IDENTIFICATION OF DOXORUBICIN DRUG RESISTANCE MECHANISMS BY USING GENOMIC TECHNIQUES

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Ayşe Banu DEMİR

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İZMİR

We approve the thesis of **Ayşe Banu DEMİR**

Examining Committee Members:

Prof. Dr. Ahmet KOÇ
Department of Molecular Biology and Genetics, Izmir Institute of Technology

Prof. Dr. Yusuf BARAN
Department of Molecular Biology and Genetics, Izmir Institute of Technology

Prof. Dr. Neşe ATABEY
Department of Medical Biology and Genetics, Dokuz Eylul University

Assist. Prof. Dr. Özden YALÇIN ÖZUYSAL
Department of Molecular Biology and Genetics, Izmir Institute of Technology

Assoc. Prof. Dr. Zekiye ALTUN
Department of Basic Oncology, Dokuz Eylul University

5 January 2015

Prof. Dr. Ahmet KOÇ
Supervisor, Department of Molecular Biology and Genetics,
Izmir Institute of Technology

Prof. Dr. Ahmet KOÇ
Head of the Department of
Molecular Biology and Genetics

Prof. Dr. Bilge KARAÇALI
Dean of the Graduate School
of Engineering and Sciences

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ABSTRACT

IDENTIFICATION OF DOXORUBICIN DRUG RESISTANCE MECHANISMS BY USING GENOMIC TECHNIQUES

Chemotherapy has been an important contributor for the treatment of cancer patients for a long time. The effectiveness of the therapies is influenced from the toxicity effects of the agents on normal cells and from the drug resistance. Therapeutic resistance is believed to cause the failure of the chemotherapy effectiveness in most cancer cases. Therefore, understanding the molecular mechanisms that underlie the drug resistance may contribute to increase the effectiveness of the chemotherapeutic treatment of cancer. Doxorubicin is a natural product that is widely used in treatment of various cancer types, yet many tumors have resistance against these agents. By using the budding yeast *Saccharomyces cerevisiae* as a model organism, we performed genome-wide screenings to identify the genes that cause resistance against this agent. Overexpression of CUE5, AKL1, CAN1, YHR177W and PDR5 genes have been identified to cause resistance against Doxorubicin at higher concentrations than the identified toxic level. Among these genes, only PDR5 overexpression was found to have cross-resistance to Cisplatin. Real-time PCR and microarray analysis for these genes were also performed. Upon 80μM Doxorubicin treatment for 2 hours, none of the CUE5, AKL1, CAN1, YHR177W and PDR5 genes showed expression changes compared to their correponding untreated wild-type status. Therefore, overexpression of these genes may not be a physiological response of yeast cells against Doxorubicin. Genome-wide microarray analysis showed changes in several cellular and biological functions upon Doxorubicin treatment. Identified genes mainly function in general stress response related events such as, filamentous growth, protein ubiquitination, autophagy, changes in membrane transportation and metabolic processes.

ÖZET

DOKSORUBİSİN DİRENÇLİLİK MEKANİZMALARININ GENOMİK YÖNTEMLERLE TESPİT EDİLMESİ

Kemoterapi, uzun zamandır kanser hastalarının tedavisine önemli katkılarda bulunmuştur. Tedavilerin etkenliği, kullanılan ajanların normal hücreler üzerindeki toksik etkilerinden ve ilaç dirençliliğinden etkilenmektedir. Teröpatik dirençliliğin, kanser durumlarının çoğunda tedavi başarısızlığının nedeni olduğuna inanılır. Dolayısıyla, ilaç dirençliliğinin altında yatan moleküler mekanizmaların anlaşılması, kanser kemoterapi tedavilerinin etkisini arttırmada önemli katkılar sağlayabilir. Doksorubisin, birçok kanser türünün tedavisinde yaygın olarak kullanılan doğal bir ajandır fakat birçok tümör bu ajana karşı dirençlilik göstermektedir. Bu çalışmada, *Saccharomyces cerevisiae* model organizması kullanılarak, BY4741 yabani tip maya suşu için Doksorubisin toksik seviyesi belirlenmiş ve bu toksik seviyeye karşı dirençlilik gösteren genleri tespit etme amaçlı genom düzeyinde tarama yapılmıştır. CUE5, AKL1, CAN1, YHR177W ve PDR5 genlerinin fazla ifadelenmesi, Doksorubisine karşı, toksik seviyeden daha yüksek ilaç seviyesinde dahi dirençlilik göstermiştir. Bu genler arasında, sadece PDR5 geninin Cisplatin ajanına karşı çapraz-dirençlilik göstermiştir. 2 saatlik, 80μM Doksorubisin muamelesinin ardından, bu genlerin eş-zamanlı PCR ve mikroarray analizleri gerçekleştirilmiş ve ekspresyon açısından anlamlı bir farklılık göstermedikleri görülmüştür. Dolayısı ile bu genlerin fazla ifadelenmesinin, maya hücrelerinde fizyolojik bir yanıt olmadığı düşünülmektedir. Tüm genom mikroarray analizleri, Doksorubisin muamelesinin çeşitli biyolojik ve hücre fonksiyonlarında farklılık yarattığını göstermiştir. Mikroarray analizlerinde bulunan genlerin genel olarak, filamental büyüme, protein übikitinasyonu, otofaji, membran geçişi değişimleri ve metabolik değişiklikler gibi genel-stres yanıtı ile ilgili olaylarda rol oynadığı görülmüştür.

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CHAPTER 1

INTRODUCTION

1.1 . Cancer

The cells of multicellular organisms not only regulate themselves, but the whole system behavior as well, by coordinating the signal receipt and interpretation among variety of cells. Molecular disturbances, such as mutations, can contribute to the deregulation of this coordination. In human body, billions of cells face with mutations every day, however they are eliminated by the proper cellular "guard-systems" unless these mutations give a selective advantage to a cell (Bruce Alberts, 2008).

Cancer is a clonal case that arises from a single cell, in which the deregulation results in an advantage for the growth of that single cell (Bruce Alberts, 2008). The uncontrolled growth of a cell may result in a mass of cells called "tumor". Some tumors are not cancerous. They do not spread to other parts of the body and can easily be removed and generally do not relapse. These tumors are called "benign tumors". However, some are cancerous that they can invade nearby tissues and spread to other parts of the body. These tumors are called "malignant tumors". Some cancers, such as the cancer of blood, even do not form tumors.

There are more than 100 different types of cancer that are named according to the cell type or the organ they arise. For example, carcinoma is the cancer that arise from the skin or tissues that cover internal organs while lymphoma and myeloma are cancers that arise from the cells of the immune system.

Rather than being a single disease, cancer can be defined as the set of diseases that results from uncontrolled growth of cells. According to National Cancer Institute statistics, in 2014, approximately 1,665,000 new cancer cases and 585,000 deaths has been recorded in the United States. When cancer incidence rates are evaluated, cancer is still the second most common cause of death in the world.

1.2 . Cancer Therapy

There are several ways to treat cancer aiming either to remove the cancerous cells from the multicellular organism. The type of treatment depends on the location and grade of the tumor, the stage of cancer and patient's other health status. Even there are variety of treatment types such as stem cell therapy, photodynamic therapy, hormonal therapy and hyperthermia, the basic treatment types can be divided into five major groups as surgery, radiation therapy, chemotherapy, immunotherapy and targeted therapy (Vincent T. DeVita, 2008).

1.2.1. Surgery

If the tumor only resides at a certain place and did not invade (metastize) other parts of the body, it can be removed by a surgical operation. In this type of treatment, the type of cancer and the stage of the tumor are important. Surgery can only be applied to non-hematological cancers, where there exists a tumor mass. Surgery removes the tumor and its surrounding tissue (Vincent T. DeVita, 2008). The taken material is then analyzed for its pathological properties and it is decided if further types of treatment, such as chemotherapy or radiotherapy, is necessary or not.

When appropriate, surgery is generally applied before other treatments, however, in some cases, the tumor mass needs to be reduced for surgery. In those cases, other therapies are applied before surgery (Enger Eldon, 2007).

1.2.2. Radiation Therapy

Radiotherapy is the use of ionizing radiation to kill the cancer cells. It only affects the region that is being treated (Enger Eldon, 2007). The ionizing radiation (such as X-rays) aims to destroy the genetic material of the tumor cells and inhibit their growth. It can either be applied from outside the body by using X-rays or a radioactive material can be injected in the tumor location. This type of treatment affects the normal cells nearby the treated area of the body. However, normal cells usually can recover from the affects of irradiation.

Radiation therapy can be used in most of the solid tumors, such as breast, brain, and lung and hematological cases, such as leukemia and lymphoma. The type of the tissue, the localization of the tumor is of great importance in the decision of radiotherapy and its dosage.

1.2.3. Chemotherapy

Chemotherapy is the treatment type in which medicines/drugs are used (Enger Eldon, 2007). The cytotoxic drugs (anti-cancer drugs) are used to target the cancerous cells. These drugs aim to kill the cells via several ways, such as intercalating the DNA, inhibiting replication, generating oxidative stress, etc. Most of the drugs target rapidly dividing cells, which is one of the properties of cancerous cells. However, these drugs affect rapidly dividing normal cells as well. Therefore, some side effects appear upon chemotherapy. Chemotherapeutic drugs are either taken as drugs or can be injected or infused into a vein.

1.2.4. Immunotherapy

In immunotherapy, the immune cells of the patient tried to be induced in such a way that the cells become able to fight with tumor cells more effectively. For this strategy, either allogeneic hematopoietic stem cell transplantation is done to generate a "graft-versus-tumor" effect on tumor cells, or patients own natural killer cells or cytotoxic T cells are used to increase them in number outside the body and re-injecting them to patient.

1.2.5. Targeted therapy

Targeted therapies have started to be used in 2000s for some types of cancer. The agents used in targeted therapy, target specific, deregulated proteins or domains of proteins in cancer cells. Tyrosine kinase inhibitors, such as imatinib, or monoclonal antibodies, such as trastuzumab, are some examples for the agents used in targeted therapy (Enger Eldon, 2007).

1.3. Chemotherapeutic Drugs

The drugs to treat cancer were first started to be used in early 20th centuries. The mustard gas used during World War I, was found to reduce the number of blood cells and be a potent supressor of blood production, upon examination of certain French patients that were gassed (Krumbhaar, 1919). Then, nitrogen mustards were studied during World War II. The first chemotherapeutic drug that was used was mustine (Joensuu, 2008). The use of general cytotoxic agents started in 1940s and the specificity of the agents used from there on increased to date (Figure 1) (Gottesman, 2002).

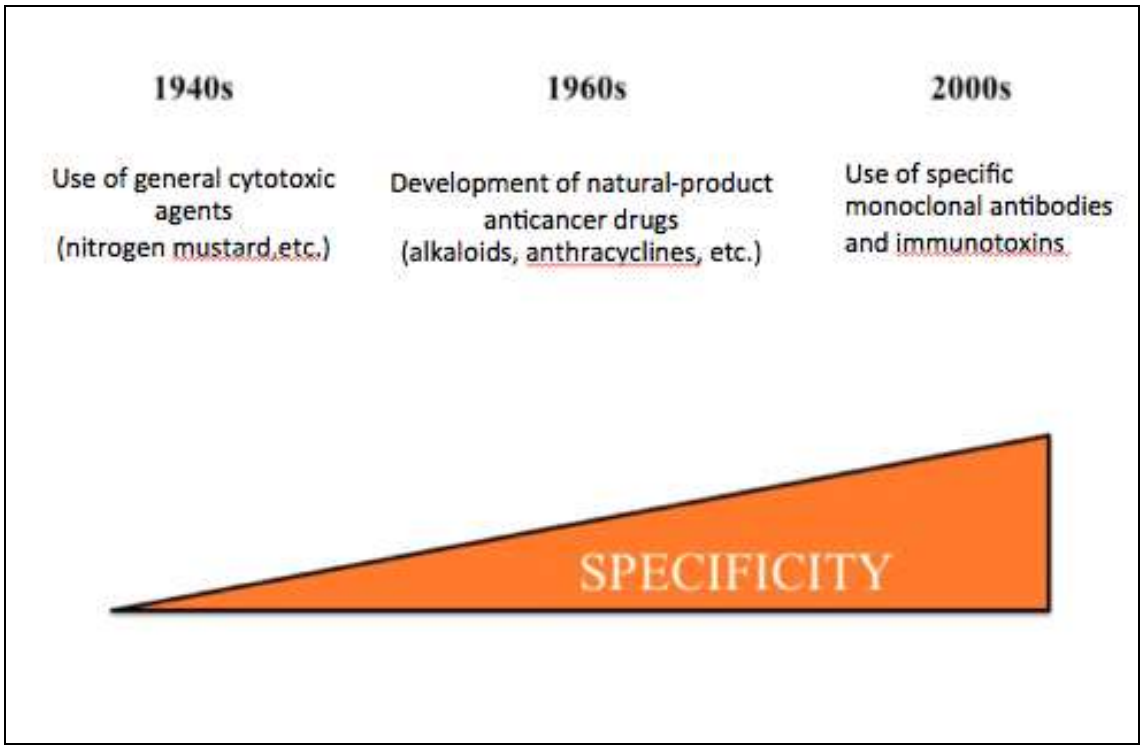


Figure 1. The Historical Chart of Chemotherapeutic Agents.

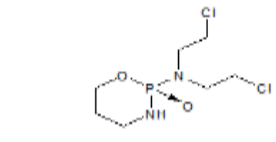
There exist several classes of drugs that can be used for various cancer types Some anticancer agents have cytotoxic effects during specific cell cycle phases and are called "Cell-cycle specific agents". For example, antimetabolites, such as 5-Flourouracil, show their effects more on S-phase cells, while vinca alkaloids or taxane group of drugs are more effective on M-phase cells (Chris H. Takimoto, 2008). Cell-cycle specific agents are plateau in their concentration-dependent effects, since only a

set of proliferating cells are fully sensitive to drug. These agents are time-dependent, in which exposure duration should be increased in order to increase cell killing. On the other hand, chemotherapeutic drugs that are not cell cycle dependent have linear dose-response curves. This means the cytotoxic effect of the drug increases linearly as the dose increases (Chris H. Takimoto, 2008).

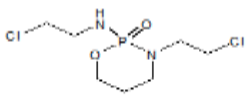
Commonly used anticancer agents can be classified into four major classes according to their mechanism of action. These are; alkylating agents, antimetabolites, natural targeted products and targeted agents, in which each class can be further divided into subcategories (Chris H. Takimoto, 2008; O'Connor et al., 2007) .

1.3.1. Alkylating Agents

Alkylating agents act by forming covalent bonds with phosphate, amino, carboxyl, and sulphydryl groups of several molecules such as DNA, RNA and proteins and alkylate them (Chris H. Takimoto, 2008). They inhibit the DNA and RNA synthesis by alkylation, prevent protein formation and therefore trigger apoptotic cell death. Some parts of DNA are particularly susceptible for alkylation. Alkylating agents are not "cell-cycle specific" but their activity depends on cell proliferation. They are more effective on rapidly dividing cells, in which there is less or no time for DNA repair. Nitrogen mustards (such as mechlarethamine (mustargen), cyclophosphamide, ifosfamide, chlorambucil), Nitrosoureas, and Platinum agents (such as Cisplatin, Carboplatin, and Oxaliplatin) are the examples for alkylating agents.



Cyclophosphamide



Ifosfamide

Figure 2. The Chemical Formulations of some Alkylating Agents

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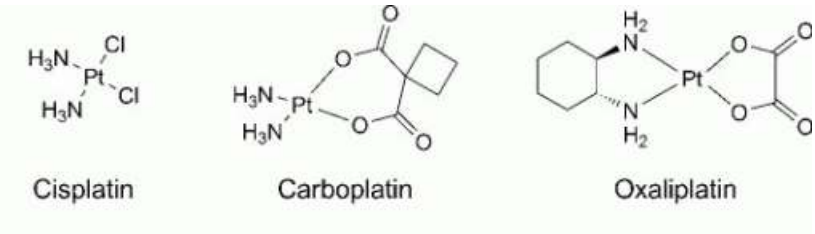

Figure 2. (cont.)

1.3.2. Antimetabolites

Antimetabolites are the analogs of the metabolites of nucleic acid synthesis (Chris H. Takimoto, 2008). They either compete with metabolites for the functional site of an enzyme (such as catalytic site, regulatory site, etc.) or substitute for a metabolite which incorporates into DNA or RNA. They are most effective for S-phase cells, so they are more active against tumor cells.

As an example to antimetabolites, Gemcitabine is one of the nucleoside analogs used in treatment of variety of solid tumors. This agent inhibits DNA synthesis by masked chain termination via ribonucleotide reductase inhibition (Philip Agop Philip, 1999).

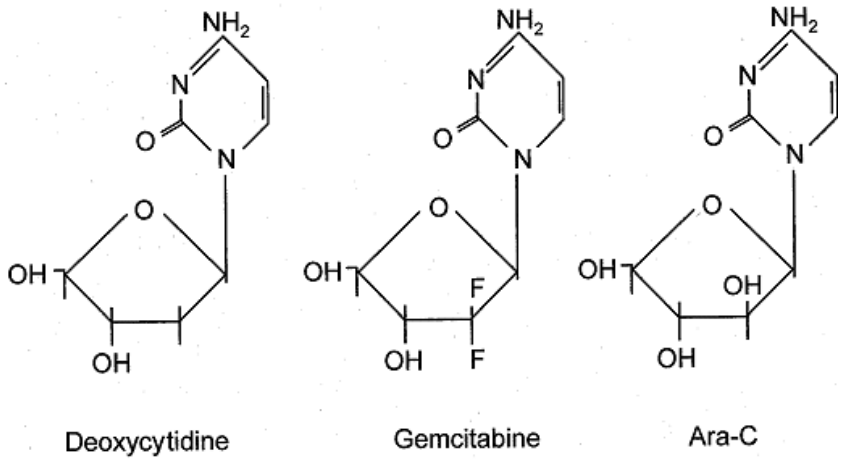

Figure 3. Structure of Gemcitabine (Gemzar), which is a Nucleoside Analong that is used in Several Solid Tumor Therapies (Source: Philip Agop Philip, 1999)

1.3.3. Targeted Agents

Targeted agents have started to be used in clinics around 2000s. These agents target specific proteins of cancer cells. They act by inhibiting the enzymatic domains of either deregulated proteins or proteins that play key roles in cellular events such as tyrosine kinases.

Targeted agent can be subdivided into two main groups as monoclonal antibodies and small-molecule-targeted agents (Chris H. Takimoto, 2008) . In monoclonal antibody therapy, the agent is an antibody that binds specifically to cancer cell proteins (Carter, 2001).

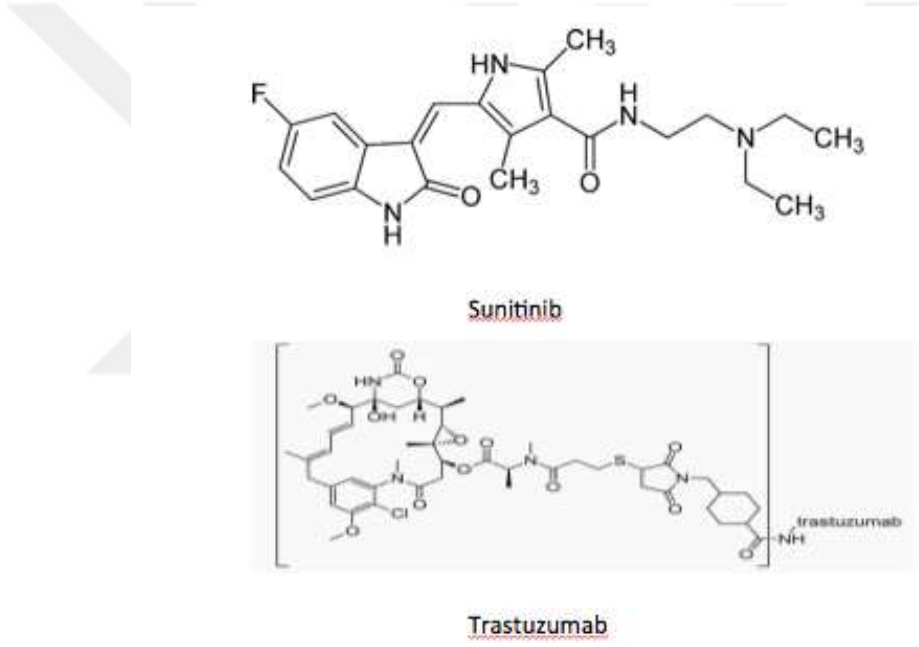

Figure 4. Structures of Sunitinib and Trastuzumab that are used in Targeted Therapies of Cancer.

Rituximab, which is an anti-CD20 antibody and Trastuzumab, which is an anti-HER2/neu antibody, are some examples for monoclonal antibodies used in clinics (Chris H. Takimoto, 2008). Sunitinib, which is a tyrosine kinase inhibitor, and Bortezomib, which is a 26S proteasome inhibitor, are examples of small-molecule targeted agents used in clinics (Chris H. Takimoto, 2008).

1.3.4. Natural Products

Discovery of the curable effects of several molecules isolated from natural products, such as foods, plants, and fungi, on variety of diseases in early 20th century, resulted in identification of several drugs that we use in the treatment of several diseases, including cancer (Susmita Mondal, 2012).

Natural products are subdivided into five groups, namely; antitumor antibiotics, anthracyclines, epipodophyllotoxins, microtubule agents (e.g. vinca alkaloids, taxanes) and camptothecin analogs (Chris H. Takimoto, 2008).

The agent Bleomycin is an example for *antitumor antibiotics*. It acts by intercalating DNA at G-C and G-T sequences, resulting in formation of free oxygen radicals and strand breakage **(Figure 5)** (Chris H. Takimoto, 2008).

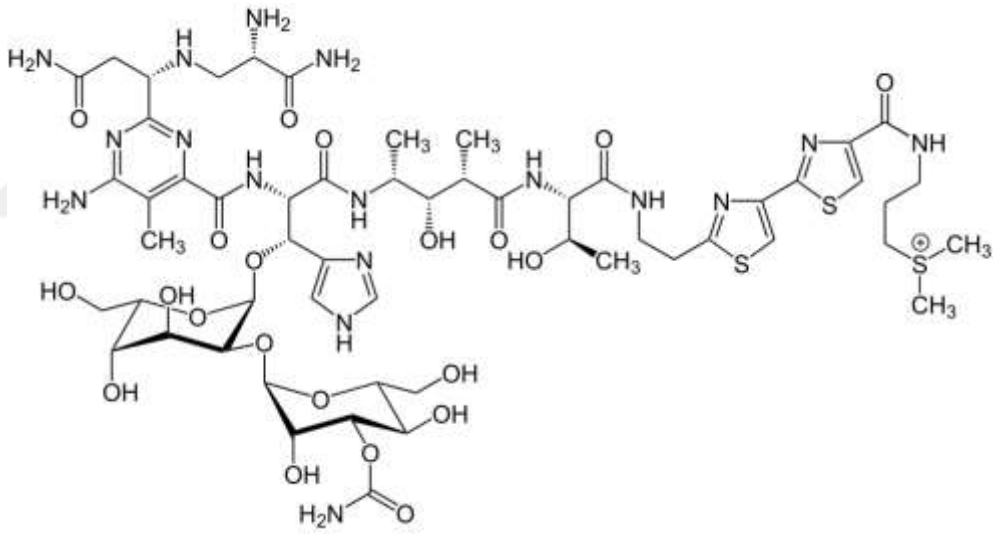

Figure 5. The Structure of Bleomycin

Epipodophyllotoxins are extracted from the root of Podophyllum peltatum (Mayapple Plant) and are inhibitors of Topoisomerase II (Chris H. Takimoto, 2008). Etoposide is an example for epipodophyllotoxins, which is used in cancer therapies (Figure 6).

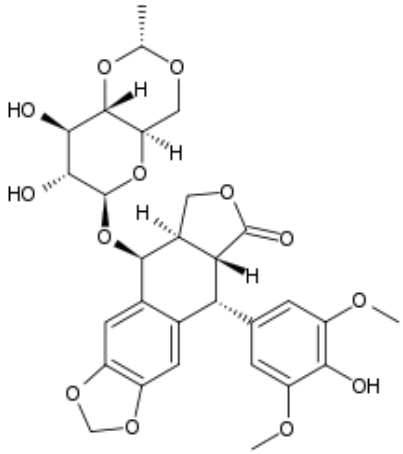

Figure 6. The Structure of Etoposide

Vinca alkaloids, which are derived from the plant vinca rosea, and taxanes, which are semisynthetic derivatives of the precursor extracts of needle of yew plants, are *microtubule agents* that are used in cancer therapy (Figure 7) (Chris H. Takimoto, 2008). Vinca alkaloids bind tubulin in S-phase cells. Taxanes, that have a 14-member ring, promote microtubule assembly and stability and block cell cycle at mitosis (Chris H. Takimoto, 2008).

A)

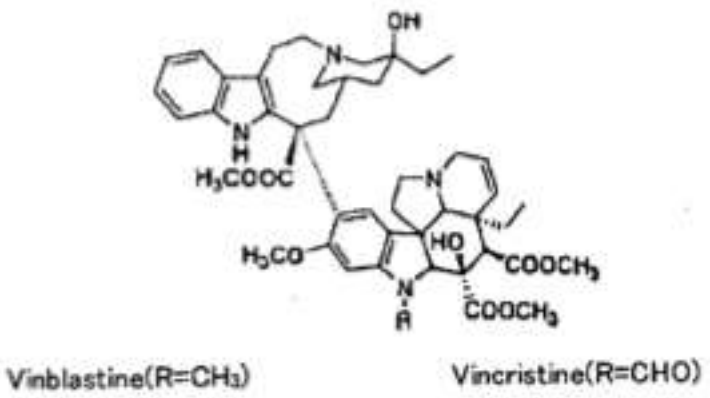

Figure 7. The Structure of A) Vinca alkaloids (Vincristine and Vinblastine) and B) Taxanes (Docetaxel and Paclitaxel).

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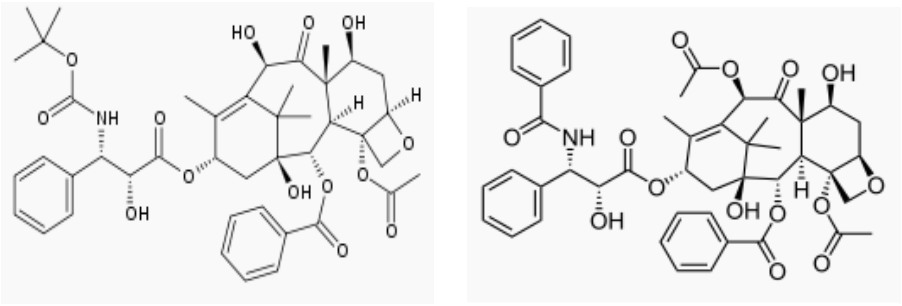
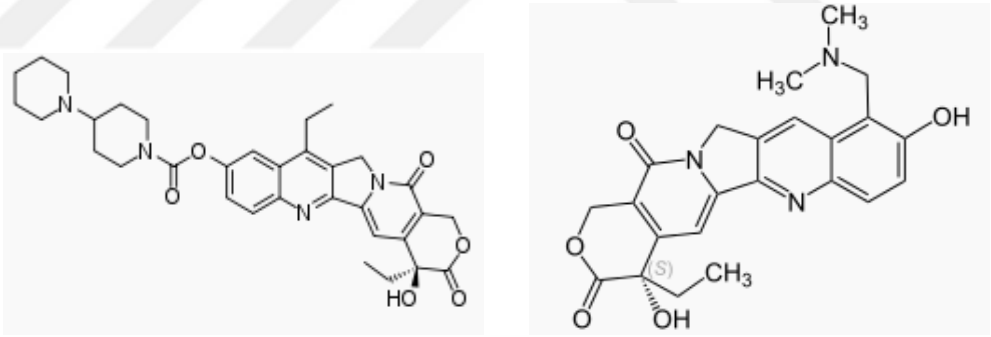
B)



Docetaxel Paclitaxel

Figure 7. (cont.)

Camptothecin analogs are semisynthetic agents derived from Camptotheca acuminate (Chris H. Takimoto, 2008). They act by inhibiting Topoisomerase I, therefore blocking the elongation phase of DNA replication. Irinotecan and Topotecan are two examples of camptothecin analogs used to treat variety of cancers.



Irinotecan Topotecan

Figure 8. The Structures of Camptothecin Analogs; Irinotecan and Topotecan

Anthracyclines, which are widely used antineoplasctic agents (Minotti et al., 2004), are the products of the Streptomyces peucetius (Chris H. Takimoto, 2008). They have variety of action but their major actions are intercalation between DNA base pairs and inhibition of topoisomerase I and II (Chris H. Takimoto, 2008). Doxorubicin and Daunorubicin are some examples for anthracyline group of drugs.

Since Doxorubicin is the focus of this study, the mode of actions of anthracyclines and Doxorubicin, will be focused on in more details.

1.4. Anthracyclines and Their Mode of Action

Anthracyclines are among the most effective anti-cancer agents that act on a large group of cancers (Weiss, 1992). The first anthracyclines were isolated in 1960s from the pigment producing Streptomyces peucetius (Minotti et al., 2004). These anthracyclines were Doxorubicin and Daunorubicin. As shown in Figure 9, the first anthracyclines, Doxorubicin and Daunorubicin, have aglyconic and sugar moieties. The aglycone moiety consists of a tetracyclic ring with adjacent quinone-hydroquinone groups, a methoxy substituent, and a side chain with a carbonyl group. The sugar moiety is daunosamine, which is bound by a glycosidic bond to the ring structure (Minotti et al., 2004). Doxorubicin terminates with a primary alcohol whereas Daunorubicin terminates with a methyl group in their side chain. This difference in side chains affects the activity of these drugs such that Doxorubicin is generally used in treatment of childhood solid tumors and breast cancers whereas Daunorubicin is used in acute lymphoblastic and myeloblastic leukemias (Minotti et al., 2004).

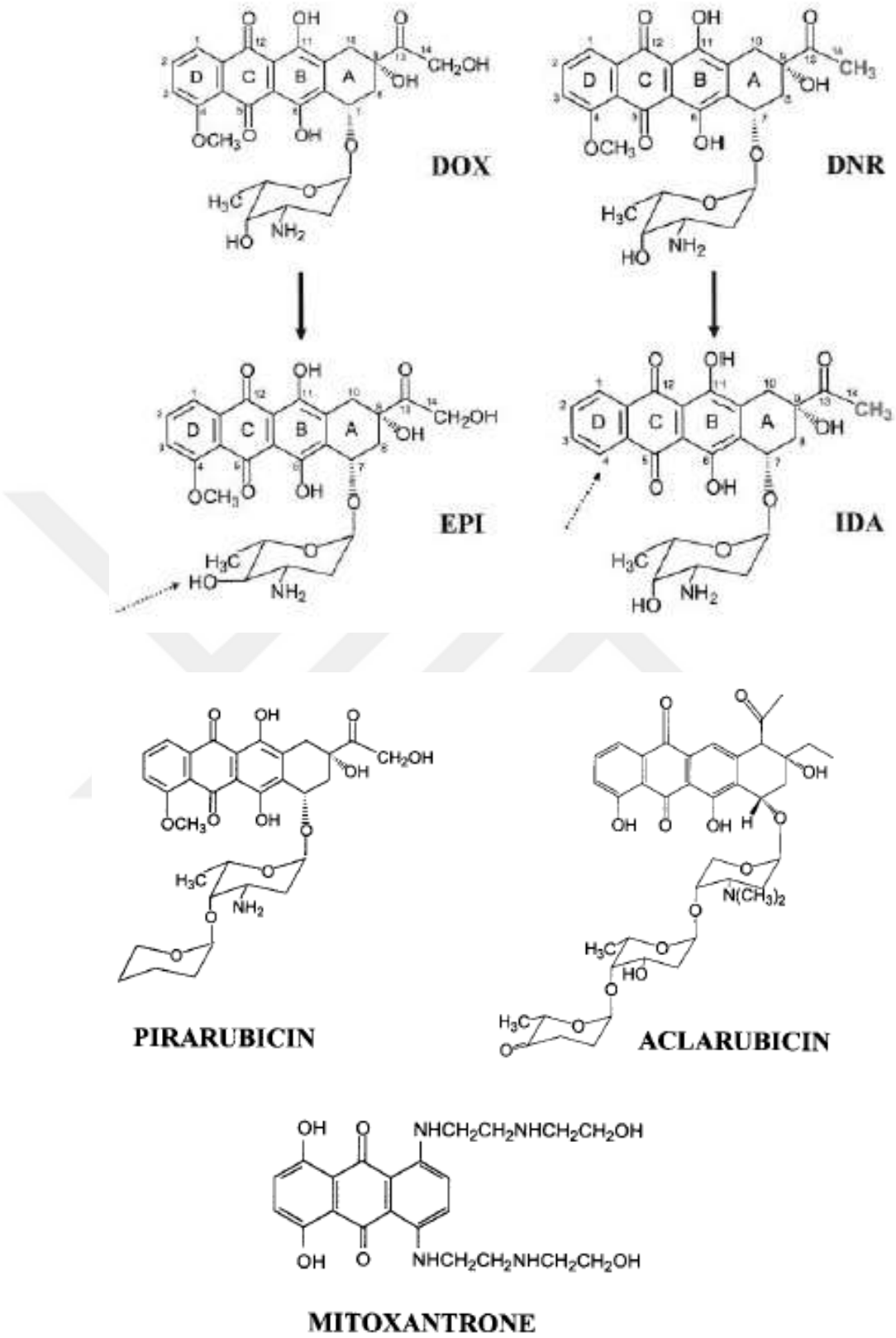


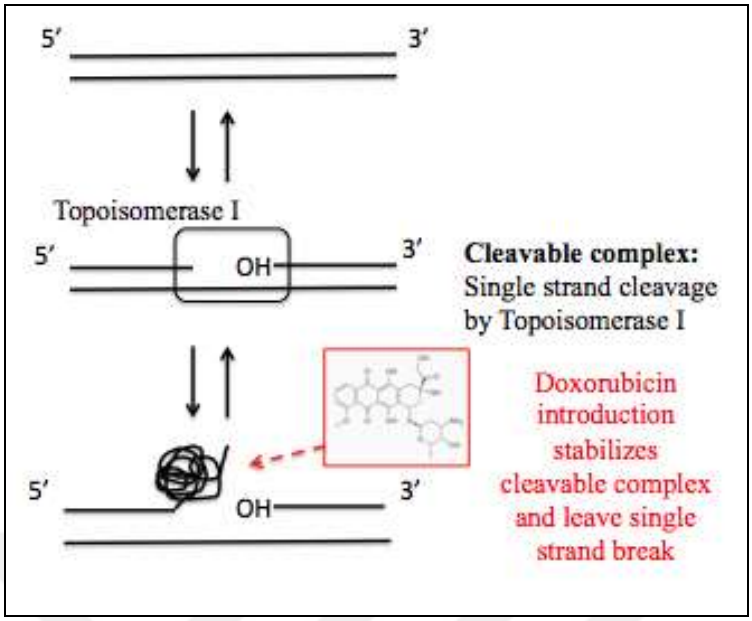
Figure 9. The Structure of Anthracyclines: Doxorubicin (DOX), Daunorubicin (DNR), Epirubicin (EPI), Idarubicin (IDA), Pirarubicin, Aclarubicin and Mitoxantrone

New anthracyclines were identified further in 2000s like Pirarubicin; Aclarubicin; Mitoxantrone; Epirubicin, which is the semisynthetic derivative of Doxorubicin; and Idarubicin, which is an analog obtained from Daunorubicin;.

The anti-cancer activity of anthracyclines can be summarized as; *intercalation into DNA* and inhibition of macromolecule synthesis; *generation of free radicals*, that results in molecular damage; *modulation of functional properties of membranes*; *inhibition of topoisomerase I and II* and leading the formation of DNA double-strand breaks as well as inducing apoptosis (Minotti et al., 2004; Tritton, 1991).

The anti-topoisomerase activity of anthracyclines results form the stabilization of the 'cleavable complex' that occurs between topoisomerase-I/ topoisomerase-II and DNA. Therefore anthracyclines are referred as topoisomerase poisons. Topoisomerases are enzymes that regulate the overwinding and underwinding of DNA during replication and transcription processes by modifying the DNA topology. Due to the double-helix nature of DNA, during replication and transcription, ahead of the replication-fork become overwound and this generates a tension on DNA. Unless this tension is released, the replication process may not progress. Therefore, in order to overcome this winding problem, topoisomerases bind DNA and generate a "cleavable complex " which results in generation of single- (Topoisomerase-I) and double- (Topoisomerase-II) strand breaks by cutting the phosphate backbone of DNA (Figure 10). These breaks are resealed afterwards (Bruce Alberts, 2008).

A)



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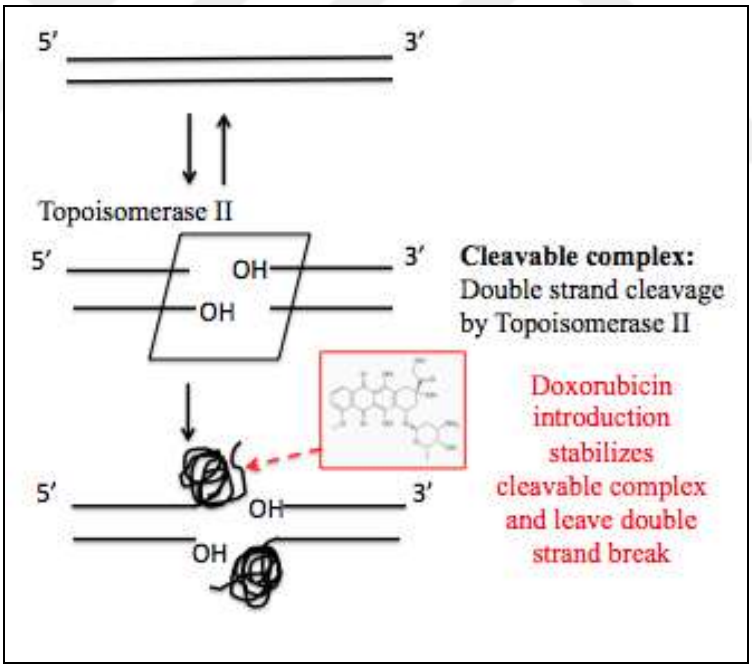


Figure 10. Doxorubicin Act on Cleavable Complex of A) Topoisomerase-I and B) Topoisomerase-II.

Anthracyclines act by stabilizing the reaction intermediate, in which the cut strands of DNA are linked to the Topoisomerase enzyme (Minotti et al., 2004). Therefore the formed strand break cannot be resealed (Figure 10). Topoisomerase

dependent DNA damage is therefore follwed by arrest in cell growth in G1 or G2 phases of cell cycle and induction of apoptosis (Minotti et al., 2004).

In addition to their topoisomerase-dependent action, anthracyclines have been known to form semiquinone upon one electron addition to their quinone moiety, which results in oxygen reduction and generation of reactive oxygen species (ROS) (Figure 11) (Minotti et al., 2004). ROS, then attacks to macromolecules and lead formation of lesions such as DNA adducts and lipid peroxidation.

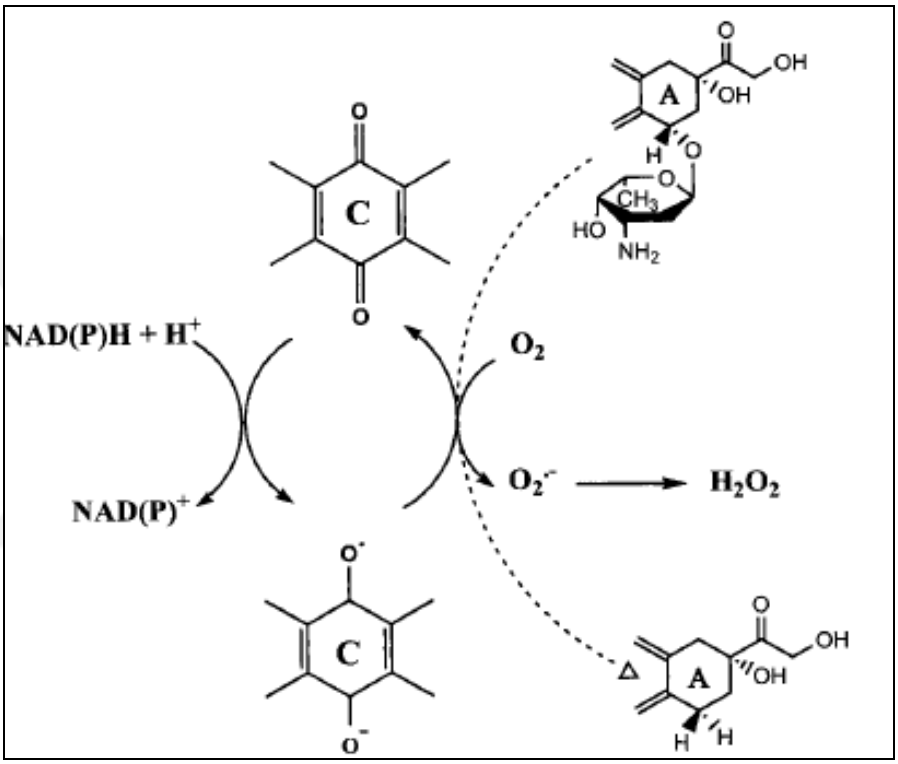


Figure 11. Redox Cycle of Anthracyclines
(Source: Minotti et al., 2004)

However, formation of free radicals, DNA damage and lipid peroxidation in tumors is still a debate. There exists both supportive and disproving evidence about the issue. For example, free radical formation in rat glioblastoma cells that are exposed to Doxorubicin and hydrogen peroxide formation in human colon adenocarcinoma cells, were both shown, while hydrogen peroxide formation was detected after 16 hours, so was said to be a delayed perturbation rather than a primary metabolic perturbation (Gewirtz, 1999). For DNA damage, non-protein-associated strand breaks were shown to occur in leukemic cells, while protein-associated strand breaks were seen at lower

concentrations of Doxorubicin (Gewirtz, 1999). Lipid peroxidation was shown to occur in rat glioblastoma cells however peroxidation event was lacking dose dependence (Gewirtz, 1999). So the action of anthracylines through free radical formation still remains to be elucidated in a more clear way.

Anthracylines also shown to act on biological membranes. The proposed model for the membrane action of Anthracylines by Tritton et. al. is as follows; anthracylines affect the membrane fluidity by promoting phosphoinositide turnover. This event, in turn leads diacylglycerol accumulation and activation of Protein kinase C, which affects topoisomerase phosphorylation and further DNA damage and cell death (Tritton, 1991).

1.4.1. Doxorubicin

Doxorubicin from anthracyclines is the focus of this study. The antitumor activity of Doxorubicin is similar to other anthracylines, including inhibition of nucleic acid synthesis by intercalating into DNA, affecting membrane properties, damaging macromolecules via formation of free radicals, promoting lipid peroxidation, and promoting DNA cleavage by inhibiting topoisomerase II (Furuchi et al., 2004b; Gewirtz, 1999). Also doxorubicin was shown to bind its RNA substrate and inhibit RH II/Gu RNA helicase activity in a dose-dependent manner (Zhu et al., 1999).

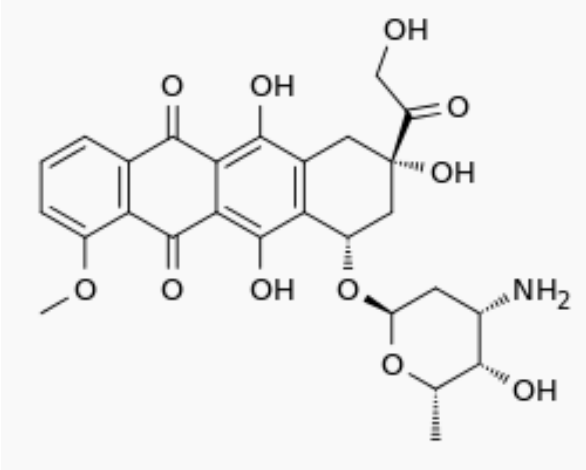


Figure 12. The Structure of Doxorubicin

DNA damage upon Quinone (which is present in anthracyclines including Doxorubicin), containing drugs is generally shown to be either due to the "DNA binding" or "Topoisomerase binding" properties of the drug. Another mechanism that is proposed to induce DNA damage is the indirect thiolation of the nuclear Topoisomerase-II protein by formation of glutathione-Topoisomerase mixed disulfides (Wang et al., 2001). As well as needed for the DNA replication and transcription events, the function of Topoisomerase-II is also necessary for chromosome condensation and decatanation of sister chromatids (Nitiss & Beck, 1996). Therefore the cellular toxicity of Doxorubicin upon Topoisomerase-II binding, also shown to come from the commitment of cells to apoptosis at G2 cell cycle check point (D'Arpa & Liu, 1989; Nitiss & Beck, 1996).

Doxorubicin is also a potent modulator of cellular membranes. It can alter the membrane fusion interactions and subsequent biological function (Tritton, 1991). It was proposed that Doxorubicin distrupts the signaling events involving phosphoinositide pathway. Agglunation properties due to lectin interactions is proposed to be affected by Doxorubicin (Tritton, 1991). Also Doxorubicin treatment increases the phosphotidylinositol turnover resulting in diacylglycerol production (Tritton, 1991). Therefore, the drug also have membrane-dependent cell-surface actions as well.

Even Doxorubicin is a widely used anthracycline to treat variety of cancer types, its use is limited due to its cadiotoxic side effects (Fanciulli et al., 1998). This side-effect is thought to be multifactorial (Minotti et al., 2004). Lipid peroxidation, inhibition of nucleic acid synthesis, abnormalities in Ca_2^+ handling, changes in membrane properties, have all thought to be effective on Doxorubicin-induced cardiomyopathy (Minotti et al., 2004). Even having a cardiotoxic side-effect, Doxorubicin is still a widely used chemotherapeutc drug and is shown to be a good "combined-therapy" partner for several anti-tumor drugs.

1.5 Drug Resistance

Since 1940s, several agents have been used in cancer chemotherapy for treatment of many cancer types. The specificity of the cancer drugs increases everyday, and the therapies become more effective. However, there is no prevalent cancer treatment yet, that shows 100% effectiveness. The limitations for the effectiveness of

the therapies arise from both the toxic effects of the agents on normal cells and drug resistance (O'Connor et al., 2007). The treatment failure for approximately 90% of the metastatic cancer patients, is believed to be caused by the therapeutic resistance (Longley & Johnston, 2005).

Cancer cells fail to respond to a specific therapy either by host-factors or by specific genetic/epigenetic changes. Poor drug absorbtion, rapid metabolism for detoxification, excretion of the drug, poor tolerance of the patient to drug effects that leads to usage of decreased doses, impairment of the drug delivery to the target site and alterations in tumor environment are among the host factors that results in therapy failure (Gottesman, 2002).

Each cancer cell has a different genetic background due to its tissue of origin, pattern of oncogene/tumor supressor genes and gene expression variations. Therefore the response of each cell may differ to a specific anti-cancer drug. Even tumor cells are not intrinsically resistant to a specific drug, the selection imposed by the drug may lead to genetic/epigenetic changes that results in drug resistance.

Resistance can apply to many drugs that differ structurally and functionally. This is called “Multidrug Resistance”. Changes in cells that lead limited accumulation of the drug, increased efflux of the drug or affect membrane fluidity, can cause multidrug resistance (Figure 13).

The drug resistance to chemotherapy, either intrinsic, which is described as the initial treatment resistance, or acquired, that originates from a cellular population not killed in previous treatments, may arise from several mechanisms. These mechanisms include; decrease in drug accumulation via increase in drug efflux by the action of ABC family of transporters, decrease in drug activation due to trapping of drug in acidic vesicles, increase in drug induced damage repair, drug target alteration due mutations, gene expression alterations and host-tumor interactions, diminished apoptotic signaling and increased tolerance of celllar damage (Chris H. Takimoto, 2008; Gottesman, 2002; Hall et al., 2009; Sood & Buller, 1998). Generally, the most common reason for the acquired resistance is the highly expression of an energy-dependent transporter to eject anticancer drugs from cells (Gottesman, 2002). Other mechanisms include induction of detoxyfication and insensitivity to drug-induced apoptosis (Gottesman, 2002).

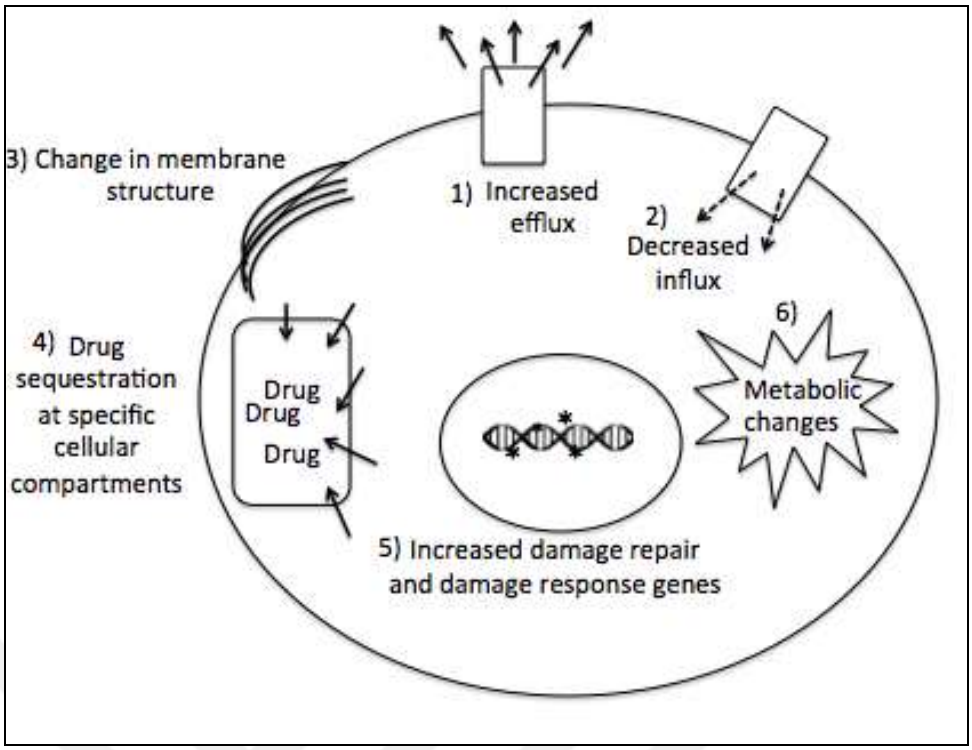


Figure 13. Several Multidrug Resistance Mechanisms.

The changes in cells mainly block apoptosis, activation of stress-response pathways (such as DNA repair mechanisms) and alter cell-cycle and its checkpoints. Therefore the cells become resistant to the treatment and keep on surviving.

1.5.1. Drug Resistance Mechanisms Against Doxorubicin

The mechanisms that cause drug resistance against Doxorubicin (Adriamycin) have been studied for a long time and several genes have been implicated to play role in Doxorubicin resistance both in mammalian cell lines and yeast cells.

In mammalian cell lines, the resistance mechanisms acquired to doxorubicin reported to date include; increase in the ABC transporters P-glycoprotein (Lincke et al., 1990; Ueda et al., 1987), multidrug resistance protein (MRP) (Cole et al., 1992; Grant et al., 1994), anthracycline resistance associated (ARA) protein MRP6 (Longhurst et al., 1996; O'Neill et al., 1998), breast cancer resistance protein (BCRP) (Allen et al., 1999; Doyle et al., 1998), and lung resistance-related protein (LRP) (Scheper et al., 1993; Slovak et al., 1995), which all promote drug efflux from the cell; changes in the topoisomerase II expression (Withoff et al., 1996; Zwelling et al., 1989);

overexpression of glutathione S-transferase (GST) (Singh et al., 1989); and changes in ERK1/ERK2 proteins (Shukla et al., 2010).

Screenings of the collection of *Saccharomyces cerevisiae* deletion strains up to date also reported the involvement of several proteins and signaling pathways in Doxorubicin resistance. These include; Sslp2 protein (Gewirtz, 1999); Bsd2 protein (Takahashi et al., 2005); SUMO pathway (Huang et al., 2007); nascent polypeptide-associated complex activity in ribosomes (Takahashi et al., 2009); extracellular signal-regulated kinases ERK1 and ERK2 (Shukla et al., 2010); endocytic Ark/Prk kinase(Takahashi et al., 2006); nitrogen permease regulator 2 (Npr2) gene (Schenk et al., 2003); cytochrome oxidase (EC 1.9.3.1) subunit IV gene (Kule et al., 1994); CLN1, CLN2 and ERG13 overexpression (Takahashi et al., 2011); checkpoint and recombination functions in G1 and/or early S phase (Westmoreland et al., 2009); and several proteins involved in DNA repair, RNA metabolism, chromatin remodeling, amino acid metabolism, and heat shock response (Xia et al., 2007).

1.6. Yeast as a Model for Drug Research

Yeast genome was the first eukaryotic genome to be sequenced in 1996 (Bharucha & Kumar, 2007; Oliver, 1997). Compared to higher eukaryotes, yeast cells are technically easier to grow, and manipulate genetically as well as being economical (Bharucha & Kumar, 2007). In addition to their practical use in laboratories, the availability of functional genomic tools for this organism makes it an ideal model for genome-wide analysis of biological, functional and chemical screenings (Menacho-Marquez & Murguia, 2007).

The budding yeast *Saccharomyces cerevisiae* is a preferred eukaryote for both developing and testing genomic technologies and approaches. It has 90 minutes of life cycle, have stable haploid and diploid forms and it is inexpensive to maintain (Menacho-Marquez & Murguia, 2007). The genome of Saccharomyces cerevisiae has approximately 6400 open reading frames (ORFs) that are ready-to-use. 31% of the proteins in yeast genome have human orthologues and 50% of the genes related to human diseases have yeast orthologues (Menacho-Marquez & Murguia, 2007). These properties make yeast cells ideal for drug research.

Several compounds are developed every year with therapeutic potential. Drugs with known mechanisms of action, are known to affect worms, yeast and flies through their therapeutic targets, which shows the utility of these organisms for drug research. Among these organisms, yeast have been used for drug screenings by several technologies, including gene expression profiling, haploinsufficiency profiling, gene deletion profiling, gene overexpression approaches, and protein chips (Menacho-Marquez & Murguia, 2007). Availability of such large set of techniques with ease of use is an advantage for drug studies on yeast.

Yeast presents a good model for drug screening since functional homologies between human and yeasts is promising and expressing drug targeted human coding sequences in yeast cells presents an opportunity for the discovery of new medicines.

1.6.1. Genome-wide Screenings by using Yeast as a Model

Several genome-wide screenings were performed by using yeast as a model organism. Pathways affected from Arcenic toxicity (Zhou et al., 2009), genes required for resistance against Cisplatin, Oxaliplatin, Mitomycin, Imatinib and Bleomycin (Aouida et al., 2004; Burger et al., 2000; dos Santos & Sa-Correia, 2009; Wu et al., 2004) , genetic requirements for resistance against several functionally distinct DNA-damaging agents (Lee et al., 2005), genes required for Gliotoxin resistance (Chamilos et al., 2008), mutations that affect resistance to Mycophenolic acid, which is an immunosuppressive drug (Desmoucelles et al., 2002) and genetic changes required for Doxorubicin (Westmoreland et al., 2009; Xia et al., 2007) have all been studied. All the genome-wide screenings found in literature was performed by using yeast deletion libraries which lack essential genes.

1.7. Aim of this Study

By genome-wide screenings, we aimed to identify new genes that play role in Doxorubicin resistance mechanisms.

Deletion of certain genes is lethal for yeast cells, so the collection of deletion libraries only covers the genes whose deletions are not lethal for the yeast cells.

Therefore, in this study, instead of screening the collection of yeast *Saccharomyces cerevisiae* deletion strains, the wild-type yeast cells were transformed by a yeast genomic library and the genes whose overexpression lead to resistance at toxic Doxorubicin concentrations were identified.

There are several studies on identification of factors that play role in drug resistance mechanisms however, data from these studies needed to be expanded. Drug resistance seems to involve multiple mechanisms and interactions of multiple factors instead of a single biochemical mechanism. Therefore, further assessments of the mechanisms of drug resistance are needed. Overcoming drug resistance will have a significant impact on survival of the patients. So, identification of new genes that play role in cancer drug resistance may provide further prognostic information as well as helping the development of new chemotherapeutic drugs and increased chemotherapeutic effectiveness.

CHAPTER 2

MATERIALS AND METHODS

2.1. Methods and the Experimental Design of the Study

The overview of the experimental design of this study is as follows:

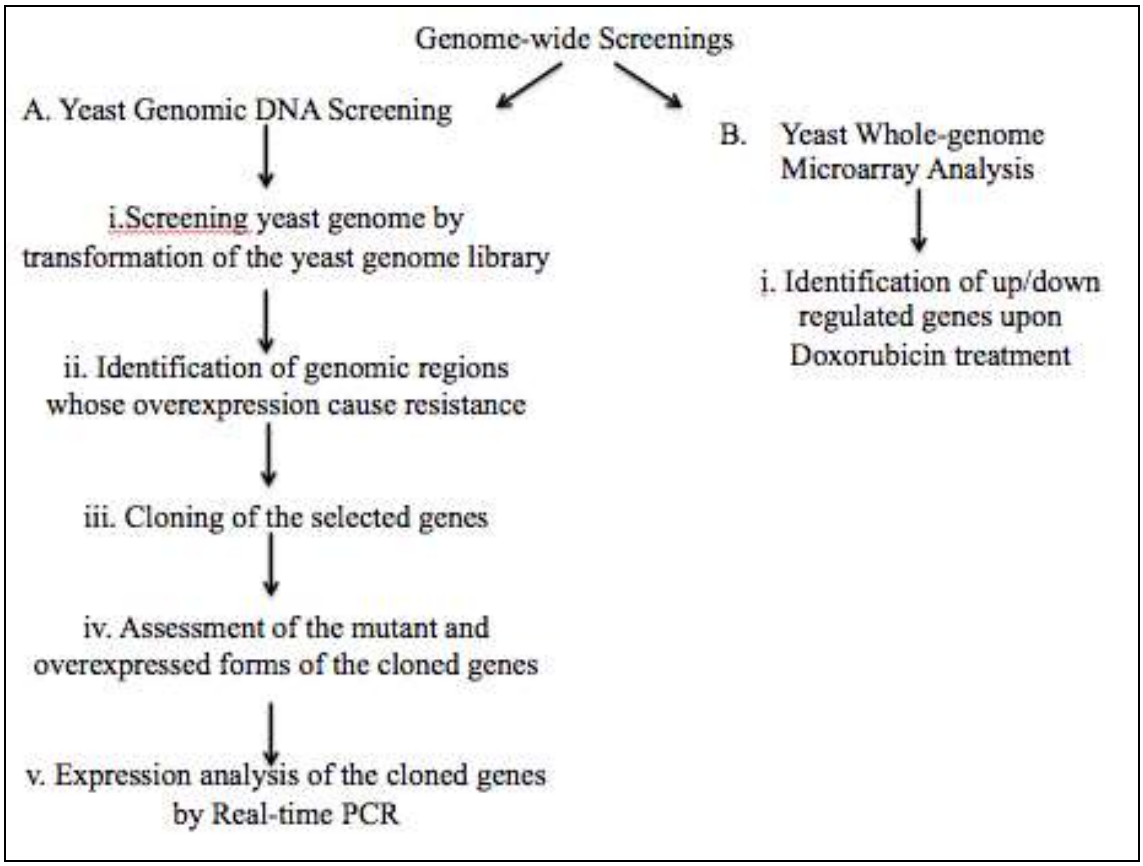


Figure 14. The Scheme for the Experimental Design of the Study

The detailed experimental procedures used in this study are indicated below and each experiment is repeated for three times.

2.1.1. Yeast Strains, Plasmids, Drugs and Growth Conditions

The BY4741 and BY4743 strains of the budding yeast *Saccharomyces cerevisiae* was used throughout this study. The wild type strain BY4741 is haploid and

is exempted from the genes that synthesize the amino acids *his3, leu2, met15*, and *ura3 (*MATa, *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0),* while the wild type strain BY4743 is diploid, and is exempted from the genes that synthesize *his3, leu2, lys2, met15* and *ura3* (MATa, *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0).* The deletion mutants used in this study do not contain the specific gene that is deleted and instead of the deleted gene, contain a kanamycin resistance gene (either MATa, *his3, leu2, met15, ura3*, ΔDeleted gene:KAN^R or MATa, *his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, LYS2/lys2Δ0, met15Δ0/MET15, ura3Δ0/ura3Δ0,* ΔDeleted gene: KAN^R).

The yeast genomic library obtained from ATCC, contains randomly restricted DNA fragments of the yeast genome (AB329 genomic library) that are integrated into YEP13 plasmids, which contains leucine (LEU2) gene as a selection marker (ATCC/ 37323).

For the cloning experiments, pAG426 plasmid (Gateway technology plasmid) was used. For the cloned PDR5 gene, the Yeplac195::PDR5 plasmid was obtained from Prof.Dr. Wenjun Guan, Zhejiang University China.

Unless indicated, all the experiments were performed by using Yeast Nitrogen Based (Synthetic dextrose (SD), 2% Glucose) media that either lacks the indicated corresponding aminoacid or not.

Doxorubicin and Cisplatin for this study was purchased from SABA pharmaceuticals (Adrimisin) and Kocak pharmaceutical company, respectively. The experiments performed with Doxorubicin were also tested on pure Doxorubicin-HCl that is purhased from European Pharmacopoeia Reference Standards.

2.1.2. Transformation of Yeast Cells

Transformations into either BY4741 or BY4743 WT cells were performed by the LiAc technique (Yeast Genetic Techniques-Cold Spring Harbour) and the transformed cells were re-inoculated into selective media that lacks the corresponding amino acid The colonies obtained from these transformations were further expanded on selective media before the drug assessment experiments.

2.1.3. Bacterial Transformation, Plasmid Isolation and Sequencing

Plasmid isolations from the resistant yeast cell colonies, were performed by using plasmid isolation kit (Thermo-Molecular Biology-GeneJET Plasmid Miniprep Kit) with additional initial lyticase treatment for 30 minutes. Each plasmid DNA isolated from these cells was transformed into E.Coli DH5α cells via heatshock procedure in order to amplify the plasmid DNA. After plasmid isolation from these DH5α bacterial cells, the isolated plasmids were re-transformed into BY4741 wild type yeast cells to confirm the resistance. After confirmation of the resistance caused by plasmid DNA, each plasmid DNA was sequenced by using YEP13 primers and DNA sequencer ABI3130xl with ABI PRISM sequencing analysis v5.1 program.

2.1.4. Cloning

By using the nucleotide-nucleotide BLAST program of NCBI, the sequenced regions were determined on yeast genome. Genes with possible resistance functions were chosen within these regions and cloned into pAG426 Gateway destination vector with uracil marker by using Gateway cloning system (Invitrogen) as described by the manufacturer. The details of the Gateway Cloning system provedure, which relies on homologous recombination, was shown below (Figure 15). E.coli OMNI cells were used for the transformations of the cloning procedure.

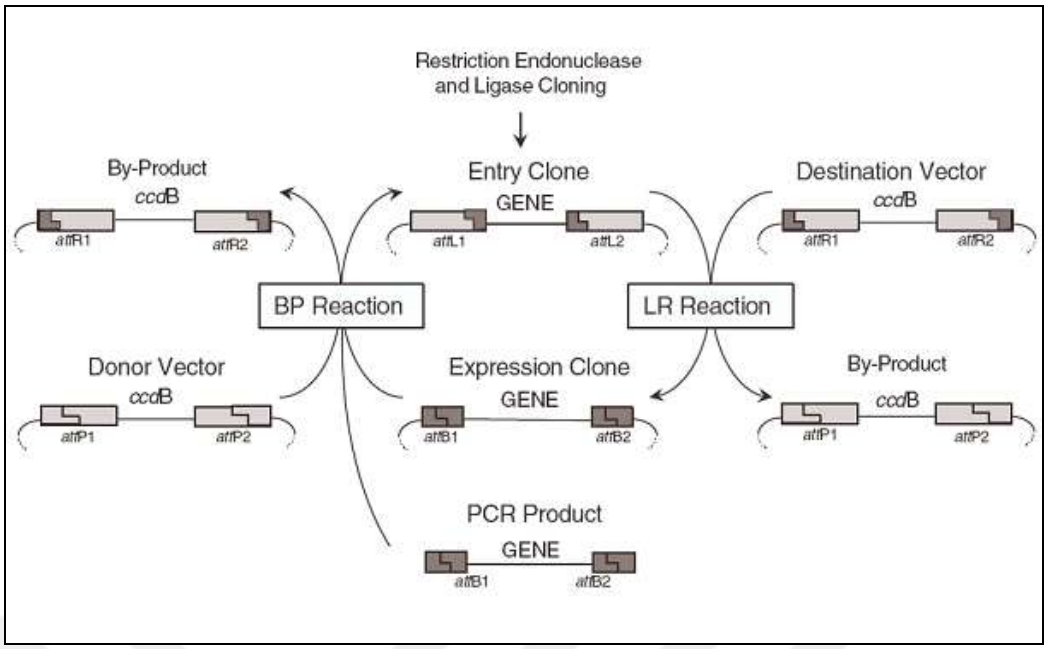


Figure 15. Gateway Cloning Technology
(Source: GIBCO Gateway Manual)

2.1.5. Overexpression Assays

For overexpression studies, the cloned genes were re-transformed into BY4741 (haploid) and BY4743 (diploid) wild type cells and plated on selective media that lacks uracil. The transformed cells were incubated at 30^{o}C for 2-3 days. The colonies that grew were assessed for their growth on selective media with toxic drug concentration by using spot assay, in order to confirm the resistance.

2.1.5.1. Gradient Spot-Assay and Growth curves

Gradient-spot assay was used for the overexpression and complementation assays. The agar plates that exclude the selected amino acid, were prepared either with or without the toxic drug level. For gradient-spot assay, the plates were prepared as having the maximum toxic level of the drug on one side and minimum level of the drug on the other side of the agar plate. The agar plates were prepared as the drug level decreased gradiently from one side to another.

The strains that were transformed with the cloned genes were grown overnight

and diluted to obtain them at log phase. After 3 hours of incubation at 30^{o}C, the cells were washed with dH_2O and their OD_{600} values were arranged into 0.2. Serial dilutions were performed with dH_2O to obtain OD_{600} values of 0.2, 0.02, 0.002, 0.0002. Then 5ul of the serial diluted cells were spotted on agar plates for the spot assays, while for gradient spot assays, only OD_{600}=0.02 cells were spotted on the plate from one side to another.

Growth curve was used both for the toxic level determination of Doxorubicin and for the confirmations of the spot assay results of overexpression strains. The cells for growth curve were grown overnight and were grown 3 more hours after dilution. OD600 values of the cells were arranged to 0.05 within selective media that either harbours 500uM Doxorubicin or not. The OD600 values of cells were recorded in every 3 hours.

2.1.6. RNA Isolation and cDNA Synthesis

RNA was isolated from log phase cells that are either treated with or without 80uM Doxorubicin by using RNA isolation kit (Thermo-Molecular Biology-GeneJET RNA Purification kit) as described by the manufacturer. The isolated RNA was transcribed into cDNA by using cDNA synthesis kit (Thermo-First Strand cDNA Synthesis kit) as described by the manufacturer.

2.1.7. Real-Time PCR Analysis

The expression of the selected genes was checked to see the response upon 80uM Doxorubicin treatment. cDNA obtained from the RNA samples of the BY4741-WT yeast cells that were either treated or not with 80uM Doxorubicin for two hours, were used as templates. The expression of AKL1, CAN1, YHR177W, PDR5, GLO4 and CUE5 genes were checked by using SyBrGreen dye (SybRGreen/ROX enzyme mix-Thermo). BioRad IQ5 program was used for real-time PCR analysis. The detailed Real-Time PCR reaction conditions was indicated in Table 1. The expression levels were determined according to ΔΔCt method.

Table 1. Real Time PCR reaction conditions.

Step 1			
	1 Cycle	95°C (initial denaturation)	10:00 min
Step 2			
	40 cycles	95°C denaturation	00:15 min
		60°C (annealing)	00:30 min
		72°C (elongation)	00:20 min

2.1.8. Cross-Resistance Analysis

For cross resistance analysis, Cisplatin, which is a platinum agent was chosen in order to see if the resistance caused by these genes can be specific for more than one cancer drug with different mechanism of action. For Cross-resistance analysis, the overexpression strains were spotted on Gradient-drug harbouring, selective SD-agar plates and incubated at 30°C for 2-3 days. Then the photos of the agar plates were taken.

2.1.9. Microarray Analysis

Microarray Analysis was performed by using 'Agilent-Yeast-One-Color' microarray. The BY4741-WT strain was compared with the 80uM Doxorubicin treated BY4741-WT cells. The bioinformatics analysis was done by using WebGestalt program.

2.1.10. Statistical Analysis

SPSS 15.0 for Windows program was used for statistical analysis. Student's T-test was used for Real-time PCR and microarray analysis data. P–value smaller than 0.05 was chosen as the significance level ($p < 0.05$) for all statistical analysis.

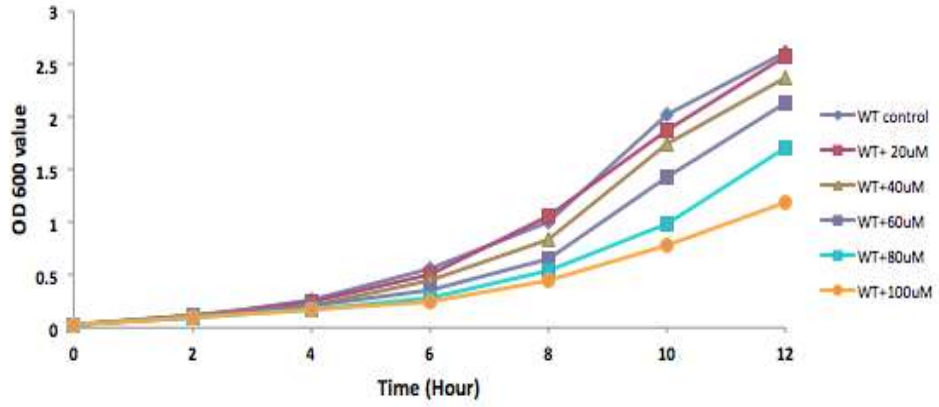
CHAPTER 3

RESULTS

3.1. Determination of the Toxic Doxorubicin Drug Concentration

The toxic level of Doxorubicin for wild type BY4741 yeast strain was determined by performing growth curves for different Doxorubicin concentrations, which were initially chosen according to the literature. First, the drug concentration interval of 20μM to 100μM was screened for 12 hours and no growth inhibition was observed (Figure 16A). Therefore, the drug concentrations between 200μM to 300μM were screened (Figure 16B). It was found that at 275μM and 300μM Doxorubicin concentration, no significant cell growth was observed and 300μM was determined as the toxic level of Doxorubicin for BY4741 wild type strain.

A)



B)

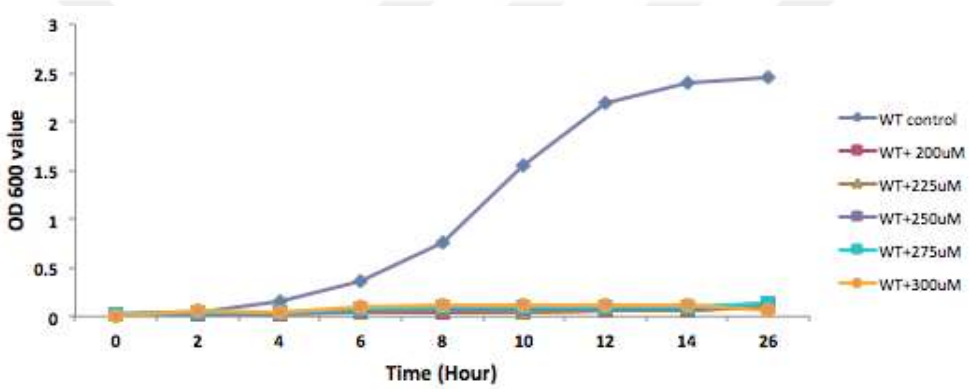


Figure 16. Growth Curves for BY4741 Wild Type Strain for Doxorubicin Drug Concentrations of **A)** 20µM, 40µM, 60µM, 80µM, and 100µM. **B)** 200µM, 225µM, 250µM, 275µM and 300µM.

3.2. Identification of the Drug Resistant Colonies by Genome-wide Screenings

The genome-wide screenings were performed by using *S.cerevisiae* yeast genome library, in which the genome of the yeast *S.cerevisiae* was randomly cut by Sau3A enzyme and each restricted fragment was ligated into YEP13 plasmids, that are restricted with the complementer BamHI enzyme (Nasmyth & Reed, 1980). The library plasmids were all sequenced and confirmed for the presence of the whole genomic regions of the yeast genome. The BY4741 WT yeast cells were transformed with this library and the transformants were grown on selective media. Each transformant was then re-inoculated into the selective SD media which contains 300µM, 400µM and 500µM Doxorubicin. The resistant colonies were chosen and expanded.

3.2.1. Resistance Against 300 and 400μM Doxorubicin Concentrations

3.2.1.1. The First Genome-wide Screen

300μM was determined as the toxic Doxorubicin concentration for BY4741 wild type cells. 400μM Doxorubicin concentration was used in screenings ,as well, in order to see if there will be genes that cause resistance against Doxorubicin concentrations higher than the toxic level.

In the first screening, 25 and 18 colonies were appeared within 36 hours after inoculation on selective media containing 300μM and 400μM Doxorubicin, respectively. 4 of these colonies were successfully sequenced and the rest were unable to grow on selective media with toxic level of Doxorubicin indicating that they either were false positive colonies or have lost their plasmids. The plasmid photos, concentrations and the genes in sequenced regions within these plasmids were shown in Table 2.

Table 2. Representative of the colonies that showed resistance to 400μM Doxorubicin and the genes that reside within these regions.

Colony ID	**Sequenced region in the genome**	**Genes in the sequenced region**
300μM resistant-colony 24	Chromosome XV- coordinates 800738 bp to 805741 bp	TMA16, NAT5, CLP, TUM1
400μM resistant-colony 10	Chromosome XVI- coordinates 414035 bp to 418995 bp	YTA6, some part of RTA16
400μM resistant-colony 11	Chromosome V- coordinates 518854 bp to 524862 bp	CCA1**,** most part of BCK2, some part of RPH1
400μM resistant-colony 12	Chromosome XIV- coordinates 72973 bp to 79780 bp	YNL295W, RIM21, Some part of MON2

3.2.1.2. The Second Genome-wide screen

In the second screening, 58 and 48 colonies were appeared on selective media within 48 hours containing 300μM and 400μM Doxorubicin, respectively. 7 of these colonies were successful in sequencing. The plasmid photos, concentrations and the genes in sequenced regions within these plasmids were shown in Table 3.

The colonies were re-confirmed by retransformation into BY4741 wild type yeast cells for their ability to grow on toxic level of Doxorubicin before sequencing.

Table 3. Representative of the colonies that showed resistance to 400μM Doxorubicin and the genes that reside within these regions.

Colony ID	**Sequenced region in the genome**	**Genes in the sequenced region**
400μM resistant-colony 3	Chromosome XV- coordinates 406267 bp to 412766 bp	GLO4, CUE5, WHI2, SNR9, SNR62
400μM resistant-colony 4	Chromosome II- coordinates 363098 bp to 368470 bp	TRM7, YBR062C, YBR063C
400μM resistant-colony 7	Chromosome II- coordinates 187482 bp to 194290 bp	FUS3**,** most of PEP1
400μM resistant-colony 24	Chromosome IX- coordinates 79406-83024	SSL2
400μM resistant colony 26	Chromosome XV-coordinates 648848-652532	GLN4, RPS28A
400μM resistant colony 38	Chromosome XIII-coordinates 38099-42313	ATR1, VAN1, ARS1704
400μM resistant colony 39	Chromosome XVI- coordinates 508147-512124	ECM23, partial RAD1, ARS1641

3.2.1.3. The Third Genome-wide screen

In the third genome-wide screening, 4 colonies showed resistance against 300μM Doxorubicin within 24 hours (Figure 17). These colonies were then retransformed into BY4741 wild type strain and checked for their resistance in both 300μM and 400μM Doxorubicin and all 4 colonies showed resistance to both 300μM and 400μM Doxorubicin. The photos of the plasmids isolated from these colonies and their concentrations were shown in Table 4.

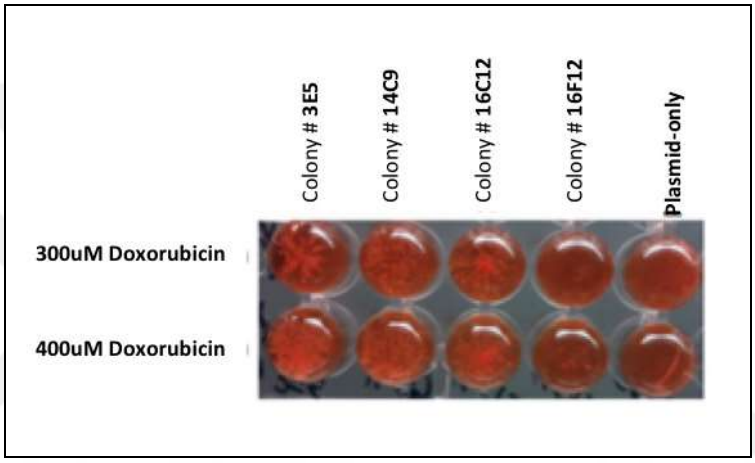


Figure 17. BY4741 wild type cell colonies transformed with genomic library and showed resistance to 300μM and 400μM Doxorubicin.

Table 4. Representative of the colonies that showed resistance to 300 and 400μM Doxorubicin and the genes that reside within these regions.

Colony ID	Sequenced region in the genome	Genes in the sequenced region
400μM resistant-colony 3E5	Chromosome XV coordinates 407059 bp to 411911 bp	GLO4, CUE5, SNR9, SNR62, part of WHI2
400μM resistant-colony 14C9	Not Sequenced	-
400μM resistant-colony 16C12	Chromosome XI coordinates 589600 bp to 595920 bp	MTD1, RPF2, most part of NUP133
400μM resistant-colony 16F12	Chromosome V coordinates 30280 bp to 33605 bp	CAN1, some part of AVT2

3.2.1.4. The Fourth Genome-wide screen

In the fourth genome-wide screening, 37 and 14 colonies showed resistance against 300 and 400μM Doxorubicin, respectively. 4 colonies among those colonies were also resistant to 500μM Doxorubicin as well (Figure 18).

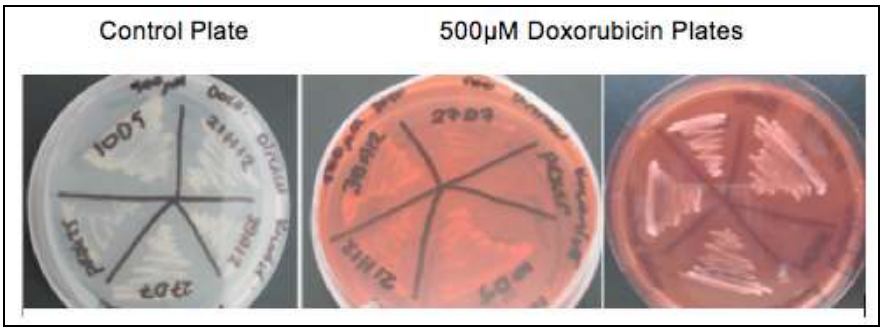


Figure 18. Colonies that showed resistance against 300,400 and 500μM Doxorubicin.

3.2.2. Resistance Against 500μM Doxorubicin

In order to identify the basic mechanism against Doxorubicin resistance, the toxic level of Doxorubicin was increased to 500μM concentration. 300μM and 400μM resistant colonies obtained from four genome-wide screenings were all screened for their resistance against 500μM Doxorubicin concentration. 6 colonies among all 300 and 400μM Doxorubicin resistant colonies, was found to be resistant against 500μM Doxorubicin. Those colonies were grown on selective media and their plasmids were isolated from yeast cells. In order to obtain required amount of plasmid DNA for sequencing, the plasmids were amplified within DH5α strain of E.coli cells and amplified plasmids were sequenced.

3.2.2.1. Sequencing Results of the 500μM Doxorubicin Resistant Colonies

The sequenced regions from 6 colonies that show resistance to 500μM Doxorubicin are shown below (Figure 19). The genome cassettes were identified via BLAST analysis of Saccharomyces Genome Database (SGD). The arrows indicate the genes that determined to be cloned primarily. The sequenced regions were also shown in Table 5.

3E5

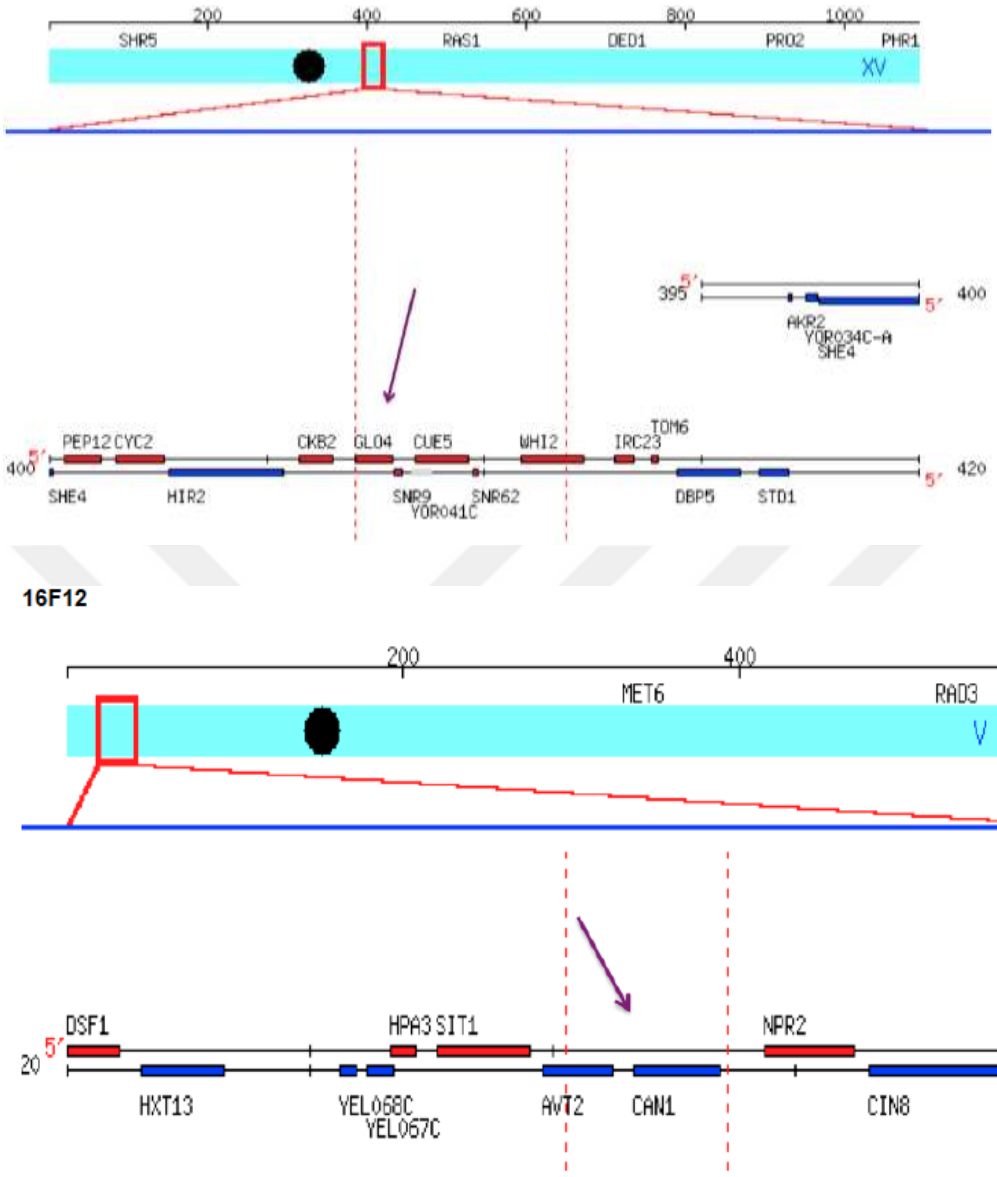


Figure 19**.** The genomic expression cassettes that show resistance against 500µM Doxorubicin.

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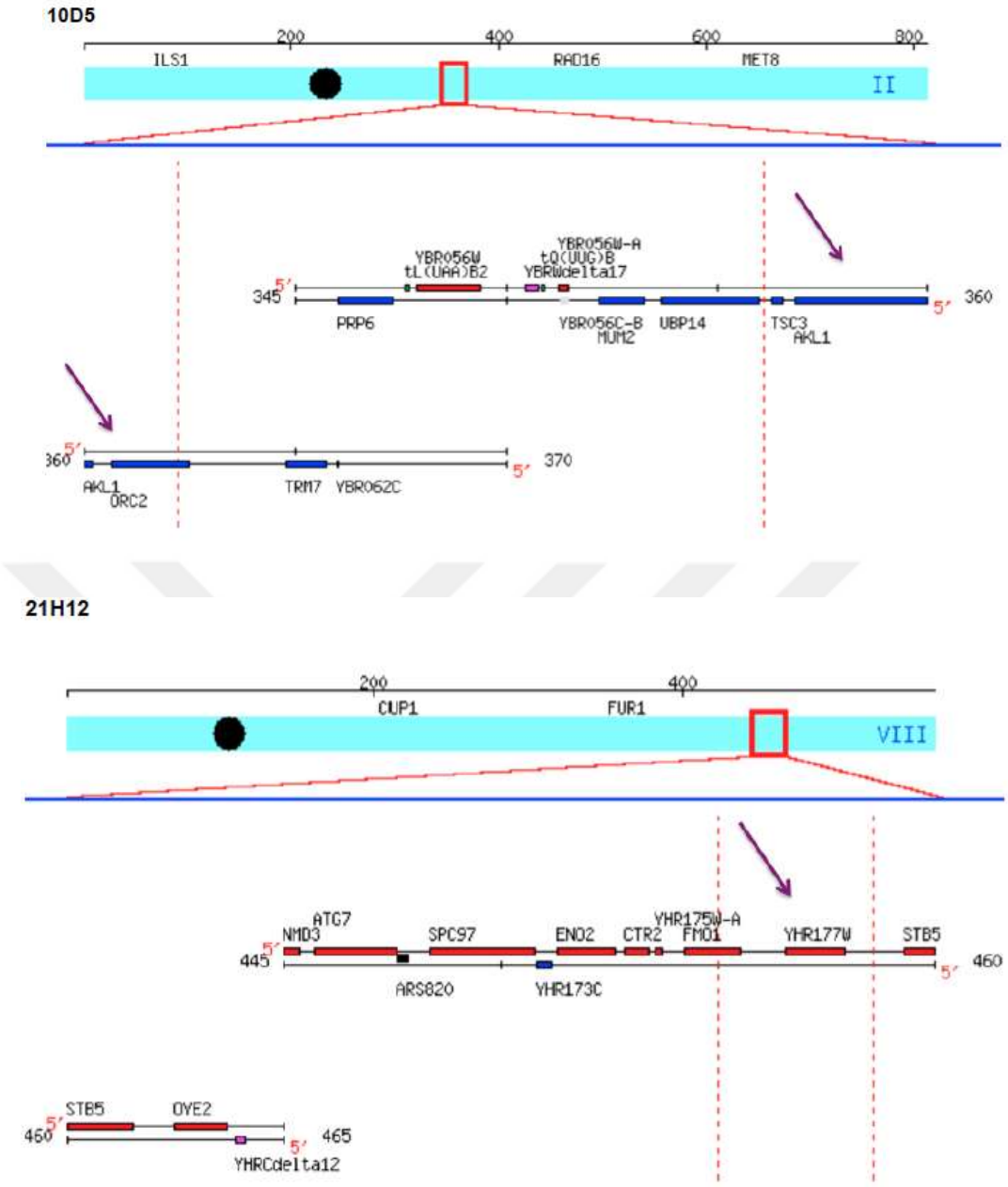

Figure 19 (cont.)

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39A12

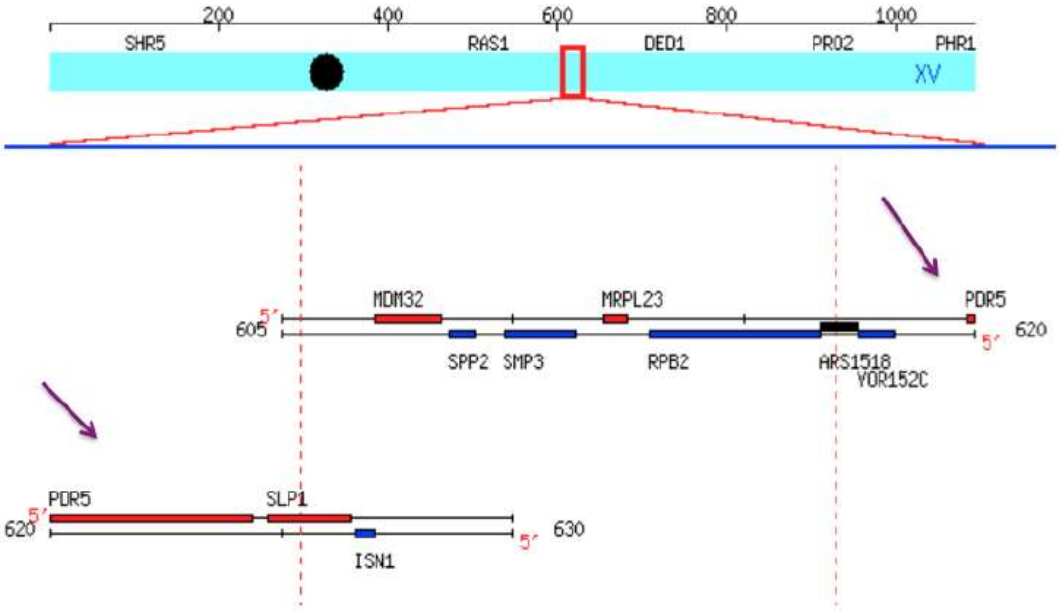

Figure 19 (cont.)

Table 5. Representative of the colonies that showed resistance to 500uM Doxorubicin and the genes that reside within these regions.

Colony ID	Sequenced region in the genome	Genes in the sequenced region
500μM resistant-colony 3E5	Chromosome XV coordinates 407059 bp to 411911 bp	**GLO4, CUE5**, SNR9, SNR62, part of WHI2
500μM resistant-colony 16F12	Chromosome V coordinates 30280 bp to 33605 bp	**CAN1,** some part of AVT2
500μM resistant-colony 10D5	Chromosome II Coordinates 346000 bp to 366000	**TSC3**, **AKL1** and part of ORC2
500μM resistant-colony 21H12	Chromosome VII Coordinates 445000bp to 465000bp	**YHR177W**
500μM resistant-colony 27D7	Chromosome IV Coordinates 1147000bp- to 1167000bp	No genes identified. Autonomously replicating sequence ARS432 is present.
500μM resistant-colony 39A12	Chromosome XV Coordinates 607000bp to 627000bp	**YOR152C**, **PDR5**, and part of SLP1

3.4. Sensitivity Assays

The sequencing results have shown that, in some cassettes, more than one gene is present. In order to identify the genes to clone primarily, the haploid and diploid mutant forms of these genes were tested for their sensitivities against Doxorubicin and

the genes whose mutant form was more sensitive to Doxorubicin, were chosen to clone primarily within those cassettes.

For the sensitivity assessments, 50µM, 100µM, 150µM, 200µM, 250µM and 300µM Doxorubicin drug concentrations were used and spotting assay was performed. PDR5, GLO4 and AKL1 shown more sensitivity agains Doxorubicin compared to WT strain. So they were chosen from their cassettes, to be cloned primarily. CAN1 and YHR177W were single genes in their expression cassettes, so they are cloned directly. The sensitivity assay results are shown in Figure 20A and 20B.

A)

Figure 20. The Sensitivity Assay for the **A)** Haploid and **B)** Diploid Mutant Forms of the Candidate Genes.

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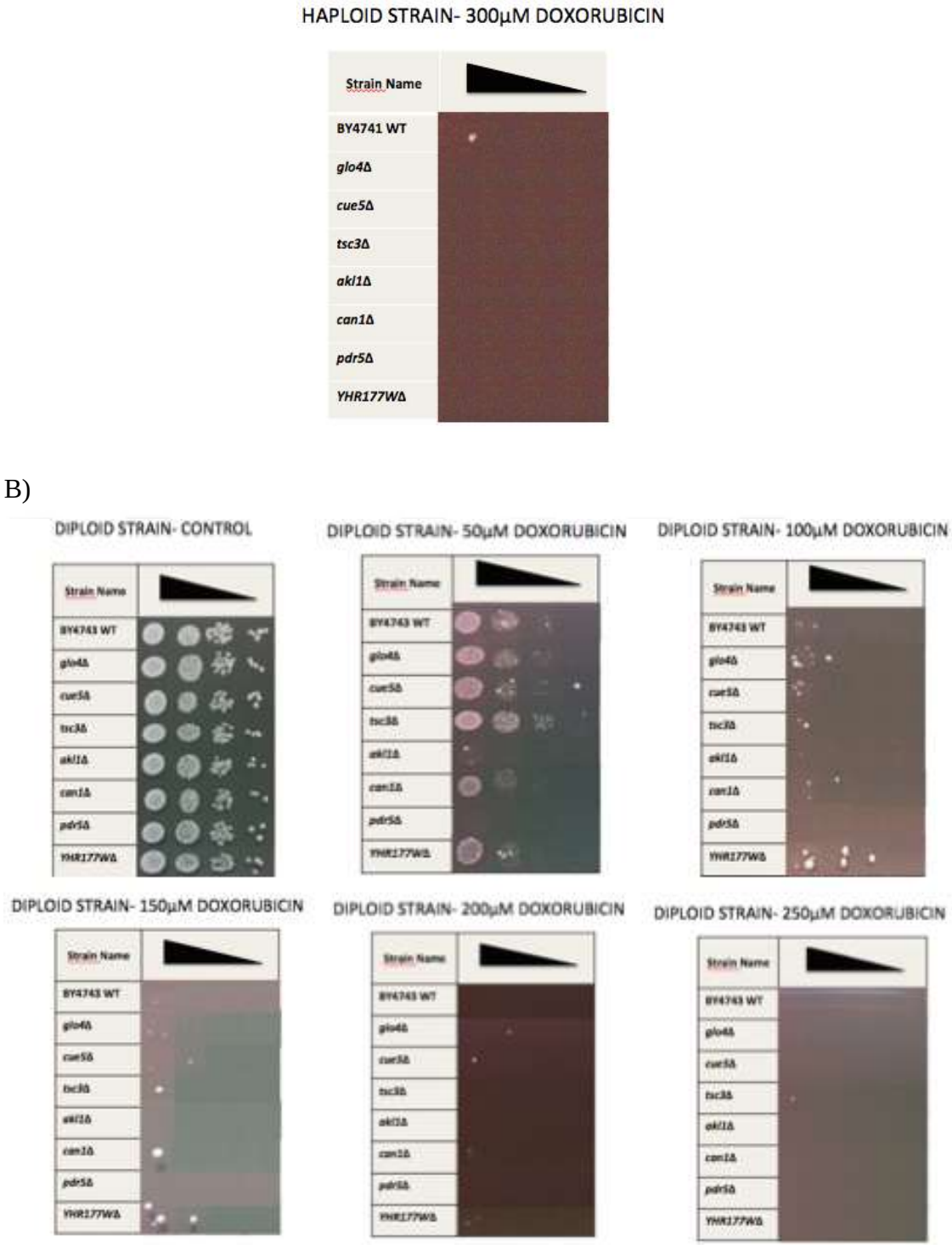

Figure 20. (cont.)

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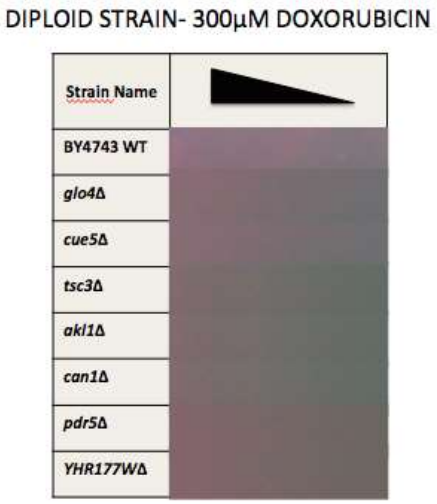


Figure 20. (cont.)

3.5. Cloning of the Selected Genes

The genes were cloned into pAG426 Gateway plasmid (with the *Ura3* selective marker) by using Gateway technology. The cloned genes were checked with BsRGI restriction enzyme (Fermentas). When empty pAG426 plasmid was restricted with BsRGI, 4 bands at 6403bp, 1281bp, 397bp and 224bp were seen. When our gene was integrated into this plasmid, a single restriction site is removed and our gene of interest is integrated instead. So the band pattern expected after BsrGI restriction is at 6403bp, 224bp and the bp of the gene of interest, unless the gene is restricted with BsrGI. Figure 19 shows the BsrGI restriction pattern of the cloned genes. The cloning of the genes AKL1, CAN1, YHR177W, CUE5, GLO4, TSC3, and WHI2 were confirmed both by restriction and sequencing analysis.

Figure 21. The BsrGI Restriction pattern of the cloned genes. AKL1 (3326 bp), CAN1 (1772bp), YHR177W (1361bp), CUE5 (1233bp), GLO4 (858bp), TSC3 (240bp), WHI2 (1462bp).

The PDR5 gene (4356bp) tried to be cloned both with Gateway Technology and regular TA cloning. However, the cloning of this gene could not be done. Therefore the gene that is cloned into Yeplac195 plasmid was taken from Zhejiang University.

3.6. Assessments of the Resistance

The resistance of the cloned genes were analysed by overexpression and complementation assays.

3.6.1. Overexpression Assay

The cloned genes were transformed back into WT BY4741 and BY4743 strains and their growth on Doxorubicin containing media was performed by gradient-spot assay. High copy expression of the genes CUE5, PDR5, YHR177W, CAN1 and AKL1, resulted in better growth in Doxorubicin (Figure 22B and 22B).

A)

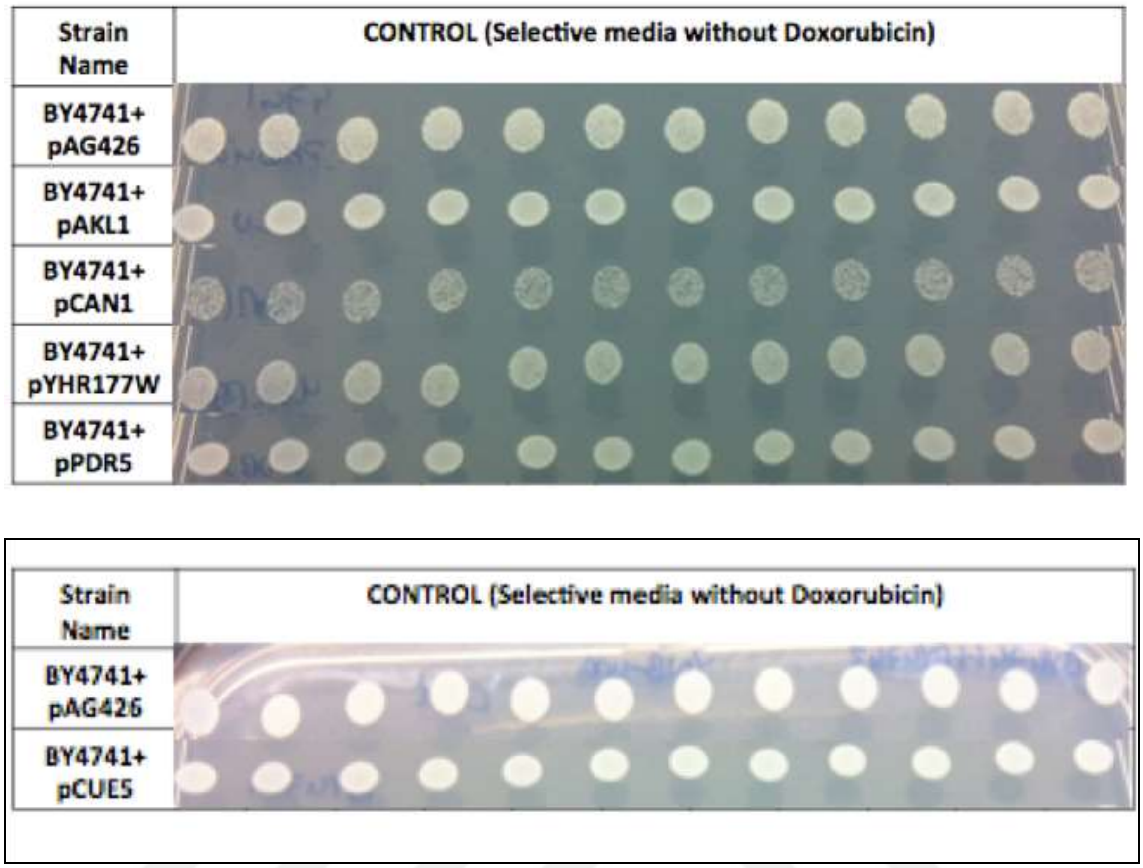
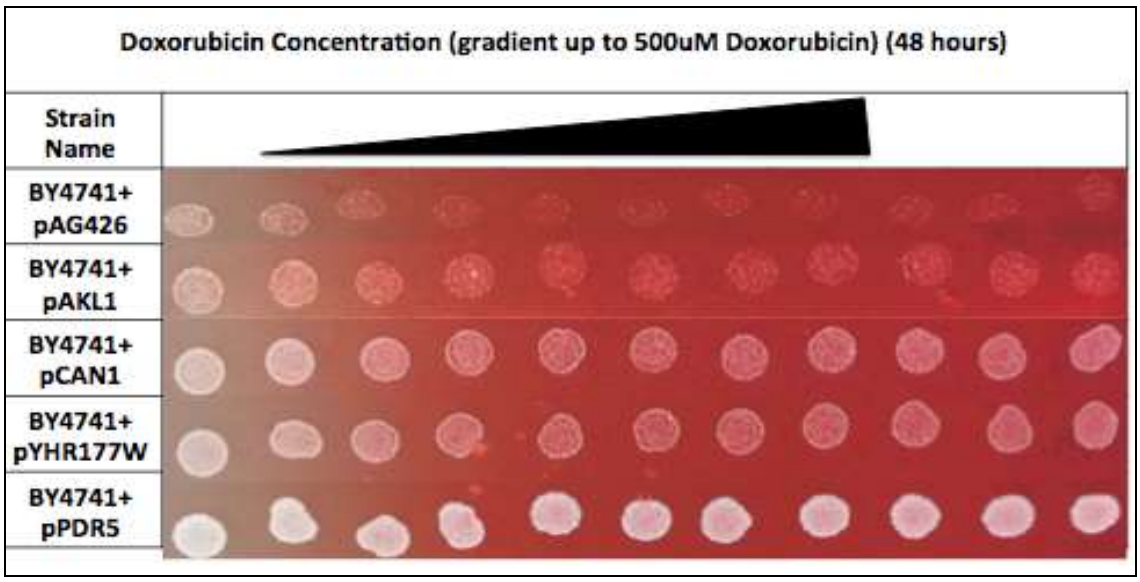


Figure 22. The Gradient-spot Assays of the haploid strains. A) Control plates without Doxorubicin. B) 500µM Doxorubicin plates.

B)



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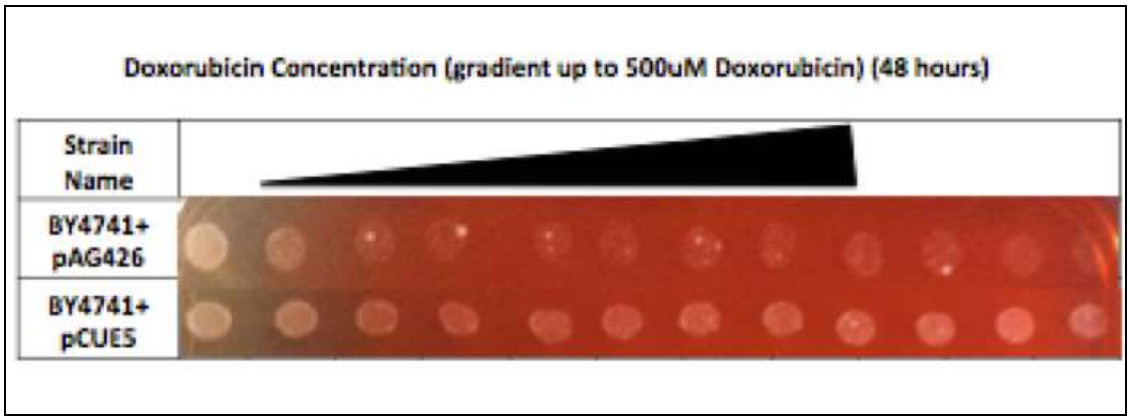


Figure 22.(cont.)

The confirmation of the spot-assay was performed by growth curve. At the end of 48 hours, WT cells with AKL1, CAN1 and YHR177W gene overexpressions resulted in 2, 5 and 6 times more growth compared to only plasmid containing WT strain (Data not shown).

A)

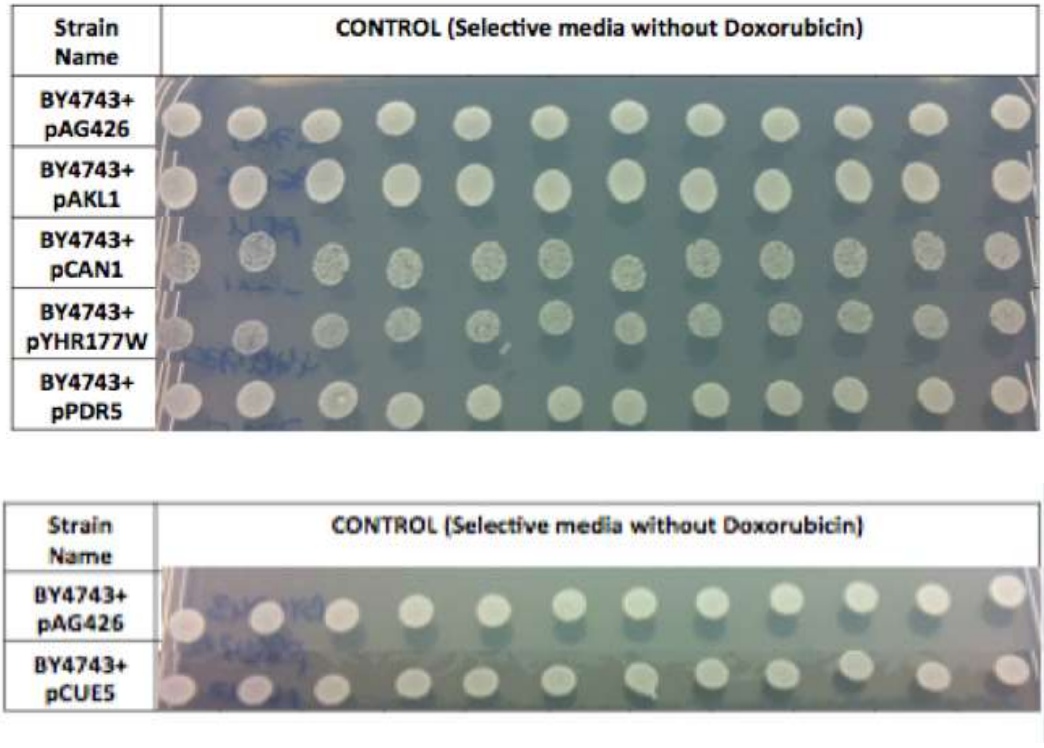


Figure 23. The Gradient-spot Assays of the diploid strains. A) Control plates without Doxorubicin. B) 500µM Doxorubicin plates.

(cont.on next page)

B)

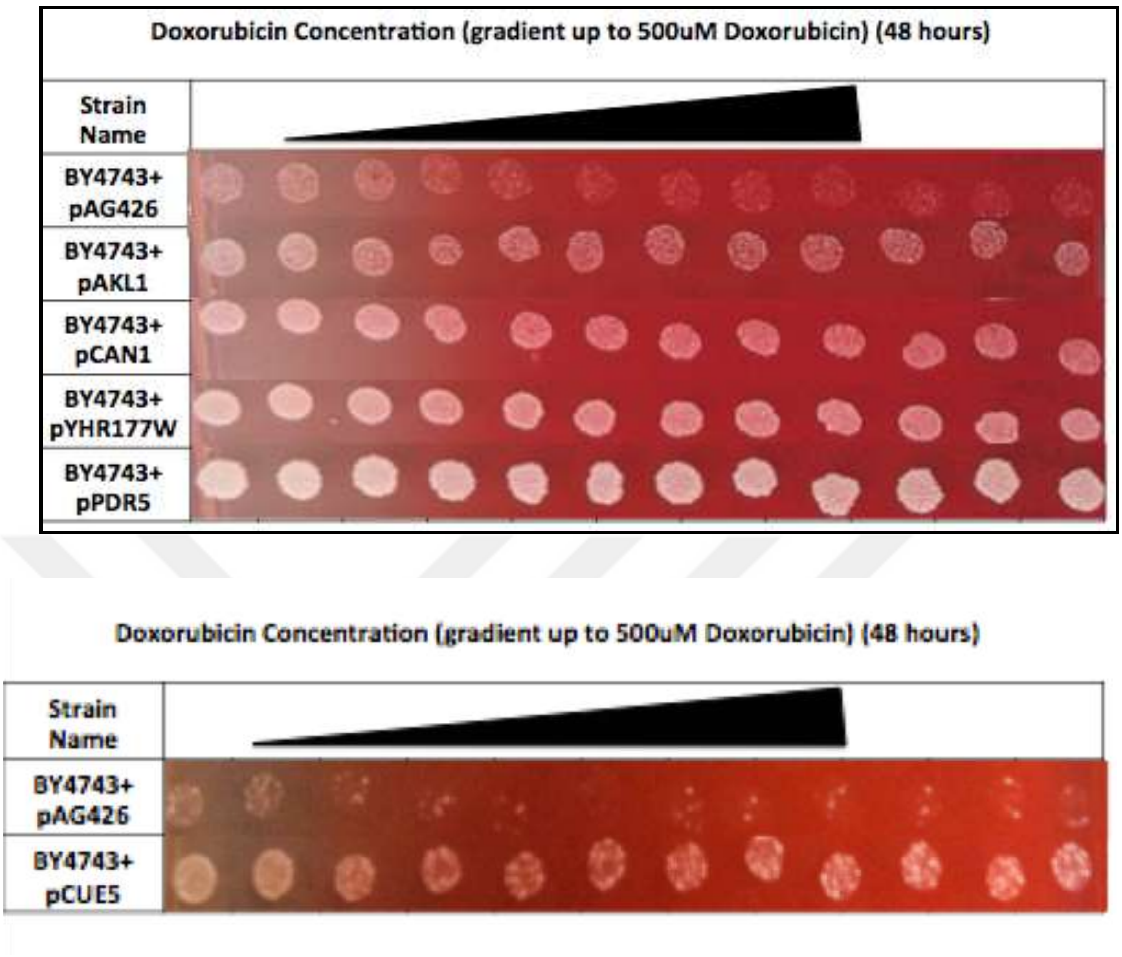


Figure 23. (cont.)

3.6.2. Real-Time PCR Analysis

For Real-time analysis, the RNA was isolated from WT cells that are either treated with 80µM Doxorubicin for two hours or not (Figure 24).

Figure 24**.** RNA samples isolated for the Real-Time PCR analysis.

cDNA from these RNA samples were synthesized with Oligo dT(18) primer and used as a template for RT-PCR analysis. Actin gene of S.cerevisiae was used as an internal control for Real-time PCR analysis and the relative expression levels of the genes compared to actin expression were shown in Figure 25. There was not any significant expression change in selected genes ($p<0.05$). The expression levels of the selected genes shown not to be affected from 2 hours of 80μM Doxorubicin treatment (Figure 25).

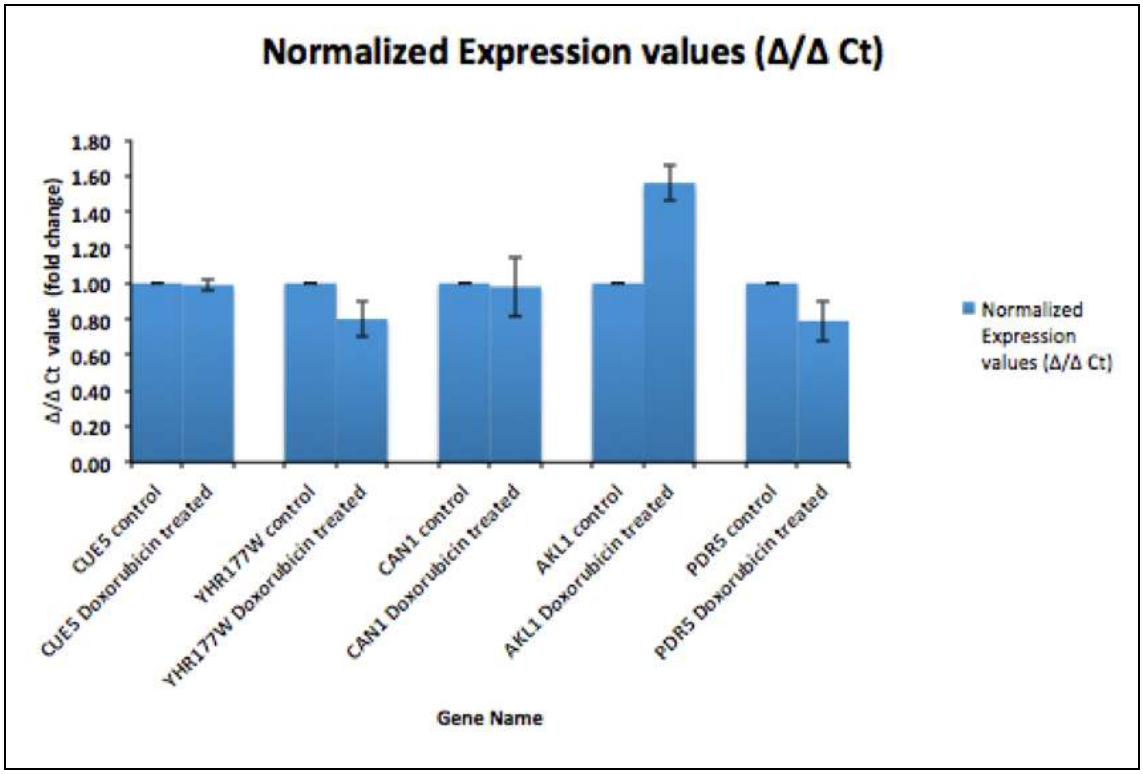
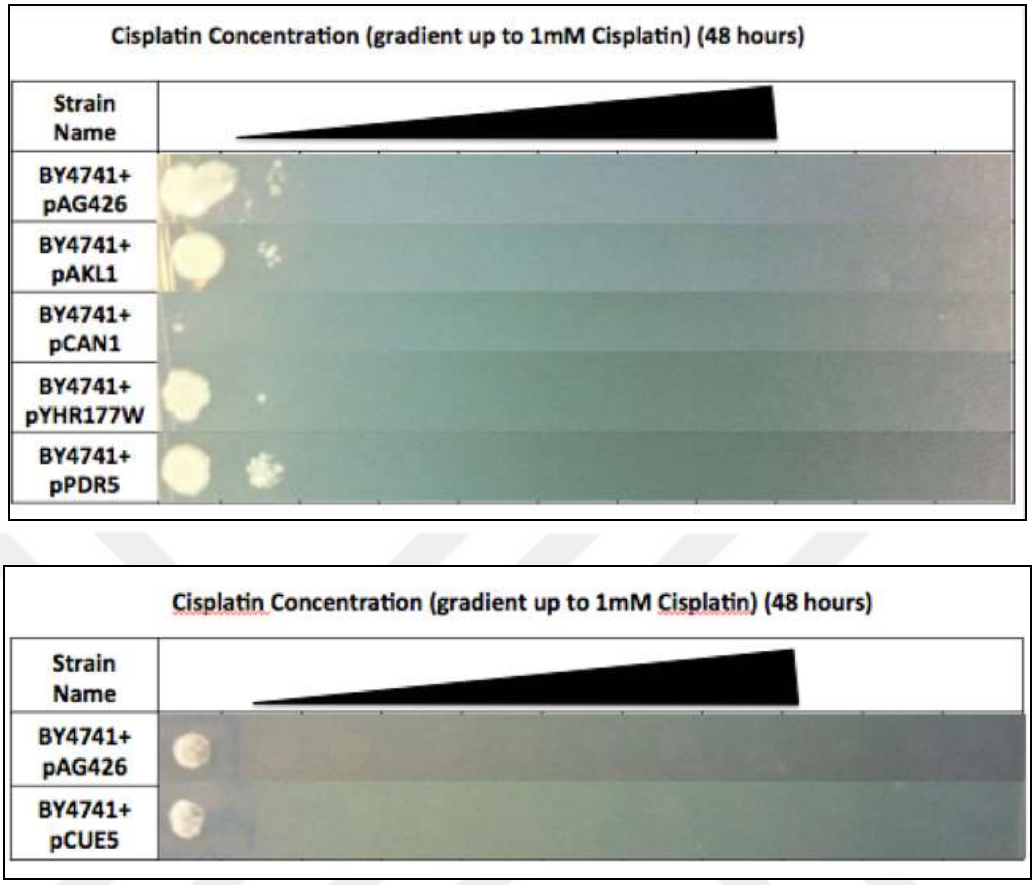


Figure 25. Relative Real-Time PCR expression levels of the identified genes upon 80μM Doxorubicin treatment. Yeast actin gene was used as an internal control for Real-time PCR analysis.

3.7. Cross-Resistance Analysis

Cisplatin was used for cross-resistance analysis. The strains that overexpress the candidate genes were spotted on Cisplatin plates. 1mM Cisplatin gradient-agar plates were used for analysis (Figure 26). Only PDR5 overexpression showed more resistance to Cisplatin. Overexpression AKL1, CAN1, and YHR177W were all showed same sensitivity to Cisplatin as wild type (plasmid-only) containing cells.

A)



B)

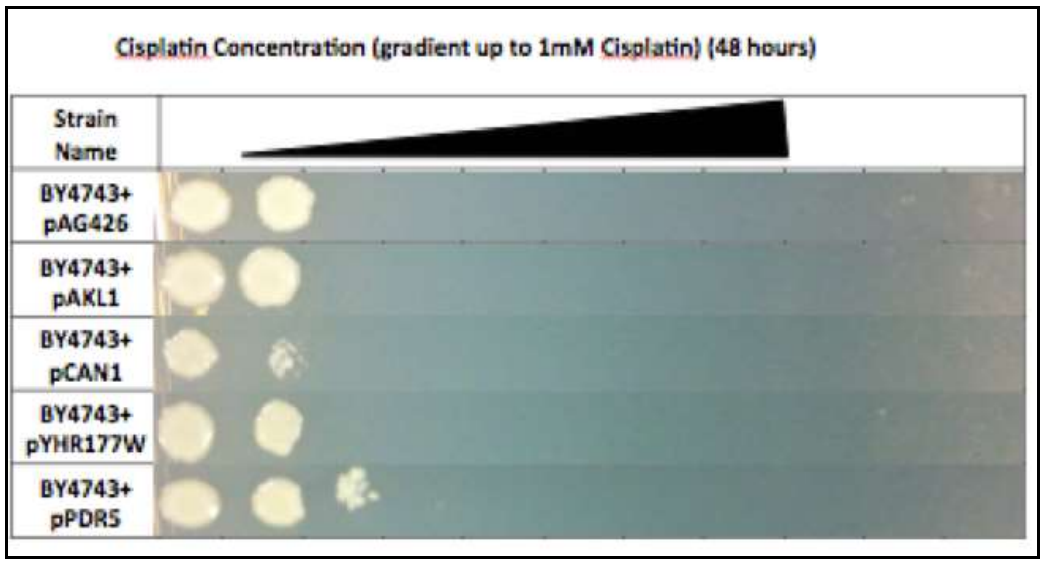


Figure 26. Cisplatin Gradient-Spot assay of A) Haploid and B) Diploid yeast strains.

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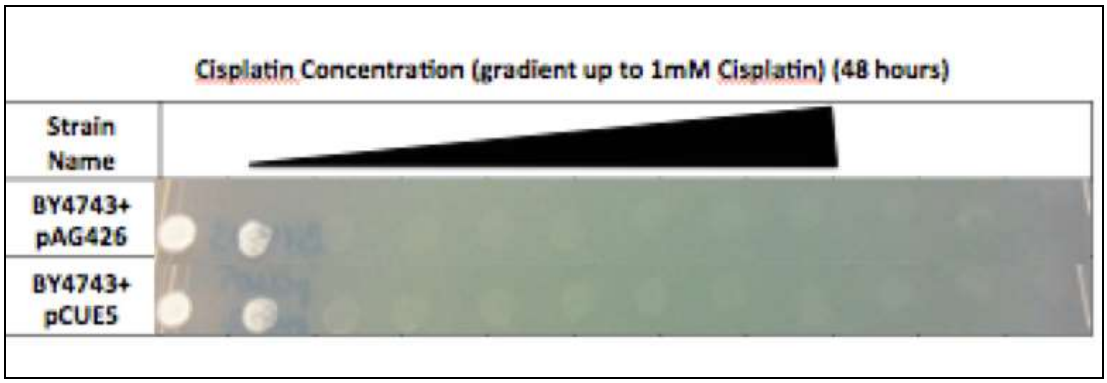

Figure 26. (cont.)

3.8. Microarray Analysis

Microarray analysis was performed with Agilent-One-Color-Yeast Expression array. The bioinformatics analysis was done via "WebGestalt" program. The genes whose expressions show significance ($p<0.005$) are listed below (Table 6). The expression changes upon 2 hours of 80µM Doxorubicin treatment were represented with the volcano plot (Figure 27). The molecular and biological functions of the genes that were affected from Doxorubicin treatment were identified with MIPS (Munih Information Center for Protein Sequences) classification using FunSpec analysis program (Figure 28).

The results indicated that general stress-response related genes are upregulated. Ubiquitination related genes also showed significant changes upon Doxorubicin treatment.

Table 6. The Representative of the Fold Changes of the genes upon 80µM Doxorubicin treatment. A) Upregulated genes. B) Downregulated genes. The changes that are found to be significant were listed only (p value< 0.05).

A)

Standard Name	Gene Name	P Value	Fold Change (log2)	Fold Change	Function
MFG1	YDL233W	0.0496	4.26	19.19	Regulator of filamentous growth
CAT2	YML042W	0.0480	2.71	6.54	Carnitine acetyl-CoA transferase
ELA1	YNL230C	0.0102	2.62	6.13	Elongin A
HRT1	YOL133W	0.0424	2.33	5.03	RING-H2 domain core subunit of multiple ubiquitin ligase complexes
SRX1	YKL086W	0.0049	2.32	5.00	Sulfiredoxin
SHH3	YMR118C	0.0336	2.30	4.91	Putative mitochondrial inner membrane protein of unknown function
ATG7	YHR171W	0.0444	2.02	4.06	Autophagy-related protein
SIZ1	YDR409W	0.0411	1.95	3.86	SUMO/Smt3 ligase
MPH2	YDL247W	0.0215	1.93	3.81	Alpha-glucoside permease
	YJL043W	0.0380	1.87	3.65	Putative protein of unknown function
PSF1	YDR013W	0.0309	1.85	3.60	Subunit of the GINS complex (Sld5p, Psf1p, Psf2p, Psf3p)
	YMR057C	0.0098	1.79	3.46	Dubious open reading frame

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Table 6. (cont.)

LEE1	YPL054W	0.0400	1.71	3.28	Zinc-finger protein of unknown function
SNX3	YOR357C	0.0016	1.59	3.02	Sorting nexin for late-Golgi enzymes
	YLR456W	0.0497	1.57	2.96	Putative pyridoxal 5'-phosphate synthase
CDC27	YBL084C	0.0293	1.54	2.91	Subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C)
RRN6	YBL014C	0.0212	1.52	2.87	Component of the core factor (CF) rDNA transcription factor complex
CPT1	YNL130C	0.0040	1.49	2.82	Cholinephosphotransferase
GRX2	YDR513W	0.0491	1.49	2.81	Cytoplasmic glutaredoxin
MNL2	YLR057W	0.0214	1.48	2.79	Putative mannosidase involved in ER-associated protein degradation
IRC23	YOR044W	0.0187	1.48	2.79	Putative protein of unknown function
URC2	YDR520C	0.0495	1.47	2.78	Putative Zn(II)2Cys6 motif containing transcription factor
ADD60	YKL206C	0.0471	1.46	2.75	Protein involved in 20S proteasome assembly
YKE4	YIL023C	0.0007	1.43	2.70	Zinc transporter
	A_06_P3974	0.0116	1.40	2.65	

(cont. on next page)

Table 6. (cont.)

LPP1	YDR503C	0.0219	1.35	2.55	Lipid phosphate phosphatase
TAH11	YJR046W	0.0102	1.31	2.48	DNA replication licensing factor
RMD9	YGL107C	0.0359	1.31	2.47	Mitochondrial protein required for respiratory growth
RPN12	YFR052W	0.0070	1.28	2.43	Subunit of the 19S regulatory particle of the 26S proteasome lid
SEC17	YBL050W	0.0194	1.27	2.41	Alpha-SNAP cochaperone
DLS1	YJL065C	0.0416	1.23	2.35	Subunit of ISW2/yCHRAC chromatin accessibility complex
APC1	YNL172W	0.0192	1.21	2.31	Largest subunit of the Anaphase-Promoting Complex/Cyclosome
YOP1	YPR028W	0.0201	1.21	2.31	Membrane protein that interacts with Yip1p to mediate membrane traffic
ELO1	YJL196C	0.0060	1.20	2.30	Elongase I, medium-chain acyl elongase
	YNL228W	0.0028	1.19	2.28	Dubious open reading frame
TFB3	YDR460W	0.0264	1.19	2.28	Subunit of TFIIH and nucleotide excision repair factor 3 complexes
	YJL068C	0.0055	1.15	2.21	Esterase that can function as an S-formylglutathione hydrolase;

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Table 6. (cont.)

TDA4	YJR116W	0.0413	1.14	2.21	Putative protein of unknown function
ATG19	YOL082W	0.0107	1.14	2.20	Receptor protein for the cytoplasm-to-vacuole targeting (Cvt) pathway
CDC4	YFL009W	0.0173	1.08	2.12	F-box protein required for both the G1/S and G2/M phase transitions

B)

Standard Name	Gene Name	P Value	Fold Change (log2)	Fold Change	Function
	YJR085C	0.0235	-1.02	0.49	Protein of unknown function
TFG1	YGR186W	0.0340	-1.09	0.47	FIIF (Transcription Factor II) largest subunit
	YLL058W	0.0357	-1.14	0.45	Putative protein of unknown function with similarity to Str2p
COQ2	YNR041C	0.0294	-1.14	0.45	Para hydroxybenzoate polyprenyl transferase
HAP5	YOR358W	0.0088	-1.14	0.45	Subunit of the Hap2p/3p/4p/5p CCAAT-binding complex
UBP16	YPL072W	0.0256	-1.14	0.45	Deubiquitinating enzyme anchored to the outer mitochondrial membrane
HOS4	YIL112W	0.0121	-1.18	0.44	Subunit of the Set3 complex

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Table 6. (cont.)

MUD2	YKL074C	0.0260	-1.27	0.41	Protein involved in early pre-mRNA splicing
PYK2	YOR347C	0.0348	-1.28	0.41	Pyruvate kinase
HHF1	YBR009C	0.0428	-1.37	0.39	Histone H4
SAT4	YCR008W	0.0473	-1.40	0.38	Ser/Thr protein kinase involved in salt tolerance
ISW2	YOR304W	0.0221	-1.41	0.38	ATP-dependent DNA translocase involved in chromatin remodeling
PRP4	YPR178W	0.0402	-1.42	0.37	Splicing factor
MGS1	YNL218W	0.0484	-1.42	0.37	Protein with DNA-dependent ATPase and ssDNA annealing activities
AVO1	YOL078W	0.0210	-1.47	0.36	Component of a membrane-bound complex containing the Tor2p kinase
	YBR124W	0.0320	-1.48	0.36	Putative protein of unknown function
FEX2	YPL279C	0.0425	-1.49	0.36	Protein involved in fluoride export
IRC2	YDR112W	0.0065	-1.52	0.35	Dubious open reading frame
	YGL185C	0.0232	-1.52	0.35	Putative protein with sequence similar to hydroxyacid dehydrogenases
PIK1	YNL267W	0.0112	-1.54	0.34	Phosphatidylinositol 4-kinase
BSC1	YDL037C	0.0342	-1.55	0.34	Protein of unconfirmed function
HTB1	YDR224C	0.0089	-1.63	0.32	Histone H2B

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Table 6. (cont.)

CFT2	YLR115W	0.0372	-1.65	0.32	Subunit of the mRNA cleavage and polyadenlylation factor (CPF)
VBA4	YDR119W	0.0418	-1.69	0.31	Protein of unknown function
	YBL062W	0.0418	-1.74	0.30	Dubious open reading frame
MET17	YLR303W	0.0421	-1.75	0.30	O-acetyl homoserine-O-acetyl serine sulfhydrylase
LUC7	YDL087C	0.0035	-1.76	0.29	Essential protein associated with the U1 snRNP complex
	YDR215C	0.0202	-1.89	0.27	Dubious open reading frame
EFG1	YGR271C-A	0.0449	-1.91	0.27	Essential protein required for maturation of 18S rRNA
	YJR011C	0.0176	-1.93	0.26	Putative protein of unknown function
PRP28	YDR243C	0.0377	-2.42	0.19	RNA helicase in the DEAD-box family
CLB6	YGR109C	0.0115	-2.75	0.15	B-type cyclin involved in DNA replication during S phase
CIN2	YPL241C	0.0079	-2.96	0.13	GTPase-activating protein (GAP) for Cin4p
	YAL042C-A	0.0094	-3.55	0.09	Dubious open reading frame
	YFR035C	0.0476	-3.78	0.07	Putative protein of unknown function

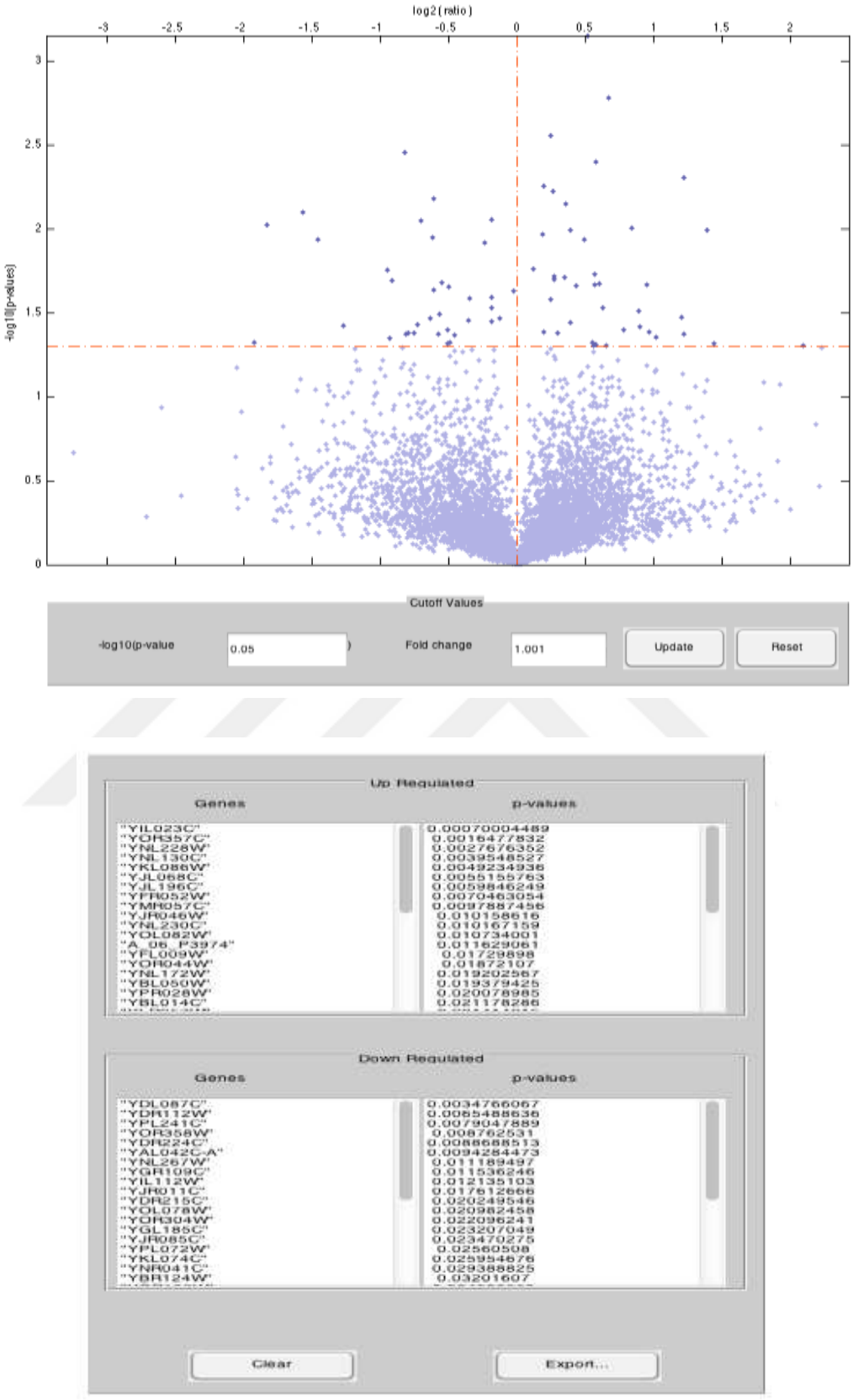

Figure 27. Volcano-plot for the expression changes upon 2 hours of 80µM Doxorubicin treatment.

Table 7. FunSpec Analysis for the MIPS functional Classification of A) upregulated genes B) downregulated genes obtained from microarray analysis. (The letter k represents the number of genes shown to function in corresponding specific functional category. The letter f represents the total number of genes in that specific functional category.)

A)

Category	p-value	In Category from Cluster	k	f
proteasomal degradation (ubiquitin/proteasomal pathway) [14.13.01.01]	9.11e-05	CDC27 CDC4 RPN12 ATG7 APC1 HRT1	6	128
glutathione conjugation reaction [32.07.07.03]	0.0003362	GRX2 YJL068C	2	5
modification by ubiquitination, deubiquitination [14.07.05]	0.001136	CDC27 CDC4 APC1 HRT1	4	79
DNA synthesis and replication [10.01.03]	0.001306	PSF1 CDC4 DLS1 TAH11	4	82
protein binding [16.01]	0.001707	SEC17 CDC27 CDC4 APC1 ATG19 HRT1 SNX3 YOP1	8	391
assembly of protein complexes [14.10]	0.005916	CDC27 TFB3 CDC4 APC1 HRT1	5	199
modification by ubiquitin-related proteins [14.07.07]	0.006651	SIZ1 ATG7	2	21
G1/S transition of mitotic cell cycle [10.03.01.01.03]	0.01988	CDC4 HRT1	2	37
ATP binding [16.19.03]	0.02534	CDC27 CDC4 APC1 HRT1	4	191
vacuole or lysosome [42.25]	0.02751	SEC17 ATG7	2	44
C-1 compound catabolism [01.05.05.07]	0.02919	YJL068C	1	5

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Table 7. (cont.)

G2/M transition of mitotic cell cycle [10.03.01.01.09]	0.03233	CDC4 HRT1	2	48
eukaryotic plasma membrane [42.02]	0.03493	YOP1	1	6
M phase [10.03.01.01.11]	0.03614	CDC27 APC1	2	51
peroxidase reaction [32.07.07.05]	0.04064	GRX2	1	7
oxidative stress response [32.01.01]	0.04148	GRX2 SRX1	2	55
detoxification by modification [32.07.03]	0.04631	GRX2	1	8

B)

Category	**p-value**	**In Category from Cluster**	**k**	**f**
splicing [11.04.03.01]	0.0007511	LUC7 PRP28 MUD2 CFT2 PRP4	5	139
C-compound binding [16.13]	0.01057	COQ2	1	2
G1/S transition of mitotic cell cycle [10.03.01.01.03]	0.01619	SAT4 CLB6	2	37
DNA conformation modification (e.g. chromatin) [10.01.09.05]	0.01673	HHF1 HTB1 HOS4 ISW2	4	188
biosynthesis of cysteine [01.01.09.03.01]	0.02104	MET17	1	4
isoprenoid metabolism [01.06.06]	0.02623	COQ2	1	5
post Golgi transport [20.09.07.06]	0.0314	PIK1	1	6
nitrogen, sulfur and selenium metabolism [01.02]	0.03288	YLL058W MET17	2	54

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Table 7. (cont.)

regulation of DNA processing [10.01.11]	0.04165	MGS1	1	8
metabolism of cysteine [01.01.09.03]	0.04673	YLL058W	1	9

CHAPTER 4

DISCUSSION

Chemotherapy is one of the major treatments used in cancer therapy. However, there is not any chemotherapy that is 100% effective. Most of the chematherapeutic failure is due to drug resistance (Longley & Johnston, 2005). The genes that play role in drug resistance have been studied for a long time. Among these studies, the ones performed at genome-wide level, represents a remarkable data to understand the mechanisms behind resistance. *Saccharomyces cerevisiae*, as a model organism, is widely used for this kind of studies.

Doxorubicin, is an anthracycline that is widely used in many cancer types (Weiss, 1992). Resistance mechanisms against Doxorubicin have been performed in various studies by using yeast cells as a model organism, including the ones at the genome-wide level (Westmoreland et al., 2009; Xia et al., 2007). However, in the genome-wide studies using yeast cells, only yeast deletion libraries were used that lack essential genes, which constitute 18% of the yeast genome (Giaever et al., 2002).

In this study, we aimed to identify Doxorubicin drug resistance mechanisms by using yeast as a model organism. However, instead of using yeast deletion library, we used yeast genome library in order to include the essential genes as well, in our screening. Therefore, all the genes that are identified in yeast genome have been included.

The toxic level for Doxorubicin differs in various yeast strains. Therefore, the primary drug levels that were chosen to test were identified according to the literature. 300μM (174 μg/ml) was chosen as being the dose in which the growth of cells was totally inhibited (Figure 16B). The yeast cells that were transformed with the genomic library were then screened for their growth on 300μM drug level. The screenings were performed on 400μM and 500μM drug concentrations, in order to identify the major mechanisms behind Doxorubicin resistance. Each transformant colony, that was able to grow on 300μM, 400μM and 500μM drug concentrations, was chosen and the plasmids they harbor were isolated and sequenced. Among the 4 different genome-wide level

screenings, the colony numbers that showed resistance to different drug concentrations were indicated in Table 8.

Table 8. The number of colonies that grew on the corresponding Doxorubicin drug concentration within 48 hours.

Genome-wide Screenings	**DOXORUBICIN DRUG CONCENTRATION**		
	300µM	**400µM**	**500µM**
1st Screening	25	18	-
2nd Screening	58	48	1
3rd Screening	4	4	2
4th Screening	37	14	4

Since genomic library is constructed by random restriction of the genome, a single plasmid may include one or more genes according to the restriction pattern. Therefore, as the sequencing results indicated, some resistance cassettes include more than one gene. Among the sequenced regions, some genes previously shown to be related with Doxorubicin resistance. These are; RAD1, which is an endonuclease functioning in double-strand DNA repair network that is shown to be related with Doxorubicin resistance (Garcia-Prieto et al., 2010); SSL2, which is known to be involved in DNA repair (Furuchi et al., 2004a; Furuchi et al., 2004b); ECM23, that function in r-RNA processing which affects Doxorubicin resistance (Rakauskaite & Dinman, 2008).

In order to identify the gene/s that causes resistance in a single cassette, each gene within the cassette must be cloned separately and analyzed for its role in resistance. When the number of colonies that showed resistance against 300µM and 400µM Doxorubicin is taken into account, the number of candidate genes increases a lot, which makes identification of a certain mechanism difficult. Therefore, all the screenings were performed on 500µM Doxorubicin and the colonies that could grow are

selected. These colonies are thought to relate with the major mechanism behind Doxorubicin resistance.

Seven colonies showed resistance against 500uM Doxorubicin in total; 1 colony from the second screening, 2 colonies from the third screening and 4 colonies from the fourth screening. The colony from the second screening was overlapping with one colony from the third screening. Therefore, overall 6 genomic cassettes have shown resistance to 500μM Doxorubicin. The sequencing results of these cassettes were shown in Table 5. Two of the genes within these cassettes, AKL1 and PDR5, were previously shown to relate with Doxorubicin resistance (Hiraga et al., 2001; Takahashi, 2013), which supports our screening results. Assesing the functions of the genes whose overexpressions caused resistance against 500μM Doxorubicin would give us a better understanding of the major mechanism behind Doxorubicin resistance.

AKL1 is a Serine-threonine protein kinase that is involved in endocytosis and actin cytoskeleton organization (Cope et al., 1999; Henry et al., 2003). Its overexpression was shown to cause Doxorubicin resistance (Takahashi, 2013), while its mutant form makes yeast cells more sensitive to Doxorubicin (Westmoreland et al., 2009). Our results support these findings as well, in which cells that overexpress *Akl1* showed 2 times more growth compared to the corresponding wild type cells (Figure 22 and 23), while Δ*akl* mutant cells were more sensitive to Doxorubicin (Figure 20). Ark/Prk kinase family proteins function in endocytosis as well as cytoskeleton formation. Sla1/Pan1/End3 complex, which is involved in endocytosis, was shown to be affected from Akl1 overexpression (Takahashi et al., 2006). Akl1 phosphorylates Pan1p and leads to dissociation of the yeast Sla1/Pan1/End3 complex, which regulates the internalization step of endocytosis (Zeng et al., 2001). Dissociation of the complex subsequently results in a decrease in endocytosis. As *Takahashi and collegues* discussed in detail, this dissociation may affect Doxorubicin resistance in several ways: either the drug internalization can be reduced since Doxorubicin crosses across the cellular membrane through diffusion, or the membrane proteins that have a role in resistance, like p95, can be more stable (Chen et al., 1990; Ross et al., 1997), or the lipid composition of the membrane can be regulated in a way to cause resistance, since Doxorubicin is known to affect the membrane compositions of cells (Tritton, 1991). The reduction in endocytosis was found not to affect the drug accumulation at all (Takahashi et al., 2006). Therefore resistance to Doxorubicin upon Akl1p overexpression may be through mechanisms other than reduced endocytosis. Also, very

few drugs were said to enter the cell through endocytosis (Gottesman, 2002). However, reduction of endocytosis may have indirect effects for drug resistance. For example, reduced endocytosis was shown to stabilize the Pdr5p, which is the main drug transporter in yeast cells (Kolaczkowski et al., 1996). Therefore, overexpression of Akl1 may affect Doxorubicin resistance indirectly through PDR5 stabilization as well. Overexpression of AAK1, which is the human orthologue of AKL1, was reported to cause Doxorubicin resistance in Hela cells (Takahashi et al., 2006). So, the mechanism/s of Doxorubicin drug resistance by Akl1 overexpression might be conserved in higher eukaryotes, too.

PDR5 is a plasma membrane ATP-binding cassette (ABC) transporter that is regulated by Pdr1p and function as a multidrug transporter for pleiotropic drug response (Ernst et al., 2005). It is known to be involved in cation resistance (Miyahara et al., 1996),and lipid transport (Decottignies et al., 1998; Kihara & Igarashi, 2004) as well. PDR5 is a well studied protein due to its function against variety of cancer drugs. Since it is the main ABC transporter in yeast cells, obtaining PDR5 from genome-wide screening was not surprising.

The major mechanism of multidrug resistance in cultured cells is the expression of P-glycoprotein, which is a multidrug transporter (Gottesman, 2002). This protein is coded by MDR1 gene, which is the human orthologue of the yeast PDR5 gene and is involved in efflux of drugs as well as transport of nutrients and important biological molecules in and out of the cells across the membrane. There are 48 known ABC transporters in human, including P-glycoprotein. It consists of two ATP binding cassettes and two transmembrane regions. Upon drug binding, one of the ATP-biding domains is activated and ATP is hydrolyzed. This event changes the confirmation of P-glycoprotein and the drug is released into the extracellular space. Subsequent hydrolysis of the second ATP, restores the original state of the transporter protein (Gottesman, 2002). P-glycoprotein, as well as PDR5, binds neutral or positively charged hydrophobic drugs, which makes it the major transport system for variety of drugs, including anticancer drugs. P-glycoprotein was shown to be expressed in many cancer types, including hematopoietic system cancers, gastrointestinal tract cancers, and childhood cancers (Gottesman, 2002). The overexpression of PDR5 resulted in the highest resistance among the identified genes. Therefore, it can be the major mechanism for Doxorubicin resistance when whole yeast genome is assessed for Doxorubicin resistance.

Next to its efflux function, overexpression of PDR5 may affect resistance through induction of changes in membrane asymmetry. Three enzyme groups are found to play a role in trans-bilayer movement of glycerophospholipids. Cross-talk between glycerophospholipids and sphingolipids were found and Pdr1 was found to be important in this crosstalk through its regulation of Yor1p, Pdr5p and Rsb1p. PDR5 mutants, showed an increase in efflux of sphingolipid long-chain bases (LCBs) (Kihara & Igarashi, 2004). Since Doxorubicin enters cell through diffusion, the changes in asymmetry between two lipid bilayers may affect the drug diffusion. Therefore, overexpression of PDR5 may function both through induction of drug effux and reduction of drug diffusion into the cell.

Plasma membrane of *Saccharomyces cerevisiae* contains stable compartments that are specialized in function (Stradalova et al., 2009). Two major compartments of the yeast plasma membrane are; membrane compartment of Can1p (MCC) and membrane compartment of Pma1p (MCP) (Stradalova et al., 2009). CAN1 is a plasma membrane arginine permease, arginine-H^+ symporter, which is exclusively associated with lipid rafts (Malinska et al., 2003). It is known to function in endocytosis as well as nitrogen signaling. Can1p is located in ergesterol-rich domains of the plasma membrane that harbors several proteins required for filamentious growth (Song & Kumar, 2012). Therefore, overexpression of CAN1 may also lead to increase in proteins related with filamentious growth and may cause Doxorubicin resistance. Microarray data suggest that, MFG1 protein which is a regulator of filamentous growth showed 19.19 fold increase in its expression (Table 6A). The common transcription factors for MFG1 and CAN1 are found to be Msn2p and Ace2p. Therefore, the overexpression of CAN1 may somehow be related with MFG1 overexpression through a pathway that includes Msn2p or Ace2p and lead to induction of filamentus growth, which can be a possible escape mechanism from drug toxicity. Another mechanism for drug resistance upon CAN1 overexpression may be related with generation of proton gradient across the membrane. Overexpression of Can1p may lead to an increase in proton gradient across the membrane and this may result either in activation of other H+-ATPases and result in efflux of the drug out of the cell or changes in signaling pathways that activates the stress-response pathways.

YHR177W (reserved name ROF1) is a putative transcription factor with a WOPR domain, whose overexpression shown to cause either cell cycle delay or arrest(Lohse et al., 2014). Proteins with WORP domains are shown to be important in

pathogenesis (Lohse et al., 2014). Filamentous growth of cells is an indicator of pathogenesis. Therefore YHR177W overexpression may lead resistance through activation of invasive growth. Also GTS1, whose known to function in endocytosis is is an interactor protein of YHR177W. In previous studies, GTS1 mutation was shown to reduce endocytosis by disrupting the dynamics of Pan1 complex and increase invasive growth (Toret et al., 2008). Therefore the effect of YHR177W overexpression may include GTS1 protein to cause Doxorubicin resistance either through endocytic changes or induction of invasive growth or both.

CUE5 is a protein that binds ubiquitin and facilitates intramolecular monoubiquitination. Ubiquitin-dependent degredation of proteins that play role in drug sensitivity may be possible mechanism of drug resistance caused by overexpression of this protein.

When we look at the transcription factors that are associated with these genes by using Yeastract program, some transcription factors are found to be common among AKL1, YHR177W, CAN1, and PDR5. The transcription factors Ste12, Sok2 and Msn2 are found to activate CAN1, PDR5 and YHR177W, while Ash1, Ace2 are found to activate PDR5 and CAN1 only. Cin5 is found to activate AKL1, CAN1 and YHR177W, while Upc2 activates PDR5 and AKL1 only and Apt23 activates PDR5, AKL1 and YHR177W. In microarray analysis, the genes CAT2, ATG19, YKE4 and SNX3, that are in the transport category of biological functions found to be affected from Doxorubicin (by Funspec analysis), are upregulated 6.54 fold, 2.20 fold, 2.7 fold, 3.03 fold respectively. These genes also have common transcription factors with AKL1, CAN1, YHR177W and PDR5. Therefore, some genes among the identified ones that cause resistance to Doxorubicin, may act on a similar pathway or may intersect at some point to cause this resistance.

When the microarray data is assessed, CUE5, AKL1, CAN1, YHR177W and PDR5 genes were not found to show a significant change upon 80uM Doxorubicin treatment. The Real-time PCR results supports this data as well (Figure 25). Therefore the genetic regulations upon Doxorubicin treatment seem to differ from overexpressions of the identified genes. However, due to the interactions indicated above, these proteins may somehow indirectly affect the other proteins that are found to be significantly up or down regulated or vice versa and result in Doxorubicin resistance. If we consider the biological and molecular functions of the genes found to be significantly affected from Doxorubicin in means of their expressions, as shown in the Volcano plot, (Figure 27).

they mainly found to be related with ubiquitin dependent events (Figure 28). This may be a stress-response event, which lead to proteosomal degradation of the oxidized proteins. Upregulation of proteasome related genes such as 2,75 fold increase in ADD60 that functions in 20S proteasome assembly and 2.43 fold increase in RPN12, which is the 19S subunit of the 26S proteasome lid may lead to a decision that the proteosomal events are activated in order to degrade the ubiquitinated proteins. However, upregulation of these genes may not be totally related with protein degradation in proteasomes. Recently, Doxorubicin was shown to translocate into the nucleus via proteosomes (Kiyomiya et al., 2001). The mechanism involves the Doxorubicin binding to the proteasome in the cytoplasm after its diffusion from the plasma membrane and the translocation of 'Doxorubicin-proteosome complex' into the nucleus through nuclear pores. Subsequently, Doxorubicin dissociates from its proteasome partner to function on DNA (Kiyomiya et al., 2001; Minotti et al., 2004).

General stress response pathways also seem to be activated upon doxorubicin treatment including overexpression of MGF1, which is a regulator of filamentious growth; ATG7, which is an autophagy-related protein; antioxidant defense systems including SRX1 (sulfiredoxin) (Table 6A). When entered into the cell, one electron addition to the quinone moiety of Doxorubicin, results in formation of 7-deoxy-doxorubicione (Thorn et al., 2011). During this change, reactive oxygen species are formed. The cytotoxicity of Doxorubicin is also caused from this reactive oxygen species formation. Therefore upregulation of genes that play role in general stress response pathways is also an expected outcome from Doxorubicin treatment.

Overall, the results show that the common factor among four of the identified genes whose overexpression leads Doxorubicin resistance seems to be the change in plasma membrane composition. This can be either through the effect of PDR5 or through other mechanisms.

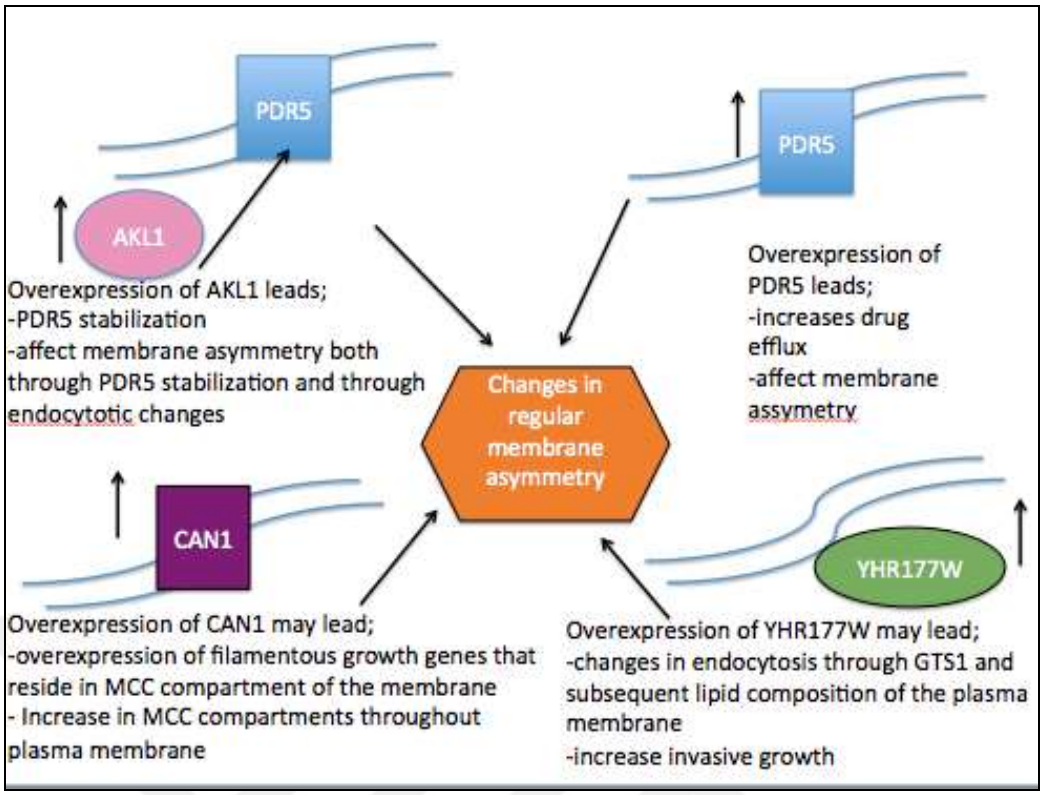

Figure 28. Possible mechanisms for the major Doxorubicin Resistance in yeast cells.

Assessing the roles of the human orthologues of the yeast genes would be helpful to understand their roles in Doxorubicin drug resistance. The human orthologues of some of these genes were shown in Table 9. As a future perspective, these genes are planned to be further cloned into human expression vectors and overexpressions, as well as siRNA silencing, of these genes in selected human cell lines will be performed. The viabilities of the constructed cell line will be assessed in the absence and presence of Doxorubicin.

Table 9. Human Orthologues of the yeast genes that caused resistance against 500μM Doxorubicin.

Systematic Name:	**Gene Name:**	**Description:**	**Function:**	**Human orthologue gene (Ensemble and MIT-Isobase databases were used)**
YOR042W	CUE5	Linking Ubiquitin conjugation to ER degredation	Ubiquitin-binding protein; CUE5 domain that binds ubiquitin is responsible from the intramolecular monoubiquitination.	Not present.
YEL063C	CAN1	Canavanine resistance	Plasma membrane arginine permease;associated exclusively with lipid rafts. Mutation leads to canavanine resistance	Solute carrier family 7, member 1. (SLC7A1) (cationic amino asid carrierı, y^+ system) (Isobase database)

(cont. on next page)

Table 9. (cont.)

YBR059C	AKL1	Ark family kinase-like protein	Ser-Thr protein kinase; Ark kinase family member (Ark1p ve Prk1p); plays role in endocytosis and actin cyto-skeleton organization.	BMP2-inducible kinase (BMP2K) (Ensembl and Isobase databases) Adaptor-associated kinase KIAA1048 protein 1.(AAK1) (Ensembl databases)
YHR177W	*ROF1* *(reserved name)*	Regulator of the Fluffy gene	Protein of unknown function; overexpression leads cell cycle delay	Not present.
YOR153W	PDR5	Pleiotropic drug resistance	Plasma membrane ATP-binding cassette (ABC) transporter; Actively regulated by Pdr1 protein and functions as a multidrug transporter; plays role cellular detoxification.	ATP- binding cassette subfamilyi G, member 2. (ABCG2) (Ensembl and Isobase databases)

CHAPTER 5

CONCLUSION

When the overall data obtained from genome-wide screenings and microarray analysis are assessed, it seems that the escape mechanism from Doxorubicin toxicity mainly includes filamentous growth, endocytosis related pathways, general stress response pathways and changes the plasma membrane composition. AKL1, CAN1, and YHR177W are related to endocytosis either directly or indirectly through other proteins. Changes in endocytosis further lead to changes in lipid composition of cell membrane.

PDR5 overexpression was shown to be the most resistant situation against Doxorubicin. Therefore, PDR5 seems to be responsible form the main escape mechanism of cells when treated with high doses of Doxorubicin. Overexpression of PDR5 both increases the efflux of the drug and changes the membrane asymmetry therefore probably reducing the intake of the drug into the cells. When microarray data are combined with the genes obtained from genome-wide screening data, the changes in plasma membrane composition/ asymmetry is the common mechanism among AKL1, CAN1, PDR5 and YHR177W overexpression (Figure 28). And these mechanisms can somehow intersect at some point via common transcription factors that regulate these genes. However, more experiments should be performed to identify the detailed major pathway that plays role in Doxorubicin resistance. Also performing further cell line studies with the human orthologues of the selected genes would be more informative about the conservation of this major resistance mechanism in higher eukaryotes.

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VITA

Date and Place of Birth: October 4, 1984 / Izmir,Turkey

Educational Institutions Attended:

- **2002-2007** I.D. Bilkent University, Faculty of Science Molecular Biology and Genetics Department, **BSc**., Ankara/ Turkey
- **June,2004-August,2004** Dokuz Eylül University, Medical Biology and Genetics Department, **Summer Internship**, Izmir/Turkey
- **June,2005-August,2005** Karolinska University Hospital-Solna, Neurogenetics Department of Center for Molecular Medicine, **Summer Internship**, Stockholm/Sweden
- **July,2006-Sept.,2006** Karolinska University Hospital-Huddinge, Hematology Laboratory,Department of Medicine, **Summer Internship**, Stockholm/Sweden
- **2007-2009** Izmir Institute of Technology, Faculty of Science, Molecular Biology and Genetics Department, **MSc**. Izmir, Turkey
- **January, 2010- 2015** Izmir Institute of Technology, Faculty of Science, Molecular Biology and Genetics Department, **PhD student**, Izmir, Turkey
- **January,2012-June,2012** University of Gothenburg, Department of Cellular and Molecular Biology,Lundbergs Laboratories, **Erasmus PhD Exchange Student**, Gothenburg/Sweden
- **March,2013-June,2013** University of Gothenburg, Department of Cellular and Molecular Biology,Lundbergs Laboratories,**PhD Student,** Gothenburg/Sweden
- **2012-2015 June(expected)** Dokuz Eylul University, Institute of Oncology, Department of Basic Oncology, **MSc,** Izmir/Turkey
- **2012-Present** **Research Assistant**, Institute of Oncology, Department of Basic Oncology, Dokuz Eylul Unviersity, Izmir/Turkey

Degrees and Honors won:

- High Honor Student at Bilkent University in 2006-2007 spring and fall semesters

Publications and Book Chapters:

- *'Assessment of chronological life span dependent molecular damages in yeast lacking mitochondrial antioxidant genes'*. **Demir AB**, Koc A. *Biochem Biophys Res Commun*. **2010** Sep10;400(1):106-10
- '*Protein oxidation and repair mechanisms in aging'*. **Ayse Banu Demir** ve Ahmet Koc, Bölüm 3, *The Field of Biological Aging: Past, Present and Future*, **2011**, ISBN: 978-81-7895-513-1.
- *'Identification of Respiratory Chain Gene Mutations That Shorten Replicative Life Span in Yeast*.' Hacioglu E[1], **Demir A.B**.[1], Koc A., *Experimental Gerontology*, **2012**; 47:149-153. ([1] Equal contribution)
- '*Linking Peroxiredoxin and Vacuolar-ATPase Functions in Calorie Restriction-Mediated Life Span Extension'*, Molin M, **Demir AB**, Int J Cell Biol., **2014**: 913071. doi: 10.1155/2014/913071