

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**THE EFFECT OF INDOLIN-2-ONE DERIVATIVES ON
BREAST CANCER**

NİMET ŞULE YÜNCÜ
**A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE**
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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THESIS SUPERVISOR
PROF. DR. ASUMAN DEMİROĞLU ZERGEROĞLU

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T.R.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

İNDOLIN-2-ONE TÜREVLERİNİN MEME
KANSERİ ÜZERİNDEKİ ETKİSİ

NİMET ŞULE YÜNCÜ
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. ASUMAN DEMİROĞLU ZERGEROĞLU

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SUMMARY

Recent analyses indicate that breast cancer has become one of the most common malignancies worldwide. Despite various types of treatment strategies, it is necessary to search for more alternatives. It is shown with several reports that indolin-2-one derivatives have anti-cancer activities. Thus, in this study, anti-cancer effect of originally synthesized different indolin-2-one derivatives; *GY342*, *GY339*, *GY306*, *GY333* were investigated in two breast cancer cells and a non-cancerous breast cell line. To expose the anti-carcinogenic effect of derivatives on cell proliferation, migration, colonisation, cell cycle and cell death; MTT, wound healing and colony formation assay, flow cytometry and double fluorescence staining (AO/EtBr and PI/DAPI) methodologies were performed, respectively. In addition, alteration of key proteins involved in those process were also evaluated by western blot techniques.

IC₅₀ and SI values of indolin-2-one compounds were calculated according to the MTT results, which indicated that all compounds reduced breast cancer cell viability in a concentration dependent manner. However, compounds were less effective on non-cancerous cells in similar concentrations indicating that compounds are more selective to cancer cells. Moreover, in a concentration dependent manner, compounds inhibited wound healing and colony formation caused accumulation of cells in S phase and activated cell death.

Keywords: Breast cancer, Indolin-2-one, Cell Death, Cell Proliferation, Cell Cycle, Signalling Pathways.

ÖZET

Son analizler, meme kanserinin dünya çapında en yaygın malignitelerden biri haline geldiğini göstermektedir. Hastalık için kullanılan çeşitli tedavi stratejilerine rağmen, hala alternatif arayışlara ihtiyaç duyulmaktadır. İndolin-2-on türevlerinin kanser önleyici aktiviteye sahip olduğu bilinmektedir. Bu çalışmada orijinal olarak sentezlenen farklı indolin-2-türevlerinin *GY342*, *GY339*, *GY306*, *GY333*, kanser önleyici etkisi iki farklı meme kanseri ve kanserli olmayan meme hücre hatlarında araştırılmıştır. Türevlerin hücre proliferasyonu, migrasyonu, kolonizasyonu, hücre döngüsü ve ölümü üzerindeki etkilerini belirlemek için MTT, yara iyileşmesi, koloni oluşumu testi, akış sitometrisi ile çift floresan boyama (AO/EtBr ve PI/DAPI) metodolojileri sırasıyla gerçekleştirilmiştir. İlaveten, bu süreçlerde yer alan anahtar proteinlerin değişimi de western blot ile araştırılmıştır.

İndolin-2-on bileşiklerinin IC_{50} değerleri ve SI değerleri MTT sonuçlarına göre belirlenmiştir. Tüm bileşikler meme kanseri hücre canlılığını konsantrasyon bağımlı bir şekilde azaltırken; kanserli olmayan meme hücre hatları üzerine benzer konsantrasyonlarda aynı etki gözlenmemiştir. Bu sonuç, türevlerin kanserli olmayan hücrelerden ziyade kanser hücrelerine karşı daha seçici olduğunu işaret etmektedir. Çalışmamızda indolin-2-on türevlerinin hücre proliferasyonu, migrasyonu ve koloni oluşumu baskılarken; hücre döngüsünü tutukladığı ve hücre ölüm yollarını aktive ettiği belirlenmiştir.

Anahtar Kelimeler: Meme kanseri, indolin-2-on, Hücre Ölümü, Hücre Proliferasyonu, Hücre Döngüsü, Sinyal Yolları.

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TABLE of CONTENTS

	<u>Page</u>
SUMMARY	v
ÖZET	vi
ACKNOWLEDGEMENTS	vii
TABLE of CONTENTS	viii
LIST of ABBREVIATIONS and ACRONYMS	x
LIST of FIGURES	xii
LIST of TABLES	xiii
1. INTRODUCTION	1
1.1. The Purpose, Contribution and Context of the Thesis	2
2. LITERATURE REVIEW	3
2.1. Breast Cancer	3
2.2. Cell Survival Pathways	3
2.2.1. MAPK Pathway	4
2.2.2. PI3K-Akt-mTOR Pathway	4
2.2.3. JAK/STAT Pathway	4
2.3. Cell Cycle	5
2.4. Apoptosis	7
2.5. Indoline	10
2.5.1. Indolin-2-one Derivatives in Breast Cancer Treatment	10
3. MATERIALS and METHODS	13
3.1. Materials	13
3.1.1. Chemicals	13
3.1.2. Solutions	14
3.1.3. Antibodies	16
3.1.4. Equipments	17
3.2. Methods	18
3.2.1. Cell Culture	18
3.2.2. Preparation of GY	19
3.2.3. Cell Viability Assay	19

3.2.4. Cell Death Analysis	19
3.2.5. Cell Morphology Analysis	20
3.2.6. Wound Healing Assay	21
3.2.7. Colony Formation Assay	21
3.2.8. Flow Cytometer	22
3.2.9. Protein Analysis, Western Blotting	23
4. RESULTS	26
4.1. Indolin-2-one Derivatives Reduce Cell Viability	26
4.2. Indolin-2-one Derivatives Inhibit MCF7 Wound Healing	28
4.3. Indolin-2-one Derivatives Inhibit Colony Formation Ability in MCF7 Cells	30
4.4. Indolin-2-one Derivatives Cause S phase arrest on MCF7 Cell Cycle	31
4.5. Indolin-2-one Derivatives Lead MCF7 Cells to Death	32
4.6. Indolin-2-one Derivatives Effect Protein Phosphorylation and Expression in MCF7 Cells	36
5. DISCUSSION	38
REFERENCES	41
BIOGRAPHY	44
APPENDICES	45

LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
μL	: Micro litre
μM	: Micro molar
ADP	: Adenosine diphosphate
AIF	: Apoptosis-inducing factor
AO	: Acridine Orange
Apaf-1	: Apoptosis protease-activating factor 1
CDK	: Cyclin dependent kinases
c-IPA	: Inhibitory PAS domain protein
CKI	: Cyclin-dependent kinase inhibitors
CO ₂	: Carbon dioxide
DAPI	: 4',6-diamidino-2-phenylindole
dH ₂ O	: Distilled water hydrogen dioxide
DMSO	: Dimethyl sulfoxide
DOX	: Doxorubicin
EndoG	: Endonuclease G
EtBr	: Ethidium bromide
FasR	: Fas receptor
GABARAP	: γ-aminobutyric-acid type A receptor-associated protein
GPCR	: G-protein coupled receptor
Grb2–SOS	: Growth factor receptor-bound protein/Son of Sevenless
I3C	: Indole-3-carbinol
IC ₅₀	: Half maximal inhibitory concentration
JAK	: Janus kinase
LIR	: LC3-interacting region
MAPK	: Mitogen-activated protein kinase
MMP	: Matrix metalloproteinase
MTT	: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MTX	: Mitoxantrone

Mw	: Molecular weight
Nbr1	: Neighbour of Brca1
NDP52	: Nuclear dot protein 52 kDa
NF- κ B	: Nuclear factor kappa B
PARP	: Poly (ADP-ribose) polymerase
PBS	: Phosphate-buffered saline
PI	: Propidium Iodine
PRRs	: Pattern recognition receptors
PTX	: Paclitaxel
RIP	: Receptor-interacting protein
RIPK	: Receptor-interacting protein kinase
ROS	: Reactive oxygen species
RTK	: Tyrosine kinase receptors
SDS-PAGE	: Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SHP2	: SH2-domain-containing tyrosine phosphatase
Smac	: Second mitochondria-derived activator of caspase
STAT	: Signal transducers and activators of transcription
TBS	: Tris-buffered saline
TBS-T	: Tris-buffered saline Tween 20
TEMED	: Tetramethyl ethylenediamine
TLR	: Toll-like receptors
TRAIL	: TNF-related apoptosis-inducing ligand
Tyk-2	: Tyrosine kinase-2

LIST of FIGURES

<u>Figure No:</u>	<u>Page</u>
2.1: Cell Survival pathways cross-talk.	3
2.2: Cell Cycle and cell cycle check points.	6
2.3: Cross-talk of cell death pathways.	7
2.4: The intrinsic and extrinsic pathways of apoptosis.	8
2.5: GY342 R= 3,5-dimethoxyphenyl; GY339 R= 2,5-dimethoxyphenyl; GY306 R= 3,4,5- trimethoxyphenyl; GY333 R= ter-butyl.	11
4.1: Dose-dependent inhibition of MCF-12A, MCF7 and MDA-MB-231 cell viability in 72h exposure of derivatives.	27
4.2: Effect of indolin-2-one derivatives on cellular wound healing in MCF7 cells between 0-48 hours.	29
4.3: Effect of indoline-2-one derivatives on colony formation ability of MCF7 cells in dose-dependent manner in 24 hours exposure.	30
4.4: The effect of indolin-2-one derivatives on cell cycle MCF7 cells.	31
4.5: Demonstration of cell death in MCF-12A and MCF7 cells.	33
4.6: MCF7 treated with indolin-2-one derivatives dead/total cells.	34
4.7: Representative images of MCF7 cells' death morphology.	35
4.8: Effect Indolin-2-one derivatives on phosphorylation of proteins and protein expression in MCF7 cells.	37

LIST of TABLES

<u>Table No:</u>	<u>Page</u>
3.1: List of equipments.	17
3.2: Cell medium ingredients.	18
3.3: Resolving gel and Stacking gel ingredients.	24
4.1: IC ₅₀ and SI values of GY342, GY339, GY306 and GY333 for MCF-12A, MCF7 and MDA-MB-231.	28

1. INTRODUCTION

Breast cancer is the most common type of cancer in women worldwide, as well as the second cause of cancer-related death after lung cancer [Dumitrescu et al., 2007]. Nowadays, treatment strategies such as surgery, chemotherapy, radiotherapy, and hormonal treatment techniques are insufficient because of multidrug resistance and side effects. Particularly, treatment efficiency is predicted as low in triple negative breast cancer types which do not express HER-2, oestrogen, and progesterone [Biersack et al., 2012]. In addition, due to non-selectivity, poor pharmacodynamics and poor oral bioavailability, current breast cancer drugs have several disadvantages. Therefore, it is a need to develop more effective and safe drugs. At the present time, doxorubicin (DOX) which is in anthracycline class, is one of the chemotherapeutic agents used for clinical treatment of breast cancers.

One chemotherapeutic agent used in breast cancer treatments is Paclitaxel (PTX), but it is known that paclitaxel may cause side effects like peripheral neuropathy and neutropenia. In addition, there are several other widely used chemotherapeutic agents such as Docetaxel, Tamoxifen, Cisplatin and Trastuzumab. Also, cyclophosphamide, fluorouracil, methotrexate, DOX or mitoxantrone (MTX) are chemotherapeutic agents which are commonly used together, but several side effects such as neuropathy, fatigue, peripheral, nausea and, weight loss were reported in patients after the chemotherapy treatments. Furthermore, serious side effects were reported in some patients as osteoporosis, lymphedema, and heart problems [Liyanage et al., 2019].

Heterocyclic compounds are anticancer agents targeting breast cancer [Kaur et al., 2019]. Indole-based derivatives are associated with pleiotropic anticancer activity by inducing apoptosis and modulating various signalling pathways. It has been reported that indole-3-carbinol (I3C) modified derivatives inhibit the growth of oestrogen-dependent (MCF7) and oestrogen-independent triple-negative (MDA-MB-231) human breast cancer cells [Biersack et al., 2012]. Moreover, another indolin-one derivative causes ROS production and mitochondrial membrane potential change in MCF7 cells, which was confirmed by Western blot analyses showing increase in Bax expression, decrease in Bcl-2 level, releasing of cytochrome C and then caused activation of caspase-9 and cleavage of Poly (ADP-ribose) polymerase (PARP)

[Nayak et al., 2016]. In another study, its effect on apoptosis was evaluated together with a series of 2-indolinone derivatives and a Pak1 inhibitor (ZMF-005) with strong anti-proliferative effect. As a result, it was observed that ZMF-005 dose-dependently induced apoptosis in Mammary Adenocarcinoma (MDA-MB-231) cells and up-regulated the expression of the pro-apoptotic protein Bax and down-regulated the anti-apoptotic protein Bcl-2. In addition, cleavage of caspase-8 and 3 proteins were detected [Yao et al., 2020].

1.1. The Purpose, Contribution and Context of the Thesis

Recent analyses indicate that breast cancer has become one of the most common malignancies worldwide. Despite various types of treatment strategies used for the disease, it is still a need to seek alternatives. It is known that indolin-2-one derivatives have a wide variety of biological effects including antimicrobial, anti-malarial and anti-cancer activities. In this study, antiproliferative and apoptotic effects of originally synthesized different indolin-2-one derivatives were investigated in two breast cancer cells and a non-cancerous breast cell line.

Within the scope of this thesis, firstly the cytotoxic effect of original indolin-2-one derivatives was investigated in two different breast cancer cells (MCF7, MDA-MB-231) and normal breast cell line (MCF-12A). Then effects of those agents to colony formation and wound healing was evaluated in breast cancer cell lines. Following investigation, the effect of agents on cell cycle profile, programmed cell death mechanism apoptosis was analysed next in these cells.

2. LITERATURE REVIEW

2.1. Breast Cancer

Breast cancer is a common type of cancer worldwide and the second most common cause of cancer-related deaths after lung cancer. Therefore, early diagnosis and therapy is vital [Dumitrescu et al., 2007] [L. Ouyang et al., 2012]. Breast cancer may occur in different parts of breast; thus, cancer types are classified in two different groups according to the tissue where the cancer begin. (i) The invasive ductal carcinoma which is the cancer cells begin in the ducts and then grow outside the ducts into other parts of the breast tissue; and (ii) the invasive lobular carcinoma which is the cancer cells begin in the lobules and then spread from the lobules to the breast tissues that are close by.

2.2. Cell Survival Pathways

The balance between cell survival and cell death is crucial for normal development and homeostasis which are unfortunately important for cancer development and progression, (Figure 2.1) [Salaheldin et al., 2021].

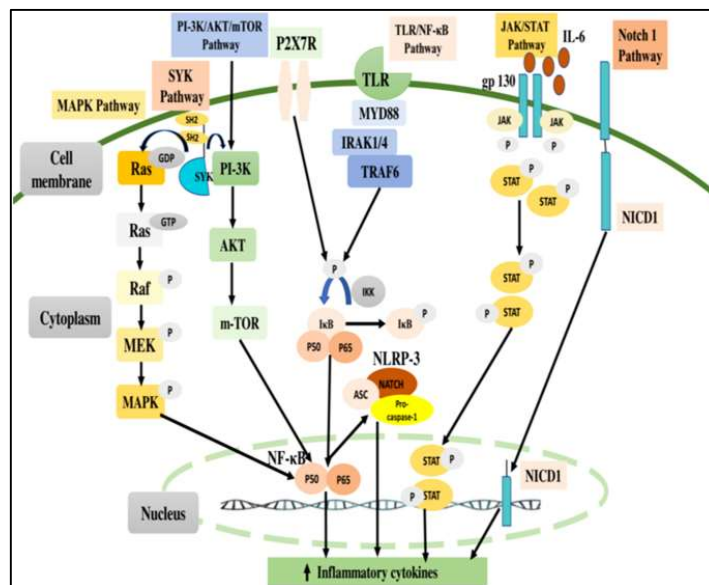


Figure 2.1: Cell Survival pathways crosstalk as JAK/STAT, MAPK, PI3K-Akt-mTOR pathways.

2.2.1. MAPK Pathway

Mitogen-activated protein kinase (MAPK) signalling pathway is activated by stimulation of numbers of receptors including tyrosine kinase, G protein, cytokine, and so on. Once MAPK pathway is induced by MAPK kinase kinases (MAPKKKs, or MAP3Ks) activation and follows activation of MAPKK such as MEK1 and MEK2. After that, MEKs phosphorylate MAPKs which are also known as extracellular signal-regulated kinases (ERK1 and ERK2), by increasing their enzymatic activity. [Zhang et al., 2002]. ERK 1 and ERK2 activation leads to activation of transcription factors such as Elk-1, c-Myc and also NF- κ B which may lead to matrix metalloproteinase (MMP) gene expression, cell survival, and resistance to apoptosis [Salaheldin et al., 2021].

2.2.2. PI3K-Akt-mTOR Pathway

Akt/PKB is a serine/threonine protein kinase which plays critical role as a regulator of cell proliferation and cell survival. Upon RTKs or G-protein coupled receptors (GPCRs) stimulation, phosphatidylinositol 3-kinase (PI3K) is activated. In this case, PIP3 is produced at the plasma membrane and conjugated with inactive Akt on PH domain, resulting in phosphorylation and full activation of Akt/PKB which promotes growth factor mediated cell survival and to block apoptosis [Song et al., 2007]. In addition, Akt leads upregulation of matrix metalloproteinase (MMP) following degradation mediated by MMP and this situation cause increase in metastasis in breast cancer [Agarwal et al., 2013].

2.2.3. JAK/STAT Pathway

JAK/STAT pathway is another important pathway which is involved in many crucial biological processes such as cell differentiation and proliferation. Upon cytokine receptor activation, JAK tyrosine kinase family members which are JAK-1, JAK-2, JAK-3 and tyrosine kinase-2 (Tyk-2) are associated and phosphorylate the receptors. Subsequently, Transcription factors STATs family members (STAT-1, STAT-2, STAT-3, STAT-4, STAT-5a, STAT-5b, and STAT-6) are phosphorylated by

JAKs which are associated with the receptor. Phosphorylation of STATs cause dimerization among themselves and translocation to the nucleus to regulate gene transcription [Salaheldin et al., 2021]. Besides, IL-6-type cytokine receptors dimerization activates both JAK/STAT pathway and MAPK cascade through recruitment of the SH2-domain-containing tyrosine phosphatase (SHP2). The phosphorylated SHP2 binds to the growth factor receptor-bound protein/Son of Sevenless (Grb2–SOS) complex and cause to Ras–Raf–MAPK pathway activation [Salaheldin et al., 2021].

2.3. Cell Cycle

Cell cycle is a period which series of events occur in the cell until cell division as shown in Figure 2.2. This cycle is formed from 4 stages as Gap-1 (G1), synthesis (S), Gap-2 (G2), and mitotic (M) respectively. G1 stage consist of the cell growth and synthesis of new organelles and proteins for daughter cells. DNA is replicated at S stage and at the G2 stage the cell synthesizes of special proteins for mitosis and carries on growing. Finally, cell division occurs by mitosis at M stage.

During cell cycle, there are checkpoints responding several events which may cause problem for cell division such as DNA damage. If DNA damage occurs during cell cycle, the cycle can be blocked at where is the damage occurred (at G1/S or G2/M phases) or DNA is repaired. In the situation when the DNA damage is irreversible and checkpoints are dysfunctional, the cell cycle is blocked, and apoptosis pathway is triggered. Therefore, cell cycle function to checkpoints control cell survival by responding to DNA damage and the cytotoxicity [Hein et al., 2014].

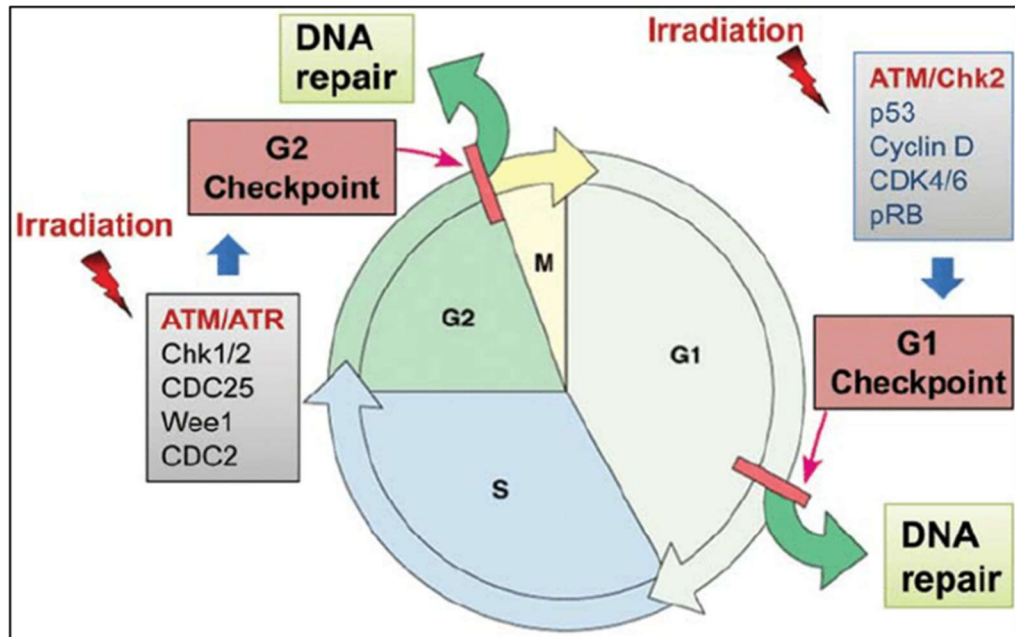


Figure 2.2: Cell Cycle and cell cycle check points

Cell cycle stages (Figure 2.2) and progression are controlled by Serine/Threonine protein kinases called as cyclin dependent kinases (CDKs). CDKs have ability of phosphorylation of specific proteins and their activity results in promotion of DNA synthesis and mitotic progression [Barnum & O'Connell., 2018].

CDKs activity is also controlled by inhibition via cyclin-dependent kinase inhibitors (CKIs). Several kinds of CKIs such as p15, p16, p18 and p19 may bind to CDKs, thus prevent CDKs to interact with cyclins. Also, there are some kind of CKIs such as p21, p27 and p57 directly bind to cyclin-CDK complexes and inhibit their activity [Rhind & Russell., 2012].

Cyclins are group of related proteins and are essential for cell cycle regulation. During cell cycle Cdk levels remain constant, but cell cycle transitions are controlled by the amount of different cyclins for each phase. Cdk4/6 kinases interact with Cyclin D and their activation which is by the p53/p21 pathway, controls G1/S transition. Cdc2/Cyclin B complex controls G2/M transition. Besides, because of DNA damage like mutations of G1 checkpoint regulators such as p53 and Cyclin D, G1 checkpoint is not well functional in many cancer types [Hein et al., 2014].

2.4. Apoptosis

Programmed cell death mechanisms are essential to adjust the balance between death and survival of normal cells. Disruption of this balance can be shown as one of the most important causes of cancer development and progression. Apart from apoptosis several programmed cell death mechanisms have been described in recent years. However, apoptosis, programmed necrosis (necroptosis) and autophagy are three main programmed cell death mechanisms that have been defined in detail so far. As shown in figure 2.3 their morphological differences can be easily distinguished [Ouyang et al., 2012].

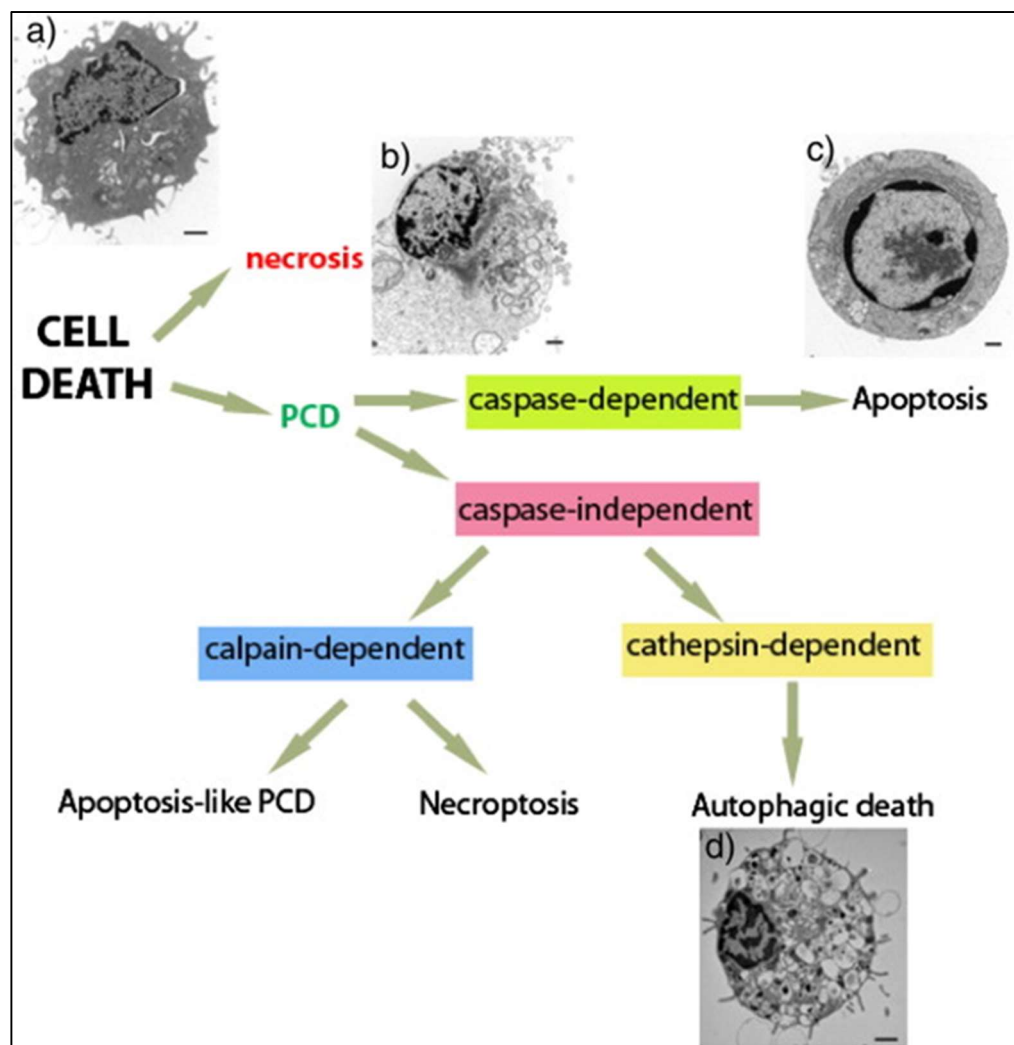


Figure 2.3: Cross-talk of cell death pathways such as apoptosis, necroptosis and autophagy.

Apoptosis can be initiated through two different pathways as intrinsic and extrinsic pathways, which are a highly regulated process.

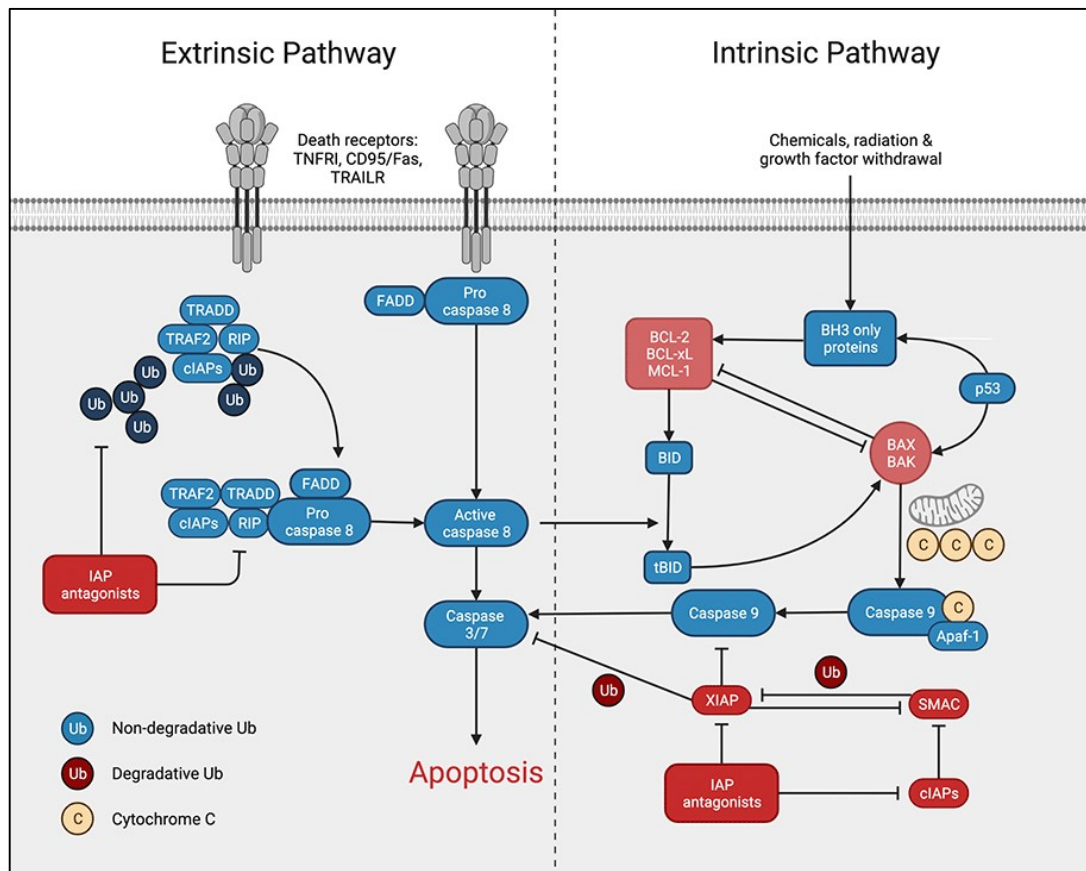


Figure 2.4: The intrinsic and extrinsic pathways of apoptosis.

In intrinsic pathway, intracellular stimuli such as irreversible DNA damage, high calcium concentrations in the cytoplasm and oxidative stress may trigger intrinsic pathway by activating one or more members of the BH3 proteins family. Bcl-2 protein family participate in this way and this family is composed of proapoptotic proteins (Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk) and antiapoptotic proteins (Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1). Once mitochondria-initiated apoptosis is stimulated by stress, c-Jun N-terminal protein kinase (JNK) and p38 which is mitogen activated (MAP) kinase are phosphorylated and target Bcl-2 [Cai, et al., 2006]. BH3 subfamily directly induce the formation of pores in the mitochondrial membrane or activate and release proapoptotic proteins to inhibit antiapoptotic proteins in indirect way. Bcl-2 and Bcl-XL which are located in outer membrane of mitochondria prevent release of cytochrome C, whereas Bad, Bid, Bax, and Bim which are located in the

cytosol translocate to mitochondria under certain stimuli and induce release of cytochrome C. When Bax and Bad are translocated to the mitochondrial membrane, mitochondrial transition permeability is activated. Because of this loss of the mitochondrial transmembrane potential causes release of cytochrome C, apoptosis-inducing factor (AIF), endonuclease G (EndoG), Second mitochondria-derived activator of caspase (Smac), and HtrA2 from the inner mitochondrial membrane to the cytoplasm and inhibition of metabolic processes, such as the respiratory chain, and overproduction of reactive oxygen species (ROS). After release of cytochrome C, it can associate apoptosis protease-activating factor (Apaf-1) and procaspase-9. This association results in formation of a molecular complex which is called as apoptosome. Activation of caspase-9 leads to a proteolytic effect and induces cell death by activating caspases-3 and 7 [Salano-Galvez SG et al., 2018].

Extrinsic pathway is activated via extracellular stress signals which are detected by specific transmembrane receptors such as tumour necrosis factor receptor (TNFR), Fas receptor (FasR), and TNF-related apoptosis-inducing ligand (TRAIL). These receptors are called as death receptors and has intracellular death domains, TNFR1 associated death domain protein (TRADD) and Fas-associated protein with death domain (FADD). When these receptors associate with their ligands, activating proteins such as receptor interacting protein kinase-1 (RIPK1), inhibitory PAS domain protein (c-IPA), and ubiquitin ligase E3 are recruited. This receptor-protein complex which is called as death-inducing signalling complex activates procaspase-8. Caspase-8 induces cell death by activating caspases-3 and 7. In addition extrinsic and intrinsic pathways may connect by caspase-8 through Bid proteolysis, turning it into tBid, which also translocates to the mitochondria and activates Bcl-2, Bax, and Bak [Salano-Galvez SG et al., 2018].

These two pathways which are induced by different stimuli, follow the same pathway at the end of execution phase starting with caspase activation (Figure 2.4). Active executioner caspases induce several cellular endonucleases starting to break down DNA and cellular proteins such as cytoskeletal proteins, Poly ADP-ribose polymerase (PARP). Consequently, apoptotic cells are characterized by cell specific morphology and biochemistry such as nuclear fragmentation, cell shrinkage and chromatin condensation.

2.5. Indoline

Indoline, which has the formula C_8H_9N , is an organic heterocyclic compound. Heterocyclic compounds are the main substance of many important organic compounds that have significant biological activity. For example, like DNA and RNA many biological molecules contain heterocyclic ring in their backbone. Also, indole based heterocyclic compounds have an ability to mimic the structure of peptides and to bind reversibly to proteins [Kaushik et al., 2013].

Different combinations of heterocyclic compounds are designed by medicinal chemists for new chemical, physical and biological properties. Heterocyclic compounds have been important subject of medical science and investigated for their anti-inflammatory, antifungal, antioxidant, antibacterial, antiallergic, anticonvulsant, herbicidal, enzyme inhibitory, antidiabetic, anti-HIV, and anticancer activity [Kaushik et al., 2013].

However, heterocyclic structures have substantially suitable framework for an effective attachment of pharmacophores to produce novel drugs. Especially nitrogen-based heterocyclic compounds form the core structure of biologically active molecules, they have been reported as active against several kinds of cancers [Sachdeva et al., 2020]. The versatility of indoline is another important property to design and develop target-based anticancer agents.

2.5.1. Indolin-2-one Derivatives in Breast Cancer Treatment

Heterocyclic compounds are anticancer agents targeting several cancers including breast cancer [Kaur et al., 2019]. Mitomycin C, beta carboline alkaloids (harmaline), bisindole alkaloids (dragmasidin D) and marine alkaloids (eudistomin K) are currently known indole-based heterocyclic compounds that exert pleiotropic anticancer activity by inducing apoptosis and modulating various signalling pathways. It has been reported that indole-3-carbinol (I3C) modified derivatives inhibit the growth of oestrogen-dependent (MCF7) and oestrogen-independent triple-negative (MDA-MB-231) human breast cancer cells [Biersack et al., 2012]. In addition, when another line of indolinone derivatives was applied to MCF7 and ROS production and Mitochondrial Membrane Potential and decreased Bcl-2 level, cytochrome C release, caspase-9 activity, and PARP cleavage has been observed [Nayak et al., 2016]. In

another study, effect of 2-indolinone derivatives was evaluated together with a Pak1 inhibitor (ZMF-005) resulting in strong anti-proliferative activity and apoptosis [Yao et al., 2020]. In addition to all these, indoles are associated with the regulation of oestrogen mediated activity and inhibition of NFκB/mTOR/PI3K/AkT pathway. For example, 3-substituted indole derivatives have been reported to have an effect on inhibition of cell proliferation of several kind of cancer cells. Especially, anticancer activity of 3-pyranyl indoles have been showed more effective than current cancer drugs on MCF7 breast cancer cells. Also, anti-proliferative activity of (E)-3-(5-substituted-1H-indol-3-yl)-1-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl) prop-2-en-1-one derivatives have been found on breast cancer cell lines. Indole-3-carbinol is another heterocyclic compound which has anti-tumour activities targeting of cell survival via Akt-NFκB pathway, regulation of cell cycle, activation of caspases, CDKs activities, oestrogen receptor signalling, oestrogen metabolism, ER stress and BRCA expression in cancer cells. Furthermore, anti-proliferative effects of pyrido[3,4-b] indoles have been also found in human breast cancer cell lines MCF7 and MDA-MB-468 [Sachdeva et al., 2020].

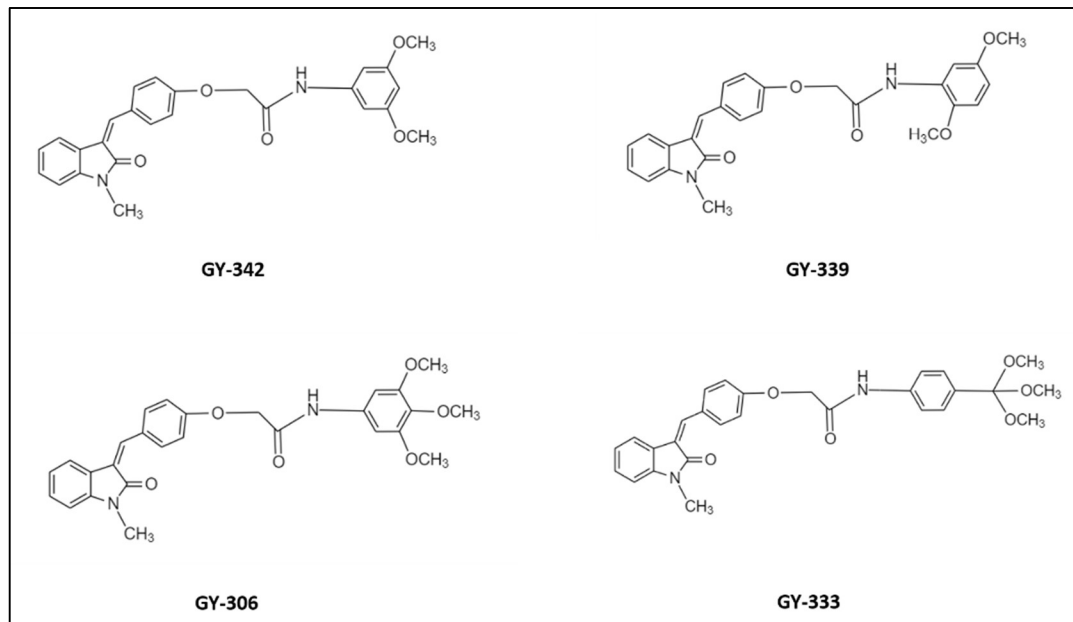


Figure 2.5: *GY342* R= 3,5-dimethoxyphenyl; *GY339* R= 2,5-dimethoxyphenyl; *GY306* R= 3,4,5- trimethoxyphenyl; *GY333* R= ter-butyl.

In figure 2.5, four indolin-2-one derivatives (*GY342*, *GY339*, *GY306* and *GY333*) that were originally synthesized by Sultan Baytaş and her team in Gazi University

Pharmacology Department are shown and within the scope of this thesis, the anti-carcinogenic effect of these four indolin-2-one derivatives were investigated on breast cancer cells.

3. MATERIALS and METHODS

3.1. Materials

3.1.1. Chemicals

The chemicals that were used in this thesis are listed below:

- Acridine Orange (Sigma-Aldrich, USA, A6014)
- Acrylamid (Applichem, USA, A4989)
- BCA Protein Assay Kit (Thermo Scientific, USA, 23225)
- DAPI (Cell Signalling Technology, USA , 4083)
- DMEM Medium (ThermoFisher/Gibco, USA, 32500-035)
- EGF (Sigma-Aldrich, USA, E9644)
- EMEM Medium (ThermoFisher/Gibco, USA, 61100-087)
- Ethidium bromide (Sigma-Aldrich, USA, E 7637)
- FBS (Gibco Invitrogen, USA, 10270106)
- Formaldehyde 37% (JT Baker, USA, 7041)
- Glycerol (Sigma-Aldrich, USA, G5516)
- Glycine 99% (MP, UK, 808822)
- HEPES (Sigma-Aldrich, USA, H0887)
- Horse serum (ThermoFisher/Gibco, USA, 16050-122)
- Hydrocortisone 98% (Sigma-Aldrich, USA, H0888)
- Insulin (Sigma-Aldrich, USA, I0516)
- Lauryl Sulphate (SDS) 95% (Sigma-Aldrich, USA, L5750)
- Luminol (Sigma-Aldrich, USA, A8511)
- L-Glutamine (Sigma-Aldrich, USA, G7513)
- p-Coumaric acid (Sigma-Aldrich, USA, C9008)
- Pen/Strep (Gibco Invitrogen, USA, 11548876)
- Propidium Iodine (PI) (Sigma-Aldrich, USA, P4170)
- Protease-Phosphatase Inhibitor Cocktail (Thermo Scientific, USA, 1862495)
- Protein ladder (Thermo Scientific, USA, 110-26616)

- PVDF Blotting Membrane (GE Healthcare, USA 10600023)
- RNase A (Sigma-Aldrich, USA, R6148)
- Sodium dodecyl sulphate (MP, UK, 808822)
- Sodium Pyruvate Sigma-Aldrich, USA, P5280)
- TEMED (Sigma-Aldrich, USA, T7024)
- Triton-x 100 (BIO FROXX, Germany, 84648)
- Trypan blue solution (Sigma-Aldrich, USA, T8154)
- Tween20 (SigmaAldrich, USA, P137 9)
- Trypsin (Thermo Fisher, USA, 25200056)

3.1.2. Solutions

All solutions and buffers used in this study are listed below including their ingredients and the final concentrations of each ingredient. All buffers were prepared with autoclaved distilled water (ddH₂O) and diluted with distilled water (dH₂O) into small amounts.

- Transfer Buffer (10x):
 - Tris Base 250 mM (final concentration)
 - Glycine 2.5 M (final concentration)
- TBS-T:
 - TBS(10X) 10% (final concentration)
 - Tween-20 0,10% (final concentration)
- TBS (10X):
 - Tris-HCl (pH 7.4) 200 mM (final concentration)
 - NaCl 1.5 M (final concentration)
- Transfer Buffer (1x):
 - Transfer Buffer (10x) 10% (final concentration)
 - Methanol 20% (final concentration)
- SDS Running Buffer (5X):
 - SDS 0.5 % w/v (final concentration)
 - Tris Base 125mM (final concentration)
 - Glycine 0.95 M (final concentration)

- Stripping Buffer:
 - Tris-HCl, pH 6.7 62.5 mM (final concentration)
 - SDS 0,02 (final concentration)
 - BME 100 mM (final concentration)
- Protein Loading Dye (5X):
 - Tris-HCl, pH 6.8 250mM (final concentration)
 - SDS 10% (final concentration)
 - Bromophenol Blue 0.5 % (final concentration)
 - Glycerol 50% (final concentration)
 - BME 25% (final concentration)
- Blocking Solution 5%:
 - Non-fat milk powder 0,05 (final concentration) dissolved in 1X TBS-T
- Cell Lysis Buffer:
 - Glycerol 10% (final concentration)
 - Tris (pH:8) 20 mM (final concentration)
 - Triton X-100 1% (final concentration)
 - NaCl 150 mM (final concentration)
 - MgCl₂ 1 mM (final concentration)
- Cell Lysis mix:
 - Cell lysis buffer 99% (final concentration)
 - Protease-phosphatase inhibitor %1(final concentration)
- PI (25 µg/ml) (for dual immunofluorescence staining):
 - PI in dH₂O 20mg/ml (final concentration) in 1X PBS
- PI (for flow cytometer):
 - PI 0,02 mg/ml (final concentration)
 - Triton-x 100 10% (final concentration)
 - PBS 1X
- DAPI (1 µg/ml):
 - DAPI in dH₂O 20mg/ml (final concentration) in 1X PBS
- AO/EtBr:
 - AO 0,01 g/ml (final concentration)
 - EtBr 0,01 g/ml (final concentration) in 1X PBS

3.1.3. Antibodies

The primary antibodies which were used in this thesis study were listed below:

- Bcl-2 (Cell Signaling Technology, 9286)
- Bax (Cell Signaling Technology, 5023)
- t-STAT3 (Cell Signaling Technology, 12640)
- p- STAT3 (Cell Signaling Technology, 9145)
- t-ERK1/2 (Cell Signaling Technology, 9107)
- p-ERK1/2 (Cell Signaling Technology, 8544)
- t-ERK5 (Cell Signaling Technology, 3372)
- p-ERK5 (Cell Signaling Technology, 3371)
- p-AKT (Cell Signaling Technology, 4060)
- t-AKT (Cell Signaling Technology, 4691)
- p21 (Cell Signaling Technology, 2947)
- p27 (Cell Signaling Technology, 3686)
- p-p38 (Cell Signaling Technology, 4511)
- t-p38 (Cell Signaling Technology, 9212)
- p-JNK (Cell Signaling Technology, 4668)
- t-JNK (Cell Signaling Technology, 9252)
- Cyclophilin B (Thermo-Fisher Scientific, PA1-027A)

The secondary antibodies which were used in this thesis study were listed below:

- Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology, 7074S)
- Anti-mouse IgG, HRP-linked Antibody (Cell Signaling Technology, 7076S)

3.1.4. Equipments

Equipments which were used during the thesis were given in table 3.1.

Table 3.1: Equipment which were used during the thesis and the companies of the equipment.

Equipment	Company
Centrifuges	Beckman Coulter, Eppendorf, VWR
Chemiluminescence Imaging System	ChemiDoc™ XRS+ System, Bio-Rad
Flow cytometer	BD Accuri C6
Heat Blocker	VWR
Incubator	Nuve EC160
Inverted Microscope	BioRad ZOE™, Leica DM IL LED
Laminar Flow Cabinet	Hera Safe
pHmeter	JENCO
Plate Reader	ThermoVarioSkan
Shaker	Biosan
Vortex	VWR
Water Bath	Memmert
Western Blotting Equipment	BioRad

3.2. Methods

3.2.1. Cell Culture

Breast cancer cells MCF7 (hormone-dependent ATCC: HTB-22) and MDA-MB-231 (triple negative, ATCC: HTB-26) were cultured in EMEM and DMEM (high glucose) cell medium respectively and non-cancerous breast cells MCF-12A (ATCC: CRL-10782) were used and cultured in DMEM: F12 as given in table 3.2.

Table 3.2: EMEM, DMEM: F12 and DMEM (high glucose) cell medium ingredients.

Ingredients	EMEM	DMEM:F12	DMEM (high glucose)
EMEM	9,52 g/L		
DMEM		12 g/L	
L-Glutamine	2 mM	2,438 g/L	2 mM
(NEAA)			0.1 mM
HEPES		0,35848 g/L	
Hydrocortisone		10 ml/L	
Sodium Pyruvate	1 mM	0,11004 g/L	
Sodium Bicarbonate	1500 mg/L		
(Pen/Strep)	1%		1%
(FBS)	1%		10%
Insulin	0,01 mg/ml	1 ml/L	
EGF		20 µl/L	
c. toxin		200 µl/L	

3.2.2. Preparation of GY

Indolin-2-one derivatives (*GY342*, *GY339*, *GY306* and *GY333*) were originally synthesized by Sultan Baytaş and her team in Gazi University Pharmacology Department. *GY342* (Mw= 444,48 g/mol), *GY339* (Mw= 444,48 g/mol), *GY306* (Mw= 474,51 g/mol) and *GY333* (Mw= 440,53 g/mol) were weighted individually and dissolved in DMSO (D5879; Sigma-Aldrich) to make 10 mM stock concentration then, subsequently diluted into several concentrations.

3.2.3. Cell Viability Assay

MCF7, MDA-MB-231 and MCF-12A cells were seeded into 96-well plates at 5×10^3 to 1×10^4 cells/well in 100 μ l total volume, incubated for 24 hours at 37°C and 5% CO₂. Then, the medium was replaced with fresh medium mixed with different concentrations of *GY342*, *GY339*, *GY306* and *GY333* (0-100 μ M) for 72 hr and then MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) analysis was performed. after old media were discarded, cells were treated with MTT solution (5 mg/mL) and incubated for 2 hr at 37°C, 5% CO₂. The wells were incubated in 100 μ l DMSO (D5879; Sigma-Aldrich) for 10 minutes at room temperature (RT) in the dark. Finally, absorbance values of samples were analysed at 470 nm wavelength (WL) by ThermoVarioSkan.

Statistical analyses were performed: IC₅₀ value of all compounds were calculated and analysed by Two-way ANOVA, SI values of *GY342*, *GY339*, *GY306* and *GY333* were calculated based on the ratio of IC₅₀ values obtained from MCF-12A cells to IC₅₀ values obtained from MCF7 and MDA-MB-231 cells.

3.2.4. Cell Death Analysis

MCF7 and MCF-12A cells were seeded into 96-well plates at $5-15 \times 10^3$ cells/well ratio which was depending on their doubling time, within 100 μ l complete medium and the cells were incubated for 24 hours at 37°C and 5% CO₂. Then, the old medium was replaced with fresh medium mixed with/without the indolin-2-one derivatives in different concentrations as 5 μ M, 10 μ M and the cells were incubated

for 24 hours under 37°C and 5% CO₂ conditions. Also, 10 µM concentration of cisplatin was used as positive control. After 24 hours treatment of the derivatives, without discarding the old media, the cells in each well were treated with 5 µl 1X AO/EtBr dye (235474/E7637; Sigma-Aldrich) which was diluted with PBS and had 100 µg/ml final concentration and the media in the wells mixed gently for 15-20 seconds with the AO/EtBr dye. Then, the media, AO/EtBr dye mixture in the well was discarded and the plates were analysed under the ZOE Fluorescent Cell Imager (ZOE™, BioRad) immediately. Images were captured at ×20 W/O filter and with FITC and Texas Red filters, respectively.

Statistical Analysis were performed: The cell number of each sample was counted by using ImageJ [Schneider et al., 2012] software programme and the ratio of dead cell/total cell number was calculated, then graphs were drawn by GraphPad Prism 8.3.0 tool of Microsoft Excel software.

3.2.5. Cell Morphology Analysis

MCF7 cells were seeded into 96-well plates at 1x10⁴ cells/well (depending on their doubling time) within 100 µl complete medium and incubated for 24 hours at 37°C and 5% CO₂. Then, the medium was replaced with fresh medium mixed with *GY342*, *GY339*, *GY306* and *GY333* at the concentration of 10 µM and incubated for 24 hr at 37°C and 5% CO₂. Afterwards, the old media was discarded from the wells and, the cells in each well were stained with 100 µl PI (25 µg/ml) and incubated for 10 minutes under dark conditions. After 10 minutes incubation with PI, the wells were washed with 100 µl 1X PBS solution once. Then, the cells in each well were fixed with 75 µl formaldehyde (4%) incubating 10 minutes in dark. After fixation of the cells, the fixative solution was removed from the wells and each well was stained with 100 µl DAPI (1µg/ml) for 10 minutes in dark. Finally, DAPI solution was discarded from each well and wells were washed with 1X PBS once and after discarding of PBS from each well, the plates were analysed under a fluorescent microscope (ZOE™, BioRad). Images were captured at 20X magnification, 25 µM scale bar. Images were arranged by ImageJ software and morphology of the cells were observed.

3.2.6. Wound Healing Assay

MCF7 cells were seeded into 96-well plates at 2.5×10^6 cells/ml in medium and incubated until cells reach 80-90% confluence at 37°C and 5% CO₂. When the cells reached 80-90% confluence, the cell layer was scraped using a 10 µl pipette tip as a thin line to form a wound area and to remove the cells detached on wound area, the wound area was washed with 1X PBS. Cells were treated with/without different concentrations of *GY342*, *GY339*, *GY306* and *GY333* (0 µM, 5 µM, 10 µM) in the media containing 1% FBS. Also, 10 µM concentration of cisplatin was used as positive control. The wound areas were imaged under inverted microscope (Leica DM IL LED) 0-48 hours after treatment and the images were captured at 5X by LAS V4.6 program. The images were analysed, wound areas were calculated using “MRI_Wound_Healing_Tool.ijm” tool of ImageJ software. Cell migration ratio and wound healing ratio were calculated by the formula:

$$\text{Wound Healing \%} = \left[\frac{A_{t=0h} - A_{t=\Delta h}}{A_{t=0h}} \right] \times 100\% \quad (3.1)$$

- $A_{t=0h}$ wound area just after scraping ($t=0h$).

- $A_{t=\Delta h}$ wound area h hours after scraping.

3.2.7. Colony Formation Assay

MCF7 cells were seeded into 6-well plates at 5×10^2 cells/ml in medium and incubated for 24 hr at 37°C and 5% CO₂. Afterwards, the cells were treated with/without a medium containing derivative at certain concentrations 0 µM, 5 µM and 10µM for 24 hours and the medium was changed at the end of the incubation. The medium was changed approximately every 3 days and colonies were allowed to form and colony formation was followed daily under the microscope. The cell mass was considered as one colony which has the minimum population of 50 or more cells. After colony formation was observed the media was discarded and the cells in the colonies were fixed by incubating with the fixative 4% formaldehyde which was dissolved in 1X PBS for 20 minutes. Then, the cells were incubated with 0.1% crystal violet which prepared in dH₂O for 15 minutes. At the end of the incubation, the wells were rinsed

with dH₂O at approximately 70 rpm shaking to remove the washed dye remains and the forming colonies were photographed by a camera. Plating efficiency was calculated by the formula:

$$\text{Plating Efficiency} = \frac{\text{Number of the colonies}}{\text{Number of inoculated cell}} \times 100 \quad (3.2)$$

3.2.8. Flow Cytometer

MCF7 cells were inoculated into 6-well plates as 3×10^5 cells/well confluence and incubated for 24 hours at 37°C and 5% CO₂. Then, the old medium was replaced with serum-free medium and after 2 hours of serum starvation, serum-free medium was replaced with serum-containing medium including *GY342*, *GY339*, *GY306* and *GY333* in different concentrations as 0 µM, 5 µM, 10 µM. The cells were incubated for 24 hours under 37°C and 5% CO₂ conditions. At the end of the incubation, the cells were collected into falcon tubes one by one by trypsinization and once washed with 1X PBS. Collected cells were centrifuged at RT and 1000 g for 5 minutes. Supernatant was discarded and pellet was washed with 1X PBS for each sample. The samples were centrifuged at RT and 1000 g for 5 minutes once again. Supernatant was discarded, the pellets were dissolved with 70% ethanol for fixation and stored at +4°C for half an hour, then -20°C until next step of the experiment.

Samples were centrifuged at RT and 1000 g for 5 minutes; supernatant was discarded, and pellets were washed with 1X PBS and were centrifuged at RT and 1000 g once again. Supernatant of each sample was discarded, the pellet in each tube was treated with 300 µl PI solution which was prepared as containing 0,5% RNase and incubated at 37°C for half an hour. After incubation, cells were centrifuged at RT and 1000 g for 5 minutes to remove dye in supernatant and cells were collected as pellets. The cell pellets were dissolved into PBS and the fluorescent light emission which was emitted by PI forming a complex with DNA of each individual cell was measured by BD Accuri C6 flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA).

3.2.9. Protein Analysis, Western Blotting

MCF7 cells were inoculated into petri dishes as 12×10^5 cells/petri confluence and incubated for 24 hours at 37°C and 5% CO₂. After 24 hr incubation, old medium was changed with fresh media containing derivatives at certain concentrations 0 μ M, 5 μ M, 10 μ M and 20 μ M. after incubation cells were collected by scrapper with ice cold 1X PBS in 24h. Collected cells were centrifuged at room temperature (approximately 25°C) and 1200 rpm to allowed them precipitated. After centrifugation, all following steps were performed in ice cold conditions. Supernatant was removed and the pellets in each sample was mixed well with 70 μ l lysis mixture. Samples were centrifuged at +4 °C and 14000 g for 10 minutes. Supernatants of each sample were collected into clean tubes individually and the cell lysates were stored at -20°C until further steps of the experiment.

Thermo BCA Protein Assay was performed to determine total protein concentration in the cell lysates. 10 μ l of each lysates were loaded into 96-well plates with 3-4 replicates of each sample. Also, for the blank 10 μ l only lysis buffer was loaded into wells. In the ratio of 50:1, Reagent A and Reagent B depending on manufacturer's instructions were prepared as BCA solution and 200 μ l of BCA solution was loaded into each well. The plate was shaken gently and incubated at 37°C in dark for half an hour. Following incubation, the plate was kept at RT for 5 minutes and then, absorbance of the protein samples was measured at 562 nm WL by microplate reader (ThermoVarioSkan).

Statistical Analysis were performed: To determine the relation between absorbance and standard protein concentration which are serially diluted, Bovine serum albumin (BSA) procedure was performed. According to BSA results, graph of absorbance-standard protein concentration was drawn, and the formula was calculated by Microsoft Excel software. Also, R² value of the BSA graph were calculated to confirm goodness-of-fit measure for linear regression between absorbance value and standard protein concentration. R² value was needed to be above 0.95 for the formula of the graph to be considered as reliable. The mean values of each absorbance of protein samples were calculated. According to the formula of the graph of standard protein concentration/absorbance, which is obtained from BSA results, protein concentrations in μ g/ml were calculated by absorbance mean of protein samples.

In purpose of comparison of protein levels between the samples, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting techniques were performed. Polyacrylamide gels were prepared as given in Table 3.3. for SDS-PAGE gel electrophoresis.

Table 3.3: Resolving gel and Stacking gel ingredients and their final volumes.

	Resolving Gel (10%)	Stacking Gel (4,5%)
Acrylamide/Bisacrylamide, 40% (w/v)	1,875 ml	337,5 µl
SDS, 10% (w/v)	75 µl	30 µl
APS, 10% (w/v)	75 µl	30 µl
TEMED, 100%	5,5 µl	3 µl
dH₂O	3,625 ml (To complete final volume)	1,865 ml (To complete final volume)
Tris-HCl 0.5 M pH 6.8		750 µl
Tris-HCl 1.5 M pH 8.8	1,875 ml	

Volume of 40 µg protein in each sample was calculated according to protein concentration of the samples and protein samples in determined amounts were mixed with certain amount of 5X loading dye to final concentration of loading dye become 1X. Proteins were denatured at 95°C for 5 minutes in Heat Blocker. After denaturation process, the samples were loaded into wells of the acrylamide gels. Protein samples were run through the stacking gel at 90 V and through the resolving gel at 120 V until loading dye reached end of the gels.

Following SDS-PAGE process, transfer of proteins from acrylamide gel to the PVDF membrane was achieved at 110 V for 1 hour 30 minutes. After transfer step completed, blocking step was performed, membranes were treated with blocking solution and shaken at 110 rpm for 1 hour at RT. After blocking step, for washing step, the membranes were washed with 1X TBS-T for 10 minutes shaking at 110 rpm three times. The membranes were treated with primary antibody overnight at +4°C shaking.

The membranes were washed with 1X TBS-T for 10 minutes shaking at 110 rpm three times again and kept into HRP-conjugated secondary antibody for 2 hours at

+4°C shaking. Then once more washing step was applied, and membranes were imaged by ChemiDoc (BioRad).

4. RESULTS

4.1. Indolin-2-one Derivatives Reduce Cell Viability

To determine the effect of the indolin-2-one derivatives on cell viability, MTT test was performed with two breast cancer cell lines MCF7, MDA-MB-231 and non-cancerous cell line MCF-12A.

Our results showed that, IC_{50} values of *GY342*, *GY339*, *GY306* and *GY333* for MCF7 cells were calculated as 15.8 μ M, 7.54 μ M, 5.78 μ M and 4.92 μ M respectively. Moreover, %50 inhibition values for MDA-MB-231 cells were 15.33 μ M, 6.68 μ M, 4.02 μ M, 5.22 μ M and for MCF-12A cells were 37.69 μ M, 26.92 μ M, 29.5 μ M, 25.14 μ M, respectively. SI values of derivatives were calculated based on the calculation of ratio of IC_{50} values obtained from noncancerous cells to IC_{50} values obtained from cancer cells. In this sense, SI values of *GY342*, *GY339*, *GY306* and *GY333* for MCF7 cells were 2.38, 3.57, 5.1 and 5.1 and for MDA-MB-231 cells were 2.45 μ M, 4.02 μ M, 5.22 μ M and 6.04 μ M respectively as given in table 4.1. and shown in Figure 4.1.

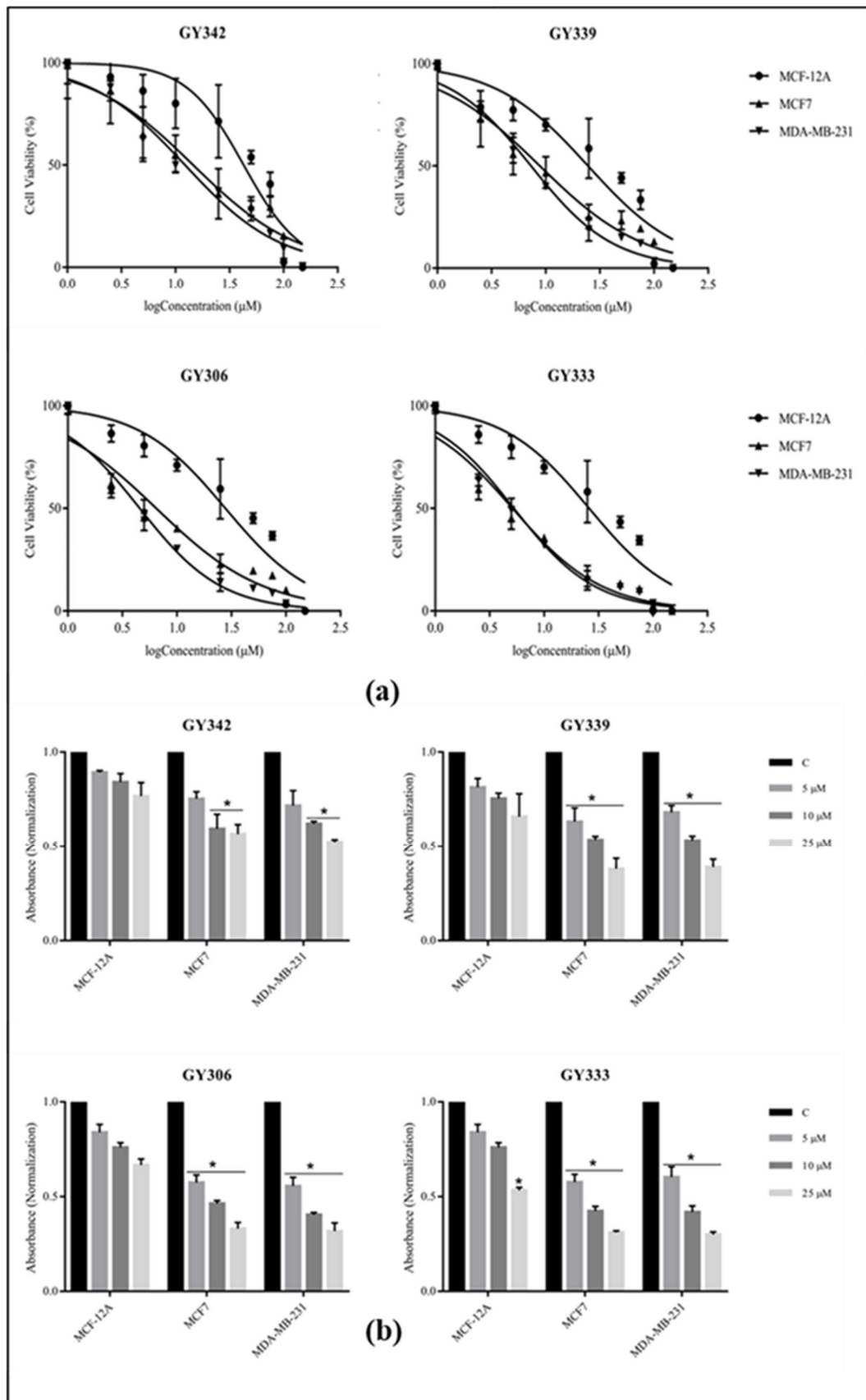


Figure 4.1: *GY342*, *GY339*, *GY306* and *GY333*, dose-dependent inhibition of MCF-12A, MCF7 and MDA-MB-231 cell viability in 72h exposure. a) Dose response graphs, b) Bar graphs.

Table 4.1: IC₅₀ and SI values of *GY342*, *GY339*, *GY306* and *GY333* for MCF-12A, MCF7 and MDA-MB-231.

	GY342		GY339		GY306		GY333	
	IC ₅₀ (μM)	SI	IC ₅₀ (μM)	SI	IC ₅₀ (μM)	SI	IC ₅₀ (μM)	SI
MCF-12A	37.69 ± 1.6		26.92 ± 1.3		29.5 ± 1.2		25.14 ± 1.5	
MCF7	15.8 ± 0.72	2.38	7.54 ± 0.65	3.57	5.78 ± 0.9	5.1	4.92 ± 0.87	5.1
MDA-MB-231	15.33 ± 0.83	2.45	6.68 ± 0.7	4.02	4.02 ± 0.65	5.22	5.22 ± 0.6	6.04

Based on these results, it can be inferred that the derivatives affect cancer cells at lower doses when compared to non-cancer cells. SI was 2 and 4 in cancer cells treated with *GY342* and *GY339*. Significantly, SI of *GY306* and *GY333* was 5< in both cancer cell lines. In other words, although the selectivity index of the *GY342* molecule is adequate, other three molecules had higher selective toxicity to both cancer cell lines. Due to having similar results for both breast cancer cells; further detailed experiments were continued with MCF7 cell line and with noncancerous MCF-12A cells.

4.2. Indolin-2-one Derivatives Inhibit MCF7 Wound Healing

The main principal of wound healing assay is to investigate movement of a cell population under a certain condition. This assay was performed based on IC₅₀ values of the indolin-2-one derivatives to observe effect of indolin-2-one derivatives on the migration ability of the cells. Results showed that, the delay of wound closure in the MCF7 cells treated with *GY342* (Figure 4.2) compared to control cells within 48 hours has started at 10 μM concentrations. However, cells treated with *GY306*, *GY339* and *GY333* delay of wound closure was observed at 5 μM concentrations.

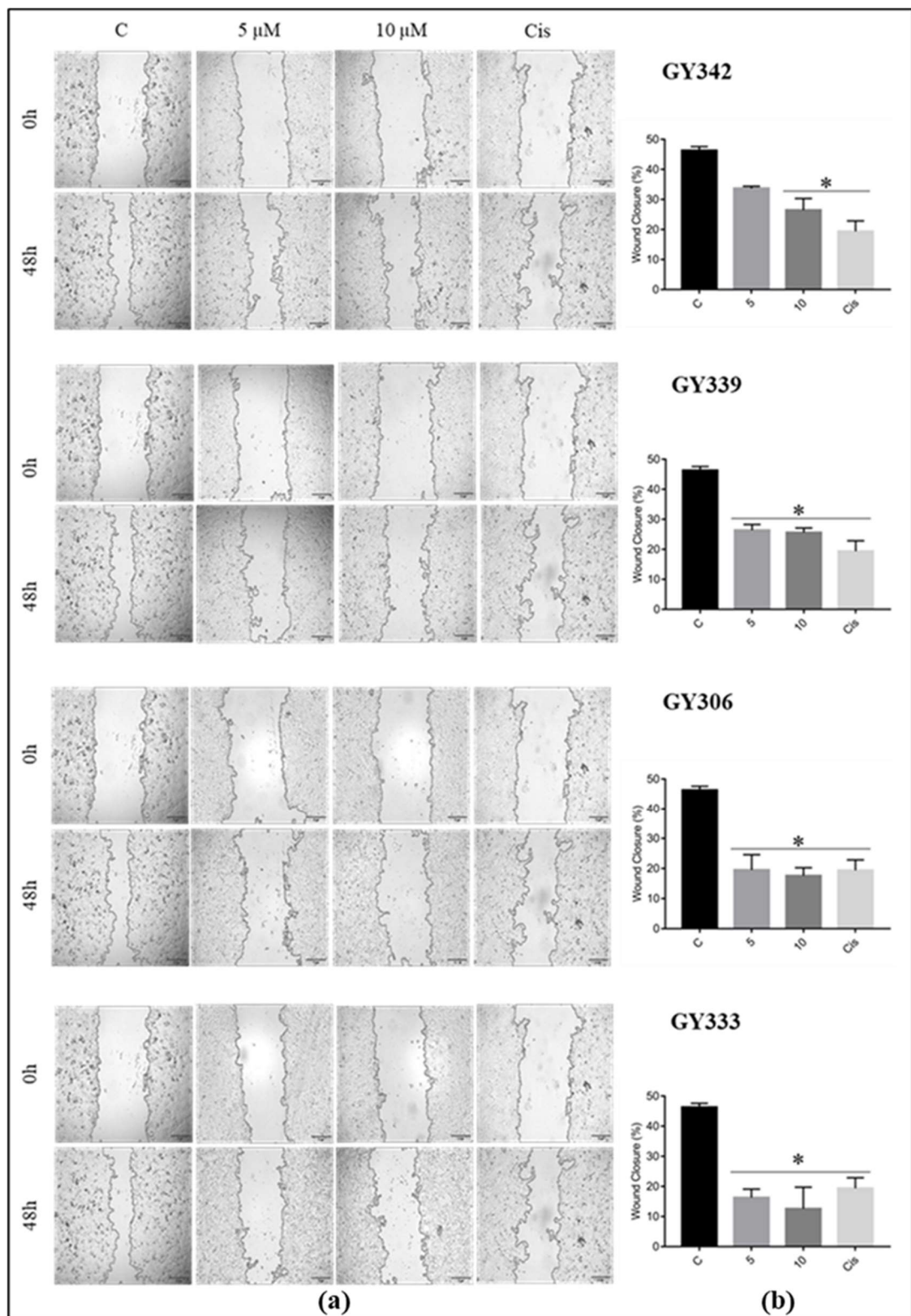


Figure 4.2: Effect of indolin-2-one derivatives on cellular wound healing in MCF7 cells between 0-48 hours. a) Clouse of wound in MCF7 cells treated with 5 and 10 μ μ M doses of derivatives between 0-48 hours. b) Wound healing ratio were calculated for each of derivatives. *p<0,01

4.3. Indolin-2-one Derivatives Inhibit Colony Formation Ability in MCF7 Cells

Colony formation assay is a kind of cell survival assay based on the ability of a single cell to grow and form a colony. This assay was performed to evaluate the indolin-2-one derivatives effect the limit of the division ability of MCF7 cells. Colony formation in MCF7 cells was inhibited starting from 10 μM dose of *GY342*, while *GY306*, *GY339* and *GY333* derivatives slowed down the colony formation at about 5 μM concentration (Figure 4.3).

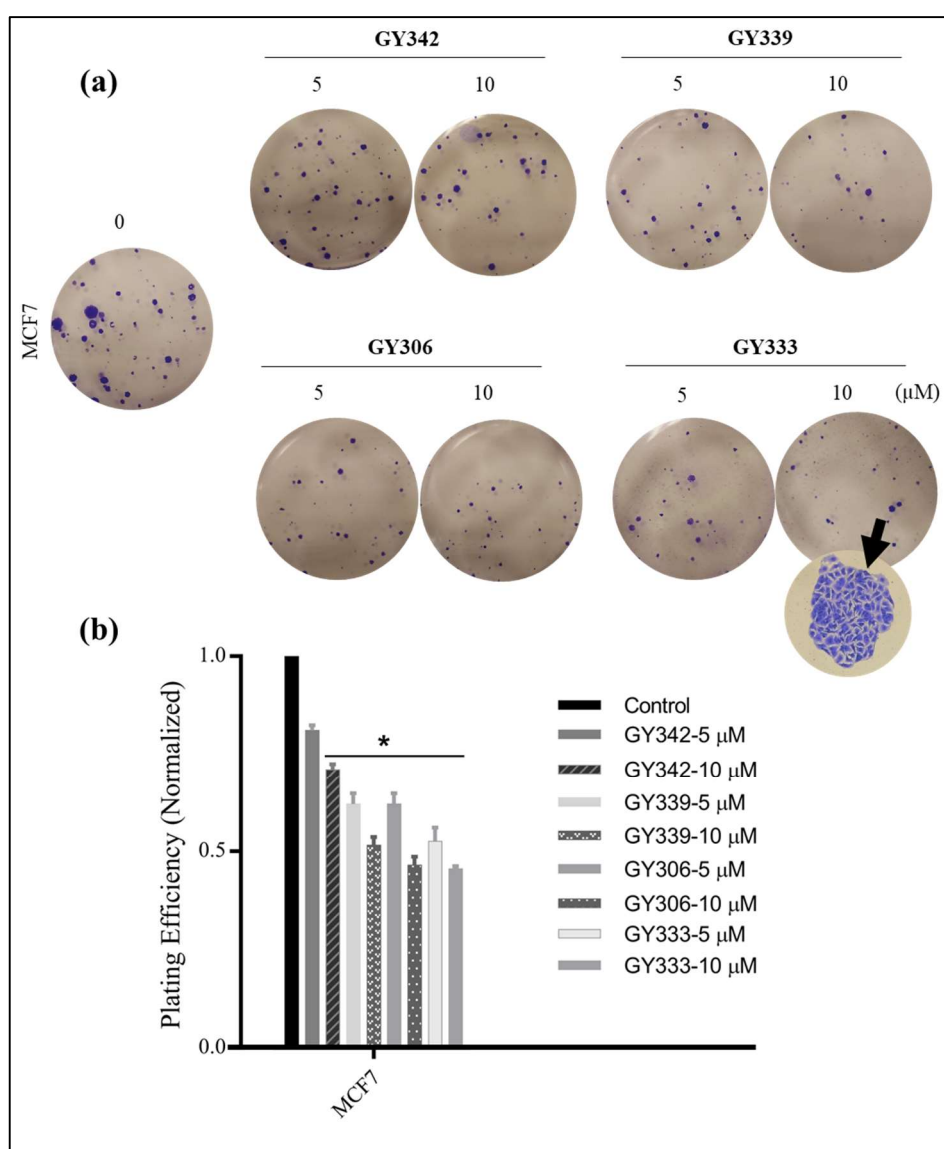


Figure 4.3: Effect of indoline-2-one derivatives on colony formation ability of MCF7 cells in dose-dependent manner in 24 hours exposure. a) MCF7 colonies. b) Graph of plating efficiency * $p < 0.01$.

4.4. Indolin-2-one Derivatives Cause S phase arrest on MCF7 Cell Cycle

PI staining and flow cytometer analysis were used to detect the effect of indolin-2-one derivatives on cell cycle of MCF7 cells. Results indicated that compare to untreated control cells three indolin-2-one derivative treated cells were accumulated at S phased in a dose-dependent manner except the *GY342*. However, both cells treated with 10 μ M doses of *GY339* and *GY306*, and with 5 μ M dose of *GY333* arrested at S phase of the cycle (Figure 4.4).

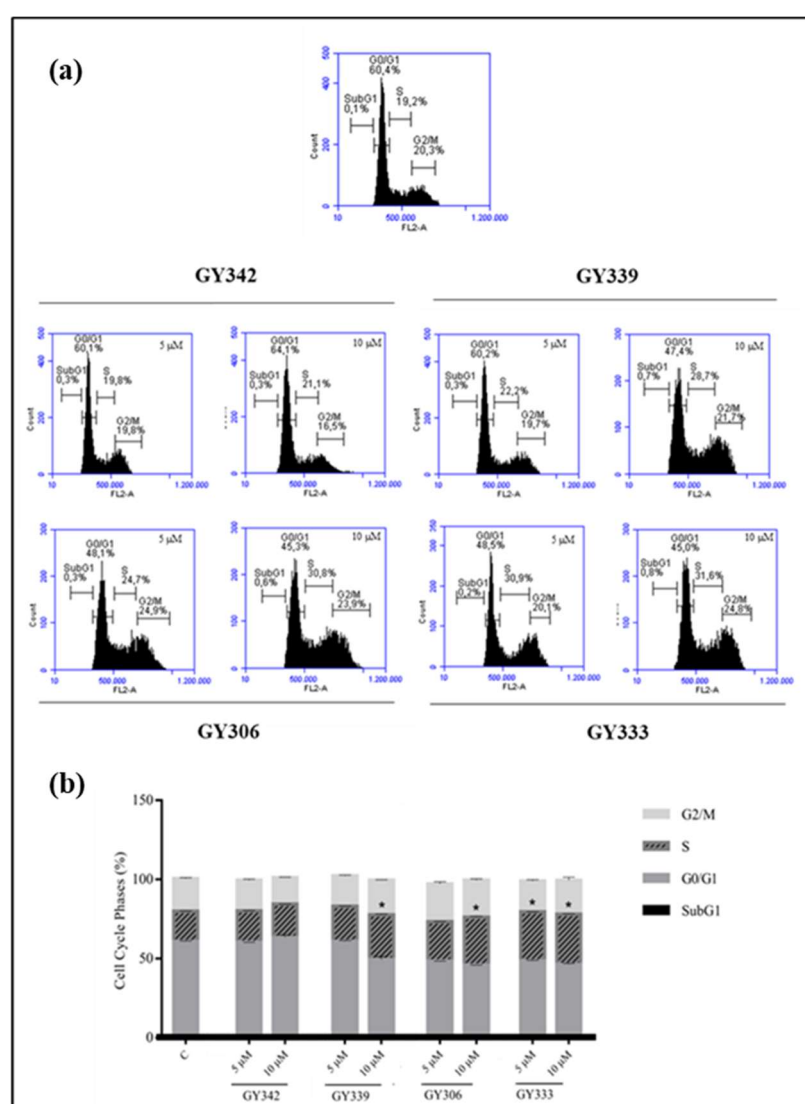


Figure 4.4: The effect of indolin-2-one derivatives on cell cycle MCF7 cells. a) The effect of indolin-2-one derivatives on cell cycle profile of MCF7 cells in concentration dependent manner. b) Graph of percentage display of cell cycle phases in MCF7 cells * $p < 0.01$.

4.5. Indolin-2-one Derivatives Lead MCF7 Cells to Death

To evaluate whether indolin-2-one derivatives can cause cell death, two different types of double staining (AO/EtBr and PI/DAPI) process were performed with MCF-7 cells and with non-cancerous cell line. MCF-12A cells were exposed to indolin-2-one derivatives showed that none of the derivatives made any lethal effect at the concentration have been used in treatments (Figure 4.5a). When microscope images were examined, it is seen that the decrease in the cell number was minimal, compared to breast cancer cell line at similar concentrations (Figure 4.5b). Number of cells were decreased when treated with compound *GY342*, however it was not found statistically significant. Nevertheless, cells treated with *GY339* and *GY306* caused significant reduction at 10 μ M and *GY333* at 5 μ M (Figure 4.6).

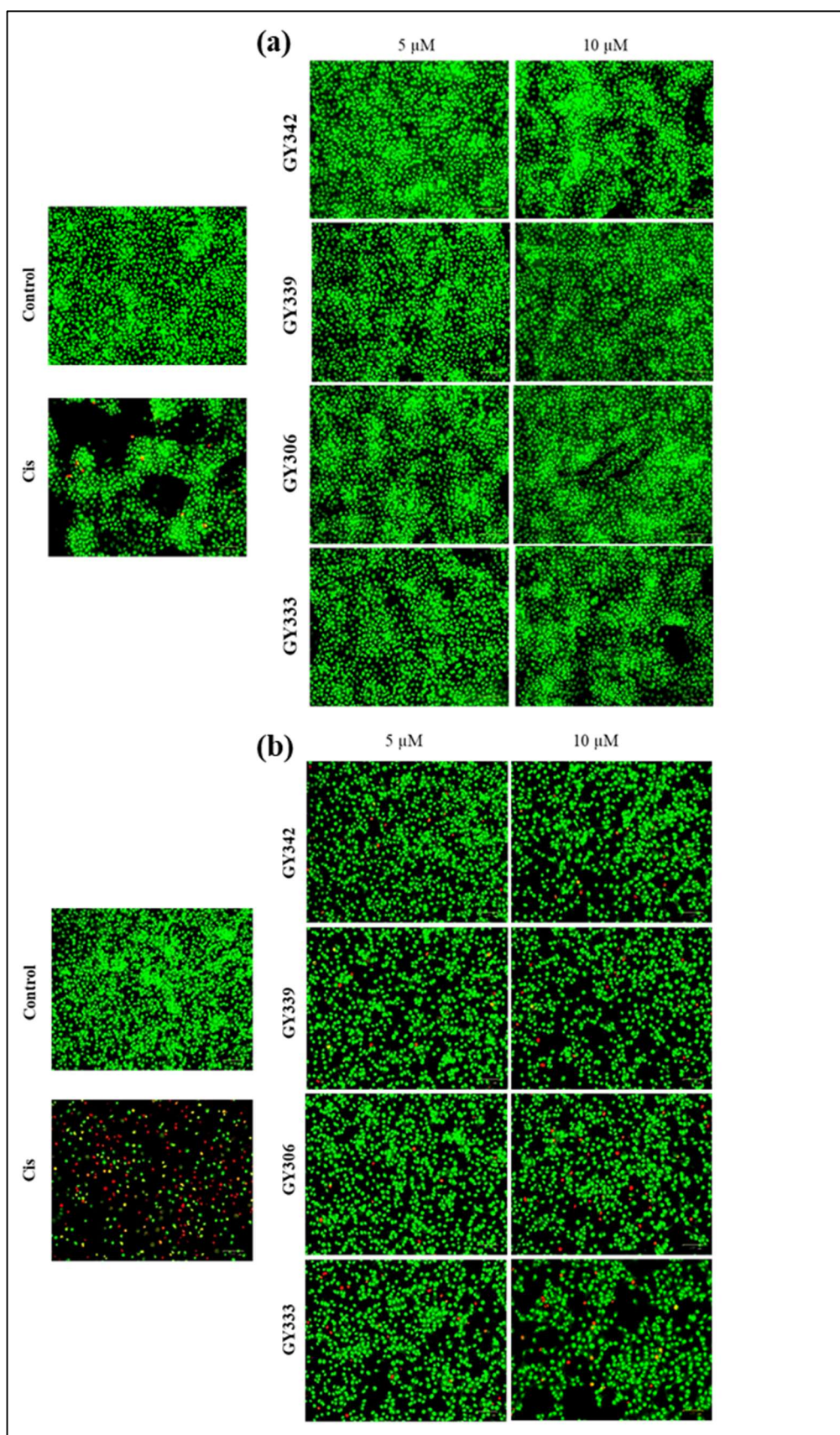


Figure 4.5. Demonstration of cell death in a) MCF-12A and b) MCF7 cells. Cell death was determined by AO/EtBr staining in 24 hours exposure of 5 and 10 μ M indolin-2-one derivatives (20X magnification, 100 μ m scale bar).

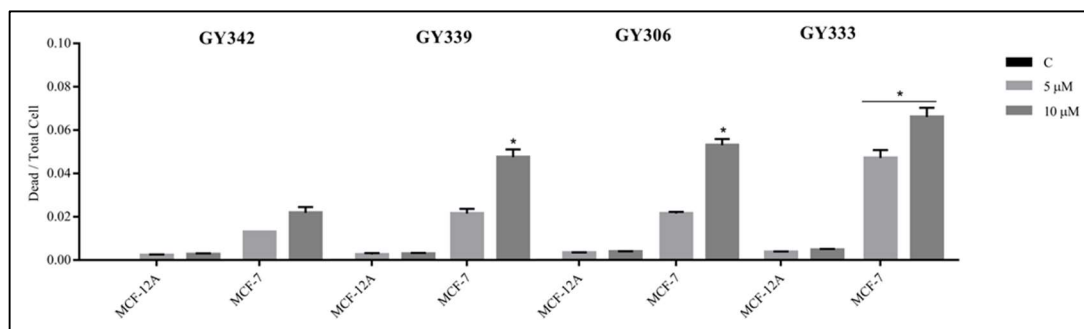


Figure 4.6: AO-EtBr dual immunofluorescence staining. After 24hour exposure of *GY342*, *GY339*, *GY306* and *GY333* to MCF7 cells, the graph shows the Dead/Total cell ratio * $p < 0.01$.

As shown in the figures above, in the first staining we observed that dose dependent apoptotic and necroptotic morphology in the cells after treatments. In addition to this staining, we also performed DAPI/PI staining with similar principle that also based on loss of cell membrane integrity. While all cells are stained by DAPI, PI can enter only the cells whose impaired integrity of cell membrane and thus, PI stains dead cells. As shown in Figure 4.7, normal nuclei without PI staining shows healthy cells, Cells that show nuclear fragmentation, chromatin condensation, or nuclear condensation without PI staining are apoptotic cells. Cells positive for nuclear fragmentation or chromatin condensation in the presence of PI typically undergo late apoptosis. In contrast, cells that are positive for PI and show normal nuclear morphology or nuclear condensation undergo necrosis. In Figure 4.7 while living cells are shown by white arrow, the yellow and the blue arrows indicate late apoptotic and red arrows show cells undergone necrosis.

As observed in Figure 4.7 except compound *GY342*, 10 μ M of all three indolin-2-one derivatives induced cell death, in which apoptotic morphology were detected such as chromatin condensation and nuclear fragmentation.

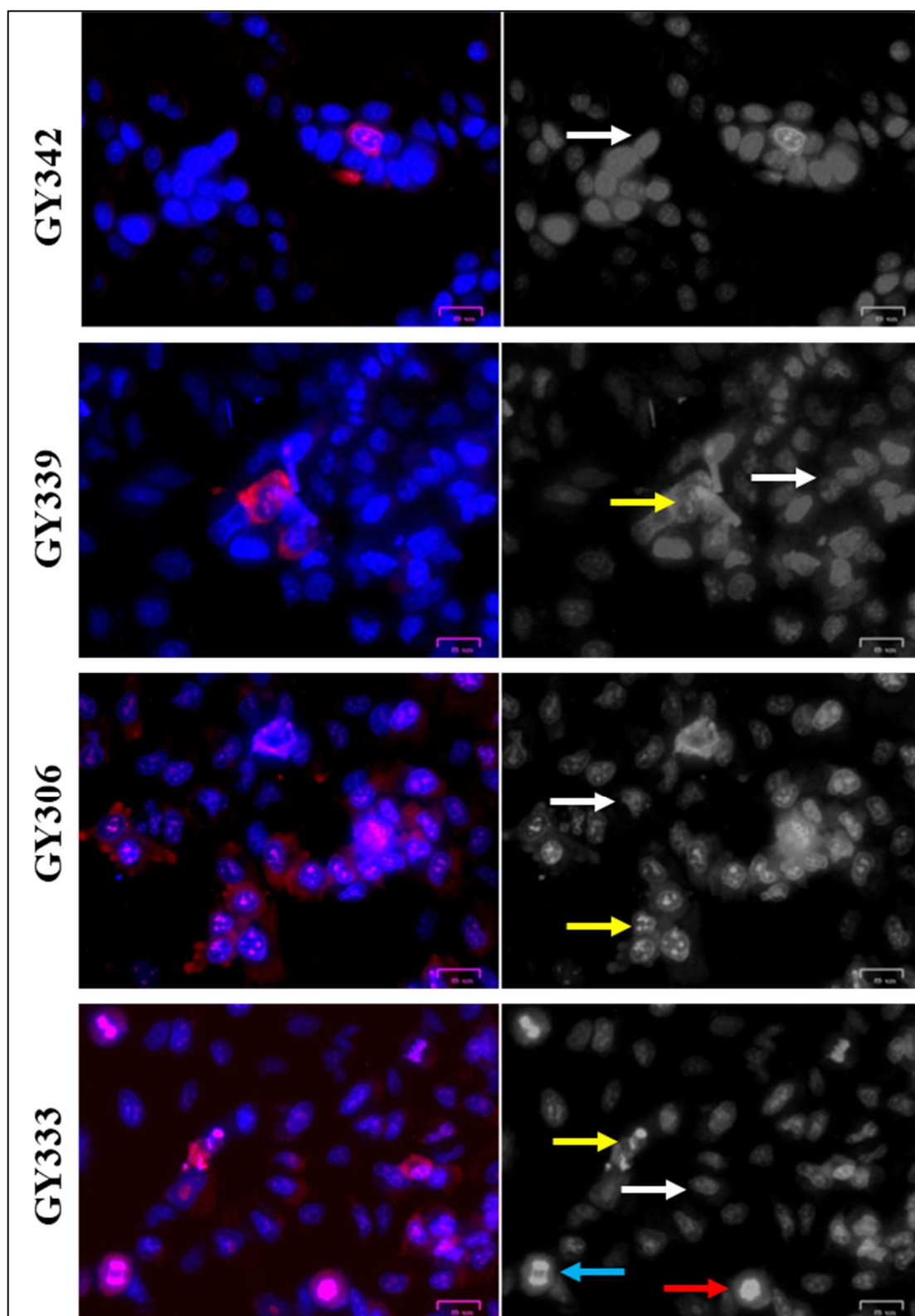


Figure 4.7: Representative images of DAPI/PI staining showing MCF cells' death after 24 hr of exposure of 10 μ M of *GY342*, *GY339*, *GY306* and *GY333* treatment, by ZOE fluorescence cell imager; 20X magnification, 25 μ M scale bar.

*Normal (not damaged) nuclei is showed by white arrow; chromatin condensation is showed by yellow arrow; nuclear condensation is showed by red arrow and nuclei fragmentation is showed by blue arrow.

4.6. Indolin-2-one Derivatives Effected Protein Phosphorylation and Expression in MCF7 Cells

In cell viability tests, derivatives were found to have a negative effect on cell proliferation in MCF7 cells, and the decrease in cell proliferation was supported by immunofluorescent staining. ERK1/2 phosphorylation, which plays a main role in cell proliferation, was significantly suppressed at a concentration of 10 μ M in MCF7 cells treated with *GY342*, *GY339*, *GY306*, and at 5 μ M in cells treated with *GY333*. Moreover, AKT phosphorylation was inhibited in the cells treated with 20 μ M of *GY342*, 10 μ M of *GY339* and *GY306* and 5 μ M of *GY333*. In addition, STAT3 phosphorylation was significantly suppressed in cells at 5 μ M concentrations of all derivatives. However, ERK5 phosphorylation was inhibited only by *GY333* starting from 10 μ M concentrations.

Cell cycle analysis showed that accumulation of cells at S phase after treatment with three of derivatives (Figure 4.4). To confirm these results, we also evaluated the expressions of CDKI proteins p21 and p27. Results indicated that both proteins were increased depending on applied derivatives and their concentrations in MCF7 cells. P21 levels were increased when cells treated with 20 μ M concentrations of *GY342*, with 10 μ M of *GY339* and *GY336* and with 5 μ M of *GY333*.

Previously morphological analysis showed that derivatives cause concentration dependent apoptotic morphology such as chromatin condensation and nuclear fragmentation (Figure 4.7). Based on this, we investigated the level of Bcl-2 and Bax proteins in MCF7 cells when exposed to derivatives. Results showed that while Bax protein levels were upregulated, bcl-2 levels were downregulated in cancer cells. Increase in Bax level and decrease in bcl-2 level were started at concentrations of 20 μ M of *GY342*; 10 μ M of *GY339* and *GY306* and 5 μ M of *GY333* (Figure 4.8.). Interestingly, the levels of JNK and p38 phosphorylation were increased in these cells exposed to derivatives. Activation of these pathways triggers intrinsic apoptosis, As seen in Figure 4.8, level of stress proteins increased with 10 μ M concentration of *GY342*, *GY306*, *GY339* and with 5 μ M concentration of *GY333*.

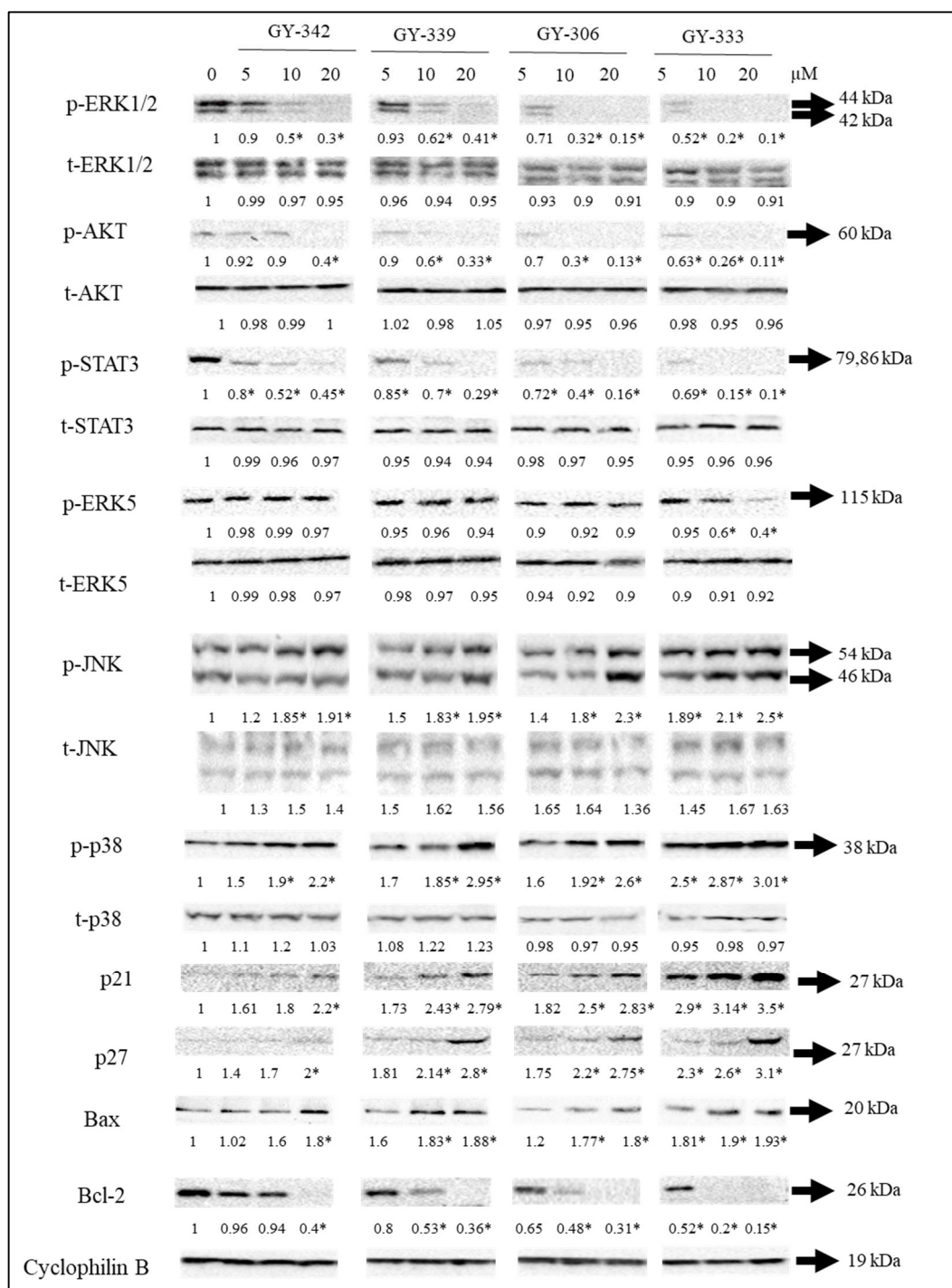


Figure 4.8: Effect Indolin-2-one derivatives on phosphorylation of proteins and protein expression in MCF7 cells *p<0,01.

5. DISCUSSION

Breast Cancer is one the most aggressive and fatal cancer type which early diagnosis and treatment is crucial. Heterocyclic compounds are anticancer agents targeting several cancers including breast cancer [Kaur et al., 2019]. Mitomycin C, beta carboline alkaloids (harmaline), bisindole alkaloids (dragmasidin D) and marine alkaloids (eudistomin K) are heterocyclic compounds which are known showing anticancer activity by inducing apoptosis and modulating various signalling pathways [Biersack et al., 2012]. Also, other 2-indolinone derivatives were reported as having anti-proliferative and apoptotic activity [Yao et al., 2020].

In our experiments originally synthesized Indolin-2-one derivatives showed cytotoxic effects at significantly lower doses in cancer cell lines compared to non-cancerous cell line. Thus, indicating that derivatives have high selectivity to cancer cells. In both breast cancer cell lines, derivatives showed similar selectivity compared to non-cancerous cell line and similar cytotoxicity effect. Therefore, further experiments were carried on with MCF7 cell line.

Decreased phosphorylation level of ERK1/2, STAT and AKT proteins, which are involved in cell proliferation were detected in MCF7 cells treated with derivatives in concentration-dependent manner. Also, there was a significant reduction in ERK5 phosphorylation in cells treated with *GY333*.

Cell migration, which is controlled by both intracellular and extracellular signals, plays an important role in cancer progression, spread and even in metastasis [Polacheck, et al., 2012]. Previously, the effect of compounds containing indolin-2-one on cell migration in hepatocellular carcinoma cells reported that cell migration was inhibited in cells treated with the compound [Wang et al., 2015]. In another study, it was reported that cell migration was inhibited in a metastatic lymph node lesion of human prostate cancer cell line treated with another compound containing an indolin-2-one domain [Saeed Nazari et al., 2020]. The cell migration inhibitory effect of the indolin-2-one derivatives in MCF7 cells showed that inhibition starting from 10 μ M concentration of *GY342*, and from 5 μ M of *GY339*, *GY306* and *GY333*.

The ability of cancer cells to divide continuously causes cell aggregation, leading to tumour formation and tumour growth in size. In a study published by C. Karthikeyan et al., the effect of derivatives of a compound containing indolin-2-one domain on the

colony forming abilities of ovarian cancer cells was investigated that these derivatives significantly inhibited colony formation of ovarian cancer cells [C. Karthikeyan et al., 2019]. In our study, when the effect of indolin-2-one derivatives on the colony forming ability of MCF7 cells was examined, it was observed that the derivatives significantly inhibited colony formation in dose-dependent manner.

Cell cycle arrest is an important mechanism for cytotoxicity studies on cancer treatments [Collins et al., 1997], [O'Grady et al., 2015]. In a previous study examining the effect of a compound containing the indolin-2-one domain was reported that the compound exposed cells arrested at G2/M phase [M. Al-Sanea et al., 2021]. In our study, Indolin-2-one derivatives (*GY339*, *GY306* and *GY333*) caused accumulation of cells at S phase and as well as increment the level of p21 and p27 proteins, which play a role in cell cycle inhibition by binding to the cyclin-CDK complex.

The cytotoxic effect of the indolin-2-one derivatives was reinforced by the effect of the derivatives using immunofluorescence imaging that was shown that the dose-related cell death rate increased, and viable-cell number decreased in the cells exposed to indolin-2-one derivatives. When the analysis of the number of dead and living cells treated with *GY339*, *GY306* and *GY333* are examined, the number of viable cells was significantly decreased in MCF7 cells in concentration-dependent manner. However, MCF-12A was not affected with those treatments and the selectivity of the cytotoxic effect of the derivatives in MCF7 cells compared to MCF-12A was supported by these results. Besides, while the indolin-2-one derivatives *GY339* and *GY306* were found to be effective in cell death, fluorescence imaging showed that *GY333* was particularly effective at lower doses. The chromatin condensation and nuclei fragmentation have been detected in MCF7 cells exposed to derivatives indicate late apoptosis which was supported via protein analysis showing an increase in the level of Bax and a decrease in the level of the anti-apoptotic protein Bcl-2. Also, phosphorylation level of apoptotic proteins p38 and JNK which regulates intrinsic pathway of stress-induced apoptosis and target bcl-2, increased in the cells treated with the derivatives. Previous studies have reported that S phase arrest is mostly associated with activation of mitochondrial apoptosis [Xu Yi, et al., 2015], [Qiaoying Zhu, et al., 2014]. It is known that by activating the phosphorylation of JNK protein, it causes upregulation of pro-apoptotic genes and activates the apoptosis signalling pathway. [N. Dhanasekaran & E. Reddy., 2011]. Another protein, mitogen-activated protein (MAP) kinase p38 plays an important role in regulation of apoptosis intrinsic-pathway [Beibei Cai, et al., 2006].

Increases in JNK phosphorylation and p38 protein level may indicate that the derivatives activate the apoptotic pathway in MCF7 cells via those stress pathways.

In summary, Indolin-2-one derivatives have a cytotoxic effect in MCF7 cells and this effect highly selective to cancer cells. The derivatives limit the colony formation and migration ability of cancer cells; able to arrest cell cycle and trigger apoptosis. Among the all four derivatives the efficacy of *GY333* was observed higher in breast cancer cells.

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APPENDICES

Appendix A: Publications Within the Scope of the Thesis

Nimet Sule YUNCU, Gulseren Turhal, Sultan Nacak Baytas, Asuman Demiroglu Zengeroglu, (2022), "Effect of indolin-2-one-based derivatives on viability and apoptosis of breast cancer cells". 5th international Eurasian conference on biological and chemical sciences, ISBN :978-605-72134-0-2 (1731) Ankara/ Turkiye, Nov 23-25 2022