REVERSAL OF MULTIDRUG RESISTANCE IN MCF-7 BREAST ADENOCARCINOMA CELL LINE BY SILENCING INTERLEUKIN 6 WITH RNA INTERFERENCE

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REVERSAL OF MULTIDRUG RESISTANCE IN MCF/7 BREAST ADENOCARCINOMA CELL LINE BY SILENCING INTERLEUKIN 6 WITH RNA INTERFERENCE

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ABSTRACT

REVERSAL OF MULTIDRUG RESISTANCE IN MCF-7 BREAST ADENOCARCINOMA CELL LINE BY SILENCING INTERLEUKIN 6 WITH RNA INTERFERENCE

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M.Sc. Department of Biology

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Multidrug resistance (MDR) in cancer is characterized by development of resistance to several unrelated drugs upon long time administration of a certain type of chemotherapeutic agent. In doxorubicin resistant MCF-7 cell line, resistance is developed mainly by upregulation of *MDR1* gene which encodes an ABC transporter protein known as P-glycoprotein. Interleukin 6 (IL-6) is a cytokine which acts as a growth factor for certain cell types including some cancer cells. IL-6 is found at high levels in cancer patients having poor prognosis and resistance to certain chemotherapeutic agent. It is known that in MCF7 cell line, IL-6 induces overexpression of *MDR1* gene by activating its transcription factor, C/EBPβ and confers resistance against Doxorubicin.

In this study, the aim is silencing *IL6* and to see the effects on MDR. Effects of *IL-6* silencing on the levels of *IL6*, *MDR1* and *MRP1* genes are determined by qRT-PCR. Further confirmation of *IL6* silencing is done by checking secreted IL-6 amount in cell culture media. Drug accumulation assay is done for evaluation of IL6 silencing on P-gp function. Moreover, to show that silencing of this particular gene sensitizes cells to chemotherapeutic drug Doxorubicin, XTT cytotoxicity assay is done.

Results indicate that *IL6* is successfully silenced by applying RNA interference which is observed by 36% decrease in IL-6 level to cell culture media. In correlation, *IL6* mRNA level decreased 0.5 fold 24 hours after treatment. In addition, *MDR1*gene is downregulated by 45% as a consequence of interruption of IL-6 signaling in cell. *MRP1*, another ABC transporter coding gene, which is not connected to IL-6 signaling pathway, is not affected by silencing showing that reversal of multidrug resistance is only dependent on decreased *MDR1* expression. Cytotoxicity assays show that cells are sensitized to Doxorubicin in an extent of 65%

It is demonstrated that silencing *IL6* gene makes Doxorubicin resistant MCF-7 cells significantly more susceptible to drug.

Keywords: IL6, MDR Reversal, MCF-7, RNA Interference

ÖZ

DOKSORUBİSİN DİRENÇLİ MCF-7 HÜCRE HATTINDA INTERLÖKİN-6NIN SİRNA İLE SUSTURULARAK ÇOKLU İLAÇ DİRENÇLİLİĞİNİN GERİ ÇEVRİLMESİ

Çakmak, Neşe

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Kanserde çoklu ilaç dirençliliği, tek bir kemoterapötik ajanın tedavi amaçlı uzun süre uygulanmasından sonra, yapısal olarak birbirinden bağımsız birden fazla ilaca direnç gelişmesi olarak tanımlanır. Doksorubisine dirençli MCF-7 meme kanseri hücre hattında direnç gelişiminin önemli sebeplerinden biri *MDR1* geni tarafından kodlanan, ATP bağlayıcı kaset taşıyıcı proteinlerinden P-glikoproteinin ifadesindeki artıştır. Interlökin 6 (IL-6) bazı kanser hücreleri de dahil çeşitli hücre tipleri üzerinde büyüme faktörü olarak etki gösteren bir sitokindir. Bu sitokinin kötü prognozla seyreden kanser hastalarının serum düzeylerinde normalden fazla bulunduğu gözlenmiştir. Ayrıca, hasta serum düzeyindeki IL-6 artışı çoklu ilaç dirençliliğinin de göstergesidir. MCF-7 hücre hattında, IL-6'nın C/EBPβ adlı transkipsiyon faktörünü aktive ederek *MDR1* gen ifadesini arttırdığı ve Doksorubisin direncine sebep olduğu bilinmektedir.

Bu çalışmanın amacı ilaç dirençli kanser hücre hatlarında *IL6* genini RNAi yöntemiyle susturmak ve bunun çoklu ilaç dirençliliği üzerindeki etkisini gözlemlemektir. *IL6* geninin susturulmasının *IL6*, *MDR1* ve *MRP1* genlerinin ifade düzeylerindeki etkisi qRT-PCR ile belirlenmiştir. Susturulmanın doğrulanması için hücre dışına salgılanan IL-6 miktarı hücre kültürü besi ortamından ELISA yöntemi ile ölçülmüştür. Ayrıca, hedef genin susturulmasının hücreleri yeniden Doksorubisine duyarlı hale getirip getirmediği XTT sitotoksisite analizi ile belirlenmiştir.

Elde edilen sonuçlara göre *IL6* geni RNAi yöntemini kullanarak başarı ile susturulmuştur. Hücre kültürü besi ortamına salgılanan IL-6 miktarı siRNA uygulamasından 24 saat sonra %36

oranında düşmüştür. Ayrıca, *IL6* gen ifadesi düzeyi 24 saatte ilk değerin %50'sine düşmüştür. *MDR1* mRNA düzeyi ise IL-6 sinyal yolağının bloke edilmesiyle %45 oranında azalmıştır. IL-6 sinyal yolağıyla düzenlenmeyen *MRP1* geninin ifadesi ise değişme göstermemiştir. Sitotoksisite analizleri dirençli MCF-7 hücrelerinin Doxorubisin'e %65 daha duyarlı oldunu göstermiştir.

Bu çalışma, *IL6* geninin susturulmasının Doksorubisine dirençli MCF-7 hücrelerinde ilaç dirençliliğini önemli ölçüde geri çevirebileceğini göstermiştir.

Anahtar kelimeler: Interlökin 6, MDR Geri çevrilmesi, MCF-7, RNA İnterferans

To my precious family

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ABC	ATP Binding Cassette
ATP	Adenosine tri-phosphate
BCRP	Breast Cancer Resistance Protein
bp	Base pair
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DMSO	Dimethyl Sulfoxide
dNTP	Deoxy Nucleotide Triphosphate
DAMP	Danger Associated Molecular Patterns
DOX	Doxorubicin
dsRNA	Double stranded RNA
DSP	Downstream promoter
EtBr	Ethidium Bromide
FR	Fold reversal
GSH	Glutathione
IC50	Inhibitory concentration 50
IL	Interleukin
kDa	kilo Dalton
MAPK	Mitogen activated protein kinase
MCF-7/Dox	Doxorubicin resistant MCF-7 Subline
MCF-7/S	Sensitive MCF-7
Mcl	Myeloid Leukemia Cell Differentiation Protein
MDR	Multidrug resistance
miRNA	Micro RNA
MRP1	Multidrug Resistance Protein 1
MSD	Membrane spanning domain
NBD	Nucleoside binding domain
NF-IL6	Nuclear Factor Interleukin 6
PBS	Phosphate buffered saline
P-gp/MDR1/ABCB1	P-glycoprotein
qPCR	Quantitative PCR
R	Relative resistance index
RISC	RNA induced silencing complex
RNAi	RNA interference
Rpm	Round per minute
RT	Reverse transcription
SEM	Standard error mean
siRNA	Small interfering RNA
shRNA	Short hairpin RNA

TAE	Tris-Acetate-EDTA
TLR	Toll-Like Receptor
USP	Upstream Promoter
w/v	Weight per volume

CHAPTERS

CHAPTER 1

INTRODUCTION

1.1 Biology of Cancer

Cancer is a group of diseases characterized by abnormal cell growth and tissue invasion (National Cancer Institute, 2012a). It is one of the most prevalent diseases in the world with expected 1.6 million new cases only in the USA in 2012 (Siegel et al., 2012).

The main cause of cancer is the mutations that change the genome of the cells. There are different types of mutations but not all of them lead to cancer. Shared features of the carcinogenic mutations can be examined in two groups: mutations that increase activity of genes known as *oncogenes* and mutations that decrease the activity of *tumor suppressor genes* (Bertram, 2000). The oncogenes are normally functioning in the proliferation of cell; a process which is tightly controlled. Once mutations either increase expression of those oncogenes or increase the activity of the oncogenic protein product, the control over proliferation is lost and cell starts to divide rapidly. Tumor suppressor genes, on the other hand, as the name implies, act as a checkpoint on cell division and suppress the cell proliferation and growth. Their loss of activity by means of mutation causes loss of control in proliferation and growth leading to formation of tumor masses. Hannahan and Weinberg stated six consequences of those mutations that are common in many cancer cells. Those are (i) escape from apoptosis, (ii) self sufficiency in growth signals, (iii) insensitivity to anti-growth signals, (iv) sustained angiogenesis, (v) limitless replicative potential and (vi) tissue invasion and metastasis (Hanahan & Weinberg, 2000).

1.2 Breast cancer

Breast cancer is the most frequently observed cancer type in women. In the USA, predictions for 2012 foresee 226,000 new cases for women with mortality rate of 15% (Siegel et al., 2012). It is also the most frequently observed cancer type among women in Turkey (Türk Halk Sağlığı Kurumu Kanser Daire Başkanlığı, 2008).

Breast cancer, as all cancer types, is not a single disease but a group of diseases with different pathologies. Although all cancers arise due to mutation, only 5-10% of breast cancer cases are

inherited. Rest of the breast cancers arise because of random mutations. Formation of tumor starts generally at the duct and lobules of breast tissue (National Cancer Institute, 2012). As it grows, it invades healthy tissue and may reach lymph nodes. It metastasizes through lymphatic to other parts of the body ("What Is Breast Cancer?," 2013).

1.3 Treatment of Breast Cancer

1.3.1 Surgery

First option for treatment of breast cancer is to remove tumor tissue from the body by surgical operation. There are different surgical methods that can be applied depending on the localization of tumor and its stage. One approach is breast conserving surgery in which only tumor and some surrounding healthy tissue is removed while rest of the breast is conserved. For the patients who undergo that operation, lymph node dissection can be done, which is the biopsy of lymph nodes that are located under arm to check presence of any cancerous cells. Other approach is to remove whole breast that has cancer, some of the lymph nodes under arm and sometimes muscles that line over the chest (National Cancer Institute, 2012c).

1.3.2 Radiation Therapy

Radiation therapy is a treatment option that uses high energy radiation that kills cancer cells. It is generally applied after a breast conserving surgery to eliminate the chances of cancer recurrence in breast tissue. It is also preferred to treat bone or brain metastasis of cancer. In general, there are two ways of giving radiation therapy. First one is external beam radiation in which radiation is focused to tumor by a machine outside of body. Second one is internal radiation that is also known as Brachytherapy. It involves administration of radioactive substances directly to breast via catheters. The type of surgery, stage of cancer and age of patient are important factors for choosing one of the options ("Radiation therapy for breast cancer," 2012).

1.3.3 Hormone Therapy

Hormone therapy is a way of treating hormone responsive cancers either by inhibiting production of hormone or blocking hormone-receptor interaction. Breast cancers bear two types of receptors that respond to hormones. They bear either estrogen receptor, progesterone receptor or both (Dunnwald et al., 2007). Breast cancer types that are negative for all hormone receptors cannot be treated with hormone therapy and have lower chance of survival compared to ER (+) or PR (+) cancers.

Hormone therapy for breast cancer is done by modulating estrogen production or blocking estrogen-estrogen receptor interaction. Estrogen production is blocked by Aromatase inhibitors (Breastcancer.org, 2013a). Aromatase is a cytochrome P450, an important enzyme that is required for synthesis of estrogen in ovaries. It catalyzes conversion of androstenedione to 17β, 16α-oestriol in a three step reaction (Ghosh et al., 2009). Its blockade interferes with estrogen production. Another approach is to inhibit ovarian function so that it can no longer produce estrogen. Gonadotropin-releasing hormone (GnRH) or Luteinizing hormone (LH) synthesized from pituitary signals ovary to produce estrogen. Blocking the synthesis of those hormones interferes with estrogen production from ovary. There are FDA approved drugs Goserelin and Leuprolide used for this purpose (National Cancer Institute, 2013). Other method is based on ablation of estrogen function. Selective estrogen receptor modulators (SERMs) are the most widely used one which binds to ER and act as Estrogen antagonist at breast tissue but act as agonist in lung or bone tissue which bear structurally different ER. This is why they are called "selective". Tamoxifen is the most famous SERM used for treatment of many ER(+) breast cancer cases (Breastcancer.org, 2013b). Estrogen receptor downregulators (ERDs) are another drug class for inhibiting Estrogen-ER interaction. They are Estrogen antagonists that are capable of binding to all types of ER (Breastcancer.org, 2013c).

1.3.4 Chemotherapy

Chemotherapy is the use of drugs or drug combinations that are capable of killing tumor cells as a treatment method. The target of that kind of drugs is dividing cells. Therefore, chemotherapeutic agents kill every type of dividing cell without making discrimination whether it is a cancer cell or normal dividing cell.

The drugs used for chemotherapy divided into several classes based on their mechanism of action (Malhotra et al., 2003) as listed below:

- *Alkylating agents* are the drugs that form covalent bonds with amino, carboxyl and phosphate groups of DNA, RNA and proteins impairing their function. These drugs kill dividing cells at any phase of cell cycle.
- *Antimetabolites* are analogs of molecules that are utilized for DNA and RNA synthesis. They show their effect at S phase of cell cycle. Therefore, that class of drugs is used generally for fast growing tumors. 5-Fluorouracil is a widely used example for this class.
- *Vinca Alkaloids* are extracted from *Vinca rosa*. Their target is tubulin. At S phase of cell cycle, they inhibit tubulin assembly blocking polymerization of microtubulins. As a result, proper spindle formation is inhibited. Vincristine and vinblastine fall into this category.
- *Taxanes* are drugs that promote microtubule assembly and stability. This blocks microtubule disassembly resulting in arrest at M phase. Docetaxel is an example.

- *Anthracyclines* are antibiotics with a characteristic structure of an amino sugar with a glycoside group. They are Topoisomerase II poisons and DNA intercalating agents. Doxorubicin and duanorubicin are two drugs in this class.

1.3.4.1 Doxorubicin

Doxorubicin is a drug that belongs to family of Anthracyclines (Octavia et al., 2012). It is used to treat some leukemias, stomach, lung, prostate, ovary and breast cancers since 1960s. *Streptomyces percetus var caesius* is the natural producer of this drug. Two mechanisms are proposed for its action. First, it intercalates into DNA and impairs topoisomerase-II mediated DNA repair leading cell to apoptosis. Second, it generates free radicals which damage DNA and proteins (D. Gewirtz, 1999).

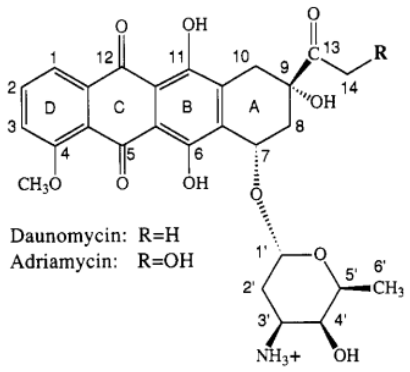


Figure 1.1 Chemical structure of Doxorubicin. Adapted from (D. Gewirtz, 1999).

It is known that Doxorubicin can cause immunogenic cell death. Normally, apoptotic cells do not evoke immune response since they are efficiently phagocytosed. In addition, apoptotic cells cause immunosuppression (Casares et al., 2005). In immunogenic cell death, however, cells release damage-associated molecular pattern (DAMPs) which stimulates immune cells, mainly neutrophils, to secrete IL-6 and Monocyte chemotactic protein-1 (Mcl-1). TLR-2 and TLR-9

are shown to be two principle sensors for activation of this immune response (Krysko et al., 2011).

1.4 Multidrug Resistance

Cancer cells may become unresponsive to several unrelated chemotherapeutic agents after long time exposure to a single drug. Moreover, those cells may be intrinsically resistant to the drugs prior to administration of the drug (Gottesman, 2002). This phenomenon is known as Multidrug Resistance (MDR) and it is the biggest obstacle of successful treatment of cancer by administration of chemotherapeutics.

Cancer cells acquire resistance by several different mechanisms. According to categorization of Longley et al., those mechanisms are *(I)* increased drug efflux or decreased drug influx, *(ii)* inactivation of drug, *(iii)* alterations of drug targets, *(iv)* increased repair of drug induced DNA damage and *(v)* evasion of drug induced apoptosis (Longley & Johnston, 2005).

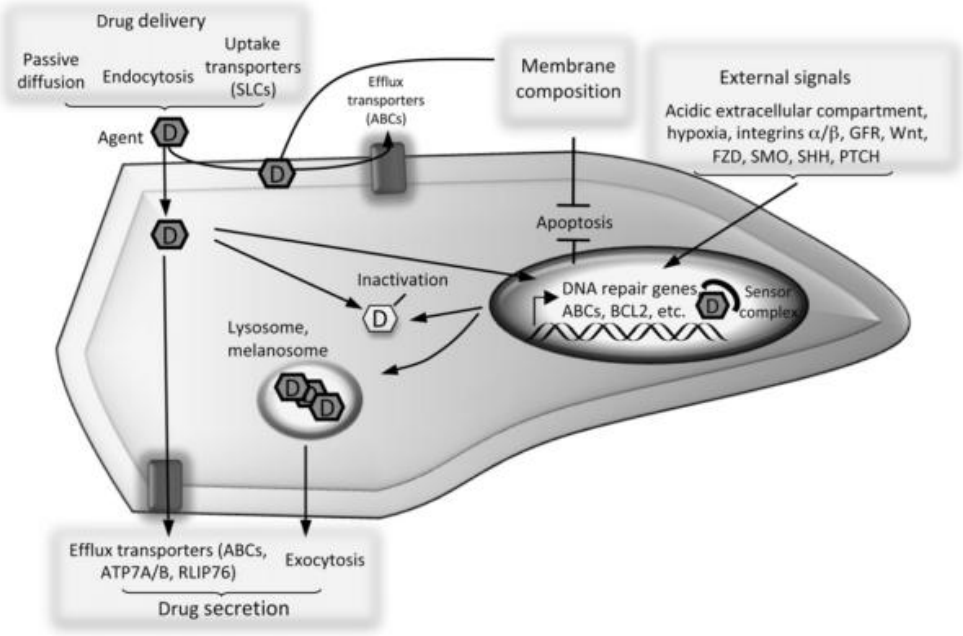


Figure 1.2 Overview of multidrug resistance mechanism in cancer cells. Adapted from (Lavi et al., 2012)

1.4.1 Transporter Based Multidrug Resistance

Although all drug resistance mechanisms are extremely important, increased drug efflux is the most widely and well studied one. Basically, cells pump the drug out of the cell with ATP Binding Cassette family transporters (ABC transporters) localized at cell membrane before drug meets its target and execute its function, thus cells stop responding to the drug.

P-glycoprotein and multidrug resistance associated protein (MRP) are two examples which play major role in drug resistance by means of drug efflux.

1.4.1.1 P-glycoprotein

Main resistance mechanism is developed by upregulation of an ATP binding cassette family transporter, P-glycoprotein (P-gp) which is encoded by *MDR1* or *ABCB1* gene (Gottesman, 2002). P-gp is the first identified mammalian ABC transporter (Juliano & Ling, 1976) and is first protein to be linked to multidrug resistance in cancer cells (Ueda, 1987).

P-gp is a 170 kDa transmembrane protein composed of 2 hydrophobic membrane spanning domains (MSD) each formed from six transmembrane α-helices and 2 nucleotide binding domains (NBD) (Choi, 2005). MSDs forms a channel through which substrates are effluxed while NBDs are site for ATP binding and hydrolysis (Hyde et al., 1990). 2 ATPs are required for complete function of P-gp. Binding of first ATP to NBD and its hydrolysis causes a conformational change (Sonveaux et al., 1996) which alters organization of MSD embedded in membrane. As a result of this conformational change, central pore is opened to allow access of the substrate to extracellular space (Rosenberg et al., 2003). Second ATP molecule is hydrolyzed to restore original conformation.

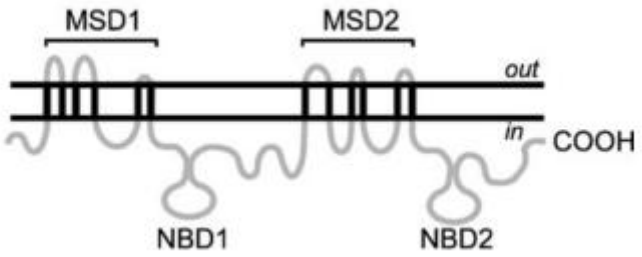


Figure 1.3 Structure of P-glycoprotein on cell membrane. Adapted from (Leslie et al., 2005)

P-gp is expressed in several non-cancerous tissues which includes blood-brain barrier epithelia cells (Cordon-Cardo et al., 1990) of small and large intestine, kidney cell and placental cells (Cordon-Cardo, 1989). The substrates of P-gp are lipophilic, electrically neutral and positively charged. Under normal conditions, this protein pump is necessary for cellular defense against hydrophobic xenobiotics (Callaghan et al., 2008). However, many of the commonly used anticancer agents such as Doxorubicin, Duanorubicine, Vincristine and Docetaxel are also substrate of P-glycoprotein (Gottesman, 2002). Therefore, over activity of P-gp results in decreased cellular availability of drugs and results in multidrug resistance in cancer cells.

The reason why P-gp expression dramatically increases after administration of chemotherapeutic agents is still poorly understood. Genetic polymorphisms in *MDR1* gene are correlated to multidrug resistance phenotype. Yet, there is not much known about the regulation of *MDR1*. Several external factors such as therapeutic agent administration and internal factors like hypoxia, glucose deprivation, free radical production are shown to induce expression of *MDR1* by altering signaling mechanisms in the cells (Gillet & Gottesman, 2010).

1.4.1.1.1 Transcriptional Regulation of P-glycoprotein

Characterization of *ABCB1* promoter regions shows that there are more than one promoter region. The major downstream promoter (DSP) of *ABCB1* is active in healthy tissues and some drug resistant cell lines. Activation of minor upstream promoter (USP) is observed to be only linked to cancerous state (Ueda et al., 1987). In MCF-7 cell line, activation of DSP but not USP

is required to develop resistance against Doxorubicin (Raguz et al., 2008). Activation of USP is observed to be linked with metastatic behavior of MCF-7 cell line (Raguz, 2004).

Major downstream promoter of *ABCB1* is very complex and there are several binding sites for different transcription factors that are activated or inactivated by different signaling mechanisms suggesting that transcriptional regulation of *ABCB1* directly depends on different states of cell (Labialle et al., 2002).

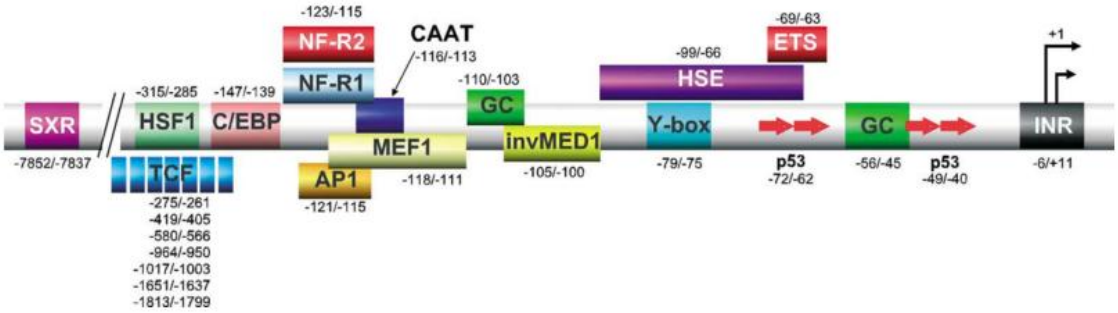


Figure 1.4: Downstream promoter structure of *MDR1* gene Adapted from (Labialle et al., 2002)

DSP of *ABCB1* is a TATA-less promoter, which contains multiple start sites as a characteristic. There is an Initiator (Inr) element which is required for proper initiation of transcription of this gene.

There are several *cis*-acting elements that are important for regulation of promoter activity. GC box is one of them. It is the G-C rich region of promoter sequence which is located at -56 to -45 upstream of +1 major start site. Sp1, one of the major transcription factors in the cell, is shown to have a binding site within GC box to drive transcription of *ABCB1* (Cornwell et al., 1993). This transcription factor binds to any TATA-less promoter region and generally drives expression of housekeeping genes.

Another important motif is an inverted CCAAT box which is also known as Y box. It is positioned at -79 to -75 upstream of the start site. NF-Y is the transcription factor that drives expression of *ABCB1* from this element (Goldsmith et al., 1993) and it is observed that stress

factors such as UV radiation and administration of anticancer agents induce activation of NF-Y (Ohga et al., 1996).

CAAT box is another important element of *ABCB1* promoter located at -116 to -113 bp upstream of major start site. It is the binding site for NF-κB/p65 and c-*fos* protein complexes which positively regulates *ABCB1* expression (Ogretmen et al., 1999).

C/EBP element is located at -147 to -139 position and provides a binding site for transcription factor CEBP/β, which is also known as nuclear factor for Interleukin (IL)-6 (NF-IL6) (Labialle et al., 2002). This transcription factor is both upregulated and activated by Interleukin 6 signaling and induces expression of *ABCB1* which caused multidrug resistance. Chen et al. demonstrated that there is another interaction site within *ABCB1* promoter that lies between -128 to -75 bases which has important role for development of multidrug resistance in MCF-7 cell line (Chen et al., 2004).

In addition to those sequences, there are binding site for p53, AP1, heat shock proteins and steroid xenobiotic receptor in the promoter region which indicates that *MDR1* transcription is regulated by various stimuli (Labialle et al., 2002).

1.4.1.2 Multidrug Resistance Associated Protein

Multidrug resistance associated protein, encoded by *MRP1* or *ABCC1* gene, is another ABC transporter that is linked to multidrug resistance in cancer cells. It belongs to MRP family of ABC transporters that have 13 members 9 of which are directly related to MDR (Chen et al., 2011). Their names, tissue localizations, substrates are summarized in Table 1.1.

MRP1, 190 kDa transmembrane protein (Cole et al., 1992), has two membrane spanning domains (MSD2 and MSD3) each composed of six transmembrane α-helices (TMs), two nucleotide binding domains (NBD1 and NBD2) and an additional MSD1 at N-terminus of the protein (Chen et al., 2011).

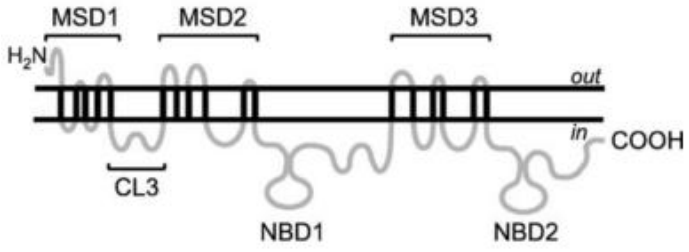


Figure 1.5: Protein structure of MRP1. Adapted from (Leslie et al., 2005)**.**

MRP1 is expressed in healthy tissues including lung, kidneys and liver (Table 1). Normal function of it is to protect tissues from cytotoxic effects of xenobiotics (Leslie et al., 2005). This protein pump has slightly different substrates than P-gp, therefore, confers resistance to different classes of anticancer agents. Most important substrates are drug metabolites which are conjugated to Glucoronides and Glutathiones (GSH).

Table 1.1: MRP family of ABC Transporters that cause multidrug resistance in cancer. (Z.-S. Chen & Tiwari, 2011)

Common Name	Gene Symbol	Tissue Expression	Physiological Substrates	Anticancer drug substrates
MRP1	ABCC1	Ubiquitous	LTC_4, sulfated bile acids, folic acid, bilirubin, GSH conjugates	Anthracyclins, Canpothecins, Vinca alkaloids, Etoposide, MTX
MRP2	ABCC2	Liver, kidney, intestine	Bilirubin conjugates, LTC_4, GSH	Anthracyclins, Canpothecins, Vinca alkaloids, Etoposide, Cisplatin, MTX
MRP3	ABCC3	Small intestine, pancreas, colon, kidneys, placenta, adrenal glands	Taurocholate, Glycocholate, LTC_4	MTX, Tenoposide, Etoposide
MRP4	ABCC4	Prostate, Testis, Ovary, Lung, Hepatocytes, Intestine, Pancreas	cGMP, cAMP, PGE1, PGE2, LTB_4, GSH and GSH conjugate bile acids	MTX, Irinotecan, Topotecan
MRP5	ABCC5	All major tissues	cGMP, cAMP, GSH	5-FU, Cisplatin, Doxorubicin, Duanorubicine, Gemcitabine
MRP6	ABCC6	Kidney, Liver	LTC_4	Doxorubicin, Duanorubicine, Etoposide, Teniposide, Cisplatin, Actinomycin-D
MRP7	ABCC10	Most tissues	LTC_4	Paclitaxel, Docetaxel, Vincristin, Vinblastin, Gemcitabine
MRP8	ABCC11	Testis, Breasts	cGMP, cAMP, LTC_4, Bile acids, Estrone 3-Sulfate, Folic acids	5-Fluorouracil, MTX

1.4.2 Interleukin-6 and Its Implication on MDR

Interleukin-6 was first discovered in late 70's and named B cell stimulatory factor-2 (BSF-2) since its first identified function was to induce differentiation into Immunoglobulin secreting B-cells (Kishimoto, 2005). The cDNA coding for this 26 kDa protein was first cloned in 1986 by Hirano and collogues (Hirano et al., 1986) accelerating the studies on this particular cytokine. The gene coding for IL-6 is located on chromosome 7p21 with 4 intronic and 5 exonic regions (Sehgal et al., 1995).

It is a pleiotropic cytokine that is involved in immune response, cell proliferation, survival and evasion from apoptosis (Grivennikov et al., 2008). It is the regulator of acute-phase responses in liver and fever (Hong et al., 2007). There are several sources of IL-6 in the body. T and B lymphocytes are the primary source while macrophages, fibroblasts, endothelial cells and synovial cells can also secrete IL-6 by various external stimuli.

1.4.2.1 Interleukin-6 Receptors and Signaling

Interleukin-6 signals via a receptor complex composed of a membrane bound 80 kDa IL-6Rα, which binds to IL-6, and glycoprotein 130 (gp130), which is the signal transducer of the complex (Heinrich et al., 2003; Hirano et al., 1997). Binding of IL-6 to IL-6Rα induces IL-6Rα - gp130 interaction leading to formation of either a tetrameric complex (Fig 1.4/a) or a hexameric complex (Fig 1.4/b) (Grötzinger et al., 1999). Gp130 is found ubiquitously in all cell types while IL-6Rα is not which may indicate that not all cells are responsive to IL-6. However, signaling via membrane bound IL-6Rα is not the only route. There is soluble form of IL-6Rα (sIL-6Rα) that is first isolated from human urine. sIL-6Rα binds to IL-6 and forms a circulating complex. When this complex encounters a gp130 positive cell, their interaction induces signaling cascade in cell which is known as *trans-signaling* (Fig 1.6/c). Therefore, almost all cell type is responsive to IL-6.

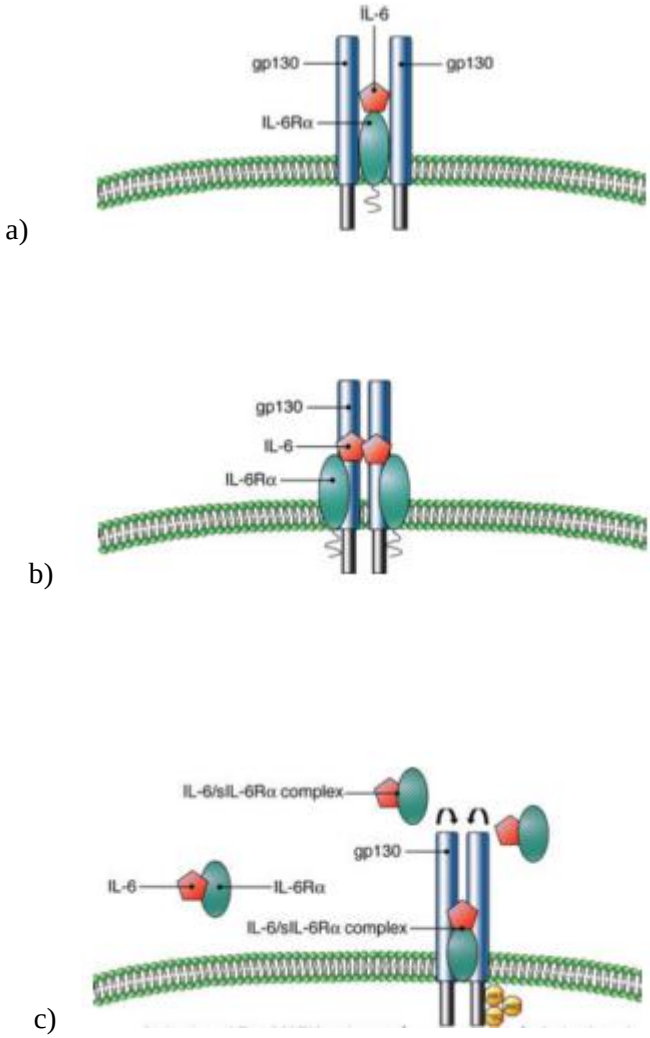


Figure 1.6 Interleukin-6/IL-6R/gp130 signaling complex a) tetrameric complex, b) hexameric complex formed in supraoptimal IL-6 concentrations, c) sIL-6Rα can activate IL-6 signaling pathway in cells that do not have IL-6Rα on their membrane. Adapted from (Hong et al., 2007).

After being a part of the receptor complex, gp130 can activate downstream JAK/STAT signaling cascade (Figure 1.7). Janus Kinase 1 (JAK1), JAK2 and tyrosine kinase 2 become activated by gp130 and further phosphorylate tyrosine residues at cytoplasmic tail of gp130 (Hong et al., 2007). This phosphorylation provides a docking site for SH2 domain of signal transducers and activators of transcription-3 (STAT-3). Recruited STAT-3s are phosphorylated by JAKs associated with gp130 and forms dimers. Dimerized STAT-3 diffuses to nucleus and activates transcription of cell cycle regulatory genes such as c-Myc and Cyclin-D, some antiapoptotic

genes like BCL2 (Taga et al., 1997). In addition, another STAT molecule, STAT-1 is activated by phosphorylation at lower level compared to previous one. It forms a heterodimer with phosphorylated STAT-3 and localizes to nucleus where it binds to response elements on DNA (Taga et al., 1997).

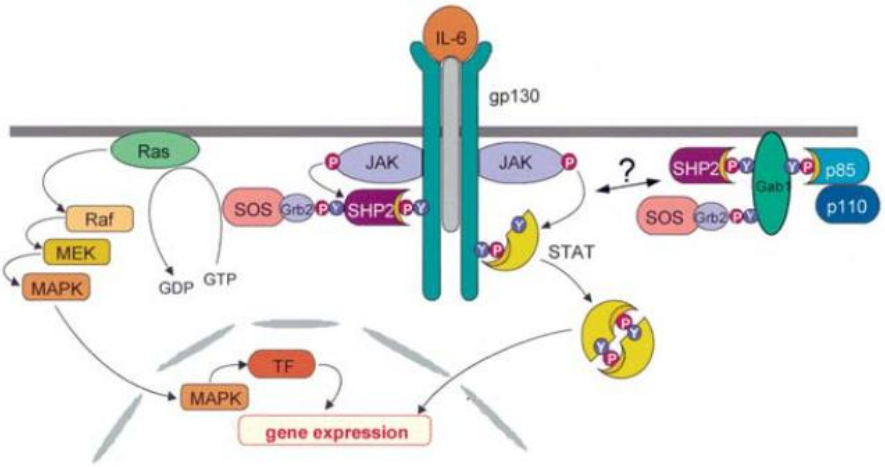


Figure 1.7 IL-6 signaling pathway. JAK/STAT and Ras-MAPK pathways are activated in response to IL-6R activation and alter target gene expression levels. Adapted from (Heinrich et al., 2003)

These are not the only signal transduction pathways activated by IL-6 binding to the receptor. Activated JAKs induce Ras mediated mitogen activated protein kinase (MAPK) signaling pathway by phosphorylating SHP2 protein which in turn activates a Ras guanine nucleotide exchange factor, Sos1, via phosphorylation (Ogata et al., 1997). Finally Ras becomes activated and downstream MAPK signaling cascade is triggered which leads to activation of certain transcription factors such as AP-1, C/EBPβ and NF-κB ("Jak / Stat Signaling : IL-6 Receptor Family," 2010).

1.4.2.2 Interleukin-6, Cancer and MDR

Interleukin-6 deregulation is linked with several cancer types including renal cancer, myelomas, ovarian cancer (Conze et al., 2001) and breast cancer (Basolo et al., 1996). There is evidence that IL-6 regulates cell growth, angiogenesis and immune activity in mammary glands (Basolo et al., 1996). However, there are also studies showing that IL-6 induces apoptosis and arrests growth in ER (+) MCF-7 cells while it has not such an effect on ER (-) cells via unknown mechanisms (Chiu et al., 1996).

Despite those diverse effects of IL-6 on different cancer cells, there is one common observation that elevated levels of IL-6 in patient serum is linked with poor prognosis of cancer. it also induces multidrug resistance in cancer cells. Wang et al. shows that autocrine IL-6 signaling causes paclitaxel and cisplatin resistance in several ovarian cancer cell lines (Wang et al., 2010). Furthermore, Conze et al. showed that MCF-7 cells exposed to recombinant human IL-6 becomes resistant against Doxorubicin. They also showed that Doxorubicin resistant MCF-7 cells secrete more IL-6 compared to drug sensitive MCF-7 cell line (Conze et al., 2001).

1.5 Multidrug Resistance Reversal Strategies

Multidrug resistance in cancer is the biggest obstacle for a successful chemotherapy and is responsible for increased death rates due to cancer. One of the most common drug resistance mechanisms is efflux of drug through membrane bound efflux pumps. There are various approaches developed to reverse MDR in order to increase success rate of chemotherapy. First, drugs that modulate ABC transporter function are used. Second, drugs that modulate signaling pathways that regulate ABC Transporter expression can be used. Last but not least is the use of RNAi mechanism in cell to prevent transcription of mRNAs coding for the pumps.

1.5.1 Use of ABC transporter Modulators

Agents falling in this class are chemicals that inhibit one or more ABC transporter to restore sensitivity of cell to the drug (Yu et al., 2013). Most widespread ones are P-pg inhibitors since P-gp is the principal pump causing MDR. Those drugs are further categorized depending on their affinity and specificity for P-gp (McDevitt et al., 2007).

First-generation P-gp inhibitors are drugs that are approved for treatment of other disease states. Those include calcium channel blockers and immunosuppressants (Abbasi, 2013). Still, there are certain drawbacks for those drugs such as high toxicity at drug doses required for P-gp inhibition. Verapamil, for example, is a calcium channel blocker which is the first drug to be

used for inhibition of MDR1 activity. Although it effectively reversed MDR, this drug caused cardiac toxicity at Phase I trials (Ozols et al., 1987). Despite other first-generation P-gp inhibitors do not cause significant toxicity; they are not effective enough to be used in clinical trials (Yu et al., 2013).

Second generation P-gp inhibitors are drug that are developed specifically for P-gp and have higher affinity compared to first generation. Verapamil analogs can be an example for this class. Those drugs, however, generally interact with P450 enzymes which are involved in metabolism of some anticancer drugs like paclitaxel (Yu et al., 2013). This makes a limitation to use second generation drugs as well because they change pharmacokinetics of anticancer drug changing their efficacy. Sometimes, they even cause increased toxicity of certain anticancer agent (Abbasi, 2013).

Third-generation P-gp inhibitors are more potent and less toxic compared to previous two generations. They also do not interfere with the pharmacokinetics of anticancer agents. Biricodar, Elcaridar and Zosuquidar are leading drugs that fall in this category (Abbasi, 2013).

Although they are promising, those drugs fail to reverse multidrug resistance occasionally. One reason for that is genetic polymorphism of ABC transporters due to single nucleotide polymorphisms (SNPs). Furthermore, multidrug resistance arises by interaction of several different pathways. Blocking one route may not be enough since there could be redundant pathways. Besides, those drugs do not specifically targeted. This results in complete blockade of ABC transporters in body even at the sites where their function is essential such as brain blood barrier. Therefore, search for alternative reversal methods continues (Abbasi, 2013).

Another approach to modulate ABC transporter function is use of monoclonal antibodies (Ab) targeting the pumps. For example, MRK16 which specifically binds to P-gp is used both *in vitro* and *in vivo* studies. Those studies show that blocking P-gp function inhibits efflux of certain chemotherapeutics such as doxorubicin, vincristine and etoposide (Iwahashi et al., 1993) (Mickisch et al., 1992). They are shown to be very specific, non-toxic and prolonged activity when introduced to organism. However, they have potential to induce immune system and cannot be targeted to cancer cells (Yu et al., 2013).

1.5.2 Modulation of Signaling Pathways Influencing ABC Transporter Expression

Transcription of *MDR1*, *MRP1* and *BCRP* are regulated by several signaling pathways including AKT (Han et al., 2007), Cyclooxygenase2-Prostoglandin E2 (COX-2/E2) (Liu et al., 2010)and Ras-MAPK (Labialle et al., 2002). Modulators of those signaling pathways can be used to alter

expression of aforementioned protein pumps. COX-2 inhibitors for example, are shown to increase sensitivity to P-gp substrates like Doxorubicin *in vivo* (Rahman et al., 2012).

Interleukin 6 signaling pathway is one of the crucial pathways regulating transcription of *MDR1* gene. Modulators of IL-6 signaling pathway are also considered as MDR reversing agents. Monoclonal IL-6 antibodies, Siltuximab for example, are used both *in vitro* and *in vivo* studies (Guo et al., 2010; Hunsucker et al., 2011). In addition, drugs that modulate the activity of downstream signaling component STAT3 are evaluated as potential reversal agents (Alas & Bonavida, 2003).

1.5.3 RNA interference

RNA interference is an intrinsic cellular mechanism that triggers sequence-specific silencing o a gene by making use of double stranded small RNA molecules. Antisense RNAs were first used to silence gene expression in muscle of *C. elegans* in 1991 (Fire et al., 1991). In 1998, the same group proposed a cellular mechanism and published details of nature of small RNAs to obtain potent and specific RNA interference (Fire et al., 1998). Those studies brought them the Nobel Prize in 2006 since understanding of RNA interference opened up new approaches in medicine.

RNA interference mechanism requires presence of a double stranded RNA (dsRNA) that is complementary to target mRNA sequence. This dsRNA can either be endogenously produced from the organism's genome itself (called microRNA) or exogenously introduced (called siRNA) by several transfection methods. Once dsRNA is in the cytoplasm, it interacts with an RNase III called Dicer and cleaved into 21-25 nt long small RNA duplexes as illustrated in Figure 1.8 (Doi et al., 2003). This duplex then is incorporated into a protein complex RNA Induced Silencing Complex (RISC). One stranded of dsRNA (sense strand) is selected by RISC complex and other strand is degraded by an Argonaute protein Ago2, which is the component of RISC. This final step activates RISC complex. Active RISC induces cleavage of mRNA molecules that are 100% complementary to guide strand. Consequently, target gene silencing is achieved (Bumcrot et al., 2006).

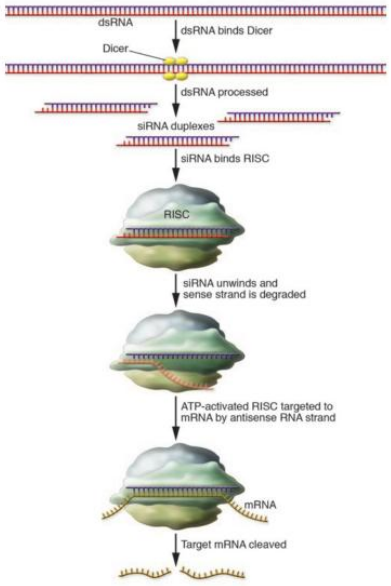


Figure 1.8 Mechanism of RNA Interference that utilized synthetic siRNAs to silence target mRNA. Adapted from (Gewirtz et al., 2007).

Although RNA interference raised great hopes for its therapeutic use, there are several limitations to that such as low stability of siRNA duplexes in serum and cytoplasm, potency and target cleavage efficiency of siRNA molecules and off target gene silencing effects.

In order to solve first problem, chemically modified siRNAs are produced. Addition of phosphorothioate backbone linkage at 3' end of duplex increases exonuclease resistance. In addition, endonuclease resistance is achieved by 2'-*O-methyl* and 2'-*fluoro* modifications. Those two modifications increased both serum and cytoplasmic stability and potency of introduced siRNA duplexes. Another approach is 4'-*thioribose* modification which also significantly increased stability. All of those modification are shown not to interfere with RISC loading and silencing efficiency (Bumcrot et al., 2006). Potency and target cleavage efficiency of siRNA is increased by rational siRNA design and some further chemical modifications increase guide strand loading to RISC. It has been shown that the structure of siRNA sequence is important for potency, target recognition and cleavage. Taking enough effort to design siRNA sequence to meet the criteria in Figure 1.9 is beneficial to have a perfectly designed dsRNA. In order to avoid off-target silencing, seed region of siRNA (Fig 1.7) should only be complementary to

target mRNA. Furthermore, 2'-O-Me modification at nucleotides neighboring seed region is claimed to reduce off-target silencing.

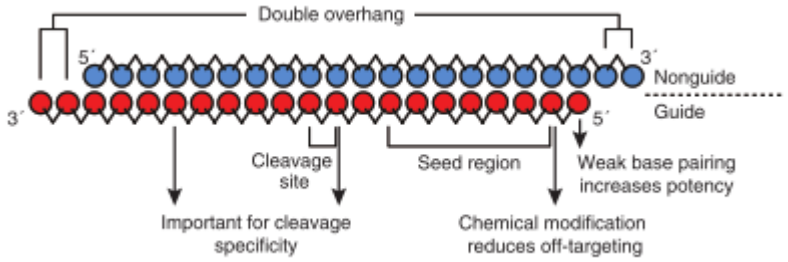


Figure 1.9 Representation of siRNA structure to achieve maximum target identification and cleavage and minimize off-target effects.

Small interfering RNAs are used for transient silencing of target genes. In order to attain stable silencing, vectors coding for siRNA sequence, known as short hairpin RNA (shRNA), are transfected into cells. Other molecules including miRNAs and hammerhead ribozymes are used for reversal of MDR. miRNAs are 18-25 nt long dsRNAs encoded by the genome itself. It is processed with the same mechanism as siRNA and incorporated into RISC. Then, it represses translation from target mRNA rather than inducing its cleavage. Hammerhead ribozymes are catalytic that cleaves target mRNA (Abbasi, 2013).

In vitro and *in vivo* studies are done showing that Multidrug resistance can be reversed via RNAi. IL6 and IL8 production was inhibited in Doxorubicin resistant MCF-7 cell line by shRNA vectors to reverse multidrug resistance (Shi et al., 2012). In another study, siRNAs targeting both Mcl-1 and P-gp were introduced into Doxorubicin resistant MCF-7 cells with polymeric carriers to reverse multidrug resistance and induce apoptosis (Aliabadi et al., 2013). Wu and colloquies directly targeted *MDR1* in same cell line using siRNAs and they achieved successful downregulation of target gene and reversal (Wu et al., 2003). An *in vivo* study showed that tumor can be re-sensitized to Doxorubicin by introducing *MDR1* antisense sequence coding shRNA vector (Stein et al., 2008). It can be inferred that RNAi is effective in reversing MDR both in cell culture and in animal models.

1.6 Aim of the Study

The aim of this study is to reverse multidrug resistance in Doxorubicin resistant MCF-7 cell line by specifically silencing IL-6. This silencing downregulates *MDR1* expression leading to increased drug accumulation in cell. In order to summarize the purposes are listed below:

- Silencing of *IL6* with siRNA to block IL-6 signaling pathway which controls *MDR1* gene expression in Doxorubicin resistant MCF-7 cells.
- Determination of *MDR1* and *MRP1* levels to see if *IL6* silencing specifically dowregulates P-gp dependent drug resistance.
- Determination of secreted IL-6 amount in cell culture medium in response to *IL6* silencing.
- Examination of intracellular Doxorubicin accumulation before and after treatment with *IL6* specific siRNA to see if our treatment effects amount of drug that accumulates in cell.
- Analysis of Inhibitory concentration 50 (IC_{50}) for Doxorubicin before and after silencing of target gene to see if cell sensitivity to drug changes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Lines

Parental MCF-7 human breast adenocarcinoma cell line was kindly donated by Şap Institute, Ankara, Turkey. 1μM Doxorubicin resistant (MCF7/Dox) subline was developed in our laboratory by stepwise increment of dose to final concentration of 1 μM (İşeri Darcansoy, 2009).

2.1.2 Chemicals and Reagents

Doxorubicin was purchased with commercial name Adrimisin (Saba İlaç Sanayi) as 10mg lyophilized drug. It was dissolved in sterile injection water as 3.44mM concentration and stored at 4^{o}C.

RPMI 1640 medium was obtained from Thermo Fisher Scientific, USA. Fetal bovine serum (FBS) and DMSO were bought from Biochrom AG, Germany. Phosphate buffered saline (PBS) and β-mercaptoethanol were purchased from Sigma-Aldrich, USA. Trypsin-EDTA, Trypan blue, Gentamycin and XTT Cell Proliferation Assay Kit were purchased from Biological Industries, Israel. 10nM dNTP set, *Taq* DNA Polymerase, DNase I, 25mM EDTA, RevertAid Reverse Transcriptase, Ribolock, 50 bp DNA ladder, 6X DNA loading dye, High Range RNA Ladder, 2X RNA loading dye were purchased from Fermentas, Lithuania. FastStart Universal SYBR Green Master Kit (Rox) was obtained from Roche Diagnostics, Switzerland. RNA-Spin RNA Isolation kit was purchased from Intron Biotechnologies Inc., Korea. Lipid based transfection reagent was obtained from QIAGEN, Dusseldorf, Germany.

2.1.3 Small Interfering RNAs (siRNAs)

Interleukin 6 siRNA and mock siRNA (Mock siRNA A) were obtained from Santa Cruz Biotechnology, California, USA. Fluorescein conjugate control siRNA was purchased from Cell Signaling Technology Inc. Massachusetts, USA.

2.1.4 Primers

IL6, MDR1, MRP1 and *β-actin* primers were purchased from Alpha DNA, Canada.

Table 2.1 Sequences and amplicon sizes of PCR primers

Gene	Primer Sequence	Amplicon Size
IL6	F: 5'-TCTCCACAAGCGCCTTCG-3'	193 bp
	R: 5'-CTCAGGGCTGAGATGCCG-3'	
MDR1	F: 5'-ACAGAAAGCGAAGCAGTGGT-3'	62 bp
	R:5'-ATGGTGGTCCGACCTTTTC-3'	
MRP1	F: 5'-TGTGGGAAAACACATCTTTGA-3'	80bp
	R: 5'-CTGTGCGTGACCAAGATCC-3'	
β-actin	F: 5'-CCAACCGCGAGAAGATGA-3'	97bp
	R:5'-CCAGAGGCGTACAGGGATAG-3'	

2.1.5 Antibodies

Human IL-6 ELISA kit was purchased from MABTECH AB, Sweden which includes monoclonal antibody (1 mg/mL), biotinylated monoclonal antibody (1 mg/mL), Streptavidin-Alkaline Phosphatase and recombinant human IL-6 standard (1μg).

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Cell line and Culture Conditions

Both parental MCF-7 cell line and drug resistant MCF-7/1μM Dox cell line are cultivated in 12 mL RPMI 1640 growth medium supplemented with 10%(v/v) FBS and 1%(v/v) Gentamycin in surface treated T-75 filter capped tissue culture flasks (Thermo Fisher Scientific, USA). For

MCF-7/Dox cell line, 24 µL of 500µM Doxorubicin is added to 12 mL of medium to reach final 1µm drug. Cells were incubated at 37°C with humidified atmosphere of 5% CO_2 in a Heraeus incubator (Hanau, Germany).

2.2.1.2 Subculturing

Cells are subcultured when 80% confluency is reached. The subculturing procedure was done as follows. First, medium was discarded and cells were washed with 5-6mL of PBS. Washing step is crucial to remove all waste products and serum ingredients which contain trypsin inhibitors. Then, 1mL of trypsin was added for T-75 filter capped tissue culture flask. Cells were incubated at 37°C, 5% CO_2 for a few minutes to allow detachment from the surface of the flask. Extensive incubation may be harmful for cells as trypsin digests surface proteins. Serum supplemented medium (3mL) was added to cells to inactivate trypsin. Cell suspension was transferred to 15 mL Falcon tube (Greiner) and centrifuged at 1000 rpm for 5 minutes to get the cell pellet. Supernatant was removed and cells were resuspended in 3 mL PBS to remove any traces of trypsin. Cells were spinned at 1000 rpm for 5 minutes one more time and resuspended in 3 mL of growth medium. Proper amount of cells were transferred to another T-75 tissue culture flask and total volume was completed to 12 mL by adding supplemented medium. Doxorubicin was added to medium of MCF-7/Dox cell line as described previously.

2.2.1.3 Storing Frozen Cells

Cells were frozen and stored in liquid nitrogen for long time storage. Briefly, cells were trypsinized and washed with PBS as previously described. After discarding PBS, cells were resuspended in optimum amount of freezing medium (10% (v/v) DMSO, 90% (v/v) FBS) to reach final cell concentration of $2x10^6$ cells/mL. 1 mL of cell suspension was transferred to one cryovials (Greiner Bio-One, Germany) and incubated at -20°C for few hours. Then, cryovials were transferred to -80°C for overnight incubation. As final step, cryovials were taken to liquid nitrogen tank where they can be stored for several years.

2.2.1.4 Thawing Cells

Cells in cryovials were obtained from liquid nitrogen tank and incubated at 37°C just to allow thawing. As soon as cells were thawed, they are transferred to a 15 mL falcon tube and 2 mL of medium was added on them. Then, they were centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded; cell pellet was resuspended in 1 mL of cell culture medium and transferred to filter capped T25 tissue culture flasks (Thermo Fisher Scientific, USA). 5mL of medium was added to reach final volume of 6mL. Cells were incubated as described previously.

2.2.1.5 Viable Cell Count

Cells were harvested with trypsin and washed once with PBS. Then, depending on the size of cell pellet, cells were resuspended in 3-4 mL of supplemented medium. Cell suspension (180µL) was taken into 1.5 mL Eppendorf tube and mixed with 20µL of Tryphan Blue with a ratio of 9/1. Thoma hemocytometer (Marienfeld, Germany) was obtained and surface was humidified to allow firm adhesion of coverslip. Stained cell suspension (10µL) was loaded to each chamber of hemocytometer. Cells were visualized under the light microscope at 40X magnification and counted. Thoma hemocytometer is composed of 16 big squares each of which is made of 16 smaller squares with volume of 0.00025 mm^3. Cells in all of those 16 big squares were counted in both chambers. Average number of cells was divided into 16 to find number of cells in one big square and further divided to 16 to find average number of cells in the smallest square with volume of 0.00025 mm^3.

2.2.2 Transfection

2.2.2.1 Optimization of Transfection Efficiency

MCF-7/Dox cells are harvested as described. Viable cell count is done and $2.0x10^5$ cells are seeded in 2300µL of medium supplemented with 10% (v/v) FBS and 1% (w/v) Gentamycin to each well of a 6-well plate (Greiner) shortly before transfection. Fluorescein conjugate control siRNA (10µM) amount was adjusted to give final concentration of 1nM, 2nM, 4nM, 5nM, 8nM and 10nM when added onto the cells. All plastic disposables used for this procedure was pre-treated with DEPC. Required amount of siRNA was mixed with 12 µL of transfection reagent (HiPerFect) in a 1.5 mL Eppendorf tube and volume was completed to 110 µL by adding serum-free culture medium. Mixture was vortexed well and incubated for 10 minutes at room temperature to allow formation of complexes. Then, 100 µL of the mixture was added drop wise onto the cells. Cells were incubated at 37°C, 5% CO_2 for 20 hours.

Once the incubation period was completed, medium was discarded and cells were washed with PBS twice. Cells were harvested and pellet was washed as described before and cell pellet was resuspended in 200 µL of PBS with 0.1 %(w/w) Sodium Azide and 1 %(w/v) Bovine serum albumin. Then, cells were analyzed with an Accuri flow cytometer (BD Biosciences, USA) using FL1 vs. side scatter dot plots.

2.2.2.2 Gene Silencing in 6-Well Format

MCF-7/Dox cells were harvested from T-75 tissue culture flask as described. Viable cell count was performed. $2.0x10^5$ cells were seeded in 2300 µL of RPMI 1640 supplemented with 10% (v/v) FBS and 1% (w/v) Gentamycin in 6-well plate format. All plastic disposables used for this procedure was treated with DEPC. Required amount of *IL6* siRNA (10µM) and mock siRNA to give 5nM final concentration in culture plate was mixed with 12µL HiPerFect in separate 1.5 mL Eppendorf tubes and volume is completed to 110µL with serum free-RPMI 1640. The mixture was vortexed well and incubated for 10 minutes at room temperature to allow formation of complexes. Then, 100 µL of the mixture was added drop wise onto the cells. Cells were incubated at 37°C, 5% CO_2 for 24, 48 and 72 hours.

2.2.2.3 Gene Silencing in 96-well format

All plastic disposables used for this procedure was treated with DEPC. Required amount of *IL6* siRNA (10µM) and mock siRNA to give 5nM final siRNA concentration in culture plate were mixed with 0.75µL of HiPerFect in separate 1.5 mL Eppendorf tubes and volume is completed to 50.7µL with serum free-RPMI 1640 for each well. Tubes are vortexed well and incubated 10 minutes at room temperature. The mixture (50µL) was added to each well of 96-well plate. MCF-7/Dox cells were harvested and viable cell count was performed. $1.0x10^4$ cells were seeded on top of siRNA-liposome complex in each well. Cells were incubated at 37°C, 5% CO_2 for 48 hours.

2.2.3 Reverse Transcription - Quantitative Polymerase Chain Reaction (RT-qPCR)

2.2.3.1 Total RNA Isolation

Total RNA was isolated with RNA-Spin Total RNA Extraction Kit (Intron Biotechnologies, Korea) according to manufacturer's instructions. Briefly, cells are harvested with trypsin and cell pellet was washed with PBS as previously described. β-Mercaptoethanol (3.5µL) was added per 350µL of lysis buffer. PBS is discarded and cell pellet was dissolved in freshly prepared lysis buffer. Vortexed vigorously for 30 seconds, incubated 10 minutes at room temperature and vortexed again for 30 seconds to ensure proper lysis of cells. Ethanol (70% v/v, 350 µL) was added onto cell lysate, mixed slowly by pipetting and loaded to silica membrane spin columns. Columns were centrifuged for 30 seconds at 13000 rpm. Flowthrough was discarded. Washing buffer A (700µL) was added to columns and centrifuged at 13000 rpm for 30 seconds, flow through was discarded. Then, columns were washed with 700µL of washing buffer B, spinned at 13000 rpm for 30 seconds. Flow through was discarded and columns were spinned again at 13000 rpm for 2 minutes to remove any residual ethanol. Columns were transferred to new 1.5

mL collection tubes. Elution buffer (40µL) was added onto the columns, incubated for 1 minute and centrifuged at 13000 rpm for 1 minute.

2.2.3.2 Quantitation of RNA

Concentration and purity of RNA was measured using Nanodrop (Thermo-Fisher Scientific, USA). RNA (1µL) was loaded on arm of Nanodrop and absorbance was determined at different wavelengths.

Nucleic acids absorb light at 260 nm. Proteins absorb light at 280 nm while small molecules absorb at 230 nm. 260/280 ratio is expected to be between 1.8 and 2.0 for an RNA solution free of proteins and 260/230 ratio is expected to be between 2.0 and 2.2 if the RNA preparate was free of salt, phenol and other chemical contamination.

2.2.3.3 Agarose Gel Electrophoresis of RNA

Agarose gel electrophoresis of RNA was carried out to check for integrity of isolated RNA. Agarose powder (1mg) was measured and dissolved in 50 mL of 1x TAE buffer by heating in microwave for 3 minutes to prepare 1.2% Agarose gel. Agarose solution was cooled down and 3.5 µL of Ethidium Bromide was added to it and mixed well. Solution was poured to casting tray avoiding formation of bubbles, comb was carefully placed. Solution was led to cool down to form agarose gel. RNA (4 µL) was mixed with 4µL of 2x RNA loading dye (Fermentas, Lithuania) and loaded to the wells. RNA was run at 80V for 40 minutes and bands were visualized by exposing to UV.

2.2.3.4 DNase Treatment

RNA was subjected to DNase I treatment prior to cDNA synthesis to remove any DNA contamination according to manufacturer's instructions. Briefly, 500ng of RNA was obtained. DNase buffer (1µL) and 1 µL DNase I (Fermentas) were added. Volume was completed to 10µL. Mixture was incubated at 37^{o}C for 40 minutes. EDTA (1µL) was added to inactivate DNase I and further incubated at 70^{o}C for 10 minutes to heat inactivate the enzyme. Preferentially, RNA samples were used for cDNA synthesis without storing but, if required, stored at -80^{o}C until need.

2.2.3.5 cDNA Synthesis (Reverse Transcription)

Gene specific primer for *IL6*, *MDR1*, *MRP1* and *β-actin* (20pmol) and nuclease free water was added to DNase treated RNA sample with final volume of 12.5 µL in 0.5 mL Eppendorf tube (Greiner). Incubated at 65^{o}C for 5 minutes to disturb secondary structures of primers. Reverse Transcriptase buffer (4µL, 5x), 2µL of 10mM dNTP mix, 0.5µL of Ribolock (Fermentas) and 1µL of RevertAid Reverse Transcriptase were added. Samples were incubated at 42^{o}C for 60 minutes, 70^{o}C for 10 minutes. cDNAs were diluted for 5 times and stored at -20^{o}C until use.

2.2.3.6 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Real time PCR was performed in Rotor Gene-Q (Qiagen, Germany). Amplification of products were observed with SYBR green, a fluorescent agent used for nucleic acid visualization (Light-Cycler – Fast Start DNA Master SYBR Green I kit, Roche Diagnostics) according to manufacturer's instructions.

Reaction mixture was prepared in sterile 0.2mL Eppendorf tubes suitable for detection of SYBR green signal. Briefly, 10µL of 2x SYBR green reaction mix, 3µL of cDNA, and 0.2µL of both forward and reverse primers of respective gene are mixed in Eppendorf tube. Cycling conditions for *IL6*, *MDR1*, *MRP1*, and *β-actin* are given in Table2.2. Each sample was prepared in triplicates. One reaction mix was prepared which contains nuclease free H_2O instead of cDNA as no template control.

In order to determine whether PCR products are expected ones, melting analysis was carried out after amplification was completed. The device was programmed to ramp the temperature from 55^{o}C to 99^{o}C by 1^{o}C and measure changes in fluorescence intensity. PCR products amplified using a particular primer were expected to have same melting temperature unless primers formed dimers, annealed to unexpected site or due to contaminants.

Table 2.2 RT-qPCR amplification conditions for *IL6*, *MDR1*, *MRP1* and *β-actin* primers.

Pre-incubation	95°C, 10 min
Denaturation	94°C, 15 sec
Annealing- Extension	60 °C, 1 min
Melting	55-99°C
Cycle number	40

2.2.3.7 Quantitation of qRT-PCR Products

Delta delta Ct ($2^{-\Delta\Delta Ct}$) relative quantitation method was used to quantify qPCR products by normalizing to *β-actin* gene. Results were expressed as fold changes.

2.2.4 Enzyme Linked Immuno Absorbent (ELISA) Assay

Gene silencing protocol was followed as described before. After 24h, 48h and 72h treatment, 200µL of culture medium was collected and stored at -80°C until use. For coating with monoclonal human IL-6 antibody, 50µL antibody solution was added to each well of 96-well Immulon 2B plates (Thermo Labsystems, USA). Plates were gently tapped for equal distribution of solution and incubated overnight at 4°C. Then, 200 µL of blocking buffer was added and further incubated for 2 hours at room temperature. Plates were washed with ELISA wash buffer for 5 times and with ddH_2O for 5 times. Plates are dried by tapping.

50 µL of supernatants and recombinant IL-6 standard was added to the wells. Recombinant IL-6 standards were serially diluted as 1:2 with 50 µL 1X PBS with 11 repeats. Plates were indubated for 24 hours and washed as before. Biotinylated-secondary antibody solution was diluted as 1:1000 in T cell buffer and 50 µL of it was added to the wells. Plates were incubated overnight at 4°C and washed as explained before. Then, 50 µL of freshly prepared, 1:5000 diluted streptavidin-alkaline phosphatase solution (SA-AP) was added to the wells, incubated for 2 hours at room temperature and washed as before.

A para-Nitrophenylphosphate (pNPP) tablet was dissolved in 4 ml ddH2O. 50 µL of this solution was added to the wells for developing the plates. Color development was determined at 405 nm at different time points with Multiskan FC Microplate Photometer (Thermo Scientific,

USA). Measurements are taken with the instrument until recombinant IL-6 standards reach a four parameter saturation forming an S-shaped curve. IL-6 concentration in supernatants was calculated using this standard curve.

2.2.5 Doxorubicin Accumulation Measurement

Gene silencing protocol was followed as described before. 24h, 48h and 72h after treatment, medium is discarded and cells were washed twice with PBS. 2 mL of culture medium was added to each well. Doxorubicin (8μM) was added to freshly added medium and incubated for 1 hour. Then, cells were harvested as done in passaging. The cell pellet was washed with PBS once and resuspended in 200 μL of PBS with 1% BSA and 0.1% NaAzide as fixative solution. Finally, cells were analyzed with Accuri Flow Cytometer in FL-2 channel.

2.2.6 Determination of Cell Proliferation with XTT Reagent

Cell proliferation was detected with XTT reagent according to manufacturer's instructions.

Briefly, $1x10^5$ MCF-7/Dox cells were seeded in 150μL culture medium to each well of flat bottomed, surface treated 96-well plate (Greiner) starting from the second column. First column contained only culture medium (medium control) and top and bottom rows contained only Doxorubicin and medium, which was used as medium control of Doxorubicin, since Doxorubicin absorbance interferes with that of formazan. In addition, second column contained untreated cells and medium only which is used as cell control.

Plates were incubated for 24 hours. Medium was discarded and cells were washed twice with 50μL of PBS. Culture medium (50μL) was added to all wells except 3rd column. Next, serial dilution of drug was performed. Simply, 200μL concentrated Doxorubicin was added to all wells of 3rd column. Then, 150μL of drug from 3rd column was taken and mixed with the medium in the next row for the rest of the plate. All volumes were completed to 150 μL by adding culture medium and incubated for 48 hours.

In addition, MCF-7/Dox cells were transfected with *IL6* or mock siRNA in 96-well format. After 24 hours, cells were subjected to same serial dilution.

Then, XTT reagent was prepared and added on cells. Incubated for 4 hours to allow formation of soluble, colored tziolium salts and absorbance was measured at 492 nm using Multiskan FC

Microplate Photometer (Thermo Scientific, USA). Background absorbance was measured at 620 nm with same device.

Cell proliferation curve was constructed for each plate using the absorbance values. Average intensity of dye was calculated for each row without including top and bottom wells. Average intensity of top and bottom wells was subtracted from calculated value. Same approach was applied to second column which contained untreated cells. Absorbance values obtained from untreated was used as 100% survival. Viability of rest calculated accordingly. IC_{50} values, resistance indices (R) and fold reversal (FR) values were calculated for each treatment and cell line. Formulas for all calculations are given below:

$$\mathbf{R = IC_{50}\ of\ resistant\ cells / IC_{50}\ of\ sensitive\ cells} \qquad \text{Equation 2.1}$$

$$\mathbf{FR = IC_{50}\ of\ resistant\ cells / IC_{50}\ of\ IL6\ siRNA\ treated\ cells} \qquad \text{Equation 2.2}$$

2.2.7 Statistical Analysis

Results of three independent experiments were analyzed with GraphPad Prism V.5 (GraphPad Software Inc, USA) with one way ANOVA and Tukey's test as post test with defined $p<0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Isolation of Total RNA

RNA is isolated with RNA Isolation kit (Intron, Korea) and visualized on 1.2% Agarose gel stained with Ethidium Bromide to see if it is suitable for cDNA synthesis (Figure 3.1). Since mRNA comprises only 4% of total RNA, they cannot be visualized on agarose gel. Therefore, characteristic bands formed by ribosomal RNA (rRNA) are used to deduce quality of RNA for subsequent cDNA synthesis. Successfully isolated intact total RNA forms two bands in agarose gel which correspond 28S and 18S rRNA ("Evrogen Technologies: RNA electrophoresis protocol," n.d.). The ratio of intensity of upper band to lower should be around 2 for a good quality of RNA. The additional band seen on very top of the gel is because of genomic DNA contamination. This RNA is isolated using silica membrane spin columns which allows filtration of nucleic acids of certain size. Therefore, only fragmented gDNA passes through column and forms this band. In order to eliminate possible contamination of gDNA, total RNA isolate is treated with DNase I prior to cDNA synthesis.

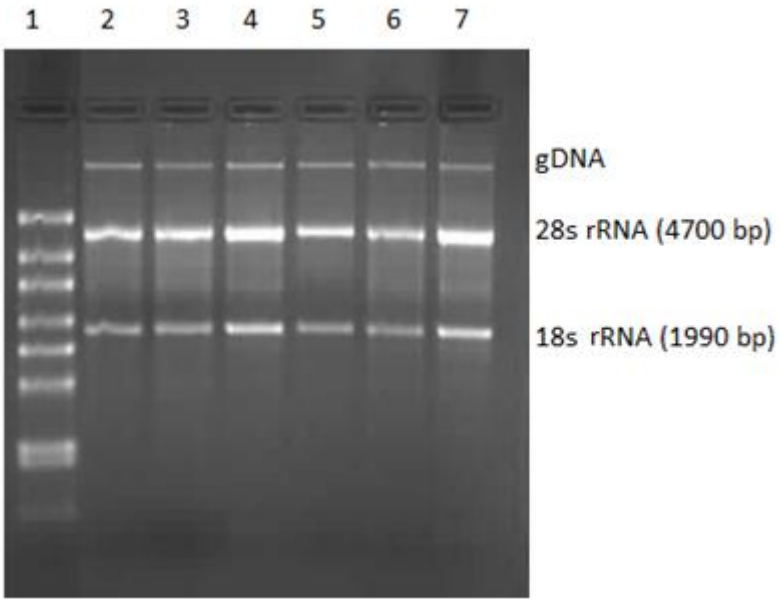


Figure 3.1 Total RNA Isolated from MCF-7/Dox cell line (Lanes 2-7). 4µL was mixed with 4µL of 2X RNA loading dye and loaded into the wells. 4µL of High range RNA Ladder was loaded to first lane.

Purity and nucleic acid concentration of RNA samples is obtained by spectrophotometric analysis by measuring absorbance at wavelengths 260nm, 280nm and 230nm which are maximum absorbance wavelengths for nucleic acids, proteins and small chemicals, respectively. Table 3.1 shows concentration of RNA isolates and 260/280 ratios as well as 260/230 ratios which are indicatives of RNA purity. The concentrations of all samples are enough to synthesize cDNA for subsequent qPCR steps. All 260/280 ratios are above 1.8 which shows that those RNA samples are free of protein contaminants. In addition, 260/230 ratios are above 2.0 which means RNA isolates do not have any salt or phenol contaminations due to RNA isolation procedure. Therefore, those RNAs are pure enough for efficient conversion to cDNA.

Table 3.1 Absorbance results of RNA isolates indicating nucleic acid concentration and purity of samples.

Sample	Nucleic Acid Conc. (ng/µl)	260/280	260/230
Control	142,7	2,15	2,33
***IL6* siRNA 24h**	154,2	2,08	3,05
IL6 siRNA 48h	203,6	2,08	3,13
IL6 siRNA72h	268,6	2,07	2,71
Mock siRNA 24h	154,6	2,05	2,36
Mock siRNA 48h	187,6	2,07	3,36
Mock siRNA 72h	255,5	2,08	2,75

3.2 Determination of *IL6* expression by Polymerase Chain reaction

A previous microarray study done at our laboratory indicates that *IL6* expression increases up to 80 fold in MCF-7/Dox cells (İşeri Darcansoy, 2009). However, this finding was not validated by PCR before. As it can be seen from Figure 3.2, parental MCF-7 cell line has very low level of *IL6* expression almost not detectable by conventional PCR and agarose gel electrophoresis. On the other hand, Doxorubicin resistant MCF-7 cells have significant expression of *IL6* as it forms very bright band in gel. IL-6 signaling was proven to control *MDR1* expression in MCF-7 cell line by activating C/EBP-β and cause MDR phenotype via *MDR1* upregulation. Therefore, we proceeded to silencing studies to observe any potential effect of *IL6* silencing on MDR phenotype.

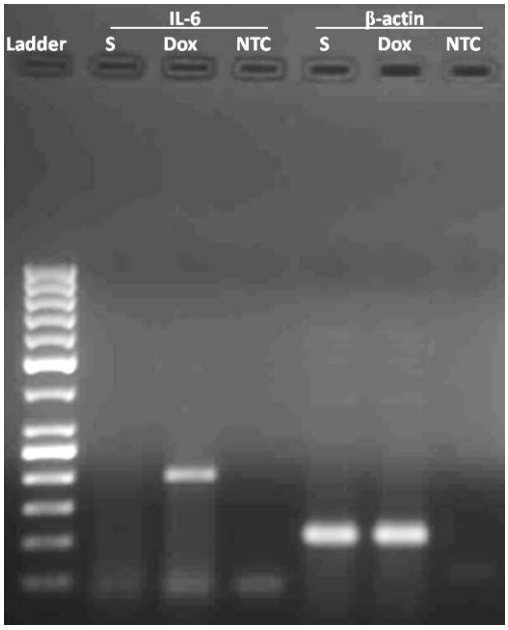


Figure 3.2 PCR products of *IL6* (192 bp) and *β-actin* (97 bp) genes from MCF-7 and MCF-7/Dox cell line run on 2% agarose gel. S: parental MCF-7 cell line, Dox: MCF-7/Dox cell line, NTC negative control. 50 bp DNA ladder is loaded to first lane.

3.3 Optimization of Transfection Efficiency

In order to proceed with gene silencing studies, transfection efficiency must be optimized first for highest level of gene silencing with minimized off-target silencing effects. In order to do that, cells were transfected with different concentrations of Fluorescein conjugate control siRNA and fluorescence emitted from cells was analyzed with flow cytometer. Figure 3.3 represents the percent of cells that gives fluorescent signal after treatment with different siRNA concentration. According to the results, significant portion of MCF-7/Dox cells receive siRNA even at lowest siRNA concentration (1nM) compared to non treated cells. The percent of cells that gives fluorescent signal keeps increasing with increasing siRNA concentration. Half of the cells are transfected successfully when 5nM siRNA concentration is used. This value reaches 60 percent for 8nM siRNA concentration.

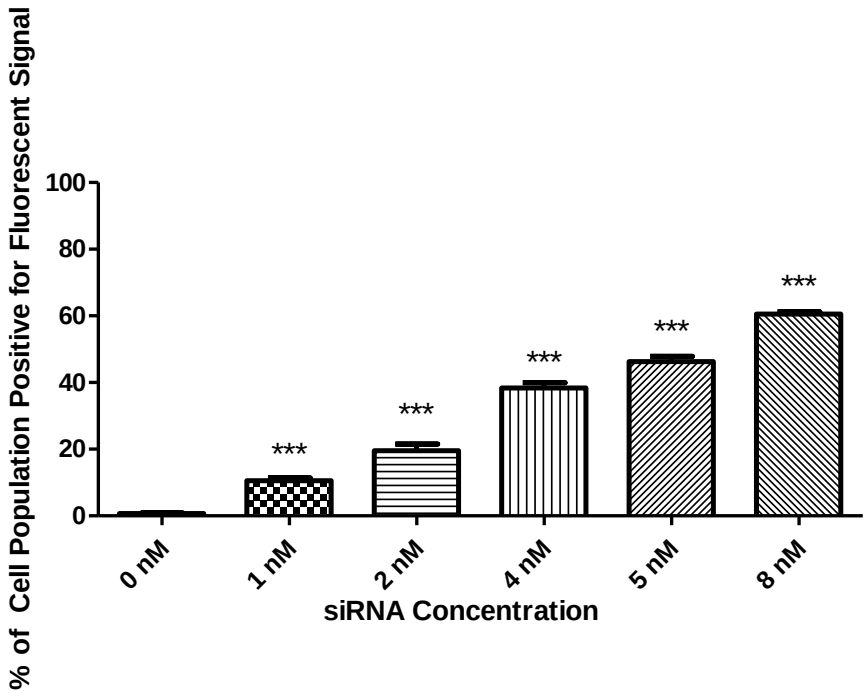


Figure 3.3 Results of transfection efficiency optimization studies. Around 50% MCF-7/Dox cells were transfected successfully with 5nM of Fluorescein conjugate siRNA. (***p<0.0001)

There are different approaches to determine optimum transfection efficiency. One method is to transfect cells with different concentrations of a siRNA targeting a known gene such as GAPDH. After proper amount of time, changes in expression level of target gene for each siRNA concentration is detected with qPCR to see which siRNA concentration is best for maximum knockdown ("Optimizing siRNA Transfection for RNAi | Life Technologies," 2013). Another approach is first to transfect cells with a vector coding Green Fluorescent Protein (GFP) and co-transfect GFP specific siRNA at different concentration. Silencing efficiency is then determined by decrease in GFP signal obtained from the cells. It reflects the effect of siRNA delivery at protein level ("How to know siRNA transfection efficiency? - siRNA, microRNA and RNAi," 2006). Final method is the one that we applied which is delivery of fluorescently labeled siRNA and measuring fluorescence intensity emitted from cells either by fluorescence microscopy or flow cytometry. This method is faster, cheaper than and as accurate as others. One drawback is that, it only tells how much of the cells receive the siRNA and does not give silencing efficiency of target gene("BLOCK-iT^{TM} Fluorescent Oligo as RNAi Transfection Control | Life Technologies," 2013).

Off-target silencing activity of siRNAs is one of the biggest problems of siRNA mediated gene silencing and it should be taken into consideration when evaluating siRNA transfection efficiency. It may lead to unintended effects of siRNA delivery. It was first discovered by Jackson et al.. They designed 8 different MAPK14 siRNA and analyzed expression profiles of HeLa cells after transfection. They also included different concentrations of siRNAs to see if high siRNA concentration significantly induces off-target gene silencing. They indeed observed significant off-target gene silencing at highest siRNA concentration (100nM), however, results also showed that even lowest siRNA concentration (1nM) was enough to induce off-target gene silencing (Jackson et al., 2003) mainly due to seed region complementarity to 5' sequence of other mRNAs (Jackson et al., 2006).

The reason for off-target activity can be examined in two groups. First, siRNAs may cause miRNA-like off target activity if seed region is complementary to 5' end of any mRNA. In addition, it is also demonstrated that high siRNA concentration is capable of inducing miRNA-like off-target activity independent from seed region complementarity. In order to avoid it, chemically modified siRNAs are used. In addition, when a pool of different siRNAs targeting same mRNA is used for silencing, it was observed that off-target silencing is reduced. Second, siRNAs may saturate RNAi machinery if very high concentration is delivered to the cell. They may block miRNA-RISC complex formation which alters miRNA mediated gene regulation leading to unintended effects. This alteration may cause significant changes in phenotype (Jackson & Linsley, 2010).

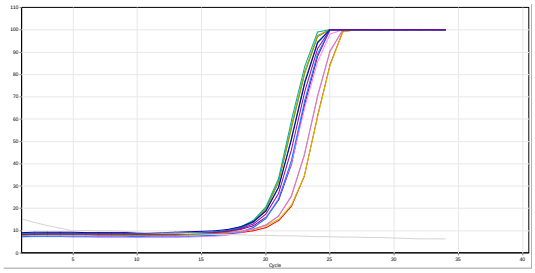
IL6 siRNA that we used is composed of a pool of three different siRNAs with length of 19-25 nucleotides all targeting *IL6*. It is a factor that reduces off target-silencing. In addition, we choose 5nM siRNA concentration for the rest of the silencing experiments since this concentration is enough to transfect 50% of cells. Subsequent qPCR studies also showed that 5nM of *IL6* siRNA is enough to induce significant target knockdown (See section 3.4).

3.4 Quantitative Real-Time Polymerase Chain Reaction (qPCR): Expression analysis of *IL6, MDR1* and *MRP1* genes

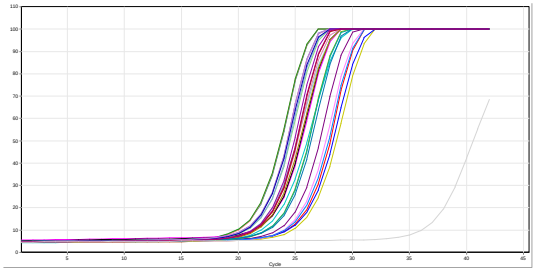
MCF-7/Dox cells are shown to express high levels of IL-6 compared to parental MCF-7 cell line (Figure 3.2). IL-6, expression of which is known to induce multidrug resistance in cancer cells, is silenced using siRNA. Amplification curves for all genes are in exponential shape and shows that fragments are amplified ideally (Figure 3.4). In order to confirm that correct fragment is amplified with qPCR reaction, melt curve analysis is done for all samples, which makes use of the fact that each fragment has a specific melting temperature depending on the GC content and length. According to Figure 3.5/a, products obtained from amplification of *IL6* yields a single peak for all tubes. Observing a single type of peak also indicates that there are not any non-specific products. This is also valid for *MDR1*, *MRP1* and *β-actin* genes. In all melt curves, there

is a wide peak at around 55^{o}C which is the indicative of formation primer dimers during last cycles of the amplification.

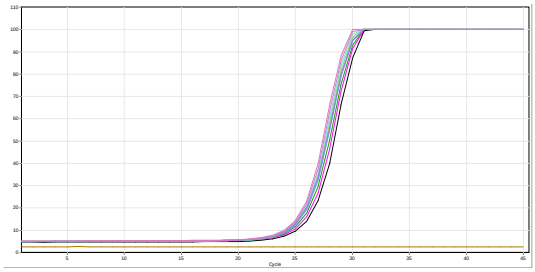
a)



b)



c)



d)

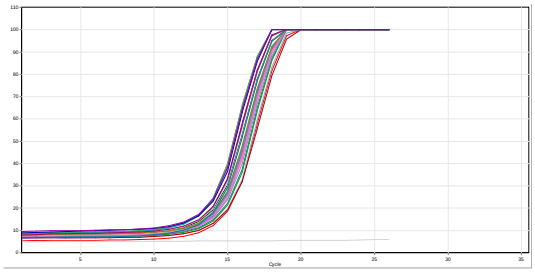
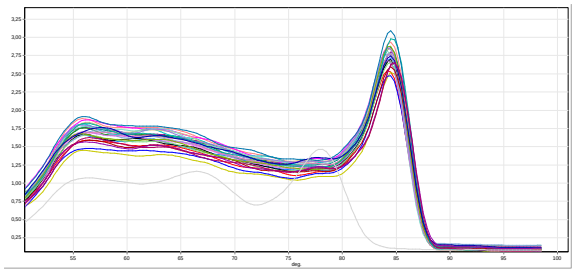


Figure 3.4 Amplification curves for a) *IL6* b) *MDR1* c) *MRP1* d) *b-actin* genes. All genes are amplified efficiently as seen from curves reaching plateau.

a)



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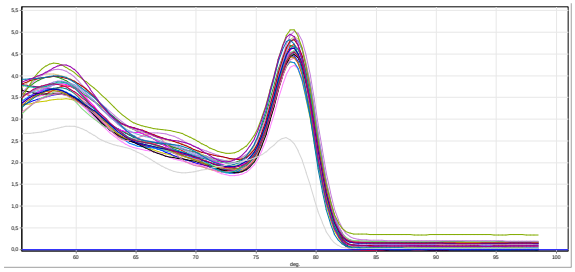

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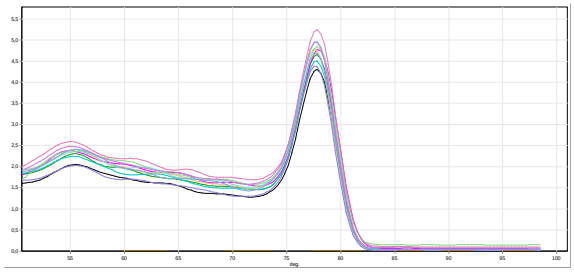

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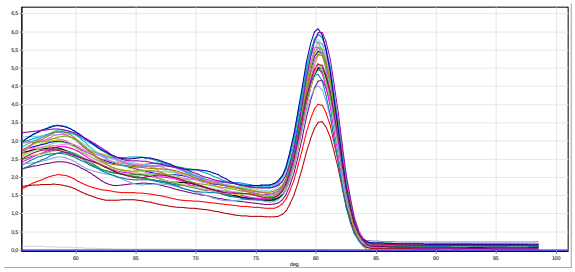

Figure 3.5 Melt curves for a) *IL6* b) *MDR1* c) *MRP1* d) *b-actin* genes. In all reactions, single peak is observed indicating that specific fragment is amplified. There is not any non-specific product amplification.

qPCR data is analyzed with $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) to obtain fold changes as *IL6*, *MDR1* and *MRP1* genes are normalized to *β-actin* gene. Results of the analysis show that treatment with siRNA effectively induces target cleavage so that levels of *IL6* mRNA decreases.

According to Figure 3.6, *IL6* mRNA levels decreases to 50% of initial value after treatment with *IL6* specific siRNA for 24 hours compared to untreated control samples. The maximum transfection efficiency is achieved when cells are treated with 5nM of siRNA. At this siRNA concentration, 44.5 % of cells receive siRNA (Figure 3.3). Since RNA is isolated from whole cell population and only half of the population receives siRNA, it is expected that maximum decrease in target mRNA level could be 0.5 fold in total cell population. In addition, statistical analysis of data indicates that 50% decrease in *IL6* level is significant. Therefore, it could be stated that siRNA treatment effectively reduces *IL6* mRNA level.

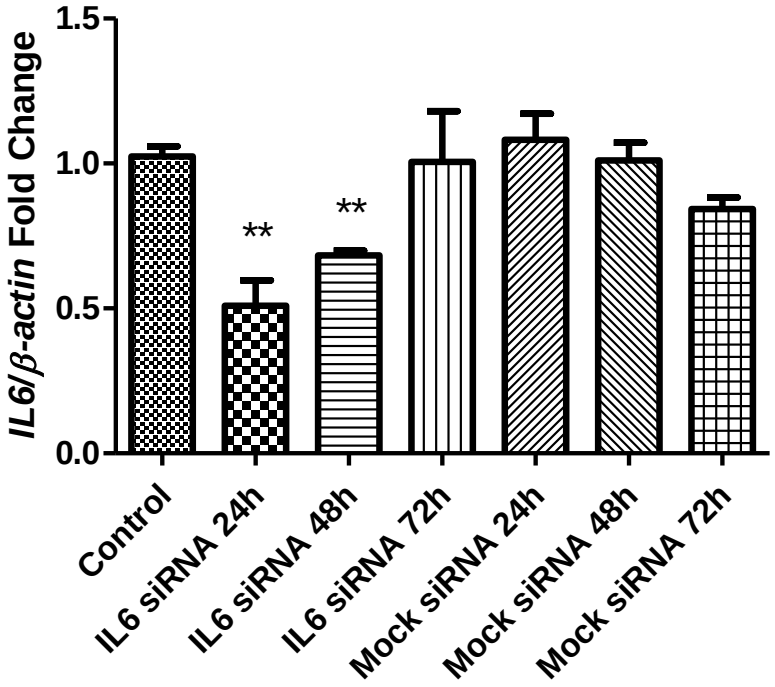

Figure 3.6 *IL6* gene expression levels at various time points after treatment with *IL6* specific siRNA. All data is represented as mean with SEM while **$p<0.05$ is compared to non treated control samples.

24 hours after siRNA delivery, level of *IL6* mRNA starts to increase. 48 hours later, the level is 68% of the control and reaches to initial value at 72 hours. There are several reasons for this increase in time even if there are anti-sense duplexes in the cell culture. First, our Doxorubicin resistant MCF-7 cell line's doubling time is 51.5h (İşeri Darcansoy, 2009). Therefore, siRNA concentration in cell remains almost same at 48 hours of treatment while it decreases at 72 hours due to cell division. Accordingly, the rate of increase in *IL6* mRNA level is more drastic between 48 hours and 72 hours time points compared to between first two time points. Analysis of siRNA concentration in non-dividing cells after transfection showed that siRNAs are still presents in cells 2 weeks after delivery showing the impact of cell division on siRNA-RISC complex decay (Mantei et al., 2008). Secondly, this rapid increase in *IL6* mRNA is due to the positive autocrine loop of IL6 signaling. IL-6 induces its own transcription. This signaling pathway operates in cell by inducing STAT3 phosphorylation. In downstream, STAT3 induces *IL6* expression leading to continuous expression and secretion of IL-6 to cell media (Grivennikov et al., 2008). As expected, mock siRNA treated samples do not exhibit any change in *IL6* expression at any of the time points.

Levels of *MDR1* mRNA positively correlates with changes in *IL6* levels as expected since its expression is regulated by IL-6 signaling. 24 hours after treatment with siRNA, *MDR1* level decreases to 55% of its initial value (Figure 3.7). It has a similar pattern to changes in *IL6* level at 48 hours and 72 hours treatment with *IL6* antisense oligonucleotide. That similarity can be explained by the fact that response to cytokine signaling is very rapid and half lives of most of the signaling components are very short. The rapid dynamics of the signaling pathway can be better understood with following examples. Transcriptional activation of targets can be observed several hours after activation of the signaling pathway (Siewert et. al, 1999). STAT3 homodimer binding to DNA occurs 15 minutes after IL-6/IL-6R association. IL-6 directed acute phase protein expression increases only several hours after induction (Wegenka et al., 1993).

Studies that reverses MDR phenotype by directly silencing *MDR1* shows that average response is obtained at mRNA level about 48 hours/72 hours time interval after transfection (Dönmez et al., 2011; Wu et al., 2003). In this study, we obtained more rapid change in *MDR1* expression in response to IL-6 signaling. Our approach may be more beneficial in terms of delivering drug and reversal agent at the same time to maximize cytotoxic effect of drug on cells.

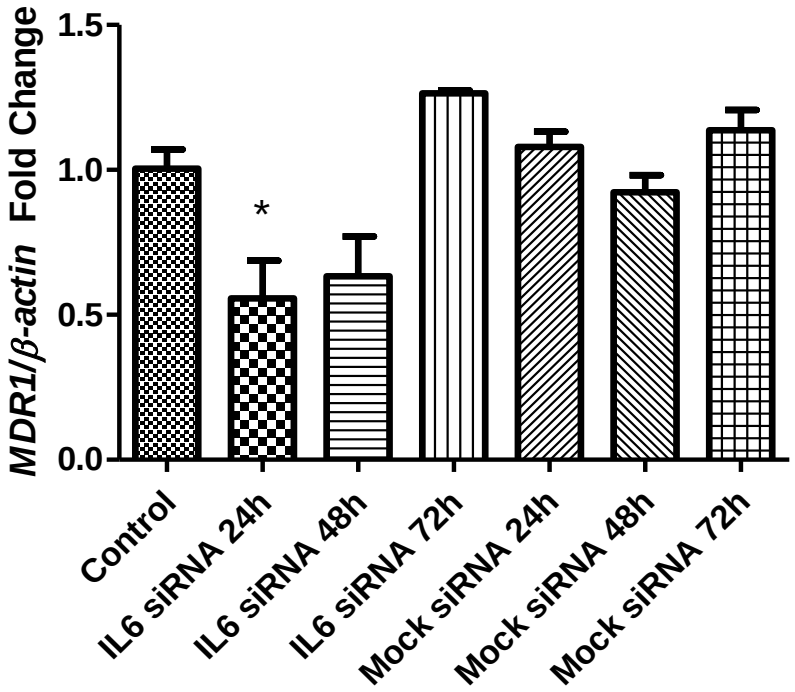

Figure 3.7 *MDR1* gene expression level after treatment with either *IL6* siRNA or mock siRNA at 24h, 48h and 72h.

On the other hand, decrease in *MDR1* level is not as considerable as *IL6* level because siRNA treatment cannot achieve complete inhibition of this signaling pathway. Some of the cells in culture plate continue to express and secrete IL-6 just because they did not receive the antisense oligonucleotide (See section 3.3). Secreted IL-6 can induce paracrine signaling in neighboring cells including the ones that received siRNA. In this case, the pathway still operates in latter cell population resulting in considerably low blockade of the signaling pathway. Therefore, *MDR1* expression is still induced but at a lower extent. In order to have more precise idea for impact of silencing in each cell, phosphorylation level of signaling components can be detected after silencing to monitor the extent of pathway blockade.

There is no change in the expression level *MRP1* gene which is not regulated with IL-6 signaling pathway in MCF-7/Dox cell line (Figure 3.8). This result fits with our expectations.

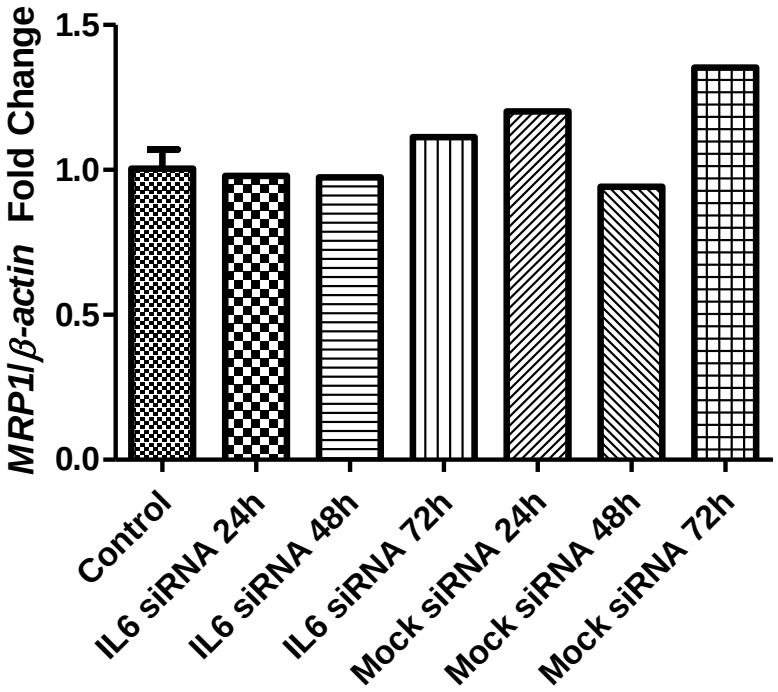

Figure 3.8 *MRP1*expression level of non-treated, *IL6* siRNA or mock siRNA treated MCF-7/Dox cells.

Interleukin-6 is associated with poor prognosis and found elevated in tumor microenvironment of primary breast tumors as well as serum of the patients. Breast tumors produce more IL-6 as tumor grade increase. It is also linked with improved metastatic ability. Normally, ER (+) breast cancer cells do not respond to IL-6 as there is no change in STAT3 phosphorylation after stimulation. Consequently, those cells do not exhibit any cell growth. Mesenchymal stem cells (MSC) are recruited to breast tumor site from bone marrow. As a normal component of tumor microenvironment, MSCs are important sources of IL-6 and promote growth of ER (+) breast cancer cells on the contrary of other evidences (Sasser et al., 2007). Therefore, it can be proposed that effect of IL-6 on ER (+) breast cancer cells is context dependent. More importantly, IL-6 is shown to promote Epithelial-Mesenchymal Transition (EMT) in MCF-7 cells which can be indicative of activation of different downstream effectors in ER (+) cells (Sullivan et al., 2009). *MDR1* expression through IL-6 signaling is mediated by Ras/MAPK pathway which do not require any STAT3 activation (G. K. Chen et al., 2004). ER (+) MCF-7 cells are responsive to IL-6 signaling as stimulation with IL-6 increases *MDR1* level and confers resistance against Doxorubicin (Conze et al., 2001). Furthermore, microarray studies shows that

MCF-7/Dox cell line lost the expression of ER during the process of resistance development (İşeri Darcansoy, 2009). This cell line can be responsive to growth promoting effect of IL-6. In addition, it is not known whether IL-6 production starts first as cells gain resistance or cells stops expressing ER first. Either way, IL-6 is a very important cytokine that influences aggressiveness and chemotherapy resistance of tumor. Targeting this cytokine is a good strategy as several mechanisms making cancer more lethal can be blocked with single type of treatment.

Another striking evidence for the significance of IL-6 signaling pathway on Doxorubicin resistance and failure of chemotherapy is the fact that Doxorubicin is a drug that causes immunogenic cell death of tumor cells (Casares et al., 2005). Generally, chemotherapeutics induces apoptosis which ends up with recruitment of macrophages and effective phagocytosis of apoptotic tumor cells. However, in case of Doxorubicin administration, tumor cells die via Caspase-3 mediated apoptosis and, unlike other apoptotic cells, cause recruitment of Neutrophils possibly due to DAMPs (Casares et al., 2005; Krysko et al., 2011). Since neutrophils are primary sources of IL-6, their recruitment site increases IL-6 level at tumor microenvironment and contribute to aforementioned effects of IL-6. *IL6* silencing agents or IL-6 signaling blockers can be co-administered with chemotherapeutics, especially with Doxorubicin, to have more prominent chemotherapeutic effect and decreased chance of drug resistance development.

RNA interference is widely applied to evaluate effects of IL-6 on disease states, cancer and Multidrug resistance. However, synthetic siRNA duplexes are not preferred frequently for this approach. Instead, short hairpin RNA (shRNA) vectors are introduced to cells in which silencing is not transient but stable. General approach is to select the cells that received the shRNA vector to have at least 95% vector positive cell population (Sakai, Miyake, Terakawa, & Fujisawa, 2011). This method is more effective in terms of cytokine silencing since those small molecules can act paracrine.

Nevertheless, there are evidences that treatment with *IL6* siRNA is significantly effective in cell culture. In a study conducted by Sansone et al., *IL6* is successfully silenced by treating mammospheres from breast cancer tissues with siRNA in *in vitro* (Sansone et al., 2007). In another study, *IL6* specific siRNA knockdown the gene to 20% of control cells in HepG2 cell line 24 hours after treatment (Yuan et al., 2011). The latter study is comparable to ours in terms of the methodology.

3.5 Enzyme Linked Immuno Absorbent (ELISA) Assay: Change in secreted IL-6 level

Enzyme Linked Immuno Absorbent Assay is done to find concentration of a specific antigen in a given sample by using antibodies and color change. In our study, we used this technique to

determine concentration of IL-6 in cell culture medium before and after treatment with *IL6* targeting siRNA or scrambled siRNA.

Figure 3.9 represents the amount of IL-6 secreted to medium by parental MCF-7 cell line. This cell line is known to produce no or very little amount of IL-6 to medium. This is also supported by our PCR results (see Section 3.2) that there is almost no expression of IL-6 by those cells. However, upon developing resistance to Doxorubicin, those cells begin to express and secrete IL-6 (Conze et al., 2001). According to our results, IL-6 level increases by 4.3 fold in drug resistant cells.

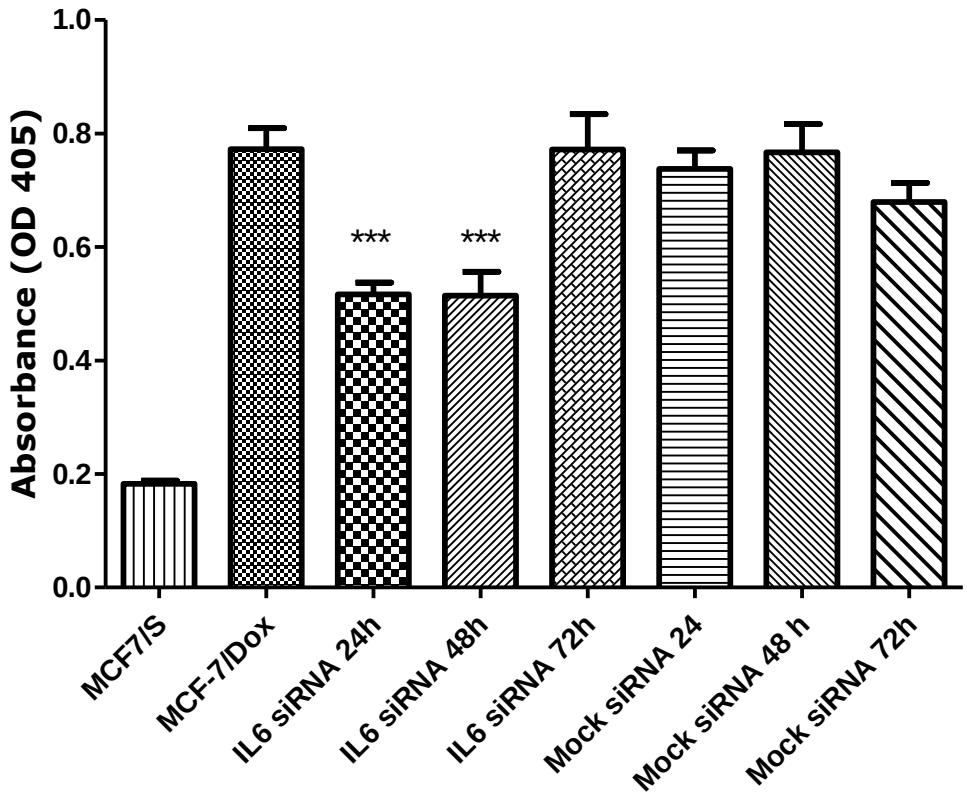

Figure 3.9 ELISA results of cell culture medium collected from MCF-7/S cells and MCF-7/Dox cells including resistant cells treated with either *IL6* siRNA or mock siRNA. *** $p<0.05$

Delivery of *IL6* siRNA to MCF-7/Dox cells decreases target protein level significantly by 36% after 24 hours. In a previous study, it was aimed to silence IL-6 to sensitize Docetaxel resistant prostate cancer cells, PC3. They used shRNA vector against *IL6* where they observed 80% reduction in IL-6 secretion in this stable knockdown system (Sakai et al., 2011). Considering this, our reduction in target level can be evaluated successful in transient knockdown system.

At 48 hours time point, there is not much increase in secreted IL-6 level which is consistent with our qPCR results (see Figure 3.6). At 72 hours, in target specific siRNA delivered cells, IL-6 level in medium reaches to control levels which correlates with gene expression levels. This increase after 48th hour can be explained by doubling time of MCF-7/Dox cells which is 51.5 hours (İşeri Darcansoy, 2009). At 51.5th hour, cells divide resulting in decreased siRNA loaded RISC concentration in each cell. As mentioned before, cytokine transcription and translation acts very quickly in cell. This, in turn, allows cells to restore their *IL6* mRNA level. As expected, mock siRNA treated cells do not display any change in secreted IL-6 level.

3.6 Drug Accumulation Assay

MCF-7 cells gain resistance to Doxorubicin by increasing the expression of P-gp which pumps the drug out of the cell resulting in decreased drug concentration(Gillet & Gottesman, 2010). Re-sensitization of cells to drug is expected to increase drug accumulation in cell. In this part of the study, we would like to observe amount of drug that accumulates in cell after treatment with *IL6* siRNA.

As stated before, blockade of this signaling pathway decreases expression of *MDR1* gene in cell. Therefore, those cells are expected to accumulate more drug due to decreased number of transporter in cell membrane. For accumulation studies, we made use of intrinsic property of doxorubicin: fluorescence. This drug is a fluorescent molecule which is excited at 480 nm wavelength of light and emits light at 580 nm (Albright et al., 2005). After treating cells with either *IL6* siRNA or mock siRNA, we exposed cells to 4μM of Doxorubicin for 1 hour. Later, we analyzed each cell by Flow cytometer at FL2 channel which detects light emission at 580 nm.

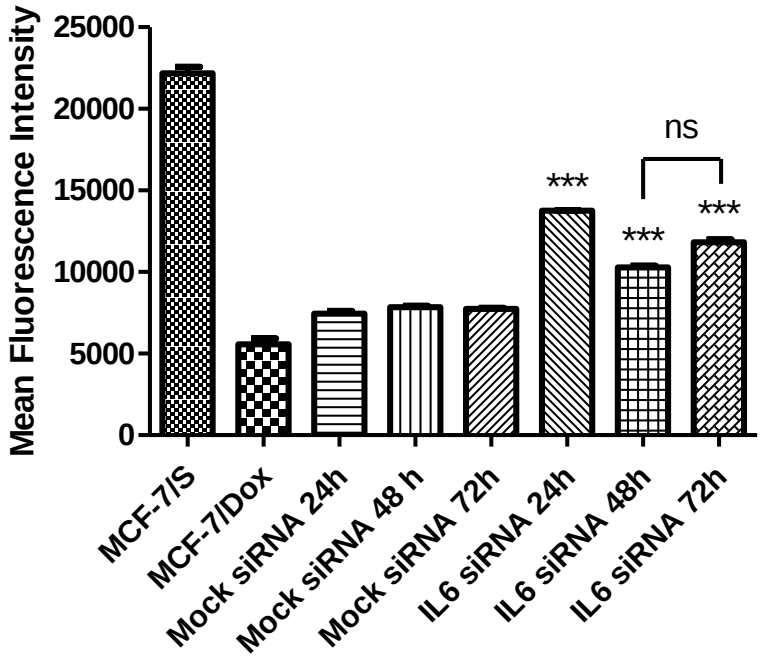

Figure 3.10 Mean fluorescence intensity of Doxorubicin in MCF-7/S cells, MCF-7/Dox cells and MCF-7/Dox cells treated either with *IL6* siRNA or mock siRNA for 24h, 48h and 72h ***p<0.0001 compared to MCF-7/Dox cells.

Doxorubicin resistant MCF-7 cells accumulate 75% less drug compared to parental MCF-7 cell line. Treatment with *IL6* siRNA for 24 hours increases the amount of drug that accumulates significantly in cell by 60%. In order to silencing be effective at drug accumulation, enough time should be passed so that P-gp level decreases at protein level on cell membrane. It has been shown that P-gp turnover rate changes between 14 hours and 17 hours when cells are cultured in normal conditions (Muller et al., 1995). According to that data, P-gp level at cell membrane is expected to change in accordance with *MDR1* mRNA level with 14hours phase difference. Indeed, maximum drug accumulation was observed 24 hours after *IL6* silencing. Since response to cytokine signaling happens very rapid, *MDR1* expression decreases possibly earlier than 24 hours and it is observed that there is enough time for P-gp turnover to take place at protein level within 24hours of treatment. Interestingly, mock siRNA treatment cells also accumulate 33% more drug compared to untreated cell. In literature, it is stated that membrane structure of cells receiving cationic liposomes is destabilized that it becomes more permeable to other molecules as well (Zelphati et al., 1996). Doxorubicin is known to enter into cell by means of diffusion due

to its lipophilic structure (Gottesman, 2002). Increased membrane permeability explains elevated drug accumulation (Figure 3.10).

3.7 Cell Proliferation Assay with XTT Reagent: Chemo-sensitivity to Doxorubicin

In order to determine chemo-sensitivity of MCF-7/Dox cells after treatment with *IL6* siRNA, XTT based cell proliferation assay was done. Change in the chemo-sensitivity due to siRNA treatment was assessed by comparing with chemo-sensitivity of MCF-7, MCF-7/Dox and mock siRNA treated MCF-7/Dox cells.

Figure 3.11 represents percent cell survival of MCF-7/Dox, Mock siRNA received MCF-7/Dox and *IL6* siRNA received MCF-7/Dox cells at increasing concentration of Doxorubicin. For all drug concentrations, *IL6* silencing causes more cell death compared to mock treated cells and untreated cells. This trend in cell death shows that after treatment with *IL6* siRNA lower Doxorubicin concentration is more effective on resistant cells, therefore, resistance can be reversed by blocking IL-6 signaling pathway.

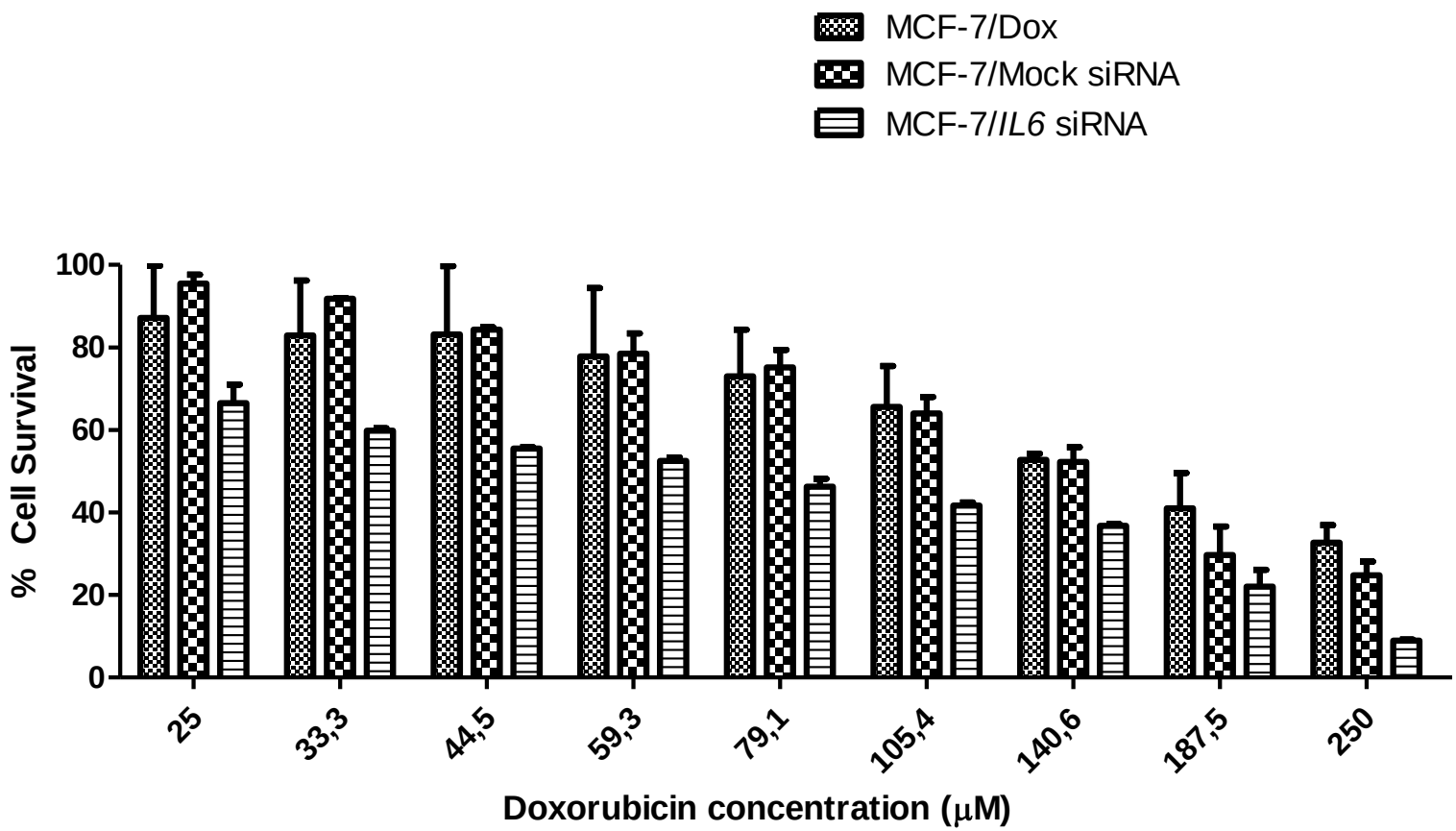

Figure 3.11 Cell survival profile of MCF-7/Dox cells and after treatment with *IL6* siRNA or Mock siRNA when increasing concentration of Doxorubicin is introduced

Table 3.2 shows IC_{50} values of resistant and sensitive cells as well as treatment groups. IC_{50} value of MCF-7/S cells was determined previously at our laboratory and this value is used for all calculations in this study (Dönmez & Gündüz, 2011). According to Table 3.1, 50% of MCF-7/S can be killed by 1.8μM Doxorubicin. For resistant cells, the dose to kill 50% of the population is about 154.28μM with resistant index of 83.84 After treatment with *IL6* siRNA, 56.54μM Doxorubicin can efficiently kill 50% of resistant cells significantly decreasing resistance index to 30.72. Mock siRNA treated MCF-7/Dox cells, on the other hand, have IC_{50} value of 131.925μM with resistance index of 71.69 compared to 83.84 of non transfected cells. It is observed that treatment of cells with a cationic liposome makes them more sensitive to Doxorubicin a slight decrease in IC50 value. Comparable result is obtained from drug accumulation studies in which mock siRNA treated cells accumulate slightly more drug compared to untreated MCF-7/Dox cells explained by same phenomena. Those values clearly show that *IL6* knockdown sensitizes MCF-7/Dox cells to Doxorubicin by downregulating *MDR1* gene as shown by q-PCR results (Section 3.3).

Table 3.2 Mean IC50 values, resistance indices and fold reversal values of MCF-7/S, MCF-7/Dox, mock siRNA treated MCF-7/Dox and IL6 siRNA treated MCF-7/Dox cells. *** p< 0.0001

	Mean IC50 (μM)±SEM	**R**	**FR**
MCF-7/S	1.84±0.07	-	-
MCF-7/Dox	154.28±2.86	83.84	-
MCF-7/Dox – Mock siRNA	131.92±0.61	71.69	1.16
MCF-7/Dox – *IL6* siRNA	56.54±0.47	30.72***	2.72

Fold reversal values also indicates the significance of re-sensitization to Doxorubicin. According to Table 3.2, MCF-7/Dox cells become 65% more sensitive after introduction of *IL6* siRNA.

There are other studies supporting our results showing that blockade of IL-6 signaling reverse multidrug resistance in cancer cells. Wang et al. shows that drug resistant ovarian cancer cells,

SKOV-3, transfected with antisense-*IL6* oligo expressing vectors become more sensitive to Cisplatin and Paclitaxel at the same time (Wang et al., 2010). Furthermore, doxorubicin resistant MCF-7 cells were treated with neutralizing antibodies for IL-6. IC50 of the drug decreased supporting the strong correlation between IL-6 and MDR phenotype (Sakai et al., 2011). Another study shows that IL-6 neutralizing antibody treated multiple myeloma cells, which are not drug resistant, becomes even more responsive to Melphalan which increases effectiveness of treatment (Hunsucker et al., 2011). IL-6 a good candidate to block in order to reverse MDR and increase response to chemotherapy even at lower drug doses.

Cells respond to several external stress signals such as chemotherapeutics and UV irradiation by differently regulating several pathways. Increased IL-6 expression is one of those responses. Along with increased survival potential and evasion from apoptosis, those cells start to gain resistance against introduced drugs (Sukhai et al., 2000). Combinatorial use of *IL6* targeting siRNA with chemotherapeutic agents starting from very beginning of treatment has the potential to increase effect and decrease the likelihood of developing drug resistance.

CHAPTER 4

CONCLUSION

1. Fluorescein conjugate siRNA transfection showed that 5nM siRNA concentration is enough deliver siRNA to 50% of the cells. This siRNA concentration is good enough to avoid off-target silencing and attain efficient and specific gene silencing.

2. *IL6* mRNA level is reduced by 50% with delivery of sequence specific siRNA.

3. *IL6* silencing downregulates *MDR1* expression because its expression level decreases to 50% of original level after siRNA treatment. *MRP1* mRNA level, on the other hand, does not change as expected since its expression is not regulated by IL-6 signaling pathway.

4. Gene specific siRNA delivery decreases secreted IL-6 level indicating that IL-6 availability decreases.

5. After treatment with *IL6* siRNA, intracellular Doxorubicin accumulation increases significantly in Doxorubicin resistant MCF-7 cells.

6. IC_{50} value of Doxorubicin decreases 2.72 folds following *IL6* silencing. This result indicates that cells are re-sensitized to Doxorubicin considerably.

Our results showa that siRNA mediated silencing of *IL6* has potential to inhibit development of MDR in cancer cells as well as it can reverse already existing MDR.

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