Impact of Aged Tumor Microenvironment and Chemosensitivity on Sub-types of Ovarian Cancer

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Article Synopsis

This study investigates how aging effects the growth of ovarian cancer, as well as sensitivity of different ovarian cancers to various chemotherapies. By growing and aging non-cancerous cells to mimic the environment ovarian cancer would see inside the human body, we were able to find that in the lab, ovarian cancer cells grew worse in the "older" condition compared to the "young" environment. Additionally, we identified a chemotherapy called ibrutinib that effectively targets slower growing ovarian cancer cell lines.

Graphic by AJ Kochuba

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ABSTRACT

Ovarian cancer (OC) is the most lethal female reproductive cancer—with a 5-year survival rate of 50.9%—and is diagnosed on average in patients in their 60s. Often OC patients enter an initial remission following chemotherapeutic or surgical interventions; however, ~75% of patients experience chemotherapy-resistant OC recurrence. This study aims to investigate the impact of aging on the tumor microenvironment (TME) and chemosensitivity of various subtypes of ovarian cancer (OC) to therapies targeting slower proliferating OC cells, which often lie dormant in patients and lead to disease recurrence. We have found slower proliferating OC cells exhibited a statistically significant higher sensitivity to the chemotherapy treatment ibrutinib compared to faster-proliferating cells in high-grade serous ovarian carcinomas (HGSOCs) in vitro. Cisplatin-resistant HGSOC cell lines displayed increased chemosensitivity to ibrutinib compared with the cisplatin-sensitive HGSOC cell lines. In clear cell ovarian carcinomas (CCOCs), we did not see dose-dependent chemosensitivity to duvelisib or ibrutinib, but there was an increased sensitivity when compared to the fast-proliferating HGSOC control. CCOC cell lines JHOC5 and TOV21-G showed increased responses to higher doses of UCN-01, but OVISE, the slowest proliferating CCOC did not. When looking at OC cell proliferation in conditioned medium from aged versus young WI-38 fibroblasts, OC cells were less proliferative in aged WI-38 medium when compared with the OC cells in young WI-38 medium in both HEYA8 and CAOV2 cell lines. Furthermore, we found that older WI-38 fibroblast coculture increased chemosensitivity for M41 HGSOC cells treated with duvelisib but not carboplatin. Future directions will be focused on the mechanisms of chemosensitivity in varying tumor microenvironments.

INTRODUCTION

Aging is a significant risk factor for many adult cancers, including ovarian cancer (OC). OC is the deadliest female reproductive system cancer, with a median diagnosis age of 63 years.[1] The 5-year survival rate for OC is 50.9%, according to the Surveillance, Epidemiology, and End Results (SEER) Data Set.[2] Most patients diagnosed with OC will go into initial remission following surgical debulking and adjuvant chemotherapy.[3] However, approximately 75% of patients will experience a recurrence of OC, often characterized by cancer cells that have become resistant to initial chemotherapies.[4] Chemotherapy resistance presents a significant issue for patients facing recurrent OC.

As the most common subtype of epithelial ovarian carcinoma, high-grade serous ovarian carcinoma (HGSOC) accounts for 70% of epithelial ovarian cancers and 90% of all ovarian cancers.[5] HGSOC is the most lethal sub-type of ovarian cancer according to the data from the SEER data set in which patients were followed four years or longer after cancer detection and treatment. HGSOCs are generally composed of solid masses of cells and are characterized by high-grade serous epithelial cell atypia and TP53 mutations.[6] It has been proposed that high-grade serous tumors originate from the epithelium of the fallopian tube.[7] There are currently five principal histotypes of ovarian carcinoma: clear cell ovarian carcinoma (CCOC), HGSOC, low-grade

serous ovarian carcinoma (LGSOC), mucinous ovarian carcinoma (MOC), and endometrioid ovarian carcinoma (EOVC).[8]

HGSOCs tend to occur in older, usually menopausal women.[9] About 80% of HGSOC cases are diagnosed at advanced stages, such as stage III and stage IV, when the tumors are aggressively growing and invading/migrating to the other organs in the peritoneal cavity or even the distant organs.[10] At these stages, the tumors are most spread out, and patients no longer have the option of effective surgical treatment. There are a variety of contributors to the high mortality rate of HGSOC: the inability to diagnose HGSOC early on due to vague symptoms, the lack of efficient screening tests, and the high frequencies of tumor recurrence.[6,11]

Tumors are comprised of cancer cells that carry the abnormal genetic and epigenetic information that contribute to cancer development and progression. Surrounding the tumor is the tumor stromal microenvironment, which is essential in supporting and regulating tumor growth and progression.[12] Over the last two decades, the focus of cancer research shifted from solely focusing on cancer cells to looking at the tissue more systematically and investigating the complex interactions between tumor cells and their surroundings—the tumor microenvironment (TME). The TME is comprised of both cellular and non-cellular components. Cellular components of the TME are tumor-associated fibroblasts (CAFs), endothelial cells, adipocytes, and immune cells, such as natural killer cells (NK), tumor-associated macrophages (TAM), tumor-associated neutrophils (TAN), myeloid-derived suppressor cells (MDSC), and adaptive immune cells like T cells and B cells.[13] The non-cellular TME consists of proteins, glycoproteins, and proteoglycans, which make the extracellular matrix (ECM).[14]

While many studies have focused on the immune cells in TME, the importance and regulation of non-immune stromal components of TME have caught researchers' attention. The non-immune tissues are composed of stromal cells, such as CAFs, tumor endothelial cells (TEC), adipocytes, and the ECM.

Among non-immune stromal cells, CAFs are among the most abundant and significant in TME tissues.[15] Compared to regular fibroblasts, CAFs are more heterogeneous and produce more collagen, ECM proteins, and pro-tumor factors. Aided by CAFs' production of enzymes, crosslinking leads to various interactions between ECM components and tumor stroma stiffness, making conditions more favorable for invasion via malignant cells during ECM remodeling. This tumor stroma stiffness can cause blood vessel collapse at the affected site and consequent hypoxia, thus impairing drug delivery and inducing the proliferation of cancer cells. Additionally, CAFs release growth factors and cytokines that can promote tumorigenesis.[16,17]

WI-38 fibroblast cells have been selected and used to model an aged TME. WI-38 cells are a human diploid lung fibroblast cell line with a finite lifetime that can be aged via passage in cell culture.[18] Senescence of cells that rely on proliferation to thrive, such as stem cells or immune cells, are limited in their capacity to repair in aging tissues; senescence in WI-38 cells has become associated with reduced DNA methylation leading to differential gene expression.[18] By culturing OC with WI-38 cells, or in WI-38 medium, we can model an aged TME to examine how age affects cell proliferation and chemosensitivity *in vitro*.

Disease recurrence is often observed in patients with platinum-resistant OC, due to low response rate[19] and the ability for platinum-resistant, slow-growing OC cells to lie dormant for years following initial platinum-based chemotherapy treatment, such as cisplatin or carboplatin. Thus, it is crucial to identify potential drug candidates that could increase the efficacy of anti-OC treatments, targeting fast and slow-growing cells, as well as platinum-sensitive and resistant cells within a patient. Analysis of the NCI-60 dataset[20] revealed that chemotherapies duvelisib, ibrutinib, and UCN-01 were more efficient at targeting slow-proliferating cell lines, while CRB was more apt at targeting fast-proliferating cell lines.[21,22] Drugs more effective on slow-proliferating OC could be used to target dormant cells, which are potentially the source of cancer recurrence.

In this study, we have tested the chemotherapeutic drugs within aged TME using the

WI-38 model. We examined the sensitivity of various sub-types of ovarian cancer to chemotherapies duvelisib, ibrutinib, and UCN-01. HGSOCs, ovarian cystadenocarcinoma, serous ovarian cancer with undetermined grade, and CCOCs were all studied in monoculture. Ibrutinib is an oral Bruton's Tyrosine Kinase (BTK) inhibitor, which in recent years has been used to treat hematological malignancies.[23] There are currently few published studies regarding the efficacy of ibrutinib in OC, as either a monotherapy or a polytherapy and chemosensitizer. However, recent clinical developments have revealed that ibrutinib may have efficacy in LGSOC.[24] UCN-01, a potent protein kinase inhibitor, shows selectivity for protein kinase C (PKC), which is a key component in the proliferation of OC via a signaling cascade involved in tumorigenesis.[25] Duvelisib is an oral phosphoinositide-3-kinase (PI3K) delta/gamma inhibitor, which currently is approved as a monotherapy for chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL).[26] AKT and mTOR, serine/threonine kinases that impact cancer cell cycling, metabolism, survival and growth,[27] work in tandem to form the PI3K/AKT/mTOR pathway, which is a frequently modified signaling pathway in OC.[19] Carboplatin (CRB) is a second-generation platinum chemotherapy drug used as a high-dose treatment for aggressive OC but eventually produces drug resistance during treatment.[28]

The test aims of this study were to examine the chemosensitivity of various HGSOCs and CCOCs to duvelisib, ibrutinib, and UCN-01. We then tested HGSOC cell proliferation in a comparison between the aged versus young TME, modeled by WI-38 medium. An initial study has been performed to evaluate the chemosensitivity of HGSOC in the aged and young WI-38 model TMEs to duvelisib, UCN-01, and CRB. Progesterone is a steroid hormone synthesized by the ovaries, corpus luteum, and placenta (J. D. Graham & C. L. Clarke, 1997) and plays a central role in the regulation of biological processes throughout uterine tissues: the two major layers of the uterus, the endometrium and the myometrium. The myometrium is the smooth muscle component of the uterus that protects the developing fetus throughout gestation and generates contractile forces at the time of labor to deliver the fetus (Carsten, 1968). The endometrium forms the inner lining of the uterus, composed of the outer epithelium and inner stromal compartment. The epithelium thickens in anticipation of pregnancy and is the site of implantation if pregnancy is established. In the absence of pregnancy, menstruation occurs, resulting in the shedding of the endometrial lining (Critchley et al., 2020). If pregnancy is established, progesterone signaling triggers the differentiation of the stromal compartment into decidual cells (a process known as decidualization) that support embryo growth and maintain early pregnancy (Ng et al., 2020). Progesterone signaling is central to the two major layers of the uterus, the endometrium and the myometrium, regulating a host of biological processes including menstruation, ovulation, embryo implantation, uterine growth, and labor (J. D. Graham & C. L. Clarke, 1997).

MATERIALS AND METHODS

HGSOC & CCOC Culture, Seeding, and Chemotherapy Treatment

The OC cell lines were cultured in RPMI medium with 10% fetal bovine serum (FBS) and penicillin-streptomycin (PS) at 37°C, 5% CO2. OC cell lines tested are listed in Table 1**.** Cell lines were seeded at 6000 cells per well into a 96-well plate for a 24-hour 37°C culture period. Cell lines were then treated with variable and increasing concentrations of ibrutinib, duvelisib, and UCN01, diluted with RPMI, and cultured for 72 hours at 37°C before 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.

WI-38 Senescence

WI-38 fibroblast cell line was obtained from Duke Cell Culture Facility with initial population doubling times (PDTs) of 29 and an initial passage of p19. The WI-38 cells were cultured with Minimum Essential Medium Eagle (MEME) with 10% FBS/PS at 37°C, 5% CO2, and passed when they reached 100% confluence. WI-38 cells were cultured over three months to obtain a

population-doubling level characteristic of an aged cell line. PDT was calculated using the formula $PDT = PDT_0 + 3.322(logC_f - logC_i)$.[29] In this formula, $PDT_0 =$ initial population doubling level, $C_f =$ final cell yield at the end of the growth period, and $C_i =$ initial cell number seeded into the vessel.

M41/WI-38 Co-culture with Chemotherapy Treatment

M41 was selected for culture as it was derived from a patient with HGSOC with a slow proliferating feature with a PDT of 40.47 hours (Table 1). M41 was cultured with RPMI medium with 10% FBS/PS and seeded at 10,000 cells per well onto 12-transwell plates bottom chamber and incubated for 24 hours at 37°C, 5% CO2. Aged (p29 and PDT 36) and young WI-38 (p19 and PDT 29) cells were seeded onto transwell plate upper chambers, allowing nutrient exchange between fibroblasts and OC lines, mimicking the TME in human patients. The co-cultured cells were incubated at 37°C, 5% CO2 for 72 hours. After 72 hours of co-culture incubation, M41 cells in both young and old WI-38 conditions were treated with high and low doses of chemotherapies: duvelisib, CRB, and UCN-01. Chemotherapy-treated plates were incubated for 72 hours at 37°C, 5% CO2. The upper chambers containing WI-38 fibroblasts were removed, and M41 cells in the bottom chamber were trypsinized and re-seeded into 96-well plates to use the plate reader for MTS assay, with six wells per treatment and control conditions. Relative cell viability was measured with MTS assay kit according to the protocol from the manufacturer (Promega).

HEYA8 & CAVOV2: WI-38 Co-culture

In Trial 1, WI-38 cell lines with PDT 36 and PDT 46 were used for young and old models respectively. $2.6x10^5$ WI-38 cells were plated into 10cm diameter culture Petri-dishes and cultured until 70% confluence in MEME 10% FBS/PS at 37°C, 5% CO2. The medium from WI-38 cell culture was collected. HEYA8 and CAOV2 cells were each seeded at 2000 cells/well into 96 well plates and cultured in both old and young WI-38 medium. The old and young WI-38 medium in the 96 well plates was replaced with fresh WI-38 medium and cultured for an additional 48 hours. After a total 96-hour culture period, cell viability was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) kit according to the protocol from the manufacturer (EMD Millipore Corporation).

In Trials 2 and 3, WI-38 cell lines with PDT 34 (36 in Trial 3) and 55 were used for young and old models respectively. $6.25x10^5$ WI-38 cells were plated into 10cm diameter culture dishes and cultured until 70% confluence in MEME 10% FBS/PS at 37°C, 5% CO2. HEYA8 at 2000 cells/well and CAOV2 cells at 4000 cells/well were each seeded into 96 well plates and cultured in either old or young WI-38 medium. After the 48-hour incubation, the old and young media in the 96-well plates were replaced by the fresh WI-38 medium. After a total 96-hour culture period, cell viability was assayed using the MTT kit.

Cell Viability Test

The MTS or MTT assays were used to measure relative cell number for proliferation studies. MTT solution was added to each tested well on the 96 well plate and incubated at 4 hours at 37°C, 5% CO2. Isopropanol was added to each well per the instruction manual on the MTT kit (Colorimetric [MTT] Kit for Cell Survival and Proliferation, EMD Millipore Corporation; 2015). For Trial 3 of the HEYA8/CAOV2 Aged vs. Young WI-38 co-culture experimentation, the plate was allowed to mix gently for an additional 20-minute period to better dissolve precipitate on the bottom of the wells. Absorbance was recorded at 570 nm using FLUOstar Omega Microplate Reader. For the MTS assay, CellTiter 96® AQueous One Solution Reagent was added to each well [30], and an MTS assay was performed after a 1 hour 37°C incubation to measure relative cell number. Absorbance was recorded at 450nm using using FLUOstar Omega Microplate Reader.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (v.9, GraphPad Software, Inc., La Jolla, CA, USA) or Microsoft Excel statistical programs. Statistical comparisons were made using a paired t-test for paired data or a two-way ANOVA testing with multiple comparisons, such as chemotherapy and

conditioned culture. A significance level of 0.05 was considered statistically significant.

RESULTS

Ibrutinib, Duvelisib, and UCN-01 Chemosensitivity in OC Subtypes

Table 1: OC Cell Lines Used in The Study And Their Population Doubling Time (PDT)

Cell Line	Histology	PDT (hrs)
SK-OV-3	NS	19.49
HEY	HGSOC	20.53
JHOC5	CCOC	30[31]
TOV21-G	CCOC	30.62
OVCA432	HGSOC	38.52
M41	HGSOC	40.47
TYKNU	HGSOC	45
M41-cisR	HGSOC	45.41
OVISE	CCOC	48[31]
PEO4	HGSOC	52.34
OVCAR3	HGSOC	53.77
TYKNU-cisR	HGSOC	–

Listed above are all OCs used in the following experimentation: High-Grade Serous Ovarian Carcinomas (HGSOC), Clear Cell Ovarian Carcinoma (CCOC), and non-serous (NC).

We predicted that the selected drugs (UCN-01, duvelisib, and ibrutinib) would more effectively target the slower growing OC cell lines (ie. cell lines with larger PDTs). Additionally, we predicted that an aged TME would cause increased OC proliferation, due to the incidence of cancer progression and development in older populations. The cell lines used in this study together with their population doubling time (PDT) are listed in Table 1. In initial experimentation, we compared chemosensitivity to ibrutinib with 8 OC cell lines. M41 (PDT = 40.47) and M41-cisR (PDT = 45.41) show consistent results with increased ibrutinib sensitivity in cisplatin-resistant cell lines (Figure 1A). Treatment with duvelisib showed a decreased HGSOC cell viability and survival with a higher drug concentration, with the exceptions of TYKNU-cisR and HEY, which showed weaker response or even no response to duvelisib (Figure 1B). CCOC TOV21-G additionally did not show a strong response when treated with duvelisib. M41 showed a stronger response to duvelisib than M41-cisR (Figure 1B).

With ibrutinib, there was a statistically significant trend ($r = 0.82$, $p = 0.0128$) of slower proliferating cells exhibiting higher sensitivity to chemotherapy treatment than more rapidly proliferating cells (Figure 2A). Unlike ibrutinib, with duvelisib, there is a weak correlation ($r = 0.16$, $p = 0.6777$) of slower proliferating cells exhibiting higher sensitivity to chemotherapy treatment than fast proliferating cells (Figure 2B).

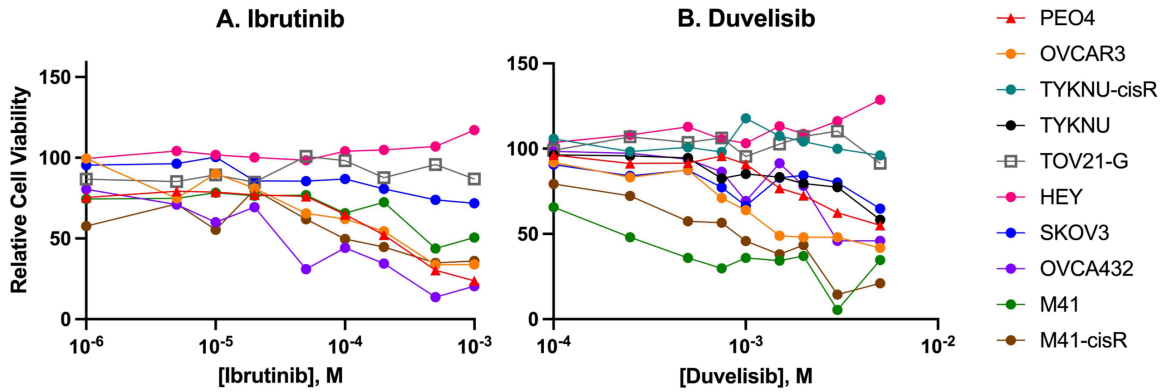

Figure 1. Chemosensitivity of OC cell lines to Ibrutinib and Duvelisib. Cell lines were plated into 96-well plates at 6000 cells per well and treated with drug for 72 hours. Readout performed with Cell Titer Glow MTS Assay. **A.** Slower proliferating cell lines are more sensitive to ibrutinib. CCOC (TOV21-G) and SOC (SKOV3) tested with ibrutinib showed weaker responses when compared to most HGSOC lines, excluding fast proliferating HGSOC HEY. M41-cisR showed a stronger response to ibrutinib than M41. **B.** No correlation between OC cell line sensitivity to duvelisib and PDT. Treatment with duvelisib showed increased HGSOC sensitivity at higher drug concentrations, with the exceptions of TYKNU-cisR and HEY, which showed weaker responses to duvelisib. CCOC (TOV21-G) did not show a strong response when treated with duvelisib. M41 showed a stronger response to duvelisib than M41-cisR.

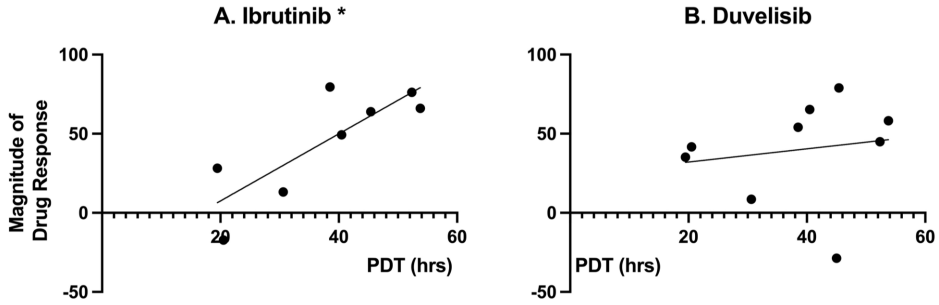

Figure 2. Positive correlation between the magnitude of relative drug responses and population doubling time (PDT) in Duvelisib and Ibrutinib conditions. Magnitude of relative drug response was defined as the greatest sensitivity from experiments in Figure 1A-B, with standard PDTs from Table 1, and each data point representing a different cell line for which we have chemosensitivity data and PDT data. **A.** With ibrutinib, slower proliferating cells exhibit a statistically significant ($p = 0.0128$) higher sensitivity to chemotherapy treatment than fast proliferating cells ($r = 0.82$). **B.** With duvelisib, slower proliferating cells exhibit higher sensitivity to chemotherapy; however, the correlation ($r = 0.16$) of slower proliferating cells exhibiting higher sensitivity to chemotherapy treatment than fast

An initial test was performed to test the chemosensitivity to ibrutinib (A), duvelisib (B), and UCN-01 (C) with 3 CCOC (JHOC5, OVISE, and TOV21G). We included the HGSOC cell line, HEYA8, as a comparison control. With the drug dose range for ibrutinib and duvelisib, we did not see dose-dependent effects, with prominent cell survival even when treated with high drug concentrations(Figure 3A-B). However, we have found the CCOC cell lines were more sensitive to ibrutinib and resistant to duvelisib compared with HGSOC HEYA8 cells (Figure 3A-B). The mixed feature was seen with UCN-01 treatment, in which the CCOC JHOC5 and TOV21-G show increased responses to UCN-01 compared to HEYA8 (Figure 3C). More tests of CCOC lines may be needed to confirm the sensitivity to duvelisib and ibrutinib compared to HGSOC using the appropriate drug doses.

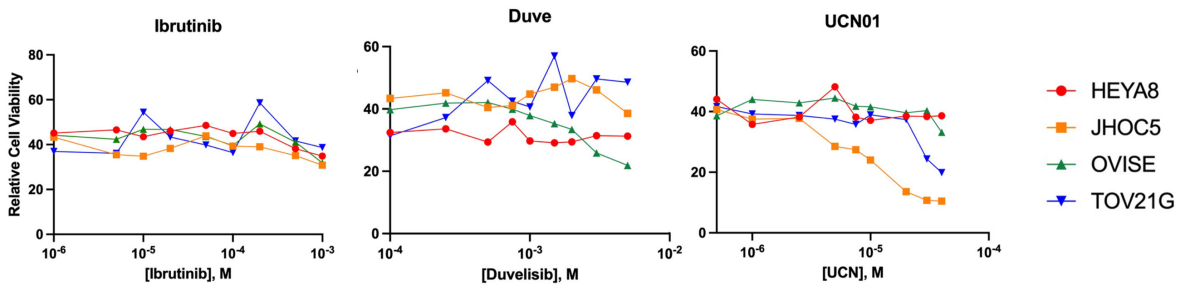

Figure 3. No response seen in CCOC when treated with Duvelisib and Ibrutinib compared to HGSOC. A. CCOCs (JHOC5, OVISE, TOV21G) did not show a greater drug response to ibrutinib doses ranging from 10^{-6}M to 10^{-3}M when compared to insensitive HGSOC control HEYA8. **B.** Additionally, CCOCs did not show a greater drug response to duvelisib doses ranging from 10^{-4}M to $5x10^{-3}$M **C.** However, CCOC cell lines JHOC5 and TOV21-G show increased responses to higher doses ($\geq 10^{-5}$M) of UCN-01 when compared to HGSOC HEYA8.

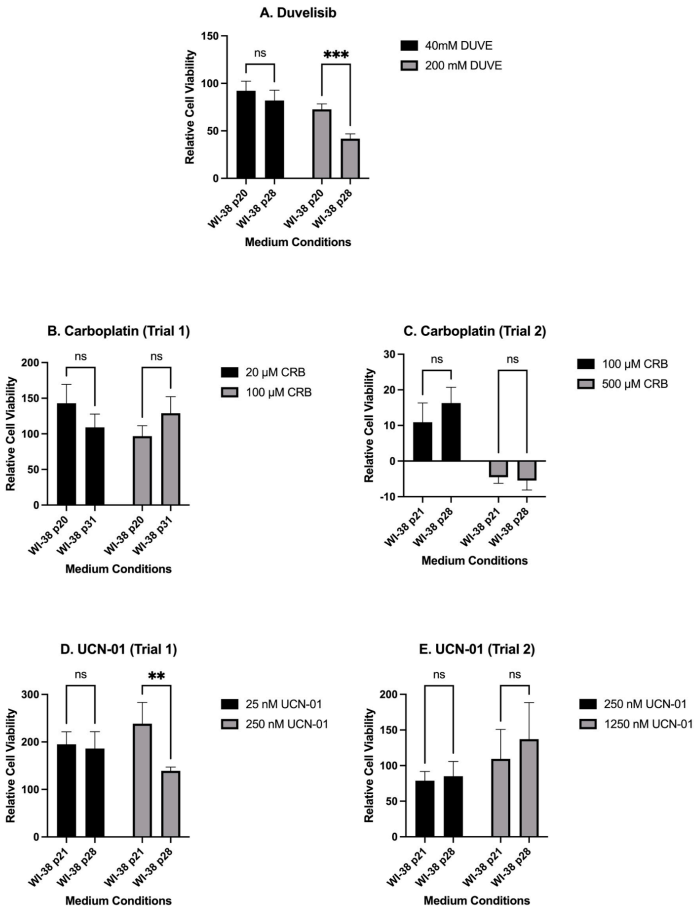

Figure 4. Older WI-38 fibroblast coculture increases chemosensitivity for M41 OC cells treated with high dose Duvelisib but not Carboplatin; results inconclusive for UCN-01 chemotherapy. In Trial 1, CRB low-dose and high-dose conditions were 20 µM and 100 µM, respectively. In Trial 2, CRB low dose and high dose conditions were 100 µM and 500 µM, respectively. For Trial 1, UCN-01 low dose and high dose conditions were 25nM and 250 nM, respectively. UCN-01 dosage was increased in Trial 2; UCN-01 low dose and high dose conditions were nM and 1250 nM, respectively. Cell proliferation was measured using an MTS cell proliferation assay compared against the control condition (was normalized to the cell proliferation = 100). Values were expressed as the mean ± standard deviation. Statistical significance was examined by analysis of a paired t-test, with $p < 0.05$ considered to indicate a statistically significant difference. **A.** High-dose (200 mM) duvelisib treatment was found to be more effective at reducing M41 cell viability in the older WI-38 fibroblast (p28) medium than young medium (p20, $p = 0.0004$). **B/C.** There is no statistically significant difference in M41's sensitivity to carboplatin when comparing the older and younger WI-38 (p20/21; p31/28) mediums at doses 20µM ($p = 0.0883$), 100µM ($p = 0.1043$, $p = 0.1387$), and 500µM ($p = 0.9314$). **C/D.** Initially found older WI-38 fibroblast (p28) coculture increases chemosensitivity for M41 OC cells treated with 250 nM UCN-01 ($p = 0.0016$), but subsequent experimentation revealed no statistically significant difference between older and younger medium conditions (p21; p28) at 250nM and 1250 nM UCN-01.

Impact of WI-38 Aging in Chemosensitivity with HGSOC-

Age being a significant risk factor for the development and progression of ovarian cancer, we wanted to investigate OC growth and chemosensitivity differences in "aged" versus "young" fibroblast coculture. The young fibroblasts consisted of the youngest passage of W-38 human fibroblasts available, while the old fibroblasts were more advanced passages where slower growth was observed in culture but varies throughout the following experiments. The duvelisib treatment condition showed increased chemosensitivity in aged WI-38 (p28) cocultures (Figure 4A) compared with

the younger WI-38 cells (p20). Duvelisib low dose and high dose conditions were 40mM and 200mM, respectively. The higher duvelisib dosage of 200mM produced the greatest decrease in cell proliferation of M41 in the aged WI-38 coculture ($p = 0.0004$). In the first CRB trial (Figure 4B), the results showed no decreased cell proliferation when the cells were treated with CRB at high (100 μM) or low (20 μM) doses suggesting the drug doses were too low for M41 cells. We then increased the dosage of CRB from 20 μM to 100 μM for the low-dose condition and from 100 μM to 500 μM for the high-dose condition. In the second CRB trial (Figure 4C), appropriate decreases in cell proliferation were observed, but the differences in chemosensitivity between aged and young WI-38 conditions were not statistically significant (p-value = 0.5298). In this test, we also found the CRB doses in trial 2 were too high, killing most cells.

In the first UCN-01 trial (Figure 4D), we found a statistically significant reduction ($p = 0.0016$) in cell viability for M41 OC cells treated with 250 nM UCN-01 in the older WI-38 fibroblast (p28) coculture compared to the young WI-38 coculture (p21). The methodology was changed to increase the dosage of UCN-01 from 25 nM to 250 nM for the low-dose condition and from 250 nM to 1250 nM for the high-dose condition. In the second UCN-01 trial, no statistically significant decreases in cell proliferation at 250nM and 1250 nM UCN-01 were observed (Figure 4E), and the differences in chemosensitivity between aged and young WI-38 conditions were not observed at both high and low chemotherapy doses.

HGSOCs HEYA8 and CAOV2 were cultured in WI-38 medium conditions for two consecutive 48-hours periods before being tested for relative cell proliferation using MTT assay. In the first trial, HEYA8 and CAOV2 were statistically significantly less proliferated (HEYA8: $p < 0.0001$, CAOV2: $p = 0.0367$) when cultured in aged WI-38 medium compared to young PDT conditions (Figure 5A). A second trial showed that HEYA8 was statistically significantly less proliferated ($p = 0.0047$) when cultured in 46-hour PDT WI-38 medium compared to 36-hour PDT conditions, but CAOV2 did not show statistically significant differences ($p = 0.2464$) between aged (PDT = 55) and young (PD = 34) medium conditions (Figure 5B). A third trial showed decreased HEYA8 and CAOV2 proliferation in aged medium conditions (Figure 5C), but the results were not statistically significant (HEYA8 $p = 0.6006$; CAOV2 $p = 0.4272$). OC cells were less proliferated when cultured in aged fibroblast (WI-38) medium in both HEYA8 and CAOV2 cell lines in all three replicate experiments, but only the first two trials had statistically significant results.

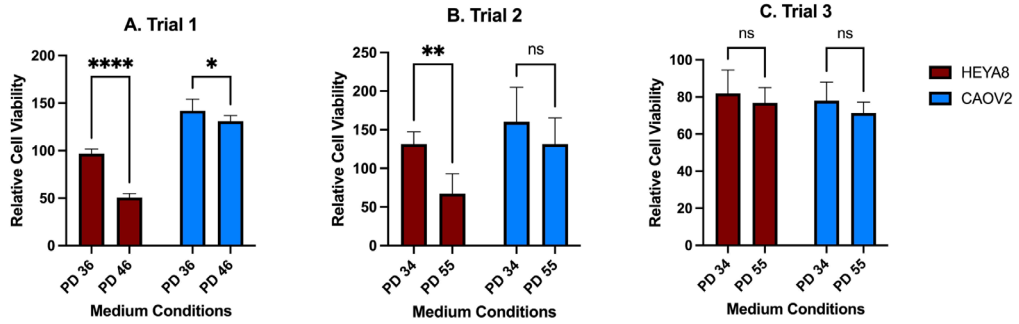

Figure 5. OC cells were less proliferated when cultured in aged fibroblast (WI-38) medium in both HEYA8 and CAOV2 cell lines. HGSOCs HEYA8 and CAOV2 were cultured in WI-38 medium conditions for two consecutive 48-hours periods before being tested for relative cell viability using MTT assay. **A.** HEYA8 and CAOV2 were statistically significantly less proliferated (HEYA8: $p < 0.0001$, CAOV2: $p = 0.0367$) when cultured in aged WI-38 medium with fibroblasts with a PDT of 46 hours compared to young, 36-hour PDT conditions. **B.** HEYA8 was statistically significantly less proliferated ($p = 0.0047$) when cultured in 46-hour PDT WI-38 medium compared to 36-hour PDT conditions. CAOV2 did not show statistically significant differences between aged and young medium conditions ($p = 0.2464$). **C.** A third trial showed decreased HEYA8 and CAOV2 proliferation in aged medium conditions, but the results were not statistically significant (HEYA8 $p = 0.6006$; CAOV2 $p = 0.4272$).

DISCUSSION

Chemosensitivity in OC Sub-types

In this study, we aimed to evaluate how sensitive various sub-types of OC would be to three different chemotherapies. Experiments were conducted that directly compared the effects of UCN-01, duvelisib, and ibrutinib on HGSOCs and CCOCs. As UCN-01,[31] duvelisib, and ibrutinib target slow proliferating cells according to the previous findings from our lab, we expected OC cells with larger PDTs (slow cell proliferation) to be more sensitive to treatment. M41-cisR is a cell line generated from M41 with a cisplatin resistance. Comparison of M41 and M41-cisR cells showed increased ibrutinib sensitivity in cisplatin-resistant cells. Overall, slower proliferating cells, including cell lines OVCA432, M41, M41-cisR, OVCAR3, and PEO4, exhibited a statistically significant higher sensitivity to ibrutinib chemotherapy treatment compared to fast proliferating cells, which included the cell lines SKOV3, TOV21-G, and HEY. These results support our hypothesis and further investigation of ibrutinib as a therapy to target slower-proliferating cells, which are often the cause of OC recurrence as they can lie dormant in patients following initial chemotherapeutic treatment. Chemotherapies such as carboplatin and paclitaxel target fast-proliferating cells and will often fail to eliminate OC cells with higher PDTs, which is characteristic of slow proliferation.

In CCOCs, we did not see dose-dependent chemosensitivity to duvelisib or ibrutinib, but there was an increased sensitivity when compared to the HGSOC control, HEYA8. CCOC cell lines JHOC5 and TOV21-G showed increased responses to higher doses of UCN-01, but OVISE showed little to no response when compared to the HEYA8 HGSOC control. These results are surprising, as OVISE had the greatest PDT among the CCOCs, but showed the weakest response to UCN-01. This preliminary data suggested that the chemotherapeutic drugs, such as duvelisib, ibrutinib, and UCN-01, have differential responses among the subtypes of OC cells. Further tests will be performed to confirm this finding with the optimal drug doses.

Recent studies have found that BTK inhibitors differentially suppress lipid accumulation in hematological malignancies,[32] but there is very little data regarding solid cell tumors and lipid interactions in the context of BTK inhibitors. In terms of future directions to pursue regarding the data collected and presented in Figures 1, 2, and 3, looking at interactions between duvelisib and ibrutinib in varying lipid environments would be worthwhile. Initial data collected by our team suggests that high-fat lipid environments cause increased HGSOC proliferation; the chemosensitivity test within a high-fat lipid environment has yet to be collected.

Many studies have shown ibrutinib's role as a platinum sensitizer,[33] suggesting the testing of ibrutinib as a polytherapy in multiple cisplatin-resistant OC cell lines should be of interest. Additionally, as OC is a cancer that often affects women in their 60s, studying differences in how duvelisib, ibrutinib, and UCN-01 interact in the aged versus young TME models—including aged lymphocytic environment as well as the other TME components—will be a future direction for this study, as we have yet to evaluate chemosensitivity in an aged versus young TME across sub-types and different OC cell lines.

Impact of Aged TME

In this experiment, we aimed to evaluate how the aged TME affects the proliferation and chemosensitivity of HGSOCs. Experiments were conducted that directly compared OC cell proliferation in an aged TME to the cell proliferation of OC found in the young TME. Then, chemosensitivity was evaluated in that aged and young TME. Previous in-lab study using rat OC xenograft models has shown increased tumor growth in aged rats when compared to young rats, which confirmed that age is a risk factor for OC development. Via RNAseq assay, we found that genes in tumor-surrounding tissues are differentially expressed between aged vs young rats. These results suggested that TME plays an important role in OC development. We therefore used aged WI-38 medium conditions to mimic the aged TME to test OC cell proliferation and chemo responses. Unexpectedly, our data showed that OC cells proliferated less when cultured in aged WI-38 medium in HEYA8 and CAOV2 cell lines (Figure 5). This seems

contradictory to our in vivo findings using rat OC xenograft models. This disagreement between the in vivo and in vitro studies was thought to be due to the single factors being tested in vitro, as the stromal fibroblasts WI-38 represent only one component of TME. In contrast, the in vivo environment is more complicated and includes multiple components, such as the immune system, blood vessels, ECM, etc. In the in vivo condition, the multiple components interact to regulate the cancer development and progress which is hard to achieve using in vitro models. The limitations have been reported by many studies that have shown the disagreement between in vitro and in vivo studies.[34]

In Figure 5, HEYA8 OC cells were consistently less proliferative in the aged TME, but the results produced using CAOV2 cell lines were statistically significant in only Trial 1. Variabilities in CAOV2 results could be due to inconsistent techniques or cell numbers from WI-38 cells, as they grow more slowly as they reach senescence. In Trial 3, the use of a WI-38 cell line that had been in culture for a long period due to slow growth may be a possible reason for the insignificant differences between old and young environment-cultured HEYA8 and CAOV2. Additionally, in Trial 2, following the 4-hour incubation with MTT assay and the addition of isopropanol, the bottom of the clear wells looked ununiform in coloring, which could have resulted in data inconsistencies. Further research is required to investigate why OC was less proliferative in aged TME conditions, which is not aligned with the in vivo data using the rat xenograft OC model.

When the chemosensitivity was tested using OC and WI-38 fibroblast co-culture model, we found increased chemosensitivity in M41 OC cells treated with duvelisib but not carboplatin. The results were conflicting for UCN-01 chemotherapy conditions; only Trial 1 produced statistical significance, with M41 being less proliferative in the aged WI-38 environment high UCN-01 dose. We expected that aged TME, being closer to a senescent and slower proliferating state, would promote a more favorable response to drugs that more effectively target slow proliferating cancer cells rather than cells growing in the young TME, which would be more proliferative. However, with statistical analysis of paired t-tests for all drug treatments, none of the results show a statistically significant difference currently (Figure 4). More experiments must be performed to further investigate the variable results seen in the CRB and UCN-01 chemotherapy treatments to rule out the possibility of technical issues contributing to the inconsistent data. Noticeably, the large standard deviation (SD) values for the CRB and UCN-01 treatments may indicate uneven cell numbers during transfer from transwell plates to 96-well plates. In Figure 4, we used WI-38 PDT 36 to represent the aged environment which may not be optimal, as higher PDT might be a better model for the test; this was implemented in the data from Figure 5. Theoretically, the larger the difference in PDT between young and aged WI-38, the more significant difference should be observed in cell proliferation between young and aged conditions of M41 culture. H higher accuracy in cell seeding and the changes in chemotherapy dosage would likely produce greater differences in the chemosensitivity of CRB and UCN-01 affected by the age of the TME as simulated by WI-38 cells, as well as more distinct cell proliferation differences in the absence of chemotherapy treatment.

In summary, we have found that slower proliferating OC cells exhibited a statistically significant higher sensitivity to chemotherapy treatment ibrutinib than faster-proliferating cells in HGSOCs. Cisplatin-resistant HGSOC cell lines displayed increased chemosensitivity to ibrutinib compared with the cisplatin-resistant HGSOC cell lines. In CCOCs, we did not see dose-dependent chemosensitivity to duvelisib or ibrutinib, but there was an increase in sensitivity when compared to the fast-proliferating HGSOC control. CCOC cell lines JHOC5 and TOV21-G showed increased responses to higher doses of UCN-01, but OVISE, the slowest proliferating CCOC did not. It is of note that there are other morphological and genetic differences—beyond speed of proliferation—between the cell lines studied that warrant investigation.

When looking at OC cell proliferation in conditioned medium from aged versus young WI-38 fibroblasts, OC cells were less proliferative in aged WI-38 medium when compared with the OC cells in young

WI-38 medium in both HEYA8 and CAOV2 cell lines. Furthermore, we found that older WI-38 fibroblast coculture increased chemosensitivity for M41 OC cells treated with duvelisib but not carboplatin; the results were inconclusive for UCN-01 chemotherapy conditions. Future studies will be focused on the mechanisms of TME in cancer cell growth and chemo-responses, utilizing aged and young lymphocytes in addition to the WI-38 fibroblasts. Future directions could also include differentiation of preadipocytes in order to manipulate lipid content in medium, so that we may better understand lipid effects on OC proliferation and chemosensitivity, as well as how BTK inhibitors differentially suppress lipid accumulation in a setting catered to solid tumors.

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