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1. Introduction

The recent addition of bevacizumab to the treatment of primary ovarian cancer and its FDA approval for this disease follow from the AURELIA and GOG/NRG 218 trials. From 1873 enrolled patients on GOG/NRG 218, it was found that the addition of bevacizumab to chemotherapy and its inclusion in the extended period resulted in a significant improvement in progression free survival (PFS) of 4 months compared to chemotherapy alone, with a hazard ratio of 0.717. The critical questions that must now be answered are 1) what are the molecular markers that predict for response on this landmark ovarian cancer trial? and 2) what are alternative treatment strategies for nonresponders? Our central ***hypothesis*** is that targeted agents including bevacizumab, chosen based upon the knowledge of the *TP53* mutational status, synergize with chemotherapy and promote catastrophic tumor cell death. The rationale for our hypothesis is that *TP53*, the ‘guardian of the genome’, is mutated in 95% of all high grade serous ovarian cancers and is considered the fundamental early genomic alteration associated with this disease. However, it is critically important to note that varying types of p53 mutant proteins exist, with different implications for cell cycle regulation and for chemosensitivity. Extensive preclinical data from our team demonstrate that the *TP53* mutations alter the master regulators of cell cycle checkpoints in predictable *yet distinct* ways that can be capitalized upon to overcome resistance to chemotherapy using targeted agents that block compensatory survival pathways. We term this principle “molecularly enhanced chemotherapy.” Our specific aims are to 1) in a retrospective study of GOG/NRG 218 clinical samples, determine the functional class of *TP53* mutation for each case and correlate with response; 2) assess the mechanisms of resistance to therapy as defined by outcomes from GOG/NRG 218 and identify alternative molecularly enhanced combinations; and 3) interrogate the function of *TP53* mutations that are *somatic variants of unknown function* (sVUF) to define the best therapy for patients with these mutations. These studies are anticipated to have a significant positive impact on the field by enhancing the design and choice of therapy for ovarian cancer based on specific *TP53* mutations. Moreover, our findings have the potential for immediate clinical application since we are performing retrospective analyses of a positive trial to better define a subgroup of patients that are more likely to respond. We will also prioritize drug choice in Aims 2 and 3 to use agents that have proven safety profiles and efficacy in other cancer types. Hence, our findings can be quickly implemented to provide personalized precision treatment options and improve outcomes for women with ovarian cancer using currently defined and FDA-approved treatments.

2. Keywords

Ovarian cancer; p53; bevacizumab; chemotherapy

3. Accomplishments

What were the major goals of the project?

Project/Grant Period: 09/30/2020-08/31/2023	Target Date (Month(s) relative to start date)	Actual Completion Date or % of Completion
Specific Aim 1: From GOG/NRG 218 clinical samples, determine the functional class of TP53 mutation for each case and correlate with response		
Major Task 1: Categorize cases in GOG/NRG 218 based upon TP53 status.		
Subtask 1: Submit institution’s IRB approval and related material for DoD’s HRPO approval	1	03/2020
Subtask 2: Receive HRPO approval or exempt finding before initiating human subjects/HAS related studies.	1	03/2020
Subtask 3: Extract de-identified TP53 sequencing data from GOG/NRG 281 BROCA sequencing files	1-4	75%
Subtask 4: Bin TP53 mutations into function categories (LOF, GOF, sVUF, WT)	5-6	50%
Milestone Achieved: Bin TP53 mutations	6	50%
Major Task 2: Correlate binned TP53 subclass designations with response, PFS and OS		

Subtask 1: Provide NRG Statistical Office with a table containing de-identified patient identifiers and TP53 functional category (LOF, GOF, sVUF, WT)	6	Projected to be completed in the next 3 months
Subtask 2: Receive a report of the correlation between <i>TP53</i> mutational status and outcomes on GOG/NRG 218	9	Projected to be completed in the next 6 months
Subtask 3: Assemble data from Major Tasks 1-2 for publication	9-12	Projected to be completed in the next 9 months
Milestone Achieved: Determine the correlation of TP53 subclass designations with PFS, OS and response overall and on each arm of GOG/NRG 218	12	Projected to be completed in the next 9 months
Specific Aim 2: Assess the mechanisms of resistance to therapy as defined by outcomes from GOG/NRG 218 and identify alternative molecularly enhanced combinations.		
Major Task 3: Determine the functional impact of GOF mutants.		
Subtask 1: Generate cell models engineered to express all GOF mutants in GOG/NRG 218 dataset	5-8	50% completed
Subtask 2: Assess the impact of each GOF mutant on canonical p53 WT function	8-12	50% completed
Subtask 3: Assess the impact of each GOF mutant on cell cycle checkpoint signaling	8-12	50% completed
Subtask 4: Define the impact of each GOF mutant on the transcriptome using RNAseq	13-16	Projected to be completed in the next 6 months
Milestone(s) Achieved: Assign function to each TP53 GOF mutant	16	Projected to be completed in the next 6 months
Major Task 4: Identify the most effective molecular inhibitor to combine with chemotherapy in order to overcome the impact of each GOF mutant		
Subtask 1: Test the impact of select agents on cell viability, cell cycle progression, percent of mitotic cells and activation of cell cycle checkpoint	17-24	50% completed
Milestone Achieved: Identify appropriate molecular agent to use in studies in Major Task 5 (Aim 2C)	24	50% completed
Major Task 5: Determine the <i>in vivo</i> efficacy of the best combinations from Aim 2B		
Subtask 1: Obtain local IACUC Approval for proposed studies in PDX mice	1	02/21/2020
Subtask 1: Submission of institution approved animal protocols and related material for DoD's ACURO approval	1	03/06/2020
Subtask 2: Receive ACURO approval before initiating animal experiments.	1	05/2020
Subtask 2: Perform drug testing studies in PDX mice	25-36	100% completed
Subtask 3: Analyze post-treatment tumor specimens from PDX mice	28-36	100% completed
Milestone Achieved: IACUC approval for animal studies	3	100% completed
Milestone Achieved: Assemble final data for Major Tasks 3-5	36	100% completed
Milestone Achieved: Manuscript preparation for data generated in Major Tasks 3-5	36	Manuscript submitted to <i>Cell Death & Disease</i> on 09/2021; currently under review
Specific Aim 3: Interrogate the function of TP53 mutations that are somatic variants of unknown function to define the best therapy for patients with these mutations		
Major Task 6: Develop a predictive algorithm to bin sVUF in GOG/NRG 218 into functional categories		
Subtask 1: Estimate mutation effect of each sVUF in the GOG/NRG 218 cohort <i>in silico</i>	6-8	25% completed
Subtask 2: Predict functional effect of mutations	6-8	25% completed
Subtask 3: Obtain FFPE slides from NRG Oncology for immunohistochemistry (IHC)	9	Projected to be completed in next 6 months
Subtask 4: Perform IHC for p53 protein expression and bin cases	10-18	Projected to be completed in next 12

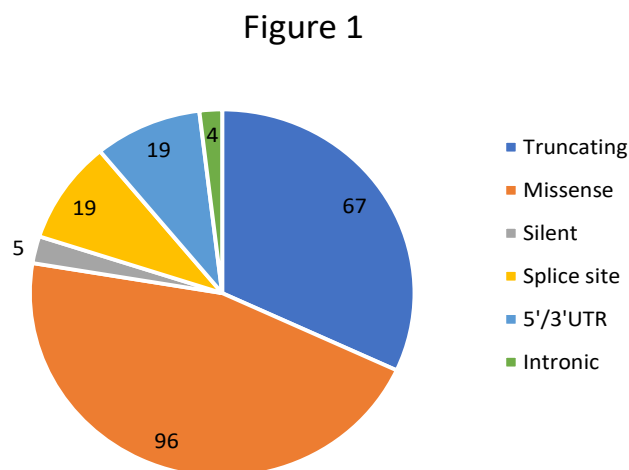
		months
Milestone Achieved: Functionally bin cases as WT, LOF or GOG		Projected to be completed in next 18 months
Major Task 7: Binning based on <i>in vitro</i> assessment of mutant p53 activity.		
Subtask 1: Generate cell models engineered to express recurrent sVUFs in GOG/NRG 218 dataset	5-8	50% completed
Subtask 2: Assess the impact of each sVUF mutant on canonical p53 WT function, cell cycle and transcriptome	8-16	50% completed
Milestone(s) Achieved: Assign function to each TP53 sVUF mutant	16	Projected to be completed in 18 months
Major Task 8: Determine the impact of TP53 sVUF on sensitivity to chemotherapy		
Subtask 1: Test the impact of select agents on cell viability, cell cycle progression, percent of mitotic cells and activation of cell cycle checkpoint	17-24	50% completed
Subtask 2: Drug testing studies in PDX mice	25-36	Projected to be completed in next 20 months
Milestone Achieved: Manuscript preparation for data generated in Major Tasks 6-7	36	Projected to be completed in next 24 months

What was accomplished under these goals? Here we detail the accomplishments by Specific Aim and Subaim.

Specific Aim 1 From GOG/NRG 218 clinical samples, determine the functional class of TP53 mutation for each case and correlate with response.

Aim 1A: Categorize cases in GOG/NRG 218 based upon TP53 status.

- We have received approval from the NRG Oncology Ancillary Projects Committee approval for TR studies (see letter of approval, **Appendix 1**).
- Our collaborators at the University of Washington have provided a list of mutations in TP53 that occurred as single events among the 713 patients with available sequencing data enrolled on Arms 1 and 3 of GOG-218.
- We have binned these cases based upon the specific type of TP53 mutation as in **Figure 1**.



Specific Aim 2: Assess the mechanisms of resistance to therapy as defined by outcomes from GOG/NRG 218 and identify alternative molecularly enhanced combinations.

Aim 2A: Determine the baseline impact of various GOF mutants on cell cycle checkpoint control and expression of canonical p53 transcriptional targets.

- We have generated both cell models and patient-derived organoid (PDO) models of ovarian cancer harboring various mutations in TP53. These data have been published in two peer-reviewed scientific manuscripts (see **Appendices 2 and 3**). **Table 1** (next page) contains a list of all ovarian PDO models annotated with TP53 mutational status determined by sequencing and p53 expression level determined by immunohistochemistry.

Table 1: Ovarian patient-derived organoid models. N.D. Not determined. WT: wild-type. OE: overexpressed.

ID	Histologic subtype	Stage	TP53 seq	p53 IHC
ONC-6695	Serous	IA	WT	WT
ONC-6163	Serous	II	WT	WT
ONC-6088	Serous	IIA	WT	OE
ONC-7188	Serous	III	N.D.	N.D.
ONC-6134	Serous	IIIA	R267G	OE
ONC-6045	Serous	IIIB	N.D.	OE
ONC-6069	Serous	IIIB	Del/splice/intron 7 retention	Null
ONC-6072	Serous	IIIB	WT	WT
ONC-7059	Serous	IIIB	N.D.	N.D.
ONC-6693	Serous	IIIC	WT	cytoplasmic
ONC-7253	Serous	IIIC	WT	N.D.
ONC-7485	Serous	IIIC	N.D.	N.D.
ONC-7705	Serous	IIIC	N.D.	N.D.
ONC-7704	Serous	IIIC	N.D.	N.D.
UBC 7391	Serous	IIIC	N.D.	N.D.
ONC-7910	Serous	IIIC	N.D.	N.D.
ONC-6061	Serous	IIIC	WT	WT
ONC-6561	Serous	IIIC	N.D.	Null
ONC-6663	Serous	IIIC	WT	OE
ONC-7052	Serous	IIIC	WT	Null
ONC-6497	Serous	IV	WT	WT
ONC-7077	Serous	IV	G266V	OE
ONC-6140	Serous	IVA	N.D.	N.D.
ONC-6562	Serous	IVA	WT	OE
ONC-7033	Serous	IVA	N.D.	cytoplasmic
ONC-5942	Serous	IVB	C275Y	OE
ONC-6007	Serous	IVB	WT	OE
ONC-6683	Serous	IVB	WT	OE
ONC-7017	Serous	IVB	N.D.	WT
ONC-7791	Serous	IVB	N.D.	N.D.
ONC-7024	Serous		R213-STOP	WT
ONC-7624	Serous		N.D.	N.D.
ONC-7627	Serous		N.D.	N.D.
ONC-7978	Endometrioid	IC	N.D.	N.D.
ONC-7477	Endometrioid	IIIA	N.D.	N.D.
ONC-7962	Adenocarcinoma	IV	N.D.	N.D.
ONC-7063	Clear cell	IA	WT	WT
ONC-5945	Clear cell	IC	WT	WT
ONC-6092	Clear cell	IC	WT	WT
ONC-7395	Mucinous	IA	N.D.	N.D.
ONC-7429	Mucinous	IA	N.D.	N.D.
ONC-7465	Mixed	IV	N.D.	N.D.
UBC 7201	Carcinosarcoma	IIIA	N.D.	N.D.
ONC-7645	Carcinosarcoma	IIIC	N.D.	N.D.
NR1	Carcinosarcoma	IVA	Δ131 mutation	OE
ONC-7234	Malignant brenner tumor	IIB	WT	N.D.

- We have examined PLAC1 transcriptional control in two ovarian cancer cell lines harboring different p53 mutations (see **Appendix 4**). PLAC1 transcription is a readout for WT p53 activity.
- Finally, we have performed Kinexus phosphoproteomic analysis of key cell cycle controllers in ovarian cancer cells (**Table 2**).

Table 2: Kinexus phosphoproteomic analysis of the impact of bevacizumab or cediranib on protein phosphorylation in CAOV3 ovarian cancer cells. Cells were treated with either bevacizumab or cediranib (1 μ M), followed by assessment of protein phosphorylation using the Kinexus KAM phosphoproteomic array. The signal intensity was normalized to internal controls and calculated relative the untreated control. Lead candidates are identified. %CFC: percent change from control; data are colored from blue to yellow to indicate the significance of the change from control. Pan-specific indicates assessment of total protein expression.

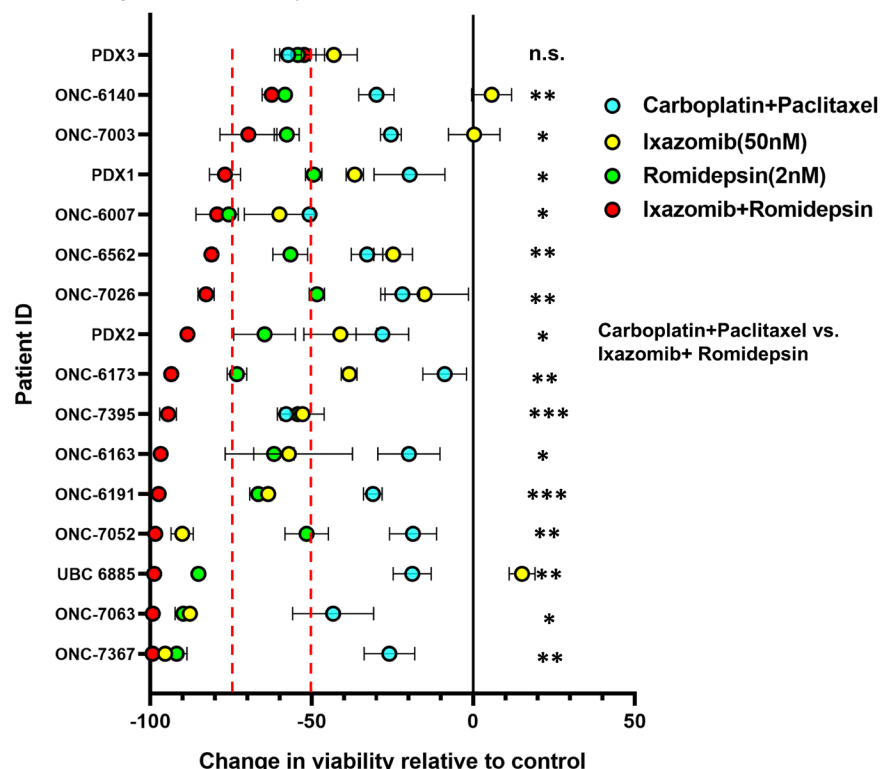
Target Name	P-Site	Control	Bevacizumab			Cediranib		
		Average Normalized Net Signal	Average Normalized Net Signal	% CFC	Lead	Average Normalized Net Signal	%CFC	Lead
Abl (Abl1)	Y257	3761	7151	90	Priority	7167	91	Priority
Abl2 (Arg)	Pan-specific	6462	8429	30		10193	58	Possible
Abl2 (Arg)	Y439+T440	6574	13841	111	Possible	15058	129	Possible
ACC1 (ACACA)	S78+S80	475	778	64	Possible	717	51	
ACC2 (ACACB)	T1342	7026	14123	101	Possible	16933	141	Priority
ACTB	Y53	2575	3771	46	Possible	1777	-31	
AMPKa2 (PRKAA2)	Pan-specific	9034	7388	-18		13189	46	Possible
AMPKa2 (PRKAA2)	S377	4704	7193	53		9907	111	Priority
Arrestin b	S412	601	940	56	Possible	580	-4	
ATM	Pan-specific	9806	10319	5		15245	55	Possible
CDK9	Pan-specific	547	1015	85	Possible	424	-23	
CLK1	S337	3336	5296	59	Possible	3033	-9	
DLK (ZPK)	S269	8521	8510	0		2626	-69	Possible
ERK5 (MAPK7)	Pan-specific	5747	8469	47	Possible	9386	63	Possible
EZH2	T487	6183	11321	83	Priority	13512	119	Priority
FAK (PTK2)	Y397	3638	8418	131	Possible	5680	56	
FGFR3	Pan-specific	7669	7854	2		12534	63	Possible
FGFR4	Pan-specific	3838	4107	7		5629	47	Possible
Fgr	Pan-specific	11578	12896	11		17239	49	Possible
Flt3 (STK1)	Pan-specific	11462	10718	-6		17339	51	Possible
FOXK1	S441+S445	6302	10532	67	Possible	11558	83	
FOXO1A (FKHR)	S256	3907	4223	8		6029	54	Possible
GFAP	Pan-specific	1925	1849	-4		2829	47	Possible
HDAC5	S498	577	907	57	Possible	437	-24	
HePTP (PTPN7)	S44	5159	8929	73	Possible	4764	-8	
HGK (ZC1)	Pan-specific	1829	9151	400	Priority	7583	315	Priority
HGK (ZC1)	T187	1966	9240	370	Priority	6767	244	Priority
HIPK1	Y352	4323	7794	80		8040	86	Possible
HRAS	Pan-specific	3839	7555	97	Priority	6829	78	
HRAS	Y157	7782	9357	20		11801	52	Possible
IGF1R	Y1161+T1163	2644	5887	123	Priority	4878	84	Priority
ILK1 (ILK)	S343	2518	3820	52	Possible	2236	-11	
Jun (c-Jun)	S243	8758	9146	4		12759	46	Possible
Ksr1	Pan-specific	913	858	-6		381	-58	Possible
MAFG	S124	6485	23087	256	Priority	5204	-20	

MEK2 (MKK2, MAP2K2)	Pan-specific	6378	7041	10		9875	55	Possible
NFKB p65 (Rel A)	Pan-specific	1439	2569	79	Priority	1140	-21	
NuaK1 (ARK5)/Nuak2	T211	7180	8503	18		10713	49	Possible
p38a MAPK (MAPK14)	T180+Y182	9035	14318	58	Possible	11614	29	
p38d MAPK (MAPK13)	T180+Y182	437	1533	250	Priority	616	41	
p53 (TP53)	Pan-specific	4531	7503	66	Possible	5429	20	
p53 (TP53)	S37	1785	2588	45	Possible	2000	12	
p53 (TP53)	S392	6873	14237	107	Priority	5913	-14	
p70S6K (S6Ka, RPS6KB1)	Pan-specific	4504	8433	87	Priority	4950	10	
p70S6K (S6Ka, RPS6KB1)	T252	8986	17413	94	Possible	15747	75	Possible
PCYT1A (CTPCT; CCTA)	T342+S343	497	1571	216	Priority	721	45	
PDK1 (PDPK1)	S241	7643	16441	115		16849	120	Priority
PFKFB3	S461	1592	3034	91	Possible	1783	12	
Pim2	Pan-specific	610	535	-12		342	-44	Possible
PKCt (PRKCQ)	Y545	3949	7233	83	Priority	6334	60	Possible
PKG1a (PRKG1A)	T515+T517	5339	11329	112	Possible	5916	11	
PLCG1	Pan-specific	1087	669	-38		496	-54	Possible
Raf-B (BRaf)	Pan-specific	5927	8916	50		13809	133	Priority
ROCK2 (ROKa)	Y722	5566	3829	-31		2926	-47	Possible
SMG1	Pan-specific	6220	8675	39		10793	74	Possible
SSRP1	Y441+S444	1527	2629	72	Possible	1479	-3	
ULK2	Pan-specific	401	1013	153	Possible	37	-91	Likely due to Ab spot mis-print

Aim 2B: Identify the most effective molecular inhibitor to combine with chemotherapy in order to overcome the impact of each GOF mutant.

- We have performed a series of studies to interrogate various molecular agents in combination with chemotherapy based upon the specific *TP53* mutational status of the models. First, we found that the antiangiogenic agent cediranib is superior to bevacizumab (see Appendix 3, Bi et al., *Pharmaceuticals*).
- Having identified this alternative angiogenic, our findings set the stage for new clinical trials using cediranib or other tyrosine kinase inhibitor antiangiogenics in place of bevacizumab with chemotherapy.
- We also found that p53 WT activity can be reactivated using a molecular reactivator that converts mutant p53 conformation back to wild type (see

Figure 2: Sensitivity was calculated relative to untreated control.



Appendix 4, Devor et al., *Pharmaceuticals*).

- We also explored agents that induce massive cell death in ovarian cancer models and identified the combination of a histone deacetylase (HDAC) inhibitor with a proteasome inhibitor. We confirmed efficacy in cell lines, PDO models, and a xenograft model of a serous endometrial tumor, a histologic subtype that is very similar to high-grade serous ovarian cancer (example data are provided in **Figure 2** on the previous page; manuscript has been submitted to *Cell Death and Disease* and currently under review).

Aim 2C: Determine the in vivo efficacy of the best combinations from Aim 2B.

- We have determined the in vivo efficacy of the combination of an HDAC inhibitor combined with a proteasome inhibitor in a xenograft model of serous endometrial cancer (**Figure 3**).

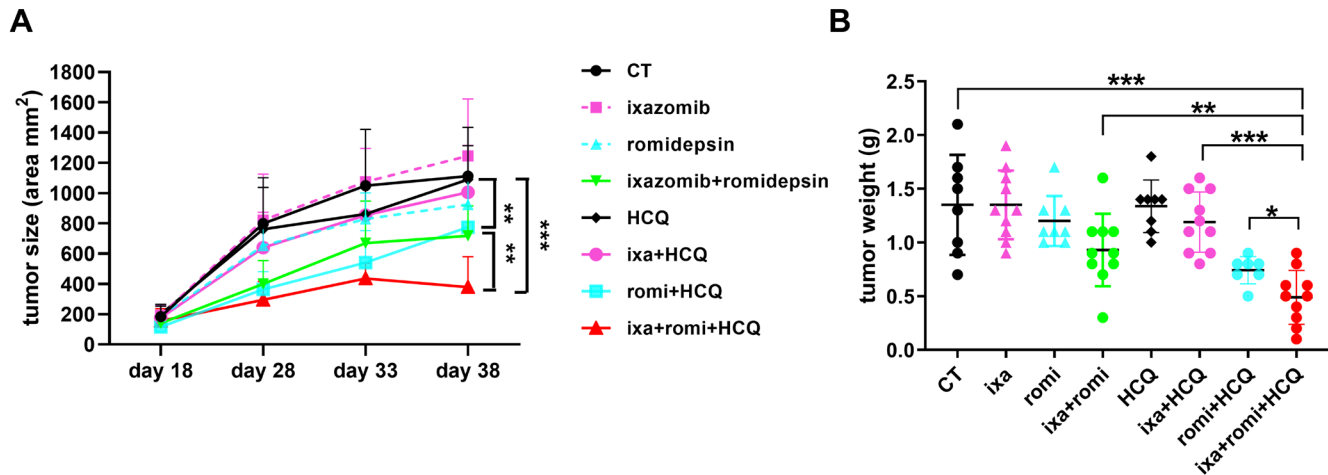


Figure 3: Sensitivity of xenograft models of Hec50 cells to ixazomib+romidespsin. A) Tumor volume; B) tumor weight at the completion of the experiment (Day 38).

Aim 3: Interrogate the function of TP53 mutations that are somatic variants of unknown function to define the best therapy for patients with these mutations.

- To date, we have binned 210 cases with *TP53* unique mutations in the GOG-218 dataset (Figure 1). Of these, nearly all of the 96 missense mutations are sVUFs. In addition, we have identified sVUFs in the PDO models of ovarian cancer that are also available for mechanistic studies. This work will follow our publication in *Clin Obstet Gynecol*, which was published between submitting this application and receiving funding (see **Appendix 5**).

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

This project was not intended to provide training and professional development opportunities. However, funds from this project have provided training opportunities for a postdoctoral fellow and a post-baccalaureate research intern. In addition, this project has supported in part the research of a clinical gynecologic oncology fellow in training. The purpose of this trainee's project was to create models of chemoresponse using patient-derived organoids. A senior scientist on this grant (Co-investigator Dr. Kristina Thiel) has now attained an NCI K22 transition career development award and is in the process of applying for a tenure-track academic faculty position.

How were the results disseminated to communities of interest?

Results have been disseminated through peer-reviewed publications as described above and in the Appendices. See also Section 6: Products. These results have also been presented in internal works-in-progress meetings at the sponsoring institution.

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

We will follow the experimental plan as outlined in the funded Project Narrative and the Statement of Work. Specific objectives in Aim 1 are to correlate p53 mutational status with outcomes through a collaboration with the NRG Oncology Statistical Office. For Aims 2 and 3, we will better characterize p53 mutations in PDO models and create additional models harboring hotspot mutations from the GOG-218 cohort.

4. Impact

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

These studies are anticipated to have a significant positive impact on the field by enhancing the design and choice of therapy for ovarian cancer based on specific TP53 mutations. Moreover, our findings have the potential for immediate clinical application since we are performing retrospective analyses of a positive trial to better define a subgroup of patients that are more likely to respond. We will also prioritize drug choice in Aims 2 and 3 to use agents that have proven safety profiles and efficacy in other cancer types. Hence, our findings can be quickly implemented to provide personalized precision treatment options and improve outcomes for women with ovarian cancer using currently defined and FDA-approved treatments.

What was the impact on other disciplines? These results pertain to other cancers because many other cancer types have identical mutations in *TP53*. For example, our group has published on p53 mutations as predictors of response to bevacizumab-containing chemotherapy in advanced or recurrent endometrial cancer (Leslie et al., Gynecol Oncol 2021 and Thiel, et al., submitted to JCO).

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

Nothing to report.

6. Products

Publications, conference papers, and presentations

Journal publications:

1. Bi J, Newton AM, Zhang Y, Devor EJ, Samuelson MI, Thiel KW, Leslie KK. Successful Patient-Derived Organoid Culture of Gynecologic Cancers for Disease Modeling and Drug Sensitivity Testing. *Cancers (Basel)*. Published on 2021 Jun 10;13(12):2901. doi: 10.3390/cancers13122901. PMID: 34200645; PMCID: PMC8229222. Acknowledgement of federal support: yes
2. Bi J, Dixit G, Zhang Y, Devor EJ, Losh HA, Newton AM, Coleman KL, Santillan DA, Maretzky T, Thiel KW, Leslie KK. Advantages of Tyrosine Kinase Anti-Angiogenic Cediranib over Bevacizumab: Cell Cycle Abrogation and Synergy with Chemotherapy. *Pharmaceuticals (Basel)*. Published on 2021 Jul 16;14(7):682. doi: 10.3390/ph14070682. PMID: 34358108; PMCID: PMC8308742. Acknowledgement of federal support: yes
3. Devor EJ, Schickling BM, Lapierre JR, Bender DP, Gonzalez-Bosquet J, Leslie KK. The Synthetic Curcumin Analog HO-3867 Rescues Suppression of PLAC1 Expression in Ovarian Cancer Cells. *Pharmaceuticals (Basel)*. Published on 2021 Sep 21;14(9):942. doi: 10.3390/ph14090942. PMID: 34577642; PMCID: PMC8465575. Acknowledgement of federal support: in progress
4. Bi J, Zhang Y, Malmrose, PK, Losh HA, Newton AM, Devor EJ, Thiel KW, Leslie KK. Blocking autophagy overcomes resistance to dual histone deacetylase and proteasome inhibition in gynecologic cancer. Under review (submitted on 10/01/2021).

Other Products

Research material: Patient-derived organoid models of ovarian cancer of differing histologies (high-grade serous, carcinosarcoma, clear cell, borderline, see **Table 1**).

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name	Role	Person months	Institution
Kimberly K. Leslie, MD	PI	No change	University of Iowa
Kristina W. Thiel, PhD	Co-I	No change	University of Iowa
Barbara Norquist, MD	Co-I	No change	University of Washington
Eric Devor, PhD	Co-I	No change	University of Iowa

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

No - Nothing to report.

What other organizations were involved as partners? Nothing to report other than the known collaboration with the University of Washington, as approved with the initial submission.

8. Special Reporting Requirements

Not applicable.

9. Appendices

*Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.***

Appendix 1: NRG Oncology Ancillary Projects Committee (APC) approval of translational research (APC #20018).

Appendix 2: Bi J, et al, Successful Patient-Derived Organoid Culture of Gynecologic Cancers for Disease Modeling and Drug Sensitivity Testing. *Cancers*. 2021

Appendix 3: Bi J, et al, Advantages of Tyrosine Kinase Anti-Angiogenic Cediranib over Bevacizumab: Cell Cycle Abrogation and Synergy with Chemotherapy. *Pharmaceuticals*. 2021

Appendix 4: Devor EJ, et al, The Synthetic Curcumin Analog HO-3867 Rescues Suppression of PLAC1 Expression in Ovarian Cancer Cells. *Pharmaceuticals*. 2021

Appendix 5: Bi J, et al, Characterization of a TP53 Somatic Variant of Unknown Function From an Ovarian Cancer Patient Using Organoid Culture and Computational Modeling. *Clin Obstet Gynecol*. 2020

Appendix 1



Advancing Research. Improving Lives.™

March 8, 2021

Kimberly Leslie, MD
Department of Obstetrics and Gynecology
University of Iowa
20 Hawkins Drive, 31140 PFP
Iowa City, IA 52242

Dear Dr. Leslie,

We are pleased to report that the NRG Oncology Ancillary Projects Committee (APC) has approved your application, *"Subanalysis of TP53 Mutations as Predictors of Outcome on GOG-0218"* (APC #20018). Austin Miller, PhD, and/or their designee of the NRG Oncology Statistical and Data Management Center (SDMC), will work through project development with you and your team as well as perform the analyses for this project.

Please note, while approval is granted, NRG Oncology cannot immediately provide a timeline for the project's completion due to the resources available and priorities of our organization. The NRG SDMC will work with you and your team to prioritize this project.

Once the analysis is completed and a report provided, you will be expected to develop an abstract for presentation at a national or international scientific session and to prepare a manuscript for publication in a peer-reviewed journal. You are required to work with the NRG Oncology Statistician and the NRG Oncology Publications Department to ensure that all presentations and manuscripts resulting from this project adhere to the NRG Oncology Publications Policy and Guidelines.

Should you have any questions, feel free to contact the APC program, at APC@NRGOncology.org.

Sincerely,

A handwritten signature in black ink that reads "M Machtay".

Mitchell Machtay, MD
Deputy Group Chair for Research Strategy
Ancillary Projects Committee Chair

cc: Austin Miller, PhD
Christopher Purdy, MS
Virginia Filiaci, PhD
Steven Waggoner, MD

Article

Successful Patient-Derived Organoid Culture of Gynecologic Cancers for Disease Modeling and Drug Sensitivity Testing

Jianling Bi ^{1,†}, Andreea M. Newton ^{1,†} , Yuping Zhang ¹, Eric J. Devor ^{1,2} , Megan I. Samuelson ³, Kristina W. Thiel ^{1,2}  and Kimberly K. Leslie ^{1,2,*}

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- † Authors contributed equally as co-first author.

Simple Summary: In the conventional treatment of gynecologic malignancies, most patients receive similar ‘one-size-fits-all’ treatment. However, it is increasingly clear that standard therapies do not work in every patient, and it would be very helpful to have pretreatment predictive assays to provide more personalized regimens. In this study, we describe the routine, successful establishment of patient-derived organoid models (PDOs) of endometrial and ovarian cancer tissues from consenting patients and provide an example of how information from drug screening in PDOs may be a useful predictor of patient response to therapy.



Citation: Bi, J.; Newton, A.M.; Zhang, Y.; Devor, E.J.; Samuelson, M.I.; Thiel, K.W.; Leslie, K.K. Successful Patient-Derived Organoid Culture of Gynecologic Cancers for Disease Modeling and Drug Sensitivity Testing. *Cancers* **2021**, *13*, 2901. <https://doi.org/10.3390/cancers13122901>

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Abstract: Developing reliable experimental models that can predict clinical response before treating the patient is a high priority in gynecologic cancer research, especially in advanced or recurrent endometrial and ovarian cancers. Patient-derived organoids (PDOs) represent such an opportunity. Herein, we describe our successful creation of 43 tumor organoid cultures and nine adjacent normal tissue organoid cultures derived from patients with endometrial or ovarian cancer. From an initial set of 45 tumor tissues and seven ascites fluid samples harvested at surgery, 83% grew as organoids. Drug sensitivity testing and organoid cell viability assays were performed in 19 PDOs, a process that was accomplished within seven days of obtaining the initial surgical tumor sample. Sufficient numbers of cells were obtained to facilitate testing of the most commonly used agents for ovarian and endometrial cancer. The models reflected a range of sensitivity to platinum-containing chemotherapy as well as other relevant agents. One PDO from a patient treated prior to surgery with neoadjuvant trastuzumab successfully predicted the patient's postoperative chemotherapy and trastuzumab resistance. In addition, the PDO drug sensitivity assay identified alternative treatment options that are currently used in the second-line setting. Our findings suggest that PDOs could be used as a preclinical platform for personalized cancer therapy for gynecologic cancer patients.

Keywords: patient-derived organoids (PDOs); endometrial cancer; ovarian cancer; drug sensitivity testing; personalized medicine



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1. Introduction

Ovarian cancer is the fifth leading cause of death of women from cancer worldwide [1]. Due to the lack of symptoms during the early development of ovarian cancer, patients are usually diagnosed at an advanced stage and have the lowest average five-year survival rate (46%) among all patients with gynecologic malignancies [2]. In 2020, over 21,000 women were diagnosed with ovarian cancer and approximately 14,000 deaths occurred from this disease in the United States [3]. Endometrial cancer is the most common gynecologic malignancy. Although many patients present at an early stage and enjoy a good prognosis,

patients with advanced or recurrent endometrial cancer have a very low five-year survival rate (<20%). In 2020, over 65,000 women were diagnosed with endometrial cancer in the United States, and nearly 13,000 women died from this disease [3]. Additionally, endometrial cancer is one of only a few cancers for which the incidence and mortality have been increasing year after year since the 1970s [4].

Current standard treatments for ovarian and endometrial cancer are very similar and use a combination of surgery and chemotherapy [5,6]. Radiation and hormone therapies are two additional methods used to treat endometrial cancer, but for high-grade serous ovarian cancers and high-risk endometrial cancers, chemotherapy with the doublet of a platinum compound and a taxane is typically recommended [7,8]. Although most patients with advanced disease respond well to initial treatment, the majority develop recurrent disease and become resistant to chemotherapy. For recurrent and platinum-resistant disease, additional cytotoxic agents such as gemcitabine, topotecan, doxorubicin and liposomal doxorubicin have been employed as the second-line chemotherapy [9].

Molecular agents which target specific molecules or pathways are also available and used in the adjuvant setting [10]. A class of targeted agents used in ovarian cancer is PARP inhibitors, which are most effective for patients with germline or somatic mutations in *BRCA1/2* [11]. In both preclinical studies and translational studies of completed clinical trials, our group has recently reported that mutations in the tumor suppressor *TP53* predict the benefit of adding the antiangiogenic compound bevacizumab to chemotherapy upfront in advanced endometrial cancer [4,12]. However, for most patients, there are no genetic markers available to predict their response to commonly used agents [13]. Today, even as our knowledge of cancer genetics has increased dramatically, our ability to treat cancers based on the presence of genetic alterations is very limited. Particularly in gynecologic oncology, personalization of medical therapy is an unrealized goal [14].

Understanding patient and tumor genetic diversity and their influence on drug responses is an important step towards personalized medicine. In addition to research models to identify biomarkers of response to novel therapeutics or combinatorial regimens, we propose that patient-derived organoids (PDOs) hold great promise as valuable preclinical models which can provide insights into drug responses that are case-specific. Accumulating evidence indicates that PDOs can predict clinical outcomes in cancer patients [15–17]. Studies in several cancer types have established that PDOs recapitulate both the histologic and genomic features of the lesion from which they were derived [18–20]. Additionally, PDOs can grow with high efficiency in a short period of time which is much faster than generating a patient-derived xenograft (PDX model), enabling a priori prediction of responsiveness to chemotherapy, with the potential to substitute other regimens if primary resistance is demonstrated [21]. PDOs can also be tested for response to novel regimens including combinations of chemotherapy with targeted agents or multiple targeted agents which can be added to the patient's initial round of therapy [22,23].

In this study, we describe progress on the generation of novel endometrial and ovarian cancer PDOs and drug testing. As an example of the power of PDOs to predict clinical outcomes, we present a case in which the PDO results reflected patient resistance to standard therapy, something we argue could be predicted upfront in the future using the PDO method for screening.

2. Materials and Methods

2.1. Clinical Features

All the studies were reviewed and approved by the University of Iowa's IRB, protocol #201809807. The electronic medical record was reviewed to determine oncologic history, diagnosis, neoadjuvant and adjuvant treatment(s) and clinical responses. For patients who received platinum-based chemotherapy, platinum sensitivity was defined as progression-free survival of at least six months after the last platinum-containing therapy. Platinum resistance was defined as recurrence or persistence of disease within six months after the last platinum-containing treatment. Outcome data had not yet matured for some cases

at the time of manuscript preparation; these cases are denoted “TBD” (to be determined). HER immunohistochemistry (IHC) was performed using clinical standards by the Central Pathology Laboratory at the University of Iowa, a CLIA-certified laboratory.

2.2. Generation of Patient-Derived Organoid Models Using Tumor and Normal Cells

Informed consent was obtained under the University of Iowa’s IRB protocol #201809807. Fresh tumor tissue was collected from each patient’s interval debulking surgery. Each patient’s ascites fluid, tumor and normal tissues were obtained, processed and cultured to create PDO cultures as previously described [20,23]; see additional details in the Supplementary Methods and Supplementary Figure S1. For H&E staining and IHC for HER2, organoids were exposed to 4% paraformaldehyde (PFA) for fixing cells and dissolving Matrigel. After pelleting by centrifugation, histologic gel was added to each sample. The samples were then transferred to a cassette, fixed in 70% ethanol overnight, sectioned and stained with either H&E or the HER2/neu antibody as for the biopsy and primary patient tumor specimens by the Central Pathology Laboratory at the University of Iowa.

2.3. Organoid Viability Assay

Viability of the tumor and normal organoids following drug treatment was performed as previously described [23]; see additional details in the Supplementary Methods. Briefly, organoids were collected with an organoid harvesting solution (Cultrex, R&D Systems, Minneapolis, MN, USA) and digested to single cells with TrypLE Express (Gibco, Waltham, MA, USA). Single cells were suspended in AdDE+++ medium with 10% Matrigel and seeded at a density of 10,000 cells/well in an ultra-low attachment 96-well U-bottom white plate. After 1–4 days, the organoids were exposed to carboplatin (1 μ M), cisplatin (1 μ M), paclitaxel (10 nM), bevacizumab (1 μ M), gemcitabine (100 nM), topotecan (100 nM) or different combinations for 72 h at 37 °C. At the end of the incubation, an equal volume of the CellTiter-Glo 3D reagent (Promega, Madison, WI, USA) was added to each well and incubated for 25 min at room temperature. Luminescence, reflective of cell viability, was measured using a Gen5 Microplate Reader (BioTek, Winooski, VT, USA). All the tests were conducted in triplicate and the data were normalized to untreated controls (set at 100% viability).

2.4. Statistical Analysis

The data were analyzed using the GraphPad Prism software. Statistical significance of differences was determined using two-way ANOVA with the Greenhouse–Geisser correction and Tukey’s multiple comparison test, with individual variances computed for each comparison, or one-way ANOVA with Tukey’s multiple comparison test. All values are expressed as the mean $\pm$ standard deviation (SD) of at least three independent experiments unless otherwise indicated; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

3.1. Feasibility of Organoid Culture Creation from Freshly Resected Endometrial and Ovarian Cancer

At the time of each patient’s surgical debulking, ascites fluid, tumor and normal tissues were obtained, processed and cultured to create PDO cultures (see the Supplementary Methods and Supplementary Figure S1).

We successfully generated tumor organoid cultures from 43 of 52 tumor samples, an establishment success rate of 83% (Table S1). Successful cultures included 21 ovarian tumors, 22 endometrial tumors and nine normal organoids from normal uterine or fallopian tube samples. While the majority of models were generated using tumor tissues, seven ascites fluid samples were used to create PDOs; among those, six PDOs were generated. Successful growth of PDOs typically occurred within two–three days of surgical resection. In 12 cases with neoadjuvant therapy, six PDOs were generated, for the establishment success rate of 50%. In 40 non-neoadjuvant therapy cases, 37 tumor organoids were generated (success

rate of 93%). We also observed that PDO generation using normal tissues was less successful than using tumor tissues, with the 33% establishment rate. The limited success rate may be due to the small size and the prevalence of stromal cells in the samples.

Next, drug screening was performed on 19 PDOs, a process that can be completed within 7–10 days from the time of surgery. Detailed information on the 19 models tested for drug sensitivity is summarized in Table 1.

3.2. Endometrial and Ovarian Cancer PDO Drug Response

Drug response assays were performed on 19 ovarian and endometrial cancer PDOs (Table 1) using therapeutic agents which are most commonly employed to treat patients with gynecologic cancer: carboplatin, paclitaxel, cisplatin, bevacizumab, gemcitabine and topotecan. PDO models that were created using ascites fluid samples are indicated with an * in Table 1.

Table 1. Patient and tumor characteristics corresponding to the 19 PDOs used for drug screening studies. IV: intravenous; IP: intraperitoneal; NED: no evidence of disease; N/A: not available; TBD: to be determined; * indicates the PDOs that were generated using the ascites fluid samples.

Patient ID	Cancer Type	Stage	Neoadjuvant Treatment	Adjuvant Treatment	Platinum-Sensitive?	Disease Status
ONC-5942	High-grade serous	IVB	Six cycles of carboplatin and paclitaxel followed by two cycles of carboplatin	Three cycles of doxorubicin and bevacizumab followed by bevacizumab	No	NED
ONC-6007 *	High-grade serous	IVB	No	Six cycles of IV/IP cisplatin and paclitaxel	Yes	Alive with disease
ONC-6045 *	High-grade serous	IIIB	No	One cycle of a single agent, carboplatin, followed by six cycles of carboplatin and paclitaxel	Yes	NED
ONC-6069	High-grade serous	Recurrent	Six cycles of carboplatin and paclitaxel followed by olaparib	Olaparib followed by gemcitabine	No	Dead of disease
ONC-6072	High-grade serous	IC	No	None	N/A	Lost to follow-up
ONC-6134	High-grade serous	IIIA	No	Six cycles of carboplatin and paclitaxel	Yes	NED
ONC-6163	High-grade serous	Recurrent	No	Six cycles of carboplatin and doxorubicin followed by olaparib	TBD	NED
ONC-7052	High-grade serous	IIIC	No	Six cycles of IV/IP cisplatin and paclitaxel	TBD	NED
ONC-6061	Low-grade serous	IIIC	No	Two cycles of carboplatin and paclitaxel followed by seven cycles of a single agent, carboplatin, followed by letrozole	No	Alive with disease
ONC-7063	Clear cell	IC1	No	Three cycles of carboplatin and paclitaxel	TBD	NED
ONC-6092	Clear cell	IC	No	Three cycles of carboplatin and paclitaxel	Yes	NED

Table 1. Cont.

	Patient ID	Cancer Type	Stage	Neoadjuvant Treatment	Adjuvant Treatment	Platinum-Sensitive?	Disease Status
Endometrial Cancer	ONC-6096	Endometrioid, grade 1	IA	No	Observation	N/A	NED
	ONC-6191	Endometrioid, grade 1	IB	No	N/A	N/A	NED
	ONC-6051	Endometrioid, grade 2	IA	No	Observation	N/A	NED
	ONC-6173	Endometrioid, grade 2	IA	No	Observation	N/A	NED
	ONC-6071	Endometrioid, grade 3	IA	No	Observation	N/A	NED
	ONC-6057	Serous	IVB	Three cycles of carboplatin, paclitaxel and trastuzumab	Three cycles of carboplatin, paclitaxel and trastuzumab followed by a single agent, bevacizumab	No	Alive with disease
	ONC-6099	Serous	IVB	Six cycles of carboplatin and paclitaxel	Three cycles of paclitaxel and bevacizumab	No	Dead of disease
	ONC-7003	Mixed serous/endometrioid	IA	No	Patient declined adjuvant therapy	N/A	NED

*: PDO models that were created using ascites fluid samples.

Carboplatin, paclitaxel and their combination are the most frequently used in first-line chemotherapeutic regimens for gynecologic malignancies. PDO responses to these agents are shown in Figure 1A. As compared to the untreated controls, nine of the 19 PDOs exhibited a notable decrease in viability when treated with the combination of carboplatin and paclitaxel, with viability ranging from 46.1% to 72.7% after 72 h of treatment. In the combinatorial setting, the effects of the taxane, and not the platinum compound, appear to be the predominant driver of therapeutic effectiveness.

Bevacizumab is an antiangiogenic agent frequently used as an adjuvant therapy in gynecologic cancer [12,24–26]. Therefore, we also screened the PDOs for sensitivity to bevacizumab alone and in combination with the first-line standard chemotherapy. In general, bevacizumab as a single agent had only a modest impact on cell viability and little additional impact on cell killing when combined with chemotherapy (Figure 1B).

As an alternative to the first-line standard chemotherapy via the intravenous (IV) route, cisplatin combined with paclitaxel provides another option for patients to receive chemotherapy by the intraperitoneal (IP) route or via heated intraperitoneal chemotherapy [27,28]. In general, compared to the combination of carboplatin and paclitaxel, cisplatin combined with paclitaxel had similar impacts on cell viability (Figure 1C).

Gemcitabine and topotecan are two commonly used second-line agents for cases of recurrent platinum-resistant gynecologic cancer [29–33]. Herein, we compared the effects of gemcitabine, topotecan and gemcitabine plus bevacizumab on cell viability. For some patients (ONC-6051, ONC-6072, ONC-6092), second-line therapies were more effective with respect to PDO cell killing than the first-line agents, but for most patients, the impact of those agents was not superior (Figure 2).

carboplatin and paclitaxel, and their combination are the most frequently used in first-line chemotherapeutic regimens for gynecologic malignancies. PDO responses to these agents are shown in Figure 1A. As compared to the untreated controls, nine of the 19 PDOs exhibited a notable decrease in viability when treated with the combination of carboplatin and paclitaxel, with viability ranging from 46.1% to 72.7% after 72 h of treatment. In the combinatorial setting, the effects of the taxane, and not the platinum compound, appear to be the predominant driver of therapeutic effectiveness.

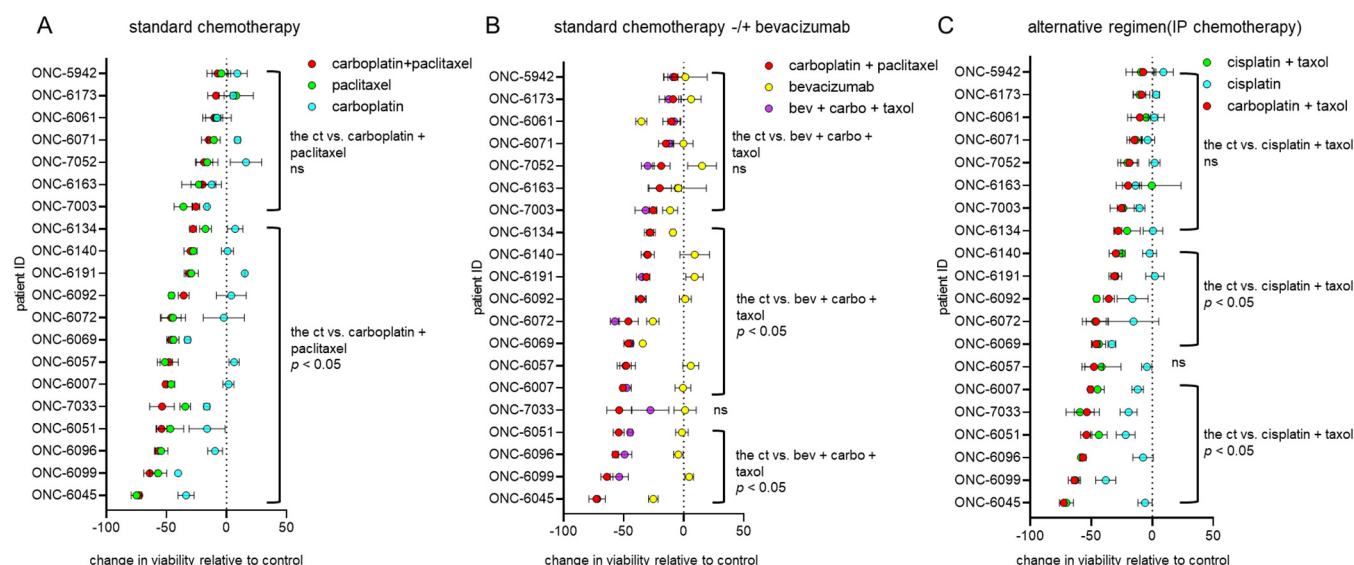


Figure 1. Therapeutic efficacy of the first-line therapies for endometrial and ovarian cancers using PDO models. Endometrial and ovarian cancer PDO responses to (A) the standard chemotherapy, (B) the standard chemotherapy +/- bevacizumab, and (C) the non-standard alternative chemotherapy regimen. PDO models were treated with the indicated agents for 72 h followed by assessment of cell viability. The data were calculated as the change in viability relative to the control which was set at 100% (i.e., no cell death). The samples are ordered based on increasing sensitivity to carboplatin + paclitaxel (red circles). Statistical significance was assessed by two-way ANOVA with the Greenhouse–Geisser correction and Tukey's multiple comparison test. Significant differences vs. the control are annotated on each panel. All the statistical comparisons are provided in Supplementary Table S2, including specific p -values and comparisons of single agents vs. control or dual treatments.

3.3. Bevacizumab as a PDGFR Inhibitor Reflects Agent Frequency Use
 Bevacizumab is a PDGFR inhibitor used as an adjuvant therapy in gynecologic cancer [12,24–26]. Therefore, we also screened the PDOs for sensitivity to

bevacizumab alone and in combination with the first-line standard chemotherapy. In general, bevacizumab as a single agent had only a modest impact on cell viability and little additional impact on cell killing when combined with chemotherapy (Figure 1B). Significant for a right pleural effusion, and the patient underwent a thoracentesis with temporary relief in symptoms. Cytology from her thoracentesis revealed TAA8-positive adenocarcinoma indicative of a gynecologic origin. Further imaging revealed multiple pulmonary emboli (PE), carcinomatosis, omental caking, ascites and a thickened endometrial stripe. Her history was negative for postmenopausal bleeding. An endometrial biopsy was obtained, which showed high-grade serous endometrial carcinoma, and HER2 immunostaining was positive (Figure 5A, pretreatment biopsy).

As an alternative to the first-line standard chemotherapy via the intravenous (IV) route, cisplatin combined with paclitaxel provides another option for patients to receive chemotherapy by the intraperitoneal (IP) route or via heated intraperitoneal chemotherapy [27,28]. In general, compared to the combination of carboplatin and paclitaxel, cisplatin combined with paclitaxel had similar impacts on cell viability (Figure 1C). Gemcitabine and topotecan are two commonly used second-line agents for cases of recurrent platinum-resistant gynecologic cancer [29–33]. Herein, we compared the effects of gemcitabine, topotecan, paclitaxel and bevacizumab on cell viability for some patients (ONC-6072, ONC-6092) who had prior platinum therapy (Figure 5B).

ONC-6072 and ONC-6092 were treated with first-line therapy over a 12-week cycle. Posttreatment imaging showed resolution of ascites, peritoneal carcinomatosis and omental cake. The patient subsequently underwent an exploratory laparotomy, total abdominal hysterectomy with bilateral salpingo-oophorectomy, infragastric and infracolic omentectomy, argon beam coagulation of tumor implants and optimal interval debulking. Final pathology from her debulking revealed her tumor to be of mixed serous and clear cell uterine histologies. Her postoperative course was uncomplicated, and she completed three more cycles of carboplatin, paclitaxel and trastuzumab with plans to continue maintenance trastuzumab until progression or toxicity. The patient received one cycle of maintenance trastuzumab, but before she was able to receive her second cycle (eighth overall), she experienced a rising CA125 and increasing shortness of breath. Imaging showed a recurrent pleural effusion, and a thoracentesis revealed recurrent adenocarcinoma.

Immunohistochemistry (IHC) performed on the cytology from this thoracentesis was estrogen receptor/progesterone receptor (ER/PR)-negative, suggesting that her pleural

effusion was from the clear cell component of her cancer since the clear cell component of the primary tumor specimen was also negative for ER and PR. The patient then received three cycles of a single agent, bevacizumab, during which her CA125 continued to rise, and her dyspnea was stable. Repeat imaging showed no evidence of intraabdominal disease, but she had persistent pleural effusions. Agents to be used for the management of the effusion were not determined (Figure 2). The patient requested a change in therapy 10 days with as-needed thoracenteses.

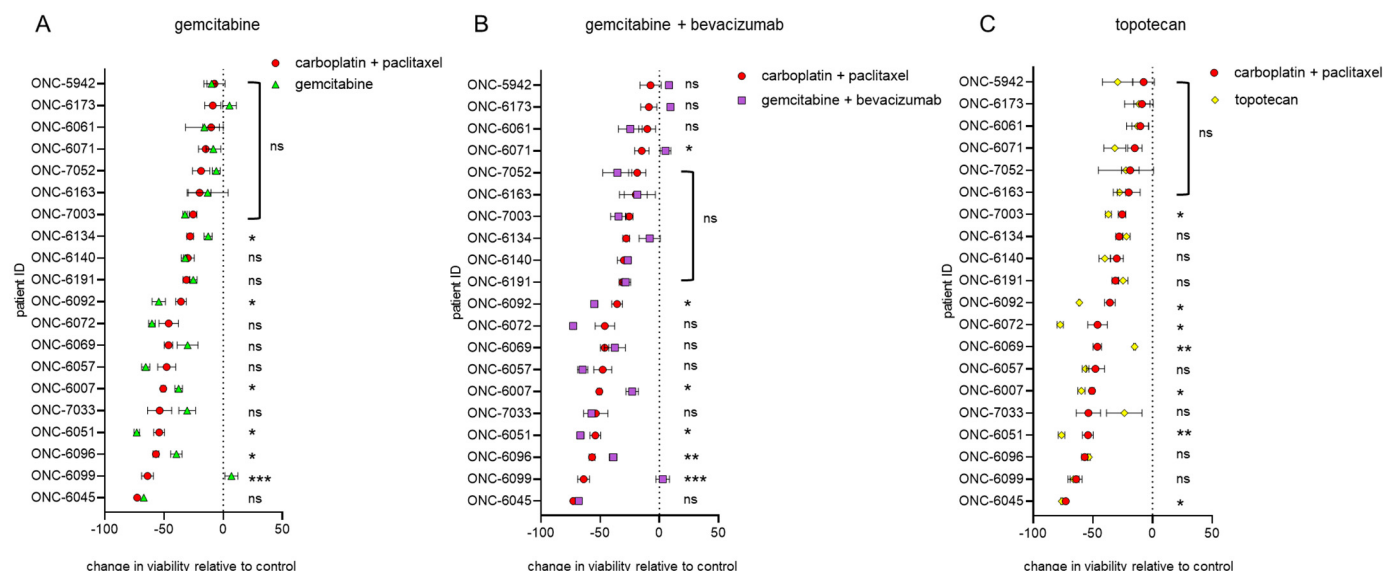


Figure 2. Therapeutic efficacy of common second-line therapies for endometrial and ovarian cancers using PDO models. Endometrial and ovarian cancer PDO response to second-line therapies: (A) gemcitabine, (B) gemcitabine + bevacizumab and (C) topotecan. PDO models were treated with the indicated agents for 72 h followed by assessment of cell viability as in Figure 1. The data were calculated as the change in viability relative to the control, which was set at 100% (i.e., no cell death). Each panel includes response to the standard chemotherapy (carboplatin + paclitaxel, red circles) for comparison, and the samples are ordered based on increasing sensitivity to carboplatin + paclitaxel. Statistical significance was assessed by two-way ANOVA with the Greenhouse–Geisser correction and Tukey’s multiple comparison test. Significant differences vs. carboplatin + paclitaxel are annotated on each panel: ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. All the statistical comparisons are provided in Supplemental Table S2, including specific p -values and comparisons vs. the untreated control. Note that some cases may have greater sensitivity to second-line therapy as compared to the standard chemotherapy. For example, ONC-6099 (panel A) was more sensitive to chemotherapy but had no change in viability when exposed to gemcitabine (panel A).

3.3. Proof-of-Concept: A PDO Model Reflects Treatment Response

3.3.2. Trastuzumab Response in the Patient-Derived Tumor Organoid Model

3.3.1. Patient Characteristics

This patient’s tumor was among the PDOs we created. We examined whether the tissues were obtained from a 70-year-old woman who initially presented with short-tumor previously exposed to trastuzumab in the neoadjuvant setting before surgical resection of breast, early safety and postprandial right upper quadrant pain. Imaging was significant for a right pleural effusion, and the patient underwent a thoracentesis with treatment would select for resistant cell clones by the time surgery was performed. Thus, temporary relief in symptoms. Cytology from her thoracentesis revealed PAX8-positive we assessed sensitivity of PDO cells to increasing concentrations of trastuzumab. Our results reflected potential resistance to trastuzumab based on a lack of reaching an IC50 pulmonary emboli (PE), carcinomatosis, omental caking, ascites and a thickened endometrial stripe. Her history was negative for postmenopausal bleeding. An endometrial biopsy was also predicted by the lack of HER2 immunostaining in the postsurgical specimen and the organoid sample (Figure 3A,B). Pathological analysis of the postsurgical specimen indicated some HER2 immunoreactivity in the cystically dilated glandular areas (Figure 3A, right margin of the posttreatment surgical specimen), whereas the stromal region and clear cell component (left side of the posttreatment surgical specimen) were devoid of HER2.

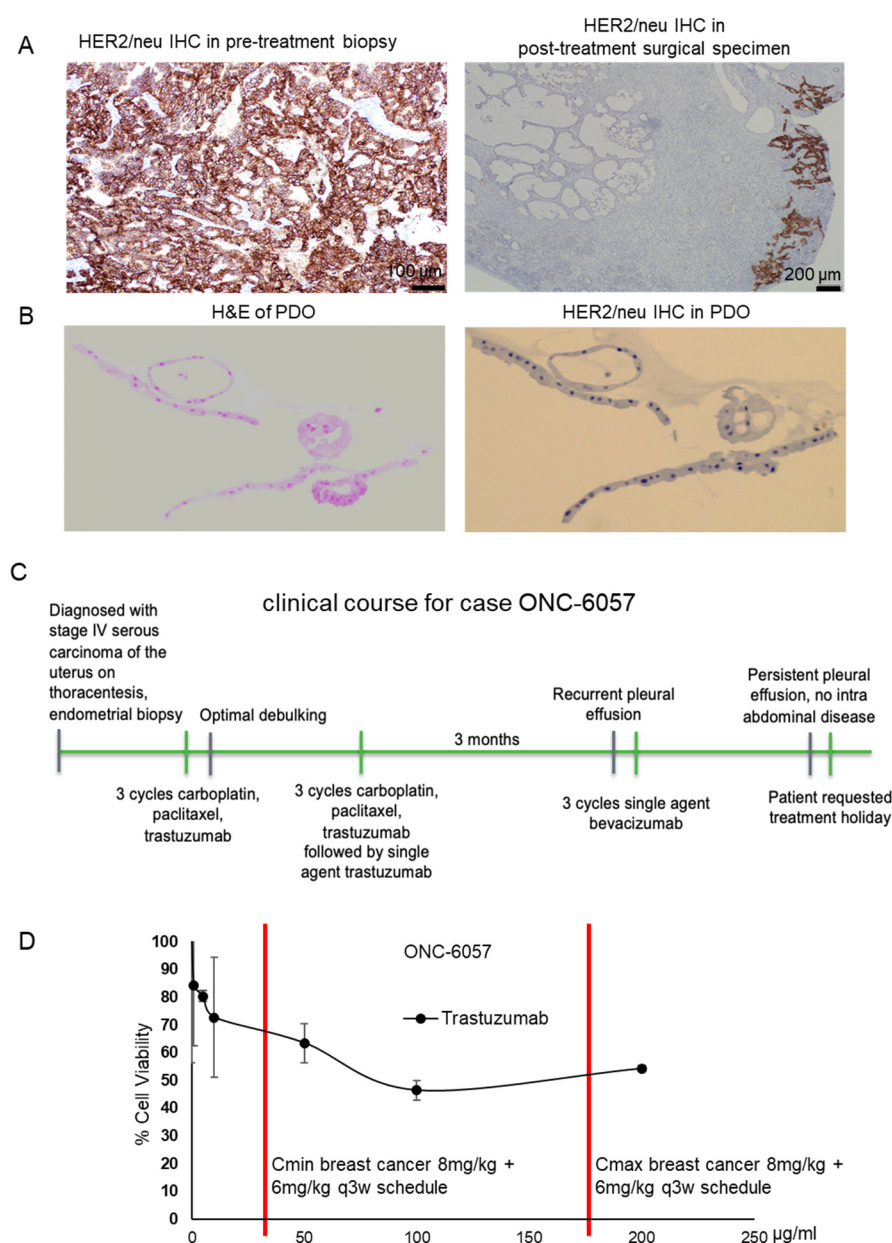


Figure 3. One advanced-stage high-grade serous endometrial carcinoma PDO model (ONC-6057) successfully predicted the patient's clinical platinum and trastuzumab resistance. (A) HER2 IHC of the patient's tumor obtained from the pre-treatment biopsy and the post-treatment surgical specimen. Additional images are provided in Supplementary Figure S2. (B) H&E staining and HER2 IHC for the corresponding PDO model. Additional images are provided in Supplementary Figure S3. (C) Timeline of the patient's clinical course. (D) PDO cells were treated with increasing concentrations of trastuzumab for 72 h, followed by assessment of cell viability. The data were calculated as the cell viability (%) relative to the untreated control which was set at 100%. The graph is annotated with the Cmin and Cmax values for trastuzumab used clinically in breast cancer. Note that an IC50 was not achieved with trastuzumab, even at concentrations greater than the Cmax.

Given her new PE diagnosis and advanced-stage disease, the patient received three cycles of neoadjuvant carboplatin, paclitaxel, and trastuzumab, a monoclonal antibody against HER2 (Figure 3B) [34]. Her CA125 decreased from 487 pre-treatment to 141 after the third cycle. Posttreatment imaging showed resolution of ascites, peritoneal carcinomatosis and omental cake. The patient subsequently underwent an exploratory laparotomy, total abdominal hysterectomy with bilateral salpingo-oophorectomy, intra-gastric patient received after failing trastuzumab. Organoid cells were more susceptible to paci-

Cancers 2021, 13, x

taxel, with 48.7% cell viability after exposure to this single agent. The patient's PDO was also more sensitive to gemcitabine (65% decrease in viability) and topotecan (56% decrease in viability) as compared to the chemotherapy regimen previously received, carboplatin + paclitaxel.

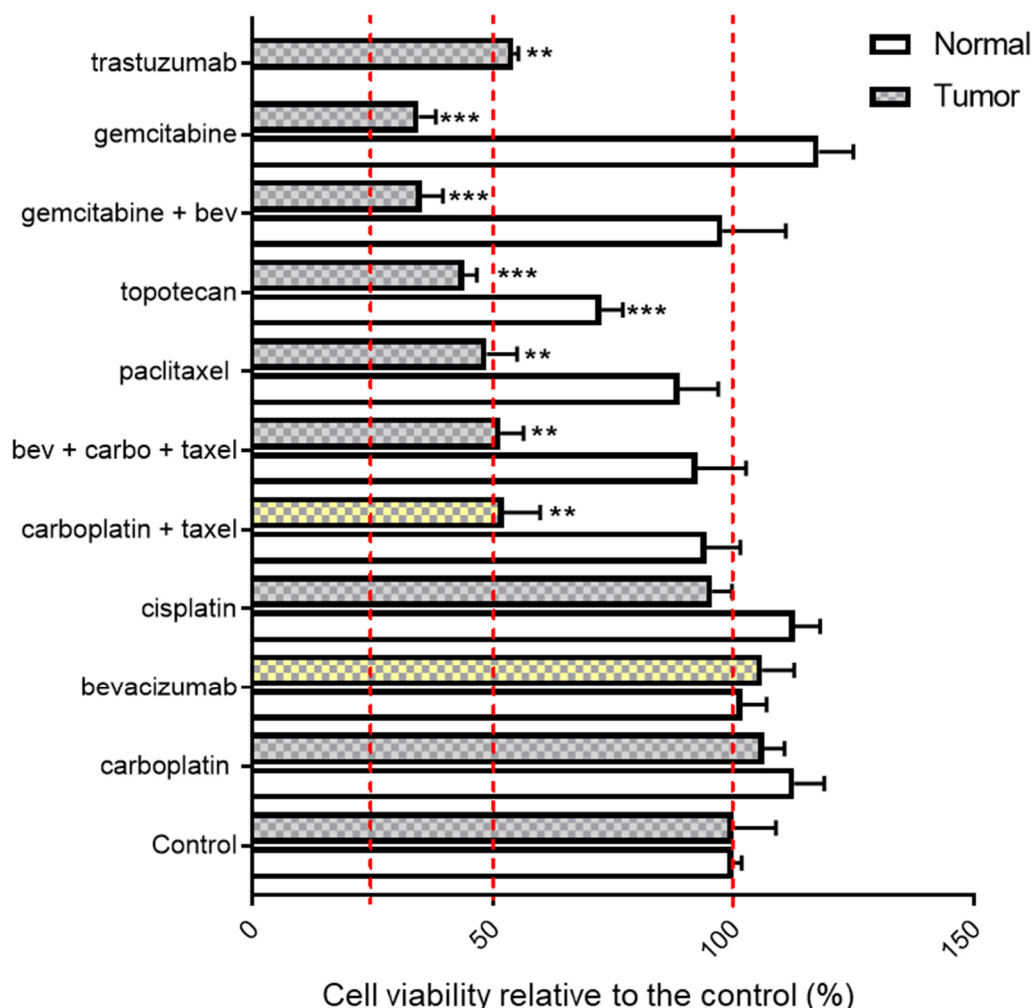


Figure 4. Varying drug sensitivity to the standard and alternative therapeutic options for the QNC-6057 PDO. Waterfall plot of the patient's QNC-6057 PDO response to various drug sensitivity with the paired normal human drug sensitivity. The activity of the agents in the chemotherapeutic regimen (carboplatin, paclitaxel, and bevacizumab) the patient received after resistance to trastuzumab was observed highlighted in yellow. The change in viability was calculated relative to the relative while the control at 100% was set at 100% (the statistical significance was assessed by ordinary one-way ANOVA with Tukey's multiple comparison test; ** $p < 0.01$, *** $p < 0.001$ vs. the paired control (normal or tumor specimen).

4. Discussion assessed drug sensitivity using a subset of agents in normal tissue organoids from the same patient (Figure 4). Gemcitabine, topotecan, and paclitaxel, which induced more than 50% cell death in tumor organoids, all resulted in minimal cell killing of normal organoids derived from non-malignant tissues.

4. Discussion We intuitively know that each patient's tumor is different, with potentially unique genetic and phenotypic characteristics. It is becoming increasingly clear that the conventional way to treat patients using a limited panel of first-line agents may miss important opportunities to optimize therapy. In contrast, personalized medicine strives to match the unique patient and tumor characteristics with innovative treatment regimens tailored to each case; however, progress in implementing precision therapies has been hampered by the lack of sufficient biomarkers that predict response to each therapeutic agent. One way to improve outcomes without the long and laborious process of biomarker identification and validation is to utilize tumor cells themselves as a patient avatar.

PDO culture technology is a novel and powerful tool for generating and maintaining three-dimensional patient tumor and normal tissues in vitro [35]. Since the time from surgical resection to drug testing results takes 7–10 days, use of organoids from primary patient tissues enables the testing of a range of compounds for efficacy even before clinical treatment decisions must be made. In addition, the establishment of organoids from non-malignant tissues from the same patient provides an opportunity to test potential drug

the lack of sufficient biomarkers that predict response to each therapeutic agent. One way to improve outcomes without the long and laborious process of biomarker identification and validation is to utilize tumor cells themselves as a patient avatar.

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Overall, our success rate in generating PDOs was quite high. Among the nine cases which failed to culture, two thirds were from the patients who received neoadjuvant chemotherapy. Neoadjuvant therapy has been shown to negatively affect the success rate of PDO generation [39]. We speculate the reason why tumor tissues from the patients who received neoadjuvant chemotherapy failed to generate organoids is because the tumors may already be undergoing chemotherapy-induced cell death. Accordingly, after we collected the tumor and isolated cells from the tumor, cell viability was too low for organoid culture. In addition, for the vast majority of tissues that were not successfully cultured, we observed that the time from surgical removal to culture was greater than 45 min, though some PDO models were successfully cultured after a delay of several hours. Nonetheless, mitigating the delay from surgical excision to tissue processing will undoubtedly improve the success rate of PDO creation. Other variables include the sample size and the percentage of viable cells within the specimen.

To illustrate how PDO data could be used in the future to predict clinical outcomes, tumor and normal PDOs were established from a patient with advanced high-grade serous endometrial carcinoma. Drug sensitivity testing reflected resistance to all agents given in the neoadjuvant setting. This raises the possibility of tumor evolution after initial exposure to drugs and brings into question the continued use of agents used in the neoadjuvant setting for ongoing adjuvant treatment, *i.e.*, after surgical removal of the tumor. We propose that drug sensitivity testing of PDOs may provide more insight into which agents are most effective at later timepoints. This is based on our proof-of-concept data that the PDO model successfully predicted the patient's clinical platinum and trastuzumab resistance despite her initial response to neoadjuvant chemotherapy.

Although PDOs have proved useful for many applications in basic research and contributed to biomedical advances [40], the road to real-life applications is long, with many technologies to be explored in this area. PDOs have not yet been widely used for translational studies yet due to some limitations [41]. For example, compared with cancer cell lines, organoid culture consumes more time and resources [40]. For most models we created, the amount of tumor tissue we received from the operating room was quite small, and we prioritized drug screening experiments. This precluded extensive molecular characterization for each model, such as comparison of biomarkers between the primary tumor and the PDO model. Given the potential for evolution with prolonged culture, we performed the bulk of experiments on passage 1–2 samples rather than expanding them to get enough cells for additional analyses. While we stored some organoids in liquid nitrogen, we found that the growth rate is significantly slower after cryopreservation. Another limitation is PDO culture may not reflect the tumor environment due to lack of stroma, blood vessels and immune cells [42]. Incomplete representation of intratumoral heterogeneity is another limitation for PDO culture. Many studies have proved that PDOs

can retain the heterogeneous genetic composition of the primary tumor [43,44]. However, each PDO is derived from a small part of the tumor and may not reflect different cell populations of the primary tumor. For example, Roerink et al. have shown the extensive genetic diversification in organoids derived from different regions of the same tumor using comprehensive genetic analysis [45]. Despite these limitations, organoids have emerged as a physiologically relevant ex vivo model to study cancer. In addition, there are several co-clinical trials ongoing in different cancer types that compare drug responses in PDOs to corresponding patient outcomes (e.g., NCT03979170, NCT04859166, NCT04555473).

In conclusion, PDOs provide a valuable preclinical model system to support new opportunities for personalized medicine. Our results demonstrate, first, that PDOs can be established in a high percentage of cases; second, that drug testing on PDOs can be performed in a timely manner and that PDOs have the potential to identify agents with the greatest efficacy in each individual case. This is an important first step in establishing a feasible functional treatment response platform to help more patients with gynecologic cancer receive precision therapeutic care.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/cancers13122901/s1>, Supplementary Methods: Protocol for Organoid Generation, Figure S1: Schematic presentation of procedures for the organoid culture of endometrial and ovarian cancer cells derived from patient tumors, Figure S2: Pretreatment biopsy specimen for ONC-6057, Figure S3: Additional H&E and HER2 IHC images of the ONC-6057 PDOs, Table S1: Characteristics of all the specimens attempted to be cultured as PDO models, including the paired normal tissue specimens, Table S2: Statistical analyses for drug screening in Figures 1 and 2.

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Conflicts of Interest: K.W.T. is a cofounder of Immortagen, Inc., Coralville, IA, USA. All other authors declare that they have no competing interests.

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Article

Advantages of Tyrosine Kinase Anti-Angiogenic Cediranib over Bevacizumab: Cell Cycle Abrogation and Synergy with Chemotherapy

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Abstract: Angiogenesis plays a crucial role in tumor development and metastasis. Both bevacizumab and cediranib have demonstrated activity as single anti-angiogenic agents in endometrial cancer, though subsequent studies of bevacizumab combined with chemotherapy failed to improve outcomes compared to chemotherapy alone. Our objective was to compare the efficacy of cediranib and bevacizumab in endometrial cancer models. The cellular effects of bevacizumab and cediranib were examined in endometrial cancer cell lines using extracellular signal-related kinase (ERK) phosphorylation, ligand shedding, cell viability, and cell cycle progression as readouts. Cellular viability was also tested in eight patient-derived organoid models of endometrial cancer. Finally, we performed a phosphoproteomic array of 875 phosphoproteins to define the signaling changes related to bevacizumab versus cediranib. Cediranib but not bevacizumab blocked ligand-mediated ERK activation in endometrial cancer cells. In both cell lines and patient-derived organoids, neither bevacizumab nor cediranib alone had a notable effect on cell viability. Cediranib but not bevacizumab promoted marked cell death when combined with chemotherapy. Cell cycle analysis demonstrated an accumulation in mitosis after treatment with cediranib + chemotherapy, consistent with the abrogation of the G2/M checkpoint and subsequent mitotic catastrophe. Molecular analysis of key controllers of the G2/M cell cycle checkpoint confirmed its abrogation. Phosphoproteomic analysis revealed that bevacizumab and cediranib had both similar and unique effects on cell signaling that underlie their shared versus individual actions as anti-angiogenic agents. An anti-angiogenic tyrosine kinase inhibitor such as cediranib has the potential to be superior to bevacizumab in combination with chemotherapy.

Keywords: endometrial cancer; bevacizumab; cediranib; patient-derived organoid models; 3D culture; personalized medicine; p53; vascular endothelial growth factor (VEGF)

1. Introduction

Endometrial carcinoma is the most common gynecologic malignancy in the United States. In 2020, over 65,000 women were diagnosed with endometrial cancer and nearly 13,000 women died of this disease [1]. Although the five-year survival rate of patients with early-stage endometrial cancer is relatively high, patients with advanced or recurrent endometrial cancer have a poor prognosis. Additionally, population studies have shown that endometrial adenocarcinoma is one of the only cancers in which prevalence and

mortality are increasing [2]. Recent progress has been made in defining the molecular biology of endometrial carcinoma, which has led to the use of targeted agents to treat this disease. The addition of targeted therapies including anti-angiogenics in conjunction with chemotherapy may be more effective than chemotherapy alone, as we have recently reported [3].

The Gynecologic Oncology Group (GOG, now NRG Oncology) has evaluated a series of anti-angiogenic agents in recurrent endometrial carcinoma. Bevacizumab (Avastin[®], Genentech) is a recombinant humanized antibody against vascular endothelial growth factor-A (VEGF-A), and it has been studied as a single agent in women with recurrent endometrial cancer in study protocol GOG-229E [4]. From this study, bevacizumab significantly improved PFS compared to additional treatment with chemotherapy [4]. GOG-229G, a Phase II trial of combination bevacizumab and temsirolimus (an mTOR inhibitor) was also deemed active in the treatment of recurrent endometrial carcinoma; however, the combination showed significant toxicity [5].

Cediranib is a small molecule multi-tyrosine kinase receptor inhibitor that targets VEGF receptors (VEGFR1/2/3), platelet-derived growth factor receptors (PDGFR α / β and c-Kit), and fibroblast growth factor receptor (FGFR1) [6,7]. GOG-229J was a study of cediranib as monotherapy in advanced endometrial carcinoma that, like bevacizumab, met the criteria for being considered an active agent in advanced endometrial cancer [8]. It was also found to be tolerable when administered as a single agent [8]. Other anti-angiogenic agents have also been investigated, but they have shown limited activity as single agents in unselected patients [9–12]. The GOG-86P trial of bevacizumab with chemotherapy in patients with advanced endometrial carcinoma did not show a statistically significantly increased PFS relative to historical controls when patients were analyzed as an entire cohort [13].

Preclinical studies have clearly demonstrated the activity of single-agent bevacizumab on a variety of tumor models, including endometrial carcinoma. In an orthotopic mouse model of endometrial cancer, bevacizumab significantly reduces tumor volume compared to control [14]. A previous study by our group also showed that bevacizumab inhibits the growth of endometrial cancer in a mouse xenograft model [15]. The anti-tumor activity of cediranib has been demonstrated in tumor xenograft models of various types of cancers including colon, lung, prostate, breast, and ovarian cancer [6,7,16]. In addition, cediranib decreases tumor vessel density and promotes vascular regression [6].

The vast majority of studies of antiangiogenic agents in endometrial cancer have focused on the inhibitory effects on the tumor vasculature. We hypothesized that therapeutic efficacy could also be derived from effects on the tumor cells. To date, cediranib has not been studied in combination with chemotherapy in endometrial cancer despite the documentation from GOG0229J that it has single-agent activity. The studies reported herein were designed to compare the impact of cediranib to bevacizumab to assess whether cediranib may also be effective as an anti-angiogenic and as an adjuvant to chemotherapy. Hence, we compared the efficacy of the two most active anti-angiogenic agents in endometrial cancer, bevacizumab and cediranib, on tumor cell survival and signaling. Whereas previous studies of bevacizumab activity were performed in vivo using xenograft models of endometrial cancer [14,15], herein we used immortalized endometrial cancer cell lines and patient-derived organoid models of endometrial cancer to specifically assess the impact of the agents on the tumor cells. Strengths and limitations of the various models of endometrial cancer have been recently reviewed [17]. Several well-characterized endometrial cancer cell lines are available for in vitro studies, including the poorly differentiated Hec50 and KLE cells. These cell lines are models of more aggressive endometrial cancer and harbor distinct mutations in *TP53* that are frequently detected in patient tumors [18]. This study also includes patient-derived organoid models of endometrial cancer whereby freshly resected tumor specimens are cultured in a three-dimensional model system [19,20]. As compared to patient-derived xenograft models, which also use fresh patient tumor tissue, patient-derived organoids are much more cost-effective with a higher

higher rate of successful culture [17]. Both patient-derived organoid and xenograft models retain intratumoral heterogeneity, which is lacking in immortalized 2D cell lines [17]. A rate of successful culture [17]. Both patient-derived organoid and xenograft models retain intratumoral heterogeneity, which is lacking in immortalized 2D cell lines [17]. A limitation of organoids as compared to xenografts is the absence of tumor vasculature. However, this provided a unique opportunity herein to study the tumor response to anti-angiogenic agents independent of vascular effects. However, this provided a unique opportunity herein to study the tumor response to anti-angiogenic agents independent of vascular effects.

2. Results

2.1. Results: Kinase Receptor Signaling Pathways Are Downregulated by Cediranib but Not Bevacizumab

2.1.1. Kinase Receptor Signaling Pathways Are Downregulated by Cediranib But Not Bevacizumab

We first established the inhibitory potential of bevacizumab and cediranib on ligand-stimulated extracellular signal-related kinase (ERK) activation in human vascular endothelial cells (HUVEC) and endometrial cells (Hec50) (Figure 1A). In contrast to robust inhibition of ERK phosphorylation at Thr202/Tyr204 by both bevacizumab and cediranib in HUVEC cells, the phosphorylation form of ERK displayed no significant induction with VEGF-A treatment and no obvious change with the addition of bevacizumab in Hec50 and KLE cells. These data suggest that the intracellular signaling impact of VEGF-A is not as robust in these tumor cells as compared to the known impact of VEGF-A on the vasculature of the surrounding tumor microenvironment (Figure 1A, middle and right panels) [6].

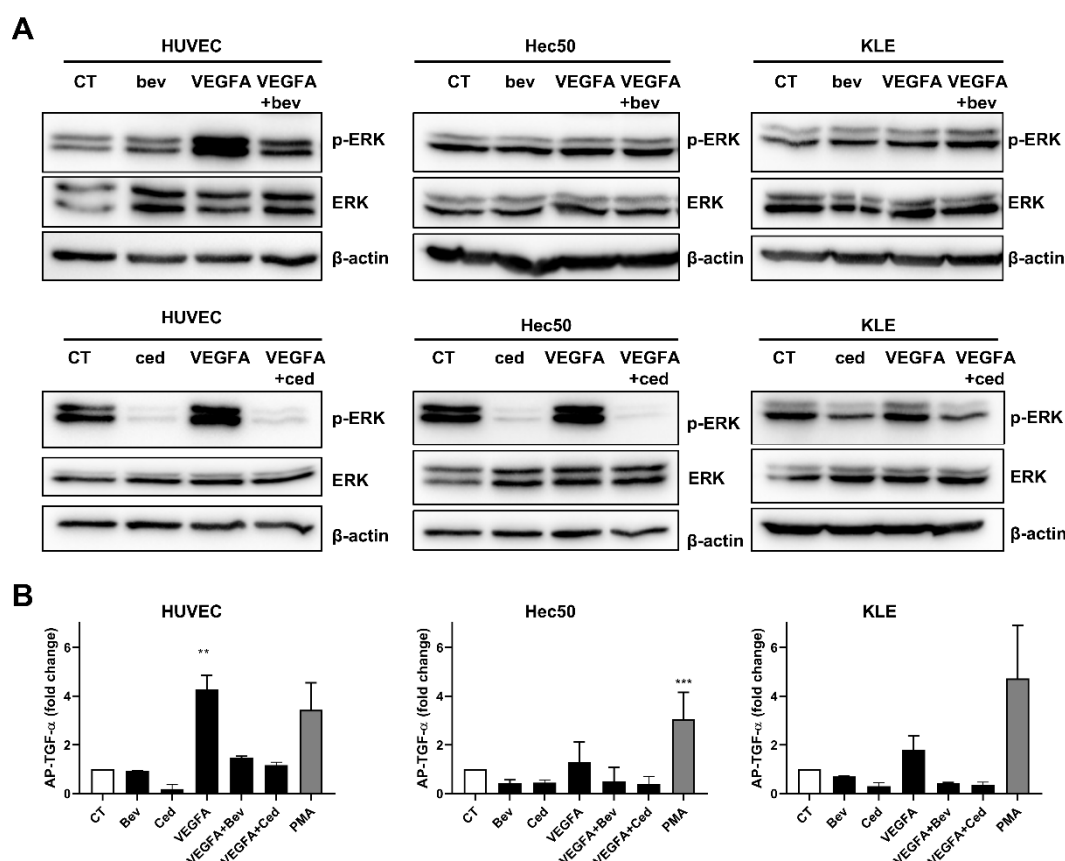


Figure 1. Impact of bevacizumab and cediranib on VEGF/ERK signaling in endothelial cells and endometrial cancer cells. (A) HUVEC, Hec50, and KLE cells were treated with vehicle control (CT), 1 μ M bevacizumab (Bev), 1000 ng/mL VEGF-A, or 1 μ M cediranib (Ced) for 1 hr, followed by assessment of ERK1/2 phosphorylation at Thr202/Tyr204 of total ERK expression by Western blotting. β -actin: loading control. (B) Cells were transfected with the alkaline phosphatase (AP)-tagged ADAM17 substrate TGF- α , and treated as in (A) for 1 hr. The change in soluble AP-TGF- α was assessed and presented as fold change compared to control (CT). PMA (25 ng/mL) served as a positive control for induction of AP-TGF- α shedding. ** $p < 0.01$; *** $p < 0.001$ versus CT by one-way ANOVA with Tukey's multiple comparisons test. ** $p < 0.01$; *** $p < 0.001$ versus CT by one-way ANOVA with Tukey's multiple comparisons test.

2.2. Cediranib but Not Bevacizumab Synergizes with Chemotherapy

assays, we next examined the impact of the 22 agents on endometrial cancer organoid models. We then examined the impact of these agents on cell originability in endometrial cancer models (Supplemental Table S1). The models represent diverse organ of origin of a cancer, from early-stage endometrial cancer to late-stage endometrial cancer, from early-stage endometrial cancer to late-stage endometrial cancer. We previously reported that the combination of tyrosine kinase inhibitors (TKIs) or antiangiogenic TKIs with the chemotherapy paclitaxel induces massive cell death via mitotic catastrophe [25–27]. We therefore screened the endometrial cancer organoid models for sensitivity to paclitaxel alone or in combination with bevacizumab or cediranib (Figure 2).

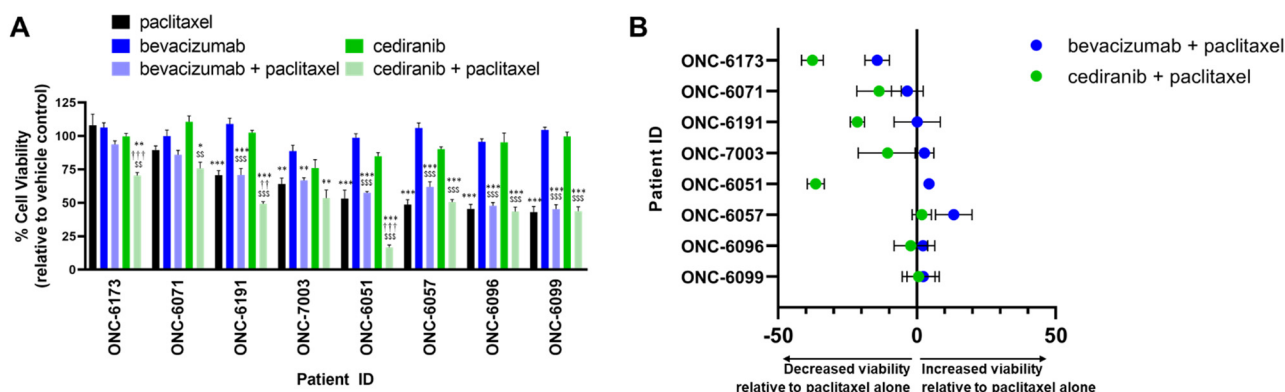


Figure 22. Effect of cediranib and bevacizumab on sensitivity to paclitaxel in patient-derived organoid cultures of primary endometrial tumors. Organoid cultures were treated with 110 nM paclitaxel, 11 μ M bevacizumab, 11 μ M cediranib, or the combination of paclitaxel with bevacizumab or cediranib for 72 hrs, followed by assessment of cell viability. (A) Data were calculated as the percent (%) cell viability as compared to vehicle control and plotted left-to-right by increasing sensitivity to single-agent paclitaxel. (B) The change in viability with the combination of paclitaxel with either bevacizumab or cediranib was calculated relative to paclitaxel alone. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus control; ++ $p < 0.01$, +++ $p < 0.001$ versus paclitaxel alone; \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ versus anti-angiogenic agent alone (bevacizumab for bevacizumab + paclitaxel or cediranib for cediranib + paclitaxel treated samples) by ordinary one-way ANOVA with Tukey's multiple comparisons test.

Compared to the untreated control, six of the eight patient-derived organoids exhibited a significant decrease in viability when treated with the paclitaxel, with a maximal decrease in viability of 57% by 72 h (Patient #ONC-6099) (Figure 2A). Both cediranib and bevacizumab as single agents had no impact on cell viability. The decrease in viability of combinatorial regimens was calculated relative to paclitaxel as a single agent.

combinatorial regimens was calculated relative to paclitaxel as a single agent (Figure 2B). In five of the models, cediranib but not bevacizumab further decreased cell viability when combined with paclitaxel as compared to treatment with paclitaxel alone (Figure 2B). In five of the models, cediranib but not bevacizumab further decreased cell viability when combined with paclitaxel as compared to treatment with paclitaxel alone (Figure 2B, ONC-6173–6071, 6191–7003 and 6051). Studies were then extended to well-characterized cell models of advanced endometrial cancer with varying baseline sensitivity to paclitaxel [26,27] (Figure 3). As in the organoid models, bevacizumab did not provide any additional cell killing when combined with paclitaxel (Figure 3A). Dose-response curves using either paclitaxel or cediranib in the presence of a set concentration of the opposing drug indicate synergy when the two agents are combined as compared to a single drug alone (Figure 3B,C). This trend was most pronounced in Hec50 cells, with no synergy observed in KLE cells treated with equivalent doses of the drugs (Figure 3B,C).

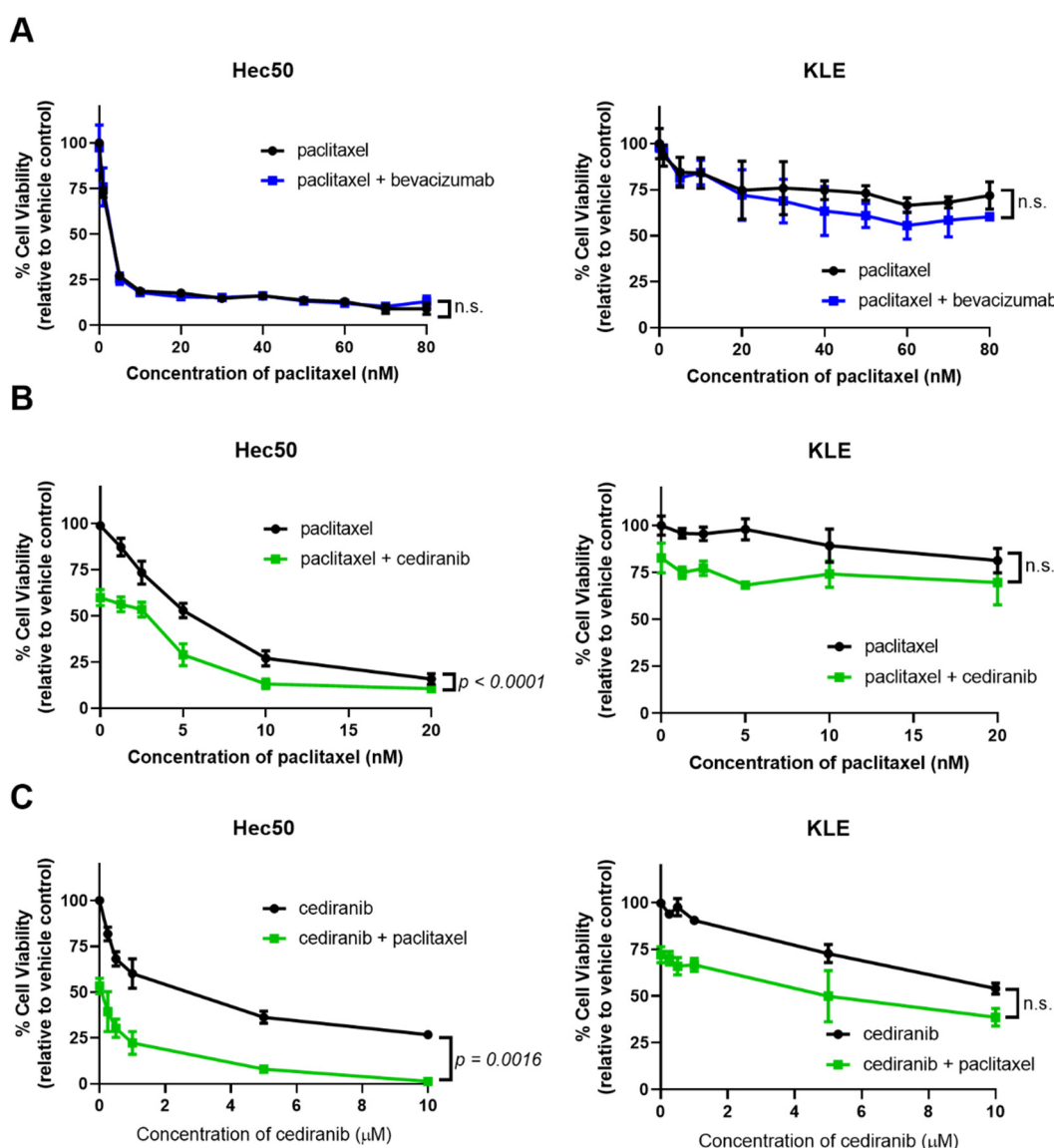


Figure 3. Cediranib but not bevacizumab increases sensitivity to paclitaxel in Hec50 endometrial cancer cells. (A) Hec50 (left) and KLE cells (right) were treated with increasing concentrations of paclitaxel in the absence or presence of bevacizumab for 72 h; cell viability was determined using WST-1 assay. (B) Hec50 (left) and KLE cells (right) were treated with increasing concentrations of paclitaxel in the absence or presence of 1 μ M cediranib for 72 h; cell viability was determined using WST-1 assay. (C) Hec50 (left) and KLE cells (right) were treated with increasing concentrations of cediranib in the absence or presence of 5 nM paclitaxel for 72 h; cell viability was determined using WST-1 assay. For the combinatorial treatments in (B,C) (green lines), the points on the y-axis at 0 reflect treatment with either cediranib alone (B) or paclitaxel alone (C). For example, single-agent cediranib at 1 μ M ((B), Hec50) results in a 40.0% decrease in cell viability as compared to untreated control. Statistical significance was assessed by two-way ANOVA with Sidak's post-hoc test. n.s.: not significant.

WST-1 assay. For the combinatorial treatments in Figure 3B,C (green lines), the points on the y-axis at 0 reflect treatment with vehicle (DMSO) or cediranib alone (3B) or paclitaxel alone (3C). For example, single-agent cediranib at 1 μ M (Figure 3B, Hec50) results in a 40.0% decrease in cell viability as compared to untreated control. Statistical significance was assessed by two-way ANOVA with Sidak's post-hoc test. n.s.: not significant.

2.3. The Combination of Cediranib But Not Bevacizumab with Paclitaxel Increases the Percentage of Cells in Mitosis

Flow cytometric measurements were performed to determine the effects of paclitaxel, cediranib, bevacizumab, and the combination treatment on cell cycle in Hec50 cells, which were more sensitive to the combination of cediranib and paclitaxel in Figure 3. Treatment with cediranib or bevacizumab alone had little effect on cell cycle distribution as compared to control treatment (Figure 4).

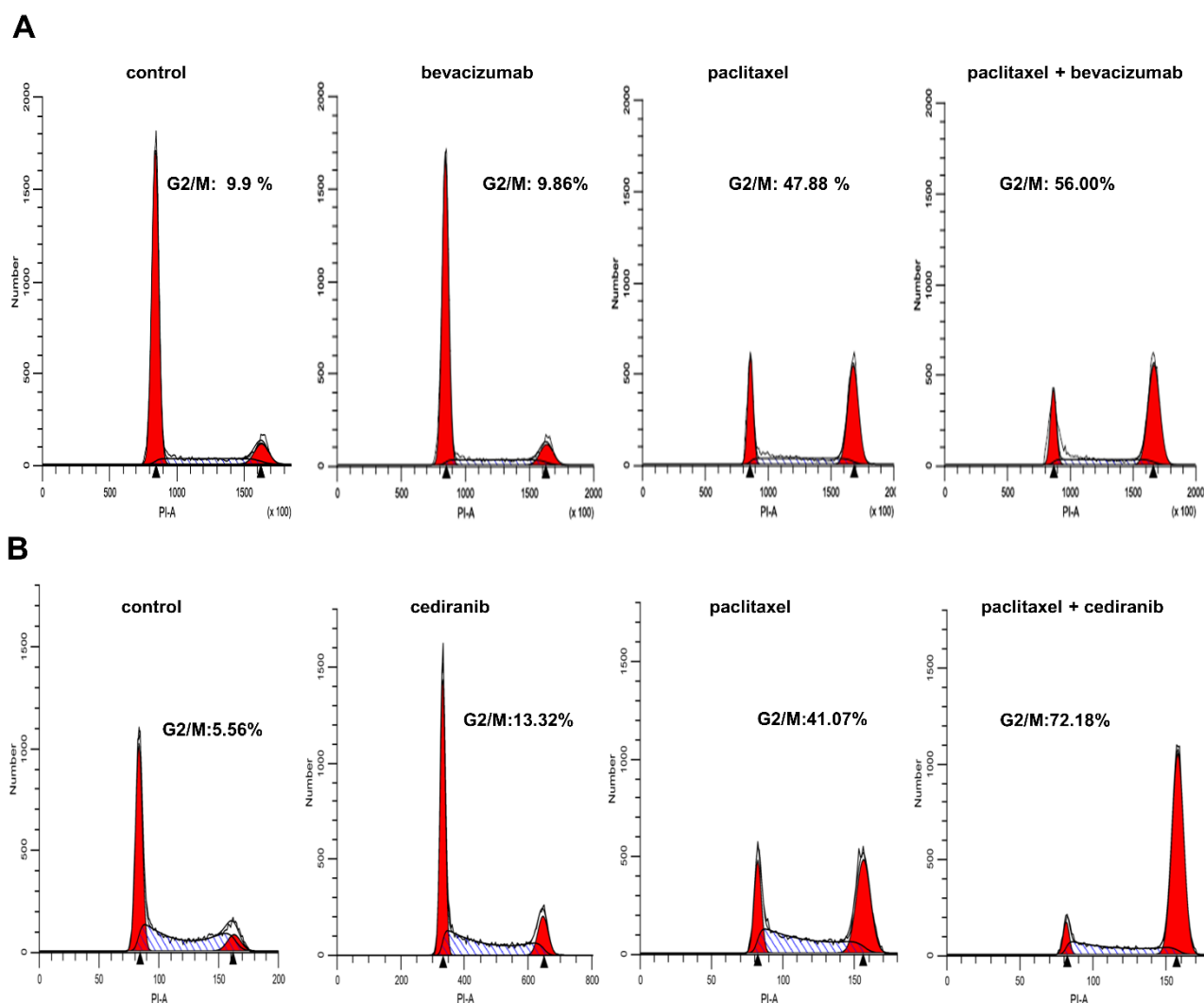


Figure 4. Comparison of the effect of cediranib and bevacizumab on cell cycle distribution in Hec50 cells treated with paclitaxel. (A) Cell cycle distribution in Hec50 cells treated with DMSO (Control), 14 nM paclitaxel, 1 μ M bevacizumab, or a combination of paclitaxel and 1 μ M bevacizumab for 24 h. (B) Cell cycle distribution for Hec50 cells treated with vehicle (DMSO), 14 nM paclitaxel, 1 μ M cediranib, or a combination of 14 nM paclitaxel and 1 μ M cediranib. Insets denote the percentage of cells in the G2/M phase of the cell cycle.

Paclitaxel is a microtubule stabilizing agent and thus is most effective in mitosis (M phase of the cell cycle). As expected, treatment with paclitaxel alone resulted in a marked shift in cells to the G2/M phase. The combination of paclitaxel and cediranib produced a profound increase in the accumulation of cells in mitosis as assessed by the percentage of cells in G2/M by flow cytometry compared to paclitaxel alone (Figure 4B). Yet there was minimal change in the percentage of cells in G2/M in bevacizumab + paclitaxel-treated cells (Figure 4A). These findings indicate that paclitaxel treatment in the presence of cediranib

results in a significant enhancement of the G2/M population by flow cytometry—the predominance of the cells being in M.

To better understand the mechanism of the mitotic cell death induction by paclitaxel and cediranib, we next examined the expression and post-translational modification of critical regulators of the G2/M checkpoint including cyclin-dependent kinase 1 (cdc2) and cell division cycle 25C (CDC25C). The effect of gefitinib, a tyrosine kinase inhibitor specific for EGFR, in combination with paclitaxel serves as a positive control based upon our previous work that gefitinib and paclitaxel promote premature entry to mitosis, especially in Hec50 cells that are devoid of functional p53 [27].

Treatment of Hec50 cells with cediranib in combination with paclitaxel decreased phosphorylation cdc2. De-phosphorylation of cdc2 is the final signaling step that opens the G2/M checkpoint, thereby allowing cells to enter mitosis. Cells that stop at G2/M have the potential to repair DNA prior to mitosis; however, when the checkpoint is abrogated as reflected by the dephosphorylation of cdc2, cells with damaged DNA enter mitosis and are more vulnerable to paclitaxel (Figure 5A).

The active form of CDC25C, a protein phosphatase responsible for dephosphorylating cdc2 (abrogating the G2/M checkpoint), was significantly increased by this treatment regimen as demonstrated by a loss of phosphorylation at Ser216 and a slower migrating band in the total CDC25C blot. These same signaling events were not observed with bevacizumab was combined with paclitaxel (Figure 5A), indicating a lack of G2/M checkpoint abrogation.

We also examined the effect of combinatorial treatment on KLE endometrial cancer cells, which we previously found to be resistant to paclitaxel + gefitinib [27]. Treatment with anti-angiogenic agents or gefitinib in combination with paclitaxel failed to override the G2/M cell cycle checkpoint, consistent with our previous findings.

To better understand why bevacizumab has no impact on cell survival or cell cycle progression in cancer cells, we performed a phosphoproteomic array using a panel of over 800 phosphorylation sites [28]. We detected similar trends in Akt and ERK/MAPK signaling and G2/M cell cycle checkpoints in response to bevacizumab or cediranib as single agents in Hec50 cells (Figure 5B). Changes in other cell cycle controllers included in the phosphoproteomic array are depicted in Figure 6A. Interestingly, bevacizumab as a single agent promoted more signaling events than cediranib, with only five changes shared between the two treatment groups (Figure 6B,C). Additionally, cediranib largely promoted decreased phosphorylation of signaling molecules, the expected therapeutic effect of a multi-TKI, whereas bevacizumab both increased and decreased phosphorylation events. We hypothesize that increased phosphorylation on pro-growth signaling molecules in response to bevacizumab treatment indicates a potential signal of developing cellular resistance.

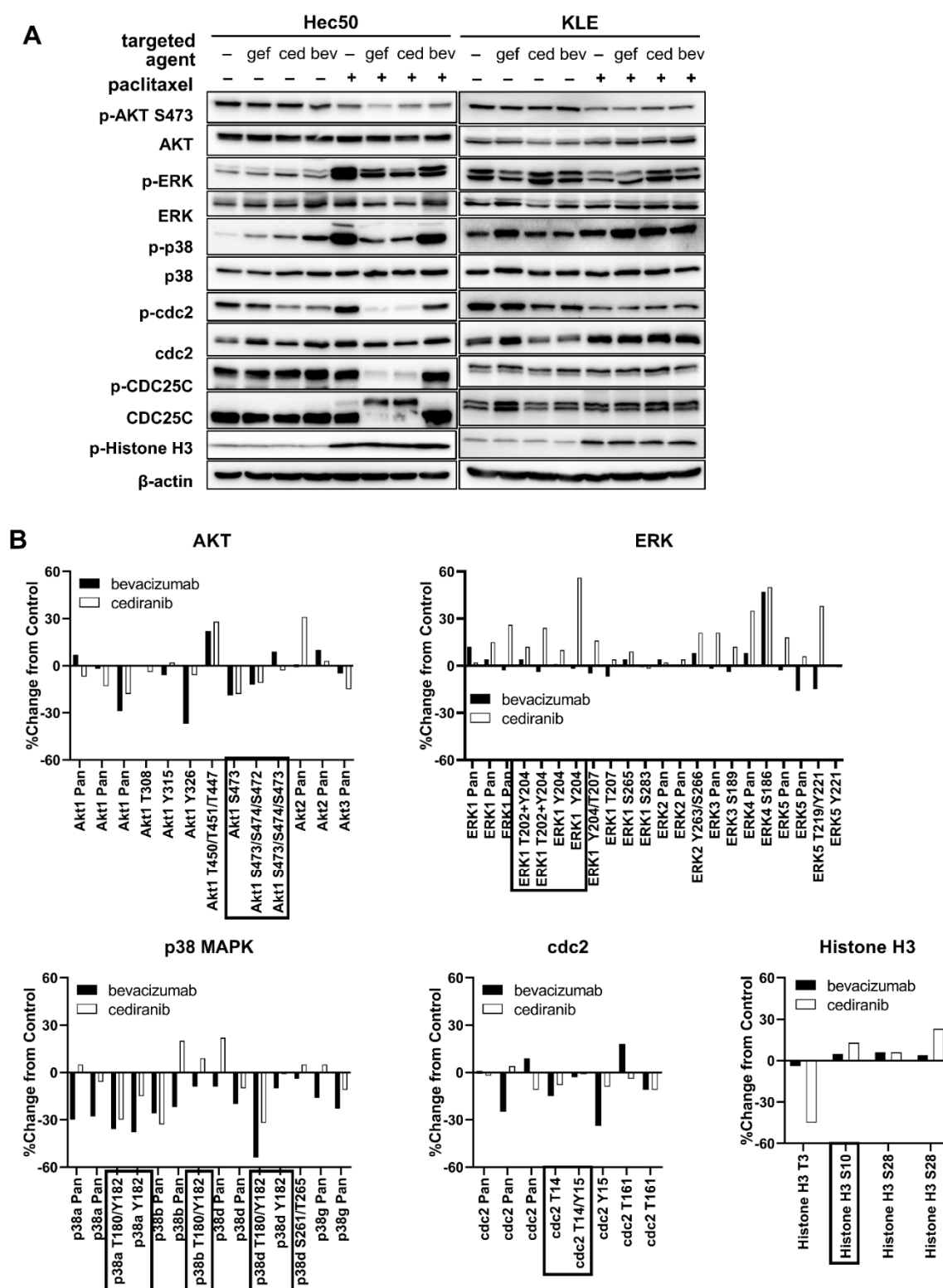


Figure 5. Molecular effects of antiangiogenic agents on C2/McM cell cycle controllers in endometrial cancer cell models (A) Hec50 (left) or KLE (right) were treated with the indicated agents either alone or in combination with paclitaxel for 24 hrs, followed by assessment of cell cycle controllers by Western blotting. Drug concentrations: 10 μ M gefitinib, 1 μ M cediranib, 1 μ M bevacizumab, 14 nM paclitaxel. (B) Expression or phosphorylation of indicated proteins was assessed by Kinex™ KAM-1325 Phosphoproteomic Antibody Microarray in Hec50 cells treated with 1 μ M bevacizumab or 1 μ M cediranib for 24 h. Data were calculated as the percent change from control (%CFC). Phosphosites corresponding to those queried in (A) are indicated with boxes. Pan: antibody total protein expression.

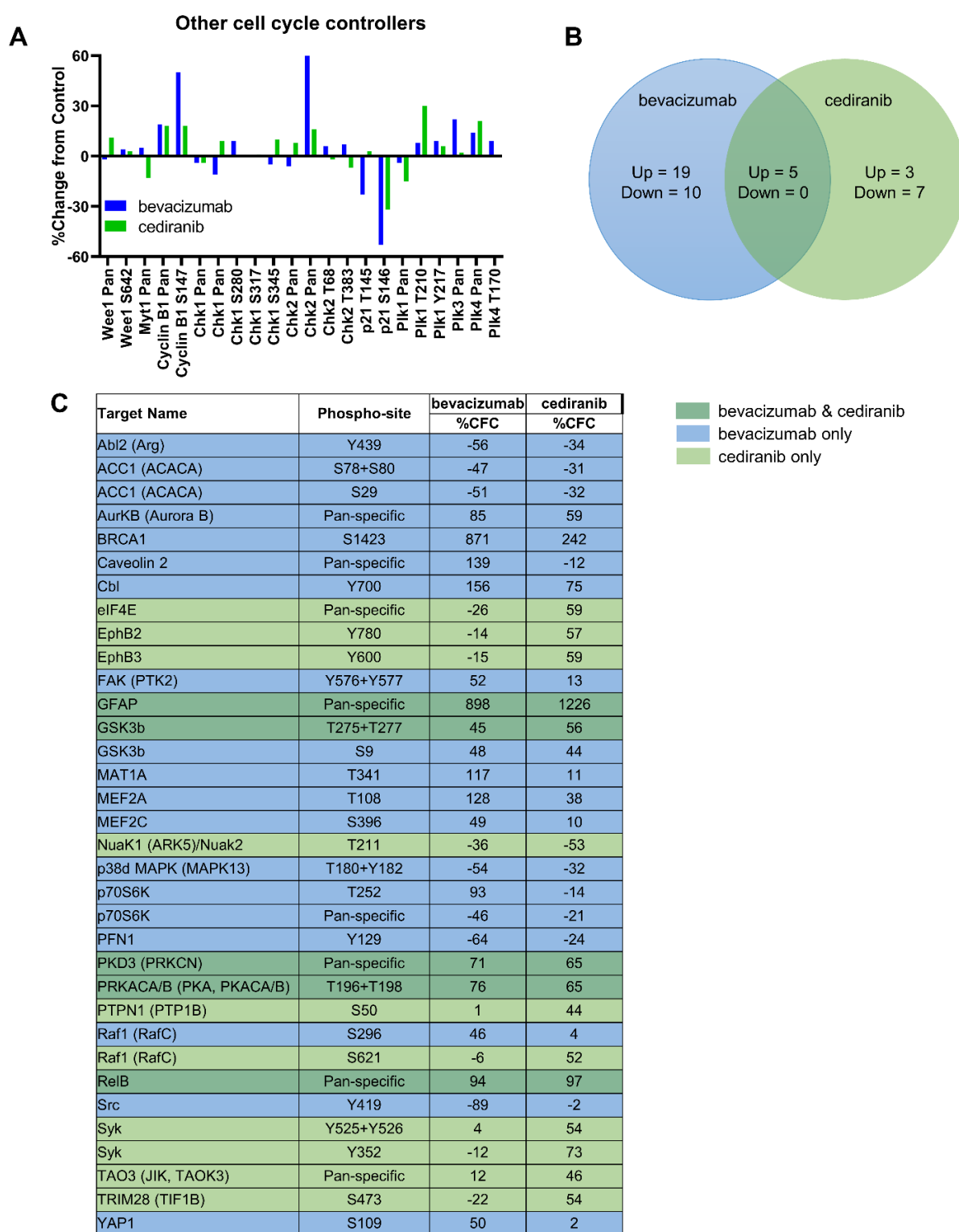


Figure 6. Bevacizumab regulates more signaling events than cediranib in endometrial cancer cells. Signaling events in response to single-agent bevacizumab or cediranib were analyzed by KinexTM KAM-1325 Phosphoproteomic Antibody Microarray after treatment of Hec50 cells with 1 μ M bevacizumab or 1 μ M cediranib for 24 h. (A) Depiction of changes in select cell cycle controllers. Data were calculated as the percent change from control (%CFC). “pan” indicates total protein expression. (B) Venn diagram of lead candidates in response to either bevacizumab or cediranib. Overlap indicates signaling events that are regulated by both agents. (C) Table of all lead up-regulated candidates identified based on phospho-%CFC ≥ 2 . Negative %CFC indicates a decrease as compared to control. Full results are provided as Supplemental Table S2. Negative %CFC indicates a decrease as compared to control. Full results are provided as Supplemental Table S2.

3. Discussion

Angiogenesis plays a crucial role in tumor development and metastasis, and cancer cells frequently upregulate VEGF-A expression to promote angiogenesis. VEGF-A binds to VEGFR1 and VEGFR2, and it is the main stimulator of tumor growth and dissemination. Accordingly, inhibitors of angiogenesis have been tested in clinical trials for a range of cancers, with FDA approval for 11 agents that inhibit either VEGF ligands or receptors [29]. Bevacizumab, which has been FDA-approved for cervical and ovarian cancers, specifically binds to the VEGF-A protein, and thereby inhibits vessel growth in the tumor. Cediranib is a tyrosine kinase inhibitor that targets VEGFR1, 2, 3, PDGFRs, and FGFRs. Cediranib is thought to be effective in the prevention of tumor progression, not only by inhibiting VEGFR2 activity and angiogenesis but also by concomitantly inhibiting VEGFR3 activity and lymphangiogenesis.

While the role of anti-angiogenic agents on tumor vascularization and endothelial cell growth is well-documented [6,7,14,16], few studies have interrogated the impact of anti-angiogenic agents on the properties of tumor cells themselves. In the present study, our objective was to compare the impact of angiogenic inhibitors on endometrial cancer cells. First, we found that cancer cells were not significantly responsive to treatment with exogenous VEGF-A despite the expression of VEGFRs 1 and 3. Bevacizumab, a monoclonal antibody specifically raised against VEGF-A, had little impact on cell viability or cell cycle progression alone as shown in Figures 2–4. In addition, bevacizumab did not synergize with chemotherapy, as we had previously found for other agents that target TKI signaling [25–27]. We provide evidence that synergy was related to the impact on the cell cycle when comparing bevacizumab, with its narrow range of activity, to a multi-targeted anti-angiogenic TKI such as cediranib. Thus, we propose that the ability of cediranib, but not bevacizumab, to inhibit multiple kinases and to dephosphorylate the G2/M gatekeeper cdc2 results in G2/M checkpoint abrogation, premature entry of tumor cells with damaged DNA into mitosis, and cell death through mitotic catastrophe. The significant enhancement of the percent of cells in G2/M when cells are treated with cediranib, and paclitaxel (as shown in Figure 4) is a potential mechanism of synergy. Another explanation for the enhanced response to cediranib versus bevacizumab is the robust expression of cediranib targets VEGFRs, PDGFRs, and FGFRs.

The phosphoproteomic array results reported are valuable as a readout of the numerous signaling events in response to bevacizumab and cediranib treatment at a single time point, 24 h (Figures 5 and 6). The inhibition of phosphorylation of multiple targets related to cediranib activity likely underlies its therapeutic effects as a tyrosine kinase inhibitor [7]. In contrast, the phosphorylation events were more often induced after bevacizumab treatment (Figure 5). Though enhanced phosphorylation is most commonly associated with signaling activation and proliferative signals, in our study, it may suggest that cells treated with bevacizumab instead induce resistance pathways by 24 h. These specific phosphorylation events are worthy of future investigation as they shed light on therapeutic agents that could block the resistance phosphorylation events when added to bevacizumab.

While no direct comparison of efficacy between cediranib and bevacizumab has been reported, we predicted cediranib would have greater benefit due to the additional blockade of all VEGFR isoforms as well as PDGF and FGF receptors. Isoforms of PDGFR and FGFR are expressed in endometrial tumors [30,31], with FGFR2 mutations occurring in ~13% of endometrial cancer cases [32]. We found that cediranib, as a multi-TKI inhibitor, had greater therapeutic effects, including blunting baseline ERK activation, synergizing with paclitaxel in organoid models and cell lines and the anticipated abrogation of the G2/M cell cycle checkpoint.

A limitation of this study, and studies of immortalized cell lines and patient-derived organoid models in general, is the absence of tumor microenvironment features, including immune cells and vasculature. Conversely, a strength of using these models is the ability to determine the impact of bevacizumab and cediranib specifically on the tumor cells without the confounding influence of the tumor microenvironment. We attempted to perform all

studies on organoids within two weeks of development (i.e., obtaining surgical specimens) in order to avoid the effects of tumor evolution in culture. Considering that the size of endometrial tumor specimens is typically very small as compared to other gynecologic cancer types (e.g., ovarian tumors), this approach precluded extensive molecular analyses. Therefore, as a companion, we assessed cell signaling using endometrial cancer cell lines. We did not extend studies to xenograft models of endometrial cancer since both bevacizumab and cediranib have been studied in this system previously by our group and others [6,7,14–16].

Findings from this preliminary study in preclinical models should be expanded to determine the mechanisms of differential sensitivity at the molecular level to cediranib, as evidenced by the lack of synergy when cediranib was combined with paclitaxel in KLE endometrial cancer cells (Figure 3) as well as some organoid models (Figure 2). We originally hypothesized that the mutations in p53, a well-established controller of the G1/S and G2/M cell cycle checkpoints, would predict for enhanced synergy. This hypothesis follows our recently published translational study of GOG-86P in which patients with mutations in *TP53* had significantly improved outcomes when bevacizumab was combined with chemotherapy as compared to other experimental agents [3]. However, the majority of endometrial organoid models used in this study had wild-type (WT) p53 (Figure 2), precluding a definitive analysis of the role of p53 in sensitivity.

4. Materials and Methods

4.1. Chemicals

Gefinitib, cediranib, bevacizumab, and paclitaxel were purchased from Selleck Chemicals, LLC (Houston, TX, USA) and suspended in DMSO.

4.2. Patient-Derived Organoid Models of Endometrial Cancer

All studies using human tissues have been approved by the University of Iowa Institutional Review Board (IRB), protocol #201809807. Patient tumor specimens were obtained within 30 min of surgical resection. Organoids were created per our protocol for ascites fluid [19], with modifications to digest tissue and isolate single cells. Specifically, freshly resected tissue was washed with 10 mL PBS and cut into small pieces. The minced fragments were collected in a 50 mL tube and digested in 5 mL AdDF+++ media (Advanced DMEM-F12 media with 1× Glutamax, 10 mM HEPES and Pen strep) supplemented with 2 U/mL Dispase II, 1 mg/mL collagenase P and 50 µg/mL Dnase I, then incubated at 37 °C for 0.5–1 h. Dissociated cells were filtered through a 40 µm cell strainer, centrifuged at 300× *g* for 10 min, washed twice with PBS, and pelleted. Erythrocytes were removed by incubating the dissociated cells with 2 mL red blood cell lysis buffer for 5 min at room temperature followed by an additional wash with 10 mL AdDF+++ and centrifugation at 300× *g* for 5 min. Finally, the cells were counted and embedded in Matrigel on ice and seeded on pre-warmed 24-well cell culture plates; 500 µL AdDF+++ media was added on the top of the Matrigel to each well.

4.3. Cell Culture

Hec50 cells were kindly provided by Dr. Erlio Gurpide (New York, NY, USA) [18] and grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco Corporation, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum. KLE cells were purchased from ATCC and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. Human umbilical vascular endothelial cells (HUVEC) were purchased from ATCC and grown in EGMTM -2 MV Microvascular Endothelial Cell Growth Medium (2 BulletKit, Lonza, Alpharetta, GA, USA). To ensure rigor and reproducibility, the identity of all cell lines was confirmed using the CODIS genotyping test (Cat. No. CL1003, Bio-Synthesis, Lewisville, TX, USA).

4.4. Western Blot

Cells were collected and lysed with NP-40 lysis buffer with protease inhibitors. Lysates were analyzed for protein expression/phosphorylation as described previously [25,27]. The following antibodies were used at the indicated dilutions; all antibodies were purchased from Cell Signaling (Danvers, MA, USA): anti-p-AKT-S473 (1:1000, #4060), anti-AKT (1:1000, #4685), anti-p-p44/42 MAPK (Erk1/2)-Thr202/Tyr204 (1:1000, #4370), anti-p44/42 MAPK (Erk1/2) (1:1000, #4695), anti-p-p38 MAPK-Thr180/Tyr182 (1:1000, #9211), anti-p38 MAPK (1:1000, #8690), anti-p-cdc2-Tyr15 (1:1000, #4539), anti-cdc2 (1:1000, #9116), anti-p-CDC25C-Ser216 (1:1000, #4901), anti-CDC25C (1:1000, #4688), anti-p-Histone H3-ser10 (1:1000, #53348).

4.5. Cell Viability Assay

4.5.1. Organoids

Viability of the tumor organoids following drug treatment was performed as previously described [19]. Briefly, patient-derived organoids were collected with organoid harvesting solution (Cultrex, Trevigen, Gaithersburg, USA), and then digested to single cells with TrypLE Express supplemented with 4 μ L 10 mg/mL DNase I stock and 4 μ L 10 mM Y-27632 stock. Single cells were suspended in AdDF+++ medium with 10% Matrigel and seeded at a density of 10,000 cells/well (50 μ L/well) in an ultra-low attachment 96-well U-bottom white plate. After 24 h, cells were exposed to paclitaxel (10 nM), bevacizumab (1 μ M), or cediranib (1 μ M) for 72 h at 37 °C. At the end of incubation, an equal volume of CellTiter-Glo 3D reagent (Promega) was added to each well and incubated for 25 min at room temperature. The luminescence was measured using the Gen5 Microplate Reader (BioTek, Winooski, VT, USA). All the tests were conducted in triplicate and data normalized to untreated control (set at 100% viability).

4.5.2. Cell Lines

Cell viability was determined by WST-1 assay as previously described [26]. Briefly, Hec50 and KLE cells were seeded into 96-well plates (10,000 cells per well) for 24 h and then cultured with increased concentrations of the drugs for an additional 72 h. Cell viability was evaluated using the cell proliferation reagent WST-1 (Roche, Pleasanton, Germany) according to the manufacturer's protocol. The absorbance was measured with a micro-plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All the tests were conducted in triplicate and data were normalized to untreated control (set at 100% viability).

4.6. Cell Cycle Analysis

Cell cycle analysis was performed by flow cytometry as described previously [25]. An equal number of cells were plated in 10 cm plates and treated with different drugs for 24 h as for cell viability assays. Cell pellets were harvested and suspended separately in Krishan's solution (3.8 mM sodium citrate, 0.014 mM propidium iodide, 1% NP-40, and 2.0 mg/mL RNase A). Cell suspensions were analyzed using a FacScan Flow Cytometer (Becton, Dickinson and Company, San Jose, CA, USA) and data were analyzed by CellQuest software version 3.3.

4.7. Shedding Assay

HUVEC, Hec50, and KLE cells were grown to 70–80% confluence and serum-starved in Opti-MEM for one hour prior to transfection. Transfection of 1 μ g alkaline phosphatase (AP)-tagged transforming growth factor (TGF)- α were performed according to manufacturer's protocols using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). Cells were starved for at least four hours before the stimulation. Recombinant human vascular endothelial growth factor (VEGF-A) was obtained from R&D Systems (Minneapolis, MN, USA) and used at a concentration of 100 ng/mL. The concentration of bevacizumab was 1 μ g/mL. PMA (phorbol-12-myristate-13-acetate) and the AP-tagged TGF- α plasmid have been previously published [33]. Evaluation of AP activity was determined by the color-

metric assay as described previously [34]. Briefly, the ratio between the total AP activity in the supernatant and the total AP activity in the cell lysate plus the supernatant was computed for normalization. The presented ratios reflect the relative proteolytic activity of a given sheddase toward the AP-tagged ligand TGF- α . No AP activity was detected in the conditioned media of untransfected cells.

4.8. Phosphoproteomic Microarray

Hec50 cells were plated in 15 cm plates and treated with DMSO, 1 μ M bevacizumab, or 1 μ M cediranib for 24 h. Cell pellets were collected and lysed with Kinexus Lysis Buffer with chemical cleavage. The microarray assay and data analysis were performed with Kinex™ KAM-1325 Antibody Microarray Kit by Kinexus Bioinformatics Corporation (Vancouver, BC, Canada). To qualify as a lead, the percent change from control (%CFC) value should be at least 45% higher or lower with fluorescent signals that were at least 1000 counts [28].

4.9. Reverse Transcription and Quantitative Polymerase Chain Reaction

Total RNA was purified from Hec50co and KLE cells using the RNeasy Plus RNA purification kit (QIAGEN, Germantown, MD, USA). Yield and purity were assessed on a Trinean DropSense 16 spectrophotometer and an Agilent Model 2100 Bioanalyzer in the Genomics Division of the Iowa Institute of Human Genetics (IIHG). The mean RNA Integrity Number (RIN) was 9.5 [35]. Next, 500 ng of QC qualified total RNA from each cell line was reverse transcribed using oligo-dT primers in the SuperScript III kit following the manufacturer's recommendations (Invitrogen). The resulting cDNAs were then amplified in the presence of SYBR Green (Thermo Fisher, Waltham, MA, USA) on an Applied Biosystems Model 7900HT platform in the Genomics Division of the Iowa Institute of Human Genetics (IIHG). Locus-specific primers are shown below (Table 1). Raw expression values (Ct) were normalized (Δ Ct) [36] against the endogenous 18S rRNA control.

Table 1. Primer sequences.

Locus	Primer Sequences	Amplicon	Tm (°C) *
VEGFA	For: GGGCAGAATCATCACGAAGT	269 bp	55.0
	Rev: AGGAAGCTCATCTCTCTATGT		54.7
VEGFR1	For: GGACAGTAGAAAGGGCTTCATC	251 bp	55.2
	Rev: CAGGGTAACTCCAGGTCATTG		55.4
VEGFR2	For: GTGGTCTCTCTGGTTGTGTATG	235 bp	55.0
	Rev: CCTCCACACTTCTCCATTCTTC		55.1
VEGFR3	For: CGAAAGTGCATCCACAGAGA	239 bp	54.9
	Rev: AGAGAGAAGATCTCCCAGAGAAG		54.9
FGFR1	For: AGGAACCTTTCAAGCTGC		
	Rev: CATCATGTACAGCTCGTTG		
FGFR2	For: ATGAGGAATACTTGGACCTC		
	Rev: TTAACACTGCCGTTTATGTG		
FGFR3	For: GAAGATGCTGAAAGACGATG		
	Rev: GCAGGTTGATGATGTTTTTG		
FGFR4	For: CTGAGGACAATGTGATGAAG		
	Rev: CCGTTGCTGGTTTTCTTCTTATAG		
PDGFRA	For: GACTTTCGCCAAAGTGGAGGAG	121 bp	58.0
	Rev: AGCCACCGTGAGTTCAGAACGC		62.0

Table 1. Cont.

Locus	Primer Sequences	Amplicon	Tm (°C) *
PDGFRB	For: TGCAGACATCGAGTCCTCCAAC	108 bp	59.0
	Rev: GCTTAGCACTGGAGACTCGTTG		57.8
18S rRNA	For: AACTTTCGATGGTAGTCGCCG	104 bp	57.3
	Rev: CCTTGGATGTGGTAGCCGTTT		57.6

* Tm calculated in the presence of 1.5 mM MgCl₂.

4.10. Statistical Analysis

Data were analyzed using GraphPad Prism software (GraphPad Software Version 0.0.0 (121), San Diego, CA, USA). Statistical significance of differences was determined using one-way ANOVA with Sidak's post-hoc test (for comparison of significance between two treatment groups with multiple independent treatment concentrations; Figure 3) or two-way ANOVA with Tukey's post-hoc test (for comparisons of means of multiple groups; Figure 2A). The normal distribution of data was assessed using the likelihood test. All values are expressed as mean $\pm$ standard deviation (SD) of at least three independent experiments unless otherwise indicated.

5. Conclusions

In conclusion, the standard of care for endometrial cancer has not changed beyond chemotherapy, the incidence is on the rise due to the obesity epidemic, and outcomes are worse now than in the 1970s. The data presented in this report are designed to identify agents that should be advanced into clinical trials to improve outcomes for women with endometrial cancer. Based on our findings, we put forth that cediranib should be investigated in future clinical trials in endometrial cancer in the upfront setting in combination with chemotherapy. Specifically, our data suggest that the anti-angiogenic agent cediranib may have additional advantages over bevacizumab as an anti-cancer therapeutic due to its dual effects on tumor cells and vasculature. Our use of endometrial tumor tissue cultured as functional three-dimensional organoids provides highly translational information on the efficacy of anti-angiogenic agents in tumor cells. These data set the stage for future clinical trials to evaluate cediranib in combination with chemotherapy as a treatment for women with endometrial cancer.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/ph14070682/s1>: Figure S1: Expression of bevacizumab and cediranib targets, Table S1: Patient characteristics for endometrial cancer organoids, Table S2: Raw data for phosphoproteomic array.

Author Contributions: Study concept and design: J.B., K.W.T. and K.K.L.; Provision of materials: J.B., A.M.N., D.A.S., K.L.C., K.W.T. and K.K.L. coordinated tumor specimen collection for organoid culture models. J.B., A.M.N. generated organoid models; Acquisition of data: J.B., E.J.D., Y.Z. characterized organoid models. J.B. performed drug response experiments in cell lines and organoid models. A.M.N., H.A.L. performed chart review for clinical characteristics. J.B. performed Western blots and/or phosphoproteomic analysis. G.D. performed AP-TGF- α shedding assays. Y.Z. performed flow cytometry; Analysis and interpretation: J.B., G.D., T.M., K.W.T., K.K.L. analyzed data and/or performed statistical analyses; Manuscript writing: J.B., E.J.D., G.D., T.M., K.W.T., K.K.L.; Critical review of the manuscript: J.B., G.D., Y.Z., E.J.D., H.A.L., A.M.N., K.L.C., D.A.S., T.M., K.W.T., K.K.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the University of Iowa (protocol code #201809807 approved on 8 March 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Article

The Synthetic Curcumin Analog HO-3867 Rescues Suppression of PLAC1 Expression in Ovarian Cancer Cells

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Abstract: Elevated expression of placenta-specific protein 1 (PLAC1) is associated with the increased proliferation and invasiveness of a variety of human cancers, including ovarian cancer. Recent studies have shown that the tumor suppressor p53 directly suppresses PLAC1 transcription. However, mutations in p53 lead to the loss of PLAC1 transcriptional suppression. Small molecules that structurally convert mutant p53 proteins to wild-type conformations are emerging. Our objective was to determine whether the restoration of the wild-type function of mutated p53 could rescue PLAC1 transcriptional suppression in tumors harboring certain *TP53* mutations. Ovarian cancer cells OVCAR3 and ES-2, both harboring *TP53* missense mutations, were treated with the p53 reactivator HO-3867. Treatment with HO-3867 successfully rescued PLAC1 transcriptional suppression. In addition, cell proliferation was inhibited and cell death through apoptosis was increased in both cell lines. We conclude that the use of HO-3867 as an adjuvant to conventional therapeutics in ovarian cancers harboring *TP53* missense mutations could improve patient outcomes. Validation of this conclusion must, however, come from an appropriately designed clinical trial.

Keywords: ovarian cancer; OVCAR3; ES-2; placenta-specific protein 1 (PLAC1); p53; HO-3867; steric hindrance; reactivator



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1. Introduction

Placenta-specific protein 1 (PLAC1) is a unique 212 amino acid secreted cell surface protein. PLAC1 was initially identified because it is located in a region of the human and mouse X-chromosome that contains other loci suspected of being involved in fetal and placental pathologies [1]. Under normal circumstances, the expression of PLAC1 is almost exclusively limited to the apical surfaces of placental trophoblasts [2]. Placenta-specific expression of PLAC1 has led to its implication in a variety of gestational disorders, including pre-term birth and preeclampsia [3–15]. In the two decades since PLAC1 was initially reported, many papers have demonstrated its co-opted expression in numerous human cancers [16–38]. In all of these studies, increased PLAC1 expression is linked to increased invasiveness, proliferation and/or aggressiveness, as well as poor prognosis, though the precise mechanisms remain poorly defined. Regardless, it is well established that *PLAC1* is an oncogene in addition to being a fetal-placental gene.

Studies of the regulation of PLAC1 expression identified two promoters, termed P1 (distal), located at exon 1, and P2 (proximal), located at exon 4. Transcripts are produced from both promoters simultaneously in cells that express PLAC1 mRNAs [39,40]. However, in placental tissues, *PLAC1* is predominantly transcribed from the P2 promoter, while in tumors and fetal tissues, *PLAC1* is predominantly transcribed from the P1 promoter. Chen et al. [39] identified RXR α and LXR β as promoters of P1 transcription, and later, Chen et al. [41] showed in vitro

that p53 protein suppresses *PLAC1* transcription by blocking the binding of these transcription factors to the *PLAC1* P1 promoter. We subsequently confirmed the p53 suppression of *PLAC1* transcription in a high-grade serous ovarian cancer (HGSOC) tumor panel [32]. However, in that study, we found that *PLAC1* transcriptional suppression was dependent upon the mutation status of the *TP53* gene. Specifically, only wild-type p53 suppressed *PLAC1* transcription, while any *TP53* mutation appears to permit P1 *PLAC1* transcription. We identified a non-canonical p53 binding site just upstream from the P1 RXR α and LXR β binding sites. These results led us to propose that P1 *PLAC1* transcriptional suppression is due to steric hindrance when wild-type p53 is bound. Conversely, P1 *PLAC1* transcription in the presence of mutant p53 is permitted due to the failure of mutated p53 to bind and block the RXR α and LXR β binding sites.

Recognition that mutant p53 permits P1 *PLAC1* transcription in HGSOC tumors and that increased *PLAC1* expression in these cancers leads to poor patient outcomes suggested that altering P1 *PLAC1* transcription can be accomplished by targeting p53 itself. *TP53* harbors both missense mutations, which result in single amino acid changes, as well as frameshift/splice site mutations that result in the loss of protein expression. p53 missense mutants can be targeted by molecules that reactivate their wild-type function. One agent that appears to be well suited to this task is the synthetic curcumin analog HO-3867. In the most extensive study to date, the treatment of various cancer cells harboring non-truncating *TP53* mutations with HO-3867 restored the wild-type transcriptional regulation of a number of key p53 target genes [42]. Herein we report that HO-3867 treatment rescues *PLAC1* transcriptional suppression in *TP53*-mutated ovarian cancer cells. Reestablishment of *PLAC1* downregulation via p53 reactivation could be used in combination with other therapies to improve outcomes for patients presenting with ovarian carcinomas harboring missense *TP53* mutations.

2. Results

2.1. HO-3867 Treatment of Ovarian Cancer Cells Restores Suppression of *PLAC1* Transcription

Our studies were carried out on two well-characterized ovarian cancer cell lines that contain *TP53* missense mutations, OVCAR3 and ES-2. OVCAR3 cells contain the frequent p53 gain-of-function mutant R248Q and were derived from a patient presenting with high-grade serous ovarian cancer, the most common and deadly type. ES-2 ovarian cancer cells are a model of clear-cell ovarian carcinoma and harbor a different p53 binding domain mutation, S241F. While not regarded as a gain-of-function mutant, S241F is classified as pathogenic in COSMIC (<https://cancer.sanger.ac.uk/cosmic> (accessed on 11 October 2019)) and is a DNA contact mutant overrepresented in ovarian cancers [43].

First, the OVCAR3 cells were exposed to 2 μ M and 5 μ M HO-3867 for 24 h and 48 h. *PLAC1* expression relative to untreated OVCAR3 cells is shown in Figure 1. In all cases, HO-3867-treated cells displayed reduced *PLAC1* transcription. Moreover, this suppression was focused at the P1 promoter.

Treatment of the ES-2 cells with 2 μ M and 5 μ M HO-3867 for 24 h and 48 h produced a similar, though not as dramatic, rescue of *PLAC1* transcriptional suppression (Figure 2). Again, transcriptional suppression appeared to be focused on transcripts originating from the *PLAC1* P1 promoter.

2.2. HO-3867 Treatment of Both OVCAR3 and ES-2 Ovarian Cancer Cells Results in a Reduction in Proliferation and an Increase in Apoptosis

Given the importance of p53 in proliferation and apoptosis, we next assessed the functional effect of HO-3867 exposure on both the OVCAR3 and ES-2 cells. Overall, the HO-3867 treatment revealed a dose-dependent decrease in proliferation and a dose-dependent increase in apoptosis in both cell lines (Figure 3). Specifically, treatment of the ES-2 cells with HO-367 showed decreased proliferation at 1.25 μ M and 2.5 μ M with no change in apoptosis, whereas with higher doses of HO-3867 (5 μ M and 10 μ M), the ES-2 cells had decreased proliferation and increased apoptosis. Interestingly, HO-3867 had a greater effect on the ES-2 cells compared to the OVCAR3 cells for both proliferation and apoptosis, especially at higher concentrations of HO-3867. The results indicate that HO-3867 is capable of arresting cell

growth and inducing cell death through the reactivation of p53 and that the effect of HO-3867 is not only cell-type-specific but also putatively dependent on p53 mutation status.

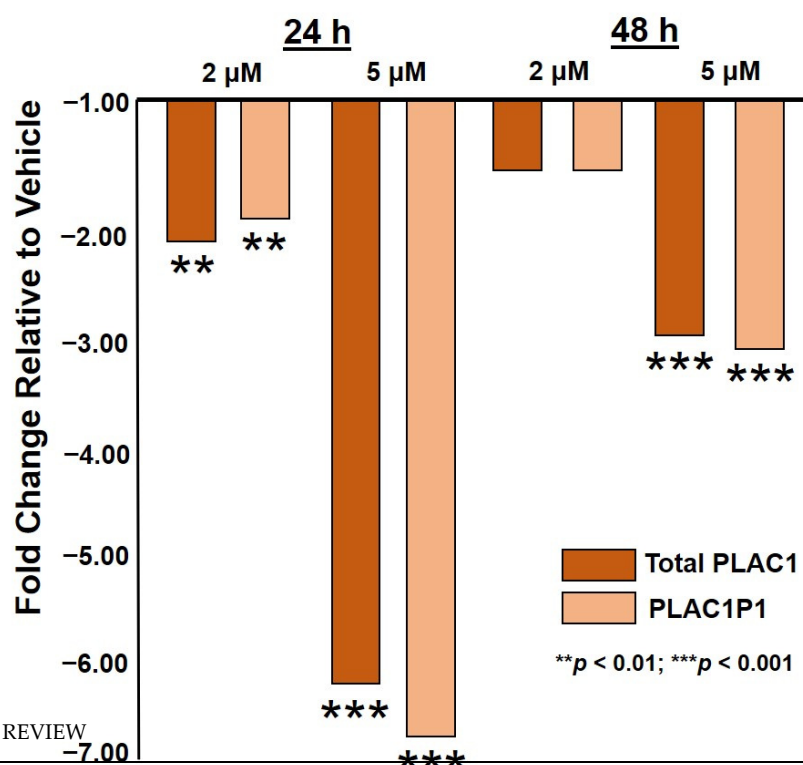


Figure 1. Total PLAC1 and PLAC1P1-specific expression in OVCAR3 ovarian cancer cells treated with 2 μM and 5 μM HO-3867 for 24 h and 48 h.

Figure 1. Total PLAC1 and PLAC1P1-specific expression in OVCAR3 ovarian cancer cells treated

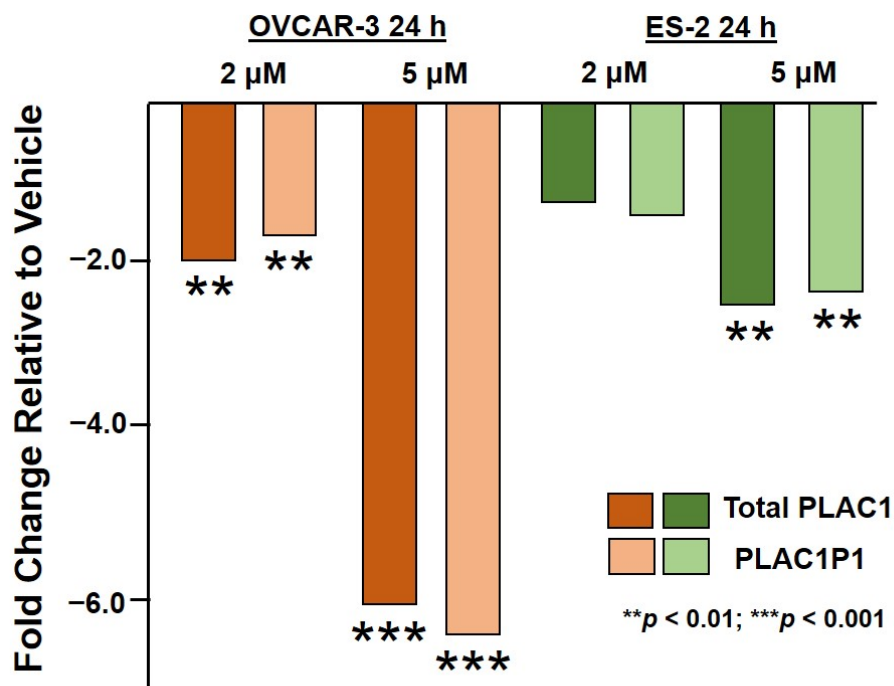


Figure 2. Total PLAC1 and PLAC1P1-specific expression in ES-2 ovarian cancer cells treated with 2 μM and 5 μM HO-3867 for 24 h. ES-2 cells exposed to 2 μM HO-3867 for 48 h did not yield RNA of sufficient quality to perform qPCR and ES-2 cells exposed to 5 μM HO-3867 for 48 h did not survive. The 24 h results for OVCAR3 cells are shown on the left for comparison.

2.2. HO-3867 Treatment of Both OVCAR3 and ES-2 Ovarian Cancer Cells Results in a Reduction in Proliferation and an Increase in Apoptosis

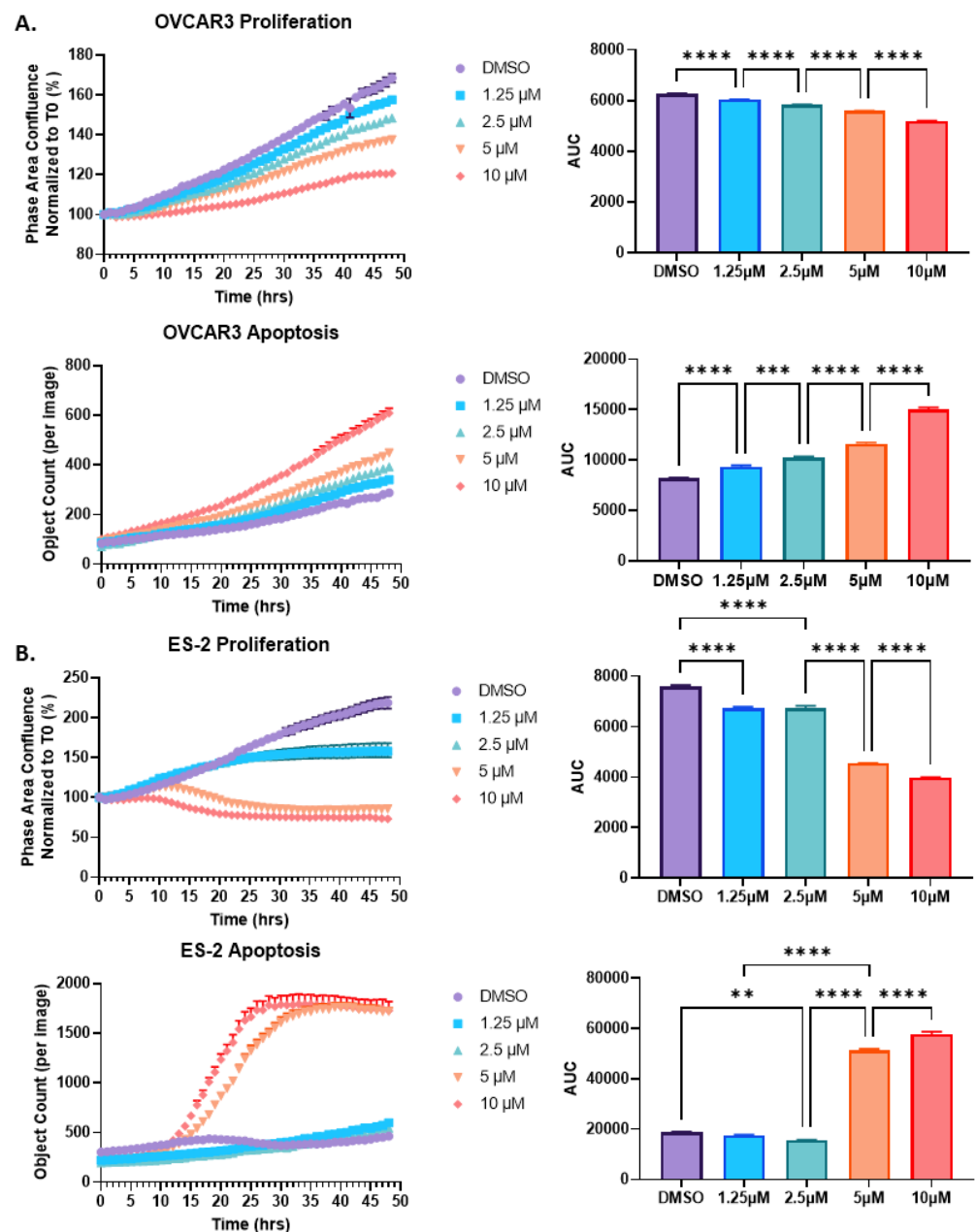


Figure 3. Proliferation and apoptosis in OVCAR3 (A) and ES-2 (B) cells. Cells were treated with increasing amounts of HO-3867, as shown. Proliferation was measured by phase contrast, and apoptosis was assessed by caspase 3/7 activation. Cells were monitored hourly for 48 h using the Incucyte S3 live-cell imaging platform. Area under the curve (AUC) values were calculated automatically by the Incucyte Software and analyzed via ANOVA. Significance values are indicated as ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3. Discussion

It has long been recognized that a TP53 mutation is a molecular vulnerability awaiting therapeutic exploitation [44–47]. Wild-type p53 has a wide variety of cellular roles, including being a powerful tumor suppressor. Thus, it follows that the re-introduction of wild-type p53 into tumor cells would result in a positive anti-tumor effect [48]. However, attempts to do this have so far proved unsuccessful [49,50]. For example, gene therapy with p53 failed in clinical trials due to its insufficient accumulation in tumors itself [49]. A promising alternative strategy would be to introduce a compound that is specific for mutant p53 and would also restore wild-type function. To this end, a number of such compounds have been developed, including quinuclidines, 2-sulfonylpyrimidines, zinc

compounds have been developed, including quinuclidines, 2-sulfonylpyrimidines, zinc metallochaperones and thiosemicarbizones [51]. Some of these are well known, such as COTI-2 [52], PRIMA1 and PRIMA1^{met}, now designated as APR-246 [53,54]. Here, we have chosen to assess the less well-known synthetic diarylidenylpiperidone curcumin analog, designated HO-3867, for the purpose of rescuing the suppression of the expression of the oncogene *PLAC1* in ovarian cancer cells harboring a missense *TP53* mutation.

HO-3867 binds p53 at Cys182 and Cys277, creating a “clamp” that holds the β -sandwich in place in a proper DNA-binding configuration while also stabilizing the Zn^{++} binding pocket [42]. The mature p53 protein in this configuration is thus able to function like wild-type p53, including in binding DNA and inducing the transcription of canonical p53 target genes. This was shown by Madan et al. [42] in a panel of 29 human cancer cell lines representing 11 different cancers. These cell lines harbor a total of 26 different missense *TP53* mutations, including six classified as “gain of function” mutants [55]. The effect of exposing these cell lines to HO-3867 was assessed via qPCR expression assays on fourteen known p53 transcriptional target loci [42,56,57]. In the vast majority of these 406 assays (29 cell lines by 14 loci), HO-3867 exposure rescued the appropriate up- or down-regulation of transcription. Indeed, the overall pattern of transcriptional changes was highly significant (5×10^{-211}) [42]. Our study adds to the literature by providing evidence for the appropriate down-regulation of another p53-regulated gene, *PLAC1*, as well as demonstrating the effect in ovarian cancer cells, which were not included in the Madan et al. study [42].

A model describing the effect of HO-3867 exposure on *PLAC1* transcription in cancer cells harboring DNA-binding domain missense mutations is presented in Figure 4. In this model the P1 promoter is sterically blocked when wild-type p53 binds to its nearby binding site. This leads to reduced *PLAC1* transcription. However, mutant p53 is unable to bind to that site, thus opening up *PLAC1* transcription. Exposure to HO-3867 will, in some mutants, restore wild-type configuration, once again sterically blocking the binding site and reducing *PLAC1* transcription.

Where ovarian cancer has been the focus of the effect of HO-3867 is a series of papers originating from The Ohio State University [58–61]. In those papers, it has been demonstrated that HO-3867 is cytotoxic to ovarian cancer cells through the induction of apoptosis and the abrogation of cisplatin resistance. Their primary readout was STAT3 protein expression and STAT3 phosphorylation. It should be noted that STAT3 is also a direct transcriptional target of p53 [56], though it remains unclear if the p53 regulation of STAT3 is the mechanism of action of HO-3867. Thus, it is possible that the changes in proliferation and apoptosis in our study may be due to the restoration of p53 regulation of genes in addition to *PLAC1*.

Finally, while it can be argued that the treatment of ovarian cancer patients with HO-3867—preferably as an adjuvant to conventional therapies—could improve outcomes for these patients, its effect is only possible for those cancers that harbor a *TP53* missense mutation. Obviously, nonsense mutants will often result in the truncation of transcripts that are likely to be eliminated by nonsense-mediated RNA decay [62] or another mechanism that would render the cells devoid of p53 protein expression (i.e., p53 null). Further, the small number of *TP53* missense mutations that occur outside the DNA-binding domain will also not be rescued by HO-3867, as the mechanism of HO-3867 action would not, for example, affect p53 tetramerization. However, taking all of the potential exceptions to the effect of HO-3867 exposure on ovarian cancer into account, there remains around 50% of all ovarian cancers that will qualify. Given the fact that the American Cancer Society estimates that there are roughly 22,000 new cases of ovarian cancer in the United States each year, some 11,000 patients could potentially benefit from adding HO-3867 to their treatment regimens. Naturally, the efficacy of using such an adjuvant therapy can only be assessed through an appropriately designed clinical trial.

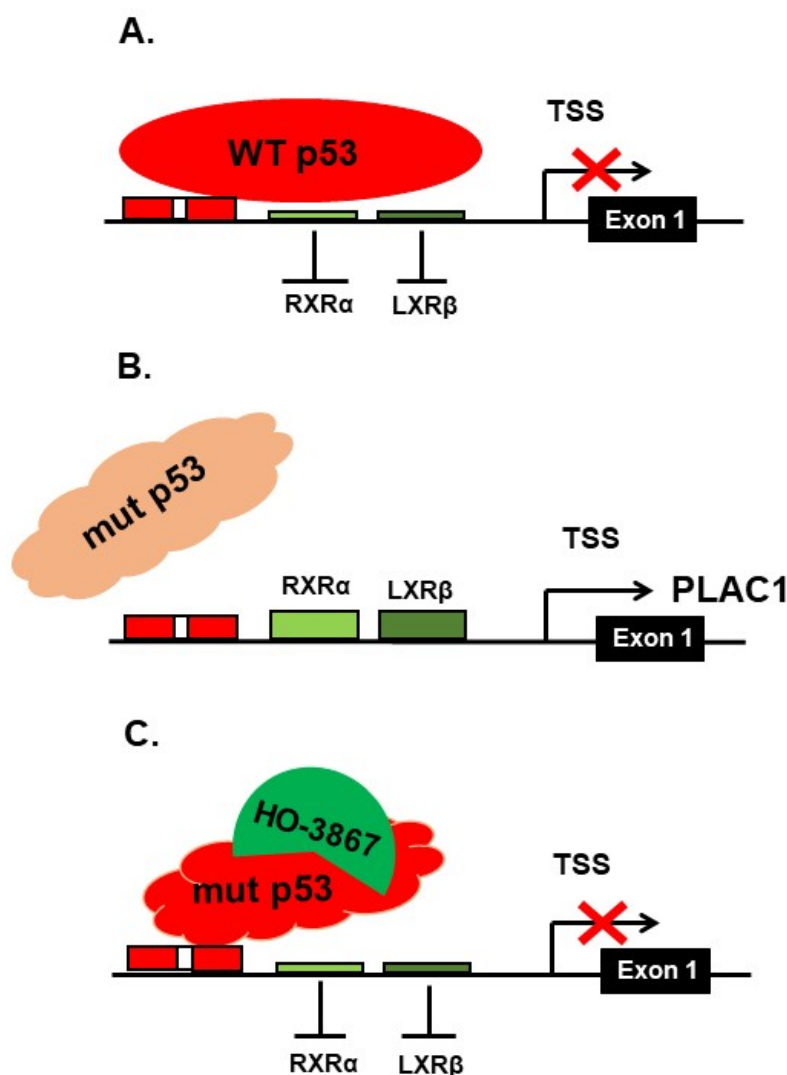


Figure 4. A model of the effect of HO-3867 on p53 binding in the P1 promoter of *PLAC1*. In the top panel (A), wild-type p53 freely binds to its binding site, sterically hindering access of the P1 transcription factors RXRα and LXRβ to their binding sites. This has the effect of suppressing *PLAC1* transcription. However, when p53 is mutated, as in the middle panel (B), p53 is unable to bind to its binding site, thus freeing the P1 transcription factors RXRα and LXRβ to bind to theirs and promoting *PLAC1* transcription. When exposed to HO-3867, as in the lower panel (C), certain p53 mutants are reactivated and will bind to the p53 binding site, once again sterically hindering access of the P1 transcription factors RXRα and LXRβ to their binding sites and hindering *PLAC1* transcription. TSS is the transcription start site.

Finally, while it can be argued that the treatment of ovarian cancer patients with HO-3867—preferably as an adjuvant to conventional therapies—could improve outcomes for these patients, its effect is only possible for those cancers that harbor a *TP53* missense mutation. Obviously, nonsense mutations will not result in the production of transcripts with a poorly differentiated ovarian adenocarcinoma [63]. We obtained these cells from the American Type Culture Collection (HTB-161) and propagated them in RPMI-1640 media, supplemented with 20% fetal bovine serum, 0.01 mg/mL bovine insulin and 1% pen/strep/antibiotic at 37 °C and 5% CO₂.

ES-2 cells were established from primary tumor tissue from a 47-year-old patient presenting with clear-cell adenocarcinoma [64]. We obtained these cells from AddexBio (C0017006) and propagated them in McCoy's 5A media supplemented with 10% fetal bovine serum and 1% pen/strep/antibiotic at 37 °C and 5% CO₂. Given the fact that the American Cancer Society estimates that there are roughly 22,900 new cases of ovarian cancer in the United States each year, some 11,000 patients could potentially benefit from adding HO-

Cell line identities were verified via CODIS DNA profiling at BioSynthesis, Inc. (Lewisville, TX, USA) and by direct *TP53* sequencing. Sequencing confirmed that our OVCAR3 cells harbor the R248Q gain-of-function mutant and that our ES-2 cells harbor the unknown function S241F missense mutant as expected.

4.2. HO-3867 Treatment

The HO-3867 reagent was purchased from Cayman Chemical (Ann Arbor, MI, USA, Item No. 21581). Five milligrams of HO-3867 crystals were dissolved in 10.76 mL of DMSO to produce a 1 mM stock solution that was then added to the appropriate cell media to generate the final experimental concentrations.

Depending upon the assay to be carried out, the OVCAR3 and ES-2 cells were seeded in optimum media in 6-well or 96-well culture plates (200,000 cells and 5000 cells per well, respectively) for 24 h prior to the HO-3867 treatment.

4.3. RNA Purification and Quality Control

The total cellular RNA was purified from cells using the RNeasy Plus Kit following the manufacturer's instructions (QIAGEN, Germantown, MD, USA). The RNA yield and purity were assessed in the Genomics Division of the Iowa Institute of Human Genetics (IIHG) using an Agilent Model 2100 DNA Analyzer and a Trinean DropSense 16 spectrophotometer. Samples with an RNA integrity number (RIN) above 7.0 were used for subsequent protocols [65].

4.4. Real-Time PCR

Equal-mass RNA aliquots (500 ng) from treated cells were reverse transcribed in the presence of SuperScript III Reverse Transcriptase and an oligo (dT) primer (Thermo Fisher Scientific, Waltham, MA, USA). The resulting cDNAs were then amplified in the presence of Power SYBR Green (Thermo Fisher) on an Applied Biosystems Model 7900HT platform in the Genomics Division of the Iowa Institute of Human Genetics (IIHG) using the locus-specific primer pairs shown in Table 1.

Table 1. Primer pairs used for SYBR Green qPCR in this study. Amplification conditions are 95 °C (10:00); 40 (95 °C (0:15), 60 °C (1:00)). A dissociation curve is run at 95 °C (0:15); 60 °C (0:15); 95 °C (0:15) following the amplification.

Locus	Sequence	Amplicon	T _m (°C) *
<i>PLAC1</i>	5'-CACCAGTGAGCACAAAGCCACATT-3'	232 bp	60.3
	5'-CCATGAACCACTCTATGGAG-3'		52.3
<i>PLAC1P1</i>	5'-AAACTTACACGAGGAGTCTGTC-3'	371 bp #	57.2
	5'-CTGTGACCATGAACCACTCTAT-3'	285 bp #	54.2
<i>18S rRNA</i>	5'-AACTTTCGATGGTAGTCGCCG-3'	104 bp	57.2
	5'-CCTTGGATGTGGTAGCCGTTT-3'		54.2

* T_m calculated at 1.5 mM MgCl₂. # Amplicons are from the simultaneously produced P1Long and P1Short transcripts [40].

The raw expression Ct values were normalized against 18S rRNA (Δ Ct). The fold change relative to the untreated cells was calculated via the conventional $\Delta\Delta$ Ct method, where fold change is $2^{-\Delta\Delta C_t}$ [66,67]. The statistical significance of fold changes was assessed via a *t*-test with unequal variances [68]. All experiments were carried out in triplicate.

4.5. Cell Proliferation and Apoptosis

Proliferation: The OVCAR3 and ES-2 cells were plated on 96-well plates. Following a 24 h incubation, the cells were treated with increasing amounts of HO-3867. The cells were imaged using the Incucyte S3 live-cell imaging platform (Sartorius AG, Göttingen, Germany) every hour for up to 48 h. The phase-contrast images were processed using masks and filters to determine cell confluence with the Incucyte Analysis Software (2019B Rev3). Proliferation was plotted as percent confluence normalized to time 0.

Apoptosis: The OVCAR3 and ES-2 cells were plated on 96-well plates. Following a 24 h incubation, the cells were treated with increasing amounts of HO-3867 in combination with 5 μ M Incucyte Caspase-3/7 Green Dye (Sartorius # 4440). The cells were imaged with the Incucyte S3 fluorescence module every hour for up to 48 h. The fluorescent images were processed with the Incucyte Analysis software (2019B Rev3). Caspase 3/7 activation was plotted as the number of fluorescent objects per image. The graphs were created using GraphPad Prism (v9.1.0). All experiments were carried out in triplicate.

5. Conclusions

We have demonstrated that the synthetic curcumin analog HO-3867 successfully rescues the p53-mediated suppression of *PLAC1* transcription in ovarian cancer cells harboring non-truncating *TP53* mutations. As a high expression of *PLAC1* is well known to be associated with poor patient outcomes in a variety of cancers, the use of this compound in conjunction with conventional therapies may serve to improve these outcomes.

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Characterization of a TP53 Somatic Variant of Unknown Function From an Ovarian Cancer Patient Using Organoid Culture and Computational Modeling

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Abstract: In our proof-of-concept study of 1 patient with stage IIIC carcinosarcoma of the ovary, we discovered a rare mutation in the tumor suppressor, *TP53*, that results in the deletion of N131. Immunofluorescence imaging of the organoid culture revealed hyperstaining of p53 protein. Computational modeling suggests this residue is important for maintaining protein conformation. Drug screening identified the combination of a proteasome inhibitor with a histone deacetylase inhibitor as the most effective treatment. These data provide evidence for the successful culture of a patient tumor and analysis of drug response *ex vivo*.

Key words: ovarian cancer, p53, organoid culture, drug screen

Introduction

Epithelial ovarian cancer is the most deadly of the gynecologic malignancies¹ and the fifth leading cause of cancer-related death among women.² Treatment normally consists of surgical debulking followed by 6 cycles of platinum and taxane-based chemotherapy. However, the asymptomatic nature of the disease partnered with a high rate of recurrence results in a long-term survival rate of only 30%.^{2,3} For these women, the evaluation of new agents must be a priority.

The classic model systems used to evaluate new therapeutic agents for ovarian cancer include immortalized cell lines, patient-derived xenograft models, syngeneic and genetically engineered mouse models, and short-term culture of primary tumor cells. Each of these methods has caveats. For example, many immortalized cell lines do not faithfully represent human disease,⁴ with significant genetic drift associated with extended culture. Patient-derived xenograft models retain many of the phenotypic and genetic features of the primary tumor. However, since these models must be created in immunocompromised mice to avoid host versus graft responses, it is not possible to study the impact of the immune system on tumor response. Although syngeneic mice and genetically engineered mouse models do allow for the evaluation of immunomodulatory agents, these models

deviate from human tumor features. The short-term culture of primary tumor cells has been attempted for decades, but recent success is attributed to optimized growth media and the use of Matrigel to provide the structural support for 3-dimensional growth.

First developed for colorectal cancer in 2011 by Clevers and colleagues in the Netherlands,⁵ patient-derived organoid culture is an emerging and novel technology to model cancer. The organoid culture technology includes a cocktail of growth factors to recreate the niche of tissue homeostasis *ex vivo*. The general protocol includes enzymatic digestion of tumor tissue into single cells, followed by embedding the cells into Matrigel and culture with specific medium optimized for the cancer type.

A handful of studies in several cancer types have established that organoids can recapitulate both histological and genomic features of the pertinent lesion from which they were derived. Very recent work by the Soragni laboratory reported a method for high throughput drug screening using organoid cultures.⁶ Importantly, Vlachogiannis et al,⁷ have reported a positive predictive value (predicting that a particular drug works) of 88% and a negative predictive value (predicting that a particular drug does not work) of 100% when comparing organoid sensitivity to the response of a patient with gastrointestinal cancer.

Herein we present proof-of-concept data that we can create an organoid model of ovarian cancer using cancer cells isolated from ascites fluid that was collected at the time of initial debulking.

Materials and Methods

HUMAN SUBJECTS

Human subjects studies were approved by the University of Iowa Institutional Review Board (IRB). Informed consent was obtained under IRB protocol #2018090807,

Molecular Genetic Study of Gynecologic Specimens and Data Repository.

SPECIMEN COLLECTION

The patient was taken to the operating room, where a midline vertical incision was made. Two hundred milliliters of ascites was collected in a sterile container and placed in a biohazard bag. This was then immediately handed off to lab personnel for processing.

ISOLATION OF TUMOR CELLS FROM ASCITES FLUID

On arrival, the ascites fluid was transferred from the vacuum container to 50 mL tubes. Next, 5 mL of 50% vol/vol Percoll was aliquoted into each of 16 to 20 15 mL conical centrifuge tubes. Five milliliters of ascites was carefully layered onto the 50% vol/vol Percoll cushion in each tube, followed by centrifugation at 1200g for 20 minutes at 4°C using a swinging bucket rotor. Erythrocytes pellet at the bottom of the tubes, while the desired ovarian cancer cells are at the interphase between the ascites fluid and Percoll. Using a 5 mL pipet, cancer cells were carefully withdrawn from the interphase fraction and transferred to a new 15 mL conical tube. To remove residual red blood cells, 2 mL of blood cell lysis buffer was added.

RNA PURIFICATION

Ascites cells were collected and total cellular RNA was purified using the mirVana RNA Purification Kit following manufacturer's (Thermo Fisher) recommendations. RNA yield and purity were determined on an Agilent Model 2100 Bioanalyzer. Sufficient yield was obtained with an RNA Integrity Number (RIN) > 9.0 to proceed with RNA sequencing.

TP53 SEQUENCING

Sanger sequencing of *TP53* was carried out using 100 ng of purified cellular RNA.

This RNA was reverse transcribed in the presence of SuperScript III reverse transcriptase (Thermo Fisher). The resulting cDNA was then amplified using the *TP53* primers p53mRNAfor: AGCCGCAGT-CAGATCCTA and p53mRNArev: GA AGTCCTGGGTGCTTCTG. The 1302 bp amplicon was prepared for Sanger sequencing using the QIAquick PCR Purification Kit following manufacturer's (Qiagen) recommendations. Using the PCR primers as sequencing primers, the mRNA amplicon was sequenced in both directions on an Applied Biosystems Model 3730XL platform and aligned against the mRNA sequence NM_000546.

COMPUTATIONAL MODELING OF p53

Simulated annealing was used to generate a plausible structure of the S2-S2' region (residues K120-Q136) with the delN131 (Δ N131) mutation. The same protocol was also applied to the WT protein to ensure a fair comparison. The 3KMD crystal structure⁸ was used as our baseline. Zinc-coordinating cysteines 176, 238, and 242 were deprotonated, and the zinc-coordinating H179 was deprotonated at the δ nitrogen. Then, H193 and H214 were deprotonated at the δ and ϵ nitrogens, respectively, as they form a buried π -stack, have a depressed pKa predicted by PROPKA,^{9,10} and were assigned as neutral by Pradhan et al.¹¹ Using Force Field X (FFX),¹² the structure was re-refined with the polarizable atomic multipole AMOEBA 2018 protein force field.^{13–15}

Subsequently, chain A was solvated in a 75.951 Å water box with a total of 13,168 water molecules (including crystal waters) with 100 mM NaCl (27 copies) and 8 additional chloride ions to neutralize the system. This was then minimized using the limited memory Broyden-Fletcher-Goldfarb-Shanno algorithm^{16,17} (LBFGS) algorithm to a convergence criterion of 0.1 kcal/(mol×Ångstrom) using FFX-OpenMM.¹⁸ This was then equilibrated for 2 nsec with 40 kcal/(mol×Ångstrom) harmonic heavy atom restraints at 298.15K. Asparagine

131 was then removed from the structure, and the structure re-minimized to the same convergence criterion while constraining all atoms outside the range Y35 to F43 to the equilibrated structure. Both the WT equilibrated structure and the Δ N131 structure were then annealed, initially at 1000K for 500 psec, then 200 psec each of 800K, 600K, 400K, 300K, 250K, 200K, 150K, and 100K, finishing with 500 psec of dynamics at 50K. During this annealing, protein-heavy atoms and the zinc ion outside the range K120 to Q136 were restrained to the equilibrated structure with 100 kcal/(mol $\times$ Angstrom) harmonic restraints.

All dynamics were performed in the NVT ensemble using FFX-OpenMM on NVIDIA Titan V accelerators. The NVT ensemble was maintained with an Andersen thermostat¹⁹ with a collision frequency of 91/psec and a RESPA multiple timestep integrator^{20,21} with 0.5 fsec inner timesteps and 2 fsec outer timesteps. The van der Waals interactions were smoothly switched off between 10.8 and 12.0 Å. The Particle Mesh Ewald algorithm^{12,22} was used for long-range electrostatics, with an Ewald coefficient of 0.545, a 7.0 Å real-space cut-off, fifth-order B-splines, and a 96 $\times$ 96 $\times$ 96 grid. Extrapolated perturbation theory²³ was used for mutual polarization.

GENERATION OF ORGANOID MODEL USING CANCER CELLS FROM ASCITES FLUID

Cancer cell pellets were suspended in AdDE+++ medium and filtered with a 40 μ m cell strainer. After centrifugation at 2500 rpm for 5 minutes, the cell pellet was suspended with cool Matrigel (1 $\times$ 10⁵ to 5 $\times$ 10⁵ cells/mL) and mixed well with a cold tip. Spheroids (20 μ L in Matrigel) were transferred to 1 well of a 96-well plate; the liquid Matrigel was allowed to solidify by placing the plate in a 37°C incubator for 15 minutes. Next, 200 μ L AdDE+++ media was added to the top of the Matrigel. Media was changed every

6 to 7 days and organoids were passaged every 2 to 4 weeks.

The AdDE+++ medium recipe (200 mL)

AdDE+++ (advanced DMEM/F12: 1x glutamax, 10 mM HEPES)	191 mL
Noggin (100 μ g/mL)	200 μ L
Rspo1 (25 μ g/mL)	2 mL
B27 (50 $\times$)	4 mL
N-Acetylcysteine (500 mM)	0.5 mL
Primocin	0.4 mL
Nicotinamide (1 M)	2 mL
A83-01 (5 mM)	20 μ L
FGF 10 (100 μ g/mL)	20 μ L
Heregulin β -1 (75 μ g/mL)	100 μ L
Y27632 (100 mM)	10 μ L
EGF (500 μ g/mL)	2 μ L
Forskolin (10 mM)	200 μ L
Hydrocortisone (250 μ g/mL)	400 μ L
β -Estradiol (100 μ M)	200 μ L
Total	200 mL

IMMUNOFLUORESCENCE IMAGING
Organoids were grown on glass coverslips and treated with indicated reagents. After fixation with 2% paraformaldehyde and permeabilization with 1% Triton X-100, cells were labeled with a p53 antibody (sc-126; Santa Cruz) and PAX8 antibody (10336-1-AP; Proteintech) followed by incubation with Alexa Fluor-555 (for p53) or Alexa Fluor-488 (for PAX8)-conjugated secondary antibody. Images were visualized by fluorescence microscopy and acquired with an Olympus BX51 camera at $\times$ 63 magnification.

HEMATOXYLIN AND EOSIN (H&E) STAINING
Formalin-fixed, paraffin-embedded organoids section were stained using H&E program 8 from Central Microscopy facility, the University of Iowa, dehydrated using 3 washes of ethanol and xylene, and mounted with coverslips.

DRUG SCREENING
The viability of the tumor organoids following drug treatment was determined by the CellTiter-Glo 3D cell viability

assay (Promega, Madison, WI). In brief, tumor cells were suspended in AdDE+++ medium with 20% Matrigel and seeded at a density of 3000 cells/well (200 μ L/well) in an ultra-low attachment 96-well U-bottom white plate. After 24 hours, cells were then exposed to cisplatin (5 μ M), carboplatin (5 μ M), paclitaxel (10 nM), bevacizumab (1 μ M), AZD1775 (125 nM), bortezomib (10 nM), belinostat (1 μ M) or olaparib (2 μ M) for 72 hours at 37°C. At the end of incubation, an equal volume of CellTiter-Glo 3D reagent was added to each well and incubated for 25 minutes at room temperature. The luminescence was measured using the Gen5 Microplate Reader (BioTek, VT). All the tests were conducted in triplicate and data normalized to untreated control (set at 100% viability).

Results

PATIENT CHARACTERISTICS

This proof-of-concept study was performed using tumor cells collected from the ascites fluid of a patient undergoing primary cytoreductive surgery. The patient is a 77-year-old woman who presented with abdominal bloating and discomfort. Her imaging was concerning for advanced stage gynecologic malignancy, and she was recommended for primary cytoreductive surgery. During surgery, she was found to have bilateral adnexal masses obscuring the gynecologic organs and obliterating the rectovaginal space, as well as an omental cake, tumor plaquing of the right hemidiaphragm, and miliary disease along the small bowel mesentery and descending colon. She underwent an optimal tumor cytoreduction consisting of a total abdominal hysterectomy with bilateral salpingo-oophorectomy with en block rectosigmoid resection with end to end anastomosis, infragastric and infracolic omentectomy, diaphragmatic peritonectomy with diaphragm resection and primary repair, and

appendectomy. Her postoperative course was complicated by a bowel leak from her rectosigmoid re-anastomosis, which required a return to the operating room for an abdominal washout and diverting loop colostomy. She recovered in the intensive care unit on broad-spectrum antibiotics and pressors, transferred to the general care unit, and finally discharged on a postoperative day 36 from her initial surgery. The patient went home on enteral nutrition based on her continued poor oral intake. Final pathology was stage IIIC carcinosarcoma of the ovary. She was recommended to complete 6 cycles of carboplatin and paclitaxel but started with single-agent carboplatin until her performance status improves.

GENERATION OF ORGANOID EX VIVO MODEL OF PATIENT TUMOR CELLS

Cancer cells were isolated from the ascites fluid and used to generate an organoid model of the patient's disease as described in *Material and Methods*. We first performed pathologic analysis of the organoids using H&E staining and found that cells form sphere-like structures (Fig. 1A). Immunofluorescent staining with protein biomarkers paired box gene 8 (PAX8) and p53 confirmed that the tumor is ovarian in origin (Figs. 1B, C). Interestingly, p53 was highly expressed in the tumor organoids by both immunofluorescence and immunohistochemistry (Figs. 1C, D).

IDENTIFICATION OF A TP53 VARIANT OF UNKNOWN FUNCTION

We next examined the *TP53* status of the tumor since *TP53* is commonly mutated in carcinosarcomas of the ovary²⁴ and was overexpressed in the tumor organoid (Figs. 1C, D). Mutations in *TP53* can be binned in a variety of ways, but the most commonly used nomenclature is to bin the mutations into 1 of 3 simplistic functional classes: (1) truncating, frameshift or splice site loss-of-function mutations that mainly result in a p53-null state, (2) missense

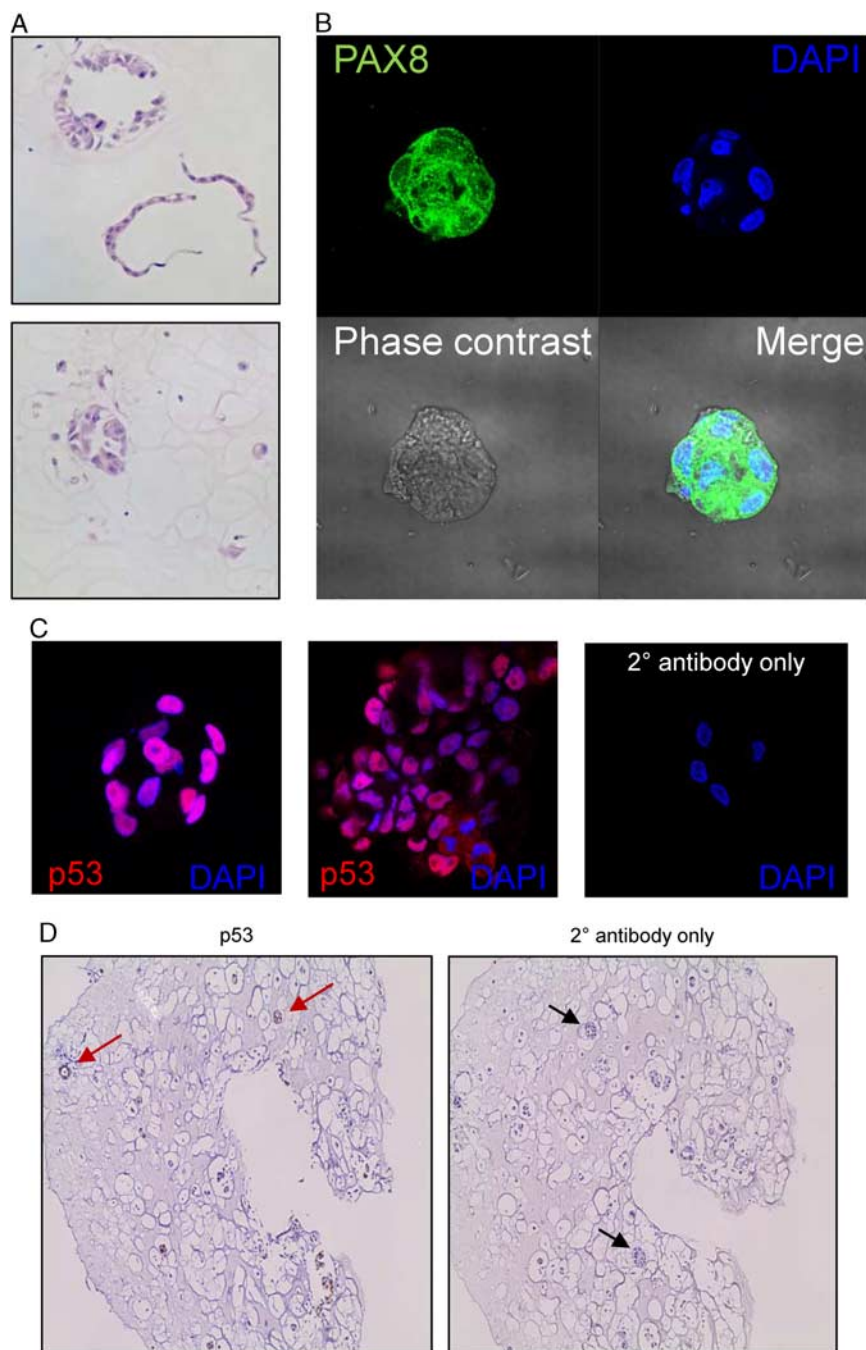


FIGURE 1. Characterization of organoid culture. A, Hematoxylin and eosin staining of paraffin-embedded section. B and C, Immunofluorescent imaging for PAX8 (B) or p53 (C). Nuclei were counterstained with DAPI. D, Immunohistochemistry for p53. Arrows indicate organoids. Other structures are Matrigel. full color online

mutations that often result in gain-of-oncogenic function (GOF), and (3) synonymous/silent mutations that are wild-type (WT) equivalent. Of the missense mutations, ~8 mutations are canonically recognized as “GOF” based on a variety of functional studies²⁵: P151S, Y163C, R175H, L194R, Y220C, R248Q, R248W, R273C, R273H, R273L, R282W.

The aligned mRNA sequence from the patient-derived tumor cells revealed a single mutation event in the *TP53* gene. A deletion of 3 bases (AAG) in Exon 5 resulted in an in-frame deletion of asparagine residue 131 (Δ N131).

In a previous analysis of The Cancer Genome Atlas (TCGA) dataset for ovarian cancer, we found that nearly one half of tumors harbor missense mutations in *TP53* that are not classic GOF mutations.²⁶ We term these missense mutations “somatic variants of unknown function” because there is not a standardized nomenclature for them. The often-used terminology “variants of unknown significance” refers to germline variants with unclear risk of disease, which is not the case when a patient already has cancer. Therefore, we characterize the Δ N131 mutation as a somatic variants of unknown function.

COMPUTATIONAL MODELING TO PREDICT THE FUNCTIONAL IMPACT OF Δ N131 MUTATION

Since the Δ N131 variant is not a traditional loss-of-function or GOF mutation, our next objective was to model the functional consequences of the deletion of N131. p53 is a well-characterized transcription factor with tumor suppressor activities. p53 GOF mutants are typically broadly divided into 2 categories: (1) *contact mutants* that have lost the ability to bind DNA (eg, R273H, R248Q/W); and (2) *structural mutants* that alter the conformation of the protein (eg, R175H, Y220C).²⁷ Our prior TCGA analysis of ovarian tumors demonstrated a high frequency in *TP53* mutations in the DNA-binding domain.²⁶

In the p53 protein structure, asparagine 131 is part of a β -turn between the S2 and S2' β -sheet segments²⁸ (Fig. 2A). The S2-S2' region lies in between sheet S10 and helix H2, and likely holds them together. The H2 helix is notable for directly contacting DNA, while the S10 helix is part of the main β -sandwich structure. As such, it is thought that without the S2 and S2' β -sheet, the H2 helix may become separated from the rest of the DBD and impact binding affinity. Numerous specific interactions hold the S10-S2'-S2-H2 bundle together (Table 1). In particular, L130 forms a hydrophobic contact with helix H2 (Fig. 2B), and it is almost certain that hydrophobic effects are central to protein folding. To confirm this, we used a global structural

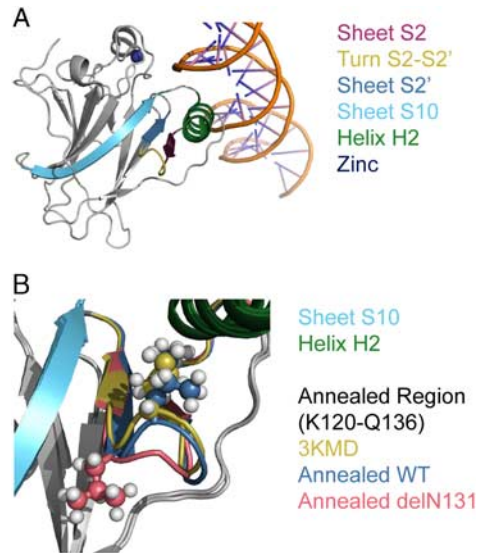


FIGURE 2. Cartoon representations of p53 (PDB ID: 3KMD). A, Zoomed out view of chain A and the DNA; protein chains B-D are excluded for clarity. B, Overlay of 3KMD chain A with optimized structures from simulated annealing of residues K120 to Q136. The side chain of L130 is shown in ball-and-stick representation. When N131 is deleted, L130 shifts downwards and fails to contact helix H2. [full color online](#)

TABLE 1. *Key Interactions Formed by Residues in S2, S2', and the Intervening Turn (T2-T2') in p53*

N-terminal Site	Location	C-terminal Site	Location	Nature of Interaction
C124	S2	C135	S2'	Potential disulfide bond site
T125	S2	F134	S2'	β -sheet H-bond
T125	S2	R282	H2	Side chain H-bond
T125	S2	Y126	S2	Side chain-main chain H-bond
Y126	S2	N131	S2'	Side chain H-bond
S127	S2	L130	T2-2'	Main chain H-bond
S127	S2	L130	T2-2'	Side chain-main chain H-bond
S127	S2	N131	T2-2'	Main chain H-bond
S127	S2	K132	S2'	β -sheet H-bond
S127	S2	K132	S2'	β -sheet H-bond
S127	S2	E286	H2	Side chain H-bond
P128	S2	N131	T2-2'	Main chain H-bond
L130	T2-2'	L289	H2	Hydrophobic contact
N131	T2-2'	S269	S10	Side chain-main chain H-bond
N131	T2-2'	E271	S10	β -sheet H-bond
K132	S2'	E271	S10	Salt bridge
K132	S2'	E271	S10	β -sheet H-bond
M133	S2'	E271	S10	β -sheet H-bond
M133	S2'	R273	S10	β -sheet H-bond
F134	S2'	R282	H2	Hydrophobic contact
C135	S2'	R273	S10	β -sheet H-bond

p53 structures used for this analysis: 3KMD, 2FEJ, 3IGK, 5BUA, 5LGY, 5MCT, and 6FJ5. The major discrepancy between structures in this region is that H2 did not appear to be as well-ordered in many members of the 2FEJ NMR ensemble.

optimization method called simulated annealing in the FFX program²⁹ to find low-energy conformations of the S2-S2' region (defined as residues K120-Q136). Although the WT structure maintained the L130-H2 interaction throughout the simulation, even at substantially elevated temperatures (Fig. 2B, dark blue), the Δ N131 mutant showed the L130 movement away from H2 within 60 psec and never returned to an H2-interacting conformation (Fig. 2B, red). This suggests that strain introduced in the β -turn by Δ N131 results in loss of key stabilizing interactions in the S10-S2'-S2-H2 region, including a hydrophobic contact made by L130.

DRUG SCREENING DEMONSTRATES SENSITIVITY OF TUMOR ORGANOID TO A RANGE OF AGENTS

To demonstrate the ovarian cancer organoids culture platform is suitable for drug screening, tumor organoids were treated

with indicated drugs for 72 hours then the cell viability assay was performed with CellTiter-Glo 3D assay (Fig. 3). The results showed that this patient's organoids were most sensitive to the combination of bortezomib and belinostat, and also quite sensitive to bortezomib, paclitaxel, and AZD1775 alone, but not sensitive to bevacizumab or olaparib. It is also important to note that the tumor organoid was relatively sensitive to the combination of carboplatin and paclitaxel, the standard front-line treatment for carcinosarcoma patients.

Discussion

To make meaningful predictions regarding how each patient is likely to respond to therapy, it is critical to have a reasonable understanding of the function of each p53 mutant. Several algorithms have been developed to predict if mutations are

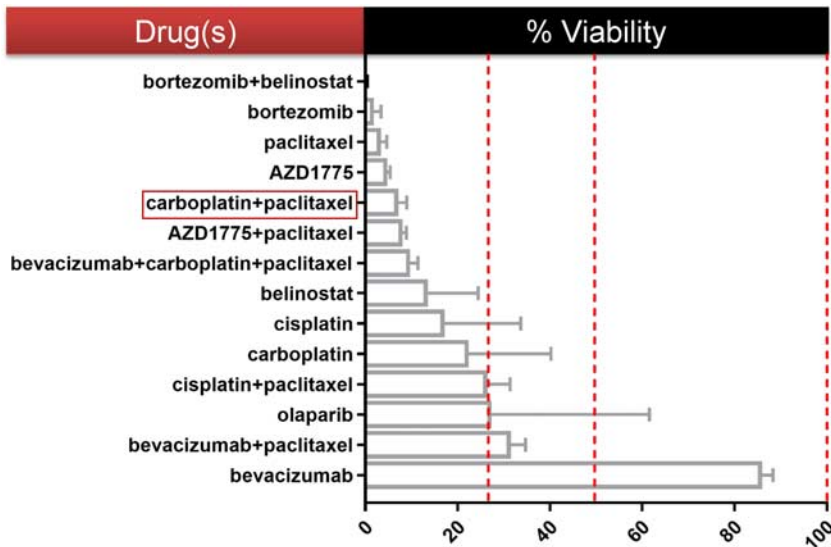


FIGURE 3. Drug screening using patient organoid. As noted with the red box, carboplatin+paclitaxel is the standard front-line treatment for ovarian carcinosarcoma. [full color online](#)

deleterious from the perspective of protein structure,³⁰ but they do not predict how the mutants affect protein function. Rather, these predictions are binary pathogenic versus benign predictions. Missense mutations may be difficult to characterize based upon predictions of structure alone, and over-expression at the protein level is not always an indicator of oncogenic GOF activity.

The ΔN131 mutant results from an in-frame deletion of nucleotides AAC at positions c.391-c.393 of the *TP53* gene (GRCh38 chromosome 17 coordinates 7675175-7675173). This mutation has been reported independently in a Wilms tumor patient,³¹ a serous ovarian cancer patient,³² a patient with neuroendocrine small cell carcinoma of the uterine cervix,³³ and a patient with adenocarcinoma of the stomach.³⁴ In the first 2 studies,^{31,32} the presence of this mutation was marked by elevated production and accumulation of p53 protein. Herein we document that p53 expression is high in the organoid culture that harbors the ΔN131 p53 mutant protein. However, additional studies are necessary to determine the mechanism of

hyperexpression, as well as functional consequences. We speculate that this indel results in a GOF-type p53 protein.

The p53 molecule contains a Zn⁺⁺ pocket in the DNA-binding domain formed by the residues C176 and H179 in the L2 loop and C238 and C242 in the L3 loop. There is evidence that disruption of optimal folding of p53 will impact retention of the Zn⁺⁺ and lead to significant loss of DNA-binding specificity.³⁵ Numerous biochemical and cellular studies have confirmed that proper binding and retention of the Zn⁺⁺ divalent cation is crucial to p53 function and that even minor alterations can significantly disrupt that function.³⁶ Our analysis of the structural impact of the N131 deletion suggests that this indel results in loss of Zn⁺⁺ binding and therefore DNA binding.

Our drug screening results may provide insights for doctors in further treatment for this patient in the clinic. GOF p53 is frequently hyper-stabilized by Hsp90, and we and others have found that strategies that promote Hsp90 acetylation (ie, histone deacetylase inhibitor) sensitize cells with

GOF p53 to chemotherapy.^{37,38} Our recent study in gynecologic cancer cells with 2 different *TP53* GOF mutations indicates that greater cell toxicity can be achieved by the addition of a histone deacetylase inhibitor to the backbone treatment of a proteasome inhibitor.³⁹ Our long-term vision is to deploy this platform to determine drug sensitivities of primary specimens, which can offer actionable information to help tailor therapy to individual cancer patients.

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