INVESTIGATION OF ANTICANCER PROPERTIES OF THE NOVEL SYNTHESIZED PYRROLE DERIVATIVES AS POTENTIAL TYROSINE KINASE INHIBITORS

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MASTER OF SCIENCE

in Chemistry

by
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İZMİR

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ABSTRACT

INVESTIGATION OF ANTICANCER PROPERTIES OF THE NOVEL SYNTHESIZED PYRROLE DERIVATIVES AS POTENTIAL TYROSINE KINASE INHIBITORS

In cancer treatment, chemotherapy has some serious side effects, because it targets active cells which might not be cancer cells. Mouth, hair, nail, bone marrow cells are some examples of active cells. For the reason that chemotherapy has side effects, targeted therapy become more important. Tyrosine kinases are most interested target, because they are necessary for cell growth and metastasis. Active form of tyrosine kinases can cause tumour growth and proliferation, angiogenesis, metastasis and antiapoptotic effects. Based on these vital role of tyrosine kinases, they became more important target in cancer treatment.

Pyrrole derivatives have been used chemotherapy drugs for years. Semaxanib and Sunitinib, indole derivatives, are tyrosine kinase inhibitors.

The main purpose of this research is to investigate the biologic activities of novel synthesized seven pyrrole derivatives, their activities on migration, apoptosis, cell cycle, and mTOR downstream as a potential tyrosine kinase inhibitor.

The results of this research proved that these seven compounds have toxicity on HeLa cells with the IC_{50} values of 140.60 µM, 382.82 µM, 366.44 µM, 542.00 µM, 255.86 µM, 148.59 µM, 171.40 µM, respectively, but toxicity effects of drugs do not depend on apoptosis mechanism. Beside this, D_1 and D_3 were able to effect cell cycle by arresting at S phase for D_1 and G1 phase for D_3. It was demonstrated that D_1 and D_3 inhibited cell migration. And this inhibition was reported as in a relation with overexpression of p-4EBP1, inhibition of p-p70S6K (Thr) and p-p70S6K (Shr) proteins. Considering all results, D_1 and D_3 might be potent inhibitory of metastasis of HeLa cells with respect to its effect on cell cycle, migration, p-4EBP1, p-p70S6K (Ser), and p-p70S6K (Thr) protein levels.

ÖZET

POTANSİYEL TİROZİN KİNAZ İNHİBİTÖRLERİ OLARAK YENİ SENTEZLENEN PİROL TÜREVLERİNİN ANTİKANSER ÖZELLİKLERİNİN ARAŞTIRILMASI

Kemoterapi aktif hücreleri hedef aldığı için kanserli olmayan hücrelere de zarar verebilir. Ağız, saç, tırnak ve kemik iliği hücreleri insan vücudundaki aktif hücrelere örnek verilebilir. Kemoterapinin bu yan etkilerinden dolayı, hedeflenmiş kanser tedavileri daha fazla önem kazanıyor. Tirozin kinazlar hücre büyümesi ve metastaz üzerindeki etkilerinden dolayı araştırmacılar tarafından en çok dikkat edilen hedefler arasındadır. Aktif formdaki tirozin kinaz tümör büyümesine, hücre çoğalmasına, anjiyogeneze, metastaza ve anti-apoptotik etkiye neden olabilir. Bu önemli etkilerinden dolayı hedeflenmiş kanser tedavisinde en önemli hedef haline gelmişlerdir.

Pirol ve türevleri yıllardır kemoterapi ilacı olarak kullanılmaktadır. Semaxanib ve Sunitinib tirozin kinaz inhibitörü olarak kullanılmaktadır ve pirol/indol halkasından türevlenmişlerdir.

Bu çalışmanın ana amacı yeni sentezlenen yedi molekülün biyolojik aktivitelerini, migrasyon, apoptoz, hücre döngüsü ve mTOR yolağına etkilerini potansiyel tirozin kinaz inhibitörü olarak incelemektir.

Araştırmanın sonucunda bu yedi yeni molekülün HeLa hücreleri üzerinde sitotoksik etkiye sahip olduğu, IC_{50} değerlerinin sırasıyla 140.60 μM, 382.82 μM, 366.44 μM, 542.00 μM, 255.86 μM, 148.59 μM, 171.40 μM olduğu, fakat bu etkinin apoptoz mekanizması ile ilgili olmadığı belirlenmiştir. D_1 molekülü hücre döngüsünde S fazında birikmeye sebep olurken, D_3 molekülü ise G1 fazında birikmeye neden olmuştur. Bunun yanında bu iki ilacın hücre migrasyonunu inhibe ettiği raporlanmıştır. Ayrıca yapılan çalışmalarda bu inhibasyonun p-p70S6K (Thr) ve p-p70S6K (Ser) proteinlerinin inhibasyonu, p-4EBP1 proteininin ekspresyonu ile alakalı olduğu raporlanmıştır. Tüm sonuçlar düşünüldüğünde D_1 ve D_3'ün HeLa hücrelerini, hücre döngüsünü, migrasyonu p-4EBP1, p-p70S6K (Ser), and p-p70S6K (Thr) protein seviyelerini etkileyerek metastazı önleyebileceği raporlanmıştır.

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HeLa	Human cervical cancer cell line
TKI	Tyrosine kinase inhibitor
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
IC_{50}	Half maximal inhibitory concentration
FDA	Food and Drug Administration
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
BSA	Bovine Serum Albumin
PBS	Phosphate buffered saline

CHAPTER1

INTRODUCTION

1.1 Cancer

All organisms are made of cells, and these cells are grow, divide and finally dead. All these events have control mechanism such as apoptosis which is named programmed cell death. But in cancer cells, these mechanisms are disabled, so they grow and divide without signals. Cancer may be defined as uncontrolled growing and proliferation of cells with insensitivity to anti-growth signals (Neal and Berry 2006). According to World Health Organization, cancer is the second leading cause of death globally and 8.8 million deaths was accounted in 2015. There were nearly 12 million cancer patients in 2008 and it was estimated that this number would double by 2020 and triple by 2030 (Lv and Xu 2009).

Tumour does not mean cancer, actually it means mass. Because tumours are divided by two, benign and malignant. Benign tumours are not cancerous, they do not spread throughout the body. Malignant tumours are cancerous, grow faster than benign tumours, resistant to treatment and spread throughout the body which is named as metastasis.

There are many types of cancer treatment and they are chosen according to cancer type. Surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy and stem cell transplants are main cancer treatment. Someone might take only one treatment but generally combination of treatments is applied to patients depending on the cancer type. For example, to remove a tumour from a body surgery can be performed, and after surgery patient might have a chemotherapy or radiotherapy treatment.

Radiotherapy uses a high dose of radiation on cancer cells, chemotherapy uses drugs to kill cancer cells.

Immunotherapy is a biological treatment which means it uses living organisms to help a body for fighting with cancer.

Targeted therapy, as it is understood from its name, takes aim at the changes in cancer cells. They can be small molecule drugs or monoclonal antibodies. Monoclonal antibodies are not small so they cannot enter the cells easily, but small molecule drugs can enter. Targeted therapy treat cancer in many different ways like helping the immune system, stopping cancer cells dividing and causing the cell death.

Stem cell transplants are a bit different, they help a body to produce stem cells after chemotherapy/radiotherapy treatment.

Nearly all cancer treatments have side effects. As radiotherapy uses radiation, it can damage/kill healthy cells too. As hormone therapy inhibit the production of hormone it has also some side effects.

Chemotherapy can treat cancer effectively but it has also some side effects. These side effects are related with some cell types which are active nearly as cancer cells, because chemotherapy targets active cells. For example, the cells in our blood, mouth, digestive system, hair, nail, and also bone marrow might be affected from chemotherapy. As chemotherapy has these side effects, scientists are developing novel drugs which is not toxic on normal cells but can destroy cancer cells. According to Winkler et al. (2014) by using targeted cancer therapy side effects can be minimalized. They also reported tyrosine kinase inhibitors as an example of targeted drug.

Chemotherapeutic agents are

- Alkylating agents
- Anthracyclines
- Cytoskeletal disruptors
- Epothilones
- Histone deacetylase inhibitors
- Topoisomerase inhibitors
- Kinase inhibitors
- Peptide antibiotics
- Platinum-based agents

1.2. Pyrrole and Derivatives

Heterocyclic compounds are the ring structures which have at least one different atom instead of carbon atom. Pyrrole is an aromatic compound, five membered heterocycle (C_4H_5N).

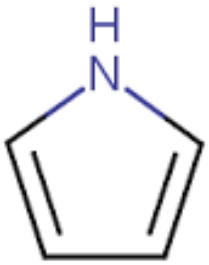


Figure 1.1 General structure of pyrrole
(Source: Toxicology Data Network)

Pyrroles are found in natural products, and basic structural element of the heme b (Figure 1.2) and chlorophyll α (Figure 1.3), vitamin B12 (Figure 1.4) and bilirubin is pyrrole (Mohamed, Abd El-Hameed, and Sayed 2017).

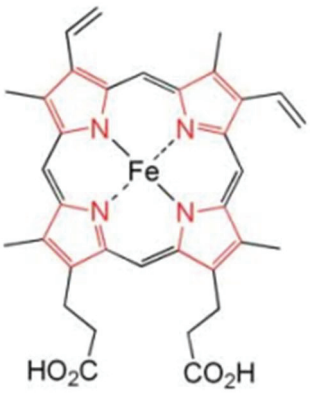


Figure 1.2 Structure of heme b
(Source: Mohamed, Abd El-Hameed, and Sayed 2017)

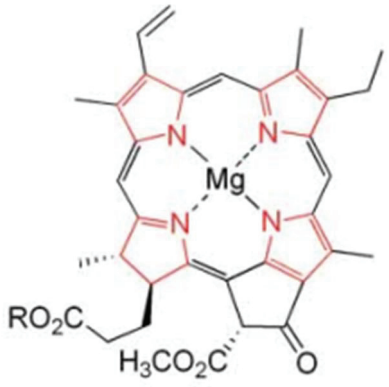


Figure 1.3. Structure of chlorophyll α

(Source: Mohamed, Abd El-Hameed, and Sayed 2017)

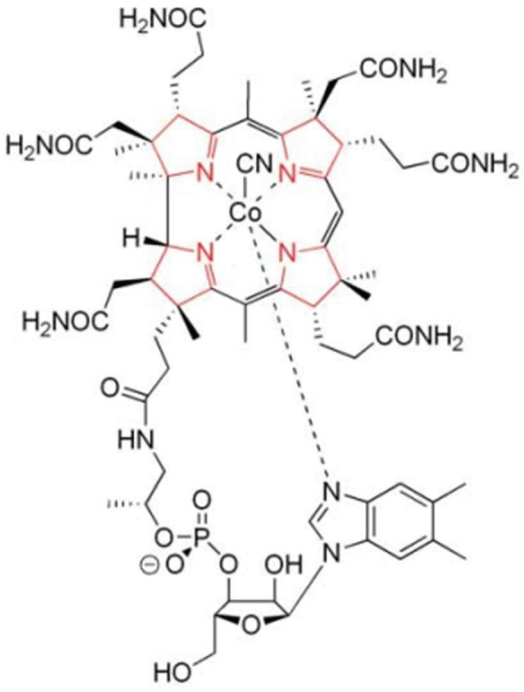


Figure 1.4 Structure of Vitamin B12

(Source: Mohamed, Abd El-Hameed, and Sayed 2017)

There are two mono-benzo derivatives of pyrrole, one is indole (2,3-benzo-), other is isoindole (3,4-benzo-).

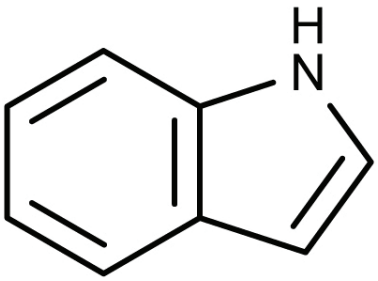


Figure 1.5 Structure of indole
(Source: About Chemistry)

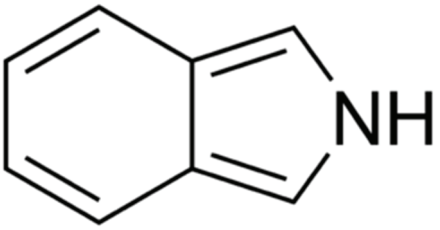


Figure 1.6 Structure of isoindole
(Source: Wikimedia Commons)

Indole takes place in the structure of tryptophan, it is the main structure of some anti-inflammatory drugs like indomethacin, and has antifungal, antimicrobial, antiviral, antioxidant, antidiabetic, antihypertensive effects (Sharma, Kumar, and Pathak 2010).

By cause of these, indole is an important heterocyclic structure. Isoindole is an isomer of indole, and can be found in pharmaceutical compounds, also in natural products. Lenalidomide and pomalidomide are anticancer drugs which have effect on multiple myeloma, and contains isoindole structure. Pazinaclone (sedative drug), thalidomide (teratogen) and apremilast (anti-inflammatory) also contain isoindole in their structure (Speck and Magauer 2013).

5-fluorouracil is a pyrimidine derivative and used as an antineoplastic agent for mainly cervical cancer, breast cancer, colon and rectal cancer, head and neck cancer, bladder cancer. Many pyrimidine analogues have been studied as chemotherapeutic agents. Both indole and pyrimidine fragments are individually potent pharmacophore, but using them in one structure will be interesting and may have better efficiency by reason of their own activity (Gokhale, Dalimba, and Kumsi). Pemetrexed (Alimta) is a FDA approved chemotherapy drug which classified as antimetabolite. It has the unique 6–5 fused pyrrolo [2,3-d] pyrimidine nucleus. It is used for the treatment of malignant mesothelioma and metastatic non-squamous non-small cell lung cancer (Hazarika et al. 2004).

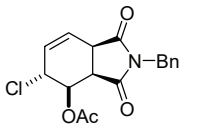
Ghorab and his co-workers synthesized series of novel 2-substituted-3-cyano-4-phenyl-pyrrole and 5-phenyl-pyrrolo[2,3-d] pyrimidine derivatives and they evaluated their cytotoxic activities on HEPG2 (liver cancer cell line) and MCF7 (breast cancer cell line). The IC_{50} values on both cell lines were identified approximately lower than 10 μM. They also combined these derivatives with radiation and observed that their activities increased which attests the significances of combination therapy to decrease side effects of drugs and radiation (Ghorab et al. 2010).

In 2014, Khayyat and Amr Ael synthesized a series of macrocyclic peptides which were based on pyridine structure and evaluated their biological activities on breast cancer cell lines and female athymic pathogen-free nude mice. Some of the molecules from the series showed both in vitro and in vivo anti-tumour activity (Khayyat and Amr Ael 2014).

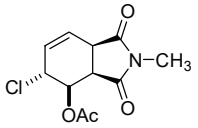
In another research in 1998, Huang and Bobek synthesized some amino-deoxy 7-hexofuranosylpyrrolo[2,3-d] pyrimidines and evaluate their antitumor activity on four different cell lines, human ovarian A121, human lung NSCLC A549, human colon HT-29, and human breast MCF7. They reported as compound 22 and 23 more active than others in terms of growth inhibition, IC_{50} ranging from 3.9 to 11.5 μM (Huang and Bobek 1998).

1.3. Chemical Structures of Drugs

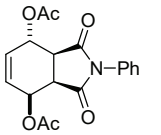
On the next page, chemical structures of seven novel synthesized isoindole derivatives were given. These molecules were synthesized by Prof. Yunus Kara in Ataturk University Department of Chemistry, Organic Chemistry Laboratory.



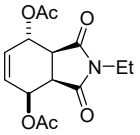
1 (3a*R*,4*R*,5*R*,7a*R*)-2-benzyl-5-chloro-1,3-dioxo-2,3,3a,4,5,7a-hexahydro-1*H*-isoindol-4-yl acetate



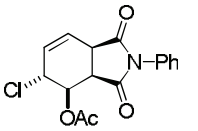
2 (3a*R*,4*R*,5*R*,7a*R*)-5-chloro-2-methyl-1,3-dioxo-2,3,3a,4,5,7a-hexahydro-1*H*-isoindol-4-yl acetate



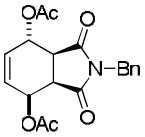
3 (3a*R*,4*S*,7*S*,7a*S*)-1,3-dioxo-2-phenyl-2,3,3a,4,7,7a-hexahydro-1*H*-isoindole-4,7-diyl diacetate



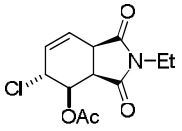
4 (3a*R*,4*S*,7*S*,7a*S*)-2-ethyl-1,3-dioxo-2,3,3a,4,7,7a-hexahydro-1*H*-isoindole-4,7-diyl diacetate



5 (3a*R*,4*R*,5*R*,7a*R*)-5-chloro-1,3-dioxo-2-phenyl-2,3,3a,4,5,7a-hexahydro-1*H*-isoindol-4-yl acetate



6 (3a*R*,4*S*,7*S*,7a*S*)-2-benzyl-1,3-dioxo-2,3,3a,4,7,7a-hexahydro-1*H*-isoindole-4,7-diyl diacetate



7 (3a*R*,4*R*,5*R*,7a*R*)-5-chloro-2-ethyl-1,3-dioxo-2,3,3a,4,5,7a-hexahydro-1*H*-isoindol-4-yl acetate

Figure 1.7. Chemical Structures of Novel Synthesized Drugs

1.4. Cervical Cancer and HeLa Cell Line

American Cancer Society identify cervical cancer as the cancer which starts in the lower part of the uterus (uterine cervix). There are two parts in cervix which are covered with two different cell lines. Endocervix, inner side, covered with glandular cells. Exocervix, next to vagina, covered with squamous cells. Because of these two different cells, cervical cancer has two different type. Squamous cell carcinoma, most common type, developed from squamous cells in the exocervix. Adenocarcinoma developed from gland cells in endocervix. Also, less commonly there is one type which consist of both cell properties, adenosquamous carcinomas (mixed carcinomas).

Human papillomavirus (type 16 and 18) are related with cervical cancer, and according to World Health Organization HPV causes 70% of cervical cancer. Although type 6 and 11 HPV is not causes cancer, they cause genital warts and respiratory papillomatosis, which is the disease tumour grows in the air passage from nose to lungs.

According to 2017 estimation of American Cancer Society for United States, about 12,820 new cases of invasive cervical cancer will be diagnosed and about 4,210 women will die from cervical cancer. Before increase of using Pap tests, dying rate of cervical cancer was 50% higher than now. Because Pap tests can realize the changes in the cervix before cancer develops.

Vascular endothelial growth factor and epidermal growth factor receptor are both play a crucial role in cervical cancer. Sunitinib, sorafenib, imatinib, pazopanib and cediranib are VEGF receptor tyrosine kinases and they have been tested in phase I-II clinical trials in cervical cancer. Phase II trial of sunitinib on advanced/metastatic cervical cancer reported 84% of stable disease in 19 enrolled patients and increase in fistula development which is a toxic effect (Mackay et al. 2010). In a phase I trial of cediranib in advanced solid tumours sVEGFR2 (soluble vascular endothelial growth factor receptor 2) concentrations were decreased (Drevs et al. 2007). Results of phase II trial of cediranib combined with carboplatin and paclitaxel in patients with metastatic/recurrent cervical cancer reported that Cediranib has significant efficacy when added to carboplatin and paclitaxel in the treatment of metastatic or recurrent cervical cancer (Symonds et al. 2015).

Henrietta Lacks was born on August 1, 1920 in Virginia. She had 5 children. On January 29, 1951, she went to Johns Hopkins Hospital because of her abnormal pain and bleeding in her abdomen. She was diagnosed with cervical cancer and doctors took two cervical samples from her without her knowledge. And unfortunately, she died at this hospital in October 4, 1951 when she was just at the age of 31. From her cervical samples, the first immortal cell line created which was named HeLa. It named as immortal because these cells can divide indefinitely unlike normal cells. HeLa cells morphology is epithelial and they grow adherently.

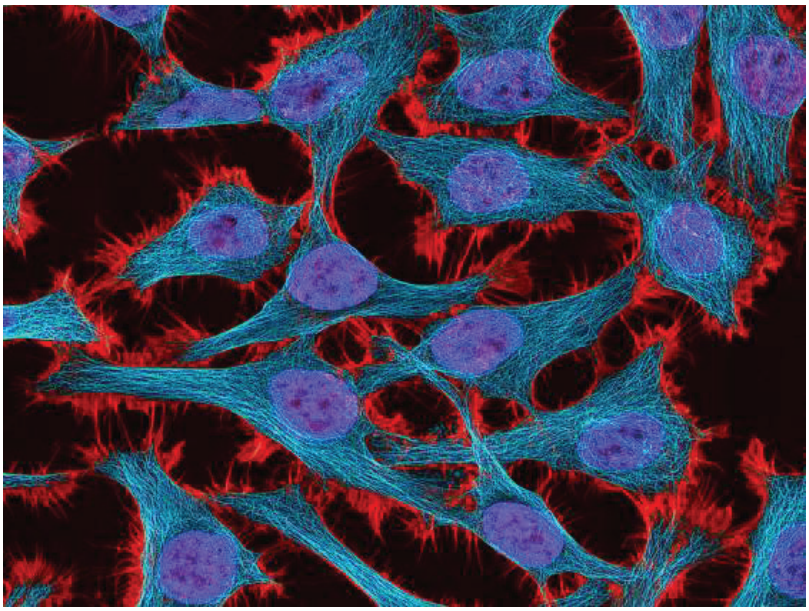


Figure 1.8. "Multiphoton fluorescence image of HeLa cells stained with the actin binding toxin phalloidin (red), microtubules (cyan) and cell nuclei (blue)"
(Source: Collins, 2013. National Center for Microscopy and Imaging Research.)

Figure 1.8 shows HeLa cells which were stained with the actin binding toxin phalloidin (red parts), microtubules (cyan parts) and cell nuclei (blue parts).

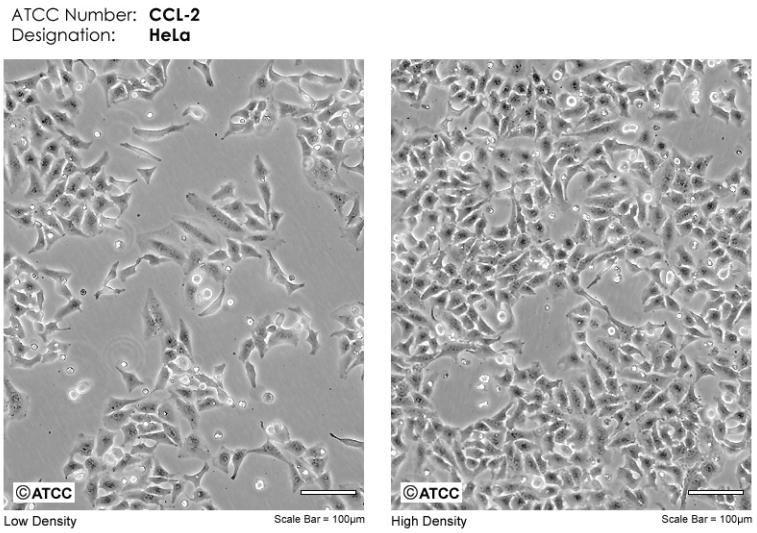


Figure 1.9. Image of HeLa cells

(Source: ATCC)

1.5. Tyrosine Kinases and Inhibitors

Cells need some signals in order to grow and divide. Growth factors are some type of chemicals and have different types, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet derived endothelial growth factor (PDGF) and fibroblast growth factor (FGF). EGF and FGF controls cell growth, VEGF controls blood vessel development, PDGF controls blood vessel development and cell growth. Epidermal growth factor binds to growth factor receptor on the cell surface and tyrosine kinases starts signal to initiate cell division.

Tyrosine kinases basically transfer the phosphate of the ATP molecule to hydroxyl group of the tyrosine residues (Figure 1.10). When growth factor attaches to receptor, tyrosine kinase starts signal for cell to divide and division occurs. Presence of tyrosine kinase inhibitor, it inhibits the signal and cell does not divide.

They can be divided into two group, receptor tyrosine kinases (RTK) and nonreceptor tyrosine kinases (NTK). Receptor tyrosine kinase, is a transmembrane

protein, take place on the cell surface, and nonreceptor tyrosine kinases take place in the cytosol, nucleus, and the inner surface of the plasma membrane.

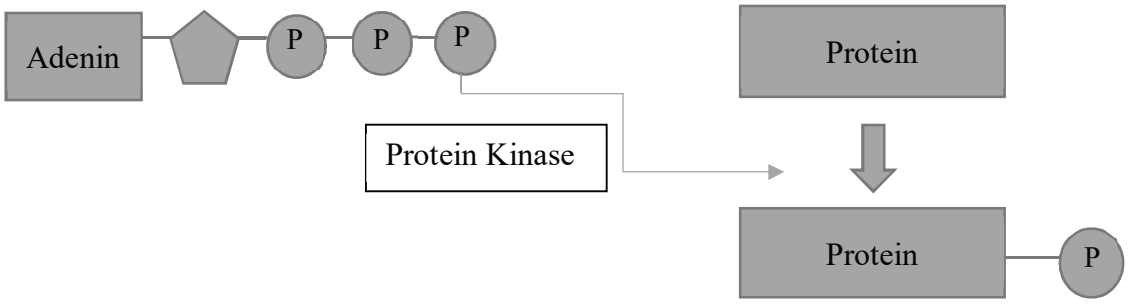


Figure 1.10. Representation of phosphorylation process by tyrosine kinase

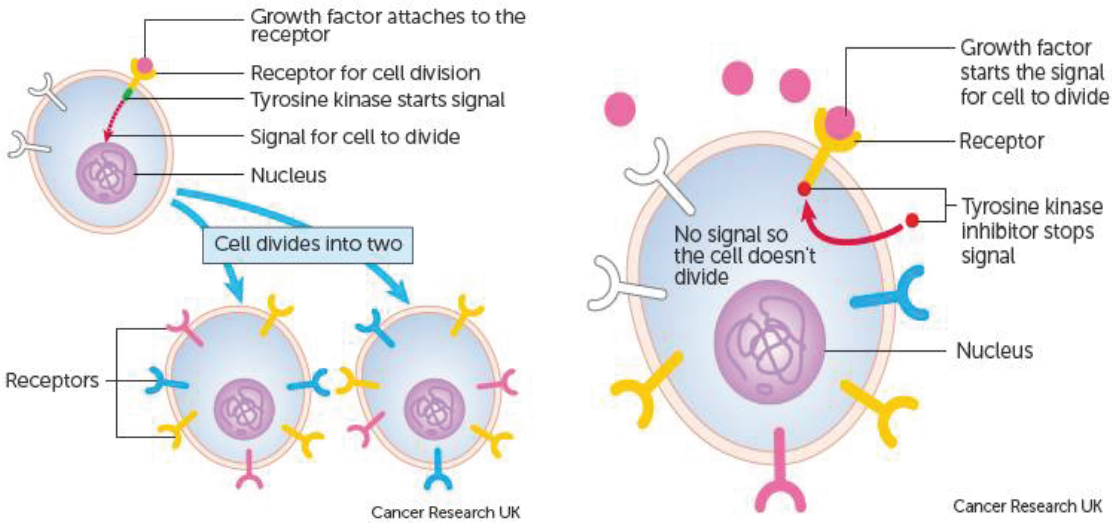


Figure 1.11. Representation of cell division control by TK and TKI

(Source: Cancer Research UK)

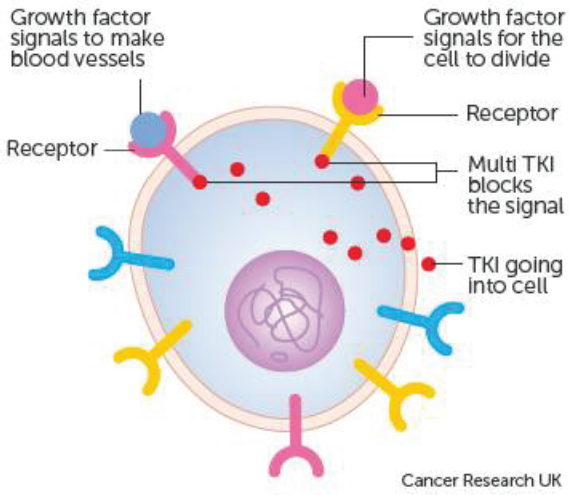


Figure 1.12. Representation of multi TKI mechanism

(Source: Cancer Research UK)

There are also multi tyrosine kinase inhibitors that seems to be advantageous according to its ability to inhibit several tyrosine kinases that overexpressed, but in some cases, it has some disadvantages also. For example, some tyrosine kinases like EGFR family increase the risk of metastasis, but some other like DKFZ1 and EPHB6 decreases risk of metastasis. For this reason, it become more important to inhibit specifically crucial tyrosine kinase without touching others. By means of this, it might be more difficult to making specific inhibition by using multi kinase inhibitor.

The main purpose of the designing tyrosine kinase inhibitor is finding a small molecule to inhibit catalytic activity of kinase.

Class I PI3K is a member of the family of intracellular lipid kinases that are activated by growth factor RTKs. This class I PI3K mediates the kinase activity of Akt by phosphorylation of threonine 308 and serine 473. Downstream substrates of activated Akt include the serine/threonine kinase mammalian target of rapamycin (mTOR) and the Forkhead family of transcription factors (FOXO).

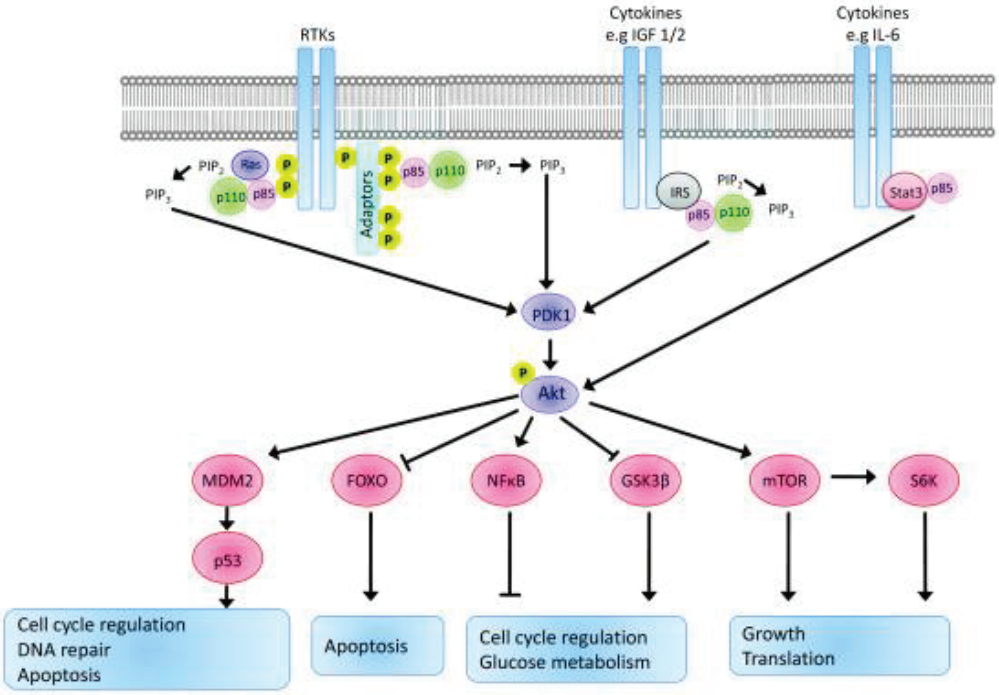


Figure 1.13. Signalling from RTK to Akt

(Source: (Wickenden and Watson 2010))

Table 1.1. Commercially available FDA approved TKI (cont. on next page)

Drug	***Commercial Name***	***FDA Approval Year***	***Target***
Afatinib	Gilotrif	2013	EGFR, HER2
Axitinib	Inlyta	2012	VEGFR, PDGFR, c-Kit
Bosutinib	Bosulif	2012	BCR-ABL, Src
Cabozantinib	Cometriq	2012	VEGFR
Crizotinib	Xalkori	2011	ALK, HGFR
Dasatinib	Sprycel	2006	BCR-ABL, c-Kit, PDGFR, Src

Table 2.1. Commercially available FDA approved TKI (cont.)

Erlotinib	Tarceva	2005	EGFR
Gefitinib	Iressa	2009	EGFR
Imatinib	Gleevec	2001	BCR-ABL, c-Kit, PDGFR
Lapatinib	Tyverb	2008	EGFR, HER2
Nilotinib	Tasigna	2007	BCR-ABL
Pazopanib	Votrient	2010	VEGFR
Regorafenib	Stivarga	2012	VEGFR
Ruxolitinib	Jakavi	2011	JAK
Sorafenib	Nexavar	2006	VEGFR, B-Raf, PDGFR
Sunitinib	Sutent	2006	PDGFR, VEGFR, c-Kit
Vandetanib	Caprelsa	2011	VEGFR, EGFR
Vemurafenib	Zelboraf	2011	B-Raf

1.6. Autophagy

One type of cell death, non-apoptotic and non-necrotic cell death, is autophagy. It means basically “eating of self”. There are 3 types of autophagy, macro-autophagy, micro-autophagy and chaperone-mediated autophagy. Macro-autophagy, thereafter referred as autophagy, is a multistep mechanism.

As shown in the Figure 1.14, autophagy begins with formation of isolation membrane or phagophore, and cytoplasmic cargo transported to lysosome by vesicle e longation and autophagosome. This autophagosome then fuse with endosome or lysosome and form autolysosome in which the transported material be degraded.

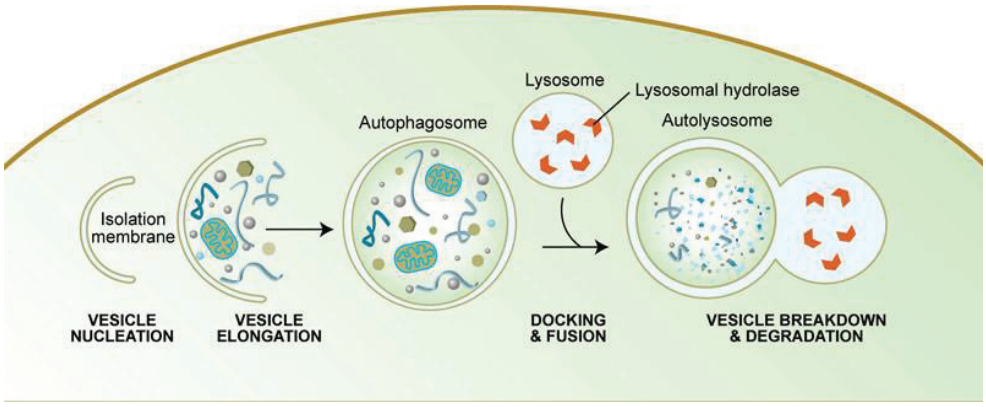


Figure 1.14. Steps of autophagy

(Source: (Melendez and Levine 2009))

Some anticancer drugs affect autophagy, histone-deacetylase inhibitors, kinase inhibitors, DNA damaging agents are some of them. There are two approaches to autophagy, some studies have concluded that inhibition of autophagy is a primarily step for chemo-resistance (Levy and Thorburn 2011), but there is also opposite claims suggest that autophagy is not a protective mechanism, it is the necessary element of the antitumor effect of a drug. For example, Wei and co-workers published a paper in 2013 concluded that EGFR inhibition requires autophagy to inhibition of lung tumour growth (Wei et al.). Their graphical abstract is shown in Figure 1.15. Active EGFR binds to Beclin 1 protein (autophagy protein) and multisite tyrosine phosphorylation occurs. This structure suppresses autophagy, induce tumour growth and cause tumour chemoresistance. EGFR activation can be inhibited by tyrosine kinase inhibitors, and as a result of this inactivation it cannot bind to Beclin 1 which means tumour growth and chemoresistance might be inhibited, autophagy might be induced.

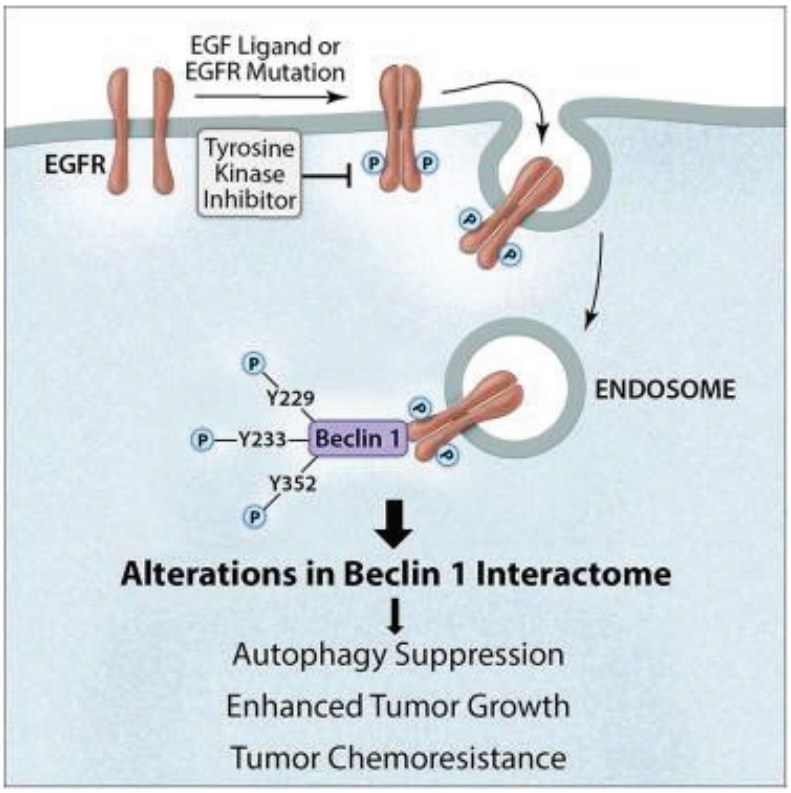


Figure 1.15. Mechanism of tyrosine kinase inhibitors on EGFR to mediate autophagy (Source: (Wei et al.))

1.7. mTOR Pathway

The mammalian target of rapamycin is a serine/threonine protein kinase. It is related with proliferation, protein synthesis, angiogenesis, autophagy and cell growth. It has two different multiprotein complexes, mTORC1 and mTORC2.

Approximately in 95% of the cervical cancer tissue mTOR pathway is active and Akt/mTOR pathway is related with invasion and metastasis of cervical cancer (Shi, Zhang, and Yin 2015).

PI3K (phosphoinositide-3 kinase), a lipid kinase, is related with Akt activation and schematic diagram of Akt activation showed in Figure 1.16 (Burnham, Shokoples, and Tyrrell 2007). They tried to investigate the role of PI3K/Akt signalling pathway in epithelial cell invasion by the group B streptococcus (bacterial pathogen) by using HeLa

cells. They demonstrated that inhibition of HeLa cells requires PI3K/Akt signalling pathway by the group B streptococcus.

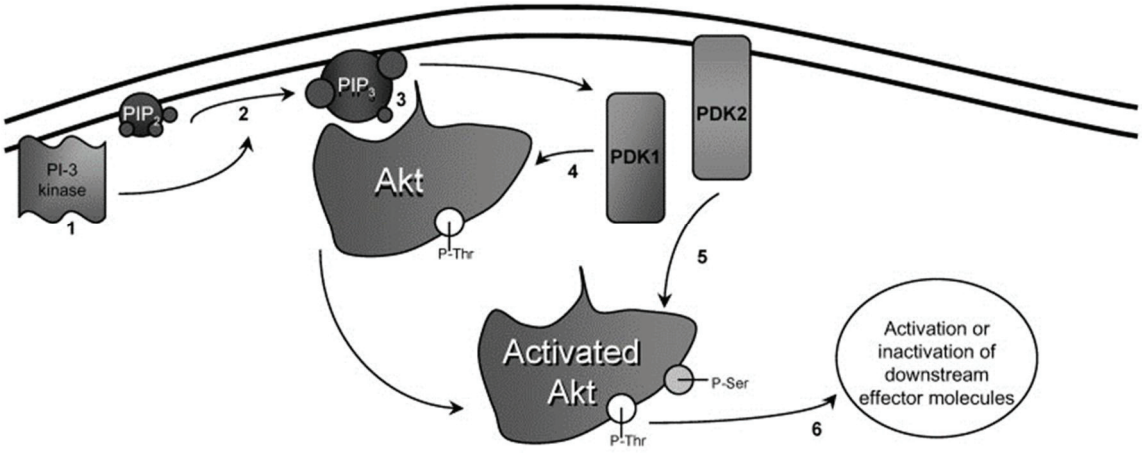


Figure 1.16. Schematic diagram of activation of Akt

(Source: (Burnham, Shokoples, and Tyrrell 2007))

Figure 1.17 shows the whole pathway of mTOR. From this figure, it is understood that autophagy is suppressed by Akt. Akt signalling increases and activates mTOR, and active mTOR inhibits autophagy. It can be concluded that MEK/ERK and autophagy is directly proportional, but PI3K/Akt/mTOR and autophagy is inversely proportional. The most important downstream of the mTOR are 4EBP1 and p70S6K which regulates cell growth and proliferation. There is inverse proportion between mTOR and 4EBP1, direct proportion between p70S6K. If mTOR inhibited there should be overexpression of 4EBP1, inhibition of p70S6K.

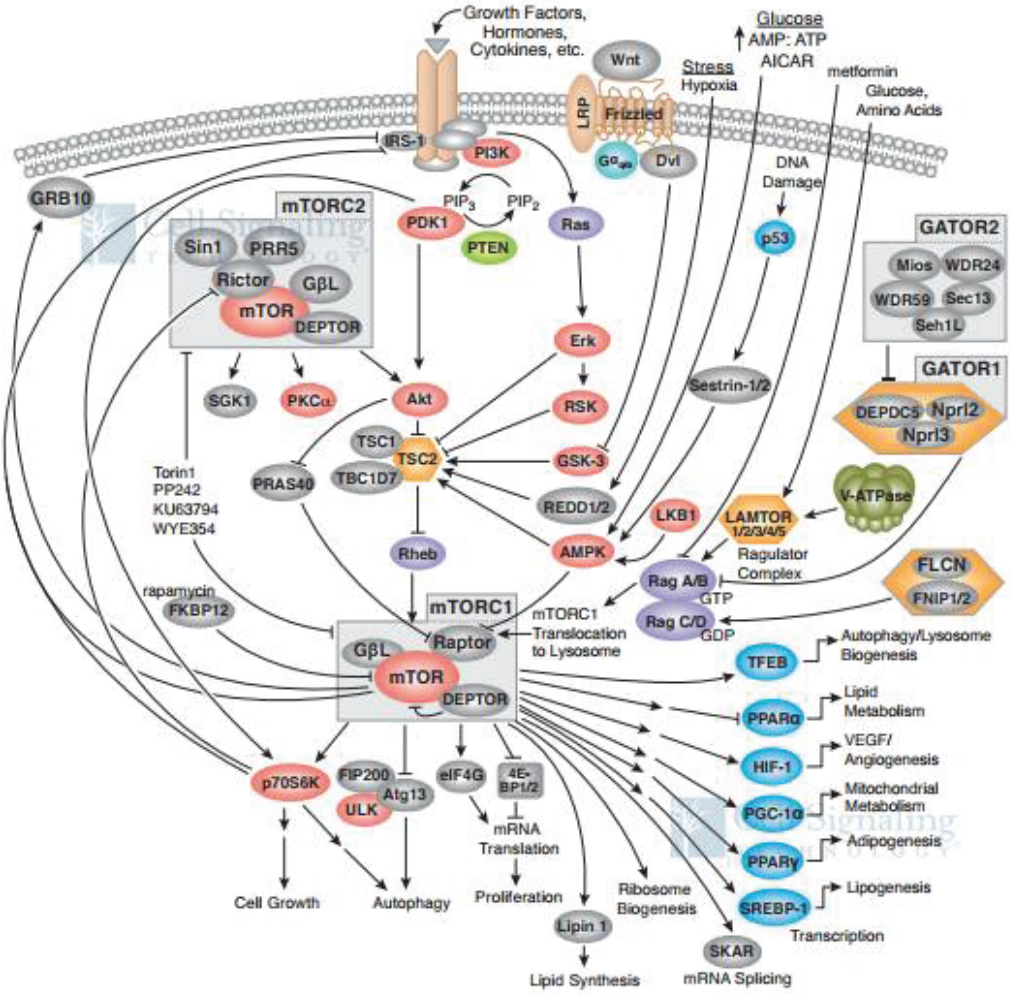


Figure 1.17. Pathway of mTOR

(Source: Cell Signalling Technology)

1.8. Aim of the Study

Due to its effect of minimizing cytotoxicity on normal cells, targeted therapy should be more considered in cancer treatments. Tyrosine kinase inhibitors have been used for targeted therapy, and some pyrrole derivatives are reported as important tyrosine kinase inhibitors.

The purpose of this study is to investigate the biological activities of novel synthesized seven pyrrole derivatives on HeLa cell line which is cervical cancer cells.

The cytotoxic effects of these molecules were investigated with MTT assay and their IC_{50} values were determined. Apoptosis analysis were performed with using Annexin-V and PI and cell cycle analysis with PI. In addition to these assays, to understand their effects on migration of HeLa cells, scratch assay was performed. Lastly, to better understand the acting mechanism of these seven molecules, western blot analysis was performed with using mTOR and its downstream substrates.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Cell Lines

HeLa cell line were kindly provided by IZTECH Biotechnology and Bioengineering Research and Application Center.

2.1.2. Drugs

Drugs were supplied in a powder form from Prof. Yunus Kara in Ataturk University Department of Chemistry, Organic Chemistry Laboratory. Stock solutions and fresh solutions were prepared with DMSO.

2.1.3. Culture Medias

Media for HeLa was DMEM (Sigma) with 4500 mg glucose/L, pyridoxine, HCl and $NaHCO_3$, without L-glutamine. DMEM was used with adding Pen-Strep Solution (Biological Industries), L-Glutamine Solution (Biological Industries), and Fetal Bovine Serum (Sigma).

2.1.4. Chemicals, Solutions and Reagents

All chemicals, reagents and solutions were listed in Appendix A.

2.2. Methods

HeLa cells were cultured in DMEM growth medium with 10% fetal bovine serum , 10% pen-strep solution and 10%L-glutamine. Cells were incubated in an incubator which provides 37°C and 5% CO_2.

Culture conditions are given in Table 2.1 as final concentrations 10% FBS, 1% pen-strep.

Table 2.1: Volume of complete media

Culture media, DMEM	450 ml
Fetal Bovine Serum, FBS	50 ml
Pen-Strep Solution	5 ml

2.2.1. Thawing the Frozen Cells

Frozen cells were taken from -80°C storage and thawed in a water bath at 37°C until nearly 90% of cells were thawed. After the ice crystals melted, cells were immediately transferred in a new complete media (10-12 ml) which was in 75 cm^2 culture flask. Then cells were incubated overnight and after 1 day the medium was changed in order to get rid of dead bodies.

2.2.2. Passaging the Cells

All solutions or medias which were used for passaging cells should be warmed in a 37°C waterbath. Flask was tkaen from incubator and media was removed, then washed with 5-8 ml PBS, and 3 ml trypsin was added to flask to detach the cells, placed in the incubator and waited approximately 2-3 minutes. When all of the cells detach, 4 ml fresh complete media added to flask and all suspension transferred to a falcon. That falcon then centrifuged at 800 rpm 5 minutes. After centrifugation supernatant was removed and cell pellet was resuspended in 2 ml fresh complete media. Some cell suspension were taken from this suspension depending on cell density and transferred new flask which contains fresh media.

Table 2.2: Volume of used growth medium and trypsin for different sizes of flasks

	Growth Medium	Trypsin
For 25 cm^2 flask	5 ml	1 ml
For 75 cm^2 flask	15 ml	3 ml
For 150 cm^2 flask	25 ml	5 ml

2.2.3. Freezing the Cells

All steps up to centrifugation are same with passaging cells. When supernatant was removed pellet was resuspended in fresh media and transferred to a cryogenic vials with final concentrations DMSO 10% (v/v), 20% FBS (v/v), 70% DMEM (v/v).

2.2.4. Counting the Cells

After centrifugation pellet was resuspended in a fresh media and 10ml of cell suspension was transferred in a microcentrifuge tube and 90ml of Trypan Blue solution (Sigma-Aldrich) was added and homogenized. Then droplets were placed in a hemocytometer (Neubar Improved-Isolab) and all the cells were counted in 4 corner squares. Blue cells were dead cells and opaque ones were viable cells. In a hemocytometer volume of the each square is 10^{-4} ml, so cell/ml can be found by (dilution factor) x (10^4) x (average number of cell/square).

2.2.5. Cell Viability Assay

In order to obtain cell viability, there are some assays depends on metabolic activity, and usually they measure mitochondrial activity. These assays use colorimetric substrate like MTT, XTT, WST-1. Tetrazolium salt MTT is widely used to detect cell viability, proliferation, and cytotoxicity. In MTT assay, viable cells reduce the tetrazolium salt (yellow colour) to formazan crystals (purple colour), because of the purple colour absorbance can be detected by a microplate reader. Non-viable cells cannot reduce tetrazolium salt, at the end of incubation period the colour cannot be observed as purple. As viability increases the signal (absorbance) increases, also colour changed to purple.

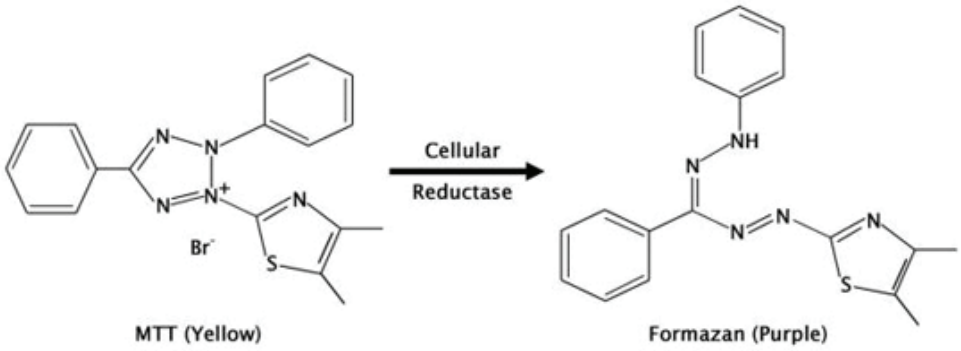


Figure 2.1 Reduction of MTT to Formazan crystals

(Source: MyBioSource)

HeLa cells were cultured in a 96-well plates (SPL Life Sciences) with $3x10^3$ cell/well and waited for 24 hours in order to attach surface of the plate bottom. Compounds were solved in sterile DMSO and stock solution of each 7 compounds were prepared as 200 mM in 100 µl DMSO. Working concentrations were prepared by diluting stock solutions with complete media and also DMSO (final DMSO concentration was 1% in a well) before the experiment. All compounds were added to wells according to 20, 50, 100, 200, 500, 1000, 2000 µM with triplicate assay. After all compound added, cells were incubated 24h, 48h and 72h in order to determine cytotoxic effects. At the end of each incubation period, media was removed from wells and MTT stock solution (5 mg/ml PBS) was diluted 1:10 ratio with complete media and added to wells. After 4 hours incubation, plates were centrifuged at 1800 rpm 10 minutes at room temperature. Supernatant was removed, DMSO was added to each well and shaked 150 rpm 15 minutes at room temperature in order to solve all formazan crystalls. Absorbance was determined at 540 nm (Thermo Electron Type 1500 Multiskan Spectrum).

2.2.6. Apoptosis Analysis

As all cells are grow and divide, they also need to die, and this programmed cell death is named as apoptosis. Apoptosis mechanism acts when cells are no longer needed. It was named firstly on 1972 by Kerr, Wyllie, and Currie, and the article which they published include morphological changes during apoptosis. They proposed that there are two stages in apoptosis, first include nuclear and cytoplasmic condensation and forming fragments, second include taking these fragments by other cells to process autolysis within phagosomes (Kerr, Wyllie, and Currie 1972).

Morphological changes in apoptosis and also other cell death mechanisms are given at the Table 2.3 (Bortner and Cidlowski 2014).

Table 2.3: Characteristics of cell death processes as a comparison

(Source: (Bortner and Cidlowski 2014))

	apoptosis	pyroptosis	autophagy	oncosis	necrosis
cellular morphology	shrinkage	swelling	no change	swelling	swelling
nuclear morphology	condensed	condensed	condensed	swollen	no change
cytoplasmic vesicles	no	no	yes	no	no
energy (ATP)	conserved	loss	conserved	loss	loss
pore formation (plasma membrane)	no	yes	no	yes	yes
caspase activity	yes	yes	no	no	no
annexin V staining	yes	yes	no	no	no
DNA degradation	internucleosomal	random	none	random	random
cellular blebbing	yes	yes	no	no	no
membrane integrity	conserved	loss	conserved	loss	loss
inflammatory response	no	yes	no	yes	yes

Necrosis described as accidental cell death and toxic because it happens when chemical damage or physical destruction is observed. There are some differences between apoptosis and necrosis. In necrosis cells are swelled, energy is lost, pores are formed, caspases are inactive, membrane integrity is lost, there are no blebbing and annexin V staining. On the other hand, in apoptosis cells are shrunk, energy is conserved, membrane integrity and energy are conserved, and most importantly caspases are active.

There are some regulatory elements to control apoptosis, cysteine proteases, caspases. Initiator caspases are activated and then they activate the executioner caspases in order to induce morphological changes which are related with phosphatidylserines. Phosphatidylserines are located on inner cell membrane, and in the case of apoptosis membrane begin to disrupt, PS flip to outer membrane. If there is any annexin V it can interact with these PS and apoptosis can be detected by this interaction.

FITC Annexin V Apoptosis Detection Kit with PI (BioLegend) was used in order to determine apoptotic effects of all 7 drugs on HeLa cells. Cells were seed in 6-well plates with volume of 1980 µl and at a density of $5x10^5$/well and incubated for 24h. After incubation period, 20µl of drugs were added to cells at their own IC_{50} concentrations with dublicate. Cells also incubated with these drugs for 48h. At the end of incubation period, all media were collected in their own tubes, wells were washed with PBS and collected, trypsin was added to wells to collect cells, and all collected cells again put in their own tubes. All tubes were centrifuges 5 minutes at 800 rpm. Supernatant were removed and

cell pellets were resuspended in 1 ml PBS, and then 4 ml PBS was added to each tubes. Tubes were again centrifuged 5 minutes at 800 rpm, supernatant was removed and pellets were resuspended in 100µl Annexin V Binding Buffer. After that, firstly 5 µl of FITC Annexin V was added to each tubes, then 10 µl of Propidium Iodide Solution was added and cells were gently vortexed and incubated for 15 minutes at room temperature in the dark. After incubation, 300µl of Annexin V Binding Buffer to each tube, and analyzed by flow cytometry (BD FACSCanto).

2.2.7. Cell Cycle Analysis

Cell cycle has four main stages, in G_1 (Gap 1) phase cell contents are duplicated, cells are active and they are at growing stage. S phase, in which DNA replication and centrosome replication occurs. In G_2 (Gap 2) phase, cell growth continues, replication of centrosomes and all preparation for division is completed and cells are ready for division. Cell division starts in M (Mitotic) phase, and this phase divided into four stages, prophase, metaphase, anaphase, telophase. Mitotic phase ends with cytokinesis which is cytoplasmic division. In Figure 2.2 general steps of cell cycle is represented.

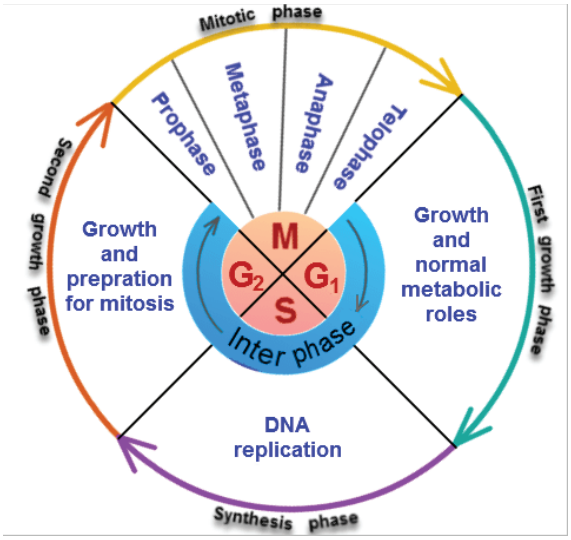


Figure 2.2 Representation of Cell Cycle

(Source: TutorVista, Biology)

Cell cycle also has some control elements and treatment with some drugs may affect these elements. As a result of this effect cell cycle may be stopped, protein synthesis, DNA replication, chromosome duplication or cell division may not be complicated.

Cell cycle analysis were performed with using PI, according to Yılmazer Çakmak (Yılmazer Çakmak 2011). HeLa cells were seeded in 6-well plates with volume of 1980 µl and at a density of $5x10^5$ cell in a well. After 24h incubation, drugs were added at their own IC_{50} concentrations and incubated 48h. At the end of incubation period cells were collected as writed in apoptosis analysis part, and tubes were centrifuged 5 minutes 800 rpm, supernatant was removed and tubes were placed on ice. Cell pellets were resuspended with 1 ml cold PBS on ice. During vortexing process, 4mL ethanol (-20°C) were added slowly to cell suspensions. This process fixed the cells, these fixed cells were waited on -20°C at least one night, most one month. After one night, cells were centrifuged at 1200 rpm 10 minutes at 4°C. Supernatant was removed and cell pellets were resuspended in 5 mL cold PBS (4°C), and centrifuged again at 1200 rpm 10 minutes at 4°C. Supernatant was removed from each tube and pellets were resuspended in 200 µl phosphate buffer which contain 0.1% Triton X-100. After that 20 µl RNase A (200 µg/ml) was added and incubated 30 minutes at 37°C. At the and of incubation period 20 µl PI (1mg/ml) was added and after incubated at room temperature for 15 minutes all tubes were analyzed by flow cytometry (BD FACSCanto).

2.2.8. Scratch Assay

Scratch assay or wound healing assay is a standard in vitro technique for observing cell migration in two dimensions. In this assay, a cell-free area is cretaed by scratching the bottom of the plate and then migration of cells through that cell-free are is detected by using microscope.

HeLa cells were seeded into 6-well plates at a density of 10^6/ml. when they reached approximately 80-90% confluency, three parallel line per well (scratch) were created by using pipette tip. The detached cells were washed with PBS carefully and fresh medium containing drugs were added to wells. All wells were contain 1% DMSO as a

final concentration. Control groups was not contain any drug. The migration were observed for 2 hours period up to 48 hours under microscope (Zeiss Z1 Inverted Fluorescence Microscope).

2.2.9. Total Protein Extraction from HeLa cells

The growth medium was removed from the flasks and washed with PBS to remove residual medium. Cells were collected by trypsination and transferred to 15ml falcon tubes. Centrifuged at 1200 for 2 minutes and supernatant was removed. 20 volume of ProteoJET Mammalian Cell Lysis Reaagent was added for each 1 volume of packed cells and resuspended by vortexing. Incubated for 10 minutes on 900-1200 rpm shaker at room temperature. The lysate was clarified by centrifugation at 16,000-20,000xg for 15 minutes. The supernatants were transferred to new tubes on ice and used immediately or stored at -70 °C for future use.

2.2.10. Bradford Protein Assay for Protein Concentration Determination

Protein standards were prepared by using BSA for 25 µg/ml to 2000 µg/ml. Protein samples (unknown) were diluted in a ratio of 1/10, 1/50, and 1/100. Dilutions were done with ultra pure water. Then all these samples and BSA standard solutions were transferred to 96-well plate and Bradford reagent was added each one and absorbance was measured at 595 nm.

2.2.11. Western Blot Analysis

Western blotting is an important technique as it gives ability to separate and identify specific proteins from a mixture pf proteins extracted from cells. There are three main steps in western blotting. First step is separation of mixture based on their molecular weight which is named as gel electrophoresis. Second step is transferring the gel to a membrane. On this membrane there is separated bands for each protein. Final step is incubating the membrane with labels antibodies specific to the protein of interest (Mahmood and Yang 2012).

Figure 2.3 shows gel electrophoresis step, separation of proteins based on molecular weight. Presteined protein ladder was used for electrophoresis which can be seen on the left side of the gel.

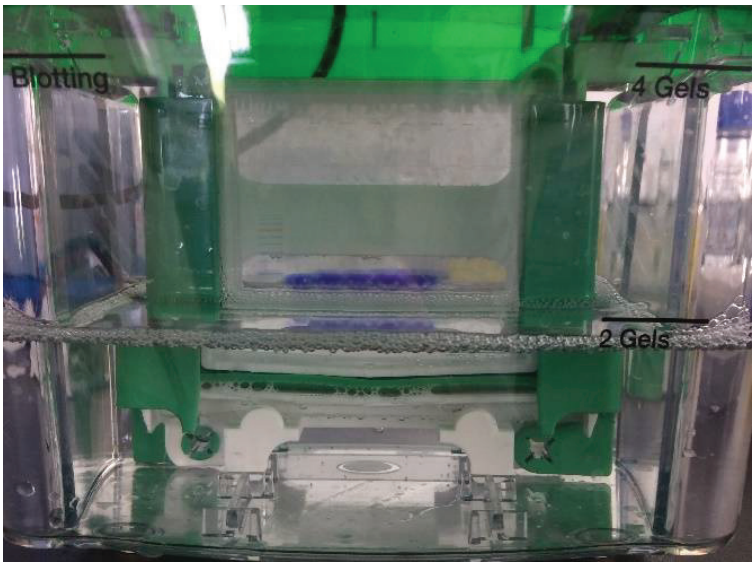


Figure 2.3. Gel Electrophoresis

Transferring the gel to membrane is important step in western blotting, because during transfer, some proteins may be lost. Figure 2.4 explains the transfer set up, there should be filter paper on top, after that gel and membrane should be placed, and finally there should be filter paper again on the bottom.

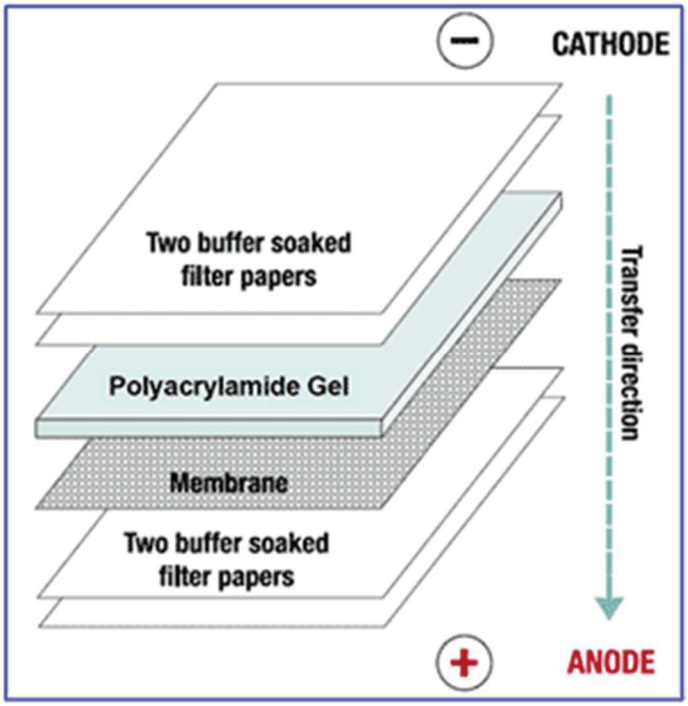


Figure 2.4. Transfer of gel to membrane

(Source: Bio-Resource)

At the first step, separating gel was prepared at the given volumes in Table 2.4. When the gel was poured trough the glass plates, isopropyl alcohol was added to correct the gel. When the gel was ready, alcohol was removed and stacking gel was added at the top of glass plates with placing plastic comb.

Table 2.4. Separating Gel

15% Separating Gel	Volume
40% Polyacrylamide	5,625 ml
1.5 M Tris (pH 8.8)	3,75 ml
10% APS*	150 µl
10% SDS	150 µl
TEMED*	6 µl
Ultra pure water	5,325 ml

(* added right before poring the gel into glass plates)

Table 2.5. Stacking Gel

5% Stacking Gel	Volume
40% Polyacrylamide	1 ml
1.5 M Tris (pH 8.8)	1 ml
10% APS*	80 µl
10% SDS	80 µl
TEMED*	8 µl
Ultra pure water	5,84 ml

(* added right before poring the gel into glass plates)

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cell Viability

In MTT assay cytotoxic effects of drugs on HeLa cells were determined and IC_{50} values of drugs were calculated. These figures were obtained by using Graphpad Prism 6 software program.

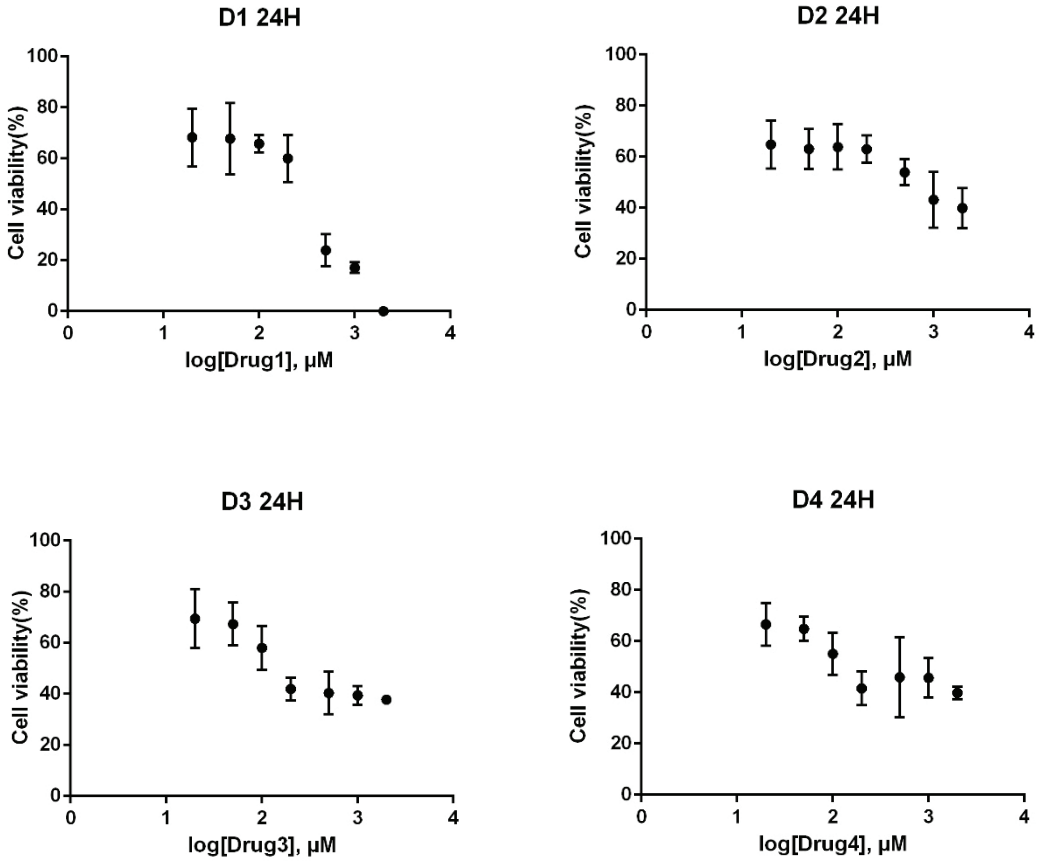


Figure 3.1. Cell viability results on HeLa cells for 24h incubation period

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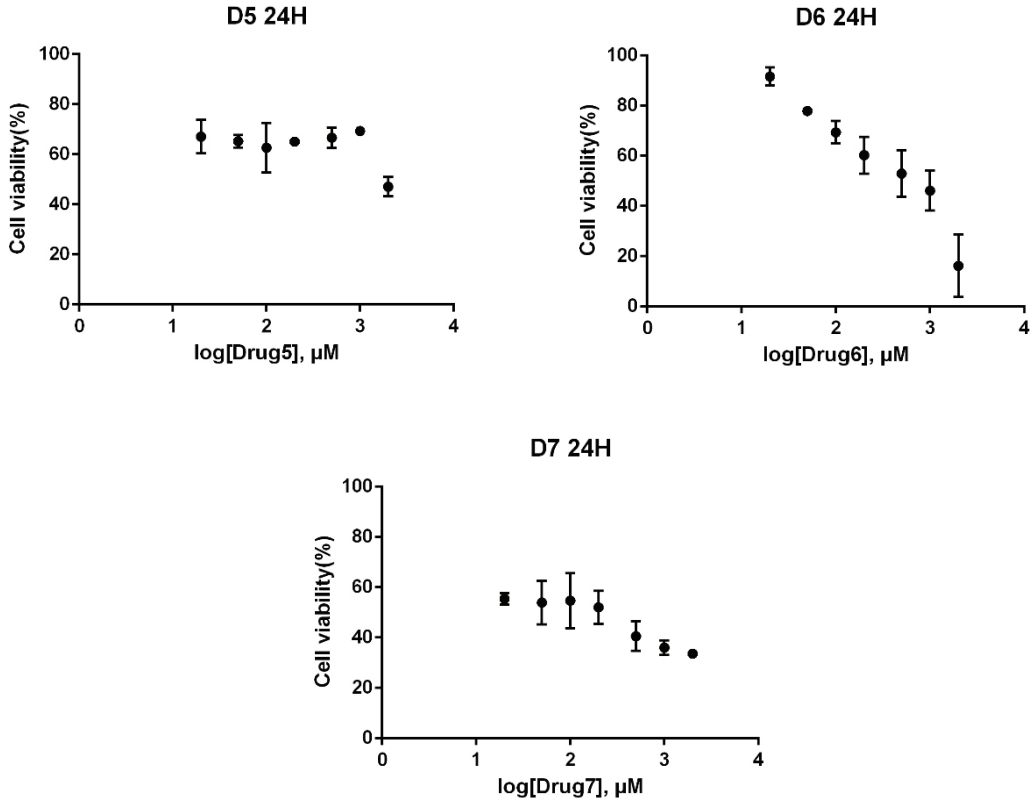

Figure 3.1. (cont.)

For this assay, three incubation times were choosen, 24h, 48h and 72h. Figure 3.1 shows the cell viability results of the cells for 24h incubation period .

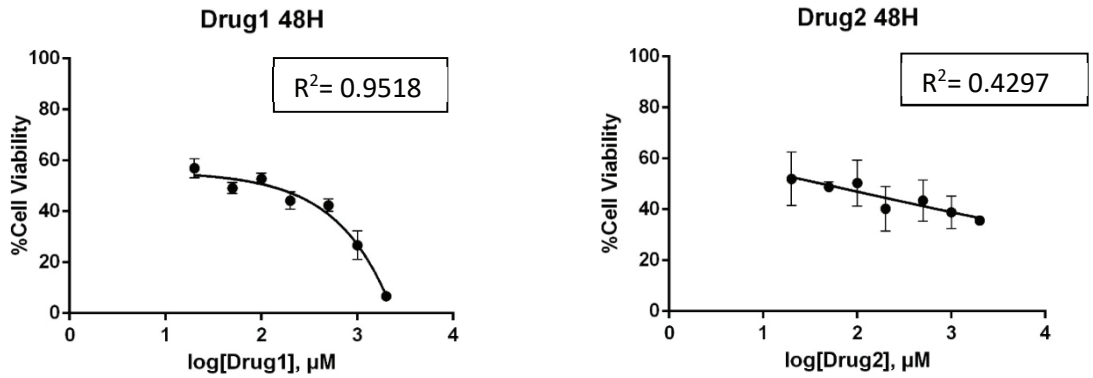

Figure 3.2. Cell viability results on HeLa cells for 48h incubation period

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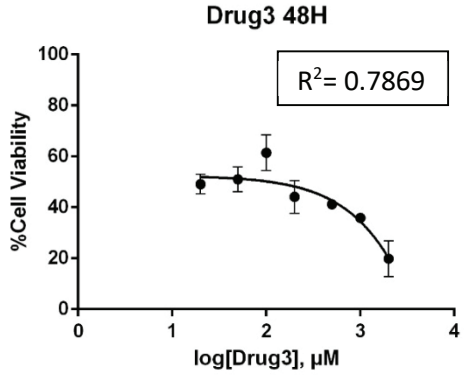

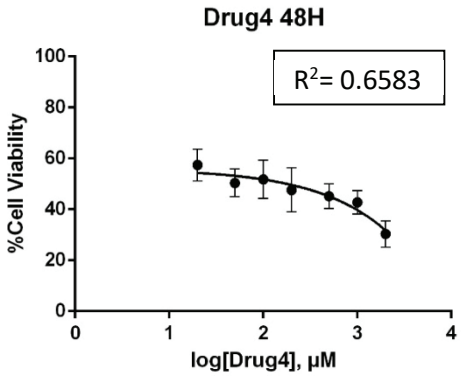

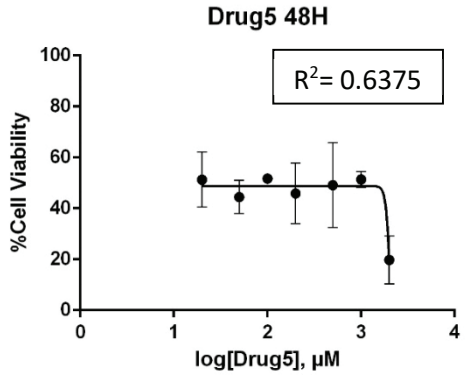

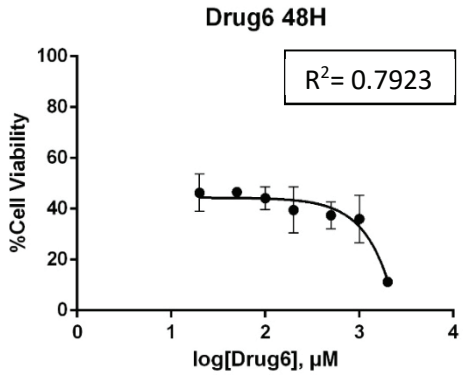

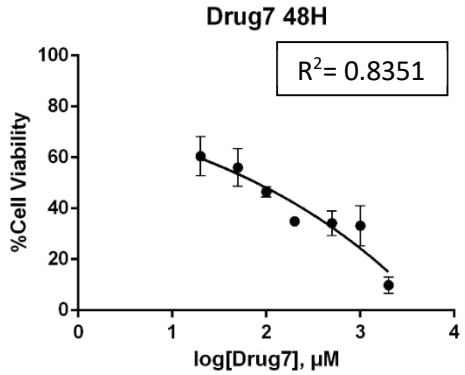

IC_{50} values:

D_1= 140.60 µM

D_2= 382.82 µM

D_3= 366.44 µM

D_4= 542.00 µM

D_5= 255.86 µM

D_6= 148.59 µM

D_7= 171.40 Mm

Figure 3.2 (cont.)

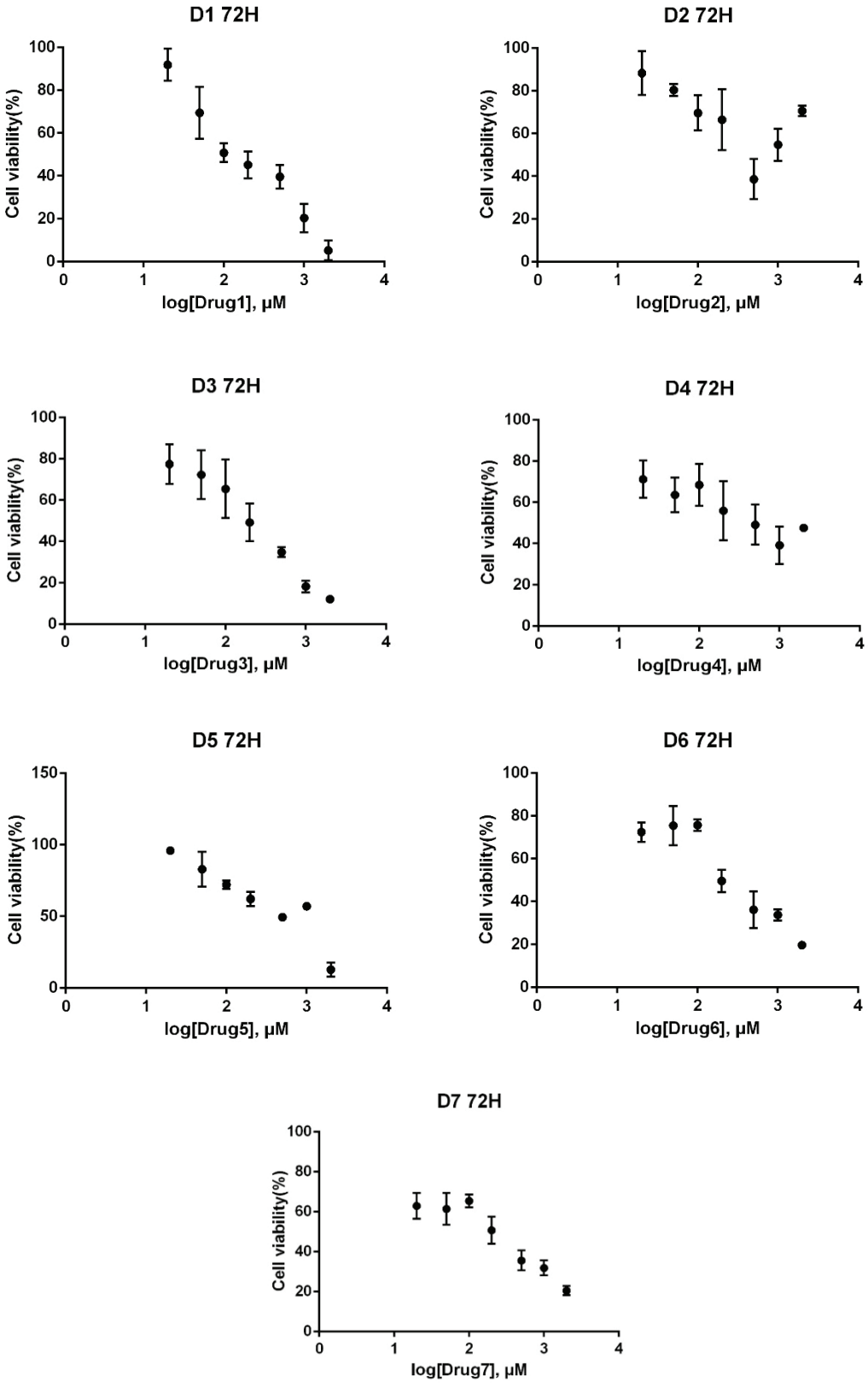

Figure 3.3. Cell viability results on HeLa cells for 72h incubation period

According to results of experiments, the optimum incubation period is determined as 48 hours. Some drugs for example drug 5 were not effective on HeLa cells when incubated 24h. The reason might be related with the dissolution of drug in the cell medium and transportation into the cell. In 2015, Mondal and his co-workers used 1-Amino-4-hydroxy-9,10-anthraquinone on MDA-MB-231 breast adenocarcinoma cells, they reported 50% inhibitory rates of this molecule as 200 μM for 24h incubation, 140 μM for 48h incubation (Mondal et al. 2015). In another research, pyrrole (1, 2, a) pyrazine 1, 4, dione, hexahydro 3-(2-methyl propyl) (PPDHMP), which was extracted from a new marine bacterium named as Staphylococcus sp. strain MB30, used on A549 (lung cancer cells), HeLa (cervical cancer cell lines) and normal peripheral blood mononuclear cells (PBMCs). PPDHMP was used various concentrations (1, 10, 25, 50 and 100 μg/ml) for 6, 12, 18, and 24 hours incubation. PPDHMP showed no toxic effect on PBMCs, but had 19.94 μg/ml IC_{50} on HeLa cells, 16.73 μg/ml IC_{50} on A549 cells for 24h incubation. The most effective inhibiton was observed on 24h incubation on their research (Lalitha et al. 2016).

On the other hand, when drugs were incubated for 72h, especially drug 2 and 4 lose their stability/effectiveness, or cells might become more resistant to drugs after long incubation periods. In 2003, Mai and his co-workers investigated the effect of pyrrole-C2 and -C4 substitutions on biological activity and they reported some molecules (compounds 3a and 1b in article) as antiproliferative agent on Friend murine erythroleukemia (MEL) cells, and also reported that cytotoxic activity of these compounds decreased after 48h incubation (Mai et al. 2004).

IC_{50} values of D_1 to D_7 were 140.60 μM, 382.82 μM, 366.44 μM, 542.00 μM, 255.86 μM, 148.59 μM, 171.40 μM, respectively. It can be concluded that D_1, D_6 and D_7 have lower IC_{50} values. Drug 4 has the maximum IC_{50} with 542 μM.

Considering the structural chemistry of these drugs, we can divide them into two groups. One having a -Cl atom in their 5^{th} position, the other having -acetate group on their 7^{th} position. First group has D_1, D_2, D_5 and D_7, second group has D_3, D_4 and D_6.

Looking deeply to first group (D_1, D_2, D_5, D_7) structures and IC_{50} values, D_1 (lowest IC_{50}) has -benzyl group, D_7 has -ethyl group, D_5 has -phenyl group and D_2 has -methyl group on their 2^{nd} position. The IC_{50} values order is $D_1 < D_7 < D_5 < D_2$. According to these results we may suggest that as the -R group on 2^{nd} position (substituent size) increases, IC50 values decreases.

In second group, D_6 has -benzyl group, D_3 has -phenyl group, D_4 has -ethyl group on their 2nd position. The IC_{50} values order is $D_6 < D_3 < D_4$ which also suggest the inverse relation between IC_{50} and substituent size , in other words as substituent size increases cytotoxicity also increases.

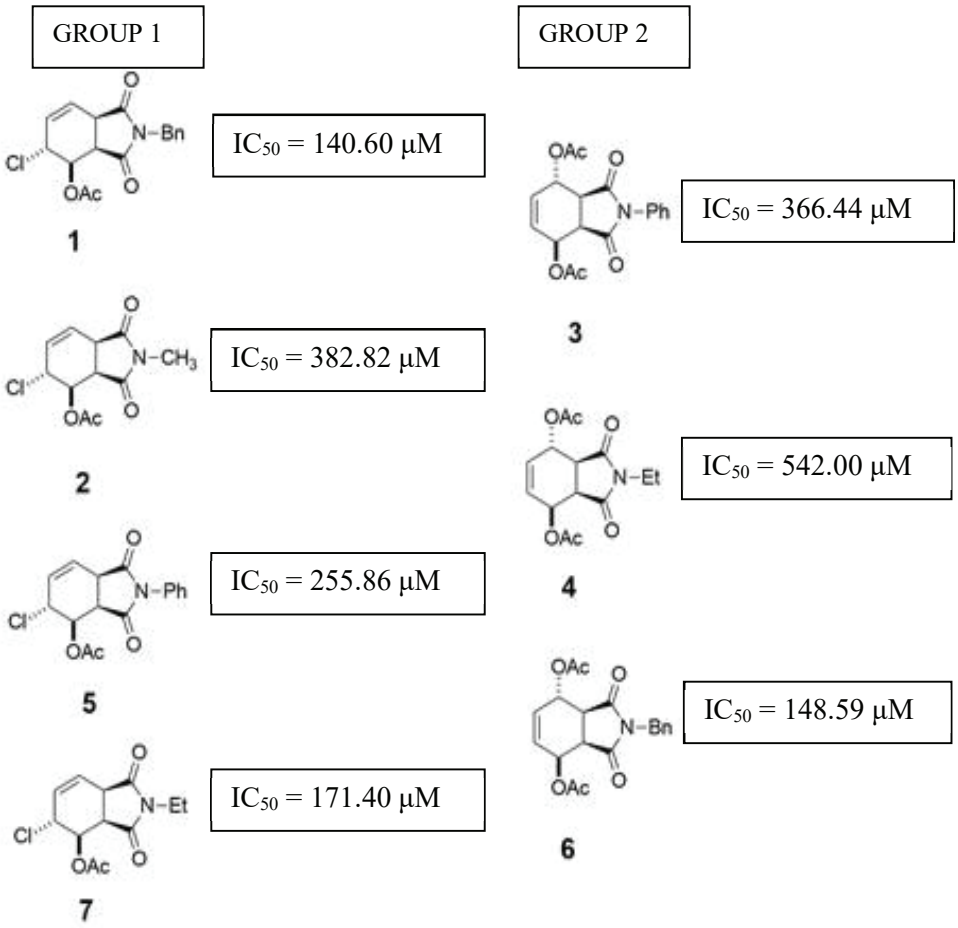

Figure 3.4. Structure of drugs as a group

3.2. Apoptosis and Cell Cycle

In order to investigate the apoptotic effects of these seven drugs on HeLa cells, each drug were applied at their own IC_{50} values with dublicate.

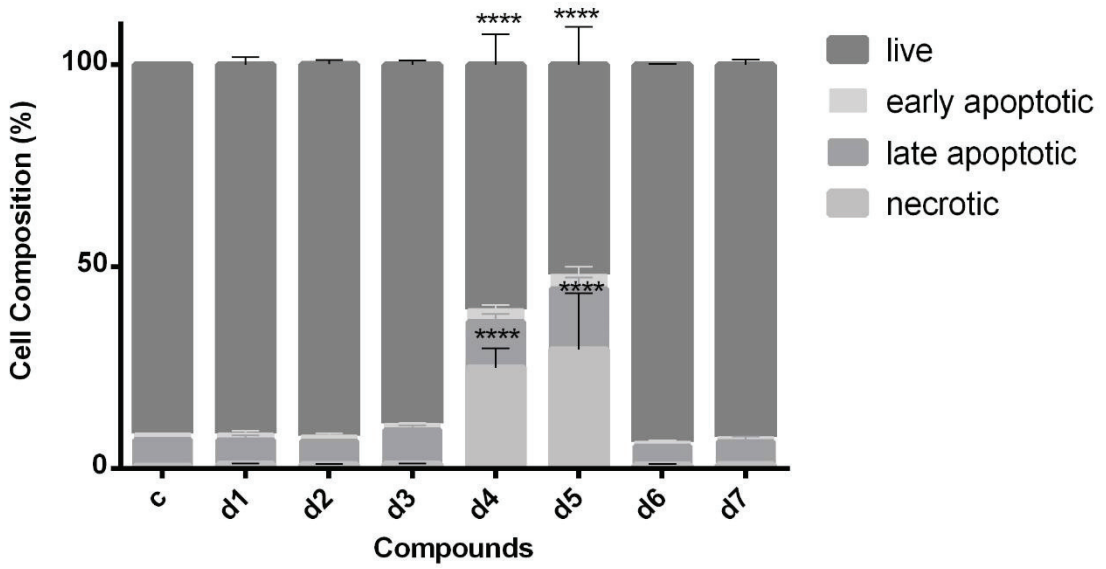

Figure 3.5. Results of apoptosis analysis on HeLa cells with the dose of IC50 for 48h incubation period. Bars indicate SD, n=3, ****p<0.0001

Figure 3.5 shows the results of apoptosis analysis and it was concluded from this result, D_4 and D_5 decreased the number of live cells significantly, because they cause approximately 25-30% necrosis at their IC_{50} doses. Other drugs are not show significant apoptotic effect on HeLa cells. Results suggest that the inhibition of proliferation of HeLa cells by these drugs was not related with their apoptosis activity. The main reason of the apoptosis resistance is reported as autophagy, and tyrosine kinase inhibitors are cause autophagy (Kung 2011). Apoptosis analysis result of these seven drugs is in agreement with this phenomenon.

Figure 3.6 shows cell cycle results which suggest that non of the drugs, except for D_3 and D_1, effects the cell cycle of HeLa cells when IC_{50} doses used. Drug 3 cause G_1 phase arrest which means cells cannot enter S phase, DNA replication phase. High cyclin D1 required for G1/S transition and it is known that Akt increase cyclin D1. Therefore, G1 arrest by D_3 might be related with mTOR, which is downstream of Akt and controlling autophagy in a negative way. In a study on human non-small cell lung cancer A549, Stellettin B induced G1 arrest and autophagy and also inhibit PI3K/Akt/mTOR pathway (Wang et al. 2016).

In 2012, 34 compounds which have diindolylmethanes were synthesized and their biological activites were investigated for HeLa, A549 and MCF-7 cancer cell lines. It was reported that HeLa and A549 cells were more sensitive than MCF-7 to these indole derivatives. Compound 7d was reported as the most promising compound for inhibiton of HeLa cells and also 7d caused G1 phase arrest on HeLa cells (Sharma et al. 2012).

Drug 1 cause S phase arrest that the cells cannot enter G2 phase, then also mitosis phase, means cells cannot dublicate its DNA. Thomas at el. were report a study in 1996, they demonstarted that when monocrotaline pyrrole used in a high concentration, it caused S phase arrest on endothelial cells. It caused S phase arrest in a dose depended manner up to 34.5 mg/ml, after this dose number of cells in S phase decreased. Also they investigated cell cycle and time dependece relation and observed 9-fold increase in S phase cells for 48h incubation. As a result they reported that monocrotaline pyrrole is able to arrest cells in S phase in a time-dependent and dose-dependent manner (Thomas et al. 1996).

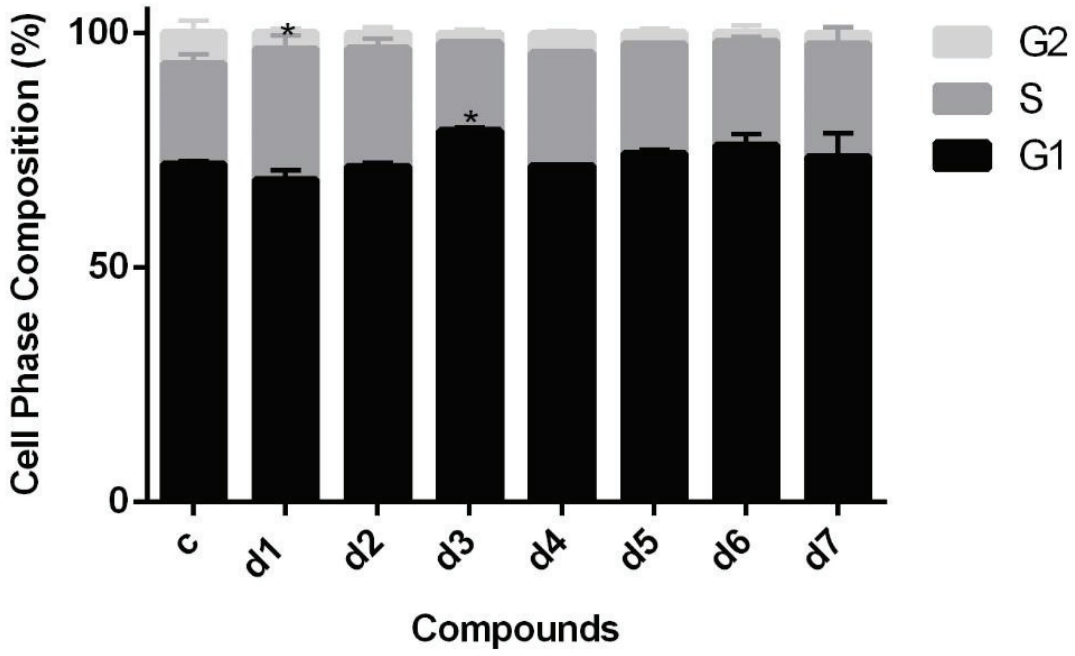

Figure 3.6. Results of cell cycle analysis on HeLa cells with the dose of IC50 for 48h incubation period. Bars indicate SD, n=3, *p<0.05

3.3. Scratch Assay

As mentioned before, these seven compounds were divided into two groups. In group 1, D_1 and D_7 have lower IC_{50}, in group 2 D_6 and D_3 have lower IC_{50} values. Besides to this, D_3 effects the cell cycle, causes G1 arrest, and D_1 causes S phase arrest. Due to these reasons, D_1, D_3, D_6 and D_7 were chosen for scratch assay analysis. Incubation period was choosen as optimum incubation period 48h according to MTT assay results. Figure 3.7 shows the results of the scratch assay. Gap closure percentages are 9.995% for D_1, 6.769% for D_3, 14.50% D_6, 19.93% for D_7 and 19.36% for control group.

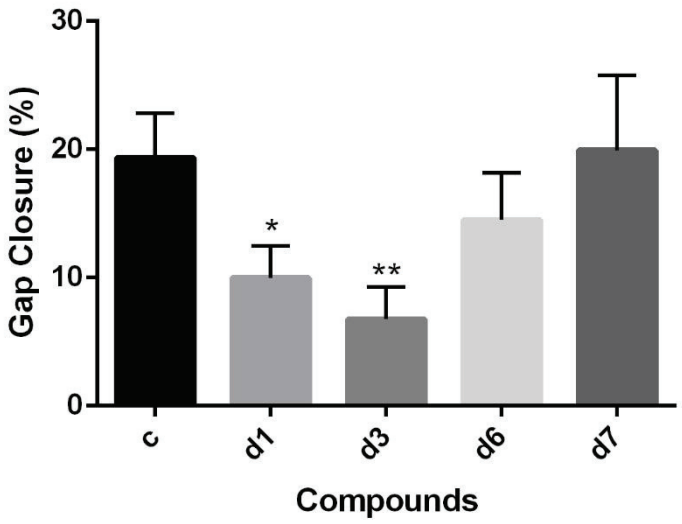

Figure 3.7. Results of scratch assay on HeLa cells with the dose of IC50 for 48h incubation period. Bars indicate SD, *p<0.05, **p<0.01

According to results, D_1 and D_3 are significantly inhibit the migration of HeLa cells which is a promising result for preventing metastasis. Metastasis of cervical cancer is related with Akt/mTOR pathway. There are some preclinical studies that evidence of the overexpression of PI3K in cervical cancer cell lines and overexpression of p-mTOR in squamous cervical tumors compared to healthy cervical epithellium (Vici et al. 2014).

In 2010, Wang et al. synthesized pyrrole-imidazole (PI) polyamide to target MMP-9 which is related with metastasis. They used HeLa cells and MDA-MB-231 cells for in vitro studies and six-week-old male athymic nude mice were used for in vivo studies. The study demosntrated that the PI polyamide has inhibitory effect on HeLa and MDA-MB-231 cell invasion and cell migration for 48h incubation. Besides this, they observed almost no effect on proliferation of these two cell lines, and they concluded that PI polyamide might inhibit metastasis. The results of in vivo studies suggested that PI polyamide suppressed liver metastasis. They explained theese strong effects of pyrrole-imidazole polyamide with penetration into nuclei and staying there for a long time (Wang et al. 2010).

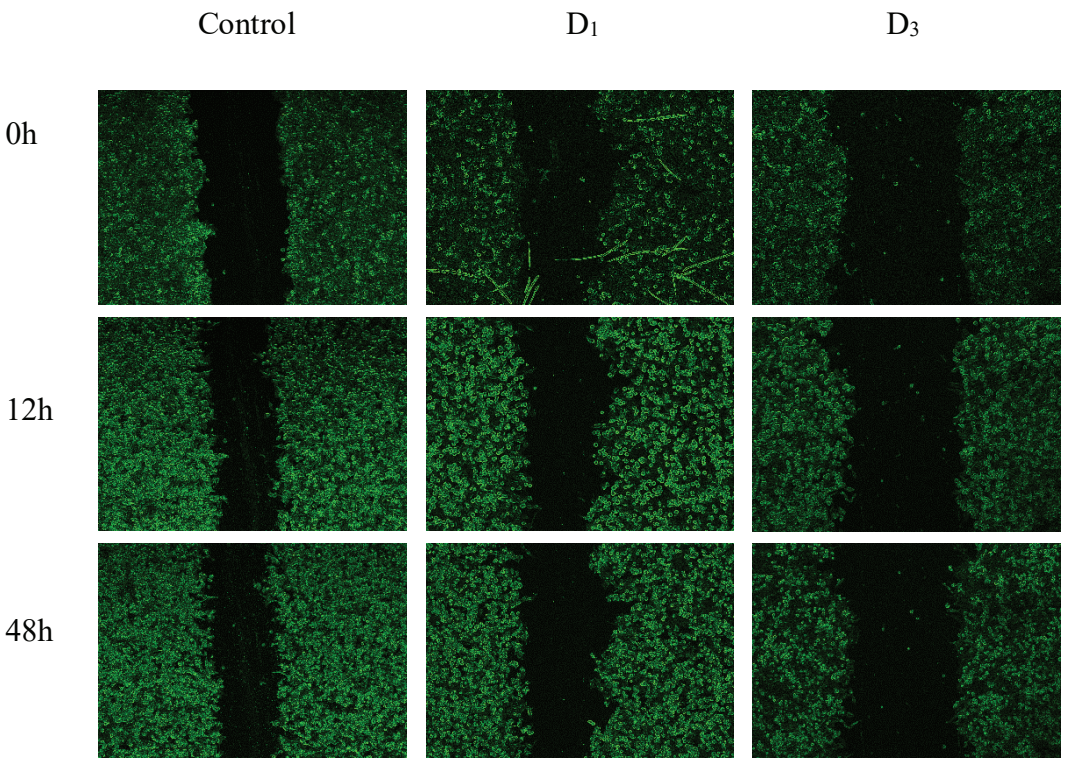

Figure 3.8. Images of gap closure of Control, D1 and D3 for 0h, 12h, 48h.

3.4. Western Blot

D_1 and D_3 showed inhibitory effect on migration and this effect might be related with mTOR pathway which is related with both metastasis and autophagy. Also according to results of MTT assay D_1, D_3, D_6 and D_7 showed more antiproliferative effect on HeLa cells and to better understand their acting mechanism, their relation with autophagy, they were chosen for Western Blot.

For western blot, Cell Cignalling Phospho-p70 S6 Kinase (Thr389) (108D2) Rabbit mAb 9234, Phospho-p70 S6 Kinase (Ser371) Antibody 9208, Phospho-4E-BP1 (Thr37/46) (236B4) Rabbit mAb 2855 proteins were chosen. For primary and secondary antibody, Anti-rabbit IgG, HRP-linked Antibody 7074 and ß-Actin (13E5) rabbit mAb were chosen.

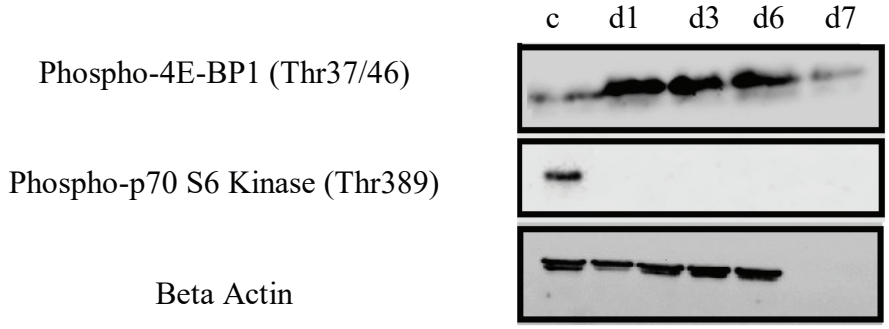

Figure 3.9. Imaging of Gel 1

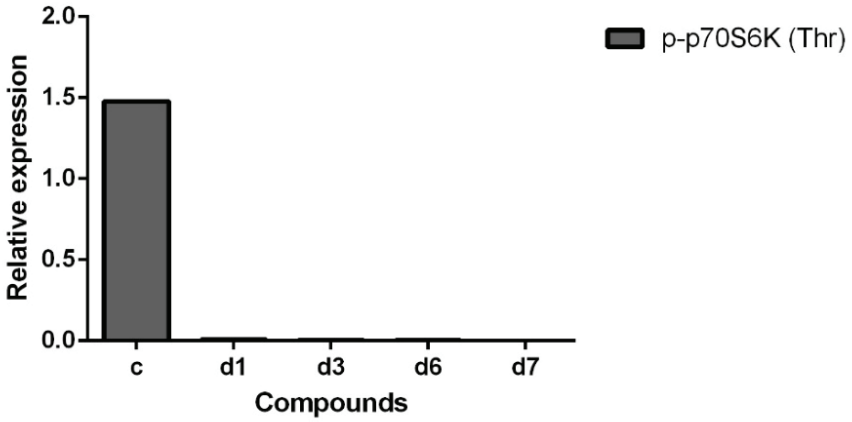

Figure 3.10. Relative Expression of p-p70S6K (Thr)

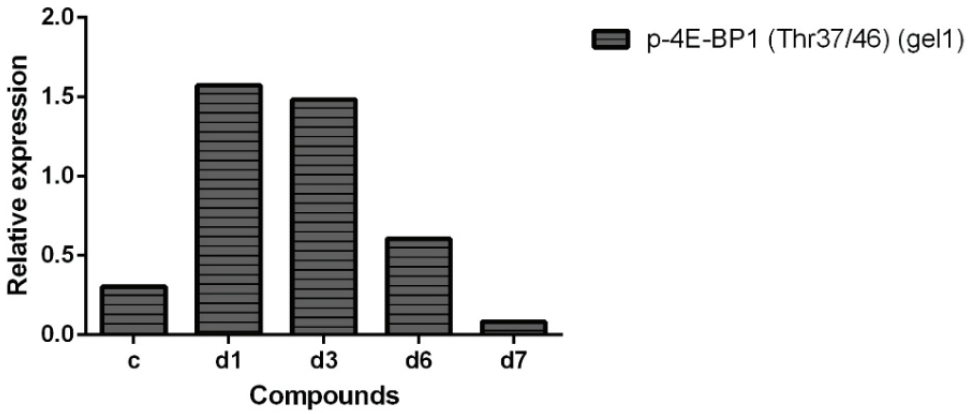

Figure 3.11. Relative Expression of p-4E-BP1 (gel1)

Two SDS-PAGE gel were performed for 3 protein, Beta Actin, p-4EBP1 and p-p70S6K. First gel imaging shows that the phosphorylated level of 4E-BP1 was overexpressed for the cells which were treated with D_1, D_3 and D_6, while control group and D_7 had approximately same protein level. Overexpression order is D_1, D_3 and D_6. It is known that if mTOR inhibited, 4E-BP1 should overexpressed according to their inversely proportional relation (Figure 3.12). Besides this, there were significant inhibiton for phosphorylated level of p70S6K (Thr) protein, suggesting there were mTOR inhibiton in the cells treated with these four drugs according to the direct proportional relation with mTOR and p70S6K (Figure 3.12). Figure 3.10 and 3.11 are quantification of the bands in the gel 1.

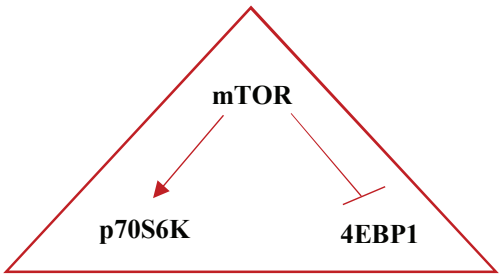

Figure 3.12 Relation of mTOR and its downstream

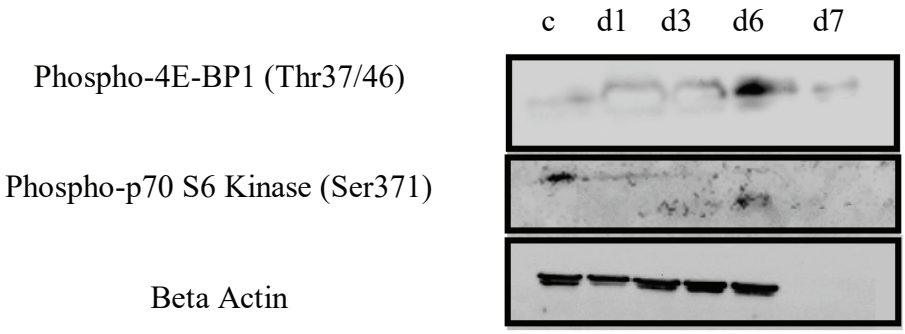

Figure 3.13 Imaging of Gel2

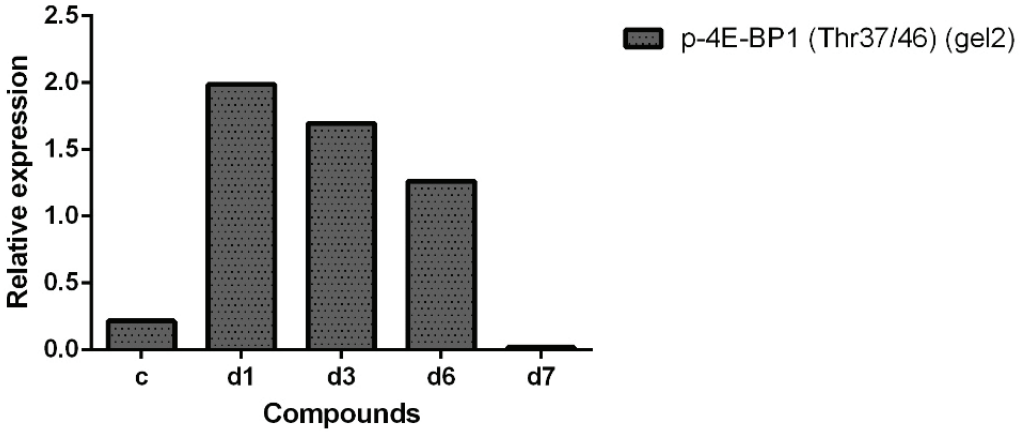

Figure 3.14 Relative Expression of p-4E-BP1 (Gel2)

For the second gel, p-4EBP1 and p-p70S6K (Ser) antibodies were used. It was observed that there were overexpression of phosphorylated level of 4EBP1 protein for the cells treated with D_1, D_3, D_6 (in order of overexpression) according to untreated control group. Also, significant inhibiton for p-p70S6K (Ser) level in cells treated with these four drugs according to control group were observed. Because of the high exposure time, there was high background for p-p70S6K (Ser) membrane. Figure 3.14 and 3.15 are quantification of gel 2.

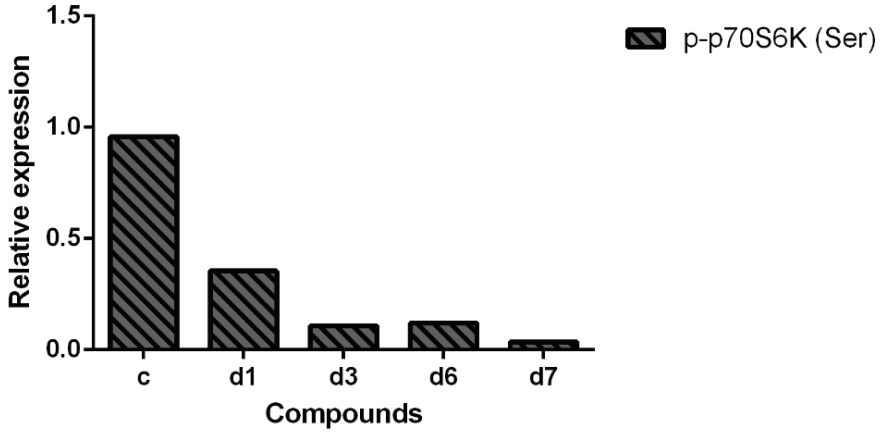

Figure 3.15 Relative Expression of p-p70S6K (Ser)

For the phosphorylated form of the 4EBP1, D1 and D3 had the maximum overexpressive effect, while they both inhibited the phosphorylated form of p70S6K (Thr). By looking the effects of each drug on protein levels, and also cell cycle results which suggest G_1 phase arrest for D_3, it might be a potent inhibitor of metastasis. In a study published on 2016 suggest that 1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione inhibits HeLa cell growth by causing a G1 arrest and by concomitantly inducing autophagy through the mediation of AMPK-mTOR and Akt-mTOR pathways, and may be a promising antitumor agent against cervical cancer (Tsai et al. 2016).

Overall these findings suggest that drugs may inhibit cervical cancer cell proliferation by repressing the mTOR pathway.

CHAPTER 4

CONCLUSION

Targeted therapies become more important day by day because of the side effects of chemotherapy drugs. Tyrosine kinases are known to be good target for cancer treatment because of their roles in cell growth signalling. Pyrroles and its derivatives, indole and isoindole are potential tyrosine kinase inhibitors which means they might be used in targeted therapy. Therefore, seven novel pyrrole derivatives were synthesized as a potential tyrosine kinase inhibitors by Prof. Yunus Kara and Aytekin Köse from Ataturk University.

In this study, cytotoxic activities, apoptosis, cell cycle effects, inhibition of migration, induction of autophagy, and involvement of mTOR pathways of these novel synthesized molecules were investigated in HeLa cell line.

These seven molecules can be examined under two groups according to their structures. The most effective ones are D_1 and D_7 from first group, D_3 and D_6 from second group considering to MTT assay results. Optimum incubation period of these molecules reported as 48h, because after 48h (72h MTT results) their activites and stabilities were decreased and before 48h (24h MTT results) they could not show efficient cytotoxic activity.

In order to investigate whether their cytotoxic activities related with apoptosis and cell cycle, drugs were incubated at their IC_{50} doses for 48h in HeLa cells then, apoptosis and cell cycles analysis were performed. D_4 and D_5 effects cells by necrosis at the rate of approximately 25-30%. There were no apoptosis detected when they were used at their IC_{50} doses on HeLa cells. On the other hand, cell cycle results reveal that D_3 causes G1 phase arrest, D_1 cause S phase arrest compared to untreated control groups.

To understand their inhibition of migration HeLa cells activities, scratch assay were performed with D_1, D_3, D_6 and D_7. The most promising molecule was D_3, shows maximum inhibitory effect on migration, and D_1 also showed significant inhibition after D_3. Results showed that these two molecules might have potentiation to prevent metastasis.

Besides that, Akt/mTOR pathway is related with both autophagy and migration of HeLa cells. Therefore to better understand the mechanism of these novel synthesized molecules, Western Blot analysis were performed by using downstream proteins of mTOR. Beta actin was used as a control protein, and other proteins were p-4EBP1, p-p70S6K (Ser), p70S6K (Thr). D_3 and D_1 had nearly same overexpression on p-4EBP1 but had higher effect compared to other two drugs. Besides this, D_3 had nearly same inhibitory effect with D_6 on p-P70S6K (Ser), and but this inhibitory effect was higher than other two drugs.

Considering all results, D_1 and D_3 might be a potent inhibitory of metastasis of HeLa cells with respect to its effect on cell cycle, migration, p-4EBP1, p-p70S6K (Ser), and p-p70S6K (Thr) protein levels.

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APPENDIX A CHEMICALS, SOLUTIONS AND REAGENTS

A.1. Cell Line

HeLa cells were supplied from IZTECH Biotechnology and Bioengineering Research and Application Center.

A.2. MTT Reaction Solution

MTT solution 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was prepared in PBS for the concentration of 5mg/ml. For 96-well plates, MTT solution was prepared with growth medium.

A3. PBS Solution

In distilled water add 0.34 g of KH_2PO_4, 1.58 g of K_2HPO_4, 8.0 g NaCl. Bring pH 7.04 and add distilled water to final volume 1 liter. Autoclave and store at 4°C.

Table A.1. Chemicals and reagents

No	Chemical	Company
1	DMEM	Sigma
2	Trypsin	Sigma

(cont. on next page)

Table A.1 (cont.)

3	Fetal Bovine Serum (FBS)	Sigma
4	DMSO	Carlo Erba Reagents
5	Trypan Blue Dye	Sigma
6	MTT Reagent	Amresco
7	Annexin-V Apoptosis Detection Kit with PI	Biolegend
8	Bovine Serum Albumin (BSA)	Sigma
9	RNase	Applichem
10	L-glutamine solution	Biological Industries
11	Pen-Strep solution	Biological Industries
12	Triton X-100	Amresco
13	KH_2PO_4	Riedel-de Haën
14	K_2HPO_4	Riedel-de Haën
15	Methanol	Sigma
16	Phosphoric Acid	Riedel-de Haën
17	Comassie Brilliant Blues G-250 dye	Sigma

A.4. Coomassie Brilliant Blue G-250 Dye

In 50 ml methanol Coomassie Brilliant Blue G-250 was dissolved and 100 ml of 85% (w/v) phosphoric acid (H_3PO_4) was added slowly to mixture and waited for dissolution of dye completely. Before use, it was filtered to remove the precipitates and stored in a dark bottle at 4 °C.