UNIVERSITY OF CALIFORNIA SAN DIEGO

Exploring Cancer Persister Cell Vulnerabilities

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by

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The Thesis of Ariel Hana West is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

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One major problem plaguing the medical community is patient relapse of cancer following targeted drug therapy. In a majority of patients with non-small-cell lung carcinoma, this process was shown to occur in as little as one year following treatment.[1] Here we investigate the role that molecules, such as antioxidants, and cellular processes, such as DNA death, damage, and response, have in the underlying mechanistic basis for acquiring drug resistance. Our findings suggest that antioxidants are not capable of adequately preventing the acquisition of drug resistance, pointing

toward R.O.S.-independent mechanisms of acquisition of drug resistance mutations. Additionally, we show that while the inhibition of DNA damage response and repair pathways significantly prevent the outgrowth of cancerous cells in the presence of drug, there is no difference in response from drug naïve versus drug-tolerant cancer cells. From these findings, we conclude that despite their reported disabled DNA repair machinery, drug-tolerant persister cells are not sensitized to death via inhibition of DNA damage response genes. Additionally, we elucidate the mechanism through which Disulfiram, a drug clinically approved for alcoholism which was recently reported to kill persister cells, induces persister cell-specific lethality. We find that Disulfiram does not kill persister cells through ALDH inhibition, as previously reported, but rather through an oxidative-apoptotic mechanism. By furthering the understanding of factors involved in the tumor's acquisition of drug resistance, we provide insight into potential mechanisms to target through the development of new treatments aimed at preventing the occurrence of cancer relapses.

INTRODUCTION

Cancer is the second leading cause of death globally,[2] but individual prognoses differ vastly depending on the type and stage of cancer at the time of diagnosis. 'Cancer' is a generic term used to describe hundreds of diseases driven by their gain of function or loss of function mutations.[3,4] Both types of molecular abnormalities promote uncontrolled cell proliferation,[4] a defining characteristic of cancer.[3]

Prior to the turn of the 21st century, treatment options included radiotherapy, surgery, and non-targeted chemotherapy.[5] While all three have been shown to prolong survival, there is often a decrease in patient quality of life due to the systemic nature and severe side-effects associated with these treatment modalities.[5] The emergence of next generation sequencing created opportunities for personalized medicine, allowing patients to be matched to specific drugs developed to target their tumor's genetic mutations. The identification of genetic abnormalities essential for tumor progression created a therapeutic window where specific drugs could be developed to impact the tumor while posing minimal cytotoxicity to normal cells.[6] 2001 marked the first ever FDA approval of a targeted molecular therapeutic to treat cancer. Imatinib mesylate, a tyrosine kinase inhibitor, was the first targeted therapeutic developed to treat the BCR-ABL oncoprotein that drives chronic myelocytic leukemia. Imatinib's ability to increase the 8 year chronic phase survival rate from 42-65% (1983-2000) to 87% demonstrated the incredible potential that targeted therapies could have within the clinical setting.[7]

Many small molecule targeted therapeutics have followed in Imatinib's wake. Afatinib, erlotinib, and gefitinib are all examples of tyrosine kinase inhibitors developed to combat overactive growth signals that promote uncontrolled proliferation.[8] Most targeted therapies induce a cytostatic effect, which prevents the tumor from expanding, rather than a cytotoxic effect which

would result in tumor shrinkage.[9] While tumor shrinkage would be preferential to cessation of growth, targeted therapies that induce a cytostatic state are valuable in providing disease stabilization, even if merely temporary.[9]

Although specialized drugs have been developed to interrupt tumor progression, cancer remains uncured.[10,11] Tumor genome sequencing has revealed that most common cancers are driven by more than one targetable mutation, increasing treatment complexity as patient toxicity increases with the amount of therapeutics given.[10,12] Irrespective of the promise targeted therapies initially showed, the emergence of drug resistance was soon to follow.[13] For non-small cell lung cancer, this process generally occurs within one year following treatment.[1,14] While some patients are exceptional responders to targeted therapies, it is not currently understood why these patients differ from others.

In light of the daunting challenge of overcoming drug resistance to prevent treatment failure, some focus has shifted to modulating the immune system as a means of combatting cancer. The primary member of the immune system responsible for an anti-tumor response is the T cell. T cell activation requires antigen presentation to its receptors by an antigen presenting cell (APC), as well as co-stimulation by molecules including B7 and CD28.[15] To avoid autoimmune responses, T cells possess inhibitory checkpoints such as cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4).[15] In 2011, scientists developed the first anti-cancer immune checkpoint antibody. Ipilimumab was designed to target the immunosuppressive T cell checkpoint receptor, CTLA4.[16,17] Tumor cells express inhibitor molecules, such as T cell exhaustion marker Programmed Cell Death Ligand 1 (PD-L1), that dampen the immune response.[8,17] Developed antibodies such as Anti-PD-1 and Anti-PDL1 block this immunosuppressive interaction between the tumor and T cell, thereby

enhancing T cell function and anti-tumor response.[8] In contrast to the specialized nature of targeted therapy, cancer immunotherapy has a broad goal of initiating an immune response toward malignant tissue.[9]

Prior to the development of immune checkpoint antibodies, the most promising treatment of BRAF-mutant melanoma, encompassing 40% of malignant melanomas, was targeted therapy with BRAF and MEK inhibitors.[18] While these therapeutics quickly achieved high response rates for most patients, their successes were plagued by the emergence of acquired drug resistance. The majority of patients with melanoma experience tumor relapse within as little as one year.[18] Patients are also capable of acquiring resistance to checkpoint blockade immunotherapy. Many melanoma patients that respond to immune checkpoint antibodies ultimately acquire resistance and relapse.[16]

Even though immune checkpoint antibodies have potential to induce an anti-tumor response, they are also associated with immune related adverse effects due to their stimulation of an overactive immune system.[19] Some patients have developed dermatitis, hepatitis, colitis, or other autoimmune-mediated effects following treatment with immune checkpoint inhibitors such as ipilimumab.[8] As promising as immune checkpoint therapy is for the minority of patients that initially respond, the remaining individuals are without successful treatment. The majority of patients across tumor types do not respond to immunotherapy and are therefore considered to have primary resistance.[15,20] In contrast, patients most commonly achieve a high primary response from targeted therapies, but later develop acquired or secondary resistance i.e. tumor relapse.[15,20] It is presently unresolved how these two therapies should be used in the clinic. However, the widely observed successful primary response to targeted therapy establishes a need to prevent acquired resistance in order to prolong progression free survival. Therefore, it is crucial for drug development research to focus on understanding and overcoming drug resistance.

Similar to cancer cells, bacteria are capable of acquiring drug resistance. Although this mechanism is poorly understood in cancer, researchers have thoroughly elucidated the mechanism of resistance acquisition in the context of bacterial cells. In E. Coli, drug induced stress causes the cell to enter a dormant state while simultaneously upregulating error-prone DNA repair machinery.[21] This tactic increases genetic diversity within the population, thereby increasing the probability that a stress-resistant population will emerge. The ability to induce mutagenesis in response to stress allows for a low mutation rate when not needed, thus avoiding a high frequency of deleterious mutations.[21]

Like bacteria, cancer cells are known to enter cell cycle arrest as a response to stress and remain dormant until either the stress is removed, or a drug-tolerant subpopulation emerges. However, the direct mechanism through which cancer cells acquire drug resistance is unknown.[22,23] Inhibition of apoptosis is believed to have an important role in resistance acquisition across multiple cell types.[24] Recent studies on a variety of cancer tissues have shown that, after initial drug treatment, cells that have initiated apoptosis are able to terminate the death process prior to its completion.[24] In these instances, the cells persist in a quiescent non-dividing state; neither fully dead nor proliferating.[25] Given time, these various cancer persister cells acquire resistance and begin to grow despite the presence of drug.[22] Proliferation in continued drug presence indicates that these drug-tolerant expanded persister cells (DTEPs) have acquired, through a mechanism which remains unknown, mutations in their genome that confer resistance.[23]

The first step toward overcoming drug resistance is to uncover mechanisms underlying resistance. Currently, the accepted hypothesis for acquired resistance focuses on the three aforementioned stages: initial drug survival, quiescence/dormancy (persister state), and exit from quiescence into a phase of drug-tolerant expansion.[23] Persister cells either preexist or emerge

during treatment and survive through nongenetic mechanisms prior to acquiring resistance mutations. How mutant resistant cells arise remains an open question. Resistance to a targeted therapeutic could emerge from the presence of pre-existing mutant drug naïve cells that initially survive targeted therapy.[26,27] Enriched through selection, these cells can eventually proliferate into drug-tolerant populations.[26,27] Alternatively, there may be scenarios in which preexisting mutant resistant cell populations do not exist. Instead, cells could gain mutations during targeted drug treatment that allow them to acquire drug resistance. Recent findings suggest that the majority of tumors do not contain pre-existing resistance mutations.[1] Therefore, I will focus on understanding the mechanism of acquired resistance rather than selection of pre-existing resistant mutant cells.

While we do not know when cells gain resistance conferring mutations, one thing is evident; mutations are required for long term drug resistance.[13] Every drug-tolerant expanding persister cell is believed to have acquired resistance mutations that allow it to grow in the presence of drug. A resistance phenotype can result from two possible mechanisms; genetic mutations and non-genetic factors.[28] Non-genetic resistance can result from epigenetic or other changes within the tumor or its microenvironment, while genetic mutations are the result of mutagenesis within the tumor.[27] There are no reported examples of durable drug resistance mechanisms in patient tumors based purely on non-genetic mechanisms, and non-genetic mechanisms are therefore thought to play a role early in drug treatment to promote survival of a surviving tumor cell reservoir which acquires resistance mutations. Instead, genetic mutations are widely accepted to drive drug resistance. For example, one study found that 49% of patients resistant to EGFR inhibitors possessed an EGFR T790M mutation which restored the tumor's EGFR ATP-binding ability.[29] Through increasing the affinity of ATP, the T790M mutation reduces the selective ability of designed ATP-competitive inhibitors, thereby conferring drug resistance.[29]

Cancer persister cells are known to undergo reversible phenotypic changes as a response to stress.[27] During initial drug treatment, proliferative drug naïve parental cells phenotypically transition into a quiescent population.[27] We hypothesize that while in this epigenetic pro-survival state, persister cells serve as a reservoir from which overtly drug-tolerant mutated cells can emerge. Of the few drug naïve tumor cells able to persist in the presence of drug, less than 25% of them are able to further expand into DTEPs.[23] Insight into the mechanism behind the acquisition of resistance conferring mutations is crucial for the development of treatment approaches without relapses.[30]

Apoptosis induced mutagenesis is one proposed mechanism for resistance acquisition.[31] Programmed cell death can be initiated by environmental stress, such as that caused by a targeted therapy.[31] During apoptosis, DNA becomes fragmented by DNAses present within the cell.[32] Cancer cells often release inhibitors of apoptosis (IAPs) to stop the process of programmed cell death at or prior to this point to survive.[33] If a cell has begun DNA fragmentation, but remains alive, it may be able to survive by repairing DNA.[33] Recent reports have demonstrated that drug-tolerant persister cells have a disabled DNA damage response resulting from downregulation of DNA repair genes and upregulation of error-prone DNA polymerases.[34] This process introduces errors, or mutations, into the genetic makeup of the persister cell which allows for the possible gain of drug resistance.[31] We have observed that persister cells undergo apoptotic signaling-mediated DNA damage. While this process promotes mutagenesis and acquired resistance, it also results in elevated levels of DNA damage. We hypothesize that a further increase in DNA damage may be toxic to persister cells and that inhibition of DNA repair enzymes may result in lethal levels of DNA damage and persister cell death. This is analogous to the use of PARP inhibitors to selectively target BRCA mutant cancers.[35] Here, we explore whether the inhibition of persister cell

DNA repair genes affect persister cell viability. I also aim to determine which DNA repair pathways, if inhibited further, represent the primary vulnerabilities of persister cells and whether any specific repair pathways differentially affect persister cell mutagenesis.

In addition to apoptosis, ferroptosis is another form of cell death resulting from a build-up of toxic lipid peroxides.[36] It is known that persister cells are susceptible to ferroptosis death through glutathione peroxidase (GPX4) inhibition.[22] GPX4 is an antioxidant enzyme responsible for reducing lipid peroxides within the cell. Inhibition of GPX4 results in higher levels of lipid peroxides and ferroptosis.[36] This observed persister cell-specific reliance upon GPX4 indicated a potential broader persister cell sensitization to oxidative stress.

Here we explore another recently discovered persister cell-specific vulnerability; Disulfiram (DSF). A 2014 study by Raha and others found that DSF selectively killed drug-tolerant persister cells with minimal effect on drug naïve parental cells.[37] Raha and others proposed that the mechanism was due to DSF inhibition of aldehyde dehydrogenase (ALDH) activity, but direct evidence that ALDH inhibition was required for persister cell death was not provided.[37] We hypothesize that DSF selectively kills persister cells through a ferroptosis-independent oxidative mechanism. Through the administration of antioxidants, aldehyde dehydrogenase inhibitors, and other experiments we elucidate the mechanism of action through which DSF selectively kills cancer persister cells.

Lastly, we aim to further understand the role that oxidative stress has in persister cell biology and subsequent resistance acquisition. Here, I will quantify the effects of antioxidants and oxidizers on persister cell outgrowth. A recent 2019 *Science* paper by Russo and others found that an antioxidant, N-Acetyl Cysteine (N.A.C.), prevents the formation of colorectal cancer drug-tolerant expanded persister (DTEP) cell colonies, the regrown colonies that form when persister

cells are exposed to drug for extended time periods.[38] The authors found that treatment of colorectal cancer persister cells with N.A.C. led to reduced cellular levels of reactive oxygen species (R.O.S.) and decreased DNA damage, changes which might potentially limit mutagenesis and resistance mutations.[38] I will test the generality of this finding using other antioxidants in multiple persister cell models. To do this, I developed a novel imaging based approach to formally count drug-tolerant colonies which is described below.

During targeted treatment persister cells exist in a quiescent state. We do not know how persister cells regain proliferative capacity to regrow.[22] I aim to identify drugs and gene vulnerabilities which can be targeted to kill persister cells or prevent persister cell regrowth. To address these goals, the remainder of my thesis is divided into 3 chapters:

1. Test Persister Cell Sensitivity to DNA Repair Inhibitors
2. Determine the Mechanism of Disulfiram-Mediated Persister Cell Death
3. Identify Processes Required for Persister Cell Regrowth

Chapter 1: Test Persister Cell Sensitivity to DNA Repair Inhibitors

Due to the persister cell's hypothesized central role in acquired resistance, there is great interest in therapeutically targeting these cells.[13] In theory, drug-tolerant expanded persister cells acquire resistance mutations which allow them to exit quiescence and form genetically diverse tumors.[13] The higher the tumor's genetic diversity is, the more difficult treatment is and worse patient outcome results.[12] Therefore, identifying targetable persister cell vulnerabilities, prior to the emergence of genetic heterogeneity, is a promising approach to prevent the acquisition of drug resistance.

Persister cells are thought to acquire resistance conferring mutations through an unknown mechanism during treatment.[13,39] One proposed mechanism for resistance acquisition is apoptosis signaling-induced mutagenesis.[31] During apoptosis, the process of mitochondrial outer membrane permeabilization (MOMP) activates DNAses responsible for initiating DNA degradation.[32] While this process typically results in cell death, it is known that sublethal stress can induce a minority of mitochondria to undergo mitochondrial outer membrane permeabilization resulting in partial activation of downstream caspases.[32] By experiencing only partial mitochondrial outer membrane permeabilization (minority MOMP), these cells avoid lethal levels of DNA damage, and are able to evade death through the activation of DNA damage response genes.[32] During minority MOMP, these damage response genes can repair the damaged DNA, allowing the cell to survive.[32] Although sublethal DNA damage provides a substrate for error-prone repair which may result in mutagenesis and provide the opportunity for resistance to emerge, elevated DNA damage may also represent a vulnerability.[31] Ichim and others propose that therapeutically inactivating DNA damage response pathways could result in an increase of minority MOMP induced DNA damage to lethal levels.[32]

Given the finding that cancer persister cells have a disabled DNA damage response due to downregulated expression of repair genes,[34] we hypothesized that inhibiting additional DNA damage response genes will increase DNA damage to lethal levels. Synthetic lethality is the circumstance where a cell can easily overcome a deficiency in one gene or another, but a combination of the deficiencies proves lethal.[35] Previously, this has been observed in BRCA1/2-mutant breast and BRCA1/2-mutant ovarian cancer, both known to have deficient DNA repair machinery.[35] Due to this disabled DNA repair, treatment with DNA repair enzyme PARP inhibitor (e.g. Olaparib) causes selective tumor cell death. Deficiencies in these genes lead to an increased mutational load and DNA damage, and further chemical inhibition of DNA repair results in apoptosis.[35] Here, we explore whether persister cells are vulnerable to inhibition of DNA repair genes including ATM, DNAPK, and PARP.

MATERIALS AND METHODS:

Cell Culture Studies:

To explore the susceptibility of BRAF-mutant A375 melanoma and EGFR-mutant PC9 non-small-cell lung carcinoma to synthetic lethality, we first performed cell culture on drug naïve parental cancer cells. Drug naïve A375 cells were cultured in DMEM medium with 5% antibacterial-antimycotic and 10% FBS. Drug naïve PC9 cells were cultured in RPMI medium with 5% antibacterial-antimycotic and 5% FBS. All cells were cultured at 37 °C with 5% CO_2.

Chemical Inhibitor Studies:

Trametinib and Olaparib (PARP inhibitor) were purchased from ApexBio. Dabrafenib, Erlotinib, and NU7441 (DNAPK inhibitor) were purchased from Selleck Chemicals. CellTiter Glo

was purchased from Promega. KU55933 (ATM inhibitor) was purchased from Sigma Aldrich. All chemicals were stored at -80 in DMSO unless otherwise specified.

Once the cells in culture were appropriately confluent (< 75%), we plated 1,000 drug naïve A375 cells per well of a 12 well plate, and 2,000 drug naïve PC9 cells per well of a 12 well plate. After 24 hours, media was supplemented with DNA damage response gene inhibitors at the concentrations listed in Figure 1 A-C and Figure 2 A-C. All experiments were performed in triplicate unless otherwise stated. Cell viability was assessed three days after initial treatment via CellTiter Glo.

We derived A375 persister cells as follows: 20,000 drug-naïve A375 cells were plated per well in 12-well plates. After 24 hours, the cells were treated with 250 nM Dabrafenib and 25 nM Trametinib media for an additional 14 days. During derivation, media was refreshed every 3-4 days. PC9 persister cells were derived as follows: 20,000 drug naïve PC9 cells were plated per well in 12-well plates. After 24hours, the cells were treated with 2.5 μM Erlotinib media for at least 9 additional days. During derivation, media was refreshed every 3-4 days.

On day 10 in drug, PC9 persister cells were co-treated with DNA damage response gene inhibitors. Cell viability was assessed three days after initial co-treatment via CellTiter Glo. After 14 days of persister derivation, we administered DNA damage response gene inhibitors to A375 persister cells for 72 hours before performing CellTiter Glo.

Statistical Analysis:

Using cell viability assays, we aimed to assess whether there exists a persister cell-specific synthetic lethality induced through the inhibition of DNA damage response. To determine this, we compared the relative cell viability of drug naïve parental cells versus drug-tolerant cancer

persister cells. All statistical analysis was performed using GraphPad Prism 9.0.1. All P-values were calculated using a two-tailed t test between the control and each individual condition.

RESULTS:

Persister cells have downregulated DNA damage response genes, and we hypothesized that further inhibition of DNA repair genes would increase persister cell DNA damage to lethal levels. To test this, we treated drug naïve and drug-tolerant EGFR-mutant non-small-cell lung carcinoma (NSCLC) and BRAF-mutant melanoma persister cells with DNA repair gene chemical inhibitors and performed cell viability assays (Figure 1 and Figure 2). Interestingly, we did not observe a persister cell-specific response to inhibition of DNA damage response genes. Instead, we observed uniform toxicity between drug naïve parental cells compared to drug-tolerant persister cells.

Specifically, we found that both melanoma and NSCLC parental and persister cells treated with ATMi (KU55933) concentrations of less than 5 μM had a relative cell viability equal to or greater than the control (Figure 1a,d and Figure 2a,d). Higher concentrations of ATMi resulted in a dose dependent decrease of cell viability of similar magnitude in both parental and persister cells compared to the control. Therefore, the treatment of non-small-cell lung carcinoma and melanoma cells with the DNA repair inhibitor ATMi did not result in persister cell-specific lethality.

The treatment of drug naïve and drug-tolerant NSCLC cells with DNAPK inhibitor (NU7441) did not significantly reduce cell viability at concentrations less than 1 μM (Figure 1b,e and Figure 2b,e). In contrast, melanoma parental cells treated with DNAPKi exhibited reduced cell viability in response to concentrations of 0.75 μM and higher. In melanoma persister cells, we observed reduced cell viability at 0.5 μM, mirroring the effect on parental cells. Therefore, DNAPKi-induced toxicity is not persister cell specific.

The cell viability of drug naïve non-small cell lung cancer cells begins to decline following treatment with 2 μM of PARPi (Olaparib) (Figure 1c,f and Figure 2c,f). However, drug-tolerant NSCLC persister cells have reduced cell viability in response to as little as 1 μM PARPi. On the other hand, melanoma parental cells experience more lethality than persister cells following PARP inhibition. Inhibition of each of all three DNA repair genes (ATM, DNAPK, and PARP) affected drug naïve parental cancer cells as much as, or more than, the drug-tolerant persister cells (Figure 1, Figure 2). Taken together, these data indicate that, contrary to our expectations, persister cells are not selectively sensitive to DNA repair gene inhibition.

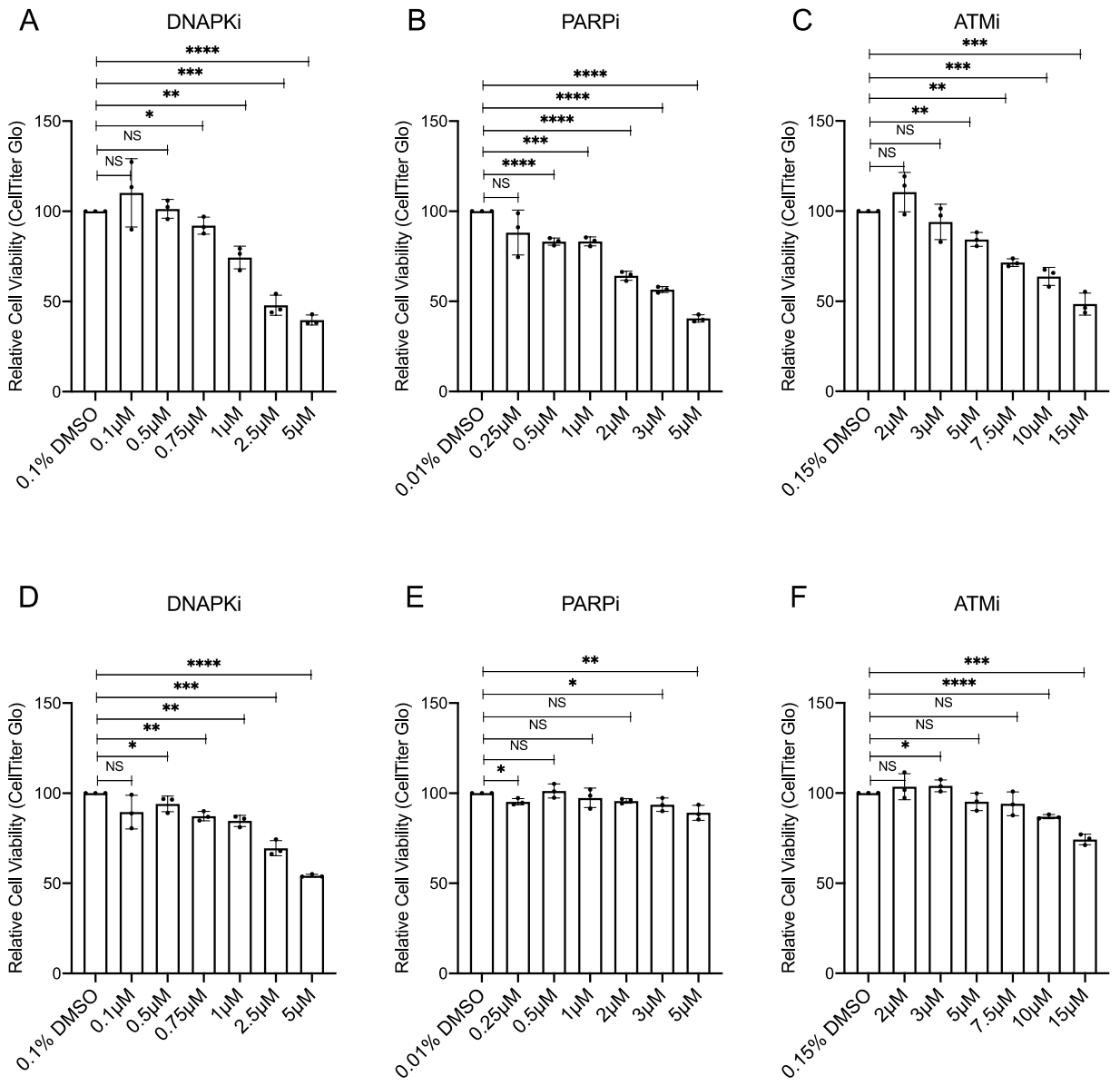

Figure 1: Melanoma persister cells are not sensitized to DNA repair gene inhibition. Cell viability of drug naïve (A-C) versus drug-tolerant persister (D-F) BRAF mutant A375 Malignant melanoma cells. A-C), Relative viability of drug naïve parental cancer cells treated with A) DNAPK inhibitor, B) PARP inhibitor, and C) ATM inhibitor. D-F), Relative viability of drug-tolerant persister cells treated with D) DNAPK inhibitor, E) PARP inhibitor, and F) ATM inhibitor. Bars represent mean with individual data points, n=3. Error bars denote standard deviation. DMSO, dimethyl sulfoxide. P-value calculated using two-tailed t test. *P≤0.05; **P≤0.01; ***P≤ 0.001; ****P≤0.0001.

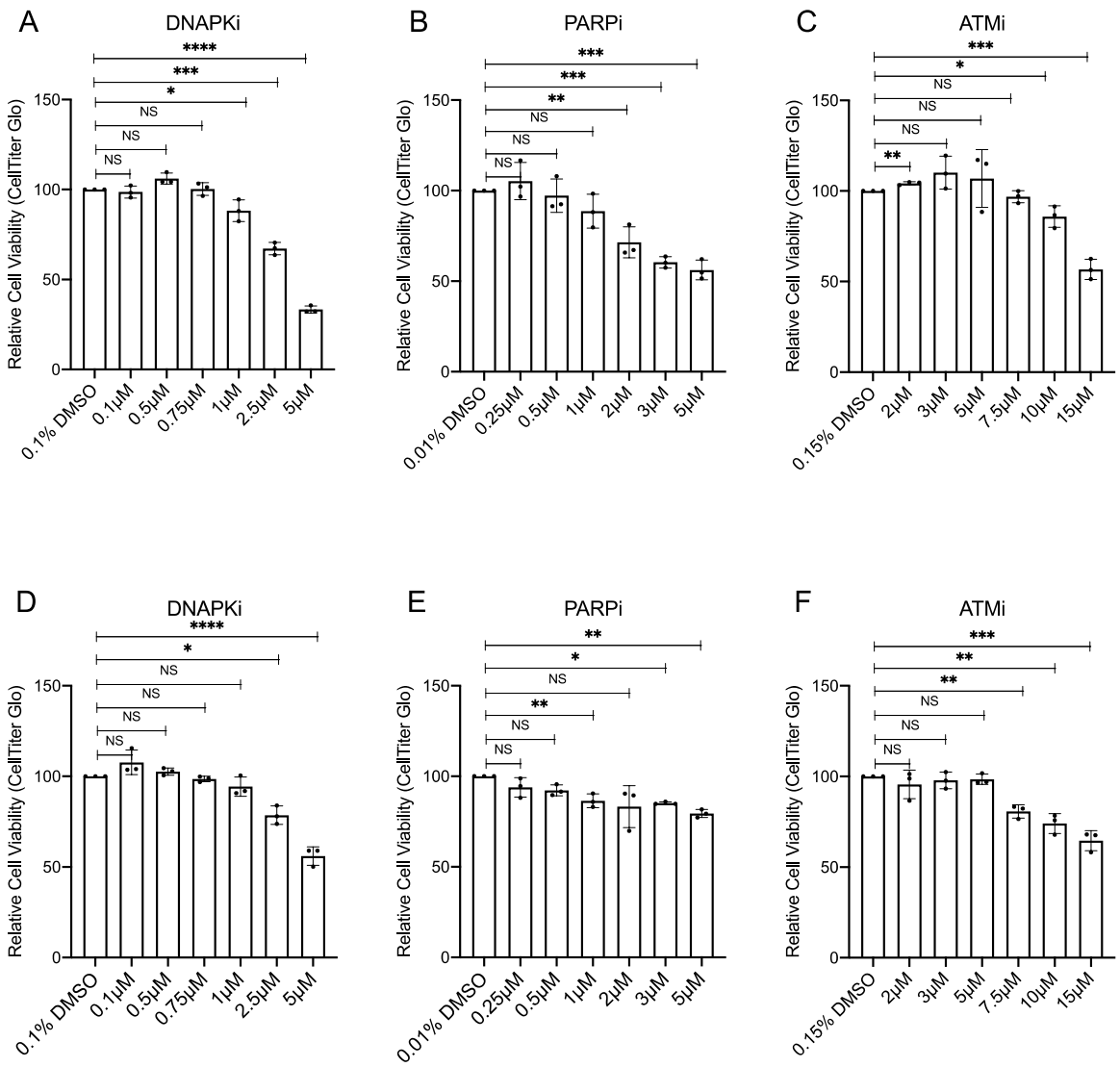

Figure 2: NSCLC persister cells are not sensitized to DNA repair gene inhibition. Cell viability of drug naïve (A-C) versus drug-tolerant persister (D-F) EGFR mutant PC9 non-small cell lung cancer cells. A-C), Relative viability of drug naïve parental cancer cells treated with A) DNAPK inhibitor, B) PARP inhibitor, and C) ATM inhibitor. D-F), Relative viability of drug-tolerant persister cells treated with D) DNAPK inhibitor, E) PARP inhibitor, F) ATM inhibitor. Bars represent mean with individual data points, n=3. Error bars denote standard deviation. DMSO, dimethyl sulfoxide. P-value calculated using two-tailed t test. *P≤0.05; **P≤0.01; ***P≤ 0.001; ****P≤0.0001.

Chapter 2: Determine the Mechanism of Disulfiram-Mediated Persister Cell Death

In the 1880s, Disulfiram functioned as a catalyst in the production of rubber.[40,41] 50 years after Disulfiram's introduction into the rubber industry, it was realized that workers exposed to DSF experienced unpleasant physical reactions after consuming alcohol.[42] Therefore, serendipitously, Disulfiram was discovered as a treatment for alcoholism. Following alcohol consumption, ethanol is converted first into acetaldehyde by alcohol dehydrogenase (ADH), and subsequently into acetic acid via aldehyde dehydrogenase (ALDH).[42] It is now known that Disulfiram inhibits ALDH activity, resulting in an accumulation of acetaldehydes in the system.[42] High levels of acetaldehydes are toxic and result in the manifestation of unpleasant physical symptoms, as noticed in the rubber workers.[42] Because of this mechanism, Disulfiram is able to help individuals overcome their alcohol dependance.[42]

Almost 40 years later, scientists identified Disulfiram as having the potential to suppress tumor growth.[41,42] In 2014, it was discovered that Disulfiram selectively kills cancer persister cells.[37] Raha et al. proposed that this selective persister cell killing occurred through ALDH inhibition[37]. However, their efforts toward identifying which of the 19 human ALDH genes were targeted by Disulfiram failed to reveal any ALDHs essential for persister cell survival. Therefore, the mechanism of Disulfiram-mediated persister cell death remains to be definitively determined. Here, we aim to further elucidate the mechanism through which Disulfiram selectively kills persister cells. We hypothesize that Disulfiram does not kill persister cells primarily through ALDH inhibition, but rather through an undetermined mechanism of ALDH inhibition-independent oxidative death. Here we report our findings from testing a variety of antioxidant compounds to clarify Disulfiram's mechanism of selective killing through oxidative stress.

MATERIALS AND METHODS:

Drug Naïve Cytotoxicity Assays:

Flavopiridol was purchased from Selleck Chemicals. Disulfiram and Gossypol (G8761) were purchased from Sigma Aldrich. 673A was purchased from Tocris Bioscience. CellTiter Glo was purchased from Promega. All chemicals were stored at -80 in DMSO unless otherwise specified.

In order to elucidate the mechanism of Disulfiram-mediated persister cell-specific killing, we performed cytotoxicity assays on both drug naïve cancer cells and drug-tolerant persister cells. Cell culture of drug naïve BRAF-mutant A375 melanoma and EGFR-mutant PC9 non-small-cell lung carcinoma cells was performed as described in Chapter 1. Drug naïve A375 cells were then plated at 33,000 cells per well of a 12 well plate in preparation for a 72-hour cytotoxicity assay. Drug naïve PC9 cells were plated at 5,500 cells per well of a 96 well plate for 24hr assays, and at 2,700 cells per well for 72hr assays. Approximately 24 hours after initial plating, all media was supplemented with Gossypol, 673A, or Disulfiram for three days before cell viability was assessed via CellTiter Glo. Cell viability of drug naïve PC9 cells treated with Flavopiridol alone and of Flavopiridol in conjunction with Disulfiram was assessed via CellTiter Glo 24 hours after treatment.

Persister Cell Formation and Studies:

Trametinib (MEKi) and Ferrostatin were purchased from ApexBio. Dabrafenib (BRAFi) and Erlotinib (EGFRi) were purchased from Selleck Chemicals. Buthionine Sulfoximine (BSO) and RSL3 were purchased from Sigma Aldrich. All chemicals were stored at -80 in DMSO unless otherwise specified.

In order to determine the mechanism through which Disulfiram selectively kills cancer persister cells, drug naïve parental cells were first transformed into persister cells and subsequently treated with aldehyde dehydrogenase inhibitors, antioxidants, or other chemical agents. In preparation for 72hr persister cell viability assays, drug naïve A375 cells were plated at 50,600 cells per well of a 12 well plate. From these plates, persister cells were formed as described in Chapter 1. On day 14 in drug, we treated A375 persister cells with Gossypol, 673A, or Disulfiram. 72 hours after treatment, we assessed their relative cell viability via CellTiter Glo.

Drug naïve PC9 cells were seeded at 1,800 cells per well of a 96 well plate for both 24- and 72-hour persister viability assays. After 24 hours, we began persister derivation as described in Chapter 1. 10 days later, we treated the PC9 persister cells with either Gossypol, 673A, or Disulfiram for 72 hours and then assessed cell viability. Also on day 10 of persister formation, we treated PC9 persister cells with Buthionine Sulfoximine, Flavopiridol, or Flavopiridol in combination with DSF for 24 hours before performing CellTiter Glo.

Statistical Analysis:

The relative cell viability of drug naïve parental cells versus drug-tolerant persister cells was analyzed and compared in order to determine the mechanism through which Disulfiram selectively kills drug-tolerant persister cells. All statistical analysis was performed using GraphPad Prism 9.0.1. All P-values were calculated using a two-tailed t test between the control and each individual condition.

RESULTS:

We sought to elucidate the mechanism of action by which Disulfiram selectively kills cancer persister cells. Raha et al. proposed that this selective persister cell killing occurred through

ALDH inhibition, although they did not identify specific ALDH which were required for persister cell survival from among the 19 human ALDHs[37]. This could indicate high functional redundancy between ALDHs, which Raha et al. proposed, or it may indicate an ALDH-independent mechanism. Indeed other groups recently published that Disulfiram-mediated killing of certain cancer cell lines is ALDH-independent.[43,44] Whether or not ALDH activity, previously known to be essential for cancer stem cells, is the relevant target of Disulfiram in persister cells therefore remains undetermined. To elucidate the mechanism of Disulfiram-mediated persister cell death, we tested the effects of aldehyde dehydrogenase inhibitors, antioxidants, and other chemical agents (Figures 3-7).

First, we confirmed that Disulfiram has a persister cell-specific lethality in our persister cell models. We found that 10 μM of Disulfiram had no significant effect on parental A375 melanoma cells, 0.5 μM Disulfiram reduced persister cell viability and 2 μM of Disulfiram resulted in over 50% of a reduction of persister cell viability compared to the control (Figure 3b,e). Disulfiram persister cell-specific killing was also observed in PC9 non-small cell lung cancer (Figure 4b,e).

We then tested whether Disulfiram-mediated persister cell death is through ALDH inhibition by testing other pharmacologic agents which induce ALDH inhibition or rescue from it. For both melanoma and NSCLC, administration of Gossypol, a pan-ALDH inhibitor , did not result in persister cell-specific lethality, with no significant reduction in cell viability following treatment with Gossypol conditions lower than 1 μM (Figure 3a,d and Figure 4a,d). Concentrations higher than 5 μM were toxic for both drug naïve parental and drug-tolerant non-small-cell lung carcinoma persister cells. Drug naïve and drug-tolerant melanoma persister cells were unresponsive to Gossypol concentrations of 2 μM or less, but both cell populations had reduced cell viability in

response to 10 μM. Therefore, pan-ALDH inhibitor Gossypol does not selectively kill persister cells, making Disulfiram-mediated persister cell death through ALDH inhibition unlikely.

Similar results were seen following the administration of 673A, an ALDH1A family inhibitor that targets ALDH1A1 which is highly expressed in cancer persister cells.[37] There was no significant reduction in the cell viability of NSCLC drug naïve nor drug-tolerant persister cells following the administration of 5 μM or less of 673A (Figure 4c,f). However, there was an observed reduction following the administration of 10 μM 673A. In contrast, treatment of both drug naïve and drug-tolerant melanoma persister cells with less than 10 μM of 673A resulted in a cell viability equal to or greater than the control (Figure 3c,f). Together, these data indicate that neither NSCLC nor melanoma cells treated with ALDH inhibitor 673A experienced a persister cell-specific reduction in cell viability.

Previous studies have shown that cell death induced through ALDH inhibition requires ALDH1A-regulated retinoic acid-mediated transcriptional changes.[45] To determine whether Disulfiram mediated persister cell death occurs through ALDH inhibition, we administered Flavopiridol, a known inhibitor of transcription previously shown to inhibit ALDH inhibitor-induced death.[45] When comparing the viability of drug naïve parental versus drug-tolerant persister NSCLC cells treated with Disulfiram with and without Flavopiridol, we found that Disulfiram-mediated persister cell death was not rescued by Flavopiridol (Figure 5). Together, these data show that ALDH inhibition alone does not kill persister cells, and that Disulfiram likely has additional mechanisms which act instead of or in addition to ALDH inhibition.

We then explored alternative mechanisms of Disulfiram-mediated killing. Disulfiram has previously been reported to directly react with glutathione leading to glutathione depletion in certain contexts.[46] To determine whether Disulfiram kills drug-tolerant persister cells via

glutathione depletion, we administered glutathione biosynthesis inhibitor Buthionine Sulfoximine (BSO) to drug-tolerant NSCLC and melanoma persister cells. We observed that glutathione depletion did not induce strong persister cell death (Figure 6a,b). For both NSCLC and melanoma persister cells, we found that up to 10 mM BSO did not reduce the relative cell viability below 50% of the control, far less toxicity than observed with Disulfiram (Figure 6). These data indicate that glutathione depletion alone is not sufficient to kill persister cells, and Disulfiram is therefore likely to have additional mechanisms promoting cell death.

To determine whether ferroptosis is involved in the mechanism of Disulfiram mediated persister cell death, we tested whether drug-tolerant NSCLC cells undergoing Disulfiram mediated cell death could be rescued by the administration of Ferrostatin, a ferroptosis inhibitor. We found that Ferrostatin treatment did not rescue persister cells from Disulfiram, while Ferrostatin does rescue persister cells from ferroptosis-inducing GPX4 inhibitor RSL3 (Figure 7). Together, these data show that Disulfiram kills persister cells through a ferroptosis-independent mechanism.

Collectively, these data point toward a mechanism of Disulfiram-mediated persister cell death which is not mediated solely through ALDH inhibition, glutathione depletion, or ferroptosis. Other data from our lab indicate that certain antioxidants rescue persister cells from Disulfiram, and experiments are underway to determine how Disulfiram kills persister cells through non-ferroptotic oxidative death.

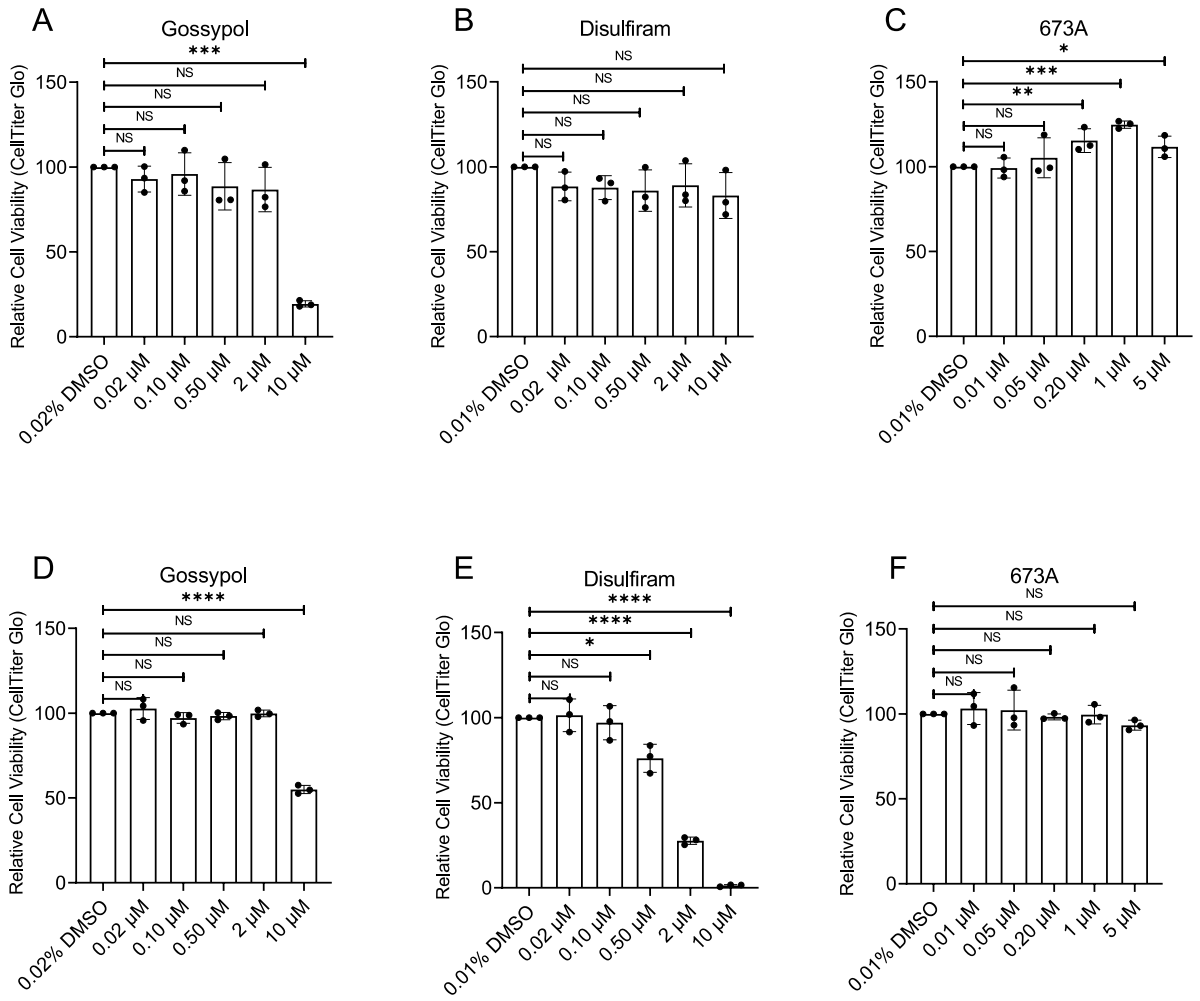

Figure 3: Disulfiram selectively kills drug-tolerant melanoma cells through an ALDH-independent mechanism. Cell viability of drug naïve (A-C) versus drug-tolerant (D-F) BRAF mutant A375 Malignant melanoma cells. A-C) Relative viability of drug naïve parental cancer cells treated with A) pan-ALDH inhibitor Gossypol, B) Disulfiram, and C) ALDH1A family inhibitor, 673A. D-F) Relative viability of drug-tolerant persister cells treated with D) Gossypol, E) Disulfiram, and F) 673A. Equal % DMSO was present in all wells per experiment. Bars represent mean with individual data points, n=3. Error bars denote standard deviation. DMSO, dimethyl sulfoxide. P-value calculated using two-tailed t test. *P≤0.05; **P≤0.01; ***P≤ 0.001; ****P≤0.0001.

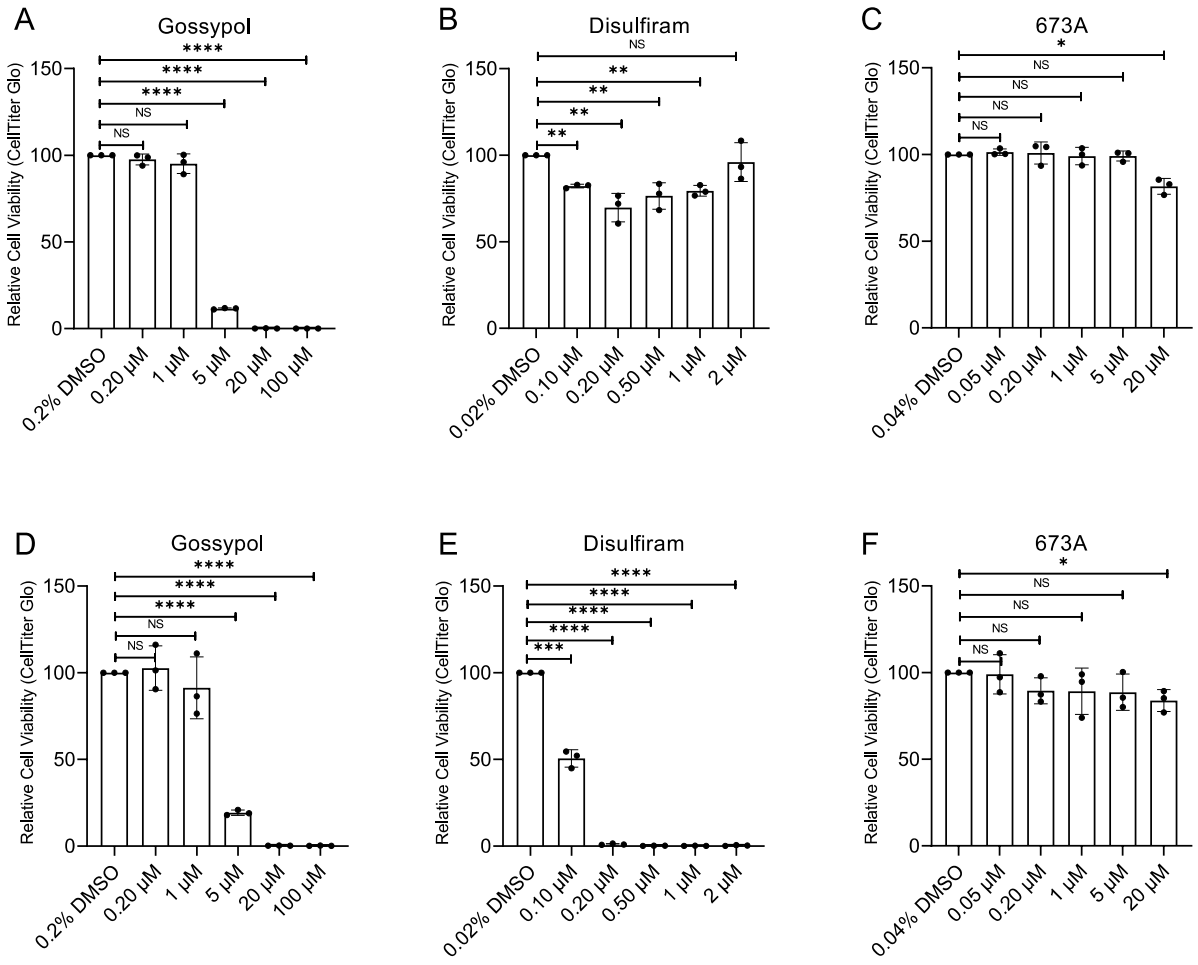

Figure 4: Disulfiram selectively kills NSCLC persister cells through an ALDH-independent mechanism. Cell viability of drug naïve (A-C) versus drug-tolerant (D-F) EGFR mutant PC9 non-small cell lung cancer cells. A-C), Relative viability of drug naïve parental cancer cells treated with A) pan-ALDH inhibitor Gossypol, B) Disulfiram, and C) ALDH1A family inhibitor, 673A. D-F), Relative viability of drug-tolerant persister cells treated with C) Gossypol, E) Disulfiram, F) 673A. Equal % DMSO was present in all wells per experiment. Bars represent mean with individual data points, n=3. Error bars denote standard deviation. DMSO, dimethyl sulfoxide. P-value calculated using two-tailed t test. *P≤0.05; **P≤0.01; ***P≤ 0.001; ****P≤0.0001.

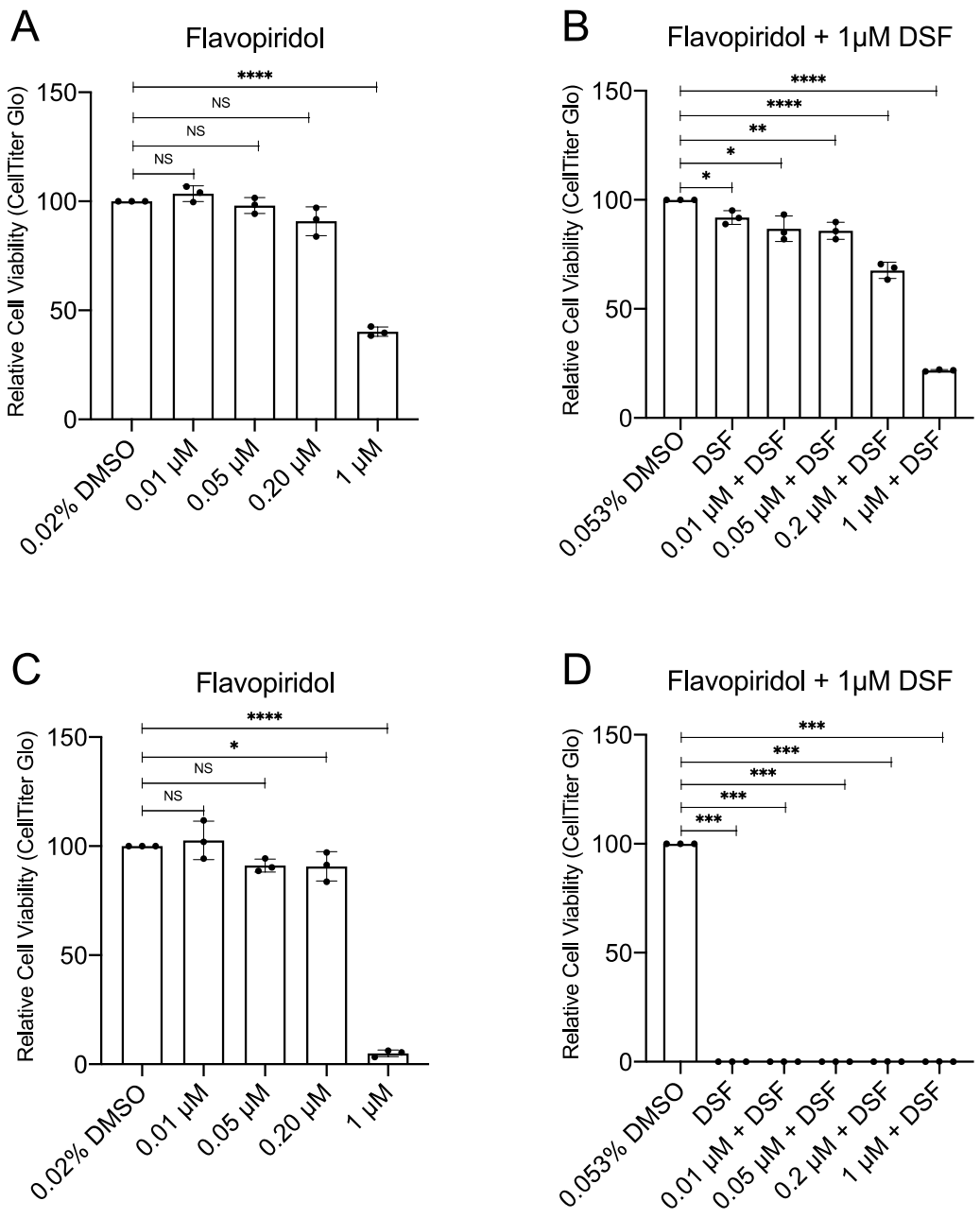

Figure 5: Flavopiridol does not rescue drug-tolerant persister cells from Disulfiram mediated cell death. To test whether Disulfiram mediated persister cell death occurs through ALDH inhibition, we administered a retinoic acid transcription inhibitor. Depicted above is the cell viability of drug naïve (A,B) versus drug-tolerant (C,D) EGFR mutant PC9 Non-small cell lung cancer cells. A-B) Relative viability of drug naïve parental NSCLC cells treated with A) transcription inhibitor Flavopiridol and B) Flavopiridol in conjunction with Disulfiram. C-D) Relative viability of drug-tolerant persister cells treated with C) Flavopiridol and D) Flavopiridol in conjunction with Disulfiram. Equal % DMSO was present in all wells per experiment. Bars represent mean with individual data points, n=3. Error bars denote standard deviation. DMSO, dimethyl sulfoxide. P-value calculated using two-tailed t test. *P≤0.05; **P≤0.01; ***P≤ 0.001; ****P≤0.0001

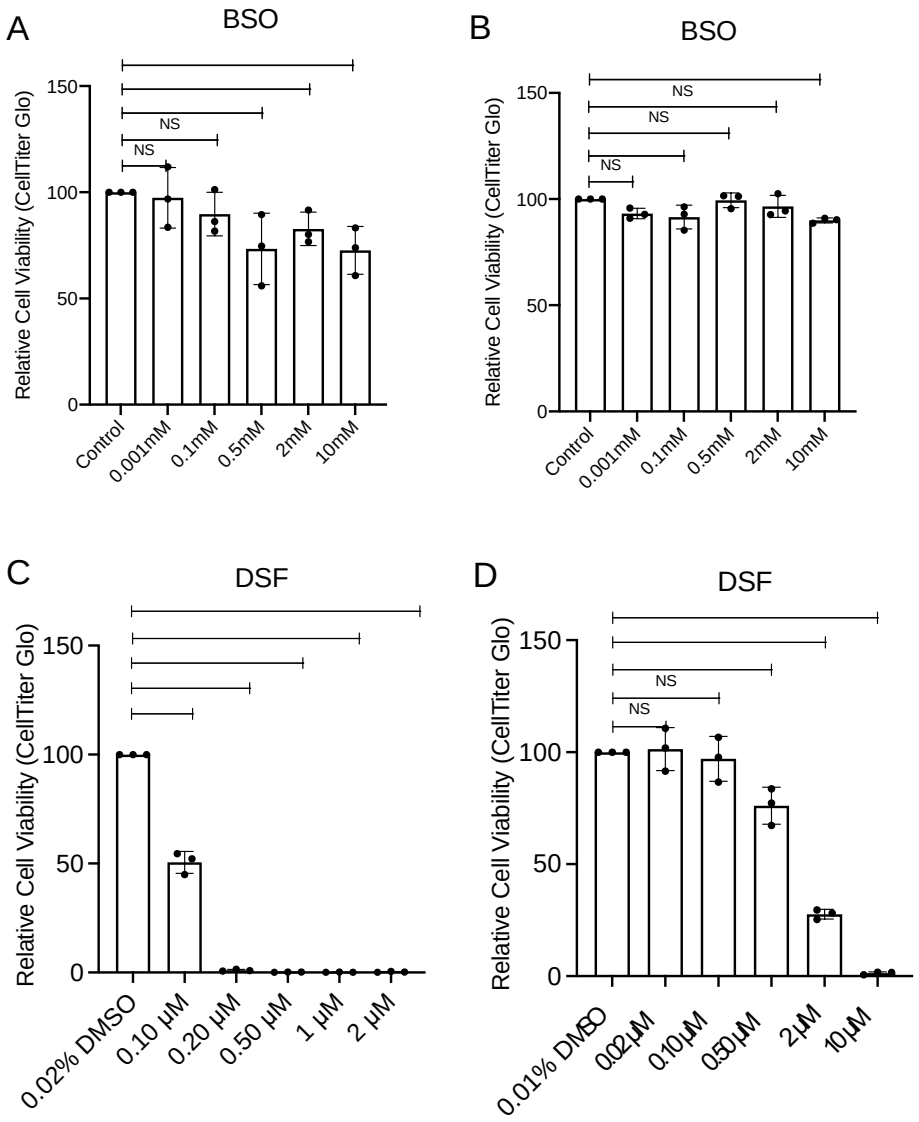

Figure 6: Disulfiram induces cell death more than glutathione depletion of Drug-tolerant persister cells. To test whether persister cell death occurs through glutathione depletion, we administered a sulfoximine derivative. Depicted above is the cell viability of drug-tolerant EGFR mutant Non-small cell lung cancer cells versus drug-tolerant BRAF-mutant melanoma. A) Relative viability of drug-tolerant NSCLC cells treated with Buthionine Sulfoximine(BSO). B) Relative viability of drug-tolerant melanoma cells treated with Buthionine Sulfoximine. C) Relative viability of drug-tolerant NSCLC cells treated with Disulfiram(DSF). D) Relative viability of drug-tolerant melanoma cells treated with Disulfiram. Equal % DMSO was present in all wells per experiment. Bars represent mean with individual data points, n=3. Error bars denote standard deviation. DMSO, dimethyl sulfoxide. P-value calculated using two-tailed t test. *P≤0.05; **P≤0.01; ***P≤ 0.001; ****P≤0.0001

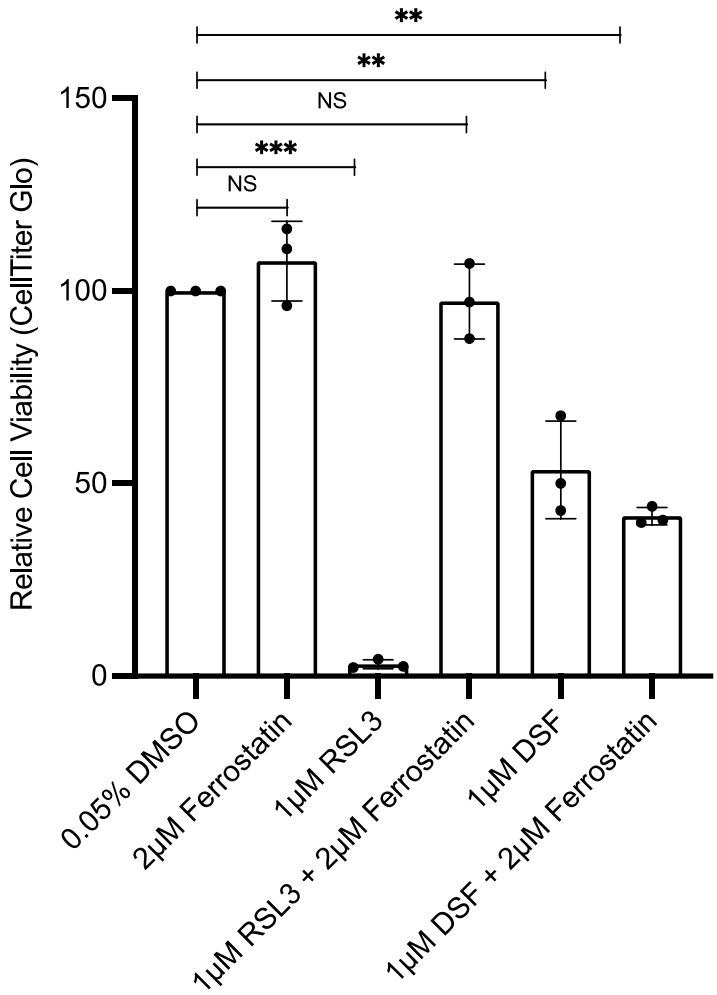

Figure 7: Disulfiram kills persister cells through a ferroptosis-independent mechanism.-Drug tolerant NSCLC cells were co-treated with Ferrostatin and Disulfiram to determine whether ferroptosis is involved in the mechanism of Disulfiram mediated persister cell death. RSL3 is known to induce lethality through ferroptosis, and therefore serves as a control for Ferrostatins ability to rescue cells from ferroptosis cell death. Depicted above is the cell viability of drug-tolerant EGFR mutant Non-small cell lung cancer cells. Equal % DMSO was present in all wells per experiment. Bars represent mean with individual data points, n=3. Error bars denote standard deviation. DMSO, dimethyl sulfoxide. P-value calculated using two-tailed t test. *P≤0.05; **P≤0.01; ***P≤ 0.001; ****P≤0.0001

Chapter 3: Identifying Processes Required for Persister Cell Regrowth

In response to initial drug treatment, drug naïve parental cells transform into a population of cell cycle arrested persister cells.[27] We hypothesize that while in this epigenetic pro-survival state, persister cells serve as a mutagenic reservoir from which overtly drug-tolerant mutated cells can emerge. Of the few drug naïve tumor cells able to persist in the presence of drug, less than 25% are able to further expand into drug-tolerant expanded persister cells (DTEPs).[23] Following continuous prolonged (> 1 month) exposure to targeted therapy, overtly drug resistant cells resume proliferation and form genetically resistant tumor cell colonies.[13] However, little is known about how persister cells acquire resistance in order to reenter the cell cycle under continued drug treatment.

Here, we investigate the role that reactive oxygen species (R.O.S) have in the transition from persister cell to DTEP cell colony. A 2019 *Science* paper by Russo et al. found that the treatment of colorectal cancer persister cells with the antioxidant N-Acetyl Cysteine (N.A.C.), in combination with an EGFR antibody and BRAF inhibitor, prevented cancer relapse.[38] The authors propose that the antioxidant, N.A.C., decreased the level of R.O.S.-induced mutagenesis, thereby preventing the acquisition of genetic resistance needed to transform from persister cells into DTEP colonies.[38] However, N.A.C. is known to covalently react with cysteines and have other functions beyond antioxidant activity, making it unclear whether R.O.S.-induced mutagenesis is genuinely involved in the acquisition of drug resistance.[47.] I aim to determine what influence R.O.S.-mediated mutagenesis has on the outgrowth of persister cells into drug-tolerant colonies.

Tumor cell oxidative homeostasis requires a balance between antioxidants and R.O.S..[48] Under hypoxic conditions, tumors are known to rely on glucose metabolism as a means of avoiding hypoxia induced cell death, referred to as the Warburg effect.[48] Here, we test the effects that

antioxidants and oxidizers have on DTEP formation. From this, we aim to determine whether R.O.S., or the lack thereof, contribute to the acquisition of drug resistance in cancer cells.

In order to test factors which affect DTEP colony growth, a novel technique for the quantification of DTEP formation was developed. Clonogenic assays are a widely accepted form of assessing cell survival responses through the quantification of colony growth. The current standardized method of quantifying the result of a clonogenic assay requires manually counting colonies.[49] This process can be time consuming and, because it relies on manual counting, prone to bias.[36] The development of a reliable way to automate the processing and analysis of the clonogenic assay would be a useful advance for DTEP assays. To this end, we establish a novel imaging-based approach to formally count drug-tolerant colonies of defined cell numbers. Using this new technique, we determine whether R.O.S. have a role in the formation of DTEPs.

MATERIALS AND METHODS:

DTEP Formation Studies:

Trametinib and Ferrostatin were purchased from ApexBio. Dabrafenib and Erlotinib were purchased from Selleck Chemicals. 3% Hydrogen Peroxide was purchased from Fischer Scientific. Alpha-Tocopherol (Vitamin E), GSH-ethyl ester, N-Acetyl Cysteine (N.A.C.), NGI-1, OSMI-1, Oseltamivir Phosphate (Tamiflu), and Tunicamycin were purchased from Sigma Aldrich. All chemicals were stored at -80 in DMSO unless otherwise specified.

To determine whether R.O.S. have a role in the transformation from drug-tolerant persister cell into overtly drug resistant cell colony, we performed clonogenic assays on A375 BRAF-mutant melanoma and PC9 EGFR-mutant NSCLC cells. All drug naïve parental cells were initially maintained in culture as described in Chapter 1.

To derive A375 drug-tolerant expanded persister cell colonies, we plated 4,000 drug naïve cells per 10cm plate. After 24 hours, the cells were treated with 250 nM Dabrafenib and 25 nM Trametinib. After two weeks of drug treatment, the A375 naïve cells had transformed into cell cycle arrested persister cells. It is at this point where we administered a variety of compounds (Figure 9) in addition to the 250 nM Dabrafenib and 25 nM Trametinib. We continued this treatment for an additional 5 weeks before quantifying colony formation. During derivation, media was refreshed every 3-4 days.

To derive PC9 drug-tolerant expanded persister cell colonies, we plated 7,000 PC9 cells per 10 cm plate. After 24 hours, the cells were treated with 2.5 μM Erlotinib media. After two weeks of treatment, the naïve PC9 cells had transformed into cell cycle arrested persister cells. We then administered a variety of compounds (Figure 9) in addition to 2.5 μM erlotinib. We continued treatment for an additional 3 weeks prior to quantifying colony formation. During derivation, media was refreshed every 3-4 days.

Automating DTEP Quantification:

Crystal Violet was purchased from Acros Organics.

In order to automate the quantification of the clonogenic assay, we established a novel imaging-based approach to formally count drug-tolerant colonies of defined cell numbers (Figure 8). Once the persister cells transformed into DTEPs at the timepoints specified previously, all plates were fixed in cold methanol for 10 minutes. We then aspirated the methanol and stained the DTEP colonies with a 0.5% Crystal Violet Solution for 30 minutes. After the plates were stained, we converted them into images using an Epson V370 flatbed scanner. To ensure a blinded analysis, all plates were scanned in a random order and were given de-identified names. Images were acquired in 48-bit color at 1000 DPI, with 50% scale. Using an Adobe Photoshop (CC 2018)

droplet, we added a 16-point circular mask in ‘pantone cool grey 8C’ to each image in order to create a uniform region of interest for analysis.

All plates were further analyzed using Genetools 4.3.9 colony analysis software by Syngene. In Genetools, we defined the region of interest to be within the applied circular mask in order to eliminate bias originating from the manual placement of the region of interest. We adjusted the sensitivity to ‘high’, and defined the size of a single cell, in number of pixels, by using the Genetools tool to select an identified cell or colony and define its pixel size. We then multiplied this value by 25 to determine a pixel range corresponding to 1-25 cells. To determine the pixel range corresponding to 26-100 cells, we multiplied the single cell pixel size by 100. Additionally, we quantified the amount of colonies greater than 100 cells, or larger than the single cell pixel size multiplied by 100. All colonies identified by Genetools were additionally visually confirmed; any false negatives or positives identified by the program were then manually addressed through the inclusion or exclusion of the identified colony with the reviewer blinded to the sample identity.

Statistical Analysis:

All results were then converted into percent of colonies formed larger than 25 cells for each condition as compared to the control. All statistical analysis was performed using GraphPad Prism 9.0.1. All P-values were calculated using a two-tailed t test between the control and each individual condition.

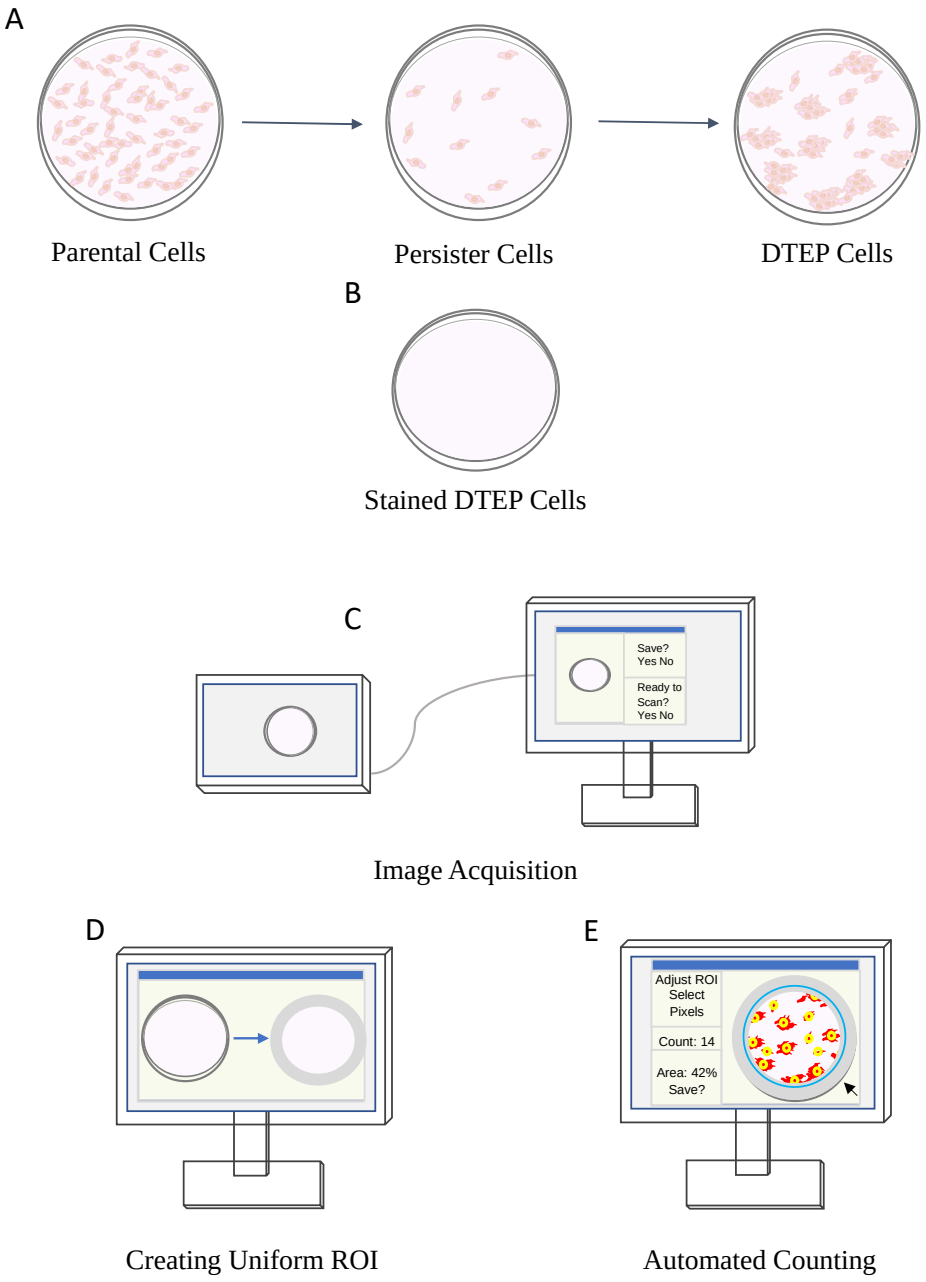

Figure 8: The automated DTEP quantification process. A) Drug treatment of the drug naïve parental cells causes tumor shrinkage. Following approximately 2 weeks of continued drug treatment, drug-tolerant persister cells remain. These cells remain in a cell-cycle arrested state until they acquire drug resistance. After an additional 3-5 weeks of continued drug treatment, these persister cells are able to proliferate into drug-tolerant expanded persister (DTEP) cell colonies. B) Cartoon depicting Crystal Violet staining of DTEP colonies. C) Cartoon depicting the scanning of stained DTEP colonies. D) Cartoon depicting the photoshop application of a uniform region of interest. E) Cartoon depicting the Genetools automated quantification of DTEP colonies.

RESULTS:

To explore the role that oxidative stress has in drug-tolerant expanded persister cell (DTEP) formation, we performed a DTEP formation assay on EGFR-mutant non-small-cell lung carcinoma and BRAF-mutant melanoma cells. Drug naïve parental cells were first treated with a targeted therapeutic causing cell death and revealing quiescent persister cells. We then administered antioxidants or oxidizers and measured DTEP formation. We also assessed glycosylation inhibitors in parallel because preliminary data from another colleague pointed toward a potential role for glycosylation in persister cell regrowth into DTEPs.

A comparison of colony formation among conditions (Figure 9) showed that antioxidants do not uniformly prevent the formation of DTEP colonies compared to the control in EGFR-mutant NSCLC or BRAF-mutant melanoma persister cells. All NSCLC persister cells treated with antioxidants (i.e. Vitamin E, N.A.C. , Ferrostatin) were capable of forming large colonies in the presence of targeted therapy at a level similar to the control, indicating that R.O.S. are not required for DTEP formation (Figure 9a). Interestingly, OSMi-1 increased DTEP formation in NSCLC persister cells, but this was not a general effect because no increase was observed in melanoma DTEPs.

Analysis of melanoma drug-tolerant expanded persister cell colony formation showed no statistically significant difference in large colony formation between any of the conditions compared to the control. We found that melanoma persister cells co-treated with antioxidants such as Vitamin E or Ferrostatin, oxidants such as H_2O_2 , or glycosylation inhibitors such as OSMi-1 and NGi-1 were equally capable of forming DTEP colonies(Figure 9b). In all DTEP formation experiments, there was a large amount of noise between replicates including in the control plates, and this is reflective of the noisy process of DTEP formation from rare persister cells. In the future,

more replicates or larger plates may be needed to derive less noisy data. Nonetheless, these experiments have indicated that antioxidants do not prevent the regrowth of persister cells into drug-tolerant expanded persister cell (DTEP) colonies, consistent with R.O.S.-independent mechanisms of DTEP formation. Therefore, these findings disagree with the report from Russo et al. which found that N.A.C. antioxidant treatment blocked DTEP formation.[38] Upon testing a wider panel of antioxidants, and also pro-oxidants, in additional persister cell models we found no role for R.O.S. in DTEP formation.

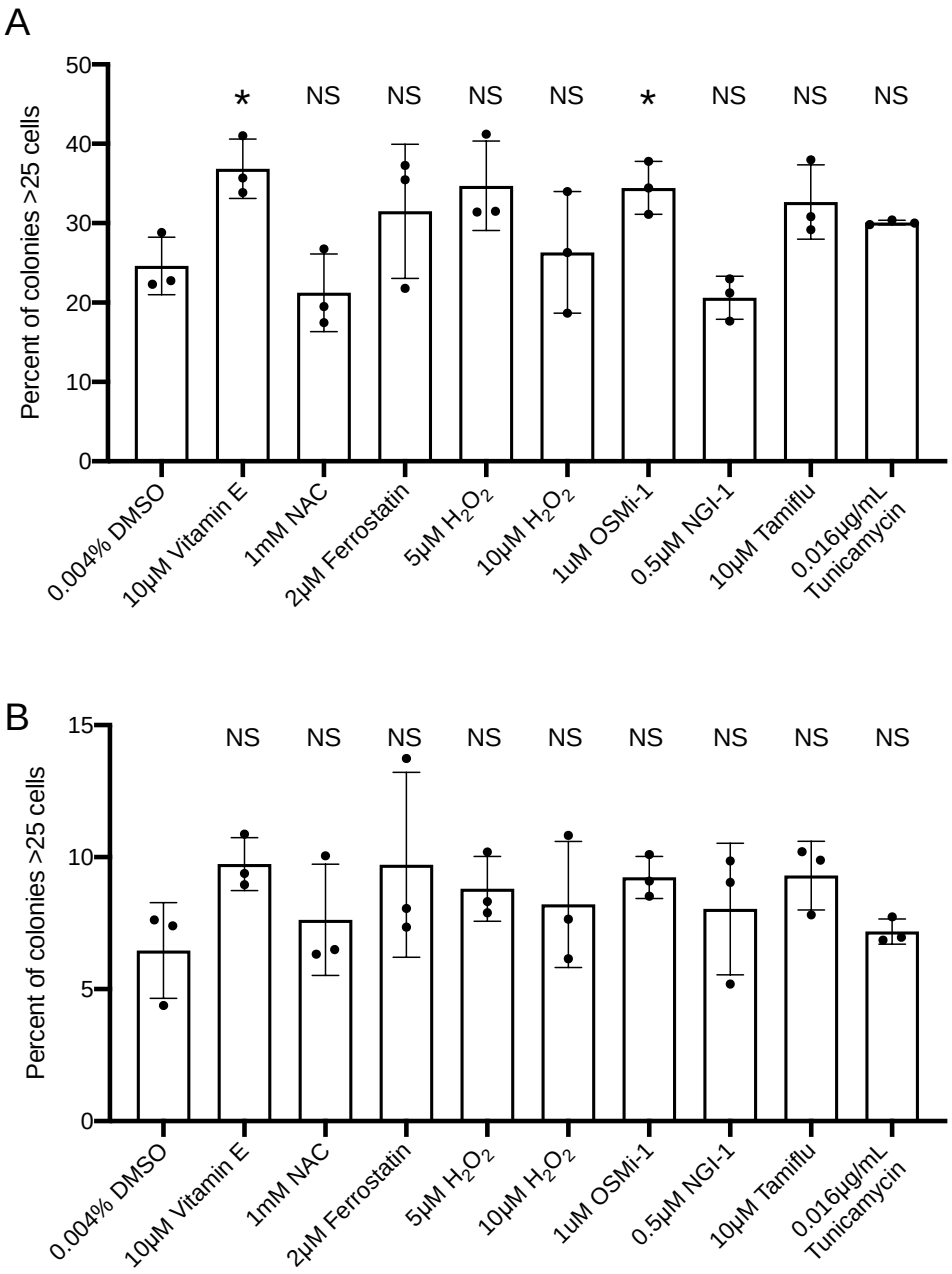

Figure 9: Antioxidants do not uniformly prevent the acquisition of drug resistance in cancer persister cells. Depicted above is the percent of colonies greater than 25 cells formed per condition following treatment with antioxidants, oxidizers, or glycosylation inhibitors. A) Percent of colonies > 25 cells formed by drug-tolerant non-small-cell lung carcinoma cells following antioxidant treatment. Vitamin E and OSMi-1 were the only conditions that resulted in a statistically significant increase in the percent of colonies >25 cells as compared to the control. B) Percent of colonies > 25 cells formed by drug-tolerant melanoma cells. There were no statistically significant differences in the percent of colonies >25 cells formed. Bars represent mean with individual data points, n=3. Error bars denote standard deviation. DMSO, dimethyl sulfoxide. N.A.C., N-Acetyl Cysteine. H_2O_2, Hydrogen Peroxide. P-value calculated using two-tailed t test. *P≤0.05; **P≤0.01; ***P≤ 0.001; ****P≤0.0001

DISCUSSION:

Although the field of cancer biology has advanced rapidly since the introduction of personalized oncogenomics, acquired resistance to targeted therapy continues to impede progression free survival.[31] During initial tumor shrinkage in response to targeted therapy, there exists a small group of cell-cycle arrested cancer cells, or persister cells, that are able to survive in the presence of drug. It is thought that persister cells phenotypically differ from their drug naïve parental cells, but do not yet possess the resistance mutations required to begin outgrowth in continued drug treatment. It is in this epigenetic pro-survival state where these drug-tolerant expanded persister cells acquire resistance mutations which allow them to exit quiescence and form genetically diverse tumors.[13] Because of the persister cell's hypothesized central role in acquired resistance, identifying targetable persister cell vulnerabilities may be the most viable way to prevent the acquisition of drug resistance.

We first explored whether there exists a persister cell-specific vulnerability to synthetic lethality induced through the inhibition of DNA damage response genes. Given the finding that cancer persister cells have a disabled DNA damage response due to downregulated expression of repair genes,[34] we hypothesized that inhibiting additional DNA damage response genes would increase DNA damage to lethal levels. Our results indicate that drug naïve parental cancer cells are affected as much as, or more than, drug-tolerant persister cells by the inhibition of the DNA repair genes ATM, DNAPK, or PARP (Figure 1, Figure 2). Contrary to our expectations, we did not observe a persister cell-specific synthetic lethality induced through the inhibition of DNA damage response genes. From this, we conclude that persister cell DNA damage is not a vulnerability. Future research is needed to determine whether persister cells are vulnerable to synthetic lethality induced through the co-inhibition of DNA damage response genes. Continued

efforts in identifying persister cell-specific vulnerabilities are essential to preventing the acquisition of drug resistance during cancer therapy.

While Disulfiram has long been a clinically approved drug indicated for treating alcoholism, it was recently identified to be a persister cell-specific vulnerability.[37] Previous studies proposed that the mechanism of Disulfiram's selective persister cell lethality was due to inhibition of aldehyde dehydrogenase (ALDH) activity; however, direct evidence of ALDH inhibition was not confirmed.[37] We hypothesized that Disulfiram does not kill persister cells through ALDH inhibition. To elucidate this further, we tested the effects of other known aldehyde dehydrogenase inhibitors, antioxidants, and other chemical agents (Figures 3-7). We observed that the administration of other aldehyde dehydrogenase inhibitors did not selectively kill persister cells (Figures 3,4).

Previous studies have shown that ALDH inhibition induced cell death requires ALDH1A-regulated retinoic acid-mediated transcriptional changes.[45] We expected that if Disulfiram killed persister cells through an ALDHi mechanism, the administration of a transcription inhibitor such as Flavopiridol would rescue the persister cells (Figure 5). The observed lack of death rescue following the inhibition of transcriptional changes required for ALDHi induced cell death, in addition to the lack of persister-specific killing following the ALDH1A inhibition both support the hypothesis that Disulfiram's persister cell-specific killing occurs through a non-ALDHi mechanism.

We have discovered that Disulfiram-mediated persister cell-specific killing can be rescued by a variety of antioxidant compounds but not by ferroptosis-rescuing lipophilic antioxidants (Figure 5,6,7). We therefore propose that Disulfiram kills persister cells through an oxidative apoptosis mechanism rather than through ALDH inhibition. Additional work is needed to elucidate

this oxidative apoptosis mechanism of Disulfiram's persister cell-specific killing. Furthermore, greater efforts are needed to determine whether Disulfiram's capability to cause the side effects used as a treatment for alcoholism is a result of its ability to induce cell death. Further elucidating Disulfiram's ability to prevent persister cells from acquiring drug resistance by inducing cell death may provide insight for other targetable vulnerabilities of the persister cell. Understanding these persister cell-specific vulnerabilities is of the utmost importance in achieving the primary goal of preventing cancer relapses.

In addition to preventing resistance acquisition, there is still much to elucidate in regard to conditions that may influence the persister cells' ability to become an overtly drug resistant proliferating colony. It is known that antioxidants reduce intracellular levels of R.O.S.,[50] and that resultant DNA damage leads to an upregulation of mutations.[51] This upregulation of mutations can allow for the introduction of drug resistance mutations into the persister cell genome, which makes it possible for them to proliferate in the presence of drug. Therefore, if R.O.S. is required for mutagenesis and acquisition of resistance mutations, direct addition of antioxidants to cancer persister cells undergoing targeted drug therapy should impede their ability to proliferate by depleting R.O.S. and blocking R.O.S.-mediated mutagenesis.

As part of determining whether R.O.S., or the lack thereof, contribute to the acquisition of drug resistance in cancer cells, we performed a colony formation assay (Figure 9). Following treatment of cancer persister cells with antioxidants such as Vitamin E, N.A.C., or Ferrostatin, we observed similar levels in the formation of colonies larger than 25 cells. This signifies that both non-small cell lung cancer and melanoma persister cells are equally capable of proliferating in continued drug presence with or without antioxidant treatment. Our findings support our hypothesis that R.O.S. is not required for the formation of drug-tolerant expanded persister cells

(Figure 9). Alternatively, we expected that if R.O.S.-mediated mutagenesis was necessary for drug-tolerant expanded persister cell (DTEP) formation, the administration of an oxidizer such as H_2O_2 would directly raise cellular levels of R.O.S. and promote mutagenesis, resistance and DTEP formation. However, we did not observe any significant differences in large colony formation between H_2O_2 treated cells and the control. These data indicate that R.O.S.-mediated mutagenesis is not required for persister cell regrowth into DTEPs.

A recent 2019 article by Russo et al. found that treating colorectal cancer persister cells with an antioxidant, N.A.C. , prevented the cells from acquiring resistance to their targeted drug therapeutic.[38] This discovery led the authors to propose that antioxidants diminish cellular levels of R.O.S., thereby preventing R.O.S.-induced mutagenesis and DNA damage.[38] Our findings in our melanoma and NSCLC models disagree with this model because none of the antioxidants administered resulted in a decrease of DTEP colony formation in persister cells (Figure 9). Therefore, the role of R.O.S.-mediated mutagenesis in acquired resistance remains to be determined and further work is needed.

We also tested whether the administered glycosylation inhibitors such as OSMI-1,NGI-1, or Tamiflu affect persister cell outgrowth into DTEPs. Our findings show that glycosylation inhibitors do not uniformly prevent the formation of DTEP colonies compared to control in both EGFR-mutant NSCLC and BRAF-mutant melanoma persister cells (Figure 9). Additional research is needed to identify processes required for persister cell regrowth.

Toward our goal of testing cellular conditions for effects on outgrowth of cancer persister cells, we developed a method for the automated quantification of DTEP formation (Figure 8). Our imaging-based approach to formally count drug-tolerant colonies of defined cell numbers allowed us to observe the influence that chemically induced oxidative changes had on persister cell

outgrowth. However, our study was limited by the inherent noise in DTEP formation, which resulted in a high variability among biological replicates. Despite this, our high throughput imaging approach is an advance in the field of DTEP assays and has the potential to expand to screen many conditions in order to observe their effect on colony formation.

Continued efforts are needed to determine what cellular conditions influence the gain of drug resistance. While we have found that antioxidants do not prevent the regrowth of persister cells into DTEP colonies, the role for oxidative stress in persister cell biology remains elusive. Additionally, we show that while the inhibition of DNA damage response and repair pathways significantly prevented the outgrowth of cancerous cells in the presence of drug, there was no difference in the response between drug naïve versus drug-tolerant cancer persister cells. From these findings, we conclude that drug naïve parental cells and drug-tolerant persister cells are equally vulnerable to the inhibition of DNA damage response genes, and that these genes are unlikely to be persister cell-specific vulnerabilities. We have also furthered the understanding of the mechanism through which Disulfiram induces persister cell-specific lethality. We found that Disulfiram does not kill persister cells through ALDH inhibition, but rather through an oxidative-apoptotic mechanism. Through further elucidating factors involved in tumor acquisition of drug resistance, we contribute to the goal of preventing the occurrence of cancer relapses by providing insight into potential therapeutic targets as well as proposing future research directions. However, far more collective effort is required to achieve this goal. While the identification of the drug-tolerant persister cells' central role in resistance acquisition was a great advance, little is currently known about the specific mechanism through which this occurs. Because overtly drug resistant cells emerge from these drug-tolerant persister cells, the identification of additional persister cell-

specific vulnerabilities is the most promising future direction toward stopping the acquisition of drug resistance and thereby preventing cancer relapse.

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