

**INVESTIGATION OF CYTOTOXIC PROPERTIES
OF NEW ISOINDOL DERIVATIVES IN LUNG AND
CERVICAL CANCERS**

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**by
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İZMİR

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ABSTRACT

INVESTIGATION OF CYTOTOXIC PROPERTIES OF NEW ISOINDOL DERIVATIVES IN LUNG AND CERVICAL CANCER

Cancer is one of the most common diseases in the world. Recently, there are many methods developed by researchers to treat this disease. One of these treatments is targeted for chemotherapy. It is preferred by researchers because it is less toxic and has fewer side effects than other cancer treatments.

This study emphasizes the anticancer properties of the newly synthesized Isoindole derivatives. Thus, it was hoped to be a significant improvement based on new generation anticancer compounds with high efficacy and fewer side effects.

The main objective of this study was to investigate the biological activity of seven newly synthesized Isoindole derivatives. The anticancer activity of these compounds was evaluated against HeLa (cervical carcinoma) and A549 (lung adenocarcinoma) cancer cell lines.

This study is divided into three parts. Firstly, the cytotoxic activity of these compounds was determined by measuring the cell viability of each compound on HeLa and A549 cell lines. The main objective of this analysis is to measure the IC_{50} value of each compound and determine which compound is best to kill at least half of the cells. Secondly, the effects of programmed cell death and cell cycle were investigated for compounds with the best IC_{50} for each cell line by using Annexin V-FITC in flow cytometry. Finally, a scratch assay was performed to investigate the effect of these new Isoindole derivatives on cell migration.

ÖZET

AKCİĞER VE RAHİM AĞZI KANSERLERİNDE YENİ İZOİNDOL TÜREVLERİNİN SİTOTOKSİK ÖZELLİKLERİNİN ARAŞTIRILMASI

Kanser, dünyadaki en yaygın hastalıklardan biridir. Son zamanlarda, bu hastalığı tedavi etmek için araştırmacıların geliştirdiği birçok yöntem mevcuttur. Bu tedavilerden biri olan hedeflenen kemoterapi; daha az toksik olması ve diğer kanser tedavilerinden daha az yan etkisi olması nedeniyle araştırmacılar tarafından tercih edilmektedir.

Bu çalışma, sentezlenen yeni Isoindole türevlerinin antikanser özelliklerini vurgulamaktadır. Böylece yüksek etkinliği ve daha az yan etkisi olan yeni nesil antikanser bileşiklere dayanan önemli bir gelişme olması umut edilmiştir.

Bu çalışmanın temel amacı, yeni sentezlenen yedi Isoindol türevinin biyolojik aktivitesini incelemektir. Bu bileşiklerin antikanser aktivitesi, HeLa (Yumurtalık kanseri) ve A549 kanser hücre hücrelerine (Akciğer kanseri) karşı değerlendirilmiştir.

Bu çalışma üç bölüme ayrılmıştır. İlk olarak, bu bileşiklerin sitotoksik aktivitesi, her bir bileşiğin HeLa ve A549 hücre hatları üzerindeki hücre canlılığı (MTT analizi) ölçülerek belirlenmiştir. Bu analizin temel amacı, her bileşiğin IC₅₀ değerini ölçmek ve hücrelerin en az yarısını öldürmek için hangi bileşiğin en iyi olduğunu belirlemektir. İkincisi, akış hücre sitometrisinde Annexin V-FITC kullanarak her hücre hattı için en iyi IC₅₀'ye sahip bileşikler için programlanmış hücre ölümünün etkisi ve hücre döngüsü üzerindeki etkisi araştırılmıştır. Son olarak, bu yeni Isoindol türevlerinin hücre göçü üzerindeki etkisini incelemek üzere çizik deneyi yapılmıştır

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CHAPTER 1

INTRODUCTION

1.1. Cancer

There is more than one definition for cancer and all these definitions indicate the same meaning and it could be formulated in a simple definition. The cancer is a type of disease that occurs when the cells going to abnormal growth. This operation has been done by changing in gene expression, which is leading to dysregulated cell proliferation and the cell will die. Consequently, the patient will favour and growth of a tumor. The tumor divide into two types the first one is called benign tumor, which is like a friend for human as it doesn't spread through the body, while the second one called malignant tumor, which is the danger than the first one as it spreads very fast through the body (Hanahan and Weinberg 2000, Pudata, Subrahmanyam, and Jhansi 2011).

There are three different types of cancer:

- **Carcinomas:** this type of cancer forms 85% in the world and develops in epithelial cell-like Lung cancer, Oral cancer, Skin cancer and Bladder cancer (Mehta 2011, Khan 2010, Farhadieh et al. 2011).
- **Lymphomas:** this type forms 7% in the world. It is cancer of lymph cells in the immune system. The structure of the lymph nodes is very small, therefore, it is along with blood in the human body (Bali et al. 2010).
- **Sarcomas:** this type of cancer formed in mesoderm tissue which is known as sarcomas (Lau, Chan, and Wei 2011). The example of this type of cancer is Bone, muscle, fat tumor of hematopoietic tissue.

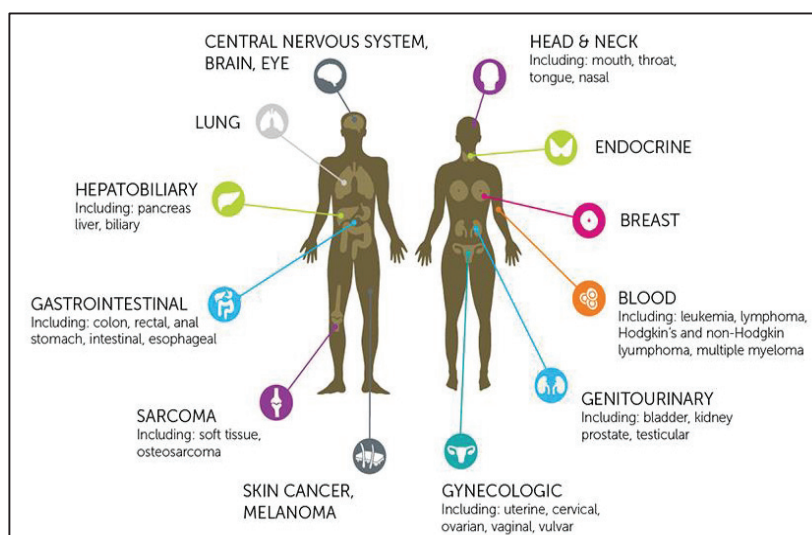


Figure 1. 1. Type of cancer
(Source: Mary Crowley Cancer Research)

There are many types of cancer treatments. These treatments, including surgery, radiation, immune therapy, targeted therapy and chemotherapy.

Surgery is a frequent and most common treatment, by removing the tumor during surgery. This type of treatment could minimize cancer, but it could not eliminate it definitively. The types of surgery are a biopsy, ultrasonography, endoscopy and open surgical exploration (Khan 2010).

Chemotherapy is one of the cancer treatment types by using anticancer drugs to kill cancer cells. Chemotherapy should use after or before surgery which gives a positive point to the patient because he does not need radiation therapy after this (Abdel Ghany et al. 2011). Chemotherapy has some side effects, these side effects will kill the normal cells that are close to cancer cells, thus, for that reason the scientist still working to develop some drugs with fewer side effects and without killing normal cells (Pöpperl et al. 2005).

Radiation therapy also one of the cancer treatments by using gamma rays, x-rays and charged particles (Wagner and Vorwerk 2011). This treatment almost using for endometrial cancer.

Targeted therapy is a small drug molecule that could enter inside the cancer cell and kill it, it also helps the immune system and stops the dividing of the cancer cells (Shaghayegh, Mahdi, and Ali 2010). The most common side effects for this treatment are hypertension, diarrhea, hyperpigmentation and headache (Nieder et al. 2010)

1.2. Lung Cancer and A549 Cell line

According to the statistic of the “American Cancer Society” lung cancer considered the most common cancer in each woman and man in the world. Where the percentage of human mortality in lung cancer rather other kinds of cancer is “154,050 deaths of 234,030 new cancer cases in 2018” (Mehta 2011)(“A.C. Society, Key Statistics for Lung Cancer, (2018) (Accessed 31 May 2018), <https://www.cancer.org/cancer/non-small-cell-lung-cancer/about/key-statistics.html>. No Title,” n.d.). The standard treatment for lung cancer is chemotherapy, it is still good therapy for this disease because it gives significant improve symptoms for a patient that has lung cancer (Eisenberg et al. 1998, Group 1995, ten Bokkel Huinink et al. 1999). Depending on the histological science, lung cancer cells classified as small cell lung cancer and non-small cell lung cancer (NSCLS). NSCLC is the most common type of lung cancer cell, and it is about 85% of all lung cancer cases (Xia et al. 2017).

The A549 lung cancer cell line was discovered in 1972 by removing and culturing of lung tissue for 58-year-old Caucasian male and it is classified through pulmonary epithelium cell type (Lieber et al. 1976).

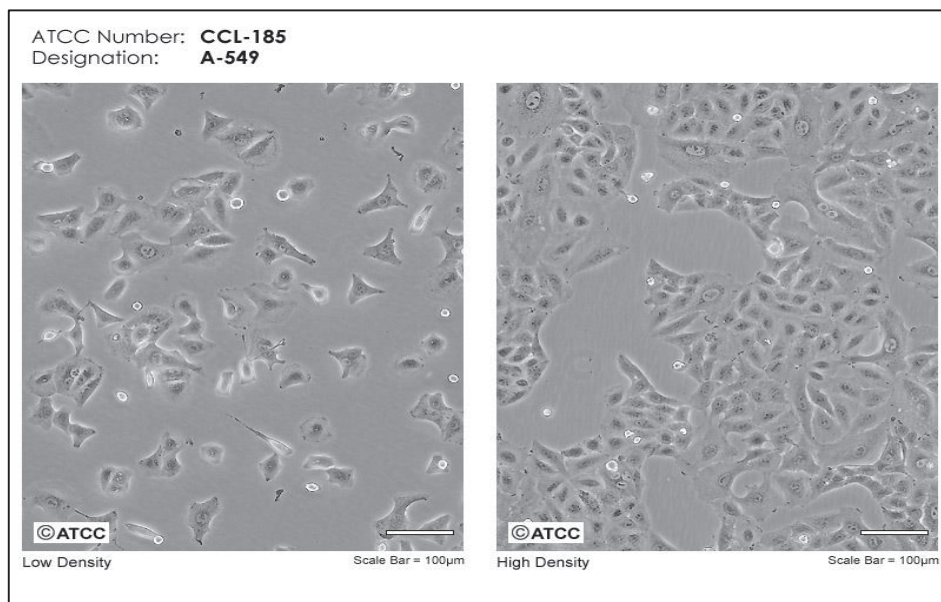


Figure 1. 2. Morphology of A549 cells monolayer
(Source: ATCC global biological materials resource)

1.3. Ovarian Cancer and HeLa Cell Line

Ovarian cancer classified as the sixth most common cancer in women worldwide and also the most common gynaecologic malignancy, it is caused around “125,000 deaths per year” (Armstrong et al. 2006, Ouyang et al. 2009, Yang et al. 2012, Gao et al. 2012). Ovarian cancer incidence has increased and it is continuing to grow to become the highest in Europe, Northern America and slightly in Japan (Rao and Pagidas 2010, Maciejczyk and Surowiak 2013). The procedure of early diagnosis for ovarian cancer is limited, therefore, the death rate of ovarian cancer did not change over decades (GERCEL-TAYLOR, Feitelson, and Taylor 2004, Luo et al. 2011). Additionally, around 80-90% of ovarian cancer is epithelial, while the nature of heterogenic for the tumor is conferring a weak prognosis and an increase in lethality (Chen et al. 2011, Mazumder et al. 2012, Solomon et al. 2008).

The HeLa cell was first found in 1951 and the name of these cells took from Henrietta Lacks, the woman that donated ovarian cancer cells, therefore, for this reason, it called by her name. She was born in Virginia 1920, and one day she felt a pain in her abdomen, after diagnosis, the result shows that she has cancer in her ovarian, and she died in 1951. The doctor took a sample from her cancer cells and do growing in the laboratory to do the research and develop a new drug for this type of cancer.

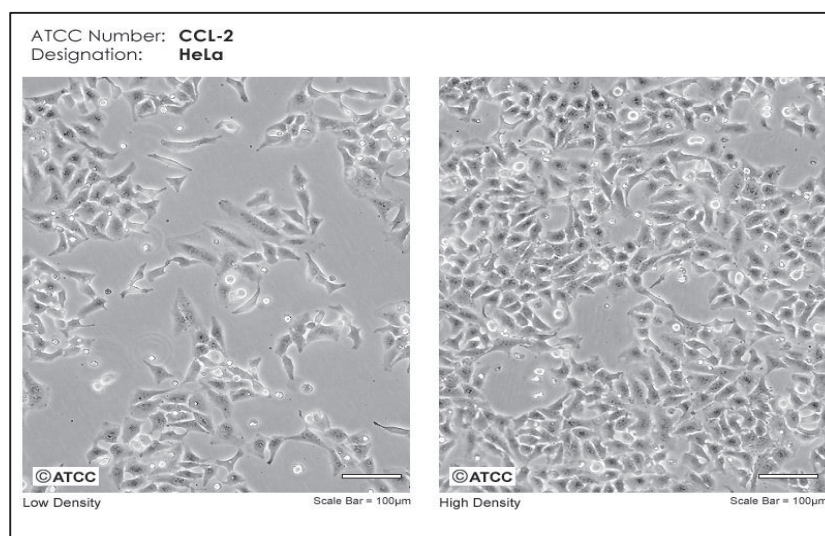


Figure 1. 3. Morphology of HeLa cells monolayer
(Source: ATCC global biological materials resource)

1.4. Cell Cycle

The first description of the cell cycle has been stated by Howard and Pele in 1951. The cell cycle has been defined that the interval between mitosis completion and the subsequent mitosis completion in one or both new cells (Gali-Muhtasib and Bakkar 2002). It is divided into four phases, mitosis (M) phase, G₁ phase, S phase and G₂ phase. The center of this process represents by the cyclin-dependent kinase (CDKs). This cyclin protein regulates cell progression during all the cycle stages.

Mitosis (M) phase contains four phases (prophase, metaphase, anaphase, and telophase). G₁ and G₂ phases describe the “gaps” between mitosis and DNA synthesis. The first stage is G₁ phase, which is the duration between mitosis and the starting of DNA synthesis. The second stage is S phase represents the interval of which of DNA is replicated. The third stage is G₂ phase represents the interval between consummation of DNA synthesis and mitosis (Alberts et al. 1986, Patt and Quastler 1963, Baserga 1968).

In addition, during the anti-cancer development for CDKs, two steps should be considered, the first one called direct strategy, which is done by inhibition the activity of CDKs, and the second one called the indirect strategy, which is done by targeting the majority of regulators activity for CDKs (Vermeulen, Bockstaele, and Berneman 2003).

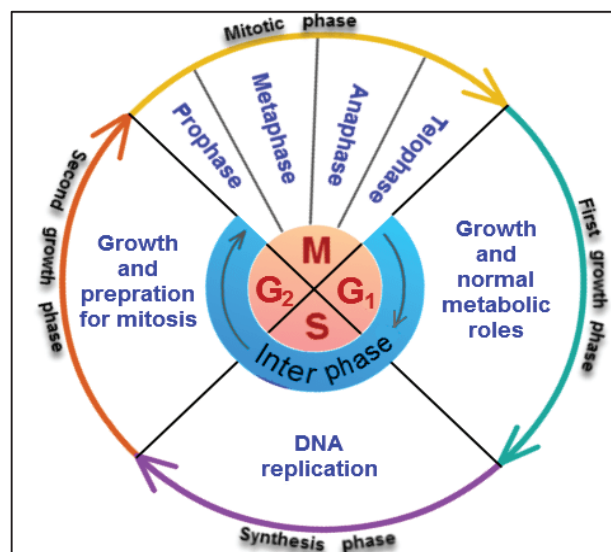


Figure 1. 4. The cell cycle stages

(Source: Tutor Vista, Biology)

1.5. Cell Viability Assay (MTT)

Any new drugs that have been produced by the scientist, the cell viability and cytotoxicity should be taken into account to check whether works for the cancer treatment or not. Thus, the MTT assay has been used for these examinations. MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) is the most common test for cell viability and cytotoxicity, which stands for Methyl-Thiazolyl-Tetrazolium (Mosmann 1983, Tully et al. 2000, Pagliacci et al. 1993).

The principle of (MTT) depends on reducing the tetrazolium salts in the formazan crystal. This process occurs by mitochondrial reductase, and the color will change from yellow to purple color, which means that all the viable cells reduce tetrazolium salts (Nikkhah et al. 1992, Van Meerloo, Kaspers, and Cloos 2011, Etxeberria, Mendarte, and Larregla 2011). Many authors prefer the (MTT) principle into two way (a) the reducing process of MTT occurs in mitochondria (b) use it as an indicator of mitochondria to evaluate the activity and functions (Saravanan et al. 2003, Van de Sandt, Rutten, and Koëter 1993, Du et al. 2010). The intensity of purple color depends on the number of viable cells, if it has a lot of viable cells, the color will be dark otherwise the color will be light, which means that the most of the cells are dead (Saravanan et al. 2003).

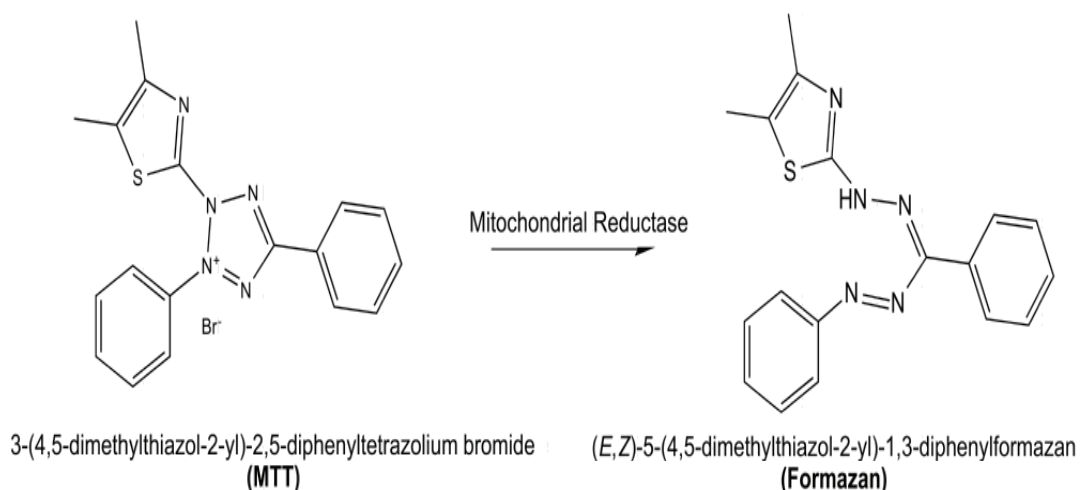


Figure 1. 5. Chemical structure of MTT and reduced to formazan

(Source: MyBioSource)

1.6. Apoptosis

Apoptosis is a process that the cells kill themselves by programmed pathways (Horvitz 1994, Vaux, Haecker, and Strasser 1994). Apoptosis has been used for the first time by Kerr, Wyllie, and Currie in 1972 for characterizing the morphological distinct of cell death (Kerr, Wyllie, and Currie 1972, Kerr 2002). One question could be raised, why the cells try to kill itself? two reasons answer this question, firstly, the cells may get the infection by Bacteria or viruses, secondly, it may be because morphological shape like a hand for mammals to have good hand shape as shown in Figure 1.6.

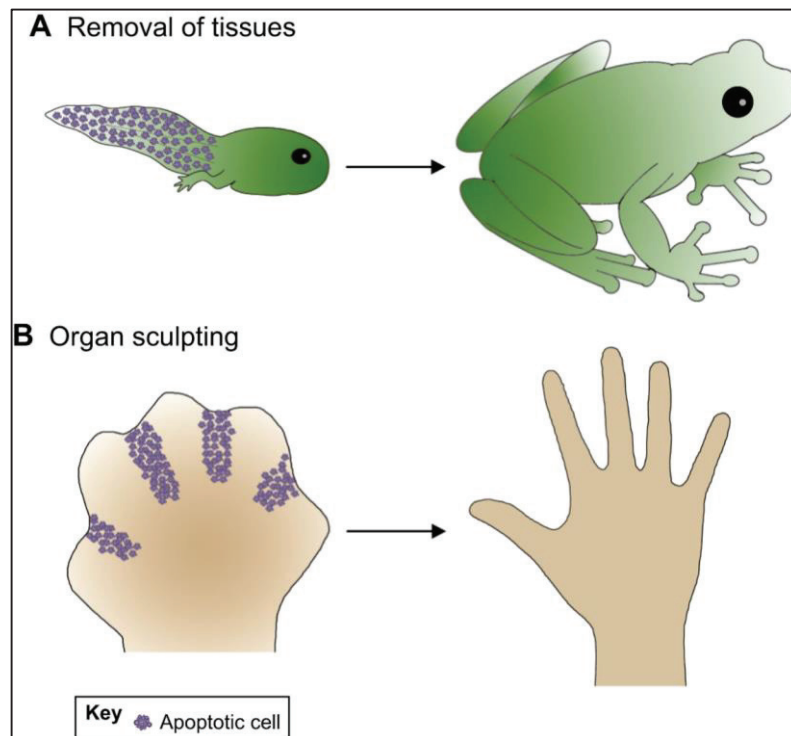


Figure 1. 6. Role of apoptosis in tissue and sculpting
(Source: The Company of Biologists)

As it is known the cancer cells grow very quickly, which may lead to a tumor and then cancer, therefore, there is a need for apoptosis to stop this spreading.

1.6.1. Mechanism of Apoptosis

First of all, all the cells contain a protein that is responsible for cell expiry (death) called Caspase-3 (cysteine-aspartic acid protease), if this protein has appeared in the cells, it will die. When this protein activated, the cell will acquire the proteases or nucleases, which means that the DNA and RNA will be destroyed (Martinvalet, Zhu, and Lieberman 2005, Hengartner 2000, McIlwain, Berger, and Mak 2015, Lakhani et al. 2006).

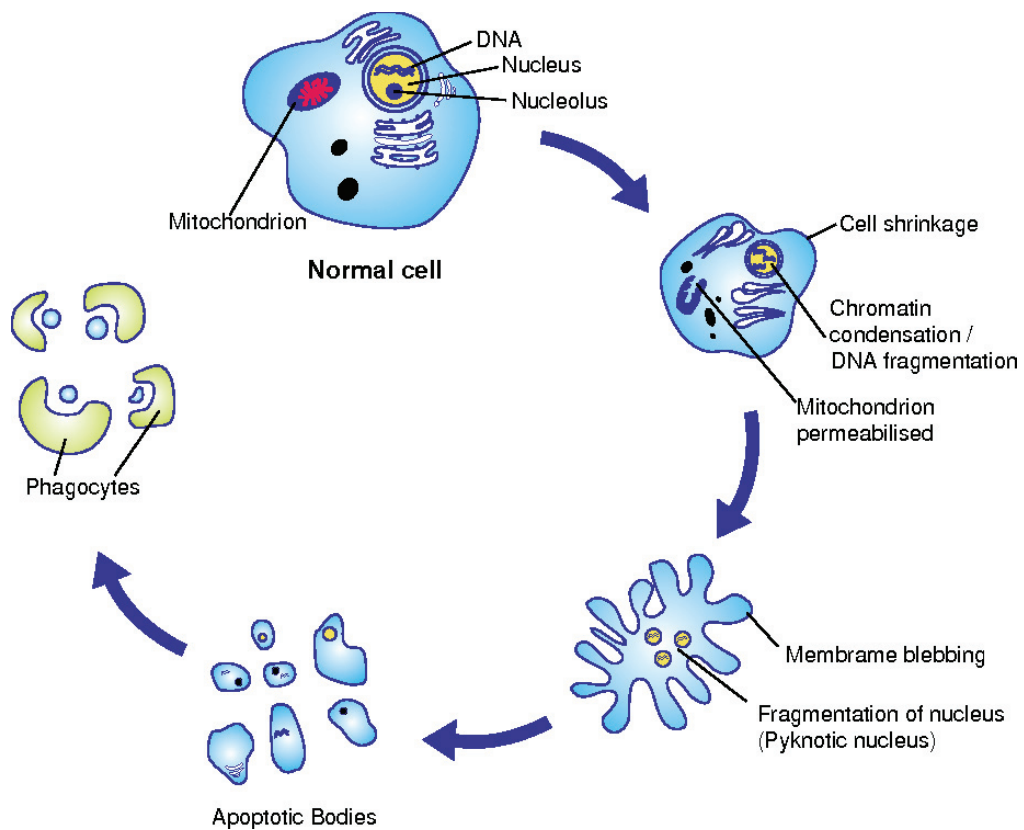


Figure 1. 7. Morphological feature of apoptosis cell

(Source: Marc, Declan, Colm and Thomas 2006)

The mechanism of apoptosis is divided into two ways:

1- Extrinsic Pathway: this pathway is happened by outside signal effect to the cell, when any weird body, for example, Bactria or virus touches the cell receptors. The cell has two receptors and these respecters are very close to each other, thus this process indicates the death-inducing signaling complex (DISC) (Wong 2011, Schneider and Tschopp 2000). DISC will activate the pro-Caspase-8 and this protein going to activate Caspase-8, Caspase-8 responsible for activating the Caspase-3 protein. Consequently, when the Caspase-3 is activated it will be deactivated of nuclease inhibitor, in this case, nuclease becomes active and has the ability to degradable the DNA of the cell (Ashkenazi and Dixit 1998,Hengartner 2000,Walczak and Krammer 2000).

2- Intrinsic Pathway: this pathway takes place inside the cell, the reason for this pathway is genetic damage. The target of this pathway is mitochondria and cytosol. As stated earlier the main reason for cell death is because of the DNA damage (Wong 2011). The DNA contains a protein like ATM and CHK, if these proteins are damaged, they will activate the P53, Thus, the cells will be in a very dangerous situation because it could give multiple proteins. After that, the P53 will activate (BAX) protein and this protein will create pores in mitochondria (Decaudin et al. 1998,Green and Kroemer 2004), Therefore, this pores will release cytochrome from mitochondria to the cytosol, which gives a direct signal that the cell will die, this is because cytochrome should present inside mitochondria. Cytochrome will bind with Apaf-1 protein and this protein will be converted to Caspase-9, when the Caspase-9 is activated the Caspase-3 will be active, and this protein will kill the cell(Tsujimoto et al. 1984, Reed 1997, Kroemer, Galluzzi, and Brenner 2007, Wong 2011).

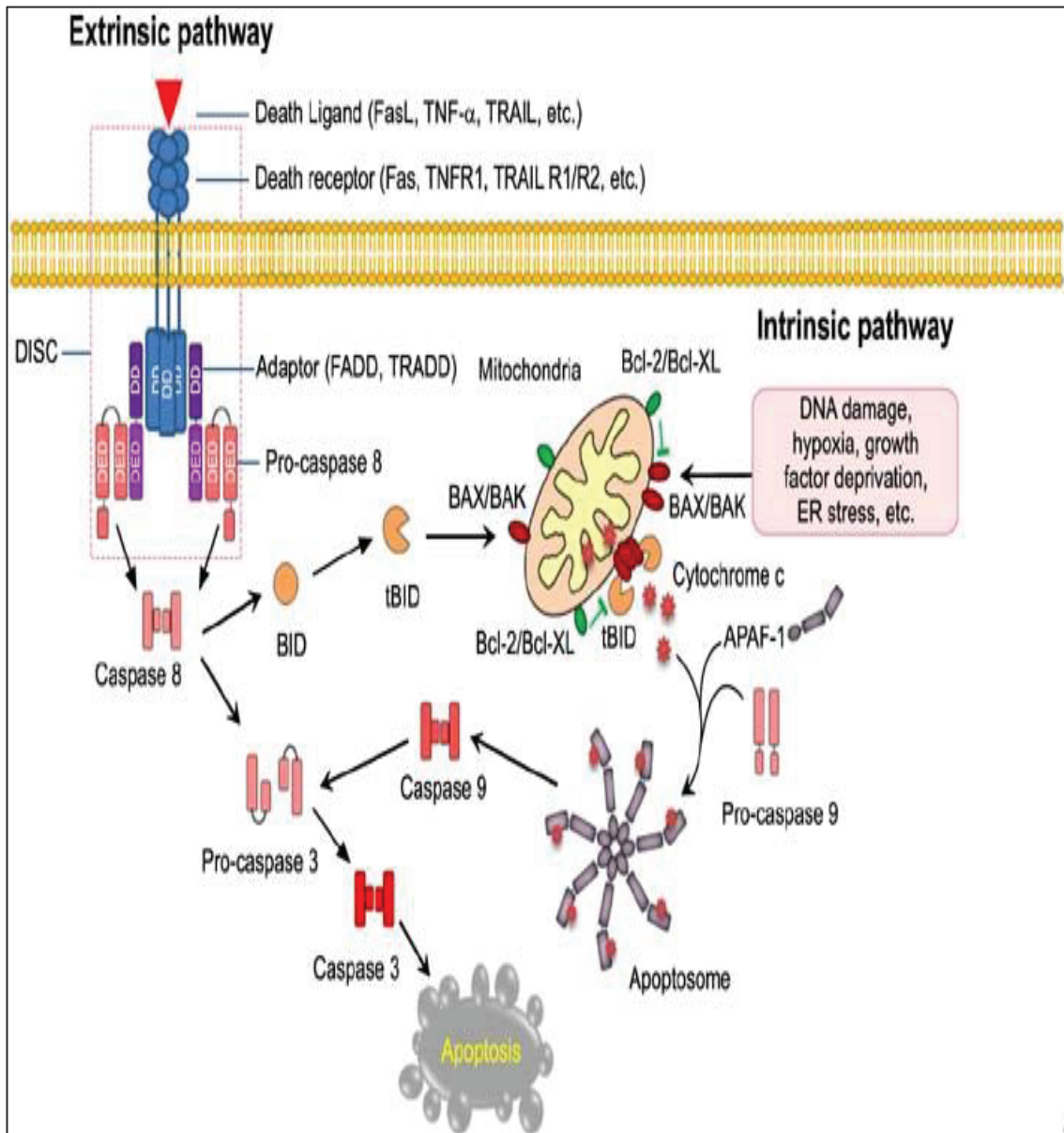


Figure 1. 8. Extrinsic and Intrinsic Apoptosis pathway
 (Source: Lorenzo, Catherine, Eugenia, Zahia and Guido 2008)

1.6.2. Difference between Apoptosis and Necrosis

Necrosis is a process for cellular death, which happens when the cells exposed to an external condition different from natural conditions (Hirsch et al. 1997). This leads to damage the internal cellular and rapid cellular tissue damage (Majno and Joris 2004). Thus, the main job of the cell is to save the cell balance and detect the hard conditions that are leading the cell to die.

The differences between Apoptosis and Necrosis based on three important points have been explained below:

- 1. The cause and presentation:** Necrosis is a pathological process; it is also harmful to the body. It happens when the cells exposed to the toxicant or hard conditions, which may increase the temperature or decrease the oxygen level (Kroemer and Dallaporta 1998). In addition, Necrosis may lead to damage to the wall or membrane of the cell and reduce its ability to maintain the normal function, which also leads to having imbalance cell (Kerr 1971). Apoptosis is a normal process which necessary and maybe a pathological or physiological process for the body. (Thompson 1995).
- 2. Structural Changes:** In necrosis, there are many integrity, which may lead to the swelling and disintegration for the small cells. However, in apoptosis, there are no disintegration or membrane but there is shrinkage, which may lead to the formation of bodies called apoptotic bodies. These bodies have been recognized by the immune system and destroyed effectually by the immune cells, thus is a well-coordinated process (Farber 1994).
- 3. Biochemical Reactions:** Necrosis is a bad process because it requires energy and it could occur randomly at any time. In Apoptosis it is an active process requires energy, but it occurs in a controllable way that makes it needs many proteins and enzymes in its operation (Ciechanover and Schwartz 1994).

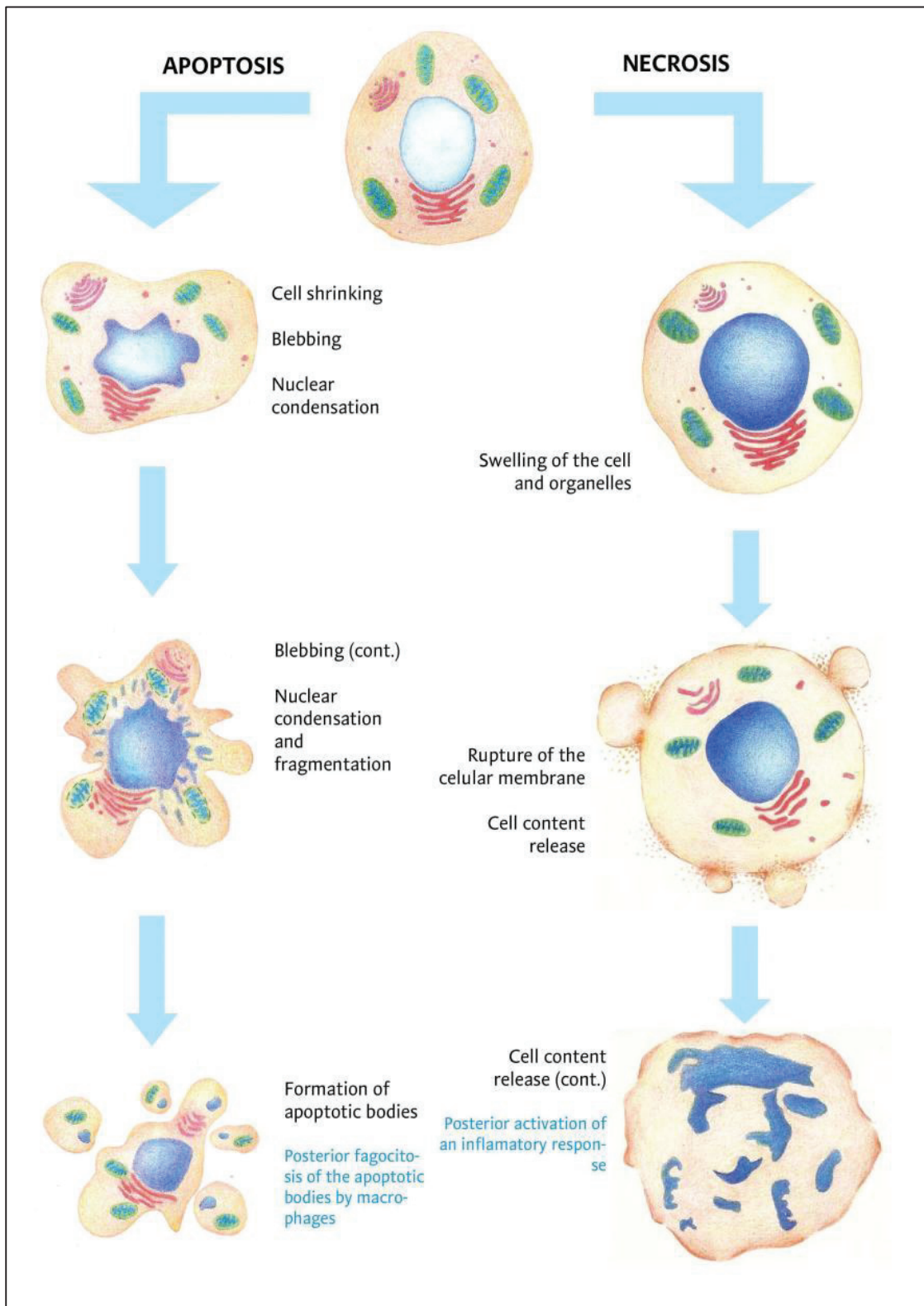


Figure 1. 9. Apoptosis and Necrosis

1.7. Wound Healing Assay (Scratch Assay)

Cell migration is a very important process for developing cells in many physiological disorders like cancer invasion, angiogenesis, metastasis and inflammatory (Klein-Soyer et al. 1994, Fischer, Stingl, and Kirkpatrick 1990). Also in the physiological repair process (Saga et al. 2003, Eccles, Box, and Court 2005, Lauffenburger and Horwitz 1996).

Furthermore, this migration is very important because it explains the parities of molecular mechanisms. Additionally, it also shows the screening of pharmaceutical compounds that are editing the migration of the cells. Thus, this process is considered as a positive chance to treat pathological cases (Decaestecker et al. 2007, Friedl, Hegerfeldt, and Tusch 2004).

Previously, people have used the plants and their preparations to accelerate the healing of the wound (Reuter et al. 2009). At that time they couldn't have any experience in medical treatments and have less knowledge about these compounds (Schmidt et al. 2009). Recently, there are many processes with the development of the new sciences to accelerate wound healing. The most common technique that has been used for this issue is wound healing assay or "scratch assay" for monolayer wound (Guan 2005). Wound healing assay is used in the studies of drug development for anticancer during a test the ability of drugs against cell migration, cell polarization, matrix remodeling and another process (Lu et al. 2004, Mc Henry et al. 2002).

The principle of wound healing assay based on a scratch on the mono cell layer in the flask piece. After this step, the open area that did will return to comeback by the migration of cells after sometimes, and the evaluation of this migration it doses by using bright-field imaging (Brooks and Schumacher 2001).

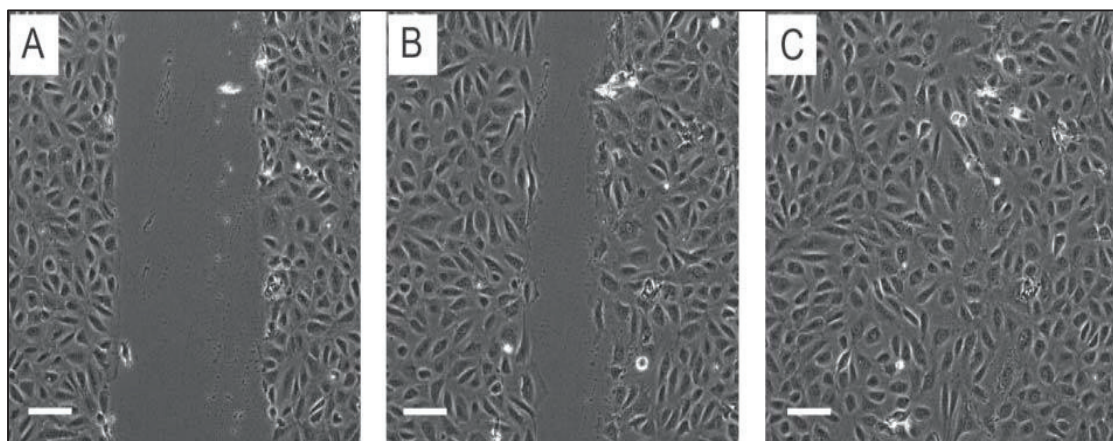


Figure 1. 10. Images of scratch assay under microscope

(Source: James, Judith, Fen, Miria, Jennifer, Katarzyna and Pina 2014)

1.8. Isoindole and Derivatives

Due to the development in the cancer treatment by chemotherapy and the malignancies. However, the anticancer agents need more studies to fill the gap of any new cancer (Bhatia 2017). Therefore, the chemist is always looking for the best compounds to have good efficacy and less toxicity to treat the cancer, and the heterocyclic compounds have proved that are good molecule for cancer (Cairns 1985, Demirbaş, Ugurluoğlu, and Demirbaş 2002).

Isoindole structure has been founded by the scientists last decades and it could be found easily in pharmaceutical and natural compounds (Liu et al. 2017). The Isoindoles is important because it has a 10π aromatic ring so it is hard to find it. Isoindoles are unstable because of the ortho-quinoid, so, for that reason, its main job is to limit the electrophilic aromatic substitution reactions. On the other hand, they have little examples of nucleophilic aromatic substitution reactions (Diana et al. 2011). Thus the uses of Isoindoles are very wide because of their efficient. Lenalidomide and pomalidomide are anticancer drugs which have effect on multiple myeloma, and contains isoindole structure.

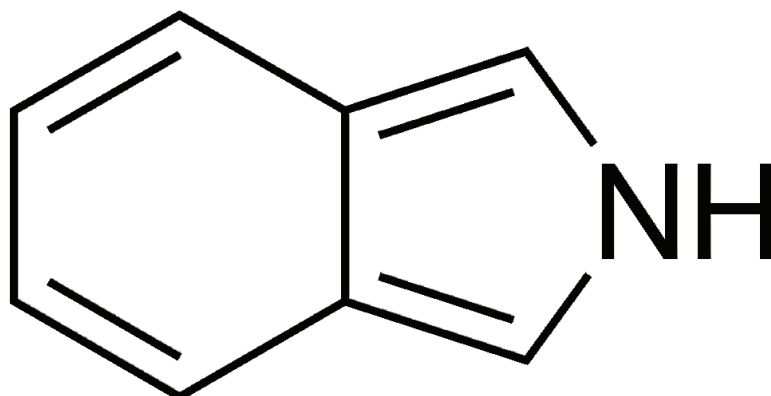


Figure 1. 11. Isoindole structure

(Source: ChemSpider Search and share chemistry)

Isoindole is one of the good heterocyclic compounds for cancer treatment (Joule and Mills 2008). The complete member of the Isoindole called isoindoline, while isoindoline and phthalimide was the oxidized form of Isoindole as shown in Figure 1.11 (Heugebaert, Roman, and Stevens 2012).

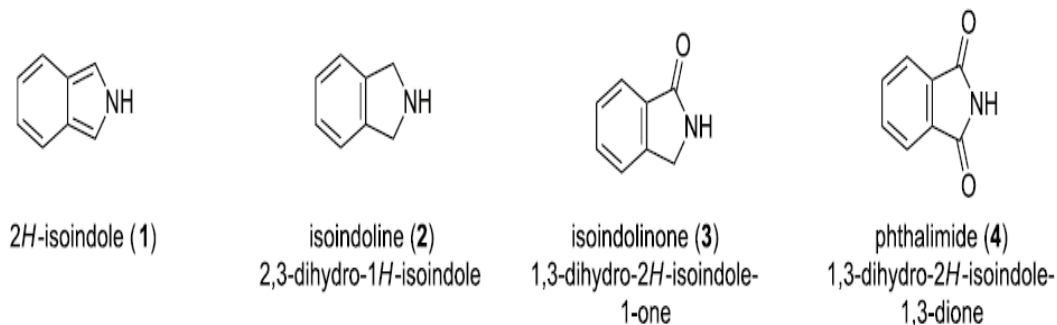


Figure 1. 12. Isoindole and related structures

(Source: Richa Kaur Bhatia 2017)

According to all features that have been stated above, the Isoindoles and derivatives have more applications especially in science (Hsu and Lin 2009). For example, they have been used in various fields of science because they contain BODIPY dyes highly fluorescent materials (Karolin et al. 1994). Also used for the biochemical process, laser dyes (Shah et al. 1990) and fluorescent sensors (Rurack et al. 2000).

The approval of Isoindoles derivatives by U.S (FDA) food and drug administration for treatment myeloma (Latif et al. 2012) makes it very interesting for the researchers to develop these derivatives for cancer treatment.

1.9. Isoindoles Derivatives as Anticancer Agents

Many studies in the literature have used the Isoindoles derivatives as anticancer because of its efficacy and less toxicity. Some of these studies have been explained below.

Diana et al. synthesized 1-acetyl-carboaldehyde-3-substituted Isoindole derivatives and the pLC₅₀ value, pTGI value and pGI₅₀ value have been measured to determine the efficacy of these compounds for antitumor. These compounds show that cytotoxic activity from micromolar to submicromolar concentration obtain good result against the cell line that investigated (Diana et al. 2011).

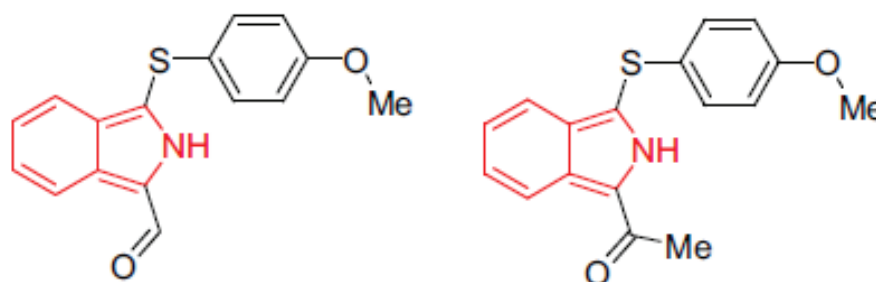


Figure 1. 13. 1-acetyl-carboaldehyde-3-substituted Isoindole derivatives
(Source: Diana et al, 2011)

Berger et al. prepared more than one compound from Isoindole alkanenitrile, α -aryl- α -thioether-alkyl and alkanecarboxylic acid methyl esters of tetrahydroisoquinoline and isoindoline. Then, these compounds have been evaluated during multidrug resistance (MDR) for human colon cancer cells in vitro. This compound has been evaluated in vivo against vincristine resistance murine P388 leukemia and also investigated for skin cancer in mice. After that, this investigation shows that these compounds produce good results in vitro and vivo (Berger et al. 1999). On the other hand, some of these compounds were very active when treated with Hela cell line while the IC₅₀ was very low between 3.16 μ M and 2.56 μ M (Muñoz et al. 2013).

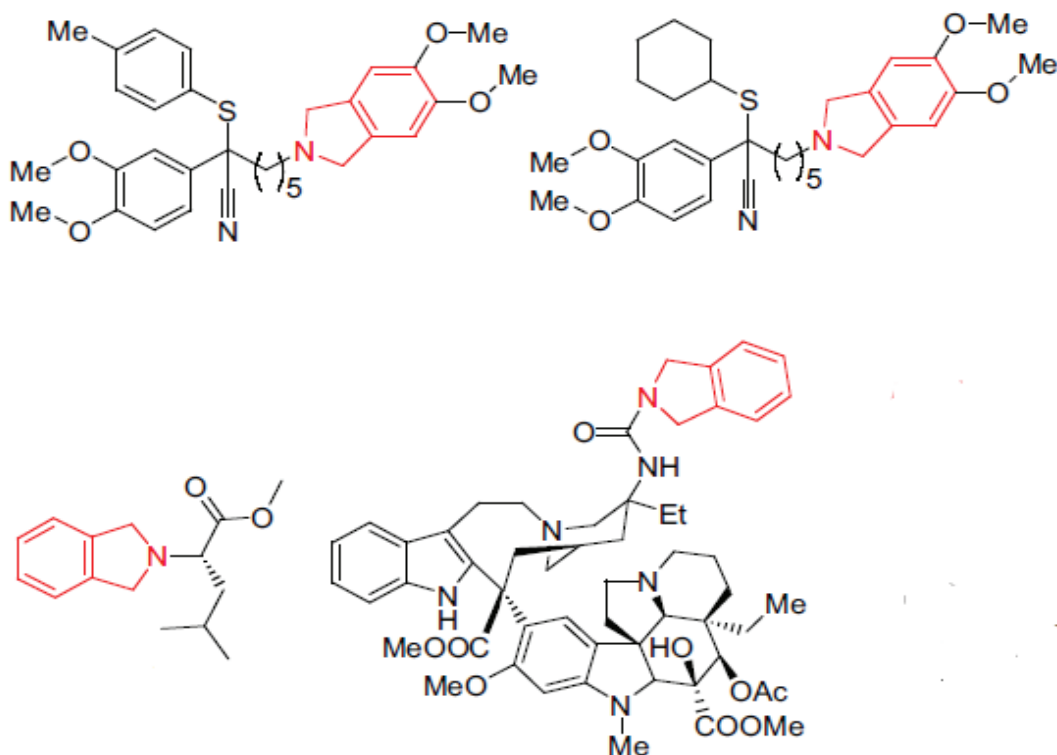


Figure 1. 14. Berger et al. Isoindoles derivatives
(Source: Berger et al. 1999)

Bhatia et al. prepared compounds from Isoindole and used them as anticancer. They synthesized “N-[4-[[2- (azepan-1-yl)ethyl]carbamoyl]phenyl]-1,3-dihydro-2Hisoindole- 2-carboxamide” and “N-[3-(isoindolin-2-yl)- 2-hydroxypropyl]aryl(alkyl) carboxamide derivatives” these two compounds have investigated for Hela cell during the measurement of IC_{50} and EC_{50} where the IC_{50} was $0.100\mu M$ and EC_{50} between $0.101\mu M$ and $10.0\mu M$ (Bhatia 2017).

In another research in 1998, Huang and Bobek synthesized some amino-deoxy 7-hexofuranosylpyrrolo[2,3-d] pyrimidines and evaluate their antitumour 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 $11.5\mu M$ (Huang and Bobek 1998).

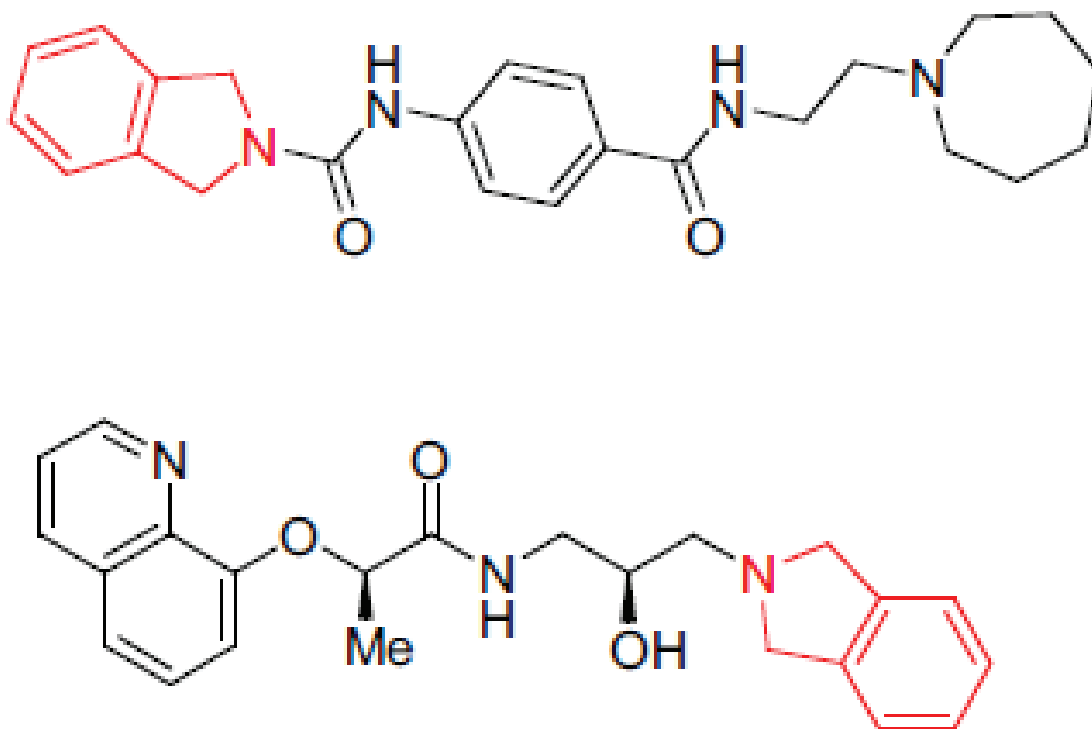


Figure 1. 15. Isoindoles derivatives

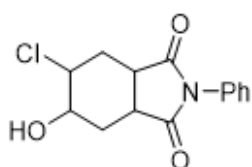
(Source: Duncan et al.2014)

Davidson et al. synthesized compounds from isoindoline and isoquinoline as an activator for apoptosis and caspase to treat the neoplasm and they obtained good results (Davidson et al. 2018). In addition, Kung and his team get a patent by using Isoindoles derivatives as PDK inhibitors and they have done several modifications to convert the Hsp90 inhibitor to all PDK isoforms with high specific (Kung et al. 2009).

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).

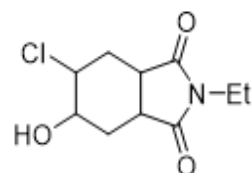
1.10. Chemical Structure of Drugs

Drugs were supplied in a powder form from Prof. Dr. Yunus Kara in Ataturk University Department of Chemistry, Organic Chemistry Laboratory.



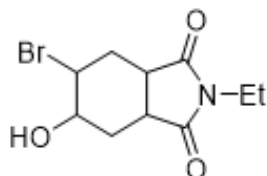
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61(2-8)



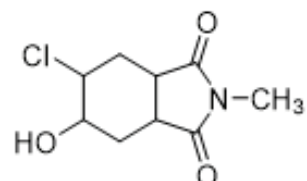
Molecular Weight: 231,68

62



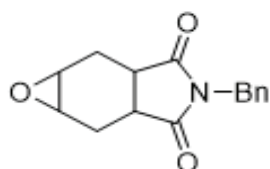
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63(4-12)



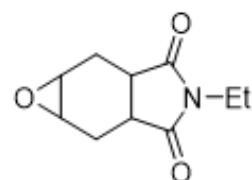
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40



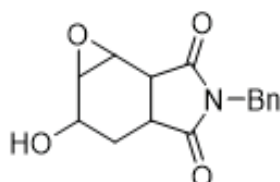
Molecular Weight: 257,29

70(23-33)



Molecular Weight: 195,22

71



Molecular Weight: 273,29

78(63-40)

1.11. Aim of the Study

The main aim of this study was to evaluate the biological activity of seven newly synthesized Isoindole derivatives. The anticancer activity of these compounds has been evaluated against HeLa (cervical carcinoma) and A549 (lung adenocarcinoma) cell lines.

The cytotoxic activity of these compounds has been determined by measuring cell viability via MTT assay on HeLa and A549 cell lines. Then, the apoptotic effect for the compounds that have the best IC_{50} on the indicated cells was investigated by using Annexin V-FITC. The cell cycle analysis of the cell lines in the presence of the most effective compounds has been performed using flow cytometry. In addition, in order to understand the effects of the compounds that have the best cytotoxicity on the migration of HeLa and A549 cells, a scratch assay was performed.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Cell Lines

The following human cancer cell lines were used for cervical carcinoma (HeLa) and lung adenocarcinoma (A549). The cell lines have been provided by IZTECH Biotechnology and Bioengineering Research and Application Center (BIYOMER).

2.1.2. Drugs

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

2.1.3. Culture Medias

The medium that is used for HeLa and A549 cell lines is RPMI 1640 (sigma) with 4500 mg Glucose/L, pyridoxine, NaHCO₃, and HCl, without L-glutamine. This medium used by adding 1% Penicillin-Streptomycin solution (Sigma), 10% Fetal Bovine Serum (Sigma) and 1% L-glutamine Solution (Sigma).

2.2. Methods

2.2.1. Cell Culture

Hela and A549 cell lines were cultured by using RPMI 1640 medium with 10% Fetal Bovine Serum (FBS), 1% Penicillin streptomycin (P/S) and 1% L-glutamine by using atmosphere condition in 5% CO₂ incubator at 37C° (Audus et al. 2002).

2.2.2. Thawing Out Cells

The frozen cells were taken from -80 C° refrigerator. Then, the cells were put in a water bath 37C° and wait until all frozen cells thawed. After thawing, all the cells have been taken carefully to the cabin and put them in a 75cm Flask, which contains 12-13 mL fresh RPMI 1640 that is already prepared during the thawing process. The cells have been placed in 5% CO₂ incubator at 37 C° for one night to grow and get more.

2.2.3. Passaging Cells

After the thawing process and waiting for one night, the cells are taken from the incubator and removed from the medium. 2-5 ml from PBS has been added to the flask in order to wash the cells. After that, 1-3 ml from sterile Trypsin is added to the flask and the cells have placed in 5% CO₂ incubator at 37 C° for 2-3 minutes to separate the cells from the flask. Later, 6-9 ml fresh RPMI 1640 has been added to the medium to inhibit Trypsin and taken all solution in 15 ml falcon and place them in the centrifuge at 800 rpm for 5 minutes. After 5 minutes the cells are taken from the centrifuge. By using mechanical pipet supernatant, the cells are removed and 2 ml fresh RPMI 1640 has been added to the falcon, after good re-homogenize, the cells with medium transported to the 75 cm flask contain fresh medium and put in 5% CO₂ incubator at 37 C°. All solutions and medium that used was put in a water bath at 37 C° and then used, the passage should do every 2-3 day.

2.2.4. Cells Counting

A 100 μL from the cells were taken and put it in a 1.5 ml microfuge tube, 900 μL of 0.4 % trypan blue solution (Sigma) was added to the cell suspension. After well mixed, a small amount from trypan blue-cell suspension was taken and put in a chamber on the hemocytometer. After this step hemocytometer was put under the microscope to count cells. The cells with blue color are non-viable cells and the viable cells remain opaque. The plate show under the microscope on four square in each corner and just the cells inside these squares will be counting. The following equation using for calculation the number of cells.

$$\text{Cells per mL} = \text{Average count of cells per square} * \text{Dilution factor} * 10^4$$

2.2.5. Freezing Cells

When a passage is doing some of the cells were taken for freezing. DMSO (Sigma) was added by 5 % concentration for these cells and re-homogenize well and transported 1 mL of cells to cryogenic vials and stored at -80 C° .

2.2.6. Cell Viability Assay (MTT Assay)

Hela and A549 cell line 95 μL were cultured in 96 well plates flat bottom (SPL Life Sciences) at 10^{-4} cell density per well with initial 5000 cell/ 100 μL complete RPMI 1640 medium and incubated in 5% CO_2 at 37 C° for 24 h to detached cells. All seven compounds were dissolved in sterile dimethyl sulfoxide DMSO and prepared 7 concentrations for each compound 20, 50, 100, 200, 500, 1000, 2000 μM . After adding all compounds cells put in the incubator in 5 % CO_2 at 37 C° for 24h,48h, and 72h to determine the cytotoxicity of this compound. MTT (Sigma) was dissolved in phosphate-buffered saline (PBS) 5mg/mL and filtered. 10 μL from MTT was added to each well and

cells were put in the incubator for 3.5 h. After incubation cells took from incubator and centrifuge for 10 min at 1800 rpm. The supernatant was removed and 100 μ L from DMSO was added to each well and cells were put on a plate shaker for 15 min to dissolve all formazan crystals. Absorbance was determined at 540 nm (Fogh and Trempe 1975, T. R. Chen et al. 1987).

2.2.7. Apoptosis Analysis

Apoptosis was detected by using compounds that give good IC₅₀ for HeLa and A549 cell lines by using an Annexin V-FITC detection kit (Bio Legend). A 6- well plate (SPL Sciences) used in this analysis and 1980 μ L from cell suspension with density 5×10^5 have been placed in each well and incubated in 5% CO₂ at 37 C° for 24 h to attachment cells. After 24 h, 20 μ L from compounds that had to choose and prepare four concentrations for each compound 20,100,250,500 μ M, after adding compounds cells incubated in 5% CO₂ at 37 C° for 48 h. Next, the incubation trypsin was added to the separated cells and centrifuge for 5 minutes at 800 rpm. Afterward, centrifuge supernatant was ignored and cells were washed with 3 mL from phosphate-buffered saline (PBS) and centrifuge again for 5 min at 800 rpm. After 5 min of centrifuging supernatant was ignored and 100 μ L from the binding buffer, 1 μ L from Annexin V-FITC, 2.5 μ L from PI were added to each tube and tubes were incubated for 15 min at room temperature in the dark. After incubation 400 μ L from binding buffer was added to each tube and all tubes analyzed by Flow Cytometry (BD FACSCanto).

2.2.8. Scratch Assay

Cell migration has been detected by using Scratch assay, glass lamellas were washed with ethanol (Sigma) and put in the autoclave for one night. After 24 h, it has been placed in 6 well plates, after putting one glass lamellae in each well HeLa cells and A549 cells were seeded 3×10^5 cells/well with a volume 1980 μ l and incubated in 5% CO₂ at 37 C° for 24 h. Next, the period of incubation is finished, the cells were scratched on each lamella with parallel lines by using a sterile 100 μ l plastic pipette tip. The old

medium was removed and changed by fresh one after cells washed by phosphate-buffered saline (PBS). Drugs were added to each well containing 1 % DMSO as a final concentration and one well leaved without drug as a control. Cells were incubated in 5% CO₂ at 37 C° for 24h and 48h. the experiments were performed at least one duplicate (Fronza et al. 2009, (Gough et al. 2011).

2.2.9. Cell Cycle Analysis

The cell cycle was detected in HeLa and A549 cell lines of compounds that gave good IC₅₀ by using propidium iodide staining (PI). The A549 and HeLa were seeded in 6 well plates at density 5×10^5 in 1980 μ l medium, and incubated in 5% CO₂ at 37 C° for 24 h. after 24 h compounds selected prepared by dissolved in DMSO and diluted for different concentrations 500,250,100,20 μ M and 20 μ l from each compound was added to cells and cells were incubated in 5% CO₂ at 37 C° for 48 h. Next, the period of incubation, the cells were washed by PBS 1 ml and suspension collected, 250 μ l from trypsin was added to cells and centrifuged for 5 minutes at 800 rpm. After centrifuge suspension was collected. After the collection process, 1 ml from cold PBS and 4 ml from -20 C° ethanol (Merck) were added by slowly to suspension. After these additions cells were incubated at – 20 C° for at least 24 h. after 24 h cells were centrifuged for 10 minutes at 1200 rpm 4 C°. cells were washed with PBS by adding 5 ml PBS to cells and centrifuged for 10 min at 1200 rpm. After centrifuge supernatant ignored and 200 μ l from phosphate buffer including 0.1% Triton X-100 was added. After this addition 20 μ l from RNase A (200 μ g/ml) was added to the cells suspension and cells incubated in 5% CO₂ at 37 C° for 30 minutes. After 30 minutes 20 μ l from propidium iodide (PI) was added to the cells and incubated at room temperature for 15 minutes. After the time of incubation is finished cell cycle was determined by Flow Cytometry (BD FACSCanto).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cell Viability

The cytotoxic effect of the newly synthesized isoindole compounds was determined on HeLa (cervical carcinoma) and A549 (lung adenocarcinoma) cell lines, IC_{50} was calculated for each compound by using Graphed prism 6 software program for at least three triplicate experiment.

The results show that the best cytotoxic one of these seven compounds is 78 because it has good IC_{50} value compared to others during all the time periods of 24, 48 and 72 h. The IC_{50} value for compound 78 was 251.4 μ M for A549 cell line and 206.9 μ M for HeLa cell line for 48 as shown in Figures 3.3 and 3.6. Compound 71 was also a reasonably effective compound after 78 on the cell lines compared to other compounds. The IC_{50} for compound 71 was 289 μ M for A549 cell line and 296.4 μ M for HeLa cell line for 48 h incubation period of time as shown in Figure 3.3 and 3.6. The compound 70 had a minimal cytotoxic effect on both cell lines. On the other hand, the other compounds did not show any significant cytotoxic effect on both cell lines. The effect of compound 78 might be related to its structure and therefore play an important role in the cytotoxicity of the cell lines.

The structure of the compounds 70, 71 and 78 were investigated and the similarities and differences were analyzed. The similarities were the benzene group between compound 78 and 71 and the hydroxyl group between compound 78 and 70. These results indicate that the benzene group and hydroxyl group together may show better effect on cytotoxicity on the cell lines among Isoindole derivatives.

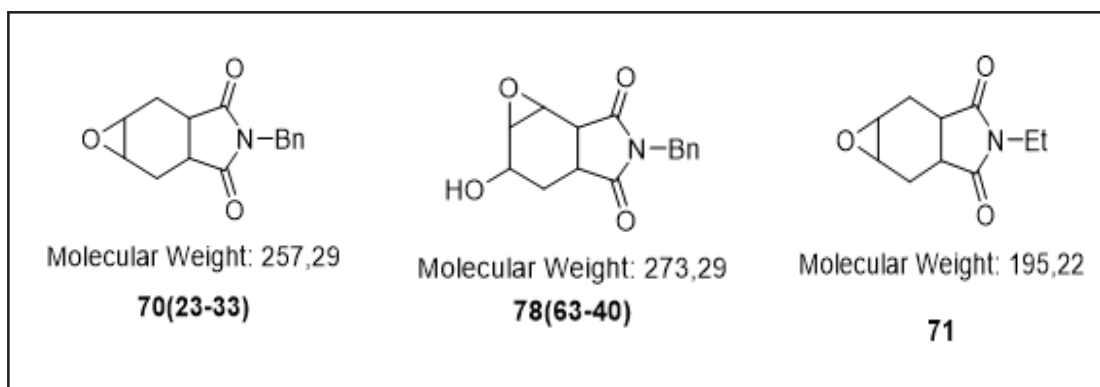


Figure 3. 1. Chemical structure of compounds 70,71 and 78.

Köse and his group worker synthesized new Isoindole derivatives 8a and 8b. They evaluated the biological activity of those compounds on A549 and MCF-7 cell lines. They found a metabolic activity as 39% for A549 cell line and 55% for MCF-7 using 50 μM concentration of 8a compound after 24 h incubation. Using the same concentration for compound 8b, the observed metabolic activity was 53% for A549 and 64% for MCF-7 cell lines after 24 h. The cytotoxicity of both compounds increased as a time-dependent manner up 72 h (Köse et al. 2017).

H.El-Diwan et al synthesized 2-arylbenzimidazole-4-carboxamides and tested this compound with 60 cell lines including HeLa and A549 in different five concentrations at 48 h incubation period-time. The results show that the best concentration was 100 μM and this compound was very potent. This effect was attributed to the structure of the compound due to the substitution of position 5 by a diazo group connected to the five-membered ring which resulted in an enhanced cytotoxic activity (El-Naem et al. 2003).

Bhatia et al. prepared compounds from Isoindole and used them as anticancer. They synthesized “N-[4-[[2- (azepan-1-yl)ethyl]carbonyl]phenyl]-1,3-dihydro-2Hisoindole- 2-carboxamide” and “N-[3-(isoindolin-2-yl)- 2-hydroxypropyl]aryl(alkyl) carboxamide derivatives” these two compounds have investigated for Hela cell during the measurement of IC_{50} and EC_{50} where the IC_{50} was 0.100 μM and EC_{50} between 0.101 μM and 10.0 μM (Bhatia 2017).

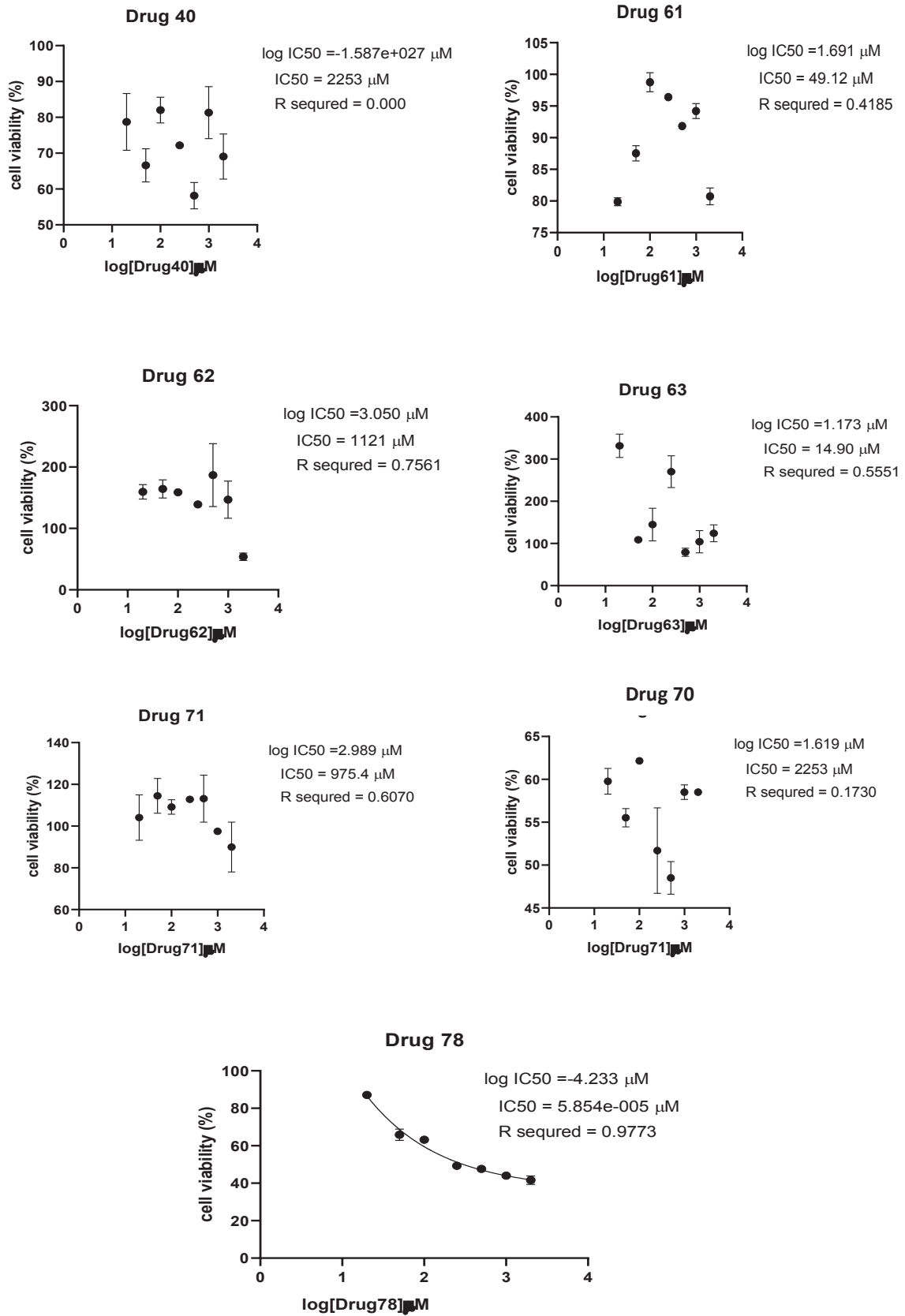


Figure 3. 2. Cell viability results on A549 cells for 24 h incubation period.

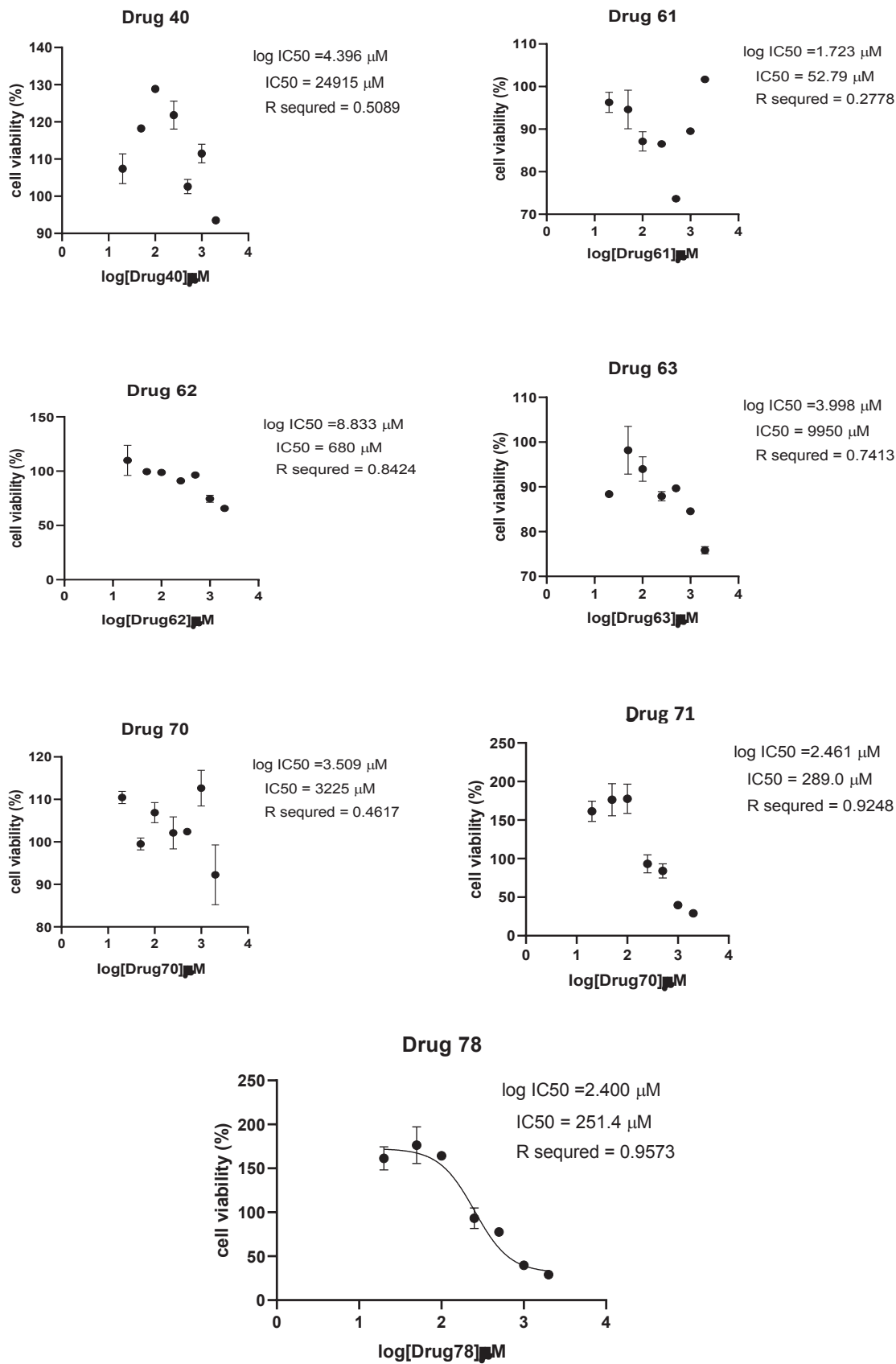


Figure 3. 3. Cell viability results on A549 cells for 48 h incubation period.

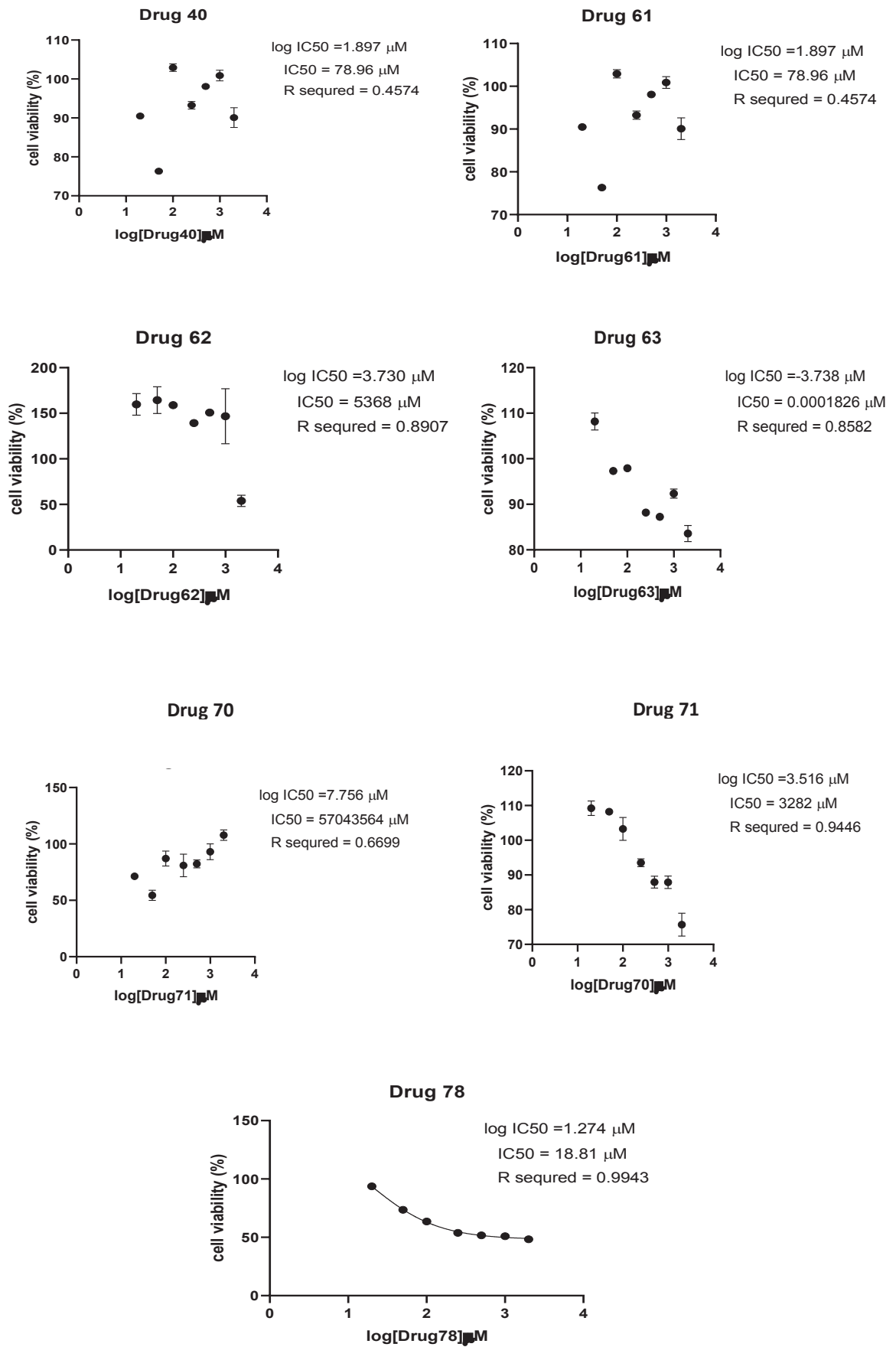


Figure 3. 4. Cell viability results on A549 cells for 72 h incubation period.

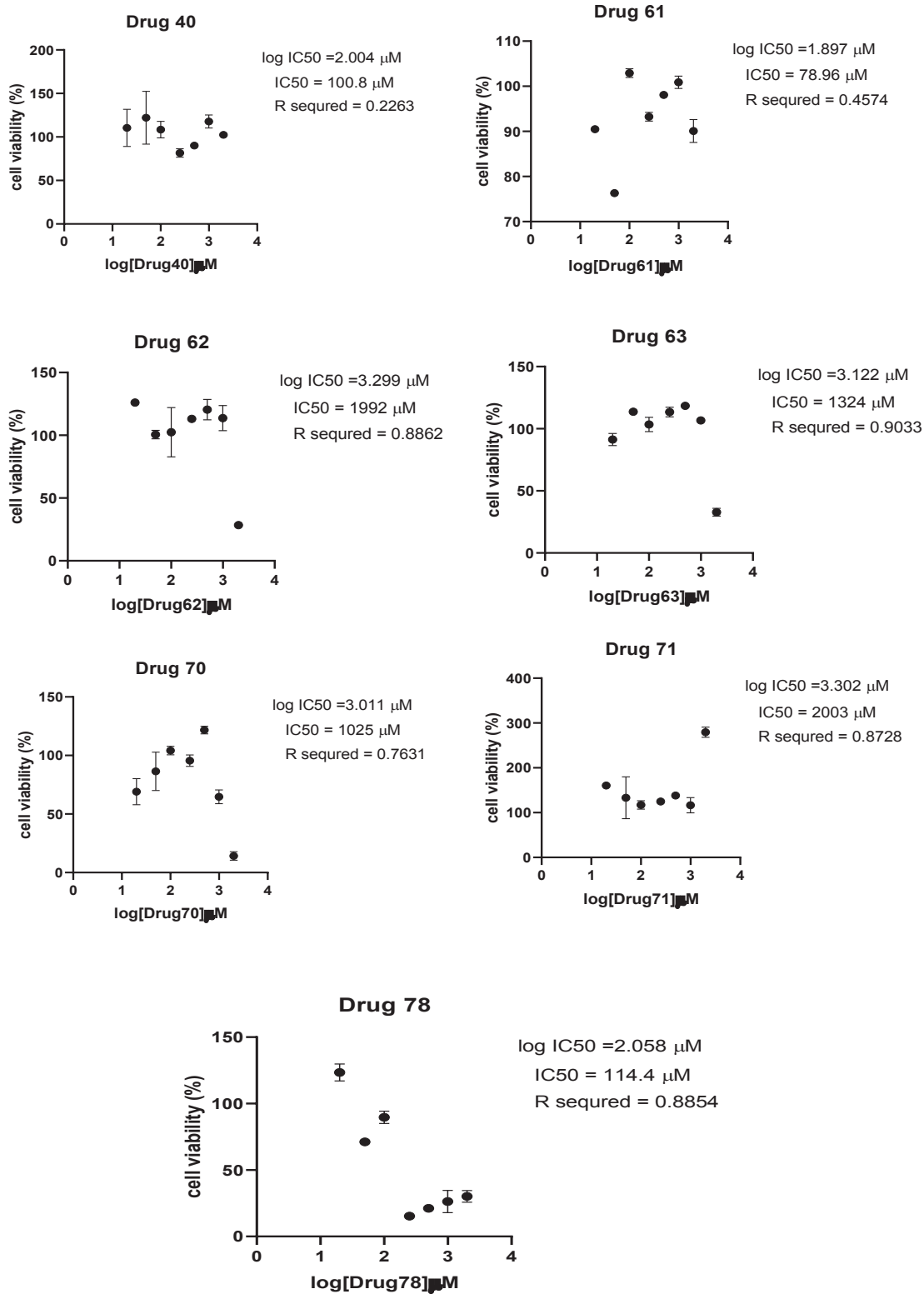


Figure 3. 5. Cell viability results on HeLa cells for 24 h incubation period.

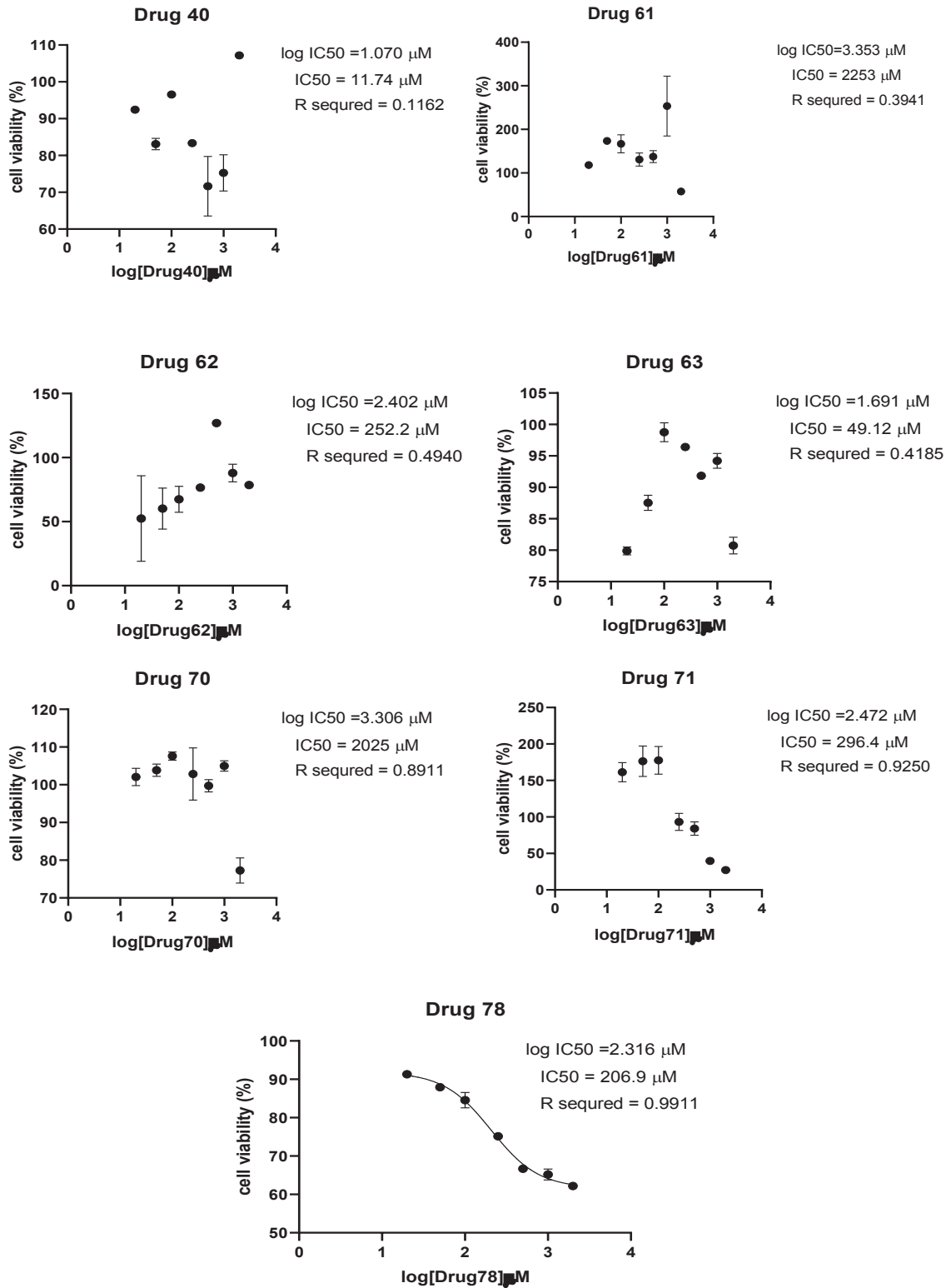


Figure 3. 6. Cell viability results on HeLa cells for 48 h incubation period.

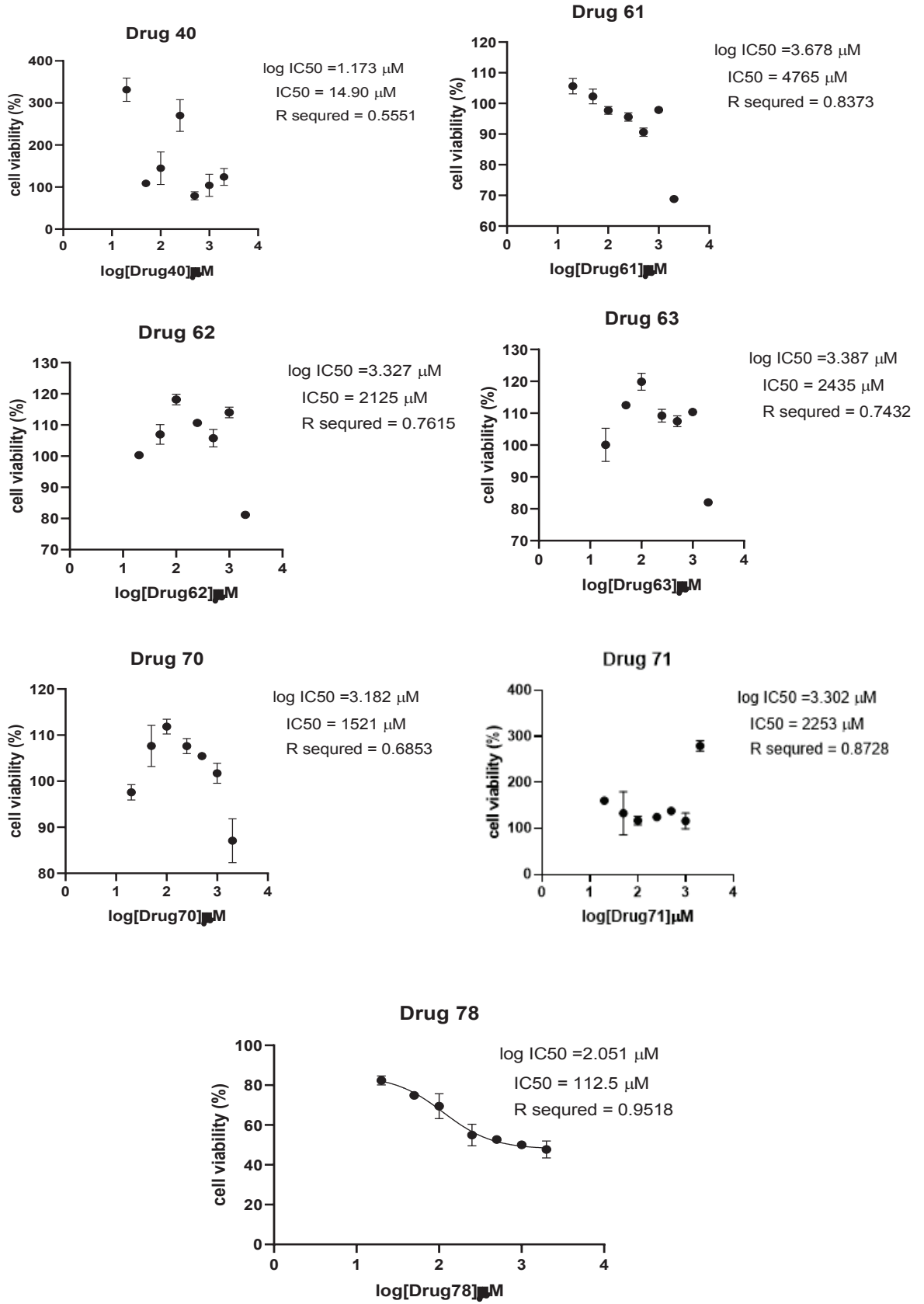


Figure 3. 7. Cell viability results on HeLa cells for 72 h incubation period.

3.2. Apoptosis Analysis

Apoptosis, which is the process of programmed cell death in a normal cell growth, is characterized by exact morphologic properties. One of the earliest features is the loss of plasma membrane asymmetry. As in normal cells the membrane phospholipid phosphatidylserine (PS) is located in the inner side of the plasma membrane, in apoptotic cells it is translocated to the outer side of the plasma membrane.

Annexin V, which is a Ca^{2+} dependent phospholipid-binding protein, has a great affinity for phospholipid phosphatidylserine (PS). When Annexin V is conjugated to fluorescein is thiocyanate (FITC), which is a fluorochrome, and Annexin V-FITC serves as a sensitive probe for flow cytometry analysis of apoptotic cells.

Apoptosis, programmed cell death effect has been investigated for the compound that gave good IC_{50} in the cell viability assay and these compounds were 78 and 71. Annexin V-FITC was used to investigate the effect of these compounds on HeLa (cervical carcinoma) and A549 (lung adenocarcinoma) cell lines.

For the A549 cell line, the results show that the compound 78 gave the best results and the cells going to death when increasing the concentration of this compound as shown in Figure 3.8. The rate was 23.8% for necrosis, 27.17% for late apoptosis, 0.4% for early apoptosis and 40.7% for live cells. All these numbers goes to decrease when decreasing the concentration of the compound, except live cells. This indicates that the compound 78 was very effective and gave significant results for A549 cancer cell line.

The second compound that chosen to investigate the apoptotic effect for the A549 cell line is compound 71. The results of compound 71 was not significant like compound 78. The rate was 2.13% for necrosis, 10.53% for late apoptosis, 4.83% for early apoptosis and 82.57% for live cells as shown in the Figure 3.9.

On the other hand, when investigating the apoptotic effect of these two compounds on the Hela cell line, the results of compound 78 were very significant according to these numbers as shown in Figure 3.10. The rate was 20.27% for necrosis, 14.63% for late apoptosis, 1.23% for early apoptosis and 64.37% for live cells. Also, these numbers decrease when decreasing the concentration of compound except for live cells. Compound 71 results was not significant with the HeLa cell line as shown in Figure 3.11. The results were 6.27% for necrosis, 6.8% for late apoptosis, 4.63% for early apoptosis and 83% for live cells.

Diana et al. investigated the Caspase-3 activation programmed cell death for compound (3-Methoxy-5H-isoindolo[2,1-a] quinoxalin-6-one) on Jurkat cell line. The results show that the compound has had the ability to induce the activation of Caspase-3 and it activates Caspase-9. This activation leads the cell to the apoptosis (Diana et al. 2008).

In another study, two new compounds of Isoindole 5a and 5g have been synthesized and evaluated based on the apoptotic effect on HepG2 cell line (human liver) and MCF7 cell line (breast cancer). The percentage of apoptotic cells have increased with HepG2 cell line when treated with compound 5a, while the percentage was at 1.65% with untreated cells, and 21.25% with compound 5a. MCF7 cell line treated with compound 5g and also noted that it has an increase in apoptotic rate from 1.92% untreated cells to 18.26% treated cells. This indicates that these two compounds are very potent for apoptotic programmed cell death (Philoppes and Lamie 2019).

Zhao et al. 2012 found that the effect of compound 2-(3-(2-Oxo-2-p-tolyethylthio)-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-4-yl) isoindoline-1,3-dione for apoptotic effect of HepG2 cell line was dose-dependent (Zhao et al. 2012).

Davidson et al. synthesized compounds from isoindoline and isoquinoline as an activator for apoptosis and caspase to treat the neoplasm and they obtained good results (Davidson et al. 2018). In addition, Kung and his team get a patent by using Isoindoles derivatives as PDK inhibitors and they have done several modifications to convert the Hsp90 inhibitor to all PDK isoforms with high specific (Kung et al. 2009).

In 2012, 34 compounds which have diindolylmethanes were synthesized and their biological activities 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 inhibition of HeLa cells and also 7d caused G1 phase arrest on HeLa cells (Sharma et al. 2012).

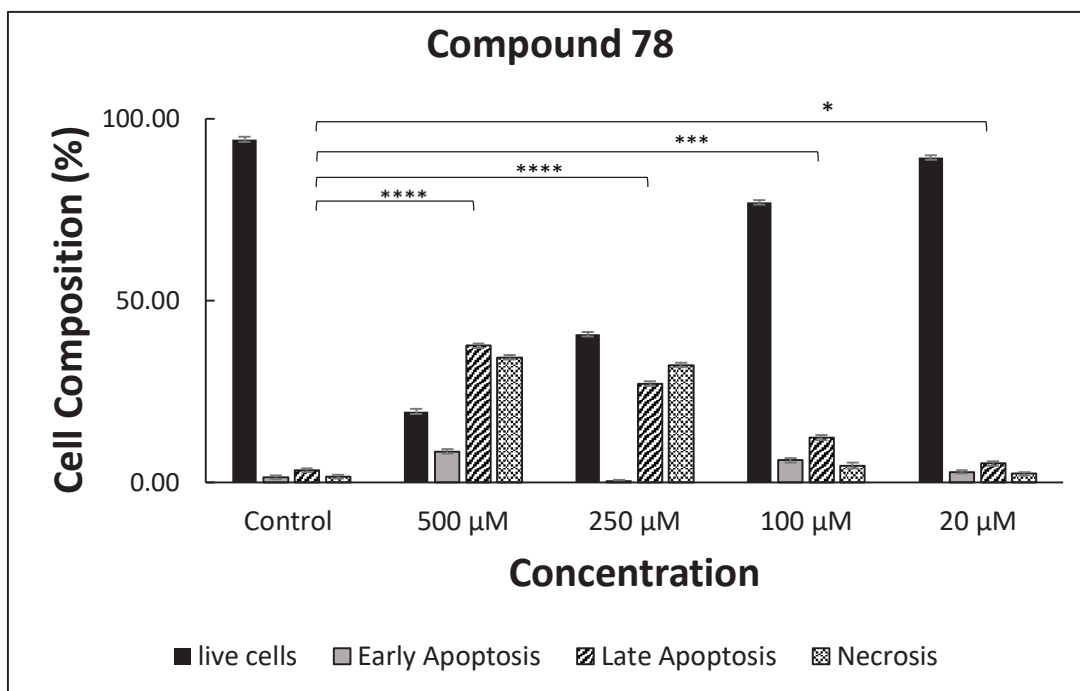


Figure 3. 8. Apoptosis analysis results for compound 78 at IC₅₀ dose in 48 h incubation period on A549 cells. Bars indicate SD, ****p<0.0001, ***p<0.001, *p<0.05

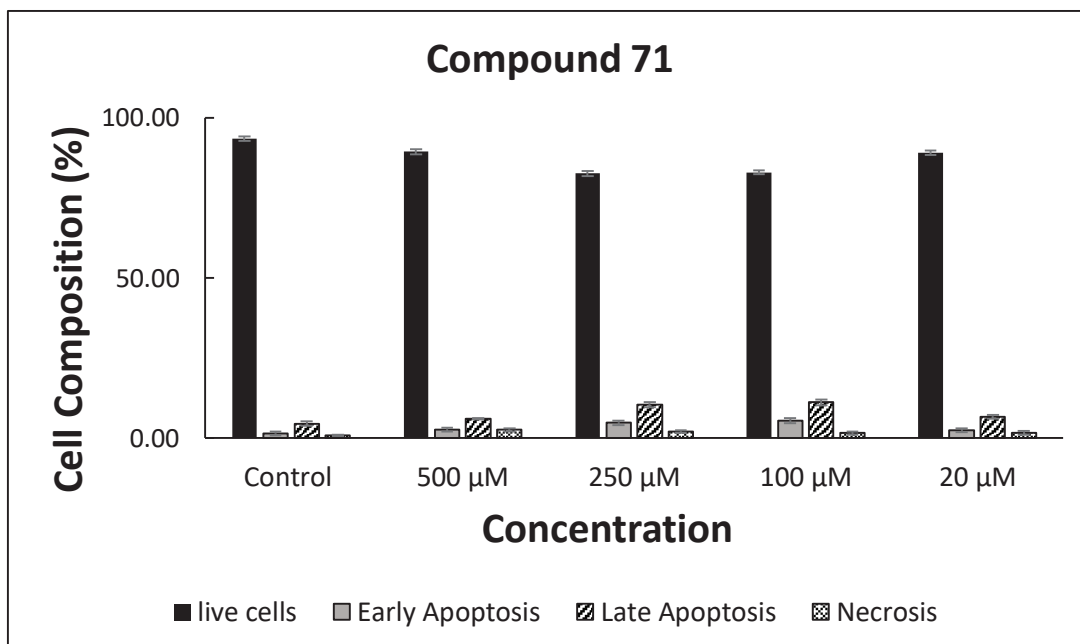


Figure 3. 9. Apoptosis analysis results for compound 71 at IC₅₀ dose in 48 h incubation period on A549 cells.

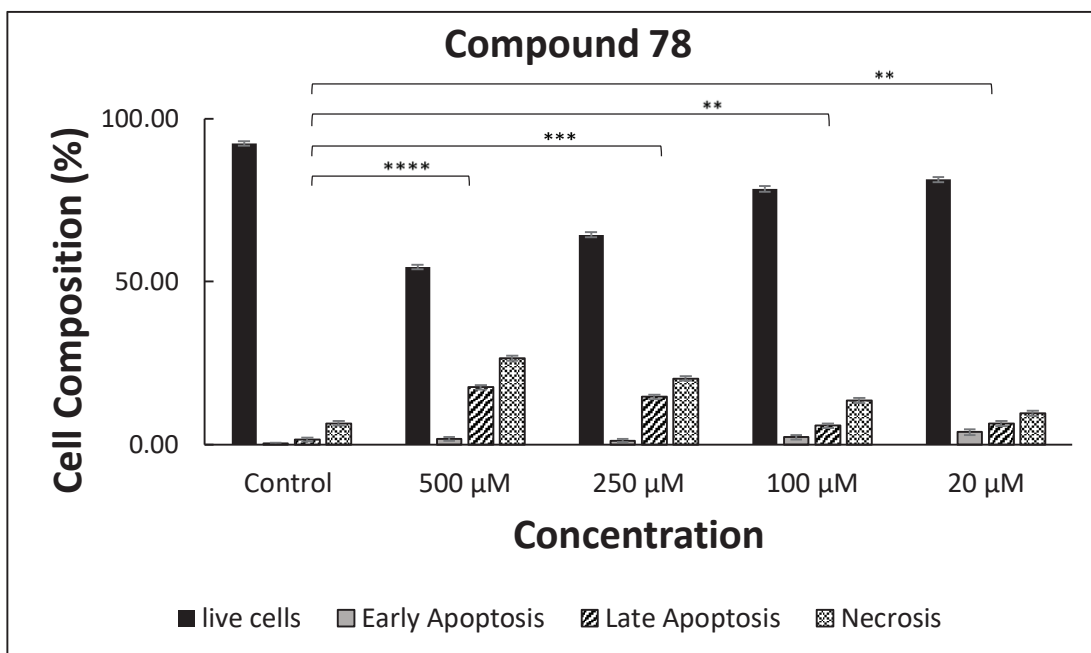


Figure 3.10. Apoptosis analysis results for compound 78 at IC₅₀ dose in 48 h incubation period on HeLa cells. Bars indicate SD, ****p<0.0001, ***p<0.001, **p<0.01.

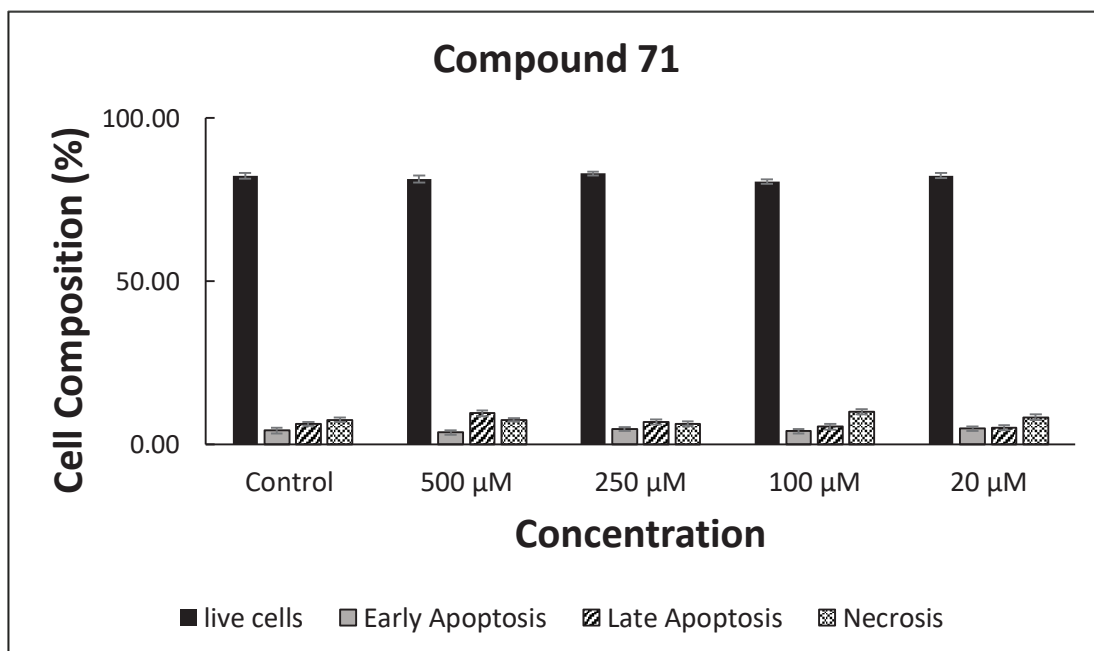


Figure 3. 11. Apoptosis analysis results for compound 71 at IC₅₀ dose in 48 h incubation period on HeLa cells.

3.3. Scratch Assay

In this study, the effect of compounds 78 and 71 on cell migration has been evaluated by using a scratch assay on A549 (lung adenocarcinoma) and HeLa (cervical carcinoma) cell lines. The effect has been evaluated for two-time periods 24 h and 48 h at IC_{50} dose. For the A549 cell line and according to the results shown in Figure 3.12, the compounds were very effective to prevent the cell migration and compound 78 was better than compound 71, the difference is obvious comparing to the control state.

On the other hand, the effect of compounds 78 and 71 was very good to prevent the cell migration on the HeLa cell line in both time period of the test as shown in Figure 3.13.

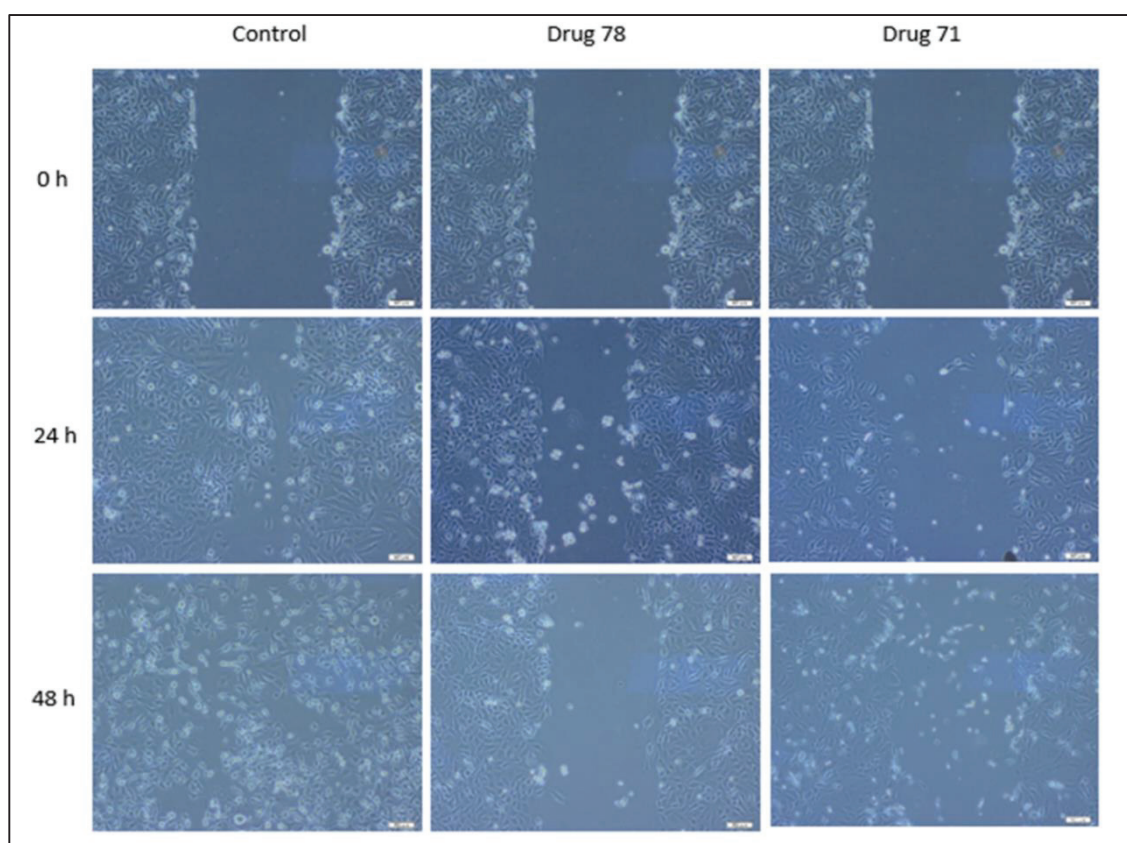


Figure 3. 12. Cell migration (Scratch assay) results on A549 cells for 0 h, 24 h and 48 h period incubation at IC_{50} dose.

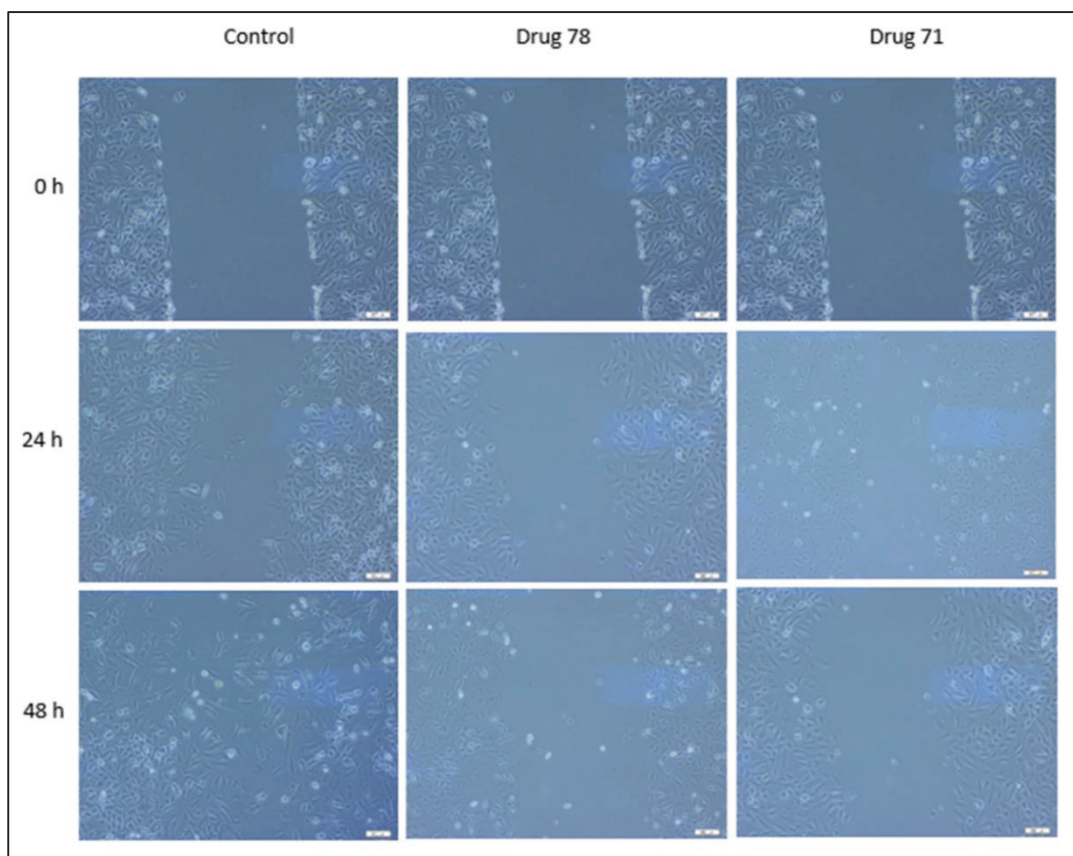


Figure 3. 13. Cell migration (Scratch assay) results on HeLa cells for 0 h, 24 h and 48 h period incubation at IC_{50} dose.

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 demonstrated 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 these strong effects of pyrrole-imidazole polyamide with penetration into nuclei and staying there for a long time (Wang et al. 2010).

3.4. Cell Cycle Analysis

During the division of the cell, it will go through four stages as is mentioned in section 1.4. These stages are G1 (growth phase), S (Synthesis phase) DNA replication, G2 the phase that cell prepares for mitosis stage and the last phase is a mitotic phase, this phase includes four phases (Prophase, Metaphase, anaphase, and Telophase).

In this study, the main aim of cell cycle analysis is to have full information about the suggested compounds to prevent the cell from dividing. In addition, the dose that has been chosen for this assay is IC_{50} for two compounds 78 and 71 at 48 h incubation period.

Figure 3.14 shows the effect of compound 78 on A549 cell line, it is very clear that is arrested the cells in S phase and the results were very significant. Also, cells could not pass to the G2 phase, which means that the cells cannot duplicate their DNA. For compound 71, Figure 3.15 shows that most of the cells arrested in G1 phase and it was not significant.

On the other hand, the effect of compound 78 on Hela cell line gave significant results and most of the cells are arrested in the S phase as shown in Figure 3.16. As for the compound 71 Figure 3.17 shows that almost the cells are staying in G1 phase, which means that the result was not significant.

Philoppes and Lamie in 2019 investigated two newly synthesized 5a and 5g for cell cycle analysis using two cell lines HepG2 and MCF7. It was found that the compounds were very potent for both cell lines and the percentage of cells in G1 phase was 55.37% for 5a and 48.22% for 5g. Also, the percentage of cells in G2/M phase was 31.22% and 33.54% for 5a and 5g, respectively (Philoppes and Lamie 2019).

Diana et al found compound (3-Methoxy-5H-isoindolo[2,1-a] quinoxalin-6-one) stop most of the cells in G2/M phase at 35% by using tubulin polymerization assay on Jurkat cell line. It was found that this compound inhibited tubulin polymerization in a concentration-dependent manner. For that reason, it could be concluded that the cells arrested in phase G2/M (Diana et al. 2008).

In 2014, 14 compounds of Isoindole derivative were investigated against HepG2 and SW620 cell lines. It has been founded that the percentage of cells is 37.3% and 41.7% of compounds 3a and 8c, respectively after 24 h and 48 h incubation. Thus, the suggested compounds are very robust and good to arrest the cells in the G2/M phase for both cell lines (1995 2014).

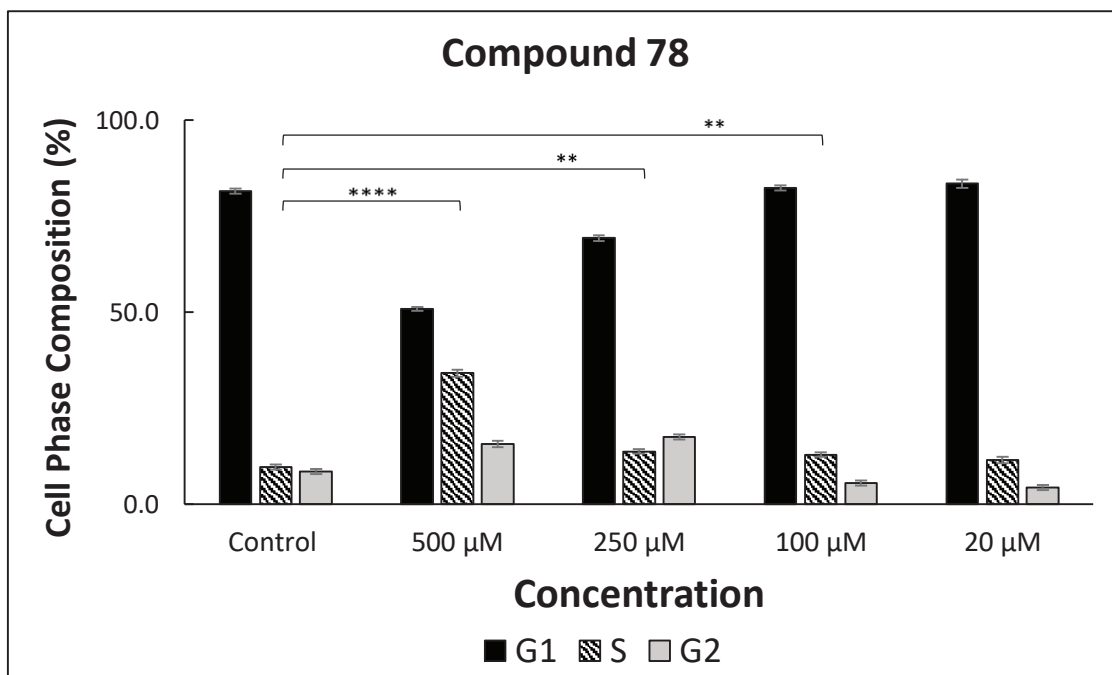


Figure 3. 14. Cell cycle analysis for compound 78 at IC₅₀ dose on A549 cells incubated for 48 h period. Bars indicate SD, ****p<0.0001, **p<0.01.

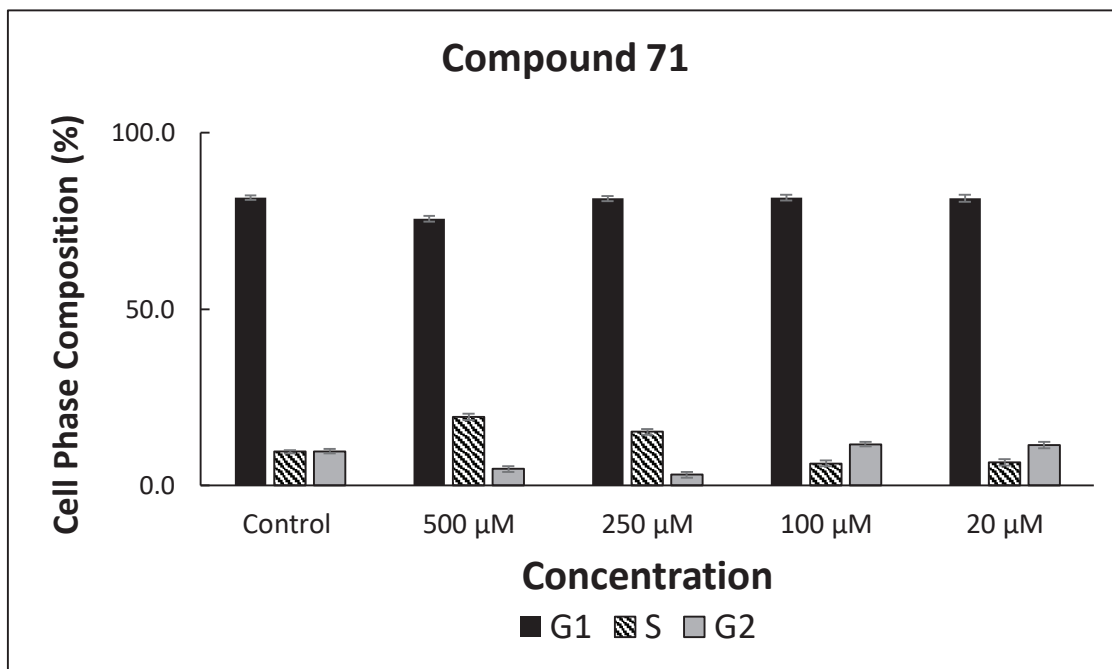


Figure 3. 15. Cell cycle analysis for compound 71 at IC₅₀ on A549 cells incubated for 48 h period.

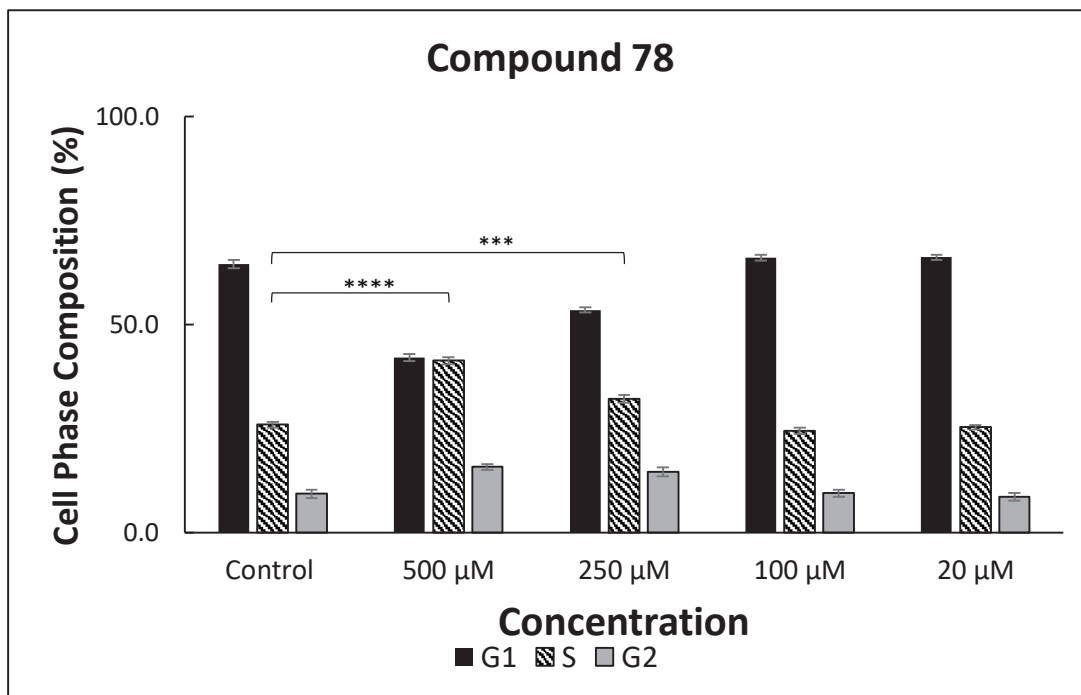


Figure 3. 16. Cell cycle analysis for compound 78 at IC₅₀ on HeLa cells incubated for 48 h period. Bars indicate SD, ****p<0.0001, ***p<0.001.

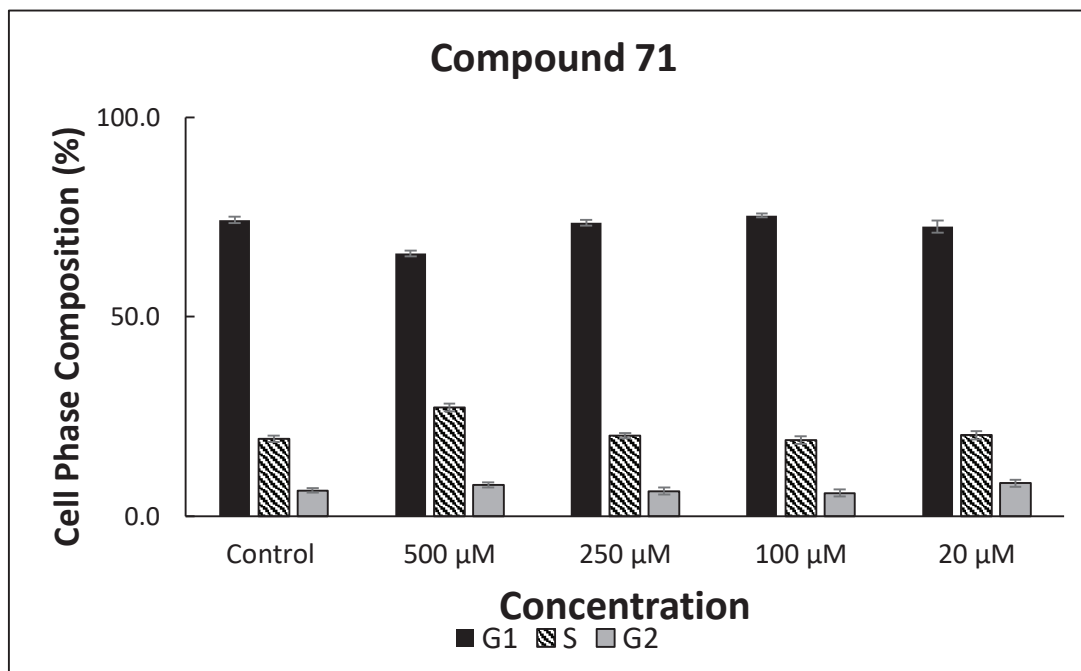


Figure 3. 17. Cell cycle analysis for compound 71 at IC₅₀ on HeLa cells incubated for 48 h period.

CHAPTER 4

CONCLUSION

Isoindole and its derivatives are one of the important biological active heterocyclic compounds. It is continued to attract the attention of many researchers because of its diversity, effectiveness as anticancer with high efficacy and fewer side effects compared to other treatments. Tyrosine kinases are known to be good target for cancer treatment because of their roles in cell growth signalling. Isoindole and its derivatives, are potential tyrosine kinase inhibitors which means they might be used in targeted therapy.

In this study, new seven compounds from Isoindole derivatives were investigated in the biological activity in order to evaluate the efficacy of these compounds.

HeLa (cervical carcinoma) and A549 (lung adenocarcinoma) cell lines have been chosen to investigate the suggested compounds for their biological activity. The cell viability and apoptosis effect have been investigated using cell cycle analysis and the effect of the best compound on cell migration assay.

Generally, the results have proved that the best compounds have good IC_{50} which were compounds 78 and 71 in the 48 h period time. IC_{50} was 251.4 μM for compound 78 and 289 μM for compound 71 on A549 cell line. For HeLa cell line, the IC_{50} was 206.9 μM for compound 78 and 296.4 μM for compound 71. Other compounds were less effective based on IC_{50} values compared to compound 78 and 71.

For the apoptosis effect, compound 78 was better than compound 71 with A549 cell line for 48 h incubation. The apoptosis rate for compound 78 was 20.4 % and 17.2 % for compound 71. For the HeLa cell line, the apoptosis rate for compound 78 was 21.2 % and 13.8 % for compound 71, which means that compound 78 was very effective with each of cell line, and compound 71 was more effective with A549 than HeLa cell line.

On the other hand, cell cycle effects on the results of the compound 78 arrest the cells in G1 phase with A549, and in G1/S phase with HeLa cell line. The compound 71 arrest the cells in G1 phase for each of cell line.

The last test in this study is the evaluation of the effect of two compounds on cell migration. The results show that each compound 78 and 71 inhibit the cell migration, and compound 78 had a significant inhibition on both cell lines. The results exhibited that two compounds could prevent cell proliferation in biological systems.

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APPENDIX A

CHEMICALS, SOLUTIONS AND REAGENTS

NSCLC	Non-small cell lung cancer
CDKs	Cyclin dependent kinase
DNA	Deoxyribonucleic acid
MTT	Methyl-thiazolyl-tetrazolium
Caspase-3	Cysteine-aspartic acid protease
RNA	Ribonucleic acid
DISC	Death-inducing signaling complex
ATM	Ataxia telangiectasia mutated
CHK	Checkpoint kinase
BAX	Bcl-2-associated x protein
APAF1	Apoptotic protease activating factor 1
MDR	Multidrug resistance
DMSO	Dimethyl sulfoxide
FBS	Fetal Bovine Serum
P/S	Penicillin streptomycin
PBS	Phosphate- buffered saline
FITC	Fluorescein isothiocyanate
PI	Propidium iodide