NAZARBAYEV UNIVERSITY

Investigating changes in cell cycle distribution of cultured cells in response to microtubule inhibitors

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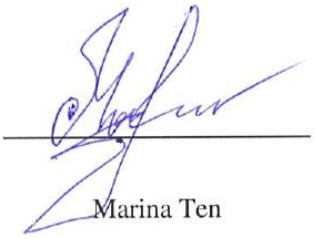
DECLARATION

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.



Marina Ten

27/05/22

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ABSTRACT

Microtubules participate in the separation of chromosomes during mitosis. Microtubule (MT) inhibitors preclude normal cell division by interfering with mitotic spindle formation during anaphase. Failure to form a mitotic spindle for a prolonged time results in prolonged mitotic arrest. The arrest in mitosis can be assessed via microscopy, where cell death in mitosis, cell survival via mitotic slippage, or unequal division outcomes can be directly observed. Different outcomes of mitotic arrest can also be analyzed at the populational level by measuring the DNA content of each cell in a population using flow cytometry. This method uses information on cell ploidy to draw a DNA content histogram, which represents the distribution of cells across different phases of the cell cycle. The normal distribution is depicted as a linear diagram of cycle phases, with the majority of cells in G0/G1 (2n), some in S (2n–4n), and others accumulating in G2/M (4n).

As a result of mitotic arrest, normal cell cycle distribution changes. Microtubule inhibitors induce prevalent accumulation in G2/M or SubG1 populations. SubG1 or "sub-diploid" population have cells with <2n ploidy. It therefore represents hypodiploid or degraded DNA cells. In the SubG1 population, cells in late apoptosis with active nucleases and cells exited via unequal division (1n) can be obtained. G2/M population cells (4n) probably indicate cells that exited from the mitotic block without division (mitotically slipped cells).

Spindle assembly checkpoint (SAC) is the cell's surveillance system during mitosis. Inability to satisfy its conditions leads to the mitotic block, as in the case with MT drugs. However, with the ability to deactivate SAC, the duration of mitotic arrest can be controlled. The duration of mitotic arrest may play a significant role in the cell's response to the treatment. Deactivation of SAC using an Aurora B kinase inhibitor (Barasertib) should theoretically result in reduced death signals, nuclear envelope re-formation even in cells with 4n set, and thus result in greater cell survival.

Mitotically slipped cells can also be regarded as survived cells. Thus, G2/M-prone cell lines that produce them should be somehow sensitized. One way to sensitize cells (enhance apoptotic response) to MT treatment- is to force cells blocked in mitosis to die. It can be achieved with the inhibition of different anti-apoptotic BCL-2 proteins (BCL-2, BCL-XL, MCL-1). Shifting equilibrium towards cell death signals should enhance caspase-dependent apoptosis, and force mitotically slipped cells to die.

In this work, we attempted to investigate the changes in cell cycle distribution that resulted from prolonged mitotic arrest in a dose-dependent manner. We tried to identify whether cell response was dependent on the nature of the mitotic inhibitor or, more probably, cell line dependent. It was obtained that all drugs exhibit similar effects within a cell line, but the inter-line response to drugs was highly variable. We found two types of responses to MT treatment: SubG1-prone and G2/M-prone accumulation of cells. In response to prolonged mitotic arrest, A549 and PC-3 cells showed accumulation in the G2/M peak, whereas HaCaT and HeLa cells accumulated in the SubG1 peak instead. We identified maximal and minimal drug threshold concentrations, which successfully describe the changes in cycle distribution that resulted from MT treatment.

We discovered that inducing short mitotic blocks with an Aurora B kinase inhibitor probably plays a role in cell survival, forcing SubG1-prone cell lines to shift to G2/M-prone with many polyploid cells. We found that maintaining the level of anti-apoptotic BCL-XL proteins but not BCL-2 or MCL-1 proteins can affect the shift of G2/M-prone cells to become SubG1-prone.

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ABBREVIATIONS

MT- microtubules

SAC- spindle assembly checkpoint

ATCC- American Type Culture Collection

HeLa- cervical carcinoma cell line

HaCaT- spontaneously transformed human keratinocytes

PC-3- human prostate cancer cell line

A549 – human lung carcinoma

DMEM- Dulbecco's Modified Eagle Medium

FBS- fetal bovine serum

PBS-Phosphate buffered saline

FSC- forward scatter

SSC- side scatter

PI- Propidium Iodide

RNAse A- Ribonuclease A

Noc- Nocodazole

Tax- Taxol

Vin- Vinorelbine

AZD1152 (Barasertib)- Aurora B kinase inhibitor

MOMP- mitochondrial outer membrane permeabilization

BCL-2 proteins– B cell lymphoma 2 proteins

A11- A1155463 BCL-XL inhibitor

S63845- BCl-2 inhibitor

Venetoclax- MCL-1 inhibitor

1. INTRODUCTION

1.1 *Microtubules and their role in mitosis*

Microtubules are key cell cytoskeleton elements with the ability to rapidly assemble and disassemble. Microtubules are polymers composed of alpha and beta-tubulin heterodimers that are non-covalently bound to each other. The continuous cycles of growth (assembly) and shortening (disassembly) at the ends of microtubules determine the dynamicity of these structures. GTP cap at the ends of microtubules stabilizes it and allows continuous assembly of tubulin bound GTP subunits (O'Brien et al, 1987). When the microtubule loses GTP-cap, its core becomes unstable leading to the rapid shortening of the microtubule, named catastrophe (Jordan and Wilson, 2004). After catastrophe microtubule can depolymerize completely or return to growth – rescue.

The phenomenon of microtubule switching between growth and shrinkage stages is called dynamic instability. It allows microtubules to rapidly assemble the mitotic spindle (Gascoigne and Taylor, 2009), which assists in chromosome alignment in metaphase plate and segregation during anaphase (Guacci et al., 1993; Cooper, 2000). The dynamic instability determines the movement of chromosomes during mitosis (McIntosh, Grishchuk and West, 2002).

There are four major cell cycle stages: G1, S, G2 and M (Nasmyth, 1996). During the G1 phase, favorable external conditions and the presence of internal growth factors stimulate cells to start the process of DNA replication. After passing a restriction point, cells enter the S phase, where the amount of nuclear material of the original cell doubles. G2 is an intermediate phase between the end of DNA replication (S) and the beginning of mitosis (M). During the M phase, the bipolar mitotic spindle is

formed. The mitotic spindle pulls apart sister chromatids into separate nuclei, and cell division occurs. Quiescent cells, which do not progress through the cell cycle, reside in the G0 phase. (Alberts et al., 2002; Israels and Israels, 2000)

1.2 *Microtubule inhibitors induce prolonged cell cycle arrest in mitosis*

Mitosis is a multistep process that includes five main stages: prophase, prometaphase, metaphase, anaphase, and telophase. In prophase, nucleolus disappears, and chromatin condensation occurs. The vanishing of nuclear envelope, chromosome condensation and movement, and kinetochore assembly and attachment to microtubules are all related to prometaphase. During metaphase, chromosomes are positioned on a metaphase plate. In anaphase, upon activation of APC/C, chromatid pair is pulled towards the opposite poles by mitotic spindle generating separate chromosomes. Finally, mitosis ends by telophase; DNA in a form of separate chromosomes locates near opposite poles of the cell, the mitotic spindle disintegrates, and small pieces of mother cell's nuclear membrane condense around chromosomes located in opposite poles (O'Connor, 2008). Phosphatases then remove phosphate groups from nuclear lamin proteins causing nuclear envelope to be restored in each daughter cell.

The onset of anaphase starts with cyclin B degradation and subsequent inhibition of CDK1. These events lead to the dephosphorylation of numerous proteins including nuclear lamins. Lamin proteins lacking phosphate group depict the formation of the nuclear envelope, and the cell exits mitosis by dividing into two descendant cells. An essential requirement for proper mitosis - is the attachment of kinetochores to microtubules. Throughout metaphase in dividing cells, cell poles pulling chromosomes to opposite directions generate tension. This force is created at the interface of kinetochore-microtubule (KT-MT) attachment. Tension on KT-MT is

essential for: appropriate bipolar orientation of sister chromatids, fulfilling the Spindle Assembly Checkpoint (SAC) requirements and for proper entry into anaphase (Salmon and Bloom, 2017). SAC complex formed by kinetochores in the prometaphase ensures correct chromosome separation. If SAC conditions are satisfied, the anaphase-promoting complex/cyclosome (APC/C) gets activated. In turn, APC/C ligase is responsible for rapid cyclin B degradation by means of ubiquitination. Finally, the destruction of cyclin B allows a cell to escape mitosis.

Microtubule stabilizing (Taxol) and destabilizing (Nocodazole, Vinorelbine) agents block cell division by reversibly binding to tubulin and interfering with mitotic spindle formation, thus halting metaphase progression (Hande, 1992). MT inhibitors suppress spindle-microtubule dynamics for a substantial amount of time (Jordan and Wilson, 2004).

When exposed to MT inhibitors, the entire process of mitosis is disrupted starting from the prometaphase. As it was mentioned above, anti-mitotic agents disrupt normal microtubule activity. Because of the loss of dynamic instability, microtubules fail to form functioning mitotic spindle and quickly respond to changes in the cell, which directly affects kinetochore-microtubules. As a result of delayed metaphase in cells, decreased kinetochore tension is observed (Mimori-Kiyosue et al., 2006). Insufficient tension forces applied to the centromeres of the chromosome are unable to pull chromosomes apart. Thus, SAC cannot be satisfied, leading to its continuous activation. Here, the process inverse to that of normal mitosis takes place. Continuous activation of SAC suppresses the activity of APC/C and prevents anaphase onset. Inhibited APC/C is unable to tag cyclin B to destroy via ubiquitination. High levels of cyclin B proteins and active CDK1, both keep nuclear lamins phosphorylated and chromosomes condensed for a long time. As a result, no nuclear envelope is formed,

and the cell gets stuck in mitosis.

However, there are different outcomes of mitotic arrest to happen.

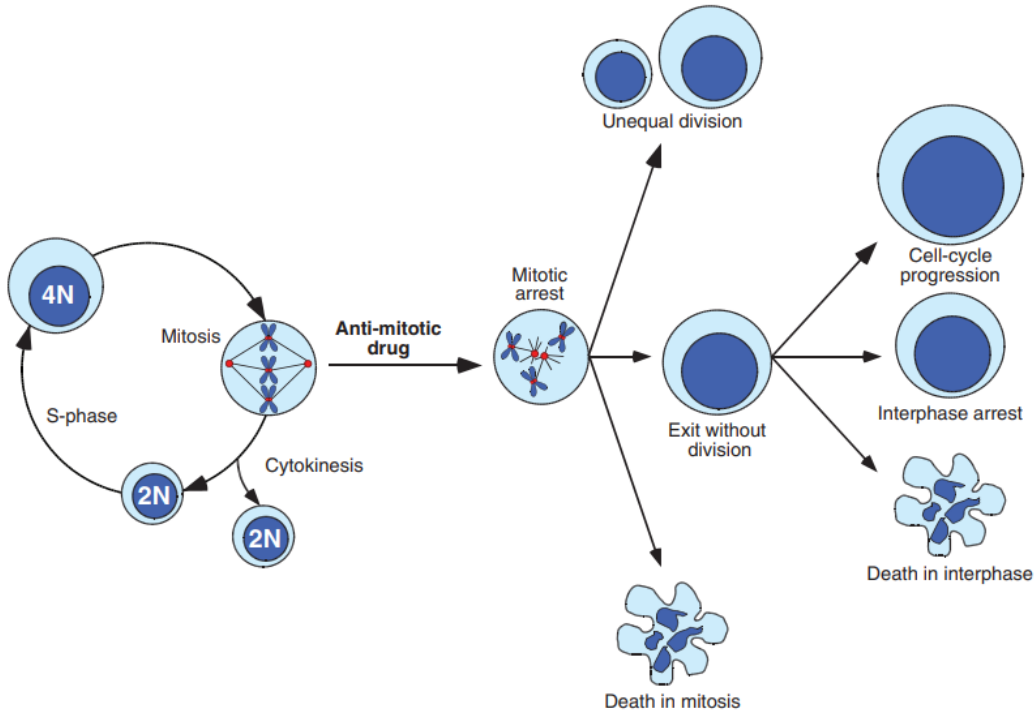

Figure 1. The process of formation of different cell responses to MT drug treatment (Gascoigne and Taylor, 2009).

In 2009, Gascoigne and Taylor stated that prolonged mitotic arrest has a complex and variable response in cells. They suggested a model of two competing networks (Gascoigne and Taylor 2008, 2009.): death in mitosis and exit from mitosis without division. The first network model suggests that mitotic catastrophe occurs as a result of prolonged mitotic arrest. The major consequence of mitotic catastrophe is apoptosis. It usually occurs via a caspase-dependent pathway (Niikura, 2007); however, it has been reported that cell death can also happen without the involvement of apoptotic caspases (Bröker et al., 2004). The gradual destruction of cyclin B can occur even in the presence of continuously activated SAC. As we know, SAC keeps the APC/C complex inactive, thus maintaining high levels of cyclin B. Nonetheless, Brito and Rieder (2006) showed cyclin B gradual degradation via proteolysis in a

proteasome-controlled pathway.

According to Gascoigne and Taylor (2009) work, both networks have threshold levels; and the future of the cell depends on which threshold is achieved first during mitotic arrest. If the death threshold is reached first, cell death in mitosis occurs. Alternatively, the cell survives via slippage if the destruction of cyclin B will bring its levels down to the threshold allowing exit into the interphase first. An unequal division can occur if the concentration of mitotic inhibitors is not sufficient to cause either of these outcomes.

Cells successfully slipped through a mitotic arrest can re-enter the cell cycle, become senescent (interphase) or die after slippage. (Rieder and Maiato, 2004; Blagosklonny, 2007).

Our mission is to investigate the outcomes of mitotic arrest comprehensively. One approach to analyzing the frequency of occurrence of different outcomes on a population level is flow cytometry.

1.3 *Flow cytometric analysis of changes in the cell cycle distribution caused by MT inhibitors*

The cell cycle illustrates the progression of a cell through a cycle of division. In the G1 phase of the cell cycle, RNA and proteins are synthesized, but the DNA content remains unchanged (2n). During the S phase, DNA synthesis occurs. Cells in the S phase have a variable genetic material (2n-4n). G2 cells with finished DNA synthesis possess twice of original DNA (4n). Both nuclear and cytoplasmic divisions occur during the mitosis (M) phase. After the cell passes the M phase, two diploids (2n) daughter cells are formed. Cells then shift to quiescence (G0 phase, 2n) or re-enter the cell cycle under proper activation signals. (Cooper, 2000)

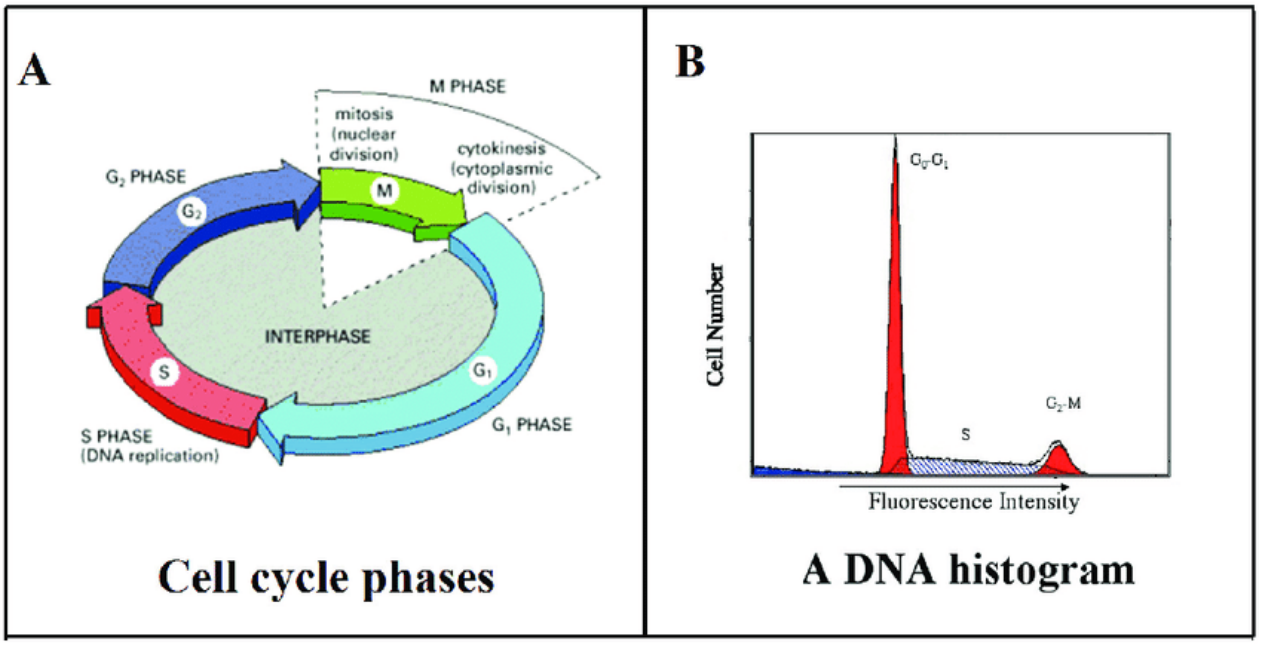

Figure 2. The correlation between cell cycle phases and DNA histogram obtained on a flow cytometer (Tabll and Ismail, 2011).

In flow cytometry, scientists analyze the distribution of cells accumulating in different nuclear states. One of the most popular flow cytometry uses is DNA content analysis. The amount of DNA in a cell suggests which proliferation state this cell belongs to. When analyzing a suspension of cells, the distribution of these cells in different cell cycle populations becomes available (Cobb and Das, 2013; Darzynkiewicz, Halicka and Zhao, 2010). Flow cytometry is widely applied to investigate the effect of drugs that affect the cell cycle.

Microtubule inhibitors act directly on the mitotic spindle appearing in the M phase. As a result of mitotic arrest, MT inhibitors should primarily induce the accumulation of cells in the G2/M phase (4n). G2/M phase can potentially contain cells blocked in mitosis; cell slipped mitosis without division. Another popular outcome for cells is SubG1 accumulation resulted from apoptotic events mainly. SubG1 possess hypodiploid amount of DNA usually produced as a result of unequal division or nucleases activity in late apoptosis. SubG1 can contain cell debris as well.

The analysis of cell cycle distribution after 24h exposure to MT inhibitors was

already published (Potashnikova et al., 2019). Briefly, during 24h exposure, cells accumulated in the G2/M phase in response to the different drug doses in a non-linear fashion.

In the present work, we attempted to investigate the changes in cell cycle distribution that resulted from the prolonged mitotic arrest by incubating cells in drugs for 48h. We also tried to identify whether cell response was dependent on the nature of the mitotic inhibitor or, probably, was cell line dependent.

1.4 *The duration of mitotic arrest and cell response*

As it was mentioned previously, the prolonged mitotic arrest involves interference with MT dynamics and continuous activation of SAC. But what happens in short-term mitotic arrest? Some studies (Huang et al., 2009; Shi et al., 2011) suggest short-term mitotic arrest involves normal entrance into M phase, but early SAC deactivation followed by nuclear envelope assembly around unsegregated chromatids. This can be achieved applying Aurora B kinase inhibitors. In a normally functioning mitosis Aurora B kinase keeps SAC proteins attached to tensionless kinetochores which are totally dependent on KT-MT interactions. It was discussed recently (Gurden et al., 2018), Aurora B kinase precludes premature dissociation of SAC proteins from malfunctioning kinetochores thus providing better surveillance and inhibition of SAC silencing.

The inhibition of Aurora B vice versa silences SAC proteins activity and leads to the activation of APC/C. Active anaphase-promoting complex/cyclosome rapidly degrades circulating cyclin B. As a result of cyclin B reduction, phosphatases dephosphorylate nuclear lamins forcing nuclear envelope to be quickly restored even in the absence of chromosome segregation. Since SAC complex is formed only at the early prometaphase and dissociated upon the completion of M phase, the inhibition

of Aurora B kinase introduces relatively short-term (compared to MT inhibitors) aberrations in mitotic events, namely SAC inhibition. As a result of the kinase blockade endocycle occurs; improper chromosome alignment during mitosis and polyploidy can be observed. Barasertib (AZD1152) is an Aurora B kinase inhibitor (Wilkinson et al., 2007).

In this study, we tried to investigate whether short-term mitotic arrest with Barasertib would cause a response in cells different from that of a prolonged mitotic arrest caused by MT inhibitors.

1.5 *The effect of anti-mitotic treatment combined with anti-apoptotic inhibitors*

Although MT drugs cause a prolonged mitotic arrest, the number of cells predetermined to death is highly variable depending on the cancer cell line. Slippage mechanism can be addressed a survival mechanism for cells to escape death in mitosis. Thus, mitotically slipped cells are "survivors" of cell death.

The preliminary data, obtained in our laboratory recently, suggests that some cell lines can successfully survive prolonged mitotic arrest and proliferate even in the presence of 1 μM of MT inhibitors. These cells appear to accumulate in the G2/M population (G2/M-prone) in flow cytometry data measurements. The G2/M peak accumulation in G2/M-prone cells probably indicates mitotically slipped cells (survivors). The rate of death in "survived" cells after mitotic slippage is uncertain.

There is evidence of non-apoptotic death (Bröker et al., 2004), however, other studies (Niikura, 2007; Shi et al., 2008; Shi et al., 2011) suggest that MT drugs induce apoptosis (during and after mitotic arrest) by the intrinsic apoptosis pathway involving loss of integrity in the mitochondrial outer membrane.

BCL-2 family of proteins are known for regulation of mitochondrial outer membrane

permeabilization (MOMP). This family have both pro- and anti-apoptotic members all of which can control MOMP. The balance between proteins inducing and inhibiting apoptosis determines cell survival. BCL-2, BCL-XL and MCL-1 members of this family are anti-apoptotic proteins.

To improve the efficacy of MT treatment, the combination of MT inhibitors with suppressors of anti-apoptotic proteins is possible (Shi et al., 2011). Shi et al. showed that Navitoclax (BCL-2 and BCL-XL proteins inhibitor) combined with paclitaxel (Taxol) illustrates diminished cell survival. Nowadays, more specific BCL-XL inhibitors were discovered (Leverson et al., 2015).

In terms of flow cytometry data, changes in cell cycle distribution regarding the combined mitotic and anti-apoptotic proteins' inhibition may appear. Therefore, in this study, we aimed to investigate effect of combined treatment on cell cycle distribution, using highly selective BCL-2, BCL-XL and MCL-1 inhibitors.

2. MATERIALS & METHODS

2.1 *Cell culture and reagents*

A549, PC-3, HeLa and HaCaT cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, United States). A549, HeLa and PC-3 are cancer cell lines and HaCaT are minimally transformed cells. PC-3 cells were grown in Nutrient Mixture F-12 Ham and other cell lines in DMEM (Thermo Fisher Scientific, United States) supplemented with 4-8mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin and 100 ug/ml strepromycin (Thermo Fisher Scientific, United States). Cells were maintained at 37°C in 5% CO_2 incubator. 0.05% Trypsin-EDTA solution (Sigma-Aldrich, United States) was applied to detach cells from the surface of cell culture dishes.

Paclitaxel (Taxol®), Nocodazole, and Vinorelbine MT inhibitors were all procured from Sigma-Aldrich, United States. A1155463, S63845 and Venetoclax BCL-2 family protein inhibitors were purchased in Sigma. Barasertib Aurora B kinase inhibitor was acquired in Sigma as well.

2.2 Seeding and incubation of cells with drugs

From T25 flask cells were seeded to 12-well plastic plate at a moderate density. The plate was left overnight for cells to grow until ~70% confluency. In Eppendorf tubes MT inhibitors were diluted in complete media using serial dilution to obtain 3-1000nM concentration range. Then, old cell culture media was aspirated and replaced with fresh media containing drugs in a wide range of concentrations. Cells were incubated in the presence of drugs in 5% CO_2 incubator at 37°C for two days. Control cells remained untreated. For experiments with A1155463, S63845, Venetoclax and Barasertib different drug concentrations/combinations, but similar procedure was

applied.

2.3 Cell cycle analysis with Flow cytometry

In the analysis of DNA content by flow cytometry, cells of interest are incubated with fluorescent dyes that stain DNA. Staining of DNA with dyes indicates the amount of DNA present in each cell. DNA content distribution in flow cytometry gives a single point measurement using linear fluorescence. If the dye is stoichiometrically bound to DNA, 2N cells will be exactly half of the 4N cells on the DNA content histogram.

To analyze cell cycle distribution of affected cells with propidium iodide staining the modified version of the procedure from the previously published paper (Potashnikova et al., 2018) was used. Briefly, cells were treated with MT inhibitors (3-1000nM) for 48h in 37°C, control cells remained untreated. After 48h of incubation cells were harvested by standard trypsinization procedure and washed with 500ul of PBS at room temperature by centrifugation. Cells were centrifuged again at 3000rpm for 5 min at room temperature. Cell pellet was resuspended with 500ul of cold 70% ethanol which fixed cells. The obtained cell suspension was incubated for at least 4h at 4°C. Cells were pelleted again and stained with 30ug/ml PI (propidium iodide) and 5ug/ml RNAse A.

Cells were analyzed using Attune NxT Flow Cytometer at Ex.488nm/Em.550-630nm for PI. Flow Jo software (BD, Ashland, OR) was used to analyze DNA content percentage in each phase of the cell cycle. Each measurement was done at least in triplicate. Over 30 000 events were analyzed using flow cytometer Similar procedure was performed for experiments with A1155463, S63845, Venetoclax and Barasertib.

2.4 DIC and microscopy data collection

Cells were seeded in 24-well plastic plates at 50% density and left overnight. On the

next day, old media was aspirated from the plates. Each well was refreshed with 500ul media containing 1μM of either Noc, Tax or Vin. Plates were placed into 5% CO_2 incubator and maintained at 37°C. After 48h and prior to microscopic investigation cells were stained with two different fluorescent dyes (TMRE and Hoechst 33342). Both probes were purchased in Abcam (Cambridge, UK). All microscopic images were obtained from Zeiss Axio Observer microscope (Carl Zeiss AG, Germany) equipped with Hamamatsu ORCA V2 CMOS camera. Microscope is supplied with heating chamber unit and Zen blue software. Cell images were acquired using EC Plan/Neofluar x20/0.5 objective. in DIC and fluorescent modes. All microscopy data was saved in czi format files and viewed with Zen blue software. Image analysis and processing was performed using ImageJ/Fiji software.

2.5 Statistical analysis

The statistical analysis was conducted using unpaired t-test (95% confidence interval) on a Graphpad Prism software.

3. AIMS OF THE THESIS PROJECT

Undertaking populational level analysis of mitotic arrest by measuring DNA content via flow cytometry we addressed the following questions:

- To perform a systematic assessment of cell response to prolonged mitotic arrest with MT inhibitors

  To determine whether there is a significant accumulation in SubG1 population in response to MT inhibitors. What are the effective concentrations causing “highest” accumulation?

  To determine whether there is a significant accumulation tetraploid population in response to MT inhibitors. What are the effective concentrations causing “highest” accumulation?

  To determine threshold concentrations where alterations in cell cycle distribution occur.

- To assess how the duration of mitotic block affects cell survival of SubG1-prone cells
- To evaluate the role of different BCL-2 protein inhibitors in the sensitization of G2/M-prone cells

4. RESULTS

4.1 Dose-dependent response of cells to microtubule inhibitors

The mitostatic effect of microtubule inhibiting drugs is well-known and widely described in the literature (Zieve et al., 1980; Jordan et al., 1993; Jordan et al., 1996). Some MT inhibitors are successfully used for cell synchronization in the M phase (Jackman and O'Connor, 2001). The effect of the mitotic block duration on cell fate has also been described (Bekier et al., 2009). However, working concentrations of MT inhibitors and target cell lines vary greatly from one study to another. To systematize the intra- and inter-line variations of the cell response to MT drugs in a dose-dependent fashion, we tested different concentrations of three MT inhibitors (Nocodazole, Taxol, Vinorelbine) on four cell lines (HaCaT, HeLa, A549, PC-3) using flow cytometry. By estimating the DNA content of each individual cell, we identified the changes in the normal distribution of cells across different phases of the cell cycle, which resulted from the drug influence.

Normal cell cycle distribution is illustrated in flow cytometry as a DNA content histogram. The G0/G1 phase represents the first peak, commonly three times higher than the second G2/M peak. S phase represents a consistent level elevation between two peaks. G2/M peak is located twice arbitrary units apart from G0/G1 peak. It is worth mentioning that flow cytometry does not allow to distinguish the G0 phase from G1 or G2 from the M phase; rather, it lines up peaks according to the cell ploidy. Each peak corresponds to a particular ploidy value. Thus, G0/G1 peak possesses 2n cells, and the G2/M peak has 4n cells. The cells containing intermediate (2n-4n) ploidy are in S phase. Cells with fractional DNA content (<2n) and polyploid cells (>4n) were defined as Sub-G1 and Above G2 populations.

There is no visible change in normal cell cycle distribution at the lowest inhibitor dose (3nM) (Figure 3). However, with the increase in concentration, the distribution of cells in cell cycle populations varies. We then defined minimal and maximal threshold doses. A minimal threshold dose is a drug concentration, where first alterations in cell cycle distribution occur (in comparison to control). Above the maximal threshold dose, the distribution of cells in the cell cycle remained highly conservative within the cell line.

From the Figure 3 and Figure 4 for we can observe changes in cell response to MT

treatment. For HeLa cells 30nM Nocodazole, 30nM Vinorelbine and 10nM Taxol are minimal effective doses. At these doses the level of SubG1 is about 13-20%, and the level of accumulation in the S phase was 2-fold increase compared to control. At the same time, the number of cells accumulating in the G0 and G2 phases decreases significantly. For HeLa cells, 100nM of any inhibitor is the maximum threshold dose where around 50-60% of cells are found in SubG1, and the remaining cells are distributed among other phases. Cell cycle distribution obtained at 100nM is conserved for 300nM and 1000nM concentrations (Figure 3,4).

For HaCaT cells 30nM of Nocodazole and 10nM of Taxol and Vinorelbine are minimal effective doses. At these concentrations SubG1 increases, however the distribution retains general trends. At 100nM of Nocodazole and Vinorelbine (80% and 70% in SubG1 respectively) as well as in 30nM of Taxol cells begin to massively die which is illustrated as sharp raise of SubG1 peak (60% in SubG1). Cell cycle distribution obtained at maximal threshold doses is conserved within higher concentrations (Figure 3,4).

At minimal threshold doses, the first changes occur with the increase of MT inhibitor's concentration, and the percentage of cells in SubG1 and S phases of HeLa and HaCaT cell lines increases. At the same time, the percentage of cells in G0/G1 and G2/M phases is decreasing accordingly. At maximal threshold dose and above, almost all cells end up in the SubG1 phase, and the remaining cells are distributed to other cycle phases (Figure 3,4).

A different picture is observed in PC-3 and A549 cell lines. Minimal threshold doses for PC-3 cells are: 100nM Nocodazole, 100nM Vinorelbine and 30nM Taxol. Here G0/G1 drops from 60% in control to 10-20% in experiments, while G2/M peak raises from 20% in control to about 40% in Taxol and Vinorelbine treatments. SubG1 peak is present as well. Maximal threshold doses are characterized by the presence of predominant peak- G2/M. In maximal threshold concentrations SubG1 peak is also present, but other G0/G1 and S peaks are almost absent (Figure 3,4).

For A549 cells minimal effective dose varies greatly among different anti-mitotic agents. As in PC-3 cells G0/G1 peak remarkably falls, but SubG1 and G2/M raise sharply. Fluctuations of percentage of S phase are also taking place. 300nM of either MT inhibitor is a maximal threshold dose for A549 cells. In A549 cells, maximal threshold doses are characterized by two equal populations of SubG1 and G2/M cell units and by the absence of G0/G1 peak (Figure 3,4).

At a minimal threshold dose, A549 and PC-3 cells tend to accumulate in the SubG1 phase and the G2/M phase. However, unlike HeLa and HaCaT cells, they behave differently at maximal threshold doses and higher. In A549 and PC-3 cells, the higher the drug concentration, the more cells accumulate in G2/M and less in SubG1. The remaining cells in a population are distributed across other cell cycle populations.

We found that all MT inhibitors exhibit similar responses within a cell line. All three drugs produced a massive SubG1 accumulation response in HeLa and HaCaT cells, which implicitly indicates a high apoptosis rate (Kajstura et al., 2007). Oppositely, in A549 and PC-3 cells, MT drugs caused a significant accumulation of cells in the G2/M phase along with moderate increase in subG1 population. Thus, we found at least two types of responses to MT inhibition: SubG1accumulation prone and G2/M accumulation prone cells. HeLa and HaCaT are SubG1-prone cell lines, while PC-3 and A549 are G2/M-prone cell lines. SubG1 peak in G2/M prone cells is significantly lower than in HeLa and HaCaT cells even at high MT doses.

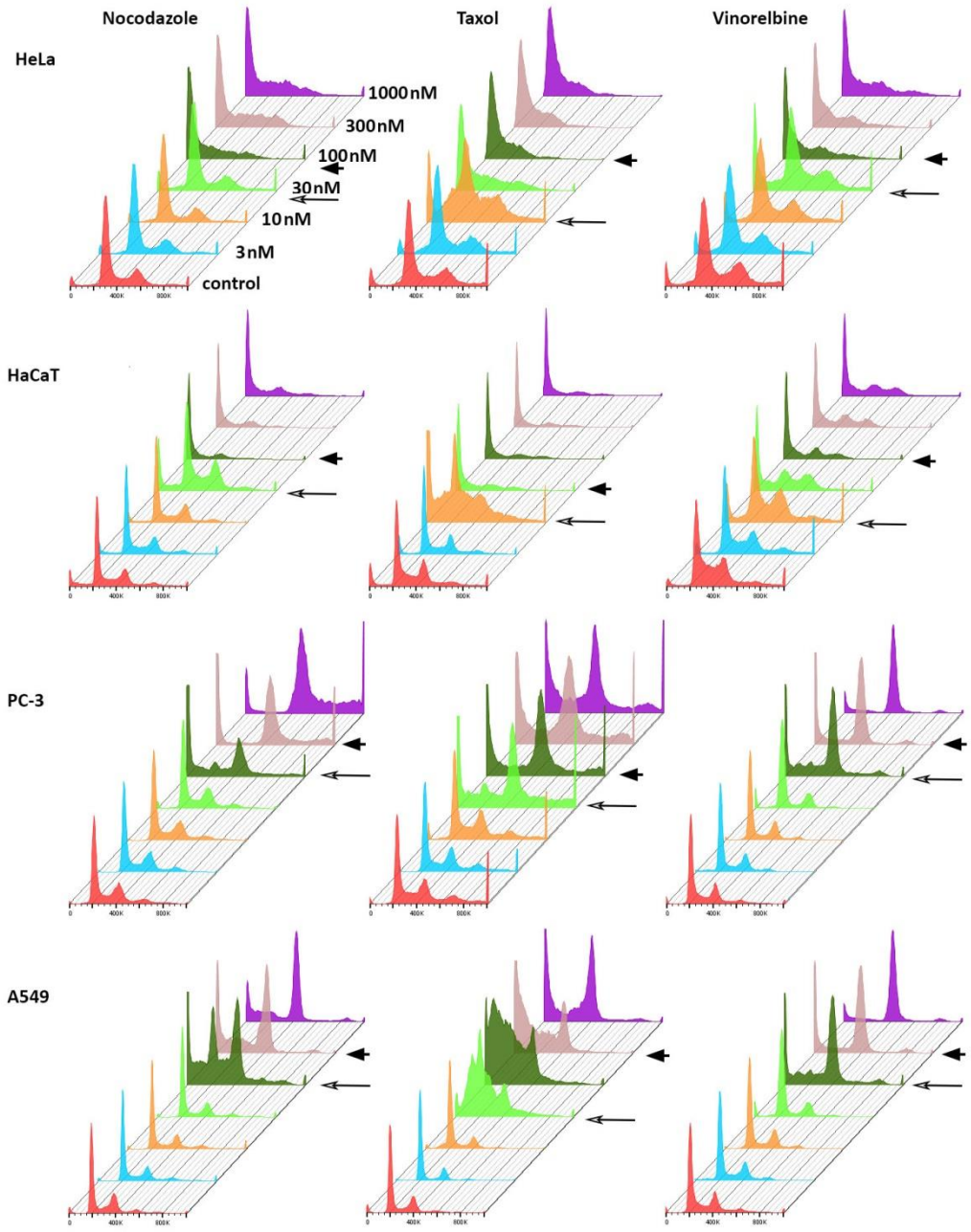

Figure 3. Stacked DNA content histograms indicating dose-dependent response of different cell lines to MT inhibitors (Nocodazole, Taxol, Vinorelbine) applied in a wide range of concentrations (3-1000nM). The duration of the treatment: 48h. Upper two rows indicate response in SubG1-prone cells, whereas lower two rows indicate G2/M-prone cells. The response of cells to the treatment changes with increasing of concentrations. Minimal threshold concentrations for each drug are indicated by long arrow, maximal threshold concentrations – by arrowhead.

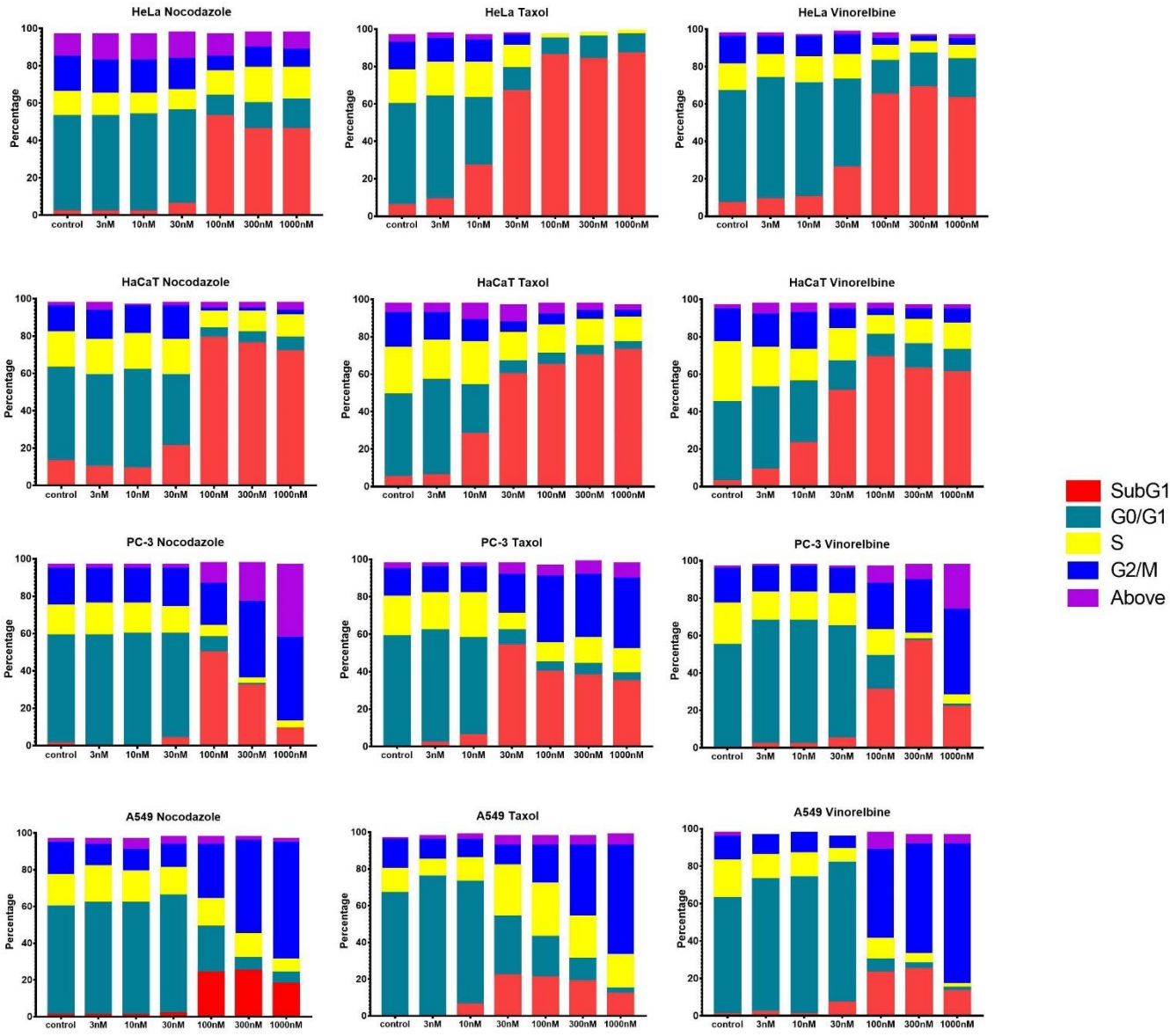

Figure 4. The distribution of cell across different phases of the cell cycle in response to MT treatment. Concentration range: 3-1000nM, treatment duration: 48h. It is a quantitative analysis of Figure 3. All bars indicate the mean of triplicate. Upper two rows indicate SubG1-prone cells showing the increase in SubG1 percentage until maximal threshold dose. Lower two rows indicate G2/M-prone cells with the increase in G2/M accumulation correlated with the increase in MT drug concentration until maximal threshold dose.

4.2 Cells showed different types of responses to prolonged treatment with 1μM MT inhibitors

To confirm the results obtained by flow cytometry, we examined the effect of 1 μM concentration of the microtubule inhibiting drugs (Nocodazole, Taxol, Vinorelbine) on our four target cell lines using microscopy. According to flow cytometry data, all doses above the maximal threshold concentration exhibit a similar effect. Therefore,

we decided to use the highest available concentration (1000nM or 1μM) for cell treatment following microscopic observations. Upon 48h incubation with 1μM of MT inhibitors, cells were stained with nuclear staining dye Hoechst 33342 and mitochondrial potential dye TMRE. Hoechst dye shows the DNA integrity and TMRE labels active mitochondria. Cells were examined on a Zeiss Cell Axio Observer microscope in DIC and fluorescence modes.

From Figure 5 (upper rows), many interphase cells, completely spread on the surface of the plate, are seen in the control wells of all cell lines. Untreated cells of all cell lines predominantly have a single nucleus with active mitochondria. A549, HeLa, and HaCaT cells in control wells express high mitochondrial transmembrane potential. Untreated PC-3 cells showed lower mitochondrial potential.

Significant differences in response to microtubule agents are observed in HeLa and HaCaT cells (Figure 5A,5B). According to DIC microscopy, interphase cells disappear when exposed to 1μM Nocodazole. The majority of cells are found in a slightly over-focus position and express many protrusions of the cell membrane, called blebbing. Cell blebbing indicates cells undergoing a late stage of apoptosis. Fluorescence microscopy images (Figure 5, lower rows) show a large number of cells with fragmented DNA and no mitochondrial potential (TMRE-) in 1μM Nocodazole. A similar effect is observed under the influence of Taxol and Vinorelbine for both HeLa and HaCaT cells. The absence of interphase cells, cell blebbing, and DNA fragmentation indicate a high apoptosis rate in these cells produced due to prolonged treatment with anti-mitotic agents. However, some cells located in close proximity to each other survive. Thus, we can assume that the most significant response of HeLa and HaCaT cells to prolonged treatment with MT drugs is apoptosis.

Under 1 μM dose of any inhibitor, PC-3 cells and A549 predominantly have intact nuclei (Figure 5C,5D) with active mitochondria around (TMRE+). The single nucleus is primarily visible in cells treated with 1μM Nocodazole and 1μM Vinorelbine. However, many multinucleated cells with more than one nucleus are seen in Taxol-treated samples. Thus, both PC-3 and A549 cells can maintain the integrity of the nucleus and actively proliferate under conditions of prolonged MT drug treatment.

We obtained two different outcomes resulting from microscopic investigations of samples subjected to 48-hour exposure with 1μM of either MT inhibitor After the treatment HeLa and HaCaT cells, showed no mitochondrial activity, fragmented,

DNA and progressive membrane blebbing, had massively died. In the other case, A549 and PC-3 show fully spread interphase cells with normal nuclei and active mitochondria. Flow cytometry and microscopy data confirm each other.

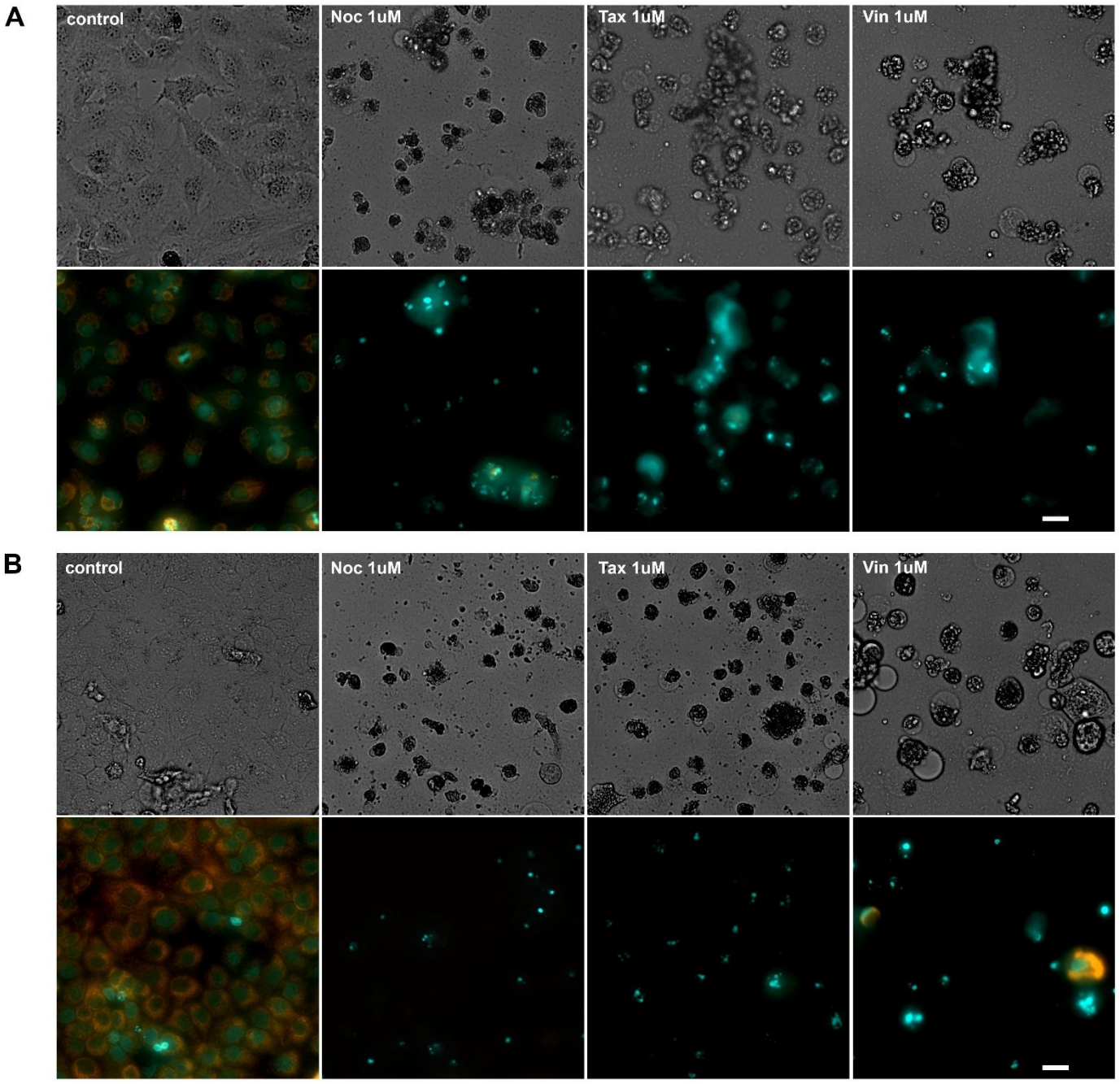

Figure 5. Apoptosis in SubG1-prone cells (from left to right: untreated cells, Noc(1 μM), Tax (1 μM),Vin (1 μM) treatments for 48h) Upper row: DIC image, lower row: overlay of two fluorescent channels (Hoechst 33342, turquoise; TMRE, orange). A- HeLa cells, B- HaCaT cells. Hoechst 33342 staining reveals intact nuclei in control, but hypodiploid and fractionated DNA in treatments. TMRE staining indicates TMRE+ cells in control, but largely TMRE- cells under treatments. Scale, 20 μm. Images were taken with EC Plan/Neofluar x20/0.5 objective.

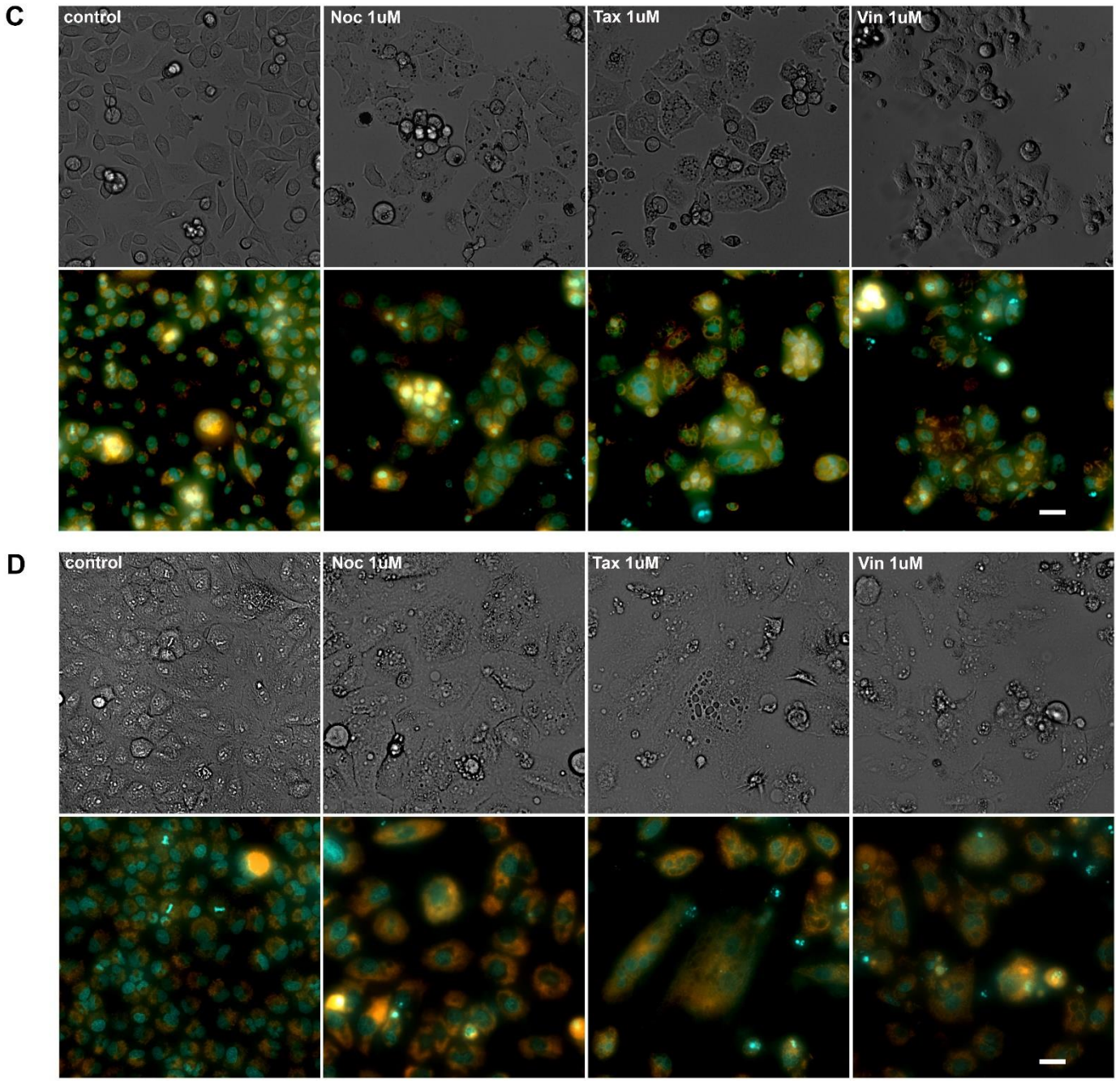

Figure 5 continued. Apoptosis in G2/M-prone cells (from left to right: untreated cells, Noc(1 μM), Tax (1 μM),Vin (1 μM) treatments for 48h) Upper row: DIC image, lower row: overlay of two fluorescent channels (Hoechst 33342, turquoise; TMRE, orange). A- PC-3 cells, B- A549 cells. Hoechst 33342 staining reveals intact nuclei in control. Nuclei integrity is preserved, predominantly in Taxol multiple nuclei are observed. TMRE staining indicates TMRE+ cells in control and under the effect of all MT treatments. Scale, 20 μm. Images were taken with EC Plan/Neofluar x20/0.5 objective.

4.3 All cells including SubG-1-prone survive in Barasertib

After we investigated the prolonged mitotic arrest, it was worthwhile to study how the short-term mitotic arrest induced by Barasertib will affect cells. In the figure 6, all cell lines were subjected to Taxol (1uM) or Barasertib (300nM) treatment. The data obtained from flow cytometry shows that A549 and PC-3 cells accumulate mainly in G2/M phase during Taxol treatment. But in Barasertib treatment these cells successfully accumulate in 8n peak as well, besides the presence of SubG1 population. G2/M prone cells better proliferate in Barasertib in polyploid states.

Intriguingly, SubG1-prone cell show similar response to Barasertib. Despite that HeLa and HaCaT massively accumulated in SubG1 (probably related to high number of apoptotic events) in Taxol-treated sample, they have readily accumulated in G2/M, 8n, and even 12n peaks in Barasertib-treated sample. Thus, SubG1-prone cells better proliferate in Barasertib in polyploid states.

Both of these results indicate that all cell lines can survive short mitotic arrest by switching to polyploid states.

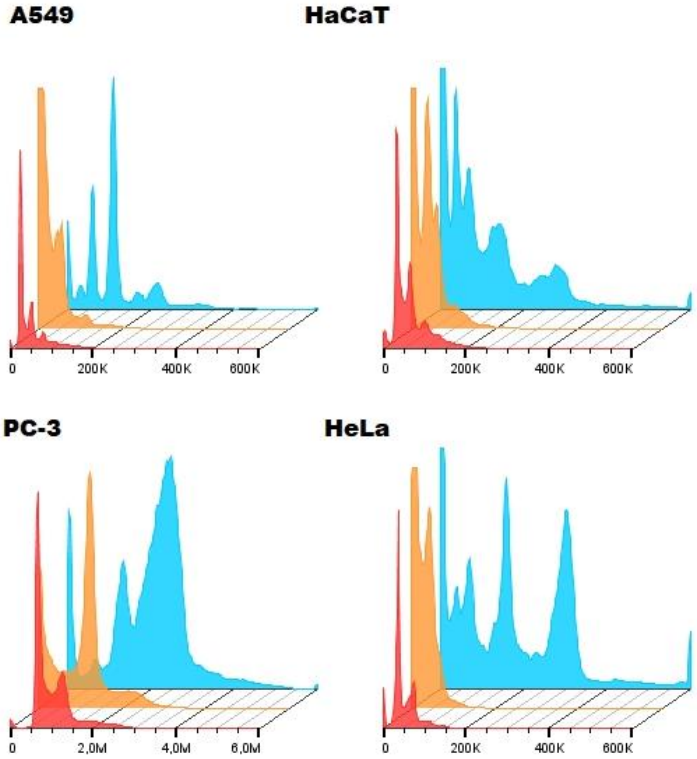

Figure 6. Cell cycle distribution in untreated (red), treated with 1uM of Taxol (orange), and treated with 300nM of Barasertib (blue) cells. Duration of the treatment: 48h.

4.4 G2/M-prone cell sensitization with BCL-2 proteins' inhibitors

Since A549 and PC-3 cells survive and continue to proliferate in high drug doses accumulating in the G2/M population, we aimed to sensitize them to the treatment using inhibitors of bcl-2 proteins. The results discovered recently in our lab suggest that mitotic arrest invariably introduces cell death via apoptotic execution. To estimate the role of different anti-apoptotic proteins in this process, we blocked their function with individual BCL-2 family protein inhibitors. In combination with mitotic arrest induced by Taxol, the inactivation of anti-apoptotic proteins should theoretically lead to an increased rate of apoptosis and SubG1 accumulation in A549 and PC-3 cells (G2/M prone) To assess how individual anti-apoptotic protein inhibitors work, we first treated A549 and PC-3 cells with 3μM A1155436 (BCL-XL inhibitor), 3μM S63845 (MCL-1 inhibitor) and 3μM Venetoclax (BCL-2 inhibitor). Figure 7A, 7B (upper row) indicates that sole action of anti-apoptotic protein inhibitors does not significantly change the normal cell cycle distribution.

We then tested the combined inhibition of microtubule dynamics and anti-apoptotic protein activity by mixing 1μM of Taxol with 3μM of different BCL-2 family protein

inhibitors Figure 7A, 7B (lower row). In A549 cells, the effect of Taxol alone and Taxol + S63845 drug combination doesn't significantly differ. In both DNA content histograms, cells readily accumulate in SubG1, and G2/M peaks and the rest of the cells are obtained between them. Some polyploid cells are also present on the histogram. Taxol and Venetoclax combined treatment illustrate controversial results, but its distribution is closer to the distribution of cells in Taxol-only sample. However, the combined Taxol and A1155463 treatment showed a G2/M peak disappearing and many cells accumulating in SubG1.

PC-3 cells treated with combinations of Taxol and S63845, Taxol and Venetoclax most likely exhibit the Taxol effect only. In these samples, small G0/G1 peaks, two prominent SubG1 and G2/M peaks and the rest of the cells located between them are observed. There is also a small number of polyploid cells. The DNA content in Taxol-treated samples and samples treated by combinations of Taxol with S63845 or Venetoclax are reminiscent. The notable change in the distribution occurs in a sample treated with Taxol and A1155463 drug combination. The combined treatment with Taxol and BCL-XL inhibitor showed a G2/M peak disappearing and many cells accumulating in SubG1.

In the samples treated with combinations of Taxol and S63845 or Venetoclax, cells arrested in mitosis by Taxol (G2/M peak) persist. The obtained data suggest that the inhibition of BCL-2 and MCL-1 proteins does not cause a massive accumulation of cells in SubG1, which is different from the usual accumulation due to exposure to Taxol alone. Accordingly, S63845 and Venetoclax most likely do not add to cell apoptosis caused by Taxol. As a result, these agents are not suitable for cell sensitization.

However, the reverse situation is observed in samples treated with the BCL-XL inhibitor. According to cytometry data, the A1155463 inhibitor forces cells arrested in mitosis (G2/M cells) to shift to the SubG1 phase inducing a higher rate of apoptosis. Thus, for both A549 and PC-3 cells, the inhibitor of BCL-XL (A1155463) is a major player in cell sensitization to MT inhibitors.

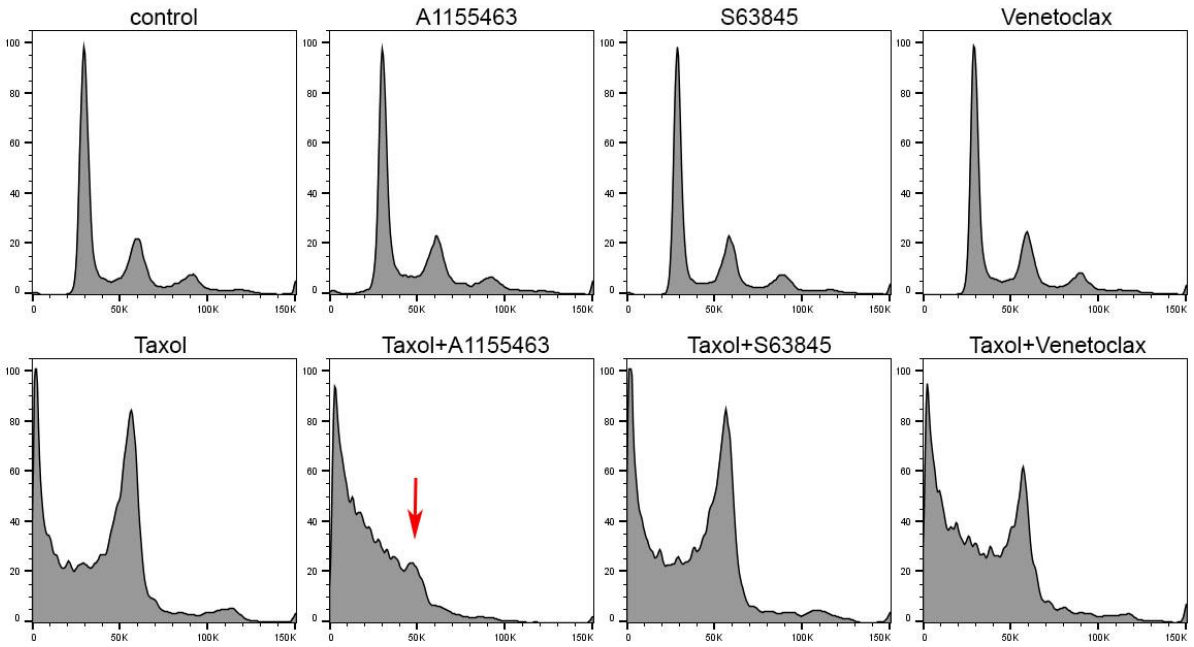

Figure 7A. DNA content histograms for A549 cells treated with Taxol(1μM), A1155463(3μM), S63845(3μM), Venetoclax (3μM) and their combinations. The duration of the treatment was 48h. Red arrow indicates decreased G2/M peak.

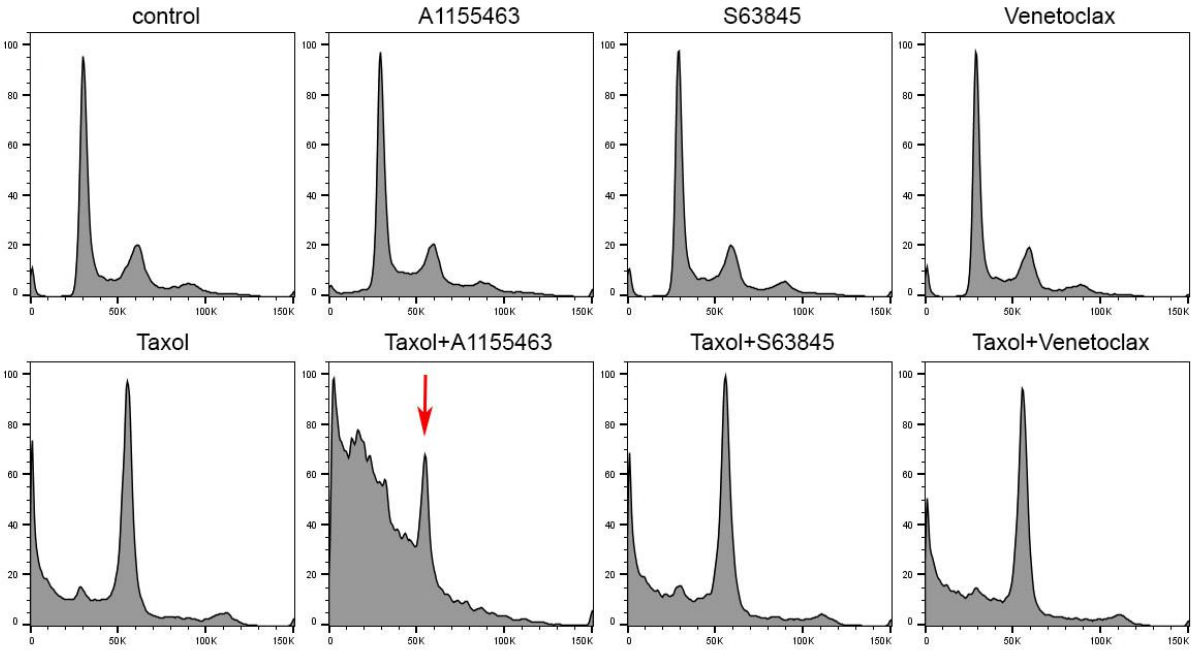

Figure 7B. DNA content histograms for PC-3 cells treated with Taxol(1μM), A1155463(3μM), S63845(3μM), Venetoclax (3μM) and their combinations. The duration of the treatment was 48h. Red arrow indicates decreased G2/M peak.

4.5 Dose-dependent response to combined treatment with MT inhibitors and BCL-XL inhibitor

Since A1155463 can sensitize A549 and PC-3 cells to MT inhibitors and the combined effect forces cells to accumulate in SubG1, we decided to investigate the dose-dependent response to A1155463. A sensitization dose is defined by more than 50% of analyzed cells accumulated in the SubG1 phase and about 10% obtained in G2/M phase.

In A549 cells 3μM A1155463 and 10μM A1155463 alone didn't cause any alterations in a normal distribution of a cell cycle (Figure 8, 9). However, even 10nM A1155463 combined with either of MT inhibitors caused 30% of all cells to accumulate in SubG1 phase, which is 30-fold higher than SubG1 percentage in control. Despite, G2/M percent in this concentration 20%-30% and was comparable to the percentage obtained after MT inhibitor alone treatment (1μM Nocodazole or 1μM Taxol or 1μM Vinorelbine). From the Figure 8 and Figure 9 it is evident that an increase in concentration of BCL-XL inhibitor led to the gradual increase in SubG1 and moderate decrease in G2/M populations until achieving plateau at sensitization dose. 100nM of A1155463 was sufficient to cause more than 50% of A549 cells to accumulate in SubG1 and less than 10% to be left in G2/M population in response to 1μM Nocodazole and 1μM Vinorelbine. Similarly, 30nM A1155463 caused the same effect when mixed with 1μM Taxol. Thus, 100nM of BCL-XL inhibitor concentration is sufficient to sensitize mitotically arrested A549 cells to Nocodazole and Vinorelbine, and only 30nM is enough to do so to Taxol.

In PC-3 cells, even 3μM of BCL-XL inhibitor was toxic enough to cause 20% accumulation in SubG1 population (Figure 8). At concentrations of 10nM of A1155463 mixed with 1μM of Nocodazole and 10nM of A1155463 mixed with 1μM of Vinorelbine, 40% of all cells were found in SubG1 phase and 20% were still accumulated in G2/M phase. Like in A549 cells an increase in concentration of BCL-XL inhibitor led to the gradual increase in SubG1 and moderate decrease in G2/M populations until achieving plateau at sensitization dose. The sensitization dose for PC-3 cells varies among different combinations of drugs. 100nM A1155463 sensitized mitotically arrested cells to Nocodazole and 30nM A1155463 to Taxol. However, only 10nM of anti-apoptotic inhibitor was required to cause a similar accumulation effect in PC-3 cells in response to Vinorelbine.

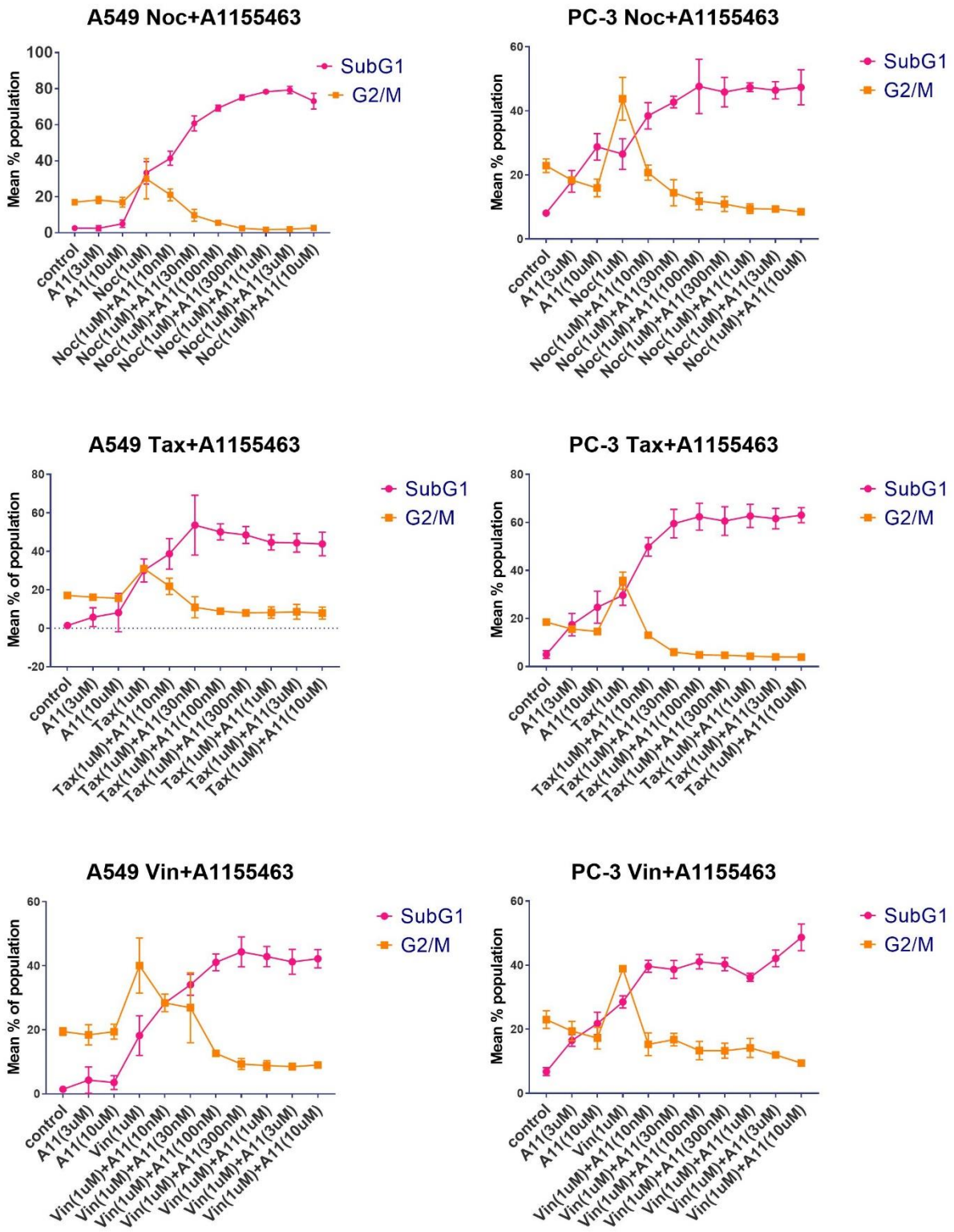

Figure 8. SubG1 and G2/M population numbers across different concentrations of a combined MT drug +BCL-XL treatment. Duration of the treatment: 48h, concentration range (10nM-10uM of A11 combined with 1uM of MT drug). All

measurements are present in mean and sd values.

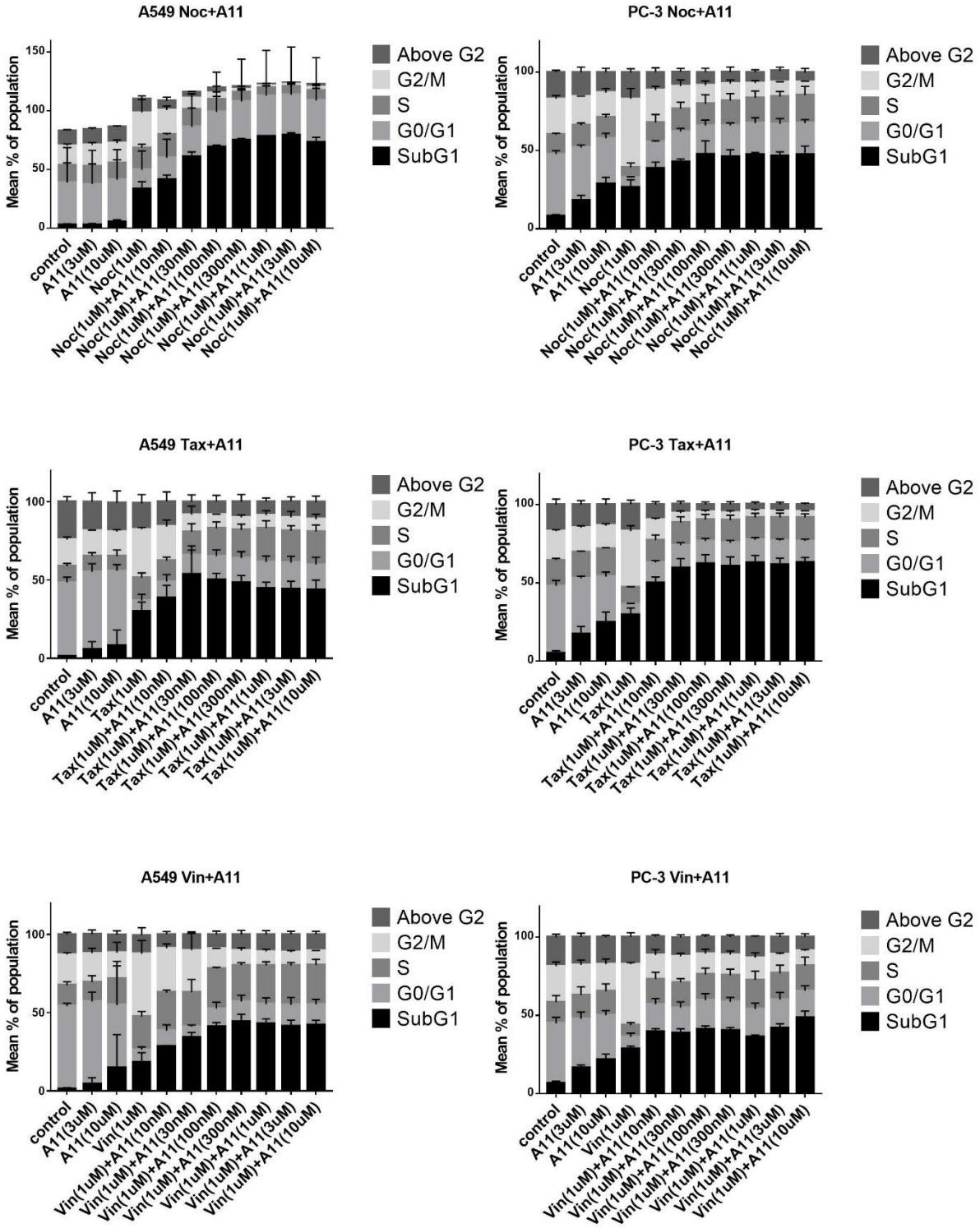

Figure 9. Cell cycle distribution across different concentrations of a combined MT drug +BCL-XL treatment. Duration of the treatment: 48h, concentration range (10nM-10uM of A11 combined with 1uM of MT drug). All measurements are present in mean and sd values.

5.DISCUSSION

Mitosis is an essential part of the cell cycle and an indicator of cell proliferation. Therefore, blocking cells in mitosis with MT inhibitors that interfere with the normal formation and functioning of the mitotic spindle is one of the main approaches in cancer therapy. Nonetheless, after reviewing different works that thoroughly measure and analyze prolonged mitotic arrest, it has become clear that the response of cells to the delayed metaphase is complex and highly variable (Gascoigne and Taylor, 2009).

Flow cytometry, a population-based quantification of physical and chemical characteristics of cells, is a sophisticated method for studying the drug-dependent response in cells. Studying the cycle of cells exposed to microtubule-inhibiting drugs or other drugs that affect the cell cycle can provide answers to what proliferation state each of the population belongs to. Studying DNA content helps to understand the drug dose-dependent response and assess the intra- and inter-line variations in responses of cells to drugs with different modes of action. Previous works on the consequences of mitotic arrest after 24 hours of inhibition via cell cycle assessment showed the high efficiency of this method (Potashnikova et al., 2018).

In this work, we tried to systematize the intra- and inter-line variations of the cell response to prolonged treatment with MT drugs described earlier (Gascoigne and Taylor, 2008). Using flow cytometry, we determined the minimal and maximal threshold doses for each cell line-MT inhibitor pair by treating cells with a wide range of concentrations (3-1000nM) of microtubule-inhibiting drugs for 48h. Minimal threshold doses showed a highly variable distribution of cells; however, above the maximal threshold dose, fluctuations in cell cycle distribution were shortened to two: predominant SubG1accumulation or prevalent G2/M accumulation. Thus, according

to the distribution of cells in response to MT treatment, we identified all cell lines as SubG1-prone and G2/M-prone.

SubG1-prone cells possess about 60% of cells in the SubG1 population at MT drug doses higher than the maximal threshold. This population of cells contains cell debris and apoptotic cells (Kajstura et al., 2007). However, they can have hypodiploid cells expressing mitochondrial activity (Potashnikova et al., 2019).

G2/M-prone cells are characterized by the accumulation of 40-50% of cells in the G2/M population at MT drug doses higher than the maximal threshold. In G2/M-prone cell lines, polyploid cells (above g2) were also present. Besides, G2/M-prone cells having a SubG1 population its percentage is at least three times less than in SubG1-prone cells.

To confirm the hypothesis of two prevailing cell responses to high concentrations of MT inhibitors obtained in cytometric data, we tested our target cell lines with 1μM of each MT inhibitor for 48 hours. Changes in cell morphology, DNA structure and mitochondrial membrane potential activity were microscopically observed. After the treatment, HeLa and HaCaT cells (SubG1-prone) showed progressive membrane blebbing, fragmented DNA and no mitochondrial activity; they had massively died. In the other case, A549 and PC-3 cells (G2/M-prone) showed fully spread interphase cells with normal nuclei and active mitochondria. Flow cytometry and microscopy data confirmed each other.

Testing the hypothesis that the duration of mitotic arrest can determine the response in cells, we treated cells with a working concentration of Barasertib (300nM) for 48h. According to the obtained results, under Barasertib treatment, all cell lines became G2/M-prone. Even HeLa and HaCaT showed significant accumulation in the G2/M peak and other polyploid peaks (8n, 12n). Thus, in Barasertib, the rate of mitotic

slippage was probably high. Although some cells were present in the SubG1 population in Barasertib only treated samples, their percentage was lower than that in Taxol only treated samples.

The results obtained in our lab recently confirmed caspase-dependent apoptosis in mitotically arrested cells described previously (Niikura, 2007). We then tried to terminate the activity of anti-apoptotic proteins with BCL-2 family protein inhibitors of high specificity, thus making G2/M-prone cells sensitive to MT treatment. Our observations showed that among three BCL-2 protein inhibitors, the inhibitor of BCL-XL (A1155463) induced high rates of apoptosis which were correlated to the increased percentage of SubG1cells. BCL-XL inhibitor is, therefore, a major player in cell sensitization to MT inhibitors. Even small concentrations of A1155463 were effective. Other inhibitors of BCL-2 (Venetoclax) and MCL-1 proteins (S63845) did not add to SubG1 accumulation caused by Taxol. As a result, these agents were not suitable for G2/M-prone cell sensitization.

Since BCL-XL inhibitor alone did not cause any dramatic alterations to the cell cycle of normally dividing cells, we decided it can only affect mitotic cells with high probability. We then concluded that cell response to MT drugs in G2/M-prone cells could be altered towards the increase in SubG1 population (sensitization) by BCL-XL inhibitor.

Taxol and Vinorelbine are widely used in the cancer treatment of patients. We established maximal threshold concentrations, different from the highest available concentration. We suggest the efficacy of cancer therapy should not be dramatically affected by the dose, as long as it is equal to or higher than the maximal threshold dose for each particular cancer type. We then claim even G2/M-prone cells can be sensitized by small doses of BCL-XL inhibitor, but not BCL-2 or MCL-1 inhibitors.

It leads us to the motivation to move away from conventional cancer therapy with one or two drugs only and move towards studying the synergy of drugs, especially researching sequential checkpoint therapy.

6.CONCLUSION

We conclude that 1) all drugs exhibit similar effects within each cell line tested, but high interline variation in response to MT treatment is evident 2) two types of responses to mitotic inhibitors are observed: SubG1 accumulation prone and G2/M accumulation prone, were obtained from flow cytometry data, 3) microscopic observations confirm two types of responses obtained in flow cytometry experiments 4) Maximal and minimal drug threshold concentrations successfully describe the changes in cycle distribution resulted from MT treatment 5) introducing short mitotic block with Aurora B kinase inhibitor probably plays a role in cell survival, forcing SubG1-prone cell lines to shift to G2/M-prone with many polyploid cells 6) maintaining the level of BCL-XL proteins, but not BCL-2 or MCL-1 proteins can affect the shift of G2/M-prone cells to become SubG1-prone.

Future perspectives. This study covers the limited number of cell lines and more other cancer cells should be tested. Also, it will be of great interest to make such analysis on the primary cultures obtained from patients.

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APPENDICES