T.C.
BAHÇEŞEHİR UNIVERSITY

DISCOVERY OF NOVEL CELL CYCLE INHIBITOR AS A CHEMOTHERAPEUTIC AGENT USING IN VITRO METHODS

Master's Thesis

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PREFACE

Above all, I would like to thank my supervisor Assist. Prof. Dr. Timuçin AVŞAR for his academical and spiritual supports.

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ABSTRACT

DISCOVERY OF NOVEL CELL CYCLE INHIBITOR AS A CHEMOTHERAPEUTIC AGENT USING IN VITRO METHODS

Berfu Nur Yiğit

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Cancer is the second cause of death after heart diseases among worldwide. According to World Health Organization (WHO) reports in 2018, one in six deaths is due to the cancer. Despite the fact that, there are a lot of researches and investments in oncology, current chemotherapeutic drugs are below the expectations to meet phenotypic and functional heterogeneities of cancers in patients. Therefore, finding new small molecule structures which can provide alternative approaches in anti-cancer treatment is important. In recent years, cell cycle inhibitors take place as hallmark strategies against cancer cells due to their relative selectivity to cancer cells compared to cytotoxic agents. These small molecules target dividing cells in G1/S, G2 or mitotic spindles checkpoints to arrest cell cycle and eventually cause apoptosis of cancer cells and inhibition of tumor growth. Suppression of cell division by G2/M arrest in cell cycle is widely used approach to treat cancer cells. These agents generally cause microtubule instability and DNA damage during cell division and cause irreversible damages which produce signals to arrest cells. Paclitaxel (PTX) is one of the anti-cancerous agent causing cell cycle arrest at mitosis, widely for various range of tumor types including lung, ovarian, breast and neck cancers. In this study, we discovered the anti-cancerous effects of a new imidazolone based small molecule that we call it as "19D". It causes G2/M phase cell cycle arrest in various cancer and healthy cell lines. To evaluate activities in cells treated with 19D, we performed many *in vitro* assays focused on cell cycle disruptions, anti-proliferative effects, apoptosis and nuclear deteriorations. The results showed that, 19D causes cell cycle G2/M phase arrest as much as PTX and activate DNA damage mechanism. In conclusion we found new chemotherapeutic drug candidate which is potential to become a novel therapeutic compound.

Key Words: Chemotherapy, Anti-Mitosis Drugs, G_2M Arrest, *In vitro* Drug Screening

ÖZET

IN VİTRO YÖNTEMLER İLE HÜCRE DÖNGÜSÜNÜ DURDURAN YENİ KEMOTERAPÖTİK AJAN KEŞFİ

Berfu Nur Yiğit

Sinir Bilim Yüksek Lisans Programı

Tez Danışmanı: Assist. Prof. Dr. Timuçin AVŞAR

Ocak 2019, 41 sayfa

Kanser dünya çapında kalp hastalıklarından sonra ikinci sırada yer alan ölüm sebebidir. Dünya Sağlık Örgütü'nün (WHO), 2018'deki raporlarına göre; altı ölümden biri kanser ile ilişkilidir. Onkoloji alanında çok fazla araştırma olmasına ve yatırım yapılmasına rağmen, mevcut kemoterapötik ilaçlar, hastalarda kanserlerin heterojenitesini karşılamak için beklentilerin gerisindedir. Bu nedenle, anti-kanser tedavisinde yeni küçük moleküller bulmak önemlidir. Son yıllarda, hücre döngüsü inhibitörleri, kanser hücrelerine karşı sitotoksik ajanlardan daha seçici olduklarından, kanserle mücadele yaklaşımında ana stratejiler olarak yerlerini almaktadırlar. Bu küçük moleküller, G1/S, G2 veya mitotik iğ iplikleri kontrol noktalarında kanser hücrelerinin bölünmesini durdurarak, apoptozunu sağlayıp tümör dokusunun büyümesinin önlenmesini hedeflemektedir. Hücre bölünmesinin, hücre döngüsündeki G2/M fazı tutulması ile baskılanması, kanser hücrelerini tedavi etmek için yaygın olarak kullanılan bir yaklaşımdır. Bu ajanlar genellikle hücre bölünmesi sırasında mikrotübül instabilitesine ve DNA hasarına neden olarak onarılmaz hasarlarla hücreleri bölünme sırasında tutuklamak için sinyaller üretilmesini sağlamaktadır. Paklitaksel (PTX), akciğer, yumurtalık, meme ve boyun kanserleri de dahil olmak üzere çeşitli tümör tipleri için yaygın olarak kullanılan G2/M fazı anti-mitoz ajanlarından biridir. Bu çalışmada, çeşitli kanser ve sağlıklı hücre hatlarında G2/M tutukluğuna neden olan yeni anti-mitotik imidazolon bazlı molekül olan 19D'nin etkileri keşfedilmiştir. 19D'ye maruz kalan hücrelerdeki aktiviteleri değerlendirmek için, hücre döngüsü, anti-proliferatif etkiler, apoptoz ve nükleer bozulmalar üzerine odaklanmış birçok *in vitro* analiz gerçekleştirilmiştir. Sonuçlar 19D'nin G2/M tutuklayıcılığının PTX kadar güçlü olduğunu ve DNA hasar mekanizmasını aktive ettiğini göstermektedir. Sonuç olarak, spesifik mekanizmayı anlamak için daha fazla araştırmaları gerektiren yeni kemoterapötik ilaç adayı bu çalışma ile bulunmuştur.

Anahtar Kelimeler: Kemoterapi, Anti-Mitoz İlaçları, G2/M Tutuklama, *In vitro* İlaç Taraması

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ABBREVIATIONS

APC	:	Anaphase-promoting complex
ATM	:	Ataxia telangiectasia mutated
ATR	:	Rab3-related ATM
AT1R	:	Angiotensin receptor II
CDK	:	Cyclin dependent kinases
DAPI	:	4’,6- Diamidibo-2-Phenylindole, Dihydrochloride
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
EDTA	:	Ethylenediaminetetraacetic acid
FBS	:	Fetal bovine serum
FDA	:	Food and Drug Administration
GTP	:	Guanosine-5'-triphosphate
HBSS	:	Hank's balanced salt solution
PBS	:	Phosphate buffered saline
PBST	:	Phosphate buffered saline with tween20
PI	:	Propidium Iodide
PrB	:	Phoshorylated retinoblastoma protein
PTX	:	Paclitaxel
rB	:	Retinoblastoma protein
RNA	:	Ribonucleic acid
SAC	:	Spindle assembly checkpoint
SDS	:	Sodium dodecyl sulfate

SYMBOLS

CO_2	:	Carbondioxyde
dH_2O	:	Distillated water
H_2O_2	:	Hydrogen Peroxide

1. INTRODUCTION

Chemotherapy is one of the most common approach to fight cancer cells in addition to surgery and radiation therapy. Today's attempts in development of new chemotherapy agents relies on previous scientific studies and confirmed drug molecules which are generally plant extracts or bacterial metabolites. The discovery and development of a new molecule requires high costs and takes long time, but diversification in these chemicals can provide more suitable therapy options to patients' subjective molecular differentiations of cancer characteristics.

New designed cancer drugs are firstly tested to find best candidates via in vitro assays and animal models. All stages in cancer drug development requires great effort to accomplish, which makes difficult challenge to translate findings to treatment process. From the cell culture experiments, most of the candidates can be eliminated before further experiments. Because of that feature, although cell lines do not really reflect differentiation and heterogeneity of cancer cell in human body, it is important to use them as a first step preclinical testing purposing.

Preclinical and clinical development of cell cycle inhibitor agents such as paclitaxel, have brought opportunity to discovery of variety of molecules that are relatively more selective to cancer cells. Although cell cycle and its control mechanisms still have unknown gaps, cell cycle inhibitor agents provide more suitable treatment approach than cytotoxic chemotherapy agents because they cause relatively lesser side effects in patients. These agents provide cancer cell arrest in cell division in either of G1/S, G2 or mitotic cell division checkpoints. The abnormal microtubule distribution across cytoplasm can trigger control of cell cycle mechanism and eventually, activate apoptosis mechanisms. Dysregulations and disruptions of cell cycle with anti-mitotic agents, lead to tumor growth arrest and apoptosis which is the main idea of chemotherapy. In this study, we discovered a novel imidazolone based molecule called as 19D which, causes cell cycle G2/M phase arrest in various cell lines and has potential as chemotherapic agent.

To understand underlying mechanisms of 19D activity, we performed many *in vitro* experiments using various healthy and cancer characterized cell lines such as HUVEC, MCF-7, HCT, HEK, RFL6 and U87-MG. The evaluation of cell cycle characterization was obtained by flow cytometric cell cycle analysis. Cellular and nuclear morphological alternations were investigated via immunocytochesmistry and immunofluorescence stainings. The target molecule of cell cycle arrest and apoptosis was investigated through caspase3/7 fluorescence staining. Finally, agent causing cell growth inhibition was determined by colorimetric MTT assay.

In general, flow cytometry results revealed that 19D inhibited cell proliferation by inducing G2/M phase arrest in a dose dependent manner. Simultaneously, we discovered that this novel molecule 19D, caused same level arrested cell population with highly used anticancer drug paclitaxel. Furthermore, we have discovered that 19D disrupts nuclear envelope of cells resulting small multiple nuclear fragments which represent subG1 populations including fragmented small DNAs and activates apoptosis mechanism. According to the morphological changes and staining results, 19D led to apoptosis over the IC50 values, following significant increase of caspase 3/7 protein levels. MTT colorimetric analysis showed that agent inhibits cell division with the relation to their division time.

Our results from this study showed that 19D induces cell growth inhibition by cell cycle G2/M phase arrest and accelerates apoptotic events in various cancer and healthy cell lines. Suppression of cell division by G2/M arrest in cell cycle is widely used approach to treat cancer cells, in our study; this novel imidazolone based small molecule has great potential as an anticancer therapy agent.

2. LITERATURE REVIEW

2.1 ANTICANCER DRUG DEVELOPMENT

Innovations and improvements to understand human genetics provides dramatic changes in the capabilities of disease treatments. Since 1990s, pharmaceutical companies have grown faster than other health science companies. Although new therapy approaches to improve patients' quality of life relies on discovery and development of new agents, this process requires long time and high costs (DiMasi et al., 1991). In the cancer field, new and more effective drug discoveries can make difference and provide alternative ways to patients' therapy, because of cancers' subjective molecular differentiations in that person. But at the same time, oncology related drugs passing to human clinical trials has been limited with a remarkably low numbers (Hutchinson and Kirk, 2011).

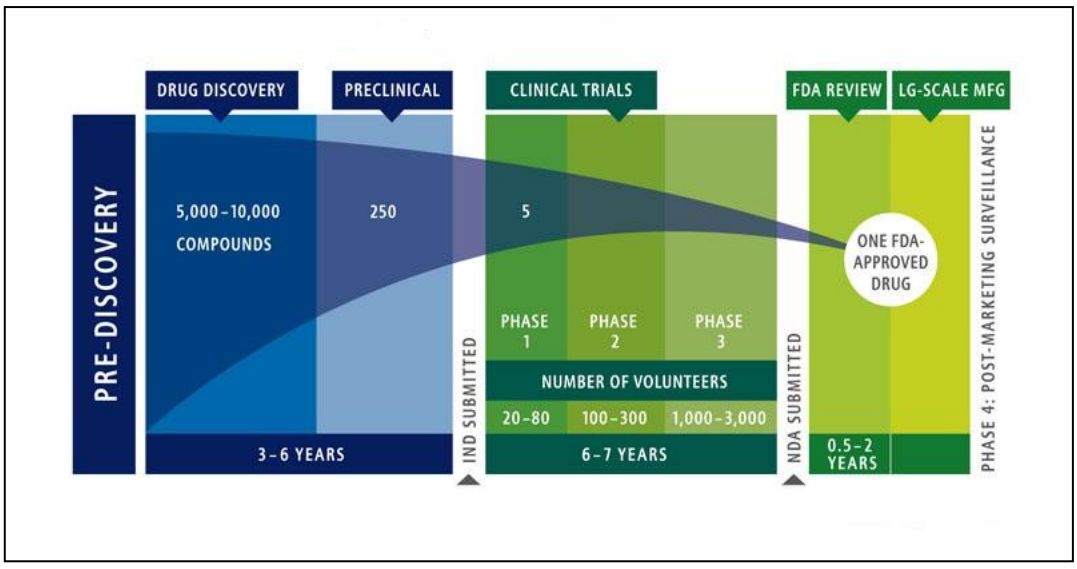
Development of new molecules in cancer therapy, mainly relies on previous scientific findings and previously tested known molecules which results high unmet needs in oncology. Discovery programs try to find best candidates in synthesized molecules firstly via cell culture assays and animal models. First of all, pre-clinical experiments in cell lines does not really reflect cancer cells' molecular differentiations and heterogeneity. Secondly although mouse models provide suitable platform to use before human trials, it is difficult to adapt these data to clinics (Francia and Kerbel, 2010). Due to obvious differences between human and animal metabolims, drugs tested on animal models might results with greater tolerance and different protein binding in chracateristics which can be concluded as imperfect reflection of pharmacokinetics of drugs in human (Chabner and Roberts Jr, 2005).

Additionally, clinical trials require great time and cost and has small success rate. To pursue a new molecule trials as a pharmaceutical, manufacturers first apply Food and Drug Administration (FDA). After confirmation to start, manufacturers can make phase I tests. The first phase requires small number of usually healthy people (20 to 100 volunteers) to test in safe dosages of molecule to get in formations about distribution, absorption, metabolic effects, toxicity and excretion. For cancer and HIV drugs, phase I

trials can be conducted on people who have specified conditions and want to try this molecule as a treatment. Usually phase I human testing generally performed outside of United States. At the next step, phase II trials try to demonstrate efficacy and safety of drug on up to several hundred people who have target disease or condition. Next step, pre-approved molecules go phase III, which is conducted with large number of disease suffering people (300 to 3000 volunteers) to state side-effects and final efficiency statement. Until this stage, ninety percent of drug candidates either fail during I-II-III phases or rejected by national regulatory agency (Benson III et al., 2004) (Figure 2.1). Only small percent of drugs can safely undergo to phase IV (post marketing surveillance) trials which conducted on several thousands of volunteers who have target disease or condition to understand long term effects of drugs (DiMasi et al., 2003). These phases can take 12 to 18 years to complete and costs nearly 1 billion dollars (Adams and Brantner, 2006).

As a conclusion, there are various factors in discovery of a new molecule and conduct this as a new therapy agent, but cancer patients' wide range heterogeneity requires new molecules to make advances and deliver which improve their lives.

Figure2.1: Stages of the drug development process



Reference: Seeland, S. 2013. How in vitro alterations in cellular energy pathways can overcome obstacles in drug research.

2.2 CHEMOTHREAPY

Chemotherapy is a way of treatment approaches in oncology which basically use chemical agents to destroy cancer cells. Today, approaches used to cure cancer in patients, still relies on most exclusively on surgical therapy in first step, but radiotherapy, chemotherapy, immunotherapy provide adjuvant theraphy to reduce the number of remnant cancer cells (Delisle and Devauchelle, 1990). According to the histological markers in lesion, location and metastases characteristics of tumor and general disease state; patients can undergo specific combined therapies.

The aims in the chemotherapy can be divided into three distinct classification based on tumor characterizations in patients. The main aim is to achieve complete recovery of cancer which is also named as curative treatment. If this aim is not a reachable, second option is that it can provide adjuvant treatment to prevent metastases of cancer. Last option is to revise the condition for palliative treatment to prolongation of life (MacEwen, 1985). Chemotherapy solely has capability to cure cancers in a way of relieved painful symptoms.

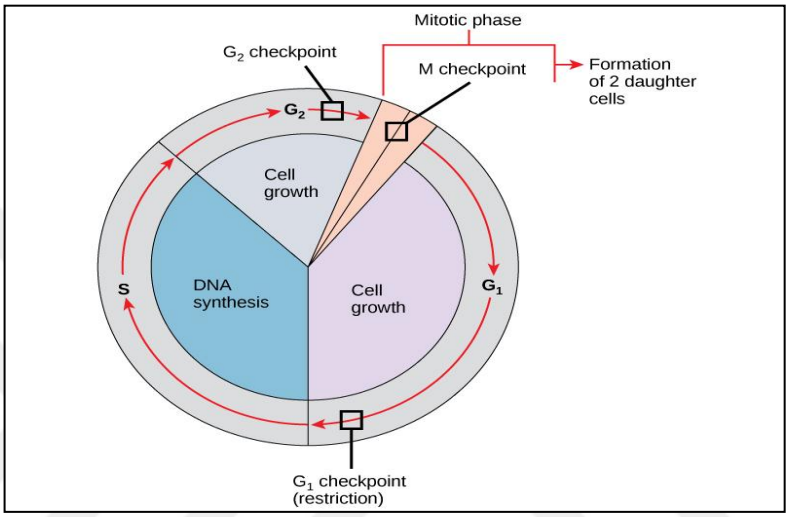
2.3 CELL CYCLE CHECKPOINTS

In eukaryotic cell division, there are signaling pathways to regulate cell cycle phase transitions to ensure correct replication of the genome. These general features in cell cycle regulations are conserved processes from yeast to human (Morgan, 2007). In these transitions, protein kinase complexes composed of cyclins and cyclin dependent kinases (CDK) provide regulation between phases G0, G1, S, G2 and M via site-specific phosphorylation and ubiqutin-mediated degradations (Koepp et al., 1999).

In cell cycle, eukaryotic cells have G1/S, G2 and mitotic spindles checkpoints (Figure 2.2). Cells can arrest at cell cycle checkpoints in response to stress factors which provide cell to time recover cellular damage in that stage. But, if the damage is irreparable, cells can activate programmed cell death to dimishes its existence at these

checkpoints. Importance of these checkpoints for proper cell division is revealed by high frequency mutations found in constituent regulatory proteins during oncogenesis (Massagué, 2004). Therefore, these processes provide more effective approach to treat cancer cells than cytotoxic drugs as well as it is important to understand genomic instability of cancer cells (Debatin, 2000).

Figure2.2: Three cell cycle checkpoint to control division

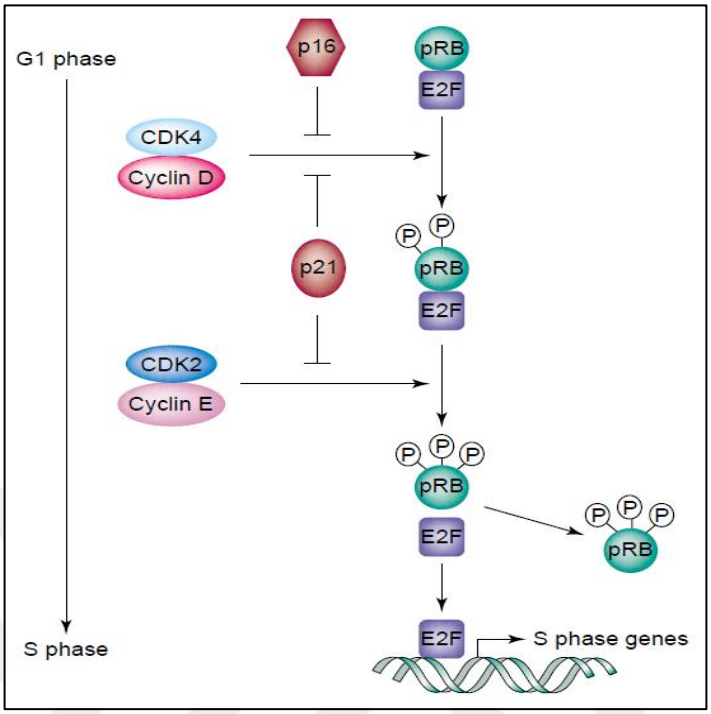


Reference: Cell cycle Checkpoints [online] https://courses.lumenlearning.com/wmopen-biology1/chapter/cell-cycle-checkpoints/ [11.12.2018]

2.3.1 G1/S Checkpoint

During G1/S checkpoint, cyclin-D-CDK4 and cyclin-E-CDK2 complexes are activated to regulate activation of genes to pass S phase to DNA synthesis correctly (Figure2.3). In G1 phase, uncommitted cells to enter S-phase, hypo-phoshorylated retinoblastoma protein (pRB) bind to E2F transcription factor to inhibit downstream transcriptional events. To enter S-phase, Rb gets sequential phosphorylation with cyclin-D-CDK4 and cyclin-E-CDK2 which dissociate repressor complex and cells led to transcriptions of required genes for DNA synthesis (Besson et al., 2008). But if cells are exposed to genotoxic agents, cyclin and CDK complexes inhibited by p21 and p53. If phoshorylated retinoblastoma protein (pRB) transcriptional factor get hypophosphorylated and bind to E2F, this binding results with inhibition of S phase genes transcription which causes cell cycle arrest at G1-S transition (Stewart and Pietenpol, 2001).

Figure 2.3: G1-S phase transition cell cycle control protein complexes



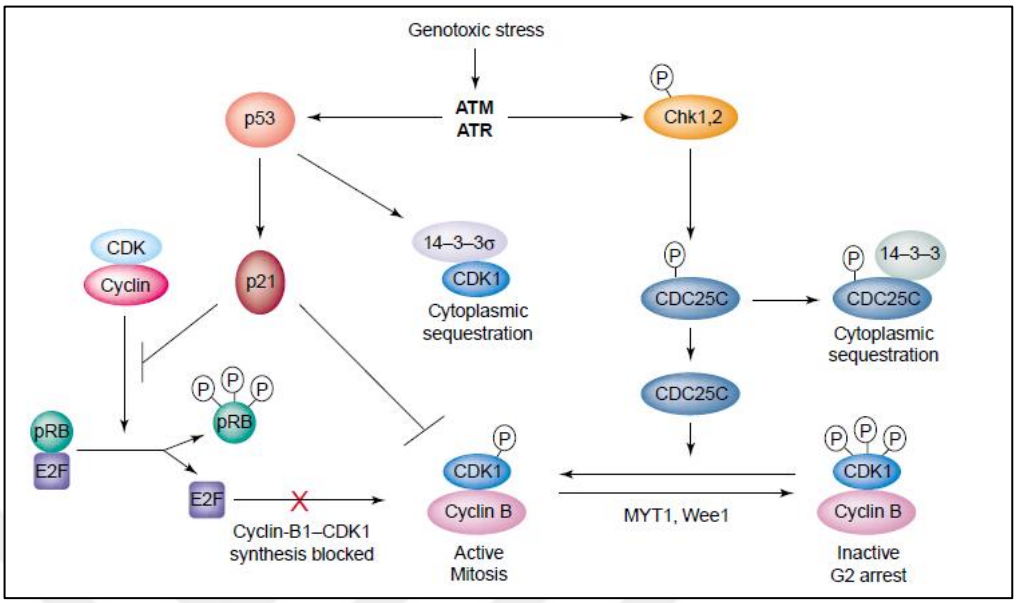
Reference: Stewartz, Z. A., Westfall, M. D. & Pietenpol, J. A. 2003. Cell-cycle dysregulation and anticancer therapy. Trends in pharmacological sciences, 24**,** 139-145.

2.3.2 G2 Checkpoint

After DNA synthesis, G2 phase checkpoint provides ensuring of cells entering mitosis without DNA damage. CDK1-Cyclin B complex meanly regulate the transition to spreading. CDK1 (cdc2) preserved in an inactive state by Wee1 and Myt1, PLK1 and Aurora A activated cdc25 phosphatase and cdc2 downstream activity (Ciccia and Elledge, 2010).

The damages to the DNA in this phase, cause activation of ataxia telangiectasia mutated (ATM) and Rab3-related ATM (ATR) pathways to inhibit Cdk-1 complex (Figure2.4). ATM signaling pathway provide synthesis of check point kinase 2 (Chk2) protein, at the same time ATR can provide check point kinase 1 (Chk1) to phoshorylate CDC25C (Abraham, 2001). Consequently, this phoshorylated CDC25C cause inactivation of CDK1-CyclinB complex and cause cell cycle arrest in G2 phase.

Figure 2.4: G2 phase cell cycle control protein complexes

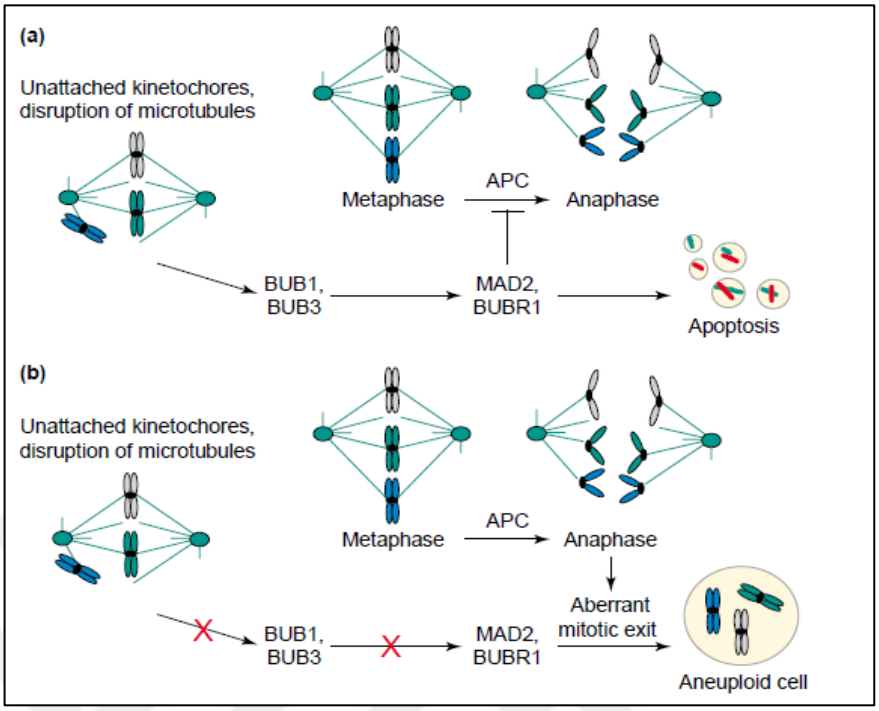


Reference: Stewartz, Z. A., Westfall, M. D. & Pietenpol, J. A. 2003. Cell-cycle dysregulation and anticancer therapy. Trends in pharmacological sciences, 24**,** 139-145.

2.3.3 Mitotic Spindle Checkpoint

The last cell cycle checkpoint in eukaryotes occurs in chromosome segregation during anaphase to control attachments of chromosome into mitotic spindle and microtubule structures (Figure2.5). The spindle assembly checkpoint (SAC) ensures kinetochores properly attached to the spindles. At the checkpoint activation, CDC20 and SAC proteins is located at the kinetochores to sense correct kinetochore-microtubule attachment. Even one unattached kinetochore can trigger checkpoint (De Antoni et al., 2005). When sister kinetochores attached to the opposite poles, forces occur two poles which incorrect tension can trigger another checkpoint. The chromosomal passenger complex Aurora-B/Ipl1 can detect and destabilized incorrect attachments at microtubule-kinetochore interface. One of the tension or orientation checkpoints is activated, cells can be blocked in anaphase by inhibiting anaphase-promoting complex (APC) and kinetochore-associated BURB1 and MAD2 to prevent further dissociations (Musacchio and Hardwick, 2002).

Figure 2.5: Mitotic spindle control protein complexes



Reference: Stewartz, Z. A., Westfall, M. D. & Pietenpol, J. A. 2003. Cell-cycle dysregulation and anticancer therapy. Trends in pharmacological sciences, 24**,** 139-145.

2.4 CELL CYCLE DISREGULATING DRUGS IN CHEMOTHERAPY

Cancer is characterized by uncontrolled proliferation of cells although there are restrictions about nutrients and space. These unlimited replication potential develops mainly because of upregulated telomerase expression. Additionally, cancer cells can mute tumor suppressor genes such as p53 which create more chronic proliferation indexes. Moreover, they can also produce their own growth factor ligand or signal to surrounding non-tumorigenic tissue to supply cancer cells with growth factors to keep them up with high energy (Musacchio and Salmon, 2007). These processes can trigger invasions and metastasis to the other sides of body.

Current chemotherapy drugs rely on interception of this catastrophic proliferation of cancer cells. Since these cells pass many cell divisions compared with health cells, arresting of cells in cell cycle provide great approach to be used in chemotherapy.

Cells arrested in cell cycle phases can go apoptosis, autophagy or remain in senescence-like G1 state. Cell cycle inhibitory drugs can be collected as; taxanes, epothilones, vinca alkaloids, anthracycline antibiotics. (Table2.4.1.)

Table 2.1: List of one of the most used chemotherapeutic agents based on chemical classes, action mechanisms and main cancer targets

Name	Class	Action mechanism	Main target cancer type
Paclitaxel	Taxanes	Microtubule stabilizing	Breast, lung, bladder, ovarian, melanoma
Cabazitaxel	Taxanes	Microtubule stabilizing	Hormones-resistant prostate cancer
Vintafolide	Vinca alkaloids	Microtubule destabilizing	Ovarian and lung cancers
Eribulin	Vinca alkaloids	Microtubule destabilizing	Breast cancer and liposarcoma
Ixabepilone	Epothilones	Microtubule stabilizing	Breast cancer
Doxorubicin	Anthracycline antibiotics	*Anthracycline Topoisomerase* Inhibitor	Bladder, breast cancers, lymphoma
Fluorouracil	Pyrimidine analog	Thymidylate synthase inhibitor	Colon, stomach, esophageal cancers
Epirubicin	Anthracycline antibiotics	Intercalating to DNA strands	Breast, ovarian, gastric, lung cancers
Vincristine	Vinca alkaloid	Microtubule destabilizing	Leukemia, neuroblastoma, and lung cancer
Cisplatin	Platinum- based alkylating agent	Intercalating to DNA strands	Testicular ovarian tumors cancers
Laulimalide (in vivo trials)	Non-taxoid microtubule stabilizer	Microtubule stabilizing	Breast cancer
Peloruside A (in vivo trials)	Non-taxoid microtubule stabilizer	Microtubule stabilizing	Lung and breast cancers
ESE-16 (clinical trials)	Estrogen derivatives	Microtubule destabilizing	Breast and esophageal cancers

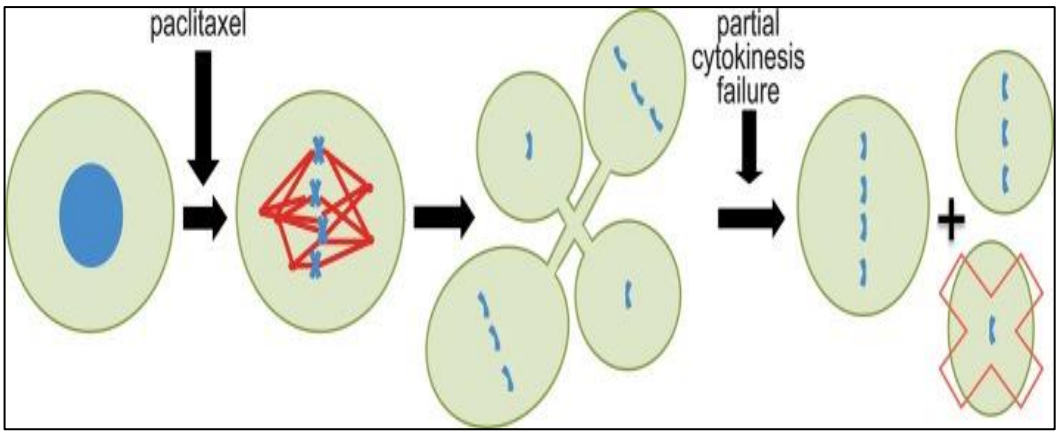
Reference: This table was prepared by Berfu Nur YİĞİT based on literature (Pryor et al., 2002, Mita et al., 2012, Stander et al., 2013, Musacchio and Salmon, 2007, Gaitanos et al., 2004)

2.4.1 Taxanes

Taxanes are class of diterpenes and originally extracted from *Taxus* (yews) plant. Paclitaxel, docetaxel are generally used against breast cancer as chemotherapy agent and cabazitaxel was developed for prostate cancer. These chemicals cause multipolar spindle formation in cell cycle and generally used in combination with alkylating agents for lung cancers (Cella et al., 2003).

Paclitaxel inhibits depolymerization of microtubules by binding to the β-tubulin. This unstable microtubule formation causes chromosome segregation into three, four or even five different directions (Figure2.6). Furthermore, this abnormal segregation causes partial cytokinesis failure and eventually activates caspase-dependent apoptosis by Bcl-2 proteins (Veldhoen et al., 2013). Although specific pathway is still unknown, one of the clinical studies suggests that, paclitaxel is only beneficial in breast cancer if tumor overexpress human epidermal growth factor receptor 2 (Bedard et al., 2010). One of the other taxane family drugs is new microtubule stabilizer cabazitaxel. This drug aims to target paclitaxel or docetaxel resistant cancer cells in prostate and breast cancers (Mita et al., 2012).

Figure2.6: Cell death mechanism activated by Paclitaxel agent



Reference : Weaver, B. A. 2014. How Taxol/paclitaxel kills cancer cells. Molecular biology of the cell, 25**,** 2677-2681.

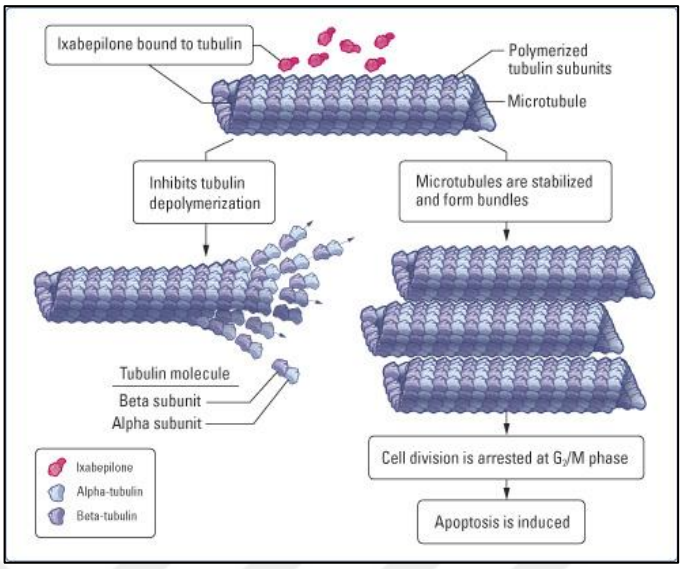
Taxane agents usually provide increased life-span for lung, ovarian and breast cancer patients. However, treatments can cause many side effects such as myelosuppression, aperiphal neuropathy, arthralgia and skin reactions (Cella et al., 2003). To increase quality of life in patients, adjunctive medications are required throughout the session of therapy.

2.4.2 Epothilone

The epothilones are other cancer drug class which is originally identified in mycobacterium *Sorangium cellulosum* as a metabolite. The epothilones works like taxane classes drugs on stabilizing microtubules. New studies suggest that these drugs are more efficient to treat cancer cells then taxanes, but it also cause higher cytotoxicity level (Risinger and Mooberry, 2012).

Ixabepilone is first candidate of this class drugs and approved in 2007 for use in clinical treatments for breast cancer especially resistant to taxanes and anthracycline (Cortazar et al., 2012). Ixabepilone and paclitaxel bind same site to β-tubulin heterodimer subunit (Figure2.7). It causes inhibition of spindle function which causes activation of apoptosis mechanism. Although exact mechanism is not known yet, one study showed that this drug is effective in over expressing class III β-tubulin whereas, class III β-tubulin over expression become taxane resistance cell population (Moudi et al., 2013). Treatment related side effects includes; myelosuppression, neuropathy, myalgia, fatigue and hypersensitive reactions. It provides alternative medicine options to patients who particularly showed resistant to taxane class agents (Cobham and Donovan, 2009).

Figure2.7: Cell death mechanism activated by Ixabepilone agent



Reference: Drug information [online] http://ixempra.com/ [11.12.2018]

2.4.3 Vinca Alkaloids

Vinca alkaloid anti-cancer drug family is originally coming from pink the periwinkle plant, *Vinca rosea.* They have been used to treat high blood pressure, diabetes and disinfections first, but 1960s they found this chemical can be used as anticancer activities (Liu et al., 2014). As an opposite of the taxane drugs, this family try to inhibit cell proliferation upon microtubule polymerization. The side of β-tubulin close to the guanosine triphosphate (GTP) binding site provides interactions in β-α-tubulin heterodimers (Liu et al., 2014). Binding occurs very rapidly and prevent straightening of curved tubulins, which in result effect assembly of microtubules and causes metaphase arrest (Toso et al., 1993).

There are various types of vinca alkaloids as anti-cancer agents such as vinblastine, vinorelbine, vincristine and vindesine. Although their structural positions are quite similar, their toxicological profiles occur at different levels. About these agents, one study suggest that vintafolide has mechanism action on folate (FR) receptor which is

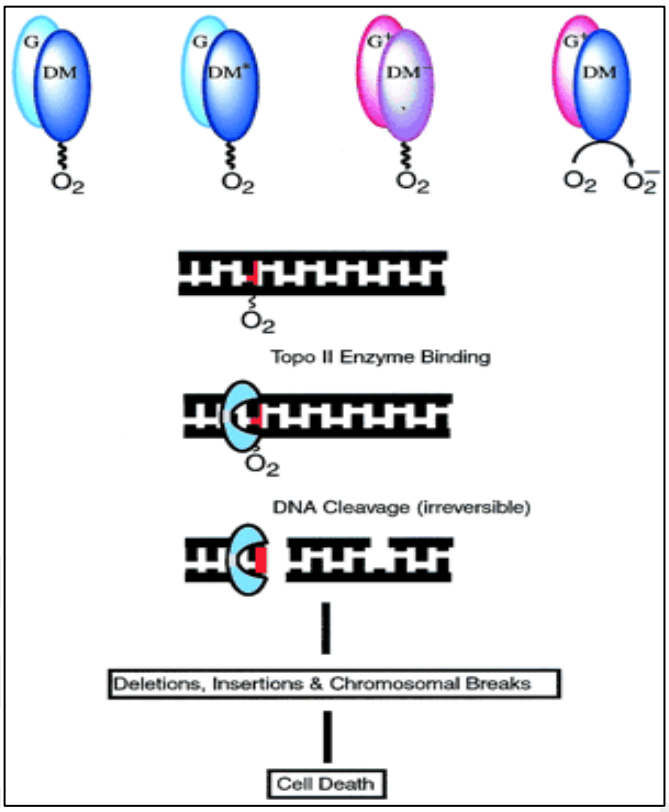
over expressed in various carcinoma and small amount in non-tumorigenic tissues (Kipp et al., 2018). This specific receptor selection of molecule provide ideal for tumor targeting strategies, but like the other chemotherapeutic drugs, this selective targeting cannot block major problems in the body's systems especially in neurotoxicity, peripheral sensory neuropathy, abdominal pain, leukopenia manners (Moudi et al., 2013).

2.5 CELL CYCLE INDIPENDENT DRUGS IN CHEMOTHERAPY

Anthracycline antibiotics are another agent class in chemotherapy which is not directly related with cell cycle downstream molecules. Although cytotoxicity of these drugs limits their use in therapy, this class provides the most effective treatment than other classes of chemotherapeutic agents (Minotti et al., 2004). These agents are extracted from *Streptomycin bacterium* and one of the most used drugs such as Doxorubicin, Epirubicin belongs this class to treat various cancers including lymphomas, breast, leukemia, stomach, ovarian, bladder and lung cancers.

In the action of mechanism, they can target many stages of cells which are correlated with DNA replication and eventually indirectly cause arresting of cells because of corruptions in DNA (Figure2.8) (Takimoto and Calvo, 2008). First of all agent can inhibit DNA or RNA synthesis by intercalating between the base pairs of strands. Secondly it can cause inhibition of topoisomerase II enzyme to block transcription and replication of DNA; they can also cause inhibition of helicase activity cause interference with DNA unwinding. Another agent induced activated mechanism is that generation of free oxygen radicals which damage cell membrane and DNA. Lastly, it can cause deregulation of histones (Pang et al., 2013). These all mechanisms contribute to cells to arrest in cell cycle. For example; Doxorubicin as a genotoxic agent, activate p55 in the basis of main role in apoptosis, which contributes to induction of WAF1/CIP1 p21 gene production to inhibit cyclin-dependent kinases involved in G1/S transitions (Bertheau et al., 2002).

Figure2.8. Cell death mechanism activated by anthracycline antibiotics



Reference: Qu, X., Wan, C., Becker, H., Zhong, D. & Zewail, A.H. 2001. The anticancer drug–DNA complex: femtosecond primary dynamics for anthracycline antibiotics function. Proceedings of the National Academy of Sciences

Although this class has strong impact to fight againist cancer cells, one of the main limitations of use is strong cardiotoxicity level including dilation of the sarcoplasmis reticulum, cytoplasmic vacuolization, loss of myofibrils and increased number of lysozymes (Zhang et al., 2012). To support patients in chemotherapy with anthracycline antibiotics, cardio protective drugs generally used to reduce the risk of cardiotoxicity.

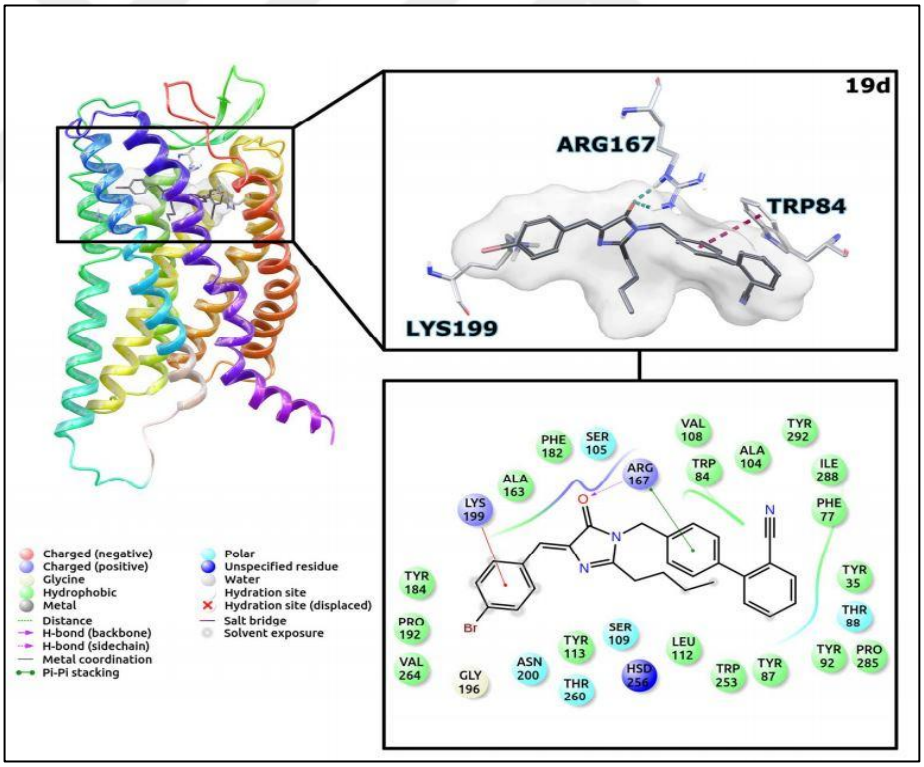
2.6 NOVEL CELL CYCLE INHIBITOR MOLECULE 19D

Angiotensin receptor antagonist is one of the molecule classes against hypertensions to treat cardiovascular diseases. Angiotensin receptor II (AT1R) blocker telmisartan drug

has been previously reported as G2/M arresting agent when it applied in high concentration. The same study is also showed that this drug causes double strand DNA breaks and activation of apoptosis mechanism thorough caspase 3/7 activation (Koyama et al., 2014).

19D molecule also is aimed to block angiotensin receptor II with *in silico* guided design (Figure2.9). 19D included designed AT1R blockers promising to hit receptor, were first investigated whether they have any effect on cell cycle or cell proliferation. In these tests, we found that imidazole based new molecule 19D has great potential as a G2/M cell cycle inhibitor agent over its angiotensin receptor blocker agent and it is effective molecule as much as currently used drugs in chemotherapy (Durdagi et al., 2018).

Figure2.9: Molecular structure of 19D



Reference: Durdagı, S., Aksoydan, B., Erol, I., Kantarcioglu, I., Acar, M., Avsar, T., Liapakis, G. & Karageorgos, V. 2018. Integration of multi-scale molecular modeling approaches with experiments for the in silico guided design and discovery of novel hERG-Neutral antihypertensive oxazalone and imidazolone derivatives and analysis of their potential restrictive effects on cell proliferation. European journal of medicinal chemistry, 145**,** 273-290.

3. MATERIAL AND METHODS

3.1 MATERIALS

3.1.1 Chemicals and Kits

All chemicals used in this research are listed in Table 3.1.

Table 3.1: List of chemicals

Chemical product	Trademark	Catalog No.
DMEM High Glucose	Biosera	LM-D1110/500
Heat-inactivated FBS	Gibco	10500-064
Penicilin-streptomycin	Multicell	450-201-EL
Dimethyl sulfoxide	Amresco	N182-6X10ML
Paclitaxel	Sigma	T7402-5mg
Staurosporine	Abcam	Ab120056
10x PBS	Biosera	XC-S2066/500
10x HBSS	Biowest	X0510-500
EDTA	Sigma-Aldrich	EDS-500G
RNAseA	Macherey-Nagel	74-505.50
Propidium Iodide	Biochemica	A2261.0025
Ethanol	Merck-EMPROVE	1.00971.2500-2.5L
Paraformaldehyde	Sigma Aldrich	P6148-1KG
DAPI	Biochemica	A4099.0005
Triton X-100	Sigma Aldrich	SIALX100-500ml
Mounting Media	Biomount	17894
Methanol	VWR	20847.320-2.5L
Hematoxylin	Biognost	DIAH09
Eosin	Ddk Italia	GTC101310
Acridine Orange	ImminoChemistry Technologies	6130
Tween-20	VWR	0777-1L
ANTI-LAMIN B1 EA	Thermo Fisher	PA519468
BETA-TUBULIN ANTIBODY (BT7R)	Thermo Fisher	MA516308
Goat anti-Rabbit IGG cross-absorbed secondary Alexa Flour 488	Thermo Fisher	A11017
F(ab') Goat anti-Mouse IGG cross absorbed secondary Alexa Flour 555	Thermo Fisher	A21425

Chemical product	Trademark	Catalog No.
Caspase3/7	Thermo Fisher	C10423
Total Protein Extraction Kit	G Biosciences	786-225
Protease Inhibitor Coctail	Cell Signaling Technology	5871S
Caspase 3 Antibody	Cell Signaling Technology	9662S
Precision Plus Protein Dual Color Standards	Biorad	1610374
Beta-Actin	Cell Signaling Technology	3700
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology	7074S
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary	Cell Signaling Technology	A11008
SuperSignal West Femto Maximum Sensitivity Substrate	Thermo Fisher	34095
Agarose	Biomax	HS-800
Generuler 1kb DNA ladder	Thermo Fisher	SM0311
Proteinase K	Sigma-Aldrich	P2308-25MG
Hydrogen Peroxide	SIGMA	18304-1L
MTT	Biomatik	A33381-1G

Reference: This table was prepared by Berfu Nur YİĞİT.

3.1.2 Equipments

All equipments used in this research are listed in Table 3.2.

Table 3.2: List of equipments

Equipment	Trademark
Panasonic cell culture incubator	Panasonic, Japan
SL 40-FR centrifuge	Thermo Fisher, USA
Microfuge 16 centrifuge	Beckman Coulter, USA
Novocyte Flow Cytometry	ACEA Biosciences, Chine
Attune NxT Acoustic focusing cytometry	Life Technologies, USA
Leica DMIL Phase Contrast Inverted Microscope	Leica, Germany
Leica DM500 Microscope	Leica, Germany
Leica Flourescence Microscope	Leica, Germany
T80+ uv/vis spectrophotometer	PG Instruments, UK
ChemiDoc MP Imaging System	Biorad, USA
Mini-PROTEAN Tetra Cell System	Biorad, USA
PowerPac Basic Power Supply	Biorad, USA
Mini Trans-Blot Cell	Biorad, USA
Mini-Sub Cell GT Cell	Biorad, USA

Equipment	Trademark
Digital heat block	Benchmark, USA
-80°C freezer	Daihan Scientific, South Korea
-20° C freezer	Vestel, Turkey
+4 °C storage	Arcelik, Turkey
MX-S vortex	Scilogex, UK
Waterbath	Daihan Scientific, South Korea
Purelab Option-Q	Elga, UK
HI-2211 ph/ORP meter	HANNA instruments, USA
Digital scale	ISOLAB, Germany

Reference: This table was prepared by Berfu Nur YİĞİT

3.2 METHODS

3.2.1 Cell Lines, Culture Conditions and Reagents

HUVEC, HCT, U-87 MG, Hek, MCF-7 and RFL6 cell lines were used in this study to evaluate our drugs' effects. Cell lines were grown in DMEM High Glucose supplemented with 10 percent heat-inactivated FBS and 1 percent penicillin streptomycin. The growth conditions were fixed in cell culture incubator 37 °C humidified environment with 5 percent CO_2.

All experiments were performed with healthy cell lines which passed two passages after frozen conditions. All cell lines were allowed to grow eighty percent confluency before drugs treatments carried out except cell viability assay.

For drug stock solution preparation; all reagents were solubilized in dimethyl sulfoxide (DMSO); 50mM 19D stock, 10mM Paclitaxel stock and 1mM Staurosporine stock. To analyze agents' specific results, 0.2 percent DMSO was introduced as reagent vehicle control into cells which has no side effect on cellular proliferation and mechanisms.

3.2.2 Flow Cytometric Cell Cycle DNA Analysis

Flow cytometry is important tool to investigate distribution of cell cycle phases based on cells' DNA content. Intercalating DNA agents allows to reveal cellular contents histograms in three major phases of cell cycle; G1, S and G2/M.

HCT, HUVEC and U-87 MG cell lines were used to analyze their DNA content changes when exposed to the 19D. To achieve exponential growths of each cell lines; $17x10^3$ HUVEC cells/well, $20x10^3$ HCT cells/well, and $28x10^3$ U-87 MG cells/well were seeded into 24-well plate. After 24 hours incubations without agents, cells were treated with 25, 50, 100µM 19D, 100nM Paclitaxel and 0.2 percent DMSO. To understand agent induced differentiation, cells were collected after 24 hours and additionally 48 hours reagent treatments.

After incubation, cells were washed with PBS and harvested with HBSS 5mM EDTA then transferred into HBSS 2mM EDTA 2 percent FBS media-like solution. Harvested cells were centrifuged at 250 g for 5 minutes. Pellets were dissolved in 70 percent ethanol and incubated in ice box for 30 minutes, which provide fixation of cells in current stage and provide analysis without further degradation of cells and DNAs. After fixation, cells were washed twice with PBS supplemented with 2 percent FBS to clarify samples from ethanol residues. As an intercataliting agent, Propidium iodide (PI) was used. Since propidium iodide (PI) bound DNA as well as RNA, samples were incubated with 200µg/ml RNAse A for 30 minutes at room temperature to degrade RNAs before staining. At the final step, DNAs were stained with PI at 25µg/ml concentration for 15 minutes in dark room. Stained cells were analyzed in Novocyte Flow Cytometry ACEA Biosiences and Attune NxT Accoustistic focusing cytometry instruments.

3.2.3 Cellular and Nuclear Morphologies Determination Analysis

3.2.3.1 Phase-Contrast Imaging

Morphological changes provide pronounced evaluation way to understand reagent causing differentiation on cells, we observed each cells before and after reagent additions via phase contrast microscope imaging. All cell lines in each experiment have captured images via Leica DMIL phase contrast inverted microscope.

3.2.3.2 Nuclear DNA Labeling

After 19D specific cellular aberration and membrane bleebing were noticed, nucleus of agent induced cells were stained with another intercalating stain**;** 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). For this purpose; 120k HUVEC cells were seeded onto glass coverslips in 6-well plate. Cells were allowed to grow for 24 hours without any reagents to obtain adherent morphologies. Then, 100µM 19D, 100nM Paclitaxel and 0.2 percent DMSO were added into wells. Furthermore, reagents were incubated for 24 hours until image collection. After incubation, cells were washed three times with PBS to aspirate cell growth medium. Live cells were fixed with 4 percent formaldehyde in PBS

for 10 minutes at room temperature. At the next step, wells were washed three times with PBS (5 minutes each) to remove fixation solution. 0.2 percent Triton X-100 was used to obtain cell membrane permeabilization, 5 minutes at room temperature. After that, cells were washed three times again (5 minutes each). At last, cells were incubated in 1:7000 diluted DAPI (10mg/mL in dH_2O) for 5 minutes at dark room. Labelling solution was washed out with three PBS washes. Then, coverslips were mounted and cells' nucleuses were visualized under Leica fluorescent microscope at violet channel.

3.2.3.3 Hematoxylin and Eosin Staining

Hematoxylin and eosin immunocytochemistry staining was another technique used in this project to label cytoplasm and nucleus of cells according to their different pH values. In this experiment, seeding of cells were repeated as in DAPI staining; $120x10^3$ HUVEC cells were grown in coverslips for 24 hours untreated and then 24 hours reagents treatment. Reagents were used in same concentration; 100µM 19D, 100nM Paclitaxel and 0.2 percent DMSO. As a fixation step in this staining, methanol and 4 percent formaldehyde hybrid method were used. Cells were firstly fixed in 4 percent formaldehyde in PBS for 5 minutes at room temperature and followed by ice cold methanol fixation for 5 minutes at -20 °C. Then, cells were washed three times with PBS to aspirate fixative solution. For staining step, cells were incubated in hematoxylin for 45 seconds and eosin for 10 seconds. Staining solution also removed from coverslips with several PBS washes. Finally, coverslips were mounted and cells' cellular and nuclear morphologies were visualized under Leica DM500 light microscope.

3.2.3.4 Acridine Orange/propidium Iodide Staining

In this staining, cell membrane integrity was tested on live cells which exposed to the 100µM 19D. For this aim, $120x10^3$ HUVEC cells were grown on coverslips in 6-well plate for 24 hour as untreated to obtain adherent morphology. Then, one well was treated with 100µM 19D and other one was treated with 0.2 percent DMSO for 24

hours. After that, live cell containing coverslips was incubated with 1 µg/mL propidium iodide and 1 µg/mL acridine orange mix for 15 minutes at cell culture incubator.

Finally, coverslips were washed with PBS supplemented with 5 percent FBS and visualized with Leica fluorescent microscope at blue channel.

3.2.3.5 Multicolor Immunofluorescent Staining

Although we tried multiple staining protocols for understanding drug interaction at cellular level, more specific markers in the nuclear membrane and cytoplasm were necessary. For serve this purpose, we performed multicolor immunofluorescent staining with nuclear membrane specific protein lamin and cytoplasmic boundary markers beta-tubulin.

In this procedure, again $120x10^3$ HUVEC cells seeded into 6-well plate over the glass coverslips and 24 hour was waited for cellular attachments. Following 24 hours cells were incubated with 100µM 19D, 100nM Paclitaxel and 0.2 percent DMSO. Following the incubation, mediums were removed with three PBS washing. In fixation step, hybrid formadehyde – methanol method was chosen to obtain more preservative morphological structures; 4 percent formaldehyde incubation at room temperature and 5 minutes methanol incubation at -20°C. Fixative solution was washed away with three PBS washes (5 minutes each). Then, cells were permeabilized with 0.2 percent Triton in PBS for 5 minutes at room temperature. After permeabilization, cells were blocked with 3 percent bovine serum albumin (BSA) for 30 minutes at room temperature. Since our primary antibodies were from different hosts, we performed simultaneous incubation with 1:500 Lamin and 1:100 Tubulin in 0.1 percent BSA and incubated for overnight at 4°C. After overnight incubation, primary antibody solutions were decant and cells were washed three times with PBST (10 minutes each wash). At secondary antibodies, cells were incubated with 1:500 Goat anti-Rabbit IGG cross-absorbed secondary Alexa Flour 488 and 1:1000 F(ab') Goat anti-Mouse IGG cross absorbed secondary Alexa Flour 555 in 0.1 percent BSA for 1 hour at dark room. Secondary antibody solutions were washed off by three times with PBST washes (10 minutes each wash). The nucleuses of cells were counterstained with 1:7000 DAPI (10mg/mL) for 5 minutes at dark room and finally before visualization slides were washed three times PBS. Mounted coverslips were visualized under Leica Fluorescent microscope at violet, blue and green channels.

3.2.4 Cell Viability Assay

To evaluate drug specific cell viability changes, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed. In this colorimetric assay, MTT reduced under the viable cells' oxireductase enzymes and results formazan crystal to reflect number of live cells in wells.

HUVEC, HCT, U-87 MG, Hek, MCF-7 and RFL6 cell lines were analyzed based on their response to 25, 50, 100μM 19D molecule and 0.2 percent DMSO reagents. At first, all cell lines were seeded at $20x10^3$cells/well, but due to low level of cell number for Hek and MCF-7 cells, we used $40x10^3$cells/well. Then, cells were incubated 24 hour without any reagent addition. After initial incubation period, 25, 50, 100μM 19D and 0.2 percent DMSO reagents were introduced into wells, each experiment groups were worked in three replicates. Three days following the reagent addition, cells were analyzed with 24 hour intervals.

For analysis of cell viability, 50μl MTT (5mg/ml in PBS) was added to each well, incubated for 4h at 37 C incubator. Formazan crystals formed in 4 hours incubation, and then solubilized with 500μl SDS solubilization buffer (0.1 M HCl 10 percent SDS) and incubated for additional 15 minutes at 37°C. Absorbences were measured at 570nm by uv/vis spectrophotometer.

3.2.5 Caspase 3/7 Analysis

Caspase 3/7 is important marker in apoptosis. To analyze presence of this protease enzyme, immunoflourecence staning was performed in live cells withouth fixation. $100x10^3$ cells/well HUVEC were seeded onto coverslips in 6-well plate. After 24 hour agent without incubation, 100 μM 19D and 0.2 percent DMSO added and cells

were further incubated for 48 hours in cell culture incubater. At the same time, in visualization day of experiment 1μM Staurosporine was added one of the wells and incubated for 4 hours. After all these reagents' incubation time were completed, 1:125 Caspase 3/7 antibodysolution in PBS supplemented with 2 percent FBS was added on the coverslips. Staining were performed for 3 hours in incubator and then cells were

pictured with Leica Leica Flourescent microscope in blue channel.

3.2.6 Protein Extraction

Protein confirmation through immunofluorescence staining in a cell is a way of proof target proteins presence, but western blot results are more appreciated. Thus, $400x10^3$ cells HUVEC were seeded in cell culture dishes and incubated for 24 hours without agent treatment. Then 100µM 19D, 100nM Paclitaxel, 0.2 percent DMSO was added to the cultures and cells incubated further 24 hours. Additionally, 1µM Staurosporine added to one of the cultured cells and incubated for 2.5 hours until extraction.

In the protein extraction method, we combined commercial total protein extraction kit's (which kit?) procedure and our methods. First of all, cells in dishes washed at least three times with PBS to wash off FBS residues. Then, cells in PBS were scraped out of dish and collected in 15mL falcon tubes. After cells were pelleted at 250g for 5 minutes, pellet was suspended in kits' 400µL TBE buffer I supplemented with protease inhibitor cocktail and waited in ice box for 2 minutes. Afterward, 240µL TBE buffer II was added and cells were heated in boiling water for 30 seconds. Next, sample was vortexed for 30 seconds and placed back into boiling water for 10 minutes. Finally, extracts were pelleted 1300xg for 5 minutes to get rid of non-protein artifacts. Supernatants were transferred to new eppendorf tubes and preserved at -80°C until experiments.

3.2.7 Western Blot

30 µg of untreated, 0.2 percent DMSO, 100 µM 19D, 100nM Paclitaxel and 1µM Staurosporine treated cell lysates were loaded into 8-15 percent SDS polyacrylamide gel and electrophoresed until bromophenol blue dye run out from gel. Proteins were

transferred to 0.45µm pore sized nitrocellulose membrane with wet transfer conditions; 90 volt 100 minutes. After transfer, blots were blocked with 5 percent non fat powdered milk in TBST at room temperature for an hour. Then, 4ml of 1:1000 diluted Caspase-3 and Actin antibodies in 5 percent non fat powdered milk were introduced into membranes for overnight incubation at 4°C. For following step; membranes were

washed four times with TBST (each 15 minutes). Secondary antibodies were diluted in 1:10000 ratio and were introduced into membranes for 1 hour at room temperature. After four TBST washes again, target of proteins' reactivity was developed by hydrogen-peroxide chemiluminescence according to the manufecturer's instructions.

3.2.8 DNA Fragmentation Assay

DNA fragmentations during apoptosis or necrosis can be visualized with agarose gel electrophoresis which represents 180-200bp fragments. To visualize this agent causing fragmentations, $400x10^3$ HUVEC cells were seeded into cell culture dishes. For negative control; we used 0.2 percent DMSO treatment for 48 hours. Our agents 19D was used at 100 μM and 50 μM concentrations for 48 hour treatment. Finally, for positive control HUVEC cells were incubated in 200μM formaldehyde for 24 hours and additionally 2mM hydrogen peroxide in other dish for 12 hours. After agents incubations were finished, cells were harvested with help of scraper and lysed in 40 μL TES (20mM EDTA, 100mM Tris, ph 8.0 and 0.8 percent SDS buffer). 5 μL RNase A was added and tubes were incubated at 37°C for 30 minutes. Then, 5 μL proteinase K was added and incubated overnight at 50°C. Samples were loaded into 1 percent agarose gel and runned at low voltage. Finally, gel was visualized under Biorad ChemiDoc MP Sytem.

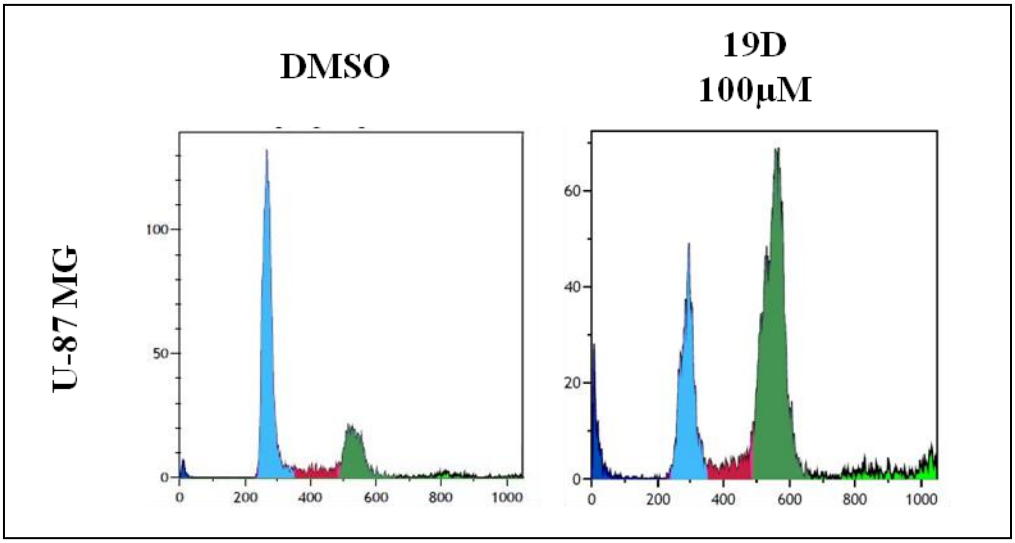
4. RESULTS AND DISCUSSION

4.1 EVALUATION OF 19D CAUSED G2/M ARRESTED POPULATION

Flow cytometric cell cycle population analysis relies on quantity of DNA molecule which is also called as DNA histograms. Cells in G1 phase contains 2n DNA, in S phase cells start to replicate their genomic content and G2/M phase these cells have 4n DNA and get bigger in size before dividing into two cells. As a result of this cellular volume and DNA content differentiation through cell cycle phase transitions provide two distinct peaks in histograms when cells DNA's stained with intercalating agents such as propidium iodide. All in all the first peak in histograms represents G1 phase, second peak showed as doubled DNA content represent G2/M phase and the area between these two peaks shows S phase.

We performed flow cytometric analysis to HUVEC, HCT and U-87 MG cell lines which requires different time to consume in cell division. At first, U-87 MG 19D caused G2/M arrest can be seen in Figure4.1 where blue area shows G1 phase cells, red area shows S phase and green phase shows G2/M populations of cells.

Figure 4.1: U-87 MG cell histogram after 19D and DMSO treatments



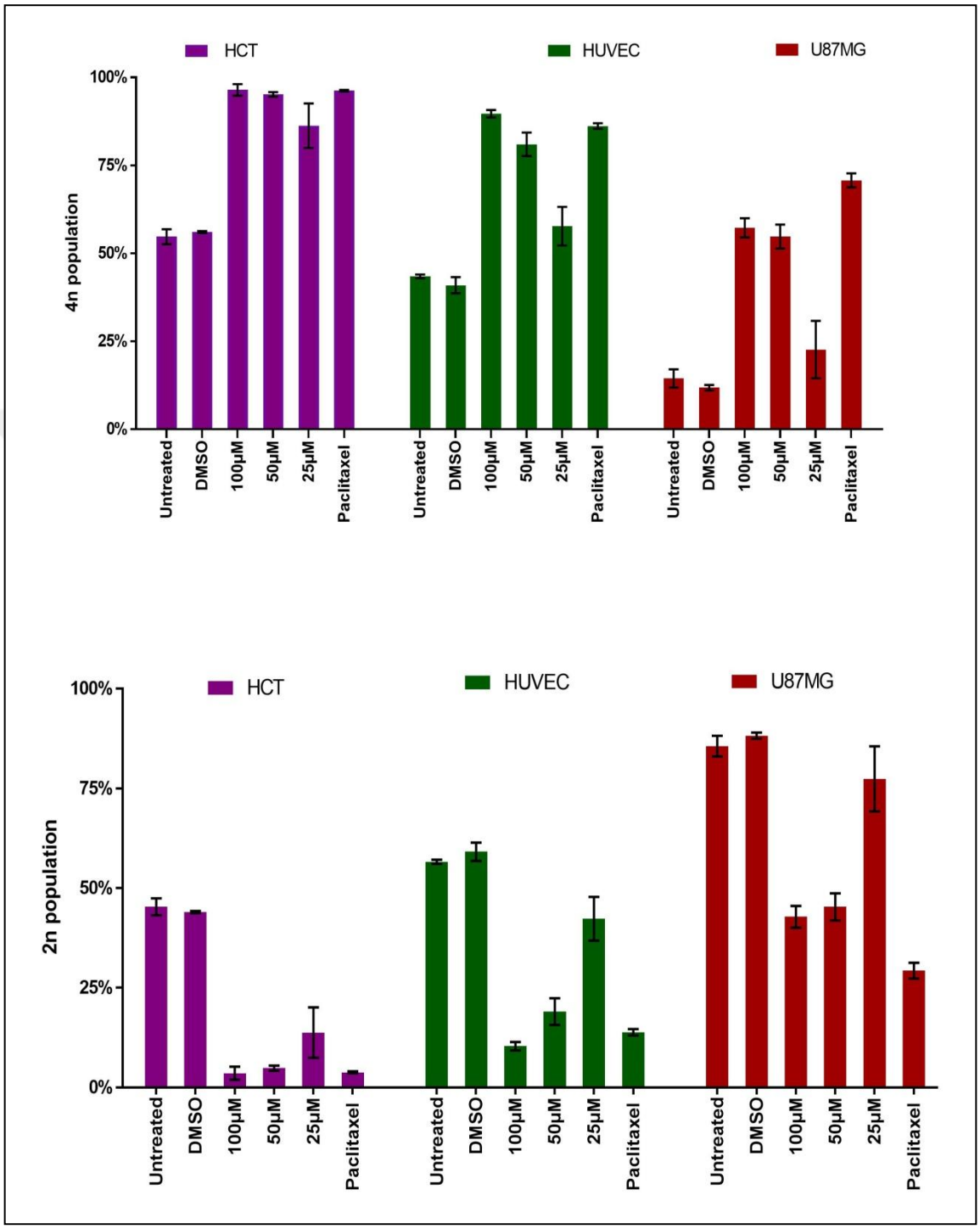
Reference: This figure was prepared by Berfu Nur YİĞİT

Then to evaluate this significant change to other cell lines 19D, Paclitaxel as a positive cell cycle inhibitor control and 0.2 percent DMSO as a negative drug vehicle control were added to other cells for 24 and 48 hour. First of our results clarified that 0.2 percent DMSO which is used as drug vehicle control, has no manipulative effect on cell cycle and act as untreated cells. Other than that our collection of all data suggests that 19D cause G2/M arrested cell population in a dose-dependent manner compared with DMSO controls and this arresting potential is strong as much as Paclitaxel commercial chemotherapeutic drug. 24 hours of 19D treatment causes significant increase in the percentage of G2/M phase in HUVEC, HCT and U-87 MG respectively 73, 90 and 59 percents compared with 0.2 percent DMSO control respectively 19, 36 and 23 percents (Figure 4.2). Additionally, Paclitaxel, the positive of arresting agent causes same level accumulation in HUVEC, HCT and U-87 MG as 60, 87 and 70 percent respectively.

Furthermore we analyzed all cell lines total distribution across cell cycle (Figure4.3.). When the apoptosis mechanism is activated, it can be also defined in flow cytometric cell cycle analysis as 180-200 bp DNA fragmentations. These fragmented DNAs is also named as subG1 population, which is shown in 19D and Paclitaxel induced cells at same level. Another important finding of these experiments is that cell cycle inhibition of each cell is related with their population doubling time requirements. When we compare fast dividing cells like HCT and HUVEC with relatively slower cells like U-87 MG our drug cause different number of cell population arrest. The 90 percent and 73 percent arrested cells in HCT and HUVEC drops to 61 percent in U-87 MG when 19D is induced. This is another evidence of that our drug causing cell cycle related distributions on cells.

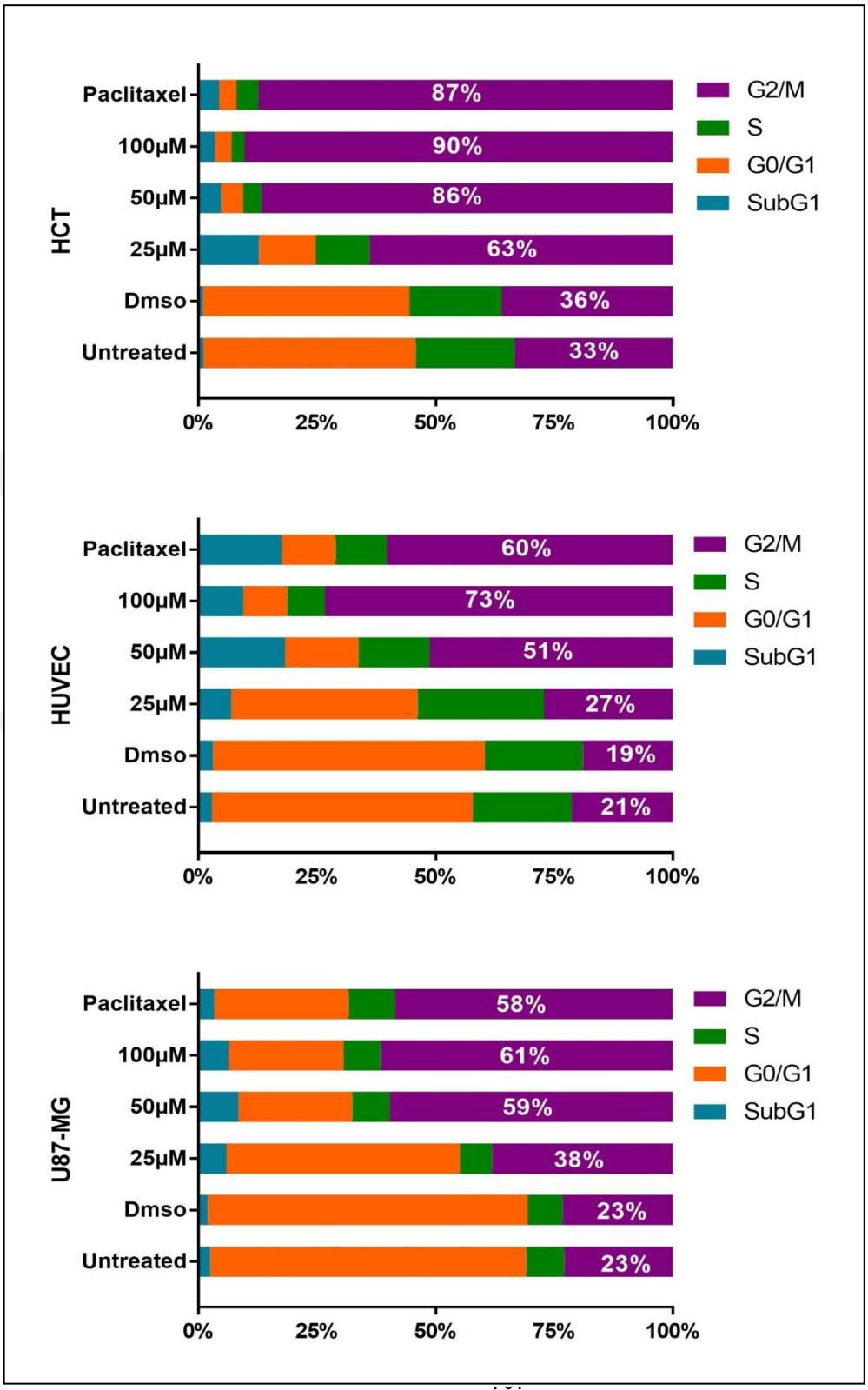
As a result of these experiments, our results showed that treatment with 19D cause G2/M arrest and further DNA fragmentation (subG1 population) in various cell lines at same level with highly used anticancer drug Paclitaxel.

Figure 4.2: DNA content 4n and 2n analysis of HCT, HUVEC and U-87 MG cell lines after 19D, DMSO, Paclitaxel treatments



Reference: This figure was prepared by Berfu Nur YİĞİT

Figure 4.3: Cell cycle phase distributions after 19D, Paclitaxel and DMSO treatments in HCT, HUVEC and U-87 MG cell lines



Reference: This figure was prepared by Berfu Nur YİĞİT

4.2 CELLULAR AND NUCLEAR MORPHOLOGICAL CHANGES IN 19D TREATED CELLS

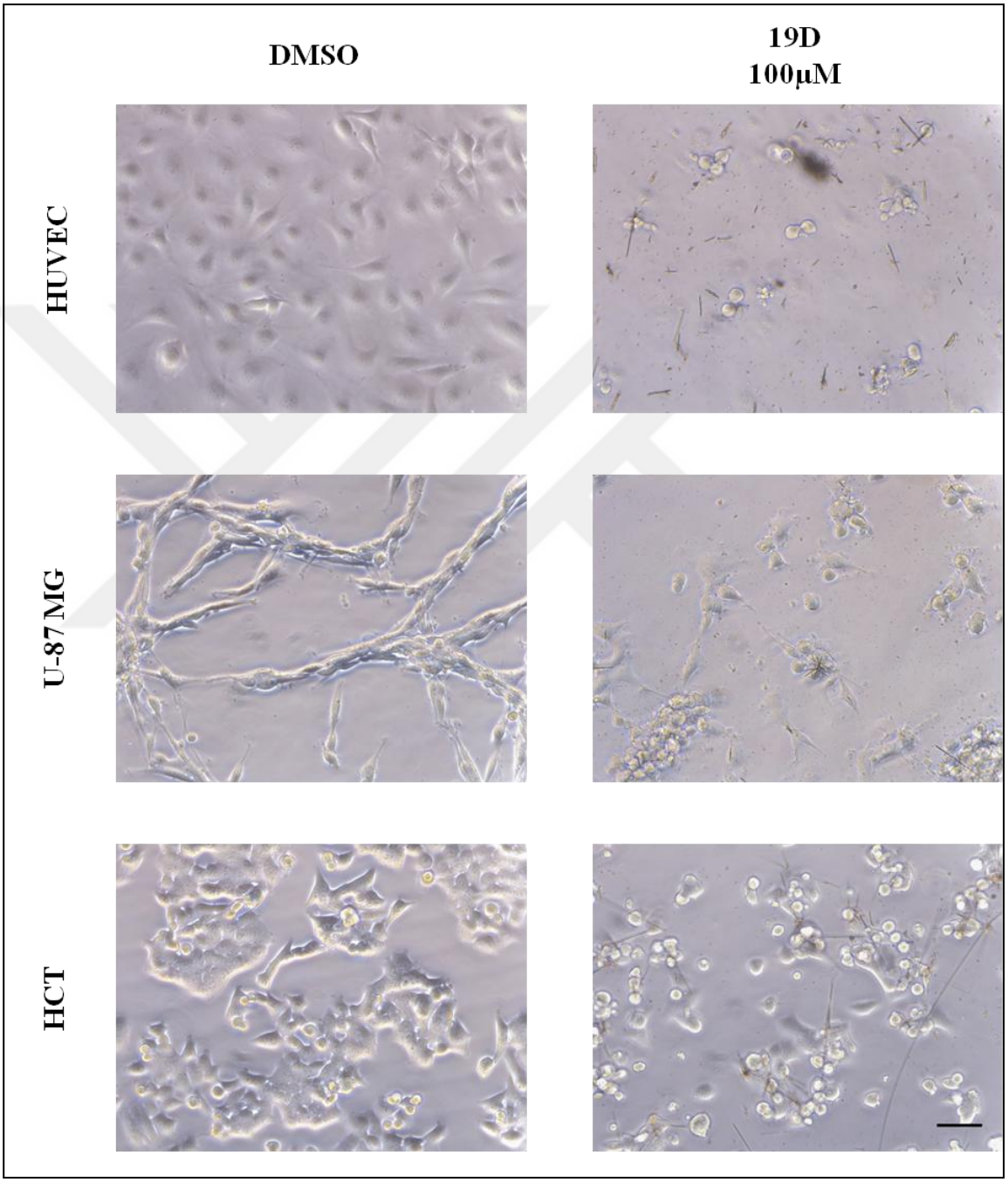
4.2.1 The First Apoptosis Evidence

Apoptosis is regulated cell death mechanism which activates many intracellular molecules and pathways to eliminate cells. But before all other evidences for apoptosis, it initially occurs on morphological level of cells. In that perspective, HUVEC, U-87 MG and HCT was visualized after 24 hour treatment with 19D and DMSO as a control (Figure4.4).

First changes in drug induced cells are that, they became smaller, lost their morphological attachments and became ball shaped. Other observation is that cell membrane get blebbing morphology which is the main evidence of apoptosis mechanism. Additionally, the number in 0.2 percent DMSO and 19D cells is quite different; 19D causes destruction of nearly all cells. After 24 hour drug incubation, it is clearly seen that, 19D can crystallize in cell culture medium which can also support cell destruction as mechanical force.

Conclusively, only phase contrast imaging can give many information about drug mechanism. With this imaging, we conclude that 19D cause possible apoptosis mechanisms which results slower number of cells compared with the control group. Additionally, these finding shape our following experiment to in-depth studies in cellular morphologies.

Figure 4.4: HUVEC, U-87 MG and HCT phase contrast images after 19D and DMSO treatment



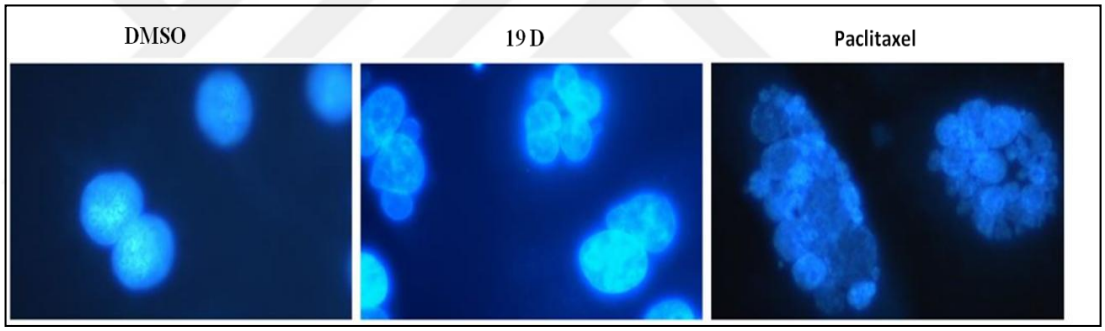
Scale bar: 200 µm

Reference: This figure was prepared by Berfu Nur Yiğit.

4.2.2 Evaluation of Cellular and Nuclear Disruptions

To get better understanding in 19D caused morphological changes, we first stained HUVEC cells' nucleuses with intercalating agent DAPI and visualized cells under fluorescent microscope. These cells are incubated with 19D, Paclitaxel and DMSO for 24 hours (Figure4.5). As a result of this experiment, we clearly saw abnormal nuclear morphologies in Paclitaxel and 19D treated cells. In normal dividing cells' nucleus duplicate to two in equal size like shown result in DMSO, but our drug and Paclitaxel showed resulting small and multiple nucleuses in a single cell because of arrested position in cell cycle.

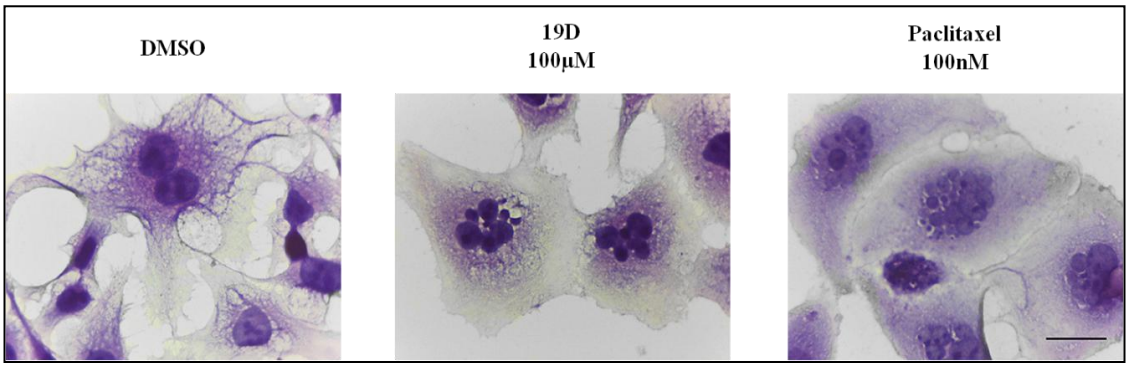
Figure 4.5: Nucleus of HUVEC cells after 24 hour 19D and Paclitaxel treatments



Reference: This figure was prepared by Berfu Nur Yiğit.

Since DAPI only stain nucleus of cells, we performed hematoxylin and eosin staining to define cellular boundaries. Hematoxylin and eosin staining perform staining of both cytoplasm and nucleus and it define them as a different colored shade in purple with the help of the pH differences across these cellular parts (Figure4.6). In these visualization, we again got normal dividing cell in DMSO control in one cell, on the other hand paclitaxel and 19D results in DAPI also conformed again and state that in agent induction one cells' nucleus clearly divided into many small nucleus fragments. As a conclusion of these two staining results, 19D treatment causes multi small nucleus formation which can be related with mitotic spindle deformation in anaphase stage like Paclitaxel action of mechanism.

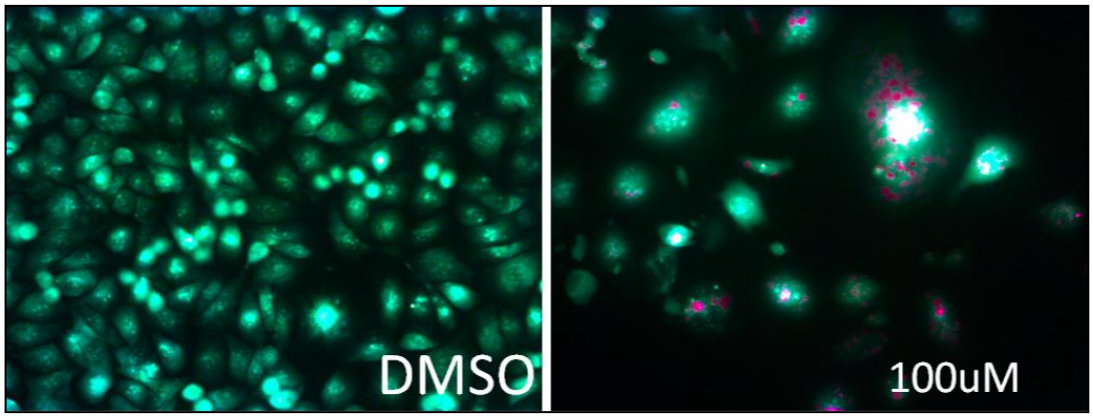
Figure 4.6: Cytoplasm and nucleus staining of HUVEC cells after 24 hour 19D and Paclitaxel treatments via hematoxylin eosin staining



Scale bar: 30 µm
Reference: This figure was prepared by Berfu Nur Yiğit.

Another experiment to understand drug mechanism was acridine orange/propidium iodide staining. Acridine orange is cell membrane permeable stain which diffuses into cytoplasm and colored cells in green. On the other hand, in normal condition propidium iodide cannot across the cell membrane without permeabilization, but when apoptosis occurs, cell membrane start to have pores. In other word, propidium iodide can across membrane only if apoptosis is initiated. To test our drug based on cell membrane changes; HUVEC cells were treated with DMSO and 19D for 24 hour and according to imaging results; 19D causes cell membrane disruptions (Figure4.7).

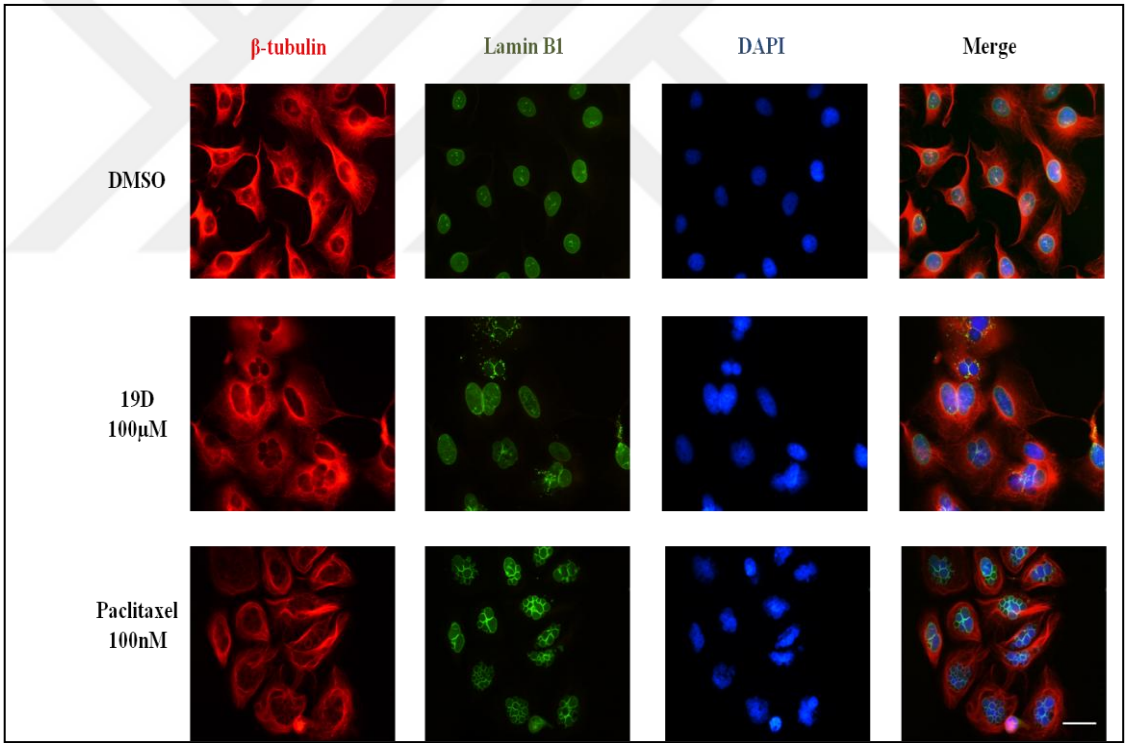
Figure 4.7: Degradation in HUVEC cell membrane after 24 hour 19D treatment



Reference: This figure was prepared by Berfu Nur YİĞİT.

In addition to these various morphology stainings, we finally performed multicolored immunofluorescence staining on HUVEC cells with nuclear envelope specific lamin antibody, beta-tubulin antibody and DAPI. One of the most interesting results is treatment with 19D caused nuclear envelope degradations in just 24h incubation in higher level than Paclitaxel treatment (Figure4.8). Paclitaxel is well known agent which act on beta-tubulin to inhibit cell proliferation. Although at first look, tubulins seem not very different compared with control group, when the distribution of tubulins in cytoplasm is checked they all have very different pattern. Firstly, DMSO group which represent negative control group, have brighter and same dispersion in cytoplasm, but Paclitaxel and 19D causes gathering of tubulin in specific location around the nucleuses.

Figure 4.8: Multicolor immunofluorescence staining on HUVEC cells after 19D and Paclitaxel treatment



Scale bar: 50 µm

Reference: This figure was prepared by Berfu Nur Yiğit.

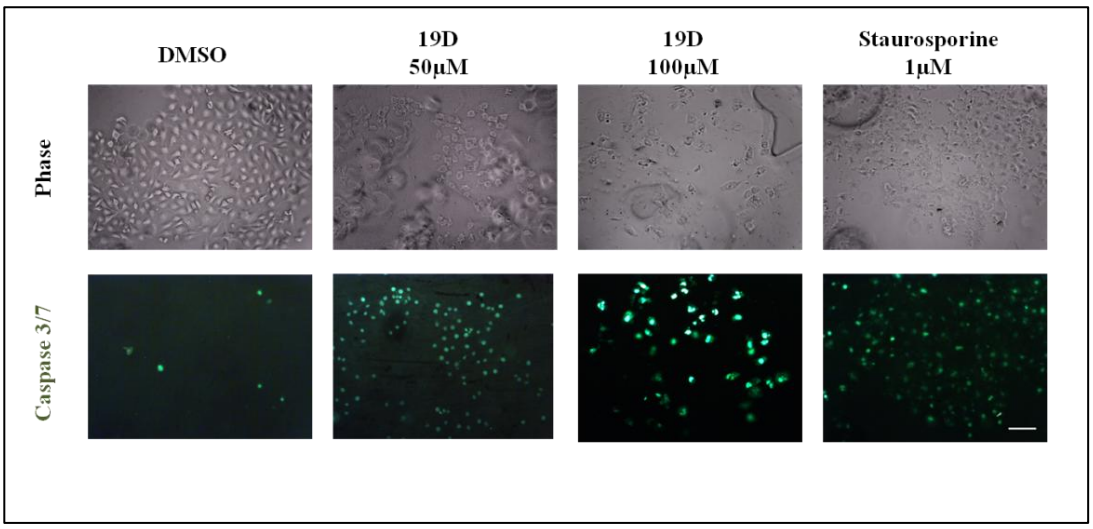
G2/M arresting agents generally act on microtubule deformations to cause mitotic spindly instabilities, 19D causing membrane blebbing, multi small nucleus formation, nuclear envelope degradation and possible tubulin instability in cells supports this agent to be used in anti-cancer treatments.

4.3 APOPTOSIS THROUGH CASPASE3/7 ACTIVATION

Caspase 3 activation is essential during apoptosis mechanism. Normally caspase 3 conserved in cells as an inactive form of procaspase state, when apoptosis signals trigger cells to the execution, this procaspase-3 is cleaved to two smaller subunits. This cleavage activates other caspase family proteins including; caspase 6 and caspase 7. There are many kits and dyes for detection of caspase 3 activity in cell population for assessing apoptotic activity. To evaluate our mechanism action based on caspase 3 activation, we performed live cell staining with of a four-amino acid peptide (DEVD) which are hydrolyzed by caspase 3 and 7 cleavage. For his experiment, HUVEC cells were treated with DMSO (negative drug vehicle control), Staurosporine (positive caspase3 activation control) and our agent 19D in two concentration. For positive control Staurosporine was applied to cells for 4 hours. Additionally, 19D incubated for 48 hours.

According to the results, 19D cause activation of caspase 3/7 apoptosis mechanism in a concentration dependent manner which is the significant result to illuminate G2/M further mechanism. and support nuclear envelope degradations.

Figure 4.9: Caspase 3/7 activation in HUVEC cells after 48 hour 19D treatment



Scale bar: 150 µm
Reference: This figure was prepared by Berfu Nur YİĞİT.

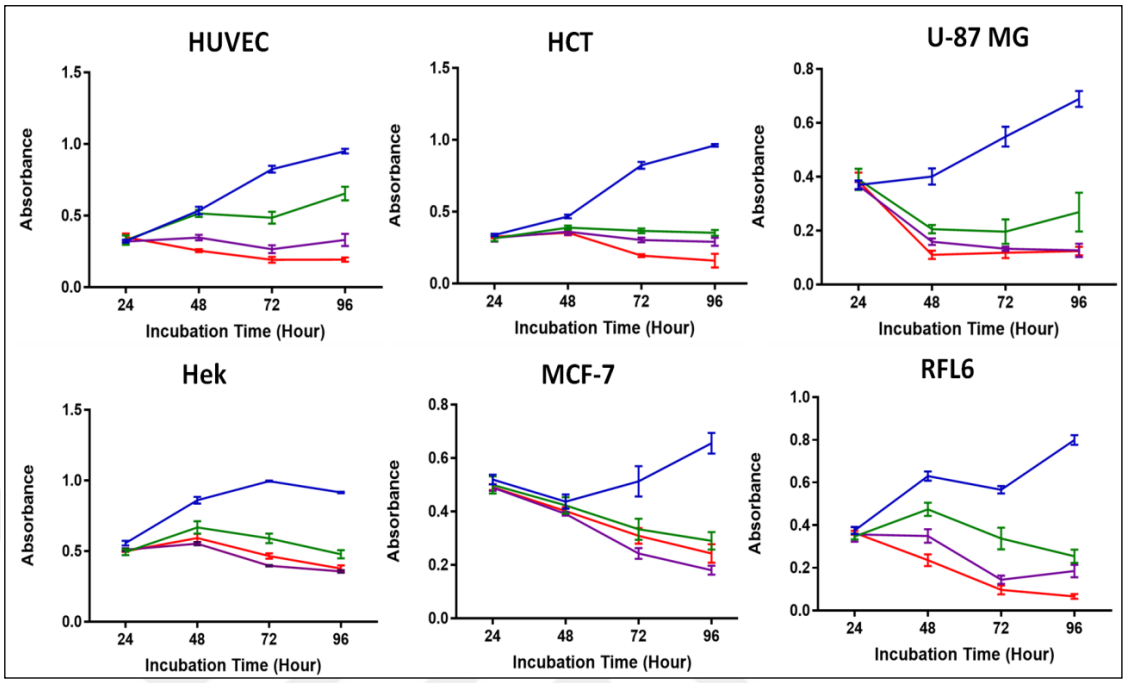
4.4 INHIBITION OF CELL PROLIFERATION BY 19D

In the 19D caused cell viability and metabolic changes analysis, we performed MTT colorimetric assay. This procedure relies on detection of NAD(P)H-dependent oxidoreductase enzymes which is the one of the markers of metabolic activity as well as cell viability. According to this assay, fast dividing cells exhibit high rates of MTT reduction by oxidoreductase enzyme. On the other hand, if there is a problem about cell viability on the culture, level of MTT tetrazolium salts become decreased. Based on this approach, we analyzed HUVEC, HCT, U-87 MG, Hek, MCF-7 and RFL6 cell lines with different concentrations of 19D (25, 50 and 100 μM) for three days.

With the help of this colorimetric assay, we evaluate that, 100 μM of 19D provide most suitable approach to reduction of cells. Especially 25 μM seems more effective in HCT, Hek, MCF-7 and RFL6, it become more tolerable for HUVEC and U-87MG where at final point of graphs cell population started to increase. Our drugs IC50 value was evaluated as 23 μM, because of this feature, cell lines can react as differently because of their characterization in cell membranes and transposition of agents. In all time points in experiment during 3 days drug treatment, there were healthy looking cells unaffected from 100 μM 19D. These cells can be called as resistance to our drug. Another theory is that, since our drugs become crystallized after 24 hour incubation effectiveness of agent may be decreasing in later times.

Because of technical issues about drug re-synthesis processes and there was no left 19D, experiments about these new theories could not be performed. In MTT assay, 100 μM 19D provide great level reduction of cell population but further results about cell resistance and crystal formation is needed for final conclusion.

Figure 4.10: Cell viability graphs of cell lines

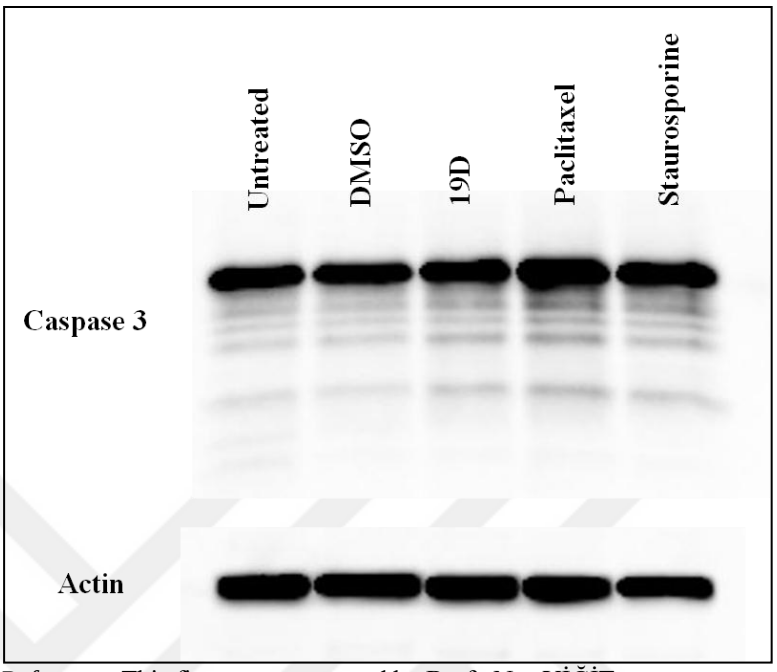


Color of lines symbolize for different experiment groups; blue line represents 0.2 percent DMSO, green line represents 25 µM 19D, purple line represents 50 µM 19D and red line represents 100 µM 19D
Reference: This figure was prepared by Berfu Nur YİĞİT.

4.5 CASPASE 3 PROTEIN ANALYSIS IN WESTERN BLOT

In the previous experiments, we investigated caspase 3/7 activation based on cell staining technique, but western blot analysis through western blot is more acceptable protein conformation techniques. Besides, caspase 3/7 activation is very important and specific execution indicator of apoptosis which can be supported by other techniques. For his analysis, equal amount of protein extracts from HUVEC cells as untreated, DMSO, 19D, Paclitaxel and Staurosporine were subjected with anti-caspase 3 and anti-actin (Figure11). According to results, we found found cleaved caspase-3 proteins in cell extracts, only procaspase inactive form of caspase-3 was found. Since, positive control subject as Staurosporine treated cells gave also no cleaved caspase-3 results, incubation times can be optimized to find active form in positive control. As a conclusion, although we state caspase 3/7 activation in live cell staining, we couldn't find in western blot which requires more experiments for optimization procedures for antibody as well as cell line.

Figure 4.11: Determination of caspase-3 and actin proteins in HUVEC cell extracts

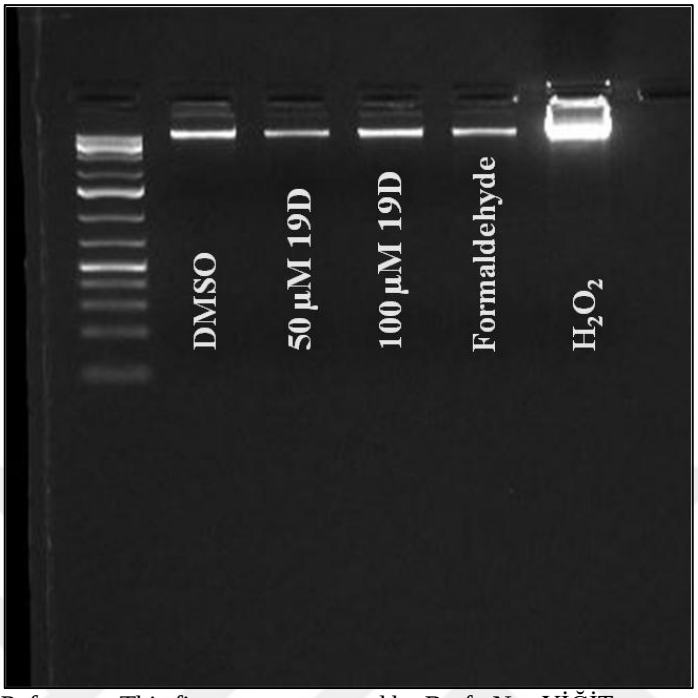


Reference: This figure was prepared by Berfu Nur YİĞİT.

4.6 APOPTOTIC DNA FRAGMENTATION

Besides from the nuclear morphological changes during apoptosis, endonuclease-mediated cleavage of nuclear DNA (180-200 bp) is an important hallmark of apoptosis. In this experiment, we treat HUVEC cells with DMSO as again a negative control, formaldehyde and hydrogen peroxide as a positive control and two concentration of our agent to see differences. The expected result was visualization of SubG1 population in agarose gel which is also confirmed with lamin antibody specific multicolor immunofluorescent staining. According to results, there were no fragmentations in our agents as well as positive controls which suggest that our protocol was not working (Figure4.12). Although we performed experiments in nearly 80 percent confluent plates, catching large number of cells in specific DNA fragmentation phase might be caused this result. Further experiments with larger number of cells in plate can be performed to analyze DNA fragmentation in agarose gel.

Figure 4.12: DNA fragmentation assay in HUVEC cell



Reference: This figure was prepared by Berfu Nur YİĞİT

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5. CONCLUSION

As a conclusion, in this study we have found novel molecule 19D which induces cell growth inhibition by G2/M phase arrest and accelerates apoptotic events in various cancer and healthy cell lines. Suppression of cell division by G2/M arrest in cell cycle is widely used approach to treat cancer cells, in our study; this novel imidazolone based small molecule has great potential as an anticancer therapy agent because of that 19D's G2/M arrest potential is strong as much as Paclitaxel chemotherapeutic drug. For 19D further studies: the drug resistance in cells and activated specific molecular pathways need to be identified. Therefore, further investigation is warranted. In addition, with this thesis, we provide wide drug screening approaches such as cycle disruptions, anti-proliferative effects, apoptosis mechanisms and nuclear deteriorations analysis which can be adaptable for other drug screening *in vitro* projects.

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