ANALYSIS OF PROTEIN AND mRNA EXPRESSIONS OF CYP1A1, CYP2E1, NQO1 AND GST ENZYMES IN LIVER, COLON AND PROSTATE CANCER CELL LINES TO STUDY DRUG AND CARCINOGEN METABOLISM

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ABSTRACT

ANALYSIS OF PROTEIN AND mRNA EXPRESSIONS OF CYP1A1, CYP2E1, NQO1 and GST ENZYMES IN LIVER, COLON AND PROSTATE CANCER CELL LINES TO STUDY DRUG AND CARCINOGEN METABOLISM

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Xenobiotic metabolism is the combination of phase I and phase II reactions which are named as modification and conjugation reactions, respectively. Most of the marketed drugs are metabolized by Cytochrome P450s (CYPs) known as phase I enzymes. CYP1A1 and CYP2E1 enzymes play role in activation/inactivation of many drugs and carcinogens. Phase II enzymes including NQO1 and GSTP1 are important for the elimination of toxic metabolites by reduction and conjugation reactions, respectively. Since *in vitro* studies are considered as the preliminary step for the discovery of new drug molecules or for the investigation of the effects of chemicals on the molecular basis, this study was aimed to show the best cell line model for studying CYP1A1, CYP2E1, NQO1 and GSTP1 enzymes playing role in drug and carcinogen metabolism. Therefore, protein and mRNA expression of these enzymes were analyzed in the HT29 and SW620 (colon); HEPG2

and HUH7 (liver); PNT1A and PC3 (prostate) cell lines. For this purpose, cell lines were grown as three replicates in 5% CO_2 incubator followed by the protein extraction and RNA purification steps. Then, mRNA and protein expressions of the enzymes by cell lines were analyzed by qRT-PCR and Western blotting techniques, respectively.

The results showed that all cell lines express CYP1A1 protein and relative protein expression is highest in the HT29 and SW620 (colon) cell lines. In addition, liver cell lines HEPG2 and HUH7 expressed highest CYP1A1 mRNA. CYP2E1 protein expression was found in all cell lines at relatively high levels except PC3. CYP2E1 mRNA expression was very high in HUH7 and HT29 cell lines. PNT1A and HEPG2 expressed NQO1 protein at relatively high levels while PC3 and HUH7 cell lines expressed NQO1 protein at significantly lower levels. NQO1 mRNA expression varies in cell lines and PNT1A showed the highest mRNA expression when compared with HUH7 cell line. HUH7 showed the highest GSTP1 protein expression whereas the HEPG2 did not show any GSTP1 protein expression. mRNA expression of GSTP1 in the descending order is PNT1A, HT29, SW620, PC3 and HUH7 while there is no mRNA expression in HEPG2 cell line.

In conclusion, CYP1A1, CYP2E1, NQO1 and GSTP1 protein and mRNA expressions showed differences in different cell lines originated from colon, liver and prostate tissues. It was found that HT29 colon cell line is the best model among these cell lines to study all of four enzymes together since it expressed both protein and mRNA of these enzymes at relatively significant levels. However, if CYP1A1, CYP2E1, NQO1 and GSTP1 would be studied individually, HT29, HUH7, PNT1A and HT29 are the best models, respectively.

Keywords: Xenobiotic metabolism, Cytochrome P450, CYP1A1, CYP2E1, NQO1, GSTP1, Western Blot, qRT-PCR, expression, cell line.

ÖZ

İLAÇ VE KANSEROJEN METABOLİZMASINI ÇALIŞMAK AMACIYLA KARACİĞER, KOLON VE PROSTAT HÜCRE HATLARINDA CYP1A1, CYP2E1, NQO1 VE GST ENZİMLERİNİN PROTEİN VE mRNA EKSPRESYONLARININ ANALİZİ

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Ksenobiyotikler faz I ve faz II enzim sistemleri tarafından metabolize edilirler. Faz I reaksiyonlar yükseltgenme, indirgenme ve hidroliz gibi modifikasyon reaksiyonlarını içermektedir. Faz II reaksiyonlar ise genel olarak faz I reaksiyonunda ortaya çıkan metabolitin amino, sulfidril, glutatyon gibi yüklü fonksiyonel gruplarla konjugasyonu sonucunda daha polar moleküllerin oluşturulmasını kapsar. Faz I enzim sistemleri arasında en bilineni ve en önemlisi Sitokrom P450 enzim sistemidir ve kısaca CYP olarak isimlendirilirler. CYP1A1 ve CYP2E1 enzimleri prokanserojenlerin ve proilaçların daha aktif moleküllere dönüştürülmesi reaksiyonlarında en önemli enzimlerdendir. NQO1 ve GST enzimleri ise sırasıyla indirgenme ve konjügasyon reaksiyonları ile ilaçların veya toksik kimyasalların atılmasına olanak sağlarlar. Bu özellikleri nedeniyle ilaç ve toksikoloji çalışmalarında bu enzimlerin incelenmesi önemlidir. Ksenobiyotiklerin metabolizmasıyla

ilgili çalışmalarda ilk basamak hücre kültürü çalışmalarıdır. Bu tezde ilaç ve kanserojen metabolizması çalışmaları için HEPG2 ve HUH7 (karaciğer); HT29 ve SW620 (kolon); PNT1A ve PC3 (prostat) hücre hatlarında CYP1A1, CYP2E1, NQO1 ve GSTP1 enzimlerinin protein ve mRNA ekspresyonlarının analizini yaparak en uygun model hücrenin tespit edilmesi amaçlanmıştır. Bu amaçla, hücreler 5% CO_2 inkübatöründe uygun besiyerleri ile büyütülüp daha sonra bu hücrelerden protein ve mRNA izolasyonu yapılmıştır. Bu aşamayı takiben protein ve mRNA ekspresyonları sırasıyla western blot ve qRT-PCR teknikleriyle ölçülmüştür. Deneyler sonucunda CYP1A1 protein ekspresyonun bütün hücre hatlarında değişken oranlarda gerçekleştiği görülmüştür. Bununla beraber en yüksek CYP1A1 rölatif protein ekspresyonunun HT29 hücre hattında olduğu ve SW620'nin de diğer hücre hatlarından istatistiksel olarak fazla ekspres ettiği görülmüştür. Ancak karaciğer hücre hatları olan HEPG2 ve HUH7 mRNA seviyesinde en fazla CYP1A1 ekspresyonu gösteren hücrelerdir. CYP2E1 protein ekpresyonunun PC3 hariç bütün hücre hatlarında yüksek olduğu bulunmuştur. En yüksek CYP2E1 mRNA ekpresyonu HUH7 ve HT29 hücre hattında gerçekleşmiştir. PNT1A ve HEPG2 hücrelerinde NQO1 protein ekspresyonu diğerlerine oranla yüksek seviyelerde görülmüştür. PC3 ve HUH7 hücrelerinde ise NQO1 protein ekspresyonu önemli oranda azdır. NQO1 mRNA ekspresyonunun HUH7 ile kıyaslanarak PNT1A'da en fazla olduğu görülmüştür. HUH7 en yüksek GSTP1 protein ekspresyonu gösteren hücre olmuştur ancak diğer karaciğer hücre hattı HEPG2'da GSTP1 ekspresyonuna rastlanmamıştır. GSTP1 mRNA ekspresyonu yüksekten düşüğe sıralandığında PNT1A, HT29, SW620, PC3 and HUH7 olarak bulunmuştur ancak HEPG2 hücre hattında GSTP1 mRNA expresyonuna rastlanmamıştır.

Sonuç olarak, CYP1A1, CYP2E1, NQO1 ve GSTP1 enzimleri protein ve mRNA seviyelerinde değişik hücre hatlarında ekspresyonel farklılıklar göstermiştir. Bu sonuçlar ışığında bütün enzimlerin aynı anda

çalışılabileceği en uygun model hücre hattının HT29 olduğuna karar verilmiştir. Ancak CYP1A1, CYP2E1, NQO1 ve GSTP1 enzimleri tek başlarına çalışılacak ise her biri için ayrı olarak, HT29, HUH7, PNT1A ve HT29 hücre hatları model olarak en uygun olanlarıdır.

Anahtar kelimeler: Ksenobiyotik metabolizması, sitokrom P450, CYP1A1, CYP2E1, NQO1, GSTP1, Western Blot, qRT-PCR, ekspresyon, hücre hattı.

To My Family,

For their endless support and love

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APS	Ammonium per sulfate
ARE	Antioxidant response element
B[a]P	Benzo[a]pyrene
BCIP	5-bromo 4-chloro 3-indoyl phosphate
BSA	Bovine serum albumin
BCA	Bicinchoninic acid
cDNA	Complementary DNA
Ct	Threshold cycle
CYP	Cytochrome P450
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dichlorodiphenyltrichloroethane
EDTA	Ethylenediaminetetraacetic acid
ERB	Electronic running buffer
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FMO	Flavin-containing monooxygenase
F-9	Ferriprotoporphyrin 9
GST	Glutathione S-transferase
kDa	kilo Dalton
Keap1	Kelchlike ECH-associated protein 1
Km	Michaelis constant
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide, reduced form

NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NBT	Nitrotetrazolium blue chloride
Nfr2	Nuclear factor erythroid 2- related factor 2
NQO1	NAD(P)H: Quinone Oxidoreductase I
OD	Optical density
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
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
XRE	Xenobiotic response element

CHAPTER 1

INTRODUCTION

1.1 Phase I and Phase II Xenobiotic Metabolizing Enzymes

1.1.1 Phase I Xenobiotic Metabolizing Enzymes

Phase I reactions include oxidation, reduction and hydrolysis which take place in the activation and/or detoxification of chemical compounds. Several enzyme systems participate in phase I metabolism including cytochrome P450 monooxygenases, flavin-containing monooxygenases (FMOs), alcohol and aldehyde dehydrogenases, epoxide hydrolases and esterases. Phase I enzymes catalyze the conversion of hydrophobic molecules to more hydrophilic form to facilitate direct excretion. The most remarkable pathway among these enzyme systems is the cytochrome P450s since it catalyzes the conversion of many chemicals to biologically active compounds.

1.1.1.1 Cytochrome P450 Enzymes

Cytochrome P450 (CYP) enzymes are a superfamily of heme-enzymes found in all organisms involved in the metabolism of most of the drugs, chemicals and endogenous compounds such as fatty acids and hormones. The term P450 refers to a pigment that absorbs light at 450nm when reduced form is exposed to carbon monoxide (Omura, 1964). The nomenclature of a cytochrome P450 enzyme includes the abbreviation for cytochrome P450 **[CYP]** followed by a number indicating for the CYP family **[2],** capital letter for subfamily **[E]** and another number representing for the individual

enzyme **[1]**; such as **CYP2E1** (D. Nelson, 1996). For being in the same family, at least 40% amino acid sequence homology is necessary while for sharing the same subfamily, the homology should be greater than 55% (Coon, 1992).

CYPs are membrane bound enzymes mainly located on the endoplasmic reticulum of the cells in a wide range of tissues. The CYPs catalyze the reactions of xenobiotics including N- and O-dealkylation, aliphatic and aromatic hydroxylation, N- and S-oxidation, and deamination by using NADPH and O_2. The active site of cytochrome P450 contains a haem structure in the center which is also called ferriprotoporphyrin 9 (F-9). This F-IX structure is bound to the P450 protein by a thiolate ligand derived from a cysteine residue. This structure is highly conserved in CYPs. The iron molecule is normally found in Fe^{2+}, ferrous, form. This structural similarity provides a common catalytic mechanism for the oxygenation of a substrate by CYPs (Figure 1.1).

The cycle begins with the binding of substrate to ferric (Fe^{3+}) form of cytochrome P450 enzyme. In the second step, electron transfer from NAD(P)H is carried out by NADPH-P450 reductase enzyme that reduces Fe^{3+} to Fe^{2+} (ferrous). In the next step, O_2 binds to the reduced enzyme-substrate complex forming an unstable structure which leads to generation of ferric iron and superoxide anion. In step 4, the newly formed unstable complex undergoes a second reduction catalyzed by the same NADPH-P450 reductase enzyme which serves to reduce molecular oxygen and forms "activated oxygen"-CYP-substrate complex. Then, O-O bond is cleaved and in step 6, H_2O is released leading to generation of high-valent FeO^{3+}. In step 7, an electron or hydrogen atom from substrate binds to FeO^{3+} while in the next step; the product is generated with the subsequent collapse of intermediate or intermediate pair. In the ninth, the final, step the product dissociates from the enzyme (Guengerich, 2001).

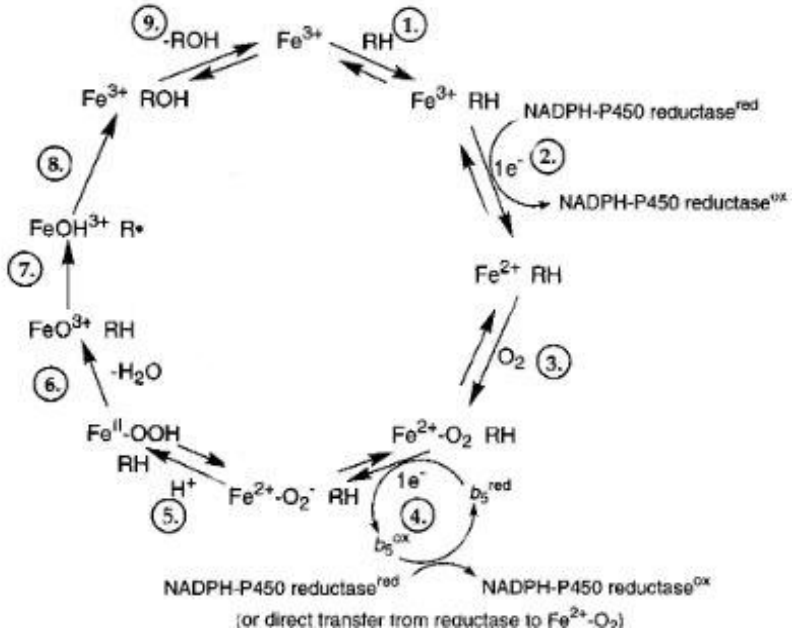

Figure 1.1 General cytochrome P450 catalytic cycle for the oxidation of substrates (Guengerich 2001).

CYP genes are expressed in mainly liver where most of the drugs and endogenous compounds are metabolized. However, it has been reported that many isoforms of CYP enzymes are expressed in wide range of tissues including kidney, lung, brain, gastrointestinal tract (Adali, 1996; Arinç, 2007; R. P. Meyer, 2002; Omiecinski, 1990). In humans, 18 families with 43 subfamilies of CYP are present. Each isoform of CYPs show different functions (Table 1.1). In total they catalyze the metabolism of thousands of endogenous and exogenous chemicals in a wide variety of tissues. However, CYP1, CYP2 and CYP3 are main families responsible for the xenobiotic metabolism. CYP1A1 and CYP2E1 are very important enzymes involved in the metabolism of xenobiotics including drugs and procarcinogens (Walsh, 2013; Y. Wang, 2009).

Table 1.1 Human cytochrome P450 genes (D. R. Nelson, 2009)

Human CYP Families	Names	Function
CYP1 (3 genes)	1A1, 1A2, 1B1	Xenobiotic metabolism
CYP2 (16 genes)	2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1	Xenobiotic and steroid metabolism
CYP3 (4 genes)	3A4, 3A5, 3A7, 3A43	Xenobiotic and steroid metabolism
CYP4 (12 genes)	4A11, 4A22, 4B1, 4F2, 4F3, 4F8, 4F11, 4F12, 4F22, 4V2, 4X1, 4Z1	Fatty acid metabolism
CYP5 (1 gene)	5A1	Thromboxane A_2 synthesis
CYP7(2 genes)	7A1, 7B1	Bile acid biosynthesis
CYP8 (2 genes)	8A1, 8B1	Prostacyclin synthesis and bile acid biosynthesis
CYP11 (3 genes)	11A1, 11B1, 11B2	Steroid biosynthesis
CYP17 (1 gene)	17A1	Estrogen and testosterone biosynthesis
CYP19 (1 gene)	19A1	Estrogen hormone biosynthesis
CYP20 (1 gene)	20A1	Drug metabolism and cholesterol biosynthesis
CYP21 (1 gene)	21A2	Steroid biosynthesis
CYP24 (1 gene)	24A1	Vitamin D degradation
CYP26 (3 genes)	26A1, 26B1, 26C1	Retinoic acid metabolism
CYP27 (3 genes)	27A1, 27B1, 27C1	Bile acid biosynthesis and vitamin D_3 activation
CYP39 (1 gene)	39A1	Cholesterol biosynthesis
CYP46 (1 gene)	46A1	Cholesterol biosynthesis
CYP51 (1 gene)	51A1	Cholesterol biosynthesis

1.1.1.1.1 CYP1A1

CYP1A1 (EC 1.14.14.1), also known as aryl hydrocarbon hydroxylase, is localized on the membrane of endoplasmic reticulum and mitochondria of extrahepatic tissues including lung and gastrointestinal tract but also it is found in cytosol of kidney and brain tissues(R. P. Meyer, 2002; Omiecinski, 1990). The gene of CYP1A1 is located on chromosome 15 and length of mRNA is 2608bp while molecular weight of the CYP1A1 protein is 58kDa and composed of 512 amino acids. The crystal structure of CYP1A1 protein is given in Figure 1.2.

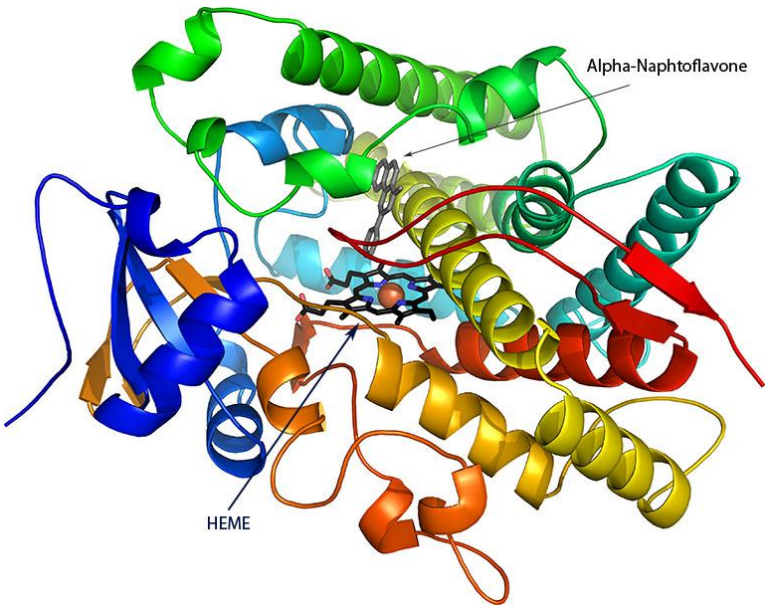

Figure 1.2 Human cytochrome P450 1A1 with alpha-naphthoflavone (Walsh et al. 2013).

CYP1A1 catalyzes the hydroxylation of many endogenous substrates including the arachidonic acid and the hormones such as melatonin (Ma,

2005; Schwarz, 2004). However, the fame of this enzyme comes from its ability to activate many procarcinogens to produce highly reactive metabolites (Rendic, 2012). CYP1A1 metabolizes polycyclic aromatic hydrocarbons (PAHs) to carcinogens by epoxidation at a vacant position of an aromatic ring that forms epoxide intermediates and this epoxide is converted to more active metabolites, diol epoxides, by the epoxide hydrolase (Wei, 1996). The best example for this catalytic activity of CYP1A1 is the conversion of inactive Benzo[*a*]pyrene (BaP) to mutagenic metabolite, BaP-7,8-diol-9,10-epoxide, forming DNA adducts mainly with deoxyguanosine (Figure 1.3) (Androutsopoulos, 2009).

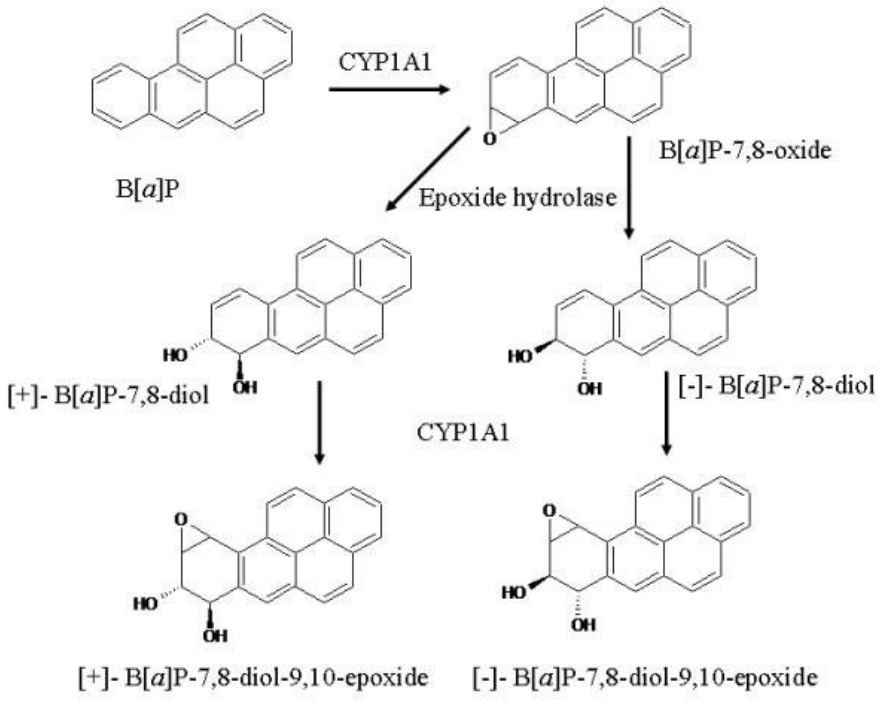

Figure 1.3 Conversion of BaP to mutagenic metabolite by CYP1A1.

Transcription of CYP1A1 is activated by aryl hydrocarbon receptor (AHR). AhR involved in the regulation of toxic responses to a number of xenobiotics, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Without any ligand AHR is located in the cytoplasm as an inactive protein complex with dimer of heat-shock protein 90, p23, AhR interacting protein (AIP) and AhR-activated 9 (ARA9) (B. K. Meyer, 1998; Whitlock, 1997). With the binding of the ligand, the protein complex dissociate and AHR forms dimer with its nuclear translocator protein (ARNT) which further binds to xenobiotic response elements (XREs), activating expression of many phase I and Phase II enzymes including CYP1A1 (Fujii-Kuriyama, 1992).

1.1.1.1.2 CYP2E1

CYP2E1 (EC 1.14.13.), ethanol inducible form, is the member of the cytochrome P450 mixed-function oxidase system. CYP2E1 gene is located on chromosome 10 while molecular weight of the human enzyme CYP2E1 is 62 kDa and composed of 493 amino acids. The crystal structure of CYP2E1 is given in the Figure 1.4. CYP2E1 is mainly found in the E.R. and mitochondria however recent studies showed that it is also found in plasma membrane of hepatocytes (Seliskar, 2007). Moreover, CYP2E1 is expressed in a range of extrahepatic tissues such as kidney, lung and brain (Botto, 1994). It catalyzes the metabolism of both endogenous substrates, such as acetone, fatty acids; and exogenous substrates, such as carbon tetrachloride, nitrosamines, chloroform (Gonzalez, 2005). Moreover, it is responsible for the formation of alcohol mediated reactive oxygen species (Cederbaum, 2006; Guengerich, 1991).

CYP2E1 is an important enzyme because it involves the conversion of drugs such as acetaminophen, pre-toxins and procarcinogens to active form (Gonzalez, 2005). It has been reported that there is significant correlation

between CYP2E1 expression and nasopharyngeal carcinoma susceptibility among cigarette smokers (Hildesheim, 1997). Cigarette smoke contains *N*-nitrosonornicotine, metabolized by CYP2E1, causes DNA methylation and carcinogenesis.

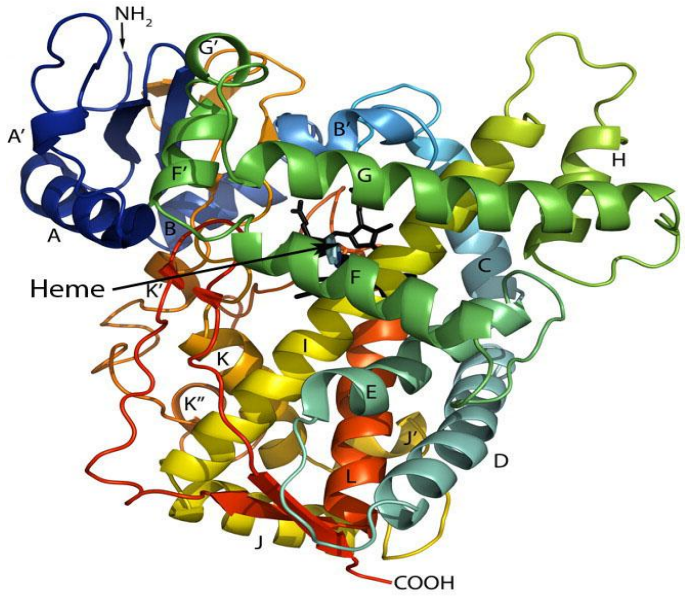

Figure 1.4 Polypeptide structure of CYP2E1 (Porubsky et al. 2008).

The CYP2El gene expression is affected by several factors including genetic factors, age, diet, drugs and chemicals. In addition, it has been reported that diabetes causes significant increase in both protein expression and catalytic activity of CYP2E1 (Arinç, 2007, 2005). As example for chemical factors, ethanol induces the expression of CYP2E1 while resveratrol, plant phenolic compound, downregulates CYP2E1 and has potential to prevent carcinogenesis caused by catalytic activity of CYP2E1 (Carroccio, 1994;

Celik, 2010). The expression of this enzyme is controlled by transcriptional, posttranscriptional and posttranslational actions. In rats, CYP2E1 gene transcription is activated with birth by a demethylation at 5' end of the gene (Umeno, 1988). In addition, CYP2E1 gene is transcriptionally upregulated in adult rats by hepatocyte nuclear factor-1 (HNF-1), a DNA binding protein, in liver (Ueno, 1990). Posttranscriptional regulation includes stabilization and transcriptional activation of CYP2E1 mRNA when induced by ethanol (Lieber, 1978). In addition to transcriptional and posttranscriptional regulations of CYP2E1 expression, the major regulation occurs at posttranslational level by increasing protein stability or rapid degradation by ubiquitin conjugation (Roberts, 1995).

CYP2E1 catalyzes the metabolism of many endogenous and exogenous chemicals with low molecular weight (Arinç et al. 2000a; Arinç, et al. 2000b; Guengerich et al. 1991). The list of the substrates, inducers and inhibitors of CYP2E1 is given in the Figure 1.5.

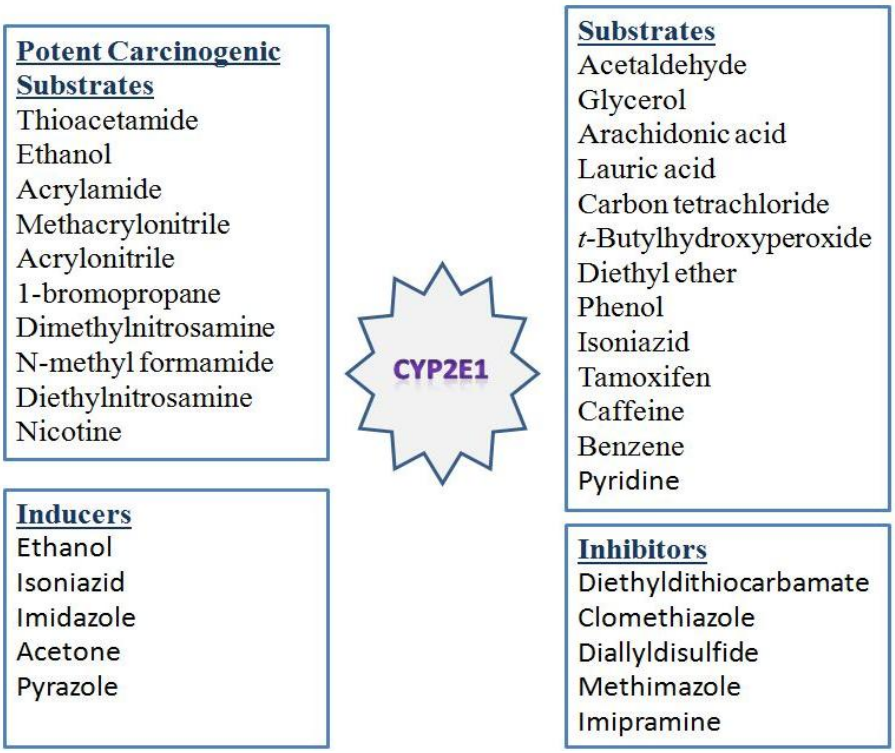

Figure 1.5 Substrates, inhibitors and inducers of CYP2E1 (Meskar, 2001; Monostory, 2004).

1.1.2 Phase II Xenobiotic Metabolizing Enzymes

In general, phase II xenobiotic metabolizing enzymes catalyze conjugation reactions including glutathione and amino acid conjugation, glucuronidation, sulfation, methylation and acetylation. As a result of these conjugation reactions the parent compounds are converted to more hydrophilic metabolites which are readily extractable. Although detoxification of drugs and carcinogenic toxic chemicals are mainly catalyzed by phase II enzymes such as Glutathione S-transferases and NAD(P)H:Quinone Oxidoreductase I, sometimes conjugation reactions may result in increased toxicity of parent compound.

1.1.2.1 NAD(P)H: Quinone Oxidoreductase I

NAD(P)H quinone oxidoreductase 1 (NQO1, EC 1.6.5.2) is a FAD-containing enzyme that is located in the cytosol and found in a wide range of eukaryotic organisms ranging from yeast to mammals (Deller, 2008). NQO1 is a homodimer enzyme with a molecular weight of 31 kDa and this homodimer structure shown in Figure 1.6 functions via ping-pong mechanism. With the binding of NAD(P)H to NQO1 the FAD cofactor is reduced and it ends up with the release of $NAD(P)^+$ which makes the enzyme available for the binding of the quinone substrate which is then reduced by NQO1. Although NQO1 is expressed in many organs including lung, liver, and colon, it has been reported that its expression in the tumor of these tissues is higher than the normal tissues (Belinsky, 1993).

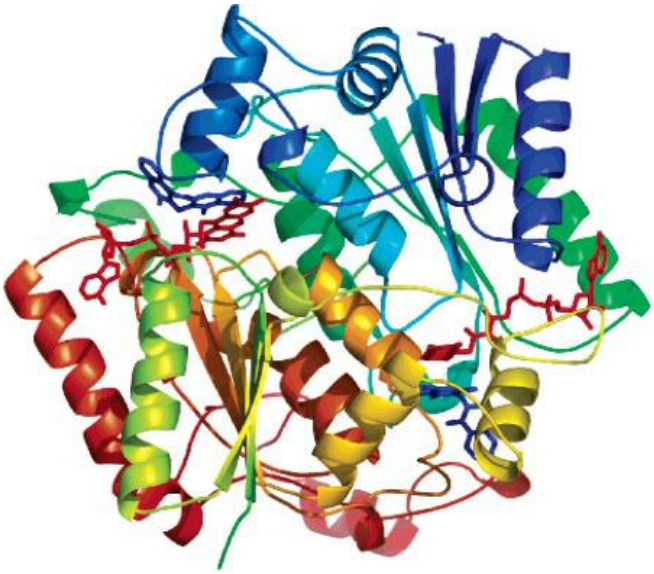

Figure 1.6 Polypeptide structure of the human NQO1 homodimer with FAD (red stick) and dicoumarol (blue stick) (Gad Asher, 2006).

NQO1 catalyzes the two-electron reduction of quinones and quinone derivatives such as plastoquinone and ubiquinone resulting in the formation of hydroquinone products (Lind, 1990). The hydroquinone production can be followed by conjugation of glucuronide and sulfate groups which facilitates their excretion (Shangari, 2005). However, the substrate of this enzyme is not only the quinones but also many other compounds including methylene blue, dichlorophenolindophenol and nitro-compounds such as dinitropyrenes, nitrophenylaziridines and nitrobenzamides which are reduced by NQO1 enzyme to less toxic metabolites (Knox, 1988; Lind, 1990). Two-electron reduction property of NQO1 avoids formation of semiquinone which leads to generation of reactive oxygen species (Lind, 1982). In addition to this ability of NQO1, its binding to tumor suppressor p53 protects this protein from proteasomal degradation (G Asher, 2001). Due to its ability to prevent formation of reactive oxygen species, toxic compounds and stabilize p53 protein, NQO1 is named as both antioxidant and anti-carcinogen enzyme (Talalay, 1988).

NQO1 activity can be regulated by several chemicals such as dicoumarol, a competitive inhibitor of NQO1 enzyme, which competes with NAD(P)H for binding to NQO1 while resveratrol is an inducer of this enzyme (Gad Asher, 2006; Floreani, 2003). Besides that transcriptional regulation of NQO1 is done by Keap1/Nrf2/ARE pathway, shown in Figure 1.7 (Dinkova-Kostova, 2010). When there is no inducer, nuclear factor erythroid 2- related factor 2 (Nrf2) is located in the cytosol through binding to Kelchlike ECH-associated protein 1 (Keap1) which leads to proteasomal degradation of Nrf2 (Itoh, 2003). When inducers are available, highly reactive cysteine residues of the Keap1 are modified and Nrf2 is stabilized and accumulated in the nucleus where it binds to antioxidant response element (ARE) that initiates transcription of antioxidant enzyme genes including NQO1 (Dinkova-Kostova, 2010).

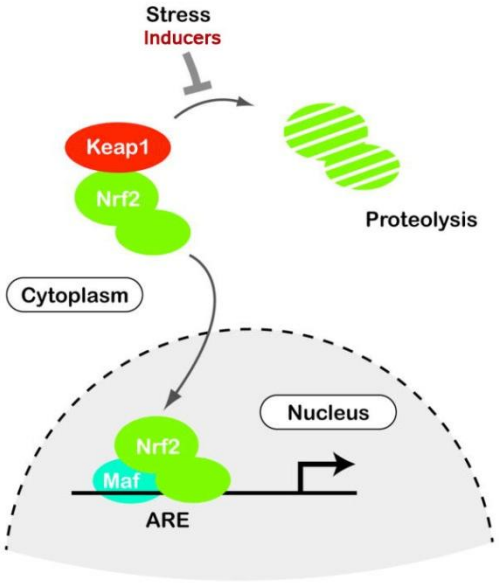

Figure 1.7 Induction of NQO1 transcription by Keap1/Nrf2/ARE pathway (Dinkova-Kostova, 2010).

1.1.2.2. Glutathione S-Transferase

Glutathione S-transferases (GSTs) (EC.2.5.1.18) are a family of eukaryotic and prokaryotic phase II metabolic isozymes which catalyze the detoxification of endogenous and exogenous electrophilic compounds by conjugating glutathione (GSH, γ-glu-cys-gly) with these electrophiles (Figure 1.8). GSTs are both located in the cytosol and on membrane of endoplasmic reticulum and mitochondria. In human, the cytosolic GSTs are divided into six groups sharing ~30% sequence similarity and represented by Greek letters (α, μ, ω, π, θ and ξ) (Townsend, 2003). Human cytosolic GSTs are homodimer enzymes each monomer of this homodimer is approximately 25 kDa in size. GSTs have two domains which are G domain for binding of GSH and H domain for binding of hydrophobic substrates (Figure 1.9) (Wu, 2012).

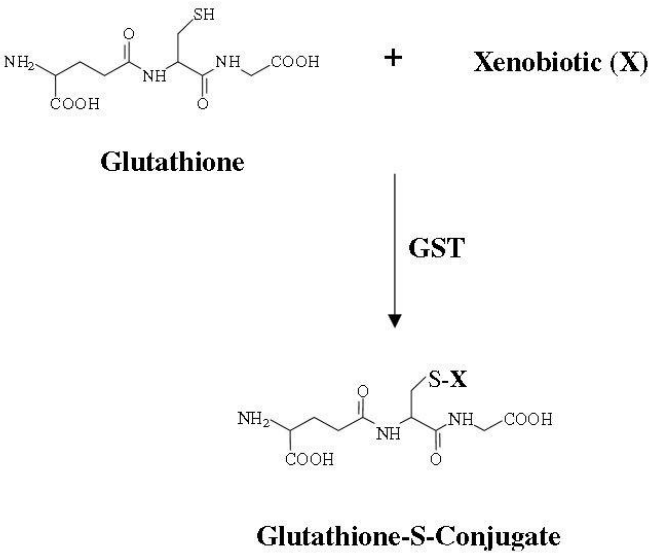

Figure 1.8 Glutathione conjugation to a xenobiotic by GSTs (Townsend, 2003).

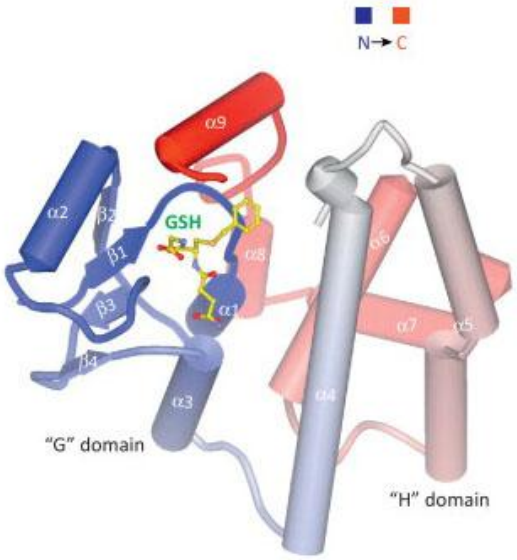

Figure 1.9 3D structure of GST enzyme (Wu, 2012).

GST enzymes use GSH as cofactor and the sulfur atom on the cysteine amino acid of GSH is activated by GSTs to generate a strong nucleophile, thiolate anion (GS^-). This thiolate anion attacks electrophilic substrates and the conjugation of glutathione to substrate is achieved. Electrophilic substrates for GST enzymes may be generated as a consequence of phase I reactions and by this conjugation reaction, toxic and carcinogenic effects of this phase I products can be eliminated. For example benzo[a]pyrene, converted to mutagenic diol epoxide by a phase I enzyme CYP1A1, can be detoxified with GSH conjugation by GST enzymes (A.-H. Wang, 2002). The GSH-xenobiotic conjugate is very hydrophilic that cannot diffuse freely through the membrane, thus the conjugate is actively transported by a transmembrane ATPase, GS-X pump (Ishikawa, 1992). Once the xenobiotic is excreted from the cell, it cannot get into the cell again because of the highly hydrophilic GSH moiety. Finally, the conjugate is excreted as mercapturic acids in urine. GST enzymes are important in the metabolism of several endogenous compounds such as biosynthesis of prostaglandins and catabolism of tyrosine (Townsend, 2003).

The role of GSTs in the elimination of xenobiotics including active form of drugs and biosynthesis of important inflammatory mediator, prostaglandin, makes this enzyme family target for the drugs (Matsuoka, 2000). For example, since GST S1-1 catalyzes the isomerization of prostaglandin H2 to prostaglandin D2, a mediator of allergy and inflammation responses; nocodazole, inhibitor of GSTS1-1, is used as anti-allergic and anti-inflammatory drug (Weber, 2010). GSTs are also believed to be associated with the drug resistance due to its role in the excretion of xenobiotics (Meijerman, 2008). Some chemotherapeutic drugs including cisplatin and carmustine are substrates of GSTP1 and GSTM1, respectively and excreted by conjugation with glutathione (Ban, 1996; M. Smith, 1989). Moreover, it has been reported that upregulation of GSTP1 may cause inhibitory effect on JNK-mediated apoptosis (Simic, 2009). Because of these properties, new

strategies are being developed for cancer therapy including inhibitors of GSTs to increase efficacy of the chemotherapeutic drug (Meijerman, 2008).

1.2 Aim of the Study

In vitro studies are the first step for the discovery of new drugs or for the investigation of effect of chemicals on the molecular basis since it enables to study individual components and the exploration of their basic biological functions while eliminating the effects of complex organ systems. Although animal models share significantly high homology with human, *in vitro* studies of human cells may provide more accurate results in the toxicology research. Moreover, *in vitro* studies give chance to control experimental environment strictly and eliminate any unwanted condition. Because of these advantages, *in vitro* method has been used in toxicology and pharmacology and cancer research. These research areas investigate the effects of xenobiotics including drugs and toxicants which are metabolized by phase I enzymes such as CYP1A1 and CYP2E1 and phase II enzymes such as NQO1 and GST. Therefore, in the present study, it was aimed to show the best cell line model for studying possible effects of xenobiotics metabolized by CYP1A1, CYP2E1, NQO1 and GSTP1 enzymes.

For this purpose human colon (HT29 and SW620), liver (HEPG2 and HUH7) and prostate (PNT1A and PC-3) cell lines were chosen and protein and total RNA were obtained from these cell cultures. After that, the protein and mRNA expressions of CYP1A1, CYP2E1, NQO1 and GSTP1 enzymes were analyzed by Western Blot technique and qRT-PCR method.

To our knowledge, this study is the first concerning the comparison of protein and mRNA expressions of drug and carcinogen metabolizing CYP1A1, CYP2E1, NQO1 and GSTP1 in liver, colon and prostate cell lines.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Lines

In this study, protein and mRNA expressions of drug and carcinogen metabolizing enzymes CYP1A1, CYP2E1, NQO1 and GSTP1 in human liver, colon and prostate cell lines were analyzed. Hepatocellular carcinoma cell line; HEPG2 (ATCC® HB-8065™) cancer cell lines was gift Prof. Dr. Tülin Güray, Biology Department, Middle East Technical University. Hepatocellular carcinoma HUH7 (JCRB0403) cancer cell line was gift from Assoc. Prof. Dr. Rengül Çetin Atalay, Department of Molecular Biology and Genetics, Bilkent University. Colorectal adenocarcinoma cell lines; HT29 (ATCC® HTB-38™) and SW620 (ATCC® CCL-227™) were gift from Assoc. Prof. Dr. Sreeparna Banerjee, Biology Department, Middle East Technical University. Normal prostate epithelium cell line; PNT1A (95012614 Sigma) and prostatic adenocarcinoma cell line; PC-3 (ATCC® CRL-1435™) were gifted from Research Assistant Bora Ergin, Department of Biophysics, Faculty of Medicine, Hacettepe University.

2.1.2 Chemicals and Materials

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), tween 20 (P1379) were purchased from 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 the products of E. Merck, Darmstadt, Germany.

Absolute ethanol (32221) and acetyl acetone (33005) were obtained from Riedel de-Haen Chemical Company, Germany. Isopropanol (AS040-L50) was the product of Atabay, Istanbul, Turkey. QIAzol® (79306) was purchased from QIAGEN, Hilden, Germany.

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

Non-fat dry milk (170-6404) and tetra methyl ethylene diamine (TEMED; 161-0801) were the products of Bio-Rad Laboratories, Richmond, California, USA.

Ethylene diamine tetra acetic acid (EDTA; A5097) and nitrotetrazolium blue chloride (NBT; A1243) were acquired from Applichem GmbH, Germany.

The CYP1A1 (sc-20772), CYP2E1 (sc-133491), GAPDH (sc-367714) and NQO1 (sc-16464) antibodies were purchased from Santa Cruz (Santa Cruz, CA). GSTP1 (ab138491) antibody was product of the Abcam, Cambridge, United Kingdom. Primers were made by Alpha DNA, Montreal, Canada.

Dulbecco's Modified Eagle's Medium (DMEM; BE12-614F), Minimum Eagle's Minimum Essential Medium (EMEM; BE12-611F), Leibovitz's L-15 (BE12-700F), McCoy's 5A (BE12-688F), Ham's F-12 (BE12-615F), RPMI 1640 (BE12-702F) and Fetal bovine serum (FBS; DE14-801FH) were purchased from 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

HT29, SW620, HEPG2, HUH7, PNT1A and PC3 cell lines were cultured in various growth mediums containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin (Pen-Strep) solution as indicated in Table 2.1. 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 mediums of culture were renewed in 2-3 days for appropriate growth conditions.

Table 2.1 Growth Medium Conditions of Cell lines.

Cell Lines	Medium	Fetal Bovine Serum	Pen-strep
HEPG2	EMEM	10%	1%
HUH-7	DMEM	10%	1%
SW620	Leibovitz's L-15	10%	1%
HT-29	McCoys's 5A	10%	1%
PC3	Ham's F-12	10%	1%
PNT1A	RPMI 1640	10%	1%

2.2.1.2 Cell Thawing

Before thawing the cells, 10 mL of pre-warmed growth medium was transferred into T75 cell culture flask. After that, cryotubes were taken from the liquid nitrogen and the cells were defrosted at 37°C water bath and immediately transferred to T75 cell culture flask containing growth medium. Cells were incubated in CO_2 incubator at 37°C. After 24 hours, medium was renewed to eliminate dimethylsulfoxide (DMSO) and placed in CO_2 incubator.

2.2.1.3 Subculturing the Cell Lines

After 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). 1:4 split of cell lines was performed by adding 2 ml of prewarmed trypsin to flask and placing the T75 flask in 37°C, CO_2 incubator until cells were detached and 6 mL of 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 3-4 days depending on the cell types.

2.2.1.4 Cell Freezing

After 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 all the cells were detached, 2 mL of 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. Then 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 after a week it was transferred to liquid nitrogen tank for longer term storage.

2.2.2 Protein Extraction

Cells were seeded to 100 x 20mm tissue culture petri dish for protein extraction. When cells were 80% confluent, growth medium in the dish was removed and the cells were washed three times by using cold (4 °C) PBS buffer. 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 just prior to use to prevent protease activity. 400 µL of the diluted RIPA buffer was added into the dish for lysis of the cells. Dish was incubated on ice for 5 minutes and the cells were scraped. The lysate was sonicated for 5 minutes and centrifuged at 14000 x g in a cold microfuge for 10 minutes. Supernatant was 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.

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

In this method, 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 by the following formula;

$$\text{Protein Concentration (mg/ml)} = \frac{[\text{OD}_{660nm}]}{\text{Slope of standards}} \times \text{Dilution}$$

2.2.4 Determination of Protein Expression

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

Protein expression of drug and carcinogen metabolizing enzymes; CYP1A1, CYP2E1, NQO1 and GSTP1 in liver, colon and prostate cancer cell lines 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, 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 8.5% separating gel for CYP1A1 and CYP2E1; and 10% separating gel for NQO1 and GSTP1 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 1.5 minutes at 100ºC heat block. Then, 10 µg of each sample was loaded on different wells. 5 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 10 mA–90 V in stacking gel and 20 mA–200 V in separating 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: 1/200 to 1/1000 dilution

Secondary Antibody: 1/500 to 1/5000 dilution

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.

ECL Substrate Solution: (Pierce ECL Western Blotting Substrate)

1 mL of Peroxide solution and 1 mL of luminol enhancer solution were mixed and 2 mL of this mixture was used for each membrane.

For western blotting, the gel was removed from the glasses and the gel was 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 prewet 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 2.1. The transfer sandwich was placed into Mini Trans-Blot module (Bio-Rad Laboratories, Richmond, CA, USA) and module was filled with transfer buffer. The transfer was carried out at 90 volt and 400 mA for 90 minutes.

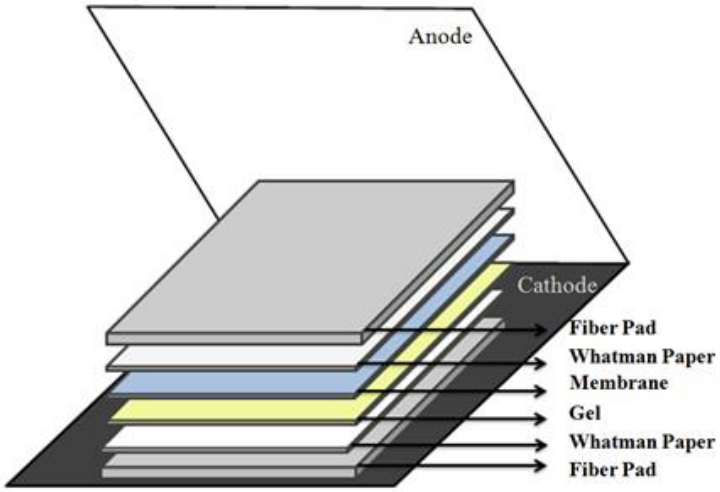

Figure 2.1 Western blot sandwich

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 1/500 dilutions of CYP1A1, CYP2E1 and NQO1 while 1/1000 dilution of GSTP1 primary antibodies for 2 hours at room temperature by shaking. 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 1/2000 dilutions of alkaline phosphatase conjugated secondary antibodies for CYP1A1 and CYP2E1; and 1/4000 dilutions of horseradish peroxidase conjugated secondary antibodies for NQO1 and GSTP1 for an hour. Finally the membrane was incubated with suitable substrate solution for the conjugated enzyme on the secondary antibody. For visualization of horseradish peroxidase conjugated secondary antibody, X-Ray Roentgen Method was used and the bands were visualized on the roentgen film. The band intensities were analyzed by Image J visualization software developed by NIH.

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 equipments were autoclaved.

Cell lines were seeded into 12 well plate for RNA isolation. 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 QIAzol®, was added into the wells and incubated for 5 minutes at room temperature. After incubation, the cells were detached by pipetting and QIAzol® 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 CYP1A1, CYP2E1, NQO1 and GST 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, 0.5 mM 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 2.3. 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 each cell lines. In the preliminary experiment, the cell line expressing the highest amount of product was selected for each gene and used as the standard sample in the subsequent assay. 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.

Table 2.3 Primer sequences, annealing temperatures and product sizes of the genes.

Gene	Forward Primer (5'→ 3')	Reverse Primer (5' → 3')	Annealing Temperature (°C)	Product Size (bp)
GAPDH	GAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG	58	197
CYP1A1	TACCTCAGCAGCCACCTCCAAG	GGCCCTGATTACCCAGAATACC	60	121
CYP2E1	AGCGCTGCTGGACTACAAGG	CCTCTGGATCCGGCTCTCAT	60	184
NQO1	AAGAGCACTGATCGTACTGGC	GGATACTGAAAGTTCGCAGGGG	60	196
GSTP1	CCTACACCGTGGTCTATTTCC	CAGGAGGCTTTGAGTGAGC	60	137

2.2.6 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). One-way ANOVA test and $p<0.05$ were chosen as the level for significance.

CHAPTER 3

RESULTS

3.1. Protein Concentrations of Lysates of the Cell Lines

In this study distribution of protein and mRNA expressions of drug and carcinogen metabolizing enzyme's; CYP1A1, CYP2E1, NQO1 and GSTP1 was carried out by using HT29 and SW620 (colon), HEPG2 and HUH7 (liver), PNT1A and PC3 (prostate) cell lines as *in vitro* model. For that purpose, protein extraction from three replicate plates of each cell line was performed by using RIPA buffer procedure and the protein concentrations were determined by BCA method as described in material and methods part. Average protein concentrations of each cell lines are listed in Table 3.1.

Table 3.1 Average protein concentrations of whole cell lysates of the cell lines.

Cell Lines	Average Protein Concentration (mg/mL)
HT29	3.18 ± 0.18
SW620	2.45 ± 0.30
HEPG2	3.12 ± 0.16
HUH7	2.55 ± 0.21
PNT1A	1.46 ± 0.04
PC3	1.73 ± 0.27

3.2 Protein Expression Analysis of CYP1A1, CYP2E1, NQO1 and GSTP1 Enyzmes in the Cell Lines

Drug and carcinogen metabolizing phase I enzymes; CYP1A1 and CYP2E1, phase II enzymes; NQO1 and GSTP1 protein expressions in colon (HT29 and SW620), liver (HEPG2 and HUH7) and prostate (PNT1A and PC3) cell lines were determined by Western blotting. Western blot experiments were performed on total cellular extracts of the cell lines and for immunochemical detection specific antibodies were used. GAPDH (37 kDa) was used as internal standard.

3.2.1 CYP1A1 Protein Expression in the Cell Lines

CYP1A1 protein expression was determined by Western blotting. Primary rabbit polyclonal anti-CYP1A1 antibody (1/500 dilution) and an alkaline phosphatase (AP) conjugated secondary goat anti-rabbit antibody (1/2000 dilution) were used for immunochemical detection of CYP1A1 protein (Figure 3.1). Band intensities were quantified by using Image J visualization software. Figure 3.2 shows the relative protein expressions where the mean of the cell line showing highest expression (HT29) was taken as 100 (n=3) and then mean of the other cell lines were calculated. Multiple comparison analysis of protein expression of the cell lines were performed by one-way ANOVA and the level of significance was chosen as $p<0.05$. Statistical results are shown in the Table 3.2.

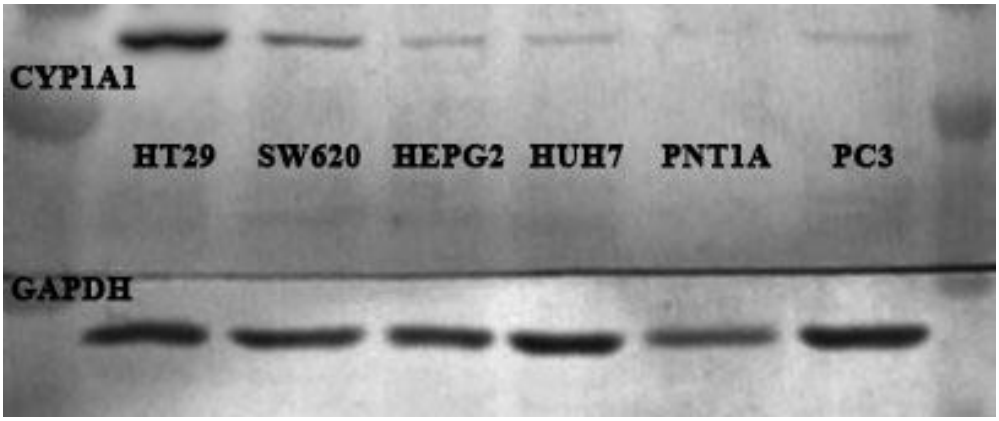

Figure 3.1 Immunoreactive protein bands of cell lines representing CYP1A1 and GAPDH used as internal standard.

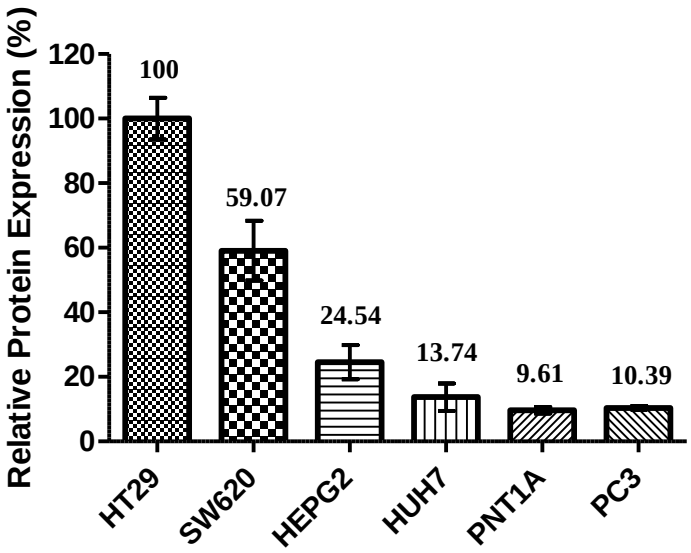

Figure 3.2 Comparison of CYP1A1 protein expression of cell lines. The band quantifications are expressed as mean ± SD of the relative intensity of three independent experiments.

Table 3.2 Statistical analysis of CYP1A1 protein expression by one-way ANOVA method.

	SW620	HEPG2	HUH7	PNT1A	PC3
HT29	***	***	***	***	***
SW620		***	***	***	***
HEPG2			NS	NS	NS
HUH7				NS	NS
PNT1A					NS

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

3.2.2 CYP2E1 Protein Expression in the Cell Lines

Primary rabbit polyclonal anti-CYP2E1 antibody (1/500 dilution) and an alkaline phosphatase (AP) conjugated secondary goat anti-rabbit antibody (1/2000 dilution) were used for immunochemical detection of CYP2E1 protein (Figure 3.3). Band intensities were quantified by using Image J visualization software. Figure 3.4 shows the relative protein expressions where the mean of the cell line showing highest expression (SW620) was taken as 100 (n=3) and then mean of the other cell lines were calculated. Multiple comparison analysis of protein expression of the cell lines were performed by one-way ANOVA and the level of significance was chosen as $p<0.05$. Statistical results are shown in the Table 3.3.

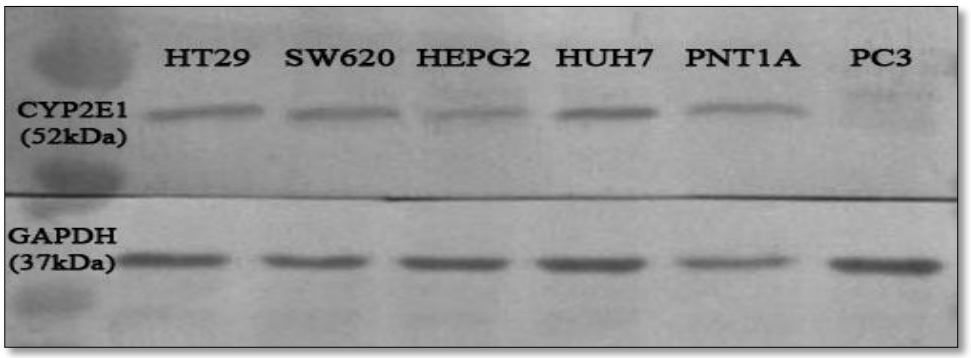

Figure 3.3 Immunoreactive protein bands of cell lines representing CYP2E1 and GAPDH used as internal standard.

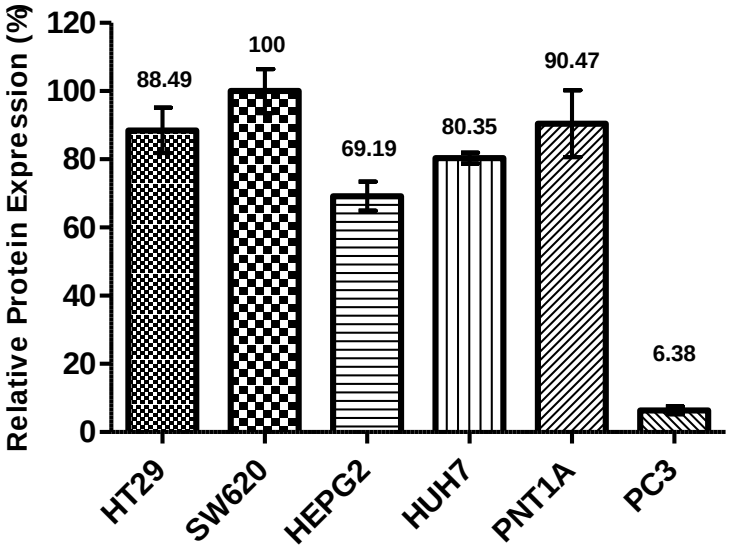

Figure 3.4 Comparison of CYP2E1 protein expression of cell lines. The band quantifications are expressed as mean ± SD of the relative intensity of three independent experiments.

Table 3.3 Statistical analysis of CYP2E1 protein expression by one-way ANOVA method.

	SW620	**HEPG2**	**HUH7**	**PNT1A**	**PC3**
HT29	NS	*	NS	NS	***
SW620		***	*	NS	***
HEPG2			NS	*	***
HUH7				NS	***
PNT1A					***

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

3.2.3 NQO1 Protein Expression in the Cell Lines

NQO1 (31 kDa) protein expression was determined by Western blotting technique. Primary goat polyclonal anti-NQO1 antibody (1/500 dilution) and a horseradish peroxidase (HRP) conjugated secondary mouse anti-goat antibody (1/4000 dilution) were used for immunochemical detection of NQO1 protein (Figure 3.5). Band intensities were quantified by using Image J visualization software. Figure 3.6 shows the relative protein expressions where the mean of the cell line showing highest expression (HEPG2) was taken as 100 (n=3) and then mean of the other cell lines were calculated. Multiple comparison of protein expression of the cell lines were performed by one-way ANOVA and the level of significance was chosen as $p<0.05$. Statistical results are shown in the Table 3.4.

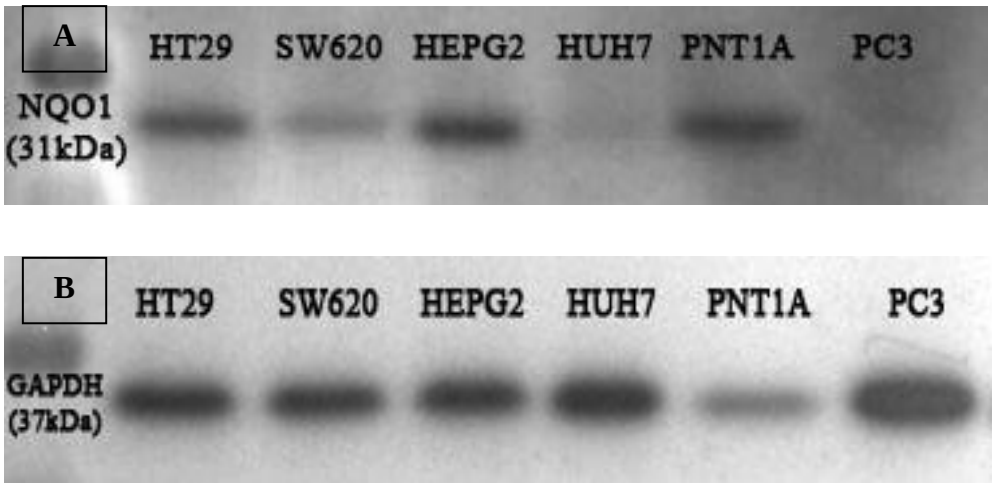

Figure 3.5 Immunoreactive protein bands of cell lines representing NQO1 (A) and GAPDH (B) used as internal standard.

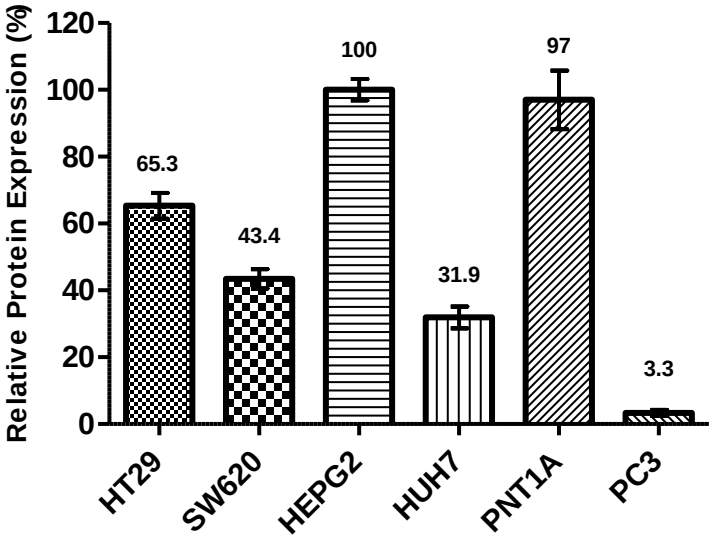

Figure 3.6 Comparison of NQO1 protein expression of cell lines. The band quantifications are expressed as mean ± SD of the relative intensity of three independent experiments.

Table 3.4 Statistical analysis of NQO1 protein expression by one-way ANOVA method.

	SW620	HEPG2	HUH7	PNT1A	PC3
HT29	NS	*	*	*	**
SW620		**	NS	**	*
HEPG2			***	NS	***
HUH7				***	NS
PNT1A					***

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

3.2.4 GSTP1 Protein Expression in the Cell Lines

GSTP1 (23 kDa) protein expression was determined by Western blotting technique. Primary goat polyclonal anti- GSTP1 antibody (1/1000 dilution) and a horseradish peroxidase (HRP) conjugated secondary mouse anti-goat antibody (1/4000 dilution) were used for immunochemical detection of GSTP1 protein (Figure 3.7). Band intensities were quantified by using Image J visualization software. Figure 3.8 shows the relative protein expressions where the mean of the cell line showing highest expression (HUH7) was taken as 100 (n=3) and then mean of the other cell lines were calculated. Multiple comparison of protein expression of the cell lines were performed by one-way ANOVA and the level of significance was chosen as $p<0.05$. Statistical results are shown in the Table 3.5.

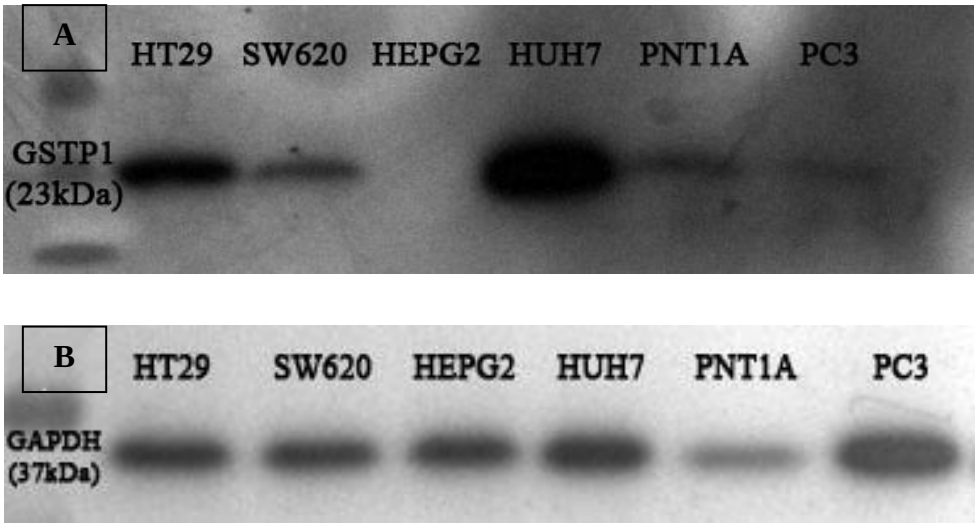

Figure 3.7 Immunoreactive protein bands of cell lines representing GSTP1 (A) and GAPDH (B) used as internal standard.

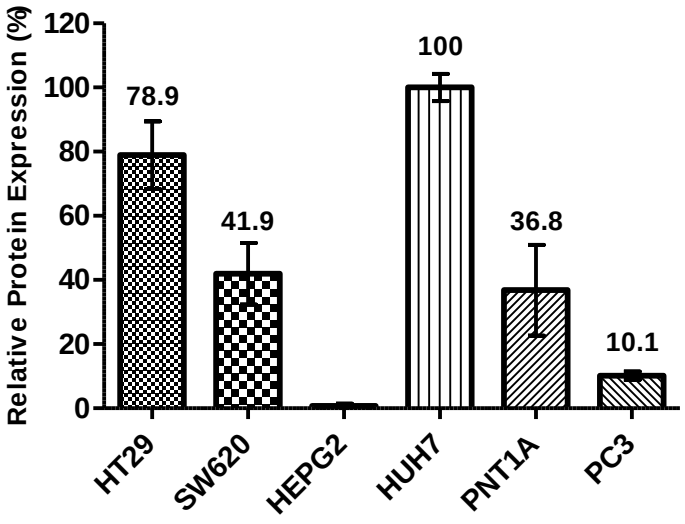

Figure 3.8 Comparison of GSTP1 protein expression of cell lines. The band quantifications are expressed as mean ± SD of the relative intensity of three independent experiments.

Table 3.5 Statistical analysis of GSTP1 protein expression by one-way ANOVA method.

	SW620	HEPG2	HUH7	PNT1A	PC3
HT29	**	***	NS	***	***
SW620		***	***	NS	**
HEPG2			***	**	NS
HUH7				***	***
PNT1A					*

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

3.3 CYP1A1, CYP2E1, NQO1 and GSTP1 mRNA Expressions in the Cell Lines

3.3.1 Quality Control of RNA Molecules by Agarose Gel Electrophoresis

Total RNA was isolated from cell lines by using Qiazol© as described in the method part. Quantity of isolated RNA was measured by reading the absorbance at 260nm. Purity of RNA molecules were checked by NanoDrop with OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios which should be in the range of 1.8-2.2 and 2.0-2.2, respectively. OD_{260}/OD_{280} ratio was used to check DNA contamination while OD_{260}/OD_{230} ratio was used to check carbohydrate and phenolic contamination. After these measurements, 28S and 18S ribosomal RNA bands were checked with agarose gel electrophoresis and showed intact, well separated 28S and 18S ribosomal RNA bands on the gel (Figure 3.9).

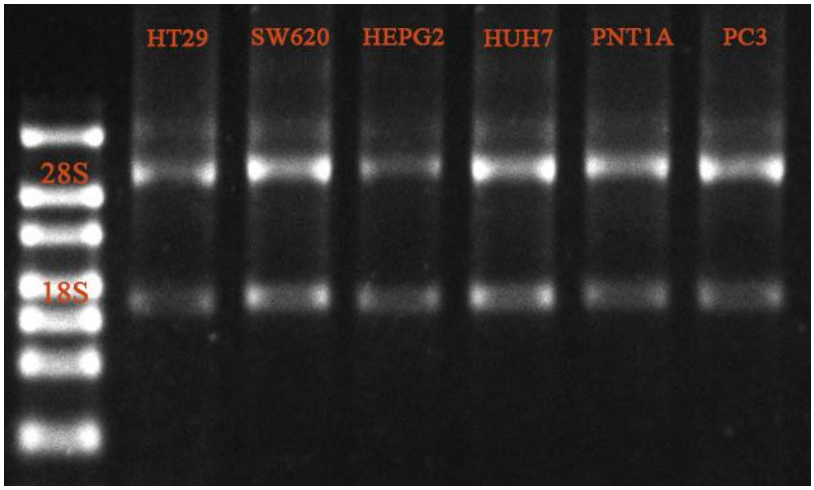

Figure 3.9 Agarose gel electrophoresis results of RNA samples isolated from cell lines.

3.3.2 CYP1A1 mRNA Expression in the Cell Lines

Quantitative real time PCR (qRT-PCR) technique was used to determine the mRNA expression of CYP1A1 in the HT29, SW620, HEPG2, HUH7, PNT1A and PC3 cell lines. GAPDH was used as internal standard to calculate relative mRNA expression of CYP1A1. Specific annealing temperatures of the primers of CYP1A1 and GAPDH were used in qRT-PCR.

Relative mRNA expression of CYP1A1 was calculated in each cell line by producing standard curve from 1:10, 1:100, 1:500, 1:1000 and 1:5000 dilutions of cDNA of HUH7 cell line (Figure 3.10). The amplification plot shows changes in fluorescence of SYBR green dye I versus cycle number of CYP1A1 gene (Figure 3.11). Melt curve analysis was performed to check amplification of specific product (Figure 3.12).

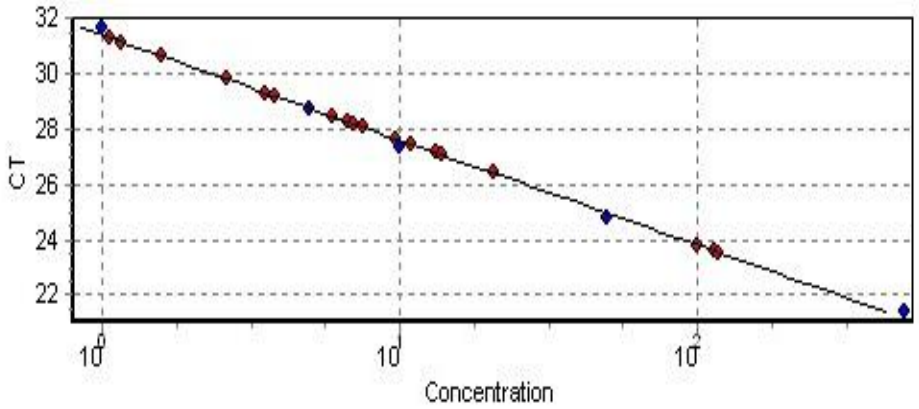

Figure 3.10 Standard curve generated from serial dilutions of chosen cDNA to calculate quantities of CYP1A1 mRNAs in the cell lines relatively.

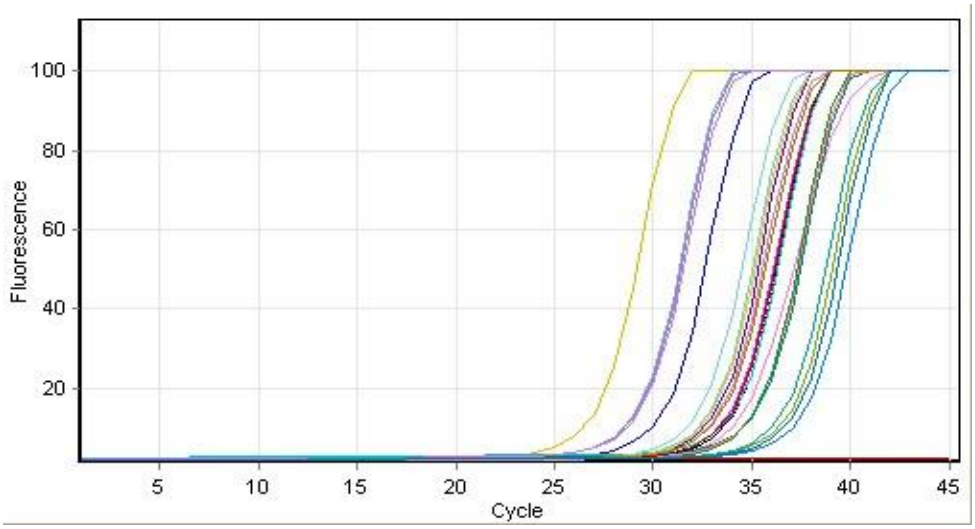

Figure 3.11 Amplification curve showing the accumulation of fluorescence emission at each reaction cycle.

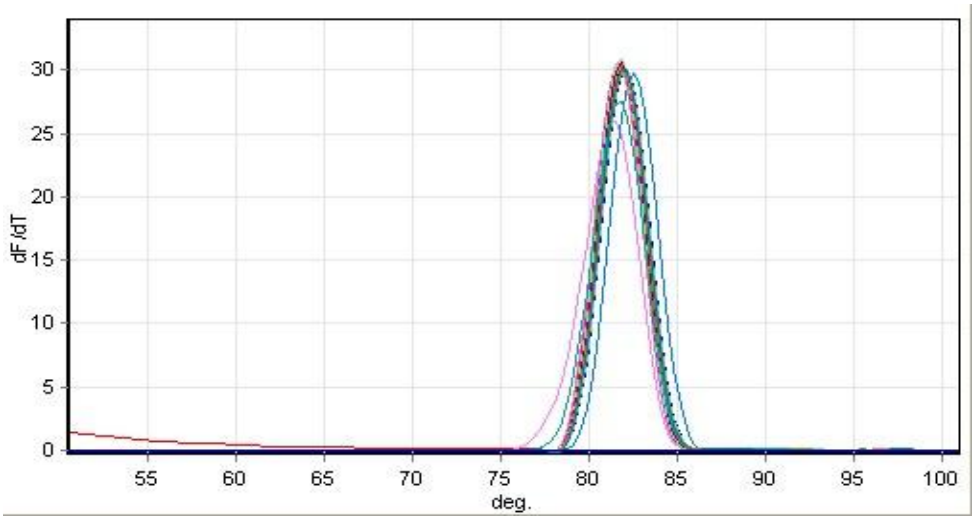

Figure 3.12 Melting curve showing the fluorescence emission change versus temperature. Detection of single peak means single PCR product.

In Figure 3.13 and Figure 3.14 the qRT-PCR products of CYP1A1 and GAPDH in cell lines are given respectively. The position of bands on the gel overlaps with expected size of the CYP1A1 and GAPDH qRT-PCR products which are 121 and 197 bp, respectively. The results obtained using Corbett Rotor-Gene 600 quantitation software were normalized with internal standard GAPDH and Livak method (Livak, 2001) was used to determine relative CYP1A1 mRNA expression in cell lines by using Ct values. Formulation for Livak ($2^{-\Delta\Delta ct}$) method is given in Table 3.6. For calculation of relative mRNA expression by using Livak method, Ct values of HUH7 cell line was used as reference since it is considered as *in vitro* model for studying drug and carcinogen metabolism.

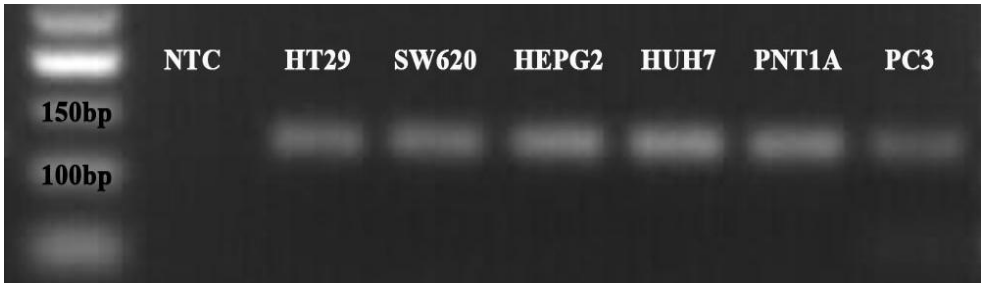

Figure 3.13 qRT-PCR products of CYP1A1 cDNA (121bp) of each cell line. 5 μL of qRT-PCR product was loaded in each well. NTC is the no template control.

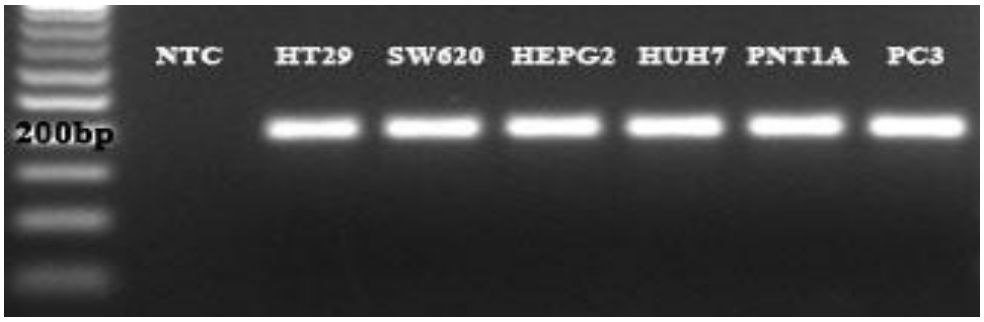

Figure 3.14 qRT-PCR products of GAPDH cDNA (197bp) of each cell line. 5 μL of qRT-PCR product was loaded in each well. NTC is the no template control.

Table 3.6 The Livak method for the calculation of relative mRNA expression using Ct values.

	HT29	**SW620**	**HEPG2**	**HUH7**	**PNT1A**	**PC3**
$\mathbf{Ct_{CYP1A1}}$	17,46	17,06	16,84	15,87	17,50	17,41
$\mathbf{Ct_{GAPDH}}$	29,44	28,26	26,27	24,64	27,77	31,03
$\mathbf{\Delta Ct_{Cells}}$ =	Ct_{GAPDH}	-	Ct_{CYP1A1}			
$\mathbf{\Delta Ct_{Reference}}$=	Ct_{GAPDH}	-	Ct_{CYP1A1}			
$\mathbf{\Delta Ct_{Cells}}$ =	-11,98	-11,20	-9,43	-8,77	-10,27	-13,62
$\mathbf{\Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{Cells}}$						
$\mathbf{\Delta\Delta Ct}$ =	3,21	2,43	0,66	0,00	1,50	4,86
$\mathbf{2^{-\Delta\Delta Ct}}$	0,11	0,18	0,63	1,00	0,36	0,04

Figure 3.15 shows the mean ± SD of the relative CYP1A1 mRNA expressions of cell lines. The data was statistically analyzed by one-way ANOVA method and multiple comparisons were performed and the results are given in Table 3.7.

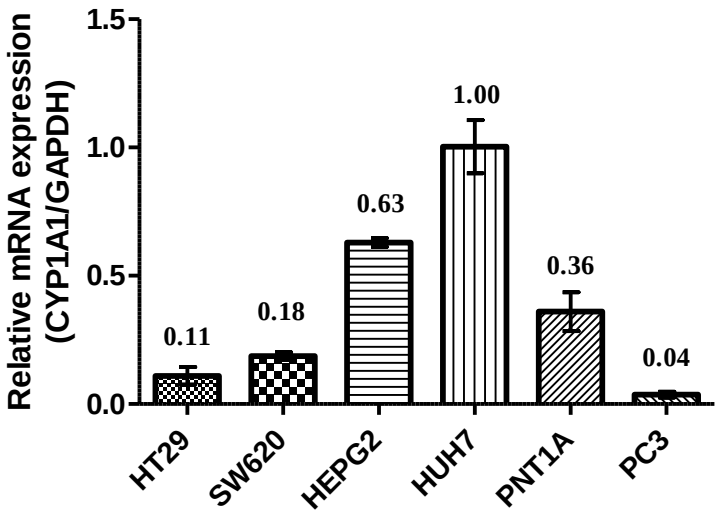

Figure 3.15 Comparison of CYP1A1 mRNA expression of cell lines. The quantifications are expressed as mean ± SD of the relative expression. Graph represents data of three independent experiments.

Table 3.7 Statistical analysis of CYP1A1 mRNA expression by one-way ANOVA method.

	SW620	**HEPG2**	**HUH7**	**PNT1A**	**PC3**
HT29	NS	***	***	**	NS
SW620		***	***	*	NS
HEPG2			***	***	***
HUH7				***	***
PNT1A					***

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

3.3.3 CYP2E1 mRNA Expression in the Cell Lines

Quantitative real time PCR (qRT-PCR) technique was used to determine the mRNA expression of CYP2E1 in the HT29, SW620, HEPG2, HUH7, PNT1A and PC3 cell lines. GAPDH was used as internal standard to calculate relative mRNA expression of CYP2E1. Specific annealing temperatures of the primers of CYP2E1 and GAPDH were used in qRT-PCR.

Relative mRNA expression of CYP2E1 was calculated in each cell line by producing standard curve from 1:10, 1:100, 1:500, 1:1000 and 1:5000 dilutions of cDNA of HUH7 cell line (Figure 3.16). The amplification plot shows changes in fluorescence of SYBR green dye I versus cycle number of CYP2E1 gene (Figure 3.17). Melt curve analysis was performed to check amplification of specific product (Figure 3.18).

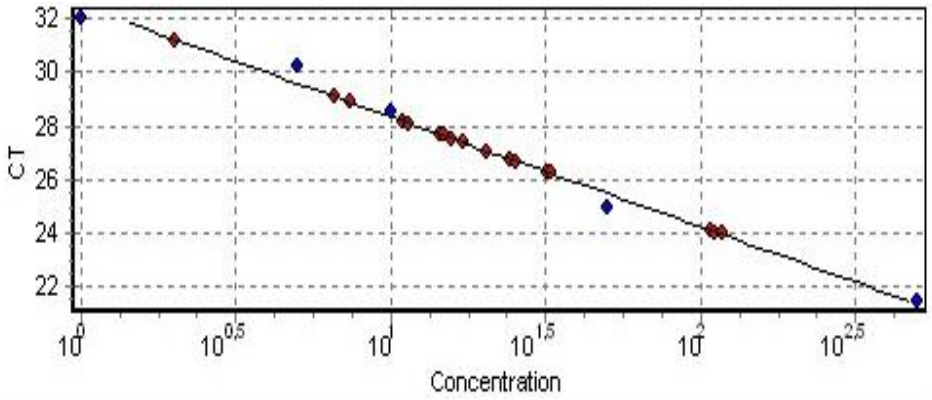

Figure 3.16 Standard curve generated from serial dilutions of chosen cDNA to calculate quantities of CYP2E1 mRNAs in the cell lines relatively.

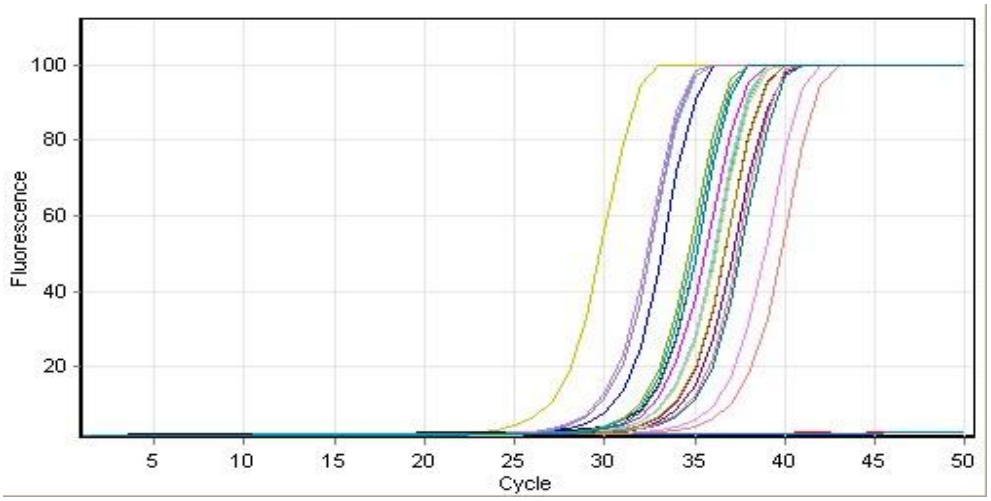

Figure 3.17 Amplification curve showing the accumulation of fluorescence emission at each reaction cycle.

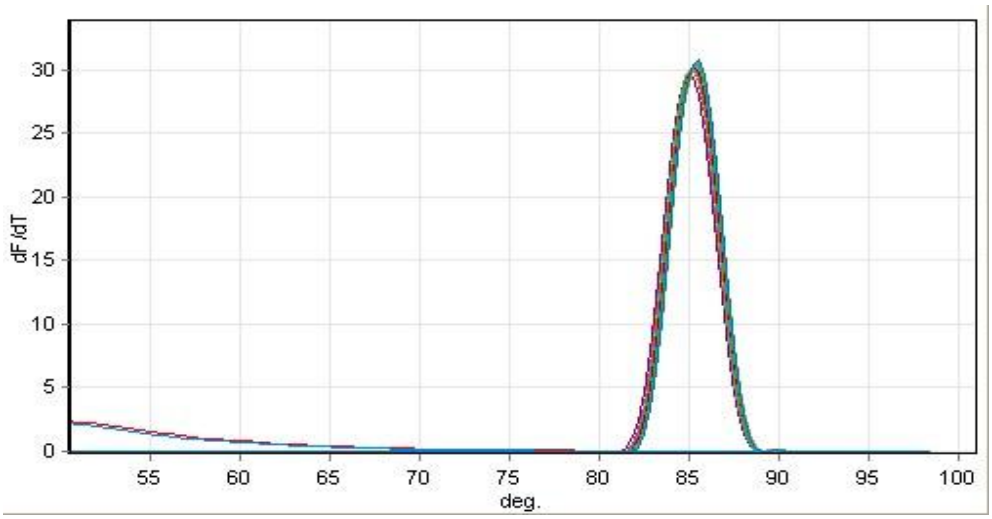

Figure 3.18 Melting curve showing the fluorescence emission change versus temperature. Detection of single peak means single PCR product.

In Figure 3.19 the qRT-PCR products of CYP2E1 in cell lines are shown. The position of bands on the gel overlaps with expected size of the CYP2E1 which is 184bp. The results obtained using Corbett Rotor-Gene 600 quantitation software were normalized with internal standard GAPDH and Livak method (Livak, 2001) was used to determine relative CYP2E1 mRNA expression in cell lines by using Ct values. Formulation for Livak ($2^{-\Delta\Delta ct}$) method is described in Table 3.6. For calculation of relative mRNA expression by using Livak method, Ct values of HUH7 cell line was used as reference since it is considered as *in vitro* model for studying drug and carcinogen metabolism.

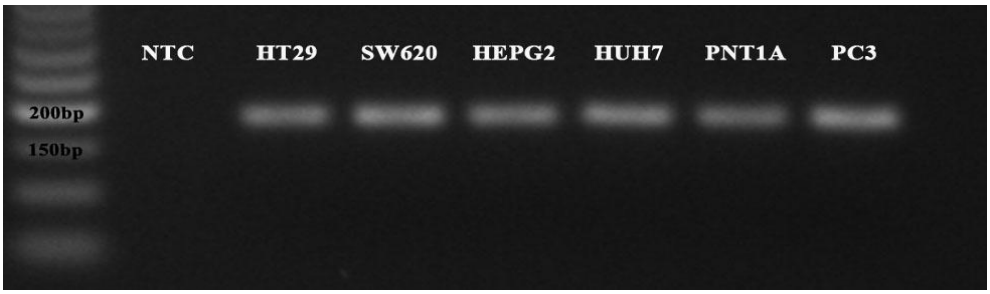

Figure 3.19 qRT-PCR products of CYP2E1 cDNA (184bp) of each cell line. 5 µL of qRT-PCR product was loaded in each well. NTC is the no template control.

Figure 3.20 shows the mean ± SD of the relative CYP2E1 mRNA expressions of cell lines. The data was statistically analyzed by one-way ANOVA method and multiple comparisons were performed and the results are given in Table 3.8.

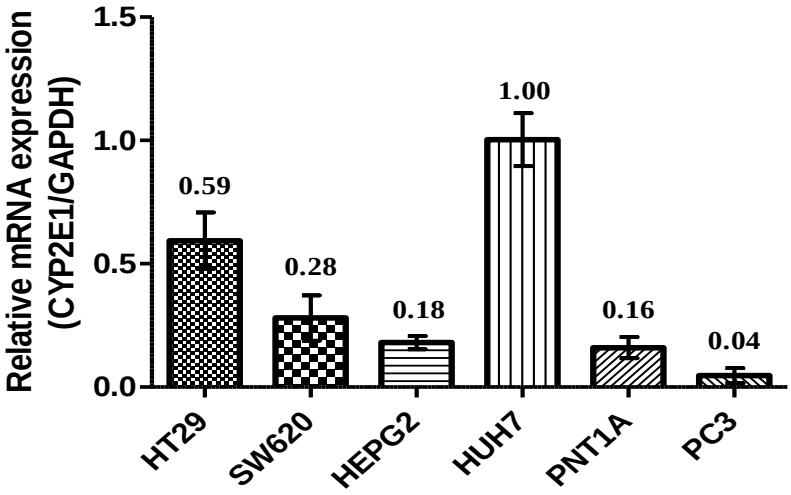

Figure 3.20 Comparison of CYP2E1 mRNA expression of cell lines. The quantifications are expressed as mean ± SD of the relative expression. Graph represents data of three independent experiments.

Table 3.8 Statistical analysis of CYP2E1 mRNA expression by one-way ANOVA method.

	SW620	**HEPG2**	**HUH7**	**PNT1A**	**PC3**
HT29	**	***	***	***	***
SW620		NS	***	NS	*
HEPG2			***	NS	NS
HUH7				***	***
PNT1A					NS

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

3.3.4 NQO1 mRNA Expression in the Cell Lines

Quantitative real time PCR (qRT-PCR) technique was used to determine the mRNA expression of NQO1 in the HT29, SW620, HEPG2, HUH7, PNT1A and PC3 cell lines. GAPDH was used as internal standard to calculate relative mRNA expression of NQO1. Specific annealing temperatures of the primers of NQO1 and GAPDH were used in qRT-PCR.

Relative mRNA expression of NQO1 was calculated in each cell line by producing standard curve from 1:10, 1:100, 1:500, 1:1000 and 1:5000 dilutions of cDNA of PNT1A cell line (Figure 3.21). The amplification plot shows changes in fluorescence of SYBR green dye I versus cycle number of NQO1 gene (Figure 3.22). Melt curve analysis was performed to check amplification of specific product (Figure 3.23).

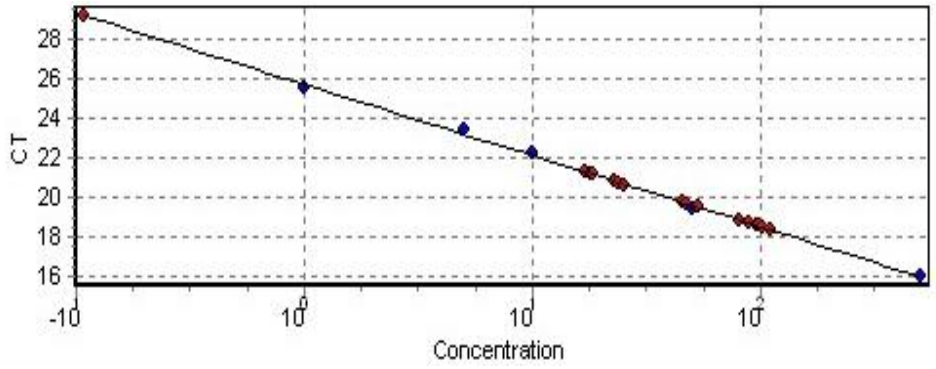

Figure 3.21 Standard curve generated from serial dilutions of chosen cDNA to calculate quantities of NQO1 mRNAs in the cell lines relatively.

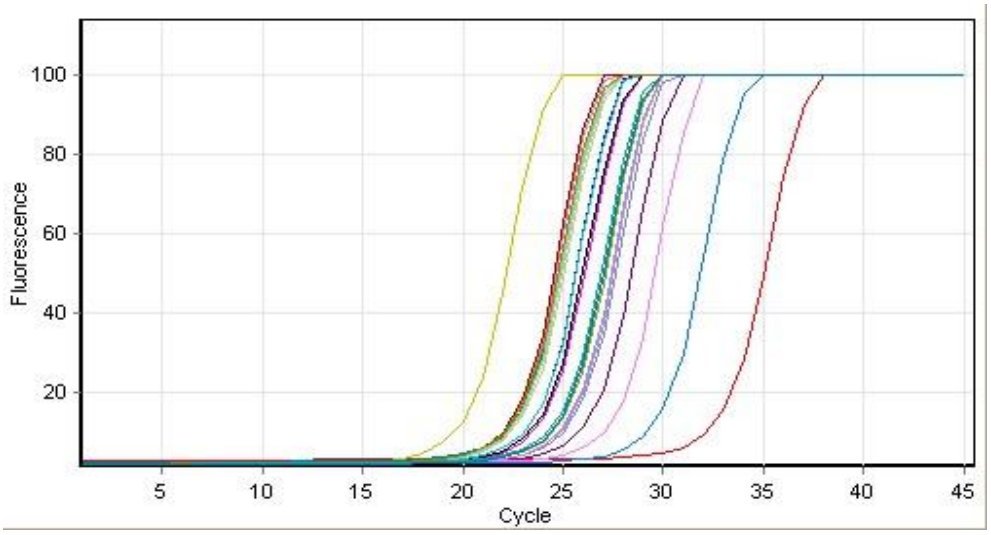

Figure 3.22 Amplification curve showing the accumulation of fluorescence emission at each reaction cycle.

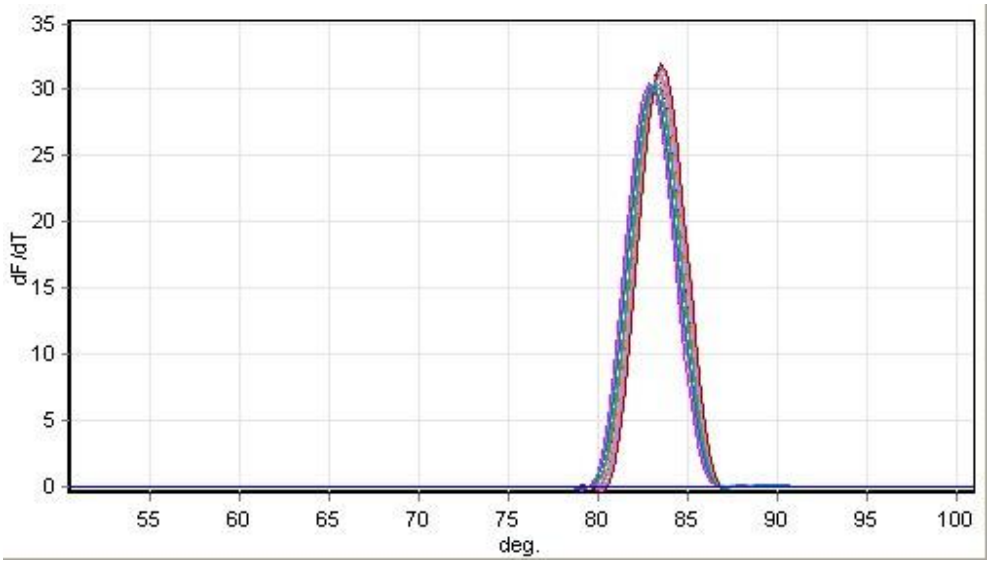

Figure 3.23 Melting curve showing the fluorescence emission change versus temperature. Detection of single peak means single PCR product.

In Figure 3.24 the qRT-PCR products of NQO1 in cell lines are shown. The position of bands on the gel overlaps with expected size of the NQO1 which is 196bp. The results obtained using Corbett Rotor-Gene 600 quantitation software were normalized with internal standard GAPDH and Livak method (Livak, 2001) was used to determine relative NQO1 mRNA expression in cell lines by using Ct values. Formulation for Livak ($2^{-\Delta\Delta ct}$) method is described in Table 3.6. For calculation of relative mRNA expression by using Livak method, Ct values of HEPG2 cell line was used as reference since NQO1 expression in HUH7 cell line is very low.

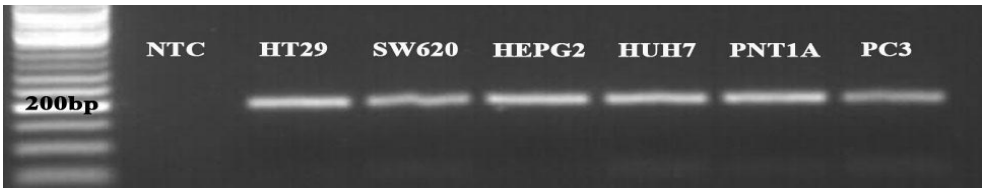

Figure 3.24 qRT-PCR products of NQO1 cDNA (196bp) of each cell line. 5 µL of qRT-PCR product was loaded in each well. NTC is the no template control.

Figure 3.25 shows the mean ± SD of the relative NQO1 mRNA expressions of cell lines. The data was statistically analyzed by one-way ANOVA method and multiple comparisons were performed and the results are given in Table 3.9.

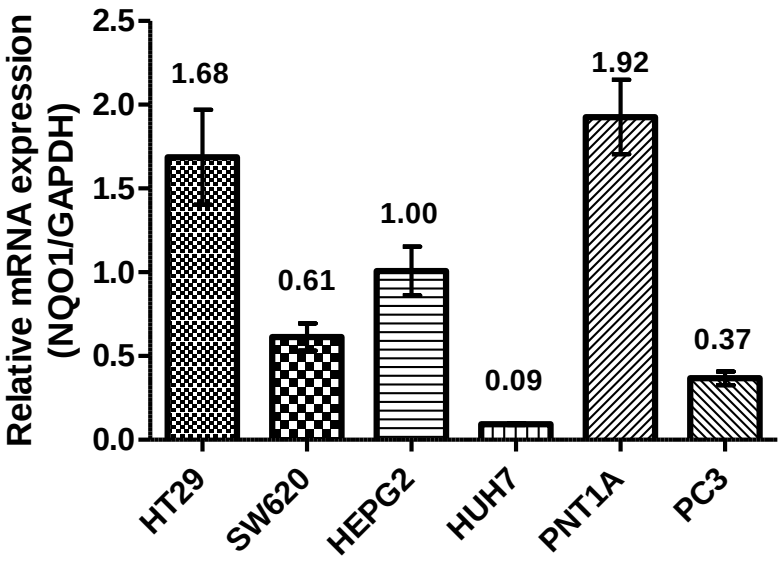

Figure 3.25 Comparison of NQO1 mRNA expression of cell lines. The quantifications are expressed as mean ± SD of the relative expression. Graph represents data of three independent experiments.

Table 3.9 Statistical analysis of NQO1 mRNA expression by one-way ANOVA method.

	SW620	**HEPG2**	**HUH7**	**PNT1A**	**PC3**
HT29	***	**	***	NS	***
SW620		NS	*	***	NS
HEPG2			***	***	**
HUH7				***	NS
PNT1A					***

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

3.3.5 GSTP1 mRNA Expression in the Cell Lines

Quantitative real time PCR (qRT-PCR) technique was used to determine the mRNA expression of GSTP1 in the HT29, SW620, HEPG2, HUH7, PNT1A and PC3 cell lines. GAPDH was used as internal standard to calculate relative mRNA expression of GSTP1. Specific annealing temperatures of the primers of GSTP1 and GAPDH were used in qRT-PCR.

Relative mRNA expression of GSTP1 was calculated in each cell line by producing standard curve from 1:10, 1:100, 1:500, 1:1000 and 1:5000 dilutions of cDNA of HT29 cell line (Figure 3.26). The amplification plot shows changes in fluorescence of SYBR green dye I versus cycle number of GSTP1 gene (Figure 3.27). Melt curve analysis was performed to check amplification of specific product (Figure 3.28).

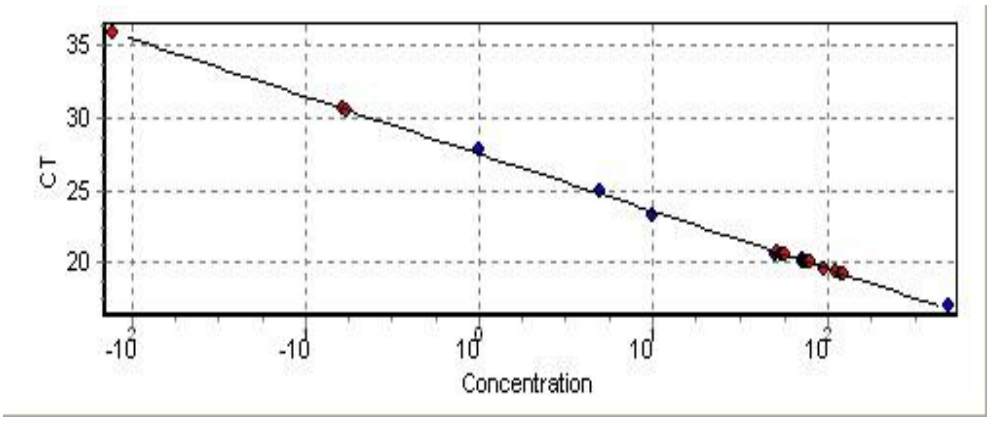

Figure 3.26 Standard curve generated from serial dilutions of chosen cDNA to calculate quantities of GSTP1 mRNAs in the cell lines relatively.

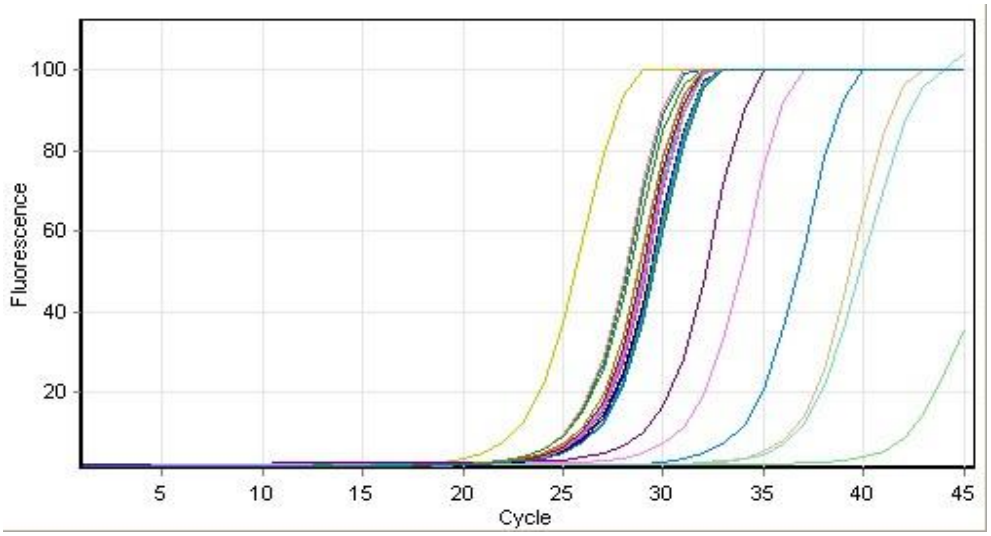

Figure 3.27 Amplification curve showing the accumulation of fluorescence emission at each reaction cycle.

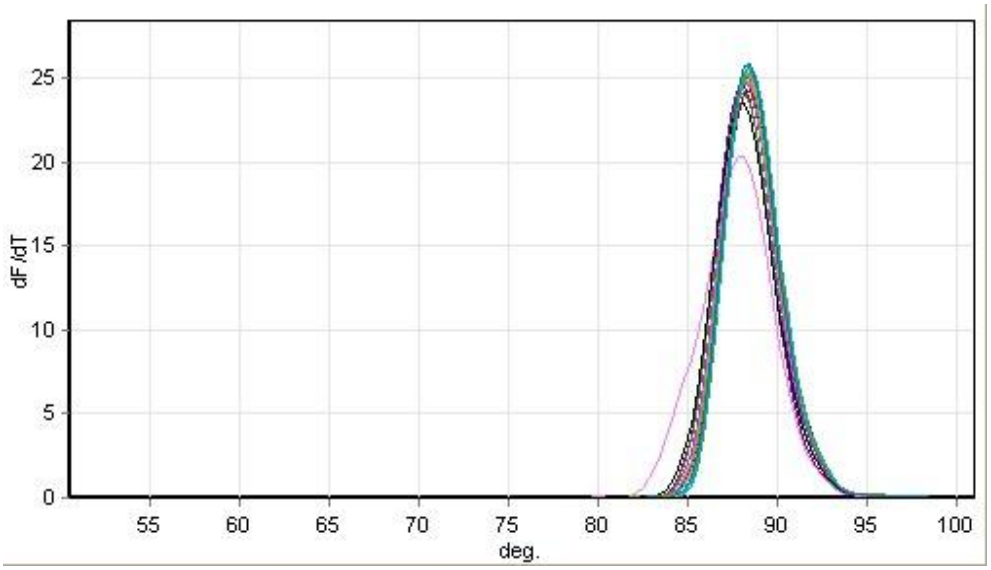

Figure 3.28 Melting curve showing the fluorescence emission change versus temperature. Detection of single peak means single PCR product.

In Figure 3.29 the qRT-PCR products of GSTP1 in cell lines are shown. The position of bands on the gel overlaps with expected size of the GSTP1 which is 137bp. The results obtained using Corbett Rotor-Gene 600 quantitation software were normalized with internal standard GAPDH and Livak method (Livak, 2001) was used to determine relative GSTP1 mRNA expression in cell lines by using Ct values. Formulation for Livak ($2^{-\Delta\Delta ct}$) method is described in Table 3.6. For calculation of relative mRNA expression by using Livak method, Ct values of HUH7 cell line was used as reference since HUH7 cell line is considered as *in vitro* model for studying drug and carcinogen metabolism.

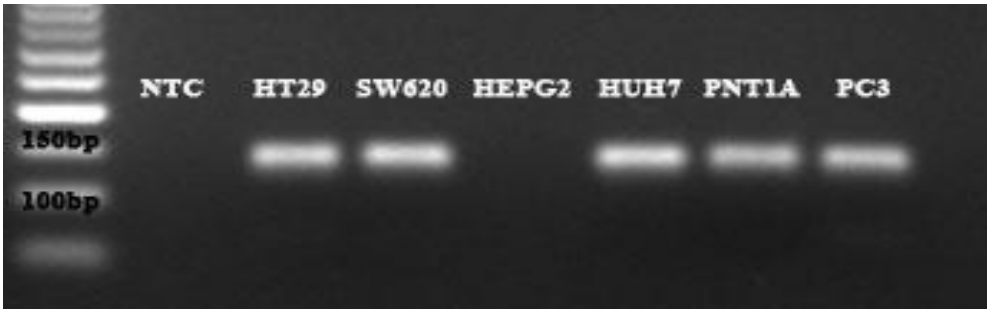

Figure 3.29 qRT-PCR products of GSTP1 cDNA (137bp) of each cell line. 5 µL of qRT-PCR product was loaded in each well. NTC is the no template control.

Figure 3.30 shows the mean ± SD of the relative GSTP1 mRNA expressions of cell lines. The data was statistically analyzed by one-way ANOVA method and multiple comparisons were performed and the results are given in Table 3.10.

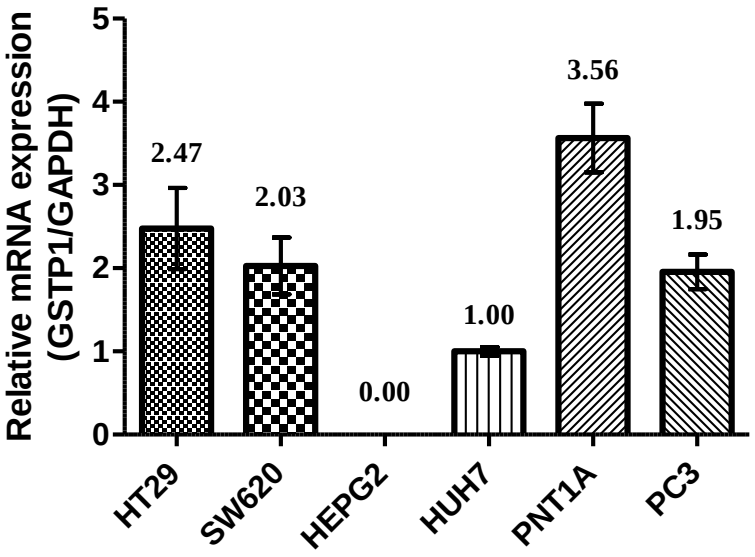

Figure 3.30 Comparison of GSTP1 mRNA expression of cell lines. The quantifications are expressed as mean ± SD of the relative expression. Graph represents data of three independent experiments.

Table 3.10 Statistical analysis of GSTP1 mRNA expression by one-way ANOVA method.

	SW620	**HEPG2**	**HUH7**	**PNT1A**	**PC3**
HT29	NS	***	**	*	NS
SW620		***	*	***	NS
HEPG2			*	***	***
HUH7				***	*
PNT1A					***

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

CHAPTER 4

DISCUSSION

Xenobiotic metabolism is the biochemical modification of xenobiotics by specialized enzymatic systems including phase I and phase II reactions. Phase I drug metabolism includes activation of an inactive xenobiotic or inactivation of an active xenobiotic by oxidation, reduction and hydrolysis reactions. Generally a phase I reaction is followed by a phase II reaction, conjugation of active compound with charged species such as carboxyl, hydroxyl, amino, sulfhydryl and glutathione group.

A phase I enzyme, CYP1A1 catalyzes the conversion of many procarcinogens to active carcinogenic compounds such as benzo[a]pyrene and hydroxylation of many endogenous substrates including the arachidonic acid and the hormones such as melatonin (Ma, 2005; Schwarz, 2004). Another phase I enzyme, CYP2E1 is also important in the conversion of drugs such as acetaminophen, pre-toxins and procarcinogens including nitrosamines to active metabolite (Gonzalez, 2005). Phase II enzymes NQO1 and GSTs catalyze the detoxification of many toxic chemicals and elimination of activated drugs by increasing their solubility.

Phase I and phase II enzymes are regulated transcriptionally, post-transcriptionally, translationally and post-translationally depending on the presence or absence of any inducer and inhibitor. It is very important to know that a xenobiotic is either inhibitor or inducer of these enzymes to estimate possible effects on metabolism of another xenobiotic when concomitantly used.

Animal models share significantly high homology with human; however *in vitro* studies of human cells may provide more accurate results in the toxicology studies. *In vitro* studies are considered as the preliminary step for the discovery of a new drug or investigation of effect of any chemicals on the molecular basis. Moreover, *in vitro* studies give chance to control experimental environment strictly and eliminate any unwanted condition. Because of these advantages, in this study, it was aimed to show the best cell line model for studying CYP1A1, CYP2E1, NQO1 and GSTP1 enzymes playing role in drug and carcinogen metabolism. For that purpose protein and mRNA expression of these enzymes were analyzed in the human cell lines. The cell lines used in this study, shown in Table 4.1, are originated from different ethnicity, gender and age. Since, generally, expression of a gene depends on age, gender and ethnicity; the cell lines may show significant expression differences although they derived from the same tissue.

Table 4.1 Name, origin and tissue cell lines

Cell Line	Origin	Derived From
HT29	Caucasian, female 44 years	Colorectal adenocarcinoma
SW620	Caucasian male, 51 years	Colorectal adenocarcinoma
HEPG2	Caucasian male, 15 years	Hepatocellular carcinoma
HUH7	Japanese male, 57 years	Hepatocellular carcinoma
PNT1A	Caucasian Male, 35 years	Normal prostate epithelial cell
PC3	Caucasian male, 62 years	Adenocarcinoma

In this study CYP1A1, CYP2E1, NQO1 and GSTP1 protein expressions were analyzed by Western blotting technique and Image J software. The overall of CYP1A1, CYP2E1, NQO1 and GSTP1 protein and mRNA expressions in the cell lines is shown in Figure 4.1 and Figure 4.2, respectively. Quantification of the relative protein and mRNA expression of these enzymes in the cell lines is expressed as mean ± SD in Table 4.2, Table 4.3, Table 4.4 and Table 4.5.

According to the results, although all cell lines show CYP1A1 protein expression, HT29 expressed CYP1A1 protein significantly higher than the other cell lines. In addition, SW620, another colon cell line, expressed CYP1A1 significantly higher than the liver and the prostate cell lines. There is no significant CYP1A1 protein expression difference between HEPG2, HUH7, PNT1A and PC3 cell lines and their expression is very low when compared with colon cell lines. However, as shown in Figure 4.2 and Table 4.2, CYP1A1 mRNA expression of HT29 and SW620 is significantly lower than the liver cell lines HEPG2 and HUH7. Although CYP1A1 protein present in the PC3 cell line, its mRNA expression is very low when compared with other cell lines. The correlation analysis between relative protein and mRNA expressions of CYP1A1 enzyme was performed and the result is given in Table 4.2 which implies there is no significant correlation.

Table 4.2 Relative CYP1A1 protein and mRNA expressions of the cell lines. The quantifications are expressed as mean ± SD of relative protein and mRNA expression. Significance level for correlation analysis is $p<0.05$.

Cell Line	CYP1A1		Correlation
	Protein	mRNA	
HT29	100.00±6.47	0.11±0.03	p = 0.395 Not significant
SW620	59.07±9.24	0.18±0.02	
HEPG2	24.54±5.33	0.63±0.02	
HuH7	13.74±4.24	1.00±0.11	
Pnt1A	9.61±0.99	0.36±0.07	
PC3	10.39±0.51	0.035±0.01	

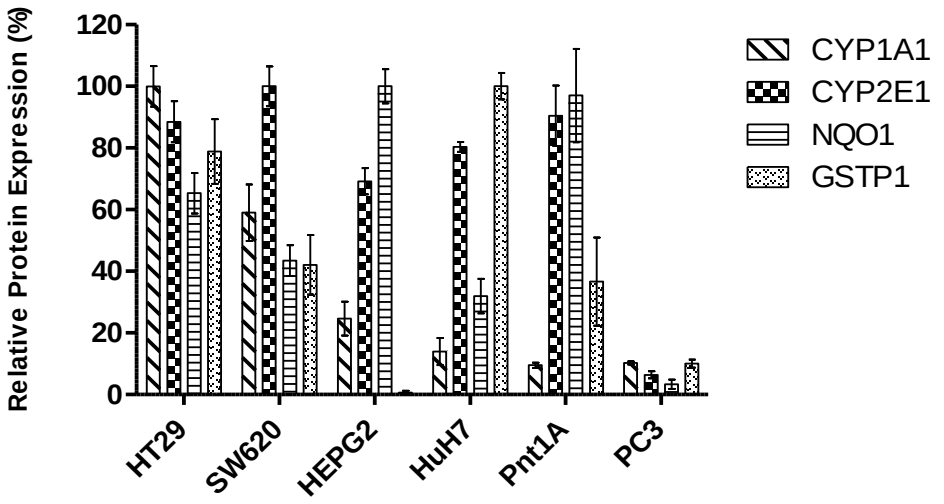

Figure 4.1 Relative protein expression of CYP1A1, CYP2E1, NQO1 and GSTP1 enzymes in HT29, SW620, HEPG2, HUH7, PNT1A and PC3 cell lines.

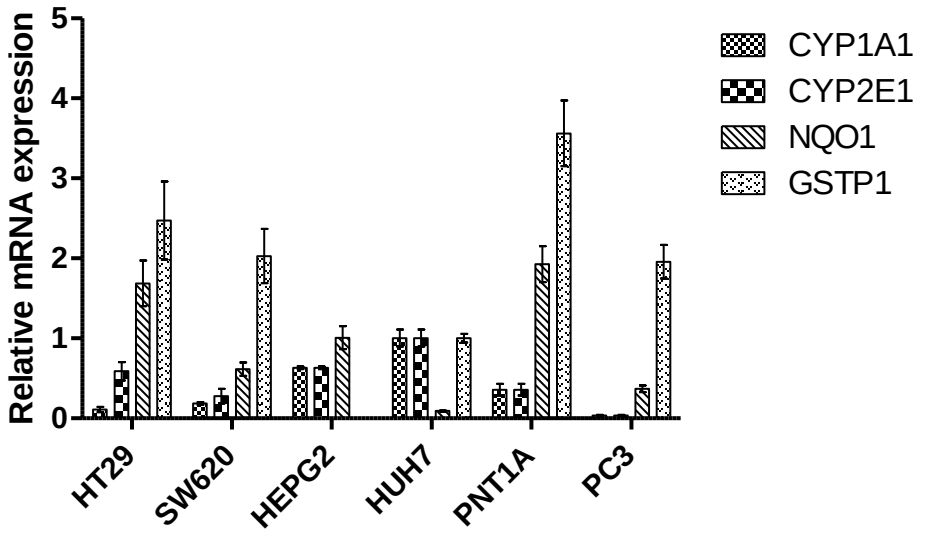

Figure 4.2 Relative mRNA expression of CYP1A1, CYP2E1, NQO1 and GSTP1 enzymes in HT29, SW620, HEPG2, HUH7, PNT1A and PC3 cell lines.

CYP2E1 relative protein expression was found at similar level in each cell line except PC3 showing very low expression. SW620 expressed CYP2E1 protein significantly higher than the HEPG2, HUH7 and PC3 cell lines. In addition, there is no significant difference between relative CYP2E1 protein expression in SW620, HT29 and PNT1A cell lines. However, as shown in Figure 4.2 and Table 4.3, CYP2E1 mRNA expression of HT29 (0.59 fold), SW620 (0.28 fold) and PNT1A (0.16 fold) is significantly lower than the liver cell line HUH7 (1.00 fold). Both protein and mRNA expression of CYP2E1 in the PC3 cell line is very low. The correlation analysis between relative protein and mRNA expressions of CYP2E1 enzyme was performed and the result is given in Table 4.3 which implies there is no significant correlation.

Table 4.3 Relative CYP2E1 protein and mRNA expressions of the cell lines. The quantifications are expressed as mean ± SD of relative protein and mRNA expression ($2^{-\Delta\Delta ct}$). Significance level for correlation analysis is $p<0.05$.

Cell Line	CYP2E1		Correlation
	Protein	mRNA	
HT29	88.49±6.62	0.59±0.12	p = 0.343 Not Significant
SW620	100.00±6.45	0.28±0.09	
HEPG2	69.19±4.29	0.18±0.03	
HuH7	80.35±1.61	1.00±0.11	
Pnt1A	90.47±9.77	0.16±0.04	
PC3	6.38±1.16	0.04±0.03	

NQO1 protein expression was found in all cell lines except PC3 showing very low or no expression. HEPG2 expressed NQO1 protein significantly higher than the HUH7, HT29, SW620 cell lines. In addition, there is no significant difference between relative NQO1 protein expression in HEPG2 and PNT1A cell lines. However, as shown in Figure 4.2 and Table 4.4, NQO1 mRNA expression of HEPG2 (1.00 fold) is significantly lower than the HT29 (1.68 fold) and PNT1A (1.92 fold) cell lines while significantly higher than the SW620 (0.61 fold) and HUH7 (0.09 fold) cell lines. Both protein and mRNA expression of NQO1 in the PC3 cell line is very low. The correlation analysis between relative protein and mRNA expressions of NQO1 enzyme was performed and the result is given in Table 4.4 which implies there is no significant correlation.

Table 4.4 Relative NQO1 protein and mRNA expressions of the cell lines. The quantifications are expressed as mean ± SD of relative protein and mRNA expression. Significance level for correlation analysis is $p<0.05$.

Cell Line	NQO1		Correlation
	Protein	mRNA	
HT29	65.30±6.60	1.68±0.28	p = 0.0836 Not Significant
SW620	43.44±5.02	0.61±0.08	
HEPG2	100.03±5.55	1.00±0.14	
HuH7	31.93±5.60	0.09±0.01	
Pnt1A	97.00±15.16	1.92±0.23	
PC3	3.33±1.53	0.37±0.04	

GSTP1 relative protein expression was found at significantly higher levels in HUH7 than the other cell lines but surprisingly there is no GSTP1 expression in the other liver cell line, HEPG2. Colon cell line HT29 also expressed GSTP1 protein significantly higher than the SW620, HEPG2, PNT1A and PC3 cell lines. In addition, there is no significant difference between relative GSTP1 protein expression in SW620, PNT1A cell lines. As shown in Figure 4.2 and Table 4.5, GSTP1 mRNA expression is significantly high in the HT29 (2.47 fold), SW620 (2.03 fold), PNT1A (3.56 fold) and PC3 (1.95 fold) when compared with HUH7 cell line. There is no mRNA and protein expression of GSTP1 in HEPG2 cell line. The correlation analysis between relative protein and mRNA expressions of GSTP1 enzyme was performed and the result is given in Table 4.3 which implies there is no significant correlation.

Table 4.5 Relative GSTP1 protein and mRNA expressions of the cell lines. The quantifications are expressed as mean ± SD of relative protein and mRNA expression. Significance level for correlation analysis is $p<0.05$.

Cell Line	GSTP1		Correlation
	Protein	mRNA	
HT29	78.92±10.54	2.47±0.49	p = 0.762 Not Significant
SW620	41.94±9.57	2.03±0.34	
HEPG2	0.76±0.66	0.00±0.00	
HuH7	100.00±4.30	1.00±0.05	
Pnt1A	36.82±14.17	3.56±0.41	
PC3	10.15±1.28	1.95±0.21	

CYP1A1 is an extrahepatic phase I enzyme. Many polycyclic aromatic hydrocarbons most of which are pro-carcinogen are activated by CYP1A1 enzyme. Because of this catalytic activity of CYP1A1 it is an important enzyme to study carcinogenetic potential of the chemicals. Although, it is expressed in liver, its constitutive and inducible expression occurs in the other tissues. CYP1A1 protein expression has been showed in normal human colon tissue by White et al. (1991). However, there are some other studies failed to observe protein presence of CYP1A1 in colon tissue (Massaad, 1992). This conflict may be caused from the genetic differences such as deletion or duplication of the gene. Also single nucleotide change in the coding region of the gene may cause an amino acid change which may be important for the protein stability and activity or this nucleotide change may cause insertion of an early stop codon resulting in formation of incomplete peptide. These genetic differences are also valid for this study since all the cell lines are originated from the genetically different persons. Also, except PNT1A, all the cell lines are cancerous and genetic origin of the cancer for this cell lines are different. Moreover, CYP1A1 expression

difference between HT29 (female) and SW620 (male) colon cell lines may be caused by the sex difference of the donors. It has been reported that in females CYP1A1 is expressed higher levels than males among lung cancer patients (Mollerup, 1999). Besides other genetic differences, since PNT1A is originated from normal prostate tissue, immortalized by Simian Virus 40, while PC3 is a cancer prostate cell line, difference in the mRNA expressions is acceptable. The surprising thing is that the liver cell lines showing lower levels of CYP1A1 protein expression, showed the higher levels of mRNA expression than the colon cell lines. According to correlation analysis, there was no correlation between mRNA and protein expression. As a conclusion the best models for studying metabolism of a chemical by CYP1A1 are the HT29 and SW620 colon cell lines. Moreover, for studying effects of a chemical at transcriptional level HEPG2 and HUH7 liver cell lines may be the best since they expressed CYP1A1 mRNA at very high levels when compared to other cell lines studied in this thesis.

CYP2E1 is a phase I enzyme also known as ethanol-inducible form. Many drugs and chemicals have been reported as the substrate, inducer and inhibitor of the CYP2E1 enzyme, given in Figure 1.5. Since it catalyzes metabolism of so many drugs, studying this enzyme is important for the drug-drug interactions. Also, because of its ability to convert inactive toxic chemicals to more active form, this enzyme has been under study of the toxicology research. In the present study, it was found that all the cell lines except PC3 are good model for studying effects of chemicals metabolized by CYP2E1 enzyme. In previous studies, it has been reported that CYP2E1 protein is expressed in colon, liver and prostate cell lines (Bergheim, 2005; Jiang, 1998; Seliskar, 2007). In this work, in liver cell line HUH7 expressed both protein and mRNA of the CYP2E1 high enough to be used as model. Like CYP1A1, there is no correlation between mRNA and protein expression of CYP2E1. Moreover, as explained before, since cell lines are

genetically different from each other, differences in CYP2E1 expressions between cell lines belonging to the same tissue are acceptable.

NAD(P)H quinone oxidoreductase 1 (NQO1) is a FAD-containing enzyme catalyzing reduction of quinones to hydroquinones to prevent one electron reduction of quinines that results in the formation of radical species (Lind, 1990). Due to its free radical preventive activity, it is known as an antioxidant enzyme. There are several studies reporting that NQO1 may serve protection against chemical carcinogenesis by preventing against radical formation and stabilizing tumor suppressor p53 protein (G Asher, 2001; Talalay, 1988). As an example, mitomycin C, drug used for treatment of non-small-cell lung cancer and activated through reduction by NQO1 enzyme (Siegel, 1992). Since mitomycin C is relatively poor substrate for the NQO1, newer compounds have been continuously tried to be generated for a better chemotherapeutic agent (Ross, 2000). Since NQO1 is an important enzyme for discovery of new drugs, it is important to determine its expression in cell lines and use as a good *in vitro* model to study drug and carcinogen metabolism. According to the results, HT29, HEPG2 and PNT1A cell lines are very good models to study drugs and carcinogens metabolized by NQO1 because they expressed NQO1 protein and mRNA at relatively high levels. Although, SW620 showed relatively lower NQO1 expression, it can be also used for this purpose. However, PC3 and HUH7 cell lines are not recommended for use as model for NQO1 studies. Since HUH7 is the liver cell line, its low NQO1 expression was not expected. However, the lack of NQO1 in HUH7 cell line which is originated from a Japanese person may be explained by C609T polymorphism, found in 39% of Japanese population while 22% of Caucasians (Gaedigk, 1998; Hishida, 2005). In this polymorphism a single nucleotide change C to T at position of 609 causes change of amino acid proline to serine at position 187 causes formation of unstable NQO1 protein which undergoes rapid proteasomal degradation (Siegel, 2001).

An enzyme family Glutathione S-transferases (GSTs) have phase II metabolic isozymes which catalyze the detoxification of endogenous and exogenous electrophilic compounds by conjugating glutathione with the electrophiles. In human, the cytosolic GSTs are divided into six groups sharing ~30% sequence similarity and represented by Greek letters (α, μ, ω, π, θ and ξ) (Townsend, 2003). In this study protein and mRNA expression of GSTP1 (π) was studied. Other than detoxification, it has been reported that elevated GSTP1 expression and activity is related to drug resistance against some chemotherapeutic agents such as adriamycin (Meijerman, 2008; Tew, 1994). In the present study, it was found that all the cell lines except HEPG2 expressed both mRNA and protein of GSTP1 enzyme. The highest protein expression was found in HUH7, liver, cell line which is an expected result however the lack of expression in HEPG2 was surprising. In a study it has been reported that the HEPG2 cell line having hepatitis B virus genome does not show any GSTP1 expression while HEPG2 cell line without this genome expressing the GSTP1 enzyme (Niu, 2009). Hepatitis B virus genome carrying cell line expresses hepatitis B virus X protein which causes hypermethylation and hypomethylation of targeted regions and in this case it has been showed that it hypermethylates the promoter of GSTP1 enzyme in HEPG2 cell line (Niu, 2009). According to our results, HT29, SW620, HUH7 and PNT1A may be used as model cell line to study drug and carcinogen metabolism by GSTP1. However, deciding to the best model for this enzyme is quite difficult because of the huge variation between protein and mRNA expression levels of these cell lines.

When mRNA and protein expressions of the cell lines were compared, there was no significant correlation. However, because of many processes of regulation of gene expression at post-transcriptional, translational and post-translational stages, low correlation between mRNA and protein expression levels is usual. One explanation for low correlation is post-transcriptional modifications such as 5' capping, 3' polyadenylation and splicing processes.

These modifications, such as polyadenylation, influences mRNA stability and translation efficiency (Preiss, 1998). Also the action of the other regulators such as RNA-binding proteins and microRNAs (miRNAs) recognize specific sequence or secondary structure and alter the interaction of the mRNA with the members of the translation machinery (Abaza, 2008). In addition, protein degradation another important regulation that causes low correlation. It has been reported that some proteins including CYP2E1 undergo rapid ubiquitin-proteasome-mediated proteolysis with the absence of its substrate (Roberts, 1995). Because of all of these regulations at different stages of expression, correlation between mRNA and protein expression levels is generally low.

In conclusion, CYP1A1, CYP2E1, NQO1 and GSTP1 enzymes showed different relative protein and mRNA expressions in different cell lines originated from colon, liver and prostate tissues. It was found that HT29 and SW620 colon cell lines expressed proteins of these four enzymes at significant level and these cell lines are the best model cell line to study all of four enzymes together. However, if CYP1A1, CYP2E1, NQO1 and GSTP1 would be studied individually, HT29, HUH7, PNT1A and HT29 are the best models, respectively. But still further studies required including determination of activities for actual effectiveness of these enzymes in the cell lines. In addition, other cell lines belonging to wide variety of tissues and the other important drug metabolizing enzymes such as CYP2D6, CYP3A4, GPx and the other classes of GST should be investigated.

CHAPTER 5

CONCLUSION

Xenobiotic metabolism is the combination of phase I and phase II reactions which are named as modification and conjugation reactions, respectively. Most of the drugs are metabolized by Cytochrome P450s (CYPs) known as phase I enzymes. CYP1A1 and CYP2E1 enzymes play role in activation/inactivation of many drugs and carcinogens. Phase II enzymes including NQO1 and GSTP1 are important for the elimination of toxic metabolites by reduction and conjugation reactions, respectively. Since *in vitro* studies are considered as the preliminary step for the discovery of new drug molecules or for the investigation of effects of chemicals on the molecular basis, this study was aimed to show the best cell line model for studying CYP1A1, CYP2E1, NQO1 and GSTP1 enzymes playing role in drug and carcinogen metabolism. Therefore, protein and mRNA expressions were determined by Western blotting and qRT-PCR methods, respectively.

The results showed that phase II enzymes NQO1 and GSTP1 may be studied best by using HT29, SW620 and HUH7 as model cell lines. Due to low expression levels of these enzymes in PC3, it is considered as a poor model. Although these genes are expressed at relatively significant levels in almost all cell lines, according to both protein and mRNA expressions of these drug and carcinogen metabolizing enzymes, HT29 is the best cell line for studying *in vitro* metabolism of xenobiotics. However, if CYP1A1, CYP2E1, NQO1 and GSTP1 would be studied individually, HT29, HUH7, PNT1A and HT29 are the best models, respectively.

For future perspective, activity assays for determination of actual effectiveness of these enzymes in the cell lines should be carried out. In addition, protein and mRNA expressions of the other important drug metabolizing enzymes such as CYP2D6, CYP3A4, GPx, FMOs and the other classes of GST should be investigated.

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