NAZARBAYEV UNIVERSITY

SCHOOL OF SCIENCES AND HUMANITIES

THE DOSE-DEPENDENT EFFECT OF ANTI-MICROTUBULE DRUGS ON CANCER CELLS

BY

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DECLARATION

I, Sultan Bekbayev, 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.

Sultan Bekbayev

Nur-Sultan, May 1, 2020

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I wish to extend my special thanks to my family, Adil, Marina, Miliana and Ramazan for their infinite support.

DEDICATION

I dedicate this work to my friend, Kuanysh.

May your soul rest in peace.

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LIST OF ABBREVIATIONS

APC/C – Anaphase Promoting Complex / Cyclosome

BUB3 - Budding Uninhibited By Benzimidazoles 3

BUBR1/Mad3 - Budding Uninhibited by Benzimidazole-Related 1 / Mitotic-arrest deficient 3

CDC20 – Cell Division Cycle 20

Cdk1 – Cyclin-dependent kinase 1

GTP - Guanosine triphosphate

IC50 – half maximal inhibitory concentration

MAD1 - Mitotic-Arrest Deficient 1

MAD2 - Mitotic-Arrest Deficient 2

MMC – Minimal Mitostatic Concentration

MPS1 – Monopolar Spindle 1

SAC – Spindle Assembly Checkpoint

T2 – the second threshold concentration

ABSTRACT

Drugs inhibiting microtubule dynamics are widely used in the treatment of various types of cancer. However, the molecular mechanisms are not fully investigated. The aim of this thesis is to study drug- and dose-dependent effect of the inhibitors of microtubule drugs on the cancer cells by treating them with the wide range of concentrations anti-microtubule drugs and observing the fate of mitotic cells directly under microscope. This will allow us to obtain systematic knowledge on the threshold concentrations of anti-microtubule drugs, which will open new prospects in disentangling molecular mechanisms of the action of these drugs.

In this work two distinct effects of anti-microtubule drugs were investigated, and they were quantified with introducing the concept of minimal mitostatic concentration, and novel second threshold concentration.

Key words: *drugs inhibiting microtubule dynamics, minimal mitostatic concentration, the second threshold concentration, mitotic arrest*

CHAPTER ONE:

INTRODUCTION

1.1 Microtubules and dynamic instability

Microtubules are essential part of cytoskeleton alongside with intermediate filaments and actin filaments. They are involved in various cellular activities including cell motility, cell spreading, intracellular transport, and mitosis. Microtubules are polymer structures composed of alpha and beta tubulin heterodimers. In mammalian cells these dimers are stacked head to tail on top of each other, and form 13 protofilaments, which compose a hollow cylindrical structure – microtubule. For the reason that in a microtubule all these heterodimers point on the same direction, microtubules are polarized structures, where alpha-tubulin is exposed on minus-end, and beta-tubulin is exposed on plus-end (Alberts, Johnson and Lewis, 2020).

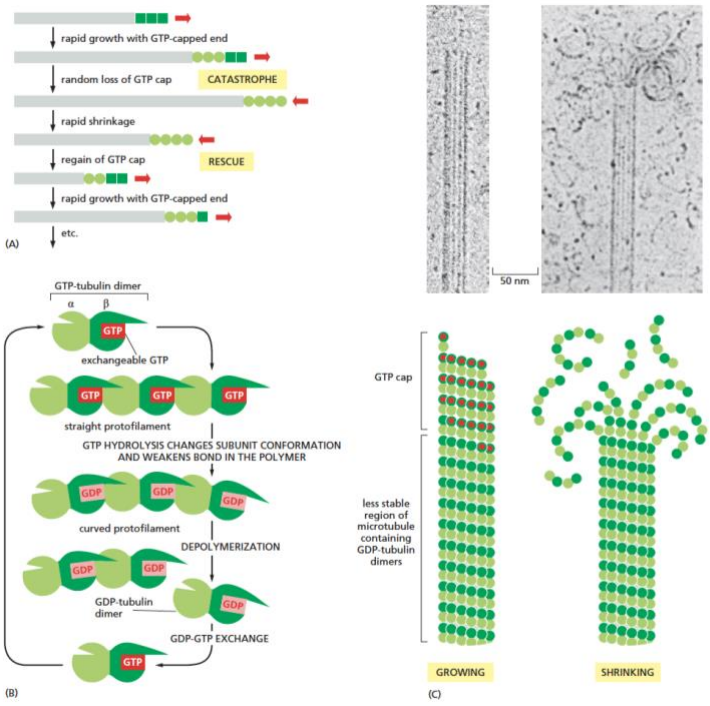

Figure 1. Dynamic instability of microtubules. Image from Alberts, B., Johnson, A. and Lewis, J., 2020. Molecular Biology of the Cell. W. W. Norton; C from E.M. Mandelkow, E. Mandelkov and R.A. Milligan, *J. Cell Biol.* 114:977-991, 1991

Microtubules are highly dynamic structures, whose normal state in the cells is dynamic instability. Both alpha- and beta-tubulins have GTP-binding site. During formation of heterodimers GTP bound to alpha-tubulin is sealed under beta-tubulin. However, GTP bound to beta-tubulin can hydrolyze. In the phase of active growth of a microtubule GTP of beta-tubulin is not hydrolyzed, and this GTP cap ensures rapid growth. When it reaches certain length, all the GTPs suddenly starts to be hydrolyzed, and this leads to change of conformation of beta-tubulin molecule. This causes curving of protofilaments, and consequently microtubule depolymerizes rapidly (Figure 1). These two phases of active growth (rescue) and rapid depolymerization (catastrophe) composes the process of dynamic instability. In all cellular processes microtubules functions in the state of dynamic instability (Alberts, Johnson and Lewis, 2020).

In mammalian cells microtubules emanate from the pericentriolar material rich in gamma-tubulin ring complexes. Gamma-tubulin ring complex provides a nucleating site for tubulin heterodimers. Therefore, in the cells minus-end of microtubules are located in the pericentriolar region, and they grow towards cell periphery with their plus ends (Alberts, Johnson and Lewis, 2020).

1.2 Mitotic spindle

One of the key cellular processes, in which microtubules play an important role is the process of division – mitosis. In prometaphase of mitosis microtubules polymerize to form three major groups of mitotic spindle: astral microtubules, kinetochore microtubules, and interpolar microtubules. Kinetochore microtubules bind with their plus-end to Ndc80 complex of kinetochores, and provide pulling force towards poles to the chromosomes during anaphase A. Interpolar microtubules from one pole connect to interpolar microtubules from the opposite pole, and actively growing towards each other during anaphase B provide pushing force to the chromosomes bound to kinetochore microtubules (Alberts, Johnson and Lewis, 2020).

In prometaphase kinetochore microtubules grow in random directions, and some of them bind to kinetochores. However, a major step in this phase of mitosis is to provide proper attachment of microtubules to both kinetochores of the sister chromatids, i.e. bi-lateral attachment. It is achieved with the help of Aurora B kinase. When microtubules attach to the kinetochore of only one chromatid, or only from one side, kinetochore is positioned laterally on the microtubule. In this lateral attachment Aurora B kinase is active, and it phosphorylates several proteins of the kinetochore, including Ndc80 complex. This inhibitory phosphorylation causes low affinity of Ndc80 complex to the microtubule. However, when microtubules are attached to both kinetochores of the sister chromatids, Aurora B kinase is inactivated, and Ndc80 complex firmly binds to the microtubule in the end-on attachment (Alberts, Johnson and Lewis, 2020).

1.3 Spindle Assembly Checkpoint and Anaphase Promoting Complex

To prevent aneuploidy and mis-segregation of chromosomes, there is a Spindle Assembly Checkpoint (SAC) – pathway, which remains active from the onset of prometaphase until all the chromosomes are attached to microtubules bilaterally. Active SAC inhibits CDC20, preventing activation of ubiquitin ligase - Anaphase Promoting Complex / Cyclosome (APC/C). The latter in an active form targets securin and cyclin B to degradation in 26S proteasome. Securin is an inhibitor of separase, an enzyme, which cleaves cohesin, which maintains sister chromatids together (Figure 2). Cyclin B is a mitotic cyclin, which binds to Cdk1, a kinase that ensures proper progression through mitosis by phosphorylating numerous key players of this process. Destruction of Cyclin B leads to exit from mitosis (Mussachio and Salmon, 2007).

BUB3, BUBR1/Mad3, and MAD2 are considered to be effectors (Mitotic Checkpoint Complex (MCC)) of SAC that interact with CDC20. In addition, Aurora B kinase, MAD1, and MPS1 are also key components of SAC. MAD1 and MAD2 are recruited to unattached

kinetochores, and removed when correct attachment occurs. The formation of MAD1-MAD2 complex is regulated by MPS1. Later MAD2 binds to CDC20, and recruits BUBR1-BUB3 complex in order to form MCC, which inhibits CDC20. Aurora B kinase as it was described above plays a role of tension-sensitive inhibitor (Mussachio and Salmon, 2007).

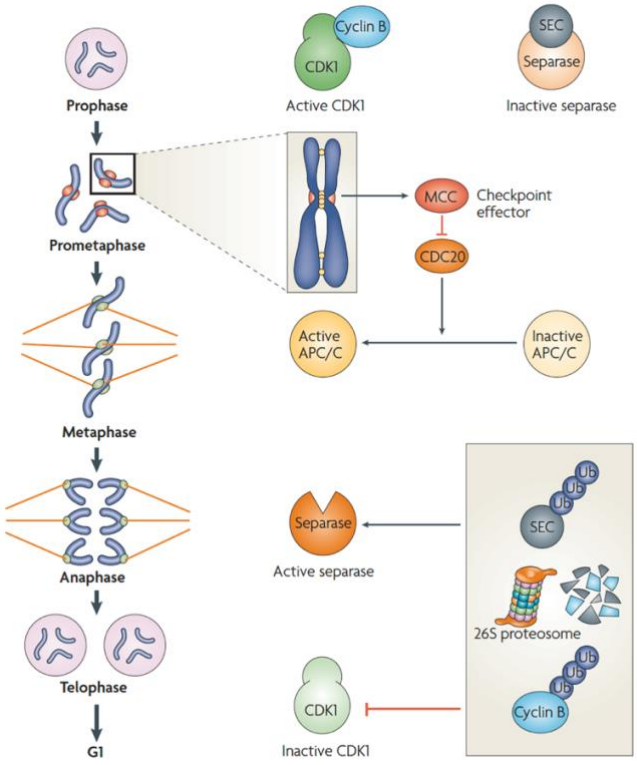

Figure 2. Targets of APC/C. Image from Musacchio, A. and Salmon, E., 2007. The spindle-assembly checkpoint in space and time. *Nature Reviews Molecular Cell Biology*, 8(5), pp.379-393.

1.4 Drugs inhibiting microtubule dynamics

Dynamic instability of microtubules plays a key role during mitosis. For the reason that one of the hallmarks of cancer cells is unlimited proliferation, drugs inhibiting microtubule dynamics are widely used in anticancer therapy. These drugs can be divided into two distinct groups by their effect on microtubules: microtubule destabilizers and microtubule stabilizers. Former includes drugs with *Vinca*-binding site and *Colchicine*-binding site. Latter includes drugs with *Taxane*-binding site, *Epothilones*, and *Taccalonolides*. In this work

Nocodazole, *Taxol*, *and Vinorelbine* are used, therefore below only these drugs will be considered. (Risinger, Giles and Mooberry, 2009)

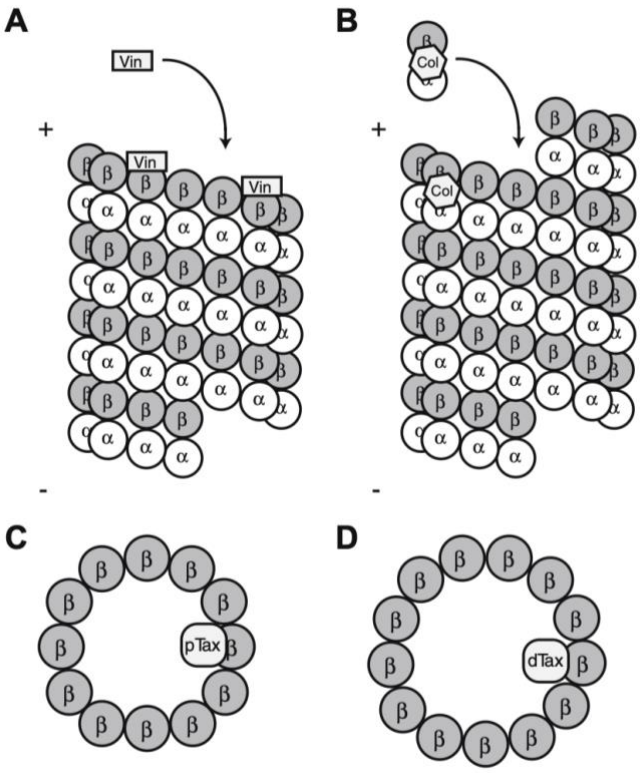

Figure 3. Binding sites of inhibitors of microtubule dynamics. Image from Risinger, A., Giles, F. and Mooberry, S., 2009. Microtubule dynamics as a target in oncology. Cancer Treatment Reviews, 35(3), pp.255-261.

Nocodazole

Nocodazole is a microtubule-destabilizer, which interacts with tubulin at *Colchicine*-binding site. It binds to beta tubulin and inhibits polymerization of microtubules. For the reason that it has failed clinical trials, *Nocodazole* is currently used as an experimental tool (Risinger, Giles and Mooberry, 2009).

Taxol

Taxol (Paclitaxel) is a microtubule-stabilizing agent, which interacts with tubulin at *Taxane*-binding site. It inhibits dynamics of microtubules by interfering with D-beta-tubulin,

preventing their change in conformation. Therefore, tubulin protofilaments containing taxol do not curve and remain stable. Under *Taxol*-treatment equilibrium between soluble tubulin heterodimers and polymerized heterodimers shifts towards the latter (Risinger, Giles and Mooberry, 2009). Taxol and its derivatives are broadly used in the treatment of Kaposi's sarcoma, prostate, lung, ovarian, breast, cervical, gastroesophageal, endometrial cancers, and different types of lymphomas and leukemia (Jordan, 2002; Weaver, 2014).

Vinorelbine

Vinorelbine is a microtubule-destabilizing agent, which interacts with tubulin through *Vinca*-binding site that is located on beta-tubulin near GTP-binding site. At low concentrations Vinca alcaloids inhibits the dynamics of plus-end, but at higher concentrations it causes microtubule depolymerization and formation of paracrystals of tubulin (Risinger, Giles and Mooberry, 2009). *Vinorelbine* is used as a chemotherapeutic agent in lymphoma, non-small cell lung cancer, advanced ovarian carcinoma, and metastatic breast cancer (Jordan, 2002).

1.5 Aim of the thesis

The first works on determining the effective concentrations of inhibitors of microtubule dynamics were based on *in vitro* studies on microtubules (Hoshino, Wilson and Muraoka, 1979; Jordan, 2002). Extracted microtubules were treated with anti-microtubule drugs, and different types of IC50 measurements were determined. When an anti-microtubule drug inhibited the shortening rate of microtubule by 50%, it was determined as IC50 of shortening rate. In addition, the effect on microtubules dynamics was calculated as a concentration when the dynamics was inhibited for 50%, i.e., IC50 of dynamicity (Jordan, 2002).

The focus of the other studies was on the effect of these drugs on cells. They have shown that Nocodazole, Vinca alkaloids and Taxol cause accumulation of cells in metaphase-like stage starting from the low (nanomolar) concentrations of these drugs. The concentration, at which 50% of cells accumulate at metaphase-like stage was determined, i.e. $K_{i,\ met}$ (Jordan 1991). Also, the concentration, which leads to 50% decrease in the anaphase to metaphase ratio ($K_{ana/met}$) was introduced (Jordan, 1991; Jordan, 1992). At these concentrations, type 1 and type 2 spindles that resemble normal spindle with metaphase plate, but with thicker microtubule bundles, shorter interpolar distance, and longer astral microtubules containing 1-3 chromosomes, were observed. However, they did not fully inhibited cell proliferation (Jordan, 1991; Jordan, 1992).

The proportional increase of the percentage of the blocked-in-mitosis cells occurred with increase in the concentration of anti-microtubule drugs. This blockage of mitosis was accompanied with a proportional inhibition of cell proliferation in the populational assay. Inhibitory constant (K_i) of proliferation for the anti-microtubule drugs was determined as a concentration of a drug, which inhibits cell proliferation by 50% ($K_{i,\ prol}$ or $K_{i,\ div}$) (Jordan, 1991; Jordan, 1992; Florian, 2016).

Further studies were done on the effect of selected concentrations of anti-microtubule drugs on the behavior of mitotic cells. Gascoigne and Taylor (2008), by analyzing the fate of cells under direct time-lapse microscopic observation of cells from fifteen cell lines, have determined profound intraline and interline variations in the outcomes of mitosis under prolonged treatment with anti-microtubule drugs (Gascoigne and Taylor, 2008). They defined the effect, when at a given dose of a drug the outcome is not uniform, i.e. some cells exit from prolonged arrest without division, and some die in mitosis or in interphase as an intraline variation. By analyzing the response of various cell lines to the same drug, they have introduced interline variation, when the same dose of a given drug induce different outcomes in various cell lines (Gascoigne and Taylor, 2008).

In addition, studies at the chosen high concentrations have revealed that the cells arrested in mitosis for prolonged time might either die in mitosis or proceed to mitotic slippage, when the cell exited from mitosis without division, mostly as a multinucleate cell (Brito and Rieder, 2009).

The aim of this thesis is to study drug- and dose-dependent effect of the inhibitors of microtubule drugs on the cancer cells by treating them with the wide range of concentrations anti-microtubule drugs and observing the fate of mitotic cells directly under microscope. This will allow us to obtain systematic knowledge on the threshold concentrations of anti-microtubule drugs.

CHAPTER TWO:

MATERIALS AND METHODS

2.1 Materials

Cell cultures were obtained from the collection of NU and ATCC: A549 – lung cancer, U-2OS - osteosarcoma, and U-118 – glioblastoma. All cell lines were maintained in DMEM, with the addition of 5% FBS, L-glutamine and an antibiotic mixture.

Drugs: Taxol (paclitaxel) and Nocodazole were obtained from Sigma-Aldrich, Vinorelbine- from Selleck Chemicals Inc. Stock solutions were prepared in DMSO stored at -20 ° C.

Microscopy for live cells imaging. DIC microscopy was performed using a Carl Zeiss Cell Observer SD automated microscope controlled by ZEN software (Carl Zeiss) using a wide-field module. For continuous monitoring of live cells in real time, a climate chamber and a fine focus system integrated with a microscope was used. Images were recorded using a 16-bit Hamamtsu ORCA-2 CCD camera (70% QY).

All statistical analyzes were performed using GraphPad software.

2.2 Methods

The cells were taken from the cell bank of the laboratory and thawed. Initially 5 mL of DMEM with 5% FBS was poured into the T25 flask, and put to the incubator for 30 minutes. After that vial with the cells was taken from the -85 freezer and thawed immediately by partially putting it to the water bath. When the last pieces of ice were melting, the vial was surface of the vial was disinfected with 70% ethanol, dried with *kimwipe* and put to the laminar flow hood. Right in this moment the flask with DMEM was taken from the incubator, disinfected, and also put to the hood. The solution of the cells were carefully taken from the

vial with 1000 uL pipette, and added to the medium, and gently resuspended. The flask with the cells was put into the incubator, and after 24 hours the medium was changed.

The cells were cultured in DMEM 10% FBS, containing pen-strep, and additionally added L-glutamine. The cells were cultured in two T25 flasks, in order to have the second one as an alternative, in case of emergencies. The trypsinization time for the cells were 2-5 minutes in 0.05% trypsin-EDTA solution. After the third passage the cells were seeded in 60% confluency onto 48-well plate for the experiment.

The drugs, inhibiting dynamics of microtubules: Nocodazole, Taxol, and Vinorelbine were used in the concentrations of 0.03-1000 nM.

After being seeded the cells were incubated in 37ºC 5% CO2 for 24 hours, in order to have them fully spread. After that medium was changed from DMEM 10% FBS to CO2-independent medium with 5% FBS containing different concentrations of the drugs (Table 1). This is done because the heated chamber of the microscope stage has no CO2-supply.

Table 1. Plate layout for the experiment. Doses are given in nM.

	Nocodazole		Taxol		Vinorelbine		Control	
A	Blank	0.03	Blank	0.03	Blank	0.03	Control	Control
B	0.1	0.3	0.1	0.3	0.1	0.3	Control	Control
C	1	3	1	3	1	3	Control	Control
D	10	30	10	30	10	30	Blank	Blank
E	100	300	100	300	100	300	Blank	Blank
F	1000	Control	1000	Control	1000	Control	Blank	Blank

Immediately after the drugs were added the plate was set into the heated chamber of the microscope and experiment was started using 10X DIC objective by taking pictures every 15 minutes for 72 hours.

CHAPTER THREE:

RESULTS

3. 1 Cell fates

The cells fates, which were observed in the wide range of all three anti-microtubule drugs were normal division (Figure 4, A), division with significant delay (Figure 4, B), abnormal/unequal division (Figure 4, C), mitotic slippage (Figure 4, D), and death in mitosis (Figure 4, E).

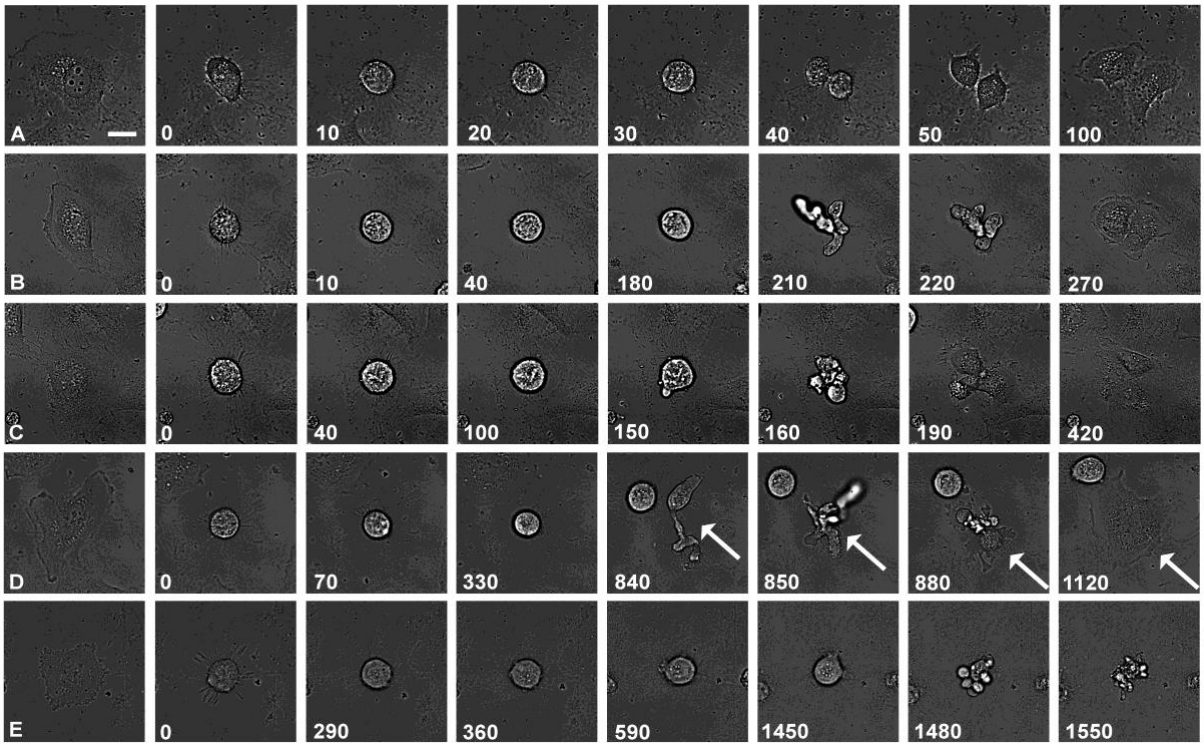

Figure 4. Panel of fates of mitotic cells. U-2OS cells under 10X DIC objective. Bar scale = 10 um, numbers indicate minutes from the moment of entering to mitosis A. Normal division. B. Division with significant delay. C. Abnormal/unequal division. D. Mitotic slippage. E. Death in mitosis.

The moment of entering to mitosis was morphologically defined as a moment when a cell became rounded, and nuclear envelope was broken down. The moment of exit from mitosis was defined when the first lamellipodium of the spreading cell emerged.

Normal division.

Cell entered mitosis and divides into two equal daughter cells in the time period equal to the duration of mitosis in control (about 1 h).

Division with significant delay.

As in normal division this fate resulted in morphologically equal two daughter cells, however the duration of mitosis for this category was at least three times longer in comparison to control.

Abnormal/unequal division.

The duration of mitosis for the cells belonging to this category was also at least three times longer in comparison to control. In addition, as a result of mitotic arrest these cells divided into two or more unequal daughter cells.

The categories ‘division with significant delay’ and ‘abnormal/unequal division’ were merged for the further analysis.

Mitotic slippage.

The duration of mitosis for this category of cells was extremely long (>5 h and sometimes up to 30 h), and as a result of mitotic arrest only one multinucleate cell was formed.

Death in mitosis.

A cell died in prolonged mitotic arrest in rounded state. The moment of death was determined when the surface of a cell started to bleb.

In addition, after division with significant delay, abnormal/unequal division, and mitotic slippage some cells died in post-mitotic interphase. However, these fates are beyond the focus of this thesis.

3.2 Analysis of dose-dependent changes in cell fates

Table 2. Summary of the results of cell fates analysis of A549 cell line under Nocodazole treatment at different doses

dose, nM	Mitotic slippage (n)	Death in Mitosis (n)	Delayed division (n)	Normal division (n)	Total (N)
30	-	-	31	20	51
100	40	1	8	-	49
300	31	17	-	-	48
1000	23	14	-	-	37

Table 3. Summary of the results of cell fates analysis of A549 cell line under Taxol treatment at different doses

dose, nM	Mitotic slippage (n)	Death in Mitosis (n)	Delayed division (n)	Normal division (n)	Total (N)
3	3	-	15	69	87
10	5	-	37	47	89
30	26	4	4	2	36
100	28	11	2	-	41
300	34	8	2	-	44
1000	31	13	-	-	44

Table 4. Summary of the results of cell fates analysis of A549 cell line under Vinorelbine treatment at different doses

dose, nM	Mitotic slippage (n)	Death in Mitosis (n)	Delayed division (n)	Normal division (n)	Total (N)
1	-	-	5	87	92
3	1	-	41	39	81
10	7	-	63	-	70
30	34	-	14	-	48
100	36	3	6	-	45
300	36	1	6	-	43
1000	43	11	-	-	54

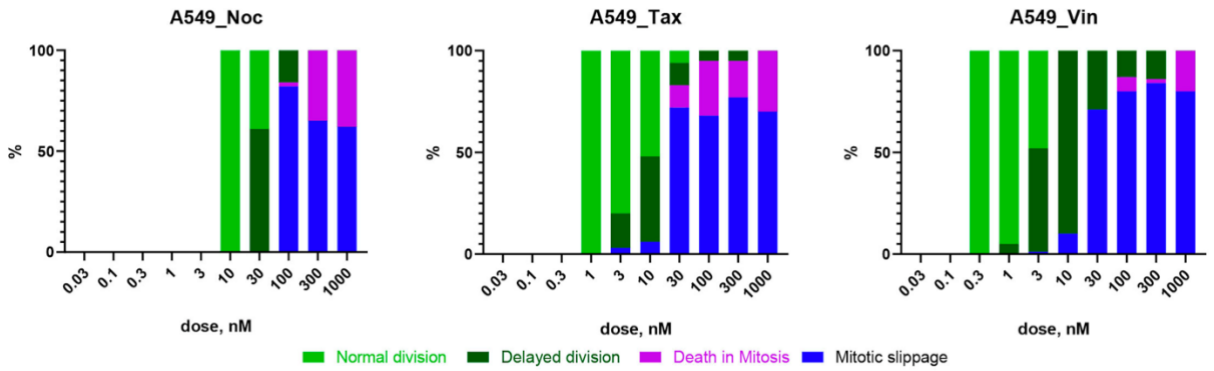

Figure 5. Cell fates profiles of A549 cell line under treatment with various inhibitors of microtubule dynamics at the wide range of concentrations. Doses are on the x-axis in nM, proportions of the fates on the y-axis. Noc – Nocodazole; Tax – Taxol; Vin – Vinorelbine.

The first significant effect of anti-microtubule drugs on A549 cells was observed at low concentrations. At the concentration of 30 nM of Nocodazole 61% of A549 cells divided with significant delay, while others divided normally. At the concentration of 10 nM of Taxol almost 50% of cells arrested in mitosis, most of these cells divided, and some of them underwent mitotic slippage. At the concentration of 3 nM of Vinorelbine exactly 50% of cells divided with significant delay, while others divided normally. Under the action of Nocodazole sharp increase in the number of divided-with-significant-delay cells was observed. However, under the action of Taxol and Vinorelbine this effect was more gradual.

Starting from the concentration of 300 nM of Nocodazole, 100 nM of Taxol, and 100 nM of Vinorelbine no significant change in the proportion of different fates was observed, and most of the cells underwent mitotic slippage.

Table 5. Summary of the results of cell fates analysis of U-2OS cell line under Nocodazole treatment at different doses

dose, nM	Mitotic slippage (n)	Death in Mitosis (n)	Delayed division (n)	Normal division (n)	Total (N)
0.3	-	-	-	50	50
1	-	-	8	42	50
3	-	-	7	33	40
10	-	-	1	39	40
30	-	-	4	48	52
100	9	-	35	-	44
300	26	6	5	-	37
1000	19	37	-	-	56

Table 6. Summary of the results of cell fates analysis of U-2OS cell line under Taxol treatment at different doses

dose, nM	Mitotic slippage (n)	Death in Mitosis (n)	Delayed division (n)	Normal division (n)	Total (N)
3	-	-	10	26	36
10	3	-	25	12	40
30	11	-	33	2	46
100	4	-	39	1	44
300	16	3	29	-	48
1000	38	8	10	-	56

Table 7. Summary of the results of cell fates analysis of U-2OS cell line under Vinorelbine treatment at different doses

dose, nM	Mitotic slippage (n)	Death in Mitosis (n)	Delayed division (n)	Normal division (n)	Total (N)
0.1	-	-	1	39	40
0.3	-	-	12	31	43
1	6	1	33	-	40
3	6	5	34	-	45
10	17	11	20	-	48
30	21	17	8	-	46
100	17	21	-	-	38
300	9	21	-	-	30
1000	6	28	-	-	34

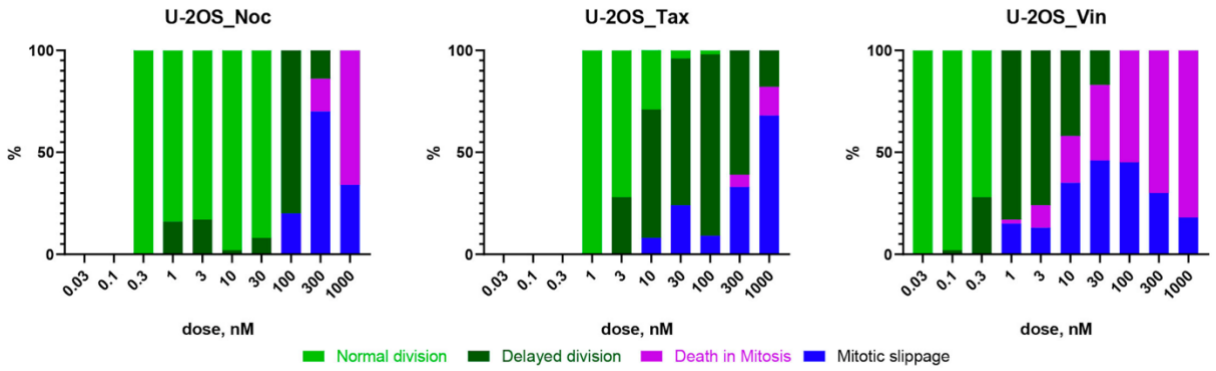

Figure 6. Cell fates profiles of U-2OS cell line under treatment with various inhibitors of microtubule dynamics at the wide range of concentrations. Doses are on the x-axis in nM, proportions of the fates on the y-axis. Noc – Nocodazole; Tax – Taxol; Vin – Vinorelbine.

At the concentration of 100 nM of Nocodazole all cells stacked in mitosis for significant time, while at 30 nM only small proportion of cells divided with significant delay. At the concentration of 10 nM of Taxol 63% of cells divided with significant delay, and some slipped cells were observed. At the concentration of 1 nM of Vinorelbine all cells were arrested in mitosis for significant time, and as a result 83% of cells divided, and other cells underwent mitotic slippage. The dose-dependent effect of Nocodazole and Vinorelbine was sharp, but dose-dependent effect Taxol resulted in gradual increase in the proportion of stacked in mitosis cells.

Starting from the concentration of 300 nM of Nocodazole, 1000 nM of Taxol, and 30 nM of Vinorelbine cells underwent mitotic slippage or died in mitosis. In Nocodazole and Vinorelbine cells mostly died in mitosis, while in 1000 nM Taxol most of cells underwent mitotic slippage.

Table 8. Summary of the results of cell fates analysis of U-118 cell line under Nocodazole treatment at different doses

dose, nM	Mitotic slippage (n)	Death in Mitosis (n)	Delayed division (n)	Normal division (n)	Total (N)
10	1	-	1	37	39
30	11	2	17	11	41
100	25	15	1	-	41
300	11	9	-	-	20

Table 9. Summary of the results of cell fates analysis of U-118 cell line under Taxol treatment at different doses

dose, nM	Mitotic slippage (n)	Death in Mitosis (n)	Delayed division (n)	Normal division (n)	Total (N)
1	-	-	4	34	38
3	2	-	7	26	35
10	2	1	10	29	42
30	16	5	29	16	66
100	20	13	13	-	46
300	15	35	2	-	52
1000	13	32	-	-	45

Table 10. Summary of the results of cell fates analysis of U-118 cell line under Vinorelbine treatment at different doses

dose, nM	Mitotic slippage (n)	Death in Mitosis (n)	Delayed division (n)	Normal division (n)	Total (N)
0.03	1	-	1	30	32
0.1	1	-	12	20	33
0.3	10	2	24	4	40
1	19	11	10	-	40
3	26	5	7	-	38
10	30	8	-	-	38
30	40	5	-	-	45
100	28	9	2	-	39
300	18	10	-	-	28

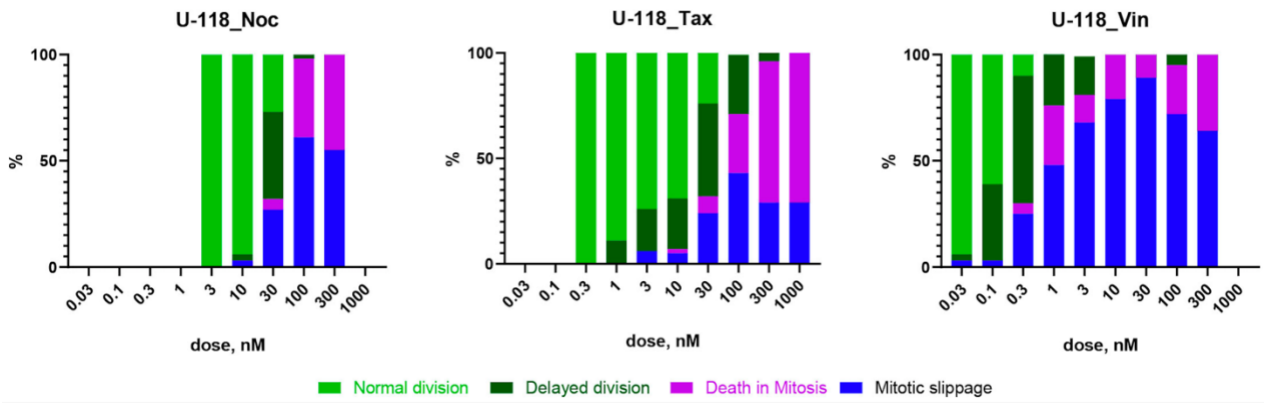

Figure 7. Cell fates profiles of U-118 cell line under treatment with various inhibitors of microtubule dynamics at the wide range of concentrations. Doses are on the x-axis in nM, proportions of the fates on the y-axis. Noc – Nocodazole; Tax – Taxol; Vin – Vinorelbine.

At the concentration of 30 nM of Nocodazole majority of cells were stacked in mitosis for significant time, while at 10 nM majority of cells divided normally. At the concentration of 30 nM of Taxol majority of cells divided with significant delay, or underwent mitotic slippage, and sometimes death in mitosis was also observed. At the concentration of 0.3 nM of Vinorelbine almost all cells divided with delay. The dose-dependent effect of Nocodazole was sharp, but dose-dependent effect of Taxol and Vinorelbine resulted in gradual increase in the proportion of stacked in mitosis cells.

Starting from the concentration of 100 nM of Nocodazole, 300 nM of Taxol, and 10 nM of Vinorelbine cells underwent mitotic slippage or death in mitosis. Under Nocodazole and Taxol most of the cells died in mitosis, while under Vinorelbine they mostly underwent mitotic slippage.

From these results two distinct effects can be determined. The first effect of inhibitors of microtubule dynamics was on the duration of mitosis: they prolonged the duration of mitosis, however, these cells divided into two or more daughter cells. Therefore, the concentration, at which the first significant effect of a drug was observed, i.e. at least 50% of

cells were arrested in mitosis for significant time, was defined as a minimal mitostatic concentration (MMC).

The second effect was observed at the higher doses, when as a result of mitotic arrest cells mostly underwent mitotic slippage or death in mitosis. Above some particular concentration the proportion of these two fates did not change significantly. This concentration was determined as a second threshold (T2). All these threshold concentrations are summarized in Table 11.

Table 11. Summary of MMC and T2 concentrations for Nocodazole, Taxol, and Vinorelbine

	Minimal mitostatic concentration (nM)			**The second threshold (nM)**		
	Nocodazole	Taxol	Vinorelbine	Nocodazole	Taxol	Vinorelbine
A549	30	10	3	300	100	100
U-2OS	30-100	10	0.3-1	300	1000	30
U-118	10-30	10-30	0.1-3	100	300	10

In addition, the effect of Nocodazole at MMC was sharp, and it independently on the cell line result in steep increase in the proportion of cells arrested in mitosis. The effects of Taxol and Vinorelbine were more gradual.

Also, for A549 cells a major outcome of prolonged mitotic arrest at the doses above T2 of all three drugs was mitotic slippage. For U-2OS under Nocodazole and Taxol treatments, and for U-118 under Nocodazole and Vinorelbine treatments the similar outcome was observed.

3.3 Analysis of dose-dependent changes on the duration of mitosis

In this part the effect of anti-microtubule drugs on the duration of mitosis was analyzed. The average duration of mitosis for all cell fates was plotted against the drug concentrations. Linear regression analysis was performed for the concentration ranges that included concentrations above T2.

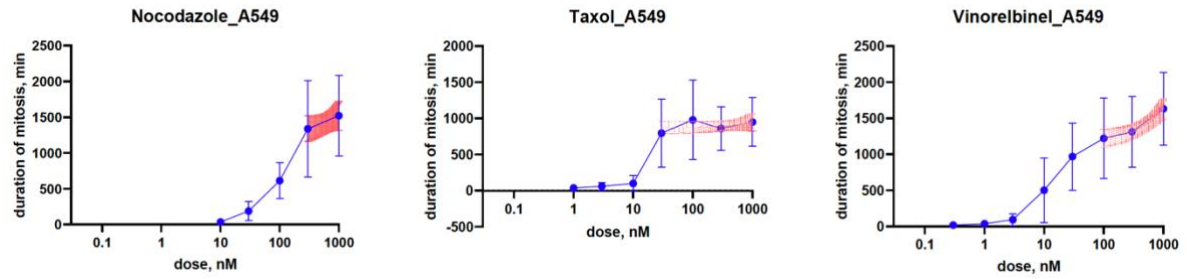

Figure 8. Dependence of the duration of mitosis of A549 cells on the concentration of an inhibitor of mitosis. Error bars indicate standard deviation. Red bars indicate 95% confidence band of the best-fit line for the chosen interval analyzed by linear regression. *p*-values are summarized on the Table 12.

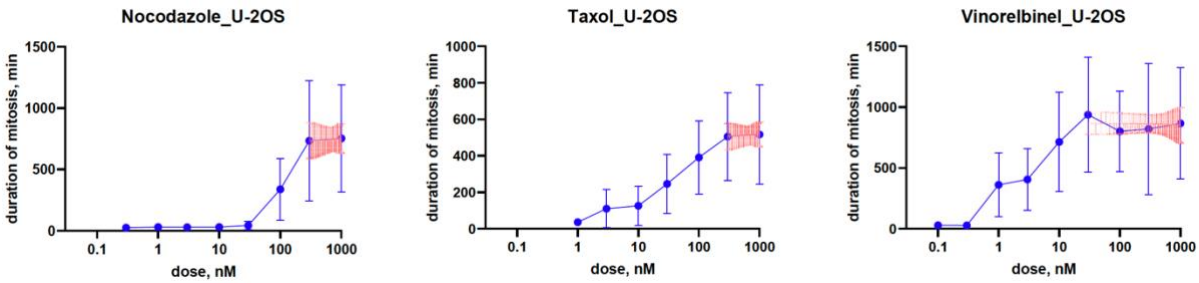

Figure 9. Dependence of the duration of mitosis of U-2OS cells on the concentration of an inhibitor of mitosis. Error bars indicate standard deviation. Red bars indicate 95% confidence band of the best-fit line for the chosen interval analyzed by linear regression. *p*-values are summarized on the Table 12.

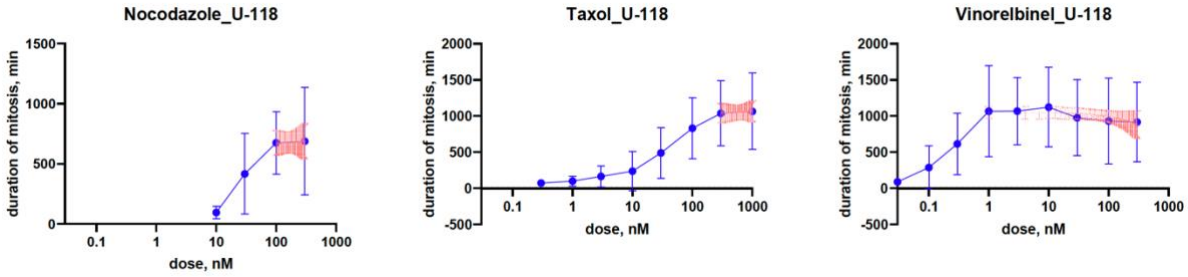

Figure 10. Dependence of the duration of mitosis of U-118 cells on the concentration of an inhibitor of mitosis. Error bars indicate standard deviation. Red bars indicate 95% confidence

band of the best-fit line for the chosen interval analyzed by linear regression. *p*-values are summarized on the Table 12.

For all cells and drugs at MMC gradual increase in the duration of mitotic arrest was observed. However, it reached plateau at the concentrations above T2.

Table 12. Summary of *p*-values indicating significance of the deviation of the slope from zero for linear regression analysis of the chosen ranges of concentrations (Detailed report of the analysis provided in Appendix 1).

	Nocodazole	**Taxol**	**Vinorelbine**
A549	0.1855	0.3753	<0.0001
U-2OS	0.8456	0.8137	0.8590
U-118	0.8863	0.7948	0.1379

CHAPTER FOUR:

DISCUSSION

The aim of this study was to study drug- and dose-dependent effect of the inhibitors of microtubule drugs on the cancer cells by treating them with the wide range of concentrations of anti-microtubule drugs and observing the fate of mitotic cells directly under microscope.

The first effect of inhibitors was observed beginning from the low concentrations. The cells were arrested in mitosis. Further increase in the concentration led to the increase in the duration of mitotic arrest. However, these cells were able to divide. In order to quantify this effect the concept of minimal mitostatic concentration was introduced, and it was defined as a concentration, which causes mitotic arrest in at least 50% of cells. This arrest mostly ended with division into two or more cells, but sometimes it could lead to mitotic slippage.

This concentration corresponds to $K_{i,\,met}$, and $K_{ana/met}$ introduced by Jordan, at which 50% of cells accumulate at metaphase-like stage, or which leads to 50% decrease in the anaphase to metaphase ratio, correspondingly (Jordan 1991, 1992). As it was reported earlier at these concentrations type 1 and type 2 spindles that resemble normal spindle with metaphase plate, but with thicker microtubules, shorter interpolar distance, and longer astral microtubules containing 1-3 chromosomes, were observed (Jordan 1991, 1992). These types of mitotic spindle eventually lead to the satisfaction of SAC (Brito et al. 2008), and the drug-treated cells divided into two or more daughter cells.

By increasing the concentration further, the dose, above which prolonged mitotic arrest led to mostly mitotic slippage or death in mitosis was determined. This concentration was defined as a second threshold (T2) concentration. It was also shown that above T2 the duration of prolonged mitotic arrest does not depend on the concentration and remains constant.

At the high concentrations of anti-microtubule drugs type 3 spindles, which are monopolar and ball shaped, with chromosomes located outside the spindle, are observed (Jordan 1991, 1992). At these concentrations SAC cannot be satisfied (Brito et al. 2008). T2 concentration might be the concentration at which type 3 spindles are formed. Since they do not lead to the satisfaction of SAC, the prolonged mitotic arrest occurs. As further increase in the concentration above T2 do not lead to the formation of spindle that can satisfy SAC, plateau in the duration of mitotic arrest is reached. However, it is unclear why at the concentrations beyond T2 some proportion of cells undergoes mitotic slippage, while others undergo death in mitosis.

For all cell lines analyzed the major outcome of prolonged mitotic arrest at the concentrations above T2 was mostly correlated between the drugs inside the same cell line. Therefore, it might be concluded that when SAC cannot be satisfied the outcome of mitotic arrest is determined by a cell line.

In contrast, the first effect, which is observed at MMC is determined by a drug. Treatment with Nocodazole at low concentrations always led to the sharp increase in the number of cells arrested in mitosis. Treatment with low concentrations of Taxol and Vinorelbine resulted in gradual increase of the proportion of cells arrested in mitosis, which mostly successfully divided after significant delay.

Based on the earliest studies done on isolated microtubules, which determined two distinct IC50 concentrations - IC50 of shortening rate and IC50 of dynamicity (Jordan 2002), it can be deduced that the effect of anti-microtubule drugs observed at MMC occurs because of the inhibition of shortening rate. However, the full dynamics of microtubules is not completely inhibited. This may lead to the proper (‘end-on’) attachment of microtubules to kinetochores, and eventually to the satisfaction of SAC.

In case of the T2 threshold concentration, it may correspond to the situation, when IC50 of dynamicity was reached inside the cells. As it inhibits the full dynamics of

microtubules, abnormal spindles (type 3) are formed, and they cannot satisfy SAC. This leads to the activation of cascades of reactions, which determines the further fate of the cell that is dictated solely by its intrinsic peculiarities.

Therefore, further studies should be focused on determining the key players determining these properties of the cell, which dictates the outcome of prolonged mitotic arrest in the concentrations above T2 threshold.

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APPENDIX 1. Detailed report of the linear regression analysis on the chosen concentrations

	A549 Noc	U-2OS Noc	U-118 Noc	A549 Tax	U-2OS Tax	U-118 Tax	A549 Vin	U-2OS Vin	3T3 Vin	U-118 Vin
Best-fit values										
Slope	0.2614	0.02714	0.065	0.07564	0.01714	0.03714	0.4572	-0.0174	0.0407	-0.5729
Y-intercept	1261	725.9	668.5	872	499.9	1026	1176	868.7	304.7	1051
X-intercept	-4822	-26742	-10285	-11528	-29158	-27619	-2572	49939	-7486	1835
1/slope	3.825	36.84	15.38	13.22	58.33	26.92	2.187	-57.49	24.57	-1.745
Std. Error										
Slope	0.1958	0.139	0.4527	0.08508	0.07255	0.1424	0.1093	0.09773	0.03633	0.3848
Y-intercept	136.5	111	86.16	46.08	55.25	101.9	70.05	48.94	20.12	43.8
95% Confidence Intervals										
Slope	-0.1280 to 0.6509	-0.2490 to 0.3033	-0.8408 to 0.9708	-0.09236 to 0.2436	-0.1268 to 0.1610	-0.2456 to 0.3199	0.2411 to 0.6733	-0.2105 to 0.1757	-0.03104 to 0.1125	-1.331 to 0.1853
Y-intercept	989.0 to 1532	505.3 to 946.4	496.1 to 840.9	781.0 to 963.0	390.3 to 609.4	823.5 to 1228	1037 to 1314	772.0 to 965.4	265.0 to 344.4	965.1 to 1138
X-intercept	-infinity to -1558	-infinity to -1715	-infinity to -528.2	-infinity to -3304	-infinity to -2482	-infinity to -2638	-5339 to -1573	4409 to +infinity	-infinity to -2444	821.9 to +infinity
Goodness of Fit										
R square	0.02103	0.0004188	0.0003494	0.004826	0.0005471	0.0007154	0.1111	0.000217	0.007688	0.009714
Sy.x	626.5	459.3	331.9	423.9	258.2	489.7	517.5	452.5	183.5	553.9
Is slope significantly non-zero?										
F	1.783	0.03813	0.02062	0.7905	0.05584	0.06801	17.5	0.03168	1.255	2.217
DFn, DFd	1, 83	1, 91	1, 59	1, 163	1, 102	1, 95	1, 140	1, 146	1, 162	1, 226
P value	0.1855	0.8456	0.8863	0.3753	0.8137	0.7948	<0.0001	0.859	0.2642	0.1379
Deviation from zero?	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Significant	Not Significant	Not Significant	Not Significant
Equation	Y = 0.2614*X + 1261	Y = 0.02714*X + 725.9	Y = 0.06500*X + 668.5	Y = 0.07564*X + 872.0	Y = 0.01714*X + 499.9	Y = 0.03714*X + 1026	Y = 0.4572*X + 1176	Y = -0.01740*X + 868.7	Y = 0.04070*X + 304.7	Y = -0.5729*X + 1051
Data										
Number of X values	93	101	68	171	112	105	149	154	170	231
Maximum number of Y replicates	1	1	1	1	1	1	1	1	1	1
Total number of values	85	93	61	165	104	97	142	148	164	228
Number of missing values	8	8	7	6	8	8	7	6	6	3

SUMMARY

The aim of this study was to study drug- and dose-dependent effect of the inhibitors of microtubule drugs on the cancer cells by treating them with the wide range of concentrations of anti-microtubule drugs and observing the fate of mitotic cells directly under microscope.

The two distinct effects of anti-microtubule drugs were determined. The first effect of inhibitors was observed beginning from the low concentrations. The cells were arrested in mitosis. Further increase in the concentration led to the increase in the duration of mitotic arrest. However, these cells were able to divide. In order to quantify this effect the concept of minimal mitostatic concentration was introduced, and it was defined as a concentration, which causes mitotic arrest in at least 50% of cells. This arrest mostly ended with division into two or more cells, but sometimes it could lead to mitotic slippage.

The second effect was observed at the high concentrations of inhibitors of microtubule dynamics. This specific concentration was defined as a second threshold (T2) concentration. It was also shown that above T2 the outcome of mitotic arrest is mitotic slippage or death in mitosis, and the proportions of these two fates do not change with further increase in the concentration. In addition, above T2 the duration of prolonged mitotic arrest does not depend on the concentration and remains constant.

For all cell lines analyzed the major outcome of prolonged mitotic arrest at the concentrations above T2 was mostly correlated between the drugs inside the same cell line. In contrast, the first effect, which is observed at MMC is determined by a drug.

ТҰЖЫРЫМ

(Summary in Kazakh)

Бұл зерттеудің мақсаты микротүтікшелер динамикасының ингибиторларының дозаға тәуелді қатерлі ісік жасушаларына әсерін, оларды микротүтікшелерге қарсы дәрілік заттардың кең концентрациясымен емдеу және митоздық жасушалардың тағдырын тікелей микроскоппен бақылау арқылы зерттеу болды.

Микротүтікшелер динамикасынының ингибиторларының екі айқын әсері анықталды. Ингибиторлардың алғашқы әсері төмен концентрациядан бастап байқалды. Жасушалар митоз кезінде тоқтатылуға ұшырады. Концентрацияны одан әрі арттыру митоздық тоқтатылу ұзақтығының өсуіне алып келді. Алайда, бұл жасушалар бөліне алды. Бұл әсерді сандық бағалау үшін ең төменгі митостатикалық концентрация (ММС) ұғымы енгізілді және ол, жасушалардың кем дегенде 50% -ында митоздық тоқтатылуды тудыратын концентрация ретінде анықталды. Бұл тоқтатылу көбінесе екі немесе одан да көп жасушаларға бөлінумен аяқталды, бірақ кейде бұл митоздық сырғуға да әкелуі мүмкін.

Екінші әсер микротүтікшелер динамикасының ингибиторларының жоғары концентрациясында байқалды. Бұл концентрация жаңа екінші шекті концентрация (Т2) ретінде анықталды. Сондай-ақ, Т2-ден жоғары концентрацияларда митоздық тоқтату нәтижесі митоздың сырғуы немесе митоз кезінде өлім болатындығы және концентрацияның одан әрі жоғарылауымен осы екі тағдырдың пропорциялары өзгермейтіні көрсетілді. Сонымен қатар, Т2-ден жоғары концентрацияларда митотикалық тоқтату ұзақтығы концентрацияға байланысты емес және тұрақты болып қалады.

Барлық жасуша желілері үшін түрлі микротүтікшелер динамикасының ингибиторларының Т2 концентрациясынан жоғары дозаларында ұзақ митоздық тоқтатылудың негізгі нәтижелері көбінесе өзара коррелятивті байланысты болды. Ал ММС кезінде байқалатын алғашқы әсер дәрі-дәрмекпен анықталады.

Кілт сөздер: *микротүтікшелер динамикасының ингибиторлары, минималды митостатикалық концентрация, екінші шекті концентрация, митоздық тоқтатылу*