# STUDIES ON THE MOLECULAR MECHANISM OF THE DRUG METABOLISM IN PROSTATE CANCER, PC3 CELL LINES

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

DERYA DİLEK KANÇAĞI

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY

SEPTEMBER 2014

**Approval of the thesis:**

**STUDIES ON THE MOLECULAR MECHANISM OF THE DRUG METABOLISM IN PROSTATE CANCER, PC3 CELL LINES**

submitted by **DERYA DİLEK KANÇAĞI** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Biochemistry Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Orhan Adalı _____
Head of Department, **Biochemistry**

Prof. Dr. Tülin Güray _____
Supervisor, **Biology Dept., METU**

Assoc. Prof. Dr. Dilek Telci _____
Co-Supervisor, **Genetics and Bioengineering Dept., Yeditepe Uni.**

**Examining Committee Members:**

Prof. Dr. Orhan Adalı _____
Biology Dept., METU

Prof. Dr. Tülin Güray _____
Biology Dept., METU

Prof. Dr. Halit Sinan Süzen _____
Pharmacy, Ankara University

Asoc. Prof. Dr. Nursen Çoruh _____
Chemistry Dept., METU

Assist. Prof. Dr. Gökhan Sadi _____
Biology Dept., Karamanoğlu Mehmetbey University

**Date: 05.09.2014**

**I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.**

Name, Last name : Derya DİLEK KANÇAĞI

Signature :

## ABSTRACT

STUDIES ON THE MOLECULAR MECHANISM OF THE DRUG METABOLISM IN PROSTATE CANCER, PC3 CELL LINES

DİLEK KANÇAĞI, Derya

PhD, Department of Biochemistry

Supervisor: Prof. Dr. Tülin GÜRAY

Co-advisor: Assoc.Prof.Dr. Dilek TELCİ

September 2014,135 pages

Metabolic changes observed in prostate cancer are very important for both the course of the event and therapeutic way to be decided. One of the effective chemotherapeutic agent showing antitumour activity against prostate cancer is Docetaxel. The metabolism of this drug is important in term of its effectiveness.

In this study we determine the effect of CYP1A1, CYP3A4 and GSTP1 enzyme inhibitors on the efficacy of the docetaxel. For this purpose, the gene expressions and activities of these enzymes were analyzed in PC3 (androgen-independent PC cell lines) and PNT1A (immortalized prostatic cell lines) cells by using real-time PCR and activity assays. Alpha-naphthoflavone, ketaconozole and quercetin were used as the inhibitors of CYP1A1, CYP3A4 and GSTP, respectively. In order to investigate the potential of these inhibitors in docetaxel metabolism, PC3 and PNT1A cells were treated with inhibitors in combination with docetaxel and the cell viability and cell death (apoptosis/necrosis) was analyzed by using WST-1 and Annexin V assays, respectively.

According to our results, the concentration which gives 70% and 60% cell viability after 24 and 48 hours applications were found as 4 μM for docetaxel, 1000 nM for alpha-naphthoflavone, 56.5 μM for ketoconazole and 300 μM for quercetin. IC50 values were calculated as 15.1 μM for alpha-naphthoflavone, 118.7 μM for ketoconazole and 692 μM for quercetin from the activity assays. Docetaxel application alone and in combination with the inhibitors showed differences in both mRNA levels and cell viability status in treated/untreated PC3 and PNT1A cells at predetermined concentrations.

In conclusion, our results showed that, the application of docetaxel with enzyme specific inhibitors is more effective by increasing the effects of docetaxel in PC3 cells. Further studies about the metabolism of drugs will help for the development of a therapy that would increase the efficacy of the drugs which might provide a better chance for the patience survival.

Keywords: Prostate cancer, docetaxel, alpha-naphthoflavone, ketaconozole, quercetin, CYP1A1, CYP3A4, GSTP1

# ÖZ

İLAÇ METABOLİZMASININ PROSTAT KANSERİ, PC3 HÜCRE HATTINDAKİ, MOLEKÜLER MEKANİZMASI İLE İLGİLİ ÇALIŞMALAR

DİLEK KANÇAĞI, Derya

Doktora, Biyokimya Bölümü

Tez Yöneticisi: Prof. Dr. Tülin GÜRAY

Ortak Tez Yöneticisi: Doç.Dr. Dilek TELCİ

Eylül 2014, 135 sayfa

Prostat kanserinde gözlenen metabolik değişiklikler hem hastalığın gidişatı hem de uygun tedavinin kararlaştırılması için çok önemlidir. Prostat kanserinde anti-tümör etki gösteren kemoterapötik ajanlardan biri de Dosetaksel'dir. Bu ilacın metabolizması etkinliği açısından önemlidir.

Bu çalışmada CYP1A1, CYP3A4 ve GSTP1 enzim inhibitörlerinin dosetaksel üzerindeki etkisini belirledik. Bu amaçla, PC3 (androjene duyarsız PC hücre hatları) ve PNT1A (ölümsüzleştirilmiş prostatik hücre hatları) hücrelerinde gerçek zamanlı PZR ve enzim aktivite analizleri kullanılarak gen ekspresyonları ve enzim aktiviteleri analiz edilmiştir. Alfa-naftoflavon, ketokonazol ve kuersetin sırasıyla CYP1A1, CYP3A4 ve GSTP için enzim inhibitörleri olarak kullanılmıştır. Dosetaksel metabolizmasında bu inhibitörlerin potansiyelini araştırmak için, PC3 ve PNT1A hücreleri dosetaksel-inhibitör kombinasyonları ile müdahale edilmiştir. Hücre canlılığı ve hücre ölümü (apoptoz/nekroz) değerlendirmeleri sırasıyla WST-1 ve Aneksin V analizleri ile gerçekleştirilmiştir.

Sonuçlarımıza göre, 24. ve 48. saatlerde sırasıyla %70 ve %60 canlılık veren konsantrasyonlar dosetaksel için 4 µM, alfa-naftoflavon için 1000 nM, ketokonazol için 56.5 µM ve kuersetin için 300 µM olarak belirlenmiştir. Aktivite analizlerine göre belirlenen IC50 değerleri, alfa-naftoflavon için 15.1 µM, ketokonazol için 118.7 µM ve kuersetin için 692 µM olarak hesaplanmıştır. Tek başına dosetaksel uygulaması ve inhibitörlerle birlikte kombinasyon terapisi, daha önceden belirlenen konsantrasyonlarda uygulanan veya uygulanmayan PC3 ve PNT1A hücrelerinde mRNA düzeylerinde ve hücre canlılığı durumlarında farklılıklar göstermiştir.

Sonuç olarak elde ettiğimiz veriler, enzim spesifik inhibitörler ile kombinasyon terapinin PC3 hücrelerinde dosetakselin etkisini artıdığını ve bu nedenle kombinasyon tedavinin daha etkili olduğunu göstermiştir. İlaç metabolizmasına ilişkin yapılan daha fazla sayıda çalışma, ilaçların etkinliğini artıracak bir tedavinin geliştirilmesi için yardımcı olacak ve böylelikle hastanın daha yüksek sağkalım şansı olabilecektir.

Anahtar kelimeler: Prostat kanseri, dosetaksel, alfa-naphtoflavon, ketokonazol, kuersetin, CYP1A1, CYP3A4, GSTP1

**Dedicated to my husband…**

## ACKNOWLEDGEMENTS

I would like to express my deep reverence to my supervisor Prof. Dr. Tülin Güray for her encouraging guidance, incredible patience and supervision throughout this study.

I am also deeply grateful to my co-advisor Assoc. Prof. Dr. Dilek Telci for her invaluable and precise commanding and guidance both in theoretical background and in experimental design of this research thesis.

I am also thankful to my other PhD Examining Committee Members: Prof. Dr. Orhan Adalı and Prof. Dr. Sinan Süzen for their advices, suggestions and constructive criticisms. I also thank to Assoc. Prof. Dr. Şevki Aslan, Assist. Prof. Dr. Gökhan Sadi and Dr. Serdar Karakurt for providing guidance in theoretical background of this research thesis.

I would like to thank to my lab mates, Deniz Hisarlı Çalık, Deniz İrtem Kartal, Merve Erdem, Ayca Zeynep Akülke, İnci Kurt, Eray Şahin and Nihan Kılınç for their sincere friendship and supports and providing me a friendly and relaxed atmosphere in the lab.

I wish to thank also to my friend Dr. İsmail Öğülür specially for being besides me and supporting me all the time during this thesis study.

I am also thankful to the Scientific and Technological Research Council of Turkey (TUBITAK) for their support by providing scholarship during my PhD study.

I am very grateful to my all family members; especially my mother Hicran Dilek, Abuzer Dilek for their eternal love, encouragement and trust.

I would like to send my ultimate appreciation to my husband Serkan Kançağı for his endless patience, support and love. Having known his presence makes me relaxed and patient all the time. I love you very much and would like to thank for always being there.

# TABLE OF CONTENTS

# LIST OF TABLES

## TABLES

## LIST OF FIGURES

**FIGURES**

# CHAPTER 1

# INTRODUCTION

## 1.1 Prostate Cancer

Prostate cancer (PCa) is the cancer that forms in the tissues of prostate and according to World Health Organizations, it is one of the most common types of cancer in men (Bott et al., 2010). In Turkey, PCa has also high incidence (24.33/100 000), such that it is the second most commonly seen cancer type. After lung and prostate cancers, skin, breast and stomach cancers are other most commonly observed ones (**Figure 1.1**) (Yılmaz et al., 2010).

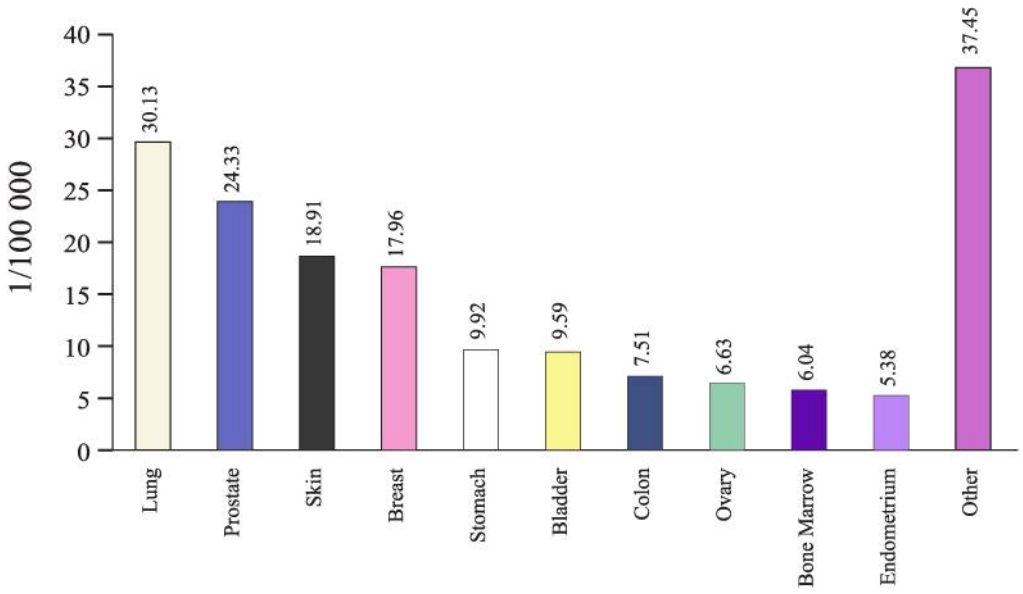



**Figure 1.1** Incidence of cancer types in Turkey (1/100.000) (Yılmaz et al., 2010)

As the diagnosis of PCa is used since 60s and 70s, the opportunity for interventions to prevent or slow the progression of the cancer is possible. Prostate cancer usually grows slowly and initially remains confined to the prostate gland, where it may not cause serious harm. Also its latent period is long and it takes 10 to 12 years to be clinically obvious (Brooks et al., 2001). Severity of the disease shows variability from patient to patient, such as indolent tumours to those aggressive cancers. This means that the disease may require little or no treatment or radical therapies (Bott et al., 2010). These treatments range from surveillance to radical local treatment or androgen-deprivation treatment. Although most tumours relapse within 2 years to an incurable androgen-independent state, in advanced PCa, symptoms are reduced by androgen deprivation in 70–80% of patients approximately (Crawford et al., 2003).

A positive family history comes with strong evidence as a risk factor, followed by age and ethnic origin (African; American) (Crawford et al., 2003; Damber et al., 2008). The development of PCa may also be related to exogenous factors, including food consumption, pattern of sexual behaviour, alcohol consumption, exposure to ultraviolet radiation, chronic inflammation, and occupational exposure (Heidenreich et al., 2014).

## 1.2 Types of Prostate Cancer

Prostate cancers have been categorized with respect to testosterone dependency. Prostate cancer cells growing in the presence of testosterone are said to be "androgen-dependent" or "androgen-sensitive". Early-stage prostate cancers depend on androgens for growth and survival. In this situation, regression is obtained via androgen ablation therapy (Feldman et al., 2001).

Prostate cancer cells, that can still grow with very, very low levels of testosterone are named as "castration-resistant" or “androgen-insensitive” or “androgen-independent” cancers. In this case prostate cancer cells can grow even when there is no longer source of testosterone from testicles. The cancer continues to grow with the rest of the testosterone remaining in a body. Prostate cancers which are not improved by

surgery become androgen insensitive in the end, resulting in anti-androgen therapy ineffective (Feldman et al., 2001).

## 1.3 Pharmacotherapy of Prostate Cancer

The purposes of pharmacotherapy for prostate cancer are induction of remission, reduction in morbidity level and prevention from complications. At present, the surgical or medical castration are main therapy options for ablation of the production of testosterone (T), dihydrotestosterone (DHT) and related androgens by the testes. Nevertheless these treatments do not affect the production of androgen by adrenal, prostate and other tissues, these agents are often used in combination with androgen receptor antagonists to prevent their action (Vasaitis et al., 2011). Luteinizing hormone–releasing hormone (LHRH) analogue, androgen antagonists, gonadotropin-releasing hormone (GnRH) agonists, bisphosphonates, antifungal agents, chemotherapeutic agents, corticosteroids and immunologic agents are used for prostate cancer treatment.

Early stages of prostate cancer are usually androgen sensitive. Therefore, the first choice of therapy is the testosterone ablation therapy. In this case, LHRH agonists are preferred for the therapy. These agonists show its effect on the pituitary-testicular axis and decrease the level of testosterone secretion (Dondi D et al., 1994).

Bone metastasis is observed approximately 75% of advanced prostate cancer patients. This results in some complications such as bone pain, hypercalcemia and skeletal related events (Fizazi et al., 2009). Zoledronic acid is a biphosphonate for the prevention or delay of the prostate cancer associated skeletal events which may require surgery or radiation therapy to bone (Schumacher et al., 2008).

Antifungals, such as ketoconazole, can take an action like antiandrogens and can be used if androgen deprivation therapy fails. These agents are also remarkable since they inhibit a variety of cytochrome P-450 enzymes (11-beta-hydroxylase and 17-alpha-hydroxylase) which in order inhibits steroid synthesis (Yap et al., 2008).

Chemotherapy is another treatment for prostate cancer if metastasis occured or hormone therapy fails. Examples of these chemotherapeutic agents are docetaxel, cabazitaxel, mitoxantrone, estramustine, doxorubicin, etoposide, vinblastine, paclitaxel, carboplatin and vinorelbine. For example, docetaxel and cabazitaxel show improvement in overall survival in patients with metastatic, castrate-resistant prostate cancer. If patient can not tolerate the taxane based treatment, mitoxantrone is another ahent preferred for palliation (Decastro et al., 2012; Huerta et al., 2013).

Docetaxel, mitoxantrone and abiraterone are used in combination with corticoteroids. Corticosteroids have an anti-inflammatory features that modify the immune system of patient. These agents result in deep and various metabolic effects, such that they counteract toxic effects related to specific cancer therapeutics and manage tumor-associated symptoms (Dorff et al., 2012).

### 1.4 Drug metabolism

Over the last decades, several studies have showed that cancer cells have a specific metabolism when compared to normal cells (Herling et al., 2011). Metabolic changes are important since it is thought that these changes affect transformation of normal cells into cancer cells and result in drug resistance during chemotherapeutic therapy (Cree, 2011). In addition to these various metabolic and signalling pathways differentiated in cancer cells, changes in the expression and activity of various drug metabolizing enzymes take important role in drug resistance (Rochat et al., 2009). Thus, metabolism of drugs by host organism is the most important determinant for the drugs' pharmacokinetic profile.

High metabolic activity usually results in poor bioavailability and high clearance. Therefore, any factor affecting the rate and extent of the drug metabolism will change the overall tendency of the xenobiotic. Metabolism of drugs results in inactivation mostly. In addition to this, active, reactive or toxic intermediates or metabolites may be also produced after the metabolic transformation (Kumar et al., 2001).

Studies showed that the expression and regulation of cytochrome P450 (CYPs) and other biotransformation enzymes have increased the role of drug metabolism in early drug development. Because they are key enzymes in cancer formation and cancer treatment. They mediate the metabolic activation of numerous precarcinogens and participate in the inactivation and activation of anticancer drugs (Rodriguez-Antona and Ingelman-Sundberg, 2006).

## 1.5 Biotransformation

Xenobiotic biotransformation is the important mechanism in order to maintain homeostasis when the organisms expose to foreign molecules, such as drugs. A limited number of enzymes with wide substrate specificities take role in xenobiotic biotransformation. Although these enzymes are expressed constitutively, the xenobiotic triggers the synthesis of these biotransformation enzymes in some cases (Parkinson et al., 2001).

Xenobiotics are mostly lipophilic in nature, so they are poorly excreted. However these xenobiotics take part in metabolic conversion reactions mediated by enzymes, termed as biotransformation, *in vivo* (Buhler et al., 1988). In biotransformation reactions most drugs and other xenobiotics become more water-soluble and efficiently excreted. In phase I biotransformation reactions, a modest increase in hydrophilicity is observed by the introduction of a functional group into the xenobiotic substrate. In phase II biotransformation reactions, highly polar endogenous moieties are added to phase I products in order to produce more readily excreted metabolites (Obligacion et al., 2006). In biotransformation reactions Phase I and Phase II enzymes usually work together in a sequential manner. On the other hand, there is a competition between Phase I and Phase II enzymes for xenobiotic or its metabolites (Buhler et al., 1988). Cytochrome P450s (CYPs) family is the most important phase I enzymes and major activating enzymes in the body. These enzymes make xenobiotics more electrophilic and reactive or in some cases more mutagenic and carcinogenic. On the other hand, in detoxification system phase I and

II drug metabolizing enzymes, including aldehyde reductase (AR), glutathione reductase (GR), epoxide hydrolase (EH), glutathione S-transferase (GST), quinone reductase (QR) and UDP-glucuronosyltransferase (UGT) can compete with these cytochrome P450s in order to eliminate reactive electrophiles by conjugation reactions. As shown in **Figure 1.2**, these reactions render electrophiles more water soluble and more readily excretable from the cell and the body ( Kensler et al., 1997; Jiang et al., 2003). Phase I or phase II drug-metabolizing enzyme can activate or detoxicate xenobiotics simultaneously relying on the catalyzed chemical reaction (Cantelli-Forti et al., 1997).

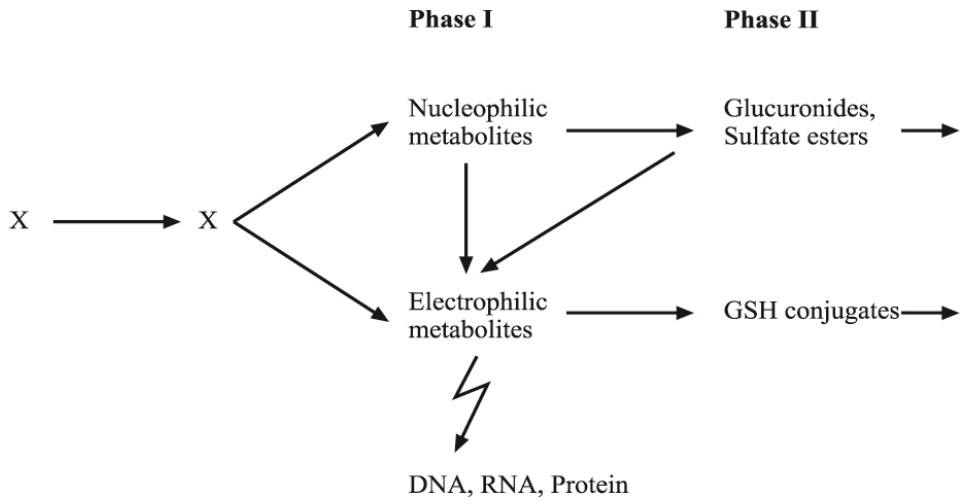



**Figure 1.2.** Scheme of cellular xenobiotic metabolism (Bock et al., 1991)

Although biotransformation reactions take place in all organs and tissues, liver has the highest concentrations of these xenobiotic metabolizing enzymes since it is the most important site for biotransformation (Buhler et al., 1988).

There are many factors affecting drug biotransformation. When the enzyme is induced by certain substrates (e.g. drugs, environmental pollutants),

biotransformation process is accelerated, causing reduction in unmetabolized drug. As a result of rapid metabolism, most drugs can show decrease in efficacy. On the other hand, if the drug metabolites are active after enzyme induction, this results in increase in drug effect and/or its toxicity. In some situations, when the drug is pro-drug, biotransformation reactions are necessary to make the drug active, but if there is an inhibition, the efficacy of drug is decreased. In addition, genetic polymorphism, disease, age, and gender are other factors affecting biotransformation process (Ogu et al., 2000).

The cytochrome P450 (CYPs), epoxide hydrolase and glutathione S-transferases (GSTs) are major groups of enzymes in activating and detoxifying therapeutic drugs. Therefore, the expression of these enzymes in malignant tumours are also related to mechanism of anti-cancer drug resistance.

According to immunohistochemical studies of Muray and his collegues, the P450 subfamilies CYP1A, CYP2C, and CYP3A were present in 63%, 25%, and 61% of prostate tumour samples, respectively. Epoxide hydrolase, another phase I enzyme, was identified in %96 of prostate tumour samples. In addition, GSTA and GSTM were observed in 29% and 41% of prostate tumour samples, respectively, but they detected no expression of the GSTP in prostate tumour samples. Although GSTP is expressed frequently in other types of malignant tumour, it is absent in prostate cancer (Murray et al., 1995). GST-P predominates in basal cells and secretory acinar epithelium of normal and benign prostatic hyperplasia tissues, but is consistently absent in incidental prostate cancer cells, in prostatic intra epithelial neoplasia and in malignant prostate tumor (Obligacion et al., 2006).

They also showed that CYP1A, CYP2C, epoxide hydrolase and the different forms of GST were expressed in non-neoplastic prostatic epithelium, but immunoreactivity for CYP3A was not the case (Murray et al., 1995). This result showed that with respect to the CYP3A prostate expression, there was found no CYP3A protein in

normal prostate tissue, but they detected expression in 61% of prostate tumor samples (Leskela et al., 2007).

Another important issue affecting drug biotransformation is pharmacogenetics. In this area, there is a few marker in order to guide physicians for the best course of treatment (Pinto et al., 2011). Polymorphism, thus genetic variability, in drug metabolizing enzymes results in large differences in the degree or rate at which a drug is transformed to its metabolites. Polymorphisms observed in drug metabolizing enzymes and transporters can affect drug efficacy and toxicity. As a result, this variability causes different responses of patients to standard dose of drug treatment. This leads to failures in the treatment or occurance of side effects (Meyer et al., 1997). Therefore it is important for physicians to undertand the relationship between the genetic variability in drug metabolizing enzymes and the efficacy and/or toxicity of a medication to optimize the therapy according to medicine (Pinto et al., 2011).

### 1.5.1 Cytochrome P450s (CYPs)

The most important of Phase I reactions are oxidation reactions catalyzed primarily by cytochrome P450-dependent mixed-function oxidases (MFOs; also referred to as monooxygenases). Hydroxylation of aliphatic and aromatic compounds, epoxidation of aliphatic and aromatic compounds, N-dealkylation of amines and O-dealkylation of ethers are cytochrome P-450-mediated oxidations.

The cytochrome P-450 MFO system is a coupled electron-transport system including cytochrome P-450 and NADPH-cytochrome P-450 reductase. The phospholipid matrix of the endoplasmic reticulum is the place for these enzymes. Electrons are transferred from NADPH through the flavoprotein to cytochrome P-450 which then inserts one atom of oxygen into the substrate and reduces the second oxygen atom to form water (**Figure 1.3**) (Buhler et al., 1988).

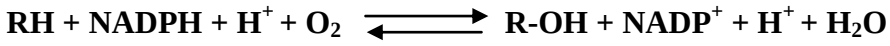



**Figure 1.3.** Generalized reaction catalyzed by the cytochrome P450 dependent monooxygenases

Characterization and classification of the human CYP genes have been performed according to their structures. The function of main CYP enzymes (CYP1, CYP2, and CYP3) is the oxidative metabolism of drugs/xenobiotics (Chauret, 1999). The important xenobiotic-metabolizing CYP families takes role in detoxification pivotally, that is why they are primarily expressed in the liver. On the other hand, these enzymes are also expressed in extrahepatic tissues, such as CYP1A1, CYP1B1, CYP2B6 and CYP3A4 expressed in the human prostate (Finnström et al., 2001; Rodriguez-Antona and Ingelman-Sundberg, 2006; Fujimura et al., 2009).

Cytochrome P450 is responsible for the biotransformation of several drugs. Drug toxicities, reduced pharmacological effect and adverse drug reactions are occurred as a result of several drug interactions and CYP enzymes are important for the determination of these drug interactions. The drug may be used as enzyme substrates, inducers or inhibitors (Ogu et al., 2000). Therefore, it is important to know its way of function in order to prevent clinically significant interactions (**Figure 1.4**).

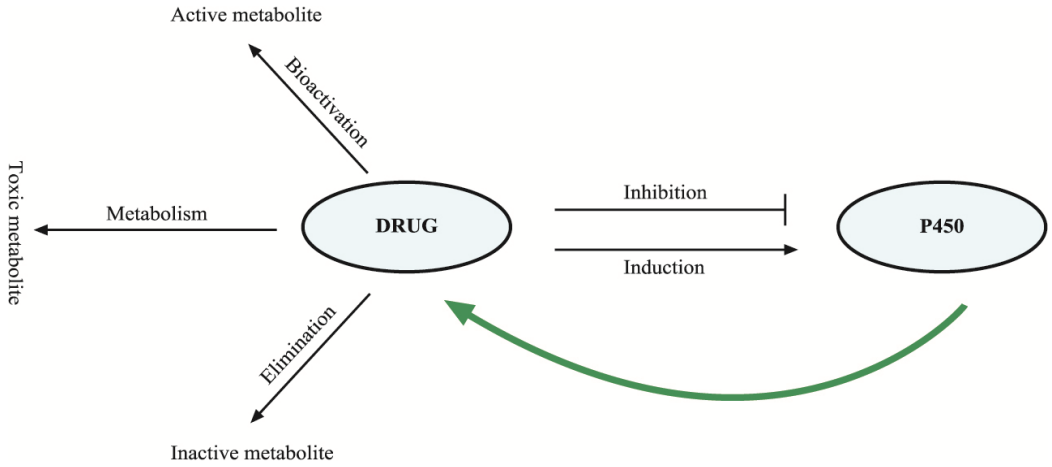



**Figure 1.4.** Several outcomes of CYP–drug interaction (Purnapatre et al., 2008).

CYP P450 enzymes have been known for their role in phase I metabolism of chemotherapeutic drugs until recently. The important issue is that these enzymes may be a target for drug development in cancer. In this respect, the first target enzyme was CYP19 (aromatase) in breast cancer therapy. In addition to this, a new strategy plan has been begun with aromatase inhibitors in hormone ablation therapy for estrogen dependent cancers (CYP17 inhibition with abiraterone). Vitamin D3 and vitamin A have anti-cancer metabolites that are inactivated by CYPs. These CYPs have also stimulated the development of new agents. Over-expression of exogenous metabolizing enzyme, CYP1B1, has evoked the development of inhibitors for chemoprevention and prodrugs designed to be activated by CYPs only in cancer cells. Another development is the bioreductive molecules which are activated by CYPs in cancer cells under hypoxic conditions (Bruno et al., 2007).

Targeting of CYPs with natural or synthetic molecules provides benefits in cancer prevention and therapy potentially. Strategies to target these enzymes include: (i) designing molecules that inhibit the enzymes; (ii) designing prodrugs activated by the enzymes; (iii) immuno-based therapies targeting immune responses toward the

enzymes; (iv) genetic therapy strategies to express specific CYPs in cancer cells (Bruno et al., 2007). The main goal of cancer search is to develop therapeutic agents which target cancerous cells and to eliminate the toxicity and the CYP P450 enzymes show such therapeutic action (Morag et al., 2004).

#### 1.5.1.1 Cytochrome P450 1A1 (CYP1A1)

Cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) has an ability to biotransform polycyclic aromatic hydrocarbons found in carcinogens such as polycyclic aromatic hydrocarbons (benzo[a]pyrene in tobacco smoke) into carcinogens. CYP1A1 expression is mainly low. However, when this enzyme interacts with its own substrates (particularly the PAHs), its expression can be increased greatly (Walsh et al., 2013). Although CYP1A1 is expressed primarily in the liver, it has been also detected in extrahepatic tissues, such as prostate (Shaik et al., 2009; Quinones et al., 2009).

CYP1A1 is an important enzyme in the metabolism due to the fact that it plays role in the conversion of pro-carcinogenic compounds to highly carcinogenic metabolites. It has an importance of being a therapeutic tool for the bioactivation of prodrugs, particularly cytotoxic agents (Anwar-Mohamed et al., 2009). Also, CYP1A1 has gained status along with other cytochrome P450 enzymes in the metabolism of drugs and mediating drug–drug interactions, such that it takes role partly in docetaxel metabolism (Anwar-Mohamed et al., 2009, Van Schaik et al., 2008; Mathijssen et al., 2006).

#### 1.5.1.2 Cytochrome P450 3A4 (CYP3A4)

CYP3A4 has the action of hydroxylation of testosterone in the liver and prostate. This function causes hormone deactivation, such that, recently it has been reported that variation in CYP3A4 increased the availability of testosterone which is related to PCa. Fujimura and coworkers (2009) demonstrated that the level of CYP3A4 mRNA expression in PCa was significantly lower than that in normal prostatic tissue,

indicating that, CYP3A4 expression was different in prostatic tissues. Therefore this decrease in CYP3A4 expression might be related to the development of cancer. Similarly, Chen and coworkers also found lower expression of CYP3A4 in PCa (Chen et al., 2012).

It is well known that CYP3A4 also has a main role in the biotransformation of pharmaceutical drugs (Fujimura et al., 2009). Likely, CYP3A4 converts docetaxel into its inactive hydroxylated metabolites. As a result, a high CYP3A4 activity causes a poor therapeutic outcome of the drug. Engels and coworkers (2004) reported that, when docetaxel was applied with the potent CYP3A4 inhibitor ketoconazole in cancer patients, docetaxel clearance was decreased with the rate of 49%. Similar results were obtained by other researchers (Akhdar et al., 2012; Rodriguez-Antona et al., 2006).

### 1.5.2 Glutathione S-transferases (GSTs)

Multi-functional glutathione S-transferases (GSTs) are Phase II enzymes which takes role in detoxification (Frova et al., 2006). These enzymes are categorized as mitochondrial, microsomal and cytosolic. Among them, cytosolic GST family is mostly found ones and according to chromosomal location and sequence similarity, cytosolic GSTs are classified as alpha (GSTA), mu (GSTM), pi (GSTP), theta (GSTT), kappa (GSTK), zeta (GSTZ) and omega (GSTO). In addition, each cytosolic class includes multiple members and is found in a specific chromosomal cluster (Polimanti et al., 2013).

Cytosolic GST reactions catalyze the nucleophilic addition of the sulfhydryl (“thiol”) group of reduced glutathione (GSH) to electrophilic centers in a large number of structurally diverse physiological and xenobiotic compounds **(Figure 1.5).**

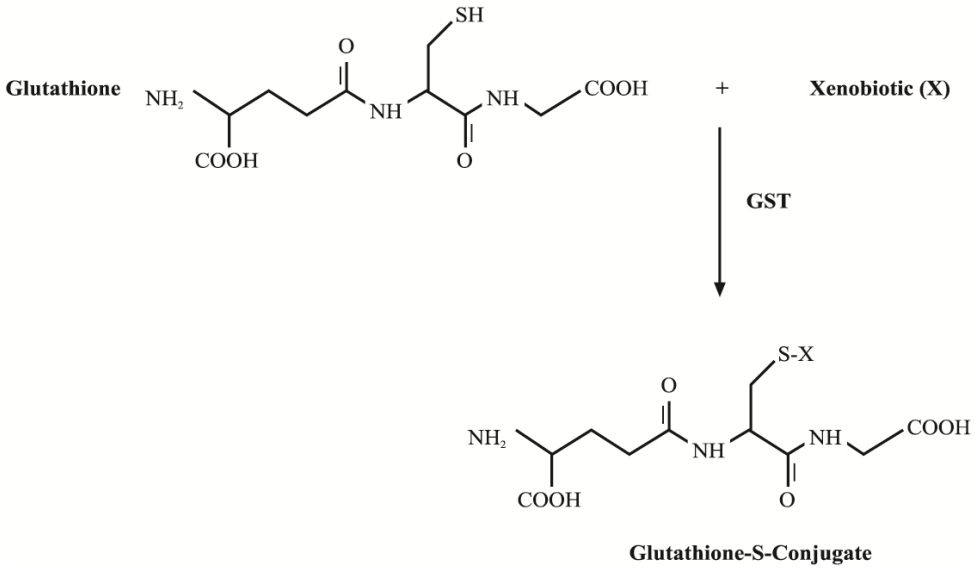



**Figure 1.5.** Glutathione conjugation reactions (Towsend et al., 2003).

The aim of cytosolic GST reactions is to make these compounds more water soluble for the elimination from the body, regarded as "detoxification" (Fischer et al., 2012). Reactive electrophiles' detoxification, biosynthesis of leukotrienes, prostaglandins, testosterone and progesterone and tyrosine degradation are also other catalytic processes in which GSTs are active (Polimanti et al., 2013).GST enzymes may also play important role in the development of drug resistance which is one of the main failure of chemotherapy treatment (Towsend et al., 2003) in cancers.

#### 1.5.2.1 Glutathione S-Transferase P1 (GSTP1)

GSTP1 encoding the pi-class glutathione S-transferase is an enzyme resposible of detoxification and protection of cellular DNA from oxidative damage (Dumache et al., 2009; Yu et al., 2005; Henrique et al., 2004). GSTP1 can detoxify several anticancer drugs by conjugating them with glutathione. This is why GSTP1 is considered in the mechanism of drug resistance to chemotherapy. For example, conjugation of docetaxel metabolites with glutathione is the second phase of docetaxel metabolism in which GSTP1 takes role. According to Park and coworkers

results (2002), the decreased anti-tumour activity could be the result of the upregulated GSTP1 expression by the metabolism of the drug. In order to understand the effect of GSTP1 on docetaxel metabolism, the relationship of intra-tumoral concentrations of docetaxel and their metabolites as a result of GSTP1 expression should be further investigated (Arai et al., 2008).

The epigenetic silencing of the glutathione-*S*-transferase P1 (GSTP1) gene is the most common (>90%) genetic alteration so far reported in prostate cancer (Dumache et al., 2009; Singal et al., 2001; Henrique et al., 2004). When GSTP1 is inactivated in prostate cells, this causes increase in vulnerability to somatic alterations as a result of chronic exposure to genome-damaging stresses such as oxidants and electrophiles (Dumache et al., 2009).

Interestingly, GSTP1 expression is increased in androgen-independent prostate cancers when compared to androgen-dependent cancers. GSTP1 is highly expressed in PC3 (human prostate cancer cell lines, androgen independent) compared to LNCaP (human prostate cancer cell lines, androgen dependent) and according to these result, Hokaiwado and coworkers suggested that, GSTP1 might play a key role in the proliferation of androgen-independent human prostate cancer cells (Hokaiwado et al., 2008).

### 1.6 Chemotherapeutic agents and inhibitors

Luteinizing hormone–releasing hormone (LHRH) analogues, androgen antagonists, gonadotropin-releasing hormone (GnRH) agonists, bisphosphonates, antifungal agents, chemotherapeutic agents, corticosteroids and immunologic agents are widely used medicines for prostate cancer treatment. Among the chemotherapeutic agents, docetaxel is also used because of its antitumoral activity against prostate cancer. Since the metabolism of docetaxel by patient is the most important determinant for its pharmacokinetic profile, it is important to know the mechanism of docetaxel metabolism well.

### 1.6.1 Chemotherapeutic agents

Conventional chemotherapeutic drugs are now becoming insufficient for the treatment of an advanced stage of prostate cancer such as metastatic androgen-independent type. As a result, new mechanism-based and targeted strategies are necessary.

#### 1.6.1.1 Docetaxel

Docetaxel is a clinically well-established anti-mitotic chemotherapy drug known as a taxane that works by interfering with microtubules, hence cell division. It shows antitumor activity against solid tumors, including PCa, and is highly effective as monotherapy and combination therapy (Li et al., 2004, Herbst et al., 2003). On the other hand, the length of the docetaxel treatment and systemic side effects result in disease relapse. Docetaxel resistance is either intrinsic or acquired due to different mechanisms which are highly related with genetic variability **(Figure 1.6)**. Several combination therapies and small P-glycoprotein inhibitors have been offered to improve the therapeutic outcome of docetaxel in prostate cancer (Ganju et al., 2014).

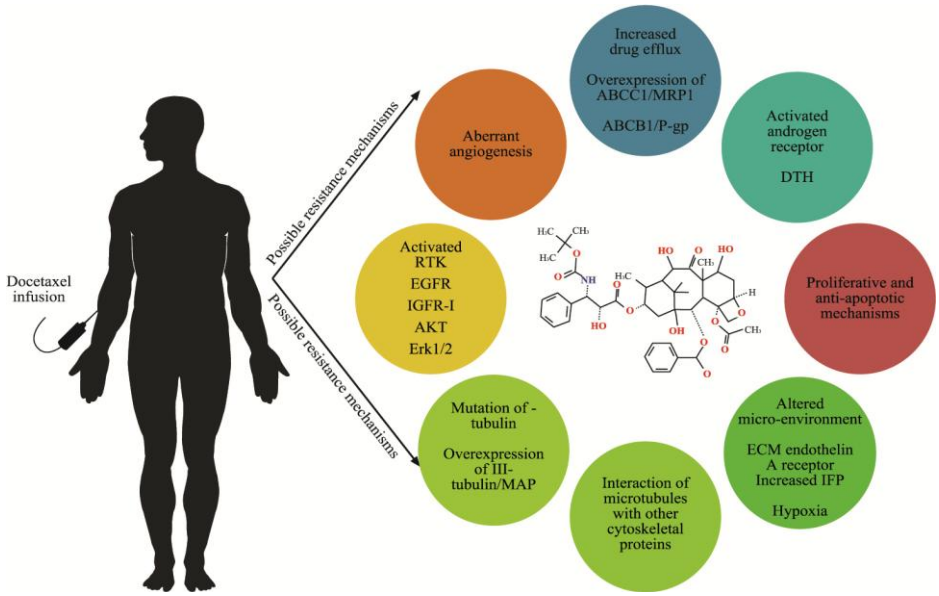



**Figure 1.6.** De novo and acquired resistance mechanisms that mediate docetaxel therapy in many prostate cancer cells and patients (Ganju et al., 2014)

Docetaxel is a semisynthetic anticancer agent derived from baccatin III of the needles of *Taxus baccata*. It was found that weekly docetaxel in patients with metastatic hormone-refractory prostate cancer is well tolerated and related to clinical benefit. Thus, docetaxel is the most important anticancer drugs in cancer treatment. Basically, it binds to tubulin and deranges the equilibrium between microtubule assembly and disassembly during mitosis. As a result, microtubules are stabilized and mitosis is corrupted (Li et al., 2004; Herbst et al., 2003).

Studies in the literature suggested that there are other mechanisms on which docetaxel show its activity. It was reported that docetaxel increases bcl-2 phosphorylation and decreases bcl-xl expression. This results in inhibition of the antiapoptotic function of bcl-2 family (Stein et al., 1999, Boudny et al., 2002, Haldar et al., 1997, Li et al., 2004). In another study with human leukemia cells, it was found that after docetaxel treatment p21 WAF1 and p53 expressions were increased and apoptosis was induced (Avramis et al., 1998, Li et al., 2004). Docetaxel has

pleiotropic effects, since it also regulates expression of cytokine genes in human cancers (Li et al., 2004). Therefore, docetaxel has multiple effector targets, such as apoptosis, angiogenesis and gene expression processes. This is why docetaxel can be used in combination with a variety of novel anticancer agents, because of its multiple functions (Herbst et al., 2003).

Li and coworkers obtained comprehensive gene expression profiles in order to show such molecular mechanism(s) in both PC3 and LNCaP cell lines following docetaxel application. After Affymetrix Human Genome U133A Array, they found that a total of 166, 365, and 1785 genes showed greater than two-fold change in PC3 cells after 6, 36, and 72 hours of treatment, respectively compared to 57, 823, and 964 genes in LNCaP cells. In the same study, decrease in microtubule-targeting effect of docetaxel tubulin expression and increase in microtubule-associated proteins expression confirmed microtubule-targeting effect of docetaxel. Expression of some genes related to cell proliferation and cell cycle were decreased while some genes related to induction of apoptosis and cell cycle arrest have higher expression profile after docetaxel application. These results showed that docetaxel effects a wide range of genes, many of which are associated with prostate cancer (Li et al., 2004). They also demonstrated that in PC3 cell lines CYP1A1 gene expression increases after docetaxel application at 6, 36 and 72 hours. In another study, induced GST expression was detected in combination treatment with estramustine in PC3 cells. On the other hand, when docetaxel or estramustine applied as a monotherapy, there was no change in the expression of GSTs (Li et al., 2005; Li et al., 2005).

CYP3A4 converts docetaxel into its inactive hydroxylated metabolites **(Figure 1.7, Table 1.1)**. As a result, a high CYP3A4 activity causes a poor therapeutic outcome of the drug (Akhdar et al., 2012) because, metabolites of docetaxel are substantially less active than the parent drug and contribute very little to docetaxel activity (Makhov et al., 2012). Engels and coworkers reported that when docetaxel was applied with the potent CYP3A4 inhibitor ketoconazole in cancer patients, docetaxel

clearance was decreased with the rate of 49% (Engels et al., 2004, Akhdar et al., 2012). CYP3A5 has also been found to function in docetaxel metabolism but its affinity is 10-fold lower compared to CYP 3A4 (Makhov et al., 2012).

**Table 1.1.** Role of CYP450 isoforms in docetaxel metabolism (Van Schaik et al.,2008)

| | 1A1 | 1A2 | 2A6 | 1B1 | 2B6 | 2C8 | 2C9 | 2C19 | 2D6 | 2E1 | 3A4 | 3A5 | 3A7 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Docetaxel | + | + | + | | + | + | + | | + | + | +++ | ++ | ++ |

+:Potential contribution; ++:Minor contribution; +++:Major contribution

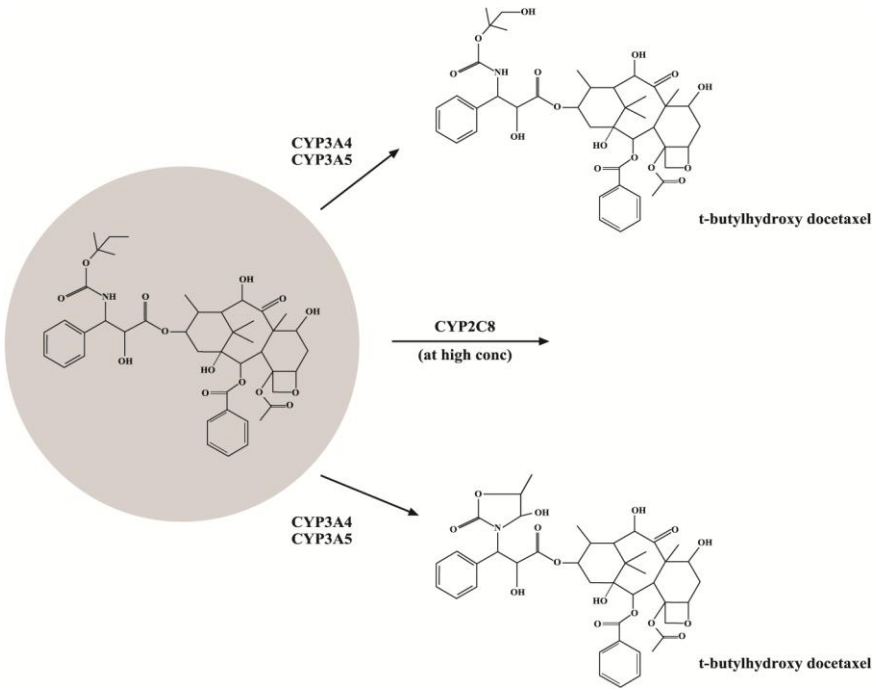



**Figure 1.7.** Metabolism of docetaxel (Van Schaik et al., 2008)

Docetaxel treatment also resulted in the induction of intracellular oxidative stress and GSH deletion (Geng et al., 2005). In order to determine the ROS and GSH levels Geng and coworkers, treated SMMC-7721 cells (hepatocellular carcinoma cells) with different doses of docetaxel. After 24 or 48 hours, they showed that drug-treated

SMMC-7721 cells increased ROS levels, but GSH levels decreased compared to that of the control group significantly. Decreased level of GSH was known to be tightly coupled with a number of downstream events in apoptosis, caspase activation and chromatin related events. Thus, GSH depletion may act as a link between oxidative stress and apoptosis (Lavrentiadou et al., 2001, Geng et al., 2005). In addition, increased ROS and GSH deletion may be involved in the action mechanism of docetaxel in SMMC-7721 cells (Geng et al., 2005).

### 1.6.2 Drug metabolizing enzyme inhibitors

Targeting drug metabolizing enzymes in tumor cells induces beneficial effects in chemoprevention and chemotherapy. One of the strategy to target these enzymes is designing molecules that inhibit the enzymes. Thus, inhibition of these endogenous enzymes in tumor cells may provide a novel target for producing new anticancer agents or new combinations. The first target enzyme was CYP19 (aromatase) in breast cancer therapy in the literature (Bruno et al., 2007). The first successful aromatase inhibitor, 4-hydroxyandrostenedione was demonstrated by Angela Brodie and colleagues to have efficacy against breast cancer tumors in 1977 (Brodie et al.,1977). Since then, several selective inhibitors of aromatase have been developed. The use of aromatase inhibitors has resulted in a new strategy plan, such as CYP17 inhibition with Abiraterone for hormone ablation therapy for estrogen dependent cancers (Bruno et al., 2007).

#### 1.6.2.1 Alpha-naphthoflavone

Flavonoids are polyphenolic molecules including three rings (A, B and C). They have supported chemopreventive activity by the inhibition of CYP1-catalyzed

carcinogenic product formation, DNA adduct production and tumor development in vitro and in vivo experiments (Androutsopoulos et al., 2011). Alpha-naphthoflavone is a synthetic flavanoid, widely studied as a modifier/inhibitor of chemically induced carcinogenesis **(Figure 1.8)** (Lundgren et al., 1987).

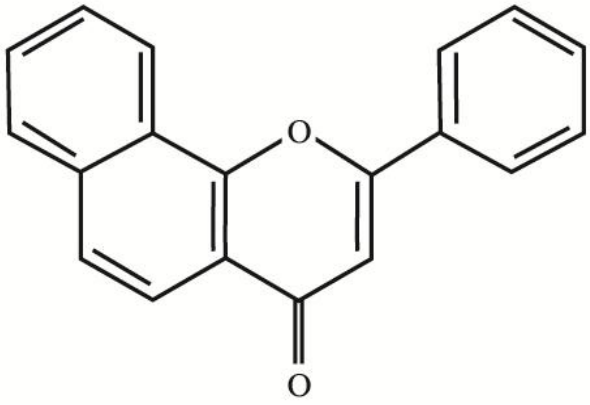



**Figure 1.8**. Structure of alpha-naphthoflavone ($C_{19}H_{12}O_2$) (Bruno et al., 2007)

It is first thought that, the influence of alpha-naphthoflavone on carcinogenesis is associated with the inhibition of specific cytochrome P-450 isozymes. These enzymes are necessary for the metabolic activation of carcinogens (7,12-dimethylbenz(a)anthracene, 3-methylcholanthrene and benzo(a)pyrene) in to reactive metabolites (Lundgren et al., 1987). Then it was found that alpha-naphthoflavone functions as an inhibitor of certain types of cytochrome P450 enzymes including CYP1A1 and as an antagonist at the aromatic hydrocarbon receptor (AhR) (He et al., 2013; Walsh et al., 2013).

#### 1.6.2.2 Ketoconazole

Ketoconazole, an antifungal drug, blocks steroidogenesis via inhibition of the cholesterol side chain cleavage so it has been used for in the treatment of hormone-dependent prostate cancer **(Figure 1.9)** (Forgue-Lafitte et al., 1992).

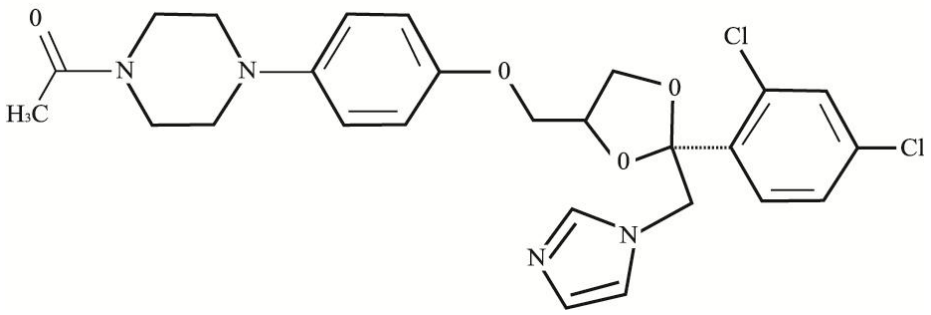



**Figure 1.9.** Structure of ketoconazole ($C_{26}H_{28}Cl_2N_4O_4$) (Bruno et al., 2007).

While ketoconazole shows indirect effect on hormone-dependent cancers, it also inhibits hepatic metastasis from a human pancreatic adenocarcinoma in the nude mouse and reduces the incidence of pulmonary metastasis in a mouse melanoma (Tzanakakis et al., 1990, Nardone et al., 1988). In addition, ketoconazole exerts a cytotoxic effect in various cancer cell lines such as breast, colon, pancreas, prostate and leukemia and potentiates the antitumor effect of interleukin1α on murine RIF tumors [mainly by inhibiting the secretion of corticosterone] (Rochlitz et al., 1988, Braunschweiger et al., 1990). Ketoconazole inhibits the activities of cytochrome P-450 enzymes and arachidonic acid lipoxygenases. In addition, it has also antiglucocorticoid properties (Forgue-Lafitte et al., 1992).

Ketoconazole has two different effects on prostate cancer according to usage as a single agent or combination with chemotherapeutic agents. If it is applied as monotherapy, it shows direct cytotoxic effects. On the other hand if it is used as a combination, it shows synergistic effects preclinically and clinically. For example, ketoconazole and doxorubicin combination therapy causes a 55% PSA response rate (Figg et al., 2010).

Figg and coworkers reported that, the addition of ketoconazole increased the antitumor effects of microtubule-active drugs, like paclitaxel or vinblastine, on prostate cancer cell lines. Same group also showed that combination of docetaxel and

ketoconazole has significant antitumor effect in castration-resistant prostate cancer with tolerable toxicities (Figg et al., 2010).

One of the preclinical study in mice showed that, docetaxel metabolism is significantly changed via ketoconazole addition (Kamataki et al., 1998, Figg et al., 2010). For example, when ketoconazole is added to primary culture, 95% of docetaxel metabolism is inhibited in human hepatocytes. Similarly, 99% of docetaxel metabolism is inhibited in human liver microsomes (Marre et al., 1996, Figg et al., 2010).

The underlying mechanism of ketoconazole-docetaxel interaction is not fully understood, but ketoconazole shows its effect in three ways: inhibiting androgen synthesis, potentiating antitumor activity of docetaxel and increasing concentration of docetaxel with CYP3A4 inhibition (Figg et al., 2010).

Ketoconazole is not highly selective and in addition to CYP3A4 it inhibits several other isoforms from the 1B, 2B and 2C family (von Moltke et al., 1998, Sevrioukova et al., 2013).

#### 1.6.2.3 Quercetin

The natural product quercetin (3,5,7,3′,4′-pentahydroxyflavone) is a flavonoid found in many fruits and vegetables The natural product quercetin (3,5,7,3′,4′-pentahydroxyflavone) is a flavonoid found in many fruits and vegetables **(Figure 1.10)**. Quercetin has been found to inhibit the onset/growth of prostate cancer in literatures. A 27% risk reduction was observed for prostate cancer when the person consumes at least 26 mg of quercetin a day. Quercetin and its metabolites are potent antioxidants with oxygen radical scavenging properties. They also inhibit xanthine oxidase and lipid peroxidation in vitro. Previous research has shown that quercetin takes role in anti-tumor, anti-inflammatory, anti-allergic and anti-viral activities. However, knowledge of the molecular mechanisms of quercetin-induced apoptosis is not well understood (Vijayababu et al., 2005; Lee et al., 2008). Quercetin has been found to inhibit the onset/growth of prostate cancer in literatures. A 27% risk

reduction was observed for prostate cancer when the person consumes at least 26 mg of quercetin a day. Quercetin and its metabolites are potent antioxidants with oxygen radical scavenging properties. They also inhibit xanthine oxidase and lipid peroxidation in vitro. Previous research has shown that quercetin takes role in anti-tumor, anti-inflammatory, anti-allergic and anti-viral activities. However, knowledge of the molecular mechanisms of quercetin-induced apoptosis is not well understood (Vijayababu et al., 2005; Lee et al., 2008).

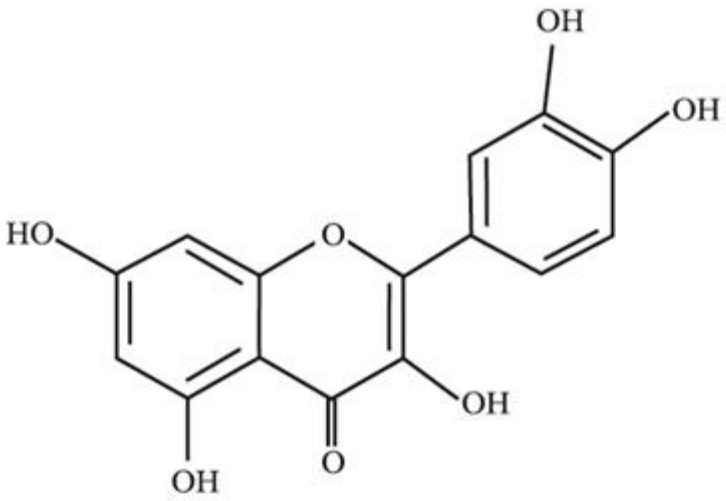



**Figure 1.10.** Structure of quercetin ($C_{15}H_{10}O_7$) (Chen et al., 2010)

Quercetin has taken much attention as a potential anti-cancer agent. There are several reports about the chemopreventive and anti-genotoxic effects of quercetin. The cancer-preventive effects have been asociated with various mechanisms: the anti-oxidative activity, the inhibition of enzymes that activate carcinogens, the modification of signal transduction pathways and interactions with receptors/other proteins. There are several cellular receptors reported to be involved in the cancer

protection of quercetin, including an aryl hydrocarbon receptor (AhR), (Fukuda and Ashida et al., 2008) the androgen receptor (Xing et al., 2001), the ErbB protein family which is also known as the epidermal growth factor receptor (EGFR) family (Kimet et al., 2005), death receptor (DR) (Chen et al., 2007), and so on.

Quercetin is multitargeted. For example, quercetin can trigger DNA fragmentation, up-regulation of Bax and posttranslational modification of anti-apoptotic Bcl-2 (Duraj et al., 2005). Quercetin can enhance TRAIL (tumor necrosis factor–related apoptosis-inducing ligand)-induced apoptosis through engagement of death receptors (Psahoulia et al., 2007) and also induce apoptosis through the activation of mitochondrial pathway by reduction in caspase-3,-9, and Bax levels and the blockade of pro-survival signaling by inhibition of Akt and ERK phosphorylation (Granado-Serrano et al., 2006). Quercetin also inhibits hGSTP1-1 irreversibly, probably leading to covalent modification of Cys47 following disulfide bond formation. The net reaction included quercetin oxidation of GST (Das et al., 2011). In addition, it was shown that quercetin decreased the formation of aberrant crypt in a rat colon cancer induction model. This suggested the importance of quercetin in the prevention of colon cancer by reducing cancer initiating events (Volate et al., 2005) (Chen et al., 2010).

Quercetin has been related with a variety of anti-cancer pathways. When it is applied with cytotoxic agent such as paclitaxel, tamoxifen and etoposide, it was found that, this combination might exert anti-cancer action through mechanisms other than the reversion of MDR (Chen et al., 2010).

## 1.7 Scope of the study

Several molecules play role in prostate cancer. In this study, we aimed to study the combined role of CYP1A1, CYP3A4 and GSTP1 in docetaxel metabolism in prostate tissue culture cells. For this purpose, the expression and the activity of these enzymes were determined in both prostate cancer cell line PC3 (androgen-independent PC cell lines) and control cells, PNT1A (immortalized prostatic cell

lines) by using real-time PCR and activity assays. Alpha-naphthaflavone, ketaconozole and quercetin were used as the inhibitors of CYP1A1, CYP3A4 and GSTP1. In order to investigate the potential of these inhibitors to overcome docetaxel resistance, PC3 cells were treated with inhibitors in combination with docetaxel and the cell viability and cell death was analyzed by using WST-1 and Annexin V assays, respectively. Results will provide further evidence for the effect of these ezymes on the mechanism of action of the chemotheurapatic agents such as docetaxel.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Instruments, Equipments, Chemicals, Kits and Solutions and Cell lines

The instruments used in this study are Laminar flow cabinet (ESCO Labculture Class II Biohazard Safety Cabinet Type 2A, Singapore), $CO_2$ incubator (Nuaire NU5510/E/G, USA), Inverted phase contrast microscope (Nikon Eclipse TS-100, USA), Centrifuge (Hettich mikro 22R, Germany and SIGMA 2-5 centrifuge, Germany), Vortex (Stuart SA8, UK), pH meter (Hanna Instruments PH211, Germany), Spectrophotometer (Implen Nanophotometer, USA), PCR Thermal Cycler (Biorad MyCycler, USA), Real-time PCR Thermal Cycler (Biorad iCycler iQ multicolor detection system, USA), Mini-PROTEAN Tetra Cell Electrophoresis System (Bio-Rad 165-8001, USA), Chemiluminescence imaging system (DNR Systems MF-ChemiBIS 3.2, Israel), -80 °C freezer (Thermo Forma -86C ULT Freezer, USA), ELISA plate reader (Bio-Tek Elx800, USA), ELISA plate reader (SpectraMax® Paradigm® Multi-Mode Detection Platform), Fluorescence spectroscopy (Cary Eclipse Fluorescence Spectrophotometer), BD FACSCalibur Flow Cytometer (BD Biosciences, USA),

The laboratory equipments used in this study are T-25/T-75 Cell culture flasks and 6 well/96-well cell culture plates (TPP, Switzerland or Grenier-Bio, Germany), Cryovials (TPP, Switzerland), Micro pipettes; 1000, 200, 100, 10, 2.5 µl (Thermo Scientific, USA), Polypropylene centrifuge tubes; 50 ml, 15 ml, 2 ml, 1 ml, 0.5 ml (Isolab, Germany), Serological pipettes; 25, 10, 5, 2 ml (Lp Italiana Spa, Italy or Axygen, USA), BrightLine Hemacytometer (Marienfeld, Germany)

The chemicals used in this study are RPMI 1640 Medium with stable glutamine (Biochrom, UK), Fetal Bovine Serum (FBS) – Cell culture tested (Sigma Aldrich,

USA), Non-essential amino acid (Sigma Aldrich, USA), Trypsin-EDTA (Biochrom, Germany), 2-propanol (AppliChem, Germany), Absolute Ethanol (AppliChem, Germany), Chloroform (Sigma Aldrich, USA), Methonol (AppliChem, Germany), Dulbecco's Phosphate Buffered Saline (DPBS) (PAN Biotech, Germany), L-Glutamine (Invitrogen, USA), Penicillin-streptomycin (Thermo Scientific SV30010, USA or Biochrom A2213, Germany), Trizol (peqGOLD RNA Pure 30-1010, UK), Docetaxel (Mustafa Nevzat), Alpha-naphthaflavone (Sigma Aldrich, USA), Ketoconazole (Sigma Aldrich, USA), Quercetin (Sigma Aldrich, USA), Glutathione (Sigma, USA), 1-Chloro-2,4-dinitrobenzene (Sigma, USA), Ethacrynic acid (Sigma, USA), 7-ethoxyresorufin (Santa Cruz, USA), Dicumarol (Sigma, USA), Resorufin (Sigma, USA), Erythromycin (Sigma, USA), HEPES (Sigma, USA), NADPH (Sigma, USA), Perchloric acid (AppliChem, Germany), Acetylacetone (Sigma, USA), Ammonium acetate (Sigma, USA), Glacial acetic acid (Sigma, USA), Formaldehyde (Sigma, USA), Bovine Serum Albumin (Santa Cruz , USA), Dimethyl Sulfoxide (Santa Cruz, USA).

The commercially available kits that were used in this study were Cell Proliferation Reagent WST-1 (Roche 05015944001, Germany), Oligo dT primer (Qiagen 79237, Germany), QuantiTect Primer Assay for CYP1A1 (Qiagen QT00012341, Germany), QuantiTect Primer Assay CYP3A4 (Qiagen QT01672608, Germany), QuantiTect Primer Assay GSTP1 (Qiagen QT00086401, Germany), QuantiTect Primer Assay β-actin (Qiagen QT01680476, Germany), QuantiTect Primer Assay GAPDH (Qiagen QT01192646, Germany), QuantiTect SYBR Green PCR Kit (Qiagen 204145, Germany), Sensiscript RT Kit (Qiagen 205213, Germany), AnnexinV-FLUOS Staining Kit (Roche 11 858 777 001, Germany), Pierce Comassie Protein Assay Kit (Pierce, Thermo Scientific, USA).

The adherent cell lines used in this study are PC3 (ATCC -- CRL-1435™) and PNT1A (ECACC) cell lines

## 2.2. Cell Culture Methods

### 2.2.1 Cells and Culture Conditions

PC3 (androgen-independent PC cell lines) and PNT1A (immortalized prostatic cell lines) cells were used. The PC3 cells were obtained from American Cell Culture Collection (ATCC; USA) and PNT1A cells were obtained from European Collection of Cell Cultures (ECACC; UK). All cell lines were kindly provided by Assoc. Prof. Dr. Elif Damla ARISAN. All cell lines were grown in RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum, 1% L-glutamine (if the medium do not include glutamine), 1% non-essential amino acids and 1% Penicillin/Streptomycin with a change of this medium every 3–4 days. All cell lines were cultured in complete medium and maintained as monolayers and incubated in a humidified atmosphere containing 5% $CO_2$ at 37$^{o}$C.

### 2.2.2 Cell Passaging

**PC3 and PNT1A Cell Lines:**

After removal of media, PC3 and PNT1A cells were washed once with DPBS solution containing 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate, pH 7.4. The cells were detached with adding 1 volume of trypsin (0.5 g/l)-EDTA (0.2 g/l) solution (1 ml for T-25 flasks, 2.5 ml for T-75 flasks) and incubating the flask for 3-5 minutes at 37°C with 5% $CO_2$ humidified incubator. The detached cells were collected in a centrifuge tube and 2 volumes of 10% (v/v) FBS supplemented medium was added to neutralize trypsin. After centrifugation of the mixture at 300 x g for 5 minutes, the supernatant was discarded and the cell pellet was resuspended in growth media to be seeded cells in a new flask.

### 2.2.3 Cell Counting

Cell counting was performed using the hemocytometer. Following cell detachment, 10 µl of cell solution was added to hemocytometer. The cells were counted using

inverted phase contrast microscope (Nikon, USA). The circled area on the hemocytometer represents 1/10000 of 1ml (**Figure 2.1**). The cells on this square were counted for 4 different times and then average of these numbers was used to calculate the number of cells per ml using the formula:

**Cells/ml = The Average Number x $10^4$ x Dilution Factor**

(**Equation 2.1**)

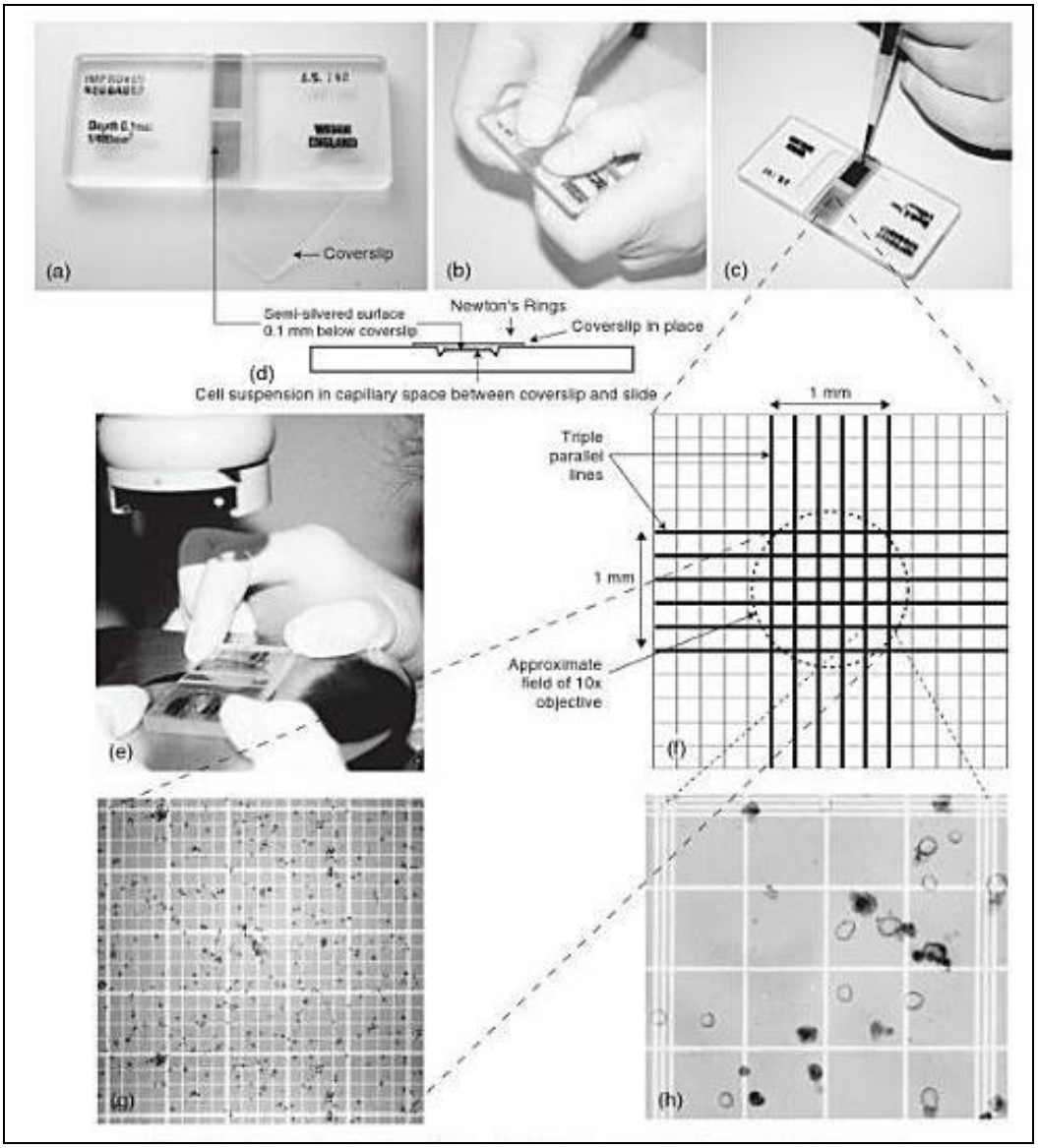



**Figure 2.1.** Hemocytometer chamber (a) showing counting chambers (b) showing coverslip in place (c) showing fill points (d) showing cell suspension in capillary space between coverslip and slide (e) showing inverted phase contrast microscope and close up view of a counting square

### 2.2.4 Cell Freezing

After trypsinization and cell counting, cell suspension was centrifuged at 300 x g for 5 minutes, supernatant was removed and cell pellet was resuspended in freezing media that contains 90% (v/v) FBS, 10% (v/v) dimethylsulfoxide (DMSO) at a concentration of $1x10^6$ cells/ml. Cells dissolved in freezing media was placed in cryovials to be transferred to -80°C for short term storage. For long-term storage, the cells were transferred to liquid nitrogen from -80°C freezer.

### 2.2.5 Cell Thawing

The frozen cell vial was taken from -80°C freezer or liquid nitrogen and was rapidly thawed at 37°C. Cell solution was transferred to a centrifuge tube and 5 ml of supplemented media was added slowly with gentle shaking. The tube was centrifuged at 300 x g for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in growth media and cells were seeded on T-25 flask. The following day, culture media was removed; cells were washed with PBS, pH 7.4 and fresh media was added.

## 2.3. WST-1 Cell Proliferation Assay

The effects of drug and inhibitors (docetaxel, quercetin, alpha naphthaflavone and ketoconazole) on cell viability were determined by the WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) assay. WST-1 is a water-soluble tetrazolium salt, the rate of WST-1 cleavage by mitochondrial dehydrogenases correlates with the number of viable cells. Exponentially growing cells were harvested and seeded at 10000 cells/100µl/well in a 96-well microtiter plate in triple combinations for each drug application. After 24 hour incubation for cell growth, agents or RPMI medium as the untreated control were added and incubated for 24, 48 and 72 hour incubation conditionally with docetaxel and inhibitors. After each incubation period, cells were treated with 50 µl WST-1 reagent and incubated for 1 hour of period. The absorbance was measured with a microculture plate reader at 450 nm. The percent of cytotoxicity was calculated using the following formula:

**Cytotoxicity% = [(absorbance of experimental well - absorbance of blank)/ (absorbance of untreated control well - absorbance of blank)] x 100%**

(**Equation 2.2**)

## 2.4. Drug and Inhibitors Application

Concentrations of drug and inhibitors, which would be applied, were determined according to % cell viability. Calculated concentration of chemicals that gave %70 and %60 and %50 cell viabilities for 24, 48 and 72 hours respectively were chosen as optimum concentrations to treat PC3 and PNT1A cell lines.

## 2.5. RNA Isolation

### 2.5.1. RNA Isolation from cells

Cells that were treated or untreated (control) with docetaxel were cultured for 24, 48 and 72 hours and then RNA isolation was carried out. To isolate RNA from cells grown in suspension, cells were sedimented and lysed by the addition of 1 ml peqGOLD RNAPureTM per 5-10 x $10^6$ cells. Cells grown in monolayer were lysed directly in the culture dish by the addition of peqGOLD RNAPure$^{TM}$ (1 ml per 3.5 cm petri dish). RNA was solubilized by passing the lysate a few times through the pipette. Then each homogenized sample was incubated for 5 minutes at +15°C to +25°C to ensure the complete dissociation of nucleoprotein complexes. Chloroform was added to each sample. 0.2 ml chloroform for each 1 ml RNAPure Isolation Reagent was required in the initial homogenization. Tube was capped securely, and shaked vigorously for 15 seconds. Tube was incubated on ice (or at 4°C) for 5 minutes. To separate the solution into three phases, tube was centrifuged at 12,000 x g for 15 minutes at 4°C. After centrifugation, the colorless upper aqueous phase was used for the RNA isolation. The lower organic phase and the interphase contained the DNA and proteins. The volume of the aqueous phase was about 50% of the initial volume of peqGOLD RNAPureTM plus the volume of tissue used for homogenization. The aqueous phase was transferred into a fresh tube, an equal volume of isopropanol was added, mixed and the samples were stored for 15 minutes at 4° C. Samples then centrifuged for 15 minutes at 12.000 g (4°C). RNA precipitate

(often invisible before centrifugation) formed a white-yellow pellet at the bottom of the tube. The supernatant was removed and the RNA pellet was washed once with 75% ethanol by vortexing and subsequent centrifugation for 10 minutes at 12,000 x g (4°C). The washing step was repeated if necessary (phenol smell!). At least 0.8 ml of ethanol per 50-100 µg RNA was used. The excess ethanol was removed from the RNA pellet by air-drying or placing the sample under vacuum for 5–10 minutes. Finally RNA pellet was resuspended in RNase-free water.

### 2.5.2. Gel Electrophpresis of RNA

RNA molecules are negatively charged, and they migrate toward the anode in the presence of an electric current during gel electrophoresis. RNA samples were heated at 70$^{o}$C and run on 1.2% agorose/0.5X TBE gel. Fifteen µl of sample was loaded into wells. 80 volts and 75 milliampere electric field were applied for 60 minutes. Gel photograph was obtained with UV transilluminator.

### 2.5.3. Quantification of RNA

The concentrations of isolated RNA samples were determined by using spectrophotometer (Implen Nanophotometer). RNAse-free water was used as blank and RNA samples were measured at 260 nm. RNA concentration was calculated from the absorbance at 260 nm using equation below:

**Concentration of RNA Sample (ng/µl)= 40 X $A_{260}$ X Dilution Factor (DF)**

(**Equation 2.3**)

As proteins give absorbance at 280 nm, the protein contamination in RNA samples was measured by calculating the A260/A280 ratio.

## 2.6. Polymerase Chain Reaction

### 2.6.1. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) for synthesis of complementary DNA (cDNA) Qiagen Sensiscript RT Kit was used. After quantification of RNA sample, 1000 ng template RNA was transferred to a new tube in 13 µl Rnase-free water. Then, 7 µl reaction mixture that contains 2 µl 10x Buffer RT, 2 µl dNTP Mix (5 mM each dNTP), 2 µl Oligo-dT primer (10 µM), 1 µl Sensiscript Reverse Transcriptase was added to the tube and 20 µl total volume was obtained. The samples were then incubated at 37°C for 60 minutes. After incubation, reaction mixture was diluted (4 fold) and samples were stored at -20°C.

### 2.6.2. Quantitative Polymerase Chain Reaction

The mRNA expression levels of GSTP1, CYP1A1, CYP3A4 in the PC3 and PNT1A cell lines with and without docetaxel application were determined using QuantiTect SYBR Green PCR Kit in Bio-Rad iCycler iQ5 instrument. Each reaction containing 6.25 µl SYBR Green PCR master mix, 1.25 µl primer mix (QuantiTect Primer Assays, GSTP1, CYP1A1, CYP3A4, β-actin as internal control) and 50 ng cDNA (2 µl) in a total volume of 12.5 µl was performed at conditions given at **Table 2.1.**

**Table 2.1.** Real-time PCR conditions

| Cycle | Temperature | Time | Phase |
|---|---|---|---|
| 1 | 94º C | 15 min | |
| 2 | 95º C | 5 min | Initial denaturation |
| 3 | 95º C | 60 sec | Denaturation |
| (40 repeat) | 55º C | 60 sec | Annealing |
| | 72º C | 60 sec | Extension |
| 4 | 72º C | 10 min | Final extension |
| 5 | 50-80º C | | Melt curve |
| (80 repeat) | 0.5º C increase | | |
| | /12 sec | | |
| 6 | 4º C | ∞ | Cooling |

**Figure 2.2** represents the PCR amplification/cycle graph or real-time PCR analysis. The specificity of PCR products were checked by melting curve analysis which was shown further in **Figure 2.3** (as an example of one of the PCR results). Each sample was studied in duplicate. The quantification of results was carried out by using standard curve method in which normalization of GSTP1, CYP1A1 and CYP3A4 expressions was performed against the β-actin expression values. **Figure 2.4** also shows a standard curve graph of one of the PCR analysis.

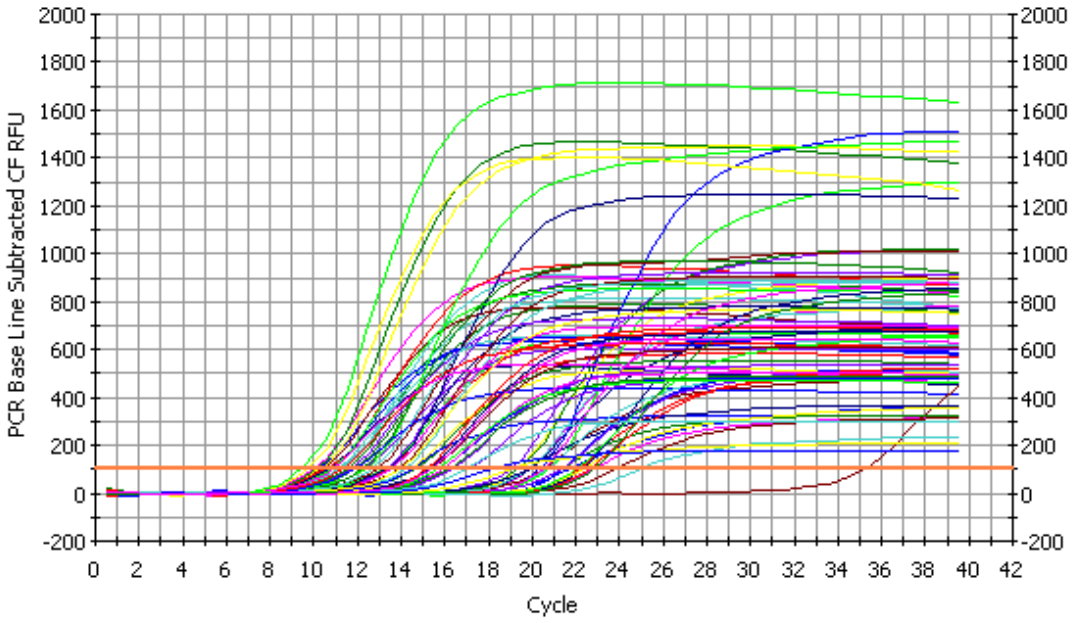



**Figure 2.2.** PCR amplification/cycle graph of one of the PCR results

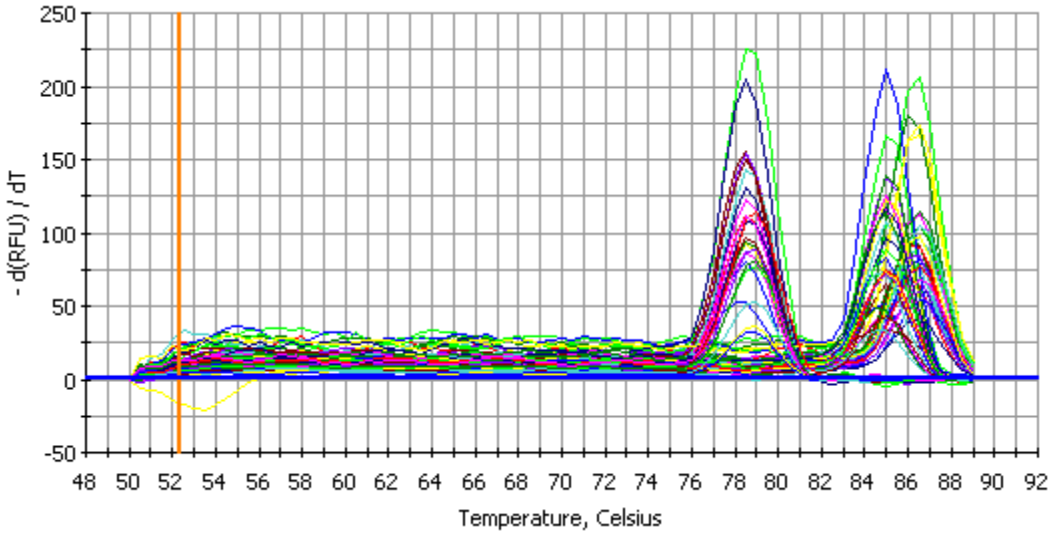



**Figure 2.3.** Melt curve analysis of one of the PCR results

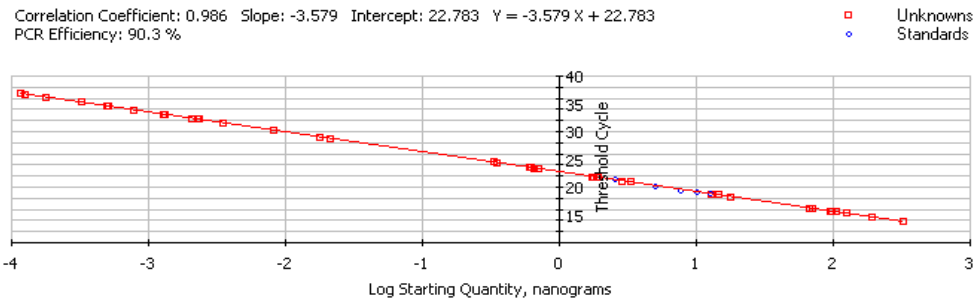



**Figure 2.4.** Standard curve of one of the PCR results in PC3 cell lines

## 2.7. Protein Isolation

### 2.7.1. Preparation of Total Cell Lysates

Whole cell lysate was obtained with lysis buffer prepared manually (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Na-deoxycholate). 300.000 cells were seeded on 6-well plates. After 24 hours, cells were applied with docetaxel or inhibitors except control wells. After 48 hours, cells were washed once with DPBS, pH 7.4 and 30 µl complete lysis buffer (1% protease inhibitor cocktail, 1% PMSF and 1% sodium orthovanadate were added freshly to lysis buffer) was added on to cell monolayers. Cells lysates were collected by a cell scraper in a new tube and homogenized on ice and stored at -80°C up to one month.

### 2.7.2. Bradford Assay

For the determination of the protein content Pierce Comassie Protein Assay Kit was used (Pierce, Thermo Scientific, USA). BSA standards were prepared by the serial dilution of 2 mg/ml BSA solution (Sigma, USA) at a working range of 0.05-0.5 mg/ml. To each well of a microtiter plate, 10 µl of each BSA solution and 10 µl unknown protein sample were added dublicately and incubated with 200 µl of Comassie reagent in dark at room temperature for 10 minutes. If necessary, unknown

protein samples were diluted with a dilution factor of 10 to 50. The absorbance was measured at 590 nm using an ELISA plate reader (Bio-Tek, USA). The standard curve graph was prepared using absorbance values of BSA standard samples vs. their concentration to calculate the protein content of samples.

## 2.8. Enzyme Activities

### 2.8.1. GST Total Activity

CDNB is a common substrate for all isozymes. GSTs catalyze glutathione (GSH) oxidation which was monitored by the increase in absorbance at 340 nm due to the 1-glutathione-2,4-dinitrobenzene-(DNB-SG) formation (**Figure 2.5**) (Habig et.al., 1974).

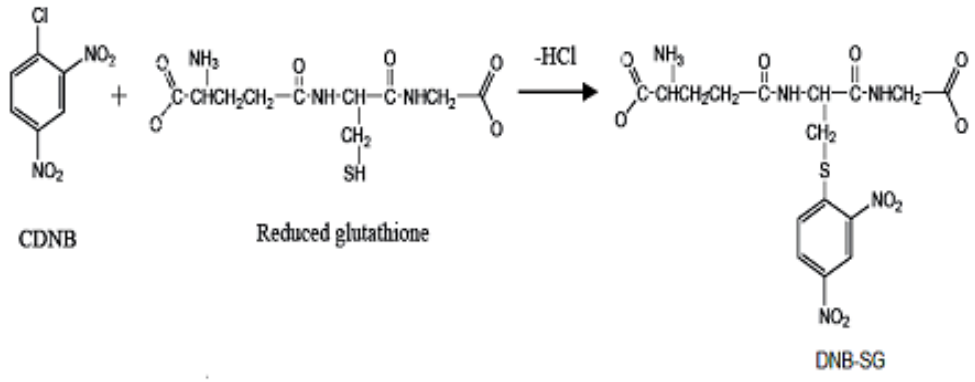



**Figure 2.5.** Common reaction catalyzed by all GST isozymes

This reaction was performed in 0.1 M potassium phosphate (pH 6.5) at 25°C in a total volume of 100 µl after optimization process. The rate of the enzyme-catalyzed reaction was determined by subtracting the rate of reaction occurring in the absence of cytosol. For total GST activity, cell lysates (40 µg of protein) was incubated with CDNB (5 mM; dissolved in 95% ethanol and stored in dark) and GSH (1 mM;

dissolved in $dH_2O$) and the time course of the reaction was monitored at 340 nm with Ɛ=0.0096 $\mu M^{-1}cm^{-1}$ (Beaumont PO, et al. 1998). The GST total activity was measured by ELISA plate reader (SpectraMax® Paradigm® Multi-Mode Detection Platform) .

$$\text{Specific Enzyme Activity (nmol/min)/mg} = \frac{\Delta OD_{340}}{\text{Extinction coefficient}} \times \frac{\text{Total Volume}}{\text{Protein Amount (mg/ml)}} \times DF$$

**(Equation 2.4)**

### 2.8.2. GSTP1 Activity

Spectrophotometric assays with GST class-selective substrates were conducted according to the method of Habig et al. (Habig et.al., 1974).

This reaction was performed in 0.1 M potassium phosphate (pH 6.5) at 25°C in a total volume of 100 µl after optimization process. The rate of the enzyme-catalyzed reaction was determined by subtracting the rate of reaction occurring in the absence of cytosol. For GSTP1 activity, cell lysates (40 µg of protein) were incubated with ethacrynic acid (1.5 mM; dissolved in ethanol:$dH_2O$ (3:2)) and GSH (0.7 mM; dissolved in $dH_2O$) and the time course of the reaction was monitored at 270 nm with Ɛ=0.005 $\mu M^{-1}cm^{-1}$ (**Figure 2.6**) (Beaumont PO, et al., 1998). The GST total activity was measured by ELISA plate reader (SpectraMax® Paradigm® Multi-Mode Detection Platform) .

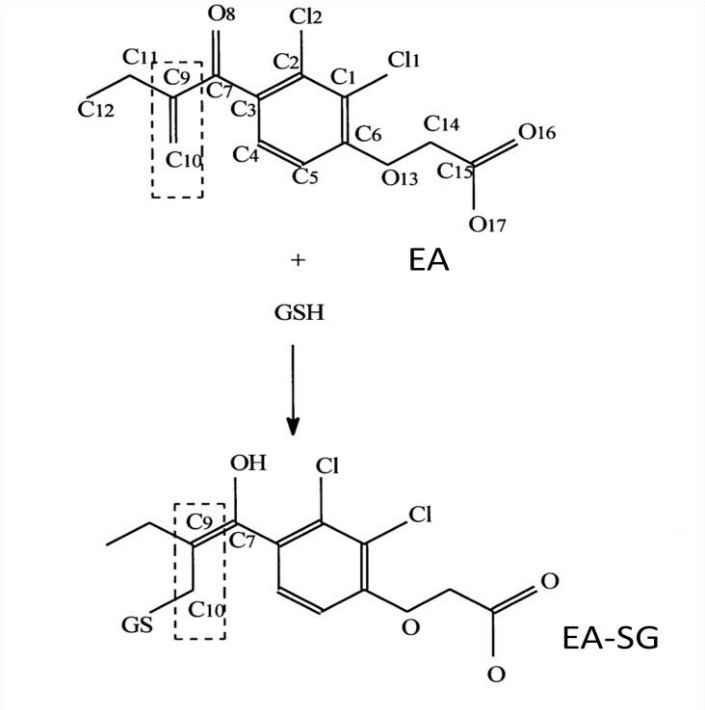



**Figure 2.6.** Reaction catalyzed by GSTP1

$$\text{Specific Enzyme Activity (nmol/min)/mg} = \frac{\Delta OD_{270}}{\text{Extinction coefficient}} \times \frac{\text{Total Volume}}{\text{Protein Amount (mg/ml)}} \times DF$$

(**Equation 2.5**)

### 2.8.3. CYP1A1 Activity

CYP1A1 enzyme activity was measured by using EROD activity assay (**Figure 2.7**) (Alfieri et al.,2011).

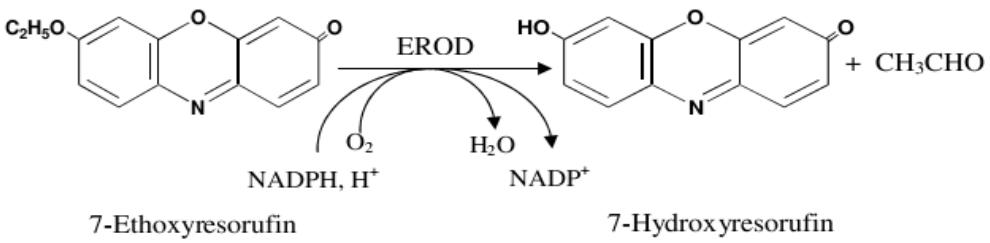



**Figure 2.7** Reaction catalyzed by CYP1A1

After the treatment on 96-well plates, cells were washed with PBS and the medium in each well was replaced with 100 µl of the assay medium containing 8 µM ethoxy-resorufin (dissolved in DMSO) and 10 µM dicumarol (to inhibit cytosolic diaphorase; dissolved in 1% NaOH in $dH_2O$). The cells were then incubated at 37$^oC$ for 60 min and the 75 µl of assay medium was transferred to another 96 well plate containing 125 µl of ice cold methanol. Resorufin-associated fluorescence was measured at excitation 550 nm and emission 585 nm. After removal of the medium, the cells were lysed and protein concentrations were measured to normalize the fluorescence intensities (Okamura K, et al., 2010; Zdarilova A, et al., 2006; Alfieri et al.,2011). A standard resorufin calibration curve was constructed and the slope of this curve was used for calculation of enzyme activity. The EROD activity was measured by Cary Eclipse Fluorescence Spectrophotometer.

$$\text{Specific Enzyme Activity } (\mu\text{mol/min/mg}) = \frac{OD_{Reaction} - OD_{ZTB}}{\text{Constant from Standard Curve}} \times \frac{1}{\text{Protein Amount (mg)}} \times \frac{1}{60\,\text{min}}$$

(**Equation 2.6**)

### 2.8.4. CYP3A4 Activity

N-demethylation of Erythromycin is associated with CYP3A isozyme of cytochrome P450 enzyme family (Combalbert et. al., 1989; Wang et. al., 1997). Erythromycin is N-demethylated by P450 dependent mixed function oxidases in the presence of molecular oxygen and formaldehyde is produced (**Figure 2.8**).

Erythromycin N-demethylase activity were determined by measuring the amount of formaldehyde formed according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). The typical assay mixture for erythromycin N-demethylation contained 100 mM HEPES buffer pH 7.8, 1.0 mM erythromycin (dissolved in DMSO), 2 mg protein and finally 5 mM NADPH (dissolved in $dH_2O$) in a final volume of 0.5 ml (Cochin and Axelrod et al., 1959).

Erythromycin N-demethylation reaction was initiated by the addition of 0.075 ml of NADPH generating system to incubation mixture and to zero time blanks to which 0.5 ml of 0.75 N perchloric acid was added just before the additon of the cofactor. The reaction was then carried out at 37 $^0$C for 60 minutes with constant moderate shaking in a shaking water bath. After 15 minutes, the enzyme reaction was finalized by the addition of 0.5 ml 0.75 N percloric acid solution. The denatured proteins were then transferred to the eppendorf centrifuge tubes, and centrifuged at 13 000xg at microcentrifuge for 25 minutes at 4 $^0$ C.

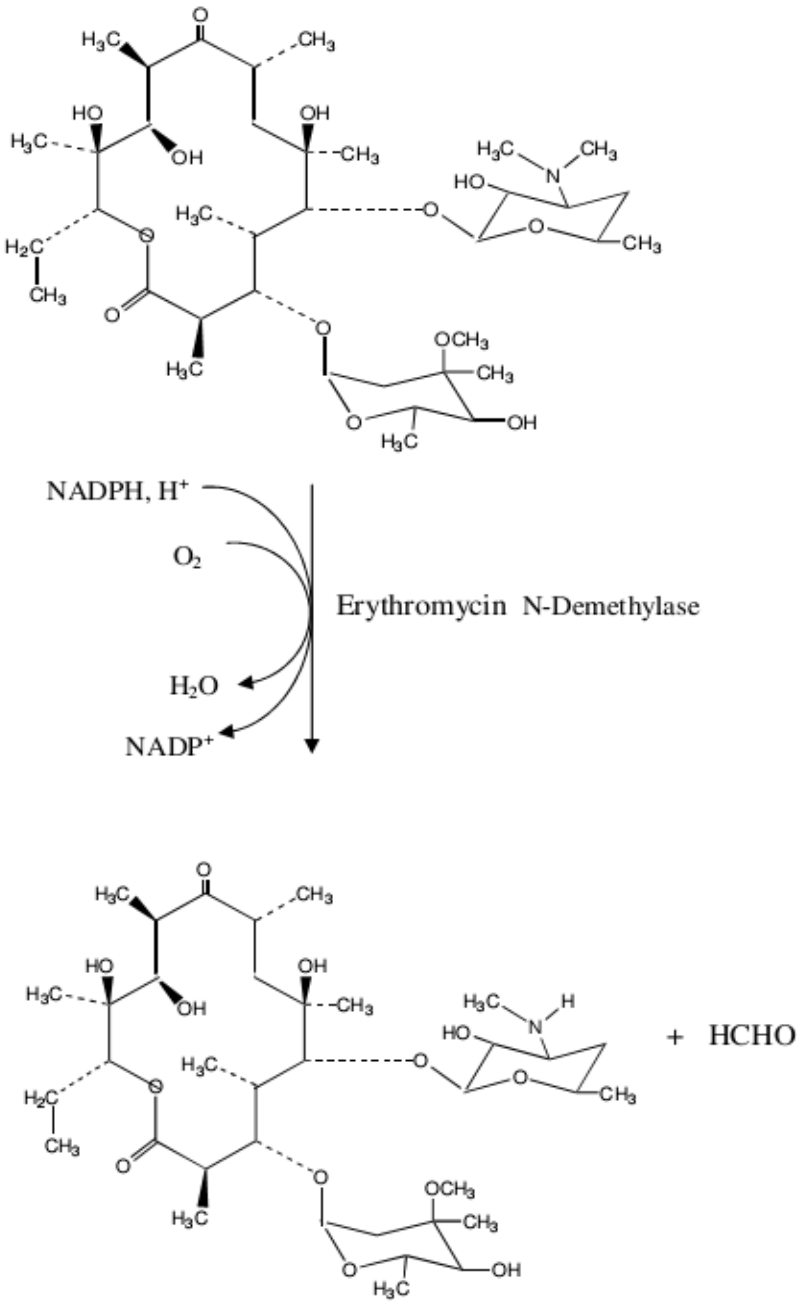



**Figure 2.8.** Reaction catalyzed by CYP3A4

After centrifugation, 0.5 ml aliquots were mixed with 0.375 ml Nash reagent (prepared by the addition of 0.4 ml acetylacetone, just before use, to 100 ml solution containing 30.8 g ammonium acetate and 0.6 ml of glacial acetic acid) again in eppendorf tubes and the mixture was incubated for 10 minutes at 50 $^{0}$C in a water bath. The formaldehyde amount was then determined by measuring the absorbance at 412 nm using ELISA plate reader (SpectraMax® Paradigm® Multi-Mode Detection Platform) .

A 0.5 mM freshly prepared formaldehyde solution was used as a standard. The tubes containing standards at four concentrations (12.5, 25, 50, 100 nmoles) with other incubation constituents were run under the same conditions as for reaction tubes. A standard formaldehyde calibration curve was constructed and the slope of this curve was used for calculation of enzyme activity, as indicated below.

$$\text{Specific Enzyme Activity } (\mu\text{mol/min/mg}) = \frac{OD_{Reaction} - OD_{ZTB}}{\text{Constant from Standard Curve}} \times \frac{1}{\text{Protein Amount (mg)}} \times \frac{1}{60\,\text{min}}$$

(**Equation 2.7**)

## 2.9. Cell Death Determination Using Annexin-V Fluos Staining Assay

For the detection and quantification of apoptotic and/or necrotic cell death initiated by the toxic effect of docetaxel, inhibitors and combination treatments. Annexin-V-FLUOS staining kit was used (Roche, Germany). Both PC3 and PNT1A cells were seeded onto the 6 -well plates at a density of 300,000 per well. After 24 hours of incubation for cell attachment, the media of the cells was changed with media containing docetaxel or inhibitors or combinations except for the control. At the end of 24 hours of incubation times, cells were collected. In order to prevent the loss of death cells, the media and the DPBS after washing step were collected in an

eppendorf tube. After trypsinization of living cells on the well surface, the trypsin solution was added into the eppendorf tube. Centrifugation at 200 xg was performed and collected cell pellet was resuspended in DPBS and the cell numbers were detected. To prepare the staining solution, 20 µL of each Annexin-V and propidium iodide (PI) solutions were mixed with 500 µL of incubation buffer. After the second centrifugation, obtained cell pellet was suspended in the staining solution of 100 µL per one million cells. Cells were incubated at room temperature for 15 minutes. 500 µL of incubation buffer was added onto the cells, and analysis was carried on flow cytometer using 488 nm excitation and a 515 nm bandpass filter for fluorescein detection and a filter greater than 600 nm for PI detection.

The working principle of this assay relies on the analysis of phosphatidylserine (PS) on the outer leaflet of apoptotic cell membranes by using Annexin-V-Fluorescein. Annexin-V is a calcium dependent phospholipid-binding protein and has a high affinity for PS. Because of that property, this protein is used to detect PS exposure upon the outer leaflet of cell membrane and therefore suited for apopototic cell detection. Since PS exposure also happens in necrotic cells because of the loss of membrane integrity, PI is used to stain DNA of leaky necrotic cells. As a result, low Annexin and low PI staining indicates the normal cells, high Annexin and low PI is for early apoptotic, and high levels of both Annexin and PI staining is for late apoptotic and/or necrotic cell determination.

### 2.10. Determination of Reduced Glutathione (GSH) Concentration

GSH determination is based on the oxidation of reduced GSH by 5,5'-dithiobis-(2-nitrobenzoic acid), [DTNB] to produce pale yellow color that gives its maximum absorbance at 412 nm. DTNB method measures the ability of SH groups to reduce DTNB to form 1 mole of 2-nitro-5-mercaptobenzoic acid (yellow) per mole of SH group (Sedlak and Lindsey, et al., 1968).

To the 0.25mL appropriately diluted cytosolic fractions (1/5 and 1/10), or GSH standard solutions ranging between 0.1 to 1mM, 0.75mL Tris buffer (0.2M Tris,

20mM EDTA, pH 8.2), 0.05mL DTNB (10mM freshly prepared) and 3.95mL methanol were added and mixed. They were then incubated at room temperature for 30 min with occasional shaking. After incubation, the samples were centrifuged at 3000g for 15 min to remove proteins having SH group and reacting with DTNB. GSH standard calibration curve was drawn and the slope was used to determine GSH concentration. The results were expressed as μmol GSH per mg protein.

### 2.11. Statistical Methods

The differences between results of the gene expressions of two cell lines were analyzed by using student's t-test. The $IC_{50}$ values of GST Total, GSTP1, CYP1A1 and CYP3A4 were calculated by using nonlinear and linear regression analysis. Nonlinear regression, linear regression and one way anova analyses for activity assays were performed in GraphPad Prism (version 5.0) analysis program. All results were considered statistically significant when $P<0.05$.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1. Effect of Docetaxel and Phase Enzyme Inhibitors on the Viability of PC3 Cell Lines

Effects of the drug, docetaxel and the inhibitors on the cell viability were analyzed by using WST-1 assay in PC3 cell lines.

### 3.1.1. Docetaxel Effect on the Viability of PC3 Cell Lines

The effect of docetaxel on cell viability of PC3 cells were analyzed by using WST-1 assay. At the end of the analysis, the concentration of docetaxel that decreased PC3 cell viability to 70%, 60% and 50% at 24 hours, 48 hours and 72 hours were determined. These percent of cell viabilities were assigned according to the fact that we should have alive cells for the rest of the analysis. Docetaxel concentration determined with respect to PC3 cells at the end of the assay was also used for PNT1A cells for the rest of the study to see the effects on immortalized prostatic cell lines which are used as control. The reason for this is that the target of our pharmacotherapy research is PC3 cells and we aimed to compare PC3 cells and PNT1A cells according to concentrations which affect the PC3 cells.

Docetaxel concentrations were used in the range of 0 μM to 100 μM for PC3 cells during this part of the study. The concentrations were tried until 70%, 60% and 50% cell viabilities were observed for 24 hours, 48 hours and 72 hours, respectively. Therefore, concentrations were reduced at every stage in order to catch these viability percentages. When the expected cell viabilities were observed, the experiment was repeated for at least three times at these concentrations to get reproducable results. The results are given in **Figure 3.1-3.4.**

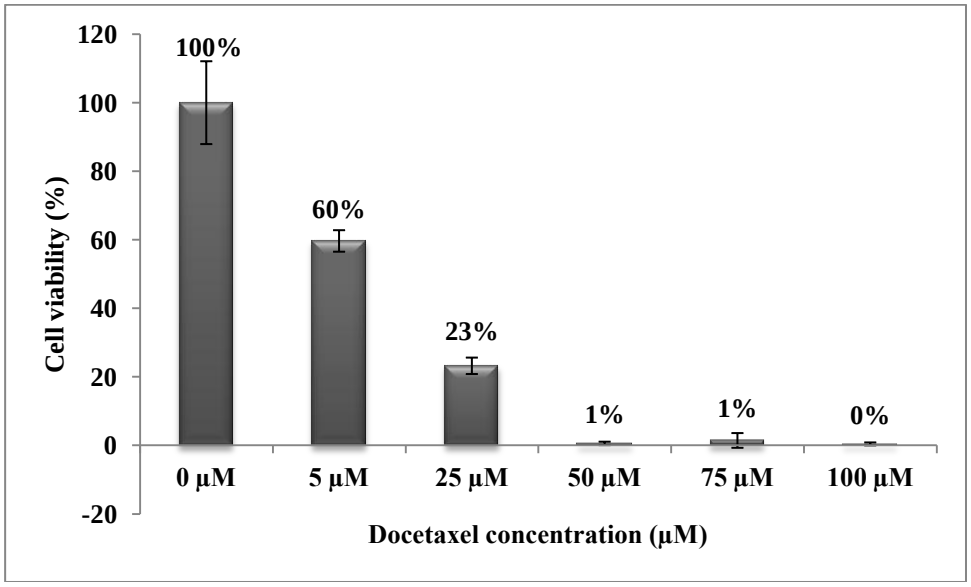



**Figure 3.1**. Docetaxel effect on cell viability of PC3 cell lines at 24 hour

In order to pick the desired concentration of docetaxel, broad range of docetaxel concentrations between 5 µM and 100 µM were analyzed in PC3 cell lines. Greater concentrations than 5 µM resulted in higher cell death ratio in PC3 cell lines. Therefore, lower concentrations than 5 µM of docetaxel were applied to PC3 cell lines as the second turn.

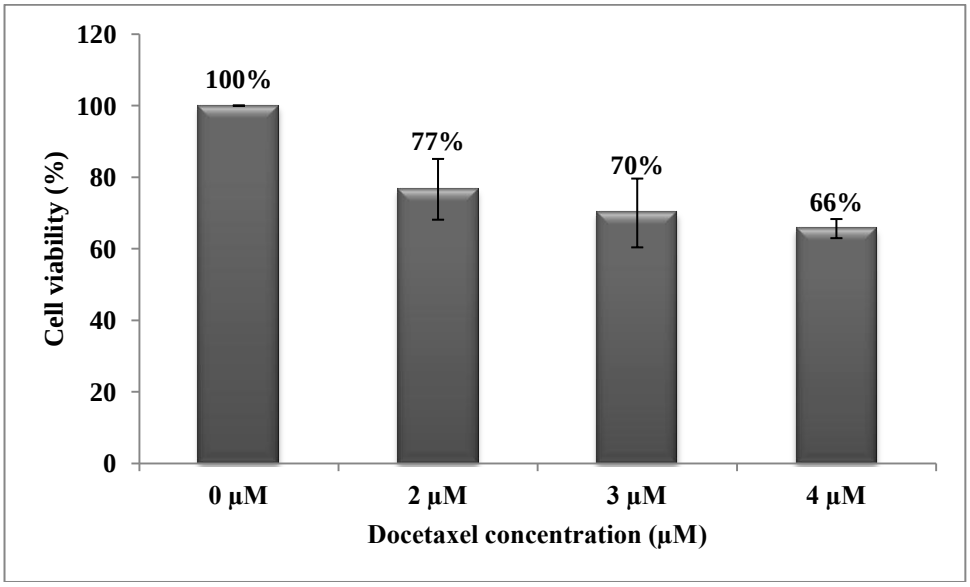



**Figure 3.2**. Docetaxel effect on cell viability of PC3 cell lines at 24 hour

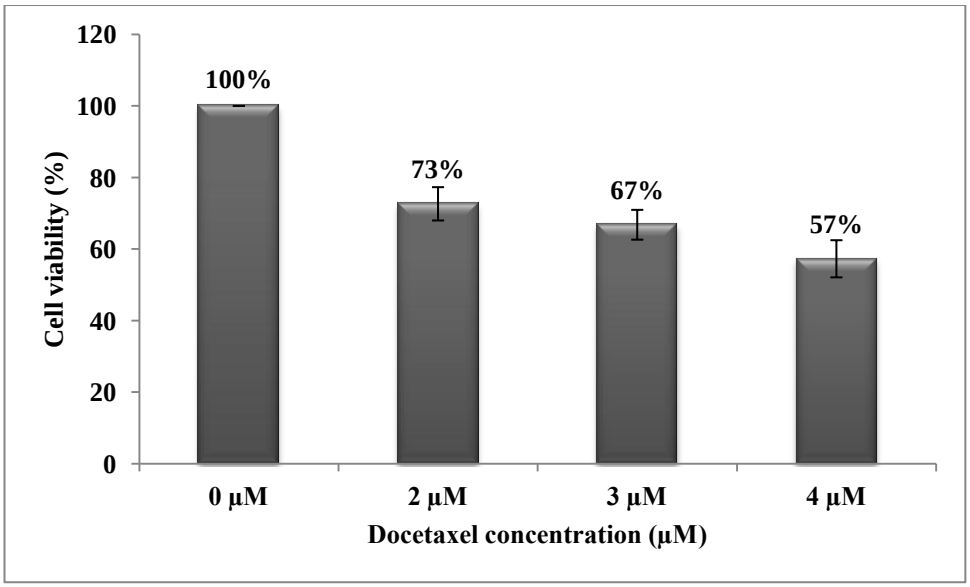



**Figure 3.3**. Docetaxel effect on cell viability of PC3 cell lines at 48 hour

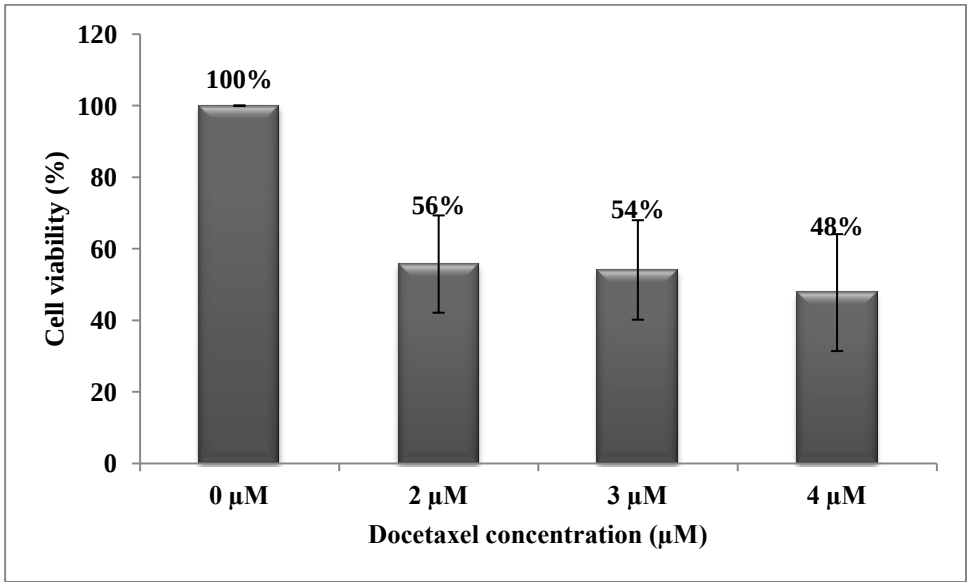



**Figure 3.4**. Docetaxel effect on cell viability of PC3 cell lines at 72 hour

Cell viability of 70% was wanted to be picked at the first 24 hour, which gave a possibility of a window to apply phase enzyme inhibitors. Four µM of docetaxel gave more or less close values to 70%, 60% and 50% cell viabilities as wanted. From these, during the rest of the study what was desired was if 70% of cell viability could be pulled down a little bit lower. If it could be pulled down, only 4 µM concentration of docetaxel in combination with phase enzyme inhibitors were enough to kill all of the cells.

### 3.1.2. Treatment with Inhibitors

The effects of inhibitors (alpha-naphthoflavone, ketoconazole and quercetin) on cell viability were also determined by using WST-1 assay in PC3 cell lines. At the end of the analysis, the concentrations of phase enzyme inhibitors that decreased PC3 cell viabilitiess to 70% and 60% at 24 hours and 48 hours were determined. Concentrations of inhibitors determined with respect to PC3 cell viabilities at the end of the assay were also used for the analysis of PNT1A cells for the rest of the study

to see the effects on immortalized prostatic cell lines which were used as control. The reason for this was that the target of this pharmacotherapy research of combination therapy was PC3 cells. Therefore, PC3 cells and PNT1A cells' comparison were performed according to concentrations which affected the PC3 cell lines.

#### 3.1.2.1. Alpha-naphthoflavone

Alpha-naphthoflavone (α-NF) concentrations were used in the range of 100 nM to 1000 nM for PC3 cell lines during this part of the study in order to pick the desired concentration of α-NF. The concentrations were tried until 70% and 60% cell viabilities were observed for 24 hours and 48 hours, respectively. Therefore, concentrations were reduced at every stage in order to catch these viability percentages. When the expected cell viabilities were observed, this concentration was also analyzed in combination with docetaxel in order to understand if docetaxel gave significant decrease in cell viabilities or not. Finally, the experiment was repeated for at least three times at these concentrations to get reproducable results. The results are given in **Figure 3.5-3.8**.

No change in PC3 cell viability was seen between 1 nM to 750 nM of α-NF for both 24 and 48 hours **(Figure 3.5-3.6)**.

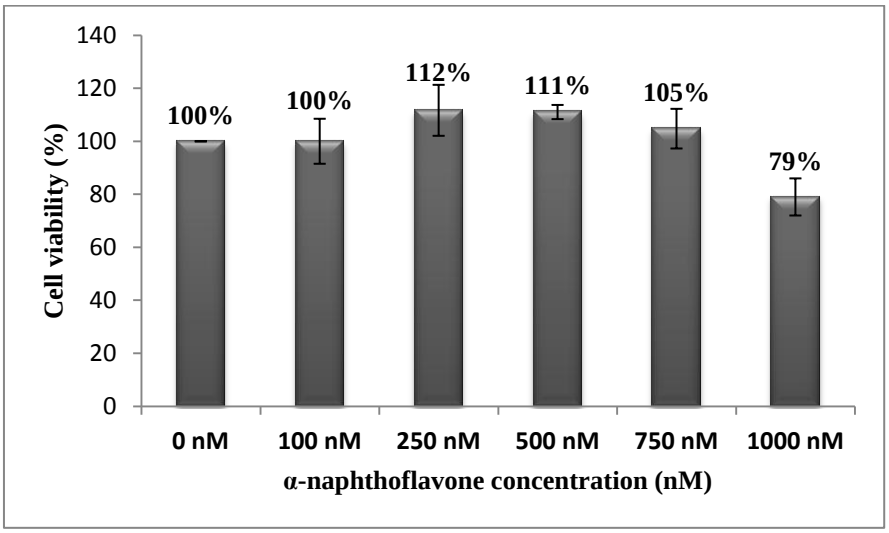



**Figure 3.5**. α-Naphthoflavone effect on cell viability of PC3 cell lines at 24 hour

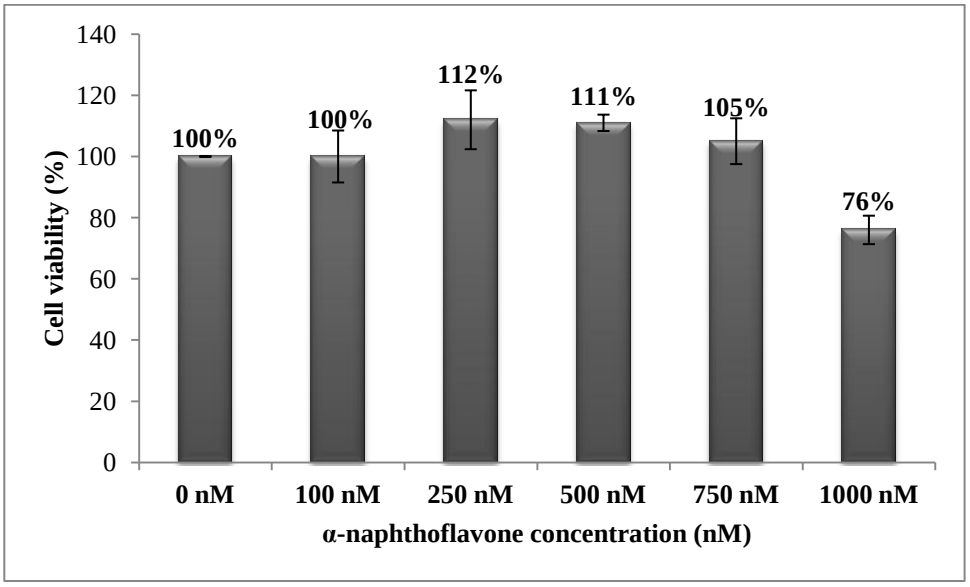



**Figure 3.6**. α-Naphthoflavone effect on cell viability of PC3 cell lines at 48 hour

As seen from **Figure 3.5-3.6**, α-NF was not physiologically toxic to the cell under normal conditions at 24 hours and 48 hours. On the other hand, 1000 nM of α-NF gave subtle changes (79% and 76%, 24h and 48h application, respectively) in the cell viability of PC3 cells **(Figure 3.5-3.6)**. In the literature, the maximum concentration of α-NF used in the analysis was 1000 nM. Therefore, 1000 nM was used in the following part of this analysis to see if combination therapy of α-NF and docetaxel would effect the cell viability of PC3 cell lines. In other words, if α-NF would increase the effect of docetaxel.

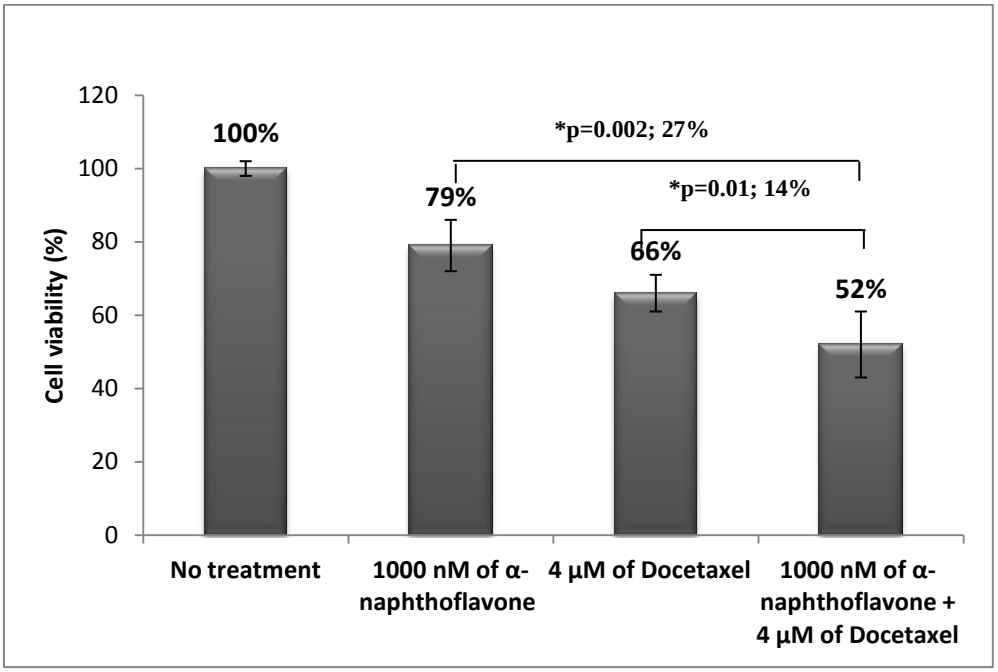



**Figure 3.7**. α-Naphthoflavone effect on cell viability of PC3 cell lines at 24 hour

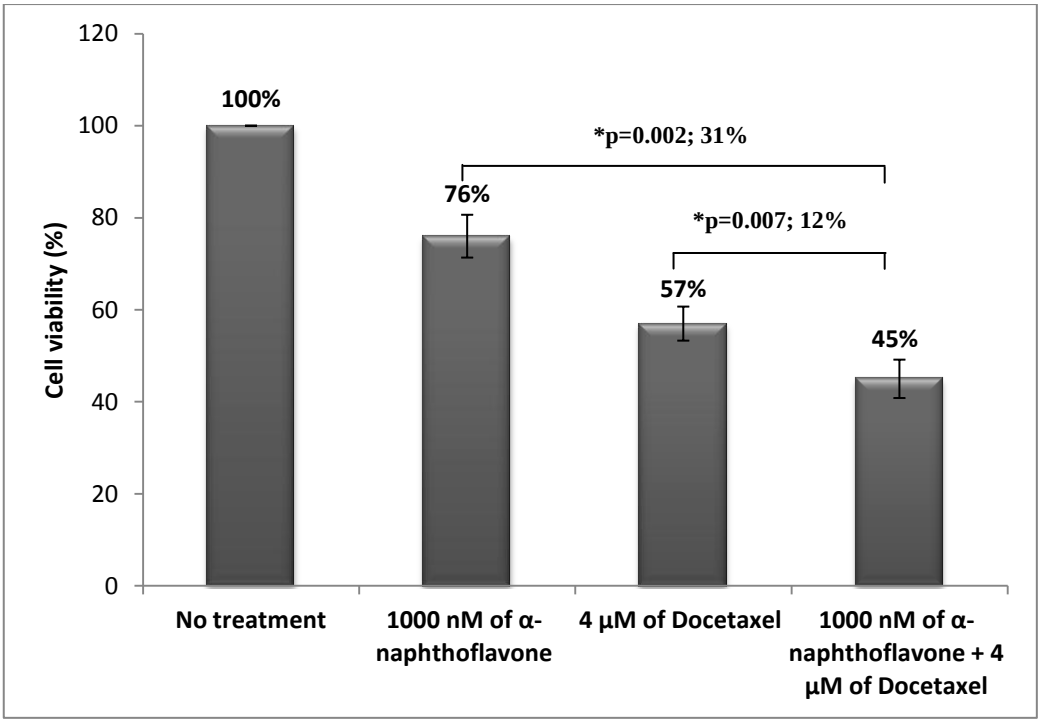



**Figure 3.8**. α-Naphthoflavone effect on cell viability of PC3 cell lines at 48 hour

As seen from **Figure 3.7- 3.8** when docetaxel was applied in combination with α-NF, docetaxel showed significant decrease in cell viability at 24 hours and 48 hours when compared to α-NF or docetaxel treatment alone ($p<0.01$). A thousand nM of α-NF determined with respect to PC3 cells was also used for PNT1A cells for the rest of the study to see the effects of α-NF on immortalized prostatic cell lines which are used as control.

#### 3.1.2.2. Ketoconazole

Ketoconazole concentrations were used in the range of 56.5 μM - 75.3 μM for PC3 cell lines during this part of the study. The concentrations were tried until 70% and 60% cell viabilities were observed for 24 hours and 48 hours, respectively. Therefore, concentrations were reduced at every stage in order to catch these viability percentages. When the expected cell viabilities were observed, this concentration was also analyzed in combination with docetaxel in order to understand if docetaxel gave extra decrease in cell viabilities or not. Finally, the experiment was repeated for at least three times at these concentrations to get reproducable results. The results are given in **Figure 3.9-3.12**.

Ketoconazole was found to be slightly toxic. 75.3 μM of ketoconazole caused 45% decrease in cell viability at 24 hours and 65.9 μM of ketoconazole caused 57% decrease in cell viability at 48 hours. On the other hand, 56.5 μM ketoconazole gave close cell viabilities to expected values of cell viabilities at 24 hours and 48 hours respectively (**Figure 3.9-3.10**). Therefore, 56.5 μM ketoconazole was used in the following part of this analysis to see if combination therapy of ketoconazole and docetaxel would effect the cell viability of PC3 cell lines. In other words, if ketoconazole would increase the effect of docetaxel.

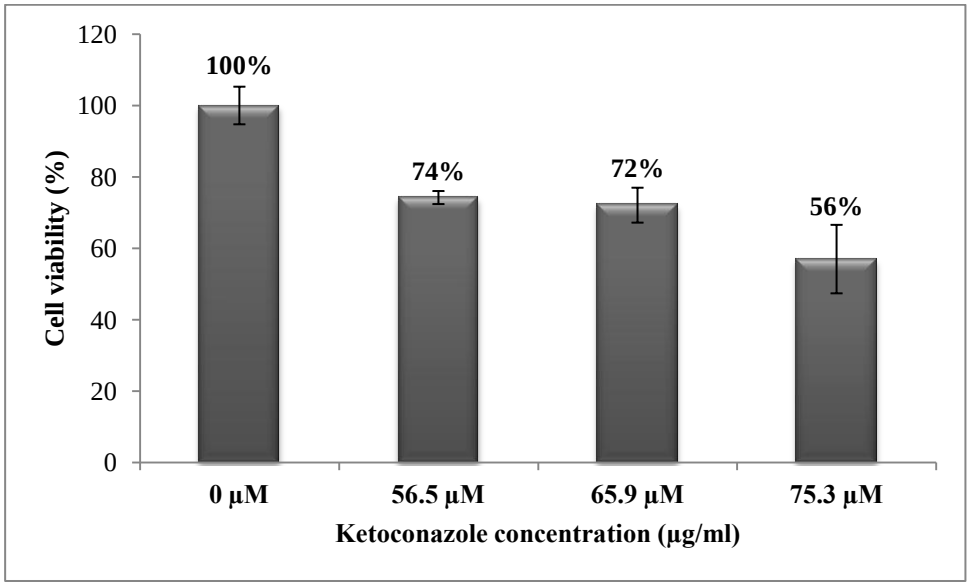



**Figure 3.9**. Ketoconazole effect on cell viability of PC3 cell lines at 24 hour

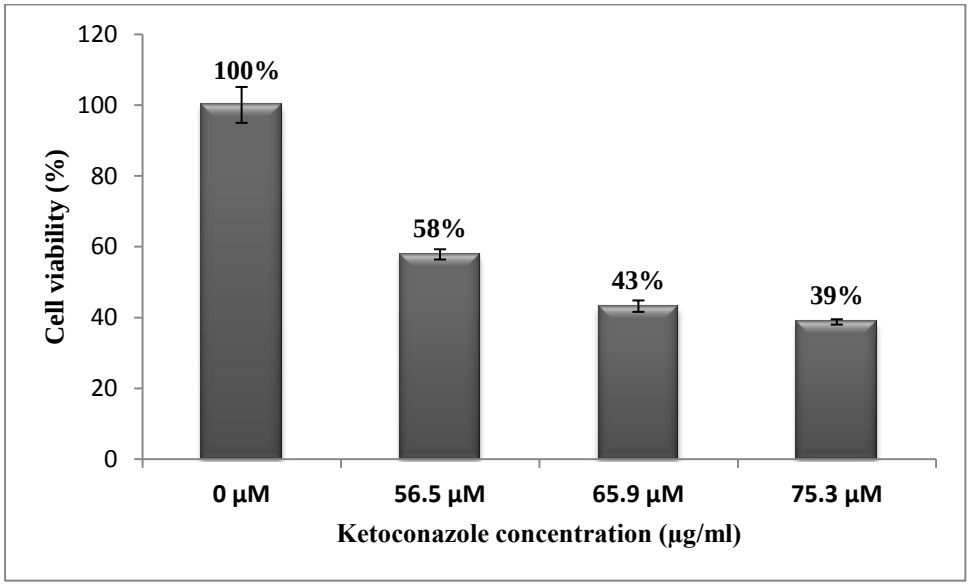



**Figure 3.10**. Ketoconazole effect on cell viability of PC3 cell lines at 48 hour

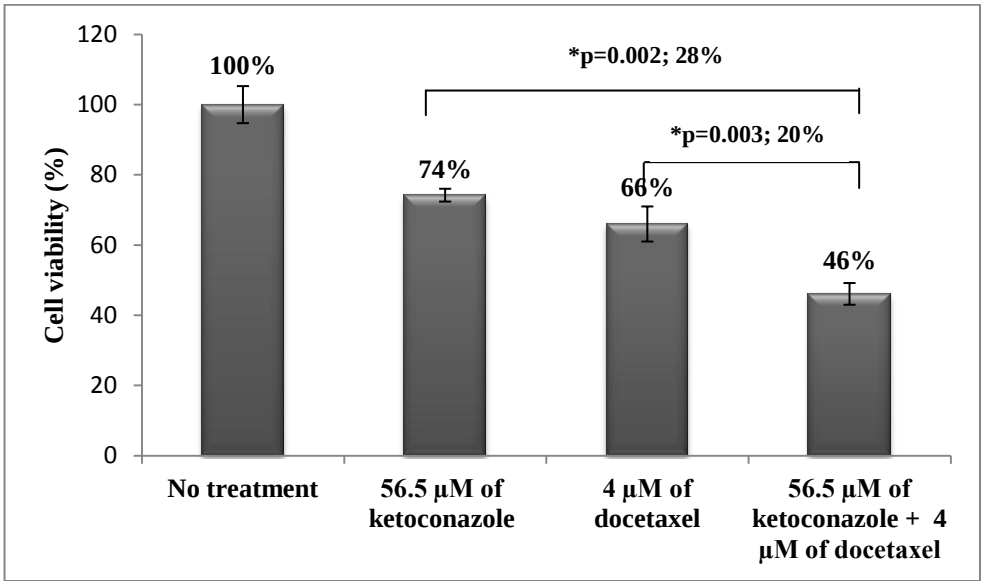



**Figure 3.11**. Ketoconazole effect on cell viability of PC3 cell lines at 24 hour

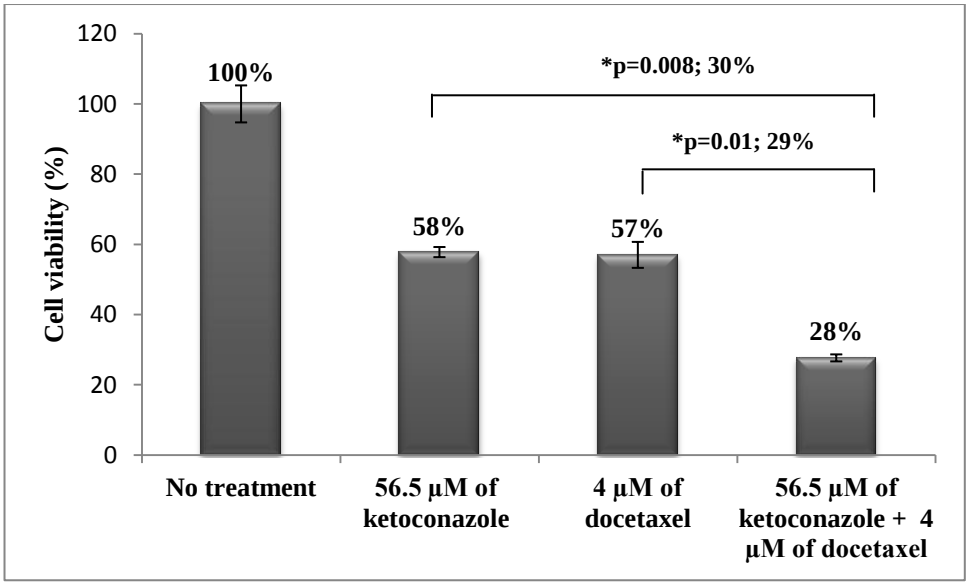



**Figure 3.12**. Ketoconazole effect on cell viability of PC3 cell lines at 48 hour

As seen from **Figures 3.11-3.12** if docetaxel was applied in combination with ketoconazole, docetaxel showed significant decrease in cell viability at 24 hours and 48 hours when compared to ketoconazole or docetaxel treatment alone ($p<0.01$). It was observed that ketoconazole showed its effect much better at 48 hours combination therapy. This indicated that 48 hours application gave prominant result with 72% decrease in cell viability. Therefore, 56.5 μM ketoconazole determined with respect to PC3 cells was also used for PNT1A cells for the rest of the study to see the effects on immortalized prostatic cell lines which are used as control.

#### 3.1.2.3. Quercetin

Quercetin concentrations were used in the range of 0 μM to 500 μM for PC3 cell lines during this part of the study. The concentrations were tried until 70% and 60% cell viabilities were observed for 24 hours and 48 hours, respectively. Therefore, concentrations were reduced at every stage in order to catch these viability percentages. When the expected cell viabilities were observed, this concentration was also analyzed in combination with docetaxel in order to understand if docetaxel gave extra decrease in cell viabilities or not. Finally, the experiment was repeated for at least three times at these concentrations to get reproducable results. The results are given in **Figure 3.13-3.17**.

In the first trial, quercetin did not decrease the cell viability up to 200 μM (**Figure 3.13**). Therefore, higher concentrations of quercetin were applied to pick up the desired concentration. In the second trial, higher concentrations of quercetin were applied to PC3 cell lines and 300 μM of quercetin showed the expected result for 24 hours (**Figure 3.14**). On the other hand, quercetin application for 48 hours gave a very drastic reduction in cell viability (**Figure 3.15**). This was why 24 hour application will be used in the next steps of this study in order to standardize the analysis.

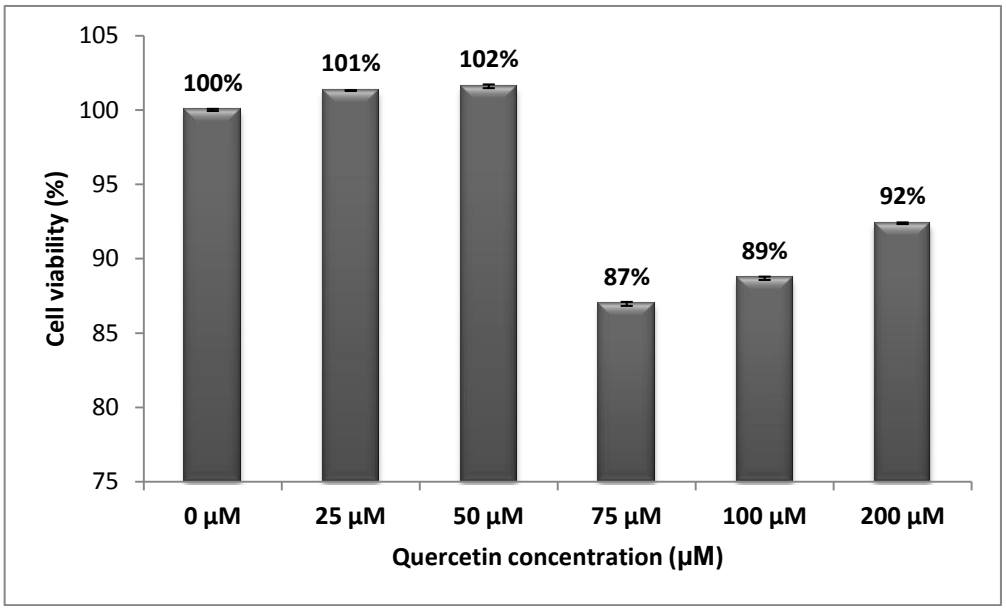



**Figure 3.13**. Quercetin effect on cell viability of PC3 cell lines at 24 hour

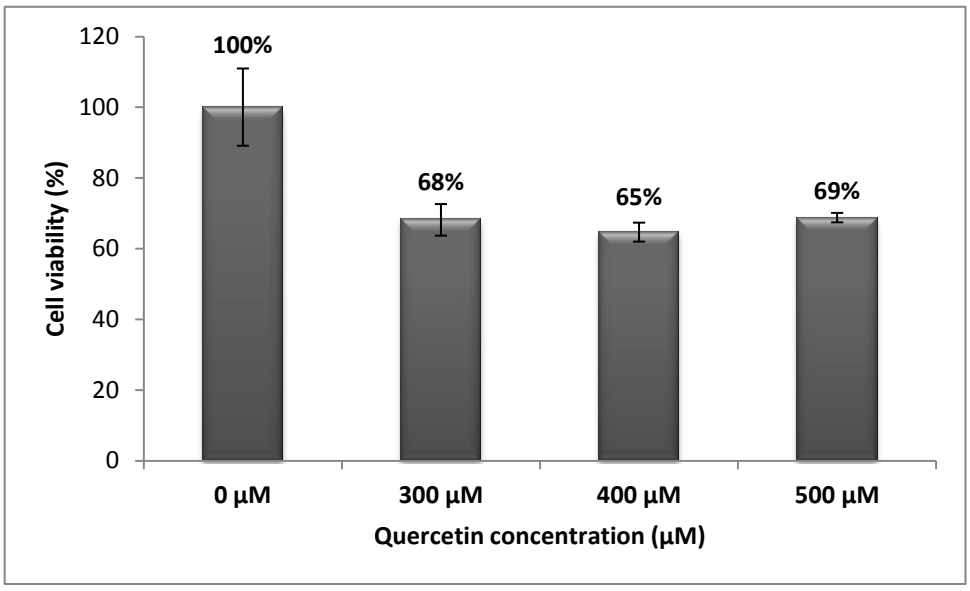



**Figure 3.14**. Quercetin effect on cell viability of PC3 cell lines at 24 hour

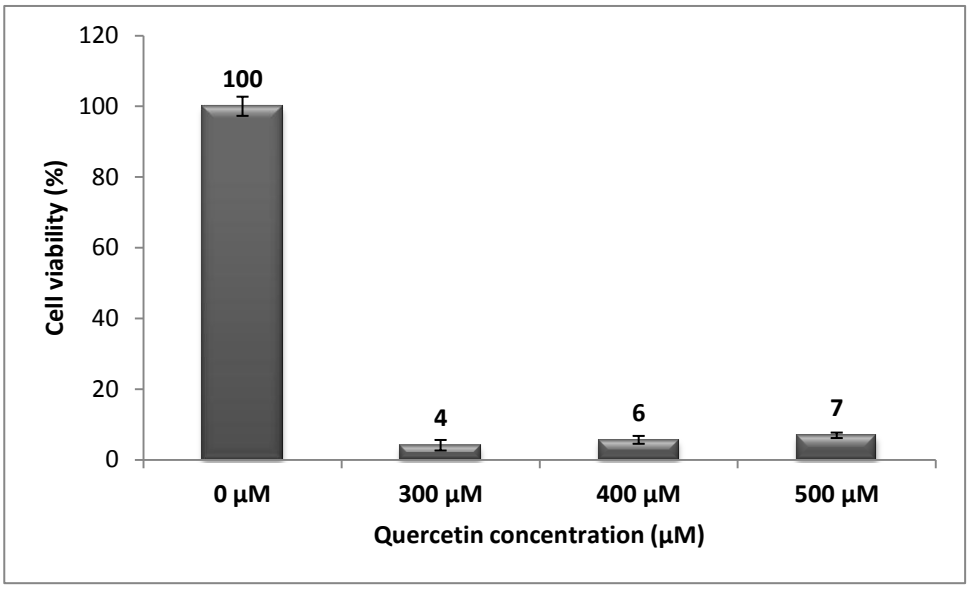



**Figure 3.15**. Quercetin effect on cell viability of PC3 cell lines at 48 hour

After determination of the concentration which was close to the desired cell viability ratio, PC3 cells were treated with the combination therapy of quercetin and docetaxel in order to observe if combination therapy of quercetin and docetaxel would effect the cell viability of PC3 cell lines

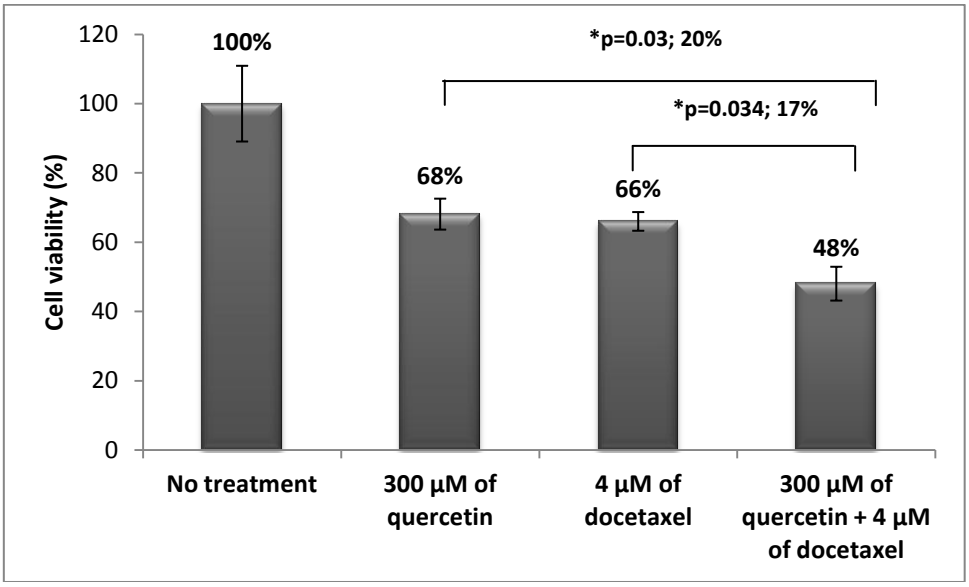



**Figure 3.16**. Quercetin effect on cell viability of PC3 cell lines at 24 hour

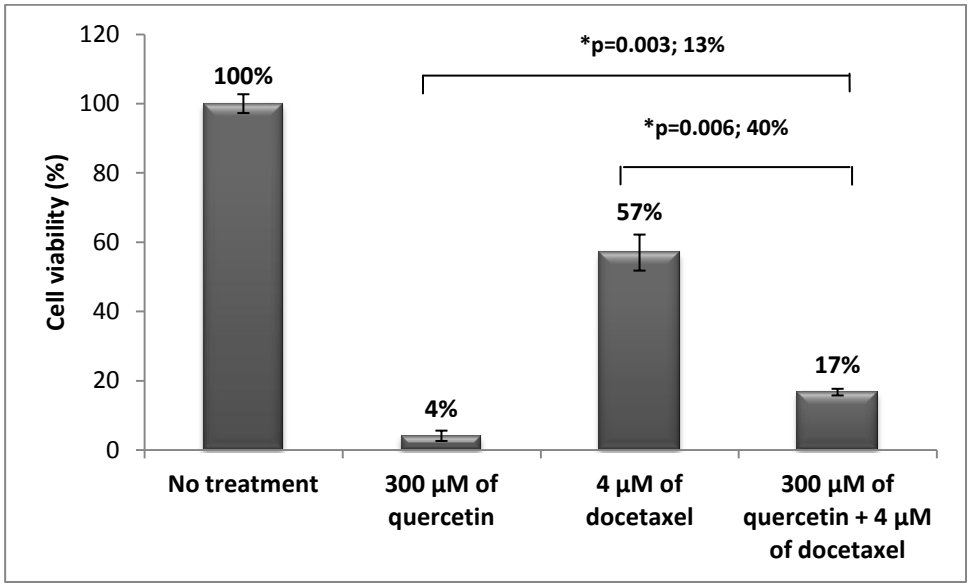



**Figure 3.17**. Quercetin effect on cell viability of PC3 cell lines at 48 hour

As seen from the **Figures 3.16-3.17** when docetaxel was applied in combination with quercetin, docetaxel showed significant decrease in cell viability at 24 hours when compared to quercetin or docetaxel treatment alone ($p<0.05$). On the other hand, docetaxel in combination with quercetin resulted in increase in cell viability at 48 hours when compared to quercetin treatment alone. This might be due to cell acivities that was overlapping. According to this result, quercetin might be used as intratumoral injection itself to kill the cells without killing normal cells. In addition, 300 µM of quercetin determined with respect to PC3 cells was also used for PNT1A cells for the rest of the study to see the effects on immortalized prostatic cell lines which are used as control.

## 3.2. Enzyme Activity Determinations

In this part of the study, $IC_{50}$ values of alpha-naphthaflavone, ketoconazole and quercetin with respect to CYP1A1, CYP3A4, total GST and GSTP1 activities in PC3 cells were analyzed in order to confirm the concentrations determined by WST-1

analysis. It was aimed to determine if these concentrations should have decreased the enzyme activities. In order to observe the effect of docetaxel on CYP1A1, CYP3A4, total GST and GSTP1 activities, docetaxel was also applied to PC3 and PNT1A cells and enzyme activities were determined.

### 3.2.1. Cytochrome P4501A1 activity in PC3 cell lines

Cytochrome P4501A1 (CYP1A1) activity in the presence of different concentrations of alpha naphthaflavone was determined as described in "Methods".

The substrate, 7-ethoxyresorufin, concentration was held at 10 μM while alpha naphthoflavone concentration was changed from 5 μM to 100 μM to investigate the effect of alpha naphthaflavone on CYP1A1 activity in PC3 cell lines.

**Figure 3.18** shows the effect of increasing concentrations of alpha naphthaflavone on CYP1A1 activity in PC3 cell lines. As seen from the figure, α-NF inhibited CYP1A1 activity in a dose dependent manner in PC3 cell lines.

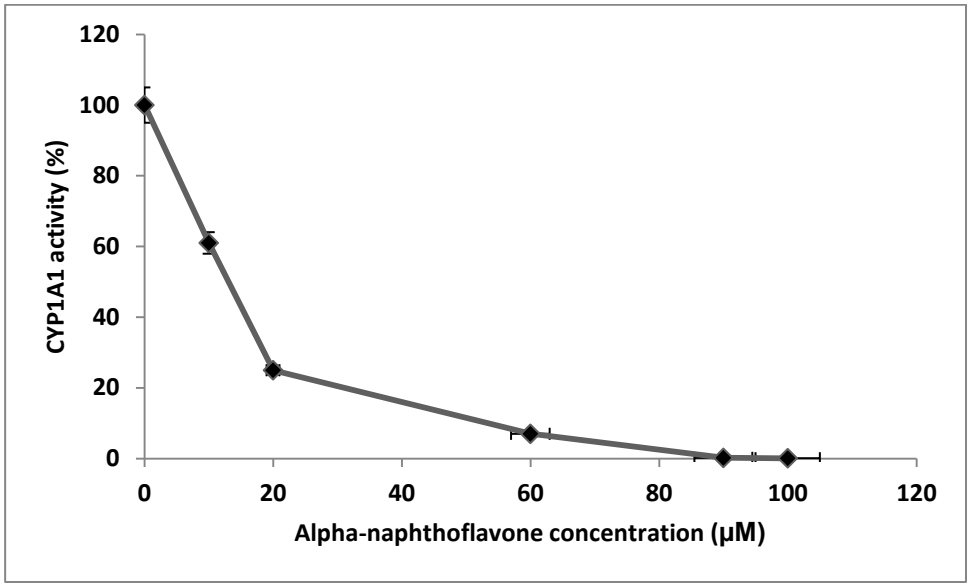



**Figure 3.18.** Effect of alpha-naphthoflavone on CYP1A1 activity. Inhibition experiments of CYP1A1 activity in order to determine $IC_{50}$ values were performed at 7-ethoxyresorufin concentration of 10 µM.

$IC_{50}$ value of alpha naphthoflavone was determined by plotting total CYP1A1 activity percent against logarithmic alpha-naphthoflavone concentration by graphpad as shown in **Figure 3.19**. Alpha-naphthoflavone inhibited CYP1A1 activity in a dose dependent manner with an $IC_{50}$ value of 15.1 µM. On the other hand, 1000 nM showed 100% activity, which was meant no decrease in CYP1A1 activity. It was concluded from this result that cellular processes might be overlapping under normal conditions.

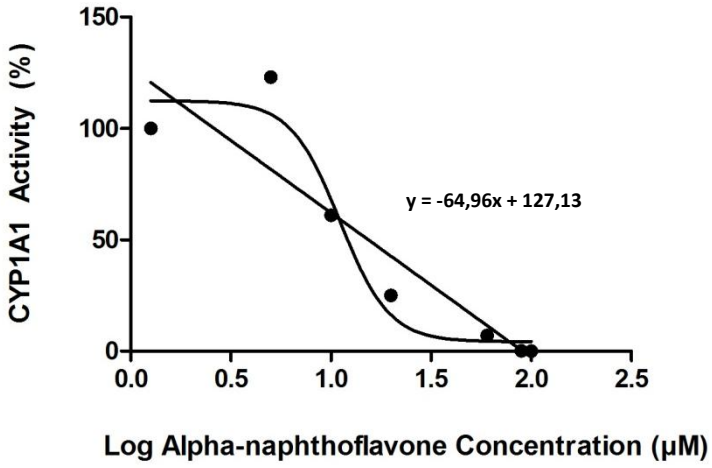



**Figure 3.19.** Determination of $IC_{50}$ value for alpha naphthoflavone

### 3.2.2. Cytochrome P4503A4 activity in PC3 cell lines

Cytochrome P4503A4 (CYP3A4) activity in the presence of different concentrations of ketoconazole was determined as described in "Methods".

Erythromycin (substrate) concentration was held at 1 mM while ketoconazole concentration was changed from 5 µg/ml to 70 µg/ml to investigate the effect of ketoconazole on CYP3A4 activity in PC3 cell lines.

**Figure 3.20** shows the effect of increasing concentrations of ketoconazole on CYP3A4 activity in PC3 cell lines. As seen from the figure, the activity started to decrease after 37.7 µM of ketoconazole.

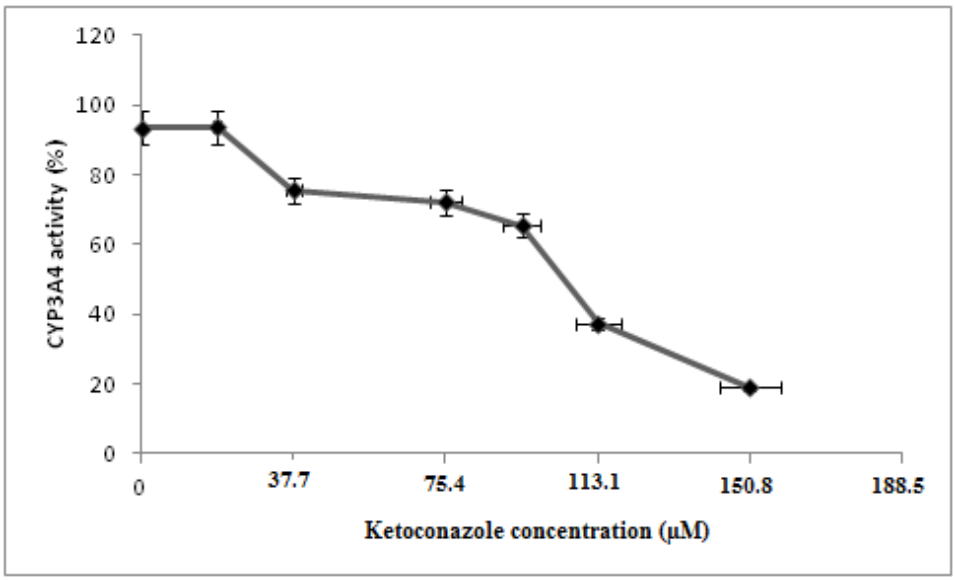



**Figure 3.20.** Effect of ketoconazole on CYP3A4 activity. Inhibition experiments of CYP3A4 activity to determine $IC_{50}$ values were performed at erythromycin concentration of 1 mM.

$IC_{50}$ value of ketoconazole was determined by plotting total CYP3A4 activity percent against logarithmic ketoconazole concentration by graphpad as shown in **Figure 3.21**. From the equation of the plot, $IC_{50}$ value for ketoconazole was calculated. Ketoconazole inhibited CYP3A4 activity in a dose dependent manner with an $IC_{50}$ value of 118.8 µM. On the other hand, 56.5 µM showed 73% activity, which was meant 27% decrease in CYP3A4 activity.

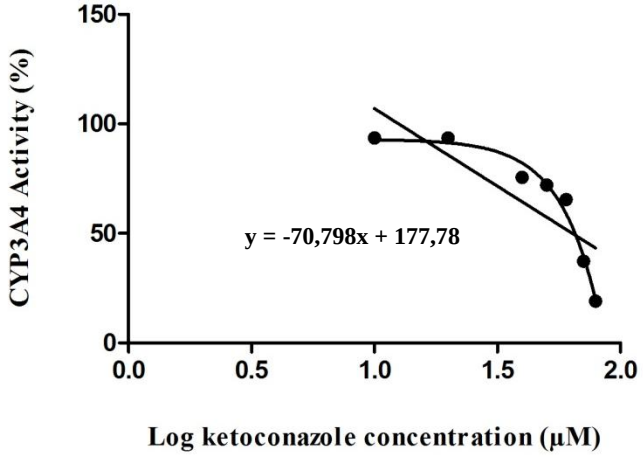



**Figure 3.21.** Determination of $IC_{50}$ value for ketoconazole

### 3.2.3. Total GST Activity

Total GST activity in the presence of different concentrations of quercetin was determined as described in "Methods".

The substrate, CDNB, concentration was held at 5 mM and GSH concentration was held at 1.0 mM while quercetin concentration was changed from 50 nM to 1000 nM to investigate the effect of quercetin on total GST activity in PC3 cell lines.

**Figure 3.22** shows the effect of increasing concentrations of quercetin on total GST activity. As seen from the figure, quercetin inhibited total GST activity in a dose dependent manner in PC3 cell lines.

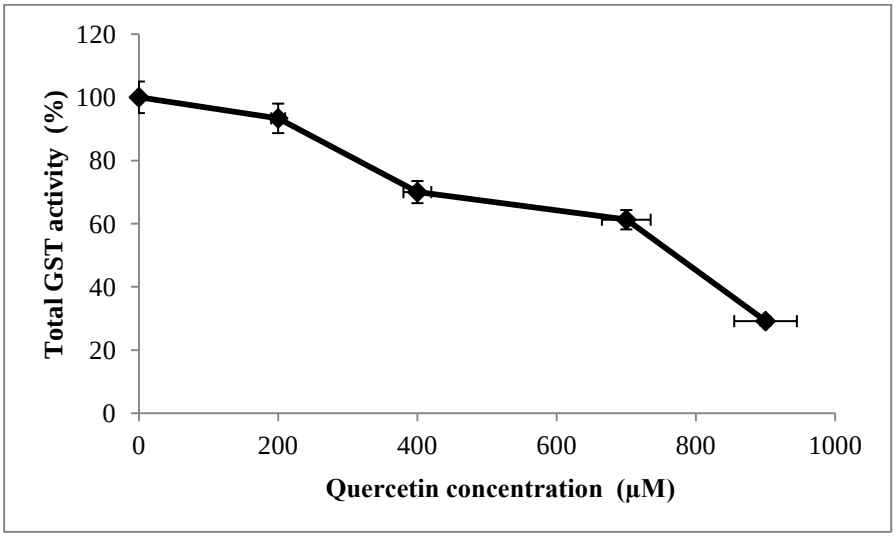



**Figure 3.22.** Effect of quercetin on total GST activity in PC3 cell lines. Inhibition experiments of GST activity to determine $IC_{50}$ values were performed at a substrate CDNB concentration of 5 mM and GSH concentration of 1 mM.

$IC_{50}$ value of quercetin was determined by plotting total GST activity percent against logarithmic quercetin concentration as shown in **Figure 3.23**. From the equation of the plot, $IC_{50}$ value for quercetin was calculated. Quercetin inhibited total GST activity in a dose dependent manner with an $IC_{50}$ value of 692 µM. On the other hand, 300 µM showed 80% activity, which meant 20% decrease in total GST activity.

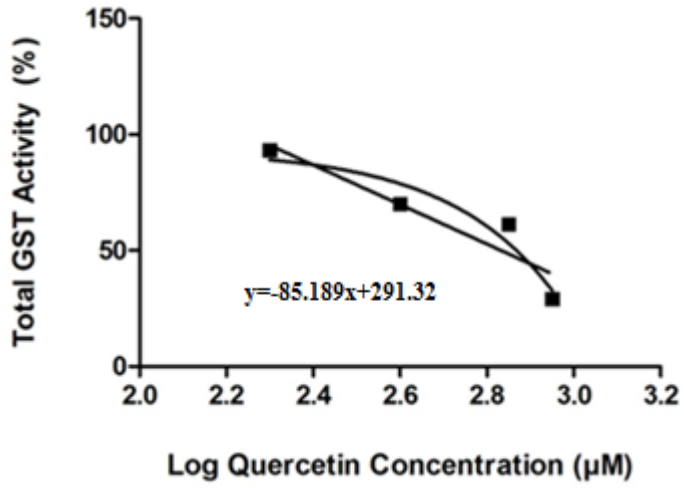



**Figure 3.23.** Determination of $IC_{50}$ value for quercetin

### 3.2.4. Glutathione S-Transferase (GST) P1 activity

GSTP1 activity in the presence of different concentrations of quercetin was determined as described in "Methods".

The substrate, Ethacrynic acid (EA), concentration was held at 1,5 mM and GSH concentration was held at 0,7 mM while quercetin concentration was changed from 50 nM to 1000 nM to investigate the effect of quercetin on GSTP1 activity in PC3 cell lines.

**Figure 3.24** shows the effect of increasing concentrations of quercetin on GSTP1 activity. Like the total GST activity, GST P1 activity was also inhibited with quercetin.

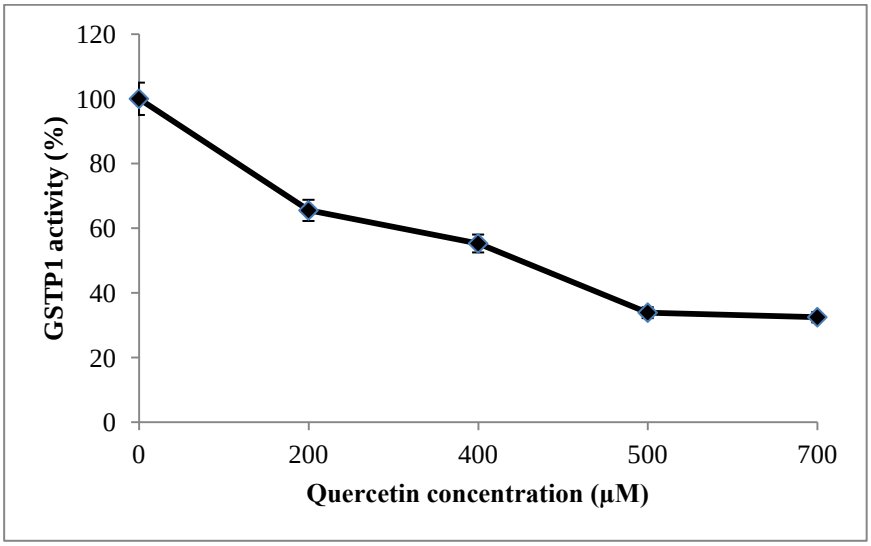



**Figure 3.24.** Effect of quercetin on GSTP1 activity. Inhibition experiments of GSTP1 activity to determine $IC_{50}$ values were performed at a substrate erythromycin concentration of 1 mM.

$IC_{50}$ value of quercetin was determined by plotting GSTP1 activity percent against logarithmic quercetin concentration as shown in **Figure 3.25**. From the equation of the plot, $IC_{50}$ value for quercetin was calculated. Quercetin inhibited GSTP1 activity in a dose dependent manner with an $IC_{50}$ value of 417,3 µM. On the other hand, 300 µM showed 56% activity, which was meant 44% decrease in GSTP1 activity.

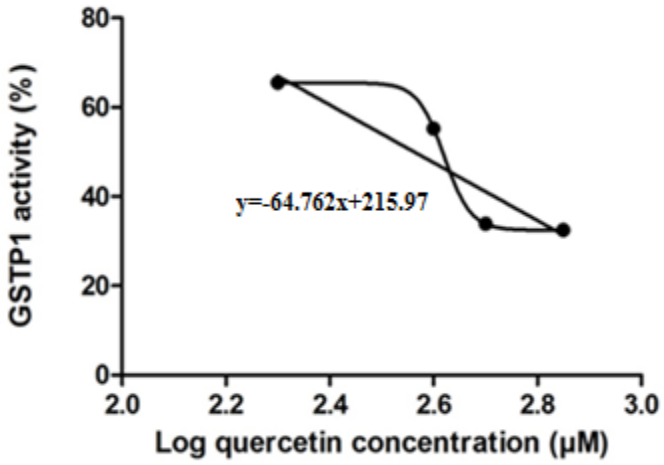



**Figure 3.25.** Determination of $IC_{50}$ value for quercetin

### 3.2.5. Effect of Docetaxel on Enzyme Activities in PC3 cell lines

#### 3.2.5.1. Docetaxel effect on CYP1A1 activity

CYP1A1 activity in the presence of 4 µM docetaxel was determined as described in "Methods" in PC3 cells. The results are given in **Figure 3.26**.

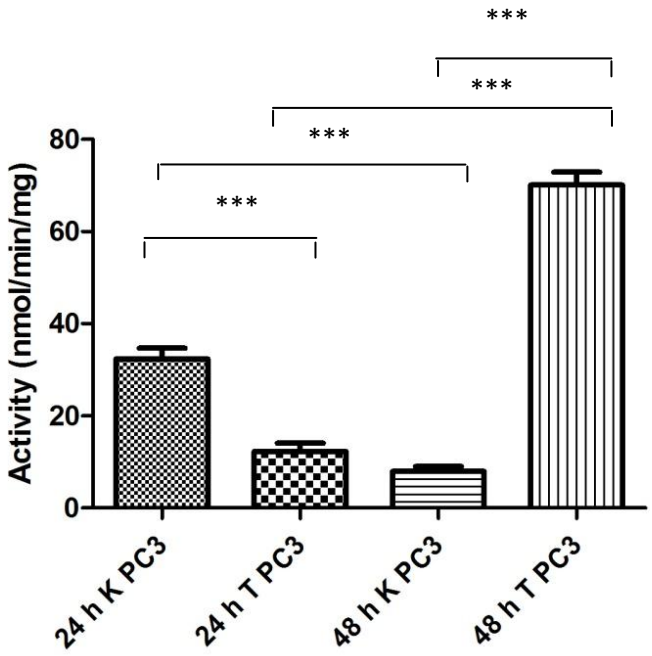



**Figure 3.26.** Docetaxel effect on CYP1A1 activity in PC3 cells. K is for untreated control cells and T is used for treated cells. ***p<0.001

Docetaxel showed time dependent effect on CYP1A1 activity in PC3 cell lines. After 24 hours application of docetaxel, CYP1A1 activity was significantly decreased compared to control ($p<0.001$). On the orher hand, after 48 hours application of docetaxel, CYP1A1 activity was significantly increased again when compared to control ($p<0.001$) cells**.** PC3 cells showed CYP1A1 activity when there was no application, since cancerous cells had been well prepared for everything. CYP1A1 activity increased more at 48 hour. Thus, PC3 cells gave late response to docetaxel. Thus, inhibition of CYP1A1 might make docetaxel stay in the cell longer. This was one explanation why docetaxel was more potent drug.

### 3.2.5.2. Docetaxel effect on CYP3A4 activity

CYP3A4 activity in the presence of 4 µM docetaxel was determined as described in "Methods" in PC3 cell lines. The results are given in **Figure 3.27**.

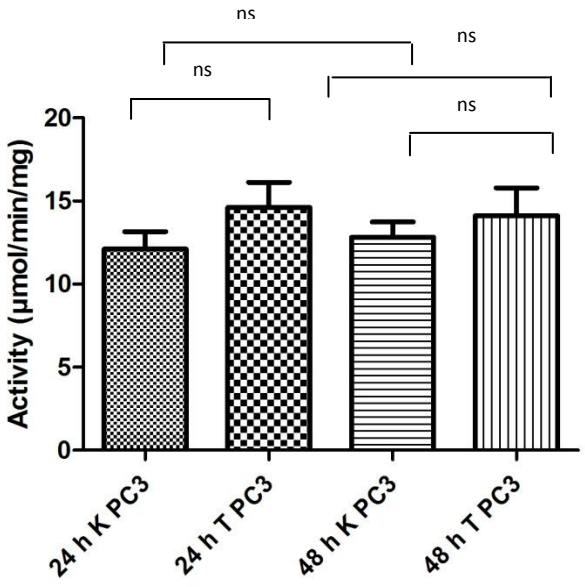



**Figure 3.27.** Docetaxel effect on CYP3A4 activity in PC3 cell lines. K is for untreated control cells and T is used for treated cells. ns: not significant

CYP3A4 was already active in the PC3 cells. After both 24 and 48 hours application of docetaxel, the observed CYP3A4 activity was slightly increased compared to control, but this change was not found statistically significant. Therefore, CYP3A4 was well responsive to docetaxel treatment

### 3.2.5.3. Docetaxel effect on GST total activity

GST total activity in the presence of 4 μM docetaxel was determined in PC3 cell lines as described in "Methods". The results are given in **Figure 3.28**.

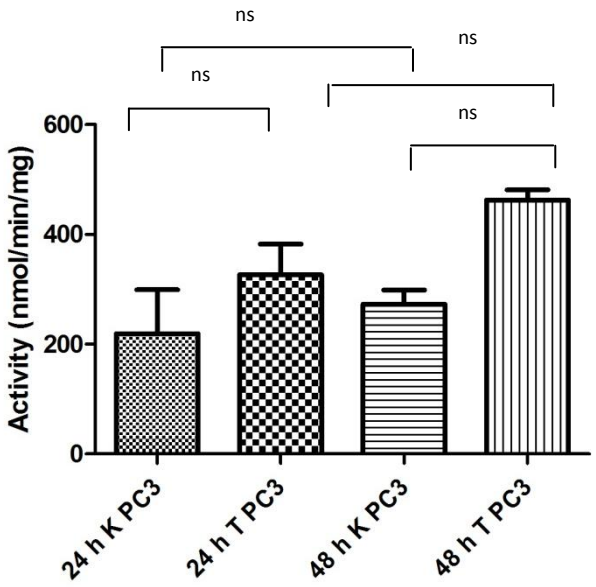



**Figure 3.28.** Docetaxel effect on GST total activity in PC3 cell lines. K is for untreated control cells and T is used for treated cells. ns: not significant.

As seen from **Figure 3.28**, docetaxel increased the GST total activity in PC3 cells slightly at 24 and 48 hour, but this increase was not statistically significant. PC3 cells showed total GST activity when there was no application, since cancerous cells had been well prepared for everything. This might be also due to the fact that, PC3 cells also had their own metabolites to be solubilized.

### 3.2.5.4. Docetaxel effect on GSTP1 activity

GSTP1 activity in the presence of 4 μM docetaxel was determined in PC3 cell lines as described in "Methods". The results are given in **Figure 3.29**.

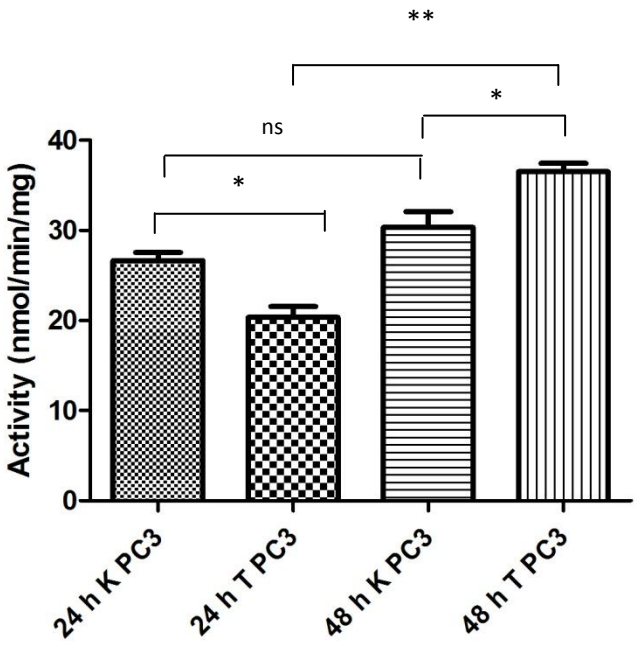



**Figure 3.29.** Docetaxel effect on GSTP1 activity in PC3 cells. K is for untreated control cells and T is used for treated cells. *p<0.05, **p<0.01, ns: not significant.

Docetaxel treatment first decreased (24 hr) then increased the GSTP1 activity with time. GSTP1 activity was significantly increased after 48 hours application compared to control cells ($p<0.05$). Interestingly GSTP1 activity also increased in control cells with increasing time

### 3.2.6. Effect of Docetaxel on Enzyme Activities in PNT1A cell lines

#### 3.2.6.1. Docetaxel effect on CYP1A1 activity

CYP1A1 activity in the presence of 4 μM docetaxel was determined in PNT1A cells as described in "Methods". Results are shown in **Figure 3.30**.

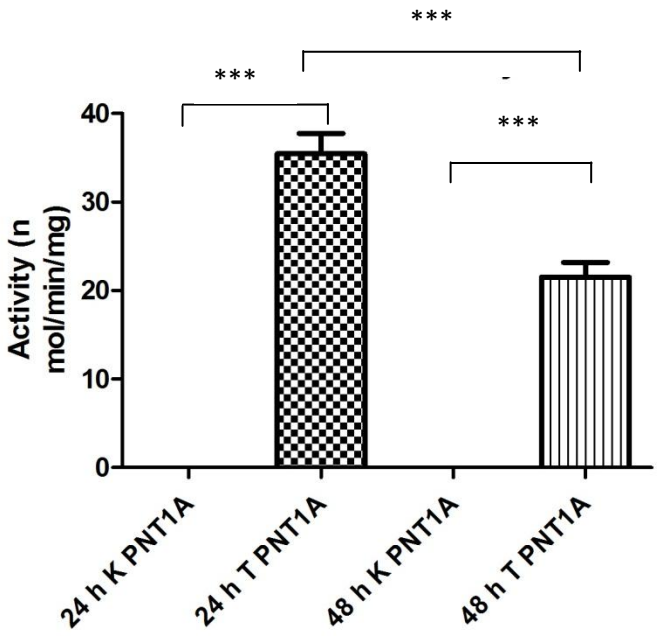



**Figure 3.30.** Docetaxel effect on CYP1A1 activity in PNT1A cells. K is for untreated control cells and T is used for treated cells. ***p<0.001

Untreated PNT1A cell lines showed no CYP1A1 activity. After 24 hours application of docetaxel, CYP1A1 activity was significantly increased compared to control cells ($p<0.001$). But then, after 48 hours application of docetaxel, CYP1A1 activity was significantly decreased ($p<0.001$). PNT1A cells gave response after docetaxel application immediately and this was normal mechanism of action.

### 3.2.6.2. Docetaxel effect on CYP3A4 activity

CYP3A4 activity in the presence of 4 μM docetaxel was determined as described in "Methods" in PNT1A cell lines, as shown in **Figure 3.31**. Pattern is similar to CYP1A1.

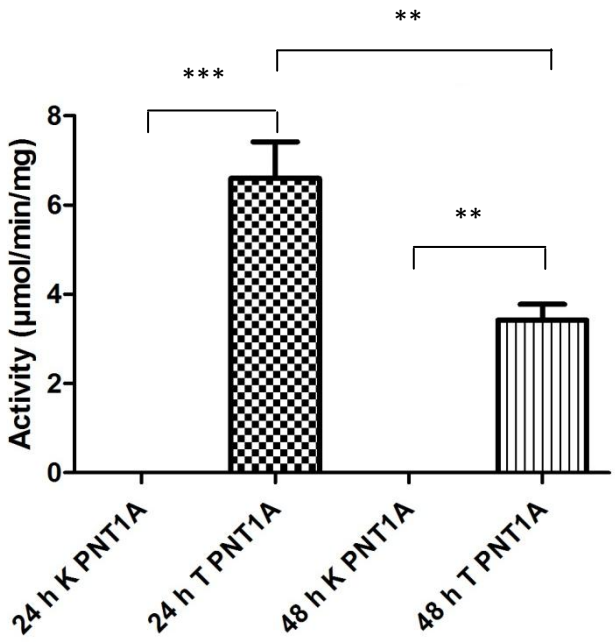



**Figure 3.31.** Docetaxel effect on CYP3A4 activity in PNT1A cell lines. K is for untreated control cells and T is used for treated cells. **p<0.01, ***p<0.001

Similarly, untreated PNT1A cell lines showed no CYP3A4 activity. After 24 hours of application of docetaxel, CYP3A4 activity was significantly increased compared to control ($p<0.001$). On the other hand, after 48 hours application of docetaxel, CYP3A4 activity was significantly decreased ($p<0.01$). PNT1A cells gave response after docetaxel application immediately and this was normal mechanism of action.

### 3.2.6.3. Docetaxel effect on GST total activity

GST total activity in the presence of 4 μM docetaxel was determined in PNT1A cell lines as described in "Methods". Results are given in **Figure 3.32**.

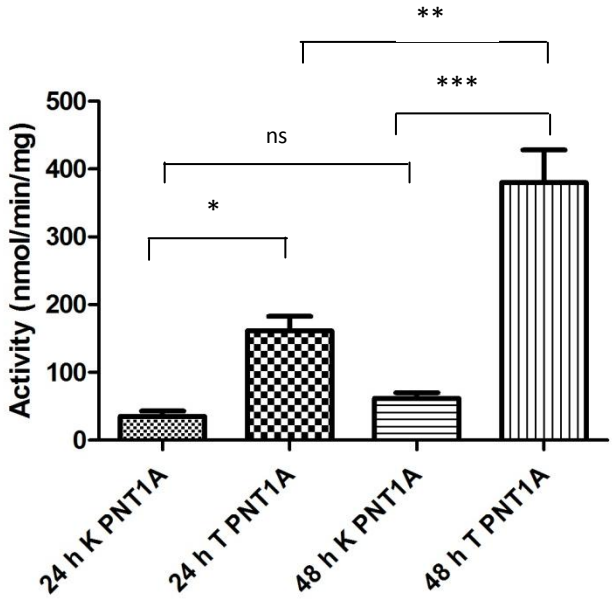



**Figure 3.32.** Docetaxel effect on GST total activity in PNT1A cell lines. K is for untreated control cells and T is used for treated cells. *p<0.05, **p<0.01, ***p<0.001, ns: not significant.

Docetaxel showed time dependent effect on GST total activity in PNT1A cell lines. After 24 hours application of docetaxel, GST total activity was significantly increased compared to control ($p<0.05$). Further application of docetaxel, increased total GST activity significantly compared to control values ($p<0.001$)**.** PNT1A cells gave response after docetaxel application, this was normal mechanism of action.

### 3.2.6.4. Docetaxel effect on GSTP1 activity

GSTP1 activity in the presence of 4 μM docetaxel was determined as described in "Methods" in PNT1A cell lines and results are given in **Figure 3.33**.

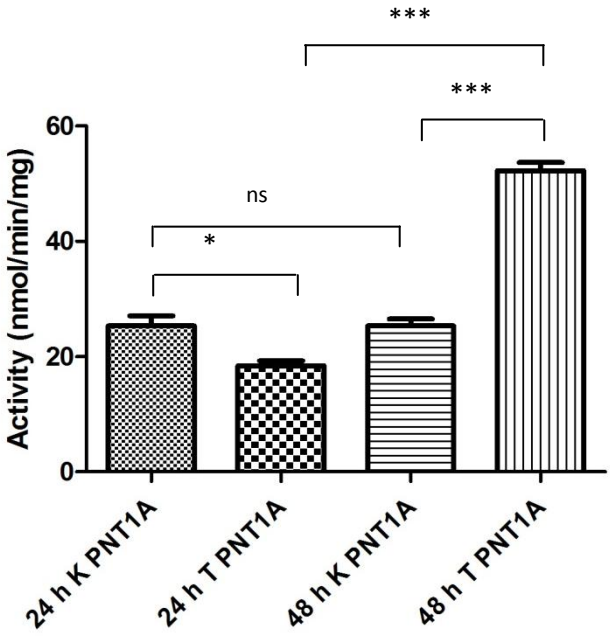



**Figure 3.33.** Docetaxel effect on GSTP1 activity in PNT1A cell lines. K is for untreated control cells and T is used for treated cells. *$p<0.05$, ***$p<0.001$, ns: not significant.

Though the total GST activity was found to be higher in untreated cells, GSTP1 izozyme is higher compared to docetaxel treated cells for 24 hours. After 24 hours application of docetaxel, GSTP1 activity was significantly decreased compared to control ($p<0.05$). Then, after 48 hours application of docetaxel, GSTP1 activity was significantly increased compared to control ($p<0.001$). PNT1A cells gave response after docetaxel application, this was normal mechanism of action.

### 3.3. RNA Isolation

RNA was isolated with peqGOLD RNAPure$^{TM}$. Gel electrophoresis and quantification of RNA were done after isolation in order to be sure if RNA is good and pure enough to study with for the following steps, thus for cDNA synthesis and quantitative real time PCR. Gel electrophoresis photograph obtained in this study is shown in **Figure 3.34.**

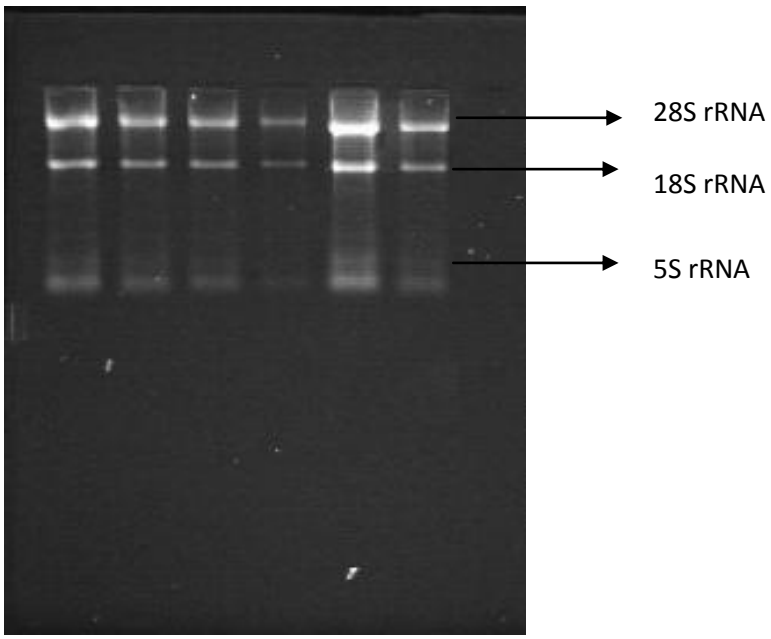



**Figure 3.34.** Gel electrophoresis of RNAs

### 3.4. Gene expression in PC3 and PNT1A Cell Lines

The mRNA expression levels of GSTP1, CYP1A1 and CYP3A4 in the PC3 and PNT1A cell lines were determined with quantitative real time polymerase chain reaction (qRT-PCR). Genes were normalized with β-actin. This housekeeping gene was chosen with respect to its stable expression level in studied cell lines, but there

were flactuations at 72 hours after docetaxel application. This is why qRT-PCR was carried out after 24 and 48 hour applications.

### 3.4.1. Gene expression in PC3 Cell Lines

#### 3.4.1.1. CYP1A1 gene expression

The results of CYP1A1 gene expressions in treated and untreated cells are given in **Figure 3.35.**

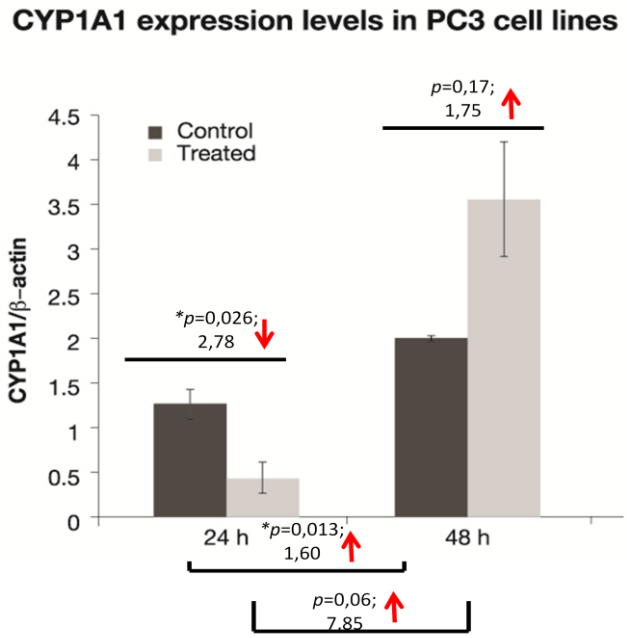



**Figure 3.35.** qRT-PCR results for CYP1A1 expression in PC3 cell lines (Excel statistics).

After 24 hours application of docetaxel, CYP1A1 expression was significantly decreased by 2.78 fold compared to control (p=0.026). On the other hand, after 48 hours application of docetaxel, CYP1A1 expression was increased by about 1.75 fold

compared to control, but the increase was not statistically significant (p=0.17). The results also showed that, time period of incubation caused increase in the expression of CYP1A1. These results were statistically significant for control group (p=0.013), but not significant for docetaxel treated group (p=0.06).

#### 3.4.1.2. CYP3A4 gene expression

**Figure 3.36** shows the results of the CYP3A4 gene expressions in PC3 cells.

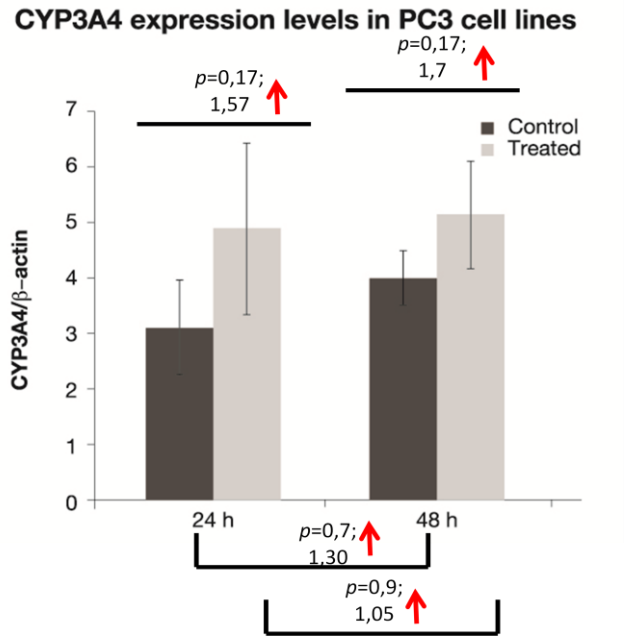



**Figure 3.36.** qRT-PCR results for CYP3A4 expression in PC3 cell lines (Excel statistics).

After 24 hour application of docetaxel, CYP3A4 expression was increased by 1.57 fold compared to control (p=0.17). Likewise, after 48 hour application of docetaxel,

CYP3A4 expression was increased by 1.7 fold compared to control (p=0.17). The results also showed that, time period of incubation caused an increase in the gene expression of CYP3A4. But these results were not statistically significant for both control (p=0.7) and docetaxel treated group (p=0.9).

#### 3.4.1.3. GSTP1 gene expression

The results of GSTP1 gene expressions in treated and untreated cells are given in **Figure 3.37.** Both cells showed decreased gene expression levels compared to controls.

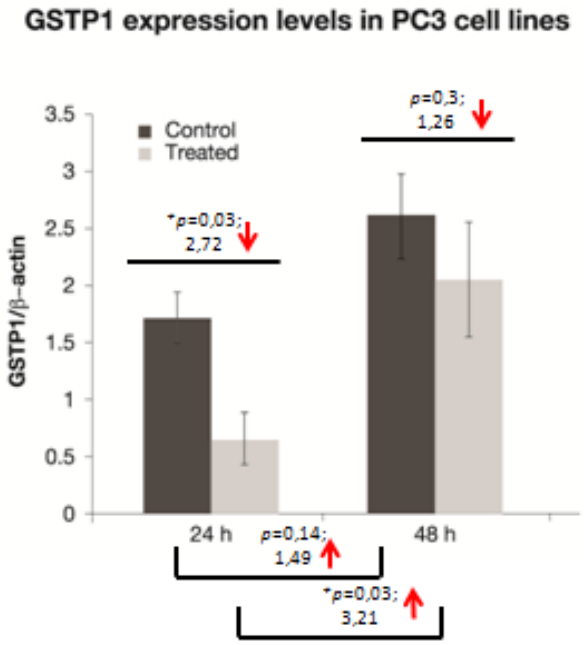



**Figure 3.37.** qRT-PCR results for GSTP1 expression in PC3 cell lines (Excel statistics).

After 24 hours application of docetaxel, GSTP1 expression was significantly decreased by 2.72 fold compared to control (p=0.03). Likewise, after 48 hour application of docetaxel, GSTP1 expression was decreased by 1.26 fold compared to control (p=0.3). It was also shown that, time period of incubation caused an increase in the expression of GSTP1. These results were not significant for control group (p=0.14), but significant for docetaxel treated group (p=0.03).

### 3.4.2. Gene expression in PNT1A Cell Lines

#### 3.4.2.1. CYP1A1 gene expression

The results of CYP1A1 gene expressions in treated and untreated PNT1A cells are given in **Figure 3.38.**

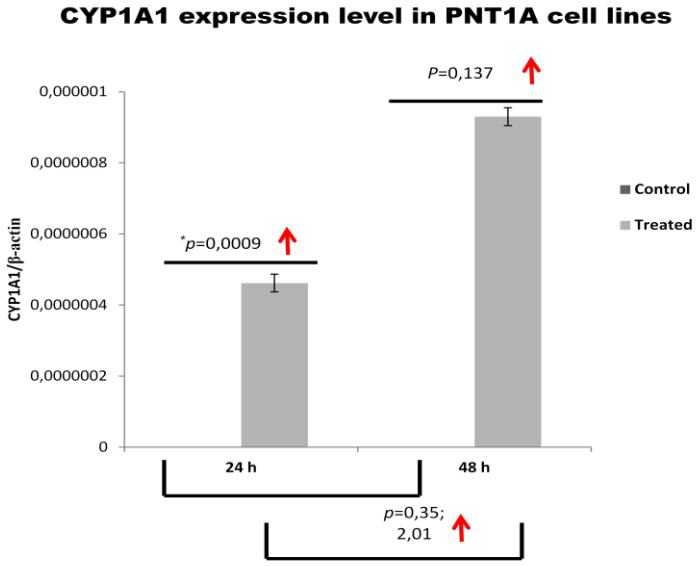



**Figure 3.38.** qRT-PCR results for CYP1A1 expression in PNT1A cell lines (Excel statistics).

After 24 hours application of docetaxel, CYP1A1 expression was significantly increased compared to control (p=0.0009), where no expression was observed in untreated PNT1A cells. Likewise, after 48 hour application of docetaxel, CYP1A1 expression was increased compared to control (p=0.3). It was important to point out that there was no expression of CYP1A1 at all before docetaxel application. It was also shown that time period of incubation caused increase in expression of CYP1A1 in treated cell lines by 2,01 fold.

#### 3.4.2.2. CYP3A4 gene expression

The results of CYP3A4 gene expressions in both treated and untreated cells are given in **Figure 3.39.**

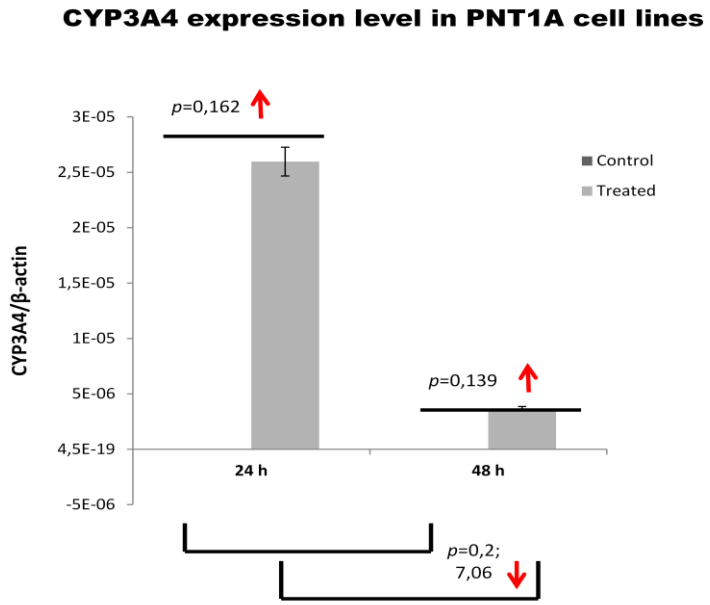



**Figure 3.39.** qRT-PCR results for CYP3A4 expression in PNT1A cell lines (Excel statistics).

Like CYP1A1, in LNT1A cells no CYP3A4 expression was observed. After 24 hours of application of docetaxel, CYP3A4 expression was increased drastically compared to control (p=0.162). Likewise, after 48 hour application of docetaxel, CYP3A4 expression was increased compared to control (p=0.139) but the increase is smaller. The time period of incubation caused decrease in the expression of CYP3A4 in treated cell lines by 7,06 fold.

#### 3.4.2.3. GSTP1 gene expression

The results of GSTP1 gene expressions in docetaxel treated and untreated cells are given in **Figure 3.40.**

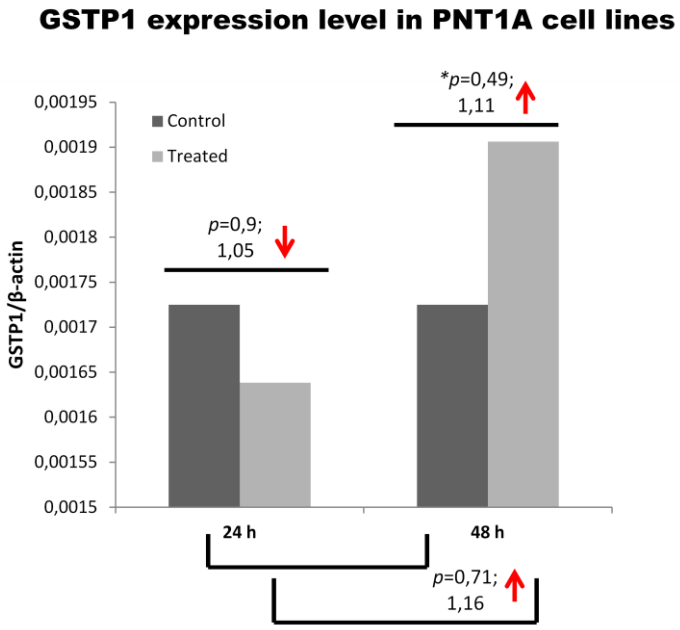



**Figure 3.40.** qRT-PCR results for GSTP1 expression in PNT1A cell lines (Excel statistics.)

After 24 hours of application of docetaxel, GSTP1 expression was decreased compared to control (p=0.9). However, after 48 hours application of docetaxel, GSTP1 expression was increased statistically, compared to control (p=0.49) cells. The results also showed that, time period of incubation caused increase in expression of GSTP1 in treated cell lines by 1,16 fold.

## 3.5. Detecion of Apoptosis by Annexin-V FLUOS Staining Assay

### 3.5.1 Cell death determination in PC3 cell lines

In order to determine the effect of docetaxel, inhibitors and their combinations on PC3 cell death, Annexin-V staining was performed after 24 hours of treatments. For that purpose, FITC-conjugated Annexin-V protein was used which has a high affinity to PI on dying cells' surface. Results are given in the following pages in **Table 3.1, Figure 3.41A-H and Figure 3.42A-D.**

When docetaxel was applied to PC3 cells, the cell viability was decreased significantly ($p<0.001$, compared to untreated PC3 cells). Similarly, the application of inhibitors alone/in combination with docetaxel showed decrease in the cell viability ($p<0.001$, compared to untreated PC3 cells). Alpha- naphthoflavone and ketoconazole in combination with docetaxel resulted in lower cell viability when compared to only inhibitor applications ($p<0.001$). On the other hand, quercetin caused lower cell viability itself compared to combination therapy with docetaxel ($p<0.001$) (**Figure 3.41A-H and Figure 3.42**).

In PC3 cells after docetaxel treatment, the early apoptosis was increased significantly ($p<0.001$, versus untreated PC3 cells). Similarly, the application of inhibitors alone/in combination with docetaxel also showed increase in the early apoptosis ($p<0.001$, versus untreated PC3 cells). Alpha-naphthaflavone and ketoconazole in combination with docetaxel resulted in higher early apoptosis when compared to

only inhibitor applications ($p<0.001$). On the other hand, quercetin caused higher apoptosis itself compared to combination therapy with docetaxel ($p<0.001$) (**Figure 3.41A-H and Figure 3.42B**).

Late apoptosis was increased significantly ($p<0.001$, versus untreated PC3 cells) when docetaxel was applied to PC3 cells, Similarly, the application of inhibitors alone/in combination with docetaxel showed increase in the late apoptosis ($p<0.001$, versus untreated PC3 cells). Alpha-naphthaflavone, ketoconazole and quercetin in combination with docetaxel resulted in higher late apoptosis when compared to only inhibitor applications ($p<0.001$) (**Figure 3.41A-H and Figure 3.42C**).

Finally, docetaxel treatment, decreased necrosis significantly ($p<0.001$, versus untreated PC3 cells). Similarly, the application of inhibitors alone/in combination with docetaxel showed a drastic decrease in the necrosis ($p<0.001$, versus untreated PC3 cells) also (**Figure 3.41A-H and Figure 3.42D**).

**Table 3.1.** Early Apoptotic, Late Apoptotic, Necrotic and Viable PC3 Cells (%)

| Applications | EA (%) | LA (%) | Nec. (%) | Viable (%) |
|---|---|---|---|---|
| Control | 0 | 0,77% | 10,75% | 88,48% |
| Docetaxel | 71,92% | 17,85% | 0% | 10,22% |
| α-NF | 60,51% | 26,70% | 0,07% | 12,71% |
| α-NF+Docetaxel | 66,13% | 30,68% | 0,03% | 3,16% |
| Ketoconazole | 48,41% | 11,77% | 0% | 39,82% |
| Ketoconazole+Docetaxel | 80,13% | 17,29% | 0,03% | 2,16% |
| Quercetin | 65,43% | 33,90% | 0,38% | 0,28% |
| Quercetin+Docetaxel | 29,45% | 39,21% | 0,28% | 31,07% |

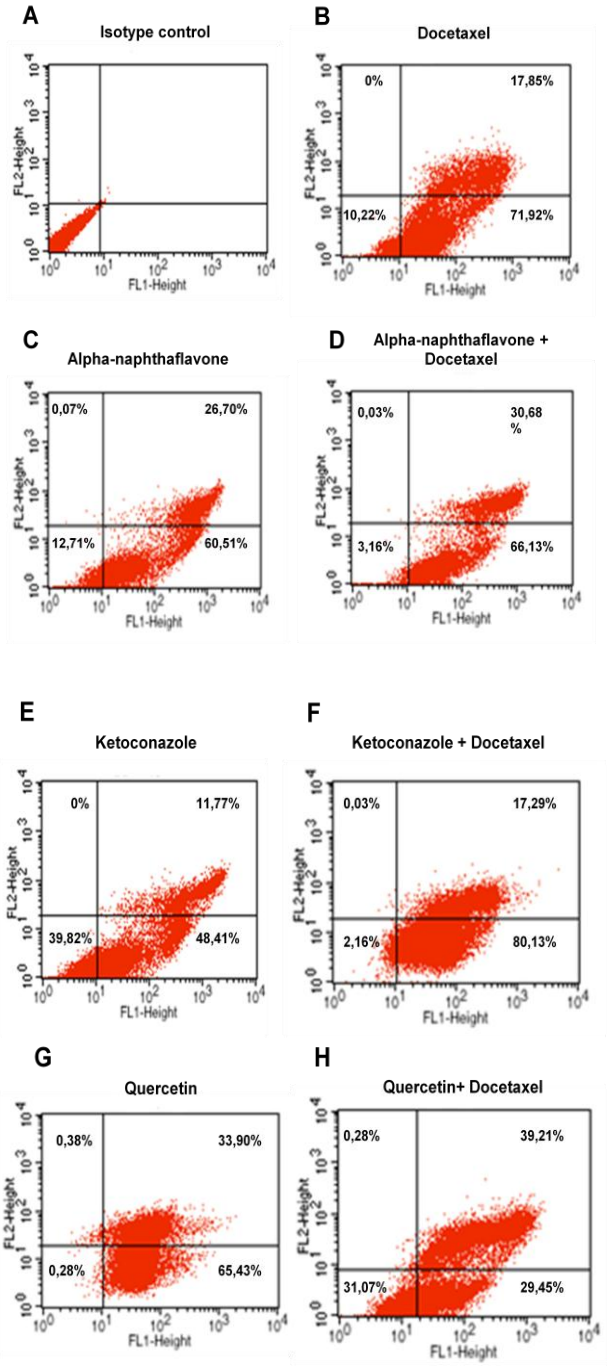



**Figure 3.41**. The effect of docetaxel (B), alpha-naphthaflavone (C), alpha-naphthaflavone + docetaxel (D), ketoconazole (E), ketoconazole + docetaxel (F), quercetin (G), quercetin + docetaxel (H) for 24 hours treatment, stained with FITC-Annexin-V.

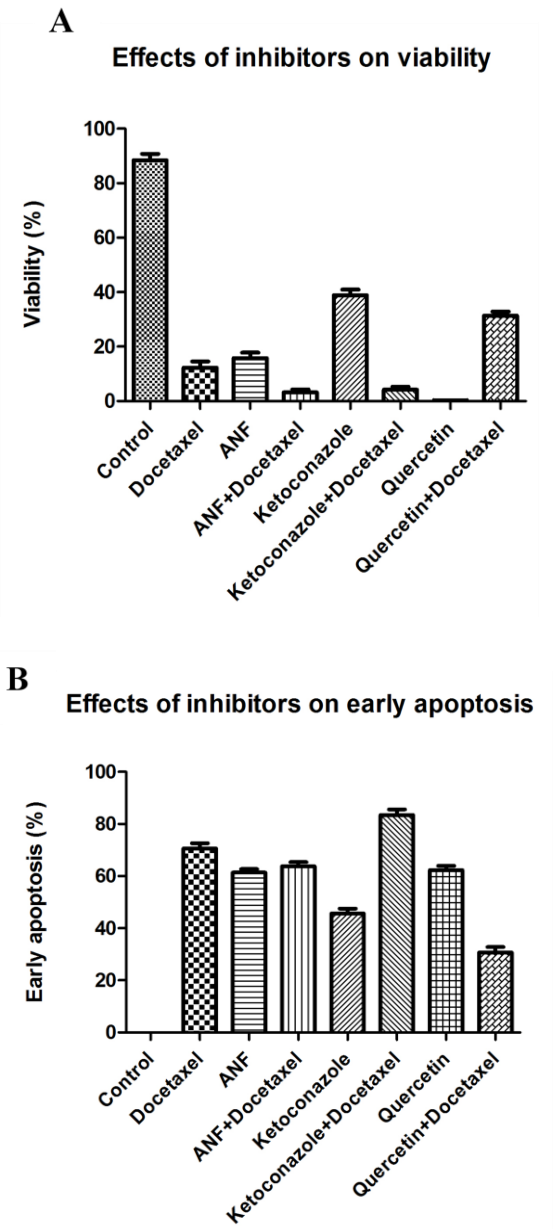



**Figure 3.42**. The effect of docetaxel, inhibitors, combination on cell death of PC3 cells. Statistical analysis of flow cytometer data of **(A)** viability, **(B)** early apoptosis **(C)** late apoptosis and **(D)** necrosis for 24 hours treatment, stained with FITC-Annexin-V.

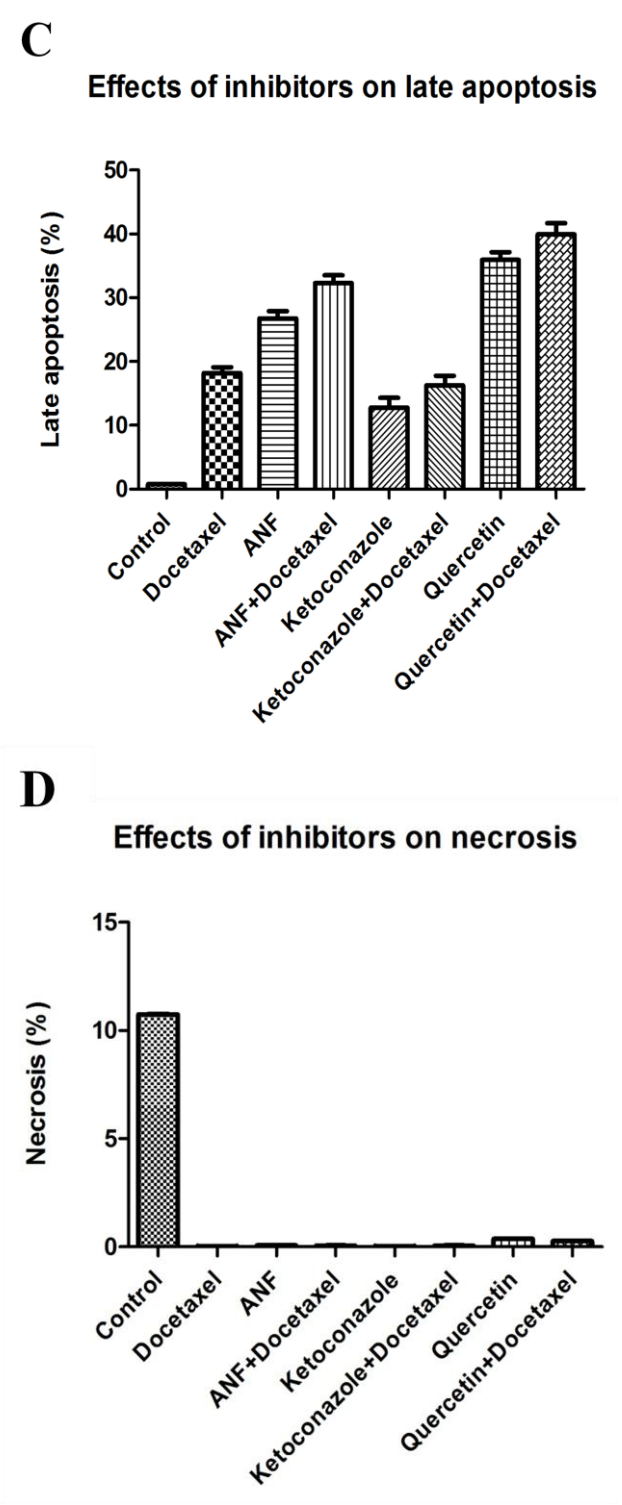



**Figure 3.42**. Continued.

### 3.5.2 Cell death determination in PNT1A cell lines

In order to determine the effect of docetaxel, inhibitors and combinations on PNT1A cell death, Annexin-V staining was used after 24 hours of treatments. For that purpose, FITC-conjugated Annexin-V protein was used. The results are given in **Table 3.2, Figure 3.43A-H and Figure 3.44A-D.**

When docetaxel was applied to PNT1A cells, the cell viability was decreased significantly ($p<0.001$, versus untreated PNT1A cells). Similarly, the application of inhibitors alone/in combination with docetaxel showed decrease in the cell viability significantly except alpha-naphthoflavone and docetaxel combination ($p<0.001$, versus untreated PNT1A cells). Alpha- naphthoflavone and its combination with docetaxel resulted in lower decrease in cell viability when compared to other applications. (**Figure 3.43A-H and Figure 3.44A**).

After docetaxel application to PNT1A cells, the early apoptosis was increased significantly ($p<0.001$, versus untreated PNT1A cells). Similarly, the application of inhibitors alone/in combination with docetaxel showed increase in the early apoptosis ($p<0.001$, versus untreated PNT1A cells). Ketoconazole alone and in combination with docetaxel resulted in higher early apoptosis when compared to other inhibitor applications ($p<0.001$). On the other hand, alpha-naphthaflavone and quercetin caused higher early apoptosis itself compared to combination therapy with docetaxel ($p<0.001$) (**Figure 3.43A-H and Figure 3.44B**).

When PNT1A cells were treated with docetaxel, the late apoptosis was increased (versus untreated PNT1A cells). Similarly, the application of inhibitors alone/in combination with docetaxel showed an increase in the late apoptosis (versus untreated PNT1A cells). Quercetin alone and in combination with docetaxel resulted in higher and significant late apoptosis when compared to other applications ($p<0.001$) (**Figure 3.43A-H and Figure 3.44C**).

When docetaxel was applied to PNT1A cells, like PC3 cells, necrosis was decreased significantly ($p<0.001$, versus untreated PNT1A cells). Similarly, the application of inhibitors alone/in combination with docetaxel showed decrease in necrosis ($p<0.001$, versus untreated PNT1A cells) (**Figure 3.43A-H and Figure 3.44D**).

**Table 3.2.** Early Apoptotic, Late Apoptotic, Necrotic and Viable PNT1A Cells (%)

| Applications | EA (%) | LA (%) | Nec. (%) | Viable (%) |
|---|---|---|---|---|
| **Control** | 0.19 | 0.08 | 25.32 | 74.41 |
| **Docetaxel** | 89.20 | 6.76 | 0.05 | 3.99 |
| **α-NF** | 55.06 | 2.06 | 0.12 | 42.76 |
| **α-NF+Docetaxel** | 28.73 | 1.36 | 0.09 | 69.81 |
| **Ketoconazole** | 93.51 | 5.03 | 0.09 | 1.37 |
| **Ketoconazole+Docetaxel** | 91.87 | 5.64 | 0.21 | 2.28 |
| **Quercetin** | 44.92 | 49.19 | 0.19 | 5.70 |
| **Quercetin+Docetaxel** | 11.65 | 85.75 | 0.1 | 3.21 |

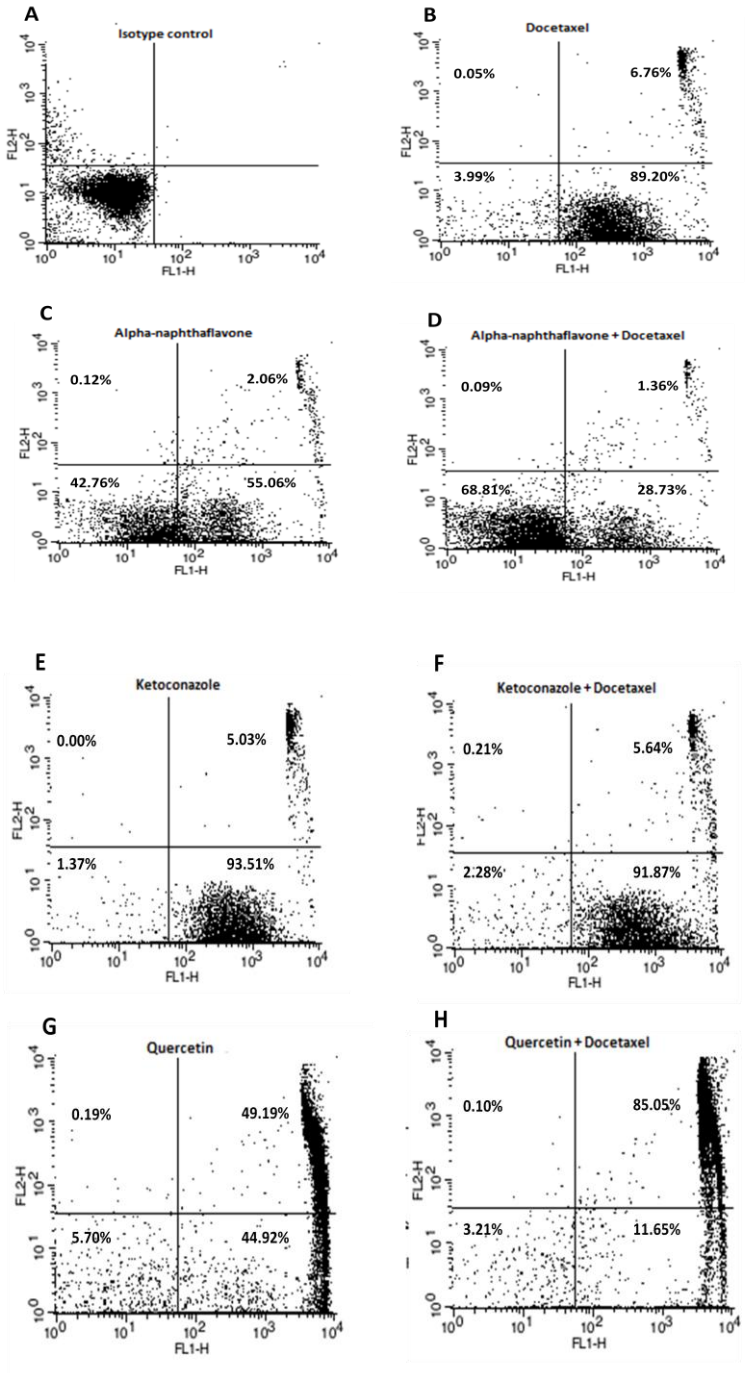
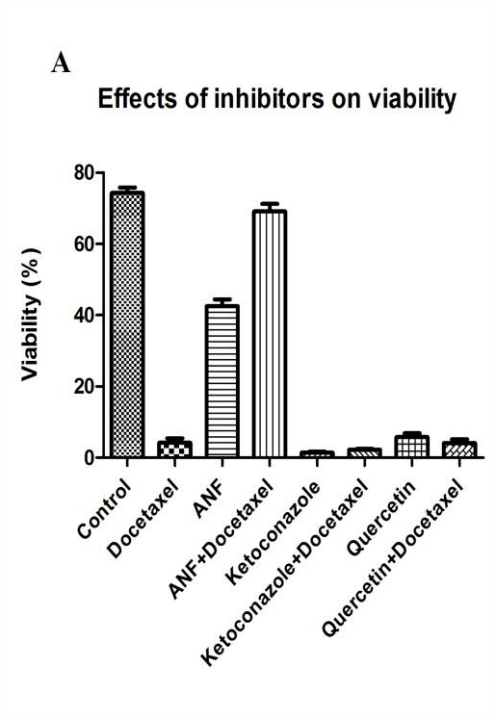



**Figure 3.43**. The effect of docetaxel (B), alpha-naphthoflavone (C), alpha-naphthaflavone + docetaxel (D), ketoconazole (E), ketoconazole + docetaxel (F), quercetin (G), quercetin + docetaxel (H) for 24 hours treatment, stained with FITC-Annexin-V.



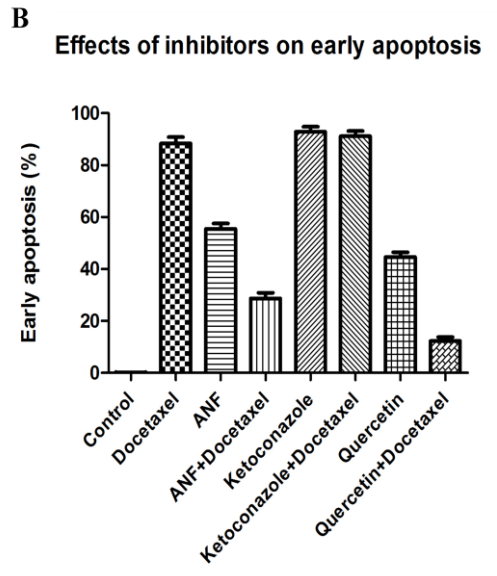




**Figure 3.44**. The effect of docetaxel, inhibitors, combination on cell death of PNT1A cells. Statistical analysis of flow cytometer data of **(A)** viability, **(B)** early apoptosis **(C)** late apoptosis and **(D)** necrosis for 24 hours treatment, stained with FITC-Annexin-V.

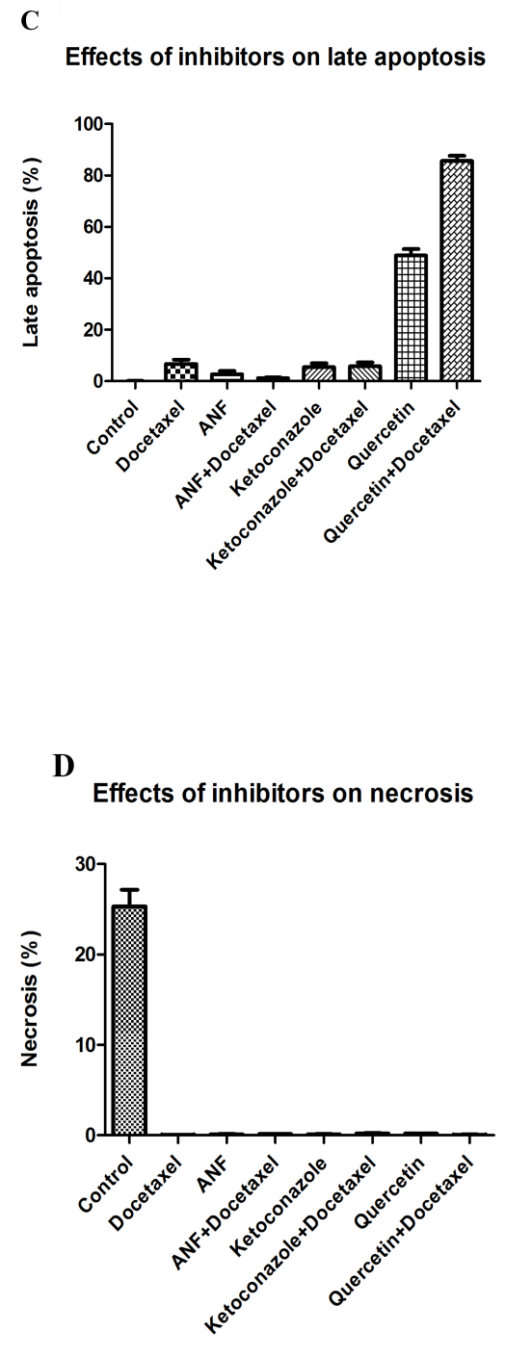



**Figure 3.44**. Continued.

### 3.6. Determination of Reduced Glutathione Concentration

Glutathione which is a tripeptide functions as a principle reducing agent in biological systems. The most important function of this tripeptide is to maintain intracellular redox status and protect cells from reactive oxygen species (Çavdar et al., 2006). In this analysis, in order to observe the oxidative stress occured after docetaxel, inhibitors and combination treatments in PC3 and PNT1A cells, reduced GSH concentrations were determined.

In PC3 cells, reduced GSH level was the highest for untreated PC3 cells (**Figure 3.45**). The application of docetaxel and inhibitor alone or combination therapies showed significant reduction in reduced GSH levels when compared to control cells ($p<0.001$). This demonstrates that after applications, there may be increased oxidative stress due to reactive oxygen species in PC3 cells.

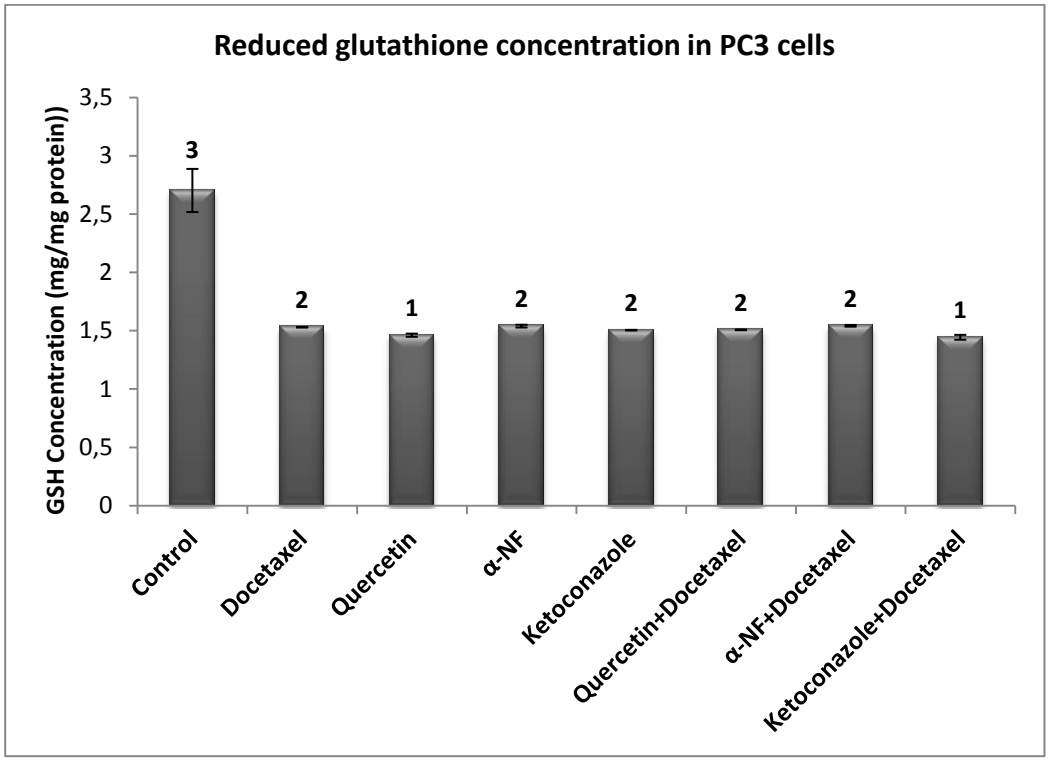



**Figure 3.45.** Reduced GSH concentration in PC3 cells at 24 hour

In PNT1A cells, reduced GSH level was the highest for quercetin+docetaxel application (**Figure 3.46**). The application of docetaxel and inhibitor alone or combination therapies showed significant reduction in reduced GSH levels when compared to contol cells except quercetin alone and quercetin+docetaxel therapies ($p<0.001$). This demonstrates that after applications, there may be increased oxidative stress due to reactive oxygen species in PNT1A cells except quercetin alone or quercetin+docetaxel applied cells.

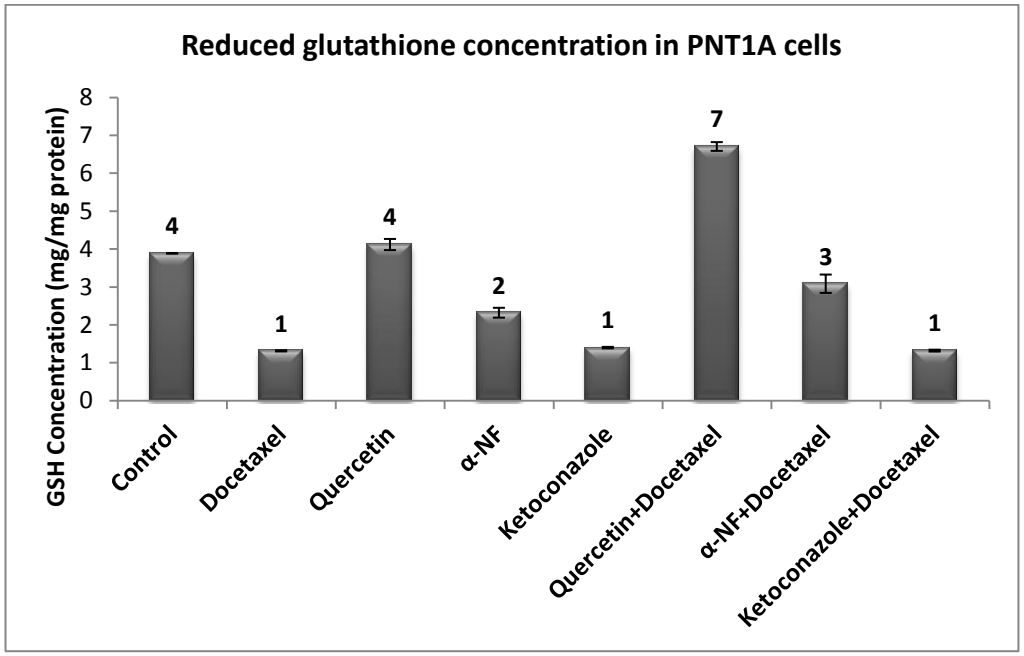



**Figure 3.46.** Reduced GSH concentration in PNT1A cells at 24 hour

As seen from the **Figures 3.45-3.46**, docetaxel and inhibitors affected reduced GSH levels as monotherapy or combination therapies in both PC3 and PNT1A cells in general.

# CHAPTER 4

# DISCUSSION

Prostate cancer is one of the mostly seen cancer type in world and in also in Turkey and for treatment it is important to use both convenient and efficient pharmacotherapy. The purpose of pharmacotherapy is the induction of remission, reduction in morbidity level and prevention of complications. Chemotherapy is one of the treatment options for prostate cancer especially if metastasis occured or hormone therapy failed. It is well known that docetaxel is one of the chemotherapy drug used as treatment for prostate cancer and it shows improvement in overall survival in patients with metastatic, castrate-resistant type prostate cancer. Several studies were carried out in order to study the docetaxel metabolism (Makhov et al., 2012, Li et al., 2004, Li et al., 2005, Li et al., 2005, Pienta et al., 2006, Petrylak, 2005, Rosing et al., 2000, Hayashi et al., 2005, Schmidt and Bastians, 2007; Pasquier et al., 2006, Baker et al., 2006, Baker et al.,2005; Goh et al., 2002; Tran et al., 2006; Yamamoto et al., 2005, Shou et al., 1998, Clarke and Rivory,1999, Shou et al., 1998, Komoroski et al., 2005, Goh et al.,2002, Puisset et al., 2004, Bosch et al., 2006, Tran et al., 2006, Amirimani et al., 2003; Schirmer et al., 2006, Baker et al., 2008, Puisset et al., 2007). In this study, we further investigate the importance of Phase I enzymes, namely, CYP1A1 and CYP3A4 and an important Phase II enzyme, GSTP1 in the metabolism of docetaxel in prostate cancer cell line, PC3 (androgen-independent PC cell lines) and in control cells, PNT1A (immortalized prostatic cell lines) by using real-time PCR and activity assays.

Novel combinations of docetaxel with biologic response modifiers are in early stages of development. Phase II studies suggest that the addition of estramustine to docetaxel results in higher response rates but also increased toxicity. Docetaxel with and without estramustine is being evaluated in Phase III studies that will provide

definitive information about its role in androgen-independent prostate cancer (Beer et al. 2003). Similarly, in metastatic breast cancer these include the monoclonal antibody agents trastuzumab and bevacizumab as well as novel agents directed at molecular targets (Tripathy et al., 2007). In addition, in a dose-dense study as neoadjuvant treatment in women with breast cancer, the combination of docetaxel and vinorelbine is currently under investigation. The combination of EGFR-TK and VEGF inhibitors was also evaluated in women with metastatic breast cancer. In the same way, a number of investigators are conducting exploratory trials that incorporate docetaxel into multimodality approaches for high-risk localized prostate cancer treatment (Beer et al. 2003).

In this study, the effects of docetaxel and phase enzyme inhibitors (quercetin, alpha naphthaflavone and ketoconazole) on cell viability were determined by the WST-1 assay. In the assay, after 24 hour incubation for cell growth, cells were incubated with the agents and analyzed after 24, 48 and 72 hours of incubation. According to WST-1 analysis, docetaxel and enzyme specific inhibitors showed cell viability of 70% and 60% with different concentrations at 24 and 48 hours, respectively. These concentrations were found to be 4 μM for docetaxel, 1000 μM for alpha-naphthaflavone, 56.5 μM for ketoconazole and 300 nM for quercetin. Our results showed that, when the combination therapy of docetaxel with inhibitors were applied to PC3 cell lines, decreases in cell viability were observed. Docetaxel and alpha-naphthoflavone combination resulted in 14% decrease in cell viability at 24 hour ($p=0.01$), while it caused 12% reduction in cell viability at 48 hour when compared to docetaxel alone treatment ($p=0.007$). Docetaxel and ketoconazole combination showed 20% decrease in cell viability at 24 hour ($p=0.003$), while 29% reduction in cell viability were determined at 48 hour when compared to docetaxel alone treatment ($p=0.01$). Docetaxel and quercetin combinations resulted in 17% decrease in cell viability at 24 hour ($p=0.034$), while it caused 40% decrease in cell viability at 48 hours when compared to docetaxel alone treatment ($p=0.006$).

Docetaxel and alpha-naphthoflavone combination resulted in 27% decrease in cell viability at 24 hour (p=0.002), while it caused 31% reduction in cell viability at 48 hour when compared to alpha-naphthaflavone alone treatment (p=0.002). Docetaxel and ketoconazole combination showed 28% decrease in cell viability at 24 hour (p=0.002), while 30% reduction in cell viability were determined at 48 hour when compared to ketoconazole alone treatment (p=0.008). Docetaxel and quercetin combinations resulted in 20% decrease in cell viability at 24 hour (p=0.003), while it caused 13% increase in cell viability at 48 hours when compared to quercetin alone treatment (p=0.003).

Because some breast cancers respond to estrogen, lowering estrogen production at the site of the cancer (i.e. the adipose tissue of the breast) with aromatase inhibitors has been proven to be an effective treatment for hormone-sensitive breast cancer in postmenopausal women. In this perspective, the first target enzyme was CYP19 (aromatase) in breast cancer therapy in the literature. The use of aromatase inhibitors has resulted in a new strategy plan, such as CYP17 inhibition with abiraterone for hormone ablation therapy for estrogen dependent cancers (Bruno et al., 2007). Similarly, inhibition of hepatic CYP3A4 activity with piperine (amajor plant alkaloid/amide) resulted in an increased area under the curve (actual body exposure to drug after administration of a dose of the drug), half-life and maximum plasma concentration of docetaxel when compared to docetaxel monotherapy. Therefore, the synergistic administration of piperine and docetaxel significantly improved the anti-tumor efficacy of docetaxel in animal model of human castration-resistant prostate cancer (Makhov, et al. 2012). When docetaxel was applied with the potent CYP3A4 inhibitor ketoconazole in cancer patients, docetaxel clearance was decreased with the rate of 49% (Engels et al., 2004, Akhdar et al., 2012).

$IC_{50}$ is commonly used as a measure of antagonist drug potency in pharmacological research. According to the FDA, $IC_{50}$ represents the concentration of a drug that is required for 50% inhibition in vitro. These analysis showed that concentration of inhibitor applications were in range for evaluating the docetaxel activity.

Blagosklonny and his collegues (2000) determined $IC_{50}$ values for ketoconazole as, 4 to 5 μg/ml, 12 μg/ml, and 25 μg /ml for LNCaP, PC3/PC3M, and DU145 cells, respectively, indicating its cytostatic effect on human different prostate cancer cell lines (Blagosklonny et al, 2000). According to CYP3A4 inhibition ketoconazole showed $IC_{50}$ value of 0.0037 – 0.18 μM in human liver microsome.

Quercetin shows inhibition on the proliferation of cell lines, with $IC_{50}$ values ranging from 0.1 pM to 45 μM, depending on the cell type and experimental conditions (van der Woude, 2006). Quercetin has $IC_{50}$ value of 45 μM in PC3 cells, 15 μM in MCF7 cells, 45 μM in Caco-2 cells, 2 μM in ALL cells, 18 μM OCM-1 cells and 15 μM in OVCA-433 cells according to growth inhibitions. In another study, it was demonstrated that $IC_{50}$ value of quercetin is 19.44 μM for LNCaP, 33.41 μM for PC3, 480 μM for MCF7 and 480 μM for PRSC cell lines (Haddad et al., 2006). In GSTP1-1 transfected MCF7 cells, quercetin showed the strongest inhibition with $IC_{50}$ value of 1.3mM (van Zanden et al., 2004). Arunakaran et al. (2013), calculated the $IC_{50}$ value of quercetin as 100 μM in PC-3 cells. In the same study, $IC_{50}$ value of breast cancer cells such as MCF-7, MDA-MB-231 were calculated as 4 μM and 6 μM, respectively. Alpha-naphthaflavone inhibited CYPs 1A1 and 1A2 with $IC_{50}$ values of 0.4-0.5 μM (Chang et al., 1994).

According to FDA, alpha-naphthaflavone inhibited CYP1A2 with an $IC_{50}$ value of 0.01 μM in human liver microsomes

According to our results, $IC_{50}$ values in PC3 cell lines were calculated as 15.1 μM for alpha-naphthaflavone, 63.1 μg/ml for ketoconazole, 692 μM and 417,3 μM for quercetin in the activity assays ($p<0,0001$). Quercetin was found to be the most potent inhibitor with respect to its concentration.

As expected, the results of activity assays and mRNA expressions were similar, thus consistent. In PC3 cell lines, all CYP1A1, CYP3A4 total GST enzyme activities increased after 48 hours treatment. On the other hand, GSTP1 showed slight decrease at 48 hour. In PNT1A cell lines, GST total and GSTP1 enzyme activities were increased in a time dependent manner. On the oher hand, no CYP1A1 and CYP3A4 enzyme activities were observed in untreated PNT1A cell lines. However, docetaxel

application stimulates the CYP1A1 and CYP3A4 enzyme activities. Thus, both activities were observed after 24 and 48 hours application of docetaxel.

Real time PCR analysis results showed differences in mRNA levels after application of docetaxel. CYP1A1 has a potential role in docetaxel metabolism (van Schaik, et al., 2008). Docetaxel was found to increase mRNA expression of CYP1A1 after treatment for both 24 and 48 hours applications in treated and untreated PC3 cell lines. Similarly, Li and his collegues found that CYP1A1 expression increased after docetaxel application at 6, 36 and 72 hours in PC3 cell lines. According to their study, docetaxel and furtulan combination therapy also showed an increase after docetaxel application (Li., et al. 2005). Thus, increase in enzyme expression can result in the decrease in docetaxel activity, however, docetaxel has antiangiogenic effects and inhibition in tumor cell proliferation can be the case (Sterling, et al., 2004). On the other hand, activation of CYP1A1 expression by drugs could also result in the metabolism of xenobiotics to which the individual may have been exposed and could result in the activation of procarcinogens. In PNT1A cell lines, no CYP1A1 expression was observed at 24 and 48 hours for untreated cells. After docetaxel application CYP1A1 expression level was increased by time. Therefore, we found that docetaxel also stimulates CYP1A1 expression in control prostate cell lines, changing the metabolic pathways and this can be very important for the activation of procarcinogens in normal cells after docetaxel treatment.

CYP3A4 has a major role in docetaxel metabolism (Fujimura et al., 2009). A low expression of CYP3A4 in breast tumors resulted in a better response of docetaxel (Miyoshi et al., 2005). According to our results, docetaxel was found to increase mRNA expression of CYP3A4 after docetaxel treatment in both 24 and 48 hours applications in PC3 cell lines. Increase in enzyme expression will result in a reduction in docetaxel activity and decrease in aggressiveness (Zeigler-Johnson, et al., 2004). In PNT1A cell lines, no CYP3A4 expression was observed at 24 and 48

hours for untreated cells. After docetaxel application, CYP3A4 expression level was increased in a time dependent manner. However, the level of mRNA expression is lower after 48 hours treatment. Therefore, docetaxel also stimulates CYP3A4 expression in control prostate cell lines changing the metabolism of drugs in these cells.

GSTP1 is a secondary enzyme in docetaxel metabolism (Arai, et al., 2008). Docetaxel was found to increase mRNA expression of GSTP1 after treatment for both 24 and 48 hours in PC3 cell lines. Similarly, Li et al. (2005), showed that GST levels were increased with docetaxel when used in combination with estaramustine. The increase in ROS which resulted in GSH depletion, can increase the GST expression (Geng, et al., 2005). In agreement with these studies, our results showed that in PNT1A cell lines, GSTP1 expression was observed in both treated and untreated cells. There is an increase in mRNA expression of GSTP1 after docetaxel treatment in PNT1A cell lines and it increases with time. Therefore, docetaxel also stimulates GSTP1 expression in control prostate cell lines, which might be effective in the removal of ROS.

The introduction of docetaxel in the treatment of hormone refractory prostate cancer (HRPC) has made a small but significant impact on patient survival. However, its effect is limited by intolerance and resistance (Howard EW., et al. 2008) In our study in order to investigate the potential of inhibitors to overcome docetaxel resistance, PC3 cells were treated with inhibitors in combination with docetaxel and the cell death was analyzed using Annexin V assays. The results of Annexin V cell death analysis showed that, in docetaxel treatment and combination with enzyme specific inhibitors resulted in differences in cell viabilities, early apoptosis, late apoptosis and necrosis. When docetaxel was applied to PC3 cells as alone or in combination with the inhibitors, the level of cell viability and necrosis was decreased significantly ($p<0.001$, versus untreated PC3 cells). On the other hand, when docetaxel was applied to PC3 cells as alone or in combination with the inhibitors, the level of early

and late apoptosis was increased significantly ($p<0.001$, versus untreated PC3 cells). Therefore, application of docetaxel with these inhibitors as a combination therapy in prostate cancer can be more effective by stimulating the apoptosis.

PNT1A cells were also treated with inhibitors in combination with docetaxel and the cell death was analyzed by using Annexin V. The results show differences in cell viability, early apoptosis, late apoptosis and necrosis. When docetaxel was applied to PNT1A cells as alone or in combination with the inhibitors, the level of cell viability and necrosis was decreased significantly ($p<0.001$, versus untreated PNT1A cells). On the other hand, the level of early and late apoptosis was increased significantly (versus untreated PNT1A cells). So the effect of the docetaxel and inhibitors on cell viability by stimulating appoptosis is not cell specific. Our results also suggest that, inhibitors and docetaxel combination can have a synergistic effect on cell viability compared to individual treatments of docetaxel and inhibitors.

Glutathione (GSH) takes an important role in several cellular processes, such as cell differentiation, proliferation, and apoptosis. Disturbances in GSH homeostasis are taken role in the etiology and progression of many human diseases including cancer. While GSH deficiency, or a decrease in the GSH/glutathione disulphide (GSSG) ratio, results in an increased susceptibility to oxidative stress implicated in the progression of cancer, increased GSH levels increase the antioxidant capacity and the resistance to oxidative stress as observed in many cancer cells (Traverso et al., 2013).

Therefore, in this analysis, reduced GSH concentrations were determined in PC3 and PNT1A cells and the results were compared. Freitas and his collegues demonstrated that PC3 cells have higher reactive oxygen species production when compared to HPV10 and RWPE1 cells but also the highest GSH levels , possibly contributing to oxidative stress resistance (Freitas et al., 2012). In this thesis study, untreated PNT1A cells have higher reduced GSH levels when compared to untreated PC3

cells. Kadam and Abhang found that administration of chemotherapeutic drugs in adjuvant setting in breast cancer patients causes increase in oxidative stress as indicated by decreased levels of antioxidant glutathione (Kadam and Abhang, 2013). In another study, quercetin treatment did not show any notable effect on intracellular levels of glutathione in human RPE cells (Kook et al., 2008), but increased GSH content in HepG2 cells In addition, co-incubation of ketoconazole and clozapine with lipopolysaccharide decreased the levels of glutathione in human precision-cut liver slices (Hadi et al., 2013). In this analysis, the application of docetaxel or inhibitor as monotherapy or combination therapies showed significant reduction in reduced GSH levels when compared to control cells. This shows that after applications, there may be increased oxidative stress due to reactive oxygen species in PC3 cells. On the other hand, in PNT1A cells, reduced GSH level was the highest for quercetin+docetaxel application. The application of docetaxel or inhibitor as monotherapy or combination therapies showed significant reduction in reduced GSH levels when compared to control cells except quercetin alone and quercetin+docetaxel therapies.

# CHAPTER 5

## CONCLUSION

In conclusion, according to our results, the application of docetaxel with enzyme spesific inhibitors is more effective by increasing the effects of docetaxel in PC3 cells. Further studies about the metabolism of drugs will help for the development of a therapy that would increase the efficacy of the drugs which might provide a better chance for the patience survival.

## REFERENCES


Akhdar H, Legendre C, Aninat C and Morel F, " Topics on Drug Metabolism", published in print edition, 2012.

Alfieri R.R., Galetti M., Tramonti S., Andreoli R., Mozzoni P., Cavazzoni A., Bonelli M., Fumarola C., La Monica S., Galvani E., De Palma G., Mutti A., Mor M., Tiseo M., Mari E., Ardizzoni A. and Petronini PG. " Metabolism of the EGFR tyrosin kinase inhibitor gefitinib by cytochrome P450 1A1 enzyme in EGFR-wild type non small cell lung cancer cell lines" Mol Cancer., 10, 143, (2011).

Amirimani B., Walker A.H., Weber B.L., Rebbeck T.R., "RESPONSE: re: modification of clinical presentation of prostate tumors by a novel genetic v variant in CYP3A4." J. Natl. Cancer Inst., 91, 1588–1590, (2003).

Androutsopoulos V.P., Tsatsakis A.M. and Spandidos D.A. “Cytochrome P450 CYP1A1: wider roles in cancer progression and prevention”, BMC Cancer, 9, 187 (2009).

Androutsopoulos V.P., Papakyriakou A., Vourloumis D. And Spandidos D.A. “Comparative CYP1A1 and CYP1B1 substrate and inhibitor profile of dietary flavonoids”, Bioorganic & Medicinal Chemistry, 19, 2842–2849 (2011).

Anwar-Mohamed A., Elbekai R.H. and El-Kadi A.O. “Regulation of CYP1A1 by heavy metals and consequences for drug metabolism”, Expert Opin Drug Metab Toxicol., 5(5), 501-521 (2009).

Arai T., Miyoshi Y., Kim S.J., Akazawa K., Maruyama N., Taguchi T., Tamaki Y. and Noguchi S. “Association of GSTP1 expression with resistance to docetaxel and paclitaxel in human breast cancers”, Eur J Surg Oncol., 34(7), 734-738 (2008).

Arunakaran J., Arunkumar R., Elumalai P. and Senthilkumar K., "Impact of Quercetin, Diallyl Disulfide and Nimbolide on the Regulation of Nuclear Factor Kappa B Expression in Prostate and Breast Cancer Cell Lines", Nat Prod Chem Res, 1,4, (2013).

Avramis V.I., Nandy P., Kwock R., Solorzano M.M., Mukherjee S.K., Danenberg P. and Cohen L.J. “Increased p21/WAF-1 and p53 protein levels following sequential three drug combination regimen of fludarabine,cytarabine and docetaxel induces apoptosis in human leukemia cells”, Anticancer Res., 18(4A), 2327-2338 (1998).

Baker S.D., Li J., ten Tije A.J., Figg W.D., Graveland W., Verweij J., Sparreboom A., " Relationship of systemic exposure to unbound docetaxel and neutropenia", Clin. Pharmacol. Ther., 77, 43–53, (2005).

Baker S.D., Sparreboom A., Verweij J., "Clinical pharmacoki-netics of docetaxel: recent developments" Clin. Pharmacokinet., 45, 235–252, (2006).

Baker S.D., Verweij J., Cusatis G., van Schaik R.H., Marsh S., Orwick S.J., Franke R.M., et al., Pharmacogenetic pathway analysis of docetaxel elimination. Clin. Pharmacol. Ther., in press, (2008).

Beaumont P.O., Moore M.J., Ahmad K, Payne M.M., Lee C. and Riddick D.S., "Role of Glutathione S-Transferases in the Resistance of Human Colon Cancer Cell Lines to Doxorubicin", Cancer Res, 58, 947-955 (1998).

Beer T.M., El-Geneidi M., Eilers K.M., "Docetaxel (taxotere) in the treatment of prostate cancer" Expert Rev Anticancer Ther., 3(3),261-8, (2003)

Blagosklonny M.V., Dixon S.C., Figg W.D., "Efficacy of microtubule-active drugs followed by ketoconazole in human metastatic prostate cancer cell lines", J Urol., 163(3), 1022-6, (2000).

Brodie A.M., Schwarzel W.C., Shaikh A.A., Brodie H.J., "The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer", Endocrinology, 100:1684, (1977).

Bock K.W., "Roles of U DP-Glucuronosyl transferases in Chemical Carcinogenesis", Critical Reviews in Biochemistry and Molecular Biology, 26(2), 129-150 (1991)

Bosch T.M., "Pharmacogenomics of drug-metabolizing enzymes and drug transporters in chemotherapy", Methods Mol Biol., 448, 63-76 (2008).

Bosch T.M., Huitema A.D., Doodeman V.D., Jansen R., Witteveen E., Smit W.M., Jansen R.L., van Herpen C.M., Soesan M., Beijnen J.H., Schellens J.H., "Pharmacogenetic screening of CYP3A and ABCB1 in relation to population pharmacokinetics of docetaxel" Clin.Cancer Res., 12, 5786–5793, (2006).

Bott S.R.J., Arya M., Shergill I.S. and Williamson M. "Molecular changes in prostatic cancer", Surgical Oncology 14, 91–104 (2005).

Boudny V. and Nakano S. "Src tyrosine kinase augments taxotere-induced apoptosis through enhanced expression and phosphorylation of Bcl-2", Br J Cancer, 86(3), 463-469 (2002).

Braunschweiger P.G., Kumar N., Constantinidis I., Wehrle J.P., Glickson J.D., Johnson C.S., Furmanski P. "Potentiation of interleukin 1 alpha mediated antitumor effects by ketoconazole", Cancer Res., 50(15), 4709-17 (1990).

Brooks J.D., Paton V.G. and Vidanes G. “Potent Induction of Phase 2 Enzymes in Human Prostate Cells by Sulforaphane”, Cancer Epidemiol Biomarkers Prev., 10(9), 949-954 (2001).

Bruno R.D. and Njar V.C.O. “Targeting cytochrome P450 enzymes: A new approach in anti-cancer drug development”, Bioorg Med Chem., 15(15),5047-5060 (2007).

Buhler D.R. and Williams D.E. “The role of biotransformation in the toxicity of chemicals”, Aquatic Toxicology, 11, 19-28 (1988).

Cantelli-Forti G., Hrelia P. and Paolini M. “The pitfall of detoxifying enzymes”, Mutation Research, 402, 179–183 (1998).

Chang T.K., Gonzalez F.J., Waxman D.J., "Evaluation of triacetyloleandomycin, alpha-naphthoflavone and diethyldithiocarbamate as selective chemical probes for inhibition of human cytochromes P450", Arch Biochem Biophys., 311(2):437-42, (1994).

Chauret N., Tremblay N., Lackman R.L., Gauthier J.Y., Silva J.M., Marois J., Yergey J.A. and Nicoll-Griffith D.A. “Description of a 96-Well Plate Assay to Measure Cytochrome P4503A Inhibition in Human Liver Microsomes Using a Selective Fluorescent Probe”, Anal Biochem., 276(2), 215-226 (1999).

Chen C., Zhou J. and Ji C. “Quercetin: A potential drug to reverse multidrug resistance”, Cancer Treat Res., 159, 185-205 (2014).

Chen J.H., He H.C., Jiang F.N., Militar J., Ran P.Y., Qin G.Q, Cai C, Chen X.B., Zhao J, Mo, Z.Y., Chen R.Y., Zhu J.G., Liu X. and Zhong W.D. “Analysis of the specific pathways and networks of prostate cancer for gene expression profiles in the Chinese population”, Med Oncol., 29(3), 1972-1984 (2012).

Clarke S.J., Rivory L.P., "Clinical pharmacokinetics of docetaxel" Clin. Pharmacokinet., 36, 99–114, (1999).

Crawford E.D., “Epidemiology of prostate cancer”, Urology, 62(Suppl 6A), 3–12 (2003).

Cree, I.A. “Cancer biology”, Methods Mol Biol,731, 1-11 (2011).

Cochin J. and Axelrod J., "Biochemical and pharmacological changes in therat following chronic administration of morphine, nalorphine, and normorphine", J Pharmacol Exp Ther, 125, 105-110 (1959).

Combalbert J., Fabre I., Fabre G., Dalet I., Derancourt J., Cano J.P. and Maurel P., "Metabolism of cyclosporin A. IV. Purification and identification of the rifampicin-inducible human liver cytochrome P-450 (cyclosporin A oxidase) as a product of P450IIIA gene subfamily", Drug Metab Dispos, 17, 197-207 (1989).

Cussenot O., Berthon P., Berger R., Mowszowicz I., Faille A., Hojman F., Teillac P., Le Duc A. and Calvo F. “Immortalization of human adult normal prostatic epithelial cells by liposomes containing large T-SV40 gene”, J Urol., 146(3), 881-886 (1991).

Çavdar Z., Yüksel Eğrilmez M., Bülbül M., Güner G, Koçtürk S., "Yüksek Performanslı Sıvı Kromatografisi İle Eritrositlerde Toplam ve İndirgenmiş Glutatyon Miktarının Tayini" Türk Biyokimya Dergisi, 31 (4), 187–193, (2006).

Damber J.E. and Aus G. “Prostate cancer”, Lancet, 371,1710–1721 (2008).

Das A.J., Chalil S., Nigam P., Magee P., Janneh O. and Owusu-Apenten R. "Glutathione transferase-P1-1 binding with naturally occurring ligands: assessment by docking simulations", Journal of Biophysical Chemistry, 2(4), 401-407 (2011).

DeCastro GJ, Jayram G, Razmaria A, Shalhav A, Zagaja GP. "Functional outcomes in African-Americans after robot-assisted radical prostatectomy" J Endourol., 26(8), 1013-9 (2012).

Dondi D, Limonta P., Moretti R.M.,. "Antiproliferative effects of luteinizing hormone-releasing hormone (LHRH) agonists on human androgen-independent prostate cancer cell line DU 145: evidence for an autocrine-inhibitory LHRH loop" Cancer Res,;54(15):4091-5, (1994)

Dorff T.B, Crawford E.D., "Management and challenges of corticosteroid therapy in men with metastatic castrate-resistant prostate cancer", Ann Oncol., 24(1):31-8 (2013).

Dozmorov M.G., Hurst R.E., Culkin D.J., Kropp B.P., Frank M.B., Osban J., Penning T.M. and Lin H.K. "Unique Patterns of Molecular Profiling Between Human Prostate Cancer LNCaP and PC-3 Cells", Prostate., 69(10),1077-1090 (2009).

Dumache R., Miclea F., Bumbacila B. and Puiu M. "Roles of glutathione-s-transferase P1 (GSTP1) gene, in prostate cancer detection", Jurnalul Pediatrului, 12, 47-48 (2009).

Duraj J., Zazrivcova K., Bodo J., Sulikova M. and Sedlak J. "Flavonoid quercetin, but not apigenin or luteolin, induced apoptosis in human myeloid leukemia cells and theirresistant variants", Neoplasma., 52(4), 273-279 (2005).

Engels F.K., Tije A.J.T., Baker S.D., Lee C.K.K., Loos W.J., Vulto A.G., Verweij J. and Sparreboom A. “Effect of Cytochrome P4503A4 Inhibition on the Pharmacokinetics of Docetaxel”, Clinical Pharmacology & Therapeutics, 75, 448-454 (2004).

Feldman BJ, Feldman D. “The development of androgen-independent prostate cancer” Nat Rev Cancer., 1(1), 34-45 (2001).

Figg W.D., Woo S., Zhu W., Chen X., Ajiboye A., Steinberg S.M., Price D.K., Wright J.J., Parnes H.L., Arlen P.M., Gulley J.L. and Dahut W.L. “A Phase I Clinical Study of High-Dose Ketoconazole Plus Weekly Docetaxel in Metastatic Castration-Resistant Prostate Cancer”, J Urol., 183(6), 2219–2226 (2010).

Finnström N., Bjelfman C., Söderström T.G., Smith G., Egevad L., Norlén B.J., Wolf C.R. and Rane A. “Detection of cytochrome P450 mRNA transcripts in prostate samples by RT-PCR”, European Journal of Clinical Investigation, 31, 880-886 (2001).

Fischer K., Da Costa L.A., García-Bailo B., Borchers C.H. and El-Sohemy A. “GlutathioneS-Transferase (GST) M1, but Not GSTT1, Genotype Influences Plasma Proteomic Profiles in Caucasian and East Asian Young Adults”, J Proteome Res., 11(10), 5022-5033 (2012).

Fizazi K, et al. Randomized phase II trial of denosumab in patients with bone metastases from prostate cancer, breast cancer, or other neoplasms after intravenous bisphosphonates. J Clin Oncol., 27(10):1564-71 (2009)

Forgue-Lafitte M.E., Coudray A.M., Fagot D. and Mester J. “Effects of Ketoconazole on the Proliferation and Cell Cycle of Human Cancer Cell Lines”, Cancer Res, 52, 6827-6831 (1992).

Freitas M., Baldeiras I., Proença T., Alves V., Mota-Pinto A., Sarmento-Ribeiro A., " Oxidative stress adaptation in aggressive prostate cancer may be counteracted by the reduction of glutathione reductase", FEBS Open Bio., 2, 119-28, (2012).

Fujimura T., Takahashi S., Urano T., Kumagai J., Murata T., Takayama K., Ogushi T., Horie-Inoue K., Ouchi Y., Kitamura T., Muramatsu M., Homma Y. and Inoue S. "Expression of Cytochrome P450 3A4 and Its Clinical Significance in Human Prostate Cancer", Urology., 74(2), 91-397 (2009).

Ganju A., Yallapu M.M., Khan S., Behrman S.W., Chauhan S.C., Jaggi M., "Nanoways to overcome docetaxel resistance in prostate cancer", Drug Resist Updat., 17(1-2),13-23 (2014)

Geng C.X., Zeng Z.C., Wang J.Y., Xuan S.Y. and Lin C.M. "Docetaxel shows radiosensitization in human hepatocellular carcinoma cells", World J Gastroenterol., 11(19), 2990-2993 (2005).

Granado-Serrano A.B., Martín M.A., Bravo L., Goya L. and Ramos S. "Quercetin induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI-3-kinase/Akt and ERK pathways in a human hepatoma cell line (HepG2)" J Nutr., 136(11), 2715-2721 (2006).

Goh B.C., Lee S.C., Wang L.Z., Fan L., Guo J.Y., Lamba J., Schuetz E., "Explaining interindividual variability of docetaxel pharma-cokinetics and pharmacodynamics in Asians through phenotyping and genotyping strategies" J. Clin. Oncol., 20, 3683–3690, (2002).

Habig W.H., Pabst M.J. and Jakoby W.B., "Glutathione-S-transferases: thefirst enzymatic step in mercapturic acid formation", J Biol Chem, 249, 7130–7139 (1974).

Haddad A.Q., Venkateswaran V., Viswanathan L., Teahan S.J., Fleshner N.E., Klotz L.H., " Novel antiproliferative flavonoids induce cell cycle arrest in human prostate cancer cell lines", Prostate Cancer Prostatic Dis., 9(1), 68-76, (2006).

Hadi M., Westra I.M., Starokozhko V., Dragovic S., Merema M.T., Groothuis G.M., "Human precision-cut liver slices as an ex vivo model to study idiosyncratic drug-induced liver injury", Chem Res Toxicol., 26(5), 710-20, (2013).

Haldar S., Basu A. and Croce C.M. "Bcl2 Is the Guardian of Microtubule Integrity", Cancer Res., 57,229-233 (1997).

He Q., Huang C., Zhao L., Feng J., Shi Q., Wang D. and Wang S. "α-Naphthoflavone inhibits 3T3-L1 pre-adipocytes differentiation via modulating p38MAPK signaling", Int J Clin Exp Pathol., 6(2), 168-178 (2013).

Heidenreich A., Bastian P.J., Bellmunt J., Bolla M., Joniau S., van der Kwast T., Mason M., Matveev V., Wiegel T., Zattoni F. and Mottet N. "EAU guidelines on prostate cancer. part 1: screening, diagnosis, and local treatment with curative intent-update 2013", Eur Urol., 65(1), 124-137 (2014).

Henderson C.J., McLaren A.W., Moffat G.J., Bacon E.J. and Wolf C.R. "Pi-class glutathione S-transferase: regulation and function" Chem Biol Interact., 111-112, 69-82 (1998).

Henrique R., Jerónimo C. "Molecular Detection of Prostate Cancer: A Role for GSTP1 Hypermethylation", European Urology, 46, 660–669 (2004).

Herbst R.S., and Khuri F.R. "Mode of action of docetaxel – a basis for combination with novel anticancer agents", Cancer Treatment Reviews, 29, 407-415 (2003).

Herling, A., Konig, M., Bulik, S., and Holzhutter, H.G., “Enzymatic features of the glucose metabolism in tumor cells” FEBS J., 278(14), 2436-2459 (2011).

Hlavna M., Raudenska M., Hudcova K., Gumulec J., Sztalmachova M., Tanhäuserova V., Babula P., Adam V., Eckschlager T., Kizek R. and Masarik M. “MicroRNAs and zinc metabolism-related gene expression in prostate cancer cell lines treated with zinc(II) ions”, Internetional Journal of Oncology, 41, 2237-2244 (2012).

Hokaiwado N., Takeshita F., Naiki-Ito A., Asamoto M., Ochiya T. and Shirai T. “GlutathioneS-transferase Pi mediates proliferation of androgen-independent prostate cancer cells”, Carcinogenesisvol., 29(6), 1134–1138 (2008).

Howard E.W., Lee D.T., Chiu Y.T., Chua C.W., Wang X., Wong Y.C., "Evidence of a novel docetaxel sensitizer, garlic-derived S-allylmercaptocysteine, as a treatment option for hormone refractory prostate cancer", Int J Cancer., 122(9), 1941-8, (2008).

Huerta A.S., Santos E.R., Campos G.B., Coloma C.S., Gómez O.N., “Pleuropulmonary and lymph node progression after docetaxel - benefits from treatment with cabazitaxel in metastatic prostate cancer” Case Rep Oncol., 6(2),391-6 (2013).

Jiang Z.Q., Chen C., Yang B., Hebbar V. and Kong A.N. “Differential responses from seven mammalian cell lines to the treatments of detoxifying enzyme inducers”, Life Sci., 72(20), 2243-2253 (2003).

Kadam C.Y., Abhang S.A., "Evaluatıon of serum levels of reduced glutathione, glutathione-S-transferase and nitric oxide in breast cancer patients undergoing adjuvant chemotherapy", IJCRR, 5(13), 51-57, (2013).

Kamataki T., Yokoi T., Fujita K. and Ando Y. “Preclinical approach for identifying drug interactions”, Cancer Chemother Pharmacol., 42, 50-53 (1998).

Kensler T.W. “Chemoprevention by Inducers of Carcinogen Detoxication Enzymes”, Environ Health Perspect., 105(Suppl 4), 965-970, (1997).

Komoroski B.J., Parise R.A., Egorin M.J., Strom S.C., Venkataramanan R., "Effect of the St. John’s wort constituent hyperforin on doc-etaxel metabolism by human hepatocyte cultures" Clin. Cancer Res., 11, 6972–6979, (2005).

Kook D., Wolf A.H., Yu A.L., Neubauer A.S., Priglinger S.G., Kampik A., Welge-Lüssen U.C., "The protective effect of quercetin against oxidative stress in the human RPE in vitro", Invest Ophthalmol Vis Sci., 49(4),1712-20, (2008).

Kumar G.N., Surapaneni S., “Role of drug metabolism in drug discovery and development” Med Res Rev.,21(5),397-411 (2001).

Lavrentiadou S.N., Chan C., Kawcak T., Ravid T., Tsaba A., van der Vliet A., Rasooly R. and Goldkorn T. “Ceramide-Mediated Apoptosis in Lung Epithelial Cells Is Regulated by Glutathione”, Am J Respir Cell Mol Biol., 25(6), 676-684 (2001).

Lee D.H., Szczepanski M. and Lee Y.J. “Role of Bax in quercetin-induced apoptosis in human prostate cancer cells”, Biochem Pharmacol., 75(12), 2345–2355 (2008).

Li Y., Li X., Hussain M. and Sarkar F.H. “Regulation of microtubule, apoptosis, and cell cycle-related genes by taxotere in prostate cancer cells analyzed by microarray”, Neoplasia., 6(2),158-167 (2004).

Li Y., Hussain M., Sarkar S.H., Eliason J., Li R. and Sarkar F.H. “Gene expression profiling revealed novel mechanism of action of Taxotere and Furtulon in prostate cancer cells”, BMC Cancer, 5, 7 (2005).

Li Y., Hong X., Hussain M., Sarkar S.H., Li R. and Sarkar F.H. “Gene expression profiling revealed novel molecular targets of docetaxel and estramustine combination treatment in prostate cancer cells”, Mol Cancer Ther., 4, 389-398 (2005).

Lo Bello M., Petruzzelli R., De Stefano E., Tenedini C., Barra D. and Federici G. “Identification of a highly reactive sulphydryl group in human placental glutathione transferase by a site-directed fluorescent reagent”, FEBS Lett., 263(2), 389-391 (1990).

Lundgren K., Andries M., Thompson C. and Lucier G.W. “α-Naphthoflavone Metabolized by 2,3,7,8-Tetrachlorodibenzo(*p*)dioxin-induced Rat Liver Microsomes: A Potent Clastogen in Chinese Hamster Ovary Cells”, Cancer Res., 47(14), 3662-3666 (1987).

Makhov P., Golovine K., Canter D., Kutikov A., Simhan J., Corlew M.M., Uzzo R.G., Kolenko V.M., " Co-administration of piperine and docetaxel results in improved anti-tumor efficacy via inhibition of CYP3A4 activity", Prostate, 72(6), 661-7, (2012).

Mannervik B., Danielson U.H. “Glutathione transferases--structure and catalytic activity”, CRC Crit Rev Biochem., 23(3), 283-337 (1988).

Marre F., Sanderink G.J., de Sousa G., Gaillard C., Martinet M. and Rahmani R. “Hepatic biotransformation of docetaxel (Taxotere) in vitro: involvement of the CYP3A subfamily in humans”, Cancer Res., 56(6), 1296-1302 (1996).

Mathijssen R.H. and van Schaik R.H. “Genotyping and phenotyping cytochrome P450: perspectives for cancer treatment.”, Eur J Cancer., 42(2), 141-148 (2006).

McFadyen M.C., Melvin W.T., Murray G.I., “Cytochrome P450 enzymes: novel options for cancer therapeutics” Mol Cancer Ther., 3(3), 363-71, (2004).

Miyoshi Y., Taguchi T., Kim S.J., Tamaki Y. and Noguchi, S., "Prediction of response to docetaxel by immunohistochemical analysis of CYP3A4 expression in human breast cancers" Breast Cancer., 12 (1), 11-15, (2005).

Murray G.I., Taylor V.E., McKay J.A., Weaver R.J., Ewen S.W., Melvin W.T. and Burke MD "The immunohistochemical localization of drug-metabolizing enzymes in prostate cancer", J Pathol., 177(2), 147-152 (1995).

Nardone P.A., Slotman G.J. and Vezeridis M.P. "Ketoconazole: a thromboxane synthetase and 5-lipoxygenase inhibitor with antimetastatic activity in B16-F10 melanoma", J Surg Res., 44(4),425-429 (1988).

Noda T., Iwakiri R., Fujimoto K. and Aw T.Y. "Induction of mild intracellular redox imbalance inhibits proliferation of CaCo-2 cells", Faseb J., 15(12), 2131-2139 (2001).

Obligacion R., Murray M. and Ramzan I. "Drug-metabolizing enzymes and transporters: expression in the human prostate and roles in prostate drug disposition", J Androl., 27(2), 138-150 (2006).

Ogu C.C. and Maxa J.L. "Drug interactions due to cytochrome P450", Proc (Bayl Univ Med Cent)., 13(4), 421-423 (2000).

Okamura K., Kizu R., Toriba A., Klinge C.M. and Hayakawa K., "Antiestrogenic activity of extracts of diesel exhaust particulate matter in MCF-7 human breast carcinoma cells", Polycyclic Aromatic Compounds, 22, 747–759 (2002).

Park J.S., Yamamoto W., Sekikawa T., Matsukawa M., Okamoto R., Sasaki M., Ukon K., Tanimoto K., Kumazaki T. and Nishiyama M. "Cellular sensitivity determinants to docetaxel in human gastrointestinal cancers", Int J Oncol., 20(2), 333-338 (2002).

Parkinson A., "Biotransformation of xenobiotics", In: Klaassen CD, editor. Casarett and Doull's Toxicology: The Basic Science of Poisons. 6th ed. New York, NY: McGraw-Hill; 2001. pp. 133–224.

Pasquier E., Honor´e S., Braguer D., "Microtubule-targeting agents in angiogenesis: where do we stand?" Drug Resist. Updates, 9, 74–86, (2006).

Petrylak D., "Therapeutic options in androgen-independent prostate cancer: building on docetaxel", BJU Int., 96(Suppl 2), 41-6, (2005).

Pienta K.J., Smith D.C., "Advances in prostate cancer chemotherapy: a new era begins", CA Cancer J Clin., 55(5), 300-18, (2005).

Pinto N., Dolan M.E., "Clinically relevant genetic variations in drug metabolizing enzymes", Curr Drug Metab., 12(5),487-97, (2011).

Polimanti R., Carboni C., Baesso I., Piacentini S., Iorio A., De Stefano G.F. and Fuciarelli M. "Genetic variability of glutathione S-transferase enzymes in human populations: functional inter-ethnic differences in detoxification systems", Gene, 512(1), 102-107 (2013).

Ploemen J.H., Van Schanke A., Van Ommen B. and Van Bladeren P.J. "Reversible conjugation of ethacrynic acid with glutathione and human glutathione S-transferase P1-1", Cancer Res., 54(4), 915-919 (1994).

Purnapatre K., Khattar S.K. and Saini K.S. "Cytochrome P450s in the development of target-based anticancer drugs", Cancer Lett., 259(1),1-15 (2008).

Psahoulia F.H., Drosopoulos K.G., Doubravska L., Andera L. and Pintzas A. "Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts", Mol Cancer Ther., 6(9), 2591-2599 (2007).

Puisset F., Chatelut E., Dalenc F., Busi F., Cresteil T., Azema J., Pou-blanc M., Hennebelle I., Lafont T., Chevreau C., Roche H., "Dexamethasone as a probe for docetaxel clearance" Cancer Chemother., Pharmacol., 54, 265–272, (2004).

Puisset F., Chatelut E., Sparreboom A., Delord J.P., Berchery D., Lochon I., Lafont T., Roche H., "Dexamethasone as a probe for CYP3A4 metabolism: evidence of gender effect" Cancer Chemother., Pharmacol., 60, 305–308, (2007).

Ricci G., Del Boccio G., Pennelli A., Lo Bello M., Petruzzelli R., Caccuri A.M., Barra D. and Federici G. “Redox Forms of Human Placenta Glutathione Transferase”, J Biol Chem., 266(32), 21409-21415 (1991).

Rochat, B., “Importance of influx and efflux systems and xenobiotic metabolizing enzymes in intratumoral disposition of anticancer agents” Curr Cancer, 2009, Drug Targets, 9,5, 652-674.

Rochlitz C.F., Damon L.E., Russi M.B., Geddes A. and Cadman E.C. “Cytotoxicity of ketoconazole in malignant cell lines”, Cancer Chemother Pharmacol., 21(4), 319-322 (1988).

Rodriguez-Antona C. and Ingelman-Sundberg M. “Cytochrome P450 pharmacogenetics and cancer”, Oncogene, 25, 1679–1691 (2006).

Rosing H., Lustig V., van Warmerdam L.J., Huizing M.T., ten Bokkel Huinink W.W., Schellens J.H., Rodenhuis S., Bult A., Beijnen J.H., "Pharmacokinetics and metabolism of docetaxel administered as a 1-h intravenous infusion", Cancer Chemother Pharmacol., 45(3),213-8, (2000).

Quiñones L.A., Irarrázabal C.E., Rojas C.R., Orellana C.E., Acevedo C., Huidobro C., Varela N.E. and Cáceres D.D. “Joint effect among p53, CYP1A1, GSTM1 polymorphism combinations and smoking on prostate cancer risk: an exploratory genotype-environment interaction study” Asian J Androl., 8(3), 349-355 (2006).

Salinas A.E. and Wong M.G. “Glutathione S-transferases--a review”, Curr Med Chem., 6(4), 279-309 (1999).

Schirmer M., Toliat M.R., Haber M., Suk A., Kamdem L.K., Klein K., Brockmoller J., Nurnberg P., Zanger U.M., Wojnowski L., "Genetic signature consistent with selection against the CYP3A4*1B allele in non-African populations" Pharmacogenet. Genom., 16, 59–71, (2006).

Schmidt M., Bastians H., "Mitotic drug targets and the development of novel anti-mitotic anticancer drugs" Drug Resist. Updates, 10, 162–181, (2007).

Schumacher M.C, Burkhard F.C., Thalmann G.N., Fleischmann A., Studer U.E., “Good outcome for patients with few lymph node metastases after radical retropubic prostatectomy”, *Eur Urol*., 54(2),344-52 (2008)

Sedlak I, Lindsay RH, "Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissues with Ellmann’s reagent", Anal Biochem, 25, 192-205,(1968).

Shaik A.P., Jamil K. and Das P. “CYP1A1Polymorphisms and Risk of Prostate Cancer-A Meta-analysis”, Urol J., 6, 78-86 (2009).

Shou M., Martinet M., Korzekwa K.R., Krausz K.W., Gonzalez F.J., Gelboin H.V., Role of human cytochrome P450 3A4 and 3A5 in the metabolism of taxotere and its derivatives: enzyme specificity, interindividual distribution and metabolic contribution in human liver", Pharmacogenetics, 8, 391–401, (1998).

Singal R., van Wert J. and Bashambu M. “Cytosine methylation represses glutathione S-transferase P1 (GSTP1) gene expression in human prostate cancer cells”, Cancer Res., 61(12), 4820-4826 (2001).

Stein C.A. “Mechanisms of action of taxanes in prostate cancer”, Semin Oncol., 26(5 Suppl 17), 3-7 (1999).

Sterling K.M., Cutroneo K.R., " Constitutive and inducible expression of cytochromes P4501A (CYP1A1 and CYP1A2) in normal prostate and prostate cancer cells", Cell Biochem., 91(2), 423-9, (2004).

Townsend D.M., Tew K.D. and Tapiero H. “The importance of glutathione in human disease”, Biomed Pharmacother., 57(3-4),145-55 (2003).

Tran A., Jullien V., Alexandre J., Rey E., Rabillon F., Girre V., et al., "Pharmacokinetics and toxicity of docetaxel: role of CYP3A, MDR1, and GST polymorphisms" Clin. Pharmacol. Ther., 79, 570–580, (2006).

Traverso N., Ricciarelli R., Nitti M., Marengo B., Furfaro A.L., Pronzato M.A., Marinari U.M., Domenicotti C., "Role of glutathione in cancer progression and chemoresistance", Oxid Med Cell Longev., (2013).

Tripathy D., " Capecitabine in Combination with Novel Targeted Agents in the Management of Metastatic Breast Cancer: Underlying Rationale and Results of Clinical Trials", The Oncologist, 12,375-389, (2007)

Tsuchida S., Kimura J., Hayakari M. and Ishikawa T. “Usefulness of glutathione S-transferase as a tumor marker”, Rinsho Byori., 45(12), 1125-1132 (1997).

Tzanakakis G.N., Agarwal K.C. and Vezeridis M.P. “Inhibition of hepatic metastasis from a human pancreatic adenocarcinoma (RWP-2) in the nude mouse by prostacyclin, forskolin, and ketoconazole”, Cancer., 65(3), 446-451 (1990).

van Bladeren P.J., van Ommen B. “The inhibition of glutathione S-transferases: mechanisms, toxic consequences and therapeutic benefits”, Pharmacol Ther., 51(1), 35-46 (1991).

van der Woude H., "Mechanisms of toxic action of the flavonoid quercetin and its phase II metabolites", ISBN 90-8504-349-2, (2006).

Van Iersel M.L., Ploemen J.P., Struik I., van Amersfoort C., Keyzer A.E., Schefferlie J.G. and van Bladeren P.J. “Inhibition of glutathione S-transferase activity in human melanoma cells by alpha,beta-unsaturated carbonyl derivatives. Effects of acrolein, cinnamaldehyde, citral, crotonaldehyde, curcumin, ethacrynic acid, and trans-2-hexenal”, Chem Biol Interact., 102(2), 117-132 (1996).

van Schaik R.H. “CYP450 pharmacogenetics for personalizing cancer therapy” Drug Resist Updat., 11(3):77-98 (2008).

van Zanden J.J., Ben Hamman O., van Iersel M.L., Boeren S., Cnubben N.H., Lo Bello M., Vervoort J., van Bladeren P.J. and Rietjens I.M. “Inhibition of human glutathione S-transferase P1-1 by the flavonoid quercetin”, Chem Biol Interact., 145(2), 139-148 (2003).

van Zanden J.J., Geraets L., Wortelboer H.M., van Bladeren P.J., Rietjens I.M., Cnubben N.H.," Structural requirements for the flavonoid-mediated modulation of glutathione S-transferase P1-1 and GS-X pump activity in MCF7 breast cancer cells", Biochem Pharmacol., 67(8), 1607-17, (2004).

Vijayababu M.R., Arunkumar A., Kanagaraj P., Venkataraman P., Krishnamoorthy G. and Arunakaran J. “Quercetin downregulates matrix metalloproteinases 2 and 9 proteins expression in prostate cancer cells (PC-3)”, Mol Cell Biochem., 287(1-2), 109-116 (2006).

Volate S.R., Davenport D.M., Muga S.J. and Wargovich M.J. “Modulation of aberrant crypt foci and apoptosis by dietary herbal supplements (quercetin, curcumin, silymarin, ginseng and rutin)”, Carcinogenesis, 26(8), 1450-1456 (2005).

von Moltke L.L., Greenblatt D.J., Schmider J., Wright C.E., Harmatz J.S. and Shader R.I. “In Vitro Approaches to Predicting Drug Interactions In Vivo”, Biochemical Pharmacology, 55, 113–122 (1998).

Walsh A.A., Szklarz G.D. and Scott E.E. “Human cytochrome P450 1A1 structure and utility in understanding drug and xenobiotic metabolism” J Biol Chem., 288(18), 12932-12943 (2013).

Wang R.W., Newton D.J., Scheri T.D., Lu A.Y., "Human cytochrome P450 3A4-catalyzed testosterone 6 beta-hydroxylation and erythromycin N-demethylation: Competition during catalysis", Drug Metab Dispos, 25, 502-507 (1997).

Vasaitis T.S., Bruno R.D., Njar V.C., “CYP17 inhibitors for prostate cancer therapy”, J Steroid Biochem Mol Biol., 125(1-2):23-31 (2011).

Yamamoto N., Tamura T., Murakami H., Shimoyama T., Nokihara H., Ueda Y., et al., "Randomized pharmacokinetic and pharmacody-namic study of docetaxel: dosing based on body-surface area compared with individualized dosing based on cytochrome P450 activity estimated using a urinary metabolite of exogenous cortisol" J. Clin. Oncol., 23, 1061–1069, (2005).

Yap T.A., Carden C.P., Attard G., de Bono J.S., “Targeting CYP17: established and novel approaches in prostate cancer” Curr Opin Pharmacol., 8(4):449-57 (2008).

Yılmaz H.H., Yazıhan N., Tunca D., Sevinç A., Olcayto E.Ö., Özgül N. and Tuncer M. “Cancer trends and incidence and mortality patterns in Turkey” Jpn J Clin Oncol. 41(1):10-6 (2011).

Yu E.Y., Hahn W.C. “Genetic alterations in prostate cancer", Clin Prostate Cancer., 3(4), 220-229 (2005).

Zdařilová A., Vrzal R., Rypka M., Ulrichova J., Dvorá´k Z., " Investigation of sanguinarine and chelerythrine effects on CYP1A1 expression and activity in human hepatoma cells", Food and Chemical Toxicology, 44, 242–249, (2006)

Zeigler-Johnson C.., Friebel T., Walker A.H., Wang Y., Spangler E., Panossian S., Patacsil M., Aplenc R., Wein A.J., Malkowicz S.B., Rebbeck T.R.., "CYP3A4, CYP3A5, and CYP3A43 genotypes and haplotypes in the etiology and severity of prostate cancer" Cancer Res., 64(22), 8461-7, (2004).

http://drugtopics.modernmedicine.com/drug-topics/news/clinical-advancements-treating-breast-cancer?page=full (Last axcess: 21.09.2014).

http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf (Last axcess: 21.09.2014).

http://digital.csic.es/bitstream/10261/60709/4/Quercetin%20modulates %20Nrf2.pdf (Last axcess: 21.09.2014).

## CURRICULUM VITAE

### PERSONAL INFORMATION


Name : DERYA DİLEK KANÇAĞI

Address :Middle East Technical University, Department of Biological Sciences

Telephone :+903122105196

Fax :+903122107976

E-mail :deryadilek1983@gmail.com


Nationality :Republic of Turkey

Date of birth :27.06.1983

MaritalStatus : Married

### EDUCATION AND TRAINING

PhD (2008 - 2014): Middle East Technical University, Department of Biochemistry

M.S (2006 - 2008): Middle East Technical University, Department of Biology

B.S (2001 - 2006): Middle East Technical University, Department of Biology

### WORK EXPERIENCE

| | | |
|---|---|---|
| Medical Manager | (2014-present) | Ghealthcare (Graphinn) Medical Agency |
| Medical Advisor | (2010-2014) | Ghealthcare (Graphinn) Medical Agency |
| Research Assistant | (2007-2010) | Middle East Technical University, Department of Biological Sciences |

### FOREIGN LANGUAGES

Advanced English

## SCHOLARSHIPS & AWARDS

| | |
|---|---|
| High Performance Award for PhD. (**METU**) | :2010 |
| High Performance Award for M.Sc. (**METU**) | :2008 |
| Dean's Honor List ( **METU**) | :2002-2003 (spring – fall) |
| | :2003-2004 (spring-fall) |
| | :2005- 2006 (spring-fall) |
| Dean's High Honor List (**METU**) | :2005 (fall) |
| **TUBİTAK** Scholorship for Master Student | **:** 2006-2008 |
| **TUBİTAK** Scholorship for Doctorate Student | **:** 2009-2013 |

## LICENCES & CERTIFICATES

**XX.Ulusal Biyokimya Kongresi,2008**
Türk Biyokimya Derneği, Kapadokya-4 days

**EMBO Young Scientists Forum**

Boğaziçi University,İstanbul (2008)-3days

**Poster**: Effects of NAT2 and GSTM1 and GSTT1 Polymorphism on Prostate Cancer Development

**DNA Damage, Repair and its Relation with Diseases**

9 Eylül University,İstanbul, (2007)-3 days

**FEBS 2006,** Federation of European Biochemical Societies, İstanbul(2006)-4 days

**High Dose Chemotherapy and Hematopoietic Stem Cell Banking**

Gulhane Military Medical Academy, Ankara-1 day

## RESEARCH TOPICS

Drug metabolism, multi drug resistance, cancer genetics, gene profiling (polymorphism)

## MEMBERSHIPS

Turkish Biochemical Society

## POSTER PRESENTATIONS

**Dilek D**, Guray T, Başal Ş (2008), Association between NAT Polymorphism and Tendency to Prostate Cancer Development. 20th National Biochemistry Congress (29 Oct 1 Nov 2008, Nevşehir, Turkey) Turkish Journal of Biochemistry.

**Dilek D**, Guray T (2008), Effects of NAT2 and GSTM1 and GSTT1 Polymorphism on Prostate Cancer Development. EMBO Young Scientists Forum (20-22 Feb. 2008, İstanbul, Turkey).

## BOOK CHAPTERS

Kançağı Dilek D., Öğülür İ. Molecular Biology. Chapter 15. Nucleic Acids: Isolation, Purification, Determination and Hybridization.ISBN: 978-605-88012-07-1.