THE EFFECT OF COMBINATION TREATMENTS ON OVARIAN CANCER CHEMORESISTANCE

ÇAĞLAR BERKEL

MASTER THESIS

DEPARTMENT OF BIOLOGY

Asst. Prof. ERCAN ÇAÇAN

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T.C.
TOKAT GAZİOSMANPASA UNIVERSITY
GRADUATE SCHOOL OF NATURAL & APPLIED SCIENCES
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ÇAĞLAR BERKEL

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ABSTRACT

MASTER THESIS

THE EFFECT OF COMBINATION TREATMENTS ON OVARIAN CANCER CHEMORESISTANCE

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Asst. Prof. ERCAN ÇAÇAN

Ovarian cancer is one of the deadliest gynecological cancers with high mortality rate and the 5-year survival rate is less than 50 %. Besides the lack of early diagnostic methods and the lack of characteristic disease symptoms, enhanced chemotherapy resistance and relapse are responsible for the high mortality rate of ovarian cancer patients. Therefore, novel therapeutic strategies for overcoming chemoresistance in ovarian cancer are urgently needed. Here, we used a combination of chemotherapeutic agents (bortezomib, olaparib and cisplatin) to overcome ovarian cancer chemoresistance. In particular drug concentrations tested, we observed that combination treatment of bortezomib and olaparib enhances cytotoxicity when compared to drugs alone. We further elucidate a potential mechanism that how combination treatment regulates expression of DYNLL1 which functions in several cellular processes including apoptosis, DNA damage response and chemoresistance. These results indicate that combination treatments have potential benefits to decrease ovarian cancer chemoresistance *in vitro*, possibly by regulating DYNLL1 expression in part.

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KEYWORDS: Ovarian cancer, Chemoresistance, Proteasome, PARP, Cisplatin, DYNLL1, Chemotherapy

ÖZET

YÜKSEK LİSANS TEZİ

KOMBİNASYON İLAÇ UYGULAMALARININ OVER KANSERİ KEMODİRENCİ ÜZERİNDEKİ ETKİLERİ

ÇAĞLAR BERKEL

TOKAT GAZİOSMANPAŞA ÜNİVERSİTESİ

FEN BİLİMLERİ ENSTİTÜSÜ

BİYOLOJİ ANABİLİM DALI

Dr. Öğr. Üyesi ERCAN ÇAÇAN

Over kanseri, jinekolojik kanserler arasında en ölümcül olanlardan biridir ve 5-yıllık hayatta kalım oranı yüzde 50'den azdır. Erken teşhiş yöntemlerinin ve karakteristik hastalık semptomlarının yokluğunun yanında, yüksek kemoterapi direnci ve hastalığın nüksetmesi, over kanseri hastalarının yüksek ölüm oranından sorumludur. Bu sebeple, over kanserinde kemodirencinin üstesinden gelmek için yeni terapötik stratejilerin geliştirilmesine acilen ihtiyaç duyulmaktadır. Bu çalışmada, over kanserinde kemodirenci azaltmak amacıyla çeşitli kemoterapi ilaçlarının (bortezomib, olaparib ve cisplatin) kombinasyonları uygulandı. Uygulanan belirli ilaç konsantrasyonlarında, bortezomib ve olaparib ilaç kombinasyonu, ilaçların tek başlarına kullanıldıkları durumlara göre sitotoksisiteyi arttırmıştır. Ayrıca, kombinasyon ilaç uygulamaları ve DYNLL1 gen ifade seviyeleri arasındaki ilişkinin kemodirenç üzerindeki karşılaştırmalı analizi de gerçekleştirilmiştir. Bu sonuçlar, kombinasyon ilaç uygulamalarının over kanserinde kemodirenci düşürmek amacıyla potansiyal etkilerini ortaya koydu ve DYNLL1 gen ifade seviyesinin kemodirenç profilleri ile ilişkisi çalışılmıştır.

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ANAHTAR KELİMELER: Over kanseri, Kemodirenç, Protozom, PARP, Sisplatin, DYNLL1, Kemoterapi

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ABBREVIATIONS

Abbreviation	Explanation
BOR	Bortezomib
CIS	Cisplatin
DYNLL1	Dynein Light Chain LC8-Type 1
FBS	Fetal Bovine Serum
HGSOC	High-grade Serous Ovarian Carcinoma
OLA	Olaparib
PARP	Poly (ADP-Ribose) polymerase
PBS	Phosphate-buffered saline
PFI	Platinum-free Survival
SRB	Sulforhodamine B
TCA	Trichloroacetic acid
UPR	Unfolded Protein Response

FIGURE LIST

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1. INTRODUCTION

1.1. Ovarian Cancer and Treatment Methodologies

Ovarian cancer has the highest mortality rate compared to other most common malignant tumors of female reproductive system, cervical and endometrial cancer (Chen *et al*., 2019). In general, it is the sixth most common cancer type in females and 4% of women diagnosed with cancer have ovarian cancer (Vargas, 2014; Ferlay *et al*., 2015). Each year, more than 200.000 women are diagnosed with ovarian cancer and approximately 100.000 people die due to this disease worldwide. The 5-year overall survival rate is less than 50 % in ovarian cancer (Siegel *et al*., 2018). Besides the lack of early diagnostic methods and the lack of characteristic disease symptoms, enhanced chemotherapy resistance and relapse (i.e. the absence of long-term and effective treatment) are responsible for the high mortality rate of ovarian cancer patients (Ali *et al*., 2013). Therefore, novel therapeutic strategies for overcoming chemoresistance in ovarian cancer are urgently needed.

The most ovarian cancers are originated from epithelial cells. Epithelial ovarian carcinoma accounts for approximately %90 of malignant ovarian tumors (Matulonis *et al*., 2016). Ovarian cancer is classified into the following five main types based on their histopathological characteristics: high-grade serous carcinoma, low-grade serous carcinoma, endometrioid carcinoma, clear cell carcinoma, and mucinous carcinoma (Lim *et al*., 2013). These ovarian cancer types differ in many aspects of cancer biology such as precursor lesions, carcinogenesis mechanisms, cells of origin, disease progression and metastasis to other tissues, chemotherapy response, clinical features and outcomes (Motohara *et al*., 2019). High-grade serous ovarian carcinoma (HGSOC) is the most frequently diagnosed form of ovarian cancer and it is typically very responsive to chemotherapy with platinum drugs at the time of diagnosis. However, similar to other histological subtypes of ovarian cancer, HGSOCs frequently relapse after several rounds of chemotherapy and become

increasingly refractory to treatment with platinum agents such as cisplatin (Matulonis *et al*., 2016).

The majority of ovarian cancer patients are diagnosed at an advanced stage (National Cancer Institute, 2016). Symptoms of the disease might be initially missed or misinterpreted and diagnosis mostly occurs when cancer has already reached the late stage where treatment success is lower. Staging of ovarian cancer is performed based on surgical assessment of cancer at initial diagnosis by using the International Federation of Gynecology and Obstetrics (FIGO) staging system. Several pathological samples including lymph nodes, tissue biopsy samples or abdominal fluid can be used in the evaluation of cancer (Pereira *et al*., 2015).

For the newly diagnosed patients, the standard treatment for ovarian cancer consists of cytoreductive surgery and platinum-based chemotherapy (platinum analogues such as cisplatin or carboplatin) with the addition of a taxane drug (paclitaxel or docetaxel) (Matulonis *et al*., 2016). The main rationale behind cytoreductive surgery for ovarian cancer is that, with the removal of tumor mass which contains cancer stem cells, chemoresistant cell subpopulations and tumor microenvironment which provides optimal conditions for tumor cells to grow, chemotherapy efficacy would be ultimately enhanced (Brand *et al*., 2017).

The first line treatment of newly diagnosed ovarian cancer patients includes either primary surgical cytoreduction followed by combination chemotherapy with platinum drugs or neoadjuvant chemotherapy where chemotherapy has been applied before cytoreduction surgery to debulk tumor mass. Recurrence after platinum-based chemotherapy is very common (up to 75 %) and this eventual development of platinum resistance is a major issue in the treatment of ovarian cancer. In the management of the recurrent ovarian cancer; chemotherapy, anti-angiogenic agents and poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi) are used. PARP inhibitors will be discussed in more detail below.

1.2. Cisplatin Treatment in Ovarian Cancer

Cisplatin (or other platinum drugs such as carboplatin) is the most commonly used first-line chemotherapeutic agent for the management of ovarian cancer progression. However, cisplatin resistance can lead to treatment failure due to decreased chemotherapeutic efficacy after several rounds of treatment with cisplatin. Therefore, chemoresistance to platinum-based drugs such as cisplatin is of critical clinical concern in the effective and long-term treatment of ovarian cancer. A better understanding of cisplatin resistance mechanisms and the development of effective strategies to increase cisplatin sensitivity in ovarian cancer management is of high importance.

Patients who initially respond to platinum-based chemotherapy and relapse after 6 months are classified as platinum-sensitive. In other words, these patients have platinum-free interval (PFI is defined as the time between the last dose of platinum drug application and the start of cancer progression) of more than 6 months (Davis *et al*., 2014). The median survival rate of ovarian cancer patients with platinum-sensitive recurrence is 2-3 years. Patients who relapse within 6 months after their initial platinum treatment (PFI of less than 6 months) are classified as platinum-resistant and their response rates to subsequent chemotherapy is as low as 15 %. Median survival for these patients is under a year. Therefore, the consideration of ovarian cancer heterogeneity between patients is compulsory in cancer research and the effective treatment of ovarian cancer.

Platinum drugs are mostly thought to result in purine base crosslinks, thus leading to DNA damage and ultimately to cell death, among other mechanisms of action such as generating cytotoxicity by inducing the production of reactive oxygen species (Dasari *et al*., 2014; Choi *et al*., 2015; Marullo *et al*., 2013). Molecular mechanisms of cisplatin resistance in cancer is multidimensional and have not been completely understood yet. Cisplatin resistance can be associated with several molecular processes including reduced uptake and increased efflux of cisplatin, increased DNA repair, inactivation of apoptosis pathways, regulation by miRNAs, DNA methylation (Cacan, 2017; Galluzzi

et al., 2012; Zhu *et al*., 2016). For instance, it was reported that ERK-mediated autophagy can lead to cisplatin resistance and inhibition of ERK decreases cisplatin-induced autophagy and ultimately sensitizes ovarian cancer cells to cisplatin induced-autophagy (Wang and Wu, 2014). In other studies, cisplatin resistance was linked with aberrant DNA methylation and histone deacetylation at the promoter region of several genes that play important roles in cell survival, proliferation and apoptosis (Cacan *et al*., 2014; Cacan, 2016). It was also shown that the presence of cancer stem cells in a tumor microenvironment could promote resistance to cisplatin. A full comprehension of cisplatin resistance in ovarian cancer will help us to design better treatment strategies to target the chemoresistance of ovarian cancer cells.

Patients with deficiencies in DNA damage response (DDR) exhibit higher chemosensitivity to platinum agents such as cisplatin. For instance, ovarian cancer patients with BRCA1/BRCA2 mutations are more responsive to platinum-based drug treatment (Konstantinopoulus *et al*., 2010). These genes play an essential role in maintaining genomic stability and act as tumor suppressors. It is found that BRCA1 levels are increased in cisplatin-resistant ovarian cancers when compared to that of cisplatin-sensitive ovarian cancer and therefore this gene is highly implicated in cisplatin cytotoxicity in ovarian cancer (Li *et al*., 2016).

1.3. Proteasome Inhibition in Cancer

Proteasome inhibitors have shown clinical benefits in the treatment of cancer at varying levels. The first FDA (US Food and Drug Administration)-approved proteasome inhibitor, bortezomib (originally PS-341 and marketed as Velcade by Millennium Pharmaceuticals), is currently used for the treatment of newly-diagnosed and relapsed multiple myeloma and mantle cell lymphoma (Kane *et al*., 2006, 2007). Bortezomib inhibits chymotrypsin like activity of 20S subunit of the 26S proteasome complex which maintains protein homeostasis by selectively degrading proteins that have (poly)-ubiquitin post translational modifications (Meyer-Schwesinger *et al*., 2019). The ubiquitylation labels misfolded, damaged, aggregation-prone or unneeded proteins for

degradation by the proteasome and this multi-enzyme system is called the ubiquitin-proteasome system. In addition to bortezomib, there are some other proteasome inhibitors developed: second generation proteasome inhibitors (carfilzomib, ixazomib, marizomib), novel proteasome inhibitors (oprozomib, delanzomib) (Kubiczkova *et al*., 2014).

Proteasome inhibition can lead to cancer cell death through multiple cellular processes including the promotion of apoptosis, disruption of cell cycle, inhibition of proliferation and angiogenesis (Boccadoro *et al.*, 2005; Cacan *et al.*, 2015; Roeten *et al.*, 2018). The main mechanism by which proteasome inhibitors induce cell death is through the accumulation of toxic (poly)-ubiquitinated proteins and misfolded protein aggregates in the absence of functional proteasomes in the cytoplasm. This accumulation of toxic proteins or protein aggregates eventually induces endoplasmic reticulum (ER)-stress which leads to the activation of the unfolded protein response (Ri, 2016; Obeng *et al*., 2006). Prolonged and strong UPR activation to the levels that it can not compensate anymore for the ER-stress which is caused by proteasome inhibition induces the upregulation of pro-apoptotic proteins to promote apoptosis. Proteasome inhibition also leads to apoptosis through the induction of reactive oxygen species due to the accumulation of unfolded proteins. Reactive oxygen species activate caspase cascade and this activation contributes to the apoptosis induced by proteasome inhibition (Perez-Galan *et al*., 2005).

Another mechanism of action of proteasome inhibition leading to cancer cell death is the inhibition of the pro-survival NF- κB pathway. As a transcription factor, NF-κB functions in the activation of pro-survival pathways, and thus, it has a role in the inhibition of apoptosis (Adams, 2004). The inhibition of proteasomal degradation by proteasome inhibitors including bortezomib leads to the cellular accumulation of IκBα, which is an inhibitor of NF-κB. This blocks the nuclear translocation of NF-κB and thus its activation in gene regulation. Although this mechanism of action is not a primary contributor of proteasome inhibition-induced cell death, it is still considerable since some cancer cells are highly dependent on NF-κB-induced pro-survival signaling (Roeten *et al*., 2018; Chaturvedi *et al*., 2011).

In cancer cells, proteasome degrades pro-apoptotic proteins such as p53 and thus blocks the programmed cell death of cancer cells. Proteasome inhibitors including bortezomib disrupt this process and the activity of pro-apoptotic proteins which were not degraded by proteasome results in cell cycle arrest and apoptosis in cancer cells. In other words, proteasome inhibition can stabilize and reactivate p53, a tumor suppressor protein, thus increasing pro-apoptotic effects caused by proteasome inhibition in cancer (McConkey *et al*., 2008). This tumor suppressor protein is inactivated in many cancer types; therefore its reactivation by the inhibition of proteasomal degradation is of high importance in the treatment of cancer.

In 2009, Uddin *et al*. reported that bortezomib inhibits cellular growth through upregulation of $p27^{kip1}$ and induction of apoptosis in epithelial ovarian cancer (EOC) (Uddin *et al*., 2009). $p27^{kip1}$ is a cyclin-dependent kinase inhibitor which is targeted by SKP2 (S-phase kinase protein 2) through ubiquitin-mediated degradation. They showed that bortezomib treatment of EOC cells leads to downregulation of SKP2 and accumulation of $p27^{kip1}$ which results in the growth inhibition of tumors. This study suggested that the ubiquitin-proteasome pathway may be a potential target for chemotherapeutic intervention in the treatment of ovarian cancer.

In 2013, bortezomib was approved by the Food and Drug Administration (FDA) in the US and became the first proteasome inhibitor authorized by this agency (Chen *et al*., 2011). Currently, this drug is licensed for the treatment of multiple myeloma and mantle cell lymphoma. However, most patients who initially respond to bortezomib treatment ultimately experience relapse and the clinical response to bortezomib is not satisfactory in other hematologic cancers and solid tumors including ovarian cancer (Ruschak *et al*., 2011; McCabe *et al*., 2006). A phase II evaluation of bortezomib as a single agent for the treatment of recurrent platinum-sensitive ovarian or primary peritoneal cancer has shown that administration of this drug as a single agent has minimal activity in this patient group (McCabe *et al*., 2006). Considering the fact that bortezomib treatment is not generally effective as a monotherapy in solid tumors, novel treatment strategies which combine bortezomib with another agent or agents have recently emerged (Wright, 2010).

1.4. Poly (ADP-ribose) Polymerase (PARP) Inhibition in Cancer

Poly (ADP-ribose) polymerases (PARP) function in a variety of DNA damage repair pathways (Konecny and Kristeleit, 2016). This family of proteins comprising 17 members catalyzes ADP-ribosylation of target proteins in response to genotoxic stress caused by DNA damage, using NAD+ as the ADP-ribose donor (Bian *et al*., 2019; Hottiger *et al*., 2010). Among all of the members of this PARP family of proteins, PARP1 is one of the most abundant and catalyze poly (ADP-ribosyl)ation (PARylation) for approximately %85 of proteins which are PARylated after DNA damage (Kim *et al*., 2005). It is involved in multiple cellular processes including the regulation of transcriptional control, DNA repair, maintenance of genomic integrity and apoptosis (Ossovskaya *et al*., 2010). PARP2 also functions in DNA damage-induced poly (ADP-ribosyl)ation (Ame *et al*., 1999). DNA nicks and double-stranded breaks (DSBs) increase PARP catalytic activity about 500-fold in the nucleus (Hassler and Ladurner, 2012).

Small molecule PARP (poly (ADP-ribose) polymerases) inhibitors (PARPi) have emerged as effective drugs in the treatment of ovarian cancer. However, this treatment efficacy has been shown to be limited to ovarian and breast cancer patients with BRCA1/2 mutations which result in a lack of homologous recombination (McCabe *et al*., 2006; Sonnenblick *et al*., 2015). Therefore, the highest efficacy of PARP inhibitors has been observed in patients with these mutations which sensitize cancer cells to the inhibition of PARP activity, by exploiting synthetic lethality. Therefore, homologous recombination deficiency is used as a prospective biomarker in the prediction of the response to PARP inhibitors such as olaparib in the treatment of ovarian cancer.

FDA has approved three types of PARP inhibitors (olaparib, rucaparib and niraparib) for the treatment of BRCA1/2 mutation-associated breast, ovarian and prostate cancers (Bian *et al.*, 2019). These pharmacological inhibitors have demonstrated anti-cancer activity alone or in combination with platinum-based chemotherapeutics such as cisplatin in several cancer types, including ovarian cancer (Audeh *et al*., 2010;

Lederman *et al*., 2014; Liu *et al*., 2013; Tutt *et al*., 2010). Among the key PARP inhibitors, the most extensive clinical investigation was performed with olaparib (Konecny and Kristeleit, 2016). The European Commission has authorized olaparib as monotherapy in the maintenance treatment of patients with platinum-sensitive, relapsed *BRCA*-mutated (germline and/or somatic) high-grade serous epithelial ovarian, fallopian tube, or primary peritoneal cancer who are in complete response or partial response following chemotherapy with platinum drugs (Lynparza prescribing information, 2014). In the US, olaparib has been approved by the Food and Drug Administration (FDA) as monotherapy in patients with deleterious or suspected deleterious germline *BRCA*-mutated (*gBRCAm*) advanced ovarian cancer and who have been treated with three or more prior lines of chemotherapy (Lynparza prescribing information, 2014). It has been shown that olaparib maintenance treatment improves progression-free survival significantly in patients with platinum-sensitive, relapsed ovarian cancer and a BRCA1/2 mutation (Pujade-Lauraine *et al*., 2017). A recent phase I/Ib study reported that olaparib treatment combined with carboplatin has clinical benefits in subsets of heavily pretreated high grade serous ovarian carcinoma patients with wild type BRCA, independent of their platinum sensitivity (Lampert *et al*., 2019). Therefore, a combination of olaparib with other chemotherapeutic agents such as bortezomib and cisplatin may have a potential benefit for advanced ovarian cancer tumors, which will be the focus of this thesis (Image 1.1).

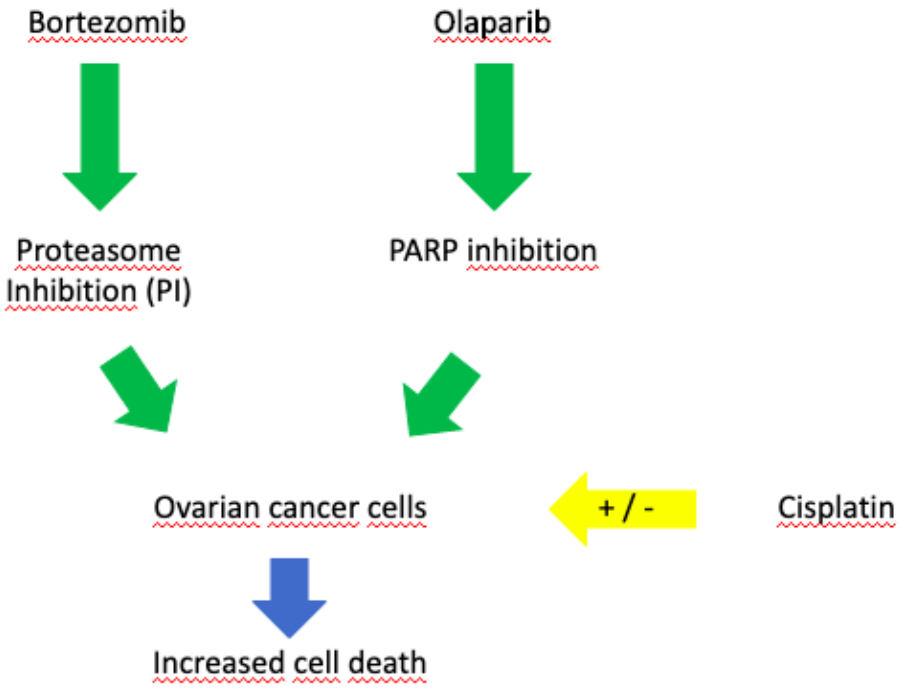


Image 1.1. Schematic representation of the current study

1.5. DYNNL1 in Ovarian Cancer Chemoresistance

Recently, He et al. used a genome-scale CRISPR-Cas9 knockout (GeCKO) library to identify genes in which loss confers resistance to PARP inhibitors (PARPi) and platinum drugs in a panel of patient-derived BRCA1-mutant high-grade serous ovarian carcinoma lines (UWB1.289, COV362 and JHOS-2) (He *et al*., 2018). In this study, they reported that loss of DYNLL1 (dynein light chain 1; also known as LC8 or PIN) in both PARPi- and platinum-treated cells correlated with resistance to these drugs. They showed that DYNLL1 inhibits DNA end resection by associating with DNA end-resection machinery (MRN complex, BLM helicase and DNA2 endonuclease) and its loss leads to restoration of DNA end resection and homologous recombination (HR), thus resistance to PARPi and platinum treatment. Since cisplatin mainly works through DNA damage and PAPR inhibitors blocks the activity of a type of DNA repair enzyme (PARPs), restoration of HR activity by the loss of DYNLL1 limits their efficacy on cancer cell death. Thus it increases chemoresistance (i.e, decreased chemosensitivity) to

these chemotherapeutic drugs. Using *in vitro* binding assays, they observed that DYNLL1 binds directly to MRE11 to limit its nuclease activity in DNA end-resection and thus it limits DNA repair by homologous recombination.

DYNLL1 functions in several cellular processes including intracellular trafficking and apoptosis (Barbar, 2018; King, 2008) and its role in DNA repair and chemoresistance has been recently identified in the study mentioned above. However, many aspects of its role in cancer chemoresistance remain unexplored. The reason behind how the chemoresistance of different ovarian cancer cell lines correlates with their DYNLL1 expression levels has not been studied yet. Whether chemotherapy with PARP inhibitors such as olaparib and proteasome inhibitors such as bortezomib (or their combination) has an effect on DYNLL1 expression levels of ovarian cancer cells remains unknown. A better understanding of the role of DYNLL1 in ovarian cancer chemoresistance has significant clinical importance.

2. MATERIAL and METHODS

2.1. Cell Culture

Human ovarian cancer cell lines (OV2008, C13, A2780 and A2780-AD) were generously provided by Dr. Shelly B. Hooks, University of Georgia, USA. These cells were maintained in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Biological Industries), 5 mM L-glutamine and 5 mM penicillin/streptomycin (Biological Industries), in a humidified 5% CO_2 incubator at 37 ºC. Media was supplemented with 2 gr/L sodium bicarbonate (Serva, Germany). Media were filtered using vacuum filters (TPP Vacuum Filtration ‘’rapid’’ Filtermax with a pore size of 0.22 um) and stored at 4 ºC for later use. Chemoresistant cell lines C13 and A2780-AD were continuously grown with 3 μM cisplatin. Confluent cells were passaged as follows: Media were aspirated from T75 flasks (Corning, 75 cm^2 cell culture flask) and 5 mL trypsin (Biological Industries) pre-warmed to 37 ºC were added for a T75 cell culture flask. Flasks were incubated for approximately 3-5 min at 37 ºC incubator until cells were completely detached from the surface of flasks and then 10 ml pre-warmed media were added to each flask. After pipetting with serological pipettes, media were transferred to 50 mL canonical tubes and centrifuged at 2000 rpm for 5 min. After discarding supernatant, cell pellets were dissolved in 10 mL media and cells were seeded to the plates in desired dilutions considering cell density. Total media volume in a T75 flask were completed to 20 mL. Cell confluency was checked continuously to avoid over confluency.

Cells were frozen for further use by using freezing media (Biological Industries) which include 10% DMSO, in 1.8 mL cryovials. Cryovials were kept at –20 ºC for 1 hour, then at –82 ºC overnight and finally moved to liquid nitrogen. In the thawing of frozen cells, after quick thawing of media + cells in cryovials, contents were transferred using Pasteur pipettes into 10 mL pre-warmed media in 50 mL canonical tubes and centrifuged at 2000 rpm for 5 minutes to remove DMSO present in freezing media. After resolving cell pellet with media without DMSO, cells were seeded in T75 flasks. Next day, flasks were checked and media were changed to remove any artifacts from freezing media.

In cell culture hood and cell culture room, UV light was turned on for approximately 30 min before and after any work performed in the cell culture. Contamination was periodically checked. ESCO class II type A2 cell culture hood was used in all cell culture experiments. 70% ethanol and 10% bleach were used to clean surfaces in the cell culture hood. Over-passaging of cells was avoided and cells were used in any experiment after at least one passage following the thawing of cell stocks stored in a liquid nitrogen tank. Cell culture room was periodically cleaned using 10% bleach in water.

2.2. Chemotherapeutic Agents

Cisplatin was purchased from Kocak Pharma (Istanbul, Turkey). Bortezomib was purchased from LC Laboratories (Woburn, MA, USA). Olaparib was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bortezomib and olaparib were dissolved in DMSO (Dimethyl sulfoxide, ultra-pure grade, Amresco, VWR) and DMSO controls were included in the assays. Bortezomib stock solution was kept at – 20 ºC and olaparib stock solution was kept at 4 ºC. Cisplatin was diluted in phosphate-buffered saline (PBS) and was kept at room temperature at dark. Extensive free-thaw cycles were avoided in the storage of drugs.

2.3. Cytotoxicity Screening: MTT and SRB Assays

2.3.1 MTT ((3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay

Cancer cell viability in response to drugs alone or in combination was measured using MTT ((3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. Metabolically active cells reduce yellow tetrazolium MTT into purple formazan in part by the action of dehydrogenase enzymes. Therefore, the spectrophotometric signal from formazan correlates with the number of cells.

In MTT assay, 10.000 cells / well were seeded in 96-well plates in 200 µL media / well. To prepare MTT solution (5 mg/ mL), MTT powder (Serva, Germany) was solved in phosphate buffered saline (PBS) and filtered using filters with 0.2 micrometer pore size (Sartorius, Germany) in cell culture hood. MTT solution was stored in the dark, at –20 ºC freezer. MTT solvent was prepared by solving 10 % SDS (sodium dodecyl sulphate) in 0.01 M HCl (w/v, in ddH_2O). 24 h after cell seeding, drug treatments were performed as triplicates for each condition and cells were further incubated for 48 hours. In MTT Assay, media were carefully aspirated from wells and 90 µL serum-free RPMI (preferably without phenol red) was added to each well. Then, 10 µL MTT solution (5 mg/mL) was added to each well (final MTT concentration in wells was 0.5 mg/mL) and plates were incubated in a humidified 5% CO_2 incubator at 37 ºC for 4 hours. After incubation, 100 µL 10% SDS in 0.01 M HCl was added to each well and plates were incubated on an orbital shaker in the dark for 3 hours. Spectrophotometric reading was performed using Multiskan Go microplate reader (ThermoScientific) at multiple wavelengths (570, 580 and 700 nm (as reference wavelength)) and absorbance values were used in data analysis. Experiment plans were provided in Supplementary Documents part.

2.3.2 Sulphorhodamine B (SRB) assay

The viability of cells after drug treatments were determined by sulphorhodamine B (SRB) colorimetric assay as previously described (Vichai and Kirtikara, 2006; Skehan *et al.*, 1990). Briefly, cells were seeded in 96-well plates (Costar, Corning, flat-bottom, NY, USA) in 200 µL media as 7.500 cells / well and allowed to adhere overnight. Cells were incubated with bortezomib and/or olaparib for 48 hours. Control cells with no drug treatment (only DMSO) were also included for each cell line in triplicates. For triple drug experiments, after bortezomib and/or olaparib addition, cells were incubated for 24 hours and then cisplatin was added. Cells were further incubated for 24 hours before SRB Assay. Cells were fixed with 100 µL cold 10% TCA (trichloroacetic acid) (w/v) per well and incubated at 4 ºC for 1.5 hour (This step prevents cells attached to the bottom of the well from washing off. Also, we have observed that flat-bottom wells show higher cell retention and decreases cell loss during washing steps). Then wells

were washed with ddH2O for four times and left to dry at 50 ºC incubator for 30 min with caps open. After wells were completely dried, 100 µL 0.057% (w/v) SRB dye dissolved in 1% acetic acid was added to each well (SRB solution can be stored at 4 ºC in dark (covered in aluminum foil) for later use). 96-well plates were kept at an orbital shaker in dark for 30 min. Following the staining step, wells were washed with 1% acetic acid for four times until no dye was left at the edges of wells. All steps following staining with SRB dye was performed by avoiding direct light exposure due to the light sensitivity of SRB dye. Then, 200 ul of 10 mM Tris base (dissolved in ddH2O) solution were added to each well and plates were placed in an orbital shaker for 30 min in order to completely solubilize the dye, at dark. Spectrophotometric reading was performed using a microplate reader (Multiskan Go, ThermoScientific, USA) at wavelengths of 492, 565 and 650 nm (control wavelength). Sulphorhodamine B dye was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.4. RNA Isolation

10^5 cells were seeded per well in 12 well plates (Costar, Corning, flat-bottom) and cells were incubated for 24 hours. Drug treatments were performed and cells were further incubated for 24 hours. Then, plates were placed on ice, media were removed from wells and wells were washed with ice-cold PBS twice. 1000 µL QIAzol lysis reagent (Qiagen, Germany) were added to each well and plates were placed at room temperature (RT) for 5 min (pipetting was performed in the middle of this incubation period to detach all the cells from the surface of the plate). The cells were then transferred to a 1.5 ml eppendorf tube and 0.2 ml chloroform (Amresco, USA) was added to each tube and tubes were shaked for 15 seconds. After placing tubes at RT for 3 minutes, centrifugation was performed at 12.000 g for 15 min at 4 C. Then, upper aqueous phase containing RNA molecules was transferred carefully (avoiding to take the interphase) to a new 1.5 ml tube labelled accordingly and 0.5 ml isopropanol (Amresco) was added per tube and tubes were briefly vortexed. After incubation at room temperature for 10 min, tubes were centrifuged at 12.000 g for 10 min at 4 ºC. Then, the supernatant was carefully aspirated and discarded. 1 ml %75 ethanol (absolute ethanol (Isolab, Germany) was dissolved in ddH2O) was added to each tube and centrifugation was

performed at 7500 g for 5 min at 4 ^{o}C. Following the removal of the supernatant completely, tubes were air-dried to remove any remaining ethanol (tubes were placed horizontally in a sterile environment). Finally, RNA pellets were re-dissolved in an appropriate volume of RNase-free water (in 30 ul RNase-free water if RNA pellet was hardly visible and in 50 µL if RNA pellet was clearly visible) and their concentrations were measured using Multiskan Go instrument at wavelengths of 260 and 280 nm. Absorbance values at 260 nm were converted to ng/µL unit by multiplying the values with 40. A260/A280 ratio was used to determine RNA purity.

Isolated RNA samples were kept at –80 ^{o}C freezer before using them in RT-PCR experiments. Extensive freeze-thaw cycles were avoided in order not to decrease RNA quality. RNA isolation experiments were performed in a fume hood to avoid the inhalation of evaporating chemicals.

2.5. Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

In RT-PCR experiments, WizPure qRT-PCR Master (Super Green) One-step Real-Time PCR kit (Wizbiosolutions, Seongnam, Korea) was used. Reaction conditions are performed as follows: qRT-PCR Master (Super Green) (10 µL, 1x), 10 uM forward primer (1 µL), 10 uM reverse primer (1 µL), template RNA (<500 ng/reaction), RNase-free water (up to 20 µL). Following One-step Real-time RT-PCR conditions were performed: reverse transcription (55 C, 30 min, 1 cycle), initial denaturation (95 C, 10 min, 1 cycle), denaturation (95 C, 15 sec, 40 cycle), annealing (detection, 55 C, 30 sec, 40 cycle), amplification (72, 35 sec, 40 cycle), cooling (37 C, 30 sec, 1 cycle). LightCycler capillaries (20 µL, Roche) were used with Roche LightCycler 1.5 Instrument (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). Instrument's software was used to acquire the data.

Relative RNA quantification was performed using the formula:

$$R = 2^{-(Cp(sample) - Cp(control))}.$$

GAPDH primers were used in all RT-PCR experiments for normalization. In each RT-PCR run, duplicates were used for each condition in addition to duplicates in which GAPDH primers were used for the same conditions. Relative RNA quantification analysis based on Cp values was performed in R.

2.5.1 qRT-PCR primers

Primers used in RT-PCR experiments are as follows:

DYNLL1 forward primer: 5′-AGATGCAACAGGACTCGGTG-3′

DYNLL1 reverse primer: 5′-CCACTTGGCCCAGGTAGAAG-3′

GAPDH forward primer: 5’-GTCTCCTCTGACTTCAACAGCG-3

GAPDH reverse primer: 5’- ACCACCCTGTTGCTGTAGCCAA-3'

Primers were purchased from Sentegen Biyotek (Bilkent, Ankara, Turkey). Primers were dissolved in nuclease-free water by pipetting without vortexing and stored at 4 ^{o}C.

2.6. Data Analysis and Visualization

The data in the graphs which show SRB Assay results were expressed as mean ± SEM. Statistical comparisons were performed using GraphPad Prism 7 software (GraphPad). Statistical significance was determined using Student’s t test (two-tailed, unpaired) or Sidak’s multiple comparisons test following ANOVA (analysis of variance) test. The data in this assay were derived from three independent biological repeats. More detail on statistical analysis is given in figure legends.

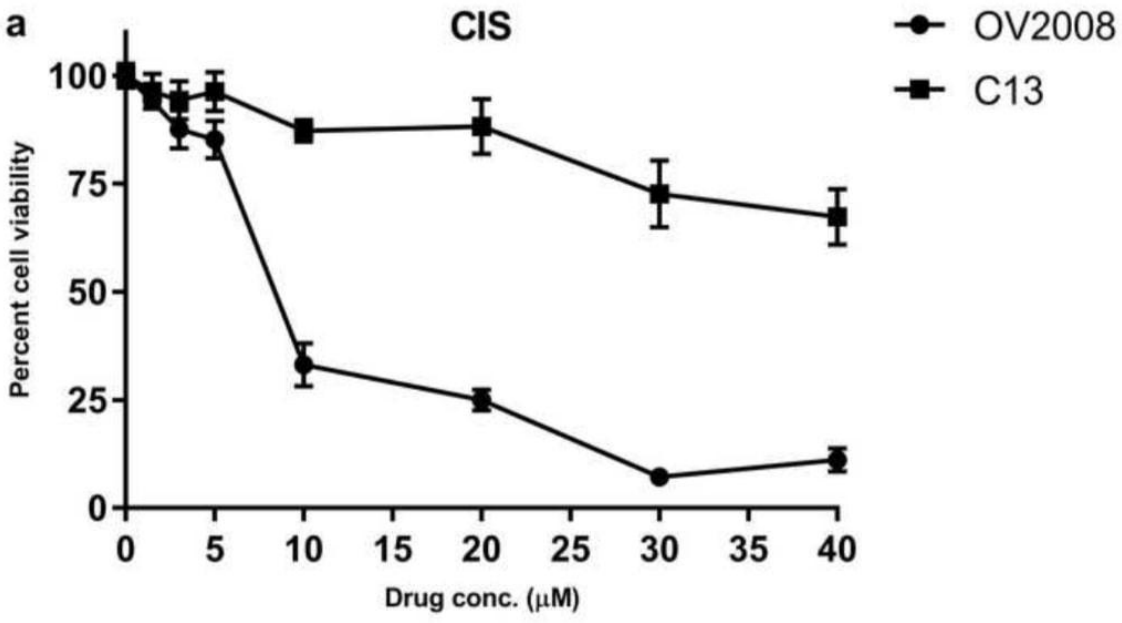
The data visualization in the other graphs were performed using R and RStudio (39, 68). R packages used in this study are as follows: tidyverse packages (ggplot, dplyr, tidyr, readr, purrr, tibble, stringr, forcats) (Wickham, 2017), readxl package (Wickham and

Bryan, 2019). RMarkdown was used to produce reproducible analysis documents (RStudio and Inc., 2014). Exemplary R codes and RMarkdown documents can be found in Supplementary Documents and additional information on experimental data and R codes are available upon request.

3. RESULTS

3.1. Response to Cisplatin in Chemosensitive and Chemoresistance Ovarian Cancer Cell Lines

Chemosensitive ovarian cancer cell lines used in this study, OV2008 and A2780, are the parent lines to chemoresistant cell lines, C13 and A2780-AD, respectively. To determine specific responses of these cell lines to cisplatin, we measured relative cell viability with Sulforhodamine B (SRB) colorimetric assay after treatment of cells with indicated concentrations of cisplatin (in 1.5 - 40 micromolar range) (Figure 3.1). As expected, this cytotoxicity screen showed that the sensitivity of chemoresistant cell lines, OV2008 and A2780, to cisplatin were significantly higher than that of their chemoresistant counterparts, C13 and A2780-AD cells, respectively. The susceptibility of A2780-AD chemoresistant cell line to platinum exposure was determined to be relatively higher than that of C13, other chemoresistance cell line (Figure 3.1a and 1b). These data indicated that these ovarian cancer cell lines preserved their chemosensitivity profiles in our laboratory conditions and experimental setup.



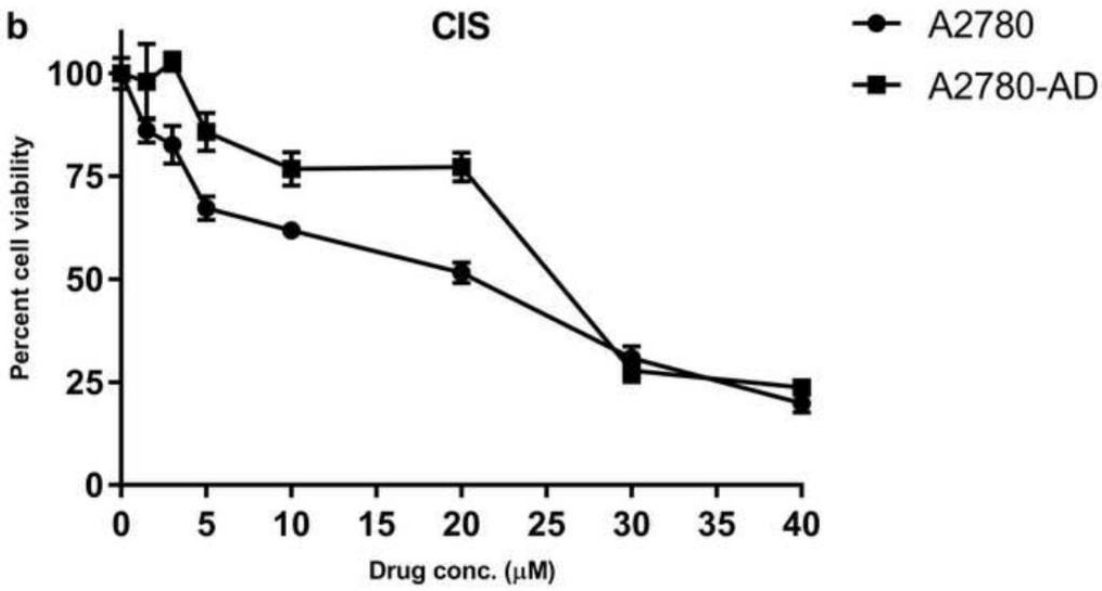
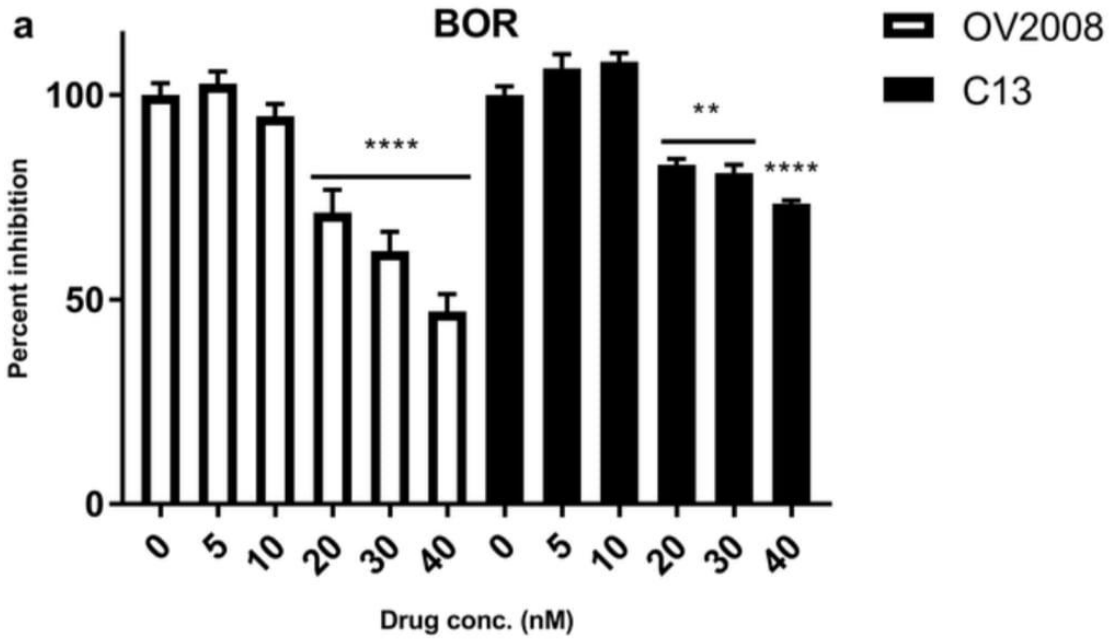


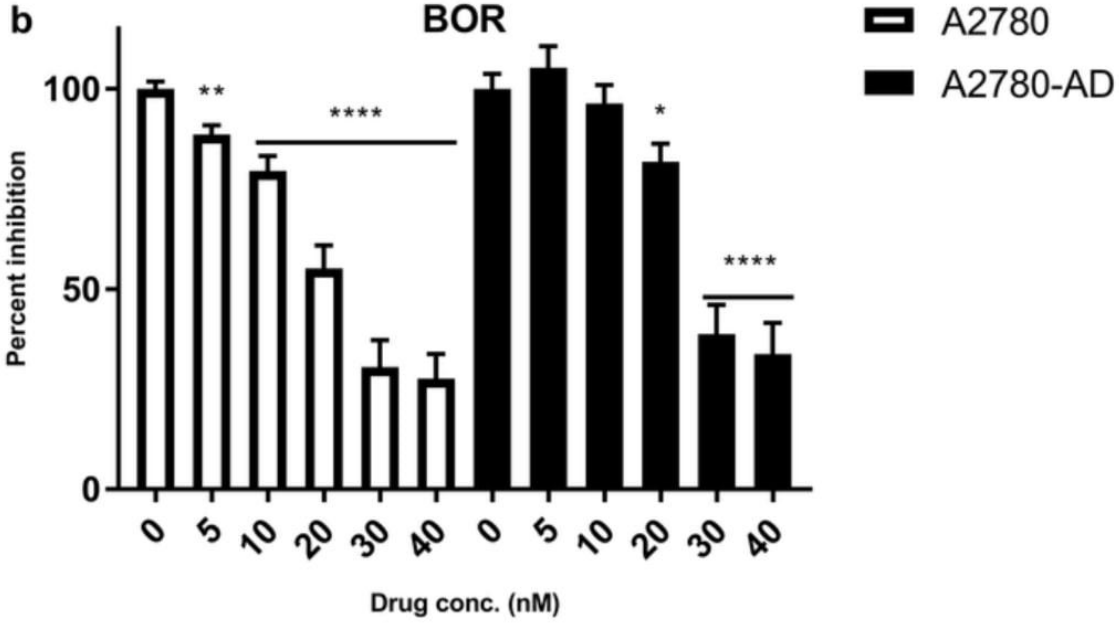
Figure 3.1. Response to cisplatin in chemosensitive and chemoresistance ovarian cancer cell lines. OV2008 – C13 (a) and A2780 – A2780-AD (b) cell line pairs were treated with different concentrations of cisplatin (CIS, μM) and the percent cell viability was measured following 48h after the treatment. Data in the graph represent an average of at least six data points. CIS: cisplatin. Drug conc.: drug concentration

3.2. Bortezomib Treatment Results in Increased Cytotoxicity in Ovarian Cancer Cell Lines in a Dose-dependent Manner

Next, we examined the cytotoxic effects of bortezomib (BOR), the proteasome inhibitor, on chemosensitive and chemoresistant ovarian cancer cell lines. Both chemosensitive and chemoresistant cells were treated with increasing concentrations of bortezomib (in 5 - 40 nanomolar range) as shown in Figure 3.2a and 3.2b. This single-agent *in vitro* treatment results demonstrated that chemosensitive cell line OV2008 were more sensitive to proteasome inhibition when compared to its chemoresistant daughter cell line C13 (Figure 3.2a). This difference in bortezomib sensitivity showed itself in concentrations higher than 10 nM for this cell line pair. Similarly, chemosensitive cell line A2780 was shown to be more sensitive to proteasome inhibition by bortezomib relative to its chemoresistant counterpart A2780-AD for which the first effective concentration of bortezomib was 20 nM, whereas it was as low as 5 nM for A2780

(Figure 3.2b). Between the two chemosensitive cell lines, cytotoxicity due to bortezomib treatment were significantly higher for A2780. Based on these cell viability experiments, two different concentrations of bortezomib (10 nM and 20 nM) were selected to be used in combination drug treatments, since 10 nM is the highest concentration of bortezomib tested with no cytotoxic effect and 20 nM is the lowest concentration tested with a significant cytotoxicity for four cell lines used in this study, except A2780 cell line whose susceptibility to bortezomib treatment is the highest among four ovarian cancer cell lines (Figure 3.2a, 3.2b).




b
BOR
A2780
A2780-AD
Percent inhibition
100
50
0
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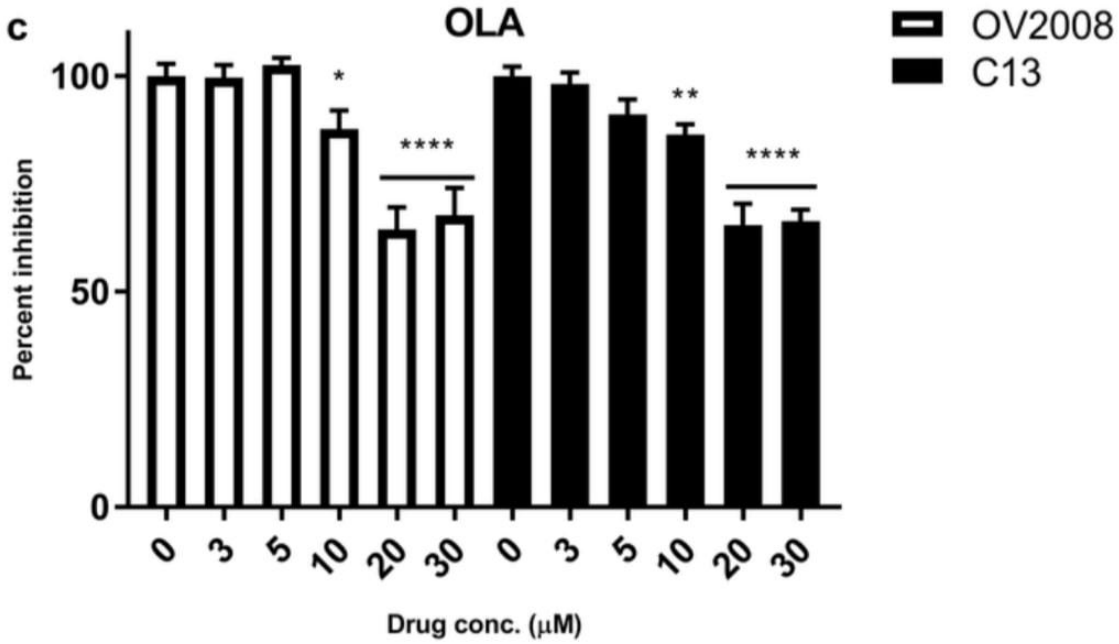
****
*
****
0 5 10 20 30 40 0 5 10 20 30 40
Drug conc. (nM)


c
OLA
OV2008
C13
Percent inhibition
100
50
0
*
****
**
****
0 3 5 10 20 30 0 3 5 10 20 30
Drug conc. (μM)

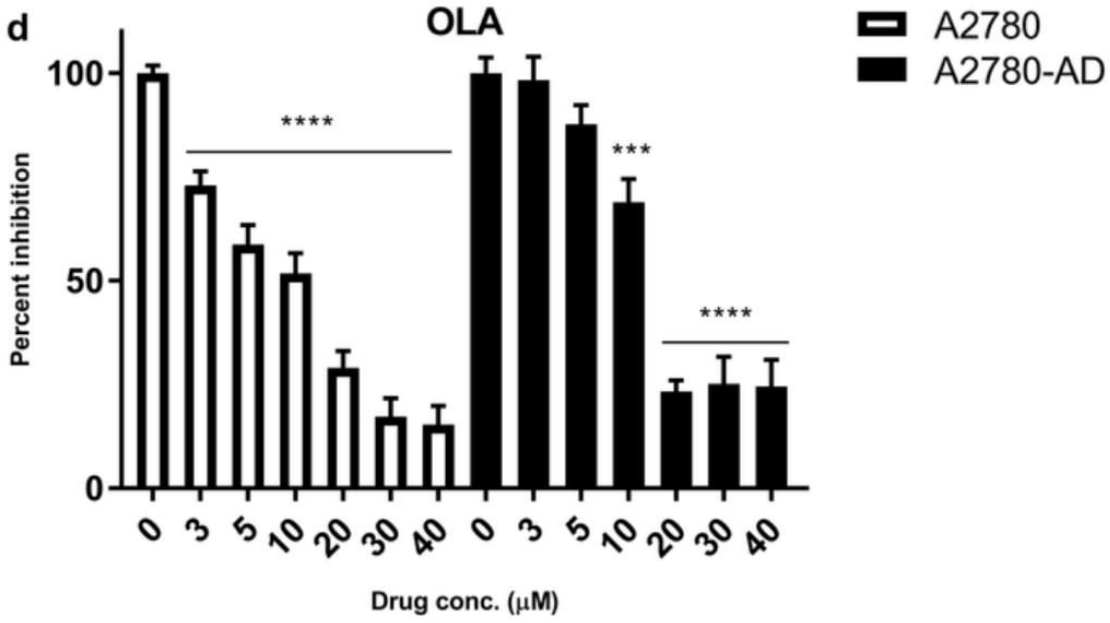


Figure 3.2. Response to bortezomib or olaparib in chemosensitive and chemoresistant ovarian cancer cell lines. OV2008 – C13 (a) and A2780 – A2780-AD (b) cell line pairs were treated with different concentrations of bortezomib (nM) for 48 h, and percent inhibition of cell viability was measured by SRB Assay. OV2008 – C13 (c) and A2780 – A2780-AD (d) cell line pairs were treated with different concentrations of olaparib (μM) for 72 h, and percent inhibition of cell viability were measured by SRB Assay. Non-significant (ns), $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ versus the control group (no drug treatment). Drug conc.: Drug concentration. BOR: bortezomib. OLA: olaparib.

3.3. Olaparib Decreases Tumor Viability in Ovarian Cancer Cell Lines in a Dose-dependent Manner

Same cytotoxicity screens were performed in ovarian cancer cell lines for the increasing concentrations of olaparib (OLA), the PARP inhibitor, as implemented with bortezomib to determine the effect of PARP inhibition on the cellular viability in these ovarian cancer cell lines. Indicated concentrations of olaparib in the micromolar range were selected to be used as shown in Figure 3.2c, 3.2d. When the two cell lines pairs were compared in terms of their sensitivity to PARP inhibition, OV2008 - C13 cell line pair were significantly more refractory to olaparib treatment than A2780 – A2780-AD pair. Among four cell lines, A2780 were shown to be the most sensitive to olaparib treatment

and even concentrations as low as 3 μM decreased cell viability around 30% in this chemosensitive cell line ($p < 0.0001$); however, the minimum effective concentration of olaparib for the other three cell lines was 10 μM (Figure 3.2c and 3.2d). This data indicates that olaparib treatment increases cytotoxicity in a dose-dependent manner in ovarian cancer cell lines used in this study.

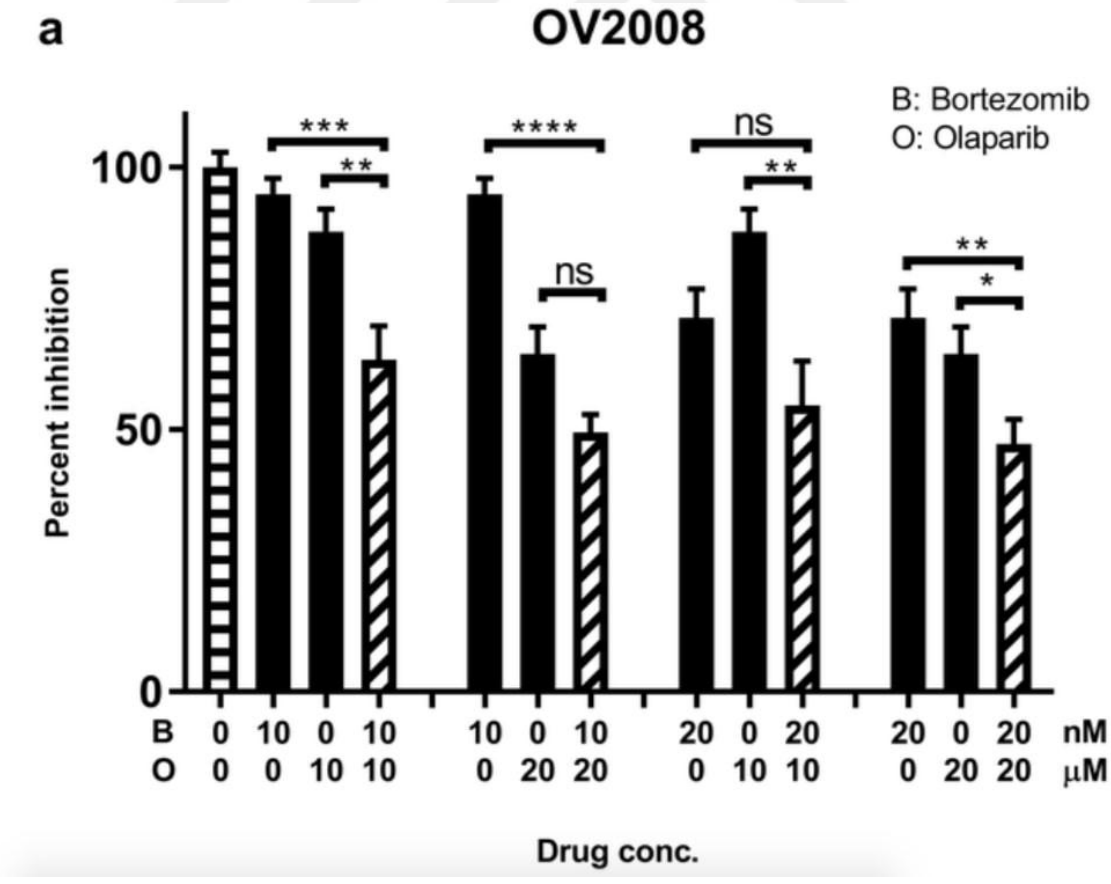
Based on the data above, two different concentrations of olaparib (10 μM and 20 μM) were chosen to be used in combination drug treatments since a dramatic change in cytotoxicity has started at these two consecutive concentrations in all four cell lines tested in this study.

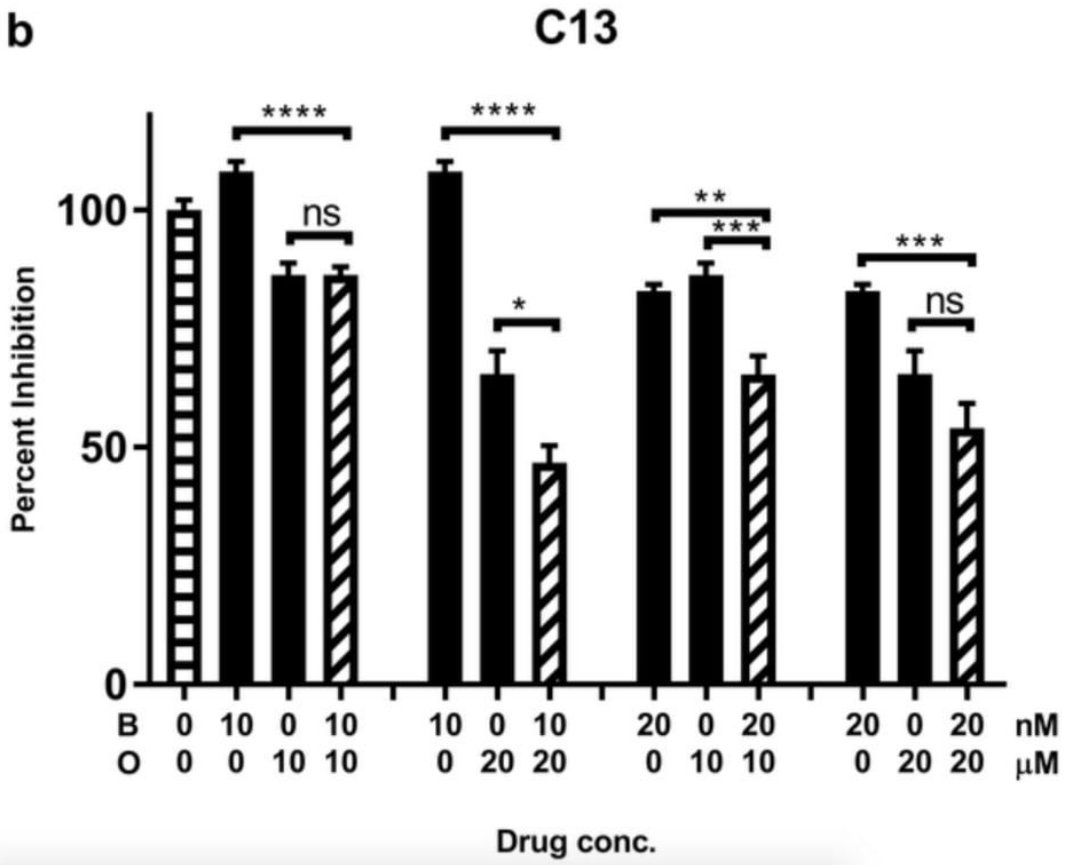
3.4. Combination Treatment Enhances Cytotoxicity as Compared to Single Drug Treatments in Ovarian Cancer Cell Lines

Upon completion of cell viability screens with either drug alone, we next examined the cytotoxic effect of the combined treatment of bortezomib (BOR) with olaparib (OLA) at previously selected concentrations against chemosensitive and chemoresistant ovarian cancer cell lines. As indicated in Figure 3.3, four different drug combinations were applied (BOR10 (nM) + OLA10 (μM); BOR 10 + OLA20; BOR20 + OLA10; BOR20 + OLA20) to all ovarian cancer cell lines and 48 hours after bortezomib plus olaparib treatment, cell viability screens were performed as previously implemented. For all the cell lines (except A2780), the cytotoxic effect on cell viability was significantly increased when compared to either drug alone, at two different drug combinations out of four tested (Figure 3.3).

Surprisingly, two different combination treatments of BOR + OLA enhanced cytotoxicity relative to either drug alone in chemoresistant cell line A2780-AD (cases with BOR10 + OLA10 and BOR20 + OLA10, Figure 3.3d), whereas chemosensitive cell line A2780, the parent line to A2780-AD, were seen to be refractory to this combination treatment when compared to the effects of either drug alone (Figure 3.3c). Combination treatment resulted in approximately 50% decrease in cellular viability for

the chemoresistant cell line A2780-AD at these two concentrations (Figure 3.3d). For the other cell line pair (OV2008 – C13), there are two co-treatment cases where the combined effect of bortezomib plus olaparib is significantly higher than that of either drug alone for both chemosensitive and chemoresistant line; although effective combination treatments are not the same in this cell line pair (BOR 10 + OLA10 and BOR20 + OLA20 for OV2008; BOR10 + OLA20 and BOR20 + OLA10 for C13) (Figure 3.3a and 3.3b). Therefore, it can be concluded that combination treatment is effective at certain concentrations for particular cell lines except for A2780. The effect of bortezomib plus olaparib cotreatment is independent of chemosensitivity profiles of cell lines for OV2008 – C13 cell line pair; however, the combination drug treatment is effective only in chemoresistant cell line A2780-AD, but no enhanced cancer cell death was observed in bortezomib plus olaparib case for its chemosensitive counterpart, A2780. Unexpectedly, for the latter cell line pair, chemoresistant line may benefit more from this combined treatment relative to its chemosensitive parent line.




b
C13
Percent Inhibition
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ns
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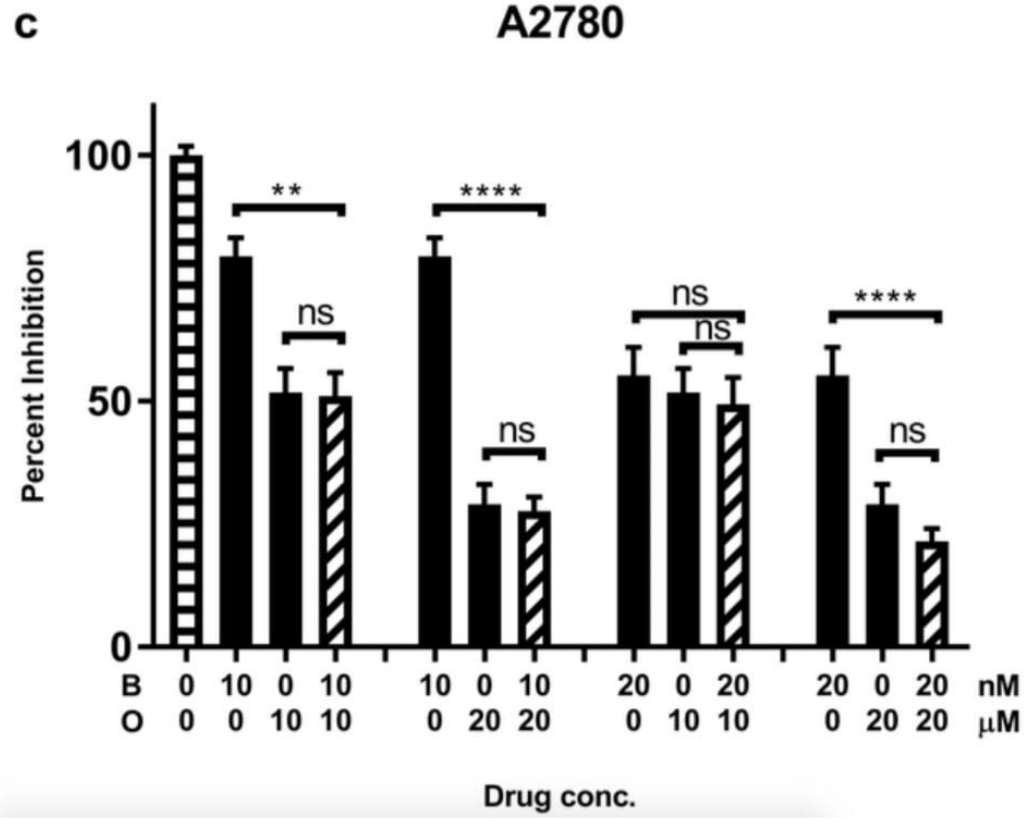
B 0 10 0 10 10 0 10 20 0 20 20 0 20 nM
O 0 0 10 10 0 20 20 0 10 10 0 20 20 μM
Drug conc.


c
A2780
Percent Inhibition
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B 0 10 0 10 10 0 10 20 0 20 20 0 20 nM
O 0 0 10 10 0 20 20 0 10 10 0 20 20 μM
Drug conc.

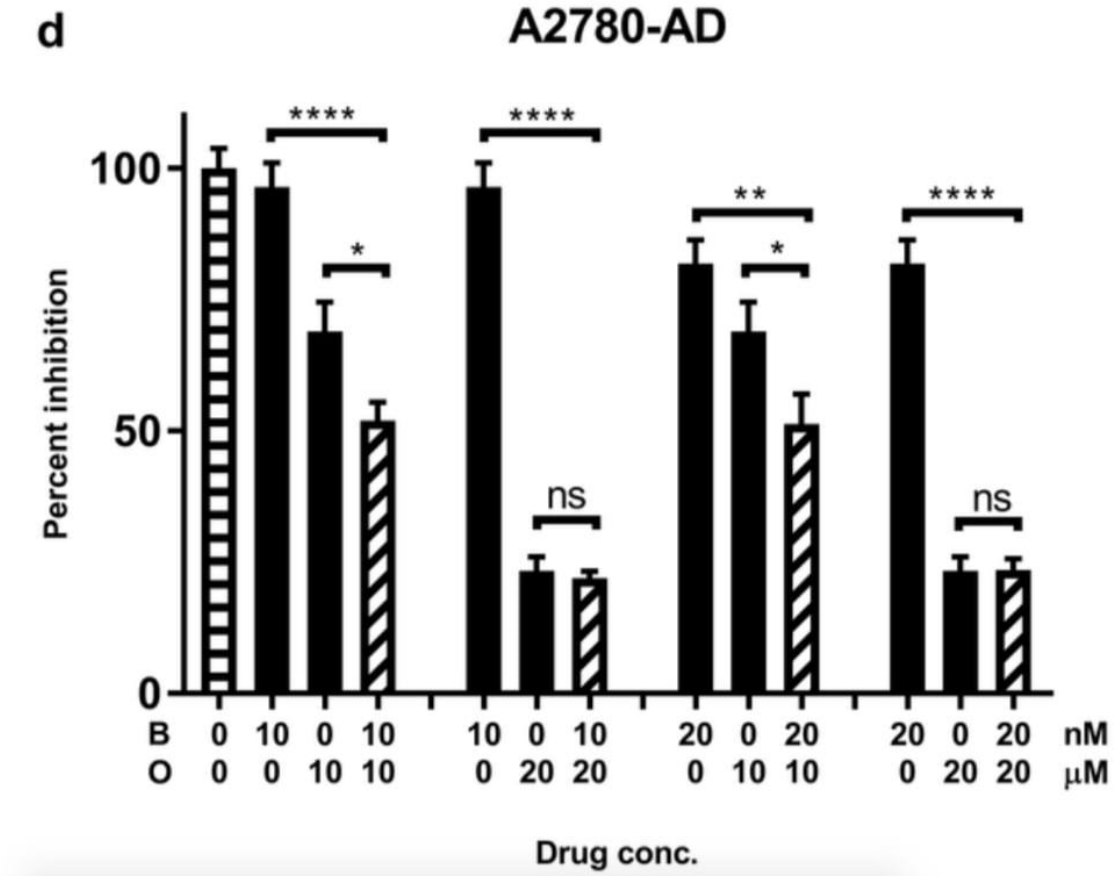


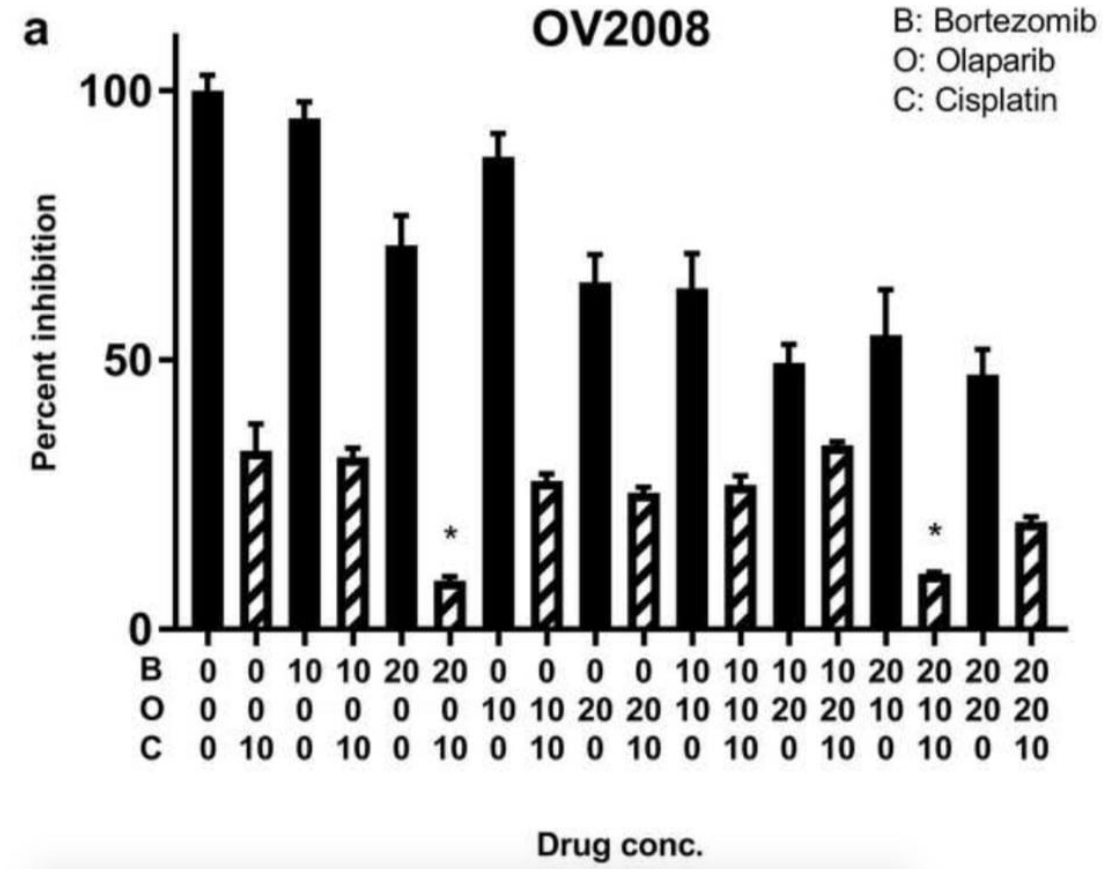
Figure 3.3. Cytotoxicity of bortezomib (B) plus olaparib (O) combination treatment on ovarian cancer cell lines. OV2008 (a), C13 (b), A2780 (c) and A2780-AD (d) cell lines were treated with indicated concentrations of bortezomib (nM) and olaparib (μM) for 72h, and percent inhibition of cell viability were measured. Non-significant (ns) $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ versus the control group (either drug alone). B: Bortezomib, O: Olaparib.

3.5. The Effect of Single and Combination Drug Treatments on Cisplatin Sensitization in Ovarian Cancer Cell Lines

Following the drug combination experiments, we examined whether drugs alone or in combination sensitizes ovarian cancer cell lines to cisplatin, a platinum-based drug which is used for the first line treatment of ovarian cancer. For this purpose, 24 hours

after bortezomib and/or olaparib administration, 10 μM cisplatin is added to the wells and cells were further incubated for an additional 48 hours, then cellular viability screen was conducted using SRB Assay. As indicated in Figure 3.4, at certain single drug concentrations, cisplatin treatment resulted in an additional decrease in cell viability when compared to bortezomib / olaparib alone and cisplatin alone (BOR20 + CIS10 for OV2008 and C13 (Figure 3.4a, 4b); BOR10 + CIS10, BOR20 + CIS10, OLA10 + CIS10 for A2780-AD (Figure 3.4d)). At BOR20 + OLA10 combination treatment in OV2008 cell line, additional cisplatin treatment (CIS10) further increased cytotoxicity in this cell line when compared to effect of BOR + OLA and of CIS alone (Figure 3.4a). Therefore, for this chemosensitive cell line, it can be argued that combination treatment (bortezomib plus olaparib) at these particular concentrations sensitized cells to cisplatin treatment. For the cell line A2780-AD, there are three different single drug concentrations (BOR10, BOR20, OLA10) where cisplatin addition further increased cytotoxicity (Figure 3.4d); and for cell lines OV2008 and C13, at one bortezomib concentration (20 nM), cisplatin further decreased cell viability relative to bortezomib and cisplatin alone (Figure 3.4a, 3.4b). There is no case for cell line A2780 where cisplatin addition further decreased cellular viability compared to drugs alone and cisplatin alone (Figure 3.4c).

As a result, A2780-AD is the cell line which can benefit most from BOR + CIS or OLA + CIS treatments within four cell lines tested; however, co-treatments with cisplatin seem to have no additional effect on cancer cell death in its chemosensitive counterpart, A2780. At one combination case in OV2008, bortezomib plus olaparib treatment sensitized cells to cisplatin; however, at none of the combination treatments in its chemoresistant daughter line C13, cisplatin treatment further resulted in enhanced cytotoxicity. Still, certain single drug treatments sensitized cells to cisplatin for OV2008-C13 cell line pair.


a
OV2008
B: Bortezomib
O: Olaparib
C: Cisplatin
Percent inhibition
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B 0 0 10 10 20 20 0 0 0 0 10 10 10 10 20 20 20 20
O 0 0 0 0 0 0 10 10 20 20 10 10 20 20 10 10 20 20
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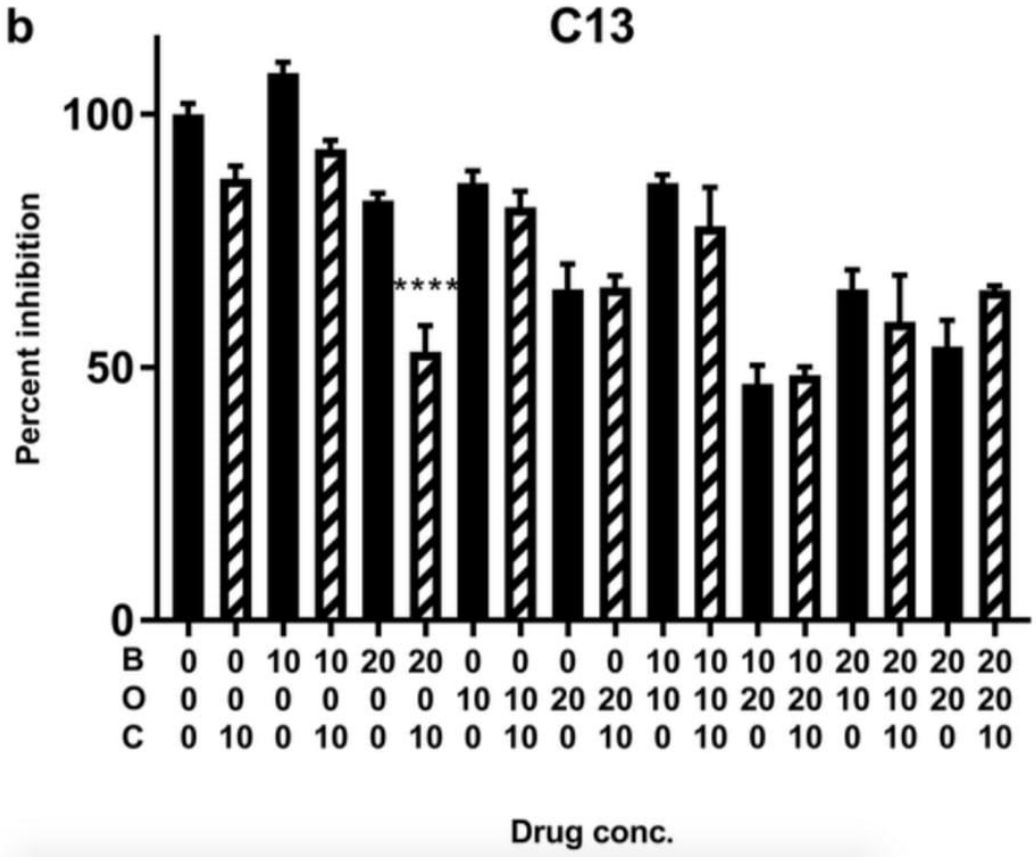
Drug conc.


b
C13
Percent inhibition
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O 0 0 0 0 0 0 10 10 20 20 10 10 20 20 10 10 20 20
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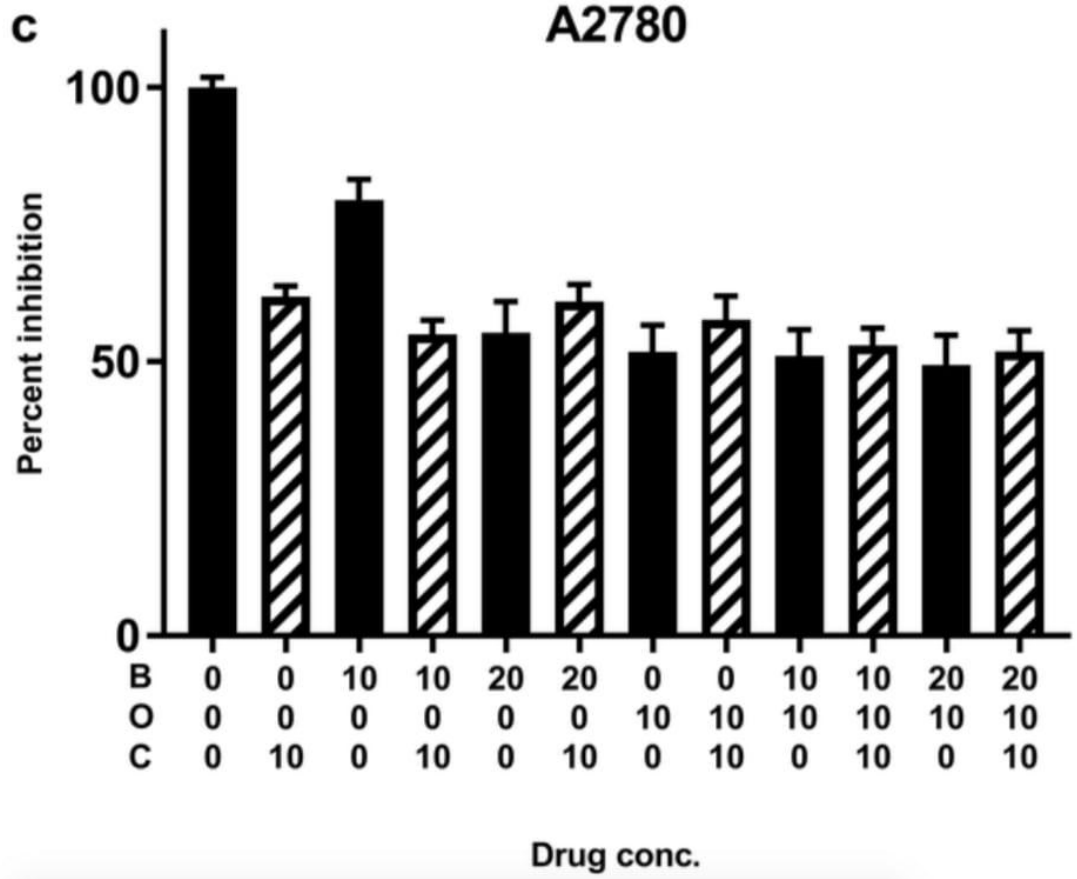
Drug conc.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A2780
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Percent inhibition
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O 0 0 0 0 0 0 10 10 10 10 10 10
C 0 10 0 10 0 10 0 10 0 10 0 10
Drug conc.

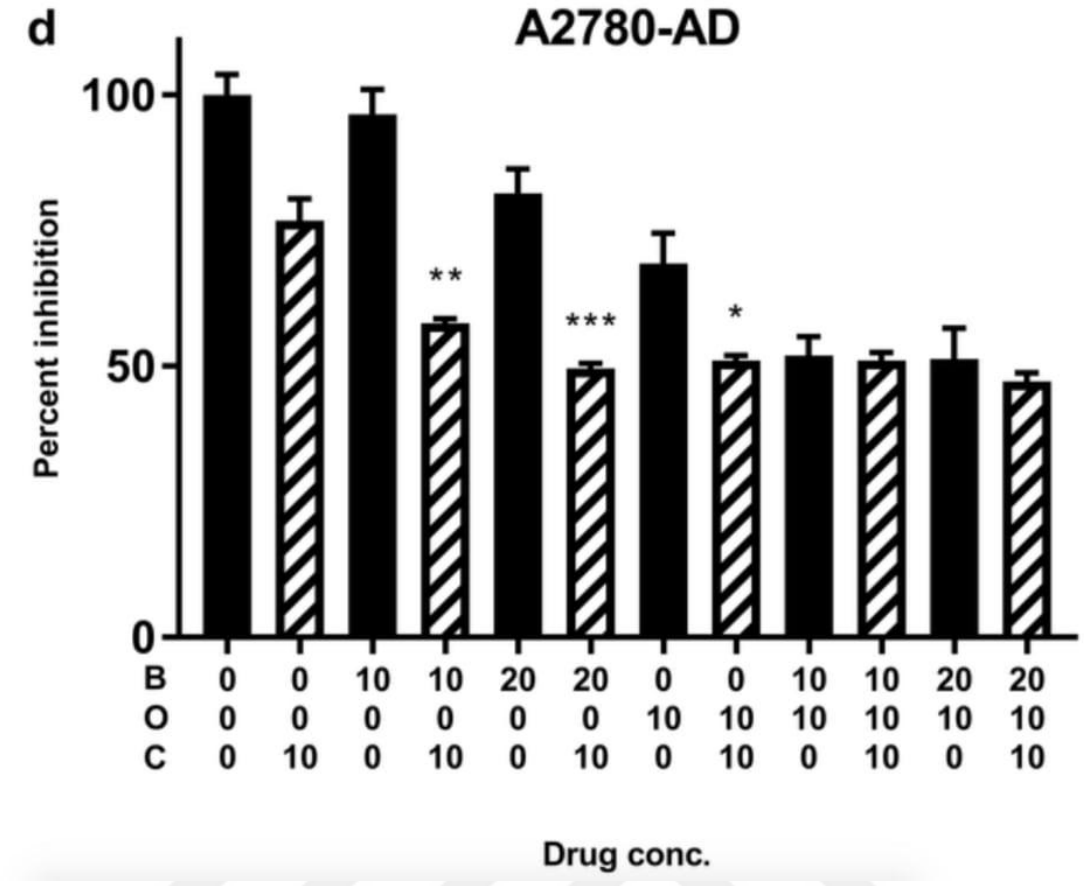


Figure 3.4. The effect of single and combination drug treatments on cisplatin sensitivity. OV2008 (a), C13 (b), A2780 (c) and A2780-AD (d) cell lines were treated with indicated concentrations of bortezomib (nM) and olaparib (μM) for 24h, and then cisplatin (10 μM) was added, cells were further incubated for an additional 24h, percent inhibition of cell viability were measured at the end of total 48h incubation period. For statistical analysis, following one-way ANOVA test, Sidak's multiple comparisons test were performed (cisplatin alone and bortezomib and/or olaparib versus cisplatin plus combination (triple drug) treatment; minimum significance value of these two comparisons were represented in the figure). Non-significant (ns) $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. B: Bortezomib, O: Olaparib, C: Cisplatin.

3.6. The Role of DYNLL1 Expression Level in The Chemoresistance of Ovarian Cancer Cells

Next, we examined how DYNLL1 expression levels correlate with chemosensitivity profiles of ovarian cancer cell lines. We observed that when cells were not treated with any of the drugs (only DMSO), expression of DYNLL1 is around 75% decreased in chemosensitive cell line OV2008 when compared with its chemoresistant subline C13

(Figure 3.5). These results are not consistent with the previous observation by He et al. (2018) that the loss of DYNLL1 expression associates with the increased chemoresistance. However, in this study, we observed that chemoresistant cell line C13 has higher DYNLL1 expression relative to its chemosensitive counterpart, OV2008 cell line (Figure 3.5).

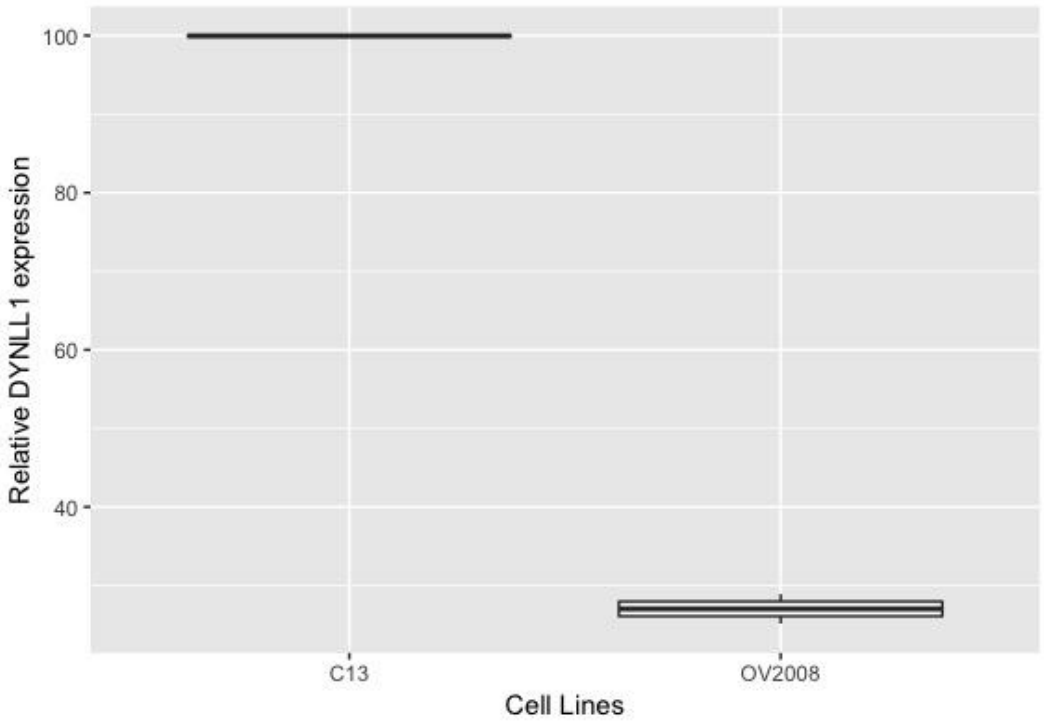
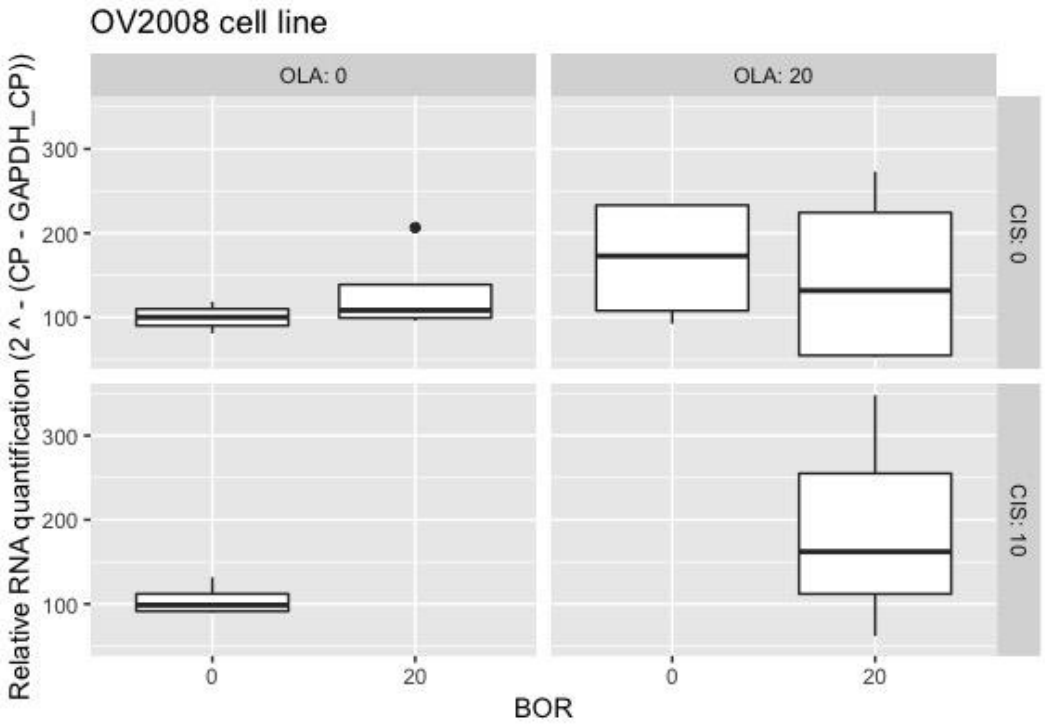


Figure 3.5. Relative expression of DYNLL1 in chemosensitive and chemoresistant ovarian cancer cell lines. Comparison of DYNLL1 expression levels in chemosensitive (OV2008) and chemoresistant (C13) ovarian cancer cell lines.

Then, we compared DYNLL1 expression levels between different drug combination conditions in each ovarian cancer cell line to see if these drug treatments have any effect on DYNLL1 expression levels. In chemosensitive OV2008 cell line, olaparib treatment seems to have an increasing effect on DYNLL1 expression levels; however, we observed that bortezomib treatment does not result in any significant change in DYNLL1 expression in this cell line (Figure 3.6a). Similarly, cisplatin treatment does not have a significant effect on DYNLL1 expression levels either when used in combination with bortezomib + olaparib or alone.

In chemoresistant C13 cell line, bortezomib treatment alone results in an increased expression of DYNLL1. Olaparib treatment alone in this cell line shows no significant change in DYNLL1 expression (Figure 3.6b). However, when olaparib is combined with bortezomib (with or without cisplatin), DYNLL1 expression decreases around 50%. In addition, cisplatin treatment alone decreases DYNLL1 expression by around 20% in this cell line (Figure 3.6b).



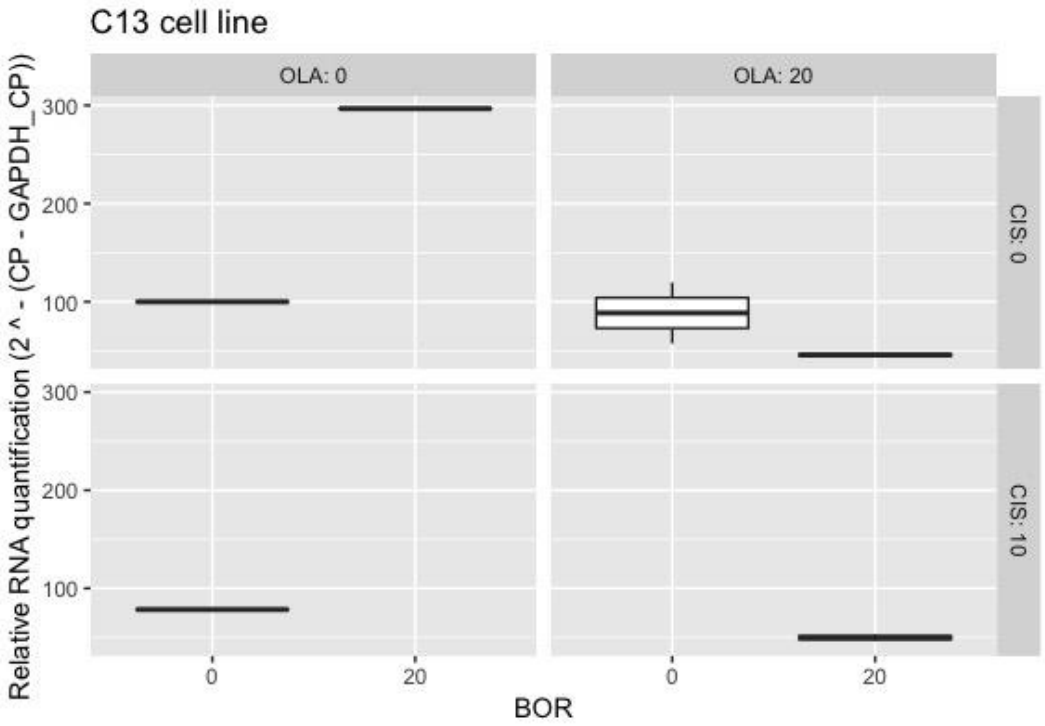


Figure 3.6. Relative expression of DYNLL1 in chemosensitive and chemoresistant ovarian cancer cell lines treated with indicated concentrations of bortezomib, olaparib and cisplatin. Relative mRNA expression of DYNLL1 gene in OV2008 (chemosensitive) (a) and C13 (chemoresistant) (b) cell lines treated with indicated concentrations of chemotherapeutic drugs. BOR: bortezomib (in nM), OLA: olaparib (in μM), CIS: cisplatin (in μM).

4. DISCUSSION

Chemoresistance is one of the major clinical problems compromising the treatment of ovarian cancer, leading to poor prognosis of patients (Jayson *et al*., 2014). The development of novel drug combination treatments with an increased combinatorial effect to overcome the drug resistance is therefore highly important for the management of ovarian cancer progression. The present study demonstrates that bortezomib and olaparib combination treatment exhibits enhanced cytotoxic effects at certain concentrations on chemoresistant ovarian cancer cell lines C13 and A2780-AD, and on chemosensitive ovarian cancer cell line OV2008, but not in chemosensitive cell line A2780. Similarly, bortezomib or olaparib co-treatment with cisplatin results in decreased cancer cell viability at particular concentrations for all four cancer cell lines tested, except A2780. Combination of bortezomib plus olaparib cotreatment with cisplatin further decreases cellular viability at a certain concentration in OV2008, suggesting that this combination case sensitizes these cells to cisplatin; however, no similar effect was observed in other three cell lines used, suggesting that the effect of bortezomib and olaparib combination treatment is independent of cisplatin sensitization.

Generally, consistent results were obtained in our study for four ovarian cancer cell lines tested except A2780, for which some cell line-dependent effects were observed. Combination treatment resulted in greater cytotoxicity in all cell lines tested, but not in A2780. This can be explained partly by the fact that olaparib alone is highly effective in this cell line and no further decrease in cellular viability is observed when combined with bortezomib. For A2780 – A2780-AD cell line pair, increased susceptibility to bortezomib or olaparib when compared to OV2008 – C13 cell line pair may be attributed to the presence of a nonsynonymous mutation in the BRCA2 gene in A2780 – A2780-AD (Beaufort *et al*., 2014), therefore limiting the activity of other DNA damage repair proteins by drug treatment has an enhanced effect on cellular viability (increased cytotoxicity) in this pair. This is in line with multiple studies which show that BRCA-deficient cells are more sensitive to inhibition of particular DNA damage repair proteins than BRCA-competent cells (O'Connor, 2015; Farmer *et al*., 2005, Bryant *et al*., 2005).

Proteasome inhibition by bortezomib has been shown to result in a decrease in BRCA1 transcription by blocking the binding of NF-KB to its promoter, thereby limiting the recruitment of this DNA repair protein to DNA damage sites (Cron *et al*., 2013). Combining this state which partially mimics BRCA mutations with the blockade of another DNA repair protein, PARP, in theory, might result in increased cytotoxicity. Indeed, we observed enhanced cell death when these two drugs are used in combination (Figure 3.3). The resultant cytotoxicity was particularly more pronounced for OV2008-C13 cell line pair which has no mutation in BRCA1/2 unlike A2780 – A2780-AD. This might be due to already high susceptibility of cell lines carrying a BRCA mutation to either drug alone (bortezomib or olaparib); however, compensation of the effect caused by bortezomib or olaparib alone in cells which do not have a BRCA mutation, therefore having a complete DNA damage response (Ledermann *et al*., 2014). This might explain why combination treatment has no enhanced effect in A2780 cell line carrying a BRCA2 mutation when compared to the effect of either drug alone. Nevertheless, its chemoresistant subline A2780-AD which also has a mutation in BRCA2 like its parental line A2780, benefits from the combination treatment relatively to treatment with drugs alone, possibly due to the chemosensitization effect of the combination treatment. Since this potential chemosensitization is not the case for intrinsically chemosensitive A2780, this additional effect might be absent in this cell line when drugs are used in combination.

In this study, we observed that chemoresistant cell line C13 has higher DYNLL1 expression compared to its chemosensitive counterpart OV2008 in contrary to the study by He *et al*., (2018). In this study, they reported that the loss of DYNLL1 expression is associated with increased chemoresistance, since the loss of DYNLL1 removes the block on HR DNA repair, therefore it results in increased DNA repair and ultimately higher chemoresistance to DNA-damaging drugs such as cisplatin. These conflicting results might be attributed to the fact that they acquired these data on BRCA-deficient cells; however, OV2008 – C13 cell line pair used in this study has no BRCA mutation. In their study, they also did not observe the same results for BRCA-wild type cells, therefore it can be concluded that the role of DYNLL1 expression on chemoresistance is dependent on BRCA status of ovarian cancer cells.

Drug treatments have cell-line specific effects on DYNLL1 expression. In OV2008 cell line, olaparib treatment seems to result in increased DYNLL1 expression; however, we did not observe the same effect for C13 cell line. In C13 cell line, bortezomib treatment alone increases DYNLL1 expression; however, when combined with olaparib, it has a decreasing effect on DYNLL1 expression independently of cisplatin treatment. Based on these data, it can be argued that the effects of drugs used in this study on DYNLL1 expression levels are highly dependent on cell lines and cell line-specific mechanisms might play roles in associations between drug treatments and DYNLL1 expression levels.

5. CONCLUSION

In conclusion, this study shows that combination treatment of bortezomib with olaparib exhibits increased cytotoxicity in ovarian cancer cell lines in vitro, particularly in cell lines which do not have any BRCA mutation, independently of their chemosensitivity profiles. For cells which have a 'BRCAness' state such as A2780 – A2780-AD, the one with chemoresistant profile might benefit more from this combination treatment. Bortezomib might be combined with olaparib in the treatment of patients with no BRCA mutation after the confirmation of the results obtained in this study, in the clinic. The cytotoxic efficacy of bortezomib and olaparib combination treatment in other ovarian cancer cell lines which have different genetic backgrounds also remains to be tested in the future. Animal experiments will also be highly valuable to comprehensively understand the effectiveness of this combination treatment in the management of ovarian cancer. In the current study, we did not evaluate the toxicity associated with this treatment strategy and drug toxicity experiments should be performed in vivo.

In this study, we observed that the effect of DYNLL1 expression levels on chemoresistance might be dependent on BRCA status of cells. Also, post-transcriptional and –translational regulation mechanisms might play a role in the functional activity of DYNLL1 in cancer chemoresistance. The effects of drugs in DYNLL1 expression levels are highly cell line-specific, therefore any treatment strategy focusing on DYNLL1 activity in the future should take cancer cell heterogeneity into account for the optimized treatment of ovarian cancer.

Considering the presence of high cellular heterogeneity in a single tumor mass, it will also be interesting to identify cell subpopulations in a tumor microenvironment, which are more responsive to this combination treatment. In addition, the identification of ovarian cancer patient groups whose possibility of response to bortezomib and olaparib combination treatment will be highly valuable in terms of personalized medicine approaches.

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7. SUPPLEMENTARY DOCUMENTS

7.1. An Exemplary Rmarkdown Document Containing R Code Which Is Used to Analyze Some Data in This Study

RT-PCR graphs - C13, OV2008

```
library("readxl")
library("tidyverse")
```

```
## ── Attaching packages ──────────────────────────────────────────────
tidyverse 1.2.1 ──

## ✓ ggplot2 3.1.1     ✓ purrr   0.3.2
## ✓ tibble  2.1.2     ✓ dplyr   0.8.1
## ✓ tidyr   0.8.3     ✓ stringr 1.4.0
## ✓ readr   1.3.1     ✓ forcats 0.4.0

## ── Conflicts ──────────────────────────────────────────────────────
tidyverse_conflicts() ──
## ✖ dplyr::filter() masks stats::filter()
## ✖ dplyr::lag()    masks stats::lag()
```

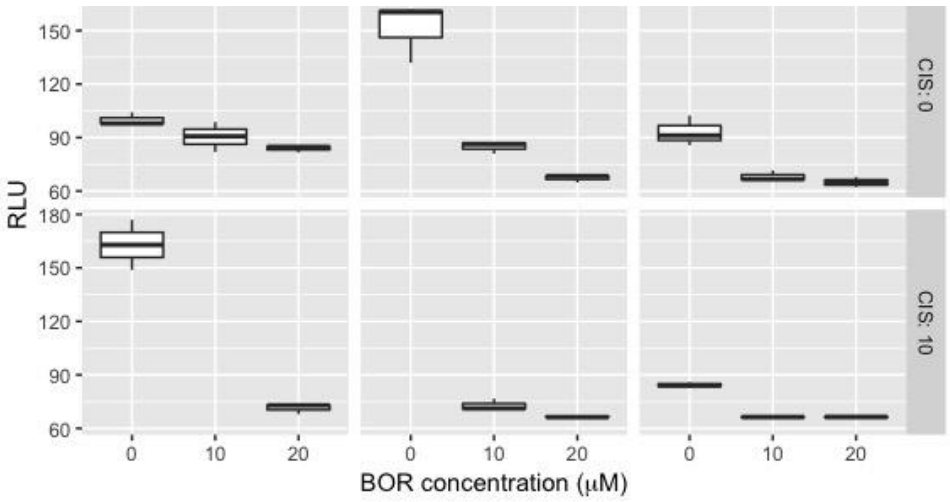
```
RTPCR_data <- read_excel(path = "/Users/caglarberkel/Desktop/LAB/Experiment data/RT-PCR data/RT-PCR_CP_values_C13_OV_2008_24.06.2019.xlsx")
names(RTPCR_data)[6] <- "CELLLINE"
RTPCR_data_C13 <- RTPCR_data %>% filter(CELLLINE == 'C13')
RTPCR_data_C13 <-  RTPCR_data_C13 %>% mutate(r_quan = 2 ^ -(CP - GAPDH_CP))
RTPCR_data_C13
```

```
## # A tibble: 12 x 7
##      BOR   OLA   CIS    CP GAPDH_CP CELLLINE  r_quan
##    <dbl> <dbl> <dbl> <dbl>    <dbl> <chr>      <dbl>
##  1     0     0     0  23.8     19.9 C13       0.0689
##  2     0     0     0  24.9     NA   C13      NA
##  3    20     0     0  18.8     19.1 C13       1.16
##  4    20     0     0  21.8     19.5 C13       0.204
##  5     0    20     0  23.5     18.8 C13       0.0396
##  6     0    20     0  22.7     19.1 C13       0.0825
##  7    20    20     0  25.9     21.0 C13       0.0328
##  8    20    20     0  26.2     21.2 C13       0.0302
##  9    20    20    10  22.8     18.0 C13       0.0367
## 10    20    20    10  22.9     18.0 C13       0.0319
## 11     0     0    10  24.5     20.2 C13       0.0540
## 12     0     0    10  15.9     20.1 C13      19.3
```

```
ggplot(RTPCR_data_C13[1:11,], aes(x = as_factor(BOR), y = r_quan)) + geom_boxplot() +
     facet_grid(CIS ~ OLA, labeller = label_both) +
     labs(x = "BOR", title = "C13 cell line", y = "Relative RNA quantification (2 ^ - (CP - GAPDH_CP))")
```

```
## Warning: Removed 1 rows containing non-finite values (stat_boxplot).
```

```
RTPCR_data_OV2008 <- RTPCR_data %>% filter(CELLLINE == 'OV2008')
RTPCR_data_OV2008 <-  RTPCR_data_OV2008 %>% mutate(r_quan = 2 ^ -(CP -GAPDH_CP))
RTPCR_data_OV2008
```




```
ggplot(RTPCR_data_OV2008, aes(x = as_factor(BOR), y = r_quan)) + geom_boxplot() +
     facet_grid(CIS ~ OLA, labeller = label_both) +
     labs(x = "BOR", title = "OV2008 cell line",
          y = "Relative RNA quantification (2 ^ - (CP - GAPDH_CP))")
```

7.2. Exemplary Experiment Plans

MTT Assay – 96 well plate – 48 wells for each cell line – 10.000 cells/well (in 200 ul media) - 48 h incubation after treatment - nM for BOR, uM for OLA!!!

	1 / 7	2 / 8	3 / 9	4 / 10	5 / 11	6 / 12
A	5 ul dmso	5 ul dmso	5 ul dmso	5 ul dmso + cis (1.23 ul from 1660 uM stock)	5 ul dmso + cis	5 ul dmso + cis
B	5 ul bor (400 nM)	5 ul bor (400 nM)	5 ul bor (400 nM)	5 ul ola (400 uM)	5 ul ola (400 uM)	5 ul ola (400 uM)
C	5 ul bor (800)	5 ul bor (800)	5 ul bor (800)	5 ul ola (800)	5 ul ola (800)	5 ul ola (800)
D	2.5 ul bor (800) + 2.5 ul ola (800)	2.5 ul bor (800) + 2.5 ul ola (800)	2.5 ul bor (800) + 2.5 ul ola (800)	2.5 ul bor (800) + 2.5 ul ola (800) + cis	2.5 ul bor (800) + 2.5 ul ola (800) + cis	2.5 ul bor (800) + 2.5 ul ola (800) + cis
E	2.5 ul bor (800) + 2.5 ul ola	2.5 ul bor (800) + 2.5 ul ola	2.5 ul bor (800) + 2.5 ul ola	2.5 ul bor (800) + 2.5 ul ola	2.5 ul bor (800) + 2.5 ul ola	2.5 ul bor (800) + 2.5 ul ola

	(1600)	**(1600)**	**(1600)**	**(1600) + cis**	**(1600) + cis**	**(1600) + cis**
F	**2.5 ul bor (1600) + 2.5 ul ola (800)**	**2.5 ul bor (1600) + 2.5 ul ola (800)**	**2.5 ul bor (1600) + 2.5 ul ola (800)**	**2.5 ul bor (1600) + 2.5 ul ola (800) + cis**	**2.5 ul bor (1600) + 2.5 ul ola (800) + cis**	**2.5 ul bor (1600) + 2.5 ul ola (800) + cis**
G	**2.5 ul bor (1600) + 2.5 ul ola (1600)**	**2.5 ul bor (1600) + 2.5 ul ola (1600)**	**2.5 ul bor (1600) + 2.5 ul ola (1600)**	**2.5 ul bor (1600) + 2.5 ul ola (1600) + cis**	**2.5 ul bor (1600) + 2.5 ul ola (1600) + cis**	**2.5 ul bor (1600) + 2.5 ul ola (1600) + cis**
H	**5 ul bor (800) + cis**	**5 ul bor (800) + cis**	**5 ul bor (800) + cis**	**5 ul ola (800) + cis**	**5 ul ola (800) + cis**	**5 ul ola (800) + cis**

B10 (bortezomib 10 nM): 5 ul BOR (400 nM) . B20 (bortezomib 20 nM): 5 ul BOR (800 nM)

O10 (olaparib 10 uM): 5 ul OLA (400 uM) . O20 (olaparib 20 uM): 5 ul OLA (800 uM)

CIS: 1.23 ul from 1660 uM stock

RNA isolation (100.000 cells/well, in 1 ml media)

12-well plate 1

	1	2	3	4
A	25 ul dmso	25 ul bor (800 nM)	25 ul ola (800 uM)	12.5 ul bor (1600 nM) + 12.5 ul ola (1600 uM)
B	25 ul dmso	25 ul bor (800 nM)	25 ul ola (800 uM)	12.5 ul bor (1600) + 12.5 ul ola (1600)
C	25 ul dmso	25 ul bor (800 nM)	25 ul ola (800 uM)	12.5 ul bor (1600) + 12.5 ul ola (1600)

12-well plate 2

	1	2	3	4
A	12.5 ul bor (1600 nM) + 12.5 ul ola (1600 uM) + cis	cis (6.17 ul from 1660 uM stock) + 15 ul DMSO		
B	12.5 ul bor (1600) + 12.5 ul ola (1600) + cis	cis (6.17 ul from 1660 uM stock) + 15 ul DMSO		
C	12.5 ul bor (1600) + 12.5 ul ola (1600) + cis	cis (6.17 ul from 1660 uM stock) + 15 ul DMSO		

8. RESUME

Çağlar Berkel received his Bachelor's degree from Department of Molecular Biology and Genetics, Bilkent University, Ankara in June, 2015. After graduation, he worked in a graduate research project on circadian clock biology in Department of Molecular Biology and Genetics, Koc University, Istanbul for 1.5 years. In 2018, he started to work as Research Assistant in Department of Molecular Biology and Genetics, Tokat Gaziosmanpaşa University.