# FINAL REPORT

# CYCLIN DEPENDENT KINASE AND POLY (ADP-RIBOSE) POLYMERASE INHIBITION IN OVARIAN CANCER


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## Abstract

Cancer is one of the most common causes of mortality worldwide [1]. Ovarian cancer is the 17th most common cancer, and the 7th most common cancer in women. Although ovarian cancer constitutes only 2.3% of all cancer deaths, there is approximately a 49% 5-year relative survival rate [2]. More than one-fifth of ovarian cancers stem from mutations in tumor suppressor genes, and BRCA gene germline mutations constitute approximately 75% of inherited ovarian tumors [3]. Dysregulated cell division and genomic instability are two segments of the hallmarks of cancer. CDK and PARP inhibitors are two pathways of therapies that are undergoing research as treatments for ovarian cancer. CDKs play an important role in the cell cycle to maintain the progression of division. CDK inhibitors have become experimental drugs in treatment of ovarian cancer by targeting dysregulated cell division. Although in development, pan-CDK inhibitors and second generational CDK inhibitors show to be promising target therapies in for the hallmark of cancer regarding evading growth suppressors [4]. PARP is involved in maintaining genomic stability through DNA repair, programmed cell death, and other functions. PARP inhibitors have indicated efficacy against several ovarian cancer cell lines, usually containing BRCA1 and BRCA2 mutations [5]. The combination of CDK inhibition and PARP inhibition in one treatment could potentially exhibit “synergistic” influence on the treatment of ovarian cancer. The influence of CDK and PARP inhibition was observed through western blotting, cell cycle analysis, and apoptosis analysis. Upon analysis of the data, there was the indication that CDK inhibition decreased the protein expression of particularly selected cancer drivers and increased the percentage of cell cycle arrest and of cell death, apoptosis. The combination of PARP inhibition and CDK inhibition indicated a greater effect on the cell cycle arrest than from each of the treatments individually. With further improvements and alterations, this research could be further advanced into animal models and eventually, clinical studies.



Key words: ovarian cancer, PARP, CDK, inhibitors, combination therapy

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| | |
|---|---|
| ADP | Adenosine Di-Phosphate |
| APC | Anaphase Promoting Complex |
| ATCC | American Type Culture Collection |
| ATP | Adenosine Tri-Phosphate |
| BCA | BiCinchoninic acid Assay |
| BER | Base Excision Repair |
| BRCA | BReast CAncer gene |
| CC | Cell Culture |
| CDK | Cyclin Dependent Kinase |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | DiMethyl SulfOxide |
| DNA | DeoxyriboNucleic Acid |
| DPBS | Dulbecco's Phosphate Buffered Saline |
| DSB | Double Strand Break |
| ECL | Enhanced ChemiLuminiescence |
| FDA | Federal Drug Administration |
| GAPDH | GlycerAldehyde-3-Phosphate DeHydrogenase |
| HR | Homologous Recombination |
| HRP | HorseRadish Peroxidase |
| IC50 | Half Maximal Inhibitory Concentration |
| ITS | Insulin Transferrin Selenium |
| NAD | Adenine DiNucleotide |
| NHEJ | Non-Homologous End Joining |
| PARP | Poly Adenosine diphosphate-Ribose Polymerase |
| PBS | Phosphate Buffered Saline |
| PFS | Progress-Free Survival |
| PI | Propidium Iodide |
| PS | Phosphatidylserine |
| RIPA | RadioImmunoprecipitation Assay Buffer |
| RNA | RiboNucleid Acid |
| RPMI | Roswell Park Memorial Institute Medium |
| SDS | Sodium Dodecyl Sulfate |
| SEER | Surveillance, Epidemiology, and End Results |
| SEM | Standard Error of Mean |
| SSB | Single Strand Break |
| TBS | Tri-Buffered Saline |
| TME | Tumor MicroEnvironment |

# 1. Introduction

Almost 1.9 million cases of cancer have been estimated for 2021 in the United States, and 1/3 of these cases are projected to result in death (Siegel et al. 2021). However, this is an improvement from previous years due to further research gaining a better understanding of the mechanisms and early detection methods. Briefly, cancer can be defined as the uncontrolled division of abnormal cells in a part of the body. According to Hanahan and Weinberg's revolutionary article on "The Hallmarks of Cancer," the foundation of cancer is based on the discovery of mutations that produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function (Hanahan and Weinberg 2000). Cancer can seem very different with each case, but all the cases are based off of the same characteristics.

## 1.1 Hallmarks of Cancer

With improved research, unique characteristics have been observed, and thus the term "hallmarks" of cancer was coined. The hallmarks of cancer and enabling characteristics include the following: sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, resisting cell death, genome instability and mutation, and deregulating cellular energetics (Hanahan and Weinberg 2011). In Figure 1, the hallmarks of cancer and their therapeutic targets are labeled.

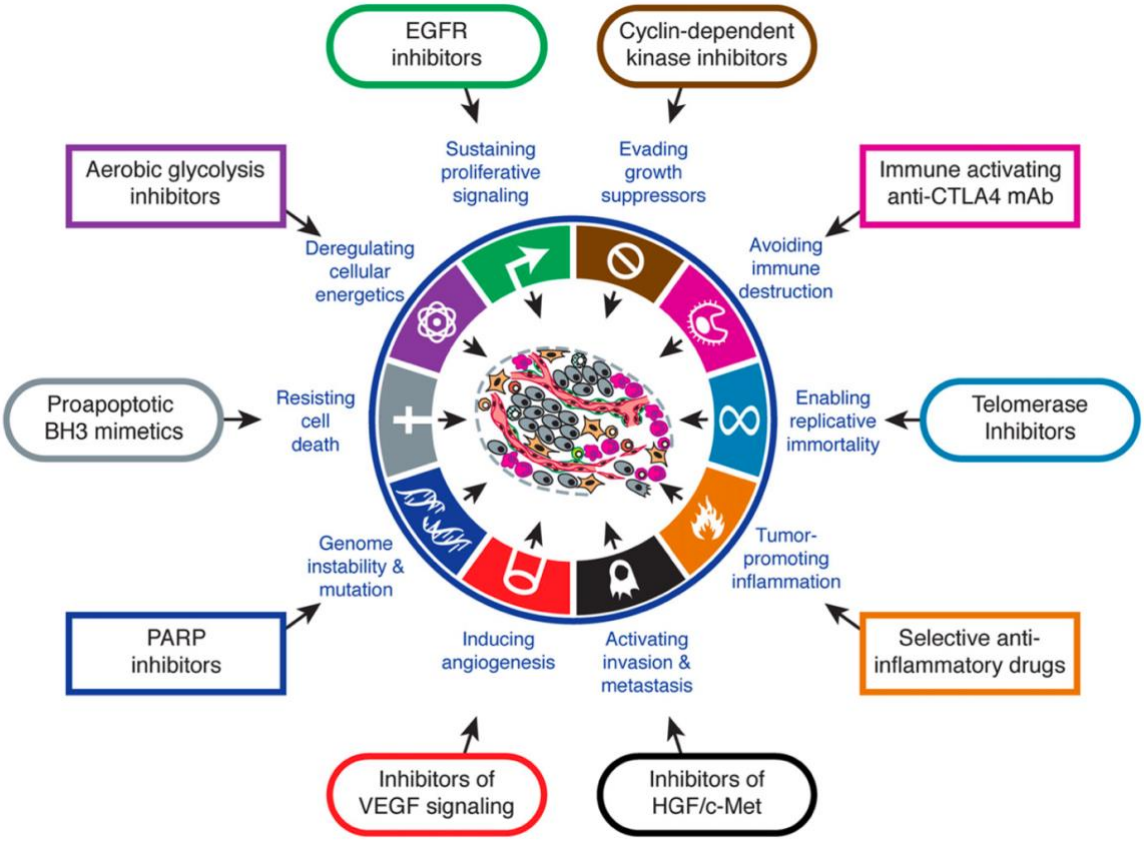


*Figure 1. Hallmarks of cancer, emerging hallmarks, enabling characteristics, and therapeutic targets [8]*

### 1.1.1 Evading growth suppressors

As seen in Figure 1, cancer cells have been able to manipulate control of several cellular processes, such as proliferation, replication, and apoptosis, to name a few. In normal cells, several antiproliferative signals control the preservation of cellular quiescence and homeostasis. The process of preventing cell growth and division is controlled by tumor suppressor genes. Tumor suppressor genes can slow down the cell cycle, repair DNA mistakes, and induce apoptosis. Apoptosis induces the removal of cells undergoing excessive proliferation, and autophagy promotes regeneration and removes abnormal proteins and cytoplasmic contents via a cellular recycling system. Antigrowth signals can inhibit proliferation by either forcing cells from the proliferative cycle to the quiescent (G0) state temporarily or inducing cells to enter postmitotic states to permanently cease their proliferative potential. In cancer cells, mutations can be found in the tumor suppressor genes, thus allowing them to overcome growth inhibition and encourage proliferation. Cancer cells resist apoptotic signaling to

prevent cell death of damaged cells and promote autophagy to increase growth and overcome nutrient-limiting condition (Hanahan and Weinberg 2011).

### 1.1.2 Genome stability & mutation

Genome instability and mutation is an enabling characteristic of cancer. Cancer cells are highly proliferative, increasing the tendency for genomic changes and mutations to occur in the cells that affect cell division and caretakers of the genome. Caretakers of the genome are components of the DNA-maintenance mechanism, which functions include detecting DNA damage and activating repair mechanisms, directly repairing DNA damage, and preventing mutations in the DNA from occurring (Negrini et al. 2010). Genetically, the caretakers of the genome behave similarly to tumor suppressor genes, in that inactivating mutations or epigenetic regression, caused by cancer, can inhibit their functions throughout tumor progression.

### 1.1.3 Overview of other hallmarks of cancer

To briefly overview the other hallmarks of cancer, there is avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, resisting cell death, deregulating cellular energetics, and sustaining proliferative signaling. First, the immune system is important to fight against pathogens, making it an immense predator against cancer cells. One way that cancer cells avoid immune destruction is by altering with immune checkpoint control mechanisms to not recognize the cancer cells and act on them as pathogens. In the tumor microenvironment (TME), immune cells secrete growth and metastasis signals, which promotes tumor survival rather than recognizing the cancerous cells and destroying them. One example of how the tumor corrupts inflammatory mechanisms is through the immune checkpoint signaling or the inflammasome signaling. Regular cells grow depend on the cell cycle to proliferate and maintain homeostasis, but cancer cells can release their own growth factors and therefore, stimulate growth. The three main signaling pathways in which the cancer cell can proliferate self-sufficiently are: Akt, MAPK/ERK, and mTOR pathways. Cancer cells are able to activate invasion and metastasis by a key process called epithelial-to-mesenchymal transition. Through this cancer cells undergo metabolic adaptation and uninhibited cell division, allowing them to survive in difficult and stressful

conditions with limited nutrients. Cancer cells are able to enable replicative immortality by several methods, such as synthesizing telomerase enzymes, which prevents telomere shortening, thus, preventing eventual cell death. They can avoid cell death by containing mutations that prevent the detection of cell damage or to prevent the signaling towards apoptosis within the cell itself. Cancer cells need a sufficient supply of oxygen and nutrients to metastasize, therefore, another characteristic of cancer is its ability to develop blood vessels, angiogenesis. VEGF is an example of a significant member in aberrant growth factor signaling to promote angiogenesis. Lastly, cancer cells require a high need for energy and nutrients to sustain their excessive growth. To satisfy this, the cellular metabolic pathways are different in cancer cells. To overcompensate for the low oxygen environment, cancer cells undergo glycolytic metabolism to gain energy, where pyruvate is changed from undergoing the Krebs cycle to the lactate production pathway.

## 1.2 Ovarian cancer and DNA repair

Ovarian cancer has a high rate of advanced disease at diagnosis, which can be explained by its early symptoms resembling common side effects, masking the characteristics of ovarian cancer and allowing it to develop increasingly. One of the main causes of ovarian cancer are mutations in the BRCA DNA repair pathway, attributing to 5-15% of all ovarian cancer cases. Mutations in the BRCA pathway result in an increase in the rate of mutations gained over time leading to a heterogenous patient tumor population that is difficult to treat due to the various driver mutations (Neff et al. 2017). DNA damage repair is essential to prevent cell death. Double strand breaks (DSBs) are a significant modification to DNA that can kill the cell if left untreated. DSBs are characterized by both reading frames of the DNA being damaged, and these breaks in the DNA make it more difficult to repair because a normal reading frame isn't available to repair nucleotides to. Mainly, there are two mechanisms that can repair a DSB: non-homologous end joining (NHEJ) and homologous recombination (HR), as seen in Figure 2.

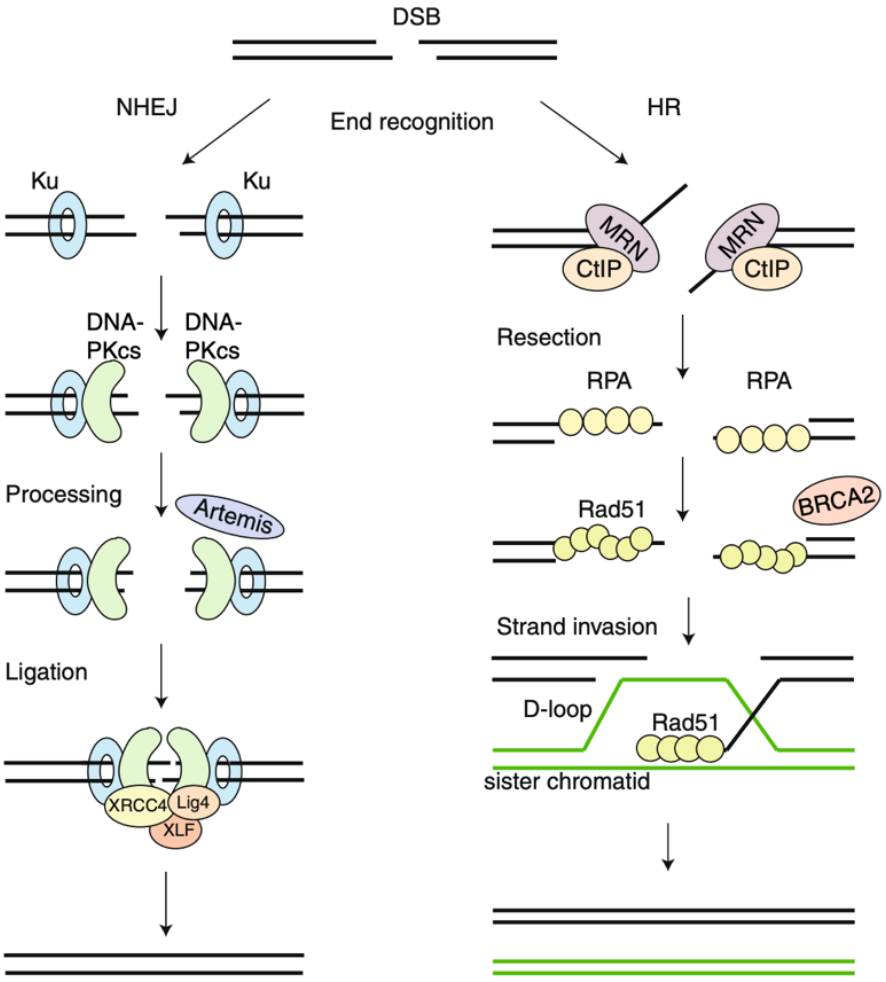


*Figure 2. NHEJ and HR DNA repair pathways [10]*

NHEJ makes the open ends of DNA attach to binding proteins for stabilization, essentially reconnecting the sides of the DNA without regard to the reading frame. In HR, a single 3' opening is created from the open ends of the DNA, allowing a series of proteins to populate and search for a compatible sequence with which to invade and create a D-loop. HR results in repairment of an unaltered reading frame (Neff 2017). BRCA1/2 have multiple roles in HR repair, where BRCA1 is believed to be part of a larger complex molecule that searches for DSB damage in DNA and BRCA2 is believed to have a direct role in repair.

### 1.2.1 PARP

Poly (ADP-ribose) polymerase (PARP) plays roles in DNA repair and genome integrity. There is a total of eighteen identified members in the PARP family, but the most important member is PARP-1 which has dominant roles in DNA repair pathways. PARP is critical in single strand break (SSB) repair and base excision repair (BER)

pathways. PARP is activated when its N-terminal zinc finger DNA binding domain recognizes and interacts with the SSB (Patel et al. 2021). PARP adds ADP-ribose to substrate protein by cleavage of adenine dinucleotide (NAD+) and release of nicotinamide (Zheng et al. 2020). This mechanism catalyzes polymers of paDPr and then transfers them to the C-terminal domain and other acceptor proteins, where the addition of pADPr adducts recruit downstream proteins that regulate DNA repair to repair the SSB (Patel et al. 2021). If these SSBs are not repaired, they lead to DSBs, which are highly cytotoxic to cells. PARP can also modulate DSB repair, in which it recruits DSB repair enzymes and regulates the expression of HR genes, such as BRCA1 and RAD51 at a transcriptional level (Patel et al. 2021).

### 1.2.2 Synthetic lethality

Synthetic lethality describes the concept of a mutation in one of two genes having no effect on the cell, but a mutation in both genes leads to cell death. This phenomenon was first observed between PARP inhibition and BRCA1/2 mutation. PARP inhibitors were observed to lead to an accumulation of SSBs and stall of replication forks, ultimately converting them into DSBs that can be lethal to the cell (Farmer et al. 2005). Therefore, tumors who lack DSB repair mechanisms, i.e. cells with BRCA1/2 mutations, would expectedly be sensitive to PARP inhibitors. Synthetic lethality has been observed in cancer cells with BRCA1/2 variants in breast and ovarian cancers, since they had shown to be prone to cell death in the presence of PARP inhibitors.

### 1.2.3 Olaparib

PARP inhibition on the enzymatic activity can be fit into two categories: PARPi can either bind to the active site of PARP and inhibit the enzymatic activity or it can bind to the PARP-chromatin complex, trapping the enzyme in a non-effective state at chromatin (Zheng et al. 2020). The PARP inhibitor, Olaparib is mostly an active site binder, as seen in Figure 3.

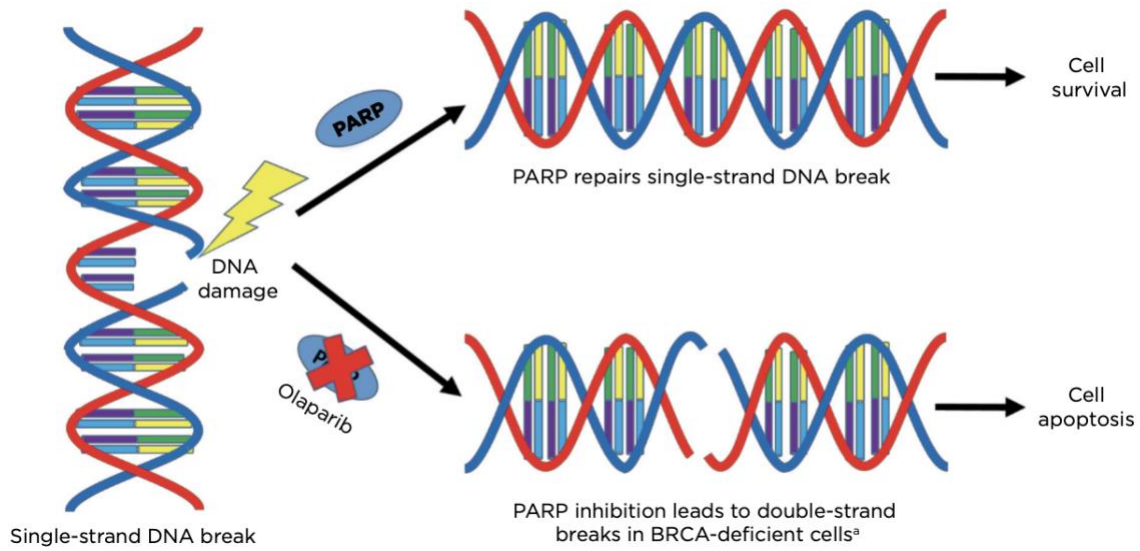


*Figure 3. Mechanism of Olaparib, specifically in BRCA-deficient cells in comparison to normal cells [13]*

Olaparib was the first PARP inhibitor to be approved, in 2014 by the FDA for treatment for germline mutated BRCA1/2 ovarian cancer patients who underwent three or more prior rounds of chemotherapy. Olaparib has shown statistical significance in progress-free survival (PFS) in several clinical trials. The results consisted of a median PFS of 19.1 months with Olaparib compared to 5.5 months with placebo (Pujade-Lauraine et al. 2017), 41% response rate for BRCA1/2 mutated patients (Gelmon et al. 2011), and a PFS of 8.4 months with Olaparib compared to 4.8 months with placebo in a study on patients with platinum-sensitive, relapsed, high-grade serous ovarian cancer, but not BRCA associated (Ledermann et al. 2012). These results influenced the FDA approval of Olaparib for maintenance therapy for ovarian cancer patients with prior platinum treatment, regardless of germline or BRCA mutations (Zheng et al. 2020).

## 1.3 Cell Cycle and CDKs

Cell division is one of the most important biological mechanisms that occurs in several processes, such as individual development, organ homeostasis, tissue regeneration, and tumorigenesis. Cell division is composed of a sequence of stages called the cell cycle, those being the synthesis phase, a mitotic segregation phase, and two intervenient phases G1 and G2. The cell cycle begins with the G1 phase where the cell enlarges itself to prepare for DNA synthesis, where there is a “restriction point.” The restriction point assesses the cell on its intrinsic and extrinsic factors, and if there is an absence of these essential factors, the cell enters the dormancy period, G0

phase. There are three checkpoints throughout the cell cycle: the G1/S, G2/M, and the mitotic spindle checkpoints.

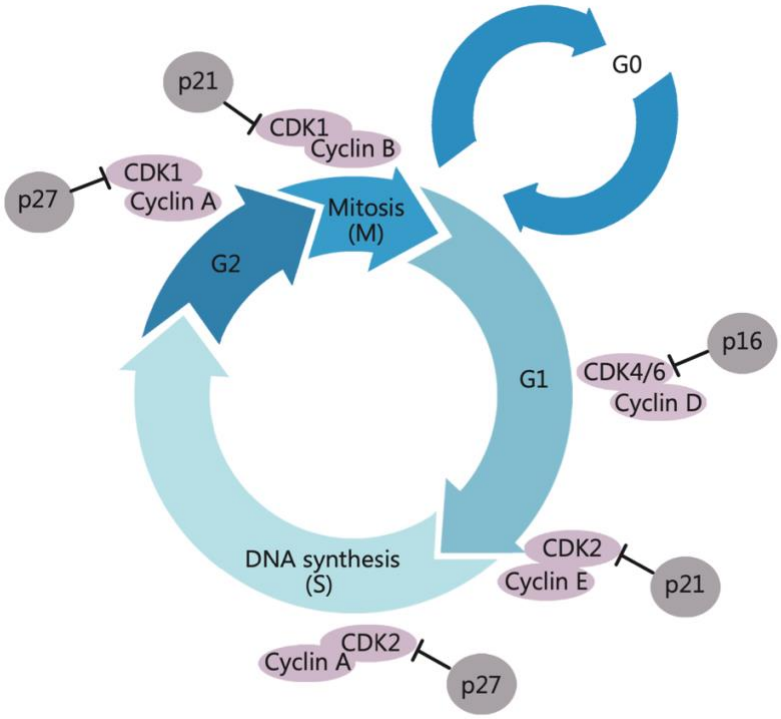


*Figure 4. Cell cycle with CDKs and their cyclins [17]*

The progression of the cell cycle is mainly controlled by two regulatory processes: phosphorylation of specific proteins by cyclin-dependent kinases (CDKs)/their dephosphorylation by phosphatases and specific proteolytic degradation by the ubiquitin-proteasome system (Bai et al. 2017). CDKs are part of the serine/threonine protein kinase family and have regulatory partners, the cyclins. CDK-cyclin complexes regulate cell cycle progression, ensuring the cell receives the appropriate growth factor signals and nutrients. Different cyclins are associated with different parts of the cell cycle. CDKs are activated when bound to their specific cyclin, but they can be negatively regulated by CDK inhibitors (Bai et al. 2017). Genes involved in the cell cycle are often mutated in tumors, which leads to unregulated cell proliferation and tumor growth. CDK and cyclin unregulated expression leads to cancer through the ability to elicit cell proliferation independent of normal extracellular stimuli or by promoting the bypass of checkpoints, initially designed to prevent the propagation of genomic change.

### 1.3.1 CDK inhibitors

CDK inhibitors can be sorted into two groups: ATP-competitive and non-ATP-competitive CDK inhibitors, based on their binding site. ATP-competitive CDK inhibitors mimic the ATP structure by binding to the ATP-binding pocket of CDK proteins. Many initial CDK compounds are pan-CDK inhibitors because of the high conservation of amino acid chains in the ATP-binding pocket. Non-ATP-binding inhibitors inhibit the cyclin binding groove/CDK-cyclin connection or stimulate the inhibitory CDK substrates. Since binding interactions and docking sites may differ among protein regulators, non-ATP competitive CDK inhibitors are more selective than ATP-competitive compounds (Bai et al. 2017).

### 1.3.2 Dinaciclib

Dinaciclib is a second-generation CDK inhibitor that mainly inhibits the activity of CDK9, in which it prevents the phosphorylation of the carboxyl terminus of RNA polymerase II, thus playing a transcriptional inhibitory role and inducing cell apoptosis.

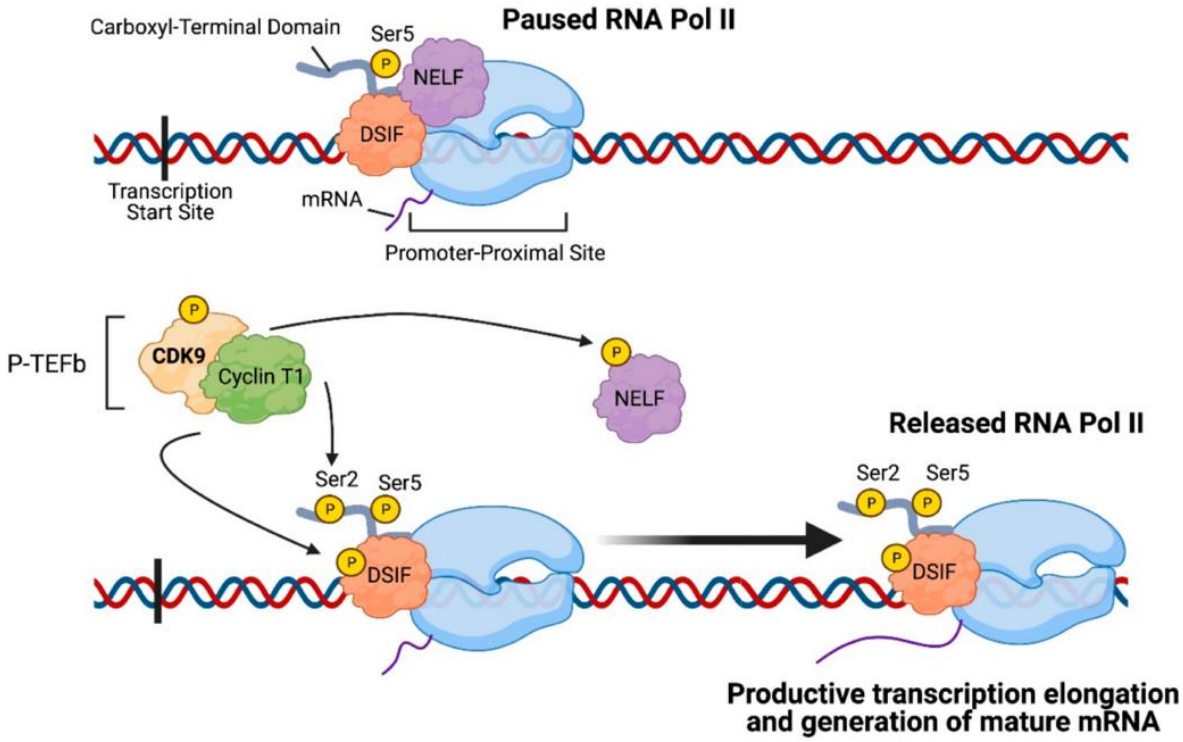


*Figure 5. Role of CDK9 [18]*

Dinaciclib also targets CDK1 and CDK2, where the cell cycle checkpoints are inhibited in the ovarian cancer cells, and thus, damaging the ability of the cells to evade growth suppressors, leading to their cell death. Dinaciclib has shown promising results in a

phase III clinical study, where a significant anti-tumor effect had been observed in treatment of melanoma, breast cancer, and leukemia. In another study, Dinaciclib showed effects of being more cytotoxic to ovarian cancer cells rather than normal cells, inducing cell cycle arrest and apoptosis (Chen et al. 2015). Further research has implemented the idea of combinational therapy as a drug delivery strategy to minimize the side effects of pan-CDK inhibitors (Zhang et al. 2021).

# 2. Aims

The objectives are to examine the protein expression of certain cancer driver's post-treatment, to measure how CDK and PARP inhibition influences the cell cycle progression, and to determine the effect of CDK inhibition on the apoptosis of ovarian cancer cells.

The overall research question is the following: Will PARP inhibitors and CDK inhibitors work better together in the treatment of ovarian cancer cells?

# 3. Materials and Methods

The research design consists of western blotting, cell cycle assay, apoptosis assay, and colony formation assay using the CAOV3 and OVCAR5 cell lines to study the influence of CDK and PARP inhibitors.

## 3.1 Cell lines and drugs

Two cell lines and two drugs are used in this study. The cell lines consist of ovarian cancer cells. The drugs are a PARP inhibitor and a CDK inhibitor.

CAOV3 cells are derived from a high grade ovarian serous adenocarcinoma in a 54-year-old white female patient. The morphology is epithelial, and the growth property is adherent.

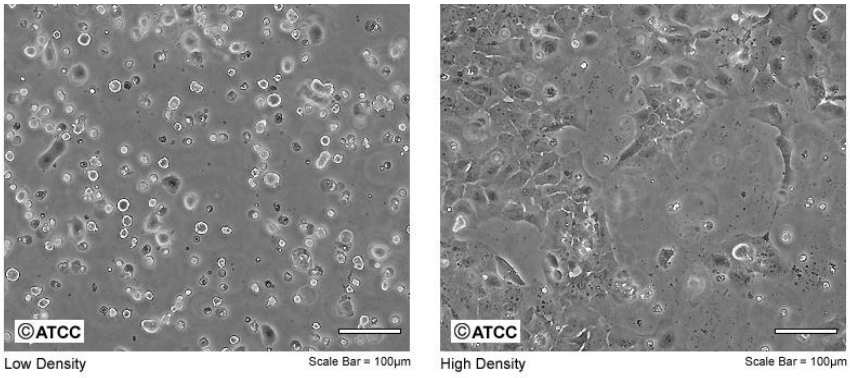


*Figure 6. Morphology of CAOV3 cell line in low density (left) and high density (right)*

OVCAR5 cells are derived from ascites fluid in a high grade ovarian serous adenocarcinoma in a 67-year-old white female patient. The morphology is epithelial, and the growth property is adherent.

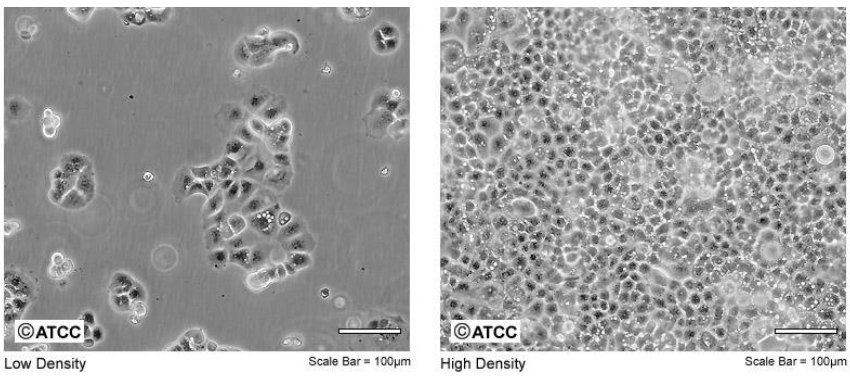


*Figure 7. Morphology of OVCAR5 cell line in low density (left) and high density (right)*

### 3.1.1 Cell culture and drugs methods

The OVCAR5 cell line (ATCC) is grown in RPMI-1640 media (Gibco) with 10% fetal bovine serum (Sigma), 1X glutamine (Gibco), 1X ITS (AR013, R&D), and 1X penicillin/streptomycin (Gibco) at 37°C in a 5% $CO_2$ incubator.

The CAOV3 (ATCC) cell line is grown in DMEM (Dulbecco's Modified Eagle Medium, Gibco) with 10% fetal bovine serum and 1X penicillin/streptomycin at 37°C in a 5% $CO_2$ incubator.

Dinaciclib (SCH727965) and Olaparib (AZD2281) are purchased from Selleck Chemicals. Dinaciclib is a novel and potent CDK inhibitor for CDK2, CDK5, CDK1 and CDK9 with IC50 of 1 nM, 1 nM, 3 nM and 4 nM in cell-free assays, respectively. Dinaciclib induces apoptosis through the activation of caspases 8 and 9. Olaparib is a selective inhibitor of PARP1/2 with IC50 of 5 nM/1 nM in cell-free assays. Olaparib induces significant autophagy that is associated with mitophagy in cells with BRCA mutations.

## 3.2 Cell culture techniques

All cell culture techniques are performed in a laminar hood environment under sterile conditions.

### 3.2.1 Thawing

Frozen cells are stored in liquid nitrogen. During cell collection, dry ice is used to transport the vials from liquid nitrogen to the cell culture laboratory. Cells are thawed in a 37C water bath. Upon completion of thawing, cells are immediately transferred to 15-milliliter confocal tubes. Then, 5 milliliters of their respective media are slowly added and mixed to counteract the toxicity from DMSO in the freezing media. The tubes are centrifuged at 300 rpm for 5 minutes to collect the cells in a pellet. The supernatant is removed, and the cell pellet is resuspended with media. A cell count can be performed at this time if needed. The necessary media is added to plate into a cell culture dish. The cells are placed in their preferred growing conditions, in this case, in the incubator at 37°C with 5% $CO_2$. The growth of cells is observed, and the media is changed as needed the following days.

### 3.2.2 Passaging

The media over the cells is aspirated, and DPBS (Gibco) is carefully added to the plate to wash off any remaining dead cells. Then, TrypLE (sensitive alternative to trypsin, Gibco) is added to cover the cells, and the cells are placed in the 37°C incubator for approximately 5-10 minutes until they have detached from the bottom of the plate. The cells are analyzed underneath a microscope to guarantee detachment of the majority of the cells. DPBS is used to wash the plate several times to detach and collect all the cells. The cells are added to a 15-milliliter confocal tube and centrifuged at 300 rpm for 5 minutes. The supernatant is collected, and the cell pellet is resuspended with the appropriate amount of media to plate as needed. A cell count can be performed at this time.

### 3.2.3 Freezing

The freezing media consists of the cell specific media and Cellbanker 1/2 (Nippon Zenkayu Kogyo Co., LTD.), which contains DMSO.

The methods in 4.2.2 are applied until the cell pellet is formed. The supernatant is aspirated, and the cell pellet is resuspended with media, where the amount depends on the cell count. Usually, $5.0x10^5$-$1.0x10^6$ cells are added to each vial. The amount of Cellbanker 1/2 is added to reach a total of 1mL of cell sample per vial. The vial is labeled accordingly with the cell line, passage number, date, and initials. The vial(s) is placed in the -80°C freezer for approximately a week before moving to the liquid nitrogen storage.

### 3.2.4 Fixing

The methods in 4.2.2 are applied until the cell pellet is formed. Then, the cell pellet is resuspended in 1 milliliter of DPBS and centrifuged at 300rpm for 5 minutes. All but 50µl of DPBS is removed, and the cell pellet is resuspended in the remaining DPBS. Cell suspension is added dropwise to its respective microcentrifuge tube containing 1 milliliter of fresh, ice-cold 70% ethanol, vortexing after every drop. The microcentrifuge tubes are stored in -20°C for a minimum of 3 hours prior to analysis. Fixed cells are stable for 2-3 months.

### 3.2.5 Lysis

Cells are resuspended in 250µl of RIPA buffer (Thermo Fisher Scientific) containing a protease inhibitor (Halt) to extract the protein and create lysates. For further lysis, cell samples are sonicated in the cold room, avoiding bubbles from forming.

## 3.3 Western blot analysis

Western blotting is a laboratory technique that detects specific protein molecules in a cell sample. They can be used to evaluate the size of a protein and to measure the protein expression in a sample. Western blotting consists of preparing the protein sample by mixing with sodium dodecyl sulfate (SDS), which makes the proteins unfold into linear chains and gain a negative charge, and then the protein molecules undergo gel electrophoresis to be separated by their size. The proteins are transferred from the gel onto a blotting membrane, which then undergoes a blocking step, preventing nonspecific bindings. Then, the membrane is incubated with a primary antibody, which binds to the protein of interest, and then incubated with a secondary antibody that is linked to a reporter enzyme that produces color or light (horseradish peroxidase, HRP), allowing it to be detected by chemiluminescent imaging.

### 3.3.1 Western blot analysis methods

CAOV3 and OVCAR5 cells are treated with Dinaciclib for 8 hours and 24 hours. After collection and lysis of the cells as described in 4.2.5, a BCA Assay (Thermo Fisher Scientific) is performed to determine the protein concentration of each lysate sample. With the obtained values, the lysates and ladder (Bio-Rad, Kaleidoscope) undergo SDS polyacrylamide gel electrophoresis (Bio-Rad) and then transfer onto a nitrocellulose membrane (Pierce). Membranes are blocked via 5% milk in Tris buffer saline (TBS, Gibco), and incubated with the following antibodies overnight at 4°C: APC2, Bad, Bcl2, Bcl-xL, Mcl1, PARP (Cell Signaling Technology), BRCA1 and GAPDH (Millipore Sigma) as a control. Cells are incubated with the appropriate secondary antibody in reference to the primary antibody for 1 hour. ECL (Pierce) or Femto (SuperSignal) is applied to each blot according to the sensitivity of the antibody, and the Bio-Rad Chemidoc MP Imaging System is used for imaging of the membranes.

## 3.4 Cell cycle assay

The Muse® Cell Cycle Assay uses a reagent that contains the nuclear DNA intercalating stain propidium iodide (PI) and RNAse A. PI discriminates cells at different stages of the cell cycle, based on differential DNA content in the presence of RNAse. In the cell cycle, resting cells (G0/G1) contain two copies of each chromosome. As the cycle continues, the chromosomal DNA synthesizes (S phase). The fluorescence intensity from PI increases until the chromosomal DNA has doubled (G2/M phase). By this stage, the fluorescence is double the intensity than from the G0/G1 population. The Guava® Muse® Cell Analyzer measures the intensity of the fluorescence to group the cells undergoing each phase of the cell cycle.

### 3.4.1 Cell cycle assay methods

The Muse® Cell Cycle Assay Kit (Luminex Corporation) is utilized as per manufacturer's instructions. Cells are plated and treated with Olaparib, Dinaciclib, and combination with their respected treatment times (8H, 24H, 72H). The cells are fixed as described in 4.2.4. Cells are centrifuged, washed with PBS, and resuspended in 200µl of cell cycle reagent provided by the manufacturer. Cells are incubated at room temperature, protected from light, for 30 minutes and analyzed via the Guava® Muse® Cell Analyzer according to the manufacturer's instructions.

## 3.5 Apoptosis assay

Apoptosis is a regulatory pathway of cell growth and proliferation. Cells respond to induction signals by inducing intracellular processes that cause physiological changes. Some of these changes are externalization of phosphatidylserine (PS) onto the cell surface, cleavage and degradation of cellular proteins, compaction and fragmentation of nuclear chromatin, and loss of membrane integrity in late stages. Annexin V has a high affinity for PS, which is a component of the membrane usually found in the inner membrane. In the beginning stages of the apoptotic pathway, molecules of PS translocate to the outer membrane, exposing them and allowing them to be bound by Annexin V. The Guava® Muse® Cell Analyzer detects the cells bound by Annexin V to differentiate between live and apoptotic cells in the samples.

### 3.5.1 Apoptosis assay methods

The Muse® Annexin V & Dead Cell Assay Kit (Luminex Corporation) is applied as per the manufacturer's instructions. Cells are plated and treated with Dinaciclib for 24 hours and 72 hours. The cells are collected as described in 4.2.2 until the cell pellet is formed. Then, the pellet is resuspended in 300µl of media. After which, 100µl of the room temperature apoptosis reagent, provided by the manufacturer, is added to 100uL of the cells resuspended in their respective media. Cells are incubated at room temperature, protected from light, for 20 minutes and analyzed via the Guava® Muse® Cell Analyzer.

## 3.7 Statistical calculations

Statistical testing consists of two-tailed t-tests in the apoptosis assay (live vs. apoptotic cells) and cell cycle assay (cycling vs. noncycling cells). P values $< 0.05$ are considered significant. The western blot images are analyzed with ImageJ through a densitogram and standard error of mean (SEM) calculations.

# 4. Results

The protein content of certain cancer protein influencers prior and post treatment with Dinaciclib are analyzed through western blotting. Then, the influence of the CDK and PARP inhibitors on the cell cycle were observed via the cell cycle assay. The apoptotic capability of CDK inhibition on ovarian cancer cells was visualized via the apoptosis assay. Finally, the ability of ovarian cancer cells treated with CDK and PARP inhibitors to form colonies was observed in the colony formation assay.

## 4.1 Western Blot Analysis

After performing the methods mentioned in 3.3, each blot was imaged, and the results are seen in Figure 8. The images on the left are of the CAOV3 cell line, and on the right, of the OVCAR5 cell line. Within each image, the left-hand side represents the cells treated as vehicle, and the right-hand side, represents the cells that underwent 24-hour drug treatment with Dinaciclib at concentration of 1μM.

GAPDH is a housekeeping gene used as a loading control which can be used to normalize the protein levels detected throughout the blot. GAPDH is one of the important enzymes involved in glycolysis, where it is continuously expressed in almost all cells in a high amount. APC2 (Apoptosis Promoting Complex 2) is involved in cell proliferation and part of the APC complex, whose main function is to trigger the transition from metaphase to anaphase in the cell cycle. The decrease in expression of the drug-treated cells would mean the drug halted the cell cycle of the ovarian cancer cells. Thus, preventing their mechanism of avoiding apoptosis and continuing proliferation. Mcl-1 is an antiapoptotic member of the Bcl-2 family, so with the drug-treatment, there should be a decrease in expression. As mentioned before, BRCA1 is frequently mutated in ovarian cancer, so the decrease of expression after drug-treatment would indicate that the drug has inhibited the mutated BRCA DNA repair system from allowing repair of double-strand breaks in mutated DNA. PARP is involved in DNA repair, and the cleavage of PARP is an indication of cells undergoing apoptosis. The absence and then presence of PARP describes the cleaving on PARP, meaning that the cells are undergoing apoptosis, which is what is expected with the drug-treatment.

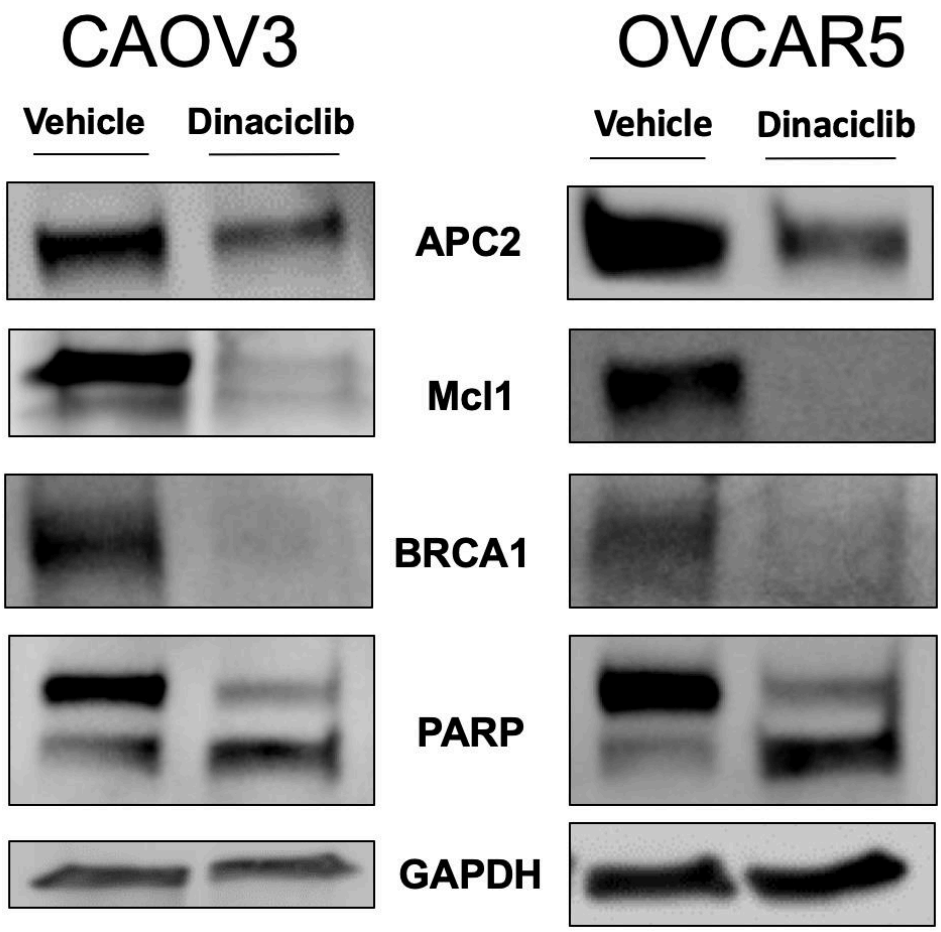


*Figure 8. Western blots*

From the images in Figure 8, a decrease in expression is seen for APC2, Mcl1, and BRCA1. The decrease in protein expression of APC2 illustrates that the drug inhibited the cell cycle from progressing in the ovarian cancer cell lines. The protein expression of Mcl1 signifies that this antiapoptotic member's signals have decreased. BRCA1 protein expression is also showing reduction, confirming that the introduction of the drug inhibited the mutated BRCA from performing the double strand break repair system. The blot for PARP is demonstrating the cleavage of PARP, as described before. GAPDH is showing equal expression, and from this it can be assumed that the protein levels are normalized.

## 4.2 Cell Cycle Analysis

The analysis of the cells, as explained in 3.4, provided information on the percentage of cells in each cell cycle phase. The results (n=3) of three trials were averaged and graphed, as seen in Figure 9. Paired two-tailed t-test statistical testing was run against the cycling vs non-cycling cells. The SEM was calculated and graphed as error bars, as seen on the graphs below. A and B display the results for the CAOV3 cell line, 8-hour and 24-hour treatment, respectively. C and D represent the results for the OVCAR5 cell line, 8-hour treatment and 24-hour treatment, respectively. In each graph, the order from left to right is the following: Olaparib, Vehicle, Dinaciclib, and Combo. Theoretically, there should be an increase in non-cycling cells (phase S, G2, and M), signifying the inhibition of ovarian cancer cells from continuing cell cycle progression.

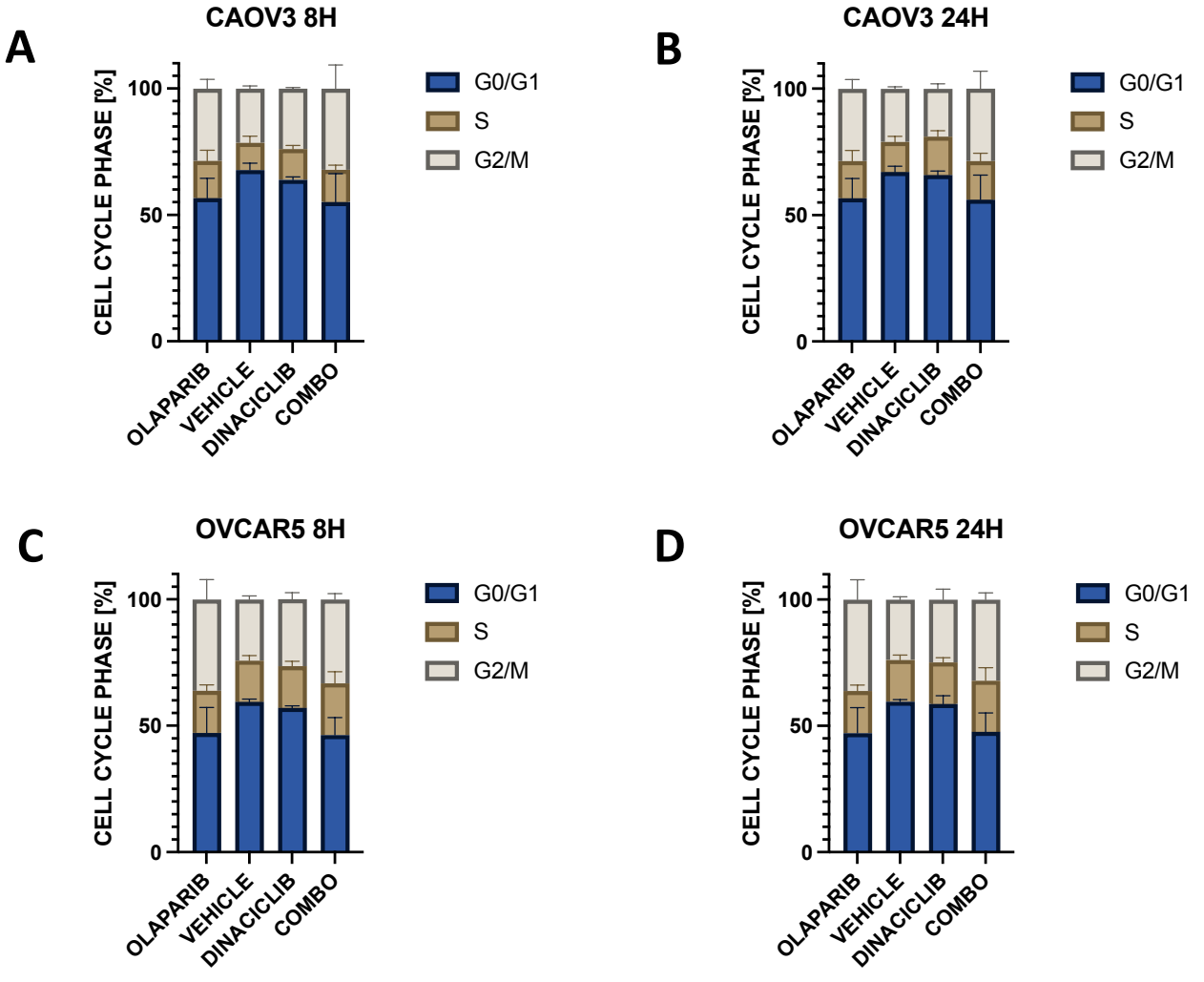


*Figure 9. Cell Cycle results*

In figure 9A, compared to vehicle, the non-cycling cell percentage increased by 11.1% with the Olaparib treatment and by 3.8% with the Dinaciclib treatment. With the Combination treatment, there was an increase of 12.6% non-cycling cells. In figure 9B, compared to vehicle, the non-cycling cell percentage increased by 10.4% with the Olaparib treatment and by 1.2% with the Dinaciclib treatment. With the Combination treatment, there was an increase of 11.0% non-cycling cells. In figure 9C, compared to vehicle, the non-cycling cell percentage increased by 2.2% with the Olaparib treatment and by 8.6% with the Dinaciclib treatment. With the Combination treatment, there was an increase of 9.5% non-cycling cells. In figure 9D, compared to vehicle, the non-cycling cell percentage increased by 1.5% with the Olaparib treatment and by 12.4% with the Dinaciclib treatment. With the Combination treatment, there was an increase of 18.8% non-cycling cells. Although there is a general trend of increased cells in non-cycling phases of the cell cycle upon treatment, there was no statistical significance found after performing paired two-tailed t-tests, as the p-value was > 0.05.

## 4.3 Apoptosis Assay Analysis

After analysis of the cell death as explained in 3.5, the data (n=3). was graphed as live cells versus apoptotic cells. Paired two-tailed t-test statistical testing was run against the live versus the apoptotic cells. The SEM was calculated and graphed, as seen below. A and B display the results for the CAOV3 cell line, 8-hour and 24-hour treatment, respectively. C and D represent the results for the OVCAR5 cell line, 8-hour treatment and 24-hour treatment, respectively. In each graph, the order from left to right is the following: Vehicle, Dinaciclib 100nM, and Dinaciclib 1μM. Theoretically, an increase of apoptotic cells should be observed with the drug, and a greater apoptotic rate from a greater concentration of the drug.

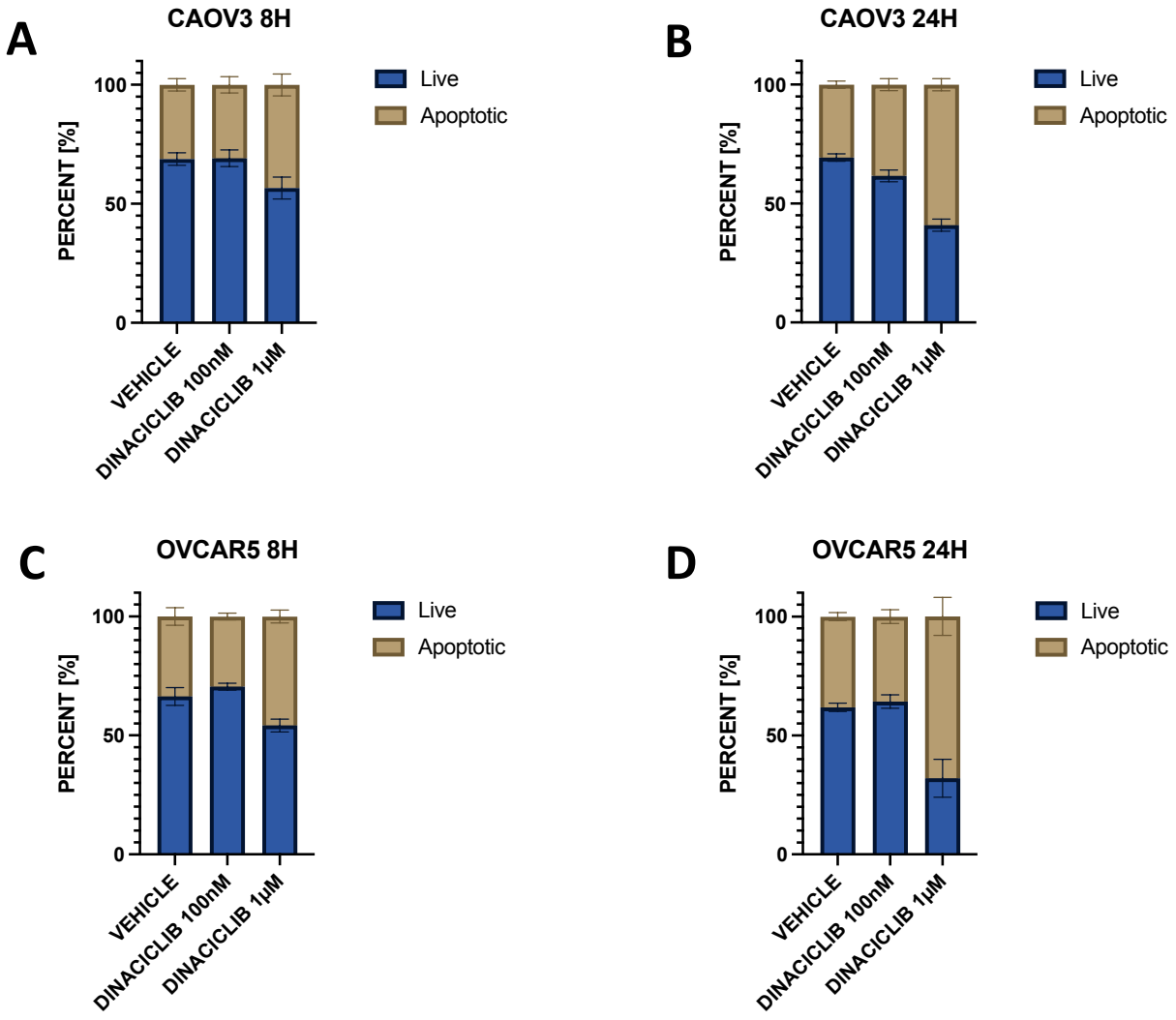


*Figure 10. Apoptosis Assay Results*

In Figure 10A, the difference between vehicle and treatment of Dinaciclib at 100nM for 8 hours is insignificant, but with the higher concentration of 1μM, there is an increase of 12.2% apoptotic cells from vehicle and an increase of 12.5% apoptotic cells from the treatment with lower concentration. In Figure 10B, the longer treatment time of 24 hours increases the apoptotic cell percentage by 7.7% (vehicle-Dinaciclib 100nM). There is an increase of 28.4% and 20.7%, comparing the vehicle to Dinaciclib 1μM and Dinaciclib 100nM to Dinaciclib 1μM, respectively. Comparing the treatment times, the longer treatment results in an increase from vehicle to Dinaciclib 100nM by 8% and Dinaciclib 1μM by 16.2%. In Figure 10C, the difference between vehicle and treatment of Dinaciclib at 100nM for 8 hours is insignificant, but with the higher concentration of 1μM, there is an increase of 12.2% apoptotic cells from vehicle and an increase of 16.3% apoptotic cells from the treatment with lower concentration. In Figure 10D, difference between vehicle and treatment of Dinaciclib at 100nM for 8 hours is also insignificant. There is an increase of 29.9% and 32.3%, comparing the vehicle to Dinaciclib 1μM and Dinaciclib 100nM to Dinaciclib 1μM, respectively. Comparing the treatment times, the longer treatment results in an increase from vehicle to Dinaciclib 100nM by 1.7% and Dinaciclib 1μM by 17.7%. Statistically, all graphs had a p-value $< 0.05$ for the testing between vehicle and Dinaciclib 1μM and between Dinaciclib 100nM and Dinaciclib 1μM, satisfying the requirement to be considered significant. As a note, the 24-hour treatment on CAOV3 cells showed statistical significance between vehicle and Dinaciclib 100nM, as well.

# 5. Discussion

## Protein expression of cancer drivers after CDK inhibition

The western blotting method and analysis was applied to two ovarian cancer cells lines: CAOV3 and OVCAR5. The blots were imaged and analyzed for protein expression of several appropriate proteins that can be found in a cancerous cell, such as APC2, Mcl1, and in this case, BRCA1. The cleavage of PARP was also analyzed for signs of cleaving, which is characteristic of a cell undergoing apoptosis. GAPDH was used as a housekeeping gene, since it is the most commonly used for comparisons of gene expression. Housekeeping genes remain constant in their expression in all cells, despite the stage or condition of the cell.

The decrease of expression of APC2 suggests that CDK inhibition by Dinaciclib had prevented APC2 from its function of transitioning cells from metaphase to anaphase. Therefore, halting the cell cycle from progressing in the ovarian cancer cells, overriding the characteristic of cancer of “evading growth suppressors.” Another protein, Mcl-1 also showed decrease in expression. This can be explained by Dinaciclib, inhibiting CDK9 from carrying out RNA phosphorylation, thus decreasing the expression of the Mcl-1 protein. This results in a reduction of this antiapoptotic protein and allowing apoptosis of otherwise concealed ovarian cancer cells. As mentioned before, BRCA1 is frequently mutated in ovarian cancer, so the decrease of expression after drug-treatment would indicate that the drug has inhibited the mutated BRCA DNA repair system from allowing repair of double-strand breaks in mutated DNA, and this could be seen in the western blot images. The cleavage of PARP signifies cells undergoing apoptosis, and the analysis of the western blot indicates this event is occurring, meaning that Dinaciclib is inducing cell apoptosis of the ovarian cancer cells. The same results were observed between CAOV3 and OVCAR5 cell lines.

To draw a conclusion from this data, the introduction of Dinaciclib, a CDK Inhibitor, as a treatment of ovarian cancer cells has shown to induce apoptosis supported in several different ways, such as the decreased expression of APC2 and Mcl-1, the cleavage of PARP, and decreased BRCA1 expression, in both CAOV3 and OVCAR5 cell lines.

## CDK inhibition and PARP inhibition on the cell cycle progression

The CDK inhibition and PARP inhibition on the cell cycle progression was analyzed through a cell cycle assay, where Olaparib, Dinaciclib, and the Combination were applied and compared to Vehicle, where the ovarian cancer cells grew with no influences. The cell sorter determined how many cells where at each phase of the cell cycle in each sample. The treatment of Dinaciclib should induce inhibition of the CDKs (CDK1, CDK2) at the S-phase and G2/M-phase checkpoints of the cell cycle. Thus, creating a greater percentage of cell cycle arrest in those phases. Olaparib is known for DNA damage, cell cycle arrest, and cell death. The purpose of this assay was to analyze the influence of CDK inhibition and PARP inhibition individually on the cell cycle, and as a Combination, to support the theory that treatments with both drugs create a better response to the progression of ovarian cancer cells.

From the data, a general trend in cell cycle arrest can be observed, where the percentage of the Combination treatment was greater than Olaparib and Dinaciclib by themselves. It can also be seen that the difference in percentages from 8 hours and 24 hours has decreased in cell cycle arrest, which can be explained by the drug possibly reaching its highest potential and declining, and in that meantime, the non-effected cancer cells could proliferate and continue with evading the growth suppressors. This could suggest that an 8-hour treatment would have a greater effect than a 24-hour treatment window. It also introduces the possibility of a serial dosing having a greater effect, and at a more constant rate, such as every 8-hours. On the contrary, in OVCAR5 cells, the cell cycle arrest percentage for the combination treatment in the 24-hour treatment (18.8%) almost doubled compared to the 8-hour treatment (9.5%). Another interesting similar observation can be seen in OVCAR5 cells having a greater percentage of cells in cell cycle arrest with treatment of Dinaciclib than in CAOV3 cells. The opposite phenomenon occurs with the Olaparib treatment, where the CAOV3 cells react more strongly (greater percentage of cell cycle arrest occurring) to the drug than OVCAR5 cells.

To summarize, CAOV3 cells showed to be more sensitive to Olaparib and OVCAR5 cells were more sensitive to Dinaciclib. This especially demonstrates how different cancer can react even though the characteristics were similar to begin with, and it’s a

good example of how the combined treatment could target a greater variety of cancer environments, despite appearing to be similar at first. The results also support the theory that treating with CDK inhibition and PARP inhibition together showed a greater increase in cell cycle arrest than if each drug was applied individually. However, in the end, the results did not prove to be statistically significant, so the experimentation would have to continue with a few tweaks to create a more solid support for this theory.

## CDK inhibition influence on cell death

The CDK inhibition influence on cell death was observed through the apoptosis assay, where the cells were determined to be either live or apoptotic. Two concentrations of Dinaciclib were applied, 100nM and 1μM, and compared to the vehicle, regular progression of ovarian cancer cells. The purpose of this assay was to determine how effective CDK inhibition was to induce cell death of ovarian cancer cells. CDK inhibition should increase cell cycle arrest through its function of targeting CDK1 and CDK2, and revealing the ovarian cancer cells as being insufficient to go through the cell cycle, and should increase apoptosis by targeting CDK9, where it prevents the phosphorylation of the carboxyl terminus of RNA polymerase II, thus playing a transcriptional inhibitory role and inducing cell apoptosis.

The data shows that the treatment with the lower concentration of Dinaciclib (100nM) is ultimately, ineffective. However, with longer treatment time and with treatment of the higher concentration (1μM), there was a greater percentage of apoptosis observed at almost 30% lower than vehicle, but at about 63.5% overall. Similar to the results from the cell cycle assay, the OVCAR5 cells showed to be more sensitive to Dinaciclib than CAOV3 cells. Other than this, there is a similar trend of influence on the CAOV3 and OVCAR5 cell lines.

To summarize, the treatment with Dinaciclib, the CDK inhibitor, was shown to induce apoptosis in the CAOV3 and OVCAR5 cell lines. A greater effect was observed with the longer treatment time and treatment with the higher concentration of Dinaciclib, 1μM. There was a problem with an already high apoptotic rate in the vehicle cells that didn’t undergo treatment. This might be explained by the cells maybe not receiving enough nutrients or possibly some rough conditions during the harvesting of cells after completion of the treatments. This data could also be supported statistically when comparing the percentage difference of apoptosis between the vehicle and higher concentration of Dinaciclib and between the two concentrations of Dinaciclib treatment.

# 6. Conclusion

The objectives of this project were to examine the protein expression of certain cancer driver's post-treatment, to measure how CDK and PARP inhibition influences the cell cycle progression, and to determine the effect of CDK inhibition on the apoptosis of ovarian cancer cells. The overall research question was the following: Will PARP inhibitors and CDK inhibitors work better together in the treatment of ovarian cancer cells?

From the discussion, we can conclude that CDK inhibitors have shown to induce apoptosis and increase cell cycle arrest in multiple ways. First, from assessing protein expression of several potential cancer drivers, decreased expression of APC2 and Mcl-1, the cleavage of PARP, and decreased BRCA1 expression, were observed in both CAOV3 and OVCAR5 cell lines. With the apoptosis assay, the CDK inhibitor was also shown to induce apoptosis in the CAOV3 and OVCAR5 cell lines, where a greater effect was observed with the longer treatment time and treatment with the higher concentration of Dinaciclib, 1μM. There was a problem with an already high apoptotic rate in the vehicle cells that didn't undergo treatment. This might be solved by ensuring the cells are receiving enough nutrients or possibly look into ways to be gentler when harvesting the cells after completion of the treatments. This data could also be supported statistically as described before, answering the question of the influence of CDK inhibition on apoptosis positively. From the cell cycle assay, the question regarding PARP and CDK inhibition influence on the cell cycle individually and the response from the combination of the treatments of CDK and PARP inhibition could be answered. In which, treating with CDK inhibition and PARP inhibition together showed a greater increase in cell cycle arrest than if each drug was applied individually. However, in the end, the results did not prove to be statistically significant, so the experimentation would have to continue with a few tweaks to create a more solid support for this theory. A few updates could include running more trials of the method system for a greater sample size, to ensure accuracy. Another change that is similar to the apoptosis assay notes is to be gentler when harvesting the cells and preparation of the samples, to prevent any unrelated cell loss and damage. Also, CAOV3 cells showed to be more sensitive to Olaparib and OVCAR5 cells were more sensitive to Dinaciclib. This especially demonstrates how different cancer can react

even though the characteristics were similar to begin with, and it's a good example of how the combined treatment could target a greater variety of cancer environments, despite appearing to be similar at first.

There is room for improvement in regard to repeating the experiments for a greater amount of data to promote accuracy and more reliable data. Another improvement would be to test Dinaciclib at different concentrations and at more treatment time periods to see the ultimate combination in which it is most effective with each cell line. If there is a stark contrast between cell lines at that point, it would also be interesting to look into what exactly makes them different and that could possibly be applied to dosing of the drug in respect to the condition of the patient when applied clinically. A future goal in this research would be to apply this theory onto mouse models to create a more similar environment to a human, where the microbiome environment is taken into account as well.

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