UNIVERSITY OF CALIFORNIA SAN DIEGO

Understanding Cancer Persister Cell Vulnerabilities and Mutagenesis

A Thesis submitted in partial satisfaction of the requirements
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by

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The Thesis of Anna Elizabeth Stuhlfire is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

This thesis is dedicated to all those who have battled or are currently fighting cancer. In unified collaboration to eliminate the cruel and invasive disease, we will win this war together.

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ABSTRACT OF THE THESIS

Understanding Cancer Persister Cell Vulnerabilities and Mutagenesis

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Master of Science in Biology

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The landscape of cancer treatment options has grown to include more precise and less invasive strategies.[8,9] However, acquired drug resistance often results in transient responses. Cancer persister cells are a heterogeneous subpopulation of tumor cells which survive lethal doses of anti-cancer therapy and seed tumor recurrence.[16] Persister cells survive treatment by

non-genetic mechanisms that are not well understood.[15] Additionally, persister cells undergo stress induced mutagenesis and rapid genetic adaptation resulting in the emergence of genetically drug resistant cells.[35] Therapeutically targeting persister cells is therefore a promising approach to prevent acquired resistance. In this thesis, we describe our investigations into persister cell vulnerabilities and mutagenesis. First, we discovered that HDAC inhibitors sensitize various types of persister cells to ferroptosis, an oxidative cell death process. Second, we describe our efforts to elucidate the mechanism by which the clinically utilized anti-alcoholism drug, Disulfiram kills persister cells.[28,42] Lastly, we explore the mechanism by which persister cells undergo mutagenesis that allows for acquired genetic resistance to cancer drugs.

INTRODUCTION:

Cancer is a category of many diseases that occur as the result of DNA damage, accumulation of mutations, and the corruption of genetic information.[1] In healthy cells, proto-oncogenes and tumor suppressor genes are tightly regulated and work in tandem to control cell proliferation, completion of the cell cycle, and apoptosis. Genetic mutations that alter the function of proto-oncogenes and tumor suppressor genes are the genetic drivers of cancer development.[1] Cancerous cells are defined by distinct "hallmarks" that describe acquired functional characteristics that set malignant cells apart from non-cancerous somatic cells.[1,2] As described by Hanahan and Weinberg in 2000, the main hallmarks of cancer are: limitless replicative potential, induced angiogenesis, self-sufficient growth signaling, insensitivity to anti-growth signals, metastasis, and resistance to cell death.[2,3] This list was expanded in 2011; avoiding immune destruction was an additional hallmark, and genomic instability and mutation was added as an enabling characteristic.[3] Nearly all human tissues and organs can develop cancer, and as of 2020, cancer was the second leading cause of death in the USA.[4,5]

There are various treatment strategies for patients following a cancer diagnosis. Surgery and radiation are effective methods for removing or shrinking a localized tumor.[6] However, to eradicate cancer cells that spread beyond the tumor site, or metastasized and invaded a distant tissue, a regimen of chemotherapy is prescribed. Chemotherapies do not carry out a mechanism of action specific to cancer cells; rather, high doses of cytotoxic therapy perturb the cell cycle and kill rapidly dividing cells, cancerous and non-cancerous.[7] The non-specific nature of chemotherapy drugs and extensive killing of healthy cells during a course of treatment motivated investigations into the development of more precise treatment avenues, such as immunotherapies and targeted therapies.[7,8,9]

As mentioned above, evasion of immune system destruction is an additional hallmark of cancer.[3] Cancer cells elude detection and destruction by the immune system through morphological changes (e.g., down regulation of major histocompatibility complex) and signaling adaptations (e.g., expression of PD-L1 to block the PD1 mechanism) to escape from immune system surveillance and diminish effector T Cell response.[9,10] To combat unimpeded tumor growth, immunotherapy is a rapidly developing class of treatment strategies that utilize the patient's own immune system in killing cancer cells by improving immune system detection.[9] Drugs known as immune checkpoint inhibitors (ICIs), such as pembrolizumab and nivolumab, can obstruct the binding between PD1 and PD-L1 secreted by the tumor cells, thereby increasing tumor sensitivity to immune system detection.[9]

Furthermore, tumor genomic sequencing advances have promoted the hypothesis that patient tumor mutations can be matched to a cognate targeted therapy.[7,12] Targeted therapies result in reduced toxicity to healthy cells when compared to chemotherapies because they inhibit pathways that are associated with mutations or genes otherwise required specifically by cancer cells, rather than mass cell cycle disruption.[11,12] With advancements in Next Generation Sequencing (NGS), we can achieve a deeper and more precise understanding of exact genetic mutations that are operative in tumors.[14] Through Whole Genome Sequencing (WGS) and Whole Exome Sequencing (WES), a genetic profile of a tumor can be constructed, and potential weaknesses can be exploited.[11,12] For example, the targeted therapy, dabrafenib, a BRAF inhibitor, is effective when treating melanoma with a BRAF-V600E mutation; whereas, a melanoma tumor that was not a BRAF-mutant, or a tumor with a different mutational profile, such as EGRF-mutation non-small cell lung carcinoma, would not be effectively killed with dabrafenib treatment.[12,13] Advancements in immunotherapy and targeted therapy research hold

the promising possibility of eliminating cancer by tailoring a treatment regimen to exploit the genetics of a patient's tumor on an individualized basis. Yet, there is a caveat; a primary cause of therapy failure and tumor relapse is the development of acquired drug resistance[7,16] (Figure 1).

In a course of highly effective anti-cancer therapy, most cancer cells will die during treatment, while a subpopulation survives.[16] Cancer persister cells survive drug treatment through poorly understood non-genetic mechanisms.[15] Despite an initial sensitivity to therapy, persister cell populations possess acquired drug-tolerance and proliferate in conditions that are lethal to most of the cell population. This causes tumor regrowth and metastasis.[15,17] Metastasis and patient death ensue in the absence of a method to kill drug-tolerant persister cancer cells.[15,16]

Similar to the aspects of antibiotic resistance observed in bacterial cells, the subpopulation of cancer persister cells that initially survive drug treatment do so without developing novel genetic mutations.[15,18] In antibiotic resistant bacteria, there is a so-called bi-phasic response to antibiotic exposure.[16] Most of the bacterial cells die from the drug while a minority persist and survive.[18] Upon isolation of the surviving bacteria, a second exposure to the same drug results in the same bi-phasic result: most of the population dying, a minority surviving.[16] The resistant population becomes re-sensitized to re-exposure of the antibiotic after a period of drug removal, demonstrating the non-genetic, reversible nature of persistence and resistance. This non-genetic, reversible resistance is also seen in persistent cancer cells.[15,16,19] Persister cells are reversibly drug-tolerant to anti-cancer drugs and can be re-sensitized to the same drugs after a period of washout.[15,19] This phenomenon of re-sensitization to available therapies is accounted for in current chemotherapy regimens. Depending on the tumor type, chemotherapy schedules are often divided into treatment periods and rest periods.[20] However, persister cells are highly adaptive, exhibit decreased proliferation rates compared to drug-naïve,

non-persister cells (parental cells), and utilize varied survival mechanisms. These characteristics make it difficult to completely eliminate persister cells (aka minimal residual disease) from a patient.[16] Understanding and obstructing the mechanisms by which drug-tolerant persister cells acquire resistance is one of the most pressing hurdles in cancer research.

Recently, others have uncovered potential persister cell vulnerabilities, characteristics of persister cell physiology or metabolism that can be exploited by therapeutic drugs and chemicals to target and kill a drug-tolerant persister cell population with more precision. In 2010, Sharma and colleagues demonstrated that the dynamic drug-tolerant persister phenotype was chromatin-mediated and dependent on enzymes responsible for histone modifications.[15] They noted the relatively rapid emergence of proliferating drug-tolerant persister cells from lone, drug-sensitive cancer cells. It was found that these drug-tolerant expanding persisters (DTEPs) were vulnerable to a knock-down of histone demethylase, KDM5A, and inhibition of histone deacetylases with HDAC inhibitors.[15] In PC9 (non-small cell lung cancer) persister cells treated with Gefitinib (EGFR inhibitor), an shRNA mediated gene knock-down of KDM5A significantly halted the emergence of DTEP colonies. Additionally, HDAC inhibition also significantly reduced the number of DTEPs that developed.[15] Given the epigenetic nature of acquired drug resistance, as stated above, these findings give support for using drugs that disrupt chromatin regulation as promising strategy for killing persister cells.[15,19]

Furthermore, research into the metabolic plasticity of persister cells has revealed that they are metabolically reliant on fatty acid oxidation (FAO).[16,21] Fatty acid β-oxidation is utilized as an energy source in mitochondrial respiration that yields high amounts of ATP in human breast cancer cells, and elevated expression of fatty acid transporter proteins were found in breast cancer persister cells that were resistant to Lapatinib (HER2 inhibitor).[21,22] Due to the

dependence on mitochondrial respiration and reliance on FAO seen in persister cells, potent anti-oxidant mechanisms are required for persister cell survival and proliferation.[16] Fatty acid oxidation produces hyperoxide species, and one of the many products of mitochondrial respiration are superoxide species.[23,24] Increased rates of mitochondrial respiration, FAO, and therefore elevated production of hyperoxides and superoxides in persister cells creates a need for effective mitigation of intracellular oxidative stress through potent anti-oxidant activity.[16,25] Work done by Hangauer *et al.* demonstrated that in many persister cell types, the harmful effect of endogenous oxidative stress is reduced by glutathione peroxidase 4 (GPX4) activity.[25] The essential role of GPX4 hyperperoxidase activity in defending against oxidative stress made it an auspicious target in identifying persister cell vulnerabilities. The chemicals RSL3 and ML210, GPX4 inhibitors, resulted in increased persister cell death, without inducing lethality in parental cells.[25] The selective killing achieved with GPX4 inhibition, and its effectiveness across persister cells of varying tissue types, give support for further investigation into use as a potential method for killing persister cells without widespread toxicity to healthy cells.[25]

Mechanistically, GPX4 inhibition is lethal to persister cells because abating GPX4 anti-oxidant activity provokes accumulation of lipid reactive oxygen species (ROS), mitochondrial membrane rupture, and oxidative cell death mediated by lipid peroxidation, known as ferroptosis.[26] Demonstrably different from the well-known programmed cell death process of apoptosis, ferroptosis is a more recently discovered form of cell death. Formally defined in 2012 by Dixon *et al.,* ferroptosis is characterized by cellular accumulation of iron and ROS and deformation of the mitochondrial cristae, but does not undergo chromatin condensation or cytoskeletal degradation, making ferroptosis biochemically and morphologically distinct from apoptosis.[26,27] Taken together, persister cells are vulnerable to selectively lethal ferroptosis

induction by chemicals (such as RSL3) that inhibit anti-oxidant mediated cellular self-protection mechanisms.[25,27]

Another potential defense mechanism against ROS in persister cells is aldehyde dehydrogenase (ALDH) activity.[28] The human hepatic system utilizes ALDH, specifically, isoform ALDHA1, to metabolize consumed alcohol. ALDHA1 reacts with ethanol to create acetaldehyde in an oxidative pathway.[29] Raha and colleagues discovered that drug-tolerant persister cells expressed elevated levels of ALDHA1, and proposed that ALDH activity is necessary for drug-tolerance to be maintained in persister cell populations.[28] They found that exposure to Disulfiram, an ALDH inhibitor, eradicated persister cells from gastric cancer and non-small cell lung cancer cell lines (MKN-45, GTL-16, and PC9 respectively).[28] Disulfiram is an FDA approved drug used to treat alcohol addiction.[29] Prescribed under the trade name Antabuse, Disulfiram's chemical inhibition of ALDH halts alcohol metabolism. If a person consumes alcohol while taking Disulfiram, they experience ethanol sensitivity and become violently ill.[30] Though currently prescribed to motivated patients who wish to incentivize themselves to remain sober, there is promising research into repurposing Disulfiram for use an anti-cancer agent to prevent tumor relapse. [28,29]

It has been shown that persister cells can survive drug-induced apoptosis.[31] Previous work has investigated the intracellular components that characterize apoptotic signaling to uncover potential causes of cancer survival and tumor regrowth following treatment. A common mammalian apoptotic pathway begins with mitochondrial outer membrane permeabilization, followed by the release of electron transport chain component, cytochrome c, and activation of caspase 3, an intracellular protease.[32] Until recently, activation of intracellular caspase 3 was considered an irreversible path to cell death. However, research conducted on human HeLa cells

by Sun *et al.* demonstrates that cell survival after caspase 3 signaling is possible through the recovery process, "anastasis."[31] Anastasis, following survival of caspase 3 activation, entails the upregulation of transcription factors and growth-associated genes in cells under the stress of apoptotic signaling. The cell cycle is resumed, and cell proliferation resumes as well. In the context of tumor regrowth following drug treatment, genetic damage caused by sublethal, or insufficient, apoptotic signaling creates a paradoxically opportunistic environment for surviving cells to acquire tolerance to anti-cancer agents.[31]

Above, we have highlighted findings for drug-tolerant persister cell vulnerabilities within the model of non-genetic mechanisms for therapy resistance. Yet, further investigations have generated evidence suggesting that advantageous genetic mutations arise in drug-tolerant persister cell populations.[33] To again draw a parallel to antibiotic resistant bacterial cell subpopulations: when under stress from antibiotic treatment, resistant bacterial cells undergo rapid adaptive mutability (rate of genetic mutation).[34] This stress induced mutagenesis (SIM) is evolutionarily favorable to bacterial populations.[33,34] Research conducted by Russo *et al.* demonstrated that human colorectal cancers (CRC cells) experienced adaptive mutability due to SIM when treated with targeted therapies.[33] Cipponi *et al.* also observed that persister cells had downregulated DNA repair machinery (i.e. homologous repair) and upregulated error-prone DNA polymerases, corroborating similar findings from Russo *et al.*.[35] In mammalian cells, the mechanistic target of rapamycin (mTOR) kinase regulates a plethora of cellular process. The metabolic mTOR pathway controls cell proliferation and apoptosis.[36] Importantly, Cipponi *et al.* discovered that cytostatic drug treatment led to DNA damage, impairment of the mTOR pathway, and subsequent upregulation of error-prone DNA polymerases, supporting the hypothesis that during treatment, persister cells accumulate genetic mutations and could adapt to

anti-cancer agents.[35] The source of DNA damage which causes mutagenesis in persister cells during drug treatment is currently unknown.

Herein, we highlight our investigations into drug-tolerant persister cell vulnerabilities and mutagenesis in the following chapters. Chapter 1: we discovered that co-treatments with HDAC inhibitors and GPX4 inhibitor, RSL3, sensitized persister cells to ferroptosis. Chapter 2: we interrogated the chemical mechanism by which Disulfiram kills persister cells. Chapter 3: we addressed the open question of the origin of DNA damage persister cells sustain during drug-treatment and discovered a possible apoptotic source of persister cell mutagenesis.

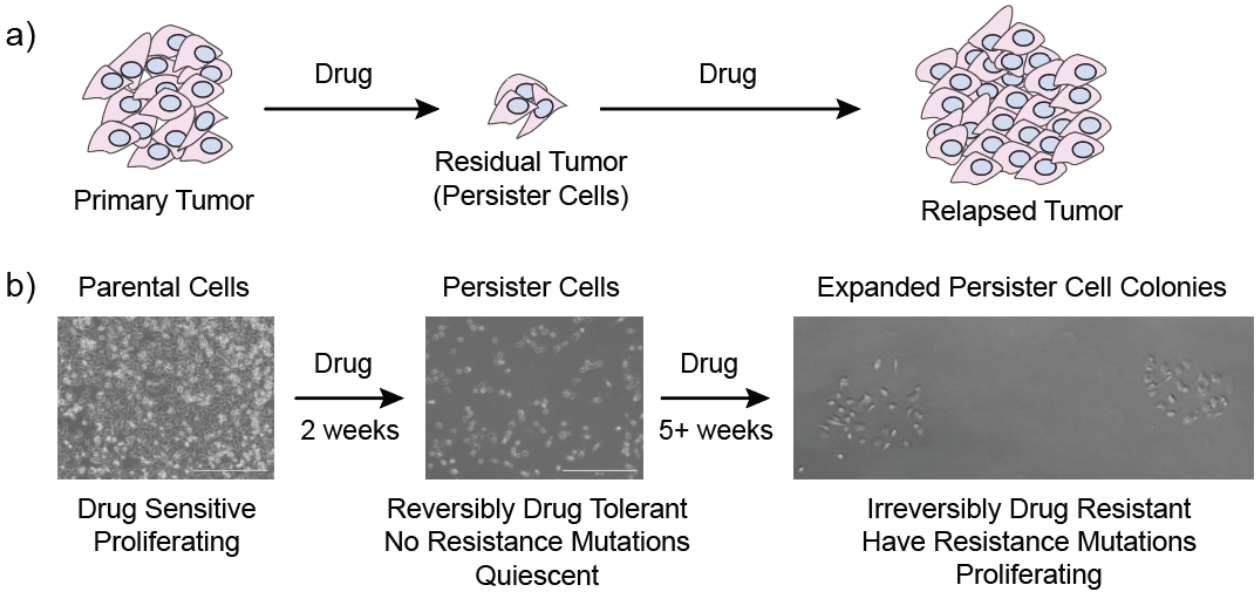

Figure 1. Persister cells survive treatment and seed tumor regrowth. a) Cartoon schematic of initial tumor response to treatment, formation of persister cells, and subsequent regrowth of drug resistant cancer cells. b) Cell culture model of acquired drug resistance in A375 BRAF V600E human melanoma cells cotreated with BRAF inhibitor Dabrafenib (250 nM) and MEK inhibitor Trametinib (25 nM). Persister cells remain alive after two weeks of cytotoxic drug treatment, and a subpopulation of persister cells regrow into expanded persister cell colonies upon further extended drug exposure.

CHAPTER 1: Discovery of HDAC Inhibitor Persister Cell Sensitization to Ferroptosis

It is known that persister cells are vulnerable to a knock-down or inhibition of chromatin regulatory enzymes, histone demethylases and histone deacetylases.[15] Our lab also performed an siRNA screen (Figure 2) and chemical screen (Figure 3) to identify additional persister cell vulnerabilities[25]. Additionally, though anti-oxidant and GPX4 activity are necessary defense mechanisms operative in drug-tolerant persister cells, persister cells have lower anti-oxidant and GPX4 cofactor (glutathione) activity compared to parental, non-persister cells.[25] This vulnerability may underly persister cell sensitivity to ferroptosis. Other studies demonstrated HDAC inhibitor, Vorinostat, has the capacity to suppress SLC7A11, a gene that codes for a cystine-glutamate transporter protein.[37,38] SLC7A11 protects against accumulation of ROS; therefore, it is an anti-ferroptosis gene, and is overexpressed in many human cancers.[38] While HDAC inhibition was shown to suppress anti-ferroptosis genes, such as SLC7A11, and increase ROS accumulation in cancer cells[37], it was not known if HDAC inhibitors (HDACi) sensitized persister cells to induction of ferroptosis.

We considered the possibility of utilizing synthetic lethality to simultaneously target more than one persister cell vulnerability as a more precise approach to inducing persister cell death. Synthetic lethality is cell death caused by a combination of inhibitory drugs, RNA interference, or deleterious mutations, where each agent is non-toxic or not sufficiently lethal when acting alone.[39] Therefore, we performed a drug synergy screen in persister cells which revealed HDACi synergize with ferroptosis inducer, RSL3, a GPX4 inhibitor (GPX4i) (Figure 3). In recent years, the concept of drug synergy has become a promising strategy for treating cancer more effectively.[39,40] Drugs are said to be synergistic if the two are more effective when used as a combination treatment than the individual effects of each one alone.[41] We therefore

further explored whether HDACi treatment sensitizes drug-tolerant persister cells derived from various cell lines to ferroptosis.

MATERIALS AND METHODS:

Cell Culture: A375 (BRAF V600E mutant) melanoma cells (purchased from ATCC) were grown in DMEM (ThermoFisher Scientific) media with 10% fetal bovine serum (ThermoFisher Scientific) and 1% antibiotic-antimycotic (ThermoFisher Scientific). To derive persister cells, A375 cells were seeded at 4000 cells per well in a 96-well plate. Cells were then treated with media containing 10 nM Dabrafenib (BRAF inhibitor) (Selleck Chemicals) 1 nM Trametinib (MEK inhibitor) (ApexBio) for two weeks. To make a parental cell population, A375 cells were seeded at 8000 cells per well in a 96-well plate and received no Dabrafenib/Trametinib treatment. A375 persister cells and A375 parental cells were co-treated for 24 hours with increasing concentrations of HDACi, LBH589 (Cayman Chemical) (0 nM – 150 nM) and GPX4i, RSL3 (Sigma Aldrich) (0 nM – 150 nM).

BT474 (HER2 L755S mutant) breast cancer cells (ATCC) were grown in RPMI (ThermoFisher Scientific) media with 10% fetal bovine serum and 1% antibiotic-antimycotic. To derive persister cells, BT474 cells were seeded at 5500 cells per well in a 96-well plate and treated with media containing 2 μM Lapatinib (HER2 inhibitor) (Sigma Aldrich) for 10 days. To make a parental cell population, BT474 cells were seeded at 3000 cells per well in a 96-well plate and received no Lapatinib treatment. BT474 persister cells and BT474 parental cells were co-treated for 24 hours with increasing concentrations of LBH589 (0 nM – 100 nM) and RSL3 (0 nM – 50 nM). BT474 persister cells and BT474 parental cells were also co-treated for 24 hours with increasing concentrations of another HDACi, Vorinostat (ThermoFisher Scientific), and

RSL3: Vorinostat (0 nM – 2500 nM), RSL3 (0nM – 50 nM). Cell media was refreshed every 3 days and cell dishes/ plates were stored in an incubator with 5% CO_2 atmosphere at 37°C.

Cell Viability: We used the CellTiter-Glo Luminescent Cell Viability assay (CTG) to determine relative cell viability following the HDACi and RSL3 24-hour co-treatments. 100 µL of CellTiter-Glo Buffer/Substrate mixture was added to each of the experimental wells. Luminescence was measured with a SpectraMax iD3 with the software SoftMax Pro. Higher luminescence is associated with higher cell viability. The CellTiter-Glo kit was purchased from Promega.

Synergy: Drug synergy was measured using the website SyngeryFinder 2 developed by Ianevski and colleagues (https://synergyfinder.fimm.fi).[40] We used the matrix format and the Bliss test of independence to obtain a synergy score. Positive synergy scores indicate synergy between the tested chemicals, whereas negative synergy scores indicate a buffering effect rather than synergy.

RESULTS:

Positive synergy between LBH589 and RSL3 was seen in A375 persister cells (Bliss synergy score: 9.32) (Figure 4b). Following the same co-treatment, weak positive synergy was observed in the A375 parental cells (Bliss synergy score: 1.53) (Figure 4d). The BT474 persister cell co-treatment of LBH589 and RSL3 yielded the highest Bliss synergy score across all cell lines (24.36) (Figure 5b). The LBH589 and RSL3 co-treatment was not synergistic in the BT474 parental cells with a Bliss synergy score of -0.62 (Figure 5d). In the Vorinostat and RSL3 co-

treatment in BT474 persister cells, we saw moderate positive synergy (Bliss synergy score: 8.12) (Figure 6b). We saw the lowest synergy score across all cell lines in the Vorinostat and RSL3 co-treatment of BT474 parental cells (Bliss synergy score: -14.05) (Figure 6d). Taken together, HDACi and RSL3 co-treatments are consistently synergistic in persister cells from different tumor types and drug treatments, but not synergistic in parental cells. Therefore, addition of HDACi sensitizes persister cells, but not parental cells, to ferroptosis induction by RSL3.

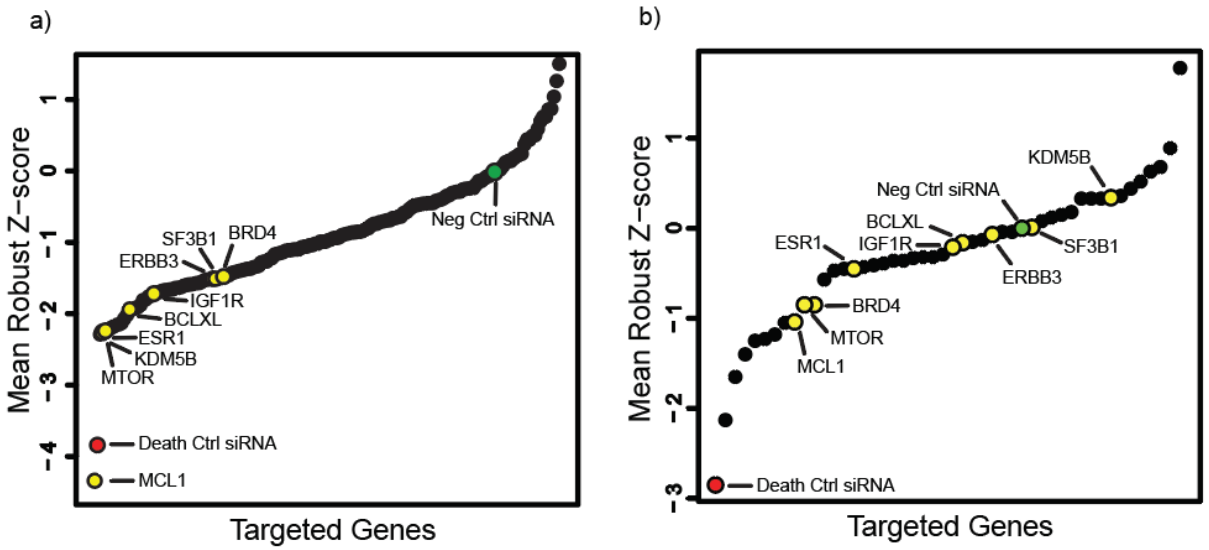

Figure 2. Discovery of persister cell vulnerabilities. a) SiRNA screen in HER2 amplified BT474 human breast cancer persister cells derived from 9 days of treatment with 2 μM HER2 inhibitor Lapatinib followed by siRNA transfection and CellTiter Glo assay after 5 additional days. Median absolute deviation Z-scores (Robust Z-score) were calculated for each siRNA replicate and all siRNA replicate z scores for all siRNAs targeting each gene (up to 6 siRNAs per gene) were averaged for each gene and plotted. Z score < 0 indicates cell death relative to the negative control siRNA. b) SiRNA counter-screen in selected genes in parental BT474 cells.

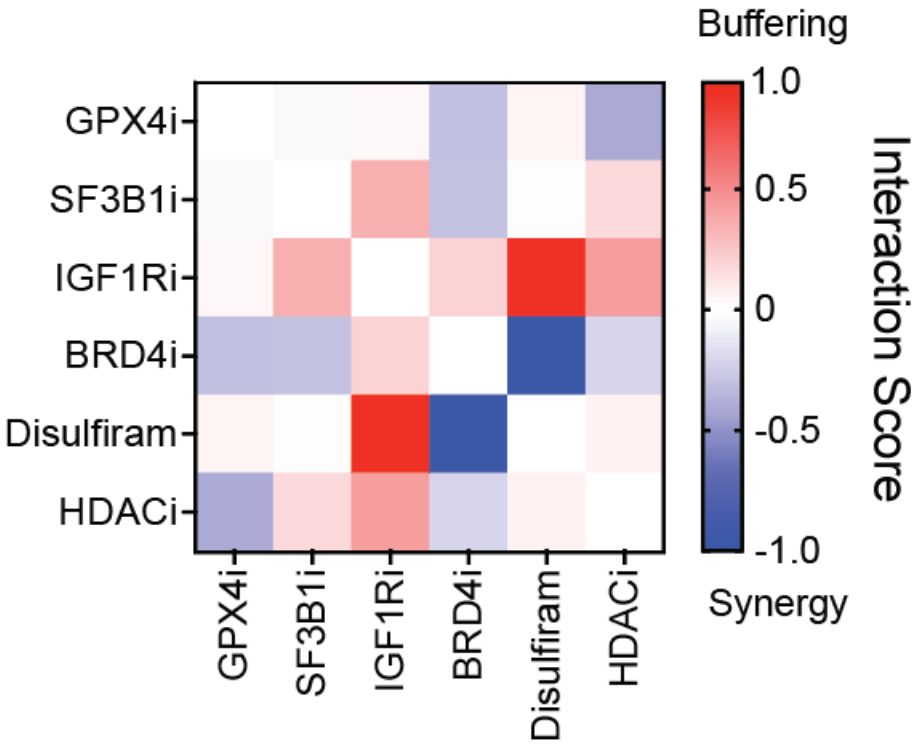

Figure 3. GPX4 inhibition synergizes with HDACi to kill BT474 persister cells. Heatmap of drug synergy screen. BT474 persister cells derived from 9 days of treatment with 2 μM Lapatinib were treated with combinations of inhibitors at IC50 concentrations in duplicate wells and cell viability was measured with CellTiter-Glo. Interaction scores were calculated for each chemical inhibitor combination using deviation of cotreatment luminescence (observed) from the quadratic fit of both single phenotypes (expected).[52] Blue squares indicate synergistic persister cell killing and red squares indicate less killing than expected (buffering).

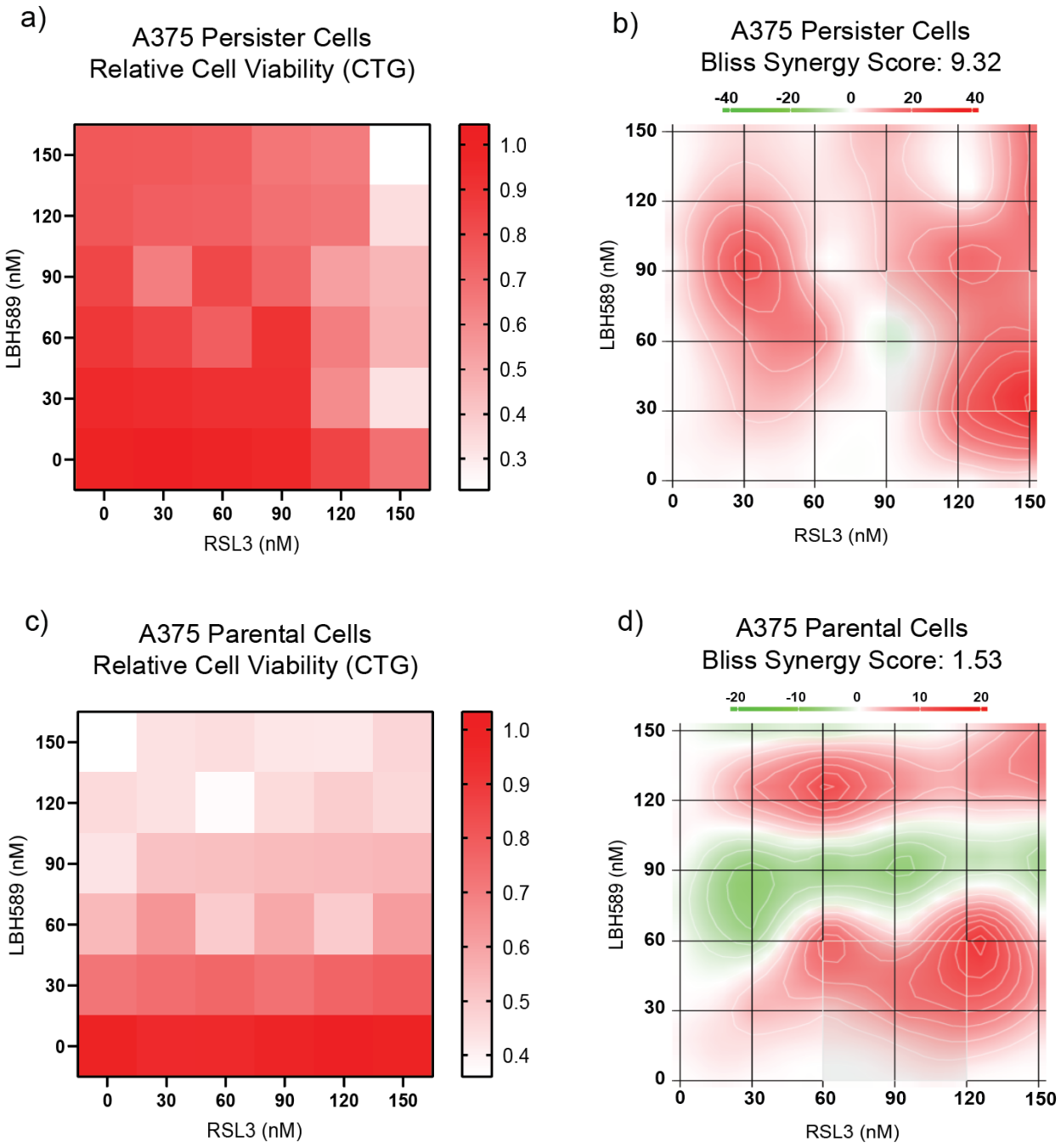

Figure 4. LBH589 partially sensitizes A375 persister cells to ferroptosis. 24-hour co-treatments performed on A375 persister and parental cells. Co-treatments were performed with various concentrations of HDACi, LBH589 and GPX4 inhibitor, RSL3. Relative CTG values are plotted on the heatmaps. a) LBH589 sensitized A375 persister cells to ferroptosis. b) Bliss Synergy Score: 9.32, indicating moderate positive synergy. c) LBH589 did not sensitize parental cells to ferroptosis. d) Bliss Synergy Score: 1.53, indicating weak synergy. Synergy scores were calculated using SyngeryFinder 2[40].

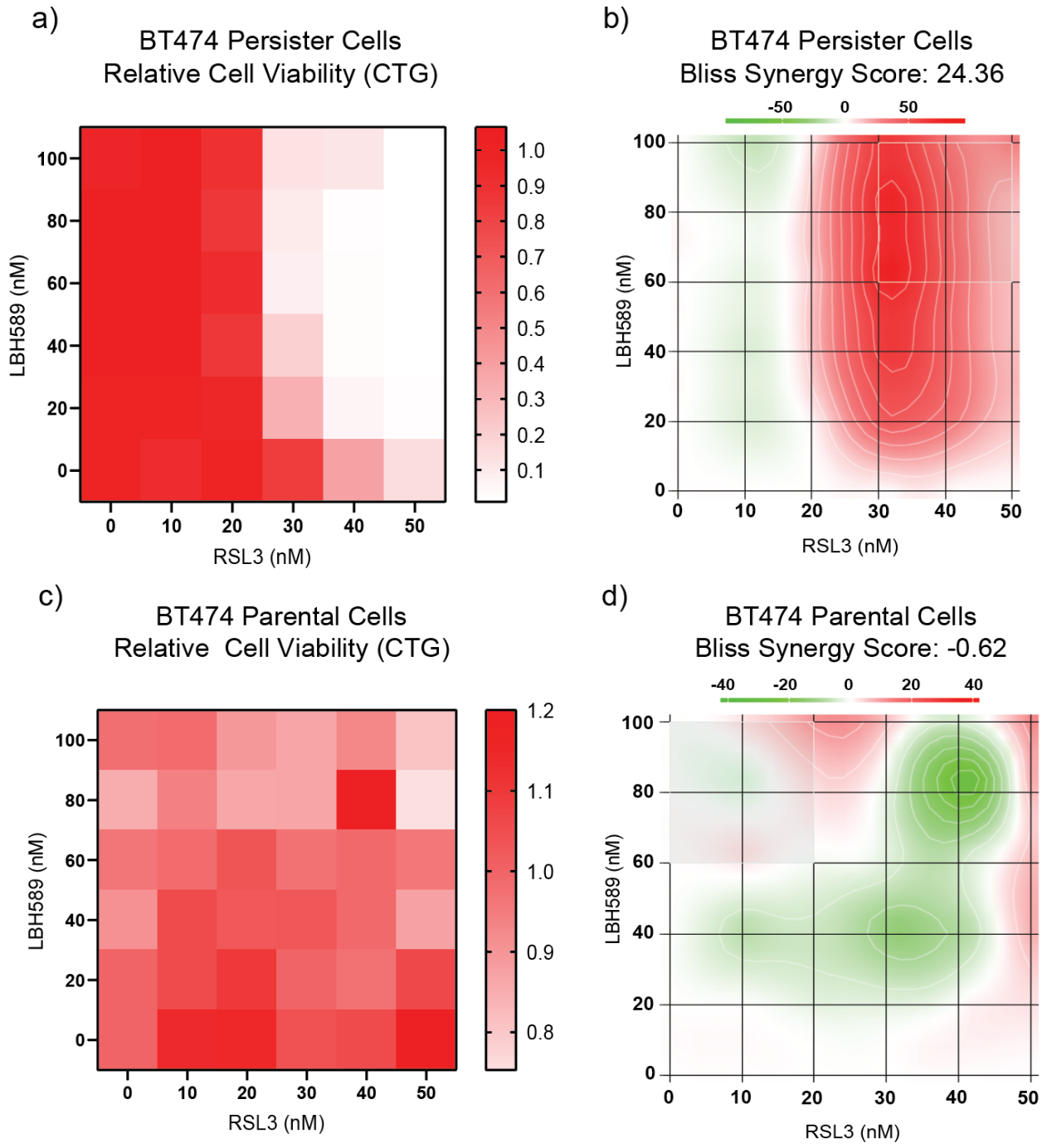

Figure 5. LBH589 sensitizes BT474 persister cells to ferroptosis. 24-hour co-treatments performed on BT474 persister and parental cells. Persister cells were pre-derived through 10-day treatment with 2 μM Lapatinib. Parental cells received no drug treatment. Co-treatments were then performed with various concentrations of HDACi, LBH589 and GPX4 inhibitor, RSL3. Relative CTG values are plotted on the heatmaps. a) LBH589 sensitized BT474 persister cells to ferroptosis. b) Bliss Synergy Score: 24.36, indicating strong positive synergy. c) LBH589 did not sensitize BT474 parental cells to ferroptosis. d) Bliss Synergy Score: -0.62, indicating lack of synergy. Synergy scores were calculated using SyngeryFinder 2[40].

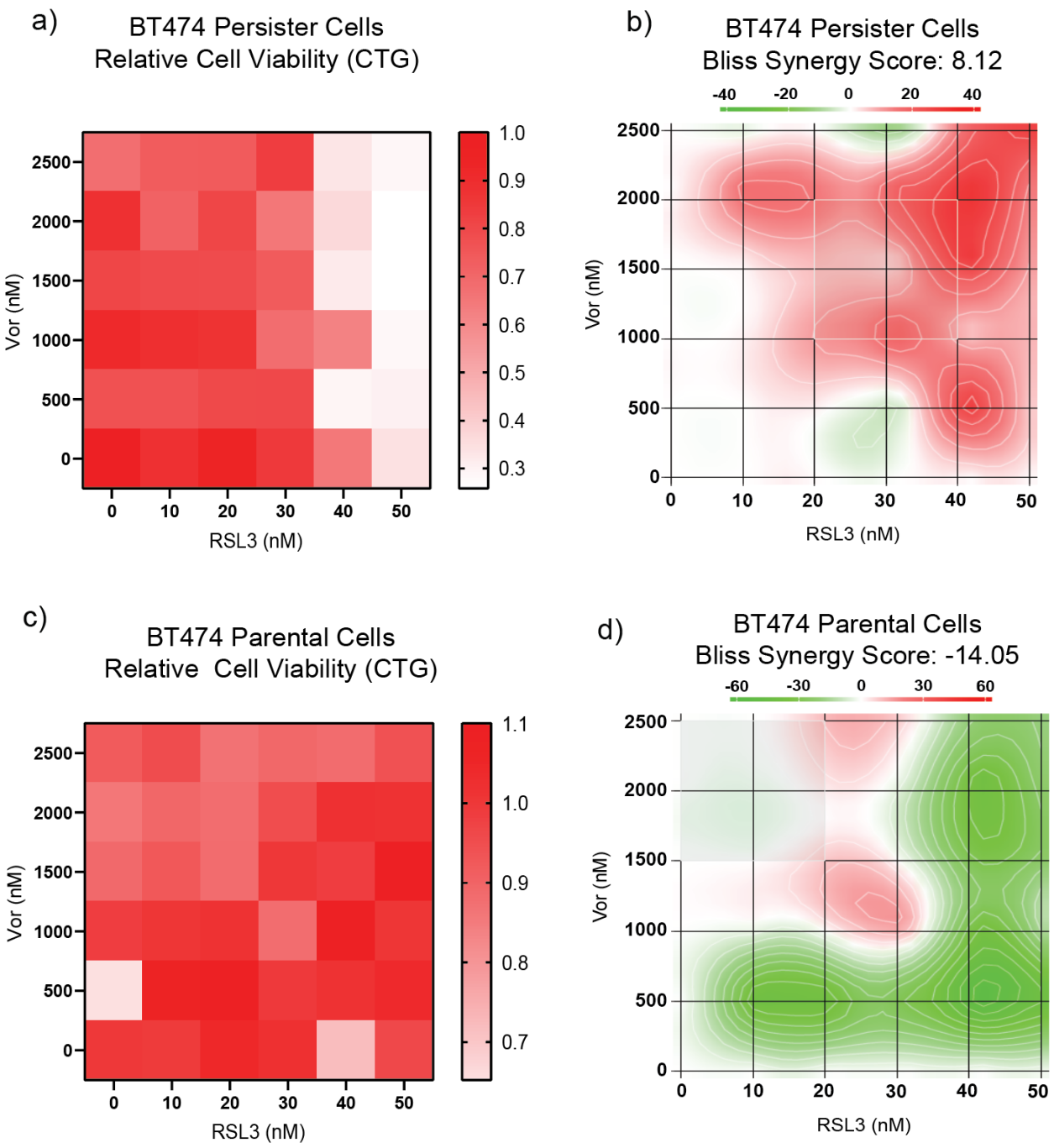

Figure 6. Vorinostat sensitizes BT474 persister cells to ferroptosis. 24-hour co-treatments performed on BT474 persister and parental cells. Persister cells were pre-derived through 10-day treatment with 2 μM Lapatinib. Parental cells received no drug treatment. Co-treatments were then performed with various concentrations of HDACi, Vorinostat (Vor), and GPX4 inhibitor, RSL3. Relative CTG values are plotted on the heatmaps. a) Vor sensitized BT474 persister cells to ferroptosis. b) Bliss Synergy Score: 8.12, indicating moderate positive synergy. c) Vor did not sensitize BT474 parental cells to ferroptosis. d) Bliss Synergy Score: -14.05, indicating buffering rather than synergy. Synergy scores were calculated using SyngeryFinder 2[40].

CHAPTER 2: Understanding the Mechanism of Persister Cell Killing by Disulfiram

Disulfiram is an FDA approved drug used to treat alcoholism. As an ALDH inhibitor, Disulfiram prevents ethanol metabolism and causes vomiting in patients who drink alcohol while taking the drug.[29] Recent studies have shown that Disulfiram may prove to be an effective anti-cancer agent, either as a therapeutic for tumor relapse or as a preventive measure to obstruct the development of drug resistance.[28] Currently, there is inconclusive evidence surrounding Disulfiram's mechanism of action. Raha *et al.* found that inhibition of ALDH activity with Disulfiram eliminated the emergence of drug-tolerant persister cell populations, and that ALDH activity serves as a protection against aldehyde by-products from lipid peroxidation and intracellular ROS accumulation.[28] However, Raha and colleagues were not able to determine the specific ALDH gene that was inhibited by Disulfiram to induce death in drug-tolerant persister cells, leaving the possibility that ALDH-independent mechanisms are also important.[28] In an effort to detail a clearer mechanism, additional research has demonstrated that downstream metabolites of Disulfiram acted as the inhibitors of ALDH.[42] Skrott *et al.* found that Disulfiram's anti-cancer activity is the result of targeting NPL4, a mitotic regulatory co-factor.[42] Disulfiram directly induces aggregation of NPL4 and death in adenocarcinoma cells (A549).[42]

Given the inconclusive and conflicting data seen in the current literature on Disulfiram, we investigated the chemical mechanism by which Disulfiram kills drug-tolerant persister cells. Disulfiram inhibits ALDH leading to an accumulation of intracellular ROS, pointing to the possibility that Disulfiram induces ROS-dependent apoptosis or ferroptosis. Disulfiram is a disulfide linked molecule that can react non-specifically with cysteine residues and other free thiols in cells; thus, we hypothesized that Disulfiram likely has a pleiotropic mechanism of action that may be specific to context or persister cell type.

MATERIALS AND METHODS:

Cell Culture: PC9 (EGFR T790M mutant) non-small cell lung cancer cells (provided by the Altschuler and Wu labs at UCSF) were grown in RPMI media with 5% fetal bovine serum and 1% antibiotic-antimycotic. PC9 persister cells were derived by seeding 1800 cells per well in a 96-well plate and treating with media containing 2.5 μM Erlotinib (EGFR inhibitor) (Selleck Chemicals) for 10 days. PC9 persister cells were then used to conduct 24-hour treatments or co-treatments with Disulfiram (Sigma Aldrich); active metabolite of Disulfiram, diethyldithiocarbamate (DDC) (Sigma Aldrich); various antioxidants at non-toxic concentrations; Staurosporine (Research Products International), an apoptosis inducer; and Q-VD-OPh (QVD) (MedKoo), a caspase inhibitor.

BT474 (HER2 L755S mutant) breast cancer cells were grown in RPMI media with 10% fetal bovine serum and 1% antibiotic-antimycotic. BT474 persister cells were derived by seeding 5500 cells per well in a 96-well plate and treating with media containing 2 μM Lapatinib (HER2 inhibitor) for 10-11 days. BT474 persister cells were then used to conduct 24-hour treatments or co-treatments with Disulfiram; RSL3; Ferrostatin-1 (ApexBio), a ferroptosis inhibitor; Liproxstatin (Sigma Aldrich), a ferroptosis inhibitor; DDC; and various antioxidants at non-toxic concentrations. Cell media was refreshed every 3 days and cell dishes/ plates were stored in an incubator with 5% CO_2 atmosphere at 37°C.

All antioxidants used, Nordihydroguaiaretic acid (NDGA), Alpha-tocopherol (A-toc), and Ethylbisiminomethylguaiacol manganese chloride (EUK) were purchased from Sigma Aldrich. Dimethyl sulfoxide (DMSO) was purchased from Invitrogen.

Cell Viability: CellTiter-Glo Luminescent Cell Viability assay (CTG) was used to determine relative cell viability. 100 μL of CellTiter-Glo Buffer/Substrate mixture was added to each of the experimental wells. Luminescence was measured with a SpectraMax iD3 with the software SoftMax Pro. Higher relative luminescence is associated with higher cell viability.

RESULTS:

Disulfiram has been previously reported to kill cancer cells via apoptosis.[28] Consistent with this, we found that Disulfiram mediated killing of PC9 persister cells was prevented by pan-caspase inhibitor QVD treatment (Figure 7). However, we have observed irreproducible results in other persister cell types and are continuing to explore whether Disulfiram mediated persister cell killing requires caspase activity.

We also found that Disulfiram-mediated persister cell death is not ferroptotic. In BT474 persister cells, ferroptosis inhibitors, Ferrostatin-1 and Liproxstatin did result in rescue from Disulfiram induced death because there was no significant difference in relative cell viability between the Disulfiram only conditions and Disulfiram co-treated with Ferrosatin-1 or Liproxstatin (Figure 8c, 8d). Also, PC9 and BT474 persister cells were not rescued from Disulfiram killing by administration of Nordihydroguaiaretic acid (NDGA) (Figure 9a, 9d). Yet, the antioxidant, Alpha-tocopherol (Vitamin E) yielded a partial rescue from Disulfiram induced death in both PC9 and BT474 persister cells (Figure 9b, 9e). Antioxidant, Ethylbisiminomethylguaiacol manganese chloride (EUK) gave mixed results when co-administered with Disulfiram: PC9 persister cells were rescued from death, whereas BT474 persister cells were not (Figure 9c, 9f).

Disulfiram contains a disulfide linkage which is chemically broken down into two molecules of Diethyldithiocarbamate (DDC). DDC has been reported to be a functionally active metabolite of Disulfiram.[29] We found that NDGA rescues PC9 and BT474 persister cells from DDC-induced death (Figure 10b, 10e) and that EUK rescues PC9 persister cells from DDC (Figure 10c). These results support a role for DDC in Disulfiram-mediated oxidative persister cell death, which is, in some contexts, apoptotic but is not ferroptotic. These context dependent findings support a pleiotropic mechanism of thiol-containing Disulfiram and DDC whereby nonspecific chemical reactions with free thiols in persister cells results in cell death through multiple mechanisms which are specific to each persister cell type or treatment context.

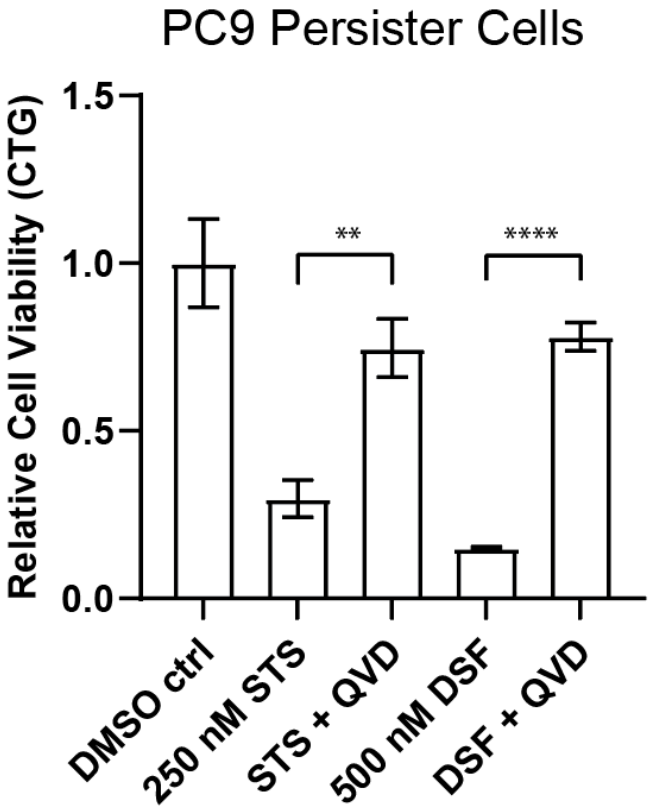

Figure 7. Disulfiram induced persister cell death is rescued by caspase inhibition. 24-hour chemical treatments performed on PC9 persister cells. Staurosporine (STS) served as the positive apoptotic death control. QVD rescues persister cells from apoptosis by caspase inhibition. QVD also rescued persister cells from death when co-administered with Disulfiram (DSF). Mean CTG values expressed relative to DMSO (dimethyl sulfoxide) control. Error bars represent standard deviation. Statistical significance determined by two-tailed t-test: *P≤0.05, **P≤0.01, ***P≤ 0.001, ****P≤0.0001. n=3.

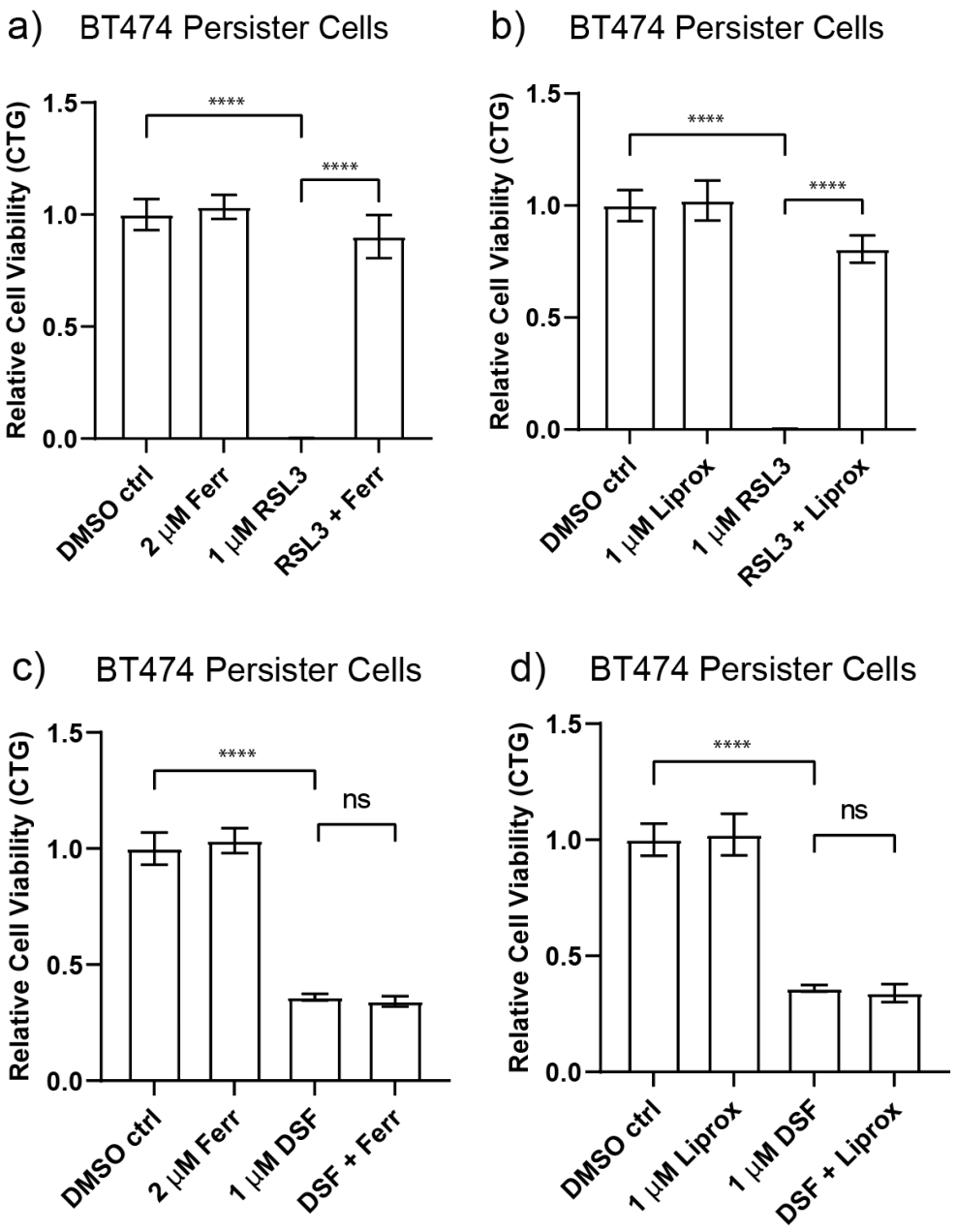

Figure 8. Disulfiram induced persister cell death is not ferroptotic. 24-hour chemical treatments performed on BT474 persister cells. a) RSL3-induced cell death was rescued by ferroptosis inhibitor, Ferrostatin-1 (Ferr). b) RSL3-induced cell death was rescued by ferroptosis inhibitor, Liproxstatin (Liprox). c) Disulfiram (DSF)-induced cell death was not rescued by ferrostatin. d) DSF induced cell death was not rescued by liproxstatin. Mean CTG values expressed relative to DMSO (dimethyl sulfoxide) control. Error bars represent standard deviation. Statistical significance determined by two-tailed t-test: *P≤0.05, **P≤0.01, ***P≤ 0.001, ****P≤0.0001. n=3.

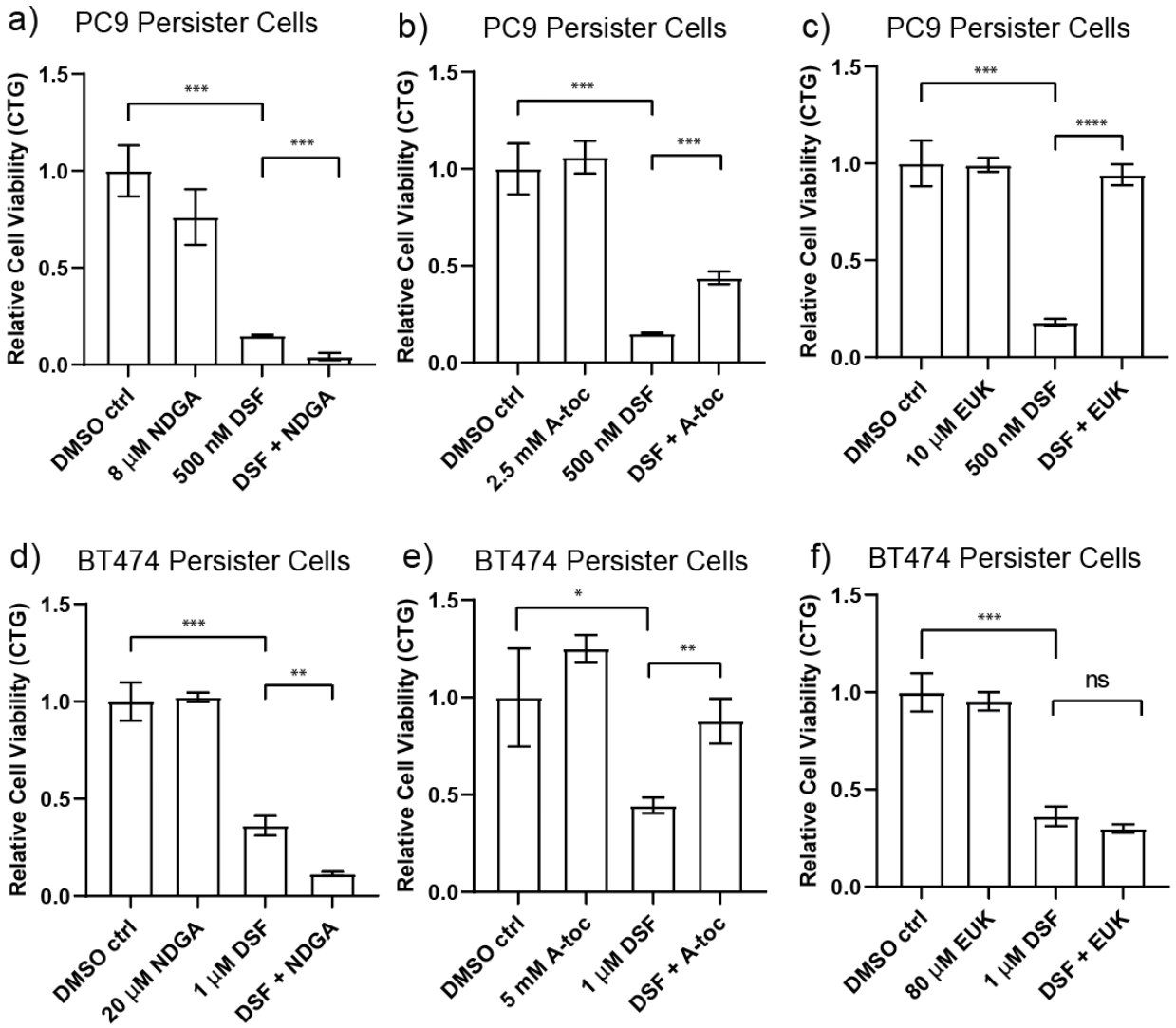

Figure 9. Antioxidant persister cell rescue from Disulfiram treatment is dependent on cell type. (a-c) 24-hour chemical treatments performed on PC9 persister cells. (d-f) 24-hour chemical treatments performed on BT474 persister cells. Non-toxic concentrations of antioxidants Nordihydroguaiaretic acid (NDGA), Alpha-tocopherol (A-toc), and Ethylbisiminomethylguaiacol manganese chloride (EUK) were co-administered with DSF to test whether DSF-mediated persister cell death is rescued by antioxidants. a) NDGA does not rescue PC9 persister cells from DSF-induced death. b) A-toc partially rescues PC9 persister cells from Disulfiram-induced death. c) EUK completely rescues PC9 persister cells from DSF killing. D) NDGA does not rescue BT474 persister cells from DSF-induced death. e) A-toc partially rescues BT474 persister cells from DSF. f) EUK does not rescue BT474 persister cells from DSF. Mean CTG values expressed relative to DMSO (dimethyl sulfoxide) control. Error bars represent standard deviation. Two-tailed t-test used to determine statistical significance: *P≤0.05, **P≤0.01, ***P≤ 0.001, ****P≤0.0001. n=3.

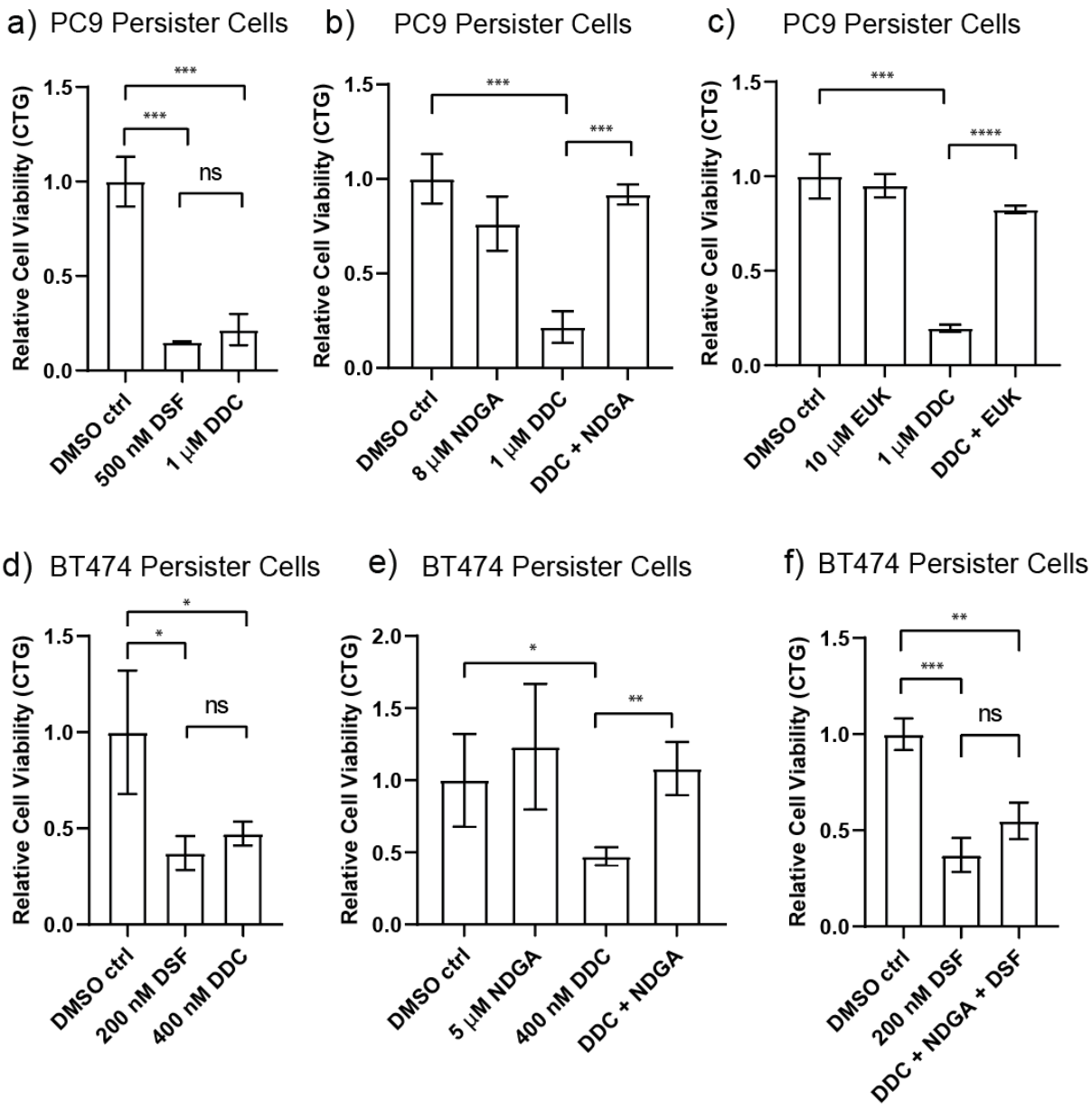

Figure 10. NDGA rescues persister cells from death induced by DDC. (a-c) 24-hour chemical treatments performed on PC9 persister cells. (d-f) 24-hour chemical treatments performed on BT474 persister cells. One DSF molecule is metabolized into two Diethyldithiocarbamate (DDC) molecules. a) Twice the concentration of DDC is sufficient for inducing the same degree of cell death as DSF. b) DDC- mediated killing of PC9 persister cells is rescued by NDGA. c) DDC-mediated killing of PC9 persister cells is partially rescued by EUK. d) Twice the concentration of DDC is sufficient for inducing the same degree of cell death as DSF. e) DDC- mediated killing of BT474 persister cells is rescued by NDGA. f) NDGA rescue of DDC induced BT474 persister cell death is reversed by introduction of DSF. Mean CTG values expressed relative to DMSO (dimethyl sulfoxide) control. Error bars represent standard deviation. Two-tailed t-test used to determine statistical significance: *P≤0.05, **P≤0.01, ***P≤ 0.001, ****P≤0.0001. n=3.

CHAPTER 3: Characterizing Persister Cell Mutagenesis

Persister cells initially survive anti-cancer therapy through non-genetic mechanisms.[15] However, recent research has revealed that established drug-tolerant persister cell populations sustain drug-induced DNA damage aided by downregulation of DNA repair genes and upregulation of error-prone DNA polymerases.[16,31,33,35] The improper repair of damaged DNA by error-prone DNA polymerases leads to subsequent mutagenesis and genetic adaptation to the anti-cancer agent.[33,35] The source of the initial DNA damage remains unknown. We hypothesize that drug stress induces sublethal, or incomplete, apoptotic signaling in persister cells, resulting in apoptotic DNase-mediated DNA damage and mutagenesis.

Apoptotic DNase, DNA fragmentation factor B (DFFB) is activated by caspase 3. Coded by the *DFFB* gene, DFFB requires its inhibitor and chaperone, DNA fragmentation factor A (DFFA), for nuclear localization.[43] When apoptosis is initiated, caspase 3 is cleaved (activated), which then cleaves DFFA, releasing DFFB to form an active homodimer.[43] Active DFFB causes double stranded breaks (DSBs) and chromosomal destabilization during apoptotic progression. The DFFB homodimer degrades genetic material, ultimately causing cell death[43,44] (Figure 12).

Survival of sublethal apoptotic signaling and DFFB-mediated mutagenesis is not unprecedented in cancer cells. For example, Sun *et al.* demonstrated that drug-naïve cancer cells can survive sublethal caspase 3 signaling through anastasis (a novel cellular recovery process), and Xiong *et al.* illustrated that sublethal caspase 3 activation results in DNA damage by DFFB.[32] Additionally, also in drug-naïve cancer cells, the "self-inflicted" DNA damage by DFFB has been shown to increase genetic diversity and tumorgenicity.[44] However, it is currently unknown whether DFFB is activated in persister cells, causes persister cell DNA damage and mutagenesis, and is required for persister cell re-growth and proliferation.

Our lab has observed sublethal apoptotic signaling in persister cells. Through western blotting, we have demonstrated that caspase 3 is cleaved, DFFA is cleaved, and DFFB is activated in A375 persister cells and DTEPs (Figure 11a, 11c). γH2AX is an intracellular marker for DNA damage, where histone H2A is phosphorylated near the region of damaged genetic material.[35] We observe caspase 3 dependent γH2AX in persister cells and DTEPs. Administration of QVD inhibits caspase 3, and downstream effects, and eliminates γH2AX in persister cells and DTEPs (Figure 11b). Importantly, a CRISPR-Cas9 mediated gene knock-out (KO) of DFFB in A375 persister cells also eliminated the γH2AX signal in persister cells and DTEPs (Figure 11d). Collectively, our preliminary findings indicate that DFFB is activated in drug-tolerant persister cells, and that the DNA damage sustained is DFFB-dependent.

We sought to perform Whole Genome Sequencing (WGS) on drug-tolerant persister cells that are DFFB WT and DFFB KO to quantify and characterize the DFFB-driven mutations in persister cells. To do so, we derived single-cell clonal populations of A375 WT, A375 DFFB KO, PC9 WT, and PC9 DFFB KO cells. Clonal populations were then split into drug-treated and untreated conditions for each cell line. After a single-cell bottle neck, the drug-treated and untreated cells from each of the four cell lines will be condensed into frozen cell pellets and submitted for sequencing (Figure 13).

Our WGS endeavor will be done in collaboration with Professor Ludmil Alexandrov's lab. To better understand cancer mutagenesis, Alexandrov *et al.,* utilized computerized modeling systems to catalog mutations (i.e., nucleotide substitutions, insertions, or deletions) occurring in human cancer genomes.[45] Mutations that were consistently identified in a genetic locus across a cell population were used to create "mutational signatures" that characterized mutagenic processes acting within cancer cells.[45] Using this approach, we will determine if persister cells

contain DFFB-specific mutational signatures. Work toward acquiring single cell bottlenecked persister cells is currently underway.

MATERIALS AND METHODS:

DFFB gene KO with CRISPR-Cas9: CRISPR-Mediated KO of the DFFB gene in A375 and PC9 cells was completed at UC San Francisco core facilities.[25] Successful gene KOs were previously confirmed by western blot. Single guide RNA used for A375: sgRNA 5'-CAGCCCGAGGAAGTTCGGCG-3'. Single guide RNA used for PC9: sgRNA 5'-GCTCCGTGCCATCCTCGTAC-3'.

Single-cell clone derivation for WGS: See Figure 13 for a comprehensive graphical representation. Through the entire process, cell media was refreshed every 3 days. A375 WT and A375 DFFB KO melanoma cells were cultured DMEM media with 10% fetal bovine serum and 1% antibiotic-antimycotic. PC9 WT and PC9 DFFB KO non-small cell lung cancer cells were cultured in RPMI media with 5% fetal bovine serum and 1% antibiotic-antimycotic. Cells from each line were seeded at 1 cell per well in a 384-well plate to derive single cells. After nine days, one single-cell derived clonal colony from each line was expanded and split into 3 10 cm plate conditions, a drug-treated condition, an untreated condition, and a starting reference genome condition that was frozen in liquid nitrogen.

In the treatment conditions A375 WT and A375 DFFB KO cells were seeded at 4000 cells per 10 cm dish and treated with media containing 250 nM Dabrafenib (BRAF inhibitor) and 25 nM Trametinib (MEK inhibitor) for 6.5 weeks; PC9 WT and PC9 DFFB KO cells were seeded at 10,000 cells per 10 cm dish and treated with media containing 2.5 μM Erlotinib (EGFR

inhibitor) for 5 weeks. The untreated conditions underwent the same respective seeding densities and timelines, but received -free, media. The drug-treated cells proliferated slowly and did not become over-confluent. The untreated cells were split once a week to prevent over-confluency. At the end of the treatment schedule, the drug-treated and untreated cells from each cell line were subjected to another single-cell bottle neck to purify each cell genotype. An additional reference genome for each condition and cell line was frozen at this step. As described above, cells were again seeded at 1 cell per well in a 384-well plate, single cells that successfully grew into colonies were monitored. Approximately 10 successfully proliferating cell clones from the drug-treated and untreated conditions of each line were expanded to obtain adequate cells for WGS. We are currently preparing frozen cell pellet samples for shipment and will be submitting them for human WGS via NovoGene in Sacramento, CA.

RESULTS:

After 6.5 weeks of drug treatment, the A375 WT cells grew into several medium to large DTEP colonies across the entire 10cm dish (Figure 14a). However, the A375 DFFB KO persister cells did not form DTEP colonies, consistent with our lab's previous findings that DFFB is required for persister cell regrowth. A375 DFFB KO cells remained as single-cell persisters for the duration of the 6.5-week drug treatment, and had distinctly different morphological features (flat, and sickle shaped) compared to the A375 WT DTEPs (Figure 14b). A similar effect on cell outgrowth was observed in the PC9 persister cells. After 5 weeks of drug treatment, the PC9 WT persister cells proliferated into several dozen large DTEP colonies (Figure 14c). The PC9 DFFB KO persister cells did not form DTEPs and stayed single-cell persister cells for the entire drug

treatment. The PC9 DFFB KO persister cells were flatter and had irregular membrane shapes compared to the PC9 WT DTEP cells (Figure 14d).

We are currently preparing frozen cell pellet samples for shipment and sequencing. We will analyze the WGS data to identify DFFB-specific mutational signatures and quantify the number of DFFB-dependent acquired mutations in persister cell populations. We predict the DFFB KO persister cells will have accumulated significantly fewer mutations than the DFFB WT persister cells over the course of drug-treatment. If our prediction is correct, our results will be a novel demonstration of a DFFB-mediated mutagenic process that drives the acquisition of mutations in cancer persister cells. Also, if we are correct, we will have illuminated the sought-after cause of drug-induced DNA damage that persister cells sustain during treatment (Figure 12).

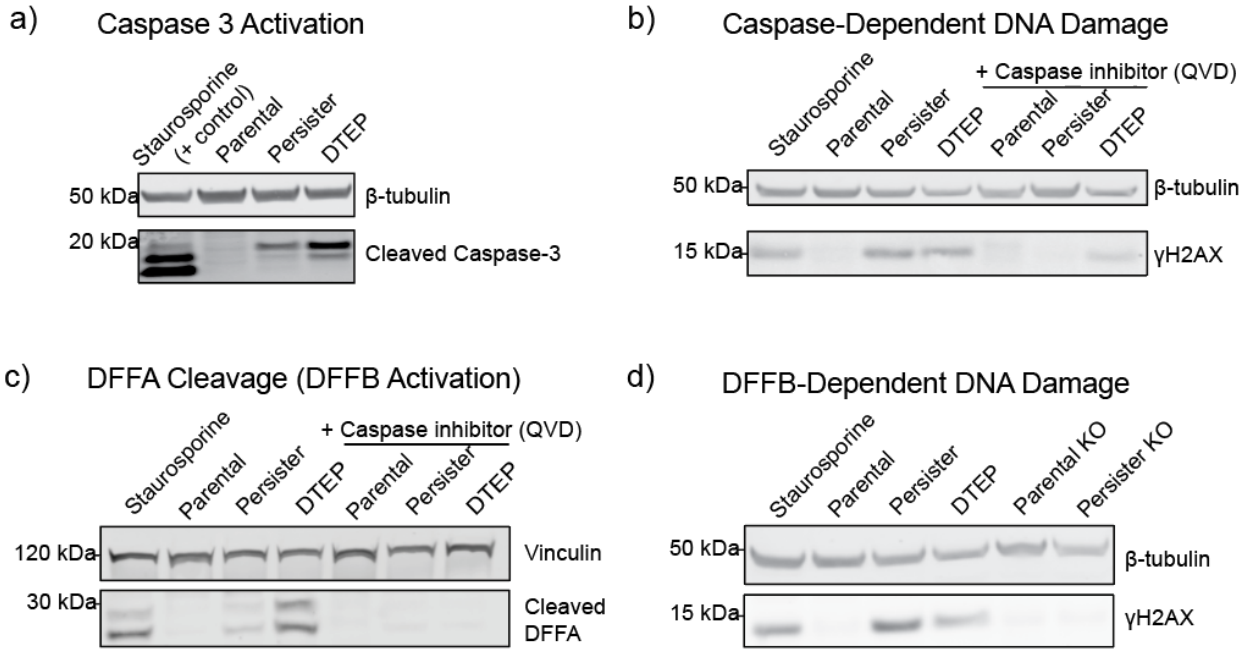

Figure 11. Persister cells undergo DFFB-mediated DNA damage. Western blots performed on A375 persister cells. a) Cleaved caspase 3 band at 20kDa indicates caspase 3 activation in persister cells and DTEPs. b) γH2AX band at 15kDa represents DNA damage. Addition of caspase inhibitor, QVD, notably decreases or eliminates the γH2AX band. c) DFFA cleavage band at 30 kDa is seen in the persister and DTEP lanes. Addition of QVD eliminates cleaved DFFA band. d) γH2AX band at 15kDa disappears upon a DFFB gene knock-out in both parental and persister cells. Loading control: β-tubulin or Vinculin.

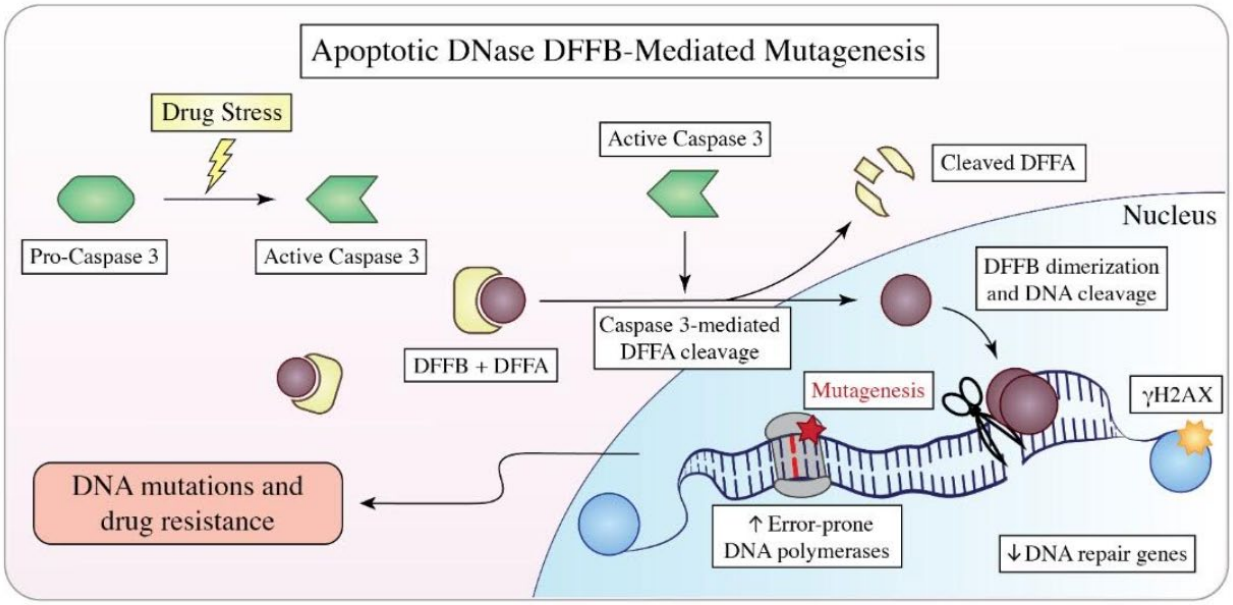

Figure 12. Cartoon depiction of the mechanism of apoptotic DNase DFFB. Drug stress activates the intracellular protease, caspase 3. Caspase 3 cleaves DFFA, allowing DFFB to dimerize. In the nucleus, the DFFB homodimer degrades DNA. γH2AX signal is located near the region of DNA damage. In this process we see a down regulation of DNA repair genes, an upregulation of error-prone DNA polymerases which lead to mutations upon repair of double-stranded breaks (DSB). Improper repair of self-inflicted DSBs by error-prone DNA polymerases is a plausible cause of genetic mutations that endow drug resistance in established drug-tolerant persister cell populations.

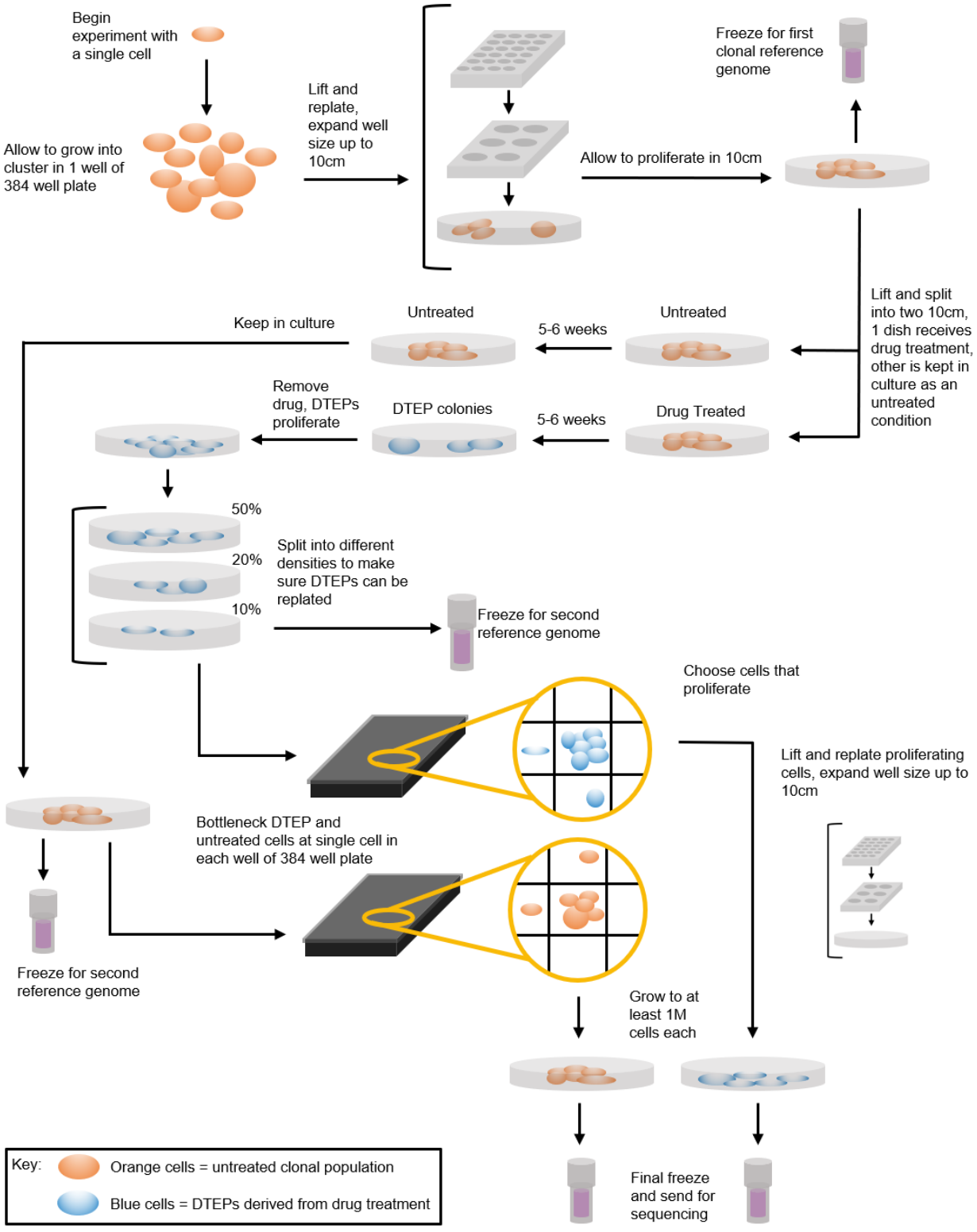

Figure 13. Flow chart for single-cell derived clonal populations of expanded persister cells for investigation of DFFB-mediated mutagenesis.

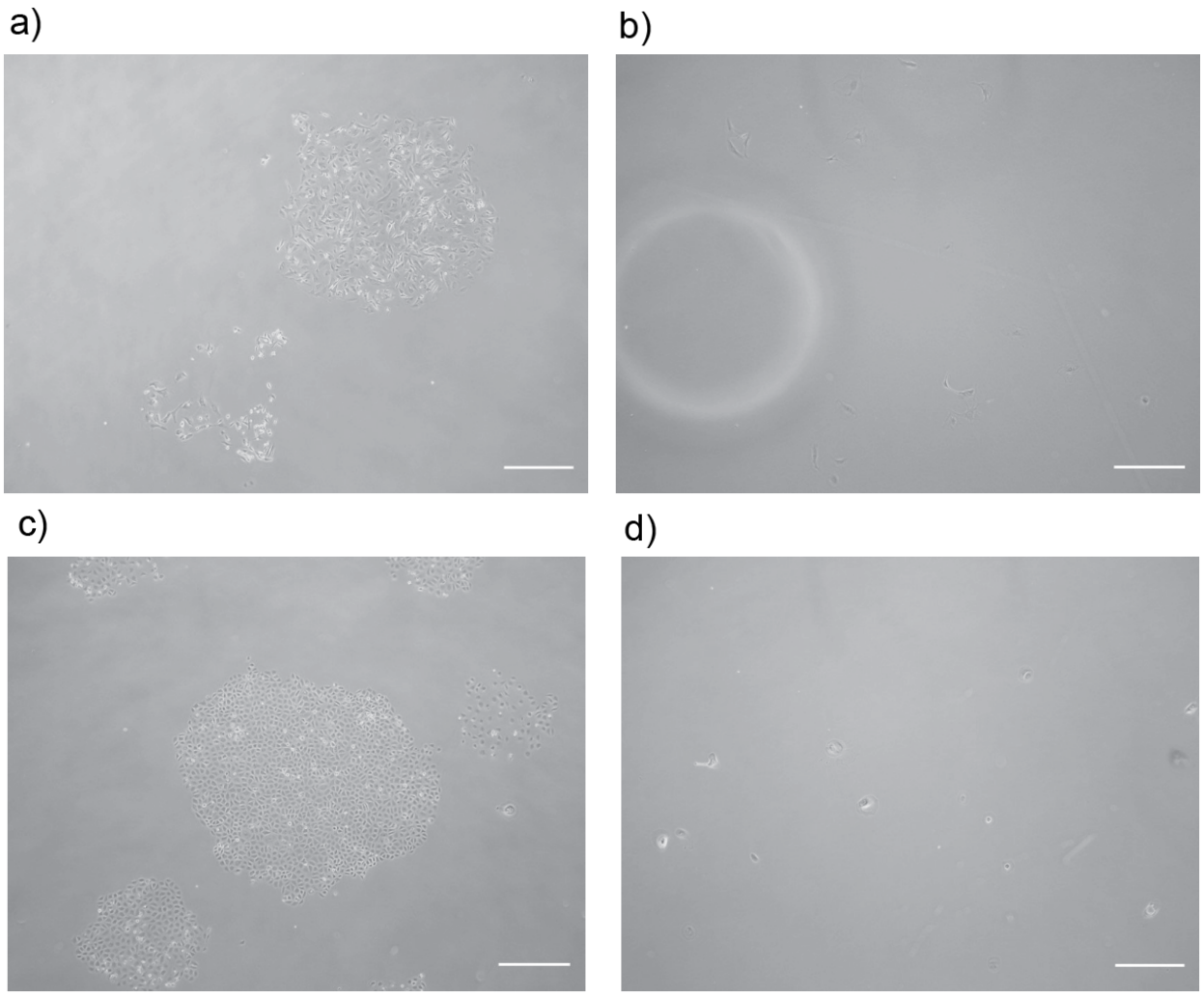

Figure 14. DFFB KO cell lines do not form of large DTEPs during drug treatment. Representative images of all cell lines during drug treatment. (a and b) Images taken after 6.5 weeks of 250 nM Dabrafenib (BRAF inhibitor) and 25 nM Trametinib (MEK inhibitor) treatment. (c and d) Images taken after 5 weeks of 2.5 μM Erlotinib (EGFR inhibitor). a) A375 WT persister cells formed many medium and large DTEP colonies. b) A375 DFFB KO persister cells did not form DTEPs, they remained as single persister cells. c) PC9 WT persister cells formed dozens of large DTEP colonies. d) PC9 DFFB KO persister cells did not form DTEPs, they remained as single persister cells. Images captured on EVOS XL Core Microscope, 4X objective, scale bar = 100 μm.

DISCUSSION:

In Chapter 1, we discovered that HDAC inhibition sensitizes drug-tolerant persister cell populations to ferroptosis. HDAC inhibitors LBH589 and Vorinostat were shown to act in synergy with GPX4 inhibitors RSL3 and ML210 in persister cells. Notably, HDACi co-treated with RSL3 did not synergistically kill parental cells. The mechanism underlying HDACi sensitization of persister cells to ferroptosis remains to be determined. However, as shown in Figure 2, compared to parental cells, persister cells are more vulnerable to siRNA knockdown of KDM5B and BRD4 (Figure 2a). Our siRNA screen findings are similar to previous research by Sharma *et al.* highlighting persister cell proliferation and DTEP formation were ablated upon an shRNA knock-down of KDM5A.[15] KDM5A and KDM5B are histone demethylases, and BRD4 is a bromodomain and extraterminal domain (BET) protein that organizes chromatin by maintaining histone acetylation status throughout the cell cycle.[46,47] It is possible that persister cells are more vulnerable to disruption of chromatin regulation and that HDAC inhibitor treatment affects anti-ferroptosis gene regulation in persister cells (Figure 2). Though the degree of positive synergy between HDACi and RSL3 varied between persister cell types and the HDACi used, our HDACi and RSL3 co-treatments demonstrated this persister cell vulnerability can be exploited without causing increased ferroptosis in parental cells (Figure 4, Figure 5, Figure 6). Therefore, HDACi sensitization of persister cells to ferroptosis could be used to selectively kill drug-tolerant persister cells without causing lethality to other healthy cells.

The HDAC inhibitors we studied are both approved for clinical use in humans to treat cancer. LBH589, also known as Panobinostat, is FDA approved for clinical use in patients experiencing myeloma tumor relapse. LBH589 is sold under the trade name Farydak.[48] Vorinostat, also called SAHA, sold as Zolinza, is FDA approved for treatment of T-cell

lymphoma.[49] Both LBH589 and Vorinostat are pan-inhibitory, making them chemically versatile.[47] Currently, ferroptosis inducers are under study and development for clinical use.[50] Taken together, our discovery indicates very promising potential co-treatment options to treat or perturb tumor relapse in cancer patients in the near future. We have ongoing experiments investigating the effects of HDACi and RSL3 in PC9 (EGFR T790M mutant) non-small cell lung cancer persister cells. We will test LBH589 and Vorinostat co-treated with RSL3 in PC9 persister cells and parental cells. Some future directions include investigating synergy between additional classes of HDACi and RSL3, as well as BET inhibitors (BETi) with RSL3. Strategies to specifically sensitize persister cells to ferroptosis through drug-synergy and synthetic lethality have promising potential for more precise and less destructive cancer treatment options.

In Chapter 2, we discussed the FDA approved drug, Disulfiram. Disulfiram was originally developed to treat alcoholism but has recently been shown to have anti-cancer activity.[28,42] Data in the field are currently inconclusive, and the exact mechanism by which Disulfiram decreases the capacity for drug-tolerance in persister cells is unknown. From our experiments, we found that Disulfiram does not induce persister cell death through ferroptosis (Figure 8). Co-treatment of Disulfiram with known ferroptosis inhibitors was not sufficient to rescue persister cells from death (Figure 8c, 8d). Rather, Disulfiram induces persister cell death through a non-ferroptotic mechanism that is partially rescuable by antioxidants and caspase inhibition, depending on the cell line (Figure 7, Figure 9). Antioxidant rescue from the Disulfiram metabolite, DDC, was also dependent on cell type (Figure 10). Notably, the antioxidant Alpha-tocopherol (Vitamin E) resulted in partial rescues from Disulfiram mediated cell death in both the cell lines tested (Figure 9b, 9e). Interestingly, antioxidant NDGA resulted in a complete rescue from DDC-mediated death in both persister cell types tested (Figure 10b,

10e). NDGA itself has been studied as a potential anti-cancer drug and is a known scavenger of superoxides.[51] In this context, future directions for experiments with Disulfiram, DDC, and antioxidants include exploring the role of superoxides, hydrogen peroxide, and superoxide dismutase in persister cell death. Disulfiram's chemical structure, a disulfide linked molecule, gives it the potential to react with free thiols or cysteine amino acids. DDC, one half of a Disulfiram molecule, has similar properties and high potential to chemically react with multiple intracellular components. Together, these data support a multimodal mechanism of Disulfiram-mediated persister cell killing, but further work is needed to fully decipher the multiple biochemical pathways utilized by Disulfiram to kill persister cells.

In Chapter 3, we sought to identify the mechanism behind drug-induced DNA damage that leads to adaptive mutability and genetically acquired drug-tolerance in persister cells. Building upon the work of others, we hypothesize that persister cells survive sublethal apoptotic signaling, tolerate genetic damage in the form of double stranded breaks (DSBs) induced by DFFB, and undergo mutagenesis when those DSBs are incorrectly repaired by error-prone DNA polymerases.[31,35] Our lab's unpublished, preliminary data give strong support for an apoptotic DNase-mediated pathway of DNA damage emerging during drug treatment (Figure 11). Still further, we aim to complete an extensive WGS on drug-treated and untreated, DFFB WT and DFFB KO cells (Figure 13). The project is nearing completion, and we anticipate receiving the sequencing results in the summer of 2021.

By analyzing the WGS data, we will gain an in depth understanding of the number and type of mutations that arose in the persister cell genomes during the drug-treatment timeline.[45] In the context of mutational signature analysis, we expect to develop a reliable catalogue of DFFB-dependent mutational signatures from the DFFB WT persister cell lines. We expect the DFFB

KO persister cells to have accumulated fewer mutations when compared to the DFFB WT persister cells. Morphologically, the DFFB KO persister cells varied greatly from their DFFB WT counterparts (Figure 14). Importantly, the DFFB gene KO was the only variable that we (knowingly) altered in the cellular physiology, and we observed a dramatic difference in persister cell proliferation while under drug-stress. Our western blot data demonstrating the necessity of DFFB for sustained DNA damage, represented by γH2AX, along with DFFB KO cell lines not forming DTEPs in the manner that the DFFB WT lines do paints a promising picture of DFFB as the cause of drug-induced DNA damage in persister cells. Therefore, DFFB may prove to be a promising target of future anti-cancer drugs.

Introduction, Figure 1; and Chapter 1, Figure 2, and Figure 3 are coauthored with Matthew Hangauer. The thesis author was the primary author of the introduction and Chapter 1.

Chapter 1, Figure 4; and Chapter 2, Figure 9 (a-c), and Figure 10 (a-c) are coauthored with Masayoshi Higuchi. The thesis author was the primary author of these chapters.

Chapter 3, Figure 11 is coauthored with David Gervasio and August Williams. The thesis author was the primary author of this chapter.

Chapter 3, Figure 12 is coauthored with Claire Turkal. The thesis author was the primary author of this chapter.

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