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# Analysis of Cancer Treatments on the Cell Cycle with Fluorescence Microscopy

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## Abstract

Cancer cells, like the Osteosarcoma cancer cell line we used in this study, are able to bypass the restraints of the cell cycle and determining possible ways to target and limit this proliferation is vital. In this study, we intend to explore the treatment options that come in the form of cell cycle inhibitors and regulators such as MLN 4924 which targets the Nedd8 pathway to cause mis-regulation of DNA replication; Hydroxyurea which targets ribonucleotide reductase to prevent DNA replication; and an unknown substance (Compound X), which we hypothesize to be a CDK4/6 inhibitor. In order to test each of these treatment options and observe their impact on the cell cycle, we used fluorescence microscopy and the Fucci system in tandem to observe the presence and relative density of CDT1 and Geminin in the cells, and where the cell cycle could possibly be interrupted. After testing, the treatment options were shown to be successful by comparing frequencies of geminin and CDT1 in the treated groups to the control. These findings will demonstrate the different possible targets for cell cycle disruption in cancer cells and given the range of targets allow for more diverse treatment paths for affected patients.


## 1. Introduction

The purpose of this experiment was to learn to use a series of fundamental laboratory techniques in order to visualize the densities and frequencies of florescent marked proteins using fluorescence microscopy. We used an Osteosarcoma cancer cell line with cells that were engineered to express mCherry-CDT1 and GFP-Geminin to visualize the dynamics of cell cycle in living cells, also called the Fucci system (Fluorescent ubiquitination-based cell cycle indicator). Cancer cells disrupt regulation of the cell cycle and replicate uncontrolled by normal regulatory mechanisms. In this experiment we investigated the effects of treatments that attempt to disrupt the replication of cells.

Fluorescent tagging, in relation to the Fucci system, refers to a technique that involves attaching fluorescent proteins, such as CDT1 or Geminin to molecules or structures within cells. Through this, the G1 phase is indicated by mCherry-CDT1 color red and the S/G2/M phases by GFP-Geminin color green. With this system, fluorescent tagging is used to visualize and track the cell cycle's progression in real-time.

The first treatment, called MLN 4924, is a Nedd8 inhibitor blocking $SCF^{skp2}$ during S and G2 phases. Nedd8 is a protein that plays a crucial role in the process of protein modification called neddylation. MLN inhibits the Nedd8-activating enzyme, preventing the neddylation process from occurring. This disruption leads to the accumulation of various proteins within the cells, inducing apoptosis.

The second treatment called Hydroxyurea (HU) is a ribonucleotide reductase (RNR) inhibitor that blocks DNA replication by preventing ribonucleotides from reducing into deoxyribonucleotides, a necessary component for DNA construction. This can lead to either cell cycle arrest at the G1/S phase boundary due to DNA replication inhibition. This can lead to incomplete DNA synthesis or DNA damage which will inhibit the growth of cancer cells.

Studying the effects of MLN and HU on the cell cycle in cancer cells using the Fucci system offers valuable insights into the mechanisms underlying cell cycle regulation and potential therapeutic strategies. By utilizing these treatment options, researchers can study these methods in relation to cell cycle progression in cancer cells, thereby aiding the development of therapies for various types of cell cycle dysregulation and the resulting cancer.

# 2. Methods

On day 1 of the experiment, the following procedures were conducted to prepare the U2OS Fucci cells for experimentation and observation. First, A 10 cm dish containing growing U2OS Fucci cells was obtained and labeled. After that, the cells in the dish were observed under a phase contrast microscope to assess their flat cellular morphology. After carefully removing the cell culture media, 5ml of pre-warmed PBS was added to the cells to wash away the serum from the media. We then removed the PBS and added 2 ml of Trypsin-EDTA which acted as a dissociation reagent. After trypsinization, the resulting suspension was collected and studied to determine their newly rounded cellular morphology. After observing that the cells were detached, we neutralized the Trypsin-EDTA with 3 ml of the cell culture media and used a 10 ml pipette to resuspend the dissociated cells. We then used Neubauer Improved cell counting chambers to count the cells in the suspension. Once our cells were prepared and accounted for, we created the proper dilution to achieve a concentration of 20,000 cells/300 µl for the ibidi slides. The first samples of U2OS Fucci cells were seeded into 8 ibidi slides with ~20,000 cells per chamber. After that, the slides were filled in sets of 2 for each group: untreated, MLN, HU, and compound X. The plates containing the seeded cells were transferred to a $CO_2$ incubator to provide suitable growth conditions for the next 24-26 hours.

On day 2 of the experiment, the following steps were preformed to investigate the effects of MLN, HU, and compound X on the U2OS Fucci cells seeded in the ibidi slides. First, our team added 3 µl of cell permeable Hoechst DNA staining dye to each chamber with the seeded cells in the ibidi slides and left to rest in a CO2 incubator for 10 minutes to allow for DNA staining. We then evaluated the cells using fluorescence microscopy and imaged the treated and untreated cells in the ibidi slides. Then images were analyzed using the software Fuji to produce clear colored images.

# 3. Results

Before beginning specific experiments untreated Osteosarcoma cancer cells were imaged using florescent microscopy (*Figure 4: Untreated Cells*) From this a frequency table (*Table 1*) was produced to be used for comparison and analysis of treated cells.

*Table 1: Untreated Cell Quantification*

| | Red | Green | Neither |
|---|---|---|---|
| Count | 20 | 27 | 66 |
| Percent Frequency | 17.8% | 23.9% | 58.4% |

Using this method, the following treatments of MLN, HU and Compound-X were observed and quantified:

## 3.1 $SCF^{skp2}$ inhibition disrupts normal cell cycle progression by preventing degradation of CDT1

The degradation of the Cdc10-dependent transcript 1 protein (CDT1), regulated by the ubiquitin ligase complex $SCF^{Skp2}$ in S and G2 phases, is crucial for proper cell cycle progression. How does the inhibition of SCF affect the degradation of CDT1 and with that the cell cycle progression?

To address this question, U2OS-Fucci cells were incubated with MLN, a nedd8 inhibitor that ultimately blocks the activity of SCF. By comparing the frequency of two Fucci markers (mCherry, marking CDT1, and GFP, marking Geminin) the relative frequency of certain cell cycle stages could be compared to untreated cells. This enabled variations to be quantified to determine what effect MLN would have on the cell cycle.

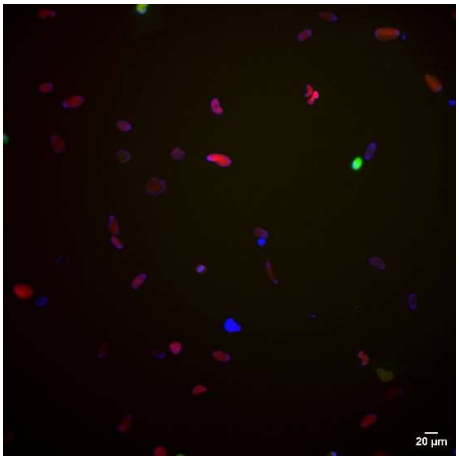


*Figure 1: MLN Treated Cells*

The red fluorescence corresponds to the cells being in the G1 phase cell cycle, while the green fluorescence represents the S/G2/M phases. The samples incubated with MLN revealed a notable increase in red cells compared to the untreated control (Figure 1: MLN Treated Cells). To verify the visual difference in cell cycle a simple difference of means was calculated comparing red MLN treated cells (Figure 1: MLN Treated Cells) and red untreated cells (Figure 4: Untreated Cells) resulting in an increase of relative frequency of 66.4% for CDT1 expressing cells (Table 2). Given the findings it can be concluded that MLN treatment inhibiting SKP2 results in an increased accumulation of CDT1 by preventing its degradation, suggesting a disruption of the normal cell cycle progression.

*Table 2: Comparison of Protein Frequency between Untreated Cells and MLN 4924 Treated Cells*

| | Cells with CDT1 | Cells with Geminin | Cells with Neither |
|---|---|---|---|
| MLN Treated Cells | 84.2% | 7.9% | 7.9% |
| Untreated | 17.8% | 23.9% | 58.4% |
| Difference | +66.4% | -16% | -50.5% |

The inhibition of $SCF^{skp2}$ by MLN has prevented the degradation of CDT1, leading to the accumulation of CDT1 and potentially causing a delay or arrest in the G1 phase. Normally the CDT1 is degraded quickly but due to the inhibiting effects of MLN it remains active causing the DNA to keep on replicating which will lead to cell cycle arrest and eventual apoptosis brought on by overproduced and damaged DNA.

## 3.2 Nucleotide depletion through RNR inhibition hinders DNA replication causing and arrest in S-phase

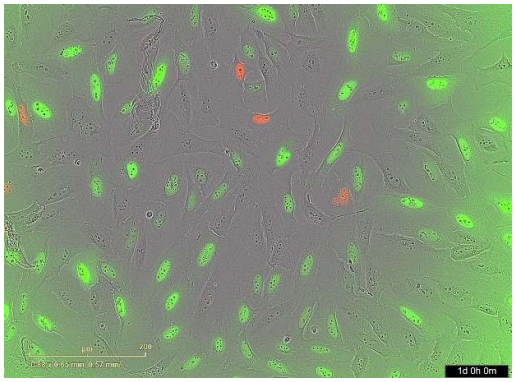


*Figure 2: HU Treated Cells 1 day Incubation*

Inhibition of ribonucleotide reductase (RNR) can lead to the depletion of nucleotides, while the availability of nucleotides is needed for DNA replication. How does nucleotide depletion through RNR inhibition affect DNA replication and cell cycle progression? To analyze this question, U2OS Fucci cells were incubated with Hydroxyurea (HU), an RNR inhibitor, and subjected to fluorescent microscopy. After HU treatment, an increase in the population of green cells was observed compared to the untreated control (Figure 2).

The inhibition of RNR by HU results in a reduction of available nucleotides. Without sufficient supply of nucleotides, the DNA replication machinery cannot proceed, stalling replication forks. Uncompleted DNA replication will activate the DNA replication checkpoint or S phase checkpoint (Nordlund & Reichard, 2006). Activation of this checkpoint halts the progression of the cell cycle. Therefore, the increase in green cells suggests that the cells are in S phase arrest.

## 3.3 Compound-X treatment arrests cell cycle progression during the G1 phase by inhibiting Cyclin-dependent Kinases 4 and 6 (CDK4/6)

Compound-X, an unknown anticancer compound, is known to cause mis-regulation of cell cycle progression. How does compound-X treatment affect cell cycle progression, and will it induce cell cycle arrest in a specific phase?

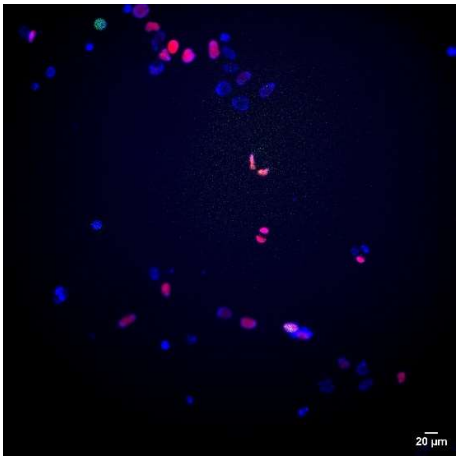


*Figure 3: Compound-X Treated Cells*

The U2OS-Fucci cancer cell line was treated with compound-X and subjected to fluorescence microscopy to assess the effects of the anticancer compound. Following treatment with compound-X, cells begin to have a notable absence of Geminin (visualized in green) in cells without an increase in CDT1 (visualized in red) presence (Figure 3: Compound-X Treated Cells). The differences where quantified by comparing relative frequencies between untreated cells (Figure 4: Untreated Cells) and compound-X treated cells (Figure 3: Compound-X Treated Cells). The resulting difference is shown in (Table 3). Given the increase in cells with CDT1 and the decrease of cells expressing Geminin it can be postulated that compound-X arrests the cell cycle in the G1 phase.

*Table 3: Comparison of Protein Frequency between Untreated cells and Compound - X Treated Cells*

| | Cells with CDT1 | Cells with Geminin | Cells with Neither |
|---|---|---|---|
| Compound-X | 41.3% | 2.2% | 56.5% |
| Untreated | 17.8% | 23.9% | 58.4% |
| Difference | +23.5% | -21.7% | -1.9% |

Given this the likely target of compound-X is the growth factor pathway activating cyclin-dependent kinases (CDKs). “Cyclin-dependent kinases 4 and 6 (CDK4/6) phosphorylate the retinoblastoma protein (Rb) to relieve repression of E2F-dependent genes and allow progression from G1- into S-phase.” (Crozier, Lisa, et al.) By downregulating this pathway, the signal to progress into the G1 phase would not activate and would trigger cell cycle arrest.

# 4. Discussion and conclusion

Treatment of cancer cells with MLN 4924, an inhibitor that blocks the $SCF^{SKP2}$ complex, resulted in a significant increase in mCherry-CDT1 red cells, indicating a disruption in the cell cycle progression. This finding is consistent with a study performed by Li et al. (Li et al., 2003). The results of this research suggest as well that the SKP2 complex may play an important role in the degradation of CDT1. Downregulation of SKP2 induces an accumulation of CDT1. Another study shows that overexpression of CDT1 causes a significant delay in the initiation and completion of the S phase. The delay indicates that the inability to degrade CDT1, hinders the normal progression through the S phase (Takeda et al., 2005). In our research, most cells did not even go into the S phase, corresponding to the finding that initiation in the S phase was hindered. Takeda et al. moreover found that CDT1 mutants that could not interact with SKP2, progressed through the S phase as normal CDT1 wildtype would do. They suggest that there is an SKP2-independent pathway that is critical for degradation of CDT1 and thus for normal progression through the S phase. This contrasts with our experiment, where the inhibition of SKP2 by MLN was most probably the reason for the disruption of the normal cell cycle.

Furthermore, treatment with HU, showed an increase in GFP-geminin green cells. HU is a potent inhibitor of ribonucleotide reductase (RNR), an enzyme responsible for synthesizing deoxynucleotides. By inhibiting RNR, HU reduces the available levels of deoxyribonucleotide triphosphates (dNTPs), which are needed for DNA replication. HU treatment has been shown to slow down replication forks and induce cell cycle arrest in the S phase (Xu et al., 2016). This is consistent with the results we observed in our experiment.

Treatment of cancer cells with unknown compound-X resulted in an accumulation in mCherry-CDT1 red cells, indicating a disruption in the cell cycle progression between the G1 to S checkpoint. Because of this, we hypothesized that the identity of this unknow substance would be Cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors. CDK4/6 works by phosphorylating the retinoblastoma protein (Rb) to reduce the repression on E2F-dependant genes to allow for the cell cycle to progress from the G1 phase to the S phase (Crozier et al.,2022). Several different types of CDK inhibitors have been developed and approved for different types of breast cancer, specifically targeting CDK4 and CDK6. These inhibitors can halt the progression of the cell cycle in cancer cells by preventing their entry into S phase and thereby decreasing cell proliferation and inducing apoptosis, which is what our team saw in our results.

In conclusion, this study provides valuable insight into the effect of MLN inhibition, HU-induced nucleotide depletion and possibly CDK4/6 inhibition in the form of compound X on cell cycle progression.

# 5. References

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# 6. Appendix

## A. Florescent Microscopy Images

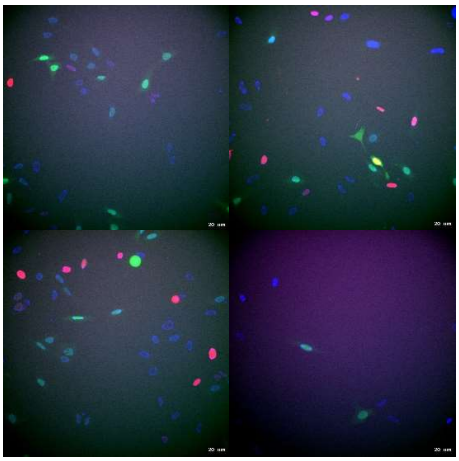
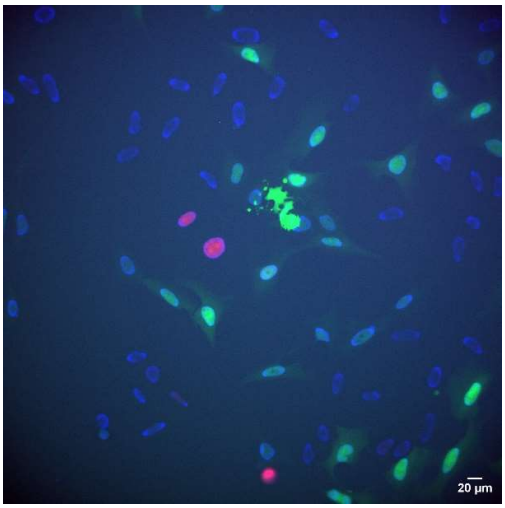


*Figure 4: Untreated Cells*



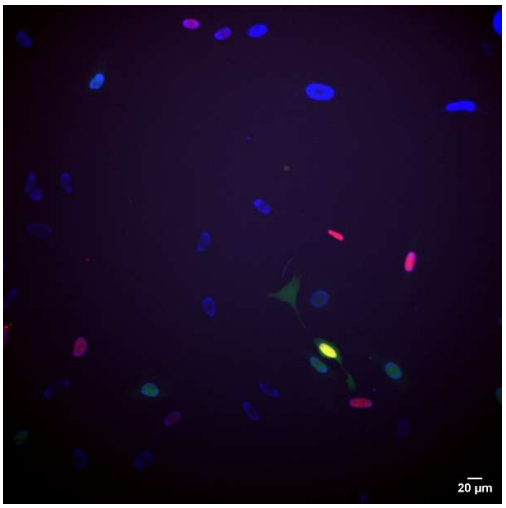




*Figure 5: Untreated Cells*

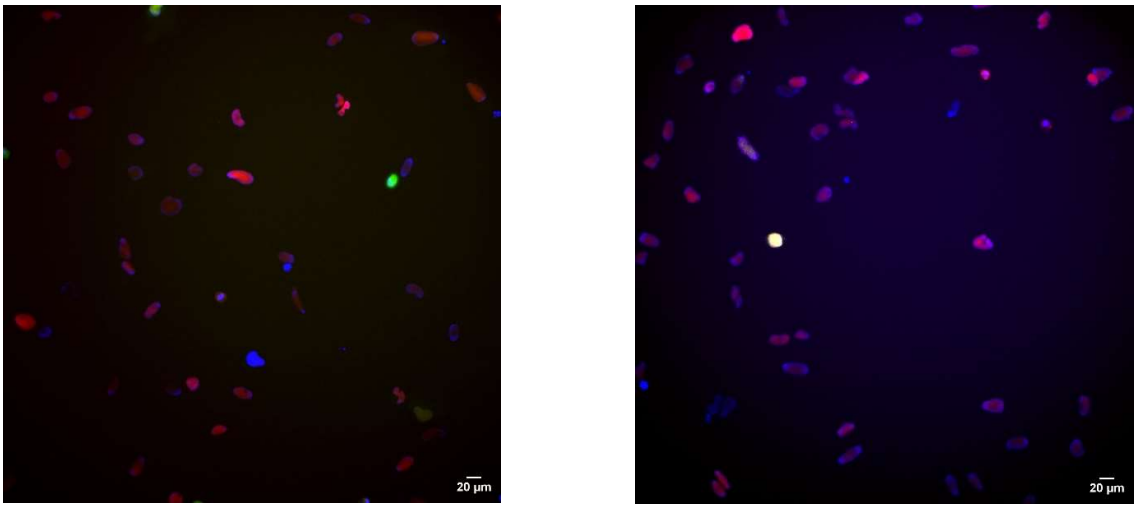



*Figure 6: MLN 4924 Treated Cells*

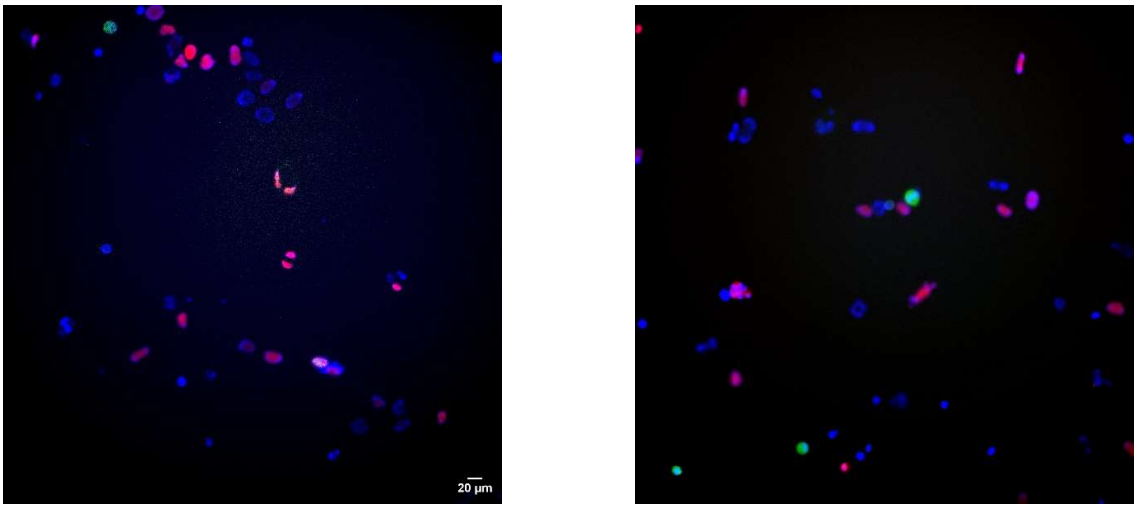



*Figure 7: Compound X Treated Cells*

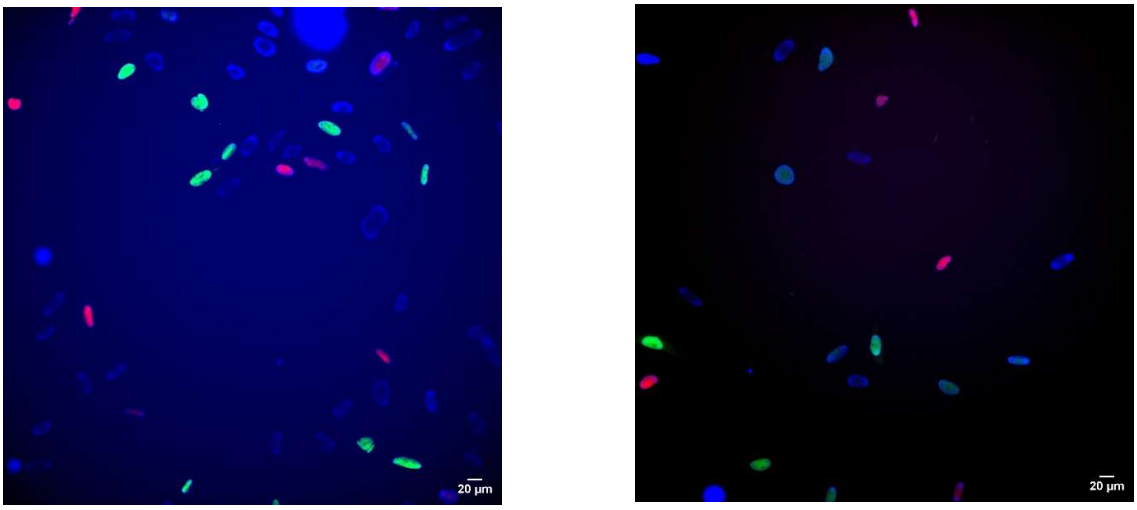



*Figure 8: HU Treated Cells*