INVESTIGATION OF THE EFFECTS OF COMBINE USE OF CISPLATIN AND METFORMIN ON PROLIFERATION OF PROSTATE CANCER CELL LINES AND GSTP, CYP17A1 AND HEXOKINASE II ENZYMES

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ABSTRACT

INVESTIGATION OF THE EFFECTS OF COMBINE USE OF CISPLATIN AND METFORMIN ON PROLIFERATION OF PROSTATE CANCER CELL LINES AND GSTP, CYP17A1 AND HEXOKINASE II ENZYMES

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Prostate cancer is the most common cancer type both in developed and developing countries. Since 2015, it has been seen as one of the main cause of death in males, both in the world and in our country. Current therapeutic approaches for prostate cancer generally have variable efficiency, develop metastasis and drug-resistance, and have high toxicity to normal tissues. Hence, the searching for more effective strategies with moderate or any adverse effects for the chemopreventive intervention of those cancers remains one of the important issues in cancer research. Prostate cancer incidence and mortality vary dramatically by geographical location. Both are higher in developed countries. Some attribute this to westernized lifestyles of high energy diets and limited physical activity with consequent obesity. This

dramatic increase in the incidence of cancer in overweight population suggests that there is a correlation between obesity and cancer. As a matter of fact, epidemiological and experimental research indicates that men with abdominal obesity is a risk factor for prostate cancer. Because in the case of obesity, people store plenty of glucose. Thus, cancer cells are more easily and quickly divide. According to this information, our aim in this study was reducing cell proliferation by reducing the blood glucose amount that passes from hepatic tissues by using metformin, a Type2 diabetes drug, hence provide the induction of cell death by creating oxidative stress in cells. Cisplatin, a cell division and growth inhibiting (antineoplastic) agent, is widely used in the treatment of prostate cancer.

Cisplatin binds to DNA and forms a DNA adduct. This adduct cause the death of cancer cells by inhibiting transcription and/or replication. Despite the high efficiency of cisplatin on prostate cancer treatment not only nephrotoxicity, neurotoxicity and gastrointestinal irritability but also azoospermia, oligospermia and infertility on reproductive age restrict the use of cisplatin. In order to reduce the side effects of cisplatin, combination therapy with other agents has been the focus of scientists in recent years. Considering the inducer effect of metformin, a type2 diabetic drug, on antiproliferative and apoptotic pathways it can provide a complementary treatment with antineoplastic agents, including cisplatin.

GST family enzymes function in the detoxification of xenobiotics, including drugs. Becide, recently it is understood that GST's also function on cell proliferation and death that controls signal transduction. Carcinogenesis, differentiation, growth and important impact on drug resistance and cell death serve GST's as an important drug targets. GST family is also involved in prostaglandin, steroid and leukotriene biosynthesis. In addition, to determination of GTSP protein and gene expression to investigate the effect of combined treatment on the role of androgen synthesis, expression of

CYP17A1 enzyme was determined in the cell lines. Furtermore, the effect of this combined treatment on hexokinase enzyme which plays an important role in the glycolysis pathway was investigated. Dose response of this combined therapy and cytotoxic effect of those drugs were measured by Alamar Blue assay. The effects of cisplatin and metformin on proliferation of cancer cell lines were detected by colony formation assay. Also, the effects of drugs on CYP17A1 GSTP and Hexokinase II enzymes will be detected by protein and mRNA expressions by using Western-Blot and qRT-PCR techniques, respectively. IC50 value of cisplatin was determined as 17µM for LNCaP and 30 µM for PC3 cell line. The cell proliferation results showed that, metformin potentiates the antiproliferative effect of cisplatin both in LNCaP and PC3 cell lines. However, according to Western-blot and qPCR analysis, this reduction can't be blamed by GSTP enzymes. Expression of GSTP enzyme increased in direct proportion to dose of metformin. At this point, metformin may antagonize the cisplatin apoptotic effect through suppression of oxidative stress in prostate cancer cells. On the other hand, cisplatin-metformin drug combination significantly decreased the CYP17A1 protein expressions in PC3 cell lines. Furthermore, Hexokinase II mRNA expressions are significantly decrease in a dose dependent manner. In the treatment of cancer, which is frightening diseases of our century, combined therapy of diabetes based drugs with chemotherapeutic drugs will be helpful not only to reduce the toxicity of the of cisplatin, but also to reduce the cost.

Keywords: Metformin, cisplatin, apoptosis, GSTP, Cytochrome P450, CYP17A1, Hexokinase II, prostate cancer

ÖZ

CİSPLATİN VE METFORMİN İİLAÇ KOMBİNASYONU UYGULAMASININ PROSTAT HÜCRE HATLARI PROLİFERASYONU VE GSTP, CYP17A1 VE HEKZOKİNAZ II ENZİMLERİ ÜZERİNE ETKİLERİNİN İNCELENMESİ

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Prostat kanseri, gelişmiş ve gelişmekte olan ülkelerde en sık görülen kanser türlerinden biri olup 2015 yılından itibaren hem dünyada hem de ülkemizde erkeklerde ilk sıradaki kanser kökenli ölüm nedenlerinden biri olarak görülmektedir. Günümüzde prostat kanseri tedavisindeki yaklaşımların yeterliliği değişken olmakla beraber bu yaklaşımlar metastaz gelişimine, ilaç direnci oluşmasına ve sağlıklı dokularda toksik etkilere sebep olabilmektedir. Bu nedenle tedavide daha etkili ancak daha az yan etkiye sahip kanser önleyici molekülleri tespit etmek kanser araştırmalarının önemli bir kısmını oluşturmaktadır. Ayrıca prostat kanserinin coğrafik lokalizasyonuna bakıldığında, batılı ülkelerde görülme sıklığının daha fazla olduğu tespit edilmiştir. Batılı yaşam tarzının getirdiği dezavantajlar olan yüksek yağ içeren diyetler (fast food alışkanlıkları) ve limitli fiziksel aktivite gibi durumlar obezite prevalansını arttırmaktadır. Aşırı kilolu popülasyonun

kanser insidansındaki bu dramatik artış obezite ve kanser arasında bir korelasyon olduğunu düşündürmektedir. Nitekim yapılan epidemiyolojik ve deneysel araştırmalar, erkeklerde abdominal obezitenin prostat kanseri için bir risk faktörü olduğunu belirtmektedir. Çünkü obezite durumunda kişi bol miktarda glikoz almakta olup kanser hücrelerinin daha kolay ve hızlı bölünmelerine neden olurlar. Bu bilgiler doğrultusunda, yapılması tasarlanan çalışmadaki amaçlardan biri Tip2 diyabet ilacı olan metformin kullanılarak, karaciğer dokularından kana geçen glikoz miktarını azaltarak hücre proliferasyonunu yavaşlatmak ve böylece hücre içerisinde oksidatif bir stres oluşturarak hücre ölümünün indüklenmesini sağlamaktır. Cisplatin prostat kanseri tedavisinde sıklıkla kullanılan antineoplastik platin bileşiği bir ajandır. Cisplatin DNA'ya bağlanarak bir kompleks oluşturur böylece normal yapısı bozularak bükülmüş DNA zinciri, normal transkripsiyonu ve/veya replikasyonunu engelleyerek kanser hücresinin ölümüne neden olur. Ancak, cisplatin yüksek etki derecesine karşın nefrotoksisite veya nörotoksisite gibi yan etkiler ile üreme çağındaki erkeklerde oligospermi, azospermi ve infertilite gelişmesine neden olmakta ve bu önemli sorunlarda ilacın kullanımını kısıtlamaktadır. Bu yüzden cisplatinin yan etkilerini azaltmak amacıyla başka ajanlar ile kombine terapiler son yıllarda bilim insanlarının ilgi odağı olmuştur. Tip 2 diyabet ilacı olan metforminin anti-proliferatif ve apoptotik yolakları indükleyici etkisi göz önünde bulundurulduğunda, antineoplastik ajan cisplatin ile tamamlayıcı bir tedavi sunabileceği açıktır. Tasarlanan çalışmada bu iki ilacın kombine kullanımının hücrede apoptoz ve detoksifikasyon enzimleri olarak bilinen Glutatyon-S Transferaz (GST) izozimleri üzerine etkilerine bakılmıştır. GST enzim ailesinin en bilinen özelliği ksenobiotiklerin detoksifikasyonunu sağlamalarıdır; ancak GST lerin son zamanlarda anlaşılmaya başlanan, hücre proliferasyonunu ve ölümünü kontrol eden sinyal transdüksiyon yolakları üzerine yapılmış çalışmalar yeterince mevcut değildir. Kanser oluşumu, büyümesi, ilaç direnci ve hücre ölümü üzerindeki bu önemli etkileri GST enzimlerini önemli ilaç hedefi

yapmaktadır. Ayrıca, cisplatin-metformin kombine kullanımının androjen sentezinde fonksiyonu olan CYP17A1 enzimi ile glikoliz yolağında önemli rol oynayan hekzokinaz II üzerine etkisi incelenmiş olup, ilaçların hücreler üzerindeki sitotoksik etkileri "alamar blue" analizi ile ölçülmüştür. Metformin ve cisplatin'in androjen sentezinde rol oynayan CYP17A1 ve apoptotik yolaklarda etkili olduğu keşfedilen GST enzimleri ile glikoliz yolağında önemli fonksiyonu olan hekzokinaz II' nin protein ve mRNA ekspresyonlarına olan etkisi sırasıyla western-blot ve qRT-PCR teknikleriyle tespit edilmiştir. Yapılan deneyler sonucunda, cisplatin-metformin ilaç kombinasyonunun hücre proliferasyonunu durdurucu özelliği olduğu her iki hücre hattında da ortaya konulmuştur. Ancak yapılan qPCR ve western blot sonuçları göstermiştir ki, bu antiproliferatif etki GSTP1 enzimleri ile ilişkilendirlendirilememektedir. Aksine, hücrelere verilen ilaç dozunun artışıyla doğru orantılı olarak GSTP1 protein expresyonu artmıştır. Bu da metforminin cisplatin ilacının hücre içinde oluşturduğu oxidatif stresi detoksifiye ettiği yönünde düşünülmesine neden olmuştur. Aynı zamanda CYP17A1 enzim expresyonunun PC3 hücre hattında anlamdı derecede azaldığı saptanmıştır. Hexokinase II gen expresyonu her iki hücre hattında da azalmıştır. Sonuç olarak hücre proliferasyonunun azalması, HKII geninin ekspresyonundaki düşüş ile ilişkilendirilebilir. Yüzyılımızın korkutucu hastalığı olan kanserin tedavisinde, diyabet temelli ilaçların, kemoterapötik ilaçlar ile kombine kullanımının etkisinin ilaç toksisitesini indirgemek ve kemoterapötik tedavinin maliyetini düşürmek açısında faydalı olacağını ümit ediyoruz.

Anahtar Kelimeler: Metformin, cisplatin, apoptosis, GST, Cytochrome P450, CYP17A1, Hexokinase II, prostate cancer

To My Grandmother,

For her endless support and love

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TABLE OF CONTENTS

ABSTRACT v

ÖZ viii

ACKNOWLEDGEMENTS xii

TABLE OF CONTENTS xiii

LIST OF TABLES xvii

LIST OF FIGURES xix

LIST OF SYMBOLS AND ABBREVIATIONS xxii

CHAPTERS

1. INTRODUCTION……………………………………………………..1

1.1 Prostate Cancer 1

1.1.1 Cisplatin 2

1.1.2 Metformin………………………………………………………6

1.2 Aim of the Previous and Present Study………………………………..8

1.2.1 Enzymes Determined in this Study………………………………10.

1.2.1.1 CYP17A1……………………………………………………..10

1.2.1.2 Glutathione-S-Transferases (GSTs)……………..………………13

1.2.1.2.1 Regulatory Role of GSTP Enzyme………………………15

1.2.1.3 Isozymes of Mammalian Hexokinase……………………..…17

1.2.1.3.1 Hexokinase II (HKII)……………………………………19

1.3 Aim of the Study…..……………………...……………………21

MATERİALS AND METHODS…………………...…………………...23

2.1 Materials……………………………………………………..23

2.1.1 Cell Line……….……………………………………………23

2.1.2 Chemicals and Materials……..……………………………………23

2.2 Methods………..…………………………………………..25

2.2.1 Cell Culture……..…………………………………………..25

2.2.1.1 Cell Culture Conditions…………………….…..……………25

2.2.1.2 Cell Thawing………………………..…………………….…..26

2.2.1.3. Subculturing the Cell Lines…………………………………26

2.2.1.4 Cell Freezing…………………………………..………..27

2.2.1.5 IC_{50} Determination for Cisplatin……………………………..27

2.2.1.6 Treatment with Metformin……………………………...…28

2.2.2 Protein Extraction……………………………………...……29

2.2.3 Determination of Protein Concentration…...................................30

2.2.4 Determination of Protein Expression……………………………..32

2.2.4.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)……………………………………………………………….32

2.2.4.2 Western Blotting…………………………………………..36

2.2.5 Determination of mRNA Expression……………………………..39

2.2.5.1 Isolation of Total RNA From Cell Lines……………………...39

2.2.5.2 Determination of RNA Concentration……………..………40

2.2.5.3 Qualification of RNA Molecules by Agarose Gel Electrophoresis 40

2.2.5.4 cDNA Synthesis 41

2.2.5.5 Quantitative Real-Time PCR 41

2.3 Statistical Analysis... 42

RESULTS……………………………………………………………….45

3.1. Cell Culture.. 45

3.1.1 IC50 Determination of Cisplatin..……………………….……....45

3.1.2 Determination of Cisplatin-Metformin Combined Effect……….50

3.2. Protein Expression Analysis of CYP17A1 and GSTP Enzymes in LNCaP and PC3 Cells…………………………..………………………54

3.2.1 CYP17A1 Protein Expressions in the Control and Treated LNCaP and PC3 Cells………………………………………...………………..….54

3.2.2 GSTP1 Protein Expression in the Control and Treated LNCaP and PC3 Cells…………………………………………………………………..59

3.3 CYP17A1, GSTP1 and Hexokinase II mRNA Expressions in the Control and Treated LNCaP Cell Lines……………………….…………63

3.3.1 CYP17A1 mRNA Expression in the Control and the Treated LNCaP and PC3 Cell Lin……………………..………………………..……………66

3.3.2 GSTP1 mRNA Expression in the Control and the Treated LNCaP and PC3 Cells……………………………………..……………..…68

3.3.3 Hexokinase II mRNA Expression in the Control and the Treated LNCaP and PC3 Cells……………………..………………………...…..71

DISCUSSION...75

CONCLUSION……………………………...………………………… 81

REFRENCES 83

APPENDİX 99

LIST OF TABLES

TABLES

Table 2.1 Growth medium conditions of cell lines.....................................26

Table 2.2 Constituents of seperating and stacking gel solutions. 33

Table 2.3 Dilutions of Primary and Secondary antibodies 39

Table 2.4 Primer sequences, annealing temperatures and product size of the genes…………… 43

Table 3.1 Percent AB reduction and and percent survival values of cisplatin treated LNCaP cells……………...46

Table 3.2 Percent AB reduction and and percent survival values of cisplatin treated PC3 cells……………..47

Table 3.3 Percent AB reduction and and percent survival values of cisplatin/metformin treated LNCaP cells……………………………......…51

Table 3.4 Statistical analysis of CYP17A1 protein expressions in LNCaP cells…………...56

Table 3.5 Statistical analysis of CYP17A1 protein expressions in PC3 cells…………………………………………………………………...58

Table 3.6 Statistical analysis of GSTP1 protein expressions in LNCaP cells…………………………………………………………………...61

Table 3.7 Statistical analysis of GSTP1 protein expressions in PC3 cells…………………………………………………………………...63

Table 3.6 The Livak method for the calculation of relative mRNA expressions using CT values...65

Table 3.9 Statistical analysis of GSTP mRNA exprssions ın PC3 cell line………………………………………………………….…...70

Table 3.10 Statistical analysis of Hexokinase II mRNA exprssions ın LNCaP cell line………… ..…………………………………………………...72

Table 3.11 Statistical analysis of Hexokinase II mRNA exprssions ın PC3 cell line………… ..…………………………………………………...74

LIST OF FIGURES

FIGURES

Figure 1.1 Structure of Cisplatin……………………………………………2

Figure 1.2 Cisplatin DNA adducts...3

Figure 1.3 Chemical formula of metformin..6

Figure 1.4 Galega officinalis……………..6

Figure 1.5 Derivatives of cisplatin...9

Figure 1.6 Human major seroidogenic pathway...11

Figure 1.7 Glutathione conjugation to a xenobiotic by GST's………………13

Figure 1.8 3D structure of GST enzyme..14

Figure 1.9 GSTP1 and regulation of kinase pathway..................................16

Figure 1.10 Phosphorylation, catalysed by Hexokinase …………………18

Figure 1.11 Hexokinase II and it's major partners in cancer promotion......20

Figure 2.1 Schematic representation of treatment……..............................29

Figure 2.2 Western blot sandwich. 38

Figure 3.1 Cell proliferation graph for cisplatin treated LNCaP cells..........48

Figure 3.2 Percent Survival graph for cisplatin treated LNCaP cells………48.

Figure 3.3 Cell Proliferation graph for cisplatin treated PC3 cells……..... 49

Figure 3.4 Percent Surival graph for cisplatin treated PC3 cell………….. .49

Figure 3.5 Cell proliferation graph for cisplatin/metformin treated LNCaP cell……………….. 53

Figure 3.6 P Cell proliferation graph for cisplatin/metformin treated PC3 cell……………….. 53

Figure 3.7 Immunorective CYP17A1 protein bands of LNCaP cells 55

Figure 3.8 Comparison of CYP17A1 protein expressions of LNCaP cells……………………………………………………………….....55

Figure 3.9 Immunorective CYP17A1 protein bands of PC3 cells 57

Figure 3.10 Comparison of CYP17A1 protein expressions of PC3 cells.....57

Figure 3.11 Immunorective GSTP1 protein bands of LNCaP cells 60

Figure 3.12 Comparison of GSTP1 protein expressions of LNCaP cells 60

Figure 3.13 Immunorective GSTP1 protein bands of PC3 cells…………...62

Figure 3.14 Comparison of GSTP1 protein expression of PC3 cells………62

Figure 3.15 Comparison of CYP17A1 mRNA expression of LNCaP cell lines……………...66

Figure 3.16 Comparison of CYP17A1 mRNA expression of PC3 cell lines...67

Figure 3.17 Comparison of GSTP1 mRNA expression of LNCaP cell lines…………..…………………………………………………………68

Figure 3.18 Comparison of GSTP1 mRNA expression of PC3 cell lines……………………………………………………………………...69

Figure 3.19 Comparison of HKII mRNA expression of LNCaP cell lines……………………………………………………………………...71

Figure 3.20 Comparison of HKII mRNA expression of PC3 cell lines……………………………………………………………………...73

.

LIST OF SYMBOLS AND ABBREVIATIONS

APS	Ammonium per sulfate
BCIP	5-bromo 4-chloro 3-indoyl phosphate
BSA	Bovine serum albumin
BCA	Bicinchoninic acid
cDNA	Complementary DNA
CRPC	Castration-resistant prostate cancer
Ct	Threshold cycle
CYP	Cytochrome P450
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
ERB	Electronic running buffer
FBS	Fetal bovine serum
GST	Glutathione S-transferase
HK	Hexokinase
kDa	kilo Dalton
mRNA	Messenger RNA
NBT	Nitrotetrazolium blue chloride
PBS	Phosphate buffered saline
PC	Prostate cancer
PCR	Polymerase chain reaction
Pen-Strep	Penicillin-Streptomycin
PMSF	Phenylmethylsulfonyl fluoride
rpm	Revolutions per minute

RNA Ribonucleic acid

SDB Sample dilution buffer

SDS Sodium dodecyl sulfate

SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TBST Tris-buffered saline and Tween 20

TEMED Tetramethylethylenediamine

CHAPTER 1

INTRODUCTION

1.1 Prostate Cancer

Prostate cancer (PCa) is the second most prevalent cancer and the sixth leading cause of cancer death in male population. Boyle and his group, 2015, showed that PCa is the most common cancer type, with an incidence rate of 214 cases per 1000 men (Boyle P., et. al., 2005). In addition to Europe, studies about Turkish population suggest that, in male population, PCa is the second common solid neoplasm after lung cancer in male population (Yılmaz H., et. al., 2010).

Androgen Deprivation Therapy (ADT) seems to be the first line treatment strategy for men with PC. Unfortunately, ADT is rarely curative because only majority of ADT responders will become castration-resistant disease (Eisenberger M.A., et. al., 1998; Eltayb A., et. al., 2005).

Than this disease will be fatal if the patients become untreated castration resistant prostate cancer ((CRPC) (Eisenberger M.A, et. al., 1998; 1985). To overcome CRPC, different treatment approaches were tested, such as investigation of new drug combinations or new doses of standart therapies. Accordingly, cisplatin is one the promising drug to treat some of the prostate cancers.

1.1.1 Cisplatin

Cisplatin (cDDP), is a metallic platinum compound as given in Figure 1.1. Cisplatin, is one of the most potent chemotherapeutic agent. For many years its activity has been demonstrated against several types of tumors (Loehrer P.J., et.al., 1984).

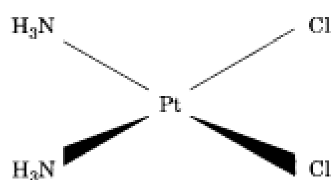

Figure 1.1. Structure of cisplatin (The chemical book database, 2014).

However, how cDDP enters the cells still unclear, a model of passive diffusion across the membrane has generally been accepted (Jung & Lippard, 2007; Kelland L., 2007). Interraction of this reactive electrophilic compound with nucleophilic substances most commonly purine nucleotides and thiol groups of proteins have been reported (Yang D. Z. et.al., 1997). Formation of the bulky adducts with DNA and/ or proteins hinders the normal cellular function and DNA repair pathways, stress signalling is activated and eventually, apoptosis will be occured.

Although cisplatin can react with the different components of the cell such as membranes, proteins and RNA, its major intracellular target is DNA (Roberts J. M., et. al, 1986). It has been shown that, cDDP exerts its cytotoxic effect by forming cisplatin-DNA adducts in the major groove of the DNA. cDDP covalently bond with the N7 residue of purine bases; frequently occured at the atoms of imidazole rings of guanine and adenine (Ahmad, 2010).

Due to reaction of cisplatin with DNA, structurally different adducts can be formed (Figure 1.2). The most common products are the intra-strand crosslinks 1, 2-guanine-guanine (~65 %) and 1,2-adenine-guanine (~25 %), which may block the replication and/or prevent transcription (Payet D., et.al., 1993). Inter-strand links between two guanine nucleotides are the only accounts for 1% of total adducts (Ahmad, 2010; Eastman, 1999; Siddik, 2003).

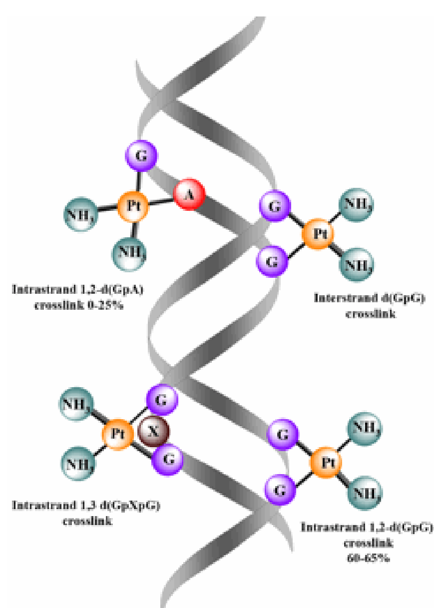

Figure 1.2. Cisplatin-DNA adducts (Boulikas T., et. al., 2007).

Activated cDDP can also react with the other amino acids, microfilaments and the abundant tripeptide glutathione (GSH) (Appleton, 1999). Despite low amount of cDDP (5% of the total cDDP in the cell) may associate with DNA (Fuertes M. A., et.al., 2003; Mandic A., et.al., 2003), active cisplatin can't be fully react with these celular components before reaching DNA. Because

these interactions are kinetically slow, and the reaction with guanine bases thermodynamically suitable (Jung & Lippard, 2007; Reedijk J., 1999).

Cisplatin inhibits DNA replication and chain elongation, by binding to DNA (Eastman A., 1990). It is believed that main reason of the antitumour effects of cisplatin is because of its inhibitory effects on DNA synthesis (Anthoney D. A., et al., 1996).

Although cisplatin often leads to an initial therapeutic success, resistance against cisplatin still a major clinical problem. Different potential mechanisms can be blamed for cisplatin resistance. These mechanisms can be divided into 2 categories those that responsible for reduction of DNA adducts and minimize the cytotoxic effect of drug (Johnson S., et al., 1994). First of them is direct detoxification by detoxification enzymes, such as GST's. Resulting in the decreasing in the drug accumulation and increasing in the drug efflux (Andrews P. A. & Howell S. B., 1990; Timmer-Bosscha H., et al., 1992; Johnson S. W., et al., 1994). The second one is, direct repairing of DNA damage by important molecular defence system against DNA adducts (Andrews P. A. & Howell S. B., 1990; Timmer-Bosscha H., et al., 1992; Johnson S. W., et al., 1994).

Different kind of human cancers, including colon, breast, liver, kidney, prostate, lung and ovarian, usually express high levels of Glutathione S-Transferases (GSTs) relative to other tissues (Tidefelt U., et al., 1992). It is not surprising that, GSTs have a role in the development of drug resistance by detoxification process (Tew K.D., 1994).

A great majority of drugs described as a multiple GST substrates. Even many of them especially alkylating agents (cisplatin, carboplatin etc.) show weak affinity to GST enzyme family, these drugs are suffering from GST dependent drug resistant phenotype. This apparent paradox may be explained by the influence of GST enzymes on signaling pathways which affect the cell

survival and death (Adler V., et al., 1999; Romero L., et al., 2006). These findings suggesting that to overcome the cisplatin induced resistance, GST enzyme family can be a useful drug target.

Beside resistance issue, cisplatin therapy can cause serious toxic effects in tissues such as kidneys (nephrotoxicity), peripheral nerves (neurotoxicity) and the inner ear (ototoxicity) (Cvitkovic E., et al., 1977; Kelland L., 2007). To overcome these side effects, cisplatin is commonly used in combination with some other drugs or natural plants. For example, in ovarian cancer cells, cisplatin used in combination with honey bee venom (Alizadehnohi M., et. al., 2012), in breast, colon, lung, prostate and pancreatic cancer cell lines with anvirzel (Apostolou P., et al., 2013) and also diabetic drug metformin has been shown to regulate cisplatin cytotoxicity by suppressing AMPK pathway (Lin C.C., et al., 2013). In recent years, in order to eliminate its toxic and resistance problem; several strategies are used. One of them is using a cispaltin with another chemical which has anti-carcinogenic potential. Metformin is a hypoglycemic agents having such a anti-carcinogenic potential.

1.1.2 Metformin

Metformin (N, N-dimethylbiguanide) that belongs to the biguanide class of oral hypoglycemic agents is widely used as an antidiabetic drug (Corriera S., et. al., 2008). The chemical structure of metformin was given in Figure 1.3.

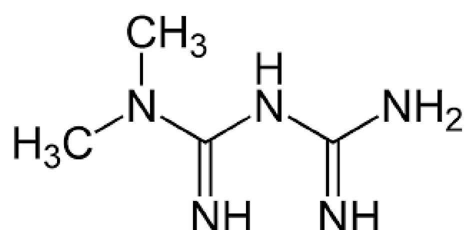

Figure 1.3. Chemical Formula of Metformin.

Metformin was first isolated from a plant Galega officinalis; a plant native to the Middle East, in 1922 by Werner and Well (Witters L. A., 2001) (Figure 1.4). But, its first glucose lowering action is described in 1929 by Slotta and Tschesche, and it was approved by the FDA in 1995 in the US (Kourelis T.V. and Siegel R.D., 2011). Now, ıt has been used by almost 120 million of people in the world for treatment of type 2 diabetes.

Figure 1.4. Galega officinalis.

Metformin exerts its glucose lowering effect by decreasing the uptake of glucose from gastrointestinal tract, by promoting peripheral glucose absorbtion and utilization by adipose tissue and skeletal muscle (Ashokkumar N., et. al., 2006). Metformin, a cationic (hydrophilic base) drug, can show its pleiotropic pharmacological effects beyond those of metabolic control (Saenz A., et. al., 2005), and this multifuncional mechanism makes researchers think that, this drug has a high probability as an anti-inflammatory and anti-oncogenic outcomes (Caballero A. E., et. al., 2004; Kim S. A., et. al., 2012). Because of these reasons, in spite of its main fuction is regulation of glucose homeostasis by inhibition of liver glucose production and an increase in muscle glucose uptake, during the last decade it has been used as an anticancer agent (Gonzalez-Angulo A. M. and Meric-Bernstam F., 2010).

This antitumorogenic potential of merformin has been first dedicated to indirect mechanisms that ability to reduce the serum levels of glucose, insulin, and insulin-like growth factor (Viollet B., et. al., 2012). However, experimental studies suggest that, it can also decrease the cellular proliferation by triggering phosphorylation of 5′ AMP-activated protein kinase (AMPK) (Zhou G., et. al., 2001). AMPK is a protein kinase that involved in regulating energy balance and ubiquitously expressed in mammalian tissues. Adenosine triphosphate (ATP)-producing catabolic pathways were stimulated by active AMPK. At the same time inhibiting ATP-consuming anabolic pathways resulting in the protection of cellular energy stores. Activated of AMPK increases the uptake of glucose and oxidation of lipids in skeletal muscle.

The problem is that how antidiabetic drug metformin activates this energy sensor pathway is still remains unclear, its believed that metformin can selectively disrupts mitochondrial respiratory chain reaction and activates the enzyme AMPK (R. J. Shaw., et. al., 2005). However mitochondrial inpairments has a limited effects on cancer because, production of ATP is

more than 50 times higher than in normal tissues (Zu XL., et. al., 2004). Recently, these experimental evidences pointed out that metformin should disrupt cancer energy asset by AMPK-independent manner through the direct enzymatic inhibition of hexokinase (HK) isoforms I and II (Salani B., et. al., 2013).

Several recent studies about metformin support these findings that direct correlation between decreased cancer incidence and treatment with biguanides in type II diabetic patients (Noto H., et. al., 2012). In addition to its anticarcinogenic outcomes, adjuvant role of metformin is being investigated in combinations for diffrent cancer types. For example, cisplatin-based chemotherapy still the first-line drug for several cancer types, however serious side effects of cisplatin, restricts the usage of this drug. The combined effects of metformin and cisplatin on lung cancer studied by Chien-Chung Lin and co-workers and significant results showed that metformin could be used as an anticancer agent in combination with cisplatin to treat lung cancer (Chien-Chung L., et. al., 2013).

However, the mechanism underlying the combined effects of metformin and other anticancer drugs on several cancer cell types remains unclear. The present studies provide combined effects of this drug as an antitumoral adjuant.

1.2 Aim of the Previous and Present Study

At the beginning of this study, several cisplatin derivatives were synthesized to analyse their anticarcinogenig effects (formulas given in Figure 1.5). Hovewer, these derivatives hast not remarkabele antiproliferative and anticarcinogenic effects on PC cell lines. Therefore, we moved on the use of cisplatin with metformin. Because of its anti-tumorogenic potential, we decided to investigate adjuvant role of metformin in combination with cisplatin in prostate cancer cell lines. By this manner, we aimed to target

cancer cell metabolism and directly induce cellular apoptosis using metformin-cisplatin combination.

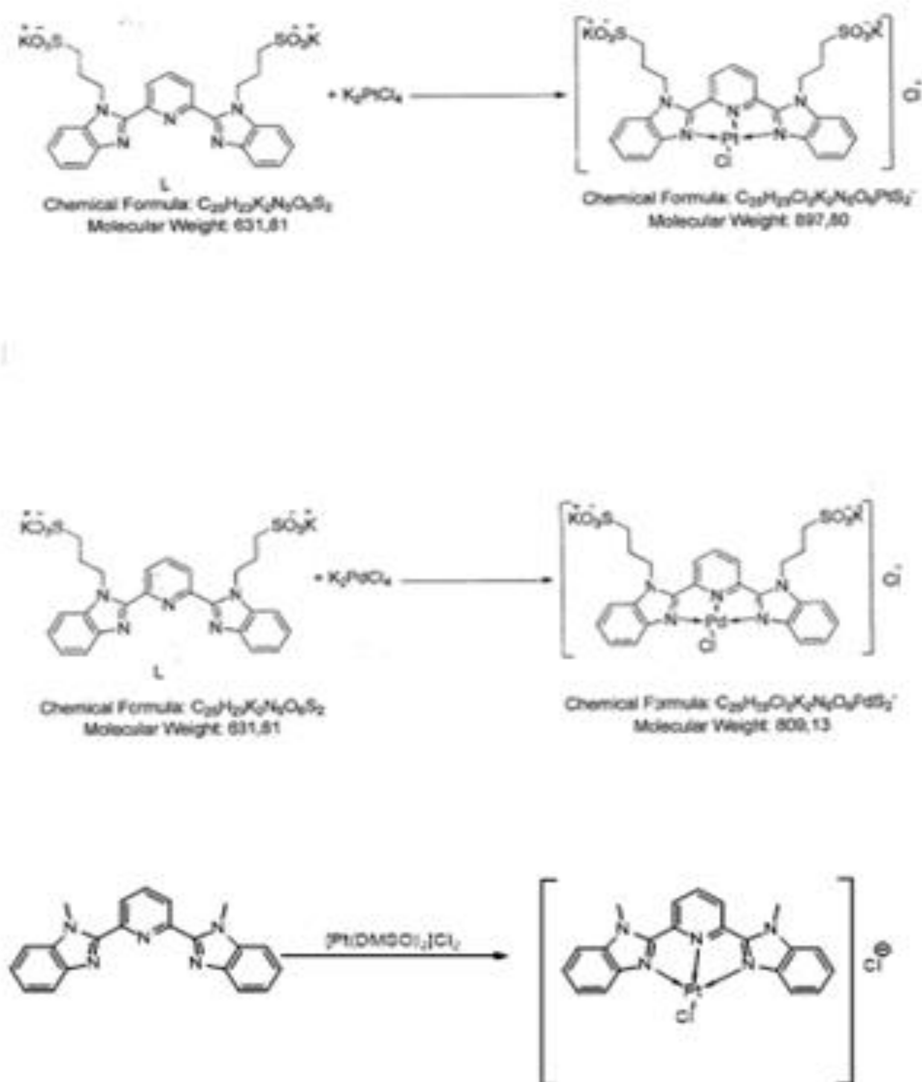

Figure 1.5 Derivatives of cisplatin.

1.2.1 Enzymes determined in this this study

It is well known that androgens play an important role in cellular proliferation of prostate carcinomas and CYP17A1 enzyme has a critical role in androgen biosynthesis. Therefore, the effects of drugs on CYP17A1 dependent production of androgens was determined in cancerous cells

In addition, it is known that elevated glucose consumption by cancer cells was associated with their invasive proliferative properties. In the glucose consumption, first enzymatic step is the phosphorylation of glucose by Hexokinase enzymes. Therefore, many anti-carcinogenic drugs are targeting these enzymes. Because of metformin's glucose lowering property, we decided to check whether hexokinase II protein and gene expression is effected by metformin and cisplatin or not.

Moreover, GST's has an important regulatory role in pathways that participates in cellular survival and death signaling. In addition, these enzymes are involved in chemotherapy resistance that was seen in cisplatin case. For this reasons, The effects of drugs on GSTP protein and mRNA levels were determined whether they have such a therapeutic effects on prostate cancer cell lines or not.

1.2.1.1 CYP17A1

The androgen biosynthesis in humans catalyzed by Cytochrome P450 17A1 (P450c17). Cytochrome P450 17A1 protein that has 17α-hydroxylase and also 17, 20 lyase activities and in the synthesis of many human steroid hormones, CYP17A1 serves a key enzyme role as a membrane-bound monooxygenase (Miller W.L., et al., 2011). To generate glucuocorticoids, such as cortisol, CYP17A1 17α-hydroxylase activity is required, while to produce androgenic and estrogenic sex steroids, hydroxylase and also 17, 20-lyase activities are required. In brief, CYP17A1 is responsible for adding a

hydroxyl group to steroid D ring of progenelone and progesterone that carbons at the position 17 (hydroxylase activity). Then, 17-hydroxyprogesterone and 17-hydroxypregnelone produced are converted to C-19 androgen precursors (the lyase activity) DHEA and androsterodione, respectively (*Boulpaep E.L. and Boron W.F., 2005;* Nimkarn S., et. al., 2000*)* (Figure 1.6).

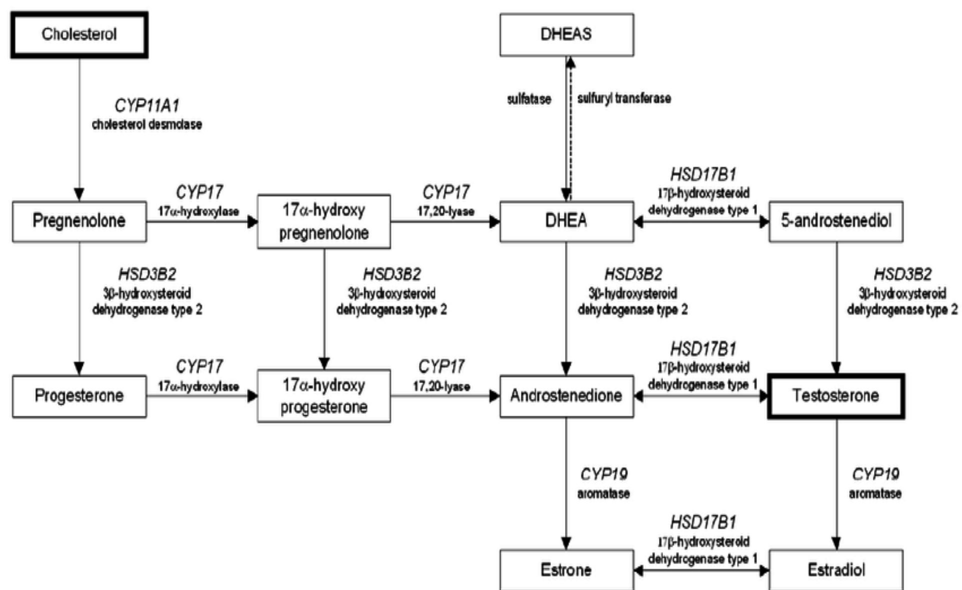

Figure 1.6 Major human steroidogenic pathway (Ananland P.C.., et. al., 2007).

Androgens play an important role in cellular proliferation and differentiation during prostate development. In healthy prostate epithelium cells, cellular growth and death are in equlibrium (Lu S., et. al., 1997). During the progression of epithelial cells into malignant state, aberrant androgen receptor (AR) activation occurs, expression of growth factors and their receptors de-regulated, at the same time proto-oncogenes up-regulated and tumor supressor genes were down-regulated (Djakiev D., et. al., 2000). It has been known that, ARs were present in the wast majority of prostate cells and the relationship between PCs and androgens has been studied for decades (Djakiev D., et. al., 2000).

In the light of the above findings, androgen deprivation therapy is the standard strategy for treatment PC. Hovewer, clinical side effects of this treatments still research subject (Bishr and Saad, 2013; Karantanos *et. al*, 2013).

Due to the importance of androgens in the development and progression of PCa, enzymes that involved in the androgen metabolism can be a target of future therapeutics for therapy of advanced cancers. Different therapeutics can be used for PCa treatment. Accordingly, metformin known as an antidiabetic drug, its newly discovered usage area as treatment of polycystic ovary syndrome because of its insulin-regulating androgen-lowering properties. According to studies of Andrea H. and her team, metformin treatment may selectively inhbits CYP17-lyase activity in adrenal NCI-H295R cells (Andrea H., et. al., 2012).

1.2.1.2 Glutathione-S-Transferases (GSTs):

Glutathione S-transferases (GSTs) are ubiquitous gene family that catalyze the conjugation of glutathione (GSH) to many exogenous and endogenous compounds including chemical carcinogens, therapeutic drugs and products of oxidative stress (Hayes et. al., 2005).

Although their well known role is catalyzing the conjugation of electrophilic substrates to GSH, recent studies demostrated that these enzyme family playing important role in stress response, apoptosis and cellular proliferation by their non-catalytic function via protein-protin interractions. Reduced glutathione (GSH) consist L-glutamine, L-cysteine, and glycinewith a linear form of tripeptide. It contains sulfhydryl sroup on the cysteinyl part which makes GSH a strong electron store (Figure 1.7).

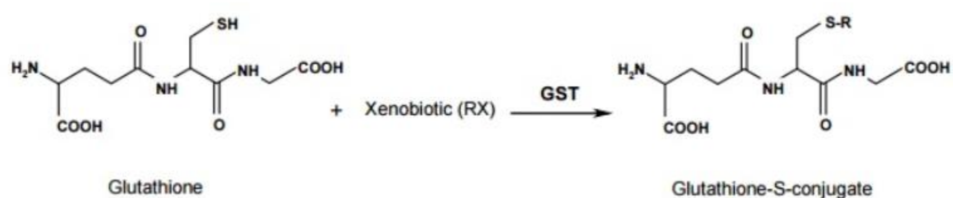

Figure 1.7 Glutathione conjugation to a xenobiotic by GSTs.

GST has 2 subunits and each GST subunit contains a GSH binding side that catalytically independent part in the N-terminal domain and in the carboxy terminal side that Hydrophobic binds the substrate as shown in Figure 1.8 (Mannervik, B. et. al., 1986; Townsend, 2003; Wu and Dong, 2012).

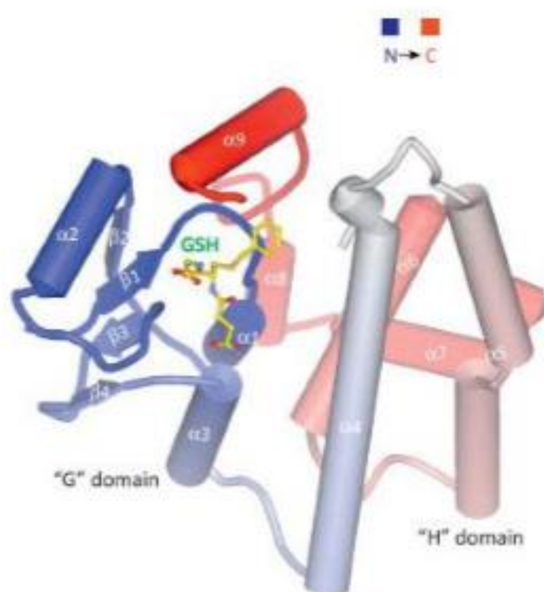

Figure 1.8 3-D structure of GST enzyme (Wu and Dong, 2012).

Detoxification process against reactive and toxic electrophiles seems to be the major biological function of GST's. During this process, in glutathione redox cycle, GST enzymes catalyze the conjugation of reduced glutathione (GSH) by formation of a thioether bond between the sulphur atom of GSH and the substrate.

1.2.1.2.1 Regulatory role of GSTP enzyme

Because of their capability to interract covalently and noncovelently with multiple compounds which were not substrates for enzymatic activity, these sequestering molecules may have a regulatory role by hindering the interactions of cytotoxic ligands with their targets. Supporting this, recent studies have demonstrated that these enzymes hs a regulatory rolein (MAP) kinase pathways that involved in cellular survival and death signalling. Especially, GSTP plays a regulatory role on this pathway via protein:protein interactions. C-Jun N-terminal kinase 1 (JNK1), a kinase known as a key mediator of stress response and apoptotic events. Accordingly, GSTP act as an endogenous inhibitor of c-Jun N-terminal kinase 1 (JNK1) (Adler et. al., 1999; Yin et. al., 2000).

In nonstressed cells, low JNK activitiy observed through the interaction of GSTP: JNK1 complex (Adler et. al., 1999). This supression will be reversed by oxidative stress conditions such as drug treatment because, dissociation of GSTP: JNK1 complex by oligomerization of GST's resulting in the induction of apoptosis as given in Figure 1.9 (Adler et .al., 1999; Laborde, E., 2010).

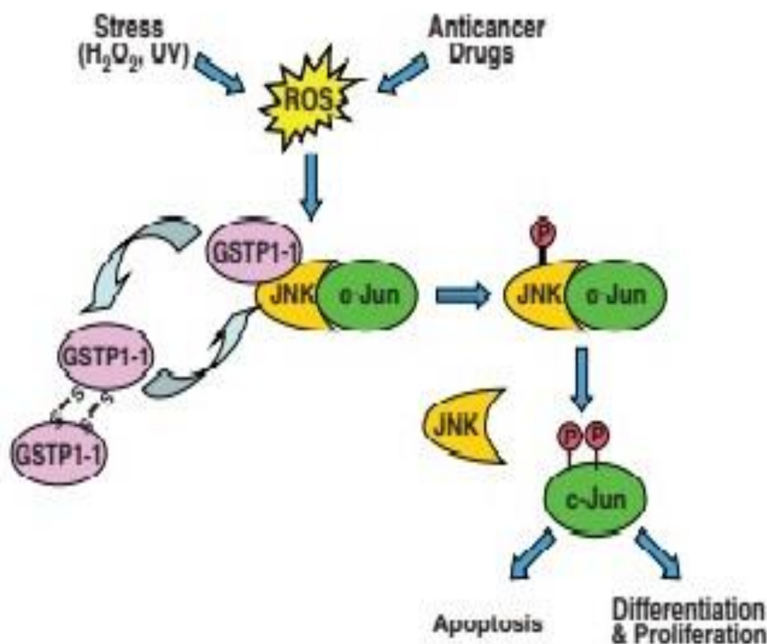

Figure 1.9 GSTP: JNK1 complex by oligomerization

However, ligand-binding mechanism is still unclear, after cells exposed to oxidative stress, GST monomers will be aggregated by disulfide bridge formation between cysteine residues of GSTP 47 and 101, which were critical for protein:protein interactions between GSTp and JNK, independantly, catalytic site of GSTP. Its clear that, there is a direct correlation between GST-overexpressing phenotypes and resistace of anticancer agents due to direct detoxification of drugs which were substrates of GST's. However there are several examples of anticancer agent resistance linking GST overexpression that are not substrates for these enzymes, one of them cisplatin, that requires JNK activation to generate cytotoxicity, is not substrate for GST enzymes but still affected by GST-overexpressing phenotypes. In view of the above information, mediatory process of GST's on apoptosis, and cellular

proliferation by direct protein:protein interractions can also initiate a reasonable explanation for this stiuation.

Because of its property as an endogenous switch for the control of signaling pathways, increased expression of GSTP can regulate the balance of kinase pathways during anticancer drug treatment, thereby conferring a potential selective advantage. This process makes these enzyme family as an impotant drug targets.

1.2.1.3 Isozymes of mammalian hexokinase

Glucose (Glc) is a ubiquitous fuel in most organisms, from microbes to humans. Glycolytic metabolism of glucose is a mojor pathway of cellular energy (ATP) process. Also, glycolytic intermediates are used for biosynthesis of other cellular components. Because of their most rapid growth rates, it is not suprising that high levels of glycolysis is observed in many cancers (Warburg et. al., 1930; Pedersen, 1978). As well, the elevated glucose consumption by cancer cells associated with the irreversible first enzymatic step of glycolysis, usually phosphorylation to glucose-6-phosphate (Glc-6-P) by hexokinase as shown in Figure 1. 10 (Bustamante and Pedersen, 1977; Bustamante et. al., 1981; Wilson, J. E., 2003). The phosphorylation process introduces a negative charge on glucose. This stiuation makes glucose difficult to transport out of the cell and entrapped for the tumor's utility, as a result specific enzyme izozymes of hexokinase involved in the cancerous glycolytic metabolism. So, while cancer cells damage to host organism; expression, regulation and localization of hexokinase isosymes provide advantages for survival and proliferation of tumor (Smith, 2000; Pedersen et. al., 2002).

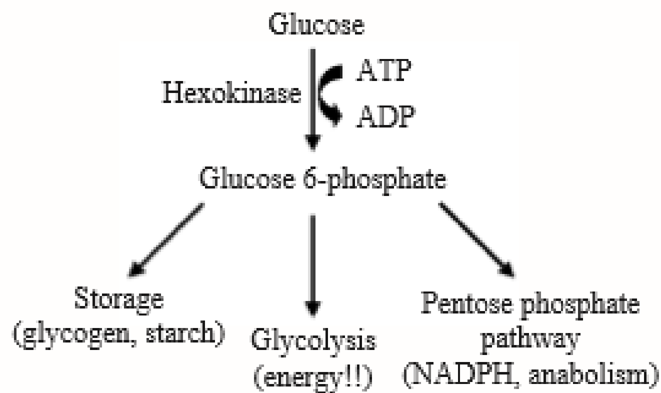

Figure 1. 10 Phosphorylation, catalyzed by hexokinase, is the initial step in common pathways of Glc metabolism (Wilson, J. E., 2003).

There are 4 different hexokinase isoforms encoded by separate genes, HKI, HKII, HKIII, and HKIV (also known as glucokinase) characterized in mammalian tissue (Wilson, 1995, 1997, 2003). Substrate (glucose) affinities are differ among these isozymes. HKI, HKII and HKIII have an approximately 250 fold higher (K_m= 0,2mM) affinity than HKIV (K_m= 5mM) (Wilson, 1995, 2003). When we analyzed the primary structure of these 3 highly affinity enzymes, it has been shown that these genes (HKI, II and III) arising via duplication of an ancestral glucokinase gene similar to that which encodes HK IV (Tsai and Wilson, 1995, 1996, 1997; Ardehali et. al., 1996; Printz et. al., 1997). Therefore, while HK IV has a molecular weight approximately 50 kDa, the other isoforms have a molecular weight approximately 100 kDa. During tumorogenesis in tissues, such as pancreas and liver, expressed HK IV as a ‘’switch-over’’ signal for expression of high affinity isoform HK II, seldomly HK I. At the same time, when these isoforms expressed, HKIV silenced (Rempel et. al., 1994; Mathupala et. al., 1997b; Mayer et. al., 1997; Pedersen et. al., 2002).

1.2.1.3.1 Hexokinase II (HKII)

Rapid and efficient phosphprilation of glucose serve a function as a precursor of glycolysis and also for biosynthesis of key metabolites through pentose-phosphate pathway and a mitochondrial tricarboxylic acid cycle, which were essential for gowth and the growth of cancer cells. Despite HK II is well known player in sustaining the highly malignant state of cancer cells, it has 4 more key protein partners (Figure 1. 11). Firstly, by glucose transporters (Glut), glucose brought across the plasma membrane and rapidly phosphorylated by HK II (Pauwels et. al., 1998; Smith, 1999; Macheda et. al., 2005). After rapid passage of glucose, HKII bound to outer membrain of mitochondria bay the pore-like outer mitochondrial membrane protein voltage-dependent anion channel (VDAC). At the same time ATP synthase generate ATP. Thus second and lesser known substrate of HKII is produced (Arora and Pedersen, 1988). Finally the adenine nucleotide translocator that transports the ATP to the VDAC-HK II complex (Arora and Pedersen, 1988; Shinohara et. al., 1997; Cesar and Wilson, 1998). Both glucose and ATP bind to active side ot HKII resulting in the elevated levels of glucose. By this adaptations cancer cells promotes the cellular proliferation and abilitiy to metstasis.

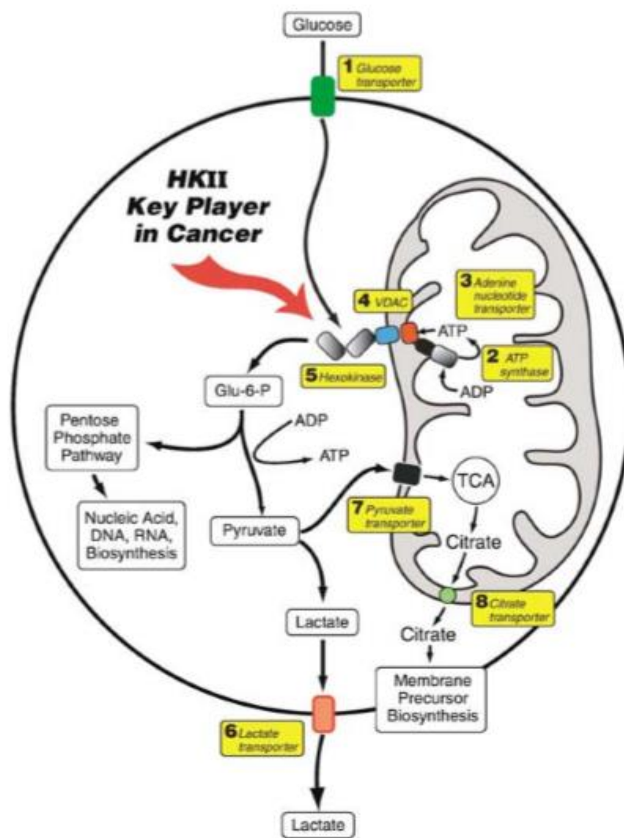

Figure 1. 11 Hexokinase II and its major partners in cancer promotion (Mathupala et. al., 2006).

As described above, uncontrolled proliferation state and apoptotic resistance is a basic definition of malignant tumor. Different approaches may involve direct inhibition of the HK isoform production, especially HKII. Such as siRNA-mediated gene silencing techniques can be applicable for that matter (Sui and Wilson, 2004). In addition, small-molecule drugs such as Lonidamine can be usable for inhibiting HK activity (Fanciulli et. al., 1996; Floridi et. al., 1998), or azole derivatives can be plausible for HK–VDAC binding disruptor (Penso and Beitner, 1998).

Together with, hexokinase plays an important role in both glucose metabolism and apoptosis. Inhibitors of this enzyme seems to have an efficient effects on cellular energy metabolism and survival. This process, makes hexokinase is an attractive target for anticancer agents.

1.3 Aim of the Study

Due to the fact that, prostate cancer growth promoted by male hormones androgens, hormonal manipulations have been the standart first-line treatment for prostate cancer since 1940's. However, this treatment option is not curative and most result in resistance. Combination therapies, which were treatment strategies that combine multiple drugs or different types of therapies, have proven effective against many types of cancer.

One of the most important mechanism for treatment of PC is to overcome the androgen production. This mechanism can be blocked with androgen synthesis inhibitors that selectively inhibits Cytochrome P450, CYP17, a key enzyme in the adrenal steroid hormone synthetic pathway. CYP17 inhibition results in a further decrease of androgen levels in the circulation and in the tumors of castration resistant prostate cancer (CRPC) patients.

The fact that, cancer cells express the highly glycolytic phenotype. Therefore modulation of glycolytic enzymes can be a good option for treatment of cancer cells. Many enzymes of the glycolytic pathway plays a significant roles that enable the cancer cells to proliferate quickly. One of them, Hexokinase II, seems to be a strong target for antitumoral agents.

Killing cancer cells by triggering cellular apoptosis seems to be the prevalent action mechanism for cancer drugs. Using substances that targeting the enzymes which paticipate in the apoptotic pathways or enzymes that triggers the cellular apoptosis can be a useful treatment option for cancer. By this

manner GSTP enzyme, its newly known role as a modulator of cellular survival and death, can be a good research topic for cancer studies.

Although, cisplatin one of the most potent alkylating agent that applied with success in vast majority of cancers, its defective side effects are still limits the usage of this drug. Concordantly, to overcome this side effects, producing less toxic and more effective drug derivatives or combination treatment options are popular research topic in recent years.

Metformin is well known drug for diabetes that is part of a group of conditions called metabolic syndrome. Recent studies demostrated the increased risk of getting prostate cancer was related with having metabolic syndrome, or some features of it. Researchers suggest that, metformin can reduce the risk of getting cancer even be able to treat it.

In the present study, in order to determine the combined effects of these drugs, cis-platin and metformin on prostate cancer, human androgen receptor dependent cell line LNCaP and androgen receptor independent PC-3 cell lines were used. To study the effects of these drugs on enzymes playing role in the metabolism of drugs and endogens and energy metabolism such as CYP17A1, GSTP1 and hexokinase II, protein and total RNA were obtained from these cell cultures. Then, the protein and mRNA expressions of CYP17A1, Hexokinase II and GSTP1 enzymes were analyzed by Western Blot and qRT-PCR techniques, respectively.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Line

In this study, protein and mRNA expressions of cyp17A1, GSTP1 and HexokinaseII enzymes determined in human androgen receptor (AR)-positive (LNCaP) and AR-negative (PC3) were analyzed. Studied cell line, LNCaP (ATCC® CRL-1740™) was gift from Assoc. Prof. Dr. Sreeparna Banerjee, Biology Department, Middle East Technical University. Prostatic adenocarcinoma cell line; PC-3 (ATCC® CRL-1435™) was gifted from Research Assistant Bora Ergin, Department of Biophysics, Faculty of Medicine, Hacettepe University.

2.1.2 Chemicals and Materials

Metformin (PHR1084), Cisplatin (1915251), Bicinchoninic acid (D8284), ammonium acetate (A7672), bovine serum albumin (BSA; A7511), phenyl methane sulfonyl fluoride (PMSF; P7626), sodium potassium tartarate (Rochella salt; S2377), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris; T1378), acrylamide (A- 8887), ammonium per sulfate (APS; A-3678), bromophenol blue (B5525), diethanolamine (D-2286), diethylpyrocarbonate (DEPC; D5758), glycerol (G5516), glycine (G-7126), ß-mercaptoethanol (M6250), methanol (34885), N'-N'-bis-methylene-acrylamide (M7256), N-N-dimethylformamide (D- 8654), phenazine methosulfate (P9625), secondary antibody AP rabbit (A3687), sodium dodecyl sulfate (SDS;

L4390), sodium-potassium (Na-K) tartarate (S-2377), and tween 20 (P1379) were the products of Sigma Chemical Company, Saint Louis, Missouri, USA.

Magnesium chloride ($MgCl_2$; 05833), potassium chloride (KCl 104935), potassium dihydrogen phosphate (KH_2PO_4; 04871), di-potassium hydrogen phosphate (K_2HPO_4; 05101), sodium carbonate (06398), sodium hydroxide (06462), boric acid (A949265), chloroform (1.02431.2500), copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$; A894987 605), folin-phenol reagent (1.09001.0500), sodium carbonate (Na_2CO_3; 1.06392), sodium chloride (NaCl; 1.06400), sodium hydroxide (NaOH; 06462), zinc chloride ($ZnCl_2$; 108815) were purchased from E. Merck, Darmstadt, Germany.

Absolute ethanol (32221) and acetyl acetone (33005) were purchased from Riedel de-Haen Chemical Company, Germany. TRIzol® (15596) was obtained from Carlsbad, CA, USA. Isopropanol (AS040-L50) was the product of Atabay, Istanbul, Turkey.

5-bromo 4-chloro 3-indoyl phosphate (BCIP; R0821), dithiothreitol (DDT; R0861), gene rulerTM 50 bp DNA ladder (SM0371), light cycler-fast start DNA MasterPlus SYBR green I (K0252), Maloney murine leukemia virus reverse transcriptase (M-MuLu-RT; K1622), pre-stained protein ladder (SM0671) were the products of MBI Fermentas, USA.

Ethylene diamine tetra acetic acid (EDTA; A5097) and nitrotetrazolium blue chloride (NBT; A1243) were obtained from Applichem GmbH, Germany. Non-fat dry milk (170-6404) and tetra methyl ethylene diamine (TEMED; 161-0801) were purchased from Bio-Rad Laboratories, Richmond, California, USA.

The CYP17A1 (sc-46081), GAPDH (sc-367714) antibodies were purchased from Santa Cruz (Santa Cruz, 27 CA). GSTP1 (ab138491) and Hexokinase II (ab-76959) antibodies were product of the Abcam, Cambridge, United Kingdom. Primers were the products of Alpha DNA, Montreal, Canada.

Ham's F-12 medium (BE12-615F), RPMI1640 medium (BE12-702F/U1) and Fetal bovine serum (FBS; DE14-801FH) were the products of Lonza, Walkersville, MD, USA. Pen-Strep solution (03-031-1B) and trypsin-EDTA solution (03-050-1B) was product of the Biological Industries, Beit-Haemek, Israel. RIPA buffer (9806) was purchased from Cell Signaling Technology, Beverly, MA

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Cell Culture Conditions

PC3 cell line was cultured in Ham's F12 Medium and LNCaP cell line was cultered in RPMI1640 medium. Both of them containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin (Pen-Strep) solution (Table2.1). Cultures were incubated at 37°C with 5% carbon dioxide (CO_2) and 95% humidity in EC 160 NÜVE incubator. The cell culture studies were carried out in NÜVE MN 090 Class II Safety Cabinet. The growth medium of culture was renewed in 2-3 days for optimum growth conditions.

Table 2.1 Growth Medium Conditions of Cell lines

Cell Lines	Medium	Fetal Bovine Serum	L-glutamine	Pen-Strep
PC3	Ham's F12	10%	1%	1%
LNCaP	RPMI1640	10%	1%	1%

2.2.1.2 Cell Thawing

Before thawing the cells, 10 mL of growth medium, which is pre-warmed to 37°C, was transferred into T75 cell culture flask. Then, cryotubes were taken from the liquid nitrogen and the cells were defrosted at 37°C water bath and transferred immediately to T75 cell culture flask containing growth medium. Cells were incubated in CO_2 incubator at 37°C. Following day from the cell thawing, medium was renewed to eliminate dimethylsulfoxide (DMSO) and again placed into CO_2 incubator.

2.2.1.3 Subculturing the Cell Lines

When the cells were 80% confluent in the T75 flask, the medium was removed and cells were washed with 10 mL of 10 mM phosphate buffered saline (PBS). 1:3 split of cell lines was performed by adding 1ml of prewarmed trypsin to flask and placing the T75 flask in 37°C, CO_2 incubator until cells were detached and 4 mL of pre-warmed growth medium was added to the flask to inactivate the trypsin and the 2 ml of this mixture was transferred into new T75 flask. Then 10 mL of growth medium was added to

new T75 flask and the culture was placed in 37°C, CO_2 incubator. This procedure was repeated in every 2-3 days.

2.2.1.4 Cell Freezing

When the cells were 80% confluent in the T75 flask, the medium was removed and cells were washed with 10 mL of PBS. 2 mL of pre-warmed trypsin was added to flask and placed in 37°C CO_2 incubator for 5 minutes. After being sure of all the cells were detached, 2 mL of pre-warmed growth medium was added to the flask to inactivate the trypsin. The cells in the flask with trypsin and growth medium were transferred into a 15 mL falcon tube and centrifuged at 400 x g for 5 minutes at room temperature. After centrifugation, supernatant was discarded and pellet was resuspended in 1 ml growth medium by pipetting. After that, the cell suspension was transferred to cryotube and 100 µL DMSO was added as cryoprotectant. Cryotube was immediately placed in the -80°C freezer and in a week it was transferred to liquid nitrogen tank for longer term storage.

2.2.1.5 IC_{50} Determination for Cisplatin

In order to determine IC_{50} value cells were inoculated to 24 well plate in 1 mL at plating density 90.000 cells per well. After cell inoculation, the microtiter plates are incubated at 37° C, 5 % CO_2, 95 % air and 100 % relative humidity for 24 h prior to addition of cisplatin. After 24 h, growthmedium was replaced with 1 mL cisplatin treated medium ranging from 5µM to 30 µM of cisplatin for PC3 cell lines and 5µM to 20 µM for LNCaP cell lines. Following cisplatin addition, the plates are incubated for an additional 48 h at 37°C, 5 % CO_2, 95 % air, and 100 % relative humidity. After 48 h, Alamar Blue Assay was performed for cell proliferation. Alamar Blue (AB) is a blue colored, water-soluble, stable and more importantly non-toxic dye to the cells, so, it is a preferred cell viability test. In addition to Alamar Blue asay, cells were counted by microscope for consolidate the data. In order to perform this

assay, cisplatin treated growth medium was discarded. Then AB treated growth medium at a final concentration of 10% was added to the wells and plate was returned to the incubator for 2-4 h. In this 2-4 time interval plate was constantly checked for color shift. When AB is added to the cell culture, the oxidized form of the AB enters the cytosol and is converted to the reduced form by mitochondrial enzymes. This reaction is easily seen by color shift in the growth medium from blue to pink and measured by colorimetric methods. The absorbance of wells was read at 570 and 600 nm by Multiskan™ FC Microplate Photometer (Thermo Scientific). The calculation of the percentage of AB reduction (%AB reduction) is as follows according to the manufacturer's protocol:

$$\%\text{AB reduction} = \frac{(\varepsilon_{ox}\lambda_2)(A\lambda_1) - (\varepsilon_{ox}\lambda_1)(A\lambda_2)}{(\varepsilon_{red}\lambda_1)(A'\lambda_2) - (\varepsilon_{red}\lambda_2)(A'\lambda_1)} \times 100$$

In the formula, $\varepsilon\lambda 1$ and $\varepsilon\lambda 2$ are constants representing the molar extinction coefficient of AB at 570 and 600 nm, respectively, in the oxidized (εox) and reduced (εred) forms. $A\lambda 1$ and $A\lambda 2$ represent the absorbance of test wells at 570 and 600 nm, respectively. $A'\lambda 1$ and $A'\lambda 2$ represent absorbance of negative control wells at 570 and 600 nm, respectively.

2.2.1.6 Treatment with Metformin

In order to compare the metformin and metformin/cisplatin effect respectively, after determination of cisplatin IC_{50} values, cells were seedet to 24 well plate in 1 mL at plating density 90.000 cells per well. Than cells were treated by different concentrations of metformin with or without cisplatin (Fig2.1).

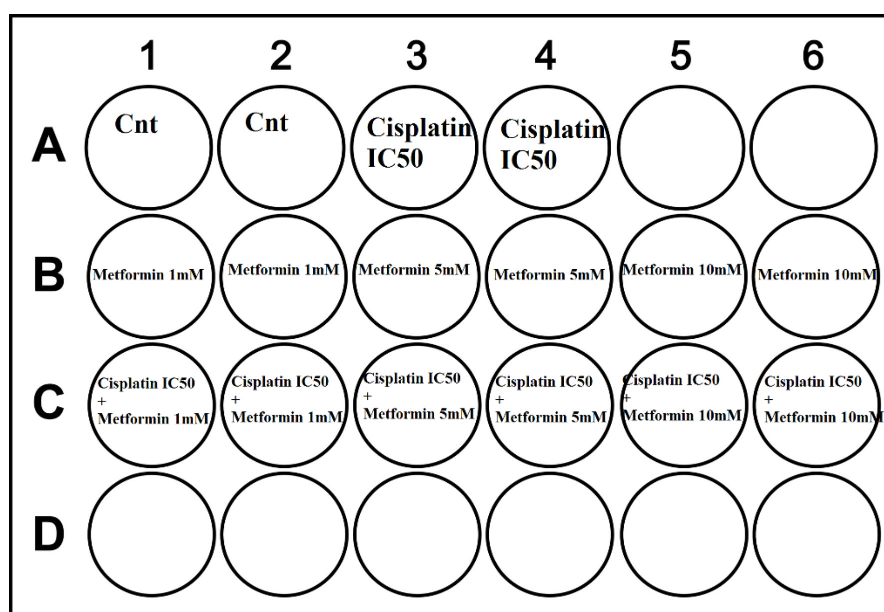

Fig2.1 The schematic representation of treatment 24 well plate.

Following metformine addition, the plates are incubated for an additional 48 h at 37°C, 5 % CO_2, 95 % air, and 100 % relative humidity. After 48 h, Alamar Blue Assay was performed for cell proliferation.

2.2.2 Protein Extraction

In order to perform protein extraction cells were seeded to 100 x 20mm tissue culture petri dishes at plating density 1.000.000 cells per petri dish. 16 petri dishes were used to culture the cells in order to duplicate the control group and the treated group. After 24 h, growth medium in the dishes were replaced with growth medium, which contains 17μM cisplatin (determined as IC_{50} of LNCaP cell lines) or 30 μM cisplatin (determined as IC_{50} of PC3 cell lines). At the same time dishes were treated with Metformine concentrations 1mM and 5mM like represented at Fig 2.1. However, 10mM concentration killed the whole cells in the dish, this concentration detected as applicable. The control groups were replaced with fresh medium. When cells were 80% confluent, growth medium in the dishes were removed and the cells were washed three times by using cold (4 °C) PBS buffer. 2 ml PBS buffer was

added into the each dish for lift the cell on the dish. Dishes were incubated on ice for 5 minutes and the cells were scraped. Cells were centrifugated at 14000 x g for 10 minutes and continued with pellets. 1X RIPA buffer was prepared by dilution of commercially available 10X RIPA buffer (Cell Signaling Technology) with distilled water and 1mM phenylmethanesulfonyl fluoride (PMSF) was added to prevent protease activity. 400 µL of the diluted RIPA buffer was added into the each group and mixed by well pipetting for lysis of the cells. After that cell lysates incubated for 5 minutes and sonicated for 4 minutes. Sonicated cell lysates centrifuged again at 14000 x g in a cold microfuge for 10 minutes. Supernatants were taken and stored at -80 °C freezer.

2.2.3 Determination of Protein Concentration

Protein concentrations of cell culture lysates were determined by the BCA (Bicinchoninic Acid) method using crystalline bovine serum albumin as a standard (P. K. Smith, 1985). This method depends on reduction of Cu^{2+} ions with peptide bonds under alkaline conditions and chelation of two molecules of bicinchoninic acid with each Cu^{+} ion, forming a purple color that absorbs light at a wavelength of 562 nm and the absorbance at this wavelength is proportional to the protein concentration.

Reagents:

Reagent A:

0.4 g of $CuSO_4.5H_2O$ was dissolved in 10 mL dH_2O.

Reagent B:

8 g of $Na_2CO_3.H_2O$ and 1.6 g of $NaKC_4H_4O_6$ was dissolved with dH_2O and titrated with $NaHCO_3$ to pH 11.25 and the volume was completed to 100 mL with dH_2O. The pH of the solution was checked at the end.

Reagent C:

4 g of BCA was dissolved in 100 mL of dH_2O.

BCA Solution:

Reagent A, Reagent B and Reagent C were mixed in the same order with the ratio of 1:25:25. 23 Bovine Serum Albumin (BSA) Protein Standards: 0.02, 0.05, 0.075, 0.1, 0.15, 0.2 mg/mL

Protein Sample: Samples were diluted 40 times.

100 μL of BSA standards and samples were added into the 96 well-plate. Then 100 μL of BCA solution was added and incubated at 60°C for 15 minutes. The absorbances of samples were measured at 562 nm with Multiskan™ GO Microplate Spectrophotometer. Protein concentration was calculated according to the following equation;

Protein Concentration (mg/ml) = [OD660nm] $slope\ of\ standards\ x\ Dilution$

2.2.4 Determination of Protein Expression

2.2.4.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein expression of mentioned enzymes in were analyzed by Western blot method as described by Towbin et al. (1979). Before western blotting, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using 4% stacking gel and 8.5% separating gel in a discontinuous buffer system as described by Laemmli (1970). Separating and stacking gel solutions were prepared freshly according to Table 2.2

Table 2.2 Constituents of separating and stacking gel solutions for two gels.

Constituents	**Separating Gel Solution**		**Stacking Gel Solution**
Monomer Concentration	8.5 %	10%	4%
Gel Solution	4250 µL	5000 µL	650 µL
dH_2O	6775 µL	6020 µL	3050 µL
Separating Buffer	3750 µL	3750 µL	---
Stacking Buffer	---	---	1250 µL
10% SDS	150 µL	150 µL	50 µL
10%APS	75 µL	75 µL	25 µL
TEMED	15 µL	15 µL	5 µL
Total Volume	15 mL	15 mL	5 mL

Reagents:

Gel Solution

14.6 g acrylamide and 0.4 g N'-N'-bis-methylene-acrylamide were dissolved separately with dH_2O then mixed and filtered through filter paper. The final volume was completed to 50 mL.

Separating Buffer (1.5 M Tris-HCl, pH 8.8)

18.15 g of tris-base was dissolved with 50 mL dH_2O, and titrated with 10 M HCl to pH 8.8. The volume was completed to 100 mL. The pH was checked at the end.

Stacking Buffer (0.5 M Tris-HCl, pH 6.8)

6 g of tris-base was dissolved with 60 mL dH_2O, and titrated with 10 M HCl to pH 6.8. The volume was completed to 100 mL. The pH was checked at the end.

Sodium Dodecyl Sulfate - SDS (10%)

1 g of SDS was dissolved with dH_2O, and the volume was completed to 10 mL.

Ammonium Persulfate - APS (10%, Fresh)

40 mg of APS was dissolved in 400 µL distilled water.

Tetramethylethylenediamine - TEMED (Commercial)

Sample Dilution Buffer-SDB (4x)

2.5 mL of 1 M tris-HCl buffer (pH 6.8), 4 mL glycerol, 0.8 g SDS, 2 mL ß-mercaptoethanol and 0.001 g bromophenol blue were used and the volume was completed to 10 mL with dH_2O.

Electrophoretic Running Buffer - ERB:

0.25 M Tris, 1.92 M glycine (10x Stock, diluted to 1x before use by adding 0.1% SDS)

15 g tris-base was dissolved with 350 mL dH_2O, and then 72 g glycine was added. The volume of the mixture was completed to 500 mL.

It was prepared as 10x stock solution and it was diluted to 1x. 1 g of SDS was added per liter of 1x buffer before use.

SDS-PAGE was performed on 10% separating gel for mentioned enzymes in a discontinuous buffer system. Vertical slab gel electrophoresis was carried out using Mini-PROTEAN tetra cell mini trans blot module (Bio-Rad, Richmond, CA). Sandwich unit of module was set up by using two glass plates with 1 cm space. Separating gel solution was prepared according to Table 2.2 and immediately the solution was transferred into the sandwich unit up to 1 cm below the comb. The top of the separating gel was covered by adding isopropanol in order to obtain smooth gel surface while providing fast polymerization of separating gel. After the polymerization of separating gel, the alcohol was removed and the stacking gel solution was poured and immediately the comb was placed. After the polymerization of stacking gel, the comb was removed. The wells were filled out with 1 x ERB and cleaned up by a syringe to remove air bubbles and remaining gel particles.

To get the 1 mg/mL concentration, the proteins were diluted with dH_2O according to the following formula;

$$V = \frac{[\text{Conc. of Protein}]}{1.333} \times 20 - 20$$

V is the volume of dH_2O to be added to dissolve 20 µL of sample.

After mixing 25 µL of 4x SDB with 75 µL of sample, the samples were incubated 1 minutes at 100ºC heat block. Then, 30 µg of each sample was loaded on different wells. 3 µL of protein ladder was loaded as marker. After loading the samples, gel running module was placed in the main buffer tank filled with ERB. The tank was connected to the Bio-Rad power supply and electrophoresis was run at 25 mA–90 V in gel.

2.2.4.2 Western Blotting

Reagents:

Transfer Buffer: (25 mM Tris, 192 mM Glycine)

3.03 g trisma-base and 14.4 g glycine was dissolved in 200 mL methanol, and the volume was completed to 1 L with distilled water.

TBST: (20 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.05% Tween 20)

9.5 g of NaCl was dissolved in some water and 6.5 mL of 1 M tris-HCl buffer was added. Then pH of the solution was adjusted to 7.4. Finally, 165 µL tween 20 was added and volume was completed to 350 mL with distilled water.

Blocking Solution: (5% Non-Fat Dry Milk)

5 g non-fat dry milk was dissolved in 100 mL TBS

Primary Antibody: 1/100 to 1/1000 dilutions

Secondary Antibody: 1/500 to 1/5000 dilutions

Alkaline Phosphatase Substrate Solution:

Solution A: 2.67 mL of 1.5 M Tris-HCl Buffer (pH 8.8), 4 mL of 1 M NaCl, 96 μL of Diethanolamine, 820 μL of 100 mM $MgCl_2$, 40 μL of 100 mM $ZnCl_2$ and 12.2 mg of Nitrotetrazolium Blue Chloride (NBT) were mixed and the pH of the mixture was adjusted to 9.55 with saturated Tris. Then the volume was completed to 40 mL with distilled water.

Solution B: 2 mg of Phenazine Methosulfate was dissolved in 1 mL of distilled water.

Solution C: 5.44 mg of BCIP (5-bromo 4-chloro 3-indoyl phosphate) was dissolved in 136 μL of N-N-dimethylformamide.

To prepare the substrate solution, 20 mL of Solution A, 68 μL of Solution C, and 134 μL of Solution B were mixed for each membrane.

HRP-ECL Substrate Solution: (Pierce ECL Western Blotting Substrate)

To get away from background and give a reliable analysis, the HRP solution mixture optimized at 750μl of Peroxide solution and 250μl of luminol enhancer solution and 1 mL of this mixture was used for each membrane.

For western blotting, the gel was removed from the glasses and placed into transfer buffer for 10 minutes. The PVDF membrane was cut as equal size with the gel and immersed in 100% methanol for a few seconds to pre-wet the membrane. Then the membrane was equilibrated in transfer buffer for 5

minutes. After that, the gel, PVDF membrane, Whatman papers and two fiber pads were placed in transfer sandwich as shown in Figure-- The transfer sandwich was placed into Mini Trans-Blot module (Bio-Rad Laboratories, Richmond, CA, USA) and module was filled with transfer buffer. Transfer was continued at 90 volt 90 minutes for CY17A1, GSTP and GAPDH. Because of its molecular weight is bigger than the other proteins Hexokinase II (100 -110 kDa) transfered at 90 volt, 180 min for efficient transfer.

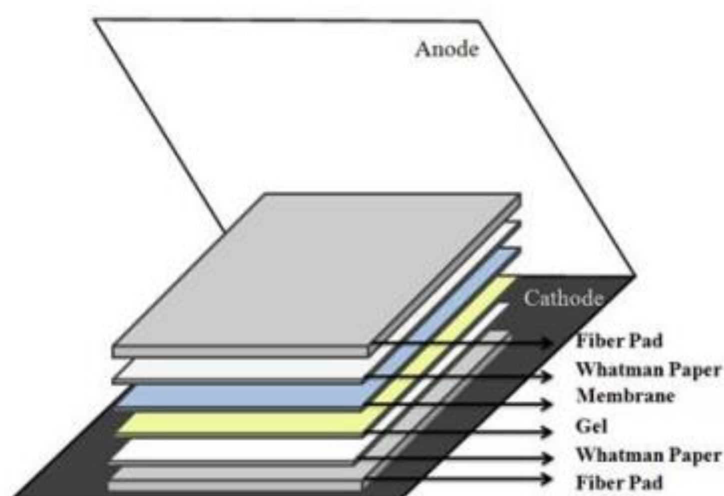

Fig 2.2 Western blot sandwich.

After transfer was completed, the membrane was washed with TBST for 10 minutes. Then membrane was incubated with blocking solution in room temperature for an hour. After that, the membrane was incubated with primary antibodies for overnight at 4ºC by shaking dilutions represented in Table 2.3. The membrane was washed with TBST for three times each of which is 10 minutes. After removal of unbound primary antibody, the membrane was incubated with alkaline phosphatase conjugated secondary antibodies or horseradish peroxidase (HRP) conjugated secondary antibodies

for an hour. Finally the membrane was incubated with suitable substrate solution for the conjugated enzyme on the secondary antibody. For visualization of HRP conjugated secondary antibody, BioRad ChemiDoc®MP Sytem was used and band intensities were analyzed by Image J visualization software developed by NIH.

Table 2.3: Dilutions of Primary and Secondary antibodies.

	PC3		LNCaP	
	Primary Antibody	Secondary Antibody	Primary Antibody	Secondary Antibody
GSTP	1/1000	1/4000 (HRP)	1/1000	1/4000 (HRP)
CYP17A1	1/250	1/2000 (AP)	1/250	1/2000 (AP)
Hexokinase	1/250	1/1000 (AP)	1/100	1/1000 (AP)
GAPDH	1/1000	1/4000 (HRP) 1/3000 (AP)	1/1000	1/4000 (HRP) 1/3000 (AP)

2.2.5 Determination of mRNA Expression

2.2.5.1 Isolation of Total RNA from Cell Lines

All plastic and glass equipments used for total RNA isolation were treated with distilled water containing % 0.1 (v/v) diethylpyrocarbonate (DEPC) in order to inhibit RNase activity. After the evaporation of excess DEPC, the equipment was autoclaved. Cell lines were seeded into 6 well plate for RNA isolation. After 24 h growth medium replaced by drug treated growth medium as explained in detail before. When cells were 80% confluent, growth medium in the wells was removed and the cells were washed three times by using PBS buffer. After that, 1 mL of Trizol®, was added into the wells and incubated for 5 minutes at room temperature. After incubation, the cells were detached by pipetting and Trizol® solution containing the cell lysate in the well was transferred into a 2 mL eppendorf tube. 200 µL of chloroform was added to tube and the tube was shaken vigorously. The tube was centrifuged

at 12000 x g for 15 minutes at 4°C which produce three layers. The upper aqueous phase containing RNA was taken and same amount of cold isopropanol was added into the tube and the tube was shaken gently. The mixture was incubated at room temperature for 10 minutes. Then, it was centrifuged at 12000 x g for 20 minutes at 4°C. The supernatant was removed and the pellet was mixed with 1 mL of 75% ethanol. The tube was centrifuged again at 7500 x g for 5 minutes at 4°C; the pellet was taken and excess amount of ethanol was evaporated in hood. Finally, RNA was dissolved in 25 μL of nuclease-free distilled water and stored at -80°C.

2.2.5.2 Determination of RNA Concentration

Concentration of the isolated RNA was quantified by measuring the absorbance at 260 nm. Purity was assessed by the 260/280 nm ratio. The ratio of OD260/OD280 must be between 1.8 and 2.2. Below 1.8 refers the DNA contamination while above 2.2 referring the protein contamination. The optical density of 1.0 corresponded to the 40 μg/mL for RNA. The concentration and purity of the RNA were measured at NanoDrop™ 2000 (Thermo Scientific).

2.2.5.3 Qualification of RNA Molecules by Agarose Gel Electrophoresis

Presence and the purity of RNA were checked on 1% (w/v) agarose gel by using horizontal agarose gel electrophoresis unit. 1% (w/v) agarose was prepared by mixing 1 g of agarose with 100 ml 0.5 X Tris –Borate-EDTA (TBE) buffer, pH 8.3. The agarose was dissolved in a microwave oven. The solution was cooled approximately 60°C. 7 μL of ethidium bromide solution (10 mg/mL) was added and the solution was mixed thoroughly. Agarose gel solution was poured into electrophoresis tray and the comb was placed for well formation. After the gel polymerization, gel tank was filled with 0.5 X TBE buffer. The comb was removed. 5 μL of RNA solution was mixed with 1 μL of 6 X loading dye and the mixture was loaded into wells.

Electrophoresis was performed at 90 mV for 1 hour. The gel was observed and photographed under UV light.

2.2.5.4 cDNA Synthesis

Reagents:

5X Reaction Buffer : 250 mM Tris-HCl pH 8.3, 250 mM KCl, 20 mM $MgCl_2$ and 50 mM DDT

M-MuLV-RT: Moloney-Murine Leukemia Virus Reverse Transcriptase

Ribolock: RNase inhibitor

dNTP: Deoxyribonucleotide triphosphate (10mM)

Reverse transcription of RNA to cDNA was performed by mixing 1 µg of total RNA isolated from cell lines and 1 µL of oligo dT primer (Fermentas, Hanover, MD, USA) in an eppendorf tube. The final volume of the mixture was completed to 12 µL with nuclease-free distilled water. The solution was mixed gently and spinned down by microfuge. Mixture was incubated at 70°C for 5 minutes and it was chilled on ice. After that, 4 µL of 5X reaction buffer, 1 µL Ribolock and 2 µL of 10 mM dNTP were added. The tube was mixed gently and spinned down by microfuge. It was incubated at 37°C for 1 hour. Finally, the reaction was stopped by keeping at 70°C for 10 minutes and chilled on ice. cDNA was stored at -20°C for further use.

2.2.5.5 Quantitative Real-Time PCR

The expressions of CYP17A1, Hexokinase II and GSTP genes in cell lines were analyzed by quantitative Real Time PCR (qRT-PCR) using Corbett Rotor Gene 6000 (Corbett life Science, PO Box 435, Concorde, NSW 2137).

The 25 µL of final reaction mixture containing 100 ng cDNA, 0.5mM reverse and forward primers and 1 X Maxima® SYBR Green qPCR Master Mix (Fermentas, Glen, Burnie, MD) and RNase free distilled water. In order to detect any contamination, no template control (NTC) was used. As an internal standard, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene was used. The DNA amplification was carried out in a reaction mixture containing specific nucleotide sequence for related gene is given in Table -- The qRT-PCR program consisted of the following cycling profile; initial melting at 95 °C for 10 minutes, amplification and quantification program repeated 45 times containing melting at 95 °C for 20 seconds, annealing at 58-60 °C (depending on the gene) for 20 seconds and extension at 72 °C for 20 seconds with a single fluorescent measurement. After cycling, melting curve program 50-99 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement was added. Melting curve analysis of the amplification product was done at the end of each amplification reaction to confirm the detection of a PCR product. Quantities of specific mRNAs in the sample were measured according to corresponding gene and relative standard curve method. In each assay, a standard curve was calculated concurrently with the control and the quercetin treated cells. Each Standard curve was derived from dilution series (1:10, 1:100, 1:500, 1:1000, 1:5000) of selected standard cDNA for each gene. Light cycler quantification software was used to draw the standard curve.

2.3 Statistical Analysis

Statistical analyses were performed by using GraphPad Prism version 6 statistical software package for Windows. All results were expressed as means with their Standard Deviation (SD). Unpaired, two-tailed ANOVA test and $p<0.05$ were chosen as the level for significance.

Gene	Forward Primer (5'→ 3')	Reverse Primer (5' → 3')	Annealing Temperature (°C)	Product Size (bp)
GAPDH	GAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG	58	157
GSTP	CTACACCGTGGTCTATTTCC	CAGGAGGCTTTGAGTGAGC	60	137
CYP17A1	GCCGCACACCAACTATCAGTGAC	GCCCTTGTCCACAGCAAACTCAC	57	152
HEXOKİNASE II	GGCATCTTTGAAACCAAGTTCTTGTC	CACACCTCCTTAACAATGATGCTGTC	54	131

Table 2.4 Primer sequences, annealing temperatures and product sizes of the genes.

CHAPTER 3

RESULTS

3.1 Cell Culture

3.1.1 IC_{50} Determination for Cisplatin

In order to study the effects of cisplatin-metformine combinations, firstly the IC_{50} value of cisplatin was determined as described previously in methods section. Table 3.1 represents the percent AB reduction and percent survival values following the cisplatin treatment ranging from 5 to 40 μM for LNCaP cell lines and Table 3.2 ranging from 10 to 40 μM for PC3 cell lines.

Table 3.1 Percent AB reduction and percent survival values of the LNCaP cell.

Cisplatin Conc. (µM)	Percent Reduction	Percent Survival
0	57,0	100
5	47,5	83,3918
10	33,8	59,3465
15	36,2	63,40082
20	26,2	45,94568
25	14,3	25,15786
30	6,9	12,20808
35	5,03	8,820287
40	4,45	7,81297

Table 3.2 Percent AB reduction and percent survival values of the PC3 cell lines

Cisplatin Conc. (μM)	Percent Reduction	Percent Survival
0	69,20491	100
10	68,94814	99,62897
20	62,76469	90,69398
30	37,72169	54,50725
40	14,0595	20,31575

By using represented values, a cell proliferation graph was drawn and the equation of this graph was used in the IC_{50} value calculation. According to the calculations, IC_{50} value for cisplatin was determined as 17μM for LNCaP cell line and 30 μM for PC3 cell line respectively. Figure 3.1 and Figure 3.2 represent the cell proliferation and percent survival graphs for cisplatin treated LNCaP cells and Figure 3.3 and Figure 3.4 for cisplatin treated PC3 cells.

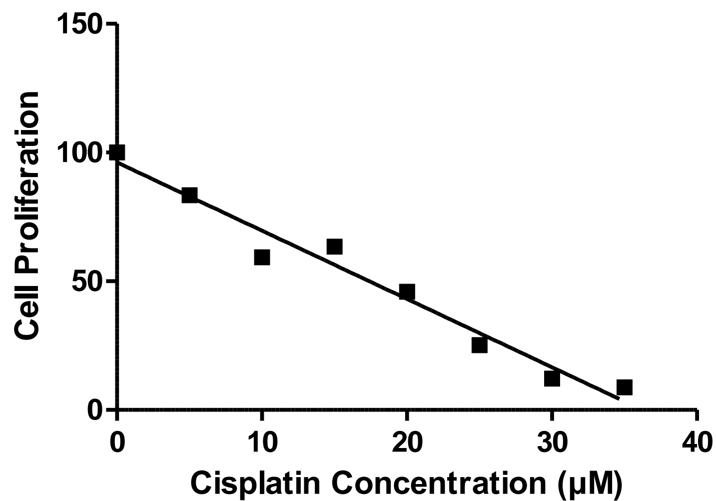

Figure 3.1 Cell proliferation graph for cisplatin treated LNCaP cells.

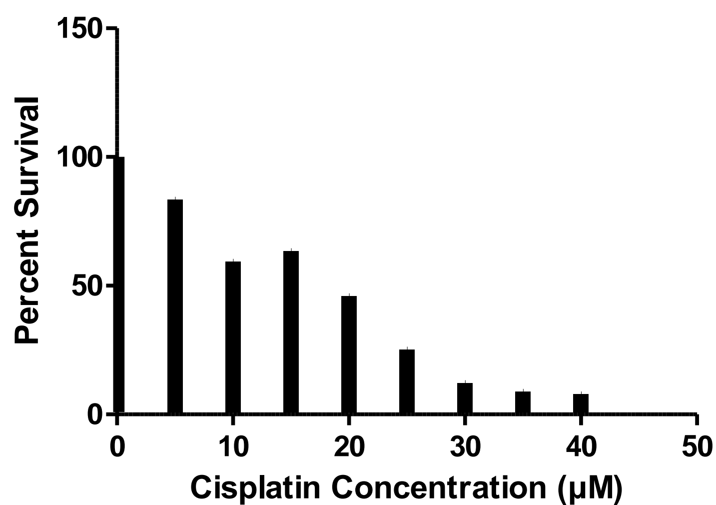

Figure 3.2 Percent survival graph for cisplatin treated LNCaP cells.

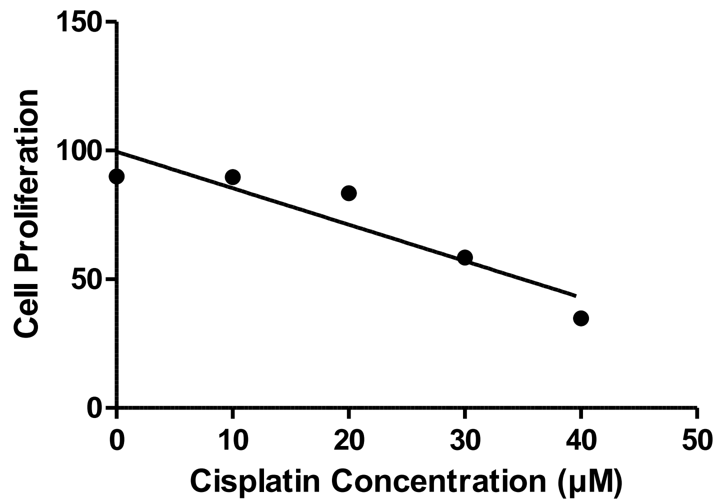

Figure 3. 3 Cell proliferation graph for cisplatin treated PC3 cells.

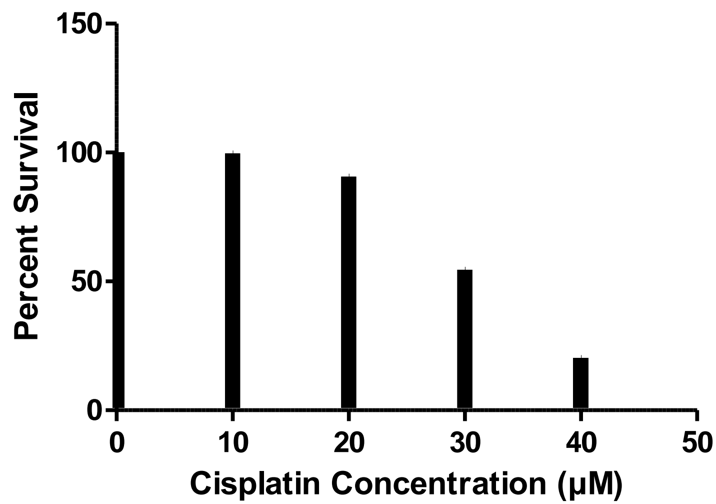

Figure 3.4 Percent survival graph for cisplatin treated PC3 cells.

3.1.2 Determination of Cisplatin-Metformin Combined Effect

After determination of cisplatin IC_{50} value , in order to demostrate the effect of cisplatin-metformin combination, cells were treated with IC_{50} value of cisplatin and at the same time different concentrations of metformine ranging from 1 to 10 mM. To control the single effects of drugs, cells were treated with drugs together or seperately as mentioned previously in methods section. After treatments, AB assay performed for cell proliferation. Percent AB reduction and percent survival values of LNCaP and PC3 cell lines shown in Table 3.3 and Table 3.4 respectively.

Table 3.3 Percent AB reduction and percent survival values of the LNCaP cell lines

Cisplatin Conc. (µM) Metformin Conc. (mM)	Percent Reduction	Percent Survival
Control	55,14221	100
Cisplatin (17µM)	23,78605	43,13583
1mM Metformin	57,08405	103,5215
5mM Metformin	55,30249	100,2907
10mM Metformin	37,77657	68,50755
Cisplatin (17µM) + 1mM Metformin	31,21129	56,60145
Cisplatin (17µM) + 5mM Metformin	25,2039	45,7071
Cisplatin (17µM) + 10mM Metformin)	11,01611	19,97764

Table 3.4 Percent AB reduction and percent survival values of the PC3 cell lines

Cisplatin Conc. (µM) Metformin Conc. (mM)	Percent Reduction	Percent Survival
Control	48,47224	100
Cisplatin (30µM)	25,29033	52,17487
1mM Metformin	47,39134	97,77007
5mM Metformin	34,8476	71,89186
10mM Metformin	22,88033	47,20295
Cisplatin (30µM) + 1mM Metformin	23,286	48,03986
Cisplatin (30µM) + 5mM Metformin	18,17242	37,49036
Cisplatin (30µM) + 10mM Metformin)	2,116424	4,366259

According to the values, which are represented in Table 3.3 and Table 3.4, combined or seperate treatment of drugs both inhibited the proliferation of LNCaP and PC3 cells in a concentration dependent manner. In fact, cisplatin-metformin combination groups significantly decrease the cellular proliferation both LNCaP and PC3 cell lines. Figure 3.5 and Figure 3.6 represent the cell proliferation graphs for cispl atin/metformin treated cells respectively.

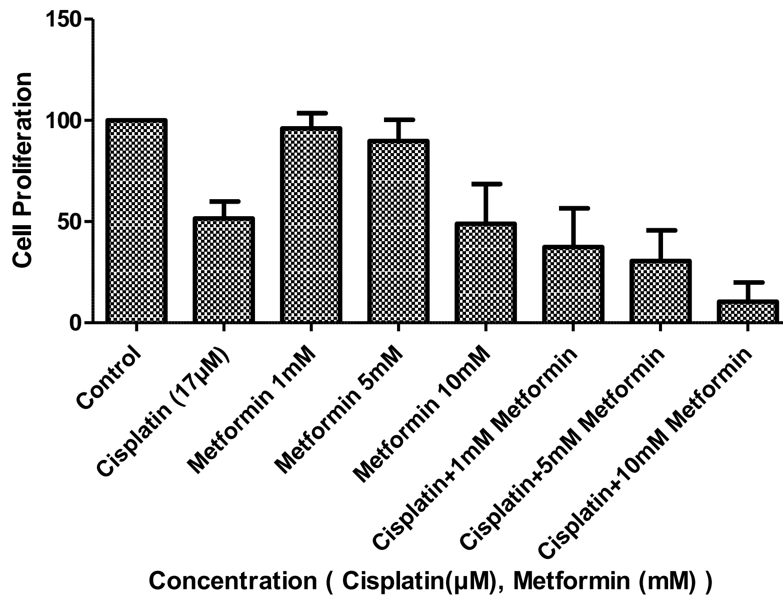

Figure 3.5 Cell proliferation graphs for cisplatin/metformin treated LNCaP cells

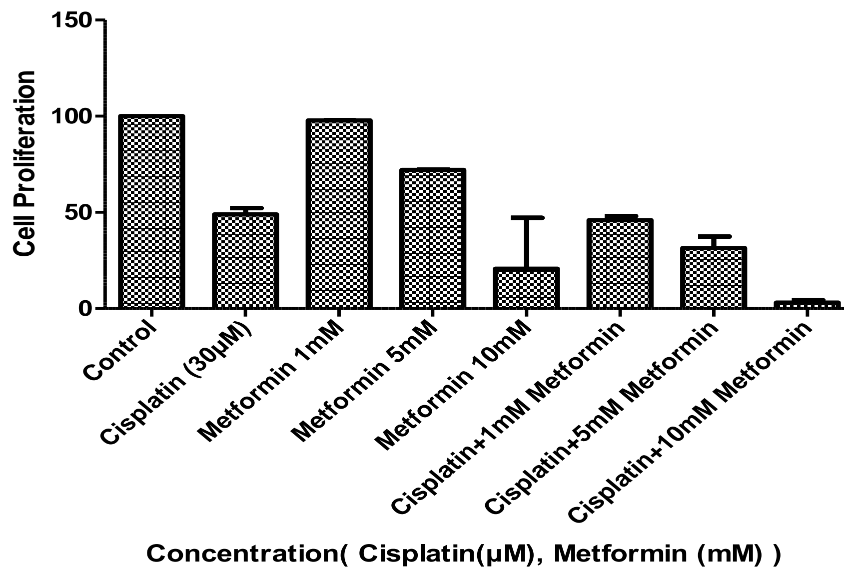

Figure 3.6 Cell proliferation graphs for cisplatin/metformin treated LNCaP cells

3.2 Protein Expression Analysis of CYP17A1 and GSTP1 Enzymes in LNCaP and PC3 Cells

CYP17A1 and GSTP enzyme expressions in prostate cancer cell lines LNCaP and PC3 were determined by Western Blotting. Immunochemical detection was performed by appropriate specific antibodies. GAPDH (37 kDa) was used as internal standard. All band intensity comparisons quantified by using Image J visualization software and One-way ANOVA method used to perform comparison analysis of protein expression and the level of significance was chosen as $p<0.05$. The band quantifications are expressed as mean ± SD and experiments were carried out in triplicate.

Each well is loaded with 30 µg protein. All experiments proteins have been loaded in the same order as; **Lane 1-3:** Control, **Lane 4-6:** Cisplatin IC50 (17µM for LNCaP cell lines and 30 µM for PC3 cell lines), **Lane 7-9** Metformin 1mM, **Lane 10-12** Metformin 5 mM, **Lane 13-15** Cisplatin + 1mM Metformin**, Lane16-18** Cisplatin + 5mM Metformin.

3.2.1 CYP17A1 Protein Expression in the Control and Treated LNCaP and PC3 Cells

In order to perform immunochemical detection of CYP17A1 protein, primary rabbit polyclonal anti-CYP17A1 antibody (1/500 dilution) and an alkaline phosphatase (AP) conjugated secondary goat anti-rabbit antibody (1/2000 dilution) were used.

For LNCaP cell lines; immunoreactive bands (Figure 3.7), results of relative protein expressions (Figure 3.8) and band intensity comparisons (Table 3.6) were shown. Also for PC3 cell lines same techniques were performed and immunochemical bands and results of relative protein expressions shown in Figure 3.9 and Figure 3.10 respectively. The statistical analysis of protein expressions was shown in Table 3.7.

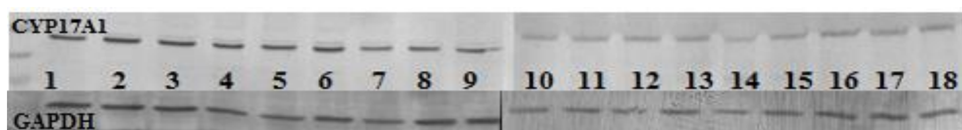

Figure 3.7 Immunoreactive protein bands of LNCaP cells representing the expression of CYP17A1 (57 kDa) and GAPDH (37 kDa).

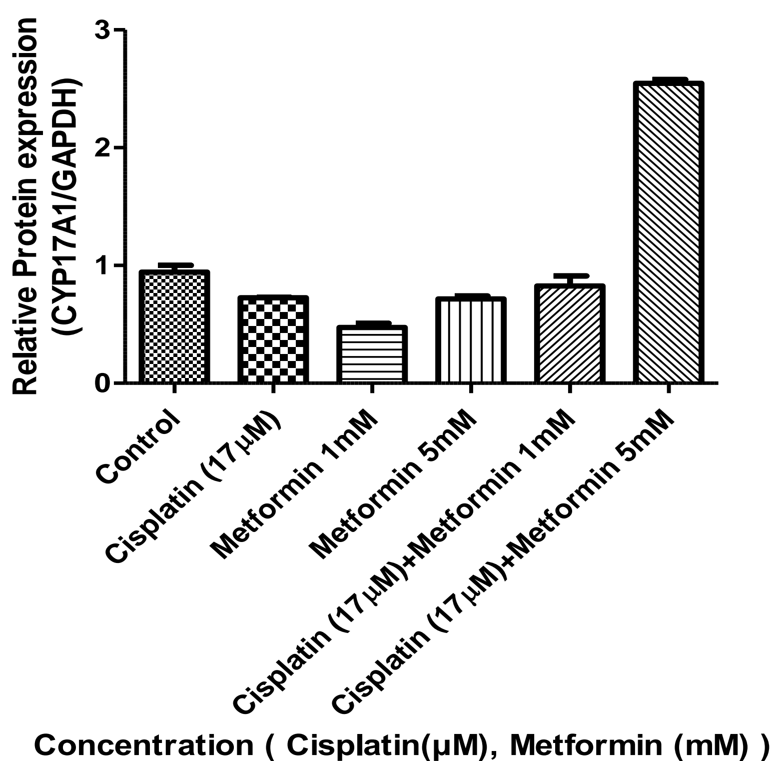

Figure 3.8 Comparison of CYP17A1 protein expression of control and treated LNCaP cells.

Table 3.4 Statistical analysis of CYP17A1 protein expression of LNCaP cell line.

	Cisplatin	**Metformin 1mM**	**Metformin 5mM**	**Cisplatin+ Metformin 1mM**	**Cisplatin + Metformin 5mM**
Control	NS	*	NS	NS	****
Cisplatin		NS	NS	NS	****
Metformin 1mM			NS	*	****
Metformin 5mM				NS	****
Cisplatin + Metformin 1mM					****

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; NS, not significant

Figure 3.9 Immunoreactive protein bands of control and treated PC3 cells representing the expression of CYP17A1 (57 kDa) andGAPDH (37 kDa).

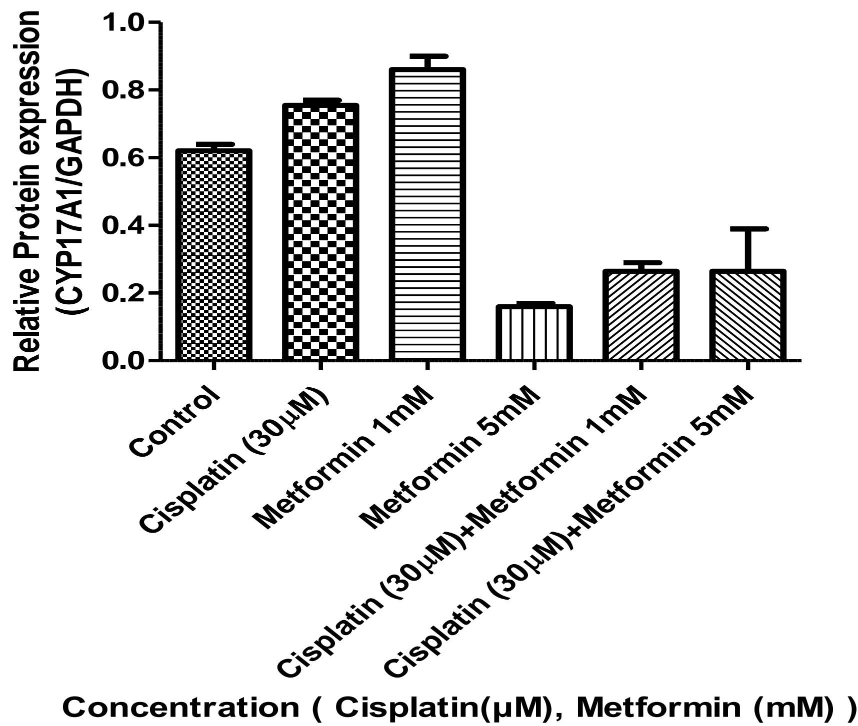

Figure 3.10 Comparison of CYP17A1 protein expression of control and treated PC3 cells.

Table 3.5 Statistical analysis of CYP17A1 protein expression of PC3 cell line.

	Cisplatin	Metformin 1mM	Metformin 5mM	Cisplatin + Metformin 1mM	Cisplatin + Metformin 5mM
Control	NS	NS	**	**	*
Cisplatin		NS	***	**	**
Metformin 1mM			***	***	***
Metformin 5mM				NS	NS
Cisplatin + Metformin 1mM					NS

* P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

3.2.2 GSTP1 Protein Expression in the Control and Treated LNCaP and PC3 Cells

As mentioned in the Chapter 1, the regulatory role of GSTP enzymes on cell proliferation and apoptotic pathways are based on the principles of dimerization of these enzymes. In this study, it's desired that, the induction of apoptosis triggered by drug combination in direct proportion to formation of dimeric bands (46 kDa).

In this information line, In order to perform immunochemical detection of GSTP1 proteins, primary goat polyclonal anti- GSTP1 antibody (1/1000 dilution) and a horseradish peroxidase (HRP) conjugated secondary mouse anti-goat antibody (1/3000 dilution) was used.

For LNCaP cell lines; immunoreactive bands (Figure 3.11), results of relative protein expressions (Figure 3.12) and band intensity comparisons (Table 3.8) were shown. Also for PC3 cell lines same techniques were performed and immunochemical bands and results of relative protein expressions shown in Figure 3.13 and Figure 3.14 respectively. The statistical analysis of protein expressions was shown in Table 3.9

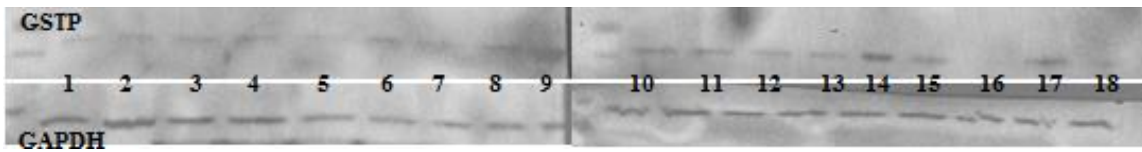

Figure 3.11 Immunoreactive protein bands of LNCaP cells representing the expression of GSTP1 (23 kDa) and GAPDH (37 kDa).

As shown in Figure 3.11 dimeric form of GSTP could not obtained in LNCaP cell lines.

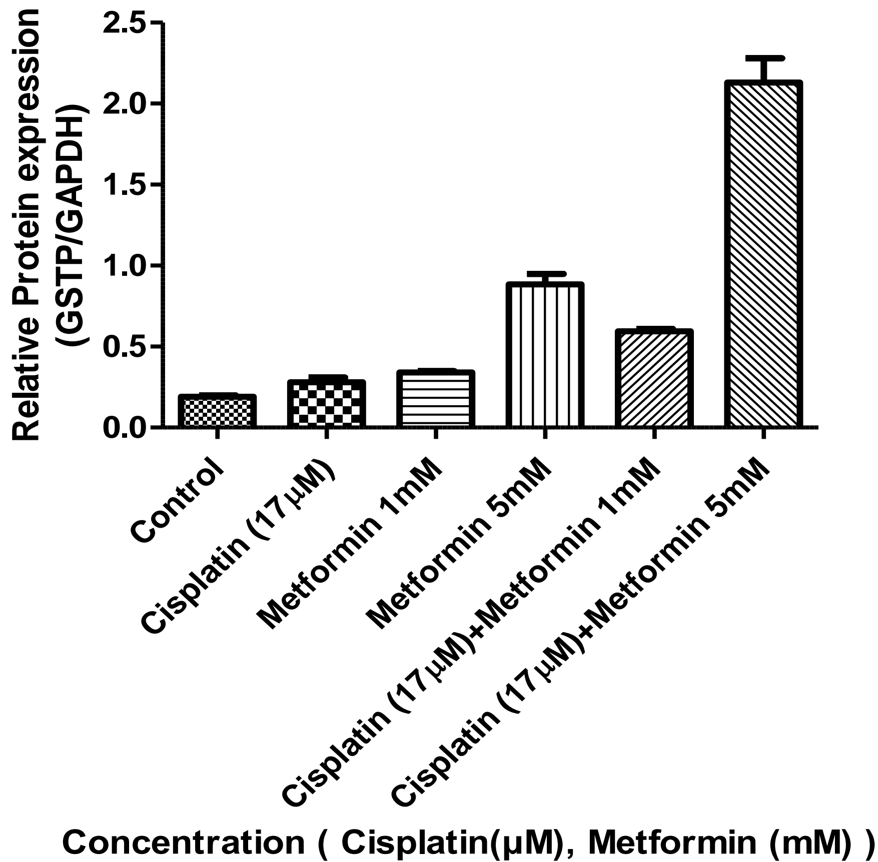

Figure 3.12 Comparison of GSTP1 protein expression of LNCaP cell

Table 3.6 Statistical analysis of GSTP protein expression of LNCaP cell line.

	Cisplatin	**Metformin 1Mm**	**Metformin 5mM**	**Cisplatin + Metformin 1mM**	**Cisplatin + Metformin 5mM**
Control	NS	NS	***	*	****
Cisplatin		NS	**	NS	****
Metformin 1mM			**	NS	****
Metformin 5mM				NS	****
Cisplatin + Metformin 1mM					****

* P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

Figure 3.13 Immunoreactive protein bands of PC3 cells representing the expression of GSTP1 (23 kDa), apoptotic bands of GSTP (46 kDa) and GAPDH (37 kDa).

According to Figure 3.13 dimeric form of GSTP obtained in PC3 cell lines. However, appearance of these dimeric bands (46kDa) disappeard by following combined tretments.

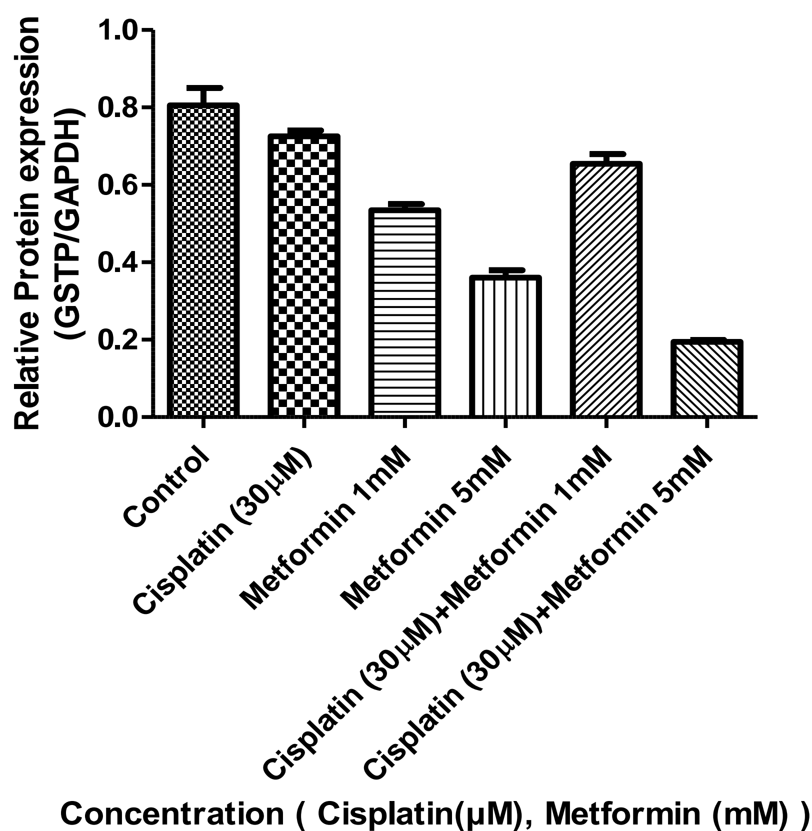

Figure 3.14 Comparison of GSTP1 protein expression of PC3 cells.

Table 3.7 Statistical analysis of GSTP protein expression of PC3 cell line.

	Cisplatin	Metformin 1mM	Metformin 5mM	Cisplatin + Metformin 1mM	Cisplatin + Metformin 5mM
Control	NS	**	***	NS	****
Cisplatin		*	***	NS	****
Metformin 1mM			*	NS	***
Metformin 5mM				**	*
Cisplatin + Metformin 1mM					***

* P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

3.3 CYP17A1, GSTP1 and Hexokinase II mRNA Expressions in the Control and Treated LNCaP and PC3 Cell Lines

In order to see the effects of drug combinations on gene expression level, mRNA expression was determined by quantitative real time PCR (qRT-PCR) technique. Total RNA isolatin was performed by Trizol© method as described in the method section. The purity, concentration and integrity of isolated RNA were checked by NanoDrop. After that, cDNA synthesis was carried out by using oligo dT primers.

For all separate genes, the standart curve, amplification curve and melting curves were generated (Datas are located in the Appendix session). The **standard curve** was generated from 1:10, 1:100, 1:500, 1:1000, and 1:5000 diluted cDNAs of the control cells used for mRNA quantifications of the samples. The **Amplification curve** showing the accumulation of fluorescence emission at each reaction cycle and **Melting curve** shows the fluorescence emission change versus temperature, that direct detection of single peak means single PCR product.

The results were normalized with internal standard GAPDH. In order to determine the relative mRNA expressions Livak method (Livak, 2001) was used. Formulation for Livak ($2^{-\Delta\Delta ct}$) method is given in Table 3.10.

Table 3.8 The Livak method for the calculation of relative mRNA expression using Ct values.

	Control	**Cisplatin**	**Metformin 1mM**	**Metformin 5mM**	**Cisplatin + Metformin 1mM**	**Cisplatin + Metformin 5mM**
$Ct_{Hexokinase II}$	20,7	22,206	24,53	20,55	22,15	23,86
Ct_{GAPDH}	14,8	14,5	17,68	13,71	14,28	15,24
ΔCt_{Cells} =	$Ct_{GAPDH} - Ct_{HexokinaseII}$					
$\Delta Ct_{Reference}$ =	$Ct_{GAPDH} - Ct_{HexokinaseII}$					
ΔCt_{Cells} =	-5,97	-7,67	-6,85	-6,84	-7,87	-8,63
$\Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{Cells}$						
$\Delta\Delta Ct$ =	0,5	2,2	1,38	1,38	2,4	3,17
$2^{-\Delta\Delta Ct}$	0,78	0,22	0,67	0,39	0,19	0,14

After performed the qRT-PCR, the PCR products were loaded in to agarose gel in order to checked the accuracy of experiment. Bands' positions on the gel overlaps with expected size of the desired genes qRT-PCR products. The agarose gel band images are located in the appendix part.

3.3.1 CYP17A1 mRNA Expression in the Control and the Treated LNCaP and PC3 Cell Lines

Figure 3.15 shows the mean ± SD of the relative CYP17A1 mRNA expressions of LNCaP cells. Experiments were carried out in triplicate.

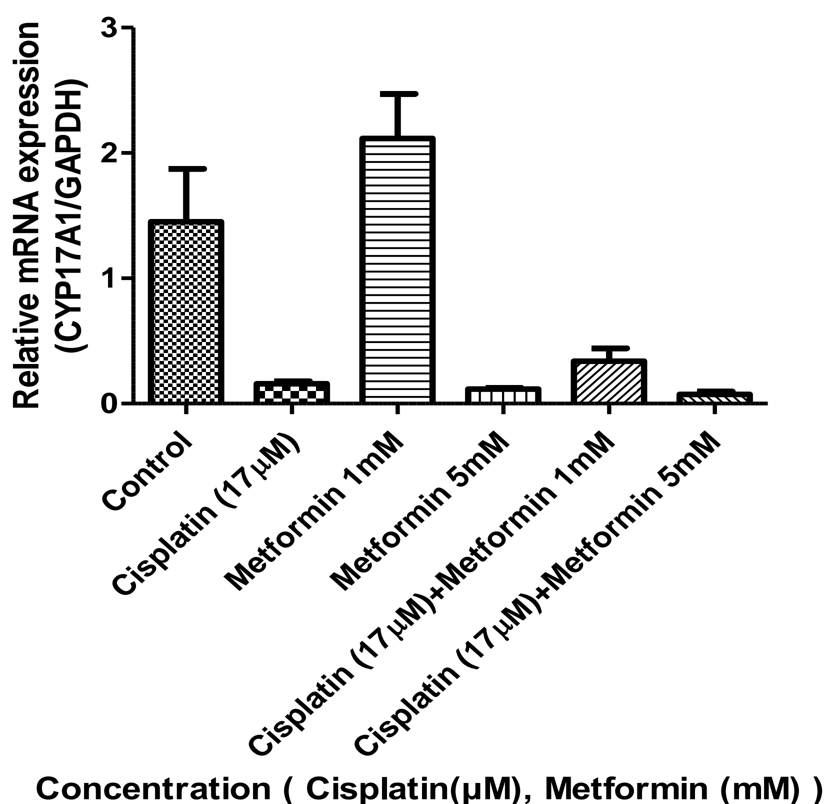

Figure 3.15 Comparison of CYP17A1 mRNA expressions of LNCaP cells.

The data was statistically analyzed by unpaired, two-tailed Anova test. According to statistical analysis, there is no significant alteration between treated and control groups of LNCaP cells.

Likewise, to observe the combined effect of drugs on CYP17A1 mRNA expressions of PC3 cells, same techniques were done by primers given previously in Table 2.4.

Figure 3.16 shows the mean ± SD of the relative CYP17A1 mRNA expressions of PC3 cells. Experiments were carried out in triplicate.

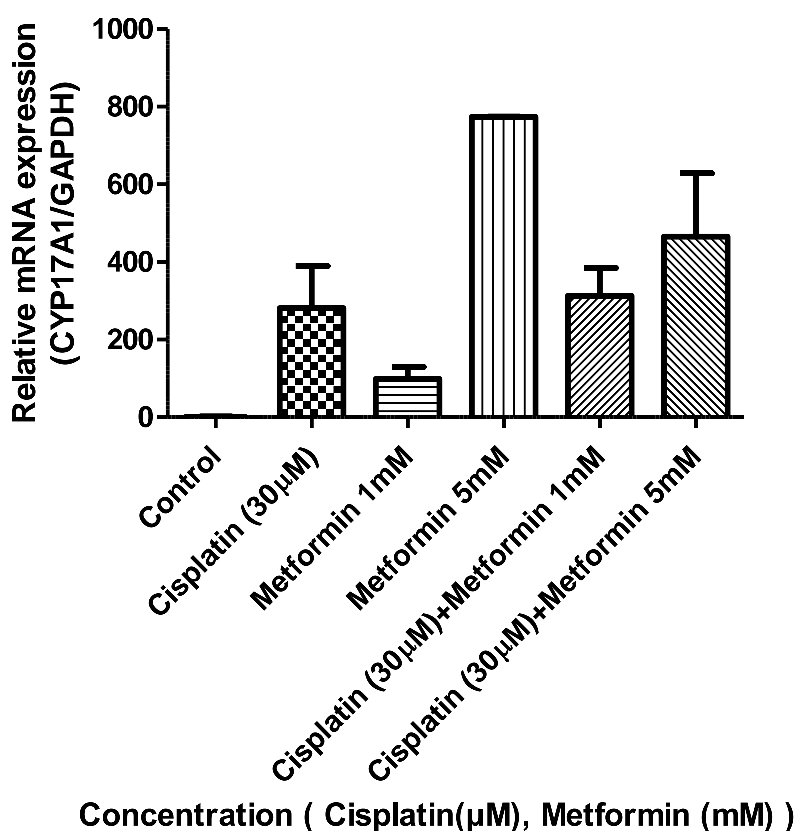

Figure 3.16 Comparison of CYP17A1 mRNA expressions between control and treated PC3 cells.

The data was statistically analyzed by unpaired, two-tailed Anova test. According to statistical analysis, there is no significant alteration between treated and control groups of PC3 cells.

3.3.2 GSTP mRNA Expression in the Control and the Treated LNCaP and PC3 Cell Lines

Figure 3.17 shows the mean ± SD of the relative GSTP mRNA expressions of control and treated LNCaP cells. Experiments were carried out in triplicate.

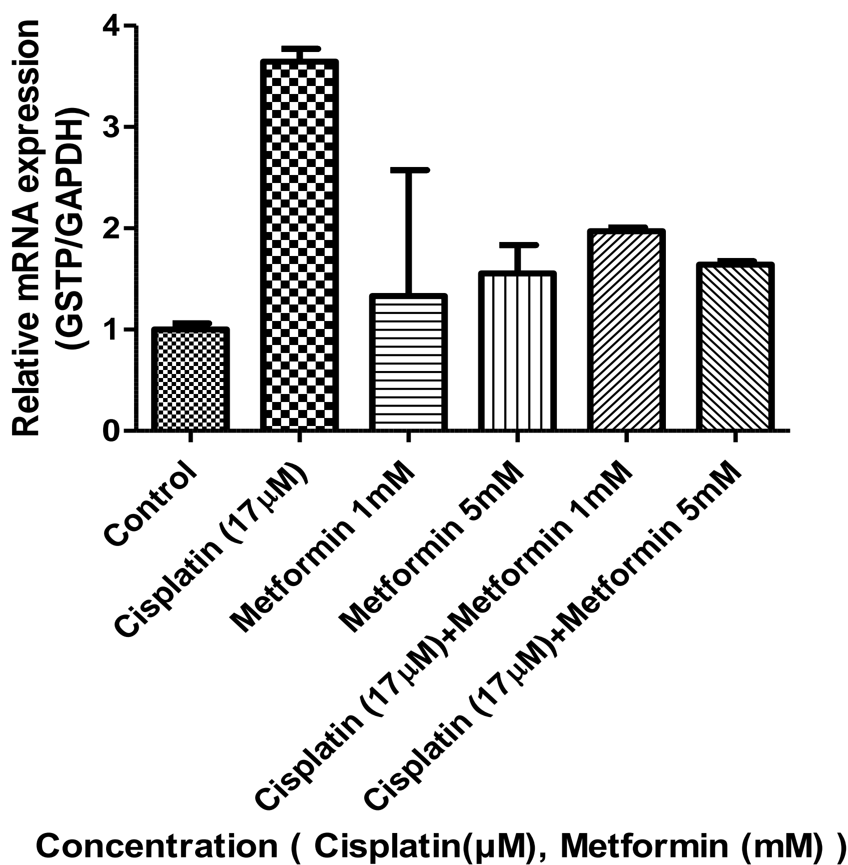

Figure 3.17 Comparison of GSTP1 mRNA expressions of LNCaP cells.

The data was statistically analyzed by unpaired, two-tailed Anova test. According to statistical analysis, there is no significant alteration between treated and control groups of PC3 cells' GSTP mRNA expressions.

Likewise, to observe the combined effect of drugs on GSTP mRNA expressions of PC3 cells, same techniques were done. Figure 3.18 shows the mean ± SD of the relative GSTP mRNA expressions of PC3 cells. Experiments were carried out in triplicate.

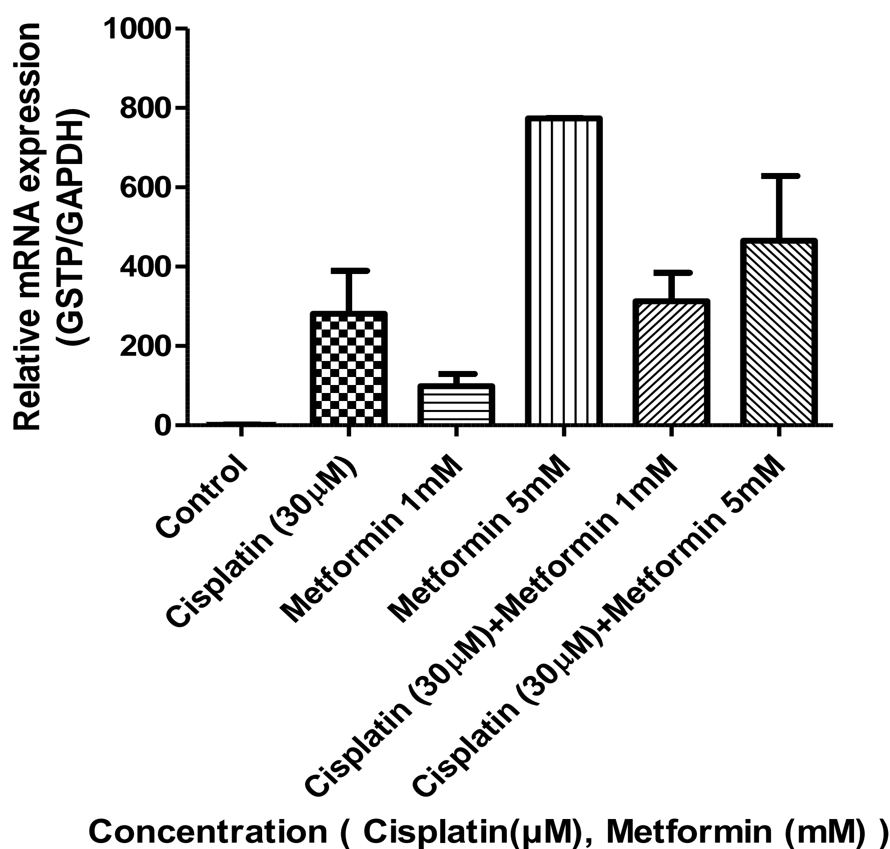

Figure 3.18 Comparison of GSTP1 mRNA expressions of PC3 cells.

The data was statistically analyzed by unpaired, two-tailed Anova test. Statistical analysis were shown in Table 3.11

Table 3.9 Statistical analysis of GSTP1 mRNA expression of PC3 cell line.

	Cisplatin	**Metformin 1mM**	**Metformin 5mM**	**Cisplatin + Metformin 1mM**	**Cisplatin + Metformin 5mM**
Control	NS	NS	NS	*	NS
Cisplatin		*	NS	**	*
Metformin 1mM			NS	NS	NS
Metformin 5mM				**	NS
Cisplatin + Metformin 1mM					NS

* P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

3.3.3 Hexokinase II mRNA Expression in the Control and the Treated LNCaP and PC3 Cell Lines

Figure 3.19 shows the mean ± SD of the relative Hexokinase II mRNA expressions of LNCaP cells. Experiments were carried out in triplicate. The datas were statistically analyzed by unpaired, two-tailed Anova test (Table 3.12).

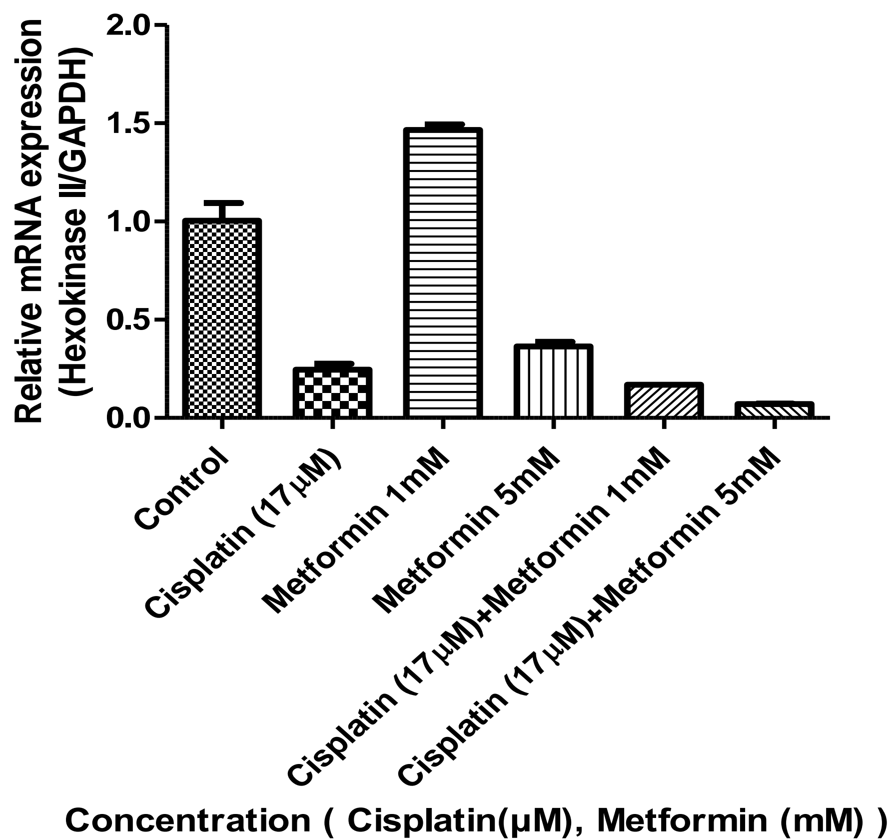

Figure 3.19 Comparison of HeksokinaseII mRNA expressions between control and treated LNCaP cells.

Table 3.10 Statistical analysis of Hexokinase II mRNA expression of LNCAP cell line.

	Cisplatin	Metformin 1mM	Metformin 5mM	Cisplatin + Metformin 1mM	Cisplatin + Metformin 5mM
Control	**	**	*	**	**
Cisplatin		NS	NS	NS	NS
Metformin 1mM			NS	NS	NS
Metformin 5mM				NS	NS
Cisplatin + Metformin 1mM					NS

* P≤0.05; ** P≤0.01; *** P≤0.001; **** P≤0.0001; NS, not significant

In order to observe the combined effect of drugs on Hexokinase II mRNA expressions of PC3 cells, same techniques were done. Figure 3.20 shows the mean ± SD of the relative Hexokinase II mRNA expressions of PC3 cells. Experiments were carried out in triplicate. The datas were statistically analyzed by unpaired, two-tailed Anova test. Results shown in Table 3.13

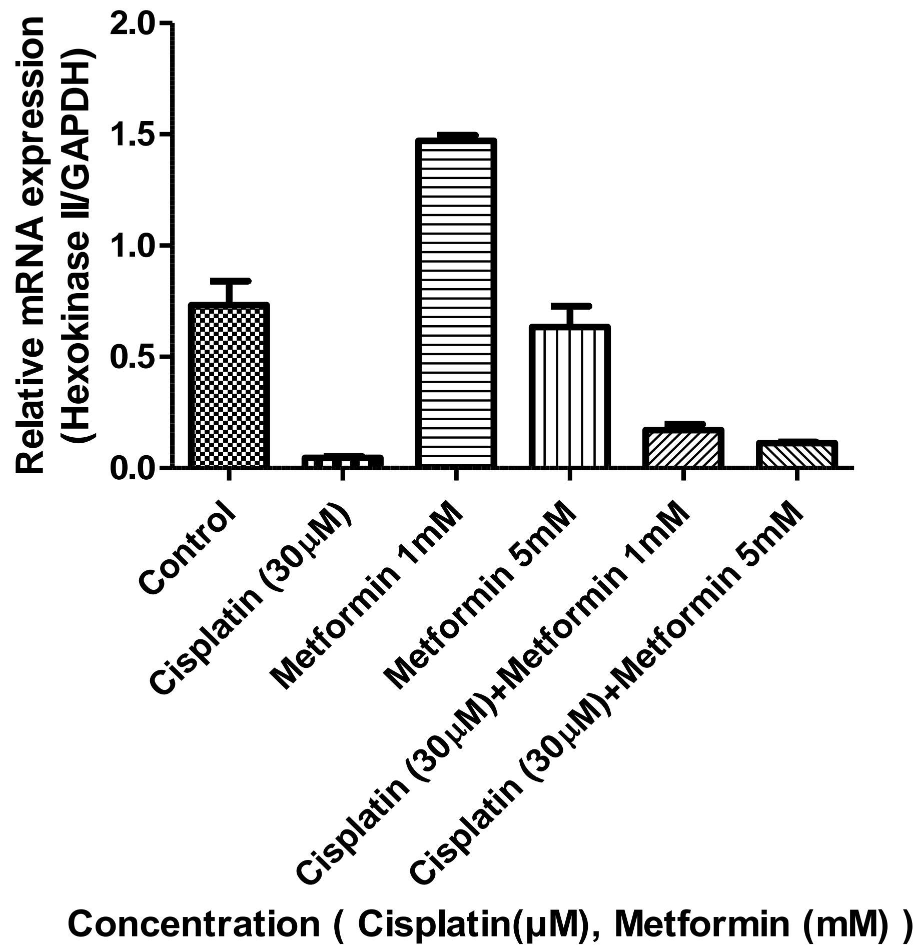

Figure 3.20 Comparison of HeksokinaseII mRNA expressions between control and treated PC3 cells.

Table 3. 11 Statistical analysis of Hexokinase II mRNA expression of PC3.

	Cisplatin (17µM)	**Metformin 1mM**	**Metformin 5mM**	**Cisplatin (17µM) + Metformin 1mM**	**Cisplatin (17µM) + Metformin 5mM**
Control	**	**	NS	**	**
Cisplatin (17µM)		****	**	NS	NS
Metformin 1mM			***	****	****
Metformin 5mM				*	*
Cisplatin (17µM) + Metformin 1mM					NS

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; NS, not significant

CHAPTER 4

DISCUSSION

Studies about prostate cancer prevalance suggest that, prostate cancer is the second most common solid neoplasm in both developed and developing countries. In order to reduced the side effects of current therapeutic strategies, using combination of different types of chemoterapeutics is still the major research topic for PC.

Platinum-based agents are very effective drugs for cancer chemotherapy because these drugs are used to treat almost 50% of cancer patients. Alkylating agents including, cisplatin, carboplatin and oxaplatin were platinum complexes that have two amine ligands and additional molecules that interract with DNA. Mechanism of action of these drugs usually start with the generation of platinum-DNA adducts. These bindigs blocks the cellular process and led to cellular apoptosis. Combination therapy using platinum drugs with other agents are major strategies develop to overcome existed problems in chemotherapy.

Cancer cells preferentially metabolize glucose and hyperglycaemia is considered a promotor of tumour growth. In this context, the anti-diabetic drug metformin is now being investigated as a potential treatment. Alone itself, metformin can reduce the cellular proliferation of cancer cells exampled in different kind of cancer types. In addition to its anticarcinogenic outcomes, adjuvant role of metformin is being investigated in combinations.

The most common problem in cancer treatment accepted as a resistance issue. Detoxification of drugs by GST enzymes, before they are acting, can cause

altered efficacy of anticancer agents. Its a clear evidence that there is a direct correlation between GST-overexpressing phenotypes and many drug-resistant tumors. But recent studies shows that several drugs, that are not substrate for GST enzymes, still effected by GST overexpression in tissues. This finding makes investigators suspicious about, these enzyme family has a different task beyond its detoxification process. Because of their ability to bind covalently and non covelently with various compound that are not substrates for anzymatic activity, its believed that, these enzymes can serve a regulatory role by organized the interractions of cytotoxic ligands with their targets.

Prostate cancer need to androgens that include testesterone and other male hormones to grow and survive. Androgen deprivation therapy (ADT) known as a hormone therapy for PC is a clinical trial that decreases the amount of androgens in a circulation. This kind of therapy reducing androgens can slow the growth of the cancer but at the same time can causes decreases in mental and emotional well-being while threatment. To reduce the side effects of this therapy, androgen production can blocked at the beggining of the sex steroid synthesis pathway by inhibitors that selectively inhibits Cytochrome P450, CYP17, a key enzyme in the production of androgens. From this point, inhibitors that selectively decrease the CYP17 activity can be useful for treatment of prostate cancer.

The fact that cancer cells express the glycolytic phenotype. Therefore modulation of glycolytic enzymes by diabetic drugs can be a good option for treatment of cancer cells. Many enzymes of the glycolytic pathway play a significant roles in several glycolytic or non-glycolytic processes. One of them, Hexokinase II, seems to be a strong target for antitumoral agents.

Because of these reasons, in this study, it was aimed to show the combined or seperate effect of antineoplastic agent cisplatin and antidiabetic drug metformin for targeting the enzymes participate in the apoptosis, androgen

metabolism and glucose metabolism. For that purpose protein and mRNA expression of these enzymes were analyzed in the human prostate cancer cell lines. The cell lines used in the study were selected due to their androgen receptor (AR) dependency. Its known that metformin reduces the AR transcriptional activity by downregulating AR proteins both in the AR dependent and independent cell lines. Especially metformin doesn’t effect the AR protein stability/degredation or nuclear stability, but by downregulating total AR mRNA levels. Wang and his coworkers suggest that, combination of low doses of metformin and bicalutamide (an anti-androgen) treatment can additively repress the growth of prostate cancer ceell prolifertion. To investigate the effect of this drugs more specifically androgen receptor dependent cell line LNCaP and androgen receptor independent cell line PC3 were used.

After calculation of cisplatin IC50 values both LNCaP (17μM) and PC3 (30μM) cell lines, the cultures were then treated with 1, 5, and 10 mM concentrations of metformin and IC50 concentrations of cisplatin. Results showed that, cellular proliferation decreased by concentration dependent manner. Its known that induction of apoptosis triggered by both of these drugs seperately. Starting from this point its investigated that, if apoptosis triggered by combination of these drugs, GSTP1 enzymes that participate in the apoptotic payhways can be a possible target of metformin. Its expected that, apoptosis will be observed after treatment of the cells, and it would be paralleled by the appearance of a dimeric form (ca. 46kDa) of GSTP1 and the intensification of its monomeric form (ca. 21.5kDa). However, in LNCaP cell line, dimeric form of GSTP can not observed. At the same time GSTP1 expressions increased, paralell with the increased concentration of metformin. Contrarily in PC3 cell lines, GSTP1 expressions shows a decline paralelled with the increased concentrations of metformin. Also, dimeric form of GSTP1 observed with treatments of metformin (1mM) but high concentration of metformin (5mM) led to moderate dimeric form and at the same time GSTP1

expressions on PC3 cell lines. These results suggest that, in contrast with our expectations, metformin can antagonize the cisplatin apoptotic effect through supression of oxidative stress in LNCaP cells. Parrelel with this idea, Jaevotic et al., showed that metformin could diminish the antineoplastic effect of SHY5Y, C6, U251, L929, and HL-60 cell lines through suppression of oxidative stress and inhibition of caspases activation (Jaevotic K. et al., 2011). Results of both protein and mRNA expressions LNCaP and PC3 cell lines seems to be support each other, PC3 cell line is more sensitive to the metformin than LNCaP cell lines. Thus, it is reasonable to conclude that the effect of metformin on cisplatin may be dependent to the cell type and possibly to the type of cancer.

By inducing expressions of lipogenic enzymes, up regulation of *de novo* lipid synthesis is the most seen change that regulated by androgens in PC cells (Moon J., et al., 2008). Because of the intermediary metabolites of glycolysis are the major carbon sources of lipid synthesis, lipogenesis directly controlled by glycolytic activity. It’s believed that, androgens play a regulatory role on glycolytic enzyme HKII. Although its unclear but, studies suggest that androgens upregulates the enzyme HKII that promotes glycolysis and directly *de novo* lipid synthesis. For this purpose, by this study its aimed that, the effects of metformin and/or cisplatin treatment on HKII enzyme and the key enzyme on androgen synthesis pathway CYP17A1.

Acritical step for highly glycolytic state is the phosphorilation of glucose by Hexokinase enzymes. Among the four hexokinase type, HKII is the overexpressed type in tumors. In fact, under hypoxic conditions, mounting evidence suggest that, HKII plays a regulatory role by promoting cellular proliferation, survival and enhancing biosynthesis and helping immortalization of the cells. Recent studies suggest that, motformin inhibits the mitochondrial respiratory chain and decreases the ATP concentration in prostate cancer cells, As menioned before, cisplatin exert its antitumorogenic

effect by producing DNA adducts and its believed that, low rate of cisplatin cytotoxicity has been correalated to the generation of mitochondrial reactive oxygen species (ROS) that influence multiple apoptotic pathways. Therefore, to evaluate the potential of metformin we decided to treat cells with cisplatin and metformin than we checked the HKII protein and mRNA expressions. Unfortunately, HKII antibody decomposed by unknown reasons. So study continued with qPCR analysis of this gene. According to mRNA expressions of the HKII gene significantly downregulating by cisplatin metformin combination treatment.

According to results represented in Figure 4.2 decline in the mRNA expressions of HKII gene may be the reason of decreasing cellular proliferaion of the cells by contrast with the GSTP1 gene. In paralell with this data, CYP17A1 enzyme and mRNA expressions were checked. CYP17A1 protein expressions significantly decreased by combined and seperate treatments of drugs. In addition to protein expressions, there is no significant difference between relative CYP17A1 mRNA expression in LNCaP and PC3 cell lines.

CHAPTER 5

CONCLUSION

This study is the first in vitro study that uses prostate cancer cell lines LNCaP and PC3 to investigate the effect of metformin–cisplatin combination on protein and mRNA expressions of GSTP1, CYP17A1 and Hexokinase II.

In conclusion, the results of this study showed that, metformin cisplatin drug combination demostrably decrease the cellular proliferation of prostate cancer cell lines. Also according to protein and mRNA expression results decline in the proliferation rate directly related with enzymes that we investigate. But, furder studies must have required to conclude that this drug combination has significant effects on prostate carcinoma. As well this study is imprtant because this application could be a challenge for cancer patients with diabetes type 2 which are treated with both cisplatin and metformin. Regarding these results, in order to conclude that, cytotoxic efficacy of cisplatin which is the most potent drug for treatment of cancer and traditionally used diabetic metformin ha not been clearly studied. But this studr considiring that, this drug combination has a high probbability to used for PC treatment.

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Jung, Y., & Lippard, S. J. (2007). Direct cellular responses to platinum-induced DNA damage. Chemical reviews, 107(5), 1387-407. American Chemical Society. doi:10.1021/cr068207j

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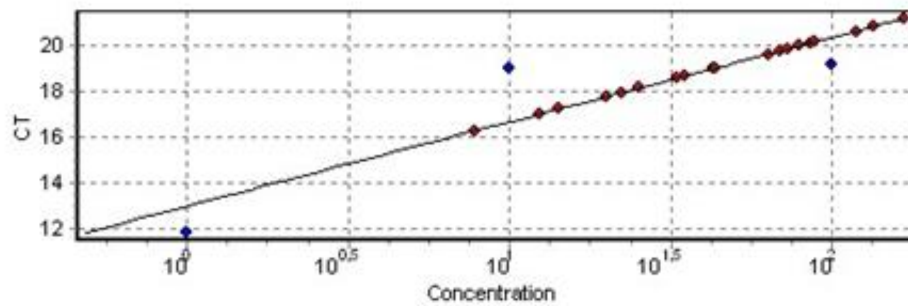
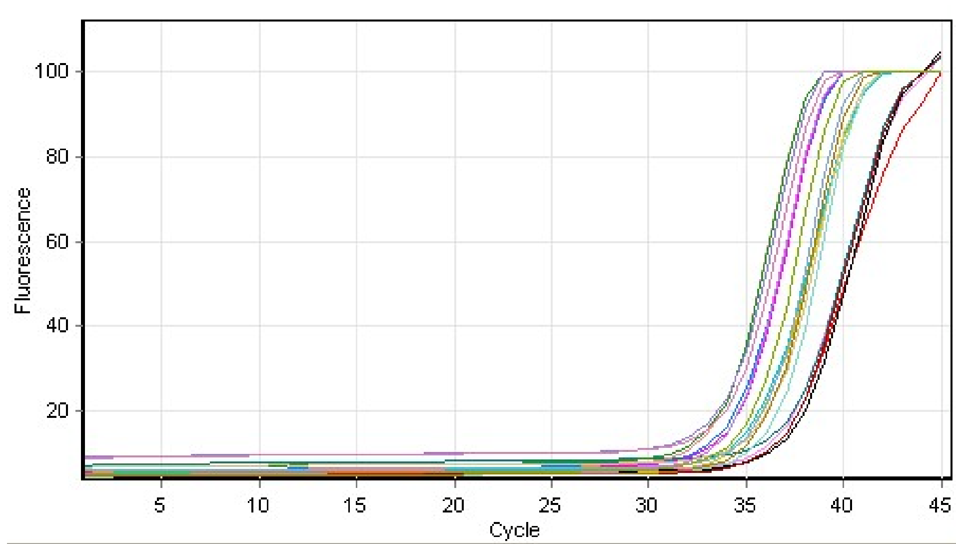
APPENDİX

A 1 Quantitative real time PCR (qRT-PCR) Curves and Agarose Gel Images of LNCaP and PC3 Cell Lines

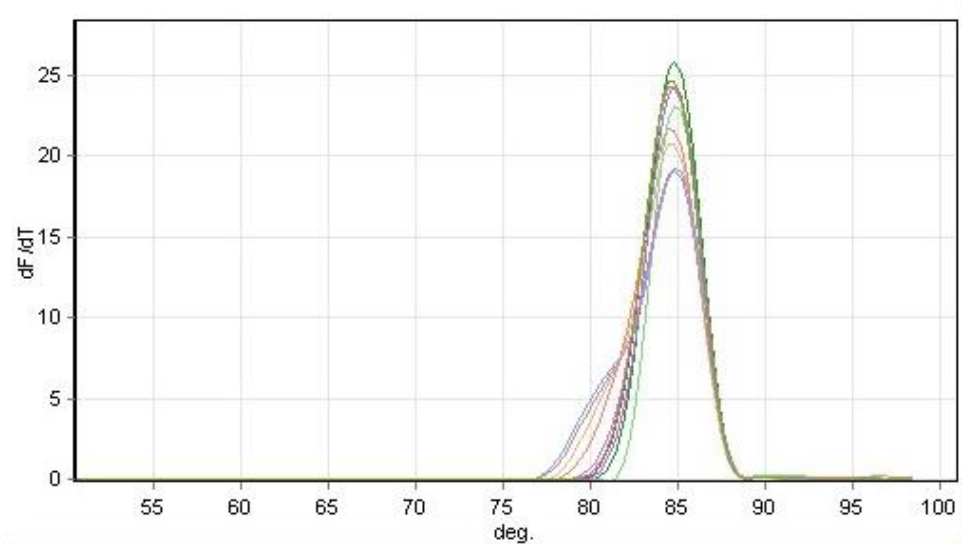
A 1.1 CYP17A1 mRNA Expression in the Control and the Treated LNCaP and PC3 Cell Lines



A 1.1 Standard curve generated from serial dilutions of control cDNA to calculate quantities of CYP17A1 mRNAs in the control and treated LNCaP cells relatively.

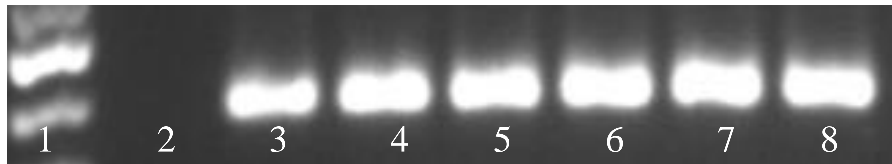


A 1.2 Amplification curve of LNCaP cells showing the accumulation of fluorescence emission at each reaction cycle.

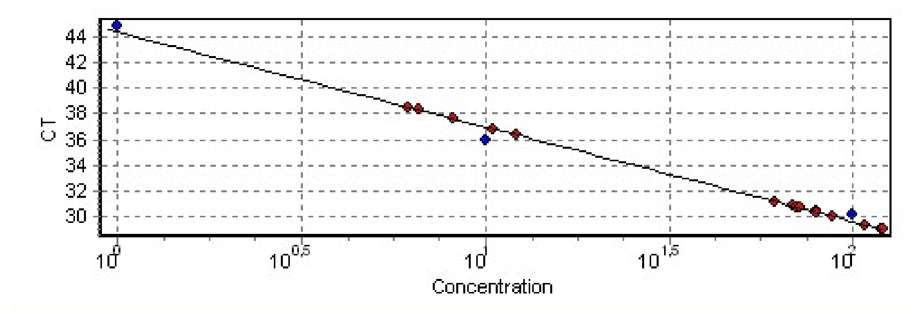


A 1.3 Melting curve showing the fluorescence emission change versus temperature of LNCaP cells. Detection of single peak means single PCR product.

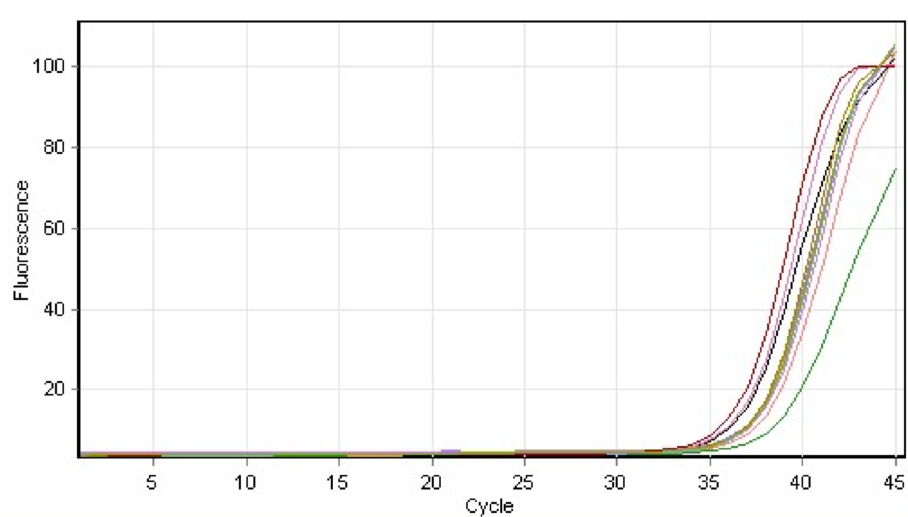
A 1.4 qRT-PCR products of CYP17A1 cDNA (152bp) of LNCaP cells. Lane 1 shows the bp markers and Lane 2 is the no template control (NTC). Lane 3: Control, Lane 4: Cisplatin (17µM), Lane 5: Metformin 1Mm, Lane 6: Metformin 5 mM, Lane 7: Cisplatin+1mM Metformin, Lane 8: Cisplatin+5mM Metformin. 5 µL of qRTPCR product was loaded in each well.



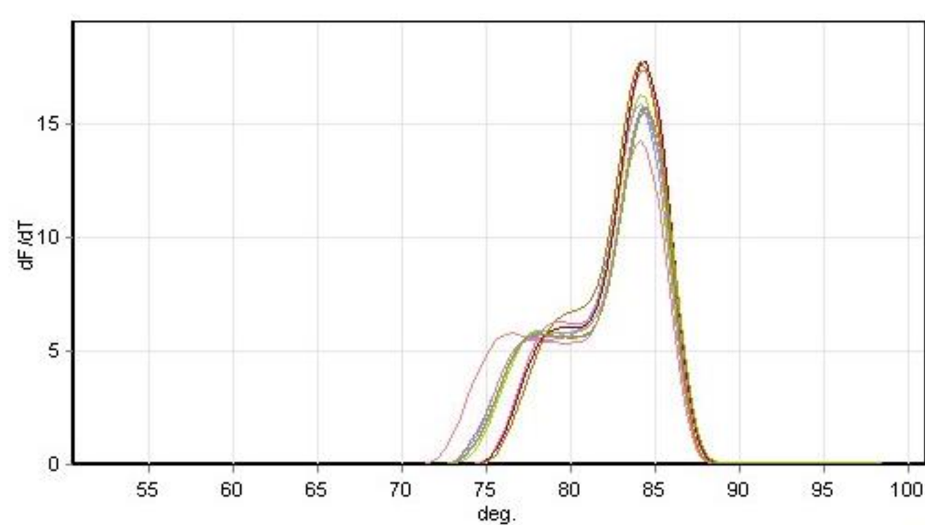
A 1.5 qRT-PCR products of GAPDH cDNA (197bp) of LNCaP cells. Lane 1 shows the bp markers and Lane 2 is the no template control (NTC). Lane 3: Control, Lane 4: Cisplatin (17µM), Lane 5: Metformin 1Mm, Lane 6: Metformin 5 mM, Lane 7: Cisplatin+1mM Metformin, Lane 8: Cisplatin+5mM Metformin. 5 µL of qRTPCR product was loaded in each well.



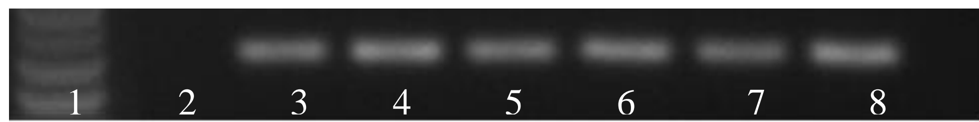
A 1.6 Standard curve generated from serial dilutions of control cDNA to calculate quantities of CYP17A1 mRNAs in the control and treated PC3 cells relatively.



A 1.7 Amplification curve of PC3 cells showing the accumulation of fluorescence emission at each reaction cycle.

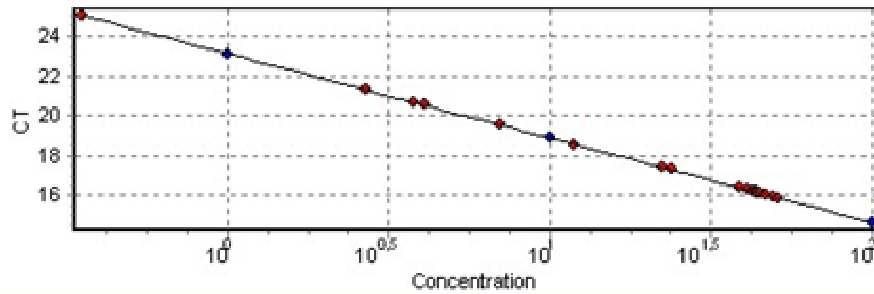


A 1.8 Melting curve showing the fluorescence emission change versus temperature of PC3 cells. Detection of single peak means single PCR product.

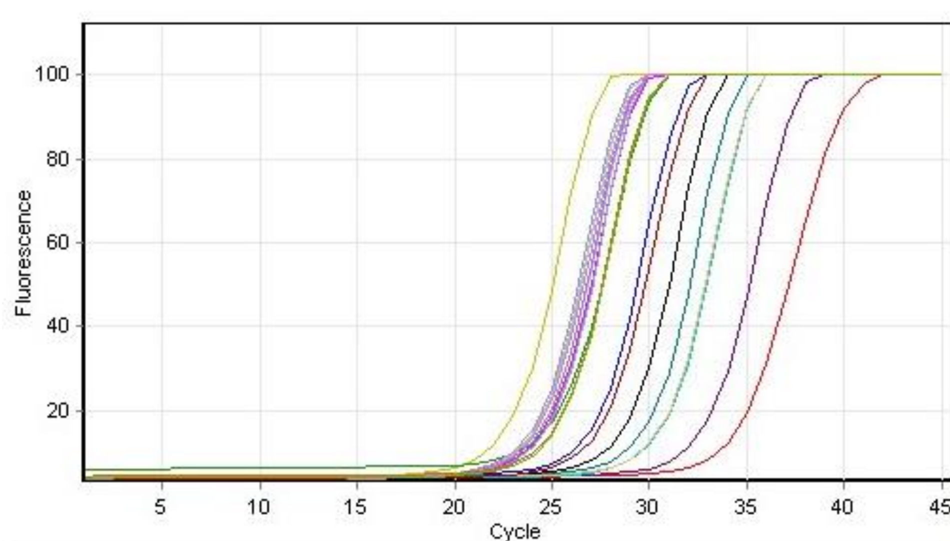


A 1.9 qRT-PCR products of CYP17A1 cDNA (152bp) of PC3 cells. Lane 1 shows the bp markers and Lane 2 is the no template control (NTC). Lane 3: Control, Lane 4: Cisplatin (17µM), Lane 5: Metformin 1Mm, Lane 6: Metformin 5 mM, Lane 7: Cisplatin+1mM Metformin, Lane 8: Cisplatin+5mM Metformin. 5 µL of qRTPCR product was loaded in each well.

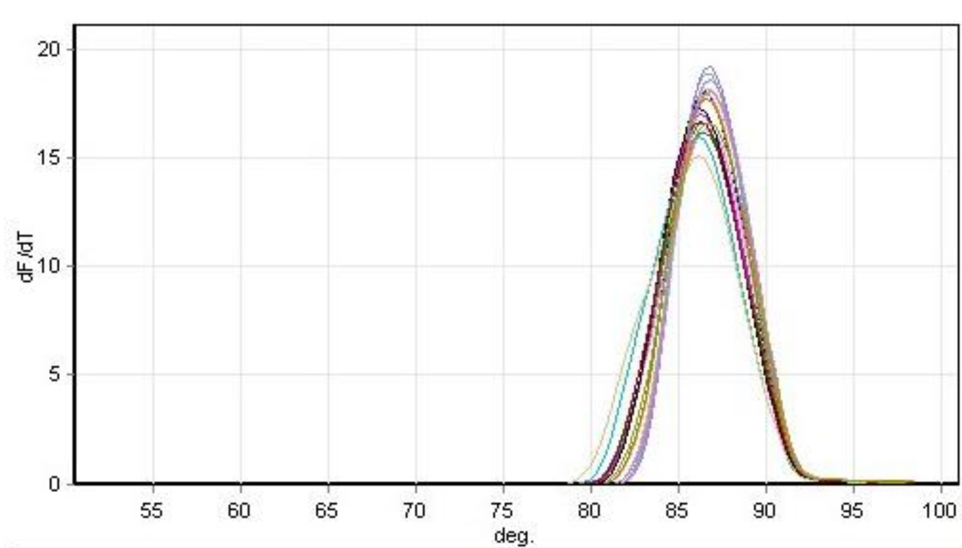
A 1.2 GSTP mRNA Expression in the Control and the Treated LNCaP and PC3 Cell Lines



A 1.10 Standard curve generated from serial dilutions of control cDNA to calculate quantities of GSTP mRNAs in the control and treated LNCaP cells relatively.

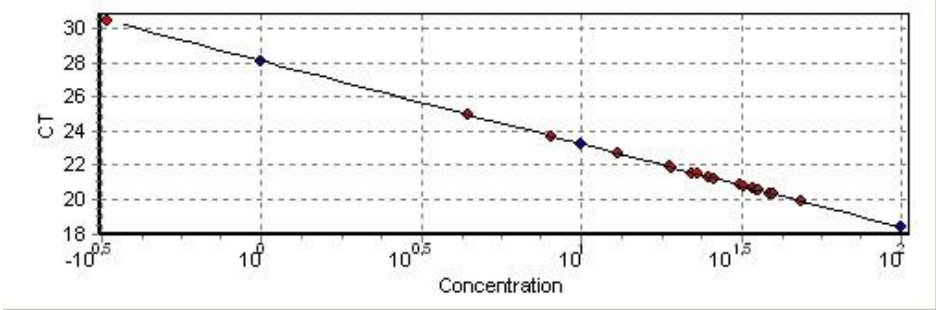


A 1.11 Amplification curve of LNCaP cells showing the accumulation of fluorescence emission at each reaction cycle.

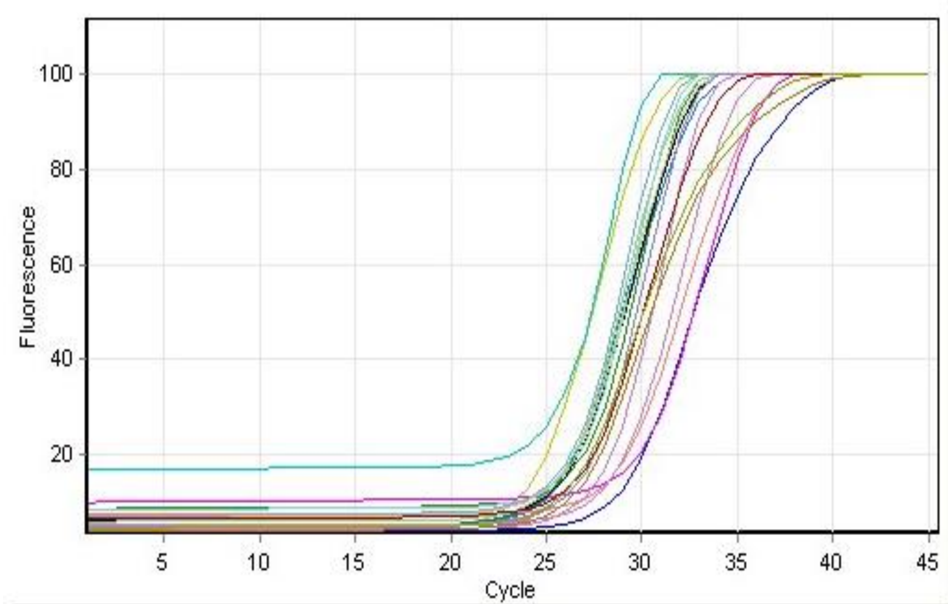


A 1.12 Melting curve showing the fluorescence emission change versus temperature of LNCaP cells. Detection of single peak means single PCR product.

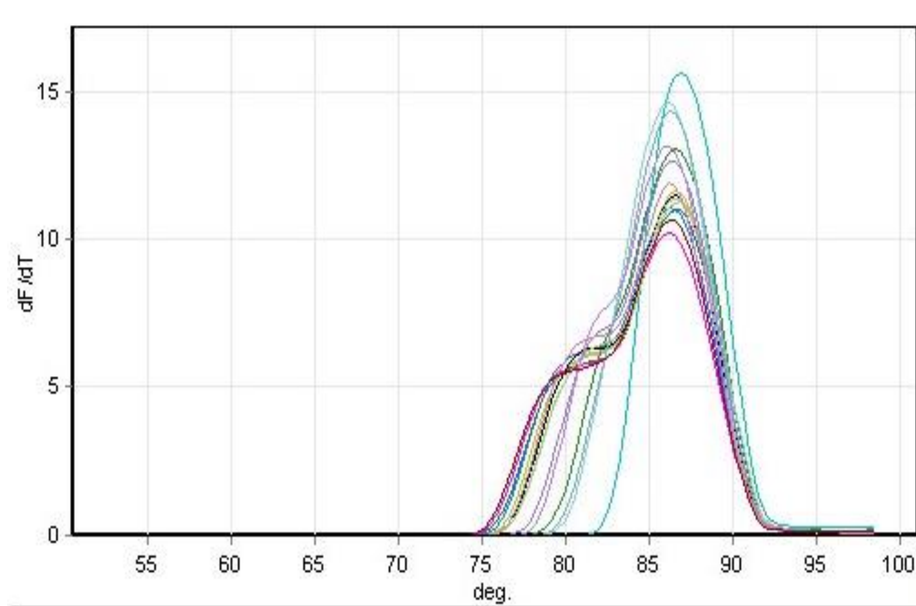
A 1.13 qRT-PCR products of GSTP1 cDNA (137bp) of LNCaP cells. Lane 1 shows the bp markers and Lane 2 is the no template control (NTC). Lane 3: Control, Lane 4: Cisplatin (17µM), Lane 5: Metformin 1Mm, Lane 6: Metformin 5 mM, Lane 7: Cisplatin+1mM Metformin, Lane 8: Cisplatin+5mM Metformin. 5 µL of qRTPCR product was loaded in each well.



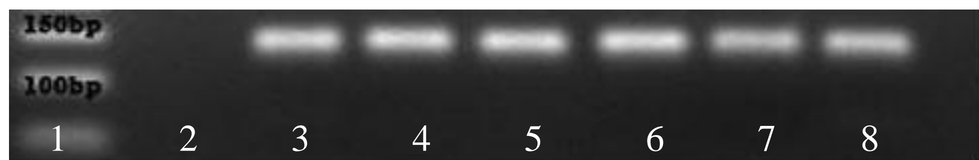
A 1.14 Standard curve generated from serial dilutions of control cDNA to calculate quantities of GSTP mRNAs in the control and treated PC3 cells relatively.



A 1.15 Amplification curve of PC3 cells showing the accumulation of fluorescence emission at each reaction cycle.

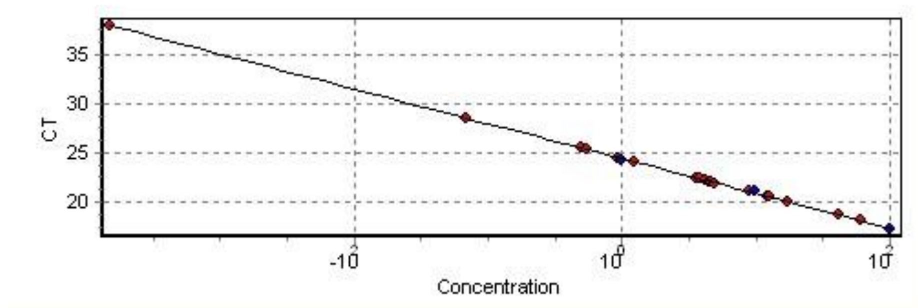


A 1.16 Melting curve showing the fluorescence emission change versus temperature of PC3 cells. Detection of single peak means single PCR product.

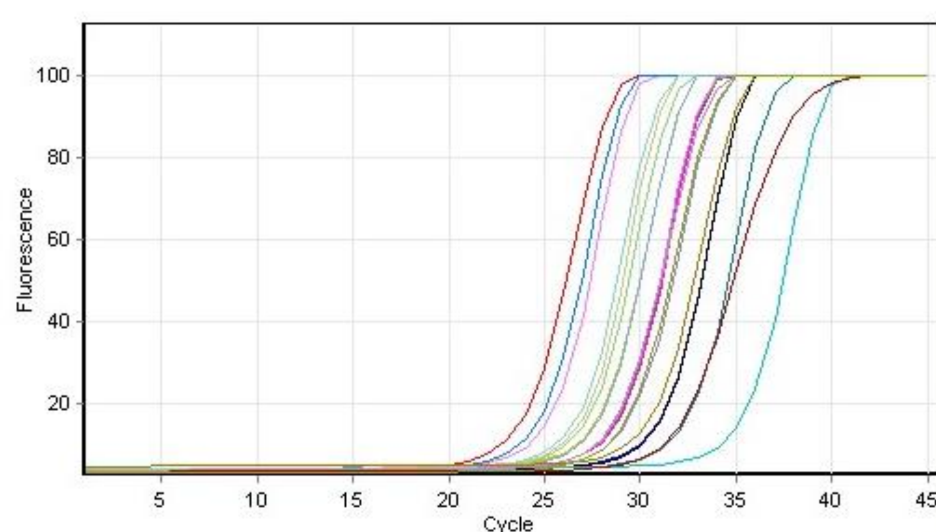


A 1.17 qRT-PCR products of GSTP1 cDNA (137bp) of GSTP cells. Lane 1 shows the bp markers and Lane 2 is the no template control (NTC). Lane 3: Control, Lane 4: Cisplatin (17µM), Lane 5: Metformin 1Mm, Lane 6: Metformin 5 mM, Lane 7: Cisplatin+1mM Metformin, Lane 8: Cisplatin+5mM Metformin. 5 µL of qRTPCR product was loaded in each well.

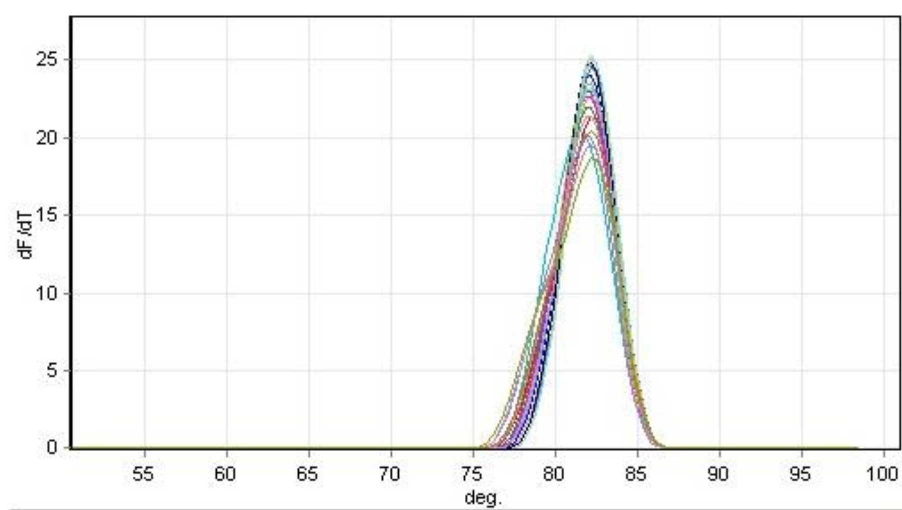
A 1.3 Hexokinase II mRNA Expression in the Control and the Treated LNCaP and PC3 Cell Lines



A 1.18 Standard curve generated from serial dilutions of control cDNA to calculate quantities of Hexokinase II mRNAs in the control and treated LNCaP cells relatively.

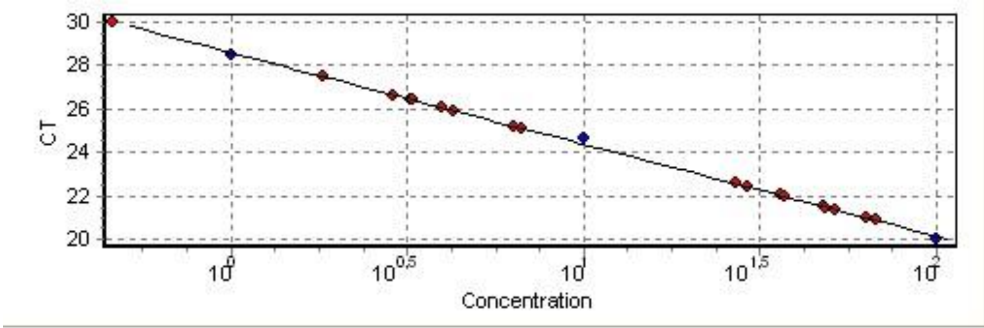


A 1.19 Amplification curve of LNCaP cells showing the accumulation of fluorescence emission at each reaction cycle.

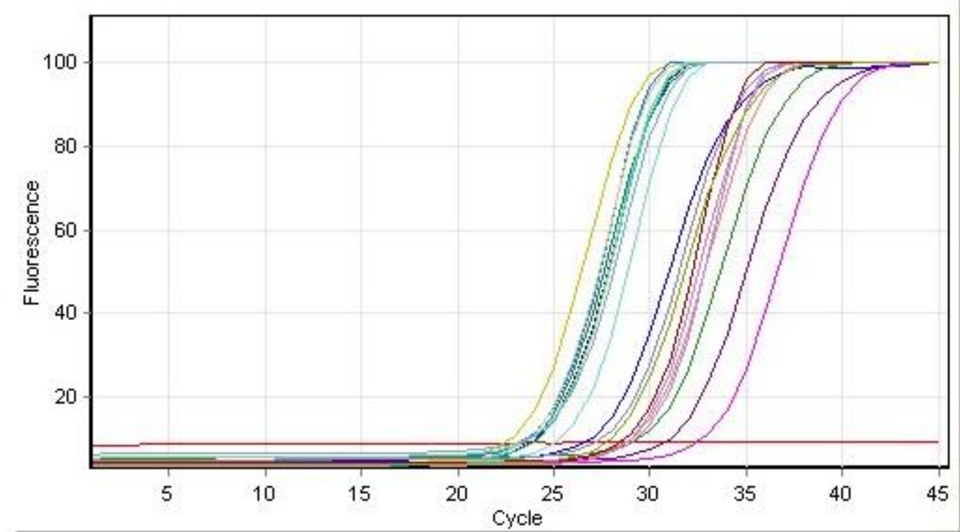


A 1.20 Melting curve showing the fluorescence emission change versus temperature of LNCaP cells. Detection of single peak means single PCR product.

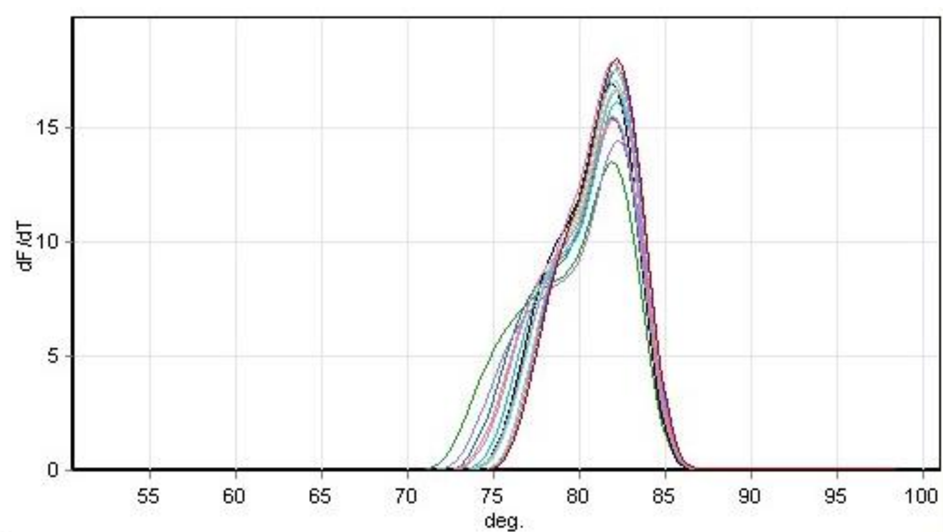
A 1.21 qRT-PCR products of HeksokinaseII cDNA (131bp) of LNCaP cells. Lane 1 shows the bp markers and Lane 2 is the no template control (NTC). Lane 3: Control, Lane 4: Cisplatin (17µM), Lane 5: Metformin 1Mm, Lane 6: Metformin 5 mM, Lane 7: Cisplatin+1mM Metformin, Lane 8: Cisplatin+1mM Metformin. 5 µL of qRTPCR product was loaded in each well.



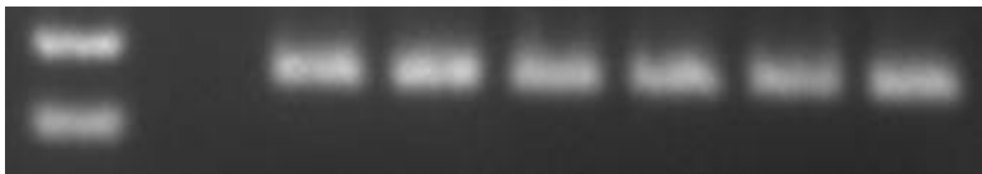
A 1.22 Standard curve generated from serial dilutions of control cDNA to calculate quantities of Hexokinase II mRNAs in the control and treated PC3 cells relatively.



A 1.23 Amplification curve of PC3 cells showing the accumulation of fluorescence emission at each reaction cycle.



A 1.24 Melting curve showing the fluorescence emission change versus temperature of PC3 cells. Detection of single peak means single PCR product.



A 1.25 qRT-PCR products of HeksokinaseII cDNA (131bp) of PC3 cells. Lane 1 shows the bp markers and Lane 2 is the no template control (NTC). Lane 3: Control, Lane 4: Cisplatin (17µM), Lane 5: Metformin 1Mm, Lane 6: Metformin 5 mM, Lane 7: Cisplatin+1mM Metformin, Lane 8: Cisplatin+1mM Metformin. 5 µL of qRTPCR product was loaded in each well