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CHARACTERIZATION OF CANCER CELL BEHAVIORS AFTER WASHOUT FROM DIFFERENT MICROTUBULE-TARGETING AGENTS

Arlan Yelzhanov
(B.Sc. in Biological Sciences, Nazarbayev University)

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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.

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Arlan Yelzhanov

2 April, 2024

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

Bachelor´s Degree Program in Biology
Bachelor´s Degree Program in Biology

Arlan Yelzhanov: Characterization of Distinct Cancer Cell Behaviors after Washout of Microtubule-Targeting Agents and Their Clonogenic Properties

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SUMMARY

Paclitaxel (Taxol), Epothilone B, Nocodazole, and the Vinorelbine are the four microtubule-targeting agents widely used in clinics and research 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 or abnormal division 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. Also, there is a scientific gap associated of how the drug clearance, a physiological washout of drug from the body, changes the cell behavior and affects clonogenic properties of cancer cells.

This thesis aimed to investigate the differences in the behavior of cancer cells as a result of mitotic arrest between the washout and non-washout cell groups. Also, the difference in the behavior seems to be related to the type of drug applied. Finally, the clonogenic potential of the cells after drug washout is

studied. Overall, there is a lack of data on the effect drug clearance has on cancer cell behavior and clonogenic capacities. Thus, the frequency of cell division, the time of first colony proliferation, and the ploidy of cancer cells treated with microtubule-targeting agents were studied in-depth. The methods included time-lapse microscopy observations, long-term microscopy observations and flow cytometry. After the drug washout the abnormal division event becomes predominant while non-washout cell groups experience more mitotic slippage. The four microtubule-targeting drugs (Nocodazole, Vinorelbine, Paclitaxel, and Epothilone B) showed different cell behavior after drug washout, various clonogenicity results due to their various modes of action on microtubules. While Nocodazole and Vinorelbine was not efficient to block normal mitosis, the Paclitaxel and Epothilone B drugs were capable to a more extent to shift cells to the abnormal division and mitotic slippage. As a result, the latter two drugs were efficient to shift cancer cell population to the multinucleated state which is featured with low clonogenic properties and rise of mononuclear colonies after relatively long period of time. The obtained results are beneficial in understanding the cell fate of cancer cells after drug washout and obtaining new insights about cancer relapse and treatment strategies.

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ABBREVIATIONS

MTOC	microtubule-organizing center
SAC	Spindle Assembly Checkpoint
PTX	Paclitaxel
NOC	Nocodazole
VIN	Vinorelbine
EPOB	Epothilone B

1 INTRODUCTION

1.1 Microtubules

Microtubules are essential structures in eukaryotic cells, involved in many biological processes such as cell motility, intracellular transport, and cell division. Microtubules originate from the microtubule-organizing center (MTOC), such as centrosome that is usually located near the nucleus. As integral components of the cell cytoskeleton, microtubules form a network critical for the intracellular transport of various molecules involved in many cellular functions.

These structures are protein polymers composed of α- and β-tubulin heterodimers. Both monomers forming the heterodimers have GTP-binding sites; however, only β-tubulin is capable of exchanging GTP for GDP, which is essential for microtubule dynamics.

In the context of this work, there is a significant emphasis on the role of microtubules in cell division. During this process, microtubules assemble into the mitotic spindle, a structure crucial for segregation of chromosomes to opposite cell poles, ultimately leading to the formation of daughter cells. This assembly requires precise features of polarity from microtubules, which have distinct ends known as the minus and plus ends. The minus end, anchored to the MTOC, is more stable, thus inhibiting its polymerization and depolymerization. Conversely, the plus end, typically located toward the cell periphery, undergoes frequent phases of polymerization and depolymerization known as dynamic instability. This end often interacts with other cellular structures such as the kinetochore during cell division (Alberts et al., 2014).

Proper chromosome segregation during cell division is a crucial event for cell cycle progression. To ensure the accuracy of this process, a regulatory mechanism known as the Spindle Assembly Checkpoint (SAC) monitors and controls the cell cycle. Activation of the SAC can induce mitotic arrest, leading to several possible cell fates, which will be explored in this work.

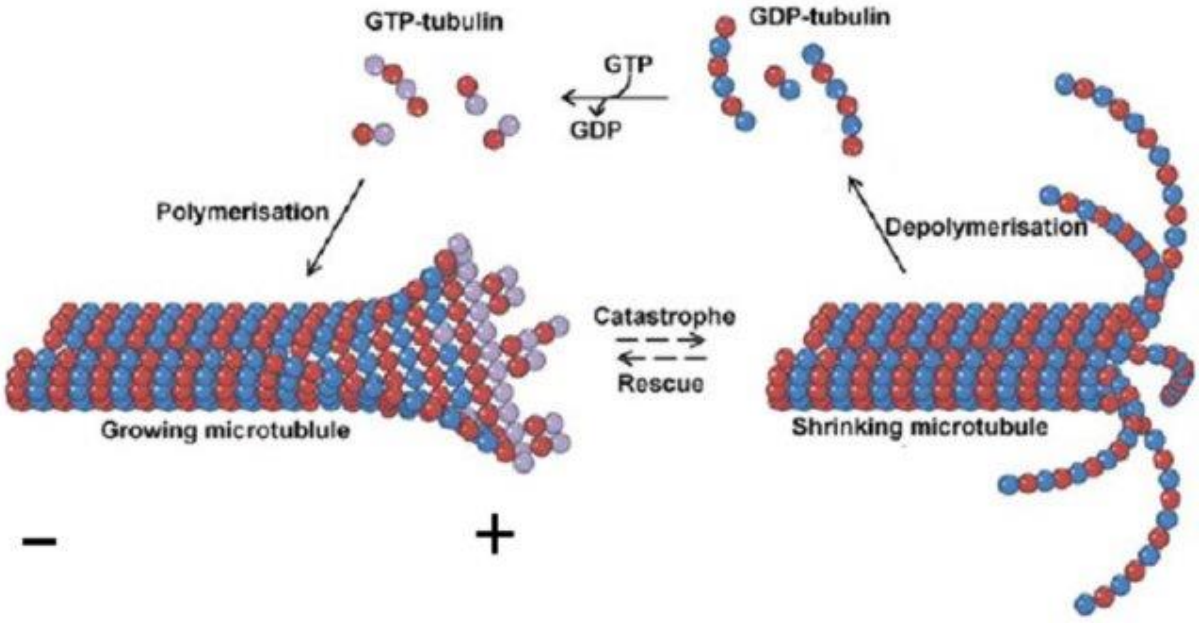


Figure 1. The assembly and disassembly of microtubule structures (Shahidi, 2013)

1.2 The importance of microtubule-targeting agents

Each year, around 19.3 million cancer cases are reported worldwide, and cancer is accounted as the second leading cause of death globally (Ferlay et al., 2020). This rises the necessity of finding effective cancer treatment methods, one of the most established methods is a chemotherapeutic agent that mainly targets cancer cells inducing their death or inhibition of proliferation. The microtubule polymerization and depolymerization are essential actions required to the proper progression during mitosis. This phenomenon is called microtubule dynamics. As microtubules polymerize to connect to chromosome kinetochores, for the correct chromosome segregation to daughter cells, these microtubules are required to depolymerize (Figure 3).

Microtubule-targeting agents have negative effects on the cancer cells by interfering the microtubule dynamics involved in the cell division. It is important to mention that this interfering effect can happen in non-cancerous cells like cells of nervous system inducing neurotoxic mechanisms (Gornstein & Schwarz, 2017). Therefore, a treatment with microtubule-targeting agents needs comprehensive and wise methodology.

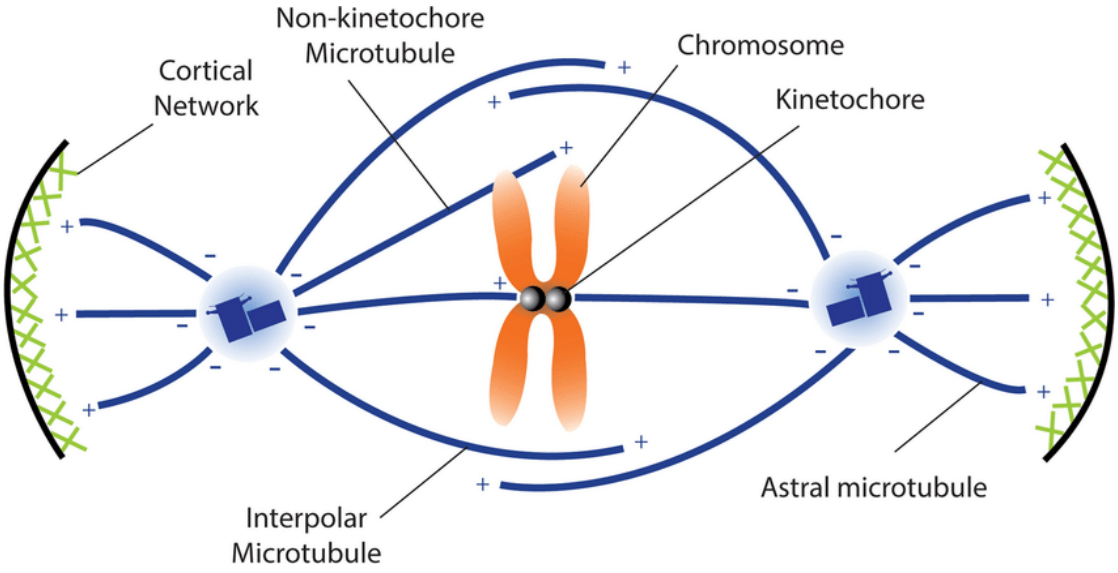


Figure 3. The mitotic spindle composed of microtubules during cell division (Ryniawec & Rogers, 2021)

1.3 Microtubule-targeting agents

The microtubule-targeting agents can be divided into two groups: microtubule-stabilizers and microtubule-destabilizers. The first group includes Paclitaxel (PTX) and Epothilone B (EPOB) and they stabilize the microtubule structure preventing its depolymerization. In contrast, Nocodazole and Vinorelbine are microtubule-destabilizers and therefore prevent microtubules polymerization (Beswick et al., 2006; Verdier-Pinard et al., 1999). Both depolymerization and polymerization are essential processes in the cell division and their inhibition results in mitotic arrest. In particular, the mitotic arrest resulted from these drugs action inhibits mitosis at the metaphase-anaphase transition (Suleimenov et al., 2022).

Nocodazole

Nocodazole acts as a microtubule-targeting agent that disrupts microtubules and prevents their polymerization, leading to mitotic arrest. It binds to the β-tubulin monomers at the same site as Colchicine, yet the two compounds differ in their chemical structure. While it is not currently approved for clinical use, Nocodazole is widely used in research settings for experimental purposes (National Center for Biotechnology Information, 2023).

Vinorelbine

Vinorelbine acts as a microtubule destabilizer by binding to tubulin subunits, which inhibits the assembly of the mitotic spindle and consequently causes cells to arrest in metaphase. It targets the Vinca-binding site on β-tubulin, near the GTP-binding site. Currently, Vinorelbine is used in clinical settings to treat various cancers, including breast cancer and non-small cell lung cancer (NSCLC) (National Center for Biotechnology Information, 2023).

Paclitaxel

Paclitaxel serves as a mitotic inhibitor, interacting with Taxane-binding sites on microtubules. It works by binding to and stabilizing microtubules, thereby blocking their depolymerization and halting cell division. Presently, Paclitaxel is utilized in the treatment of ovarian, breast, and lung cancers, according to the National Center for Biotechnology Information (2023)

Epothilone B

Epothilone B is known for inducing apoptosis by stabilizing microtubules, much like Paclitaxel, which it resembles in preventing microtubule depolymerization. What sets it apart from Taxane-based treatments is its cytotoxicity in cells that overexpress P-glycoprotein. Currently in clinical trials, Epothilone B has demonstrated greater effectiveness and fewer side effects compared to Paclitaxel, according to the National Center for Biotechnology Information (2023).

1.4 Drug Clearance

The drug clearance process, also known as washout, is a crucial factor in the results that are seen after cells are incubated with chemotherapeutic drugs. The process of drug washout accounts as a physiological phenomenon that does not allow cells to be under the constant treatment of chemotherapeutic medication (Horde and Gupta, 2023). When medications are administered to cells, especially ones that target cellular structures like microtubules, the effects that are seen are a function of both the drug's immediate activity and the efficiency with which the drug is eliminated from the body. For example, the length of exposure to microtubule-targeting medicines like Vinorelbine,

Epothilone B, Nocodazole, and Taxol can have a substantial effect on cell survival, division, and potential drug resistance mechanisms. Reduced drug exposure duration from effective drug clearance can lessen toxicity and adverse effects. In contrast, a prolonged duration of medication effect may arise from a lack or partial washout.

1.5 Mitotic Slippage

Normal mitotic cells divide into two diploid daughter cells when they exit mitosis, but microtubule-targeting agents stop cells in mitosis, leading to several cell fates. One such fate is mitotic slippage, escape into the interphase, and creation of polyploid multinucleated cells (Figure 2). Mitotic slippage is the main event as a result of microtubule-targeting agents, which is an exit event from mitosis without appropriate chromosomal segregation (Lok et al., 2020). Cheng and Crasta (2017) claim that microtubule-targeting agents prevent the commencement of anaphase by degrading normal mitotic spindles. Treatment with microtubule-targeting agents causes cells to remain in mitotic arrest for a longer period of time because anaphase cannot be proceeded.

Most cells undergo apoptosis and perish, however some cells enter interphase without properly segregating their chromosomes. Microtubule-targeting agents prevent the restoration of a normal mitotic spindle, resulting in polyploid multinucleated cells. For a long time, it was believed that cells that experienced mitotic slippage would only live for a little while before dying. Nonetheless, certain research findings indicate that polyploid multinucleated cells resulting from mitotic slippage may have the ability to endure and maybe generate viable mononucleated aneuploid offspring.

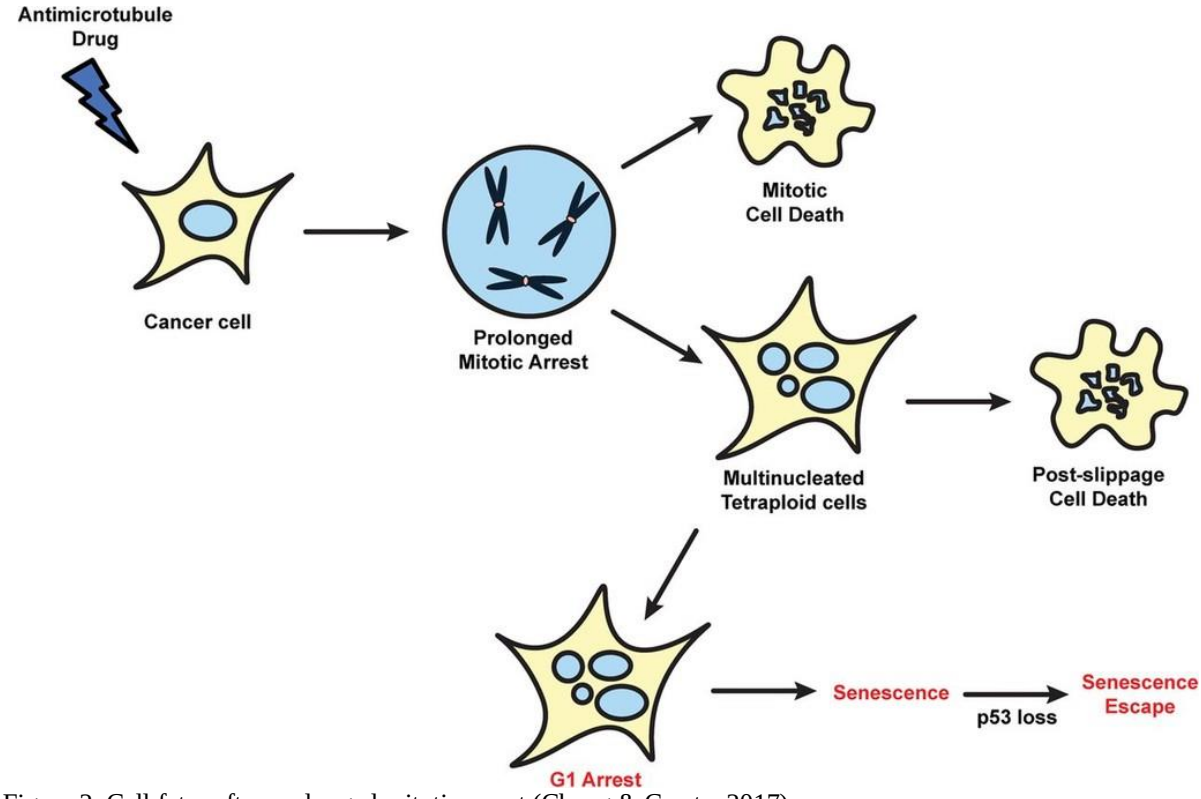


Figure 2. Cell fates after prolonged mitotic arrest (Cheng & Crasta, 2017).

1.6 Abnormal division

Microtubule-targeting agents, such as Paclitaxel, Vinorelbine, and Nocodazole, Epothilone B have profound impacts on cell division, one of which includes inducing multipolar spindle formation, leading to abnormal mitotic division (Scribano et al, 2021). This specific type of abnormal division can significantly affect the fidelity of chromosome segregation during mitosis and has notable implications for cancer progression and treatment.

During mitosis, chromosomes should be evenly distributed between two daughter cells. However, a multipolar spindle can lead to uneven distribution and missegregation of chromosomes (Figure 3). This results in daughter cells with unequal and abnormal numbers of chromosomes (aneuploidy), significantly increasing genomic instability (Bakhoum and Cantley, 2018).

The genomic instability resulting from abnormal chromosome segregation can lead to tumorigenesis and the progression of existing cancers. Cells with abnormal karyotypes may acquire growth advantages, resistance to apoptosis, and other cancer-promoting traits.

1.7 Colony formation

In the practice of chemotherapy, there is a phenomenon of a cancer relapse when resistant cancer cells give rise of the resistant cancer cell population. Initially heterogeneous cancer population can generate resistant sub-clone under the pressure of chemotherapeutic treatment (Aktipis et al, 2011). These sub-clones become highly proliferative generating similar progeny. The microtubule-targeting agents mainly inhibit cell proliferation and even though the cancer relapse through colony formation can be observed.

Cancer cell colonies are defined as a dense population of cancer cells that consist of at least 50 cells (Franken et al., 2006). These colonies probably originated from one cancer cell. The colony formation of cancer cells is key component of cancer biology, reflecting the ability of cancer cells to survive, multiply, and form colonies. This process is known as colony formation. This process is an important marker of a cell's clonogenic potential, or its capacity to proliferate and survive in order to create new colonies. Clonogenic potential is important for determining the aggressiveness of tumors, the potential for metastasis, and the general malignancy of cancer cells. The colony formation phenomenon assesses the efficacy of anticancer drugs. The formation of colonies after the treatment shows to what extent the effect of the drug is maintained in the population. The higher clonogenic potential of cancer cells corelates with more aggressive types of cancer cells. On the example of Paclitaxel research, it can be seen that the colony formation is highly inhibited in several cancer cell lines after the exposure to this microtubule-targeting agent (Zasadil et al., 2014).

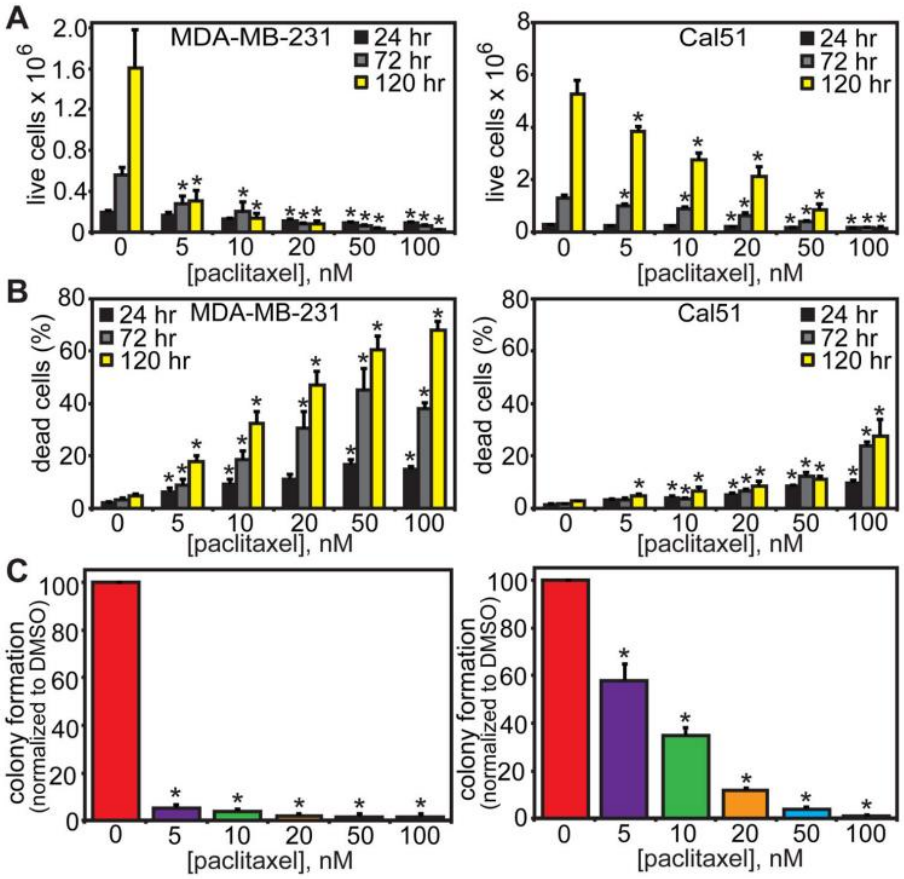


Figure 4. Clinically relevant concentrations of paclitaxel cause cell death. (Zasadil et al., 2014)

2 MATERIAL AND METHODS

2.1 Reagents and buffers

1) For cell culturing and general use

Name	Composition or use	Source
Dulbecco's Modified Eagle Medium (DMEM)	General media used for cell growth. Composition also involves:	Thermo Fisher Scientific
	Fetal bovine serum 10%	Thermo Fisher Scientific
	L-glutamine 2mM	Sigma Life Science
	1% penicillin-streptomycin-amphotericin B	Sigma Life Science
		Sigma Life Science
		In house made

PBS 1x	150 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4	Sigma Life Science
Trypsin 1x	Cell detachment during cell passaging	Sigma Life Science
Paclitaxel	Microtubule-stabilizing agent	Sigma-Aldrich
Epothilone B	Microtubule-stabilizing agent	Fisher Scientific
Nocodazole	Microtubule-destabilizing agent	Fisher Scientific
Vinorelbine	Microtubule-destabilizing agent	BenchChem

2) For light microscopy experiments

Name	**Composition or use**	**Source**
CO_2 independent media	Medium needed for cell growth without CO_2 generator (light microscopy chamber lacks CO_2 generator). Composition also involves: Fetal bovine serum 10 L-glutamine 2mM 1% penicillin-streptomycin-amphotericin B	Thermo Fisher Scientific Sigma Life Science Sigma Life Science Sigma Life Science In house made
TMRM	A dye that stains cells with active mitochondria with intact membrane potentials. The cell viability dye.	Abcam
Hoechst 33342	Staining cells' nucleuses	Invitrogen
Immersion Oil	Increases the resolving power of microscope	Fisher Scientific

1) For flow cytometry experiments

Name	Composition or use	Source
Ethanol	A 70% ethanol solution needed for cell fixation	In house made
Propidium Iodide	A red-fluorescent nuclear and chromosome counterstain	Thermo Fisher Scientific
RNAse A	Removes RNA by digestion	Thermo Fisher Scientific

2.2 Cell culture

All cells growth in T-25 flask and are plated or passaged once they reach a cell confluency of 80%. Gibco Dulbecco's Modified Eagle Medium (DMEM) is modified with fetal bovine serum (FBS) (10% of total volume), L-glutamine, and 1% Penicillin/Streptomycin/Amphotericin B (Pen/Strep/Amp). Cells are stored in the incubator at 37 °C with CO_2 supply (5%). A549 were chosen for its high survivability in response to the applied drugs. This is an important feature to study colony formation properties of cancer cells.

2.3 Cancer cell treatment

After a day of passaging on either 6-well or 12-well lpates, A549 cells are treated with microtubule-targeting agents (NOC 1μM, VIN 1μM, PTX 100nM, 300nM, EPOB 10nM , 30Nm). The concentrations were based on the previous experiments of our laboratory workers that are considered enough to induce mitotic arrest (Suleimenov et al. (2022)). In our experiments we have used two time periods of drug incubation (2h and 16h), and after this time of incubation the washout procedure is performed. The

washout consists of the consequent change of media with PBS (two times by 1 ml) and DMEM media (one time by 1 ml).

2.4 Time-lapse observation under the light microscope

Some of our experiments required a time-lapse observation of cancer cells. For that purpose, the cell was placed under the ZEISS Axioscope 5 microscope light microscope observation (20x magnification) instantly after washout were performed. Due to the lack of CO_2 generator in the light microscope chamber cells' media is replaced with CO_2 independent media with addition of TMRM (50 nM). Primarily, the TMRM staining allows to distinguish viable cells from dead ones, but also help to determine exact number of nuclei for each cell. The Hoechst staining is inefficient in this case, due to its DNA-interfering effect and cell cycle progression. Therefore, time-lapse observation operates for 48 hours with image taken every 15 minutes in both phase-contrast mode and Rhodamine B (to detect TMRM associated fluorescence). For each of the concentrations used seven different positions were taken for further analysis. The analysis involves manual calculation of events that happen with 100 cells in the ImageJ software.

2.5 The long-term observation under the light microscope

To explicitly characterize the colony formation abilities of cells after the treatment and washout from microtubule-targeting agents, the long-term observation is required. The colonies are characterized by relatively small size and high density of mononuclear cells. To do so, A549 cells are seeded on 6-well plates in the center, and image is taken every day or every third day (10x magnification). As a result, there is no need to keep cells in the light microscope chamber and consequently no need of CO_2 independent media. To keep cells viable every third day media is replaced in each well with addition of TMRM (50 nM). Minimum five positions were taken in order to gather information

about three hundred cells for statistical accuracy. The three biological repeats were obtained.

2.5 The Flow Cytometry

As alternative method to observe the dynamic changes in A549 population, the fixed cells after treatment and washout are analysed on flow cytometry. Cells are passaged on several 6-well plate, each well dedicated to a particular time point (a day of observation) in the long-term analysis. On the corresponding day, cells are centrifuged two times and fixed with ethanol. Prior the flow cytometry procedure, cells are stained with Propidium Iodide (PI) and its RNA content is digested by RNAse. Furthermore, samples were analysed on flow cytometry based on the distribution of cells in accordance with the proportion of cells with certain sets of DNA (G1, S, G2/M).

3 AIMS OF THE THESIS PROJECT

I. To investigate the main events that occurs with the cells' mitotic progression after washout from antimitotic drugs.

II. To compare the difference in the cell division of cells incubated in drugs and cells experiencing washout.

III. To compare the effects of the Nocodazole, Taxol, Epothilone B and Vinorelbine treatment and washout through estimating the time when mononucleated colonies start to accumulate.

4 RESULTS

4.1 Time-lapse observation demonstrates other events than mitotic slippage in cells experiencing drug washout.

The mitotic arrest induced by both cells incubated with the drug for 48 hours and those after a 16-hour drug treatment and washout resulted in abnormal cell behaviors due to the effects of microtubule-targeting agents. As depicted in Figure 5, outcomes for mononucleated cells include: 1) normal division; 2) abnormal division (including tripolar divisions); and 3) mitotic slippage. Two latter outcomes are the main contributors to the formation of multinucleated cells.

While all these outcomes are observed in both the drug-incubated and washout cells, their proportions differ significantly (Figures 6 and 7). For instance, in cells continually exposed to 100 nM Paclitaxel, 100% exhibited mitotic slippage post-arrest (Figure 6A). Conversely, in the washout group at the same concentration, 95% of cells divided abnormally, with the remainder undergoing mitotic slippage (Figure 7A).

As the concentration of the administered drug increases, so does the proportion of abnormal cell behaviors post-mitotic arrest. For cells incubated with the lowest concentration of EPOB (0.3 nM), 100% resulted in normal cell division (Figure 6B). However, at higher concentrations (1 nM and 3 nM), abnormal division rates increased to 78% and 83%, respectively, and at EPOB 10 nM, all cells progressed to mitotic slippage. Similarly, all tested concentrations of PTX led to more than 80% of cells experiencing mitotic slippage (Figure 6B). In contrast, cells undergoing drug washout displayed distinct behaviors. At the minimal concentration of EPOB (0.3 nM), 84% of cells divided normally, but the next two higher concentrations resulted in 50% abnormal divisions (Figure 7B). For PTX washout, more than 60% of the cells predominantly exhibited abnormal divisions. Notably, the increase in concentrations of both EPOB and PTX correlated with a higher frequency of mitotic slippage events. It is also noteworthy that 30% and 34% of cells washed out from EPOB 1 nM and 3 nM , respectively, died during mitosis.

Another significant difference is the duration of mitotic arrest between drug-incubated cells and those undergoing washout. As illustrated in Figure 8, the average duration of mitotic arrest, before mitotic slippage, for cells treated with 100 nM PTX and 10 nM EPOB was 16 ± 4.6 and 8 ± 5 hours, respectively. However, for the washout

group, these figures were reduced to 4 ± 2.28 and 2.5 ± 0.75 hours, aligning the washout group more closely with control cells in terms of mitotic arrest duration.

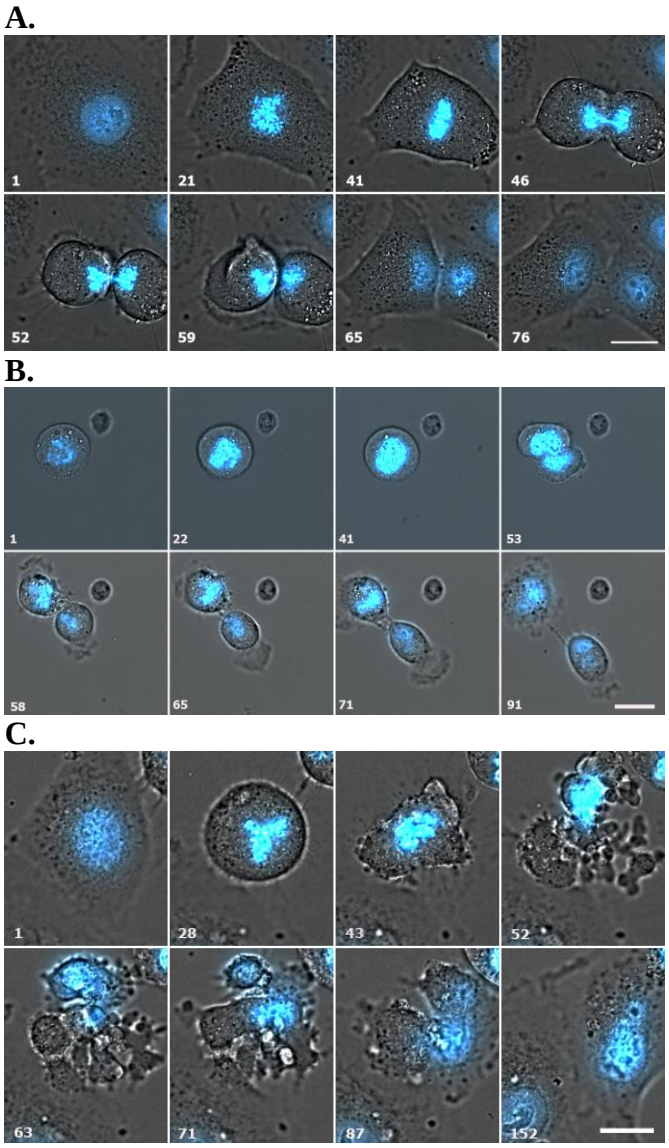


Figure 5. Time-lapse sequences illustrating major fates exhibited by A549 cells entering mitosis after sixteen hours treatment with Paclitaxel 300 nM. Number indicate time passed since the beginning of time-lapse observation in minutes. The presence and number of nuclei are visualized after staining with 180 nM Hoechst 33342(blue). The cell fates fall into the following categories: (A) normal division; (B) abnormal division (can be multipolar); (C) mitotic slippage. The images were acquired using a Plan-Neofluor 40x oil immersion objective with NA = 1.30. Scale bar = 20 μm.

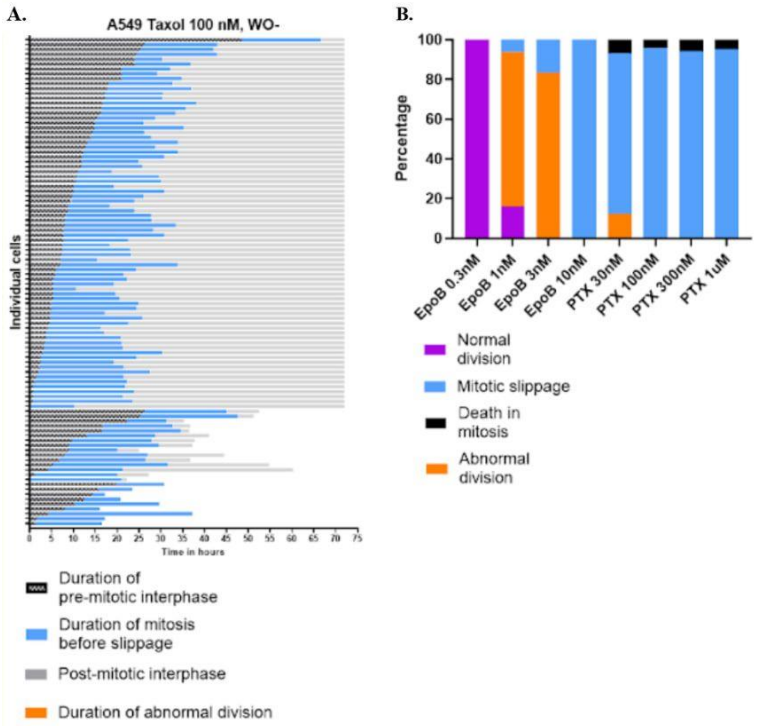


Figure 6. The calculation of events after mitotic arrest for cells incubated in the drugs for 48 hours. (A) Individual A549 cell life history in the presence of the drug (WO-) 100 nM Paclitaxel. (B) Proportion of mitotic cells from WO- group that i) divide normally, ii) experience mitotic slippage, iii) die during mitosis, iv) experience abnormal division. (N = 100 cells)

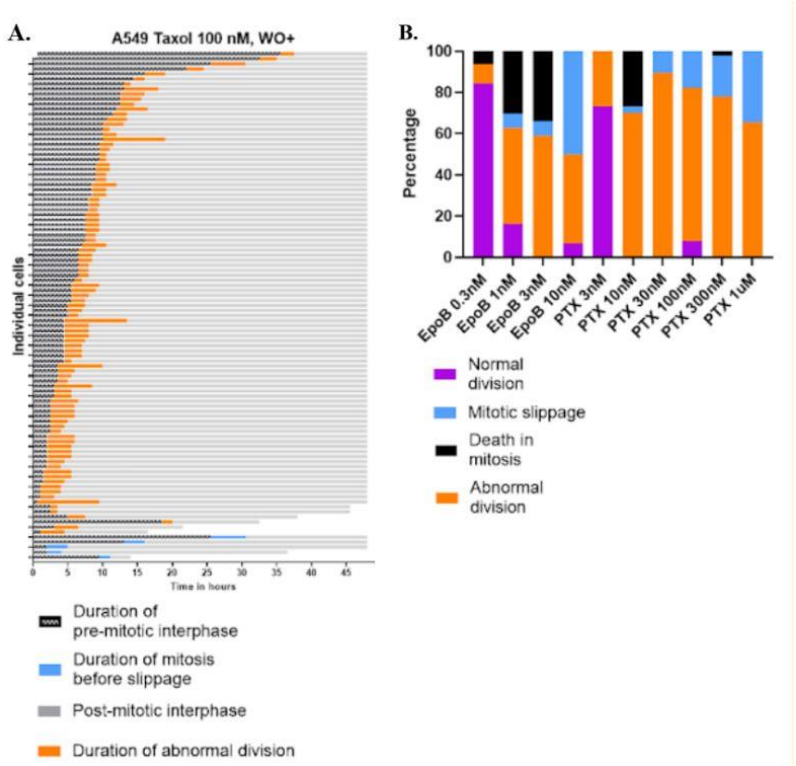


Figure 7. The calculation of events after mitotic arrest for cells treated 16 hours with subsequent washout (WO+). (A) Individual A549 cell life history after the Paclitaxel 100 Nm drug washout (WO-). (B) Proportion of mitotic cells from WO+ group that i) divide normally, ii) experience mitotic slippage, iii) die during mitosis, iv) experience abnormal division. (N = 100 cells)

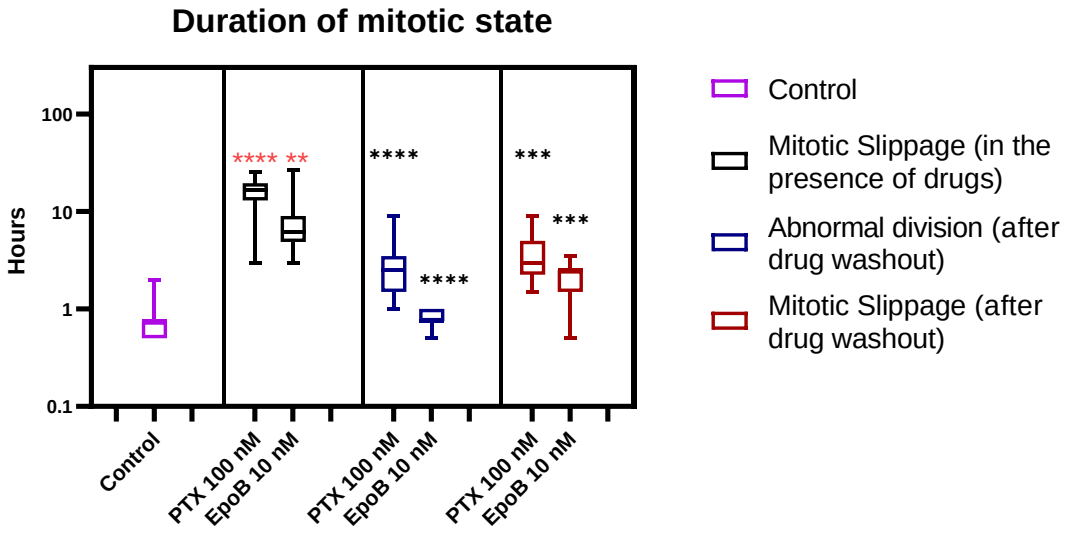


Figure 8. Durations of mitotic state (in hours) of A549 cell line in control, without washout, and after the washout of the 100 nM Paclitaxel (PTX) or 10nM Epothilone B (EPOB). Red stars represent statistical significance from control, black stars represent statistical significance. between WO- and WO+ groups.

4.2 Microtubule-stabilizing agents' treatment lowers tendency for cell to undergo normal cell division.

The life histories of individual cells reveal that cell behavior varies with the type and concentration of the drug applied (Figure 9). From Figure 9, we observe that a two-hour treatment with NOC followed by washout does not significantly impact the normal division process, mimicking the behavior of the control group with 100% progression to normal division. In contrast, cells treated with VIN not only undergo normal division but also experience a considerably longer mitotic state, averaging 8.4 hours—substantially longer than that of the control and NOC-treated cells. Normal division occurs in 32% of VIN-treated cells, accompanied by 41% undergoing abnormal division and 26% experiencing mitotic slippage, indicating that VIN is a more severe disruptor of normal cell behavior compared to NOC.

The microtubule-stabilizing agents, PTX and EPOB, to most extent was more effective at disrupting normal cell behavior. Concentrations of EPOB at 10 nM and 30 nM led to approximately 50% of cells undergoing mitotic slippage and 40% demonstrating abnormal division. At a PTX concentration of 300 nM, a significant majority (65% of cells) proceed with abnormal division, with an additional 10% of cells undergoing mitotic slippage. In contrast, a washout from a two-hour treatment with

PTX at 100 nM was insufficient to prevent normal cell division, with about 40% of mitotic arrests resulting in this outcome. The second major event was abnormal division with frequency of about 35%. Consequently, mitotic slippage was more frequent in cells treated with EPOB than with PTX, as the latter resulted in more frequent normal and abnormal divisions.

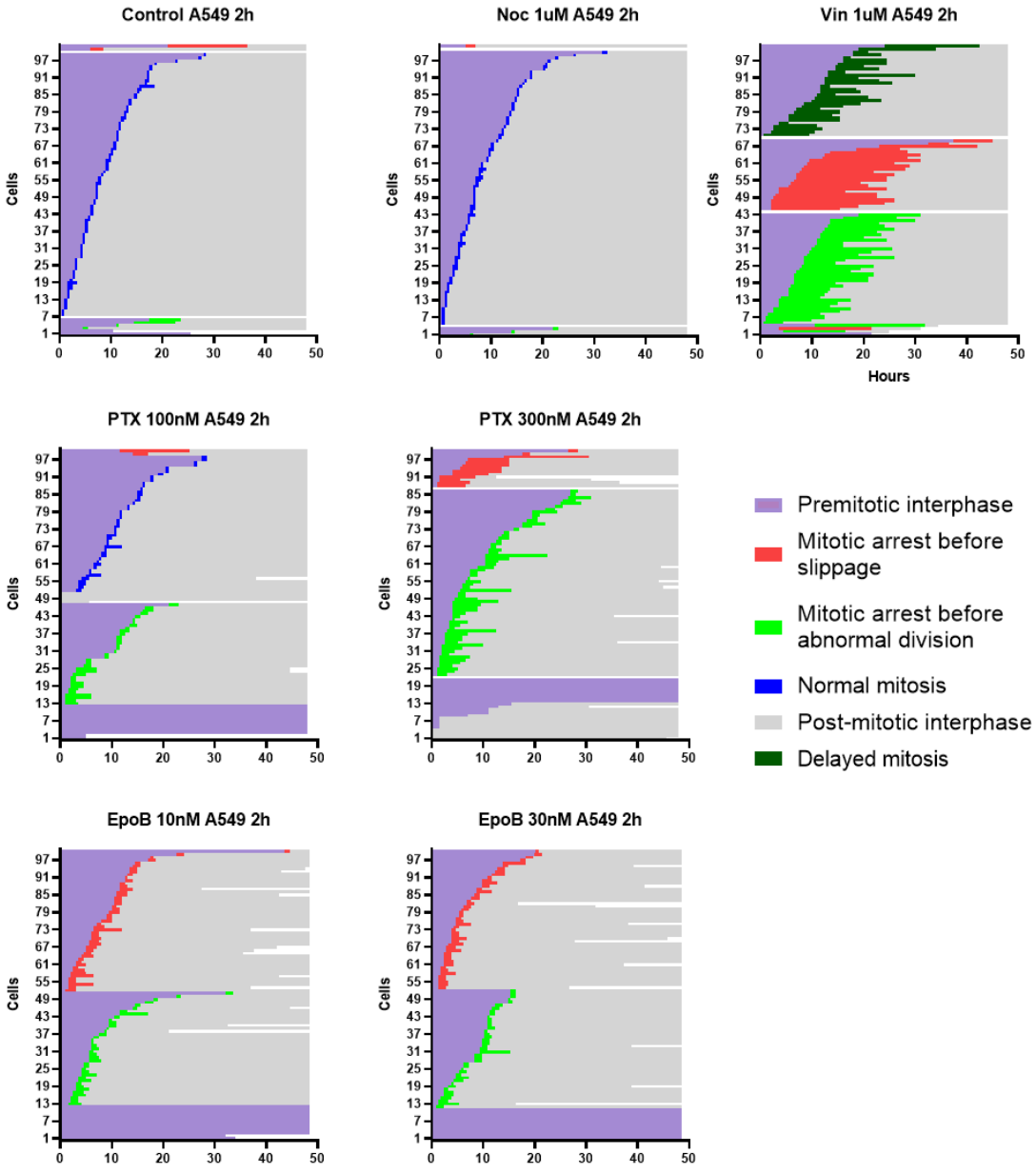


Figure 9. The life histories of individual cells from WO+ group in the time-lapse observation (N = 100). The duration of distinct cell state (in hours) can be visualized by the length of color line. The purple line shows time before mitotic phase; the blue line shows duration of normal mitosis; the red line shows duration of mitotic slippage; the green color shows duration of abnormal division; the grey color shows duration of post-mitotic state. In vinorelbine a distinct category of normal division, called delayed mitosis, contributed significantly to the cell behavior.

4.3 The cancer cell population experiences dramatic changes in the percentage of mono- and multinucleated cells after drug washout

If it is observed for a long period of time, the cancer cells treated and washed out from relatively high concentrations of microtubule-targeting agents experience dramatic changes in its population (Figure 10). Initially, after the washout, the majority of the cells remain spread out and mononuclear. However, the residual effects of the drug encourage most of these cells to become multinucleated, either through abnormal division or mitotic slippage. These multinucleated cells, characterized by elevated survivability, rarely proceed to divide. Several days later, the first mononuclear colonies emerged, resembling the original mononuclear cells in size, and exhibiting high proliferative capacity. Over the following days, these cells begin to occupy a larger area and significantly contribute to the overall population of the cancer cells.

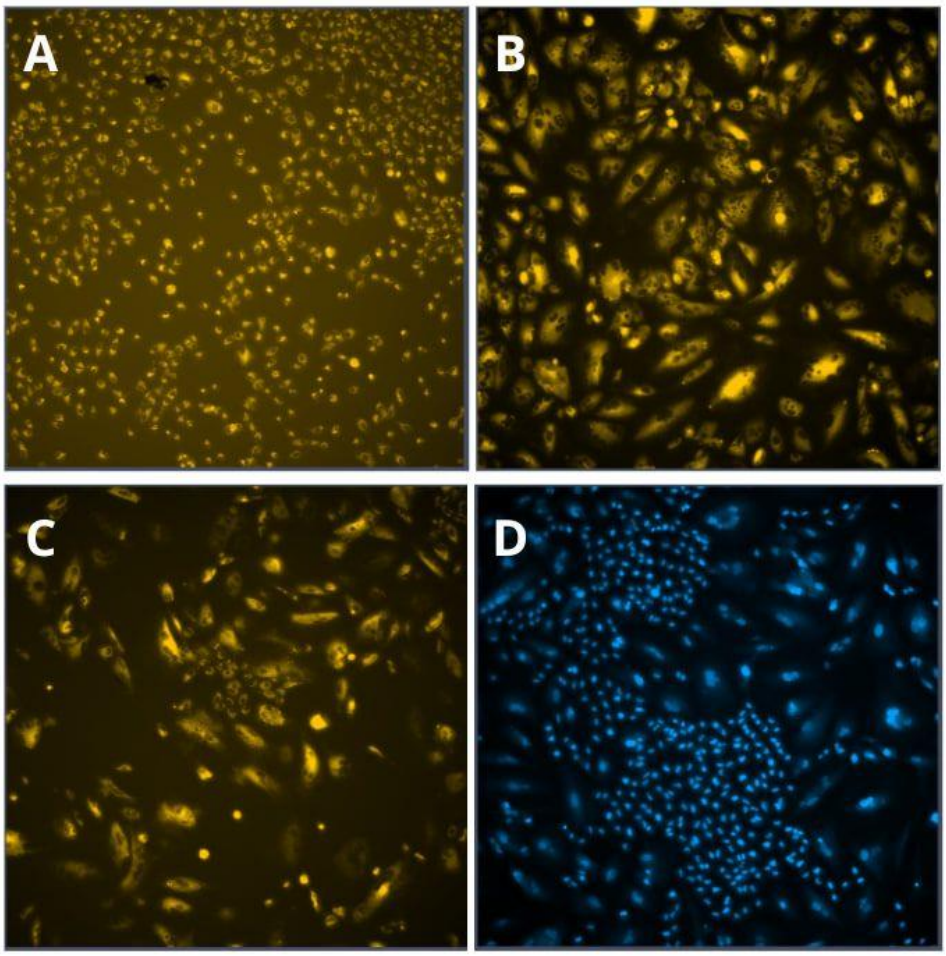


Figure 10. Snapshots sequences of A549 cells after two hours treatment with Epothilone B 10 nM and immediate washout. (A) The snapshot of A549 cells at Day 0 (the day at which washout was performed). (B) The snapshot of A549 cells at Day 3. (C) The snapshot of A549 cells at Day 15. (D) The snapshot of A549 cells at Day 21. Number indicate time passed since the beginning of time-lapse observation in hours. Cell with active mitochondrial membrane potential can be seen after staining with 50 nM TMRM

(green) solution. The nuclei can be visualized by the dark areas within cells. In picture D, the presence and number of nuclei are visualized after staining with 178 nM Hoechst 33342(blue). The images were acquired using a Plan-Neofluor 10x oil immersion objective with NA = 0.3. Scale bar = 50 μm.

4.4 The significant shift to the multinuclear state in cancer cell population elongates the period of colony formation.

Observing A549 cells for 21 days after a two-hour treatment and subsequent washout with various drugs revealed distinct population changes depending on the drug and its concentration (Figure 11). For instance, the majority of cells treated with NOC 1uM and VIN 1 uM demonstrated control-like behaviour. At least 70% of the cells remained mononuclear, a state that persisted for the first four days and beyond. This stability correlates with normal mitotic activity observed in these cells post-treatment. Approximately 15% of the population consisted of multinucleated and rounded (mitotic) cells, leading to a predominance of mononuclear cells over time. Similarly, treatment with 100 nM and 300 nM PTX did not significantly reduce the proportion of mononuclear cells, which comprised about 40% of the population. However, the percentage of multinucleated cells increased, reaching around 40% by day three post-washout. Then, the colony formation process take place by days 6 and 9 for PTX 100 nM and PTX 300 nM, respectively, resulting in an overall increase in mononuclear cell dominance. Regarding epothilone B (EPOB), concentrations of 10 nM and 30 nM transformed nearly the entire A549 cell population into a multinucleated state, which persisted through day 15 and beyond day 21, respectively. These multinucleated cells are substantial resistant to cell death and does not enter mitotic state. Notably, the few remaining mononuclear cells showed negligible proliferation until the abovementioned days, indicating a delayed colony formation phase following EPOB treatment and wash

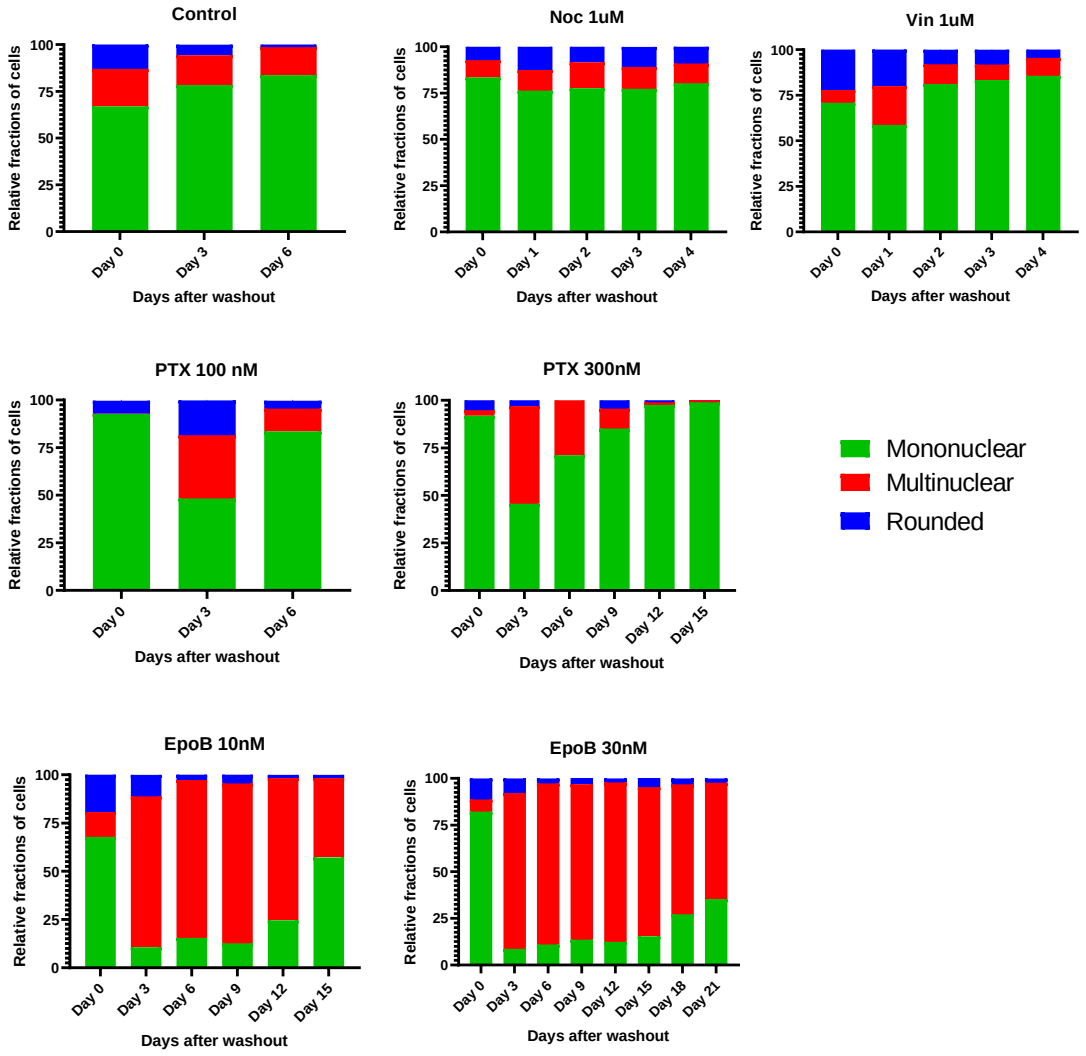
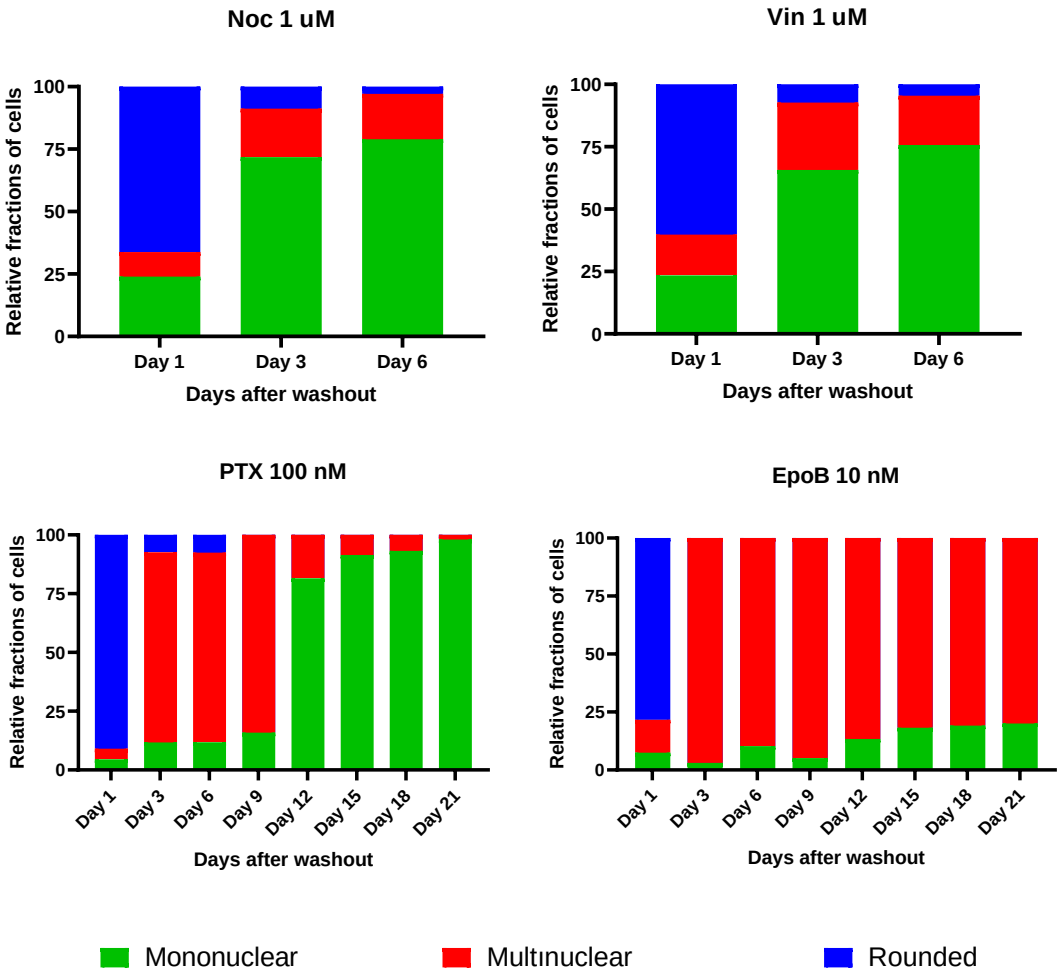


Figure 11. The dynamics of A549 mononucleated, multinucleated, and rounded cell populations in control and after washout treatment groups (2 hours of treatment). The relative fraction of cells is portrayed next to the days passed from drug washout. (N = 300).

To investigate the effects of extended incubation times on colony formation from predominantly multinucleated cell populations, the incubation period was increased to 16 hours. This adjustment led to a more pronounced shift to a multinuclear state in cells treated with PTX 100 nM, as evidenced by over 80% of the cells being multinucleated (Figure 12A). This significant shift to a multinuclear state, induced by microtubule-targeting agents, restricts the formation of mononuclear colonies for twelve days. In cells pre-treated with PTX 100 nM, mononuclear colony emergence began on Day 12, with mononuclear cells constituting 82% of the population, overtaking the multinucleated cells. A longer incubation period with EPOB 10 nM resulted in a more

extended shift to a multinuclear state, persisting throughout the 21 days of observation. For instance, a two-hour incubation resulted in mononuclear cells comprising 50% of the cell population by Day 15; however, with a 16-hour incubation, this proportion reduced to 18% on the same day. These observations, based on microscopy, were further validated by flow cytometry analysis (Figure 12B). While cells treated with PTX 100 nM showed a complete restoration of normal cell cycle distribution by Day 12, those treated with EPOB 10 nM did not show restoration at any point during the observation period. It can be observed by the presence of control-like high G1 peak and lower, in comparison to it, G2/M phase peak.

A.



B.

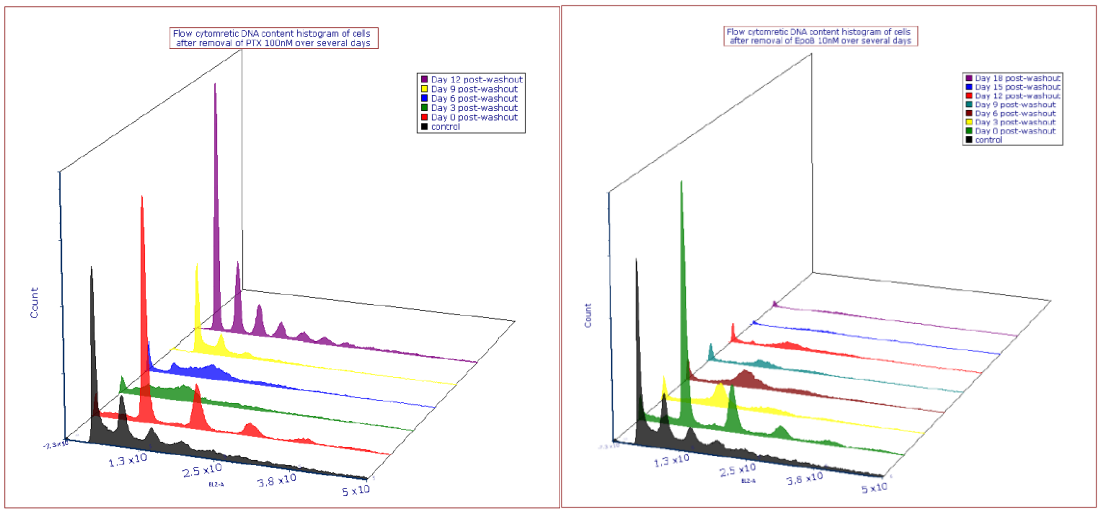


Figure 12. (A) The dynamics of A549 mononucleated, multinucleated, and rounded cell populations in control and after washout treatment groups (2 hours of treatment). The relative fraction of cells is portrayed next to the days passed from drug washout. (N = 300). (B) The flow cytometry plots representing the possible restoration of mononuclear population of cancer cells. The peaks correspond to the amount of DNA contained within the cells. (N = 30 000)

5 DISCUSSION

In the literature, there are a few papers that describe how cellular behavior is altered by the drug clearance as a result of drug washout. Moreover, few studies find the linkage between the cell's behavior as a response to microtubule-targeting agents and their further clonogenic abilities. The study conducted by Zasadil et al. (2014) examined that the long-term action of Paclitaxel which induces multipolar spindles assembly, significantly lowers the clonogenic abilities of cancer cells. By observing that different microtubule-targeting agents and their doses encourage cells to proceed with different events in the end of mitotic arrest, we aimed to follow up the possible correlation between the behaviour of cancer cells and their clonogenic abilities.

Comparing the effect of certain microtubule-targeting agents (PTX and EPOB) in the presence of the drug and those experiencing drug washout allowed us to observe the distinct behaviour cells demonstrate in the end of mitotic arrest. While normal division and abnormal division were present in cells incubated in the drug, the mitotic slippage was the major event that happens with cells especially when the concentrations of drug used was relatively high. In contrast, the washout group reveal a more diverse cell behaviour: cells were able to divide although abnormally, keeping mitotic slippage the second frequent event that happens with arrested cells. This could be explained by the less extent action of the drug that was not enough to block microtubule-dynamics in cell division completely. The microtubule-targeting agents had a modest effect on spindle assembly yet it cells were managed to proceed with division even abnormally. Moreover, the statistical difference between the duration of mitotic state between the incubated in drug cells and washout group reveals different effect of their treatment. The washout group's mitotic arrest was shorter making it closer to control group. Finally, the occurrence of cell death was more evident in the washout cells than in cells incubated in the drug that can be explained by formation of aneuploid cells with chromosome instability as a result of abnormal division (Bakhoum and Cantley, 2018).

There were also difference in the events and their proportion between different microtubule-targeting agents. While treatment with NOC and washout does not disrupt cells' normal division, VIN treatment and washout result in normal division, mitotic slippage, and abnormal division. Therefore, even similar in action drugs (both NOC and VIN are microtubule-destabilizers) can have different effect on the cell behaviour after

treatment and washout. Microtubule-stabilizers exposure however led to a more sever effect on cell behaviour. Overall, high concentrations of both PTX and EPOB encourage cells to proceed with a more frequent abnormal division and mitotic slippage. Moreover, both EPOB treatment with 10 Nm and 30 Nm leads to the more frequent mitotic slippage even in comparison with PTX 300 Nm.

The last observation made us realize that almost complete shift of cancer cell population to multinuclear state is the result of both abnormal division and mitotic arrest triggered by PTX and EPOB drugs. Within first three days after drug washout, almost whole population becomes multinuclear, and cells stay intact for a relatively long period of time. After certain days after drug washout, the mononuclear colonies arise eventually overweighting the number of multinuclear cells. These data resonate with findings of Zasadil et al. (2014), where they examine no colony formation because of presence of PTX drug, emphasizing the importance of drug clearance in the colony formation abilities. In contrast, NOC and VIN treatment is not enough to effectively shift population to the multinuclear state allowing mononuclear cells to proliferate widely within a few days after drug washout.

It is worthwhile to mention that PTX and EPOB are also differentiate from each other in terms of this multinuclear shift with former be less effective to shift population to the multinuclear state. The presence of approximately half of mononuclear cells allows the population after PTX treatment and washout lead to the faster cancer relapse as a result of colony formation. In contrast, cells treated with EPOB are capable to produce colonies after several weeks of drug washout. These data is also proven by flow cytometry analysis where the emergence of normal cell cycle distribution is achieved in the same day where colonies proliferate widely. The elevation of cells proportion with 2n set of chromosomes coincides with the mononuclear colonies' formation. The possible explanation to this phenomenon may be that almost half of the multinuclear cells generated after treatment with EPOB drug are the result of mitotic slippage. At the same time, the mitotic slippage was not as evident after PTX treatment and majority of multinuclear cells generated are the results of abnormal division. When the time of incubation in PTX increased to 16 hours, the colony formation was suppressed similar to the EPOB treatment. It can be suggested that longer incubation in the microtubule-targeting agent may encourage shift to the multinuclear state of population through

mostly mitotic slippage. Multinucleated cells generated from this event maybe more efficient to inhibit colony formation for a long period of time in comparison with those generated by abnormal division.

These findings are crucial, as they demonstrate the high efficacy of EPOB treatment than PTX treatment in terms of colony formation period. It also suggests that effect of microtubule-targeting agents on population of cancer cells by blocking normal mitosis shifting population to the multinuclear state limits clonogenic properties of these cells. The better the extent of this shift the longer time will be required to form colonies for cells.

As a future direction of this research, there is necessity to expand study to different cancer cell lines to test cell-dependency effect on clonogenicity. It is also important to research in detail what is the state of cell mononuclear colonies find their origin from: whether it mononuclear or multinuclear. The comparison between multinuclear shift generated as a result of mitotic slippage or abnormal division in terms of colony formation properties may also create a space for further studies. Finally, in vivo experiment could be carried on animal models where the tumorigenicity and the colony formation results of this work could be studied in a more details.

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7 APPENDICES

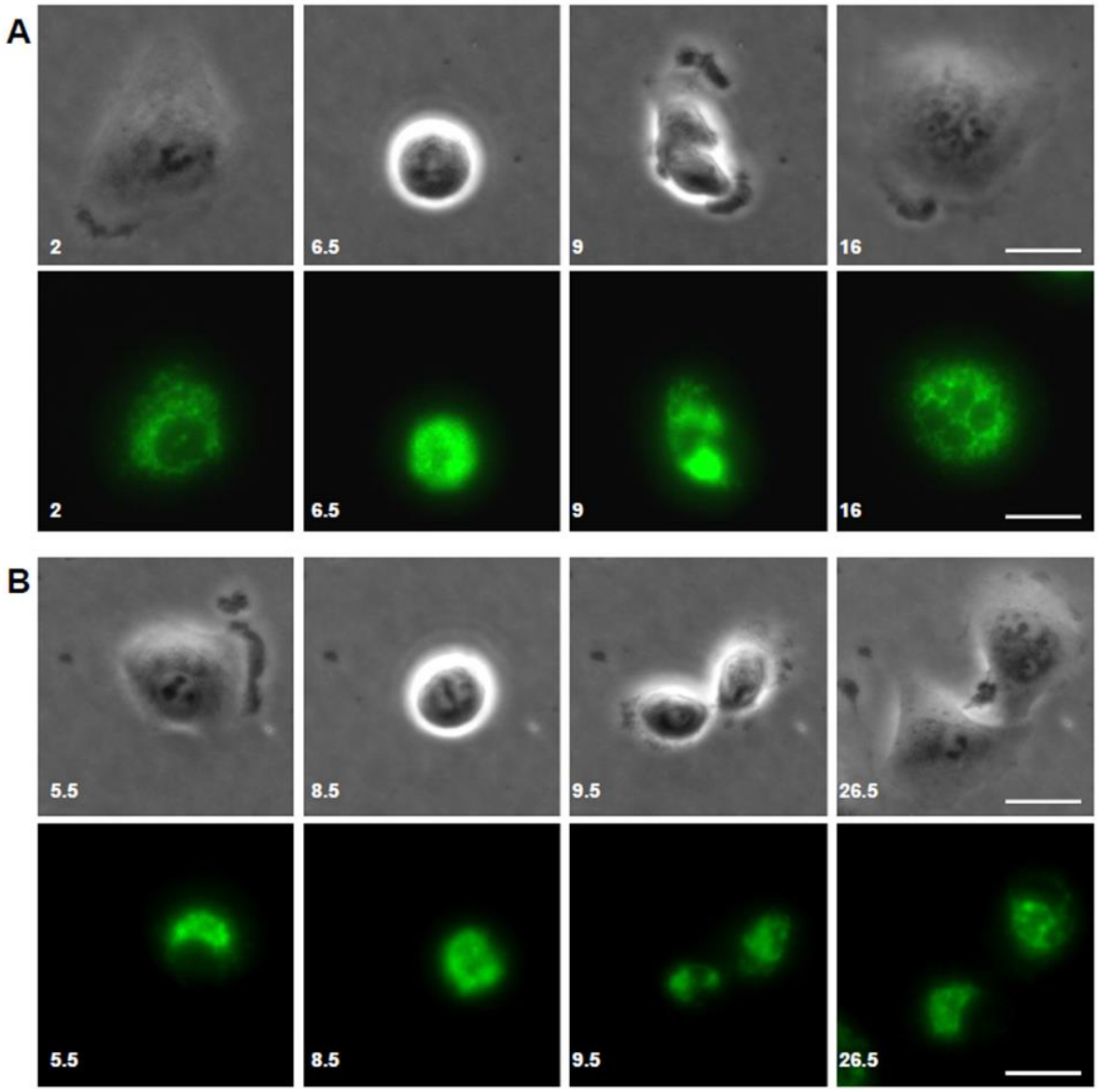

Figure 13. Time-lapse sequences illustrating two major fates he phase contrast and fluorescent time lapse images of mitotic slippage (A) obtained during 16 h treatment with either Paclitaxel or Epothilone B, and abnormal division (B) that was predominant event in cells after washout from the drug. Cell with active mitochondrial membrane potential can be seen after staining with 50 nM TMRM (green) solution. The nuclei can be visualized by the dark areas within cells. The images were acquired using a Neofluor ×20 oil immersion objective with NA = 0.5. Scale bar = 20 μm. Time is given in hours.