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TITLE: Overcoming Chemoresistance in Ovarian Cancer by Targeting a Novel Lipid Hydroperoxidase Pathway

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14. ABSTRACT Chemoresistance remains a critical challenge in ovarian cancer that has stymied the achievement of cures. Dysregulation of apoptosis has been proposed as a mechanism underlying this phenomenon, and methods to re-sensitize chemoresistant ovarian cancer cells to apoptosis are being explored. In this project, we seek to explore a novel avenue that builds upon preliminary results showing therapy-resistant ovarian cancer cells to be highly susceptible to ferroptosis, a non-apoptotic form of cell death. The goals of this proposal are primarily to validate the preliminary findings in physiologically-relevant models of ovarian cancer which include patient-derived xenograft cells and fresh patient ascites-derived cells. A secondary aim is to gain initial clues into the mechanisms that may underlie ferroptosis sensitivity in these therapy-resistant ovarian cancer cells.		

15. SUBJECT TERMS

Chemoresistance, ferroptosis, apoptosis, patient-derived xenograft, ascites, GPX4, lipid peroxidation

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1. INTRODUCTION:

Chemoresistance remains a critical challenge in ovarian cancer that has stymied the achievement of cures. Dysregulation of apoptosis has been proposed as a mechanism underlying this phenomenon, and methods to re-sensitize chemoresistant ovarian cancer cells to apoptosis are being explored. In this project, we seek to explore a novel avenue that builds upon preliminary results showing therapy-resistant ovarian cancer cells to be highly susceptible to ferroptosis, a non-apoptotic form of cell death. The goals of this proposal are primarily to validate the preliminary findings in physiologically-relevant models of ovarian cancer which include patient-derived xenograft cells and fresh patient ascites-derived cells. A secondary aim is to gain initial clues into the mechanisms that may underlie ferroptosis sensitivity in these therapy-resistant ovarian cancer cells.

2. KEYWORDS:

Chemoresistance, ferroptosis, apoptosis, patient-derived xenograft, ascites, GPX4, lipid peroxidation

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of the project were as follows:

Specific Aim 1: To validate GPX4 dependency of chemoresistant persister cells generated from ovarian cancer patient-derived cells

1.1 Survey sensitivity of ovarian cancer patient-derived cells to carboplatin-paclitaxel therapy and GPX4 inhibitors (proposed timeline: 1-3 months; final status: completed)

1.2 Establish *ex vivo* model of chemoresistant persister cells from ovarian cancer patient-derived cells (proposed timeline: 3-9 months; final status: completed)

1.3 Characterize GPX4 dependency of chemoresistant persisters cells generated from ovarian cancer patient-derived cells (proposed timeline: 9-12 months; final status: completed)

Specific Aim 2: To perform modeling of GPX4 inhibition in patient-derived cells using *ex vivo* sensitivity profiling (modified aim)

2.1 Generate luciferized GPX4 -WT and GPX4-KO CAOV3 cells for in vivo experiment (proposed timeline: 3-6 months; final status: completed)

2.2 Use ascites from patient-derived xenograft models to test *ex vivo* efficacy of GPX4 inhibition in killing chemoresistant ovarian cancer cells (Modified from: Perform xenograft experiment to test efficacy of GPX4 loss in preventing emergence of chemoresistant disease in vivo) (proposed timeline: 9-15 months; current status: complete)

2.3 Use patient-derived ascites cells removed directly from patients during radiological procedures to test the efficacy of GPX4 inhibition in killing ovarian cancer cells (Modified from: Test in vivo efficacy of a small-molecule GPX4 inhibitor in a patient-derived xenograft model of the GPX4-dependent, chemoresistant disease) (proposed timeline: 18-24 months; current status: complete)

Specific Aim 3: To probe the mechanisms underlying GPX4 dependency of chemoresistant ovarian cancer cells

3.1 Probe the correlative and causal role of autophagy in GPX4 dependence of ovarian cancer persister cells (proposed timeline: 12-18 months; current status: not started)

3.2 Perform transcriptomic characterization of ovarian cancer parental and persister cells (proposed timeline: 18-24 months; current status: 50% completed)

3.3 Perform metabolomics profiling of persisters and parental cells (proposed timeline: 18-24 months; current status: completed)

What was accomplished under these goals?

Please see appendix A

What opportunities for training and professional development has the project provided?

Both I, and my research assistant, who is an aspiring physician, benefitted from numerous opportunities to interact closely with ovarian cancer physician-scientists. In addition, our work with fresh patient-derived ascites integrated us into aspects of patient care, allowing us a critical glimpse into the realities of the clinical context that have influenced our growth and scientific thinking.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report. This is the final report.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Ferroptosis is a newly-discovered cell death mechanism that has been postulated to have significant medical potential, particularly in oncology. However, ferroptosis vulnerability of cancer cells depends heavily on their metabolism and chemical make-up. These are aspects of cell biology that are fraught with challenges when modeling using cancer cell lines or even xenograft systems. As a result, an open question concerning ferroptosis is whether vulnerability to this cell death is actually present in human cancers.

A key achievement of this project has been to innovate and establish an ex vivo platform for assessment of ferroptosis sensitivity in cancer cells harvested freshly from patient-derived xenografts and patient ascites. The results of experiments using this platform have yielded compelling evidence that ferroptosis sensitivity does exist with considerable uniformity in ovarian cancers of certain patients. Companion metabolomic profiling has identified specific lipid species that have the potential to serve as predictive biomarkers for ferroptosis sensitivity with potential for integration into the clinical workflow.

While highly relevant to ovarian cancer contexts, which are the focus of this work, the general findings and platforms established here are of broad value across oncology, immunology and other disease areas where ferroptosis induction may have utility.

What was the impact on other disciplines?

The paradigms advanced in this work may broadly support the use of functional precision medicine to guide cancer care (<https://www.sfpm.io/>).

What was the impact on technology transfer?

-
-
-

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Progress during the funding period was impacted by two events: the birth of a child and maternity leave of the primary PI, and later a shutdown in lab activities related to COVID-19.

As a result, in vivo experiments were deprioritized slightly, and instead patient-derived xenograft data were further bolstered with analogous data generated from fresh patient-derived ascites. In addition, experiments addressing mechanistic questions (e.g. metabolomics) were initiated earlier than indicated in the original statement of work.

A revised SOW that reflects these changes has been included with the final report for incorporation as part of the termination modification. In addition, correspondence with HRPO and ACURO were established to communicate changes to experimental plans.

Actual or anticipated problems or delays and actions or plans to resolve them

Delays associated with maternity leave were mitigated by continued progress by the supporting research assistant and a request for a no-cost extension to complete the necessary work. In addition, cost-intensive experiments involving in mice were put on hold and additional patient-derived models (such as fresh patient-derived ascites) were investigated to bolster findings.

Departure of the PI to a new position in the private sector will be addressed by termination of the grant and submission of a manuscript reporting all findings to date.

Changes that had a significant impact on expenditures

Expenditures were below the expected level for a period due to the maternity leave of the primary PI. In addition, projected spending related to animal experiments were not incurred as those experiments have not yet been initiated.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

During the course of the work, to supplement *ex vivo* work with patient-derived xenograft cells, the investigator had the opportunity to use patient derived ascites cells removed directly from patients during radiological procedures. This work was covered by an IRB at Dana Farber that facilitates similar work for a number of labs at the Dana Farber Cancer Institute.

This change was communicated to HRPO following the termination of the award. The investigator was informed that HRPO does not complete reviews on terminated awards. As a result, a brief memorandum for record was drafted and included in the HRPO record; the record was then closed.

Significant changes in use or care of vertebrate animals

Animal studies proposed in Specific Aim 2 of the original SOW (Perform xenograft experiment to test efficacy of GPX4 loss (through both genetic and pharmacological perturbations) in preventing emergence of chemoresistant disease *in vivo*) were not performed due to challenges addressed in section #5 as well as the discovery of poor PK properties of BRD-1331, the small molecule proposed to be used *in vivo*. An alternative *ex vivo* approach, using ascites cells from patient derived xenografts, that circumvented these challenges was adopted instead. These changes were reported to the IACUC and ACURO.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Iruvanti, S., Cai, L., Deik, A., Clemons, P., Zeng, Q., Zhou, S., Pacheco, J., Kirschmeier, P., Gokhale, P., Clish, C., Matulonis, U., Schreiber, S., Liu, J., Viswanathan, V. Assessment of ferroptosis sensitivity and lipid correlates in patient-derived ovarian cancer cells. *In preparation*

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

While not formally deployed or covered by intellectual property filings, the ex vivo platform we have established for assessment of ferroptosis sensitivity in cancer cells harvested freshly from patient ascites is one that will be shared via our manuscript and adopted by the ferroptosis field.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: *Mr. Suvruta Iruvanti*

Project Role: *Research Associate*

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: *12*

Contribution to Project: *Mr. Iruvanti has performed most wet-lab experiments and generation of metabolomic data.*

Funding Support:

Name: *Dr. Joyce Liu*

Project Role: *Collaborator*

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: *<1*

Contribution to Project: *Dr. Liu has facilitated access and use of patient-derived materials, and has provided input on experimental methods and data interpretation.*

Funding Support:

Name: *Dr. Paul Clemons*

Project Role: *Bioinformatician*

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: *<1*

Contribution to Project: *Dr. Clemons has assisted with data analysis and appropriate use of statistical methods.*

Funding Support:

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Joyce Liu, MD, MPH

ACTIVE

BCRF (Matulonis)

10/1/2019-9/30/2020

Breast Cancer Research Foundation

Award Title: Genomic relationship between ovarian and breast cancers: Predictors for chemotherapy and targeted therapy response

Role: Co- Investigator

Effort: 0.36 calendar months

Level of Funding:

Overlap: None

(Liu, J)

01/01/2020 - 12/31/2020 0.12

Calendar Months

Susan F. Smith Center for Women's Cancers Award

Dissecting mechanisms of replication stress in uterine serous carcinoma

Role: Co- Investigator

Effort: 0.12 calendar months

Level of Funding:

Overlap: None

PENDING

(Dinulescu)

09/30/2020- 09/29/2023

Department of Defense (CDMRP)

Combinatorial Epigenetic-Based Approach to Enhance Ovarian Cancer Immunotherapy

Role: Co- Investigator

Effort: 0.48 calendar months

Level of Funding:

Overlap: None

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Appendix A. Key results from the previous reporting period

The results described below reveal that there exist both ferroptosis-sensitive and ferroptosis-insensitive ovarian cancer tumors across patient-derived xenografts and freshly-harvested patient ascites in the context of recurrent ovarian cancer. These states are stable and reproducible over the course of months, during which patients receive standard-of-care cisplatin-based treatments. These results establish the rationale for developing ferroptosis-inducing therapies and using them in a targeted fashion to eliminate recurrent ovarian cancer bearing the hallmarks of ferroptosis sensitivity. Relevant to the latter point, we have performed metabolomic measurements of ferroptosis-sensitive vs. insensitive patient samples and are exploring metabolite profiles that appear to show promising as biomarkers.

patient	attributes
DF3922	metastatic endometrial carcinoma of serous and endometrioid type
DF3926	advanced stage Mullerian adenocarcinoma
DF4044	recurrent high-grade serous peritoneal cancer
DF2462	recurrent ovarian adenocarcinoma
DF3029	recurrent ovarian cancer
DF3284	recurrent ovarian cancer
DF3706	recurrent ovarian carcinosarcoma

Table 1. List of patient de-identification codes and salient attributes for the ascites samples that were studied. Entries colored in black are sensitive to GPX4 inhibition while those colored in gray are not.

Patient-derived models allow moving beyond cell lines to obtain an improved perspective on *in vivo* tumor genetics and histology [Hidalgo et al, Cancer Discovery, 2014; Weroha et al, Clinical Cancer Research, 2014]. However, the use of patient-derived organoids presents a set of challenges that differ from those of cell lines. Only short-term *ex vivo* culture of the organoids is possible, with limited replicative potential and competence for genetic manipulation. These organoids are maintained in three-dimensional (3D) cultures that are highly heterogeneous in size and morphology. This aspect complicates cell counting and seeding, viability assessments, and drug response. In addition, patient-to-patient variability may influence ascites biology.

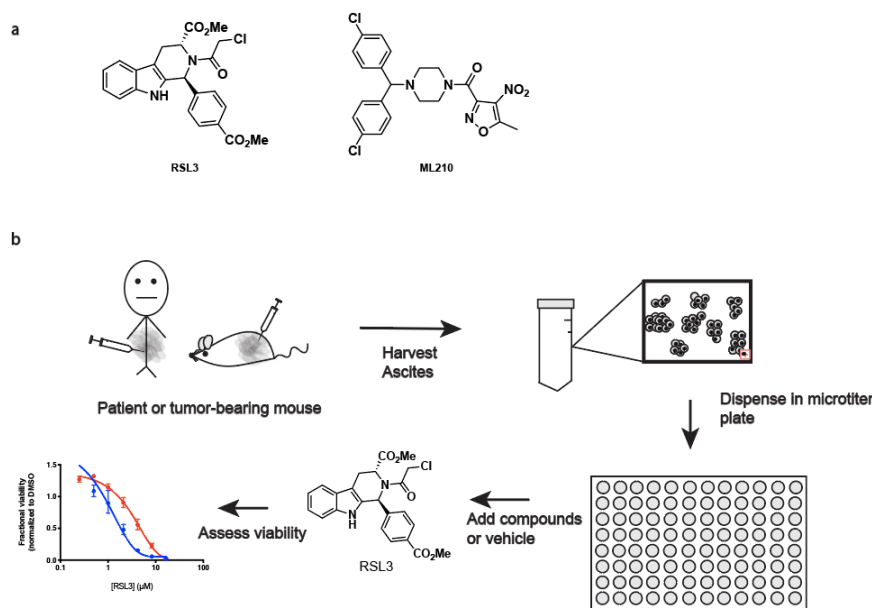
At the start of this work, we aimed to optimize culture parameters for PDXs to enable assessment of sensitivity to GPX4 inhibitors that trigger ferroptosis (Fig. 1). PDX cells were developed from the ascites of patients with high-grade serous ovarian cancer. These PDXs maintain molecular, genetic, immunohistologic, and clinical traits of the original tumor tissue.

Optimized PDX culture parameters show range of sensitivities to GPX4 inhibition

One factor that can affect cellular responses is seeding density. When optimizing parameters for PDX cells, we selected a seeding density that was most similar to that of standard two-dimensional (2D) culture (Fig. 2a). Generally, in 2D cell

cultures, a single-cell layer is generated. While this experimental design allows for standardization of surface areas of cells exposed to media, it does not necessarily recapitulate the true 3D structure of a tumor *in vivo*. At the outset of our work, we attempted to use the tissue dissociation reagents Accutase and trypsin to achieve de-clumping of the organoids. This approach was unsuccessful even after 20 minutes of Accutase treatment at 37 °C followed by vigorous micropipette aspiration. As the 3D structure of the organoids may be important to their faithfulness to *in vivo* disease states, we did not proceed with further de-clumping efforts.

Figure 1. a, structures of the covalent GPX4 inhibitors (1*S*,3*R*)-RSL3 and ML210. **b**, Process-flow diagram of the procurement of PDX cells and ascites; seeding in microplates; treatment with compound; and viability assessment.



We next assessed the contributions of plate format on sensitivity profiles (Fig. 2b). Plate format can alter experimental results because organoids are large in size, and the reduced volumes of 384-well formats can change the cells:medium:drug ratio. While 384-well format allows for the interrogation of an increased number of dose points, it masks sensitivity to certain compounds such as ML210. This caveat is likely due to differences in the cells:medium:drug ratio. Ultimately, 96-well format was chosen for subsequent experiments. Adherent conditions appear to lead to a slight increase in compound sensitivity (Fig. 2c). Therefore, non-adherent plates were chosen to ensure that any signal observed was robust and the result of non-artefactual phenomena. PDX organoids were previously engineered with a luciferase reporter. Such a feature allowed for several methods for assessing viability following treatment with compound, two of which we explored. When compared against each other, the readouts from the CellTiter-Glo assay and the ONE-Glo luciferase assay showed high concordance (Fig. 2d). We chose to use CellTiter-Glo. The same PDX models were grown in different mice as part of the organoid propagation process at the Belfer Institute. Sensitivity profiles were robust across cultures harvested from different mice (Fig. 2e). Using a set of optimized parameters, we profiled the panel of PDX cultures. We identified that a range of sensitivities to the GPX4 inhibitors RSL3 and ML210 existed (fig. 2g). DF118Luc, DF101Luc, DF181Luc, DF68Luc and DF86Luc PDX models were sensitive to GPX4 inhibition and subsequent ferroptotic cell death (fig. 2g).

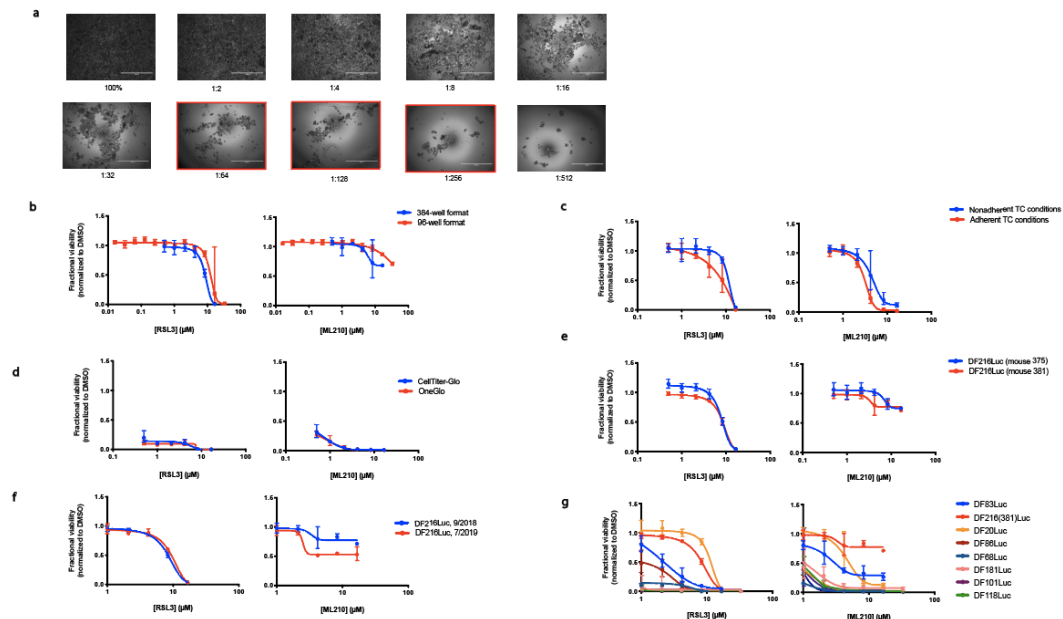


Figure 2. **a**, dose dilution of ovarian PDX cells DF83Luc. **b**, Comparison of 96-well and 384-well tissue-culture-treated, adherent plate format using DF216Luc PDX cells. **c**, Comparison of adherent and non-adherent plates using DF20Luc in 96-well format. **d**, Assessment of CellTiter-Glo and One-Glo luciferase assays. **e**, Assessment of mouse-to-mouse variability using DF216Luc in 96-well and adherent culture conditions shows a high degree of response profile reproducibility between PDX cells grown in different mice. **f**, Comparison of DF83Luc over a one-year gap in profiling shows that response profiles remain concordant. **g**, Summary of optimized parameters. **h**, PDX cells treated with optimized culture parameters show a range of sensitivities to GPX4 inhibition. Data are presented as mean \pm s.d., $n = 3$ technical replicates.

parameter	optimization
dissociation reagent	none
medium	WIT-T culture medium
format	96-well
culture surface	non-adherent
seeding density	moderate
treatment time	PDX: 72 h ascites: 7 days
assay	CellTiter-Glo

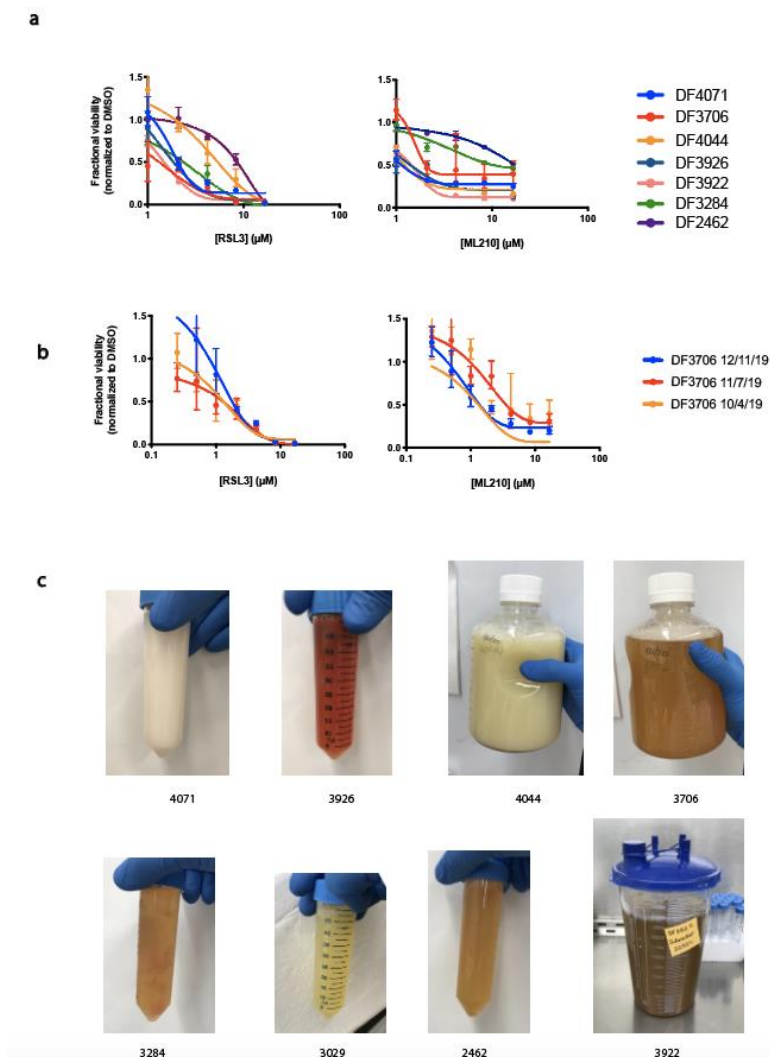
Table 2. Summary of optimized parameters for PDX organoids and ascites cells.

Freshly-harvested patient ascites exhibit a diverse but reproducible range of sensitivities to GPX4 inhibition

Given that a range of sensitivities existed among the PDX models, we extended this study to patient tissue removed directly from patients during radiological procedures. Using the PDX parameters as a baseline, similar optimized experimental procedures were developed for treating ascites. We found that comparable to early studies in PDX models, patients exhibited diverse but reproducible responses to GPX4 inhibition with the compounds ML210 and RSL3 (Fig. 3a). Similar to what was observed with the PDX organoids, the ascites cells from patients exhibited sensitivity profiles to RSL3 and ML210 that were reproducible over multiple paracentesis procedures spanning multiple months (fig. 3b). Furthermore, the appearance of the peritoneal ascites fluid from which tumor cells were isolated had large differences in appearance (fig. 3c). As is seen in fig. 3c, some patients' fluids were fattier and heterogenous (DF3284, fig. 3c), while others contained what appeared to be more blood cells and were more homogeneous (DF3926, fig. 3c).

From our finding that patients exhibited differential yet reproducible patterns of sensitivity to GPX4 inhibition, we classified patient samples as either ferroptosis-sensitive or ferroptosis-insensitive. Sensitive samples included DF4071, DF3706, DF3922, and DF3284 (fig. 3a). Insensitive samples included DF2462 and DF4044 (fig. 3a). All patients received carboplatin-paclitaxel treatment in the years prior to our experiments. Clinically, the identities of the sensitive and insensitive groups of patients do not show any clear trends (tab. 3). However, the fact that there exist individuals who have a consistently reproducible sensitivity to GPX4 inhibition suggests that there may be other factors at play that can be of clinical significance.

Figure 3. a, Range of dose-responses to RSL3 and ML210 among seven patients. **b**, Sensitivity profiles between different procedure dates for the same patient. All data are presented as mean \pm s.d., $n = 3$ technical replicates. **c**, Appearances of ascites fluid upon collection from patient and before processing for drug treatment.



Lipidomic analysis is being explored to identify biomarkers of ferroptosis-sensitive state in ovarian cancer

A high phosphatidylcholine-derived polyunsaturated fatty acid (PUFA) state is known to correlate with susceptibility to ferroptosis. In order to determine if predictive differences exist between the lipid profiles of patients whose tumors exhibit ferroptosis sensitivity *versus* those that do not, we have performed metabolomic analysis. We are still in the midst of data analysis, but a preliminary view points to arachidonic acid as a potential biomarker of the ferroptosis sensitive state in recurrent ovarian cancer patient ascites and patient-derived xenografts.

Materials and Methods

Compounds. The compounds (1*S*,3*R*)-RSL3 and ML210 were synthesized as previously described (Yang 2014; Eaton 2020). Erastin and paclitaxel were purchased from SelleckChem. Ferrostatin-1 (fer-1) was purchased from Sigma-Aldrich.

Compound treatments. Cells were diluted and seeded into tissue culture plates at a density determined to be similar to that of two-dimensional culture using visual inspection. Cells were exposed to compounds for 72 h for PDX assays and 7 days for ascites assays. Viability was assessed by measuring cellular ATP levels using CellTiter-Glo. Raw data were log transformed and normalized to DMSO controls. The standard deviation was calculated in log space to yield dose response values \pm standard deviation. These values were then re-exponentiated and plotted in GraphPad PRISM.

Ovarian patient-derived xenograft cells (PDXs). PDXs were propagated as described [Liu et al 2016; Zervantonakis et al 2017] and obtained from the Belfer Center. Once obtained from the Belfer Center, PDX cells were kept on ice. Medium was changed to WIT-T (Cellaria Biosciences) as soon as possible, within 4 h. Seeding and compound addition were performed on the same day as PDX harvest. Cells were centrifuged at 1000 RPM for 5 min, after which the medium was aspirated and the organoids washed with PBS. Organoids were once again centrifuged at 1000 RPM for 5 min. PBS was aspirated and the organoids were resuspended in 10 mL of WIT-T medium. This suspension was stored on ice until proceeding to the next step. In one row of a 96-well plate, a serial dilution of the cells in WIT-T was performed and visually inspected under a microscope. A seeding dilution was selected in a dilution range of 1:64–1:256 in relation to the original organoid suspension. An organoid suspension was prepared in WIT-T medium using the identified dilution. The suspension was mixed thoroughly through inversion. Two-hundred microliters of this suspension was added to each well of a 96-well ultra-low adherence plate (Costar), with frequent mixing by pipetting up and down and stirring the solution between dispenses. Only the suspension needed to seed half a plate at a time was added to a reagent reservoir during the seeding process to ensure even seeding, as the organoids rapidly fell out of suspension. The original stock suspension of cells was kept on ice while seeding plates, and plates were transferred to a 37° C incubator immediately after seeding. Compound addition was performed by creating dose dilutions of compounds in WIT-T such that 50 μ L of compound-containing medium was added to each well. For some samples, compound addition was conducted using a D300e digital dispenser (Tecan). Viability was assessed following 72 h of treatment via CellTiter-Glo (Promega) using an Envision plate reader (PerkinElmer).

Patient ascites. Patient ascites were obtained from interventional radiology paracentesis procedures at the Dana-Farber Cancer Institute's Department of Gynecologic Oncology in concurrence with DFCI IRB protocols. Patient ascites were treated in the same method as the PDX organoids. Drug additions were performed using a D300e digital dispenser (Tecan).

Imaging. Ascites dilution images were taken on an upright microscope at 4x magnification. Images of patient peritoneal ascites fluid were taken on an iPhone 7.

Metabolomics. Metabolomic profiling and raw data processing were performed as described in [Zou, Y., Li, H., Graham, E.T. *et al*; Zou *et al* 2019 *Nat Commun*]. Median-normalized data were subsetted by lipid head group and normalized to the sum of the values in each head group. Once head-group-normalized, Student's *t*-tests with Bonferroni correction were performed between the set of sensitive and insensitive samples, and lipids were identified that significantly varied between the two groups ($p < 0.05$).