MOLECULAR MECHANISMS OF VINCRISTINE AND PACLITAXEL RESISTANCE IN MCF-7 CELL LINE

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MOLECULAR MECHANISMS OF VINCRISTINE AND PACLITAXEL RESISTANCE IN MCF-7 CELL LINE

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ABSTRACT

MOLECULAR MECHANISMS OF VINCRISTINE AND PACLITAXEL RESISTANCE IN MCF-7 CELL LINE

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Resistance to broad spectrum of chemotherapeutic agents in cancer cell lines and tumors has been called multiple drug resistance (MDR). In this study, the molecular mechanisms of resistance to two anticancer agents (paclitaxel and vincristine) in mammary carcinoma cell line MCF-7 were investigated.

MCF-7 cells were selected in the presence of paclitaxel and vincristine by stepwise dose increments. The cell viability and growth profiles of resistant sublines were examined. As the resistance indices increased, the growth rates of sublines were found to decrease. Gene and protein expression levels of the basic drug resistance proteins P-gp and MRP1 were studied in sensitive and drug resistant MCF-7 cells. It was shown that P-gp overexpression is significantly contributing to the developed drug resistance phenotype.

Mutation analysis of *beta tubulin* gene which encodes the target of paclitaxel and vincristine was performed. Single histidine to proline mutation was identified near GTP binding site of beta tubulin in vincristine resistant subline which was not reported before.

Apoptosis related *BCL-2* and *BAX* were examined at both gene and protein expression levels and they were not found to be significantly related to the developed resistance in the sublines.

The reversal of drug resistance by various inhibitory agents of P-gp and MRP1 was investigated by using flow cytometry. Synthetic silicon compounds were found to be the most effective MDR reversal agents. The effects of various combinations of anticancer drugs and reversal agents on cell proliferation were examined by checkerboard microplate method. ALIS409-paclitaxel and paclitaxel-doxorubicin pairs seem to have highest antiproliferative effects on resistant sublines.

The microarray expression profiling of sensitive and resistant MCF-7 cells was performed for a much detailed and comprehensive analysis of drug resistance. The results indicated that the upregulation of *MDR1* gene is the dominating mechanism of paclitaxel and vincristine drug resistance. Additionally up regulation of the genes encoding the detoxifying enzymes (i.e. GSTP1) was observed. Significant down regulation of apoptotic genes (i.e. *PDCD2/4/6/8*) and alterations in expression levels of genes related to invasion and metastasis (*MMPs, ADAMs, COL4A2, LAMA* etc.) were detected. Upregulation of some oncogenes (i.e. *ETS, RAS*) and cell cycle regulatory genes (*CDKN2A, CCNA2* etc.) was seen which may be in close relation to MDR in breast cancer. Further studies will demonstrate the relationship between the components contributing to drug resistance phenotype in breast cancer cells.

Key words: MCF-7, multiple drug resistance, apoptotic pathway, beta tubulin, MDR reversal, microarray analysis

ÖZ

MCF-7 HÜCRE HATTINDA PAKLİTAKSEL VE VİNKRİSTİN DİRENÇLİLİĞİNİN MOLEKÜLER MEKANİZMALARI

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Kanser hücre hatlarında ve tümörlerde kemoterapötik ajanlara karşı gelişen dirençliliğe çoklu ilaç dirençliliği (MDR) denir. Araştırmada, meme kanseri hücre hattında MCF-7 hücre hattında iki antikanser ajana (paklitaksel ve vinkristin) karşı gelişen dirençliliğinin moleküler mekanizmaları araştırılmıştır.

MCF-7 hücreleri paklitaksel ve vinkristin doz artışlarıyla seçilerek dirençli hatlar geliştirilmiştir. Dirençli hücrelerde hücre yaşam ve üreme profilleri incelenmiştir. Hücre hatlarında dirençlilik düzeyleri arttıkça hücre üreme hızlarının azaldığı bulunmuştur. Dirençli MCF-7 hücre hatlarında ilaç dirençliliği proteinleri olan P-gp ve MRP1'nin gen ve protein düzeylerindeki değişimler araştırılmıştır. P-gp'de aşırı gen ifadesinin gelişen dirençliliğe önemli ölçüde katkısı olduğu bulunmuştur.

Paklitaksel ve vinkristin ilaçlarının hedefi olan beta tübülin proteinini kodlayan gende mutasyon analizi yapılmıştır. Vinkristine dirençli hücre hatlarında tübülinin GTP bağlanma bölgesinde bulunan histidini proline döndüren, tek amino asit değişikliği bulunmuştur. Bu mutasyon literatürde ilk defa gösterilmektedir.

Apoptoz yolağında rol oynayan *BCL-2* ve *BAX* ,gen ve protein ifade düzeyleri açısından incelenmiş ve dirençliliğe anlamlı derecede katkı yapmadıkları gösterilmiştir.

P-gp ve MRP1 ile oluşan ilaç dirençliliğinin kimyasal modülatörler kullanılarak geri çevrilmesi denenmiş ve modülatörlerin etkisi flow sitometrik ölçümler ile belirlenmiştir. Sentetik silikon bileşikleri dirençliliği geri çeviren en etkili ajanlar olarak belirlenmiştir. Farklı antikanser ajanlar hücrelere birarada eklendiklerinde, veya antikanser ajanlar ile dirençliliği geri çeviren ajanlar birlikte kullanıldıkları zaman hücre çoğalmasına olan etkileri checkerboard microplate yöntemi ile araştırılmıştır. ALIS409-paklitaksel ve paklitaksel-doksorubisin çiftlerinin hücre çoğalmasını engellemede en büyük etkilerinin olduğu bulunmuştur.

MCF-7 hücre hatlarında ilaç dirençliliği mekanizmalarını daha detaylı ve kapsamlı olarak anlayabilmek amacıyla mikrodizin analizi yapılmıştır. Bu analiz yönteminde de *MDR1* ifadesinin artışı en önemli ilaç dirençliliği mekanizmalarından birisi olarak belirlenmiştir. Ayrıca ,hücrelerde toksik maddelerin metabolize edilmesi ile ilgili genlerin (GSTP1 vb.) ifade düzeylerinde ciddi artışlar gözlenirken,apoptoz ile ilgili bazı önemli genlerin (*PDCD2/4/6/8*) ifade düzeylerinde önemli azalmalar bulunmuştur. Metastaz ile ilgili birçok genin (*MMPlar, ADAMlar, COL4A2, LAMA* vb.) ifade düzeylerinde değişimler gözlenmiştir. Bazı onkogenlerin (*ETS, RAS vb.*) ve hücre döngüsünü düzenleyen genlerin (*CDKN2A, CCNA2* vb) ifade düzeylerinde önemli değişimler gözlenmiştir. Bahsedilen genlerin meme kanserinde ilaç dirençliliği gelişimine katkıları olduğunu söylemek mümkündür. Ancak, yeni yapılacak araştırmalarla, etkili görülen bu genlerin ilaç dirençliliğinde ne derece önemli oldukları ve birbirleri ile olan ilişkileri açığa çıkarılacaktır.

Anahtar kelimeler: MCF-7, çoklu ilaç dirençliliği, apoptotik yolak, beta tübülin, MDR geri çevirilmesi, mikrodizin analizi

To Fatma and Hüseyin Demirel

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MDR	Multiple Drug Resistance
P-gp	P-glycoprotein
MRP1	Multiple Drug Resistance Associated Protein 1
BCRP	Breast Cancer Resistance Protein
ABC	ATP Binding Casette
TUBB	Beta Tubulin
MCF-7/S	Sensitive MCF-7 Cell Line
MCF-7/400nMPac	400 nM Paclitaxel adapted resistant MCF-7 cell line
MCF-7/120nMVinc	120 nM Vincristine adapted resistant MCF-7 cell line
IC_{50}	Inhibitory Concentration 50
GTC	Guanidium Thiocyanate
EtBr	Ethidium Bromide
RT-PCR	Reverse Transcription Polymerase Chain Reaction
Q-PCR	Quantitative Polymerase Chain Reaction
dH_2O	Distilled water
dNTP	Deoxy ribonucleotide triphosphate
DMSO	Dimethyl sulfoxide
IVT	*In vitro* Tanscription
DI water	Double ionized water
EtOH	Ethanol
FAR	Fluorescent Activity Ratio
R123	Rhodamine 123
CF	Carboxy Fluorescein
ATRA	All trans Retinoic Acid
cGy	Centi-Gray

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells (American Cancer Society, 2006). Cancer is defined as tumor formation in any part of the body that is resulted from unregulated cell division. It is one of the most threatening health problems that cause death among populations. Cancer is caused by external factors (tobacco, chemicals, radiation and infectious organisms) or internal factors (inherited mutations, hormones, immune conditions and mutations) (American Cancer Society, 2006). As tumor mass continues to grow, tumor cells invade the surrounding tissues and get access to the vascular system. This causes spread of tumor cells to distant organs and metastasis (Cancer Research UK, 2007).

Cancer cells develop because of damage to DNA. Most of the time when DNA becomes damaged the body is able to repair it. In cancer cells, the damaged DNA is not repaired. While solid cancers form tumor. Some cancers, like leukemia, do not form tumors. Instead, these cancer cells involve the blood and blood-forming organs and circulate through other tissues where they grow. Often, cancer cells travel to other parts of the body where they begin to grow and replace normal tissue. This process is called metastasis. Regardless of where a cancer may spread, it is always named for the place it began. For instance, breast cancer that spreads to the liver is still called breast cancer, not liver cancer.

Not all tumors are cancerous. Benign (noncancerous) tumors do not spread (metastasize) to other parts of the body and, with very rare exceptions, are not life threatening. Different types of cancer can behave very differently. For example, lung

cancer and breast cancer are very different diseases. They grow at different rates and respond to different treatments (Croce, 2008). Most cancer types affect regulatory pathways and DNA fidelity in the cell. Most of the studies are concentrated on correction of molecular impairments which are caused by cancer cells and tumor mass. According to statistics worldwide, about 10 million people are diagnosed with cancer every year and 6 million people die from it. There are currently about 22 million cancer patients in the world. The National Cancer Institute assumes that the annual number of diagnoses will reach 15 million by 2050. Colon, lung, breast and prostate cancer types are mostly seen cases among populations (The National Cancer Institute, http://www.cancer.gov/cancertopics).

1.2 Breast Cancer

Breast cancer originates from terminal ducto-lobular unit of breast tissue. Breast cancer that has not invaded the basement membrane is termed as *in situ* carcinoma; on the other hand breast cancers that invade the basement membrane are called invasive cancers. Invasive type results in distant organ metastases (The National Cancer Institute, http://www.cancer.gov/cancertopics ; Atalay, 2004).

Breast cancer is the most commonly diagnosed type among women worldwide. Although mortality rates are declining, breast cancer ranks second among cancer deaths in women (Cancer Research UK, 2007).. Women who have a personal or family history of breast cancer, whose menstrual periods started early and ended late in life, with obesity after menopause, who recently used oral contraceptives or postmenopausal estrogens and progestins, who have never had children or had their first child after age 30 and who consume alcoholic beverages are under risk. (Cancer Research UK, 2007).

The reduction of mortality is possible by identification of risk factors, early diagnosis and improvement of treatment strategies (American Cancer Society, 2006). The factors influencing breast cancer are summarized in Figure 1.1.

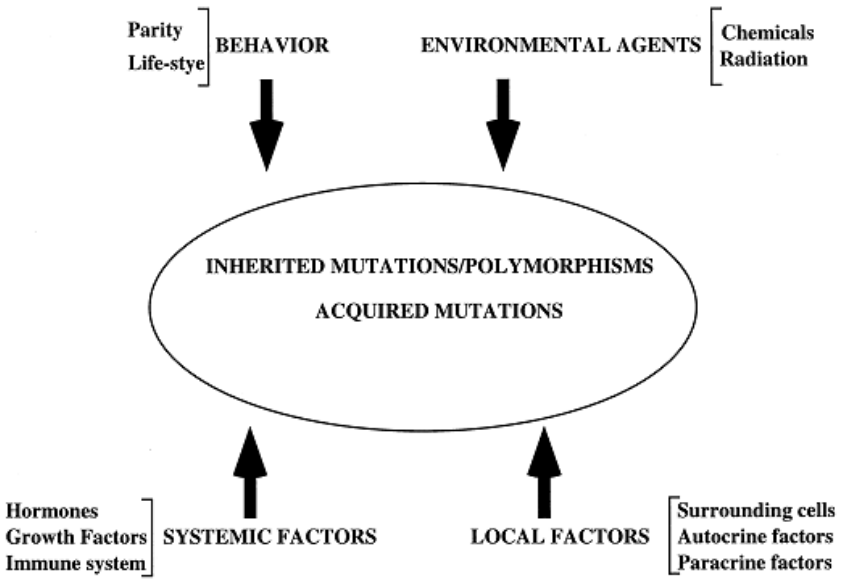


Figure 1.1 Summary of factors influencing breast cancer (Figure was taken from Polyak, 2001).

Treatment of breast cancer may involve local removal of tumor, surgical removal of breast, radiation therapy, chemotherapy, hormone therapy (American Cancer Society, 2006). Tumor size, stage and other characteristics in addition to patient preference are important factors during selection of treatment type.

In *in vitro* studies scientists need model cell lines for tumor types, so that some molecular analysis is performed on the model cell lines. MCF-7 is a model cell line for mammary epithelium carcinoma (Zava *et al*., 1977).

1.3 Chemotherapy

Chemotherapy is the treatment of cancer with drugs that can destroy cancer cells. These drugs - often called anticancer drugs - destroy cancer cells by stopping them from growing or multiplying (Hirsch, 2006). Healthy cells can also be harmed, especially those that divide quickly. These cells usually repair themselves after chemotherapy. Because some drugs work better together than alone, often two or

more drugs are applied at the same time. This is called combination chemotherapy. Chemotherapy could be applied to patients either before (neoadjuvant) or after surgery (adjuvant).

Chemotherapy drugs are divided into several categories based on how they affect specific chemical substances within cancer cells, which cellular activities or processes the drug interferes with, and which specific phases of the cell cycle the drug affects (Hirsch, 2006).

Alkylating agents work directly on DNA to prevent the cancer cell from reproducing. As a class of drugs, these agents are not phase-specific (in other words, they work in all phases of the cell cycle). These drugs are active against chronic leukemias, non-Hodgkin's lymphoma, Hodgkin's disease, multiple myeloma, and certain cancers of the lung, breast, and ovary. Examples of alkylating agents include busulfan, cisplatin, carboplatin, chlorambucil, cyclophosphamide, ifosfamide, dacarbazine (DTIC), mechlorethamine (nitrogen mustard), and melphalan (Rummel, 2008).

Antimetabolites are a class of drugs that interfere with DNA and RNA growth. These agents work during the S phase and are used to treat chronic leukemias as well as tumors of the breast, ovary, and the gastrointestinal tract. Examples of antimetabolites include 5-fluorouracil, capecitabine, methotrexate, gemcitabine, cytarabine (ara-C), and fludarabine (Ewald *et al*., 2008).

Antitumor antibiotics interfere with DNA by stopping enzymes and mitosis or altering the membranes that surround cells. These agents work in all phases of the cell cycle. Thus, they are widely used for a variety of cancers. Examples of antitumor antibiotics include dactinomycin, daunorubicin, doxorubicin (Adriamycin), idarubicin, and mitoxantrone (Galm *et al*., 2005).

Antimitotic agents are plant alkaloids and other compounds derived from natural products. They inhibit mitosis or inhibit enzymes for making proteins needed for reproduction of the cell. These work during the M phase of the cell cycle. Examples

of mitotic inhibitors include paclitaxel, docetaxel, etoposide (VP-16), vinblastine, vincristine, and vinorelbine (McGrogan *et al*., 2008).

Among hormone receptor targetting drugs, tamoxifen is a synthetic non-steroidal anti-estrogen that is used in the treatment of estrogen receptor-positive breast cancer patients (Love, 1989). Tamoxifen is currently used for the treatment of both early and advanced ER+ (estrogen receptor positive) breast cancer, it is also approved by the FDA for the prevention of breast cancer in women at high risk of developing the disease.

Retinoids, alone or in combination, have been promising agents for treatment of some cancers including breast cancer. All-trans-retinoic acid is a natural ligand of nuclear retinoic acid receptors (RARs) that activate RA-responsive genes which have effects on cell proliferation, differentiation and apoptosis (Wu *et al*., 1997).

1.3.1 Antimitotic Agents

Tubulin, a protein of molecular weight 100,000 Da, is the principal protein subunit of microtubules. It is the cellular target for antimitotic agents (Drukman and Kavallaris, 2002). Some of the known anti tubulin agents are vinblastine, vincristine, maytansine, rhizoxin, paclitaxel (Taxol) and docetaxel (Taxotere). The first four are anticancer agents that inhibit tubulin polymerization and are thought to bind to the same site of tubulin. The mechanism of actions of paclitaxel and docetaxel are quite different from that of the other four, for it promotes the assembly of microtubules, resulting in highly stable, nonfunctional polymers (Fitzpatric and Wheeler, 2003).

1.3.1.1 Vincristine (ONCOVIN®)

Vincristine belongs to the general group of chemotherapy drugs known as plant (vinca) alkaloids. Vinca alkaloids are found in the Madagascar periwinkle, *Catharanthus roseus* (formerly classified as *Vinca rosea*). The vinca alkoloids bind to tubulin dimers at a specific recognition site on tubulin. The tubulin- drug complex

is able to form paracystalline aggregates. This reduces the concentration of the dimers and pushes the equilibrium between growth and shrinking of the microtubules in favor of shrinking. Cells treated with vincristine loose the ability to progress through mitosis correctly because of poorly formed mitotic spindles. The demaged cells then die (Drukman and Kavallaris, 2002).

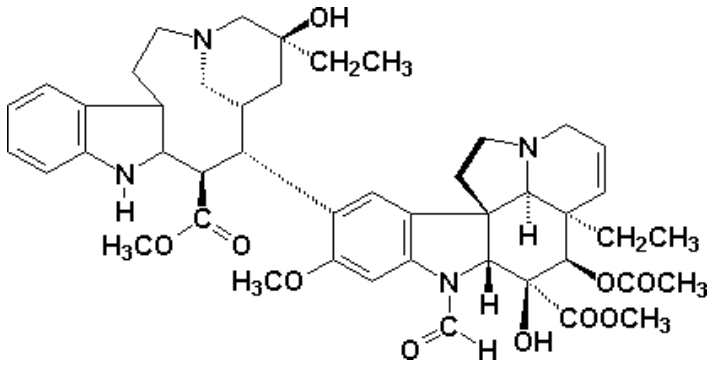


Figure 1.2 Chemical structure of vincristine.

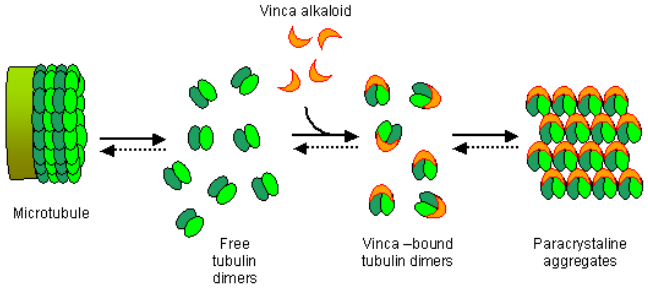


Figure 1.3 Mechanism of action of vincristine.

1.3.1.2 Paclitaxel (TAXOL®)

Paclitaxel (Taxol) is a taxoid drug, extracted from the bark of the Pacific yew and needles of English yew (Fitzpatric and Wheeler, 2003). Paclitaxel is among the most

unique, successful chemotherapeutic agents used for treatment of breast and ovarian cancer intravenously. Paclitaxel binds microtubules and causes kinetic stabilization of microtubule dynamics that result in inhibition of mitosis.

Paclitaxel binds to tubulin dimers at a different site from vincristine. In this case, formation of abnormal microtubules is promoted. Cell death occurs during G1 phase and during G2-M transition which are caused by disorganized microtubule filaments. In December 1992, the U.S. Food and Drug Administration (FDA) approved the use of paclitaxel for ovarian cancer that was resistant to treatment. Paclitaxel was later approved as initial treatment for ovarian cancer in combination with cisplatin. Women with epithelial ovarian cancer are now generally treated with surgery followed by a taxane and a platinium therapy. The FDA has also approved paclitaxel for the treatment of breast cancer that recurred within 6 months after adjuvant chemotherapy (chemotherapy that is given after the primary treatment to enhance the effectiveness of the primary treatment), or that spread (metastasized) to nearby lymph nodes or other parts of the body. Paclitaxel is also used for other cancers, including AIDS-related Kaposi's sarcoma and lung cancer.

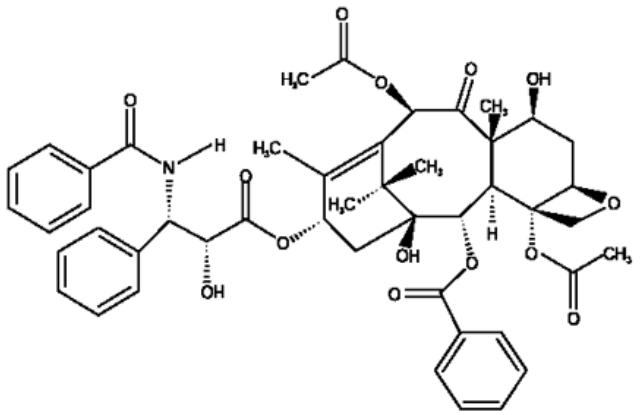


Figure 1.4 Chemical structure of paclitaxel.

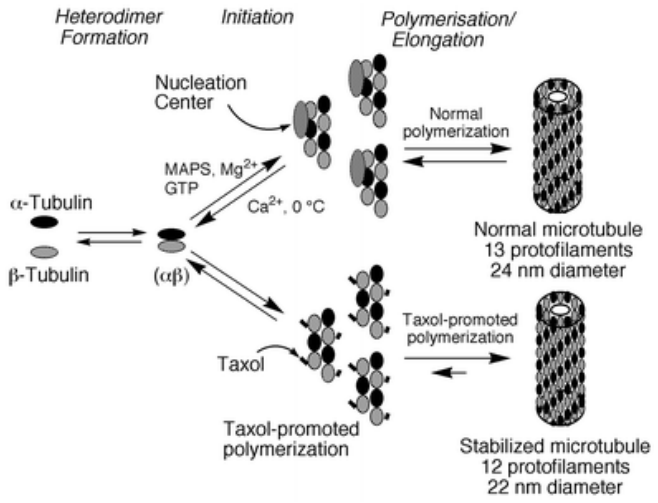


Figure 1.5 Mechanism of action of paclitaxel. (Fitzpatric and Wheeler, 2003)

1.4 Multiple Drug Resistance

In vitro studies demonstrated that cancer cells might become relatively resistant to chemotherapeutics through a variety of mechanisms. Increased drug efflux that results from up-regulation of ATP binding cassette (ABC) transporters such as multidrug resistance protein 1 (MDR1, P-gp), multidrug resistance associated protein 1 (MRP1), breast cancer resistance protein (BCRP) and lung resistance protein (LRP) (Bodo *et al*, 2003; Lage, 2003), mutations in genes encoding drug target proteins (Giannakakau et.al., 2000), differential expressions of drug target proteins, up-regulation of anti-apoptotic BCL-2 family members and downregulation of BAX pro-apoptotic protein (Lilling et.al., 2000) are some known mechanisms of drug resistance.

These mechanisms lead to development of a phenotype known as 'multidrug resistance (MDR)' (Ueda *et al*., 1987). MDR may be intrinsic, making malignant cells resistant to numerous unrelated drugs or may be acquired after exposure to chemotherapy (Krishan *et al*., 1997). The genes represent the largest family of transmembrane proteins that bind ATP and use energy to drive the transport of various molecules across all cell membranes. P-gp is thought to play a role in

protecting cells from chemical toxicity and oxidative stress and to mediate inflammatory responses involving cysteinyl leukotrienes (Sparreboom *et al*., 2003). MRP1 confers resistance to several compounds, with similar profile to that of P-gp. However, unlike P-gp, MRP1 transports drugs (i.e. chlorambucil, melphalan) that are conjugated to glutathione (Zaman *et al*., 1994). Consistent with P-gp and the MRPs, BCRP also confers resistance to a variety of drugs. Significant resistance is observed with mitoxanthrone, anthracyclines, camptothecins and its derivatives, mainly topotecan (Tan *et al*., 2000). The substrates shared by three ABC transporter proteins (P-gp, MRP1, BCRP) are daunorubicin, doxorubicin, and epirubicin.

Clinical resistance to chemotherapy results from the interaction of numerous biological variables. Overproduction of BCL-2 protein prevents cell death induced by almost all cytotoxic anticancer drugs and radiation, and contributes to drug resistance in patients with some types of cancer (Reed, 1995). BAX is a cell death protein with homology to the anti-apoptotic BCL-2 protein and decrease in *BAX* expression promotes drug resistance (McCurrach *et al*., 1997). Alterations in drug target genes and/or their expression levels are also other mechanisms for drug resistance development. Mutations in amino acid residues of β-tubulin which abolish taxol and vincristine binding (Giannakakou *et al*., 1997) and or differential expression of various tubulin isotypes (Kavallaris *et al*., 2001) may promote MDR phenotype.

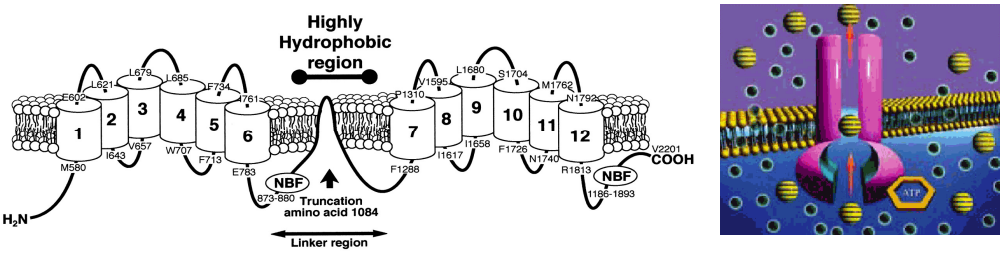
1.4.1 Up-regulation of ABC Transporter Protein Family Members

Hydrophobic drugs enter the cells by diffusion across plasma membrane. Energy dependent transport systems are needed to expel these drugs out of the cell. These pumps namely ATP binding cassette (ABC) transporters work by coupling the hydrolysis of ATP to substrate transport accross the cell membrane. In human, there are 49 members in this ABC transporter superfamily which are divided into seven subfamilies (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCG) (Liu et.al., 2005).

Among these ABC transporter members, several are known to efflux anticancer drugs and, thus, cause drug resistance when over-expressed in model cancer cell lines. On the other hand, two of the 49 members of the human ABC transporter family, ABCE1 (also known as RNase Li and OABP) and ABCF1 (also known as ABC50), are known to lack transmembrane domains and localized in cytosolic fractions (Dean and Allikmets, 2001). Therefore, they are unlikely to be membrane transporters. The highly recognized members are P-glycoprotein (P-gp, ABCB1) and multidrug resistance associated protein (MRP1, ABCC1), breast cancer resistance protein (BCRP, ABCG2) have been identified by Chen *et al*., 1986; Cole and Deeley, 1996; Doyle *et al*., 1998 respectively. Increase in expression levels cause drug efflux. So the cells are namely drug resistant.

1.4.1.1 P-glycoprotein (P-gp)

P-glycoprotein is encoded by *MDR1* (ABCB1) gene. The gene is located on chromosome 7q21.1 with 28 exons. P-gp is 170 kDa protein of 1280 aa polypeptide (Safa, 2004; Germann, 1996). The protein contains 12 transmembrane segments, 6 domains and 2 nucleotide binding domains. Structute of P-gp is represented in Figure 1.6.



(a) (b)

Figure 1.6 P-glycoprotein (a) transmembrane segments (Germann, 1996) (b) as efflux pump.

Intestine, liver, kidney, placenta and brain intrinsically express P-gp. In patients during chemotherapy, P-gp functions as a drug pump extruding drug molecules through the plasma membrane. Uncharged drug molecules entering the cells by passive diffusion are removed from membrane before they can enter the cell (Mechetner *et al*. 1998; Filipits *et al*., 1996). Some of the substrates are listed in Table 1.1.

Table 1.1 Some of the substrates of P-glycoprotein and MRP1.

Protein	**P-glycoprotein**	**MRP1**
Anticancer drugs	Doxorubicin	Doxorubicin
	Daunorubicin	Daunorubicin
	Vinblastin	Vincristine
	Vincristine	Etoposide
	Actinomycin D	Methotrexate
	Paclitaxel	Melphalan

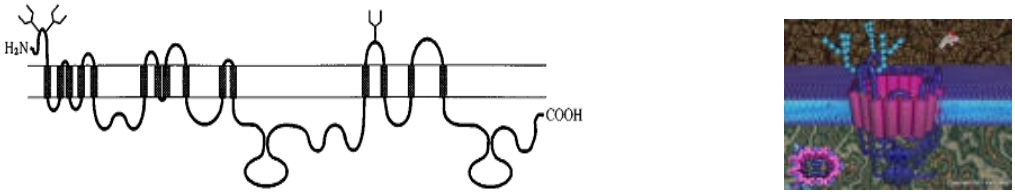
1.4.1.2 Multidrug Resistance Associated Protein 1 (MRP1)

The *MRP1* (ABCC1) gene maps to chromosome 16p13.1 and is expressed in many resistant tumor cells (Cole *et al*, 1992). It encodes a full transporter that is the principal transporter of glutathione-linked organic compounds from cells.

The glutathione S-transferase (GST) family of enzymes comprises a long list of cytosolic, mitochondrial, and microsomal proteins that are capable of multiple reactions with a multitude of substrates, both endogenous and xenobiotic. These enzymes can constitute up to 10% of cytosolic protein in some mammalian organs (Boyer 1989). GSTs catalyse the conjugation of reduced glutathione via the sulfhydryl group, to electrophilic centers on a wide variety of substrates. This

activity is useful in the detoxification of endogenous compounds such as peroxidised lipids, as well as the metabolism of xenobiotics (Douglas, 1987).

The ABCC1 pump (multidrugresistance-associated protein-1, (MRP1)) confers resistance to several compounds, with similar profile to that of P-gp (Table1.1). However, unlike P-gp , MRP1 transports drugs that are conjugated to glutathione. The MRP1 protein is thought to play a role in protecting cells from chemical toxicity and oxidative stress and to mediate inflammatory responses involving cysteinyl leukotrienes. Figure 1.7 represents the topology of MRP1 protein.



(a) (b)

Figure 1.7 MRP1 protein (a) transmembrane segments (Cole *et al*, 1992) (b) as efflux pump.

1.4.1.3 Breast Cancer Resistance Protein (BCRP)

Breast cancer resistance protein (BCRP) is a newly discovered ABC transporter isolated from human breast cancer cells that were selected with doxorubicin in the presence of verapamil. BCRP is encoded by the ABCG2 gene which is mapped on chromosome 4 (4q22) (Kawabata *et al*., 2001). One difference between BCRP and the other ABC transporters is the fact that it is composed of only one transmembrane region and one ATP-binding domain while other transporters are composed of two transmembrane regions and two ATP-binding domains (Brangi *et al*., 1999). BCRP's homology with the Drosophila white gene family, a white eye pigment gene, suggests that BCRP requires heterodimerization or homodimerization in order to

function in the transport activity of cytotoxic agents (Scheffer *et al*., 2001). For this reason, BCRP is referred to as a half transporter. Consistent with P –gp and the MRPs, BCRP also confers resistance to a variety of drugs. The drug displaying the highest resistance due to BCRP appears to be mitoxantrone. Lesser but still significant resistance is observed with anthracyclines, daunorubicins, doxorubicin, camptothecins (Tan *et al*., 2000) and its derivatives, mainly topotecan and SN-38. BCRP also displays cross resistance to many topoisomerase inhibitors.

1.4.1.4 Lung Resistance Protein (LRP)

The lung resistance protein (LRP) was initially identified in an anthracycline-resistant, non-small cell lung cancer cell line that lacked P-gp overexpression (Scheper *et al*., 1993). The LRP gene is located on chromosome 16 (16p11.2), close to the MRP1 and protein kinase C-b gene, and encodes a110-kDa protein (Slovak et.al., 1995). Based on the LRP amino acid sequence, no transmembrane fragments or ATP binding sites, characteristic for ABC transporters, were identified. Therefore, LRP is not considered to be a member of the ABC transporter family. Screening of an expression library identified LRP as the major vault protein (MVP) (Scheffer *et al*., 1995). Vaults are highly conserved ribonucleoprotein organelles that are found in all higher eukaryotes. They are localised in cytoplasmic vesicles and nuclear membranes and form the transporter core of the nuclear pore complex. Vaults are composed of the major vault protein, vault poly (ADP-ribose) polymerase, telomerase associated protein1 and small untranslated RNA (Chugani *et al*., 1993). The functional role of vaults in MDR is still unclear but it was proposed that they act by transporting drugs away from their subcellular targets by mediating the extrusion of cytostatics from the nucleus.

1.4.1.5 Human ABC Genes and Physiological Functions

A list of all known human ABC genes is displayed in Table 1.2 (Dean, 2002). Some of the genes in the list has physiological function of drug efflux that leads to drug resistance. The others also mostly have transport and efflux functions.

Table 1.2 List of human ABC genes, chromosomal locations, and functions.

Symbol	Alias	Location	Function
ABCA1	ABC1	9q31.1	Cholesterol efflux onto HDL
ABCA2	ABC2	9q34.3	Drug resistance
ABCA3	ABC3, ABCC	16p13.3	Surfactant secretion
ABCA4	ABCR	1p21.3	N-Retinylidiene-PE efflux
ABCA5		17q24.3	
ABCA6		17q24.3	
ABCA7		19p13.3	
ABCA8		17q24.3	
ABCA9		17q24.3	
ABCA10		17q24.3	
ABCA12		2q34	
ABCA13		7p12.3	
ABCB1	PGY1, MDR	7q21.12	Multidrug resistance
ABCB2	TAP1	6p21.3	Peptide transport
ABCB3	TAP2	6p21.3	Peptide transport
ABCB4	PGY3	7q21.12	PC transport
ABCB5		7p21.1	
ABCB6	MTABC3	2q35	Iron transport
ABCB7	ABC7	Xq21-q2	Fe/S cluster transport
ABCB8	MABC1	7q36.1	
ABCB9		12q24.3	
ABCB10	MTABC2	1q42.13	
ABCB11	SPGP	2q24.3	Bile salt transport
ABCC1	MRP1	16p13.1	Drug resistance
ABCC2	MRP2	10q24.2	Organic anion efflux
ABCC3	MRP3	17q21.3	Drug resistance

Table 1.2 continues

Symbol	Alias	Location	Function
ABCC4	MRP4	13q32.1	Nucleoside transport
ABCC5	MRP5	3q27.1	Nucleoside transport
ABCC6	MRP6	16p13.1	
CFTR	ABCC7	7q31.31	Chloride ion channel
ABCC8	SUR	11p15.1	Sulfonylurea receptor
ABCC9	SUR2	12p12.1	K(ATP) channel regulation
ABCC10	MRP7	6p21.1	
ABCC11		16q12.1	
ABCC12		16q12.1	
ABCD1	ALD	Xq28	VLCFA transport regulation
ABCD2	ALDL1, ALDR	12q11	
ABCD3	PXMP1,PMP70	1p22.1	
ABCD4	PMP69, P70R	14q24.3	
ABCE1	OABP, RNS4I	4q31.31	Oligoadenylate binding protein
ABCF1	ABC50	6p21.1	
ABCF2		7q36.1	
ABCF3		3q27.1	
ABCG1		21q22.3	Cholesterol transport
ABCG2		4q22	Toxin efflux, drug resistance
ABCG4		11q23	
ABCG5		2p21	Sterol transport
ABCG8		2p21	Sterol transport

1.5 Down Regulation of Drug-Target Interaction

Since most anticancer agents target DNA or nuclear enzymes, sequestration of drug in cytoplasmic organelles, such as the trans-Golgi network, the recycling endosomes, and the lysosomes, will lead to decreased drug-target interaction and thereby, decreased cytotoxicity, even if the total intracellular drug concentration remains unchanged. However, altered intracellular drug distribution is often closely linked to alterations in drug accumulation, making it difficult to separate the two processes, which seem to be among the most common resistance mechanisms occurring in tumor cells (Larsen *et al*., 2000). Cell division materials and DNA replication materials are the most known anticancer drug targets.

Microtubules are regulated dynamic cytoskeletal polymers. Suppression of microtubule dynamics is important for antimitotic action of antitubulins (Giannakakau *et al*., 2002). Development of drug resistance to antitubulin agents is a major clinical problem. Several mechanisms of resistance have been investigated, including changes in the expression levels of β -tubulin isotypes and mutations in the β -tubulin gene in several studies (Berrieman *et al*., 2004).

Although positive results have been obtained with established cell lines, the size of the β-tubulin gene family has complicated the process of studying the functional gene in clinical samples, and these findings remain controversial. Tumor cell lines resistant to tubulin binding agents such as paclitaxel and vinca alkaloids presented altered tubulin composition (Giannakakau *et al*., 1997). The resistant sublines exhibit impaired paclitaxel driven tubulin polymerization caused by acquired β-tubulin mutations (Giannakakau *et al*., 1997). The functional protein is mostly encoded by the 4^{th} exon of the gene. The β-tubulin mutations cause inhibition of paclitaxel and vincristine binding, so that the drug-target interaction is downregulated. Inhibition of paclitaxel or vincristine binding will cause survival of tumor cells which results in drug resistance phenotype.

A large variety of microtubule associated proteins (MAPs) have been identified in many different cell types. They function in both stabilizing and destabilizing microtubules, guiding microtubules towards specific cellular locations, cross-linking microtubules and mediating the interactions of microtubules with other proteins in the cell (Honore *et al*., 2005). MAP1a (MAP1A) and MAP1b (MAP1B), which make up the MAP1 family, bind to microtubules differently than other MAPs, utilizing charged interactions. While the C-terminals of these MAPs bind the microtubules, the N-terminals bind other parts of the cytoskeleton or the plasma membrane to control spacing of the microtubule within the cell. Within the cell, MAPs bind directly to the tubulin monomers of microtubules. This binding can occur with either polymerized or depolymerized tubulin, and in most case leads to the stabilization of microtubule structure.

MAP4 is ubiquitously found in all cell types (Honore *et al*., 2005). It stabilizes microtubules by increasing the rescue frequency (transition from a sudden change in microtubule shortening to growth) and has been postulated to be involved in the regulation of microtubule dynamics in mitosis (Hyman and Karsenti, 1996; Dehmelt and Halpain, 2004). The activity of MAP4 within the cell is controlled by phosphorylation (Honore *et al*., 2005). When MAP4 is phosphorylated/inactivated it dissociates from the microtubule allowing mitosis to proceed (Poruchynsky *et al*, 2001). Thus, theoretically, downregulation or inactivation of MAP4 may increase the dynamic nature of microtubules therefore having effects on paclitaxel resistance (Orr and Pinard, 2003).

1.5.1 Paclitaxel and Vincristine Binding Sites on ß-tubulin

The chemotherapetuic drugs paclitaxel and vicristine are known to interact within a specific site on β-tubulin (Kavallaris *et al*., 2001; Huzil *et al*.,2006). The sites are determined by electron crystallography and photoaffinity labelling (Snyder *et al*., 2001). For paclitaxel, the binding sites were found to be amino acid residues 22–26,172–177, 214–217, 223–235, 270–280 and 357-360 (Huzil *et al*.,2006) and can be schematically demonstrated in Figure 1.8 as:

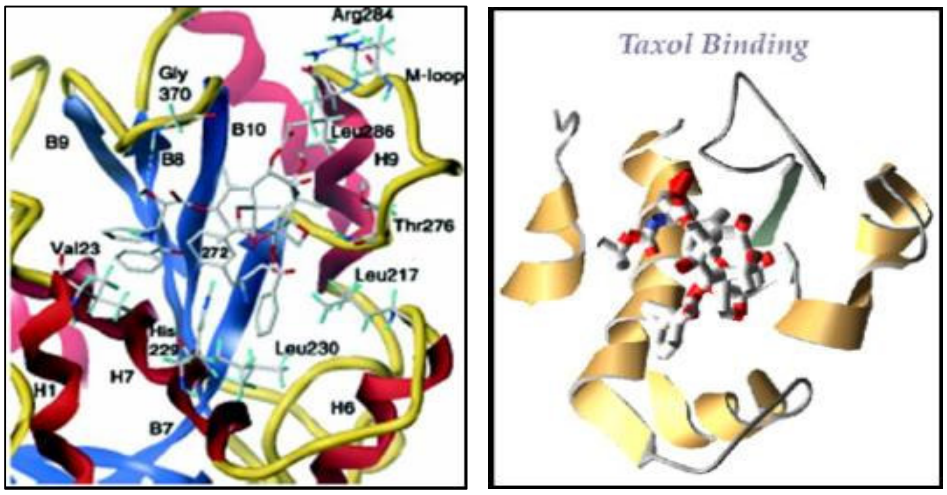


Figure 1.8 Taxol binding sites on β-tubulin (Snyder *et al*., 2001).

Also for vinka alkaloids, at high concentrations they bind to microtubules and results in depolymerization. However, at low concentrations, vinblastine is thought to bind to microtubule tips and suppresses their dynamic instability, leading to stabilization (Toso *et al*., 1993). As for paclitaxel treatment, the expression of specific ß tubulin isotypes is influenced by vincaalkaloid exposure in cancerous cell lines (Ferguson *et al*., 2005). Yet, unlike the isotype interaction with paclitaxel, this effect seems to be much less pronounced and is only observed following exposure to the vinca alkaloid vincrisine (Lobert *et al*., 1998).

The structure of tubulin bound to vinblastine identified its binding site at the inter-dimer interface between the α/ß heterodimers in the protofilament (Figure 1.9) (Gigant *et al*., 2005). In the analysis of this structure, Gigant *et al.* identified several important interactions between ß tubulin and vinblastine. First, several contacts with residuesVal175, Asp177, Tyr208 and Phe212 were shown to be in agreement with previous photoaffinity labelling. The vinblastine binding site is composed of only two contiguous stretches of sequence within ß tubulin that include residues 172–177 and 208–225 (Huzil *et al*., 2006).

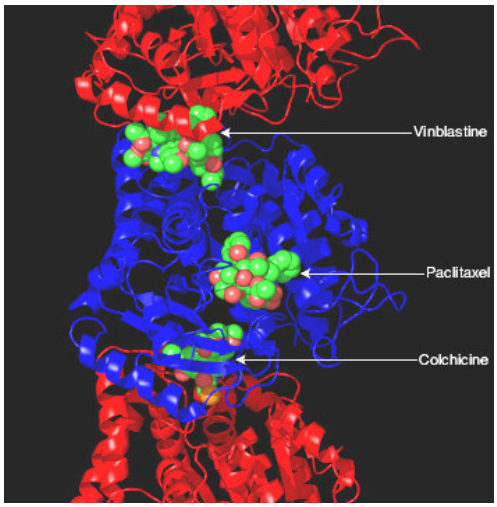


Figure 1.9 Vinblastine, taxol and colchicines binding sites on β-tubulin.

Class I *β tubulin* is the most commonly expressed isotype in human beings, and the most common isotype in cancer cells. The class-I isotype is encoded by the *TUBB* gene located at 6p25, which has four exons and encodes a protein 444 aminoacids long. The protein can be divided into three functional domains: an N-terminal domain (aminoacids 1–205, encoded by exons 1, 2, 3, and part of 4), an intermediate domain (aminoacids 206–381, encoded by part of exon 4), and a C-terminal domain, encoded by the remainder of exon 4 (aminoacids 382–444) (Shalli *et al*., 2005). The acquired mutations on β-tubulin were found to be Phe-270 → Val, Ala-364→Thr, Thr-274→Ile, and Arg-282→Gln before (Berrieman *et al*., 2004). This mutation types may also differ among cells.

1.6 Apoptosis Related Pathways and Down-regulation of Apoptosis

Apoptosis, or programmed cell death, has an essential role in controlling cell number in many developmental and physiological settings and in chemotherapy-induced tumour-cell killing. It is a genetically regulated biological process, guided by the ratio of pro-apoptotic and anti-apoptotic proteins. Apoptosis is a genetically regulated biological process. An apoptotic cell is characterized by the loss of cell volume, plasma membrane blebbing, nuclear condensation, chromatin aggregation,

and endonucleocytic degradation of DNA into nucleosomal fragments. These cell changes occur after a cascade of cell signalling and caspase-mediated events that regulate pro-apoptotic and anti-apoptotic proteins, and are triggered by two major pathways: the death-receptor-induced extrinsic pathway and the mitochondria apoptosome mediated apoptotic intrinsic pathway. Both of these pathways lead to caspase activation and cleavage of specific cellular substrates. The receptor-triggered-apoptosis pathway includes ligands and their receptors such as FAS, TNF, TRAIL, and downstream molecules, such as caspases and BCL2 family members (Hu *et al*., 2003). The mitochondria-apoptosome-mediated pathway includes apoptotic stimuli induced by radiation therapy and chemotherapy, mitochondria, apoptosome, and key effector caspases. Caspases are activated in a cascade-like fashion.

Although chemotherapeutic drugs vary greatly in structure and cellular targets it is now well established that many initiate a highly organized and distinct molecular process known as apoptosis to quickly and efficiently kill the target cell (Janicke *et al*., 1998). Figures 1.10 and 1.11 demonstrate the mechanisms that bring cells to the apoptotic death.

Resistance to apoptosis was correlated with the reduced caspase-3 activation in human cervical multi-drug resistant cells (Cheng *et al*., 2001). Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis (Janicke *et al*., 1998). Downregulation of caspase-3 through mutational or expressional alterations will cause survival and resistance to apoptotic death. Also downregulation of apoptotic pathways will lead cell survival so that drug resistance phenotype is a consequence.

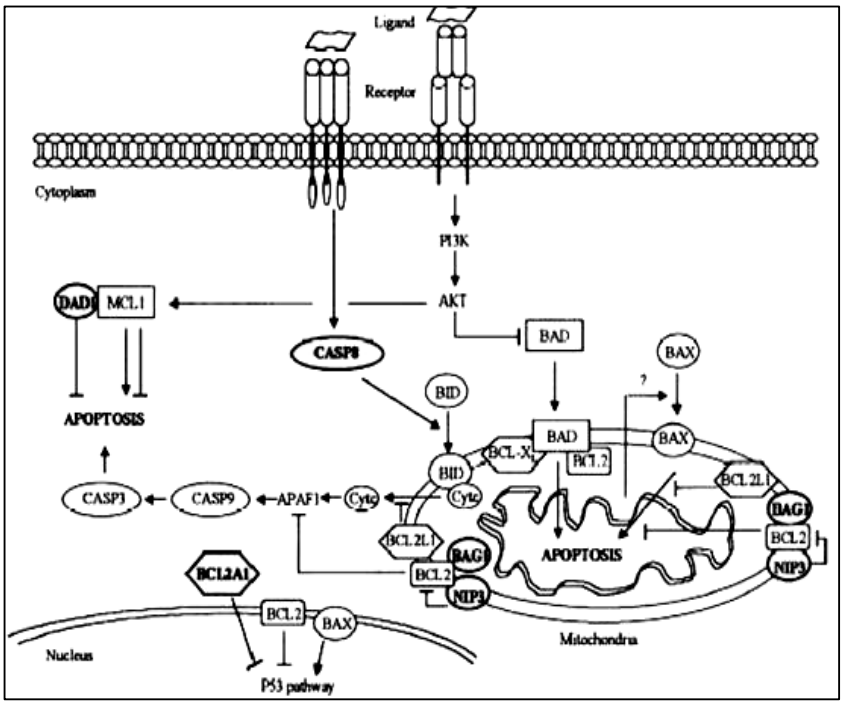


Figure 1.10 The general scheme of mechanisms that are related with apoptosis (Janicke *et al*., 1998).

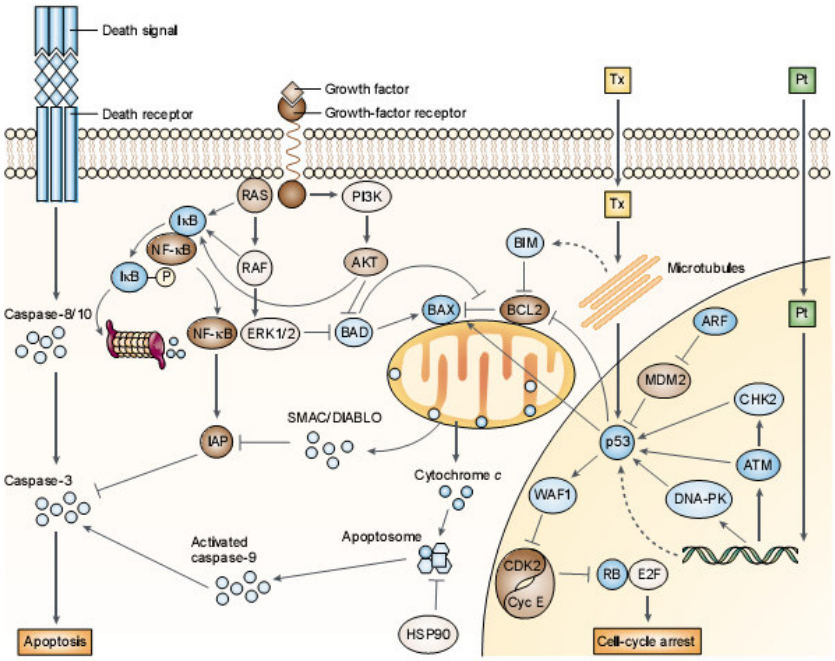


Figure 1.11 Signalling pathways involved in taxane and platinum-therapy-induced apoptosis and cell-cycle arrest. (Agarwal and Kaye, 2003).

In Figure 1.11 proteins in blue promote, whereas those in brown inhibit apoptosis and/or cell-cycle arrest in response to chemotherapy (Agarwal and Kaye, 2003).

1.6.1 Up-regulation of Anti-apoptotic BCL-2 family

Human BCL-2 protein functions to promote cell survival by inhibiting apoptosis. Taxol induces transient BCL-2 phosphorylation in hepatocellular carcinoma cell line which leads to a number of pathways or death mechanisms (Wang *et al*., 1999). Microtubule interacting agents including paclitaxel have been known to cause significant activation of JNK (Jun N terminal kinase) (Vasilevskaya and O`Dwyer, 2003). Loss of the BCL-2 phosphorylation loop domain increases resistance to human leukemia cells to paclitaxel mediated mitochondrial dysfunction and apoptosis (Vasilevskaya and O`Dwyer, 2003).

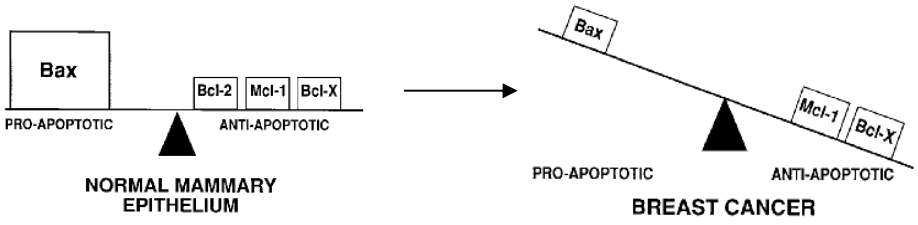


Figure 1.12 The representation of balance between pro-apoptotic and anti-apoptotic proteins (Reed, 1996).

An appropriate balance of pro-apoptotic (BAX) and anti-apoptotic (BCL-2, BCL-X_L, MCL-1), BCL-2 family member proteins is present in normal mammary epithelium (Figure 1.12). During the pathogenesis of adenocarcinoma of the breast, BCL-2 expression is sometimes lost in combination with reduced levels of Bax. Since other anti-apoptotic proteins such as BCL-X_L and MCL-1 may still be present, the balance between pro- and anti- apoptotic proteins seems to be upset in favor of death blockers. This may also result in resistance to cell death (Reed, 1996).

In a viable cell, the pro-apoptotic BCL-2family members BAX, BAK, and BH3 are antagonized by anti-apoptotic members such as BCL-2. Activated BH3 prevents anti-apoptotic BCL-2 members from inhibiting pro-apoptotic members. In addition, they might directly induce a conformational change of BAX and BAK which subsequently oligomerize and insert into the mitochondrial membrane where they form pores either by themselves or by associating with the permeability transition pore complex. In consequence, pro-apoptotic factors are released from the inner mitochondrial membrane into the cytosol, such as cytochrome c which contributes to the activation of the caspase cascade. Increase in BCL-2/BAX ratio will cause inhibition of apoptosis so the cells will be resistant to death at any drug concentrations.

1.7 Drug Metabolism via Cytochrome P450 Family Members

Several cytochrome P450 enzymes are involved in the metabolism of a range of anticancer drugs, including cyclophosphamide, paclitaxel, and docetaxel (Le Blanc and Waxman, 1989; Kivisto *et al*., 1995; Iyer and Ratain., 1998). Cytochrome P450-mediated metabolism usually results in reduced activity or inactivation of the anticancer drugs, but in some cases bioactivation to a more cytotoxic metabolite occurs. One example of detoxification of anticancer drugs is shown by the taxanes. The major pathway of metabolism of paclitaxel, an anticancer drug used in the treatment of breast, ovarian and non-small cell lung cancer, is catalysed by CYP2C8 and involves the hydroxylation of position 6 on the taxane ring (Rahman *et al*., 1994). The metabolite 6-hydroxytaxol is 30-fold less cytotoxic than the parent compound paclitaxel and this metabolite is further metabolised by CYP3A4 (Crommentuyn *et al*., 1998). Docetaxel, a semisynthetic taxane currently under going phase II and phase III trials for use in first-line therapy of ovarian cancer, is metabolised by CYP3A to apparently less cytotoxic metabolites (Marre F *et al*., 1996). Several anticancer drugs are metabolically activated by P450s. In particular, cyclophosphamide, an inactive pro-drug used in the treatment of a number of different cancers, including ovarian, must first undergo a 4-hydroxylation reaction to 4-hydroxycyclophosphamide by cytochrome P450 enzymes (CYP2B6 and CYP3A4) (Chang *et al*., 1993) before becoming cytotoxic. Vincristine is metabolized by CYP3A4 and CYP3A5.

Cytochrome P450 NADPH reductase (P450R) is a flavin containing monooxygenase that acts as an electron bridge between NADPH and electron acceptors, using its cofactors, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), as electron transporters. The main electron acceptors of P450R are cytochrome P450, although other microsomal proteins such as cytochrome b5, heme oxygenase and fatty acid elongase might also be reduced by P450R. P450R expression is abundant in the liver, although it is widely expressed in the body, present in all different types of tissue studied; within the cells, it is located in the endoplasmic reticulum. P450R

activity has also been detected in tumors; activity was measurable in microsomes obtained from renal cell carcinoma samples (McFadyen *et al*., 2004).

1.8 Correlation Between MDR and Tumor Microenvironment

Many soluble factors have been related to survival and growth in cancer cells, including VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor); TNF super family members and many others. These soluble factors stimulate cell survival and growth, and thereby confer drug resistance (Li and Dalton , 2006). The effect of soluble factors depends on the expression of the appropriate receptor on malignant cells. bFGF, is also important for the pathogenesis of malignant cells (Menzel *et al*, 1996). The upregulation of BCL-2 by bFGF underlies the resistance to apoptosis and prolongs CLL cell survival. (Konig *et al*., 1997) Certain growth factors, such as PDGF (platelet derived growth factor) and TGF-b (transforming growth factor-b), induce the expression of VEGF and bFGF, and thereby stimulates cell proliferation.

Interleukin-6 (IL-6) is a cytokine that provokes a broad range of cellular and physiological responses (Akura *et al*, 1993). In addition to playing role in inflammation and hematopoiesis, IL-6 is involved in other processes such as neuronal differentiation and bone loss. To produce these effects IL-6 signals through a receptor composed of two different subunits, an alpha subunit that produces ligand specificity and gp130, a receptor subunit shared in common with other cytokines in the IL-6 family. Binding of IL-6 to its receptor initiates cellular events including activation of JAK kinases and activation of ras-mediated signaling.

IL-18 is a pro-inflammatory cytokine similar in structure and mechanism of action to IL-1 beta. Formation of active IL-18 by macrophages requires cleavage of an inactive precursor by caspase-1 protease, also termed the IL-1 converting enzyme (ICE). One of the key biological responses induced by IL-18 is that in combination with IL-12 it stimulates Th1 cell differentiation and involvement in immune responses. IL-18 was originally known as interferon-gamma-inducing factor, named

after its induction of interferon-gamma production (Duan *et al*., 1999). Up-regulation of Fas ligand in Th1 cells by IL-18 may increase apoptosis of Fas receptor expressing cells that interact with the activated Th1 cells. This induction of apoptosis may allow IL-18 to have anti-tumor activity and may also play a role in chronic inflammatory and autoimmune conditions.

Cellular transformation is accompanied by many cellular changes, including uncontrolled proliferation, loss of the differentiated cell morphology, and invasion of the extracellular matrix (Hazlehurst *et al*, 2003). Degradation of the extracellular matrix is a key component of tumor cell invasion into surrounding tissues. Matrix metalloproteinases (MMPs) are a class of proteases secreted by tumor cells, degrading the proteins of the extracellular matrix and allowing metastasis. Extracellular matrix (ECM) made up of collagens, fibronectin, laminins, proteoglycans and other macromolecules, controls many aspects of cells such as gene expression, differentiation and proliferation, and invasive/metastatic phenotype of tumor cells. Altered expression levels of these molecules in tumor cells was reported to affect their sensitivity to drug induced apoptosis and drug resistance through activation of survival pathways of cells (Hoyt *et al*.,1996; Kraus *et al*., 2002). Tumor cells remodel their microenvironment for survival and invasion. Differential expression of ECM components, matrix metalloproteinases (MMPs) and adamalysins (a disintegrin and metalloproteinase; ADAMs) may contribute the remodeling of ECM, cancer invasion and metastasis. According to a previous report (Mitsumoto *et al*., 1998) on invasive/metastatic behavior of drug resistant tumor cells, carboplatin resistant mouse epithelial cells had increased levels of ECM components (fibronectin, laminin, collagen type IV) with enhanced MMP-2 activity. In addition, changes in ECM can also affect the sensitivity of the tumor cells to apoptosis through growth factors dependent on cell-matrix and cell-cell interactions through ECM-integrin signaling (Morin , 2003) as well as development of cell adhesion-mediated drug resistance (CAM-DR).

Communication between cells is essential for normal as well as tumor cell growth. Cancer cells are influenced by the extrinsic factors provided by the

microenvironment through intercellular communication, including direct contact. The tumor microenvironment plays an important role in mediating *denovo* drug resistance (Hazlehurst *et al*, 2003). The tumor microenvironment consists of stromal cells, extracellular matrix (ECM) and soluble factors such as cytokines and growth factors (Li and Dalton , 2006). Soluble factors in the tumor microenvironment provide further signals for tumor cell growth and survival. Environment mediated-drug resistance (EM-DR) in tumor cells therefore could be considered as a synergism between cell adhesion mediated-drug resistance (CAM-DR) and soluble factor mediated-drug resistance (SM-DR).

1.9 Cell Cycle Regulation in MDR

The cell cycle is regulated by cyclin-dependent kinases (CDKs), cdk1 (cdc2), cdk2, cdk4/6 that bind to various cyclins during cell cycle progression. The CDK complexes are regulated by phosphorylation and are involved in the regulation of transcription of a variety of genes (McGrogan *et al*., 2008). Figure 1.13 represents the cell ceyle check points and related proteins schematically.

During interphase microtubule turnover is relatively slow, with half-lives ranging from several minutes to several hours (Jordan and Wilson, 2004). During G1 to S phase the two centrioles of the centrosome separate and duplicate and by late G2 phase have enlarged in size but remain attached together until mitosis begins. In eukaryotic cells, mitosis is initiated when the regulatory subunit cyclin B1 complexes with the cyclin-dependent kinase (cdk1) p34cdc2 forming the maturation promoting factor (MPF).

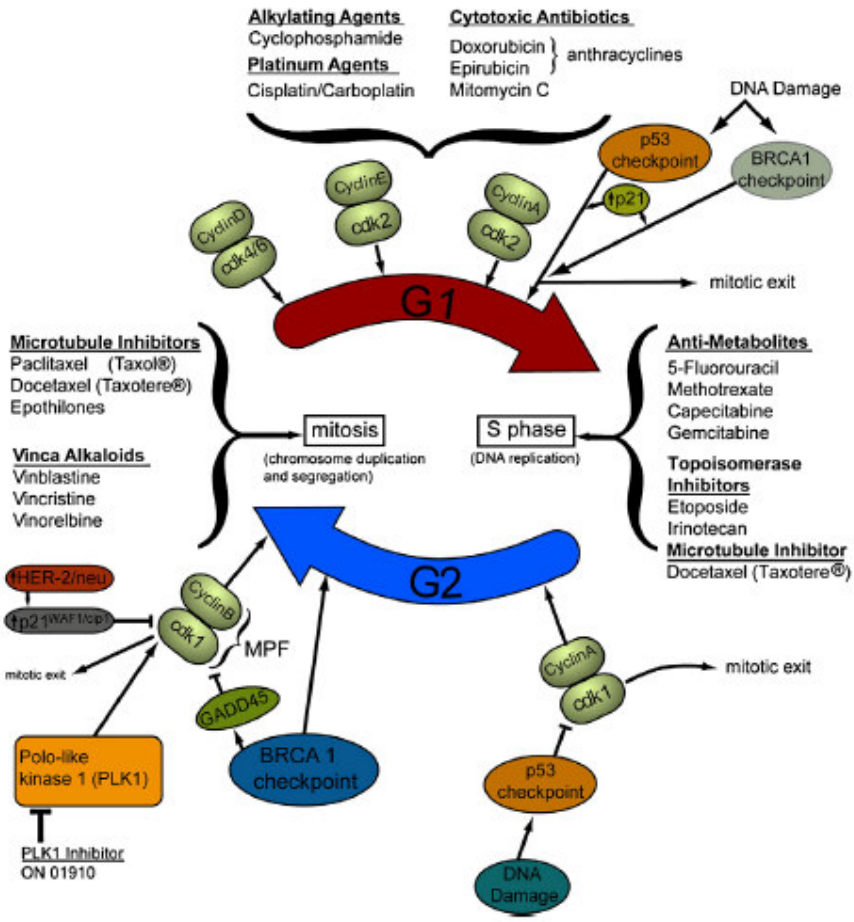


Figure 1.13 Cell cycle check points (McGrogan *et al*., 2008)

Breast cancer susceptibility gene 1 (BRCA1) BRCA1 is a tumor suppressor gene with pleiotropic functional roles including DNA repair, transcriptional regulation and maintenance of genomic stability (Venkitaraman, 2002). It functions by sensing cellular stress and transducing signals either to the cell cycle or the apoptotic machinery initiating mitotic arrest and apoptosis. BRCA1 is associated with the stress-response pathway p38/MAPK (Mullan *et al*., 2001) and can activate the G2M and spindle checkpoints in response to mitotic inhibitor drugs that disrupt the mitotic spindle. BRCA1 also induces apoptosis by activating the C-Jun N-terminal kinase/stress-activated protein kinase JNK/SAPK pathway (Harkin *et al*., 1999) and

additionally through enhanced activation of Fas and caspase 9 (Thangaraju *et al*., 2000). Following paclitaxel treatment BRCA1 activates the upstream regulator MEKK3 of both the p38/MAPK and JNK/MAPK pathways inducing apoptosis. However the exact mechanism by which BRCA1 activates MEKK3 is unclear (Gilmore *et al*., 2004) and both pathways have been implicated in mediating paclitaxel-induced apoptosis. So the important role played by BRCA1 in modulating cellular stress response has implications for chemoresistance to mitotic inhibitor drugs, which affect the mitotic spindle (Mullan *et al*., 2006).

1.10 Effects of Ras/Raf/MEF/ERK Pathway on Cell Survival

The Ras/Raf/MEK/ERK cascade couples signals from cell surface receptors to transcription factors, which regulate gene expression (Cornwell and Smith 1993). Furthermore, this cascade also regulates the activity of many proteins involved in apoptosis. A diagrammatic overview of the Ras/Raf/MEK/ERK pathway is presented in Figure 1.14 This pathway is often activated in certain tumors by chromosomal translocations such as BCR-ABL, mutations in cytokine receptors or overexpression of wild type or mutated receptors, e.g., EGFR.

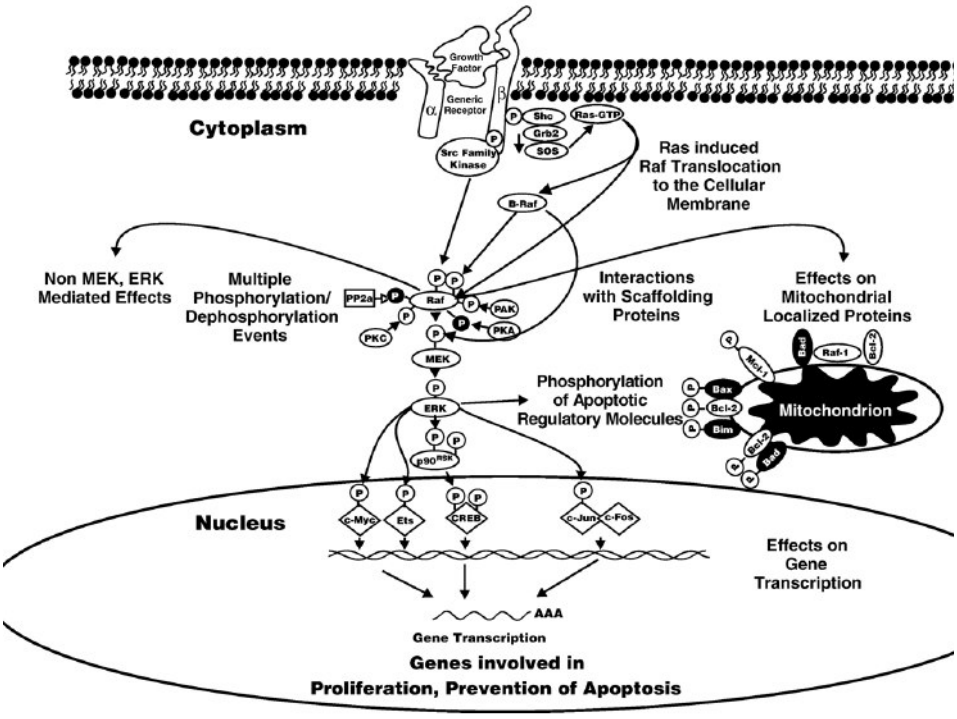


Figure 1.14 Overview of Ras/Raf/MEK/ERK pathway (Morrison and Cutler, 1997).

1.11 Reversal of *MDR1/MRP1* Mediated Drug Resistance

Design of nontoxic agents that would overcome the MDR of tumors has been a challenging area of pharmaceutical research. This effort combines several strategies like; rational design of agents that retain their cytotoxic activities towards tumor cell, development of augmenting compounds that restore cyototoxicity of available antitumor drugs against cells (Borowski *et al*., 2005), downregulation of *MDR1* expression through antisense or small interfering RNA (siRNA) strategies (Stierle *et al*., 2005). The function of drug pumps can be effectively inhibited by substrate analogs (i.e. verapamil), inhibitors of ATP binding or utilization, specific monoclonal antibodies and by various agents with non-specified mechanisms (Sarkadi and Müller, 1997).

The compounds that would reverse resistance against anticancer drugs are called MDR inhibitors, MDR modulators, MDR reversal agents or chemosensitizers. They may modulate more than one transporter (Özben, 2006). The process of chemosensitization involves the co-administration of a P-gp inhibitor (MDR modulator) with an anticancer drug in order to cause enhanced intracellular anticancer drug accumulation via impairing the P-gp function. Numerous compounds have been shown to inhibit the drug efflux function of P-gp and therefore, reverse cellular resistance. There are many studies to overcome MDR by inhibiting MDR transporters, to suppress or circumvent MDR mechanisms. The use of anticancer drugs that could escape from the ABC transporters might be a solution to avoid drug resistance. Modulators of P-gp directed MDR belong to a number of chemical classes including calcium channel blockers, calmodulin inhibitors, coronary vasodilators, indole alkaloids, quinolines, hormones, cyclosporines, surfactants, and antibodies (Ford and Hait, 1993). The reversal of MDR may also be at expression level, such that application of some MDR modulating agents results in decrease in *MDR1* gene expression (Molnar *et al*., 1998). Some strategies for MDR reversal are summarized in Figure 1.15, 1.16.

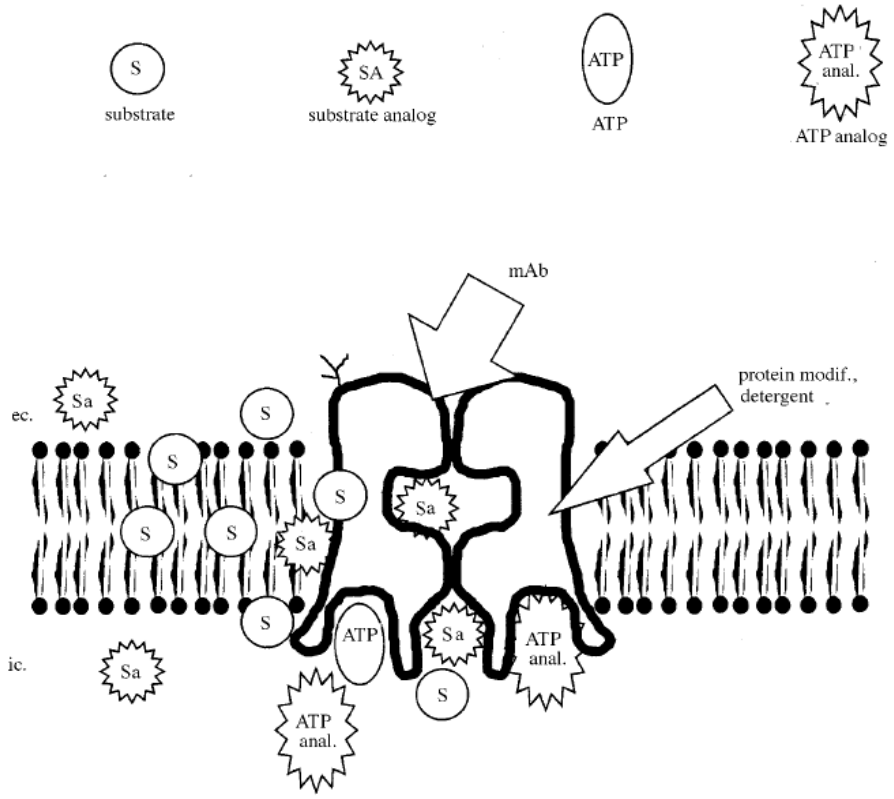


Figure 1.15 Schematic representation of P-gp modulation strategies. The function of pumps can be inhibited by substrate analogs, inhibitors of ATP binding or utilization, specific monoclonal antibodies (Sarkadi and Müller, 1997).

First generation MDR modulators such as verapamil (VRP) and cyclosporin (CsA) exhibit inherent pharmacological acitivity (cardiovascular, immunosuppression respectively). Both have been shown to require high doses to achive sufficient plasma concentrations to reverse MDR (Ozols *et al*., 1987, Fisher *et al*., 1994). Effective concentration of VRP (2-6 μM) was found to be clinically toxic (arteio-ventricular block) (Anderson *et al*., 1987). Due to such complications of first generation MDR modulators, MDR modulators with lower inherent toxicities were developed. Some of them are dex-VRP (R-enantiomer of VRP), dex-niguldipine (R-enantiomer of niguldipine), PSC 833 (CsA analog) which exhibited decreased inherent toxicity and higher MDR reversal efficacy (Krishna and Mayer, 2000).

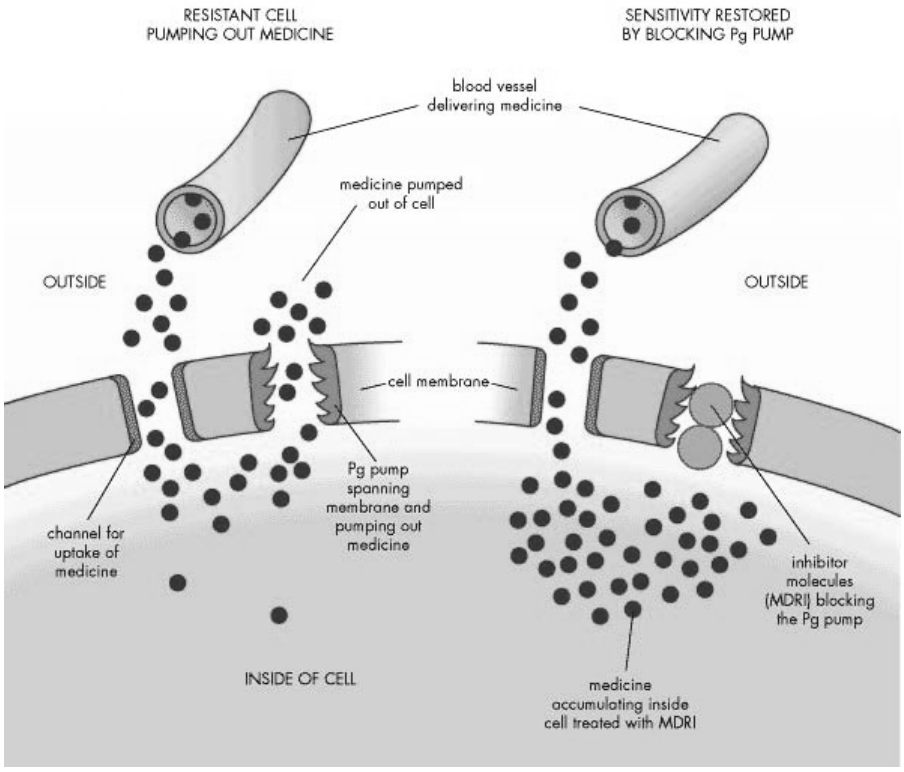


Figure 1.16 Schematic representation of P-gp pump blockage (Drori *et al*., 1995).

In additional to the interaction of modulators with ABC transporter proteins, their interaction with the lipid bilayers of the plasma membrane is essential. So, membrane lipids are also regarded as one of the targets for MDR reversing agents. Most of the MDR reversing compounds are soluble in lipids and they may also exert an influence on the physical properties of lipid bilayers. Most of them alter membrane fluidity and increase membrane permeability (Drori *et al*., 1995; Callaghan *et al*., 1993). Alterations in the physical state of plasma membrane lipids influence a number of important carrier-mediated processes and they appear to be important factors modulating efflux pump systems (Shin *et al*., 2006).

According to literature, phenothiazines can be used as MDR reversal agents as a P-gp suppressor (Motohashi *et al*. 2001). It was also proved that, some flovanoids

(Zhang *et al*., 2004) and carotenoids exhibit anticarcinogenic and resistance modulating effects by modification of lipid bilayer in which P-gp is embedded and the transport activity of P-gp can slow down depending on the conformational changes induced by resistance modifiers (Molnar *et al*., 2006).

Table 1.3 Major ABC transporters and some of the related inhibitors (Özben, 2006).

Common Name	**Systematic name**	**Inhibitors**
P-gp	**ABCB1**	
		Phenothiazine
		CyclosporineD
		NSC-38721(mitotane)
		Pipecolinate
		Quinoline
		PSC-833(valspodar)
		LY-335979(zosoquidar)
		XR-9576(tariquidar)
		R-101933(laniquidar)
		VX-710(biricodar)
		GF-120918(elacridar)
		Isothiocyanates
		Diallylsulfide
		siRNA
		Flavonoids
MRP1	**ABCC1**	
		Indomethacine
		VX-710(biricodar)
		Isothiocyanates
		XR-9576(tariquidar)
		AgosterolA
		Rifampicin

1.12 Combination Chemotherapy

The use of multiple drugs in the treatment and prevention of diseases can be traced back for thousands of years to the use of herbal medicines. Modern medicine uses defined ingredients or compositions, optimizes treatment schedules and combination proportions, refines routes of drug delivery, and combines different modalities of treatments (Chou and Talalay, 1983; Chou *et al*., 1994). The popularity of using drug combinations for the treatment of cancer and other diseases necessitates the research at the laboratory level for the best choice of drugs to be used, for the quantitative evaluation of experimental data to provide the bases for the design of clinical trial protocols, and finally for the implementation of the rationale into clinical practice.

Combination drug regimens for the treatment of cancer, AIDS, and tuberculosis often achieve a therapeutic efficacy greater than that achieved with monotherapy. Other benefits may include decreased toxicity, the delay or prevention of drug resistance development, and the favorable effects of synergistic drug interactions (Fivelman *et al*., 2004). The rationale for combination chemotherapy is to use drugs that work on different parts of the cancer cell's life cycle, thereby increasing the likelihood that more cancer cells will be killed. When drugs with different toxicities are combined, each drug can be used at its optimal dose, helping avoid intolerable side effects. Finally, drugs with very different properties are sometimes combined. For example, drugs that kill tumor cells may be combined with antibodies or with drugs that stimulate the body's immune system against cancer (biologic response modifiers). The stage of the cancer often determines whether single therapy or a combination is needed (Fisher and Sikic, 1995). There is much interest in the use of modulators clinically, in combination with standard chemotherapy regimens, to circumvent the effects of Pgp (Efferth *et al*., 1995; Germann and Harding, 1995). The application of anticancer drugs with MDR modulators may be easily acceptable for clinicians under controlled condition. Combined application is used if the drug interaction is synergistic, but not acceptable if the interaction is antagonistic.

Synergism is a positive interaction between two agents, that is, combined effect of two agents is significantly greater than expected results based on their independent effects when they are used separately. Antagonism is a negative interaction, that is, combined effect of anticancer drug and modulator being examined is significantly less than their independent effects when they are tested separately (Kars *et al*, 2007).

So far, only two methods namely, the isobologram and the combination index (CI) method of Chou-Talalay, are actually derived equations for defining what "additive effect" is (Chou and Talalay, 1983; Chou, 1991). The multiple drug-effect equation was derived from the inhibition of enzyme kinetic models via mathematical induction and deduction (Chou *et al*., 1997; Berenbanm, 1989; Chou and Talalay, 1987). The resulting general equation was then used to define the CI equation of Chou and Talalay, in which the equations CI = 1, CI < 1, and CI > 1 indicate additive effect, synergism, and antagonism, respectively. This phenomenon can be demonstrated also graphically (Figure 1.17).

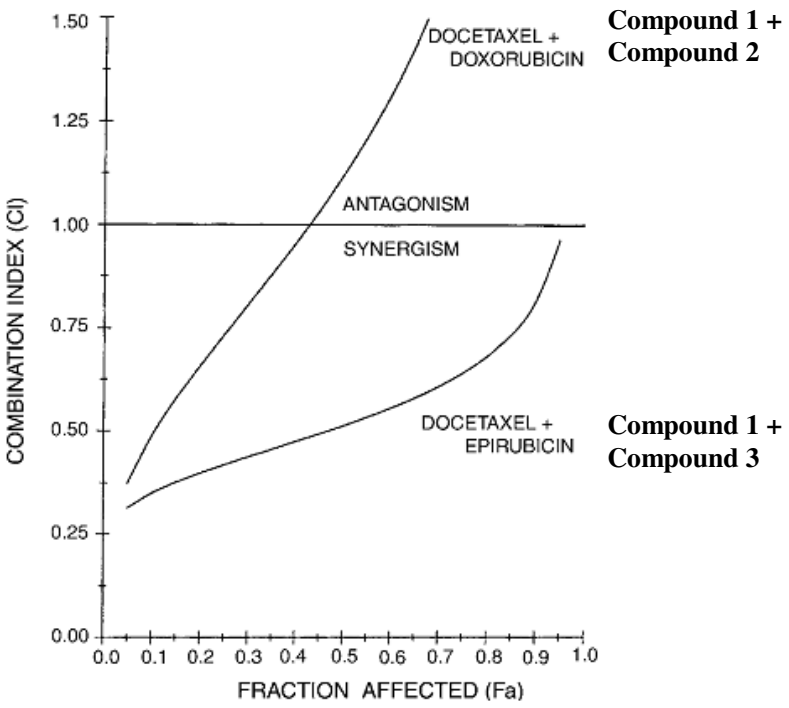


Figure 1.17 Synergism and antagonism of cytotoxic effect as measured over the full range of cell kill (Fa) (Budman and Calabro, 2002).

1.13 Advantage of Gene Expression Profiling in Cancer Biology

One of the most important issues facing the pharmaceutical and biotechnology industry is finding new drug candidates. Several new genomic technologies have been developed in hopes of addressing the issues of target identification and lead candidate optimization (Cunningham, 2000). Gene expression microarray is one of these technologies. Microarray aims discovering new genes and proteins, quantifying and analyzing gene and protein expression. Being able to compare levels of gene and protein expression between diseased and normal cells or cells treated with compounds, which vary in their efficacy and toxicity, could prove valuable in identifying new drug targets and optimizing the choice of lead compound candidates by more closely predicting their success or failure.

The gene expression profile of a cell determines its phenotype, function, and response to the environment. The complement of genes expressed by a cell is very dynamic and will respond rapidly to external stimuli (Clarke *et al*., 2001). Therefore, measurement of gene expression can potentially provide clues about regulatory mechanisms, biochemical pathways, and broader cellular function. Analysis of global gene expression patterns has the potential to predict biological behavior and clinical consequences, an expectation that will revolutionize cancer diagnosis and treatment.

1.14 Objectives of This Study

The major goal of the study is to investigate the molecular mechanisms of paclitaxel and vincristine resistance in MCF-7 breast cancer cells. Development of paclitaxel and vincristine resistant MCF-7 cell lines as *in vitro* models for acquired drug resistance was the first approach of this study. Determination of degree of developed resistance to selective agents and other unrelated chemotherapeutics was the second step of the work to identify proliferation profiles of drug resistant sublines with respect to sensitive MCF-7. Investigation of growth profile and doubling time differences between sensitive cell line and resistant sublines clarified the variations in doubling periods as resistance developed.

To understand the effects of drug application with dose increments on the *MDR1* and *MRP1* gene expressions mRNA and protein analysis techniques and to investigate MDR-survival relationship in dose dependent manner were the other important objectives. Analysis of *β-tubulin* gene mutations and analysis of microtubule associated genes in terms of mRNA expression level let to understand the impact of target alteration on MDR phenotype. Microarray analysis let to clarify the complete expression profiles of the resistant cell lines in relation with cell survival.

Reversal of drug resistance by inhibiting the P-gp and MRP1 activities and *MDR1* and *MRP1* expression levels by chemical blocking agents let to identify effective MDR blocking agents.

Another purpose was the application of drug combinations to investigate the antiproliferative effects of MDR modulating agents with anticancer drugs; or interactions of two anticancer agents when applied together.

CHAPTER 2

MATERIALS AND METHODS

2.1 MCF-7 Cell Line, Anticancer Drugs and Development of Resistant MCF-7 Cell Lines

MCF-7 cell line, which is a model cell line for human mammary carcinoma, was used as parental cell line. MCF-7 cell line was established from 69 year female Caucasian. The cell line exhibits some features of differentiated mammary epithelium and was donated by ŞAP Institute, Ankara-Turkey. MCF-7 cells were maintained as an attached type monolayer culture in RPMI 1640 (Biochrom AG, Berlin) medium supplemented with 10 % heat inactivated fetal bovine serum (FBS) (Biochrom AG, Berlin), 1.5 % L-glutamine and 0.1 mg/ml gentamicin (Biological Industries, Israel). Incubation conditions at 37 °C in humidified atmosphere of 5 % CO_2 were maintained in Heraeus incubator. Cultures were passaged by trypsinization when cells reach 70% confluency under sterile conditions in Bioair Laminar Flow (Euroclone, Pavia, Italy). MCF-7 cells were frozen in freezing medium (10 % DMSO+ 90 % FBS) incubating firstly at 4 °C for one hour, at -20 °C for 2-3 hours, at -80°C for days stepwise, finally in liquid nitrogen for long periods.

Paclitaxel and vincristine were applied separately in dose increments to MCF-7 cell line for stepwise selection of resistant cells. The resistant sublines to paclitaxel (MCF-7/400nMPac) and vincristine (MCF-7/120nMVinc) from parental MCF-7 cell line (MCF-7/S) were developed by increasing the doses, stepwise, from 0.1 nM to 400 nM paclitaxel and from 2 nM to 120 nM vincristine. The drug concentrations were determined according to cellular viability and morphology by microscopic observations. The doses were arranged by preventing complete killing of the cells and maintaining little survival. The cells were selected with that dose for about 3-4 weeks. The mechanisms of drug resistance were investigated on MCF-7/S, MCF-

7/50nMPac, MCF-7/120nMPac, MCF-7/200nMPac, MCF-7/400nMPac, MCF-7/20nMVinc, MCF-7/40nMVinc, MCF-7/80nMVinc, MCF-7/120nMVinc cell lines. Paclitaxel (TAXOL®) was purchased from Sigma (St. Louis, MO, USA) and drug was dissolved in DMSO. Vincristine (ONCOVIN®) was denoted by Gülhane Military Medical Academy, School of Medicine as injection form and the drug was diluted with deionized water.

2.2 Viability and Proliferation Assays

2.2.1 Doubling Time Determination, Growth Curve Construction

The cell number was adjusted to $3.0x10^4$ cells /mL in growth media and distributed to 24 well plates. The cells were seeded in drug containing medium. Every corresponding day counts were made in triplicates from three different wells. Cells were trypsinized, stained with 10 % tryphan blue (Biological Industries, Israel) and counted in a Neubauer counting chamber (Bright-line, Hausser Scientic, Horseam, PA, USA) under light microscopy. The viable cell counts were expressed as the percentage of the initial cell seeding concentration ($3.0x10^4$ cells /mL) which was set as 100% growth. The cells were counted until the zero viability was reached. The specific growth rate (μ) was determined from the slope of the ln (cell number/mL) versus time plot. The doubling time (t_d) that was the required time period for cells to complete one cell cycle, was calculated as follows:

$$t_d = \ln 2 / \mu; \qquad \text{[Equation 1]}$$

where μ=specific growth rate.

The changes in doubling time of the resistant cell lines were compared with the parental cell line in terms of significance of the change by student t-test (SPSS Inc.,USA). $p<0.05$ represents significant difference between groups.

2.2.2 XTT Cytotoxicity Analysis

The use of tetrazolium salts, such as XTT, was first described by Scudiero *et al*. in 1988 (Scudiero *et al*,1988), and is based on the fact that live cells reduce tetrazolium salts into colored formazan compounds. The effects of the chemotherapeutic agents, MDR modulators and their combinations on the proliferation of sensitive and resistant MCF-7 cell lines were evaluated by means of the Cell Proliferation Kit (Biological Industries, Israel) in 96 well flat bottomed microtiter plates. (Eskiocak *et al*., 2008). In summary, first well is filled with 150 µl and all the wells except the cell control column (second) were filled with 100 µl medium. 200 µl of concentrated anticancer agent (prepared in medium) was added in to the third column and the drug was diluted horizontally by taking 100 µl portion of drug solution from the third column and putting in to the next column. Finally the cells were seeded in to 96-well microtiter plates ($5x10^3$ cells /well) and incubated for 72 h in medium containing horizontal dilutions of compound (except for medium control wells). Then, XTT reagent was applied to form a soluble dye. After incubation at 37 ºC for 4 h, the dissolution of formazan crystals that were produced by mitochondrial enzymes of the living cells occurred, the optical density of chromogenic product was measured at 500 nm with an Spectromax 340 96-well plate reader (Molecular Devices, Sunnyvale, CA, USA). The inhibition of cell proliferation and IC_{50} (inhibitory concentration 50) values were determined for each cell line. The resistance indices of each cell line to the agents were calculated to determine the degree of acquired resistance of each cell line to selecting drugs or antiproliferative effects of compounds. The resistance indices (R) were evaluated according to the following expression:

$$R = \frac{IC_{50}\ \text{resistant cell line}}{IC_{50}\ \text{sensitive cell line}} \qquad \text{[Equation 2]}$$

2.3 Expression Analysis of Drug Resistance Related Genes

2.3.1. RNA Isolation with GTC Single Step RNA Isolation Method

RNA isolation from the MCF-7 cell line was performed using the guanidium thiocyanide / phenol-chloroform single-step RNA isolation method (Chomczynski, 1987), (Appendix A). Cells were pelleted at 800 rpm and then lysed with 4M Guanidium thiocyanate (Applichem, Germany). The RNA was extracted with acidic phenol (pH 4) and chloroform on ice and centrifugated at 13000 rpm, 15 minutes. The upper RNA phase was collected in a seperate tube and RNA was precipitated with cold absolute ethanol. After washing and drying RNA, absorbance values (260 nm, 280 nm) were measured for RNA quantification with Shimadzu (Columbia, U.S.A.) spectro photometer. RNA intactness was checked on native agarose (1% w/v) gel electrophoresis (70 V, 90 min) with ethidium bromide staining. The prescription of agarose gel in Tris Acetate EDTA buffer system is given in Appendix B.

2.3.2 RT-PCR (Reverse Transcription-Polymerase Chain Reaction)

cDNA synthesis (reverse transcription) reaction was performed with 5 µg of total RNA, 1 pmole gene specific primer, 40 units M-MuLV Reverse Transcriptase for each sample (42 ºC, 1 hour), according to the manufacturer's instructions (MBI Fermentas, Vilnius, Lithuania). Briefly, RNA, primer and dH_2O were added in to tube with a total amount of 11 µL. The tube was incubated at 70 ºC for 5 minutes to let RNA supercoil degradation. Next step was adding buffer (4 µL of 5X reaction buffer), dNTP (2 µL from 10 mM mix), and 2.7 µL of dH_2O. The reaction mixture was incubated at 37 ºC for 5 minutes to let primer annealing. Finally the enzyme M-MuLV Reverse Transcriptase was added to the reaction tube and the mixture was incubated at 42 ºC for 60 minutes for synthesis. The reaction was terminated by incubating the reaction mixture at 70 ºC for 10 minutes. The cDNA was kept at -20 ºC for long periods.

cDNA was used as template for the RT-PCR reaction; 1 unit Taq DNA polymerase was used for 50 µL of reaction volume. *MDR1, MRP1, Bcl2, Bax*, and *β-2microglobulin* gene specific primers were used for expression analysis. PCR conditions were optimized by performing cycle tests initially. The control cDNA for each primer set was amplified at different PCR cycles. The optimal cycle conditions were determined. The product of RT-PCR performed with *β-2microglobulin* primers was used as constitutively expressed common internal control.

The optimum PCR conditions were as follows; initial denaturation at 94 ºC for 5 min, denaturation at 94 ºC for 30 s, annealing at 55 ºC for 30 s (*MRP1* and *beta-2 microglobulin*), 56 ºC for 25 s (*MDR1*), 53 ºC for 30 s (*Bcl-2 and Bax*), extension at 72º C for 30 s (*MDR1, MRP1, Bcl-2, Bax* and *beta-2 microglobulin*) and final extension at 72 ºC for 10 min. PCR cycles were set as 30 cycles for *MDR1, Bcl-2* and *beta-2 microglobulin* primers, 25 cycles for *MRP1* and *Bax* primers. List of primers and amplicon sizes are represented in Table 2.1. The list of optimum PCR contents is also given in Table 2.2. PCR products were examined by native agarose (2 % w/v) gel electrophoresis (90 V, 60 min) and visualized with ethidium bromide staining. RT-PCR experiments were performed twice independently. Densitometric measurements of band intensities were performed using Scion Image Software (Scion Corporation, USA). The band intensities of resistant sublines were compared with that of MCF-7/S statistically by student t-test using SPSS Software.

Table 2.1 Primers sets and amplicon sizes for RT-PCR

Primer List	Sequence	Location	Product
[a]*MDR1 Sense*	5'GAGCCTACTTGGTGGCACAT 3'	Exon 24	295 bp
MDR1 Antisense	5'AGGCTCAGTCCCTGAAGCAC3'	Exon26	
[b]*MRP1 Sense*	5'CTGGCATTCAAGACAAGGT 3'	Exon 6	217 bp
MRP1 Antisense	5'ACCGGAGGATGTTGAACAAG 3'	Exon 7	
[c]*Bcl-2* Sense	5' GGATTGTGGCCTTCTTTGAG 3'	Exon 1	219 bp
Bcl-2 Antisense	5'TCTTCAGAGACAGCCAGGAGA 3'	Exon2	
[d]*Bax Sense*	5' TCTGACGGCAACTTCAACTG 3'	Exon 4	188 bp
Bax Antisense	5' TTGAGGAGTCTCACCCAACC3'	Exon5	
[e]*β-2microglobulin* Sense	5' TCTCTCTTTCTGGCCTGGAG 3'	Exon 1	122 bp
β-2microglobulin Antisense	5' GGATGGATGAAACCCAGACA 3'	Exon2	

[a] NCBI accession number: NM_000927

[b] NCBI accession number: NM_004996

[c] NCBI accession number: NM_000633

[d] NCBI accession number: NM_004324

[e] NCBI accession number: NM_004048

Table 2.2 The reaction ingredients of PCR. The PCR reaction was performed either in 25 or 50 µl reaction volumes.

Ingredients	**Volume (µl)**				
	***MDR1**	***MRP1***	***Bcl-2***	***Bax***	***β-2microglobulin**
Buffer (10X *Taq* buffer)	5	5	5	5	5
$MgCl_2$ (25 mM)	4	4	4	4	4
dNTP mix (10 mM each)	1	1	1.6	1.6	1
Forward Primer, (25 pmol/µl)	1	1	1	1	1
Reverse Primers (25 pmol/µl)	1	1	1	1	1
DMSO	5	-	-	-	2.5
cDNA	6	2-6	8	6	5
Enzyme (*Taq* DNA polymerase, 5u/µl)	0.3	0.3	0.3	0.3	0.2
Complete w/ dH_2O	to50 µl	to50 µl	to50 µl	to50 µl	to50 µl

* The reaction is hot start.

2.3.3 Agarose Gel Electrophoresis and Quantification of Band Intensities

Detection of RT-PCR products was performed through agarose gel electrophoresis with TAE (Tris-Acetate-EDTA) buffer system. The samples were run on 2% (w/v) agarose (for PCR products) in TAE buffer at horizontal electrophoresis system. Ethidium bromide was added to the gel to provide visualization under ultra violet light. The bands were detected with Vilber Lourmat gel documentation system (Cedex, France). The band intensities of RT-PCR products were quantified using Scion Image Software (Scion Corporation, USA). The band intensities of RT-PCR products performed with resistance related genes were divided with band intensity of *β-2microglobulin* gene to normalize the densitometric measurements. The dose dependent changes in expression levels of the genes were analyzed according to the variations of densitometric ratios among MCF-7 cell lines that developed drug resistance at different levels.

2.3.4 Real-time PCR (Q-PCR)

RT-PCR is a semiquantitative detection system for gene expression levels, Real-time PCR is a absolute quantitative technique. cDNA was used as template for the Real-time PCR reaction. Roche Light Cycler Taqman Master ready to use hot start reaction mix (Basel, Switzerland) was used for amplification. 0.5 µM primer (Table 2.3), 0.2 µM probe (Roche universal probe library), 1X master mix, 7 µL cDNA were the components of the 20 µL reaction. The conditions were optimized by performing Real-time PCR with the cDNA of known concentrations. Finally the reaction was performed with five standard cDNAs, one non-template control (all the reaction components except cDNA) and the unknowns. The total RNA concentrations of five standarts are presented in Table 2.4. The unknown samples were cDNA from MCF-7/S, MCF-7/50nMPac, MCF-7/400nMPac, MCF-7/20nMVinc, MCF-7/120nMVinc. Each sample was run twice in reaction. The Real-time PCR reaction was performed in Roche LightCycler®2.0 Instrument (Basel, Switzerland). The optimal cycle conditions are given in Table 2.5.

Table 2.3 Primers sets and amplicon sizes for real-time PCR

Primer List	**Sequence**	**Location**	**Product**
MDR1 Sense	5'AAGGCATTTACTTCAAACTTGTCA 3'	Exon 16	77 bp
MDR1 Antisense	5' GGATTCATCAGCTGCATTTTC 3'	Exon 17	
MRP1 Sense	5' TGTGGGAAAACACATCTTTGA 3'	Exon 18	80 bp
MRP1 Antisense	5' CTGTGCGTGACCAAGATCC3'	Exon 19	

Table 2.4 RNA concentrations of five standarts and dilution numbers

	RNA concentration	**cDNA dilution number**
Standart 1	1.75 µg	$Log10^{7} = 7$
Standart 2	0.573 µg	$Log10^{6.5} = 6.5$
Standart 3	0.175 µg	$Log10^{6} = 6$
Standart 4	0.0573 µg	$Log10^{5.5} = 5.5$
Standart 5	0.0175 µg	$Log10^{5} = 5$

Table 2.5 Real-Time PCR conditions

Analysis Mode	Cycles	Segment	Temperature ºC	Time
Pre-Incubation				
	1		95 ºC	15 min
Amplification				
Quantification	45	Denaturation	95 ºC	10 s
		Annealing	56 ºC	30 s
		Extension	72 ºC	1 s
Cooling				
	1		40 ºC	30 s

The results were supplied automatically as fluorescent values that were obtained from unknown samples. The expression levels were quantified by converting fluorescent values to RNA amounts. The standard deviations of the values were also supplied automatically. The significant RNA level variations between cell lines were demonstrated graphically.

2.3.5 Microarray Analysis

2.3.5.1 RNA Isolation, cDNA Synthesis and Target Preparation

RNA isolation form MCF-7/S, MCF-7/400nMPac and MC-F-7/120nMVinc were performed using TRI Reagent (Sigma, St. Louis, MO, USA) according to manufacturer's instructions. Absorbance values (260 nm, 280 nm) were measured for RNA quantification by spectrophotometry. RNA intactness was checked on denaturating agarose (1% w/v) gel electrophoresis (70V, 90min) in MOPS buffer system (Appendix C). Quality of RNA was adjusted to ratio between 1.8-2.0 and concentration was at least 2.5 µg/ µL. All RNA samples were prepared as duplicates

to provide biological replicates for microarray assay. The microarray experiment steps are summarized in Table 2.6. cDNA was synthesized from total RNA by One-Cycle Target Labelling Assay® (Affymetrix, Santa Clara, CA, USA) according to manufacturer's instructions. Second strand cDNA synthesis, biotin labelled cRNA synthesis (IVT Labelling), fragmentation of cRNA were performed by Affymetrix GeneChip® kit reagents according to procedure as described in detail in the Affymetrix GeneChip® Expression Analysis Technical Manual (Santa Clara, CA, USA).

2.3.5.2 Target Hybridization and Scanning

Biotin labelled and fragmented target cRNA samples were loaded into 49/64 format type Affymetrix GeneChip® (Human Genome U133 Plus 2.0 Array) together with control cRNAs and oligo B2. Target hybridization and scanning procedures were performed in Molecular Biology and Biotechnology R & D Center (METU-Central Laboratory, Ankara, Turkey). Hybridization procedure was conducted at 45 ºC, 60 rpm for 17 h in Affymetrix GeneChip® Hybridization Oven 640. The required solutions are given in Appendix D. Washing and staining procedure was performed in Affymetrix GeneChip® Fuidics Station 450 with Euk Ge-WS2v5 fluidics script according to the instructions in Affymetrix GeneChip® Expression Analysis Technical Manual. Affymetrix GeneChip® Scanner 3000 device was used for array scanning according to technical instructions. The prescriptions of solutions for washing and staining step are described in Appendix E.

Table 2.6 Target preparation, hybridization, scanning steps are summerized

Components	Amount (µL)	Total (µL)	T (ºC)	Time
One Cycle cDNA Synthesis				
Total RNA (15µg)	Variable			
Diluted Poly-A Control (1: 3.33)	2	11	70	10 min
T7-Oligo(dT), 50 µM	2			
RNase-free Water	variable			
Cool, 4ºC, 2 min				
5X 1st strand Reaction Mix	4			
DTT, 0.1 M	2	18	42	2 min
dNTP, 10 mM	2			
Superscript II	2	20	42	60 min
Cool, 4ºC, 2 min				
Second Strand cDNA Synthesis				
RNase-free Water	91			
5X 2nd strand Reaction Mix	30	150	16	2 h
dNTP, 10 mM	3			
E.coli DNA ligase	1			
E,coli DNA polymerase	4			
RNase H	1			
1st strand cDNA	20			
Cool, 4ºC, 2 min				
T4 DNA polymerase	2	152	16	5 min
EDTA, 0.5 M	10	162	4	2 min
Clean-up of Double Stranded cDNA				
IVT Labeling (synthesis of Biotin Labelled cRNA)				
Double strand cDNA	6			
RNase-free water	14	40	37	17 h
10X IVT Labelling Buffer	4			
IVT Labeling NTP mix	12			
IVT Labelling Enzyme mix	4			
Clean-up and Quantification (UV spectroscopy) of Biotin Labelled cRNA				
Fragmenting cRNA				
cRNA (20µg)	1-21			
5X Fragmentation Buffer	8	40	94	35 min
RNase free water	variable			
Store at -20ºC				

Table 2.6 continues

Components	Amount (µL)	Total (µL)	T (ºC)	Time
Target Hybridization (Application in to Array)				
Fragmented cRNA (15µg)	30	300	45	60 rpm,17 h
Control oligonucleotide B2 (3 nM)	5			
2X Euk Hybridization Control	15			
Herring Sperm DNA (10 mg/mL)	3			
BSA (50mg/mL)	2			
2X Hybridizaiton Buffer	150			
DMSO	30			
RNase-free Water	64			
Washing				
Wash A Buffer	200			
Staining (SAPE soln)				
2X Stain Buffer	600	1200	Insert solution in washing- staining station, (25 ºC)	
50 mg/mL BSA	48			
Streptavidin	12			
DI Water	540			
Staining (Antibody soln)				
2X Stain Buffer	300	600	Insert solution in washing- staining station, (25 ºC)	
50 mg/mL BSA	24			
10 mg/mL Goat IgG	6			
0.5 mg/mL Biotinylated antibody	3.6			
DI Water	266.4			
Insert Array in Washing, Staining Station (2 h) **Insert Array in Scanner (15 min)**				

2.3.5.3 Data Analysis and Preparation of Gene Lists

Preliminary analysis of the scanned chips was performed using Affymetrix GeneChip® Operating Software (GCOS). The quality of gene expression data was checked according to quality control criteria which are based on background signal of the chip, noise, signal levels of polyA controls and percentage of signals that demonstrate the genes whose expression is present (Yılmaz *et al*., 2008). Then, GeneSpring GX 7.3.1 Software (Agilent Technologies, Inc., Santa Clara, CA, USA) was used for further data analysis and evaluation. Agilent GeneSpring GX is a powerful visualization and analysis solution designed for use with genomic expression data. The GeneSpring GX platform is designed to break through bottlenecks in the analysis process and to help identify genes/pathways that are truly relevant to the biological question by comparing analysis results from expression, genotyping, protein, metabolite and other data types. The program allows scientists to identify targets quickly and reliably; cover statistically meaningful results, using techniques such as 1-way and 2-way ANOVA, multiple testing corrections, or Student-Newman-Keuls post hoc tests; display expression data with customizable visualization tools; create complex experiments that link trends in expression; data to a variety of test parameters.

The data was initially normalized by Robust Multichip Average (RMA) normalization algorithms. Statistically significant data were selected by independent sample t-test (α=0.05) between duplicate data for resistant cell lines with respect to data of MCF-7/S. Significantly altered genes between resistant sublines and MCF-7/S were listed and gene trees were constructed from these lists by standard correlation (Figure 2.1, 2.2). The red lines are display the upregulated genes, blue lines are for downregulated genes, yellow lines are for the genes that did not change. Upregulated and downregulated genes were selected from gene trees. The genes were filtered by volcano plots (Figure 2.3., 2.4) the red dots are the single 2 fold upregulated and downregulated genes and the yellow ones are the genes whose expression levels are at between 2 and 0.5 fold with respect to sensitive cell line. The genes upregulated and downregulated more than 2-fold were considered in

constructing gene lists. The gene lists were classified with respect to “biological processes” (i.e. cell growth and maintenance etc.) and “molecular functions” (i.e. apoptosis regulator activity, binding activity, catalytic activity etc.) of the proteins that are encoded by the genes. Finally the genes were grouped specifically and new lists were generated to evaluate the relation of the genes between multiple drug resistance.

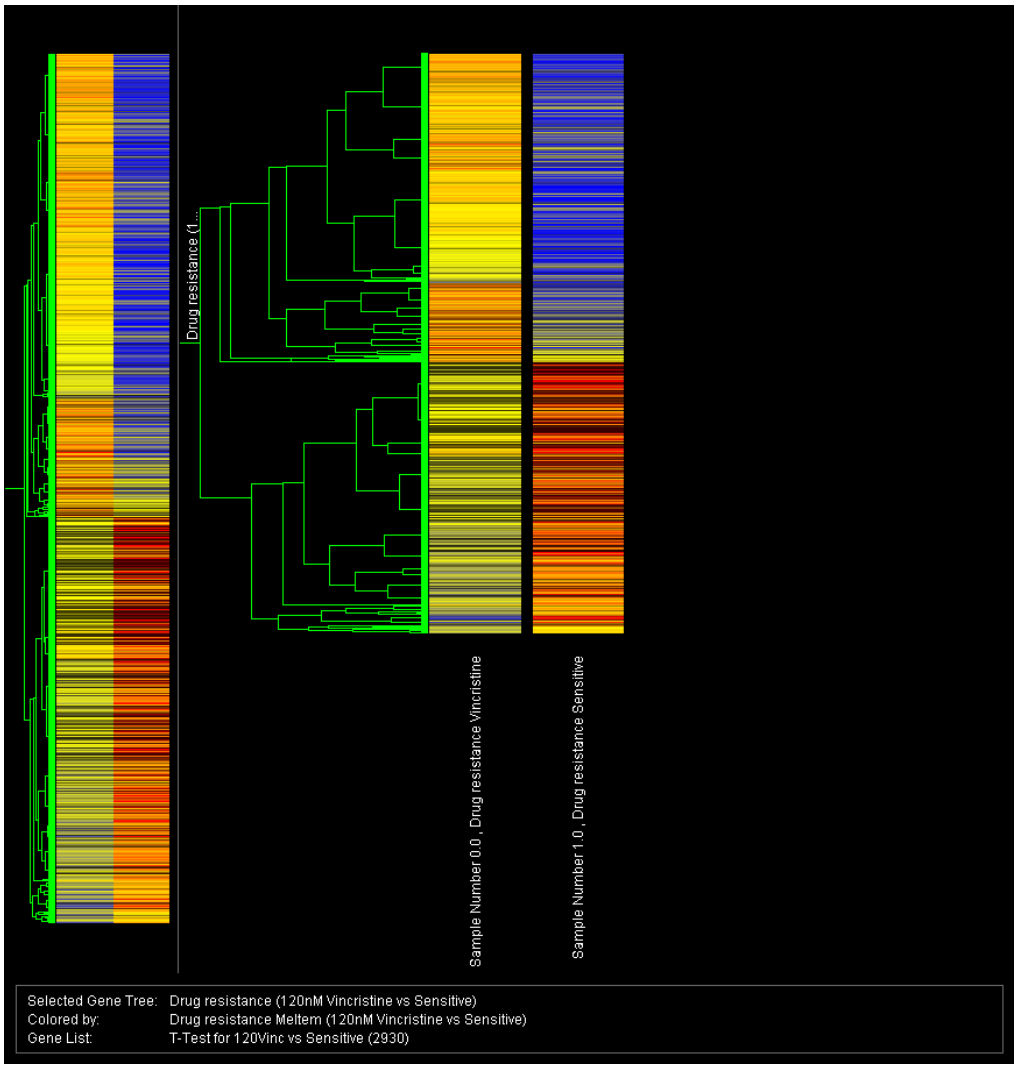


Figure 2.1 Gene tree that compares MCF-7/120nMVinc and MCF-7/S

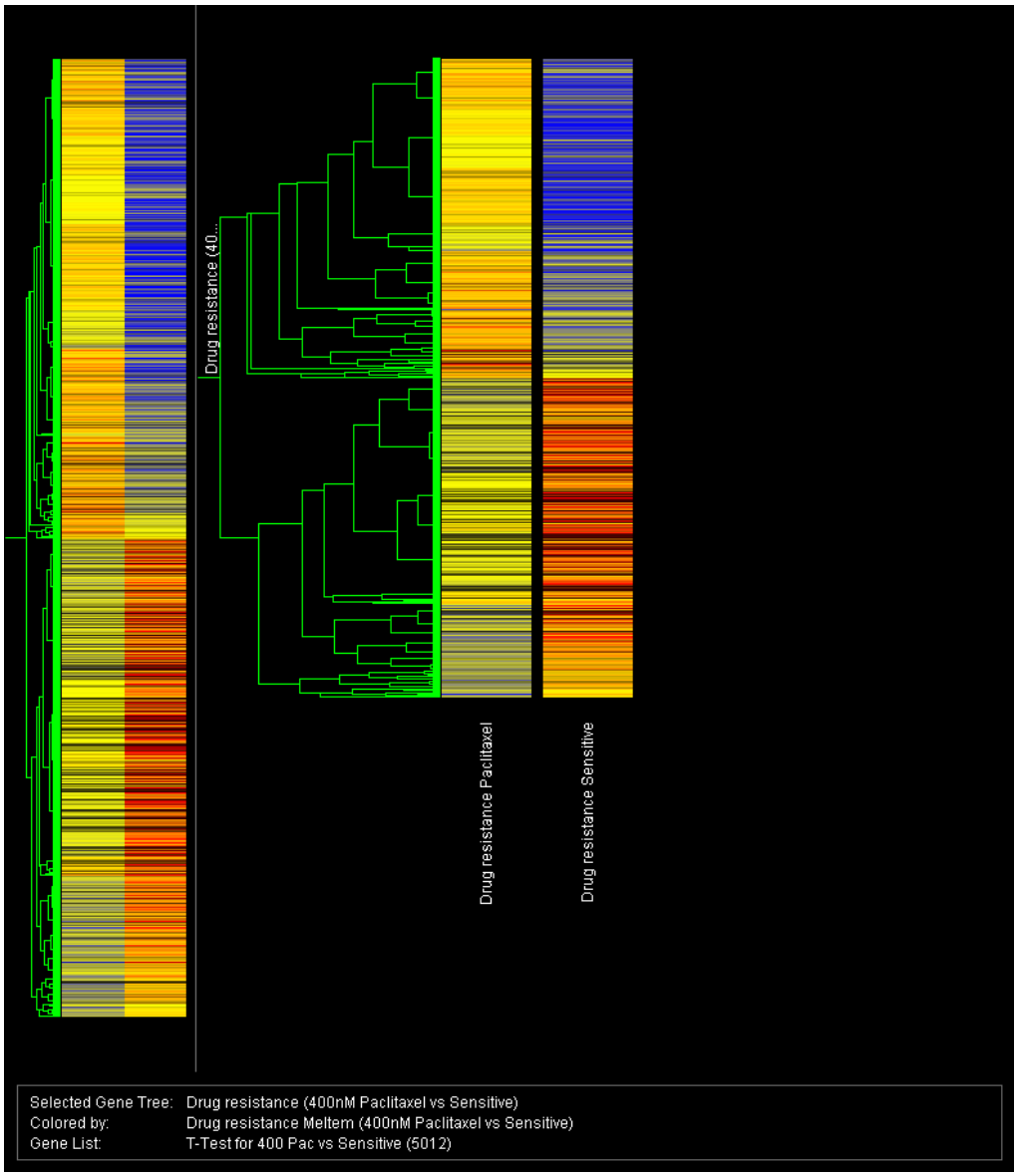


Figure 2.2 Gene tree that compares MCF-7/400nMPac and MCF-7/S

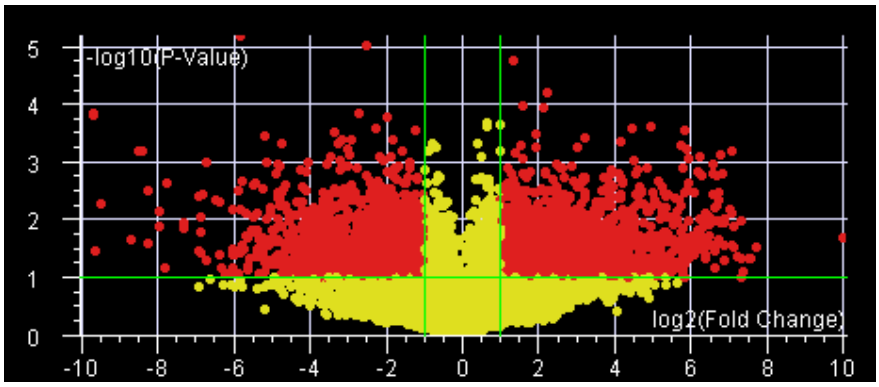


Figure 2.3 Volcano plot that displays 2 fold and more upregulated and downregulated genes of MCF-7/120nMVinc with respect to MCF-7/S

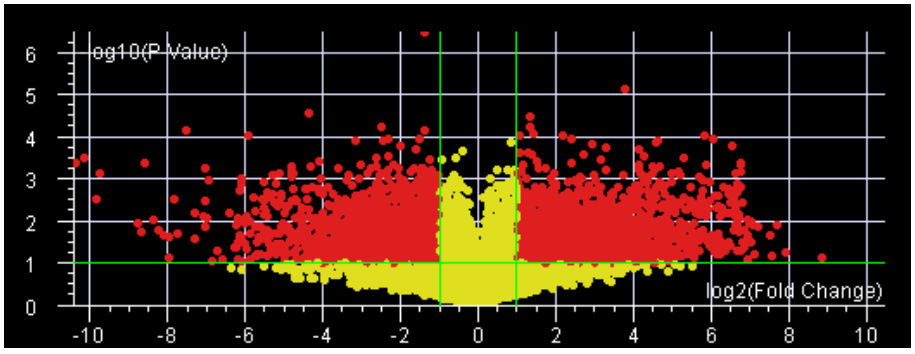


Figure 2.4 Volcano plot that displays 2 fold and more upregulated and downregulated genes of MCF-7/400nMPac with respect to MCF-7/S

2.4 Expression Analysis of Drug Resistance Related Proteins

2.4.1 Immunocytochemisrty

Cells were harvested and resuspended in serum free media; the density of cell suspension was $1x10^6$ cell /mL. 200 µL of suspension was cytocentrifugated for 5 minutes on 1000 rpm (Cytospin-3 Shandon). The samples were fixed in cold acetone for 10 minutes and were washed in PBS buffer (pH 7.6) for 5 minutes. Endogenous peroxidases were quenched in 0.3 % H_2O_2 for 5 minutes. The samples were

incubated in primary antibodies (P-gp: JSB1, MRP1, monoclonal (Novocastra), LRP: LRP, monoclonal (Novocastra)) in suitable dilution (1:25) for 30 minutes at room temperature. The samples were washed in PBS buffer for 2x2 minutes and were incubated with the secondary antibody (DAKO EnVision TM System, CA, USA) for 30 minutes. The samples were washed in PBS for 2x2 minutes. Diaminobenzidine (DAB-brown) was used as chromogen. Slides were counterstained with hematoxylin and mounted.

2.4.2 Protein Isolation and Bradford Assay

Protein isolation was performed according to total cell lysate extraction, described by Han et al. (Han *et al*., 2000). Cell number was adjusted to $3x10^6$ cells and pelleted. Lysis buffer containing a cocktail of protease inhibitors (PMSF; 1 mM, aprotinin; 2 µg/mL, pepstatin; 1 µg/mL) was added onto the pellet. Following 30 min ice incubation, nuclei were pelleted at 3500 rpm for 5 min at 4 ºC. Supernatant containing cell lysate was collected and the protein concentration was measured by Bradford assay (Bradford, 1976). In order to draw a calibration curve, seven standards were prepared. And then, the concentrations of unknown samples to be used in SDS-PAGE were calculated from the standard curve. The solutions prepared for protein isolation and bradford assay are described in Appendix F.

2.4.3 SDS Poly-acrylamide Gel Electrophoresis and Western Blot

The optimized amounts of cell lysates were run in 7.5 % polyacrylamide gel SDS-PAGE using vertical electrophoresis system (40 mA-60 mA, 5 h) and were stained with Comassie Blue. (See Appendix G for details).

Total cell lysates were electrophoretically transferred to a 0.45 µm nitrocellulose membrane (Protran BA 85, Schleicher & Schuell) at 25 V for overnight using BioRad ElectroBlot System. The membrane was washed with TBST buffer and the nonspecific binding sites were blocked with 5% non fat dry milk in TBST. The membrane was then incubated with glyceraldehydes-3-phosphate dehydrogenase

(GAPDH) (Chemicon, 1:200), Bcl-2 (Biovision, 4 µg/mL), Bax (Chemicon, 4µg/mL) monoclonal antibodies. 144 kDa tetrameric GAPDH enzyme is constitutively expressed in mammalian cells and was used as loading control in Western blotting. Other monoclonal antibodies react with 26 kDa Bcl-2 and 21 kDa Bax proteins. Membranes were incubated in primary antibody solution for 2 hours. Detection was possible by incubation with peroxidase conjugated goat anti mouse IgG secondary antibody (Chemicon, 1:5000) 1 hour. Finally the membranes were applied with diaminobenzidine (DAB-brown) solution (1:20). Bands became visible by conversion of DAB substrate to chromogenic brown product on membrane. The detection of P-gp (170 kDa) and MRP (190 kDa) proteins were performed by incubation with primary antibodies (P-gp: JSB1, MRP1, monoclonal (Novocastra), in suitable dilution (1:25) for 30 minutes at room temperature. The membranes were incubated with the secondary antibody (DAKO EnVision TM System, CA, USA) for 30 minutes. The samples were washed in PBS for 2x2 minutes. Diaminobenzidine was used as chromogen. The solutions used for Western Blot are described in Appendix F.

2.5 Investigation of Mutations on *Beta-tubulin* Gene

Functional beta tubulin protein is mostly encoded from 4th exon of the class I isotype of the *beta tubulin* gene (Hasegawa *et al*., 2002). In this part of the investigation any mutations in the fourth exon were determined.

The experimental steps were DNA isolation, PCR for the amplification of 4th exon, nested PCR for the amplification of 4th exon as four fragments, precipitation of the PCR products, elution from the gel and finally sequencing the fragments. The sequence ofthe fragments from MCF-7/120nMVinc, MCF-7/400nMPac cell lines were compared with that of MCF-7/S and also with the gene sequence that was obtained from databases. Any changes in the gene sequence were determined for the resistant sublines.

DNA isolation was performed with RTA DNA isolation kit (Sacem,Turkey). All the PCR reactions were performed with error free high fidelity DNA polymerase enzyme, Phusion (Finnzymes, Espoo, Finland) at very stringent PCR conditions to eliminate mis-incorporations. PCR products were eluted from the gel by using Mo-Bio Ultra Clean Gel Elution Kit (Carlsbad, CA) and were sent for sequencing (İontek, Istanbul, Turkey). The experiment set-up is represented in Figure 2.5. Primer sets, annealing temperatures and amplicon sizes are given in Table 2.7.

Table 2.7 Primer sets and PCR conditions for amplification of 4th exon of beta-tubulin gene and for nested PCR.

	Primers	Ann T°C	# cycle	Amplicon (bp)
TUBB 4th exon	F: 5' TGTATTGGAGTGCTAATACAG 3' R: 5' CTCCCTTGAAGCTGAGATGG 3'	58	35	1810bp
Fragment1	F: 5' CATGTATCTTCCATACCCTG 3' R: 5' CTGAAGGTATTCATGATGCG 3'	59	30	278bp
Fragment2	F: 5' GAATGGGCACTCTCCTTATC 3' R: 5' GGACCATGTTGACTGCCAAC 3'	59	30	339bp
Fragment3	F: 5' ATGAGTGGTGTCACCACCTG 3' R: 5' GACTGCCATCTTGAGGCCAC 3'	59	30	399bp
Fragment4	F:5' CCCAACAAT GTCAAGACAGC 3' R:5'CAAGATAGAGGCAGCAAACAC 3'	59	30	401bp

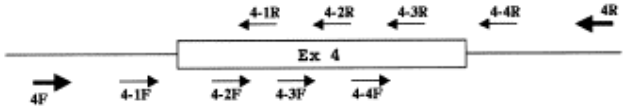


Figure 2.5 Demonstration of nested PCR procedure.

2.6 Drug Accumulation Assays

Fluorescent dye accumulation assays were performed to measure the drug efflux activities of P-gp and MRP1 proteins in drug resistant MCF-7 cell lines. In order to assay the efflux activity, parental and resistant cell lines were trypsinized and the cell concentration was adjusted to $2x10^6$ cells/mL. The cells were then suspended in serum-free RPMI 1640 medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. Rhodamine-123 (Sigma , 5.2 μM) was added to test drug efflux through P-gp, while carboxyfluorescein (Sigma, 5.2 μM) was added to test efflux through MRP1. The cells were incubated for 20 min at 37 ºC then centrifuged, washed twice in 0.5 mL PBS and finally resuspended in 0.5 mL PBS. The fluorescence of the cell population was measured using a flow cytometer (BD FACScan, Franklin Lakes, NJ, UK). The cells were selected by dose increments until resistant sublines were generated. The cell lines were named as resistant when the drug accumulation assays resulted in single-peaked histograms in the low fluorescence region. The resistant sublines served as models and were used to investigate molecular mechanisms of acquired drug resistance.

2.7 Reversal of P-gp and MRP1 Related MDR by Modulators

Parental and resistant cell lines were trypsinized and the cell concentration was adjusted to $2x10^6$cells/mL. The cells were suspended in serum free RPMI 1640 medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. Compounds to be tested were added (40 μg/mL) and samples were incubated for 10 min at room temperature (25 ºC). Rhodamine-123 (P-gp substrate) or carboxyfluorescein (MRP1 substrate) fluorescent indicators were added (5.2 μM

final concentration) to samples and cells were incubated for 20 min at 37º C. The cells were centrifuged, washed twice in 0.5 mL PBS and finally resuspended in 0.5 mL PBS for assay. The fluorescence of the cell population was measured using flow cytometry. Verapamil was used in the rhodamine-123 exclusion assays, and indomethacin in the carboxyfluorescein exclusion assays, as positive control modulator compounds. The fluorescent activities for the treated MCF-7 cell lines were calculated by comparing them with the fluorescent activities of the untreated cells.

The ratio was calculated using the following expression (Fakla *et al*., 1998) on the basis of the measured fluorescence intensities (F):

$$FAR = \frac{\text{mdr treated / mdr control}}{\text{parental treated / parental control}} \qquad \text{[Equation 3]}$$

Compounds were judged to be active modulators if the ratios (fluorescence activity ratio, FAR) were greater than 1.10 (Ugocsai *et al*., 2005). The compounds that were used as MDR reversal agents are listed in Table 2.8.

Table 2.8 MDR Reversal Agents

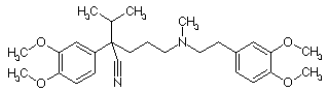
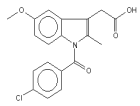
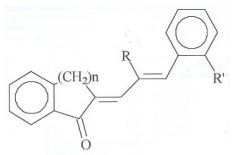
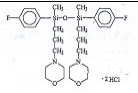
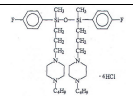
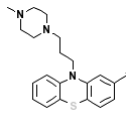
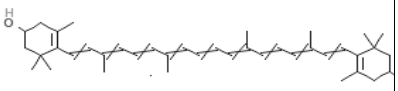
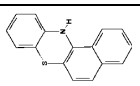
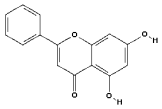
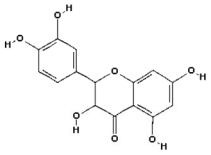
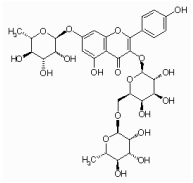
Compounds	**Functions**	**Structures**
Verapamil	Calcium channel blocker	
Indomethacine	Anti-inflammatory, agent	
Promethazine	Phenothiazine, calmodulin inhibitor	
Capsanthin	Carotenoid, antioxidant	
Cinnamylidene ketone-1	Cinnamylidene ketone, synthetic R=H, R'=Ome	
Cinnamylidene ketone-2	Cinnamylidene ketone, synthetic R=H, R'=H	
ALIS 409	Silicon compound	
ALIS 421	Silicon compound	

Table 2.8 continues

Compounds	**Functions**	**Structures**
Silyl-phenothiazine	Phenothiazine, calmodulin inhibitor	
Zeaxanthin	Carotenoid, natural extract	
12H-benzo(a)phenothiazine	Phenothiazine, calmodulin inhibitor	
Chrysin	Flovanoid, antioxidant	
Dihydroquercetin	Flovanoid, antioxidant, plant coloring material	
Robinin	Flovanoid, antioxidant *Robinia pseudoacacia*	

2.8 Combined Application of MDR Modulators with Anticancer Drugs

Checkerboard micro plate method was applied to study the effects of drug interactions between resistance modifiers and anticancer drugs on resistant MCF-7 cell lines. The dilutions of anticancer drugs (A) were made in horizontal direction and the dilutions of resistance modifiers (B) vertically in microtiter plate in 100 µL. The cells were distributed to each well in 50 µL containing $5x10^3$ cells. The cells were incubated for 72 h at 37 ºC in CO_2 incubator.

The cell growth was determined after XTT staining and intensity of colored formazan crystals was measured on ELISA reader. Drug interaction was evaluated according to the following expressions:

$FIC_A = IC_{50A \text{ in combination}} / IC_{50A \text{ alone}}$ [Equation 4]

$FIC_B = IC_{50B \text{ in combination}} / IC_{50B \text{ alone}}$ [Equation 5]

where FIC is fractional inhibitory concentration.

Fractional inhibitory index, $FIX = FIC_A + FIC_B$ [Equation 6] demonstrates the effect of combination of anticancer drug and resistance modifier. It is accepted that if FIX value is 0.51-1, it is an additive effect; if FIX value is less than 0.5 it is a synergism. FIX value in between 1-2 is considered an indifferent effect while the value greater than 2 indicates antagonism (Eliopoulos and Moellering, 1980).

Since cancer therapy is already a combination approach, the application of anticancer drugs with MDR modulators may be easily acceptable for clinicians under controlled conditions. Combination test by checkerboard micro plate method was used to test the effects of combined application. Paclitaxel and vincristine were applied in combination with selected effective MDR modulator compounds on the sublines.

2.9 Reversal of *MDR1* and *MRP1* Gene Expression by Application of MDR Modulating Agents

Promethazine was previously proved as an inhibitor for *MDR1* gene expression in mouse lymphoma cell line (Molnar *et al.*, 1998). In this study, the resistant MCF-7/400nMPac and MCF-7/120nMVinc cell lines were treated with 1.6 µM and 4.8 µM promethazine for 0, 12, 24, 48, 72 hours and with 9.6 µM promethazine for 72h. RNA isolation and RT-PCR for *MDR1* and *beta2-microglobulin* genes were performed for the treated and untreated cell lines. RT-PCR were repeated two times and the PCR products were run in 2% agarose gel. The dose dependent variations of gene expression levels among cell lines were determined by densitometric analysis.

Verapamil was previously proved as inhibitor for MDR1 protein activity. The resistant MCF-7/400nMPac cell line was treated with 50 µM (IC_{50}/2) of verapamil for 0, 48, 72 hours. MCF-7/120nMVinc cell line was treated with 60 µM (IC_{50}/2) verapamil for 0, 48, 72 hours. RNA isolation and RT-PCR for *MDR1*, *MRP1* and *beta2-microglobulin* genes were performed for the treated and untreated cell lines. RT-PCR were repeated two times and the PCR products were run in 2 % agarose gel. The dose dependent variations of gene expression levels among cell lines were determined by densitometric analysis.

2.10 Investigation of Cross Resistance Development to Various Anticancer Agents and Gamma Irradiation in MCF-7/400Pac and MCF-7/120Vinc Cell Lines

The effects of the chemotherapeutic agents (paclitaxel, vincristine, doxorubicin, tamoxifen, ATRA- all-trans retinoic acid) on proliferation of sensitive and resistant MCF-7 cell lines (MCF-7/400nMPac and MCF-7/120nMVinc) were tested in 96-well microtiter plates as previously described in section 2.2.2. Paclitaxel, tamoxifen, all-trans retinoic acid (ATRA) (Sigma, St. Louis, MO, USA) were dissolved in DMSO to prepare stock solutions. Vincristine and doxorubicin were obtained from Gülhane Military Academy, School of Medicine, Ankara, Turkey. Vincristine and doxorubicin were diluted in deionized water. The resistance indices of each cell line to anticancer agents were calculated to determine the degree of acquired resistance or cross-resistance of each cell line to anticancer drugs. The resistance indices were evaluated according to the Equation 2.

Sensitive and resistant MCF-7 were seeded to 96-well microtiter plates (5000 cells/well) and after 24 h incubation in standard culture conditions irradiated with doses of 200 and 800 cGy by a Theratron 780 Cobalt 60 Teletherapy Unit (AECL Medical, Ontario, Canada). After irradiation, all plates were incubated at 37°C in a 5% CO_2 atmosphere for an additional 24 hour. Then cell proliferation was evaluated using XTT Cell Proliferation Kit (Biological Industries, Israel). IC_{50} values represent inhibitory doses of irradiation in cGY to evaluate antiproliferative effects of

irradiation on cells and were calculated from cell proliferation curves as described in XTT cytotoxicity analysis section before.

2.11 Combined Application of Anticancer Agents to MCF-7/400Pac and MCF-7/120Vinc Cell Lines

Checkerboard micro plate method was applied to study the effects of drug interactions between two anticancer drugs on resistant MCF-7 cell lines. The dilutions of anticancer drugs (A) were made in horizontal direction and the dilutions of second drug (B) vertically in microtiter plate as previously described in section 2.7. The combined effects of paclitaxel with vincristine, doxorubicin, tamoxifen and ATRA on MCF-7/400Pac and that of vincristine with paclitaxel, doxorubicin, tamoxifen and ATRA on MCF-7/120Vinc cell lines were evaluated according to Equations 4, 5 and 6.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Doubling Time Determination, Growth Curve Construction

When the td values were considered, it may be concluded that paclitaxel and vincristine resistant cell lines (MCF-7/400nMPac and MCF-7/120nMVinc) have developed different growth profiles and division intervals. All the newly generated sublines have different and significantly higher doubling times ($p<0.05$) with respect to parental cell line (Table 3.1). Also the growth profiles of the cell lines are different form the parental cell line significantly (Figure 3.1, 3.2, 3.3, 3.4). As a result, it can be concluded as, resistant sublines are derivatives of MCF-7/S.

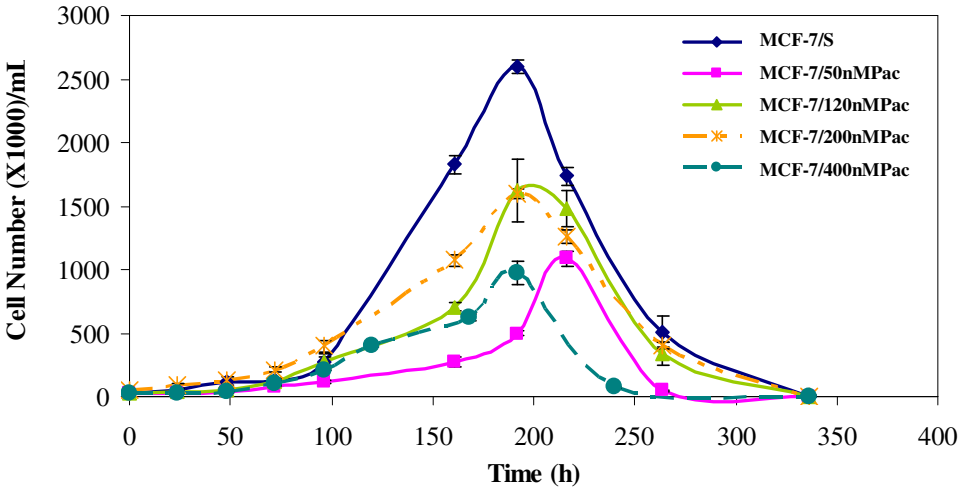


Figure 3.1 Graphical representation of growth profiles of MCF-7/Pac sublines.

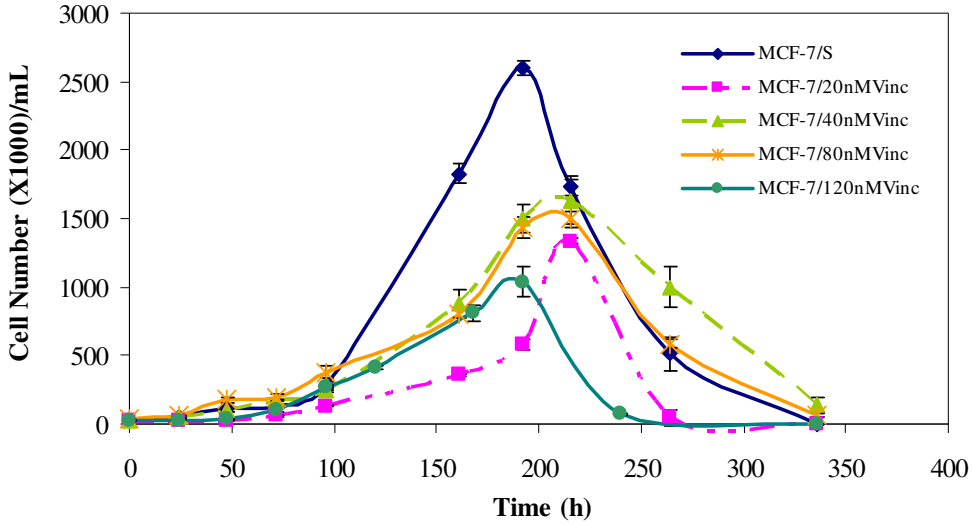


Figure 3.2 Graphical representation of growth profiles of MCF-7/Vinc sublines.

The increase in doubling time brought the slow generation of the resistant sublines which may be a result of variation in expression levels of regulatory genes in cell cycle check points. This point will be discussed later during evaluation of microarray data (cell cycle related genes part).

Table 3.1 Doubling time values for a) MCF-7/Pac cell lines

a)

	MCF-7/S	MCF-7/50nMPac	MCF-7/120nMPac	MCF-7/200nMPac	MCF-7/400nMPac
td ± SEM (h)	28.55± 1.57	35.63 ± 2.07	40.24 ± 2.10	46.94 ± 1.76	52.68 ± 1.36
p value		**p<0.05**	**p<0.05**	**p<0.05**	**p<0.05**

Table 3.1 b) Doubling time values for b) MCF-7/Vinc cell lines

	MCF-7/S	MCF-7/20nMVinc	MCF-7/40nMVinc	MCF-7/80nMVinc	MCF-7/120nMVinc
td ± SEM (h)	28.55± 1.57	35.88 ± 1.14	41.99 ± 2.18	43.49 ± 1.90	53.94 ± 3.62
p value		**p<0.05**	**p<0.05**	**p<0.05**	**p<0.05**

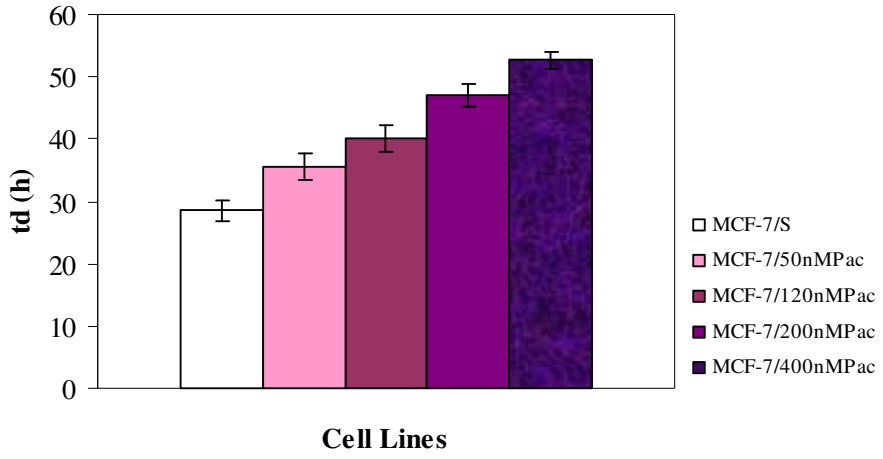


Figure 3.3 Graphical representation of td for MCF-7/Pac cell lines. SEM values (standart error of means) were derived from the results of four independent experiments.

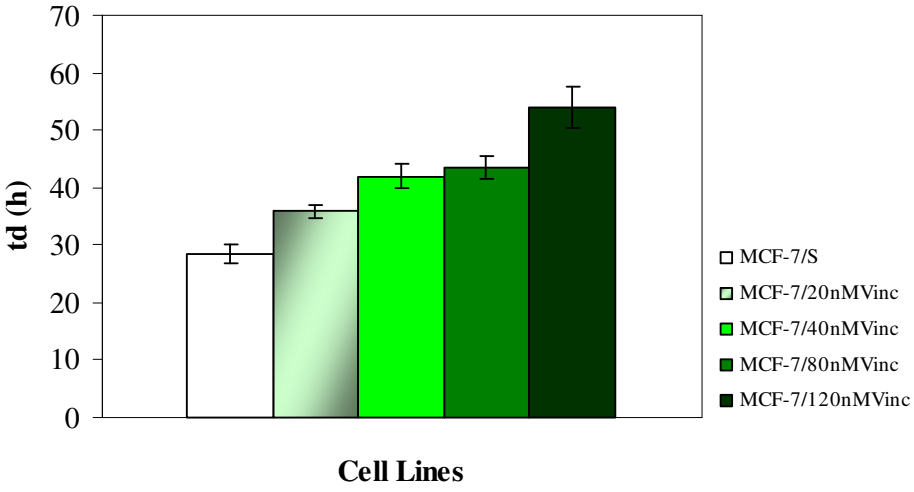


Figure 3.4 Graphical representation of td for MCF-7/Vinc cell lines. SEM values (standart error of means) were derived from the results of four independent experiments.

3.2 XTT Cytotoxicity Analysis

3.2.1 Cell Proliferaiton Profiles and IC_{50} Values for Paclitaxel Adapted MCF-7 Cell Line

Cell proliferation profiles, IC_{50} values and resistance indices of the paclitaxel adapted sublines are presented in Figure 3.6, 3.7 and Table 3.2 According to results, resistance index of MCF-7/50nMPac was not significantly higher than the MCF-7/S. On the other hand, MCF-7/120nMPac, MCF-7/200nMPac, MCF-7/400nMPac sublines developed significant resistance with respect to MCF-7/S ($p<0.05$). IC_{50} value of MCF-7/400nMPac was about 150 folds more than the MCF-7/S, which means that the 400nM paclitaxel adapted cell line acquired that much resistance with respect to the parental cell line.

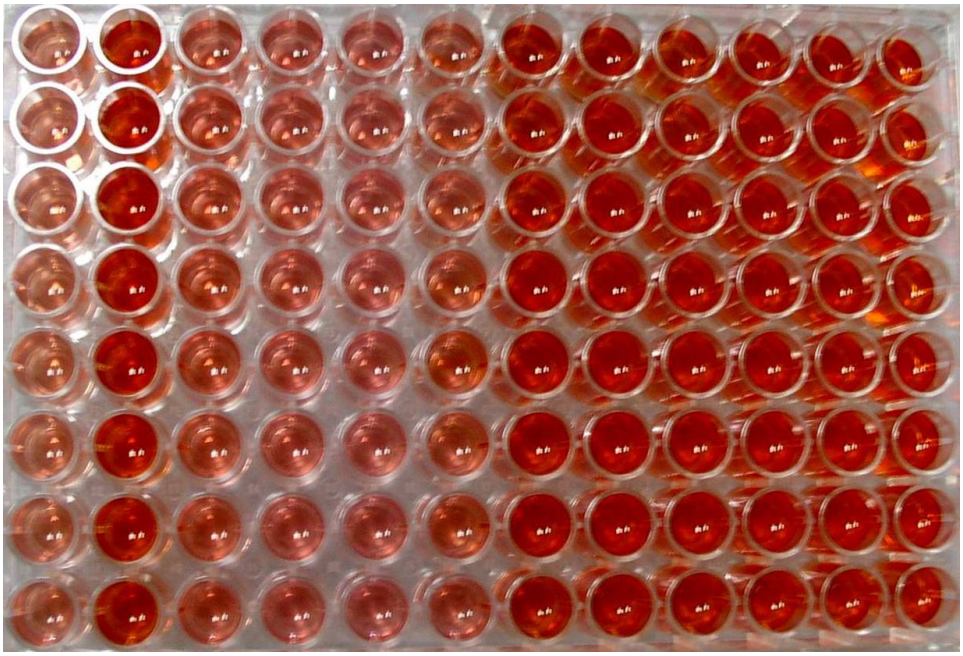


Figure 3.5 96 well microtiter plate, 1st column: medium control, 2nd column: cell control, 3rd-12th column contain equal amount of cell, medium, XTT reagent but each column contained anticancer drug as horizontally decreasing concentrations.

The colorimetric assay plate also reveals that the high drug concentration inhibited cell growth so as the color intensity was lower than the wells that contain low concentration of drug in medium.

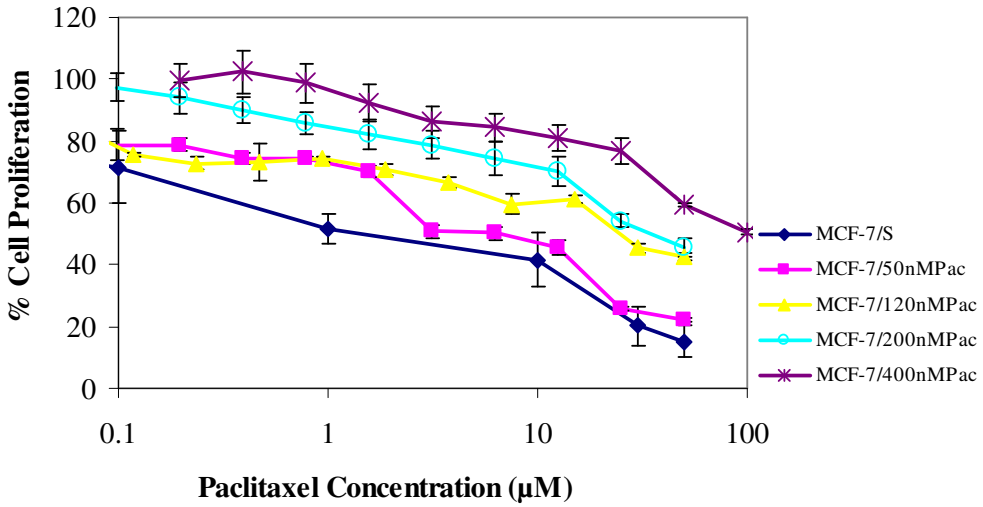


Figure 3.6 Graphical demonstration of inhibition of cell proliferation in MCF-7/Pac sublines.

The findings indicate that the sublines were increasingly resistant to paclitaxel from eachother. It is obviously seen in Table 3.2 and Figure 3.10 that the resistance indices were also increasing exponentially with the values 9, 13, 36, 150. During dose increaments sublines may have developed new escape shortcuts by differential expressions of survival genes or the genes that allow the cells to live in that new condition.

Table 3.2 IC_{50} values and resistance indices of the MCF-7/Pac sublines. The values were calculated from three independent experiments. IC_{50} values of sublines were compared with respect to MCF-7/S by student t-test.

	MCF-7/S	**MCF-7/50nMPac**	**MCF-7/120nMPac**	**MCF-7/200nMPac**	**MCF-7/400nMPac**
IC50± SEM(µM)	2.12±0.23	19.61±0.20	28.04±4.21	76.14 ±5.96	317.94± 0.20
Resistance index		**9.25**	**13.23**	**35.92**	**149.98**
p value		**p>0.05**	**p<0.05**	**p<0.05**	**p<0.05**

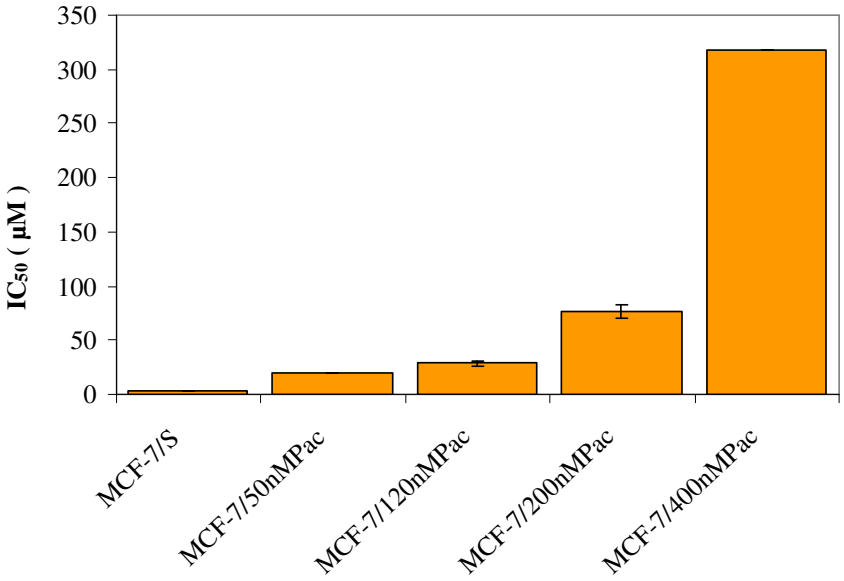


Figure 3.7 Graphical demonstration of IC_{50} values for MCF-7/Pac sublines. SEM values were derived from three independent experiments.

3.2.2 Cell Proliferation Profiles and IC_{50} Values for Vincristine Adapted MCF-7 Cell Line

Cell proliferation profiles, IC_{50} values and resistance indices of the vincristine adapted sublines are presented in Figure 3.8, 3.9 and Table 3.3. According to the results, resistance indices of MCF-7/20nMVinc, MCF-7/40nMVinc, MCF-7/80nMVinc, MCF-7/120nMVinc sublines developed significant resistance with respect to MCF-7/S ($p<0.05$). On the other hand, IC_{50} value of MCF-7/120nMVinc was about 30 folds more than the MCF-7/S, which means that the 120nM vincristine selected cell line acquired that much resistance with respect to the parental cell line.

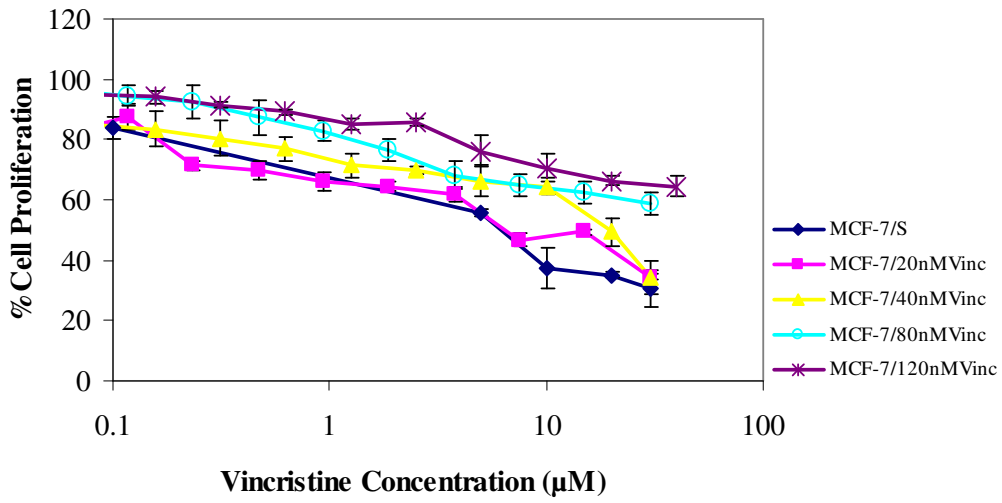


Figure 3.8 Graphical demonstration of inhibition of cell proliferation in MCF-7/Vinc sublines.

According to Table 3.3 it is seen that the vincristine resistant MCF-7 sublines developed significant level of resistance during dose increaments. However the increase in resistance indices is not as high as that of paclitaxel resistant sublines. The values are 10, 14, 17 and 30 that indicates the MCF-7/120Vinc subline is not as much resistant as MCF-7/400nMPac to its selective agent. This difference in resistance indices between two sublines may be due to the inverse effects of two drugs. The parental cell line may have become resistant to two different drugs through diverse mechanisms, so that the sublines responded differently. Several studies previously showed that paclitaxel and vincristine cause the development of acquired drug resistance in various tissue types during clinical chemotherapy (Valero *et al* ,1998; Salmon *et al*, 1991). Understanding the mechanisms of drug resistance in paclitaxel and vincristine resistant breast cancer sublines will clarify the causes of clinical breast cancer recurrence.

Table 3.3 IC_{50} values and resistance indices of sublines. The values were calculated from three independent experiments. IC_{50} values of sublines were compared with respect to MCF-7/S by student t-test.

	MCF-7/S	MCF-7/20nMVinc	MCF-7/40nMVinc	MCF-7/80nMVinc	MCF-7/120nMVinc
IC50± SEM(µM)	5.45±0.66	53.79 ±2.96	74.67±1.36	93.81±1.12	162.29± 2.19
Resistance index		**9.87**	**13.70**	**17.21**	**29.78**
p value		**p<0.05**	**p<0.05**	**p<0.05**	**p<0.05**

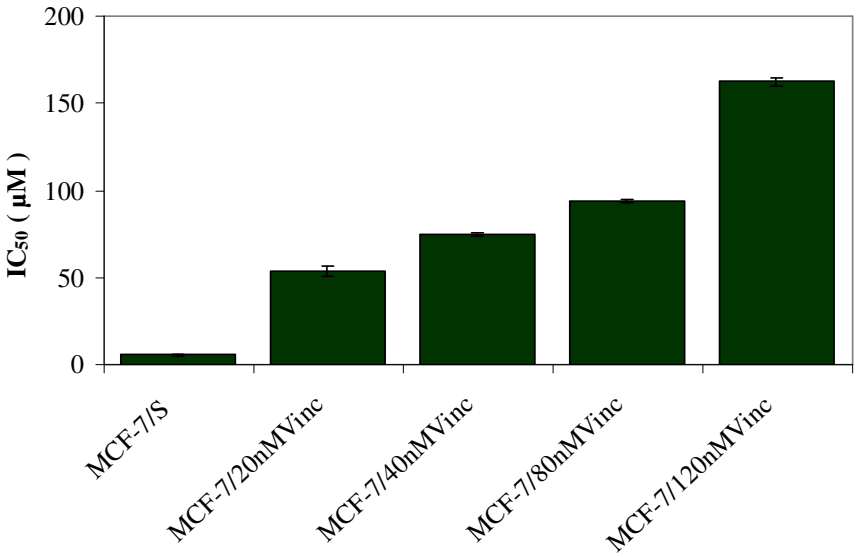


Figure 3.9 Graphical demonstration of IC_{50} values for MCF-7/Vinc sublines. SEM were derived from three independent experiments.

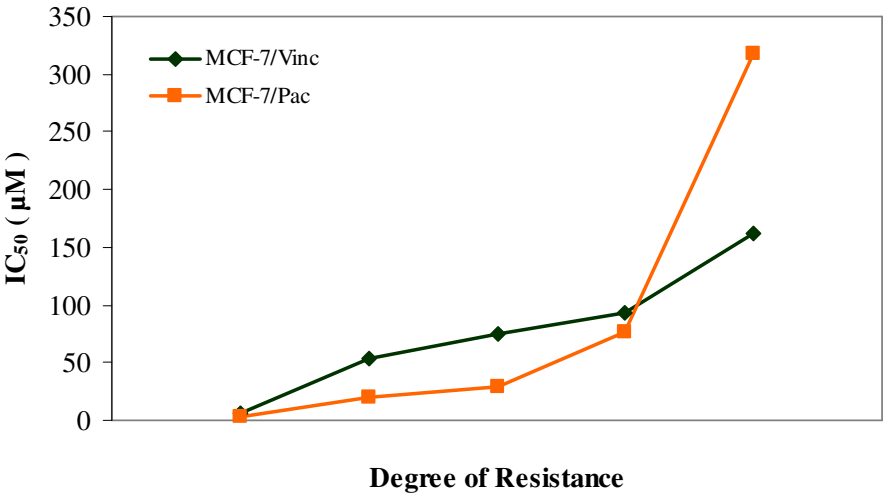


Figure 3.10 Changes in IC_{50} values in MCF7/Pac and MCF-7/Vinc sublines during stepwise dose increases.

3.3 Expression Analysis of Drug Resistance Related Genes

3.3.1 Total RNA Isolation with Guanidium Thiocyanate (GTC) Phenol Chloroform Single Step RNA Isolation Method

In our experiments we obtained intact, high cocentrated total RNA samples with GTC RNA isolation method.

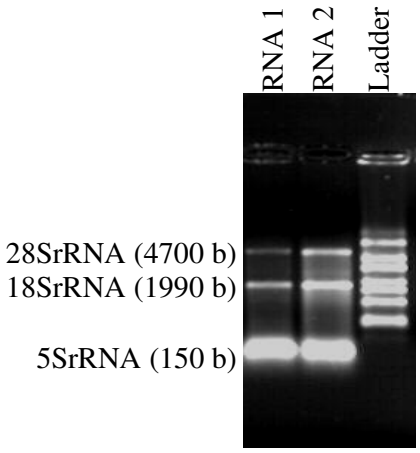


Figure 3.11 Ribosomal RNA and subunits are observed in 1 % agarose gel.

The purpose of selection of this method was that during experiment the procedure was easily modified since every ingredient were prepared manually. This made the procedure cheap and practical. Also we could obtain intact RNA without DNA contamination. The concentration of RNA was applicable for RT-PCR amplification.

3.3.2 RT-PCR (Reverse Transcription-Polymerase Chain Reaction)

The optimal cycle numbers were determined for PCR conditions. The optimal cycles before saturation phase of PCR reactions were 30, 25, 25, 30, 30 for amplification of *Bcl-2, Bax, MRP1, MDR1* and *β-2 microglobulin* genes respectively. Then RT-PCR were performed after optimizations. The Figures 3.12 - 3.20 display the gel photographs and densitometric ratio graphs.

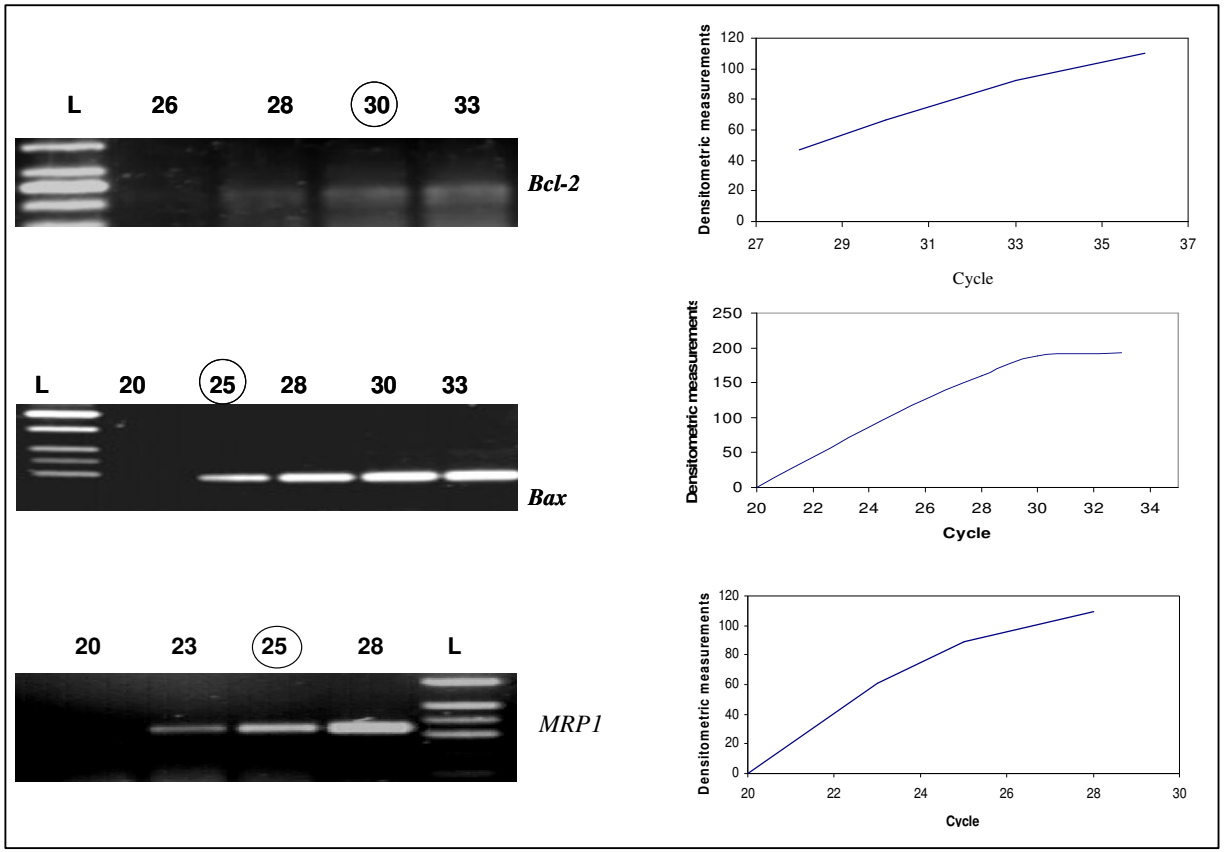


Figure 3.12 a) RT-PCR products in 2% gel and densitometric masurements of band intensities of the genes amplified at varying PCR cycles.

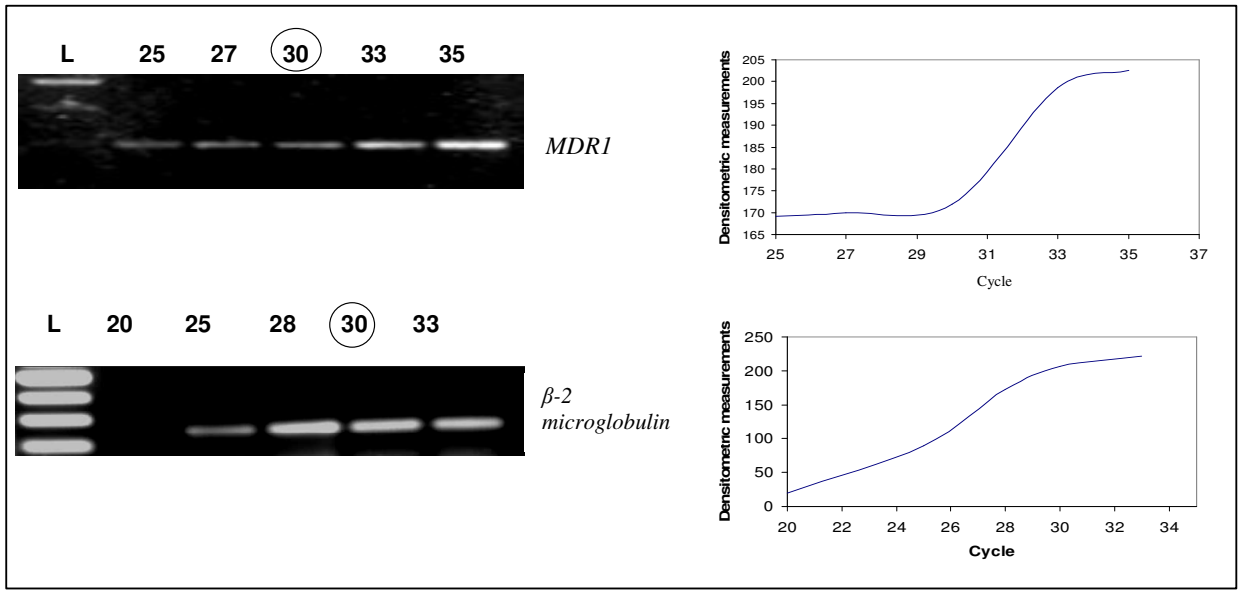


Figure 3.12 b) RT-PCR products in 2% gel and densitometric masurements of band intensities of the genes amplified at varying PCR cycles.

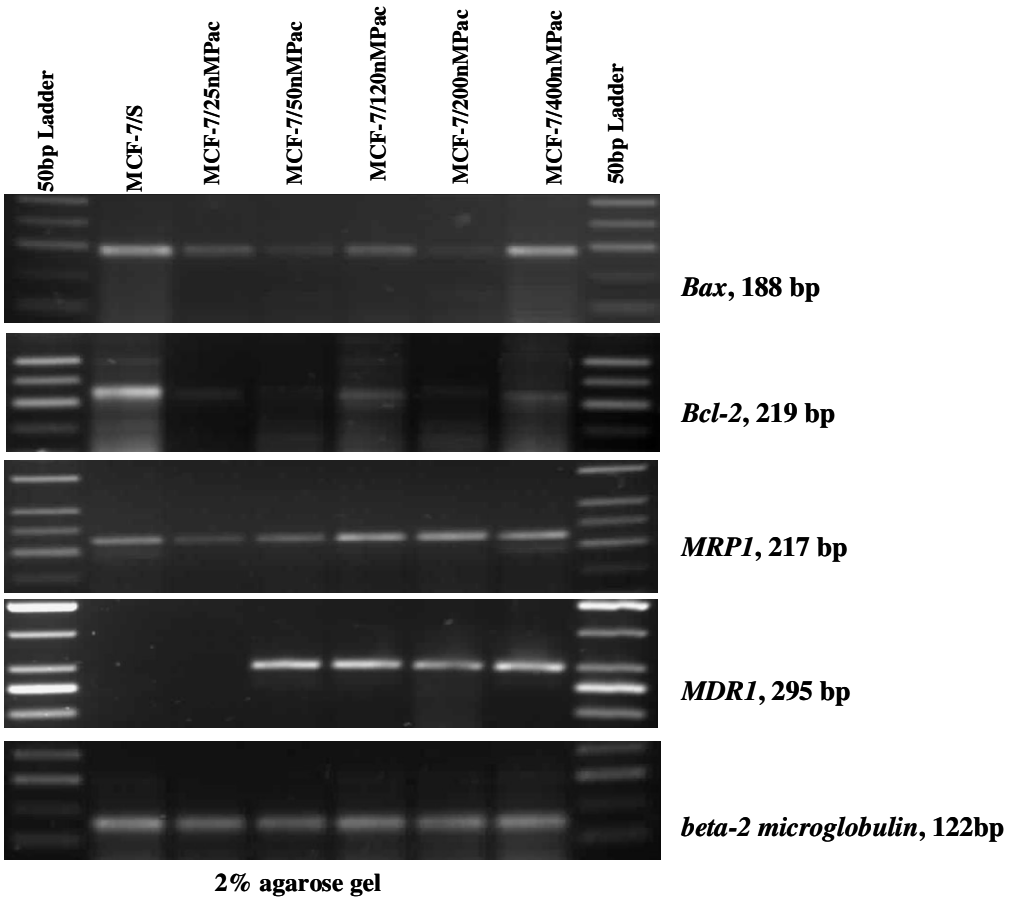


Figure 3.13 Schematic representations of RT-PCR products for sensitive and paclitaxel resistant MCF-7 cell line.

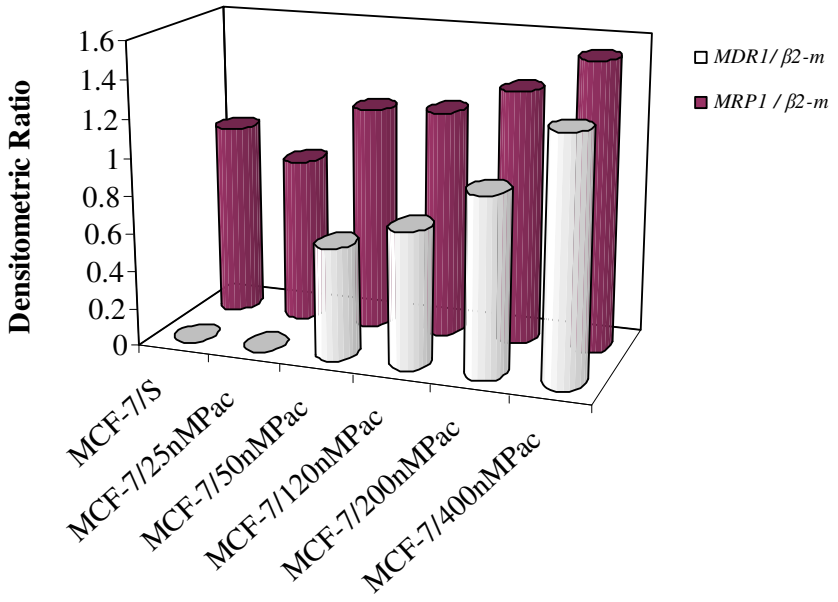


Figure 3.14 Densitometric ratio graph for *MDR1* and *MRP1* gene expressions in MCF-7/Pac cell lines with respect to MCF-7/S.

MCF-7/50nMPac, MCF-7/120nMPac, MCF-7/200nMPac, MCF-7/400nMPac sublines acquired *MDR1* gene expression. The expression levels are significantly different from MCF-7/S, MCF-7/25nMPac and also significantly different from eachother ($p<0.05$). On the other hand *MRP1,* and *Bcl-2* gene expression levels did not change significantly ($p>0.05$) when sublines were compared with MCF-7/S. However, *MRP1* gene expression of MCF-7/25nMPac is significantly higher than MCF-7/400nMPac. *Bax* gene expression decreased significantly in MCF-7/200nMPac cell line with respect to MCF-7/S ($p<0.05$). Also MCF-7/400nMPac overexpressed *Bax* gene significantly different from MCF-7/S ($p<0.05$). Fold changes in gene expressions are represented in Figure 3.14-3.16 and Table 3.4. Consequently, *Bcl-2/Bax* expression level ratio did not change significantly in MCF-7/Pac cell lines.

The results are consistent with literature that, the paclitaxel resistant cell lines have *MDR1* overexpression (Kamazawa *et al*., 2002). There are also studies that represent

importance of increase in Bcl-2/Bax ratio in paclitaxel resistance development which is contradictory to the results reported here. Although Chun *et al*. reported that overexpression of Bcl-2 and Bcl-x_L is important in induction of drug resistance in hepatocellular carcinoma (Chun and Lee, 2004), this survival mechanism seems to not have been used by MCF-7/Pac sublines at least according to RT-PCR results.

When the RT-PCR results are considered, *MDR1* gene overexpression seems the most significant mechanism of paclitaxel resistance in MCF-7 sublines. Since there is not any increase in Bcl-2/Bax expression ratio it can be said that MCF-7 sublines may have used pathways by overexpressing other survival related genes.

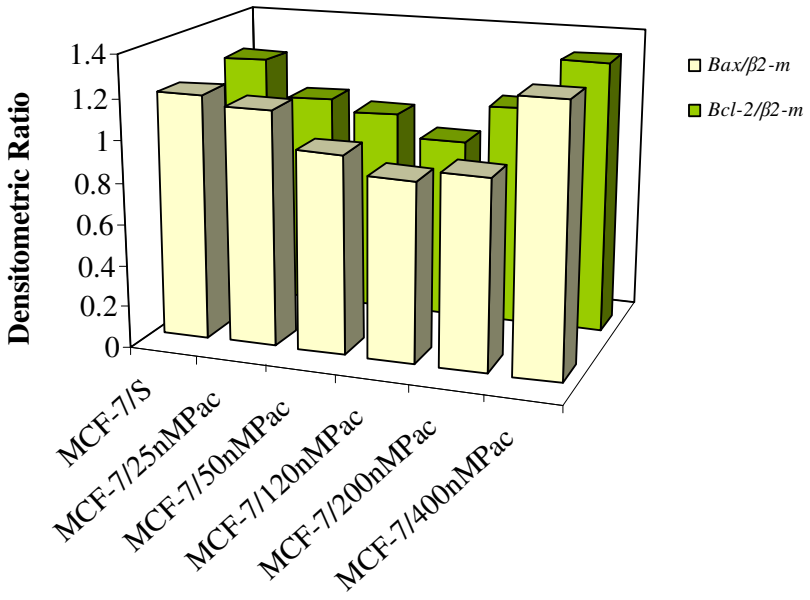


Figure 3.15 Densitometric ratio graph for *Bcl-2* and *Bax* gene expressions in MCF-7/Pac cell lines with respect to MCF-7/S.

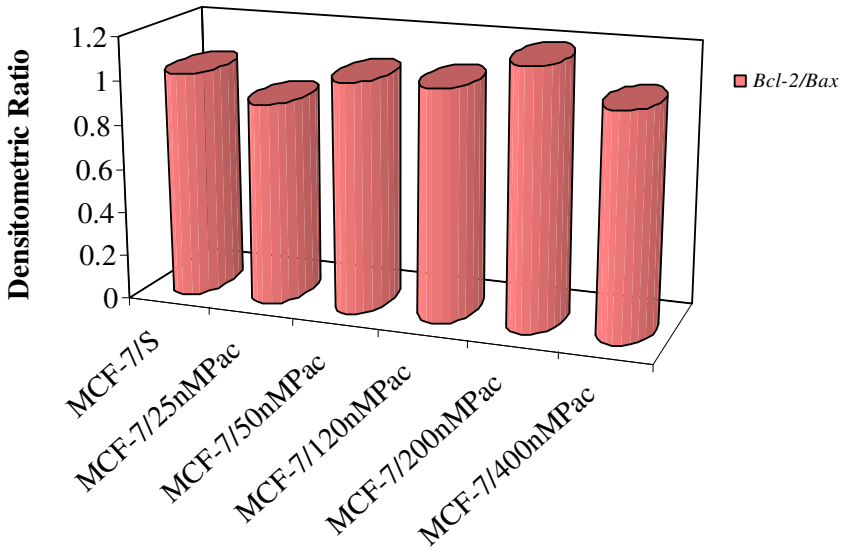


Figure 3.16 Densitometric ratio graph for *Bcl-2 /Bax* gene expressions in MCF-7/Pac cell lines with respect to MCF-7/S.

When the vincristine resistance in MCF-7 cell line is considered; MCF-7/20nMVinc, MCF-7/40nMVinc, MCF-7/80nMVinc, MCF-7/120nMVinc sublines acquired *MDR1* gene expression.

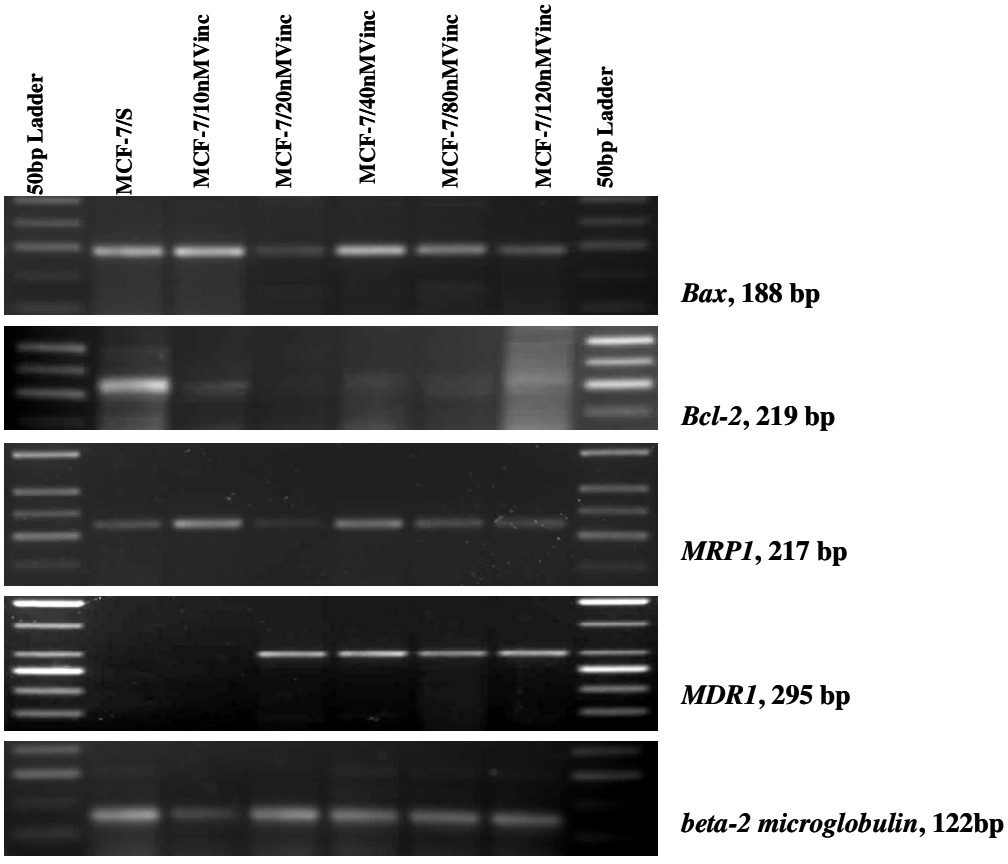


Figure 3.17 Schematic representation of RT-PCR products for sensitive and vincristine resistant MCF-7 cell line.

The expression levels are significantly different from MCF-7/S, MCF-7/10nMVinc and also significantly different from eachother ($p<0.05$). On the other hand *MRP1* gene expression only increased significantly in MCF-7/80nMVinc and MCF-7/120nMVinc ($p<0.05$). *Bcl-2* gene expression only increased significantly form 80nMVinc to 120nMVinc adapted MCF-7 cell line ($p<0.05$). *Bax* gene expression decreased significantly form MCF-7/10nMVinc to MCF-7/120nMVinc ($p<0.05$), but

there is not significant difference between other sublines. Consequently, *Bcl-2/Bax* expression ratio did not change significantly in MCF-7/Vinc cell lines except MCF-7/80nMVinc and MCF-7/120nMVinc. Only the so called sublines have higher *Bcl-2/Bax* expression level ratio than the MCF-7/S significantly. Fold changes in gene expressions are represented in Figure 3.17-3.19 and Table 3.4.

According to literature, the vincristine resistant cell lines and tissues have *MDR1* overexpression as a predominant resistance mechansim (Sanfilippo *et al*., 1991; Muller *et al*., 1992). Consistent with literature, RT-PCR results demonstrate that, *MDR1* overexpression is the most significant mechanism of vincristine resistance in MCF-7 sublines.

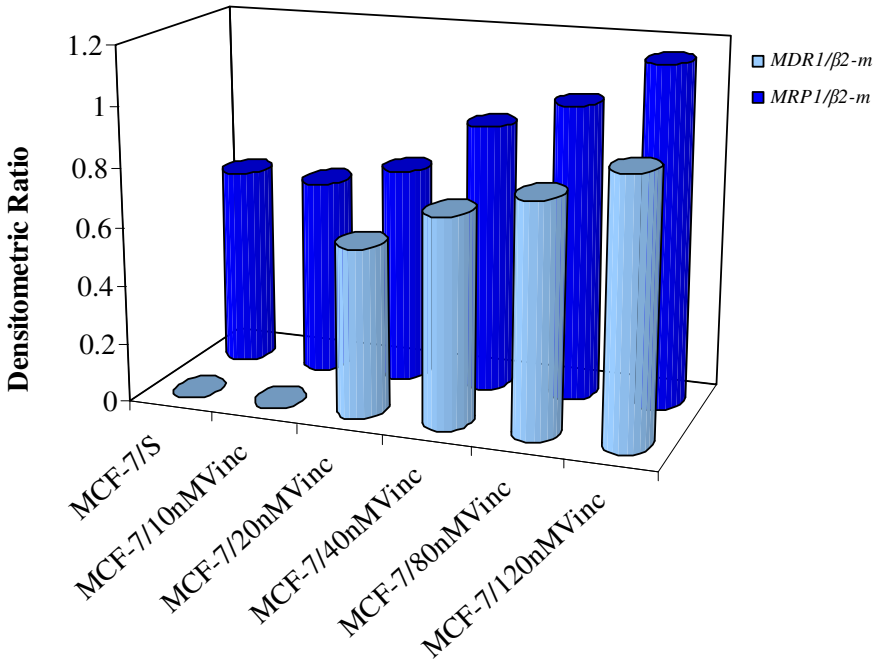


Figure 3.18 Densitometric ratio graph for *MDR1* and *MRP1* gene expressions in MCF-7/Vinc cell lines with respect to MCF-7/S.

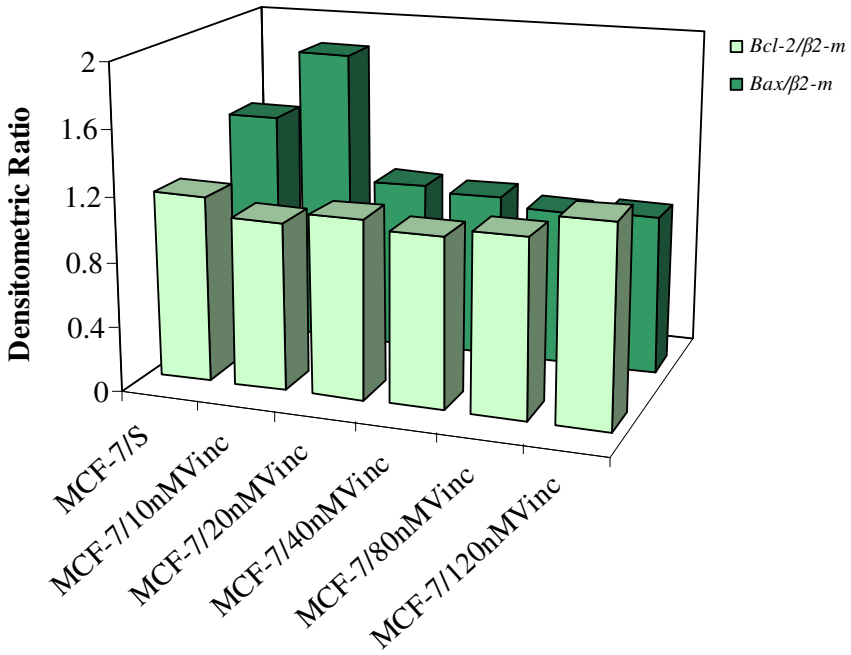


Figure 3.19 Densitometric ratio graph for *Bcl-2* and *Bax* gene expressions in MCF-7/Vinc cell lines with respect to MCF-7/S.

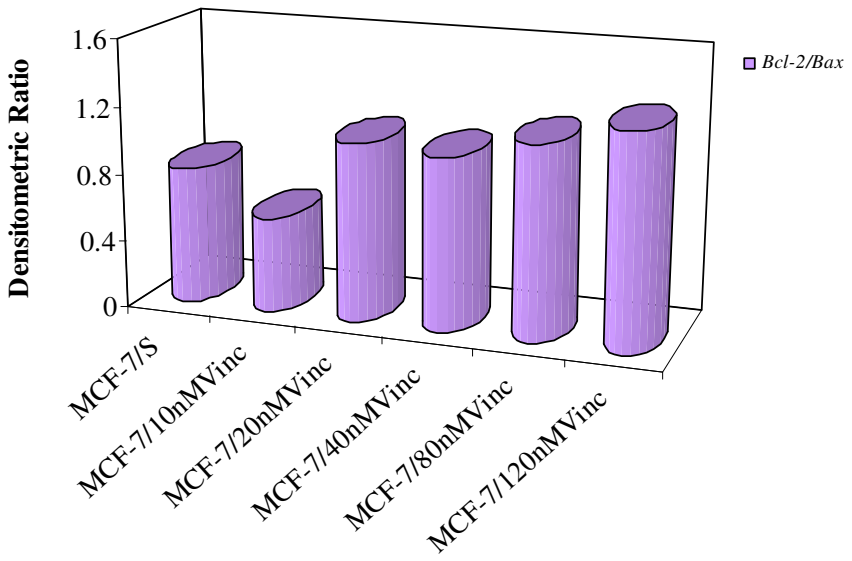


Figure 3.20 Densitometric ratio graph for *Bcl-2 /Bax* gene expressions in MCF-7/Vinc cell lines with respect to MCF-7/S.

When gene expression analyses in MCF-7/Pac and MCF-7/Vinc sublines are compared (Table 3.4) similar trends in expression levels are observed (for *MDR1, MRP1, Bcl-2* and *Bax* genes). Although resistance indices of the MCF-7/Pac and MCF-7/Vinc sublines are different from each other, *MDR1* and *MRP1* expression levels are similar respectively. To conclude, *MDR1* gene overexpression with 2.16 and 1.72 fold increase in MCF-7/400nMPac and MCF-7/120nMVinc cell lines respectively seems the important mechanism for development of MDR phenotype in paclitaxel and vincristne resistance in MCF-7 cells. Also when the most resistant sublines are considered, *MRP1* gene expression increased 1.51 and 1.70 fold increase in MCF-7/400nMPac and MCF-7/120nMVinc cells respectively. Overexpression of *MRP1* seems to be the other dominant mechanism among the four genes. Two sublines may have survived by overexpressing or regulating other suvival genes than *Bcl-2*. This pathway will be clarified during discussing microarray data related with apoptotic genes.

Table 3.4 The table represents the changes in gene expressions in resistant sublines in terms of fold change. "+" values represent increases, "-"values represent decreases in gene expression. * demonstrates the values significantly different from that of MCF-7/S ($p < 0.05$)

Fold change / *Cell line*	*MDR1/β2-m*	*MRP1/ β2-m*	*Bcl-2/ β2-m*	*Bax/ β2-m*	*Bcl-2/Bax*
MCF-7/S	-	1.00	1.00	1.00	1.00
MCF-7/25nMPac	-	-0.84	-0.82	-0.96	0.90
MCF-7/50nMPac	1.00*	+1.17*	-0.87	-0.81	1.02
MCF-7/120nMPac	+1.22*	+1.19	-0.73*	-0.73	1.02
MCF-7/200nMPac	+1.60*	+1.33	-0.90	-0.78*	1.16
MCF-7/400nMPac	+2.16*	+1.51*	+1.09*	+1.09*	0.99
MCF-7/S	-	1.00	1.00	1.00	1.00
MCF-7/10nMVinc	-	0.98	0.90	+1.31*	-0.70
MCF-7/20nMVinc	1.00*	+1.08	0.96	-0.76	+1.29
MCF-7/40nMVinc	+1.20*	+1.34	0.91	-0.73	+1.25
MCF-7/80nMVinc	+1.39*	+1.48*	0.96	-0.69	+1.40
MCF-7/120nMVinc	+1.72*	+1.70*	1.08	-0.70	+1.54

3.3.3 Real-time PCR (QPCR)

Previously diluted standart templates were amplified with *MDR1* gene specific primers by Real Time PCR reaction. The standart curve was plotted by software program. Figure 3.21 presents amplification curves and standart curve plotted during reaction. The fluorescent values and crossing point (threshhold cycle) values for *MDR1* standarts are demonstrated in Table 3.5.

a) b)

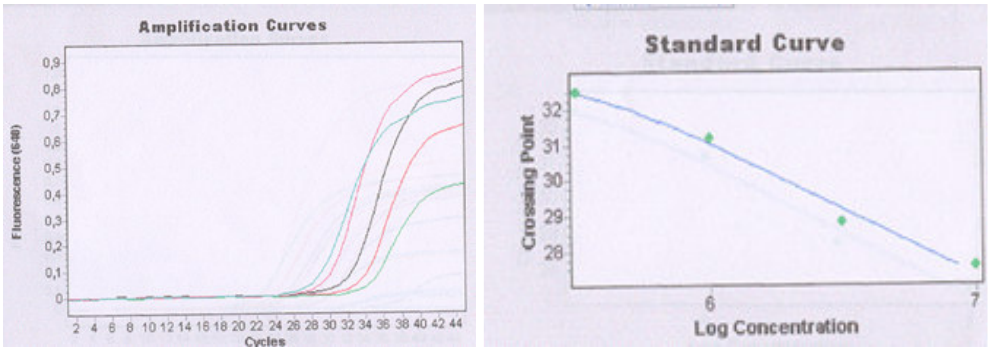


Figure 3.21 a) Amplification curves for standarts for *MDR1* gene b) standart curve.

Table 3.5 Table displays samples and results for standarts of *MDR1* gene

Samples				Results		
Color	**Position**	**Name**	**Total RNA**	**CP**	**Fluorescence Value**	**cDNA dilution number**
	2	Standart1	0.0573 µg	31.49	3.16×10^5	3.16×10^5
	3	Standart2	0.175 µg	31.16	8.78×10^5	1.00×10^6
	4	Standart3	0.573 µg	28.79	3.99×10^6	3.16×10^6
	5	Standart4	1.75 µg	27.55	8.59×10^6	1.00×10^7

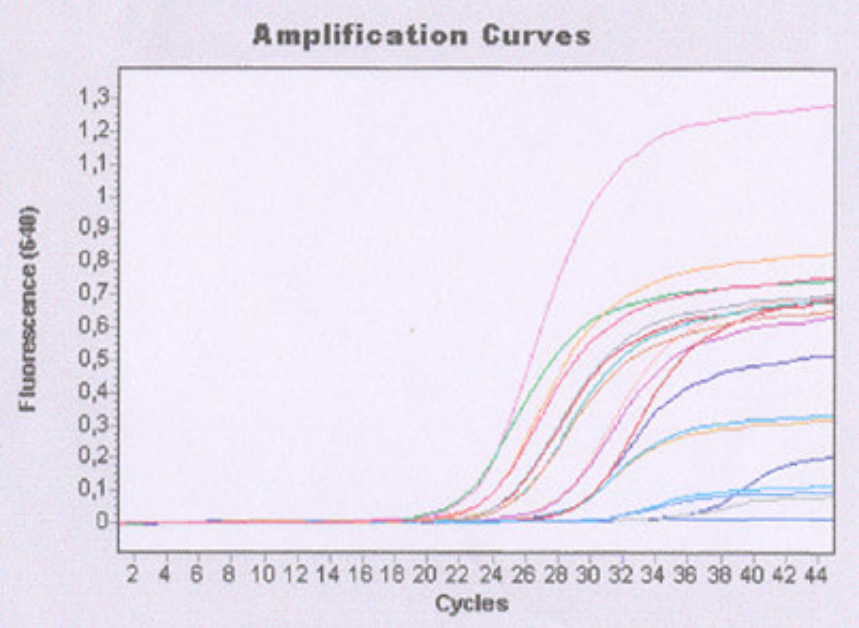


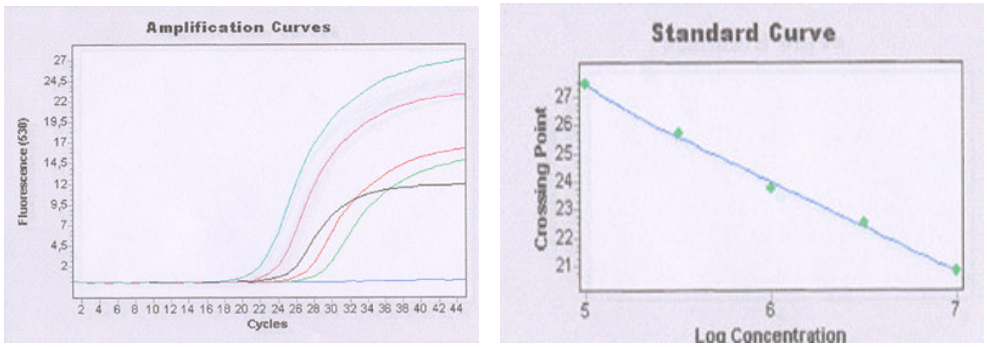
Figure 3.22 Amplification curves of MCF-7/S, MCF-7/Pac and MCF-7/Vinc cell lines for *MDR1* amplification.

The amplification profiles of the samples from MCF-7/S, MCF-7/50nMPac, MCF-7/400nMPac, MCF-7/20nMVinc and MCF-7/120nMVinc are shown in Figure 3.22. Table 3.6 summarizes the results obtained after the reaction. According to mRNA amounts, it is obvious that the *MDR1* gene expression was upregulated in resistant cell lines. MCF-7/120nMVinc cell line has the highest *MDR1* gene expression level among the other sublines. This result is different from the results of semiquantitative RT-PCR. QPCR is more reliable than the RT-PCR. Still it can be concluded that *MDR1* gene was overexpressed in both MCF-7/400nMPac and MCF-7/120nMVinc cells as the resistance indices increased.

Table 3.6 Table displays samples and results for *MDR1* gene

Samples	Mean Cp	STD Cp	Mean Fluorescence	STD Fluorescence	Mean RNA (µg)	STD RNA (µg)
MCF-7/S	34.40	1.00	4.27×10^4	4.81×10^4	0	0.004
MCF-7 /50nMPac	26.82	0.05	1.34×10^7	3.99×10^5	0.135	0.001
MCF-7 /400nMPac	27.25	0.03	1.03×10^7	1.70×10^5	0.103	0.002
MCF-7 /20nMVinc	23.54	0.03	1.02×10^8	1.69×10^6	0.102	0.014
MCF-7 /120nMVinc	21.44	0.79	5.29×10^8	0.21×10^6	0.543	0.002

Standart templates were amplified with *MRP1* gene specific primers by Real Time PCR reaction. The standart curve was plotted by software program. Figure 3.23 demonstrates amplification curves and standart curve plotted during reaction. The fluorescent values and crossing point (threshhold cycle) values for *MRP1* standarts are shown in Table 3.7.



a) b)

Figure 3.23 a) Amplification curves for standarts for *MDR1* gene b) standart curve.

Table 3.7 Table displays samples and results for standarts of *MRP1* gene

Samples				Results		
Color	**Position**	**Name**	**RNA**	**CP**	**Fluorescence value**	**cDNA dilution number**
	1	NTC*				
	2	Standart1	0.0175 µg	27.47	1.00×10^5	1.00×10^5
	3	Standart2	0.0573 µg	25.69	2.91×10^5	3.16×10^5
	4	Standart3	0.175 µg	23.72	1.20×10^6	1.00×10^6
	4	Standart4	0.573 µg	22.47	2.94×10^6	3.16×10^6
	6	Standart5	1.75 µg	20.81	9.74×10^6	1.00×10^7

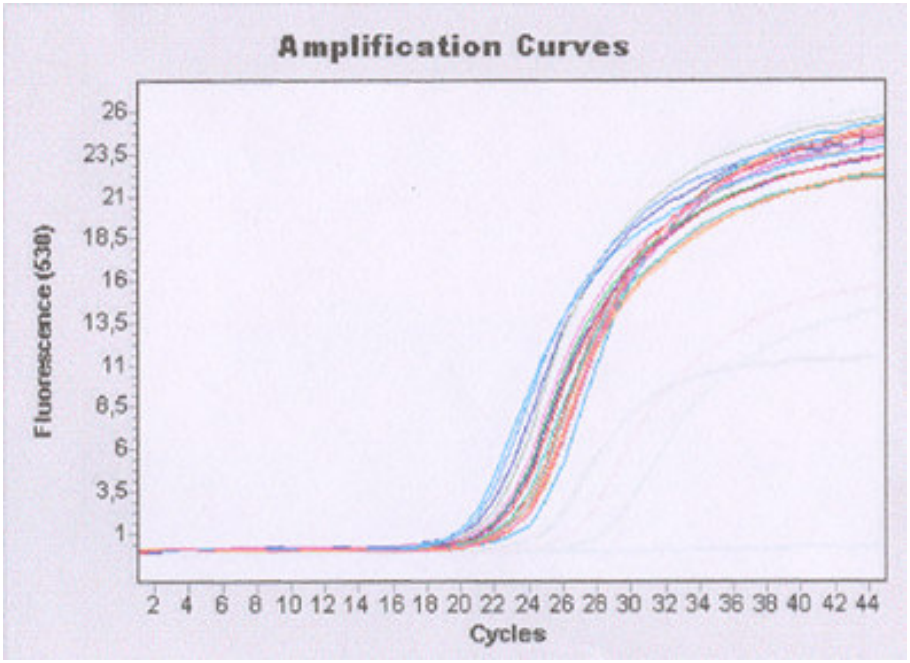


Figure 3.24 Amplification curves of MCF-7/S, MCF-7/Pac and MCF-7/Vinc cell lines for *MRP1* amplification.

Table 3.8 Table displays samples and results for *MRP1* gene

Samples	Mean Cp	STD Cp	Mean Fluorescence	STD Fluorescence	Mean RNA (µg)	STD RNA (µg)
MCF-7/S	20.34	0.34	1.39×10^7	3.39×10^6	0.125	0.030
MCF-7 /50nMPac	22.55	0.11	2.78×10^6	2.19×10^5	0.025	0.002
MCF-7 /400nMPac	22.92	0.36	2.17×10^6	5.53×10^5	0.019	0.005
MCF-7 /20nMVinc	22.07	0.08	3.93×10^6	2.29×10^5	0.035	0.002
MCF-7 /120nMVinc	20.92	0.05	8.97×10^6	3.35×10^5	0.081	0.003

The amplification profiles of the samples from MCF-7/S, MCF-7/50nMPac, MCF-7/400nMPac, MCF-7/20nMVinc and MCF-7/120nMVinc for *MRP1* are shown in Figure 3.24. Table 3.8 summarizes the results obtained after the reaction. According to mRNA amounts, *MRP1* gene expression levels were decreased in resistant cell lines with respect to sensitive MCF-7 cell line.

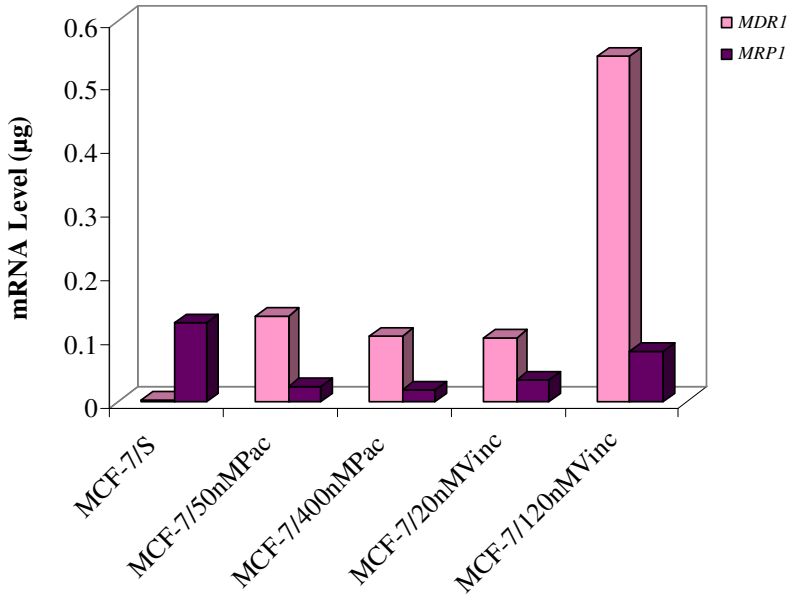


Figure 3.25 The bar graph that demonstrates the *MRP1* and *MDR1* gene expression levels.

According to Figure 3.25, MCF-7/50nMPac, MCF-7/400nMPac, MCF-7/20nMVinc, MCF-7/120nMVinc sublines acquired *MDR1* gene expression. The expression levels are significantly different from MCF-7/S and also significantly different from eachother ($p<0.05$). On the other hand *MRP* gene expression decreased significantly ($p<0.05$) when sublines were compared with MCF-7/S. So this data is contradicting with semiquantitative RT-PCR results. Since the QPCR data is more reliable it can be said that *MRP1* gene seems not important in MDR development in paclitaxel and vincristine resistant MCF-7 sublines. As a conclusion, Real-time PCR results demonstrate that, *MDR1* overexpression is the significant mechanism of paclitaxel and vincristine resistance in MCF-7 sublines.

3.3.4 Microarray Analysis

At this point, microarray study enabled us detailed analysis of gene expression in sensitive and drug resistant cell lines to understand the mechanisms of drug resistance. Relationships between many biological pathways and drug resistance will be evaluated.

The analysis of results with GeneSpring GX 7.3.1 Software was conducted firstly by downloading the human genome database to the computer. Then the attributes were identified as given in Appendix H.

The microarray analysis was performed by plotting different graphs to compare the expression profile of the genes between sensitive and resistant MCF-7 cell lines. Figures 3.26, 3.27, 3.28 represent the scatter plot of genes which were upregulated (red dots), down regulated (blue dots) and not changed (yellow dots) between MCF-7/400nMPac-MCF-7/S, MCF-7/120nMVinc-MCF-7/S, MCF-7/400nMPac-MCF-7/120nMVinc pairs respectively.

When the three plots are compared, similar scatter profiles are observed in Figure 3.26 and Figure 3.27. On the other hand Figure 3.28 demonstrates that MCF-7/400nMPac and MCF-7/120nMVinc have similar expression profiles that the genes are not scattered as much as that of in Figure 3.26 and Figure 3.27 plots.

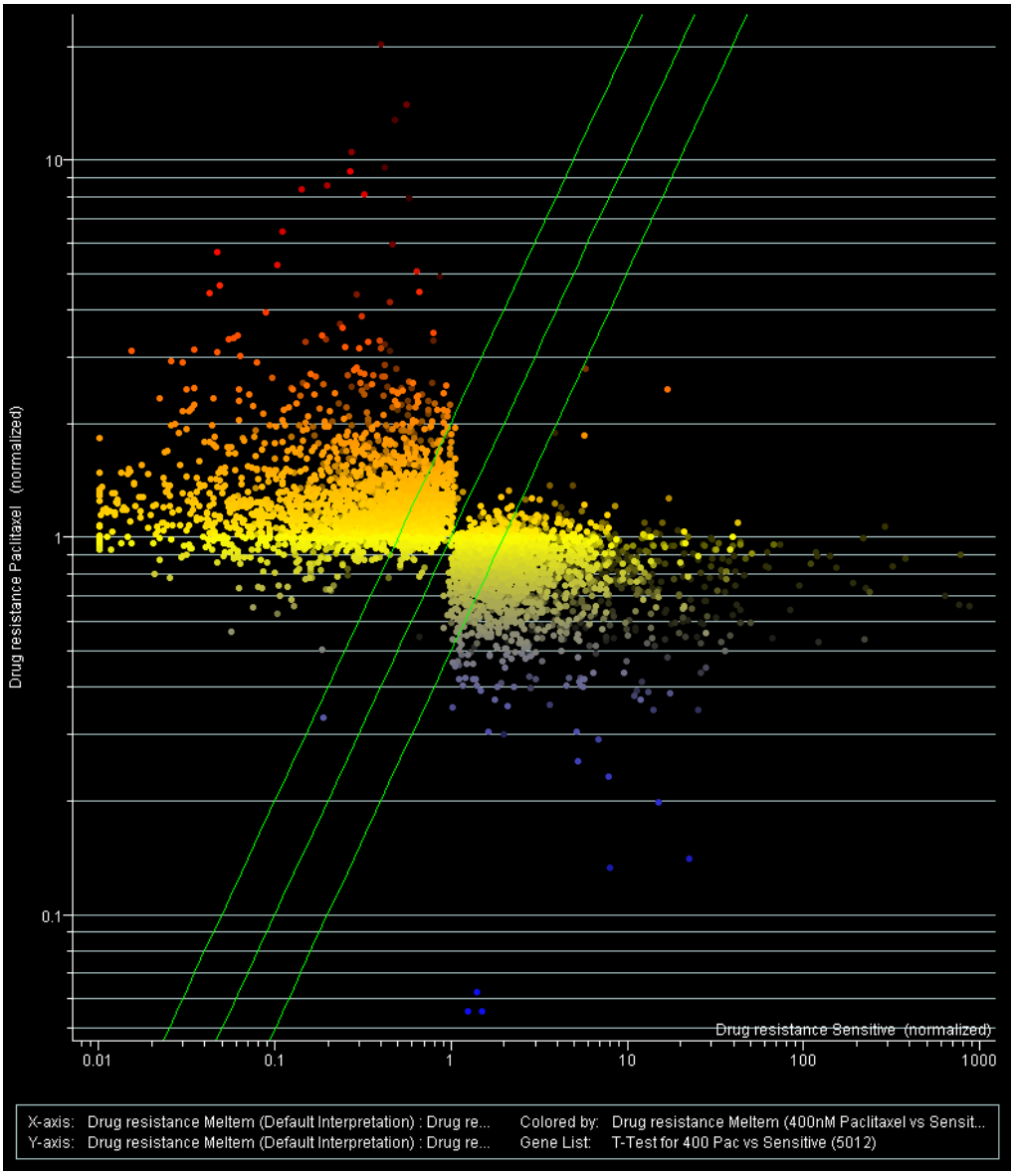


Figure 3.26 Scatter plot compares MCF-7/400nMPac and MCF-7/S.

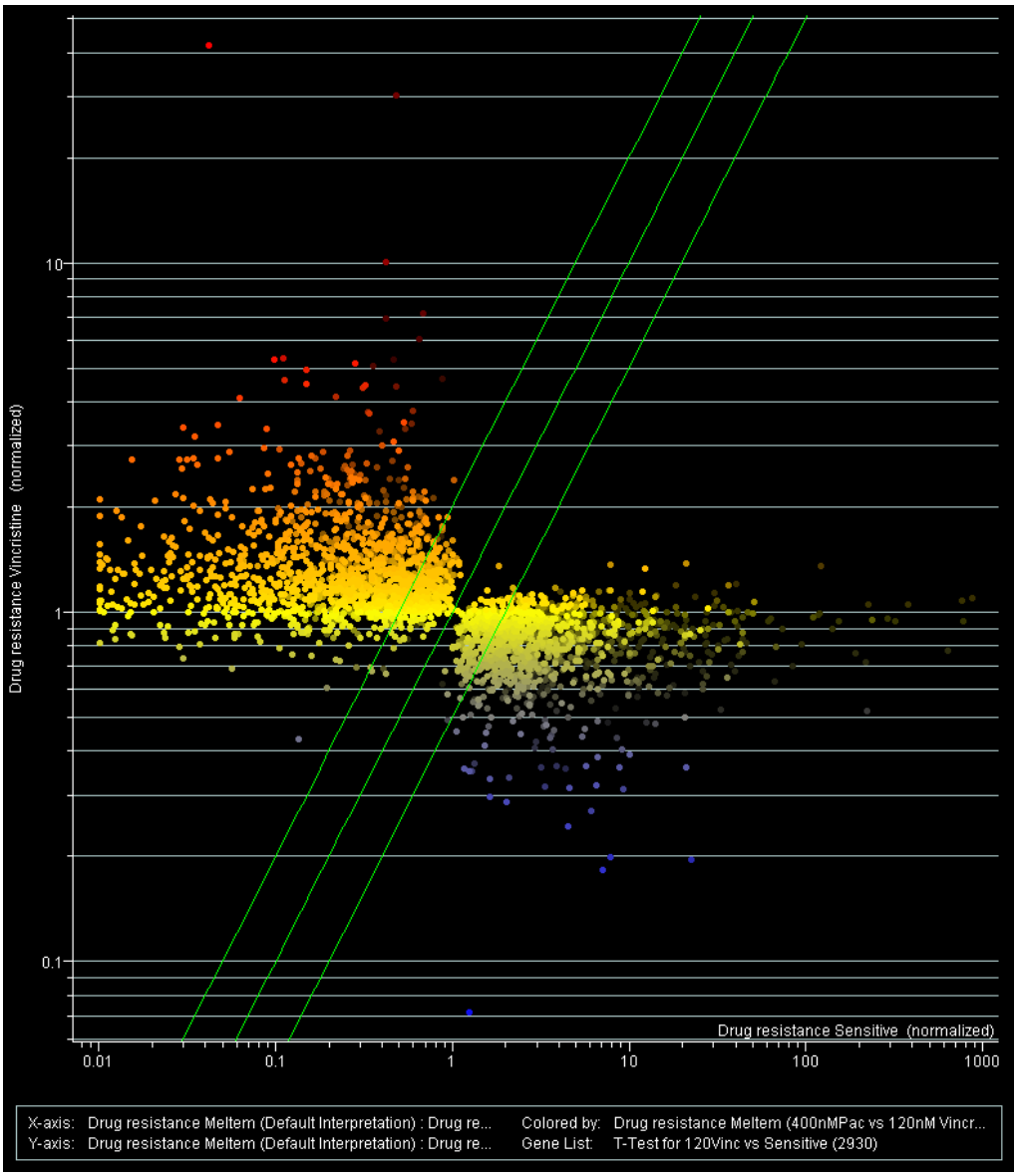


Figure 3.27 Scatter plot compares MCF-7/120nMVinc and MCF-7/S.

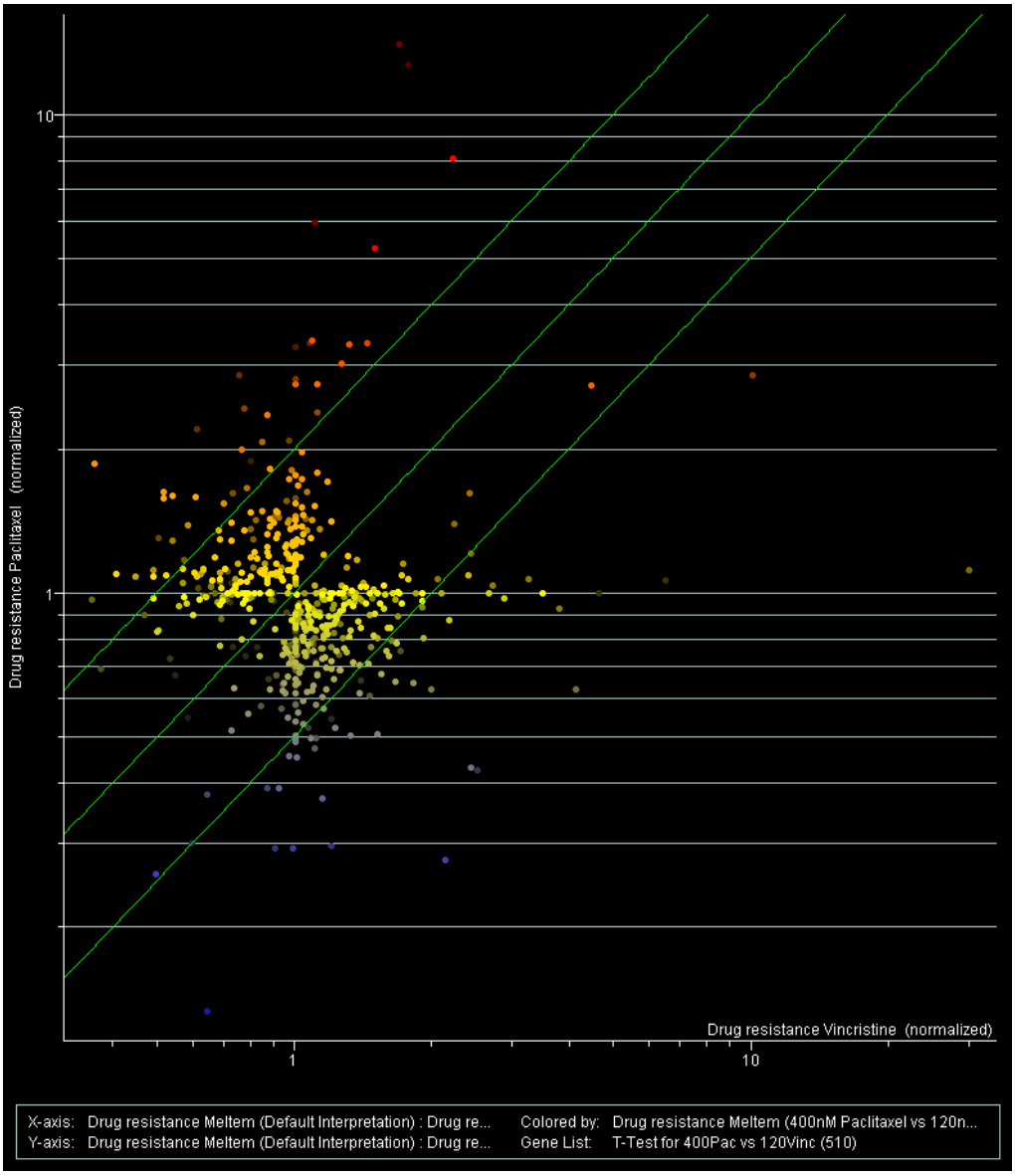


Figure 3.28 Scatter plot compares MCF-7/400nMPac and MCF-7/120nMVinc.

Box plot in Figure 3.29 demonstrates the fluorescent intensity distribution among three cell lines. It is observed in the plot that gene expression intensity distribution of paclitaxel and vincristine resistant sublines have similar profiles but different from MCF-7/S.

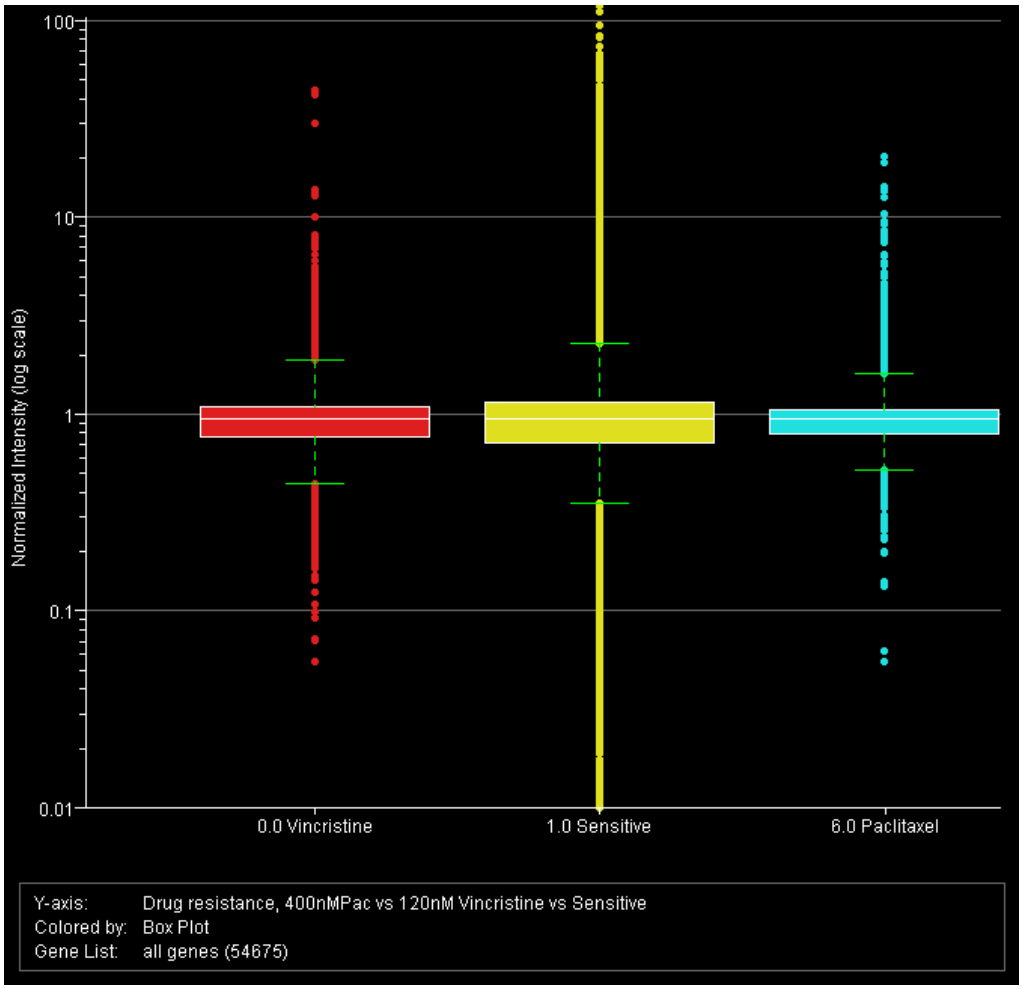


Figure 3.29 Box plot demonstrates the fluorescent intensity distribution among MCF-7/S, MCf-7/400nMPac and MCF-7/120nMVinc.

Figure 3.30 is a Venn diagram presentation of the number of genes which have common or different expression levels in between MCF-7/S, MCF-7/400nMPac, MCF-7/120nMVinc. According to Venn diagram, 115 genes have the same expression levels in three cell lines. On the other hand 3092 and 1015 genes have significantly different epression levels when MCF-7/400nMPac and MCF-7/120nMVinc were compared with MCF-7/S respectively.

Figure 3.30 The Venn Diagram that shows the number of genes that have different and same expression levels among cell lines.

According to functions of the proteins, the genes that were selected from the main list were grouped in to ontology lists by use of software.

First grouped genes were related to biological processes (BP) includes cell growth and maintenance and death. The second group involves molecular functions (MF) including, apoptosis regulator activity, binding, cell adhesion molecules, catalytic activity, defense and immunity proteins, signal transducer activity, structural molecule activity, transporter activity. Among the groups 9 tables (3.9-3.17) were prepared by selecting the genes related to relevant functions such as ABC transporter genes, microtubule related genes, apoptosis related genes, detoxification related

genes, growth factor, interleukin, interferon encoding genes, extracellular matrix related genes, cell cycle components and oncogenes.

It is important to relate the data obtained by microarray by other techniques such as RT-PCR, Western Blot and Immune Cytochemistry. Therefore expression level of *MDR1* gene obtained by microarray and other techniques mentioned were compared and it was observed that mRNA and protein expression data were well correlated.

According to expression profile in Table 3.9, it is obvious that *MDR1* gene was highly overexpressed in paclitaxel and vincristine resistant cell lines. Overexpression of *MDR1* gene in the resistant sublines was previously demonstrated by RT-PCR by our group (Kars *et al*, 2006). The most well known mechanism of drug resistance is the increased drug efflux that results from up-regulation of ABC transporters such as P-gp and MRP1 (Lage, 2003). So the result is parallel to literature also.

The protein encoded by *ABCB4* gene is located in liver and is involved in the secretion of bile acids. According to Table 3.9, the gene is overexpressed 27 fold in only MCF-7/400nMPac subline. Although it has not a direct correlation with drug resistance overexpression of the gene may be due to the chromosomal localization. The *ABCB4* gene is localized exactly at the same locus with *ABCB1* (*MDR1*) that is 7q21.12 (Dean and Allikmets, 2001). Knowlegde about regulation of *MDR1* gene expression suggest highly complex pattern extending from regulation through binding sites on the promoter to the role of binding elements and competition of several factors for DNA binding (Labialle *et al*, 2002). One of these mechanisms may involve in *ABCB4* co-expression.

Gluthatihone S-transrease (GST) belongs to a family of important detoxifying enzymes. They catalyze the conjugation of glutathione with a wide variety of hydrophobic compounds bearing an electrophilic center, including chemical carcinogens and mutagens (Boyer, 1989). Multi drug resistance associated protein 1 (MRP1) efflux drugs which are conjugated with gluthathione by GST. Although overexpression of MRP1 encoding gene was not observed in Table 3.9, upregulation

of the gene encoding detoxifiying GST enzyme *GSTP1* may have increased the efflux of MRP1 substrates. And this phenomenon may suggest GSTP as an enzyme involved in the mechanism of drug resistance in MCF-7/120nMVinc and MCF-7/400nMPac. It is interesting to observe about 4-5 fold downregulation of *GSTM 3* which encodes GST enzyme, found maily in brain, in breast cancer cells.

ABCG1 facilitates cholesterol efflux with this function; *ABCG1* is linked to both obesity and atherosclerosis but not linked to MDR phenotype (van Veen and Konnings, 1997). *ABCG1* was highly downregulated in both drug resistant sublines. *ABCG1* contains a TATAless, GC-rich promoter that contains silencing elements that can mediate transcriptional repression (Langmann *et al*, 2000). *ABCG1* gene expression may have been down regulated by the silencing elements during drug resistance development does not seem to be directly related to drug resistance development. Pallis and Russel reported that some of the drug resistant acute myeloblastic leukemia patients had high level of cholesterol (Pallis and Russel, 2004). This may be due to downregulation of ABCG1 in drug resistant patients.

Table 3.9 Expression levels of ABC transporters in MCF-7/Vinc and MCF-7/Pac cell lines with respect to MCF-7/S

Gene Name	Gene Symbol	Description	Ontology	Fold change	
				MCF-7/Vinc	MCF-7/Pac
209993_at	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Growth Main, Binding, MF transporter activity, MF catalytic activity	+146.5	+93
1570505_at	ABCB4	ATP-binding cassette, sub-family B (MDR/TAP), member 4	BP Growth-M, MF binding, MF catalytic activity,	NS	+26.55
214033_at	ABCC6	ATP-binding cassette, sub-family C (CFTR/MRP), member 6	BP Growth-M, MF Binding, MF transporter activity, MF catalytic activity	+2.921	+5.491
213485_s_at	ABCC10	ATP-binding cassette, sub-family C (CFTR/MRP), member 10	BP Growth-M, MF binding, MF catalytic activity,	NS	-2.31
211113_s_at	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	BP Growth-M, MF Binding, MF transporter activity	-8.93	-12.89
200824_at	GSTP1	glutathione S-transferase pi	BP Growth-M, MF catalytic activity	+49.71	+47.77
202554_s_at	GSTM3	glutathione S-transferase M3 (brain)	BP Growth-M, MF catalytic activity	-3.88	-4.89

Table 3.10 indicates the expression levels of microtubule associated genes in paclitaxel and vincristine resistant sublines. The anticancer drugs paclitaxel and vincristine are microtubule inhibiting agents. While paclitaxel is stabilizing, vincristine destabilizes microtubules. As a result, both anticancer agents inhibit mitosis.

Microtubule associated proteins (MAPs) bind to the tubulin subunits that make up microtubules to regulate their stability. Table 3.10 indicates that both MCF-7/400nMPac and MCF-7/120nMVinc overexpressed several MAP genes (*MAP1A, MAP1B, MAP4*). *MAP4* gene was upregulated 3.3 and 2.4 fold in paclitaxel and vincristine resistant cells respectively. Theoretically downregulation or inactivation of MAP4 may increase the dynamicity of microtubules therefore having effects on paclitaxel resistance (Orr and Pinard, 2003). According to previous findings, *in vitro*, *MAP4* overexpression correlates with increased resistance to paclitaxel (Zhang *et al*., 1998; Murphy *et al*., 1998). The correlation between paclitaxel and vincristine resistance and *MAP1A* and *MAP1B* overexpression was not reported before. This is the first study that proposes overexpression of *MAP1B* may have an implication for paclitaxel and vincristine resistance in breast cancer. Also *MAP1A* overexpression may be related with vincristine resistance only.

Beta-tubulin is encoded by a large multi-gene family. Cleveland and Sullivan (Cleveland and Sullivan *et al*., 1985) distinguished tubulin isotypes in vertebrates. In humans, at least six possible beta-tubulin isotypes have been identified and characterized in different tissues (Mozzetti *et al*., 2005). An additional mechanism of resistance is the selective expression of beta-tubulin isotypes. Table 3.10 indicates that the isotypes *TUBB3,4* and particularly *TUBB6* were overexpressed in both sublines. βIII-tubulin isotype (*TUBB3*) has been previously characterized for its low polymerization rate (Banerjee *et al*., 1990) and tumor cells overexpressing this isotype have been shown to overcome stabilizing effects of paclitaxel (Hari *et al*., 2003). Overepression of *TUBB4* gene was also demonstrated in paclitaxel resistant breast cancer cells before which is paralel to our results (Hasegawa *et al*., 2002). However, alterations in expression of *TUBB6* gene have not been correlated with

antimicrotubule resistance, previously. This is the first report that shows that *TUBB6* overexpression may have implications for paclitaxel and vincristine resistance.

The tau protein binds longitudinally to the inner and outer surface of the microtubule, between two adjacent tubulin dimers on the protofilaments, and has been found to bind to the paclitaxel binding site on the inner surface of the microtubule (Dehmelt *et al*., 2004). Tau activity is controlled by phosphorylation of the microtubule- binding domain and various kinases have been shown to phosphorylate/ dephosphorylate tau (Merrick *et al*., 1996). It was also suggested that (Rouzier *et al*., 2005) microtubule associated protein tau is a marker of paclitaxel sensitivity in breast cancer. In this respective study high levels of tau were associated with residual tumor and resistance to treatment in breast cancer patients. Our results are also parallel to these findings. Overexpression of gene encoding tau kinase was observed in paclitaxel resistant MCF-7 cell line but not in vincristine resistant cell line. So accordingly it can be proposed that overexpression of *TTBK* gene (Tau tubulin kinase) may be associated with paclitaxel resistance but not with vincristine resistance in breast cancer.

According to results, expression levels of all the selected microtubule associated genes were upregulated except *TUBD1* and *MAP7* (Table 3.10) in resistant sublines. So it can be proposed that alterations in microtubule dynamics may have close relationship with the development of drug resistance in these sublines.

Table 3.10 Expression levels of microtubule associated genes in MCF-7/Vinc and MCF-7/Pac cell lines with respect to MCF-7/S

Gene Name	Gene Symbol	Description	Ontology	Fold change	
				MCF-7/Vinc	**MCF-7/Pac**
212233_at	MAP1B	Microtubule-associated protein 1B ; Homo sapiens, clone IMAGE:5535936, mRNA	BP Growth-M, MF motor activity, MF structural molecule	+18.96	+14.82
203151_at	MAP1A	microtubule-associated protein 1A	BP Growth-M, MF motor activity, MF structural molecule	+12.57	NS
200836_s_at	MAP4	microtubule-associated protein 4	BP Growth-M, MF Binding, MF motor activity, MF structural molecule	+2.401	+3.278
202890_at	MAP7	microtubule-associated protein 7	BP Growth-M, MF motor activity, MF structural molecule	-34.97	-34.01
209191_at	TUBB6	tubulin, beta 6	BP Growth-M, MF Binding, MF motor activity, MF structural molecule	+10.61	+11.85
212664_at	TUBB4	tubulin, beta 4	MF Binding, MF motor activity, MF structural molecule activity	+3.847	+3.421
202154_x_at	TUBB3	tubulin, beta 3	BP Growth-M, MF Binding, MF motor activity, MF structural molecule	+2.597	+3.675

Table 3.10 Continues

1557073_s_at	TTBK2	Tau tubulin kinase 2	MF catalytic activity, MF structural molecule	NS	+7.421
221326_s_at	TUBD1	tubulin, delta 1	BP Growth-M, MF Binding, MF motor activity, MF structural molecule	-17.83	-17.12

Table 3.11 represents the expression levels of the genes selected and grouped according to relevance to apoptosis. Apoptosis can be thought of as a 'default' process, intrinsic to all cells. Apoptosis is triggered by a variety of stimuli, including cell surface receptors like FAS, mitochondrial response to stress, and cytotoxic T cells (Hu and Kavanagh, 2003). The pro and anti-apoptotic members of the Bcl-2 and associated proteins are integrated with survival signals and coupled to the release of mitochondrial cytochrome c and the activation of caspases (Chinnaiyan, 1999)

The highest expression levels are observed in some of tumor necrosis factors (TNF) and their receptors (TNFR). According to the results Table 3.11, *TNFRSF6B/10D, TRAF7* genes were upregulated in MCF-7/120nMVinc and MCF-7/400nMPac. Particularly *TNFR* was significantly overexpressed in both sublines. On the other hand *TNFRSF9* was upregulated only in MCF-7/120nMVinc cells. *TNFSF10* and *TNFSFR11B* were downregulated in both cell lines. A tumor necrosis factor receptor (TNFR), or death receptor, is a cytokine receptor that binds tumor necrosis factors (TNF) (Gaur and Aggarwal, 2003). One of the members of tumor necrosis factor receptor superfamily known as *TNFRSF6B* encodes protein which plays a regulatory role in suppressing FasL mediated cell death (Harper and MacFarlane, 2008). Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain, is also known as *TNFRSF10D* gene. The protein encoded by this gene is a receptor containing an extracellular TRAIL-binding domain, a transmembrane domain, and a truncated cytoplasmic death domain. This receptor does not induce apoptosis, and has been shown to play an inhibitory role in TRAIL-induced cell apoptosis. *Park et al* reported that overexpression of *MDR1* gene enhanced the TRAIL apoptosis in drug resistant cells (Park *et al*., 2006). TNF and its family members activate both apoptosis and antiapoptosis pathways simultaneously (Schwarz, 2005; Kimberley and Screaton, 2005). So both upregulation and downregulation of TNF and TNFR family members in MCF-7/120nMVinc and MCF-7/400nMPac indicates that sublines may have been trying to balance the components in paralel. On the other hand it seems that the genes that supress cell death are significantly upregulated which may be underlying the MDR phenotype.

Caspase-3, -6 and -7 are downstream caspases which are activated by the upstream proteases and act themselves to cleave cellular targets leading to apoptosis. Among caspases, C*ASP4* and *CASP1* which are not effector or initiator caspases and are not involved in apoptosis were upregulated. However expression levels of other important caspases did not change significantly in both sublines.So caspases may not be directly correlated with MDR development in MCF-7/400nMPac and MCF-7/120nMVinc cells.

According Table 3.11 the Bcl related genes, *BCL11A, BAG4, BNIP2* were upregulated in MCF-7/120nMVinc and MCF-7/400nMPac. On the other hand *BIK* was upregulated only in MCF-7/120nMVinc cells. *BAG3* was downregulated only in MCF-7/400nMPac. The transcription factor BKLF (basic Kruppel-like factor, KLF3) is a member of the Kruppel-like factors (KLF) family. KLF members harbor a characteristic C-terminal zinc-finger DNA-binding domain and bind preferentially to CACCC-motifs. BCL11A is one of the Kruppel-like family has been implicated in tumorigenesis (Black *et al*., 2001). BcL-2 associated genes *BAG4* and *BNIP2* overexpression and particularly *BCL11A* upregulation may also have taken role in survival of the resistant cells. Also here it is seen that *Bax* and *Bcl-2* genes are not in the gene list, which means there is not a statistically significant alteration in mRNA level. This is a confirmation of the results we obtained by RT-PCR and Western Blot.

Death-associated protein kinase (DAPK) is a multidomain protein kinase with an important role in apoptosis regulation and tumor suppression. Anjum *et al*. proposed that DAPK is a suppressor of apoptotic activity (Anjum *et al*., 2005). The encoding gene *DAPK* was overexpressed in both sublines 17-18 fold, which may have acted as a suppressor of apoptosis in drug resistant cells also.

The programmed cell death gene (*PDCD10;* previously known as the *CCM3* gene) encodes a protein called programmed cell death 10. Although the exact function of this protein is unclear, it is believed that it plays several roles in cells. This protein is thought to be involved in pathways that signal cells to die when they have completed

a certain number of cell divisions or accumulated errors in their DNA. The other correlated proteins *PDCD2/4/6/8* also function in signalling pathways of programmed cell death (Lee *et al*., 2005; Baron *et al*., 2002). *PDCD* (programmed cell death gene family, *PDCD*-2, 4, 6, 8, 10) were downregulated which may be correlated with resistance to apoptosis and MDR phenotype.

Table 3.11 Expression levels of apoptosis and death associated genes in MCF-7/Vinc and MCF-7/Pac cell lines with respect to MCF-7/S

Gene Name	Gene Symbol	Description	Ontology	Fold change	
				MCF-7/Vinc	MCF-7/Pac
206467_x_at	TNFRSF6B ; RTEL1	tumor necrosis factor receptor superfamily, member 6b, decoy ; regulator of telomere elongation helicase 1	BP Growth-M, MF Binding, MF signal transducer, MF catalytic activity, MF apoptosis regulator activity	+26.67	+31.14
227345_at	TNFRSF10D	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	BP death, MF signal transducer, MF apoptosis regulator activity	+18.38	+8.337
202644_s_at	TNFAIP3	tumor necrosis factor, alpha-induced protein 3	BP death, MF catalytic activity, MF apoptosis regulator	+12.13	+13.06
207536_s_at	TNFRSF9	tumor necrosis factor receptor superfamily, member 9	BP death, MF signal transducer, MF apoptosis regulator activity	+10.6	NS
206907_at	TNFSF9	tumor necrosis factor (ligand) superfamily, member 9	BP Growth-M, BP death, MF Binding, MF apoptosis regulator	+3.906	+2.32
202687_s_at	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10 ; tumor necrosis factor (ligand) superfamily, member 10	BP death, MF signal transducer, MF apoptosis regulator activity	-5.38	-10.88

Table 3.11 continues

204933_s_at	TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	BP Growth-M, BP death, MF signal transducer	-26.46	-25.84
223029_s_at	TRAF7	TNF receptor-associated factor 7	BP death, MF signal transducer, MF apoptosis regulator activity	+2.707	+2.174
209310_s_at	CASP4	caspase 4, apoptosis-related cysteine peptidase	BP Growth-M,F Binding, MF apoptosis regulator	+23.01	+27.63
209970_x_at	CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	MF binding, MF catalytic activity, BP death, MF apoptosis regulator	NS	+4.978
222891_s_at	BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	MF binding, MF transcription regulator	+12.36	+9.76
219624_at	BAG4	BCL2-associated athanogene 4	BP death, MF Binding, MF signal transducer	+3.238	+5.333
226280_at	BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2	BP death, MF apoptosis regulator	+2.907	+3.944
205780_at	BIK	BCL2-interacting killer (apoptosis-ind)	BP death, MF Binding, MF apoptosis	+2.715	NS
217911_s_at	BAG3	BCL2-associated athanogene 3	MF binding, MF apoptpsis regulator	NS	-2.702
203139_at	DAPK1	death-associated protein kinase 1	BP Growth-M, BP death, MF Binding, MF catalytic activity, MF apoptosis regulator	+17.04	+18.46
222595_s_at	DATF1	death associated transcription factor 1	BP death, MF apoptosis regl	-2.985	-2.439

Table 3.11 continues

224461_s_at	AMID	apoptosis-inducing factor (AIF)-like mitochondrion-associated inducer of death ; apoptosis-inducing factor (AIF)-like mitochondrion-associated inducer of death	BP Growth-M, MF catalytic activity, BP death, MF apoptosis regulator, MF cell adhesionmolecule	NS	-3.389
213581_at	PDCD2	programmed cell death 2	BP death, MF apoptosis regulator	-2.232	-2.801
205512_s_at	PDCD8	programmed cell death 8 (apoptosis-inducing factor)	BP Growth-M, MF binding	NS	-2.463
212594_at	PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)	BP death, MF apoptosis regulator	NS	-4.405
1569110_x_at	PDCD6	programmed cell death 6	BP death, MF apoptosis regulator, MF transcription regulatory	-62.50	-34.13
210907_s_at	PDCD10	programmed cell death 10	BP death, MF transporter activity, MF apoptosis regulator activity	-118.62	-130.38

Table 3.12 lists the gene expression levels of cytochrome b, cytochrome P450 related genes. Cytochrome b/b6 is main subunit of transmembrane cytochrome bc1 and b6f complexes. These complexes are involved in electron transport and the generation of ATP and thus play a vital role in the cell (Blakely *et al*., 2005). According to microarray data (Table 3.12), genes encoding cytochrome b reductase encoding genes 1 (*CYBRD1*) and 2 (*CYB5R2*) were upregulated in MCF-7/120nMVinc and MCF-7/400nMPac cells (about 21 fold and 9 fold respectively) but gene encoding cytochrome b reductase 3 (*CYB5R3*) was upregulated 2 fold only in MCF-7/400nMPac. Cytochrome b5 (*CYB5*) was downregulated in both cell lines about 2 fold. Overexpression of cytochrome b reductase 1 (*CYBRD1*) gene may have been a consequence of increase in ATP requirement to survive under drug stress.

In breast cancer CYP1B1 was identified as a marker for progression free survival (Marsh *et al*., 2007). CYP1B1 is the most prominent human cytochrome in breast tissue (McFadyen *et al*., 2001) and is often over-expressed in tumor cells. Cytochrome P450-mediated metabolism usually results in reduced activity or inactivation of the anticancer drugs, but in some cases bioactivation to a more cytotoxic metabolite occurs. One example of detoxification of anticancer drugs is shown by the taxanes. The major pathway of metabolism of paclitaxel is catalysed by CYP2C8 (Rahman *et al*, 1994). Vincristine is metabolised by CYP3A4 and CYP3A5 (van Schaik, 2008). The genes encoding cytochrome P450 famliy members 26 (*CYP26B1*) and 27 (*CYP27B1*) were upregulated in both sublines. However the genes encoding cytochrome P450 family members 1 (*CYP1B1*) and 51 (*CYP51A1*) were downregulated in MCF-7/120nMVinc cells 3.3 and 3.8 fold respectively. CYP26A1 and CYP26B1 have been identified as members of a new family of P450 enzymes that seem dedicated to ATRA metabolism (Njar *et al*, 2006). This phenomenon appears to be implicated in clinically acquired resistance to ATRA. In the literature there is not any reports about the overexpression of P450 family member 27B1 (*CYP27B1*) may be related with drug resistance development. McFadyen *et al*. previously reported that CYP1B1 protein over-

expression has a correlation with drug resistance development (McFadyen *et al*., 2001). According to our results the gene encoding CYP1B1 protein was downregulated in MCF-7/400nMPac but did not significantly change in MCF-7/120nMVinc.So here it can be proposed that there is not any correlation with drug resistance in MCF-7/400nMPac or MCF-7/120nMVinc and *CYP1B1* gene expression.

Table 3.12 Expression levels of cytochrome related genes in MCF-7/Vinc and MCF-7/Pac cell lines with respect to MCF-7/S

Gene Name	Gene Symbol	Description	Ontology	Fold change	
				MCF-7/Vinc	**MCF-7/Pac**
222453_at	CYBRD1	cytochrome b reductase 1	MF transporter activity	+22.56	+20.52
220230_s_at	CYB5R2	cytochrome b5 reductase 2	MF Binding, MF transporter activity	+8.764	+8.635
201885_s_at	CYB5R3	cytochrome b5 reductase 3	BP Growth-M, MF binding, MF catalytic activity,	NS	+2.145
209665_at	CYB561D2	cytochrome b-561 domain containing 2	MF transporter activity	+2.016	NS
209366_x_at	CYB5	cytochrome b-5	BP Growth-M, MF binding	-2.16	-2.0
202263_at	CYB5R1	cytochrome b5 reductase 1	MF catalytic activity,	NS	-2.90
209164_s_at	CYB561	cytochrome b-561	MF transporter activity, MF catalytic activity	-3.85	-3.57
219825_at	CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1	MF transporter activity, MF catalytic activity	+6.192	+8.842
205676_at	CYP27B1	cytochrome P450, family 27, subfamily B, polypeptide 1	BP Growth-M, MF Binding, MF transporter activity, MF catalytic activity	+2.185	+3.513
202434_s_at	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	BP Growth-M, MF binding, MF catalytic activity,	NS	-3.23
216607_s_at	CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	BP Growth-M, MF Binding, MF transporter activity, MF catalytic activity	NS	-3.79

Table 3.13 indicates the expression levels of growth factor related genes in drug resistant sublines. The genes encoding transforming growth factor beta (*TGFB1I1),* was upregulated in paclitaxel and vincristine resistant sublines about 60 fold. However the receptor *TGFBR2* was upregulated only in MCF-7/400nMPac. TGF beta induces the expression of vascular endothelial growth factor (*VEGF*) in human cancer cells (Shih and Claffey, 2001). So overexpression of *TGF* may be an indicator of cell proliferation, angiogenesis and increased ability of cell proliferation.

Some fibroblast growth factors and receptors display altered gene expression levels. *FGF2* was upregulated about 30-40 fold in both sublines. *FGFR* was upregulated only in MCF-7/400nMPac and *FGF18, FGFBP1* genes were up regulated only in MCF-7/120nMVinc.The genes encoding platelet derived growth factors *PDGFC, PDGFD* were upregulated in paclitaxel and vincristine resistant sublines. Upregulation of the so called growth factors and receptors may induce cellular proliferation as previously reported (Shih and Claffey, 2001).

The epidermal growth factor receptor (EGFR) signaling pathway is one of the most important pathways that regulate growth, survival, proliferation, and differentiation in mammalian cells. It has been investigated in depth, both experimentally and computationally (Wiley *et al*., 2003).The epidermal growth factor (EGF) peptide induces cellular proliferation through binding the EGF receptor (EGFR), which has a tyrosine kinase cytoplasmic domain, a single transmembrane domain and an extracellular domain involved in EGF binding and receptor dimerization. According to Table 3.13, *EGFR* was upregulated 15 fold in resistant MCF-7 cell lines. *EGFR* seems to have taken role in induction of cellular proliferation in drug resistant cell lines.

Insulin like growth factor binding protein and receptor encoding genes *IGFBP6, IGFBP3* and *IGFBPL1* were upregulated. *IGF1R, IGFBP5, IGFBP2* genes were down regulated in both sublines when compared to MCF-7/S. Insulin like growth factor 1 (IGF-1) and its receptor (IGF-1R) provide a potent proliferative signaling system that stimulates growth in many different cell types and blocks apoptosis. *In*

vivo IGF-1 acts as an intermediate of many growth hormone responses, and may stimulate the growth of some types of cancer (Bhat and Singh, 2008). There are several reports investigated the relationship between estrogen receptor (ESR) and insulin like growth factor binding protein (IGFBP-2) (Sheikh *et al*., 1993; Kim *et al*., 1991; Jensen *et al*., 2006). It was proposed that estrogen receptor negative breast cancer cell lines do not express *IGFBP-2* in the respective studies. Breast cancer progression is often associated with the loss of nuclear estrogen receptors. Estrogen receptor-negative breast cancers are generally more aggressive and have poor prognosis (Sabbah *et al*, 2008). The resistant cells become more independent of hormonal regulations which may be one of the indicators of epithelial-to-mesenchymal transitions in epithelial cancers. In this study *ESR1* downregulation (80 fold) was observed which is in parallel with *IGFBP-2* gene downregulation confirming the previous results. Anticancer agent tamoxifen is an estrogen receptor analog. MCF-7/400MPac and MCF-7/120nMVinc sublines developed cross resistance to tamoxifen which which may be explained by very dramatic downregulation of *ESR1* expression.

Table 3.13 Expression levels of growth factor related genes in MCF-7/Vinc and MCF-7/Pac cell lines with respect to MCF-7/S

Gene Name	Gene Symbol	Description	Ontology	Fold change	
				MCF-7/Vinc	MCF-7/Pac
209651_at	TGFB1I1	transforming growth factor beta 1 induced transcript 1	MF signal transducer	+59.78	+69.91
207334_s_at	TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	BP Growth-M, MF binding, MF catalytic activity, MF signal transducer activity	NS	+4.332
204422_s_at	FGF2	fibroblast growth factor 2 (basic)	BP Growth-M,	+34.45	+44.73
211535_s_at	FGFR1	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	BP Growth-M, MF binding, MF catalytic activity, MF defense immunity protein, MF signal transducer activity	NS	+10.46
205014_at	FGFBP1	fibroblast growth factor binding protein 1	BP Growth-M, MF Binding	+3.993	NS
218718_at	PDGFC	platelet derived growth factor C	BP Growth-M,	+30.49	+31.3
219304_s_at	PDGFD	platelet derived growth factor D	BP Growth-M, MF Binding	+6.922	+4.908
232541_at	EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	BP Growth-M, MF binding, MF catalytic activity, MF cell adhesion, MF signal transducer act	+15.12	+15.91

Table 3.13 continues

203851_at	IGFBP6	insulin-like growth factor binding prot 6	BP Growth-M,	+22.8	+14.33
210095_s_at	IGFBP3	insulin-like growth factor binding protein 3	BP death, MF catalytic activity, apoptosis regulator activity	+10.16	+12.53
227760_at	IGFBPL1	Insulin-like growth factor binding pro-like1	MF catalytic activity, MF defense immunity proteins	+9.264	+6.217
237377_at	IGF1R	Insulin-like growth factor 1 receptor	BP Growth-M, BP death, MF Binding, MF signal transducer, MF apoptosis regulator activity	-5.32	-3.97
211959_at	IGFBP5	insulin-like growth factor bindingprotein5	BP Growth-M,	-9.52	-9.345
202718_at	IGFBP2	insulin-like growth factor binding protein 2, 36kDa	BP Growth-M, MF binding	-37.17	-43.103
205225_at	ESR1	estrogen receptor 1	BP Growth-M, MF Binding, MF signal transducer, MF catalytic activity, MF structural molecule,	-81.97	-76.34

The genes encoding interleukins, interferons and related receptors are listed in Table 3.14 and seem to be highly correlated with drug resistance development. The expression values are very impressive among the other grouped gene expressions. The interleukin encoding genes *IL6* (60-120 fold), *IL8* (65-115 fold), *IL32* (157-100 fold), *IL18* (59-70 fold) were significantly upregulated in both vincristine and paclitaxel resistant breast cancer cells. Interleukin-6 (IL-6) is a cytokine that provokes a broad range of cellular and physiological responses (Akura *et al*., 1993). In addition to playing a role in inflammation and hematopoiesis, IL-6 is involved in other processes such as neuronal differentiation and bone loss. High serum IL-6 levels have been associated with poor prognosis in several solid and hematopoietic neoplasms (Blay *et al*, 1992). Induction of apoptosis by transforming growth factor beta 1, wild type p53 and cytotoxic agents is suppressed by IL-6. *IL-6* and *IL-8* genes have been reported as overexpressed in paclitaxel and doxorubicin resistant cell lines (Duan *et al*, 1999) In addition, several investigations have reported that in cancer patients elevated serum IL-6 or IL-8 levels are correlated with advanced disease status and shortened survival time (Zhang and Adachi,1999). Duan Z *et al* previously reported that transfection to U-2OS human osteosarcoma cells increased paclitaxel resistance but *IL-8* was not related with paclitaxel resistance (Duan *et al*., 1999). In this respective study it was proposed that expression of *IL-6* gene resulted in drug resistance phenotype (decreased sensitivity of cells to paclitaxel) but is not related with overexpression of *MDR1* gene. The results presented here are parallel to literature and additionally presents that the elevated interleukin amount in vincristine resistant MCF-7 cells may also have implications in drug resistance. Overexpression of *IL-32* is known to povoke IL-8 secretion so increase in *IL-8* expression level may be due to increase in *IL-32* expression but there is not any information about the relationship between IL-32 and drug resistance yet. On the other hand IL-8 is known as one of the factors that mediate tumor angiogenesis. So this relationship may be an indication of increased metastatic properties of the resistant cells.

The genes encoding interleukin receptors IL7R, IL1R2, ILRB2, IL31RA, were upregulated but IL13RA1 was downregulated in resistant sublines. Knockout studies in mice suggested that blocking apoptosis is an essential function of IL7R during differentiation and activation of T lymphocytes (Al-Rawi *et al*, 2004).Also the interferon IFI16 was 57-78 fold upregulated in resistant cells. Increased expression of IFI16 protein (encoded by the *IFI16* gene) in normal human prostate epithelial cells is associated with cellular senescence-associated cell growth arrest. (Alimirah *et al*, 2007)

The genes encoding some of the cytokines (interleukins, interferons) were upregulated in paclitaxel and vincristine resistant MCF-7 cell lines. The tumor microenvironment consists of stromal cells, extracellular matrix (ECM) and soluble factors such as cytokines and growth factors (Li and Dalton , 2006). The tumor microenvironment plays an important role in mediating *de novo* drug resistance (Hazlehurst *et al*, 2003). Although cell lines do not have microenvironment and extracellular matrix, it can be supposed that in the paclitaxel and vincristine resistant breast tumors, overexpression of the so called genes may become more significant. Also elevation of the genes related to tumor microenvironment may be observed in breast cancer patients as a signature of drug resistance phenotype which may be used as a marker of tumor and resistance progression.

Table 3.14 Expression levels of interleukin and interferon encoding genes in MCF-7/Vinc and MCF-7/Pac cell lines with respect to MCF-7/S

Gene Name	Gene Symbol	Description	Ontology	Fold change	
				MCF-7/Vinc	MCF-7/Pac
205207_at	IL6	interleukin 6 (interferon, beta 2)	BP Growth-M, MF binding, MF structural molecule, MF signal transducer activity	+59.38	+122.4
202859_x_at	IL8	interleukin 8	BP Growth-M, MF binding, MF cell adhesion molecule, MF signal transducer activity	+64.96	+115.8
203828_s_at	IL32	interleukin 32	MF catalytic activity, MF cell adhesion molecule, MF defense immunity protein	+157.5	+99.73
206295_at	IL18	interleukin 18 (interferon-gamma-inducing factor)	BP Growth-M, MF binding, BP death, MF apoptosis regulator, MF cell adhesion molecule, MF structural molecule, MF signal transducer activity	+53.08	+72.03

Table 3.14 continues

226218_at	IL7R	Interleukin 7 receptor	MF signal transducer activity	+14.69	+25.41
205403_at	IL1R2	interleukin 1 receptor, type II	MF defense immunity protein,MF signal transducer activity	+6.502	+6.075
206999_at	IL12RB2	interleukin 12 receptor, beta 2	BP Growth-M,	NS	+3.926
243541_at	IL31RA	interleukin 31 receptor A	BP Growth-M, MF catalytic activity, MF apoptosis regulator, MF structural molecule, MF defense immunity protein, MF transcription regulator	NS	+3.633
201888_s_at	IL13RA1	interleukin 13 receptor, alpha 1	MF signal transducer	-2.55	-2.48
208966_x_at	IFI16	interferon, gamma-inducible protein 16	MF binding, BP death, MF apoptosis regulator, MF transcription regulator	+56.92	+78.5
201422_at	IFI30	interferon, gamma-inducible protein 30	MF catalytic activity,	NS	-2.96
1552477_a_at	IRF6	interferon regulatory factor 6	BP death, MF apoptosis regulator	-5.56	-4.31

Table 3.15 lists expression levels of some of the extracellular matrix (ECM) related and structural genes. Cellular transformation is accompanied by many cellular changes, including uncontrolled proliferation, loss of the differentiated cell morphology, and invasion of the extracellular matrix (Hazlehurst *et al*, 2003). Degradation of the extracellular matrix is a key component of tumor cell invasion into surrounding tissues.

Matrix metalloproteinases (MMPs) are a class of proteases secreted by tumor cells, degrading the proteins of the extracellular matrix and allowing metastasis. Drug inhibitors of MMPs are one strategy to control cancer and block metastasis. Soluble secreted MMP inhibitors have also been identified, (Tissue inhibitor matrix metalloproteases, TIMPs), which appear to be less active at inhibiting MMPs and even perhaps to be essential for MMP maturation. MMPs are responsible for cleavage of cell surface receptors, release of apoptotic ligands, and chemokine in/activation. They are thought to play a major role on cell behavior such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis and host defense. MMP1 and MMP14 are able to degrade interstitial fibrillar collagens to form gelatin for further degradation by gelatinase MMPs (Lovejoy *et al*., 1999). They have also been known to enhance cancer cell migration. MMP1 gene is drastically overexpressed in MCF-7/120nMVinc cells (1000-fold) and increase in this gene may be correlated to invasive ability of these cells. In a previous report on gene expression analysis of drug resistant cells by microarray, overexpression of MMP1 was also demonstrated (Turton *et al*., 2001) and correlated to invasive property of drug resistant cells. Among MMPS, two gelatinases MMP2 and MMP9 are distinguished both structurally and functionally. MMP9 is rapidly available upon activation for any remodeling events (Somerville *et al*., 2003). Additionally, knockout mice lacking MMP9 have shown decreased incidence of skin and pancreatic carcinogenesis and metastasis (Bergers *et al*., 2000; Coussens *et al*., 2000).

The adhesion of cells to each other or to the ECM is responsible for stimulating signals that regulate migration of immune cells, invasion and metastasis of

tumor cells, and angiogenesis. Previous reports demonstrated the association between MDR phenotype with P-gp expression and increased invasive ability (Li *et al*., 2007; Weinstein *et al*., 1991). Laminins, fibronectins and collagens are among attachment proteins that mediate cell survival via integrin interactions (Ahmed *et al*., 2005; Morin, 2003; Sethi *et al*. 1999).

LAMA1 gene encoding the α1 chain of laminin was drastically overexpressed in drug resistant cells (140-, 73-, -fold in MCF-7/Vinc and MCF-7/Pac respectively). *LAMA4* and *LAMC3* were also upregulated in both sublines. *FN1* gene encoding the type I domain of fibronectin was significantly upregulated in drug resistant cells (88-30 fold). Other ibronectin encoding genes *FLTR2, FNDC6, FSD1, FNDC3B* were also upregulated in resistant sublines. The collagen encoding genes *COL4A2, COL4A1, COL6A1, COL6A2, COL12A1* and *COL27A1* were overexpressed in resistant sublines, among them *COL4A2* is highly overexpressed in resistant cells. Overexpression of the genes encoding the important ECM remodeling proteins may have contributed to the invasive behavior of tumor cells.

Among genes encoding the integrins, gene encoding the α6 integrin (*ITGA6*) was highly overexpressed in all drug resistant sublines. To a lesser extent genes encoding the α5 (except for in MCF-7/400nMPac) and β1 integrins (*ITGA5* and *ITGB1*, respectively) were also upregulated. Several reports have demonstrated differential expression of integrin subunits in drug resistant variants of tumor cell lines exhibiting altered binding efficiencies to ECM ligands such as fibronectin, laminin and collagen IV (Narita *et al*. 1998; Liang *et al*., 2001). In addition, Aoudjit *et al*., (Aoudjit and Vuori, 2001) identified the inhibitory role of β1 integrin signaling in paclitaxel and vincristine induced apoptosis of breast carcinoma cells which was related to integrin-mediated inhibition of cytochrome c release from mitochondria dependent on activation of the PI3-kinase/Akt pathway.

A disintegrin and metalloproteinase (ADAMs) family takes role in the control of membrane fusion, cytokine and growth factor shedding, and cell migration (Seals and Courtneidge, 2003). As it is seen in Table 3.15, ADAM9 was overexpressed 3-4 fold in drug resistant sublines. It can modulate EGF receptor activity upon shedding of heparin-binding EGF, cause transactivation of EGFR which in turn activates pathways to promote cell survival (Fischer *et al*., 2004). So besides playing role in ECM remodeling, invasion and metastasis, modulation of growth factor activity seems to be directly related to cell survival. The adamalysins also contain the ADAMTS family proteinases; members having a variable number of thrombospondin-like (TS) motifs. *ADAMTS1* gene was upregulated in vincristine and paclitaxel resistant sublines. Liu *et al*. (Liu *et al*., 2006) have demonstrated that overexpression of full length *ADAMTS1* in mammary carcinoma cells promoted tumor angiogenesis and invasion and activation of the EGFR leading to cell proliferation and survival. *ADAMTS3* and *ADAMTS6* were upregulated in vincristine resistant cells. The protein function of *ADAMTS6* product has not been well characterized yet. However, Porter *et al*. (Porter *et al*., 2004) reported an association of expression between *ADAMTS6* and *MMP9* which is also upregulated only in MCF-7/Vinc cells.

The tissue inhibitors of metalloproteinases (TIMPs) regulate extracellular matrix turnover and tissue remodeling by forming tight binding inhibitory complexes with the MMPs and ADAMs directly interacting with their active sites (Somerville *et al*., 2003). *TIMP4* was upregulated in MCF-7/120nMVinc, MCF-7/400nMPac cells. *TIMP4* has been shown to have anti-apoptotic activity and tumor-stimulating effect in breast cancer cells (Jiang *et al*., 2001). So overexpression of TIMP4 gene may be another cause of survival in resistant cell lines.

ECM1 gene, upregulated in MCF-7/120nMVinc cells ,encodes the extracellular matrix protein 1. Stimulatory effect of ECM1 on proliferation of endothelial cells and angiogenesis of breast carcinoma cells with its increased expression was identified (Han *et al*., 2001).

Table 3.15 presents the differential expression of ECM component and related genes in drug resistant and sensitive breast carcinoma cells. It was previously reported that overexpression of the multidrug resistance (MDR) gene increases aggressive behavior of MCF-7 cells (dit Faute *et al*., 2002). This finding is also proved by the results presented in this work. As mentioned before, the cell lines do not have microenvironment and extracellular matrix *in vitro*. Here it can be proposed that in paclitaxel and vincristine resistant breast tumors overexpression of the ECM components and related genes may become more significant in its own environment. Also elevation of these gene expressions may be observed in breast cancer patients as a marker of tumor progression and resistance phenotype.

Table 3.15 Expression levels of extracellular matrix related genes in MCF-7/Vinc and MCF-7/Pac cell lines with respect to MCF-7/S

Gene Name	Gene Symbol	Description	Ontology	Fold change	
				MCF-7/Vinc	**MCF-7/Pac**
204475_at	MMP1	matrix metallopeptidase 1 (interstitial collagenase)	BP Growth-M, BP death, MF Binding, MF catalytic activity	+1,013	NS
207118_s_at	MMP23B ; MMP23A	matrix metallopeptidase 23B ; matrix metallopeptidase 23A	MF Binding, MF catalytic activity, MF defense immunity proteins, MF structural molecule	+15.94	+15.07
202827_s_at	MMP14	matrix metallopeptidase 14 (membrane-inserted)	MF binding, MF catalytic activity	NS	+5.048
203936_s_at	MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase,	MF binding, MF catalytic activity	+2.368	NS
227048_at	LAMA1	laminin, alpha 1	BP Growth-M, MF structural molecule, MF signal trandsducer,MF cell adhesion molecule	+140.5	+72.65
202202_s_at	LAMA4	laminin, alpha 4	MF binding, MF catalytic activity	+13.77	+13.77
219407_s_at	LAMC3	laminin, gamma 3	BP Growth-M, MF structural molecule, MF signal trandsducer,MF cell adhesion	NS	+8.87
212464_s_at	FN1	fibronectin 1	BP Growth-M,MF Binding, MF signal transducer, MF transporter activity, MF cell adhesion molecule, MF structural mol	+88.28	+30.34
228575_at	FNDC6	fibronectin type III domain containing 6	MF signal transducer	+63.42	NS

Table 3.15 continues

204359_at	FLRT2	fibronectin leucine rich transmembrane protein 2	MF Binding,	+16.44	+8.913
219170_at	FSD1	fibronectin type III and SPRY domain containing 1	MF signal transducer	+7.541	+7.363
222692_s_at	FNDC3B	fibronectin type III domain containing 3B	MF signal transducer	+5.678	+4.128
219250_s_at	FLRT3	fibronectin leucine rich transmembrane protein 3	MF signal transducer	-8.264	-13.51
211964_at	COL4A2	collagen, type IV, alpha 2	MF transporter activity, MF structural molecule	+77.66	+76.95
213428_s_at	COL6A1	collagen, type VI, alpha 1	BP Growth-M, MF transporter activity, MF structural molecule	+17.58	+14.29
211980_at	COL4A1	collagen, type IV, alpha 1	MF transporter activity, MF structural molecule	+17.47	+16.39
225664_at	COL12A1	collagen, type XII, alpha 1	BP Growth-M, MF structural molecule, MF cell adhesion molecule	NS	+14.35
213290_at	COL6A2	collagen, type VI, alpha 2	BP Growth-M, MF transporter activity, MF structural molecule	+8.124	+7.15
225292_at	COL27A1	collagen, type XXVII, alpha 1	MF transporter activity, MF structural molecule	+2.785	+2.351
215177_s_at	ITGA6	integrin, alpha 6	MF structural molecule	+39.68	+23.96
201389_at	ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	BP Growth-M, MF structural molecule, MF signal trans	+5.591	+NS
1553678_a_at	ITGB1	integrin, beta 1 (fibronectin recept)	BP Growth-M, MF structural molecule, MF signal trans	+2.55	+3.013

Table 3.15 continues

202381_at	ADAM9	ADAM metallopeptidase domain 9	MF catalytic activity	+3.13	+4.517
217007_s_at	ADAM15	ADAM metallopeptidase domain 15 (metargidin)	MF binding, MF cell adhesion molecule	NS	-2.61
222162_s_at	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	MF Binding, MF catalytic activity	+11.79	+5.799
214913_at	ADAMTS3	ADAM metallopeptidase with thrombospondin type 1 motif, 3	MF Binding, MF catalytic activity	+5.949	NS
1570351_at	ADAMTS6	ADAM metallopeptidase with thrombospondin type 1 motif, 6	MF Binding, MF catalytic activity	+9.276	NS
206243_at	TIMP4	TIMP metallopeptidase inhibitor 4	MF catalytic activity	+12.07	+6.733
209365_s_at	ECM1	extracellular matrix protein 1	BP Growth-M, MF transporter activity, MF structural molecule	+11.7	NS

Expression analyses of the genes related with cell cycle regulation are listed in Table 3.16. The cell cycle is regulated by the interplay of many molecules (Mullan *et al*., 2001). Keys among these are the cyclins which are expressed and then degraded in a concerted fashion to drive the stages of the cell cycle. Cyclins combine with cyclin dependent kinases (cdks) to form activated kinases that phosphorylate targets leading to cell cycle regulation. A breakdown in the regulation of this cycle can lead to out of control growth and contribute to tumor formation. Defects in many of the molecules that regulate the cell cycle have been implicated in cancer. Key among these are p53, the cdk inhibitors (p15, p16, p18, p19, p21, p27), and Rb, all of which act to keep the cell cycle from progressing until all repairs to damaged DNA have been completed. Cyclin dependent kinase inhibitor encoding gene (CDKN2A, p16) was significantly upregulated in MCF-7/120nMVinc and MF-7/400nMPac (70-100 fold). However *CDK6* was only induced in MCF-7/400nMPac. These increases may be correlated with the slow growth and increase in doubling time of the resistant cells that we have previously shown by constructed growth curves (Figure 3.1, 3.2, 3.3, 3.4).

The cell cycle regulatory cyclin encoding genes *CCNE1, CCNA2* were induced in MCF-7/400nMPac and MCF-7/120nMVinc cells. But *CCNE2* was downregulated only in MCF-7/400nMPac. Differential expression of cyclins in drug resistant sublines may be due to effects of mitotic inhibitor drugs paclitaxel and vincristine. The both anticancer agents are acting on G2-M phase transition in cell cycle. So it may be proposed that these expression level alterations may have been due to the continuing exposure to antimitotic agents. The results presented in Table 3.16 suggest that during the development of drug resistance, paclitaxel and vincristine adapted cells have presented decreased cell division rate or prolonged doubling time.

Breast cancer susceptibility gene 1 (*BRCA1*) is a tumor suppressor gene. It was prevously shown that BRCA1 sensitizes breast cancer cells to apoptosis induced by both paclitaxel and vinorelbine (Quinn *et al.* ,2003) and reduced BRCA1 activity confers resistance to these mitotic inhibitor agents (Lafarge *et*

al., 2001; Tassone *et al*., 2003). Here the results are also parallel to literature that, the gene encoding a BRCA1 associated protein (*BARD1*) and the gene encoding the BRCA1 interacting protein (*BRIP1*) were significantly downregulatd in both paclitaxel and vincristine resistant MCF-7 sublines.

The retinoblastoma protein (abbreviated pRb or *Rb*) is a tumor suppressor protein that is dysfunctional in many types of cancer. One highly studied function of pRb is to prevent excessive cell growth by inhibiting cell cycle progression until a cell is ready to divide (Murphree and Benedict, 1984). *RB1* was down regulated about 9 fold in paclitaxel and vincristine resistant MCF-7 sublines. The decrease in expression level may have a role in cell proliferation. Banerjee *et al*. reported that when Rb is deleted or rendered, nonfunctional levels of E2F (transcription factor) are high resulting in enhanced transcription of genes (Banerjee *et al*., 1995). O'Loughlin *et al* (O'Loughlin *et al*., 2000) previously reported that drug exposure and resistance altered the cell cycle response in lung carcinoma cell line.

Table 3.16 Expression levels of cell cycle related genes in MCF-7/Vinc and MCF-7/Pac cell lines with respect to MCF-7/S

Gene Name	Gene Symbol	Description	Ontology	Fold change	
				MCF-7/Vinc	**MCF-7/Pac**
209644_x_at	CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	BP Growth-M, BP death,MF Binding, MF catalytic activity, MF apoptosis regulator, MF transcription regulatory	+71.11	+100.2
224848_at	CDK6	cyclin-dependent kinase 6	BP Growth-M, BP death,MF Binding, MF catalytic activity, MF apoptosis regulator, MF transcription regulatory	NS	+2.503
212897_at	CDC2L6	cell division cycle 2-like 6 (CDK8-like)	BP Growth-M, MF Binding	-3.17	-3.07
213523_at	CCNE1	cyclin E1	BP Growth-M, MF binding, MF signal transducer activity	+3.911	+2.468
213226_at	CCNA2	Cyclin A2	BP Growth-M, MF defense immunity proteins	+2.165	+3.489
205034_at	CCNE2	cyclin E2	BP Growth-M, MF binding, MF catalytic activity,	NS	-7.04
227545_at	BARD1	BRCA1 associated RING domain 1	BP Growth-M, MF apoptosis regulator activity	-4.05	-3.11
221703_at	BRIP1	BRCA1 interacting protein helicase 1	BP Growth-M, MF apoptosis	-10.96	-3.115
203132_at	RB1	retinoblastoma 1 (including osteosarcoma)	MF Binding, MF signal transducer	-8.77	-8.695

Table 3.17 presents the alterations in expression levels of some of the oncogenes in drug resistant cell lines.

Ets1 proto-oncoprotein is a member of the Ets family of transcription factors that share a unique DNA binding domain, the Ets domain. The DNA binding activity of Ets1 is controlled by kinases and transcription factors Ets1 plays a key role in the acquisition of an invasive behavior. Among the genes that respond to Ets1 are those that code for certain proteases such as matrix metalloproteases MMP-1, MMP-3, MMP-9, and urokinase type plasminogen activator (uPA). These proteases are known to be involved in ECM (extracellular matrix) degradion, a key event in invasion (Dittmer, 2003). When overexpressed in endothelial cells or hepatoma cells, Ets1 induced the production of MMP-1, MMP-3 and MMP-9 or MMP-1, MMP-9 and uPA, respectively (Sato *et al*., 2000; Jiang *et al*., 2001). Overexpression *ETS1* gene in resistant cells around 80 fold, may have caused the upregulation of *MMP1* (1000 fold) and *MMP9* (2.4 fold) in MCF-7/120nMVinc. However this interaction was not valid for paclitaxel resistant breast cancer cells. ETS1 and p53 interaction is reported as one of the major mechanism to up regulate MDR1 overexpression at transcriptional level. Mutant, but not wildtype, p53 was found to cooperate with Ets1 to increase the transcription from the *MDR1* gene (Sampath *et al*, 2001). This finding is also parallel to our results that *MDR1* gene was overexpressed 146-93 fold in MCF-7/120nMVinc and MCF-7/400nMPac sublines (Table 3.9).

Ras is a small GTP-binding protein, which is the common upstream molecule of several signalling pathways including Raf/MEK/ERK, PI3K/Akt and RalEGF/Ral (Peyssonnaux *et al*., 2000). The Ras/Raf/MEK/ERK cascade couples signals from cell surface receptors to transcription factors, which regulate gene expression.This pathway is often activated in certain tumors by mutations or overexpression of upstream molecules such as BCR-ABL and epidermal growth factor receptor (EGFR). It was suggested in a previous study that Ras/Raf pathway may be involved in the regulation of P-glycoprotein extrusion pump expression (Cornwell and Smith, 1993). According to our

results (Table 3.17), *Ras* gene family member expression levels significantly changed. These alterations may have caused the upregulation of *MDR1* gene and also many survival pathways. In a study by Fan *et al*, knockdown of *RAB25* expression by RNAi inhibited growth of human ovarian cancer cells *in vitro* and *in vivo* (Fan *et al*, 2007). The so called gene was down regulated dramatically in both sublines.

The oncogenes *ABL1/2, MRAS* and *JUN* were upregulated in resistant sublines. *MYB , AKT, ERBB3* were down regulated. MYB is overexpressed in the majority of colo rectal cancers and estrogen receptor alpha positive breast cancers (Drabsch *et al*, 2008). Activation of *MYB* transcription occurs at the earliest stages of adenoma formation in the colon and progressively increases in primary and finally metastatic adenocarcinoma. The decrease in estrogen receptor gene expression about 80 fold may have resulted in downregulation of *MYB* oncogenes expression (57-73 fold).

Table 3.17 Expression levels of oncogenes in MCF-7/Vinc and MCF-7/Pac cell lines with respect to MCF-7/S

Gene Name	Gene Symbol	Description	Ontology	Fold change	
				MCF-7/Vinc	MCF-7/Pac
224833_at	ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	BP Growth-M	81.87	75.01
1555630_a_at	RAB34	RAB34, member RAS oncogene family	BP Growth-M,MF Binding, MF transporter activity	77.74	84.36
227123_at	RAB3B	RAB3B, member RAS oncogene family	BP Growth-M,MF Binding, MF transporter activity	31.05	23.63
219412_at	RAB38	RAB38, member RAS oncogene family	BP Growth-M, MF transporter activity, MF catalytic activity	15.55	10.76
204214_s_at	RAB32	RAB32, member RAS oncogene family	MF Binding, MF transporter activity	5.731	4.736
229504_at	RAB23	RAB23, member RAS oncogene family	BP Growth-M, MF Binding, MF transporter activity	4.009	NS
210127_at	RAB6B	RAB6B, member RAS oncogene family	MF Binding, MF transporter activity	3.855	4.183
226633_at	RAB8B	RAB8B, member RAS oncogene family	MF transporter activity	3.095	4.363
218700_s_at	RAB7L1	RAB7, member RAS oncogene family-like 1	BP Growth-M, MF binding	NS	-2.11
239409_at	RAP1A	RAP1A, member of RAS oncogene family	BP Growth-M	-2.07	-2.43
219807_x_at	RAB4B	RAB4B, member RAS oncogene family	BP Growth-M, MF binding	NS	-2.12

Table 3.17 continues

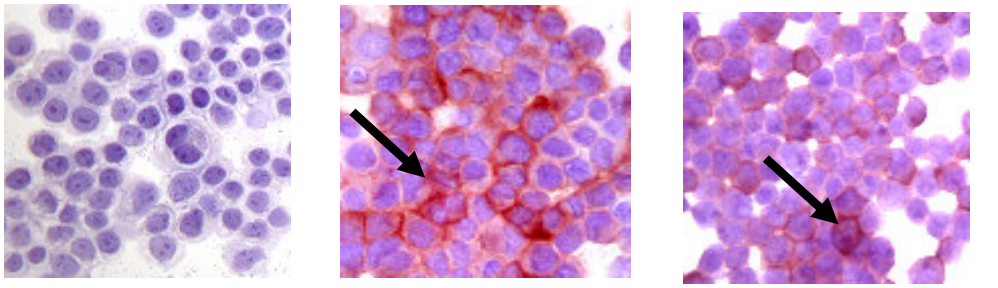
201048_x_at	RAB6A	RAB6A, member RAS oncogene family	BP Growth-M,	NS	-2.14
219622_at	RAB20	RAB20, member RAS oncogene family	BP Growth-M, MF transporter activity	-2.28	-2.40
221808_at	RAB9A	RAB9A, member RAS oncogene family	BP Growth-M, MF transporter activity	-2.30	-2.36
213405_at	RAB22A	RAB22A, member RAS oncogene family	BP Growth-M, MF transporter activity	-2.58	NS
203885_at	RAB21	RAB21, member RAS oncogene family	BP Growth-M, MF binding	NS	-2.04
208466_at	RAB3D	RAB3D, member RAS oncogene family	BP Growth-M, MF transporter activity	-2.69	NS
204547_at	RAB40B	RAB40B, member RAS oncogene family	BP Growth-M, MF transporter activity	-3.021	NS
235059_at	RAB12	RAB12, member RAS oncogene family	MF binding	-4.48	-3.73
218931_at	RAB17	RAB17, member RAS oncogene family	BP Growth-M, MF Binding, MF transporter activity	-22.27	-35.09
218186_at	RAB25	RAB25, member RAS oncogene family	BP Growth-M, MF Binding, MF transporter activity	-113.6	-129.54
207163_s_at	AKT1	v-akt murine thymoma viral oncogene homolog 1	BP Growth-M, BP death	-3.46	-3.09
226213_at	ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	BP Growth-M	-4.27	NS
204798_at	MYB	v-myb myeloblastosis viral oncogene homolog (avian)	BP Growth-M	-56.82	-73.53

Table 3.17 continues

225185_at	MRAS	muscle RAS oncogene homolog	BP Growth-M, MF transporter activity	8.949	NS
202647_s_at	NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	BP Growth-M	NS	-6.54
206411_s_at	ABL2	v-abl Abelson murine leuk v-oncog	BP Growth-M	3.044	NS
202123_s_at	ABL1	v-abl Abelson murine leukemia viral oncogene homolog 1		NS	2.255
201464_x_at	JUN	v-jun sarcoma virus 17 oncogene homolog (avian)		NS	2.005

3.4 Expression Analysis of Drug Resistance Related Proteins

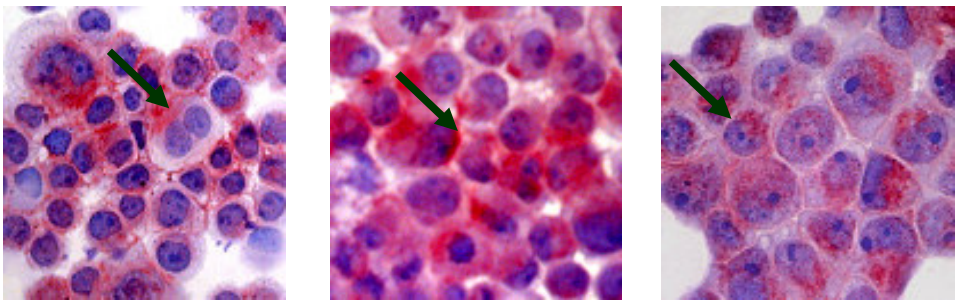
3.4.1 Immunocytochemisrty



MCF-7/S, P-gp, HRP-DAB MCF-7/Pac, P-gp, HRP-DAB MCF-7/Vinc, P-gp, HRP-DAB

Figure 3.31 Immunocytochemistry staining results (P-gp)

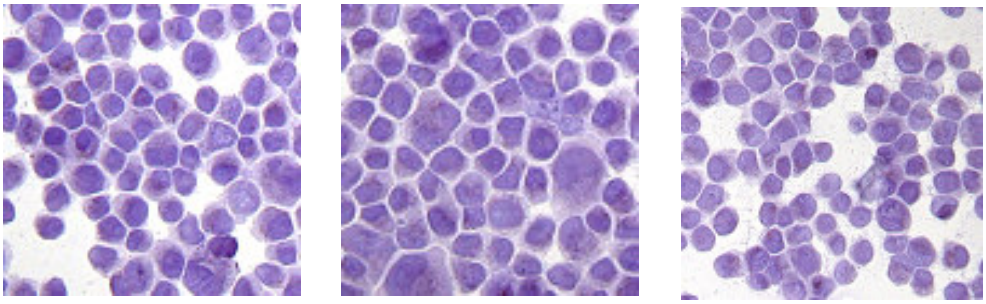
Immunocytochemistry results for P-gp identification are correlated with the Real-Time PCR and microarray results. The findings clearly represent that the parental cell line is P-gp negative but the sublines express P-gp. Moreover, DAB staining clearly indicated the localization of the protein. The localization of the protein is mostly on the cell membranes. Also it is interesting that the P-gp expression is not uniform in the sublines, that is P-gp is not expressed by all the cell population at the same level.



MCF-7/S, MRP1, HRP-AEC MCF-7/Pac, MRP1, HRP-AEC MCF-7/Vinc, MRP1, HRP-AEC

Figure 3.32 Immunocytochemistry staining results (MRP1)

Immunocytochemistry results for MRP1 protein identification clearly represent that the parental cell line and the resistant sublines are MRP1 positive. Moreover, AEC staining clearly indicated the localization of the protein. The localization of the protein is on the cell membranes and in cytoplasm also. The cell lines seem to express the protein more or less at the same level.



MCF-7/S, LRP, HRP-DAB MCF-7/Pac, LRP, HRP-DAB MCF-7/Vinc, LRP, HRP-DAB

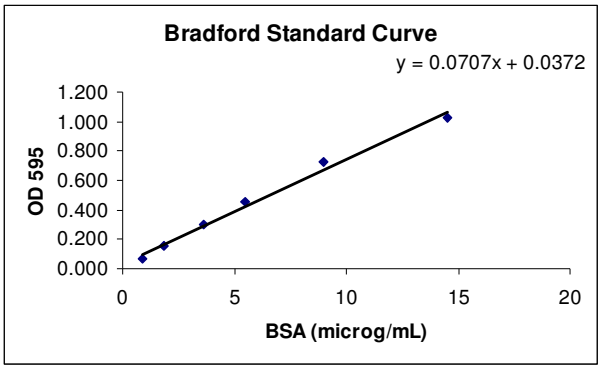
Figure 3.33 Immunocytochemistry staining results (LRP)

According to the results, all the cell lines are LRP negative. This means that *LRP* gene does not contribute to the resistance mechanisms in the MCF-7 sublines.

3.4.2 Western Blot Analysis

The protein amounts were determined by Bradford assay. Figure 3.34 represents a representative standart curve driven from OD vs BSA concentration graph and an SDS poly acrylamide gel on which different amount of cell lysates were run.

a)



b)

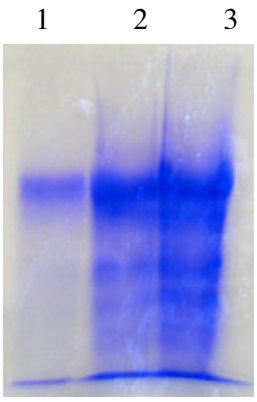


Figure 3.34 a)Bradford standart curve b)MCF-7 cell lysate PAGE 1: 10 µg of total protein, 2: 30 µg of total protein, 3: 50 µg of total protein.

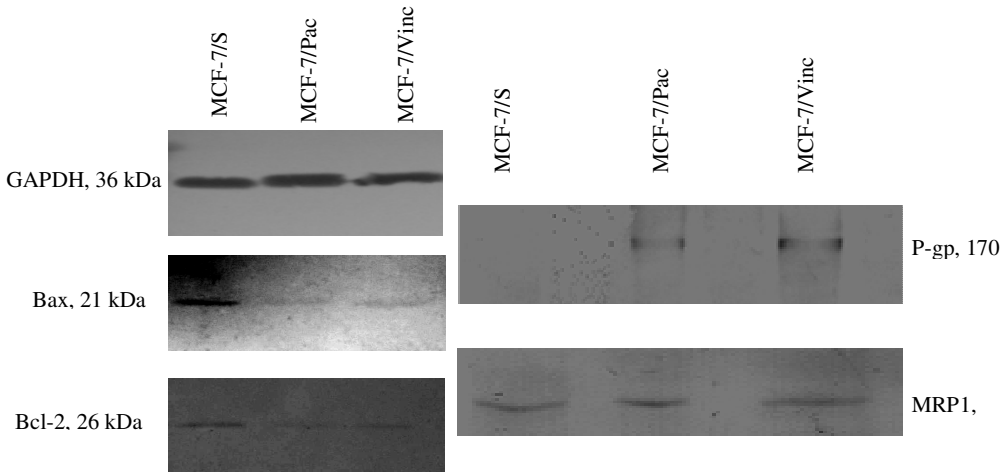


Figure 3.35 Blot membrane photographs demonstrate GAPDH, Bcl-2, Bax, P-gp and MRP1 protein levels in MCF-7/S, MCF-7/400nMPac and MCF-7/120nMVinc cell lines.

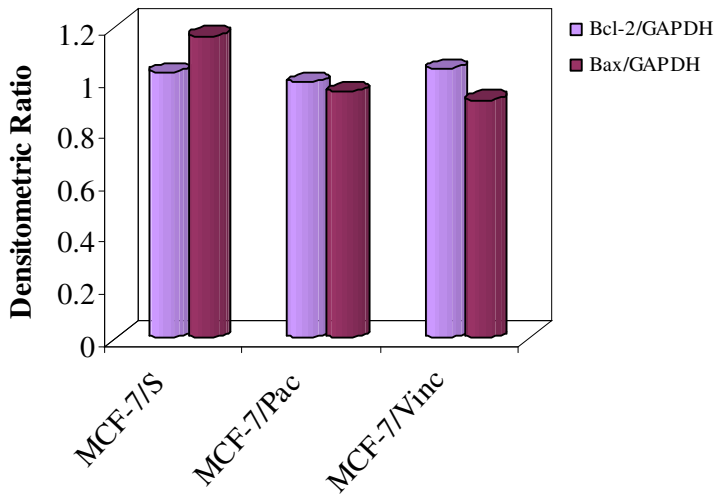


Figure 3.36 Densitometric ratios demonstrate Bcl-2 and Bax expression levels in MCF-7/400nMPac and MCF-7/120nMVinc cell lines with respect to MCF-7/S

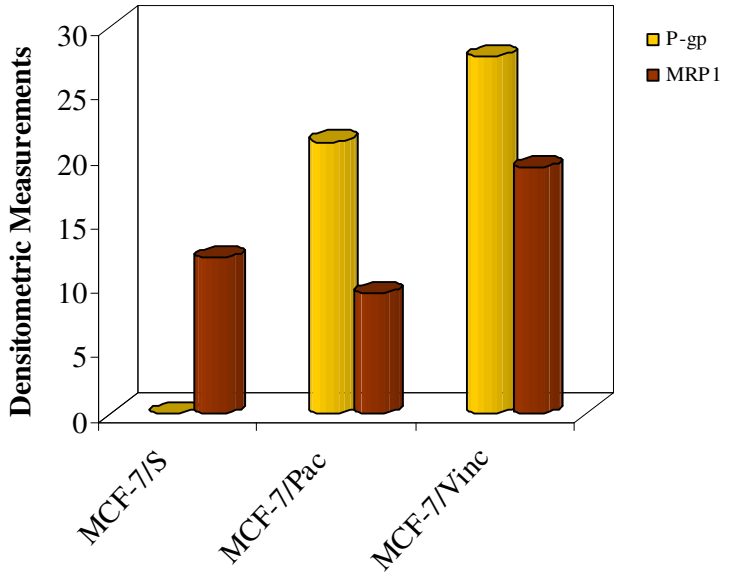


Figure 3.37 Densitometric ratios demonstrate P-gp and MRP1 expression levels in MCF-7/400nMPac and MCF-7/120nMVinc cell lines with respect to MCF-7/S

Western blot analysis also confirms the real-time PCR, microarray and immunocytochemistry results. According to densitometric measurements (Figure 3.36, 3.37), overexpression of P-gp was the dominant drug resistance mechanism in both MCF-7/400nMPac and MCF-7/120nMVinc sublines among four proteins (P-gp, MRP1, Bcl-2 and Bax).

3.5 Investigation of *Beta-tubulin* Gene Mutations

Sequence differences between sensitive and resistant cells were evaluated and only differences in MCF-7/120nMVinc cells made sense (Table 3.18). Modeling of these differences revealed that there was a His179 to Pro179 change that was interpreted from CAT → CCT change in nucleotide sequence of MCF-7/120nMVinc. The sequence data sheet is given in Appendix I.

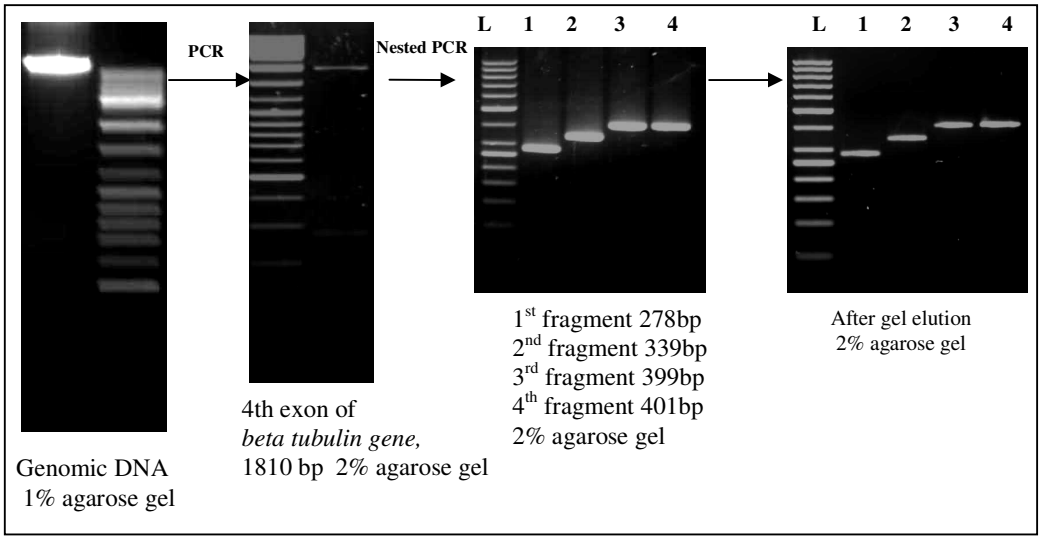


Figure 3.38 Gel photographs obtained to detect *Beta-tubulin* mutations in MCF-7/400nMPac, MCF-7/120nMVinc with respect to MCF-7/S.

Table 3.18 Aminoacid differences in MCF-7/120nMVinc and MCF-7/400nMPac with respect to MCF-7/S.

	Location	Mutation	Codon	Effect
MCF-7/ 120nMVinc	4-3	CAT to CCT	179	His to Pro
MCF-7/ 400nMPac	-	No change	-	

There is evidence that mutations in class I *β-tubulin* may play an important role in resistance to drugs that target the tubulin/microtubule system in resistant cell lines (Drukman and Kavallaris, 2002). In this study mutational analysis revealed single base substitutions in MCF-7/120nMVinc cells resulting in missense mutations at amino acid level. MCF-7/120nMVinc cells acquired His-179 → Pro-179 mutation in class I *β-tubulin.* This mutation is near GTP binding site (β173) and possibly alters microtubule stability (Kavallaris *et al*., 2001). Since GTP binding is an essential regulatory mechanism of microtubule stability, changes in that site may seriously influence microtubule stability (He *et al*., 2001). The cells may have developed this mechanism to rearrange microtubule stability and compensate for the effect of presence of microtubule disassembly agent vincristine. This amino acid alteration may be one of the mechanisms of drug resistance in MCF-7/120nMVinc cells. According to previous reports, important β-tubulin amino acids for paclitaxel binding were identified by electron crystallography and these were located between 1-31 and 217-233 (Rao *et al*., 1994; Snyder *et al*. 2001) amino acids. In this study, MCF-7/400nMPac cell line did not have any *TUBB* gene mutation. Although there are several cases that represent base substitutions in *TUBB* gene in paclitaxel resistant cell lines (Giannakakou *et al*., 1997; He *et al*., 2001), it has been also reported that alterations in this gene sequence have not been correlated to paclitaxel resistance in patients (Sale *et al*., 2002). Furthermore; acquired *β-tubulin* mutations may differ among cell and tissue types.

3.6 Drug Accumulation Assays

The original sensitive culture contained only P-gp negative cells (Figure 3.39 a). As the cells were developed resistance *via* the stepwise increments of paclitaxel from 0.1nM to 100nM, and vincristine from 2nM to 30nM in media, the culture was found to contain both P-gp positive and negative cells when the sublines were assayed (indicated by the rhodamine-123 fluorescence assay). The P-gp positive cell numbers were not dominantly present therefore the histograms presented two peaks (Figure 3.39 b), one belonging to the P-gp negative and the other to the P-gp positive cells. The continuing drug dose increments (120nM paclitaxel, 40nM vincristine) resulted in resistant MCF-7 sublines, of which a high percentage of the cells had active P-gp. The portion of cells that had the P-gp activity was greater than the P-gp negative cells in number, so that the histogram had a single peak of low fluorescence (Figure 3.39 c). Similarly, carboxyfluorescein is a substrate for MRP1 and used to determine its efflux activity. Figure 3.40 represents fluorescent intensities of the cell lines obtained from various flow cytometry measurements. From the fluorescent activity values, it is apparent that the parental MCF-7/S cells express inherently the *MRP1*, but not the *MDR1* gene. On the other hand, the resistant sublines MCF-7/Pac and MCF-7/Vinc express both of the genes so that the resistant sublines can efflux both rhodamine-123 and carboxyfluorescein from the cells effectively. The rhodamine-123 efflux activity of P-gp is higher than the carboxyfluorescein efflux activity of MRP1 in the drug resistant sublines (Figure 3.40). In fact, the activity of MRP1 was already high in the original sensitive cells and did not change significantly in resistant sublines (Kars *et al*, 2006).

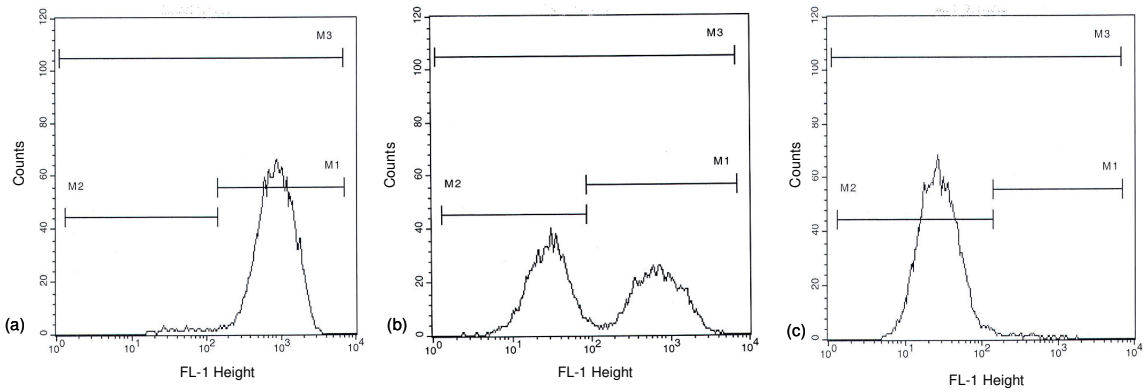


Figure 3.39 Flow cytometry histograms of rhodamine 123 accumulation assay: (a) Sensitive MCF-7 cell line; (b) MCF-7 cell line with non-uniform cell population; (c) Resistant MCF-7 subline.

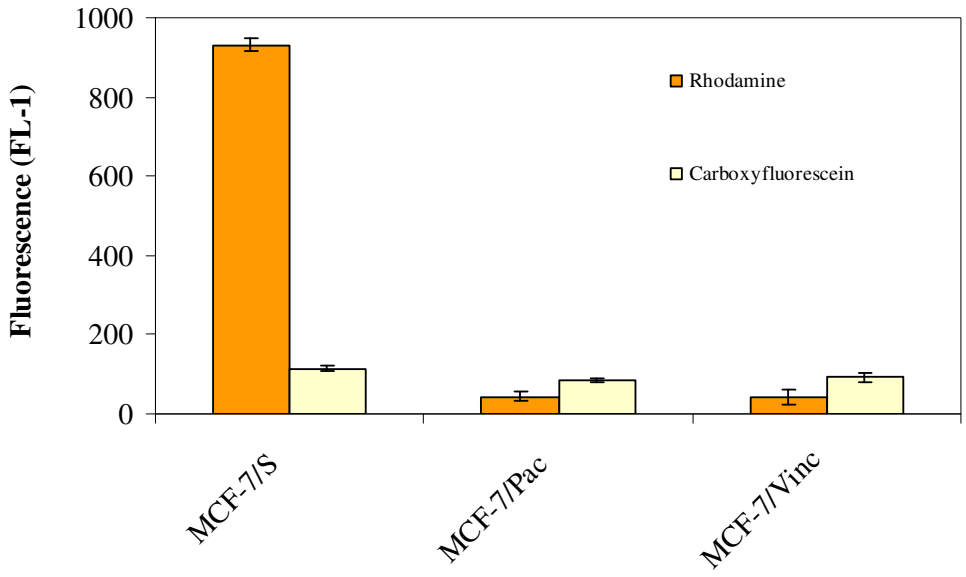


Figure 3.40 The scheme illustrates the fluorescent dye R123 and CF accumulation in each MCF-7 cell line. SEM (standard error of the means) values were derived from results of three independent experiments.

3.7 Reversal of P-gp and MRP1 Related MDR by Modulators

The flow cytometry results demonstrate that the inhibition of P-gp resulted in cellular accumulation of the rhodamine-123 and peak shifts form low fluorescent region (M2) to high fluorescent region (M1) in histograms (Figure 3.41, 3.42). Inhibition of MRP1 protein also resulted in accumulation of carboxyfluorescein.

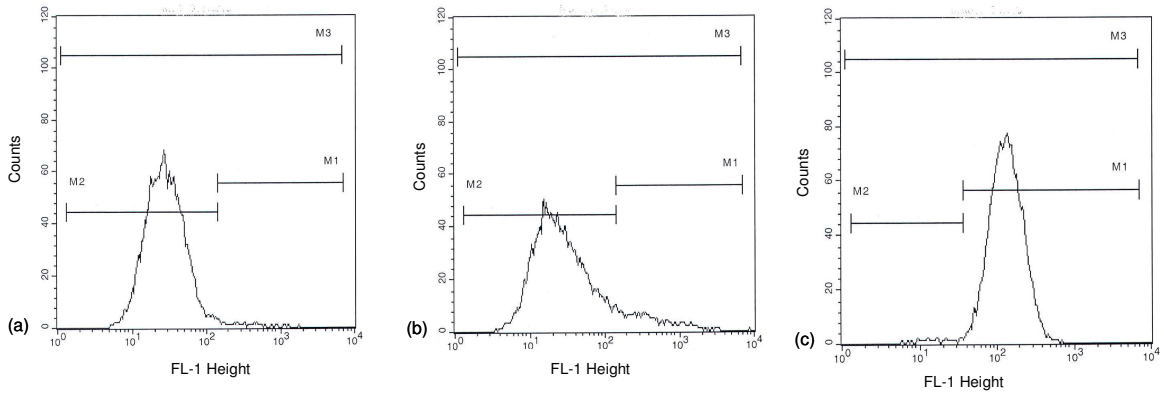


Figure 3.41 Flow cytometry histograms of rhodamine 123 accumulation assay of modulated resistant MCF-7 cell lines: (a) Resistant MCF-7 subline; (b) Cinnamylidene-2 modulated resistant MCF-7 subline; (c) ALIS 409 modulated resistant MCF-7 subline.

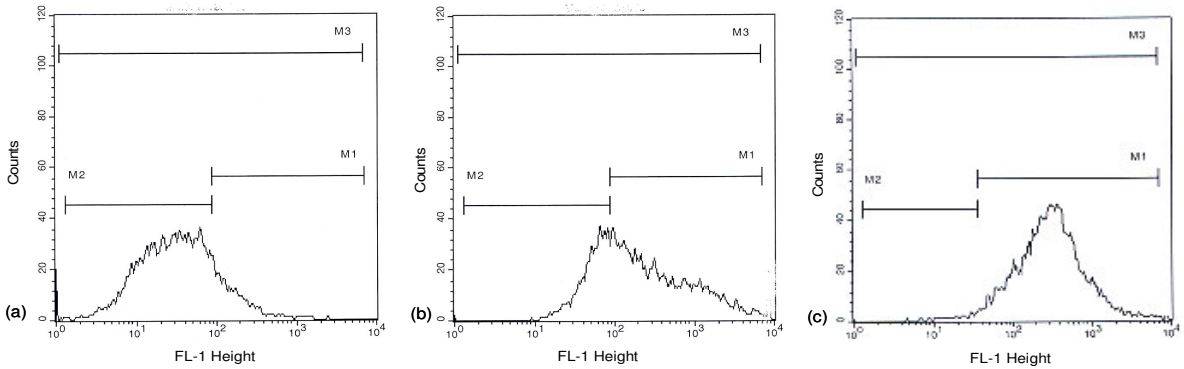


Figure 3.42 Flow cytometry histograms of the carboxyfluorescein accumulation assay of modulated resistant MCF-7 cell lines: (a) Resistant MCF-7 subline; (b) Indomethacin modulated resistant MCF-7 subline; (c) ALIS 409 modulated resistant MCF-7 subline.

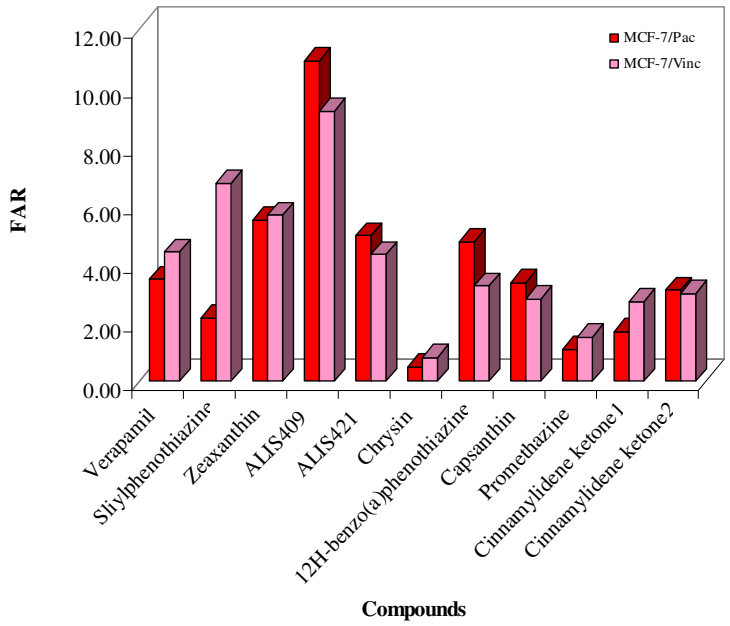


Figure 3.43 Graphical representation of R123 accumulation ratio in MCF-7/400nMPac and MCF-7/120nMVinc cell lines by use of MDR reversal agents.

Reversal of MDR is an area of intense research. The fluorescent activity ratios (FAR) are demonstrated in Figure 3.43, 3.44. The fluorescent activity ratios indicate that all of the applied compounds were effective as P-gp modulators except a flavonoid, chrysin. Also the compounds except robinin and cinnamylidene ketone compounds are effective MRP1 modulators.

The development of pharmacological agents that reverse drug resistance is a very promising way to overcome the difficulties in chemotherapy. Resistance to anticancer drugs such as vinblastine, vincristine or paclitaxel can be reversed, at least *in vitro*, by a variety of resistance modifying agents. In addition to the interaction of modulators with ABC transporter proteins, their interaction with the lipid bilayers of the plasma membrane may be essential. So, membrane lipids can be regarded as one of the targets for MDR reversing agents (Kars *et al*, 2008). Most of the MDR reversing compounds are soluble in lipids and they may also exert an influence on

the physical properties of lipid bilayers. Most of them can alter membrane fluidity and increase membrane permeability (Drori *et al*., 1995; Callaghan *et al*., 1993). Here it is supposed that changes in the lipid membrane can modify the functional conformation of the P-gp or MRP1 protein. Alterations in the physical state of plasma membrane lipids can influence a number of important carrier-mediated processes and they appear to be important factors modulating efflux pump systems (Shin *et al*., 2006). The different degrees of modulator effects of these compounds in sublines adapted to paclitaxel and vincristine may be due to differences in cell membrane structure, in P-gp conformations in the sublines and also due to other drug resistance mechanisms developed in the sublines at different levels. Based upon transport inhibition hypothesis, the MDR modulators can bind into trans-membrane domains on three different ways, where the drug transporter shifts electrical charges while going through transporter cycle. The transporter related electric currents can be modified after incorporation of resistance modifiers into the P-glycoprotein, MRP1 or into the membrane bilayer around the proteins (Molnar *et al*., 2006). Different levels of modulator effects may also have resulted from modulation of electric charges and different modifications in membrane bilayer.

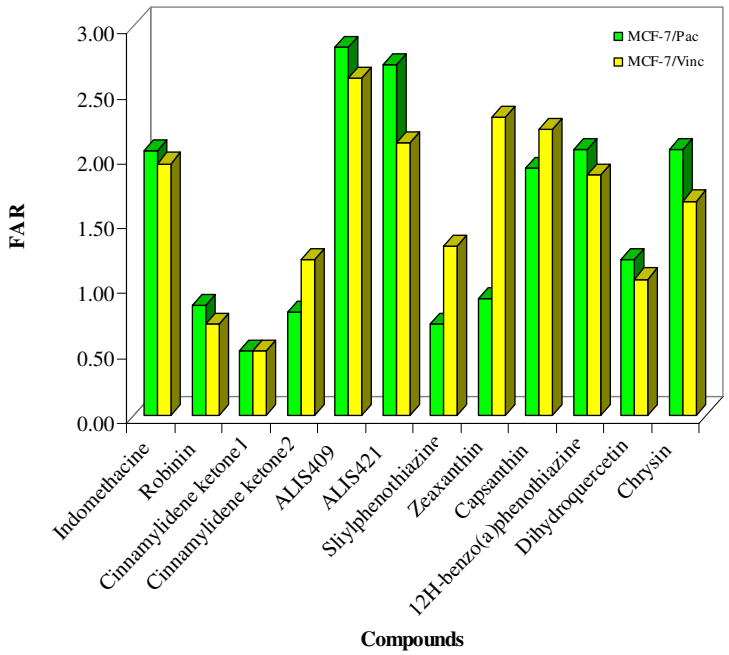


Figure 3.44 Graphical representation of CF accumulation ratio in MCF-7/400nMPac and MCF-7/120nMVinc cell lines by use of MDR reversal agents.

According to literature, phenothiazines can be used as MDR reversal agents as a P-gp suppressor (Motohashi *et al*., 2001), which supports the results obtained with promethazine. It was proved that, some flovanoids (Zhang *et al*., 2004) and carotenoids exhibit anticarcinogenic and resistance modulating effects by modification of lipid bilayer in which P-gp is embedded and the transport activity of P-gp can slow down depending on the conformational changes induced by resistance modifiers (Molnar *et al*., 2006). The results also suggest that capsanthin and zeaxanthin, which are carotenoids, are good natural MDR modulators with their reasonable antiproliferative effects when combined with anticancer drugs. Chrysin was previously proven as the most potent BCRP inhibitor, producing significant increases in mitoxantrone accumulation in BCRP overexpressing MCF-7 cell line (Zhang *et al*., 2004). MCF-7/400nMPac and MCF-7/120nMVinc cells are BCRP negative, so it may be proposed that chrysin is not an inhibitor for P-gp activity which is also supported by the results.

3.8. Combined Application of MDR Modulators with Anticancer Drugs

The IC_{50} values of the modulating agents on the resistant cell lines were determined from proliferation curves and the results are listed in Table 3.19. Concerning these values, the modulating agents and were applied in combination with paclitaxel or vincristine and the effects on sublines were studied separately. The results are represented in Table 3.20.

Table 3.19 IC_{50} values (μM) that were calculated from proliferation curves, resistant cell line vs reversal agents.

Compounds	MCF-7/400nMPac	MCF-7/120nMVinc
Verapamil	100.37	112.68
Promethazine	48.33	54.56
Cinnamylidene-1	182.53	115.38
Cinnamylidene-2	80.41	41.01
ALIS409	11.58	8.06
ALIS421	5.65	4.98
Zeaxanthin	26.95	50.37
Capsanthin	20.30	35.37

The application of anticancer drugs with MDR modulators may be easily acceptable for clinicians under controlled conditions. The results show that, paclitaxel affected in synergism or additive antiproliferative effect with seven compounds except promethazine. Additionally, vincristine and combination of six components exerted synergistic and additive antiproliferative effects. On the contrary, ALIS 409 and capsanthin did not enhance antiproliferative effect when applied in combination with vincristine to the MCF-7/120nMVinc subline.

The application of anticancer drugs with MDR modulators may be acceptable if the drug interaction is synergistic and additive, but not acceptable if the interaction is antagonistic. Synergism is a positive interaction, that is, combined effect of two compounds is significantly greater than expected results based on their independent effects when they are used separately. Antagonism is a negative interaction, that is, combined effect of two drugs being examined is significantly less than their independent effects when they are measured separately.

So accordingly, all the drug-modulator combinations, except paclitaxel-promethazine, vincristine-capsanthin and vincristine- ALIS-409 pairs, exerted the significant antiproliferative effects, which makes them candidates as MDR reversal agents in combination therapy of resistant breast cancer (Table 3.20) .Sample curves that demonstrate the growth profile of the cells in combined application of anticancer agents and modulators with synergistic, additive and antagonistic effect are given in Appendix J.

Table 3.20 Effects of resistance modifier and anticancer drug interactions on MCF-7 sublines. *SEM values were derived from the standard errors of the means of at least three FIX values.

Cell lines	**Treatment**	**FIX ± SEM***	**Comments**
MCF-7/			
400nMPac	Paclitaxel (+)		
	Verapamil	0.13 ± 0.04	Synergism
	Promethazine	5.02 ± 1.88	Antagonism
	Cinnamylidene-1	0.45 ± 0.11	Synergism
	Cinnamylidene-2	0.40 ± 0.05	Synergism
	ALIS 409	0.44 ± 0.04	Synergism
	ALIS 421	0.49 ± 0.02	Synergism
	Capsanthin	0.88 ± 0.09	Additive
	Zeaxanthin	0.83 ± 0.088	Additive
MCF-7/			
120nMVinc	Vincristine (+)		
	Verapamil	0.62 ± 0.14	Additive
	Promethazine	0.61 ± 0.06	Additive
	Cinnamylidene-1	0.17 ± 0.02	Synergism
	Cinnamylidene-2	0.44 ± 0.04	Synergism
	ALIS 409	1.94 ± 0.31	Indifferent
	ALIS 421	0.87 ± 0.02	Additive
	Capsanthin	1.96 ± 0.05	Indifferent
	Zeaxanthin	0.90 ± 0.08	Additive

3.9 Reversal of *MDR1* and *MRP1* Gene Expression by Application of MDR Modulating Agents

The reversal *MDR1* and *MRP1* gene expressions were studied by application of promethazine and verapamil at different concentrations for varying time periods. Promethazine and verapamil are MDR modulators and they inhibit P-gp and MRP1 activity on cell membrane.

Figure 3.45 and 3.47 show the band intensities of the RT-PCR products for *β-2 microglobulin* and *MDR1* genes after promethazine treatment in MCF-7/400nMPac and MCF-7/120nMVinc cell lines. The primers were were designed by our group from different exons of the encodinggenes to prevent amplification from DNA if any.

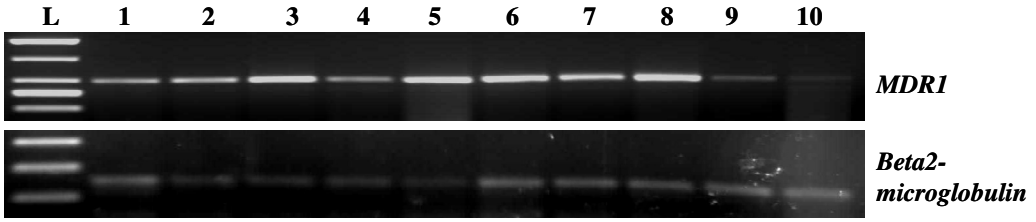


Figure 3.45 RT-PCR products of *MDR1* and *Beta-2 microglobulin* gene amplifications from promethazine applied MCF-7/400nMPac cell line.

Lanes are; L:50bp DNA ladder, 1: no treatment, 2: 1.6 µM promethazine 12h, 3: 1.6 µM promethazine 24h, 4: 1.6 µM promethazine 48h, 5: 1.6 µM promethazine 72h, 6: 4.8 µM promethazine 12h, 7: 4.8 µM promethazine 24h, 8: 4.8 µM promethazine 48h, 9: 4.8 µM promethazine 72h, 10: 9.6 µM promethazine 72h

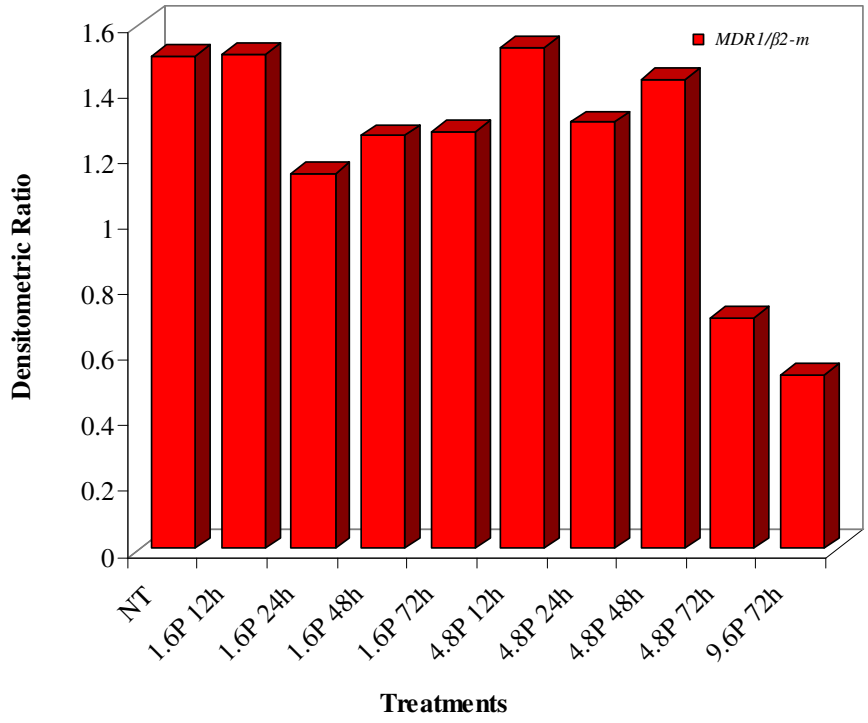


Figure 3.46 Graphical demonstration of *MDR1/β2-m* densitometric ratio for MCF-7/400nMPac cell line upon promethazine treatment

According to densitometric measurements, 4.8 and 9.6 μM promethazine application for 72 hours inhibited the *MDR1* gene expression in MCF-7/400nMPac and MCF-7/120nMVinc cell lines (Figure 3.46, 3.48).

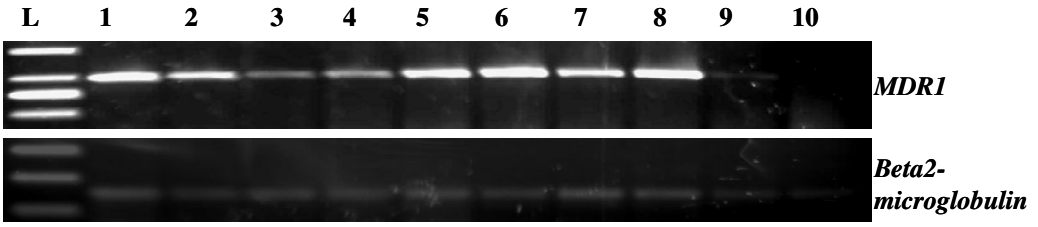


Figure 3.47 RT-PCR products of *MDR1* and *Beta-2 microglobulin* gene amplifications from promethazine applied MCF-7/120nMVinc cell line.

Lanes are; L:50bp DNA ladder, 1: no treatment, 2: 1.6 µM promethazine 12h, 3: 1.6 µM promethazine 24h, 4: 1.6 µM promethazine 48h, 5: 1.6 µM promethazine 72h, 6: 4.8 µM promethazine 12h, 7: 4.8 µM promethazine 24h, 8: 4.8 µM promethazine 48h, 9: 4.8 µM promethazine 72h, 10: 9.6 µM promethazine 72h

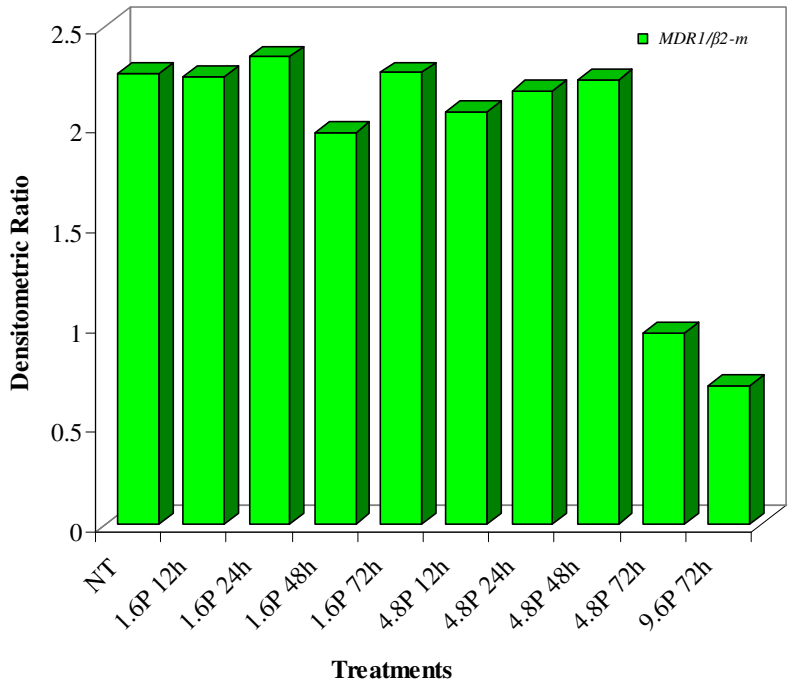


Figure 3.48 Graphical demonstration of *MDR1/β2-m* densitometric ratio for MCF-7/120nMVinc cell line upon promethazine treatment

Promethazine was previously proved as an inhibitor for *MDR1* gene expression in mouse lymphoma cell line (Molnar *et al*., 1998).It was proposed that promethazine was able to bind on promoter region of *MDR1* gene and the gene expression was reduced.

Figure 3.49 and 3.50 show the band intensities of the PCR products for *β-2 microglobulin*, *MDR1* and *MRP1* genes after verapamil treatment in MCF-7/400nMPac and MCf-7/120nMVinc cell lines. According to densitometric measurements, verapamil application for 72 hours downregulated the *MDR1* gene expression in MCF-7/120Vinc and MCF-7/400Pac cell lines 2.0 and 1.6 fold respectively (Figure 3.51). On the other hand, verapamil application for 72 hours did not inhibit the *MRP1* gene expression significantly in MCF-7/120nMVinc and MCF-7/400nMPac cell lines (Figure 3.52).

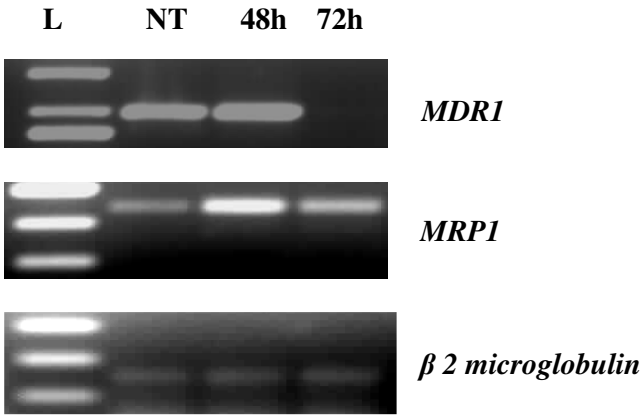


Figure 3.49 Agarose gel photograph (2%) for *MDR1, MRP1, β 2 microglobulin* RT-PCR products of MCF-7/400nMPac. L: 50bp DNA ladder, NT: no treatment, 48h verapamil treatment, 72h verapamil treatment.

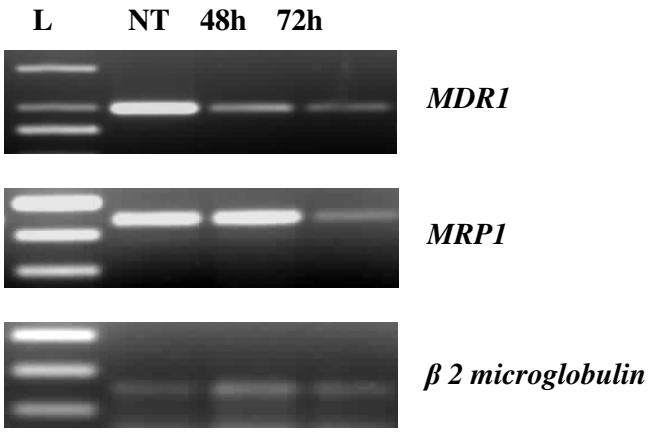


Figure 3.50 Agarose gel photograph (2%) for *MDR1, MRP1, ß 2 microglobulin* RT-PCR products of MCF-7/120nMVinc, L: 50bp DNA ladder, NT: no treatment, 48h verapamil treatment, 72h verapamil treatment.

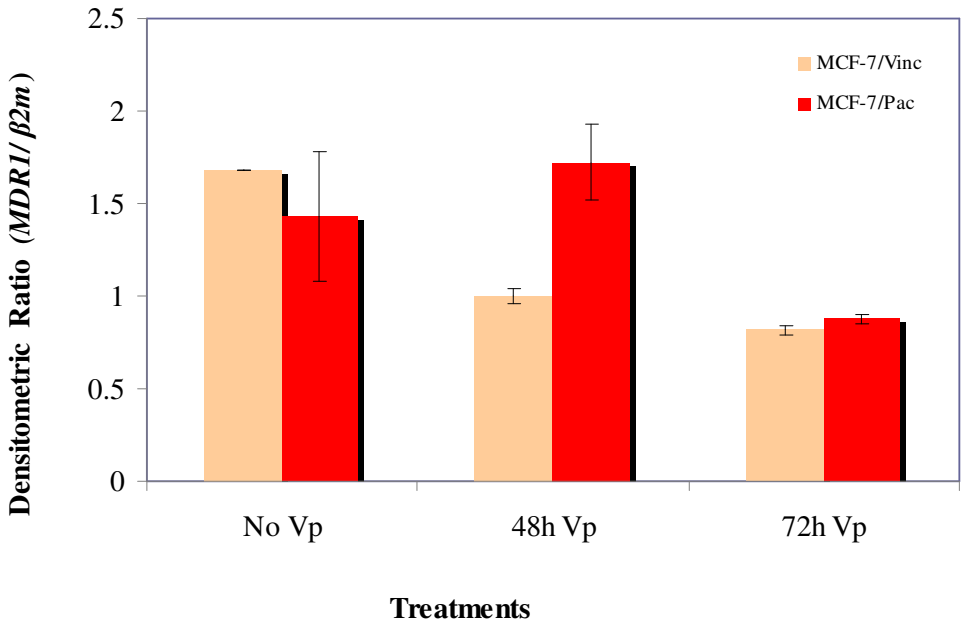


Figure 3.51 Densitmetric ratio graph presents the effect of verapamil (Vp) application on *MDR1* gene expression levels in MCF-7/400nMPac and MCF-7/120nMVinc cell lines.

Verapamil also has P-gp reversal effect by chemical means as represented in previous sections. However *MRP1* gene expression was not affected from verapamil application. So we can also propose verapamil as a reversal agent both as P-gp blocker and as *MDR1* gene inhibitor.

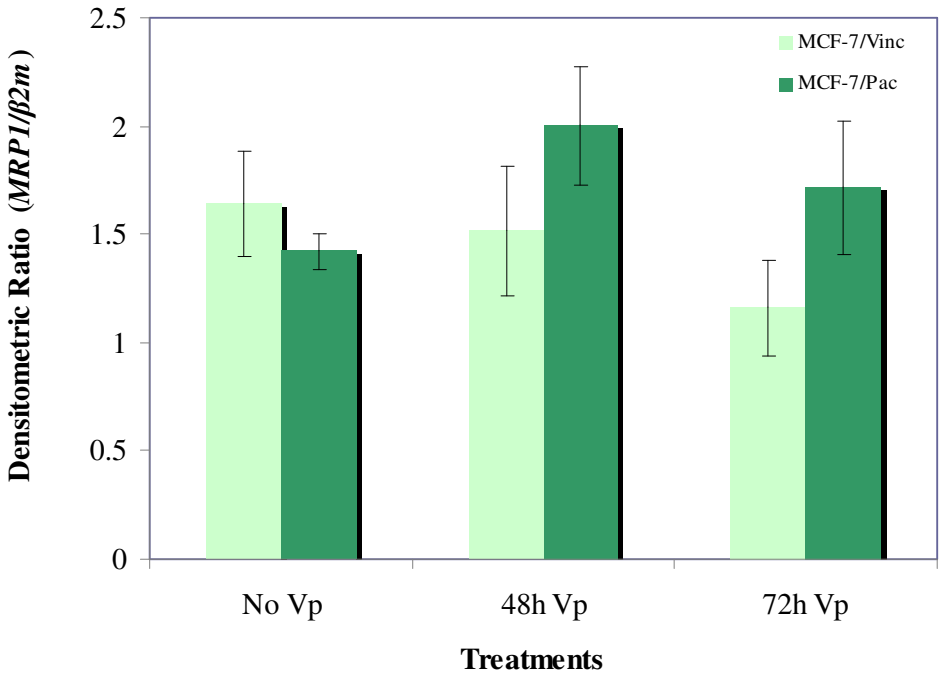


Figure 3.52 Densitmetric ratio graph presents the effect of verapamil (Vp) application on *MRP1* gene expression levels in MCF-7/400nMPac and MCF-7/120nMVinc cell lines.

3.10 Investigation of Cross Resistance Development to Various Anticancer Agents and Gamma Irradiation in MCF-7/400nMPac and MCF-7/120nMVinc Cell Lines

3.10.1 Cross Resistance Development to Various Anticancer Agents in MCF-7/400nMPac and MCF-7/120nMVinc Cell Lines

Development of cross-resistance to different anticancer agents (other than respective selective agent) was also investigated for each resistant subline. The cross resistance level of MCF-7/400nMPac to vincristine and tamoxifen is 13 fold and around 2 fold respectively. However MCF-7/400nMPac subline did not develop statistically significant cross-resistance to doxorubicin and ATRA. MCF-7/120nMVinc cell line developed 48 fold cross resistance to paclitaxel which is higher than the resistance against vincristine (30 fold) itself. This cell line is also cross-resistant to doxorubicin and tamoxifen about 6 and 2 folds significantly; however did not developed cross-resistance to ATRA (Table 3.21).

Table 3.21 Antiproliferative effects of anticancer drugs on sensitive and resistant MCF-7 cell lines and resistance indices.

Cell Line	Anticancer Drug	IC_{50} (μM) ± SEM*	Resistance Index
MCF-7/S	Paclitaxel	2.12 ± 0.23	-
	Vincristine	5.45 ± 0.66	-
	Doxorubicin	1.14 ± 0.38	-
	Tamoxifen	6.02 ± 1.30	-
	ATRA	40.78±9.42	**-**
MCF-7/Pac	Paclitaxel	317.94± 0.20	**149.98***
	Vincristine	71.64 ± 11.59	13.14*
	Doxorubicin	1.64 ± 0.33	1.44
	Tamoxifen	15.06 ± 0.75	2.5*
	ATRA	36.76 ± 9.05	0.90
MCF-7/Vinc	Vincristine	162.29± 2.19	**29.78***
	Paclitaxel	101.99 ± 13.85	48.11*
	Doxorubicin	6.98 ± 1.64	6.12*
	Tamoxifen	12.24 ± 0.75	2.03*
	ATRA	54.82 ± 8.14	1.34

*SEM (standard error of means) values were derived from the IC_{50} values of three independent experiments. * Represents significant difference between groups with $p<0.05$.

The results are consistent with literature that the breast cancer cells developing resistance to an anticancer drug may also develop cross-resistance to another agent (Guo *et al*., 2004). In clinic, development of cross-resistance affects the success of chemotherapy and some patients become refractory to treatment (Marty *et al*., 1997; Adamo *et al*., 2007). In resistant model cell lines, development of cross-resistance to another anticancer agent is a frequently observed situation *in vitro* (Schafer *et al*., 2003). The paclitaxel and vincristine resistant sublines express *MDR1* and *MRP1*

genes so it is natural for the sublines to develop cross-resistance to P-gp and MRP1 substrates (paclitaxel, doxorubicin and vincristine). Senstive MCF-7 cell line may have become resistant to different drugs through diverse mechanisms, so that the sublines may have responded differently. However tamoxifen and ATRA are not the substrates of efflux pumps so this fact may be the major cause for the sublines developing no or very low level of cross resistance to these agents. Retinoids are known to suppress carcinogenesis in various epithelial tissues. Among them, ATRA is recognized as active retinoid (Choi *et al*., 2006). However, despite the known anticarcinogenic activity of ATRA, it exhibits its short plasma half-life during repeated oral administration due to the acute retinoid resistance (Choi *et al*., 2006). Drug resistance to tamoxifen is a significant clinical problem but the mechanism through which this occurs was not well understood (Riggins *et al*., 2006). Approximately 30% of estrogen receptor α-positive breast cancers do not respond to tamoxifen treatment. In addition, the majority of tumors that initially respond to treatment develop resistance over time (Schafer *et al*., 2003; Riggins *et al*., 2006).

3.10.2 Cross Resistance Development to Gamma Irradiation in MCF-7/400nMPac and MCF-7/120nMVinc Cell Lines

The sensitive paclitaxel and vincristine resistant cell lines MCF-7/S, MCF-7/400nMPac and MCF-7/120nMVinc were treated with 200 and 800 cGy γ irradiation. Non-treated cells served as control for the experiment. According to Figure 3.53, cell proliferation decreased as a function of increased radiation dose in sensitive and resistant cell lines with similar proliferation profiles.

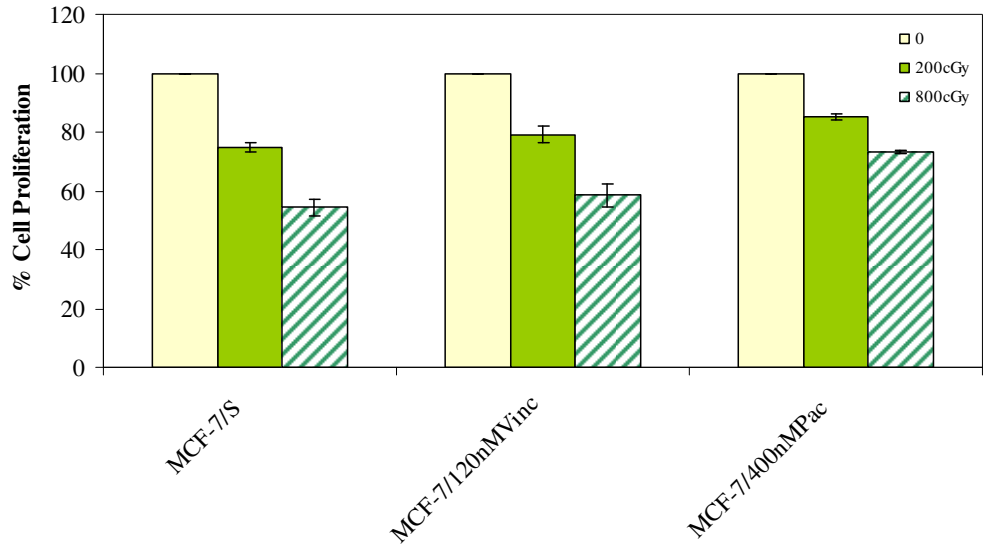


Figure 3.53 Cell proliferation profile of MCF-7/S, MCF-7/120nMVinc and MCF-7/400nMPac with increased γ irradiation.

According to IC_{50} values that were driven from % proliferation versus radiation dose graph, MCF-7/400nMPac is 1.59 fold cross resistant to γ irradiation but MCF-7/120nMVinc did not develop cross resistance (Table 3.22).

Table 3.22 IC_{50} (cGy) values and resistance indices of the cell lines

Cell Line	**IC_{50} (cGy)**	**Resistance index**
MCF-7/S	967	-
MCF-7/400nMPac	1534	1.59
MCF-7/120nMVinc	967	1.00

Breast cancer is the most common and frequently diagnosed cancer at a median age in women (Ries *et al*, 2008). Even though substantial advances in therapy and diagnosis have enhanced the survival rate of patients with breast cancer, late recurrences of the disease account for deaths from breast cancer (Widakowich *et al*., 2007) So, further studies are needed to optimize therapeutic applications in patients with metastatic breast cancer.

In clinical applications, development of cross-resistance affect the success of chemotherapy and some patients become refractory to treatment (Marty *et al*., 1997; Adamo *et al*., 2007). This part of the work demonstrates dose dependent cytotoxic effect of γ-radiation on drug resistant breast cancer cells and development of cross resistance in MCF-7/400nMPac cell line to irradiation.

When the microarray data were re-considered, the cell cycle regulatory genes *CDKN2A, CCNE1, CCNA2* were induced in MCF-7/400nMPac and MCF-7/120nMVinc. However, *CDK6* and *CHEK1* were only induced in MCF-7/400nMPac. *CDKN2A* is the gene encoding a cyclin dependent kinase inhibitor and it was significantly upregulated (71 fold) in MCF-7/400nMPac. In a previous report, levels of *DDB2* (involved in DNA repair) and *CDKN1A* (cell cycle regulator- Cyclin dependent kinase inhibitor 1A) genes were significantly induced by irradiation in breast cancer tissue samples (Helland *et al*., 2006). The respective study proposed that these genes were modulated by p53 and altered the radiosensitivity/resistance profiles in tissues following radiotherapy. In addition, both *DDB2* and *CDKN1A* were previously identified as radiation-induced in peripheral white blood cells (Amundson *et al*., 2004) and fibroblasts (Rodningen *et al*., 2005). In the MCF-7/400nMPac there is not any significant alteration in *DDB2* and *CDKN1A* genes in MCF-7/400nMPac however there are numerous cell cycle regulatory genes that were downregulated or upregulated in the resistant cells. Differential expression of the cell cycle regulatory genes may have affected the radioresistance profile of the cells.

According to the results, MCF-7/400nMPac cells are resistant to γ-radiation and therefore the treatment of the paclitaxel resistant breast cancer with radiotherapy may

not be useful. On the other hand MCF-7/120nMVinc cells did not develop cross resistance to irradiation. Therefore the γ-radiation may show its cytoreductive effect on vincristine resistant breast cancer cells. If it was considered that vincristine resistant breast cancer cell line as model to clinically resistant breast carcinoma, it may be stated that γ-radiation may be a therapeutic protocol for metastatic and resistant breast cancer patients. However this finding should be confirmed with drug resistant breast cancer patients that were exposed to radiation after chemotherapy.

3.11 Combined Application of Anticancer Agents to MCF-7/400nMPac and MCF-7/120nMVinc Cell Lines

Results represent that, combinations of paclitaxel with doxorubicin exerted significant synergic antiproliferative effects on MCF-7/400nMPac. However doxorubicin combination with vincristine did not interact effectively on the MCF-7/120nMVinc. Tamoxifen had significant synergic effects in combined applications with vincristine. It also had additive antiproliferative effects when applied with paclitaxel. According to results (Table 3.23), paclitaxel, has shown indifferent interaction with retinoic acid while vincristine was antagonistic.

Table 3.23 Effects of anticancer drug interactions on MCF-7 sublines. *SEM values were derived from the standard errors of the means of at least three FIX values.

Cell Line	Anticancer Drug	FIX ± SEM*	Interaction
MCF-7/Pac	Doxorubicin	0.43 ± 0.07	Synergistic
	Vincristine	0.92 ± 0.04	Additive
	Tamoxifen	0.84 ± 0.14	Additive
	ATRA	1.55 ± 0.39	Indifferent
MCF-7/Vinc	Paclitaxel	0.70 ± 0.18	Additive
	Doxorubicin	1.60 ± 0.51	Indifferent
	Tamoxifen	0.44 ± 0.08	Synergistic
	ATRA	3.56 ± 0.64	Antagonistic

Modern medicine uses defined ingredients or compositions, optimizes treatment schedules and combination proportions, refines routes of drug delivery, and combines different modalities of treatments (Chou *et al*., 1994). The results obtained show that tamoxifen can be used in combination with paclitaxel, and vincristine. Paclitaxel and tamoxifen were previously applied in combination *in-vivo* (Fine *et al*., 2006). Also effective combinations of paclitaxel with doxorubin or vincristine *in vitro* may be good models for *in vivo* combined chemotherapy. Different sensitivities of the sublines to the anticancer agents and to their combined applications can be explained by cellular differences in the resistance mechanisms and differences in the expression levels of the resistance related genes in the sublines. Effective antiproliferative effects of paclitaxel-doxorubicin, paclitaxel-vincristine, paclitaxel-tamoxifen, vincristine-tamoxifen pairs may also be related to the independent effects of anticancer agents on different pathways in the resistant sublines and their combined cytotoxic effects.

CHAPTER 4

CONCLUSION

The term multidrug resistance was defined as "cellular resistance to anticancer agents due to a decreased concentration of active drug at the target sites that is caused by increased metabolism or altered transport or routing of the active drug species" (Broxterman *et al*., 1995). Multidrug resistance has been mostly studied as a major obstacle to successful cancer chemotherapy, whereby tumor cells become resistant to a range of diverse drugs after exposure to a single cytotoxic agent.

In this study paclitaxel and vincristine resistant breast cancer models were generated from the original model cell line MCF-7. The developed sublines MCF-7/400nMPac and MCF-7/120nMVinc are resistant to their selective agents 150 and 30 fold respectively. Meanwhile, MCF-7/400nMPac cells developed cross resistance to vincristine, tamoxifen and γ irradiation to a lesser extend. MCF-7/120nMVinc also developed cross resistance to paclitaxel, doxorubicin and tamoxifen. This information may be used in clinic chemotherapy to suggest new therapy regimens for paclitaxel or vincristine resistant breast cancer patients. The paclitaxel and vincristine resistant cells also have different growth profiles from their parental cell line MCF-7/S with doubling times 53 and 54 hours respectively. Here it can be proposed that MCF-7/400nMPac and MCF-7/120nMVinc are differentiated derivatives of the original MCF-7/S with different growth profiles in their selective media or in some other therapeutic agents. So it is obvious that MCF-7/400nMPac and MCF-7/120nMVinc are "multiple drug resistant", that is sublines are resistant to more than one kind of chemotherapeutic drug.

Modern medicine uses defined ingredients or compositions, optimizes treatment

schedules and combination proportions and combines different modalities of treatments. The results obtained from combined applications of anticancer agents to MCF-7/400nMPac and MCF-7/120nMVinc showed that tamoxifen can be used in combination with paclitaxel, and vincristine. Also effective combinations of paclitaxel with doxorubin or vincrsitine *in vitro* may be good models for *in vivo* combined chemotherapy. Effective antiproliferative effects of paclitaxel-doxorubicin, paclitaxel-vincristine, paclitaxel-tamoxifen, vincristine-tamoxifen pairs may be related to the independent effects of anticancer agents on different pathways in the resistant sublines and their combined cytotoxic effects. These effective combinations may be suggested in clinical chemotherapy applications. Here the most powerful combination seems to be paclitaxel-doxorubicin pair because paclitaxel resistance caused development of cross resistance to vincristine and tamoxifen *in vitro*. Also vincristine resistant cells developed cross resistance to paclitaxel and vincristine. So their combinations will also not be successful in clinic.

Several sets of experiments were performed to answer the question what the molecular mechanisms of paclitaxel and vincristine resistance in breast cancer are. During the research the genes that have altered expression levels were also clarified. As a result, some of the mechanisms that are the reasons or the outcomes of paclitaxel and vincristine resistance were identified.

According to the results, overexpression of *MDR1* gene seems to be the most dominating mechanism causing the multidrug resistance phenotype in MCF-7/400nMPac and MCF-7/120nMVinc. Additionally upregulation of the gene encoding detoxifiying GST enzyme *GSTP1* is one of the mechanisms of drug resistance in MCF-7/120nMVinc and MCF-7/400nMPac.

An additional mechanism of resistance is the selective expression of *beta-tubulin* isotypes. Interestingly, overexpression of gene encoding tau kinase was observed in paclitaxel resistant MCF-7 cell line but not in vincristine resistant cell line. So accordingly it can be proposed that overexpression *TTBK* may be associated with paclitaxel resistance but not with vincristine resistance in breast cancer. Expression

levels of microtubule associated genes were upregulated except *TUBD1* and *MAP7* in resistant sublines. As a conclusion, alterations in microtubule dynamics may have changed the affinity of drugs to microtubules and caused drug resistance in these sublines.

Mutational analysis revealed MCF-7/120nMVinc cells acquired His-179 → Pro-179 mutation in class I β-tubulin. This mutation is near GTP binding site (β173) and possibly alters microtubule stability. This amino acid alteration may be one of the mechanisms of drug resistance in MCF-7/120nMVinc cells.

Some genes that are involved in apoptotic pathways, that encode growth factors and receptors, that encode tumor micro environment related proteins and that are involved in metastaci property and cell cycle regulation of the cells have some roles in increased proliferation ability of resistant cells. The genes which have significant correlation with multiple drug resistance are listed in Table 4.1 and 4.2. Then the genes were put in to schemes and some pathways were proposed for the mechanisms and outcomes of paclitaxel and vincristine resistance in breast cancer (Figure 4.1, 4.2). These relations and correlations may be used to predict the response of breast cancer patients at molecular level and to apply effective chemotherapeutic regimens.

Table 4.1 The genes which are supposed to take role in molecular mechanisms of drug resistance.

Gene Symbol	**Description**
ABCB1	ATP-binding cassette, sub-family B (MDR1)
GSTP1	glutathione S-transferase pi
MAP1B, MAP1A,MAP4	Microtubule-associated proteins
TUBB6, TUBB2, TUBB4	Tubulin isotypes
TTBK2	Tau tubulin kinase 2
TNFRSF6B, 10D	tumor necrosis factor receptor superfamily
DAPK1	death-assoc protein kinase1
PDCD2/4/6/8/10	programmed cell death
TGFB1I1	transforming growth factor beta 1
FGF2/18	fibroblast growth factor 2/18
PDGFC/D	platelet derived growth factors
EGFR	Epidermal growth factor receptor
IL6	interleukin 6 (interferon, beta 2)
TIMP4	TIMP metallopeptidase inhibitor 4
RAB oncogenes	RAB oncogenes, member RAS oncogene family

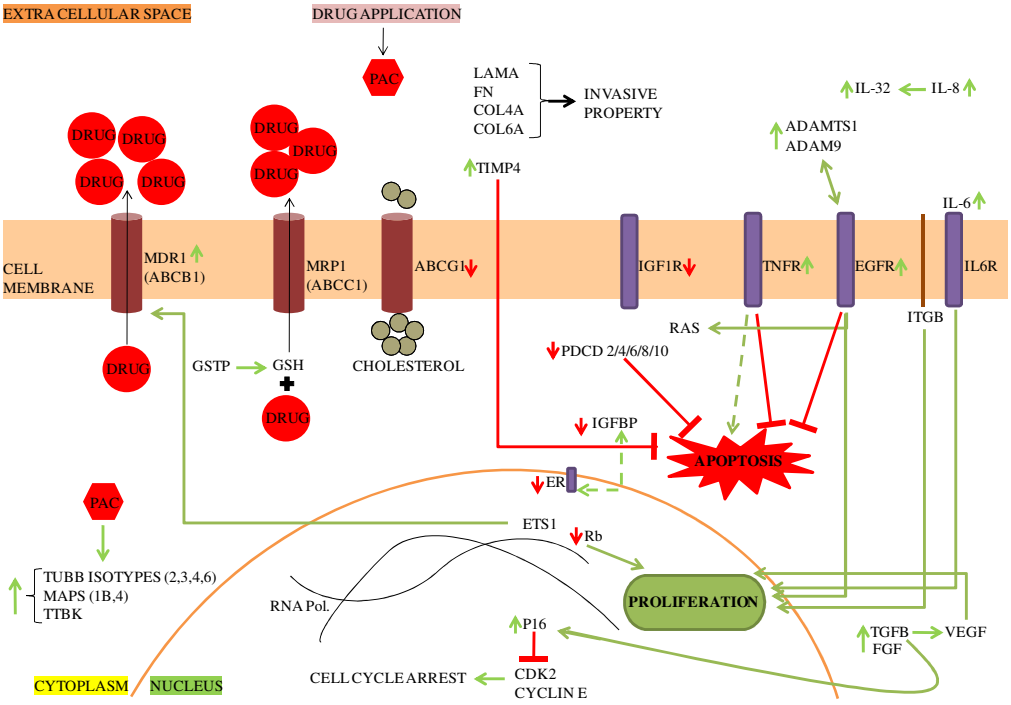


Figure 4.1 Major signatures of paclitaxel resistance in MCF-7 cells. Red arrows and line represent downregulations or inhibitions, green arrows and lines indicate the upregulations and inductions.

Table 4.2 The genes which are supposed to have altered expression levels during MDR phenotype development (outcomes of MDR).

Gene Symbol	Description
ABCB4	ATP-binding cassette, sub-family B MDR 4
ABCG1	ATP-binding cassette, sub-family G 1
CYBRD1	cytochrome b reductase 1
IGFBP2	insulin-like growth factor binding protein 2
ESR1	estrogen receptor 1
IL8/32	interleukin 8/32
MMP1/9	matrix metallopeptidase 1/9 (interstitial collagenase)
LAMA1	laminin, alpha 1
FN1	fibronectin 1
COL4A2/6A1/4A1/12A1	Collagen
ADAMTS1/TS6/TS3	ADAM metallopeptidase (TS)
ADAM9	ADAM metallopeptidase
ITGB1	integrin, beta 1
CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
CCNE1/A2	cyclin E1/A2

Table 4.2 continues

Gene Symbol	Description
CDK6	cyclin-dependent kinase 6
RB1	retinoblastoma 1 (including osteosarcoma)
ETS1	erythroblastosis virus E26 oncogene

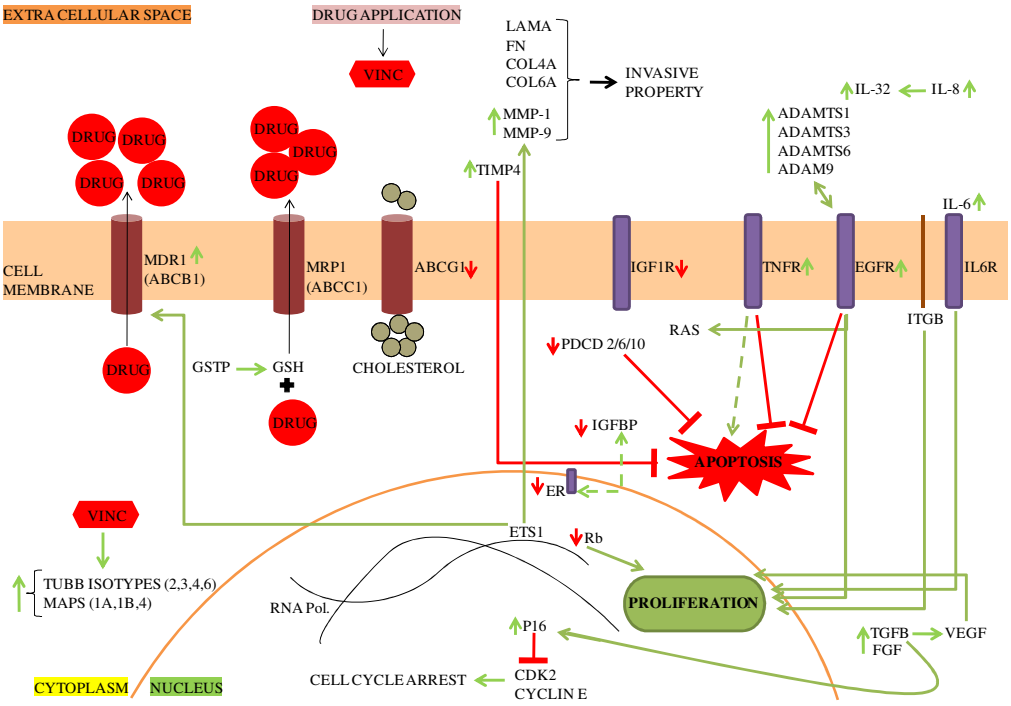


Figure 4.2 Major signatures of vincristine resistance in MCF-7 cells. Red arrows and line represent downregulations or inhibitions, green arrows and lines indicate the upregulations and inductions.

According to drug accumulation assay results, it is apparent that the parental MCF-7/S cells express inherently the *MRP1*, but not the *MDR1* gene. The rhodamine-123 efflux activity of P-gp is higher than the carboxyfluorescein efflux activity of MRP1 in the drug resistant sublines.

Reversal of P-gp and MRP1 mediated resistance was also the other objective of this study. Alterations in the physical state of plasma membrane lipids can influence a number of important carrier-mediated processes and they appear to be important factors modulating efflux pump systems. Drug efflux of resistant cell lines through P-gp and MRP1 was inhibited by MDR modulating agents successfully. Combination tests were also confirmed that combined application of effective modulators may be possible in drug resistant mammary carcinoma cell line. *MDR1* gene was also inhibited by application of promethazine and verapamil. So the developed resistance due to *MDR1* overexpression may be successfully inhibited by chemical treatment of the resistant cell lines.

For clinical applications, the genes which are suggested as signatures of paclitaxel and vincristine resistance here, may be used as targets to inhibit proliferative and metastatic nature of resistant breast cancer cells.

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APPENDIX A

PRESCRIPTIONS FOR THE SOLUTIONS USED IN DEPC TREATMENT AND GUANIDUM THIOCYANATE PHENOL CHLOROFORM SINGLE STEP RNA ISOLATION

DEPC (diethylpyrocarbonate)

0.01 % (v/v) DEPC in dH_2O

All the materials (tubes, tips etc) are washed with DEPC treated water and DEPC is let evaporate overnight in fume hood. The remaining water is discarded and the materials are autoclaved.

Guanidium Thiocyatane Solution, pH 7

4 M Guanidium thiocyatane (9.45 g)

25 mM Sodium citrate, pH 7 (0.147 g)

0.5 % (w/v) N- Lauroylsarcosine (0.1 g)

The solution is completed to 20 mL with DEPC treated dH_2O and should be filtered through coarse filter.

0.1 M β-mercaptoethanol (4 µL to 596 µL RNA isolation solution during RNA experiment)

Sodium Acetate, 2M, pH 4, (1.64 g /10mL DEPC water)

Citrate Saturated Phenol, pH 4.3

Sodium citrate buffer , 0.1 M, pH 4.3

Liquid phenol (sodium citrate saturated liquid phenol, pH 4.3)

The solution should be stored at 2-8 ^{o}C and kept away from light.

EtOH , 95 % (v/v) and 70 % (v/v)

APPENDIX B

SOLUTIONS REQUIRED FOR PREPARATION OF TAE BUFFER AND AGAROSE GEL IN TAE BUFFER SYSTEM

TAE Buffer, 50X

Tris-Base (242 g)

Glacial acetic acid (57.1 mL)

$Na_2EDTA.\ 2\ H_2O$ 0.5 M, pH 8 (100mL)

The solution is completed to 1 L with dH_2O.

Agarose Gel

2 % (w/v) or 1 % (w/v) agarose in TAE buffer for PCR products or RNA/ DNA samples respectively.

EtBr solution

10 mg EtBr is dissolved in 1 mL d H_2O. The solution is kept at 2-8ºC and away from light. (7 µL of EtBr solution is added in 100 mL warm agarose gel.)

APPENDIX C

SOLUTIONS REQUIRED FOR MOPS (MORPHOLINOPROPANESULPHONIC ACID) BUFFER SYSTEM AND DENATURATING AGAROSE GEL

MOPS buffer (10X)

0.2 M MOPS (10.46 g)

0.05 M NaOAc (1.025 g)

0.01 M EDTA (2.92 g)

The solution is completed to 250 mL with dH_2O, pH is adjusted to 7 and sterilized through filter.

Denaturing buffer

1X MOPS buffer

Reduced formaldehyde denaturating gel

1 % Agarose

1X MOPS

3 % (v/v) 37 % formaldehyde

Sample loading solution (for 5 µL RNA)

1X MOPS buffer (2 µL)

5.5 % (v/v) 37% formaldeyde (3 µL)

50 % (v/v) formamide (10 µL)

RNA (5 µL)

Loading dye (3 µL)

APPENDIX D

SOLUTIONS REQUIRED FOR TARGET HYBRIDIZATION

MES (2-(N-morpholino)ethanesulfonic acid) stock buffer, 12X

1.22 M MES

0.89 M [Na^+]

Hybridization buffer, 2X

200 mM MES

2 M [Na^+]

40 mM EDTA

0.02 % Tween-20

The solutions should be stored at 2-8 ^oC and kept away from light.

APPENDIX E

MICROARRAY WASHING- STAINING SOLUTIONS

SSPE, 20X

3 M NaCl

0.2 M NaH_2PO_4

0.02 M EDTA

Stain buffer, 1X

100 mM MES

1 M [Na^+],

0.05 % Tween-20

Wash A buffer (1000 mL, 200 µL / array)

6X SSPE

0.01 % Tween-20 (Pierce Chemical)

The solution should be filtered through a 0.2 µM filter.

Wash B buffer (1000mL, 200 µL / array)

100 mM MES

1 M [Na^+]

0.01 % Tween-20

The solution should be filtered through a 0.2 µM filter, stored at 2-8 ^oC and dark.

SAPE solution mix (for one array)

1X stain buffer

2mg/mL BSA (Invitrogen)

10 µg/mL (v/v) SAPE (R-Phycoerythrin Streptavidin, Molecular Probes)

Deionized H_2O (complete to 1200 µL)

Antibody solution (for one array)

1X stain buffer

2 mg/mL BSA

10 mg/mL Goat IgG (Sigma, Reagent Grade)

3 µg/mL biotinylated antibody (Vector Laboratories)

Deionized H_2O (complete to 600 µL)

APPENDIX F

SOLUTIONS REQUIRED FOR TOTAL PROTEIN (CELL LYSATE) EXTRACTION

Lysis Buffer (400 µL/sample)

1 M Tris-HCl, pH 7.5, (4 µL)

1 M $MgCl_2$, (0.4 µL)

0.5 M EDTA, (0.8 µL)

5 % CHAPS, (40 µL)

87 % Glycerol, (46 µL)

100 mM PMSF, (4 µL)

0.1 mg/mL Aprotinin, (8 µL)

0.2 mg/mL Pepstatin, (2 µL)

0.5 M β-mercaptoethanol, (5 µL)

dH_2O (290.8 µL)

Solutions Required for Bradford Assay and Procedure

5X Bradford reagent

Commasie Brillant Blue G-250 (500 mg)

95 % EtOH (250 mL)

85 % (w/v) phosphoric acid, (500 mL)

Total solution is completed to 1 Liter,

The solution should be filtered through coarse filter and kept at 2-8^{o}C.

1mg / mL BSA

Procedure

5X Bradford reagent is diluted to 1X and filtered. To draw a standard curve, the dilutions represented in Table F1 are prepared. The dilutions are mixed by vortexing and incubated for 10 minutes at room temperature. Absorbances of the tubes are read at 595 nm. The standard curve is plotted as OD_{595nm} vs BSA concentration (µg/mL). The concentration of unknown samples are calculated from the equation of standard curve (y = mx+n) taking unknown protein concentration x and OD_{595nm} of the total lysates as y. Finally the x value is multiplied with dilution factor (volume of unknown in the Bradford reagent/ total volume (5500 µl)) to obtain the actual protein concentration. The standard deviation is calculated from triplicate values for each sample.

Table F.1 Chart of BSA dilutions for preparation of standart curve.

Tube # (prepared as triplicates)	dH_2O (µl)	BSA , 1 mg/mL (µl)	1X Bradford Reagent (mL)
Blank	500	0	5
1	495	5	5
2	490	10	5
3	480	20	5
4	470	30	5
5	450	50	5
6	420	80	5

APPENDIX G

SDS- POLY ACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOT SOLUTIONS

G.1 SDS-PAGE solutions

40 % Gel Solution

Acrylamide (29.2 g)

Biscrylamide (0.8 g)

Volume is completed to 75 mL with dH_2O.

Stacking gel buffer

Tris base (6 g)

pH is adjusted to 6.8 and volume is completed to 100 mL with dH_2O.

Separating gel buffer

Tris base (18.15 g)

pH is adjusted to 6.8 and volume is completed to 100 mL with dH_2O.

10% SDS

1 g SDS is dissolved in 10 mL dH_2O.

10% (w/v) Ammonium persulfate

100 mg APS is dissolved in 1 mL dH_2O. The solution is freshly prepared.

10X running buffer

0.25 M Tris base (15 g)

1.92 M Glycine (72 g)

Volume is completed to 100 mL with dH_2O.

1X running buffer

10X runnig buffer (70 mL)

SDS (0.1g)

Volume is completed to 700 mL with dH_2O.

G.2 Gel preparation

Table G.1 Ingredients of stacking and separating gels.

Reagents	Seperating gel (7.5 %)	Stacking gel (4 %)
Gel solution	11.25 mL	1.95 mL
dH_2O	21.795 mL	9.15 mL
Seperating buffer	11.25 mL	------
Stacking buffer	-----	3.75
10 % SDS	0.45 mL	0.15 mL
Total	44.745 mL	15 mL
10 % APS	0.225 mL	0.075 mL
TEMED	0.0225 mL	0.015 mL

Solution required for staining SDS PAGE

Commasie brillant blue R (0.2 g)

Acetic acid (24 mL)

MetOH (100 mL)

Volume is completed to 200 mL withdH_2O.

G.3 Preparation of solutions for Western Blot

Blot buffer

25 mM Tris base (12.144 g)

192 mM glycine (57.65 g)

20 % (v/v) MetOH (800 mL)

The solution is completed to 4 L with dH_2O.

TBST equilibration buffer

500 mM NaCl (29.22 g)

20 mM Tris-HCl (3.16 g)

0.05 % (v/v) Tween (0.5 mL)

The solution is completed to 1 L with dH_2O.

Blocking buffer

5% (w/v) non fat dry milk (15 g)

The solution is completed to 300 mL with TBST buffer.

DAB chromogen/substrate solution

Concentrated DAB solution (DBS, Pleasanton CA ; 50 µL)

DAB buffer (DBS, Pleasanton CA ; 1mL)

APPENDIX H

ATTRIBUTES USED IN MICRORARRAY ANALYSIS

Table H.1 Attributes given for microarray samples

Attribute name	Array Design	Experiment Type	Labeling Protocol	Organism	RNA type	Tissue Type	Probe Level Analysis	CEL File
120 VINCRISTINE 2.txt	HG-U133_Plus_2	Drug resistance	IVT labelling	Human	total RNA	Breast epithelial	RMA	120 VINCRISTINE 2.CEL
120 VINCRISTINE I.txt	HG-U133_Plus_2	Drug resistance	IVT labelling	Human	total RNA	Breast epithelial	RMA	120 VINCRISTINE I.CEL
400 PACLITAXEL 1.txt	HG-U133_Plus_2	Drug resistance	IVT labelling	Human	total RNA	Breast epithelial	RMA	400 PACLITAXEL 1.CEL
400 PACLITAXEL 2.txt	HG-U133_Plus_2	Drug resistance	IVT labelling	Human	total RNA	Breast epithelial	RMA	400 PACLITAXEL 2.CEL
NORMAL 1.txt	HG-U133_Plus_2	Drug resistance	IVT labelling	Human	total RNA	Breast epithelial	RMA	NORMAL 1.CEL
NORMAL 2.txt	HG-U133_Plus_2	Drug resistance	IVT labelling	Human	total RNA	Breast epithelial	RMA	NORMAL 2.CEL

Table H.1 continues

Attribute name	Algorithm Name	Strain/ Cell-line	Growth Conditions	Drug Resistance	Conce	Disease Normal	Genetic Characteristic	Sampling Method	Treat type
120 VINCRISTINE 2.txt	Percentile	MCF-7	RPMI 1640, 10% FBS, 2 mM L-glutamine, 1% Gentamycin	Vincristine	120	Cancer	Wild type	Cell pellet	Drug applic
120 VINCRISTINE I.txt	Percentile	MCF-7	RPMI 1640, 10% FBS, 2 mM L-glutamine, 1% Gentamycin	Vincristine	120	Cancer	Wild type	Cell pellet	Drug applic
400 PACLITAXEL 1.txt	Percentile	MCF-7	RPMI 1640, 10% FBS, 2 mM L-glutamine, 1% Gentamycin	Paclitaxel	400	Cancer	Wild type	Cell pellet	Drug applic
400 PACLITAXEL 2.txt	Percentile	MCF-7	RPMI 1640, 10% FBS, 2 mM L-glutamine, 1% Gentamycin	Paclitaxel	400	Cancer	Wild type	Cell pellet	Drug applic
NORMAL 1.txt	Percentile	MCF-7	RPMI 1640, 10% FBS, 2 mM L-glutamine, 1% Gentamycin	Sensitive	0	Cancer	Wild type	Cell pellet	No drug applic
NORMAL 2.txt	Percentile	MCF-7	RPMI 1640, 10% FBS, 2 mM L-glutamine, 1% Gentamycin	Sensitive	0	Cancer	Wild type	Cell pellet	No drug applic

APPENDIX I

TUBB GENE SEQUENCE DATA SHEET OF MCF-7/120NMVINC

Exon 4-1

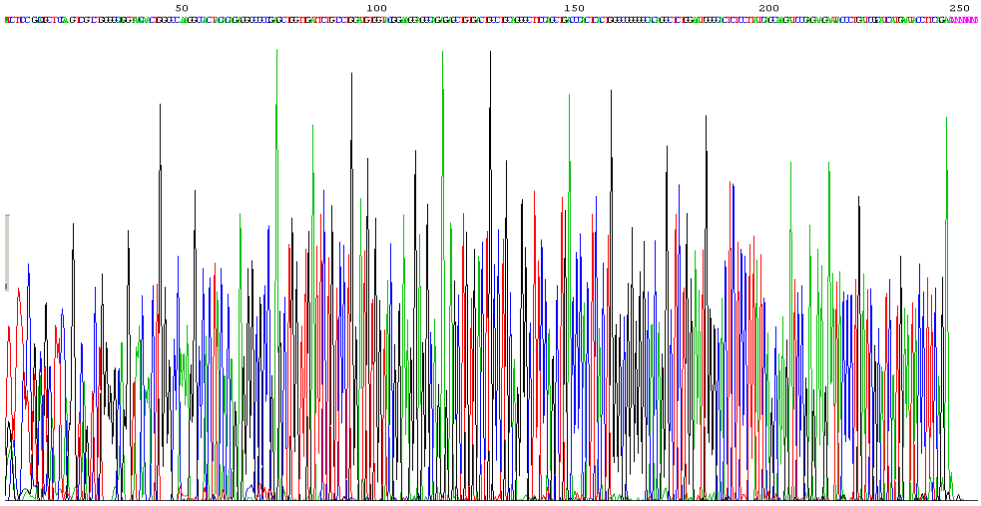

Figure H.1 Sequence data sheet of the first part of 4th exon of *TUBB* gene.

NNNAATCTTCCGACTGCTCCAGTCGTCTGGGGCAGGTAACAACTGGGCCA
AGGCCACTACACAGAGGGCGCCGAGCTGGTTGATTCTGTCCTGGATGTGG
TACGGAAGGAGGCAGAGAGCTGTGACTGCCTGCAGGGCTTCCAGCTGAC
CCACTCACTGGGCGGGGGCACAGGCTCTGGAATGGGCACTCTCCTTATCA
GCAAGATCCGAGAAGAATACCCTGATCGCATCATGAATACCTTCAGAAN
N

Exon 4-2

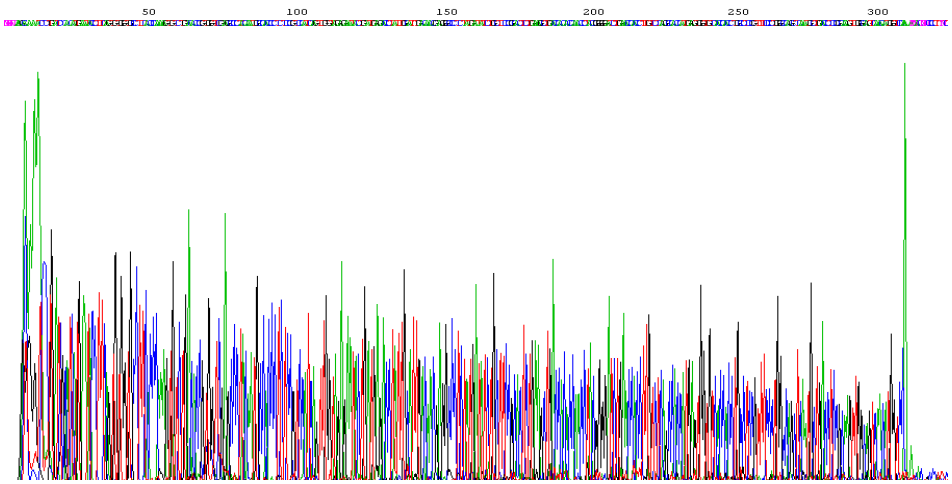

Figure H.2 Sequence data sheet of the second part of 4th exon of *TUBB* gene.

NNNAAGACAAATCCTGATCCACATGAATACCTTCAGTGTGGTGCCTTCAC
CCAAAGTGTCTGACACCGTGGTCGAGCCCTACAATGCCACCCTCTCCGTC
CATCAGTTGGTAGAGAATACTGATGAGACCTATTGCATTGACAACGAGG
CCCTCTATGATATCTGCTTCCGCACTCTGAAGCTGACCACACCAACCTAC
GGGGATCTGAACCACCTTGTCTCAGCCACCATGAGTGGTGTCACCACCTG
CCTCCGTTTCCCTGGCCAGCTCAATGCTGACCTCCGCAAGTTGGCAGTCA
ACATGGTCCAAANNACNNCCCTTNCTCCCTACATNTCNTGCTTTACCATC
CTTTCACCTTCTTCTCATACTCTACATCTCTTCATCAAGCTCTCCCCCTTTG
TCCTATAATCCCTTCTGCTGCCAACTCCTAGACTCTAGAACGGGGGTCAG
TTCAATAGTAACTGATCATCCTCCCCGACGAATTACCAAACTTCCCCTTG
GTGAGCTAGTAGCTCACCAACCTAATCATCCATACTTAGCCCTTCTTATTT
CACTATGCCCGAAAAGACCACCGCTTTCTTCCGATAAAAAATAGACGGGT
CTAGCCTCCGTATCCCATCTTTATACTCCTATTAACAGGATTATTTTTAAG
TTTTAATTCTATTAAATTGCCACTCGCCCCCTGAGAAAAAAAACCTAATT
TGTCCCTTTTATTTAAAGAGAAAAGCCCTTTCCCAAAGTTTTATCTTAGAC

CCTGAGTCATTCTGTAAAAAGGCTTTGAGGTACTCATTTCTTCATTCCGTC
CTATCCACTACTAAAATATTC

Exon 4-3

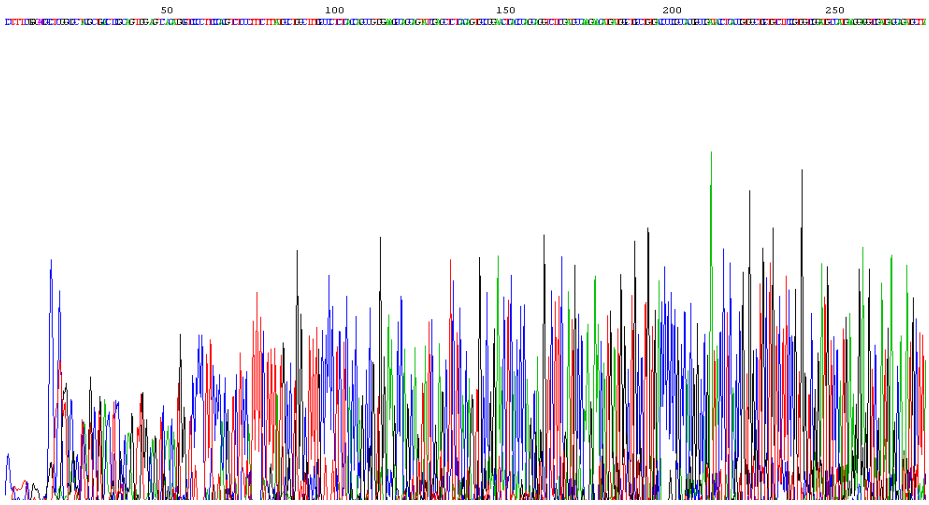

Figure H.3 Sequence data sheet of the third part of 4th exon of *TUBB* gene.

NNAAAAGGGGATGAGGCTGTCTGACTTGTTGGGGATCCATTCCACAAGT
AGCTGCTGTTCTTGTTCTGCACGTTAAGCATCTGCTCATCGACCTCCTTCA
TGGACATCCGACCACGGAAGACAGCAGCCACGGTGAGGTATCGGCCGTG
GCGGGGGTCACAGGCAGCCATCATGTTCTTGGCATCGAAGACCTGCTGG
GTGAGTTCCGGCACTGTGAGAGCTCGATACTGCTGGCTTCCACGGCTGGT
GAGAGGGGCAAAGCCAGGCATAAAGAAATGGAGACGTGGGAAGGGGAC
CATGTTGACTGCCAACTTGCGGAGGTCAGCATTGAGCTGGCCAGGGAAA
CGGAGGCAGGTGGTGACACCACTCATAAANN

Exon 4-4

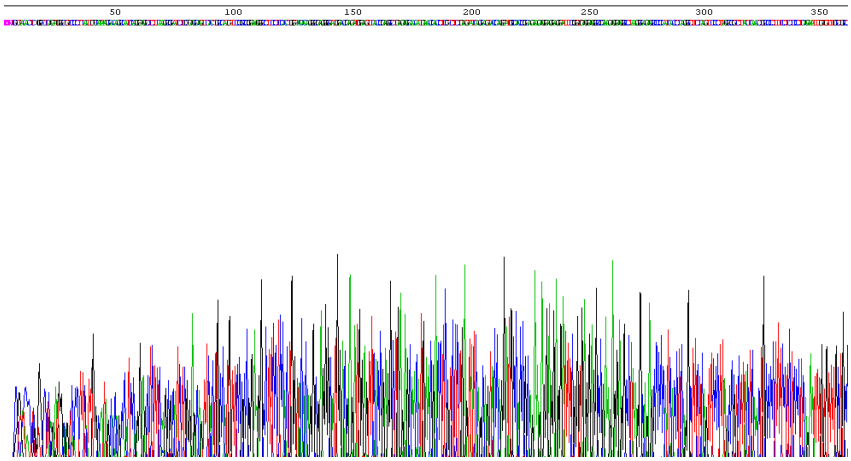

Figure H.4 Sequence data sheet of the fourth part of 4th exon of *TUBB* gene.

NGCGTACACTCGGGCTCAGATGGCGTCCCTTCATTGGCAATAGCACAGCC
ATCCAGGAGCTCTTCAAGCGCATCTCGGAGCAGTTCACTGCCATGTTCCG
CCGGAAGGCCTTCCTCCACTGGTACACAGGCGAGGGCATGGACGAGATG
GAGTTCACCGAGGCTGAGAGCAACATGAACGACCTCGTCTCTGAGTATC
AGCAGTACCAGGATGCCACCGCAGAAGAGGAGGAGGATTTCGGTGAGGA
GGCCGAAGAGGAGGCCTAAGGCAGAGCCCCCATCACCTCAGGCTTCTCA
GTTCCCTTAGCCGTCTTACTCAACTGCCCCTTTCCTCTCCCTCAGAATTTG
TGTTTGCTGCCTCTATCTTGANNN

APPENDIX J

SAMPLE COMBINATION GRAPHICS

J.1 Synergic Antiproliferative Effect of Verapamil on MCF-7/400nMPac

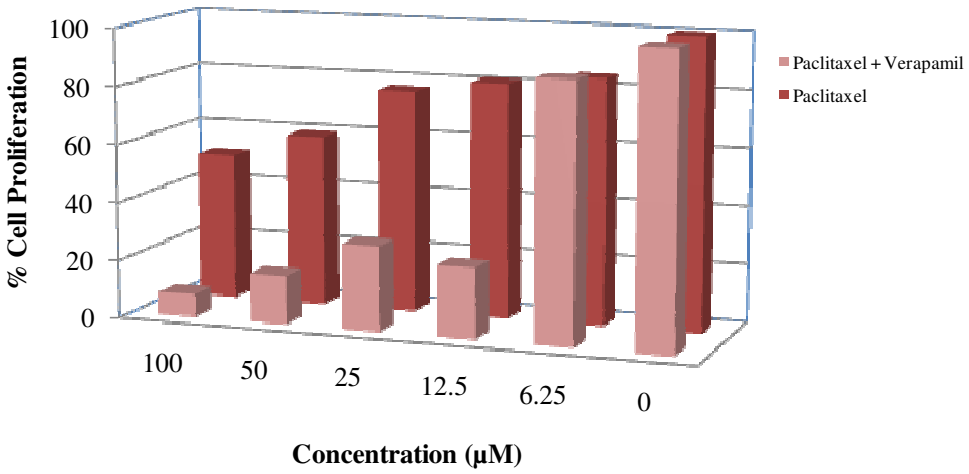

Figure J.1 The graph demonstrating synergic antiproliferative effects of Verapamil on MCF-7/400nMPac.

J.2 Antagonist Antiproliferative Effect of Promethazine on MCF-7/400nMPac

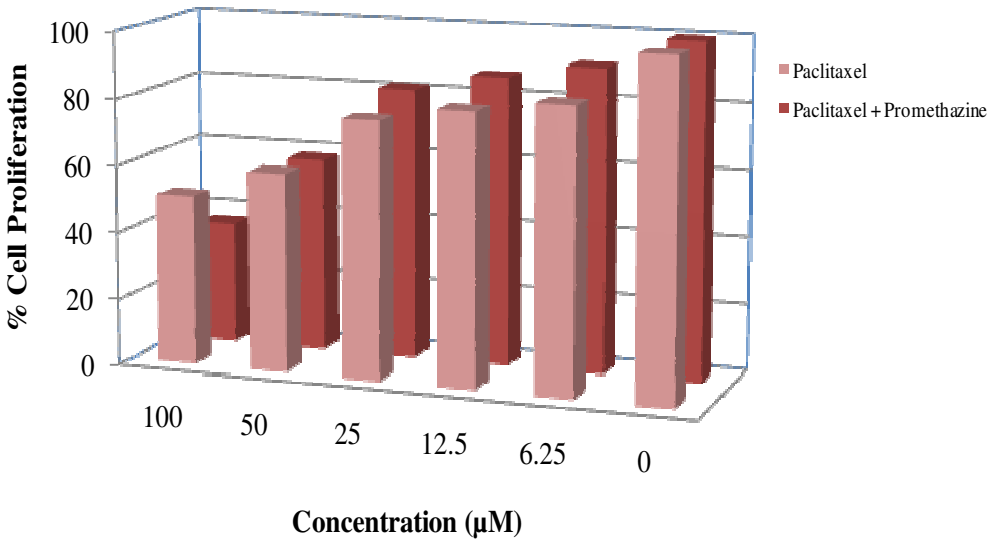

Figure J.2 The graph demonstrating antagonist antiproliferative effect of promethazine on MCF-7/400nMPac.

Additive Antiproliferative Effect of Promethazine on MCF-7/120nMVinc

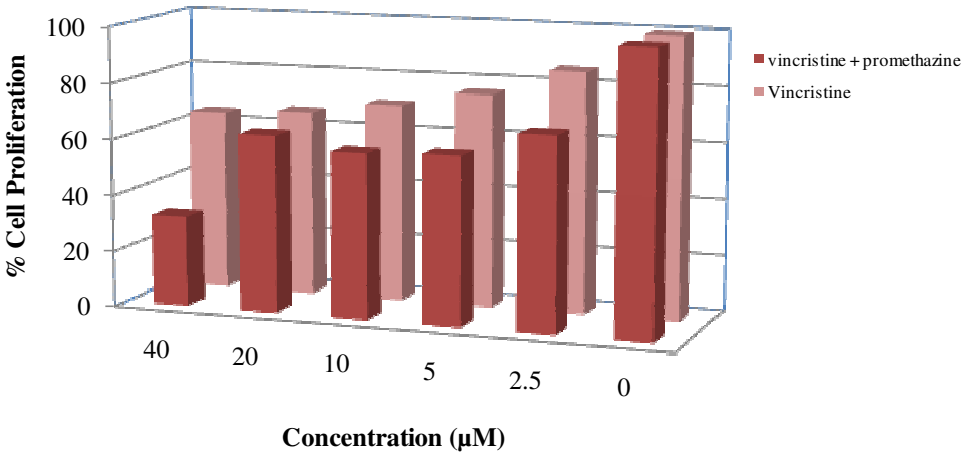

Figure J.3 The graph demonstrating additive antiproliferative effect of promethazine on MCF-7/120nMVinc.

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PhD	METU Biotechnology	2008
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	METU Department of Chemistry (Minor program)	2001
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WORK EXPERIENCE

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2001 (Three weeks)	TUBITAK, Gene and Tissue Research Laboratory, Ankara	Internship
2005-2006	University of Szeged, Faculty of Medicine, Department of Medical Microbiology and Immunobiology, Szeged, Hungary	Research Scientist
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FOREIGN LANGUAGES

Advanced English

PUBLICATIONS

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4. Iseri ÖD, **Kars MD**, Gündüz U: ATP Bağımlı Taşıyıcı Poteinlerinin Gen Ekspresyon Düzeylerinin MCF-7 Meme Kanseri Hücre Hatlarında İncelenmesi. Poster sunumu. Multidisipliner Kanser Araştırma Sempozyumu 12-15 March 2006, Uludağ-TÜRKİYE, p200.

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6. Eskiocak U, İşeri ÖD, **Kars MD**, Gündüz U: Telomeraz aktivitesinin TRAP yöntemiyle incelenmesi. Poster sunumu, 14. Ulusal Biyoteknoloji Kongresi, 31 August-2 September 2005 Eskişehir-TÜRKİYE, p 545.

7. İşeri ÖD, **Demirel M**, Arpacı F, Gündüz U: Meme kanseri doku örneklerinde ve MCF-7 model hücre hattında ilaç dirençliliği genlerinin ekspresyon analizi. Sözlü ve poster sunumu, Türk Biyokimya Derneği Moleküler Kanser Sempozyumu, 6-7 April 2004, İzmir-TÜRKİYE, p 26.

8. **Demirel M**, İşeri ÖD, Ünal N, Ural AU, Gündüz U: Taksoid grubu ilaçların MCF-7 meme kanseri hücre hattında sitotoksisite ve gen ekspresyonu analizleri. Poster sunumu (Special Poster Award), Klinik Biyokimya Uzmanları Derneği 1. Ulusal Kongresi, 1-4 November 2003, Kapadokya-TÜRKİYE, p173.

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1. TUBİTAK (Researcher): "Anti-Kanser İlaç Salımına Yönelik Dendritik Polimers Sentezi ve Hücre Kültürlerinde Uygulanması" 107T179, Haziran 2007-.Ocak 2009.

2. TUBİTAK (Researcher): "Farklı Kanser Tiplerine Model Hücre Hatlarında İlaç Dirençlilik Mekanizmalarının Moleküler Yöntemlerle İncelenmesi ve Geri Çevirilmesi" SBAG-3297, Haziran 2006-Haziran2008.

3. DPT (Researcher): "Meme Kanserinde Antikanser İlaçlara Karşı Dirençlilik Mekanizmalarının İncelenmesi ve İlaç Hedeflenmesi" BAP 07-02-DPT.02K120540-14, Haziran 2002-Haziran 2005.

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