DEVELOPMENT AND INVESTIGATION OF ETOPOSIDE RESISTANCE IN MCF-7 BREAST CANCER CELL LINE

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ABSTRACT

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Failure of chemotherapy in cancer patients because of development of drug resistance is a major problem. Alterations of DNA repair mechanisms and drug targets are among the important resistance mechanisms which are developed against topoisomerase II inhibitors etoposide and doxorubicin. Modifications in the expression levels of mismatch repair (MMR) genes due to resistance to topoisomerase II inhibitors are involved in breast cancer.

In this study, etoposide resistant sublines were developed from MCF7 breast cancer cell line (MCF7/S) and the expression levels of *TOP2A* and two important MMR genes *MSH2* and *MLH1* were examined by real time qPCR. Previously developed doxorubicin resistant cells were also studied for comparison. Etoposide resistant sublines MCF7/1000E, MCF7/1250E and MCF7/2000E were approximately 2, 3 and

4 fold resistant relative to parental MCF7/S cells, respectively. *MLH1*, *MSH2* and *TOP2A* expressions decreased in both etoposide and doxorubicin resistant sublines relative to MCF7/S cells. Expression levels of *TOP2A* in resistant sublines differ between 10-95 percent of the expression levels in the parental cells. In the sublines MCF7/200E, MCF7/500E, MCF7/750E and MCF7/1000E a decrease in *TOP2A* gene expression was determined. In sublines MCF7/1250E and MCF7/2000E fluctuations in the expression levels were observed. Among the doxorubicin resistant sublines (MCF7/600D and MCF7/1000D), in MCF7/1000D which is more resistant to doxorubicin, *TOP2A* expression level was higher. Expression levels of *MSH2* decreased regularly as the resistance increased. However, in MCF7/1250E significant increase relative to MCF7/1000E was observed. In MCF7/2000E, expression levels of *MSH2* again significantly decreased to 41 percent of the levels in parental cell line. Expression levels of *MLH1* decreased significantly (18-58 percent) in etoposide resistant sublines relative to MCF7/S cells. In doxorubicin resistant sublines, a decrease in *MLH1* gene expression was observed in MCF7/1000D.

It can be concluded from the results that decrease in the expression levels of *TOP2A*, *MSH2* and *MLH1* genes may contribute to resistance together. Above a certain resistance level, sublines may develop new strategies for acquiring higher resistance. Whenever a strategy becomes limited, new strategies emerge. New approaches developed to overcome resistance in cancer chemotherapy should consider the molecular basis of resistance in different stages of the disease.

Key words: Breast cancer, drug resistance, etoposide, doxorubicin

ÖZ

MCF-7 MEME KANSERİ HÜCRE HATTINDA ETOPOSİT DİRENCİNİN GELİŞTİRİLMESİ VE İNCELENMESİ

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Kemoterapideki başlıca sorunlardan biri kanser hastalarında ilaç dirençliliğinin gelişmesi nedeniyle tedavinin başarıya ulaşamamasıdır. DNA tamir mekanizmalarının ve ilaç hedeflerinin değiştirilmesi, topoizomeraz inhibitörleri etoposit ve doksorubisine karşı geliştirilen önemli direnç mekanizmalarındandır. Meme kanserinde topoizomeraz II inhibitörlerine karşı geliştirilen dirençlilikten dolayı hatalı eşleşme tamir (HET) genlerinin ifade düzeylerinde farklılıklar görülmektedir.

Bu çalışmada, MCF7 meme kanseri hücre hattından (MCF7/S) etoposide dirençli alt hücre hatları geliştirilmiş, *TOP2A* ile iki önemli HET geni *MSH2* ve *MLH1*'in ifade düzeyleri gerçek zamanlı PZR kullanılarak incelenmiştir. Karşılaştırma amacıyla, önceden geliştirilmiş olan doksorubisine dirençli hücreler de incelenmiştir. Etoposide

dirençli MCF7/1000E, MCF7/1250E ve MCF7/2000E hücre hatlarının, ana hücre hattına göre sırasıyla yaklaşık 2, 3 ve 4 kat dirençli olduğu bulunmuştur. Hem etoposite hem de doksorubisine karşı dirençli hatlarda *MLH1*, *MSH2* ve *TOP2A* ifade düzeyleri MCF7/S hücrelerine göre düşmüştür. Dirençli hücre hatlarında *TOP2A* ifade düzeyleri ana hücre hattındakine göre yüzde 10-95 arasında değişmektedir. MCF7/200E, MCF7/500E, MCF7/750E ve MCF7/1000E alt hücre hatlarında *TOP2A* gen ifadesinde düzenli bir azalma belirlenmiştir. MCF7/1250E ve MCF7/2000E alt hücre hatlarında gen ifade düzeylerinde dalgalanmalar görülmüştür. Doksorubisine dirençli hücre hatları (MCF7/600D ve MCF7/1000D) arasında, doksorubisine daha dirençli olan MCF7/1000D hücrelerinde *TOP2A* geninin ifadesi daha yüksek bulunmuştur. *MSH2* ifade düzeyleri dirençlilik arttıkça düzenli olarak azalmıştır. Ancak MCF7/1250E alt hücre hattında MCF7/1000E alt hücre hattına göre anlamlı bir artış görülmüştür. MCF7/2000E hücrelerinde *MSH2* ifade düzeyleri yine anlamlı bir şekilde ana hücre hattındaki düzeylerin yüzde 41'ine düşmüştür. *MLH1* ifade düzeylerinin etoposite dirençli bütün alt hücre hatlarında ana hücre hattına göre anlamlı olarak (yüzde 18-58) azalmıştır. Doksorubisine dirençli hücre hatlarında, MCF7/1000D'de *MLH1* ifadesinde azalma görülmüştür.

Buna göre, *TOP2A, MSH2* ve *MLH1* genlerinin ifade düzeylerinin paralel olarak azalmasından bu genlerin dirençliliğe ortak olarak katkıda bulunduğu sonucu çıkarılabilmektedir. Belli bir dirençlilik seviyesinin üzerinde, hücrelerin daha yüksek dirençliliğin kazanılması için yeni stratejiler geliştirdiği düşünülmektedir. Dirençlilik mekanizmalarından birisi yeterli olmadığında, yeni stratejiler ortaya çıkmaktadır. Bu nedenle, kemoterapide dirençliliğin engellenebilmesi için geliştirilen yaklaşımlar, hastalığın farklı evrelerindeki değişken moleküler mekanizmaları göz önünde bulundurmalıdır.

Anahtar kelimeler: Meme kanseri, ilaç dirençliliği, etoposit, doksorubisin

To my adored family

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ABC	ATP-Binding Casette
cDNA	Complementary Deoxyribonucleic Acid
DEPC	Diethyl Pyrocarbonate
dH_2O	Distilled water
DMSO	Dimethyl Sulfoxide
dNTP	Deoxy Nucleotide Triphosphate
DOX	Doxorubicin
EDTA	Ethylene Diamine Tetraacetic Acid
ETO	Etoposide
FBS	Fetal Bovine Serum
FC	Fold Change
HET	Hatalı Eşleşme Tamir
IC_{50}	Inhibitory Concentration Fifty
MCF7	Orijinal Rapid-Growing Breast Cancer Cells
MCF7/S	MCF7 cell line sensitive to drugs
MCF7/200E	MCF7 subline resistant to 200 nM etoposide
MCF7/500E	MCF7 subline resistant to 500 nM etoposide
MCF7/750E	MCF7 subline resistant to 750 nM etoposide
MCF7/1000E	MCF7 subline resistant to 1000 nM etoposide
MCF7/1250E	MCF7 subline resistant to 1250 nM etoposide
MCF7/2000E	MCF7 subline resistant to 2000 nM etoposide
MCF7/600D	MCF7 subline resistant to 600 nM doxorubicin
MCF7/1000D	MCF7 subline resistant to 1000 nM doxorubicin
MDR	Multidrug Resistance
MDR1/ P-gp	Multidrug Resistance Protein 1/ P-glycoprotein

min	Minute
MLH1	MutL Homolog 1
MMR	Mismatch Repair
MRP1	MDR-Associated Protein 1
M-MLV	Moloney Murine Leukemia Virus
MSH2	MutS Homolog 2
MSH6	MutS Homolog 6
hMutSα	Human MutSα consists of MSH2-MSH6 heterodimer
hMutSβ	Human MutSβ consists of MSH2-MSH3 heterodimer
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PZR	Polimeraz Zincir Reaksiyonu
qPCR	Quantitative Real Time Polymerase Chain Reaction
R	Relative resistance index
RNA	Ribonucleic Acid
rpm	Revolution per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TAE	Tris Acetate EDTA
TOP2A	DNA topoisomerase II alpha
V	Volt
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Definition and History of Cancer

Hippocrates (460-370 B.C.) used the words c*arcinoma* and *carcinos* for cancer which express crab in Greek. The term *cancer* was firstly used by Celsus (28-50 B.C.), a Roman physician, which again refers to crab in Latin. Roman physician Galen (130-200 A.D.) used *oncos* which means swelling (http://www.cancer.org/Cancer). Today, the term *cancer* is used to define a group of disease characterized by abnormal growth and uncontrolled spread of cells as a result of genetic and epigenetic changes. The definition of the word *cancer* generally includes invasion and metastasis of the abnormal cells.

The definition of cancer dates back to about 3700 years ago. In the Edwin Smith Papyrus which belongs to old Egyptians (1600 B.C.), cancer is described as an untreatable disease. Although centuries have passed over the foundation of cancer in Egypt, in the present day, there have still been very limited treatments of cancer. According to the reports of World Health Organization (WHO) in 2004, 13% (7.4 million) of deaths was due to cancer globally. Unfortunately, death rates continue to increase. It is estimated for the year 2030 that there will be 12 million deaths because of cancer worldwide (http://www.who.int). In Turkey 13.1% of all deaths are due to cancer, in 2000. 15.2% and 16.2% of all deaths are the estimated ratios for cancer in 2020 and 2030, respectively (Turkish Ministry of Health, 2005). Environmental alterations, such as increasing exposure to various carcinogens or UV in parallel with

technological development seem to increase the cancer rates. Even though the treatments are restricted, there are many promising developments on prevention, early diagnosis and treatment recently.

1.2 Cancer Biology

Cancer has been known for centuries; however the developments to understand cancer genetics initiated with the release of completed draft of Human Genome Project in 2003. After deciphering the human genome, researchers started genome-wide association studies (GWAS) which involve rapid scanning of known genetic markers of diseases. GWAS provide information about altered genes in cancer by comparing different groups and give insight about the underlying molecular reasons. Therefore, GWAS and other genetic studies lead to new discoveries and developments on therapeutic applications and on prevention from cancer (http://plan2009.cancer.gov/Genetics_of_Cancer.htm). Although the data about cancer genetics opens the gates for developments, a detailed understanding of cancer biology is also required for development of more adequate and applicable strategies.

Carcinogenesis, cancer formation, is a process involving initiation, promotion and progression stages. The development of cancer begins with transformation of a normal cell into a cancer cell as a result of sequential inherited and/or acquired mutations (Rieger, 2004). The genotoxic factors can be external, such as tobacco smoke, diet, irradiation, viruses, or internal like inherited mutations, repair mechanisms or hormones. Because of a prolonged exposure to mutators, by time the mutant cell becomes more susceptible for further transformation acquiring abnormal characteristics that alter the gene expressions through an unregulated growth. Along with series of divisions of mutant cell(s), a tumor consisting of many mutually dependent cell types is developed (http://plan2009.cancer.gov/Understanding_the_Biology_of_Cancer.htm). Some tumors, called “benign tumors”, do not invade the surrounding tissue and they can be removed surgically. However, some of the tumors go into a progression stage

representing genomic instability. They further gain advantageous characteristics, such as limitless replication, sustained angiogenesis, apoptosis avoidance, insensitivity to anti-growth signals and self-sufficiancy in growth (Hanahan and Weinberg, 2000). The tumors containing most of these characteristics have the capability to invade surrounding tissue and metastasize to other organs and they are classified as malignant type tumors. The term "cancer" is usually used to identify malignant tumors.

1.3 Types of Cancer

More than 200 different types of cancers are identified in literature and they are named according to the site of origin (organ or tissue) where they begin to grow. Cancer of leukocytes (white blood cells) is called "leukemia", while cancer of connective tissue including specialized white blood cells lymphocytes (lymphoid tissue) is called "lymphoma". The origin of melanomas is pigment cells of the skin, namely melanocytes. Carcinomas are types of cancer which originate in epithelial tissue lining the cavities, such as stomach or lung. The kinds of cancer which begin in muscle or connective tissue (such as bone and cartilage) are named as "sarcomas" (Cooper, 2000). In terminology, in order to indicate a specific type of cancer, generally name of the organ in which it initiates is used, like breast, colon, lung and prostate cancers.

1.4 Breast Cancer

The starting points of breast cancer may be different types of cells of breast. Breast is composed of lobules which are milk producing glands, ducts formed to carry milk, blood and lymph vessels and connective tissue. Breast cancer may develop in each of these structures and specifically named according to the place of origin. Previously, ductal carcinoma was known to develop in the epithelial cells lining ducts, and lobular cancer was believed to originate in the lobules. However, it is now known that both of the cancer types begin at the terminal lobular unit of terminal duct

(Tyczynski *et al*., 2002; Sainsbury *et al*., 2000). Ductal carcinoma is the most common breast cancer. Lobular and ductal breast cancers are usually invasive. There are also other types of breast cancer detected rarely. Inflammatory breast cancer (IBC) which is diagnosed with red-looking, warm and itchy breast is an example of unusual types of breast cancer (American Cancer Society, 2010).

Breast cancer is the mostly observed among females worldwide and cancer-associated deaths of women are mainly due to breast cancer. One out of nine women suffer from breast cancer every year (Köhrmann *et al*., 2009). According to the WHO reports, cancer was responsible from 962,000 deaths globally in 2004 and breast cancer contributes to overall cancer mortality with 519,000 deaths, annually (World Health Organization, 2010). In United States (US), 28% of cancer cases are expected to be breast cancer among women and the estimated death rate of new breast cancer cases is 15% (American Cancer Society, 2010). Similarly, in Europe, breast cancer cases constitute 26.5% of all cancer cases with a cancer death rate of 17.5% (Tyczynski *et al*., 2002). Developing countries contribute to more than 70% of all cancer cases. As a middle-income country, breast cancer incidence and mortality rates are high, in Turkey. It is indicated that incidence of breast cancer has been increasing in Turkey, and for the year 2007, estimated number of breast cancer cases was around 44,253 (Özmen *et al*., 2009). Although lung cancer is in the first place with an incidence of 30.13% over 100,000 cases, when the data is evaluated according to the gender breast cancer is the most common cancer (36.47% over 100,000 cases) among women (Yılmaz *et al*., 2010). Having these statistics, breast cancer stands to be a big problem waiting for new therapeutic strategies.

There are many external and internal factors causing breast cancer. Exposure to tobacco smoke, early menarche, giving birth at old ages, using hormone therapy during or after menopause, physical inactivity, alcohol use and fat-rich diets are some of the external factors (Tyczynski *et al*, 2002; World Health Organization, 2009). People having family history and genetic predisposition are under an internal risk (Tyczynski *et al*, 2002). People having *BRCA1* and *BRCA2* gene mutations, have

a high risk of developing breast cancer during their lifetime (Emery *et al*., 2001). Elucidating these factors and eliminating them is very important to prevent breast cancer.

1.5 Treatment of Breast Cancer

There are improvements in the treatment of breast cancer recently. Treatments composed of surgery, chemotherapy, radiotherapy and targeted therapy. A cancer specialist together with the patient decides the type of therapy according to the stage of cancer and physiologic and/or psychological condition of the case.

Surgery including lumpectomy or mastectomy is usually the choice of primary therapy. Lumpectomy is removal of only tumor with a little of surrounding tissue, also called as breast-conserving surgery. In the advanced or risky cases sometimes mastectomy, which is the removal of breast tissue, is the decision. Formation of new tumors mostly prevented by means of mastectomy.

Chemotherapy is defined as a systemic therapy; because the drugs used for cancer treatment are injected to the bloodstream, therefore they affect the entire body. In order to eliminate all the possible cancer cells in the body, chemotherapy generally used for invasive breast cancer after surgery. However, chemotherapy sometimes also used to decrease the size of tumor before surgery (http://www.breastcancer.org/treatment/chemotherapy/). Since chemotherapy affects the whole body, the patients encounter significant side effects.

In radiotherapy, in order to destroy the cancer cells high energy beam, namely ionizing radiation is utilized. During radiotherapy normal cells as well as cancer cells are affected by radiation. However, because the repair and regulation mechanisms of healthy cells are stronger than cancer cells, normal cells are able to overcome the side effects. Radiation therapy can be used at all levels of breast cancer. It is an effective and reliable method to treat the possible remaining cancer cells after

surgery. Therefore, recurrence of cancer is highly impeded. It is shown by Clarke *et al*. (Clarke *et al*., 2005) that radiotherapy applied after lumpectomy significantly decreased the 5-year risk of relapse of breast cancer with a rate of 19%.

Hormonal therapy is another type of therapy used for estrogen receptor positive breast cancers. Estrogen causes progression of estrogen-positive breast cancer, for this reason, hormone therapy targets estrogen. There are three groups of drugs used in hormone therapy. One group is aromatase inhibitors which blocks the production of estrogen. This group of drugs decreases the amount of estrogen and blocks the stimulation of estrogen-positive breast cancer cells to proliferate. Aromatase inhibitors are not able to block estrogen production in ovaries; hence they are used in post menopausal women. Selective estrogen receptor modulators and estrogen receptor downregulators are other two groups used in hormone therapy. Both groups work in a similar manner. Their structure resembles estrogen and by mimicking estrogen they bind to estrogen receptors inhibiting estrogen binding, since blocking proliferation of cancer cells (http://www.breastcancer.org/treatment/hormonal/).

A new promising therapy in breast cancer is targeted therapy. In targeted therapy, aim of the developed drugs is disrupting specialized characteristics of cancer cells. This group of therapy includes the use of monoclonal antibodies (MAb) mimicking the natural antibodies, protein kinase inhibitors or inhibitors of repair enzymes. For instance, in HER-2 receptor positive cells trastuzumab (Herceptin) is used as a monoclonal antibody to inhibit the receptor functioning and signal transduction through growth and proliferation (National Cancer Institute, Breast Cancer Treatment, 2010). Poly ADP-ribose polymerase (PARP) inhibitors are another example as targeted therapy drugs which are in clinical trial phase II. PARP is a nuclear enzyme which recognizes DNA nicks caused after chemotherapy or radiotherapy and facilitates the DNA repair. When it is halted by an inhibitor, repair cannot be processed and cancer cells go into apoptosis (Low and Ratnam, 2007). PARP inhibitors seem to be effective especially in BRCA1 and BRCA2-deficient hereditary breast cancer (Miles DW, 2009).

1.5.1 Chemotherapy

Cancer chemotherapy is application of anti-cancer drugs to a patient in order to destroy the cancer cells or reduce their proliferation. In chemotherapy, the drugs travel throughout bloodstream, hence all over the body, so the metastatic tumors can also be treated. In breast cancer, chemotherapy is used to prevent relapse in early-phase invasive breast cancer after surgery and to destruct cancer cells as much as possible in advanced-phase breast cancer. Drugs are mostly given through infusion in a vein, injection to a muscle or orally as a pill. The drugs and combinations can change according to the stages or features of breast cancer, such as invasive-noninvasive, her2+/her2- etc. Additionally, age and health state of the patient and the drugs applied previously must be considered. Drug interactions are also among the important factors to be considered while deciding the combinations. Although chemotherapy is a beneficial method of treatment in breast cancer and used generally, it has many side effects that decrease the life quality of the patients. Chemotherapeutics weakens the healthy cells as well as cancer cells; therefore the side effects can be devastating. Especially, fast-dividing cells, like hair follicles are negatively influenced from chemotherapeutics.

Today, clinically alkylating agents, topoisomerase inhibitors, antimetabolites and plant alkaloids are used alone or in combination in breast cancer. Alkylating agents causes strand breakage in nucleic acids by binding to N7 position of guanine and causing latter miscoding of thymine or depurination. This leads to interference in transcription and DNA replication. Cisplatin and carboplatin are widely used drugs as alkylating agents. Topoisomerase inhibitors composed of topoisomerase I and topoisomerase II inhibitors. These chemotherapeutics stop functioning of topoisomerases which make the complex structure of DNA open for transcription, replication or repair. Etoposide and doxorubicin are used as topoisomerase II inhibitors in chemotherapy. Topotecan is one of the examples of topoisomerase I inhibitors. Antimetabolites are purine or pyrimidine mimicking agents which incorporate into DNA during S phase. This group of drugs is called “cytostatics”

since they halt the cell cycle. Plant alkaloids include podophyllotoxins, vinca alkaloids and taxanes. Vinca alkaloids inhibit the function of microtubules by interfering with the assembly of microtubules whereas function of taxanes is stabilizing their structure. Podophyllotoxins are antimitotic agents which inhibit cells to go into G1 phase. Etoposide is one of the phodophyllotoxins which inhibits topoisomerase II activity.

1.5.1.1 Topoisomerase II Inhibitors

Topoisomerases are the nuclear enzymes which are involved in processes such as chromosome condensation/decondensation and the relief of tensional stress that occurs during replication and transcription. There are two isoforms of topoisomerase II: topoisomerase II alpha (TOP2A) and beta (TOP2B). These enzymes are differentially expressed in different tissues. In proliferating cells TOP2A is highly expressed, whereas TOP2B expression does not change in proliferating and nonproliferating cells. Therefore, TOP2A is assumed to be a specific marker of proliferation (Capranico, 1992). Cancer cells have also high proliferating rates and high expression of TOP2A may be used as cancer marker. However, decrease in *TOP2A* gene expression was shown in many studies (Takano *et al*., 1992; Hasegava *et al*., 1995).

Topoisomerase II catalyzes the transient breaking and rejoining of two strands of double helix allowing the strands to pass through one another. With this mechanism topoisomerase II controls and modifies the topologic state of DNA in nucleus. The mechanism of action of topoisomerase II is summarized in figure 1.1.

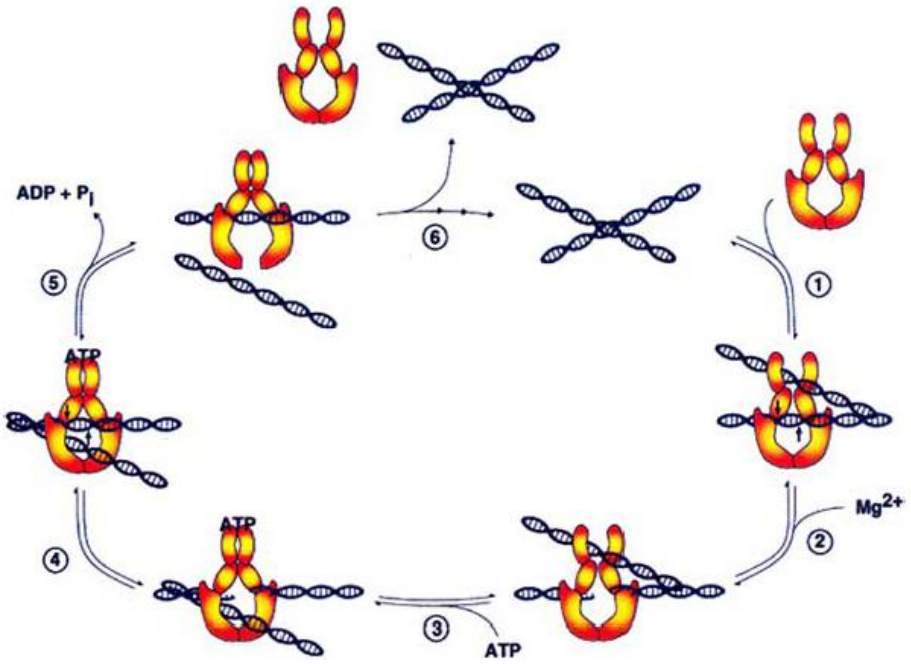


Figure 1. 1 Mechanism of action of topoisomerase II
(1) Topoisomerase II-DNA binding (2) pre-strand passage DNA cleavage-religation equilibrium (3) ATP binding and DNA strand passage (4) post-strand passage DNA cleavage-religation equilibrium (5) ATP hydrolysis and gate opening (6) DNA release and enzyme turnover (Baldwin and Osheroff, 2005).

Some topoisomerase inhibitors blocks religation of broken DNA by stabilizing topoisomerase II-DNA cleavage complex while others mainly act by accelerating the enzyme rate to generate more DNA cleavage as well as little inhibition of ligation. Former group of drugs increase concentration of topoisomerase-DNA cleavage complexes and additionally extend the half life of them. (Baldwin and Osheroff, 2005). These mechanisms of both groups of drugs cause permanent double strand breaks in DNA and enhance apoptotic signals leading to an effective death of cancer cells. The fate of the cancer cells affected by topoisomerase II inhibitors is shown in Figure 1.2.

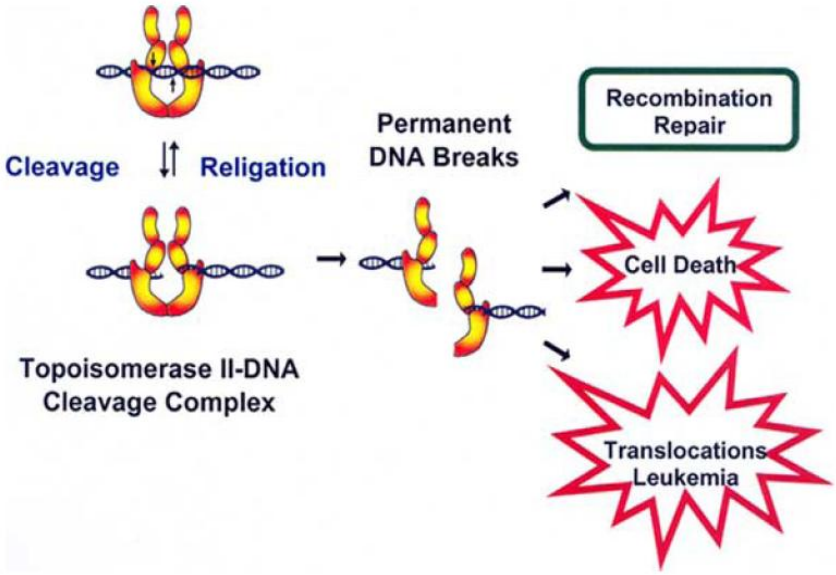


Figure 1. 2 Results of accumulated permanent topoisomerase II-DNA complexes created by topoisomerase II inhibitors (Baldwin and Osheroff, 2005).

1.5.1.1.1 Etoposide (Vepesid, Toposar®)

Etoposide is an effective antimitotic and antineoplastic agent which has a clinical usage for over two decades. Podophyllotoxins were first discovered in 1950s and since they were very toxic to be used clinically a serious of semisynthetic derivatives of them were generated. In 1960s, etoposide was found to be a good alternative of podophyllotoxins for clinical use. After preclinical studies and clinical trials for over 20 years, Food and Drug Administration (FDA) approved use of etoposide in clinics for cancer chemotherapy (Baldwin and Osheroff, 2005).

Podophyllotoxins are known to inhibit microtubule assembly on the mitotic spindle apparatus resulting with an arrest of cells in mitosis. Initially, etoposide function was believed to be similar with podophyllotoxins from which it was derived. However, it was discovered that etoposide caused reduced fractions of mitotic cells in etoposide-exposed cancer cells (Grieder *et al.*, 1974). Recently, it is known that main mechanism of action of etoposide is topoisomerase II inhibition. However, binding

of etoposide to topoisomerase II is reversible; hence the longevity of the effectiveness of etoposide is due to the exposure time (Hainsworth and Greco, 1995). Beside topoisomerase II inhibition function, some metabolic products of etoposide directly bind to DNA causing single strand breaks (Baldwin and Osheroff, 2005).

Regarding its antineoplastic properties, etoposide inhibits topoisomerase II and its function in ligating broken DNA molecules via binding it. Finally, it causes the accumulation of single and/or double-strand breaks in double helix, therefore, inhibition of DNA replication and transcription is provided. Apoptotic cell death can also be stimulated by accumulation of breaks. Etoposide mainly show its effect in the G2 and S phases of the cell cycle (http://www.cancer.gov/Templates/drugdictionary.aspx?CdrID=39207).

Structure of etoposide is highly important for its function. As represented in the Figure 1.3b, the domain composed of A, B and E rings is proposed to act on drug-enzyme interactions. –OH and $–COH_3$ groups of the domain are thought to take place in drug function. It is also thought that D-ring and sugar moiety in the structure of etoposide possibly have role in its interactions with DNA (Bender *et al*., 2008). However, this proposal has not been clarified yet.

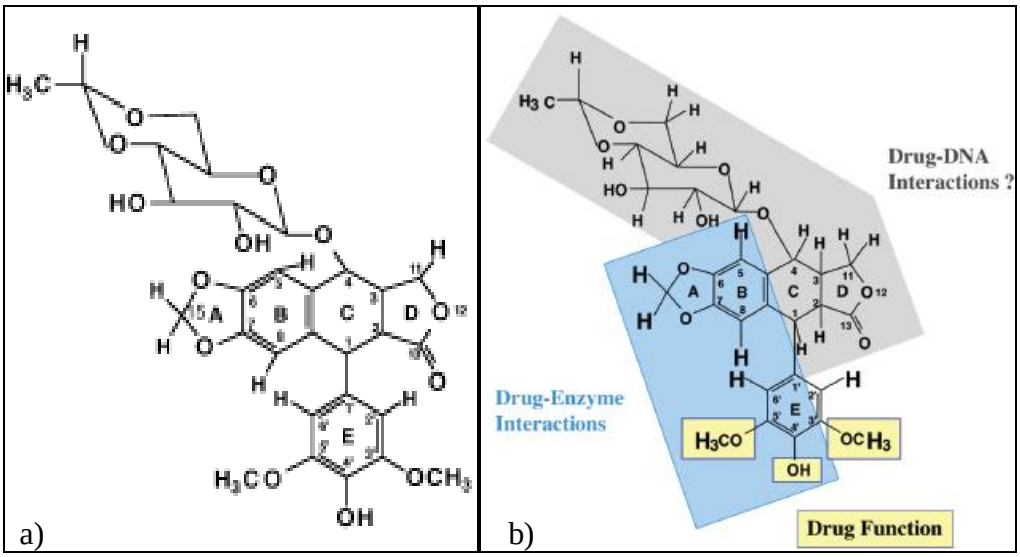


Figure 1. 3 (a) Structure of etoposide (b) structural domains that interact with topoisomerase II alpha (Bender *et al*., 2008).

1.5.1.1.2 Doxorubicin (Adriamycine®)

Doxorubicin is an antracycline antibiotic isolated from the bacterium *Streptomyces peucetius var. caesius* which was obtained from *S. peucetius* by mutagenic treatment (Arcamone *et al*, 1969).

Doxorubicin acts generally with three direct mechanisms. First mechanism of action is intercalation between base pairs. By means of this action, doxorubicin interferes with DNA and RNA synthesis. Doxorubicin also acts as a topoisomerase II inhibitor. Similar to etoposide, it stabilizes the enzyme-DNA cleavage complex by binding, thereby causes accumulation of double strand breaks which are signals for programmed cell death (Swift *et al*., 2006). Another mechanism for doxorubicin is interaction with cell membrane. By binding to negatively charged phospholipids, it changes the membrane dynamics and fluidity (Speelmans *et al*., 1994). An indirect mechanism for doxorubicin action is also proposed. During metabolism of doxorubicin inside cell, free radicals are produced by means of redox reactions. These reactive oxygen species also harm DNA helix by oxidizing bases that form

DNA. Additionally, free radicals cause lipid peroxidation in cell membrane (http://www.cancer.gov/Templates/drugdictionary.aspx?CdrID=38860). This mechanism also contributes to the indirect cytotoxicity of doxorubicin.

Doxorubicin is a drug which is soluble in water and in aqueous alcohols; however it is not soluble in nonpolar organic solvents (Arcamone *et al*., 1969). This feature of doxorubicin favors the usage of it and it is a widely used chemotherapeutic in clinic in various cancers, such as breast, ovarian, bladder, lung, thyroid and gastric cancers, as well as neuroblastoma, lymphoma, leukemia, and Kaposi's sarcoma (http://breastcancer.about.com/od/chemotherapydrugs/p/adriamycin.htm).
The structure of doxorubicin can be seen in Figure 1.4.

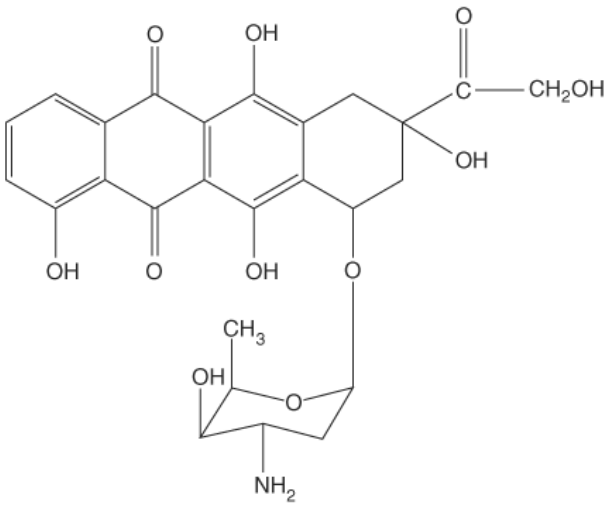


Figure 1. 4 Structure of doxorubicin

Doxorubicin is used in the treatment of breast cancer as combinations with other drugs. Etoposide is used in metastatic breast cancer especially after recurrence of cancer following treatment with other chemotherapeutics. Even though both of the drugs are effective, they have drawbacks in case of chemotherapeutic resistance.

1.6 Drug Resistance in Chemotherapy

Chemotherapy offers a significant qualified life advantage to some patients, however response to chemotherapy differs from patient to patient and treatment is frequently failed because of the drug resistance feature of tumors. Indeed, drug resistance mechanisms arise from the normal detoxification function of metabolism, however in cancer cells those mechanisms are used much more than the normal cells, and the cancer cells take the advantage of survival by means of resistance to drugs (Liscovitch and Lavie, 2002).

Multidrug resistance (MDR) is ability of tumor cells to be or become resistant against several unrelated drugs given during chemotherapy. While some types of tumors intrinsically resistant, the others gain MDR subsequent to chemotherapy (Kufe *et al.*, 2003). It is very important to get information about the MDR mechanisms of tumor cells. If the mechanisms of MDR are understood, alternative treatments such as targeting delivery of anti-cancer medicines with novel combinations can be improved in order to circumvent MDR. There are many cellular and biochemical resistance mechanisms that are tried to be unraveled and these mechanisms are generally interrelated. Also, several independent resistance mechanisms can be found together in a specific tumor population.

1.6.1 Mechanisms of Drug Resistance

1.6.1.1 Decreased Drug Accumulation

Decreased drug accumulation is one of the most common MDR mechanisms. Influx and efflux pumps on the membrane are involved in decreased drug accumulation. For example, hydrophilic drugs cannot pass through lipid bilayer; hence they are uptaken by special influx pumps. Downregulation of activity of these membrane proteins diminishes the accumulation of related drugs in the cell and as a result, resistance to that drug emerges. Furthermore, hydrophobic drugs, which can easily penetrate into

cell through membrane, are pumped out the cell by efflux pumps. Upregulation of this mechanism causes again decreased drug accumulation in the cell, hence emergence of resistance.

P-glycoprotein (P-gp) is the most important and most known example of efflux pumps involved in MDR. P-gp belongs to ATP-binding cassette (ABC) transporters, a family of transporter proteins that contribute to drug resistance via ATP-dependent drug efflux pumps, and normally found on cell membrane for excretion of toxins from cells. However, P-gp is overexpressed on resistant tumor cells (Leonard *et al*., 2003). Increased levels of P-gp cause raise in the levels of efflux of chemotherapeutic agents, hence the levels of drug accumulation decreases. The mechanism can be seen in Figure 1.5.

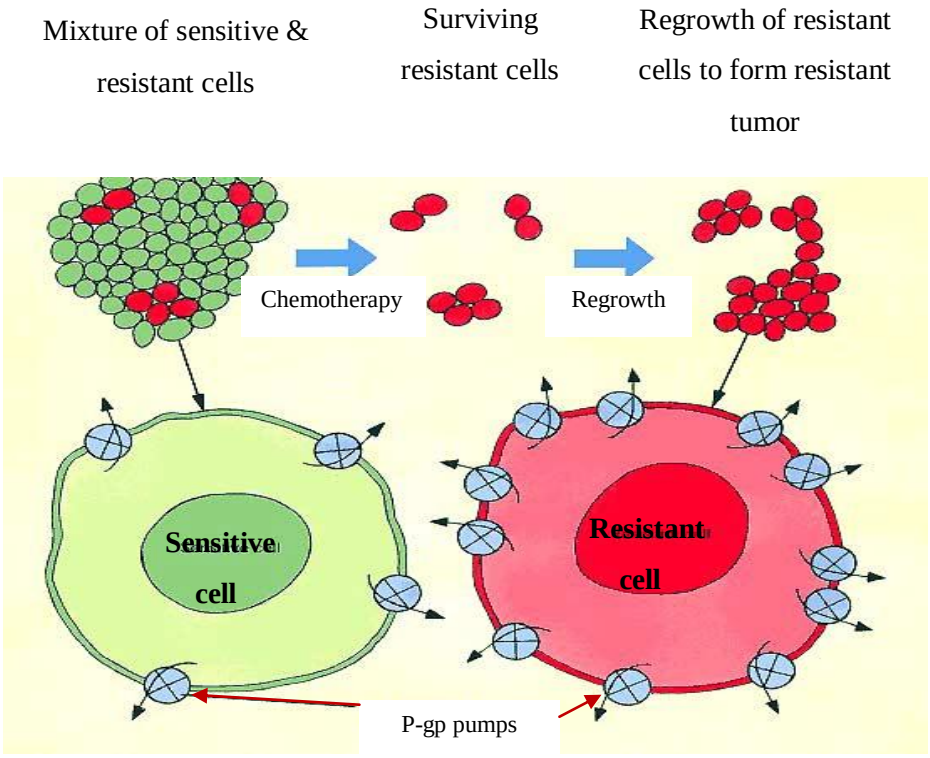


Figure 1. 5 Working mechanism of P-gp pumps and resistant tumor formation (Kufe *et al*., 2003)

There is also a study that shows a high correlation between MRP2 mRNA and etoposide efflux. This evidence suggests that MRP2 can be an efflux pump for topoisomerase inhibitors and if so, it should be a candidate in reversing strategies of MDR (Matsumoto et al., 2005). It was shown by Kurosawa and coworkers (Kurosawa *et al*., 2001) that verapamil when used with vincristine or doxorubicin had partially reversed MDR of leukemia cells developed against these drugs. It was also verified that SNF4435C and D (immunosuppressants produced by a strain of *Streptomyces spectabilis*), completely reversed MDR against vincristine in various tumor cells overexpressing P-glycoprotein *in vitro*. There are many other approaches to reverse P-gp-mediated MDR. Besides pharmacological compounds, monoclonal antibodies, immunotoxins and bispecific antibodies to target directly P-gp, antisense oligodeoxynucleotides (Dönmez *et al.*, 2010), and ribozymes in order to suppress P-gp in expression level, and albumin-conjugated drugs in *in vitro* and *in vivo* assays have also been examined for reversal of MDR (Volm, 1998). There are also other efflux pumps which belong to ABC transporters and involved in MDR. MRP1 is one of these pumps which belong to multidrug resistance associated protein (MRP) family. Similar therapeutic strategies mentioned above have been tried against these pumps. Here is a table that shows substrates and MDR inhibitors used against some major transporters:

Table 1. 1 Chemotherapeutics and MDR inhibitors for major transporters (Kufe *et al.*, 2003)

Common name	Other names	Systematic name	Substrates	Inhibitors
P-gp	MDR1	ABCB1	Adriamycin Actinomycin-D Bisantrene Daunorubicin Docetaxel Doxorubicin Etoposide Epirubicin Homoharringtonine Mitoxantrone Paclitaxel Teniposide Topotecan Vinblastine Vincristine Vinorelbine VP-16	Anthranilamide Cyclosporine D NSC-38721 (mitotane) Pipecolinate Quinoline OC-144-093 PSC-833 (valspodar) MS-209 LY-335979 (zosoquidar) XR-9576 (tariquidar) R-101933 (laniquidar) VX-710 (biricodar) GF-120918 (elacridar) ONT-093 Isothiocyanates Diallyl sulfide PK11195 Amooranin siRNA tRA 98006 Agosterol A Flavonoids
MRP1	–	ABCC1	Doxorubicin Daunorubicin Etoposide Epirubicin Methotrexate Paclitaxel Vincristine Vinorelbine	MS-209 XR-9576 (tariquidar) VX-710 (biricodar) Isothiocyanates tRA 98006 Agosterol A Rifampicin NSAIDs
MRP2	CMOAT	ABCC2	Cisplatin CPT-11 (irinotecan) Doxorubicin Etoposide Methotrexate Mitoxantrone Vincristine Vinblastine SN-38	XR-9576 (tariquidar) VX-710 (biricodar) Isothiocyanates tRA 98006
BCRP	MXR1, ABC-P	ABCG2	Bisantrene Camptothecin Daunorubicin Doxorubicin Epirubicin Flavopiridol Mitoxantrone SN-38 Topotecan CPT-11 (irinotecan)	GF-120918 (elacridar) tRA 98006 Flavonoids Phytoestrogens Imatinib mesylate Fumitremorgin C TAG- 139

1.6.1.2 Altered Drug Targets

The other mechanism of resistance is alteration of drug targets. Some of the chemotherapeutics interact with special enzymes in cancerous tissue in order to exhibit their effects. When expression of those enzymes is changed, effect of the drug also changes. Topoisomerase poisons stabilize the DNA-Topoisomerase complex and because topoisomerase cannot leave DNA, it cleaves the DNA continuously by forming lethal DNA breaks. At last, the tumor cells cannot proliferate and die. Quantitative and/or qualitative alterations in topoisomerase activity were demonstrated in resistant cell lines. It has been thought that reduction in the level of activity of topoisomerase is related with decreased drug-induced DNA strand breaks, hence decreased drug cytotoxicity (Per *et al*., 1987; Deffie *et al*., 1989).

1.6.1.3 Altered Drug Metabolism

Altered drug metabolism is the third reason of MDR. When chemotherapeutic drugs are administered to body, changes in drugs' metabolisms may occur at any point along the pathway to tumor cells or in the tumor cell. Unwanted metabolisms of drugs may cause a reduction in cytotoxicity. For instance, antimetabolites and alkylating agents are given as prodrugs and they must be converted into cytotoxic forms in the body, generally in the target tumor cells. However, in resistant tumor cells this mechanism does not work efficiently due to deficiency in prodrug converting enzymes or overexpression of enzymes used in pyrine or pyrimidine synthesis (Hunt and Hoffee, 1983).

Fate of chemotherapeutic drugs is generally determined by Cytochrome P450 enzymes (CYPs) (Ioannides, 2008). Metabolism and/or bioactivation of anticancer agents are mainly carried out by different types of CYPs. Some of these enzymes are involved in biotransformation of drugs leading to reduced cytotoxicity, while others cause activation of prodrugs by converting them into their active compounds

(Guengerich, 2008). While 90% of CYPs are expressed in liver in the body, there are many evidences that they also present in tumor cells. Furthermore, in cancer cells which are resistant to chemotherapy it was shown that ATP-binding cassette B1 (ABCB1) transporters and some CYPs were co-expressed. It was also demonstrated by some studies that CYP3A and ABCB1 had overlapping substrate specificities (Rochat, 2005). These evidences show that activity of both efflux proteins and metabolizing enzymes synergistically reduces drug toxicity and concentration in cancer cells which contributes to resistance. Namely, CYPs which are related to the detoxification of the drugs may be expressed in resistant tumor cells. It was demonstrated in a CYP1B1 expressing Chinese Hamster Ovary (CHO) cell line that the cells gained 5 fold resistance against paclitaxel (McFadyen *et al*., 2001). Interestingly, expression of some of the CYPs causes sensitivity to some anticancer agents in cancer cells. This is due to the prodrug converting activity of that specific CYP which generates active cytotoxic drug. In a study performed by using a few cell lines it was shown that only CYP1A1, 1A2 and 1B1 expressing cell lines could respond to candidate anticancer drug 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (DF 203). In this study, the cells which did not express the related enzymes were seemed to be resistant because cytotoxic effect of DF 203 is exposed by activity of CYP1A1, 1A2 and 1B1 enzymes (Rochat, 2005; Bazzi 2009).

1.6.1.4 Alteration of DNA Repair Mechanisms

Increased DNA repair together with increased cellular tolerance is also among the mechanisms of MDR. Cancer cells can circumvent the affect of chemotherapeutic drugs by increasing their repair mechanisms. After administration of cisplatin, which is a cytotoxic drug thought to be involved in intrastrand DNA cross-linkage, some cancer cells increase the activity of their repair system in order to cicumvent cisplatin cytotoxicity. Sometimes, intrinsically resistant cells display cisplatin resistance. The resistant cells tolerate cisplatin-induced DNA damages because of their mismatch repair defects. The repair system cannot recognize platinum-DNA adducts and the cell is not directed to normal apoptosis, hence the cancer cells continue to proliferate

easily and quickly. However, resistant cells generally suppress their apoptotic pathways directly instead of increasing their repair mechanisms (Topping *et al.*, 2009).

1.6.1.4.1 Role of Mismatch Repair in Drug Resistance

During lifetime, a cell undergoes many DNA damages which threaten its survival. These damages not only caused by chemicals or physical factors such as tobacco smoke, drugs or UV light, but also generated by endogenous reactive metabolites. The mechanisms related to DNA processing are also responsible from the damages as internal factors. During recombination, repair or replication some mistakes of protein machinery causes DNA damages. These damages accumulated over time ending up with cell death or accumulation of mutations that cause abnormal cells. In order to survive healthy, cells evolved different DNA repair mechanisms for different types of damages. Mismatch repair (MMR) is one of the specialized repair mechanisms.

1.6.1.4.1.1 Mechanisms of Mismatch Repair (MMR)

Mismatch repair is a conserved repair mechanism which is thought to be involved not only in repair of some kind of DNA damages, but also in other DNA processing events and cell death. Mismatch repair system prevents cell from lethal mutations by its repair function, however on the other hand, it contributes to genetic diversity by its role in homologous recombination or meiotic chromosome segregation.

MMR was first discovered in bacteria (*Streptococcus pneumonia*) as an evolutionarily well conserved system. After the discovery, detailed studies were performed in *Escherichia coli (E. coli)* (Silva *et al*., 2009). Then, more advanced partial homologous system was found in yeasts. Human MMR system was firstly described in 1993. In humans, more specialized MMR is observed meaning that the

proteins belong to MMR are different in mitochondria and nucleus (Kruh and Tew, 2000).

Incorrect integration of bases by some types of DNA polymerases (e.g. Translesion DNA polymerases) into DNA during replication or alteration in the structure of bases by some drugs or free radicals lead to mismatches and/or insertion/deletion loops (IDLs). The main role of MMR is fixing the mismatches and IDLs, thereby maintaining DNA integrity and preventing DNA from accumulation of mutations.

In eukaryotes, MMR proteins act as heterodimers. hMutSα heterodimer consists of MSH2 and MSH6 proteins which are present in nucleus. Eighty to ninety percent of total MSH2 is sequestered in MSH2-MSH6 complex and it is responsible for recognition of base-base mismatches and short IDLs (Silva *et al*., 2009; Li, 2008). On the other hand, MSH2-MSH3 heterodimer called hMutSβ recognizes larger IDLs. Both of the heterodimers are responsible for damage recognition. There are also human MutL homologs; MLH1, MLH3, PMS1 and PMS2. When MLH1 heterodimerizes to PMS2 hMutLα is formed and it is needed for continuation of MMR. The second heterodimer of MLH1 is hMutLβ (MLH1-PMS1) function of which has not been described yet. The other known heterodimer is MutLγ (MLH1-MLH3) which is thought to have roles in meiosis (Li, 2008; Schofield and Hsieh, 2003). In general, repair mechanism starts with recognition of mismatches/IDLs by one of the hMutS heterodimers containing MSH2 and recruitment of MLH1-containing hMutLα heterodimer. hMutLα regulates downstream processes for excision of damaged bases. By the recruitment of exonucleases (EXO1) damaged part excised and new strand is generated by means of polymerases. A generalized mechanism for MMR is demonstrated in Figure 1.6.

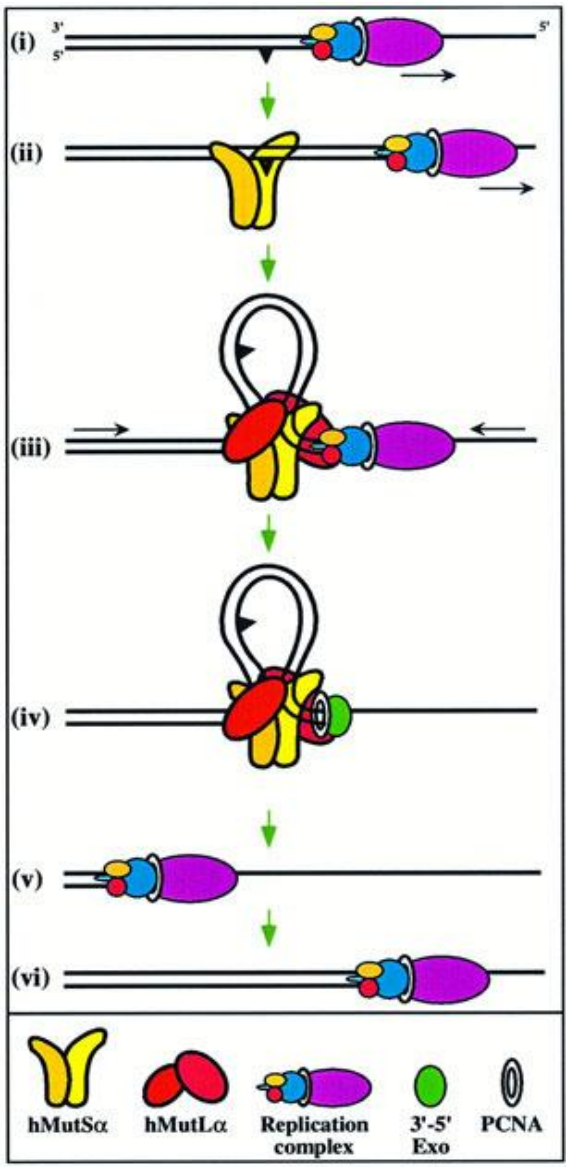


Figure 1. 6 A proposed model for MMR mechanism
(i) Generation of mismatch in the newly synthesized strand during replication by the replication complex (pol-δ, PCNA, RFC and other replication factors). (ii) Recognition of damage by hMutSα. (iii) ATP driven assembly of the MMR complex and the bi-directional threading of DNA through the hMutSα heterodimer until interaction of the replication complex with PCNA leading to replication arrest and dissociation of pol-δ. (iv) Excision of damage containing strand by exonuclease. (v) Dissociation of the MMR complex and recruitment of replication complex by PCNA (vi) Initiation of new replication. Replication protein A (RPA), which is not shown here, covers the single-stranded DNA throughout the process. As an alternative model, MMR proteins interact with replication complex throughout replication in order to stop the complex and repair the damage when mismatch is formed. (Jiricny, 1998)

There are also other mechanisms proposed for MMR. MMR is involved in DNA damage signaling, cell cycle arrest and apoptosis as well as in some metabolic pathways such as homologous recombination, interstrand crosslink repair, meiotic chromosome segregation or immunoglobulin class switching (Li, 2008).

Involvement of MMR in checkpoint arrest and apoptosis is explained with two possible model mechanisms. An altered base generated by alkylating agents or reactive oxygen species is mispaired with a wrong base causing mismatches. In the first model, if the damage is in template strand, due to the proposed strand specific activity of MMR, it tries to repair newly synthesized strand and is not able to remove damage triggering new repair cycle. This cycle becomes permanent, thereby, stimulate ATR/ATM damage signaling cascade resulting with cell cycle arrest of apoptosis. In the second model, it was shown that recruitment of MutSα/MutLα on the O6-meG/T mispairs produced by alkylating agents, initiated direct signaling of ATR and phosphorylation of Chk1 resulting with a checkpoint response (Yoshioka *et al*., 2006). Accordingly, MMR proteins can serve as direct sensors in recognition of methylation damage. Figure 1.7 represents the mentioned model mechanisms.

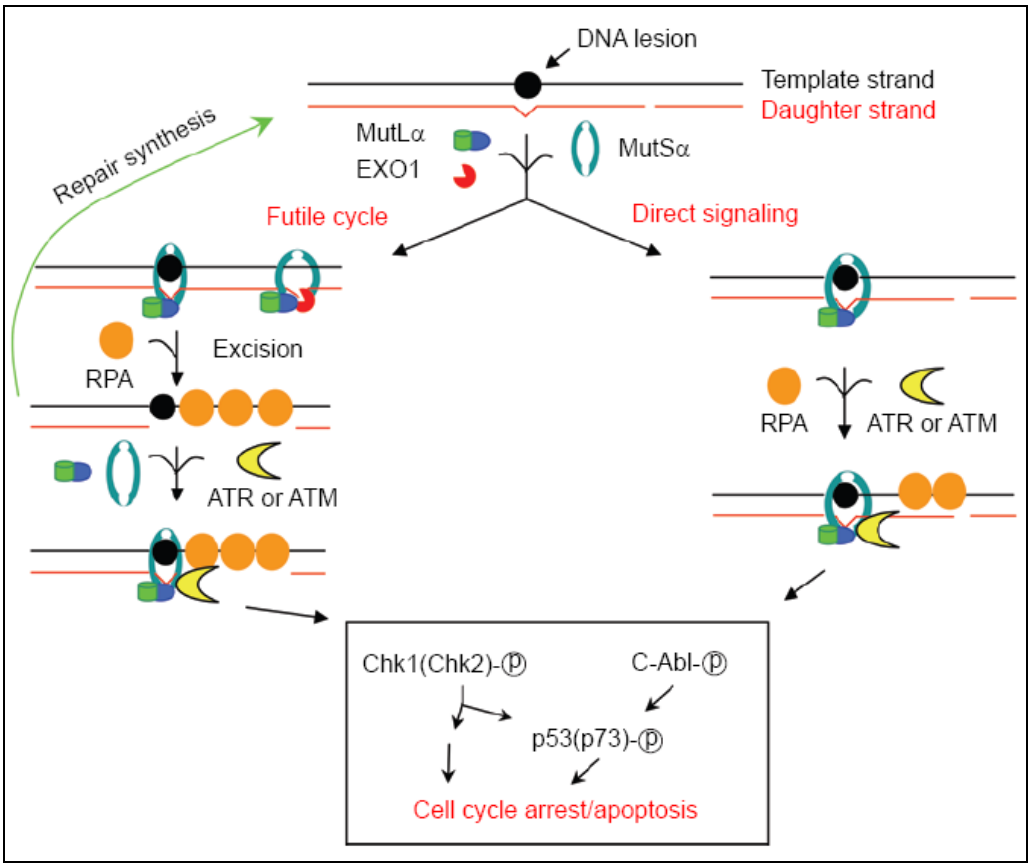


Figure 1. 7 Two model mechanisms of involvement of MMR in checkpoint response (Li, 2008)

The heteroduplex generated as a result of homologous recombination, generally has mismatches and IDLs. MMR is also implicated in the repair of these damages. Beside repair function in heteroduplexes, MMR thought to have role in the inhibition of homologous recombination at the sites of double strand breaks (DSBs) due to an interaction with a helicase, called BLM (Smith *et al.*, 2007). Therefore, improper recombination which possibly leads to huge mutations or fatal errors is obstructed. Additionally, it is proposed by Sugawara and colleagues (Sugawara *et al.*, 1997) that MMR acts in nonhomologous end joining by favoring the recruitment of Rad1-Rad10 endonucleases for trimming of nonhomologous DNA ends.

Because of different roles of MMR in a broad range of processes, its deficiency is discussed to be the reason for various distinct diseases. For instance, in Huntington's or Fragile X diseases trinucletide repeats (CAG and CGG, respectively) are increased due to defects in MMR proteins. Furthermore, deficiency in MSH2 and MLH1 is discussed to be underlying cause of some cancers such as colorectal cancer and lymphoma (Li, 2008). MMR is a highly sophisticated system which has many unsolved functions standing to be clarified.

1.6.1.5 Implications on Resistance Mechanisms

Besides all mentioned ways of resistance above, there are many other complicated mechanisms developed and utilized by resistant cancer cells. Cancer cells, by altering their mechanisms take the advantage of predominance, at the same time they introduce themselves to the immune system as normal cells. In a period of time, they proliferate and become metastatic and invade the other tissues. Chemotherapy generally does not efficiently treat all of the cancer cells because of their complicated intrinsic or acquired resistance mechanisms mentioned above. Furthermore, many of the chemotherapeutic medicines damage the normal, healthy cells beside cancer cells making the course of treatment more difficult and decreasing the life quality of patients. Moreover, the drugs sometimes cause other diseases. For example, some of the drugs that target P-glycoprotein efflux pump cause neurodegeneration in the brain because of inhibiting toxin efflux in the healthy brain cells which have more P-gp than the other cells (Kufe *et al*., 2003). In order to avoid these types of problems many approaches are developed by researchers all over the world. Using liposomes and antibodies to target only the cancer cells is among the most challenging studies, however to improve the efficiency of liposome or antibody targeted therapies, finding specialized cancer markers is very important not to target the healthy cells. Also, study of targeting of defense systems of the melanoma cells which metastasize to brain, by engineered stem cells can be given as an example of different studies (Dimitrov *et al*., 2007).

It is also important to determine whether the resistance is intrinsic or acquired. If the resistance is acquired during chemotherapy, the drug which causes that specific resistance should be determined and should not be given to that patient. In this case, patient-specific therapies maybe required. According to cancer stem cell hypothesis, "The cancer-initiating cell is a transformed tissue stem cell which retains the essential property of self protection through the activity of MDR transporters" (Donnenberg and Donnenberg, 2005). From this hypothesis, it is understood that the resistance exhibited with MDR transporters is intrinsic. In this case, the ways of the treatment strategies to circumvent resistance may change. For the development of novel treatment strategies to destroy all the cancer cells in the future the entire above hypothesis, observations as successes and failures must be considered.

1.7 Objectives of the study

Drug resistance against chemotherapy is an important clinical problem which prevents treatment of cancer and makes the life harder for patients. The underlying mechanisms in drug resistance remain to be elucidated. Topoisomerase II inhibitors are a group of drugs used in breast cancer and resistance to these agents in breast cancer is reported in literature. Changes in expression levels of various genes that are involved in DNA/RNA processing and DNA repair is thought to be a reason of drug resistance against topoisomerase II inhibitors. In order to elucidate the effect of *TOP2A*, and two crucial mismatch repair genes *MSH2* and *MLH1* on resistance to topoisomerase II inhibitors, the genes were evaluated in expression levels. TOP2A is the target of the two topoisomerase II inhibitors used in this study and decrease in the expression of this enzyme is reported to be a reason of resistance (Takano *et al*., 1992; Hasegava *et al*., 1995). Similarly, deficiency in MSH2 and MLH1 proteins is also reported to be related to the resistance to various chemotherapeutics (Aebi *et al*., 1997).

The goal of this study can be listed as below:

- Development of etoposide resistant sublines from drug sensitive MCF7 breast cancer cell line (MCF7/S).
- Evaluation of antiproliferative effect of etoposide on MCF7/S and etoposide resistant sublines by calculating inhibitory concentration 50 (IC_{50}) and determination of the degree of resistance of the developed sublines.
- Determination of the expression levels of *TOP2A* in MCF7/S, etoposide and doxorubicin resistant sublines by using qPCR.
- Determination of quantitative changes in the expressions of *MSH2* and *MLH1* as chief proteins of MMR by qPCR in MCF7/S, etoposide and doxorubicin resistant sublines
- Comparison of gene expression changes between etoposide resistant sublines, doxorubicin resistant sublines and MCF7/S
- Comparison of resistant sublines in terms of gene expression levels in order to evaluate the effects of different drugs.

In summary, this study aims to evaluate mechanisms of resistance developed against topoisomerase II inhibitors by analyzing the expression changes of drug target and two basic MMR genes after development of resistant sublines.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell Lines

Parental MCF7 human breast adenocarcinoma cell line was donated by Şap Institute, Ankara, Turkey. Doxorubicin resistant MCF7 cell lines, MCF7/600D and MCF7/1000D, were developed previously by Dr. Özlem Darcansoy İşeri from the parental MCF7 cell line (MCF7/S) by stepwise selection in dose increments. MCF7/600D and MCF7/1000D are resistant to 600 nM and 1000 nM doxorubicin, respectively (İşeri, 2009). Etoposide resistant cell lines were gradually selected by application of increasing doses of etoposide to MCF7/S about a year.

2.1.2 Chemicals, Reagents and Kits

Doxorubicin-HCl (D) and etoposide (E) were kindly provided by Prof. Dr. Fikret Arpacı (Gülhane Military Medical Academy, School of Medicine, Department of Oncology) and stored at +4°C.

RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Biochrom AG, Germany. Phosphate buffered saline (PBS) tablets, dimethyl sulfoxide (DMSO) and total RNA isolation reagent (TRI Reagent®) were commercially obtained from Sigma-Aldrich, USA. PBS solution was prepared by dissolving tablets in distilled

water; pH was adjusted to 7.2, and autoclaved at 121°C for 20 minutes. Trypsin-EDTA, tryphan blue and XTT Cell Proliferation Kit were acquired from Biological Industries, Israel. Diethyl pyrocarbonate (DEPC), isopropanol, ethanol and agarose were purchased from AppliChem, Germany. Nuclease-free water, Moloney-Murine Leukemia Virus Reverse Transcriptase, *Taq* DNA polymerase, dNTP mix, $MgCl_2$, RiboRuler High Range RNA ladder, GeneRuler 50bp DNA ladder, 2X RNA loading dye and 6X DNA loading dye were obtained from Fermentas, Lithuania. FastStart Universal SYBR Green Master (ROX) was purchased from Roche Diagnostics, Germany.

2.1.3 Primers

β-actin, TOP2A and *MSH2* primers were purchased from Alpha DNA, Canada. *MLH1* primers were synthesized by Iontek, Istanbul, Turkey. Primer sequences, location and amplicon sizes are represented in Table 2.1.

Table 2. 1 Primers used in PCR

Primer	Sequence (5'->3')	Location	Amplicon Size
***TOP2A* Forward**	ATTCCCAAACTCGATGATGC	Exon 11	136 bp
***TOP2A* Reverse**	CCCCATATTTGTCTCTCCCA	Exon 12	
***MLH1* Forward**	TTATCCAGCGGCCAGCTAATG	Exon 1	95 bp
***MLH1* Reverse**	GCCTCCCTCTTTAACAATCACTT	Exon 3a	
***MSH2* Forward**	GGAGGAGAGACTGCTGGAGA	Exon 3	144 bp
***MSH2* Reverse**	TCCCTTTTTGCCTTTCAACA	Exon 4	
***β-actin* Forward**	CCAACCGCGAGAAGATGA	Exon 3	97 bp
***β-actin* Reverse**	CCAGAGGCGTACAGGGATAG	Exon 4	

2.2 METHODS

2.2.1 Cell Culture

2.2.1.1 Growth Conditions

Parental MCF7 (MCF7/S) cells, doxorubicin resistant cells and etoposide resistant cells were maintained as an attached type monolayer culture in 15ml RPMI 1640 medium (Appendix A) supplemented with 10% (v/v) fetal bovine serum (FBS) in $25cm^2$ and $75cm^2$ surface-treated filter cap tissue culture flasks (Greiner Bio-One, Germany). Cells were incubated in a 95% (v/v) humidified atmosphere of 5% (v/v) CO_2 at 37°C in an Heraeus incubator (Hanau, Germany). Cell culture was performed in Bioair Aura 2000 M.A.C Class II Safety Cabinet (Bioair Instruments, Italy). The solutions were cell culture grade quality and disposable equipments were commercially presterilized. Nondisposable materials were presterilized at 121°C for 20 min using Hirayama Hiclave autoclave (Hirayama Manufacturing Corp., Japan).

2.2.1.2 Subculturing of Cells

For a proliferating cell culture when the cells reach a confluence which causes slower growth and finally cell death, the culture must be divided to provide the cells an adequate environment (Freshney, 2005). Passaging cells when they reach a confluence where the cells exponentially grow is a crucial process for a healthy cell growth. Subculturing process for adherent cell culture includes detaching the cells from the surface of the flask and transferring them to another flask with reduced cellular densities. Cells were grown in both $25cm^2$ and $75cm^2$ surface-treated filter cap flasks. When the cells reach 80-90% confluence, medium was discarded and PBS (2 ml for $25cm^2$ and 5ml for $75cm^2$ flasks) was used to wash away the FBS-containing medium not to cause inactivation of trypsin. Trypsin-EDTA (0.5 ml for $25cm^2$ and 1ml for $75cm^2$ flasks) was added and the cells were incubated at 37°C for 5-10 min. to activate the trypsin. When the cells detached, they were resuspended in

FBS-containing medium to inactivate trypsin and homogenize cells. Depending on the downstream process, the cells were transferred to another flask(s) for propagation, separated for further experiments or frozen. For the ongoing culture of resistant cell lines, required amounts of drugs were added (Appendix B, Table B.2).

2.2.1.3 Cell Freezing and Thawing

Eighty to ninety percent confluent healthy cells were detached from 75cm^2 flasks by trypsinization. Detached cells were homogenized in 5 ml FBS-containing medium prior to centrifugation at 1000 rpm for 5 min at 4°C. After discarding supernatant, cell pellet was resuspended and washed in 5 ml cold PBS. Cells were again precipitated by centrifugation at 1000 rpm for 5 min at 4°C. Supernatant was disposed and cell pellet was homogenized in freezing medium (10% (v/v) DMSO + 90% (v/v) FBS). Cell suspension of at most 5x10^6 cells/ml were immediately transferred to cryovial (Greiner Bio-One, Germany) and kept at -20°C for 1-2 h. The vial was incubated at -80°C overnight prior to transferring it to liquid nitrogen for long term storage.

For thawing the cells, the required cryovial was taken from the liquid nitrogen and incubated at 37°C. Immediately after cell thawing, 1 ml cell suspension in freezing medium was transferred to a 15 ml falcon tube (Greiner) and 4 ml medium was added to the suspension to dilute DMSO. It is important to note that DMSO is toxic to cells at 37°C and it is crucial to keep the cell suspension cold not to kill or harm the cells. Cell suspension in falcon tube was centrifuged at1000 rpm for 5 min at 4°C and cells were precipitated. Supernatant was discarded, cells were homogenized in FBS-containing medium and transferred to culture flask.

2.2.1.4 Cell Viability Determination by Trypan Blue Exclusion Method

Viable cells were counted using hemocytometer-based trypan blue exclusion method. Healthy cells were detached using trypsin and homogenized in medium as described

previously (refer to 2.2.1.2). 450 µl cell suspension (90% (v/v)) was mixed with 50 µl trypan blue solution (0.5% solution) (10% (v/v)) in an eppendorf tube and cells were counted in a Neubauer hemocytometer (Bright-line, Hausser Scientific, USA) under light microscope (Olympus, USA).

Hemocytometer is composed of a ruled large 1 x 1 mm square which is subdivided in 16 0.25 x 0.25 mm squares that are further divided in 16 0.05 x 0.05 mm squares. The volume of the smallest square is 0.00025 mm^3. In case of crowded cells 5 x 16 cells were counted, however 16 x 16 squares were counted when there were a few cells. The number of cells in 1 ml was determined using the formula below (Equation 2.1):

Cell number / ml = Average cell number per square x Dilution factor x 4 x 10^6 (2.1)

2.2.2 Establishment of Etoposide Resistant Sublines

2.2.2.1 Determination of Starting Dose

In order to determine the appropriate starting dose which allows growth of parental MCF7 cell line sensitive to etoposide (MCF7/S), a wide range of etoposide concentrations were assayed. Five thousand cells/well were seeded in two 6-well plates and they were supplemented with 2 ml 10% FBS containing medium. First well was designated as control and the others were experimental samples. Etoposide was added into the experimental samples in 2-fold increasing concentrations (0.015-7.680 µM). Media were renewed in every 3-4 days during 20 days cultivation. Effects of different concentrations on growth were observed under inverted light microscope and appropriate initial concentration was determined accordingly.

2.2.2.2 Development of Resistant Sublines

Six etoposide-resistant sublines (MCF7/200E, MCF7/500E, MCF7/750E, MCF7/1000E, MCF7/1250E and MCF7/2000E) were established by stepwise selection in dose increments over 1.5 year. At the beginning, 50 nM etoposide was applied to MCF7/S. After adaptation of cells, concentration was increased gradually. Periodically, resistant sublines were chosen, reproduced and stored in liquid nitrogen after freezing. During cultivation, developed sublines were exposed to their specific selective drug concentration, in order to retain the resistance level.

2.2.3 Determination of Drug Resistance

2.2.3.1 Cell Proliferation Assays

2.2.3.1.1 XTT Cell Proliferation Assay

Antiproliferative effects of etoposide on parental and resistant cell lines were assessed using XTT Cell Proliferation Kit according to the manufacturer's instructions. Assay is based on a colorimetric change after incubation of the cells with XTT reagent. The change in the color results from the reduction of tetrazolium salt XTT in the mitochondria of the metabolically active cells into orange colored formazan compounds. In contrast to MTT based assays, the formazan products of XTT are water-soluble giving the opportunity to measure the absorbance with spectrophotometer directly (Scudiero, 1988). The absorbance of the compounds is proportional to the number of metabolically active cells.

Briefly, the medium of healthy cells intended to be used in proliferation assay was renewed one day before the assay. The next day, cells were trypsinized and counted. Starting from the second column, 5000 cells/well in 50 µl medium were reseeded to attach type 96-well plates. The plates were incubated at 37°C overnight for attachment of the cells to the surface. Since the membrane proteins and growth factor

receptors may be damaged because of the proteolytic activity of trypsin, metabolic activity of the cells are negatively affected and most of these proteins are expressed again in 24 hours after seeding (Huang *et al*., 2010). Hence, overnight incubation let the cells to recover before drug exposure. The next day, media were poured out and drug was added to experimental samples in serial dilutions in the ratio of 2:3. Firstly, 100 µl medium was added to first and second columns designated as medium control composed of only medium and cell control composed of 5000 cells/well plus medium without drug, respectively. Afterwards, 150 µl 2x concentrated drug was put in each well of the third column which contains the highest drug concentration among the serial dilutions. 50 µl medium/well was added to columns from 4 to 12. For serial dilution, 100 µl concentrated drug was taken from 3rd column and transferred to the next column sequentially. Finally, volume of each of the wells were completed to 100 µl by adding 50 µl medium/well except medium control (1st column) and cell control (2nd column) columns. Starting with the time of completion, plates were incubated at 37°C for 72 hours. 2% activation reagent was mixed with XTT reagent just before addition and 50 µl of reaction solution was added to all of the wells containing 100 µl medium. Following 4 hour of incubation at 37°C, absorbance was measured at 500 nm with a SpectraMax 340 96-well plate reader (Molecular Devices, USA).

Microsoft Excel program was utilized to generate % cell proliferation versus dose curves and IC_{50} (50% inhibitory concentration) values were calculated from the logarithmic trendline of the graphs. Resistance indices (R) were calculated according to the formula below (Equation 2.2)

$$R = IC_{50} \text{ resistant subline} / IC_{50} \text{ parental (sensitive) cell line} \quad (2.2)$$

2.2.3.1.2 Statistical Analysis

All cell proliferation assays were carried out in triplicate and IC_{50} values were stated as mean ± SEM. Statistical significance of resistance was determined by one way

ANOVA test and to compare the degree of significance between groups Tukey's Multiple Comparison Post Test was applied using GraphPad Prism software.

2.2.3.2 Growth Curve Analysis

Growth curve analysis was performed for sublines MCF7/1000E and MCF7/600D to calculate doubling times. Growth curves of MCF7/S and MCF7/1000D were previously constructed by Dr. Özlem Darcansoy İşeri (İşeri, 2009). Briefly, healthy cells were trypsinized and a cell suspension containing $5x10^4$ cells/ml was prepared for each cell line. 1 ml cell suspension was distributed to each well of surface-treated 6-well plates and 2 ml medium/well was added on the cell suspensions. Cells in each well were trypsinized in every corresponding one or two days and suspended in 2 ml medium. Every count was carried out 8-20 times by using trypan blue exclusion method as described in Section 2.2.1.4. Cell number per ml was calculated according to the equation 2.1. Total cell number was determined by multiplying the cell number per ml with volume of medium in which the cells were suspended after trypsinization. MCF7/1000E was counted every day during 12 days and MCF7/600D was counted during 18 days. Experiments were performed in duplicates. Growth curves and logarithmic plots for calculation of doubling times were drawn by means of Microsoft Excel. Growth curves were constructed by plotting total cell number against time. Logarithmic plots were generated from the data of logarithmic phase of growth. Doubling times (t_d) were calculated using the slope of linear trendline of logarithmic plots with the equation below (Equation 2.3)

Slope of the linear trendline = μ

$$t_d = \ln(2)/\mu \qquad (2.3)$$

2.2.3.3 Isolation of RNA

2.2.3.3.1 Total RNA Isolation Using TriReagent

In order to deactivate RNases which cause degradation of RNA, all the equipments were exposed to 0.1% diethyl pyrocarbonate (DEPC) treated dH_2O and residual DEPC was evaporated under hood prior to autoclaving at 121°C for 20 min. DEPC-treated dH_2O was also used for preparation of 75% ethanol. All the experiment was performed in cold environment to avoid RNases to be active. Therefore, centrifuges were performed at 4°C and tubes were kept on ice throughout the isolation.

In brief, 5-10 x 10^6 healthy cells in the logarithmic phase were trypsinized as explained previously, homogenized in 5 ml medium and transferred to 15 ml falcon tube. Cells were precipitated at 1000 rpm for 5 min and supernatant was poured out. In order to clean the residual drug and medium, pellet was washed with 5 ml cold PBS and again cells were precipitated at 1000 rpm for 5 min at. After PBS was discarded, cells were homogenized well in 1 ml TriReagent solution by pipetting in order for lyses of cells. The lysate was transferred to a DEPC-treated RNase-free eppendorf tube and centrifuged at 12000g for 10 min to remove insoluble material (extracellular membranes, polysaccharides, high molecular mass DNA). Supernatant was transferred to a new tube and allowed to stand at room temperature for 5 min to assure complete dissociation of nucleoprotein complexes. Two hundred µl of chloroform was added and samples were incubated for 15 min on ice prior to centrifuge at 12000 g for 15 min. After centrifugation, phase separation occured, 3 phases were seen: colorless upper aqueous phase containing RNA, white intermediate phase containing DNA and red lower organic phase (phenol-chloroform phase) containing proteins and DNA. Since upper aqueous phase (~500 µl) contains RNA, it was taken to a new tube separately from the intermediate phase carefully. Five hundred µl of 100% isopropanol was added to the sample to isolate the RNA from solution, and mixture was kept at room temperature for 10 min. After a 10 min centrifugation at 12000 g RNA precipitate was appeared at the bottom. Supernatant

was removed, RNA pellet was washed with 1 ml of 75% ethanol and RNA was precipitated at 12000g for 5 min. Supernatant was discarded, RNA was dried near flame for 10-15 min and solved in nuclease-free water by incubation at 55°C for 15 min in thermostat (Biosan Medical Biological Research and Technologies, Latvia). Isolated RNA was stored at -80°C.

2.2.3.3.2 RNA Quantitation and Determination of Purity

For determination of the purity and the amount of isolated total RNA, absorbance measurement was performed using Shimadzu UV-1208 spectrophotometer (Shimadzu Corp., Japan), at the wavelengths of 260, 280, 270, 230 and 330 nm. Nucleic acids and protein give absorbance at 260 and 280 nm, respectively. Measurements at 270 and 230 nm determine the presence of phenol and organic contaminations in the RNA solution. Particulates contaminated the solution give absorbance at 330 nm; hence, the absorbance at this wavelength must be close to zero. Purity of RNA sample can be found by dividing absorbance at 260 to others. Optimal ratios of 260/280, 260/270 and 260/230 are approximately 1.7, 1.2 and 1.8, respectively. For measurement of optical density, 2 µl of RNA solution was diluted in 998 µl DEPC-treated water (with a dilution factor of 500) in a quartz spectrophotometer cuvette, and 1 ml DEPC-treated water was used as blank. RNA concentration was calculated using the following formula (Equation 2.3):

[RNA] µg/ml = Absorbance at 260 nm x Dilution Factor x 40 µg/ml (2.3)
(40 µg/ml is the average extinction coefficient of RNA)

2.2.3.3.3 Determination of Quality of RNA

Agarose gel electrophoresis was used for the quality control of RNA. Agarose gel was prepared by dissolving 1% (w/v) agarose in 1X Tris Acetate EDTA (TAE) buffer (Appendix C). Agarose in buffer was dissolved completely by boiling in microwave oven for 1-2 min. After ethidium bromide solution was mixed

homogenously, the gel solution was poured into the gel tray, the comb was placed, and gel was left for solidification. Solidified gel was placed in the electrophoresis tank (Bio-Rad Laboratories, France) containing 1X TAE buffer. Five µl RNA sample was mixed with 6 µl formamide and 1 µl 6X loading dye (Fermentas) in an eppendorf tube and loaded. Ten µl High Range RNA Ladder Ready-to-Use (Fermentas) was used. The samples were run for 80 min at 70V and the gel was visualized and photographed using an ultraviolet transilluminator (Vilber Lourmat, Marine la Valeé, France).

2.2.3.4 Polymerase Chain Reaction (PCR)

2.2.3.4.1 Primer Design

Gene specific primers for *TOP2A, MSH2* and *MLH1* genes were designed using PrimerBLAST (National Center for Biotechnology Information (NCBI)) and Primer3 (Massachusetts Institute of Technology) programs. The compatibility of homodimer, heterodimer and hairpin structures of primers were analyzed with OligoAnalyzer (Integrated DNA Technologies). Nucleotide-BLAST (NCBI) was used to control similarity of the sequences. Primers were designed for different exons of the genes to make certain of amplification only of cDNA. *β-actin* was previously purchased from Alpha DNA, Canada.

2.2.3.4.2 Reverse Transcription

Rnase-free equipments and nuclease-free dH_2O (Fermentas) were used for cDNA synthesis. Five µg isolated total RNA was reverse transcribed into cDNA by Moloney-Murine Leukemia Virus reverse transcriptase (M-MLV RT) (Fermentas) which is isolated from *Escherichia coli (E. coli)* having cloned fragment of *pol* gene that encodes M-MLV RT (Kotewicz, *et al.*, 1988). Twenty pmol gene specific primers were used for reverse transcription of each of the *β-actin, TOP2A, MSH2* and *MLH1* genes. For a 20 µl reaction mixture, in 0.5 ml eppendorf tube, 5 µg RNA and

20 pmol primer was mixed in nuclease-free water with a final volume of 11 µl. The tube was incubated at 72°C for 5 min to melt the secondary structures of RNA and primer. Then, 4 µl 5X reaction buffer, 2 µl 10 mM dNTP mix (final concentration: 1 mM) and 2.5 µl nuclease-free water were added to the tube. The tube was incubated at 37°C for 5 min to allow annealing of primer to RNA. After addition of 0.5 µl reverse transcriptase, reaction was performed at 42°C for 1 hour to obtain cDNA. Finally, reaction was terminated by the deactivation of enzyme at 72°C for 10 min. All the reaction was performed in thermal cycler (Apollo ATC 401, Belgium). In order to prevent the misleading results arising from different reaction efficiencies, cDNAs of all transcripts were obtained at the same reaction conditions. Synthesized cDNAs were stored at -20°C until use.

2.2.3.4.3 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

2.2.3.4.3.1 Method of qPCR

Expressions analyses of the genes were studied by means of quantitative real-time polymerase chain reaction (qPCR) using SYBR Green chemistry. SYBR Green I dye is an intercalating agent and fluoresces when bind to double stranded DNA. During PCR, as the amplified product accumulates, fluorescence increases and increase can be detected and displayed in a graph in real time by the real time qPCR instrument.

cDNAs generated from RNAs of all cell lines (MCF7/S, MCF7/200E, MCF7/500E, MCF7/750E, MCF7/1000E, MCF7/1250E, MCF7/2000E, MCF7/600D and MCF7/1000D) were used as template. For amplification of cDNAs, Rotor-Gene 6000 (Corbett Research, Australia) and FastStart Universal SYBR Green Master (ROX) kit (Roche Diagnostics, Germany) were used. Optimal primer and cDNA concentrations and annealing temperatures for each gene were determined using conventional PCR. *β-actin* was used as an internal control gene and the cDNA obtained from the RNA of MCF7/S cell line was used as calibrator. The reactions for each sample were performed as triplicates in each run. A negative control which did

not contain cDNA was included in each reaction for the control of DNA contamination and background signal.

qPCR was performed in 10 µl reaction solutions in sterile 0.2 ml Eppendorf tubes. Five µl 2X SYBR Green master mix (final concentration: 1X) was mixed with optimal amounts of reverse and forward primers and appropriate amount of nuclease-free water were added. Optimized amount of DNA was added to each tube separately. PCR steps include a pre-incubation at 95°C for the activation of *Taq* DNA polymerase, a step including amplification cycles where the fluorescence is detected and a melting step enabling determination of presence of unexpected DNA products such as primer dimer and amplification products generated from foreign or misprimed DNA. Amplification step was composed of a denaturation step at 95ºC for 15 sec, an annealing step at optimized temperature for each gene for 60 sec and an extension step, where the fluorescence was acquired, at 72ºC for 30 sec. Amplification step was repeated for 45 cycles. After completion of cycles, melting step was performed by increasing temperature from 55ºC to 99ºC with 1ºC increments. The optimized conditions for each gene are represented on Table 2.2.

Table 2. 2 Conditions for qPCR

	TOP2A	***MSH2***	***MLH1***	***B-actin***
Primers (25µM each) (µl)	0.10	0.16	0.10	0.16
cDNA (µl)	1.80	1.40	1.20	1.40
Annealing Temperature (°C)	61	62	62	62
Number of Cycles	45	45	45	45

2.2.3.4.3.2 Quantitation of qPCR Products by ($2^{-\Delta\Delta Ct}$) Method

Relative quantitation in gene expression was achieved using ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). Reactions having efficiency close to 100% were chosen for a reliable quantitation. In the ($2^{-\Delta\Delta Ct}$) method, relative change in gene expression is stated as fold change. Fold changes can be obtained by normalization of quantitation data to an internal control and comparison of experimental (e.g.: drug treated) samples relative to a calibrator (e.g.: untreated reference sample). Fold changes of gene expressions were determined using the formula below (Equation 2.4):

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta C_t = (C_{t\ \text{target}} - C_{t\ \text{internal control}})_{\text{treatment}} - (C_{t\ \text{target}} - C_{t\ \text{internal control}})_{\text{no treatment}} \quad (2.4)$$

Fold changes were obtained by normalization of quantitation data to the internal control gene *β-actin* and comparison of resistant sublines relative to MCF7/S cell line.

2.2.3.4.3.2 Statistical Analysis of qPCR Data

All data were obtained from three independent experiments with three samples for each cell line in each of the runs. Totally nine quantitation data were used for every cell line for the quantitation of expression of each gene. Fold changes were expressed as mean ± standard error of the means (SEM). The results were subjected to one way ANOVA test using GraphPad Prism software. For comparison of significance between groups, Tukey's Multiple Comparison Post Test was applied following ANOVA The mean differences were found to be significant at the 0.01 level (Appendix E).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Development of Etoposide Resistance

3.1.1 Determination of Starting Dose

In order to establish etoposide resistant sublines, a starting dose was first determined. For making a rough estimation of cytotoxicity of etoposide, MCF7/S cells were seeded to 6-well plates, observed for 20 days and photographed (Figure 3.1).

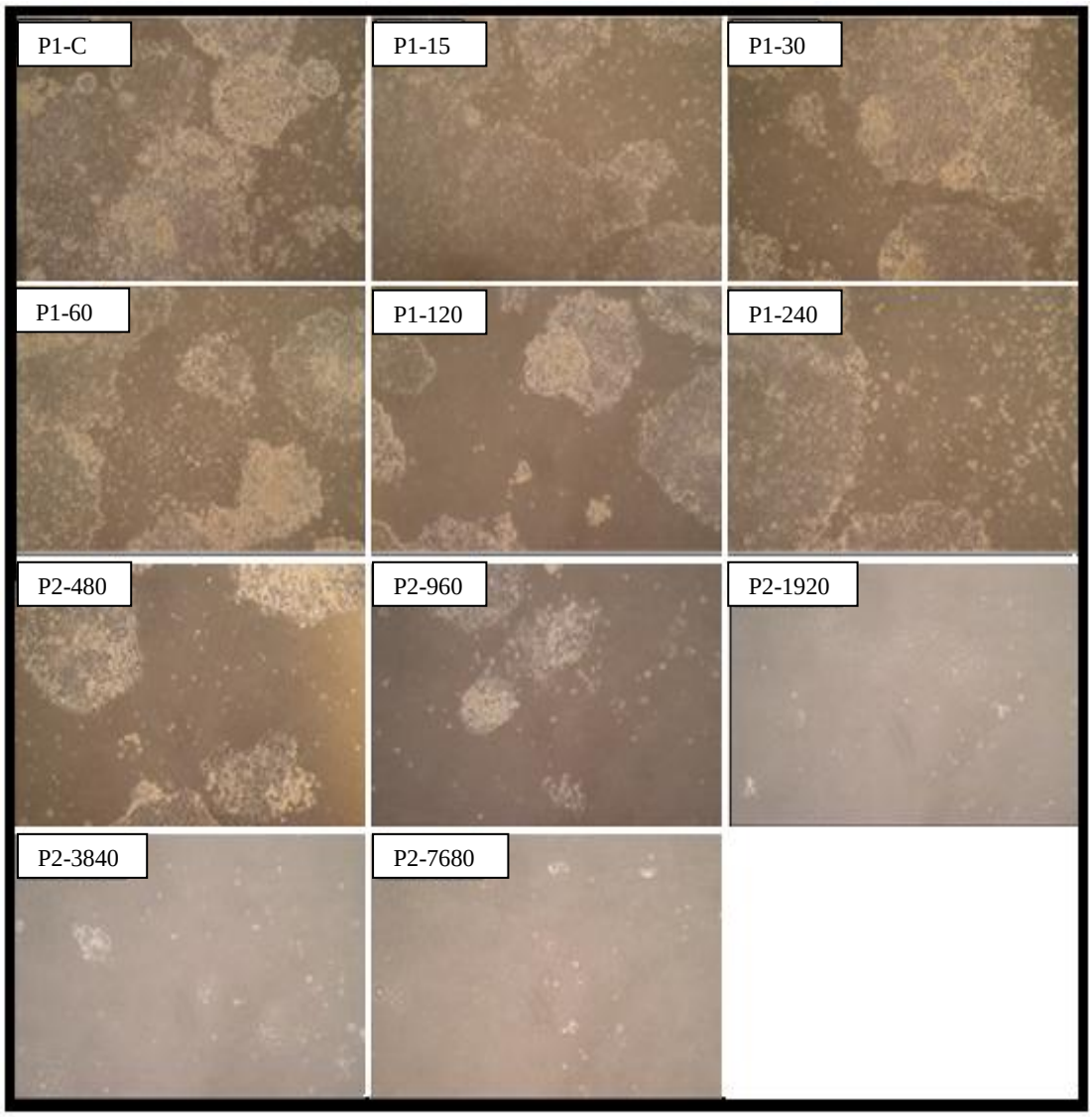


Figure 3. 1 Effect of different concentrations of etoposide on MCF7/S cells

Figure 3.1 shows the cytotoxic effect of different concentrations of etoposide on MCF7/S cells. A control well which did not contain etoposide was included. The other wells of the 6-well plates were designated as test samples. Etoposide was applied to the test samples in two fold increasing concentrations in a range of 15 nM-7.7 μM. According to the observations (Figure 3.1), cells could proliferate between 30 and 60 nM easily despite a little cytotoxic effect of etoposide. Starting from 120 nM, etoposide showed more dramatic cytotoxic effects. According to the micrographs, at the highest 3 concentrations almost no cell could survive. In compliance with these results, initial etoposide concentration was determined as 50 nM and increased up to 2 μM. The resistant sublines MCF/1000E, MCF7/1250E and MCF7/2000E were developed in about a year. The duration of etoposide applications and the concentration of drug required for sustainable resistance are indicated in appendix B Table b.1 and Table b.2, respectively.

3.1.2 Determination of Resistance

3.1.2.1 Determination of Antiproliferative Effects of Etoposide by XTT

Cell proliferation assays were performed using XTT cell proliferation kit. Different concentrations were applied to each of MCF7/S, MCF7/1000E, MCF7/1250E, and MCF7/2000E cell lines in 96-well plates separately. After 72 hour of exposure to drug, optical density was measured and cell proliferation curves were created. Assays were performed in triplicates for each cell lines. IC_{50} values for each plate were calculated from the equation of logarithmic trendline of the curves individually. Final IC_{50} was determined by taking the average of 3 separate plates. IC_{50} values were indicated as IC_{50} ± SEM. Resistance indices (R) were determined by using the equation 2.2 described in Section 2.2.3.1.1 for each cell line.

Starting concentration was 200 µM and dilutions were done at 2:3 dilution ratios for parental MCF7/S cell line. The lowest concentration was 5 µM.

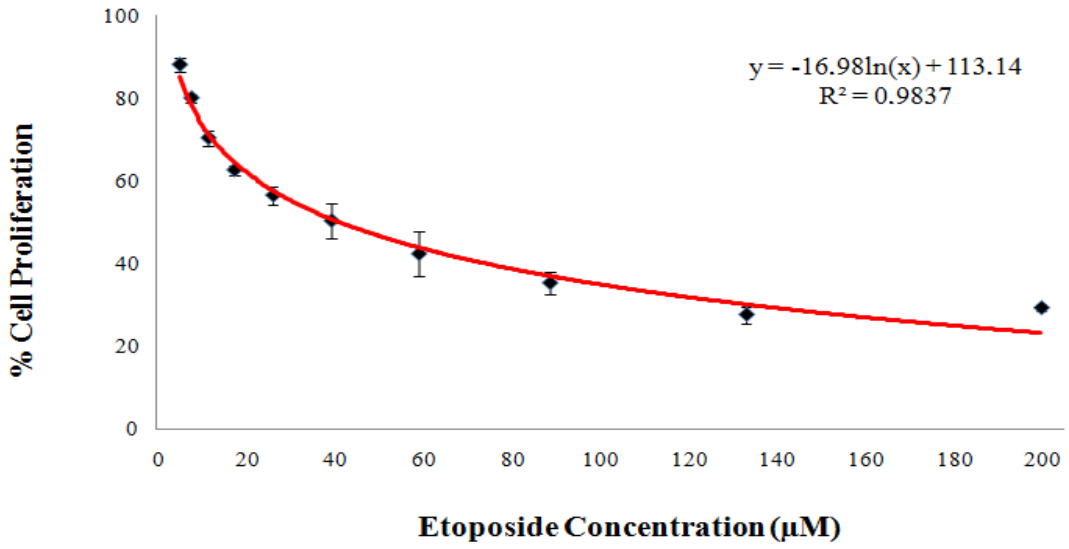


Figure 3. 2 Antiproliferative Effect of Etoposide on MCF7/S

In Figure 3.2 a graph of percent cell proliferation with respect to etoposide concentration is shown for parental MCF7/S cells. IC_{50} was calculated as 42.0 ± 4.2 µM. This means that at 42 µM concentration, etoposide kills 50% of the MCF7/S cells. This value was taken as 1 for the calculation of relative resistances. It can also be concluded that with a 42µM of IC_{50}, cytotoxic effect of etoposide is about 20 fold lower than that of doxorubicin which was previously found approximately 2µM (Dönmez, 2010).

MCF7/1000E cell line was exposed to concentrations of etoposide 5-200 µM at 2:3 dilution ratios.

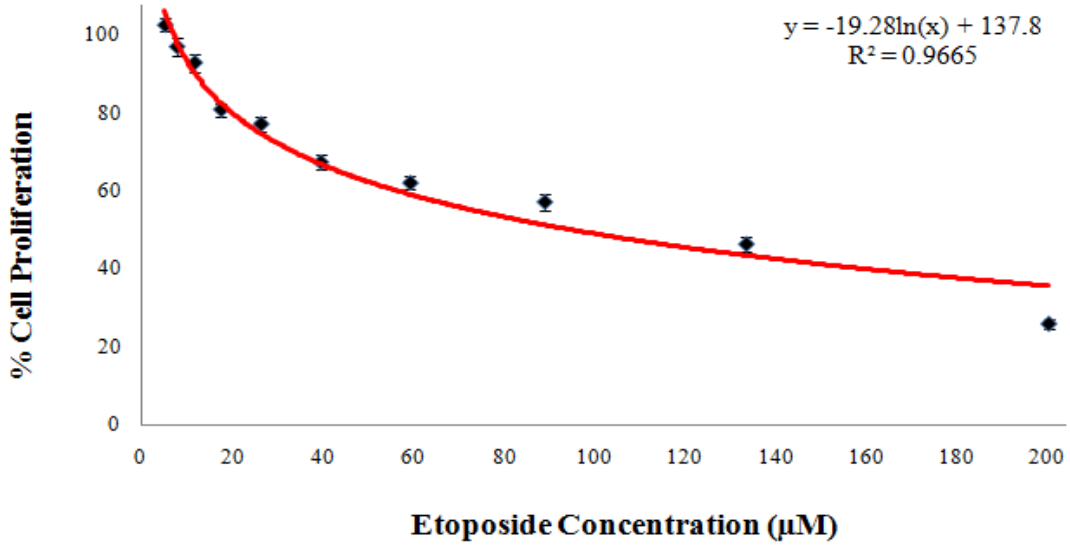


Figure 3. 3 Antiproliferative Effect of Etoposide on MCF7/1000E

Figure 3.3 represents proliferation of MCF7/1000E cells at different etoposide concentrations. IC_{50} was calculated as 95.0 ± 2.9 µM meaning that 95 µM etoposide kills 50% of the cells. According to this result, MCF7/1000E is 2.3 fold more resistant to etoposide with respect to original MCF7/S cells. It is clear that resistance was attained after about 22-week continuous exposure of cells to etoposide successfully and the resistance was proved to be significant compared to parental cell line (Figure 3.6) according to one-way ANOVA at the $p<0.01$ level with a 99% confidence interval.

MCF7/1250E cell line was exposed to concentrations of etoposide 8-300 µM at 2:3 dilution ratios.

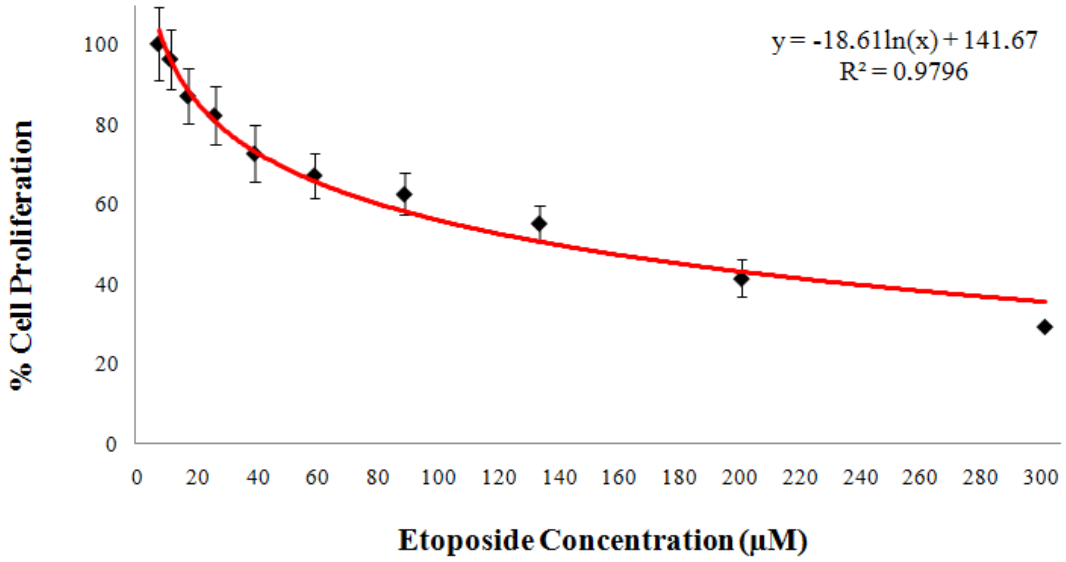


Figure 3. 4 Antiproliferative Effect of Etoposide on MCF7/1250E

Figure 3.4 shows the antiproliferative effect of etoposide on MCF7/1250E. With respect to MCF7/1000E, applied etoposide dose in culture was increased 1.25 fold. IC_{50} was calculated as 127.5 ± 8.8 µM. This result demonstrates that the effectiveness of etoposide on MCF7/1250E was less compared to previous subline, MCF7/1000E. MCF7/1250E was 3 fold more resistant to etoposide than MCF7/S cells. Relative to MCF7/S, resistance was significant (Figure 3.6) according to one-way ANOVA at the $p<0.01$ level. However, it is not significant relative to MCF7/1000E according to Tukey's post-test at the $p<0.01$ level. This means that MCF7/1250E could not gain a significant resistance than that of MCF7/1000E, although cytotoxic effect of etoposide became lower. Even though resistance is not significantly higher than MCF7/1000E, differences in the mechanisms contributing to resistance can be expected due to decrease in cytotoxic effect of etoposide. In order to evaluate the changes in resistance mechanisms in a subline with higher

resistance index, MCF7/2000E was developed and its antiproliferative effect was tested.

MCF7/2000E cell line was exposed to concentrations of etoposide between 5-200 µM at 2:3 dilution ratios.

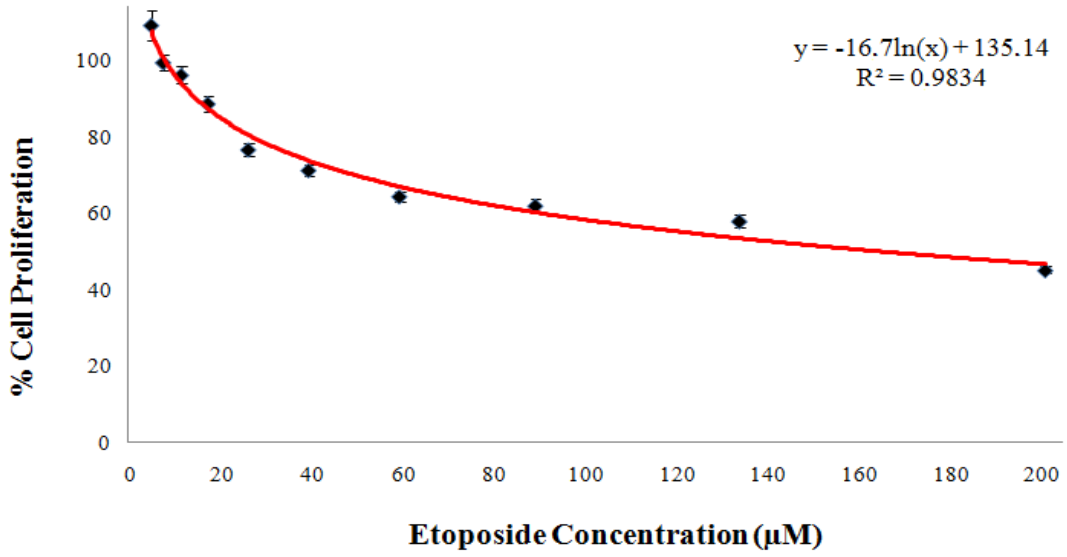


Figure 3. 5 Antiproliferative Effect of Etoposide on MCF7/2000E

Figure 3.5 corresponds to the antiproliferative effect of etoposide on MCF7/2000E. With respect to MCF7/1000E, applied etoposide dose was increased 2 fold. IC_{50} was calculated as 164.0 ± 3.4 µM. This result demonstrates that the effectiveness of etoposide on MCF7/2000E further decreased relative to previous sublines, MCF7/1000E and MCF7/1250E. MCF7/2000E is about 4 fold more resistant to etoposide compared to original MCF7/S cells and resistance was significant according to one-way ANOVA at the $p<0.01$ level. According to Tukey's post test, resistance level of MCF7/2000E was also significant (Figure 3.6) relative to MCF7/1000E, however it was not significant relative to MCF7/1250E at the level of $p<0.01$.

Degree of statistical significance of resistance between groups determined by Tukey's Multiple Comparison Post Test following one-way ANOVA and it is described by means of bar graph in Figure 3.6.

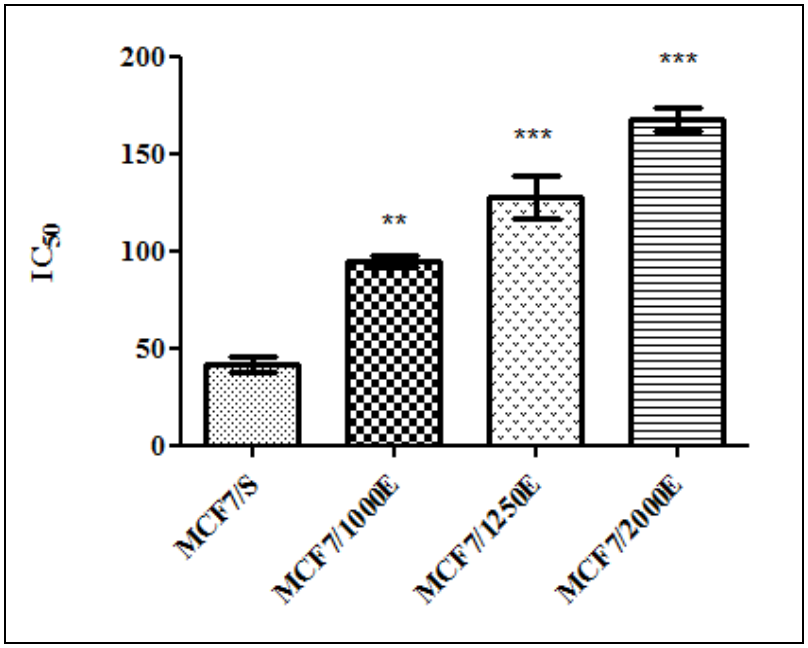


Figure 3. 6 IC_{50} values of MCF7/S and resistant sublines. ** Represents significant difference relative to MCF7/S cell line at the level $p<0.01$ and *** represents significant difference relative to MCF7/S cell line at the level $p<0.001$.

Figure 3.6 demonstrates a linear relationship between IC_{50} and continuously applied dose. IC_{50} increases as the continuously applied dose of etoposide increases. IC_{50} and cytotoxic effect of drug is inversely proportional such that increasing IC_{50} shows decreasing cytotoxicity, hence increasing resistance. It can be concluded from the Figure 3.6 that the degree and significance of resistance relative to MCF7/S increased as the applied etoposide increased. Cells seemed to be adapted to environmental changes by modifying their mechanisms in order to survive. Decreasing cytotoxic effect of etoposide is an indication of alteration in cellular mechanisms in resistant cells to adapt the increasing dose of drug. As the resistance

level increases, surviving cells develop new strategies which are more efficient than the former one.

Table 3. 1 IC_{50} and resistance indices of MCF7 cell lines resistant to etoposide

Cell Line	IC_{50}±SEM[a] (μM)	Resistance Index
MCF7/S	42.0 ± 4.2	1
MCF7/1000E	95.0 ± 2.9	2.3[b]
MCF7/1250E	127.5 ± 8.8	3[b]
MCF7/2000E	164.0 ± 3.4	3.9[b]

a - Mean IC_{50} and SEM values were obtained from three independent experiments
b - Significant according to the one way ANOVA (Tukey's Multiple Comparison Test, $p<0.01$) compared to MCF7/S

3.1.2.2 Determination of Cross Resistance to Etoposide in Doxorubicin Resistant Cell Line

In order to determine the cytotoxic effect of etoposide on doxorubicin resistant subline MCF7/1000D XTT cell proliferation assay was performed with MCF7/1000D as described previously. The aim of this assay was to evaluate the cross resistance of MCF7/1000D to etoposide. MCF7/1000D subline was exposed to concentrations of etoposide between 4.5-180 μM at 2:3 dilution ratios.

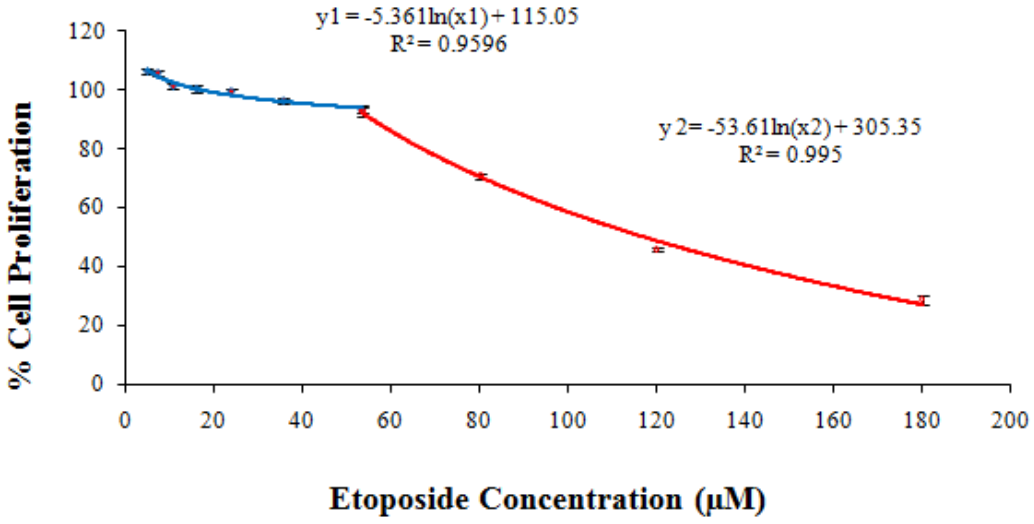


Figure 3. 7 Antiproliferative effect of Etoposide on MCF7/1000D

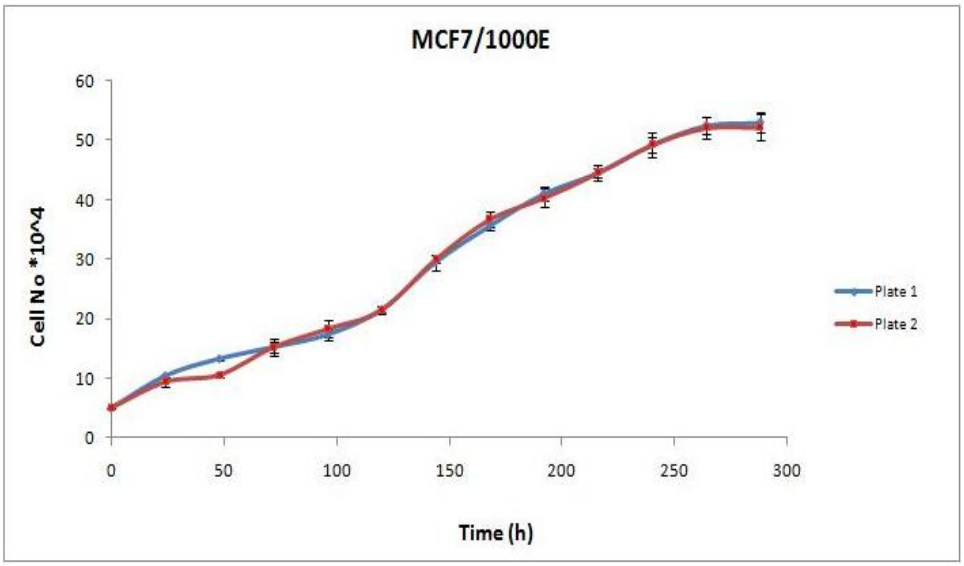
Figure 3.7 represents the antiproliferative effect of etoposide on doxorubicin resistant MCF7/1000D cell line. According to Figure 3.7, the cells demonstrated two different trends between the indicated concentrations of etoposide. In the first part, between 4.5-50 µM drug concentrations, etoposide had almost no effect on MCF7/1000D. This indicates that almost all of the cells have the ability to survive in this range of concentrations. In the second part, between 50-180 µM drug concentrations a noticeable decrease in the cell proliferation showing a higher cytotoxicity of etoposide on MCF7/1000D was observed. The IC_{50} value for the second part of the plot, between concentrations 50-180 µM was calculated from 3 independent assays and found as 117.1 ± 1.2 µM. These results revealed that doxorubicin resistant MCF7/1000D cell line could endure up to 50 µM etoposide; however over 50 µM, MCF7/1000D started to be affected by etoposide. MCF7/1000D was shown to overexpress *MDR1* and develop cross resistance to other P-gp substrates (İşeri, 2009). Etoposide is one of the substrates of P-gp (Dantzig *et al.*, 1996; Kandimalla and Donovan, 2005). However, it was previously revealed in a study which was performed using paclitaxel that P-gp mediated efflux could be dose dependent (Jang *et al.*, 2001). It may be concluded from Figure 3.7 that as a substrate of P-glycoprotein (P-gp), etoposide can be pumped out due to the increased P-gp in

MCF7/1000D. However, at higher doses of the drug these pumps may be saturated by etoposide which results in accumulation of the drug inside the cells. Overexpression of P-gp may decrease the requirement for some resistance mechanisms inside the cell; hence altered expression of resistance-related genes such as *TOP2A* (Figure 3.13) may be restored to original expression levels. Thereby, accumulated etoposide in the cell may cause sudden death of doxorubicin resistant subline MCF7/1000D over 50 μM concentrations. Although causing sudden death, with 117.1 ± 1.2 μM IC_{50} value MCF7/1000D is highly resistant to etoposide comparable to etoposide resistant subline MCF7/1250E with an IC_{50} of 127.5 ± 8.8 μM.

3.1.2.2 Generation of Growth Curves

Growth curve of MCF7/1000E and MCF7/600D were constructed by plotting the cell number against time. Cells were counted as described in Section 2.2.1.4 and 2.2.3.2. Linear plots of growth curves were obtained from the logarithmic phase of growth. Doubling time(t_d)s were calculated using the slope of linear trendline of logarithmic phase by using equation 2.3.

a)



b)

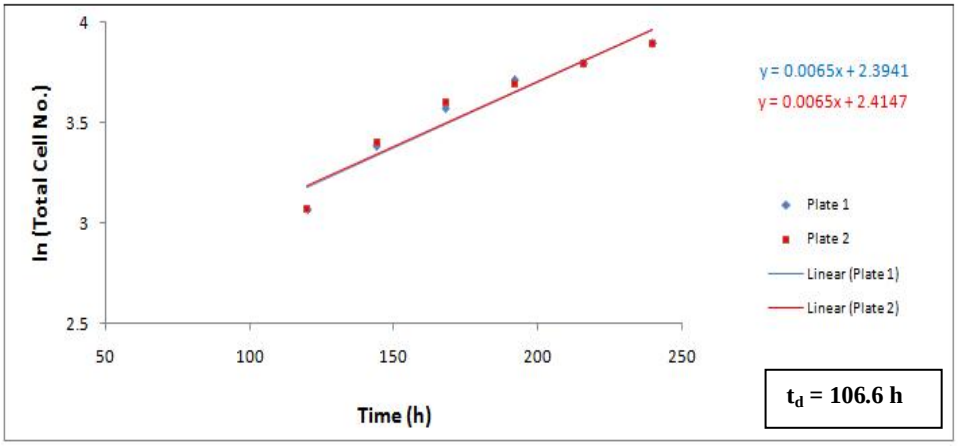
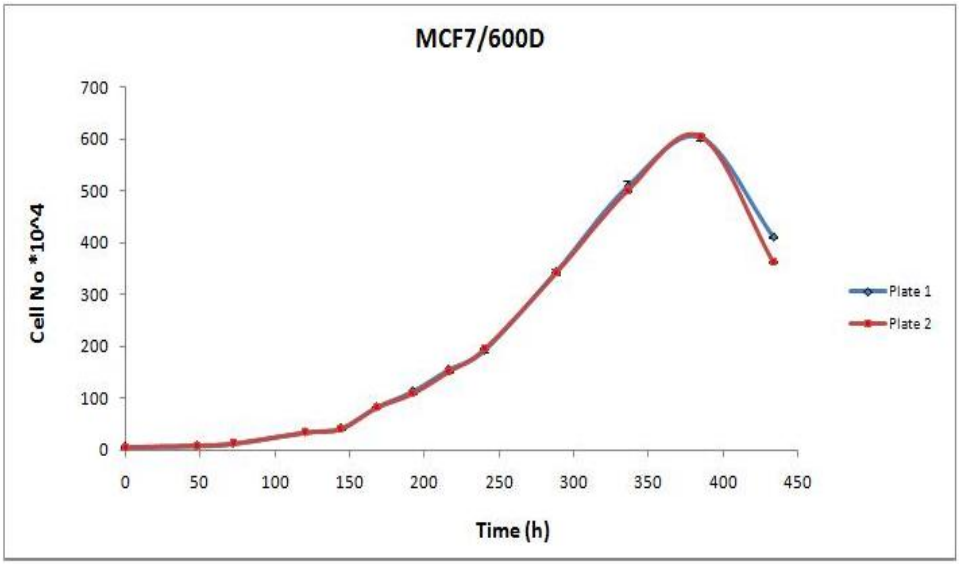


Figure 3. 8 Growth curve (a) and linear plot (b) representing the logarithmic phase of MCF7/1000E. Data was obtained from 8-20 times counting of duplicate samples.

MCF7/1000E cells were followed by 12 days. Growth curves were obtained from two separate plates. Logarithmic growth was determined between 120 and 240 hours. Using the data from logarithmic growth phase, linear plots were generated in order to calculate the doubling times. Doubling times were determined for two plates separately and mean t_d was stated as "t_d ± SEM". Doubling time of MCF7/1000E was found as 106.6 hours. Higher t_d value is probably due to the effect of etoposide on cell cycle. Etoposide is effective in G2 and S phases (http://www.cancer.gov/Templates/drugdictionary.aspx?CdrID=39207) and it

reversibly binds to TOP2A (Hainsworth and Greco, 1995). Binding of etoposide to TOP2A may block the cell cycle. However, considering its reversible binding and decreased *TOP2A* expression, it cannot be cytotoxic to MCF7/1000E, and instead it causes a prolonged proliferation relative to MCF7/S (Ohishi *et al.*, 1996). t_d for MCF7/S was previously found as 27.9 ± 0.7 hours in our laboratory (İşeri, 2009).

a)



b)

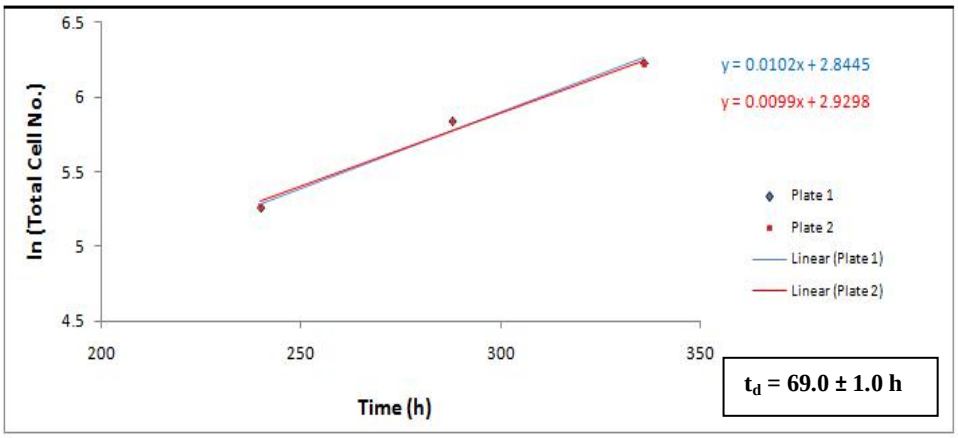


Figure 3. 9 Growth curve (a) and linear plot (b) representing the logarithmic growth of MCF7/600D. Data was obtained from 8-20 times counting of duplicate samples.

Growth curve of MCF7/600D cells were constructed over 18 days as duplicate experiments. Logarithmic growth was determined between 240 and 336 hours. From logarithmic growth phase data, linear plots were generated in order to determine the t_ds for two plates and mean t_d was stated as "t_d ± SEM". Doubling time of MCF7/600D was found as 69.0 ± 1.0 hours.

3.1.2.3 Expression Analysis of Resistance Genes

3.1.2.3.1 Total RNA Isolation

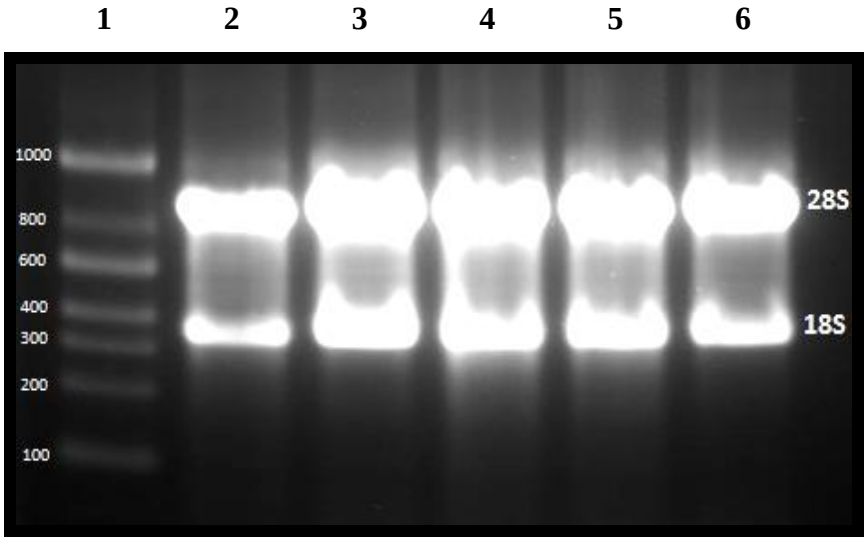


Figure 3. 10 Representative image of agarose gel (1%) of total RNAs isolated from various MCF7 sublines. Lane 1: High range RNA Ladder; Lanes 2 - 6: various RNA samples isolated with TriReagent.

In Figure 3.10 total RNA of various MCF7 sublines isolated with TriReagent (Sigma Aldrich, Germany) are demonstrated. Sharp bands corresponding to 28S and 18S rRNAs without any smear of broken RNAs, indicated that intact RNAs were isolated. Isolated RNAs were further analyzed by spectrophotometry for their purity. The RNA samples having nucleic acid/protein (A_{260}/A_{280}) ratios greater than 1.7, and

with nucleic acid/phenololic compounds (A_{260}/A_{230}) ratios greater than 1.8 were used in expression analysis.

3.1.2.3.2 Expression Analysis of *TOP2A*, *MSH2* and *MLH1* genes

Expression analysis of *TOP2A*, *MLH1*, *MSH2* and *β-actin* genes for MCF7/S parental cell line and doxorubicin and etoposide resistant sublines were carried out by qPCR and relative expressional changes were determined using $\Delta\Delta C_t$ method as described in Section 2.2.3.4.3.2. Briefly, the expression level of each of *TOP2A*, *MSH2* and *MLH1* genes in each cell line was normalized to expression level of *β-actin.* Fold changes in expression levels were found by comparing normalized data of resistant sublines with that of untreated MCF7/S cells by using equation 2.4. Amplification curves were plotted as fluorescence versus threshold cycle number (Figure 3.11). Following the completion of each run, melting curve analysis was performed in order to visualize the dissociation kinetics of the products amplified, and to discriminate the product of interest from other unexpected products, if there exist (Figure 3.12). Melting curves are displayed by plotting first derivative of fluorescence with respect to temperature (dF/dT) against temperature (Smith, 2009). If there are sharp peaks at the same Tm, it is recognized that only the product of interest has been amplified. Figure 3.12 represents the melting curves for *TOP2A*, *MSH2, MLH1* and *β-actin* genes, respectively.

a)

b)

c)

d)

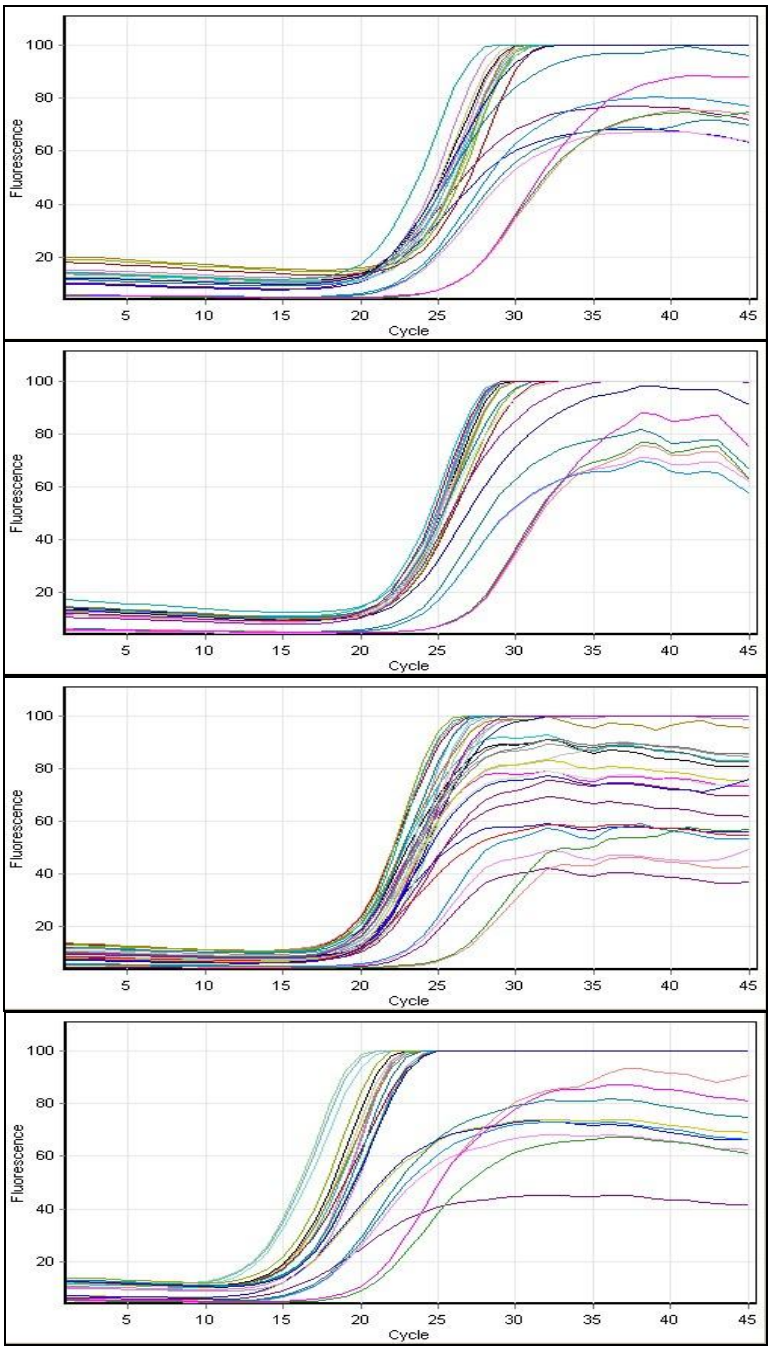
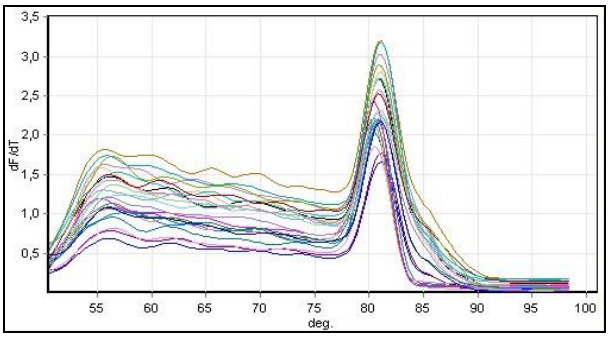
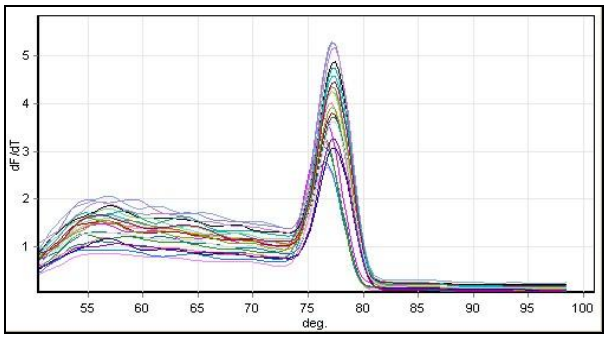


Figure 3. 11 Amplification Curves in qPCR for a) *TOP2A* b) *MSH2* c) *MLH1* and d) *β-actin* genes for parental subline MCF7/S, etoposide and doxorubicin resistant sublines.

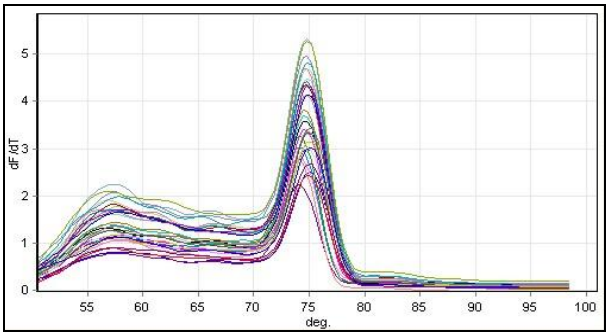
a)



b)



c)



d)

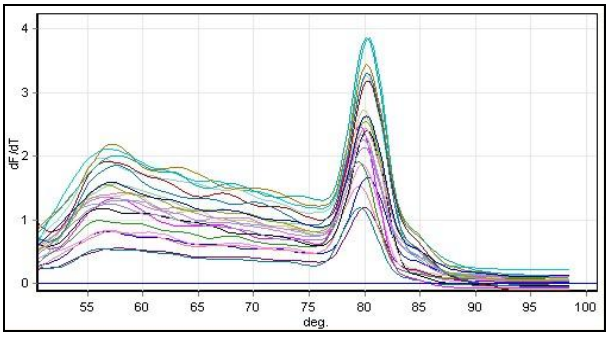


Figure 3. 12 Melting Curve Analysis for qPCR of a) *TOP2A* b) *MSH2* c) *MLH1* and d) *β-actin* genes in MCF7/S parental subline MCF7/S, etoposide and doxorubicin resistant sublines.

According to the Figure 3.12, the sharp peaks for each run were produced at the same melting temperature, and no other sharp peak indicating unexpected products were generated during the melting curve analysis. From this data it is figured out that at the end of the reactions, only the products of interest were amplified by the specific primers, and there were no other product to which SYBR green could bind.

Raw gene quantitation data was obtained using Rotor-Gene software (Qiagen, Australia) after each qPCR. The quantitation data of *TOP2A*, *MSH2* and *MLH1* were normalized to internal control *β-actin*, and fold changes in the expression of *TOP2A*, *MSH2*, and *MLH1* were calculated for each of the resistant subline with respect to MCF7/S using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Fold changes in each cell line were represented with bar graphs for *TOP2A*, *MSH2* and *MLH1* transcripts in Figures 3.13, 3.14 and 3.15, respectively.

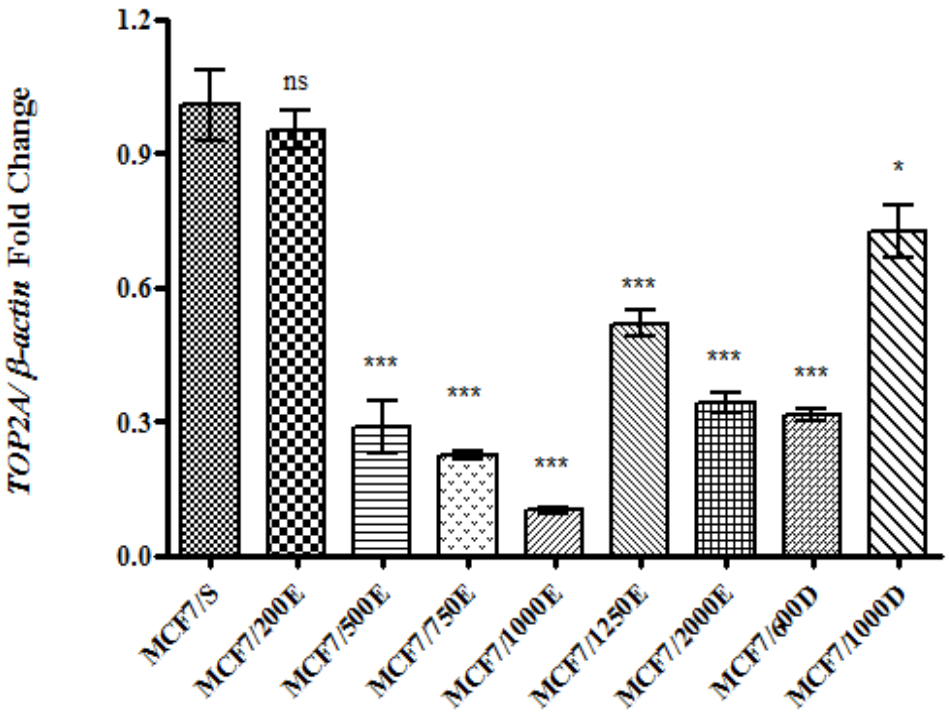


Figure 3. 13 Relative gene expression levels for *TOP2A* in each cell line.
ns: non-significant change compared to MCF7/S
*** significant difference with compared to MCF7/S $p<0.001$
* significant difference with compared to MCF/S $p<0.05$
SEM values were determined from three independent experiments.

Figure 3.13 indicates relative gene expression levels for *TOP2A* in parental MCF7/S cells and resistant sublines. All resistant sublines expressed *TOP2A* at significantly lower levels than MCF7/S cell line. In etoposide resistant sublines, a regular decrease in the expression of *TOP2A* was observed as the resistance level increased including MCF7/1000E. However, in cells resistant to 1250 μM and 2000 μM etoposide, MCF7/1250E and MCF7/2000E, *TOP2A* expression increased slightly. Decrease in the expression of *TOP2A* was also recognized in doxorubicin resistant sublines. Highly significant decrease was observed in MCF7/600D compared to parental cell line at level $p<0.001$. The decrease in *TOP2A* expression levels in MCF7/1000D was also significant compared to parental cell line at the level $p<0.05$ with a lower decrease than MCF7/600D with compared to parental cell line. This indicates that decrease in the expression levels of *TOP2A* in MCF7/600D is more significant

compared to MCF7/S than in MCF7/1000D. Also a significant increase approaching to the expression level of parental cell line was observed in MCF7/1000D relative to MCF7/600D. These results are generally consistent with literature. Downregulation of *TOP2A* is a known response of resistant cell lines against topoisomerase inhibitors (Takano *et al*., 1992; Hasegava *et al*., 1995). Considering the direct inhibitory effect of etoposide on TOP2A, lower expression of *TOP2A* may be expected as a first line resistance mechanism in etoposide resistant sublines. The cells may decrease *TOP2A* expression as a response to presence of drug; therefore the drug cannot show its cytotoxicity due to lower amount of TOP2A probably leading to formation of lower amounts of double strand breaks. Thereby, the cells can overcome cytotoxic effect of etoposide which results in development of resistance. Low level of resistance is generally related with this phenomenon, however it is proposed that more striking effects on TOP2A with several modifications of the enzyme is required for higher levels of resistance (Larsen and Skladanowski, 1998). A cell has a dynamic environment. The fluctuation in the expression of *TOP2A* at higher resistance levels (MCF7/1250E and MCF7/2000E) may be due to development of other resistance mechanisms in order to overcome higher etoposide cytotoxicity. The stress on *TOP2A* may decreased due to development of other resistance mechanisms in subline MCF7/1250E. However, for higher resistance levels to etoposide *TOP2A* downregulation is again required, as it is observed in MCF7/2000E.

In doxorubicin resistant sublines, when two sublines were compared, *TOP2A* expression was significantly lower in MCF7/600D than in MCF7/1000D. This may be explained by the development of other strategies for higher resistance levels that decrease the stress on *TOP2A* and the requirement of cells for downregulation of *TOP2A*. One of the explanations for downregulation of *TOP2A* may be overexpression of *MDR1* gene which encodes P-gp. P-gp is an ATP-dependent efflux pump and doxorubicin is one of the substrates of P-gp (Paul and Cowan, 1999). It was previously clarified that both MCF7/600D and MCF7/1000D overexpress *MDR1* gene. Moreover, it was reported in the same study that the expression levels of *MDR1* was lower in MCF7/600D than in MCF7/1000D (İşeri, 2009). Furthermore, it

was demonstrated in our laboratory that doxorubicin accumulation in doxorubicin resistant cells (MCF7/1000D) decreased significantly compared to parental cells (Dönmez, 2010) due to the overexpression of *MDR1* and production of P-gp (İşeri, 2009). Taken together, it may be concluded that *TOP2A* is downregulated as a first response mechanism to the presence of drug. However, when new resistance strategies emerge, such as overexpression of *MDR1*, due to higher drug concentrations, accumulation of doxorubicin in the cell decreases. Consequently, selective pressure for downregulation of *TOP2A* decreases and *TOP2A* expression is restored to its initial levels.

In etoposide resistant sublines, as the resistance increased a regular decrease in the expression of topoisomerase occurred until a certain level. However, in doxorubicin resistant sublines an increase was observed at the higher resistance level. The most resistant subline among etoposide resistant sublines is MCF7/2000E and it is only 4 fold resistant compared to MCF7/S. On the other side, MCF7/1000D is 110 fold more resistant than MCF7/S against doxorubicin (Dönmez, 2010). Very high resistance level of MCF7/1000D may indicate development of more effective resistance mechanisms than downregulation of *TOP2A*. Also, it was previously demonstrated that *MDR1* overexpressed in doxorubicin resistant sublines at higher levels of resistance, including MCF7/600D and MCF7/1000D. Furthermore, *MDR1* gene expression was upregulated with increasing drug concentrations (İşeri, 2009). P-gp which is a product of *MDR1* gene, decreases the accumulation of doxorubicin inside the cells probably contributing to the restoration of *TOP2A* expression levels in MCF7/1000D. However, etoposide resistant cell lines do not seem to have a significant *MDR1* overexpression according to the unpublished observations. Instead of *MDR1*, increased expression of *MRP1* was observed (Appendix D, Figure D.1). *MRP1* codes a different ATP-dependent efflux pump, which was described by Cole and co-workers (Cole *et al*., 1992). *MRP1* overexpression instead of *MDR1* was reported in etoposide resistant cell lines previously (Schneider *et al*., 1994). Among etoposide resistant sublines, *MRP1* expression was increased in sublines MCF7/1000E, MCF7/1250E and MCF7/2000E. Although increase in *MRP1*

expression, decrease in the expression of *TOP2A* remained in these sublines. This result may indicate an insufficient expression of *MRP1* to fully account for resistance. Downregulation of *TOP2A* and upregulation of *MRP1* probably contribute to resistance collaboratively. Different mechanism of action of two drugs may also be a reason of different expression pattern of two groups of sublines. Since etoposide is mainly a TOP2A inhibitor, downregulation of *TOP2A* in all of the etoposide resistant sublines is an expected response. However, doxorubicin has many other action mechanisms in cells and may evoke many other different mechanisms for development of resistance (İşeri *et al*., 2010).

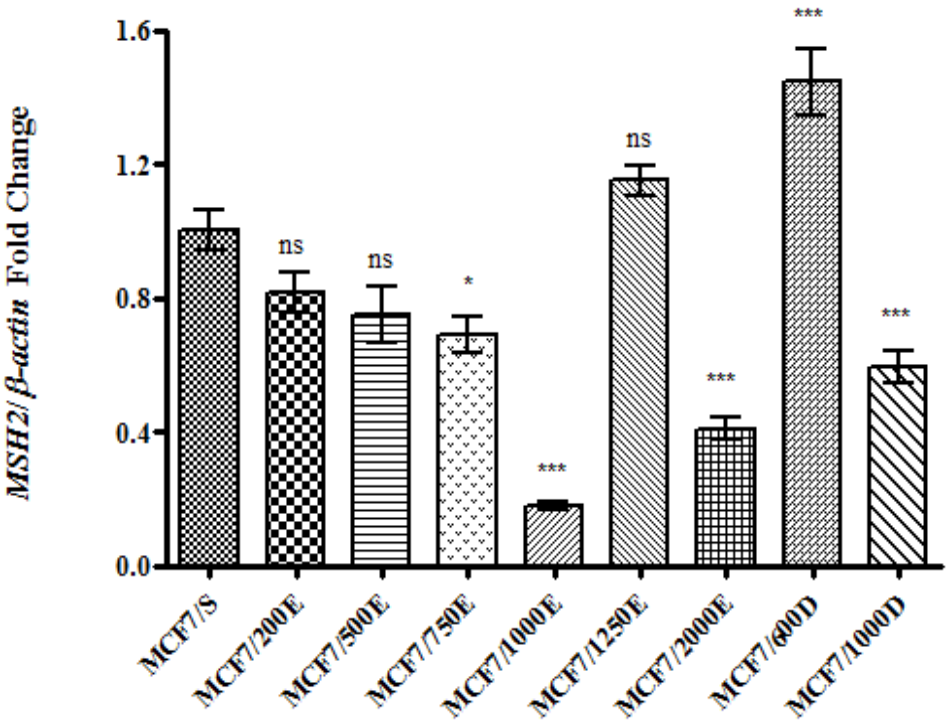


Figure 3. 14 Relative gene expression levels for *MSH2* for each cell line.
ns: non-significant change compared to MCF7/S
*** significant difference relative to MCF7/S $p<0.001$
** significant difference relative to MCF7/S $p<0.01$.
SEM values were determined from three independent experiments.

Figure 3.14 represents relative gene expression levels of *MSH2* in etoposide and doxorubicin resistant sublines. *MSH2* expression was significantly lower in sublines MCF7/750E, MCF7/1000E, MCF7/2000E. Until MCF7/1000E a regular reduction in the expression levels of *MSH2* gene was observed, however in MCF7/200E, and MCF7/500E decrease was not so significant. MCF7/1000E sublines expressed significantly low levels of *MSH2*. However in MCF7/1250E, there was a sudden increase in expression levels of *MSH2* followed by a decline in MCF7/2000E. In doxorubicin resistant sublines, a dramatic increase in expression in MCF7/600D and a significant decrease in expression in MCF7/1000D were observed.

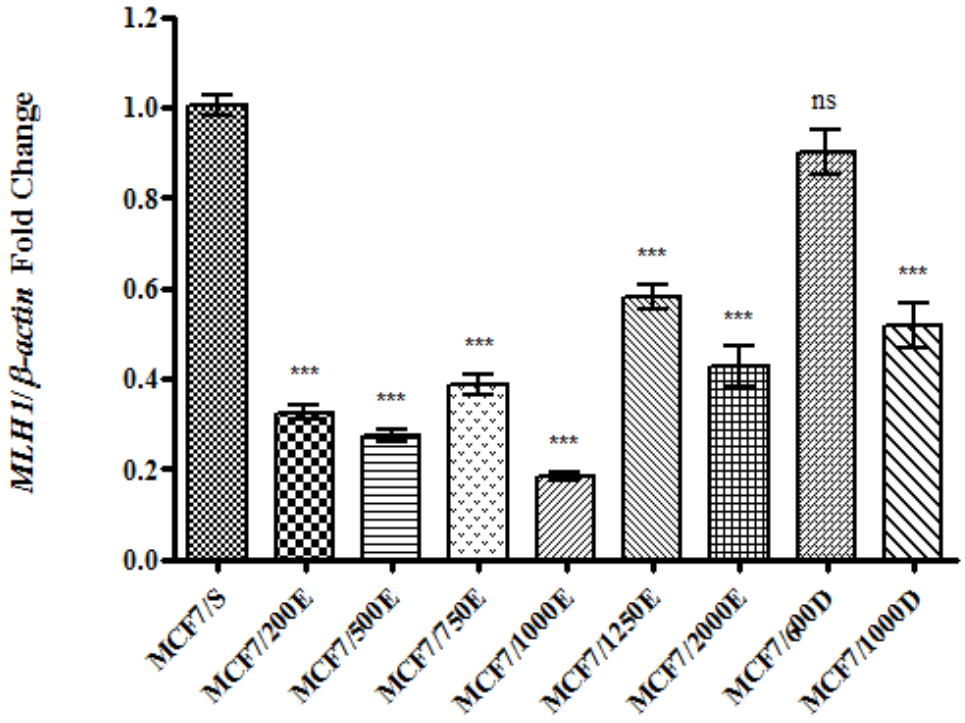


Figure 3. 15 Relative gene expression levels for *MLH1* for each cell line.
ns: non-significant change compared to MCF7/S
*** significant difference relative to MCF7/S $p<0.001$
SEM values were determined from three independent experiments.

Figure 3.15 indicates expression level of *MLH1* for each of the parental MCF7/S cell line and resistant sublines. General observation for etoposide resistant sublines was reduction in the expression of *MLH1* gene as the resistance increased. *MLH1* downregulation was significant at the level $p<0.01$. However, in doxorubicin resistant sublines there was no significant change in MCF7/600D, while there was very significant decrease in the expression levels of *MLH1* in MCF7/1000D.

Table 3. 2 Fold changes in gene expression (*TOP2A*, *MSH2* and *MLH1*, left to right)

Cell Line	**FC (*TOP2A*)**	**FC (*MSH2*)**	**FC (*MLH1*)**
MCF7/S	1.01 ± 0.08	1.00 ± 0.06	1.00 ± 0.02
MCF7/200E	0.95 ± 0.04	0.82 ± 0.06	0.32 ± 0.02
MCF7/500E	0.29 ± 0.06	0.75 ± 0.08	0.27 ± 0.01
MCF7/750E	0.22 ± 0.01	0.69 ± 0.06	0.39 ± 0.02
MCF7/1000E	0.10 ± 0.01	0.18 ± 0.01	0.18 ± 0.01
MCF7/1250E	0.52 ± 0.03	1.15 ± 0.05	0.58 ± 0.03
MCF7/2000E	0.34 ± 0.02	0.41 ± 0.03	0.43 ± 0.04
MCF7/600D	0.31 ± 0.01	1.45 ± 0.10	0.90 ± 0.05
MCF7/1000D	0.72 ± 0.06	0.60 ± 0.05	0.52 ± 0.05

Fold changes are stated as "mean ± SEM". SEM values were obtained from triplicate samples of three independent experiments

Cells can sometimes tolerate to some DNA damaging agents by means of mismatch repair pathways (Karran, 2001). There are many studies that propose a relationship between deficiency in MMR proteins and drug resistance (Fink *et al*., 1998; Bock *et al*., 2000). Especially microsatellite instability (MSI) which is utilized as a marker for resistance is thought to be based on deficiency in MMR proteins, MSH2 and MLH1, in human nonpolyposis colorectal cancer (HNPCC). MMR deficiency is also

correlated with inhibition of apoptosis due to the reduced function of MMR in recognition or signaling of DNA damage. When the apoptotic signals are restricted due to insufficient MMR system, cancer cells may tolerate cytotoxicity of some chemotherapeutics. However, the detailed mechanism of the involvement of MMR in apoptotic signaling has not been fully elucidated yet. Considering its mismatch and insertion/deletion loop (IDL) recognition function, presence of MMR deficiency in resistant cells against platinating or alkylating agents is reasonable. MMR deficiency is additionally associated with resistance against topoisomerase II inhibitors (Aebi *et al*., 1997). This finding is noticing because, double strand breaks are generated due to topoisomerase II inhibitors and these types of breaks are primarily repaired by homologous recombination and nonhomologous end joining (Sonoda, 2006). It is proposed by Chen and Liu (Chen and Liu, 1994) that MMR may also recognize cleavable complex produced after binding of etoposide to topoisomerase II a-cleaved DNA complex. Also, alternative mechanisms of topoisomerase II inhibitors such as intercalation of doxorubicin into DNA creating crosslinks, may be recognized by MMR. Due to lower expression or deficiency of *MSH2* or *MLH1*, the damages cannot be recognized by the cell and the cell continues to proliferate by accumulating mutations that may cause development of more aggressive, so called resistant, tumors which may develop a "mutator phenotype". MMR is involved in various mechanisms in the cells. The association of main MMR proteins with resistance to topoisomerase II inhibitors may be explained by its involvement in homologous recombination. MSH2 containing heteroduplex MutSα was previously observed to decrease recombination at double strand breaks (DSBs) due to interaction with BLM helicase (Smith *et al*., 2007). BLM helicase acts in the processing of double strand break ends and it is possibly served as a sensor for aberrant double stranded DNA structures (Wang *et al*., 2000). BLM also directly interacts with topoisomerase II (Russel, 2002). It may be concluded from this information that both TOP2A and MSH2 may act together as a sensor of double strand breaks by interacting with BLM helicase. In a huge complex composed of repair and apoptotic proteins, BLM transmits the apoptotic or checkpoint signal to ATM or ATR and cell fate is determined. Therefore, decrease in the expression levels of both *MSH2* and *TOP2A*

supports the resistance of cancer cells which become tolerant to topoisomerase II inhibitors. Affectivity of homologous recombination (HR) is also increased by increasing the expression of HR-related genes in resistant cells. By reducing *MSH2* expression, the resistant cells provide additional increase in the affectivity of HR because of eliminating the inhibitory effect of MMR.

Loss of *MLH1* expression also appears to be one of the results of resistance to chemotherapy. Decreased level of expression in *MLH1* is proposed to be based on methylation on the promoter of *MLH1* (Mackay *et al*., 2000). Mutant yeast strains of *MLH1* were previously shown to have identical phenotypes with *MSH2* mutants; however antirecombination function of *MSH2* mutants was more effective than that of *MLH1* mutants (Chen and Robertson, 1999). This means that MLH1 may have a similar but a decreased function in apoptotic response after inhibition of homologous recombination. Hence, decrease in the expression of *MLH1* in resistant sublines is reasonable. MLH1 is also involved in apoptotic response via acting as a proapoptotic signal in cytoplasm as a result of a caspase-3 cleavage (Chen *et al*., 2004). MCF7 cell line does not express caspase-3 (Jänicke, 2009). Therefore, a caspase-3 cleavage of MLH1 is not expected. However, interestingly it is reported that doxorubicin resistant cell lines derived from parental MCF7 cell line has full length caspase-3 (Mehta *et al*., 2002). Decrease in the expression of *MLH1* in MCF7/1000D may inhibit cleavage-dependent MLH1 induced apoptosis. It is also proposed that caspase-3-like proteases are activated in MCF7 cell line as a response to etoposide application (Benjamin *et al*., 1997). It can be proposed that these caspase-3-like proteases may be activated in response to prolonged etoposide application in order to trigger an apoptotic response via MLH1 cleavage in etoposide resistant sublines. However, this mechanism may be inhibited by significant decrease in *MLH1* expression. Hence, *MLH1* decrease in etoposide resistant sublines may also be a response to the apoptotic effect of MLH1. In its first intron, *MLH1* gene also has p53-response element indicating that it is regulated by p53 (Chen and Sadowski, 2005). This information may further increase the importance of MMR and specifically MLH1 involvement in apoptosis signaling as a sensor. Therefore, it can

be concluded that due loss of MLH1, p53-dependent apoptosis or cell cycle arrest signaling can be inhibited in resistant cell lines leading to survival in the presence of drugs.

Mismatch repair system is a complex mechanism and its crucial proteins such as MSH2 and MLH1 are involved in many different pathways in a cell. Loss of these proteins may cause a huge difference in the fate of cells due to their direct involvement in pathways such as repair and cell death as well as due to their indirect effects. MMR deficiency causes microsatellite instability (MSI) which leads to mutations in specific parts of genome composed of nucleotide repeats. *TOP2A* gene has repeat regions which can be altered due to MSI. Therefore, loss of *MSH2* and *MLH1* may lead to *TOP2A* gene to become a target for mutations in resistant cell lines (De las Alas, 1997). This effect of MMR system may also lead to alterations in other repair mechanisms causing resistance to chemotherapy.

In this study, *TOP2A*, *MSH2* and *MLH1* were chosen for investigation of their differential expression in resistant cell lines. However, there are also many proteins and systems that affect the resistance. The cells develop resistance by means of changing most of their cellular pathways. For instance, differential expression of basic apoptosis related genes such as *p53* or survival associated genes such as *EGFR* are reported as the reasons or results of resistance. Moreover, cytochrome P450 enzymes (CYPs) and the enzymes related to glutathione metabolism which regulate the drug cytotoxicity or elimination are some of the major proteins, expression of which is altered due to drug resistance (Kars, 2008; İşeri, 2009). In order to obtain a general view of expression changes in various genes due to drug resistance, a genome-wide microarray following by PCR arrays which give information about the expression of genes in a specific pathway can be performed. Furthermore, studies at the protein level should also be carried out for a more accurate assessment. By protein detection studies such as Western blotting, the expression of proteins of interest can be confirmed. MALDI-TOF-MS (Matrix-Assisted Laser Desorption/Ionization - Time Of Flight – Mass Spectrometry) analyses may also be

used to determine the post-translational modifications in the structures of proteins if there exist. Moreover, because confirmation of expression and structural intactness of some proteins such as enzymes is yet not enough, activity tests should also be performed to determine whether the enzyme functions accurately.

CHAPTER 4

CONCLUSION

1. Significantly different IC_{50} values compared to original MCF7/S cell line demonstrated that etoposide applied cells developed varying degrees of resistance to etoposide. Degree of resistance enhanced with increase in drug concentration. Three resistant sublines were obtained and they were named as MCF7/1000E, MCF7/1250E and MCF7/2000E. These sublines had different appearances and they also showed different growth characteristics.

2. According to the growth curve analysis of MCF7/1000E, doubling time was found to be approximately 106 hours. Doubling time of MCF7/1000E was determined to be approximately 4 fold longer than of parental cells. Long proliferation time for this subline may be due to the cell cycle arrest effect of etoposide.

3. According to expression analyses, expression of *TOP2A*, *MSH2* and *MLH1* in resistant sublines was found to be significantly different from those in parental MCF7/S cell line.

4. Expression analyses of *TOP2A* indicated that in etoposide and doxorubicin resistant sublines *TOP2A* expression was significantly lower than parental MCF7/S cell line. Decrease in *TOP2A* expression levels in resistant cell lines verified the contribution of differential expression of *TOP2A* in resistance. It may be concluded that as a drug target, decreasing the level of topoisomerase II in the cell can be the first line response of cancer cells against topoisomerase II inhibitors.

5. In etoposide resistant sublines, as the resistance increased expression levels of *MSH2* decreased, except the subline MCF7/1250E. Accordingly, it can be concluded that *MSH2* is involved in resistance to etoposide at least partially. Also, over a resistance level, the expression can change due to development of other resistance strategies.

6. *MLH1* expression analyses demonstrated that in resistant sublines the expression levels of *MLH1* significantly decreased. This decrease in resistant cells was possibly related to a primary role of *MLH1* in apoptosis signaling.

7. *TOP2A*, *MSH2* and *MLH1* expression levels were the lowest in MCF7/1000E subline which may lead to a mutator phenotype in these cells due to a significant deficiency in mismatch repair system.

8. Differential gene expressions of *TOP2A*, *MSH2* and *MLH1* indicated that these genes contribute to resistance against topoisomerase II inhibitors, etoposide and doxorubicin. Furthermore, decrease in the expression levels of all these genes may indicate a collaborative contribution to resistance.

Consequently, alterations of *TOP2A*, *MSH2* and *MLH1* expression levels were demonstrated to be involved in resistance against topoisomerase II inhibitors etoposide and doxorubicin. These genes may be considered for development of new strategies to overcome resistance against topoisomerase II inhibitors.

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APPENDIX A

CELL CULTURE MEDIUM

Table A. 1 Composition of RPMI 1640 Medium (Biochrom AG, Germany)

Substance	Concentration (mg/l)	Substance	Concentration (mgl/l)
NaCl	6000	L-methionine	15
KCl	400	L-phenylalanine	15
$Na_2HPO_4 \cdot 7H_2O$	1512	L-proline	20
$MgSO_4 \cdot 7H_2O$	100	L-serine	30
$Ca(NO_3)_2 \cdot 4H_2O$	100	L-threonine	20
D-glucose	2000	L-tryptophane	5
Phenol red*	5	L-tyrosine	20
$NaHCO_3$	2000	L-valine	20
L-arginine	200	Glutathione	1
L-asparagine	50	Biotin	0.2
L-aspartic acid	20	Vitamin B_{12}	0.005
L-cystine	50	D-Ca-pantothenate	0.25
L-glutamine	300	Cholin chloride	3
L-glutamic acid	20	Folic acid	1
Glycine	10	Myo-inositol	35
L-histidine	15	Nictoninamid	1
L-hydroxyproline	20	p-amino benzoic acid	1
L-isoleucine	50	Pyridoxin·HCl	1
L-leucine	50	Riboflavin	0.2
L-lysine·HCl	40	ThiamineHCl	1

* (1x) liquid medium contains 10 mg/l phenol red

APPENDIX B

DEVELOPMENT AND CULTURING OF RESISTANT SUBLINES

Etoposide resistance was developed by stepwise selection in dose increments. Adapted cells were chosen at some levels and maintained to become confluent before application of increased dose. Total time required to develop maximum resistance level (MCF7/2000E) was approximately 1.5 years. After development of resistant sublines photographs were taken of both parental cell line (MCF7/S) and resistant sublines under inverted light microscope (Figures B.1, B.2, B.3, B.4, B.5 and B.6).

Table B. 1 Durations (week) of etoposide application for the development of resistant sublines

Development of Etoposide Resistant MCF7 Cell Lines								
[Eto (µM)]	0.05	0.1	0.2	0.5	0.75	1	1.25	2
Weeks	1	2	2	4	3	10	8	9

In order to maintain the resistance level of etoposide and doxorubicin resistant sublines, required amounts of drugs were added to media in which they were cultured (Table B.2). No drug was added to parental MCF7/S cell line.

Table B. 2 Concentrations of the drugs added to the medium for maintenance of resistance

MCF7/S	MCF7/200E	MCF7/500E	MCF7/1000E	MCF7/1250E	MCF7/2000E	MCF7/600D	MCF7/1000D
	Etoposide (μM)					Doxorubicin (μM)	
0	0.2	0.5	1	1.25	2	0.6	1

After development of resistant cell lines micrographs of parental cells and resistant sublines were taken in 40x, 100x, 200x and 400x magnifications (Figure B.1 - B.6)

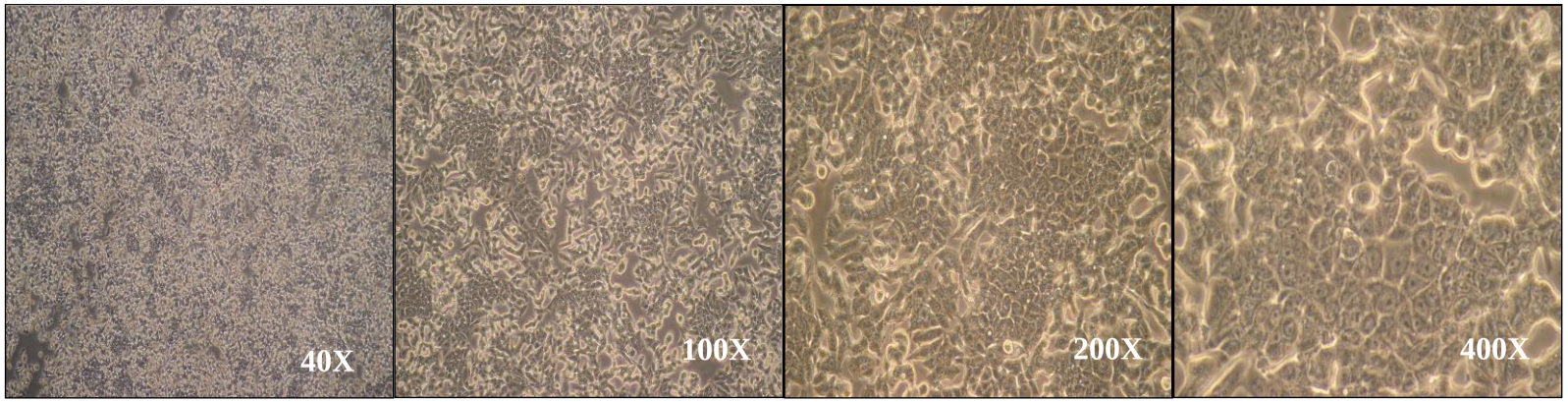

Figure B. 1 Microscopic images of **MCF7/S** (Magnifications: 40X, 100X, 200X and 400X from left to right)

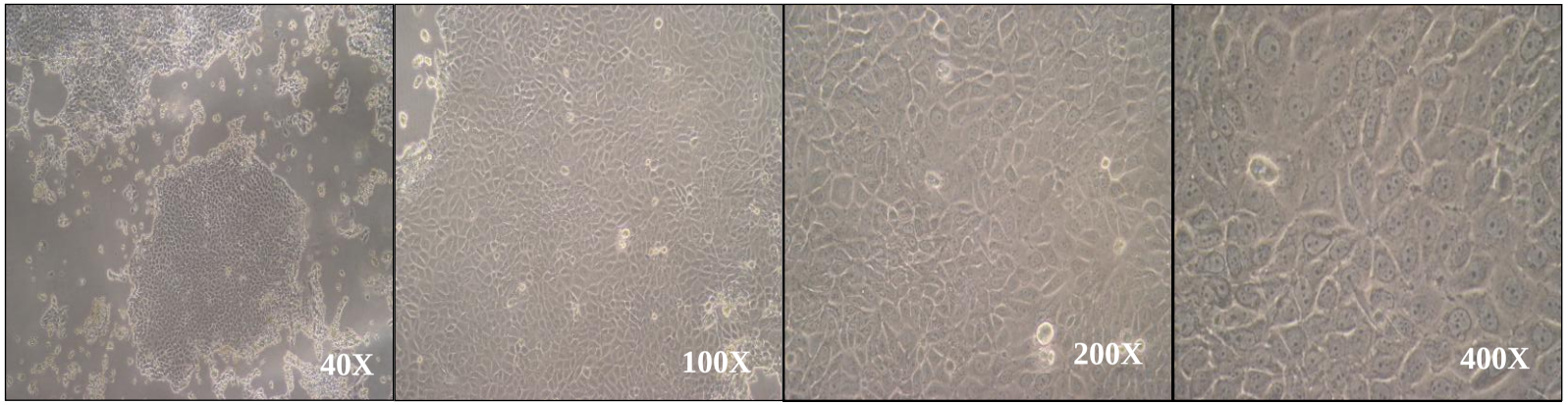

Figure B. 2 Microscopic images of **MCF7/1000E**

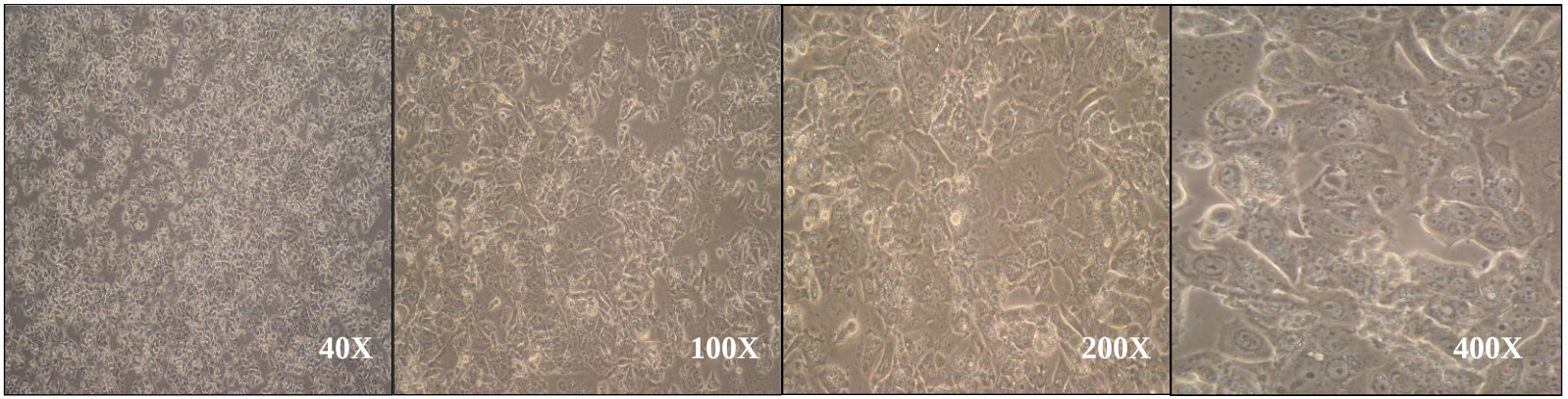

Figure B. 3 Microscopic images of **MCF7/1250E**.

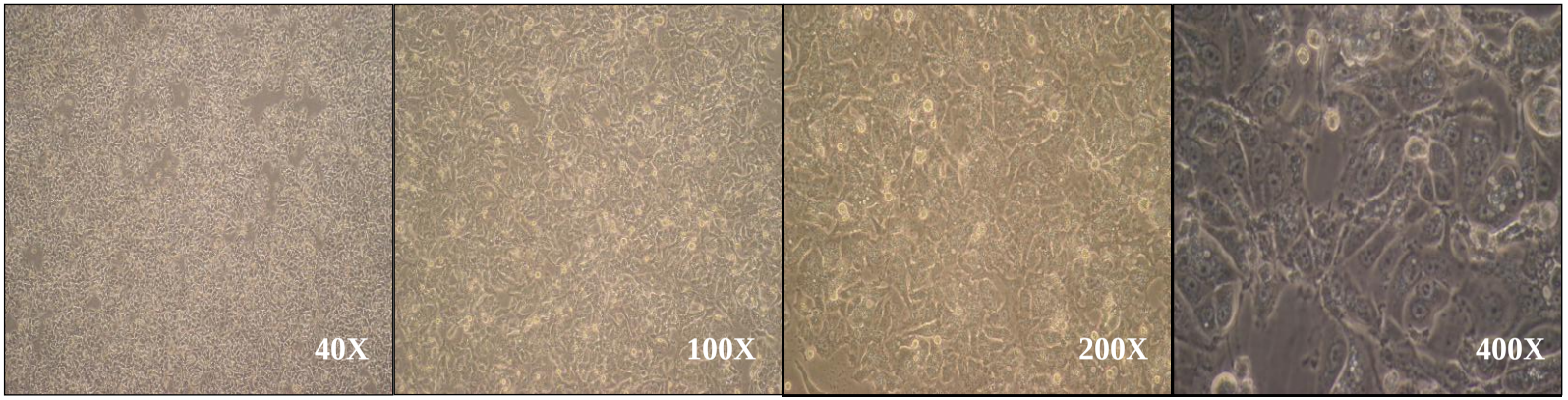

Figure B. 4 Microscopic images of **MCF7/2000E**

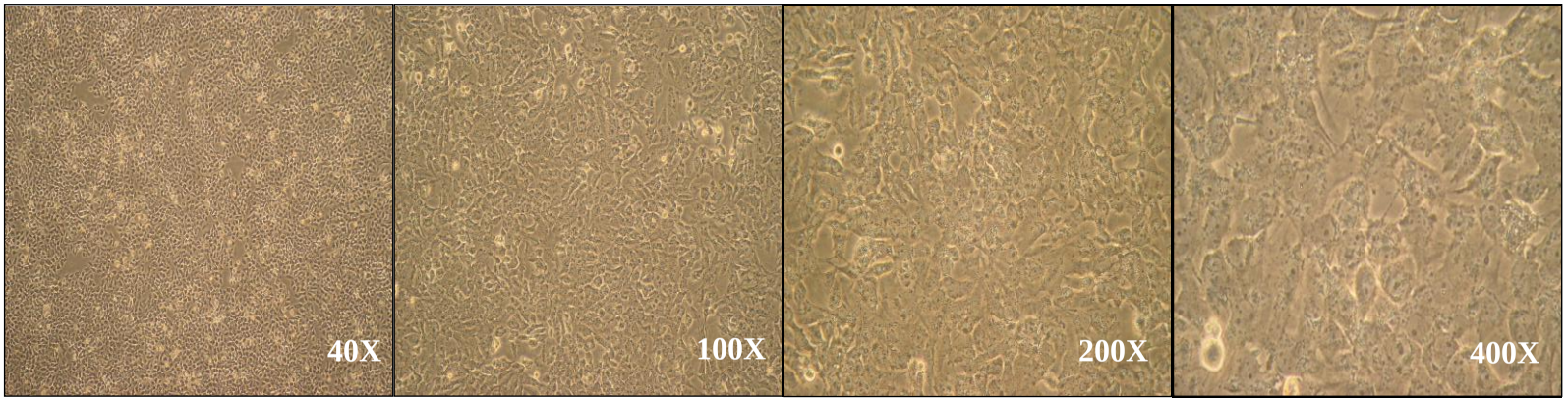

Figure B. 5 Microscopic images of **MCF7/600D**

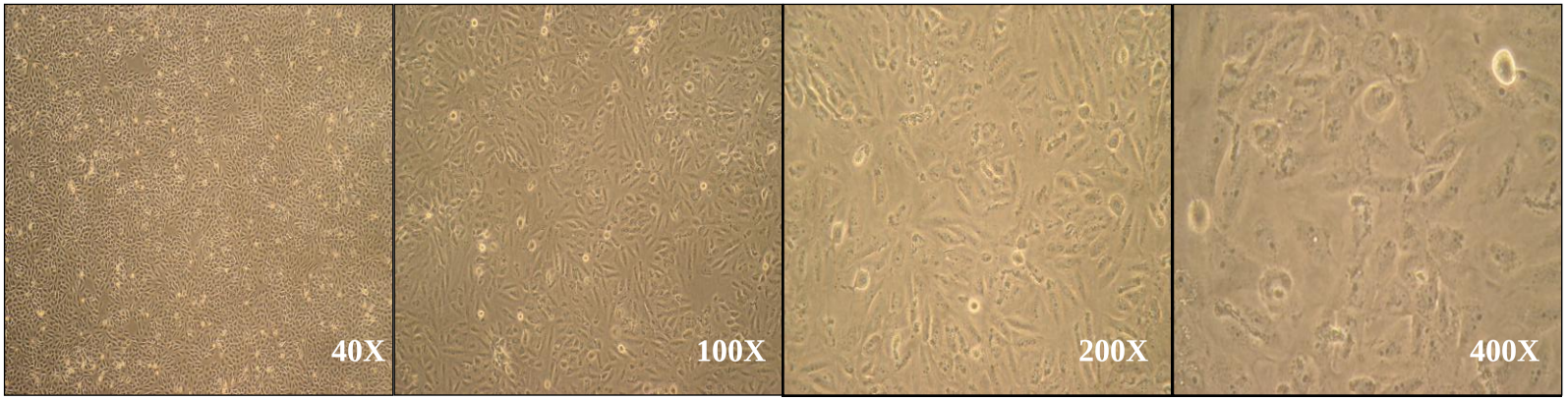

Figure B. 6 Microscopic images of **MCF7/1000D**

APPENDIX C

BUFFERS AND SOLUTIONS

- **Freezing Medium**

DMSO (Cell Culture Grade, 10%)	1 ml
FBS (Heat-inactivated, 90%)	9 ml

- **Diethylpyrocarbonate (DEPC) treated dH_2O (0.1% (v/v), 1 L)**

DEPC	1 ml
dH_2O	1 L

DEPC was mixed vigorously with dH_2O. After overnight incubation it was autoclaved at 121°C for 20 min.

- **Ethanol (75% (v/v), 100 ml)**

Ethanol (100% Molecular Biology Grade)	75 ml
DEPC-treated dH_2O	25 ml

- **50X Tris-Acetate-EDTA (TAE) Buffer (1 L)**

Tris base (MW: 121.14 g/mol)	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA disodium dehydrate (MW: 372.24 g/mol)	100 ml

Volume was completed to 1 L with dH_2O and pH was adjusted to 8.5. After autoclaving, solution was diluted to 1X with dH_2O to use in agarose gel preparation.

- **Ethidium bromide (EtBr) solution (10 mg/ml)**

EtBr	10 mg
dH_2O	1 ml

EtBr was dissolved in dH_2O and stored in dark at 4°C.

- **Agarose Gel (100 ml)**

	1%	**2%**
Agarose	1 g	2g
1X TAE Buffer	100 ml	100 ml

Agarose was mixed in 1X TAE buffer and boiled in microwave oven for 2 min. After cooling, 5-10 µl 10 mg/ml EtBr was added and homogenized. Solution was poured into gel tray with combs and cooled.

- **2X RNA Loading Dye (Fermentas)**

0.5 mM EDTA
95% formamide
0.025% SDS
0.025% bromophenol blue
0.025% xylene cyanol FF
0.025% ethidiumbromide

- **6X DNA loading dye (Fermentas)**

60 mM EDTA
10 mM Tris-HCl (pH 7.6)
0.03% xylene cyanol FF
0.03% bromophenol blue
60% glycerol

APPENDIX D

MRP1 EXPRESSION IN RESISTANT SUBLINES

In order to determine the expression levels of *MRP1* in parental MCF7/S cells and in etoposide and doxorubicin resistant sublines, RT-PCR was performed. cDNA synthesis was carried out as described in Section 2.2.3.4.2 by using specific primer (Table D.1). PCR was performed in duplicates for cDNAs obtained from MCF7/S, MCF7/200E, MCF7/500E, MCF7/750E, MCF7/1000E, MCF7/1250E, MCF7/2000E, MCF7/600D and MCF7/1000D. PCR conditions were demonstrated in Table D.2. PCR reaction was performed in 25 µl reaction mixture. 2% Agarose gel electrophoresis was carried out at 100V for 40 min (Figure D.1).

Table D. 1 Primer sequences of *MRP1*

Primer	Sequence (5'->3')	Location	Amplicon Size
***MRP1* Forward**	TGTGGGAAAACACATCTTTGA	Exon 18	80 bp
***MRP1* Reverse**	CTGTGCGTGACCAAGATCC	Exon 19	

Table D. 2 PCR Conditions for *MRP1*

$(NH_4)_2SO_4$ Buffer (10x)	2.5 µl
$MgCl_2$ (25 mM)	2 µl
dNTP (10 mM each)	0.5 µl
Primers (25 µM each)	0.3 µl
cDNA (reverse transcribed from 0.25 µg/µl RNA)	3 µl
Taq DNA Polymerase (5 u/µl)	0.2 µl
Initial Denaturation	94ºC, 5’
Denaturation	94ºC, 30’’
Annealing	62ºC, 30’’
Extension	72ºC, 30’’
Final extension	72ºC, 10’
Number of cycles	30

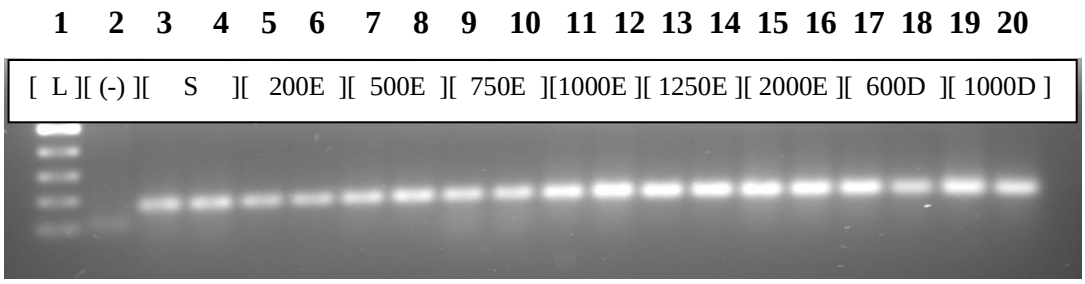

Figure D. 1 Expression levels of MRP1 in parental MCF7/S and etoposide and doxorubicin resistant sublines. Lane 1: Low range DNA ladder; lane2: negative control; lanes 3-4: *MRP1* in MCF7/S cell line, lanes 5-6: *MRP1* in MCF7/200E subline; lanes 7-8: *MRP1* in MCF7/500E subline; lanes 9-10: *MRP1* in MCF7/750E subline; lanes 11-12: *MRP1* in MCF7/1000E subline; lanes 13-14: *MRP1* in MCF7/1250E subline; lanes 15-16: *MRP1* in MCF7/2000E subline; lanes 17-18: *MRP1* in MCF7/600D subline; lanes 19-20: *MRP1* in MCF7/1000D subline.

APPENDIX E

SIGNIFICANCE OF GENE EXPRESSIONS IN DIFFERENT CELL LINES

In order to determine the significant differences in *TOP2A*, *MSH2* and *MLH1* gene expressions in different cell lines one way ANOVA test was used. For comparison of significance between groups, Tukey's Multiple Comparison Test was applied as a post-test following ANOVA. Significant differences in gene expressions were evaluated at $p<0.01$ level. Table E.1, table E.2 and table E.3 represent the significance of *TOP2A*, *MSH2* and *MLH1* genes expressions between indicated groups in the tables. In "Summary" part in the table, "ns" represents non-significant difference, and *, **, *** represent significant difference at $p<0.01$, $p<0.05$, and $p<0.001$ levels, respectively.

Table E. 1 Significance of different expression levels of *TOP2A* gene between different cell lines

Groups	**Significant? (P<0.01)**	**Summary**
MCF7/S vs MCF7/200E	No	ns
MCF7/S vs MCF7/500E	Yes	***
MCF7/S vs MCF7/750E	Yes	***
MCF7/S vs MCF7/1000E	Yes	***
MCF7/S vs MCF7/1250E	Yes	***
MCF7/S vs MCF7/2000E	Yes	***
MCF7/S vs MCF7/600D	Yes	***
MCF7/S vs MCF7/1000D	No	*
MCF7/200E vs MCF7/500E	Yes	***
MCF7/200E vs MCF7/750E	Yes	***
MCF7/200E vs MCF7/1000E	Yes	***
MCF7/200E vs MCF7/1250E	Yes	***
MCF7/200E vs MCF7/2000E	Yes	***
MCF7/200E vs MCF7/600D	Yes	***
MCF7/200E vs MCF7/1000D	No	ns
MCF7/500E vs MCF7/750E	No	ns
MCF7/500E vs MCF7/1000E	No	ns
MCF7/500E vs MCF7/1250E	No	ns
MCF7/500E vs MCF7/2000E	No	ns
MCF7/500E vs MCF7/600D	No	ns
MCF7/500E vs MCF7/1000D	Yes	***
MCF7/750E vs MCF7/1000E	No	ns
MCF7/750E vs MCF7/1250E	No	ns
MCF7/750E vs MCF7/2000E	No	ns
MCF7/750E vs MCF7/600D	No	ns
MCF7/750E vs MCF7/1000D	Yes	***
MCF7/1000E vs MCF7/1250E	Yes	***
MCF7/1000E vs MCF7/2000E	No	ns
MCF7/1000E vs MCF7/600D	No	ns
MCF7/1000E vs MCF7/1000D	Yes	***
MCF7/1250E vs MCF7/2000E	No	ns
MCF7/1250E vs MCF7/600D	No	ns
MCF7/1250E vs MCF7/1000D	No	ns
MCF7/2000E vs MCF7/600D	No	ns
MCF7/2000E vs MCF7/1000D	Yes	**
MCF7/600D vs MCF7/1000D	Yes	**

Table E. 2 Significance of different expression levels of *MSH2* gene between different cell lines

Groups	**Significant? (P<0.01)**	**Summary**
MCF7/S vs MCF7/200E	No	ns
MCF7/S vs MCF7/500E	No	ns
MCF7/S vs MCF7/750E	No	*
MCF7/S vs MCF7/1000E	Yes	***
MCF7/S vs MCF7/1250E	No	ns
MCF7/S vs MCF7/2000E	Yes	***
MCF7/S vs MCF7/600D	Yes	***
MCF7/S vs MCF7/1000D	Yes	***
MCF7/200E vs MCF7/500E	No	ns
MCF7/200E vs MCF7/750E	No	ns
MCF7/200E vs MCF7/1000E	Yes	***
MCF7/200E vs MCF7/1250E	No	*
MCF7/200E vs MCF7/2000E	Yes	**
MCF7/200E vs MCF7/600D	Yes	***
MCF7/200E vs MCF7/1000D	No	ns
MCF7/500E vs MCF7/750E	No	ns
MCF7/500E vs MCF7/1000E	Yes	***
MCF7/500E vs MCF7/1250E	Yes	**
MCF7/500E vs MCF7/2000E	No	*
MCF7/500E vs MCF7/600D	Yes	***
MCF7/500E vs MCF7/1000D	No	ns
MCF7/750E vs MCF7/1000E	Yes	***
MCF7/750E vs MCF7/1250E	Yes	***
MCF7/750E vs MCF7/2000E	No	ns
MCF7/750E vs MCF7/600D	Yes	***
MCF7/750E vs MCF7/1000D	No	ns
MCF7/1000E vs MCF7/1250E	Yes	***
MCF7/1000E vs MCF7/2000E	No	ns
MCF7/1000E vs MCF7/600D	Yes	***
MCF7/1000E vs MCF7/1000D	Yes	***
MCF7/1250E vs MCF7/2000E	Yes	***
MCF7/1250E vs MCF7/600D	No	ns
MCF7/1250E vs MCF7/1000D	Yes	***
MCF7/2000E vs MCF7/600D	Yes	***
MCF7/2000E vs MCF7/1000D	No	ns
MCF7/600D vs MCF7/1000D	Yes	***

Table E. 3 Significance of different expression levels of *MLH1* gene between different cell lines

Groups	**Significant? (P<0.01)**	**Summary**
MCF7/S vs MCF7/200E	Yes	***
MCF7/S vs MCF7/500E	Yes	***
MCF7/S vs MCF7/750E	Yes	***
MCF7/S vs MCF7/1000E	Yes	***
MCF7/S vs MCF7/1250E	Yes	***
MCF7/S vs MCF7/2000E	Yes	***
MCF7/S vs MCF7/600D	No	ns
MCF7/S vs MCF7/1000D	Yes	***
MCF7/200E vs MCF7/500E	No	ns
MCF7/200E vs MCF7/750E	No	ns
MCF7/200E vs MCF7/1000E	Yes	**
MCF7/200E vs MCF7/1250E	Yes	***
MCF7/200E vs MCF7/2000E	No	ns
MCF7/200E vs MCF7/600D	Yes	***
MCF7/200E vs MCF7/1000D	Yes	***
MCF7/500E vs MCF7/750E	No	ns
MCF7/500E vs MCF7/1000E	No	ns
MCF7/500E vs MCF7/1250E	Yes	***
MCF7/500E vs MCF7/2000E	No	*
MCF7/500E vs MCF7/600D	Yes	***
MCF7/500E vs MCF7/1000D	Yes	***
MCF7/750E vs MCF7/1000E	Yes	***
MCF7/750E vs MCF7/1250E	Yes	***
MCF7/750E vs MCF7/2000E	No	ns
MCF7/750E vs MCF7/600D	Yes	***
MCF7/750E vs MCF7/1000D	No	ns
MCF7/1000E vs MCF7/1250E	Yes	***
MCF7/1000E vs MCF7/2000E	Yes	***
MCF7/1000E vs MCF7/600D	Yes	***
MCF7/1000E vs MCF7/1000D	Yes	***
MCF7/1250E vs MCF7/2000E	No	*
MCF7/1250E vs MCF7/600D	Yes	***
MCF7/1250E vs MCF7/1000D	No	ns
MCF7/2000E vs MCF7/600D	Yes	***
MCF7/2000E vs MCF7/1000D	No	ns
MCF7/600D vs MCF7/1000D	Yes	***