SCREENING OF NEW DRUG CANDIDATES WITH ANTITUMOR ASSAYS

by

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This thesis is dedicated to my family…

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ABSTRACT

SCREENING OF NEW DRUG CANDIDATES WITH ANTITUMOR ASSAYS

Drug development process consists of discovery and research, preclinical studies, clinical trials and approval phases. During discovery and research process, one of the main issues is pharmacological activity determination of either synthesized, proposed or screened compounds. During our study, selected candidates having chemical structures that were 4-(1,3-dioxoisoindolin-2-yl) benzenesulfonamide and 4-(4,5,6,7-tetrafluoro-1,3-dioxoisoindolin-2-yl) benzenesulfonamide structured thalidomide derivatives, 4-phenylpiperidine derivatives for sigma receptor ligands, 1-(naphthalen-2-ylmethyl) piperazine derivatives synthesized in Yeditepe University, Faculty of Pharmacy were screened for their anti-tumor activity by using SH-SY5Y human neuroblastoma cell line, HT29 human colon cancer cell line, MDA-MB-231 human breast cancer cell line. As a healthy control, cytotoxic effects of compounds were assed using L929 mouse fibroblast cell line by MTT experiment. The results showed that some compounds belonging to all three of these groups have significant anti-tumor activity that may qualify them to be suitable candidates for clinical use. In order to understanding the mode of action of these putative drug candidates, examination of expression levels of different genes in cells' DNA after the exposure has been carried out; our preliminary work has at this stage concentrated on RT-PCR analysis of common cell cycle-related genes such as *p21*, *cyclin D1*, *c-fos* and *elk-1*. However, further detailed molecular analysis will be required to get an in-depth understanding of the molecular mechanisms.

ÖZET

ANTİ TÜMÖR DENEYLERİYLE YENİ İLAÇ ADAYLARININ TARANMASI

İlaç geliştirme süreci, keşif ve araştırma, klinik öncesi çalışmalar, klinik çalışmalar ve onay olmak üzere dört ana fazdan oluşur. Keşif ve araştırma süreci boyunca ele alınan temel konulardan biri sentezlenmiş ya da taranmış bileşiklerin farmokolojik aktivitelerinin belirlenmesidir. Çalışmamız sırasında Yeditepe Üniversitesi Eczacılık Fakültesi tarafından sentezlenen, 4-(1,3-dioksiisoindolin-2-yl) benzensülfonomid ve 4-(4,5,6,7-tetrafloro-1,3-dioksiizoindolin-2-yl) benzensulfonamid kimyasal yapısındaki talidomid türevleri, 4-fenilpiperidin türevi olan sigma reseptör ligandları, 1-(naftalin-2-ylmetil) yapısındaki piperazin türevleri, SH-SY5Y insan nöroblastoma hücre hattı, HT29 insan kolon kanseri hücre hattı, MDA-MB-231 insan meme kanseri hücre hatları kullanılarak anti-tümor akitivitelerinin belirlenmesi için taranmıştır. Bileşiklerin sitotoksik etkileri sağlıklı fare fibroblast hücresi olan L929 hücre hattı üzerinde MTT deneyleri ile taranmıştır. Elde edilen sonuçlar bu üç gruptan bazı bileşiklerin klinik kullanımlar için uygun olarak nitelendirilebilecek önemli antitumor aktiviteye sahip olduğunu göstermiştir. Bu ilaç adaylarının etki mekanizmasını anlamak için, hücrelere uygulandıktan sonra hücrelerin DNAsındaki çeşitli genlerin ekspresyon seviyeleri incelenmiştir. Bu aşamada ön çalışma olarak *p21, cyclinD-1, c-fos* and *elk-1* gibi belli başlı hücre döngüsü genlerinin PZR analizlerine odaklanılmıştır. Ancak moleküler mekanizmaların detaylı bir şekilde anlaşılabilmesi için daha kapsamlı moleküler analizler gerekmektedir. Bu aşamada ön çalışma olarak *p21, cyclinD-1, c-fos* and *elk-1* gibi belli başlı hücre döngüsü genlerinin PZR analizlerine odaklanılmıştır. Ancak moleküler mekanizmaların detaylı bir şekilde anlaşılabilmesi için daha kapsamlı moleküler analizler gerekmektedir.

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LIST OF SYMBOLS / ABBREVIATIONS

ADME	Absorption, distribution, metabolism and excretion
ATCC	American Type Culture Collection
cDNA	Complementary deoxyribonucleic acid
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's Minimal Eagle Medium
DMSO	Dimethylsulfoxide
DMT	Dimethlytryptamine
ECG	Electrocardiogram
EDTA	Ethylenediaminetetracetic acid
EMA	European Medicine Agency
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITM	First in man study
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HT29	Human Colon Adenocarcinoma Cell Line
IMIDs	Immınomodulatory drugs
L929	Mouse Fibroblast Cell Line
MDA-MB-231	Human Breast Adenocarcinoma Cell Line
ml	Milliliters
µl	Microliters
mM	Millimolar
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
NCI	National Cancer Institute
NMR	Nuclear Magnetic Resonance
RNA	Ribonucleic acid
SH-SY5Y	Human Neuroblastoma Cell Line
TM	Transmembrane
VEGF	Vascular Endothelial Growth Factor
UV	Ultraviole

1. INTRODUCTION

The purpose of this study was to identify anti-proliferative and anti-tumorigenic properties of potential novel drug candidates through a preliminary cell-based screening. This step is only the beginning of overall drug development process, therefore, a detailed explanation of both conventional design and high throughput screening processes will be reviewed before detailing our approach to this study.

1.1. THE GENERAL PROCESS OF DRUG DEVELOPMENT

Drug development is a long process that includes different phases in which a new drug is launched to the market. The length of the whole process, from the discovery of a single drug to its regulatory approval takes approximately 12 to 15 years according to data taken from US Food and Drug Administration (FDA). In pharmaceutical industry, the overall spent costs during this process estimated from US$ 802 million to 1 billion per year [1]. The challenge about this process is only 1/10 drugs enter clinical trials will make it to the market. The rate of success for therapeutic areas ranging from 20% in cardiovascular drugs to only 5–8% for oncology and central nervous system disorder drugs in an average of approximately 11% overall [2]. Medical requirements and general existence of the disease are the factors that determine therapeutic areas for discovering new drugs [3].

After selecting the therapeutic area, the second step of the drug development process is target identification and validation. Targets can be enzymes, receptors, particular components of intracellular signaling cascades, or components with in gene expression systems [4].

The next step in drug development project is development of an activity assay for screening of new compounds and eliminating the inactive ones. Two main assay types are available for screening: low throughput and high throughput. Low throughput assays run on cells, tissues, organs and animals involving detailed procedures that take several weeks to months [5]. The term “high throughput screening” is testing a large number of various chemical structures against disease targets to identify “hits” [6]. This technique based on

interaction with selected targets, which allow the researcher to check affinity/activity of large libraries of compounds very quickly [7]. Advances in proteomic research provide an understanding of the physiological and metabolic pathways of cells [8]. Using the knowledge gained through sequencing of DNA various functions of several molecular targets have been identified. In addition, cellomics and metabolomics enable detailed pictures of cells under complex parameters. All of these advances accelerate the high throughput techniques used in the pharmaceutical industry [9].

Drug screening panel of 60 tumor cell lines conducted by National Cancer Institute (NCI) is an example of high throughput screening. In 1989 the NCI designed 'disease oriented' screening panel includes 60 cell lines derived from different human solid tumors. Compounds with different concentrations are screening for their antitumor effects [10].

On the other hand a technique opposes high throughput screening is virtual screening. This process is a computational technique and involves the rapid *in silico* screening of potential drug candidates. Molecular docking is the most frequently used structure-based virtual screening method [11]. In docking, with the structure of protein targets obtained from either X-ray or NMR, a library of ligands are virtually "brought" to the proximity of the specific binding site of the target and followed by applying a scoring function to estimate the possibility that the ligand in question will bind to the protein with acceptable affinity [12].

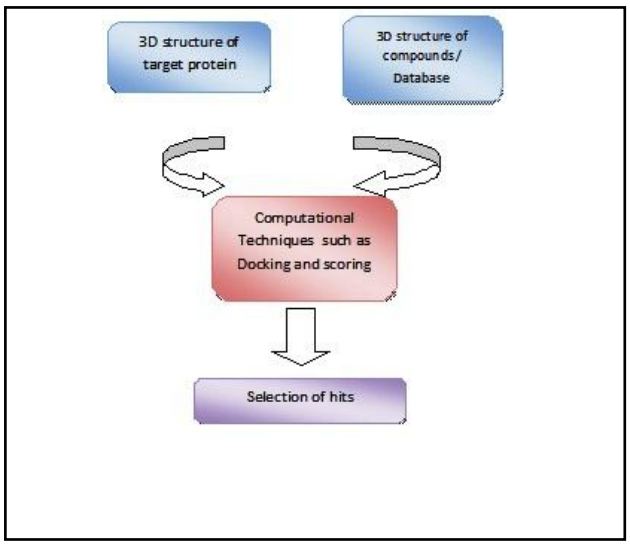


Figure 1.1. Schematic flow chart of virtual screening [13]

After finding the hits, next step is confirming their activity and estimating potency. This step includes different steps such as re-testing using the same conditions, such as drug concentration –response assay, specificity of the drug candidate to different targets, etc. Lead optimization step aims to investigate analogs with improved potency, such as more selective compounds, or compounds with suitable pharmaceutical properties such as absorption, distribution, metabolism and excretion (ADME) through modification of chemical structure [14,15].

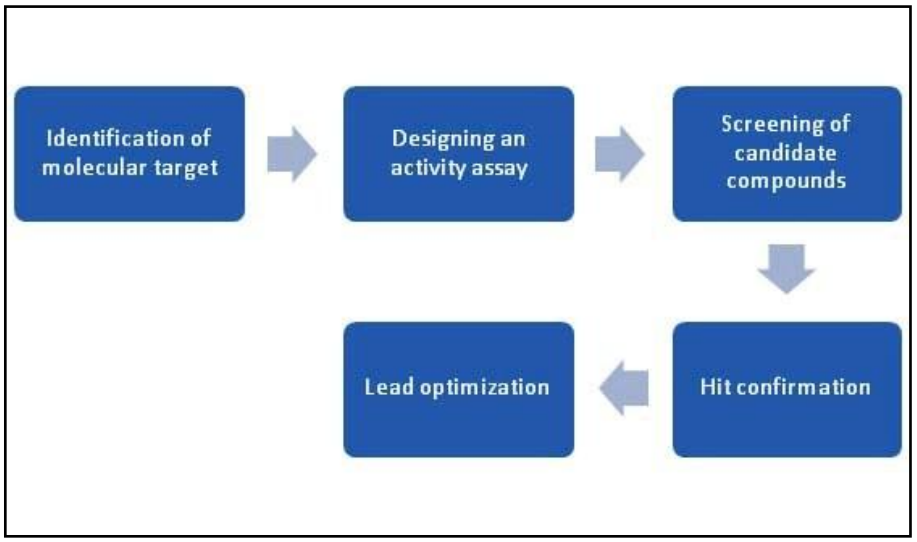


Figure 1.2. General process for high throughput drug discovery [16]

In vitro activity experiment follows toxicological studies in animals to determine the maximum tolerated dose and likely areas of toxicity [17].

Reproductive toxicology in male and female animals (required prior to testing in women of childbearing potential) and long-term carcinogenicity testing must be performed before clinical trials [18].

1.2. CLINICAL TRAILS

In clinical trials, newly synthesized experimental drugs tested over human volunteers for their activities and adverse effects before launchment as therapeutic agents. There are four phases of clinical trials each having their own regulations for safety measures and efficiency. Experimental protocols belonging to these phases must be approved by regulatory agencies specialized in medicines such as Food and Drug administration (FDA) in USA and European Medicines Agency (EMA) in Europe before prior to use [19].

1.2.1. Phase I

Phase I trials are designed to evaluate the tolerability and safety of a single dose of a potential lead [20]. This phase starts with the first administration of the new medicinal product to humans, which involves healthy volunteers with the exception of cytotoxic drugs (e.g. oncology drugs) [21]. A first in man study (FTIM) involves small number of volunteers (usually 20 to 100) who monitored very closely. In Phase I studies the pharmacodynamic effect of the drug on the body, e.g. effect on heart rate, blood pressure, electrocardiogram (ECG), etc and pharmacokinetic effect of the body on the drug, i.e. absorption, distribution, metabolism and excretion, are monitored [22].

1.2.2. Phase II

Once the drug's safety, pharmacokinetics and dose selection are established in healthy volunteers, the next step is to investigate some preliminary data on the effectiveness of the drug for a particular indication or indications in patients with the disease or condition [23]. This phase can also measure the short-term side effect and risks associated with the drug.

Phase IIa trials are generally interested in assessment of safety and tolerability of the new drug in the target patient population. **Phase IIb** follows to determine the minimally effective or non-effective dose and to decide the optimal dose for phase III trials [24].

1.2.3. Phase III

This is the last step of drug development prior to registration, and intended to gather additional information about effectiveness and safety that is needed to evaluate the overall benefit-risk relationship of the drug. Phase III studies are usually expensive and include several hundred to several thousand patients depending on the protocol and design of the study [25].

1.2.4. Phase IV

Phase IV is the post-marketing surveillance that include several thousand people, therefore conducted after a drug is approved by FDA. The aim of this phase is to obtain data for long-term effectiveness and safety, to monitor the impact of the drug on patients, to observe any other side effects, and to investigate new therapeutic areas. This phase of the clinical trial may result in commercially available drug to be restricted for use or removal from market [26].

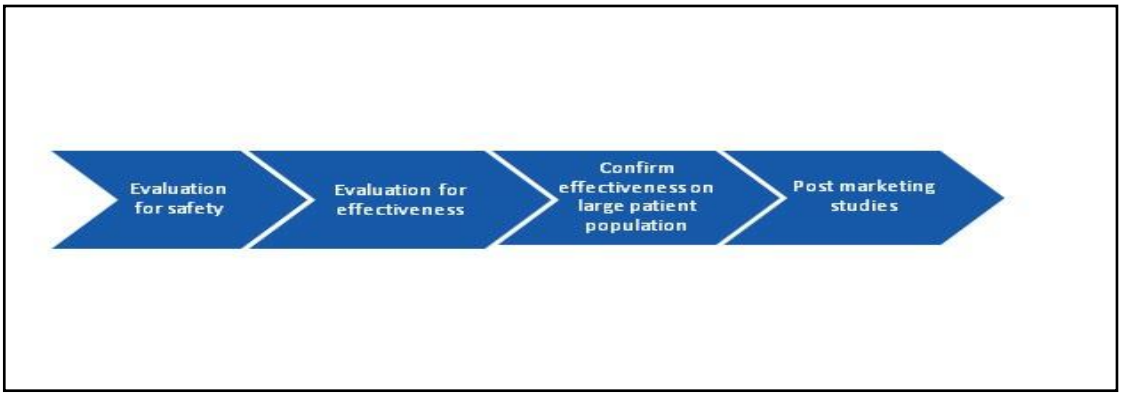


Figure 1.3. Various stages of drug development [13]

1.3. DRUG SCREENING FOR POTENTIAL NEW ANTI-CANCER COMPOUNDS

Cancer is one of the most common diseases in the world. In developing and advanced countries, cancer remains the second leading cause of death after heart disorders [27, 28]. Although many anti-cancer agents have been investigated for cancer therapy over the years, their long-term use is generally restricted and only a small proportion of the anti-cancer drugs in the market have success in treatment. Thus discovering new anti-cancer agents remains critically important [29, 30].

A successful approach for anti-cancer drug discovery generally covers either cell-free or cell-based assays.

1.3.1. Cell-free assays

These assays based on molecular targeted assays such as screening of enzyme or receptor inhibitors (e.g gyrase inhibitors, kinase inhibitors, etc). Although these assays are simple and biochemically precise, they eventually require cell- based assays, as *in vitro* and *in vivo* effects of compounds can disagree dramatically. In fact, most of the drug candidates identified in cell free assays do not exhibit any significant effect in subsequent cell culture assays for lack of cellular uptake or metabolic conversion prior to reaching their intracellular target [31].

1.3.2. Cell-based assays

These screens make it possible to assess *in vivo* activity of a candidate compound, intracellular target specificity, target-unrelated cytotoxicity, metabolic stability and bioavailability [32]. However, cell-based screening assays also require validation experiments such as polymerase chain reaction (PCR), Western blotting to understand the molecular mechanism of drug's action against target, as well as further animal studies in order to confirm activity in live organisms. In most recent studies, cell-free and cell based assays are complementary to each other.

Table 1.1. Comparative Features of Cell-Free and Cell-Based Screening Models [6]

Cell-Based Assays	**Cell-Free Assays**
Reflect cellular metabolism	Simple
Predictive model of *in vivo*	Accurate
Modelling cellular uptake	Conformable to viable assay types
Candidate drug require secondary experiment to understand the mechanism of target action	Candidate drug imply additional cell-based assays

Although cell–based assays depend on several essential elements, cell culture model is the most important component. For providing flexibility in the choices of assay chemistry and instrumentation, culture model must be a good representation of events to analyze. In development of the cell-based assays for anti-cancer drug screening, a number of important criteria have to be addressed such as choosing the type of cells, culture conditions, cells concentration, concentration of the test compound, positive and negative controls, the exposure time of the test compound and assay safety etc [33].

1.4. CELL CULTURE MODEL SYSTEMS

Studies on both anti-proliferation and cytotoxicity mainly rely on cell lines derived from tumors, which refers as "cancer lines" throughout this text.

A cancer cell line is a permanently established cell culture that will proliferate indefinitely when given appropriate fresh medium and space. Those cell lines are easily propagated and genetically manipulated, pure and in theory should give the same results under the same assay conditions. Results obtained from cell lines assumed to resemble tumors in vivo, although the tests validated and confirmed using primary tumor cells prior to testing the compounds in animal assays [34].

Cell-based assays mainly depend on cell viability/cytotoxicity or cell death. Testing the compounds with cell viability assays are based on the ability of the cell population to proceed to divide and proliferate [35]. Counting the number of cells using a counter or

microscope is dependable but it is a time consuming procedure. Advanced procedures are based on measurement with markers that reflect the total biomass or cell growth, such as measuring cell membrane integrity, events related to DNA synthesis, selective staining (e.g trypan blue, calcein-AM, sulforhodamine B, neutral red and others) or viability markers (tetrozolium reduction assays, aminopeptidase markers, ATP assays etc) indicating active metabolism [36-38]. In our study, we have routinely used the MTT assay for viability/cytotoxicity.

1.4.1. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

Measuring cell viability with metabolic markers commonly used in screening studies [39]. The aim of the MTT assay is to detect active cell metabolism as a marker for cell viability by supplying the cells with a substrate molecule that only converted into a detectable product by viable cells [40].

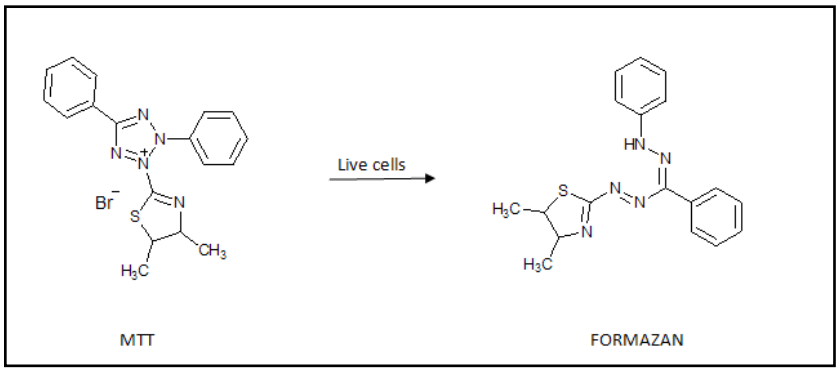


Figure 1.4. Basis of MTT assay [41]

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a yellow colored water soluble tetrazolium salt, which has the ability to enter the cells by endocytosis [42]. The mitochondrial reductase enzymes cleave the tetrazolium ring and convert MTT to an insoluble purple formazan, which can be quantified by recording changes in absorbance at 540 nm.wavelengths [43].

1.5. CANDIDATE COMPOUNDS ANALYZED IN THIS STUDY

In anti-cancer screening, we collaborated with Yeditepe University's Faculty of Pharmacy department by analyzing the bioactivity assays of their newly synthesized compounds in cell based systems.

These compounds are categorized into three major groupsaccording to their chemical structure;

- 4-(1,3-dioxoisoindolin-2-yl)benzenesulfonamide and 4-(4,5,6,7-tetrafluoro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide structured thalidomide derivatives synthesized for antimicrobial, anti-inflammatory and antitumoral purposes.
- 4-phenylpiperidine derivatives for sigma receptor ligands.
- 1-(naphthalen-2-ylmethyl)piperazine derivatives for antitumoral activities.

1.5.1. Thalidomide derivatives

Thalidomide (phthalimidoglutarimide) is a synthetic glutamic acid derivative and is formulated as a racemic mixture of two active enantiomers, S (−) and R (+) [44]. Initially, the S (−) isoform was thought to be the enantiomer primarily responsible for the teratogenic effects and the R (−) isoform for the sedative properties. Thalidomide and its metabolites quickly eliminated in the urine, with a mean elimination half-life of 5 hour [45].

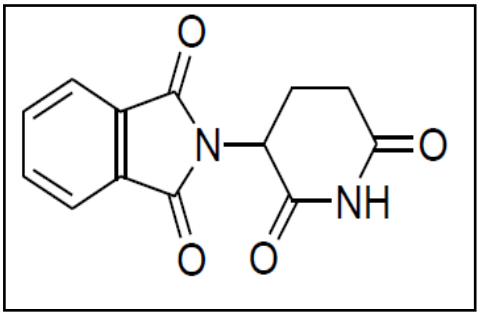


Figure 1.5. The chemical structure of thalidomide [46]

Thalidomide introduced to the drug market in 1957 for the treatment of epilepsy. Due to its insufficient efficacy as an anti-epileptic, its marketing strategy eventually changed as a sleep aid and widely used as an antiemetic during pregnancy. It was used as a sedative in many countries until 1963 and withdrawn from the market due to its teratogenic and neuropathic effects [47]. Thousands of infants were born with arm and leg malformations since pregnant women used it in the late 1950s. Even a single dose of thalidomide taken during pregnancy can cause severe birth defects or death of the unborn baby. The teratogenicity of the drug is due to its ability to inhibit angiogenesis and neovascularization.

Although its teratogenic effects have shown in zebrafish, chickens, rabbits and primates, it reviwed for some interest as an antitumor agent in 1990s. Currently thalidomide is being used to treat various cancers and inflammatory diseases. Thalidomide is an anti-angiogenic drug which inhibits the secretion of Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF) from the tumor cells resulting in reduced endothelial cell migration and adhesion [48]. Thalidomide has been shown to be effective in the treatment of multiple myeloma, prostate, renal cell carcinoma and Kaposi's sarcoma. Thalidomide was approved for the treatment of multiple myeloma in May 2006.

Thalidomide and its many analogs are immunomodulatory drugs (IMiDs) that show a multitude of biologic effects on cytokine and cell-mediated responses [49]. Thalidomide and derivatives induce immune response by the induction of T cells and natural killer cells (NK cells), thus increasing IL-2 and IFN- γ secretion which leads to lysis of multiple myeloma cells [50]. In addition, thalidomide derivatives inhibit IL-6 production which in turn inhibits the proliferation of the tumor cells. Thalidomide derivatives also inhibit TNF-α, which is a proinflammatory cytokine produced by monocytes, macrophages and lymphocytes. These effects are largely responsible for the clinical efficacy seen in conditions such as lupus erythematosis, apthous ulcers that occur in human immunodeficiency virus and Behcet's disease, ENL, graft versus host disease and others [51].

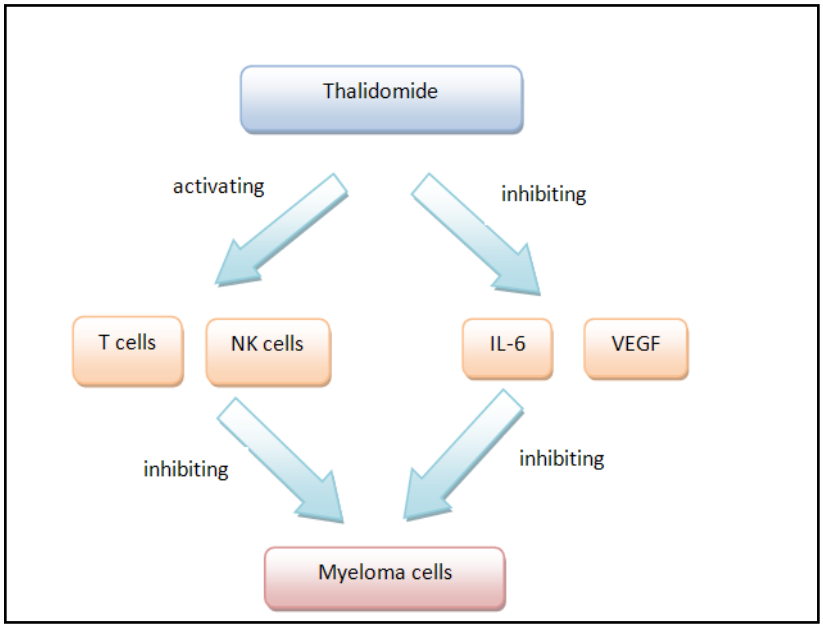


Figure 1.6. Proposed mechanism of action of thalidomide in cancer illustrated using myeloma as an example [46]

1.5.2. Sigma Receptors Ligands

Sigma receptors were discovered in 1976 and first classified as a subtype of opioid receptors [52,53]. After its binding affinity to various drugs, they are recognized as a distinct class of receptors [54]. Two major subclasses of sigma receptors, sigma-1 (σ1) and sigma-2 (σ2), differ in their molecular weights, tissue distributions, and pharmacological profiles.

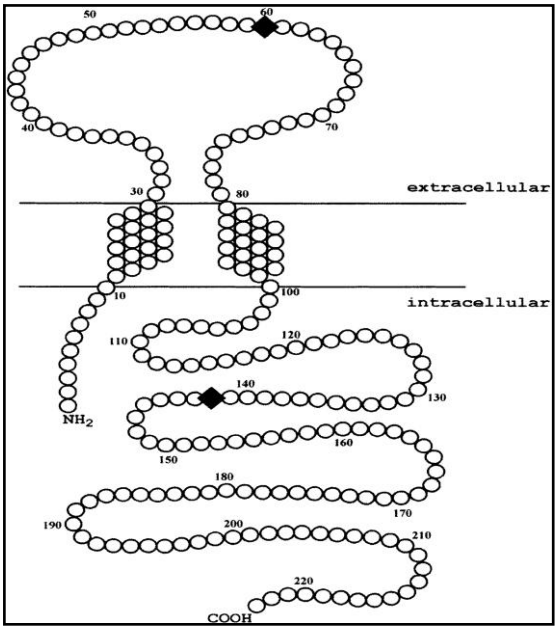


Figure 1.7. A structural model for the σ1 receptor. This model contains two transmembrane (TM) segments as determined by TM homology plots. The NH_2 and COOH termini are shown on the intracellular side of the membrane [55]

The sigma (σ) receptor and its agonists implicated in a many of cellular functions, biological processes and diseases in central nervous, endocrine, motor, and immune systems. Sigma receptors are also widely distributed throughout the brain, particularly in the motor regions including cerebellum, brainstem, motor nuclei, and substantia nigra. Besides, high levels of both subtypes of sigma receptors expressed in tumor cell lines such as neuroblastomas, glioma, melanoma, as well as cell lines derived from breast, prostate, and lung carcinomas [56]. These observations lead to the development of sigma ligands that can be used as molecular probes for diagnosis, molecular-targeted therapy and augmentation of standard cancer therapy.

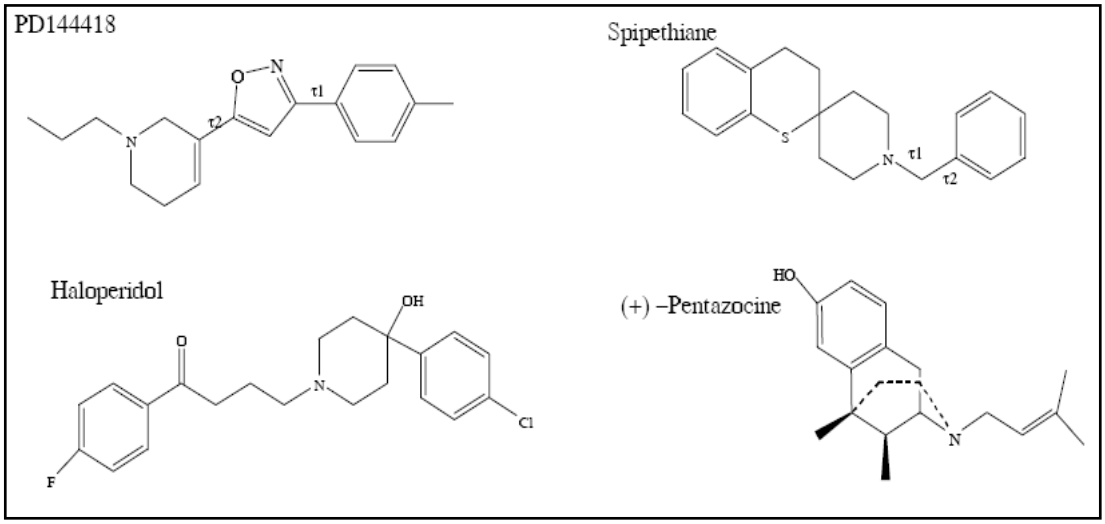


Figure 1.8. Example of sigma receptor ligands [57]

The sigma-1 receptor (σ_1R) modulates calcium signaling through the Inositol trisphosphate (IP3) receptor. Dimethyltryptamine (DMT) and neuroactive steroids such as dehydroepiandrosterone (DHEA) and pregnenolone, are ligands of the sigma 1 receptor [58].

Calcium takes an important role in cell proliferation. An increase of calcium release causes activation of enzymes such as proteases and nucleases, and this leads to degradation of key components and subsequently cell death. In recent studies selective sigma-2 receptor agonist or selective sigma-1 receptor antagonist (exp:haloperidol and IPAG) cause Ca^{+2} release to cytosol in different cancer cell lines. This free intracellular modulation affects to protein kinase C activity and leads to apoptosis of cancer cells [59].

In addition, different sigma ligands disrupt lipid rafts by cholesterol depletion [60]. This disruption leads to an increase of ceramide, which is a second manager of spingomyelin, also involved in induction of apoptosis [61].

1.5.3. Piperazine Derivatives

Piperazine is an organic compound that consists of a six-membered ring with two nitrogen atoms located oppositely. The name of piperazine is coming from the pepper plant from which the compound is extracted [62].

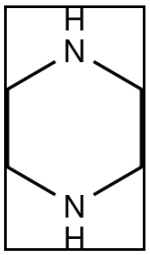


Figure 1.9. Molecular structure of piperazine [62]

Piperazine and its derivatives primarily used in treatment of motion sickness and vertigo and suppression of nausea and vomiting [63]. Because of its small and rigid heterocyclic backbone acting on various pharmacological targets, these agents are widely present in several drugs such as calcium channel blockers, histamine antagonists, and anticancer agents [64]. The studies of National Cancer Institute (NCI) have demonstrated that lead piperazines suppress and eliminate tumors in small-animal models. Recent experiments showed that the piperazine analogues have potent anti-proliferative activity against breast, colon, prostate, lung and leukemia tumors not also inhibition of cell cycle progression and angiogenesis but induction of apoptosis [65].

In addition, piperazine derivatives were found to induce apoptosis by down regulation of Bcl-2 protein and G2/M arrest, thereby to inhibit the growth of various types of human cancers [66].

2. MATERIALS

2.1. CELL LINES

- SH-SY5Y - Human Neuroblastoma Cell Line (ATCC number: CRL-2266)
- MDA-MB-231 –Human Breast Adenocarcinoma Cell Line (ATCC number: HTB-26)
- HT29 –Human Colon Adenocarcinoma Cell Line (ATCC number: HTB-38)
- L929-Mouse Fibroblast Cell Line (ATCC number: CCL-1)

2.2. REAGENTS AND COMMERCIAL KITS

- MTT Cell Growth Assay Kit (Millipore CTO1, USA)
- The PureLink RNA Mini Kit (Invitrogen 12183-018A, USA)
- DyNAmo cDNA Synthesis Kit (Thermo Scientific F-470L, USA)
- DNA Ladder (1 Kb and 100 Bp) (Invitrogen 15628-050, USA)
- 5X Loading Dye (Sigma G7654, USA)
- Agarose (Sigma A9539, USA)

2.3. CELL CULTURE

- Dulbecco’s Modified Eagle Medium (DMEM)- high glucose (Invitrogen 31966-047, USA)
- Dulbecco’s Modified Eagle Medium (DMEM) low glucose (Invitrogen 10567-14, USA)
- Fetal Bovine Serum (FBS) (Sigma F9665, USA)
- Penicilin/Streptomycin Solution (100X) (Biochrom A2213, Germany)
- 0.05 % Trypsin-EDTA Solution (Thermo Scientific SH30042-01, USA)
- Phosphate Buffered Saline (PBS) (Invitrogen 14190-136, USA

2.4. LABORATORY TECHNICAL EQUIPMENT

- Cell culture flasks, T-25, T-75, T-150 and cell culture plates, 6-well, 96-well, (TPP, Switzerland or Grenier-Bio, Germany)
- Cryovials (TPP, Switzerland)
- Micro pipettes 1000, 200, 100, 10, 2.5 µl (Thermo Scientific, USA)
- Polypropylene centrifuge tubes, 50 ml, 15 ml, 2 ml, 1 ml, 0.5 ml (Isolab, Germany)
- Serological pipettes 25, 10, 5, 2 ml (Lp Italiana Spa, Italy or Axygen, USA)
- CO_2 incubator (Thermo Scientific EW-39320-08, USA)
- Inverted Microscope (Eclipse TC 100 - Nikon, USA)
- Hemocytometer (Hausser Bright-Line, USA)
- PCR Thermal Cycler (Biorad MyCycler, USA)
- -80 °C freezer (Nuare ULT Freezer, USA)
- ELISA plate reader (Bio-Tek Elx800, USA)
- Vortex (Stuart SA8, UK)

3. METHODS

3.1. MTT Assay

In our study MTT assay was used for measuring antiproliferative and cytotoxic effects of the synthetic compounds. Cell lines SH-SY5Y, MDA-MB-231, HT29 and L929 cell line were used for antiproliferative activity and cytotoxicity assays respectively. Cells were cultured in a humidified atmosphere of 5% of CO_2 and 95% air at 37ºC in a minimum essential medium supplemented with fetal bovine serum. The cell were used during their exponential growth phase.

When the cells reached confluency, they were detached with Tyripsin (0,05%)-EDTA treatment for few minutes at 37º C. After detachment, tyripsin was deactiveted with fresh growth medium. Cell suspension (10 µL) was placed on the hemocytometer chamber and covered with a cover slip and completely filled the hemocytometer chamber by capillary action. Further, cells on the multiple squares of the hemocytometer were counted by inverted microscope and the average of 3 count were taken and calculated.

For anti-proliferative activity assays, the cell lines were plated at 50 x 10^3 cell per well for SH-SY5Y cells and 5x 10^3 cell per well for MDA-MB-231 and HT29 cells with 100 µL of growth medium. Control wells were plated with growth medium without any cells. After 24 h incubation the cells were treated with the synthetized compounds. The synthetized compounds were dissolved in less than 0,1% DMSO. These compounds were diluted in the range of 10^{-4} M to 10^{-9}M. Each experiment was carried out 3 times. After 24 h incubation the MTT reagent were added to each wells and the cells were incubated 4 h for color formation than color change in 96-well plates were detected with Elisa plate reader at 540nm. Cell viability was expressed as the percent absorbance relative to that obtained for cells not exposed to synthetic compounds.

In measuring cytotoxicity effect we used L929 cell line at a concentration of 3x 10^3 cell /well. The procedure was the same with anti-proliferative activity assay.

3.2. REVERSE TRANSCRIPTASE POLYMERASED CHAIN REACTION EXPERIMENTS

3.2.1. Total RNA isolation

SH-SY5Y and L929 cells were cultured in 10 mm cell culture petri dishes as 1,4 x 10^6 cell per one dish. After 24 h incubation, synthetic compounds were administrated into the petri dishes in 100 µl volume in order to achieve10^{-4} M drug concentration. This concentration was chosen as the most effective synthetic sample concentration.

After 24 h incubation with synthetic compounds, cells were prepared for total RNA isolation. RNA isolation was done with Invitrogen RNA Mini kit according to the manifacturer instructions. Basically cells were trypsinized in (0,05%). Tryripsin-EDTA and Lysis buffer was added to cell pellets. Cells were vortexed and collected in spin coloumn tubes with silico semi membrane provided with the kit. Ethanol was added to perform precipitation of RNA. Samples in spin coloumns were washed several times with wash buffers and centrifugated in 12000 g. Then with adding elution buffer RNA was eluated and collected in collection tubes. Total RNA concentration was calculated with Nanodrop spectrophotometer using 260/280 wavelength .

3.2.2. Synthesis of cDNA by reverse transcription

cDNA was synthesized from isolated RNA using Finnzymes DyNamo cDNA synthesis kit according to the manufacturer's instructions. Total RNA, oligo DT and nuclease free water was added to eppendorf tubes and incubated at 65 º C for 15 min. After then tubes were incubated with reaction buffer and RNase H^+ reverse transcriptase (includes RNase inhibitor) enzyme at room temprature for 10 minute and 37º C for 2 h respectively. Final incubation period was at 65º C for 5 minute. Synthesized c DNA were stored at – 20º C for further use.

3.2.3. PCR –reaction

For PCR reactions, dNTP, distilated water, 10X buffer, forward and reverse primers, template and I –Tag DNA Polymerase enzyme were mixed in an eppendorf tube. Primers and template DNA concentrations varied according to optimization with GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) and β- actin which are a house keeping gene for normalization of the data. Total reaction volume was 50 µl and the PCR program was carried out.

Table 3.1. The content for PCR mixtures

Component	**Volume**	**Final Concentration**
Template	Varies	varies
10X buffer	5 µl	1X
10Mm dNTP	1µl	0,5 mM
Forward Primer	0,4 µl	0,2 µM
Reverse Primer	0,4 µl	0,2 µM
DNA polymerase	0,2 µl	
dH_2O	Up to 50 µl	

Table 3.2. The program for PCR reaction

Predenaturation	95° C	2 min
Denaturation	95° C	30 second
Annealing	varies	30 second
Extension	72° C	2 min
Last extension	72° C	5 min

Table 3.3**.** Sequences of designed forward and reverse primers

Gene Name	**Forward primer**	**Reverse primer**
elk- 1	5’ CCT TGC GGT ACT ACT ATG AC 3’	5’ GGC TGC GGC TGC AGA GAC TG 3’
c-fos	5’ GAATTAACCTGGTGCTGG 3’	5’ CAG AAC ATT CAG ACC ACC 3’
p21	5’ TGC CCA AGC TCT ACC T 3’	5’ TCT GCC GCC GTT TTC G 3’
cyclinD 1	5’ GCC CTC TGT GGC ACA GAT GT 3’	5’ CTG CTG GGC CTG GCG CAG GC 3’
β -actin	5’ AGC GAG CAT CCC CCA AAG TT 3’	5’ GGG CAC GAA GGC TCA TCA TT 3’
gapdh	5’TGA TGA CAT CAA GAA GGT GGT GAA G 3’	5’ TCC ACC ACC CTG TTG CT 3’

The PCR samples checked on 1.5% agarose gel prepared with TAE buffer and agarose staining with ethidium bromide. Samples were run at 100 volt for 30 minutes using electrophoresis system and gel was visualized under ultraviolet with Biorad® Gel Imager.

4. RESULTS AND DISCUSSION

4.1. RESULTS OF CELL VIABILITY

SH-SY5Y human neuroblastoma, MDA-MB-231 human metastatic breast cancer, and HT 29 human colon adenocarcinoma cell lines used for for anti-cancer lines and L929 mouse fibroblast cell line as control. SH-SY5Y are widely used human neurablastoma cell lines which are derived from SK-N-SH cells taken from bone marrow biopsy of a neuroblastoma patient. MDA-MB-231 cell lines are the epithelial cell lines of the mammary gland tissue. Since these cell lines have the aggressive metastatic properties of breast adenocarcinoma cells they are widely used in anti-cancer studies. HT29 colon adenocarcinoma cell lines is often used as a model for colorectal cancer studies.

In our study, we used three different group of synthetic drugs with different chemical and structural properties;

- 4-(1,3-dioxoisoindolin-2-yl)benzenesulfonamide and 4-(4,5,6,7-tetrafluoro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide structured thalidomide derivatives synthesised for antimicrobial, antiinflammatory, antitumoral purposes
- 4-phenylpiperidine derivatives for sigma receptor ligands
- 1-(naphthalen-2-ylmethyl)piperazine derivatives for antitumoral activities.

4.1.1. Thalidomide Derivatives

Thalidomide derivatives consists of 15 different compounds and their anti-tumor activities first tested in SH-SY5Y neuroblastoma cell line.

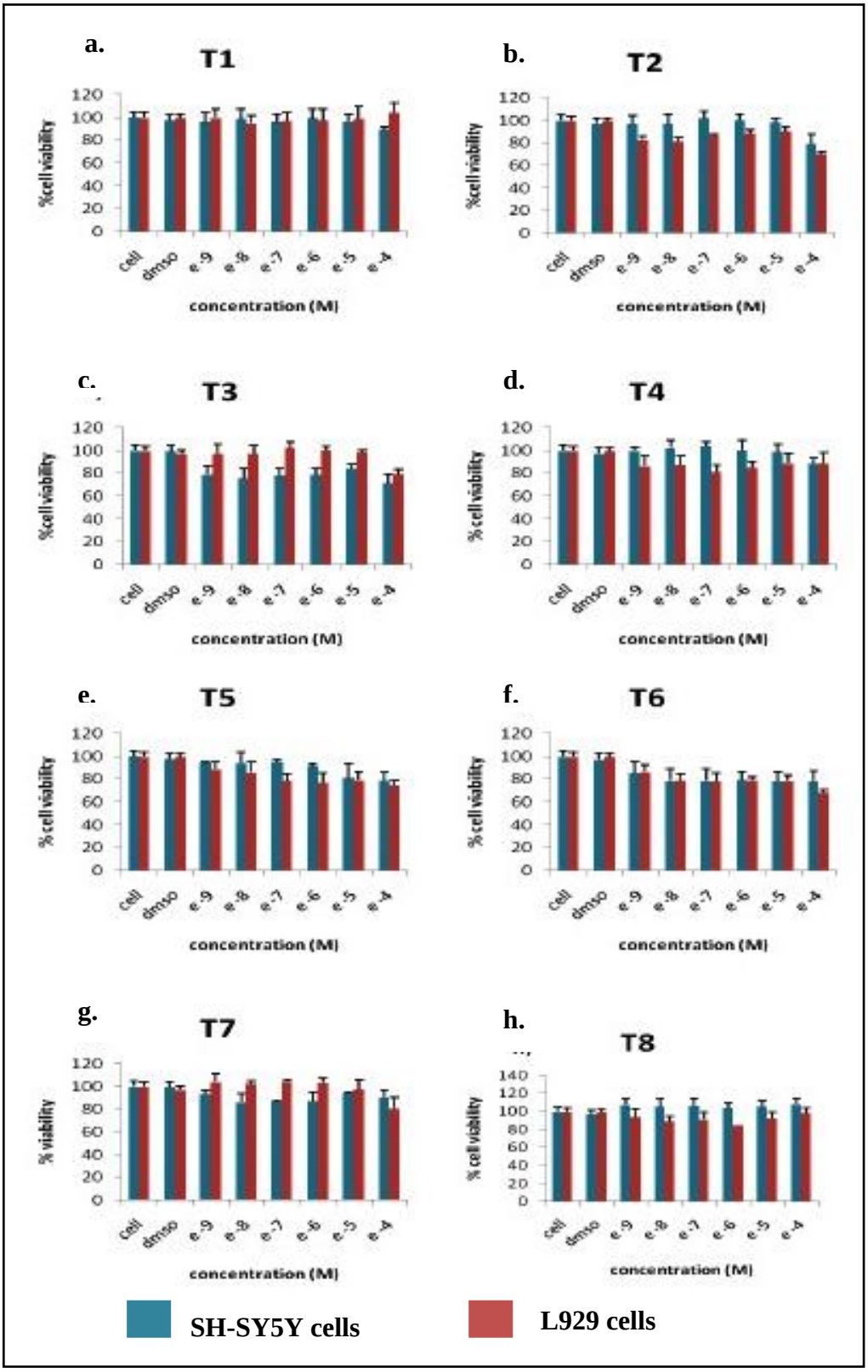


Figure 4.1. The effects of a range of concentration of synthetic compounds T1, T2, T3, T4, T5, T6, T7 and T8 on cell viability in SH-SY5Y and L929 cells

As shown in Figure 4.1, these compounds did not showed significant reduction on SH-SY5Y cells. Also they showed decrease in cell viability on L929 cell lines except T1. Compound T2, T5 and T6 reduced the viability of SH-SY5Y cell in the ratio of 20% on average where they reduced viability of L929 cell line in the ratio of 30% ($p<0.05$). Moreover, a cytotoxic effect was observed in T5 besides its anti-proliferative effect ($p<0.05$). T8 increased the cell viability in SH-SY5Y cells furthermore reducing the cell viability in L929 cells in the ratio of 20%. According to these results, none of the compounds this series selected for further evaluation.

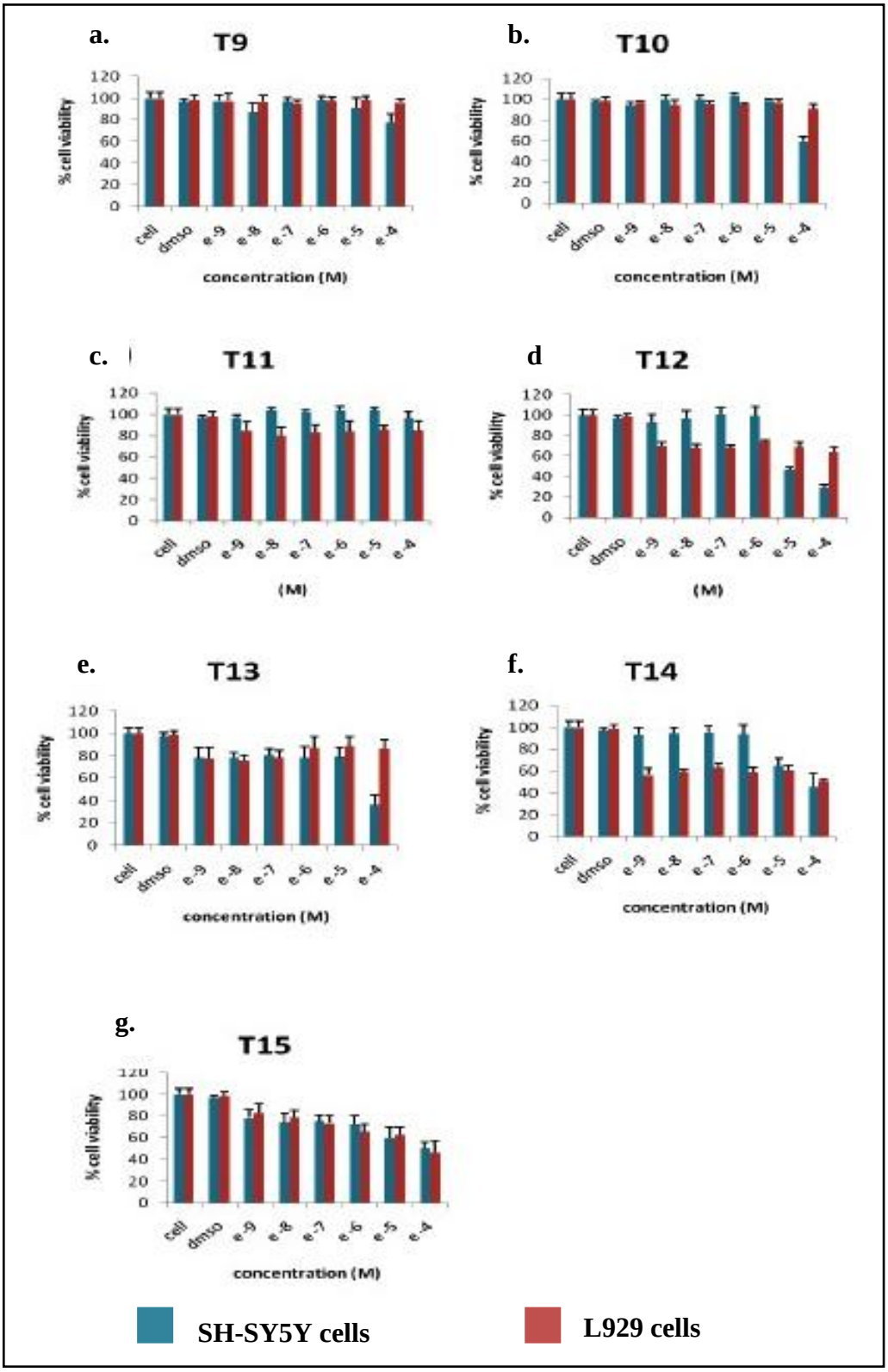


Figure 4.2. The effects of a range of concentration of synthetic compounds T9, T10, T11, T12, T13, T14 and T15 on cell viability in SH-SY5Y and L929 cells

As shown in Figure 4.2., all of these compounds showed anti-proliferative effect except T9 and T11. T9 showed little effect on SH-SY5Y and L929 cells where T11 showed better effect on L929 cells. Compound T10 showed reduction on SH-SY5Y with the ratio of 40% but it did not showed cytotoxic effect on L929 cells ($p<0.05$). T12 showed 70% reduction in SH-SY5Y cells same time with its reduced cell viability down to 60% in L929 cells ($p<0.05$). Although T14 and T15 showed the same effect on L929 and SH-SY5Y cells with the raito of 50%, they are the most potent candidates in this group because while they reduced the viability with the ratio of 70% in SH-SY5Y cells, it showed little effect on control cells ($p<0.05$). When we compared the compounds chemically, one explanation of why T12 compound is more active than T14 compound could be the presence of additional methyl groups. Thus the lipophilicity may be increased and the cellular uptake may be increased also (although our studies do not address the issue of cellular uptake at this point). When we compared T10 and T11, in T10 replacement of pyrimidin with phenyl group may be leading to more anti-proliferative activity than T11.

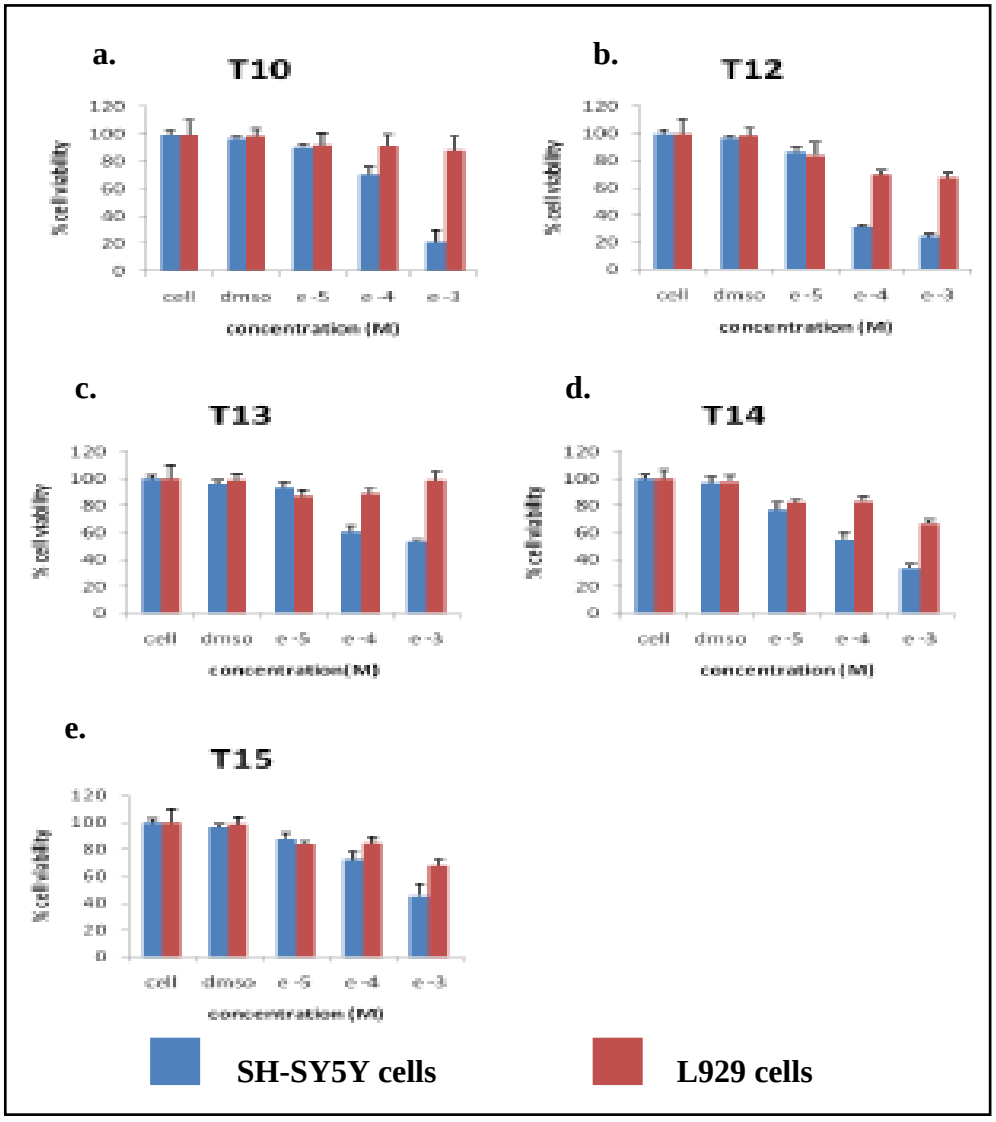


Figure 4.3. The effects of a range of concentration of compounds T10, T12, T13, T14 and T15 on cell viability in SH-SY5Y and L929 cells with upper concentration

We tested the higher concentration of the potential compounds in series T. The increase in concentration causes an additional 20% reduction in cell viability in SH-SY5Y whereas an insignificant decrease occurred in L929 cell lines. In compound T12, SH-SY5Y cell viability decreased from 30 % to 20 %, on the other hand in L929 cell lines, no remarkable change was observed. T13 caused a slight change in SH-SY5Y cell viability but raised the cell viability in L929 cells ($p<0.05$). Based on these results, compound T10, T12, T15 can potentially be used as an anti-tumor drug candidate at this higher concentration.

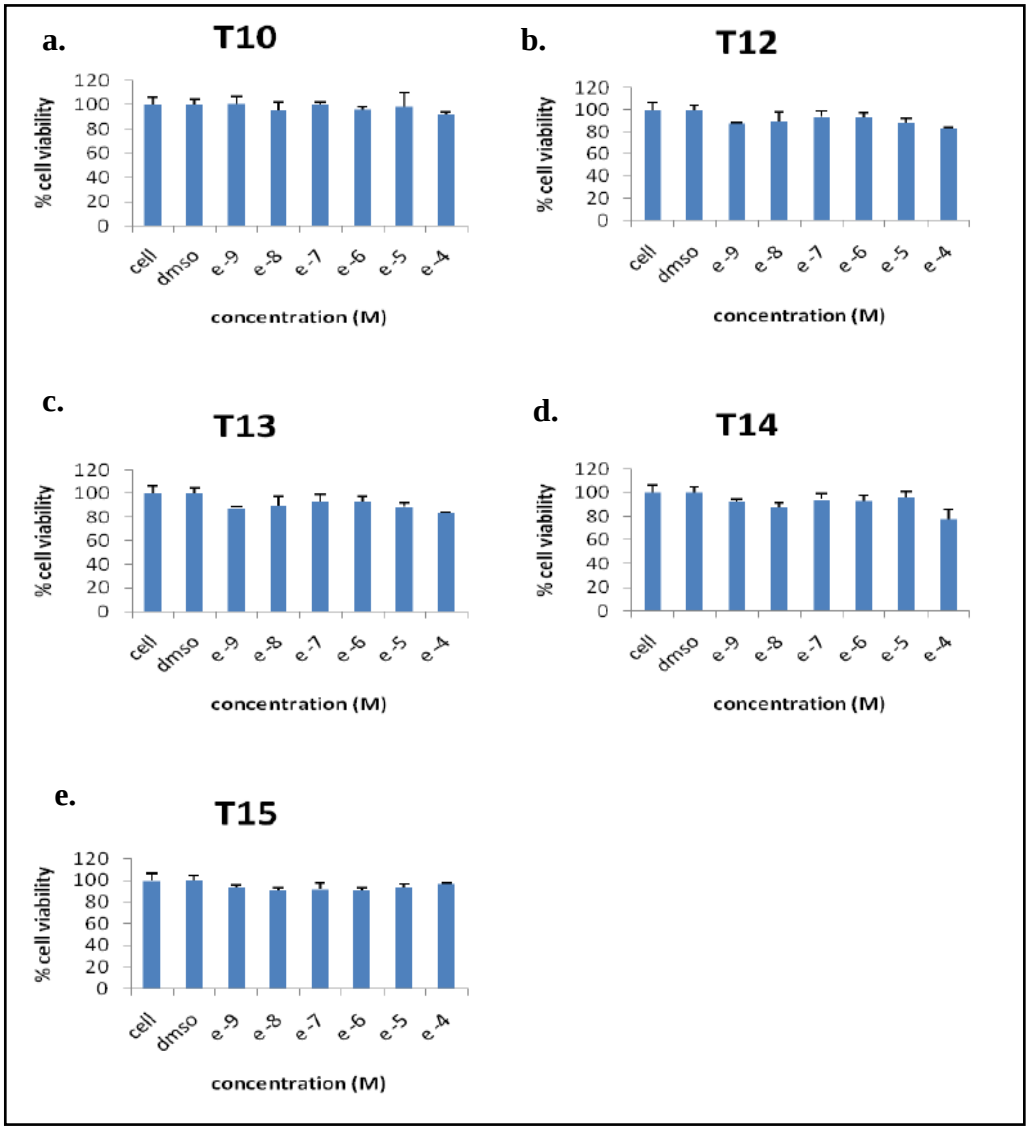


Figure 4.4. The effects of synthetic compounds T10, T12, T13, T14 and T15 on cell viability in MDA-MB-231 cells

As shown in figure 4.4. drugs that are effective on SH-SY5Y cells did not show significant reduction in cell viability on MDA-MB-231 cells. Compound T10 caused a decrease of 10% in cell viability; whereas T12, T13 and T14 caused a decrease of 20% in average. When we look T15, no anti-proliferative effect was observed. Therefore, these compounds do not present a viable drug candidate for medical use in breast cancer.

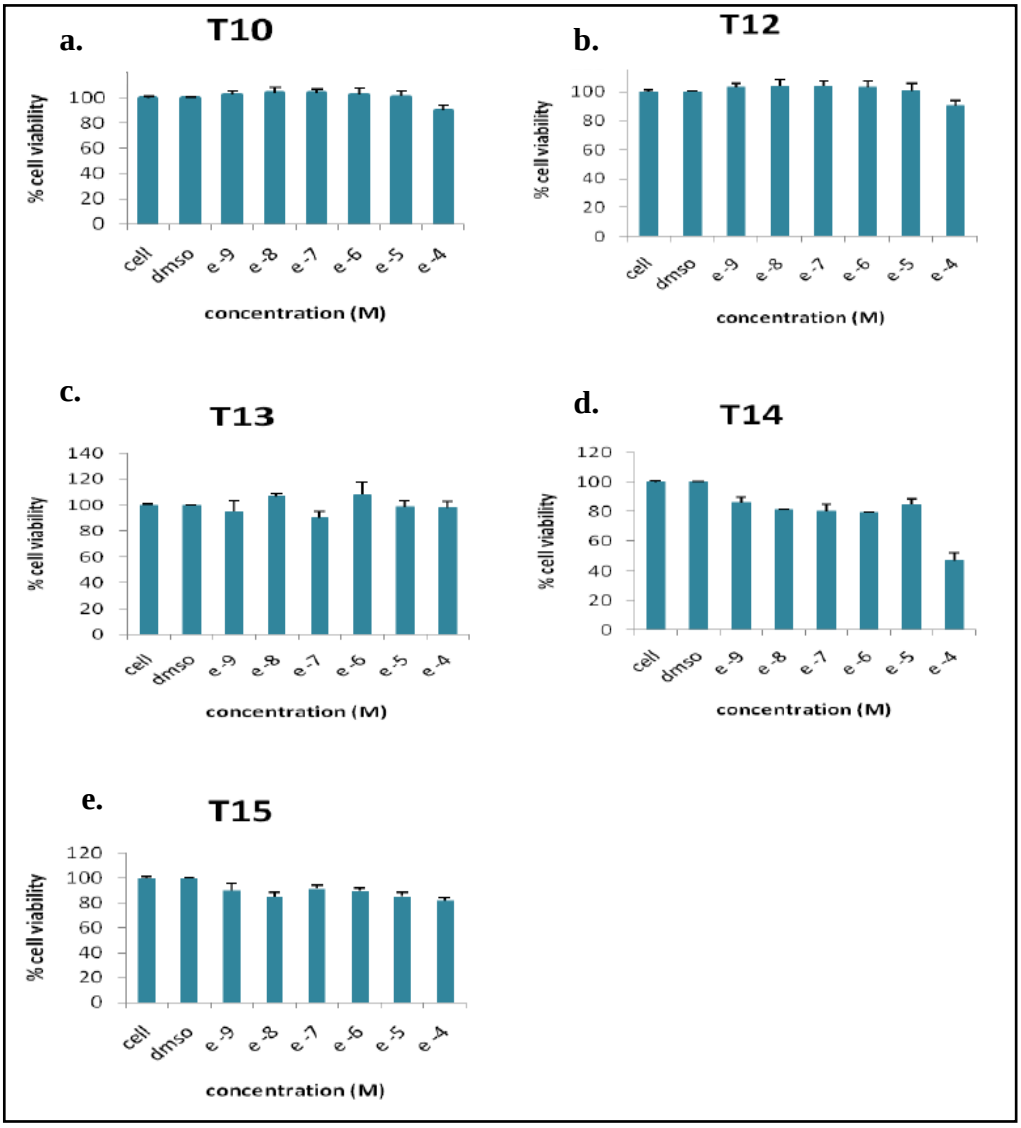


Figure 4.5. The effects of synthetic compounds T10, T12, T13, T14 and T15 on cell viability in HT29 colon carcinoma cells.

As shown in figure 4.5. these drugs did not showed significant reduction on viability in HT29 cell lines except T14. T14 caused a decrease 60% in HT29 cell line. While T10 and T12 showed a decrease of 12%; T15 showed a 20% decrease in cell viability. T13 did not show any anti-proliferative effect. Although T14 showed significant reduction in SH-SY5Y cells and HT29 cells, it showed cytotoxic effect on L929 cell lines. Therefore T14 cannot be regarded as an ideal drug candidate.

In recent years, thalidomide and derivatives have gained significance in drug development research not only with their anti-inflammatory effects but also with their anti-tumor effects. Thalidomide is approved to be a treatment for multiple myeloma. At the same time it is realized to be effective on prostate and renal cell carcinoma. Therefore, in our study we decided to test our thalidomide smilar derivatives on different cell lines. When compared to HT29 and MDA-MB-231 cell lines we can say that these drug candidates showed the most anti-proliferative effect on SH-SY5Y cell line. Thalidomide is a derivative of glutamic acid and due to its neurotransmitter feature in neurons, cellular uptake of drug candidates in SH-SY5Y cell lines may be easier than other cell lines. In a recent study, thalidome showed more antiproliferative activity on SH-SY5Y cells than on MDA-MB-231 cells [67]. It is seen that in xenograft model of human neurobalstoma, thalidomide showed anti-angiogenic effect by blocking VEGF and induced apoptosis in another study [68]. In synthesized thalidomide derivatives, because of the less cytotoxicity on L929 cell lines and selective anti- proliferative effects on SH-SY5Y cell lines, T13 can be considered as a serious anti-cancer drug candidate on neuroblastoma.

4.1.2. Sigma receptor ligand

This part covers some compounds possessing sigma receptor ligand activities. These compounds were screened on SH-SY5Y, HT29, MDA-MB-231 and L929 cell lines.

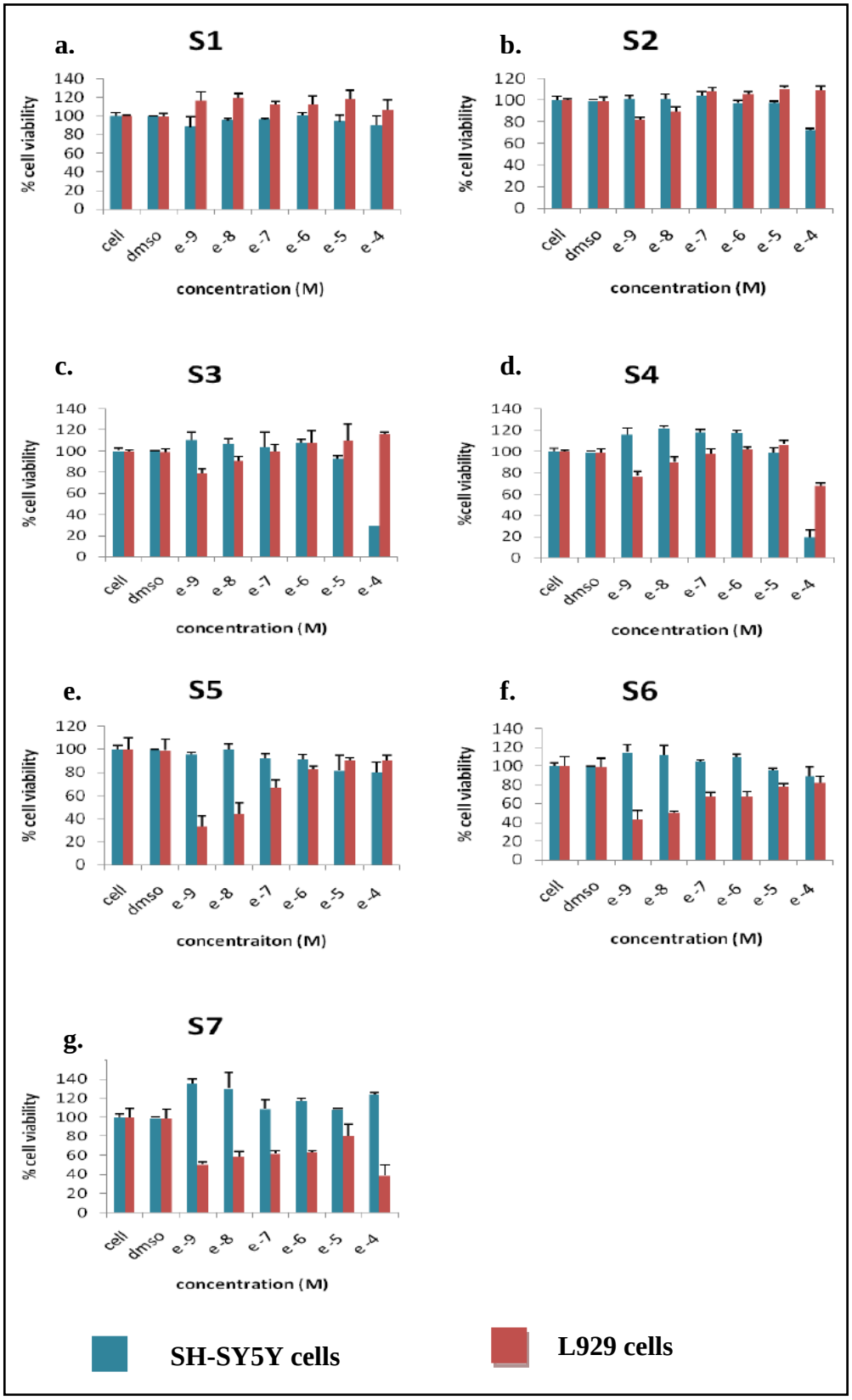


Figure 4.6. The effects of S1-S7 compounds on cell viability in SH-SY5Y and L929 cells

As shown in Figure 4.6. no significant cytotoxicity was observed in S1, S2, S3 class of drugs. Although S4, S5, S6 showed little cytotoxicity, (viability around 80%) values were in acceptable range. However, increased cytotoxicity was observed in compound S7 ($p<0.05$).

When the compounds tested for their effect of in SH-SY5Y human neuroblastoma cell lines S1, S5, S6 and S7 showed no effect on the viability of these cells. Altough S7 increased the cell viability, S3 and S4 showed significant reduction on cell viability ($p<0.05$). So these drugs can be drug candidate because of their anti-proliferative activity. When we compare S3 and S4, S4 is more effective on SH-SY5Y cells but this drug showed cytotoxicity on L929 cell lines. Although S3 showed less reduction of viability on SH-SY5Y cell line ($p<0.05$). Therefore, S3 selected as potential candidate.

All S series compounds contain cylopentanone ring as S4 contains cylohexanone ring. This change in structure may increase lipofility of the compound where this can lead to an effective cell penetration through membrane. As a result, antiproliferative effect of S4 can be expectd to be higher than the other members of this series. As the carbonil group changes into hydroxyphenyl on cyclopentananone ring of S7; cytotoxic effect increases while antiproliferative effect disappears.

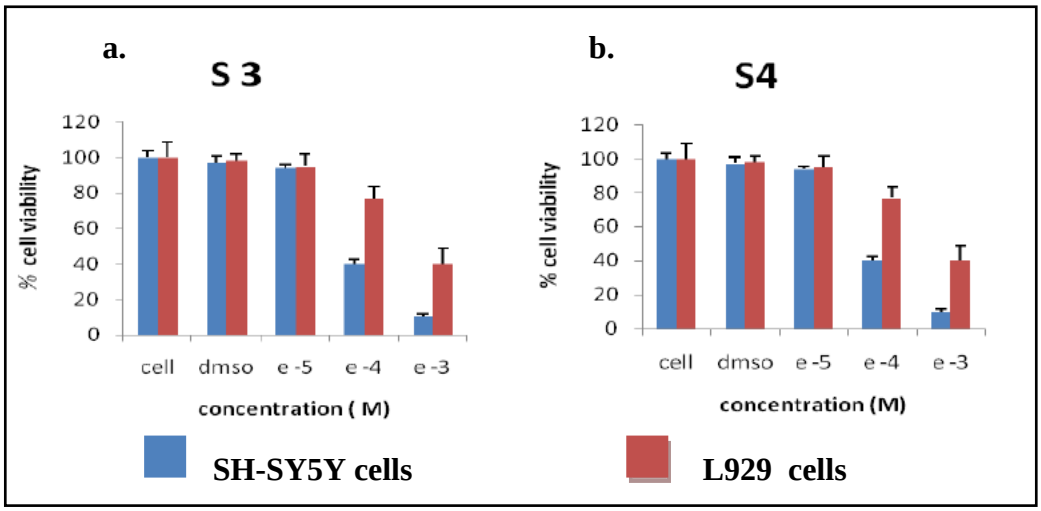


Figure 4.7. The effects of compound S3 and S4 on cell viability in SH-SY5Y and L929 cells with upper concentration

Compound S3 and S4 with upper concentrations, which are the most effective compounds of this series, were tested again. Both of the compounds showed significant reduction on SH-SY5Y at the highest concentration but at the same time showed more cytotoxicity on control cells. An increase of concentration may lead to loss of selectivity to SH-SY5Y cells and from the results, we can say that the suitable concentration of both drugs is 10^{-4} M.

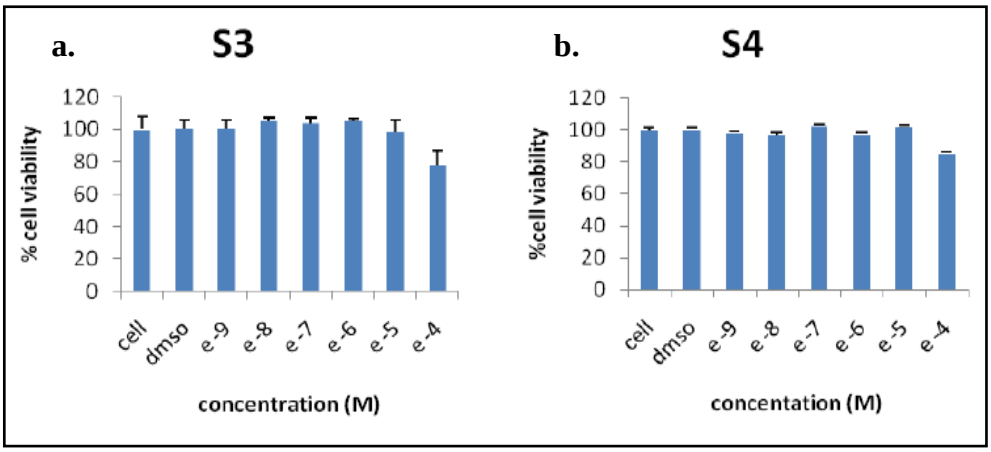


Figure 4.8. The effects of compound S3 and S4 on cell viability of MDA-MB-231 cells

In Figure 4.8., S3 and S4 showed approximately 20% reduction on cell viability at the highest concentration. They cannot be considered as potential drugs for MDA-MB-231 cells.

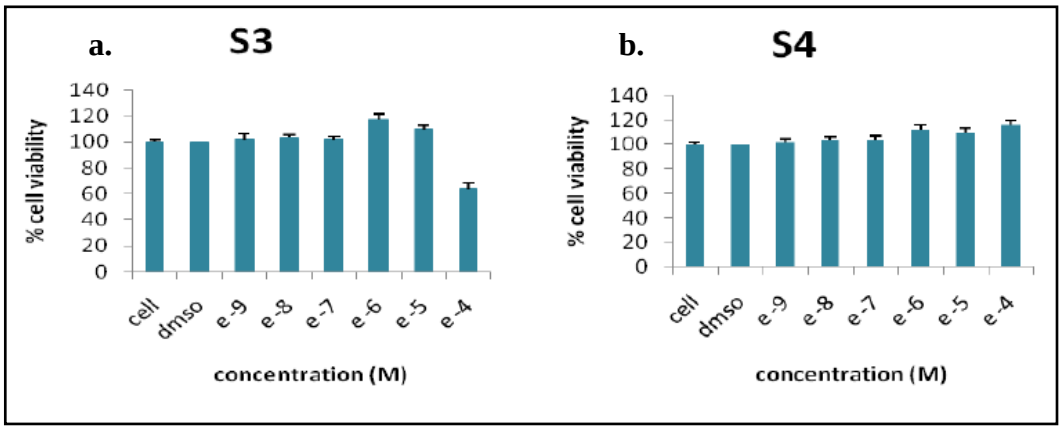


Figure 4.9. The effect of compound S3 and S4 on cell viability in HT29 cells

As shown in Figure 5.8., S3 showed some decrease the cell viability (viability around 60%) when S4 showed positive effect on the viability. Since S4 cannot be drug candidate for HT29 cells, H3 can.

Recent studies showed that sigma receptors are highly expressed in various cancer cells. When we compare all of sigma receptor ligands on SH-SY5Y, MDA-MB-231 and HT29 cells, the most effective reduction on viability was observed in SH-SY5Y cells. This can be commended as sigma receptor ligands may show more selectivity to SH-SY5Y cells. Sigma α-1 receptors are well characterized in the central nervous system thus it may involved in nervous system disease. Sigma ligands that we used in this study are sigma α-1 selective ligands. This situation might be the answer why these compounds are more selective to neuroblastoma cells.

4.1.3. Piperazine derivatives

Ten compounds from this group tested on HT29 and MDA-MB-231 cell lines for anti-proliferative activity where L929 cell line used as control.

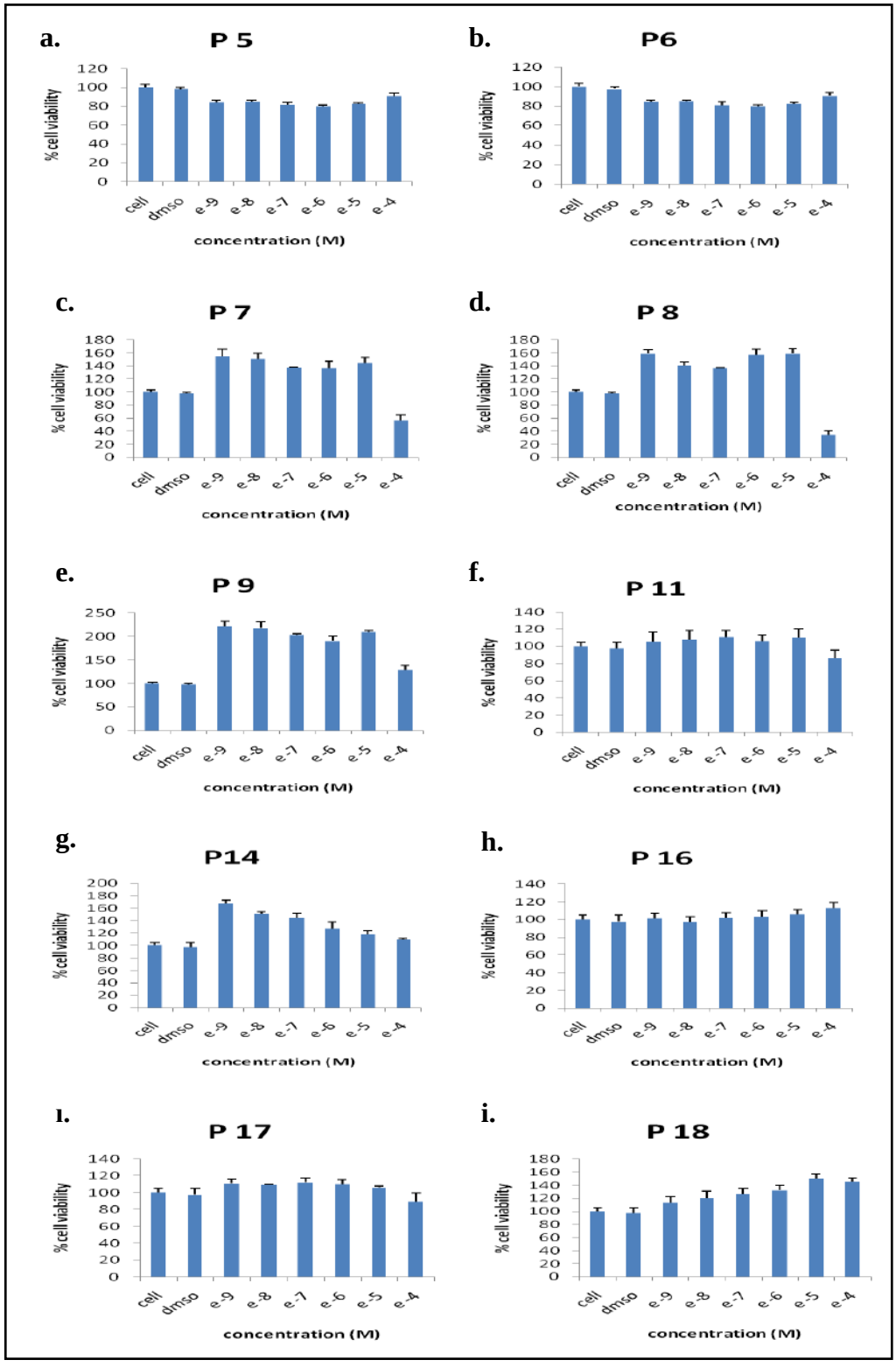


Figure 4.10. The effects of compound P5, 6, 7, 8, 9, 10, 11, 14, 16, 17 and 18 on cell viability in L929 cells

In this figure the compound P5, P6, P17 and P11 showed reduction in cell viability in the ratio of 10-20%. However, P7 caused 50% reduction on cell viability in L929 cells. But when we compared with others, P8 is the most cytotoxic compound in L929 cell line and reduced the cell viability around to 30%. However P9, P14, P16 and P18 showed positive effect on L929 cell viability. When we compare all the compounds of this series, all of these drugs except P8 and P7 can be potential drug candidate because of their less cytotoxicity on control cells

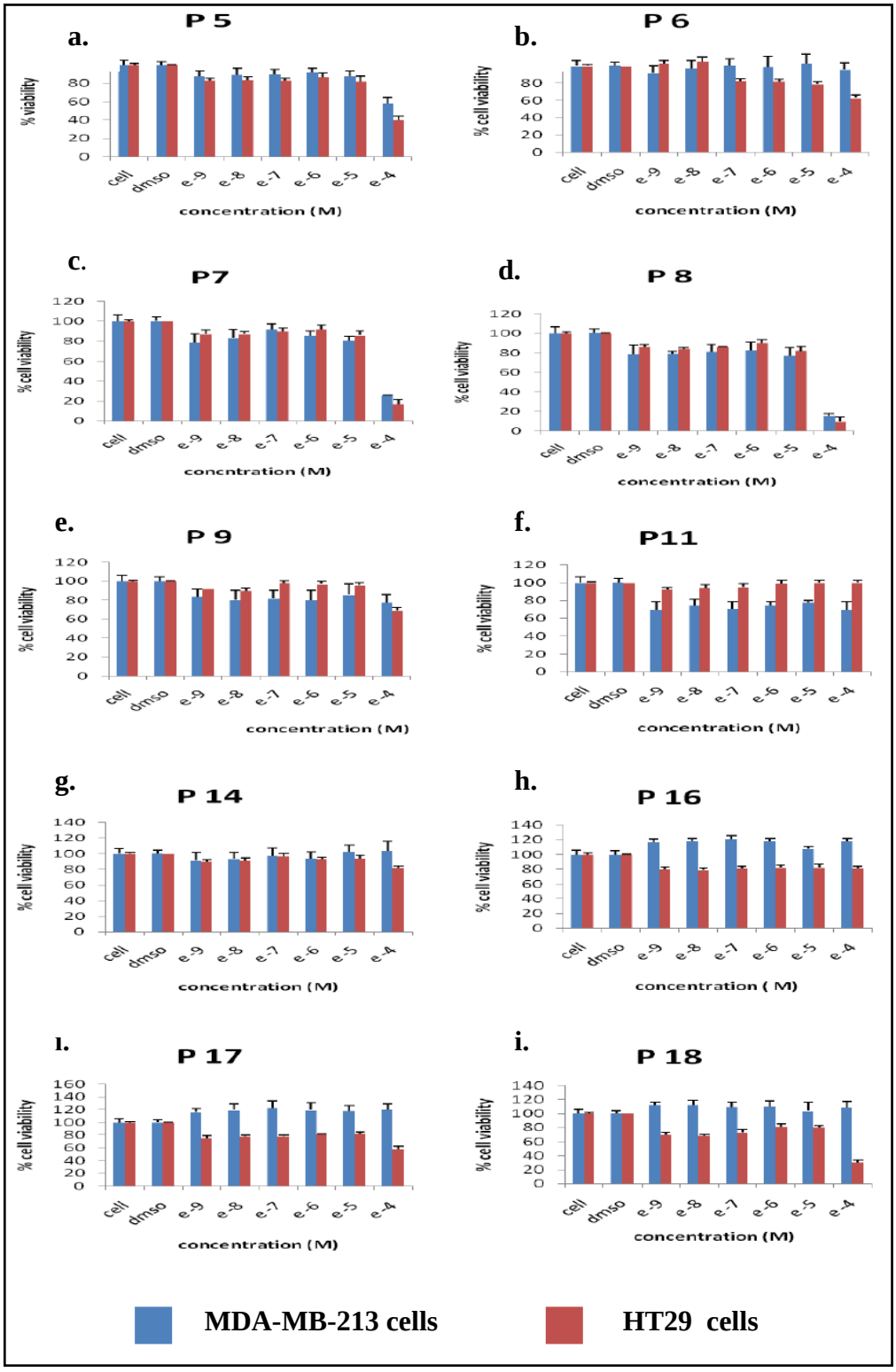


Figure 4.11. The effects of compound P7 - P18 on cell viability in MDA-MB-231 and HT29 cells

As shown in Figure 4.11., this group of compounds showed anti-proliferative activity on HT29 cells except P11 and P17. P8 and P7 are the most effective drugs in HT29 cells.

When we looked at MDA-MB-31 cells P8 and P7 again showed significant reduction on cell viability. However, P16 and P17 showed proliferative effect. On the other hand P18 and P14 showed no effect on MDA-MB-231 cells.

When we compared HT29 and MDA-MB-231 cell lines, they seemed to be more effective on HT29 cells showing cytotoxic effect on L929 cells. As a result, this cytotoxic effect decreased the potential of the compounds for medical use. On the other hand compound P5 is both effective on HT29 and MDA-MB-231 cells, and furthermore it did not showed cytotoxic effect on L929 cells. Thus, it can be considered as a potent anti-tumor drug candidate.

Since piperazine has a small and stable structure, it is usually combined with many drugs. Recent studies showed that when it is combined with other active substances, such as epoxide in epoxide containing piperazines, they are effective on MCF-7 and prostate cancers [69]. In another study a new flavonoid with a piperezine substitution, YG-202, induces p53 and p21 expression leading to apotosis [70]. As a conclusion, it is possible that piperazine substituents can be potent anti-tumor drug. And they can be applied to other cancer types.

Table 4.1. List of IC_{50} values for tested compounds on all cell lines

IC_{50} VALUES FOR TESTED COMPOUNDS ON ALL CELL LINES				
Compound	**SH-SY5Y cells**	**L929 cells**	**MDA-MB-231 cells**	**HT29 cells**
T1	>100μM	>100 μM	-	-
T2	>100 μM	>100 μM	-	-
T3	>100 μM	>100 μM	-	-
T4	>100 μM	>100 μM	-	-
T5	>100 μM	>100 μM	-	-
T6	>100 μM	>100 μM	-	-
T7	>100 μM	>100 μM	-	-
T8	>100 μM	>100 μM		
T9	>100 μM	>100 μM	-	-
T10	>100 μM	>100 μM	>100 μM	>100 μM
T11	>100 μM	>100 μM	-	-
T12	10,2 μM	>100 μM	>100 μM	>100 μM
T13	39,8 μM	>100 μM	>100 μM	>100 μM
T14	33,1 μM	>100 μM	>100 μM	79,4 μM
T15	>100 μM	44,6 μM	>100 μM	>100 μM
S1	>100 μM	>100 μM	-	-
S2	>100 μM	>100 μM	-	-
S3	53,7 μM	>100 μM	>100 μM	>100 μM
S4	42,6 μM	>100 μM	>100 μM	>100 μM
S5	>100 μM	-	-	-
S6	>100 μM	-	-	-
S7	>100 μM	-	-	-
P5	-	>100 μM	>100 μM	74,1 μM
P6	-	>100 μM	>100 μM	>100 μM
P7	-	>100 μM	57,5 μM	51,1 μM
P8	-	77,6 μM	47,8 μM	47,8 μM
P9	-	>100 μM	>100 μM	>100 μM

Table 4.1. List of IC_{50} values for tested compounds on all cell lines (continue)

P11	-	>100 μM	>100 μM	>100 μM
P14	-	>100 μM	>100 μM	>100 μM
P16	-	>100 μM	>100 μM	>100 μM
P17	-	>100 μM	>100 μM	>100 μM
P18	-	>100 μM	>100 μM	53,7 μM

$IC_{50\ value}$ is determined as a concentration of 50% growth inhibition effect of compounds on cell lines. In this project the maximum treatment concentration of compounds were 100μM. IC_{50} values of some compounds cannot be calculated because they could not inhibit 50% growth of cells even in 100 μM.

4.2. ANALYSIS OF CELL CYCLE RELATED GENE EXPRESSION IN RESPONSE TO TREATMENT WITH COMPOUNDS STUDIED

We first isolated total RNA from SH-SY5Y and L929 cells which were treated with compounds and then we syntnhesized cDNA and used them in PCR experiments. The control cells were exposed to 0,1% DMSO solution. Drugs were applied at appropriate concentration.

For normalising RT-PCR datas, primers of *gapdh* and *β-actin* ‘house- keeping genes‘ assumed to be expressed in all cell types to similar levels are used to equalize cDNAs of all samples.

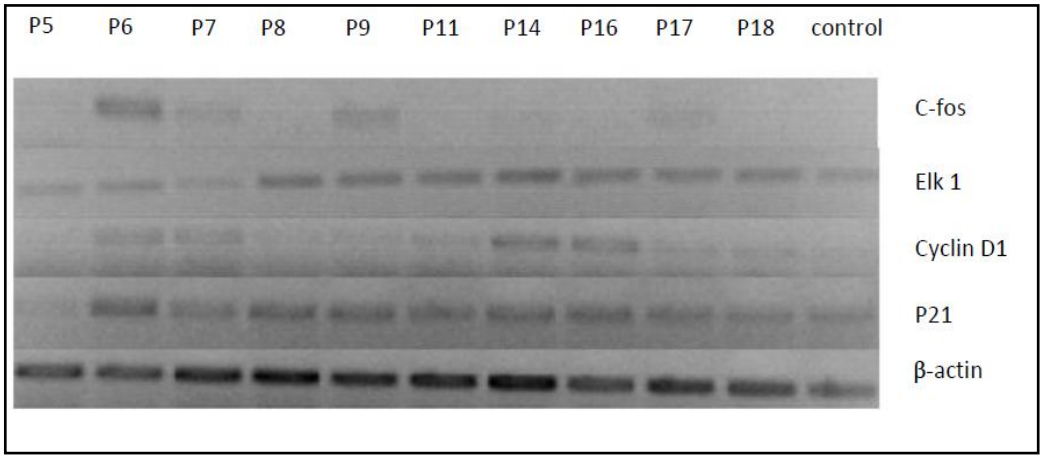


Figure 4.12. RT-PCR results for comparing the expression levels of cell cycle related genes on SH-SY5Y cells which were exposured with P compounds

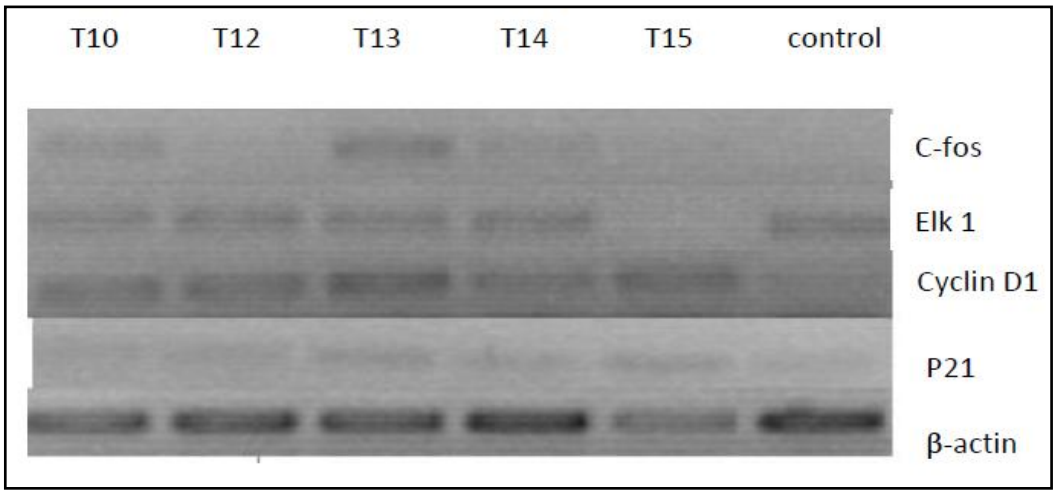


Figure 4.13. RT-PCR results for comparing the expression levels of cell cycle related genes on SH-SY5Y cells which were exposured with T compounds

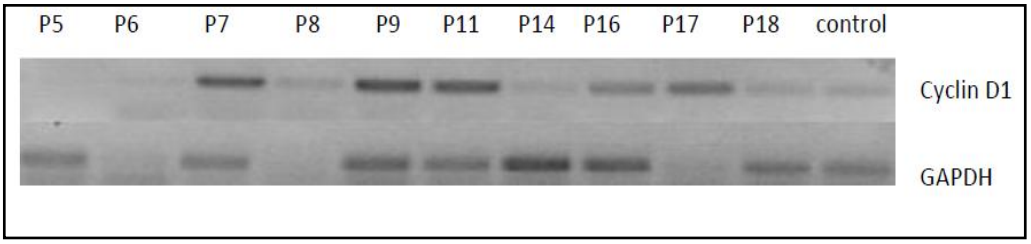


Figure 4.14. RT-PCR results for comparing expression levels of *cyclin D1* and *gapdh* gene on L929 cells which were exposured with P compounds

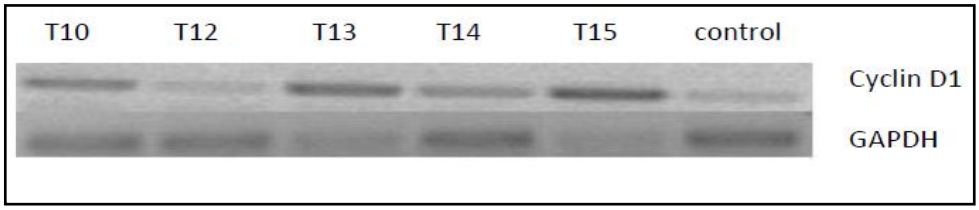


Figure 4.15. RT-PCR results for comparing expression levels of *cyclin D1* and *gapdh* gene on L929 cells which were exposured with T compounds

As seen in Figures we cannot equalize the house keeping gene expression levels of all samples due to high toxicity of the compounds (not enough cells = not enough RNA).
After normalizing the data to the best of our ability, gene expression ratios was calculated according to the ratio of interested gene density / house keeping gene density of control cells (not exposed to the synthetic compounds).

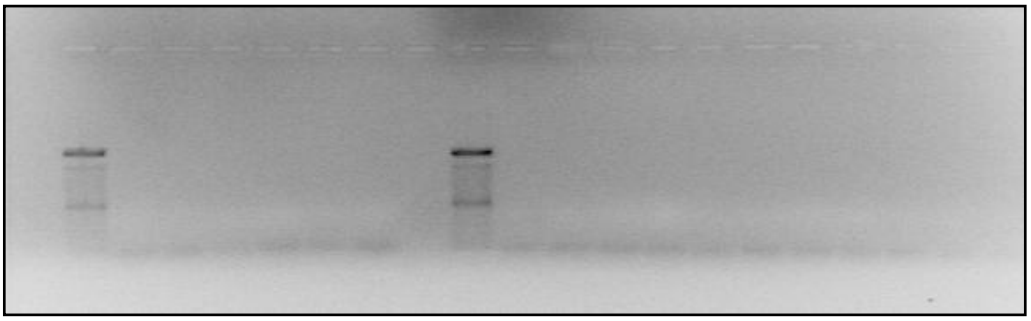


Figure 4.16. RT-PCR results for comparing expression levels of *elk-1* gene on L929 cells which were exposured with P and T compounds

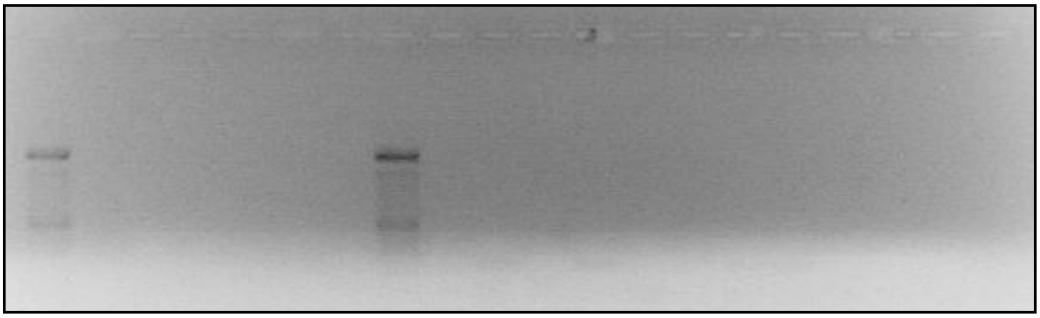


Figure 4.17. RT-PCR results for comparing expression levels of *c-fos* gene on L929 cells which were exposured with P and T compounds

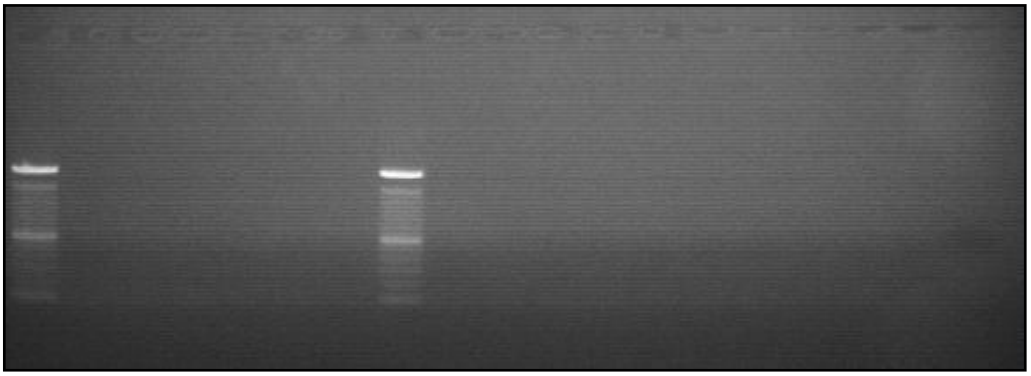


Figure 4.18. RT-PCR results for comparing expression levels of *p21* gene on L929 cells which were exposured with P and T compounds

As seen in Figure 4.16., 4.17. and 4.18.; we cannot observe any bands on the gel we cannot calculated the ratio of gene expressions of *c-fos* , *elk-1* and *p21* on L929 cells. Although these genes are uniquitous, they were not detected in L929 cells. Primer degradation might be an explanation of these results.

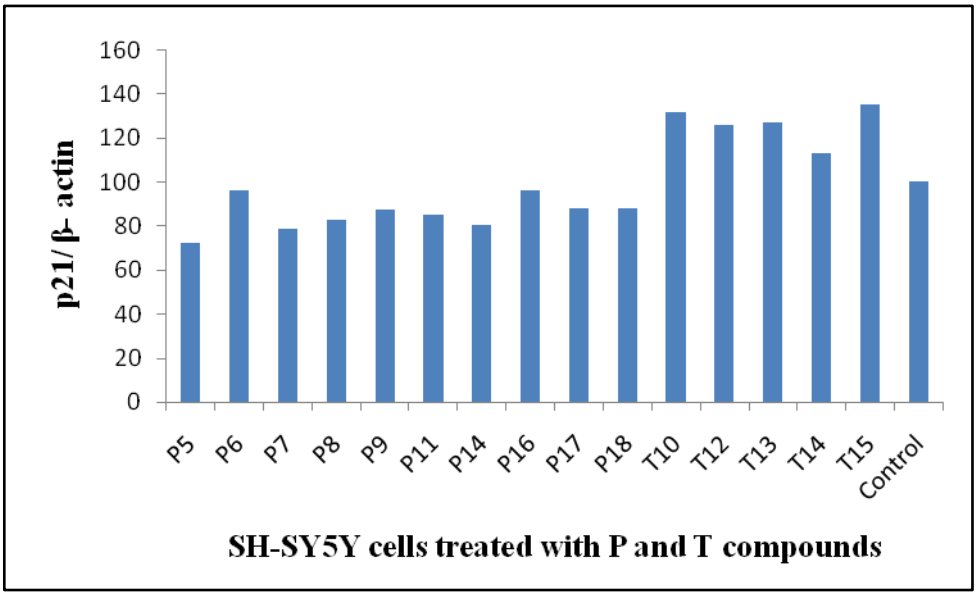


Figure 4.19. The ratios of *p21* gene expression / house-keeping genes expression in SH-SY5Y cells treated with P and T compounds (control sample were considered as 100% of gene expression ratio)

As shown in Figure 4.19, P compounds generally decreased the *p21* gene expression levels. It is observed that compound P6 and P15 did not alter this gene expression level. However P5 showed the most significant reduction, down to nearly 70 % of control. As for T compounds, it is seen that all variations of the series, especially T15 increased p21 expression levels to almost 130 % of control.

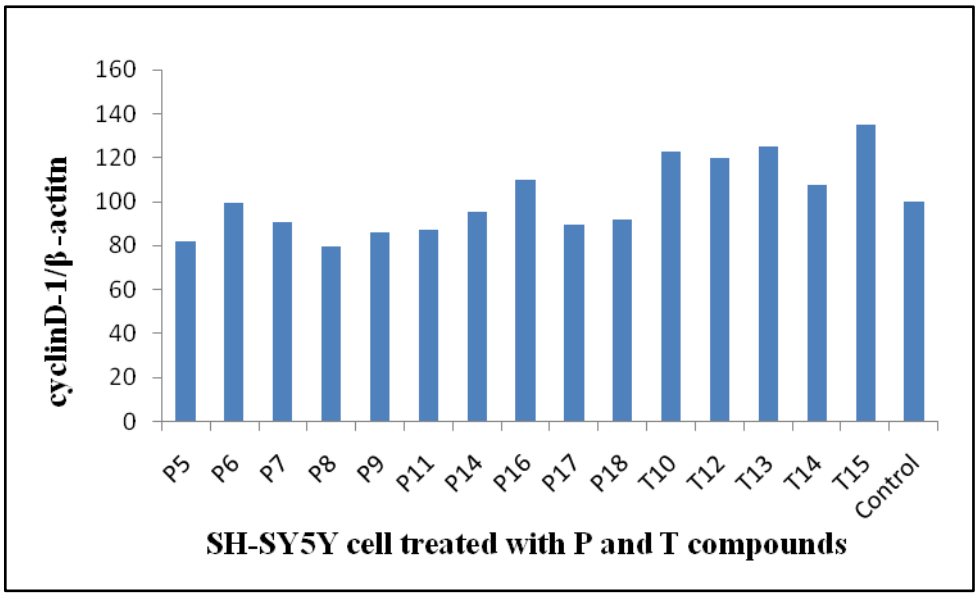


Figure 4.20. The ratios of *cyclin D1* gene expression/house keeping genes expression in SH-SY5Y cells treated with P and T compounds (control sample were considered as 100% of gene expression ratio)

In Figure 4.20., it is clearly seen that P compounds except p15 generally reduced the p21 gene expression levels. P6 compound did not significantly change the expression level of *cyclin D1*. Nevertheless in cells treated with T series, *p21* expression levels rised .to around 120% ratio.

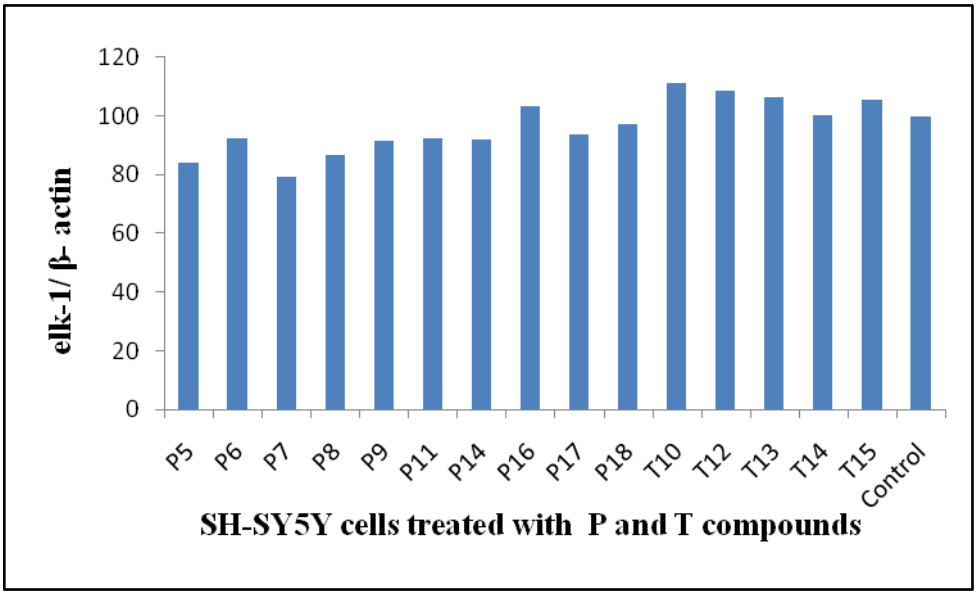


Figure 4.21. The ratios of *elk 1* gene expression / house-keeping genes expression in SH-SY5Y cells treated with P and T compounds (control sample were considered as 100% of gene expression ratio)

As shown in Figure 4.21, in the cells treated with P compounds, decrease in the level of *elk1* expression was not significant. As for T series, except for effect of T14, a little increase of *elk-1* expression level were observed, but it did not go beyond around 110 %. The significance of this increase needs to be confirmed.

.

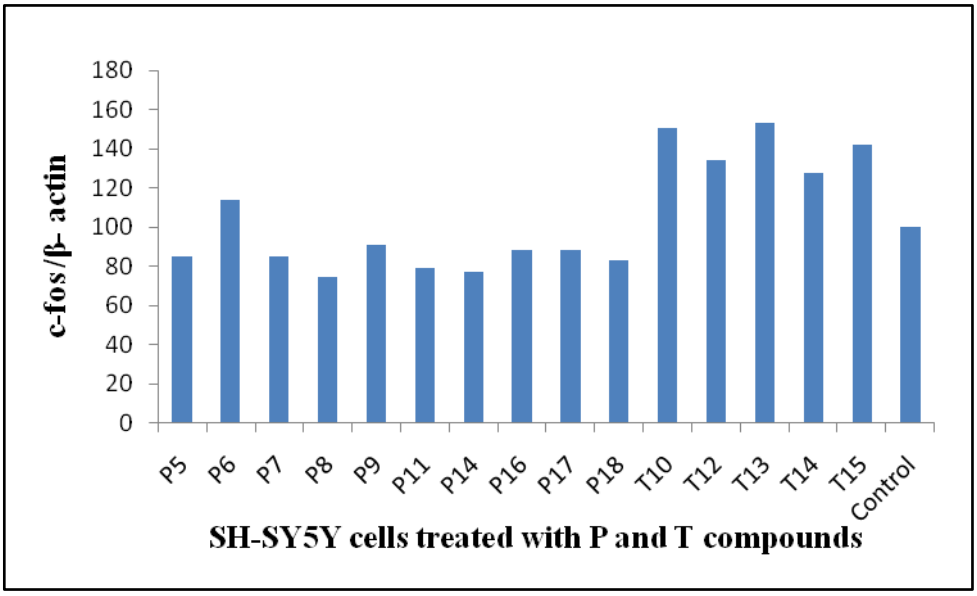


Figure 4.22. The ratios of *c-fos* gene expression / house-keeping genes expression in SH-SY5Y cells treated with P and T compounds (control sample were considered as 100% of gene expression ratio)

In Figure 4.22., T series especially T13, increased the *c-fos* expression level to 30-50%. In contrast, in P series only P6 increased the expression level of *c-fos* to 10%. Other compounds showed reduction on *c-fos* expression levels.

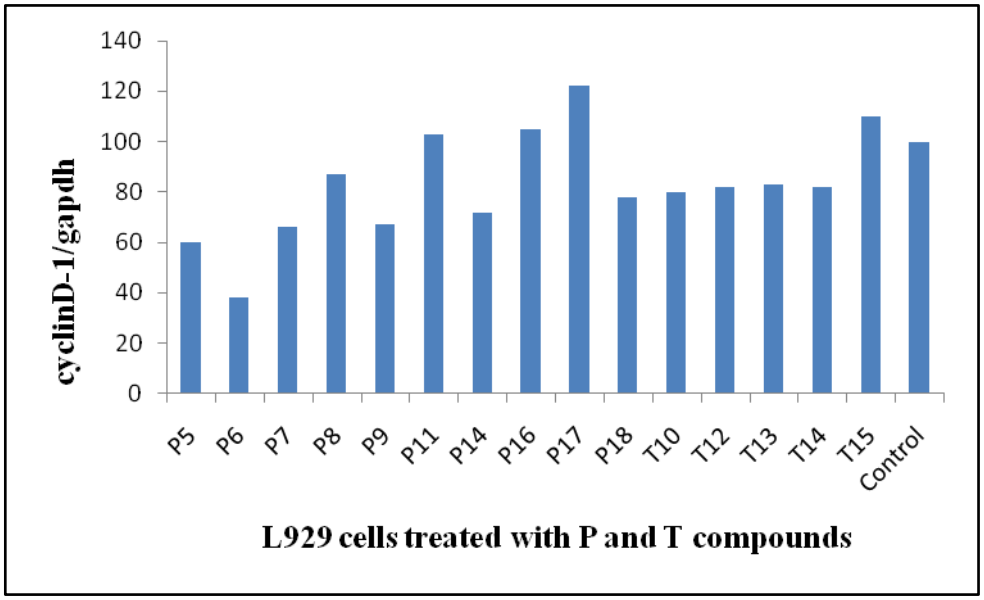


Figure 4.23. The ratios of *cyclinD1* gene expression / house keeping genes expression in L929 cells treated with P and T compounds (control sample were considered as 100% of gene expression ratio)

In figure 4.23, all of the compounds except P17 and T15 decreased the *cyclin D1* expression levels. However, P17 increased *cyclinD1* expression to 20%.

p21 has a role in cell cycle arrest and DNA repair. It inhibits several cyclin / CDK complexes and cause a pause in G1-S phase transition of the cell cycle through p53 tumor suppressor gene [71]. If there is a significant damage that could not be repaired, it will lead to activation of pro-apoptotic genes such as Noxo, Puma and Bax and inhibits anti-apoptotic genes such as Bcl-2 and Bcl-xL [72].

In our results, *p21* expression levels were reduced in P series and increased in T series. Hence, it could be that during the experiments (which rely on non-synchronous population of cells) we may not have treated the cells all at the same phase of the cell cycle, therefore may not have observed proper regulation of *p21* expression at the onset of G1/S phase. As our treatment lasts 24 hours, P series may have already initiated apoptosis in these cells, and by the time we have obtained RNA there may have remained no adequate living cells to compare expression levels of the gene. Another reason of this effect might be that these drugs do not act through the *p21* gene and may be using as yet unidentified pathways.

c-Fos is a cellular proto-oncogene and a member of immediate early gene family of transcription factors. Binding to c-Jun protein, it forms the AP-1 transcription factor which upregulates transcription of a diverse range of genes involved in everything from proliferation and differentiation to defense against invasion and cell damage [73].

c-Fos and c-Jun proteins are the first proteins transcribed in G0/S phase and it is seen that they also initiate the cell cycle. C-Jun / c-Fos complex binds to specific promoter regions of DNA and trigger DNA transcription [74].

It is expected to observe a reduction in gene expression of c-Fos. However, in our results, T series increased the expression levels of *c-fos.* This can be explained that the compounds leaded to cell death and little amount of remaining cells increased the *c-fos* expressions to rise their survivals. Another alternative idea might be that these drug concentrations are not sufficient to decrease the expression levels of the gene.

elk1 (E twenty-six (ETS)-like transcription factor 1) which belongs to ETS oncogene family is a protooncogene and functions as a transcription activator. It has significant role in various diseases such as Alzheimer's disease, Down syndrome, cancer, drug addiction and depression and also in long-term memory formation. One of the downstream target of *elk*-1 is the gene for the *c-fos* proto-oncogene [75].

Some of the compounds we tested in this PCR analysis showed a cytotoxic effect on SH-SY5Y cells. Due to the cytotoxic effect on SH-SY5Y cells I expected a decrease in *elk-1* expression levels as well. However the compounds did not decrease elk-1 expression. One explanation could be that these drugs did not inhibit the proliferation by targeting this gene.

In P series except P6 and P16, the expression levels of *c-fos* and *elk 1* are decreased. It could be suggested that P series compounds could inhibit cell proliferation by targeting these protooncogenes. Reduction in expression levels of *cyclinD-1* and *p21* could be due to the initiation of cell death pathways (either necrotic or apoptotic) already before it could be detected in our assays by the expression of genes analyzed.

cyclinD-1 is a protein which specific to G1/S in cell cyle and protein expressed by this gene is the member of cyclin family which characterized being in large amount in throughout the cell cycle. Mutations and overexpression of this gene constantly observed in different tumor types and may contribute to tumorigenesis [76]. It is expected that the compounds exhibiting a high anti-proliferative effect might decrease *cyclinD-1* expression. Nevertheless, in our results we could not observe a significant decrease in the expressionon SH-SY5Y cells. Hence, it is suggested that the cells could die because of necrosis or they undergo apoptosis already before it could be detected in G1/S checkpoint. This circumstance may also affect the results of other gene expression analyses, since we have incubated the cells for 24 hours with the compounds. Incubation period of 6h and 12 h respectively could be included in the assay so as to provide more detailed analysis and to possibly get data at earlier expression time points.

Another explanation of these results could be that these compounds might bind or interact with the *cyclinD-1* and other proteins instead of regulating gene expression. Therefore, studies at the protein level could also be conducted in the long term.

In Figure 4.23., we observed that compounds generally decreased the *cyclinD-1* expression levels. It is expected that the cytotoxic compounds might decrease the expression. Although P6, T10, and T13 did not show cytotoxic effect on L929 cells, they decreased *cyclinD-1* expression. The reduced levels of *cyclinD-1* of P6, T10 and T13 does not necessarily mean the less amount of proteins, the efficiency of translation of *cyclinD-1* mRNA to protein might have been diminished. In contrast, the translation efficiency could have decreased in the cells treated by as P18, whose *cyclinD1* expression appears to be relatively higher. In this case, the protein levels ought to be measured by Western Blotting or mRNA levels should be tested more precisely by Real Time PCR in order to compare the effects of the drugs in more details.

5. CONCLUSION

The objective of this study was to screen the anti-proliferative activity of new synthesized compounds. For this purpose we first tested activities on cell viability with MTT assays. According to the results it can be concluded that S3, S4 compounds in sigma receptor ligands; P5, P6, P7, P8, P9, P11, P14, P16, P17 and P18 in piperazine derivatives (unpublished results) and T10, T12, T13, T14 and T15 in thalidomide derivatives have specific anti-proliferative activities on neuroblastoma cell line so that they can be used for anticancer drug development processes. In addition, P10 and P13 showed proliferative effect on L929 healthy fibroblast control cells and can be used for drug development process for wound healing in the long term.

The aim of the second part of this study was to understand the mechanism of action of these compounds. To that end, we compared the expression levels of some of the cell cycle related genes using RT-PCR experiments. This was a preliminary study, and so far our results have been largely inconclusive. However, most of these cell cycle-related genes are actually regulated at the protein level, therefore future experiments should address the effect of these compounds on the protein level of these genes. Additionally, as part of the drug development process, these drug candidates should be screened on primary tumor cells, followed by *in vivo* tumor growth assays in animals.

APPENDIX A: CHEMICAL STRUCTURES OF ANALYZED COMPOUNDS

Table A.1. Chemical Sturctures of Thalidomide Derivatives

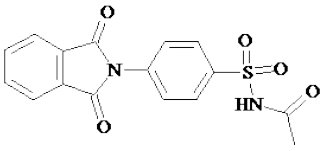
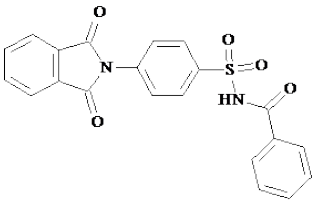
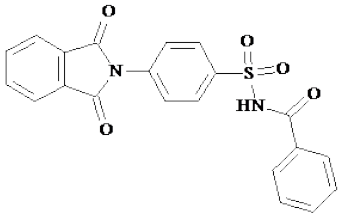
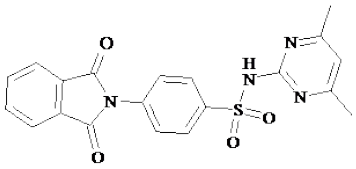
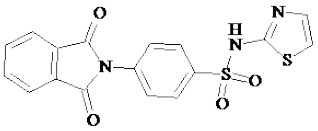
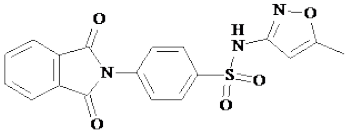
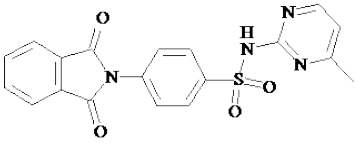
Code	Compound Name	Structure
T1	N-([4-(1,3-Dioxoisoindolin-2-yl)phenyl]sulfonyl)acetamide	
T2	N-benzoyl-4-(1,3-dioxoisoindolin-2-yl)benzene sulfonamide	
T3	N-benzoyl-4-(1,3-Dioxoisoindolin-2-yl)benzenesulfonamide	
T4	N-(4,6-Dimethylpyrimidin-2-yl)-4-(1,3-dioxoisoindolin-2-yl)benzene sulfonamide	
T5	4-(1,3-Dioxoisoindolin-2-yl)-N-(2-thiazolyl)benzenesulfonamide	
T6	4-(1,3-Dioxoisoindolin-2-yl)-N-(5-methylisoxazole-3-yl)benzene sulfonamide	
T7	4-(1,3-Dioxoisoindolin-2-yl)-N-(4-methylpyrimidin-2-yl)benzene sulfonamide	

Table A.1. Chemical Stuructures of Thalidomide Derivatives (continue)

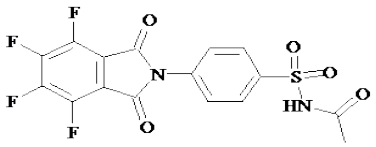
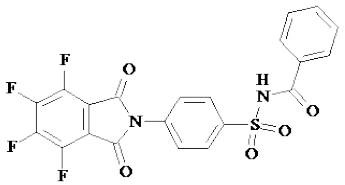
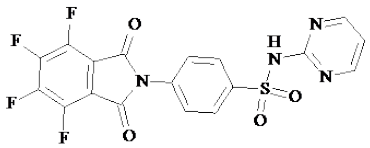
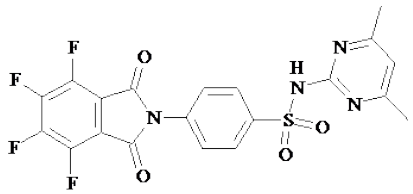
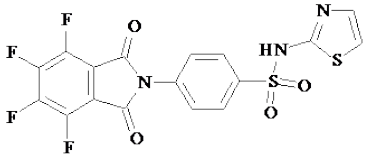
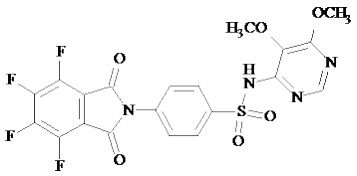
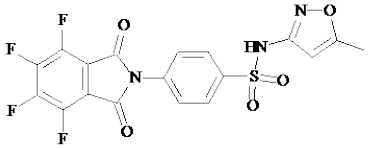
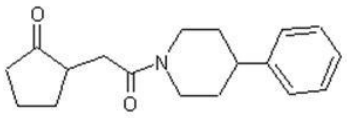
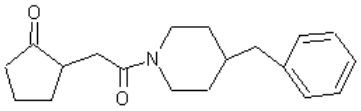
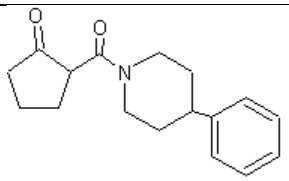
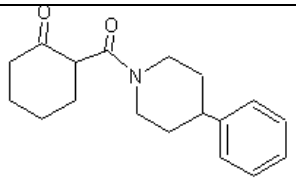
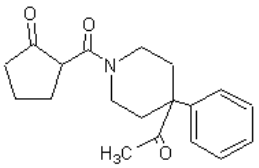
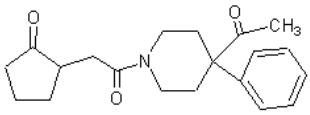
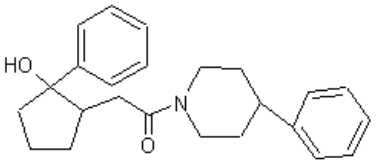
Code	Compound Name	Structure
T9	N-([4-(4,5,6,7-Tetrafluoro-1,3–dioxoisoindolin-2-yl)phenyl]sulfonyl)acetamide	
T10	N-benzoyl-4-(4,5,6,7-Tetrafluoro-1,3-dioxo–isoindolin-2-yl)benzenesulfonamide	
T11	4-(4,5,6,7-Tetrafluoro-1,3-dioxo-isoindolin-2-yl)-N-pyrimidin-2-yl-benzenesulfonamide	
T12	N-(4,6-Dimethylpyrimidin–2-yl)-4-(4,5,6,7-tetrafluoro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide	
T13	4-(4,5,6,7-Tetrafluoro-1,3-dioxo-isoindolin-2-yl)-N-(2-thiazolyl)benzenesulfonamide	
T14	N-(5,6–Dimethoxypyrimidin-4-yl)-4-(4,5,6,7-tetrafluoro-1,3–dioxoisoindolin-2-yl)benzenesulfonamide	
T15	N-(5–methylisoxazol-3–yl)-4-(4,5,6,7-tetrafluoro-1,3-dioxoisoindolin-2-yl)benzenesulfonamide	

Table A.2. Chemical structure of Sigma Receptor Ligands

Code	Compound Name	Structure
S1	2[2-oxo-2-(4-phenyl-1-piperidyl)-ethyl]cyclopentanone	
S2	2-(2-(4-benzilpiperidin-1-il)-2-oksoetil)siklopentanon	
S3	2-((4-phenylpiperidinil)carbonyl)cyclopentanone	
S4	2-((4-Phenylpiperidinil)carbonyl)cyclohexanone	
S5	2-(4-acetyl-4-phenylpiperidin-1-carbonyl)cyclopentanone	
S6	2-(2-(4-acetyl-4-phenylpiperidin-1-il)-2-oxoethyl cyclopentanone	
S7	2-(2-hydroxy-2-phenylcyclopentyl)-1-(4-phenylpiperidin-1-yl)ethanone	

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