NAZARBAYEV UNIVERSITY

EFFECT OF INHIBITION OF ANTI-APOPTOTIC BCL-2 PROTEINS ON THE OUTCOME OF MITOTIC ARREST INDUCED BY MICROTUBULE TARGETING DRUGS

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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.

Mereke Suleimenov

April 8, 2022

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SUMMARY

Anti-mitotic drugs induce prolonged mitotic arrest followed either by death in mitosis (DiM) or by mitotic slippage (Rieder and Maiato, 2004). The model explaining DiM versus mitotic slippage proposed by Gascoigne and Taylor in 2008 states about the two competing networks between well-documented cyclin B1 degradation and undefined pro-death signal (Brito and Rieder, 2006; Terrano et.al, 2010). The major purpose of this work is to study the nature and regulation of the mechanism of cell death after treatment with MT drugs.

Using fluorescent time-lapse microscopy, we showed that cells respond to treatment with MT inhibitors in a threshold dependent manner. We defined two threshold doses starting from which cell's response to MT drugs changes significantly. The first threshold dose (T1) was defined as the maximal ineffective dose, wherein normal division is observed in >90% of cases and the second threshold dose (T2) was defined as the minimal dose, wherein >90% mitotic cells are arrested in mitosis for prolonged time. In doses below T2, most cells were able to divide either normally or abnormally. In T2 and higher doses, duration of mitotic arrest and relative frequency of cell fates after mitotic arrest varied greatly among the cell lines but were similar within the cell line under the action of different drugs. Prolonged mitotic arrest in T2 and higher doses resulted in one of three outcomes: (i) DiM, (ii) mitotic slippage followed by death in interphase or (iii) mitotic slippage followed by cell survival for indefinite time. Based on the outcome of mitotic arrest in T2 and higher doses, we categorized HeLa and HaCaT cells as DiM-sensitive and A549 and PC-3 cells as mitotic slippage prone.

Using specific markers, we demonstrated that cell death during mitotic arrest and after mitotic slippage proceeded through apoptotic pathway, involving mitochondrial outer membrane permeabilization (MOMP) followed by activation of executive caspases 3/7.

We determined that specific inhibition of anti-apoptotic Bcl-xL protein is sufficient to induce MOMP in the slippage prone cell lines. Inhibitors of Bcl-xL (Navitoclax and A-1155463) but not inhibitors of Mcl-1 (S63845) or Bcl-2 (Venetoclax) sensitized slippage prone A549 and PC-3 cells to DiM. With increase in the dose of Bcl-xL inhibitors, proportion of cells undergoing DiM increased and duration of mitotic arrest decreased. On the other hand, treatment of slippage-prone cells with each Bcl-2 inhibitor in combination with a well-known apoptotic inducer Staurosporine significantly (~3 times) accelerated MOMP and was followed by activation of caspases 3/7 in a similar way, suggesting that there is a special regulation of apoptosis by Bcl-xL during mitotic arrest.

We conclude that different cell cultures have intrinsic mechanism of Bcl-xL based sensitivity to pro-death signal during mitotic arrest.

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ABBREVIATIONS

1. MT drugs – microtubule targeting drugs
2. SAC – spindle assembly checkpoint
3. DiM – death in mitosis
4. MOMP – mitochondrial outer membrane permeabilization
5. Bcl – B cell lymphoma
6. Mcl – myeloid cell leukemia
7. TMRE – tetramethylrhodamine, ethyl ester
8. GTP – guanosine triphosphate
9. MCC – mitotic checkpoint complex
10. Cdc 20 – cell division cycle protein 20 homolog
11. APC/C – anaphase promoting complex/ cyclosome
12. CDK1 – Cyclin Dependent Kinase 1
13. WHO – World Health Organization

1. INTRODUCTION

1.1 Microtubules

Microtubules are an integral part of the cytoskeleton and are highly conservative among most eukaryotes. Microtubules are prolonged, hollow structures made of αβ-tubulin heterodimers (Figure 1). Most microtubules originate from the centrosome and grow towards the cell membrane. Polymerization occurs at the expense of GTP hydrolysis, which allows α-tubulin from one heterodimer to bind β-tubulin of another heterodimer. The growing end of a microtubule with an exposed β-tubulin is known as "+" or plus end, whereas the end which is embedded into the centrosome is denoted as "-" or minus end. Inside a cell, microtubules are very dynamic and undergo rapid and frequent polymerization and depolymerization events, known as dynamic instability. Normally, microtubules exist in the state of dynamic instability, which is controlled by different factors, including the availability of GTP and interaction of microtubules with a variety of regulatory proteins. Properly operating microtubules are essential for a normal cell function as microtubules (i) interact with other cytoskeletal proteins such as actin and myosin and contribute to the maintenance of cell shape and control of cell migration; (ii) serve as a substrate for motor proteins called kinesin and dynein that move along the microtubules to facilitate intracellular transport of vesicles and organelles; (iii) are the major building block of mitotic spindle, that plays major role in chromosome segregation (Alberts, et al., 2014).

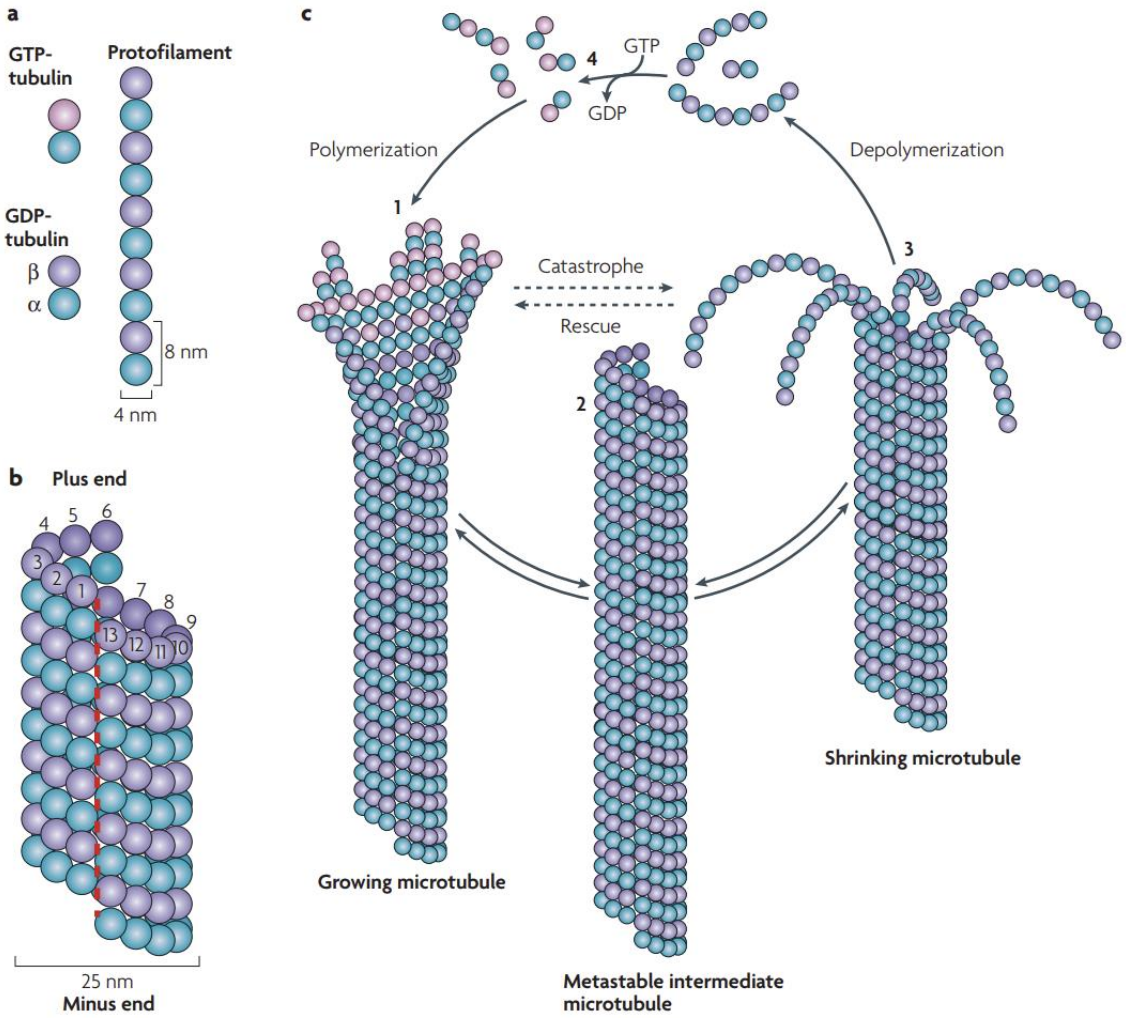

Figure 1. Microtubule structure and dynamic instability. (Taken from Akhmanova and Steinmetz, 2008)

1.2 Mitotic Spindle

During normal mitosis, microtubules originate from two centrosomes located at the opposite ends of a cell, forming mitotic spindle (Figure 2). Mitotic spindle operates throughout different stages of mitosis and its main function is to segregate chromosomes during anaphase. This task is performed by cooperative work of spindle microtubules and involved proteins. While astral and interpolar microtubules coordinate the spindle positioning and provide a platform for pulling force, kinetochore microtubules attach to kinetochores, large sized protein structures located at the

centromeres of sister chromatids, to segregate chromosomes. To avoid improper chromosome segregation and aneuploidy, precise connection between microtubules and kinetochores must be established before the onset of anaphase (Alberts, et al., 2014).

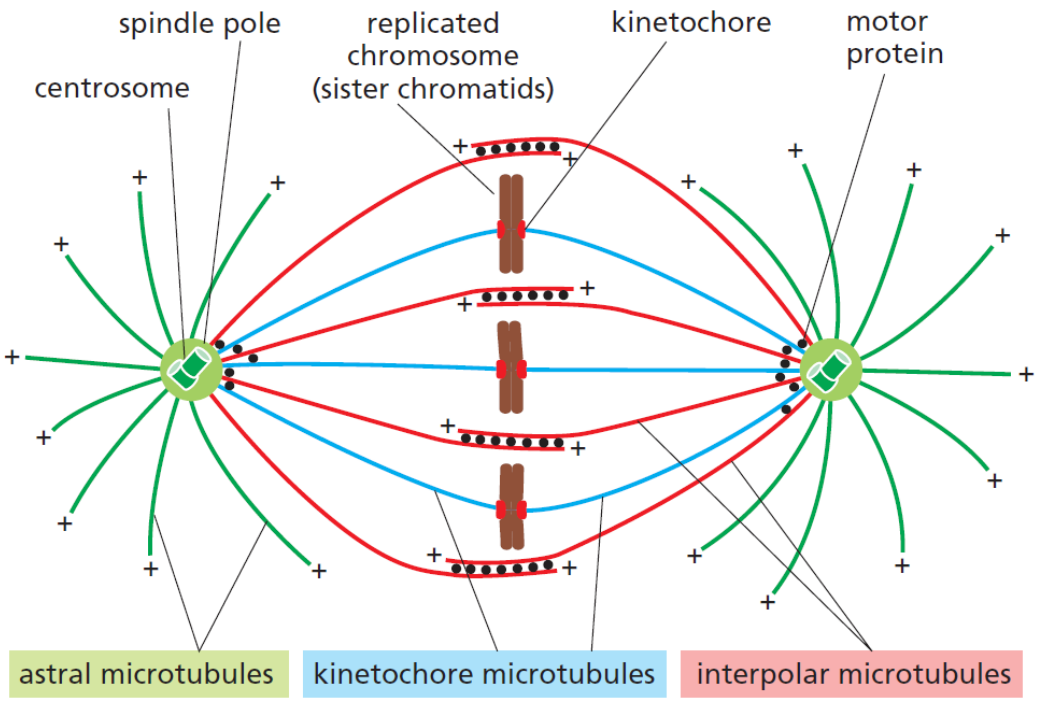

Figure 2. Scheme of the mitotic spindle. (Alberts, et al., 2014)

1.3 Spindle Assembly Checkpoint

Spindle assembly checkpoint (SAC) is a pathway that helps cells to avoid improper chromosome segregation and aneuploidy. When a kinetochore is not attached to the spindle microtubules, SAC effector called mitotic checkpoint complex (MCC), which consists of several proteins, binds to Cdc20 and keeps anaphase promoting complex/ cyclosome (APC/C) inactive. Inactive APC/C cannot promote securin, which inhibits separase, and CyclinB1, which keeps CDK1 active, for ubiquitination and

proteolysis. As a result, chromosomes do not segregate, and a cell remains in mitotic state.

When all kinetochores are attached, MMC releases Cdc20. Interaction with Cdc20 activates APC/C, which leads to ubiquitination and proteolysis of securing and cyclinB1. In the absence of securin, separase removes cohesin that binds sister chromatids, and allows chromosome segregation. On the other hand, degradation of cyclinB1 results in Cdk1 inactivation, which leads to mitotic exit (Musacchio and Salmon, 2007; Barbosa, et al., 2011; Musacchio 2015).

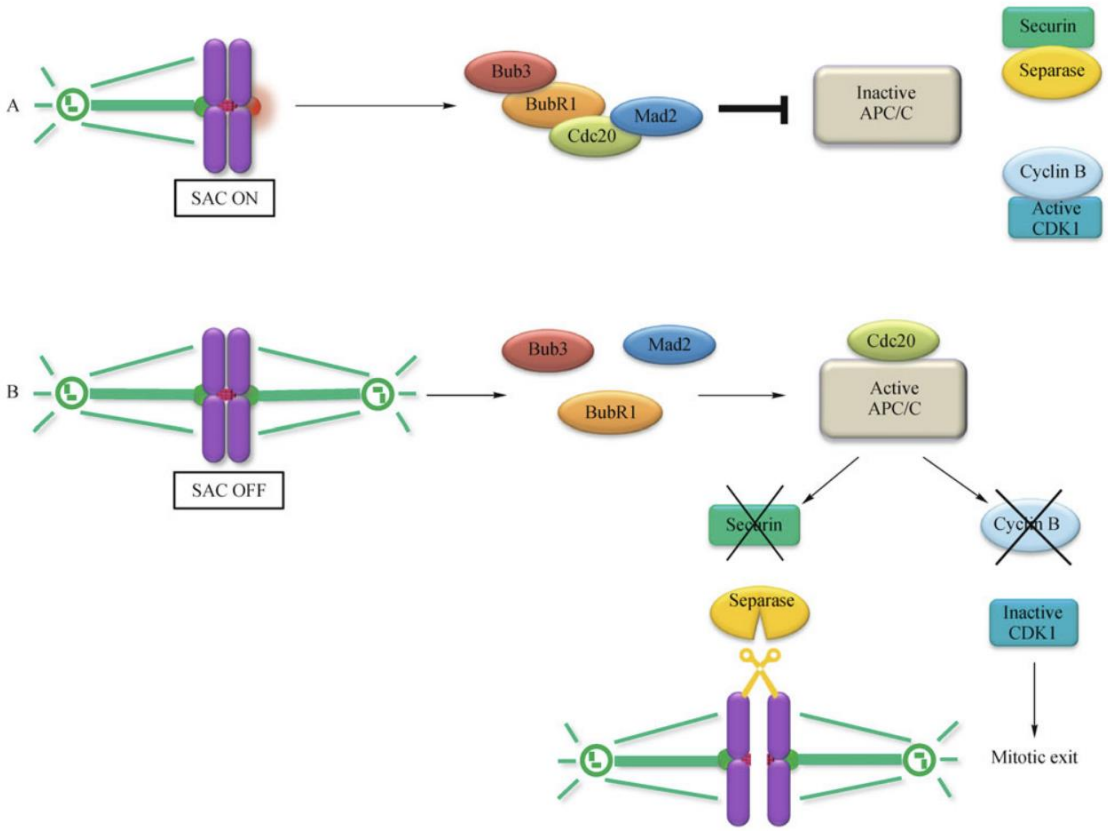

Figure 3. Scheme of the Spindle Assembly Checkpoint (SAC). (Barbosa, et al., 2011)

1.4 Microtubule Targeting Drugs

Since cancer cell have unlimited proliferative capacity, microtubule-based mitotic spindle has been chosen as a target to treat cancers. Microtubule targeting (MT) drugs have been extensively used in chemotherapy for more than 30 years and drugs such as paclitaxel (taxol) and vinorelbine have been included in the WHO's list of essential medicines (McGuire, et al., 1989; Rowinsky et al., 1993).

Today, there are many available MT drugs that were obtained both naturally and synthetically. Despite their differences on the molecular level, most of these drugs interfere with microtubule dynamics by inhibiting the process of either microtubule polymerization or depolymerization. For example, Taxol inhibits microtubule depolymerization, while Vinorelbine and Nocodazole inhibit microtubule polymerization (Perez, 2010).

1.5 Literature review

Sufficient doses of MT drugs show high anti-proliferative activity against cancer cell lines. Drugs such as Taxol and Nocodazole interfere with microtubule dynamics, which leads to the activation of spindle assembly checkpoint (SAC) during mitosis (Jordan et al., 1992, Jordan et al., 1996; Musacchio and Salmon, 2007). Inability of dividing cell to satisfy SAC in the presence of MT drugs results in prolonged mitotic arrest (Rieder and Maiato, 2004). Two major outcomes following prolonged mitotic arrest have been described: death in mitosis (DiM) and escape into the interphase without proper chromosome segregation and cytokinesis, the process known as mitotic slippage. Cells after mitotic slippage are tetraploid and are often multinucleated.

Following mitotic slippage some cells die relatively soon after mitotic slippage, while other cells survive for indefinite time (Blagosklonny, 2007; Ikui, et al., 2005).

Several previous studies attempted to understand why sensitivity to MT drugs varies among different cell lines as well as within a single cell line (Gascoigne and Taylor, 2008; Shi and Zhou et al., 2011). Longer duration of mitotic arrest has been associated with higher chances of cell death. However, the evidence has been provided for a single cell line only, leaving the question of inter-line variation widely open (Bekier et al., 2009). On the other hand, to explain both intra- and inter-line variations, the model of two competing networks between well-documented cyclin B1 degradation and undefined pro-death signal has been proposed (Gascoigne and Taylor, 2008; Brito and Rieder, 2006). Currently operating evidence associates cell death induced by MT drugs with standard apoptotic pathway. The role of anti-apoptotic Bcl-2 proteins (Bcl-2, Mcl-1, Bcl-w and Bcl-xL) in cell survival during mitotic arrest has been of particular interest. These proteins are known to block apoptotic pore formation on the mitochondrial membrane by pro-apoptotic Bcl-2 proteins, such as BAK and BAX. Several studies have already shown that depletion of anti-apoptotic Bcl-2 proteins sensitizes cells to death during mitotic arrest, while their overabundance results in mitotic slippage. However, results regarding the role of individual members of Bcl-2 family are not consistent among different studies (Shi and Zhou, 2011; Hellmuth and Stemmann, 2020; Huang et al., 2016; Bennet et al., 2016). Moreover, how cell's fate is regulated after mitotic slippage is also unclear. Death in some post-slippage cells has been associated with accumulation of DNA damage while cells are arrested in mitosis (Orth et al., 2011). Whether sensitivity to cell death after mitotic slippage depends on the activity of anti-apoptotic Bcl-2 proteins is yet to determine.

2. MATERIALS AND METHODS

Cell lines:

4 cancer cell lines: HeLa – cervical cancer cells, U-118 – glioblastoma cells, A549 – non-small lung cancer cells, PC-3 – prostate cancer cells

2 minimally transformed cell lines: HaCaT – minimally transformed human keratinocytes, NIH 3T3 – minimally transformed mouse embryo fibroblast cells.

All cell lines, except PC-3, were grown in DMEM with 10% FBS and 4-8 mM L-Glutamine. PC-3 were grown in F12 medium with 10% FBS and 4-8 mM L-Glutamine.

Reagents

Nocodazole – microtubule depolymerizing drug, Taxol – microtubule stabilizing drug, Vinorelbine – microtubule depolymerizing drug. Staurosporine – pan-kinase inhibitor (widely used as apoptotic inducer), TMRE – dye for visualization of mitochondrial membrane potential, caspase 3/7 detecting ready probe reagent – reagent for visualization of active caspases 3/7, sir-DNA – dye for visualization of DNA, Venetoclax – selective Bcl-2 inhibitor, S63845 – selective Mcl-1 inhibitor, Navitoclax – non-specific inhibitor of anti-apoptotic Bcl-2 proteins, A-1155463 – selective Bcl-xL inhibitor.

Data acquisition and data analysis

High throughput time-lapse imaging (Zeiss Cell Observer microscope) was used as the major tool during this project. 6cell lines were seeded into a 48-well plate and incubated overnight. Then, the cells were treated with 1-1000 nM doses of MT drugs (diluted in CO-2 independent medium with 10% FBS and 4-8 mM L-glutamine) and tracked for 72 hours with a 15-minutes interval between the frames under an objective

with 10X magnification factor (Figure 4). Acquired time-lapse series were first converted from .czi into .tiff format. Then, time-lapse series were manually analyzed in ImageJ software. Acquired data was saved as .xls file and then used to construct representative figures in GraphPad Prism 8 software.

All calculations and comparisons were performed in GraphPad Prism 8 software based on the assumptions that (i) frequency distributions for the duration of mitotic arrest follows normal distribution (Figure A1) and (ii) variances for the duration of mitotic arrest in different treatment groups were similar (Table A1). All tables were constructed in Microsoft Excel software.

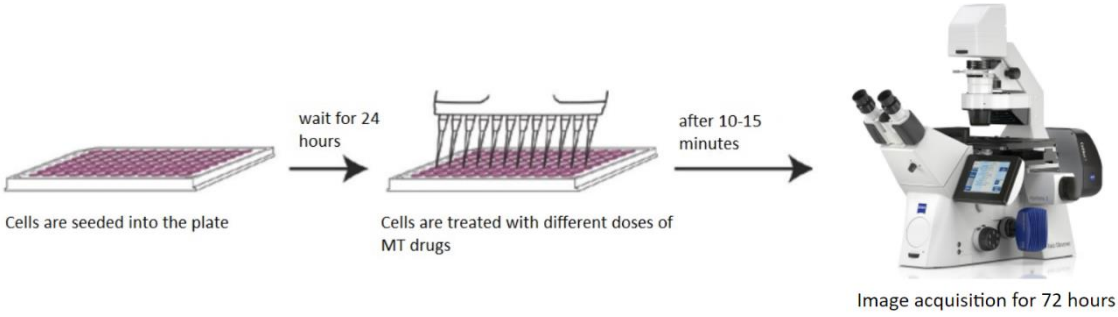

Figure 4. Scheme illustrating the major experimental setup used for completion of the current project. (Modified from Thermo Fisher)

3. AIMS OF THE THESIS PROJECT

1. Substantial amount of data regarding the inter- and intra-line variation following mitotic arrest has already been reported (Gascoigne and Taylor, 2008; etc.). However, because most studies prefer to use overly high and single doses of MT drugs to ensure the occurrence of prolonged mitotic arrest, variation in cell response to different doses of MT drugs has not been studied enough. **Thus, we aim to systematically assess the degree of variation with regards to MT drugs by treating HeLa, HaCaT, U-118, A549, PC-3 and 3T3 cell lines with a wide range of doses of MT drugs Nocodazole, Taxol and Vinorelbine using high-throughput time-lapse microscopy.**
2. Recent evidence suggests that cell death during mitotic arrest and after mitotic slippage proceeds via mitochondria dependent cell death (). However, terms such as “mitotic catastrophe” and caspase independent cell-death have also been commonly used. **One of the aims of this work is to study the nature and regulation of cell death after treatment with MT drugs using specific markers.**
3. It has also been reported that anti-apoptotic Bcl-2 proteins play major role in the regulation of MOMP during mitotic arrest. However, there is no clear understanding on the importance of particular members, especially of Bcl-2, Mcl-1 and Bcl-xl (Shi and Zhou, 2011; Hellmuth and Stemmann, 2020; Huang et al., 2016; Bennet et al., 2016). **We aim to assess the role of individual anti-apoptotic Bcl-2 members in the regulation of cell death during mitotic arrest by applying specific small-molecule inhibitors of Bcl-2 proteins.**

4. RESULTS

4.1 Cell fates after treatment with different doses of MT drugs

Depending on the drugs' dose, for all cell lines mitosis always resulted in one of the following: (i) normal division, (ii) abnormal division or (iii) prolonged mitotic arrest followed by either death during mitotic arrest (DiM) or mitotic slippage. Division observed in the control group was considered as normal. Most cells in control group, regardless of the cell line, underwent division within ~1 hour and were able to undergo several rounds of division during the 72 hours of observation. All divisions that deviated from the control group were classified as abnormal. Abnormal divisions include unequal division, death after division, division after mitotic arrest (when duration of mitosis was 3 times longer than average duration of mitosis in a respective control group) and division followed by cell cycle arrest. After mitotic slippage, the cells either died or survived until the end of the observation. Mitotic entry was identified by the moment of cell rounding. Cell death was distinguished by cell blebbing and discharge of the cytoplasm.

4.2 Cells show threshold dependent response to the treatment with different doses of MT drugs

For each drug type - cell line pair, we observed threshold dependent response. More detailed microscopic analysis showed the presence of two critical doses. The first threshold dose (T1) was defined as the maximal dose cells can tolerate undergoing normal division in >90% of cases and the second threshold dose (T2) as the minimal mitostatic dose, wherein >90% mitotic cells are arrested in mitosis. In T1 and lower doses, the cells divided normally. In between T1 and T2 many cells divided abnormally.

In T2 and higher doses, all mitotic cells were arrested in mitosis followed by either death during mitosis or mitotic slippage. T1 and T2 doses of three MT drugs for six cell lines are shown in the Table 1. For each cell line, except highly resistant 3T3 cells, T1 doses of Taxol and Vinorelbine were ~3-10-fold lower than of Nocodazole. T1 doses were in the range of 10-30 nM for Nocodazole, in the range of 1-3 nM for Taxol and 3 nM for Vinorelbine.

Table 1. Threshold doses T1 and T2 of MT drugs Nocodazole, Taxol and Vinorelbine for HeLa, HaCaT, U-118, A549, PC-3 and 3T3 cell lines.

	Nocodazole		Taxol		Vinorelbine	
	T1	T2	T1	T2	T1	T2
HeLa	10 nM	100 nM	1 nM	30 nM	3 nM	30 nM
HaCaT	30 nM	300 nM	3 nM	30 nM	3 nM	100 nM
U118	30 nM	100 nM	3 nM	30 nM	3 nM	10 nM
A549	10 nM	100 nM	3 nM	100 nM	3 nM	30 nM
PC-3	10 nM	300 nM	3 nM	30 nM	10 nM	300 nM
3T3	30 nM	300 nM	100 nM	1000 nM<	30 nM	300 nM

Distribution of cell fates after treatment with T2 and higher doses remained highly conservative within each cell line regardless of drug type (Figure 5). Detailed analysis was performed on A549 cells and U-118 cells, which often showed all three fates following mitotic arrest. We observed that response of A549 and U-118 changed insignificantly even after 30-fold and 100-fold increase in the dose of Vinorelbine respectively.

Relative frequency of cell fates in T2 and higher doses varied greatly among the cell lines studied. During microscopic analysis of the inter-line variation, the most sensitive lines with the highest frequency of DiM were HeLa (~90% DiM) and HaCaT (~70% DiM). The resistant cell lines with high frequency of mitotic slippage were U-118 (~70% slipped), A549 (~85% slipped) PC-3 (~95% slipped) and 3T3 (~100% slipped).

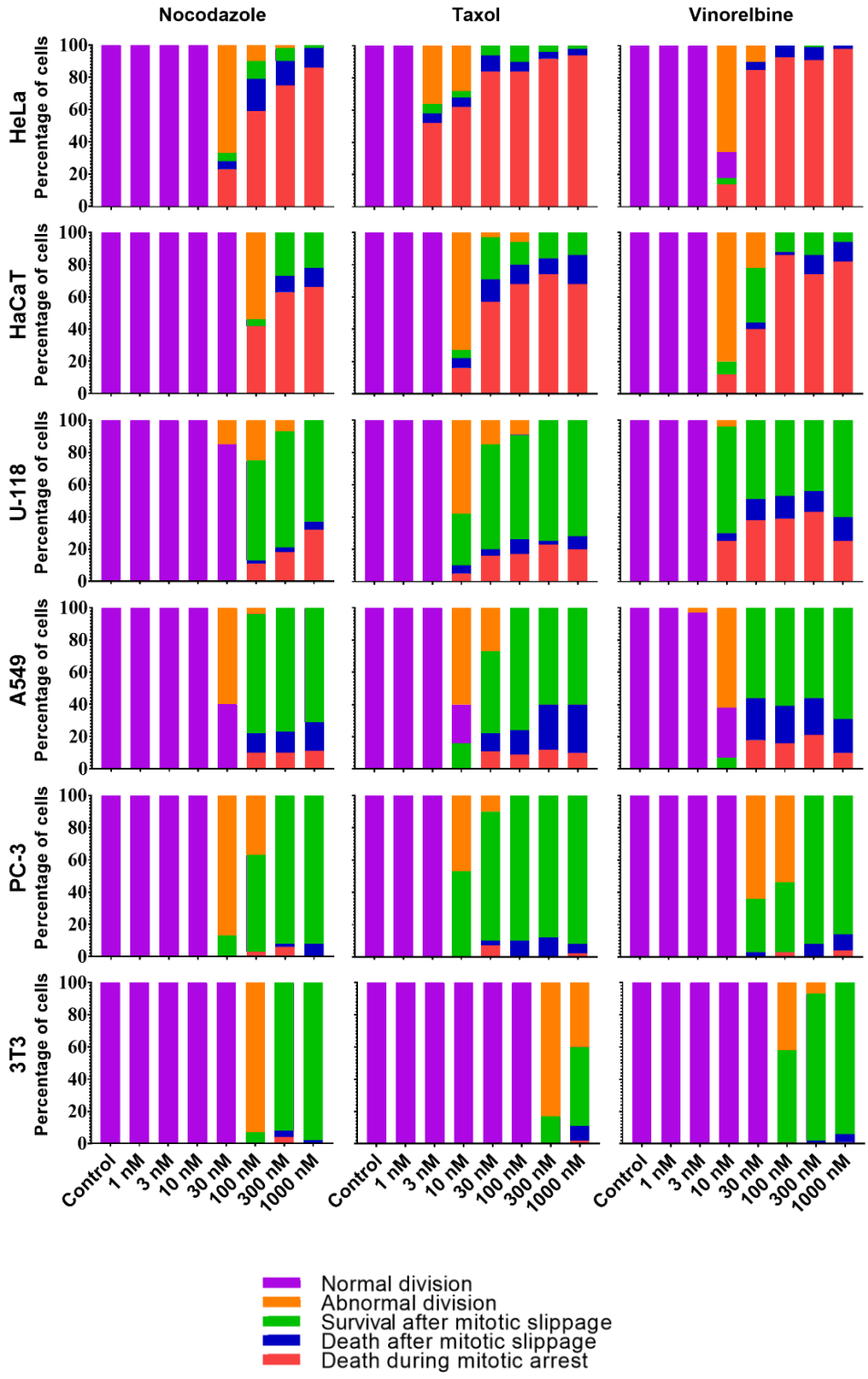

Figure 5. Threshold dependent response of HeLa, HaCaT, U-118, A549, PC-3 and 3T3 cell lines after 72 hours of treatment with 1-1000 nM doses of Nocodazole, Taxol and Vinorelbine. From top to bottom cell lines are placed in order of decreasing sensitivity to DiM. For each treatment group N = 30-50 cells.

4.3 Longer duration of mitotic arrest does not sensitize cells to DiM.

During mitosis transcription and translation are almost fully blocked, suggesting that longer duration of mitotic arrest should decrease the chances of cell survival. However, when we calculated and compared average durations of mitotic arrest among six cell lines after treatment with post T2 doses of MT drugs, no direct correlation between the duration of mitotic arrest and probability of cell death was found. Depending on drug type, average durations of mitotic arrest were in the range of 13-15 h. for HeLa, 8-9 h. for HaCaT, 13-15 h. for U-118, 17-21 h. for A549, 17-19 h. for PC-3 and 6-8 h. for 3T3 cells (Figure 6, Tables A1-3). Slippage prone A549 and PC-3 cells were arrested in mitosis longer than DiM sensitive HeLa and HaCaT cells. Similarly, slippage prone U-118 were arrested in mitosis approximately the same time as HeLa and more than HaCaT.

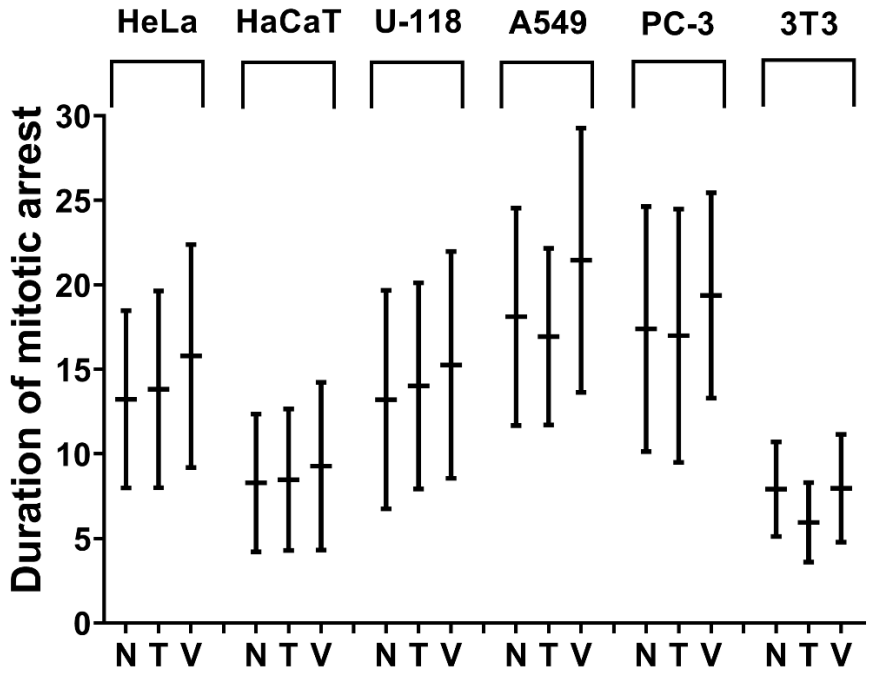

Figure 6. Average durations of mitotic arrest (represented as mean + SD in hours) in post-T2 doses of Nocodazole (N), Taxol (T) and Vinorelbine (V) for HeLa, HaCaT, U-118, A549, PC-3 and 3T3 cell lines. N= >100 cells for each treatment group.

4.4 Cell death during mitotic arrest and after mitotic slippage proceeds via standard apoptotic pathway

Progression of apoptosis was studied in a detailed manner by tracking MOMP and caspase 3/7 activity in HeLa and A549 cells after treatment with apoptotic inducer Staurosporine or Taxol. In both cell lines, death after treatment with Staurosporine and Taxol started with MOMP and was followed by activation of caspases 3/7 in >99% of cases (Figure 7).

Soon after addition of 1 uM Staurosporine, cells started to shrink but retained mitochondrial membrane potential. MOMP occurred after ~4 hours in HeLa and after ~11 hours in A549 cells from the moment of Staurosporine addition. For both cell lines, active caspases 3/7 were detected ~2 hours after MOMP. Maximal caspase 3/7 intensity was recorded ~5 hours after MOMP for HeLa cells and ~3 hours for A549 cells (Figure 8).

For Taxol treated HeLa cells, MOMP started ~10 hours after the mitotic entry. MOMP occurred in parallel with cell shrinkage and was completed within ~10 minutes. It was followed by activation of caspases 3/7 after the delay of ~2 hours. Activation of caspases proceeded gradually and peaked ~5 hours after MOMP. Maximal intensity from the caspases 3/7 was recorded at the moment of cytoplasm discharge.

For Taxol treated A549 cells, MOMP started ~19 hours after mitotic entry or within first 24 hours after mitotic slippage. Like for HeLa cells, MOMP occurred in parallel with cell shrinkage and was completed within ~10 minutes. It was followed by activation of caspases 3/7 after the delay of ~2 hours. Activation of caspases proceeded gradually and peaked ~4 hours after the MOMP. Maximal intensity from the caspases 3/7 was recorded at the moment of cytoplasm discharge.

For both cell lines, we observed manifestation of apoptotic events in similar order and timing as after treatment with Staurosporine, confirming that execution of apoptosis during mitotic arrest and after mitotic slippage follows standard apoptotic pathway.

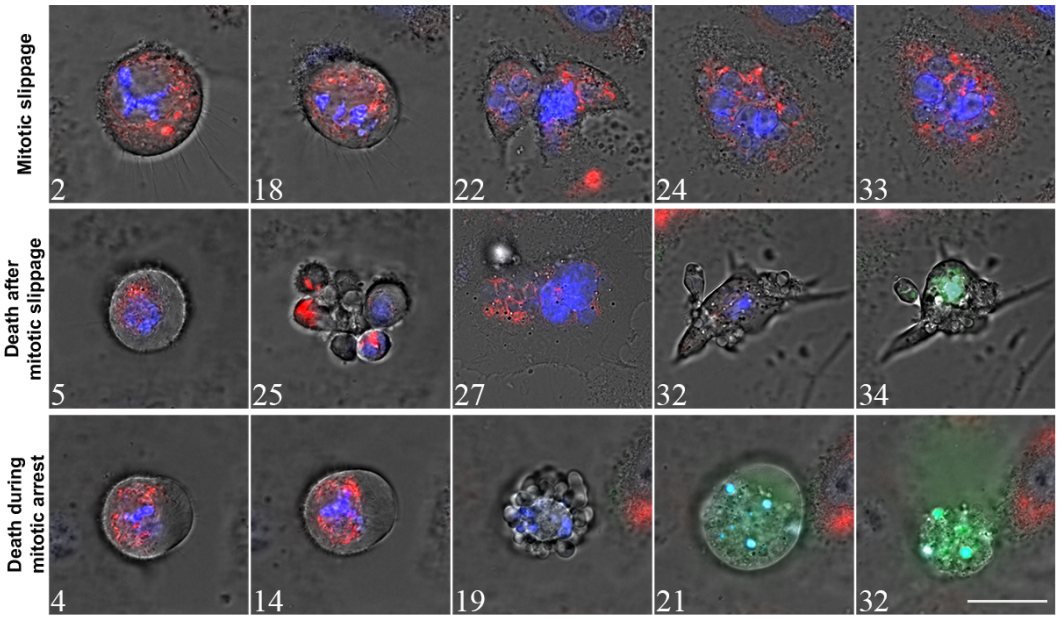

Figure 7. Time-lapse sequences illustrating three fates (mitotic slippage, death after mitotic slippage and death in mitosis) exhibited by A549 cells under the treatment with 1 uM Taxol. Numbers indicate time from the moment of mitotic entry in hours. Mitochondrial membrane potential was visualized after staining with 50 nM TMRE (red), active caspases 3/7 with caspase 3/7 detecting reagent (green) and nuclei with 200 nM siR-DNA (blue). The images were acquired using 63X oil immersion objective with NA=1.46. Scale bar = 20 μm.

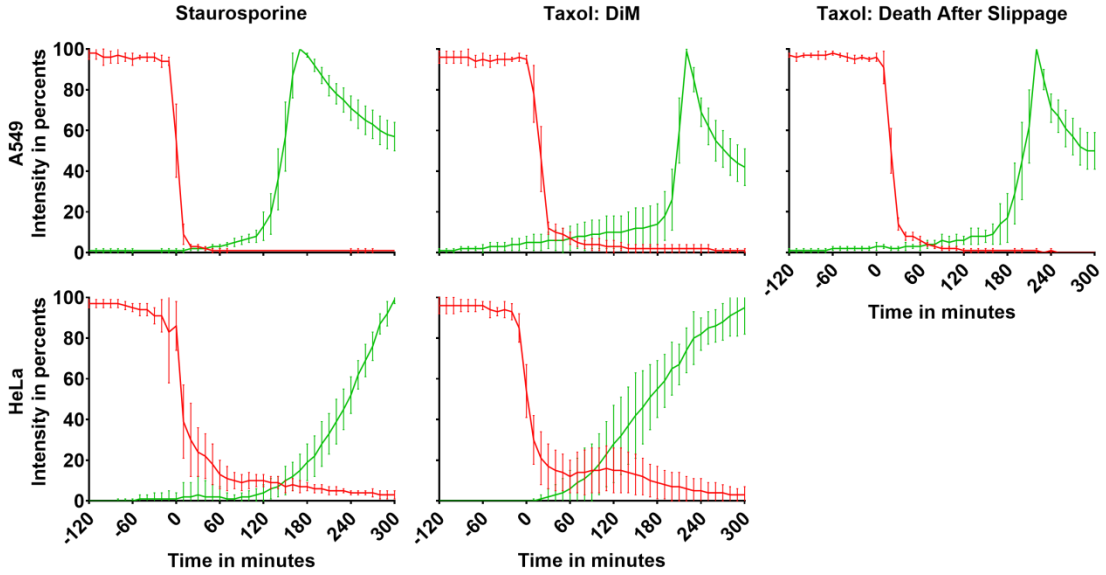

Figure 8. Averaged and normalized intensity values from TMRE (red) and caspase 3/7 detecting reagent (green) for slippage prone A549 and DiM sensitive HeLa cells after treatment with 1 uM Staurosporine (positive control for apoptosis) or 1 uM Taxol. Vertical bars represent SD. N=10-15 cells for each category.

4.5 Inhibition of Bcl-xL, but not other anti-apoptotic Bcl-2 proteins, sensitizes resistant cells to DiM.

To assess the role of individual anti-apoptotic Bcl-2 members in the regulation of apoptosis during mitotic arrest, resistant PC-3 and A549 cells were treated with Taxol in combination with 10 uM S63485 (Mcl-1 inhibitor), 10 uM Venetoclax (Bcl-2 inhibitor), 10 uM A-1155463 (Bcl-xL inhibitor) or 10 uM Navitoclax (Bcl-2/Bcl-xL/Bcl-w inhibitor). When were used alone, each Bcl-2 inhibitor had no visible effect on cells' survival and proliferation. However, PC-3 showed moderate sensitivity to the treatment with 10 uM Navitoclax or A-1155463 alone, with ~20% of the cells dying in interphase.

Inhibition of Bcl-xL sensitized resistant cells to apoptosis and accelerated DiM. When A549 and PC-3 cells were treated with Taxol in combination with Venetoclax or S63485, most A549 and PC-3 cells underwent slippage and survived until the end of the

observation. When slippage prone A549 and PC-3 cells were treated with Taxol in combination with Navitoclax or A-1155463, >90% of mitotically arrested cells died in mitosis. For both cell lines average time spent in mitosis shortened from ~20 hours under the action of Taxol only to ~10 hours under Taxol + A-1155463, whereas duration of mitotic arrest after inhibition of Bcl-2 or Mcl-1 was similar to the duration of mitotic arrest under the action of Taxol only (Figure 9 and Table A4).

On the other hand, inhibition of each anti-apoptotic Bcl-2 member in A549 and PC-3 cells accelerated MOMP during Staurosporine treatment. After treatment with Staurosporine only, MOMP was observed on average after ~11 hours in A549 cells and ~10 hours in PC-3 cells. When Staurosporine was used with each inhibitor of Bcl-2 proteins, average time until MOMP in A549 and PC-3 cells shortened ~3 fold (Figure 9)

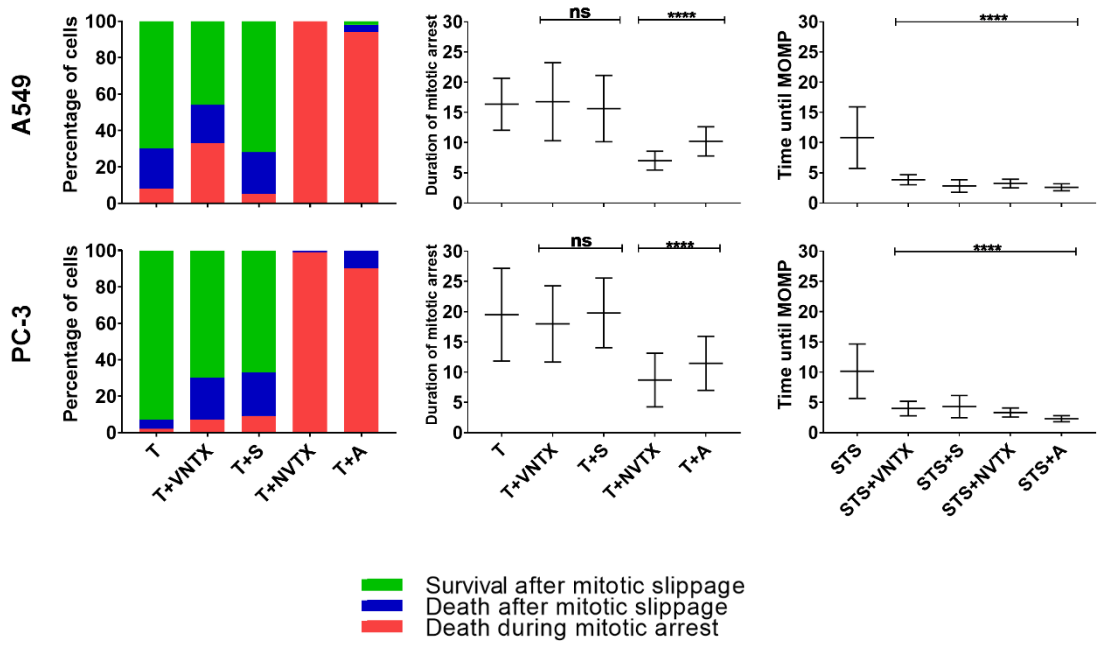

Figure 9. Frequency of fates and respective durations of mitotic arrest after treatment with Taxol (T) in combination with Venetoclax (V), S63845 (S), Navitcolax (N) or A-1155463 (A) and time until MOMP after treatment with Staurosporine (STS) in combination with the same inhibitors of anti-apoptotic Blc-2 proteins for A549 and PC-3 cell lines. Data is shown in the form of mean ± SD. * - p-value < 0.05, ** - p-value < 0.01, *** - p-value < 0.001, **** - p-value < 0.0001.

4.6 Sensitivity to DiM and duration of mitotic arrest depends on the level of Bcl-xL

Since inhibition of Bcl-xL effectively sensitized cells to DiM and accelerated MOMP in slippage prone A549 and PC-3 cells, we assumed that sensitivity to DiM and duration of mitotic arrest could depend on the concentration of available Bcl-xL within the cell. To test this, A549 and PC-3 cells were treated with Nocodazole, Taxol or Vinorelbine in combination with 10-10000 nM A-1155463. We observed that mitotically arrested A549 and PC-3 cells were sensitized to DiM under treatment with A-1155463 in a dose-dependent manner. In both cell lines, with increasing the dose of A-1155463 cell fates shifted from survival after mitotic slippage to death after mitotic slippage to DiM (Figure 10). Effect of Bcl-xL inhibition on survival of mitotic cells was evident starting from 10-30 nM and saturation effect was observed at 300-1000 nM of Bcl-xL inhibitor. For both cell lines average duration of mitotic arrest decreased with increasing the dose of Bcl-xL inhibitor as shown on Figure 11 and Tables A4-5.

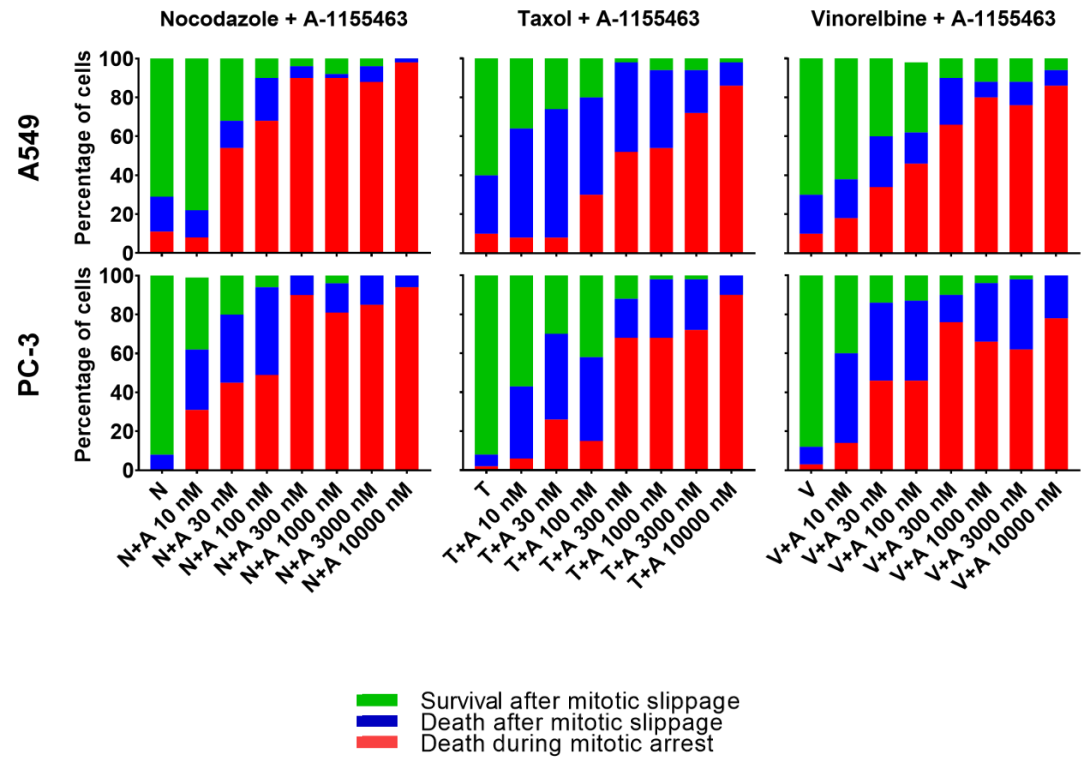

Figure 10. Distribution of fates for slippage prone A549 and PC-3 cell lines after the treatment with 1 uM doses of Nocodazole (N), Taxol (T) or Vinorelbine (V) in combination with 10-10000 nM doses of A-1155463.

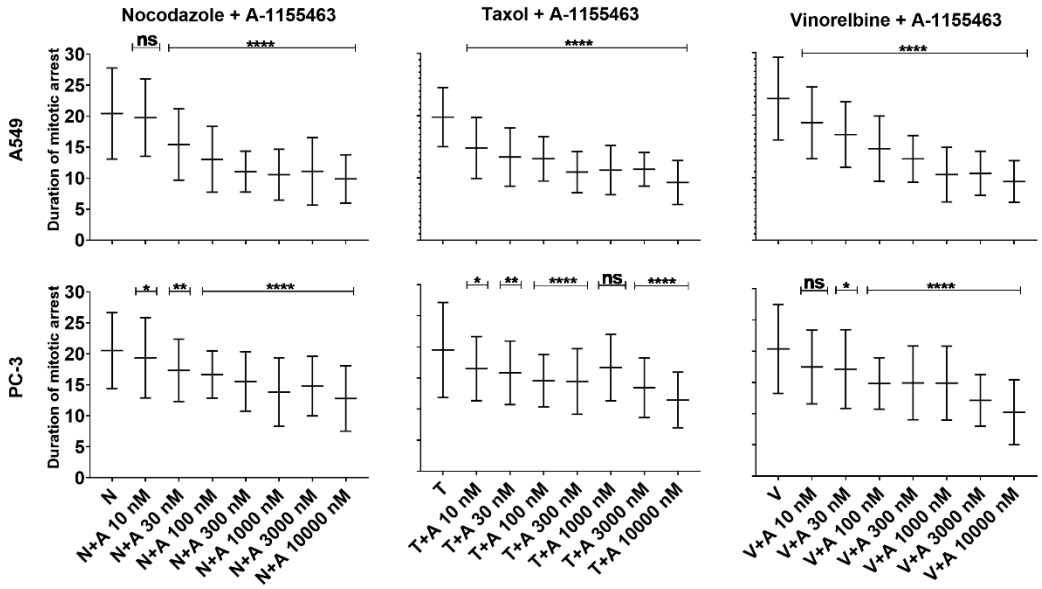

Figure 11. Average durations of mitotic arrest (in hours) of slippage prone A549 and PC-3 cell lines after the treatment with 1 uM doses of Nocodazole (N), Taxol (T) or Vinorelbine (V) in combination with 10-10000 nM doses of A-1155463. Data is shown in the form of mean ± SD. * - p-value < 0.05, ** - p-value < 0.01, *** - p-value < 0.001, **** - p-value < 0.0001

5. DISCUSSION

Substantial amount of data regarding the inter- and intra-line variation following mitotic arrest has already been reported (Gascoigne and Taylor, 2008; etc.). However, because most studies prefer to use overly high and single doses of MT drugs to ensure the occurrence of prolonged mitotic arrest, variation in cell response to different doses of MT drugs has not been studied enough. Thus, to systematically assess the degree of variation with regards to MT drugs, we determined reference doses (T1 and T2) for each cell line-drug type pair by treating each cell line with a wide range of doses (1-1000 nM) of each MT drug. By studying dose-dependent cell response we explicitly showed the existence of two threshold doses, which might be highly relevant to the clinical use of the drugs. Considering increased neurotoxicity of MT drugs, accurate determination of T2 doses could be especially beneficial for the patients. From the point of view of general knowledge, titration of the drugs and identification of references doses allowed us to assess both inter- and intra-line variations from most if not all relevant perspectives: (i) dose-dependent variation within the line after treatment with different doses of a single drug, (ii) drug dependent variation within the line after treatment with equivalent doses of different drugs, (iii) cell-line dependent variation after treatment with equivalent doses of the same drug.

The main focus of this work, as well as most of the others in the field, was to study how cell fate following prolonged mitotic arrest is regulated. Our results also suggest that abnormal division after treatment with lower doses of MT drugs might interfere with the further progression of cell cycle and potentially slow down proliferation of resistant cancer cells.

To confirm apoptosis, approaches like measuring cleaved caspases or PARP and staining with Annexin V or Propidium Iodide have been commonly used. However, these methods are often not sufficient enough as they provide data mostly for fixed specimen and at a single time point only. In our experiments, tracking the dynamics of MOMP and caspase activity provided with detailed insights of the events taking place during the transition from mitosis or post-slippage interphase to apoptosis.

Despite a clear consensus on the importance of anti-apoptotic Bcl-2 proteins in the regulation of MOMP, there is no clear understanding of the role of particular members, especially of Bcl-2, Mcl-1 and Bcl-xl, during mitotic arrest (Shi and Zhou, 2011; Hellmuth and Stemmann, 2020; Huang et al., 2016; Bennet et al., 2016). It has been previously shown that A549 and PC-3 cells have significantly higher intracellular Bcl-xL concentration than HeLa cells (Shi and Zhou, et al., 2011). We propose that Bcl-xL acts as the major regulator of MOMP during mitotic arrest because out of the three studied anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL and Mcl-1) only inhibition of Bcl-xL accelerated the onset of MOMP and sensitized slippage prone cells to death after slippage and DiM after treatment with MT drugs, while during Staurosporine induced apoptosis inhibition of each of the three anti-apoptotic Bcl-2 members significantly accelerated initiation of MOMP.

6. CONCLUSION

In this work we (i) systematically assessed the degree of variation in cell response to the treatment with MT drugs by defining two threshold doses T1 - maximal ineffective dose and T2 - minimal mitostatic dose; (ii) determined that cell death during mitotic arrest and after mitotic slippage proceeds via standard apoptotic pathway. Cell death always starts with MOMP and is followed by activation of caspases 3/7; (iii) determined that Bcl-xL is the major anti-apoptotic protein that defines sensitivity of cultured cells to DiM.

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8. APPENDICES

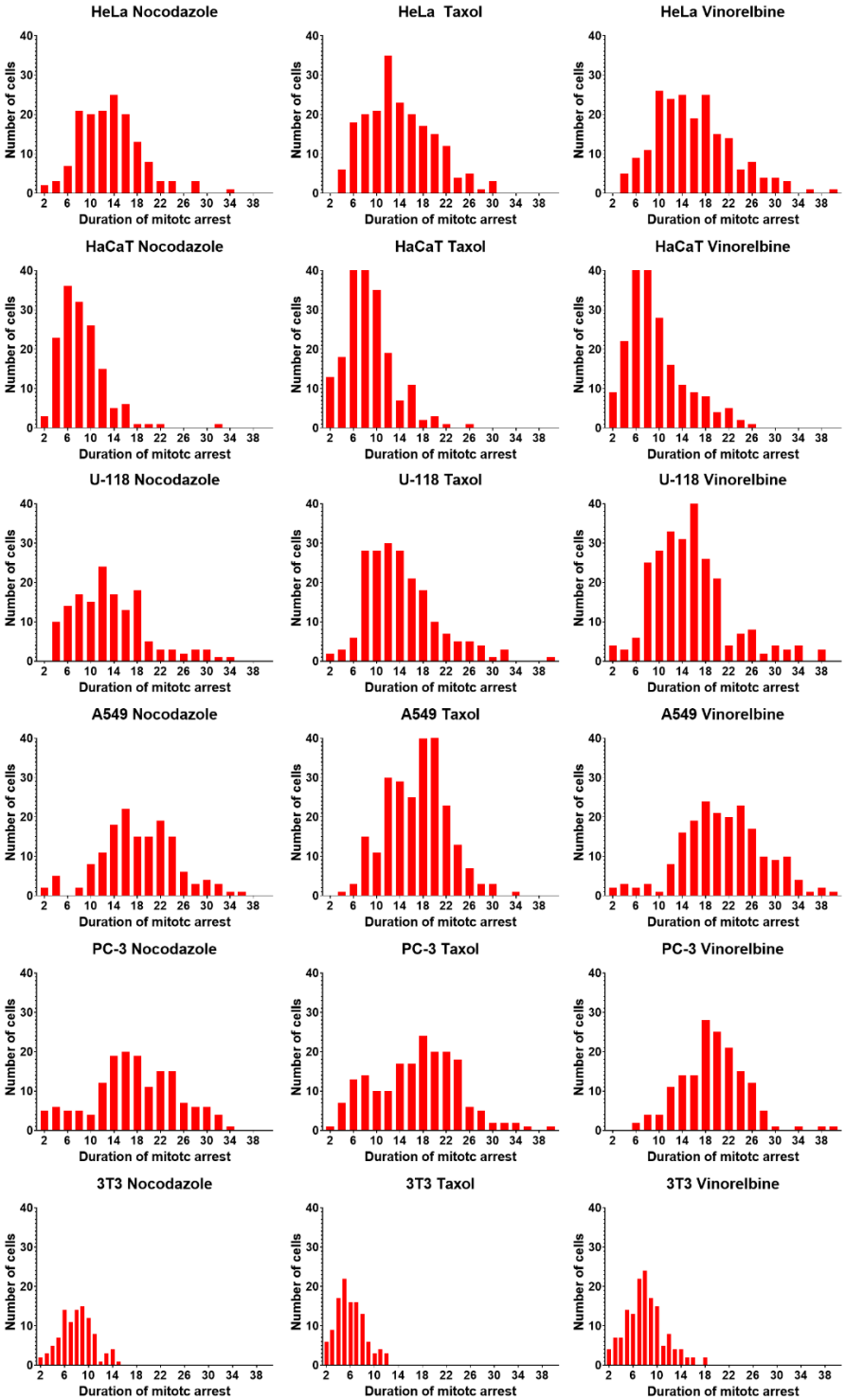

Figure A1. Frequency distributions of the duration of mitotic arrest for HeLa, HaCaT, U-118, A549, PC-3 and 3T3 cell lines treated with post-T2 doses of MT drugs.

Table A1. Average durations of mitotic arrest (in hours) in post-T2 doses of Nocodazole, Taxol and Vinorelbine for HeLa, HaCaT, U-118, A549, PC-3 and 3T3 cell lines. N= >100 cells for each treatment group.

	Nocodazole		Taxol		Vinorelbine	
	mean	SD	mean	SD	mean	SD
HeLa	13,23	5,25	13,82	5,82	15,79	6,59
HaCaT	8,28	4,08	8,48	4,19	9,27	4,97
U-118	13,21	6,47	14,02	6,10	15,26	6,71
A549	18,11	6,43	16,94	5,22	21,45	7,82
PC-3	17,39	7,25	16,99	7,49	19,37	6,08
3T3	7,91	2,80	5,95	2,35	7,96	3,19

Table A2. Results from ANOVA analysis. Comparison of durations of mitotic arrest within the same cell line after treatment with Nocodazole (N), Taxol (T) or Vinorelbine (V).

	Significant?	p-value
N HeLa vs. T HeLa	No	0,6302
N HeLa vs. V HeLa	Yes	0,0002
T HeLa vs. V HeLa	Yes	0,003
N HaCaT vs. T HaCaT	No	0,9159
N HaCaT vs. V HaCaT	No	0,1011
T HaCaT vs. V HaCaT	No	0,1784
N U-118 vs. T U-118	No	0,474
N U-118 vs. V U-118	Yes	0,0061
T U-118 vs. V U-118	No	0,107
N A549 vs. T A549	No	0,1863
N A549 vs. V A549	Yes	<0,0001
T A549 vs. V A549	Yes	<0,0001
N PC-3 vs. T PC-3	No	0,8565
N PC-3 vs. V PC-3	Yes	0,0303
T PC-3 vs. V PC-3	Yes	0,0044
N 3T3 vs. T 3T3	Yes	<0,0001
N 3T3 vs. V 3T3	No	0,9908
T 3T3 vs. V 3T3	Yes	<0,0001

Table A3. Results from ANOVA analysis. Comparison of durations of mitotic arrest between different cell lines after treatment with Nocodazole, Taxol or Vinorelbine.

	Nocodazole		Taxol		Vinorelbine	
	Significant?	p-value	Significant?	p-value	Significant?	p-value
HeLa vs. HaCaT	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001
HeLa vs. U-118	No	>0,9999	No	0,9991	No	0,9468
HeLa vs. A549	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001
HeLa vs. PC-3	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001
HeLa vs. 3T3	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001
HaCaT vs. U-118	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001
HaCaT vs. A549	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001
HaCaT vs. PC-3	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001
HaCaT vs. 3T3	No	0,9962	Yes	0,0016	No	0,368
U-118 vs. A549	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001
U-118 vs. PC-3	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001
U-118 vs. 3T3	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001
A549 vs. PC-3	No	0,8771	No	>0,9999	Yes	0,0194
A549 vs. 3T3	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001
PC-3 vs. 3T3	Yes	<0,0001	Yes	<0,0001	Yes	<0,0001

Table A4. Average durations of mitotic arrest (in hours) for A549 cells treated with 1 uM Taxol (T), Nocodazole (N) or Vinorelbine (V) + 10-10000 nM A-1155463 (A). For each treatment group N = 30-50 cells.

A549																
A-1155463	0		10 nM		30 nM		100 nM		300 nM		1000 nM		3000 nM		10000 nM	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
N	20,43	7,35	19,76	6,24	15,43	5,75	13,04	5,30	11,05	3,30	10,56	4,11	11,10	5,45	9,88	3,88
T	19,77	4,75	14,82	4,91	13,36	4,69	13,08	3,56	10,94	3,32	11,25	3,95	11,39	2,71	9,26	3,53
V	22,68	6,62	18,79	5,75	16,91	5,23	14,64	5,22	13,01	3,73	10,50	4,38	10,71	3,53	9,39	3,33

Table A5. Average durations of mitotic arrest (in hours) for PC-3 cells treated with 1 uM Taxol (T), Nocodazole (N) or Vinorelbine (V) + 10-10000 nM A-1155463 (A). For each treatment group N = 30-50 cells.

PC-3																
A-1155463	0		10 nM		30 nM		100 nM		300 nM		1000 nM		3000 nM		10000 nM	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
N	20,52	6,14	19,34	6,46	17,33	5,02	16,68	3,81	15,54	4,79	13,83	5,51	14,80	4,82	12,80	5,26
T	19,51	7,65	16,51	5,14	15,84	5,11	14,57	4,19	14,45	5,27	16,70	5,35	13,44	4,78	11,47	4,48
V	20,37	7,12	17,48	5,91	17,14	6,30	14,83	4,12	14,92	5,91	14,90	5,93	12,14	4,13	10,23	5,21