NAZARBAYEV
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# CLONOGENIC CAPACITY OF CANCER CELLS AFTER MITOTIC SLIPPAGE

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**DECLARATION**

I hereby declare that the thesis is my original work, and it has been written by me in its entirety.

I have duly acknowledged all the sources of information which have been used in the thesis. This thesis has also not been submitted for any degree in any university previously.

Raushan Yeskendirova

28 April, 2023

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| TAX | Taxol |
| EpoB | Epothilone B |
| NOC | Nocodazole |
| VIN | Vinorelbine |
| SAC | Spindle Assembly Checkpoint |

Nazarbayev University, School of Science and Humanities, Department of Biology

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Raushan Yeskendirova: Clonogenic Capacity of Cancer Cells after Mitotic Slippage

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## SUMMARY

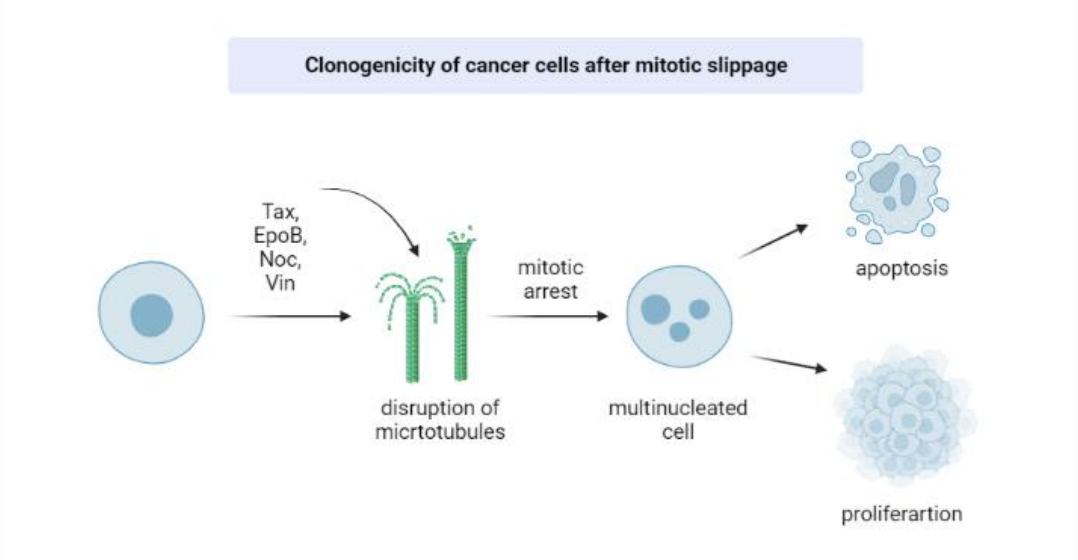


**Figure 1.** Graphical abstract on clonogenicity of cancer cells after mitotic slippage

Paclitaxel (Taxol), Epothilone B, Nocodazole, and the Vinca alkaloids are the four antimicrotubule agents widely used in clinics as anticancer chemotherapeutic drugs. These drugs have microtubules stabilizing and destabilizing effects, inducing mitotic arrest. As a result of prolonged mitotic arrest, some cells undergo apoptosis in their mitotic stage, whereas surviving cells undergo mitotic slippage and become multinucleated. It was generally thought that multinucleated cells are short-lived and eventually die. However, some studies reported that multinucleated cells produce mononucleated aneuploid progeny, which might be linked to a cancer relapse and tumorigenesis.

This thesis aimed to investigate the differences in the clonogenic potential of the cells after being arrested in mitosis by Taxol (TAX), Epothilone B (EpoB), Nocodazole (NOC), and Vinorelbine (VIN), as there is a lack of data on comparing the clonogenic capacities and efficiencies of the drugs. Thus, the frequency of cell division, the time of first colony proliferation, and the ploidy of cancer cells treated with antimicrotubule drugs were studied in-depth. The methods included long-term microscopy observations and cell viability assay. The three antimicrotubule drugs (Taxol, Nocodazole, and Vinorelbine) showed various clonogenicity results due to their various modes of action on microtubules. Cancer cells after TAX washout showed minor clonogenic potential, whereas VIN and NOC washout resulted in extensive colonies in the cell population. The obtained results are beneficial in understanding the cell fate of cancer cells after mitotic slippage and obtaining new insights about cancer relapse and treatment strategies.

**Key words:** *drugs inhibiting microtubule dynamics, antimicrotubule agents, mitotic arrest, mitotic slippage, multinucleated cells, mononucleated cells.*

# 1. INTRODUCTION

## 1.1 Microtubules

Microtubules are a vital part of eukaryotic cells that assist in many processes, such as cell motility, spreading, intracellular transport, and mitosis. They are polymer structures that comprise α- and β-tubulin heterodimers and form 13 protofilaments. Each α and β monomer has a GTP-binding site. However, GTP bound to the α-tubulin is trapped and never hydrolyzed, whereas GTP on the β-tubulin could be either in GTP- or GDP-bound form and, thus, is exchangeable. The hydrolysis of GTP on the β-tubulin site impacts the microtubular dynamics.

In a normal cell, microtubules arise from the cellular structure called centrosome near the cell center. The ability to quickly reassemble and disassemble allows them to develop a network within which cellular components are transported to various compartments. Being an essential part of the cell cytoskeleton, microtubules assist in the intracellular transport of macromolecules within the cytoplasm.

During mitosis, microtubules disassemble and reassemble into the mitotic spindle, a structure that guides the segregation of chromosomes, allowing the cell to form two daughter cells. The hollow tube structure of microtubules and polarized protofilaments provide overall polarity to the microtubule. The β-tubulin end is called a plus end, whereas the α-tubulin end is referred to as a minus end. Microtubules can grow from both ends. However, the polymerization happens faster at a faster rate at its plus end side. Both the polarity and the fact that its structure has a clear orientation with chemically and functionally distinct two ends ensure proper cell division and other functions. Microtubules depolymerize around 100 times quicker from the GDP-bound tubulin end, while the GTP-cap favors growth. Once a GTP cap is lost, depolymerization takes the lead. Thus, microtubules can switch from a state of slow growth to a period of dynamic disassembly, called dynamic instability (Alberts et al., 2014).

Spindle Assembly Checkpoint (SAC) is one of the pathways that regulates cell cycle by ensuring a proper chromosome segregation during mitosis. Any issues associated with a

proper chromosome segregation by microtubules activates SAC during mitosis, hence cell cycle arrest can be initiated. There might be two outcomes: cell death and mitotic slippage, an escape to interphase without proper segregation of chromosomes. Multinucleated cells can be a result of the mitotic slippage.

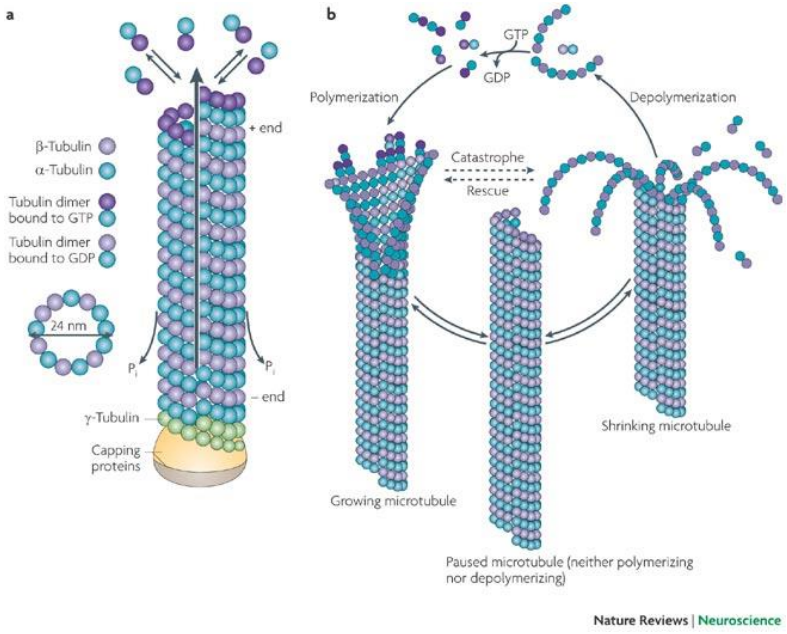


**Figure 2.** Microtubule assembly and disassembly. *Nature Reviews. Neuroscience*

## 1.2 Relevance of the antimicrotubule drugs

Cancer is one of the most widespread diseases, causing approximately 10 million deaths in 2020, around 1 in 6 (Ferlay et al., 2020). Drugs affecting cancer cells' proliferation are extensively used to treat many cancer types. Among them, antimicrotubule drugs, targeting cancer cell proliferation by influencing the polymerization of microtubules, are of high scientific interest.

Many chemotherapeutic drugs used in clinics generate resistant cancer cell populations that contribute to a cancer relapse and decrease the efficiency of currently employed treatment strategies. According to Aktipis et al. (2011), chemotherapeutic treatment selects for drug-resistant cancer cells originating from an initially heterogeneous

population. During the cancer relapse, the tumor's composition mainly consists of a new diverse population of resistant sub-clones generated by extensive genetic alterations.

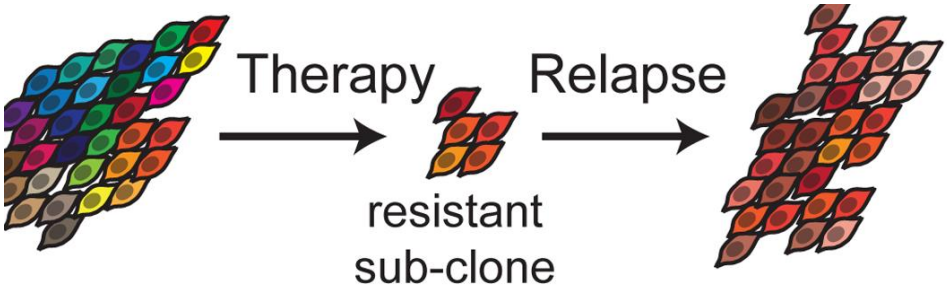


**Figure 3.** Generation of resistant sub-clones after therapy results in cancer relapse. Aktipis et al. (2011).

All microtubule inhibitors cannot completely kill the cancer population, but they are capable of inhibiting cell proliferation. The consequence of this inhibition might be interesting to explore at the cellular level.

### 1.3 Antimicrotubule drugs

Paclitaxel, Epothilone B, Nocodazole, and the Vinca alkaloids (Vinorelbine) represent two different classes of antimicrotubule agents that effectively interfere with the polymerization of microtubules. Paclitaxel and Epothilone B act on microtubules by stabilizing them, whereas Vinca alkaloids and Nocodazole destabilize microtubules and inhibit polymerization (Beswick et al., 2006; Verdier-Pinard et al., 1999). The alteration of spindle microtubule dynamics leads to mitotic arrest in many cell lines by inhibiting mitosis at the metaphase-anaphase transition at concentrations ≥ 100 nM (Suleimenov et al., 2022).

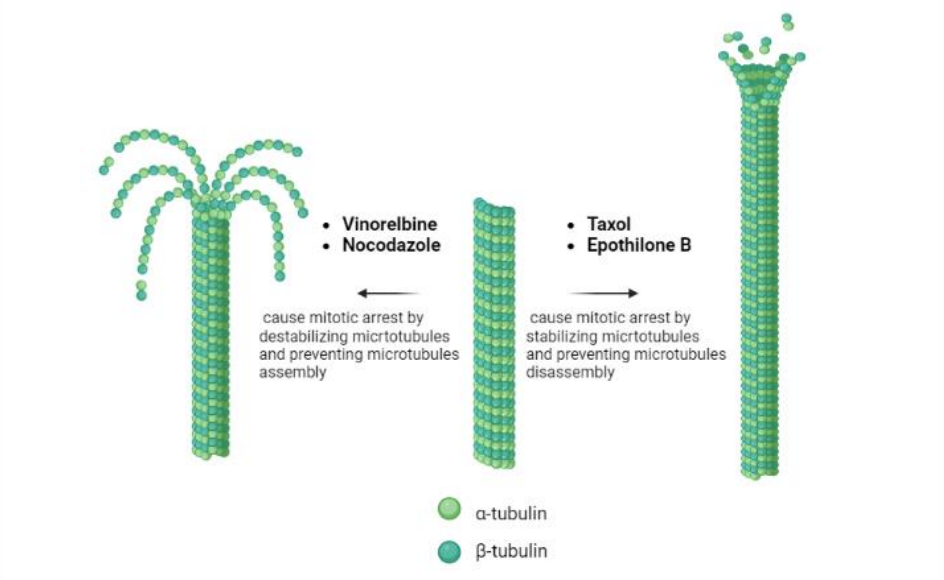


**Figure 4.** The inhibition of microtubule polymerization by antimicrotubule drugs

*Nocodazole*

*Nocodazole* refers to antimicrotubule agents that cause mitotic arrest by destabilizing microtubules ad halting their polymerization. It binds to β-tubulin monomers at the Colchicine binding site. Although the binding sites of the two drugs overlap, they are structurally distinct from each other. Currently is not used in clinics but is heavily employed as an experimental tool in many laboratories (National Center for Biotechnology Information, 2023).

*Vinorelbine*

*Vinorelbine* is a microtubular destabilizer of a vinca alkaloid nature that binds tubulin subunits and prevents the formation of the mitotic spindle, resulting in mitotic arrest in metaphase. It attaches the Vinca-binding site located on the β-tubulin close to the GTP-binding site. Vinorelbine is currently employed in the treatment of several malignancies, such as breast cancer and non-small cell lung cancer (NSCLC) (National Center for Biotechnology Information, 2023).

*Taxol*

*Taxol* is a mitotic inhibitor that binds at Taxane-binding sites. Taxol acts by binding and stabilizing microtubules and preventing their depolymerization, thus, inhibiting cellular division. It is currently being used to treat ovarian, breast, and lung cancer (National Center for Biotechnology Information, 2023).

*Epothilone B*

*Epothilone B is* an apoptosis inducer that acts by stabilizing the microtubules. Has a similar effect on cells as Taxol by stabilizing microtubules against depolymerization. However, it is cytotoxic for cells overexpressing P-glycoprotein, which makes it distinct from Taxane-based agents. Epothilone B is currently undergoing clinical trials. It has shown better efficacy and milder adverse effects than Taxol (National Center for Biotechnology Information (2023).

### 1.4 Mitotic slippage

Cells that undergo normal mitotic division produce two diploid daughter cells while exiting mitosis, whereas cells treated with antimicrotubule agents are arrested in mitosis and might have several cell fates. Some cells treated with microtubule-targeting agents die in a mitotic state, while others escape from mitosis into the interphase, undergo mitotic slippage, and produce polyploid multinucleated cells (Figure 1). Mitotic slippage is a mitotic exit event without proper chromosome segregation induced by antimicrotubule drugs (Lok et al., 2020). According to Cheng and Crasta (2017), antimicrotubule agents deteriorate normal mitotic spindles and preclude anaphase onset. Prolonged treatment of cells with antimicrotubule drugs results in prolonged mitotic arrest due to the impossibility of proceeding into anaphase. A vast majority of cells die undergoing apoptosis, whereas others enter interphase without proper chromosome segregation. A normal mitotic spindle cannot be restored in the presence of antimicrotubule drugs, thus forming polyploid multinucleated cells.

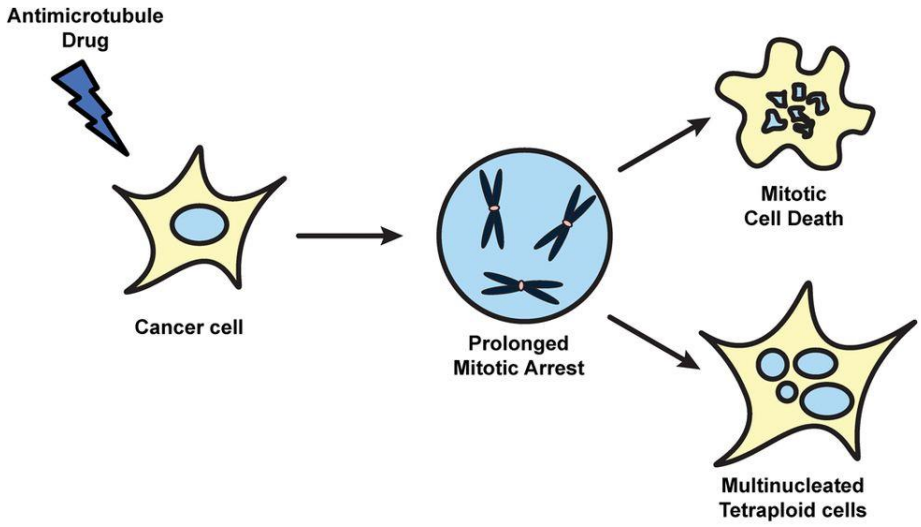


**Figure 5.** Cell fates after prolonged mitotic arrest (Cheng & Crasta, 2017).

It was long assumed that cells that underwent mitotic slippage survive for a short period and eventually die. However, some studies revealed that polyploid multinucleated cells formed after mitotic slippage could survive and might have produced viable mononucleated aneuploid progeny.

## 1.5 Literature review

There were several attempts to study the clonogenic capacity of cancer cells after mitotic arrest. Mittal et al. (2017) made important observations about multinucleated polyploidy-driven docetaxel (paclitaxel derivative) resistance. They found that PC-3 cells treated with docetaxel generate large multinucleated polyploid (MP) cells. Moreover, these cells contribute to the generation of mononucleated aneuploid cells via abnormal cell division. Zhang et al. (2014) also concluded that polyploid giant cancer cells generate cancer stem-like cells. Similar findings were reported by Rajaraman et al. (2006) that cells escape mitotic catastrophe-induced cell death by turning into polyploid cells. These cells give rise to cells with viable genomes and display some stem cell-like characteristics.

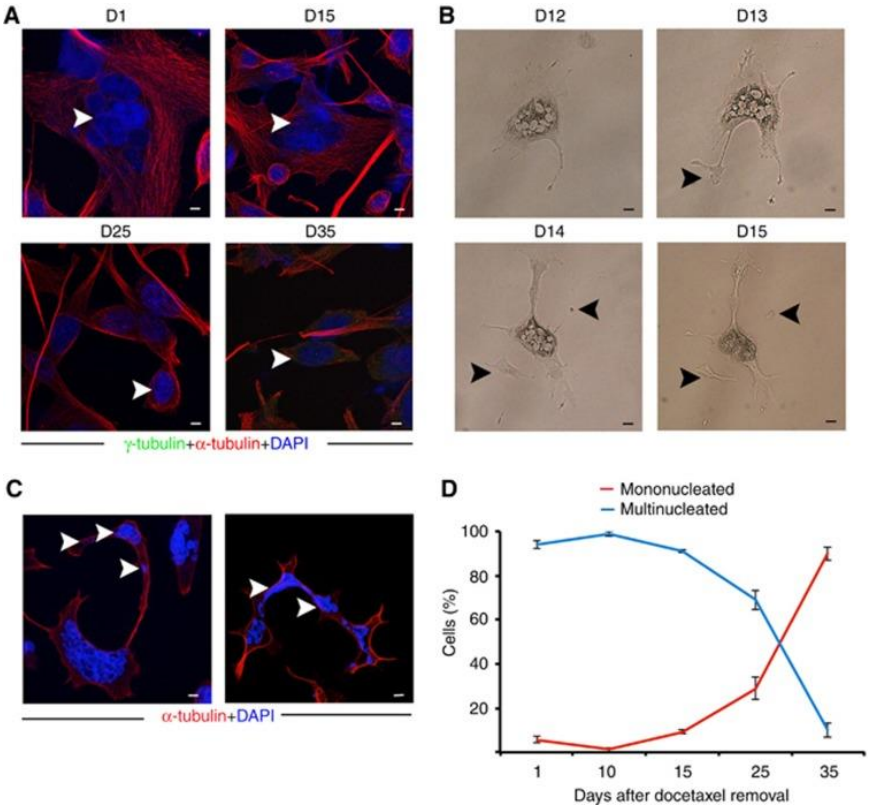


**Figure 6.** Giant MP cells undergo asymmetric cell division via neosis. (A) Confocal micrographs of docetaxel treated PC-3 cells. Cells are stained with α-tubulin (red) and DNA (blue) and showed emergence of small mononucleated cells on different days (D) after drug removal. Scale bar (white) 5 μm. (B) Time lapse images of giant MP cells generating small-sized daughter cells via budding (black arrows) over a 7-day period. Scale bar (black), 5 μm. (C) Confocal immunographs of cells stained with α-tubulin (red) and DNA (blue) showing transport of DNA from the branches of the giant MP cells (white arrows). (D) Line graph representing the total number of small sized nucleated cells and giant MP cells at different days after drug removal. (Mittal et al., 2017)

Although cancer and its treatment are the subjects of extensive studies, the cell fates of multinucleated cells and proliferation dynamics are not well understood. Thus, this study aims to investigate the long-term effects of microtubule-targeting drugs on cancer cells and observe the clonogenic potential of cancer cells that underwent mitotic slippage events. These findings will allow us to assess the clone formation dynamics of different cancer cell lines while comparing the effects of four microtubule-directed agents: TAX, Epothilone B, Nocodazole, and Vinorelbine. The obtained results might be beneficial in broadening our understanding of cancer relapse and becoming closer to effective treatment against drug-resistant cancer cells.

## 2. AIMS OF THE THESIS PROJECT

The primary aim of the research is to compare the effects of NOC, TAX, EpoB, and VIN by estimating the frequency of multinucleated cells' proliferation. Moreover, to compare the effects of the NOC, TAX, and VIN by estimating the time when multinucleated cells start proliferating (the detection of the first colonies and divisions). Finally, to conduct a ploidy analysis of cells at different time points.

As a result of mitotic arrest, many cancer cells do not die but instead experience mitotic slippage and become multinucleated. Cell fates of multinucleated cells have formed after mitotic slippage is poorly understood. Thus, the goal of the current study is to follow up directly on the behavior of multinucleated cells and study whether the fate of multinucleated cells depends on the drug used for their formation. The formation of long-living multinucleated cells might have further potential for the formation of aneuploid clones. Those clones might be beneficial for cancer progression.

## 3. MATERIALS AND METHODS

**Table 1.** List of microtubule-targeting agents and cell lines

| Name | Abbreviation | Catalog Number | Source |
|---|---|---|---|
| Taxol | TAX | PHL89806 | Sigma-Aldrich |
| Epothilone B | EpoB | 325001 | Fisher Scientific |
| Nocodazole | NOC | M1404 | Fisher Scientific |
| Vinorelbine | VIN | B000268 | BenchChem |

The cell line was purchased from ATCC. A549 is lung carcinoma epithelial cells with a doubling time of approximately 22 h.

These cell lines were chosen because they had higher survival chances after TAX, NOC, and VIN treatment.

The complete medium was prepared to ensure the long-term survival of cell lines. The optimal composition of complete media for A549 cells in accordance with ATCC guidelines was the following:

A549 cells: DMEM, 10% FBS, 2mM L-glutamine, and 1% penicillin and streptomycin mixture

To investigate the cells' fates after exposure to antimicrotubule drugs, first, cancer cells were unfrozen. Then, the cell lines were passaged 3-4 times to test the viability and normal morphology of cells. After approximately 90% confluency was reached, the cells were treated with 1 μM 0 nM TAX, 1 μM VIN, and 1 μM NOC antimitotic agents separately to induce mitotic arrest and induce mitotic slippage event. The concentrations were chosen by the method of trial and error based on the concentration-dependent experiments by Suleimenov et al. (2022), revealing that they are optimal concentrations to both induce almost complete mitotic arrest and slippage events and ensure cells' survival following drug treatment. Experiments by Suleimenov et al. (2022) also showed that 24 h incubation post-drug treatment was sufficient to obtain many mitotic cancer cells.

For prolonged studies of mitotically arrested cells, it was important to decrease the drug's concentration to its minimum. Thus, a washing-out step of drugs by change of medium was performed. After that, the cancer cells were seeded on a 6-well plate for microscopic studies. The medium was changed every 3-4 days to keep cells viable. The microscopic observations were conducted on a ZEISS Axioscope 5 microscope every 24-48 h to study how cancer cells behave post mitotic arrest and slippage. The colony formation patterns were observed on lower magnifications, whereas the analysis of nuclearity was carried out on high magnifications. The images were taken and further corrected with ImageJ and ZEISS applications for better analysis. The number of colonies and percentages of multinucleated and mononucleated cells' was manually counted to study the clonogenic capacities of cancer cells.

To observe the patterns and frequency of divisions, in addition to the prolonged manual observation on a ZEISS Axioscope 5 microscope, a time-lapse experiment for 7 days at 15-30 min intervals was conducted using a CO2-independent medium. The time series were analyzed by adjusting the contrast of films and counting the number and timing of divisions. In all experiments, negative control, a cell line without drug treatment, was used.

## 4. RESULTS

### 4.1 Multinucleated cells can proliferate.

After the 16-day treatment of A549 with antimicrotubule drugs, following was observed: (i) large multinucleated cells, (ii) large mononucleated cells, and (iii) small mononucleated cells. As depicted in Figure 7, mononucleated and multinucleated cells were differentiated based on their cell size and nuclei number. Small mononucleated cells were similar in size to control group (untreated cells), while large mononucleated cells differed.

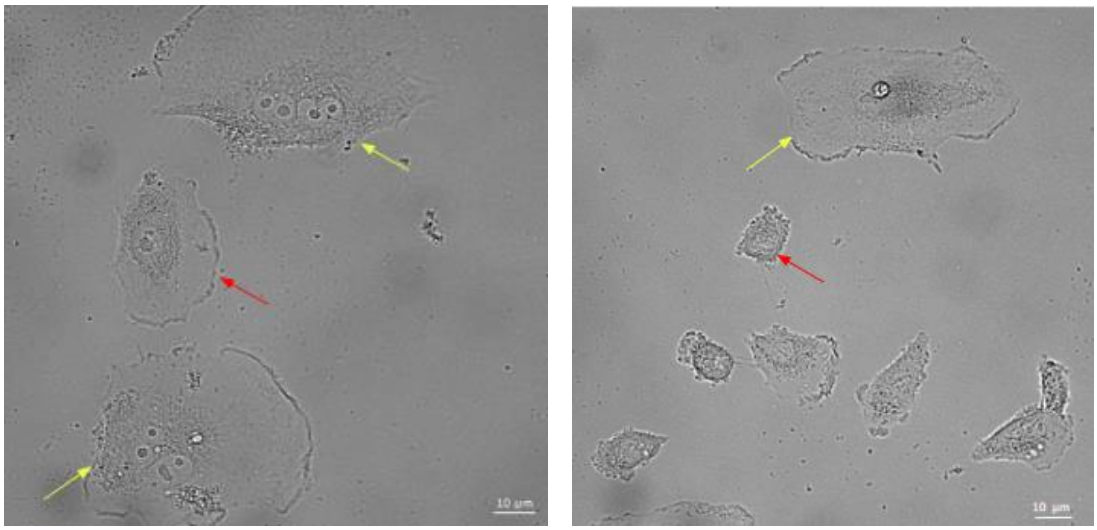

**Figure 7.** Microscopy results (40x) for A549 cells 16 days post-VIN (1 μM) washout (left image: yellow arrows indicate multinucleated cells, red arrow - mononucleated cell; right image: yellow arrows indicate large mononucleated cell, red arrow – small mononucleated cell). Scale bar (white) 10 μm.

With regards to a time-lapse experiment, it was observed that multinucleated cells can indeed produce progeny (Figure 8). However, this event is relatively rare. Compared to that, the division of mononucleated cells was observed far more frequently. Both multi and mononucleated cells divide by mitosis, forming a mitotic bubble and then dividing their cytoplasm to produce two or rarely three daughter cells.

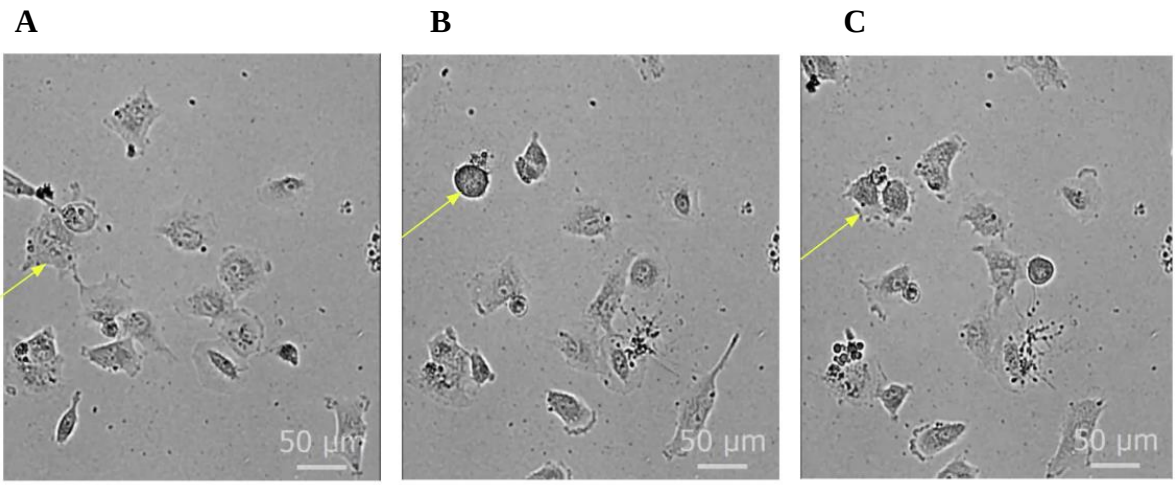


**Figure 8.** Timelapse results (10x) for A549 cells proliferation 21 h post NOC (1µM) washout. (A) multinucleated cell. (B) mitotic bubble. (C) daughter cells. Scale bar (white) 50 µm.

### 4.2 The percentages of mono- and multinucleated cells changes over time.

As it is evident from Figures 9-11, after the washout of all drugs, the percentage of multinucleated cells decreases, whereas the percentage of mononucleated cells increases with time. However, the change in the percentage of mono- and multinucleated cells progressed the fastest after the NOC washout, then in VIN. As for TAX, it took the longest time to observe a significant increase in the percentage of mononucleated cells. Similar results were obtained for prolonged life observations and time-lapse experiments. As can be seen from Figures 12-14, the fastest occurrence of mononucleated cells proceeded after the NOC washout, whereas, VIN was the second fastest in the proliferation of multinucleated cells. In contrast, life observations after the TAX washout showed that the colonies emerged between 200-300 h. The time-lapse experiment was unable to capture the moment of emergence of mononucleated cells, suggesting the window for divisions for cancer cells after washout occurs later, as evident by life observations. Thus, the time-lapse experiment for TAX washout should be conducted later, after around a week of life observations.

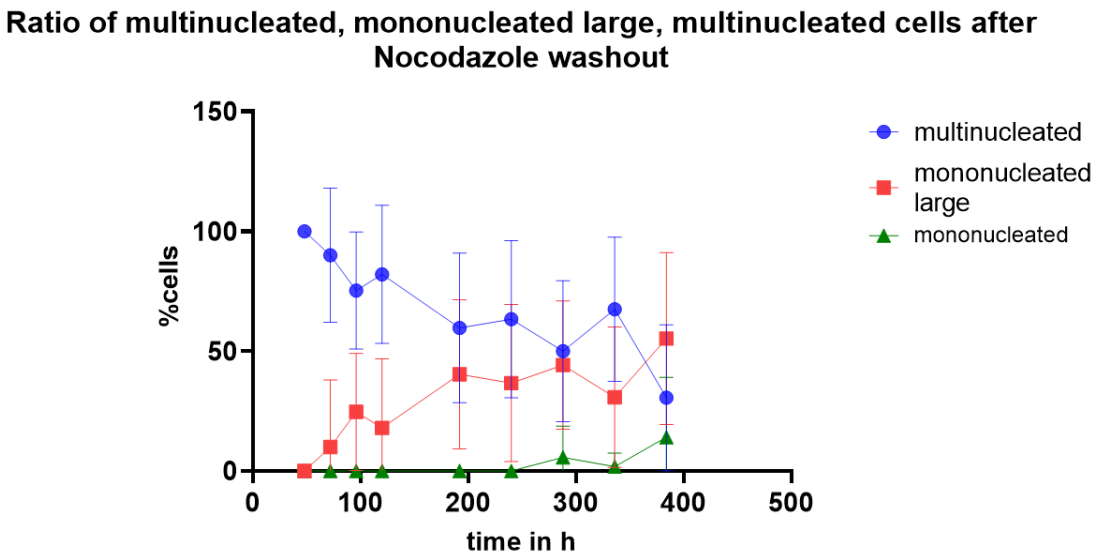


**Figure 9.** The ratio of multinucleated, mononucleated large, multinucleated cells after NOC washout.

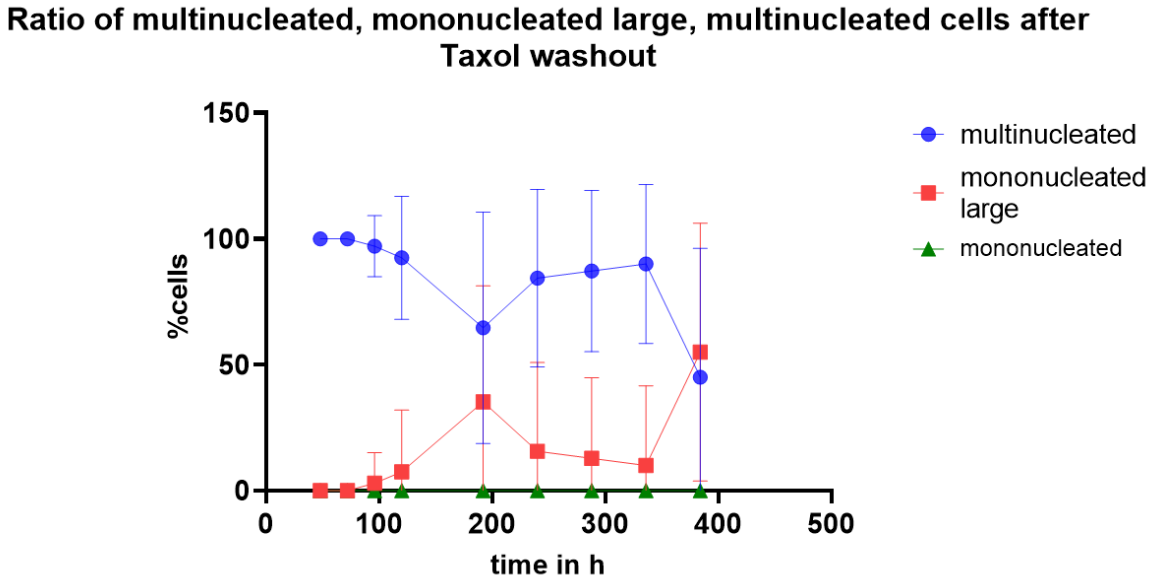


**Figure 10.** The ratio of multinucleated, mononucleated large, multinucleated cells after TAX washout.

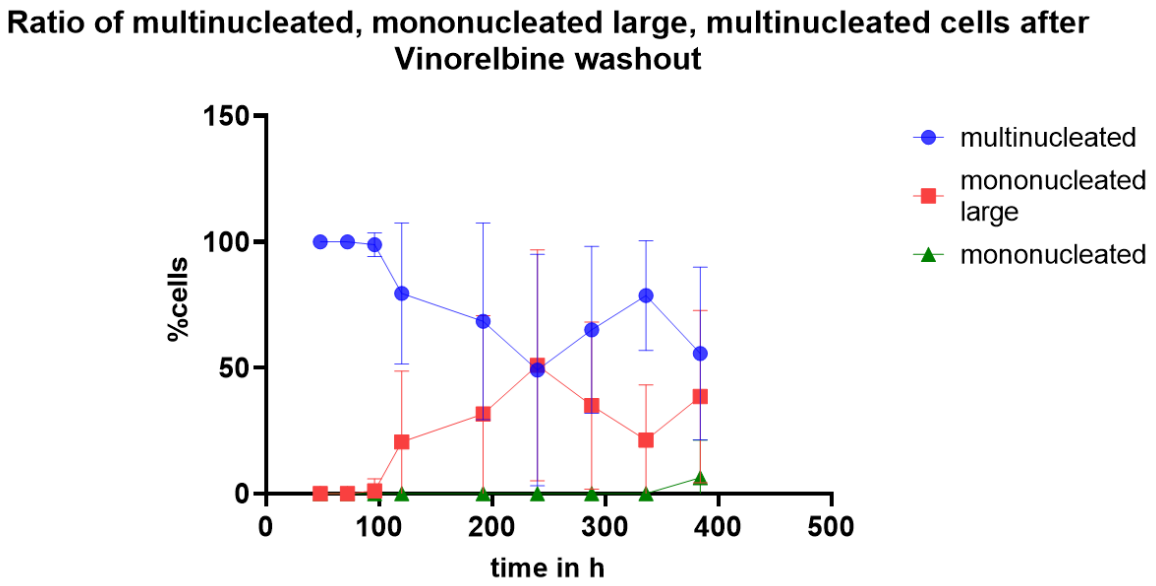


**Figure 11.** The ratio of multinucleated, mononucleated large, multinucleated cells after VIN washout.

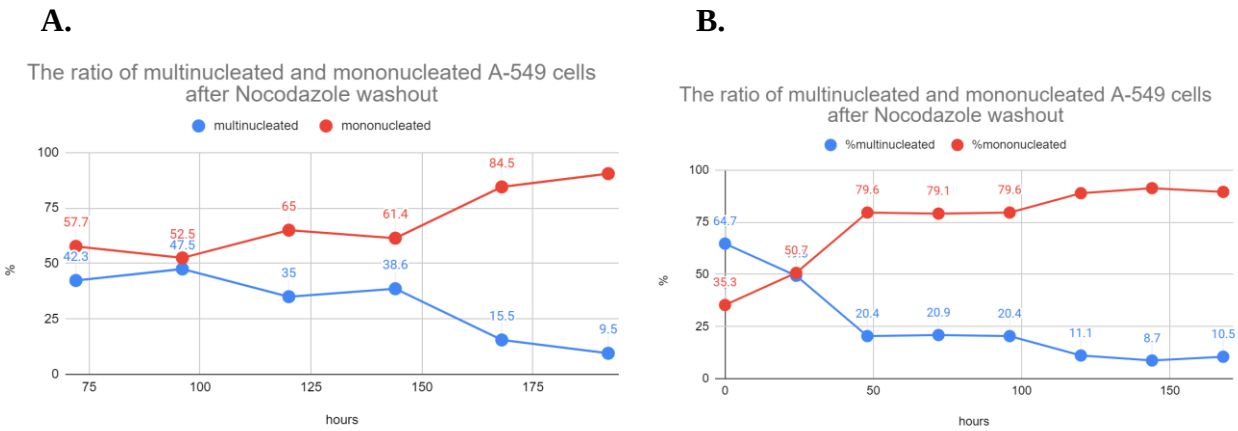


**Figure 12.** The ratio of multinucleated and mononucleated cells after NOC washout. (A-life observation, B-time-lapse experiment).

**A.** **B.**

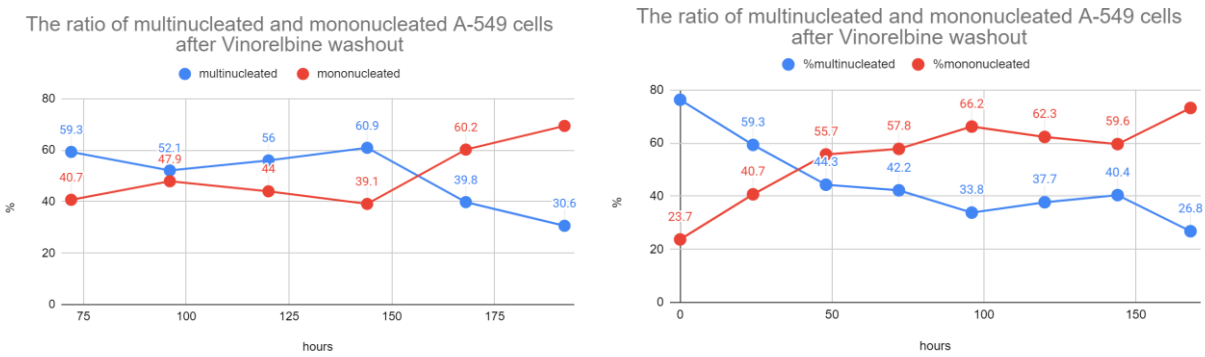


**Figure 13.** The ratio of multinucleated and mononucleated cells after VIN washout. (A-life observation, B-time-lapse experiment).

**A.** **B.**

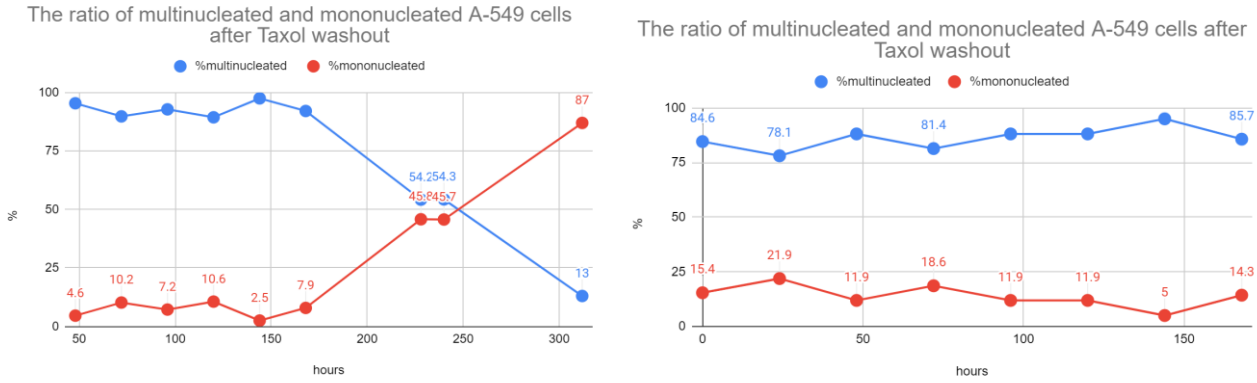


**Figure 14.** The ratio of multinucleated and mononucleated cells after TAX washout. (A-life observation, B-time-lapse experiment).

### 4.3 Potential for Colony Formation

As evident from Figure 15, the greatest potential for colony formation was observed after NOC washout as it was supported by the evidence from time-lapse and life observation experiments. The second proliferative potential refers to the VIN washout, whereas after 12 days of observations, no colonies were observed after the TAX washout, suggesting that it has a later proliferative window and more time for cell recovery.

The colonies' appearance was characterized by numerous small mononucleated cells and fewer multinucleated cells that still persisted in the colony after 12 days of observation. The summary of observations of life observation experiments is shown in Table 2. After TAX washout, 16 days were required to detect the presence of emerging mononucleated cells, as opposed to VIN and NOC washout, where mononucleated cells were observed at Day 13.

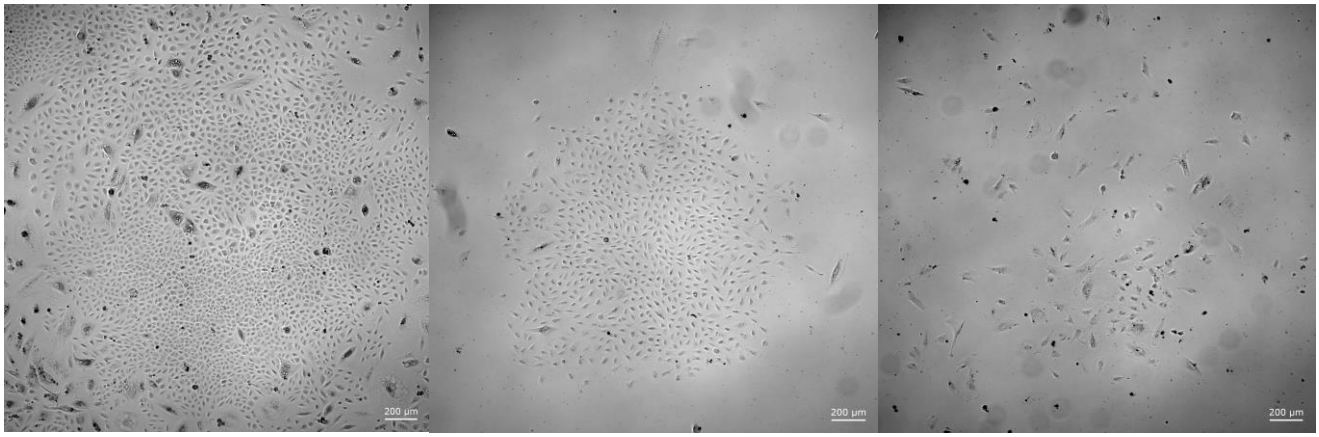


**Figure 15.** Snapshots (10x) of A549 cells 12 days post-Nocodazole, Vinorelbine, and Taxol washout

**Table 2.** The Summary of life observations experiments

| **Events** | **TAX** | **Vinorelbine** | **Nocodazole** |
|---|---|---|---|
| | **A549** | **A549** | **A549** |
| Cells appearance | mainly multinucleated | mainly multinucleated | mainly multinucleated |
| Time when the first mononucleated colonies appeared | Day 16 | Day 13 | Day 13 |
| Cells appearance | large multinucleated and mononucleated | large multinucleated and mononucleated | large multinucleated and mononucleated |
| Numerous colonies | Day 18 | Day 16 | Day 16 |
| Cells appearance | mainly mononucleated | mainly mononucleated | mainly mononucleated |

### 4.4 Flow Cytometry Analysis of Cell Populations

Concerning the Flow Cytometry experiment, it was concluded that after the washout of all drugs, the initial point (Day 0) had a >4n peak, suggesting the presence of aneuploid multinucleated cells. On Day 9, cells after the NOC and VIN washout showed a normal distribution, which could be linked to the emergence of mononucleated cells. Ambiguous data was recorded after the TAX washout, which could be linked to the extensive death of cells that resulted in an insufficiency of cells for further analysis. The ploidy experiment with TAX washout has to be reproduced with a higher concentration of cells and a more prolonged period of observations.

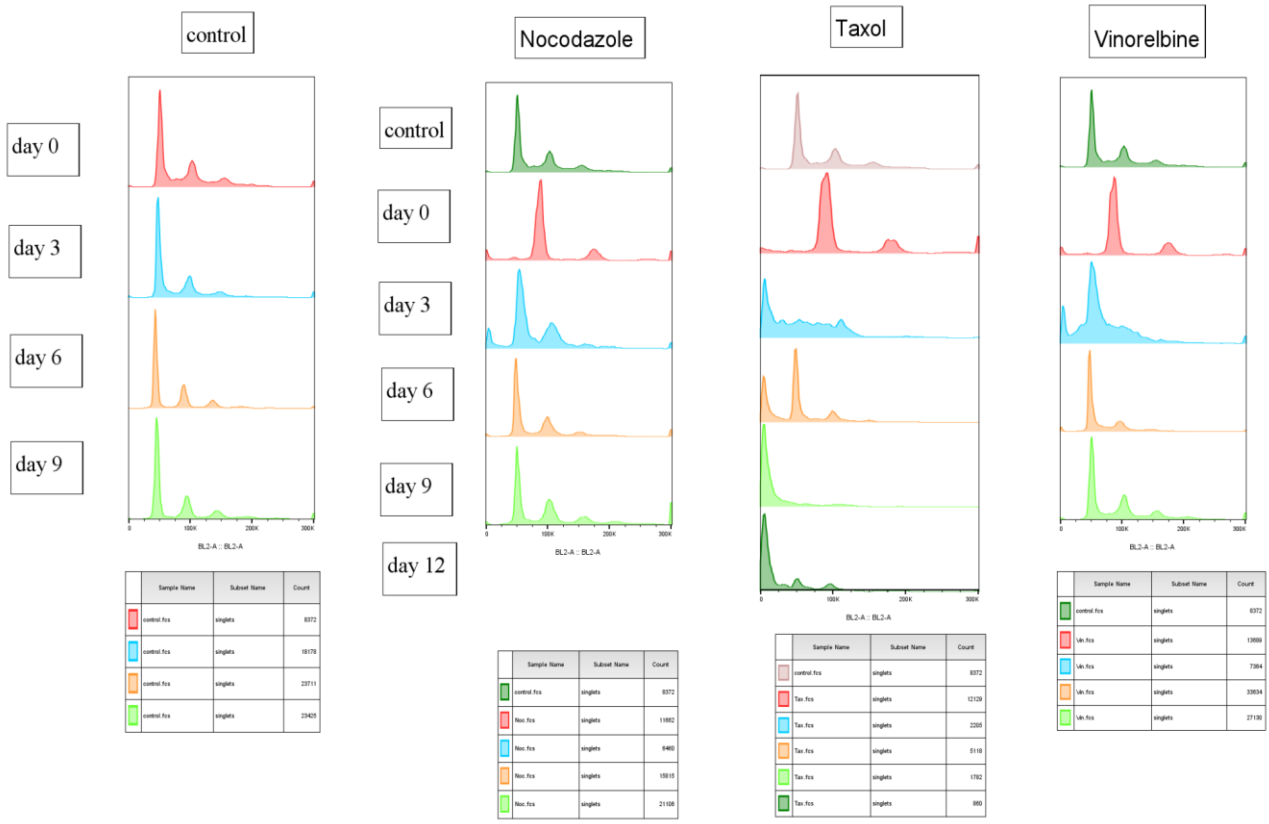


**Figure 16.** Flow Cytometry Analysis of cell populations after NOC, TAX, and VIN washout.

## DISCUSSION

Few papers discuss clonogenicity of cancer cells after mitotic arrest and mitotic slippage as scientific phenomenon in detail. The only paper that touches upon the topic of clonogenicity after administration of antimicrotubule drugs is Mittal et al. (2017) that reveals the emergence of small mononucleated cells from giant aneuploid multinucleated cells formed as a result of a prolonged mitotic arrest. Therefore, we aimed to follow up cell fates of multinucleated cells directly. We wanted to complement Mittal et al. (2017) findings by testing different antimicrotubule drugs on the basis of their effects on proliferation of multinucleated cells. Also we aimed to compare their findings with our obtained results to study the reproducibility of their research outputs.

Three out of four mentioned drugs (NOC, VIN and TAX) demonstrated different effects on cancer cells. It took the longest for cells after TAX to form the mononucleated colonies compared to NOC and VIN washout, suggesting that cells recovered faster after NOC and VIN washout. This could be explained by the difference in their modes of action: TAX is a microtubule-stabilizer, whereas NOC and VIN are microtubule-destabilizers. The flow cytometry experiment confirmed the emergence of the mononucleated cells, where the >4n peak that was evident at the initial point disappeared, and the ploidy curve returned to the normal distribution, similar to the control group.

This was evident by the Mittal et al. (2017) findings, where 35 days of observations were required to get almost 100 % of multinucleated cells. Moreover, Mittal et al. (2017) observed an increase in the percentage of mononucleated cells opposed to a decrease in the percentage of multinucleated cells.

Regarding the division of multinucleated cells, it was observed that they proliferate via mitosis. This was confirmed by time-lapse experiments by forming a mitotic bubble and dividing into two and rarely three daughter cells. However, these findings contradict Mittal et al. (2017) observations, which claim a new type of division of multinucleated cells via so-called "neosis," similar to budding. Therefore, the new phenomenon of neosis is less evident, and hence, the Mittal et al. (2017) findings require reconsideration of the results on division of multinucleated cells.

Different results were obtained for four drugs, showing various colony formation dynamics. The highest potential for colony formation was evident for NOC and VIN washout, whereas TAX had the lowest capacity for colony formation. The multinucleated cells were still persistent after the long observation period, meaning the experiment could even be prolonged for a month to analyze the cells' fates in more detail. Among the four drugs tested, TAX was more suitable model for clonogenicity studies since most cells after treatment and washout are multinucleated.

These findings are crucial, since they demonstrate that TAX might have a higher efficacy against proliferation of cancer cells. Moreover, the emergence of mononucleated cells could be one of the contributing factors to cancer relapse and a new direction for chemotherapeutic therapy targets. Currently, EpoB is being tested on clonogenicity, and its preliminary observations show that it has similar effect on cancer cells as TAX, which could be explained by its microtubule-destabilizing effect.

As a future direction, the study could be expanded to different cancer cell lines and drugs to test the cell- and drug-dependency in terms of clonogenicity. As suggested by the research findings, TAX and EpoB experiments could be prolonged to observe the fates of progeny cells in detail. Experiments could be carried on animal models to investigate the tumorigenicity of multinucleated and progeny cells. Also, a combinative drug treatment might be a subject for clonogenicity studies. Moreover, a more detailed study on the progeny of multinucleated cells can be conducted in the long-term.

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## APPENDICES

**Table 3.** The mean and standard deviation of cell distribution after TAX washout

| time | multinucleated | | | mononucleated large | | | mononucleated small | | |
|---|---|---|---|---|---|---|---|---|---|
| X | Mean | SD | N | Mean | SD | N | Mean | SD | N |
| 48.000 | 100.000 | 0.000 | 15 | 0.000 | 0.000 | 15 | 0.000 | 0.000 | 15 |
| 72.000 | 100.000 | 0.000 | 15 | 0.000 | 0.000 | 15 | 0.000 | 0.000 | 15 |
| 96.000 | 97.059 | 12.127 | 17 | 2.941 | 12.127 | 17 | 0.000 | 0.000 | 17 |
| 120.000 | 92.500 | 24.468 | 20 | 7.500 | 24.468 | 20 | 0.000 | 0.000 | 20 |
| 192.000 | 64.706 | 45.978 | 17 | 35.294 | 45.978 | 17 | 0.000 | 0.000 | 17 |
| 240.000 | 84.375 | 35.208 | 16 | 15.625 | 35.208 | 16 | 0.000 | 0.000 | 16 |
| 288.000 | 87.179 | 32.026 | 13 | 12.821 | 32.026 | 13 | 0.000 | 0.000 | 13 |
| 336.000 | 90.000 | 31.623 | 10 | 10.000 | 31.623 | 10 | 0.000 | 0.000 | 10 |
| 384.000 | 45.000 | 51.235 | 5 | 55.000 | 51.235 | 5 | 0.000 | 0.000 | 5 |

**Table 4.** The mean and standard deviation of cell distribution after NOC washout

| time | multinucleated | | | mononucleated large | | | mononucleated small | | |
|---|---|---|---|---|---|---|---|---|---|
| X | Mean | SD | N | Mean | SD | N | Mean | SD | N |
| 48.000 | 100.000 | 0.000 | 13 | 0.000 | 0.000 | 13 | 0.000 | 0.000 | 13 |
| 72.000 | 90.000 | 28.031 | 15 | 10.000 | 28.031 | 15 | 0.000 | 0.000 | 15 |
| 96.000 | 75.305 | 24.484 | 16 | 24.695 | 24.484 | 16 | 0.000 | 0.000 | 16 |
| 120.000 | 82.045 | 28.851 | 17 | 17.955 | 28.851 | 17 | 0.000 | 0.000 | 17 |
| 192.000 | 59.688 | 31.164 | 16 | 40.313 | 31.164 | 16 | 0.000 | 0.000 | 16 |
| 240.000 | 63.333 | 32.773 | 16 | 36.667 | 32.773 | 16 | 0.000 | 0.000 | 16 |
| 288.000 | 50.056 | 29.474 | 15 | 44.222 | 26.836 | 15 | 5.722 | 13.028 | 15 |
| 336.000 | 67.447 | 30.081 | 20 | 30.720 | 29.351 | 20 | 1.833 | 5.669 | 20 |
| 384.000 | 30.548 | 30.443 | 15 | 55.273 | 35.879 | 15 | 14.179 | 24.860 | 15 |

**Table 5.** The mean and standard deviation of cell distribution after VIN washout

| time | multinucleated | | | mononucleated large | | | mononucleated small | | |
|---|---|---|---|---|---|---|---|---|---|
| X | Mean | SD | N | Mean | SD | N | Mean | SD | N |
| 48.000 | 100.000 | 0.000 | 15 | 0.000 | 0.000 | 15 | 0.000 | 0.000 | 15 |
| 72.000 | 100.000 | 0.000 | 15 | 0.000 | 0.000 | 15 | 0.000 | 0.000 | 15 |
| 96.000 | 100.000 | 0.000 | 14 | 0.000 | 0.000 | 14 | 0.000 | 0.000 | 14 |
| 120.000 | 79.474 | 28.071 | 19 | 20.526 | 28.071 | 19 | 0.000 | 0.000 | 19 |
| 192.000 | 68.431 | 39.076 | 17 | 31.569 | 39.076 | 17 | 0.000 | 0.000 | 17 |
| 240.000 | 49.074 | 45.901 | 18 | 50.926 | 45.901 | 18 | 0.000 | 0.000 | 18 |
| 288.000 | 65.032 | 33.212 | 15 | 34.968 | 33.212 | 15 | 0.000 | 0.000 | 15 |
| 336.000 | 78.690 | 21.849 | 16 | 21.310 | 21.849 | 16 | 0.000 | 0.000 | 16 |
| 384.000 | 55.623 | 34.348 | 13 | 38.516 | 34.252 | 13 | 5.861 | 14.438 | 13 |

**Table 6.** The % of multinucleated and mononucleated cells after TAX washout

| hours after TAX treatment | % of multinucleated cells | % of mononucleated cells |
|---|---|---|
| 48 | 95.4 | 4.6 |
| 72 | 89.8 | 10.2 |
| 96 | 92.8 | 7.2 |
| 120 | 89.4 | 10.6 |
| 144 | 97.5 | 2.5 |
| 168 | 92.1 | 7.9 |
| 228 | 54.2 | 45.8 |
| 240 | 54.3 | 45.7 |
| 312 | 13 | 87 |
| 48 | 95.4 | 4.6 |

**Table 7.** The % of multinucleated and mononucleated cells after NOC washout

| hours after NOC treatment | % of multinucleated cells | % of mononucleated cells |
|---|---|---|
| 72 | 42.3 | 57.7 |
| 96 | 47.5 | 52.5 |
| 120 | 35 | 65 |
| 144 | 38.6 | 61.4 |
| 168 | 15.5 | 84.5 |
| 192 | 9.5 | 90.5 |

**Table 8.** The % of multinucleated and mononucleated cells after VIN washout

| hours after Vinorelbine treatment | % of multinucleated cells | % of mononucleated cells |
|---|---|---|
| 72 | 59.3 | 40.7 |
| 96 | 52.1 | 47.9 |
| 120 | 56 | 44 |
| 144 | 60.9 | 39.1 |
| 168 | 39.8 | 60.2 |
| 192 | 30.6 | 69.4 |