REVERSAL OF PACLITAXEL RESISTANCE IN MCF-7 CELL LINE BY A CHEMICAL MODULATOR ELACRIDAR

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REVERSAL OF PACLITAXEL RESISTANCE IN MCF-7 CELL LINE BY A CHEMICAL MODULATOR ELACRIDAR

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ABSTRACT

REVERSAL OF PACLITAXEL RESISTANCE IN
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The phenomenon called multi drug resistance (MDR) is the resistance of cancer cells to anticancer drugs before or during chemotherapy. One of the mechanisms causing MDR is the upregulation of efflux pumps. The overexpression of MDR1 and MRP1 results in increased efflux of anticancer agents.

The aim of this study was to reverse MDR1-mediated paclitaxel resistance in MCF7 breast cancer cell line by a chemical MDR modulator elacridar. In this study, cytotoxicity and the reversal effect of elacridar on sensitive and paclitaxel resistant cells were investigated. The effect of elacridar on *MDR1* and *MRP1* gene expressions were also determined.

Results indicated *MDR1* gene was highly overexpressed (208 fold) in MCF7/Pac cells compared to MCF7/S cells. Elacridar was not found to be cytotoxic in MCF7/Pac cells up to 30μM. XTT results demonstrated 0.5μM elacridar

concentration was able to restore the antiproliferative effect of paclitaxel by 94% in MCF7/Pac cells. Complete MDR reversal was achieved at 5μM elacridar concentration. qPCR results revealed dose dependent upregulations in *MDR1* and *MRP1* gene expression levels after elacridar treatment which did not prevent reversal of MDR by elacridar.

Elacridar was shown to be very effective against paclitaxel resistance in MCF7/Pac cells at low concentrations. Therefore, it can be a suitable candidate for therapeutic applications in patients who developed paclitaxel resistance. Nevertheless, dose dependent upregulations in *MDR1* and *MRP1* gene expressions should be taken into consideration and overdose elacridar administration should be avoided.

Keywords: P-glycoprotein, paclitaxel, elacridar, MDR reversal, breast cancer

ÖZ

MCF-7 HÜCRE HATTINDA PAKLİTAKSEL DİRENCİNİN KİMYASAL MODÜLATÖR ELAKRİDAR İLE GERİ ÇEVİLMESİ

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Çoklu ilaç dirençliliği (MDR) kanser hücrelerinin antikanser ilaçlarına önceden dirençli olması veya kemoterapi sırasında direnç kazanmasıdır. MDR'a sebep olan mekanizmalardan biri atılım pompa genlerinin ifadesinin artışıdır. MDR1 ve MRP1 proteinlerinin aşırı ifadesi antikanser ajanlarının hücreden atılımını artırır.

Bu çalışmanın amacı MCF7 meme kanseri hücre hattında MDR-1'den kaynanlanan paklitaksel direncinin, kimyasal modülatör elakridar ile geri çevirilmesidir. Çalışmada, elakridarın sitotoksisitesi ve geri çevirim etkisi incelenmiştir. Ayrıca, elakridarın *MDR1* ve *MRP1* genlerinin ifadesine olan etkisi de belirlenmiştir.

Bu çalışmada, MCF7/Pac hücrelerindeki *MDR1* geninin MCF7/S hücrelerindekine oranla aşırı ifade edildiği (208 kat) bulunmuştur. XTT sonuçlarına göre, MCF7/Pac hücrelerinde elakridar 30μM'a kadar sitotoksik etki göstermemektedir. Sitotoksisite

analizleri 0,5µM elakridarın, MCF7/Pac hücrelerindeki paklitaksel direncini %94 oranında, 5µM elakridarın ise tamamen geri çevirdiğini göstermiştir. Kantitatif eş-zamanlı PZR sonuçlarına göre elakridar muamelesinden sonra *MDR1* ve *MRP1* gen ifadelerinde doza bağımlı biçimde artışlar meydana gelmiştir. Ancak bu artışlar elakridarın geri çevirim etkisini engellememiştir.

Düşük konsantrasyonlardaki elakridarın MCF7/Pac hücrelerindeki paklitaksel direncine karşı çok etkili olduğu gösterilmiştir. Bu yüzden elakridarın paklitaksele dirençli hastalarda tedavi amaçlı kullanılabileceği düşünülmektedir. Yine de, doza bağımlı *MDR1* ve *MRP1* gen ifadesi artışları göz önünde bulundurulmalı ve aşırı dozda elakridar uygulamalarından kaçınılmalıdır.

Anahtar kelimeler: P-glikoproteini, paklitaksel, elakridar, çoklu direnç geri çevirimi, meme kanseri

To my devoted family

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LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
ATP	Adenosine Triphosphate
BCRP	Breast Cancer Resistance Protein
bp	Base Pair
cDNA	Complementary Deoxyribonucleic Acid
CYP	Cytochrome P450
DEPC	Diethylpyrocarbonate
dH_2O	Distilled Water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxy Nucleotide Triphosphate
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
FR	Fold Reversal
hr	Hour
IC_{50}	Inhibitory Concentration 50
MCF7/Pac	Paclitaxel Resistant MCF-7 Cell Line
MCF7/S	Sensitive MCF-7 Cell Line
MDR	MDR Multidrug Resistance

mRNA	Messenger Ribonucleic Acid
MRP1	Multidrug Resistance Associated Protein1
M-MLV	Moloney Murine Lukemia Virus
NBD	Nucleotide Binding Domain
Pac	Paclitaxel
PBS	Phosphate Buffered Saline
P-gp/MDR1	P-glycoprotein/Multidrug Resistance Protein 1
qPCR	Quantitative Real-Time Polymerase Chain Reaction
RI	Resistance Index
RNA	Ribonucleic Acid
rpm	Revolution per Minute
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM	Standart Error of the Mean
TAE	Tris-acetate-EDTA
TMD	Transmembrane Domain
v/v	Volume per Volume
w/v	Weight per Volume

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a group of diseases characterized by uncontrolled growth and metastasis of abnormal cells to distant sites. It is the leading cause of death worldwide as stated by World Health Organization (World Health Organization, 2008). In 2008, 7.6 million of all deaths in the world were due to cancer and this number is expected to reach 13.1 million by the year 2030 (World Health Organization, 2008). In Turkey deaths caused by cancer has the second place after heart related diseases according to Turkish Ministry of Health reports (Turkish Ministry of Health, 2006).

The development of cancer starts with a single cell which transforms into a cancer cell (Reiger, 2004). Usually this transformation is a consequence of multiple factors that accumulate and disrupt cells normal behavior. These factors may be external such as UV, chemicals, radiation, infectious organisms, diet, tobacco or internal such as inherited mutations, mutations in the cell causing alterations in oncogenes and tumor suppressor genes, free radicals formed during metabolic processes and mutations in the genes that regulate cell proliferation and apoptosis (American Cancer Society, 2011).

1.2 Breast Cancer

Most frequently seen cancer type among women is breast cancer (World Health Organization, 2008). In 2011, approximately 231.000 new breast cancer cases and 40.000 deaths due to breast cancer occurred in United States only (American Cancer Society, 2011). Furthermore, according to Turkish Ministry of Health reports in 2006, breast cancer has the most incidence among Turkish women with 25% of all cancer cases (Turkish Ministry of Health, 2006).

Human breast consists of blood vessels, lymph vessels, connective tissue, lobes and ducts. Each lobe is formed from lobules which have bulbs that produce milk. Lobes, lobules and bulbs are connected to each other with ducts. The most common breast cancer type is ductal carcinoma which begins in duct cells (National Cancer Institute, 2011). Both lobular and ductal cancers are usually invasive.

There are numerous factors that cause breast cancer. Older ages, familial history of breast cancer, taking hormones or drugs like oral contraceptives, having menstruation at early ages, being Caucasian, alcohol consumption, giving birth at old ages are some of them (National Cancer Institute, 2011). In order to prevent breast cancer, these factors should be avoided and regular monitoring of the risk groups is crucial.

1.3 Breast Cancer Treatments

In order to treat breast cancer, a cancer specialist should decide on which treatment or treatments to use. The age of the patient, phase of the disease, size and location of the tumor, patient preference and other tumor characteristics are important in this selection. Mainly there are five main types of breast cancer treatments.

1.3.1 Surgery

Surgery can be performed on breast cancer patients in order to remove the tumor. Lumpectomy is a kind of surgery that conserves the breast. In this type, only tumor site is removed. Lumpectomy is usually followed by radiation in order to kill the possible remaining cancer cells to prevent reformation of the tumor. In more invasive cases mastectomy, removal of whole breast, is performed to prevent the spread of cancer cells further into the body (National Cancer Institute, 2011).

1.3.2 Radiotherapy

X-rays, gamma rays and charged particles are used in radiotherapy in order to cause DNA damage in cancer cells (Lawrence , Ten Haken, & Giaccia, 2008). When this treatment is used as a neoadjuvant therapy, radiation is applied on tumor cells before the surgery in order to shrink the tumor size. Radiotherapy can also be used after surgery (adjuvant therapy) in order to destroy any remaining cancer cells especially after lumpectomy.

1.3.3 Hormonal Therapy

In case of estrogen receptor positive breast cancers, estrogen promotes cancer cells growth. Thus the blockage of estrogen action or reducing estrogen amounts is crucial. Aromatase inhibitors, estrogen receptor modulators and estrogen receptor downregulators are used for hormonal therapy. For instance, hormone therapy with tamoxifen can block the effect of progesterone or estrogen. Pre-menopausal women can also be subjected to ovary removal in order to stop production of these hormones (Medline Plus, 2012).

1.3.4 Targeted Therapy

Targeted therapy is a relatively new therapy type used in breast cancer. In this type of therapy monoclonal antibodies, protein kinase inhibitors and repair enzyme inhibitors are used. Most commonly used drugs in targeted therapy are lapatinib, trastuzumab, pertuzumab and bevacizumab. Lapatinib blocks proteins that cause uncontrolled cell growth, bevacizumab acts by blocking angiogenesis, whereas trastuzumab and pertuzumab exert their effects on HER-2 positive breast cancers by blocking cell signaling pathways (Breastcancer.org, 2012).

1.3.5 Chemotherapy

Chemotherapy is the application of anticancer drugs to destroy cancer cells. This therapy can also be combined with local treatments like radiotherapy and/or surgery. Since anticancer drugs are given intravenously or orally, it is a systemic treatment. Anticancer drugs travel throughout the body to reach the cancer cells, making it possible to reach to metastasized tumors (American Cancer Society, 2011).

Alkylating agents, antimetabolites, anthracyclines, topoisomerase inhibitors, mitotic inhibitors are the five major types of anticancer drugs used in chemotherapy. Alkylating agents like cisplatin and carboplatin directly damage DNA and prevent cell proliferation. Antimetabolites are purine or pyrimidine mimicking agents that damage the cells in S phase by interfering DNA and RNA synthesis. Anthracyclines are very commonly used type of anticancer drugs that act on enzymes involved in DNA replication. For instance, the binding of doxorubicin to DNA inhibits DNA polymerase (Swift & Rephaeli, 2006). Topoisomerases separate the strands of DNA in order to enable DNA replication, repair or transcription. Therefore, topoisomerase inhibitors like etoposide and topotecan prevent the functioning of these enzymes in order to stop cell proliferation. Anticancer drugs that are derived

from natural products or plant alkaloids are mostly in the group of mitotic inhibitors. These drugs interfere with mitosis or inhibit synthesis of proteins needed in cell reproduction (American Cancer Society, 2011). Taxanes like docetaxel and paclitaxel stabilize the microtubule formation whereas vinca alkaloids like vinblastine and vincristine interfere with the assembly of them.

1.3.5.1 Paclitaxel (Taxol®)

Paclitaxel is a plant alkaloid isolated from the bark of the pacific yew tree *Taxus brevifolia*. It is used against breast cancer, ovarian cancer, AIDS-related Kaposi's sarcoma, non-small cell lung cancer, head and neck cancer (National Cancer Institute, 2012). Paclitaxel is also used with carboplatin to treat different types of cancer like ovarian cancer or non-small cell lung cancer (Meier, *et al*., 2012).

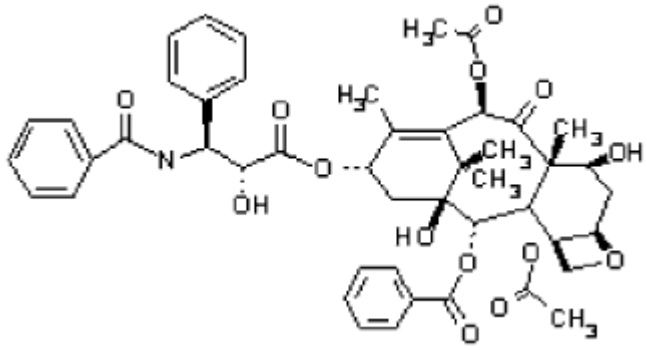


Figure 1.1 Chemical structure of Paclitaxel (Fitzpatrick & Wheeler, 2003)

Paclitaxel's mechanism of action is to stabilize microtubules and to block chromosome segregation (Manfredi & Horwitz, 1984). In order to achieve this, paclitaxel binds to β-tubulin subunits and stabilizes microtubule formation. Cells

are arrested at G2/M phase and eventually apoptosis occur. Cytotoxic effect of paclitaxel is not only limited to microtubule stabilization. Disruption of microtubules (Wang & Liu, 1999) and/or the binding of paclitaxel leads to Bcl-2 phosphorylation, inducing apoptosis (Ferlini & Cicchillitti, 2009). Paclitaxel also enhances apoptosis independent from p53. It is suspected that *Bax* gene may have a role in this regulation (Smoter & Bodnar, 2011).

Among ABC transporters, paclitaxel is a substrate of MDR1, MDR2 and bile salt export pump (BSEP) proteins (Gottesman, 2002). MDR1 protein is found in intestine, liver, kidney, placenta and blood-brain barrier whereas MDR2 and BSEP proteins are located in the liver. Since paclitaxel is a substrate of MDR related efflux pump MDR1, the upregulation of *MDR1* gene is usually associated with paclitaxel resistance (Kars, 2008). The two other important proteins which are related to multidrug resistance (MDR) are MRP1 and breast cancer resistance protein (BCRP). Therefore, the fact that paclitaxel is not a substrate of either MRP1 or BCRP is important to understand the mechanism of paclitaxel resistance.

1.4 Multidrug Resistance (MDR)

In chemotherapy, cancer cells may become resistant to a wide range of structurally and functionally unrelated drugs due to constant exposure of a single agent. This phenomenon is known as multidrug resistance (Biedler & Riehm, 1970). The resistance of the tumor cells to chemotherapeutic agents lowers the effectiveness of the anticancer drug. Following resistance development, patients need to take higher doses of the agent or they need to change the anticancer drug they are using to gain benefit. However, this is not always feasible due to increased adverse effects in higher doses of chemotherapeutic agents or due to cross resistance mechanisms.

Cells have detoxification systems in order to remove any toxic product produced during cell metabolism or to remove any toxic chemicals administered by an external source (such as drugs). However, cancer cells are able to use these mechanisms more extensively than normal cells, thus are able to evade toxic effects of anticancer drugs. These detoxification mechanisms form the basis of multidrug resistance (Liscovitch & Lavie, 2002).

There are many mechanisms that result in multidrug resistance such as decreased drug influx, increased drug efflux, reduced apoptosis, altered drug metabolisms or increased DNA repair mechanisms. Such diversity makes this phenomenon more complex and harder to overcome. A schematic representation of mechanisms involved in multidrug resistance is presented in Figure 1.2.

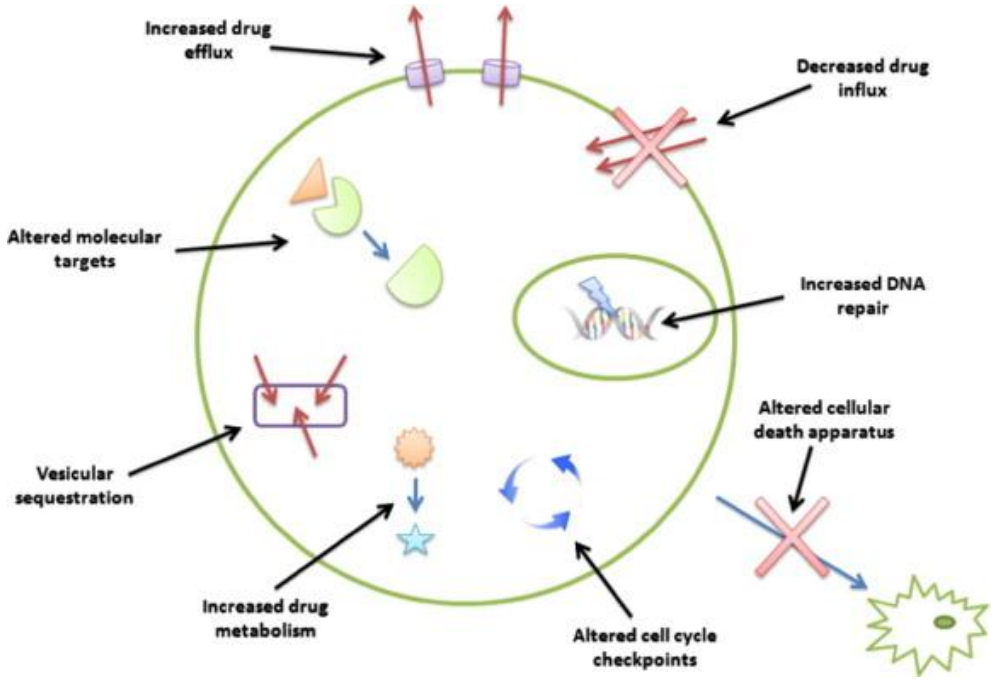


Figure 1.2 Mechanisms involved in multidrug resistance (Gong & Jaiswal, 2012).

1.4.1 Decreased Drug Influx

Cells have influx and efflux pumps on their membrane which form the basis of transport based multidrug resistance. Due to the chemical character of the lipid bilayer, hydrophilic drugs are taken up into the cells by influx pumps. Moreover some anticancer drugs enter the cell via binding to receptors or transporters. Downregulation of the genes encoding influx proteins or decreased activity of these proteins results in MDR (Gottesman, 2002).

1.4.2 Altered Drug Metabolism

Altered drug metabolism is another reason for MDR. This type of MDR is especially important in drugs administered in prodrug form. Prodrugs are metabolized into their active form inside the cells thus deficiency in prodrug converting enzymes prevents them from turning into their effective metabolite forms.

Cytochrome P450 superfamily members (CYPs) are the most important enzymes in this kind of MDR. These enzymes are involved in the metabolism of anticancer drugs. In this family, CYPs like CYP1A1 and CYP1A2 increase the sensitivity to anticancer agents (Rochat, 2005). This may be due to the pro-drug/drug conversion ability of CYPs. Therefore, downregulation of these proteins activity results in MDR. On the other hand overexpression of some CYPs like CYP3A and CYP1B1 cause MDR resistance. Rochat *et al.* claimed that some CYPs and efflux proteins work together causing MDR and such result may be due to the overexpression of CYPs that are involved in detoxification pathways (Rochat, 2005).

1.4.3 Alterations in DNA Repair Mechanisms

DNA is the site of action for many anticancer drugs such as anthracyclines, alkylating agents and platinum containing compounds. They disrupt DNA by forming DNA adducts and direct cells to apoptotic pathways. DNA repair mechanisms are naturally found in healthy cells in order to protect the cells from alterations that can be formed by free radicals, DNA breakages and mutations that can occur during replication. Due to prevention of apoptosis, the superior functioning of the DNA repair mechanisms is not desired in cancer cells (Lage & Dietel, 1999). Table 1.1 is presented for an overview of complex DNA repair mechanisms involved in cancer drug resitance (Table 1.1).

Table 1.1 DNA repair mechanisms and their proteins that take part in cancer drug resistance (Lage, 2008)

DNA repair mechanism	Corresponding DNA repair pathways	Participating proteins
Reversion repair	Single-step repair by MGMT	MGMT
	Repair by AlkB homologous	ABH1; ABH2; ABH3
Base excision repair (BER)	Short patch repair (SPR)	Glycosylases I, II; Polβ; XRCC1; PARP-1; Lig III
	Long patch repair (LPR)	Glycosylases I, II; Polβ; RF-C; FEN1; Pol δ; Pol ε; PCNA
Nucleotide excision repair (NER)	Global genomic repair (GGR)	DDB1; DDB2; RPA; HR23B; ERCC1; XPA; XPB (ERCC3); XPC; XPD (ERCC2); XPE; XPF; XPG; GTFH1; GTFH2; GTFH3; GTFH4; CDK7; CCNH; MNAT1; Pol δ; Pol ε; PCNA; Lig I
	Transcription-coupled repair (TCR)	GTFH1; GTFH2; GTFH3; GTFH4; CDK7; CCNH; MNAT1; XPB (ERCC3); XPD (ERCC2); FFIIS; CSA; CSB; XPF; XPG; Pol δ; Pol ε; Lig I
Mismatch repair (MMR)	Mismatch repair (MMR)	hMSH2; hMSH6; hMLH1; hPMS2; Pol δ; Exo I; Lig
Double-strand break repair (DSB)	Homologous recombination (HR)	MRE11; NBS1; Rad50; Rad51; Rad51B; Rad51C; RAd51D; Rad52; RPA; XRCC2; XRCC3
	Non-homologous end-joining (NHEJ)	Ku70; Ku80 (XRCC5); DNA-PKCs; FEN1; MRE11; NBS1; Rad50; XRCC4; XRCC7; Artemis; Lig IV

An example of a DNA repair protein that causes MDR is MGMT (O6-methylguanine-DNAmethyltransferase). This protein is able to recognize and repair DNA adducts formed by methylating agents. Thus, the overexpression of MGMT results in drug resistance (Hegi, *et al*., 2005).

Topping *et al.* stated the cells with defect DNA repair systems may develop drug resistance due to the nonrecognition of DNA damages caused by anticancer agents. Since these tumor cells can not notice the damaged DNA formation, they are not directed to apoptosis and cell proliferation continues (Topping, Wilkinson, & Scarpinato, 2009).

1.4.4 Increased Drug Efflux

MDR caused by drug efflux is mediated by proteins which belong to ATP-binding cassette (ABC) transporters superfamily. ABC transporters are transmembrane proteins which have hydrophilic nucleotide-binding domains (NBDs) and hydrophobic transmembrane domains (TMDs). These proteins need ATP to gain energy for the transport of their substrates (Higgins, 1992).

Normal functions of ABC transporters' are uptake, transportation and distribution of a wide range of substrates. They are important in detoxification processes, hence found in many tissues like kidney, liver, blood-brain barrier, intestine and placenta (Gottesman, 2002). Oral bioavailability of many anticancer drugs are also altered by ABC transporters (Wu, Calcagno, & Ambudkar, 2008). Since these transporters are able to transport many chemotherapeutic agents, tumors derived from these tissues are intrinsically resistant to these anticancer agents. Moreover, cancer cells without intrinsic MDR may also gain resistance during continuous chemotherapy (Ejendal & Hrycyna, 2002).

Up to now, 48 ABC transporter proteins have been discovered and especially three of them namely MDR1, MRP1 and BCRP play an important role in MDR development (Wu, *et al.*, 2008). Localization and the substrates of common ABC transporters are listed on Table 1.2.

Table 1.2 Localization and substrates of ABC transporters, adapted from Gottesman *et al.* (Gottesman, 2002)

Common Name	**Systematic Name**	**Tissue**	**Non-chemotherapy Substrates**	**Chemotherapy Substrates**
PGP, MDR1	ABCB1	Intestine, liver, kidney, placenta, blood-brain barrier	Natural and cationic organic compounds, many commonly used drugs	Doxorubicin, daunorubicin, vincristine, vinblastine, actinomycin-D, paclitaxel, docetaxel, etoposide, teniposide, bisantrene, homoharringtonine (STI-571)
MDR2	ABCB4	Liver	Phosphatidylcholine, some hydrophobic drugs	Paclitaxel, vinblastine
MRP1	ABCC1	All tissues	Glutathione and other conjugates, organic anions, leukotriene C4	Doxorubicin, epirubicin, etoposide, vincristine, methotrexate
MRP2, cMOAT	ABCC2	Liver, kidney, intestine	Similar to MRP1, non-bile salt organic anions	Methotrexate, etoposite, doxorubicin, cisplatin, vincristine, mitoxantrone

Table 1.2 (Continued)

MRP3	ABCC3	Pancreas, kidney, intestine, liver, adrenal glands	Glucuronate and glutathione conjugates, bile acids	Etoposide, teniposide, methotrexate, cisplatin, vincristine, doxorubicin
MRP4	ABCC4	Prostate, testis, ovary, intestine, pancreas, lung	Nucleotide analogues, organic anions	Methotrexate, thiopurines
MRP5	ABCC5	Most tissues	Nucleotide analogues, cyclic nucleotides, organic anions	6-Mercaptopurine 6-Thioguanine
MRP6	ABCC6	Liver, kidney	Anionic cyclic pentapeptide	Unknown
MXR, BCRP, ABC-P	ABCG2	Placenta, intestine, breast, liver	Prazosin	Doxorubicin, daunorubicin, mitoxantrone, topotecan, SN-38
BSEP, SPGP	ABCB11	Liver	Bile salts	Paclitaxel
ABCA2	ABCA2	Brain, monocytes	Steroid derivatives, lipids	Estramustine

1.4.4.1 P-glycoprotein (P-gp/MDR1)

ABCB1 also known as MDR1 or P-glycoprotein (shortly P-gp) is the first human ABC drug transporter identified. It transports various types of anticancer drugs such as taxenes, anthracyclines and vinca alkaloids (Wu *et al.*, 2008). A schematic representation of P-gp is given in Figure 1.3.

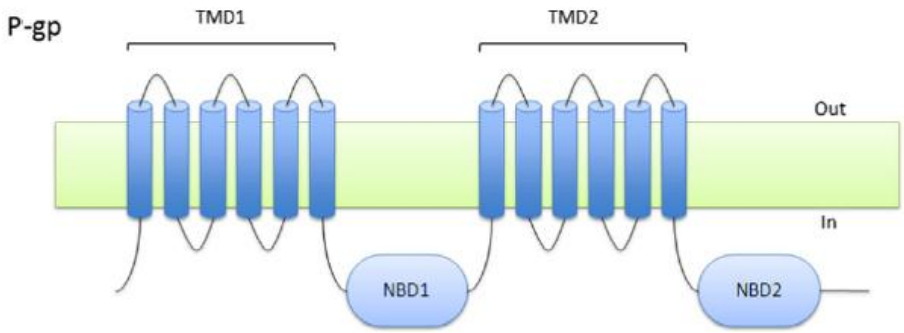


Figure 1.3 Structure of P-glycoprotein (Gong & Jaiswal, 2012).

P-gp is encoded by the *MDR1* gene which is located at 7q21. This protein has 170kDa molecular weight, 2 transmembrane domains (TMDs) each containing six helices and 2 nucleotide binding domains (NBDs) (Clarke & Loo, 1999). When a substrate binds to P-gp, ATP is hydrolyzed to obtain energy. After conformational changes, substrate is carried to a lower affinity binding site and released into the extracellular space. In order to return to the initial conformation of the protein, another ATP hydrolysis is needed at the second binding site (Leonard & Fojo, 2003).

P-glycoprotein is expressed in healthy cells of intestine, liver, kidney, placenta and blood-brain barrier (Gottesman, 2002). Although P-gp usually takes part in detoxification system of the cell, its' overexpression leads to MDR in many cancer types.

1.4.4.2 Multidrug Resistance Associated Protein (MRP1)

ABCC1 is the first MRP family member that was found to be associated with MDR (Cole, *et al*., 1992). ABCC1 is a 190-kDa protein with 3 transmembrane domains and 2 nucleotide binding domains. Although the structure of MRP1 is similar to

P-gp, MRP1 has one extra TMD which consists of 5 transmembrane segments. Another difference among MRP1 and MDR1 is related to substrate recognition. While P-gp recognizes mostly hydrophobic substrates, MRP1 recognizes hydrophilic substrates, organic anions as well as glutathione and its conjugates (Gottesman, 2002).

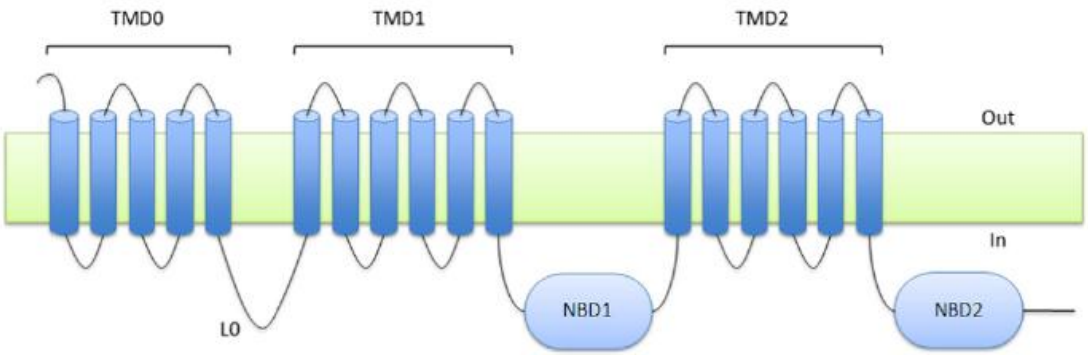


Figure 1.4 Structure of MRP1. Transmembrane domain (TMD), nucleotide binding domain (NBD), L0 linker region (Gong & Jaiswal, 2012)

MRP1 is encoded by *MRP1* gene located on 16p13 and primarily expressed in plasma membrane (Zaman, Flens, & van Leusden, 1994). Overexpression of MRP1 is associated with MDR in breast cancer, non-small cell lung carcinoma and prostate cancer (Gong & Jaiswal, 2012). Some of the anticancer agents related to MRP1 mediated drug resistance are doxorubicin, epirubicin, etoposide, vincristine and methotrexate (Gottesman, 2002).

1.5 Reversal of ABC Transporter Mediated MDR in Cancer Cells

As previously stated, MDR development can be caused by various types of changes in cell mechanism. In cases of breast cancer and paclitaxel resistance, P-gp overexpression is the main reason of multidrug resistance (Gottesman *et al.,* 2002; Kars, 2008). Therefore in this section, reversal strategies on ABC transporter mediated MDR will be further discussed.

Approaches for reversing ABC transporter mediated MDR can be classified into two sections. While one group of approach acts on the expression of ABC transporters, the other group controls the function of these proteins (Borowski *et al.*, 2005).

1.5.1 Targeting Expression of ABC Transporters

In order to target mRNA of ABC transporters; antisense oligonucleotides, hammerhead ribozymes and RNA interference strategies have been developed. Ribozymes are catalytic RNAs which have intrinsic endoribonucleolytic cleavage activity. This property can be used to target ABC transporters for specific cleavage. Indeed, Kowalski *et al.* successfully designed ribozymes that target ABCB1, ABCC1 and ABCG2 (Kowalski, *et al*., 2005). These kinds of ribozymes were able to cleave ABC transporter specific transcripts in drug resistant cancer lines.

Small interfering RNAs (siRNAs) are also used to target ABC transporters mRNAs. Since low concentrations of siRNA is very effective for gene silencing (Dönmez, *et al.*, 2011), this strategy is advantageous over antisense oligonucleotides. However, transient effect of siRNAs is a limiting factor that should be kept in mind. Another way to circumvent MDR is the use of repressors

against ABC transporters' promoter regions. For instance Jin *et al.* showed a natural marine product Et743 is able to inhibit MDR1 transcription via blocking its promoter activation (Jin, *et al*., 2009).

1.5.2 Modulating MDR Transporters Functions

The agents that directly act on or block ABC transporters activity are called MDR modulators. These modulators are used with anticancer drugs in order to reverse MDR. To this date many chemosensitizers have been developed and they are grouped in four generations. Some of the P-gp modulators and the generations they belong to are listed in Table 1.3. Moreover, some of the most commonly used MDR reversal agents and their targets are presented in Table 1.4.

Table 1.3 Summary of MDR modulators that inhibit P-gp (adapted from Morjani & Madoulet, 2010)

Generation	P-gp Inhibitor
First	Amiodarone, Cyclosporin A (CSA), Quinidine, Quinine, Verapamil, Nifedipine, Dexniguldipine
Second	PSC833 (Valspodar) VX-710 (Biricodar)
Third	GG918 (Elacridar), LY475776, LY335979 (Zosuquidar), XR-9576 (Tariquidar), V-104, R101933 (Laniquidar), S9788
Fourth	Curcumin Flavonoids

Table 1.4 Commonly used MDR reversal agents and their targets (adapted from Morjani & Madoulet, 2010)

Compound	P-gp	MRP1	BCRP
Verapamil	+	-	-
Quinine, Quinidine	+	-	-
Cyclosporin A (CSA)	+	-	-
PSC833 (Valspodar)	+	-	-
Biricodar (VX-710)	+	+	-
Elacridar (GG918)	+	-	+
Zosuquidar (LY335979)	+	-	-
Tariquidar (XR-9576)	+	-	-
S9788	+	-	-

1.5.2.1 First Generation Inhibitors

First generation inhibitors were substrates of ABC transporters. Their mechanism of action usually comprised of competing with anticancer drugs for ABC transporter efflux. The first MDR modulator discovered was verapamil (Tsuruo *et al.,* 1981). Verapamil was a calcium channel blocker which is able to increase anticancer drug accumulation inside the cells. However, it was not a very potent P-gp inhibitor. Another important first generation MDR modulator was cyclosporine A. This reversing agent was very successful in *in vitro* trials with complete MDR restoration. Although the effectiveness of first generation inhibitors were exciting, the need for high dose administrations caused serious side effects in clinical trials (Tan *et al.,* 2000).

1.5.2.2 Second Generation Inhibitors

After the failure of verapamil and cyclosporine A, second generation inhibitors were developed. Generally, these drugs were the modified forms of first generation inhibitors. Among them valspodar was the most successful one according to *in vitro* and *in vivo* trials. This MDR modulator was found to be 10 to 20 fold more potent than cyclosporine A (Twentyman & Bieehen, 1991). Although valspodar was less toxic than its ancestors, its pharmacokinetic interactions impaired drug metabolism and elimination (Bates & Kang *et. al*, 2001). This was a common problem of second generation inhibitors since many of them were substrates of cytochrome P450-3A isoform. The metabolization of the reversing agents by this protein resulted in unpredictable pharmacogenetic interactions (Özben, 2006).

1.5.2.3 Third Generation Inhibitors

Third generation MDR reversal agents were developed by combinatorial chemistry. This group of modulators differed from previous generations in their high potency and selectivity. Most of these drugs were highly effective in nanomolar concentrations and they were not affected by pharmacokinetic interactions since they were not metabolized by cytochrome P450 (Lee, 2010). Due to the fact that low concentrations of these modulators were adequate for complete MDR reversal, they were less likely to cause toxicity in clinical trials.

LY335979 (Zosoquidar) was a selective MDR modulator that acts on P-glycoprotein but not on MRP or BCRP (Lee, 2004). Currently LY335979 is on Phase III trial stage, after being successful in preclinical studies with mice and in clinical studies with non-Hodgkin's lymphoma patients (Morschhauser & Zinzani, 2007). Moreover Gerrard *et al.* stated that 75% response rate was observed in patients with acute myeloid leukemia (Gerrard, 2004).

XR9576 also known as Tariquidar was another potent MDR reversal agent. This chemosensitizer fully restored anti-tumor activity of many anticancer drugs in mice with resistant tumors (Abraham & Edgerly, 2001). Phase I trials were also promising. XR9576 did not show any unwanted pharmacokinetic effect on patients when co-administered with paclitaxel and doxorubicin (Steward & Steiner, 2000). Despite of these successful trials, tariquidar studies in Phase II and Phase III were terminated due to severe toxicity seen in patients (Nobili & Landini, 2006). Some of the third generation MDR modulators and their current stages in clinical trials are given in Table 1.5.

Table 1.5 Third generation MDR modulators, their targets and current stages in clinical trials (Lee, 2010).

MDR Modulator	Targeted ABC Transporter(s)	Current Stage of Studies
CBT-1	Pgp	III
Tesmilifene	?	III
MS209 (Dofequidar)	Pgp, MRP1	III
PSC833 (Valspodar)	Pgp	III
ONT-093	Pgp	II
Annamycin	?	II
Mitotane	Pgp	II
R101933 (Laniquidar)	Pgp	II
VX710 (Biricodar)	Pgp, MRP1	II
LY335979 (Zosuquidar)	Pgp	I, II
XR9576 (Tariquidar)	Pgp, MRP1	I, II
GF120918 (Elacridar)	Pgp, BCRP	I
Sulindac	MRP1	I
S9788	Pgp	I

1.5.2.3.1 GF120918 (Elacridar)

Another member of third generation inhibitors was GF120918 also known as GG918 or Elacridar. Like the other members of the group, elacridar was a very

potent MDR modulator that exerted its MDR reversal effect in nanomolar concentrations. However, there were evidence showing in some cases macromolar concentrations of this reversal agent might be needed for complete MDR reversal. De Bruin *et al.* suggested the need for high levels of elacridar may be related to very high levels of transporters in some resistant cell lines (de Bruin, 1999).

Different from XR9576 and LY335979, this reversal agent could reverse multidrug resistance mediated by MDR1 and also mediated by BCRP. Therefore, elacridar had a broader range of effectiveness than many other third generation inhibitors (Maliepaard, *et al*., 2001). On the other hand, this MDR modulator was not effective against MRP1 mediated MDR (de Bruin, 1999). Another important property of elacridar as stated by Hyafil *et al.* was that this chemosensitizer was not a substrate of P-gp thus it was not pumped out from the cells by this protein (Hyafil, 1993).

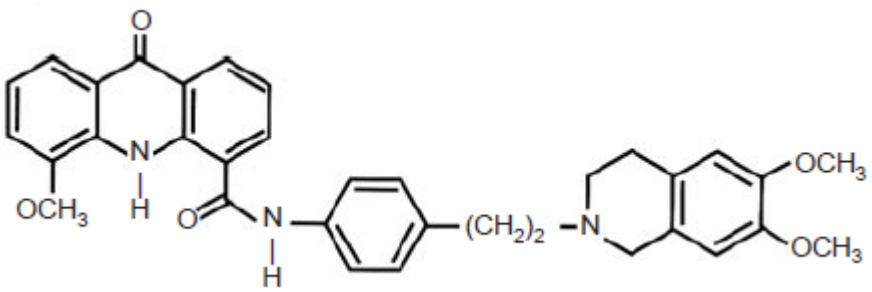


Figure 1.5 Chemical structure of Elacridar (Wallstab, 1999)

Elacridar's mechanism of action on P-glycoprotein is proposed to be in a non-competitive manner (de Bruin *et al.*, 1999). A study by Martin *et al.* indicated

that elacridar binds to a modulatory site on P-gp which anticancer drugs can not interact with (Martin *et al.*, 2000).

Currently elacridar is in Phase I trial. According to many studies, GF120918 did not make undesired pharmacokinetic interactions with anticancer drugs (Lee, 2010). In phase I studies, both oral and intravenous injections of elacridar were successfully administered to patients showing its convenient usage in clinical areas. Many of the studies involving elacridar in clinical trials, aimed increasing the oral bioavailability of anticancer drugs. As in the case of paclitaxel, high affinity of anticancer drugs to P-gp lowers their bioavalibility. However in combination with elacridar, P-gp pumps in intestinal track were blocked which significantly increased the effect of paclitaxel and its rate of absorbtion in intestines (Malingre, *et al*., 2001).

GF120918's effect on other tissues and organs which have high P-gp content, like blood-brain barrier, were also investigated. In a study by Hubesack *et al.* tariquidar and elacridar were shown to be very effective in modulating blood-brain barrier in nude mice models (Hubesack, 2008). Recent studies that involve elacridar and encapsulation strategies were also reported. For instance, by cytotoxicity analyses Wong *et al.* evidenced that the encapsulation of elacridar and doxorubicin into one polymer-lipid hybrid nanoparticle was more efficient than administering them separately (Wong, 2006).

1.5.2.4 Fourth Generation Inhibitors

Fourth generation inhibitors were originated from natural products. Studies reported that fruits like orange, grapefruit and strawberry were able to inhibit P-gp function (Deferme, 2002). Moreover, natural products like curcumin and flavonoids were also shown to be effective against MDR (Limtrakul, 2005). Among the fourth generation inhibitors, possibly the most studied compound was curcumin. Limtrakul

et al. reported curcumin and its derivatives could inhibit MDR1, MRP1 and BCRP (Limtrakul, 2007). However when administered orally, curcumin's low bioavailability was a limiting factor.

Although the toxicities of this group of inhibitors are low, their low potencies for MDR reversal makes them less likely to be candidates for clinical trials (Coley, 2010).

1.6 Aim of the Study

The aim of this study is to reverse P-gp mediated multidrug resistance in paclitaxel resistant cells by using MDR modulator elacridar in MCF7 subline. The treatment by elacridar can re-sensitize paclitaxel resistant cells to anticancer drug paclitaxel. The gene expression of efflux proteins MDR1 and MRP1 were determined in sensitive and paclitaxel resistant cells at mRNA level, in order to better understand the reversal mechanism of elacridar.

The objectives of this study are:

- Determination of inhibitory concentration 50 (IC_{50}) of paclitaxel for MCF7 parental cell line and MCF7/Pac paclitaxel resistant cell line.
- Investigation of elacridar toxicity on MCF7/S and MCF7/Pac cell lines.
- Determination of IC_{50} of paclitaxel for MCF7/Pac cell lines which are treated with various concentrations of elacridar and paclitaxel combinations.
- Evaluation of MDR reversal effect of elacridar.
- Determination of expression levels of *MDR1* and *MRP1* in parental and paclitaxel resistant MCF7 cell lines before and after elacridar treatment.

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. Paclitaxel resistant MCF7/Pac cell line was developed previously in our laboratory, from the parental cell line (MCF7/S) by stepwise selection of the cells in increasing drug concentrations. In this study the most resistant subline developed, MCF7/Pac400 cell line, was used which is resistant to 400nM of paclitaxel (Kars *et al.*, 2006).

2.1.2 Chemicals, Disposables, Kits and Reagents

Paclitaxel was purchased from Sigma-Aldrich, USA. 30 mM stock solution was prepared with dimethyl sulfoxide (DMSO) and stored at 4°C. Elacridar was obtained from Santa Cruz Biotechnology, USA. Stock solution of 2.8 mM elacridar was prepared with DMSO and stored at -20 °C. Both of the solutions were further diluted with DMSO just before cytotoxicity experiments.

RPMI 1640 medium and heat inactivated fetal bovine serum (FBS) were obtained from Biochrom AG, Germany. Gentamycin, tryripsin-EDTA, tryphan blue and XTT Cell Proliferation Kit were purchased from Biological Industries, Israel. Diethyl pyrocarbonate (DEPC), agarose, isopropanol and ethanol were acquired from Applichem, Germany. Phospate buffered saline (PBS), DMSO and TRIzol® were purchased from Sigma-Aldrich, USA. Nuclease-free water, Taq DNA polymerase, dNTP mix, $MgCl_2$, Moloney-Murine Leukemia Virus reverse transcriptase, RiboRuler High Range RNA ladder, 2X RNA loading dye, GeneRuler 50bp DNA ladder and 6X DNA loading dye were obtained from Fermantas, Lithuania. Disposable materials were acquired from Greiner Bio-One, Germany. FastStart Universal SYBR Green Master Kit (ROX) was purchased from Roche Diagnostics, Switzerland.

2.1.3 Primers

MDR1, MRP1 and β-actin primers were obtained from Alpha DNA, Canada. Primer sequences, locations and the amplicon sizes are given in Table 2.1.

Table 2.1 Primers used in qPCR

Primer	Sequence	Location	Amplicon Size
MDR1 Sense	5'ACAGAAAGCGAAGCAGTGGT3'	Exon 15	62 bp
MDR1 Antisense	5'ATGGTGGTCCGACCTTTTC3'	Exon 16	
MRP1 Sense	5'TGTGGGAAAACACATCTTTGA3'	Exon 18	80 bp
MRP1 Antisense	5'CTGTGCGTGACCAAGATCC3'	Exon 19	
β-actin Sense	5'CCAACCGCGAGAAGATGA3'	Exon 3	97 bp
β-actin Antisense	5'CCAGAGGCGTACAGGGATAG3'	Exon 4	

2.2 METHODS

2.2.1 Cell Culture

2.2.1.1 Cell Culturing Conditions

MCF7 parental cells (MCF7/S) and MCF7 paclitaxel resistant cells (MCF7/Pac) were both maintained in 15mL RPMI 1640 medium (Appendix A) supplemented with 10% (v/v) fetal bovine serum and 1% (w/v) gentamycin in T75 filter capped tissue culture flasks (Thermo Fisher Scientific, USA) that are surface treated in order to help cell attachment. Cells were incubated at 37°C in a humidified atmosphere with 5% (v/v) CO_2 in a Heraeus incubator (Hanau, Germany). All cell culture experiments were performed in Bioair Aura 2000 M.A.C Class II Safety Cabinet (Bioair Instruments, Italy).

2.2.1.2 Passaging

In cell culture, subculturing (passaging) is essential when the cells reach to %80 confluency in order to keep the cells healthy (Freshney, 2010). Having confluency more than %80 leads to slower cell growth and eventually to cell death. Passaging cells in adherent cell culture involves releasing the cells from the flask surface and transferring them into a new flask thus lowering the cellular density. When the cells reached %80 confluency, passaging was performed. Briefly, medium was discarded then in order to remove dead cell remainings and waste materials cells were washed with 5mL PBS (Appendix B). 2mL trypsin-EDTA was added and cells were incubated at 37°C for 5 minutes in order to activate trypsin. When cells were detached 4mL FBS-containing medium was added in order to inactivate trypsin. After suspension, cells were distributed into other flasks. In order to maintain the

resistance of MCF7/Pac cell line, after each medium change or passaging, final concentration of 400nM paclitaxel was added.

2.2.1.3 Freezing Cells

After cells reached 80-90% confluency, they were trypsinized as described. After 4mL of FBS containing medium addition and suspension steps, cells were taken into 15mL falcon tubes (Greiner Bio-One, Germany). Cell suspension was centrifuged at 1000 rpm for 5 min at 4°C. Supernatant was discarded and the cells were resuspended in 5mL PBS. Cells were subjected to centrifugation at 1000 rpm for 5 min 4°C once more and the supernatant was discarded. Pellet was resuspended in 1mL freezing medium (Appendix B) and transferred into cryovials (Greiner Bio-One, Germany). The cryovials were kept at -20°C for 2 hours followed by overnight incubation at -80°C. Lastly, vials were transferred into liquid nitrogen for long time storage.

2.2.1.4 Thawing Cells

Cryovial was taken from liquid nitrogen and incubated at 37°C. As soon as frozen cells melted, they were taken into 15mL falcon tubes. Since DMSO is toxic to cells in temperatures above 4°C, it should be removed immediately. For this reason, cells were suspended in 4ml medium and centrifuged at 1000 rpm for 5 min at 4°C. Supernatant was discarded and cells were suspended in 2ml medium containing FBS. The suspension was taken into T75 flasks (Thermo Fisher Scientific, USA) and the volume was completed to 15mL.

2.2.1.5 Cell Viability Assessment by Trypan Blue Exclusion Method

Trypan Blue is a dye used in the determination of cell viability. Since living cells have intact cell membranes they prevent the entry of the dye into the cell (Freshney, 2010). Cells which are blue are considered to be dead, whereas colorless cells are viable.

In order to count the viable cells, cells were trypsinized as described previously. After the centrifugation of PBS-cell suspension, the supernatant was discarded. Pellet was dissolved in 2mL medium and mixed throughly. 180µL cell suspension was taken into an 1.5mL Eppendorf tube and mixed throughly with 20µL trypan blue solution to have a ratio of 9:1. 10µL of the suspension was taken onto Neubauer hematocytometer (Bright-line, Hausser Scientific, USA) and cells were counted under phase contrast microscopy (Olympus, USA).

The hematocytometer used was composed of 16 large squares, each having 16 smaller squares within. Each small square volume was 0.00025 mm^3. 16 x 16 squares were counted three times and the average value was calculated. The cell number per mL was calculated according to formula below (Equation 2.1):

$$\text{Cell number/mL} = \text{Average cell count per square} \times \text{Dilution factor} \times 4 \times 10^6 \qquad (2.1)$$

2.2.2 Determination of Antiproliferative Effects by XTT Cell Proliferation Assay

2.2.2.1 Determination of Antiproliferative Effect of Paclitaxel

In order to determine antiproliferative effect of Paclitaxel, XTT cell proliferation assay was carried out by using XTT Cell Proliferation Kit (Biological Industries, Israel). XTT reagent is a tetrazolium salt that is taken up into the living cells where it gets reduced by mitochondrial enzymes to yield orange colored formazan compounds. Since mitochondrial enzymes get inactivated shortly after cell death, only living cells can make this reduction (Scudiero, *et al*., 1988). The formazan compounds formed are water soluble and their intensities can be measured by spectrophotometer. The absorbance of the dye is proportional to number of the metabolically active cells.

Briefly, MCF7/S cells were trypsinized and counted as described previously. 5000 cells per well were seeded into 96-well plates starting from the second column. After overnight incubation, medium was discarded to remove unattached cells. To the first and second columns 150 µL medium was added (medium control column and cell control column respectively). 50 µL of medium was added to wells from 4 to 12. First and last rows starting from third column were left as DMSO control. To these columns 200 µL of DMSO and medium solution was added. The volume of DMSO in this solution was the same volume of DMSO in diluted paclitaxel and DMSO solution used as the highest drug concentration. For the highest drug concentration preparation, paclitaxel was taken from the stock solution and diluted with medium. To the third columns' wells (highest drug concentration column) from 2 to 7, 200 µL paclitaxel was added in final concentration of 12 µM. In order to make serial dilution, 150 µL of the solutions in the third row was taken and transferred into the next column sequentially. Finally, all rows were completed to 150 µL by 100 µL of medium addition. Following the cell seeding procedure, cells were incubated at 37°C for 72 hours. XTT reagent and activator reagent was thawed

and mixed just before addition to the 96-well plate according to the manufacturer's instructions. 75 µL of XTT reagent and activator mixture was added to the wells and the cells were incubated at 37°C for 4 hr. The absorbances were measured at 492 nm with Anthos2010 96-well plate reader (Biochrom, Germany).

In order to determine antiproliferative effect of paclitaxel on MCF7/Pac cells the same procedure was followed. However as the highest drug concentration, 100 µM paclitaxel was tested.

Inhibitory concentration 50 (IC_{50}) is the concentration of a drug which inhibits 50% of a specific biological activity. In order to calculate IC_{50} values, the data obtained from spectrophotometric measurements were copied into Microsoft Excel. Percent cell proliferation versus drug concentration curves were plotted and the IC_{50} values were calculated from the formula of the logarithmic trendline of the graphs. The medium control columns' data was subtracted from other columns values to omit medium absorbance. Cell control column was taken as 100% cell proliferation and the data was adjusted accordingly.

The ratio of IC_{50} value of a resistant cell line to IC_{50} value of a sensitive is known as resistance index (RI) and calculated as in the formula below (Equation 2.2):

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

2.2.2.2 Determination of Antiproliferative Effect of Elacridar

The antiproliferative effect of elacridar was determined by XTT cell proliferation assay, and IC_{50} values were calculated as described previously. However, instead of

paclitaxel, 100μM elacridar was used as the highest drug concentration for both MCF7/S and MCF7/Pac cells.

2.2.2.3 Determination of the Reversal Effect of Elacridar

In order to determine reversal effect of elacridar, cells were treated with paclitaxel and elacridar combinations. Firstly, cells were seeded and incubated into 96-well plates, DMSO control rows were prepared in the previously described manner. The highest paclitaxel concentration for MCF7/Pac cells was 37.5 μM. Elacridar was taken from the stock solution and diluted with medium. After the addition of paclitaxel and serial dilution steps, either 0.5, 1, 2.5 or 5μM elacridar was added to the wells which contained paclitaxel. Since elacridar was dissolved in DMSO, the volume of DMSO in elacridar solution was added onto DMSO control columns. IC_{50} values were calculated.

In order to define reversal effect, the term fold reversal (FR) was used. FR is the ratio of IC_{50} of the resistant cell line to IC_{50} of the resistant cell line after a certain kind of treatment (Wu *et. al*, 2003). In this case, the treatment was the addition of elacridar on MCF7/Pac cells. The equation for determining fold reversal was as follows (Equation 2.3) :

Fold reversal= IC_{50} of the resistant cells/IC_{50} of the elacridar treated resistant cells (2.3)

2.2.2.4 Statistical Analysis

Statistical analyses were carried out by using GraphPad Prism 5.0 Software (GraphPad Software Inc., USA). All cytotoxicity experiments were performed in triplicates. SEM values were derived from the data and the data was subjected to

one-way ANOVA test to assess the degree of significance. Different groups were compared with Tukey's Multiple Comparison Test. Results were considered as significant when p value was smaller than 0.05 ($p<0.05$).

2.2.3 Gene Expression Analyses

2.2.3.1 Total RNA Isolation

All equipment used in RNA isolation were DEPC treated (Appendix B) in order to inactivate RNases and to prevent RNA degradation. Prior to RNA isolation, all of the plastic and glassware were treated with 0.1% DEPC treated dH_2O. Afterwards, materials were left under hood overnight for evaporation of residual DEPC. Following day, equipment was autoclaved at 121°C for 20 min. For preparation of DEPC water, 50µL DEPC was added to 50mL dH_20 and the mixture was shaken vigorously. Solution was evaporated overnight and autoclaved at 121°C for 20 min. All RNA isolation steps were carried on ice unless stated otherwise. Centrifugation steps were performed at 4°C.

Total RNA isolation was performed by using TRIzol® reagent (Sigma-Aldrich, USA) according to manufacturer's instructions. Briefly, when cells reached 80% confluency they were tryripsinized and suspended in 4mL medium. Suspension was taken into 15mL falcon tube and cells were centrifuged at 1000 rpm for 5 min at 4°C. After discarding supernatant, pellet was resuspended in 5mL PBS. Another centrifugation step was carried out at 1000 rpm for 5 min at 4°C. Supernatant was removed and 1mL TriReagent was added. Cells were suspended thoroughly and taken into DEPC treated Eppendorf tubes. After 5 min room temperature incubation, cells were centrifuged at 12000 g for 10 minutes to precipitate any insoluble materials and some of the genomic DNA. Supernatant was transferred into DEPC treated Eppendorf tubes and 200µL chloroform was added. Cell lysates were

mixed slowly for 15 min and immediately put on ice for 15 min incubation. On the next step, cell lysates were centrifuged at 12000 for 15 min. After this step three layers were formed. Upper layer was transparent and consisted of RNA. The middle white layer was the precipitated DNA whereas bottom pink layer was the part which contained organic molecules. Upper aqueous phase was carefully removed to another DEPC treated Eppendorf tube. 500µL absolute ice-cold isopropanol was added and pipetted a few times slowly. Samples were left for incubation at room temperature for 10 min. Following incubation, samples were centrifuged at 12000g for 15 min. In this step RNA pellet could be seen at the bottom corner of the tubes. Isopropanol was removed and 1mL 75% ethanol was added (Appendix B). Samples were left at -20°C for 3-5 days to obtain pure RNA isolates. After that, the samples were centrifuged at 12000g for 5 min. Ethanol was removed and any remaining ethanol trace was evaporated. RNA was dissolved in RNase free water and incubated at 55°C for 15min to break secondary structures and was kept at -80°C.

2.2.3.2 Total RNA Isolation from Elacridar Treated Cells

In order to mimic XTT conditions, cells were seeded into new T75 flasks and either final concentration of 0.5, 1, 2.5 or 5µM elacridar was added. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 72 hours before RNA isolation, in the aforementioned manner.

2.2.3.3 Quantification of RNA

In order to determine the purity of the isolated RNA, NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, USA) was used. Measurements at 260nm, 280nm determined the presence of nucleic acids and proteins respectively whereas phenol and other organic contaminations absorbations could be measured at 230nm. The purity of the RNA sample could be checked by calculating A_{260}/A_{280}

and A_{260}/A_{230} ratios. The sample was considered to be pure when A_{260}/A_{280} ratio was between 1.8-2.0 and A_{260}/A_{230} ratio was between 2.0-2.2 (Thermo Scientific Technical Bulletin, 2011).

Concentration of the RNA isolate was assessed by Equation 2.4.

[RNA] µg/mL= Absorbance at 260nm x Dilution Factor x 40 µg/mL (2.4)

Where 40 µg/mL is the average extinction coefficient of RNA.

2.2.3.4 Agarose Gel Electrophoresis of RNA

In order to check any DNA contamination and the integrity of RNA isolate, agarose gel electrophoresis was carried out. Briefly, 0.5 g agarose was dissolved in 50 mL 1X TAE buffer (Appendix B) and boiled in microwave oven to completely melt the agarose in the mixture. After cooling the mixture, 3.5 µL EtBr (Appendix B) was added and the mixture was shaken to homogenize. Gel solution was poured into gel tray with the comb placed and left to solidify. After the solidification step, the gel was taken into electrophoresis tank (Bio-Rad Laboratories, France) containing 1X TAE buffer. 4 µL RNA sample was mixed with 4 µL 2X RNA loading dye (Appendix B) and loaded. Samples were run on 1% (w/v) agarose gel at 80V for 60 min and visualized by UV gel acquisition system (Vilber Lourmat, France).

2.2.3.5 cDNA Synthesis

All of the plastic and glassware used in cDNA synthesis were DEPC treated. cDNA synthesis from 5µg total RNA was performed in thermal cycler (Apollo ATC 401,

Belgium) with Moloney-Murine Leukemia Virus reverse transcriptase (Fermentas) and 20 pmol of either *MDR1*, *MRP1* or *β-actin* gene specific antisense primers.

5µg total RNA, 20 pmol gene specific antisense primer were put in 0,5mL Eppendorf tube and the volume was completed to 11µL with nuclease free water. Sample was incubated at 70°C for 5min to disrupt any secondary structures of RNA. 4µL of 5X reaction buffer and 2µL of 10mM dNTP mix was added and the total volume was completed to 19.5µL with nuclease free water. Sample was incubated at 37°C for 5min to allow primer binding followed by 0.5 µL M-MLV RT addition. Reaction mixture was incubated at 42°C for 60 min for cDNA synthesis. Lastly, the reaction was terminated by incubation at 72°C for 10min. cDNA was kept at -20°C for long term storage.

To avoid any misleading results that may arise from different reaction efficiencies, cDNAs were synthesized at the same time. For all genes examined, master mixes and same RNA sample were used (when applicable).

2.2.3.6 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Quantitative Real-Time Polymerase Chain Reaction (qPCR) enables detection and quantification in real time rather than traditional end-point approach. SYBR Green I is a dye that gives fluorescent signal when it intercalates the double stands of DNA. As the products accumulate in each cycle of PCR, dye binds to more DNA double strands and the signal increases. The changes in the signal intensity are detected and converted into graphs by the software of the qPCR instrument.

qPCR experiments were carried out in Rotor-Gene 6000 (Corbett Research, Australia) instrument. 10µL of 2X SYBR Green master mix, 2µL template cDNA,

0.2µL forward and reverse primers (25µM each) and 7.6µL nuclease free water was mixed and prepared in 0.2µL PCR tubes (Greiner Bio-One, Germany). In order to determine DNA contamination and to detect background signal, no template control (NTC) was used. NTC was prepared in the same manner however it contained water instead of the template. Each sample was prepared in triplicates. After amplification, melting analysis was performed in order to check any non-specific product formation. In the melting step, temperature was rised from 50°C to 99°C gradually and the change in the fluorescent signal was detected. Since the same PCR products have the same melting temperature, they give the same melting peak in melt-curve analysis. qPCR conditions for each gene examined are shown below (Table 2.2):

Table 2.2 qPCR conditions for *MDR1*, *MRP1* and *β-actin* genes

	MDR1	**MRP1**	**β-actin**
Activation	95°C, 10 min	95°C, 10 min	95°C, 10 min
Denaturation	95°C, 20 sec	95°C, 20 sec	95°C, 20 sec
Annealing	60°C, 15 sec	57°C, 15 sec	60°C, 15 sec
Extension	72°C, 15 sec	72°C, 15 sec	72°C, 15 sec
Melting	50°C-99°C	50°C-99°C	50°C-99°C
Cycle Number	45	45	40

2.2.3.6.1 Confirmation of the qPCR Products by Agarose Gel Electrophoresis

After melting analysis it is possible to see primer dimers, foreign DNA amplifications and non-specific product formations. While peaks more than 1 are indicative of undesired PCR product, having one peak does not confirm that the product generated is the desired one. In order to control the sizes of the amplified

products, they were run on agarose gel electrophoresis and the product sizes were compared to that of expected product size.

Briefly, 2 g agarose was dissolved in 100 mL 1X TAE buffer (Appendix B) and boiled in microwave oven to completely melt the agarose in the mixture. After cooling the mixture, 7 µL EtBr (Appendix B) was added and the mixture was mixed to homogenize. Gel solution was poured into gel tray with a comb placed and left to solidify. After the solidification step, gel was taken into electrophoresis tank (Bio-Rad Laboratories, France) containing 1X TAE buffer. 10 µL DNA product was mixed with 2 µL 6X DNA loading dye (Appendix B) and loaded. Samples were run on 2% (w/v) agarose gel at 100V for 90 min and visualized by UV gel acquisition system (Vilber Lourmat, France).

2.2.3.6.2 Quantitation of qPCR products

Quantitation analyses were made according to $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). In this method the relative changes in gene expression were described as fold changes. The fold change in relative quantification method was calculated by normalization with respect to an internal control gene and a reference group (i.e. untreated control group).

In order to calculate gene expression changes, *β-actin* was chosen as an internal control gene. Fold changes of *MDR1* and *MRP1* expression was normalized to the control gene and relative to a reference group.

2.2.3.7 Statistical Analysis

Statistical analyses were carried out by using GraphPad Prism 5.0 Software (GraphPad Software Inc., USA). qPCR experiments were performed in triplicates and were repeated three times. Standard curves and Ct values were obtained with Rotor-Gene 6000 version 1.7 software. Fold changes were expressed as mean and ± standard error of means (SEM). Data was subjected to one-way ANOVA test and the different groups were compared with Tukey's Multiple Comparison Test. Results were considered as significant when p value was smaller than 0.05 ($p<0.05$).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Determination of the Resistance Level in Paclitaxel Resistant MCF7/Pac Cells by XTT Cell Proliferation Assay

In this study paclitaxel resistant MCF7/Pac cell line was used. This subline was previously developed in our laboratory by Kars *et al.*(Kars, *et al.,* 2008). In that study, in order to develop MCF7/Pac subline, MCF7 cells were subjected to paclitaxel in increasing drug concentrations for two years. Highest drug concentration used was 400 nM of paclitaxel which developed MCF7/Pac 400 nM resistant subline. This subline was found to be BCRP negative and expressing high levels of P-gp (detailed information of this cell line and its paclitaxel resistance characteristics are given in Appendix C). In order to confirm the resistance of the MCF7/Pac subline, IC_{50} value was recalculated by XTT cell proliferation assay.

MCF7/Pac resistant subline was treated with a concentration gradient of paclitaxel as described previously. Highest drug concentration administered was 100 μM. % cell proliferation versus drug concentration graphs were plotted and the IC_{50} values were calculated from the formula of the logarithmic trendline of the graphs. The mean value was expressed as the final IC_{50} value which was 42.52 $\pm$ 4.79 μM.

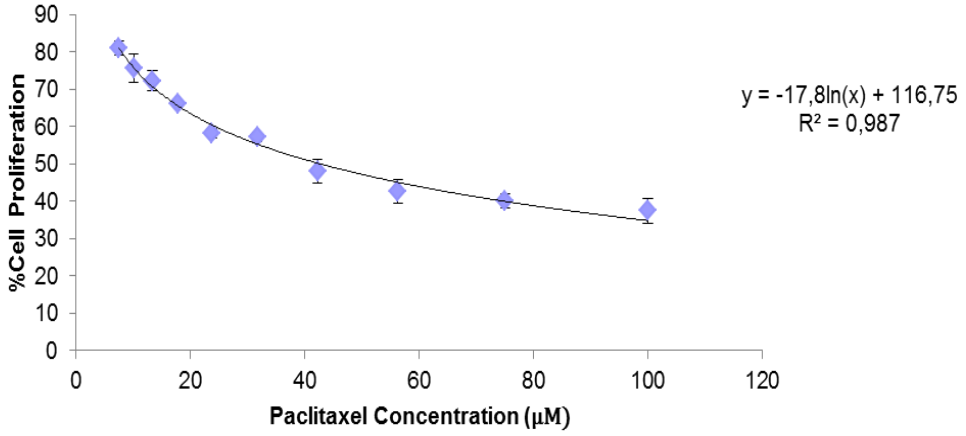


Figure 3.1 Antiproliferative effect of paclitaxel on MCF7/Pac cells

In order to determine the resistance index value, MCF7/S cells were also subjected to XTT cell proliferation assay as described previously. The highest drug concentration administered was 12µM and IC_{50} value for MCF/S cells was 3.64 ± 0.73 µM.

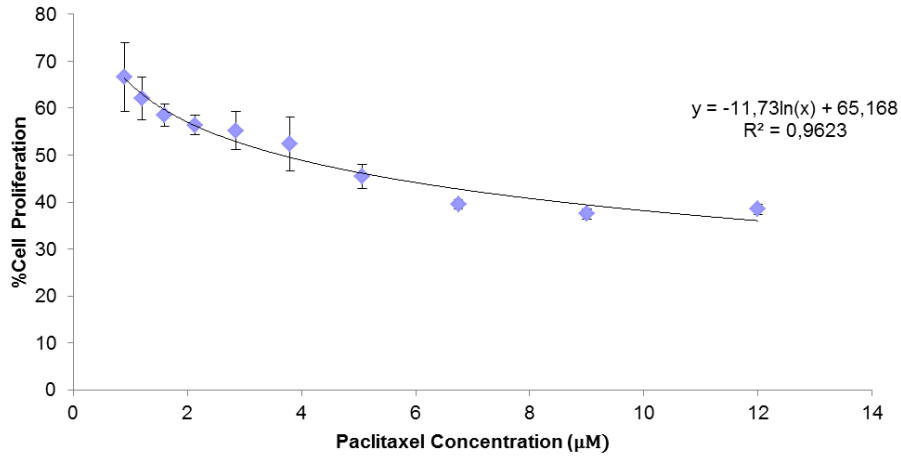


Figure 3.2 Antiproliferative effect of paclitaxel on MCF7/S cells

According to the results, parental cell line had slightly higher IC_{50} value than it had in previous findings (IC_{50} value for MCF7/S cells was 2.12 ±0.23 μM (Kars, 2008)). The reason behind this might be the increased passage number overtime. In each passaging, there is a slight probability that mutations can occur. These mutations may alter the metabolism of the cells (Hughes & Marshall, 2007) and change their response to drugs. Therefore over subculturing should be avoided as much as possible.

After the determination of IC_{50} values for paclitaxel in MCF7/Pac and MCF7/S, resistance index of the cells were calculated according to Equation 2.2 and it was found that the MCF7/Pac cells were 11.68 fold resistant to paclitaxel compared to sensitive MCF7/S cells ($p< 0.05$).

3.2 Cytotoxicity Determination of Elacridar on MCF7/S and MCF7/Pac Cells

In the current study both paclitaxel and elacridar were dissolved in DMSO. Choosing a solvent which can dissolve both of the chemicals was important to have less variables in XTT cell proliferation assay. However, DMSO was toxic to cells at 37°C therefore it was important to keep DMSO volume lower than 2 % (v/v) per well in 96-well plates to avoid any cytotoxicity. This was achieved by dissolving paclitaxel and elacridar in minimum DMSO volume possible and further dilute them in medium so that the final volume of DMSO in a well of 96-well plate did not exceed 2% (v/v). Furthermore, upper and bottom rows were assessed as DMSO control rows to confirm no toxicity was exerted on the cells by DMSO. The cytotoxicity of both DMSO only and DMSO elacridar combinations were determined by XTT cell proliferation assay.

In order to determine the cytotoxicity of elacridar, high concentrations of the modulator (starting from 100 μM) were used. For elacridar, preparation of very

concentrated stock solutions was not possible due to the fact that, to fully solubilize 3mg of elacridar, at least 2 mL of DMSO was needed even after intense pipetting and vortexing steps. Taken these into consideration, XTT cell proliferation assay was performed in the previously described manner (Section 2.2.2.2). Figure 3.3 illustrates the graph of % cell proliferation versus antiproliferative effect of DMSO alone and elacridar DMSO combinations in MCF7/S cells.

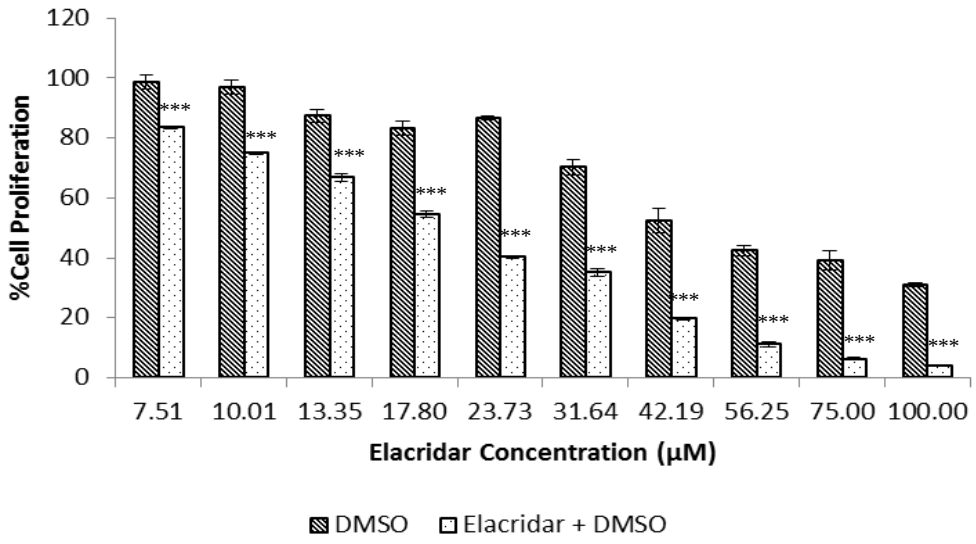


Figure 3.3 Antiproliferative effect of elacridar and DMSO on MCF7/S cells. For DMSO bars data was derived from DMSO control wells. All data represent significant values with $p < 0.001$.

Since elacridar was dissolved in DMSO, cells were also subjected to this chemical whenever elacridar was added. In order to see any toxic effect that might have been caused by DMSO, the data from DMSO control wells were calculated. When the % cell survival of DMSO and elacridar treated cells were determined, it was observed

that as the concentration of elacridar was increased % cell proliferation was decreased. Moreover, the proliferation percentage values were significantly different from each other at each concentration. Furthermore, when DMSO treated cells were compared to DMSO and elacridar treated cells, again % cell proliferation values were significantly different with a p value < 0.001 (Figure 3.3).

In order to have a concentration of elacridar and DMSO mixture that will not cause any cytotoxicity on the cells, elacridar concentrations lower than 7.5μM were used in reversal experiments (highest elacridar concentration administered was 5μM). At this concentration DMSO did not exert any cytotoxic effect on MCF7/S cells. Hence, elacridar and DMSO cytotoxicity was minimized. Since the aim of the current study is to see the reversal effect of elacridar, cell line used in reversal studies was MCF/Pac cell line. MCF7/Pac cells had much higher tolerance to the toxicity of elacridar and to DMSO (refer to next paragraph). Hence, any interference of toxicities with the antiproliferative effect of paclitaxel was avoided.

The cytotoxicity of elacridar was also tested on MCF7/Pac cells. Cells were subjected to XTT cell proliferation assay in the same manner as MCF/S cells. The results for the cytotoxicity of DMSO alone and elacridar DMSO mixture are illustrated in Figure 3.4

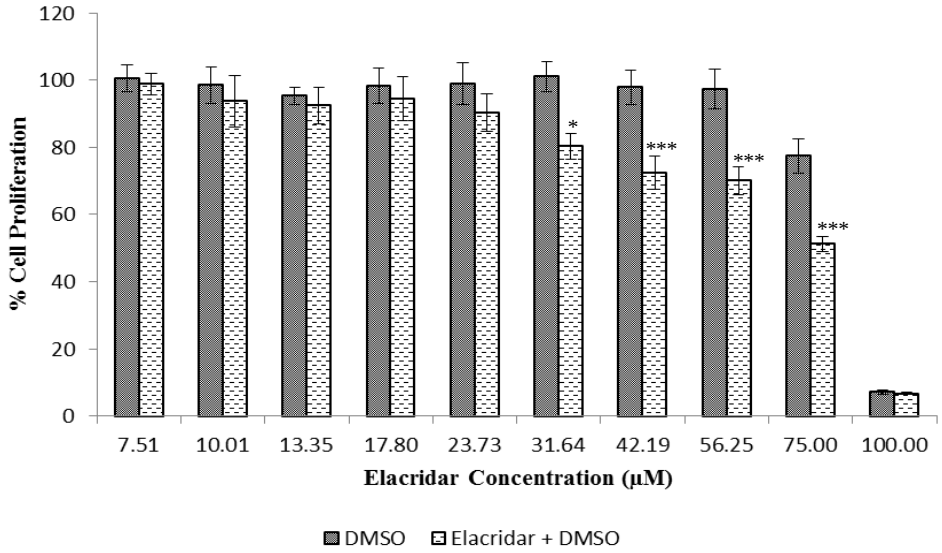


Figure 3.4 Antiproliferative effect of elacridar and DMSO on MCF7/Pac cells. * Results were significant with $p<0.05$ compared to % cell proliferation after DMSO treatment. *** Results were significant with $p<0.001$ compared to % cell proliferation after DMSO treatment

According to cytotoxicity results, MCF7/Pac cells were less affected by the toxicity of DMSO and elacridar. Since these cells have an improved resistance mechanism it is possible that they tend to evade toxicity of chemicals better than MCF7/S cells which indicates MCF7/Pac cells have more developed detoxification systems than MCF7/S cells. A similar relation between MCF7/S cell line, MCF7 resistant cell line and an MDR modulator; was also reported in a previous study in our laboratory (Urfalı, 2012).

Neither DMSO nor elacridar exerted any significant cytotoxic effect at concentrations below 30µM of elacridar in paclitaxel resistant MCF7/Pac cells. Above this concentration cytotoxic effect of elacridar and DMSO combination compared to only DMSO administration was significant. At 100µM elacridar concentration, the antiproliferative effects of DMSO only and elacridar DMSO combinations were both very evident. At this concentration % cell proliferation was

as low as 6.5%. It is also possible that the 96-well plate reader was not able to give lower absorbance results due to the self-absorbance of the medium, thus calculating lower % cell proliferation values was not possible.

3.3 The Effect of Elacridar on the Reversal of Paclitaxel Resistance in MCF7/Pac Cells

The current study aims achieving multi drug resistance reversal by elacridar. In order to detect the increased antiproliferative effect of paclitaxel by MDR reversal, the cytotoxic effect should only be caused by the drug. Any toxicity caused by DMSO or elacridar may interfere with the results. Therefore elacridar concentrations less than 7.5μM were chosen for fold reversal studies to ensure neither elacridar nor DMSO was toxic to MCF7/Pac cells.

Four different concentrations of elacridar were chosen for fold reversal determination experiments. MCF7/Pac cells were treated with 0.5μM, 1μM, 2.5μM or 5μM elacridar together with a concentration gradient of paclitaxel as previously described (Section 2.2.2.3). The antiproliferative effect of paclitaxel and elacridar combinations on MCF7/Pac cells were illustrated in Figure 3.5.

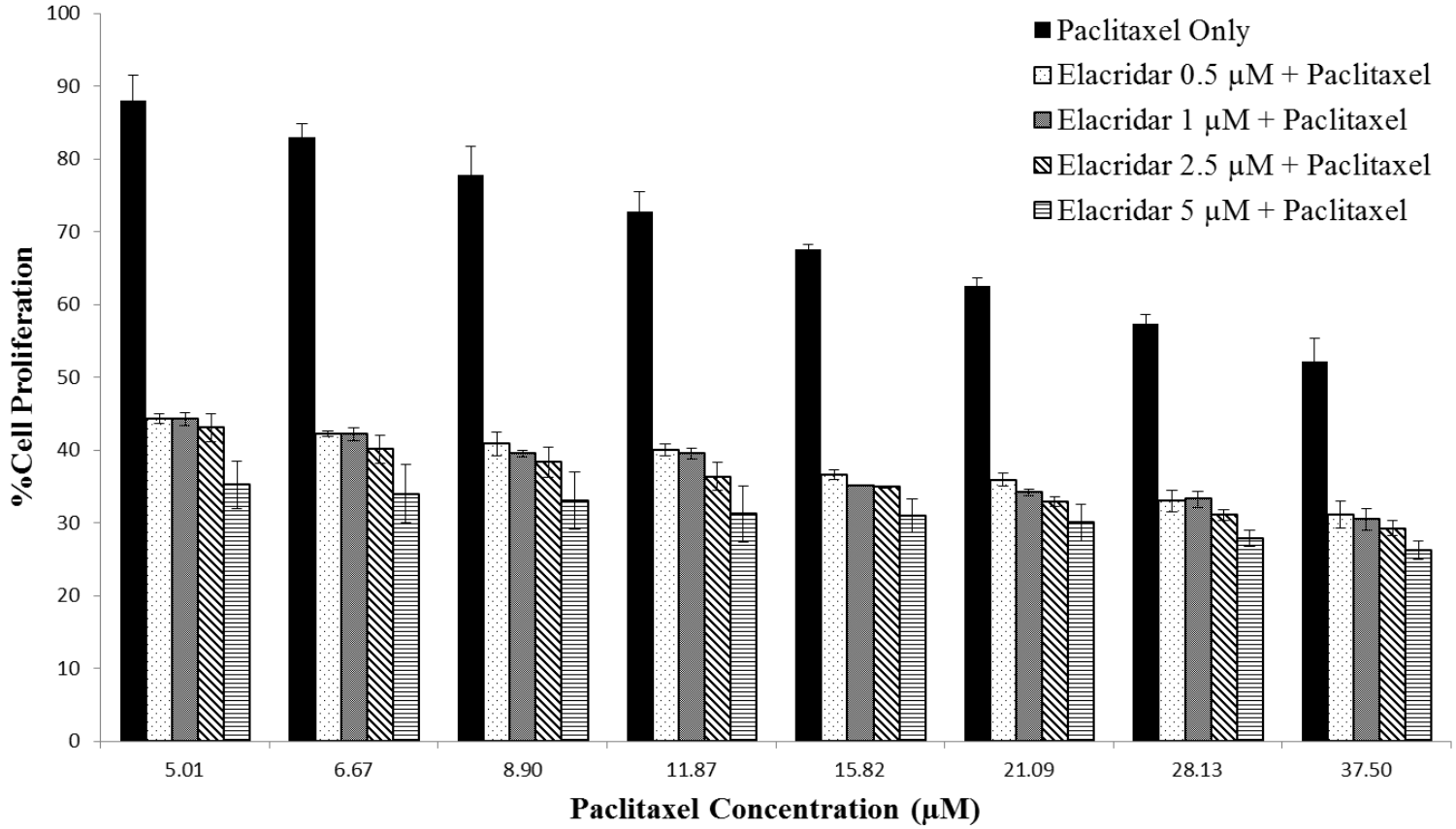


Figure 3.5 Profile of cell proliferation of untreated or elacridar treated MCF/Pac cells at increasing concentrations of paclitaxel. All data compared to "Paclitaxel only" group is statistically significant with $p < 0.001$.

Figure 3.5 demonstrates proliferation of MCF7/Pac cells decreased dramatically when elacridar was coadministered together with the anticancer drug paclitaxel. All of these decreases were statistically significant compared to only paclitaxel treated cells, in all paclitaxel and elacridar combinations examined ($p<0.001$). However, at a given paclitaxel concentration, increasing the concentration of elacridar did not make any significant change in % cell proliferation. Also the IC_{50} values for paclitaxel after 0.5μM, 1μM and 2.5μM elacridar treatment were not significantly different from each other. On the other hand, the IC_{50} value for paclitaxel obtained after 5μM elacridar treatment was significantly different from the IC_{50} values obtained after 0.5μM and 1μM elacridar treatments ($p<0.05$). The reason why non-significant changes were observed in IC_{50} values for paclitaxel among 0.5μM, 1μM and 2.5μM elacridar treated cells, may be due to the fact that in all of the investigated concentrations of elacridar in this study, more than 90% reversal was achieved. In the literature there are reports which indicate that elacridar at lower concentrations exerts its effect in a concentration dependent manner on different cell lines (Hyafil, 1993).

Interestingly, elacridar treatment was more effective in lower concentrations of paclitaxel. For instance, when 5μM paclitaxel was combined with elacridar (from 0.5μM to 5μM elacridar) the % cell death was increased approximately 45% more than the paclitaxel alone treatment in paclitaxel resistant MCF7/Pac cells. However, 20μM of paclitaxel coadmistered with elacridar resulted in approximately 25% increase in cell death compared to only paclitaxel administration at the same concentration. This result suggests it would be a better approach to use low concentrations of paclitaxel and elacridar combinations in order to have the higher efficiency in MDR reversal. Moreover, to avoid toxicity on healthy cells, administering low concentrations of any drug that makes efficient treatment is more preferable in clinical trials.

3.3.1 Determination of Fold Reversal Values of Elacridar

IC_{50} values were calculated after each elacridar treatment from three separate plates. Fold reversal values were derived from Equation 2.3. IC_{50} values for paclitaxel in untreated/elacridar treated MCF7/Pac cells and the fold reversal values are represented below (Table 3.1)

Table 3.1 Fold reversal values and IC_{50} of paclitaxel in elacridar treated and untreated MCF7/Pac cells

Treatment	**IC_{50} ± SEM† (µM)**	**Fold Reversal (FR)**
Paclitaxel only	42.52 ± 4.79	---
0.5 µM Elacridar + Paclitaxel	2.15 ± 0.09 ***	19.79 ***
1 µM Elacridar + Paclitaxel	2.06 ± 0.42 ***	20.64 ***
2.5 µM Elacridar + Paclitaxel	1.58 ± 0.78 ***	26.89 ***
5 µM Elacridar + Paclitaxel	0.46 ± 0.43 ***	95.52 ***

† SEM values were obtained from three independent experiments.

*** $p < 0.001$ compared to "paclitaxel only".

As represented in Table 3.1, at 0.5 µM and 1 µM elacridar concentrations MCF7/Pac cells were re-sensitized to paclitaxel's antiproliferative effect 20 and 21 fold respectively. When the concentration of elacridar was increased to 2.5µM and 5µM the fold reversal values were approximately 27 and 96 fold compared to untreated control. In terms of percentages, IC_{50} value of MCF7/Pac cells were decreased 94% and 95% more when 0.5 and 1 µM elacridar was administered. Approximately 96% and 99% more decrease in the IC_{50} values were observed when the cells were treated with 2.5 and 5 µM elacridar respectively. Since complete

restoration in paclitaxel cytotoxicity was achieved with 5μM elacridar, higher doses of elacridar treatment were not performed.

In the literature elacridar is reported as an anticancer drug that can usually make full MDR reversal in nanomolar concentrations (Hyafil, 1993). However in most of these studies cell lines that overexpress P-gp were constructed by cloning. Therefore, only P-gp expression levels were altered. In the current study the MCF7/Pac resistant cell line was built in two years by the stepwise increments of paclitaxel, hence many gene expression levels have changed. Resistance was not a result of only P-gp overexpression but a series of alterations in metabolic pathways. Indeed in patients who acquire paclitaxel resistance the whole mechanism of the cells change thus working with cells which gained resistance in stepwise manner could give more realistic results. In this study although 94% restoration of the antiproliferative effect of paclitaxel was accomplished with 0.5μM elacridar, for complete MDR reversal 5μM elacridar was needed. The need of higher concentrations of elacridar for full reversal than the amounts used in literature (0.1-2.5μM) could be related to overall altered resistance mechanisms of MCF7/Pac and/or high expression levels of P-gp in this cell line. There are reports that state elacridar interacts with P-gp although the binding site is not exactly determined yet (Martin, *et al*., 2000). As elacridar interacts with its binding site on P-gp, the presence of large amounts of P-glycoprotein requires more elacridar molecules to bind and inhibit the pumping completely. This proposal is in line with the evidence of de Bruin's study. In this study, instead of nanomolar concentrations, 1μM elacridar was needed for complete reversal in colon cancer cell line which expressed very high amounts of BCRP (de Bruin, 1999). As previously mentioned, elacridar is an inhibitor of both BCRP and MDR1. Although elacridar's mechanism of action might not be the same for BCRP and P-gp the need of high amounts of elacridar for complete MDR reversal is reasonable.

Another explanation for the need of higher concentrations of elacridar could be due to variable efficiency of elacridar with anticancer drugs. Traunecker *et al.* studied

elacridars' effect with doxorubicin, vinblastine, docetaxel and paclitaxel in P-glycoprotein expressing human sarcoma cell line MES-Dx5. The concentration of elacridar that is needed to reverse the resistance to paclitaxel by 50% was fivefold higher than the concentration needed to reverse 50% resistance to doxorubicin or to vinblastine. Similarly, the concentration of elacridar that is needed to reverse the resistance to docetaxel by 50% was eightfold higher than the concentration needed to have the same effect with etoposide (Traunecker H. *et al*., 1999). According to these results, it could be derived that anticancer drugs that act on microtubules have less efficiency with elacridar than other anticancer agents thus the need for higher concentrations is consistent. An explanation for this could be related to the communication between drug binding sites. Elacridar binds to P-gp in an allosteric fashion and acts as a non-competitive inhibitor (de Bruin, 1999). A study by Martin *et al.* indicated at least four different sites exist on P-glycoprotein for binding of various anticancer drugs and MDR modulators. The site where elacridar interacted with P-gp was suggested to be a regulatory site since only modulators but not substrates were able to interact with it. These four sites were also able to allosterically communicate with each other in a negative heterotropic manner. According to this study; paclitaxel, vinblastine and elacridar had distinct binding sites on P-gp thus differences in the communication of these sites could be a factor influencing efficiency of MDR reversal (Martin, *et al*., 2000).

Although above considerations should be kept in mind these results do not indicate elacridar was ineffective in nanomolar concentrations in this study. As mentioned before, the lowest concentration examined 0.5μM of elacridar, resulted in 94% MDR reversal. In order to fully reverse multidrug resistance, dose increments were carried out until %99 MDR reversal was achieved at 5μM elacridar concentration.

The effectiveness of elacridar can be better understood when compared to other MDR modulators that have been investigated in other studies in our laboratory. Although the sublines used were resistant to different cytotoxic agents, all of the resistant cell lines were developed in stepwise manner from MCF7 parental cell

line. Recently, Urfalı *et al.* demonstrated the MDR reversal effect of biochanin A on MCF7 zoledronic acid resistant cell line. This cell line had acquired resistance to zoledronic acid in a BCRP mediated manner. Biochanin A is an isoflavonoid type of MDR reversal agent and it is one of the most potent BCRP modulators. In the stated study, at 5 μM of biochanin A concentration no resistance reversal effect was seen (Urfalı, 2012). Another study by Dönmez *et al.* reported the MDR reversal effect of verapamil and promethazine on MCF7 doxorubicin resistant subline. In this study in order to effectively inhibit P-gp activity, 60 μM of verapamil and 9.6 μM promethazine were necessary (Dönmez *et al.*, 2011). Although comparing the efficiency of MDR modulators among cell lines with different resistance mechanisms may not be very informative, the results still suggest elacridar's efficiency is superior due to the fact that in lower concentrations of the modulator, higher MDR reversal rates were obtained.

3.4 *MDR1* and *MRP1* Gene Expression Analyses in MCF7/S and MCF7/Pac Cell Lines Upon Elacridar Treatment

MDR1 and MRP1 are two important efflux pumps expressed in MCF7/Pac. The changes in the expression levels of these genes may be important in multidrug resistance characteristics of MCF7/Pac. In order to see any alterations on *MDR1* and *MRP1* gene expression levels that may be caused by elacridar treatment, MCF7/Pac cells were treated with four different concentrations of elacridar (0.5μM, 1μM, 2.5μM or 5μM) 72 hr prior to total RNA isolation. Total RNA from MCF7/S cells and MCF7/Pac cells were also extracted for comparison. cDNA were synthesized from total RNA isolates. In order to quantitatively analyze the expression levels of *MDR1* and *MRP1*, qPCR was performed. The qPCR results obtained from MCF7/S cells expression levels were compared to the results of MCF7/Pac cells to determine the changes in gene expression levels of *MDR1* and *MRP1* due to paclitaxel resistance. In order to see any alterations on *MDR1* and *MRP1* expressions caused by elacridar treatments, untreated MCF7/Pac cells

expressions for *MDR1* and *MRP1* genes were compared to elacridar treated MCF7/Pac cells gene expression levels.

3.4.1 Total RNA Isolation From MCF-7 Cell Lines

For gene expression studies, firstly total RNA was isolated from MC7/S and MCF7/Pac cells which were untreated or treated with elacridar. The RNA samples were subjected to electrophoresis on 1% agarose gel. Sharp bands of 28S rRNA and 18S rRNA without smear formation indicated that the isolated RNAs were intact (Figure 3.6).

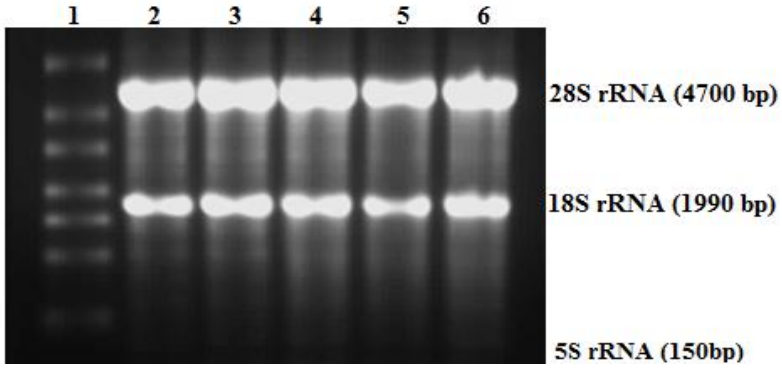


Figure 3.6 Representative figure of total RNA isolates. High Range RNA ladder (Lane 1), MCF7/Pac (Lane 2), 0.5µM elacridar treated MCF7/Pac (Lane 3), 1µM elacridar treated MCF7/Pac (Lane 4), 2.5µM elacridar treated MCF7/Pac (Lane 5), 5µM elacridar treated MCF7/Pac (Lane 6)

After intactness of RNA samples were confirmed, they were further investigated by NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, USA). In gene expression analyses only the samples which have A_{260}/A_{280} ratio of 1.8-2.0 and

A_{260}/A_{230} ratio of 2.0-2.2 were used. The exact A_{260}/A_{280} and A_{260}/A_{230} ratios of the RNA samples used in cDNA synthesis are given in Table 3.2.

Table 3.2 The A_{260}/A_{280} and A_{260}/A_{230} ratios of the RNA samples used in cDNA synthesis

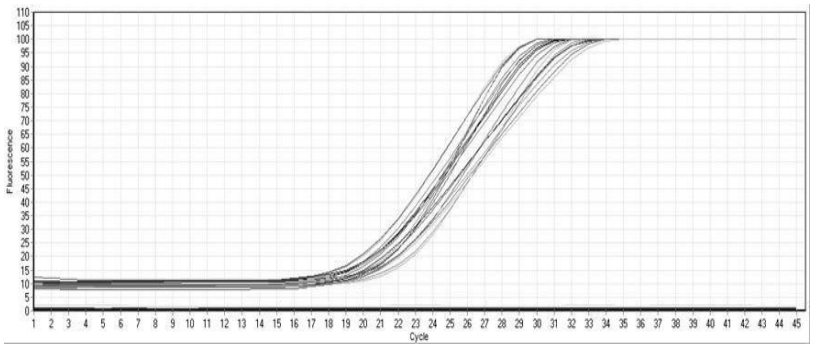
Sample	**A_{260}/A_{280} Ratio**	**A_{260}/A_{230} Ratio**
MCF7/S (untreated)	2.00	2.14
MCF7/Pac (untreated)	1.98	2.14
MCF7/Pac (0.5 μM elacridar treated)	1.98	2.11
MCF7/Pac (1 μM elacridar treated)	1.98	2.08
MCF7/Pac (2.5 μM elacridar treated)	1.97	2.08
MCF7/Pac (5 μM elacridar treated)	1.97	2.04

3.4.2 Expression Analyses of *MDR1* and *MRP1* Genes by Quantitative Real-Time Polymerase Chain Reaction (qPCR)

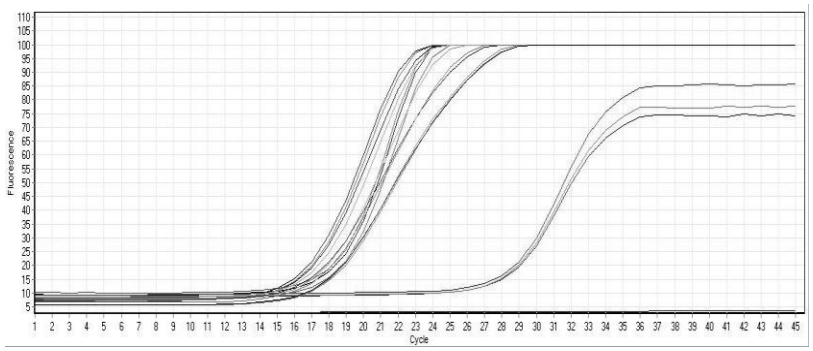
Following total RNA isolation, cDNA synthesis was performed as described in Section 2.2.3.5. Synthesized cDNA was subjected to qPCR. Expression analyses were performed in Rotor-Gene 6000 (Corbett Research, Australia) instrument and the quantitation data was determined by Rotor-Gene 6000 version 1.7 software. Amplification curves were generated with fluorescence versus threshold cycle number. The data were normalized according to expression level of *β-actin* and the $2^{-\Delta\Delta Ct}$ method was used to calculate relative fold changes (Livak & Schmittgen, 2001). The qPCR results obtained from MCF7/S cells expression levels were compared to the results of MCF7/Pac cells to determine the changes in gene expression levels of *MDR1* and *MRP1* due to paclitaxel resistance. Untreated MCF7/Pac cells expressions for *MDR1* and *MRP1* genes were compared to

elacridar treated MCF7/Pac cells gene expression levels to detect any alterations in these gene expressions. Amplification curve graphs for *MRP1*, *MDR1* and *β-actin* are shown in Figure (3.7):

a)



b)



c)

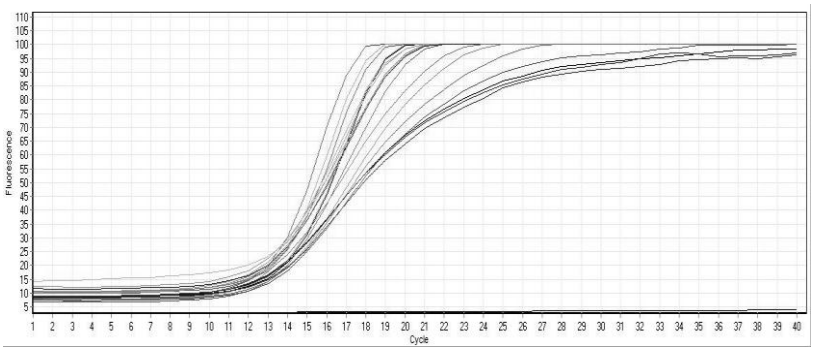
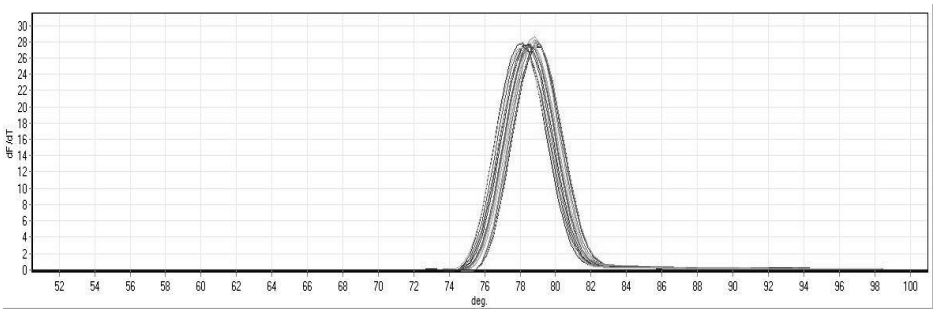


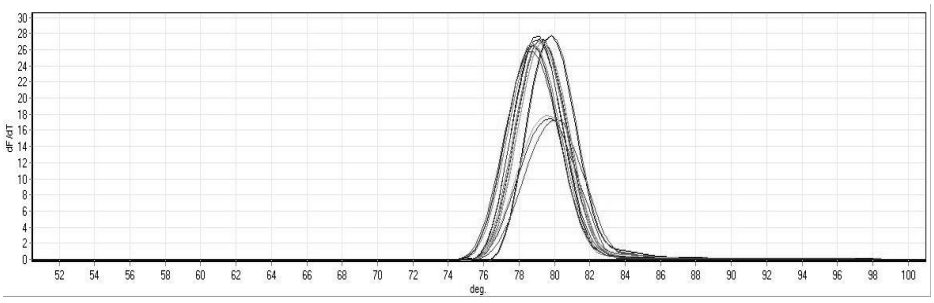
Figure 3.7 Amplification curves for a) *MRP1* b) *MDR1* and c) *β-actin* genes in MCF7/Pac and MCF7/S cell lines

In order to confirm that the product of interest was amplified only, melting analyses were performed after each run. A single sharp peak in melt curve graphs indicates only the product of interest had been amplified. As seen in Figure 3.8 the products gave a single sharp peak which indicated a single product was amplified in each reaction. Products were further examined in agarose gel electrophoresis and their agarose gel photographs are presented in Appendix E. Melt curve graphs were plotted by taking the first derivative of fluorescence intensity with respect to temperature by Rotor-Gene 6000 version 1.7 software.

a)



b)



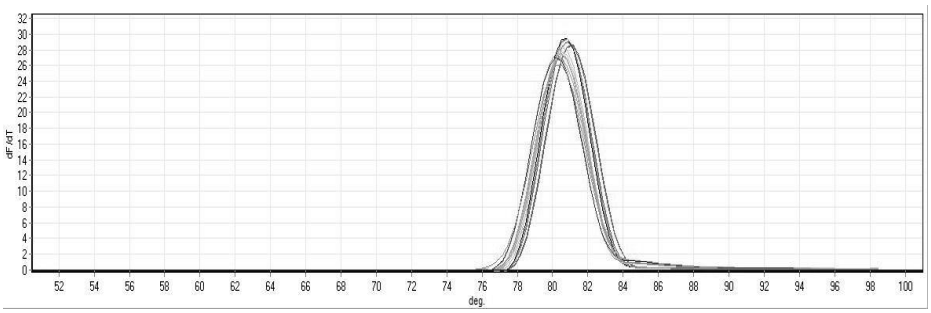
c) 

Figure 3.8 Melting curve analysis of a) *MRP1* b) *MDR1* c) *β-actin* genes in MCF7/Pac and MCF7/S cell lines

According to qPCR results *MDR1* gene expression was approximately 200 fold higher in MCF7/Pac cells relative to MCF7/S cells (Figure 3.9). This result is in-line with previous findings (Kars, 2008) where the upregulation of *MDR1* gene was stated as the main reason of MDR in paclitaxel resistant cell line. Results are also consistent with the literature where upregulation in *MDR1* gene overexpression was reported in paclitaxel resistant cells (Kamazawa, *et al*., 2002).

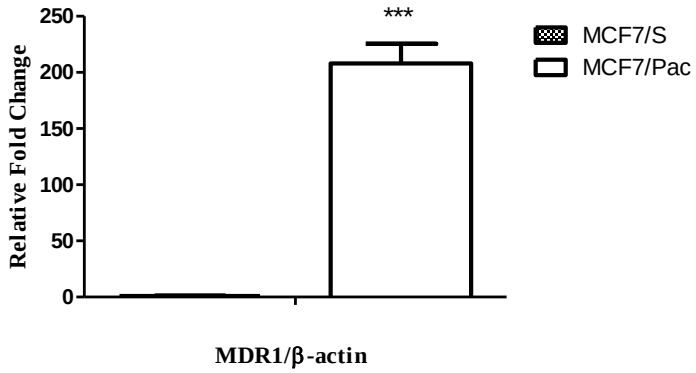


Figure 3.9 Relative gene expression level of *MDR1* in MCF7/Pac and MCF7/S cell lines (*** Results were significant with $p < 0.001$compared to MCF7/S)

The expression level of *MRP1* gene was also examined by qPCR. The results indicated *MRP1* gene expression in MCF7/Pac cells was significantly downregulated when compared to MCF7/S. Results were consistent with the previous findings (Kars, 2008).

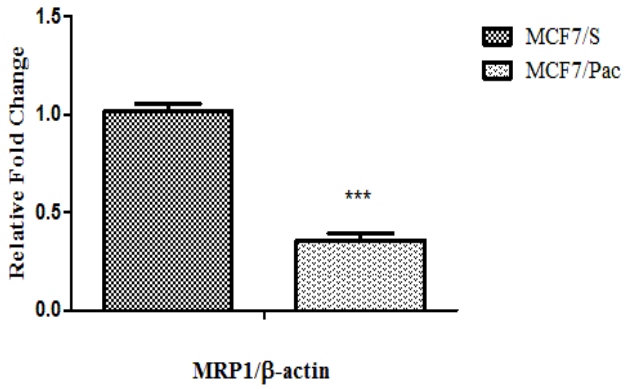


Figure 3.10 Relative gene expression level of *MRP1* in MCF7/Pac and MCF7/S cell lines (*** Results were significant with $p < 0.001$compared to MCF7/S)

In the current study, elacridar was used to reverse MDR and complete restoration of paclitaxel cytotoxicity was achieved. In order to further analyze the effect of elacridar qPCR analyses were performed. The relative *MDR1* gene expression levels of elacridar treated MCF7/Pac cells compared to untreated MCF7/Pac cells are presented in Figure 3.11.

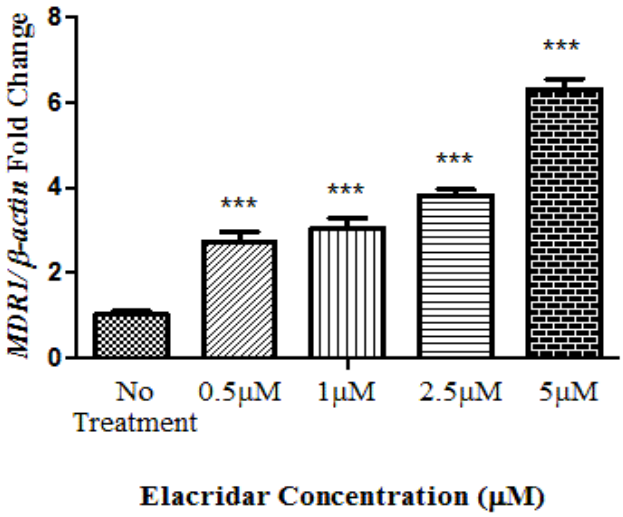


Figure 3.11 Relative gene expression levels of *MDR1* gene in various concentrations of elacridar treated MCF7/Pac cells (*** Results were significant with $p < 0.001$compared to MCF7/Pac)

Interestingly, in all of the concentrations examined, *MDR1* gene expression was significantly upregulated after each elacridar treatment when compared to no treatment control. Moreover, this increase was dose dependent. In this study, used elacridar concentrations were more than the concentrations usually administered in the literature due to aforementioned reasons. Presumably in the concentrations usually used in literature, i.e. as low as 100nM, elacridar would not make any change on *MDR1* expression. Although all of the elacridar concentrations used were non-cytotoxic, this foreign compound administration could have triggered pathways such as detoxification pathways in the molecular level. Possibly, cells were trying to escape from the toxic effect of this foreign compound by increasing their expression levels of efflux pumps**.** Nonetheless, very efficient (up to 99%) MDR reversal which was observed by XTT cell proliferation assay analyses proves that the increase at mRNA level was not sufficient to prevent elacridar's reversal effect. This result suggested the upregulation in *MDR1* gene expression level may not be increasing the MDR1 gene product directly. Martin *et al.* stated that elacridar exerts

its reversal effect on protein level by binding to P-glycoprotein (Martin *et al*., 2000) and preventing its activity. Moreover, it had been reported by Hyafil *et al.* that elacridar is a poor substrate of MDR1 protein and thus is not pumped out rapidly from the cells (Hyafil, 1993). This could be the reason why increased *MDR1* expression was not enough to stop the reversal effect of elacridar. On the other hand, paclitaxel is a substrate of P-glycoprotein (Gottesman, 2002) and such increase may result in increased paclitaxel efflux. Despite the increase in P-gp expression, given elacridar concentrations were still quite adequate to efficiently block MDR1 protein and prevent paclitaxel efflux. Another suggestion may be that elacridar's binding to P-gp led to changes on paclitaxel's binding site on P-gp, preventing efficient efflux. That could also be the reason why increased P-gp expression did not affect the efflux of paclitaxel.

After *MDR1* gene expression analyses, *MRP1* gene expression was also investigated. The relative *MRP1* gene expression levels of elacridar treated MCF7/Pac cells compared to untreated MCF7/Pac cells are presented in Figure 3.12.

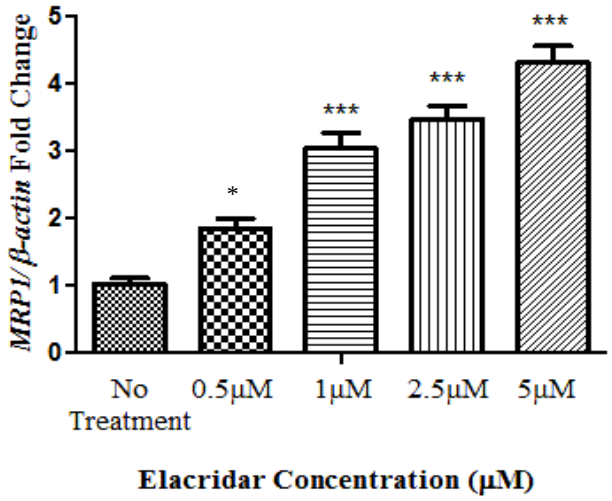


Figure 3.12 Relative gene expression level of *MRP1* gene in various concentrations of elacridar treated MCF7/Pac cells (*Results were significant with $p<0.05$, ***results were significant with $p<0.001$ compared to MCF7/Pac)

As illustrated in Figure 3.12, *MRP1* gene expression was significantly upregulated in all of the elacridar treatments compared to no treatment control. Moreover, these results indicated that the increase in MRP1 expression was also dose dependent. However as previously suggested, such increments may not be seen in elacridar's usually administered dose ranges in the literature. In deed, when 0.5μM elacridar treatments' and no treatment controls' fold change results for *MRP1* gene expression were compared, a much lower level of statistical significance was observed.

In the literature there are a few compounds such as curcumin, verapamil and promethazine that potentially reverse MDR by downregulating *MDR1* or *MRP1* gene expression (Molnar, *et al*., 1998). However in the current study such decreases in *MDR1* and *MRP1* gene expressions were not observed. Therefore these results indicate elacridar is not a modulator that exerts its MDR reversal effect by inhibiting gene expression. Fold changes in expression levels of *MDR1*, *MRP1* and *β-actin* after elacridar treatment are demonstrated in Table 3.3.

Table 3.3 Fold changes in expression levels of *MDR1* and *MRP1* genes

Elacridar Treatment	**Fold Change (*MDR1*)**	**Fold Change (*MRP1*)**
No treatment	1.02 ± 0.09	1.03 ± 0.10
0.5 μM	2.73 ± 0.24 ***	1.86 ± 0.14 *
1 μM	3.05 ± 0.25 ***	3.04 ± 0.24 ***
2.5 μM	3.83 ± 0.14 ***	3.48 ± 0.19 ***
5 μM	6.32 ± 0.24 ***	4.32 ± 0.25 ***

Any outlier value was omitted. Fold change values were represented as ''mean ± SEM". SEM values were obtained from three individual experiments, each run in triplicates. (* Results were significant with $p<0.05$ compared to MCF7/Pac. *** Results were significant with $p<0.001$compared to MCF7/Pac)

When the results were taken together, elacridar treatment led to the upregulation of *MRP1* and *MDR1* gene expressions in a dose dependent manner, i.e. higher concentrations of elacridar caused higher fold changes in expression levels. Although these increases in mRNA levels may or may not be a transient response which occurs in the presence of elacridar, the upregulation in the gene expression levels of both *MDR1* and *MRP1* supported the idea that elacridar treated MCF7/Pac cells were under stress and trying to avoid cytotoxic effects of the modulator. Although cytotoxic effect of elacridar was not detected on XTT cell proliferation assay in the administered concentrations (i.e was not fatal) of elacridar, it is possible that the cytotoxicity was still exerted on the cells on molecular basis. Presumably, since elacridar is not a substrate of P-gp, this toxic effect was not reduced and the cells began to increase the expression of another detoxification related pump MRP1. The effect of such increase was not reflected onto XTT assay results since paclitaxel is not a substrate of MRP1 protein. Therefore, such increase was irrelevant in terms of paclitaxel resistance. Nevertheless, this study suggests it would be a better approach to use lesser amounts of elacridar (0.5 μM) with low concentrations of paclitaxel and obtain 94% MDR reversal instead of causing significant changes in expression levels and obtain full reversal.

As previously stated, although the expression of *MDR1* and *MRP1* genes, which are related with MDR development, were upregulated after elacridar treatment; XTT cell proliferation assay results clearly indicated that none of these gene expression changes were able to prevent elacridar from reversing resistance. The reason behind this may also be related to lack of correlation between mRNA levels and protein levels. Bailly *et al.* evidenced, high *MDR1* expression at mRNA levels did not mean high amounts of efficient P-glycoprotein production (Bailly & Muller, 1995). In the study, seven acute myeloblastic leukemia (AML) sublines expressing different amounts of MDR1 were investigated. Highest *MDR1* gene expressing subline's expression was 13 fold more than TF1 subline's. Afterwards P-glycoprotein function was investigated by Rhodamine123 assay. When the rhodamine efflux capacities were compared TF1, KG1a and KG1 sublines which had much lower

levels of *MDR1* expression than three other sublines, had shown significantly higher values of P-gp activity. In order to confirm resistance, MTT assay was carried out. Once again, KG1a and KG1 sublines were resistant to daunorubicin 10 to 15 fold more than other sublines, despite the fact that they expressed lower amounts of *MDR1*. From this study it can be derived that mRNA expression levels and dynamic function of P-gp pump may not always be correlated. Although elacridar caused upregulation in *MDR1* expression, it may have also caused an alteration on P-gp that lowers its efficiency. Hence, elacridar was still able to restore cytotoxic effect of paclitaxel. For instance, such alteration can be a conformational change on paclitaxel's binding site. Since elacridar and paclitaxel does not bind on the same site of P-gp (Martin *et al.*, 2000) elacridar can still maintain its function while paclitaxel efflux is inhibited.

The increased expression levels of *MDR1* and *MRP1* may be due to increased mRNA stability, suggesting elacridar's effect on posttranscriptional mechanisms. Alternatively, these increased mRNA levels may not reflect the exact increase in protein level. A study by Tian *et al.* estimated correlation of mRNA and protein levels to be 40% at the most (Tian, *et al.*, 2004). Such differences among mRNA levels and protein levels may be due to post translational modifications (Brockmann & Beyer, 2007). Any change that may have been caused by elacridar in degradation rate or translation rate of MDR1 and MRP1 mRNAs may alter their protein levels. Consequently, increases in mRNA level may not have changed the protein levels enough to prevent MDR reversal by elacridar.

In the current study, the effect of elacridar on paclitaxel resistant MCF7/Pac subline was investigated. MCF7/Pac had been developed from MCF7/S parental cell line by stepwise dose increments of paclitaxel (Kars, 2008). Therefore MCF7/Pac was a subline that had developed resistance to paclitaxel by changing many MDR related mechanisms of the cell. Nevertheless, elacridar concentrations between 0.5µM and 5µM were very efficient to reverse multidrug resistance in MCF7/Pac despite many altered resistance related mechanisms. Elacridar treatment resensitized MCF7/Pac

cells to the cytotoxicity of paclitaxel up to 96 fold. The lowest elacridar concentration examined was 500nM which resulted in 94% MDR reversal. Highest examined concentration of elacridar was 5μM which restored full antiproliferative effect of paclitaxel.

MDR1 and *MRP1* genes were known to be expressed in MCF7/Pac subline (Kars, 2008). Since these two genes encode two major MDR related proteins P-gp and MRP1, the effect of elacridar on these genes were investigated. qPCR results indicated dose dependent increases in the expression levels of these genes after elacridar treatment. On the other hand, these increases in gene expression levels were not sufficient to prevent elacridar from restoring antiproliferative effect of paclitaxel as can be derived from XTT cell proliferation assay results. In order to assess the significance of such increase in mRNA level further analyses on protein level must be carried out. Moreover, in order to understand whether produced efflux proteins are active or not, drug efflux assays must be performed.

The increases in *MDR1* and *MRP1* gene expression levels in a dose dependent manner may be important in clinical studies, thus should be carefully investigated. For instance, administration of elacridar 72 hours prior to paclitaxel administration could elevate *MDR1* and *MRP1* gene expression levels in patients according to this study's results. Although such increase did not cause ineffective MDR reversal by elacridar in the current study, it may reduce paclitaxel's or other anticancer drugs efficiency in clinical trials in long term treatments. Therefore administering paclitaxel and elacridar simultaneously could be more reasonable to effectively restore paclitaxel's antiproliferative effect without being effected by the increased *MDR1* and *MRP1* gene expression levels.

This study suggests, scheduling paclitaxel and elacridar administrations in time intervals may be a better approach to avoid any transient effect caused by the elevation in the gene expression levels of efflux pumps.

Finally, one must be careful not to administer high doses of elacridar since low concentrations are effective for reversal and there is risk for upregulation of *MDR1* and *MRP1* gene expressions which can result in multidrug resistance in long term chemotherapy.

CHAPTER 4

CONCLUSION

1. The IC_{50} value of paclitaxel resistant MCF7/Pac subline was significantly higher than that of MCF7/S parental cell line. Results indicated MCF7/Pac cell line was 11.7 fold resistant to paclitaxel compared to drug sensitive (parental) MCF7/S cell line.

2. The toxicity of elacridar on MCF7/S cells started at around 7.5μM whereas elacridar concentrations up to 32μM were not toxic to MCF7/Pac cells. This result suggested MCF7/Pac cell line had an improved detoxification mechanism against elacridar.

3. Various elacridar concentrations were administered on MCF7/Pac cell line (from 0.5μM to 5μM) in combination with paclitaxel. The lowest administered dose of elacridar (0.5μM) reduced the IC_{50} value of paclitaxel by 94% whereas the highest administered dose (5μM) achieved 99% reduction which corresponded to almost complete reversal of the resistance.

4. According to XTT cell proliferation assay results, elacridar was more effective at lower concentrations of paclitaxel.

5. qPCR results indicated expression level of *MDR1* was significantly upregulated in MCF7/Pac cell line compared to MCF7/S cells. It was found that MCF7/Pac cell line expressed 208 fold more *MDR1* mRNA than parental cell line. This result is in line with literature and with previous

findings, demonstrating *MDR1* overexpression is the main reason of paclitaxel resistance.

6. Gene expression analyses indicated *MRP1* expression was significantly downregulated in MCF7/Pac compared to MCF/S parental cell line. It may be concluded that MCF7/Pac cell line adapted its gene expression profile in the favor of *MDR1* expression in order to gain resistance to paclitaxel.

7. Expression analyses indicated elacridar treatment significantly upregulated *MDR1* and *MRP1* expression in MCF7/Pac cell line in a dose dependent manner. Such increase could be due to a transient response mechanism against elacridar application to compensate the inhibition of already existing efflux pump P-gp.

8. Despite the fact that examined concentrations of elacridar caused upregulation of *MDR1* and *MRP1* gene expressions, this could not prevent elacridar from reversal of the resistance in MCF7/Pac cell line. One plausible reason is that elacridar is a very potent inhibitor of P-gp that exerts its effect on protein level and it can maintain its effectiveness even at high levels of *MDR1* and *MRP1* gene expressions.

9. In order to have the most efficient reversal by elacridar and to avoid excessive increases in expression levels of *MDR1* and *MRP1* genes, low concentrations of elacridar should be combined with low concentrations of paclitaxel. Such approach would also be beneficial to avoid cytotoxic effects in clinical applications.

Elacridar is a potent MDR modulator that can efficiently reverse P-glycoprotein mediated multidrug resistance at low concentrations in paclitaxel resistant cell line MCF7/Pac. This MDR reversal agent has potential to be effective without causing toxicity in breast cancer patients who developed paclitaxel resistance. On the other

hand, high concentrations of elacridar cause upregulation in *MDR1* and *MRP1* gene expression levels in a dose dependent manner. The significance of this finding should be further investigated by clinical studies. Moreover in clinical applications, careful dosing of the modulator should be administered and overdosing must be avoided.

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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 (mg/l)
NaCl	6000	L-methionine	15
KCl	400	L-phenylalaline	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
Pheneol red	5	L-tryosine	20
$NaHCO_3$	2000	L-valine	20
L-arginine	200	Glutathionie	1
L-asparagine	50	Biotin	0.2
L-aspartic acid	20	Vitamin B_{12}	0.005
L-cysteine	50	D-Ca-pantothenate	0.25
L-glutamine	300	Choline 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

Biochrom: RPMI 1640, retrieved from http://www.biochrom.de/fileadmin/user_upload/service/produktinformation/englisch/BC_catalogue_62_63_RPMI1640.pdf. Last accessed date: 2012, July 10.

APPENDIX B

BUFFERS AND SOLUTIONS

Phosphate buffered saline (Sigma-Aldrich, USA):

Phosphate buffered saline	1 tablet
dH_2O	200 mL

1 tablet of PBS was dissolved in dH_2O with the help of a magnetic stirrer and a magnetic stir bar. The solution was autoclaved at 121°C for 20 min. After cooling PBS solution was stored at 4°C.

Freezing medium:

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

Freezing medium was stored at 4°C.

Diethylpyrocarbonate (DEPC) treated water:

DEPC	1 mL
dH_2O	1 L

1 mL of DEPC was vigorously mixed with 1 L dH_2O. Solution was left for overnight incubation. Afterwards, DEPC treated water was autoclaved at 121°C for 20 minutes.

Ethidium bromide (EtBr) solution:

EtBr	10 mg
dH_2O	1 mL

Dissolved EtBr solution was kept in dark at 4°C.

Agarose Gel (For RNA sample loading):

Agarose	1 g
1X TAE Buffer	100 mL

Agarose Gel (For DNA sample loading):

Agarose	2 g
1X TAE Buffer	100 mL

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

Tris base (Mw: 121.14 g/mol)	242 g
Acetic acid	57.1 mL
0.5 M EDTA disodium dehydrate (Mw: 372.24 g/mol)	100 mL
dH_2O	842.9 mL

pH was adjusted to 8.5 and solution was autoclaved at 121°C for 20 min. 50X TAE buffer was diluted to 1X with dH_2O for use in electrophoresis tank and agarose gel preparations. The solution was stored at 4°C.

6X DNA Loading Dye (Fermentas, Lithuania):

60 mM EDTA, 10 mM Tris-HCl (pH 7.6), 0.03% Xylene cyanol FF, 0.03% Bromophenol blue, 60% Glycerol.

2X RNA Loading Dye (Fermentas, Lithuania):

0.5 mM EDTA

95% Formamide

0.025% SDS

0.025% Bromophenol blue

0.025% Xylene cyanol FF

0.025% Ethidium bromide

APPENDIX C

MCF7/Pac SUBLINE AND ITS PROPERTIES

The IC_{50} value of the MCF7/Pac subline was determined by XTT cell proliferation assay and reported as 317.94 ± 0.20 μM by Kars previously (Kars *et al.*, 2008). However, MCF7/Pac cells used in this study had been stored in liquid nitrogen for 6 years. In order to confirm the resistance of the MCF7/Pac subline, IC_{50} value was recalculated by XTT cell proliferation assay and the IC_{50} value was found to be 42.52 ± 4.79 μM in this study.

The dramatic decrease in the IC_{50} value of MCF7/Pac cell line indicates it has partially lost resistance to paclitaxel over time. This may be due to its long term storage in liquid nitrogen. Although the cells in liquid nitrogen should keep their properties, it is suggested in the cells which gain resistance to paclitaxel in a stepwise manner, the resistance may not be very stable. In case of constant exposure to paclitaxel in cell culture studies, it is expected that the cells will not lose their resistance. However, when the cells are kept in liquid nitrogen in a freezing medium which does not contain paclitaxel, the resistance seems to be partially reverted. The change in the IC_{50} value might be an indicator of changes in metabolism of the cells under storage conditions.

Since P-gp overexpression was the main reason of paclitaxel resistance in this subline (Kars *et al.*, 2008) most probable explanation of IC_{50} reduction was the downregulation of P-gp in either mRNA or protein level. Nevertheless, there are many other mechanisms contributing to resistance besides MDR1 upregulation.

For instance, any mutation in the genes that are related to detoxification mechanisms may have lowered the resistance of MCF7/Pac cells. *GSTP1* gene which encodes Glutathione S-transferase P, was significantly upregulated in previous report (Kars *et al.,* 2011). Since this protein is responsible from detoxification, downregulation of its expression could be a reason for lower IC_{50} value. Again in the previous report it was stated that, the genes that suppress cell death were upregulated (Kars *et al.*, 2011). If these gene expression levels were downregulated in the storage period without paclitaxel exposure, lower cell death avoidance could explain reduced IC_{50} value.

Kars *et al.* had also stated that *BRCA1* expression was significantly downregulated in MCF7/Pac cell line when compared to MCF7/S cell line becoming an important factor causing resistance (Kars *et al.*, 2011). This was due to the fact that, BRCA1 is a tumor suppressor that directs the cells to apoptosis after paclitaxel treatment (Quinn, *et al*., 2003). The reduced activity of BRCA1 is an indicator of resistance for mitotic inhibitor agents (Lafarge, 2001). According to these informations, upregulation of this gene could also reverse resistance in this cell line.

Paclitaxel resistance can also be due to the changes in microtubules. Since this anticancer drug exerts its effect by binding to microtubules, alterations in microtubule related genes may change microtubule dynamics and result in decreased paclitaxel efficiency (Cheung, 2010; İşeri *et al.* 2010). When the expression levels of MCF7 parental and MCF7/Pac cells for microtubule associated genes were compared by İşeri *et al.,* upregulation was seen in most of the microtubule related genes such as tubulins. The changes in their expression levels were proposed as another reason for paclitaxel resistance (İşeri *et al.*, 2010). Since these changes in the microtubules were due to the exposure of mitotic agent paclitaxel, it is possible that these alterations were reverted in an environment without the drug. Hence, long time storage in liquid nitrogen where paclitaxel is not present, may have altered the microtubule related gene expression levels back to their parental form.

In addition to these possible changes, new pathways or genes which were not found to be significantly altered in this cell line might have been activated (such as upregulation of Caspase 3) or deactivated (such as Bcl-2 downregulation) causing loss of resistance.

According to IC_{50} values for paclitaxel derived from XTT cell proliferation assay results, MCF7/Pac cells' overall resistance to paclitaxel was much lower than previously reported (Kars *et al.*, 2008). However, gene expression studies indicated MCF7/Pac cells relative *MDR1* gene expression in this study was higher than the relative *MDR1* gene expression in the same subline compared to previous report (Kars *et al.*, 2008). This supports the idea that decreased IC_{50} value during storage time was not due to reduced amounts of P-gp expression at mRNA level. It is more likely that the reduction in resistance level was a result of other altered resistance mechanisms of MCF7/Pac cell line. In any case, the results indicated that the resistance mechanism of MCF7/Pac cell line had changed over the years. In order to understand these changes and to predict the reasons behind better, a microarray study can be performed and compared to the previous microarray results (Kars *et al.*, 2008) of the same subline. Furthermore, this study suggests that when using drug resistant cell lines that had been stored in liquid nitrogen for a long time, at least the IC_{50} values of the corresponding drugs of resistant sublines should be recalculated in order to detect a possible decrease in the resistance levels of the cells.

APPENDIX D

LIGHT MICROSCOPY IMAGES OF

MCF7/S AND MCF7/Pac CELLS

MCF7/Pac cells can be easily distinguished from MCF/S cells under light microscope. As the cells gain resistance to paclitaxel many changes occur in their morphology as well as changes in their metabolism. In order to illustrate, photographs of MCF7/S and MCF/Pac cells were taken under inverted light microscope.

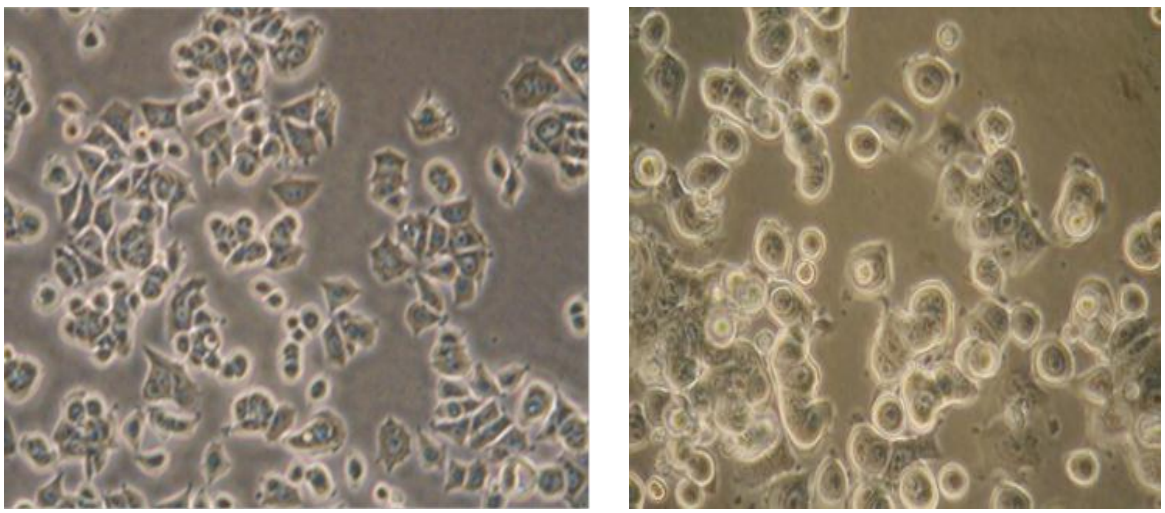

Figure D.1 Microscopic images of MCF/S cells. Magnifications are 200X and 400X respectively.

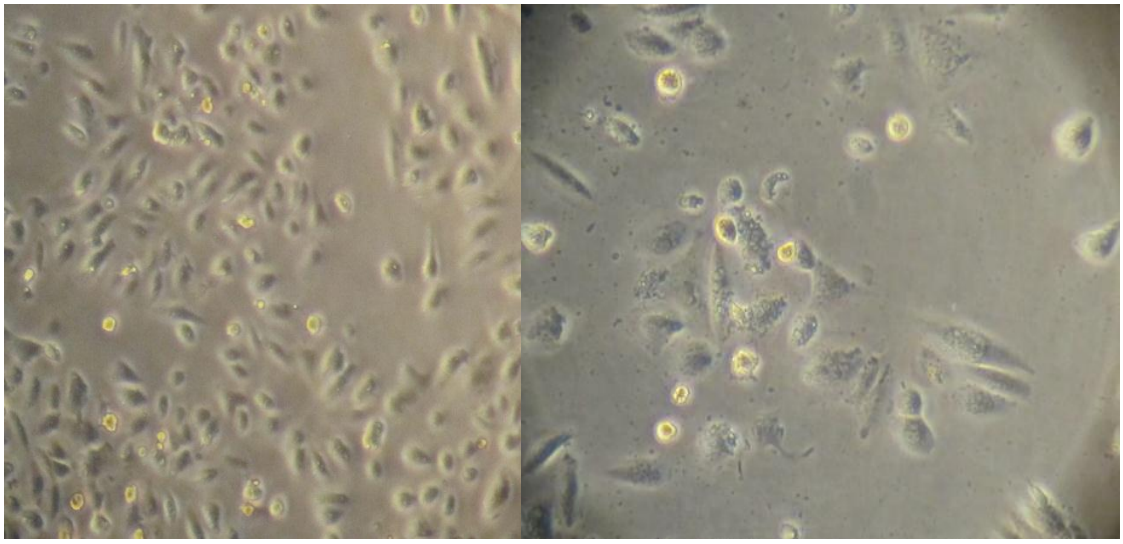

Figure D.2 Microscopic images of MCF/Pac cells. Magnifications are 200X and 400X respectively

When the cell pictures in Figure D.1 and Figure D.2 are compared the altered morphology can easily be seen. MCF/S cells are more round shaped cells whereas MCF7/Pac cells are thinner and longer. These visual changes in the morphology are only some of many changes that occured during multi drug resistance development.

In order to illustrate the resistance of MCF7/Pac cell line, different concentrations of paclitaxel was administered on MCF7/S and MCF/Pac cells. 72 hours later, cells were washed with PBS and microscopic images under inverted light microscope were taken.

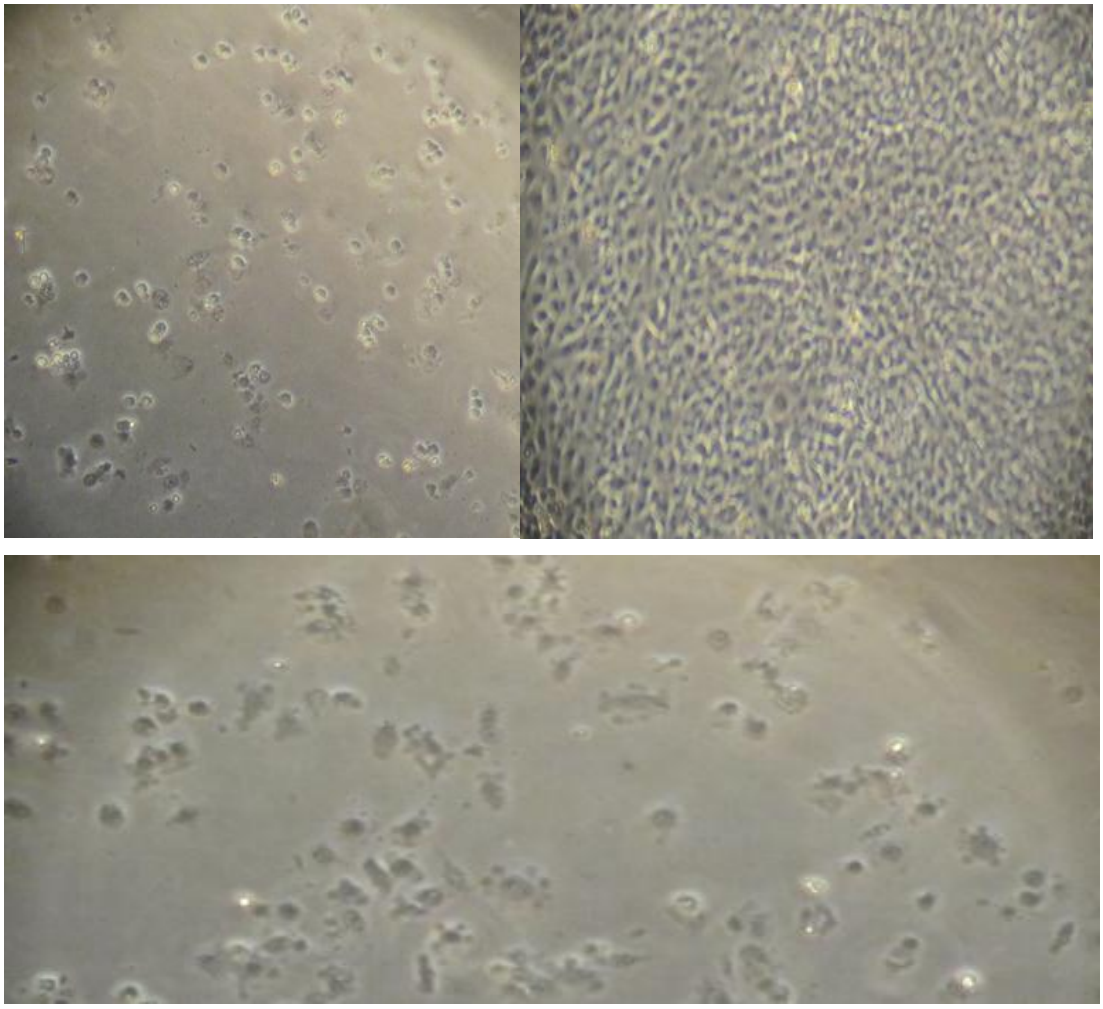

Figure D.3. Microscopic images of MCF7/S and MCF7/Pac cells after paclitaxel administration. Upper photographs show cell proliferation after 10 μM paclitaxel administration on MCF7/S (left) and MCF7/Pac cells (right). Bottom figure illustrates cell proliferation after 100 μM paclitaxel administration on MCF/Pac cells.

As seen in Figure D.3, administration of 10 μM paclitaxel on MCF/S cells dramatically lowered cell proliferation (left). On the other hand, 100% confluency observed in MCF7/Pac cells indicated the same concentration of paclitaxel administration did not cause any antiproliferative effect (right). Bottom picture

represents 100µM paclitaxel treated MCF7/Pac cells. In this picture MCF7/Pac cells' shape is similar to disrupt MCF7/S cells. Loss of characteristic shape and excessive cell death can be observed.

Although examination under light microscope may give an idea about cytotoxicity of an anticancer drug, for certain assesments cell proliferation assays should be performed. In the current study, this measurement was carried out by XTT cell proliferation assay. Figure D.4 represents a typical 96-well plate used in an XTT assay experiments.

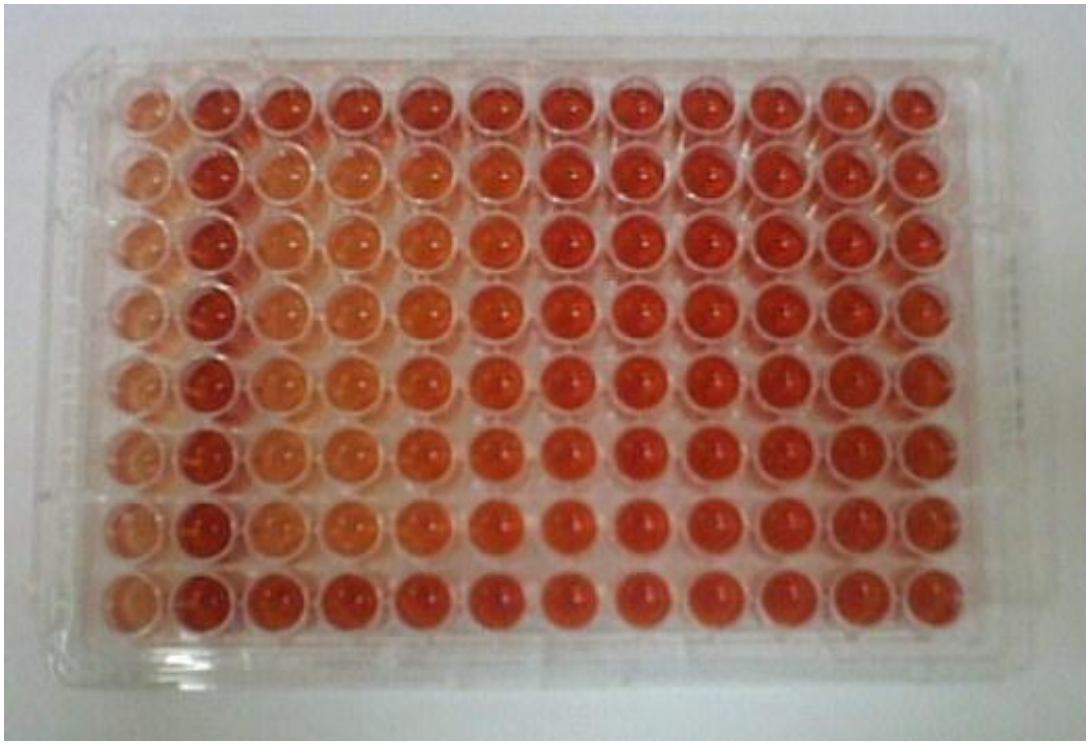

Figure D.4 A 96-well plate photograph. First column medium control, second column cell control, third column highest drug concentration column. From left to right drug concentration decreased. Upper and bottom rows 3-12 were DMSO control wells.

In Figure D.4, first column is the medium control column where no cells were seeded. Second column is the cell control column where cells were seeded but no drug was administered. The red color demonstrated the cells were healthy and metabolized tetrazolium salts into formazan compounds. Lighter colors as can be seen in columns 3-4-5-6 indicated in these wells some of the cells were dead. Therefore metabolization of tetrazolium salts was less than cell control column. Higher concentrations of the drug resulted in more death and formed lighter colors, whereas lower concentrations of the drug was less toxic and colors similar to cell control column were formed. Hence, the gradual change from orange to red among columns colors suggested dilution of the drug was successfully carried out. Upper and bottom rows which contained cells and DMSO only, were in the same color with cell control columns. This observation partially proved DMSO administered with paclitaxel and/or elacridar was not toxic to cells in the investigated concentrations, since cells in DMSO control rows were able to efficiently metabolize tetrazolium salts as much as the cells in cell control column.

APPENDIX E

EXPRESSIONS OF *MDR1*, *MRP1* and *β-actin*

In gene expression studies, the expression levels of *MDR1*, *MRP1* and *β-actin* were identified with qPCR. In order to control whether the amplified products were the product of interest, samples were run on 2% agarose gel in the previously described manner (section 2.2.3.6.1) for 90 min at 100 V.

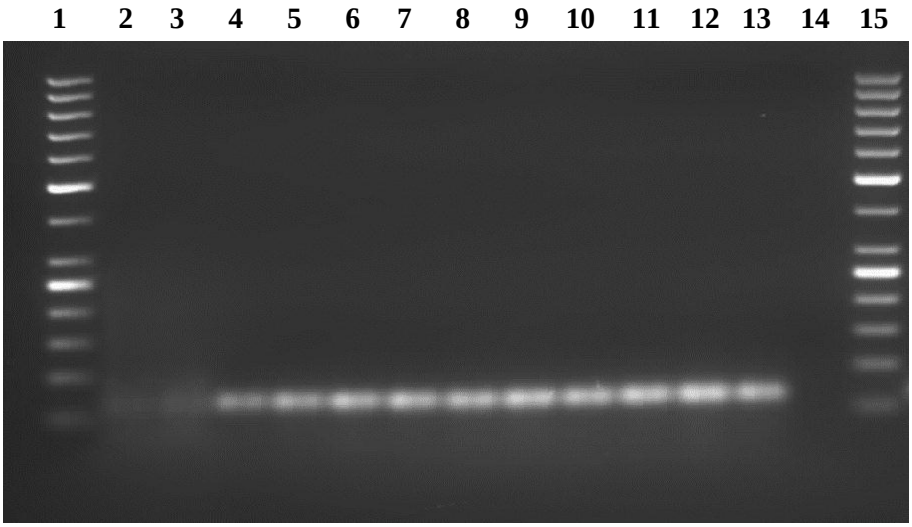

Figure E.1 Expression of *MDR1*. Lane 1 and 15: 50bp DNA ladder (Fermantas, Lithuania), Lane 2 and 3 *MDR1* expression in MCF7/S, Lane 4 and 5 *MDR1* in MCF7/Pac, Lane 6 and 7 *MDR1* in 5μM elacridar treated MCF7/Pac, Lane 8 and 9 *MDR1* in 2.5μM elacridar treated MCF7/Pac, Lane 10 and 11 *MDR1* in 1μM elacridar treated MCF7/Pac, Lane 12 and 13 *MDR1* in 0.5μM elacridar treated MCF7/Pac, Lane 14 Negative control

Due to very low expression level of *MDR1* in MCF7/S cells, their corresponding bands were hardly visible. This result was consistent with late Ct values obtained for MCF7/S in qPCR experiments.

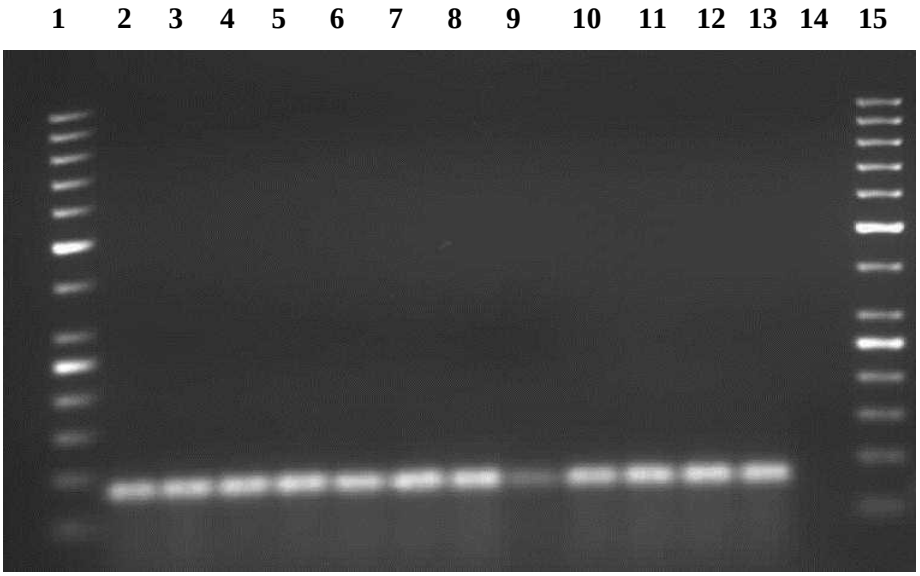

Figure E.2 Expression of *MRP1*. Lane 1 and 15: 50bp DNA ladder (Fermantas, Lithuania), Lane 2 and 3 *MRP1* expression in MCF7/S, Lane 4 and 5 *MRP1* in MCF7/Pac, Lane 6 and 7 *MRP1* in 5μM elacridar treated MCF7/Pac, Lane 8 and 9 *MRP1* in 2.5μM elacridar treated MCF7/Pac, Lane 10 and 11 *MRP1* in 1μM elacridar treated MCF7/Pac, Lane 12 and 13 *MRP1* in 0.5μM elacridar treated MCF7/Pac, Lane 14 Negative control

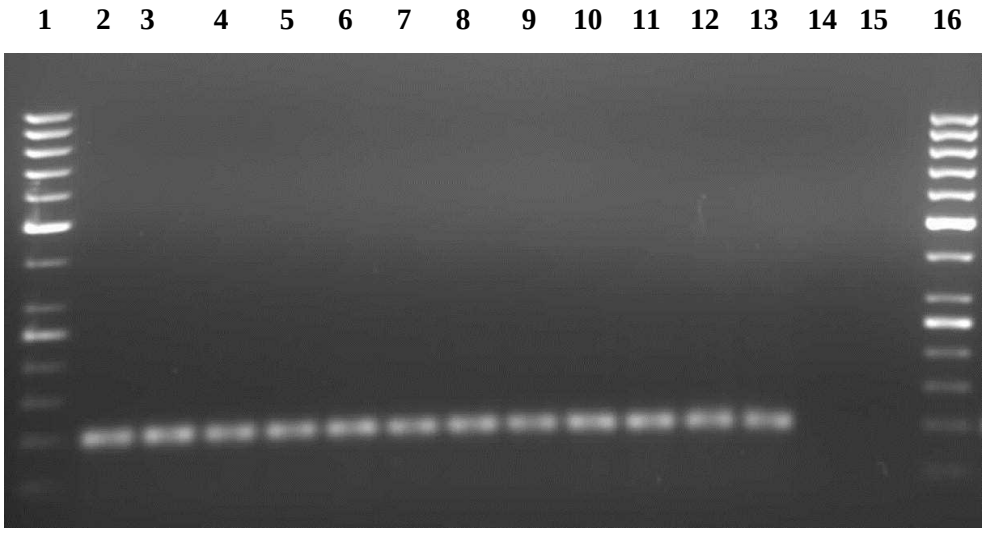

Figure E.3 Expression of *β-actin.* Lane 1 and 16: 50bp DNA ladder (Fermantas, Lithuania), Lane 2 and 3 *β-actin* expression in MCF7/S, Lane 4 and 5 *β-actin* in MCF7/Pac, Lane 6 and 7 *β-actin* in 5μM elacridar treated MCF7/Pac, Lane 8 and 9 *β-actin* in 2.5μM elacridar treated MCF7/Pac, Lane 10 and 11 *β-actin* in 1μM elacridar treated MCF7/Pac, Lane 12 and 13 *β-actin* in 0.5μM elacridar treated MCF7/Pac, Lane 14 and 15 Negative controls