a patient's likelihood of developing metastatic disease would drop. Involving the immune system overcomes one of the requirements of cancer development—evasion of the immune system.

By stimulating the body's endogenous response, cancer development is slowed and prevented in the future. Identifying which chemotherapeutic agents that create this response and also provide a potent cytotoxic effect would be show ideal agents for use in various cancers, especially those more likely to metastasize.

If developing and modifying new cancer chemotherapeutics that maintain cytotoxicity as well as causing an immune response is too costly, it could also be possible to investigate or modify older agents, such as niclosamide, not traditionally used for cancer chemotherapy. If these agents successfully create an immune response via immunogenic cell death, they could be added in to current regimens. Induction of immunogenic cell death is one new mechanism of action that should be considered when creating regimens for cancer chemotherapy. Regimens containing mechanisms of actions were originally designed to prevent resistance, but in this case, multiple mechanisms of action could also improve killing strategies. Causing ICD may actually be more beneficial when done by a nontraditional cancer chemotherapeutic, as those agents tend to have an improved side effect profile and do not typically cause bone marrow suppression resulting in an impaired immune system.

Rapid, low-cost detection of ICD remains a problem that slows development and further characterization of this response as well as the agents that potentially cause it. If ICD could be detected without using animal models, the current gold standard, and could take less than 2-4 weeks, it would be more feasible for all agents to be tested for this response. Additionally, it would be realistic to re-test agents that may have been shown not to cause it in the past. It is possible that some of these agents, particularly the ones that cause immunosuppression, must be used at lower doses to accurately assess if they cause ICD. In this work, a rapid and relatively

low-cost method of detection using transient transfection of murine cells was identified, which could be transferred to immune-competent murine models to detect ICD via the gold standard method.

4.2 Feasibility of a Pre-Clinical Model

This work, while identifying cell lines as a potential pre-clinical model for immunogenic cell death using fluorescent stains and tags, has much to be improved on. Lipofectamine is not a realistic transfection agent for generation of a stably transfected cell line with this line and plasmid construct. However, the transient transfections were able to accurately predict generation of immunogenic cell death as shown by the positive and negative controls, oxaliplatin and cisplatin. Use of the transient transfection model should not be entirely dismissed as it is relatively cheap and can be completed in four days. These pre-clinical models should not be used alone to definitively say whether an agent does or does not induce immunogenic cell death. Rather, these models could be used as more high-throughput method to determine the potential and likelihood of an agent to induce immunogenic cell death. It is considerably easier to test wide varieties of concentrations, exposure times, and drug classes with this model than with expensive murine models, and the utility should not be entirely dismissed.

4.3 Future Directions

a. Lentiviral transfection

Generation of a stable cell line using another, slightly more expensive cell line will be done using lentiviral transfection methods. This will likely be superior to the lipofectamine and

plasmid method as the lentivirus is significantly harder to remove from the cell and cannot simply be shuffled out via efflux. Lentiviral transfections modify the DNA within the nucleus and zip the plasmid construct into the host DNA, making it almost impossible for the host cell to remove. There are optimization methods to accompany this form of transfection as well, but after the transfection is found to be successful, generation and maintenance of the stable cell line is considerably more likely. Testing of agents could be done in two days—half the time—and as the transfection efficiency is higher than the transient lipofectamine transfections, could provide a stronger suggestion as to the potential of inducing immunogenic cell death. In the transient transfections, there is a higher chance for error because there are fewer cells even available for imaging.

b. Murine models

The murine model remains the gold standard to prove a drug causes ICD. After generation of a stable murine cell line that can detect ICD potential, continuity can be maintained by using the same transfected cell line to prove the agent causes ICD in mice. The continuity from cell line to cell line increases the likelihood that an agent will be able to cause ICD at doses already tested in murine cells. It is a low-cost step that prevents optimization at higher cost models, and will be essential as the final step of the assay. Though the murine model does have its own problems as far as generalizability to human cell lines, addition of this step will make it easier to test from compound to compound, mouse to mouse. The niclosamide analog should be further investigated using this gold-standard method based on the strong evidence in this study.

c. Testing of a modified Platinum compound

Collaborators in our lab have been working on a modified platinum compound that should be tested for its capability to induce immunogenic cell death. The compound was not available for testing at the time of this work, but will be tested with the new transfection method to assess ICD.

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